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Genetic Incorporation of ε-*N*-2-Hydroxyisobutyryl-lysine into Recombinant Histones

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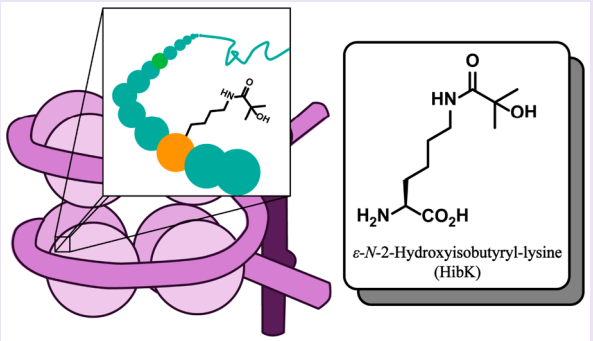
Supporting Information

ABSTRACT: Here, we report the evolution of an orthogonal amber suppressor pyrrolysyl−tRNA synthetase (PylRS)/$tRNA_{CUA}^{Pyl}$ pair that genetically encodes the post-translationally modified amino acid, ε-*N*-2-hydroxyisobutyryl-lysine (HibK), in bacteria and mammalian cells. HibK is a new type of histone mark that is widely distributed in histone proteins. The ability to site-specifically incorporate HibK into proteins provides a useful tool to probe the biological function of this newly identified post-translational modification.



Post-translational modifications (PTMs) in histone proteins play a critical role in cellular processes including DNA replication, DNA repair, chromosome condensation, gene regulation, spermatogenesis, and apoptosis.[1−5] In particular, lysine residues are subject to a diverse array of PTMs including methylation, acetylation, crotonylation, propionylation, butyrylation, ubiquitination, biotinylation, glutarylation, succinylation, malonylation, and ADP ribosylation.[6,7] These chemical modifications can affect chromatin folding and function by altering the net charge of histones, recruiting PTM-specific binding proteins or functioning as an inhibitor for the binders with chromatin.[7−10] Recently, Dai and co-workers reported the identification of a new lysine modification, 2-hydroxyisobutyrylation.[11] This novel PTM was found in mouse and human cells at the N-terminal tail regions of histones and at surface sites involved in inter- or intranucleosome interactions. Modification of lysine with a 2-hydroxyisobutyrl group (K_{hib}) not only abolishes the positive charge of Lys, but it also significantly introduces the steric bulk of the side chain, which is likely to affect the association of histones with DNA. Indeed, dramatic changes in K_{hib} distribution were observed during the development of sperm cells.[11]

The ability to site-specifically incorporate this novel PTM into proteins in living cells should greatly facilitate studies of its function. Both biosynthetic and semisynthetic strategies have been developed to prepare homogeneous, post-translationally modified proteins; however, semisynthetic methods can be challenging with larger proteins and are less compatible with cellular studies.[12−17] An alternative approach is to genetically encode the post-translationally modified amino acid in the host of interest, which involves the generation of orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pairs that site-specifically incorporate unnatural amino acids (UAAs) into proteins in response to the amber codon, TAG. Indeed, this strategy has been used to genetically encode ε-*N*-acetyl-lysine, ε-*N*-methyl-lysine, ε-*N*,*N*-dimethyl-lysine, and ε-*N*-crotonyl-lysine in order to probe the roles of these modified amino acids in regulating cell function.[15,18−21] For example, the site-specific incorporation of ε-*N*-acetyl-lysine into H3K56 revealed that H3K56 has no direct effect on the compaction of chromatin but increases DNA breathing 7-fold,[22] and the introduction of ε-*N*-methyl-lysine into H3K9 allowed determination of its effects on the binding specificity of heterochromatin protein 1.[19] Here, we report that a similar approach can be used to site-specifically incorporate the newly reported post-translationally modified amino acid, ε-*N*-2-hydroxyisobutyryl-lysine (HibK), into recombinant proteins in both *Escherichia coli* and mammalian cells.

