A unique histone 3 lysine 14 chromatin signature underlies tissue-specific gene regulation

Graphical Abstract

Authors

Isabel Regadas, Olle Dahlberg, Roshan Vaid, ..., Sebastian Deindl, Jiayu Wen, Mattias Mannervik

Correspondence

jiayu.wen@anu.edu.au (J.W.),
mattias.mannervik@su.se (M.M.)

In Brief

Regadas et al. report a chromatin state consisting of H3K14ac but lacking H3K9ac, H3K27ac, and H3K4me3. Using histone replacement technology in *Drosophila*, they found that this state is required for tissue-specific gene expression, larval development, and wing patterning. The SWI/SNF protein Brahma recognizes H3K14ac and maintains chromatin accessibility.

Highlights

- Histone 3 lysine 14 is essential and required for developmental patterning
- H3K14ac decorates a set of tissue-specific genes that lack canonical histone marks
- H3K14 is necessary for expression of genes marked uniquely by H3K14 acetylation
- H3K14ac is recognized by the Brahma bromodomain

Article

A unique histone 3 lysine 14 chromatin signature underlies tissue-specific gene regulation

Isabel Regadas,¹ Olle Dahlberg,^{1,4} Roshan Vaid,^{1,4} Oanh Ho,² Sergey Belikov,¹ Gunjan Dixit,³ Sebastian Deindl,² Jiayu Wen,^{3,*} and Mattias Mannervik^{1,5,*}

¹Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, 10691 Stockholm, Sweden

²Department of Cell and Molecular Biology, Science for Life Laboratory, Uppsala University, 75237, Uppsala, Sweden

³Department of Genome Sciences, The John Curtin School of Medical Research, Australian National University, Canberra, ACT 2600, Australia

⁴These authors contributed equally

⁵Lead contact

*Correspondence: jiayu.wen@anu.edu.au (J.W.), mattias.mannervik@su.se (M.M.)

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SUMMARY

Organismal development and cell differentiation critically depend on chromatin state transitions. However, certain developmentally regulated genes lack histone 3 lysine 9 and 27 acetylation (H3K9ac and H3K27ac, respectively) and histone 3 lysine 4 (H3K4) methylation, histone modifications common to most active genes. Here we describe a chromatin state featuring unique histone 3 lysine 14 acetylation (H3K14ac) peaks in key tissue-specific genes in *Drosophila* and human cells. Replacing H3K14 in *Drosophila* demonstrates that H3K14 is essential for expression of genes devoid of canonical histone modifications in the embryonic gut and larval wing imaginal disc, causing lethality and defective wing patterning. We find that the SWI/SNF protein Brahma (Brm) recognizes H3K14ac, that *brm* acts in the same genetic pathway as *H3K14R*, and that chromatin accessibility at H3K14ac-unique genes is decreased in *H3K14R* mutants. Our results show that acetylation of a single lysine is essential at genes devoid of canonical histone marks and uncover an important requirement for H3K14 in tissue-specific gene regulation.

INTRODUCTION

Cellular differentiation and the response to environmental cues require altered gene expression and are associated with changes in chromatin state (Allis and Jenuwein, 2016; Atlasi and Stunnenberg, 2017). Various histone post-translational modifications correlate with gene activity. For example, histone 3 lysine 27 trimethylation (H3K27me3) is found at Polycomb group (PcG)-repressed genes, whereas histone 3 lysine 4 trimethylation (H3K4me3) is often present at the promoters of transcriptionally active genes (Black et al., 2012). Although histone methylation can decorate active or inactive genes, histone acetylation is invariably associated with active transcription (Verdin and Ott, 2015). Histone 3 lysine 27 acetylation (H3K27ac) is a hallmark of active enhancers, and histone 3 lysine 9 acetylation (H3K9ac) can be found at active promoters. However, some developmental genes lack these canonical histone marks but are nonetheless transcriptionally active (Pérez-Lluch et al., 2015). Whether these developmental genes bear another histone mark or whether they are completely devoid of histone modifications altogether is not known. Here we investigate the role of histone 3 lysine 14 acetylation (H3K14ac) in developmental gene expression and find that a group of tissue-specific genes that lack canonical histone marks are decorated uniquely with this histone modification.

The biological functions of individual histone modifications are difficult to decipher in multicellular organisms because histones are typically encoded by multi-copy genes dispersed over several chromosomes. The functions of individual histone marks have therefore mainly been inferred indirectly from phenotypes produced by mutations in histone-modifying enzymes. Because these enzymes also have non-histone substrates, such phenotypes may not be related to the histone mark. Therefore, the direct biological roles of histone modifications in metazoans are largely unknown. In *Drosophila melanogaster*, however, the replication-dependent histone genes are clustered together in one single chromosomal locus, the histone complex (HisC). It consists of tandem arrays of ~100 histone gene units (His-GUs), each encoding the histone proteins H2A, H2B, H3, and H4 and the linker histone H1 (Bongartz and Schloissnig, 2019; Lifton et al., 1978; McKay et al., 2015). A histone deficiency that deletes the entire HisC results in lethality at an early embryonic stage as soon as the maternally provided histones are depleted, but transgene cassettes consisting of 12 His-GUs in total rescue Δ HisC into viable adults (Günesdogan et al., 2010). Introduction of specific point mutations in these transgenes allows direct study of the function of different post-translational modifications of histone amino acids (Copur et al., 2018; Graves et al., 2016; Günesdogan et al., 2010; Hödl and Basler, 2012; McKay et al., 2015; Meers et al., 2017; Pengelly et al., 2013, 2015; Penke et al., 2016; Yung et al., 2015; Zhang et al., 2019).

Figure 1. Gut-specific genes contain a unique H3K14ac chromatin state in *Drosophila* embryos

(A) Genomic distribution of H3K14ac ChIP-seq peaks in *w¹¹¹⁸* 12- to 13-h-old embryos.
(B) Metagenome analysis comparing enrichment of 12- to 13-h H3K14ac with H3K9ac, H3K27ac, and H3K4me3 12- to 16-h ChIP-seq signals from transcription start sites (TSSs) to transcription end sites (TESs).
(C) Venn diagram indicating the number of total and unique H3K14ac ChIP-seq peaks and those overlapping 12- to 16-h H3K9ac, H3K27ac, and H3K4me3 embryo peaks (Roy et al., 2010).
(D) Genomic distribution of unique H3K14ac peaks, overlapped peaks, and peaks with no H3K14ac.
(E) Expression of genes containing unique H3K14ac, overlapped, or no H3K14ac ChIP-seq peaks in tissues of larvae and adult flies (modENCODE RNA-seq data). Error bars show standard error.
See also Figure S1.

Here we leverage this approach to examine the role of H3K14ac in developmental gene expression and address whether H3K14ac is functionally redundant with other histone acetylation marks or whether it has unique functions. We find that H3K14 is required for expression of genes uniquely decorated with H3K14ac and that this is essential for wing patterning and animal survival.

RESULTS

A unique H3K14ac chromatin state

The presence of H3K14ac in promoter regions is positively correlated with active gene expression in yeast and in mammalian cells (Karmodiya et al., 2012; Pokholok et al., 2005), suggesting

an important biological role of H3K14ac in transcription activation. To investigate its role in developmental gene expression, we performed H3K14ac chromatin immunoprecipitation sequencing (ChIP-seq) at the time of organogenesis in stage 15 *Drosophila* embryos. In total, 7,254 H3K14ac peaks were identified (Figure 1A). Most of the peaks were located in promoters, introns, and intergenic regions, similar to previous reports of H3K14 acylations in other models (Karmodiya et al., 2012; Kebede et al., 2017; Li et al., 2017). However, in stark contrast to these reports and to other histone modifications associated with active transcription (modENCODE ChIP-seq data for H3K9ac, H3K27ac, and H3K4me3; Figure S1A; Roy et al., 2010), 30% of H3K14ac is found in exons in *Drosophila*

embryos (Figure 1A). A similar distribution of peaks was obtained with a different anti-H3K14ac antibody (Figure S1B). A metagene analysis also demonstrated H3K14ac enrichment over gene bodies (Figure 1). Although this resembles histone 3 lysine 36 trimethylation (H3K36me3) and histone 4 lysine 16 acetylation (H4K16ac), which are also present in gene bodies (Figure S1C), these modifications are absent from promoters and do not overlap H3K14ac to a large extent (Figure S1D). Thus, the genomic distribution of H3K14ac in embryos differs from that of other promoter marks, such as H3K9ac and H3K4me3.

More interestingly, comparison of the H3K14ac peaks with H3K9ac, H3K27ac, and H3K4me3 from a similar embryo stage (Roy et al., 2010) showed that most H3K14ac peaks are unique (81%, $n = 5,923$; Figure 1C). The predominant fraction of these unique H3K14ac peaks is present in exons (34%), whereas overlapping peaks and peaks without H3K14ac are mainly found in promoters (87% and 66%; Figure 1D; Figures S1E and S1F). A Reactome pathway enrichment analysis showed that the genes associated with unique H3K14ac peaks are enriched in several metabolic pathways, G protein coupled receptor (GPCR) signaling, and digestion events (Figure S1G). Furthermore, these unique H3K14ac peak-containing genes display increased expression in the digestive system of larvae and adult flies, whereas genes with overlapping peaks or no H3K14ac peaks are expressed broadly across tissues (modENCODE RNA sequencing [RNA-seq] data; Figure 1E). This suggests that genes decorated with unique H3K14ac peaks are mainly expressed in the gut and may have a metabolic function. Our data are consistent with emerging evidence suggesting that epigenetic regulation and metabolic state are interconnected (Li et al., 2018) and suggest a tissue-specific function for a novel chromatin state featuring H3K14ac unique peaks.

H3K14 is essential in *Drosophila*

To directly probe the biological role of this histone modification, we turned to a transgenic histone replacement approach in *Drosophila melanogaster* (Günesdogan et al., 2010; Figures 2A–2C). We created transgenic flies with 12 His-GUs in which every histone H3K14 was substituted for arginine (H3K14R; Figure 2C) to prevent acetylation and other modifications at this position while maintaining the positive charge. We crossed these transgenic flies with a histone deficiency mutant where all endogenous histones were removed ($\Delta HisC$). Homozygous $\Delta HisC$ individuals containing 12 $\times$ His-GU^{H3K14R} on the third chromosome (hereafter called *H3K14R*) did not survive to adulthood but died as second-instar larvae (Figure 2D). In contrast, $\Delta HisC$ heterozygous offspring and $\Delta HisC$ homozygous animals rescued by 12 $\times$ His-GU with wild-type histone H3 (referred to as *H3WT*) survived and generated fertile adults. We conclude that the H3K14 residue is critically required for larval development in *Drosophila*.

As expected, *H3K14R* animals display diminished global levels of H3K14ac (Figures 2E and 2F). We observed a reduction of 70% relative to *H3WT* individuals or heterozygous siblings ($\Delta HisC/+$; *H3K14R*), although they all display comparable expression of histones H3 and H4 (Figure S2A). We attribute the remaining acetylation to variant histone H3.3, shown previously to be enriched in this modification (McKittrick et al., 2004), because the antibody recognizes both histone variants.

Other histone H3 modifications remained unchanged (Figure S2B), and because we could not detect H3K14 trimethylation in *Drosophila* (Figure S2C), we conclude that the H3K14R substitution specifically affects H3K14ac and possibly other H3K14 acylations. Immunostaining showed that maternally deposited H3K14ac is depleted by stage 14 in *H3K14R* embryos (Figure 2E), demonstrating that reduced H3K14ac is compatible with late embryonic and early larval development.

