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Report

Transcription in the Absence of Histone H3.3

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Summary

Di- and trimethylation of histone H3 lysine 4 (H3K4me2 and H3K4me3) are hallmarks of chromatin at active genes [1]. The major fraction of K4-methylated histone H3 is the variant H3 (termed H3.3 in *Drosophila*) [2], which replaces canonical H3 (H3.2) in transcribed genes [3, 4]. Here, we genetically address the in vivo significance of such K4 methylation by replacing wild-type H3.3 with a mutant form (H3.3K4A) that cannot be methylated. We monitored the transcription that occurs in response to multiple well-described signaling pathways. Surprisingly, the transcriptional outputs of these pathways remain intact in H3.3K4A mutant cells. Even the complete absence of both H3.3 genes does not noticeably affect viability or function of cells: double mutant animals are viable but sterile. Fertility can be rescued by K4-containing versions of H3.3, but not with mutant H3.3 (H3.3K4A) or with canonical H3.2. Together, these data suggest that in *Drosophila*, presence of H3.3K4me in the chromatin of active genes is dispensable for successful transcription in most cells and only plays an important role in reproductive tissues.

Results and Discussion

A major open question in our understanding of the design and evolution of multicellular organisms is how cells activate and maintain gene expression in response to extracellular cues. The transcription of genes is a DNA-templated process, which requires dynamic changes at the chromatin level. Posttranslational modifications on the histone tails are critical to regulating chromatin composition (reviewed in [5]). Acetylation and methylation are two well-established modifications of certain histone residues and form the basis of a "histone code," which has been postulated to modulate gene transcription. A prominent hallmark of active transcription is the trimethylated state of lysine 4 in histone H3 (H3K4me3; for review, see [6, 7]).

In most eukaryotes, there are two different noncentromeric H3 proteins, and these appear to fulfill distinct functions. The canonical histone H3 (termed H3.2 in *Drosophila*) is synthesized only during S phase, when it is deposited onto newly replicated DNA. The variant histone H3 (H3.3 in *Drosophila*) is synthesized in a replication-independent manner and has been shown to replace canonical H3 at actively transcribed genes in *Drosophila* [3, 4, 8]. The major fraction of K4-methylated histone H3 is the variant H3 (H3.3) [2].

Together, these lines of evidence attribute a key role to the methylation state of K4 in variant H3.3 for the transcription of eukaryotic genes. However, the basis for this notion is mainly of a correlative nature, and no direct causal relationship has yet been established between H3K4me3 and gene transcription. Here, we set out to examine this important issue by genetic means in the model organism *Drosophila*. We aimed to replace H3.3 with a mutant form of H3.3 in which methylation of residue 4 could no longer take place and then to examine the extent to which initiation and maintenance of gene transcription would be affected. We focused our analysis on the transcriptional state of target genes of well-established developmental signaling pathways; recent findings indicate that the regulation of such genes involves histone-modifying enzymes (e.g., the lysine methylases Trx and Ash1 in *Drosophila* [9–12] or Bre1, the E3 ubiquitin ligase required for the monoubiquitination of histone H2B and subsequent methylation of H3K4 [13–15]), as well as "readers" of such modifications (e.g., PHD fingers recognizing the H3K4 methylation state [16–20]).

To produce a situation in which H3.3K4 cannot be methylated, we needed the following three genetic tools: First, we generated null alleles for both *Drosophila* genes that encode H3.3 (*His3.3A* on chromosome 2 at position 25C and *His3.3B* on the X chromosome at position 8C) by imprecise excision of a P element [21] and Flp recombinase-mediated gene deletion [22], respectively (Figures 1A, 1B, and 1E). Second, we assembled transgenes that expressed H3.3 under the native regulatory elements of the *His3.3B* gene. These transgenes were designed to allow the in vivo replacement of the wild-type coding region with that of a mutant version (e.g., H3.3K4A) by inducible Flp-mediated recombination (Figure 1C). Third, we inserted short, distinct epitope tag sequences into the wild-type and mutant coding regions so that the resulting proteins could be identified in vivo (Figure 1C).

