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Identification and characterization of 'readers' for novel histone modifications

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Histone readers recognize histone modifications and mediate downstream biological events. A series of strategies to identify new histone readers have been developed and improved recently. Asides from the traditional pull-down methods and protein structure/function based educated guess, crosslinking and high-throughput screening based strategies led to the discovery of many new histone readers. In this review, we reviewed the rationale and applications of photo-affinity lysine based crosslinking strategies and array/designer nucleosome libraries based high-throughput screening strategies. Epigenome editing technologies to incorporate histone modifications in cells were also discussed. Finally, we summarized the newly identified histone readers (e.g. ZZ domain and Agenet domain) and histone modifications (e.g. serotonylation and benzoylation).

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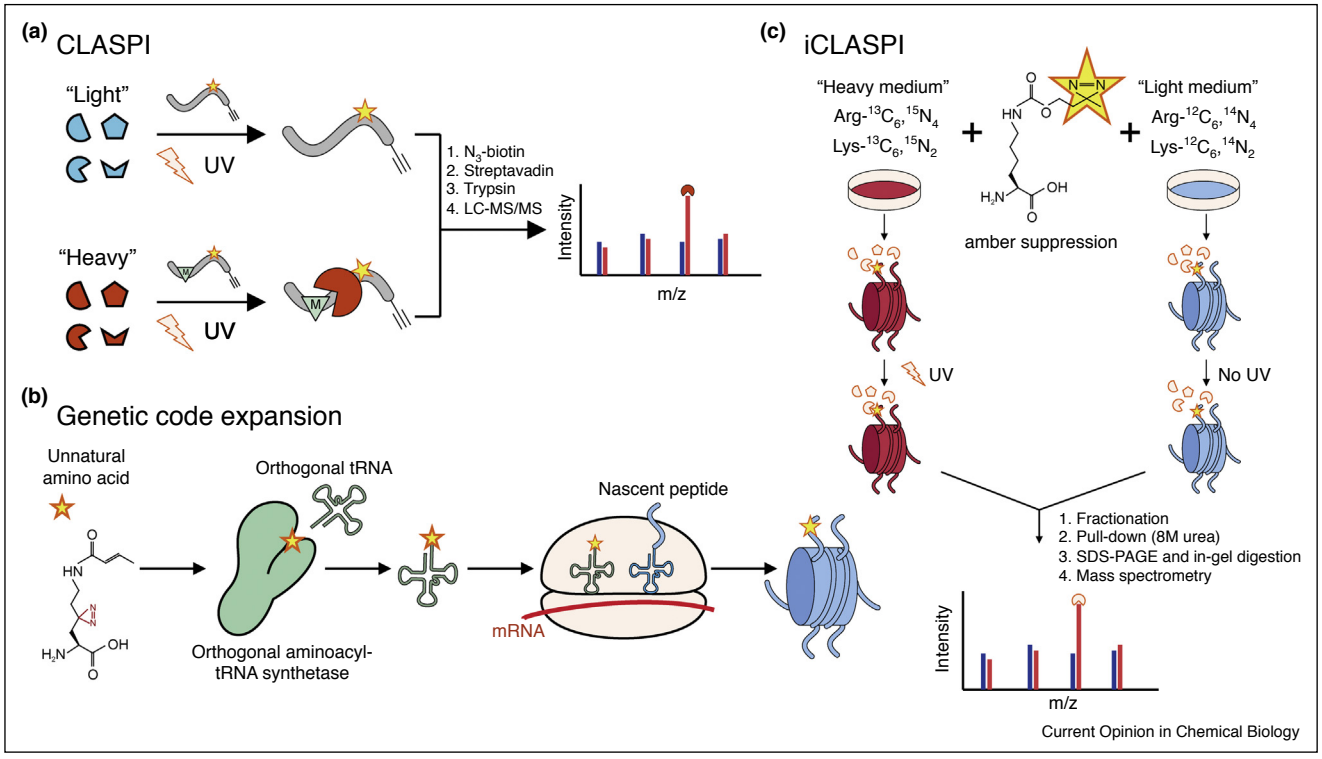
Introduction

