Sex-specific phenotypes of histone H4 point mutants establish dosage compensation as the critical function of H4K16 acetylation in *Drosophila*

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Acetylation of histone H4 at lysine 16 (H4K16) modulates nucleosome–nucleosome interactions and directly affects nucleosome binding by certain proteins. In *Drosophila*, H4K16 acetylation by the dosage compensation complex subunit Mof is linked to increased transcription of genes on the single X chromosome in males. Here, we analyzed *Drosophila* containing different H4K16 mutations or lacking Mof protein. An H4K16A mutation causes embryonic lethality in both sexes, whereas an H4K16R mutation permits females to develop into adults but causes lethality in males. The acetyl-mimic mutation H4K16Q permits both females and males to develop into adults. Complementary analyses reveal that males lacking maternally deposited and zygotically expressed Mof protein arrest development during gastrulation, whereas females of the same genotype develop into adults. Together, this demonstrates the causative role of H4K16 acetylation by Mof for dosage compensation in *Drosophila* and uncovers a previously unrecognized requirement for this process already during the onset of zygotic gene transcription.

chromatin | histone modification | H4K16 acetylation | dosage compensation complex | *Drosophila*

In eukaryotic chromosomes, the genomic DNA exists in the form of chromatin with the nucleosome as its basic building block. Nucleosomes consist of an octamer assembly of the four core histone proteins H2A, H2B, H3, and H4, around which 147 bp of DNA are wrapped in two-helical turns. Every nucleosome is separated from its neighboring nucleosomes by a stretch of linker DNA that, on average, is between 20 and 40 base pairs long. This organization of genomic DNA into arrays of nucleosomes affects almost all processes that occur on DNA. Consequently, a wide repertoire of proteins that modulate chromatin structure has evolved in eukaryotes. These proteins fall into two broad categories: those that make chromatin more accessible and those that make it more compact. At the molecular level, these proteins include factors that remodel nucleosomes, enzymes that add or remove posttranslational modifications on histone proteins, or proteins that bind to these modifications.

Among the different posttranslational histone modifications, acetylation of histone H4 at lysine 16 (H4K16ac) has long been of particular interest because of its roles in chromatin fiber folding, gene silencing in yeast, and dosage compensation in *Drosophila*. The importance of H4K16 in chromatin folding emerged from structural studies on nucleosomes that showed that a region of the N-terminal tail of H4 encompassing K16 interacts with the acidic patch formed by H2A and H2B residues on the octamer surface of a neighboring nucleosome (1, 2). This direct nucleosome–nucleosome contact is critical for compaction of nucleosome arrays in solution in vitro and, importantly, compaction is disrupted if H4K16 is acetylated (3, 4). In the case of gene silencing in yeast, the specific requirement for H4K16 was first uncovered through genetic studies that showed that yeast cells with H4K16 point mutations are viable and proliferate normally but show defective silencing at the mating type loci (5–7). Subsequent biochemical and structural studies then established that deacetylation of H4K16ac by the SIR protein silencing complex and binding of the complex to nucleosomes with deacetylated H4K16 are key for creating a transcriptionally inactive chromatin structure at the mating type loci and at telomeres (8, 9). In the case of dosage compensation in *Drosophila*, finally, it was the observation that H4K16ac is specifically enriched on the single X chromosome in males that first indicated that this modification might be linked to X-chromosome dosage compensation (10). Analyses of the dosage compensation regulatory proteins first identified through genetic approaches then revealed that the acetyltransferase Mof, a subunit of the dosage compensation complex (DCC), hyperacetylates H4K16 on the X chromosome in males, and this established a functional connection between H4K16ac and increased transcription of X-chromosomal genes (11–13). Taken together, these studies therefore shaped the view that nonacetylated H4K16 generates compact and transcriptionally repressed chromatin, whereas acetylation of H4K16 provides a mechanism for generating less compact and transcriptionally active chromatin. In both biological settings—that is, for silencing of mating type loci by SIR protein complexes in yeast and for X-chromosome dosage compensation by the DCC in *Drosophila*—it is the site-specific targeting of these protein complexes to the relevant genomic locations that provides the basis for localized deacetylation and acetylation of H4K16, respectively (reviewed in refs. 14 and 15). For example, while H4K16 acetylation in

Significance

The posttranslational modification of nucleosomes is implicated in the regulation of gene expression and chromatin packaging in all eukaryotes. In this study, we investigate the function of histone H4 lysine 16 (H4K16) and its acetylation in *Drosophila* by generating strains in which lysine 16 is mutated to arginine, glutamine, or alanine. The main conclusion of our paper is that even though H4K16 acetylation was reported to be a critical regulator of chromatin folding in vitro and has therefore been assumed to affect many different nuclear processes, its essential function in *Drosophila* is in one process: X-chromosome dosage compensation in males.

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Drosophila is present at the 5′ transcription start sites of active genes on all chromosomes in both sexes, it is the targeted binding of the dosage compensation complex to X-linked genes in males that results in the specific enrichment of this modification on X-linked gene bodies in this sex (16).

