OPINION

Is there a code embedded in proteins that is based on post-translational modifications?

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Abstract | Covalent post-translational modifications (PTMs) provide vast indexing potential and expanded protein use. The 'histone code' hypothesis has inspired rapid advances throughout chromatin biology, and has recently been tapped for its relevance to non-histone proteins. Comprehensive analyses suggest that rather than constituting a general code, the covalent modifications of proteins (including histones) provide surfaces that are recognized by effectors that can give rise to intricate interactions and downstream events. These are reminiscent of other regulatory cascades in transcription and cell signalling.

Biological complexity cannot simply be defined by the number of genes that an organism possesses. We have learned that more advanced species evolved additional regulatory features that allow for an increased number of functions and increased adaptability. Ample evidence suggests that these additional modes of functional indexing are not only diverse, but that they work together to accomplish a specific biological outcome.

In eukaryotic organisms, covalent post-translational modifications (PTMs) on proteins result in many specific functions. Histone polypeptides, which are intimately associated with DNA to form chromatin, are subject to many PTMs. It has been speculated that histones provide an additional layer of indexing potential to the genome, primarily through epigenetic phenomena (heritable changes in genomic function that do not occur through alterations in the DNA sequence)[1,2]. The 'histone code' hypothesis predicts that diverse covalent modifications within the highly accessible histone tails are read by effector molecules, which in turn mediate distinct outcomes. It has been suggested that these chromatin marks might function in a combinatorial manner, thereby increasing their indexing potential or capacity to store information. If these PTMs should distinguish themselves as components of a code, they could be indicative of specific biological outcomes, presuming that these patterns of marks are fully characterized at a molecular level. The 'epigenetic code' is an extension of this idea: it suggests that the downstream events that stem from code recognition facilitate epigenetic processes[2].

Histone methylation has received special attention with respect to its indexing potential because it is available in three distinct forms (mono-, di- and trimethylated forms), and has slow turnover *in vivo* under normal cellular conditions[3–7]. This allows for the long-term propagation of information to progeny. More recently, a growing number of reports have shown that non-histone proteins are methylated[8,9] (TABLE 1). Some of these methylated targets are also subject to different types of PTMs, thereby drawing comparisons to histone tails. In some examples, single Lys residues within non-histone proteins can be targeted by varying types of PTMs that provide additional regulatory layers. As such, it has been speculated that the same principles that apply to a putative histone code can also apply to non-histone proteins[10,11]. It is important to examine the evidence that supports the existence of a histone and/or protein code in consideration of the consequences that this framework would have for predicting downstream events.

The protein code concept

Protein phosphorylation is the most extensively studied PTM and has been shown to have important roles in most, if not all, cellular processes. In particular, phosphorylation has been well established to promote or inhibit protein–protein interactions. For example, the Src-homology-2 (SH2) domain selectively recognizes phosphorylated Tyr residues in signalling molecules and facilitates distinct cellular pathways in response to external cues[12]. Under defined conditions, these phosphorylation events can be predictive of distinct biological events. For example, Tyr autophosphorylation of the platelet-derived growth factor (PDGF) receptor allows the docking of SH2-domain-containing phospholipase Cγ kinase to these modified Tyr residues, which in turn activates the inositol phospholipid-signalling pathway[13]. Therefore, from a restricted point of view, non-histone-protein phosphorylation abides by the basic principle of a code — that PTMs can be predictive of biological function.

By definition, a protein code suggests that PTMs (either single PTMs or PTMs in combination) are read by effector molecules that are predictive of specific downstream events. In essence, these principles are no different than those proposed for the histone code. PTMs of histones have been clearly shown to create binding surfaces for effector molecules that mediate specific biological events[14,15]. However, most experimental data suggest that PTMs on histones are poor predictors of function at a molecular level.

PTMs can present chemical surfaces that can be recognized by particular effector molecules. In some examples, these PTM-recognition surfaces might be combinatorial in nature. However, this is consistent with typical protein–protein interactions. For example, if transcription factor X binds to a specific DNA element within a promoter region, a particular coregulator might be recruited by associating with factor X. However, if a second transcription factor, Y, binds to the same promoter through a different DNA element, the outcome to transcription could be markedly different. The interaction between factors X and Y could create a protein surface that recruits a

