Reading the maps: Organization and function of chromatin types in Drosophila
Braunschweig, U.

Citation for published version (APA):
Amsterdam: Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis

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Chapter 1

Histone H1 binding is inhibited by histone variant H3.3

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EMBO Journal 2009 Dec 2;28(23):3635-45

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Introduction

Nuclear DNA in eukaryotic cells is packaged in a complex fiber called chromatin. Chromatin plays 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; Wolffte, 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). Aggregation of nucleosome arrays into 30 nm fibers 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 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, i.e., the spacing of nucleosomes is reduced (Lu et al., 2009; Woodcock et al., 2006). Taken together, these data provide compelling evidence that H1 is a major modulator of chromatin structure.

Linker histones must fulfill important regulatory functions in multicellular organisms. Mice which lack three out of six somatic H1 subtypes die in midgestation, and fruit flies with low amounts of H1 have reduced viability and defects in pericentric heterochromatin (Fan et al., 2005; Lu et al., 2009; Siriaco 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 depletion of H1 suggests that H1 may bind to only a 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 know that H1 associates primarily with transcriptionally inactive regions of the genome (Jamrich et al., 1977; Chiu et al., 1977). Drosophila H1 binds to DNA-dense bands on polytene chromosomes in a manner that is dependent
on the ATP-dependent remodeling enzyme ISWI (Corona et al., 2007; Siriaco et al., 2009). In a human breast cancer cell line, H1 has been found by chromatin immunoprecipitation to be depleted from a set of active promoters where poly(ADP-ribose) polymerase-1 (PARP) is enriched (Krishnakumar et al., 2008), but it remains 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 system, because this organism has only one isoform of H1. We generated whole genome, high resolution maps of H1 in the Kc167 cell line using DNA adenine methyltransferase identification (DamID) (Greil 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 cohybridized with Dam-only control material to NimbleGen oligonucleotide microarrays with 300 bp median resolution covering the entire nonrepetitive 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 (Fig. 1A,B,E).

Because of 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, which are called dips hereafter. To verify our DamID results, we performed chromatin immunoprecipitation (ChIP) with an affinity purified H1 antiserum for selected loci. DamID and ChIP measurements were in good agreement with each other (Supplementary Fig. 1A,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 = 7.8x10^{-47}; Fig. 1A,B,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 (Fig. 1A,C). This also holds for regions known to coincide with large Polycomb (Pc) domains (Tolhuis et al., 2006) (Fig. 1B,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 (Fig. 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 in euchromatin (Tolhuis et al., 2006; Schwartz 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 due to 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 the 40% 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 centered over the transcription start site (TSS) (Fig. 2A,B and Supplementary Fig. 2A-D).

In order to investigate the relationship between H1 occupancy and transcription at a higher resolution, we measured occupancy of RNA polymerase by DamID of RpII18, the 18 kDa subunit that is common to the three nuclear RNA polymerases. RpII18 occupancy was in good agreement with mRNA levels (Spearman’s $\rho = 0.66$ after averaging the DamID signal per gene). Indeed, RpII18 signals were strongly anticorrelated with H1 (Spearman’s $\rho = 0.58$, $p < 2.2 \times 10^{-16}$; Fig. 1E and Supplementary Fig. 3A). The group of active genes, but not the inactive genes, exhibited high average RpII18 binding with a pronounced enrichment at the TSS (Fig. 2A and Supplementary Fig. 2C). This relationship was retained when the analysis was restricted to tRNA genes (Fig. 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; Mavrich et al., 2008; Barski et al., 2007). To investigate whether the observed H1 dips were simply attributable to NFRs, we generated a map of 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 methods). While the group of active genes showed a well defined NFR, inactive genes did not, as anticipated (Supplementary Fig. 2A,C). The first nucleosome upstream and downstream from the TSS were spaced about 350 bp from each other, a similar value to what was found in *Drosophila* embryos (Mavrich et al., 2008). This is likely too small to be the cause for H1 dips at TSSs (Fig. 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 (Fig. 2A,B, Supplementary Fig. 2). Transposable elements (TEs) are an example of genomic features that are less bound by RpII18 than the genome median (Fig. 2D). In contrast, H1 levels are above median, while 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 where nucleosomes are less frequently bound by H1.
Figure 2: H3.3 enrichment is inversely correlated with H1 binding

Average binding profiles aligned to genomic features. Colored lines represent running means of 2% of the probes in the window region except where indicated otherwise. Datasets are the same as in Fig. 1.

