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Histone acetylation as an epigenetic determinant of long-term transcriptional competence

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Abstract. All four histones of the nucleosome core particle are subject to post-translational acetylation of selected lysine residues in their amino-terminal domains. The modification is ubiquitous and frequent. Steady-state levels of acetylation have been shown to vary from one part of the genome to another and to be maintained by a dynamic balance between the activities of two enzyme families, the histone acetyltransferases (HATs) and deacetylases (HDAs). The recent demonstration that some at least of these enzymes are homologous to, or identical with, known regulators of transcription, has renewed interest in the involvement of histone acetylation in transcriptional control. Acetylation might influence the initiation and/or elongation phases of transcription in a chromatin context, possibly by regulating the accessibility of nucleosomal DNA to transcription factors or the displacement of histones by the progressing transcription complex. But there is also evidence to suggest that acetylation might be involved in the longer-term regulation of transcription, acting as a marker by which states of genetic activity or inactivity are maintained from one cell generation to the next. This review outlines the evidence for such a role, using centric heterochromatin and the dosage-compensated male X chromosome in *Drosophila* as model systems, and suggests possible mechanisms by which it might operate.

Key words. Histone acetylation; chromatin; *Drosophila*; dosage compensation; cell memory.

Histone acetylation and chromatin structure and function

Post-translational acetylation of the histones which organize the nucleosome core particle has been found in all animal and plant species so far examined. Acetylation occurs at specific lysine residues, all of which are located in the amino-terminal domains of the core histones [1]. The transfer of acetate groups from acetyl coenzyme A (acetyl CoA) to histones and their subsequent removal is catalysed by specific enzymes, the histone acetyltransferases (HATs) and deacetylases (HDAs). An association of some sort between acetylation and gene expression has been discussed over many years, and this relationship has attracted increasing attention recently with the discovery that these enzymes are often identical to, or associated with, known regulators of transcription [2–7].

One way of rationalizing the association between acetylation and transcription is to propose that increasing levels of histone acetylation somehow loosen the interaction between histones and DNA and thereby improve access of transcription factors to their cognate sequences on nucleosomal DNA. Evidence for this comes from the finding that acetylation of lysines in the N-terminal tail domains of the core histones does, as expected, weaken their association with DNA and directly influences nucleosome structure [8]. High levels of acetylation increase the binding of some transcription factors to nucleosomal DNA in vitro [9]. A related mechanism is suggested by recent crystallographic analysis that defines the structure of the nucleosome core particle at 2.8 Å resolution [10]. It appears that, in crystals, the N-terminal tail of one of the two H4 molecules in each core particle associates with an

H2A:H2B dimer in an adjacent particle. The cross-linking of nucleosomes via the tail domains, if it occurs in vivo, raises the interesting possibility of a direct role for histone tails, and possibly their acetylation, in maintenance of higher-order structure, a role initially suggested by the experiments of Allan and co-workers [11].

An alternative view of the mechanism by which histone acetylation influences chromatin structure and function is that its effects are indirect. This model proposes that acetylation of the exposed tail domains leads to the creation of markers on the nucleosome surface that can be recognized by nonhistone proteins [12, 13]. It is the association of such proteins with chromatin that leads to functional change. This idea predicts that, in some situations at least, it will prove to be not the level of histone acetylation that matters (i.e. the number of sites on any given histone that are acetylated), but which specific lysines are acetylated. This mechanism is considered further below.

Histone acetylation as an epigenetic marker

Histone acetylation has been most often thought of in terms of its potential to alter chromatin structure, either directly or indirectly, and thereby exert an effect on transcription or DNA replication. However, histone acetylation can also be considered as a marker of chromosome domains; a signal by which information about the functional status of a particular region of the genome is transmitted from one cell generation to the next. To act in this way, as a component of cell memory or genetic imprinting, acetylation itself need have no effect at all on chromatin structure or function. It acts simply to transmit information.

The potential information content of histone acetylation is enormous. Histones H2B, H3 and H4 each have 4 acetylatable lysines and therefore 16 possible isoforms (i.e. 1 nonacetylated, 4 monoacetylated, 6 diacetylated, 4 triacetylated and 1 tetra-acetylated). H2A has just two isoforms (nonacetylated and monoacetylated) – altogether 50 histone isoforms that differ in their acetylation status. We therefore have 50 'bits' of information, each with the potential ability to define a specific functional state. If combinations of acetylated isoforms are considered, then the information content goes up to $16 \times 16 \times 16 \times 2 = 8192$ possible combinations within an individual nucleosome. This takes no account of the fact that there are two copies of each histone per nucleosome. If this is allowed for, then the potential number of differentially acetylated nucleosomes becomes $8192 \times 8192 = 6.7 \times 10^7$, which exceeds the number of nucleosomes in the nucleus of a typical eukaryotic cell. So there is more than enough information potential in histone acetylation to specify as many functional states as the cell is likely to need.

