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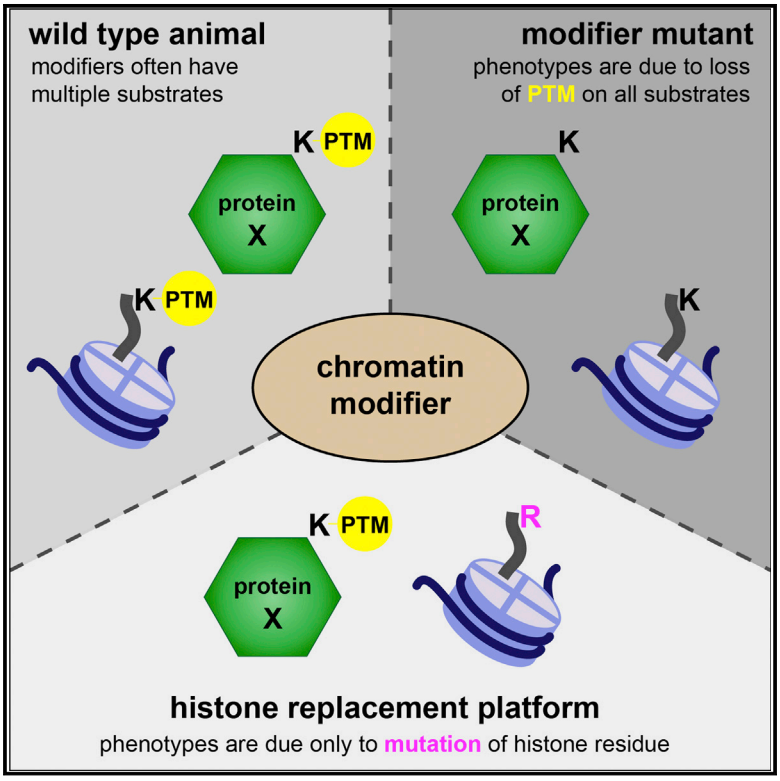
Developmental Cell

Interrogating the Function of Metazoan Histones using Engineered Gene Clusters

Graphical Abstract



Highlights

- Specific histone genotypes can be engineered in *Drosophila* using BAC transgenes
- Histone gene expression is controlled by an active dosage compensation mechanism
- Posttranslational modification of H4K20 is not required to complete development
- H3K27 is required for Polycomb target gene repression but not for gene activation

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In Brief

McKay et al. describe a platform for histone gene engineering in *Drosophila* that allows direct genetic assessment of histone residue function in vivo. Using the system, they demonstrate that, contrary to inferences based on analysis of H4K20 methyltransferases, H4K20 is not essential for DNA replication or completion of development.

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Interrogating the Function of Metazoan Histones using Engineered Gene Clusters

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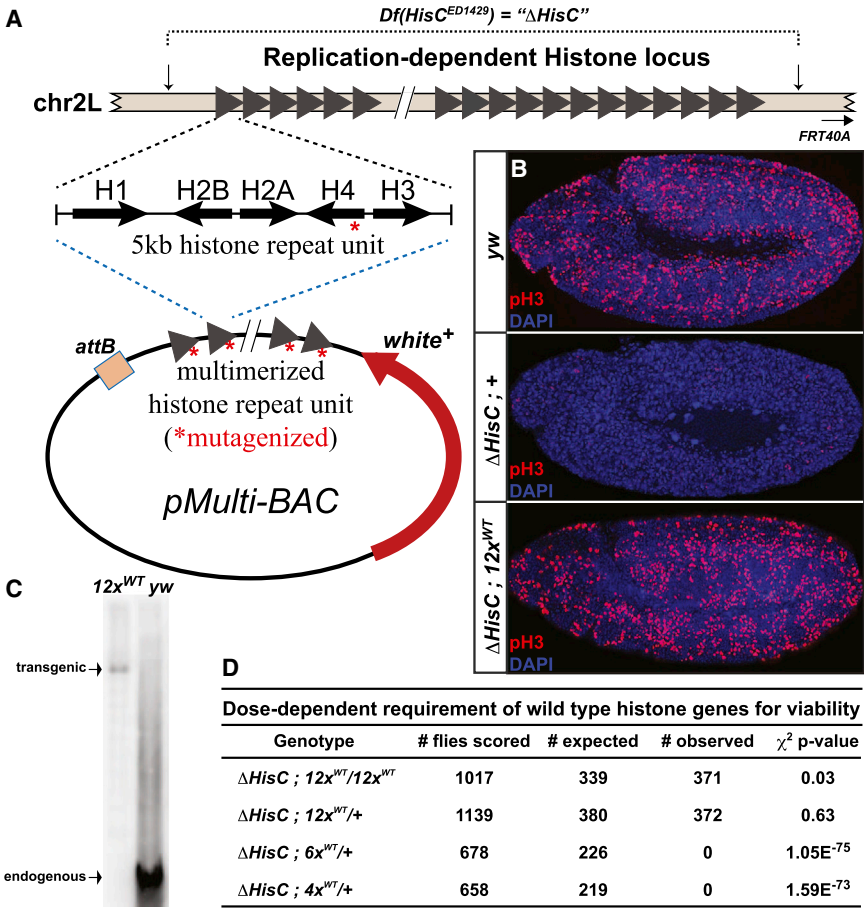
SUMMARY

Histones and their posttranslational modifications influence the regulation of many DNA-dependent processes. Although an essential role for histone-modifying enzymes in these processes is well established, defining the specific contribution of individual histone residues remains a challenge because many histone-modifying enzymes have nonhistone targets. This challenge is exacerbated by the paucity of suitable approaches to genetically engineer histone genes in metazoans. Here, we describe a platform in *Drosophila* for generating and analyzing any desired histone genotype, and we use it to test the in vivo function of three histone residues. We demonstrate that H4K20 is neither essential for DNA replication nor for completion of development, unlike inferences drawn from analyses of H4K20 methyltransferases. We also show that H3K36 is required for viability and H3K27 is essential for maintenance of cellular identity but not for gene activation. These findings highlight the power of engineering histones to interrogate genome structure and function in animals.

INTRODUCTION

During animal development, a single genome gives rise to a wide diversity of cells. Each cell type differentially regulates genome activity to accurately execute a particular program of gene expression, cell-cycle progression, and DNA replication. Failure of this execution can lead to developmental defects or disease states that reduce organismal fitness. Because the genome sequence is essentially identical in most cell types, epigenetic mechanisms have been proposed to bring about cell-type specific regulation of genome activity (Margueron and Reinberg, 2010). Such mechanisms require a substrate that carries regulatory information and a means of propagating this information over time. Histone proteins are particularly attractive candidates for carriers of epigenetic information because they can fulfill both of these criteria. First, histone proteins have the potential to be dynamic regulators of genome activity because they are subject to a broad range of posttranslational modifications (PTMs), including phosphorylation, acetylation, and methylation (Rothbart and Strahl, 2014). Histone PTMs are thought to contribute to regulation of genome activity by controlling chromatin packaging (Shogren-Knaak et al., 2006), and by serving as binding sites for protein complexes that control a variety of DNA-dependent processes including transcription, replication, and repair (Lachner et al., 2001). Second, histone proteins provide a potential means of propagating information over time through their partitioning to daughter cells during each cell division (Margueron and Reinberg, 2010).

Whereas critical roles for histone-modifying enzymes in the regulation of genome activity have been clearly demonstrated in a variety of species, the specific contribution of histone residues is less well understood (Henikoff and Shilatifard, 2011). Systematic mutagenesis in the budding yeast *Saccharomyces cerevisiae* has identified histone residues essential for viability and for response to environmental challenges (Dai et al., 2008; Nakanishi et al., 2008). However, there are likely to be additional roles for histone residues in multicellular organisms, which exhibit diverse regulation of genome activity across different cell types and developmental stages. In multicellular organisms, the function of histone residues has largely been inferred from phenotypes caused by mutation of histone-modifying enzymes rather than by mutation of histone residues themselves. Examination of phenotypes caused by mutations in histone modifiers is not sufficient to make conclusions regarding causality because many of these enzymes have multiple substrates, including nonhistone proteins (Glozak et al., 2005; Huang and Berger, 2008; Sims and Reinberg, 2008). More recently, substitution of methionine for lysine residues in histone proteins has been used to test the function of histone residues in animals (Herz et al., 2014). However, these efforts are also insufficient to test causality because the methionine mutants are thought to act by dominantly interfering with histone methyltransferase



D

Dose-dependent requirement of wild type histone genes for viability

Genotype	# flies scored	# expected	# observed	χ^2 p-value
ΔHisC ; 12x^{WT}/12x^{WT}	1017	339	371	0.03
ΔHisC ; 12x^{WT}/+	1139	380	372	0.63
ΔHisC ; 6x^{WT}/+	678	226	0	1.05E^{-75}
ΔHisC ; 4x^{WT}/+	658	219	0	1.59E^{-73}

Figure 1. A Single Histone Replacement Transgene Rescues Deletion of the Endogenous Histone Locus

(A) Schematic of the endogenous replication-dependent histone locus. Breakpoints of the deficiency are indicated by vertical arrows. Each triangle represents a single 5 kb histone repeat unit, which was cloned and multimerized into a BAC vector for transgenesis. *white*$^+$, mini-white cassette; *attB*, site-specific recombination sequence.