H3K14 is required for gene expression in the gut

To examine the effects of *H3K14R* on gene expression, we performed RNA-seq in stage 15 embryos 12–13 h after egg laying (AEL). We selected this time point because we wanted to identify primary H3K14 target genes that are deregulated soon after depletion of maternal H3K14ac. To minimize differences arising from genetic background, we compared *H3K14R* embryos with *H3WT* and with heterozygous $\Delta HisC/+$; *H3K14R* animals and considered genes whose differential expression overlaps between *H3K14R* and both controls. Thus, although more genes change expression when comparing *H3K14R* and *H3WT* or *H3K14R* and $\Delta HisC/+$; *H3K14R* individually, only 10 genes were commonly upregulated, and 50 showed decreased expression in *H3K14R* embryos (false discovery rate [FDR] < 0.1, fold change ≥ 2 -fold; Figure 2G; Figures S3A and S3B; Table S1). Gene Ontology (GO) analysis for common upregulated genes showed that they were weakly enriched for odorant binding, whereas downregulated genes are involved in sugar metabolism and signaling (Figures S3C and S3D). A gene set enrichment analysis (GSEA) showed similar results (Figures S3E and S3F). Intriguingly, we observed that 46% of these downregulated genes were expressed in the midgut or hindgut (Figure 2H; Figure S3G), the same tissues where unique H3K14ac peaks are enriched. We conclude that H3K14 is required for tissue-specific expression of a key set of genes in embryo development.

H3K14 target genes lack canonical histone marks

To investigate the relationship between H3K14 target genes and H3K14ac genomic distribution, we compared our ChIP-seq and RNA-seq data in stage 15 embryos. Importantly, most of the genes downregulated in *H3K14R* mutants feature unique H3K14ac peaks (56%; Figure 3A). This is an underestimation because some of the downregulated genes contain an H3K14ac signal below our peak calling cutoff (Figure 3C). Strikingly, all H3K14 targets lack the three histone marks H3K9ac, H3K27ac, and H3K4me3 (Figures 3A–3C; Figure S1E). This shows that H3K14ac is required for expression of genes that lack canonical histone marks. In support of this finding, average expression was reduced in *H3K14R* embryos for genes associated with unique H3K14ac peaks, whereas expression of genes that lack H3K14ac or feature peaks that overlap with other histone marks showed little change (Figure 3D).

Our evidence shows that, during embryonic development, H3K14 is required for expression of genes that are expressed preferentially in the midgut and hindgut (Figures 2H, 3B', and 3C'; Figure S3G) and exhibit well-defined features: they (1) lack canonical modifications associated with active transcription (H3K9ac, H3K27ac, and H3K4me3; Figure 3A) and (2) contain H3K14ac unique peaks located predominantly in exons. This

Figure 2. H3K14 is required for viability and gene expression in the embryonic gut

(A) Representation of the histone complex (HisC) cluster in chromosome 2 of *Drosophila melanogaster*. The HisC consists of tandem arrays of ~100 replication-dependent histone gene units (His-GUs), each encoding the histone proteins H2A, H2B, H3, and H4 and the linker histone H1.

(B) The histone deficiency that deletes the entire HisC from chromosome 2 (Δ HisC). Offspring that are homozygous for Δ HisC die at an early embryonic stage (Günesdogan et al., 2010).

(C) Scheme of a cross between flies containing Δ HisC and a chromosome 2 balancer expressing GFP (GFP balancer, +). Transgene cassettes (orange boxes) were inserted in both arms of chromosome 3 and consist of three copies of WT His-GU (3xHis-GU^{H3wt}) or His-GU in which every histone H3K14 was substituted with arginine (3xHis-GU^{H3K14R}), providing a total of 12 copies of transgenic His-GUs. The genotypes of the offspring resulting from the cross are depicted below the arrow.

(D) Quantification of the number of surviving embryos from three different genotypes and distinguished from heterozygous individuals by the absence of GFP. WT, N = 1,519; Δ HisC; 12xHis-GU^{H3wt} (here called H3WT), N = 2,183; Δ HisC; 12xHis-GU^{H3K14R} (here called H3K14R), N = 2,620. Error bars show standard deviation.

(E) Stage 14 embryos immunostained for H3K14ac, GFP, and DE-cadherin (DCAD2, control staining). GFP expression distinguishes individuals with transgenic histones that are homozygous for endogenous histones (Δ HisC, GFP negative) from heterozygotes (GFP positive, Δ HisC/+). Nuclei are stained by DAPI. Scale bar, 100 μ m.

(F) Western blots in early first-instar larvae in the denoted genotypes. Nuclear protein extracts corresponding to approximately 25 larvae were loaded per lane. The graph shows quantification of H3K14ac levels relative to H3. The blot is representative of six independent biological replicates. Two-tailed, unpaired Student's t test was applied to compare Δ HisC;H3WT with Δ HisC;H3K14R. $p < 0.0001$.

(G) Volcano plot of differentially expressed genes between H3K14R and H3WT, identified by RNA-seq analysis (gray). Genes highlighted in blue and red are changed significantly (fold change ≥ 2 -fold, FDR < 0.1) in comparison with H3WT and Δ HisC/+;H3K14R. Common up, n = 10; common down, n = 50.

(H) Pie chart showing adult and larval tissues where each of the 50 downregulated genes has the highest expression.

Data were retrieved from FlyAtlas and FlyAtlas 2 (Leader et al., 2018). N.A., data not available. See also Figures S2 and S3 and Table S1.

Figure 3. H3K14 target genes lack canonical histone marks but contain H3K14ac peaks

For a Figure360 author presentation of this figure, see <https://doi.org/10.1016/j.molcel.2021.01.041>.

(A) Overlap analysis between unique H3K14ac peaks, no ChIP-seq peaks, no H3K14ac, and overlapped peaks with the 50 commonly downregulated genes in H3K14R embryos.

(B, C, and E) Genome browser snapshots showing two downregulated genes (CG13492 and CG18585) and one unaffected gene (CG3309) with ChIP-seq tracks for H3K14ac, H3K9ac, H3K27ac, and H3K4me3 (immunoprecipitation [IP]/input) and RNA-seq signal (H3WT and H3K14R). All tracks show reads per million.

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demonstrates a unique and essential function of H3K14ac or other H3K14 acylations in tissue-specific gene expression. In contrast, many other active genes contain H3K14ac peaks that overlap with canonical histone marks (Figure 3E; Figure S1F). In this case, however, functional redundancy between histone modifications is likely observed because, in the absence of H3K14ac, these genes are nonetheless transcriptionally active.

Decreased chromatin accessibility and RNA polymerase II (RNA Pol II) occupancy at H3K14 targets in H3K14R mutants

To identify the mechanism by which the H3K14R mutation diminishes gene expression at genes lacking canonical histone marks, we performed Assay for Transposase-Accessible Chromatin (ATAC-seq) in WT and *H3K14R* stage 15 embryos. We calculated the fold change in accessibility over gene bodies and compared this with changes in gene expression. As shown in Figure 3F, chromatin accessibility decreased slightly at genes that are downregulated in *H3K14R* mutants but was not reduced at genes with no change in gene expression or at upregulated genes. Similar results were obtained when comparing accessibility at the promoter-proximal region (Figure S4A). Decreased accessibility at genes downregulated in *H3K14R* embryos is illustrated for gene *CG13492* (Figure S4C) and validated further by ATAC-qPCR (Figure S4D). We also compared the ATAC-seq and ChIP-seq data, which demonstrated that genes uniquely decorated with H3K14ac are less accessible in *H3K14R* mutants, whereas chromatin accessibility is not diminished at genes lacking H3K14ac or at genes where H3K14ac overlaps with other histone marks (Figure 3G; Figure S4B). These results suggest that H3K14ac contributes to maintenance of an open chromatin state at genes lacking canonical histone marks.

To investigate whether decreased accessibility affects RNA Pol II occupancy, we performed Cleavage Under Targets and Tagmentation (CUT&Tag) with a Rpb3 antibody in WT and *H3K14R* stage 15 embryos (Figure S4E). This showed that the amount of RNA Pol II at downregulated gene promoters is reduced in *H3K14R* embryos, whereas promoters with no change in expression had similar amounts of RNA Pol II in WT and mutant embryos (Figure 3H). The RNA Pol II signal was not strong enough to measure occupancy at gene bodies, but occupancy at promoters associated with H3K14ac unique genes was reduced significantly compared with promoters associated with genes lacking H3K14ac ($p = 5.1 \times 10^{-4}$) or where H3K14ac overlaps other histone marks ($p = 4.3 \times 10^{-5}$) (Figure 3I). Our results suggest

that lack of H3K14ac results in decreased chromatin accessibility, diminished RNA Pol II levels, and, as a consequence, reduced transcription of H3K14 target genes.

Patterning of the adult wing margin depends on H3K14

We next sought to examine the function of H3K14 during later stages of development. To observe *H3K14R* mutant cells in heterozygous third-instar larvae, we used FLP-FRT mitotic recombination and clonal analysis. Such clones are homozygous for the $\Delta HisC$, have 12 copies of *H3WT* or *H3K14R* transgenes inserted on chromosome 3, and do not express GFP (Figure 4A). The remaining cells contain at least one copy of the endogenous *HisC* cluster and are marked by GFP expression. Analysis of wing imaginal discs showed that *H3K14R* clone cells have reduced levels of H3K14ac but proliferate similarly as *H3WT* cells (Figure 4B; Figures S5A–S5D; no significant difference between *H3WT* and *H3K14R* clone areas, $p = 0.39$). This demonstrates that although H3K14 is indispensable for animal survival, the *H3K14R* mutation is not cell lethal. However, adult flies carrying *H3K14R* mutant clones showed defects specifically in the wing margin, such as lack of bristles, notches, and necrotic spots (Figure 4C), whereas other parts of the wing were unaffected. In accordance with this phenotype, the Notch target gene *cut* and the Wingless target gene *senseless* were not expressed in *H3K14R* clones in the dorsal/ventral (DV) boundary of the developing wing imaginal disc (Figure 4D). Both genes are necessary for proper wing margin formation and development of non-innervated and mechanosensory bristles in this region (Jack et al., 1991; Jafar-Nejad et al., 2006). Defective expression of these genes was specific to the developing wing margin because *cut* expression was not affected in *H3K14R* cells in the notum of the disc, and *senseless* was still expressed in other sensory organ precursors located throughout the wing disc (Figures S5F and S5G). Other genes that are part of these signaling pathways, such as the ligands *wingless* and *Delta*, were correctly expressed in *H3K14R* mutants, suggesting that the effect of H3K14 on *cut* and *senseless* expression is locus specific and not due to a disturbance of upstream signaling components (Figures S6A and S6B). Furthermore, the expression of *patched* and *spalt*, targets of Hedgehog and Dpp signaling, respectively, which, in turn, are involved in anterior-posterior patterning of the wing (Strigini and Cohen, 1999), was unaffected in *H3K14R* clones (Figure 4E). *H3K14R* clones were also observed in other imaginal discs (Figures S5B–S5E) but did not result in visible phenotypes in adult flies. These results show that H3K14 is essential for expression of patterning genes in wing DV boundary cells.

(B' and C') *In situ* hybridization in stage 16 embryos (anterior to the left) using antisense probes for *CG13492* and *CG18585*. Expression of these genes is restricted to the midgut. An asterisk marks the *GFP* antisense probe signal, which was used to distinguish between homozygous and heterozygous $\Delta HisC$ animals. Scale bars, 50 μm . (D) Mean RNA-seq expression fold change in *H3K14R* versus *H3WT* and *H3K14R* versus $\Delta HisC/+;H3K14R$ for genes associated with unique H3K14ac, no H3K14ac, or overlapped ChIP-seq peaks. Error bars show standard error.

(F) ATAC-seq fold change in *H3K14R* versus *H3WT* stage 15 embryos at gene bodies of the 50 commonly downregulated genes, genes downregulated 1.5-fold, unchanged genes, and genes upregulated 1.5-fold in RNA-seq. Error bars show standard error.