Histone modifications constitute one of the crucial epigenetic mechanisms regulating gene expression. Proteins recognizing histone modifications are called 'histone readers'. A plethora of histone modifications have been identified till now, such as methylation, acetylation and phosphorylation. Histone readers recognizing these modifications have also been identified and proved to be crucial in regulating transcription and human disease. Several excellent reviews written earlier are available on systematically summarizing histone readers and histone combinatorial readout [1–3]. The traditional pull-down method using modified histone peptides or tagged proteins led to the discovery of many histone readers, such as WDR5, BPTF and ING2 [4–6]. The educated guess based on protein structure and function also led to the discovery of various histone readers. For example, the first histone lysine acetylation (Kac) reader module bromo domain was identified based on its structure and functional relationship to histone acetylation [7]. There were also many other examples such as the identification of the BPTF as an H3K4me3-H4K16ac reader, the Spindlin1 as an H3K4me3R8me2a reader and the AF9 as a histone lysine crotonylation (Kcr) reader [8–10]. Asides from traditional pull-down based strategies and educated guess, many new methods were developed to identify histone readers with better sensitivity, accuracy and throughput. In this review, we focused on chemical biology based technologies to identify new histonereaders. We also highlighted newly identified histone readers and histone modifications in recent two years.

Photo-affinity lysine based crosslinking strategy

The photo-crosslinking based pull-down could convert transient interactions into irreversible covalent bonds, making the identification of weak interactions more efficient. Li and Kapoor proposed the rationale of developing photo-affinity probes to identify histone PTM readers [11]. They then combined photo-crosslinking strategy with SILAC (stable isotope labeling with amino acids in cell culture) and developed an approach termed crosslinking-assisted and SILAC-based protein identification (CLASPI) [12]. As shown in Figure 1a, the photo-affinity group labeled unmodified and modified histone peptides are pull-downed with 'light' and 'heavy' labeled nuclear extract, separately. The pull-downed proteins are further analyzed by LC–MS/MS. By adopting this strategy, they identified several histone readers such as MORC3 and Spindlin1 [12]. Similarly, Zhang group utilized the self-assembled multivalent photo-crosslinking technique and designed multivalent probes to more efficiently enrich histone readers [13••]. Li group further developed a photo-affinity lysine amino acid by incorporating the photo-crosslinker diazirine into the side chain of lysine. The photo-lysine could be recognized by the native protein biosynthesis machinery. The photo-affinity lysine could thus replace natural lysine in the medium and UV irradiation could facilitate crosslinking *in vivo* [14••]. However, this incorporation of photo-lysine is residue-specific instead of site-specific incorporation. In order to incorporate photo-affinity lysine into specific site of histone, Chen and Li group

Figure 1



The schematic diagram of photo-crosslinking based strategies. **(a)** The flowchart of CLASPI technology. The yellow star represents the photo-crosslinker. The green triangle represents the histone modification. **(b)** The flowchart of genetic code expansion technology. The yellow star represents the K*cr residue. **(c)** The flowchart of iCLASPI technology. The photo-crosslinker is highlighted under the background of a yellow star.

