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Expression of Histone H3 Tails with Combinatorial Lysine Modifications under the Reprogrammed Genetic Code for the Investigation on Epigenetic Markers

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SUMMARY

We report the ribosomal synthesis of N-terminal peptides of histone H3, so-called H3 tail (H3t), with combinatorial methyl and acetyl modifications of selected lysine residues, and the application of such peptides to studying the influence of lysine modification on H3t binding to chromodomain of heterochromatin protein 1 (chromoHP1). Genetic code reprogramming was employed to reassign four codons to acetylated, mono-, di-, and trimethylated lysines, and 38-mer H3t peptides containing modified lysines at designated sites were expressed from the corresponding mRNA sequences. Using a series of H3t constructs, we show complex crosstalk among methylated lysine 9 and 27, and acetylated lysine 14 for binding to chromoHP1. This proof-of-concept study offers a unique means for the synthesis of not only an H3t library containing modified lysines but also other classes of peptides bearing posttranslational methylation and acetylation.

INTRODUCTION

A nucleosome consists of 146 base pairs of DNA wrapped around an octamer of core histone proteins, forming the basic repeating unit of chromatin. Each of the four core histones, H2A, H2B, H3, and H4, contains a structural domain consisting of three α helices and an unstructured domain at the N terminus or C terminus, referred to as the histone tail. Enzyme-catalyzed chemical modification of certain residues in the histone tails serves to regulate the local structure of chromatin, altering the accessibility of various proteins to histone tails as well as to DNA. Thus, histone tail modification represents one of the important epigenetic marks linked to various cellular processes, such as replication, transcription, and DNA repair (Kouzarides, 2007).

The histone tails of core histones, especially that of H3 (H3t), consists of about 40 amino acid residues and are subjected to various chemical modifications such as acetylation, methylation, phosphorylation, and possibly ubiquitylation (Kouzarides, 2007; Peterson and Laniel, 2004). Lysines (Ks) in H3t can be acetylated (ac; acetylated lysine, acK) or methylated (me), and the methylation can be one of mono-, di-, or trimethylation (me1, me2, and me3, respectively; Figures 1A and 1B). In general, hyper- and hypoacetylation of H3t are linked with transcriptional activation and repression, respectively. Methylation can influence transcription in various ways, depending on the site of the modification (Kouzarides, 2007; Martin and Zhang, 2005). Linking a particular modification or set of modifications to a particular cellular function involving effector proteins postulated a "histone code" hypothesis (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Thus, modifications at H3t can be viewed as special marks modulating the interaction among histones or between the histone and nonhistone proteins. Certain modifications at H3t can also affect the consequences of others, and evidence of such crosstalk between modifications has been accumulating in the past few years (Fischle et al., 2005; Kouzarides, 2007; Latham and Dent, 2007; Seet et al., 2006). Moreover, recent findings of the combinatorial linkages among modifications in H3t also suggested possible long-range crosstalk among modifications of H3t (Garcia et al., 2007; Taverna et al., 2007). However, our current understandings of their interplay at the molecular level are limited, partly because there is no reliable and readily accessible method to prepare the full-length H3t with multiple modifications in a defined and controlled manner.

Genetic code reprogramming is a technique where codons usually assigned to proteinogenic amino acids are reassigned to nonproteinogenic amino acids, including those with unique side chains (Forster et al., 2003; Josephson et al., 2005; Murakami et al., 2006). This enables the expression of peptides containing multiple nonproteinogenic amino acids using a translation system. We envisioned that this technique is suitable for the synthesis of H3t with sparsely positioned multiple modifications. We here report the mRNA-directed synthesis of 38-mer H3t containing Ks modified with N^{ε}-mono-, di-, trimethyl and/or acetyl group(s) and its application to studies on the specific interaction between chromoHP1 and H3t modified at positions 9, 14, and 27. This proof-of-concept study offers a unique means for the synthesis of an H3t library containing modified Ks to investigate the role of combinatorial modifications in histone tail structure and function.

RESULTS

Translatability of Methylated or Acetylated Lysine upon Genetic Code Reprogramming

The 38-mer H3t is composed of 10 members of the proteinogenic amino acids (alanine, arginine, glutamine, glycine, leucine,

