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## Multivalent engagement of chromatin modifications by linked binding modules

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### Abstract

Various chemical modifications on histones and regions of associated DNA play crucial roles in genome management by binding specific factors that, in turn, serve to alter the structural properties of chromatin. These so-called effector proteins have typically been studied with the biochemist's paring knife — the capacity to recognize specific chromatin modifications has been mapped to an increasing number of domains that frequently appear in the nuclear subset of the proteome, often present in large, multisubunit complexes that bristle with modification-dependent binding potential. We propose that multivalent interactions on a single histone tail and beyond may have a significant, if not dominant, role in chromatin transactions.

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The eukaryotic genome is assembled into chromatin, and the nucleosome serves as its fundamental organizational unit. This unit is composed of an octamer of core histone proteins (two copies of H2A, H2B, H3 and H4) encircled by ~146 bp of DNA. Histones project unstructured N-terminal 'tails' from the  $\alpha$ -helical protein core of the nucleosome through the superhelical turns of DNA that enshroud the radial surface of the histone octamer. The majority of known histone post-translational modifications (PTMs) localize to residues in the unstructured tails, particularly at the N termini, yet a burgeoning number of modifications also appear to reside within the helical secondary structure and loops of folded histones<sup>1</sup>. Further diversifying the nucleosome core particle is a set of histone isoforms known as histone variants, some of which appear to have essential roles in various stages of DNA management<sup>2–5</sup>.

The lowest order of chromatin structure is the nucleosomal unit iterated in extended conformation to resemble 'beads on a string', which can be consolidated into higher-order structures through the intermediacy of attendant proteins, RNA and cations. Physiological chromatin structure is a vital arbiter of DNA function, in that structural variation appears to regulate the accessibility of underlying DNA, ranging from condensed heterochromatin to more 'open' euchromatin<sup>6,7</sup>. Rather than mere static packaging of the genome, the spatial arrangement of chromatin serves as an information carrier that may help to preserve cell

identity through mitotic division<sup>8</sup>, and yet the local structure is sufficiently dynamic that it may be rapidly modulated by signalling cascades in response to external stimuli<sup>9–11</sup>.

Phenotypic traits that are not encoded in the Watson–Crick base pairing of the genome are collectively referred to as epigenetic phenomena and appear to manifest physically as the faithful heritability of chromatin states by daughter cells<sup>12–14</sup>. The precise mechanisms of epigenetic phenomena are poorly understood, but causal connections between chemical modifications to DNA<sup>15</sup> and histone proteins<sup>16–18</sup>, as well as other non-histone proteins<sup>19–21</sup> and resultant local chromatin structure, are increasingly recognized as crucial intermediaries. It is becoming clear that chromatin modifications rarely occur in isolation — rather, new patterns of covalent modifications are emerging rapidly from mass spectrometry and large-scale ‘epigenomics’ efforts<sup>1,3,22–27</sup>.

How are these patterns interpreted? There are several non-mutually exclusive mechanisms by which DNA methylation and histone PTMs may produce crucial structural transformations in the chromatin polymer. First, direct nucleosome-intrinsic effects alter the physical properties of individual nucleosomes, particularly by neutralization or addition of charge, which enhances nucleosome mobility by abrogating individual histone–DNA contacts within a given nucleosome<sup>28–30</sup>. Second, direct nucleosome-extrinsic effects of chromatin modifications toggle the ability of nucleosomes to form higher-order structures through the modulation of internucleosomal contacts<sup>31</sup>. Third, effector-mediated consequences are changes that are elicited in the chromatin fibre due to specific binding events that couple a particular histone modification with a cognate non-histone binding partner, termed an effector<sup>32</sup>. Effector proteins may alter the properties of chromatin by crosslinking two or more nucleosomes<sup>33–35</sup>, by enhancing the occupancy of the RNA polymerase complex and related factors<sup>36</sup> or by recruiting active structure remodelling or further chemical modification activities<sup>32</sup>.

Since the landmark discovery that bromodomains may specifically engage acetylated Lys residues, particularly in histone sequence contexts<sup>37</sup>, the chromatin field has focused heavily on elucidating additional effector-mediated pathways. Subsequent studies have revealed a wealth of protein folds that bind various histone PTMs<sup>17,38,39</sup>. The focus of most of these efforts has been pairing a single PTM with a cognate effector module, followed by examination of the functional significance of this association event. Cellular processes as varied as transcription<sup>40</sup>, replication<sup>23</sup>, stem-cell pluripotency<sup>26</sup>, gene silencing<sup>40,41</sup>, X-chromosome inactivation<sup>24</sup>, DNA repair<sup>42</sup>, apoptosis<sup>43,44</sup>, certain cancers<sup>45,46</sup>, epigenetic inheritance<sup>47</sup> and gene-expression programmes during development<sup>48,49</sup> all appear to require effector–chromatin-modification interactions in their course or causation.

Early formulations of the histone/epigenetic code hypothesis suggested that distinct functional consequences result from histone PTMs and that a given outcome is encoded in the precise nature and pattern of marks<sup>18,32,50</sup>. In subsequent years, the discovery of many novel histone-binding modules has fuelled much attention and considerable interest in this general area. For example, certain PHD finger domains have recently been identified as ‘readers’ of the trimethylated Lys4 mark on histone H3 (H3K4me3)<sup>51–54</sup>. However, despite these advances, some have concluded that there is an inherent flaw in the logic underlying

the histone code hypothesis<sup>55–57</sup>. one criticism is that multiple binding partners have been reported for a single histone PTM<sup>55</sup>. Furthermore, some bromodomains are somewhat promiscuous with regard to the sequence context of sub-strate acetylation marks<sup>37,58</sup>, which plausibly accounts for the functional redundancy observed for some acetylation marks in yeast<sup>56</sup>. This apparent redundancy confounds a simple one-mark-to-one-module type of decoding. others have questioned the functional significance of molecular interactions that are individually weak in nature<sup>14</sup>. These valid concerns prompt us to re-evaluate the original hypothesis by asking: is there a theoretical framework that accommodates these issues without abandoning the core of the original histone code hypothesis? It is our contention that the phenomenon of multivalency<sup>59,60</sup> — that is, the cooperative engagement of several linked substrates by a species with more than one discrete interaction surface — may be widespread in chromatin transactions and that this biophysical effect may allay some of the above concerns.

Here, we seek to apply to chromatin biology the general concepts of multivalency, the biophysics of which is amply developed in other areas of biology and chemistry<sup>57,59–62</sup>. Indeed, there are hints of multi-valency littered throughout the vast body of chromatin literature: certain nuclear proteins are replete with predicted effector domains, and many coincident predicted binding modules occur within the subunits of chromatin-associated macromolecular complexes. However, direct proof in the form of a systematically characterized example is still lacking. This review examines the literature that establishes the coexistence of certain chromatin modifications, briefly describes the thermodynamics of multivalency and marshals the available evidence to support the proposed hypothesis, with the hope of stimulating further efforts to study the extent and magnitude of these putative effects. In trying to describe these concepts, we seek to advance a theoretical framework for what may be thought of as a ‘nucleosome code’; we note that variations of this theme have been proposed by others<sup>32,50,63</sup>. We hope that the biophysics of multivalency will provide valuable new mechanistic insights into the signalling potential of chromatin modification patterns.

