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Systematic analysis of histone modification readout

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To faithfully execute diverse biological programs all cells need to access and distribute their genomes in a highly organized way. In the nucleus of eukaryotic cells DNA is packed with histone proteins into chromatin. The originating nucleo–protein complex is the regulatory platform for all genetic processes. Of these, posttranslational modifications of the histone proteins play a key role as they are thought to direct different chromatin states. Most histone modifications appear to not have a direct effect onto chromatin structure, but work *via* recruitment of specific binding proteins. A large number of such individual factors interacting with diverse histone marks have been identified and characterized. Also, global approaches have been established that aim to define the interactome of histone modifications or patterns thereof. We summarize the experimental approaches that are used to determine histone modification readout and discuss complexities that are emerging within this regulatory system.

Introduction

Over a lifetime, any cell needs to carry out a wide array of diverse functions. These require that the DNA as inherited material is maintained, read and translated in a highly organized manner. Cells need to react rapidly to external stimuli, but also have to faithfully execute long-term differentiation and developmental programs. While the DNA in its base sequence contains the blueprint for all these processes, various systems have evolved to ensure that this stored information is used and handled in an appropriate manner corresponding to the specific needs of the cell.

The molecular target of many of these processes is chromatin, the packaging form of the genome in eukaryotic cells. Its basic structural element – the core nucleosome particle, is formed of 147 bp of DNA wrapped around an octamer of histone proteins (H2A, H2B, H3, H4). Histones are relatively small (*ca.* 100–150 amino acids), highly conserved proteins. Their C-terminal domains fold into compact units that mediate interaction with each other forming a wedge like structure around which DNA is wrapped. The N-terminal regions, in contrast, are intrinsically unstructured and protrude out from the nucleosome core. Addition of short stretches of linker DNA that complex with linker histone (H1/H5) establishes the repeating fundamental unit of chromatin, the nucleosome. The architecture of all nucleosomes is essentially the same. Variation is achieved by the exact positioning of nucleosomes on the DNA,[1] the ordered and localized incorporation of sequence variants of the core and linker histones[2] as well as in particular by a large number of diverse post-translational modifications (PTM) of the histone proteins. These PTMs mark individual nucleosomes or stretches of nucleosomes, thereby potentially controlling the biology of the underlying DNA.

Despite intensive work in this area for the past 50 years, new histone modification sites as well as types of modification are still being identified.[3] Main histone modifications include acetylation (Kac), crotonylation (Kcr), monomethylation (Kme1), dimethylation (Kme2), trimethylation (Kme3), ubiquitylation (Kub) and sumoylation (Ksu) of the ε-amino group of numerous lysine residues, monomethylation (Rme1), symmetric dimethylation (Rme2s) and asymmetric dimethylation (Rme2a) of the guanidine group of several arginine residues as well as phosphorylation of some serine (Sph) and threonine (Tph) residues and few tyrosine (Yph) residues. While many sites of modification cluster on the histone N-terminal tails, core regions can also carry marks. Some PTMs appear to be established on the histones before incorporation, but the majority is found in the context of chromatin.[2,4,5]

Due to the large number of modification types and sites, the potential signaling functions of histone PTMs and in particular their potential combinations are enormous. While concepts postulating a code like behavior of these marks have been put forward,[6] other studies point to a more restricted and redundant function of the many PTMs.[7] Irrespective of the concepts used to describe the signaling principles of histone modifications, it is now clear that these affect all processes targeting

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the genome including transcription, repair, cell cycle, recombination, *etc.*

For the comprehension of the function and signaling of histone modifications it is essential to define their molecular working mechanisms. Few histone modifications have been shown to directly affect chromatin structure. For example, histone tail hyperacetylation in general interferes with higher order folding of chromatin in *in vitro* test systems.[8] And in particular, H4K16ac and H4K20me2 have been found to limit transition of elongated arrays of nucleosomes into 30 nm fibers.[9,10] It is assumed that such effects directly impact on biological machinery such as polymerases that need to get access to the chromatin-underlying DNA. However, the majority of histone modifications appear to work indirectly. These recruit or repel specific proteins that interact with the histones in a PTM directed manner. While several recent reviews have summarized the structural and functional properties of the many histone modification-binding proteins,[11–15] we summarize here the techniques and methods that have been employed to identify such factors. Particular emphasis is given to the emerging complexity of the histone modification readout systems.

Identification of histone modification binding proteins

As for many protein–protein interactions directed by PTMs the binding strength of histone modification-binding proteins to their targets is not very strong (in the range of μM to mM). Therefore, identifying such factors is not always simple and straightforward (Fig. 1). Due to the specific nature of the interaction directed by a chemical and not genetically encoded modification, genetic approaches can only provide limited indication of histone PTM directed interactions and pathways. In screens for suppressors of variegation – a phenomenon of heterochromatin spreading best studied in *Drosophila* – connections between modifying enzymes (Suv39 as H3K9 methyltransferase) and a binding protein Suv25 (= heterochromatin protein 1 binding to H3K9me3) could be established.[16–18]

Educated approaches

The analysis of histone modification binding proteins has established that a number of specialized protein domains mediate interaction (see Table 1). These modules of *ca.* 50–150 amino acids are found in numerous chromatin-associated factors in different organisms and model systems. While this list of domains is constantly growing, only in few cases have all the members of a protein family systematically been analyzed for their binding properties. The PHD (plant homeo domain) finger containing factors in *S. cerevisiae* (14 proteins with 18 PHD fingers in total) were studied in their interaction with methylated lysines.[19] More recently, all members of the human bromo domain family were fully characterized in their biochemistry and structural details of recognition of acetylated lysines.[20] Also, several mammalian tudor (named after the tud factor in *Drosophila*)

