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Histone probes for reader and eraser investigations

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Histone modifications critically regulate chromatin architecture, gene transcription, and epigenetic inheritance. These modifications are dynamically regulated by writers and erasers. In addition, readers recognize site-specific histone marks to mediate downstream cellular activities. Therefore, precise identification and functional investigation of these regulatory enzymes and proteins are essential for elucidating the biological roles of histone modifications. Chemical probes, due to their target-specificity, serve as indispensable tools for investigating readers and erasers in chemical biology. This perspective summarizes the previous and cutting-edge development of histone probes. We first discuss affinity-based probes, which exploit binding interactions to proteins of interest (POIs) but suffer from limited efficacy for enrichment of low-abundance proteins and proteins with transient interactions. Covalent crosslinking probes could overcome these limitations. We thus next introduce photoreactive probes that crosslink POIs from highly reactive intermediates generated under UV irradiation. In addition, we summarize activity-based probes that selectively bind and crosslink readers and erasers inside pockets due to the unique warheads. At the end, we discuss our perspective on the field in the future, touching on key challenges and emerging directions.

1. Introduction

In eukaryotic cells, genetic information encoded by DNA is packaged into chromatin through its association with proteins in the nucleus. The fundamental structural unit of chromatin is the nucleosome, comprising a histone octamer formed by histones H3, H4, H2A, and H2B with wrapped DNA (Fig. 1A).[1,2] Histones undergo diverse post-translational modifications (PTMs) that play essential roles in the regulation of chromatin

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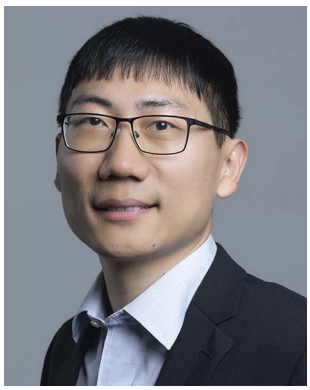

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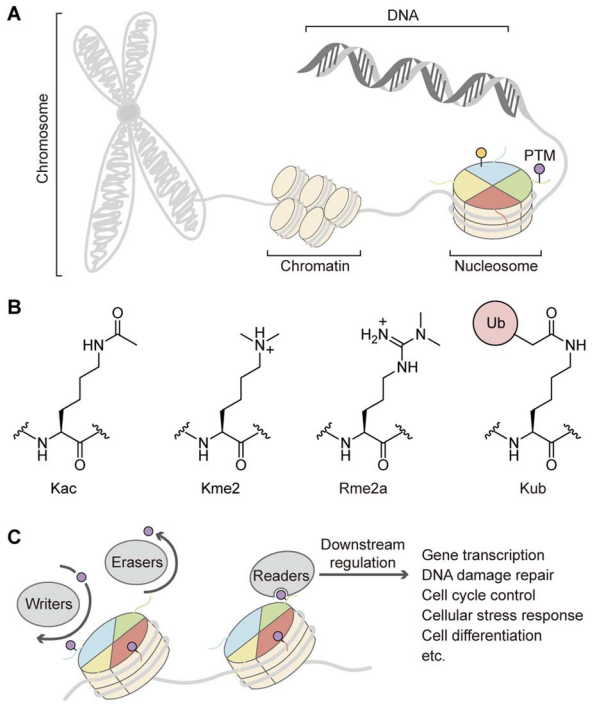

Fig. 1 Schematic diagram of chromatin architecture and histone post-translational modification (PTM) regulations. (A) Chromatin architecture in eukaryotic cells. The fundamental unit of chromatin is a nucleosome, which consists of a histone octamer with wrapped DNA. Histone tails or globular domains of the nucleosome are subject to various post-translational modifications. (B) Representative types of histone PTMs, including lysine acetylation (Kac), lysine dimethylation (Kme2), arginine asymmetric dimethylation (Rme2a), and lysine ubiquitination (Kub). (C) Dynamic regulatory mechanism of histone PTMs. Writers catalyse the addition of modifications at specific sites, erasers remove existing modifications, and readers recognize these modifications to regulate downstream biological processes.

structure, gene transcription, and epigenetic inheritance (Fig. 1B).[3–6] For example, histone H3 serine 10 phosphorylation (H3S10ph) promotes chromosome condensation during mitosis.[7,8] Histone H3K9 methylation drives the formation of heterochromatin to silence genes.[9–12] Poly acetylation on N-tails of histone H3 and H4 serves as a signature of gene activation and transcription.[13,14] Therefore, elucidating the biological functions of histone modifications is of paramount importance.

There are three functionally distinct protein classes that dynamically regulate histone modifications: writers, erasers, and readers (Fig. 1C). Writers catalyse the addition of histone marks to specific sites, such as lysine acetyltransferases (KATs) and lysine methyltransferases (KMTs).[15–18] On the contrary, erasers remove histone marks back to unmodified status, such as histone deacetylases (HDACs) and lysine demethylases (KDMs).[19–24] In addition, reader proteins recognize and bind to site-specifically modified histones.[25,26] In general, writers and erasers dynamically modulate the levels of histone modifications, while readers mediate downstream regulation. In consequence, identification and functional manipulation of writers, erasers, and readers are essential for deciphering the histone code.

Conventional biochemical approaches initiated the study of histone-modifying enzymes and readers. Researchers first cloned and expressed a protein of interest (POI) *in vitro*, then inferred its function through phenotypic analysis or sequence alignments, followed by screening histone-derived peptides and proteins for validation. This strategy led to seminal discoveries, including the first acetyltransferase Tetrahymena HAT A, the first methyltransferase Suv39H1, and the first lysine demethylase LSD1, as well as the first acetyllysine reader bromodomain and the first methyllysine reader chromodomain.[10,27–30] Complementing this protein-centric methodology, chemical biology strategies employ functionalized ligands as probes to identify interacting proteins. For example, the first class I HDAC was identified by a trapoxin-based probe, due to the high binding affinity and target-selectivity.[31] Critically, such probes permit *in situ* manipulation of protein function in cell cultures and animal models, offering advantages over genetic knockout in terms of temporal control and reversibility.[32–35] Therefore, the development of chemical probes targeting writers, erasers, and readers provides indispensable tools for mechanistic investigation within chemical biology.

This perspective summarizes key advances and persistent challenges in developing histone-targeted probes for erasers and readers, focusing on peptide- and protein-based scaffolds. Our analysis progresses through three strategic categories. First, affinity-driven probes that exploit binding interactions with POIs; second, photo-crosslinking probes incorporating photoreactive groups to covalently capture POIs; third, activity-based probes that selectively engage active sites to achieve POI-specific crosslinking. Collectively, these case studies, including contributions from our laboratory, demonstrate how chemical biology principles guide the rational design and application of histone probes for site-specific eraser and reader proteins. Such probes provide versatile toolkits for dissecting histone modification mechanisms, and the developed novel crosslinking chemistries may extend to other target classes beyond epigenetic regulation.

2. Affinity probes

2.1 General strategy

Since readers exhibit specificity for modified proteins, affinity probes for readers are typically designed as a peptide or a protein with the modification to mimic the binding partner. In comparison, probes targeting erasers act as substrate mimics. As a result, unique functional groups are required so that the probes confer high binding affinity while maintaining resistance to enzymatic activity. In addition, an affinity tag (*e.g.*, biotin) is usually incorporated to facilitate enrichment and analysis. Given their distinct design strategies, affinity probes for readers and erasers are reviewed separately in subsequent sections.