What are the consequences of mutating H4K16 in higher organisms? Here, we generated *Drosophila* in which H4K16 was substituted by amino acids with different chemical properties. Depending on the amino acid substitution, these mutations cause embryonic lethality in both sexes, male-specific lethality, or permit the mutant animals to develop into adults. Importantly, these studies reveal a stringent requirement for H4K16 acetylation specifically in males but not in females, suggesting that dosage compensation is the main process that critically requires this modification. Moreover, we show that males that lack maternally deposited Mof protein arrest development during gastrulation, whereas females develop into adults. This suggests that H4K16 acetylation by Mof may be critical for a previously unexplained phase of dosage compensation reported to occur during the onset of zygotic gene transcription (17).

Results

H4K16 Point Mutants Reveal Essential Functions of This Residue in Metazoans. We generated *Drosophila* in which H4K16 was mutated to arginine, glutamine, or alanine and shall refer to these animals as $H4^{K16R}$, $H4^{K16Q}$, and $H4^{K16A}$ mutants, respectively. Each of these amino acid substitutions changes the chemical character of residue 16 in H4 in a different way. In the $H4^{K16R}$ protein, the long aliphatic side chain and the positively charged head that are characteristic of lysine are retained but, unlike lysine, the arginine cannot be acetylated, and H4 therefore constitutively contains a positive charge at residue 16. In the $H4^{K16Q}$ protein, the long aliphatic side chain with a polar head group of glutamine has chemical properties similar to an acetylated lysine and can therefore be regarded as a constitutive acetyl-mimic substitution. In the $H4^{K16A}$ protein, finally, the substitution with the short apolar side chain of alanine causes the most drastic change to the chemical properties of the side chain at this position.

The following genetic strategy was used to produce $H4^{K16R}$, $H4^{K16Q}$, or $H4^{K16A}$ mutants. We generated animals that were homozygous for genomic deletions that remove all wild-type H4 gene copies and instead contained transgenes that expressed either wild-type or the above-mentioned H4 mutant proteins under the control of their native promoters. It is important to note that the *Drosophila* genome contains two types of histone H4 genes. First, there are the canonical histone H4 genes that are expressed during S phase, and these are all contained in a single large locus called *HisC* (18). *HisC* comprises 23 repeats of the so-called histone gene unit (HisGU), each of which harbors one copy of the genes for the core histones H2A, H2B, H3, H4, and for the linker histone H1. Second, the *Drosophila* genome also contains *histone H4 replacement* (*His4r*), a single copy gene that encodes an H4 protein with the same amino acid sequence as canonical H4 but is expressed also outside of S phase (19). Because H4 and His4r are identical proteins and therefore subject to the same modifications also at K16, it was essential to create strains with deletions lacking both the canonical *H4* genes and the *His4r* gene. We first generated a $His4r^{\Delta}$ deletion allele (*SI Appendix*, Fig. S1). $His4r^{\Delta}$ homozygous animals were viable and fertile and could be maintained as a healthy strain. We next introduced the $His4r^{\Delta}$ mutation into a strain carrying a deletion of the entire *HisC* cluster ($HisC^{\Delta}$) (18) to generate $HisC^{\Delta}$ $His4r^{\Delta}$ mutant animals. Previous studies showed that $HisC^{\Delta}$ homozygotes arrest development at the blastoderm stage after exhaustion of the pool of maternally deposited histones but that transgene cassettes providing 12 copies of a wild-type *HisGU* fragment (*12xHisGU*) rescue $HisC^{\Delta}$ homozygotes into viable adults (18). We found that the wild-type *12xHisGU* cassette also rescued $HisC^{\Delta}$ $His4r^{\Delta}$ homozygotes into viable adults (Fig. 1). Below, we shall refer to individuals with this genotype as $H4^{wt}$ animals (Fig. 1) because they served as control for the $H4^{K16R}$, $H4^{K16Q}$, and $H4^{K16A}$ mutant animals that we generated by introducing $12xHisGU^{H4K16R}$, $12xHisGU^{H4K16Q}$, or $12xHisGU^{H4K16A}$ mutant cassettes, respectively, into $HisC^{\Delta}$ $His4r^{\Delta}$ homozygotes. Below, the phenotypes of these $H4^{K16R}$, $H4^{K16Q}$, or $H4^{K16A}$ mutant animals are discussed in turn.

$H4^{K16R}$ mutants showed sex-specific lethality. In particular, $H4^{K16R}$ mutant males survived up to the end of larval development and then died but, remarkably, their $H4^{K16R}$ mutant sibling females developed into adults that showed no obvious morphological defects but survived only up to 7 d after eclosion (Fig. 1, see figure legend for details). In females, chromatin that exclusively contains H4R16 nucleosomes therefore permits normal gene regulation and progression of development up to the adult stage. The lethality of males with H4R16 chromatin on the other hand suggests that this chromatin fails to support dosage compensation.

