Histone H1 binding is inhibited by histone variant H3.3

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Linker histones are involved in the formation of higher-order chromatin structure and the regulation of specific genes, yet it remains unclear what their principal binding determinants are. We generated a genome-wide high-resolution binding map for linker histone H1 in *Drosophila* cells, using DamID. H1 binds at similar levels across much of the genome, both in classic euchromatin and heterochromatin. Strikingly, there are pronounced dips of low H1 occupancy around transcription start sites for active genes and at many distant *cis*-regulatory sites. H1 dips are not due to lack of nucleosomes; rather, all regions with low binding of H1 show enrichment of the histone variant H3.3. Knockdown of H3.3 causes H1 levels to increase at these sites, with a concomitant increase in nucleosome repeat length. These changes are independent of transcriptional changes. Our results show that the H3.3 protein counteracts association of H1, providing a mechanism to keep diverse genomic sites in an open chromatin conformation.

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Introduction

Nuclear DNA in eukaryotic cells is packaged in a complex fibre called chromatin. Chromatin has an active role in regulating all DNA-related processes, like transcription, replication, and DNA repair. The fundamental building block of chromatin is the nucleosome, consisting of an octamer of core histones with 146 bp of DNA wrapped around it (van Holde, 1988; Wolffe, 1998). At specific sites in the genome, core histones are replaced with variant histones that have distinct regulatory functions. Furthermore, specialized proteins, named linker histones, associate with nucleosomes near the entry–exit point of linker DNA, protecting another ~20 bp (Simpson, 1978; Travers, 1999). In most eukaryotic cells, linker histones are present in concentrations similar to that of nucleosomes (Woodcock *et al*, 2006), suggesting that a

large proportion of the nucleosomes is bound by a linker histone molecule. Histone H1 and its isoforms are linker histones that are commonly found throughout the eukaryotic kingdom.

Linker histones are believed to be necessary for folding of nucleosomes into higher-order structures. The presence of linker histones renders chromatin assembled *in vitro* more compacted and more refractory to transcription and nucleosome remodelling by ATP-driven remodelling enzymes (Osipova *et al*, 1980; Shimamura *et al*, 1989; Horn *et al*, 2002). The aggregation of nucleosome arrays into 30-nm fibres can be observed in the presence of linker histones (Huynh *et al*, 2005), and similar structures have been isolated from cells (Gilbert and Allan, 2001). Furthermore, polytene chromosomes of mutant fruit fly larvae expressing ~20% of the wild-type amounts of H1 appear generally decondensed (Lu *et al*, 2009; Siriaco *et al*, 2009). Finally, H1 also controls the length of the linker DNA that separates two nucleosomes. At lower occupancy of H1, linker DNA tends to be shorter, that is, the spacing of nucleosomes is reduced (Woodcock *et al*, 2006; Lu *et al*, 2009). Taken together, these data provide compelling evidence that H1 is a major modulator of chromatin structure.

Linker histones must fulfil important regulatory functions in multicellular organisms. Mice that lack three out of six somatic H1 subtypes die at midgestation, and fruit flies with low amounts of H1 have been shown to have reduced viability and defects in pericentric heterochromatin (Fan *et al*, 2005; Lu *et al*, 2009; Siriaco *et al*, 2009). In embryonic stem cells derived from mice with lethal H1 depletion, transcriptional changes are observed for various genes (Fan *et al*, 2005). A recent study in human cells suggests that each of the six somatic H1 variants controls a distinct set of genes (Sancho *et al*, 2008).

The fact that not all genes are equally affected by the depletion of H1 suggests that H1 may bind to only a particular subset of genes. However, the precise binding pattern of H1 in the genome is still poorly understood. From early microscopy and chromatin fractionation studies, we have come to know that H1 associates primarily with transcriptionally inactive regions of the genome (Chiu *et al*, 1977; Jamrich *et al*, 1977). *Drosophila* H1 binds to DNA dense bands on polytene chromosomes in a manner that is dependent on the ATP-dependent remodelling enzyme ISWI (Corona *et al*, 2007; Siriaco *et al*, 2009). In a human breast cancer cell line, H1 has been observed, by chromatin immunoprecipitation (ChIP) experiments, to be depleted from a set of active promoters at which poly (ADP-ribose) polymerase-1 (PARP) is enriched (Krishnakumar *et al*, 2008), but it is yet to be determined to what extent this pattern is isoform specific.

Here, we characterize the genomic binding of histone H1 in detail. Our aims were to identify signals that might regulate H1 binding and to understand the functional relationships of H1 with other chromatin components that mark repressed or active parts of the genome. We used *Drosophila* as a model

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system, as this organism has only one somatic isoform of H1. We generated whole genome, high-resolution maps for H1 in the Kc167 cell line using DNA adenine methyltransferase identification (DamID) (Greif *et al*, 2006). We then compared the binding sites of H1 with those of Polycomb (Pc) and Heterochromatin protein 1 (HP1) as markers of heterochromatin, and with RNA polymerases and the core histone variant H3.3 as markers for active sites. This revealed that H1 binds pervasively throughout the genome, without clear differences between classic euchromatin and heterochromatin. However, H1 is excluded from thousands of active promoters and other regulatory regions. We demonstrate that H3.3 contributes to the exclusion of H1 from these sites. This antagonism provides a mechanism that helps to maintain active regulatory regions in an accessible state.