2.2 Affinity probes for readers

Based on the natural affinity between readers and post-translationally modified proteins, affinity probes for readers

can be constructed as peptide fragments with a site-specific PTM, along with an affinity tag (Fig. 2A). Such probes are routinely synthesized by well-established solid-phase peptide synthesis (SPPS).[36] A variety of Fmoc-protected building blocks with PTMs are commercially available, such as Fmoc–Ser(-PO(OBzl)OH)–OH, Fmoc–Lys(Ac)–OH, Fmoc–Lys(me3)–OH, *etc.* This synthetic accessibility permits site-specific incorporation of PTMs at any desired peptide position. Biotin is the most frequently used affinity tag and can be introduced either at the *N*-terminal or on a lysine side chain. Such peptide probes with PTMs and biotin were applied to pull-down POIs from cell lysates (Fig. 2B). Furthermore, peptide microarray technology enables high-throughput screening of reader binding affinity and selectivity using diverse PTM peptide libraries. Notably, this approach has facilitated the identification of multiple readers, including acetyllysine reader BRD4, and methyllysine readers BPTF, mORC1, WDR5.[37–41]

Although peptide-based probes were initially successful in finding new readers, they have certain limitations. In eukaryotic cells, histone modifications occur on nucleosomes with a complicated quaternary structure assembled by a histone octamer and double-stranded DNA (dsDNA).[1,2] In contrast, histone peptides represent only a tiny fragment of nucleosomes. Although such peptides might effectively mimic modification sites located at the disordered histone tails, they fail to mimic local microenvironments at globular domains, which also interact with other core histones and DNA. Consequently, nucleosome-based probes are preferred as more accurate mimics for investigating the functional implications of histone modifications within their native chromatin context.

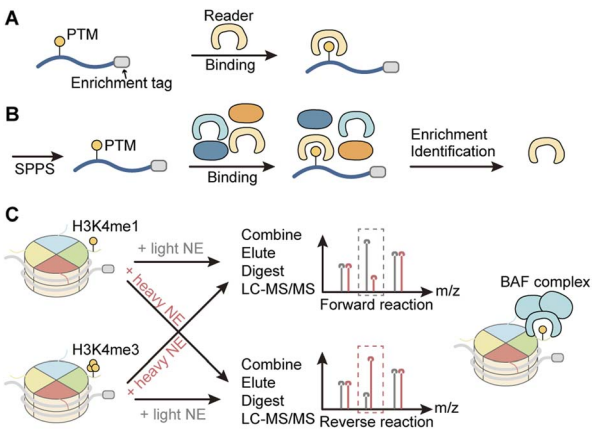

Fig. 2 Affinity probes for the detection and characterization of histone modification readers. (A) General design strategy of affinity probes targeting readers that contain PTM as a mimic of a natural ligand. (B) Schematic workflow of peptide-based affinity probes. Probes are synthesized by solid-phase peptide synthesis (SPPS) and incubated with complex biological systems such as cell lysates to bind potential readers. Subsequent enrichment and mass spectrometric identification enable the characterization of these binding proteins. (C) Schematic workflow of nucleosome affinity probe-based stable isotope labelling by amino acids in cell culture (SILAC) mass spectrometry. Using this approach, the BAF complex was identified as a reader of H3K4me1. NE: nuclear extract.

However, preparation of full-length histone with site-specific PTMs is not as straightforward as peptide synthesis. Chemists have established several beautiful strategies, including semi-synthesis, residue-specific modification, genetic codon expansion, *etc.* More chemistry details could be found in several excellent reviews elsewhere.[42–50] Once the unique engineered histones are available, the next step involves nucleosome reconstitution with other core histones and 601 DNA. Based on the quaternary structure of nucleosome, the affinity tag may be incorporated into any histone or DNA.

Once the nucleosomes are prepared, they are employed to pull down binding proteins from cell samples using a similar protocol for peptide probes. For example, Local *et al.* prepared two types of nucleosomes that contained H3K4me1 or H3K4me3, and they investigated H3K4me1-associated proteins using SILAC (stable isotope labelling by amino acids in cell culture) (Fig. 2C).[51] This work revealed that the chromatin-remodelling complex BAF specifically recognizes H3K4me1 rather than H3K4me3 through the component BAF45C, thereby elucidating distinct regulatory mechanisms at enhancers. With advances in designer nucleosomes, the Muir lab developed a nucleosome library.[52] A key innovation lies in the integration of unique DNA barcodes within each nucleosome variant, enabling PCR amplification and high-throughput sequencing of enriched nucleosomes, significantly enhancing screening sensitivity. This platform has been successfully applied for studying oncohistone variants.[53]

2.3 Activity-based affinity probes for erasers

Although reader probes also exhibit affinity to erasers, they have major limitations. First, the substrate binding affinity (estimated by K_m) of erasers is typically lower than the ligand binding affinity of readers (evaluated by K_d). Second, the modification on the probe can be removed by these enzymes, generating unmodified products that lose their binding affinity. Therefore, an ideal eraser probe should fulfill dual criteria: it should structurally mimic the native enzyme substrate to ensure high affinity while incorporating chemical modifications that render the probe inert to the enzymatic catalysis (Fig. 3A).

Histone lysine acetylation is generally associated with chromatin relaxation and promotes gene transcription. Since hypoacetylation is a hallmark in some cancer cells, histone deacetylases (HDACs) have emerged as high-priority therapeutic targets.[54,55] Class I, II, and IV histone deacetylases (HDACs) employ Zn^{2+} to catalyse the removal of acetyl groups from acetyllysine substrates by water (Fig. 3B).[56] Among the HDAC inhibitors for drug development, hydroxamic acid is a widely used warhead, as it mimics acetyllysine and exhibits chelating activity toward the Zn^{2+} inside the HDAC pocket (Fig. 3C).[57] The Schwarzer group thus developed a series of unnatural amino acids that contain hydroxamic acid to mimic acetyllysine, with 2-aminosuberic acid ω-hydroxamate (AsuHd) showing the highest binding affinity. They subsequently prepared histone and non-histone peptides with AsuHd, and these probes enriched distinct HDACs based on their probe selectivity.[58] In addition, Wu *et al.* prepared AsuHd-containing nucleosome

