Inventory of Supplemental Materials

Figure S1, related to **Figure 1.** Map of the BAC vector used for transgenesis of histone gene arrays and details on the cloning scheme used to multimerize the 5kb histone repeat unit. Also included is an image of a Southern blot demonstrating the *in vivo* stability of histone transgenes.

Figure S2, related to **Figure 3.** Plots of survival rates of $12x^{WT}$ resuce flies after exposure to Hydroxyurea.

Figure S3, related to **Figure 5**. Immunofluorescence images showing that nurse cell nuclei from $12x^{H4K20A}$ rescue flies progress through the cell cycle similar to wild type nurse cell nuclei. Also included is characterization of the *His4r* gene and the $His4r^{PB}$ allele.

Figure S4, related to **Figure 6**. Evidence of genetic interactions between *Polycomb* and the $8x^{H3K27A}$ transgene. Also included is an image showing derepression of *Ubx* in *ΔHisC* clones covered by $8x^{H3K27R}$.

Supplemental Experimental Procedures

Supplemental References

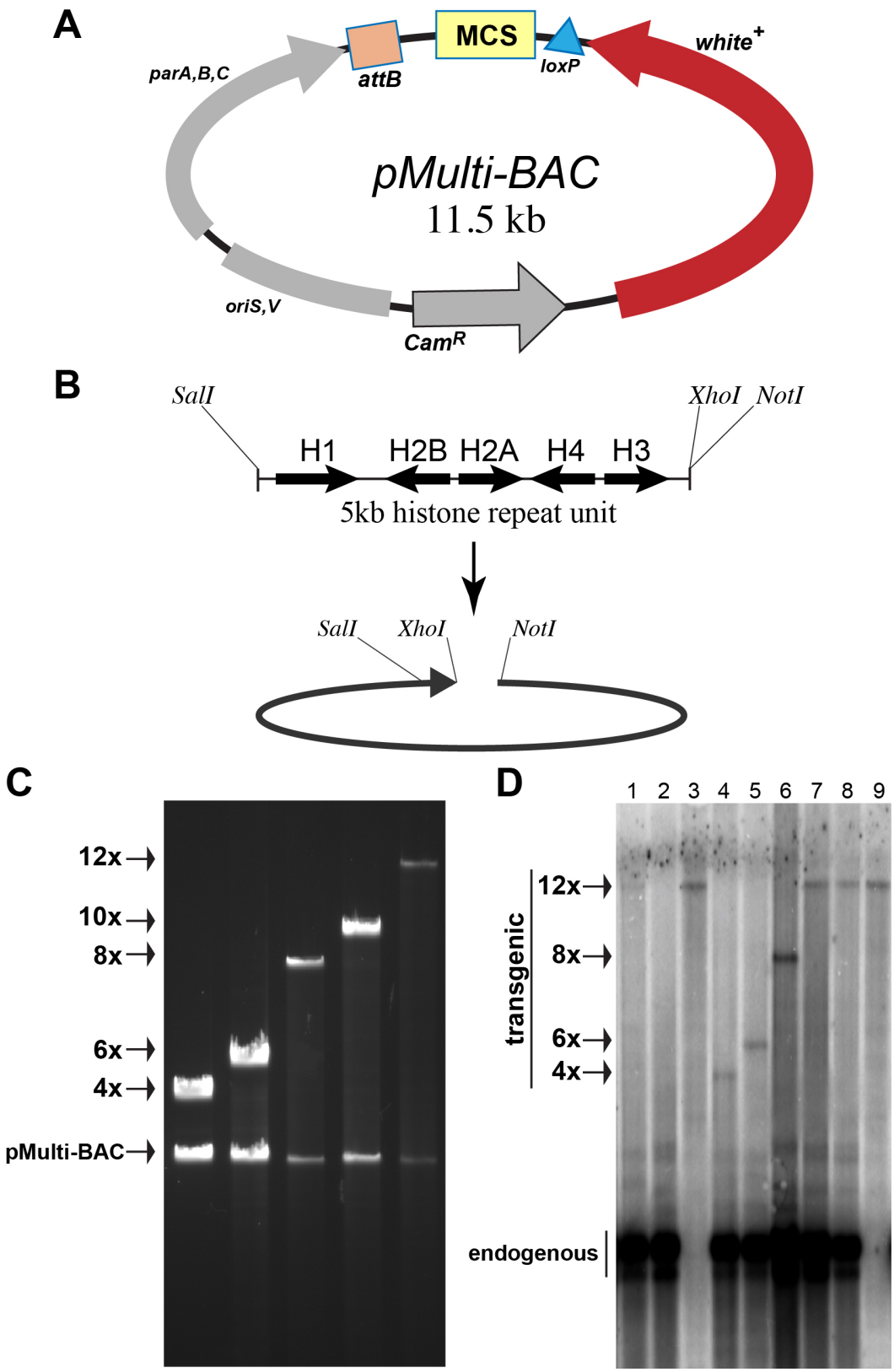

A
MCS
attB
loxP
white+
parA,B,C
pMulti-BAC
11.5 kb
oriS,V
CamR
B
SalI
XhoI
NotI
H1
H2B
H2A
H4
H3
5kb histone repeat unit
SalI
XhoI
NotI
C
12x
10x
8x
6x
4x
pMulti-BAC
D
1 2 3 4 5 6 7 8 9
12x
8x
6x
4x
transgenic
endogenous

