The Constantly Changing Face of Chromatin

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Many recent findings have altered our vision of chromatin and its role in the regulation of cellular functions. Our perspective concerning chromatin has changed to a much more complex, but also more dynamic, view of chromatin as an entity that is intimately involved in the regulation of a variety of cellular functions. In this review, we describe the various types of proteins that alter the structure and, therefore, the function of chromatin and discuss the possible role of chromatin in cell aging. The elucidation of the mechanisms that link chromatin to aging will be one of the most exciting and striking advancements in the coming years

In the past several years, many findings have challenged dramatically our vision of chromatin and its role in the regulation of cellular functions. Our perspective of chromatin as static and structural has changed to a much more complex view of chromatin as a dynamic entity that is intimately involved in the regulation of a variety of cellular functions. Thus, eukaryotic DNA metabolism cannot be understood outside of the context of chromatin packaging, where multiple specific interactions are established between DNA and its associated proteins.

These dynamic properties of chromatin are achieved by several different molecular "machines" that cause the state of chromatin to remain fluid. A number of chromatin remodeling complexes that use adenosine 5′-triphosphate (ATP) to alter chromatin structure have been identified. Enzymes that modify the N-terminal tail of histones also play key roles in generating the dynamic state of chromatin. And histone chaperones along with histone variants have been implicated in the regulation of chromatin dynamics and, therefore, gene expression.

These chromatin regulatory factors are the link between chromatin structure and cellular processes such as transcription regulation, DNA replication and repair, cell cycle control, and cell aging. In this review, we discuss these factors in detail and provide an overview of the possible connection between chromatin and aging.

Nucleosome Structure: Packing the Seemingly Unpackable

In each organism, the length of the genomic DNA is considerably longer than the diameter of the cell. For instance, human DNA is nearly 94 cm long [2.75 x 10^9 base pairs (bp)], almost 10^7 times the diameter of an average cell. Therefore, genomic DNA must be efficiently compacted. Prokaryotic and eukaryotic cells have developed different mechanisms for compacting DNA. In bacteria, compacting is accomplished mainly by supercoiling of circular DNA and formation of DNA loops of about 40 kb in length [reviewed in (*1*)].

In eukaryotic cells, the DNA is compacted into a complex structure known as chromatin. The individual unit of chromatin is the nucleosome, which is composed of 146 bp of DNA wrapped 1.75 times around a protein complex called the core histone octamer. The octamer is formed by two copies each of the core histone proteins H2A, H2B, H3, and H4 (Fig. 1A). These core histones have two domains: a globular domain involved in histone-histone interactions through the histone fold

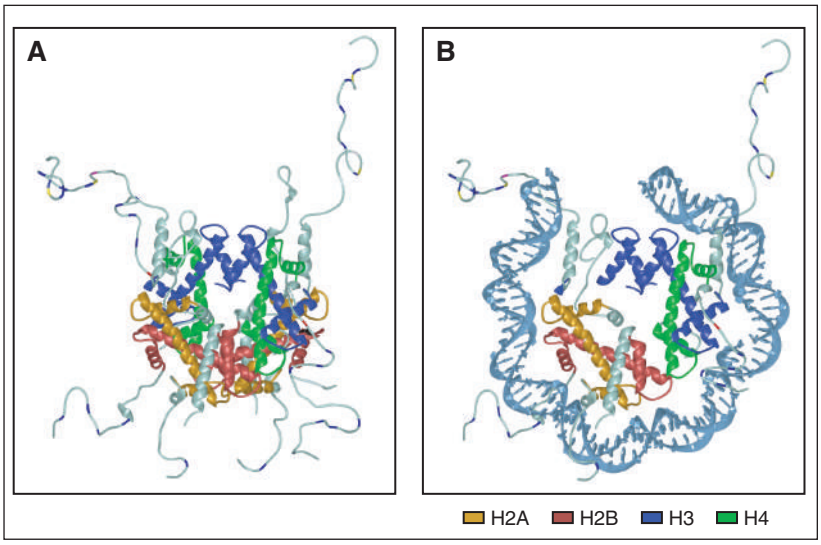

Fig. 1. Nucleosome core particle structure. (**A**) Core histone octamer. The figure shows the core histone octamer structure obtained from crystallographic studies of the nucleosome core particle. There is a shared structural motif at the center of all four histones, which consists of three α helices connected by two loops, forming what is known as the "histone fold domain." The histone fold domain is shown in several colors that correspond to each of the histones: orange, histone H2A; red, H2B; blue, H3; and green, H4. The rest of the protein is represented by white, including the N-terminal histone tails protruding from the center of the core particle. The association through the histone fold domains of each of the histone pairs can be observed: H2A/H2B and H3/H4. The figure was kindly provided by K. Luger. (**B**) Nucleosome core particle. The figure shows DNA (73 base pairs) and one copy of each of the core histone proteins, which corresponds to half of the nucleosome core particle. The DNA, shown in light blue, wraps 1.75 times around the core histone octamer. The DNA and histones interact mainly via the DNA phosphodiester backbone and each pair of histone folds. The N-terminal region of the histones (shown in white) protrudes from the nucleosome particle. The figure was kindly provided by K. Luger.

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domain; and the N-terminal histone tail, which is composed of approximately 15 to 30 amino acids that are mostly basic in nature and highly modified posttranslationally (see below) (*1*).

Recently, the crystal structure of the nucleosome was resolved to a resolution of 2.8 Å (Fig. 1B) (*2*). The crystals provided detailed information about interactions within the histone fold domains, as well as between histones and DNA. The nucleosome structure is chiefly maintained by interactions between the DNA phosphodiester backbone and each pair of histone folds within the core histone proteins. The N-terminal tails of the histones have been shown to be unorganized in these crystals, although several biochemical studies disagree on this point. For example, circular dichroism analysis revealed that at least half of the tail residues in histones H3 and H4 adopt an α-helical structure (*3, 4*). Moreover, cross-linking experiments showed that histone tails within the nucleosome interact with the linker DNA (that is, the DNA segments between nucleosomes) (*5*). The discrepancies among these various findings might have resulted from the stringent conditions used to obtain the nucleosome crystals and/or the absence of linker DNA in the nucleosome preparation used in the crystallization procedure.

In vitro experiments have demonstrated that the nucleosome structure is very stable. However, in vivo experiments using green fluorescent protein (GFP)-histone fusion proteins showed that about half of the total histone H2B pool and 20% of the histone H3 and histone H4 pools are turned over by passive and transient mechanisms involving incoming histones. Consequently, this exchange could provide a useful way to maintain the nucleosome in a dynamic state (*6*). Nonetheless, it is important to mention that nucleosomes are maintained on the chromatin fiber during processes that involve DNA metabolism, including replication, transcription, etc. (see below). Therefore, this dynamic state of the nucleosome might still allow important information to be passed on from one cell generation to the next. This form of inheritance, which does not involve the DNA sequence per se, is referred to as epigenetic inheritance.

A series of consecutive nucleosomes produces an array known as "beads on a string" or the 11-nm fiber; both designations are derived from electron micrographs (Fig. 2). The average length between nucleosomes is about 10 bp in *Saccharomyces cerevisiae* and 30 to 50 bp in metazoans. The nucleosomal array compacts the DNA about sevenfold (*1*).

The next level of compaction is the 30-nm fiber, in which the DNA is condensed another sixfold. The N-terminal tails of the histones are essential for this higher degree of compaction, in part caused by the neutralization of the DNA charge by the positively charged tails [reviewed in (*7*)]. But the histone tails are not *only* involved in electrostatic interactions, an idea supported by the finding that, in competition experiments, the addition of free H2B tail completely inhibits chromosome com-