(B) Confocal images of cycle 15 embryos stained with antibodies for phospho-histone H3 (red) and DAPI (blue). Genotypes (top to bottom): wild-type (*yw*); homozygous histone deletion (*ΔHisC;+*); homozygous histone deletion with two copies of the 12x wild-type histone transgene (*ΔHisC;12x^{WT}*).

(C) Southern blot of SalI/XhoI digested genomic DNA from *12x^{WT}* and wild-type (*yw*) flies, hybridized with an H2A probe.

(D) Table of viability tests for various wild-type histone arrays. The p value for the chi-square test is shown.

See also Figure S1.

activity (Lewis et al., 2013), which would likely affect all substrates of the methyltransferases.

A particularly powerful approach for studying the biological function of specific histone PTMs is to change the acceptor residue to an amino acid that cannot be appropriately modified and then to engineer a complete gene replacement for phenotypic analysis. Implementing this strategy in animals is technically challenging because metazoan histones are typically encoded by gene clusters found at multiple chromosomal locations (Marzluff et al., 2008). For example, the human genome has 64 histone genes, clustered at three different loci (Marzluff et al., 2002). In contrast, the *Drosophila* replication-dependent histone genes are found at a single locus (Lifton et al., 1978). Recently, Herzig and colleagues created a system for complementing deletion of the endogenous *Drosophila* histone gene cluster with plasmid-based transgenes (Günesdogan et al., 2010), allowing for the first analysis of histone residue function in animal development (Hödl and Basler, 2012; Pengelly et al., 2013). However, a minimum of four transgenes was required to rescue the histone locus deletion phenotype, limiting the ease with which this strategy can be used in combination with other genetic tools to study histone gene function in *Drosophila*.

Here, we present a BAC-based platform that can rescue deletion of the endogenous *Drosophila* histone locus with a single transgenic insertion, allowing us to study not only the regulation of histone genes themselves, but also the specific contribution of histones to the regulation of DNA-dependent processes. After demonstrating its in vivo functionality, we used this platform to directly test the function during animal development of three posttranslationally modified histone residues: H3K36, H3K27, and H4K20. Unlike results obtained in yeast, we show that H3K36 is required for viability in *Drosophila*. Consistent with current models, we find that H3K27 is required for the maintenance of Polycomb target gene repression, demonstrating that histone residues can perform an essential function in gene regulation. These results underscore the essential roles played by these two histone residues in gene expression and animal development. Finally, in contrast to current models, we show that a modifiable H4K20 residue is neither required for DNA replication nor for completion of *Drosophila* development. Together, these studies demonstrate the importance of directly testing the function of individual histone residues in animal development, and highlight the potential of this approach to test the role of histones in metazoan genome structure and function.

RESULTS

A BAC-Based Platform for Histone Gene Replacement

The *Drosophila melanogaster* replication-dependent histone genes are tandemly arrayed at a single locus on chromosome 2L (Figure 1A). Each 5kb repeat unit contains one copy of each of the four core histone genes (*His2A*, *His2B*, *His3*, *His4*), plus the linker histone, *His1*. Using the *DrosDel* system (Ryder et al., 2004), Herzig and colleagues (Günesdogan et al., 2010) generated a precise deletion of the histone gene complex, termed *Df(2L)HisC^{ED1429}* (hereafter *ΔHisC*). Because zygotic transcription of histone genes is first required during S phase of cell cycle

15 (Smith et al., 1993; Günesdogan et al., 2014), *ΔHisC* homozygotes cannot complete this cycle and die as embryos following depletion of the maternal histone contribution (Figure 1B).

In the Herzig approach, four independent plasmid-based transgenes bearing three copies of the 5 kb histone repeat unit were used to rescue *ΔHisC* (Günesdogan et al., 2010). To create a more genetically facile system, we cloned tandem arrays of the native 5 kb histone gene repeat unit into a BAC-based vector capable of site-specific transgenesis (Figure 1A; Figure S1 available online). The tandemly repeated organization of the native histone gene repeat sequence in these constructs maintains the *cis*-regulatory information required for proper histone gene expression, thereby avoiding potential cell toxic effects of histone overexpression or expression outside of S phase (Gunjan and Verreault, 2003; Singh et al., 2010). Resupplying zygotic histone expression with a BAC-based transgene fully rescues the embryonic cell-cycle arrest phenotype, as depicted by phospho-histone H3 staining during cell cycle 15 (Figure 1B) and supports development to adulthood. Thus, the BAC-based, transgenic histone arrays are functional in vivo.

To define the minimal number of transgenic histone genes needed for full rescue of the histone deletion phenotype, we generated tandem arrays with different numbers of repeat units (Figure 1A). These vectors were integrated into the same genomic location, thereby eliminating position-dependent effects on transgene expression. Southern blots of genomic DNA from histone replacement flies propagated for more than 50 generations show that the transgenic histone arrays are stable after integration in the genome (Figures 1C and S1). Viability tests showed that six or fewer histone gene repeats are insufficient to rescue lethality of *ΔHisC* (Figure 1D), whereas 12 or more repeats fully rescues lethality. When homozygous, the 12x transgene supports the propagation of a stable stock lacking all endogenous histone genes. We refer to these genotypes as "*12x-Rescue*" and "*24x-Rescue*" strains, respectively. Importantly, we did not observe any developmental delays in *12x-Rescue* flies, either in the timing of larval hatching or adult eclosion. We note that the fertility of *24x-Rescue* and *12x-Rescue* females is somewhat decreased relative to wild-type flies; however, the basis for this defect is not known.

The *Drosophila* Genome Contains 100 Copies of the Histone Repeat Unit

The ability of a single transgene containing 12 histone repeats to support development to adulthood is somewhat surprising, given that original estimates suggested that there are upward of 100 copies of the histone repeat unit on chromosome 2L (Lifton et al., 1978). In contrast, current genome annotations (FlyBase release version FB_2014_1) list only 23 histone repeats. Thus, the precise number of histone genes in the *Drosophila* genome remains an open question.

We took two complementary approaches to directly measure the number of endogenous histone genes. First, we reasoned that transgenic histone arrays could be used as in vivo calibrators to accurately measure the endogenous gene copy number by PCR. To discriminate between endogenous and transgenic *His2A* DNA, we engineered a silent mutation in an XhoI site within the transgenic *His2A* gene (Figure 2A). Using PCR primers that recognize both endogenous and transgenic templates, we amplified *His2A* genomic DNA from four genotypes and digested the PCR products with XhoI, cutting the endogenous *His2A* fragment in two equal halves while leaving the transgenic *His2A* product intact. Following electrophoresis, quantification of band intensities revealed that the endogenous *His2A* template is 8-fold more abundant than the transgenic *His2A* template (Figure 2A). Importantly, semiquantitative PCR reactions from both the endogenous and transgenic *His2A* templates are within the linear range, as shown by XhoI digestion assays using genomic DNA from four genotypes with different histone gene copy numbers (Figure 2A). Consistent with measurements from the XhoI digestion assay, real-time PCR indicates that the *His2A* and *His3* genes are 7-fold more abundant in wild-type flies than *24x-Rescue* flies (Figure 2B). These experiments indicate that the haploid *Drosophila* genome contains approximately 100 histone repeats.

Second, we calculated the histone gene copy number using high-throughput sequencing analysis. We reasoned that the abundance of histone sequences relative to those of other genes on chromosome 2L would reflect the number of copies of histone genes in the genome. To accurately measure their abundance, we sequenced genomic DNA from two different strains and mapped reads to a custom *Drosophila* genome containing a single histone gene repeat unit (see Experimental Procedures). Comparison of the average read density across the coding sequence of each histone gene to the average read density across coding sequences of the remaining annotated genes on chromosome 2L revealed that the histone genes are ~100-fold more abundant (Figure 2C), consistent with our PCR assays and the original estimates (Lifton et al., 1978).

A Histone Gene Dosage Compensation Mechanism

The preceding experiments show that wild-type diploid flies contain ~200 copies of the histone repeat unit, and yet a single 12x histone transgene is sufficient to support development of flies lacking all endogenous histone genes. We therefore compared expression levels between the endogenous and transgenic histone genes. Western blot and RT-PCR analysis at two stages of embryogenesis (0–1 hr and 4–6 hr) showed no significant differences in histone protein or mRNA levels between wild-type and *24x-Rescue* flies (Figures 3A–3C). Because the zygotic histone genes are not active in 0–1 hr embryos, histone levels at this time point reflect maternal protein and mRNA derived from the activity of the histone genes during oogenesis. The 4–6 hr time point includes cell cycle 15, when zygotic histone gene activity is first required due to destruction of the maternal histone supply. Despite different demands on histone gene activity between these two stages, the 24x transgenic histone genes produce the same amount of protein and mRNA as 200 copies of the endogenous histone genes (Figures 3A–3C). Thus, histone replacement flies express equivalent steady-state levels of histones as wild-type flies, despite a 10-fold difference in gene copy number.