Results

Histone H1 binds universally with characteristic dips

To map histone H1 binding in the *Drosophila* genome, we generated a DamID profile in Kc167 cells. In short, trace amounts of H1 tagged with Dam methylase were transiently expressed, thereby methylating adenines in the vicinity of binding sites. Methylated DNA fragments were selectively amplified and co-hybridized with Dam-only control material to NimbleGen oligonucleotide microarrays with 300-bp median resolution covering the entire non-repetitive part of the fly genome. The resulting map shows that H1 binding is similar at the majority of probes, with the exception of conspicuous dips that are up to a dozen kb wide (Figure 1A, B and E). Owing to the near-stoichiometric nuclear abundance of H1 relative to nucleosomal histones (Woodcock *et al*, 2006), we interpret this pattern as a uniform global binding of H1 interrupted by local gaps that are called dips hereafter. To verify our DamID results, we performed ChIP with an affinity purified H1 antiserum for selected loci. DamID and ChIP measurements were in good agreement with each other (Supplementary Figure 1A and B).

Using a simple running median-based algorithm, we identified 4792 dips in the DamID binding profile with a median length of 1867 bp. Of these, 4319 dips (90%) had at least 1-bp overlap with genes annotated in FlyBase release 5.8 (total overlap 71% compared to 57% expected by chance, $P = 7.8 \times 10^{-47}$; Figure 1A, B and E). Thus, most of the fly genome is decorated with H1, and dips are relatively short and predominantly localized at genes.

The baseline H1 level outside dips was similar in pericentric heterochromatin known to be bound by HP1 (de Wit *et al*, 2007) and in regions traditionally denoted as euchromatin (Figure 1A and C). This also holds for regions known to coincide with large Pc domains (Tolhuis *et al*, 2006; Figure 1B and C). A minor difference between these diverse chromatin compartments was the number of H1 dips. The frequency of H1 dips was lower in pericentric heterochromatin than in euchromatin, and lowest in Pc domains (Figure 1D). HP1-marked heterochromatic regions are known to be relatively gene-poor (Hoskins *et al*, 2007), and genes in Pc domains are on average more silent than those in euchromatin (Schwartz *et al*, 2006; Tolhuis *et al*, 2006). These results suggest that in general, H1 binds in a similar manner in all classic chromatin types and differences exist only in dip

frequency because of regional differences in gene density and activity.

H1 dips are mainly active TSSs

H1 has been shown to be absent from a set of active promoters in human cells (Krishnakumar *et al*, 2008). To test whether most H1 dips in our *Drosophila* profile also coincide with active promoters, we compared 40% of the most active and inactive genes (Pickersgill *et al*, 2006) in alignments of their 5' and 3' ends. This showed that H1 levels are clearly lower throughout the transcribed region of active genes, with a pronounced dip centred over the transcription start site (TSS) (Figure 2A and B and Supplementary Figure 2A–D).

To investigate the relationship between H1 occupancy and transcription at a higher resolution, we measured the occupancy of RNA polymerase by DamID of Rpl18, the 18-kDa subunit that is common to the three nuclear RNA polymerases. Rpl18 occupancy was in good agreement with mRNA levels (Spearman's $\rho = 0.66$ after averaging the DamID signal per gene). Indeed, Rpl18 signals were strongly anti-correlated with H1 (Spearman's $\rho = -0.58$, $P < 2.2 \times 10^{-16}$; Figure 1E and Supplementary Figure 3A). The group of active genes, but not the inactive genes, exhibited high average Rpl18 binding with a pronounced enrichment at the TSS (Figure 2A and Supplementary Figure 2C). This relationship was retained when the analysis was restricted to tRNA genes (Figure 2C), illustrating that H1 and RNA polymerases bind to chromatin in an opposite manner.

In many organisms, active genes have a short nucleosome-free region (NFR) upstream of their TSSs at the site of RNA polymerase assembly (Yuan *et al*, 2005; Barski *et al*, 2007; Mavrich *et al*, 2008). To investigate whether the observed H1 dips could be simply attributed to NFRs, we generated a map for nucleosome occupancy in Kc cells with 10 bp resolution for a total of 5.2 Mb of the fly genome, distributed over multiple regions (see Materials and methods). Although the group of active genes showed a well-defined NFR, inactive genes did not, as anticipated (Supplementary Figure 2A and C). The first nucleosome upstream and downstream from the TSS were spaced about 350 bp from each other, a value similar to what was observed in *Drosophila* embryos (Mavrich *et al*, 2008). This is probably too small to be the cause for H1 dips at TSSs (Figures 1E and 2A). Furthermore, along bodies of active genes the average nucleosome occupancy was not different than outside genes or along inactive genes, whereas H1 levels were lower than median (Figure 2A and B; Supplementary Figure 2). Transposable elements (TEs) are an example of genomic features that are less bound by Rpl18 than the genome median (Figure 2D). In contrast, H1 levels are above median, whereas average nucleosome density does not differ significantly. All these differences underline that histone H1 binding is not simply determined by nucleosome occupancy. Instead, H1 dips are regions at which nucleosomes are less frequently bound by H1.