## Coexistence of chromatin modifications

Proximal modifications that constitute a putative ‘code’ need not be restricted to a single histone tail as originally anticipated<sup>18</sup>, but may span two or more tails on a given nucleosome, adjacent nucleosomes, or nucleosomes that are discontinuous in primary DNA sequence but spatially colocalized in a chromatin territory<sup>64</sup>. Patterns of native chromatin modifications are studied principally through mass spectrometry<sup>1,27</sup> and chromatin immunoprecipitation (ChIP) methods<sup>65</sup>. New genome-scale extensions of ChIP, such as ChIP-chip<sup>66,67</sup> and ChIP-seq<sup>68</sup>, have enabled a transition from investigating single marks at discrete loci to correlating complex epigenetic signatures, expression patterns and other physical features at the megabase level and beyond with spatial resolution well below a single nucleosome footprint<sup>22,23,25,68,69</sup>.

The first well-studied histone modification class, Lys acetylation, is correlated with transcriptional activation through direct alteration of the physical properties of chromatin<sup>70</sup> and recruitment of bromodomain-containing effectors<sup>37</sup>. Histone hyperacetylation is

correlated positively with actively transcribed chromatin<sup>71,72</sup>, and biophysical studies reveal a more translationally mobile octamer<sup>73–75</sup>, as well as inhibition of 30-nm packing contacts in the case of acetylated Lys16 of histone H4 (H4K16ac)<sup>31</sup>. Alternatively, there are numerous bromodomain effector-mediated pathways that also generally serve to enhance transcriptional activation<sup>37,76–80</sup>.

Conversely, Lys methylation and Arg methylation events are more varied in their correlated functions. Some are generally associated with gene activation, and others are involved with repression and silencing — all known examples of which are effector mediated (for recent reviews that catalogue individual marks and associated functions, see REFS 1,17). However, the greater the resolution and percentage of the genome that is covered by epigenomics, the more these canonical associations between a given mark and gene expression become nuanced and idiosyncratic<sup>16,25,81</sup>. For example, H3K4me3 is traditionally associated with the promoter-proximate regions of genes undergoing active transcription, yet in embryonic stem cells, there is a significant colocalization of this mark with a canonical heterochromatic PTM, H3K27me3, at developmentally regulated loci<sup>22,26</sup>. These so-called bivalent domains are largely transcriptionally repressed. Moreover, the H3K4me3 modification is found at nearly all gene start sites in embryonic stem cells, only a subset of which are producing full-length transcripts, so it may now be thought of as principally associated with transcription initiation<sup>25</sup>. A collection of spatially juxtaposed chromatin marks is presented in TABLE 1.

Although epigenomics-level efforts strongly imply mark coexistence, most do not definitively prove it because the ChIP method yields relative ensemble measurements. That is, heterogeneity in the cell population and cell cycle may cause a mixture of different chromatin states at a locus that might be averaged together inappropriately. (When collections of cells with uncertain degrees of homogeneity are used, the resulting net signal may be susceptible to large fluctuations in a small subpopulation.) Further complicating analyses in diploid genomes is the mixture of allele-specific marks, an issue that may be partially resolved by allele-specific ChIP-seq<sup>26</sup>. Furthermore, because of variable immunoprecipitation efficiencies and inconsistent antibody quality, these measurements must be normalized in such a way that makes them relative measurements without clear relation to an actual number of modifications over a given chromatin span. Without measuring an absolute number of modifications per nucleosome, it is unclear whether two marks actually simultaneously exist, or on average are both biased towards a particular region but occur at a frequency well below one mark per locus. BOX 1 lists methods for studying this coincidence of marks and presents ways of addressing these problems that have been recently reported in the literature.

Many questions remain about the patterns of modification discovered thus far. To what extent does the appearance of proximate ‘opposing’ marks coexist for extended periods of time? Do some apparent bivalent domains represent transient intermediate chromatin states that are in transit from one limiting state to another in a developmental context (simplistically, off-to-on)? The finding that the jumonji domain-containing protein-2A (JMJD2A) enzyme demethylates H3K9me3 and is recruited by binding the H3K4me3 mark suggests that such incipient states exist<sup>82,83</sup>. Mediators and intermediaries in this process have also been implicated in the particulars of establishing, maintaining or modulating

different chromatin structural states — the collections of marks themselves may serve as signalling platforms, integrate inputs (embodied by individual marks), and recruit chromatin-modulating or -stabilizing factors. Indeed, ample circumstantial evidence links distinct patterns of chromatin modifications to different chromatin structural states. How specific congregations of marks might physically collaborate to achieve these outcomes is the subject of the remainder of this article.

## A physical basis for the histone code?

Mainly ignored in the feverish elucidation of chromatin modifications and their correlation to genome function are the details by which the physical processes stimulated by the modifications actually transpire. Often, the problem of quantitative attribution of the individual binding contributions may not be experimentally tractable. But frequently, when one specific interaction is discovered that has some role in locus targeting, it is deemed sufficient even though the studied interaction is too weak and/or too non-specific to explain the observed phenomenon adequately. We propose that the multivalent nature of many complexes that manage chromatin may explain the magnitude of the isolated binding affinities and their individually modest substrate specificities, as well as serving a crucial role in chromatin fibre dynamics. As we have recently outlined the theoretical argument for widespread multivalency in chromatin biology in some detail<sup>38</sup>, only a brief treatment will be undertaken here.

In well-characterized systems outside of chromatin biology, multivalent binding results in dramatic affinity enhancements and additional specificity while remaining much more dynamic and susceptible to competition than a correspondingly tight monovalent interaction<sup>59,60</sup>. The physical basis of the multivalency phenomenon is attributable to both thermodynamic and kinetic effects. In thermodynamic terms, binding enhancement is caused by roughly additive enthalpies of each binding event (assuming no strain is introduced) with the concomitant reduction of entropy loss<sup>59</sup> (FIG. 1). This reduction of the entropy term relative to a similar number of uncoupled interactions is the result of a sacrifice of rotational and translational degrees of freedom in binding that occurs as a group (FIG. 1b). Under optimal conditions of little conformational flexibility and no introduced strain, much of the entropy loss on binding is 'prepaid' by the initial complex assembly and is not levied against the enthalpy during association<sup>61</sup>. Thus, the free energies for multiple individual effector–substrate binding events can achieve levels of significant synergy that are dependent on the degree of valence and the spatial organization of effectors relative to the arrangement of substrates<sup>61</sup>.

The kinetic explanation arises from the significant enhancement of individual rates of association, not by altering individual rate constants, but by enhancing the local concentration of a given substrate and cognate effector module. Local concentration increases are caused by effective tethering of an effector complex to a locus through other similar interactions. This sort of cooperativity in binding has been widely observed in DNA annealing, gene activation by sequence-specific DNA-binding factors<sup>57</sup> and other chemical and biological systems<sup>59</sup>. When multiple association events between chromatin-modification complexes and chemically modified chromatin substrates are coupled and

elaborated by the degree of valence, substantial additive or super-additive binding may ensue. Several unusual aspects of this phenomenon are worth noting because they are likely to be crucial for the functional mechanisms of chromatin transactions.

First, not all contacts need to be modification dependent; for example, contacts with DNA<sup>84</sup> (sequence dependent and independent) or contacts with a region of the histone octamer surface that is not subject to modification may have significant roles in dictating ensemble association<sup>85</sup>. If the overall binding energetics are distributed among several non-specific and specific interactions, the non-specific interactions may not be sufficient to drive binding on their own, so that the crucial free-energy balance may be dictated by one or more specific contacts.

Second, the higher the degree of valence, the more profound the effect; for example, moving from bivalent to trivalent oligosaccharide ligand binding to mammalian hepatic GalNAc lectin produces apparent affinity enhancement over monovalent galactose of  $10^3$ -fold and  $10^6$ -fold, respectively<sup>62</sup>. Similar energetic consequences might be expected in multivalent interactions with the chromatin fibre<sup>38</sup>.