Acetylation-sensitive histone-binding proteins

For the information content of acetylated histones to be useful, the cell must have some method of reading it and translating it into structural or functional terms. As noted earlier, it could be that acetylation itself influences the structure of chromatin in a functionally significant way. But to take advantage of even a small fraction of the total potential information content, it would be necessary for combinations of acetylated histones to impose distinct and different structural changes on the nucleosome. These in turn would have specific functional effects, such as suppressing or facilitating the binding of particular transcription factors to nucleosomal DNA or influencing the formation of more open or more compact higher-order structures. However, even accepting that acetylation can effect nucleosome structure and that this is likely to be significant in some aspects of the transcription mechanism, the proposal that acetylation can regulate chromatin function by generating a spectrum of subtly different structural states seems intrinsically unlikely. A much more efficient way for histone acetylation to realize its full potential as an epigenetic marker would be by generating a family of markers on the nucleosome surface that could be recognized by acetylation-sensitive, histone-binding proteins.

There is no doubt that such proteins can exist. Work over several years with antibodies to different acetylated histone isoforms has shown that these proteins can be exquisitely sensitive to differences in acetylation [12, 14, 15]. For example, antibodies can distinguish between H4 isoforms acetylated at lysines 5 and 12, even though in each case the acetylated lysine is part of a GKGG motif. If immunoglobulins are capable of binding selectively to H3 and H4 isoforms acetylated at specific residues, presumably other proteins can do the same. But do such proteins actually occur in the nuclei of living cells? As yet, most of the evidence comes from work on the budding yeast *Saccharomyces cerevisiae*. There is strong genetic evidence that binding of the silencing proteins SIR3 and SIR4 to chromatin in yeast occurs through association with the N-terminal domains of histones H3 and/or H4. Mutations in the N-terminal domains of H3 and H4 can lead to derepression of genes normally silenced by SIR3/SIR4 and associated proteins, namely the mating-type genes HMLα and HMRa and genes adjacent to telomeres [16–19]. Two regions are specifically involved, namely amino acids 4–20 of H3 and 16–29 of H4. Single amino acid substitutions in SIR3 can suppress mutations in the H4-silencing domain, suggesting that the two proteins interact, directly or indirectly, in vivo. Further, both SIR3 and SIR4 can bind selectively to the amino-terminal regions of H3 and H4 in vitro, but not to H2A or H2B [20]. Mutants in which H4 lysine 16 (but not other lysines) is replaced by a neutral amino acid, i.e. substi-

tutions that mimic the neutralization of H4lys16 by acetylation, show both a loss of silencing of mating-type genes and reduced SIR3/SIR4 binding in vitro [20]. H4lys16 seems to have a central role in regulating gene silencing in yeast. Further evidence for selective binding to the histone tails comes from studies on another yeast repressor, Tup1. This protein too has been shown in vitro to interact specifically with the amino-terminal tail domains of H3 and H4 (but not H2A or H2B) and seems to bind preferentially to the less-acetylated isoforms [21].

An alternative approach to demonstrating the existence of acetylation-dependent binding of nonhistone proteins to the nucleosome is to search for situations in which lysine-specific histone acetylation is associated with defined functional effects. Two particularly striking examples of selective acetylation of H4 lysines have been noted in polytene chromosomes of the fruit fly *Drosophila*. By immunolabelling with antisera specific for H4 acetylated at lysine 12 (H4Ac12) or lysine 16 (H4Ac16), it was found that the former was preferentially associated with centric heterochromatin, while the latter was found almost exclusively on the X chromosome in male (but never female) cells [12]. In *Drosophila*, genes on the single male X chromosome are transcribed twice as rapidly as those on the two X chromosomes in female cells, leading to equalization of gene products between the sexes, i.e. dosage compensation. This provides a particularly good model system in which to test the possible role of lysine-specific histone acetylation in bringing about a defined change in chromatin function.

Dosage compensation in *Drosophila*

Dosage compensation occurs in organisms that use chromosomal methods of sex determination, i.e. in which the two sexes have different chromosome complements. It has been most widely studied in mammals and in flies of the genus *Drosophila*. In both mammals and *Drosophila*, males have one copy of each of two different sex chromosomes, designated X and Y, while females have two copies of the X. In both groups of organisms, the Y is gene poor, containing just one or a very few genes needed for initiating the male developmental pathway (mammals) or for fertility (*Drosophila*). In contrast, the X is a relatively large, gene-rich chromosome, and a twofold difference in copy number would result in a twofold difference between the sexes in the intracellular concentrations of several hundred gene products. Given that almost all metabolic and developmental pathways are common to both sexes, it is not surprising that evolution has been unable to accommodate such a difference. It has instead developed ways of eliminating it through mechanisms of dosage compensation.

From first principles, the three most straightforward ways in which dosage compensation can be achieved are by (i) switching off genes on one of the two female X's, (ii) doubling the rate of transcription of genes on the single male X and (iii) halving the rate of transcription on each of the two female X's. Examples of all three mechanisms have been observed. Mammals use the first, *Drosophila* the second and the nematode worm *Chaenorhabditis elegans* the third [22, 23]. The dosage-compensation strategies adopted by these widely divergent groups of organisms, while fundamentally different, show fascinating similarities when examined in detail. A comparison of these similarities and differences can provide valuable insights into the molecular mechanisms that underly epigenetic change [22, 24], but the point that should be emphasized here is that three very different mechanisms of dosage compensation have evolved, apparently independently, an observation that confirms the importance of the end result, i.e. equalization of the levels of X-linked gene products.