$H4^{K16Q}$ mutants differed from $H4^{K16R}$ mutants in that not only females but also males developed into adults (Fig. 1). While the fraction of surviving $H4^{K16Q}$ adult males was smaller compared with females, neither of the sexes showed any detectable morphological defects, but the animals survived only up to 7 d after eclosion (Fig. 1, see figure legend for details). Chromatin containing only H4Q16 nucleosomes is therefore also able to support normal gene regulation and progression of development.

$H4^{K16A}$ mutants, finally, were strikingly different in that both males and females arrested development by the end of embryogenesis (Fig. 1). The embryonic cuticles of these animals showed no obvious morphological defects and looked comparable to wild-type embryos (*SI Appendix*, Fig. S2).

Taken together, the comparison of these three different H4 mutants yields two main conclusions. First, the presence of an amino acid containing a long aliphatic side chain with a polar group at the end (i.e., either K, R, or Q) permits normal progression of development, whereas the short apolar side chain of alanine fails to do so. It may appear surprising that *Drosophila* in which all nucleosomes are positively charged (i.e., H4R16) or in which all nucleosomes are uncharged (i.e., H4Q16) both contain chromatin that is functional to support normal development. We note, however, that both $H4^{K16R}$ and $H4^{K16Q}$ mutant adults had a short lifespan (Fig. 1*A*), suggesting that presence of a lysine at this position is essential for normal adult viability. The second main conclusion comes from the finding that only H4Q16 chromatin supports the survival of males into adults. The most straightforward explanation of this result is that the glutamine substitution mimics acetylation of H4K16 and thereby permits dosage compensation to occur and allow at least a fraction of males to survive into adults.

H4K16 Acetylation by Mof Early in Embryogenesis Is Critical for Dosage Compensation in Males but Dispensable for Development in Females. The sex-specific developmental defects of H4 mutants prompted us to compare their phenotypes with those of *mof* mutants. Previous studies had focused on the analysis of *mof* zygotic mutant animals (mof^{z-}) (11, 20). These studies showed that mof^{z-} males develop up to the late larval stage and arrest development shortly before pupation (20). Here, we investigated the phenotype of animals that lacked not only zygotically expressed but also maternally deposited Mof protein (Mof^{m-z-}). Using mof^{2}, a presumed null allele that lacks acetyltransferase activity (20, 21), we generated $mof^{2\ m-z-}$ animals from females with mof^{2} mutant germ cells. Interestingly, $mof^{2\ m-z-}$ mutant males arrested development shortly after gastrulation, whereas their $mof^{2\ m-z-}$ mutant female siblings developed into viable adults (Fig. 1 and *SI Appendix*, Fig. S3 and Table S1). Like $mof^{2\ z-}$ mutant females (16, 21), $mof^{2\ m-z-}$ mutant females had a short lifespan and exhibited very low fertility (Fig. 1). We next generated $mof^{2\ m-z+}$ males that we obtained as the progeny from mothers with mof^{2} mutant germ cells and fathers that provided a wild-type copy of the mof^{+} gene (*SI Appendix*, Table S1). Such

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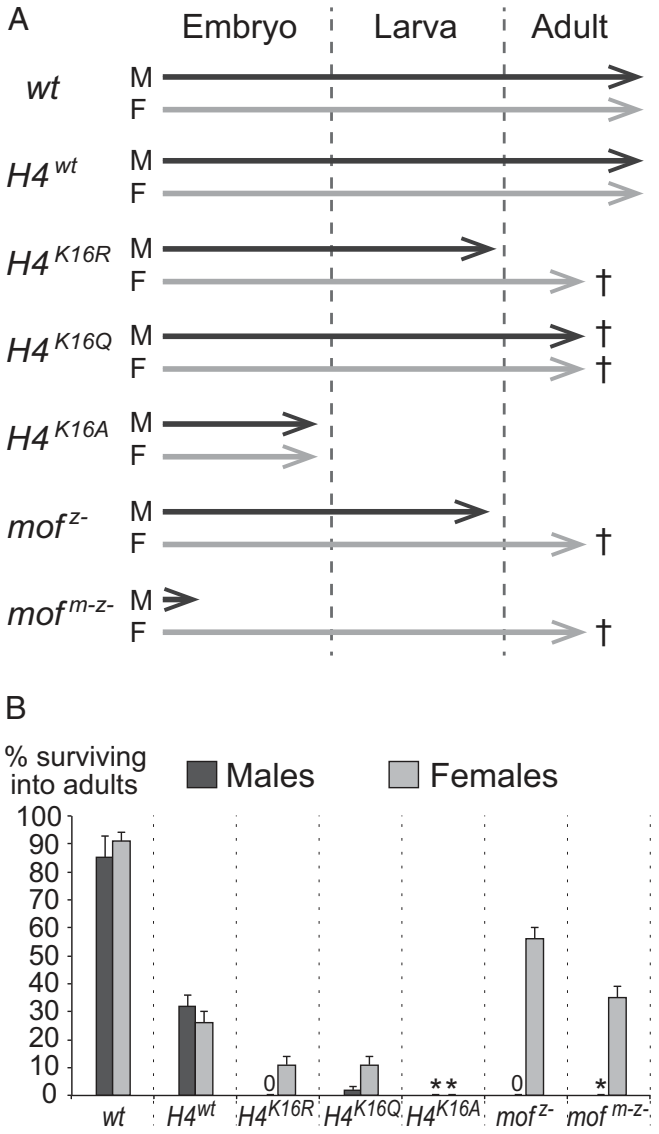