A-B, Transcription start sites (A) and transcript 3’ ends (B) of the 40% most active genes. Expression profile taken from (Pickersgill et al., 2006). Arrow indicates the location of genes. C, Centers 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).

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 not yet annotated TSSs or cis-regulatory regions. In both cases, they would be expected to often occur close to known genes. Indeed, intergenic H1 dips were more 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 that of known TSSs or regulatory...
sites. We used formaldehyde-assisted isolation of regulatory elements (FAIRE) to test if 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; Giresi 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 (Fig. 2E,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) (Fig. 3A). Also Z and E(Z)/PSC sites were clearly enriched in FAIRE signal, suggesting that these sequences are binding sites for their cognate...
**A**

H3.3A

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H3.3B

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**B**

log2(Dam-H1/Dam)

**C**

Dependency of H1 change on H3.3

**D**

5' alignment of active genes

**E**

Alignment to centers of intergenic H1 dips

**F**

5' alignment of transposable elements

**G**

RNAi: w H3.3

| H1 | H3 | tubulin |

**H**

RNAi: width w H3.3 width w H3.3 width w H3.3

80 mM 600 mM 600 mM SN SN PEL
proteins and in an open chromatin conformation also in Kc cells (Fig. 3B,C). Intergenic H1 dips gave similar FAIRE signals as Z binding sites (Fig. 3D). Additionally, while H1 was depleted similarly at intergenic dips and active TSSs, RpII18 enrichment was between that of active TSSs and Z binding sites (Supplementary Fig. 2), suggesting that many of them are not active TSSs. Thus, it appears likely that at least a subset of the intergenic H1 dips are indeed regulatory elements.

Binding of H3.3 and H1 are negatively correlated

We observed that at binding sites of Z as well as E(Z) and PSC, H1 dips did not coincide with enrichment of RpII18. 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, which is known to be enriched at both active genes as well as 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 resampled the H3.3 dataset to the same resolution as the H1 data (see methods). Strikingly, there was a strong negative correlation (Spearman’s $\rho = -0.49$, $p < 2.2x10^{-16}$; Supplementary Fig. 3B), even though the H3.3 and H1 profiles were obtained with different techniques and on different microarray platforms. Almost all sites with low H1 signals were enriched in H3.3 (Fig. 1E). In alignments for genomic features, H3.3 was enriched on average at transcriptionally active sites (Fig. 2A-C). H3.3 was depleted at TEs, which are lowly transcribed and bound by H1 throughout (Fig. 2D). A notable exception were regulatory sites: there, H3.3 is enriched while H1 is depleted although RNA polymerase is not (Fig. 2E,F). Thus, while the presence or activity of RNA polymerase cannot explain the low levels of H1 at regulatory sites, H3.3 and H1 levels were inversely correlated at all investigated classes of genomic features.

Depletion of H3.3 RNAi leads to increased H1 binding

Because of the clear negative correlation between the H3.3 and H1 binding patterns, we wondered whether H3.3 might play 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 which 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 noninvolved white gene (Fig. 4A, average of two experiments). We then again mapped H1 by DamID in both conditions. While the general pattern of H1 binding was preserved, binding levels in many, but
not all H1 dips increased (Fig. 4B). These changes were relatively mild, possibly owing to the limited degree of H3.3 knockdown, but nevertheless reproducible. 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 (Fig. 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 H3.3 specifically affected the group of dips with low initial H3.3 levels, but not dips with high initial H3.3 levels (Supplementary Fig. 4A,D). The differences between these two groups were also observed when replicate experiments were analyzed 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 < 4.4x10⁻¹⁶, Mann-Whitney U-test; Fig. 4E,F). In comparison, no change could be observed at TEs, where H3.3 is not enriched (Fig. 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 (Fig. 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 prior 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 (Fig. 4H).