Because both the protein levels and the amino acid sequences of the endogenous and transgenic histones are identical, we infer that the nucleosome and higher-order chromatin organization is similar across the genome in wild-type and *24x-Rescue* flies. In addition, *12x-* and *24x-Rescue* flies show no increase in sensitivity to the DNA-replication inhibiting agent hydroxyurea (Figure S2, and data not shown), as we hypothesize would occur

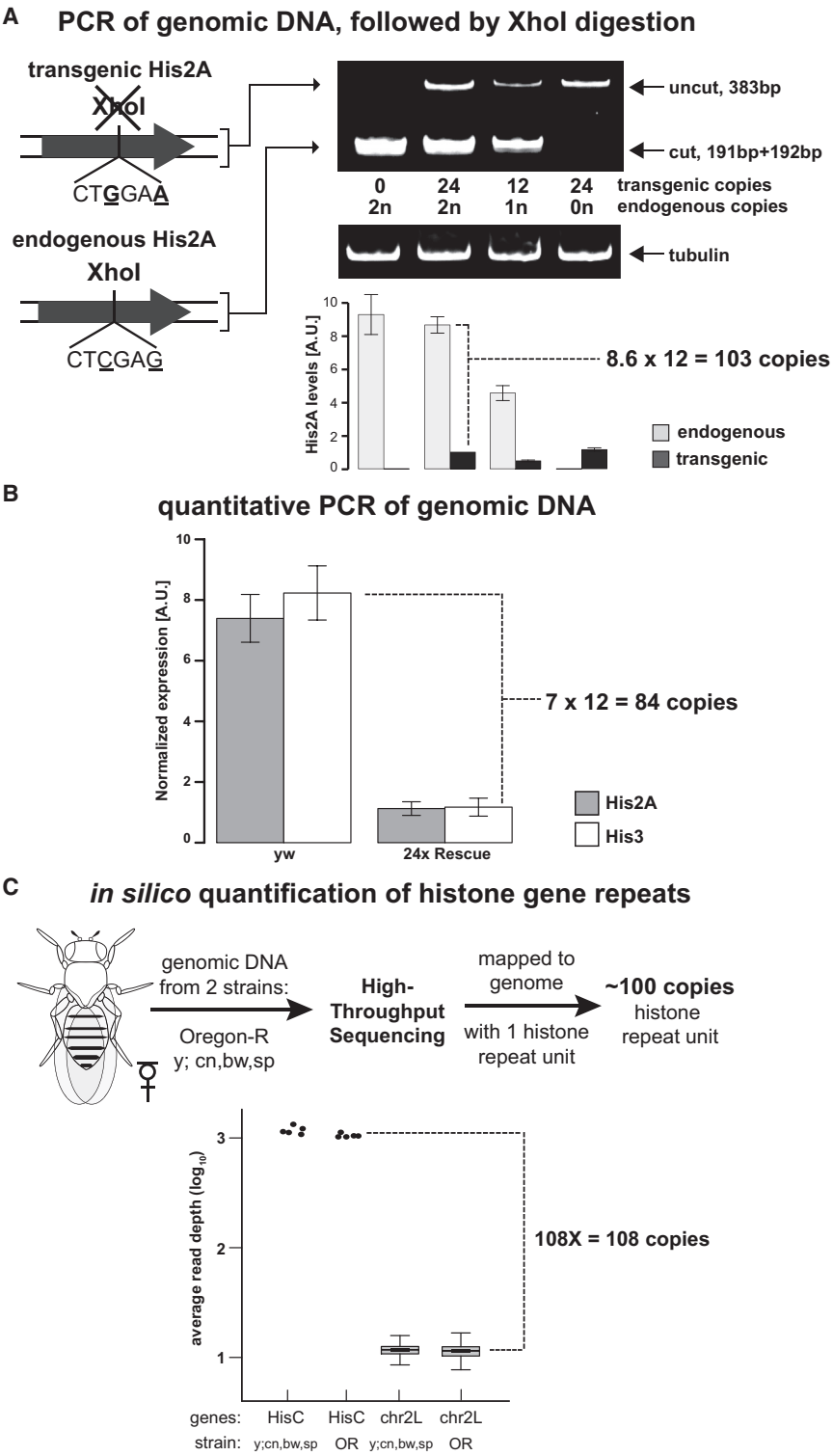


Figure 2. The Haploid *Drosophila* Genome Contains 100 Copies of the Histone Repeat Unit

(A) Ethidium bromide-stained gel of XhoI-digested PCR products of endogenous and transgenic *His2A* genes. For each of the four genotypes, barplots of normalized band intensity are shown below each lane. Error bars represent SEM.

(B) Barplots of normalized real-time PCR results for wild-type (*yw*) and *24x Rescue* genotypes using primers to *His2A* and *His3*. Error bars represent SEM.

(C) Flow chart and plots of in silico quantification of histone gene repeats for two wild-type strains (*Oregon R* [*OR*], and *y;cn,bw,sp*). HisC: total read depth for each of the five replication-dependent genes; chr2L: box plots of average read depth for the remaining genes on chromosome 2L. The box represents the inner quartile range (IQR), and whiskers represent 1.5-times IQR. For clarity, outliers were not plotted.

if histone production during S phase was limiting in these animals.

The similar amount of mRNA produced in *24x-Rescue* and wild-type flies suggests the existence of a histone gene dosage compensation mechanism. To test whether such a mechanism exists, we compared the levels of mRNA in wild-type and *24x-Rescue* flies to those in flies containing both endogenous and transgenic histone genes ("endogenous + 24x"), discriminating between them using the XhoI digestion assay described above (Figure 3D). Similar to the results from undigested samples (Figure 3B), His2A mRNA levels are the same in wild-type and *24x-Rescue* embryos (Figure 3D, lane 1 and lane 3). In contrast, His2A mRNA levels originating from both endogenous and ectopic histone genes are reduced in "endogenous + 24x" embryos compared with wild-type and *24x-Rescue* embryos (Figure 3D, lane 2). Importantly, the sum of endogenous plus ectopic His2A mRNA in "endogenous + 24x" flies equals the levels observed in wild-type or *24x-Rescue* embryos. Thus, the total amount of histone mRNA at a given stage of embryogenesis is the same for each genotype, suggesting that the steady state level of RNA expressed from individual histone genes is scaled to the total number of histone genes present in the genome.

Transgenic Histone Gene Arrays Assemble HLBs that Accurately Process Histone Transcripts

The endogenous histone locus assembles a nuclear subcompartment termed the histone locus body (HLB), which is thought to facilitate efficient transcription and mRNA processing during S phase (Liu et al., 2006; Salzler et al., 2013). To test for HLB assembly at the transgenic arrays, we performed immunofluorescence on salivary gland polytene chromosome spreads. In these polyploid cells, the genome is amplified up to 1000-fold, and sister

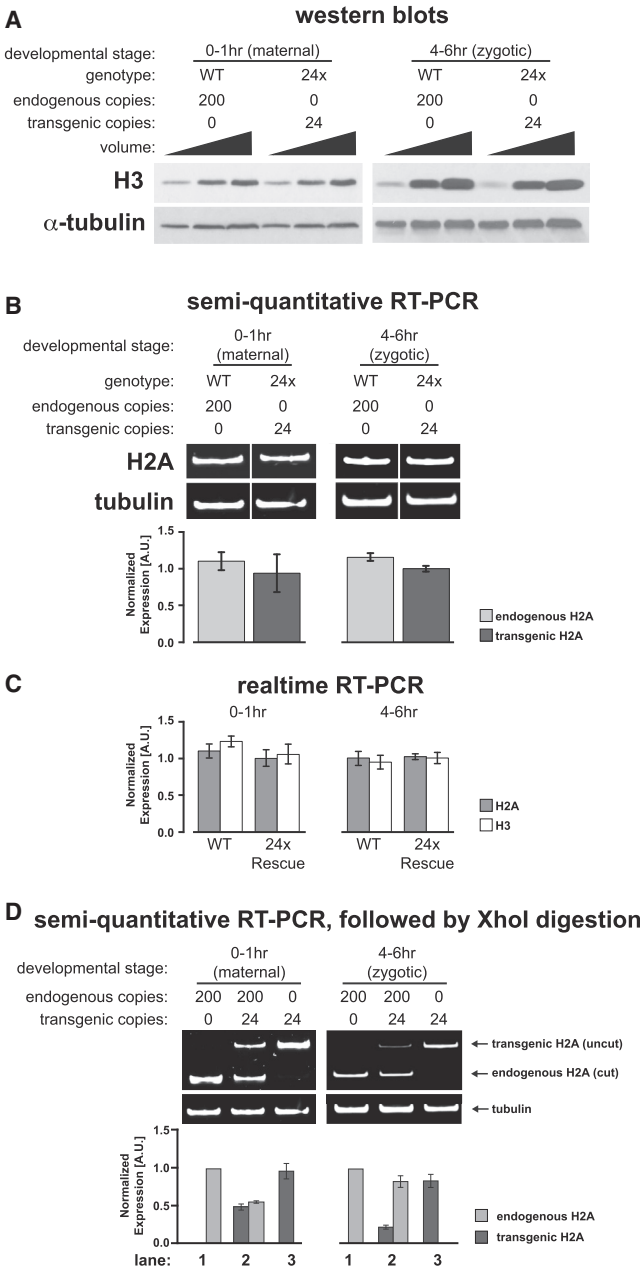


Figure 3. Transgenic Histone Arrays Are Expressed at Levels Similar to the Endogenous Genes

(A) Western blot of wild-type (WT) and *24x Rescue* genotypes at 0–1 hr and 4–6 hr after egg laying.

