

Communication

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Engineering Methyllysine Writers and Readers for Allele-Specific Regulation of Protein-Protein Interactions

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Supporting Information Placeholder

ABSTRACT: Protein-protein interactions mediated by methyllysine are ubiquitous in biological systems. Specific perturbation of such interactions has remained a challenging endeavor. Herein, we describe an allele-specific strategy towards an engineered protein-protein interface orthogonal to the human proteome. We develop a methyltransferase (writer) variant that installs aryllysine moiety on histones that can only be recognized by an engineered chromodomain (reader). We establish biochemical integrity of the engineered interface, provide structural evidence for orthogonality and validate its applicability to identify transcriptional regulators. Our approach provides an unprecedented strategy for specific manipulation of the methyllysine interactome.

Lysine methylation in histones is a posttranslational modification that regulates gene expression.¹ This process is catalyzed by lysine methyltransferases (KMTs), classified as 'writers', using S-adenosylmethionine (SAM) (Figure 1).²⁻³ Biological functions of methyllysine are manifested via specific recognition by conserved protein modules termed 'readers' (Figure 1).⁴⁻⁶ In humans, an array of ~60 KMTs and >200 proteins with reader domains are involved in establishing and recognizing >5000 methyllysine sites.⁷ Context-dependent function of the methyllysine network is largely unexplored, mainly due to a lack of tools capable of interrogating various components of the 'interactome' simultaneously.⁸⁻¹⁰ Probing how a specific writer-histone-reader axis contributes to chromatin-dependent processes requires precise and sequential perturbation of the methyllysine writers and readers.

Allele-specific chemical genetics (often called the 'bumpand-hole' tactic) has emerged as a powerful tool to examine member-specific protein function.¹¹⁻¹⁶ We envisioned developing allele-specific writer-histone-reader pairs, fully orthogonal to the human proteome, for precise manipulation of methyllysine interaction (Figure 1). We reasoned a 'holemodified' KMT could accept SAM analogues to modify histone with bulky groups that would perturb the binding of a wild type reader through steric repulsion. Interaction with the 'bumped' histone could be restored with a 'hole-modified' reader variant, thus providing control over methyllysine-dependent interactions. Although engineered KMTs are known,¹⁷ evolving a reader to recognize non-native protein modifications is unexplored. We hypothesized that the hydrophobic 'aromatic cage' in reader domain that recognizes methyllysine⁵ could be expanded to accommodate alkyllysine in histone and create an orthogonal interface.



Figure 1. A strategy for allele-specific protein-protein interactions. Methyllysine writer (KMT) is first engineered to introduce 'bumped' lysine and disrupt interaction with wild-type readers. Binding is restored using 'hole-modified' reader, allowing conditional manipulation of methyllysine pathways.

We chose trimethylation on H3 at lysine 9 (H3K9me3) as a host system to test the above approach. H3K9 methylation is a marker of transcriptional repression.¹ In humans, a large set of KMTs methylate H3K9, and the modification is 'read' by chromodomain and PHD finger containing proteins.^{5,18} Herein, we first develop 'hole-modified' H3K9me3 methyltransferases, G9a and Suv39H2, to install lysine modifications that are not recognized by cognate wild-type readers CBX1, 3, and 5; we go on to engineer CBX proteins that bind 'bumped' histone and restore the lost interaction. Finally, we provide structural evidence for orthogonality and demonstrate suitability of the engineered system to control a methyllysine-dependent signaling pathway.