Reduced H3.3 levels might impact transcription and thereby lead to gain of H1 at formerly active TSSs as a secondary effect. To test if 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 ρ = 0.02, Supplementary Fig. 4C), indicating that altered transcription could not have been responsible for H1 changes upon H3.3 RNAi. The transcriptional changes were not specific for genes enriched in any functional annotation. However, they were connected to the H3.3 knockdown, because the aforementioned weaker knockdown yielded similar transcriptional changes (Supplementary Fig. 4B). In summary, we conclude that reduction of H3.3 leads to increased association of H1 at sites where 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 with increasing amounts of micrococcal nuclease (MNase). In mild digestions yielding nucleosome ladders, we observed a small increase in NRL in H3.3 vs. white knockdown cells in four out of four experiments (Supplementary Fig. 5A; see Supplementary Fig. 5B for quantitation). It should be noted that with the partial H3.3 knockdown, a strong change in NRL could not be expected given that even almost complete reduction of H1 itself causes only a 14-bp difference in NRL (Siriaco et al., 2009).

To verify this increase with a more sensitive method, we hybridized DNA from more complete digestions yielding mostly mononucleosomes to high density microarrays as previously for wildtype cells (Supplementary Fig. 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 Fig. 6 and Materials and Methods). The resulting normalized autocorrelation plots are depicted in Fig. 5.

While NRL in control treated cells closely resembled wildtype 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.
Figure 5: Depletion of H3.3 leads to increased nucleosome repeat length

Mononucleosomal DNA from RNAi-treated cells (Supplementary Fig. 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 Fig. 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.

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 is 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 behavior 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 heterochromatin and euchromatin, even though chromatin in these regions is thought to form different structures (Sun et al., 2001). Instead, we find 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. Nucleosome repeat length (NLR) increases concomitantly, in line with the known linear relationship of NLR to the cellular concentration of H1 (Lu et al., 2009; Woodcock et al., 2006).

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. Also 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). It is probable that the process of local chromatin disruption, caused by passing RNA polymerase or by the action of remodeling 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 process of RI chromatin reassembly 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 remodeling 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 notion is in line with the fact that in murine cells that have only half of 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 tissuespecific transcription patterns in somatic cloning experiments (Ng and Gurdon, 2008). We therefore speculate that a possible mechanism of active site maintenance involving H1 may only be required 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. Firstly, amino acids and posttranslational 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 (Ser31 of H3.3, corresponding to alanine in H3) is located in the flexible N-terminal tail which 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 posttranslational
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 decreased effective affinity of H3.3-containing nucleosomes for H1.

Thirdly, 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 remodeling complexes that can remodel and assemble H1-containing chromatin in vitro, respectively, is required for association of H1 to polytene chromosomes (Siriaco et al., 2009). In a human cancer cell line, where 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: dimethylation at H3K9 is strongly reduced in larval cells and HP1 does not localize to the chromocenter, indicating that H1 is necessary for heterochromatin establishment or maintenance.

Yet 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 play 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 work, 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.

**Acknowledgments**

We thank Marja Nieuwland, Wim Brugman and Ron Kerkhoven for microarray hybridizations; Daan Peric Hupkes and Wendy Talhout for initial MNase experiments; Steven Henikoff for helpful discussions and sharing unpublished data; Peter Becker for sharing reagents; Steven Henikoff, Kami Ahmad, Fred van Leeuwen and members of the van Steensel lab for critical reading of the manuscript. Supported by a European Young Investigator Award to BvS.

Author contributions: UB performed experiments and analyzed 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 manuscript. All authors read and approved the final manuscript.
Material and Methods

Constructs
Rpll18-Dam and control Dam-only constructs have been described previously (van Steensel and Henikoff, 2000; 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 insertion of a GATEWAY recombination cassette (Invitrogen).