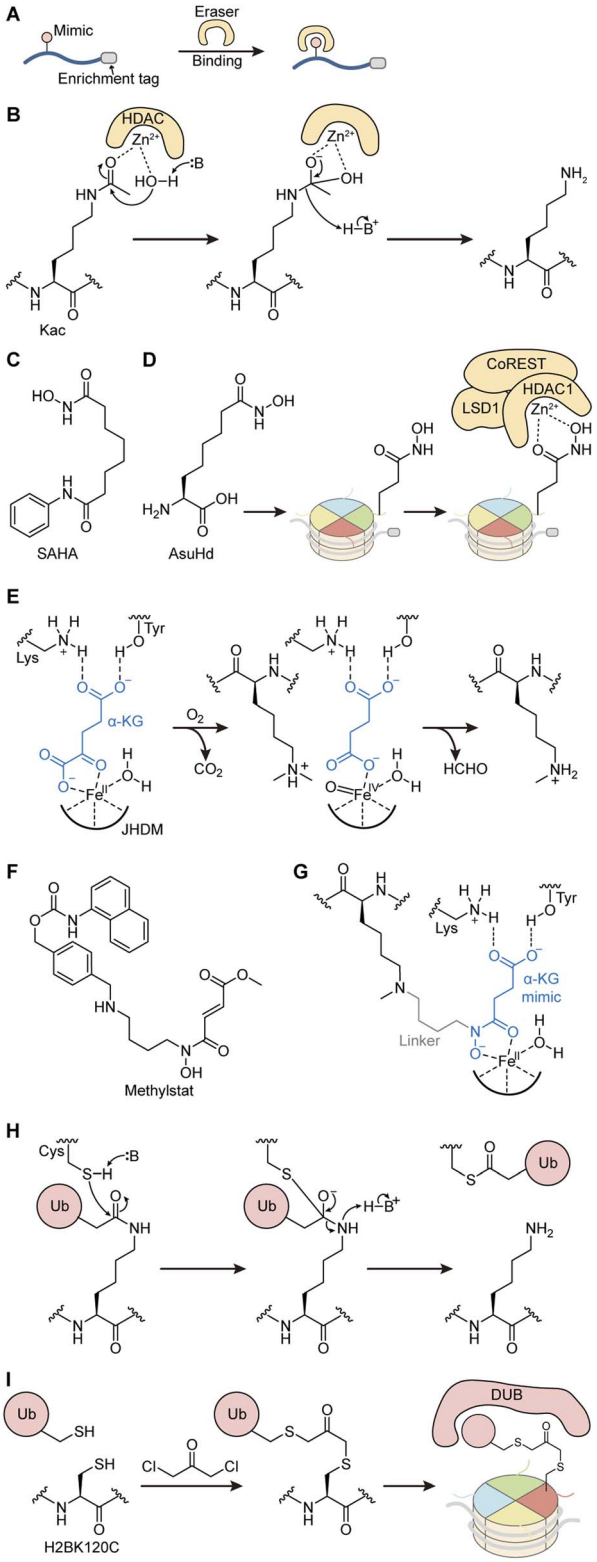

Fig. 3 Affinity probes for the study of histone modification erasers. (A) A structural mimic of PTM that cannot be removed by erasers is generally developed for affinity probes targeting erasers. (B) Mechanism of lysine deacetylation by Zn^{2+}-dependent HDACs. (C) Chemical structure of SAHA as an HDAC inhibitor. (D) Nucleosome probes containing 2-aminosuberic acid ω-hydroxamate (AsuHd) for investigation of the CoREST complex. (E) Mechanism of lysine demethylation catalysed by JmjC domain-containing KDMs. (F) Chemical structure of methylstat as a KDM inhibitor. (G) Working principle of KDM-targeting affinity-based peptide probes. (H) Mechanism of lysine deubiquitination by DUB. (I) Ubiquitin-nucleosome probe with a nonhydrolyzable linkage.

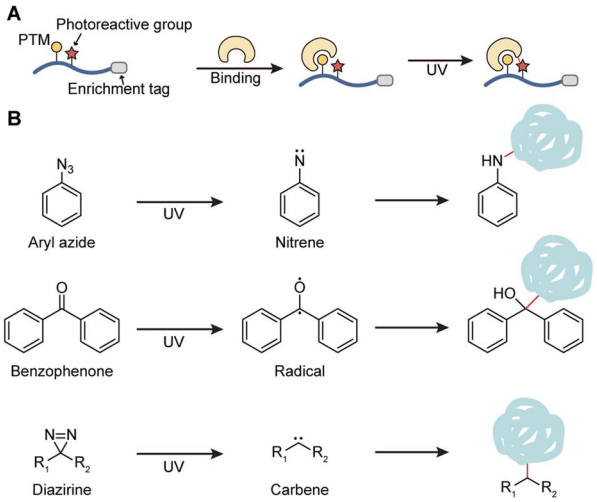

Fig. 4 (A) General design strategy of photoreactive crosslinking probes. A photoreactive group is placed close to PTM, which crosslinks the PTM binder upon UV irradiation. (B) Structures and mechanisms of three major photoreactive groups.

probes to investigate Kac site-selectivity mechanism of the CoREST complex, which comprises HDAC1, LSD1, and CoREST1. These probes were obtained from SPPS of H3(1–34) peptide with AsuHd at K9 or K14. The synthetic peptide was then subjected to traceless sortase-mediated ligation with the recombinant H3(33–135) fragment, followed by nucleosome reconstitution (Fig. 3D).[59]

Jumonji C (JmjC) domain-containing KDMs are Fe^{2+} and α-ketoglutarate (α-KG) dependent oxygenases (Fig. 3E). A more detailed introduction can be found later in Section 4.3. Since these enzymes bind Fe^{2+}, α-KG, and methyllysine substrates, the Wang lab reported methylstat as a small molecule with a substrate mimic motif and a cofactor mimic motif (Fig. 3F).[60] Later, they developed KDM affinity-based peptide probes based on a bisubstrate analog strategy (Fig. 3G). For example, a histone H3K36me2-JHDM probe contains a histone H3 peptide backbone with H3K36 methylation as a lysine-substrate-mimicking motif, an α-KG cofactor-mimicking motif, and a short methylene linker between them. This design confers selective binding affinity for JHDM1A, preventing catalytic turnover, as the α-KG binding pocket was occupied by the cofactor mimic, thereby blocking enzymatic activity.[61]

In addition to post-translational modifications by small chemical groups, histone can also be modified by proteins such as ubiquitin. For example, histone H2BK120 ubiquitination promotes gene transcription while histone H2AK119 ubiquitination acts as a repressive mark.[62–66] Deubiquitinases (DUBs) are an important class of enzymes that catalyse the removal of ubiquitin modifications and regulate chromatin

function. The catalytic mechanism is similar to cysteine proteases, which cleave the isopeptide bond between ubiquitin and the lysine side chain of histones (Fig. 3H).[67] In order to bind a specific DUB, an ideal probe would be ubiquitin–nucleosome complex with a nonhydrolyzable linkage. One great example was reported by the Wolberger lab. Histone H2BK120C and UbG76C were coupled by dichloroacetone, and the resulting H2B–Ub conjugate was applied to nucleosome reconstitution (Fig. 3I). Due to the unnatural linkage from dichloroacetone, the nucleosome–ubiquitin complex is an excellent affinity-based probe without hydrolysis by DUB. Therefore, a stable quaternary complex was obtained, and the 3D structure was well resolved at atomic resolution.[68]

2.4 Limitations of affinity-based probes

Due to the relatively simple design and synthesis, affinity-based probes have been widely used to study readers and erasers. However, there are some limitations. First, many affinity-based probes face the challenge that insufficient binding affinity may impede the enrichment of POIs with low abundance. Weakly bound proteins may dissociate during washing steps, resulting in eluates with concentrations below the detection threshold of mass spectrometry. Second, proteins with transient interactions cannot be stabilized throughout pull-down over the protocol steps. These constraints necessitate the development of covalent probes capable of forming irreversible linkages with target proteins.