(G) ATAC-seq fold change at gene bodies with unique H3K14ac peaks, no H3K14ac peaks, and peaks overlapping other histone marks. H3K14ac unique versus no H3K14ac, $p = 3.94 \times 10^{-111}$; H3K14ac unique versus overlapping peaks, $p = 1.93 \times 10^{-129}$. Error bars show standard error.

(H) CUT&Tag promoter (± 100 bp around the TSS) fold change for RNA Pol II (Rpb3 antibody) occupancy in mutant versus WT (w^{1118}) stage 15 embryos compared with RNA-seq data. Error bars show standard error.

(I) RNA Pol II promoter fold change (CUT&Tag) at genes with unique H3K14ac peaks, no H3K14ac peaks, and peaks overlapping other histone marks. H3K14ac unique versus no H3K14ac, $p = 5.1 \times 10^{-4}$; H3K14ac unique versus overlapping peaks, $p = 4.3 \times 10^{-5}$. Error bars show standard error.

Statistical tests were performed by two-sided t tests. See also Figure S4.

Figure 4. Patterning the adult wing margin requires H3K14

(A) Representation of a cell in mitosis that has replicated its DNA and undergoes FLP-mediated recombination between homologous chromosomes where an FRT sequence is present (pink box, chromosome 2) (i) and illustration of, from top to bottom, heterozygous cells that have not recombined and show intermediate levels of GFP (dark green), cells WT for the HisC that show strong levels of GFP (bright green, twin spots), and homozygous Δ HisC cells that do not express GFP (black, mutant clones) (ii). All cells contain 12 copies of $H3WT$ or $H3K13R$ transgenes inserted on chromosome 3.

(B) Third-instar wing imaginal discs containing $H3WT$ or $H3K14R$ mutant clones (white arrows, no GFP expression) and twin spots (white arrowheads, strong GFP expression) stained for H3K14ac.

(C) Wings from adult flies containing $H3WT$ or $H3K14R$ mutant clones and percentage of flies showing defects in the wing margin (lack of bristles, notches, and necrosis). N of flies counted: $H3WT$, 223; $H3K14R$, 221. Scale bar, 200 μ m.

(D) Expression of Cut and Senseless in the wing disc of third-instar larvae with $H3WT$ or $H3K14R$ mutant clone cells (no GFP expression).

(E) Expression of Patched and Spalt in the wing disc of third-instar larvae with $H3WT$ or $H3K14R$ mutant clone cells (no GFP expression). White arrows indicate regions where $H3K14R$ or $H3WT$ clones are observed. Merged panels show the expression of Cut, Senseless, Patched, or Spalt in mutant clones. Dashed squares indicate the region that is magnified in the rightmost panels in (D) and (E).

Scale bars in (B), (D), and (E), 50 μ m. See also [Figures S5](#) and [S6](#).

H3K14ac unique peaks in H3K14 wing disc targets

Considering our observation that, in embryos, H3K14 targets contain H3K14ac unique peaks in exons, we predicted that H3K14 targets in the wing would show a similar pattern. We therefore performed ChIP-seq in dissected wing imaginal discs of w^{1118} third-instar larvae to determine whether target genes

in this tissue were also occupied by H3K14ac. In the wing disc, out of 13,064 H3K14ac peaks, 18% were found in exons ([Figure 5A](#)). We found that 5,571 peaks overlap with published H3K27ac and H3K4me2/3 data ([Schertel et al., 2015](#); [Vizcaya-Molina et al., 2018](#)), whereas 7,493 peaks (57%) were unique ([Figure 5B](#)). As in the embryo, a large fraction of unique peaks

Figure 5. H3K14 target genes in the wing disc contain unique H3K14ac peaks

(A) Genomic distribution of H3K14ac ChIP-seq peaks in *w¹¹¹⁸* third-instar larvae wing disc. (B) Venn diagram indicating the number of total and unique H3K14ac ChIP-seq peaks and those overlapping H3K27ac (Vizcaya-Molina et al., 2018) and H3K4me2/3 (Schertel et al., 2015). (C) Pie charts showing the genomic distribution of unique H3K14ac peaks, overlapped peaks, and peaks with no H3K14ac. (D–F) Genome browser snapshots showing the ChIP-seq signal for H3K14ac, H3K27ac, and H3K4me2/3 (IP/input, except H3K27ac) in the H3K14 target genes (D) *cut* and *senseless*, (F) *pebbled/hindsight*, and in other active genes in the wing disc (E) *patched*, *salr*, and *salm*. All tracks show reads per million. (G) Projection image of *Pebbled/Hindsight* in the wing disc of third-instar larvae with *H3WT* or *H3K14R* mutant clone cells (no GFP expression). White arrows indicate regions where *H3K14R* or *H3WT* clones are observed. Asterisk points to a cell expressing *Pebbled* outside of the dorsal-ventral boundary. Dashed squares indicate the region that is magnified in the rightmost panels. Scale bar, 50 μ m. See also Figure S6.

is located in exons (25%), whereas the majority of overlapping peaks are found in promoters (64%; Figure 5C).

As predicted, H3K14ac was found in exons of *cut* and *senseless*, whereas H3K4me2/3 and H3K27ac were absent from these loci (Figure 5D). In contrast, non-targets of H3K14 in the wing, such as *patched*, *spalt* (*salm*), *wingless*, and *delta* were co-occupied by H3K14ac, H3K27ac, and H3K4me2/3 (Figure 5E; Figures S6C and S6D). In *H3K14R* cells, the inability to acetylate H3K14 at loci such as *cut* and *senseless* that lack other histone marks results in functional impairment of wing development.

We next reasoned that we could predict other targets of H3K14 based on the genomic occupancy of these three histone marks. Because the DV boundary appears to be particularly sensitive to the H3K14R mutation, we searched for genes expressed

in this position. *Pebbled* (also known as *Hindsight*) is expressed anterior to sensory organ precursor cells in the DV boundary (Giráldez et al., 2002), and contains a strong unique H3K14ac exonic peak (Figure 5F). Indeed, we confirmed by immunostaining that *Hindsight/Pebbled* is not expressed in *H3K14R* mutant cells in the DV boundary of third-instar larvae (Figure 5G). In contrast, *Deadpan*, which is also expressed in the DV boundary (San Juan et al., 2012) but is occupied by H3K27ac and H3K4me2/3, is unaffected in *H3K14R* clones (Figures S6E and S6F).

We then compared the H3K14ac peaks between embryos and wing discs. Fifty-two percent of the unique H3K14ac peaks in embryos are also unique in wing discs, although many of the associated genes are not expressed in the wing (Figures S6G and S6H). This shows that the unique H3K14ac chromatin state

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is not sufficient for gene activation, consistent with the wing disc phenotype being restricted to the DV boundary. In conclusion, H3K14 is essential, but not sufficient, for expression of genes that are decorated with H3K14ac but devoid of canonical histone marks in the embryonic gut and in a specific population of cells in the wing disc.

Brahma and Chameau are required for expression of H3K14 targets in the wing disc

To identify potential mediators of H3K14ac, we incubated *Drosophila* S2 cell nuclear extracts with biotinylated H3K14ac or unmodified H3 peptides. We then purified bound proteins with streptavidin beads, labeled them with tandem mass tags, and identified differentially bound proteins by mass spectrometry (Figure 6A). We found 12 proteins to be enriched by the H3K14ac peptide bait compared with unmodified H3 peptide (Figures 6A and 6B; Figure S7A). Intriguingly, 11 of these proteins are components of the SWI/SNF chromatin remodeler complexes Brahma-associated protein (BAP) and Polybromo-BAP (PBAP) (Bracken et al., 2019; Figure 6B). In addition, we identified the bromodomain-containing protein BR140 (Figures 6A and 6B). We confirmed selective binding of the BAP/PBAP subunits Brahma (Brm) and Moira (Mor) to the H3K14 acetylated peptide by western blot in pull-down experiments with embryo nuclear extracts (Figure 6C). The catalytic subunit Brm contains a bromodomain, and the homologous bromodomain in mammalian BRG1 has a higher affinity for H3K14ac peptide than for histone peptides acetylated at other positions (Shen et al., 2007). A sequence alignment showed that the residues involved in selective binding to the H3K14ac peptide are conserved between human BRG1 and the *Drosophila* Brm bromodomain (Figure S7B). To examine whether an H3K14ac peptide could indeed be bound by the Brm bromodomain, we expressed and purified it from *E. coli* and carried out circular dichroism measurements (Figures S7C and S7D). Addition of H3K14ac peptide did not change the secondary structure of the bromodomain (Figure S7C) but induced a shift in the thermal unfolding profile (Figure S7D), which demonstrates peptide binding. Fluorescence anisotropy-based titration experiments showed that the Brm bromodomain binds to H3K14

acetylated peptide with a K_d of 317 μ M, whereas no specific binding was observed with non-acetylated peptide (Figure 6D). Our data show that the bromodomain in *Drosophila* Brm mediates specific recognition of H3K14ac.

To examine whether Brm is involved in regulating H3K14 target genes, we used RNA interference (RNAi) in wing imaginal discs. Using a Nubbin-Gal4 driver expressed in the wing pouch, Brm protein levels were reduced efficiently (*nub>brm-RNAi*; Figure 6E). Strikingly, Cut and Senseless expression was eliminated in the wing pouch of *nub>brm-RNAi* flies, whereas Patched and Wingless expression was essentially normal (Figure 6F). This is consistent with a previous study reporting decreased Senseless expression in wing discs with *brm* RNAi (Zhu et al., 2015). However, Senseless and Cut are not affected in *polybromo* RNAi wing discs (Figures 6G and 6H), indicating that BAP, but not the PBAP complex, is involved in expression of these genes. The *brm* RNAi phenotype resembles the one in *H3K14R* mutant cells (Figure 4), strongly suggesting that *brm* acts in the same genetic pathway as H3K14. Moreover, *brm* and *mor* interact genetically with *cut* during development of the wing margin (Krupp et al., 2005), further supporting a function for the BAP complex in formation of the DV boundary.

We next sought to identify the enzyme responsible for H3K14ac, and performed RNAi in the wing disc with a Patched-Gal4 (*ptc-Gal4*) driver, followed by Cut immunostaining. Although Gcn5 is the major H3K14 acetylase in flies (Carré et al., 2005), and its depletion resulted in reduced H3K14ac levels in the wing disc, Cut expression was only partly reduced (Figures 6I and 6J). This indicates that there may be additional H3K14 acetyltransferases. We focused on Chameau (Grienerberger et al., 2002), the fly homolog of mammalian HBO1, which is known to acetylate H3K14 (Kueh et al., 2011). *chameau* RNAi did not result in a global reduction in H3K14ac, but it did lead to reduced Cut expression and expanded Wingless expression in the Patched domain (Figures 6K and 6L). The gene expression phenotypes of Chameau depletion closely resemble that of H3K14R mutant cells, where Cut is reduced and Wingless expanded (Figure 6M), suggesting that it is the major H3K14 acetylase at H3K14ac-unique genes.