Cell culture and DamID
Kc167 cells were cultured and transiently transfected with DamID vectors as described (Moorman et al., 2006). RNA interference experiments were performed with dsRNAs directed against white, His3.3B, or His3.3A and His3.3B that were in vitro transcribed with the RiboMax kit (Promega) from PCR amplicons as published (white) (Greil et al., 2003), or designed by the Harvard Drosophila RNAi Screening Center www.flyrnai.org; His3.3A with crossreaction to His3.3B, DRSC03343; His3.3B, DRSC28380). 5x10^6 cells were seeded on day 1 with 150-200 μg dsRNA in 5 ml serum-free BPYE. After one hour, 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 24h in BPYE supplemented with dsRNA. Cells were harvested 24 h after transfection. In vivo methylated DNA was amplified as described (Moorman et al., 2006) and hybridized to microarrays carrying 380,000 60-mer DNA oligos (Choksi et al., 2006) (Roche-NimbleGen Inc.). 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 TRlzol (Invitrogen) and treated with DNasel. For H3.3 knockdown control, RNA was reverse transcribed and analyzed 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 cohybridized and two replicates were done 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 the synthetic peptides EP062866 (NH2-CAGTKAKKASATPSHP-CONH2) and EP062867 (NH2-CATAKKPKAKTTAAKK-CONH2) and was affinity purified against only peptide EP062866 (Eurogentec). Specificity for H1 was tested in peptide dot blots, Western blots, and immunofluorescence microscopy (Supplementary Fig. 1A and not shown).

Chromatin immunoprecipitation (ChIP)
About 2.4x10^6 growing cells were crosslinked 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 log_2 ratios over ChIP input.

Salt extraction of chromatin
Nuclei were isolated from untreated or RNAi-treated Kc cells as follows: All steps were done on ice. Cells were washed 3 times with PBS and pelleted 3 times 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 (Henikoff et al., 2009). 1/40th of spin pre-cleared supernatants and pellets were used for Western blots with H1 and 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 addition of formaldehyde to 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 (0.1-0.15g of cells) was resuspended in 3 ml of buffer L1 (50 mM Hepes pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 5% NP-40, 0.25% Triton X-100) and incubated 10 min. on ice with occasional inverting to mix. Cells were pelleted, resuspended in 3 ml of buffer L2 (10 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA), and incubated 10 min. at room temperature with agitation. Cells were pelleted and resuspended 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 Qiagen’s MinElute kit. Purified DNA was labeled and hybridized to microarrays as for DamID. Control material in these hybridizations was genomic DNA from cells that had not been crosslinked.

Nucleosome spacing analysis

Nuclei from 13x10^6 cells were isolated as described above and were taken up 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, 20% glycerol) and 150 units Micrococcal nuclease (Worthington) was added at room temperature followed by 20 min. incubation to yield predominantly mononucleosomes. Reactions were stopped by addition of 1 μl 0.5 M EDTA and 5 μl 20% SDS. Chromatin was digested with RNaseA and Proteinase K, extracted with phenol and precipitated with ethanol. Digestion was checked by running on 1.2% agarose gels. DNA was labeled according to NimbleGen’s protocol and hybridized to custom microarrays as for DamID. Control material in these hybridizations was genomic DNA from cells that had not been crosslinked.
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 centers), probes were assigned to the closest feature. Where datasets from diverse platforms are shown in the same panel, alignment was done separately for each dataset in the native resolution to all the 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 (Pickersgill et al., 2006).

Supplementary information is available at The EMBO Journal Online.


Supplementary Figure 1: H1 chromatin immunoprecipitation (ChIP) and antibody

A, Comparison of H1 DamID and ChIP at selected genes. ChIP values are relative enrichment in TaqMan PCR; DamID values represent mean of microarray probes within 250 bp of the TaqMan amplicons. For both, lace has been set to 1 and values are the mean of two experiments.