Intergenic H1 dips may be regulatory elements

About 10% of the H1 dips are located outside of annotated genes. We hypothesized that they may represent the TSSs or *cis*-regulatory regions those have not been annotated yet. In both cases, they would be expected to often occur close to known genes. Indeed, intergenic H1 dips were more

Figure 1 Characteristics of the H1 binding profile. (A) Profiles of H1 and HP1 (de Wit *et al*, 2007) at the boundary of pericentric heterochromatin on chromosome 2R. Red bars indicate H1 dips as defined in Materials and Methods section. Hatched bar indicates heterochromatin according to Release 5 of the *Drosophila* genome (Hoskins *et al*, 2007). Black and white bars indicate genes on the top and bottom strand, respectively. Profiles are depicted as three-probe running median. (B) H1 and Pc (Tolhuis *et al*, 2006) across a region of chromosome 2L with Pc domains. Hatched bars indicate Pc domain as annotated in the study by Tolhuis *et al* (2006). Other details as in (A). (C) DamID binding values of H1, Pc (Tolhuis *et al*, 2006), and HP1 (de Wit *et al*, 2007) outside of annotated H1 dips in pericentric heterochromatin (defined as in the study by Hoskins *et al* (2007) and including the whole of chromosome 4), Pc domains, or euchromatin (remaining parts of the genome). Only probes overlapping with the design regions of the microarray used in the previous studies (Tolhuis *et al*, 2006; de Wit *et al*, 2007) were analysed. Lower end, thick line, and upper end of the boxes indicate 1st quartile, median, and 3rd quartile, respectively; whiskers extend to the most extreme data points with a distance to the box at most 1.5 times the box height. (D) Frequency of genes and H1 dips in the same genomic regions as defined in (C). (E), Detail of genomic profiles. Red, H1 DamID; black, Rpl18 DamID; grey, nucleosome occupancy (MNase digestion); turquoise, enrichment of H3.3 over H3 in biotin ChIP (Mito *et al*, 2007). Pale red vertical bars highlight H1 dips. Black and white boxes denote genes on the top and bottom strand, respectively, with introns depicted as lines.

Figure 2 H3.3 enrichment is inversely correlated with H1 binding. Average binding profiles aligned to genomic features. Coloured lines represent running means of 2% of the probes in the window region except where indicated otherwise. Datasets are the same as in Figure 1. (A, B), (A) Transcription start sites and (B) transcript 3' ends of the 40% most active genes. Expression profile taken from the study by Pickersgill *et al* (2006). Arrow indicates the location of genes. (C) Centres of tRNA genes. (D) 5' ends of naturally occurring transposable elements (running mean of 0.5% of depicted probes). Arrow indicates the location of TEs. (E) Binding sites of the trxG protein Zeste in *Drosophila* embryos (Moses *et al*, 2006). (F) Co-binding sites of the PcG proteins Enhancer of Zeste and Posterior sex combs in S2 cells (Schwartz *et al*, 2006).

frequently positioned within 2 kb of a gene than expected by chance ($P < 0.002$).

Furthermore, if intergenic H1 dips represent functional genomic elements, their chromatin state might resemble those of known TSSs or regulatory sites. We used formaldehyde-assisted isolation of regulatory elements (FAIRE) to test whether intergenic H1 dips were enriched in regulatory elements. FAIRE enriches for DNA sequences that are relatively free of bound protein and have been shown to overlap with active regulatory sites and DNaseI hypersensitive sites (Hogan *et al*, 2006; Giressi *et al*, 2007). We compared intergenic H1 dips with TSSs of active genes or two classes of regulatory sites: putative Polycomb response elements (PREs) marked by Enhancer of Zeste (E(Z)) and Posterior sex combs (PSC) (Schwartz *et al*, 2006), and known embryonic binding sites of the trithorax group (trxG) protein Zeste (Z) (Moses *et al*, 2006). Both of these classes of sites coincided with low levels of H1 relative to flanking regions (Figure 2E and F). Active TSSs gave the highest average FAIRE signals with a peak just upstream of the TSS as has been reported to be the case for DNaseI hypersensitivity (Sabo *et al*, 2004;

Figure 3A). Furthermore, Z and E(Z)/PSC sites were clearly enriched in FAIRE signal, suggesting that these sequences are binding sites for their cognate proteins and in an open chromatin conformation also in Kc cells (Figure 3B and C). Intergenic H1 dips gave similar FAIRE signals as Z-binding sites (Figure 3D). In addition, although H1 was depleted to a similar extent at intergenic dips and active TSSs, Rpl118 enrichment was found to be between that of active TSSs and Z binding sites (Supplementary Figure 2), suggesting that many of them are not active TSSs. Thus, it seems likely that at least a subset of intergenic H1 dips are indeed regulatory elements.