Fig. 1. Differential viability of *Drosophila* with H4R16, H4Q16, or H4A16 chromatin support a specific role for H4K16 acetylation in dosage compensation. (*A*) Graphic representation illustrating the stage of developmental arrest of $H4^{K16R}$, $H4^{K16Q}$, $H4^{K16A}$, and mof^{2} mutants. Detailed information about each genotype and how it was generated is presented in *SI Appendix*, Table S1. In the case of $H4^{K16R}$ mutants, males did not develop beyond the third larval instar. In the case of $H4^{K16A}$ mutants, males and females all arrest development at the end of embryogenesis and no larvae were obtained. In the case of $mof^{2\ m-z-}$ mutants, only female and no male larvae were obtained; male embryos were analyzed as described in *SI Appendix*, Fig. S3. $mof^{2\ z-}$ males develop to the end of the third larval instar, as previously reported (20). Crosses indicate that adults with these genotypes had a reduced life span and typically died within a few days after eclosion; moreover, these animals were generally weak and we therefore could not assess their fertility. (*B*) Quantitative analysis of the capacity of the different *H4K16* and mof^{2} mutant genotypes to develop into viable adults. For every genotype that developed beyond embryogenesis, six independent batches with 50 first instar larvae were collected in each case and reared separately in vials. Histograms represent the mean ± SD of the percentages of larvae in individual vials that developed into viable adults. Asterisks show genotypes that arrest development during embryogenesis (*A*). The diminished viability of $H4^{wt}$ adult animals likely reflects incomplete rescue of $HisC^{\Delta}$ $His4r^{\Delta}$ homozygotes by the $12xHisGU^{wt}$ transgene cassettes. It should be noted that $H4^{wt}$ adult males and females showed normal lifespan and were fertile.

$mof^{2\ m-z+}$ males also failed to develop into adults. This shows that zygotic expression of Mof protein is insufficient to compensate for defects caused by the lack of maternally deposited Mof protein during the earliest stages of embryogenesis. In summary, these analyses reveal that, in males, H4K16 acetylation by Mof is critically required already during the onset of zygotic gene transcription and that in its absence, males are unable to develop past the gastrulation stage. Furthermore, these data demonstrate that H4K16 acetylation by Mof is fully dispensable for development of female zygotes into adults.

Is Mof the principal H4K16 acetyltransferase in *Drosophila*? Previous analyses in *mof* mutants led Conrad et al. (21) to propose that Mof creates most H4K16 acetylation in both males and females, whereas studies by Gelbart et al. (16) and Feller et al. (22) found that H4K16ac levels in *mof* mutant females are only about twofold reduced compared with wild type. Here, we analyzed imaginal discs with clones of mof^{2} mutant cells to directly compare H4K16ac levels in wild-type and mof^{2} mutant cells in the same tissue. While mof^{2} mutant cells in males showed a drastic reduction of H4K16ac signal, mof^{2} mutant cells in females still contained substantial levels of H4K16ac and the signal appeared only mildly reduced compared with wild-type cells (*SI Appendix*, Fig. S4*A*). Similarly, H4K16ac levels also appeared only mildly reduced in imaginal discs from $mof^{2\ m-z-}$ mutant female larvae that had never contained any Mof activity (*SI Appendix*, Fig. S4*B*). These observations are consistent with the findings from Gelbart et al. (16) and Feller et al. (22) and support the view that a considerable fraction of total H4K16 acetylation in females is generated by acetyltransferases other than Mof. Considering that females with H4R16 chromatin that cannot be acetylated also develop into adults (Fig. 1), the role of this Mof-independent H4K16 acetylation in females currently remains enigmatic. We conclude that the essential function of H4K16 acetylation in *Drosophila* is for male-specific X-chromosome dosage compensation.