Third, composite specificity, which is greater than the intrinsic specificity of any of the discrete binding interactions, may also arise from multivalent interactions. In principle, the apposition of effector modules in an optimal alignment creates surfaces that are complementary to the spatial positions of each of the substrate elements when they are displayed in chromatin, so that the distances between discrete interactions become additional specificity determinants. Thereby, greater net specificity may be imparted by constraining productive binding to the specific spatial relationships among each substrate element in its native context (or the ability to attain an ideal conformation). This phenomenon is how type IIP restriction enzymes build up huge sequence specificity from a symmetric bivalent interaction with two half-sites<sup>86</sup>.

Finally, a system of recognition and localization that results from a collection of individually weak interactions may also be more readily competed because the individual dissociation rates are high relative to comparably strong monovalent binding interactions<sup>59,87</sup>. During development, the plasticity of chromatin structure and attendant modifications requires dynamics: with multivalent interactions, high affinity can be coupled with a susceptibility to competition, as well as a greater potential specificity through the synchronous recognition of several marks. However, to our knowledge, no PTM-dependent multivalent effect has been reported that systematically and quantitatively measures the isolated affinities of each effector–substrate interaction as well as the net affinity. Thus, the magnitude of the effect, even in the form of a proof-of-principle experiment, still remains obscure for chromatin transactions. BOX 2 details empirical approaches to rectify this dearth of empirical evidence.

## Linked effectors engaging chromatin

Examination of the predicted domains in most enzymatic complexes that operate on chromatin (many of which contain several putative modification-dependent binding modules) shows that the combinatorial engagement of chromatin through numerous

modalities may indeed be the rule rather than the exception (FIG. 2a,c). Structural details of the molecular discrimination of individual effector domains have been extensively reviewed<sup>38,39,80,88</sup> and will not be addressed here. Distinguishing among several possible modes of synchronous chromatin mark binding is crucial to understanding the actual mechanisms of multivalent chromatin mark ‘readers’. In particular, simultaneous tail binding may occur at the level of a single tail or at two tails of the same nucleosome, adjacent nucleosomes or discontinuous nucleosomes (FIG. 3).

Additional recognition of covalent modifications on other surfaces of the folded core of the histone octamer and DNA contacts (including sequence-specific recognition, 5-methylcytosine recognition and general DNA affinity) can presumably occur in an intra- or internucleosomal manner. These multivalent mode distinctions are important because they are thought to yield different functional consequences — ranging from gripping a particular histone tail in a multidentate fashion while ratcheting on DNA to enhance nucleosomal mobility, to crosslinking two nucleosomes in disparate chromosomal positions so that a heterochromatic region is bridged and compacted<sup>34,35</sup>. examples could include effector modules with conserved PTM recognition elements that coexist within a given polypeptide or complex, but at present there is little direct evidence to support these claims (FIG. 2a,b). Nevertheless, the sheer number of such associations tempts speculation that this phenomenon is general. The remainder of this section will examine numerous cases in which multivalency may be inferred, organized according to the putative mode of the interaction.

### Cooperative binding of PTMs on a single histone tail

Elegant work by the Tjian laboratory first discerned modest cooperativity in binding multiply acetylated histone peptides in tandem bromodomain proteins, human **TAF1** (TATA-binding protein-associated factor-1) and a partial yeast homologue, bromodomain-containing factor-1 (**Bdf1**)<sup>76,78</sup>. In particular, the TAF1 tandem bromodomains display moderate affinity increases for multi-acetylated histone H4 peptides that may arise from the synergistic binding energetics that are typical of multivalent systems. The initial work centred on the structural characterization of the tandem bromodomain module of TAF1, the largest subunit of the TFIID basal transcription factor (FIG. 3a; *cis*-histone, intranucleosomal). The two bromodomains appear to be rigidly confined in a relative orientation that positions each acetyl-Lys-binding pocket on roughly the same face, such that a single peptide with two acetylated Lys residues positioned ~25 Å apart could bridge the two pockets<sup>76</sup>. Typical single bromodomain–acetylated-peptide interactions are so weak (with a dissociation constant ( $K_d$ ) of 100–350  $\mu\text{M}$ <sup>37,79</sup>) that their *in vitro* significance comes into question, whereas the  $K_d$  for double bromodomains that bind doubly acetylated counterparts is considerably lower (~1–20  $\mu\text{M}$ ).

There is some apparent specificity for acetylated H3 and H4 tails, and in the case of TAF1, a potential preference for a certain spacing of acetylated Lys residues; however, the extent to which this preference is truly restricted to two particular acetyl-Lys residues remains unclear<sup>58,76,78</sup>. It is possible that the magnitude of cooperativity of this bivalent interaction may be even greater with other intranucleosomal substrates. Regardless of the magnitude of

the effect, cooperative binding was deemed vital to the function of these two tandem bromodomain proteins. Bromodomain pocket mutations and mutation of potential H4 acetylated Lys residues also negatively influence the efficacy of TFIID in promoting transcription, particularly when the TATA box is obscured by nucleosome wrapping<sup>89,90</sup>. Although the precise role, specificity and molecular detail for each of the two bromodomains involved in composite binding have not been systematically detailed, the current data attest to significant cooperativity in the binding.

**CMT3**, a homodimeric *Arabidopsis thaliana* DNA methyltransferase that is implicated in gene silencing, bears two copies of a chromodomain that together bind well only to the combination of H3K9me3 and H3K27me3 in the same polypeptide<sup>91</sup>. Although neither individual mark is bound tightly enough to be measured, nor is sufficient to recruit CMT3 *in vivo*, it seems that the combination of the two trimethyl-Lys residues within a single polypeptide satisfies this requirement<sup>91</sup>.

A third example of the *cis*-histone phenomenon involves negative cooperativity of adjacent modifications within the histone H3 tail in the binding and expulsion of the chromodomain of heterochromatin protein-1 (HP1)<sup>92,93</sup>. The affinity of HP1 for H3K9me2 or H3K9me3 is in the low micromolar range; however, when this modification is paired with a subsequent phosphorylation of H3S10 mediated by Aurora B kinase, the affinity of the chromodomain is diminished by several orders of magnitude (an effect referred to as phosphomethyl switching)<sup>92</sup>. Repulsive forces in biology can easily achieve much greater magnitude than the corresponding attractive forces. HP1 binding to H3K9me3 is thought to be crucial for the formation of pericentric heterochromatin, and its subsequent ejection triggered by phosphorylation appears to be necessary for mitotic progression<sup>92,93</sup>.

### Harnessing the nucleosome unit

There are several potential modification-dependent binding modules that paradoxically associate with complexes that have opposing enzymatic activities. one such example is provided by the **Eaf3** protein that resides in both the histone deacetylase Rpd3S and the histone acetyltransferase NuA4 complexes. The chromodomain of Eaf3 alone displays a modest preference for several methyl forms of H3K36 *in vitro* (H3K36me3 binds approximately two-fold tighter than the unmodified peptide in a pull-down assay, although no quantitative binding experiments have yet been reported)<sup>94,95</sup>. In the Rpd3S complex, this chromodomain appears to engage enzymatically modified nucleosomes as a function of methylation at H3K36, whereas no such nucleosome binding was observed with the NuA4 complex under the same conditions<sup>84</sup>.