Does *Drosophila* dosage compensation need epigenetics?

A fundamental requirement of dosage-compensation mechanisms, whatever their details, is that elements must be present that recognize the X chromosome and, directly or indirectly, alter the transcription of its genes in one sex but not the other. In theory, this could be brought about by restricting the presence of these elements to just one sex, something that could be done through the sex-determining mechanism itself. These elements must be able to distinguish the X chromosome from the autosomes, and the most straightforward way of doing this is through X-specific DNA sequences. This logic dictates that a dosage-compensation system of the type used in *Drosophila* has no a priori need for an epigenetic component. A conceptually simple system can be devised whereby elements present only in male cells recognize X-specific DNA sequences and thereby trigger the doubling of transcription of X-linked genes. But is this what happens in the real world?

Components of the *Drosophila* dosage-compensation pathway

The requirements of the *D. melanogaster* dosage-compensation system are that (i) the dosage-compensation pathway should be activated in males but not females, (ii) the mechanism is such as to lead to an almost exact doubling of transcription of genes on just one of the four *Drosophila* chromosomes (in itself an interesting mechanistic problem) and (iii) this transcriptional state is stably transmitted from one cell generation to the next. Components involved in dosage compensation in *Drosophila* have been identified by a combination of biochemical and genetic approaches and are listed in

Table 1. Components of the dosage-compensation pathway in *Drosophila melanogaster*.

Component and gene (chromosome)	Putative function	Present in		Present on male X
		male	female	
MLE *maleless* (2)	RNA helicase*	+	+	+
MSL-1 *male-specific lethal 1* (2)		+	+/−	+
MSL-2 *male-specific lethal 2* (2)	DNA binding by RING finger	+	−	+
MSL-3 *male-specific lethal 3* (3)		+	+/−	+
MOF *males absent on the first* (X)	acetyltransferase	+	+	?
roX1 RNA on the X (X)	X-specific protein-DNA binding	+	−	+
H4Ac16	chromatin structure epigenetic marker	+	+/−	+
SXL *Sex lethal* (X)	regulator of RNA splicing/stability	−	+	−

*MLE can be removed from the male X by RNAase treatment [55].

table 1. Details can be found in reviews that manage to be both comprehensive and accessible to the nonspecialist [25–27].

The genetic switch that turns on the dosage compensation pathway in *Drosophila* is a gene called Sex lethal (*Sxl*). The expression of *Sxl* and formation of a functional protein product (SXL) is regulated by a system that measures the ratio of X chromosomes to autosomes. How this is done is still not clear, though some of the X-linked and autosomal genes involved have been identified [28]. SXL initiates developmental pathways that lead to both male sexual characteristics and dosage compensation. Note that in *Drosophila*, unlike mammals, the Y chromosome has no role in sex determination. XO flies are sterile, but otherwise normal males.

The first components of the *Drosophila* dosage-compensation system to be identified were the products of the male specific lethal genes *mle*, *msl-1*, *msl-2* and *msl-3*. These proteins have all been characterized in recent years and all four have been shown to associate with several hundred specific sites on the X chromosome in male, but not female, cells [29–34a]. It therefore seems likely that the MSLs have a direct effect on the structure and function of the male X chromosome. The fact that all four are located at the same X chromosome sites suggests that they act in a coordinated manner, possibly as a multisubunit complex. This possibility is consistent with the finding that different MSL proteins coimmunoprecipitate [34]. So are these the male-specific elements that mediate the simple model of *Drosophila* dosage compensation spelled out earlier? The immediate answer is no. Of the four MSLs, three are present in female cells, either at similar levels to males (MLE) or at 5–10% of male levels (MSL-1 and MSL-3). Further, there is evidence to suggest that reduced levels of MSL-1 and MSL-3 in females may be due to their more rapid degradation, possibly as a result of the absence of a stabilizing multiprotein complex, rather than to a more specific regulatory effect at the transcriptional level [26]. Only MSL-2 is truly male-specific. It may be significant that MSL-2 contains amino acid sequence motifs that can be used for protein-protein and protein-DNA interactions, i.e. coiled-coil and RING finger domains, respectively [33].

Of the other components listed, only the recently identified RNA roX1 is male-specific and also localizes specifically to the X chromosome [35]. Unfortunately, mutants in which roX1 RNA is not produced are viable and healthy, in contrast to the uniform lethality of mutations to other components of the dosage-compensation system. Either roX1 has no essential function, or its function can be carried out by other RNAs in the absence of roX1 itself [36]. However, of the presently known members of the dosage-compensation pathway, only MSL-2 is both sufficiently male-specific and has the necessary biochemical and genetic properties to be able to mediate a DNA sequence-based dosage-compensation system.