To access the 'bumped' histone peptides, we synthesized a series of SAM analogues carrying bulky sulfonium alkenyl and aryl groups (Figure 2A, Scheme S1).^{17,19} Each of the SAM derivatives was incubated with previously developed Y1154A mutant of G9a¹⁷ and tetramethylrhodamine (TAMRA) labeled H3K9 peptide. The products were purified and confirmed by MALDI-MS (Figure S1). The mutant showed complete alkenylation of the substrate (Figure S1); however, it was not efficient at incorporating benzyl modification. We envisioned an aryl group as a lysine modifier would improve binding with engineered CBX by maintaining π - π interaction in the remodeled aromatic cage. We surmised that expanding the pocket of G9a further could facilitate its use of benzyl-SAM. Indeed, Y1154G mutation led to complete substrate benzylation (Figure 2B), demonstrating that G9a can be engineered to

accept aryl SAM analogues for expanding the repertoire of 'bumped' histone variants. Under our assay conditions, we did not observe any dibenzylation of the substrate by G9a mutant (Figure S2).



Figure 2. Engineering of G9a and CBX1. (A) G9a-Y1154A mutant transfers alkenyl groups from the corresponding SAM analogues to TAMRA-labeled H3K9 peptide. (B) MALDI-MS showing benzylation of the substrate peptide by G9a-Y1154G mutant using benzyl-SAM. (C) Heat-map diagram showing dissociation constants (K_d) for the binding of modified H3K9 peptides by wild-type CBX1 and 'hole-modified' mutants.

As a model reader domain for allele-specific engineering, we selected the CBX1 chromodomain, which recognizes H3K9me3 through its aromatic cage.²⁰ First, using a fluorescence polarization assay, we determined the dissociation constants (K_d) of wild-type CBX1 chromodomain from TAMRA-labeled H3K9me3 and various 'bumped' variants (Figure 2C, S3). The reader protein bound H3K9me3 tightly, with a K_d (2.0±0.1 µM) close to the reported value.²¹ CBX1 showed 53-fold weaker binding affinity ($K_d = 106\pm 20 \mu$ M) towards allyl-modified peptide. Importantly, it failed to bind peptides with modifications bulkier than allyl ($K_d > 500 \mu$ M, Figure S3), presumably due to a steric clash inside the aromatic cage.

In order to develop an orthogonal reader, we analyzed a published structure of the CBX1 chromodomain and identified residues Y26, W47, F50, D54 and T56 that recognize H3K9me3.22 We generated the corresponding alanine mutants and measured their binding affinity towards all the modified peptides (Figure 2C, S4-8). Compared to wild type CBX1, the mutants showed decreased binding towards the trimethylated peptide, indicating that an intact hydrophobic pocket is essential for optimum recognition of H3K9me3.21 Among the CBX1 mutants examined, F50A showed significant gain in binding affinity towards the pentyl and benzyl peptides (K_d of 14.1±2.8 and 18.5±4.5 µM, respectively), while affinity for H3K9me3 decreased by almost 32-fold (K_d of 63.5±6.2 µM) compared to wild-type reader (Figure 2C, S6). Encouraged by this result, we prepared F50G mutant with a larger cavity in an effort to further improve the binding efficiency. F50G indeed showed a 3-fold improvement in affinity towards H3K9bn (Figure 2C, S9).

To improve orthogonality of the engineered readers, we generated several double mutants by combining F50G with mutations at Y26. Most mutations led to complete loss of

binding (Figure S10-12); however, a Y26F/F50G variant with intact aromatic sidechain at F26 maintained robust affinity towards benzyl peptide ($K_d = 5.5\pm0.9 \mu$ M; Figure 2C, S10). Importantly, interaction with H3K9me3 peptide was reduced to a K_d of ~80 μ M. We further confirmed the binding constants by isothermal titration calorimetry using H3K9me3 and benzylated (H3K9bn) peptides. Both native and engineered readers showed comparable affinity to their cognate peptides (Figure 3A, B, S13, Table S1). Furthermore, when tested against the H3K9me2 peptide, a major enzymatic product of G9a, wildtype and engineered CBX1 bound with K_d of 18.3 μ M and >300 μ M, respectively (Figure S14). Taken together, our mutational study led to an orthogonal chromodomain capable of recognizing 'bumped' histone peptide—a new approach to modulate histone-reader interactions.