3. Photoreactive crosslinking probes

3.1 General strategy

In order to overcome the limitations of affinity-based strategies, covalent crosslinking would empower the enrichment and identification of POIs. Therefore, chemical probes equipped with photoreactive crosslinkers have been developed (Fig. 4A). In general, such probes retain the structure of affinity probes while adding a photoreactive group, such as arylazide, benzophenone (BPA), and diazirine (Fig. 4B).[69,70] In principle, the probes bind to POIs through specific affinity, and subsequent irradiation causes the formation of highly reactive intermediates that crosslink POIs. While this perspective summarizes the key progress of this strategy, more details could be found in excellent reviews by Dr Xiang D. Li.[71,72]

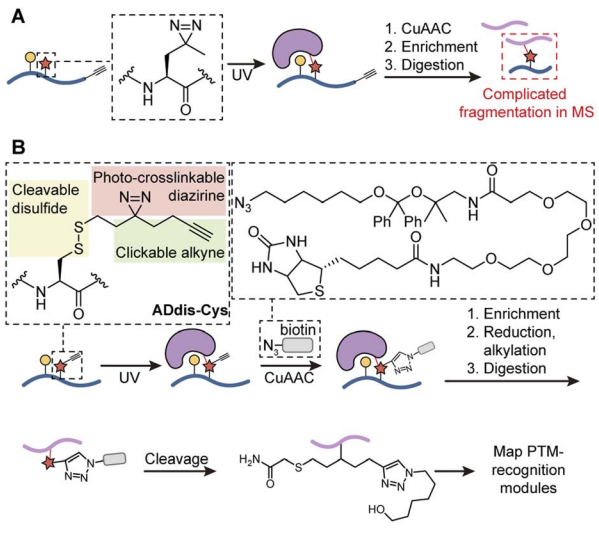

Fig. 5 Peptide probes with photoreactive groups. (A) Diazirine-based photoreactive probes for reader identification. The crosslinked peptide fragments present analytical challenges for mass spectrometry. (B) ADdis-Cys-based photoreactive probes for identifying readers and mapping PTM recognition modules.

3.2 Peptide probes

Photoreactive probes were initially developed as peptide-based constructs leveraging the straightforward synthesis by SPPS, similar to affinity-based peptide probes. Commercially available Fmoc-protected photoreactive unnatural amino acids such as Fmoc–4-azido-phenylalanine–OH and Fmoc–Bpa–OH enable direct site-specific incorporation during SPPS.[36] For example, Li *et al.* developed quantitative chemical proteomics by usage of BPA-based histone peptides.[73] Kleiner *et al.* used a BPA-based probe to identify readers of γH2AX.[74]

However, arylazide generally requires short UVA wavelengths that may cause severe damage to biological samples, and benzophenones exhibit slow crosslinking kinetics. Diazirine as a carbene precursor was later reported with fast UVC activation and rapid crosslinking.[75] In addition, the small size of diazirine minimizes potential interference with POI binding. As a result, diazirine-containing photoreactive probes are currently the most widely used (Fig. 5A). So far, a number of diazirine-containing amino acids have been commercialized, including photo-leucine, photo-methionine, photo-proline, *etc.* Beyond SPPS using Fmoc-protected building block,[76–78] diazirines may also be introduced through cysteine modifications, including alkylation by alkylhalide[79,80] and disulfide linkage by ADdis (alkynyl diazirino disulfanyl).[81,82] Notably, ADdis-Cys also enhances the identification of crosslinking sites by mass spectrometry, owing to the combination of the diazirine, an alkyne handle, and the cleavable disulfide bond (Fig. 5B). For example, ZMYND8 was identified as a reader of a dual histone modification H3K4me0/1-H3K14ac using this strategy.

Since photo crosslinkers may capture dynamic and transient protein–protein interactions, substrate-based probes can be applied to crosslink erasers. For example, H3K4Kcr probes were applied to identify SIRT3 as an eraser of H3K4 crotonylation.[83] As introduced in Section 2, chemists have developed unique functional groups to enhance the binding affinity of erasers. These groups can also be combined with photo-reactive groups to improve crosslinking efficiency. For example, a peptide probe containing Kme2-α-KG bisubstrate analogue and BPA was synthesized to selectively crosslink JHDM1A, an eraser of H3K36 methylation.[61]

3.3 Nucleosome probes

As introduced in Section 2.2, histone peptide probes cannot mimic nucleosomes in chromatin, especially for the PTMs on the globular domain. However, there are several critical histone

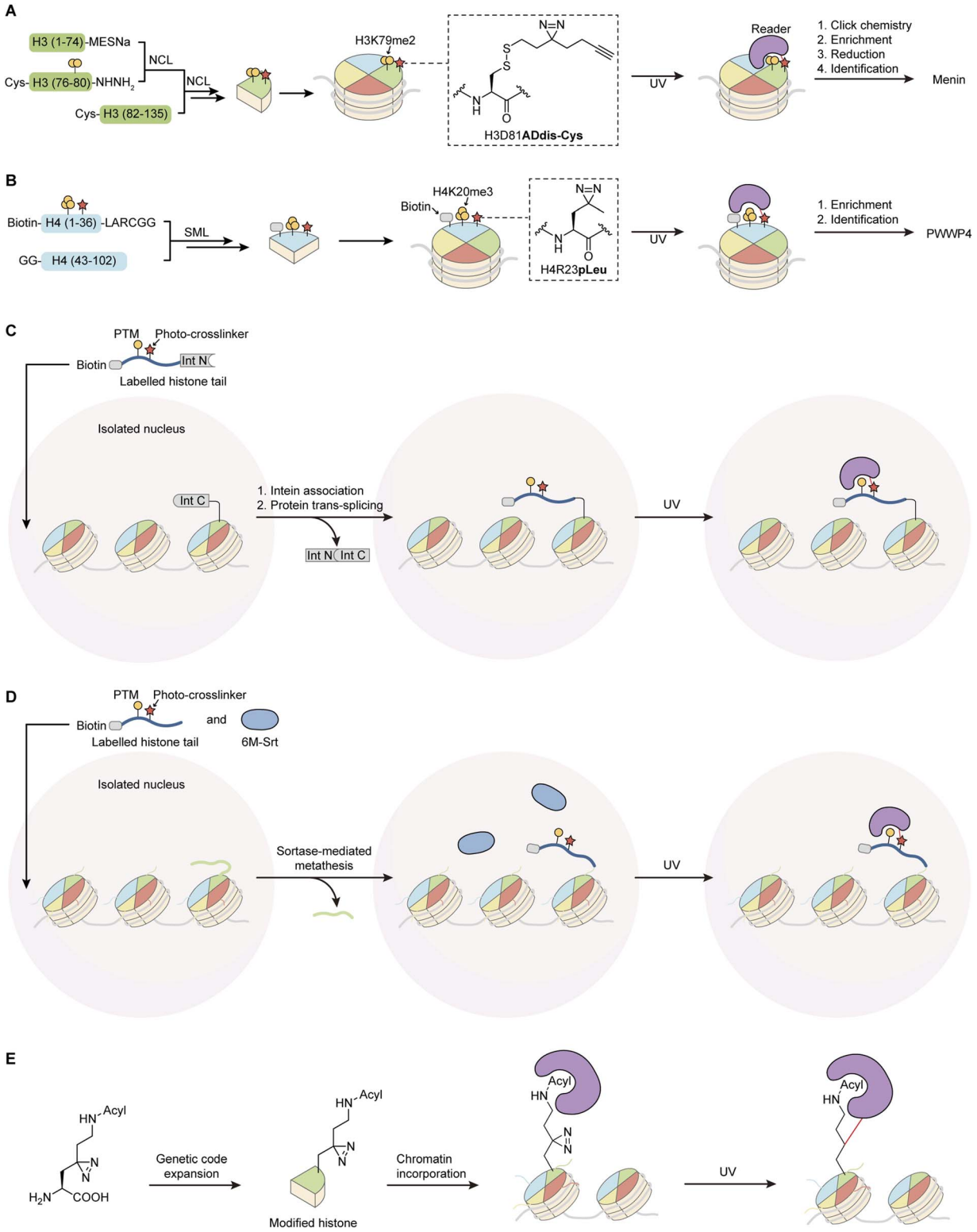

Fig. 6 Nucleosome probes with photoreactive groups. (A) Synthesis of full-length histone H3 containing H3K79me2 and ADdis-Cys *via* native chemical ligation (NCL), assembly into nucleosome probes, and identification of menin as a novel H3K79me2 reader. (B) Synthesis of full-length H4 containing H4K20me3, diazirine, and biotin tag *via* sortase-mediated ligation (SML), assembly into nucleosome probes, and identification of PWWP4 as a novel H4K20me3 binder. (C) *In situ* capture of PTM-specific readers *via* protein trans-splicing-mediated modification of native chromatin with PTM- and photocrosslinker-containing histone tails. (D) Sortase-mediated histone tail editing in isolated nuclei with sorting motif-containing histone tail peptides bearing modified N-fragments. (E) Genetic code expansion enables incorporation of photoreactive unnatural amino acids into chromatin in cells.