(B) Ethidium bromide stained gel of RT-PCR products from 0–1 hr and 4–6 hr wild-type (WT) and *24x Rescue* embryos. Barplots of normalized band intensity are shown below each lane. Error bars represent SEM.

(C) Barplots of normalized real-time RT-PCR results for H2A and H3 in 0–1 hr and 4–6 hr wild-type (WT) and *24x Rescue* embryos. Error bars represent SEM.

(D) Ethidium bromide stained gel of XhoI-digested RT-PCR products from 0–1 hr and 4–6 hr embryos for three genotypes: wild-type (lane 1); +/+;$12x^{WT}$/$12x^{WT}$ (lane 2); *24x Rescue* (lane 3). Barplots of normalized band intensity are shown. Error bars represent SEM.

See also Figure S2.

chromatids are aligned in register, allowing for sensitive and high-resolution cytology. Using antibodies to HLB components FLASH, Mxc, and Mute (Figure 4A, and data not shown), we observed a single HLB in wild-type polytene chromosomes at the endogenous histone locus on chromosome 2L (Figure 4A′). In "endogenous + 12x" larvae we observed two HLBs: one assembled at the endogenous histone locus and a second (smaller) one at an ectopic location corresponding to the 12x wild-type transgene inserted on chromosome 3L (Figure 4A″). Finally, in *12x-Rescue* larvae, which contain no endogenous histone genes, we only detected a single HLB at the ectopic site (Figure 4A‴). We conclude that HLBs assemble at transgenic, ectopic histone loci in salivary gland polytene chromosomes, similar to previous findings (Salzler et al., 2013).

To test whether we could also detect HLBs in *24x-Rescue* diploid cells, we performed immunofluorescence on post-blastoderm stage embryos. In *24x-Rescue* embryos, we detected Mxc and FLASH foci in 100% of nuclei (Figure 4B″). Consistent with these cytological results, and with the absence of defects in viability (Figure 1C), S1 nuclease protection assays showed that histone mRNAs were processed normally in *24x-Rescue* animals (Figure 4C). However, we note that HLB assembly is not fully recapitulated in histone replacement animals. Localization of Mute reveals diffuse nuclear staining in 2–4 hr *24x-Rescue* embryos, in contrast to the discrete foci observed when detecting Mute in wild-type embryos at this stage (Figure 4D, 2–4 hr). In *24x-Rescue* embryos, Mute becomes more concentrated in the HLB as embryogenesis proceeds, and its localization resembles that of wild-type embryos by 6–8 hr (Figure 4D), although it never fully achieves the wild-type pattern. Previous work has shown that Mute is a repressor of histone gene expression in *Drosophila* (Bulchand et al., 2010), raising the possibility that Mute's localization to the HLB in *24x-Rescue* embryos helps regulate histone gene transcription.

Together, these data show that we can engineer a functional replacement of the endogenous replication-dependent histone genes, presenting an opportunity to interrogate the function of individual histone residues in animal development. To test this premise, we engineered genotypes that prevent posttranslational modification of three different histone residues with proposed roles in well-characterized epigenetic pathways.

H4K20 Is Dispensable for DNA Replication and Viability

Histone H4 lysine 20 (H4K20) can be mono-, di-, or tri-methylated, and mono-methylation is cell-cycle regulated (Jørgensen et al., 2013). During DNA replication, newly incorporated histones have low levels of H4K20 methylation because of S phase-coupled destruction of PR-Set7/SET8, the enzyme that catalyzes H4K20 mono-methylation (H4K20me1; Havens and Walter, 2011). During G2, H4K20me1 levels rise, allowing for di- and tri-methylation by Suv420H1/H2 enzymes during the subsequent G1 phase. Experimental manipulation of H4K20 methyltransferases suggests that H4K20 methylation contributes to multiple DNA-dependent processes, including replication, maintenance of genomic integrity, and chromatin condensation (Beck et al., 2012b). For example in *Drosophila*, PR-Set7 mutants display defects in heterochromatin silencing, and PR-Set7 null animals die during larval stages, exhibiting cell proliferation defects (Karachentsev et al., 2005). In

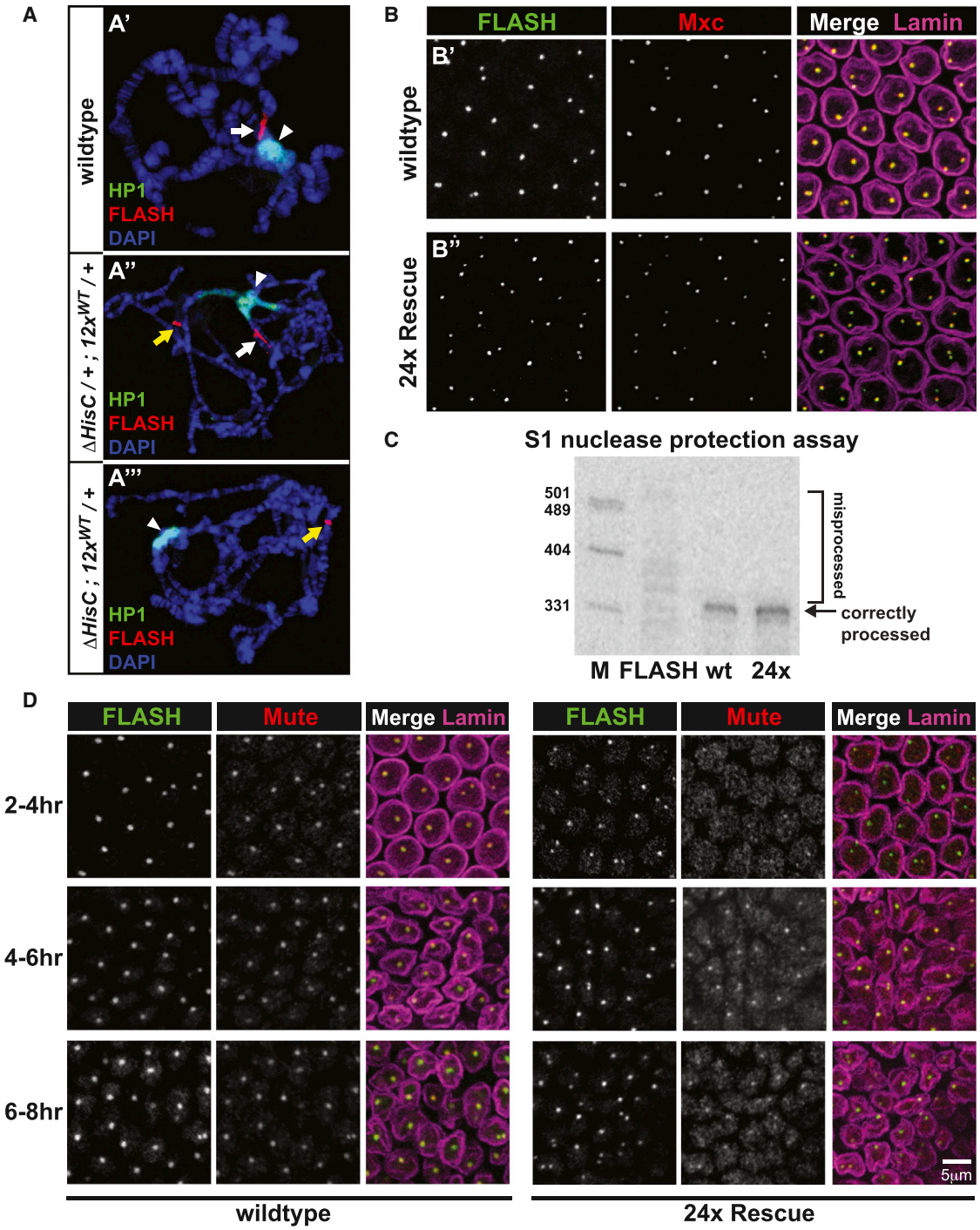


Figure 4. The Transgenic Histone Gene Locus Assembles an HLB that Accurately Processes Histone Transcripts

(A) Confocal images of salivary gland polytene chromosome squashes stained for FLASH (red), HP1 (green), and DAPI (blue) for the three indicated genotypes: wild-type (A′); *ΔHisC*/+;$12x^{WT}$/+ (A″); and *ΔHisC*;$12x^{WT}$/+ (A‴). White arrow, endogenous HLB; white arrowhead, chromocenter; yellow arrow, transgenic HLB.

(B) Confocal images of blastoderm stage embryos stained for FLASH (green), Mxc (red), and Lamin (magenta) for the two indicated genotypes: wild-type (B′) and *24x Rescue* (B″).

(C) Phosphorimager scan of S1 nuclease protection assay performed on total RNA from three genotypes: $FLASH^{PBac}/FLASH^{Df}$; *Oregon R* (wt, 4–6 hr); *24x Rescue* (24x, 4–6 hr). M, markers. Note that FLASH function is required for normal processing of histone mRNA.