Figure 3. Characterization of the histone-reader pairs. (A, B) Isothermal titration calorimetric (ITC) measurements for binding between wild-type CBX1 and H3K9me3 (A), and Y26F/F50G-CBX1 and H3K9bn (B). (C, D) View of the aromatic cage from crystal structures of wild type CBX1 chromodomain bound to H3K9me3 peptide (C) and 'hole-modified' CBX1 (Y26F/F50G) bound to H3K9bn (D).

To gain further insight into the orthogonality of the bumphole pair, we grew crystals of the complex of native CBX1 with H3K9me3 as well as the complex of Y26F/F50G-CBX1 with H3K9bn by hanging drop vapor diffusion. We collected diffraction data on each complex, solved the native structure (PDB 6D07) by molecular replacement with a model derived from a structure of CBX5 bound to H3K9me3,²¹ and solved the mutant structure (PDB 6D08) using refined model of the native CBX1 complex. Both structures were refined to 2.1 Å resolution (Figure 3C, D, Table S2). The overall fold and binding interactions observed for native CBX1 in complex with H3K9me3 are identical to those noted in closely related members CBX3 and CBX5 (Figure S15).²¹ The backbone coordinates for the bump-hole mutant complex were virtually identical to native CBX1 (0.6 Å C_{α} rmsd, Figure S15). Inspection of the aromatic binding pocket shows that the benzyl 'bump' effectively fills the hole created by the F50G mutation (Figure 3D) and that removal of the side chain in CBX1 has no measurable effect on local backbone conformation. Superimposition of the two complexes revealed that F50 of wild

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type CBX1 undergoes steric interference with benzyl group of H3K9bn (Figure S15), explaining the observed orthogonality.

Sequence analysis of the major KMTs revealed that Y1154 in G9a is highly conserved among writers (Figure 4A) and an aromatic residue, equivalent to F50 in CBX1, is present in all of the readers analyzed (Figure 4B).²³ These bulky amino acids are acting as gatekeepers that preclude binding of SAM and methyllysine analogues to writers and readers, respectively. We focused on another H3K9me3 methyltransferase, Suv39H2, and the cognate reader CBX3.²⁴⁻²⁵ The hole-modified Y372G mutant of Suv39H2, equivalent to G9a-Y1154G, efficiently utilized benzyl-SAM to modify substrate peptide as well as fulllength H3 (Figure 4C, S16, 17).

To demonstrate generality among reader domains, we prepared F44G mutant of CBX3, equivalent to Y26F/F50G-CBX1. Conveniently, CBX3 already carries a phenylalanine at the position equivalent to Y26 in CBX1. Wild type CBX3 recognized H3K9me3 peptide effectively as judged by fluorescence anisotropy, but no binding was observed towards the benzylated homologue (Figure 4D). In contrast, the 'holemodified' CBX3 exhibited enhanced affinity for H3K9bn with significant loss of binding towards the trimethyl mark (Figure 4E). These results establish that multiple histone writers and readers can be engineered at gatekeeper sites to access orthogonal protein-protein interfaces.



Figure 4. Generality in methyllysine writer-reader engineering. (A, B) Sequence alignments of KMTs (A) and methyllysine readers (B) showing Y1154 of G9a and F50 in CBX1 are highly conserved. (C) Substrate benzylation by Suv39H2-Y372G mutant on full-length histone H3. (D) Binding of wild-type CBX3 towards H3K9me3 (K_d = 12.2±0.2 µM) and H3K9bn ($K_d > 1$ mM). (E) Binding of CBX3-F44G mutant towards H3K9me3 ($K_d > 1$ mM) and H3K9bn (($K_d = 49\pm2.4 \mu$ M).

To determine if the engineered writer and reader could act in tandem, we first benzylated full-length H3 and purified mononucleosomes using Suv39H2 mutant followed by enrichment with the hole-modified CBX1. We observed signal for H3 only in the presence of benzyl-SAM as evident from western blot data (Figure S18), demonstrating successful reading and writing of H3K9bn on full-length substrates.