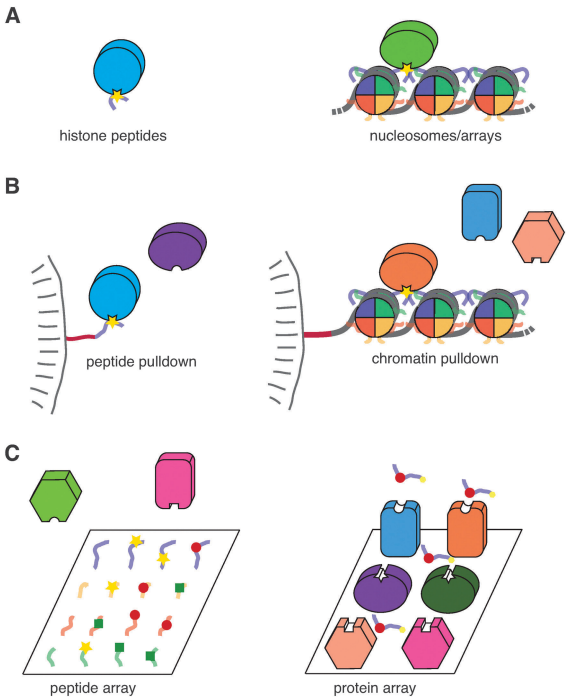

Fig. 1 Different methods for identifying and defining histone modification binding proteins. (A) Educated guesses – mainly based on sequence and structural homology are directly tested in peptide (left) and chromatin (mono-/oligonucleosome, right) based assays. (B) Affinity purification schemes for the unbiased enrichment of histone modification binding proteins make use of immobilized histone modification peptides (left) or reconstituted chromatin (mono-/oligonucleosome, right) templates. (C) Arrays with many different histone modification peptides (left) or recombinant protein domains (right) immobilized on cellulose or glass supports are used to determine relative interaction specificities.

domain factors were analyzed in their binding to methylated arginines.[21] While algorithms for sequence comparison and similarity search can group different proteins and regions thereof into different classes, the conserved sequence information provides an important but sometimes limited element of predictive power. For example, while the first PHD fingers studied were found to interact with H3K4me3, further analysis of this large protein family indicated members with binding to H3K36me or preferring unmodified histones over modified targets.[19] Similarly, tudor domain containing factors were found to interact with H3K4me2/3 (*e.g.* JMJD2A[22,23]), H4K20me1/2 (*e.g.* 53BP1[24]), H4K20me2/3 (*e.g.* JMJD2A[23]) and H3K9me2/3 (*e.g.* UHRF1[25]).

In the case of the methylation binding factors structural studies have implied three to four aromatic residues (forming a so called aromatic cage) in recognition of the modification. The presence of such residues at conserved positions at least appears to be an indication of potential to recognize methyl-marks. In addition, structural similarity in overall domain folds can be helpful in defining new histone modification binding proteins. Detailed comparison grouped the chromo, tudor, MBT (malignant brain tumor) and PWWP (proline–tryptophan–tryptophan–proline) protein folds into one 'Royal' superfamily.[26]

Table 1 Paradigm protein domains recognizing histone modifications. The listing is not complete, but contains examples of factors containing distinct modules for histone modification recognition

Modification	Module/domain	Protein	Recognized site(s)	Repelling effects of neighboring modifications	References
Kac					
	bromo				
		TAF1	H4hyperac		90
		TRIM24	PHD: H3K4me2/3 bromo: H3K23ac working as one functional unit		75
		BPTF	PHD: H3K4me2/3 bromo: H4K16ac simultaneous binding has been shown	PHD: H3R2me2a	29, 80, 88
	PHD				
		DPF3b	PHD1: H3K14ac PHD2: H3unmod working as one functional unit	H3K4me3	108, 109
		MOZ	PHD1: H3K14ac PHD2: H3unmod working as one functional unit	H3R2me2	110
Kme					
	chromo				
		HP1 (Cbx1, Cbx3, Cbx5)	H3K9me2/3, H1.4K26me2/3, H3K23me2/3	H3S10ph, H1.4S27ph	16–18, 24, 55, 83, 84, 111–115
		Polycomb (Cbx2, Cbx4, Cbx6, Cbx7, Cbx8)	H3K9me3, H3K27me2/3	H3S28ph	86, 87, 111, 116–118
		Tip60	H3K4me1, H3K9me2/3		96, 97
		CHD1 (double chromo domain)	H3K4me2/3	H3R2me2a H3T3ph	100, 119–121
		CMT3	chromo: H3K9me2 BAH: H3K9me2 simultaneous binding to two H3 tails within one nucleosome		77
	chromobarrel				
		EAF3	H3K36me2/3		122–127
		MSL3	H4K20me1/2 (+DNA) unclear whether DNA binding is required	H4K16ac	128, 129
	tudor				
		53BP1	H4K20me1/2 weak: H3K79me2		24, 41, 49
		JMJD2A	H3K4me2/3, H4K20me2/3	H3T3ph	22, 23, 49, 60
		UHRF1,2	PHD: H3unmod tudor: H3K9me2/3 working as one functional unit	PHD: H3R2me1, H3R2me2a H3R2me2s tudor: H3K4me2/3	25, 130–137
	MBT 3xMBT PWWP	L3MBTL1	general histone Kme1/2		49, 93, 138–141
		Dnmt3a	H3K36me3		99
	PHD				
		ING1,2,4,5	H3K4me2/3	H3R2me2a H3T3ph, H3T6ph, H3R2me2s	30, 89, 142–145
		TAF3	H3K4me2/3	H3T3ph, H3R2me2a	37, 146, 147
		RAG2	H3K4me2/3 (note: binding is slightly increased by the presence of H3R2me2s, H3R2me2a has a weaker effect)	H3T3ph, H3T6ph	60, 78, 148, 149
		TRIM24	PHD: H3K4me0 bromo: H3K23ac working as one functional unit		75
		BPTF	PHD: H3K4me2/3 bromo: H4K16ac simultaneous binding has been shown	PHD: H3R2me2a	29, 80, 88
		UHRF1,2	PHD: H3unmod tudor: H3K9me2/3 working as one functional unit	PHD: H3R2me1, H3R2me2a H3R2me2s tudor: H3K4me2/3	25, 130–137