H4 Point Mutants Highlight Dosage Compensation as the Key Cellular Process Requiring K16 Acetylation. To compare *H4* and *mof* mutants at the cellular level, we generated clones of $H4^{K16R}$, $H4^{K16Q}$, $H4^{K16A}$, or mof^{2} mutant cells in wild-type animals (*Materials and Methods*). We induced such clones in first instar larvae and analyzed them 96 h after induction in third instar larvae. Immunofluorescence labeling of imaginal discs with clones of $H4^{K16R}$ mutant cells showed that 96 h after clone induction, H4K16ac was no longer detectable in these mutant cells (Fig. 2*A*). However, the size of these $H4^{K16R}$ mutant clones in males was smaller than in females (Fig. 2*B*, first row and Fig. 2*C*). This is best quantified by comparing the size of the mutant clones relative to that of their "twin spot" control clones that were generated by the reciprocal recombination event (Fig. 2*C*). In males, $H4^{K16R}$ mutant clones were smaller than their twin spot control clones, whereas in females, $H4^{K16R}$ mutant clones and twin spot control clones were more similar in size (Fig. 2*B*, first row and Fig. 2*C*). Similarly, clones of $H4^{K16Q}$, $H4^{K16A}$, or mof^{2} mutant cells were also smaller in males compared with females (Fig. 2*B*, second, third, and fourth rows and Fig. 2*C*). No obvious sex-specific differences in clone size were observed in $H4^{wt}$ clones, consistent with the similar percentage of surviving $H4^{wt}$ males and females (Fig. 1). Interestingly, when we compared the clone sizes between males with different H4 mutant genotypes, we found that $H4^{K16Q}$ mutant clones were on average larger than $H4^{K16R}$, $H4^{K16A}$, or mof^{2} mutant clones (Fig. 2). The most straightforward explanation for these observations is that $H4^{K16R}$, $H4^{K16A}$, or mof^{2} mutant cells in males are more severely compromised for proliferation and/or survival because of defective dosage compensation. On the other hand, $H4^{K16Q}$ mutant cells, containing H4Q16 acetyl-mimic chromatin, proliferate and survive better because dosage compensation is sustained in these cells, in agreement with the observation that males consisting solely of $H4^{K16Q}$ mutant cells can survive into adults (Fig. 1). A final point