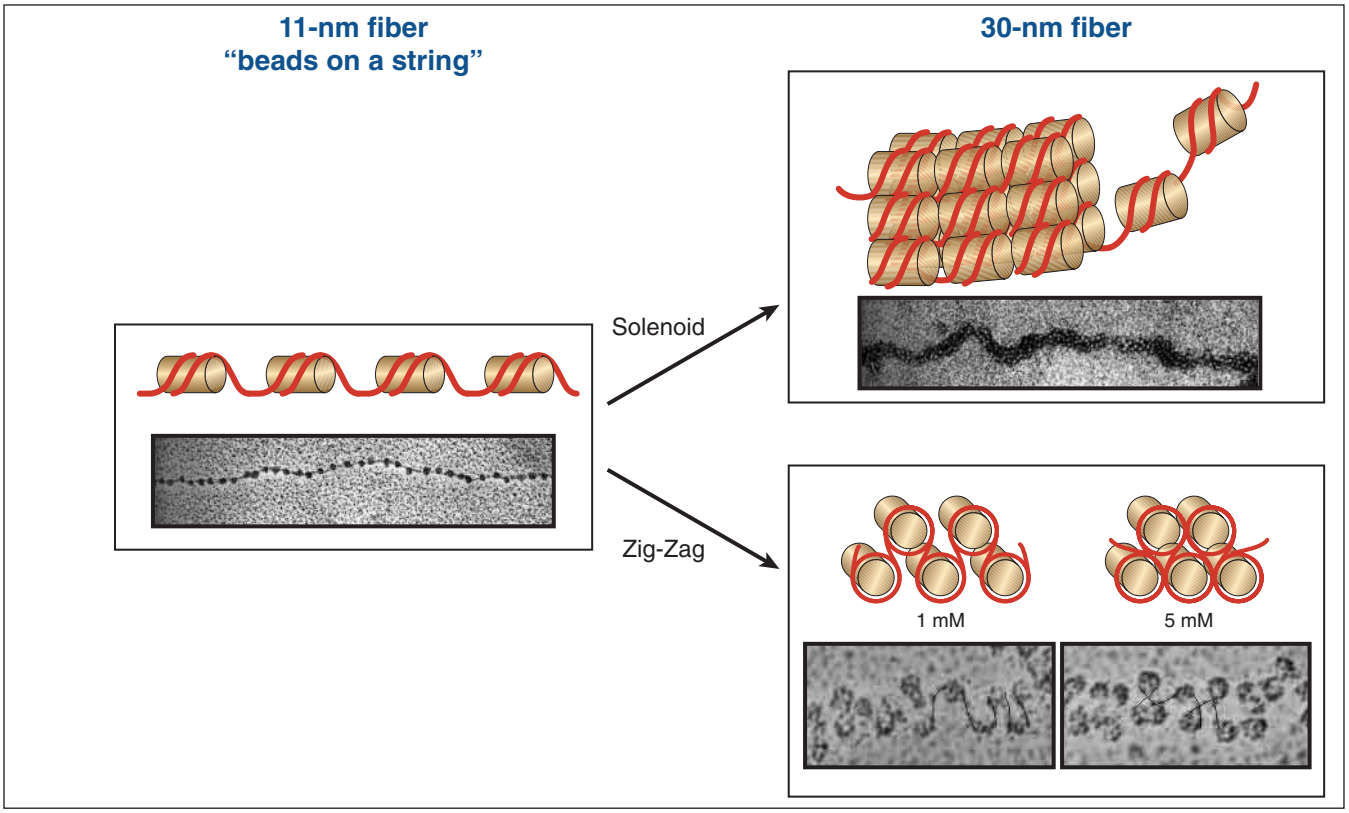

Fig. 2. Models for the 30-nm fiber. The figure shows a schematic representation of two models that have been proposed to explain the 30-nm fiber, as discussed in the text. The electron micrographs of the 11-nm fiber and solenoid were adapted and modified from *Molecular Biology of the Cell* (B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Garland Science Publishing, London, ed. 4, 2002). The zig-zag electron micrograph was adapted and modified from *J. Cell Biol.* **83**, 403 (1979). Two concentrations of TEA-Cl (trieathanolamine-HCl) are shown, 1 and 5 mM, to emphasize the importance of the salt concentration in the compaction of the nucleosomal arrays.

paction of *Xenopus* sperm DNA in vitro (*8*). This observation suggests the presence of a factor involved in condensation that is titrated out by the free H2B tail. The 30-nm fiber is further stabilized by the binding of the linker histone H1 to the nucleosome dyad (that is, the intersection between the DNA entering and the DNA leaving the nucleosome) [reviewed in (*7*)].

Because of the lack of appropriate genetic and biochemical techniques, the study of chromatin compaction has been difficult. In contrast, computer modeling as well as biophysical techniques, such as velocity sedimentation (*9, 10*) and scanning force microscopy (*11*), have accelerated research on chromatin compaction tremendously and will probably provide important structural and mechanistic information in the near future. To date, at least two models have been proposed to define the 30-nm fiber [reviewed in (*1*)]. The first is the solenoid model, which consists of an arrangement of six nucleosomes per DNA turn (*12*). The other is the zigzag model, which predicts that the linker DNA forms a straight path between successive nucleosomes (*13*) (Fig. 2).

Chromatin Assembly

The current model for nucleosome assembly proposes that histone chaperones (that is, protein complexes that associate with histones in order to prevent the positively charged histones from making nonspecific associations with the highly negatively charged DNA) escort the histones to the DNA in such a way that the H3/H4 tetramers are first deposited onto the DNA. This is a slow reaction and probably the rate-limiting step in nucleosome formation. The rapid association of two H2A/H2B dimers with the H3/H4 tetramers, mediated by histone chaperones, follows. The final step is the wrapping of the DNA around the histone octamers (*14, 15*). However, nucleosomes in the cells are regularly spaced; that is, the linker DNA length is kept constant (about 10 bp in *S. cerevisiae* and 30 to 50 bp in metazoans). Therefore, histone chaperone proteins must work in concert with chromatin spacing activities, which mobilize nucleosomes to obtain regularly spaced arrays (*16, 17*).

The bulk of chromatin assembly occurs during the S phase of the cell division cycle, when the cell's DNA is being replicated. Replication-dependent chromatin assembly is mediated by at least two histone chaperone complexes, CAF-1 (chromatin assembly factor 1) and RCAF (replication-coupling assembly factor). Both proteins interact with H3/H4 tetramers that contain a specific, newly synthesized acetylation pattern (acetylated lysine 5 and lysine 12 in the N-terminal tail of histone H4) created by the cytoplasmic histone acetyltransferase (HAT) 1 [(*18*), and see below for details on posttranslational histone modifications]. It has been proposed that RCAF is the histone donor for CAF-1, which is localized at sites of replication-dependent chromatin assembly by its interaction with PCNA (proliferating cell nuclear antigen), a subunit of the DNA replication machinery (*19*) (see Shcherbakova Review: http://sageke.sciencemag.org/cgi/content/full/sageke;2003/8/re3). The fact that yeast strains lacking these two histone chaperone complexes are still viable suggests that there are other chaperones that can substitute for them during DNA replication, although no others have yet been identified (*17, 19, 20*).

In addition to DNA replication-dependent chromatin assembly, minor amounts of histone deposition take place outside of S phase. Regions of the genome that undergo DNA repair and actively transcribed segments of DNA are examples of sites where nucleosomes might be altered and need to be reestablished. For example, CAF-1 and RCAF have been found to mediate synergistic nucleosome deposition after ultraviolet (UV) damage (*21, 22*).

Several other histone chaperones as well as chromatin spacing activities that function independently of DNA replication have also been identified. HIRA (histone regulation A), a newly discovered H3/H4 histone chaperone complex, can deposit nucleosomes on DNA. In vitro experiments showed that HIRA-depleted *Xenopus laevis* egg extracts are incompetent for chromatin assembly independent of DNA replication, whereas DNA replication-dependent assembly remains unaffected. HIRA is essential for embryonic development in mice (*23*), yet its precise function is unknown.

As mentioned above, histone chaperones require chromatin spacing factors to achieve regularly spaced nucleosomal arrays. These factors, initially identified in *Drosophila*, are highly conserved among species. All of them utilize ATP to mobilize nucleosomes within an array, and all share the adenosine triphosphatase (ATPase) subunit ISWI. ACF (ATP-utilizing chromatin assembly factor) and CHRAC (chromatin accessibility complex) assemble chromatin in vitro in the presence of the histone chaperone NAP-1 (nucleosome assembly protein-1) [reviewed in (*17, 24*)]. RSF (remodeling and spacing factor) is another chromatin spacing factor that was initially identified as an activity that allows the formation of competent RNA polymerase II transcription initiation complexes on chromatin templates (*25*). RSF is also able to assemble nucleosomes, but unlike ACF and CHRAC, RSF does not require an additional histone chaperone. Indeed, it has been suggested that the largest subunit of the RSF complex has a histone chaperone activity (*26*).

Investigations into the mechanism of ACF-mediated chromatin assembly show that ACF is a highly processive enzyme. Moreover, results from these studies suggest that chromatin assembly occurs through a tracking mechanism; ACF appears to translocate along the DNA in an ATP-dependent manner similar to that observed with helicase enzymes (*27*) (Fig. 3) (see Fry Review: http://sageke.sciencemag.org/cgi/content/full/sageke;2002/13/re2).

The identification of the components of these various histone chaperone complexes and cloning of the genes encoding them, together with the use of bacterially expressed histones, has made it possible to obtain recombinant chromatin in a completely defined biochemical system. This approach has allowed researchers to study the functions of various histone modifications in a variety of reactions, such as chromatin assembly. For example, it was shown that acetylation of the H2A and H2B tails is essential for RSF-dependent chromatin assembly (*26*). Moreover, ACF-directed chromatin assembly is also stimulated by acetylation, although it is not yet clear whether this effect is caused by the acetylation of the histones themselves or acetylation of the histone chaperone NAP-1 (*28*).