modifications that occur at the interface among core histones and DNA, such as H3K56 acetylation, H3K79 methylation, and H4K20 methylation.[84–87] Therefore, nucleosome probes with photoreactive groups have been developed. However, the key challenge arises from the significantly increased complexity of nucleosome preparation compared to peptide probe synthesis.

One strategy is semisynthesis by ligation of synthetic peptide fragments and recombinant protein fragments. An outstanding example is the identification of menin as a H3K79me2 reader by nucleosome probes that contain H3K79me2 for reader binding and H3D81ADdis-Cys for crosslinking and enrichment (Fig. 6A).[88] Our group also made some efforts to develop nucleosome probes to crosslink binding proteins.[89,90] One successful example was the identification of PWWP4 as a reader of H4K20me3 using nucleosome probes that were prepared by sortase-mediated ligation of histone H4 fragments (Fig. 6B).[91,92] Although JMJD2A was reported as a H4K20me3 peptide reader, our data indicated that JMJD2A was not a H4K20me3-nucleosome reader. The negatively charged residues around the methyllysine binding pocket of JMJD2A are not likely to accommodate the DNA around H4K20me3 on nucleosomes. Such comparison further demonstrated the significance of using nucleosome probes to investigate histone modification at globular domains.

Although nucleosome tools have demonstrated much greater advantages over peptide-based approaches, the methods mentioned above are limited to *in vitro* applications. Chemists have therefore developed novel strategies to incorporate photo crosslinkers into chromatin in the cell nucleus. The Muir lab developed an intein splicing-mediated method so that synthetic histone peptide fragments containing an Int N sequence react with chromatin histone bearing an Int C sequence to yield photo-crosslinker-containing chromatin *in situ* (Fig. 6C).[93,94] Our lab reported a sortase-mediated metathesis method for editing histones in live cells (Fig. 6D). Histone tail peptides with a sorting motif and a cell-penetrating sequence can enter cells, and the N-fragments with modifications can be metathesized to chromatin histone by sortase.[95,96] In addition to protein semisynthesis, chromatin with photo crosslinkers can be generated by genetic code expansion (Fig. 6E). Unnatural amino acids such as photo-lysine and photo-arginine can be incorporated into histones during protein translation, with the resulting modified histones subsequently integrated into cellular chromatin.[97] Furthermore, a series of modified photo-lysines as lysine acylation analogues were applied for systematic mapping and profiling of the dynamic modifications in chromatin.[98]

3.4 Limitations of crosslinking probes with photo-reactive groups

Although such crosslinking probes bring advantages of covalent linkage to POIs over affinity-based probes, there are still some limitations. First, the synthesis of some amino acids with photo crosslinkers requires plenty of steps and suffers from low overall yield. Second, the placement of photo crosslinker often requires laborious optimization. The photo crosslinker groups may disrupt the native interactions with readers/erasers. As a result, several probes with distinct crosslinker placements are usually carried out to optimize binding and crosslinking efficiency. Third, under UV irradiation, photoreactive groups are converted to highly reactive intermediates that may cause extensive nonspecific crosslinking. Such background reactions can complicate the identification of true POIs. In consequence, there is a demand for probes with facile synthesis and precise crosslinking capabilities to enable reliable protein identification from cellular samples.

4. Activity-based crosslinking probes

4.1 General strategy

Suicide irreversible inhibitors serve as valuable tools in basic research and clinical therapies. These inhibitors mimic the natural substrates and covalently conjugate to key residues inside the protein pocket. A classic example is penicillin and other β-lactam antibiotics, which structurally mimic the D-alanine–D-alanine peptide terminus and irreversibly conjugate to the catalytic serine of glycopeptide transpeptidase.[99] Another example is fluorophosphonate-based probes that conjugate catalytic serine of serine proteases for activity-based protein profiling (ABPP).[100] Therefore, activity-based crosslinking offers opportunities for precise covalent crosslinking of proteins of interest, owing to its dependence on enzymatic activity.

In principle, a warhead of an activity-based probe plays dual roles as a natural substrate/ligand analogue and as a reactive group capable of crosslinking within the protein pocket (Fig. 7A). To enhance affinity and selectivity to POI, the warhead is typically attached to the side chain of a key residue in peptides or proteins. Depending on the binding and catalytic activity, the crosslinking site might be a conserved amino acid residue or a cofactor.

4.2 Sirtuin probes by thioacetyl and *N*-methylthiourea-containing histone

Sirtuins belong to class III histone lysine deacetylases and play a critical role in epigenetic regulation, participating in various biological processes such as gene silencing, chromatin structure regulation, DNA damage repair, cellular stress response, *etc.*[101] Sirtuins employ the cofactor nicotinamide adenine dinucleotide (NAD^+) to remove acetyl groups from substrates (Fig. 7B). The unique electrophilic character of NAD^+ in deacetylation transition state has inspired development of activity-based crosslinking probes targeting the cofactor. Initially, thioacetyl lysine was developed as an inhibitor of sirtuins.[102,103] The discovery of an ADP-ribose-peptide intermediate revealed a stalling mechanism. Building on the initial success of activity-based inhibition, a series of warheads were reported by several labs, including thiocarbamoyl-lysine,[104] azalysine analogues,[105] lysine-based *N*-alkyl thiourea,[106] *etc.* In addition to peptide backbone-based inhibitors, several small molecules as covalent inhibitors have been developed based on these warheads, exhibiting broad anticancer activity through selective inhibition of SIRT2.[107] Furthermore, general probes that broadly target sirtuins were developed for activity-based protein profiling with