Table 1 (*continued*)

Modification	Module/domain	Protein	Recognized site(s)	Repelling effects of neighboring modifications	References
	ADD				
		ATRX	H3K9me3	H3K4me3	150–152
	WD40				
		Eed	H1K26me2/3, H3K9me2/3, H3K27me2/3, H4K20me2/3		92, 153
		Nurf55	H3K4unmod	H3K4me1/2/3	91
	ankyrin				
		G9a/GLP	H3K9me1/2		98
	BAH				
		ORC1	H4K20me1/2/3 with preference for me2		28
		CMT3	chromo: H3K9me2 BAH: H3K9me2 simultaneous binding to two H3 tails within one nucleosome		77
Rme					
	tudor				
		TDRD3	H3R17me2a, H4R3me2a		21, 154
	WD40				
		WDR5	H3R2me2s, H3K4me0/1/2/3 from the structure unclear how binding to Kme is achieved		31, 34, 155–158
Sph					
	BRCT				
		MDC1	H2AXS139ph		159
		MCPH1	BRCT2: H2AXS139ph BRCT3: H2AXY142ph simultaneous recognition of both marks has been shown		76
	14-3-3				
		14-3-3	H3S10ph, H3S28ph		32, 79, 160

The initial finding that chromo domains interact with histone methyl-lysine marks sparked numerous studies investigating the histone modification binding potential and properties of members of the other domain classes of this superfamily.[16–18]

In some instances, the biological context has pointed to new histone modification interactions. For example, H4K20me was known to be a histone mark fluctuating during the cell-cycle.[27] As origin binding proteins need to be loaded onto DNA before replication, it could be recently shown that the BAH domain factor ORC1 interacts with this modification.[28] In all cases, the factors suspected to interact with a particular histone PTM are directly tested using different qualitative and quantitative assays (see below) (Fig. 1A).

Affinity purification

Unbiased approaches can identify novel interactions (Fig. 1B). Since the interaction interfaces of histone modification binding proteins appear to be relatively small covering five to ten amino acids of the histone sequence, peptide based affinity enrichment schemes have proven to be very valuable. Synthetic histone peptides carrying the modification of interest as well as an anchoring moiety are readily accessible and can be immobilized on a solid support (*e.g.* biotin–streptavidin, Cys *via* sulfolink). When incubated with nuclear or chromatin extract these can be used to affinity purify specific modification binding proteins when comparing to an unmodified control.[29] Due to the relative low binding affinities, the single step purification method often produces low signal to noise ratios and high background. With the development of sensitive detection and especially identification methods in mass spectrometry and the introduction of quantitative proteomics these approaches have nevertheless proven to be extremely useful. A number of factors were identified to interact with different histone modifications this way. These include WDR5, BPTF and ING2 binding to H3K4me3,[29–31] UHRF1 binding to H3K9me3[25] and 14-3-3 binding to H3S10ph.[32] More recently, global description of the interactome of defined histone modifications was done.[33–40] The experiments show that individual marks recruit a number of factors in direct or indirect interaction. Independent verification, binding profiling of recruited factors and genome wide localization studies in combination could establish networks of histone modification interacting complexes.[36]

In a reverse approach of the affinity purification scheme, proteins suspected to interact with histone modifications are used as baits and are incubated with histone peptides carrying different modifications. Binding of 53BP1 to H3K79me2 was identified by this method.[41]

Peptides only reflect a minimal part of the native environment of histone modification-binding proteins. In chromatin these might be influenced by direct or indirect interaction with other histones as well as DNA. To mimic this complexity of the binding substrate, affinity purification schemes using recombinant chromatin templates were recently established.

These use either uniformly modified mononucleosomes[39] or oligonucleosomal arrays[33] both immobilized *via* biotinylated DNA on streptavidin beads. In these experimental schemes novel and already known interaction partners to different histone methylation marks (H3K4, H3K9 and H3K27) could be found.