Closer examination reveals that several additional contacts are made by the Rpd3S complex with the nucleosome that appear to tip the balance of binding energetics through multivalency. The complex has overall affinity for linker DNA and histone hyperacetylation that is not yet traceable to a particular subunit, and a PHD finger in the Rco1 subunit has an as-yet-undefined role in enhancing binding<sup>84</sup>. The PHD finger of Rco1 and the chromodomain of Eaf3 have significant roles in targeting the complex to genes or maintaining complex stability *in vivo*, because individual domain deletions phenocopy Set2 (the K36 methyltransferase) null strains in their inability to suppress aberrant transcripts as a



downstream consequence of localized histone deacetylase activity<sup>84</sup>. Despite the incomplete characterization of each module's preferred portion of the modified nucleosomal substrate, this work is a pioneering demonstration that the product of a series of individually weak contacts, when assembled into a multivalent complex, can be important for *in vivo* chromatin function (FIG. 3a; oligovalent complex).

What is the role of Eaf3 in NuA4 binding to nucleosomes? A plausible interpretation is that a suboptimal nucleosomal substrate methylated only at H3K36 may explain the lack of affinity under the conditions described. The absence of the H3K4me3 mark in this nucleosomal species precludes a potential second modification-dependent interaction with Yng2, a known H3K4me2/3 effector and NuA4 subunit<sup>52</sup>, from productive participation. Furthermore, it would be interesting to examine whether the PHD finger within the Pho23 subunit of the related Rpd3L complex (which also has demonstrated intrinsic H3K4me3-binding capacity<sup>52,96</sup>) has an analogous role to Eaf3, supporting binding for H3K4me3-modified nucleosomes rather than H3K36me3-modified ones. This study demonstrates that it is not always an individual constituent of a complex that dictates the overall binding capacity; rather, it is the ensemble of all different binding elements in varying complexes that may give rise to differing specificities. Other examples of common subunits shared by oligovalent protein complexes with opposing activities have been documented in the literature<sup>41,97,98</sup>, which is suggestive of a more general theme operating in chromatin biology.

Several models for multivalent engagement of a single nucleosome have been suggested, yet still none has been tested for actual cooperativity. A striking example is provided by the Lys-specific demethylase-1 (LSD1)-corepressor to the Re1 silencing transcription factor (CoREST) complex. Structural analysis reveals that LSD1 projects an extended helical stalk from the amine oxidase-histone H3 recognition centre that binds to CoREST, thereby positioning a second SANT domain at the apex of the stalk >100 Å away<sup>99</sup> (FIG. 4a). Given the intrinsic DNA-binding capacity of this SANT domain<sup>99</sup> and the H3 Lys demethylase function of LSD1, a model has been presented that is consistent with the noted requirement for DNA binding by CoREST in the processing of nucleosomal substrates<sup>100</sup>. This situation appears to reflect the composite recognition of a particular histone modification and sequence non-specific binding of DNA, which together collaborate to yield favourable nucleosomal-binding free energy.

Furthermore, BHC80, another subunit of some LSD1 complexes, has recently been demonstrated to bind specifically to non-methylated H3 tails, providing yet another point of contact with substrate nucleosomes<sup>101</sup>. Interestingly, a similar unmodified H3 tail interaction was uncovered within a Cys-rich (PHD finger-like) domain of the DNA methyltransferase subunit DNMT3L, providing a link between a lack of methylation at H3K4 and *de novo* methylation of DNA in mammals<sup>102</sup>. In addition, pull-down assays with an epitope-tagged version of DNMT3L indicate an association with all four core histones, foreshadowing possible nucleosomal recognition. The energetics of these and other similar modules have been individually investigated, but how these discrete interactions assemble into presumptive multivalent associations with nucleosomal units remains to be elucidated (see below and BOX 2).

A final example of intranucleosomal engagement is provided by the bromodomain PHD finger transcription factor (BPTF) component of the nucleosome remodelling factor (NURF) complex. In particular, the structurally characterized PHD–bromodomain module can bind H3K4me3 (REFS 51,54) and shows acetylation-dependent H4 recognition (H.L., A.J.R., C.D.A and D.J.P., unpublished observations). The structure suggests that these two effector domains are rigidly spanned by an  $\alpha$ -helix that enforces a particular spatial disposition of the binding pockets (FIG. 4b). Interestingly, modelling of this bivalent module by superimposing the two histone tail substrates provides a plausible model for bivalent nucleosomal engagement that predicts additional DNA contacts due to close proximity of the helical linker to the DNA major groove (FIG. 4b) (H.L., A.J.R., C.D.A and D.J.P., unpublished observations). In this case, we predict a clear bivalent enhancement that is well beyond the sum of the individual affinities. Further experiments are currently underway to measure precisely the exact energetic contributions and spatial requirements of the interaction through perturbations to both the substrate and the dual-effector module.

It should be noted that there are likely to be two copies of BPTF within the NURF complex<sup>103</sup>; in addition, there are further putative effector modules within the BPTF polypeptide and in other subunits of the complex (FIG. 2b). Moreover, two other complex members, the WD40-repeat proteins RBBP4 and RBBP7 (formerly known as RbAp48 and RbAp46, respectively), can bind histones through the first helix of the histone fold<sup>98</sup> (FIG. 4b). An intriguing facet of this minimally bivalent interaction is the pre-organized rigidity of the  $\alpha$ -helical linker between effector modules. This fixed linkage appears to reduce the conformational entropy of the module before binding so that less entropy is lost in the act. Nevertheless, by inspection of polypeptides with several putative binding modules, one can envision that flexible linkers are also likely to be used and, in such cases, the accompanying induced fit during complex formation on the nucleosome must be energetically offset by greater enthalpies of binding.

### Beyond the nucleosome

The formation and stabilization of higher-order chromatin organization requires several architectural chromatin proteins. The archetypal example is HP1, which has been implicated in numerous protein–protein associations, including the H3K9me-mediated histone binding described above. Several studies have suggested that HP1 crosslinks H3K9me-rich chromatin domains by virtue of dimerization — a known property of its chromoshadow domain<sup>34,35</sup>. Whether this phenomenon occurs at adjacent nucleosomes along the same chromatin fibre or is able to span large sections of unconnected chromosomal arrays remains an open question (FIG. 3b; compare the panels labelled ‘adjacent bridging’ and ‘discontinuous bridging’), as does the actual importance and energetic consequences of this bridging.

A related chromodomain complex is similarly associated with chromatin crosslinking; the Polycomb repressive complex-1 (PRC1) appears to bind approximately three nucleosomes simultaneously and, consequently, compacts arrays of nucleosomes in a histone-tail-independent manner (as shown by electron microscopy)<sup>33</sup>. Intriguingly, the Polycomb protein (Pc) of PRC1 is known to specifically engage K27me3 in the H3 tail<sup>104</sup>, although

this interaction is dispensable in this *in vitro* experiment. The precise molecular contacts that underlie this essential heterochromatin complex that engages chromatin remain enigmatic.

Beyond the well-studied chromatin regulators described above, other examples of potential multivalency may easily be proposed. For example, 8 out of the 15 annotated bromodomains in yeast reside in RSC, a chromatin remodelling complex of the SWI/SNF family. Many of these bromodomains bear the canonical residues that are implicated in acetyl-Lys binding. One subunit, *Rsc4*, has a tandem bromodomain architecture that is similar to human TAF1 (FIG. 3a) and exhibits a preference for binding H3K14ac<sup>77</sup>. Remarkably, mutants in both bromodomains are required to expose synthetic lethality with mutations of H3K14 (REF. 77). This paradox is resolved by a recent study that revealed a surprising autoinhibitory role for the first bromodomain — it binds an N-terminal peptidic extension of *Rsc4*, but only when this substrate has been acetylated at a conserved Lys by the histone acetyltransferase GCN5 (REF. 105). It is suggested that autoinhibition of *Rsc4* binding to H3K14ac is involved in destabilizing the binding of the RSC complex to attenuate its residence time at promoters. In the RSC complex, it is likely that there is synergistic binding with other affinity determinants outside the *Rsc4* subunit; in particular, it is tantalizing to speculate that other bromodomains in the complex also have roles in a multivalent system.

