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# A genetic system to assess *in vivo* the functions of histones and histone modifications in higher eukaryotes

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**Despite the fundamental role of canonical histones in nucleosome structure, there is no experimental system for higher eukaryotes in which basic questions about histone function can be directly addressed. We developed a new genetic tool for *Drosophila melanogaster* in which the canonical histone complement can be replaced with multiple copies of experimentally modified histone transgenes. This new histone-replacement system provides a well-defined and direct cellular assay system for histone function with which to critically test models in chromatin biology dealing with chromatin assembly, variant histone functions and the biological significance of distinct histone modifications in a multicellular organism.**

Keywords: histone deletion; histone transgenes; functional assay; *Drosophila melanogaster*

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## INTRODUCTION

Eukaryotic genomes are packaged into arrays of nucleosomes composed of 147-base-pair DNA intervals wrapped around an octameric complex of the core histones H2A, H2B, H3 and H4 (Luger *et al*, 1997). Together with the H1-type linker histones, these proteins are termed canonical histones, as they constitute the vast majority of histone proteins in chromatin (Marzluff *et al*, 2008). Concomitant with genome duplication during S-phase of the cell cycle, disassembled parental nucleosomes and *de novo* synthesized histones are used as distinct sources for replication-coupled nucleosome assembly (Corpet & Almouzni, 2009). In a subset of nucleosomes, the canonical histones are subsequently replaced by histone variant proteins by replication-independent processes, which can specify functionally distinct chromatin regions (Henikoff & Ahmad, 2005). The complexity of chromatin diversification is further increased by the many post-translational modifications of histones, which are thought to constitute an epigenetic 'histone code' (Jenuwein & Allis, 2001; Kouzarides, 2007). Despite the central role of histones in chromatin assembly and diversification, no experimental tools have been developed so far to directly address canonical histone divergence from replacement variants and the post-translational modifications in multicellular organisms. In fact, genetic analysis has been limited to lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*, in which histone genes are encoded by tandem repeats (Marzluff *et al*, 2008). In all higher eukaryotes, however, canonical histones are encoded by multiple gene units of between 10 and 400 copies that are mostly distributed over several chromosomes (Marzluff *et al*, 2008). The high number of histone genes and their distribution within the genome prevent straightforward functional genetics and explains the correlative nature of many previous studies in the field (Kouzarides, 2007). As the yeast model system is clearly not suitable to address chromatin diversification and its function in the context of the complex multicellular development of higher organisms, we developed an experimental system for the model organism *Drosophila melanogaster* that serves as a tool to directly assess histone functions by molecular genetics and by transgene-dependent rescue.

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## RESULTS AND DISCUSSION

### Generation of a molecularly defined histone deficiency

*D. melanogaster* is an ideal model organism in which to develop an experimental system to address fundamental questions regarding histone function for several reasons. The genetic toolset in *D. melanogaster* is highly advanced, the fly undergoes a complex but well-characterized development and, most importantly, all canonical histone genes are clustered in a single chromosomal locus. This arrangement is distinct from that in other multicellular organisms, including genetically accessible systems such as the mouse and nematodes, in which the histone genes are distributed throughout the genomes (Marzluff *et al*, 2008). According to the genome annotation, the histone gene complex of *D. melanogaster* is composed of 23 canonical histone gene repeat units (His-GUs), each bearing a gene array encoding the five canonical histones