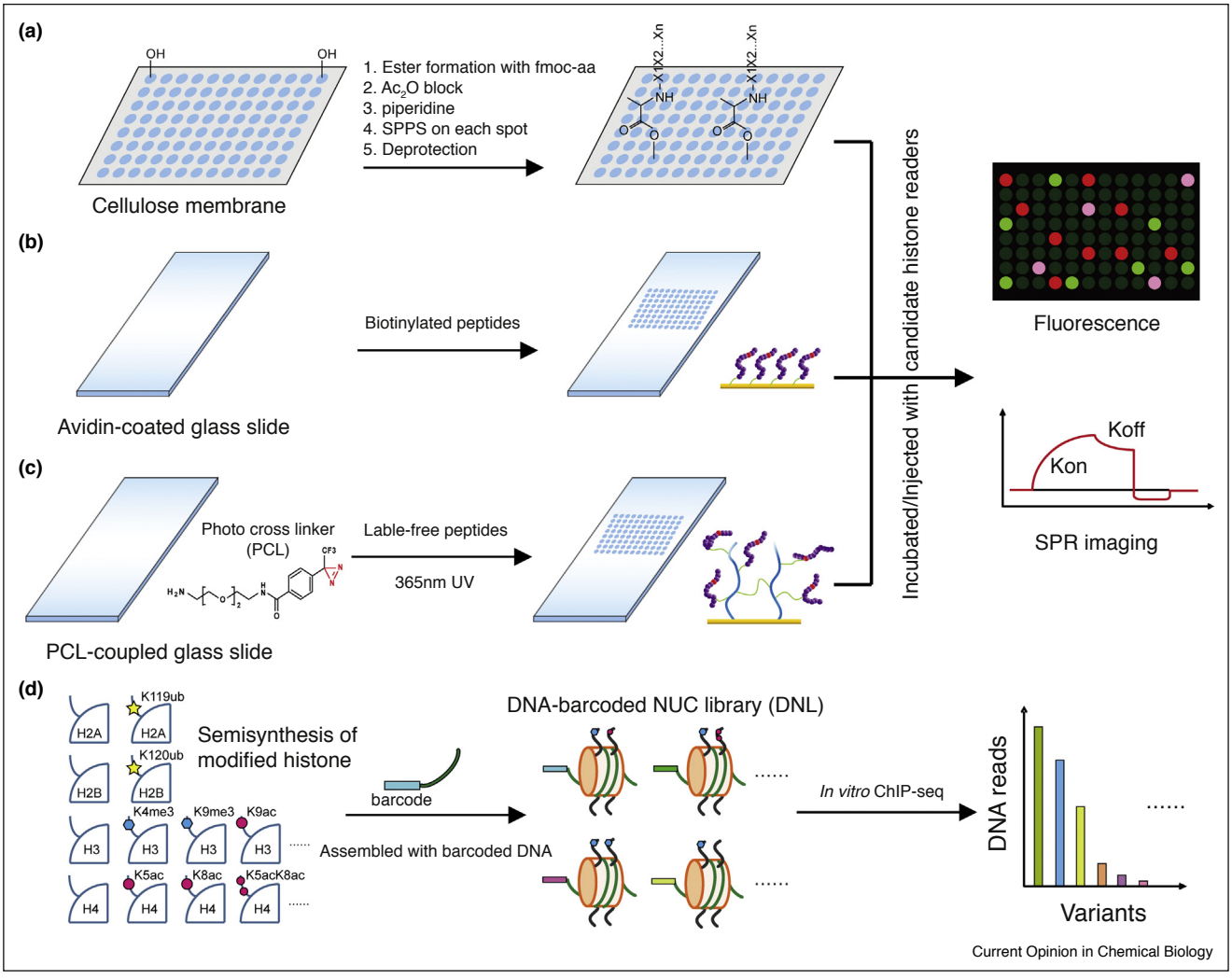
adopted the genetic code expansion strategy and found the K*cr-PylRS bio-orthogonal pair [15••]. The K*cr (chemical structure shown in Figure 1b) was the photo-affinity Kcr analogue that could crosslink binding effectors upon UV irradiation. The screened aminoacyl-tRNA synthetase(L309A/C348F/Y384W) could efficiently incorporate K*cr into the nascent peptide (Figure 1b). This strategy could be further developed to incorporate more photo-active lysine modifications into histone. Recently, a technology termed iCLASPI (*in vivo* CLASPI) combined CLASPI and genetic code expansion strategies was capable to site-specifically introduce photo-active lysine modifications *in vivo* and identify histone effectors in proteomic level [16••]. The authors site-specifically introduced adiazirine-modified lysine derivative (chemical structural shown in Figure 1c) to histones H3 and H4 and profiled interactomes by the SILAC-based proteomics (Figure 1c). By using this strategy, the authors profiled cell-cycle-specific interactions in cells arrested in mitosis and identified ANP32A as a chromatin effector at this stage [16••]. With the fast development of these photo-affinity probe based crosslinking strategies, we envision that the identification of transient and cell-type/stage-specific histone 'mark-reader' interactions would be much more facilitated.

Array/DNLs based high-throughput screening

Array-based technologies are able to identify histone readers in high-throughput. Both potential histone reader proteins and modified histone peptides could be immobilized on the chip. When potential histone reader proteins were immobilized on the chip, the protein array could be used to screen modified histone peptides or small molecules. Bedford group developed a chromatin-associated domain array (CADOR) chip, which contained bromo, chromo, tudor, PHD, SANT, SWIRM, MBT, CW, and PWWP domains. By utilizing this CADOR chip, they discovered TDRD3 was a reader of H3R17me2a and H4R3me2a [17]. Recently, they also demonstrated that the protein array was able to discover small molecule inhibitors [18•].