The simplest explanation for the association of the MSLs with the male X chromosome remains that one or more of the components of the putative multisubunit complex (most likely MSL-2) recognizes X-specific DNA sequence motifs. If this is the case, then it is predicted that genes translocated from the X chromosome to an autosome should, in general, remain dosage-

compensated. Many studies have shown that this is indeed the case and have provided strong evidence for the existence of *cis*-acting elements on the X chromosome that are required for dosage compensation [26, 27]. Exceptions can be accommodated by proposing that the translocation did not involve the necessary element. Autosomal genes translocated to the X are often dosage-compensated, presumably because they come under the influence of these same *cis*-acting elements [26, 27]. However, the situation is not entirely straightforward. First, it has not yet been possible to identify any consensus sequence or sequence motif that initiates dosage compensation. (Hence the use of the deliberately vague term '*cis*-acting elements'). Second, in genes for which the DNA regions responsible for dosage compensation have been closely located, they have been found to lie within the gene itself, in flanking regions or some way from the gene [26]. This variability suggests that these elements may act not through a direct effect on transcription of a specific gene but through their influence on the higher-order structure of chromosome domains. This is also more likely in view of the fundamental requirement that the dosage-compensation mechanism must be applicable to a large number of different genes, each with their own distinctive promoter and enhancer elements and idiosyncratic control mechanisms. Dosage compensation must be able to override these disparate mechanisms and impose a blanket, twofold upregulation of transcription. In accordance with this higher-order-structure model, it has been found that the expression of any given gene can depend on its chromosomal location. X-linked genes plus flanking DNA translocated to autosomes can show incomplete dosage compensation that varies depending on the exact location of the translocated gene [26]. To summarize, the evidence is strong that dosage compensation involves *cis*-acting elements on the X chromosome, but it is not clear whether these elements are based on DNA sequence itself.

Chromatin structure and dosage compensation

In polytene chromosome squashes from third instar female larvae, the two X chromosome homologues are wrapped around one another to form what appears to be a single polytene chromosome. This should, in theory, be twice as thick as the male polytene X. In fact, the male and female X's are of similar thickness, the reason being that the male X has a visibly more diffuse structure [37]. There is clearly a difference in higher-order chromatin structure betwen male and female X's. The two chromosomes also differ in a more specific feature of their chromatin, namely the pattern of acetylation of histone H4. By immunostaining of polytene chromosome squashes with antisera to acetylated H4, it was shown that the male X chromosome was uniquely marked by a high level of a specific acetylated isoform, namely H4 acetylated at lysine residue 16, H4Ac16 [12]. Significantly, this H4 isoform was distributed along the male X in a pattern of discrete bands that corresponded almost exactly to the bands containing MLE and other protein components of the dosage-compensation pathway [29]. A model consistent with the data so far available is shown in figure 1.

Histone acetylation and dosage-compensation

The presence of a specific acetylated isoform of histone H4 on the dosage-compensated male X chromosome is one of the first examples of the association between a functionally defined chromatin domain and a specific acetylated histone isoform. A second example, also from work on *Drosophila*, is noted below. The crucial question now becomes whether this chromatin characteristic is an integral component of the dosage compensation pathway itself, or simply a secondary event contingent upon the increased level of transcription along this chromosome.

The evidence available so far indicates that the increased level of H4Ac16 is not transcription-dependent. First, there is no evidence for increased levels of H4Ac16 in highly transcribed (puffed) regions of polytene chromosomes [1]. Second, the male X chromosome in diploid larval neuroblasts is also marked by increased levels of H4Ac16 at metaphase [38], a stage of the cell cycle when transcription is minimal. Third, the male X chromosome is indistinguishable from the autosomes in the intensity with which it labels with antisera to H4 acetylated at lysines 5, 8 and 12, i.e. it is not hyperacetylated in any general sense [12]. An example is presented in figure 2, which shows part of a polytene chromosome squash from a male larva that has been double-labelled with antibodies to H4Ac8 and H4Ac16. The X chromosome is immunostained with both antibodies, though it is clear that each antibody defines a different pattern of bright and dim bands along the X. It can be concluded from this that while H4Ac16 colocalizes with components of the dosage-compensation system [29], other acetylated H4 isoforms do not. Finally, it is important to emphasize that genes on the male X chromosome are not 'transcriptionally hyperactive', a misleading term that is often used as a convenient shorthand for the twofold increase in transcription on the male X. Though crucial for dosage compensation, such an increase would be considered insignificant in most systems used for studying transcriptional control.

Circumstantial evidence that H4Ac16 has a central role in dosage compensation comes from the finding that its presence exclusively on the male X has been highly conserved through evolution. *D. pseudoob*-