Binding of H3.3 and H1 are negatively correlated

We observed that at binding sites for Z as well as those for E(Z) and PSC, H1 dips did not coincide with the enrichment of Rpl118. Therefore, presence of RNA polymerase or active transcription cannot generally explain H1 depletion at regulatory sites. As an alternative, we considered the histone variant H3.3 that is known to be enriched at both active

Figure 3 Intergenic H1 dips are reminiscent of regulatory elements. Alignment of FAIRE data (gold), H1 (red), Rpl18 (black), and nucleosome occupancy (grey) to genomic features as in Figure 2. (A) TSSs of the 40% most active genes as measured in the study by Pickersgill *et al* (2006); (B) Embryonic Z-binding sites (Moses *et al*, 2006); (C) Co-binding sites of E(Z) and PSC (Schwartz *et al*, 2006); (D) geometric centres of H1 dips that have no overlap with genes.

genes as well as at regulatory sites (Ahmad and Henikoff, 2002; Mito *et al*, 2007).

To compare the genomic localization of H3.3 and H1, we used a published high-resolution map of H3.3 enrichment over the main H3 isoform in S2 cells (Mito *et al*, 2007), which are known to have a similar expression pattern as Kc167 cells (Muller *et al*, 2008). For direct comparison, we re-sampled the H3.3 dataset to the same resolution as the H1 data (see Materials and methods). Strikingly, there was a strong negative correlation (Spearman's $\rho = -0.49$, $P < 2.2 \times 10^{-16}$; Supplementary Figure 3B), even though H3.3 and H1 profiles were obtained using different techniques and on different microarray platforms.

Almost all sites with low H1 signals were enriched in H3.3 (Figure 1E). In alignments for genomic features, H3.3 was observed to be enriched on average at transcriptionally active sites (Figure 2A–C). H3.3 was depleted at TEs that are lowly transcribed and bound by H1 throughout (Figure 2D). A notable exception were regulatory sites at which H3.3 is enriched, whereas H1 is depleted, although RNA polymerase is not (Figure 2E and F). Thus, although the presence or activity of RNA polymerase cannot explain the low levels of H1 at regulatory sites, H3.3 and H1 levels were observed to be inversely correlated at all the investigated classes of genomic features.

Depletion of H3.3 RNAi leads to increased H1 binding

Owing to the clear negative correlation between H3.3 and H1 binding patterns, we sought to determine whether H3.3 might have an active role in the local exclusion of H1. To test this hypothesis, we studied the genome-wide changes in H1 binding after depletion of H3.3 by RNA interference (RNAi). To simultaneously knock down both H3.3 genes that code for identical polypeptides, we treated Kc167 cells with long dsRNAs for 5 days. Total H3.3 mRNA was reduced by 54% relative to treatment with dsRNA against the non-involved *white* gene (Figure 4A, average of two experiments). We then mapped H1 by DamID again under both conditions. Although the general pattern of H1 binding was preserved, binding levels in many, but not all H1 dips increased (Figure 4B). These changes were relatively mild, possibly due to the limited degree of H3.3 knockdown, but were nevertheless reproducible. A detailed analysis indicated that the local increase in H1 binding after H3.3 knockdown was related to the initial magnitude of the H1 dip, as well as to the initial levels of H3.3 in the dip (Figure 4C). In particular, dips with relatively low initial H3.3 levels showed more prominent changes in H1 levels, suggesting that a threshold level of H3.3 may be needed for effective exclusion of H1 over the course of the experiment. Consistent with this threshold model, we observed that a more modest knockdown of

Figure 4 Depletion of H3.3 causes increased binding of H1. (A) Quantitative RT-PCR of *His3.3A* and *His3.3B* mRNA after knockdown of *white* or simultaneous knockdown of *His3.3A* and *His3.3B*. Average of cumulative mRNA concentration (*His3.3A* + *His3.3B*) after knockdown was 46%. (B) Examples of H1 levels at H1 dips on H3.3 knockdown. DamID binding values after knockdown of *white* (black) and H3.3 (red). Black and white boxes denote genes on top and bottom strand, respectively, with introns depicted as lines. (C) Scatter plots for H1 change ($\log_2$ ratio of DamID values of H3.3 knockdown over *white* knockdown) versus H1 binding after *white* knockdown for all microarray probes. Colours are according to H3.3 enrichment ($\log_2$ of H3.3 ChIP over H3.1 ChIP (Mito *et al*, 2007)) re-sampled to the same resolution with blue ≤ -1 and yellow ≥ 1.5 . Blue and yellow lines are regression lines of the points associated with the lowest 20% and highest 20% H3 enrichment, respectively. (D–F) Alignment of H1 DamID values after *white* knockdown (black) and H3.3 knockdown (red) as in Figure 2. (D) TSSs of the 40% most active genes; (E) geometric centres of intergenic H1 dips; (F) 5' ends of transposable elements. Asterisks indicate statistical significance of difference in window ± 1 kb ($P < 4.4 \times 10^{-22}$, Mann-Whitney *U*-test). (G) Western blots for whole-cell extracts after knockdown. (H) Western blots for supernatants (SN) and pellets (PEL) after sequential extraction using 80 mM and 600 mM salt containing buffers. One-fortieth of the sample was loaded in each lane. — indicates whole cell lysate of untreated cells.

H3.3 specifically affected the group of dips with low initial H3.3 levels, but not dips with high initial H3.3 levels (Supplementary Figure 4A and D). The differences between these two groups were also observed when replicate experiments were analysed separately.