Table 1 | **Protein Lys methylation in chromatin and transcription**

Enzyme	Histone target	Non-histone target	Methyl effector	Downstream effect
SET1*	H3K4me1		BPTF	Chromatin remodelling
			CHD1	Post-initiation events
			ING2	Histone deacetylation
			JMJD2A	Demethylation?
			RAG2	V(D)J recombination
			Yng2 (yeast)	Histone acetylation
		Dam1K233me1 (yeast)	None?	Antagonizes Dam1 phosphorylation
SET9	H3K4me1		?	As for SET1 (see above)
		TAF10K189me1	?	Stabilizes protein associations
		p53K372me1	TIP60	p53 activation
		ERαK302me1	?	ER activation
SMYD2	H3K36me1		Eaf3 (yeast)	Chromatin maintenance
		p53K370me1	?	p53 repression
?		p53K370me2	53BP1	p53 activation
SMYD3	H3K4me1		?	As for SET1 (see above)
		VEGFR1K831me1	?	Enhanced VEGFR1 activity
PR-SET7	H4K20me1		L3MBTL1, others	Chromatin compaction
		p53K382me1	?	p53 repression
G9a‡	H3K9me1		HP1, others	Gene silencing
	H1K26me1		L3MBTL1	Chromatin compaction
		G9aK94me1	?	?
		G9aK165me1	HP1, CDYL	?
		GLPK133me1	?	?
		GLPK185me1	HP1, CDYL	?
		Others‡	?	?

*In higher organisms, H3K4 methylation is also carried out by the MLL family of methyltransferases. ‡G9a was recently shown to methylate an assortment of substrates, although whether these targets are methylated by G9a in cells remains unknown[49]. Rubisco, cytochrome *c*, RPL23AB, and RPL12 are also methylated, although the biological significance of these modifications is unknown[9]. CHD1, chromo-domain-helicase-DNA-binding protein-1; ER, endoplasmic reticulum; HP1, heterochromatin protein-1; SET1/9, SET-domain-containing protein-1/9; SMYD1/2, SET and MYND domain-containing protein-1/2.

distinct coregulator (FIG. 1a). Given the data that has been accumulated thus far, PTMs on histones and non-histone proteins are analogous to the example discussed above as the combinatorial influence of multiple proteins, their interactions and activities can account for disparate outcomes. The context of the PTM is a pivotal consideration; for example, the distance between the two DNA elements in another promoter might deter contact between factors X and Y.

In isolation, trimethylated histone H3 at K4 (H3K4me3) is an ideal modification to examine the principles of a code because so much is known about its global positioning, recognition and downstream consequences[14,16–19]. H3K4me3 is a histone modification that is typically associated with active transcription[14,20]. During transcription, H3K4me3 recognition facilitates subsequent histone acetylation and post-initiation events, such as transcript elongation and pre-mRNA processing[18,19] (FIG. 1b). Chromatin remodelling, methyl propagation and histone demethylation during active transcription are probably also supported by H3K4me3, although there is a lack of direct evidence for this[14]. Under conditions of DNA damage, H3K4me3 is used to silence transcription through the recruitment of a repressor complex[17]. Apart from gene expression, recent studies have identified that H3K4me3 recognition can facilitate V(D)J recombination[21], a mechanism of DNA recombination that occurs in vertebrates and in which segments of genes that encode specific proteins with important roles in the immune system are assembled. Therefore, this single modification regulates transcription in a positive and negative manner, and regulates DNA recombination — a completely dissimilar process. Thus, H3K4me3 cannot be predictive of function without considering cellular context.

A second example is provided by histone H3K9me1, which is associated with constitutive heterochromatin — regions of chromatin that are refractory to active transcription[22]. However, this modification is also present at several actively transcribed genes[23]. Therefore, a unique modification (H3K9me1) can have very different meanings, depending on the location of the modification. We can attempt to explain the presence of H3K9me1 at several active genes by inferring chromatin context, but how can this be consistent with the basic principle of a code?

A third example is provided by phosphorylation at histone H3 S10 (H3S10ph). This PTM was known to be a marker of chromosome condensation during metaphase of mitosis and meiosis. It also has a role in transcriptional activation, and is induced at gene promoters. Chromosome condensation and transcriptional activation are unrelated processes and presumably involve very different chromatin effects (broad compaction compared to localized decompaction, respectively). Therefore, functional and structural predictability cannot be based solely on the presence of the H3S10ph PTM.

These examples indicate that if modifications are predictive, then additional layers of specificity must exist that are either embedded within histones (combinatorial histone marks) or that use entirely different mechanisms. Not one known combination of modifications that includes H3K4me1 can explain the diversity of H3K4me1 function. Increasing complexity of PTMs requires a more general concept that expands on the concepts suggested by the term 'code', as the term inevitably invokes comparisons with the universal triplet genetic code that dictates protein composition (see below).