Figure 6. Brahma and Chameau are required for expression of H3K14 targets in the wing disc

- (A) Volcano plot showing *Drosophila* S2 cell nuclear extract proteins enriched in H3K14ac peptide affinity purification relative to unmodified H3 peptides. The $-\log_{10}$ Benjamini-Hochberg-corrected p value is plotted against the $\log_2$ fold change: H3K14ac/H3K14 unmodified peptide pull-down. Proteins with an FDR of less than 0.05 and a fold change of at least 100% are considered hits and labeled red.
- (B) Table listing proteins preferentially bound by the H3K14ac peptide and schematic drawings of the Brahma-associated protein (BAP) and Polybromo-BAP (PBAP) complexes in *Drosophila*. Subunits enriched by the H3K14ac peptide are labeled with a red star.
- (C) Affinity purification from *w¹¹¹⁸* embryo nuclear extracts using unmodified (K14un) or acetylated (K14ac) H3 peptide followed by western blot. The blot is representative of two independent biological replicates.
- (D) Fluorescence anisotropy of TAMRA-labeled unmodified or H3K14ac peptide in the presence of varying amounts of Brm.
- (E) Expression of Brm in the wing disc of *w¹¹¹⁸* (top) control and in *nub-Gal4>UAS-brm-RNAi* (bottom) third-instar larvae. The dashed line indicates the expression domain of Nub.
- (F) Expression of Cut, Senseless, Patched, and Wingless in the wing disc of *nub-Gal4>UAS-brm-RNAi* third-instar larvae.
- (G) Expression of Polybromo in the wing disc of *w¹¹¹⁸* (top) control and in *nub-Gal4>UAS-polybromo-RNAi* (bottom) third-instar larvae.
- (H) Expression of Cut, Senseless, Patched, and Wingless in the wing disc of *nub-Gal4>UAS-polybromo-RNAi* third-instar larvae.
- (I) H3K14ac and expression of Cut and Wingless in the wing disc of *ptc-Gal4>UAS-gcn5-RNAi* third-instar larvae.
- (J) Quantification of Cut and Wingless signal within and outside the *ptc* domain (dashed lines).
- (K) H3K14ac and expression of Cut and Wingless in the wing disc of *ptc-Gal4>UAS-chameau-RNAi* third-instar larvae.
- (L) Quantification of Cut and Wingless signal within and outside the *ptc* domain.
- (M) Cut and Wingless expression in H3K14R mutant clones.
- (i)–(iv) in (E)–(H) show the same wing disc. Scale bars in (E)–(M), 50 μ m. See also Figure S7.

Figure 7. A unique H3K14ac chromatin state in human cells

(A) Venn diagram indicating the number of total and unique H3K14ac ChIP-seq peaks and those overlapping H3K9ac, H3K27ac, and H3K4me3 in human mesenchymal stem cells (MSCs) from ENCODE data (Moore et al., 2020).

(B) Genome browser snapshots showing two genes with H3K14ac unique peaks (*NPY* and *GATA3*) with ChIP-seq tracks for H3K14ac, H3K9ac, H3K27ac, and H3K4me3 (IP/input) and RNA-seq signal. All tracks show reads per million.

(C) MSC genes with the unique H3K14ac chromatin state are more strongly expressed in these cells compared with human embryonic stem cells (ESCs) and neural stem cells (NSCs). Error bars show standard error.

(D) Schematic model showing active genes containing canonical histone modifications associated with gene expression and H3K14-target genes that lack these promoter modifications and are decorated with H3K14ac in exons.

See also Figure S8 and Table S2.

A unique H3K14ac chromatin state in human cells

We mined publicly available datasets from human cells to search for the presence of genes decorated with H3K14ac without canonical histone marks. We compared ENCODE H3K14ac ChIP-seq peaks with H3K9ac, H3K27ac, and H3K4me3 in embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), and IMR-90 lung fibroblasts (Moore et al., 2020; Figure 7A; Figure S8A). At the gene level, we found 202 genes bearing H3K14ac but lacking canonical histone marks in MSCs but only 26 genes in ESCs, 55 in NSCs, and 123 in IMR-90 cells. The H3K14ac-unique genes in MSCs include cytokines and growth factors known to be secreted from MSCs (Pittenger et al., 2019), such as interleukin 1 beta and hepatocyte growth factor as well as the tran-

scription factor *GATA3* and neuropeptide Y (*NPY*) (Figure 7B; Table S2). Interestingly, the *NPY* homolog *NPF* is also uniquely decorated with H3K14ac in the *Drosophila* embryo (Figure S8E). As in *Drosophila*, a large fraction (40%) of the H3K14ac peaks that overlap other histone marks are located in promoters, whereas only 4% of the unique H3K14ac peaks are found in promoters (Figures S8B and S8C). Unlike in *Drosophila*, many of the unique H3K14ac peaks are located in introns and fewer peaks appear in exons (Figure S8B). The MSC genes associated with unique H3K14ac peaks are more strongly expressed in MSCs than in ESCs and NSCs (Figure 7C; Figure S8D), indicating that the unique H3K14ac chromatin state may function in cell type-specific gene expression in both *Drosophila* and human cells.

DISCUSSION

We discovered a set of genes that are decorated with unique H3K14ac peaks whose expression is dependent on H3K14 and that are required for proper *Drosophila* development (Figure 7D). Although H3K14 is essential in *Drosophila melanogaster*, H3K14R substitutions are viable in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Dai et al., 2008; Mann and Grunstein, 1992; Mellone et al., 2003; Nakanishi et al., 2008). However, H3K14ac is important for the response to nutrient stress in these yeasts and regulates transcription elongation (Church et al., 2017; Johnsson et al., 2009). Although H3K14ac is required for tissue-specific expression of a few genes lacking canonical histone marks, the histone variant H3.3 is intact in *H3K14R* flies, and H3K14ac is reduced but not eliminated. It is therefore likely that H3K14ac has an even more pervasive role in development than the one revealed by our study. The presence of a unique H3K14ac chromatin state in MSCs suggests that this chromatin state is also important for the cell type-specific expression of key genes in some human cell types.

Gcn5 and HBO1 are enzymes known to acetylate H3K14, and Gcn5 is the major H3K14 acetylase in flies (Carré et al., 2005). However, Gcn5 also acetylates H3K9 (Torres-Zelada and Weake, 2020), and H3K14 target genes contain H3K14ac but lack H3K9ac. This suggests that the substrate preference of Gcn5 is altered at H3K14 targets or that another enzyme is responsible for H3K14ac at these genes. We favor the latter possibility because inhibition of the HBO1 homolog Chameau in the wing disc results in gene expression phenotypes reminiscent of those observed in *H3K14R* mutant clones.

In contrast to the compelling phenotypes observed in *H3K14R* mutants, elimination of canonical histone marks has not shown such drastic phenotypes in flies. Despite decoration of enhancers with H3K4me1 and most active promoters with H3K4me3, developmental genes such as *Cut*, *Senseless*, *Patched*, *Spalt*, and *Hox* genes are expressed normally in mutant clones in which H3K4 has been replaced with arginine or alanine in H3 and H3.3 simultaneously (Hödl and Basler, 2012). Furthermore, a small percentage of flies with a H3K9R substitution survive to adulthood, and expression of most genes is normal in these animals (Penke et al., 2016), although *H3K9R*; *H3.3K9R* double mutants die as first-instar larvae (Penke et al., 2018). Similarly, *H3K27R* animals show defects in Polycomb silencing because of reduced H3K27me3 levels but not in gene activation (McKay et al., 2015; Pengelly et al., 2013). Thus, elimination or reduction in H3K4me3, H3K9ac, or H3K27ac has less dramatic consequences for *Drosophila* development than reduced H3K14ac levels.

Using mass spectrometry, we identified potential mediators of H3K14ac. Of 12 proteins that bind a H3K14ac peptide better than unmodified H3 peptide, 11 are confirmed or likely subunits of the SWI/SNF chromatin remodeler complexes BAP and PBAP (Bracken et al., 2019). Our results are consistent with a study using mammalian cell extracts showing that the corresponding BAF and PBAF complexes are bound preferentially by acetylated H3K14 peptide (Kebede et al., 2017). Three of the subunits, Polybromo, BRD7, and Brm, contain bromodo-

domains that are known to recognize acetylated histones (Fujisawa and Filippakopoulos, 2017). We demonstrate that the *Drosophila* Brm bromodomain binds to an H3K14ac peptide and, importantly, show that *brm* acts in the same genetic pathway as *H3K14R*. In addition, one more bromodomain-containing protein was enriched by the H3K14ac peptide, BR140, the homolog of mammalian BRPF1/BRD1. It will be interesting to investigate whether this protein also has a function in regulating H3K14R target genes.

What could be the role of the Brm-H3K14ac interaction? It could help recruit BAP/PBAB to H3K14 targets, but this is a low-affinity interaction, and transcription factors may be more important for recruitment (Ho et al., 2019). Nevertheless, interacting with H3K14ac could stabilize BAP/PBAB on chromatin. Alternatively, the Brm-H3K14ac interaction could modulate chromatin remodeling activity. Recent cryoelectron microscopy (cryo-EM) structures of BAF and SWI/SNF complexes have failed to resolve the bromodomain (Han et al., 2020; He et al., 2020), and the structural basis for such an allosteric effect remains unclear. Notably, H3K14 target genes become less accessible in the absence of H3K14, suggesting that the Brm-H3K14ac interaction is important for maintaining these genes in an open chromatin state.

An open chromatin state could be critical for proper transcription elongation and consequent gene expression. Another possibility is that sequences uniquely decorated with H3K14ac function as transcriptional enhancers and that activity of these enhancers depends on H3K14ac. Understanding the precise molecular function of H3K14ac in transcriptional regulation remains a challenge for future studies.

Control of gene expression during animal development is a complex process that involves multiple layers of regulation. Knowledge of the molecular mechanisms that determine the expression of genes at a given time or in a specific population of cells is of crucial importance for an in-depth understanding of cell differentiation and tissue diversity in animals. Using a histone replacement system and biochemical reconstitution, we probed the function of a single lysine in organismal development and tissue patterning. We found that H3K14ac in genes devoid of canonical histone marks is essential for expression of these genes in the embryonic gut and the wing DV boundary. Our study unravels an unexpectedly specific and context-dependent role of H3K14ac in tissue-specific gene expression.

Limitations of study

We employed histone replacement technology in *Drosophila* to interrogate the function of H3K14 in developmental gene expression. These flies express transgenes with a K14R mutant histone H3 in the absence of endogenous H3 but contain the H3.3 histone variant. H3K14 modifications are therefore reduced severely but not eliminated in these animals. Here we examined H3K14 acetylation but not other H3K14 acylations, such as, e.g., butyrylation or crotonylation, that may also contribute to the H3K14R phenotypes. Although the affected genes are mainly restricted to the gut in the embryo and to the dorso-ventral boundary in the wing imaginal disc, our genome-wide methods were performed in whole embryos and imaginal discs. Cell

sorting or single-cell methods may provide a clearer picture of which cells feature this H3K14ac-unique chromatin state.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.molcel.2021.01.041>.