(D) Confocal images of embryos at 2–4 hr, 4–6 hr, and 6–8 hr stained for FLASH (green), Mute (red), and Lamin (magenta) for wild-type and *24x Rescue* embryos. For (B) and (D), the maximum projection of four 0.5 μm slices is shown.

mammalian cells, expression of a nondegradable PR-Set7 induces re-replication of DNA due to aberrant licensing of replication origins (Beck et al., 2012b; Tardat et al., 2010). Although these experiments demonstrate the essential role of H4K20 methyltransferases in DNA replication and other processes, the specific function of the H4K20 residue remains untested.

To directly test the requirement for H4K20 in vivo, we generated a histone replacement transgene in which the H4K20 residue is mutated to alanine (*12x*^{H4K20A}). Expression of H4K20A histones in an otherwise wild-type genetic background had no observable phenotype. Given the putative critical role of H4K20 methylation in chromosome duplication and condensation, we were surprised to find that 56% (n = 554) of homozygous *ΔHisC* files with a *12x*^{H4K20A} transgene survive to adulthood (hereafter, H4K20A replacement flies). Although H4K20A replacement flies exhibit a significant developmental delay (24–48 hr), their survival is in direct contrast to expectations, because 100% of PR-Set7 null animals die as larvae (Karachentsev et al., 2005).

To explicitly test the requirement of H4K20 modification in DNA replication, we performed 5-ethynyl-2′-deoxyuridine (EdU) incorporation studies in combination with H4K20me1 antibody staining of egg chambers from wild-type, *12x*^{WT}, and *12x*^{H4K20A} adult ovaries (Figures 5A and 5B). The germline nurse cells and somatic follicle cells of *Drosophila* egg chambers are polyploid, making them amenable to cytological assays. In addition, these cells endoreduplicate asynchronously, allowing for direct comparisons between different stages of the cell cycle within a single egg chamber. We detected robust EdU incorporation in wild-type and *12x Rescue* nurse and follicle cell nuclei that is inversely correlated with H4K20me1 levels (Figures 5A and 5B), consistent with previous findings that PR-Set7 activity is lowest during S phase. We corroborated this result using MPM-2 monoclonal antibody staining of the histone locus body, a readout of CycE-Cdk2 activity that is commonly used as a proxy for active DNA replication (Figures 5C, 5D, S3A, and S3B). In egg chambers from H4K20A replacement flies, there is no detectable H4K20me1 signal in nurse cell or follicle cell nuclei (Figures 5A and S3B). Nevertheless, we detected robust EdU incorporation in these nuclei (Figures 5A, 5C, and S3A), demonstrating that they are capable of DNA replication in the absence of histone H4K20 modification. Because cells lacking detectable H4K20 mono-methylation can actively incorporate EdU during S phase and progress to gap phases, we conclude that canonical H4K20 methylation is not essential for viability, cell-cycle progression, or DNA replication.

The *Drosophila* genome contains a single-copy gene encoding a replication-independent version of histone H4 (*His4r*) that is identical in amino acid sequence to canonical H4 (Akhmanova et al., 1996). Although immunofluorescence and western blot experiments (Figures 5A and 5E) could not detect methylation of H4K20, expression of His4r could, in theory, provide a sufficient amount of modifiable H4K20 to compensate for the absence of replication-dependent H4K20. RT-PCR showed that His4r mRNA levels are unchanged in 12x^{H4K20A} replacement flies, arguing against the bulk replacement of canonical H4 with His4r (Figure S3D). To genetically test for the requirement of His4r in 12x^{H4K20A} replacement flies, we obtained a fly line with a Piggy-Bac transposon insertion near the 5′ end of *His4r* (hereafter *His4r*^{PB}) (Figure S3C). Sequencing of PCR amplicons from *His4r*^{PB} flies showed that the transposon is inserted in the first intron or 5′ UTR of *His4r*, depending on the gene isoform (Figure S3E). *His4r*^{PB} homozygotes are viable at submendelian ratios, but they are sterile. RT-PCR of whole *His4r*^{PB} adults or dissected ovaries showed no detectable *His4r* mRNA (Figure S3F). Thus, *His4r*^{PB} is a null or strong hypomorphic allele of *His4r*.

We next took advantage of the facile genetics afforded by our histone replacement platform by recombining the *His4r* mutant allele with the *12x*^{H4K20A} transgene (Figure 5F). Third instar larvae homozygous for both *His4r*^{PB} and *ΔHisC* covered by *12x*^{H4K20A} showed no expression of *His4r* by RT-PCR (Figure 5G). Despite this fact, ∼85% of these flies pupate and 35% develop until late pharate adults (Figure S3G). In one instance, we obtained an overtly healthy adult fly that contained no wild-type copies of H4, demonstrating that H4K20 is not essential for completion of development. We note that this experiment may overestimate the importance of H4rK20 because it was performed in the absence of any H4r expression, and thus in the absence of any function of H4r independent of its K20 residue. Together, these findings demonstrate the importance of directly testing the functions ascribed to histone residue PTMs, rather than relying on indirect inferences from mutant phenotypes of the enzymes that catalyze their modification.

H3K27 Is Required for Heritable Silencing of Polycomb Target Genes

The signaling events that specify cell fates during development of multicellular organisms are often transient, yet these decisions need to be remembered over time, through multiple rounds of cell division. Methylation of histone H3 on lysine 27 (H3K27me3) is associated with heritable repression of Polycomb group (PcG) target genes (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002). Genetic and biochemical studies have identified multiple protein complexes that are required for PcG target gene repression (Klymenko et al., 2006; Kuzmichev et al., 2002; Shao et al., 1999). However, the specific role of H3K27 in PcG target gene repression is less well understood.

We generated histone replacement constructs with either 8 or 12 histone gene repeat units in which H3K27 is mutated to alanine (H3K27A). We recovered transgenic flies with eight H3K27A tandem gene repeats (*8x*^{H3K27A}), but did not recover a *12x*^{H3K27A} transformant. The presence of *8x*^{H3K27A} transgenes in an otherwise wild-type genetic background elicited several dominant phenotypes, including decreased viability and diminished fertility. A hemizygous *ΔHisC* background enhanced these phenotypes, and also resulted in new phenotypes, including aberrant leg and wing morphogenesis (Figure S4, and not shown). The posterior wing phenotype is reminiscent of gain-of-function alleles of *Ultrabithorax* (*Ubx*), in which *Ubx* is ectopically active in cells of the second thoracic segment where it is normally inactive in wild-type animals (Lewis, 1978). *Ubx* is a well-characterized target of PcG complexes in the wing (Christen and Bienz, 1994). To determine whether expression of H3K27A histones results in ectopic expression of *Ubx*, we performed immunofluorescence experiments on third instar imaginal wing discs. Similar to wild-type flies, we detected no Ubx expression in the main epithelium of flies bearing *12x*^{WT} transgenes