Despite the variation in the effects on H1 binding between individual dips, depletion of H3.3 by roughly 50% caused a significant overall change in H1 levels in dips located at TSSs as well as in intergenic regions (both $P < 4.4 \times 10^{-16}$, Mann-Whitney *U*-test; Figure 4D,E). In comparison, no change could be observed at TEs, at which H3.3 is not enriched (Figure 4F).

These comparisons rely on the assumption that overall H1 levels in the cells did not change globally during H3.3 knockdown. Indeed, cellular levels of H1 and H3 did not change during treatment as tested by western blotting (Figure 4G). To investigate whether the fraction of H1, that was associated with chromatin, might have decreased, we sequentially extracted nuclei of treated cells with buffers containing increasing salt concentrations. After initial incubation with 15 mM salt buffer, no H1 could be detected in supernatants containing 80 mM salt, whereas comparable amounts of H1 were released with 600 mM salt in both H3.3 knockdown and control cells (Figure 4H).

Reduced H3.3 levels might impact transcription, and thereby lead to a gain of H1 at previously active TSSs as a secondary effect. To test whether that was the case, we generated mRNA expression profiles from *white* and H3.3 RNAi-treated cells. Transcript changes were not correlated with H1 changes per gene (Spearman's $\rho = 0.02$, Supplementary Figure 4C), indicating that altered transcription could not have been responsible for H1 changes on H3.3 RNAi. Transcriptional changes were not specific for genes enriched in any functional annotation. However, they were related to the H3.3 knockdown, because the aforementioned weaker knockdown yielded similar transcriptional changes (Supplementary Figure 4B). In summary, we conclude that reduction in H3.3 levels leads to increased association of H1 at sites at which H1 was previously depleted, in a manner that is mostly independent of the local activity of transcription.

Increased nucleosome repeat length after H3.3 RNAi

A well-established property of H1 is the linear relationship between the H1:nucleosome ratio and nucleosome repeat length (NRL) (Woodcock *et al*, 2006; Siriaco *et al*, 2009). We reasoned that if H1 binding to chromatin is increased in cells with reduced H3.3, we should be able to observe an increase in NRL. Towards this end, we isolated nuclei from Kc167 cells treated with H3.3 or *white* dsRNA as before, and digested the chromatin using increasing amounts of micrococcal nuclease (MNase). In mild digestions yielding nucleosome ladders, we observed a small increase in NRL in H3.3 versus *white* knockdown cells in four out of four experiments (Supplementary Figure 5A; see Supplementary Figure 5B for quantification). It should be noted that with the partial H3.3 knockdown, a strong change in NRL could not be expected given the fact that even almost complete reduction of H1 causes only a 14-bp difference in NRL (Siriaco *et al*, 2009).

Figure 5 Depletion of H3.3 leads to increased nucleosome repeat length. Mononucleosomal DNA from RNAi-treated cells (Supplementary Figure 5C) was hybridized to tiling microarrays together with *in vitro*-digested control DNA. Signals were subjected to autocorrelation analysis (see Materials and methods and Supplementary Figure 6) to yield average nucleosome spacing. Probes in HP1-bound regions (de Wit *et al*, 2007) have been excluded because nucleosome spacing is different in these regions. Dotted lines at multiples of the estimated nucleosome repeat length (NRL) indicate positions of peaks and valleys corresponding to nucleosomes and linker DNA in *white* (black) and H3.3 (grey) knockdown cells.

To verify this increase using a more sensitive method, we hybridized DNA from more complete digestions yielding mostly mononucleosomes to high-density microarrays as previously for wild-type cells (Supplementary Figure 5C). As a sensitive tool to detect patterns in nucleosome spacing, we calculated autocorrelations for each treatment and subtracted baseline fluctuations that are derived from regional differences in accessibility to MNase (Supplementary Figure 6 and Materials and methods). The resulting normalized autocorrelation plots are depicted in Figure 5. Although NRL in control-treated cells closely resembled wild-type cells at about 189 bp, it increased to 193 bp in H3.3 RNAi-treated cells. These results are consistent with the enhanced binding of H1 after H3.3 depletion, and suggest that H3.3 affects nucleosome packaging by modulating H1 levels. We expected that the changes in nucleosome spacing would be most prominent near the H1 dips. Unfortunately, we were unable to confirm this, because the autocorrelation analysis lost its power to resolve periodic signals when applied to short data segments and much fewer data points. It is thus possible that the effect of H3.3 on nucleosome spacing is not restricted to H1 dip regions, but is rather more global. This could be the case if H3.3 is present in low but non-zero amounts along the entire genome.

Discussion

H1 distribution along the genome

We present a detailed analysis of the genome-wide distribution of linker histone H1 in *Drosophila*, which we obtained using the DamID method. A characteristic feature of the H1 pattern its pervasive binding interrupted by thousands of local dips that mostly coincide with TSSs of active genes and putative regulatory elements. This is remarkably similar to the pattern of H1 as detected by ChIP in human cells (Krishnakumar *et al*, 2008), suggesting the evolutionarily conserved behaviour of H1 proteins, and at the same time providing cross-validation between the fundamentally different DamID and ChIP methods.

