**Uncovering combinatorial interactions in chromatin**

**Summary.**

It was suggested eighteen years ago that post-translational modifications of specific residues in histones could have unique functional roles [1]. A decade ago Brian Strahl and David Allis [2] proposed the "histone code" hypothesis – that post-translational modifications on histones are read, either individually or in combination, by other proteins to bring about distinct downstream events. Since then, much work has focussed on cracking such a code and some progress has been made in understanding the roles of some individual modifications. For example, the associations between histone acetylation and Histone H3 lysine 4 trimethylation (H3K4me3 [3]) with gene expression and H3K9 methylation with gene repression. However it has become increasing clear that there is substantial cross-talk between individual modifications and between histone modifications and DNA methylation. Development of new data sets using whole genome approaches, next generation sequencing and quantitative proteomic methods have shed new light on some of the connections between individual modifications. These studies are uncovering groups of chromatin modifications associated with specific genomic regions. Together such new approaches should help us understand the language of epigenetics* .

* A formal definition of an epigenetic mark is that it is a heritable mark that does not alter the DNA sequence. DNA methylation is an epigenetic mark and we understand how the pattern of DNA methylation is passed on to daughter cells. Modifications of histone proteins are widely referred to as epigenetic marks, though we currently do not understand how such modifications may be passed on to daughter cells. Additionally, some modifications play transient roles in cellular process such as replication, DNA repair and gene transcription and therefore do not meet the formal definition of epigenetic. A more detailed discussion of the issues can be found here [4].

**Keywords**: Cross-talk, bioinformatics, proteomic, machine learning, mass spectrometry, chromatin modifications.

## Executive summary

### Association of chromatin marks using computational methods.

- There is a wealth of data on the location of specific histone modifications within the genome, generated from high throughput chromatin immunoprecipitation (ChIP) studies.
- Computational methods are now being used on these data to look at the extent of overlap and association between individual marks.
- Clustering of chromatin marks has identified sets of chromatin modifications associated with individual genomic features (eg transcription initiation sites, enhancers and transcribed sequences).
- Several sets of modifications have been found associated with each feature suggesting either some redundancy within these sets and/or functional variation between related sets.
- Machine learning methods focussed on uncovering connections between two or three individual marks has identified a widespread association between active and repressive marks.
- In depth analysis of some of associations has identified some potentially subtle roles of individual modifications in regulating gene expression.

### Identifying linked modifications using quantitative proteomics

- Recent advances in mass spectrometry techniques coupled with labelling and/or purification are allowing the quantitative analysis of mixtures of peptides.

- One advantage of this technique is the ability to identify whether multiple modifications occur on the same histone molecule.
- One use of this method has been to determine the extent of modifications on a specific histone has and has shown that only subsets of the potential variation is found *in vivo*.
- Another approach is to quantify the changes in histone modifications in response to a loss of histone modifying enzymes which has provided some hints at mechanisms by which those enzymes achieve their functional roles.

**Cross-talk in generating chromatin changes**

- Understanding relationships between individual chromatin modifications does not on its own provide us mechanistic understanding of the function of those modifications..
- Understanding the mechanisms will require functional and biochemical approaches.
- From what we currently know, it appears that many of the chromatin modifications promote further modifications by recruiting specific enzymes or complexes that recognise one mark and modify another.

In eukaryotes, genomic DNA does not exist as a naked molecule, but is wrapped around histone proteins to form chromatin. The unit of chromatin is the nucleosome which consists of DNA wrapped around a histone octamer consisting of 2 copies each of histone H2A, H2B, H3 and H4. Interaction of DNA with histones is structurally important as it facilitates the packing of genomic DNA into a cell nucleus. The histone proteins can also be post translationally modified at specific residues which provides signals that are recognised by proteins which regulate processes (eg transcription) acting upon the underlying DNA

sequence, The modifications that have been identified on histone proteins include acetylation of lysine residues, methylation of lysine and arginine residues and phosphorylation of serine. These modifications have been shown to participate in diverse functions including transcription, DNA repair, DNA replication and apoptosis. For a detailed discussion of the different modifications and their roles in cellular physiology and disease see [5, 6]