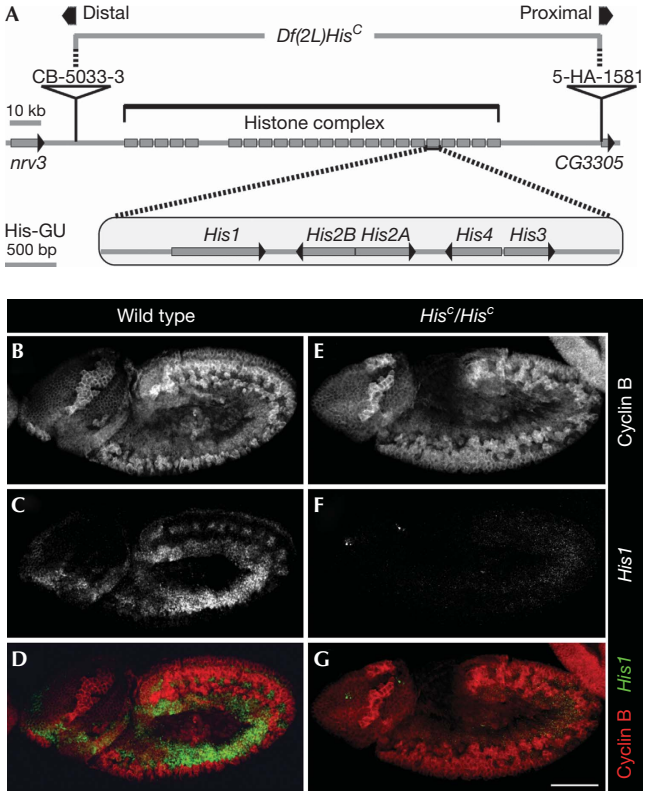


**Fig 1** | *Df(2L)His^C* deletes all canonical histone genes. (**A**) Schematic representation of the canonical histone gene organization in *Drosophila melanogaster*. A total of 23 histone gene repeat units, each containing a single *His1*, *His2B*, *His2A*, *His4* and *His3* gene (His-GU), are clustered in the histone complex. The deletion of the histone complex includes the region between the distal rearrangement screen element CB-5033-3 and the proximal rearrangement screen element 5-HA-1581. Neighbouring genes are *nrv3* and *CG3305*. (**B–D**) Wild-type histone H1 (*His1*) expression in S-phase 15 ($S_{15}$). Cyclin B labels cells in $G2_{14}$; cells in early $S_{15}$ show low or absent Cyclin B staining (**B**). $S_{15}$ cells expressed *His1* mRNA as detected by *in situ* hybridization (**C**). Expression was absent from G2 cells (**D**, Cyclin B red, *His1* green). (**E–G**) Corresponding staining showed that *His1* expression was undetectable in homozygous *His^C* mutant embryos (*His^C/His^C*). Wild type refers to internal control. Scale bar, 100 μm; anterior, left. mRNA, messenger RNA.

(for simplicity we refer to them as 'histones'; for details of the histone complex see http://flybase.org; Fig 1A). Owing to this arrangement, we were able to use the *DrosDel* system (Ryder *et al*, 2004) to generate the deletion *Df(2L)His^C*, which precisely uncovers the entire histone complex and thus all histone genes in the genome. We call this molecularly defined histone null mutation '*His^C*' (Fig 1A).

## Replacement of the *Drosophila* histone complement

Homozygous *His^C* mutant individuals die during embryogenesis. This allows validation of a transgene-based histone-replacement system by its ability to rescue the lethality associated with the *His^C* deletion. Our strategy included three important technical aspects. We used the genuine His-GUs for histone expression instead of an artificial expression system, thereby maintaining S-phase-specific histone expression and avoiding histone overexpression. We used the 'Multisite Gateway' system (Invitrogen) for a modular transgene design from single His-GUs (supplementary Fig S1 online). Finally, we used the ϕ*C31* site-specific recombination system (Bischof *et al*, 2007), which allowed us to target transgene integrations to defined genomic locations (landing sites), an essential prerequisite for drawing conclusions from comparing the effects of modified and wild-type histone transgenes that could suffer from variable position effects if transgenes were randomly integrated.