To genetically encode ε-*N*-2-hydroxyisobutyryl-lysine, the pyrrolysyl-tRNA synthetase (PylRS)/$tRNA_{CUA}^{Pyl}$ pair, which incorporates a number of Lys derivatives, was used.[12] On the basis of the crystal structure of the PylRS·pyrrolysyl-AMP complex, a *Methanosarcina barkeri* PylRS library containing four randomized positions (Leu270, Tyr271, Leu274, and Cys313; Figure 1B) involved in recognition of pyrrolysine was generated.[23] Each position was randomized to NNK (N = any nucleotide, K = G or T) by site-saturation mutagenesis. Tyr349 was fixed as Phe by site-directed mutagenesis, since this substitution was previously shown to increase aminoacylation efficiency.[24] More than 10^9 transformants were generated for the library, and no significant sequence bias was observed by sequence analysis of individual clones. This library was then

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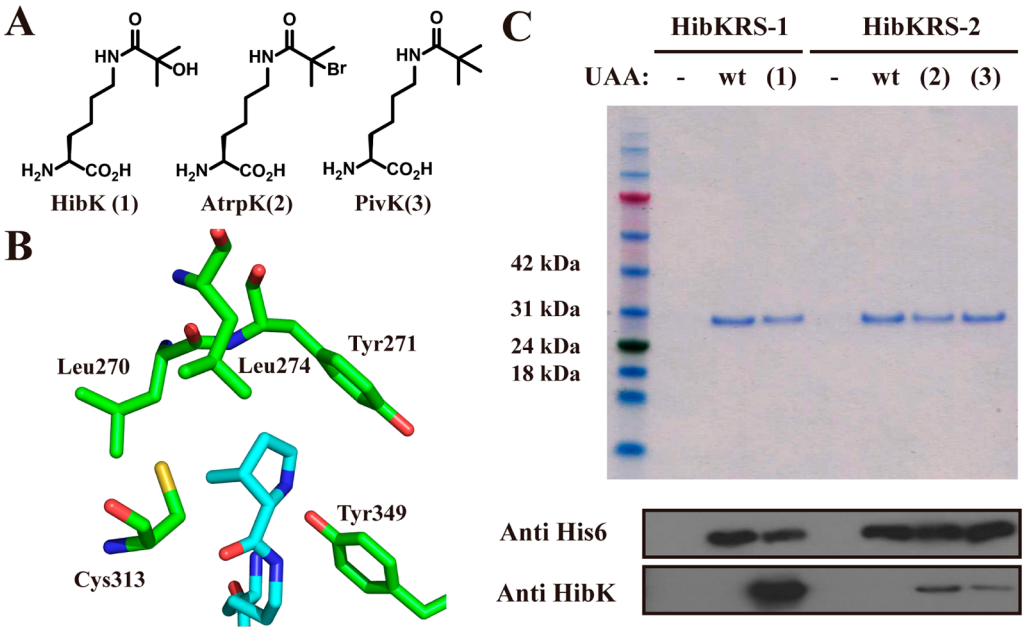

Figure 1. (a) Structures of *ε-N*-2-hydroxyisobutyryl-lysine (HibK, 1), *ε-N*-2-bromoisobutyryl-lysine (AtrpK, 2), and *ε-N*-pivaloyl-lysine (PivK, 3) used in this study. (b) X-ray crystal structure of the MmPylRS complex with pyrrolysyl-AMP. The structure is from PDB entry 2ZIM. (c) Expression of the GFP mutants analyzed by SDS-PAGE in the presence or absence (−) of 1 mM HibK, AtrpK, or PivK. Western blot analysis of GFP mutants with anti-His6 antibody and anti-2-hydroxyisobutyryl-lysine antibody confirmed the incorporation of HibK into GFP.