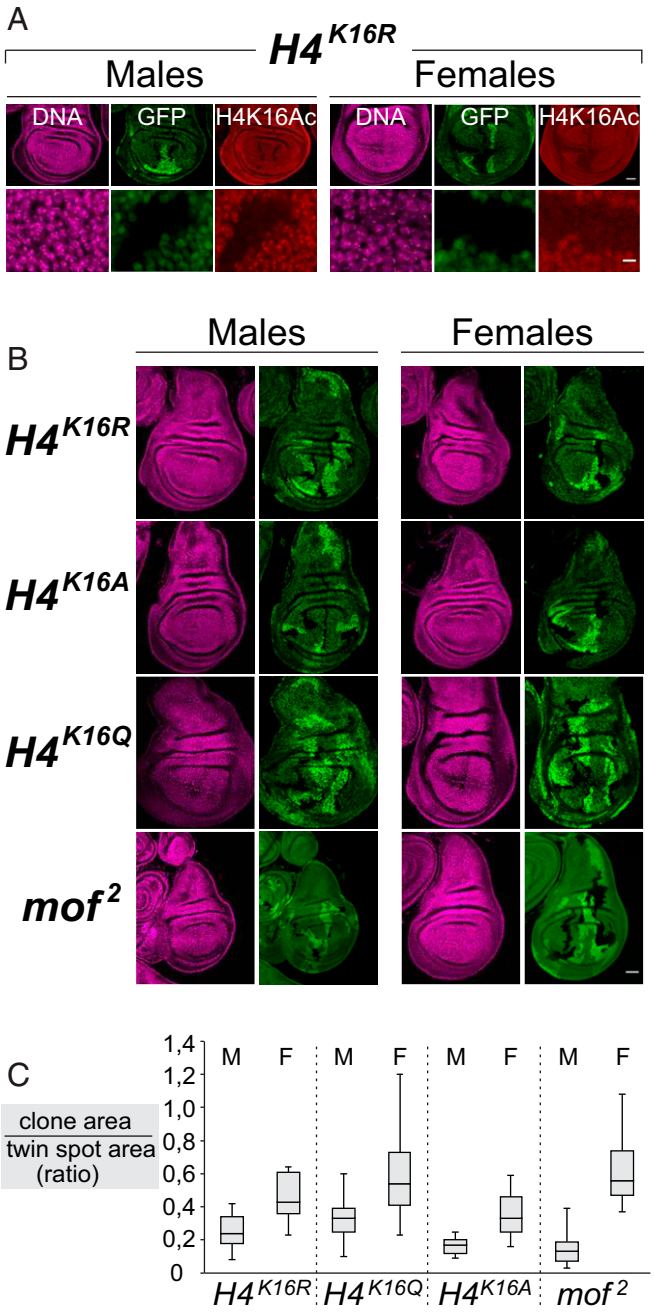

Fig. 2. Clones of cells with H4R16, H4Q16, or H4A16 chromatin show male-specific growth defects. (*A*) Imaginal wing disc from male (*Left*) and female (*Right*) larvae with clones of $H4^{K16R}$ mutant cells that are marked by the lack of GFP, stained with antibody against H4K16ac, and with Hoechst (DNA) to visualize nuclei. Clones were analyzed 96 h after clone induction. (Scale bar: 30 μm.) *Lower* row shows representative area of disc with wild-type and $H4^{K16R}$ mutant cells at higher magnification. (Scale bar: 5 μm.) In wild-type cells in males, the brightly labeled H4K16ac spot superimposed on the more uniform H4K16ac signal in every nucleus corresponds to the X-chromosome territory (10); wild-type nuclei in females only show the more uniform H4K16ac signal. Note that $H4^{K16R}$ mutant cells lack H4K16ac signal. (*B*) Imaginal wing discs from male (*Left*) and female (*Right*) larvae with clones of $H4^{K16R}$ (first row), $H4^{K16Q}$ (second row), $H4^{K16A}$ (third row), and mof^2 (fourth row) mutant cells, in each case marked by the lack of GFP; Hoechst staining labels all nuclei. Clones were analyzed 96 h after induction. Note that in males, the mutant cell clones are significantly smaller compared with their juxtaposed brightly labeled GFP^+/GFP^+ twin spot clones induced by the reciprocal recombination event. This smaller size of the mutant cell clones is most notable in the case of $H4^{K16R}$, $H4^{K16A}$, and mof^2, and less pronounced in the case of $H4^{K16Q}$. (Scale bar: 30 μm.) (*C*) Quantitative analysis of the results shown in *B*, in box plot representation. For each genotype and sex, the total area of GFP-negative mutant clone tissue and of GFP^+/GFP^+ twin spot clone tissue was measured in individual imaginal discs using ImageJ. The ratio of total clone area to total twin spot area was calculated in individual discs, and for each genotype and sex, the results from 15 imaginal discs were plotted. These results are consistent with males being more sensitive than females to H4K16 mutations in all cases, but also with decreased viability of females with mutant H4 compared with wild type (Fig. 1).

worth noting is that, in females, $H4^{K16A}$ mutant cells proliferate to form large patches of mutant tissue (Fig. 2*B*, third row), suggesting that chromatin consisting entirely of H4A16 nucleosomes permits cells to proliferate for multiple generations.

In Males, Cells with H4K16 Mutant Chromatin or Lacking Mof Protein Differentiate to Form Tissues with Normal Morphology. The results described above show that growth of $H4^{K16R}$, $H4^{K16A}$, and mof^2 mutant cell clones in male larvae is impaired. We next investigated whether such mutant cells are able to differentiate. We induced clones of $H4^{K16R}$, $H4^{K16A}$, mof^2, or also $H4^{K16Q}$ mutant cells in male larvae and tested whether they differentiate to form epithelial structures in adults. As shown in Fig. 3, mutant cells of all four genotypes formed epidermal structures with sensory bristles that looked indistinguishable from neighboring wild-type tissue. In conclusion, even though cells that contain H4R16 or H4A16 chromatin, or that lack the Mof acetyltransferase, are all impaired in proliferation in males, they nevertheless have the capacity to undergo differentiation and build tissues with apparent wild-type morphology.

Discussion

Mutational analyses of histone amino acid residues that are subject to posttranslational modifications provide a direct approach for probing the physiological role of these residues and their modification. Here, we investigated the function of H4K16 and its acetylation in *Drosophila* by generating animals in which all nucleosomes in their chromatin were altered to constitutively carry a positively charged H4R16, an acetyl-mimic H4Q16, or a short apolar H4A16 substitution. These three types of chromatin changes have different physiological consequences that lead to the following main conclusions. First, H4R16 and H4Q16 chromatin both support development of female zygotes into adults. This suggests that, in females, modulation of H4K16 by acetylation is a priori not essential for the regulation of gene expression and the chromatin folding that occurs during development of the zygote. Second, unlike in females, only H4Q16 but not H4R16 chromatin supports development of male embryos into adults. This difference between males and females directly supports the critical role of H4K16 acetylation for dosage compensation in males. Third, cells with H4A16 chromatin are viable, proliferate, and can differentiate to form normal tissues in both males and females, but animals that entirely consist of cells with H4A16 chromatin arrest development at the end of embryogenesis. This lethality contrasts with the viability of animals with H4K16, H4R16, or H4Q16 chromatin and suggests that presence of a long aliphatic side chain with a polar group (i.e., either K, R, or Q) at residue 16 is more important for H4 function than the ability to regulate the charge of this residue by acetylation. A fourth main conclusion of this work comes from the finding that males that completely lack Mof protein (i.e., mof^{m-z-} males) arrest development during gastrulation, whereas females of the same genotype develop into morphologically normal adults. This uncovers a previously unknown critical requirement of Mof acetyltransferase activity in males, already during the onset of zygotic gene transcription. In the following sections, we discuss the results reported here in the context of our current understanding of the role of H4K16 and its acetylation.