Lethality of *His^C* was not rescued by two or six transgene-encoded His-GUs. The respective transgenes carried one or three His-GUs integrated into the same landing site on chromosome 3R and were assayed as homozygous individuals. Whereas *His^C* mutant embryos lacking His-GU transgenes did not develop a larval cuticle, individuals bearing two His-GU transgenes developed a defective cuticle, and individuals bearing six His-GU transgenes developed a wild-type cuticle pattern but failed to hatch (supplementary Fig S2 online). These results indicate a dose-dependent partial rescue with increasing copy numbers of His-GU transgenes. Finally, we increased the His-GU copy numbers by integrating three His-GUs into an additional landing site on chromosome 3L to generate flies that carried 12 transgene-encoded His-GUs in their genome by recombination. *His^C* mutant individuals with 12 His-GUs were rescued to become fertile adults with no visible morphological defects. Furthermore, when we compared the protein expression of transgene-encoded histones from rescued flies with endogenous histones from wild-type flies, we did not find a significant difference (supplementary Fig S3 online). The complete rescue of the *His^C* mutation by 12 His-GU transgenes indicates that the deletion does not affect any essential genes other than the canonical histones. The combination of histone transgenesis with the *His^C* mutation is, therefore, an effective system in which to replace endogenous histones with transgene-encoded histones in a multicellular organism.

## Assay for cell cycle related histone function

To validate the system as a cellular assay for histone function, we characterized the embryonic lethal phenotype of the *His^C* mutation by studying cell cycle progression. For this, we used antibodies of the cyclin B protein as a cell cycle marker. Cyclin B reaches peak levels in G2-phase, is degraded during the metaphase-to-anaphase transition in mitosis and re-accumulates gradually during the next S-phase (Lehner & O'Farrell, 1990). Homozygous *His^C* mutant embryos lack zygotic histone synthesis, but as they derive from heterozygous females they receive maternal histone proteins and mRNAs (Lanzotti *et al*, 2002). These embryos complete the first 14 cell cycles of embryogenesis with no scorable defects and undergo mitosis 14 ($M_{14}$) similarly to the wild type (Fig 1B,E). Thus, the maternal store of histone mRNA and histone protein is sufficient to drive the early embryonic cell cycles and development up to the blastoderm stage. Histone mRNA is unstable outside the S-phase of the cell cycle (Marzluff *et al*, 2008). Therefore, the maternal store of histone mRNA is degraded completely during the G2-phase of cell cycle 14 ($G2_{14}$) in both wild-type embryos and the *His^C* mutants (Lanzotti *et al*,