subjected to a double-sieve selection to identify PylRS variants that can charge HibK onto pyrrolysyl tRNA.[25] In the positive selection, the library was transformed into *E. coli* DH10B cells containing a pRep plasmid encoding $tRNA_{CUA}^{Pyl}$ and a chloramphenicol acetyltransferase (CAT) gene with an amber codon at a permissive site (Asp112TAG). The cells were grown in the presence of 1 mM HibK and 40 μg mL^{-1} chloramphenicol to select aaRS mutants that efficiently incorporate HibK into the CAT gene in response to the amber codon. In the negative selection, the surviving library variants were introduced into cells harboring a plasmid containing a toxic barnase gene with amber mutations at three permissive sites (Gln2TAG, Asp44TAG, and Gly65-TAG). The cells were grown in the absence of HibK to remove all aaRS variants that recognize the 20 canonical amino acids. After two rounds of positive selections and one round of negative selection, single colonies that survived 60 μg mL^{-1} chloramphenicol only in the presence of 1 mM HibK were further characterized. Sequencing of 20 colonies revealed the existence of two unique sequences (HibKRS-1: Leu270, Tyr271, Leu274, Cys313Thr, Tyr349Phe and HibKRS-2: Leu270, Tyr271, Leu274, Cys313Ser, Tyr349Phe; Supporting Information Figure S1). The mutation of Cys313 to Thr or Ser may result in hydrogen bond formation between HibK and the mutant PylRSs.

To investigate the efficiency and specificity of these two synthetases, *E. coli* DH10B cells containing the plasmid pLeiG-GFP-Asp134TAG were independently transformed with pBK-HibKRS-1 and pBK-HibKRS-2.[20,26] pLeiG-GFP-Asp134TAG encodes a proK promoter-driven $tRNA_{CUA}^{Pyl}$ expression cassette and a GFP variant with a C-terminal hexahistidine-tag and an amber codon at a permissive site, Asp134. A quantitative fluorescence assay was carried out in minimal media in the presence and absence of 1 mM HibK, and an increase in fluorescence was observed for both HibKRS-1 and HibKRS-2 in the presence of the HibK. HibKRS-1 exhibited the higher activity (Supporting Information Figure S2) and was therefore used to express a GFP containing an amber codon at Asp134 in Luria−Bertani (LB) medium in the presence or absence of 1 mM HibK, followed by Ni-NTA affinity purification. SDS-PAGE analysis of the GFP mutants revealed that full-length GFP was only expressed in the presence of 1 mM HibK. ESI-MS analysis afforded two observed masses of 27 866 Da and 27 997 Da (with N-terminal methionine) in agreement with a calculated mass of 27 864 Da, which confirmed site-specific incorporation of HibK into GFP (Figure 1C, and Supporting Information Figure S3). In addition, an anti-2-hydroxyisobutyryl-lysine polyclonal antibody was also used to confirm the incorporation of HibK by Western blot analysis. Both wild type GFP and GFP containing HibK with a C-terminal hexahistidine-tag revealed a band using an anti-His_6 antibody, but a band was only observed for the GFP containing HibK with the anti-2-hydroxyisobutyryl-lysine antibody (Figure 1C).[11] The yield of the purified GFP mutant containing HibK was 11.9 mg L^{-1} in LB.

The evolved aaRS variants may be substrate poly-specific (i.e., aminoacylate various UAAs, but not canonical amino acids), since the selection scheme was not designed to select against other UAAs. To determine if the aaRSs selected against HibK can charge other analogues, two other UAAs with similar structures (AtrpK: *ε-N*-2-bromoisobutyryl-lysine; PivK: *ε-N*-pivaloyl-lysine) were synthesized (Figure 1A). The 2-bromoisobutyryl group is known as an efficient initiator in atom-transfer radical polymerization (ATRP) reactions, and 4-(2′-bromoisobutyramido)phenylalanine has been used to afford a stable linkage between proteins and a growing polymer.[27,28] We expressed GFP variants in *E. coli* DH10B cells containing pLeiG-GFP-Asp134TAG transformed with pBK-HibKRS-1 or pBK-HibKRS-2. Protein expressions were carried out in GMML media supplemented with 1 mM AtrpK or PivK, and the relative fluorescent intensities of these cell cultures were measured (Supporting Information Figure S2). Based on the