Contrary to our expectations, we could not find general differences in H1 binding between regions classically denoted as heterochromatin and euchromatin, even though chromatin in these regions is thought to form different structures (Sun *et al*, 2001). Instead, we found that the H3.3 protein, or functional properties inherent to it, locally restricts H1 association. This result is supported by the fact that H3.3 depletion leads to increased H1 binding at sites with previously low H1. NRL increases concomitantly, in agreement with the known linear relationship of NRL to the cellular concentration of H1 (Woodcock *et al*, 2006; Lu *et al*, 2009). The data provided by our MNase microarrays did not provide the resolving power required to discern whether the NRL changes occur specifically in H1 dips, as might be expected, or globally. We conclude that H3.3 contributes to a mechanism whereby active genomic sites, both genes and regulatory elements, are maintained in an H1-free state, and thus remain readily accessible to regulatory factors.

Our results refine the long-standing paradigm that H1 is depleted from actively transcribed genomic regions that are visible as interbands on polytene chromosomes (Jamrich *et al*, 1977). Mutually exclusive binding of H1 with different RNA polymerases does not encompass whole genomic regions, but happens at the scale of transcription units.

At many H1 dips, but also along the bodies of active genes, H1 signals are intermediate. This may be interpreted as partial occupancy in both time and space. At such sites, H1 may be bound at only a fraction of the nucleosomes, or alternatively it may only be bound sometimes. Furthermore, the fact that loci with high or low initial H3.3 react differently after H3.3 RNAi should be interpreted in that way. In a fraction of cells within the population, sites with low exchange rates (and thus low H3.3 levels) may not be able to acquire H3.3 at all in between two rounds of replication, leading to increased H1 binding.

Relationships between H1, transcription and H3.3

Our data show that H1 binding is counteracted by H3.3. Replication-independent (RI) incorporation of H3.3 has been shown to occur in the wake of transcription (Schwartz and Ahmad, 2005). Probably, the process of local chromatin disruption, caused by passing RNA polymerase or by the action of remodelling enzymes, and subsequent RI chromatin assembly may expel H1, leaving dips. When H3.3 levels are reduced, other H3 isoforms may to some extent be used for RI chromatin assembly, leading to increased H1 binding. Technically, it is difficult to assess the magnitude of the effect of the RI chromatin reassembly process relative to the effect of H3.3 itself, as this would require experimental manipulation of transcription without affecting H3.3 incorporation.

We have not addressed whether the presence of H1 also has repercussions for H3.3 incorporation, or indeed for histone turnover and the processes that cause it. *In vitro*, H1 is a strong suppressor of nucleosome remodelling and transcription. We could not achieve significant knockdown of H1 (data not shown). In our H3.3 RNAi experiments, the observed transcriptional changes were not correlated with H1 changes, suggesting that H1 does not influence transcription in a simple, direct manner *in vivo*. This idea is in agreement with the fact that in murine cells that have only half the normal H1, transcriptional changes are limited to specific sets of genes (Fan *et al*, 2005). In addition, drastic reduction of H1 is not lethal in flies, which might be expected if transcription was globally affected (Lu *et al*, 2009).

H3.3 has been implicated in maintaining tissue-specific transcription patterns in somatic cloning experiments (Ng and Gurdon, 2008). We therefore speculate that a possible mechanism for active site maintenance involving H1 may be required only during differentiation.

Possible mechanisms of H1 exclusion by H3.3

The restriction that H3.3 exerts on H1 binding may occur by several possible mechanisms. First, amino acids and post-translational modifications that distinguish H3.3 from H3 may regulate H1 binding to the nucleosome. Although the two H3 isoforms differ in only four amino acids, one of them (Ser 31 of H3.3, corresponding to alanine in H3) is located in the flexible N-terminal tail that is thought to exit the nucleosome near the dyad, and could potentially be in contact with bound H1 (Davey *et al*, 2002). Furthermore, the two isoforms are known to carry distinct post-translational modifications before and after they are assembled into nucleosomes (McKittrick *et al*, 2004; Loyola *et al*, 2006). Different modifications that exist in non-nucleosomal mammalian H3 and H3.3 have been shown to impact their later modification states (Loyola *et al*, 2006). In such a way, different sets of

modifications could permanently differentiate the two H3 isoforms.

A second possibility is that nucleosome stability determines H1 binding. Jin and Felsenfeld (2007) showed that nucleosomes with H3.3 and especially with both H3.3 and the H2A replacement histone, H2A.Z, are biophysically less stable. A greater propensity for disruption of these nucleosomes may result in unstable H1 docking sites and thus in decreased effective affinity of H3.3-containing nucleosomes for H1.

Third, H3.3 may promote binding of other proteins that are known to affect H1 localization. ISWI, the ATPase subunit of the CHRAC and ACF chromatin remodelling complexes that can remodel and assemble H1-containing chromatin *in vitro*, respectively, is required for the association of H1 to polytene chromosomes (Siriaco *et al*, 2009). In a human cancer cell line, in which H1 is absent from a subset of TSSs that are occupied by PARP, it has been demonstrated that PARP depletion can cause some genes to be downregulated and acquire H1 (Krishnakumar *et al*, 2008). PARP has also been found to directly regulate ISWI, suggesting that ISWI may globally promote H1 binding except where it is specifically inhibited (Sala *et al*, 2008). It remains to be investigated whether PARP and H3.3 each independently contribute to the local exclusion of H1, or are part of the same mechanism.