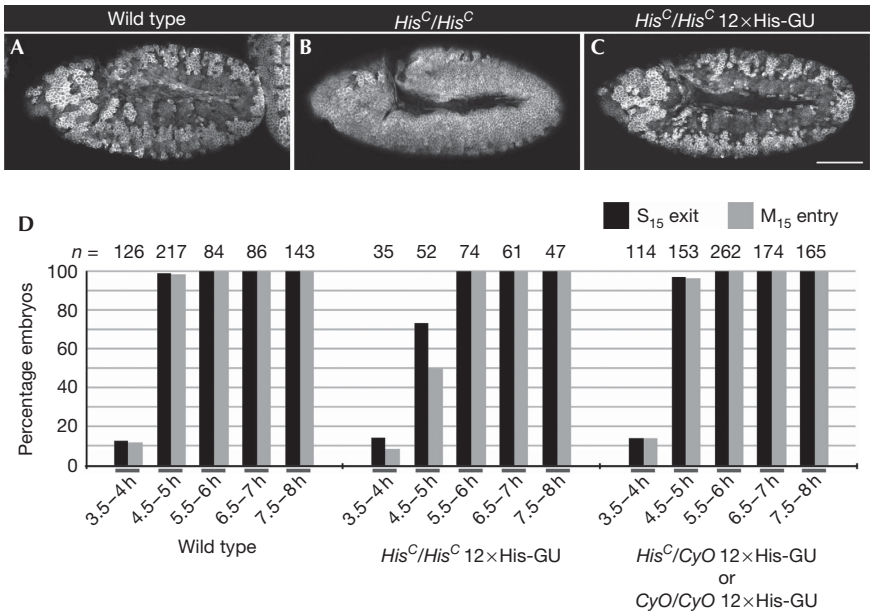


**Fig 2** | Rescue of *His*$^{C}$ by 12 His-GUs. (**A–C**) Representative embryos from time-matched collections 4.5–5 h after egg laying stained for Cyclin B. Wild-type embryos undergo $M_{15}$ in the dorsal epidermis (**A**), whereas *His*$^{C}$ mutant embryos are blocked with high levels of Cyclin B before $M_{15}$ (**B**). In the presence of 12 transgene-based His-GUs, *His*$^{C}$ mutant embryos display a wild-type $M_{15}$ pattern (**C**); wild type refers to $w^{-}$ control. (**D**) The percentage of embryos that completed $S_{15}$ ($S_{15}$ exit) or progressed into $M_{15}$ ($M_{15}$ entry) in the dorsal epidermis was determined. Embryonic collections were time matched for the indicated time interval after egg laying. The left set of columns are wild type. The middle display *His*$^{C}$ homozygotes rescued by 12 His-GUs and the right display embryos from the same collection, which were not homozygous *His*$^{C}$ mutant. These embryos acted as internal controls to ensure reproducible timing of the collections. For details on classification of embryos see supplementary Fig S5 online. Scale bar, 100 μm (**A–C**); anterior left (**A–C**), His-GU; histone gene repeat unit; $M_{15}$, mitosis 15; *n*, number of embryos.

2002). Hence, newly synthesized histone mRNA was observed in S-phase 15 ($S_{15}$) of wild-type cells (Fig 1B–D), whereas no histone mRNA was present in the corresponding *His*$^{C}$ mutant cells (Fig 1E–G; supplementary Fig S4 online).

The pattern of epidermal cell divisions is stereotyped in wild-type fly embryos (supplementary Fig S5 online). Mitosis 15 ($M_{15}$) first takes place in the dorsal epidermis about 4.5–5 h after egg laying, as marked by Cyclin B degradation in those cells (Fig 2A). By contrast, *His*$^{C}$ mutants maintained uniformly high levels of Cyclin B in all parts of the epidermis, both at this stage (Fig 2B) and during the later stages of embryogenesis (supplementary Fig S6 online). These results indicate that loss of *de novo* synthesized histones abrogates the completion of cell cycle 15 in all epidermal cells. The *His*$^{C}$ phenotype is fully penetrant and invariant. Thus, a possible rescue of the mutant phenotype into a wild-type pattern by transgene-derived histone synthesis can be reliably scored. In fact, the presence of 12 transgenic His-GUs in the genome of the *His*$^{C}$ mutants causes a reversion of the mutant $M_{15}$ pattern to that of wild-type embryos (Fig 2C; supplementary Fig S7 online). Quantitative estimates on rescued *His*$^{C}$ mutant embryos confirmed that not only the pattern, but also the timing of $M_{15}$ was fully restored (Fig 2D). This result shows that multiple His-GUs are required to ensure normal development of a multicellular organism, such as *Drosophila,* when expressed under the control of endogenous regulatory elements. This finding is consistent with the phenomenon that all multicellular organisms contain multiple histone gene units, ranging from about 10 up to several hundred copies per genome (Marzluff *et al*, 2008).

## Introduction of modified histone transgenes

Previous studies, which targeted histone synthesis in higher organisms by reducing histone translation and transcript stability, have yielded pleiotropic effects (Lanzotti *et al*, 2002; Pettitt *et al*, 2002; Zhao *et al*, 2004). In view of the clearly defined phenotype of the *His*$^{C}$ mutation obtained here, these earlier observations might be due to incomplete deprivation of the newly synthesized histones or off-target effects, highlighting the value of the newly established mutation. In fact, the complete lack of *de novo* histone synthesis, similar to that in *His*$^{C}$ mutants, is unprecedented for eukaryotes and will enable not only direct and detailed studies of chromatin assembly, but also the assessment of the function of histones in cell cycle progression.