The concept of a protein code has gained most attention when relating to proteins that are modified by multiple PTMs, because these are suggestive of increased indexing potential. Both p53 and the C-terminal domain (CTD) of the largest subunit of RNA polymerase II are targeted by multiple modifications within a defined region and both of these proteins have evoked histone code

comparisons. Although numerous non-histone proteins are subject to methylation and other modifications[8,9] (TABLE 1), p53 is methylated on at least three sites within the middle of its highly modified regulatory domain. We therefore consider p53 to be a model for examining the principles of a protein code.

p53 as a model

p53 is the most commonly mutated gene in all forms of cancer[24] and is therefore likely to be the most scrutinized protein in biology. p53 has an N-terminal transactivation domain, a central DNA-binding domain and a C-terminal regulatory domain that is subject to a high degree of PTM (FIG. 2a). p53 can be modified by methylation, acetylation, phosphorylation, ubiquitylation, sumoylation and neddylation[25]. Many of the enzymes that methylate and acetylate histones also target the C-terminal domain of p53 (REFS 9,26). Similar to histones, several of these individual p53 sites can be modified by multiple types of PTM. This means that the presence of one modification excludes or prevents the addition of another modification at that same site and therefore dictates the function of that residue. A clear example from histone biology is that acetylation on H3K9 (described above) prevents its methylation at this same position. Thus, the mere presence of the activating H3K9ac mark blocks a cascade of H3K9 trimethylation, its subsequent recognition by heterochromatin protein-1 (HP1) and ultimately gene silencing[27].

p53 is acetylated by an assortment of histone acetyltransferases (HATs), including CREB-binding protein (CBP), p300/CBP-associated factor, and TIP60 (REF. 26). p53 activation correlates with its acetylation, which stabilizes DNA binding and facilitates interactions between p53 and its co-activators[28,29]. Similarly, histone acetylation near promoters corresponds with active transcription, and is thought to function in part by reducing histone–DNA interactions. In addition, effector molecules contain bromodomains, which recognize acetylated Lys residues[30,31]. Chromatin-remodelling complexes often contain subunits with bromodomains that are anchored to hyper-acetylated nucleosomes[32]. In *Saccharomyces cerevisiae*, the tandem bromodomain of the Rsc4 subunit of the chromatin-remodelling complex RSC recognizes acetylated Lys residues on both histones and the non-histone protein Rsc4 (REF. 33). Thus, non-histone proteins can also signal through acetyl-Lys recognition to mediate specific molecular events.