It is becoming clear that such chromatin modifications do not act in isolation but that there is cross-talk between individual modifications such that the presence of one modification may influence the activity of other modifications (for a comprehensive overview of discovery and potential function of combinatorial chromatin modifications see [7]). Cross-talk between chromatin marks can be manifested in different ways and can result in a positive link, one modification enhancing the deposition or activity of another, or a negative outcome, one modification inhibiting the deposition or activity of another. Further, such interactions can take place in cis, between modifications on the same histone or in trans, between modifications on different histones [8, 9]. The molecular mechanism underlying much of the currently known interactions relies on the fact that many chromatin modifying proteins exist as part of larger complexes that contain proteins that recognise specific chromatin modifications (readers) as well as proteins that deposit (writers) or remove (erasers) chromatin marks. Such complexes thus have the potential to produce large scale changes to the chromatin landscape to which they are recruited. Combinatorial activity is not confined to protein complexes as some individual proteins contain domains that can recognise specific marks as well as enzyme activities that can modify chromatin. For example the chromatin remodelling enzyme Brg1 contains a bromodomain that can recognise and bind to acetylated lysine residues as well as an ATPase domain that remodels nucleosome positions [10, 11] while the histone methyltransferase G9a can bind mono- and dimethylated lysine residue via

a domain containing ankyrin repeats [12] and methylate H3K9 and H3K27 via its methyltransferases domain [13-15].

**Association of chromatin marks using computational methods.**

The advent of next generation sequencing coupled to chromatin immunoprecipitation (ChIP-seq) has allowed researchers to interrogate the locations of specific histone modifications across entire genomes. In combination with gene expression data, researchers are now in a position to quantify associations of individual marks, not only with each other but also with mRNA levels and other features of genes such as promoters, coding regions and enhancers. One early breakthrough in this area was the observation that H3K4me1 is found enriched and H3K4me3 depleted at enhancer regions and such a chromatin signature was able to predict novel enhancer regions [16]. Though they predict enhancer sequences with relatively high accuracy, H3K4me levels on their own do not discriminate active enhancers from poised enhancers. More recent work has shown that the presence of H3K27ac correlates with active enhancers providing a set of modifications, acting as a signature, by which these two enhancer types can be distinguished [17].

Studies correlating specific chromatin marks with known functional sites are a useful way to identify potentially important modifications. An alternative approach is to identify any patterns of chromatin modifications occur repeatedly and more often than expected by chance throughout the genome which has the advantage that it does not require prior knowledge of specific functional sites [18, 19]. Using such an unsupervised approach with genomic location data for 9 histone modifications (H3ac, H4ac, H3K9ac, H3K18ac, H3K27ac, H3K4me, H3K4me2, H3K4me3 and H3), Hon *et al.* identify eight chromatin signature patterns [18], significantly fewer than the 362,880 potential patterns that arise from 9 independent modifications. Given that the number of potential combinations of all known histone

modifications exceeds the number of nucleosomes in the human body, it is not feasible that the entire potential repertoire of chromatin modification patterns could be utilised by the cell. This suggests we will only find a subset of the potential modification patterns. In addition there is some evidence that, like the triplet code for amino acids, there is redundancy in chromatin marks [19, 20]. The individual signatures identified do correlate very well with specific functional elements such as known promoters and enhancers suggesting that distinct chromatin signatures are associated with distinct functions [18]. Using an expanded set of 21 histone modifications the same group identified signatures associated with promoters, enhancers, insulators and exons in CD4+ T cells [19]. In each case multiple signatures were found associated with each feature though whether this is due to redundancy within the patterns or reflects subtle functional variation awaits experimental validation.

Using machine learning methods, on published genome wide Chip-seq data, Xu *et al.* [20] identified 7 monovalent modifications and 16 multivalent (8 bivalent and 8 trivalent) marks associated with gene expression. The ChIP approach used to generate the original dataset utilised micrococcal nuclease to fragment the genome into single nucleosomes, thus individual marks that overlap are presumed to be derived from the same nucleosome. However it is possible that individual modifications are present in different cells within the population of cells used for the study. Such uncertainties could be resolved by the use sequential ChIP to determine if the multiple modifications are indeed present on the same nucleosome. One surprising finding of this study is the extent of overlap between apparently opposing marks. In Embryonic stem cells many groups have reported the widespread use of the bivalent mark consisting of a repressive mark (H3K27me3) and activating mark (H3K4me3) to produce a poised state in many genes [21]. Upon differentiation, such bivalent marks are resolved so that in differentiated cells a given gene is associated with either H3K4me3 or H3K27me3 depending on whether or not it is expressed in the differentiated cell