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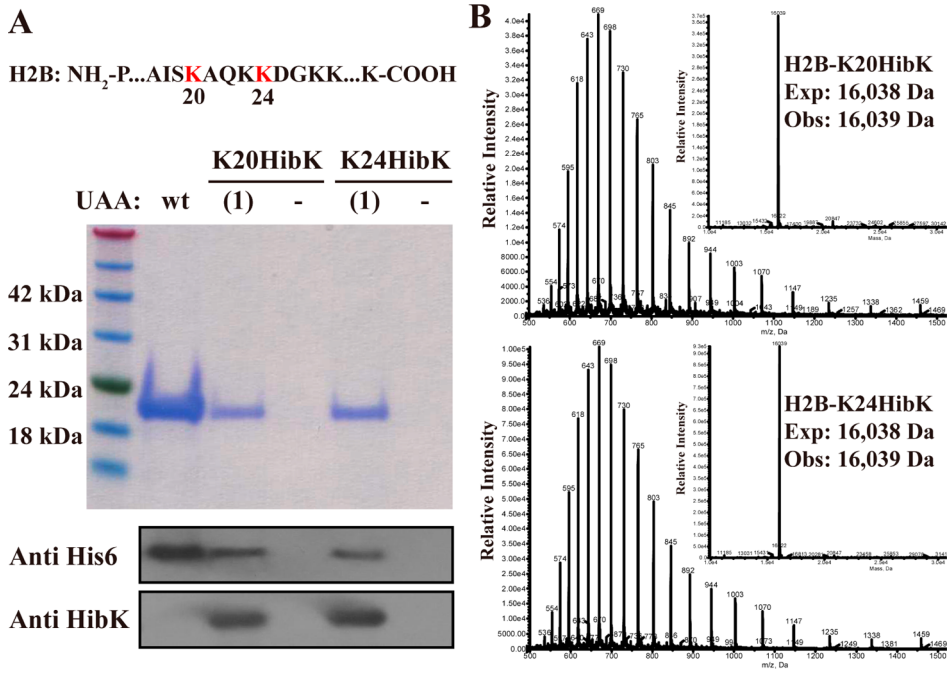

Figure 2. Incorporation of HibK into recombinant human histone H2B. (a) Expression of the H2B mutants analyzed by SDS-PAGE in the presence or absence (−) of 1 mM HibK. Western blot analysis of GFP mutants with anti-His6 antibody and anti-2-hydroxyisobutyryl-lysine antibody confirmed the incorporation of HibK. (b) ESI-MS analysis of H2B mutants (H2B−K20HibK and H2B−K24HibK).

GFP expression levels observed in the presence or absence of UAAs, *ε-N*-2-bromoisobutyryl-lysine (AtrpK (**2**)) and *ε-N*-pivaloyl-lysine (PivK (**3**)) were found to be efficiently incorporated by HibKRS2 (Figure 1A and C). In the Western blot analysis, weak bands were also observed for the GFP mutants with AtrpK or PivK using an anti-2-hydroxyisobutyryl-lysine antibody, which suggests this antibody cannot efficiently distinguish the substitution of the isobutyl moiety of HibK. Yields of GFP mutants expressed in LB substituted with AtrpK and PivK were 4.3 mg L^{-1} and 4.9 mg L^{-1}, respectively. ESI-MS analysis of Ni^{2+}-NTA affinity chromatography purified proteins confirmed the incorporation of these two UAAs (Supporting Information Figure S3). Calculated mass of AtrpK-containing GFP is 27 927 Da and observed masses are 27 929 Da and 28 060 Da (with N-terminal methionine). Calculated mass of PivK-containing GFP is 27 862 Da and masses of 27 865 Da and 27 996 Da (with N-terminal methionine) were observed.