Figure S1, related to Figure 1. Design, construction and *in vivo* stability of histone transgenes. (**A**) Detailed map of *pMulti-BAC* vector, including the following features: MCS (Multiple Cloning Site); *loxP* recombination site; *mini-white* cassette for detection of transgenic flies; Cam^R for chloramphenicol resistance; *oriS,V* origins of replication, *oriS* is constitutive, *oriV* is inducible; *par* elements for faithful partitioning to daughter cells; and *attB* recombination site. (**B**) Schematic for multimerization of histone gene repeat units by sequential rounds of cloning. Digestion with SalI and XhoI generates non-re-cleavable compatible cohesive ends after ligation. (**C**) Agarose gel stained with ethidium bromide showing SalI/NotI fragments resolved by Pulsed Field Gel Electrophoresis for five pMulti-BAC histone transgenes with different array lengths. (**D**) Southern blot of SalI/XhoI digested genomic DNA resolved by Pulsed Field Gel Electrophoresis using a Histone H2A probe that recognizes both endogenous and transgenic histone genes. The XhoI site in the endogenous locus collapses the histone gene array into individual repeats. Nine genotypes used in the manuscript are shown: (1) *DHisC/+;12x*^{H3K36R}*/+* (2) wild type *(yw)* (3) *DHisC/DHisC;12x*^{WT}*/+* (4) *DHisC/+;4x*^{WT}*/+* (5) *DHisC/+;6x*^{WT}*/+* (6) *DHisC/+;8x*^{H3K27A}*/+* (7) *DHisC/+;12x*^{WT}*/+* (8) *DHisC/+;12x*^{H4K20A}*/+* (9) *DHisC/DHisC;12x*^{H4K20A}*/+*.