The methods for establishing uniformly modified histone proteins for incorporation into recombinant chromatin are more laborious compared to the simple peptide based approaches. Native chemical ligation of synthetic modified peptides to histone cores was used in both cases.[33,39] Alternatively, chemical mimicry based on derivatized Cys residues (for methylated[42] and acetylated[43] lysines) as well as stop codon suppression in genetic code expansion[44] may also be used for introducing specific marks into recombinant histones. The chromatin-based methods provide access to putative combinatorial readout of histone modifications that are not in close enough proximity to fit on a single histone peptide or that are on different histones altogether. Also, methylation and hydroxymethylation of DNA can be factored in. Indeed, one of the chromatin based affinity purification studies used chromatin material reconstituted with methylated (CpG) or unmethylated DNA. However, a putative combinatorial contribution of the different chromatin marks was not analyzed.[39]

There are obvious differences in the peptide and chromatin based affinity purification approaches of histone modification-binding proteins (see Table 2). One study directly compared the interactome of H3K4me3 and H3K9me3 on peptides or oligonucleosomal array templates.[33] Surprisingly, the overlap of factors found with either method was limited. Besides some common factors, each approach identified proteins not recuperated by the other method. In general, the number of factors found with peptides appeared to be larger compared to the chromatin templates. When comparing independent peptide[36] and mononucleosome[39] studies on the same histone modifications this trend appears to reproduce. Likely, varying density of the modifications in the different templates plays a role. Locally, this might be higher on the peptides directly immobilized on the streptavidin matrices. Also, accessibility of the marks in the context of chromatin *vs.* peptide might not be comparable.

Variations of the affinity approaches include the expression of short histone fragments in conjunction with modifying enzymes that establish modification marks *in situ* in a cellular context. *Via* an affinity tag on the hybrid histone–histone modifying enzyme construct associated factors binding to the histone modification can be purified (mammalian tethered catalysis, MTec).[45] While candidate factors have been tested using this method, only identification of a single new factor has been reported. Since purification of the complex of hybrid histone–histone modifying enzyme construct and associated factors from cells under conditions that preserve the binding appears difficult, it has to be seen whether the approach is applicable to global identification of novel factors and high throughput. Similar expression of histone fusion proteins has also been applied for unmodified histone tails.[46,47] Further, reprogramming of the genetic code has been used to establish multiple sites of acetylation and methylation in the full H3 tail (residues 1–38) thereby bypassing synthesis of a complex library of long and potentially difficult to make peptides.[48]

Array based methods

Due to the immense number of histone modifications and the many possibly binding proteins, methods that can analyze multiple interactions at once are desirable (Fig. 1C). In a candidate approach 109 putative histone modification binding domains including many members of the Royal superfamily, which were expressed as GST-fusions in bacteria, were spotted on a microarray chip (CADOR, chromatin-associated domain array).[49,50] These were probed with differentially modified histone peptides bearing a fluorescent marker. Different already known and novel interactions could be established this way, which could also be verified in independent assays. The approach was later extended to binding of labeled mononucleosomes that were modified *in vitro* using specific enzymes.[51] More recently, the first histone methyl-arginine binding protein, TDRD3 could be identified on an expanded version of the CADOR chip, probing it with a labeled H3R17me2a peptide.[21]

Table 2 Comparison of peptide and chromatin based approaches for affinity purification and characterization of histone modification binding proteins

Synthetic modified histone peptides	Recombinant chromatin templates of defined modification status
+ Straightforward peptide synthesis and immobilization	− Complicated reconstitution (use of native chemical ligation, mimetics, manipulation of the genetic code)
− Limitation to relative short patches of histone sequence	+ Physiological substrate combining DNA and different histones
+ Relative high density of peptides immobilized on surfaces/ matrices can be achieved	− In general lower density on the surface/matrices used for immobilization ⇒ select for different/stronger interaction partners
+ Straight forward quantitative binding assays available	− Quantitative interaction parameters difficult to determine
− Singular interaction modes	+ Multiple different interactions (with histones and DNA) possible
	+ Putative identification of histone code readers that interact with more than one modification
	+ Cooperativity or multivalency might result in stronger binding
	+ Possibility to combine specific DNA elements and sequences with defined histone modifications
	− For stable incorporation of nucleosomes generally DNA sequences with strong positioning propensity are used
	+/− Combinations of effects: direct interaction and recognition of modification-induced chromatin conformations

While the protein chips are useful in identifying new histone modification binding proteins, these require the expression of different factors or domains in a native and active state. In a reverse manner, peptide arrays containing a large number of different histone modification sites and marks have been used. These can be synthesized directly on functionalized membranes in solid phase peptide synthesis (SPOT).[52] Since verification of peptide sequence and modification status requires cleavage from the membrane, an alternative and more widely used approach uses spotting and immobilization of individually synthesized peptides *via* added biotin tags on streptavidin-functionalized glass slides.[53–55] Celluspots peptide arrays represent a combination of the two approaches as peptides synthesized on a cellulose solid support are dissolved and spotted on different glass surfaces.[56]

The peptide arrays have generally been used to define the exact interaction profile of known binding proteins, one at a time. This includes simultaneous analysis of many different modification types and sites as well as different degrees of methylation.[53–55] In addition, the effects on modifications adjacent to the marks targeted by a particular binding protein have been probed.[56] Since the analysis is mostly qualitative and allows only semi-quantitative comparison of spots on the same membrane, it is often combined with independent quantitative verification of the findings in solution. Antibody recognition of histone modification marks is also affected by adjacent modifications.[57] The approach has therefore also been applied to characterize the specificity of these reagents.[58]

An alternative experimental scheme used libraries of small beads coupled to individual peptides of histone modification sites.[59] Due to the spilt-pool methodology used, an immense number of 5000 randomized combinations of histone H3 PTMs were accessible.[60] In a direct comparison of the peptide- and protein-based arrays the latter were found to be less sensitive, putatively because coupling/printing of the protein domains interrupts function/folding. Also, due to the necessary washing steps of the heterogeneous surface assays the overall sensitivity of the arrays is relatively low (detection of interactions stronger than *ca.* 100 μM).[19] This results, in general, in relatively few false positive detections (high stringency), but comes with a high rate of false negatives, where bona fide interactions might not be detected.