The ability to obtain recombinant chromatin will continue to be of great help in the study of histone modifications and the interplay between different factors in a variety of DNA-dependent reactions.

Histone Variants

As mentioned in the previous section, the bulk of nucleosome assembly occurs during DNA replication; therefore, a large amount of histones are needed at this time of the cell cycle. To fulfill this requirement, the bulk of histone gene expression also occurs during S phase. However, outside of S phase there is a

low, but constant, expression of histone proteins. These histones, called "replacement histones" or "histone variants," are used for nucleosome assembly independent of DNA replication.

All of the histones except H4 are encoded by several different genes. In some cases, the various genes encode histones that differ in only a few amino acids. For example, the histone H3.3 variant differs from the major H3 at only four amino acid positions. In other cases, the differences are more extensive, such as in the case of macroH2A, where about 60% of its sequence is unrelated to the sequences of any of the other histones. It is not known whether all of these sequence variations have functional significance or are simply the result of genetic diversity. However, for some of the histone variants, their role in the regulation of gene expression is becoming clearer.

ATPase family	Complex	Species	Subunits	Function
ISWI	ISWI1	Yeast	ISWI1 + p110 + p105 + p74	Nucleosome remodeling and spacing factor (29).
	ISWI2	Yeast	ISWI2 + p140	Nucleosome remodeling and spacing factor (29). In vivo repression of early meiotic genes upon recruitment by Ume6 to promoter regions (30).
	ACF	*Drosophila, Xenopus*, human	Acf-1 + ISWI (hSNF2H in human)	Assembles chromatin in vitro in the presence of the histone chaperone NAP-1. Slides nucleosomes and activates chromatin transcription (31, 32).
	WICH	*Xenopus*, mouse	WSTF + hSNF2H	Chromatin remodeling complex, stably associated with mitotic chromosomes (33)
	CHRAC	*Drosophila,* human	Acf-1 + ISWI (hSNF2H in human) + CHRAC16 + CHRAC14	Shares ACF activities (34).
	NURF	*Drosophila*	NURF301 + ISWI + NURF55 + NURF38	Slides nucleosomes, facilitates transcription initiation through GAGA interaction (35).
	RSF	Human	Rsf-1 (p325) + hSNF2H	Allows formation of competent transcription initiation complexes in vitro (25). Assembles chromatin in vitro. Binds to H3/H4 (26).
	NCoRC	Human	Tip5 + hSNF2H	Induces nucleosome sliding, Tip5 interacts with the RNA Polymerase I terminator factor TTF-I (36).
	ISWI-D	*Xenopus*	p195 + ISWI	Remodel chromosome structure of somatic nucleus upon nuclear reprogramming (37)
SWI2/ SNF2	SWI/SNF	Yeast, *Drosophila,* mouse, human	Brg1/Brm + about 10 subunits, depending the species.	Alters nucleosome structure. Contains actin related proteins (38).
	RSC	Yeast	Sth1 (ATPase) + ~15-polypeptide complex	Alters nucleosome structure. Essential in yeast (39)
INO	INO80	Yeast	12-polypeptide complex	Chromatin remodeling, facilitates transcription in vitro, contains 3' to 5' DNA helicase activity. Possibly involved in transcription as well as DNA repair (40).
CHD	CHD1	Yeast, human	CHD1	Interacts with the human FACT subunit SSRP1 (41). Chromatin remodeling activity (42).
	NuRD	*Drosophila, Xenopus,* human	CHD4 (Mi2) + MTA2 + MBD3 + HDAC1/2 + RbAp48/46	Histone deacetylase and chromatin remodeling activity. Interacts with methylated DNA through interaction with MBD2 (43, 44).

Fig. 3. ATP-dependent chromatin remodeling factors

The histone H2A family is the most diverse among the histones. The human genome contains 16 genes that encode the various forms of histone H2A. Six of them are identical at the amino acid sequence level, and five differ in only a few amino acids. These 11 genes are responsible for the bulk of histone H2A expression. The remaining five genes encode H2A variants with more diverse amino acid sequence differences (*45, 46*):

(i) H2AX differs mainly in the C-terminal region of the protein, and its sequence is highly conserved throughout evolution. Phosphorylation of a serine residue in the C-terminus of H2AX takes place when double-stranded DNA breaks occur; double-stranded breaks are a common product of chemical drugs that sever DNA and many metabolic reactions that involve DNA, such as homologous V(D)J recombination. It has been suggested that H2AX phosphorylation might be a mark for sites of DNA breakage.

(ii) H2AZ has a small number of amino acid differences throughout its sequence, but the differences are concentrated in the H3/H4 binding domain. Mice with the H2AZ gene knocked out die early in embryogenesis. In vitro as well as in vivo studies suggest that nucleosomes carrying the H2AZ variant are less stable and tend to lose the H2AZ/H2B dimers easily, compared with nucleosomes that carry H2A/H2B dimers.

(iii) MacroH2A1 and MacroH2A2 are the most diverse of the H2A variants. Both are found in the inactive X chromosome in human females and female mice, suggesting a role in transcriptional silencing.

(iv) The recently identified H2A-Bbd (Barr body-deficient) variant is excluded from inactive X chromosomes in mammalian cells and colocalizes with acetylated histone H4 (*47*), suggesting that it might be associated with transcriptionally active regions of the genome.

Histone H3 has two major variants (*1*):

(i) CENP-A is a highly conserved variant that localizes at centromeres. The N-terminus is unrelated to the major histone H3. CENP-A knockout mice die early in embryogenesis.

(ii) H3.3. In vivo studies using H3- and H3.3-GFP fusion proteins revealed that histone H3 is assembled into nucleosomes exclusively during DNA replication, whereas H3.3 is assembled only outside of S phase. Moreover, H3.3 is deposited onto DNA mainly at highly transcribed loci. If just one residue in the histone-fold domain of H3 is mutated to the corresponding amino acid residue in the H3.3 variant, deposition of the mutated H3 is no longer restricted to S phase (*48*). This result suggests that histone deposition is tightly regulated and highly specific, and that H3.3 might mark transcriptionally active genes.

Histone H1 has many more variants than do the core histones, and it has been suggested that the ability of H1 to condense chromatin depends on which H1 variant is present. A very nice example of this tenet is the developmental regulation of the 5*S* ribosomal DNA (rDNA) gene in frogs. The histone H1 variant B4 allows expression of the 5*S* gene from the oocyte cluster in the oocyte and early embryo. In late-stage embryos, B4 is replaced by H1, which represses the oocyte 5*S* gene and activates the somatic 5*S* gene cluster (*49*).

Although the functions of the histone variants are still largely uncharacterized, more and more evidence indicates that at least some of the variants play specific physiological roles (*1, 45, 46*). It will be interesting to determine how these variants are targeted to specific regions of the genome at precise times in the life of the cell. Researchers are also investigating how the variants affect chromatin function and whether they affect nucleosome stability and higher order chromatin structure directly or indirectly by interaction with other proteins.

Chromatin Remodeling Factors: Flexibility is Key

The fact that the DNA is highly compacted into chromatin has profound effects on DNA metabolism. Many of the processes in which DNA acts as the substrate are repressed by chromatin (*50*). It was thought initially that this repression was simply due to the blocking of DNA by core histones so that other proteins (for example, the transcription machinery) had restricted access to the DNA. However, it was shown recently that chromatin has a more participatory function in regulation, rather than simply occluding the binding of other proteins (*51*).

To circumvent the inhibitory effects of chromatin, cells use a variety of factors to make the structure of chromatin more dynamic. Two families of factors have been described. One is the family of ATP-dependent chromatin remodeling factors, which will be discussed in this section (*52, 53*). The other family is composed of enzymes that covalently modify the N-terminal residues of histones, and will be discussed below.

Chromatin remodeling is usually associated structurally with chromatin opening and functionally with transcriptional activation. But not all remodeling factors have this effect. Some of these factors alter chromatin by forming transcriptionally repressed structures. This is true for NuRD, a chromatin remodeling complex that is associated with histone deacetylases (HDACs) (*43, 44*). Therefore, the true definition of chromatin remodeling is a change in the state of chromatin that results in either an activated or a repressed structure.

ATP-dependent chromatin remodeling factors are divided into four families according to similarities in their ATPase subunit: the ISWI (imitation of switch), SWI/SNF (switch/sucrose nonfermenting), INO (inositol), and CHD (chromodomain helicase/ATPase DNA binding protein) families. Fig. 3 shows a summary of these families and their members.