By no means does the above discussion provide a comprehensive examination of prospective multivalent chromatin associations. Undoubtedly, as more modules in multivalent chromatin complexes succumb to detailed structural and biophysical analyses, a clearer picture of the importance of cooperativity and putative combinatorial readout of nucleosomal units will emerge. We look forward to experimental tests of these and other theories of multivalent behaviour that potentially govern the association of key regulators (proteins or complexes) in chromatin biology, some of which are outlined in BOX 2.

## Conclusion

Large-scale experiments that have examined chromatin modification patterns through evolutionary space suggest a strong conservation of chromatin signatures and nucleosome positioning biases<sup>69,106</sup>. Chromatin modifications serve as an effective and economic way to regulate gene expression through the alteration of modification patterns, which, in turn, modulate the higher-order structure of the fibre and/or govern recruitment of effector modules. local patterns of epigenetic marks appear to dictate transcriptional activity at particular regulons and chromatin territories as well as sequential firing of developmental loci<sup>69,107</sup>. More long-lived and self-reinforcing patterns are involved in silencing phenomena — DNA methylation is heritable, and certain histone marks may be effectively heritable<sup>108</sup>. Histone modifications do not operate alone; rather, they appear to act in concert with other putative epigenetic information carriers and a host of DNA-sequence-specific factors to constitute another level of regulation that governs DNA-templated processes. More complex histone or nucleosomal code combinations may provide an important distinction between lower and higher eukaryotes. Indeed, there is evidence for more sophisticated patterns of histone modification and histone variant utilization in metazoans<sup>3,27</sup>.

We propose that combinations of effectors may greatly increase the reading specificity, affinity and dynamics of chromatin-associated macromolecular assemblages. The promiscuity of certain marks with regard to binding partners (there are now >10 effector proteins that are known to bind H3K4me3) suggests that, in many cases, one histone modification is not sufficient to recruit a given complex. Instead, we suspect that it is the collusion of chromatin marks and possible sequence-specific DNA-binding factors that all contribute significantly to recruitment and stabilization. The ‘one mark, many partners’ promiscuity paradox<sup>55,56</sup> may be resolved by the addition of binding constraints and free energies that are imparted by other domains within each distinct complex. The converse of this idea, that a single effector protein may serve as a subunit in several different complexes, even those with opposing enzymatic activity, has recently been examined<sup>84</sup> and we expect more examples of this phenomenon to be discovered in the near future. There is some evolutionary economy to both phenomena in that they represent combinatorial diversification from a smaller set of interaction elements that maximizes the signalling potential of a given mark or module capable of binding it.

When the overall affinity and specificity are divided into a modular set of interactions, the individual importance of any one interaction can either be vital or dispensable for binding. It has been suggested that the ‘code’ of covalent chromatin modifications is highly redundant<sup>109</sup>. Some of this apparent redundancy may actually be instances of multivalent recognition where not every constituent interaction is necessary to transduce an apparent effect, while no single interaction is individually sufficient. Perhaps there are even instances of multivalent chromatin association when only subsets of competent binding modules are engaged at a given time — this would allow readout of different combinations of marks in different temporal and tissue contexts. An example of broadened specificity as a consequence of distinct combinations of interaction modules is provided by the CCCTC-binding factor (CTCF): when CTCF binds to sequence-heterogeneous insulator elements, different combinations of its 11 zinc-finger binding modules are used depending on the precise sequence and DNA methylation pattern of the substrate<sup>110</sup>.

In some cases, one mark may be sufficient to elicit a specific biologic output<sup>42</sup>; in other cases, it appears that multiple marks are required<sup>9</sup>. How do we begin to think about these multiple marks with the goal of providing crucial experimental tests of the multivalency-based theories provided here? We must venture beyond the reductionist conventional wisdom that largely restricts studies to a single mark considered in isolation. Rather, we should begin to explore how the coexistence of marks within a given tail, within a given nucleosome and within a given chromatin domain may serve to dictate functional outcomes in combination. Here, we hope to emphasize that multivalent assemblages of effector domains are emerging as crucial interpreters of chromatin modification patterns. Their study will undoubtedly be central to enhancing our understanding of genome management mechanisms. Although direct evidence may still be lacking, assembled genetic and biochemical data suggests the widespread use of multivalent recognition that governs many interactions with chromatin. We suspect that the nucleosomal unit, with all its rich binding surfaces, promises to have many more secrets left to share with far-reaching implications for human biology and disease.

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## Glossary

<b>PTM</b>	A post-translational modification or chemical alteration of an amino acid residue (or residues) that occurs subsequent to its
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	translation, introducing a new functional group into a given protein.
<b>Histone variant</b>	An isoform of a particular histone type that is encoded as a distinct gene and denoted by an additional designation (for example, the bar body-deficient histone H2A gene product is called H2A.bbd). Some variants have specialized functions: notably, H2A.X is involved in DNA repair and H2A.Z is involved in gene regulation.
<b>Heterochromatin</b>	A highly condensed and transcriptionally less active form of chromatin that occurs at defined sites, such as centromeres, silencer DNA elements or telomeres.
<b>Euchromatin</b>	Chromatin that appears to be less compact than condensed mitotic chromosomes. Active genes are contained within euchromatin.
<b>DNA methylation</b>	A covalent modification of DNA at the 5-position of the cytosine nucleobase that is coupled to transcriptional repression. CpG sequence elements in gene regulatory regions are often modified in this manner to attenuate gene transcription.
<b>Direct nucleosome-intrinsic</b>	A histone modification-dependent process that alters the physical properties of the nucleosome by modulation of interactions of the core histone octamer with DNA.
<b>Direct nucleosome-extrinsic</b>	A histone modification-dependent process that alters the physical properties of chromatin by modulation of interactions between nucleosomes.
<b>Effector mediated</b>	A chromatin modification-dependent process that changes or stabilizes the chromatin architecture through modification-dependent recruitment of accessory factors.
<b>Effector</b>	A protein domain that binds a particular histone modification to transduce a downstream function. effector proteins themselves or other subunits of complexes in which they reside can be histone-modifying enzymes or chromatin-remodelling factors, or they can be involved in stabilization of heterochromatin or have some other gene regulatory role.
<b>Bromodomain</b>	A domain with sequence conservation that is found in several transcriptional regulatory proteins involved in gene activation, and that has acetyl-Lys-binding activity.
<b>PHD finger domain</b>	The plant homeodomain (PHD) zinc finger is found in many nuclear proteins that are thought to be involved in chromatin transactions.
<b>ChIP-chip</b>	Chromatin immunoprecipitation followed by amplification and microarray hybridization.