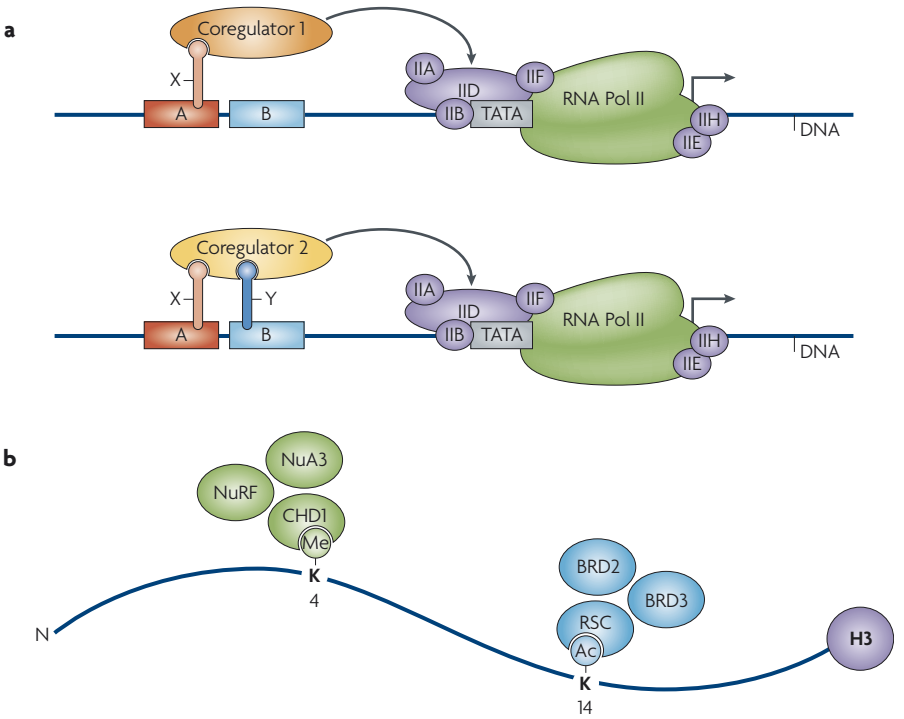

Figure 1 | **Different modes of functional readout during transcription. a** | The single or combinatorial presence of DNA-binding proteins (orange or blue lollypops) can dictate specific functional outcomes, depending on the binding surfaces that are present for recognition by different coregulators with varying transcriptional effects. RNA polymerase II and the general transcription factors are indicated. **b** | One histone modification (trimethylated histone H3 at K4 (H3K4me3) or acetylated histone H3 at K14 (H3K14ac)) can be read by several proteins or protein complexes, each of which results in different downstream events. The NuA3 histone acetyltransferase complex, nucleosome-remodelling factor (NuRF) and chromodomain helicase DNA-binding protein-1 (CHD1) chromatin remodellers individually associate with H3K4me3, whereas the RSC remodelling complex and the transcript elongation-associated factors bromodomain-containing protein-2 (BRD2) and BRD3 each associate with acetylated Lys residues through their bromodomains. Ac, acetylation; Me, methylation.

By contrast to acetylation, p53 methylation can either activate or suppress p53-related activities, depending on the site and degree of methylation (mono-, di- or trimethylation) (FIG. 2a). The first enzyme to be shown to methylate p53 was the methyltransferase SET-domain-containing protein-9 (SET9), which targets K372 (REF. 34). SET9 was originally shown to monomethylate Lys residues of histone H3K4, although this activity is prevented by nucleosomes[35,36]. More recently, SET9 has been shown to target various non-histone substrates in addition to p53 (TABLE 1). Monomethylation of K372 by SET9 facilitates the stabilization and activation of p53 (REF. 34). Genetic studies in mice corroborated the role of SET9 and of K372 methylation in p53 activation. Furthermore, elimination of K372 methylation also resulted in diminished recruitment of the TIP60 HAT complex and subsequent p53 acetylation[37]. This crosstalk of p53 PTMs draws clear analogies with that of histones, as site-specific methylation on histone H3 facilitates acetylation on nearby residues[18] (FIG. 2b). SET9 also methylates the transcription-initiation factor TAF10, the oestrogen receptor and presumably other non-histone proteins. The only general statement that can be drawn regarding SET9-mediated methylation of proteins is that they function in activating pathways, but whether this constitutes a more general, predictive signal for TIP60-mediated acetylation is currently unknown.

p53 K372 methylation has been shown to prevent SET and MYND domain-containing protein-2 (SMYD2)-mediated monomethylation of p53 at K370, which otherwise serves to repress p53-related activities[38]. SMYD2 is a Lys methyltransferase that also targets histone H3 on K36 (REF. 39). SMYD2-independent dimethylation of p53 K370 by an unknown methyltransferase activates p53, a process that is reversed by LSD1, the first Lys demethylase to be discovered[40]. In addition to p53K370me1, monomethylation of p53 on K382 also functions to repress