Detection and quantification of histone modification binding protein interaction

Based on the discussed assay methods for analyzing binding of proteins to histone modifications, different methods are used for the identification and detailed analysis of the interactions (Fig. 2).

Identification by mass spectrometry-based proteomics

Mass spectrometry has become the method of choice for identifying proteins recruited to histone modification peptides or chromatin templates. The many applications of MS technology to chromatin research have recently been reviewed[61] and we only briefly summarize the main implications for the analysis of histone modification binding proteins. On a singular level, factors recruited to a modified histone template (peptide or chromatin) are directly compared to those recruited to the unmodified counterpart by running the material on SDS-PAGE. Individual protein bands are cut out off the gel and are digested yielding complex peptide mixtures. When these are separated by chromatography and subjected to MS/MS fragmentation the identity of proteins can be determined from the derived peptide sequence in comparison to protein databases.[62] With the sequences and therefore protein templates of the genome of many organisms now known, such mapping has become routine. Several histone modification-binding proteins have been identified in this way (see for example ref. 29–32).

Since the histone modification affinity purification approaches do however often not produce highly enriched and purified factors (*i.e.* individual bands on a protein gel), but result in complex samples containing many more or less abundant proteins, global sample analysis without selection of individual protein bands has been applied. For example, in MudPIT approaches the complete set of proteins bound in experiment and control samples are each digested in solution, separated by liquid chromatography in two dimensions and analyzed by MS.[63] An alternative and very common approach is separation of the interacting proteins by SDS-PAGE, excision of the whole experiment and control lanes into a defined number of pieces and in-gel digestion. These are then processed for analysis of all proteins contained in the slices (Fig. 2A, left). Factors recuperated on modified and unmodified histone samples are then subtracted resulting in a modification dependent interactome. While such non-quantitative approaches have been applied in few cases, these have the problem that any factors present in both samples in different amounts (*i.e.* weak binding to one modification state of the template and strong binding to the other modification state of the template) will nevertheless be excluded by the analysis. Therefore, the false negative rate of the method is relative high. Further, no information on strong or weak enrichment of factors can be deduced.

Spectral counting, which uses the number of fragment spectra acquired for each protein, is one of several label-free techniques for quantitative MS.[64,65] It has been applied to comprehensively analyze the interaction of proteins from nuclear extracts with histone H3 tail peptides bearing H3K4me2, H3K9me2 and H3K9ac modifications.[35] SILAC (stable incorporation of labeled amino acids in cell culture) in contrast requires differential labeling of the starting material used in histone modification affinity purification schemes (Fig. 2A, middle). The method has been more widely used to provide global interactome analysis of the factors interacting with different histone modifications on peptides (H3K4me3,[33,36,37] H3K9me3,[33,36] H3K27me3,[36] H3K36me3,[36] H4K20me1,[40] H4K20me3,[36] H3R2me2s,[34]) mononucleosomes (H3K4me3, H3K9me3, H3K27me3[39]) and oligonucleosomal