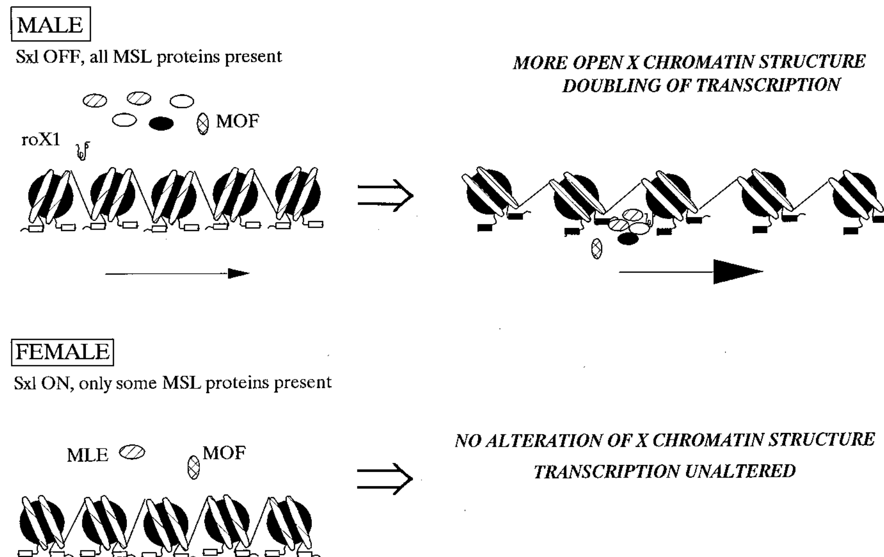

Figure 1. Dosage compensation in *Drosophila*. Diagram showing how components of the dosage-compensation system might assemble on the male X chromosome, with a consequent decondensation of chromatin and overall upregulation of transcription. Assembly on the X chromosome occurs only when all components are present. Some components are present in female cells but neither form multisubunit complexes nor assemble on the X chromosome. Despite the presence of the putative histone acetyltransferase MOF in female cells, the N-terminal domain of histone H4 (open rectangles) on the female X is not selectively acetylated at lysine 16, as it is in male cells (closed rectangles).

scura and *D. miranda* are closely related species that diverged from the lineage leading to *D. melanogaster* about 46 million years ago. While *D. melanogaster* has just one X chromosome, representing about 20% of the genome, translocations in which the original X chromosome was fused with autosomal material have resulted in *D. pseudoobscura* and *D. miranda* having the equivalent of two and three X chromosomes, respectively. In the latter species, these X chromosomes represent almost half the genome. The extra X chromosome material in *D. miranda* and *D. pseudoobscura* is both dosage-compensated [39, 40] and enriched in H4Ac16 [41]. Further, Bone and Kuroda [42] have shown that the dosage-compensated chromosomes in *D. miranda*, *D. pseudoobscura* and *D. americana americana* contain MSL proteins and that these proteins, as in *D. melanogaster*, colocalize with H4Ac16. Thus, H4Ac16 is a highly conserved component of the *Drosophila* dosage-compensation system.

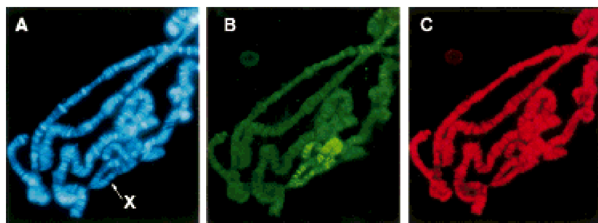

Figure 2. Distribution of acetylated H4 on polytene chromosomes from a male *Drosophila* larva. Part of a polytene chromosome spread prepared from the salivary glands of a male third instar *D. melanogaster* larva and double-labelled with antisera to H4 acetylated at lysine 16 (panel *B*, detected with FITC-conjugated second antibodies) and lysine 8 (panel *C*, detected with Texas Red-conjugated second antibodies). Panel *A* shows the Hoechst 33342 counterstain. H4Ac16 is present primarily on the X chromosome, of which only part is present in the section of the spread shown in the photograph, while H4Ac8 is present on all chromosomes at comparable levels. Note that the banding patterns produced by the two antisera along the X chromosome are quite different. H4Ac16 has been shown to colocalize with components of the dosage-compensation system, so H4Ac8 clearly does not.

However, there is as yet little evidence as to how H4Ac16 might be incorporated into the dosage-compensation complex on the male X (fig. 1). One attractive possibility is that it serves as a marker to which one or more of the other proteins might bind. This is still possible, but analysis of loss-of-function mutants of each of of the four MSLs has shown that H4Ac16 is only seen on the male X when the dosage-compensation system is complete and functional [29]. H4Ac16 cannot therefore be a primary and independent signal which serves to initiate assembly of the dosage-compensation complex on the male X, although it may be involved in tethering the complete complex to chromatin or in maintenance of dosage compensation from one cell generation to the next. This is considered further below.