H1 and heterochromatin

In a recent publication, Lu *et al* (2009) have shown that flies with strongly diminished H1 levels have heterochromatin defects: trimethylation at H3K9 is strongly reduced in larval cells and HP1 does not localize to the chromocentre, indicating that H1 is necessary for heterochromatin establishment or maintenance. Nonetheless in Kc cells we did not observe a difference in H1 baseline levels between regions that are bound or not bound by HP1. It follows that although H1 is necessary, it cannot be sufficient for targeting of H3K9 methylation and HP1 to heterochromatin. Possibly distinct modifications or accessory factors of H1 exist in these regions. Alternatively, folding of chromatin into higher-order structures may have a role in heterochromatin definition, and higher-order structures may require long uninterrupted nucleosome arrays. Genes and associated H1 dips are scarcer in pericentric heterochromatin, and it may be this fact that allows higher-order structures to form.

In this study, we show that local inhibition of *Drosophila* H1 association with the genome by the histone variant H3.3 is a means of modulating H1 binding at many places in the genome. This is the first case of regulation of linker histone binding through a histone variant. Further studies will be required to determine the molecular mechanism of this regulation, and to elucidate functional consequences of this negative interaction.

Materials and methods

Constructs

RpII18–Dam and control Dam-only constructs have been described previously (Moorman *et al*, 2006). To obtain pN-GW-DamMyc-H1, the *His1* open reading frame was amplified from genomic DNA from Kc167 cells, inserted into pENTR/D-TOPO (Invitrogen) and recombined with a variant of pNDamMyc that had been made compatible by the insertion of a GATEWAY recombination cassette (Invitrogen).

Cell culture and DamID

Kc167 cells were cultured and transiently transfected with DamID vectors as described by Moorman *et al* (2006). RNAi experiments were performed using dsRNAs directed against *white*, *His3.3B*, or *His3.3A* and *His3.3B* that were *in vitro* transcribed using the RiboMax kit (Promega) from PCR amplicons as published (*white*) (Greil *et al*, 2003), or designed by the Harvard *Drosophila* RNAi Screening Centre (www.flyrnai.org; *His3.3A* with cross-reaction to *His3.3B*, DRSC03343; *His3.3B*, DRSC28380). A total of 5×10^6 cells were seeded on day 1 with 150–200 µg dsRNA in 5 ml serum-free BPYE. After 1 h, 5 ml BPYE with 10% serum was added. The procedure was repeated on day 3. Cells were transfected with DamID constructs on day 5 and grown for another 24 h in BPYE supplemented with dsRNA. Cells were collected 24 h after transfection. *In vivo* methylated DNA was amplified as described earlier (Moorman *et al*, 2006) and hybridized to microarrays carrying 380 000 60-mer DNA oligos (Choksi *et al*, 2006) (Roche-NimbleGen). For each protein and RNAi treatment, material from two independent experiments were hybridized in opposite dye orientations over Dam controls.

Expression analysis

Total RNA was extracted with TRIzol (Invitrogen) and treated with DNaseI. For H3.3 knockdown control, RNA was reverse transcribed and analysed by TaqMan qPCR using an amplicon for *Fmo-2* for normalization. See Supplementary Table 1 for primer and probe sequences. Genomic transcription profiles were generated using INDAC oligo arrays version (<http://www.indac.net>) printed at the NKI Central Microarray Facility, with each oligonucleotide spotted twice. RNA from the same H3.3 RNAi- and *white* RNAi-treated cell cultures that were also used for H1 DamID was co-hybridized and two replicates were made for each condition. The Rosetta error model was applied to assign statistical significance (Weng *et al*, 2006).

H1 antibodies

Polyclonal rabbit anti-H1 antiserum was raised against a mixture of synthetic peptides EP062866 (NH₂-CAGTKAKKASATPSHP-CONH₂) and EP062867 (NH₂-CATAKKPKAKTTAAKK-CONH₂) and was affinity purified against peptide EP062866 only (Eurogentec). Specificity for H1 was tested in peptide dot blots, western blots, and immunofluorescence microscopy (Supplementary Figure 1A and not shown).

Chromatin immunoprecipitation (ChIP)

About 2.4×10^8 growing cells were cross-linked and treated for ChIP according to protocol PROT03 on the Epigenome Network of Excellence website (www.epigenome-noe.net). Chromatin was fragmented to 300–600 bp in a Bioruptor (Diagenode) and immunoprecipitated with affinity-purified H1 antiserum. Relative DNA concentrations were measured by TaqMan qPCR (see Supplementary Table 1 for primer and probe sequences) and represented as log₂ ratios over ChIP input.

Salt extraction of chromatin

Nuclei were isolated from untreated or RNAi-treated Kc cells as follows: all steps were performed on ice. Cells were washed thrice with PBS and pelleted thrice in lysis buffer (10 mM Tris-HCl (pH 8.0), 0.4% Triton X-100, 0.5 mM DTT, 15 mM NaCl, 4 mM MgCl₂, 2 mM CaCl₂, Complete protease inhibitors (Roche)). Nuclei were sequentially extracted with 80 mM buffer and 600 mM buffer as described by Henikoff *et al* (2009). One-fortieth of spin pre-cleared supernatants and pellets were used for western blots using anti-H1 and anti-H3 (Abcam ab1791) antibodies.