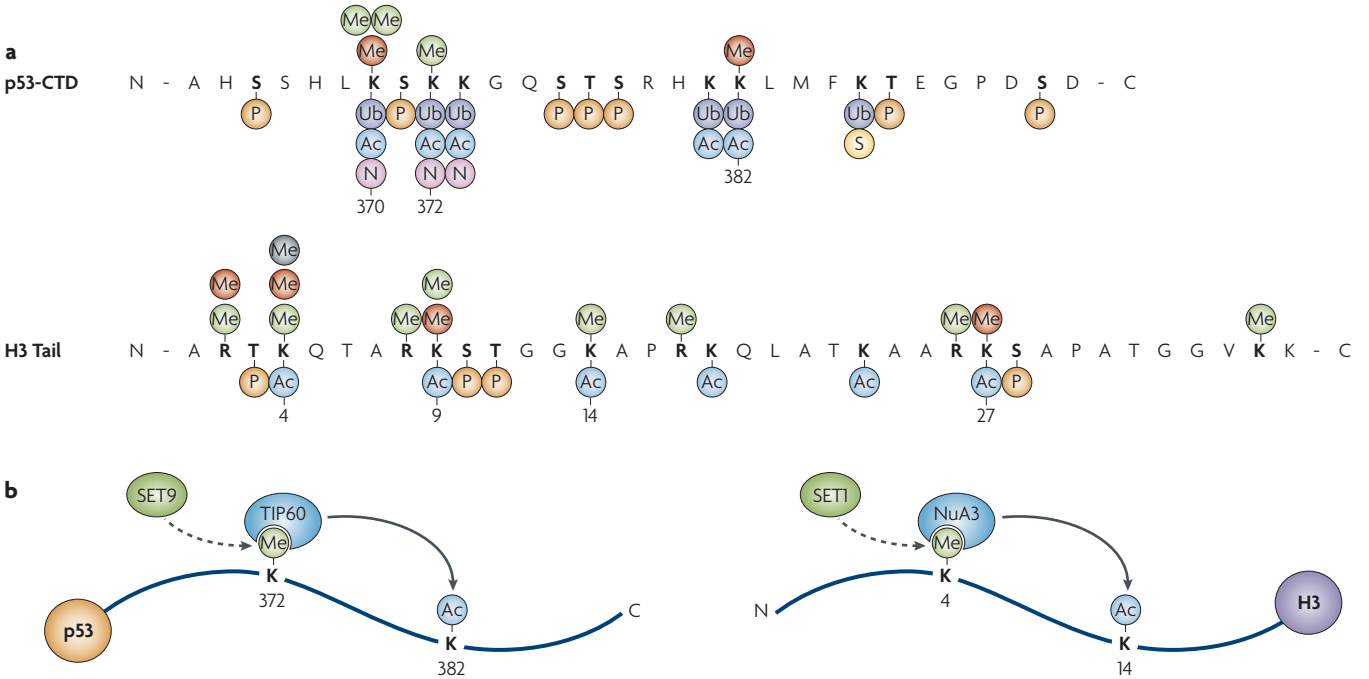

Figure 2 | **Post-translational modifications. a** | Schematic diagram of the covalent post-translational modifications (PTMs) of p53 and the histone H3 N-terminal tail. The different modifications are indicated (P, phosphorylation, shown in orange; Ub, ubiquitylation, shown in purple; Ac, acetylation, shown in blue; S, sumoylation, shown in yellow; N, neddylation, shown in pink). Methylation (Me) is shown on top, with green and red indicating the methyl marks that are associated with activation or repression, respectively. Methyl marks that are shown in black are associated with processes that are not associated with transcription. **b** | The functional interplay between methylation and acetylation that occurs in p53 and histones. Methylation of p53 K372 (p53K372me1) by SET-domain-containing protein-9 (SET9) facilitates the recruitment of theTIP60 histone acetyltransferase (HAT) complex and the subsequent acetylation of p53K382. Similarly, the SET1 histone methyltransferase methylates H3 K4 (H3K4me1), thereby facilitating the recruitment of the NuA3 HAT complex and the acetylation of H3K14 (see main text for details). Thus, signalling pathways that involve Lys methylation and acetylation occur on both histone and non-histone proteins in a similar fashion. Figure part **a** is modified, with permission, from REF. 25 © (2007) Cold Spring Harbor Laboratory Press.

p53 activities[11]. p53K382me1 is catalysed by PR-SET7, an enzyme that was previously shown to target nucleosomal H4K20 (REF. 41). Accordingly, PR-SET7 protein levels decrease following DNA-damage-induced p53 activation[11]. Thus, at least four distinct Lys methyltransferase enzymes target p53 for methylation, each of which directs different downstream events (TABLE 1). Overall, p53 shares striking similarities with histones in that it can be regulated by varying degrees of methylation, it has site-specific functional activities and because its regulation can be reversed by demethylation. Do these observations support the existence of a protein code?

Is there a protein code?

The defining principles of the term 'protein code' will undoubtedly draw comparisons to the genetic code, which has a strict amino acid readout that is dependent on the integrity of the tRNA (for example, AUG codes for a Met residue). Can the principles of a protein code be as universally predictable as the genetic code? The functional consequences of p53 methylation (or histone modifications) seem to be quite specific and direct with respect to either activation or repression activities. In this scenario, some PTM patterns could be viewed as an accurate predictor of biological outcome (either activation or repression). Is this simply because we know less about the mechanistic causes of downstream events? As we uncover how H3K4me3 is involved in biological functions, it has become clear that this modification signals in many different directions. We predict that p53 methylation will be no different. However, it is reasonable to assume that some protein modifications are predictive of function under carefully defined settings. How general is this predictability?