The functional replacement of endogenous histones by histone transgenes offers a unique opportunity to directly address the relevance of specific histone modification sites in a multicellular organism. Such an approach requires that transgenes carrying histones with altered modification sites do not interfere with the development or viability of the *His*$^{C}$/+ heterozygous individuals bearing the modified His-GU transgenes. As a proof of principle, we targeted the methyl-modified Lys 27 residue in histone H3 (H3 K27), a well-characterized epigenetic modification site involved in Polycomb-dependent gene regulation (Müller & Verrijzer, 2009). We replaced a lysine with an arginine (H3 K27R) in a single His-GU to prevent lysine methylation, and subsequently generated transgenes containing three repeats of H3 K27R. The resulting transgenes were integrated into the same landing sites on chromosome 3L and R, which were used for the wild-type

transgenes. After recombination and the necessary crosses to obtain $His^{C}/+$ heterozygous transgene-bearing flies, we investigated whether H3 K27R expression has any effects that would preclude the functional analysis of this specific modification site. No such effects were observed, indicating that the expression of H3 K27R would interfere in a dominant manner with fly development, viability and fertility. We note, however, that this result does not exclude the possibility that other histone modification mutations would not cause dominant effects. Therefore, each histone modification mutation to be characterized needs to be tested as shown for H3 K27R here. In a similar approach with the unicellular organism yeast, Dai *et al* (2008) used constitutive expression of mutated histones to study the relevance of individual histone modifications. Of the 486 mutations targeting histones H3 and H4, none caused a dominant effect against a background of reduced endogenous histone expression. Thus, it seems unlikely that the experimental system introduced here will be limited by dominant effects from transgene-encoded mutated histones. The experimental system described in this study should extend beyond yeast studies and allow examination of the effects of designed histone mutants on the development and homeostasis of an entire multicellular organism. It will also benefit from the advanced genetic methodology and tools for stage and tissue assessment of histone functions and cell clone analysis by mitotic recombination that are available for the fly (Ashburner, 1998). Biological questions of high relevance, such as histone-dependent chromatin assembly, the effect of histone deprivation on transcriptional regulation, functional differences between canonical and replacement histones, as well as concepts including the 'histone code' hypothesis, can now be examined and put to a critical test *in vivo*.

## METHODS

**Plasmid construction.** Plasmid construction is schematically included in supplementary Fig S1 online. Using the oligonucleotides 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACGGTACCGGACAATTGACACTGTCCCTTCAAACGCCTG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAACCGGTCGTACGACCTCTTCAATAATAACACTTTCTTCAGTTAACACATG-3′ as primers for PCR, the His-GU was amplified from $w^{1118}$ genomic DNA, recombined into pDONR221 (Invitrogen) leading to the 'Gateway' entry vector pENTR221-*HisGU* and verified by DNA sequencing. In addition, we generated pENTRL4R1-*504bpseq* and pENTRR2L3-*504bpseq* entry vectors by recombining a 504-bp noncoding PCR product into pDONRP4P1R and pDONRP2RP3, respectively. The oligonucleotides used as PCR primers were 5′-GGGGACAACTTTGTATAGAAAAGTTGACGGTACCGCATAGCTAACGAATGTAACTG-3′ and 5′-GGGGACTGCTTTTTTGTACAAACTTGCACCGGTGCACACGAATAAGACGGTC-3′ for pDONRP4P1R; 5′-GGGGACAGCTTTCTTGTACAAAGTGGACGGTACCGCATAGCTAACGAATGTAACTG-3′ and 5′-GGGGACAACTTTGTATAATAAAGTTGAACCGGTGCACACGAATAAGACGGTC-3′ for pDONRP2RP3.