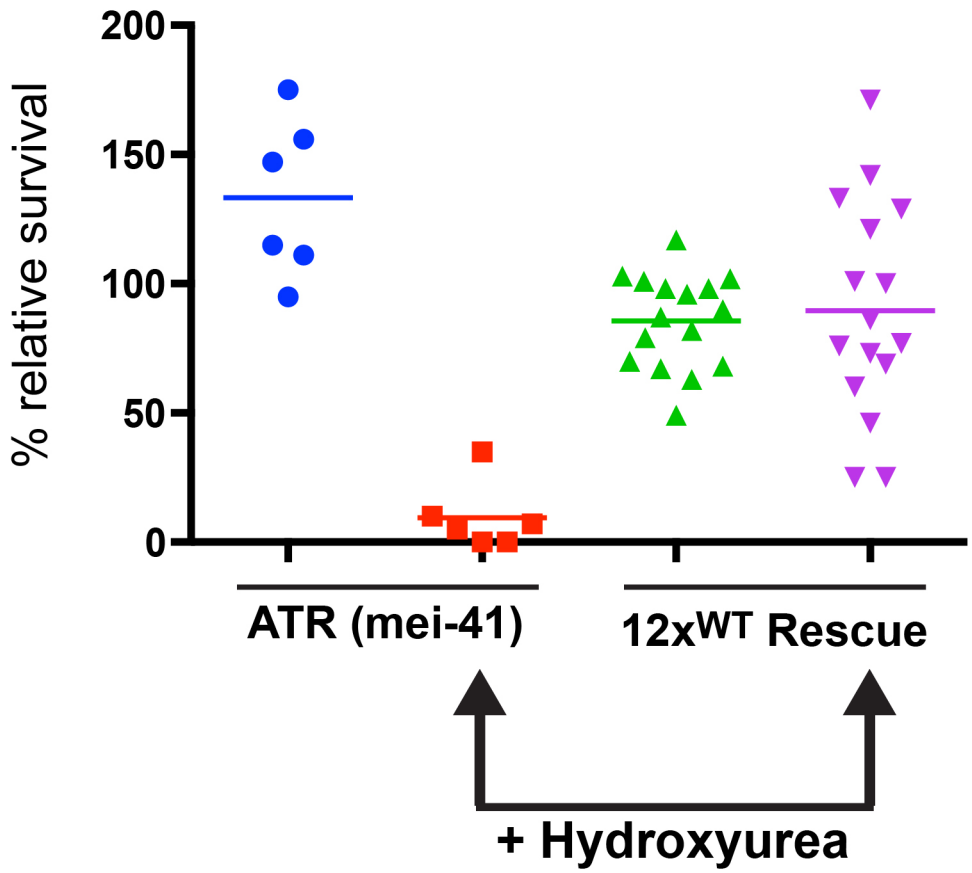
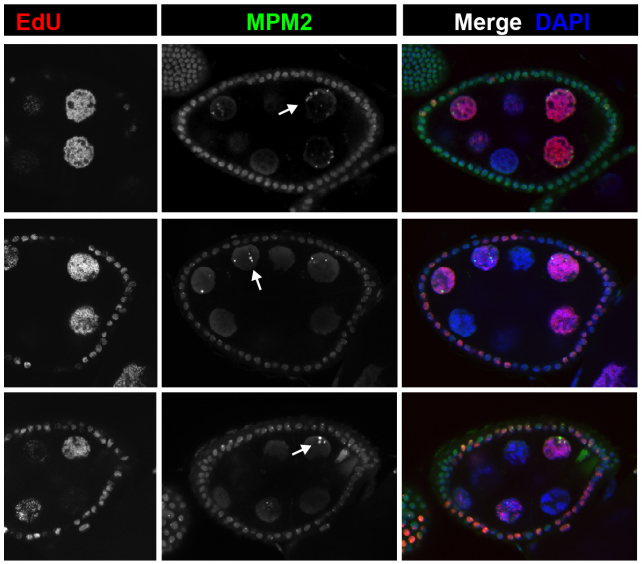


Figure S2, related to Figure 3. 12x^{WT} Rescue flies do not exhibit increased sensitivity to HU-induced DNA damage. Plot of survival to adulthood of two crosses (x-axis). (**left**) "ATR (mei-41)": *mei-41^{29D}/FM7* x *mei-41^{29D}/y*. (**right**) "12x^{WT} Rescue": *ΔHisC/CyO* x *ΔHisC/ΔHisC;12x^{WT}/12x^{WT}*. Each point represents a vial of flies. The y-axis represents the ratio of experimental (no balancer chromosome) to control (balancer chromosome) siblings within each vial. Half of the vials from each cross were treated with 250ul of 70mM hydroxyurea, as indicated. The horizontal bar indicates the average survival rate.



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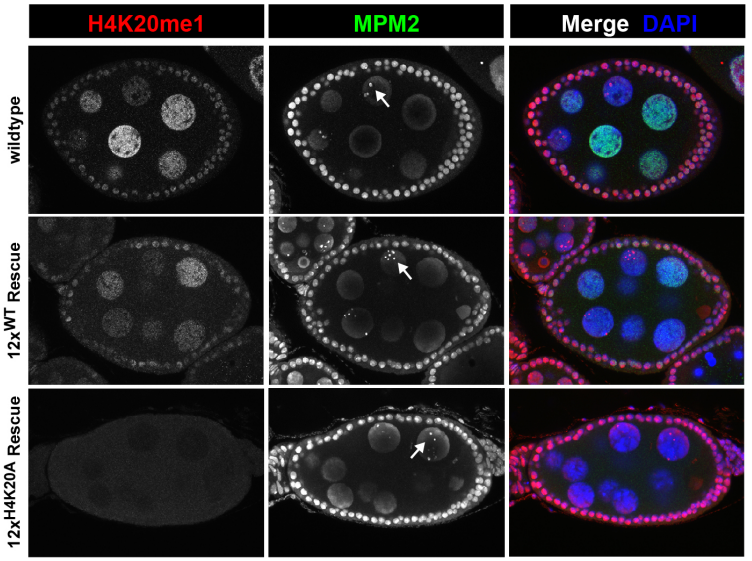