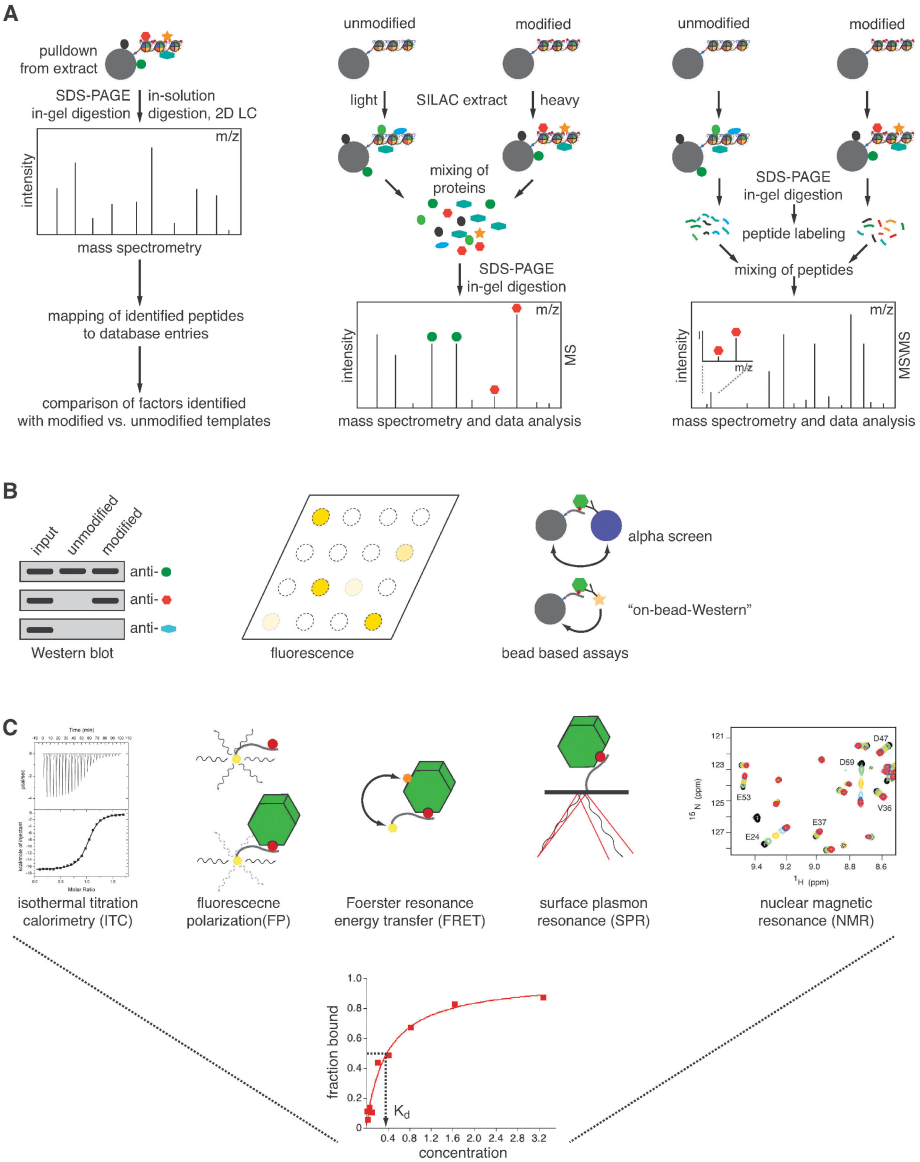

Fig. 2 Detection methods of interaction of histone modification binding proteins. (A) Proteomics approaches make use of mass spectrometry for identifying proteins bound to affinity matrices containing histone modifications (left). Relative quantification of recruitment of factors to modified and unmodified templates is possible using different labeling techniques. In SILAC approaches (middle) extracts from cells grown in normal (Lys+0, Arg+0) and heavy (Lys+6, Arg+10) medium are used. Post-purification peptide labeling (right) using *e.g.* iTRAQ or dimethyl approaches could also be applied. Note, for simplicity only affinity matrices containing nucleosomal templates are shown. The approaches for peptide-based experiments are identical. (B) Qualitative analysis of interaction can be done using standard Western blot experiments after affinity purification (left). Arrays can be developed using fluorescently labeled ligands (peptide or protein). Spot intensities allow relative comparison of interaction (middle). Bead based assays work in solution and allow selection of species containing a particular modified peptide (right). (C) Quantification of histone modification binding requires titration of the interacting protein optimally over a two log concentration range around the K_d. The dissociation constant equals the concentration at half-maximal binding. The indicated biophysical methods have been used to determine the fraction bound at a given concentration of ligand and binding protein.

arrays (H3K4me3, H3K9me3[33]). Other methods of quantitative MS that require labeling of the samples after affinity purification such as iTRAQ[66] and dimethyl[67] chemical reaction could also be useful in the global analysis of the histone modification binding interactomes but have not yet been used (Fig. 2A, right).

All MS based methods can be limited by the protein abundance in the sample, the efficiency of digestion and peptide extraction and the sampling rate and dynamic range of MS measurements. Therefore, the results contain an uncertain bias and independent methods are used to verify the interactions.

Qualitative methods

Once interaction partners of histone modifications have been identified these can be further validated using classical Western blot analysis of the SDS-PAGE gels on which samples from different affinity purifications have been run. Similar detection is also used for the peptide arrays usually employing affinity

tagged (GST or similar) recombinant histone modification-binding proteins or domains, which can be detected using antibodies against the tag (Fig. 2B, left).[53–55] In the reverse protein arrays, the histone peptides or nucleosomes bearing different modification patterns are usually marked with a fluorescent dye for direct detection (Fig. 2B, middle).[21,49,51] Alpha screen[68] and 'on bead Western'[60] assays have been used to directly mark micron sized beads carrying peptides of histone modification in solution once a specific interaction partner is bound (Fig. 2C, right).

Quantitative methods

To determine strength of interaction of histone modification-binding proteins to different marks quantitative methods are required. These allow comparison and discrimination of interactions that cannot be resolved by qualitative assays. While different physical principles form the basis for detecting the histone modification binding protein complexes formed, all approaches that have been used have in common that reactants need to be titrated below and above the concentration range of the interaction. This way the dissociation constant (K_d) can be determined (Fig. 2C, bottom). ITC (isothermal calorimetry) directly measures the heat (enthalpy ΔH) of the binding reaction. Since the K_d and stoichiometry of the interaction can be directly deduced from the titration curve, it presents the only method that provides full thermodynamic parameters including the entropy of the reaction ($nRT\ln K_d = \Delta G = \Delta H - T\Delta S$). Since it is also a label-free method, it is the golden standard for determining protein–ligand interactions.[69] Fluorescence polarization[70] and Förster energy transfer measurements,[71] in contrast, require labeling of one or both binding partners, respectively, with a fluorescent dye. This might interfere with the interaction. For surface plasmon resonance measurements ligands have to be immobilized on surfaces, which might also impede interaction. In NMR the increasing shifts of defined amino acids participating in the interaction are quantified. Since the method requires labeled (^{15}N) samples, it has not been applied widely. While most methods have been used with histone peptides, ITC and SPR have recently also been shown to be applicable to measure interaction of modified nucleosomes with binding proteins.[72,73]

Complexity of histone modification readout

From the analysis of different histone modifications as well as the study of distinct binding proteins a complex picture of readout mechanisms is emerging. The idea of one PTM mark solely responsible for recruiting a single factor for a defined downstream function is clearly far too simple.