B, H1 antibody quality control. Left, Western blots with crude polyclonal antibody for H1 fraction from bovine pancreas, Kc cells transiently expressing Dam-myc and Kc cells transiently expressing Dam-myc-H1 from DamID vectors after induction by heat shock. Right, dot blots with crude H1 serum, affinity purified H1 serum, and flow-through of affinity purification against two H1 peptides. For ChIP and Western blots shown in fig. 4, affinity purified serum was used.
Supplementary Figure 2: Alignment of genomic features

Binding profiles of chromatin proteins at genomic features as shown in fig.2 of the main text. A and B, TSSs and 3’ ends of the 40% most active genes. C and D, TSS and 3’ ends of the 40% most inactive genes. E, centers of intergenic H1 dips. F, Zeste (Z) binding sites. G, binding sites of both Enhancer of Zeste (E(Z)) and Posterior Sex Combs (PSC).
Supplementary Figure 3: Correlation of H1 with Rpl118 and H3.3

A, Scatterplot of H1 and Rpl118; B, scatterplot of H1 and H3.3 ChIP in S2 cells (Mito et al., 2007) resampled to the resolution of DamID microarrays. Spearman’s $\rho$ and associated p-values are indicated.

Supplementary figure 4: H3.3 knockdown

A, Quantitative RT-PCR of His3.3A and His3.3B mRNA levels after knockdown of white ($w$), His3.3B alone (H3.3B RNAi) and simultaneous knockdown of His3.3A and His3.3B (H3.3A+B). Values are represented as relative concentration with respect to cumulative concentration of His3.3A and His3.3B after white knockdown. B, Expression changes after knockdown of His3.3B or His3.3A and His3.3B represented as log$_2$ change in each knockdown over white knockdown. Colored dots are genes changing significantly with p < 0.01. C, Expression changes (log$_2$ change in each knockdown over white knockdown) and H1 changes in knockdowns of His3.3B or His3.3A and His3.3B. Average H1 change for each gene is represented as log$_2$ of H1 in His3.3 knockdown over H1 in white knockdown. D, Scatter plots of H1 change (log$_2$ ratios of DamID values after H3.3 knockdown and white knockdown) vs. H1 binding after white knockdown for all microarray probes. Details as in fig. 4E.
Supplementary Material

A  Relative H3.3 mRNA

B  Expression change

C  H3.3B RNAi

D  H3.3B RNAi

H3.3 highest 20% 
(R² = 0.02)  
H3.3 lowest 20% 
(R² = 0.54)

H3.3 highest 20% 
(R² = 0.45)  
H3.3 lowest 20% 
(R² = 0.55)
**Supplementary figure 5: MNase digestion of chromatin after depletion of H3.3**

A. Quantification of the agarose gel in fig. 5A. Lanes corresponding to MNase digested material from *white* and H3.3 RNAi treated cells were analyzed with ImageJ software using the ‘Plot Profile’ analysis tool. Black triangles indicate peaks corresponding to tetraneucleosomes. B. DNA from MNase digestions of untreated (−), *white* knockdown (w) or H3.3 knockdown (H3.3) cells that was used to generate microarray profiles analyzed in fig. 5B. MNase digested purified genomic DNA (gDNA) served as control material in hybridizations.
Supplementary figure 6: Details of normalized autocorrelation analysis of nucleosome occupancy

Microarray signals from hybridizations of MNase digestions over in vitro digested genomic DNA were processed as follows: Per cluster of probe regions on the array, autocorrelation was calculated for lags between 0 and 1500 bp in steps of 10 bp (correlation of each probe with the probe shifted to the “right” by the indicated lag, top panel). Probes that were not in the 10 bp register were omitted. To calculate background autocorrelation that is not derived from nucleosome positions but from regional differences in MNase sensitivity, autocorrelations were calculated as above, but for each probe not with one probe, but a window of probes whose center is shifted by lag (middle panel). Probe-window combinations with windows that extended beyond the cluster of probes and thus overlapping with fewer probes were omitted. Normalization was done by subtracting background from single-probe autocorrelations, facilitating the determination of peak positions (bottom panel). For all panels, values calculated for each cluster of probes were added per lag and divided by the number of contributing clusters.