<b>ChIP-seq</b>	Chromatin immunoprecipitation followed by massively multiplexed sequencing.
<b>Lys acetylation</b>	Acetylation of the $\zeta$ -amine of the Lys side chain, a PTM that is catalysed by histone acetyltransferases.
<b>Lys methylation</b>	A PTM at the $\zeta$ -amine of Lys that adds one, two or three methyl groups.
<b>Arg methylation</b>	A PTM of the $\eta$ -nitrogens of Arg to introduce one or two methyl groups.
<b>Cooperativity</b>	Binding enhancement caused by one or more discrete binding interactions that further assist such interactions, although not necessarily as a consequence of a conformational change.
<b>Chromodomain</b>	The canonical methyl-Lys-binding protein fold that was initially characterized in HP1 and Polycomb proteins. A member of the royal superfamily of folds.
<b>Negative cooperativity</b>	Antagonism in binding that leads to sub-additive or repulsive binding energies.
<b>WD40 repeat</b>	A protein motif that is composed of a 40-amino-acid repeat that forms a four-stranded antiparallel $\beta$ -propeller sheet. WD40 proteins that contain 5–7 WD40 repeats may form $\beta$ -propeller structures that participate in many cellular functions, including G-protein-mediated signal transduction, transcriptional regulation, rRNA processing and regulation of vesicle metabolism.

**Box 1****Experimental approaches to characterize epigenetic signatures****Epigenomics**

High-resolution chromatin immunoprecipitation (ChIP) technologies (ChIP-chip and ChIP-seq) imply the coexistence of various marks but do not simultaneously and directly establish the residence of two or more marks within the same nucleosome. Furthermore, because the information gleaned from epigenomics experiments crucially depends on the quality of the antibody used in ChIP experiments, more robust reagents that are specific for given modifications are needed. There are many identified modifications for which there are no ChIP-grade antibodies; consequently, the patterns of these modifications have not been spatially correlated with genomic elements. Thus, in addition to continued progress in epigenomics approaches, parallel efforts are necessary to implicate directly the concurrence of patterns at the nucleosome level.

**Mass spectrometry**

Although several proximate post-translational modifications within particular histones have been identified from tryptic digest mass spectrometry (MS)/MS<sup>1</sup>, new forms of high-resolution MS/MS (such as electron transfer and electron capture dissociation) permit the examination of modifications present on residues that are distal in primary sequence on fragment sizes approaching whole histones<sup>27,111,112</sup>. More whole-histone MS/MS from staged or select cell (that is, stem cell) populations should dramatically enhance the current set of coincident post-translational modifications within a given histone<sup>113</sup>.

**Acid-urea gels**

Various forms of acid-urea gels are sensitive enough to provide single-charge difference resolution of modified histones. When coupled with western blot analysis, this method can effectively discern multiple modifications on a given histone<sup>9,10,114</sup>.

**Sequential IP and rechIP**

Sequential immunoprecipitation (IP) methodologies permit the investigation of chromatin modifications that occur within a single nucleosome<sup>22,24,115,116</sup>. This technique, which is often performed on mononucleosomes, entails a second IP after elution from an initial IP with an orthogonal antibody, such that two marks can be simultaneously investigated<sup>116</sup>. More widespread application of this technically demanding technique, in concert with PCR using locus-specific probes, should reveal more precise patterns of nucleosome modification mark coexistence<sup>22,115,116</sup>. The systematic combination of all of the above approaches with synchronized and staged cell populations should reveal more histone modification patterns with higher resolution.

**Box 2****Methods for examining nucleosome engagement****'Designer chromatin' from semi-synthetic histones**

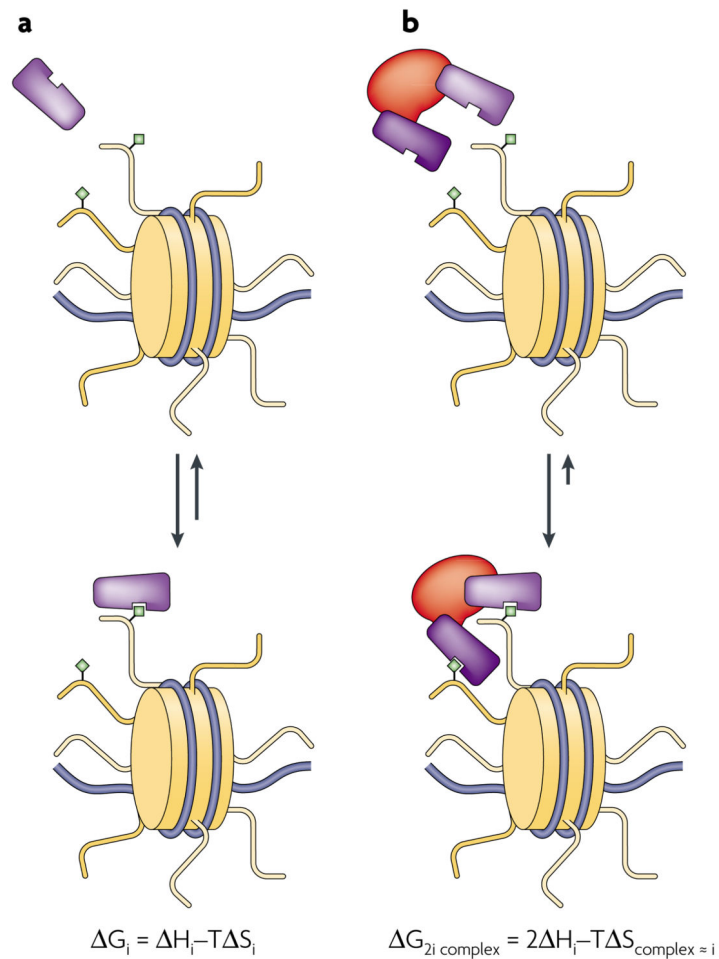
Any of the known histone modifications may be introduced semisynthetically by native chemical ligation of a recombinant histone fragment and the appropriate peptide<sup>117–119</sup>. A simpler route to methyl-Lys analogues is through direct alkylation of the appropriate Cys mutant in a given histone background<sup>120</sup>. Histones with chemically incorporated modifications may then be assembled with recombinant histones and DNA to form mononucleosomes or arrays of 'designer chromatin'<sup>31</sup> that bear multiple specific modifications at once. The advantage of chemical methods over the enzymatic production of modified histones lies primarily in the 90–100% homogeneity of the resultant species. By contrast, enzymatic installation of particular modifications often suffers from poor control of the extent and degree (in the case of methylation) of modification, with additional concerns regarding the specificity of a given modification enzyme under the forcing *in vitro* conditions. In the latter case, resultant heterogeneity often makes qualitative analysis difficult and quantitative analysis impossible. Quantitative mono- and dinucleosome binding assays, nucleosomal array compaction assays<sup>121</sup>, electron microscopy<sup>33</sup> and even transcription assays with multiply modified designer chromatin can be profitably deployed to understand the physical basis of a given mark in the presence of multivalent effector modules.

**Structures of multi-effector unit fragments**

Although it is often more convenient to solve individual domain structures in complex with their respective substrates, this level of structural analysis reveals little about the crucial spatial relationships between modification-dependent binding pockets of multi-module protein fragments or multi-effector subunit complexes. Moving structural analysis away from this reductionist routine will invariably enhance our understanding of chromatin engagement mechanisms by these complexes.

***In vivo* approaches**

Once the particulars of a multivalent interaction have been well characterized *in vitro*, appropriate mutations (which are preferable to domain deletions) may be introduced, either in a null genetic background or in a tagged form (especially in dominant-negative cases). Spatial requirements for effector-module juxtaposition can be probed by insertion or deletion of rigid protein spacer modules, for example, creating a longer helix between two effectors. Because putative multivalent interactions are implicated in recruitment or stabilization of chromatin-modifying complexes to particular genomic regions, chromatin immunoprecipitation experiments with mutants should recapitulate the *in vitro* quantitative binding experiments. Combinatorial assemblies of effectors can also be probed by swapping analogous portions or domains with different binding specificities<sup>84</sup>, or by serial duplication of a putative effector domain, and examining locus residence times at a related inducible gene target following synchronized activation.