Formaldehyde-assisted isolation of regulatory elements (FAIRE)

The FAIRE protocol for human cells (Giresi *et al*, 2007) was adapted for Kc cells as follows: cells grown in suspension were fixed by the addition of formaldehyde at a final concentration of 2%. After incubating for 15 min at room temperature with agitation, glycine was added to a final concentration of 125 mM, and samples were incubated with tumbling for 5 min at room temperature. Cells were pelleted and washed twice with ice-cold PBS. Each pellet (0.1–0.15 g of cells) was re-suspended in 3 ml of buffer L1 (50 mM HEPES (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 5% NP-40, and 0.25% Triton X-100) and incubated for 10 min on ice

with occasional inversion to mix. Cells were again pelleted, re-suspended in 3 ml of buffer L2 (10 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA), and incubated for 10 min at room temperature with agitation. Cells were pelleted and re-suspended in 3.9 ml of buffer L3 (10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na deoxycholate, 5 mg/ml N-lauroyl sarcosine, with Complete protease inhibitors (Roche)) and sonicated using the Bioruptor to yield mainly fragments smaller than 500 bp. DNA was isolated by two extractions in buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated, RNA was removed with RNase A, and DNA was purified using the MinElute kit (Qiagen). Purified DNA was labelled and hybridized to microarrays as for DamID. Control material in these hybridizations was genomic DNA from cells that had not been cross-linked.

Nucleosome spacing analysis

Nuclei from 13×10^6 cells were isolated as described above and were suspended in 200 µl MNase buffer (15 mM Tris-HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 2 mM CaCl₂, 0.5 mM DTT, and 20% glycerol) and 150 U MNase (Worthington) was added at room temperature followed by 20-min incubation to yield predominantly mononucleosomes. Reactions were stopped by the addition of 1 µl 0.5 M EDTA and 5 µl 20% SDS. Chromatin was digested using RNaseA and proteinase K, extracted with phenol, and precipitated with ethanol. Digestion was checked by running on 1.2% agarose gels. DNA was labelled according to the manufacturer's protocol and hybridized to custom microarrays featuring 380 000 probes clustered in 18 regions with a median 10-bp spacing. These regions were chosen to represent diverse parts of the genome in terms of chromosomal location and gene density. Genomic DNA from Kc cells digested *in vitro* with MNase to a size of 100–1000 bp served as control material.

Analysis of microarray experiments

Normalization and analyses of DamID, FAIRE, and nucleosome position microarrays were done using custom R scripts. Raw data from each array were loess normalized, median centred, and dye-swap arrays averaged. H1 dips were defined as follows: after applying a running median over windows of 5 probes, dips were defined as regions of, at least, 600 bp length with log₂ DamID ratios below –1, whereby at most one consecutive position above –1 was permitted. This set of parameters yielded an FDR ≈ 0.087. Downstream analyses were robust with respect to parameter settings. Pc domains were taken from Tolhuis *et al* (2006) and transposed to FlyBase release 4. The definition of heterochromatin was taken from FlyBase release 5 and transposed to release 4. To calculate baseline H1 binding and H1 dip/gene frequencies, all probes outside dips or H1 dips/genes that overlapped with design regions of microarrays used for HP1 and Pc data were considered.

H3.3 and H3 maps resulting from the study by Mito *et al* (2007) were taken from GEO dataset GSE4091. Only reporters in the regions with 50-bp spacing were used. To calculate correlation with DamID data, H3.3/H3 data were re-sampled to the resolution of the microarrays used for DamID by averaging values for reporters falling into the space of one reporter of the DamID array ± half the median reporter distance with linear distance weights.

Normalized autocorrelations of nucleosome positions were calculated from normalized log₂ signals by subtracting background autocorrelation derived from accessibility differences on larger scales from ordinary autocorrelations. This background was calculated as the correlation of each probe with a 19-probe (180 bp) window as described in Supplementary Figure 5. Periodicities were estimated from the resulting graphs.

Microarray platform specifications, raw and normalized datasets have been deposited in the Gene Expression Omnibus, under the accession number GSE16885.

Alignment plots

Custom Perl scripts were used to align probes to indicated genomic features. For direction features (genes, TEs), probes upstream, downstream, or within a feature until the next feature were assigned to each feature. Probes that could be assigned to more than one feature were recycled. For nested features, probes within one feature but outside another feature were assigned to the former. For undirected features (binding sites, tRNA centres), probes were assigned to the closest feature. In the case in which datasets from diverse platforms are shown in the same panel, alignment was

performed separately for each dataset in the native resolution to all genomic features overlapping with data available for this dataset. Active and inactive genes were defined as the bottom and top 40% active genes as measured in the study by Pickersgill *et al* (2006).

Supplementary data

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

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Author contributions: UB performed experiments and analysed the data. GH performed FAIRE experiments. LP designed the nucleosome positioning microarray and developed the H1 dip finding algorithm. BvS and UB conceived of the study, designed the experiments, and wrote the paper. All authors read and approved the final paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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