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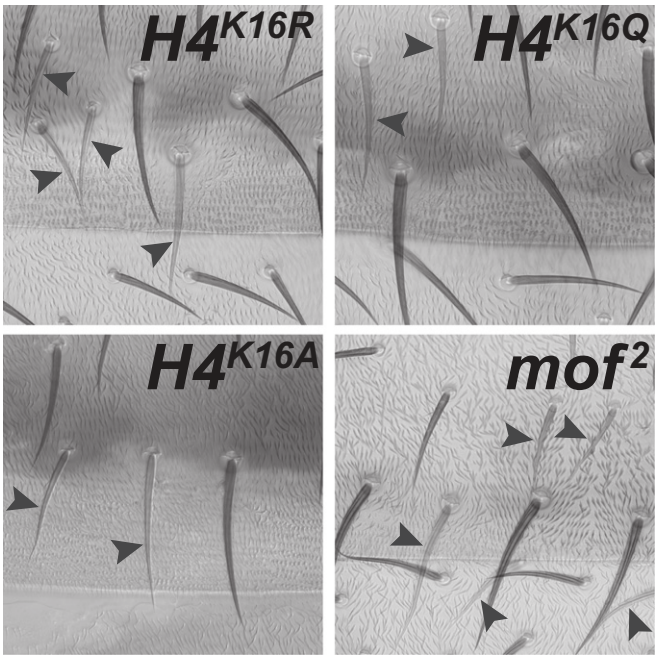

Fig. 3. In males, cells with H4R16 or H4A16 chromatin or lacking Mof protein retain the capacity to differentiate normally. Portions of abdominal segment A2 from adult males with clones of $H4^{K16R}$, $H4^{K16Q}$, $H4^{K16A}$, or mof^2 mutant cells. In each case, mutant clone cells are marked by the yellow mutation, and bristles in the mutant clone tissue therefore are more lightly pigmented than bristles in the neighboring wild-type tissue. Note that the morphology of the mutant bristles (arrowheads) and associated tissue is in all four cases comparable to the neighboring wild-type tissue. $H4^{K16Q}$ mutant clones were analyzed for comparison and as control, recall that males consisting solely of $H4^{K16Q}$ mutant cells can survive into adults with wild-type morphology (Fig. 1).

Differences in H4K16R, H4K16Q, and H4K16A Mutant Phenotype Severity in Flies Versus Yeast Reflect Organism-Specific Chromatin Functions of H4K16. In yeast and flies, the comparison of the severities of the phenotypes caused by different amino acid substitutions at H4K16 highlights how the two organisms have evolved to use this conserved residue and its modification in different ways. In yeast, H4K16ac is present genome-wide and SIR silencing is the key physiological process that requires H4K16, in its deacetylated state (5–7). Yeast cells with *H4K16R*, *H4K16Q*, or *H4K16A* mutations are viable but they show defective SIR silencing. Silencing is much more strongly impaired in *H4K16Q* or *H4K16A* mutants than in *H4K16R* mutants (5–7). This is because SIR3 protein binding to deacetylated H4K16 (9), a prerequisite for silencing, is probably less severely impaired by the arginine substitution than by the alanine or glutamine substitutions. In *Drosophila*, the phenotypic differences between *H4K16R*, *H4K16Q*, and *H4K16A* mutants suggest that H4K16 is associated with two other, distinct physiological functions that are critical for the organism. The male-specific lethality of *H4K16R* mutants and the restoration of male viability in *H4K16Q* mutants demonstrate that dosage compensation is one essential process that critically requires the acetylated form of H4K16. A reduction of internucleosomal contacts by H4K16ac (3) to generate chromatin that is more conducive to gene transcription on the male X chromosome currently is the simplest mechanistic explanation for how H4K16 acetylation enables dosage compensation. The observation that an *H4K16A* mutation causes lethality in both sexes suggests that, unlike in yeast, a long aliphatic side chain at this residue is essential for H4 function in *Drosophila*. It is currently not known why *Drosophila H4K16A* mutants die. However, it is important to note that *H4K16A* mutant cells retain the capacity to proliferate and differentiate and the mutation therefore does not disrupt any fundamental process required for cell survival.

Mutation of His4r Is Critical for Assessing the Consequences of K16 Mutations in Canonical Histone H4. Previous studies that investigated the function of histone H3 modifications by histone replacement genetics showed that for modifications associated with transcriptionally active chromatin it is essential to remove not only the wild-type copies of the canonical histone genes but to also mutate the histone H3.3 variants (23, 24). The analyses of *H4K16R*, *H4K16Q*, and *H4K16A* mutant phenotypes reported here were all performed in the genetic background of animals lacking His4r, the only histone H4 variant in *Drosophila*. Importantly, we found that in a $His4r^+$ background, where only the canonical H4 proteins are replaced with mutant H4, the modifiable His4r protein permitted *H4K16R* $His4r^+$ mutant males and, surprisingly, also *H4K16A* $His4r^+$ mutant females and males to develop into adults. These animals were therefore not analyzed further. Supporting these observations, a recent study by Armstrong et al. (25) that used a similar strategy for replacing canonical histone H4 with $H4^{K16R}$ also found that *H4K16R* $His4r^+$ mutant males develop into normal adults. This suggests that, like His3.3 (26, 27), the His4r protein might also preferentially be incorporated into transcriptionally active chromatin and become acetylated by Mof. Although the viable *H4K16R* $His4r^+$ males have been reported to show a significant reduction of X-linked gene expression (25), a full assessment of transcriptional defects in animals containing only H4R16 nucleosomes would require that such molecular analyses be performed in *H4K16R* $His4r^{\Delta}$ mutant males.