The peptide arrays are widely used in identifying new histone readers. The SPOT synthesis peptide array was generated by direct synthesis of peptides on the cellulose sheets (Figure 2a). By utilizing this strategy, Jeltsch group identified the PWWP domain of Dnmt3a as an

Figure 2



The schematic diagram of peptide-arrays/DNLs based high-throughput screening strategies. **(a)** The flowchart of SPOT synthesis peptide array technology. **(b)** The flowchart of biotin-labeled peptide array technology. Purple strings represent the synthesized peptides. These peptides are immobilized on the array in the same orientation. **(c)** The flowchart of carbene-based SPRi technology. The chemical structure of the photo cross linker (PCL) is shown with the photo-affinity group highlighted in red. Purple strings represent the synthesized peptides. These peptides are immobilized on the array in random orientations. **(d)** The flowchart of DNA-barcoded nucleosome library strategy.

H3K36me3 reader and the ADD domain of Dnmt3a/3L as an unmodified H3 reader [19,20]. An impressive work using SPOT synthesis peptide array was the large-scale binding and structural analysis of human bromo domain proteins [21]. The authors screened more than 30 representative bromo domain proteins binding to the peptide array containing all possible human histone Kac sites. Through this screening work, the authors identified 485 linear Kac-dependent bromo domain binding motifs and further confirmed representative interactions by isothermal titration calorimetry and crystallography.

The avidin array could immobilize biotinylated peptides efficiently in high-throughput. The printed peptide arrays were incubated with candidate reader proteins and the signal was detected by fluorescence (Figure 2b). Because of the convenience and high-throughput of the peptide array strategy, many histone readers were identified through this method. For example, Gozani group identified the BAH domain of ORC1 as an H4K20me3 reader by screening BAH domains binding to the peptide microarray containing 82 types of histone peptides [22]. Shi group utilized peptide array and identified several histone readers such as H3.3K36me3 reader ZYMND11 and histone acetylation reader AF9 [23,24]. Recently, Strahl groups utilized the peptide array and identified many new interactions, including interactions between H3K23me3 and chromo domain proteins [25•].

By combining photo-crosslinking and peptide array strategies, we developed a 3D-carbene based SPR imaging (SPRi) platform to identify histone readers [26••]. The 3D-carbene surface was constructed on the gold slide and the label-free peptides could be cross-linked by radiation at 365 nm UV light (Figure 2c).The carbene crosslinking resulted in random immobilization and the SPRi platform could detect binding events in real time and high-throughput. The random immobilization could help eliminate the tag or labelling induced false positive and false negative results. Weak interactions ($K_D > 100\ \mu M$) could also be effectively detected due to the kinetic detection. The tudor domain of plant MSH6 was identified as an H3K4me3 reader in this work.

The high-throughput screening of histone readers could also be performed at the nucleosome level. Muir group reported the platform identifying chromatin effectors at the nucleosome level by the combination of chemically defined DNA-barcoded nucleosome libraries (DNLs) and the next-generation sequencing [27]. The semi-synthesized modified histones were reconstituted into nucleosomes with barcoded DNA (Figure 2d). *In vitro* ChIP-seq was performed based on the designed DNLs and the chromatin effector of interest. The authors demonstrated that the DNLs based strategy was efficient in screening histone modification readers and studying histone cross-talk. This platform showed unique advantages on studying histone modifications cross-talk and combinatorial readout at the nucleosome level. Collectively, these array/DNLs based high-throughput screening strategies facilitated the identification of histone readers provided that the candidate proteins have been identified.