pENTRL4R1-*HisGU* and pENTRR2L3-*HisGU* were generated by replacing the noncoding sequences in pENTRL4R1-*504bpseq* and pENTRR2L3-*504bpseq* with the His-GU from pENTR221-*HisGU* through *Acc*65I and *Age*I. pENTR221-*HisGU.H3K27R* was generated by replacing an *Nco*I/*Sac*I fragment in pENTR221-*HisGU* with a synthetic fragment (Mr Gene, Regensburg, Germany) containing an AAG into CGC codon exchange leading to the H3 K27R mutation. The pENTRL4R1-*HisGU.H3K27R* and pENTRR2L3-*HisGU.H3K27R* entry vectors were generated analogously to the wild-type variants. The pDESTR3R4-ϕ*C31att*B destination vector was created from $p^{wP\text{-}Ex2}$*UASTattB* (Bischof *et al*, 2007) by replacing a *Bam*HI fragment (UAS elements, basal promotor, multiple cloning site and SV40) with a *Bgl*II fragment containing the *att*R4-*ccdB*-$Cm^{R}$-*att*R3 cassette for 'MultiSite Gateway' that was amplified from pDESTR4-R3 using the oligonucleotides 5′-GTCAGATCTACCTAGGAGATACCAGCGGATAACAATTTCAC-3′ and 5′-ACCAGATCTATCTAGAGGCAAGGCGATTAAGTTGGGTAAC-3′ as primers for PCR. Recombination of pENTR221-*HisGU*, pENTRL4R1-*HisGU* and pENTRR2L3-*HisGU* with pDESTR3R4-ϕ*C31attB* resulted in the transgene integration construct pϕ*C31attB3xHisGU*. Analogously, pENTR221-*HisGU*, pENTRL4R1-*504bpseq* and pENTRR2L3-*504bpseq* led to pϕ*C31attB1xHisGU* and pENTR221-*HisGU.H3K27R*. pENTRL4R1-*HisGU.H3K27R* and pENTRR2L3-*HisGU.H3K27R* led to pϕ*C31attB3xHisGU.H3K27R*.

**Fly strains.** *Df(2L)*$His^{C}$ was constructed from $w^{1118}$*; P{FRT, w*$^{+mc}$*}5-HA-1581* (Szeged Stock Center) and $w^{1118}$*; P{FRT, w*$^{+mc}$*}CB5033-3* (provided by G. Reuter) following a previously described scheme (Ryder *et al*, 2004). *Df(2L)*$His^{C}$ was kept heterozygous over *CyO, P{ftz/lacB}E3* to identify mutant embryos by lacking β-galactosidase expression, or *CyO, P{ActGFP}JMR1* to identify mutant embryos lacking green fluorescent protein expression. Wild-type controls were either nonmutant sibling embryos (internal control) or $w^{1118}$ embryos ($w^{-}$ control) as indicated in the respective figure legends. ϕ*C31*-mediated transgenesis using landing sites *M{3xP3-RFP.attP}ZH-86Fb* or *M{3xP3-RFP.attP}ZH-68E* (Bischof *et al*, 2007) was purchased from BestGene, Chino Hills, CA. pϕ*C31attB1xHisGU* was used to obtain *M{1xHisGU.wt}ZH-86Fb*; pϕ*C31attB3xHisGU* for *M{3xHisGU.wt}ZH-86Fb* and *M{3xHisGU.wt}ZH-68E*; pϕ*C31attB3xHisGU.H3K27R* for *M{3xHisGU.H3K27R}ZH-86Fb* and *M{3xHisGU.H3K27R}ZH-68E*. Transgenes were recombined and crossed into the *Df(2L)*$His^{C}$ mutant background.

**Embryo collection, classification, fixation and mounting.** Time-matched embryonic collections were obtained by restricting egg deposition to 30 min with subsequent ageing at 25 °C. Embryos were dechorionated with 50% bleach, fixed in 1:1 heptane/4% paraformaldehyde, 50 mM ethylene glycol tetraacetic acid (pH 7.4) for 20 min and devitellinized in a 1:1 heptane/methanol followed by washes and storage in methanol. After staining, embryos were mounted in ProlongGold (Invitrogen). Staining was visualized with TCS-SP2 AOBS or TCS-SP5 AOBSconfocal laser-scanning microscopes (Leica).

**Fluorescence *in situ* hybridizations.** Dioxigenin-labelled antisense RNA probes for *His1*, *His2A*, *His2B*, *His3* and *His4* were prepared using standard methods and hybridized to embryos at 57 °C overnight as described previously (Lécuyer *et al*, 2008). Probes were detected by sheep anti-digoxigenin-POD $F_{ab}$ fragments (1:300; Roche) and signal amplification with TSA Cyanine3 reagent (Perkin Elmer) for 40 min. Horseradish peroxidase was inactivated for 10 min at 70 °C for subsequent stainings.