Even though all of these ATPases are involved in chromatin remodeling, they exhibit mechanistic differences (*54*). For example, the ATPase activity of the SWI/SNF complex is stimulated by naked DNA and does not require histone tails. The ATPase of Mi2 (a member of the CHD family) is stimulated by oligonucleosomes, but not by naked DNA, and does not require the presence of histone tails. The ISWI ATPase is slightly stimulated by naked DNA and strongly stimulated by oligonucleosomes, and the histone H4 tail is essential for this activity (*55, 56*). Detailed analysis of the H4 tail requirement has shown that tail amino acid residues 17 to 19 are essential for ISWI ATPase activity. This region of H4 was previously shown to contact nucleosomal DNA (*57*). In addition, acetylation of H4-K12 and H4-K16 inhibits the ISWI ATPase activity (*58*).

The final remodeled chromatin state also differs among the various remodeling machineries. In a process known as *trans*-transfer, SWI/SNF, under certain circumstances, transfers the entire core histone octamer to another region of DNA. It can also disrupt nucleosomes, although, in this case, the histones are not removed from the DNA and the result is a topological change in the chromatin structure. In contrast, the ISWI machinery does not disrupt nucleosomes; instead, it slides them along the DNA, a process known as *cis*-transfer. As a consequence, ISWI changes the organization of the nucleosomal array (*53*).

Precisely how chromatin remodeling occurs is not yet clear, but two models have been considered: the twisting model and the bulge model (*54*). The first model is supported by experiments using cruciform DNA. This assay measures the superhelical tension created in the DNA in vitro. Three of the ATPases families have been shown to create such superhelical tension: SWI/SNF, ISWI, and Mi2. This observation suggests that the DNA torsion angle (http://www.imb-jena.de/~csc/NANA.html) produced can alter the DNA twist (http://www.imb-jena.de/~csc/NANA.html), and the propagation of this new twist through the nucleosome would result in nucleosome sliding. However, nicking of nucleosomal circular DNA, which destroys the superhelical torsion, does not inhibit nucleosome sliding, as would be expected if the superhelical torsion were required for sliding (*59*). Therefore, the bulge model was proposed, which suggests that a small DNA loop is formed upon binding of the chromatin remodeling factors at two nearby sites, probably at the linker DNA and at the nucleosome. The propagation of this loop through the nucleosome would explain the occurrence of sliding. Electron microscopic studies show that SWI/SNF binds naked DNA at two positions, creating loops of different sizes. Nucleosome remodeling occurs only in the loop (*60*). In a similar way, RSF (a member of the ISWI family) is able to form loops during chromatin assembly (*26*). Although chromatin assembly and chromatin remodeling seem to be opposite reactions, there is a common step between them, the sliding of nucleosomes on a DNA array. It has not yet been shown that the loops formed upon binding of the remodeling factors are actually involved in nucleosome movement.

RSC (remodel the structure of chromatin) belongs to the SWI2/SNF2 family of remodeling factors. Mechanistic analyses of RSC alone, as well as with its ATPase subunit Sth1, suggest that its remodeling mechanism also involves an ATP-dependent DNA translocation (*61*). From these experiments, it was proposed that RSC remains fixed to the histone octamers and translocates through the DNA, breaking histone/DNA contacts within the entire nucleosome. This suggests that the various members of the SWI2/SNF2 family of ATPases utilize similar mechanisms to mobilize or alter nucleosomes.

The in vivo role of ATP-dependent chromatin remodeling factors is still under investigation. However, there are some hints as to their physiological functions. Yeast cells contain two ISWI complexes, ISWI1 and ISWI2. A knockout of either of the two ISWI genes is not lethal, whereas a *chd* (which encodes a member of another ATP-dependent chromatin remodeling complex family; see Fig. 3), *iswi1*, and *iswi2* triple knockout shows a synthetic lethal phenotype under different stress conditions, including high temperature and the presence of formamide in the media (*29*). In contrast, a knockout of the *ISWI* gene in *Drosophila* (the only member of this family in *Drosophila*) results in lethality at the late larval stage. The X chromosome in male ISWI mutants is less condensed than in the wild type, suggesting that ISWI is required for higher order chromatin structure (*62*). Interestingly, in *Drosophila*, the X-chromosome structure is crucial for dosage compensation, which results in a twofold increase in transcription of the X chromosome in males, as compared with that of the X chromosome in females (*63*). In the mouse, an analysis of the expression of the two ISWI homologs, SNF2H and SNF2L, during development showed that in the brain, SNF2L is predominantly expressed in differentiated neurons, whereas SNF2H is found mostly in proliferating cells. These observations suggest that SNF2H is involved in chromatin assembly in proliferating cells and SNF2L functions in differentiated cells (*64*). Recent experiments in human cells have started to clarify the role of the chromatin remodeling complex ACF. The use of RNA interference (RNAi) revealed that ACF plays a role in the replication of heterochromatin at pericentromeric regions, allowing DNA replication to occur through highly condensed, chro-

matin-packaged regions (*65*).

In the case of the SWI2/SNF2 family, RSC is essential in yeast (*39*), whereas the other member of the family, SWI/SNF, is not. Genome-wide gene expression analyses in yeast indicate that about 6% of the genes in this organism are regulated by SWI2/SNF2. This low percentage could be due to the redundancy between SWI/SNF and the Gcn5p histone acetyltransferase (HAT), which is also involved in keeping the structure of chromatin dynamic (see below) (*66*).

Chromatin remodeling complexes are inhibited by the linker histone H1. However, upon phosphorylation of H1, chromatin becomes accessible to the chromatin remodeling complex SWI/SNF (*67*). This example illustrates the complexity of chromatin, but it also demonstrates the many forms of regulation that this structure can provide.

We expect that in the near future, the chromatin field will benefit greatly from the new biochemical and in vivo approaches that are being used to understand chromatin remodeling. In addition, the deciphering of the in vivo functions of these complexes and the interplay between them, together with the study of their function in the context of higher order chromatin structure, will provide a fuller picture of chromatin remodeling complexes and their functions.

Histone Chaperones and Chromatin Dynamics

Histone chaperones, as mentioned earlier, can directly regulate chromatin structure through their association with core histones. This regulation is seen in processes as diverse as chromatin assembly, chromosomal decondensation, and transcription. A variety of fine examples illustrate this effect.

At the time of fertilization in the *Xenopus laevis* oocyte, the sperm chromosomes decondense. This process occurs in two steps. The first is the rapid, but limited, sperm decondensation that is dependent on cytoplasmic proteins. This rapid step is followed by a slower decondensation that occurs when the nuclear membrane is assembled on the surface of the chromosomes. The first step is dependent on the histone chaperone nucleoplasmin. Nucleoplasmin removes from chromatin two specific proteins present in the sperm, X and Y, and replaces them with H2A and H2B. Depletion of nucleoplasmin from the cytoplasm of the *Xenopus* oocyte inhibits sperm decondensation (*68, 69*).

Another example is the effect of the H2A/H2B histone chaperones NAP-1 and nucleoplasmin on the binding of transcription factors to nucleosomal DNA. These chaperones stimulate the in vitro binding of transcription factors by disrupting core histone octamers and thus increasing the accessibility of the DNA (*70*).

FACT is another H2A/H2B histone chaperone that was identified as an RNA polymerase II transcription elongation factor specific for chromatin templates (*71*). It has been proposed that the transcriptional stimulation mediated by FACT is due to its binding to H2A/H2B, which causes destabilization of the nucleosome structure, allowing RNA polymerase II to transcribe on the chromatin template (*72*).

The recent finding regarding the deposition of the histone variant H3.3 mainly in highly transcribed loci (*48*) led to the hypothesis that the dynamic exchange of histones by chaperones might function in epigenetic regulation—that is, transcriptional regulation via changes in chromatin state. Therefore, histone H3.3 deposition might be a mark for transcriptional activation. Consequently, one of the goals of future experiments is to characterize histone chaperones that specifically recognize histone variants and to determine their function.

Histone-Modifying Enzymes: Tailing the Chromatin

Although histones can be posttranslationally modified in different regions of their amino acid sequence, the N-terminal regions or N-terminal "tails" are preferentially modified. The reason is obvious if we consider the nucleosome structure and the protruding position of the tails from the nucleosome core. The possible modifications include acetylation, methylation, phophorylation, ADP-ribosylation, ubiquitination, and biotinylation (*1, 7*).

In the past several years, our view of the function of histone tails and their modifications has changed drastically. The various findings and theories that make up our current vision are summarized in a general biological principle known as the histone code hypothesis (*73*).