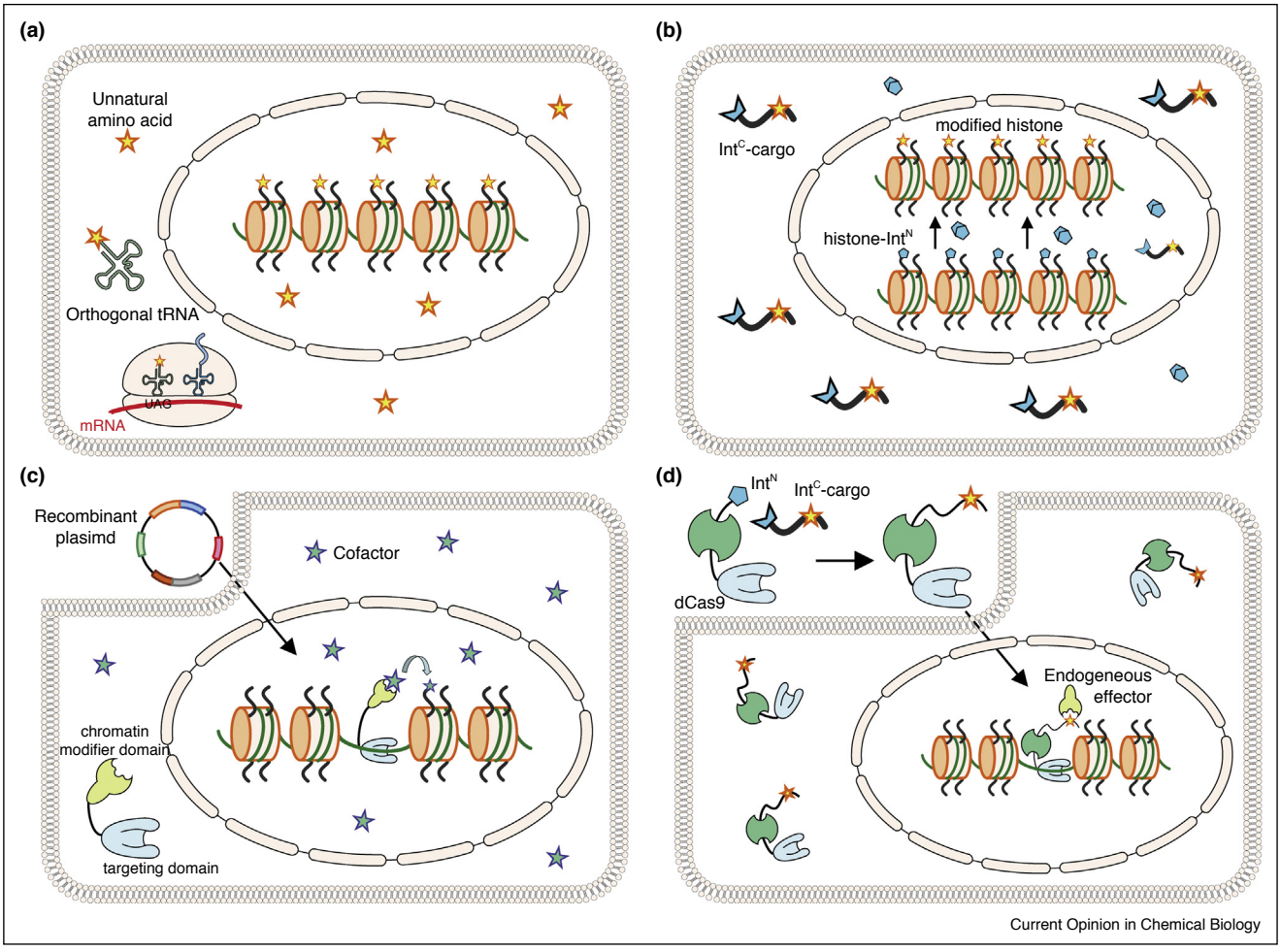
Modifying chromatin in cells and chromatin phase separation