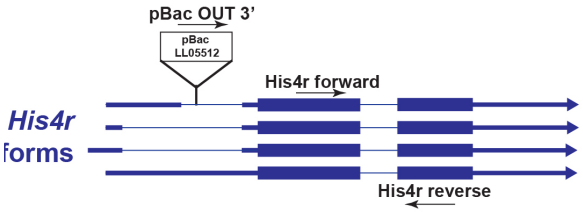

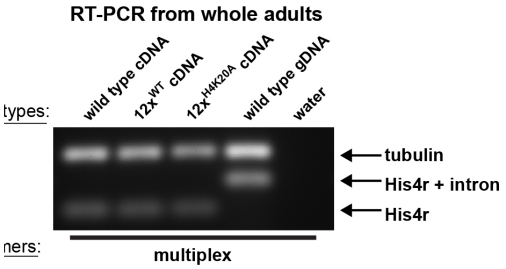

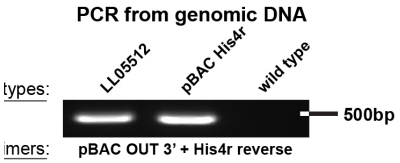

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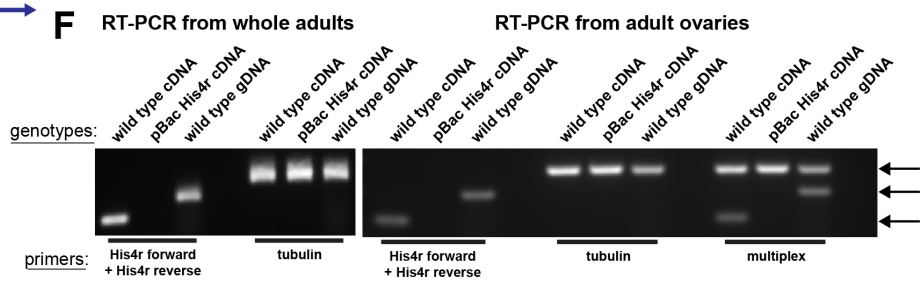
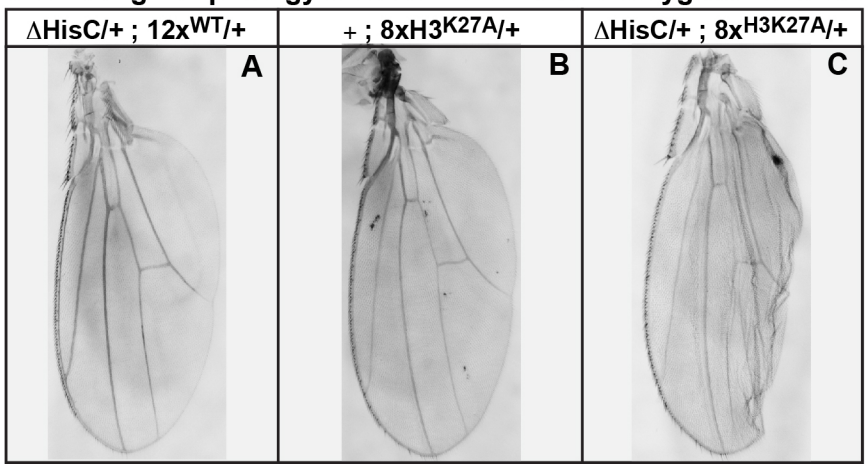