Modification patterns

The density of PTMs on histones is extremely high. Therefore, it is not surprising that binding of a defined factor to a given histone modification can be influenced by other marks (see Table 1 and Fig. 3, left). Such higher order effects can be additive, *i.e.* a histone modification binding protein contains multiple domains for interaction with more than one PTM mark. While true cooperative binding has not yet been found, multivalent interaction[74] has been demonstrated in the case of TRIM24. The factor binds H3K4me0 *via* a PHD finger simultaneously to recognition of H3K23ac *via* a bromo domain for regulation of estrogen dependent genes.[75] Also, the DNA damage response protein MCPH1 was found to be capable of binding dual phosphorylation on H2A.XS139 and H2A.XY142 *via* adjacent BRCT domains.[76] In other cases the effects are more subtle. In the VDJ recombination factor RAG2 binding of H3K4me2/3 by a PHD finger is slightly enhanced by methylation of the neighboring H3R2 *via* a specific additional contact point in the complex.[78] Similarly, interaction of 14-3-3 proteins with H3S10ph is strengthened by adjacent acetylation of H3K9 or H3K14.[32,79] As it turns out, such additive effects are not limited to histone modifications that are in close vicinity on the same histone tail. It could be shown that in BPTF, which is a component of chromatin remodeling complexes, interaction of a PHD finger with H3K4me3 cooperates with binding of a bromo domain to

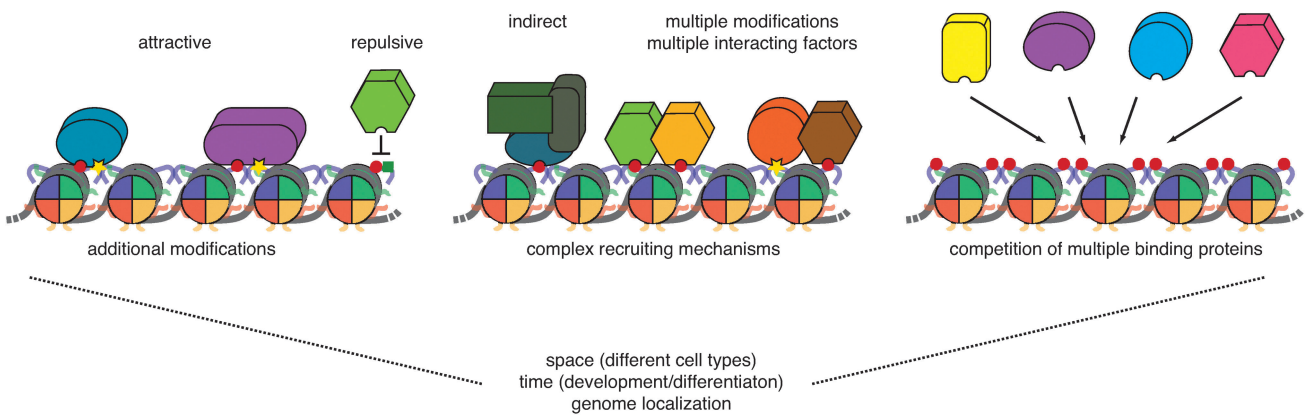

Fig. 3 Complexity of histone modification binding. Additional modifications on the same or different histones within a stretch of chromatin can have enhancing or repelling effects on the interaction of a binding protein with its cognate mark (left). Within multiprotein complexes factors can be recruited indirectly or multiple modifications might be recognized (middle). Several different binding proteins have been identified for distinct histone modifications (right). The different phenomena shown need to be integrated on different levels for highly organized chromatin regulation.

H4K16ac in the context of nucleosomes.[80] Also, the DNA methylase CMT3 binds two H3K9me2 marks on different H3 tails within a nucleosome *via* a chromo and a BAH domain.[77]

Effects of other modifications can also be negative, resulting in repulsion of a binding protein from a cognate mark (see Table 1). The phenomenon has been conceptualized as binary switching and instances of methyl–phos and methyl–methyl switches have been described.[81,82] For example, binding of the chromo domain protein HP1 to H3K9me3 is impaired by H3S10ph at the entry of mitosis[83,84] or in cellular senescence.[85] Also, interaction of Polycomb-type factors with H3K27me3 is inhibited by H3S28ph in stress and mitogenic signaling, retinoic acid-induced neuronal differentiation and myogenesis.[86,87] Similarly, methylation of H3R2 has been shown to interfere with binding of BPTF to H3K4me2/3 *in vitro.*[88]

The complexity in readout introduced by patterns of histone modifications is enhanced by the fact that their behavior cannot really be predicted. Proteins containing the same binding module can behave very differently. So does H3R2me2a/s enhance binding of RAG2 to H3K4me2/3,[78] while it inhibits interaction of ING2 with the same mark.[89]

Complex recruitment mechanisms

Different marks not only influence the recruitment of individual binding proteins, but can also contribute to simultaneous targeting of multiple factors (Fig. 3, middle). In a direct manner multiple histone modifications synergize in recruitment of more than one binding protein. These can be present in stable multiprotein complexes or transient binding of factors might be stabilized in a ternary complex. For instance, TAF3 binds H3K4me2/3 while TAF1 interacts with hyperacetylated histones both within the TFIID basal transcription factor complex.[37,90] In the PRC2 complex Eed and Nurf55 proteins read different histone methylation marks.[91,92] Transient association of HP1 with L3MBTL1 might be stabilized in the context of chromatin.[93] Other additional contacts might be formed with distinct chromatin components such as DNA or RNA. HP1 heterochromatin association seems to be dependent on RNA – albeit *via* an unknown mechanism,[94] as well as could be enhanced by the ACF1 auxiliary factor.[95] Other histone modification-binding proteins like TRIM24 appear to make contacts with transcription factors.[75]