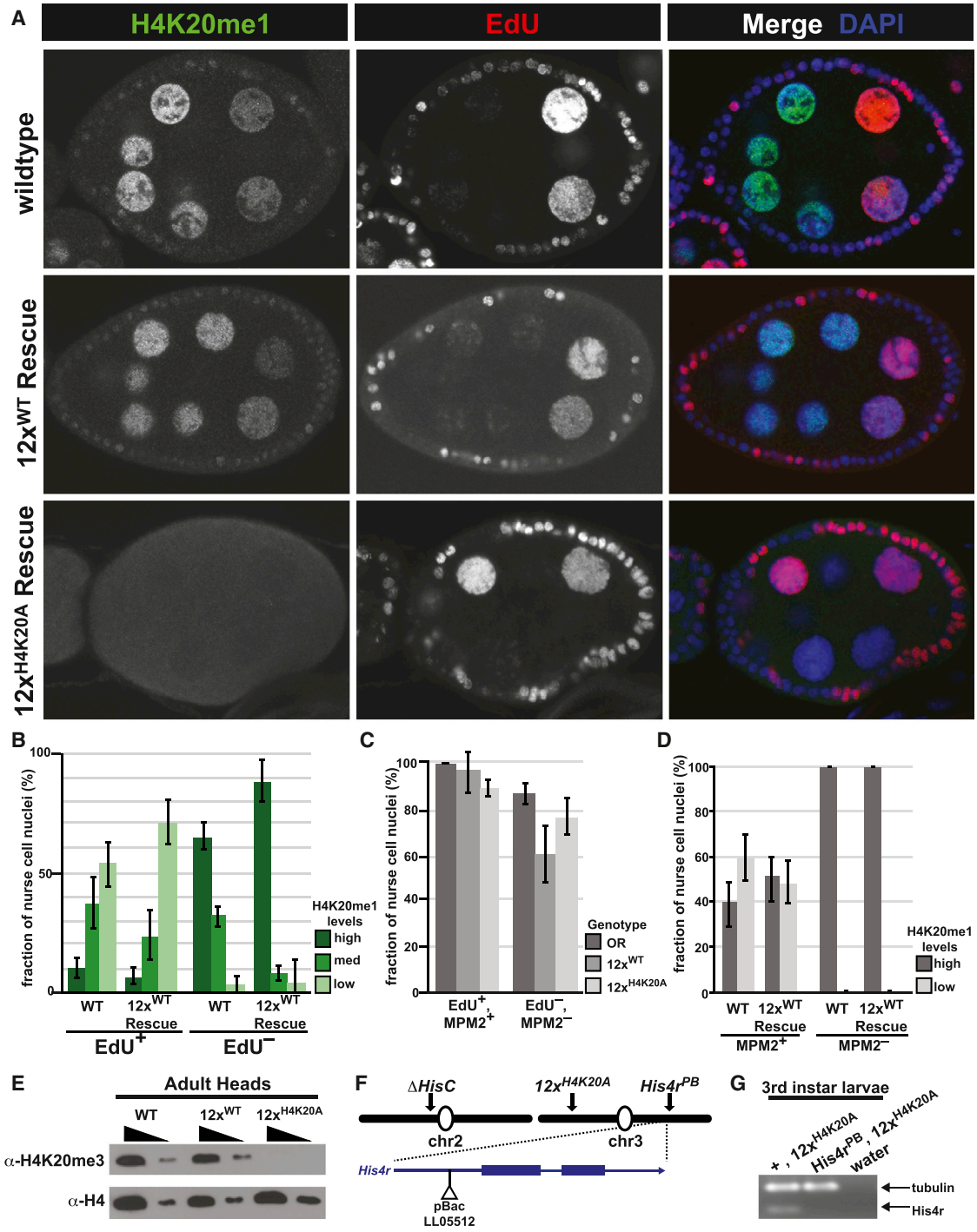


Figure 5. H4K20 Is Dispensable for DNA Replication and Viability

(A) Confocal images of stage 6–8 egg chambers stained for H4K20me1 (green), EdU (red), and DAPI (blue).

(B) Barplot of the fraction of nurse cell nuclei with indicated H4K20me1 levels for each category of EdU incorporation. Error bars represent SEM.

(C) Barplot of the fraction of nurse cell nuclei with shared EdU and MPM2 status for each genotype. Error bars represent SEM.

(D) Barplot of the fraction of nurse cell nuclei with the indicated H4K20me1 levels for each category of MPM2 staining. Error bars represent SEM.

(E) Western blots of adult head extracts from three genotypes: wild-type (WT), *12x Rescue* ($12x^{WT}$), and H4K20A replacement flies ($12x^{H4K20A}$).

(F) Genetic scheme for analysis of *His4r* function in H4K20A replacement flies.

(G) Ethidium bromide stained gel of RT-PCR products from H4K20A replacement flies. Genotypes: (lane 1) *ΔHisC/ΔHisC; $His4r^{PB}$, $12x^{H4K20A}$/TM6B*. (lane 2) *ΔHisC/ΔHisC; $His4r^{PB}$, $12x^{H4K20A}$/ $His4r^{PB}$*.

See also Figure S3.

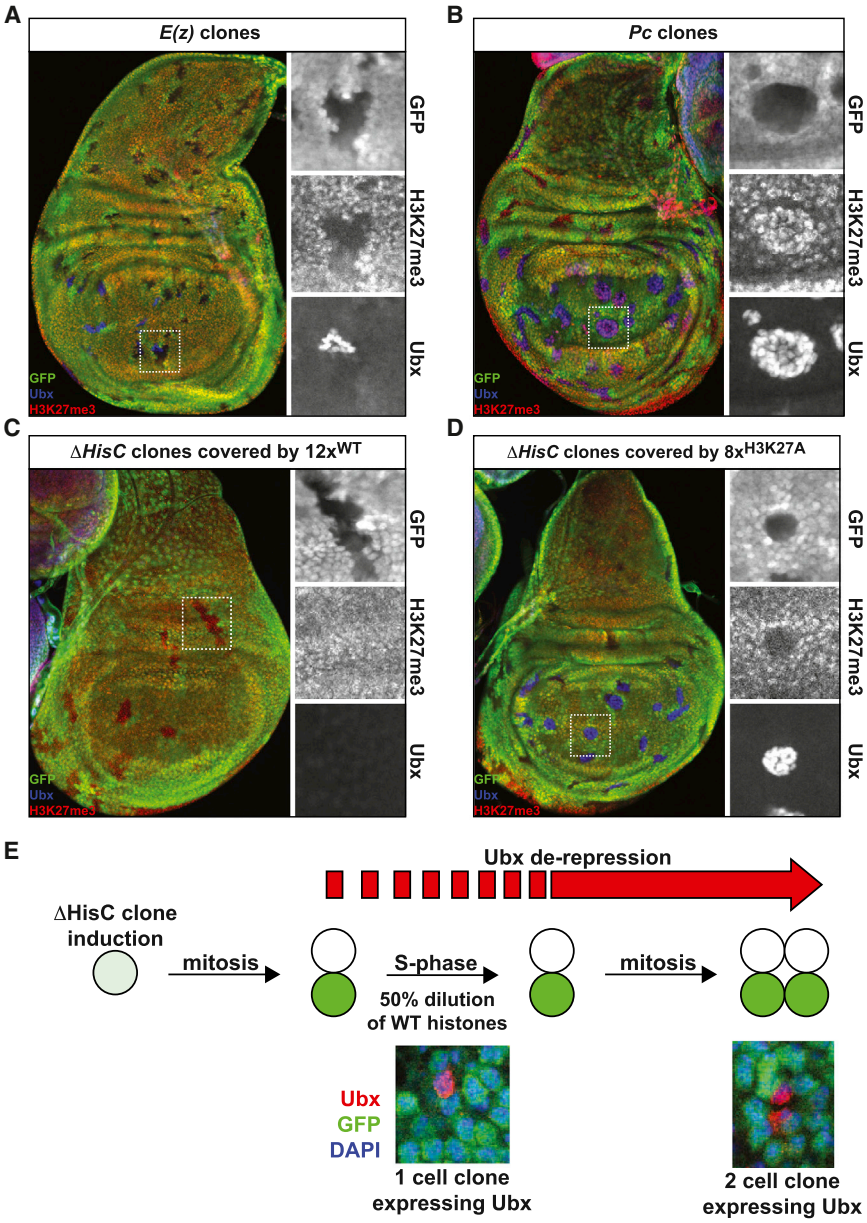


Figure 6. H3K27 Is Required for Polycomb Target Gene Repression

(A–D) Confocal images of imaginal wing discs of the indicated genotypes with 48–60 hr mitotic clones stained for Ubx (blue) and H3K27me3 (red). Mutant and *ΔHisC* clones are marked by absence of GFP (green). The square outlines the magnified area at the right.

(E) Schematic of clone induction and confocal images of two different clones induced 24 hr prior to dissection. GFP (green), Ubx (red), DAPI (blue). See also Figure S4.

(Figure S4D). In contrast, we detected low-level ectopic expression of *Ubx* in the posterior compartment of $8x^{H3K27A}$ third instar imaginal wing discs (Figure S4E), and this ectopic expression of *Ubx* is enhanced in *ΔHisC* hemizygous animals (Figure S4F). These experiments are consistent with a dose-dependent requirement for modified H3K27 histones in PcG target gene repression, and indicate that incorporation of nonmodifiable H3K27A into chromatin interferes with the ability of PcG complexes to repress target genes.

Because methylation of H3K27 is found at target genes repressed by PcG complexes, we tested for a genetic interaction between a *Pc* loss of function allele (Pc^{XT}) and the $8x^{H3K27A}$ transgene. The *Pc* gene is haploinsufficient, and both Pc^{XT} heterozygous flies and $8x^{H3K27A}$ transgenic flies exhibit ectopic sex combs at low frequency (8% and 1%, respectively; Figure S4G). However, $8x^{H3K27A}$ transgenic flies that are also heterozygous for Pc^{XT} exhibit ectopic sex combs at a much higher frequency (68%; Figure S4G). Thus, the presence of H3K27A histones enhances the *Pc* ectopic sex comb phenotype, consistent with a role for modified H3K27 in PcG target gene silencing.

To directly test the requirement of H3K27 in PcG target gene repression, we used the FLP-FRT system (Xu and Rubin, 1993) to generate and compare cell clones that express nonmodifiable H3K27A to those that are mutant for *E(z)*, the enzyme that catalyzes methylation of H3K27 (Czermin et al., 2002; Müller et al., 2002), as well as for *Pc*, which specifically binds to H3K27me3 at PcG target genes (Cao et al., 2002). Clones of *E(z)* mutant wing imaginal disc cells show ectopic expression of *Ubx* in the wing pouch near the dorsal-ventral boundary (Figure 6A), consistent with previous findings (Müller et al., 2002). Clones of cells lacking *Pc* function also ectopically express *Ubx* in the same spatial pattern as *E(z)* mutant clones (Figure 6B). Because these clones were made at the same time (48–60 hr after egg laying), the increased *Ubx* levels in *Pc* clones relative to *E(z)* clones may be due to differences in Pc and E(z) protein stability. Strikingly, *ΔHisC* clones containing an $8x^{H3K27A}$ transgene (hereafter, H3K27A clones) show robust ectopic expression of *Ubx* in the same spatial pattern as do *E(z)* and *Pc* mutant clones, unlike *ΔHisC* clones containing a $12x^{WT}$ or $6x^{WT}$ transgene that do not express Ubx (Figures 6C and 6D). H3K27A clones also exhibit ectopic expression of *Abd-B* and *En*, two other PcG target genes in wing imaginal discs (not shown). The restriction of ectopic PcG target activity only to clones near the D/V boundary demonstrates that replacement of wild-type H3 with H3K27A does not result in widespread deregulation of gene expression. Instead, there is a specific deregulation of PcG target genes in the expected cell populations within wing imaginal discs. *ΔHisC* clones covered by an 8x histone replacement transgene in which H3K27 was mutated to

an arginine instead of an alanine ($8x^{H3K27R}$) deregulate PcG target genes in the same spatial profile (Figure S4H), demonstrating that loss of a modifiable H3K27 is responsible for the phenotype rather than loss of the positive charge on the lysine side chain, consistent with another recent report (Pengelly et al., 2013). These experiments provide direct evidence that H3K27 is required for regulation of PcG target genes. Activation of PcG target genes in the absence of a modifiable H3K27 residue also demonstrates that other modifications of H3K27 (e.g., acetylation) are not required for expression per se.