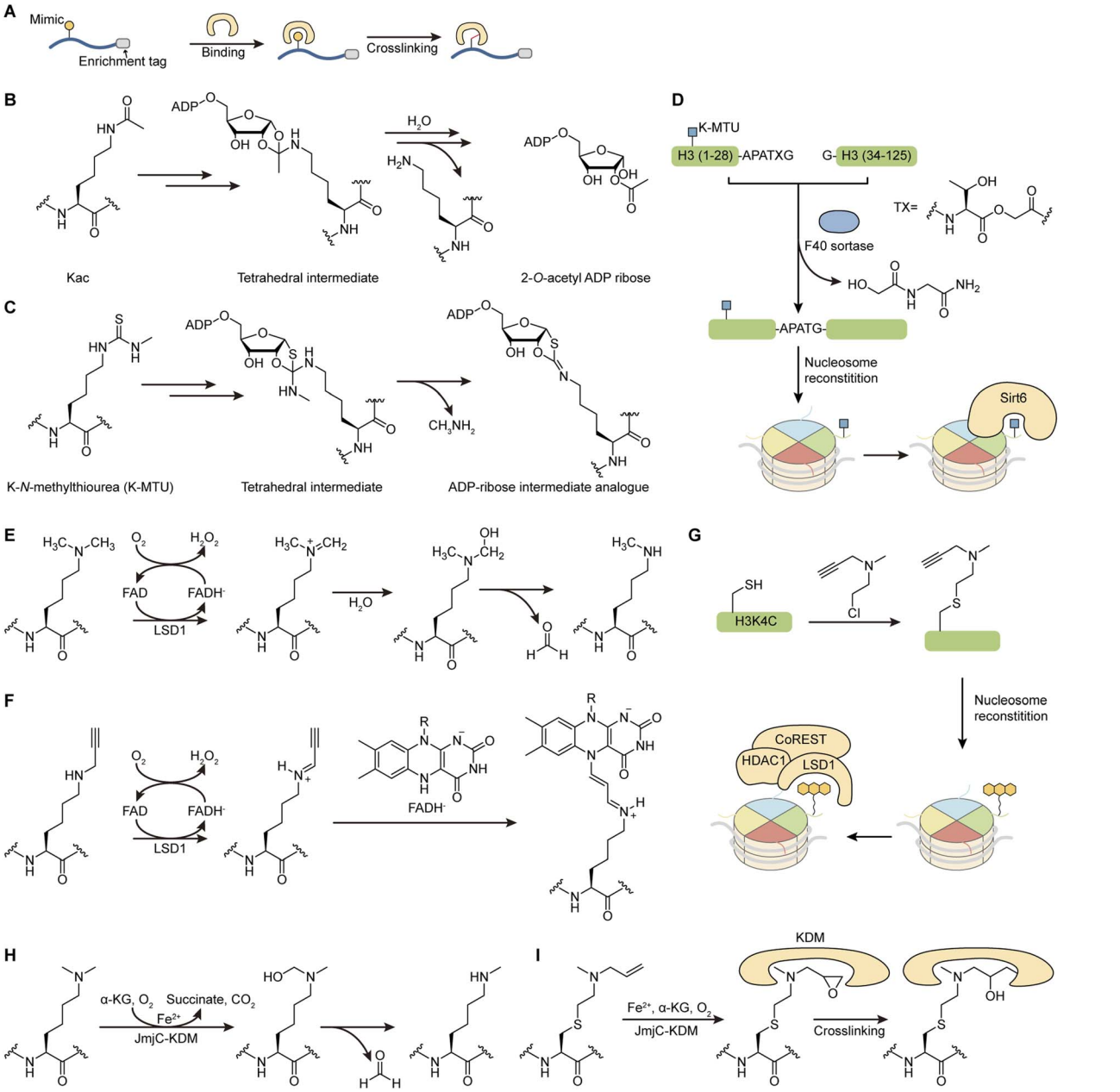

Fig. 7 Activity-based crosslinking probes for studying erasers. (A) General design strategy of activity-based crosslinking probes. A warhead as a PTM mimic binds to POI and subsequently crosslinks to a residue inside the binding pocket. (B) Mechanism of lysine deacetylation catalysed by sirtuins. (C) Proposed reaction mechanism of K-MTU. (D) Preparation of nucleosomes with K-MTU at H3K9 by sortase-mediated ligation, facilitating structural resolution of the SIRT6-nucleosome complex. (E) Mechanism of lysine demethylation catalysed by LSD1 with FAD as a cofactor. (F) Development of propargyl lysine as a covalent probe to conjugate FAD. (G) Incorporation of propargyl lysine at H3K4 *via* cysteine alkylation enabled structural investigation of LSD1-HDAC-CoREST complex with nucleosome. (H) Mechanism of lysine demethylation catalysed by JmjC domain-containing KDMs. (I) Proposed crosslinking mechanism of the allyllysine probe.

additional photo-reactive groups.[108,109] In general, such warheads were introduced during SPPS from either special Fmoc-protected building blocks or selective derivatization of lysine using orthogonal protection strategies.

Due to the selective crosslinking to NAD^+ in sirtuins, these methods have been well applied to investigate co-structures of the deacetylases and their substrates. For example, an AceCS2 peptide that contains thioacetyl lysine at K642 was applied to conjugate SIRT3, revealing the interactions at an atomic level through the crystal structure.[110] Similarly, the structure of SIRT4 was studied by application of thioacetyl H3K9 peptide.[111] Furthermore, the Cole lab prepared designer nucleosomes with *N*-methyl thiourea at H3K9 by sortase-mediated traceless semi-synthesis, and the 3D structure of SIRT6-nucleosome was well resolved by cryo-EM (Fig. 7C and D).[112] All cases highlight the advantages of site-selective crosslinking in the field of structural biology.