A male-specific, H4-lysine-16-specific, acetyltransferase?
When the presence of H4Ac16 on the male X was first described, it was noted that this result predicted the existence of a male-specific, H4lys16-specific histone acetyltransferase activity [12]. None of the original MSLs show any sign of such activity, but the gap may be filled by a newly discovered component of the dosage-compensation pathway encoded by an X chromosome gene named *mof* (*males absent on the first*) [43]. Mutants of *mof* lead to a phenotype of male-specific lethality very similar to that produced by the other *msls*, including the absence of detectable H4Ac16 on the X chromosome. The *mof* gene has an open reading frame that encodes a protein of 827 amino acids. This protein has an extended region of homology with the human proteins Tip60 and MOZ and with the yeast protein SAS2 [44]. Within this region is a C2HC/H zinc finger and a sequence of about 20 amino acids that is found in acetyltransferases from several different organisms and with specificities for a variety of substrates, including histones. The domain is believed to be required for binding of the acetate donor acetyl CoA. Crucially, the mutation that led to the discovery of the *mof* gene is a single-base change resulting in the substitution of a glutamic acid residue for a glycine. This glycine is present within the putative acetyl CoA-binding region, is highly conserved and has been shown to be necessary for acetyltransferase activity [43, 45]. These findings strongly suggest that the acetyltransferase activity of MOF is necessary for its function. It is also significant that MOF is present in both males and females, but that loss of MOF function is lethal only in males [43]. This suggests that the HAT activity of MOF is essential for its role in dosage compensation but that this activity is not necessary for functions common to both males and females.
So, is MOF the predicted male-specific, H4lys16-specific acetyltransferase? The answer is still not clear. MOF is clearly not, itself, male specific, but its acetyltransferase activity may be mediated by interaction with components that are, just as other MSLs are functionally regulated by protein-protein and possibly protein-RNA interactions. The specificity of MOF also remains to be determined, and here too getting the answer to the question may not be straightforward. The specificity of the protein in vitro in the absence of its normal protein or RNA partners, e.g. when using the powerful 'in gel' assay [46], may be very different to that in vivo.

Evidence for regionally localized, lysine-specific acetyltransferases in *Drosophila*

The dynamic nature of the histone acetylation/deacetylation cycle is readily seen by treating cells with inhibitors of histone-deacetylating enzymes, such as the salts of short-chain fatty acids or more specific inhibitors such as the fungal antibiotic trichostatin A [47]. In most species and cell types, these inhibitors lead to the progressive accumulation of the most highly acetylated isoforms. However, this is not the case in *Drosophila* cells. In both the Kc and SL2 cultured cell lines, treatment with either butyrate or TSA leads to accumulation of acetylated H4 isoforms that is always significantly less than in mammalian cells treated in the same way [48]. Irrespective of the length of exposure to the inhibitors or their concentration, the level of acetylation never reached a stage at which the most acetylated isoforms (i.e. tri- and tetra-acetylated) were the most frequent. Western blotting and immunostaining with site-specific antibodies was used to show that, in *Drosophila* cells, monoacetylated H4 ($H4Ac_1$) was acetylated at lysines 5, 8 or 12 with approximately equal frequency [48]. This is in complete contrast to other species tested, in which $H4Ac_1$ is acetylated preferentially at a single lysine, often lysine 16, and in which the other lysines are then acetylated in a more or less fixed sequence through the di-, tri- and tetra-acetylated isoforms [1, 14, 49].
These observations on the pattern of acetylation of H4 and its response to deacetylase inhibitors suggest that, in *Drosophila*, histone acetyltransferases are both specific for H4 lysines and are located in different regions of the nucleus where they act on different H4 subpopulations. If this were the case, then exposure to deacetylase inhibitors would not lead to H4 hyperacetylation. Instead, in different regions of the nucleus, H4 would show increased levels of acetylation at lysines 5 or 8 or 12 or 16, depending on which HAT was located in that region, but only in regions where two or more HATs were located together could the more highly acetylated isoforms be generated. If it can be shown that MOF, like other components of the dosage-compensation system, is located on the male X chromosome, this will provide the first clear example of this prediction.

H4Ac12 provides a second example of an acetylated H4 isoform associated with a specific chromatin domain

Labelling of polytene chromosome squashes from *Drosophila* larvae with antisera to specific acetylated H4 isoforms reveals that each isoform has a characteristic distribution along the chromosomes. H4Ac16, as noted above, is found predominantly, though not exclusively, on the X chromosome in male cells. H4Ac5 and H4Ac8 are distributed along the arms of both autosomes and X chromosomes in a reproducible pattern of bright and dim bands which does not correspond in detail to the conventional banding pattern seen by DNA staining (fig. 2). Both these isoforms are present at only low levels in the constitutive heterochromatin of the chromocentre [12]. In contrast, H4Ac12 is present at both