A hallmark of epigenetic regulators like Polycomb is their ability to propagate gene regulatory states through many cell divisions (Simon and Kingston, 2013). Histone modification levels, chromatin composition, and chromosome structure all change dramatically through the cell cycle (Follmer et al., 2012; Fonseca et al., 2012), raising the possibility that changes in epigenetic states may depend on passage through a particular stage of the cell cycle. We took advantage of the high temporal resolution of mitotic clone analysis to determine when ectopic expression of *Ubx* is first detectable following removal of wild-type histones. Consistent with a cell-doubling time of 12–24 hr in wing discs (Shibutani et al., 2008), we observed many H3K27A clones consisting of only two cells 24 hr after clone induction. Many of these clones show high levels of ectopic *Ubx* expression (Figure 6E). Remarkably, we also found single cell H3K27A clones with robust ectopic *Ubx* activity (Figure 6E). Single cell H3K27A clones have yet to divide following induction of the clone. Because the cell cycle-dependent histones are only expressed during S phase, the observed ectopic expression of *Ubx* indicates that these clones have completed DNA replication and are in the G2 phase of the cell cycle. Thus, we conclude that cell division is not necessary for ectopic activation of *Ubx*, and that a 50% dilution of wild-type histones by H3K27A histones after a single S phase results in derepression of *Ubx* in the wing.

H3K36 Is Required for Completion of Development

Methylation of lysine 36 on histone H3 (H3K36) is a PTM associated with actively transcribed genes (Bannister et al., 2005; Strahl et al., 2002). Studies in the budding yeast *S. cerevisiae* have demonstrated a role for H3K36 methylation in suppression of spurious transcription initiation in the wake of transcribing RNA polymerase by recruitment of histone deacetylases to gene bodies (Carrozza et al., 2005; Keogh et al., 2005). In animals, depletion of SETD2, the enzyme that catalyzes H3K36me3, is reported to cause dysregulation of alternative exon inclusion, implicating H3K36 methylation in the regulation of pre-mRNA splicing (Luco et al., 2010). A role for H3K36 methylation in regulation of sex chromosome dosage compensation has also been reported (Larschan et al., 2007).

We generated a 12x histone replacement transgene in which H3K36 is mutated to arginine ($12x^{H3K36R}$). When carried in a wild-type or *ΔHisC* hemizygous background, $12x^{H3K36R}$ transgenes cause no discernible developmental or fertility defects, demonstrating that expression of H3K36R histones does not result in overtly dominant phenotypes. By contrast, 100% of homozygous *ΔHisC* animals containing one $12x^{H3K36R}$ histone replacement transgene (hereafter H3K36R replacement flies) die before the end of pupal development (Figure 7A). Western blots of wild-type and H3K36R replacement larvae show that H3K36R histones are expressed and are not recognized by anti-H3K36me3 antibodies (Figure 7B). To examine the cellular basis for the requirement of H3K36 in development, we generated clones of wing imaginal disc cells lacking endogenous histones. Although we observed a marked decrease in H3K36me3 levels in *ΔHisC* clones covered by a $12x^{H3K36R}$ transgene (Figure 7C), we did not observe a significant difference in the size of these clones relative to their wild-type twins (not shown). We also observed a dramatic decrease in H3K36me3 levels in polytene chromosomes from H3K36R replacement third instar larvae (Figure 7D). Despite this depletion, we detected no apparent defects in chromosome structure. Together, these experiments demonstrate that H3K36 is essential for viability in *Drosophila*, in contrast to results from budding yeast, where H3K36 mutations show no overt growth defects (Kizer et al., 2005).

DISCUSSION

A Facile Genetic Platform to Study Histone Function

Distinguishing direct from indirect effects caused by mutations in histone-modifying enzymes can be difficult because histone modifiers can have multiple substrates, including nonhistone proteins (Glozak et al., 2005; Huang and Berger, 2008; Sims and Reinberg, 2008). The more direct approach of investigating the role of histone PTMs by mutating residues of interest is intractable in most animal systems. Here we demonstrate functional histone gene replacement in *Drosophila* with a single BAC-based transgene, improving upon a previously described method requiring multiple transgenic insertions (Günesdogan et al., 2010). Using this platform, we provide evidence for a mechanism that scales histone expression to compensate for changes in gene number, and demonstrate that the biological function of histone tail residues can be ascertained either in whole animals or specific tissues. The data presented here reveal that phenotypes caused by mutation of histone-modifying enzymes will not always be recapitulated by mutation of the corresponding histone target residue.

Histone Residue Mutant Phenotypes Can Differ between Yeast and Animals

Histone gene replacement strategies in budding yeast have identified multiple histone residues that are essential for viability and responses to environmental challenges (Dai et al., 2008; Nakanishi et al., 2008). Multicellular animals require a greater range of genome regulation to create a diversity of cell types in development, which may result in a greater reliance on posttranslationally modified histone residues that affect epigenetic regulation of genome activity. Our data support this hypothesis. We found that H3K36R replacement flies die before completing development, whereas the same mutation in yeast is viable and shows no overt growth defects (Kizer et al., 2005). These findings suggest a greater dependency on H3K36 in the development and survival of multicellular organisms. Thus, our histone replacement platform has the potential to identify additional histone residues that perform essential functions in animals but have not been discovered in previous studies.

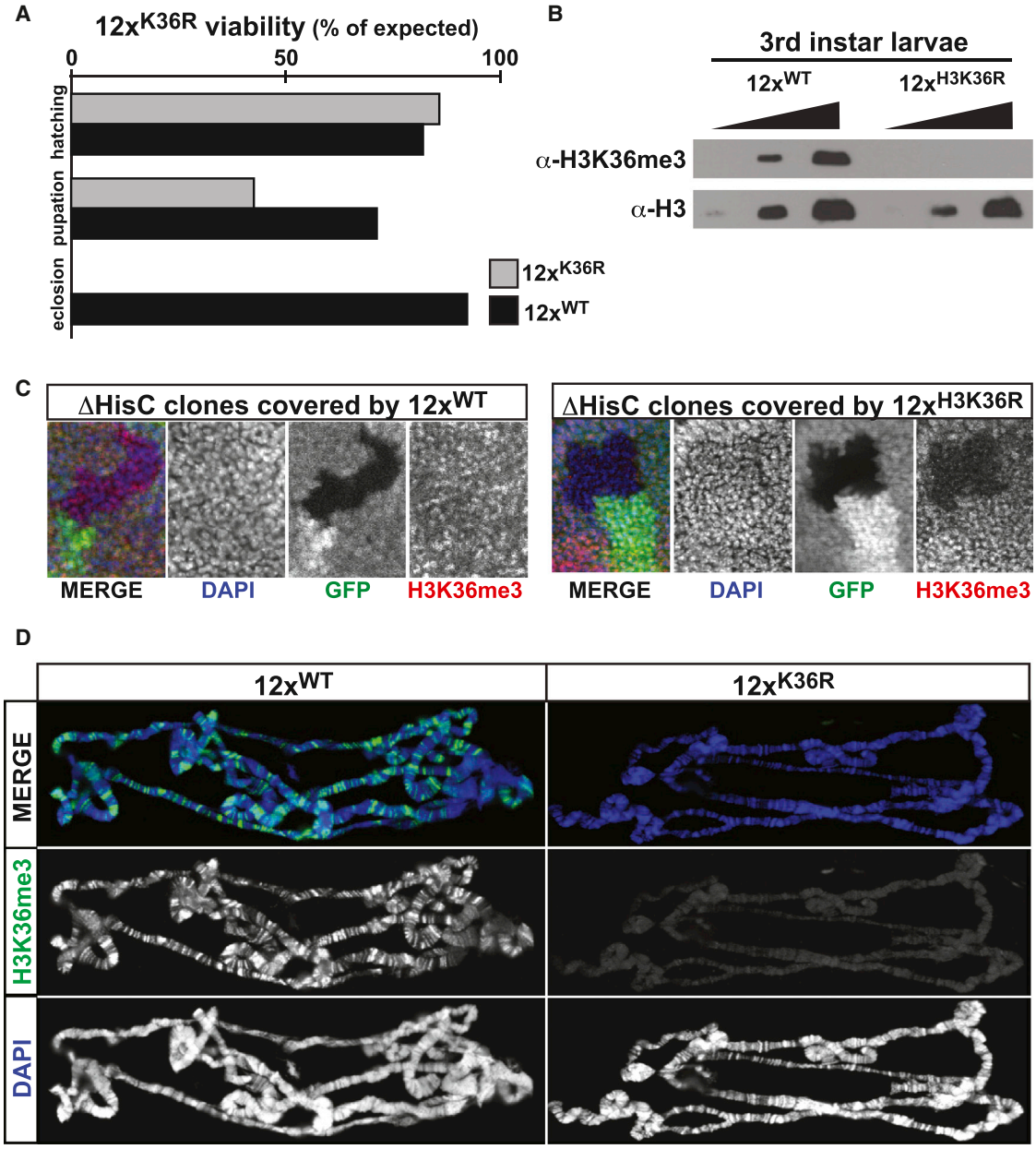


Figure 7. H3K36R Replacement Flies Are Inviable and Lack H3K36me3

(A) Barplots of the expected fraction of viable flies at each indicated stage. We used the denominator from the previous stage to calculate the percentage observed at each stage.