We examined adult head extracts, larval polytene chromosomes from transgenic animals, and imaginal wing discs to ascertain that the tagged H3.3 wild-type protein, as well as the K4A version, was expressed (Figure 2A and data not shown), incorporated into chromatin (Figure 2C and data not shown), and clonally expressed upon Flp-mediated recombination (Figure 2D).

Next we combined the *His3.3A* and *His3.3B* null alleles and the $His3.3B_{promoter} > H3.3WT^{FLAG}$,*ubiGFP* > $H3.3K4A^{HA}$ transgene to generate animals in which imaginal disc cell clones could be induced that express the K4A version instead of the wild-type form (see Experimental Procedures). The transcriptional output of four major signaling pathways was analyzed in such clones: Wnt/Wingless (Wg), Notch, TGF-β/Decapentaplegic (Dpp), and Hedgehog (Hh). As a readout for Wg signaling, we monitored Senseless (Sens) expression, which occurs in two narrow stripes of cells along the dorsoventral (DV) boundary of the mid- to late-third-instar wing disc [23]. *cut* (*ct*) is a target gene for Notch signaling expressed in the cells along the DV boundary [24]. *spalt* (*sal*) represents a downstream target of Dpp signaling and is active in response to the inverse Dpp and Brinker gradients in the central region of the wing pouch [25]. Finally, *patched* (*ptc*) served as a representative target gene of the Hh pathway. It is expressed in a stripe along the anteroposterior (AP) boundary [26].

Surprisingly, we did not observe a change in the level or pattern of target gene expression for any of these pathways in H3.3K4A mutant cells (Figure 3). This indicates that the

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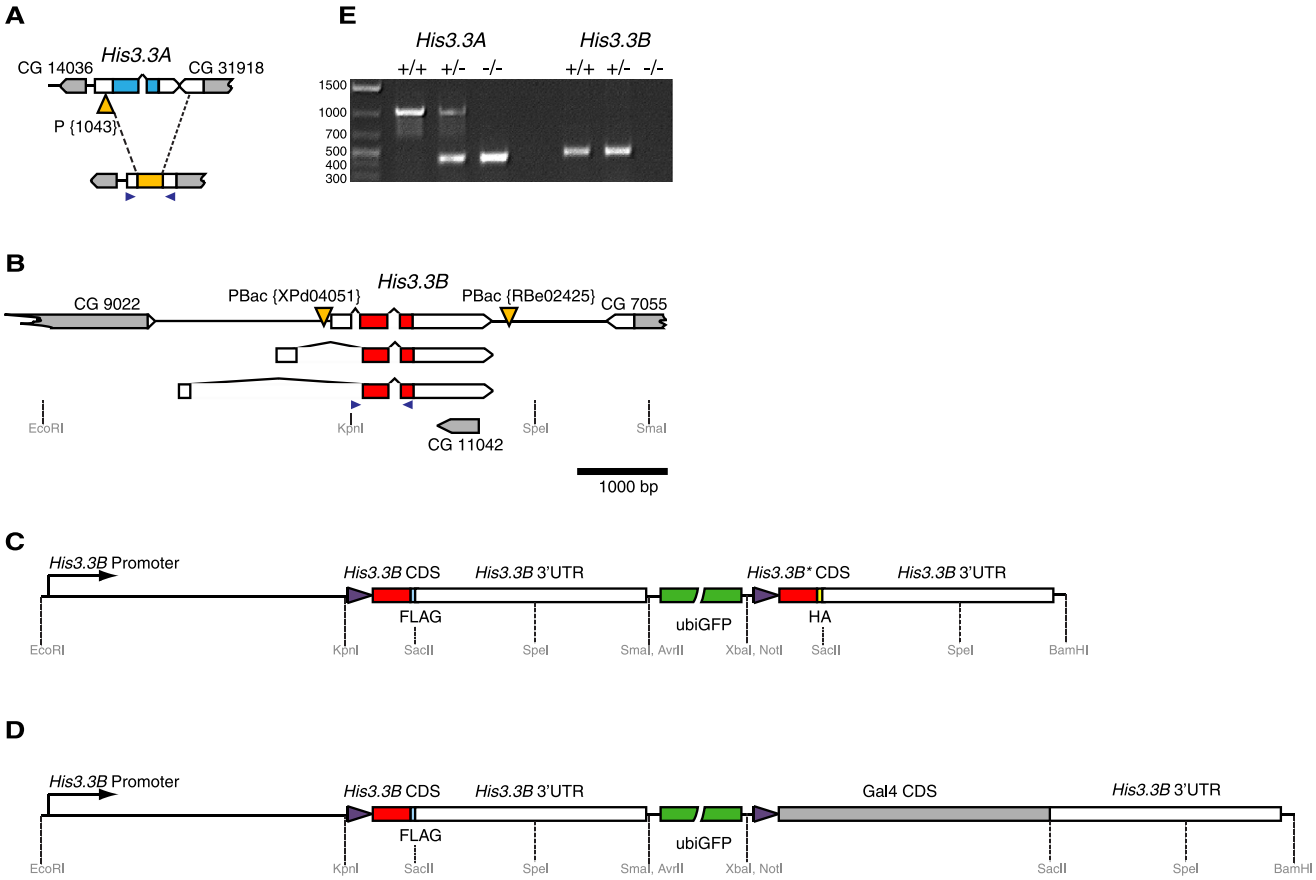