The Histone Code

Histone tail modifications that regulate chromatin structure are, in general, dynamic, highly regulated, and transient, so as to adapt to physiological changes in the cell. An exception to this general rule is methylation, which appears to be stable and irreversible. Histone tail modifications can be viewed as "letters" of a new biological code that ultimately determines which proteins will specifically recognize and bind to the tail. Different combinations of modifications will result in different effects (see Animation 1: http://sageke.sciencemag.org/cgi/content/full/sageke;2003/14/re4/DC1). Thus, protein domains that recognize and bind to specific histone tail modifications have been identified, such as the bromodomain and chromodomain, which interact directly with lysine residues in histone tails that have been acetylated and methylated, respectively. Chromatin becomes a functional panel where combinations of multiple modifications produce distinct outputs that can differ among species. An example of this is histone acetylation, which can give rise to transcriptional activation or repression depending on the context and the species. For instance, H4-K12 is hyperacetylated in transcriptionally silent regions of the yeast and *Drosophila* genomes, but this is not the case in humans (*74*). To add another level of precision and sophistication, each successive modification produces a new context where other modifications can now take place. Thus, the order and positions of the modifications, in addition to the type, determine the context in which these alterations exist and then synergize to produce a new regulatory effect.

Histone Acetylation and Deacetylation

Acetylation is the most extensively studied posttranslational modification to date and involves the transferring of an acetyl group from the metabolic intermediary acetyl coenzyme A to the ε-amino group of a lysine residue. It is a highly dynamic reversible modification that was originally linked to transcriptional activation (*75*). Although it is known that the four core histones (H2A, H2B, H3, and H4) can be acetylated in their N-terminal tails, the H3 and H4 modifications are mainly responsible for the effect on transcription.

Histones H2B, H3, and H4 can be acetylated on four lysine residues in their N-terminal tails (H3: K9, K14, K18, and K23; H4: K5, K8, K12, and K16; H2B: K5, K12, K15, and K20), whereas H2A can be acetylated only at K5 and K9 (*75, 76*). It is generally accepted that the primary effect produced by acetylation is the partial neutralization of the positive charge of the histones, thus decreasing their affinity for the DNA (*77, 78*) and altering nucleosome-nucleosome interactions (*79*). After acety-

HAT family	HAT enzyme	Organisms known	Complex	Specificity	Function
GNAT	Gcn5	Yeast to humans	SAGA, ADA ADA2	H3, H2B	Coactivator
	PCAF	Human, mice	PCAF	H3, H4	Coactivator
	Hat1	Yeast	HatB	H4 [K5, K12], H2B	Histone deposition, silencing
	Elp3	Yeast to humans	Elongator	H3, H4	Transcriptional elongation
	Hpa2	Yeast		H3, H4	Unknown
	ATF-2	Humans, mice		H2B, H4	Sequence-specific transcription factor
MYST	Sas2	Yeast		H4 [K16]	Silencing
	Sas3	Yeast	NuA3	H3, H4, H2A	Silencing
	MORF	Humans		H4>H3	Unknown
	TIP60	Humans	TIP60	H4>>H3, H2A	HIV Tat interaction, DNA repair, Apoptosis
	Esa1	Humans	NuA4	H4, H2A	Cell cycle progression
	MOF	Drosophila	MSL	H4 [K16]	X- chromosome hyperactivation dosage compensation
	HBO1	Humans	HBO1	H3, H4	DNA replication
	MOZ	Humans	AML1	H3, H4> H2A	Transcription activation, others?
P300/CBP	p300	Multicelular organisms		H2A, H2B, H3, H4	Coactivator
	CBP	Multicelular organisms		H2A, H2B, H3, H4	Coactivator
Hormone Receptor Coactivators	ACTR	Humans, mice		H3>H4	Hormone Receptor coactivators
	SRC-1	Humans, mice		H3>H4	Hormone Receptor coactivators
	TIF2	Humans, mice			Hormone Receptor coactivators
TAFII250	TAFII250	Yeast to humans	TFIID	H3>H4,H2A	TBP-associated factor/ Cell cycle progression
TFIIIC	TFIII220	Humans	TFIIIC	H3, H4 >H2A	RNA polymerase III –mediated transcription
	TFIII110	Humans			
	TFIII90	Humans			
Nut1	Nut1	Humans	Mediator	H3>>H4	RNA polymerase II –mediated transcription

Fig. 4. Histone acetyltransferases.

lation, weak interactions still exist between DNA and the histone tails that protrude from the nucleosome (*80*); however, the changes brought about by acetylation are enough to generate a permissive structure for the binding of proteins, such as those of the transcriptional machinery, to DNA (*81, 82*). Consistent with this observation, deacetylation produces a more compacted structure that is refractive to the binding of factors (*83*). In addition, acetylated tails can directly recruit other components of the chromatin machinery to DNA, because certain protein domains, such as the bromodomain (*84*), can bind on their own to specific acetylated lysines in the tails of histones (*85*). Bromodomains are present in many chromatin-associated factors and in almost all HAT enzymes, including the TATA-binding protein (TBP)-associated factor $TAF_{II}250$ (*86*), which is involved in transcription. Histone acetylation has also been implicated in DNA repair and replication (*87*).

K14 acetylation in histone H3 is one of the better (if not the best) examples of the interrelationship among histone modifications and can provide us with a glimpse of the complexity of the histone code. K14 acetylation is involved in transcriptional activation and can be regulated positively by serine 10 phosphorylation in H3 and negatively by methylation in lysine at K9 of H3. Thus, because several modifications can coexist in a single histone tail at any given time, the complexity of the regulation established by the histone tails of a particular nucleosome is considerable.

Histone Acetyltransferases (HATs)

Multiple enzymes that display HAT activity have been described. Most of them are present in multimeric complexes and are involved mainly in transcription as coactivators (*75, 76*). However, some also participate in transcriptional silencing, cell cycle progression, DNA replication, and development (*88*). Because HAT proteins do not contain DNA binding domains, other factors are required for their proper recruitment to specific locations in the genome. This requirement establishes a new point of transcriptional regulation and generates substantial flexibility in terms of the number of effects that can be achieved with a few factors (*76, 78*). Some of the HATs, such as CBP/p300 (CREB-binding protein) or PCAF (p300/CREB-binding protein-associated factor), can also acetylate other nonhistone proteins (*88, 89*), allowing the HATs to act not only as transcription factors, but also as mediators of most of the cellular signaling that affects chromatin.

One way to distinguish between the various HAT activities is on the basis of subcellular localization. Using this criterion allows us to define two types of HATs: type A, or nuclear, and type B, or cytoplasmic. The type A HATs are present in the nucleus and are responsible for the acetylation of the nuclear histones (*76, 90*). Type B HATs are, in general, H3 and H4 specific; these HATs exist in the cytoplasm and are implicated in histone deposition (*91*).

Another way to classify HATs is on the basis of sequence homology and substrate. Using these criteria, we can define at least six different families of HAT activities (*76, 92*). The members of these families are shown in Fig. 4. Recently, however, new unrelated HATs have been described, so that this classification will have to be reviewed after further analysis of these new activities (*93*).

Histone Deacetylases (HDACs)

The functional importance of acetylation depends completely

HDAC group	HDACs members	TSA sensitivity	NAD^+ dependence	Localization	Function
Class I (Rpd3)	HDAC1, HDAC2, HDAC3, HDAC8	Yes	No	Nuclear and ubiquitous localization.	Involved in a variety of functions, such as transcriptional repression and cell differentiation.
Class II (Hda1)	HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10	Yes	No	Tissue-specific expression.	Transcriptional repression, microtubule regulation
Class III (yeast Sir2)	Sir2, HST1-4 Homologues in different organisms from yeast to humans. (Arabidopsis, C. elegans, D. Melanogaster, etc…) e.g. Human Sirt1-7	No	Yes	Some are nuclear, others cytoplasmic and mitochondrial.	Involvement in silencing. Connection with aging. Function in development, gene repression and DNA repair.

Fig. 5. Histone deacetylases [from (*99, 102, 112, 115, 117, 122*)]. TSA, HDAC inhibitor trichostatin A.

on the accuracy and efficiency of the reverse reaction, histone deacetylation.

Although it is clear that, in general, histone hypoacetylation is related to repression of transcription, some genetic studies in yeast suggest a role for HDACs in the activation of transcription (*94*). HDACs have been divided into three different classes: I, II, and III (Fig. 5). The first two classes are sensitive to the HDAC inhibitor TSA (trichostatin A) (*95*), whereas the third class is insensitive, and its activity requires the coenzyme NAD^+ (nicotinamide adenine dinucleotide) as a cofactor (*96*).

Classes I and II

Class I includes HDAC 1, 2, 3, and 8, while class II includes HDAC 4, 5, 6, 7,9, and 10. Both classes are defined by their homology to two yeast HDACs: Rpd3 in the case of class I and Hda1 in the case of class II (*97, 98*).