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AUTHOR CONTRIBUTIONS

O.D., I.R., R.V., and M.M. conceived the project. I.R. performed immunofluorescence, *in situ* hybridization, imaging, western blots, RNA-seq, ChIP-seq, CUT&Tag, and peptide pull-down. R.V. performed survival and ATAC-seq assays. S.B. performed ChIP-seq in embryos. O.D., R.V., and I.R. generated transgenic flies. S.D. designed and O.H. performed circular dichroism and fluorescence anisotropy measurements and peptide pull-down for mass spectrometry analysis. J.W. and G.D. performed bioinformatics analyses. I.R. and M.M. wrote the paper with help from J.W. and S.D. and comments from all the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-H3K14ac	Abcam	Cat# ab52946, RRID:AB_880442
Rabbit anti-H3K14ac	Millipore	Cat# 07-353, RRID:AB_310545
Rabbit anti-H3K14me3	Fei Lan	N/A
Rabbit anti-H3K4me1	Abcam	Cat# ab8895, RRID:AB_306847
Rabbit anti-H3K4me2	Millipore	Cat# 07-030, RRID:AB_310342
Rabbit anti-H3K4me3	Abcam	Cat# ab8580, RRID:AB_306649
Rabbit anti-H3K9ac	Abcam	Cat# ab4441, RRID:AB_2118292
Rabbit anti-H3K9me3	Abcam	Cat# ab8898, RRID:AB_306848
Rabbit anti-H3K18ac	Abcam	Cat# ab1191, RRID:AB_298692
Rabbit anti-H3K27ac	Abcam	Cat# ab4729, RRID:AB_2118291
Mouse anti-H3K27me3	Abcam	Cat# ab6002, RRID:AB_305237
Rabbit anti-H3K36me3	Abcam	Cat# ab9050, RRID:AB_306966
Mouse anti-H3	Millipore	Cat# 05-499, RRID:AB_309763
Rabbit anti-H3	Abcam	Cat# ab1791, RRID:AB_302613
Rabbit anti-H4	Abcam	Cat# ab10158, RRID:AB_296888
Mouse anti-Lamin Dm0	DSHB	Cat# adl67.10, RRID:AB_528336
Chicken anti-GFP	Abcam	Cat# ab13970, RRID:AB_300798
Rat anti-DE-Cadherin	DSHB	Cat# DCAD2, RRID:AB_528120
Mouse anti-Cut	DSHB	Cat# 2b10, RRID:AB_528186
Mouse anti-Patched	DSHB	Cat# Drosophila Ptc (Apa 1), RRID:AB_528441
Mouse anti-Wingless	DSHB	Cat# 4d4, RRID:AB_528512
Mouse anti-Delta	DSHB	Cat# c594.9b, RRID:AB_528194
Mouse anti-Pebbled	DSHB	Cat# 1g9, RRID:AB_528278
Guinea pig anti-Senseless	Hugo J. Bellen	N/A
Rabbit anti-Sal	Adi Salzberg	N/A
Rabbit anti-Brahma	Lei Zhang	N/A
Rabbit anti-Polybromo	Peter Verrijzer	N/A
Rabbit anti-Deadpan	Sarah Bray	N/A
Rabbit anti-RPB3	John Lis	N/A
Rabbit anti-Moira	Peter Verrijzer	N/A
IRDye® 680RD Goat anti-Rabbit IgG	LI-COR Biosciences	Cat# 925-68071, RRID:AB_2721181
IRDye® 800CW Goat anti-Mouse IgG	LI-COR Biosciences	Cat# 925-32210, RRID:AB_2687825
Alexa Fluor 488-conjugated donkey anti-chicken	Jackson ImmunoResearch Labs	Cat# 703-545-155, RRID:AB_2340375
Cy3-conjugated donkey anti-mouse	Jackson ImmunoResearch Labs	Cat# 715-165-151, RRID:AB_2315777
Cy3-conjugated donkey anti-guinea pig	Jackson ImmunoResearch Labs	Cat# 706-165-148, RRID:AB_2340460
Cy5-conjugated donkey anti-mouse	Jackson ImmunoResearch Labs	Cat# 715-175-151, RRID:AB_2340820
Alexa Fluor 647-conjugated donkey anti-rat	Jackson ImmunoResearch Labs	Cat# 712-605-153, RRID:AB_2340694
Alexa Fluor 647-conjugated donkey anti-rabbit	Jackson ImmunoResearch Labs	Cat# 711-605-152, RRID:AB_2492288
Alexa Fluor 568-conjugated donkey anti-rabbit	Thermo Fisher Scientific	Cat# A10042, RRID:AB_2534017
HRP-conjugated goat anti-Rabbit IgG	Dako	Cat# P0448, RRID:AB_2617138

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
BL21(DE3)pLysS competent <i>E.coli</i>	Novagen	Cat# 69451
Chemicals, peptides, and recombinant proteins		
H3K14 peptide (N-term Biotin)	Peptide2.0	N/A
H3K14ac peptide (N-term Biotin)	Peptide2.0	N/A
H3K14 peptide (C-term Biotin)	Peptide2.0	N/A
H3K14ac peptide (C-term Biotin)	Peptide2.0	N/A
H3K14 peptide (C-term TAMRA)	Peptide2.0	N/A
H3K14ac peptide (C-term TAMRA)	Peptide2.0	N/A
Dynabeads M-280 Streptavidin	Thermo Fisher Scientific	Cat# 11205D
Agencourt AMPure XP beads	Beckman Coulter	Cat# A63881
Phusion® High-Fidelity PCR Master Mix with GC Buffer	NEB	Cat# M0532L
PCR Primer Cocktail	Illumina	Cat# 15026786
5x HOT FIREPol® EvaGreen® qPCR Mix Plus	Solis BioDyne	Cat# 08-24-0000S
BioMag®Plus Concanavalin A	Polysciences	Cat# 86057-3
ERCC RNA Spike-In Mix	Thermo Fisher Scientific	Cat# 4456740
Tn5 (Tagment DNA Enzyme 1 (TDE1))	Illumina	Cat# 15027865
Tagment DNA Buffer	Illumina	Cat# 15027866
Critical commercial assays		
TruSeq Stranded mRNA LT Library Prep Kit	Illumina	Cat# RS-122-2101
Direct-zol RNA Microprep	Zymo Research	Cat# R2060
ChIP DNA Clean & Concentrator™	Zymo Research	Cat# D5205
NEBNext® Ultra II DNA Library Prep Kit for Illumina®	NEB	Cat# E7645L
Nextera® Index Kit	Illumina	Cat# 15055290
Deposited data		
Embryo 12-13h ChIP-seq H3K14ac, ATAC-seq, RNA-seq and CUT&Tag	This study	GEO: GSE149343
Embryo 12-16h ChIP-seq input	Roy et al., 2010	GEO: GSM432634
Embryo 12-16h ChIP-seq H3K9ac	Roy et al., 2010	GEO: GSM439458
Embryo 12-16h ChIP-seq H3K27ac	Roy et al., 2010	GEO: GSM432582
Embryo 12-16h ChIP-seq H3K4me3	Roy et al., 2010	GEO: GSM432580
Embryo 14-16h ChIP-seq H3K36me3	Roy et al., 2010	GEO: GSE47256
Embryo stage 15 ChIP-seq H4K16ac	Samata et al., 2020	GEO: GSE130335
Wing disc ChIP-seq H3K27ac	Vizcaya-Molina et al., 2018	GEO: GSM3185650
Wing disc ChIP-seq H3K4me2/3	Schertel et al., 2015	GEO: GSM1446261
Wing disc RNA-seq	Vizcaya-Molina et al., 2018	GEO: GSM2747068
ChIP-seq ESC, NSC, MSC, IMR-90	Moore et al., 2020	https://www.encodeproject.org/
Original imaging files	This study	https://dx.doi.org/10.17632/r4k2wx3p4x.1
Experimental models: cell lines		
Drosophila Schneider 2 (S2) cells	Drosophila Genomics Resource Center	Cat# 006
Experimental models: organisms/strains		
<i>w¹¹¹⁸</i>	Bloomington Drosophila Stock Center	Cat# 3605
+/ <i>CyO Dfd-GFP</i>	This study	N/A
<i>w; Dfd(2L)His^CFRT40A/CyO;TM3Sb/Vno</i>	Jürg Müller	N/A
<i>w; Dfd(2L)His^CFRT40A/CyO Dfd-GFP;TM3Sb/Vno</i>	Jürg Müller	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
w; <i>Cyo Dfd-GFP/Sp; 6XHis-GU</i>	Alf Hertzig	N/A
w; <i>Cyo Dfd-GFP/Sp; 6XHis-GU^{H3K14R}</i>	This study	N/A
w; <i>Df(2L)His^CFRT40A/CyO Dfd-GFP;6XHis-GU</i>	This study	N/A
w; <i>Df(2L)His^CFRT40A/CyO Dfd-GFP;6XHis-GU^{H3K14R}</i>	This study	N/A
yw <i>hs-FLP122; hs-nGFP FRT40A; 6XHis-GU</i>	Jürg Müller	N/A
yw <i>hs-FLP122; hs-nGFP FRT40A; 6XHis-GU^{H3K14R}</i>	This study	N/A
<i>UAS-brm-RNAi</i>	Bloomington Drosophila Stock Center	Cat# 31712
<i>UAS-polybromo-RNAi</i>	Bloomington Drosophila Stock Center	Cat# 32840
<i>UAS-dcr-2; nub-Gal4</i>	Bloomington Drosophila Stock Center	Cat# 25754
<i>ptc-Gal4</i>	Bloomington Drosophila Stock Center	Cat# 2017
<i>UAS-gcn5-RNAi</i>	Bloomington Drosophila Stock Center	Cat# 9332
<i>UAS-chameau-RNAi</i>	Bloomington Drosophila Stock Center	Cat# 32484
Oligonucleotides		
Oligonucleotides and primers are listed in Table S3	Eurofins Genomics	N/A
Recombinant DNA		
pENTR 221-HisGU	Alf Hertzig (Günesdogan et al., 2010)	N/A
pENTR L4R1-HisGU	Alf Hertzig (Günesdogan et al., 2010)	N/A
pENTR R2L3-HisGU	Alf Hertzig (Günesdogan et al., 2010)	N/A
pENTR 221-HisGU ^{H3K14R}	This study	N/A
pENTR L4R1-HisGU ^{H3K14R}	This study	N/A
pENTR R2L3-HisGU ^{H3K14R}	This study	N/A
phiC31attB 3xHisGU ^{H3K14R}	This study	N/A
pET-15b	Novagen (EMD Millipore)	69661
Software and algorithms		
R (3.6.1) R packages: tidyverse	a free software environment for statistical computing and graphics	https://www.r-project.org/
Bioconductor packages: ggplot2, DESeq2, Biostrings, Rsubread, venneuler, UpSetR, pheatmap, fgsa	Tools for the analysis and comprehension of high-throughput genomic data.	https://www.bioconductor.org/
Trimmomatic	Bolger et al., 2014	http://www.usadellab.org/cms/?page=trimmomatic
Bowtie2	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
HISAT2	Kim et al., 2015	http://www.ccb.jhu.edu/software/hisat/index.shtml
HOMER2 (v4.11)	a suite of tools for peak calling and motif Discovery and next-gen sequencing analysis	http://homer.ucsd.edu/homer/index.html
deeptools	tools for exploring deep sequencing data (Ramírez et al., 2016)	https://deeptools.readthedocs.io/en/develop/
samtools	Li et al., 2009	http://samtools.sourceforge.net/
UCSC kent-tools	A collection of UCSC binary tools	http://hgdownload.soe.ucsc.edu/admin/exe/linux.x86_64/
IGB	Integrated Genome Browser	https://www.bioviz.org/
IGV	Integrative Genomics Viewer	http://software.broadinstitute.org/software/igv/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
UCSC Genome Browser	UCSC browser for interactively visualizing genomic data	https://genome.ucsc.edu/
GraphPad Prism Version 5.00	GraphPad Software	https://www.graphpad.com:443/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mattias Mannervik (mattias.mannervik@su.se).

Materials availability

Drosophila stocks generated in this study are available from the Lead Contact without restriction.

Data and code availability

The dataset generated during this study are available at Gene Expression Omnibus with the Accession Number GEO: GSE149343. Original imaging data have been deposited at Mendeley Data with the URL <https://dx.doi.org/10.17632/r4k2wx3p4x.1>

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster strains and genetics

*w*¹¹¹⁸
 +/CyO *Dfd-GFP*
w; *Df(2L)His^C-FRT40A/CyO*; -*TM3Sb/Vno*
w; *Df(2L)His^C-FRT40A/CyO Dfd-GFP*; -*TM3Sb/Vno*
w; *CyO Dfd-GFP/Sp*; 6*XHis-GU*
w; *CyO Dfd-GFP/Sp*; 6*XHis-GU*^{H3K14R}
w; *Df(2L)His^C-FRT40A/CyO Dfd-GFP*; -6*XHis-GU*
w; *Df(2L)His^C-FRT40A/CyO Dfd-GFP*; -6*XHis-GU*^{H3K14R}
yw hs-FLP122; *hs-nGFP FRT40A*; 6*XHis-GU*
yw hs-FLP122; *hs-nGFP FRT40A*; 6*XHis-GU*^{H3K14R}
UAS-dcr-2; *nub-Gal4* (BL 25754)
UAS-brm-RNAi (BL 31712)
UAS-polybromo-RNAi (BL 32840)
ptc-Gal4 (BL2017)
UAS-gcn5-RNAi (BL9332)
UAS-chameau-RNAi (BL32484)

The *Df(2L)His^C* allele (referred to as Δ *HisC/CyO* in this manuscript), construction of 12*xHisGU*, and the *Df(2L)His^CFRT40A* recombinant were previously described (Günesdogan et al., 2010; Pengelly et al., 2013). They were balanced with *CyO Dfd-GFP* (Le et al., 2006).