**BrdU incorporation.** The incorporation of bromodeoxyuridine (BrdU) was carried out as described previously (Lehner *et al*, 1991). Embryos were air-dried for 5 min, permeabilized in octane for 6 min and incubated in Schneider's medium (Gibco) with 1 mg/ml BrdU (Sigma) for 15 min at 25 °C followed by fixation.

The incorporation of BrdU was included in the ageing period for time-matched embryonic collections. Embryos were re-hydrated in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS-T), treated with 2 N HCl for 40 min, washed twice with 0.1 M $NaBrO_3$ for 2 min and with PBS-T for 15 min. BrdU was detected by a mouse BrdU antibody (1:80; Becton Dickinson).

**Antibody staining.** Embryos were re-hydrated in PBS with 0.1% Triton X-100 (PBTx), blocked for 30 min at room temperature and labelled with primary antibodies at 4 °C overnight in PBTx and 10% goat serum (Sigma). Secondary antibody incubation was in PBTx, 10% goat serum for 2 h at 25 °C. Washes after antibody incubations were 20 min in PBTx, performed three times. Primary antibodies were rabbit Cyclin B (Jacobs *et al*, 1998; 1:3,000) and chicken β-galactosidase (1:1,000; Abcam). Secondary antibodies were goat anti-mouse IgG Alexa Fluor488 (1:400; Invitrogen), goat anti-rabbit IgG Alexa Fluor633 (1:400; Invitrogen) and goat anti-chicken IgY Cy3 (1:400; Jackson ImmunoResearch). For fluorescence *in situ* hybridizations and BrdU incorporations, chicken β-galactosidase antibody was detected by donkey anti-chicken IgY Biotin-SP (1:500; Jackson ImmunoResearch) followed by VectastainABC (Vector Laboratories) incubation and TSA (Perkin Elmer) detection for 5 min.

**Western blot analyses.** Pools of six male flies, aged 7 days, were frozen in liquid nitrogen, disrupted with a pestle in 75 µl PBS with 0.1% Tween-20 (PBS-T) containing Complete Protease Inhibitor (Roche) and lysed by adding 25 µl of 4 × sodium dodecyl sulphate sample buffer, 5-min incubation at 95 °C and sonication for 5 min at low power in a Bioruptor (Diagenode). Samples were centrifuged for 15 min, at 14,000 *g* at 25 °C and the supernatant was incubated for 10 min at 95 °C before loading onto 15% polyacrylamide Tris/Glycin gels. After blotting, nitrocellulose membranes were blocked with 1% bovine serum albumin and 3% dry milk powder in PBS-T at 4 °C overnight. Incubation with primary or secondary antibodies was in blocking buffer for 2 h or 1 h, respectively, at 25 °C. Washes after incubations were three changes 10 min each in PBS-T. Primary antibodies were rabbit histone H4 (1:1,000; Abcam), rabbit histone H2A (1:1,000; upstate), rabbit histone H3 (1:20,000; Abcam), mouse histone H2B (1:1,000, Abcam) and mouse α-tubulin (DM1A, 1:2,000; Sigma). Secondary antibodies were goat anti-rabbit IgG horse-radish peroxidase conjugated and goat anti-mouse IgG horse-radish peroxidase conjugated (Thermo Scientific). Signals were detected using the ECL Western Blotting Substrate Kit (Thermo Scientific). Membranes were re-used after stripping with the Restore Western Blot Stripping Buffer (Thermo Scientific).

**Cuticle preparations.** Mutant embryos homozygous for *Df(2L)His*$^{C}$ and carrying two or six transgene-encoded His-GUs were identified by lacking green fluorescent protein expression, selected from 22- to 24-h embryo collections, dechorionated, fixed for 10 min in 1:1 heptane/methanol and mounted in 1:1 Hoyers medium/lactic acid.

**Supplementary information** is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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