Class I HDACs are all nuclear and ubiquitous activities (*99*) associated with a variety of complexes, for example, the complex containing the general transcriptional co-repressor Sin3 (*100*) and the ATP-dependent chromatin remodeling complex NuRD (*101*, *102*). These complexes can interact with a wide variety of transcriptional regulatory proteins, most of them DNA binding proteins, such as the repressor Mad (*103*), the transcription factor YY1 (*104*), the insulator factor CTCF (*105*), the hormone receptor-dependent co-repressors NCoR and SMRT (*106*), and others (*107*). Class I HDACs have also been shown to interact with factors involved in other functions, such as DNA or histone methyltransferases [Dnmt3a or Su(Var)3-9] (*108, 109*), developmental regulatory factors (for example, the Polycomb group proteins) (*110*), and cell growth regulators such as p33ING1 (*111*). Class I HDACs themselves are involved in multiple functions, such as transcriptional repression, development (*112*), cell differentiation (*113*), X-inactivation in mammalian females, epigenetic silencing (*114*), cell cycle progression (*115*), and position effect variegation in *Drosophila* (*116*).

In contrast, class II HDACs are expressed in a tissue-specific manner, and some of them (for example 4, 5, and 7) are mobilized from the cytoplasm to the nucleus upon phosphorylation. Class II members participate in skeletal muscle differentiation by interaction with members of the muscle-specific MEF2 transcription factor family (*117*), and they also interact with the NCoR, SMRT, and the *Bcl6* gene-specific co-repressor BCoR (*118, 119*).

Class III

This group is defined by homology with the catalytic domain of the *S. cerevisiae* transcriptional silencing factor Sir2p (silent mating type information regulator (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;137) (*120, 121*). Sir2p was isolated genetically as a member of the SIR (*122*) family of proteins. The four Sir proteins (Sir1p, 2p, 3p, and 4p) are involved in the formation of highly compacted silenced chromatin that is hypoacetylated in histones H3 and H4 (*123, 124*). Sir proteins have also been shown to be involved in double-stranded DNA repair (*125, 126*), recombination (*127*), cell cycle progression (*128*), DNA replication (*129*), chromosome stability (*130*), and meiotic checkpoint control (*131*).

Three different genomic regions are known to be silenced by the Sir complex in yeast: the mating type loci, telomeres, and rDNA. However, not all of the Sir proteins are required for the silencing of all three of these regions. Interestingly, only Sir2p is required in all of them (*122, 132*) (see Animation 2: http://sageke.sciencemag.org/cgi/content/full/sageke;2003/14/re4/DC1).

Sir2p was reported to have a NAD^+-dependent HDAC activity, which is conserved among the Sir2p homologs found thus far from yeast to humans (*133, 134*) and is required for the silencing function of Sir2p (see Animation 3: http://sageke.sciencemag.org/cgi/content/full/sageke;2003/14/re4/DC1) (*133*). In addition, Sir2p was also shown to contain an NAD^+-dependent ADP-ribosylase activity (*120*). Sir2 deacetylates several acetylated residues in H3 and H4, although other possible nonhistone substrates cannot be excluded.

The Sir2p family members are found in many eukaryotes from yeast to humans, and even bacteria contains proteins with some homology to Sir2p, such as CobB, which is related to the bacterial enzyme CobT, a protein that is involved in cobalamin biosynthesis (*135*).

Although we do not have much functional information about Sir2p homologs, some clues as to function do exist: First, of the four Sir2p homologs in yeast [that is, the HSTs (Hst1p-4p), or

"homologs of Sir2"]. Hst1p and Hst2p can partially replace some of the silencing defects in *sir2* mutants (*130, 136*). Hst1p appears to be recruited by transcriptional silencing factors, such as Sum1p and the repressor Set3p-containing complex, and participates in transcriptional silencing of meiosis-specific sporulation genes (*137, 138*). Hst2p, on the other hand, localizes in the cytoplasm and appears to be linked somehow to the metabolic requirement of these proteins for NAD^+ (*136*). Thus far, there is no evidence that Hst2p is ever transported to the nucleus.

Second, *Drosophila* dSir2 has been recently found to be involved in heterochromatin formation and euchromatic gene repression by interaction with transcription factors (*139*). Third, in humans, there are at least 7 Sirts or Sirtuins (Sirt1-7), and, in the case of Sirt1, some nonhistone substrates, such as the tumor suppressor p53 and the transcriptional regulatory protein TAF_I68, have been described (*140, 141*). Sirt1 deacetylates p53 at lysine 382 and represses its transcriptional activation activity, as well as p53-induced senescence and apoptosis (*142*). Finally, the fact that the Sir2p family is conserved among eukaryotes suggests a much more complex set of functions, such as chromatin regulation (A.V. and D.R., unpublished results).

Sir2p and Metabolism: The Aging Connection

Chromatin has been linked to the biology of aging in several ways (Fig. 6). Aging produces phenotypic chromatin defects such as telomere shortening (*143*) and general heterochromatinization, which correlates with a decrease in the repair of chromatin aberrations (*144*). The first clear link between chromatin-modifying activities and the aging process was described in yeast and pointed to Sir2p (see Kaeberlein Perspective: http://sageke.sciencemag.org/cgi/content/full/sageke;2001/1/pe1). Yeast *SIR2* mutants show a decreased life-span (as measured by the number of mitotic divisions), whereas an additional copy of the gene has the opposite effect (*145*). In *Caenorhabditis elegans*, a similar effect has been observed with Sir-2.1 (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;136), the worm Sir2p homolog. Increased amounts of Sir-2.1 promote longevity in adult worms and formation of the long-lived dauer intermediate. The insulin-like signaling pathway in worms has been shown to be involved in the determination of life-span (see Johnson Review: http://sageke.sciencemag.org/cgi/content/full/sageke;2002/34/re4 and "All For One" http://sageke.sciencemag.org/cgi/content/full/sageke;2002/49/nf15). Mutations that decrease signaling through this pathway enhance longevity (*146*). Recently, Sir-2.1 was shown to repress insulin signaling in worms (*146*). However, Sir-2.1 has not been implicated in silencing or chromatin structure in worms. Finally, deletion of *RPD3* in yeast both activates *SIR2* (*147*) and extends life-span (*148*), and the same appears to be true in flies (*149*).

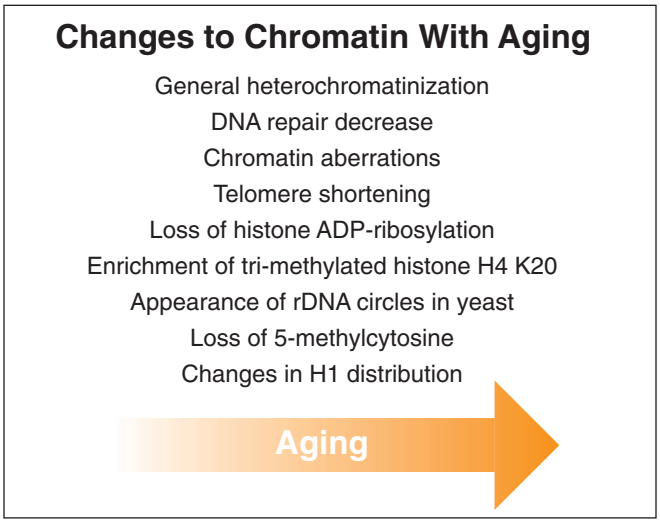

Fig. 6. Aging and chromatin. The main changes that chromatin undergoes upon aging are indicated, and most of them are discussed further in the text.

There are three features that make Sir2p especially interesting with respect to aging (see Animation 4: http://sageke.sciencemag.org/cgi/content/full/sageke;2003/14/re4/DC1). First, the NAD^+ requirement provides a connection between aging and the metabolic state of the cell, and this connection may be related to the effects of calorie restriction (CR) on aging. In general, CR correlates with an increased the life-span in many organisms, such as yeast (*150*), nematodes (*151*), rodents (*152*), and possibly primates (*153*) [see also (*154*), Masoro Review: http://sageke.sciencemag.org/cgi/content/abstract/sageke;2003/8/re2 and "Monkey in the Middle": http://sageke.sciencemag.org/cgi/content/abstract/sageke;2002/31/nw108]. In yeast, CR promotes oxidative metabolism (respiration), and NAD^+ is a product of this reaction. SIR2 is activated by increased respiration through a mechanism involving NAD^+ (see "High-Octane Endurance—Yeast in the Metabolic Fast Lane Live Longer": http://sageke.sciencemag.org/cgi/content/full/sageke;2002/28/nf9). The activation of SIR2 by CR has been demonstrated by the increase in SIR2-dependent silencing at telomeres and rDNA observed calorie-restricted yeast (*155*) or HAP4-overexpressing cells (*156*).