More works were reported to incorporate histone modifications in cells or even at specific chromatin locus. Genetic code expansion strategy has been proved to be an efficient way to site-specifically introduce histone modifications *in vivo*. By developing orthogonal tRNA/tRNA-synthetase pairs, modified residues could be introduced into various cell types (Figure 3a). By developing an integration expression system, Chin group replaced six lysine residues with acetylated lysine residues, demonstrating its powerful ability to study histone combinatorial readout [28]. Asides from the genetic code expansion system, split inteins based strategy could also site-specifically incorporate histone modifications *in vivo* without additional chemical groups [29]. Inteins are autocatalytic proteins that could function as a 'protein ligase'. The intein could be fractured into two pieces, Int^N and Int^C, and the two subdomains could be expressed or synthesized in fusion with two proteins/peptides/small-molecules (Figure 3b). Muir group developed a method to engineer histones *in vivo* based on the ultrafast trans-splicing inteins [30]. In this work, the C-terminus of histone H2B was fused with Int^C and various histone modifications were fused with Int^C. The authors also achieved the modification of histone N-terminus using the same scheme. One advantage of split inteins strategy is that one does not need to screen orthogonal tRNA/tRNA-synthetase pairs upon different histone modifications. Moreover, some large size modifications such as ubiquitin are hard to be incorporated by the aminoacyl-tRNA synthetase. These manipulation methods of chromatins pave the way for characterizing the mechanism of histone readers *in vivo*.

With the development of genome editing techniques such as dCas9 system, the locus-specific modification of chromatin regions become much more efficient. Cas9 could be able to target specific chromatin locus through designed guide RNA [31]. The locus-specific chromatin manipulation is achieved by combining chromatin modifying enzymes (chromatin modifier domain) with targetable DNA binding domain (targeting domain) (Figure 3c). Through this strategy, various chromatin modifiers such as histone methyltransferase and HDACs, were introduced into specific chromatin locus *in vivo* [32]. Muir group combined the dCas9 system and the split inteins strategy and reported a method to incorporate synthetic molecules into the specific chromatin locus [33••]. The authors fused dCas9 system with Int^N and deliver small molecules/peptides with Int^C by chemical ligation (Figure 3d). By utilizing this method, they introduce (+)-JQ1, a potent bromo domain inhibitor, into specific genome locus. This introduced (+)-JQ1 could be viewed as a Kac modification with stronger binding affinities and specificities to bromo domains but could not be removed by HDACs. More importantly, multiple (+)-JQ1 molecules could be introduced *in vivo* as a branched cargo, creating a multivalent modification chromatin locus. These multivalent and stable modifications are important for histone reader studies, and may have intriguing potential for chromatin phase separation research.

Phase separation is a widely observed phenomenon in various biological events [34]. Recently, histone reader mediated chromatin phase separation phenomena were reported at many chromatin regions, such as HP1 mediated heterochromatin phase separation and BRD4-MED1 mediated super enhancers phase separation [35–37]. The multivalent recognition of nucleosome arrays by histone readers is the biophysical basis of phase separation [34]. By locus-specific incorporation of multivalent modifications at chromatins *in vivo*, the epigenome editing technologies provide powerful tools to study histone readers mediated chromatin phase separation and co-separated chromatin effectors.