The attB 3*xHisGU*^{H3K14R} construct was introduced at the two same third chromosome attP landing sites as 3*xHisGU*^{wt} using phiC31-mediated integration, *y¹ M{vas-phiC31}ZH-2A w**; *attP-68E* and *y¹ M{vas-phiC31}ZH-2A w**; *attP-86Fb* (Bischof et al., 2007). The two transgenic chromosomes were combined by meiotic recombination, and PCR was used to confirm the presence of both transgenes on the recombinant chromosome. The recombinant chromosome was combined with the histone deficiency *Df(2L)His^CFRT40A/CyO Dfd-GFP* (Δ *HisC*) on the second chromosome.

To generate clones containing either transgenic wild-type H3 or the H3K14R mutation in a histone deficiency background, the *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6*xHisGU* or *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6*xHisGU*^{H3K14R} strains were crossed respectively to *yw hs-FLP122*; *hs-nGFP FRT40A*; 6*XHis-GU* or *yw hs-FLP122*; *hs-nGFP FRT40A*; 6*XHis-GU*^{H3K14R} and FLP expression activated by heat shock during late embryonic or early larval stages to induce mitotic recombination events. Wandering third instar larvae lacking the *CyO Dfd-GFP* balancer chromosome were heat-shocked one hour prior to dissection to induce GFP expression.

The expression of *brm-RNAi* or *polybromo-RNAi* was induced by crossing these strains with *nub-Gal4* flies at 25°C, whereas *gcn5-RNAi* and *chameau-RNAi* were crossed to *ptc-Gal4* flies at 18°C.

Drosophila cell line

Drosophila Schneider 2 (S2) cells were maintained in Schneider's Insect Medium supplemented with 10% heat inactivated FBS (GIBCO) and 1% Penicillin-Streptomycin (GIBCO) at 25°C and without CO₂. Cells were harvested for peptide pull-downs at a 90 to 95% confluency.

METHOD DETAILS

Molecular Cloning

Three different pENTR vectors A, B and C (pENTR 221-HisGU, pENTR L4R1-HisGU, and pENTR R2L3-HisGU) containing the histone gene unit that consists of histones H1, H2A, H2B, H3 and H4, were previously described (Günesdogan et al., 2010), and kindly provided by Alf Hertzog. Each pENTR vector was mutated at H3K14 based on the QuikChange Multi site-directed mutagenesis principle using the primer ATCGACTGGTGGACGCGCGCCACGCAAAC. A PCR reaction was set up using Phusion flash polymerase (NEB) together with Taq ligase (NEB) and phosphorylated primer, creating single stranded circular plasmids with a H3K14R mutation. Next, the reaction mix was DpnI-treated and used to transform DH5-alpha cells. Sequencing of the vectors confirmed the H3K14R substitution. The same procedure was repeated for the three different pENTR vectors. MultiSite Gateway cloning (Life Technologies) using the three mutated pENTR vectors and the phiC31attB destination vector resulted in the phiC31attB 3xHisGU^{H3K14R} plasmid.

Survival assay

Embryos from three fly strains, +/CyO *Dfd-GFP* (denoted as wild-type), *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6xHisGU and *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6xHisGU^{H3K14R} were collected for 1 hour, counted, and distributed on new apple juice agar plates supplemented with yeast. For each fly line 10 plates were prepared to monitor their survival for 5 days. The number of embryos developing into different stages was assessed every 24 h. Homozygous and heterozygous offspring from the *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6xHisGU and *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6xHisGU^{H3K14R} strains were differentiated based on GFP expression. Percent survival was calculated at each developmental stage relative to the number of embryos plated on day 0. On day 5, a few larvae were transferred to food vials and monitored for development into pupae and adults.

Western blots

First instar larvae (approximately 25 to 28h AEL) from the *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6xHisGU and *Df(2L)His^CFRT40A/CyO Dfd-GFP*; 6xHisGU^{H3K14R} strains were hand sorted based on GFP expression. Nuclear protein extracts were prepared by grinding these larvae with a dounce homogenizer in Buffer A1 (60 mM KCl, 15 mM NaCl, 15 mM HEPES pH 7.9, 4 mM MgCl₂, 0.5 mM DTT, 0.5% Triton X-100, supplemented with Protease inhibitor cocktail tablets (Roche)). The sample was spun down at 5,000 rpm for 5 min and the pellet washed in the same buffer once. The pellet was then dissolved in SDS-PAGE loading buffer (50 mM Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT). All samples were heated to 95°C for 5 min prior to loading. Proteins were separated in a 15% SDS-PAGE and transferred to a nitrocellulose membrane. The following primary antibodies were used: rabbit anti-H3K14ac (ab52946, Abcam 1:500), rabbit anti-H3K14me3 (gifted by Fei Lan, 1:200), rabbit anti-H3K4me1 (ab8895, Abcam 1:500), rabbit anti-H3K4me2 (07-030, Millipore 1:2,000), rabbit anti-H3K4me3 (ab8580, Abcam 1:1,000), rabbit anti-H3K9ac (ab4441, Abcam 1:5,000), rabbit anti-H3K9me3 (ab8898, Abcam 1:1,000), rabbit anti-H3K18ac (ab1191, Abcam 1:1,000), rabbit anti-H3K27ac (ab4729, Abcam 1:1,000), mouse anti-H3K27me3 (ab6002, Abcam 1:1,000), rabbit anti-H3K36me3 (ab9050, Abcam 1:1,000), mouse anti-H3 (05-499, Millipore 1:1,000), rabbit anti-H3 (ab1791, Abcam 1:2,000), rabbit anti-H4 (ab10158, Abcam 1:1,000) and mouse anti-Lamin Dm0 (ADL67.10, DSHB 1:250). The secondary antibodies IRDye® 680RD Goat anti-Rabbit IgG (925-68071, LI-COR Biosciences) and IRDye® 800CW Goat anti-Mouse IgG (925-32210, LI-COR Biosciences) were used at a 1:10,000 dilution. The Odyssey FC (LI-COR Biosciences) dual-mode imaging system was used for signal acquisition and the Image Studio Lite (LI-COR Biosciences) software for quantification of band intensities.

Immunostaining and RNA *in situ* hybridization

For whole-mount immunostaining and RNA *in situ* hybridization, embryos were collected on apple juice agar plates supplemented with yeast for 6 hours and aged an additional 10 hours. The embryos were washed, dechorionated in bleach and fixed in formaldehyde as previously described (Haecker et al., 2008). Immunostaining of embryos was done in PBS containing 0.3% Triton X-100 and 0.5% BSA using rabbit anti-H3K14ac (ab52946, Abcam 1:500), chicken anti-GFP (ab13970, Abcam 1:2,000) and rat anti-DE-Cadherin (DCAD2, DSHB 1:50). For immunostaining of wing discs, inverted carcasses of wandering third instar larvae (approximately 120h AEL) were fixed in 4% formaldehyde, washed and stained in PBS containing 0.1% Triton-X and 0.5% BSA with the following antibodies: rabbit anti-H3K14ac (ab52946, Abcam 1:1,000), chicken anti-GFP (ab13970, Abcam 1:2,000), mouse anti-Cut (2B10, DSHB 1:40), mouse anti-Patched (Apa 1, DSHB 1:50), mouse anti-Wingless (4D4, DSHB 1:100), mouse anti-Delta (C594.9B, DSHB 1:100), mouse anti-Pebbled (1G9, DSHB 1:15), guinea pig anti-Senseless (gifted by Hugo J. Bellen, 1:800), rabbit anti-Sal (gifted by Adi Salzberg, 1:500), rabbit anti-Brahma (gifted by Lei Zhang, 1:100), rabbit anti-Polybromo (gifted by Peter Verrijzer, 1:400) and rabbit anti-Deadpan (gifted by Sarah Bray, 1:500). Secondary antibodies used were Alexa Fluor 488-conjugated donkey anti-chicken (703545155, Jackson ImmunoResearch Labs), Alexa Fluor 568-conjugated donkey anti-rabbit (A10042, Thermo Fisher

Scientific), Cy3-conjugated donkey anti-mouse (715165151, Jackson ImmunoResearch Labs), Cy3-conjugated donkey anti-guinea pig (706165148, Jackson ImmunoResearch Labs), Cy5-conjugated donkey anti-mouse (715175151, Jackson ImmunoResearch Labs), Alexa Fluor 647-conjugated donkey anti-rat (712605153, Jackson ImmunoResearch Labs) and Alexa Fluor 647-conjugated donkey anti-rabbit (711605152, Jackson ImmunoResearch Labs) at a 1:800 dilution. DAPI was used at a concentration of 1 μ g/mL. Imaginal discs were dissected out and mounted using PBS with 0.1% Triton X-100 and 60% glycerol. Images were acquired with a Zeiss LSM 780 or LSM 800 confocal microscope and sections shown for all stainings except Pebbled/Hindsight which is a projection image.

The whole-mount embryo RNA *in situ* hybridization protocol was adapted from the previously used protocol (Haecker et al., 2008). Anti-sense probes against *CG13492*, *CG18585*, *Mal-A3*, *CG4288*, *CG16712*, *CG31300* and *GFP* (*eYFP*) were generated by PCR from genomic DNA (Table S3) followed by *in vitro* transcription using DIG-UTP labeled RNA. *GFP* was used in combination with *CG13492* or *CG18585* probes to distinguish between homozygous and heterozygous animals for Δ *HisC*. Images were acquired with a Leica DMLB microscope.

Quantification of fluorescence intensity

The fluorescence signal of Cut and Wingless was measured with ImageJ by manually drawing with the Freehand Line tool a 30-pixel line over the region in the wing disc dorsal-ventral boundary where these genes are expressed. The “raw integrated intensity” (the sum of all fluorescence intensity of the selected region) was obtained and this signal was normalized to the total length of the drawn line. To determine the background signal, a 30-pixel line of similar length was also drawn in a region above or below the Cut and Wingless expression domain in the wing disc and the “raw integrated intensity” normalized to the total length of the line. The background signal was subtracted from the fluorescence signal and the resulting values are shown in a scatterplot. The fluorescence intensity of Cut and Wingless from the region within the Patched-expression domain was compared to regions of similar length on the left and right side of this domain using unpaired Student’s *t* test.

Quantification of mutant clone area

The area of mutant clones and twin spots in the wing imaginal discs was quantified using ImageJ. The Freehand tool was used to select the area of the mutant clones or the corresponding twin spots and the Measure function was used to obtain the total area of each. The mutant clone area was plotted relatively to the twin spots area and the size of H3wt clones was compared to H3K14R clones using unpaired Student’s *t* test.

Wing pictures

Wings were isolated from *yw hs-FLP122; Df(2L)His^CFRT40A/hs-nGFP FRT40A; 6XHis-GU* and *yw hs-FLP122; Df(2L)His^CFRT40A/hs-nGFP FRT40A; 6XHis-GU^{H3K14R}* adult flies and mounted in PBS with 0.1% Triton X-100 and glycerol. Images were acquired with a Zeiss Axioplan 2 microscope.