[22]. In the study by Xu *et al.* 8 of the 16 multivalent marks consist of a mixture of activating and repressing marks with one mark apparently acting in a dominant way to the other. Such ability of one mark to override another appears to some extent to be concentration dependant. Gene expression levels associated with the bivalent mark H3K36me3/H3K27me2 are higher when the ratio of H3K36me3 to H3K27me2 is high and lower when the ratio is low. There is a threshold level of H3K27me2, above which high levels of H3K36me3 do not appear to enhance gene expression suggesting H3K27me2 is a dominant mark to H3K36me3 (Figure 1). In a second bivalent mark studied, H3K36me3/H4R3me2, the positive H3K36me3 mark strongly overrides the repressive H4R3me2 [20] suggesting there may be a hierarchy of influence among histone modifications (Figure 1). The single mark most strongly associated with a repressive effect was found to be the symmetrically methylated arginine H4R3me2. The role of H4R3me2, suggested by its association with gene expression and active histone marks, is that it is involved in further repressing low to moderately expressed genes [20]. A biochemical mechanism underlying H4R3me2 repression has previously been identified which involves the recruitment of DNA methyltransferase 3A (DNMT3A, a *de novo* DNA methyltransferase) and couples histone methylation to DNA methylation [23]. Such a subtle role for H4R3me2 may go some way to explaining why a previous genome wide study did not identify any association of H4R3me2 with either active or silent promoters [24].

Though genomewide studies generate a lot of data, care should be taken that the datasets are truly representative. Assessing the quality of the data can be difficult, though some potential problems can be overcome by using multiple reads, a requirement facilitated by next generation sequencing approaches. The wealth of genome wide datasets that are currently being generated open the door to more sophisticated analyses that should hopefully uncover some more of the complexity of interactions and associations between histone modifications and provide some clues to their functional roles in regulating gene expression.

**Identifying linked modifications using quantitative proteomics**

One potential limitation of ChIP studies is that it is not possible to know whether two modifications occur within the same histone or in different histones within the same nucleososme. Another limitation is the specificity of the antibodies used to isolate the chromatin fractions – though many antibodies used have been well characterised with respect to known modifications problems may arise due to an antibody recognising more than one modification or the presence of a second modification masking an antibody binding to its epitope [25, 26]. Complementary approaches such as mass spectrometry are thus particularly useful because they are not dependant on antibody specificity. Recent advances in proteomic technologies have allowed the modification state of histone tails to be interrogated and these studies are highlighting some potential functional connections between individual modifications (for a review on the use of quantitative mass spectrometry in epigenetic studies see [7, 27]). Such studies cannot however be a direct replacement, because unlike ChIP, the proteomic approach does not allow the specific modifications identified to be mapped to genomic regions, associated with open/closed chromatin or with active/repressed genes and it is difficult to look at associations between modifications occurring on different histones. Thus any functional consequences of interactions between modifications can only be inferred by what we know of the individual component marks. Two groups have used mass spectrometry to interrogate the combinations of post translational modifications on histone H4 [28-30]. Pesavento *et al.* used two-dimensional liquid chromatography to separate multiply modified forms of Histone H4, obtained from HeLa cells, into fractions which were then individually subjected to mass spectrometry analysis [29] while Phanstiel *et al.* used HPLC to separate differentially acetylated H4 peptide fractions from human ES cells and a human fibroblast