We further investigated whether the orthogonal pairs are functional in cellular milieu (Figure 5A, S19). HEK293T cells were cultured to express full-length hole-modified Suv39H2 variant and the isolated nuclei were incubated with benzyl-SAM in a pulldown assay. Only the benzylated H3 was found to be specifically enriched with the CBX1 mutant (Figure S19), indicating that engineered pair could act on chromosomal histone. Next, we expressed full-length CBX1 variant carrying the engineered chromodomain.²⁶ Nuclear extracts were incubated with biotinylated histone peptides, each carrying either unmodified, methylated or benzylated lysine, pulled down with avidin beads and analyzed using antibodies for CBX1, 3 or 5 (Figure 5B, S20). While the trimethylated peptide enriched all three wildtype CBX proteins, the benzylated bait was specific for hole-modified CBX1 (Figure 5B, S21). Furthermore, biotinylated peptides carrying methyllysine at a site different from H3K9 failed to interact with the mutant (Figure 5C, S21), confirming competency of the engineered system to mediate allele-specific histone-reader interactions. Although, we only examined CBX1, 3 and 5, detailed proteomic analysis of enriched proteins will be required to evaluate specificity of H3K9bn across all the methyllysine readers.

Finally, we examined interactions between the CBX1 variant and transcriptional complexes (Figure 5A). Binding of full-length CBX proteins to H3K9me3 creates a docking site for transcriptional regulators such as TIF1B.26,27 We incubated biotinylated H3K9bn peptide with nuclear extract of HEK293T cells expressing the 'hole-modified' reader, and enriched the 'interactome' using avidin and analyzed by western blotting with TIF1β antibody (Figure 5D, S21). We observed selective pull-down of TIF1B by benzylated peptide, but not the unmodified peptide, only when cells expressed the 'holemodified' CBX1 mutant. The 'bumped' peptide failed to interact with transcriptional regulators without the engineered reader. As a positive control, trimethylated peptide was shown to enrich TIF1ß using endogenous CBX proteins. This set of results demonstrated that the engineered system maintains native-like interactions between a specific methyllysine reader and transcriptional regulators.



Figure 5. Allele-specific interactions demonstrated in cell extracts. (A) Interaction between 'hole-modified' CBX1 and 'bumped' histone in presence of endogenous methyllysine readers (step 1). The engineered complex binds to transcriptional regulators (step 2). (B) Western blot (indicated antibody) of nuclear extracts pulled-down using biotin-attached H3K9me3 or H3K9bn peptide. CBX1 mutant carries HA tag. (C) Western blot (anti-HA antibody) of nuclear extracts pulled-down using biotin-attached H3K9me3 or H3K9bn peptide (D) Western blot (anti-TIF1 β antibody) of nuclear extracts pulled-down using biotin-attached H3K9me3 or H3K9bn peptide.

In summary, we offer a strategy to modulate protein-protein interactions particularly those governed by lysine methylation. Development of orthogonal pairs for protein-protein interactions is rarely attempted.²⁸⁻³⁰ Using a set of H3K9me3 writers and readers as paradigm, we showed that the remodeled interface is orthogonal to the wild type. We established biochemical integrity of the engineered interface, provided structural rationale for orthogonality, demonstrated generality of the approach, and validated functional compatibility of the synthetic interface in recognizing transcriptional regulators. We anticipate developing an in-cellulo assay to synthesize benzyl-SAM by promiscuous SAM synthetase mutant using benzyl methionine precursor.³¹ The engineered benzyllysine apparatus would allow installing the unique modification on chromosomal histone to interrogate a specific methyllysine pathway within cell for orthogonal manipulation of mammalian gene expression.