Figure 3



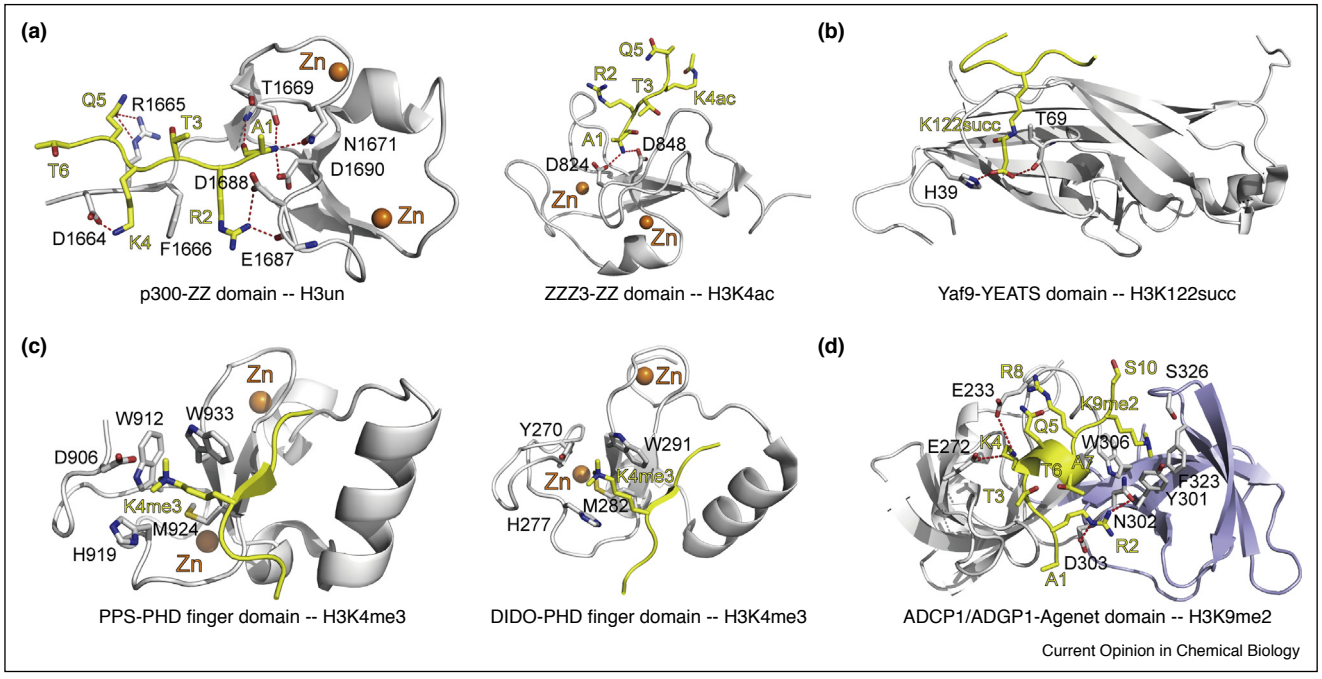
The schematic diagram of introducing engineered histone modifications and effectors in cells (figure adapted from David and Muir [29]). **(a)** The rationale of incorporation of histone modifications in cells by the genetic code expansion strategy. The yellow stars represent the modified histone residues. **(b)** The rationale of incorporation of histone modifications in cells by the split inteins based strategy. The yellow stars represent the modification histone residues. The cyan polygons represent Int^N, Int^C, and split Inteins. **(c)** The rationale of chromatin locus-specific incorporation of chromatin effectors in cells. The blue stars represent cofactors for the chromatin effectors. **(d)** The rationale of chromatin locus-specific incorporation of small molecules/peptides in cells by the split inteins based strategy. The yellow stars represent the small molecules/peptides. The cyan polygons represent Int^N, Int^C, and split Inteins.

Newly identified histone readers

Several new histone 'mark-reader' recognition pairs have been identified in recent two years. ZZ domain was identified as a histone H3 reader module. The ZZ domain of p300 recognizes the N-terminus of histone H3 [38•]. The H3A1 is recognized by a hydrogen bond network formed by p300 and H3R2, K4, Q5 also involved in the interactions (Figure 4a). The reader function of p300-ZZ domain is essential for its catalytic activities and promotes the acetylation on H3K18 and K27 site [38•]. The ZZ domain of ZZZ3 also recognizes the N-terminus of H3 with a preference toward H3K4ac [39•]. The recognition of histone H3 by ZZZ3 is essential for ATAC-dependent promoter H3K9ac and gene activation [39•].

The YEATS domain proteins were identified as histone acetylation and crotonylation readers before [10,40–43]. Recently, the YEATS domain of GAS41 was identified as a reader of histone H3K122 succinylation [44•]. The complex structure showed that a conserved protonated histidine recognized the carboxyl group of the succinylation (Figure 4b). Because of the protonation of histidine residue, the recognition of H3K122succ is influenced by the pH value. Similarly, the recognition of H3K4me3 by PHD-finger domain proteins PPS and DIDO is also influenced by the pH value *in vitro* and *in vivo* [45]. The aromatic cage recognizing H3K4me3 in PPS and DIDO contains a conserved histidine residue and the binding affinities were enhanced at high pH values (Figure 4c). The acidification of cells resulted in the

Figure 4



The structures of histone reader — modified histone peptide complex. **(a)** The structures of histone H3 peptide in complex with ZZ domain containing proteins p300 (PDB code: 6DS6) and ZZZ3 (PDB code: 6E86). The protein domains are shown as white cartoons and the histone peptides are shown as yellow sticks. **(b)** The structure of yeast Yaf9 in complex with histone H3K122succ peptide. (PDB code: 5WYI). **(c)** The structures of histone H3K4me3 peptide in complex with the PHD finger domain of PPS (PDB code: 5WLE) and DIDO (PDB code: 4L7X). **(d)** The structure of *Arabidopsis* ADCP1 in complex with histone H3K9me2 peptide (PDB code: 6IE6).

accelerated efflux of DIDO, indicating an *in vivo* function of pH-dependent histone recognition.