G

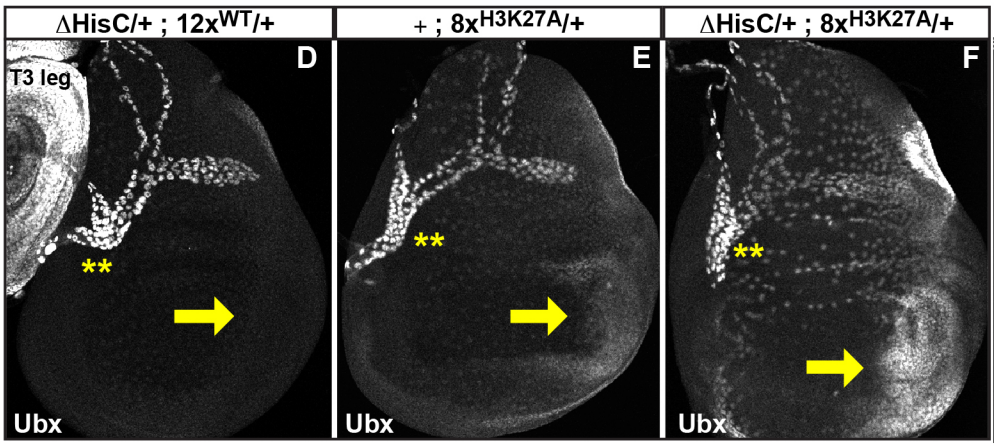
	$\frac{\Delta HisC\ His4r^{PB},12x^{H4K20A}}{\Delta HisC\ ;\ TM6B}$	$\frac{\Delta HisC\ His4r^{PB},12x^{H4K20A}}{\Delta HisC\ ;\ His4r^{PB}}$	$\frac{\Delta HisC\ 12x^{H4K20A}}{\Delta HisC\ ;\ TM6B}$	$\frac{\Delta HisC\ 12x^{H4K20A}}{\Delta HisC\ ;\ His4r^{PB}}$
# larvae	113	153	90	102
# pupae	97	133	85	96
# pharates	75	46	62	86
# adults	11	1	4	66

Figure S3, related to Figure 5. H4K20 is not required for cell cycle progression. (**A**) Confocal images of stage 6-8 egg chambers stained for EdU (red), MPM2 (green) and DAPI (blue). Arrows indicate a subset of MPM2 foci. (**B**) Confocal images of stage 6-8 egg chambers stained for H4K20me1 (red), MPM2 (green), and DAPI (blue). Arrows indicate a subset of MPM2 foci. (**C**) Depiction of the four RefSeq *His4r* isoforms, and the insertion location of PBac LL05512. Primers are indicated with black horizontal arrows. (**D**) Ethidium bromide stain of an agarose gel containing RT-PCR products from adult male total RNA from the three indicated genotypes: lane 1: wild type (*yw*), lane 2: $12x^{WT}$ (12x Rescue flies), lane 3: $12x^{H4K20A}$ (H4K20A replacement flies). Genomic DNA from wild type flies is also included in lane 4. The PCR was a multiplex reaction containing primer pairs for α-tubulin and His4r. (**E**) Ethidium bromide stained agarose gel containing PCR products from genomic DNA of the three indicated genotypes: lane 1: Kyoto Stock #141607 (LL05512), lane 2: a derivative of the stock used in lane 1, in which the other transgenes were recombined away from PBac LL05512 (PBac His4r), lane 3: *yw* (wild type). The primers used are diagrammed in **Figure S3C**. (**F**) Ethidium bromide stained agarose gels showing RT-PCR products from whole adults (left) or adult ovaries (right). Two genotypes were used: wild type (*yw*), and PBac His4r homozygotes. The gel on the right contains both singleplex and multiplex PCR reactions. Primers used are diagrammed in **Figure S3C**. (**G**) Table showing the number of flies of the four indicated genotypes at each of the four indicated developmental stages. See **Experimental Procedures** for more details.

Posterior wing morphology is aberrant in HisC hemizygous H3K27A flies



K27A histones de-repress Ubx in wing discs in a dose-dependent manner



G

H3K27A genetically interacts with Polycomb		
genotype	# legs with ectopic sex combs	total # legs
+/+	0	212
Pc^{15}/+	25	304
H3K27A/+	2	144
Pc^{15}/H3K27A	99	146