RNA extraction and sequencing

Embryos from the *Df(2L)His^CFRT40A/CyO Dfd-GFP*; *6xHisGU* and *Df(2L)His^CFRT40A/CyO Dfd-GFP*; *6xHisGU^{H3K14R}* strains were collected on apple juice agar plates supplemented with yeast for one hour and aged an additional 12 hours. After dechoriation in bleach, they were hand-sorted according to GFP expression, collected in 300 μ L TRIzol LS Reagent, snap-frozen in liquid N₂ and stored at -80°C until further use. On the day of the extraction, the embryos were homogenized in TRIzol LS Reagent using a 1.5 mL tube pestle and the total RNA was purified using the Direct-zol RNA MicroPrep kit (R2060, Zymo Research). Twenty embryos were used in each replicate. Libraries were prepared using the TruSeq Stranded mRNA LT Library Prep Kit (Illumina), spiked-in with ERCC RNA Spike-In Mix (Invitrogen) and single-end (1x75 bp) sequenced in the NextSeq 550 Sequencing platform (Illumina) at BEA core facility, Stockholm.

ChIP sequencing

For embryo ChIP sequencing, *w¹¹¹⁸* embryos were collected on apple juice agar plates supplemented with yeast for one hour and aged an additional 12 hours. After dechoriation in bleach they were resuspended in a mixture of 2 mL of PBS containing 0.5% Triton X-100 and 6 mL of Heptane. One hundred μ L of 37% formaldehyde solution (F8775, Sigma-Aldrich) was added and embryos were rigorously vortexed for 1 min and then rotated at room temperature for 10 min followed by a spin at 1,000 g for 1 min. Pelleted embryos were collected in 1.5 mL tube and the excess of formaldehyde containing solution was discarded. Finally, 15 min after addition of formaldehyde, crosslinking was halted by resuspension of embryos in 1 mL of PBS containing 0.5% Triton X-100 and 0.25 M Glycine for 5 min at room temperature. After washing three times in PBS containing 0.5% Triton X-100 embryos were snap-frozen and stored at -80°C until further use. Frozen embryos were washed and then homogenized in 1 mL of buffer A1 (see western blots) with 1% Triton X-100 and protease inhibitor (up to 30 μ L embryos/1 mL buffer A1). Chromatin was pelleted and washed three times with buffer A1 and twice with Lysis buffer (15 mM HEPES at pH 7.6, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate). The chromatin was sonicated in 600 μ L of Lysis buffer supplemented with 0.25% SDS and 0.5% N-lauroyl-sarcosine in 15 mL Falcon tubes to obtain average size of $\sim$ 250 bp. Sonication was performed in a Bioruptor (Diagenode) using high

power setting, intervals of 30 s of burst/pause for a total time of 20–25 min. Concentration of detergents was lowered 2.5 times and after clearing by centrifugation at 14,000 rpm for 10 min at 4°C, soluble chromatin was used for ChIP.

For wing disc ChIP sequencing, inverted carcasses of wandering *w¹¹¹⁸* third instar larvae (approximately 120h AEL) were fixed in 1% formaldehyde in PBS supplemented with protease inhibitors for 12.5 min. Formaldehyde was quenched by addition of 0.125 M glycine/PBS. Carcasses were washed with PBS and left in Lysis buffer supplemented with protease inhibitor O/N at 4°C. On the next day, wing discs were dissected in ice cold Lysis buffer and pooled ($\approx$ 80 wing discs) in LoBind tubes (Eppendorf). Samples were sonicated for five min on high power mode (with intervals of 30 s of burst/pause) using a Bioruptor (Diagenode) and afterward SDS and N-lauroylsarcosine were added to a final concentration of 0.5%. Samples were again sonicated on high power for 7.5 min (with intervals of 30 s of burst/pause) $\times$ 6 cycles until an average fragment size range of 200–500 bp was obtained. The solubilized chromatin fraction was cleared by centrifugation, diluted 5 times with Lysis buffer and used for immunoprecipitation. Immunoprecipitation with respective antibodies (1 or 2 μ g anti-H3K14ac, ab52946, Abcam (wing disc or embryo, respectively), 2 μ g anti-H3K14ac, 07-353, Millipore) was carried out at 4°C O/N. A mix of 15 μ L Protein A and 15 μ L G Dynabeads (Invitrogen) blocked with BSA (1 mg/mL) was used to capture the antibody-chromatin complexes. Chromatin and antibody were allowed to form complexes with beads for 6 hours, followed by 10 min washes with Lysis buffer with 0.1% SDS and 0.1% Sodium Deoxycholate, Wash A (Lysis buffer with 0.1% SDS, 0.1% N-lauroylsarcosine and 500 mM NaCl), Wash B (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Sodium Deoxycholate) and Tris-EDTA. Beads were resuspended in 100 μ L TE supplemented with 20 μ g/mL RNase A and incubated at 50°C for 30 min. Tris pH 8.0 and SDS were added to these tubes to a final concentration of 50 mM and 0.1% respectively, and reversal of the cross-links performed at 65°C O/N, followed by protein digestion by Proteinase K treatment. Finally, DNA was purified using ChIP DNA Clean & Concentrator™ (D5205, Zymo Research) and eluted in 65 μ L of DNA elution buffer.

ChIP-seq libraries were prepared using the NEBNext® Ultra II DNA Library Prep Kit for Illumina® (NEB) and single-end (1 $\times$ 75 bp) sequenced in the NextSeq 550 Sequencing platform (Illumina) at BEA core facility, Stockholm.

ATAC-seq and ATAC-qPCR

Embryos from the *Df(2L)His^CFRT40A/CyO Dfd-GFP; 6xHisGU* and *Df(2L)His^CFRT40A/CyO Dfd-GFP; 6xHisGU^{H3K14R}* strains were collected on apple juice agar plates supplemented with yeast for one hour and aged an additional 12 hours. After dechoriation and hand-sorting of embryos according to GFP expression, crude nuclei were isolated by homogenization using a motor pestle in ATAC lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂ and 0.1% IGEPAL) and centrifugation at 700 g for 10 min. The nuclear pellet was resuspended in 22.5 μ L ATAC lysis buffer, 2.5 μ L Tn5 (Tagment DNA Enzyme 1 (TDE1) (Illumina)) and 25 μ L Tagment DNA Buffer (Illumina) and incubated at 37°C for 30 min on thermomixer at 800 rpm. Following transposition, the DNA was purified with Agencourt AMPure XP beads (Beckman Coulter) using a 2:1 volume of beads to sample. Purification was performed according to manufacturer instructions and purified DNA was eluted from beads with 22 μ L Tris-EDTA. Following purification, tagmented DNA was amplified using 1 $\times$ Phusion® High-Fidelity PCR Master Mix with GC Buffer (NEB), 1.25 μ M of the PCR Primer Cocktail (Illumina) and 1.25 μ M of Nextera PCR primers i5 and i7 (Nextera® Index Kit (Illumina) as previously described (Buenrostro et al., 2013). PCR conditions were: 72°C for 5 min; 98°C for 30 s; and thermocycling for 5 cycles at 98°C for 10 s, 63°C for 30 s and 72°C for 1 min. Following amplification, libraries were purified with Agencourt AMPure XP beads (Beckman Coulter) using a 1.5:1 volume of beads to sample. Ten embryos were used in each replicate. Libraries were sequenced (paired-end, 2 $\times$ 75 bp) in the NextSeq 550 Sequencing platform (Illumina) at BEA core facility, Stockholm.

The ATAC libraries prepared above were used in the qPCR reaction, which was performed using the 5 $\times$ HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biotec) and primers in Table S3. Three biological replicates were used per genotype. All samples were normalized to *Actin5C* and a *Rab11* intronic region was used as control.

CUT&Tag

Embryos from the *w¹¹¹⁸* and *Df(2L)His^CFRT40A/CyO Dfd-GFP; 6xHisGU^{H3K14R}* strains were collected on apple juice agar plates supplemented with yeast for one hour and aged an additional 12 hours. After dechoriation and hand-sorting of embryos according to GFP expression, crude nuclei were isolated by homogenization using a glass douncer and a pestle in Nuclear Extraction buffer (20 mM HEPES pH 7.9, 10 mM KCl, 0.5 mM spermidine, 0.1% Triton X-100, 20% glycerol) (Hainer and Fazio, 2019) and centrifugation at 700 g for 10 min. The nuclear pellet was spiked-in with nuclei from HeLa cells (2% of the estimated number of *Drosophila* nuclei) prepared in a similar way and incubated with 20 μ L of BioMag®Plus Concanavalin A beads (Polysciences) for 10 min at 4°C. The resulting nuclei/beads complex was incubated with 1 μ L of primary antibody (rabbit anti-RPB3, gifted by John Lis) in 100 μ L of Antibody buffer (Kaya-Okur et al., 2019), overnight at 4°C. Afterward, the experimental procedure was followed strictly as previously described (Kaya-Okur et al., 2019), using pA-Tn5 kindly provided by Steve Henikoff. Tagmented DNA was amplified using 1 $\times$ Phusion® High-Fidelity PCR Master Mix with GC Buffer (NEB), 1.25 μ M of the PCR Primer Cocktail (Illumina) and 1.25 μ M of Nextera PCR primers i5 and i7 (Nextera® Index Kit, Illumina). PCR conditions were: 72°C for 5 min; 98°C for 30 s; thermocycling for 13 cycles at 98°C for 10 s and 63°C for 10 s and finally one step at 72°C for 1 min. Following amplification, libraries were purified with Agencourt AMPure XP beads (Beckman Coulter) using a 1.1:1 volume of beads to sample. Sixteen embryos were used in each replicate. Libraries were sequenced (paired-end, 2 $\times$ 75 bp) in the NextSeq 550 Sequencing platform (Illumina) at BEA core facility, Stockholm.

Synthetic H3 tail peptides

Non-acetylated and acetylated tail peptides corresponding to residues 3-19 from *Drosophila* histone H3, denoted as H3K14 and H3K14ac, were purchased from Peptide2.0 (95% purity). For peptide pull-down experiments, both H3K14 and H3K14ac were N-terminally (Western-blot) or C-terminally (Tandem mass spectrometry) biotinylated and dissolved in 150mM NaCl, 50 mM Tris 8.0, and 0.1% NP-40. For circular dichroism (CD) spectroscopy, peptides were dissolved and dialyzed overnight at 4°C against CD buffer (20 mM sodium phosphate pH 7.5, 100 mM NaCl, 0.2 mM TCEP) using Biotech CE Dialysis Tubing (VWR). Peptides C-terminally labeled with TAMRA were used for fluorescence anisotropy binding assays. Lyophilized peptides were dissolved in 100 mM HEPES, pH 7.5 at a concentration of 1 mM and stored at –20°C.

Peptide pull-downs

Drosophila S2 cells were washed in PBS and nuclei isolated with a dounce homogenizer in Buffer A1 (see western blots). The sample was spun down at 4,000 rpm for 10 min and the pellet containing the intact nuclei was washed in the same buffer once. For mass spectrometry, the nuclear pellet was snap-frozen in liquid N₂ or freshly analyzed. The frozen or fresh pellet was resuspended in binding buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.25% NP-40, 1 mM DTT, pH 7.5), supplemented with protease inhibitor, 2.5 mM MgCl₂ and Benzonase® Nuclease (Sigma-Aldrich). The sample was then sonicated, centrifuged and the supernatant with approximately 0.8 mg/mL in 1 mL buffer was loaded onto 100 μ L Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) that were saturated with biotinylated H3K14 unmodified or acetylated peptides. After incubation for 2 hours at 4°C, the magnetic beads were washed 3 times with binding buffer (5 min each) at 4°C, and 2 times with high-salt buffer (400 mM NaCl) for 5 min at 4°C. The bound proteins were eluted by boiling at 95°C for 5 min in SDS-PAGE loading buffer. Both frozen and fresh nuclear extracts were used in 3 replicates. However, the eluted samples from fresh nuclear extracts were pooled together, whereas samples from frozen nuclear extracts were analyzed separately by the EMBL Proteomics Core Facility.