We next demonstrated that we can substitute either Lys20 or Lys24 of human histone subunit 2B (H2B) with HibK in *E. coli*. Lys24 is known to be only modified by 2-hydroxyisobutyrylation, while Lys20 residue can be acetylated, crotonylated, or 2-hydroxyisobutyrylated.[11] A pLeiG-H2B plasmid encoding a proK promoter-driven $tRNA_{CUA}^{Pyl}$ expression cassette and a H2B variant with an N-terminal hexahistidine-tag was constructed. To express HibK-containing H2B mutants, *E. coli* DH10B cells cotransformed with the plasmids pLeiG-H2B and pUltra-HibKRS-1 were grown in 2xYT media supplemented with 1 mM HibK.[29] Protein expressions were induced with 1 mM IPTG, and proteins were harvested after 20 h at 37 °C followed by Ni^{2+} affinity chromatography purification under denaturing conditions. Both H2B variants, H2B−K20HibK and H2B−K24HibK, were expressed with good yields of 3.6 mg L^{-1} and 5.6 mg L^{-1}, respectively. SDS-PAGE analysis showed that the full-length H2B mutants were expressed only in the presence of 1 mM HibK (Figure 2A). The incorporation of HibK was further confirmed by Western blot using an anti-2-hydroxyisobutyryl-lysine polyclonal antibody. Wild type H2B and H2B mutants revealed a band with anti-His6 antibody, but only HibK-containing H2B variants (H2B−K20HibK and H2B−K24HibK) were detectable using an anti-2-hydroxyisobutyryl-lysine polyclonal antibody (Figure 2A). ESI-mass spectrometric analysis of H2B mutants afforded an observed mass of 16 039 Da, in agreement with the calculated mass (Figure 2B).

It has been shown that the MbPylRS/$tRNA^{Pyl}$ pairs evolved in bacteria can be directly imported into mammalian cells and are functional.[20,26] To test the feasibility of incorporating HibK in mammalian cells using the HibKRS/$tRNA^{Pyl}$ pair, we constructed a plasmid encoding a U6-driven *Methanosarcina mazei* pyrrolysyl tRNA ($MmtRNA^{Pyl}$) cassette, as well as the HibKRS-1 gene expressed under a CMV promoter (Figure 3). This plasmid was cotransfected with pAcBac2.tR2-EGFP into HEK293T cells, and protein expression was carried out in

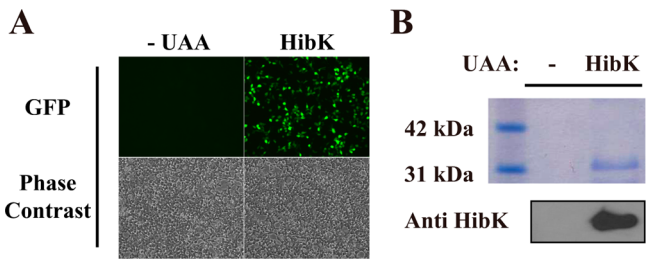

Figure 3. Incorporation of HibK into EGFP-Y39TAG expressed in mammalian cells. (a) Expression of the following EGFP mutants in HEK293T cells analyzed by fluorescence microscopy in the presence (+UAA) or absence (−UAA) of 1 mM HibK. (b) Expression of the aforementioned EGFP mutants analyzed by SDS-PAGE in the presence (+) or absence (−) of 1 mM HibK. The incorporation of HibK into EGFP was confirmed by Western blot analysis with anti-2-hydroxyisobutyryl-lysine antibody.

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Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in the absence or in the presence of 1 mM HibK.[30,31] Full-length EGFP mutant containing HibK was only observed in the presence of 1 mM HibK, and the incorporation of HibK into EGFP in mammalian cells was confirmed by Western blot using anti-2-hydroxyisobutyryl-lysine polyclonal antibody and ESI-MS (Supporting Information Figure S5). ESI-mass spectrometric analysis afforded an observed mass of 29 736 Da, in agreement with the calculated mass. The isolated yield of HibK-containing EGFP from 10^6 HEK293 cells was 3.0 μg, approximately 10% of the yield of wild type EGFP.