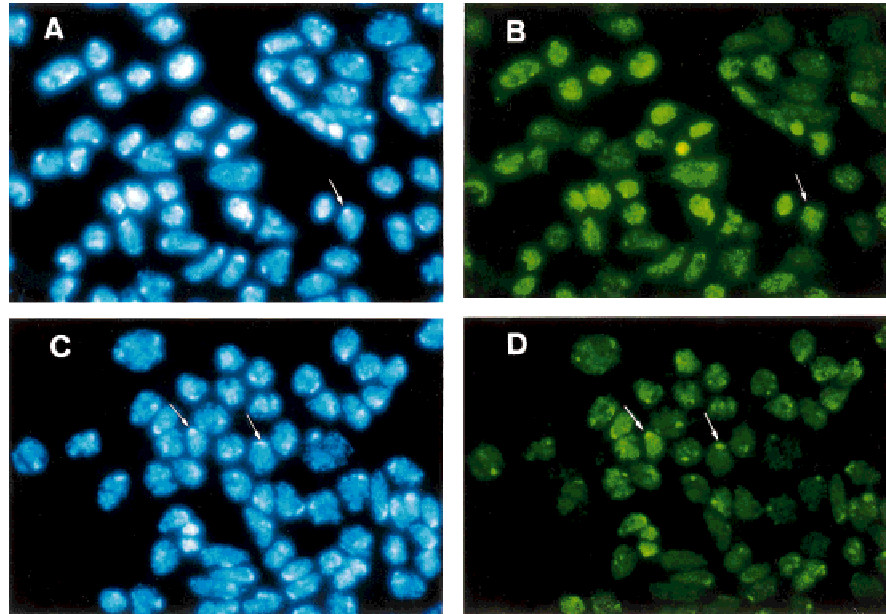

Figure 3. Distribution of acetylated H4 in *Drosophila* interphase nuclei. Neuroblasts from third instar *D. melanogaster* larvae were squashed, immunostained with antisera to H4Ac8 (*B*) or H4Ac12 (*D*) and counterstained with Hoechst 33342 (*A*, *C*). Whereas H4Ac8 is distributed throughout the nucleus, H4Ac12 is located preferentially in regions of constitutive heterochromatin (small arrows), recognizable by their bright Hoechst fluorescence. Note that (i) H4Ac12 is not exclusively located in heterochromatin, but is present also in other regions of the nucleus; (ii) in some nuclei patches of heterochromatin label relatively brightly with the antibody to H4Ac8 (*B*), though the difference between heterochromatin and euchromatic regions is never so marked as it is with the antibody to H4Ac12. This result suggests that underacetylation of H4 in the visible clumps of heterochromatin in diploid nuclei may not be so marked as that of H4 in the chromocentre of polytene nuclei.

euchromatin sites along the chromosome arms and in heterochromatin. In some cases, the heterochromatic regions actually stain more brightly with antisera to H4Ac12 than euchromatin. This is particularly true in diploid interphase neuroblasts, in which clumps of heterochromatin stain particularly brightly with antibodies to H4Ac12 but not with antibodies to the other acetylated isoforms. An example is shown in figure 3. Intriguingly, a relatively increased level of H4Ac12 also seems to be a characteristic of heterochromatin in yeast. It has been shown that chromatin packaging the silent-mating type genes HMLα and HMRa in *S. cerevisiae* is underacetylated at lysines 5, 8 and 16, but not at lysine 12 [50].

Speculations on the role of H4 acetylation in dosage compensation

Although an accumulation of circumstantial evidence suggests that H4Ac16 plays a central role in dosage compensation in *Drosophila*, the mechanism by which it does this remains a mystery. The fact that H4Ac16 is seen on the male X chromosome only when all the other (known) components of the dosage-compensation system are present argues against a role as the primary signal for location of a dosage-compensation complex on the male X. It may instead serve to (i) open up the higher-order chromatin structure of the male X (either directly or indirectly) and thereby facilitate binding of transcription factors and other proteins, (ii) alter DNA-H4 contacts as part of the (unknown) mechanism that brings about the doubling of transcriptional activity of X-linked genes in male cells, (iii) tether the dosage-compensation complex to chromatin or (iv) provide a marker that maintains the exact location of the dosage-compensation complex from one cell generation to the next. These possibilities are not mutually exclusive. Nor are they limited to the problem of dosage compensation. With only minor variations in wording, they can also be applied to the role of H4Ac12 in heterochromatin structure and function.

There are two stages of the cell cycle during which multicomponent complexes regulating levels of gene expression are liable to be lost or reprogrammed. These are DNA-replication and chromatin assembly during S phase and chromatin condensation during mitosis. The fact that both H4Ac16 and MLE are present at easily detectable levels on the male X chromosome at metaphase [38] suggests that H4Ac16 may help retain at least some components of the dosage-