(B) Western blots from wild-type (*12x*^{WT}) and H3K36R replacement (*12x*^{H3K36R}) third instar larval nuclei.

(C) Confocal images of imaginal wing discs with 48–60 hr mitotic clones stained for H3K36me3 (red) and DAPI (blue). *ΔHisC* clones are marked by absence of GFP (green).

(D) Confocal images of salivary gland polytene chromosomes from *12x Rescue* (*12x*^{WT}) and H3K36R replacement flies (*12x*^{H3K36R}) stained for DAPI (blue) and H3K36me3 (green).

The Requirement of Histone Residues in Heritable Regulation of Gene Expression

The co-occurrence of H3K27me3 and transcriptional repression by Polycomb proteins has led to the widely held hypothesis that H3K27me3 contributes to repression of PcG target gene expression. However, a direct test for the requirement of H3K27me3 in PcG target gene repression has been lacking. Our data, as well as those of Müller and colleagues (Pengelly et al., 2013), demonstrate that H3K27 performs an essential function in maintaining PcG target gene repression. Thus, at least in certain circumstances, posttranslational modification of a histone residue is directly required for regulation of gene expression.

Direct comparison of *ΔHisC* clones covered by *H3K27A* and *H3K27R* transgenes shows the same pattern of PcG target

gene derepression in wing imaginal discs. However, the dominant effects we observe in H3K27A transgenic flies are notably absent from the H3K27R transgenic flies, suggesting that expression of H3K27A histones results in a more severe phenotype. Because both mutations introduce nonmodifiable residues, the difference in their phenotypes may result from the charge at this residue, which is maintained as positive by substitution with arginine, but not alanine. The decrease in positive charge in H3K27A histones may decrease the affinity of the interaction between the histone tail and the negatively charged DNA backbone, potentially altering chromatin structure. Polycomb target genes may be particularly sensitive to changes in chromatin structure, as one proposed mode of PcG action is through chromatin compaction (Simon and Kingston, 2013). Thus, the amino acid used to mutate a particular histone residue could distinguish between roles of histone PTMs to serve as binding sites for *trans*-acting proteins and to act as regulators of chromatin packaging.

H4K20 Methylation in Metazoans

The data definitively show that canonical H4K20 is dispensable for DNA replication and *Drosophila* development. This finding is surprising, given that genetic studies manipulating PR-Set7/Suv420H1/Suv420H2 activity in cell culture and animal models have demonstrated a consistent correlation of deleterious and lethal phenotypes with loss of H4K20 methylation (Beck et al., 2012b). In both mouse and *Drosophila* a complete loss of the PR-Set7 mono-methyltransferase, which is required for all H4K20 methylation states, results in a failure to complete development (Huen et al., 2008; Karachentsev et al., 2005; Oda et al., 2009). In addition, tethering active, but not catalytically inactive, PR-Set7 to DNA results in the recruitment of components of the ORC and MCM complexes, which are essential DNA replication factors (Beck et al., 2012a; Tardat et al., 2010). Although flies lacking canonical H4K20 are viable, we note that they are not phenotypically wild-type. For example, they are sensitive to culture conditions and exhibit a significant developmental delay, even when H4K20A replacement larvae are cultured separately from their wild-type siblings. Thus, posttranslational modification of H4K20 is likely one of multiple mechanisms that ensures fidelity and robustness to the processes of DNA replication, chromatin condensation, and heterochromatin maintenance.

Several lines of evidence demonstrate that the *Drosophila* replication-independent H4 gene (*His4r*) does not compensate for the absence of replication-dependent H4K20. First, we saw no increase in *His4r* mRNA levels in H4K20A replacement animals. Second, we cannot detect H4K20 PTMs in H4K20A replacement animals by immunofluorescence or by western blot. Third, we show that when covered by $12x^{H4K20A}$ transgenes, flies homozygous for both $His4r^{PB}$ and *ΔHisC* can survive until late pharate stages and complete development. We cannot detect *His4r* mRNA in flies homozygous for $His4r^{PB}$, demonstrating that this is a strong allele. Any maternally contributed wild-type H4 would either be absent or greatly diluted by these late stages of development. Thus maternal H4 is unlikely to have any impact on processes that require a significant concentration of H4K20 PTMs. The decrease in the ability of $12x^{H4K20A}$ transgenes to rescue *ΔHisC* in $His4r^{PB}$ homozygotes may be due to a function for *His4r* that becomes necessary only after mutation of canonical H4K20. *His4r* rescue experiments will be required to definitively test this possibility.

Altogether, our data demonstrate that H4K20 is neither essential for DNA replication nor for completion of development. One explanation for this finding is that nonhistone substrates of PR-Set7/SET8 are essential for DNA replication and cell-cycle progression. Comparative phenotypic analysis of mutations in histone modifying enzymes and mutations in their cognate histone residues should provide answers to this question. More generally, our findings raise the important possibility that nonhistone proteins can function as carriers of epigenetic information that is required for proper animal development.

EXPERIMENTAL PROCEDURES

Histone Locus Transgene Construction

The pMulti-BAC vector was generated by combining components from pBAC/oriV (Wild et al., 2002) and pattB (Bischof et al., 2007), followed by extensive site-directed mutagenesis. The 5 kb histone repeat unit was subcloned into pBluescript prior to multimerization in pMulti-BAC. See Supplemental Experimental Procedures for additional details.

Fly Strains and Genetic Crosses

A list of all fly strains and genetic crosses is included in the Supplemental Experimental Procedures.

Immunofluorescence, Clone Induction, and Confocal Microscopy

Salivary gland polytene chromosome squashes were performed on wandering third instar larvae, as previously described (Salzler et al., 2013). Embryo stains and mitotic recombination experiments in imaginal discs were performed as previously described (Estella et al., 2008). Egg chamber immunofluorescence was performed as previously described (Deng et al., 2001). EdU incorporation was performed using the Click-iT EdU Alexa Fluor 555 imaging kit (Invitrogen) according to the manufacturer's instructions. The maximum projection from two adjacent z-slices from stage 6–8 egg chambers was used as representative images for each genotype. For quantification of signal intensities in nurse cell nuclei, each channel was scored independently of the other channels. See Supplemental Experimental Procedures for details and for a list of antibodies used.

In Silico Quantification of Endogenous Histone Gene Repeats

High-throughput genomic DNA sequencing libraries were generated from adult virgin females, as previously described (McKay and Lieb, 2013; see genomic input files under GEO accession number GSE38727). The following exceptions were made to unambiguously map reads to the histone locus. A custom reference genome was created by removing all replication-dependent histone gene repeat sequences from genome version dm3, and by adding back a single 5 kb histone gene repeat unit. An unlimited number of reads were then mapped to this custom genome using bowtie (version 0.12.3) using the options "–nomaqround" and "–best" (Langmead et al., 2009). Coverage values were then calculated for each base in the genome. The average read depth across each gene's translated sequence was calculated for the five replication-dependent histone genes, and the remaining refSeq genes on chromosome 2L. Data were plotted in R (http://www.R-project.org).

S1 Nuclease Protection Assay

S1 assays were performed as described in (Salzler et al., 2013) using total RNA isolated from 4 to 6 hr embryos.

Western Blotting

Immunoblot analyses were performed as described (Fuchs et al., 2012) with the following exceptions. Embryos were lysed by bead-beating for 3 min in SUTEB buffer (1% SDS, 8M urea, 10 mM Tris [pH 6.8], 10 mM EDTA, 0.01% bromophenol blue). Lysates were then boiled for 10 min and the supernatant was clarified by centrifugation. We could not obtain a reproducible

anti-H4K20me1 signal on western blots, so anti-H4K20me3 was used. Adult heads were used for this analysis because they expressed high levels of H4K20me3.

Reverse Transcription and PCR Assays

For histone gene expression analyses, total RNA was isolated using Trizol, and reverse transcription was performed using random hexamers and Superscript II (Invitrogen), according to the manufacturer's protocols. RT-PCR was performed using gene-specific primers to *His2A*, *His3*, *His4r*, and α-*tubulin*. Genomic DNA from 15 adult males was used for histone gene copy-number assays. For semiquantitative analysis of PCR products, amplicons were run on 8% acrylamide gels, and bands were quantified using ImageJ. iTaq Universal SYBR Green Supermix (Bio-Rad) was used for real-time PCRs. Additional details and primer sequences are available in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.12.025.

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