In conclusion, we have evolved a MbPylRS/$tRNA_{CUA}^{Pyl}$ pair to genetically encode HibK in both *E. coli* and mammalian cells with good yield and high fidelity. The ability to prepare homogeneous histones with defined PTMs provides a useful tool to decipher the biological functions of these PTMs.

METHODS

Determination of aaRS Polyspecificity. pLeiG-GFP-Asp134TAG and pBK-HibKRS-1 or pBK-HibKRS-2 plasmids were transformed into *Escherichia coli* DH10B. Cells were grown in GMML media, supplemented with chloramphenicol (25 μg/mL), kanamycin (25 μg/mL), and 1 mM various unnatural amino acids at 37 °C to an OD_{600} of 0.6, at which point IPTG was added to a final concentration of 1 mM. After 16 h of expression at 30 °C, the cells were harvested by centrifugation at 4700*g* for 10 min and washed three times with PBS. Cells resuspended in PBS were transferred to a clear bottom 96 well plate, and GFP fluorescence was measured using a plate reader (485 nm excitation and 515 nm emission).

Expression and Purification of GFP. pLeiG-GFP-Asp134TAG and pBK-HibKRS-1 or pBK-HibKRS-2 plasmids were transformed into *Escherichia coli* DH10B. Cells were grown in 2× YT media, supplemented with chloramphenicol (25 μg/mL), kanamycin (25 μg/mL), and 1 mM unnatural amino acids at 37 °C to an OD_{600} of 0.6, at which point IPTG was added to a final concentration of 100 μM. After 20 h of expression at 30 °C, the cells were harvested by centrifugation at 4700*g* for 10 min. To purify the protein, the cell pellets were resuspended in BugBuster protein extraction reagent and lysed at 30 °C. The resulting cell lysate was clarified by centrifugation at 18 000*g* for 30 min, and the proteins were purified on Ni^{2+}-NTA resin following the manufacturer's (Qiagen) instructions.

Expression and Purification of Histone H2B. pUltra-HibKRS-1 and pLeiG-H2B−K20HibK or pLeiG-H2B−K24HibK plasmids were transformed into *Escherichia coli* DH10B. Cells were grown in 2× YT media, supplemented with chloramphenicol (25 μg/mL), spectinomycin (25 μg/mL), and 1 mM unnatural amino acids at 37 °C to an OD_{600} of 0.6, at which point IPTG was added to a final concentration of 100 μM. After 20 h of expression at 37 °C, the cells were harvested by centrifugation at 4700*g* for 10 min. To purify the protein, the cell pellets were resuspended in BugBuster protein extraction reagent and lysed at 30 °C. The resulting cell lysate was clarified by centrifugation at 18 000*g* for 30 min, and the proteins were purified on Ni^{2+}-NTA resin under denaturing conditions following the manufacturer's (Qiagen) instructions.

Expression and Purification of EGFP Mutants in HEK293T Cells. Adherent HEK293T cells were maintained in DMEM media supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37 °C in a humidified chamber with 5% CO_2. HEK293T cells were seeded in a six-well plate at 5 × 10^5 cells/well in DMEM media, supplemented with 10% FBS and 0.5% antibiotic−antimycotic. Transfection was performed with pShax-$HibK^{CUA}$ and pAcBac2.tR4-EGFP* using FuGENE HD Transfection Reagent (Promega) according to manufacturer's protocol. Cells were lysed with 250 μL of CelLytic M buffer (Sigma-Aldrich) supplemented with 10 unit/mL benzonase (EMD) for 1 h. Cell extract was clarified by centrifugation at 14 000*g* for 3 min, and EGFP was purified by Ni^{2+}-NTA affinity chromatography following the manufacturer's (Qiagen) instructions. The isolated protein was characterized by SDS-PAGE analysis followed by coomassie staining, and by ESI-MS analysis using an Agilent 1100 series LC/MS instrument. Protein concentrations were measured using a Coomassie Plus (Bradford) Protein Assay kit from Pierce.