Second, yeast aging correlates with the appearance of extrachromosomal rDNA circles (ERCs), which are circular DNAs excised from the yeast rDNA (see Kaeberlein Perspective: http://sageke.sciencemag.org/cgi/content/full/sageke;2001/1/pe1). The Sir2p concentration is inversely related to the amount of ERCs, which could reach more than 1000 copies per cell. This is because the silencing of rDNA by Sir2p inhibits the recombination event that produces the ERCs (*157*). In accordance with these findings, in aged yeast cells, there is a general redistribution of Sir proteins from the mating type loci and telomeres to the nucleolus, which houses the rDNA (*158*). This observation may reflect the fact that Sir proteins are involved not only in generating epigenetic information, but also in conserving the integrity of the genome. ERCs have not been found in higher eukaryotes, and it is possible that they never will be (*121*). But the general model of chromatin and aging is still valid. That is, cellular aging occurs when the chromatin machinery is unable to maintain intact chromatin structure and the regulatory status quo.

Third, a connection exists between human Sirt1 and apoptosis induced by p53 (see "Death and Aging, Together at Last": http://sageke.sciencemag.org/cgi/content/full/sageke;2001/4/nf2). In mammalian cells, Sirt1 promotes cell survival by inhibiting p53-directed apoptosis thorough deacetylation of p53 (*140-142*) (see Animation 4: http://sageke.sciencemag.org/cgi/content/full/sageke;2003/14/re4/DC1). However, because these experiments were performed in cells that overexpress Sirt1, additional studies are required to establish the precise physiological conditions under which this effect takes place. In addition, be-

cause p53 was also found to interact with the Class I HDAC complex NuRD (*159*), a clear delimitation of the effects of the various HDACs on p53 function should be addressed.

Given these provocative findings and the obvious expectations of a clear-cut connection between cell aging and the mammalian Sir2 homologs, this area of research will be one of the most exciting over the next few years. Many aspects of this connection are completely unknown and will have to be addressed, such as the biochemical characterization of these proteins, the functions of Sir proteins in chromatin modification, and the relation between the Sir proteins and other cellular functions.

Methylation

Histone methylation was reported many years ago (*160*), but its function has remained obscure for almost 40 years. HMTs (histone methyltransferases) catalyze the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to either arginine or lysine residues in histones. Arginines can be mono- or dimethylated in a symmetric or asymmetric way by the HMT family of proteins arginine methyltransferases (PRMTs), whereas lysines can be mono-, di-, or trimethylated by the SET-containing HMT family (*161*). Only H3 and H4 have been found to be methylated at lysine residues in their tail domain (H3: K4, K9, K27, and K36; H4: K20). In addition, H3 can be methylated in the globular domain at lysine 79. A general overview of the HMTs is shown in Fig. 7.

One aspect of methylation that is radically different from acetylation is the apparent lack of reversibility of the reaction (*162*). Although indirect evidence points toward their existence, and theoretical studies have predicted them (*163*), no histone demethylases have yet been identified.

HMT group	HMT	Specificity	Function
Arginine	PRMT1	H4-R3 (non-histone proteins)	Transcriptional activation (signal transduction, etc)
	PRMT2	Unknown	Coactivator of estrogen receptor
	PRMT3	Unknown	Cytoplasmic (mitosis?)
	PRMT4/ CARM1	H3-R2, -R17, -R26. (Also at the C-terminal)	Transcriptional coactivator
	PRMT5	H2A, H4 (non-histone proteins)	Transcriptional repressor and spliceosome formation
Lysine SET domain	Suv39H1, Suv39H2	H3-K9	Heterochromatin formation, silencing
	G9a	H3-K9, H3-K27	Early embryogenesis role, transcriptional repression
	ASH1	H3-K4, -K9, H4-K20	Establishment of epigenetic, active transcription patterns
	Set1	H3-K4	Silencing
	Set2	H3-K36	Silencing, transcription
	Set7	H4-K20	Development, silent chromatin. Involved in aging
	Set9	H3-K4	Transcriptional activation
	ESC-E(z)	H3-K27	Polycomb-mediated silencing
	SETDB1	H3-K9	Silencing-mediated by the corepressor KAP-1
Dot	Dot1	H3-K79	Silencing by precluding Sir binding to bulk chromatin

Fig. 7. Histone methyltransferases.

Although histone methylation has been largely associated with transcriptional repression and epigenetic regulation, it is also involved in transcriptional activation. The function of methylation is dependent not only on the amino acid residues affected, but also on the interplay established between methylation and other histone modifications, such as acetylation, ubiquitination, and phosphorylation (*162*, *164*).

The apparent functional contradiction related to methylation function is well represented by methylation of K9 and K4 in H3. K9 methylation is associated with heterochromatin formation and transcriptional repression, whereas the K4 methylation is involved in the activation of gene transcription. Both modifications are mutually excluded from the regions where the other is present.

Five PRMTs [protein arginine methyltransferases] (PRMT 1-5) are found in humans, but only PRMT1 and 4 (CARM-1) have been shown to have in vivo histone-methylation activity (*161*). PRMT1 methylates histone H4 on R3, is widely conserved from yeast to humans, and participates in transcriptional activation (*165*). PRMT4 methylates histone H3 on R17, R3, and R26, as well as other nonhistone proteins such as the HAT CBP/p300, and seems to have an important role in transcription activation by methylation of nuclear hormone coactivators. The methylation of CBP/p300 produces an intriguing functional switch that inhibits the recruitment of CBP/p300 to CREB-dependent genes, freeing up CBP/p300 for recruitment to nuclear hormone receptor-dependent genes (*166*).

SET-containing HMTs are defined by the presence of a SET [Su(var)3-9, Enhanzer of zeste E(z), Trithorax, TRX] domain. Although the HMT activity resides in the SET domain, not all SET-containing proteins show HMT activity (*167*). In yeast, seven different SET-containing proteins have been identified (*161*), and they are generally involved in transcriptional regulation (*161, 168, 169*).

The first HMT described was the human homolog of *Drosophila* Su(var)3-9, a suppressor of position effect variegation [PEV] (*170*). Su(var)3-9 specifically methylates H3 at the K9 position, is important for heterochromatin formation, and is required for the proper localization of the heterochromatin protein HP1. HP1 is a constitutive element of heterochromatin in higher eukaryotes and is recruited by the binding of its chromodomain to methylated K9 in H3 (*171, 172*). Su(var)3-9 is also linked to other functions, such as transcription regulation, genomic imprinting, and epigenetic silencing (*173*).

Among the other SET-containing HMTs described in mammalian cells thus far are the following:

(i) G9a, which modifies K9 and, to a minor extent, K27 in vitro, participates in transcription regulation and development, and is the most important K9 activity in euchromatic regions of the genome (*174*).

(ii) hSet9, which methylates K4 in H3. Methylation of this residue is important for transcriptional activation and prevents the binding of NuRD to the H3 tail, which also leads to the hyperacetylation of chromatin and, consequently, transcription activation (*169, 175*).

(iii) PR-SET7, a histone H4 HMT that methylates K20 and appears to be present in transcriptionally silenced domains within euchromatin. In regions that contain methylated H4-K20, H4-K16 acetylation (which is involved in transcriptional hyperactivation of the X chromosome in *Drosophila* males) is excluded, suggesting that K20 is an important epigenetic marker for transcriptional silencing (*176*). Very recently, an increase

in H4-K20 trimethylation has been correlated with aging (*177*), which might partially explain the relationship between genome compaction and aging.

One additional histone H3 HMT, Dot1p, has been described in yeast (*178, 179*) (see "Tangled Up In Spools": http://sageke.sciencemag.org/cgi/content/abstract/sageke;2002/24/nw84). Dot1p does not contain a SET domain and can trimethylate K79 in the globular domain of H3. Dot1p was originally shown to be involved in Sir-mediated silencing with an important role in Sir recruitment, because mutations in *DOT1* produce a loss of localization of Sir2p and Sir3p at nucleoli and telomeres, respectively (*180*). Active homologs of Dot1p have been found in higher eukaryotes, but their functions are still unknown. One interesting possibility would be that Dot1p defines specific chromosomic domains involved in epigenetic regulation. In addition, there is an obvious possible connection between the Sir2 homologs and Dot1p that should be addressed in the future.

Because methylated K79 is present in 90% of the yeast H3 pool, an interesting theory has been proposed whereby the Sir complex could be formed only in regions that contain hypomethylated H3-K79. This hypothesis attributes to Dot1p an active role in transcriptional silencing. However, although it is still possible that Dot1p actively regulates silencing, we cannot exclude the possibility of an indirect effect, such as the existence of a hypothetical demethylase activity that would allow recruitment of the Sir silencing complex.