Several proteins recruited to histone modifications contain besides binding modules enzymatic domains that appear to mediate their effects on chromatin. In contrast, other readout factors have indirect mechanisms of function. These bridge separate effector proteins to specific sites of histone modification. Factors with histone modification binding intrinsically paired with enzymatic activity include the histone acetyltransferase TIP60 that binds H3K9me3 in DNA repair[96] or/and H3K4me1 in estrogen mediated transcriptional activation,[97] the histone demethylase JMJD2A that demethylates H3K9 and binds H3K4me2/3 and H4K20me2/3 *via* a PHD finger,[22,23] the H3K9 methyltransferase G9a that binds H3K9me1/2 *via* ankyrin repeats,[98] the Dnmt3 DNA methyltransferase that binds H3K36me3 *via* a PWWP domain[99] or the chromo domain containing remodeling factor CHD1, which binds H3K4me3.[100] Indirect recruitment of enzymatic activity has been shown for HP1, which binds the Suv39 H3K9 methyltransferase,[101] ING2 that binds HDAC1,[30] L3MBTL, which binds RPD3,[102] the WD40 protein WDR5 that binds H3R2me2s and is part of the COMPASS H3K4 methyltransferase complex,[34] or BPTF, which is a component of the chromatin-remodeling complex NURF.[29]

While higher order and indirect interactions are emerging for several individual histone modification binding proteins, the hierarchies of binding seen in affinity purification approaches with global proteomics analysis are far less clear. Database comparison and sequence analysis allow in some cases to suggest direct interaction due to the presence of modules that have been implicated in histone modification recognition in other proteins. Known protein–protein interactions determined in other experiments might also help to further deduce the networks underlying the observed recruitment.[33]

Multiple binding proteins

The readout of histone marks is not fully linear, with one modification always recruiting a singular protein for downstream function. Indeed, cases where the same modification can be associated with opposing activities, *e.g.* transcriptional activation and repression have been found. H3K4me2/3 are generally considered as activating marks but these can also be linked to transcriptional repression.[30,103] The difference might be due to recruitment of distinct binding proteins, an idea that is supported by more than twelve factors that have independently been shown to bind H3K4me3 (Table 1).[19,104] Of these, ING2, for instance, brings about a histone deacetylase complex, whereas the related ING1, 4, and 5 connect with activating histone acetyltransferase activities. The global proteomics analyses of histone modification readout support this complex picture of multiple binding proteins of individual histone modifications (Fig. 3, right).[33–37,39,40,55]

While we are far from comprehending the exact mechanisms that direct recruitment of specific factors to defined histone modifications in a time (*i.e.* cellular differentiation), space (*i.e.* different cell types), and localization (*i.e.* defined regions of the genome bind a certain factor through a histone modification, but not others) specific manner, some regulatory principles are emerging. As discussed before, the modification status of adjacent sites might exert additive positive or negative effects depending on the particular factor that is recruited. Further, post-translational modification of the binding proteins can modulate the interaction with a certain PTM mark. It has for example been shown that phosphorylation of a threonine residue within the chromo domain of HP1 results in dissociation from chromatin after DNA damage.[105] In contrast, distinct phosphorylation at two serine residues at the N-terminus appears to increase affinity for H3K9me3.[106] Lastly, the expression levels of the histone modification binding proteins have to be considered. Not all factors will be present at all times. For example, during ES cell differentiation different factors of the mammalian Polycomb proteins (CBX2, CBX4, CBX7 and CBX8)

might mediate readout of H3K27me3.[38] In the future, it has to be seen how these and other regulatory mechanisms interface to direct the overall specificity of histone modification readout.

Perspective

The number of possible combinations of histone modifications within a single histone, an individual nucleosome and a short array of nucleosomes is astronomical. However, it becomes more and more clear that different groups of marks work in concert and are partially redundant.[7] Defining the set of factors that is necessary and sufficient to mediate precise downstream functions on a local (*i.e.* a transcription unit) or global (*i.e.* defined chromatin domains such as heterochromatin) level is a major challenge in the analysis of histone modification readout. Combinations of marks can be directly rebuild and globally analyzed in the newly described chromatin affinity purification schemes in combination with quantitative proteomics.[33] Producing histones of defined and uniform modification states for these analyses is, however, not (yet) straightforward.[107] Since it can be hypothesized that essential factors are simultaneously recruited directly and indirectly by different marks, systematic comparison of the interactomes of individual histone modifications that colocalize and that appear to functionally cooperate might also provide interesting insight by defining interaction networks.

Surprisingly, the available data from global analysis of affinity purification experiments targeting the same histone modifications using mammalian cell lines do only partially overlap in the interaction proteins identified.[33] This might be due to different experimental conditions, different approaches (peptide *vs.* chromatin affinity matrices) and/or the use of different cell systems and extract preparation procedures. Use of simpler model organisms (*e.g.* yeast, *C. elegans*) could be advantageous in this regard, since not only the complexity of the histone modification system is reduced in these organisms, but also because these are easily accessible to genetic manipulation for dissecting the organization and function of the histone modification interaction networks.

Besides systematic analysis of patterns of modifications and the global interplay of interaction factors, full biochemical characterization of the molecular details of interaction of individual factors with target sites is essential. Here, positive and negative effects on interaction by adjacent modifications (*i.e.* within the same histone protein) but also in the context of chromatin need to be mapped more systematically. The availability of arrays of histone peptides with multiple modification patterns is a first step in this regard.[56] Since these provide, however, only qualitative comparison, adaptation and development of high throughput quantitative assays is a major challenge. Bead based assays in combination with automated readout[60] are a first step towards full characterization of the interaction space of defined binding proteins with combinations of histone modifications.

Despite the identification and initial characterization of many histone modification binding proteins, the complexity of their interactions, the principles of their regulation and their putative interplay is only beginning to emerge. The field will without doubt benefit from further technical, experimental as well as conceptual advances.

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