Figure 1. Genetic Tools for Analysis of H3.3K4me

(A) The endogenous *His3.3A* locus (coding sequence [CDS] shown in blue) was deleted via imprecise excision of the P element *P{1043}*. The deletion allele retains 300 bp of left P element flank (orange) joined to the 3′ untranslated region (3′UTR) of the downstream gene *CG31918*. Primers used for verification of the mutant allele are indicated by blue arrowheads.

(B) The endogenous *His3.3B* locus comprising three transcript isoforms. A deletion was generated via FRT-mediated recombination between P elements *{RBe02425}* and *{XPd04051}*, resulting in loss of the complete H3.3B CDS (red). The gene *CG11042* is also deleted but is reintroduced in the genomic *His3.3B* rescue construct. The indicated restriction sites were used for cloning. Primers used for verification of the mutant allele are indicated by blue arrowheads.

(C) Rescue construct assembled from *His3.3B* sequences. Wild-type FLAG-tagged H3.3B is expressed under its endogenous regulatory sequence. *ubiGFP* (*ubiquitin* promoter, EGFP CDS, *Tubulin*α*1* 3′UTR) was inserted to mark cell clones. FRT sites flanking the wild-type *His3.3B* and the *ubiGFP* allow recombination-mediated expression of a promoterless, modified HA-tagged *His3.3B**. For cloning strategy, see Experimental Procedures.

(D) Transgene version in which the second copy of the H3.3B CDS is replaced by the yeast transcription factor Gal4 CDS (gray). After FRT-mediated recombination, cell clones lose GFP and H3.3B expression from the transgene but gain Gal4-driven expression of additional UAS transgenes.

(E) PCR verification of *His3.3A* and *His3.3B* mutant loci. PCR for the *His3.3A* mutant allele amplifies a shorter product compared to wild-type. Heterozygous animals show both products; the unequal intensity in bands most likely reflects a competitive advantage of the short fragment during PCR. Primers for *His3.3B* detect the major part of the H3.3B CDS, present in wild-type and heterozygous mutant animals but absent in *His3.3B* homozygous mutant animals.

transcriptional output of these signaling pathways is not significantly influenced by the methylation state of H3.3K4. A potential caveat to this interpretation is perdurance of wild-type H3.3 protein in mutant cell clones. Based on incorporation studies of GFP-tagged H3.3, the half-life of bulk chromatin-bound H3.3 protein in postmitotic cells has been estimated to be about 24 hr [4]. From the time point of clone induction (12–36 hr after egg deposition) to dissection of third-instar larvae, cells in the imaginal disc undergo massive proliferation. We believe that wild-type H3.3 protein levels must be negligibly low in the cells that we analyzed because even the dilution caused by cell proliferation alone must lead to a decrease of two to three orders of magnitude.