Phosphorylation

All histones, including H1, have been shown to be substrates for phosphorylation in vivo. Phosphorylation of H1 and H3 has been associated with chromosome condensation and segregation (*181*), and this function is highly conserved among species from *Tetrahymena* to mammals (*182, 183*). However, H3 phosphorylation is not absolutely required for chromosome condensation in all species, because in yeast, unlike in *Tetrahymena* (*184*), mutations in phosphorylated serine residues in H3 tails do not cause any mitotic defects. This difference is likely due to the absence of H3-K9 methylation in yeast, a hypothesis that stems from several observations. First, there are substantial levels of K9 methylation in mammalian heterochromatin. Second, in mice, both in vivo and in vitro, methylation of H3-K9 inhibits phosphorylation of H3-S10, and vice versa (*170*). The concentrations of phosphorylated H3-S10 in vivo are abnormally increased in mice carrying mutations in *Suv39h*, which in turn induce chromatin aberrations. Thus, phosphorylation of H3 tails might not be required for chromosome condensation in yeast because the antagonistic effects of H3-K9 methylation are not present in this organism.

An additional role in transcriptional activation has been described for H3 phosphorylation in mammals (*184, 185*). In eukaryotes, H3 phosphorylation occurs in two serine residues, S10 and S28. S10 phosphorylation appears early in the G_2 phase of the cell cycle, first in the pericentromeric heterochromatin of all chromosomes and then spreading, by metaphase, to the rest of the chromosome (*186*). Several kinases are involved in H3 phosphorylation. Among them is Aurora-B, a member of the Aurora/AIK kinase family, which participates in mitotic regulation. Aurora-B localizes with phosphorylated H3, and the down-regulation of Aurora-B by RNAi in *Drosophila* (*187*) and *C. elegans* (*188*) produces a significant loss of H3 phosphorylation. Genetic studies in yeast and *C. elegans* have pointed out that protein phosphatase 1 is involved in the reverse reaction (*189*).

As we mentioned earlier, phosphorylation of S10 affects K14 acetylation. In yeast, K14 acetylation is promoted by phosphorylation of S10. In accordance with this observation, a subset of the genes activated by the transcription factor Gcn5p (but not all Gcn5p-dependent genes) requires the phosphorylation of S10 for normal amounts of transcription in vivo (*190*).

Ubiquitination

Ubiquitination is the process by which a lysine residue is modified covalently by conjugation to the 76-amino acid (~8.5 kD) protein ubiquitin (*191*). Thus far, H2A, H2B, H3, and H1 were all found to be ubiquitinated in vivo, although H2A and H2B are modified far more frequently than are H3 and H1 (*192*). Proteins can be mono- or polyubiquitinated, and each of these forms is related to different functions and substrates.

Polyubiquitination marks proteins for degradation by the 26*S* proteasome complex (*193*), linking this process with multiple cellular functions, such as cell cycle regulation, DNA repair, cell differentiation, stress response, and others (*194*). In contrast, monoubiquitination has been linked to transcriptional activation, because actively transcribed chromatin was found to contain monoubiquitinated histones in *Tetrahymena* (*195*). Supporting this idea, an H1-monoubiquitination activity has been reported for the transcriptional coactivator $TAF_{II}250$ (*196*). Conversely, monoubiquitination also has been linked to transcriptional silencing and DNA repair in yeast (*197*). Several pieces of evidence suggest a parallel between acetylation and monoubiquitination: First, H2A and H2B ubiquitination can destabilize the interaction of H2A-H2B dimers with the H3-H4 tetramers in the nucleosome (*198*), linking the modification to a general structural effect on chromatin. Second, a new character in the histone language associated with monoubiquitination was reported recently. Yeast Rad6p, a factor involved in DNA repair that can monoubiquitinate H2B at K123, is necessary for the methylation of H3-K4 by Set1p (*199*). A mutation in H3-K4 does not prevent the ubiquitination, but mutations in H3-K123 or Rad6p suppress K4 methylation. Thus, H3-K4 methylation requires the monoubiquitination of H3-K123. However, we still do not know much about monoubiquitination in higher eukaryotes, and the search for histone-specific monoubiquitinase activities in mammals must still be undertaken.

ADP-Ribosylation

ADP-ribosylation implies the transfer of ADP-ribose molecules to either glutamic acids in a polyglutamate stretch or single arginine residues (*200*). All histones types appear to be ADP-ribosylated, although H1 and H2B are the most highly modified (*201*).

The ADP-ribosylation of histones is interesting for many reasons: First, ADP-ribosylation of histones clearly declines with age (*202*). Second, the source of ADP-ribose is NAD^+, thus relating this reaction to that of class III HDACs. In addition, the requirement for NAD^+ as a cofactor in the ADP-ribosylation reaction links the metabolic state of the cell with a wide variety of functions. Third, several observations implicate this modification in the histone code hypothesis; that is, ADP-ribosylation is preferentially found in hyperacetylated histones, and this is especially clear in the case of H4 (*203*).

Both mono- and poly-ADP-ribosylation have been described. Mono-ADP-ribosylation has been implicated in cell signaling and function of the immune system (*204*). However,

members of the yeast Sir2p family have been reported to mono-ADP-ribosylate histones, and this activity seems to be involved in the Sir2p transcriptional silencing function. These findings suggest that mono-ADP-ribosylation of chromatin has a repressive effect on transcription (*205*). In any case, the Sir2p ribosylation activity is weak and nonspecific, because it modifies all four histones, Sir2p itself, and bovine serum albumin in vitro (*120*), and it cannot be excluded as a consequence of the general NAD^+-dependent mechanism of deacetylation.

Poly-ADP-ribosylation is clearly related to histone modification. It is a radical modification that has effects on DNA repair, gene expression, DNA replication, apoptosis, genomic stability, and other processes (*206*). Poly-ADP ribose is added as a branched polymeric structure that can contain over 100 ADP-ribose molecules (*200*). The modification appears to be tightly regulated, and, as degradation of these structures by poly-ADP-ribose glycohydrolase (PARG) is rapid, poly-ADP-ribosylation could have an important role in adaptation of the cell to environmental changes (*207*).

Among the various factors that poly-ADP-ribosylate proteins, only PARP1 can clearly modify histones (*208*). PARP1 is an abundant nuclear protein involved in base excision repair (BER), cell death, and transcription regulation. Upon DNA damage, PARP1 recognizes DNA nicks and recruits the DNA repair machinery (*209*).

Several lines of research suggest a contradictory effect of poly-ADP-ribosylation in the regulation of gene expression. Early studies reported that in the absence of DNA damage, PARP1 is localized mainly in actively expressed chromatin, where poly-ADP-ribosylated nucleosomes are found (*210*). Consistent with a role in gene activation, PARP1 appears to modify transcription factors, such as Oct-1, NF-κB, p53, AP-2, and others (*206*), thus stimulating their transcriptional activity. In contrast to this evidence, other evidence suggests that PARP1 is able to repress transcriptional elongation by RNA polymerase II (*211*).

The effect of this extensively branched structure on chromatin is believed to be mainly structural. The modification generates an open chromatin conformation favorable, in general, to transcription (*212*). DNA is also affected, as ADP-ribosylation can produce bending of the DNA due to the presence of a massive amount of negatively charged ADP-ribose (*213*).

Taken together, the evidence points to an important function for this modification in chromatin regulation, in addition to its role in DNA repair. The identification of novel, specific histone ADP-ribosylases and the characterization of their functions with respect to their interplay with other histone modifications will be important landmarks for future studies. In addition, the clear link between ADP-ribosylation and aging, including the connection to Sir2p, should be addressed.

Biotinylation

The last of the modifications is also the one we know least about. Biotin or vitamin B was discovered almost 70 years ago as a coenzyme for CO_2-fixing carboxylases (*214*). Studies have shown that, in the nuclei of cells, histones are biotinylated, and although we know almost nothing about this modification, some observations point to a role in cell cycle progression, transcription regulation, and chromatin stability. Biotinidase appears to be the enzyme responsible for biotinylation of histones, because it catalyzes the transfer of biotin to lysine residues in all four histones and also carries out the reverse reaction (*215*). Given the current lack of data on the biotinylation reaction, we expect to see, in the near future, further studies to characterize the functions of biotinylation, as well as to identify other enzymes involved in the modulation of chromatin.

Summary

Understanding and stalling the aging process has been an aspiration of human beings for eons. Only now are we beginning to decipher the complex nature of this process and the variety of elements that contribute to its manifestation. A key discovery in the study of aging is its direct connection to chromatin metabolism and all of the regulatory machinery associated with proper integrity and expression of the genome, which in turn regulates cellular viability.

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