ASSOCIATED CONTENT

Supporting Information

Supporting Figures S1−S5, supporting Table S1, and more experimental procedures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/cb501055h.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Grunstein, M. (1997) Histone acetylation in chromatin structure and transcription. *Nature 389*, 349−352.

(2) Jenuwein, T., and Allis, C. D. (2001) Translating the histone code. *Science 293*, 1074−1080.

(3) Peterson, C. L., and Laniel, M. A. (2004) Histones and histone modifications. *Curr. Biol. 14*, R546−551.

(4) Shahbazian, M. D., and Grunstein, M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annu. Rev. Biochem. 76*, 75−100.

(5) Sterner, D. E., and Berger, S. L. (2000) Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev. 64*, 435−459.

(6) Martin, C., and Zhang, Y. (2007) Mechanisms of epigenetic inheritance. *Curr. Opin. Cell Biol. 19*, 266−272.

(7) Ruthenburg, A. J., Li, H., Patel, D. J., and Allis, C. D. (2007) Multivalent engagement of chromatin modifications by linked binding modules. *Nat. Rev. Mol. Cell Biol. 8*, 983−994.

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

(9) Wysocka, J., Swigut, T., Milne, T. A., Dou, Y., Zhang, X., Burlingame, A. L., Roeder, R. G., Brivanlou, A. H., and Allis, C. D. (2005) WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development. *Cell 121*, 859−872.

(10) Zeng, L., and Zhou, M. M. (2002) Bromodomain: an acetyl-lysine binding domain. *FEBS Lett. 513*, 124−128.

(11) Dai, L., Peng, C., Montellier, E., Lu, Z., Chen, Y., Ishii, H., Debernardi, A., Buchou, T., Rousseaux, S., Jin, F., Sabari, B. R., Deng, Z., Allis, C. D., Ren, B., Khochbin, S., and Zhao, Y. (2014) Lysine 2-hydroxyisobutyrylation is a widely distributed active histone mark. *Nat. Chem. Biol. 10*, 365−370.

(12) Groff, D., Chen, P. R., Peters, F. B., and Schultz, P. G. (2010) A Genetically Encoded epsilon-N-Methyl Lysine in Mammalian Cells. *Chembiochem 11*, 1066−1068.

(13) Bernardes, G. J. L., Chalker, J. M., Errey, J. C., and Davis, B. G. (2008) Facile conversion of cysteine and alkyl cysteines to