H

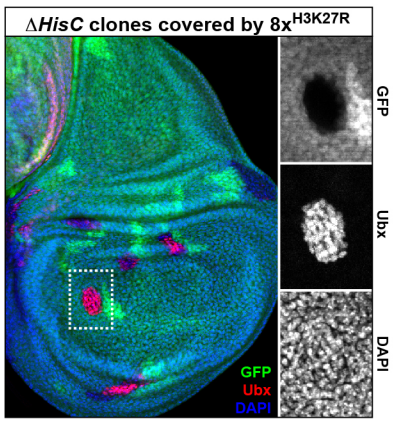


Figure S4, related to Figure 6. H3K27A expression causes dominant phenotypes. (**A-C**) Whole-mount images of adult wings from the three indicated genotypes. Expression of H3K27A histones in an otherwise wild type genetic background (**B**) causes occasional adhesion defects between the dorsal and ventral wing surfaces in the posterior portion of the wing blade, and dark patches/necrotic cells. Reduction in the endogenous histone gene copy number by half (**C**) enhances the penetrance of these phenotypes. (**D-F**) Confocal images of wing imaginal discs stained for Ubx from the three indicated genotypes. The arrow indicates ectopic expression of Ubx in the main epithelium of the posterior wing. Asterisks indicate normal expression of Ubx in the trachea. Normal expression of Ubx in the peripodial cells of the wing is also visible in panel **F.** (**G**) Table of the number of T2 or T3 legs from male flies with at least one ectopic sex comb for each indicated genotype. (**H**) Confocal image of a wing imaginal disc containing *ΔHisC* clones induced at 48-60hr covered by the $8x^{H3K27R}$ histone replacement transgene, stained for Ubx (red) and DAPI (blue). Clones are marked by the absence of GFP (green).

Supplemental Experimental Procedures

Vector and artificial histone array construction

To generate the pMulti-BAC vector (sequence available on request), we deleted the cos and loxP sites, along with the lacZ gene and multiple cloning sequence (MCS) from pBAC/oriV (Wild et al., 2002), Epicentre Biotechnologies) by *Sal*I digestion and religation of the vector. We then removed all but one of the *Xho*I or *Sal*I restriction sites from pBAC/oriV and pattB (Bischof et al., 2007), respectively, using Quickchange site-directed mutagenesis (Agilent Technologies). In order to finally assemble pMulti-BAC, a unique *Pac*I site was added to each vector in such a way as to allow us to combine the *mini-white* cassette, attB recombination site, MCS, and loxP site from pattB with the L-arabinose inducible *E. coli* replication genes and chloramphenicol resistance cassette from pBAC/oriV.

The 5kb endogenous histone repeat unit was subcloned into pBSK (Agilent) by PCR and the *Xho*I site within the *His2A* coding region was then mutated by site-directed mutagenesis (as shown in **Fig. 2A**). Additional site-directed mutations of histone N-terminal tails were incorporated into the histone repeat unit prior to tandem array construction. Multimerization of the 5kb insert was accomplished by iterative cloning of SalI/NotI-digested histone repeats (n = 2, 4, etc.) into an XhoI/NotI-digested pMulti-BAC vector (**Fig. S1**). Insert sizes were verified by pulsed-field gel electrophoresis. All histone replacement transgenes were inserted into the VK33 *attP* site on chromosome 3L (65B2) (Venken et al., 2006).

Fly strains and genetic crosses

The following genotypes were used (with origin):

ΔHisC, UAS-2xEYFP / CyO (gift from Alf Herzig)

ΔHisC, twi-GAL4 / CyO (gift from Alf Herzig)

Oregon R (Bloomington Stock Center # 25211)

y;cn,bw,sp (BSC# 2057)

Df(2R)8057 (BSC# 7871) (aka *FLASH*^{Df})

yw; FRT40A, PBac{DsRed}LL01602 (DGRC# 140418) (aka *FLASH*^{PBac})

yw122; ΔHisC, FRT40A / CyO twi-GAL4, UAS-GFP (gift from Alf Herzig)

yw122; Ubi-GFP^{S65T}*nls, FRT40A / CyO* (BSC# 5629)

w; Ubi-GFP.nls X2, FRT2A (BSC# 5825)

yw122; E(z)^{731}*, FRT2A / TM6B* (BSC# 24470)

yw122; hsp70-CD2, Pc^{15}*, FRT2A / TM6B* (BSC# 24468)

mei-41^{21D} (gift from Jeff Sekelsky)

yw; PBac{SAstopDsRed}LL05512 (Kyoto Stock Center #141607)

All stocks were maintained on standard corn media. *H4K20A* replacement stocks were raised on standard molasses media.