line, IMR90, which were the subjected to mass spectrometry [30]. Pesavento *et al.* identified 42 different isoforms of Histone H4 in HeLa cells while Phanstiel *et al.* identified 72 isoforms in ES cells. These differences could be due to different sensitivities, the different ways in which histone proteins were purified or due to the different cell types used. In support of the latter explanation, Phanstiel *et al.* identified greater number of modification patterns in the H4 peptides which contained H3K20me and were multiply acetylated. Overall the ES H4 peptides were hyperacetylated compared to IMR90 cells and acetylation levels were reduced upon ES cell differentiation [30]. The Histone H4 N–terminal region (amino acids 1-20) contains 4 lysine residues at positions 5, 8, 12 and 16, which can all be acetylated. Consistent with previous ChIP data both studies identified that the levels of acetylation of K16 were the highest [29, 30]. Previous data has suggested that K12 is acetylated at the next highest level and data from Pesavento *et al.* was consistent with this idea. However the study by Phanstiel *et al.* showed K5 to be the next most highly acetylated H4 lysine in ES cells. Furthermore the ratio of lysine acetylation was found to be affected by the methylation of K20 in HeLa cells by Pesavento *et al.* Indeed in monoacetylated H4 that contains K20me3, all of the acetylation is found at K16 leading them to conclude that the presence of K20me increases the likelihood of acetylation occurring on at a more proximal lysine [29]. How such an influence is brought about is not clear but may for example involve interactions of the K20me with histone acetyltransferases, deacetylases or result in a histone tail structure more readily acetylated at K16. Also, such a interaction does not seem to occur within ES cells; in H4K20me3 peptides monoacetylation marks are found at each of the four lysines in approximately the same ratio as found for non-methylated H4 peptides [30]. Both studies did find that H4R3me was only present on peptides containing a K20me2 mark suggesting that H4R3 methylation requires prior methylation of K20. A connection between H4 acetylation and H4K20me was previously identified by Scharf *et al.* using an in vitro chromatin assembly assay (Figure 1)

[31]. During DNA replication the newly deposited Histone H4 carries acetylation marks at lysine 5 and 12 which are removed within minutes of chromatin formation. Inclusion of H4 into nucleosomes stimulates the ability of the methyltransferase PR-SET7 to methylate H4K20. In turn the H4K20me provides a binding site for an MBT domain containing protein and an associated histone deacetylase [31]. These data suggest a pathway whereby newly deposited Histone H4 is monomethylated at lysine 20 which then acts as a binding site for a deacetylase containing complex and deacetylation of K5 and K12 (Figure 1) [31].

Clearly the ability to quantify the levels of individual modification patterns is a major strength of an MS approach and may uncover associations not predicted by previous analysis. For example, previous work has suggested that H4K20me3 is associated with heterochromatin formation and whilst overall H4K20me3 is associated with a hypoacetylated H4 tail, 30% of the H4 containing K20me3 is also acetylated at K16 in HeLa cells [29], a mark not consistent with its proposed role. A potential requirement for H4K20me2, which is linked to transcriptionally inactive regions, for deposition of H4R3me, which is linked to transcriptionally active regions, is also intriguing. The functional significance of these potential interactions awaits experimental interrogation and future work aimed at identifying the association of these variants with specific chromatin or genomic features should hopefully uncover this.

A recent proteomic study by Plazas-Mayorca *et al.* [32] used mass spectrometry of purified histone tails from HEK293 cells to identify potential cross-talk between H3K9me and other modifications. The authors used siRNA to knock down G9a and Glp1, two histone methyltransferases that interact and mono- and di-methylate H3K9 [14] and H3K27 [15, 33], in HEK293 cells. Upon knockdown of G9a and Glp1, levels of both H3K9me1 and H3K9me2 were reduced, the levels of non-methylated H3K9 doubled while levels of H3K9me3 were not affected [32]. With respect to modifications at other positions, the level of the repressive mark

H3K27me2 showed a slight decrease which may reflect the extent to which H3K27 is methylated by G9a/Glp1 [15] in HEK293 cells while the levels of H3K36me2 were slightly increased. H3K36me2 is associated with transcriptional activation when it is located in the 3' region of genes but with transcriptional repression when found in the coding region [34, 35]. Perhaps more significantly the levels of H3K79me2, a mark associated with gene activation, approximately doubled suggesting there may be some antagonism between H3K9me2 and H3K79me2 [32]. As well as being a substrate for methylation, H3K9 can also be acetylated, a mark associated with gene activation. After knockdown of G9a/Glp1, though the levels of non-methylated H3K9 increased, there was no significant increase in the levels of acetylation at this position. There was however an increase in the levels of acetylation at H3K14, another active mark [36, 37], leading the authors to suggest that the presence of H3K9me2 inhibits H3K14 acetylation (Figure 1 ) [32]. One caveat to this study is that a study of gene expression levels showed that knockdown of G9a/Glp1 led to the altered expression of 5201 genes [32], some of which are known to modify chromatin and the altered levels of chromatin modifying enzymes could contribute to the changes in histone in response to the loss G9a/Glp1. In a similar approach, Pasini *et al.* [38] used embryonic stem cells lacking the essential polycomb repressive complex 2 (PRC2) subunit, Suz12 to investigate the changes resulting from loss of PRC2 mediated H3K27me3. In ES cells lacking Suz12, levels of H3K27me2 and H3K27me3 were reduced and levels of H3K27ac increased about four fold suggesting that one way PRC2 represses gene activity is by preventing H3K27 acetylation.