To further address the requirements of H3.3 methylation, we also assayed lysine 9 (K9), another prominent site of histone H3 protein methylation, which is associated with gene repression rather than active transcription [2]. Substituting wild-type H3.3 by H3.3K9A also did not cause any change in the above-described signaling readouts (data not shown).

Because K4A and K9A mutations did not show any effect on transcriptional activation, repression, or maintenance, we next asked whether any other modification or activity of H3.3 might be required for cells to properly control gene expression in response to extracellular signals. For this, we clonally removed the entire H3.3 coding sequence and replaced it with that of the yeast transcription factor Gal4 (Figure 1D). Just like substitution of H3.3 with its K4A or K9A derivatives, complete removal of H3.3 had no discernible effect on the output of the Wg, Notch, Dpp, and Hh pathways (Figures 3D and 3H).

Although it was unlikely, we wanted to rule out the possibility that previously incorporated H3.3 protein might account for a functional chromatin composition at actively transcribed loci in mutant cells. To this end, we generated animals that were entirely null for H3.3, lacking both *His3.3A* and *His3.3B*

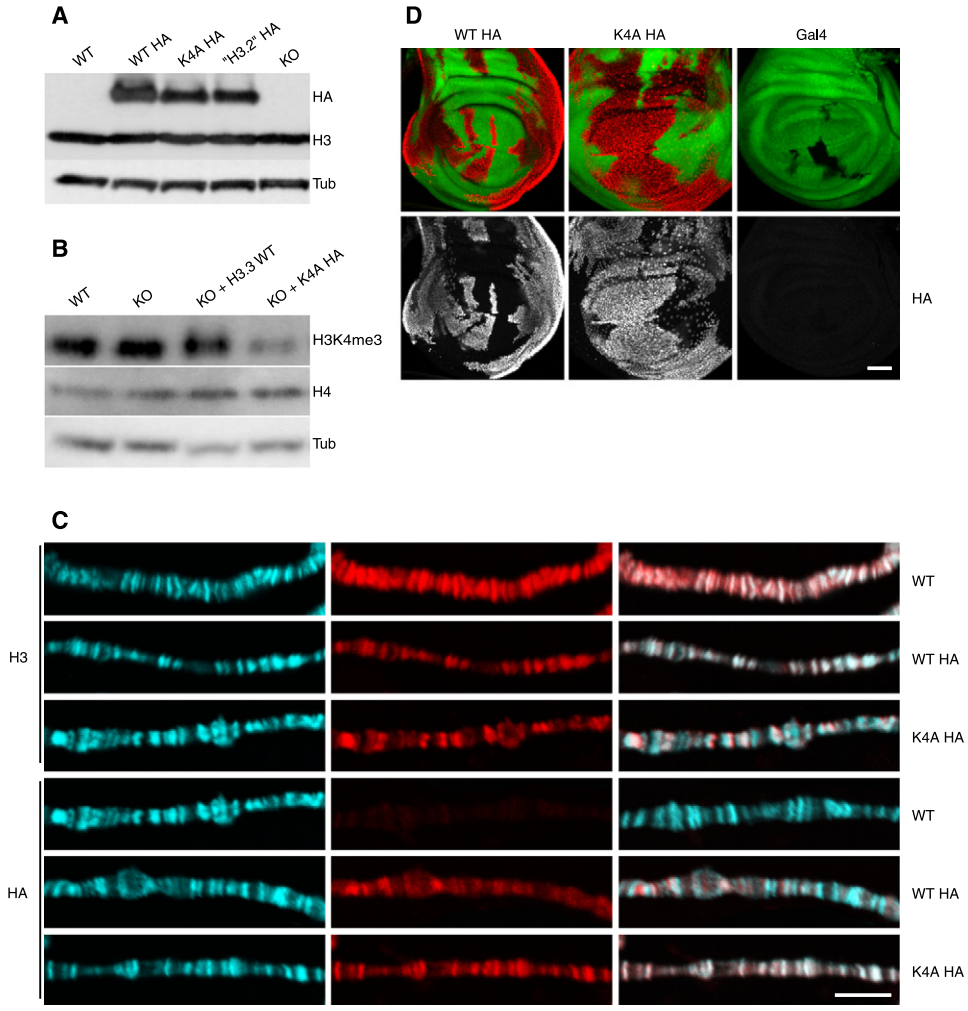

Figure 2. Transgene Versions Are Expressed, Incorporated into Chromatin, and Specifically Expressed after Flp-Mediated Recombination

(A) Western analysis of adult head extracts monitors expression of HA-tagged wild-type, K4A, or "H3.2" versions of H3.3. Transgenes were expressed in the wild-type background. Tubulin and total H3 serve as loading controls. KO indicates double mutants of the genotype *His3.3B*$^{-}$*hsp70-flp;His3.3A*$^{-}$*/His3.3A*$^{-}$.

(B) Analysis of bulk H3K4me3 levels by western blotting. Head extracts of wild-type and mutant animals were probed with antibodies against H3K4me3, H4, and tubulin.

(C) Immunostaining of polytene chromosomes reveals incorporation of the tagged H3.3 versions into chromatin. Transgenes were expressed in the wild-type background. DNA is stained with DAPI (cyan); total H3 and the HA epitope are stained with antibodies (red). Scale bar represents 10 μm.