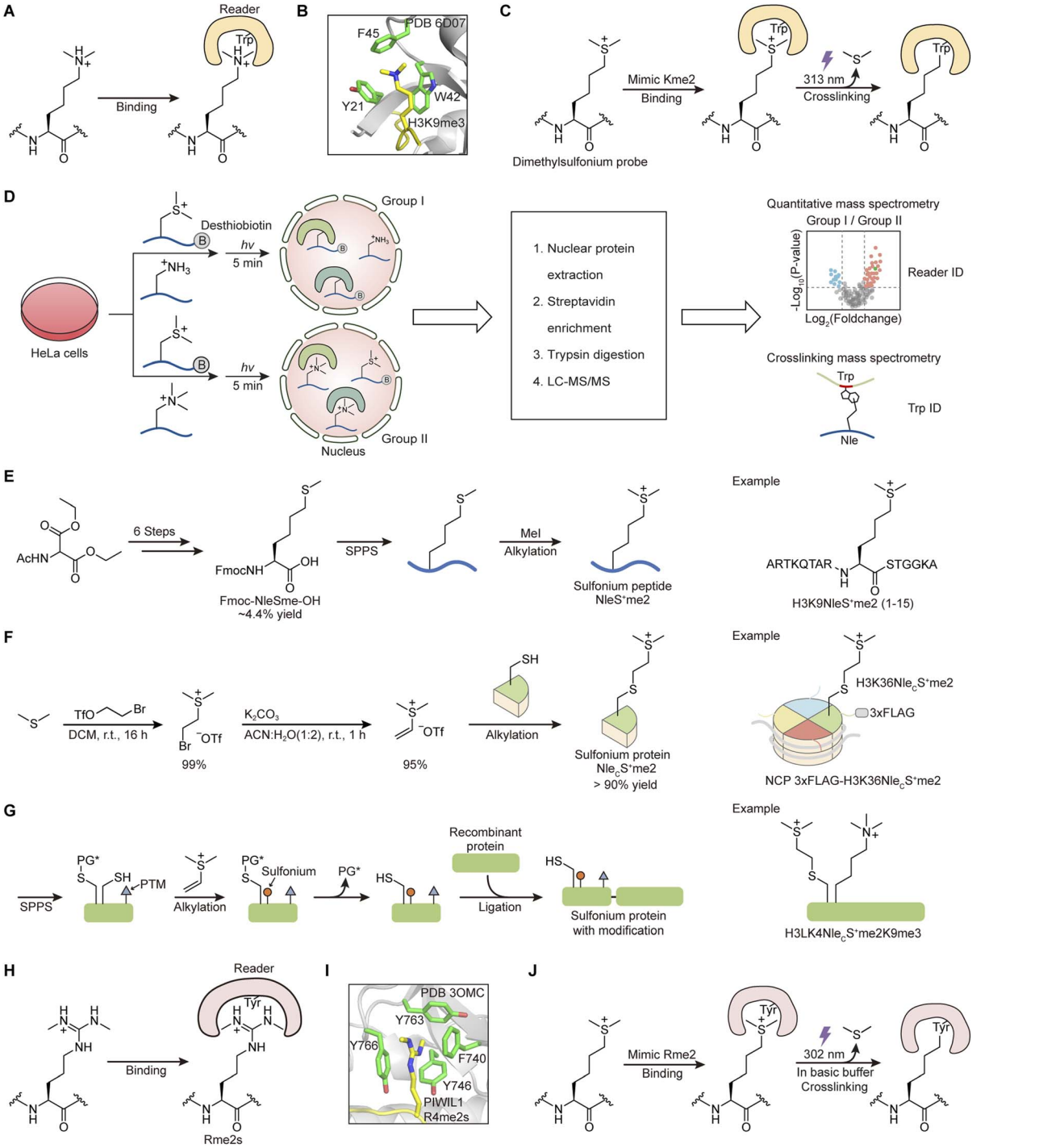

Fig. 8 Synthesis and applications of dimethylsulfonium probes as mimics of methyllysine and methylarginine. (A) Methyllysine readers recognize lysine methylation *via* aromatic cages, most of which contain conserved tryptophan residues. (B) PyMOL structure of CBX1 binding with H3K9me3 peptide. (C) Dimethylsulfonium mimics methyllysine, binds with readers, and crosslinks tryptophan under UV irradiation. (D) Workflow of proteomic analysis with dimethylsulfonium probes. Selectively crosslinked proteins could be identified by quantitative mass spectrometry. The key crosslinked tryptophan site could be identified by crosslinking mass spectrometry. (E) Synthesis of sulfonium peptides from the synthesis of Fmoc–NleSme–OH, SPPS, to sulfide alkylation with iodomethane, and an example of sulfonium peptide probe synthesized by this method. (F) Preparation of sulfonium proteins by cysteine alkylation with dimethylvinylsulfonium, and an example of sulfonium nucleosome probe prepared by this method. (G) Semisynthesis of sulfonium proteins with diverse modifications, and an example of sulfonium protein with lysine trimethylation obtained by semisynthesis. (H) Methyl arginine readers recognize arginine methylation *via* aromatic cages, some of them containing tyrosine but no tryptophan. (I) PyMOL structure of SND1 binds with PIWIL1 R4me2s peptide. (J) Dimethylsulfonium also serves as an arginine methylation analogue, crosslinks tyrosine in the aromatic cage pocket of arginine methylation readers under 302 nm irradiation and in basic buffer.

4.3 KDM probes by unsaturated methyllysine mimic

Lysine demethylation plays a crucial role in downregulating the histone marks and is catalysed by lysine demethylases.[19] There are two types of KDMs. LSD1, the first discovered lysine demethylase by the Shi lab, and LSD2 (also known as KDM1A and KDM1B) use flavin as cofactor to oxidize methylamine to imine, which was subsequently converted to formaldehyde and free lysine by water (Fig. 7E).[30,113] Following the discovery of LSD1, another class of KDMs, represented by JHDM1, was identified by the Zhang lab.[114] The Jumonji C (JmjC) domain-containing KDMs are non-heme oxygenases that use ferrous, α-ketoglutarate (α-KG), and oxygen to transform methylamine to hydroxylmethylamine. The intermediate was later converted to formaldehyde and unmodified lysine in aqueous solution.[115–117]

Since flavin is the key cofactor for dehydrogenation of methyllysine, early inhibitor designs focused on methyllysine mimics capable of reacting with flavin. The propargyl group was initially applied in monoamine oxidase (MAO) inhibitors, such as pargyline, which covalently conjugates flavin.[118] Due to the high structural homology between LSD1 and MAO, propargyl lysine was developed as a covalent probe to conjugate FAD (Fig. 7F).[119] Later, more functional groups from MAO inhibitors, such as cyclopropane and hydrazine, were also developed.[120] In cells, LSD1 is a unit of chromatin remodelling complexes, which also contain CoREST and HDAC1/2 to interact with nucleosomes for selective demethylation.[121–123] To study the molecular mechanisms, covalent crosslinking provides a strategy for the preparation of a stable quaternary complex for structural analysis. Therefore, propargyl lysine was incorporated at H3K4C *via* cysteine alkylation, and the CoREST complex and nucleosome co-structures were elucidated by the Mattevi lab and Schwabe lab (Fig. 7G).[124,125]

Given that JmjC-KDMs are non-heme iron-dependent oxygenases (Fig. 7H), our group reported unsaturated methyllysine analogues as warheads to covalently conjugate KDMs,[126] inspired by the probe development cases of P450 oxygenases and DNA demethylase TETs.[127,128] We found allyllysine offered the best crosslinking reactivity and specificity. Interestingly, a +16 Da mass product was observed upon incubation of the H3K4 allyllysine probe with KDM5B, which was later confirmed as an epoxide from subsequent functionalization by HCl and thiols. Based on these findings, we proposed an activity-based crosslinking mechanism (Fig. 7I). The allyllysine probe, acting as a methyllysine mimic, was first oxidized to epoxide by KDMs. A nucleophilic residue, such as cysteine, then attacks the three-membered epoxide ring, resulting in the formation of stable peptidyl conjugates. Since the allyllysine can be incorporated *via* cysteine alkylation, this strategy has the potential to prepare allyllysine-containing nucleosome probes for novel demethylase identification.

4.4 Methyllysine readers by dimethylsulfonium peptides and nucleosomes

Since readers bind to ligands rather than catalyse chemical transformations, identifying a suitable residue within the binding pocket for crosslinking is not trivial. Although numerous methyllysine reader inhibitors have been developed, no covalent inhibitors have been reported to date.[129,130] Through pull-down assays by affinity-based probes, diverse methyllysine readers have been identified, and their protein structures were resolved. Despite the diversity of folding in reader domains, including chromodomain, Tudor domain, PHD domains, *etc.*, they adopt an aromatic cage that binds to methyllysine *via* π-cation interactions, van der Waals interactions, as well as hydrophobic effects (Fig. 8A and B).[131–133] The tryptophan residue is highly conserved in approximately 90% of known cases, due to its electron-rich aromatic ring.[134] The indole ring of tryptophan is reactive in redox reaction and substitution, thus providing an opportunity for the development of selective crosslinking probes.

Feng, Gao, and Zhao *et al.* reported dimethylsulfonium peptide probes that crosslink readers in a binding-dependent manner (Fig. 8C).[135] Norleucine-ε-dimethylsulfonium ($NleS^{+}me2$) serves as a structural mimic of Kme2, binding to site-specific readers, and the sulfonium thereby closely interacts with the tryptophan inside the binding pocket. Under UV-B irradiation, the excited indole ring undergoes single electron transfer (SET) to the adjacent sulfonium, leading to selective crosslinking and the release of dimethylsulfide. Since the formation of an electron donor–acceptor (EDA)-complex driven by the reader-probe binding is crucial for efficient single-electron transfer, the tryptophan–sulfonium crosslinking exhibits high site-selectivity. Given that tryptophan is a highly conserved residue in the aromatic cages of methyllysine readers, sulfonium probes demonstrate broad applicability across diverse readers. In addition, due to their biocompatibility, sulfonium peptide probes have been applied to HeLa cell nuclei, enabling the selective crosslinking and identification of readers *via* quantitative mass spectrometry (Fig. 8D). The tryptophan site-selective crosslinking also enabled the identification of key tryptophan residues in readers through crosslinking mass spectrometry (Fig. 8D). Thus, the sulfonium probes showed significant potential for reader-selective crosslinking and identification.