For the viability tests presented in **Figure 1C**, males of the genotype *ΔHisC, twi-GAL4 / CyO; 12x*^{WT} */ 12x*^{WT} were crossed to females of the genotype *ΔHisC, UAS-YFP* / CyO or *ΔHisC, UAS-YFP / CyO; 12x*^{WT} */ 12x*^{WT}. The number of flies scored represents the total number of progeny from this cross. The expected number represents the number *ΔHisC* homozygous progeny expected if the histone transgene fully rescued lethality. Vials were maintained at 25C and were flipped every 12 hours.

To test the ability of H4K20A transgenes to rescue viability of histone locus deletion, 4 females of the genotype *ΔHisC, UAS-YFP / CyO* were crossed to 4 males of the genotype *ΔHisC, twi-GAL4 / CyO; 12x*^{H4K20A} */ 12x*^{H4K20A}*.* Vials were maintained at 25C and flipped every 24 hours. For the data presented in **Figure S3G**, females of the genotype *yw; ΔHisC, twi-GAL4 / CyO; His4r*^{PB} */ TM6B* were crossed to males of genotype *yw; ΔHisC, UAS-YFP / CyO; His4r*^{PB}*, 12x*^{H4K20A} *or yw; ΔHisC, UAS-YFP / CyO; 12x*^{H4K20A}*.* Crosses were flipped every 24 hours, and 36-48 hours later, all GFP-positive larvae were transferred to new vials. The number of pupae,

pharate adults, and eclosed flies was scored 14-18 days later. Other transgene insertions were recombined away from *PBac{SAstopDsRed}LL05512* to generate stock *His4r*^{PB}.

Immunofluorescence, clone induction, and confocal microscopy

For mitotic recombination experiments in imaginal discs, embryos were collected for 12 hours in vials, and larvae were heat-shocked for 8 minutes at 37C at 48-60hr AEL. Wandering 3rd instar larvae were dissected in PBT (0.15% Triton X-100) and fixed for 25 minutes in 4% para-formaldehyde, 1xPBS. Imaginal disc antibody stains were performed as previously described (Estella et al., 2008). Immunofluorescence of egg chambers was performed essentially as previously described, with the following changes. Dissection, fixation (10 minutes), and antibody staining of ovaries were performed using PBS-A (135 mM NaCl, 3.7 mM KCl, 5.3 mM Na_2HPO_4, 1.6 mM NaH_2PO_4) without blocking. Secondary antibodies were incubated overnight at 4C. EdU incorporation was performed using the Click-iT EdU Alexa Fluor 555 imaging kit (Invitrogen). Ovaries were dissected in Grace's medium and incubated in the presence of EdU for 1hr prior to fixation for 20 minutes with 4% PFA in 1xPBS. After antibody staining, the samples were fixed again with 4% PFA in 1xPBS, and washed in 1xPBT. The Click-iT reaction was then performed according to the manufacturer's instructions before DAPI staining and mounting. For the EdU/H4K20me1 experiment, a 10-minute EdU pulse was performed with a 5-minute chase in PBS-A prior to fixation. All egg chamber images were acquired with z-stacks using an LSM710 confocal microscope with ZEN DUO software. For semi-quantitative imaging of H4K20me1 and EdU levels, imaging parameters were optimized by normalizing gain and offset on wildtype egg chambers, and then using the same parameters for other genotypes, allowing 5% adjustment of gain levels. For quantification of EdU, H4K20me1, and MPM2 signal intensities in cycling nurse cells, 90 nurse cell nuclei from stage 6-8 egg chambers were scored for each genotype. To minimize bias, each channel was scored independently of other channels.

Antibodies

Histone H3 (rabbit,(Fuchs et al., 2012)) (1:50,000)

α-tubulin (mouse, Sigma T6074) (1:30,000)

Phospho-histone H3 (rabbit, Millipore 06-570) (1:1000)

GFP (mouse, Abcam ab1218) (1:1000)

HP1 (mouse, DSHB C1A9) (1:1000)

DLG (mouse, DSHB 4F3) (1:1500)

FLASH (rabbit,(Yang et al., 2009)) (1:2000)

Mxc (guinea pig,(White et al., 2011)) (1: 1000)

Lamin (mouse, DSHB ADL84.12) (1:500)