(D) Flp-mediated recombination within the rescue construct leads to expression of H3.3HA (red) in cell clones. Clones were induced 12–36 hr after egg deposition. Third-instar wing imaginal discs were stained with anti-HA antibody. Loss of GFP indicates the recombination event. Induced, HA-tagged versions of H3.3 are either wild-type or K4A. Gal4 (untagged) serves as a negative control for the specificity of the antibody staining. Scale bar represents 50 μm.

genes. Astonishingly, although such animals show a sterility phenotype in both sexes, they are viable to the adult stage, with no obvious morphological defects. Maternal H3.3 product can be ruled out as an explanation because the rare offspring from homozygous double-mutant female germline clones are phenotypically normal, irrespective of whether they paternally inherit *His3.3* function or not (see Experimental Procedures). Thus, both constitutive transcription and signal-induced transcription of *Drosophila* genes occur apparently normally in the absence of variant histone H3.3.

H3.3 has previously been implicated in germline functions in flies [27, 28], worms [29], plants [30], ciliates [31], and mice [32]. Because H3.2 can apparently take over H3.3 function in somatic cells, we reasoned that one explanation for the failure to rescue germline function may be expression levels. To investigate this, we converted H3.3 into "H3.2" by altering the four amino acids in which the proteins differ (S31A, A87S, I89V, and G90M). Although now expressed like H3.3 outside the S phase, the "H3.2" from the *His3.3B*$_{promoter}$ > *H3.2WT*^{HA} transgene still failed to rescue the fertility of *His3.3* double-mutant animals, although the equivalent control construct (*His3.3B*$_{promoter}$ > *H3.3WT*^{HA}) restored fertility (Table 1). Hence, it is not a mere quantitative insufficiency of generic H3 function that causes sterility of *His3.3* double mutants. Rather, it appears that the variant histone H3.3 plays a specific role in male and female reproductive development.

Finally, we asked whether this latter, specific function of H3.3 depends on its methylation state and tested the rescue activity of the H3.3K4A^{HA} and H3.3K9A^{HA} transgenes (Table 1). Whereas H3.3K9A restored male fertility, H3.3K4A did not, suggesting that K4, although dispensable in most of the soma, plays an important role in germline function.