ASSOCIATED CONTENT

Supporting Information. Methods for protein expression and crystallization, biochemical assays, supplementary figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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The authors declare no competing financial interest

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REFERENCES

(1) Kouzarides, T., Chromatin modifications and their function. *Cell* **2007**, *128*, 693-705.

(2) Luo, M., Chemical and Biochemical Perspectives of Protein Lysine Methylation. *Chem. Rev.* **2018**, *118*, 6656-6705.

(3) Lin, H., S-Adenosylmethionine-dependent alkylation reactions: when are radical reactions used? *Bioorg. Chem.* **2011**, *39*, 161-70.

(4) Taverna, S. D.; Li, H.; Ruthenburg, A. J.; Allis, C. D.; Patel, D. J., How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Na. Struct. Mol. Biol.* **2007**, *14*, 1025-40.

(5) Patel, D. J., A Structural Perspective on Readout of Epigenetic Histone and DNA Methylation Marks. *Cold Spring Harbor Perspec. Biol.* **2016**, *8*, a018754.

(6) Beaver, J. E.; Waters, M. L., Molecular Recognition of Lys and Arg Methylation. *ACS Chem. Biol.* **2016**, *11*, 643-53.

(7) Murn, J.; Shi, Y., The winding path of protein methylation research: milestones and new frontiers. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 517-527.

(8) Arkin, M. R.; Tang, Y.; Wells, J. A., Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. *Chem. Biol.* **2014**, *21*, 1102-14.

(9) James, L. I.; Frye, S. V., Chemical probes for methyl lysine reader domains. *Curr. Opin. Chem. Biol.* **2016**, *33*, 135-41.

(10) Milosevich, N.; Hof, F., Chemical Inhibitors of Epigenetic Methyllysine Reader Proteins. *Biochem.* **2016**, *55*, 1570-83.

(11) Islam, K., The Bump-and-Hole Tactic: Expanding the Scope of Chemical Genetics. *Cell Chem. Biol.* **2018**, *25*, 1171-1184.

(12) Belshaw, P. J.; Schoepfer, J. G.; Liu, K.-Q.; Morrison, K. L.; Schreiber, S. L., Rational Design of Orthogonal Receptor–Ligand Combinations. *Angew. Chem. Int. Ed.* **1995**, *34*, 2129-2132.

(13) Bishop, A. C.; Ubersax, J. A.; Petsch, D. T.; Matheos, D. P.; Gray, N. S.; Blethrow, J.; Shimizu, E.; Tsien, J. Z.; Schultz, P. G.; Rose, M. D.; Wood, J. L.; Morgan, D. O.; Shokat, K. M., A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* **2000**, *407*, 395-401. 14.

(14) Baud, M. G.; Lin-Shiao, E.; Cardote, T.; Tallant, C.; Pschibul, A.; Chan, K. H.; Zengerle, M.; Garcia, J. R.; Kwan, T. T.; Ferguson, F. M.; Ciulli, A., Chemical biology. A bump-and-hole approach to engineer controlled selectivity of BET bromodomain chemical probes. *Science* **2014**, *346*, 638-41.

(15) Qiao, Y.; Molina, H.; Pandey, A.; Zhang, J.; Cole, P. A., Chemical rescue of a mutant enzyme in living cells. *Science* **2006**, *311*, 1293-7.

(16) Breski, M.; Dey, D.; Obringer, S.; Sudhamalla, B.; Islam, K., Engineering Biological C–H Functionalization Leads to Allele-Specific Regulation of Histone Demethylases. J. Am. Chem. Soc. **2016**, *138*, 13505-13508.

(17) Islam, K.; Zheng, W.; Yu, H.; Deng, H.; Luo, M., Expanding cofactor repertoire of protein lysine methyltransferase for substrate labeling. *ACS Chem. Biol.* **2011**, *6* 679-84.

(18) Allis, C. D.; Muir, T. W., Spreading chromatin into chemical biology. *ChemBioChem.* **2011**, *12*, 264-79.