One technical issue with using mass spectrometry is the requirement for purified fragments derived from a trypsin digestion of the parent molecule. Young *et al.* [28] developed an automated high throughput method that couples liquid chromatography to electron transfer dissociation mass spectrometry. With this approach they were able to identify 70 forms of H4 and 200 forms of the histone variant H3.2 and, importantly, examine

the combinatorial modifications across large peptide fragments [28]. More progress in this area should allow for the identification of combinatorial sets of modifications which include individual marks that occur some distance from each other.

**Cross-talk in generating chromatin changes**

Recent studies described above have begun to use discovery approaches to identify combinatorial chromatin marks, though the mechanisms generating these combinations and their functional consequence will require more focused biochemical assays. Some interactions, both in cis and trans, have been identified using candidate approaches and the molecular mechanisms responsible for their generation or underlying their function have been ascertained (see table 1).

In some cases an order has been assigned to sets of chromatin modifications that are made in response to some change within the cell. The first example of cross-talk identified involves the ubiquitination of H2BK123 which influences methylation of H3K4 and H3K79 in yeast (Figure 1) [39, 40]. H2BK123 is ubiquinitinated by the protein Rad6 which provides a binding site for Cps35, a subunit of the yeast COMPASS complex. COMPASS also contains H3K4 methyltransferase activity and thus, once recruited, results in methylation of H3K4 [39, 40]. Cps35 also interacts with the H3K79 methyltransferase Dot1 and H2BKl123ub is required for H3K79 methylation [40]. Another example of trans cross-talk is provided by the transcription factor REST. REST is a transcriptional repressor associated with two protein complexes containing multiple chromatin modifying enzymes [41]. Recruitment of REST to chromatin is mediated at least partly by the chromatin remodelling enzyme, Brg1 and is enhanced by binding of the Brg1 bromodomain to acetylated H4 [42]. The ensuing chromatin remodelling enhances the stability of REST:chromatin interaction and leads to histone deacetylation by the recruited histone deacetylases. One result of histone

deacetylation is a stimulation of the histone demethylase enzyme LSD1 and a reduction in H3K4 methylation. The levels of the acetylation and methylation are tightly linked in chromatin regulated by REST [43-45]. Many of these functional interactions appear to result from the substrate specificity of the modifying enzyme, the H3K4 demethylase, LSD1, demethylates a non-acetylated template much more readily than an acetylated template [44, 45] while several of the H3K9 methyltransferases, (SETDB1, G9a/ GLP and SUV39H1) cannot bind and methylate histone tails containing H3K4 methylation [46]. Cross-talk is not restricted to interactions between histone modifications - biochemical studies have also identified cross-talk between hypoacetlyation, H3K9, H3K27 or H4K20 methylation and DNA methylation (for a more in depth discussion see [8, 9, 47]). More recently symmetrical dimethylation of arginine at H4R3 by the protein arginine methyltransferases PRMT5 has been shown to lead to DNA methylation because a domain within DNMT3A binds the symmetrically methylated H4R3me2 [23].

Even though we have likely only scratched the surface of the interactions between individual chromatin modifications these studies highlight chromatin as a very dynamic entity. Individual modifications are, on their own, likely to have quite subtle effects. A series of cascading modifications, on the other hand, where one change initiates another could result in a dramatic shift in the chromatin landscape and facilitate the ability of, for example, individual genes to be expressed highly in one cell type but not in another.