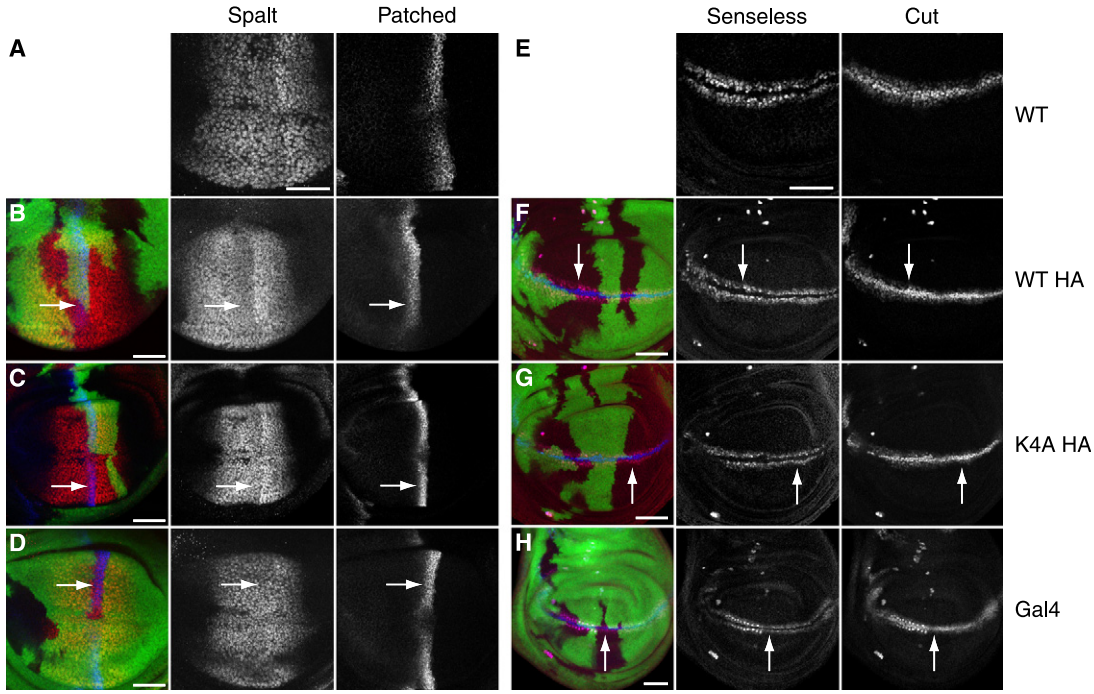

Figure 3. Transcriptional Outputs of Signaling Pathways Are Not Affected in Mutant H3.3 Clones

Clones were induced 12–36 hr after egg deposition. Third-instar wing imaginal discs were stained with antibodies against Spalt (red) and Patched (blue) (A–D) and against Senseless (red) and Cut (blue) (E–H). GFP (green) is specifically lost in clones, which express a modified version of H3.3. Wild-type H3.3 was replaced by another wild-type H3.3 (B and F), H3.3K4A (C and G), or Gal4 (D and H). Fluorescence levels are not altered in GFP^+ versus GFP^- clones. Arrows indicate representative positions in clones. Discs are shown with the anterior to the left and dorsal on top. Scale bars represent 50 μm.

Conclusions

Because variant histone H3.3 is highly enriched in transcribed regions and trimethylation of H3K4 is a hallmark of transcriptionally active genes, K4 of variant H3.3 has emerged as a major focal point in understanding the mechanistic interdependence between transcription and chromatin modification. In our genetic attempts to address the functional significance of H3.3K4me, we found that it is dispensable for most transcriptional events throughout development of *Drosophila*, including the upregulation and maintenance of gene expression that occurs in response to extracellular cues. In the course of these experiments, we found that even H3.3 itself is nonessential in these transcriptional events. Importantly, our assays do not allow us to extend this conclusion to K4 methylation per se, because we cannot, for technical reasons, replace canonical H3 with a nonmethylatable K4A form (the *Drosophila* genome harbors 23 copies of *His3.2*). Biochemical analysis indicates that the levels of H3K4me3 are unchanged in H3.3 null mutant animals (Figure 2B); this must stem from H3.2 substituting for the absent H3.3. Interestingly, H3.3K4A mutants show significantly reduced H3K4me3 levels, indicating that, when present, the variant H3.3 functions as the major carrier for H3K4me. Because the H3.3K4A mutants are morphologically normal, it seems that bulk H3K4me levels can be reduced without obvious consequences for somatic transcription.