A final point that should be noted here is that during the early stages of embryogenesis, *H4K16R*, *H4K16Q*, or *H4K16A* mutants also still contain maternally deposited wild-type H4 protein that becomes incorporated into chromatin during the preblastoderm mitoses and only eventually becomes fully replaced by mutant H4 proteins during postblastoderm cell divisions. During the earliest stages of embryogenesis it has therefore not been possible to assess the phenotype of animals with chromatin containing exclusively H4R16, H4Q16, or H4A16 nucleosomes. This needs to be kept in mind when considering comparisons between the phenotypes of *H4K16* point mutants and mof^{m-z-} mutants.

Mof Acetyltransferase Activity in the Early Embryo Is Essential to Permit Male Development Beyond Gastrulation. We found that males without Mof protein (i.e., mof^{m-z-} males) arrest development during gastrulation while their female siblings develop into adults. Moreover, mof^{m-z+} males also fail to develop, demonstrating that zygotic expression of Mof protein is insufficient to rescue male embryos that lacked maternally deposited Mof protein. The most straightforward explanation for these observations is that H4K16 acetylation by Mof is critically required for hypertranscription of X-chromosomal genes that has been reported to occur already during the onset of zygotic gene transcription (17) and that the early developmental arrest of males is a direct consequence of failed dosage compensation.

How does this early requirement for Mof activity at the blastoderm stage relate to our current understanding of the temporal requirement for the DCC for dosage compensation? Previous studies showed that males lacking the DCC subunits Msl-1, Msl-2, Msl-3, or Mle complete embryogenesis and arrest development much later, around the stage of puparium formation (28). For example, Msl-1 protein null mutants (i.e., $msl\text{-}1^{m-z-}$ mutants) die as late third instar larvae, yet Msl-1 directly interacts with Mof to incorporate it into the DCC (29) and is critical for targeting the complex and H4K16ac accumulation on the X chromosome in larvae (30, 31). One possible explanation for the conundrum that the lack of Mof but not that of Msl-1 or other

DCC subunits results in lethality during gastrulation could be that during these early stages, H4K16 acetylation by Mof for dosage compensation is not as strictly dependent on the other DCC subunits as during later developmental stages, or that there is redundancy between Msl-1, Msl-2, or Msl-3 for targeting Mof to the X chromosome in the early embryo.

A final point worth noting is that Mof is also present in another protein assembly called the NSL complex (32). NSL was reported to act genome-wide for regulating housekeeping gene transcription in both sexes (33, 34) and several NSL subunits are essential for *Drosophila* viability (35). The finding that *mof* $^{m-z-}$ mutant females develop into morphologically normal adults shows that the NSL complex must exert regulatory functions that are essential for viability independently of Mof H4K16 acetyltransferase activity.

Concluding Remarks. The acetylation of lysine residues in the N termini of histones is generally associated with chromatin that is conducive to gene transcription. Mutational studies in yeast showed that there is substantial functional redundancy between most of the different acetylated lysine residues in the N termini of histone H3 and H4 but that H4K16 has unique effects on transcriptional control (36), with well-defined phenotypic consequences (5–7). Here, we show that in *Drosophila* the principal function of H4K16 acetylation is X-chromosome dosage compensation in males.

Materials and Methods

***Drosophila* Stocks and Genetic Analyses.** Genotypes of fly strains and of the animals shown in each figure are listed in *SI Appendix*, Table S1. *SI Appendix*, Table S1 also contains information on how *mof*$^{2\ m-z-}$ males and females were generated. Induction and analyses of mitotic cell clones in imaginal discs and in the adult epidermis were performed as described (37). Discs were stained with Hoechst and rabbit anti-H4K16ac antibody (Active Motif no. 39167).

Generation of Histone Transgenes. Site-directed mutagenesis on *pENTR221-HisGU.WT*, *pENTRL4R1-HisGU.WT*, and *pENTRR2L3-HisGU.WT* (18) was used to convert the *AAG* codon for H4K16 to *CGT* (Arg), to *CAG* (Gln), or *GCC* (Ala). The final constructs *ϕC31-attB-3xHisGU.H4K16R*, *ϕC31-attB-3xHisGU.H4K16Q*, and *ϕC31-attB-3xHisGU.H4K16A* were generated by Gateway LR recombination of the above vectors. The transgene cassettes were integrated into the attP sites VK33, 68E, or 86Fb, as described (37). Standard meiotic recombination was then used to generate chromosomes carrying appropriate combinations of these transgene insertions and the *His4r*$^{\Delta}$ deletion allele, as listed above.

Generation of *His4r*$^{\Delta}$. The H*is4r*$^{\Delta}$ deletion allele was generated by imprecise excision of the P{EPgy2}His4r^{EY06726} P-element; this resulted in a deletion from 3R:14,625,816–3R:14,626,410 (Berkeley *Drosophila* Genome Project, Release 6) that removes the entire His4r coding region.

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