A variety of histone readers in plants have also been identified in two years. In accordance with the SPRi platform, we systematically identified histone readers in *Arabidopsis thaliana* [46]. Several new histone readers were discovered such as EML1 as an H3K4me3 reader. Among those new histone readers, a novel domain type of histone H3K9me2 reader was discovered. The Agenet domain is a plant-specific 'Royal family' module, but its reader activity was much less known. Two recent papers reported the tandem Agenet domain protein as the H3K9me2 reader [47•,48•]. In the complex structure, the histone peptide was induced to form an alpha-helix conformation and H3K9me2 residue inserted into an aromatic cage (Figure 4d). Functionally, ADCP1 is essential for the establishment of H3K9me2 modification and non-CG region DNA methylation. ADCP1 also mediates phase separation of the nucleosome array in an H3K9me2-dependent manner [48•]. Du group also systematically reported the reader properties of plant BAH-PHD cassettes in recent two years. The BAH-PHD cassette of ORC1b was identified as an unmodified H3 reader in *Arabidopsis* [49]. In contrast, the BAH-PHD cassette of EBS and SHL were bivalent histone readers that recognized H3K4me3 and K27me3 dual modifications [50,51].

Newly identified histone modifications

New histone modification types were also being identified such as H3 glutamine 5 serotonylation (H3Q5ser) and lysine benzoylation (Kbz). Histone serotonylation is the first report of monoamine modification on histone residue, establishing a direct link between neurotransmitter and gene regulation [52••]. Histone serotonylation is catalyzed by the tissue transglutaminase 2 (TGM2). H3Q5ser can co-exist with H3K4me3 and is enriched in euchromatin. The H3K4me3Q5ser dual modifications could more efficiently recruit TFIID complex and correlate with permissive gene expression. Moreover, this study also demonstrated the possibility that other monoamine modifications could also occur on histones such as dopaminylation and histaminylation. Obviously, the regulatory effectors of these interesting new modifications await further studies.

A repertoire of acylation types occurs on histone, such as acetylation and crotonylation [53]. Recently, a new type of histone acylation—benzoylation was discovered at 22 sites in mammalian cells [54••]. The cellular benzoyl CoA could be generated from the metabolization of sodium benzoate. The ChIP-seq and RNA-seq results showed that Kbz and Kac were associated with different sets of genes. The authors also discovered that SIRT2 was

the eraser of Kbz modification *in vitro* and *in vivo*. The corresponding readers of lysine benzoylation have not been identified yet. Considering the large size and aromatic group of benzoylation, we guess that Kbz might also be recognized through an aromatic cage.

Conclusions

Various histone readers recognizing methylation/acylation/phosphorylation have been identified till now. However, there are still challenges in identifying the transient and low binding-affinity interactions. Moreover, the identification of cell type-specific, cell stage-specific and chromatin locus-specific interactions is also desperately needed. With the development of photo crosslinking-based strategies, the capture of transient interactions becomes easier than before. Also, iCLASPI technology enables the identification of cell type-specific and cell stage-specific interactions in cells. However, the crosslinking strategies to identify weak interactions also result in many artificial results. Till now, no confirmed weak histone modification reader has been identified through this strategy. The high-throughput screening strategies lead to the identification of many histone readers. With the introduction of SPRi platform, the *in vitro* screening strategy is able to detect weak interactions. The DNLs based strategy enables the histone reader screening at the nucleosome level, which are crucial to the study of histone combinatorial readout. However, all these screening strategies require candidate reader proteins and are hard to identify novel module domains without previous indications. Recently, the epigenome editing technologies provide new methods to introduce histone modifications *in vivo* and identified histone readers in a chromatin locus-specific way. But the epigenome editing technologies are still in infancy and how to utilize these methods to efficiently identify histone readers are challenging. Importantly, new histone modifications are being discovered. Whether every histone modification has its specific readers still need much more researches.

Conflict of interest statement

Nothing declared.

Acknowledgements

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- •• of outstanding interest

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