### Figure 1. Thermodynamics of multivalent binding

**a** | Monovalent association of a hypothetical effector module (purple) to a chromatin substrate (yellow tail with green diamond) is simplistically compared to a bivalent association of the same effector in a complex, representing the lowest order of multivalent interactions. The change in free energy  $\Delta G_i$  for the monovalent system undergoing binding is indicated by the change in enthalpy  $\Delta H_i$  minus the change in entropy  $\Delta S_i$ , scaled by the temperature ( $T$ ). **b** | By tethering the two effector modules, the entropy term may be, to a first approximation, similar for each of the binding equilibria in panels **a** and **b** ( $T \Delta S_{\text{complex}} \approx T \Delta S_i$ ). For our purposes, this example assumes that hydrogen-bonding electrostatic interactions dominate and desolvation is negligible, so that  $\Delta S$  for the system will be negative. Thus, the entropic penalty to binding is lessened approximately twofold by pre-organizing the effector domains in a complex ( $T \Delta S_{\text{complex}} \approx T \Delta S_i$ ), while the enthalpy of the bivalent domain interaction is effectively double that of the monovalent case ( $\sim 2 \Delta H_i$ , if enthalpic penalties due to the strain induced by bivalent binding are negligible). In this manner, the reduced net entropy loss for the binding process can be a significant determinant of free energy, especially in low-binding enthalpy regimes. Losses of entropy on the substrate side would be expected to be minimal due to the low intrinsic rotational and

translational freedom of chromatin; however, conformational entropy losses here are assumed to be negligible for simplicity.

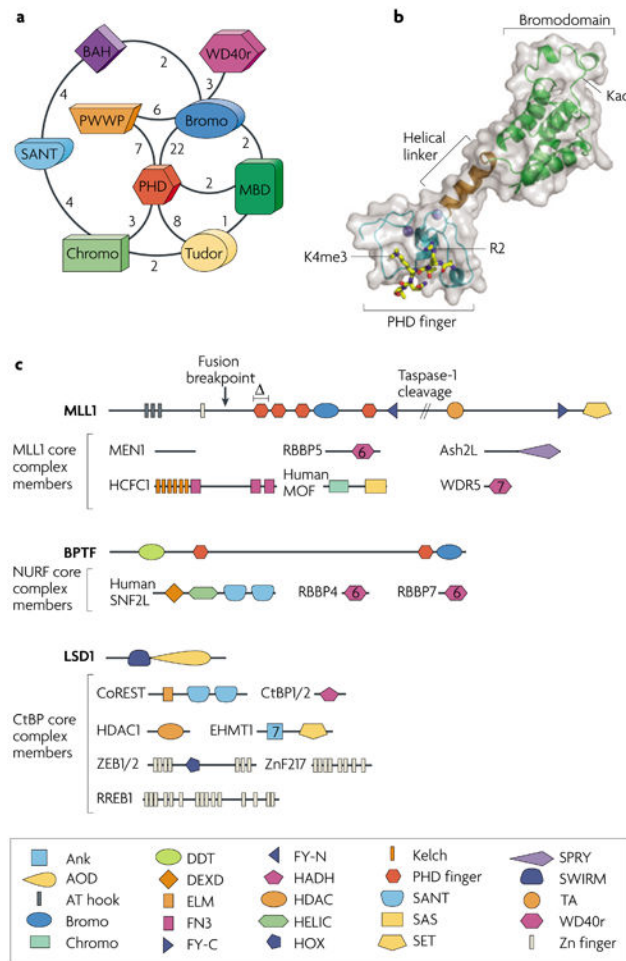
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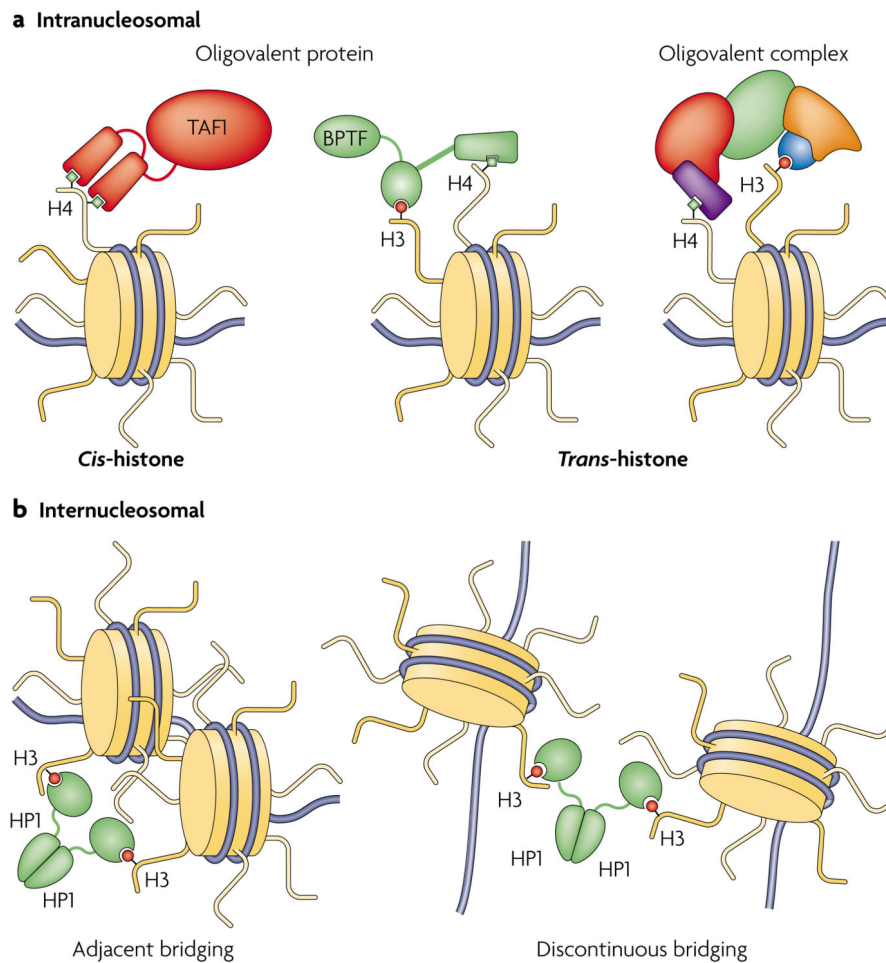