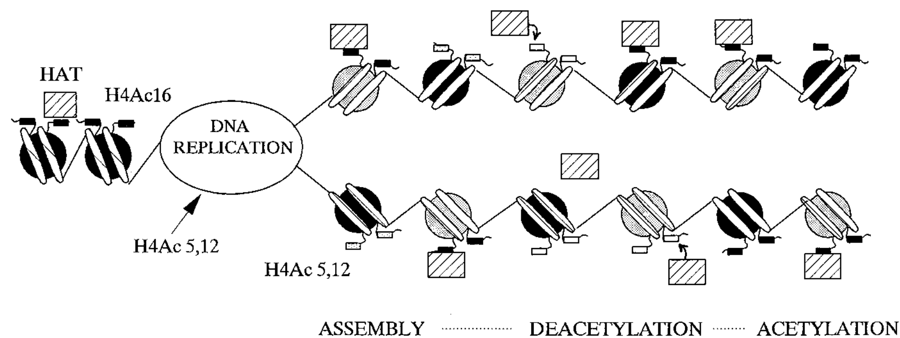

Figure 4. How a distinctive pattern of histone acetylation might be retained through DNA replication and chromatin assembly. The diagram shows a replication complex passing through a region of the male X chromosome in which the amino-terminal domain of histone H4 is acetylated selectively at lysine 16 (small, filled rectangles). An H4lys16-specific histone acetyltransferase (HAT, large shaded rectangle) is associated with the H4 amino-terminal domain, either through direct association with it, or as part of a larger complex. Following DNA replication, nucleosomes containing either newly synthesized H4 (speckled) or the original H4 (filled) are distributed between the two daughter strands. (The diagram assumes that the two H4 molecules in each original nucleosome are reassembled together, probably as an H3/H4 tetramer, but this is not essential for the model.) At this stage nucleosomes will be acetylated at either H4lys16 (original) or other lysines, most often 5 and 12 (newly synthesized, small shaded rectangles). There then follows a deacetylation step, during which histone acetates are removed by histone deacetylases. These enzymes certainly remove the deposition-related acetates and may remove others as well. The model proposes that the H4lys16-specific HAT remains in the immediate vicinity during the deacetylation step, possibly through continued association with the H4 amino-terminal domain, and is immediately available to reacetylate H4 at lysine 16, thus restoring the original acetylation pattern.

compensation complex on the male X through mitosis and facilitate its reassembly as cells exit mitosis and transcription is reinitiated. H4 acetylation can also provide a means for addressing what may be a more difficult problem, namely reassembly of a functional complex on each of the two daughter chromosomes following DNA replication and chromatin reassembly.

Patterns of H4 acetylation after DNA replication and chromatin assembly

Figure 4 shows a simple model that summarizes how specific patterns of H4 acetylation might be retained through DNA replication and assembly of new chromatin. Replication of a region on the male X chromosome enriched in H4 acetylated at lysine 16 is used as an example. The model proposes that an H4lys16-specific HAT is tethered to chromatin via the H4Ac16 tail, possibly as part of the complete dosage compensation complex. Following DNA replication, nucleosomes reassemble on the two daughter strands, some containing the original H4Ac16 and some containing new H4 molecules with the deposition-related pattern of acetylation, usually diacetylation at lysines 5 and 12 [51, 52]. Next comes a period of deacetylation during which histone deacetylases, subunits of which may be derived from the replication complex itself [53], remove acetate groups from the newly deposited histone and possibly the parental histones as well. There then follows a period of reacetylation. The two requirements for the model presented are (i) that the H4lys16-specific HAT should be present in association with H4Ac16 prior to replication and (ii) that it should remain in the vicinity during the post-replication phases so that it can reacetylate H4 at lysine 16 and thereby initiate reassembly of the complex. Requirement (i) is entirely consistent with the results summarized earlier on colocalization of H4Ac16 and components of the dosage-compensation complex and, given (i), (ii) is a reasonable assumption. Note that there is no requirement for the HAT to remain associated with histones during or after replication, or for it to protect H4Ac16 from deacetylation, though it may do either or both of these things. The major advantage of this system is that because it uses an enzyme, levels of the marker (i.e. H4Ac16) on the daughter chromosomes can be quickly restored to those present prior to DNA replication. Assembly of the rest of the complex can proceed as the necessary components become available. Because the marker is securely in place, there is no pressure for this assembly to occur rapidly to prevent the signal being lost. An alternative, and superficially simpler, mechanism whereby components of the original complex are simply distributed between the daughter chromosomes suffers from the inevitable drawback that the marker density on each daughter chromosome will be halved, and will remain so until such time as the numbers are made up by recruitment of newly synthesized components.

Essentially the same mechanism may be used to maintain H4Ac12 on heterochromatin post-replication. We need only substitute an H4lys12-specific HAT which is retained at locally high levels by association with pre-

existing H4Ac12. In the case of H4Ac12, there is also the theoretical possibility of an even simpler mechanism for maintaining this pattern of H4 acetylation, one that requires no lysine-specific HAT activity. It requires instead a specific pattern of post-assembly H4 deacetylation, namely removal of the deposition-related acetate group at lysine 5 but not that at lysine12. This requires an H4lys5-specific histone deacetylase. Unfortunately, there is as yet little useful information on the number of HDAs in *Drosophila* or their specificities. It should also be noted that, although, overall, newly deposited H4 is enriched in the diacetylated isoform H4Ac5,12, the degree of enrichment, or the lysines acetylated [53], may not be uniform across the whole genome. Indeed, H4 acetylation is not essential for chromatin assembly either in vivo or in vitro [54].

In summary

Selective acetylation of specific lysines on the core histones has the potential to provide a stable epigenetic marker, or imprint, from one cell generation to the next. Information can be encoded in the pattern of lysine-specific acetylation on the nucleosome surface and can be read and converted into structural and functional effects by nonhistone proteins. If the acetylation of a specific histone at a specific lysine (e.g. H4Ac16) can also serve to locate, directly or indirectly, the appropriate, lysine-specific acetyltransferase, then the imprint can be maintained through DNA replication and the assembly of new chromatin on the daughter strands. As both acetyltransferases and deacetylases are involved in these closely coupled processes, the enzymatic machinery for such a mechanism is already in place. What remains is the substantial task of identifying (i) the histone-specific and lysine-specific enzymes that can generate the specific imprint, (ii) the mechanisms by which these enzymes are located to the appropriate genomic regions and (iii) the nonhistone proteins that can read the imprint and convert it into functional changes.

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