The sterility of our double-mutant animals confirms the proposed role of H3.3 in reproductive development [27, 28]. Our results suggest not only a specific requirement of variant histone H3.3 but also a dedicated function of K4, indicating that methylation of this residue is critical for this role of H3.3. It seems that correctly balanced levels of H3K4me are an important feature in the germline of *Drosophila*: decreased levels of H3K4me appear to be detrimental, as are increased levels, because male sterility and severely impaired ovary development have also been observed in mutants for the H3K4 demethylase Lsd1 [33].

Finally, H3.3K4 has been found to serve as the transmitter of preexisting epigenetic information during nuclear transplantation experiments [34]. It is tempting to speculate that a major function of H3.3K4me could be to provide subsequent generations with an epigenetic memory.

Table 1. Ability of H3.3 Transgenes to Rescue Fertility

Male × WT Female	Number of Offspring
WT	220
His3.3 double mutant	0
H3.3 untagged	243
H3.3 wtHA	278
H3.3K4A HA	0
H3.3K9A HA	212
"H3.2" HA	0

Transgenic rescue constructs are present in a single copy in an otherwise *His3.3* double-mutant background. Seven virgin WT females were precrossed with seven males of the indicated genotypes for 3 days and then allowed to deposit eggs for 24 hr in a fresh tube. Offspring were counted in larval stage (second and third instar).

Experimental Procedures

Drosophila Stocks and Genetics

P element insertion line *P{RS5}5-HA-1043* (Szeged Drosophila Stock Centre; Szeged, Hungary) was used to generate the imprecise excision line for *His3.3A*. P element insertions *PBac{RB}e02425* and *P{XP}His3.3B d04051* (Harvard University) were used to generate the FRT-mediated deletion allele of *His3.3B*. The following primers were used to verify the deletions: *His3.3A*,

5′-TGAATGCATTTACTACATGG-3′ and 5′-TAGGGTCACACTGAGCAGAC GC-3′; *His3.3B*, 5′-GCGAATTCGAGAAATTCATATGAGTTGGATAACC-3′ and 5′-TCTGTGTCAATCTGGAACGC-3′.

Germline clones were induced by Flp-mediated mitotic recombination in females of the genotype *His3.3B^{-}hsp70-flp/His3.3B^{-}hsp70-flp;His3.3A^{-} FRT40A/P[ovo^{D1}]FRT40A*, which were fertilized with males of the genotype *His3.3B^{-}hsp70-flp;His3.3A^{-}/SM6B*.

Cloning

For the untagged rescue construct, a 9 kb endogenous sequence for *His3.3B* was retrieved from BAC *N01M09* (Children's Hospital Oakland Research Institute) in a BamHI digest, from which a final fragment (EcoRI-SmaI, 6.5 kb) was subcloned.

Modifications (FRT sites and FLAG/HA epitopes) were introduced via polymerase chain reaction (PCR) with the following primer sets: KpnI-SacII FLAG, 5′-ATTTGCGGCCGCGGTACCAACCAAAGAAGTTCCTATACTTTCTAGAGA ATAGGAACTTCCAATGATTGTAGAAGTGTTT-3′ and 5′-GACCCGCGGTTA CTTGTCATCGTCGTCCTTGTAGTCGCTAGCAGCACGCTCGCCACGGATG CG-3′; KpnI-SacII HA, 5′-ATTTGCGGCCGCGGTACCAACCAAAGAAGTTC CTATACTTTCTAGAGAATAGGAACTTCCAATGATTGTAGAAGTGTTT-3′ and 5′-GACCCGCGGTTATGCATAGTCCGGGACGTCATAGGGATAGCTAGCAG CACGCTCGCCACGGATGCG-3′; SacII-SpeI, 5′-GACCCGCGGGGTGGATC AGCAGGAACGCCAGATTCG-3′ and 5′-ATACGAATAGACACATATTTAGT CCG-3′.