**Figure 2. Polypeptides with many putative effector modules and representative complexes**

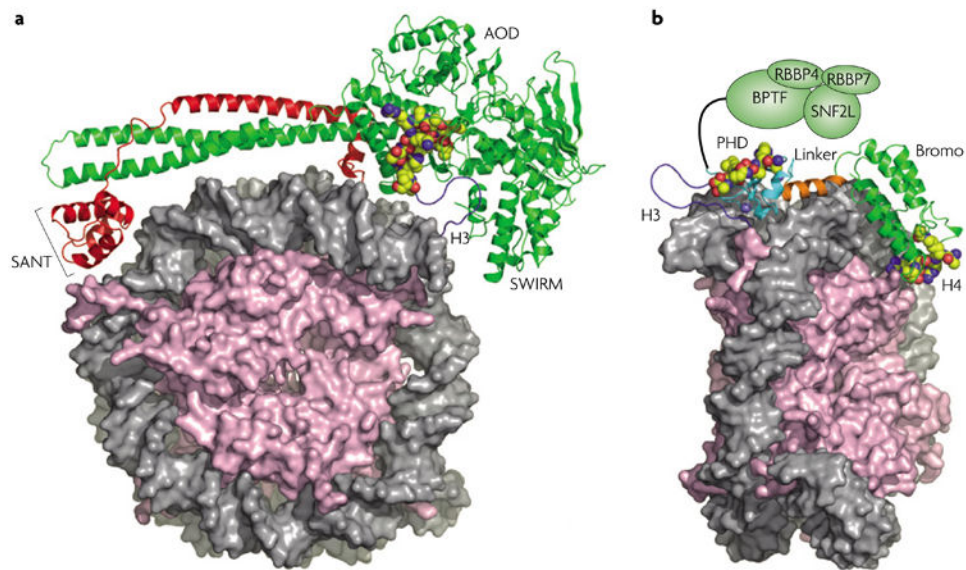
**a** | The coexistence of possible effector module domains within single polypeptides is depicted schematically, with the number of instances of linkage for any two domains within the human proteome listed near the line connecting them. The [SMART](#) database was used as the source of these linkages, and redundant entries were removed. **b** | A structurally characterized example of two linked effector domains is provided by the structure of a BPTF module that comprises a PHD finger, a helical linker and a bromodomain, with a trimethylated Lys4 of histone H3 (H3K4me3) peptide bound to the PHD finger<sup>85</sup>. The acetyl-Lys (Kac)-binding pocket on the bromodomain is shown, as well as residues R2 and K4me3 of the H3 peptide. **c** | Chromatin metabolism complexes, exemplified by the MLL1 (ref. 122), NURF<sup>103,123</sup> and CtBP<sup>11</sup> core complexes, have multiple putative effector domains. The predicted domain structure of subunits of the complex members are shown as a linear arrangement from N to C terminus. Chromatin-associated domains, most of which are modification sensitive, are coloured as in panel **a**, and are shown with additional predicted domains given in the key. The portion of the MLL1 protein that is cleaved by taspase-1 to yield two functional fragments (MLL1-N and MLL1-C) is shown. A frequent breakpoint at which fusion partners are appended and a domain deletion ( ) that causes certain leukaemias are also depicted on the MLL1 domain structure. Ash2L, Set1–Ash2

histone methyltransferase complex subunit; BAH, bromo-adjacent homology domain; BPTF, bromodomain PHD finger transcription factor; Bromo, bromodomain; Chromo, chromodomain; CoREST, corepressor to the RE1 silencing transcription factor; CtBP, C-terminal binding protein; EHMT1, euchromatic histone-Lys *N*-methyltransferase-1; HCFC1, host cell factor C1; HDAC1, histone deacetylase-1; LSD1, Lys-specific demethylase-1; MBD, methyl-CpG binding domain; MEN1, multiple endocrine neoplasia-1; MLL1, mixed lineage leukaemia; MOF, males absent on first histone acetyltransferase; NURF, nucleosome remodelling factor; PHD, plant homeodomain; PWWP, PWWP motif protein of the Royal superfamily; RBBP, retinoblastoma binding protein; RREB1, Ras responsive element binding protein-1; SNF2L, sucrose non-fermenting-2-like ATPase; WD40r, WD40 repeat; WDR5, WD repeat domain-5; ZEB1/2, zinc finger E-box binding homeobox-1/2; ZnF217, zinc finger protein-217.



### Figure 3. Modes of multivalent chromatin engagement

To distinguish among several potential mechanisms of multivalent association, we propose the following nomenclature. **a** | Intranucleosomal association can be subdivided into two distinct classes<sup>124</sup>: *cis*-histone, when more than one discrete binding contact is made to a single histone, in particular the same tail; and *trans*-histone, whereby contacts are made to different histone protomers or attendant DNA within the same nucleosome. **b** | By contrast, internucleosomal binding modes crosslink two nucleosomes that are either adjacent or discontinuous in DNA sequence. Most of these crucial interactions are envisioned as modification dependent; however, DNA interactions and modification-independent contacts may have a vital energetic role. BPTF, bromodomain PHD finger transcription factor; HP1, heterochromatin protein-1; TAF1, TATA-binding protein-associated factor-1.



**Figure 4. Models of nucleosomal engagement**

**a** | A model of the LSD1–CoREST complex docked with the nucleosome<sup>99</sup> in a bipartite manner with the second SANT domain binding to DNA and the AOD and SWIRM domains binding an H3 tail bearing Lys methylation. LSD1 is shown in green and CoREST in red. Adapted from REF. 99. **b** | A model of the PHD–bromodomain module of BPTF (green) binding a nucleosome with modifications in the tails of two different histones, H3K4me3 and H4ac (the precise site is unknown but H4K16ac is modelled here). The remaining portion of the NURF complex is shown as green ovals, including the N terminus of BPTF, SNF2L and RBBP4 and -7. In both panels, core histones are pink with tail cartoons in dark blue. The modification recognition epitope is shown in space-filling spheres (carbon, yellow; nitrogen, blue; oxygen, red) with DNA in grey. AOD, amine oxidase domain; BPTF, bromodomain PHD finger transcription factor; Bromo, bromodomain; CoREST, corepressor to the RE1 silencing transcription factor; LSD1, Lys-specific demethylase-1; NURF, nucleosome remodelling factor; PHD, plant homeodomain; RBBP, retinoblastoma binding protein; SNF2L, sucrose non-fermenting-2-like ATPase.

**Table 1**  
**Notable patterns of coexisting histone marks**

Histone marks	Locus/chromatin state	Method
H3K4me2/3 + H4K16ac	Transcriptionally active homeotic genes	ChIP <sup>122</sup>
H3K4me2/3 + H3K9/14/18/23ac	Transcriptionally active chromatin	MS <sup>125</sup>
H3S10ph + H3K9/14ac	Mitogen-stimulated transcription	Ab <sup>9,10</sup> and MS <sup>126</sup>
H3R17me1/2a + H3K18ac	Oestrogen-stimulated transcription	Ab <sup>127</sup>
H4K5ac + H4K12ac	Pre-deposition	Ab and MS <sup>128</sup>
H3K4me3 + H3K27me3	'Bivalent domains' at key developmental genes	ChIP-chip, reChIP <sup>22</sup>
H3K9me3 + H3K27me3 + 5-MeC	Silent loci	ChIP, bisulphite sequencing <sup>91,129</sup> , MS
H3K27me3 + H2AK119ub1	Silent homeotic genes	ChIP, ChIP-chip <sup>41,69</sup>
H3K9me3 + H4K20me3 + CpG 5-MeC	Heterochromatin	IF and nucleosomal CoIP <sup>130</sup> , ChIP-seq <sup>26</sup> and bisulphite sequencing
H3K9me2/3 + H4K20me1 + H3K27me3 + CpG 5-MeC	Inactive X-chromosome	ChIP, ChIP-chip, reChIP <sup>24</sup> , bisulphite sequencing

5-MeC, 5-methylcytosine; Ab, specific antibody in western blot; ac, acetyl; ChIP, chromatin immunoprecipitation; ChIP-chip, ChIP followed by amplification and microarray hybridization; ChIP-seq, ChIP followed by massively multiplexed sequencing; CoIP, co-immunoprecipitation; CpG, the DNA sequence that is often targeted for epigenetic 5-cytosine methylation; IF, immunofluorescence; me, methyl; MS, mass spectrometry; ph, phosphoryl; reChIP, ChIP with two sequential immunoprecipitations with different antibodies; Rme2a, asymmetric dimethylated Arg; ub, ubiquitin.