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

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Contributed by Mitzi I. Kuroda, November 7, 2018 (sent for review October 8, 2018; reviewed by Judith A. Kassis and Erica Larschan)

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.

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 non-acetylated 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.

Author contributions: Ö.C., A.G., M.I.K., and J.M. designed research; Ö.C., A.G., and K.F. performed research; Ö.C. and J.M. analyzed data; and Ö.C., M.I.K., and J.M. wrote the paper.

Reviewers: J.A.K., NIH; and E.L., Brown University.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1817274115/-/DCSupplemental.

Published online December 10, 2018.
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 Metazoa. We generated Drosophila in which H4K16 was mutated to arginine, glutamine, or alanine and shall refer to these animals as H4K16R, H4K16Q, and H4K16A mutants, respectively. Each of these amino acid substitutions changes the chemical character of residue 16 in H4 in a different way. In the H4K16R 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 constitutionally contains a positive charge at residue 16. In the H4K16Q 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 H4K16A 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 H4K16R, H4K16Q, or H4K16A 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 mutant-mimicked Drosophila H4K16 protein 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, and 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 His4rΔ deletion allele (SI Appendix, Fig. S1). His4rΔ homozygous animals were viable and fertile and could be maintained as a healthy strain. We next introduced the His4rΔ mutation into a strain carrying a deletion of the entire HisC cluster (HisCΔ) (18) to generate HisCΔ His4rΔ mutant animals. Previous studies showed that HisCΔ homozygous 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 HisCΔ homozygotes into viable adults (18). We found that the wild-type 12xHisGU cassette also rescued HisCΔ His4rΔ homozygotes into viable adults (Fig. 1).

Below, we shall refer to individuals with this genotype as H4K16R, H4K16Q, and H4K16A mutant animals that we generated by introducing 12xHisGU12K16R, 12xHisGU12K16Q, or 12xHisGU12K16A mutant cassettes, respectively, into HisCΔ His4rΔ homozygotes. Below, the phenotypes of these H4K16R, H4K16Q, or H4K16A mutant animals are discussed in turn.

H4K16R mutants showed sex-specific lethality. In particular, H4K16R mutant males survived up to the end of larval development and then died but, remarkably, their H4K16R 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. H4K16Q mutants differed from H4K16R mutants in that not only females but also males developed into adults (Fig. 1). While the fraction of surviving H4K16Q adult males was smaller compared with females, neither of the sexes showed any detectable morphological defects but survived only up to 7 d after eclosion (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 H4K16R and H4K16Q mutant adults had a short lifespan (Fig. 1A), 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−/−) (11, 20). These studies showed that mof−/− 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−/+). Using mof−, a presumed null allele that lacks acetyltransferase activity (20, 21), we generated mof−/+ males and females from females with mof−/− mutant germ cells. Interestingly, mof−/+ mutant males arrested development shortly after gastrulation, whereas their mof−/+− mutant female siblings developed into viable adults (Fig. 1 and SI Appendix, Fig. S3 and Table S1). Like mof−/− females (16, 21), mof−/−− mutant females had a short lifespan and exhibited very low fertility (Fig. 1). We next generated mof−/−− males that we obtained as the progeny from mothers with mof−/− mutant germ cells and fathers that provided a wild-type copy of the mof gene (SI Appendix, Table S1). Such
mutant cells in males are more severe than in females. H4K16ac levels in mutant clones and twin spot control clones were almost equal, consistent with the similar percentages of larvae in control clones that were generated by the reciprocal maternal and paternal H4R16 alleles. Interestingly, H4K16ac levels also appeared only mildly reduced in mutant cells from mof mutant females that had never contained any Mof activity. (SI Appendix, Fig. S4C). The observations are consistent with the findings of Oldham et al. (16) and Feller et al. (22) and suggest that the 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 H4K16R, H4K16Q, H4K16A, or mof 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 H4K16R mutant cells showed that 96 h after clone induction, H4K16ac was no longer detectable in these mutant cells (Fig. 2A). However, the size of these H4K16R mutant clones in males was smaller than in females (Fig. 2B, first row and Fig. 2C). 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. 2C). In males, H4K16R mutant clones were smaller than their twin spot control clones, whereas in females, H4K16R mutant clones and twin spot control clones were more similar in size (Fig. 2B, first row and Fig. 2C). Similarly, clones of H4K16Q, H4K16A, or mof mutant cells were also smaller in males compared with females (Fig. 2B, second, third, and fourth rows and Fig. 2C). No obvious sex-specific differences in clone size were observed in H4wt clones, consistent with the similar percentage of surviving H4wt males and females (Fig. 1). Interestingly, when we compared the clone sizes between males with different H4 mutant genotypes, we found that H4K16R mutant clones were on average larger than H4K16Q, H4K16A, or mof mutant clones (Fig. 2). The most straightforward explanation for these observations is that H4K16R, H4K16A, or mof mutant cells in males are more severely compromised for proliferation and/or survival because of defective dosage compensation. On the other hand, H4K16Q 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 containing solely of H4K16Q mutant cells can survive into adults (Fig. 1). A final point
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 of male embryos into adults. This difference between males and females directly supports the critical role of H4K16 acetylation for dosage compensation in males. 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.

The results described above show that growth of H4K16R, H4K16A, and mof- mutant cell clones in male larvae is impaired. We next investigated whether such mutant cells are able to differentiate. We induced clones of H4K16R, H4K16A, mof-2, or also H4K16Q 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.
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 also to 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 H4K16R 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 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−/− 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−/− males) arrest development during gastrulation while their female siblings develop into adults. Moreover, mof m−/+ 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-1 m−/− 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 mofΔmz– 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−/mz− males and females were generated. Induction and analysis 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-HisGlu.WT, pENTRL4R1-HisGlu.WT, and pENTR2L33-HisGlu.WT (18) was used to convert the AAG codon for H4K16 to CGT (Arg), to CAG (Gin), or GCC (Ala). The final constructs pC31 attB 3XHisGU.H4K16R, pC31 attB 3XHisGU.H4K16Q, and pC31 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 His4− deletion allele, as listed above.

Generation of His4Δzm. The His4Δzm deletion allele was generated by imprecise excision of the P[Epgy2]His6+Δ5272 element; this resulted in a deletion allele, as listed above.

ACKNOWLEDGMENTS. We thank Frank Schnorrer for providing the M[attP] ZH86Fb stock lacking the RFP marker gene and Shigeo Hayashi for the Y–GFP stock. This work was supported by the Max Planck Society (J.M.) and by NIH Grant GM126944 (to M.I.K.).

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