In a comparable way, phosphorylation of the CTD of the largest subunit of RNA polymerase II occurs in distinct patterns that result in clear functional processes. These processes are related to mRNA maturation events[42]. Similar to histone methylation, CTD phosphorylation creates a binding platform for the effector molecules that selectively recognize these PTMs. In turn, this recognition allows the nucleation of many factors that facilitate elongation, pre-mRNA capping, splicing, polyadenylation and termination[42]. Extensive analysis of CTD phosphorylation has shown a tight association with active transcription. In general, this PTM is also a relatively good predictive indicator of function with respect to gene expression. However, when looking at specific downstream activities on a mechanistic level, different patterns of CTD phosphorylation result in varying processes. In this context, specific CTD patterns do not predict what molecular events will follow. How specifically must we define the readout of PTMs to generalize the existence of a protein code?

Evidence that supports the existence of a code comes from phosphorylation events adjacent to histone-tail Lys residues that are subject to methylation or acetylation. In this example, factors that recognize methyl-Lys residues fail to bind their substrates owing to steric interference of nearby phosphorylated Ser or Thr residues. Indeed, trends detecting these outputs have been observed[43]; however, this phospho–methyl interplay[44] is simply chemical alterations in

binding surfaces, and is no different from the example discussed above that describes SH2-domain binding to phosphorylated Tyr. Again, the best indicator of whether a protein code is truly predictive of function comes from the examples illustrated above for histone H3K4me3, H3K9me1, and H3S10ph. In each of these examples, the PTM cannot be viewed in the same light as the genetic code, because cellular context dictates the functional readout. If the histone code fails to be an absolute predictor of function, then so does the protein code. As argued above, the PTMs on proteins seem to function with similar biological complexity as the PTMs on histones.

When considering PTMs as indicators of function, it should be noted that unmodified Lys residues have been shown to behave in much the same way as modified Lys residues. Unmodified histone H3K4 is selectively recognized by a subunit of the LSD1 demethylase complex, which facilitates its repressor activities *in vivo*[45]. Modification of H3K4 disrupts the association of this LSD1 sub-module, and structural studies indicate that the unmodified H3K4 residue is 'read' in much the same way as its methylated version[45]. In addition, some factors recognize methylated H3K4 only when H3 is unmodified at R2 (H3R2), which suggests that specific pathways of H3K4 methyl signalling should be examined in the context of unmodified H3R2 (REFS 46–48). Thus, the functional predictability of PTMs must also incorporate the patterns of unmodified amino acids when required. A protein code that must consider unmodified residues in a combinatorial manner would be difficult to process on a genome-wide scale because of its enormous complexity.

The concept of a code was initially appealing because it proffered predictability and a set of basic principles that can be reasonably defined. The term code implies a broad scope to its meaning. Although specific examples of PTM-driven predictability exist when using both molecular and more generalized readouts, ample evidence from histone biology show the importance of cellular context when considering the 'meaning' of a covalent modification. In light of data accumulated, the patterns of protein (specifically histone) modification seem to be consistent with their being protein signatures with varying readouts. Importantly, the term signature does not elicit direct comparisons to the genetic code, but rather relies on well-appreciated regulatory mechanisms that involve protein–protein interactions, protein conformational changes and/or cascades of activities. It might remain useful to consider isolated examples of PTM predictability, but we feel it is not useful to over-generalize the meaning of PTMs, as we clearly have much to learn regarding their function.

Conclusions

The word code in conjunction with protein or histone PTMs raises comparisons with the genetic code or the transfer of information (DNA–RNA–proteins). However, such a general designation is not corroborated by the reported outputs that are associated with different protein modifications (including histone modifications). The surfaces presented by unmodified proteins and modified proteins in isolation, particularly within the context of other surfaces, constitute a gamut of potential targets for effector molecules that can then give rise to a range of outputs. Although exceptions to every rule in biology undoubtedly exist, it is important to refrain from over-generalizing concepts that limit our scope of their meaning. At the same time, the challenge remains to find unifying themes in biology that further our understanding of a particular area, as the concepts underlying the protein and histone codes have done in the past.

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DATABASES

InterPro: http://www.ebi.ac.uk/interpro/SH2
UniProtKB: http://ca.expasy.org/sprot
CBP | histone H3 | LSD1 | p53 | Rsc4 | SET9 | SMYD2 | TAF10 | TIP60

FURTHER INFORMATION

Danny Reinberg's homepage: http://www.med.nyu.edu/biochem/ReinbergLab/index.html

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