For western blot experiments, *w¹¹¹⁸* embryos were collected for 8 hours on yeast supplemented apple juice agar plates, washed and dechorionated in bleach. Nuclear protein extracts were prepared by grinding the embryos with a dounce homogenizer in Buffer A1 (see western blots). The sample was spun down at 5,000 rpm for 5 min and the pellet containing the intact nuclei was washed in the same buffer once. The pellet was resuspended in a hypertonic buffer containing 420 mM NaCl, 20 mM HEPES pH 8.0, 25% glycerol, 15 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitor. The resulting extract of nuclear proteins was used in the peptide pull-down experiments, which were performed according to this protocol (Vermeulen, 2012), with the following conditions: each pull-down reaction contained 100 μ L of Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) saturated with biotinylated H3K14 unmodified or acetylated peptides, and 400 μ g of nuclear extracts in 600 μ L of protein binding buffer (200 mM NaCl, 50 mM Tris pH 8.0, 0.25% NP-40, 0.5 mM DTT and 200 mM TSA). The reaction was incubated for 2 hours at 4°C. Afterward, beads were washed five times for 5-10 min with protein binding buffer with 350 mM NaCl. Proteins were eluted by boiling with SDS-PAGE loading buffer and resolved in a 7.5% SDS-PAGE. For western-blot, rabbit anti-Brahma (1:500) and rabbit anti-Moira (1:500, gifted by Peter Verrijzer) were used to probe the nitrocellulose membrane. The secondary antibody HRP-conjugated goat anti-Rabbit IgG (P0448, Dako) was used at a 1:2,000 dilution and signal was detected using the ECL Select Western Blotting Detection Reagent (Sigma-Aldrich) with a Chemi Doc XRS+ with Image Lab Software (BioRad).

Tandem mass spectrometry

For identification and relative quantification of proteins, samples were subjected to tandem mass spectrometry using Tandem Mass Tag (TMT) Reagents (Thermo Fisher Scientific). The raw protein.txt output files generated by IsobarQuant (Franken et al., 2015) were analyzed using the R programming language (ISBN 3-900051-07-0). For further analysis, only proteins that were quantified with at least two unique peptides were retained. Summed raw TMT reporter ion intensities (signal_sum columns) were first cleaned for batch effects using limma (Ritchie et al., 2015) and subsequently normalized using vsn (variance stabilization normalization - (Huber et al., 2002)). Differential expression of proteins was examined using the limma package. A protein was annotated as a hit with a false discovery rate of less than 5% and a fold-change of at least 100%.

Expression and purification of Brahma

DNA encoding an N-terminal TEV protease cleavage site, followed by residues 1421-1540 of the *Drosophila* ATP-dependent helicase Brahma (Brm), was cloned into pET-15b. Using this construct, Brm protein expression from *E. coli* BL21(DE3)/pLysS (Novagen), grown in Terrific Broth medium at 37°C, was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at OD₆₀₀ = 0.6. Following overnight protein expression at 18°C, bacteria were harvested by centrifugation for 30 min at 5,000 g and pellets were frozen at –80°C. Cell pellets were resuspended in 20 mM HEPES pH 7.5, 300 mM NaCl, 2 mM DTT, 20 mM imidazole, 2.5 mM MgCl₂, supplemented with EDTA-protease inhibitor cocktail (Sigma-Aldrich) and Benzonase® Nuclease (Sigma-Aldrich). Cells were lysed using a sonicator and centrifuged for 30 min at 30,000 g and 4°C. The resulting supernatant was filtered (0.45 μ m filter) and loaded onto a 5 mL-HisTrap HP column (GE Healthcare) pre-equilibrated with the binding buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 2 mM DTT, 20 mM imidazole), and the His-tagged Brm was eluted using a linear imidazole gradient (20–800 mM). The pooled Brm-containing fractions were then injected onto a HiPrep 26/10 desalting column (GE Healthcare) and eluted in binding buffer (with 20 mM imidazole). The 6xHis-tag was cleaved by overnight incubation at 12°C with TEV protease. Following a second, subtractive HisTrap affinity purification to remove the cleaved 6xHis-tag, the Brm-containing flow-through was buffer-exchanged (HiPrep 26/10

desalting column) into cation exchange buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT). The sample was then applied to a HiTrap SP HP (GE Healthcare) column and eluted using a linear salt gradient (100–800 mM NaCl). Brm-containing fractions were loaded onto a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) size exclusion column equilibrated with 20 mM HEPES pH 7.5, 150 mM NaCl, and 1 mM TCEP. Brm was concentrated to 50 mg/mL, snap-frozen in liquid N₂, and stored at –80°C.

Circular dichroism (CD) spectroscopy and fluorescence anisotropy peptide binding assay

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter, equipped with a Peltier element for temperature control. The proteins were measured in a 0.1 cm quartz cuvette at 10 μM in CD buffer (20 mM sodium phosphate pH 7.5, 100 mM NaCl, 0.2 mM TCEP). To measure the binding of H3K14ac peptide, far-UV measurements (260–190 nm) for Brm in the absence and presence of 200 μM H3K14ac peptide were acquired at 20°C in continuous scanning mode with 0.5-nm steps, a bandwidth of 2 nm, and a scan speed of 50 nm/min. Five spectra per sample were accumulated and averaged to improve the signal-to-noise ratio. Thermal dissociation experiments were performed by monitoring the CD signal at 220 nm as a function of temperature. All thermal profiles were acquired in the 20°C–90°C interval. A thermal scan rate of 1°C/min was used for all CD spectroscopy-based temperature perturbation experiments. For both secondary structural and thermal unfolding comparison, the appropriate solvent/peptide spectrum background was subtracted from each of these spectra. Fluorescence anisotropy-based titration experiments were carried out on CLARIOstar Plus microplate reader (LabVision Instruments) at 25°C. Individual wells contained 15 nM TAMRA-labeled peptide and the indicated concentration of Brm in 20 mM HEPES, 100 mM NaCl, 0.5 mM TCEP, pH 7.5. Dissociation constants were determined by fitting the fluorescence anisotropy data assuming a one-site binding isotherm.

Differential expression analysis

After trimming the adaptor sequences using Trimmomatic, the RNA-seq reads from four replicates of mutant *Df(2L)His^CFRT40A;6x-HisGU^{H3K14R}* (H3K14R) embryos, *Df(2L)His^CFRT40A; 6xHisGU* (H3wt), and heterozygous *Df(2L)His^CFRT40A/CyO Dfd-GFP; 6xHisGU^{H3K14R}* (Δ HisC/+;H3K14R) samples were mapped to the *Drosophila melanogaster* (dm6) genome assembly using HISAT2 with default parameters. One of the four replicates of Δ HisC/+;H3K14R sample was an outlier detected by PCA analysis and was removed from the subsequent analysis. We performed RNA-seq differential expression analysis (DE) of H3K14R versus H3wt and H3K14R versus Δ HisC/+;H3K14R using DESeq2 Bioconductor package. Differentially expressed genes were identified and the false discovery rate (FDR, Benjamini-Hochberg) was estimated (presented in Table S1). We identified a set of highly differentially expressed genes between H3K14R and both controls with a FDR < 10% and fold change $\geq$ 2-fold. A pathway enrichment analysis was performed for these highly up- and downregulated genes by HOMER2. To test whether a set of genes are significantly changed (up- or downregulated as a gene set) among the differentially expressed (DE) genes from controls and mutant RNA-seq data, the gene set enrichment fgsa Bioconductor package was used.

ChIP-seq analysis

H3K14ac ChIP and Input reads from *w¹¹¹⁸* 12–13h AEL embryos and *w¹¹¹⁸* third instar larvae wing discs were mapped to the *Drosophila melanogaster* (dm6) genome assembly using Bowtie2 with default parameters, after adaptor trimming by Trimmomatic. The uniquely mapped reads with a mapping quality MAPQ > 20 were used for further analysis. We generated coverage tracks for ChIP samples, which were normalized to the library sizes and Input samples. We performed peak calling of the ChIP-seq reads against the Input reads with the HOMER2 peak caller, using the parameters “-style histone -fdr 0.001 -F 4 -L 4 -C 2” for embryos, and the parameters “-style histone -fdr 0.05 -F 1.8 -L 2 -C 2” for wing discs. For comparison, ChIP-seq data for H3K9ac, H3K27ac and H3K4me3 from 12–16h AEL embryos was obtained from modENCODE (GSE16013). The data was mapped to dm6 and the same parameter as H3K14ac (“-style histone -fdr 0.001 -F 4 -L 4 -C 2”) was used for peak calling. Similarly, we obtained H3K27ac (GSM3185650) and H3K4me2/3 (GSM1446261) ChIP-seq data for wing discs and used the parameters “-style histone -fdr 0.05 -F 1.8 -L 2 -C 2” for peak calling. As H3K27ac wing disc ChIP-seq data does not have the Input sample as control, we selected the peaks with > 100 peak scores for further analysis. Peaks were annotated using HOMER2 package, and the genomic locations where defined as: 1. Promoter-TSS (–2k to +1kb), 2. TTS (–100 bp to +1kb), 3. CDS, 4. 5'UTR, 5. 3'UTR, 6. Introns, 7. Upstream 10k (–10k to –2k) 8. Intergenic. In case of the overlapping annotations for a peak, the priority is given to 1–8 as above. To simplify genomic locations of the peaks in the pie chart, we combined peaks in CDS and UTRs as exons, and exclude the peaks in TTS and non-coding RNAs which consists of small fraction of peaks. To overlap peaks from different factors, we merged the peaks by “mergePeaks” function in the HOMER2 package and set a maximum distance to “-d given” which requires the peak regions to overlap. For comparison, we reanalyzed public ChIP-seq data for H3K36me3 14–16h embryo (GSE47256) and H4K14ac 14–16h embryo (GSE130335), using the same parameters for mapping and peak callings as H3K14ac embryo ChIP-seq. We further analyzed human ENCODE H3K14ac ChIP-seq peaks with H3K9ac, H3K27ac and H3K4me3 in embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs) and IMR-90 lung fibroblasts. We used the peak calls from the ENCODE analysis pipeline (<https://www.encodeproject.org/>) and overlapped peaks from different modification factors using “mergePeaks” in the HOMER2 package.

ATAC-seq analysis

The ATAC-seq samples of *Df(2L)His^CFRT40A; 6xHisGU* (H3wt) and *Df(2L)His^CFRT40A; 6xHisGU^{H3K14R}* (H3K14R) stage 15 embryos in three biological replicates were mapped to the *Drosophila melanogaster* (dm6) genome assembly using Bowtie2 with default

parameters after adaptor trimming by Trimmomatic. The high quality and uniquely mapped reads (MAPQ $\geq$ 20) were used for further analysis. ATAC-seq differential accessibility analysis between mutant and wild-type on the promoter regions (–100 bp to +100 bp of TSS) and gene bodies (500 bp from TSS to 100 bp upstream of TES) was performed with the DESeq2 package.

CUT&Tag analysis

The Pol II CUT&Tag samples in wild-type (*w¹¹¹⁸*) and *Df(2L)HisCFRT40A; 6xHisGU^{H3K14R} (H3K14R)* stage 15 embryos in three replicates were mapped to dm6 using Bowtie2 (bowtie2–end-to-end–very-sensitive–no-mixed–no-discordant–phred33 -l 10 -X 700). Spike-in reads were mapped to the human (hg38) genome assembly. CUT&Tag differential analysis between *H3K14R* and wild-type on the promoter regions (–100 bp to +100 bp of TSS) was performed with the DESeq2 package. The spike-in reads were used for normalization in the differential analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

For all quantification analyses, statistical tests performed are described in the corresponding figure legends or in the methods.