(19) Dalhoff, C.; Lukinavicius, G.; Klimasauskas, S.; Weinhold, E., Direct transfer of extended groups from synthetic cofactors by DNA methyltransferases. *Nat. Chem. Biol.* **2006**, *2*, 31-2.

(20) Lachner, M.; O'Carroll, D.; Rea, S.; Mechtler, K.; Jenuwein, T., Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **2001**, *410*, 116-20.

(21) Kaustov, L.; Ouyang, H.; Amaya, M.; Lemak, A.; Nady, N.; Duan, S.; Wasney, G. A.; Li, Z.; Vedadi, M.; Schapira, M.; Min, J.; Arrowsmith, C. H., Recognition and specificity determinants of the human cbx chromodomains. *B. Biol. Chem.* **2011**, *286*, 521-9.

(22) Nielsen, P. R.; Nietlispach, D.; Mott, H. R.; Callaghan, J.; Bannister, A.; Kouzarides, T.; Murzin, A. G.; Murzina, N. V.; Laue, E. D., Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature* **2002**, *416*, 103-7.

(23) Liu, L.; Zhen, X. T.; Denton, E.; Marsden, B. D.; Schapira, M., ChromoHub: a data hub for navigators of chromatin-mediated signalling. *Bioinformatic*. **2012**, *28*, 2205-6.

(24) Rea, S.; Eisenhaber, F.; O'Carroll, D.; Strahl, B. D.; Sun, Z. W.; Schmid, M.; Opravil, S.; Mechtler, K.; Ponting, C. P.; Allis, C. D.; Jenuwein, T., Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **2000**, *406*, 593-9.

(25) Canzio, D.; Larson, A.; Narlikar, G. J., Mechanisms of functional promiscuity by HP1 proteins. *Trends Cell Biol.* **2014**, *24*, 377-86.

(26) Lechner, M. S.; Schultz, D. C.; Negorev, D.; Maul, G. G.; Rauscher, F. J., 3rd, The mammalian heterochromatin protein 1 binds diverse nuclear proteins through a common motif that targets the chromoshadow domain. *Biochem. Biophys. Res. Commun.* **2005**, *331*, 929-37.

(27) Miyagi, S.; Koide, S.; Saraya, A.; Wendt, G. R.; Oshima, M.; Konuma, T.; Yamazaki, S.; Mochizuki-Kashio, M.; Nakajima-Takagi, Y.; Wang, C.; Chiba, T.; Kitabayashi, I.; Nakauchi, H.; Iwama, A., The TIF1beta-HP1 system maintains transcriptional integrity of hematopoietic stem cells. *Stem Cell Rep.* **2014**, *2*, 145-52.

(28) Koh, M.; Nasertorabi, F.; Han, G. W.; Stevens, R. C.; Schultz, P. G., Generation of an Orthogonal Protein-Protein Interface with a Noncanonical Amino Acid. J. Am. Chem. Soc. **2017**, *139*, 5728-5731.

 (29) Palmer, A. E.; Giacomello, M.; Kortemme, T.; Hires, S. A.; Lev-Ram, V.; Baker, D.; Tsien, R. Y., Ca²⁺ indicators based on computationally redesigned calmodulin-peptide pairs. *Chem. Biol.* **2006**, *13*, 521-30.

(30) Rooklin, D.; Modell, A. E.; Li, H.; Berdan, V.; Arora, P. S.; Zhang, Y., Targeting Unoccupied Surfaces on Protein-Protein Interfaces. J. Am. Chem. Soc. **2017**, 139, 15560-15563. (31) Wang, R.; Islam, K.; Liu, Y.; Zheng, W.; Tang, H.; Lailler, N.; Blum, G.; Deng, H.; Luo, M., Profiling genome-wide chromatin methylation with engineered posttranslation apparatus within living cells. *J. Am. Chem. Soc.* **2013**, *135*, 1048-56.