DOI: 10.1021/cb501055h
ACS Chem. Biol. 2015, 10, 1599−1603

dehydroalanine on protein surfaces: Versatile and switchable access to functionalized proteins. *J. Am. Chem. Soc. 130*, 5052.
(14) Guo, J., Wang, J., Lee, J. S., and Schultz, P. G. (2008) Site-specific incorporation of methyl- and acetyl-lysine analogues into recombinant proteins. *Angew. Chem., Int. Ed. Engl. 47*, 6399−6401.
(15) Nguyen, D. P., Garcia Alai, M. M., Virdee, S., and Chin, J. W. (2010) Genetically directing varepsilon-N, N-dimethyl-L-lysine in recombinant histones. *Chem. Biol. 17*, 1072−1076.
(16) Simon, M. D., Chu, F. X., Racki, L. R., de la Cruz, C. C., Burlingame, A. L., Panning, B., Narlikar, G. J., and Shokat, K. M. (2007) The site-specific installation of methyl-lysine analogs into recombinant histones. *Cell 128*, 1003−1012.
(17) Ai, H. W., Lee, J. W., and Schultz, P. G. (2010) A method to site-specifically introduce methyllysine into proteins in E. coli. *Chem. Commun. (Cambridge, U. K.) 46*, 5506−5508.
(18) Neumann, H., Peak-Chew, S. Y., and Chin, J. W. (2008) Genetically encoding N(epsilon)-acetyllysine in recombinant proteins. *Nat. Chem. Biol. 4*, 232−234.
(19) Nguyen, D. P., Garcia Alai, M. M., Kapadnis, P. B., Neumann, H., and Chin, J. W. (2009) Genetically encoding N(epsilon)-methyl-L-lysine in recombinant histones. *J. Am. Chem. Soc. 131*, 14194−14195.
(20) Kim, C. H., Kang, M., Kim, H. J., Chatterjee, A., and Schultz, P. G. (2012) Site-specific incorporation of epsilon-N-crotonyllysine into histones. *Angew. Chem., Int. Ed. Engl. 51*, 7246−7249.
(21) Gattner, M. J., Vrabel, M., and Carell, T. (2013) Synthesis of epsilon-N-propionyl-, epsilon-N-butyryl-, and epsilon-N-crotonyl-lysine containing histone H3 using the pyrrolysine system. *Chem. Commun. (Cambridge, U. K.) 49*, 379−381.
(22) Neumann, H., Hancock, S. M., Buning, R., Routh, A., Chapman, L., Somers, J., Owen-Hughes, T., van Noort, J., Rhodes, D., and Chin, J. W. (2009) A method for genetically installing site-specific acetylation in recombinant histones defines the effects of H3 K56 acetylation. *Mol. Cell 36*, 153−163.
(23) Kavran, J. M., Gundliapalli, S., O'Donoghue, P., Englert, M., Soell, D., and Steitz, T. A. (2007) Structure of pyrrolysyl-tRNA synthetase, an archaeal enzyme for genetic code innovation. *Proc. Natl. Acad. Sci. U. S. A. 104*, 11268−11273.
(24) Yanagisawa, T., Ishii, R., Fukunaga, R., Kobayashi, T., Sakamoto, K., and Yokoyama, S. (2008) Multistep engineering of pyrrolysyl-tRNA synthetase to genetically encode N(epsilon)-(o-azidobenzyloxycarbonyl) lysine for site-specific protein modification. *Chem. Biol. 15*, 1187−1197.
(25) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem. 79*, 413−444.
(26) Xiao, H., Peters, F. B., Yang, P. Y., Reed, S., Chittuluru, J. R., and Schultz, P. G. (2014) Genetic incorporation of histidine derivatives using an engineered pyrrolysyl-tRNA synthetase. *ACS Chem. Biol. 9*, 1092−1096.
(27) Matyjaszewski, K., and Xia, J. (2001) Atom transfer radical polymerization. *Chem. Rev. 101*, 2921−2990.
(28) Peeler, J. C., Woodman, B. F., Averick, S., Miyake-Stoner, S. J., Stokes, A. L., Hess, K. R., Matyjaszewski, K., and Mehl, R. A. (2010) Genetically Encoded Initiator for Polymer Growth from Proteins. *J. Am. Chem. Soc. 132*, 13575−13577.
(29) Chatterjee, A., Sun, S. B., Furman, J. L., Xiao, H., and Schultz, P. G. (2013) A versatile platform for single- and multiple-unnatural amino acid mutagenesis in Escherichia coli. *Biochemistry 52*, 1828−1837.
(30) Xiao, H., Chatterjee, A., Choi, S. H., Bajjuri, K. M., Sinha, S. C., and Schultz, P. G. (2013) Genetic incorporation of multiple unnatural amino acids into proteins in mammalian cells. *Angew. Chem., Int. Ed. Engl. 52*, 14080−14083.
(31) Chatterjee, A., Xiao, H., Bollong, M., Ai, H. W., and Schultz, P. G. (2013) Efficient viral delivery system for unnatural amino acid mutagenesis in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A. 110*, 11803−11808.

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ACS Chem. Biol. 2015, 10, 1599−1603