**Future perspective**

In the decade since the histone code was first proposed we have made great strides in understanding the mechanisms by which the chromatin marks are read and how they are written or erased. Clearly there is still much to learn but with the ability to generate large datasets using ChIp-seq we can expect to have a much greater understanding of how the

chromatin landscape relates to specific features within the genome. Using proteomic approaches it is currently only possible to correlate modifications within a single peptide. Purification of individual nucleosomes carrying a specific modification would be the next logical step to try and probe for trans interactions using mass spectrometry. Understanding the underlying molecular mechanisms is likely to be more difficult to progress rapidly given the requirement for biochemical assays that do not lend themselves well to automation or high throughput. We should expect to see more insights into structures of chromatin modifying complexes which would hopefully shed light on how isoforms within such complexes alter their function and/activity [48, 49]. Though we may not understand entirely how the chromatin landscape is sculpted we should hopefully have a good appreciation of what it means after another decade of research in the histone code.

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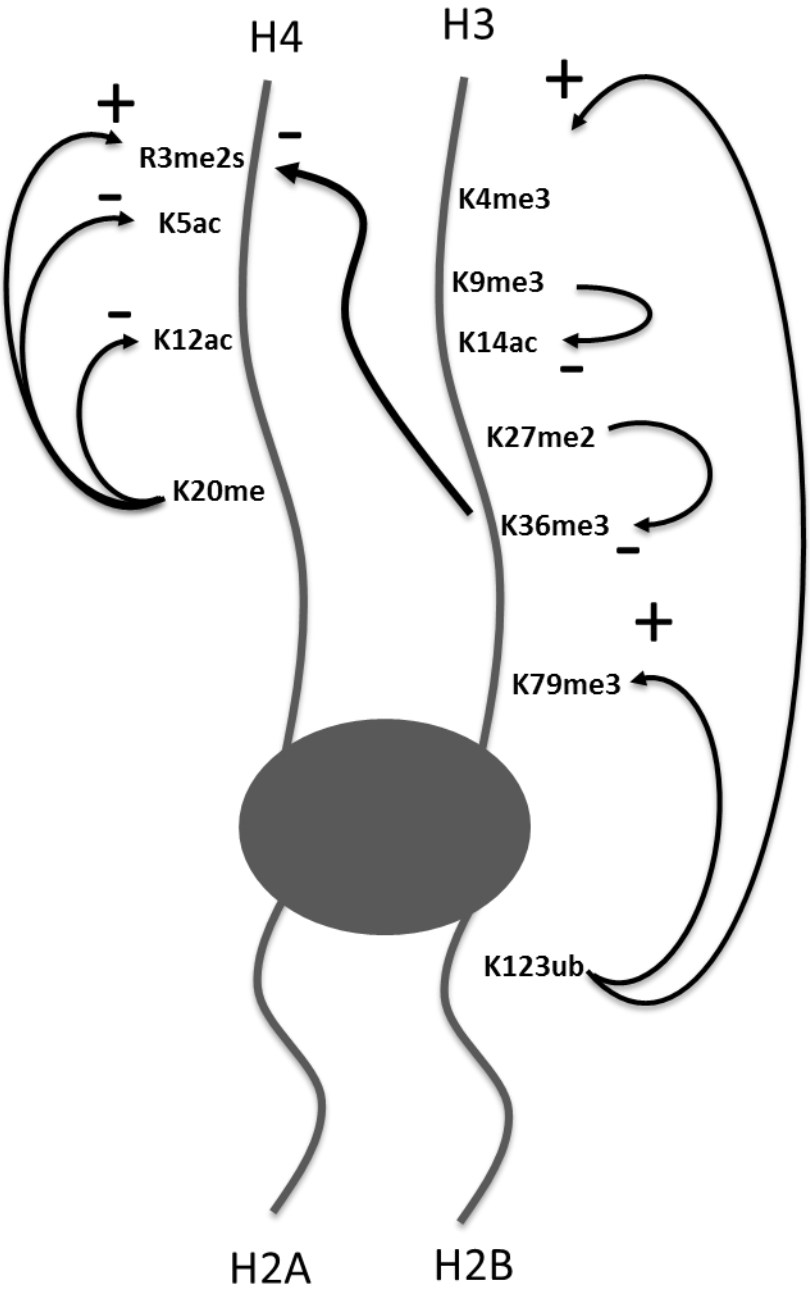


**Figure 1**

A representation of a nucleosome showing some of the in cross-talk between individual marks discussed within the text. For clarity only one of each Histone protein is represented in the nucleosome and although many of the interactions have been suggested, and are shown, between residues within the same nucleosome and/or within the same histone protein this has not been proven for all interactions.