QuikChange Site-Directed Mutagenesis (Stratagene) was used to introduce the following amino acid changes: K4A mutation, 5′-CAAACATG GCTCGTACTGCGCAGACTCCCGTAAGTC-3′ and 5′-GACTTACGGGCAGT CTGCGCAGTACGAGCCATGTTTG-3′; "H3.2" construct S31A, 5′-GTAAAT CGGCGCCAGCCACCGGCGGAG-3′ and 5′-CTCCGCCGGTGGCTGGCGCC GATTTAC-3′. A87S, I89V, and G90M were introduced via two PCR products and a triple ligation because the newly established sequence harbors an EaeI site: PCR 1, 5′-ATTTGCGGCCGCGGTACCAACCAAAGAAGTTCCTATA CTTTCTAGAGAATAGGAACTTCCAATGATTGTAGAAGTGTTT-3′ and 5′-CC TGCAAGGCCATGACGGCCGACGACTGGAAACGCAG-3′ (cut KpnI, EaeI, 430 bp); PCR 2, 5′-GACCCGCGGTTATGCATAGTCCGGGACGTCATAGGG ATAGCTAGCAGCACGCTCGCCACGGATGCG-3′ and 5′-CTGCGTTTCCAG TCGTCGGCCGTCATGGCCTTGCAGG-3′ (cut EaeI, AatII, 295 bp).

For introducing the Gal4 coding sequence, the H3.3B start codon was converted into a SacI restriction site (QuikChange Site-Directed Mutagenesis Kit, Stratagene) with the primers 5′-CAGTTTAAAAAAAAAAGCTAAGAAAA GAGCTCTCGTACTAAGCAG-3′ and 5′-CTGCTTAGTACGAGAGCTCTTTTC TTAGCTTTTTTTTTTAAACTG-3′. The Gal4 coding sequence was amplified with the primers 5′-ACGAGCTCAGCCTCCTGAAAGATGAAGC-3′ and 5′-AG CCGCGGAGCTTCTGAATAAGCCCTCG-3′ and cloned into the vector via SacI and SacII sites in the PCR overhang.

A *ubiGFP* fragment (ubiquitin promoter, EGFP coding sequence, *Tubulinα1* 3′ untranslated region) was integrated via NheI and XbaI sites.

Rescue constructs were integrated into the fly genome at landing site 51D via the phiC31/attB integration system [35].

Immunohistochemistry

Polytene chromosome staining was performed as described previously [36]. Antibodies used were mouse anti-HA (HA.11, Covance, 1:500), rabbit anti-H3 (Abcam 1791, 1:500), and Alexa Fluor 594 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (Molecular Probes, 1:500).

Immunostaining of wing imaginal discs was performed via standard protocols. Antibodies used were guinea pig anti-Sens (GP55, 1:800; gift from H. Bellen, Baylor College of Medicine, Houston), mouse anti-Ct (Developmental Studies Hybridoma Bank [DSHB], 1:20), rabbit anti-Sal (1:50; gift from R. Schuh, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), mouse anti-Ptc (DSHB, 1:100), and secondary antibodies Alexa Fluor 594 goat anti-mouse, Alexa Fluor 594 goat anti-rabbit, and Alexa Fluor 568 goat anti-guinea pig (Molecular Probes, 1:400) and goat anti-mouse Cy5 (Jackson ImmunoResearch, 1:400).

Images were acquired with Leica TCS SP1 and TCS SP5 confocal microscopes, and image processing was performed in Adobe Photoshop 7.0.

Western Blotting

Crude protein extracts were obtained by directly grinding 20 adult fly heads in 70 μl NuPAGE LDS sample buffer (Invitrogen). Western blotting was performed with mouse anti-Tub (DM1A, Sigma, 1:5,000), rabbit anti-H3 (Abcam 1791, 1:10,000), mouse anti-HA (HA.11, Covance, 1:5,000), rabbit anti-H3K4me3 (Abcam 8580, 1:5,000), and rabbit anti-H4 antibodies (Upstate 05-858, 1:50,000) followed by chemiluminescence detection.

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