Mute (guinea pig,(Bulchand et al., 2010)) (1:6000)

H3K36me3 (rabbit, Abcam ab9050) (1:2000 for IF, 1:5000 for westerns)

Ubx (mouse, DSHB FP3.38) (1:30)

H3K27me3 (mouse, Abcam ab6002) (1:200)

H4K20me1 (rabbit, Millipore NL314) (1:200)

H4K20me3 (rabbit, Diagenode C15410207) (1:1000)

MPM2 (mouse, Millipore 05-368) (1:1000)

Antibodies from the Developmental Studies Hybridoma Bank (DSHB) were created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

PCR-based assays

Histone gene copy number analyses. Genomic DNA from 15 adult males was prepared using phenol:chloroform extraction. For semi-quantitative analysis (**Figure 2A**), PCR was used to amplify His2A, His3, or α-tubulin, and the resulting amplicon was ethanol precipitated and subjected to digestion with XhoI. DNA was subsequently run on an 8% acrylamide gel and

bands quantified using ImageJ. Quantifications were made relative to the wild type (yw) band. Average values were calculated based on experiments performed in biological triplicate and technical duplicate. The same calculations were performed for the tubulin loading control. For real-time PCR assays (**Figure 2B**), absolute values of His2A, His3, α-tubulin, and actin5C were calculated for experiments performed in biological triplicate and technical duplicate. Values were normalized to tubulin or actin with similar results. The resulting values were made relative to one 24x Rescue value to calculate fold change. The average fold change was subsequently calculated along with the standard error of the mean.

RT-PCR. Embryos, adults, or dissected ovaries were flash frozen in liquid nitrogen. Total RNA was prepared in Trizol and cDNA synthesized using random hexamers. RT-RCR was performed using gene-specific primers to H2A, H3, His4r, and α-tubulin. PCR products were run on 1.5% agarose gels, with the following exceptions. For semi-quantitative analysis shown in **Figure 3B**, PCR products were run on an 8% acrylamide gel and quantified using ImageJ. For semi-quantitative analysis shown in **Figure 3D**, PCR products were ethanol precipitated and digested using XhoI. Digested amplicons were run on an 8% acrylamide gel and bands quantified using ImageJ. For both **Figures 3B** and **Figure 3D**, quantifications were normalized to tubulin and set relative to the wildtype (yw) value. Average values of relativized band quantifications were calculated based on experiments performed in biological triplicate and technical duplicate. For real-time PCR assays (**Figure 3C**), the $\Delta\Delta$Ct values of H2A and H3 were calculated using tubulin as a loading control. Average fold change was calculated from experiments performed in biological triplicate and technical duplicate.

Southern Blots

Genomic DNA was digested with SalI/XhoI for a minimum of 12 hours and separated using pulsed-field gel electrophoresis. DNA was denatured in 0.5M NaOH and blotted to a PVDF membrane overnight in 20X SSC buffer. DNA was crosslinked to the membrane using UV light,

and then hybridized with an α-^{32}P CTP H2A probe labeled with Klenow. After washing in 2X SSC buffer, the membrane was exposed to a phosphor screen and imaged on a Typhoon phosphorimager.

Primers

H2A Gel Forward 5'-GGCCATGTCTGGACGTGGAAAAGG T-3'

H2A Gel Reverse 5'-GGCCTTAGGCCTTCTTCTCGGTCTT-3'

H2A Real-time Forward 5'-GCCGTATTCACCGTTTGC-3'

H2A Real-time Reverse 5'-GACGCCGGAGAGCAGCTT-3'

H3 Real-time Forward 5'-TCTGGTGCGTGAAATCGCT-3'

H3 Real-time Reverse 5'-GACACGCTTGGCATGAATGG-3'

α-tubulin Forward 5'-AGATGCCGTCTGACAAGACC-3'

α-tubulin Reverse 5'-GACCACAGTGGGTTCCAGAT-3'

Actin5C Forward 5'-AGGAGGAGGAGGAGAAGTCG-3'

Actin5C Reverse 5'-TGGTTCCGCTCTTTTCATCT-3'

His4r Forward 5'-GGGTATCACCAAGCCTGCTA-3'

His4r Reverse 5'-GTGACAGCGTCACGGATAAC-3'

PBac OUT 5'-CCGATAAAACACATGCGTCA-3'

Supplemental References

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