The initially reported synthesis of sulfonium peptide probe requires 6-step preparation of Fmoc–NleSme–OH as an SPPS building block, and the resulting sulfide peptides are then converted to sulfonium by iodomethane (Fig. 8E). However, this synthetic method is overall time-consuming and not applicable to the synthesis of sulfonium proteins.[135,136] Zou and Yang *et al.* reported a cysteine alkylation method to install sulfonium to peptides and proteins (Fig. 8F).[137] Vinylsulfonium as a Michael acceptor alkylates cysteine with high selectivity and yield. This method enables facile preparation of sulfonium-containing full-length proteins. For example, recombinant histone H3K36C was converted to $H3K36Nle_{C}S^{+}me2$ followed by nucleosome reconstitution. A few known H3K36me3 readers were selectively crosslinked by sulfonium nucleosome probes.

However, the cysteine alkylation method is not compatible with proteins containing multiple cysteines. Additionally, methyllysine-modified proteins typically coexist with diverse post-translational modifications in cellular contexts. To advance functional sulfonium-based protein tools, Yang *et al.*

developed a semisynthetic method (Fig. 8G).[138] This approach employed an orthogonal cysteine protection strategy, enabling the preparation of peptides with both sulfonium and free cysteine. Subsequently, they established methods for protein fragments ligation using both native chemical ligation (NCL) and sortase-mediated ligation (SML). This semisynthetic method facilitates mechanistic investigation of crosstalk between lysine methylation and other PTMs using functionalized sulfonium protein tools.

4.5 Methylarginine readers by dimethylsulfonium

Arginine methylation is another important type of histone methylation.[139,140] This modification shares key features with lysine methylation, as both modified residues contain positively charged groups with methyl marks (Fig. 8H). Therefore, it is not surprising that methylarginine readers also contain aromatic cages for specific binding (Fig. 8I).[141,142] Luo *et al.* synthesized and screened several different sulfonium residues with different spacers between the dimethylsulfonium moiety and peptide backbone. Among these, the Nle_CS^+me2-containing peptide showed the highest efficiency, indicating its ability to partially mimic methylarginine and selectively crosslink to the readers.[143]

Among both methyllysine readers and methylarginine readers, some of them utilize aromatic cages devoid of tryptophan but only contain phenylalanine and tyrosine (Fig. 8I).[144–146] To achieve broader coverage across these readers, Gao *et al.* developed a strategy to crosslink tyrosine by sulfonium probes (Fig. 8J).[147] First, they switched UV lamp from 313 nm to 302 nm, which covers the absorbance range of tyrosine, so that tyrosine can be excited for single-electron transfer. Next, they elevated buffer pH to 9 to enhance deprotonation of tyrosine. Due to higher absorbance of phenoxide than phenol, the crosslinking was further improved. Finally, they tested several readers with tryptophan-free binding pockets, all of them were readily crosslinked by sulfonium probes. Although the efficiency was lower than crosslinking to tryptophan, such a strategy enables a broader coverage of aromatic cages of methyllysine and methylarginine readers by sulfonium probes.

4.6 Limitations

Activity-based probes exhibit superior crosslinking selectivity with low non-specific crosslinking background, but this method also has limitations. First, the crosslinking rate is constrained by the natural enzyme kinetics. For example, the demethylation by KDMs is far slower than deacetylation by sirtuins, and the allyllysine probes exhibit slow crosslinking rate and low conversion. Second, the development of such probes is complicated. Distinct mechanisms demand tailored warhead design, synthesis, and validation for each POI. It requires a deep understanding of the protein function mechanism and developing unique warheads for potential selective crosslinking. Therefore, the development is not as straightforward as photoreactive probes.

5. Future outlook

Significant progress in histone probe development over the past decade reveals a paradigm shift from affinity-based probes to covalent crosslinking strategies. The transition addresses critical limitations in capturing low-abundant proteins and POIs with transient interactions through irreversible target engagement. Driven by deepened mechanistic understanding of eraser catalysis and reader recognition, and innovations in organic chemistry, more activity-based, precise crosslinking probes are being developed. These probes bring new insights into both organic chemistry and biochemistry.

However, there are still some challenges in the field, and here we would like to propose some future directions. First, many histone probes are based on UV-driven photocrosslinking, however, UV has poor tissue penetration and causes DNA/protein damage. With the development of photoredox catalysis, proteins can be selectively crosslinked under light with longer wavelengths.[148] For example, based on the microMap (μMap) technology, Muir and MacMillan groups used engineered split inteins to tracelessly incorporate iridium-based photocatalysts into nuclear proteins, which enable the activation of diazirines under blue light and nanoscale crosslinking of histone-proximal proteins.[94] In addition to blue light, near-infrared (NIR) red light exhibits significantly deeper tissue penetration and minimal phototoxicity. Recent advances have reported red light activation photocatalysts to achieve proximity labelling, such as μMap-Red.[149,150] These systems could be adapted for histone probes in the future. Alternatively, irradiation-independent crosslinking probes would also be highly desired for *in vivo* applications. Although activity-based probes targeting HDACs, sirtuins, DUBs, and KDMs mentioned above have made certain progress, there is still a need to develop more irradiation-independent probes for diverse enzyme crosslinking with higher efficiency. Besides, sulfur(VI)-fluoride exchange (SuFEx) chemistry shows promise for application in histone research due to its employment in the investigation of PPIs.[151–153]

Second, current probes are mainly based on peptide and protein backbones, which exhibit poor cell permeability, restricting applications to cell lysates or extracted cell nuclei. Although efforts are being made in the field, such as intein and sortase-based methods, a significant improvement for wide applications is still challenging. One alternative strategy is to develop small molecule-based probes. Currently, numerous small-molecule inhibitors targeting KMTs, KDMs, HDACs, Kac readers, and Kme readers have been developed.[154–158] These inhibitors exhibit high affinity and specificity toward their targets, providing important insights for the design of small molecule probes. However, it is challenging to study crosstalk since such probes usually only target one pocket.

Third, even though the field has moved from peptide-based probes to nucleosome-based probes, NCPs are still a far simplified model for chromatin that contains multiple nucleosomes bound with large chromatin remodelling complexes. Several methods have been developed to prepare nucleosome

arrays with specific modifications. These modified nucleosome arrays have been employed to investigate nucleosome dynamics, the crosstalk of acetylation and ubiquitylation and higher-order chromatin compaction, the regulation of methyltransferase Suv39h1, and the effects of modifications in DNA damage repair.[159–162] With the advancement of nucleosome arrays, this approach more precisely elucidates the regulatory mechanisms of histone modifications. Therefore, large probes as nucleosome arrays that mimic local chromatin and crosslinking large chromatin complexes would bring more insights into readers and erasers.

Finally, PTMs of nonhistone proteins are also very important for cellular activities. Given homology of erasers and readers, histone probes might be expanded to investigate important biological questions from nonhistone modifications. In addition, the activity-based crosslinking chemistry may be used to develop chemoproteomic methods rather than site-specific probes. In this case, general probes could be applied to profile erasers and readers, just like ABPP of serine proteases by fluorophosphonate.

Author contributions

M. W. conceived the structure of the manuscript. J. Y. prepared the figures. J. Y. and M. W. wrote and edited the perspective.

Conflicts of interest

There are no conflicts to declare.

Data availability

No new data were generated in this perspective.

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