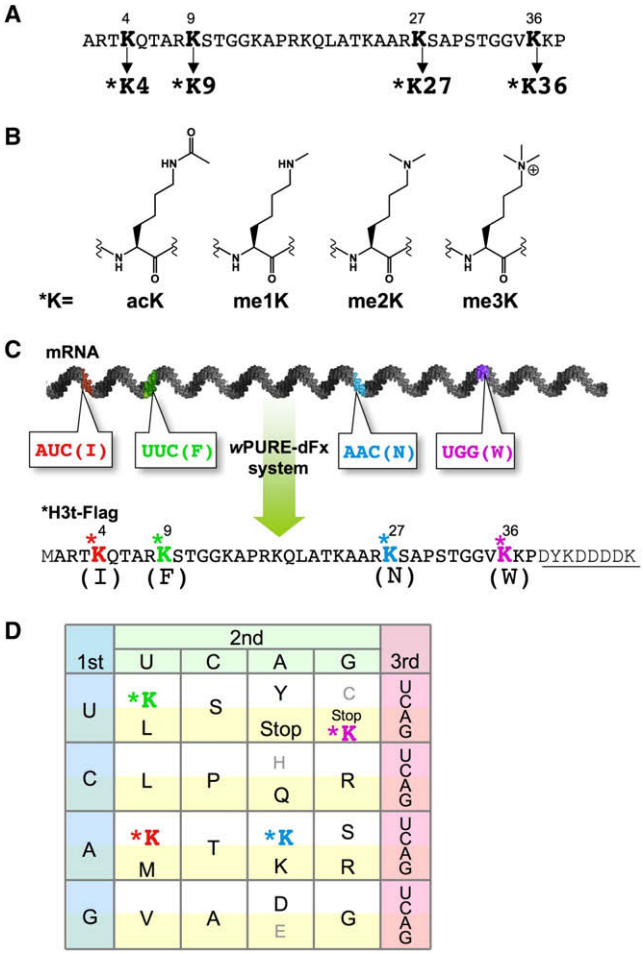

Figure 1. Genetic Code Reprogramming for the Preparation of *H3t

(A) The primary sequence of H3t used in this study. *Ks were incorporated into positions indicated.

(B) *Ks used in this study. Note that both me1K and me2K are protonated in physiological conditions, and thus all the meKs are positively charged.

(C) An example of codon assignments for the genetic code reprogramming. Codons for K4, K9, K27, and K36 were changed to those for I, F, N, and W, respectively. Color codes used here represent the codons in use throughout the paper.

(D) A representative reprogrammed genetic code table. Four reprogrammed codons are in colors, whereas three unused codon boxes, shown in gray, were left vacant.

lysine, proline, serine, threonine, and valine), including eight Ks. Among these Ks, four Ks at positions 4, 9, 27, and 36 (K4, K9, K27, and K36, respectively) were often found methylated in vivo; therefore, we were interested in synthesizing H3t peptides containing Ks specifically modified at these positions (abbreviated to *Ks; Figure 1A). In addition to the these 10 amino acids, methionine, aspartate, and tyrosine (M, D, Y, respectively) were included in the peptide chain to assign the initiation (M) and Flag peptide tag (DYKDDDDK; D could be [^{14}C]-D) at the C terminus for purification, immobilization, and radioisotope labeling of the expressed H3t peptides. Consequently, seven amino acids were yet unused, and four of these codons were reassigned to *Ks modified with me1, me2, me3, or ac on the ε-amino group (Figure 1B). We chose codons of AUC (isoleucine, I), UUC (phenylalanine, F), AAC (asparagine, N), and UGG (tryptophan, W) and reassigned these codons to code for *Ks (Figures 1C and 1D). Thus, these amino acids as well as other unused amino acids were withdrawn from the PURE (protein synthesis using recombinant elements) system to generate a *w*PURE system that contained all the aminoacyl-tRNA synthetases but only the essential 13 amino acids, for genetic code reprogramming. Derivatization of *Ks to 3,5-dinitrobenzyl esters (DBE) made these amino acids compatible with a dinitro-flexizyme, a ribozyme-based tRNA acylating catalyst (dFx; see Murakami et al., 2006). dFx was able to aminoacylate tRNA$^{Asn\text{-}E2}_{GAU}$ with the respective modified *K-DBE (see Figure S1 available online). Because dFx is inherently able to recognize tRNAs bearing any anticodon, this finding ensured that any pair of *K and tRNA$^{Asn\text{-}E2}_{NNN}$ (NNN denotes an anticodon complementary to the reprogrammed codon) could be prepared by the dFx technology.

Though it was known that acK could be incorporated into a nascent peptide chain efficiently by translation under the reprogrammed genetic code (Murakami et al., 2006), no information was available for the incorporation efficiency of meKs. To verify the translatability of the individual *Ks at each position, we designed a template containing codons for I F, N, and W at positions 4, 9, 27, and 36, respectively (Figure 1C). Prior to the suppression experiments, two control experiments were performed: as a positive control, the corresponding DNA template was transcribed and translated in the *w*PURE system that was supplemented with I, F, N, and W. An intense band of a [^{14}C]-labeled peptide was observed on SDS-tricine-PAGE (Figure 2A, lane 1), and MALDI-TOF analysis of the Flag-purified peptide revealed a molecular weight (MW) consistent with that of the expected peptide containing I, F, N, and W at the designated positions instead of Ks (Figure 2B, P-WT). As a negative control to verify the background expression at position 4 (I) without *K4 suppression, the same template was translated in a *w*PURE system supplemented with F, N, and W only. A faint band with nearly the same mobility as the P-WT was observed (Figure 2A, lane 2), and the MALDI-TOF analysis of the Flag-purified peptide suggested the misincorporation of other amino acids (Figure S2). However, this background expression could be readily competed out by suppression using *K-tRNA$^{Asn\text{-}E2}_{GAU}$; upon the addition of the respective *K-tRNA$^{Asn\text{-}E2}_{GAU}$ to the *w*PURE system supplemented with F, N, and W, an intense band appeared irrespective of *K used (lanes 3–6), and the MALDI-TOF analysis of the respective Flag-purified peptide confirmed a peak of the main product with the expected MW containing the designated *K (Figure 2B, P-K4me1, P-K4me2, P-K4me3, and P-K4ac). Likewise, the background peptide expressions at the other three positions, K9, K27, and K36 (lanes 8, 14, and 20), were also suppressed by the addition of the respective *K-tRNA$^{Asn\text{-}E2}_{NNN}$ (NNN denotes the corresponding anticodon) to afford an intense band in the tricine-SDS PAGE (Figure 2A, lanes 9–12, 15–18, and 21–24), and the MW of the respective peptides were also confirmed (Figure 2B, P-KXme1, P-KXme2, P-KXme3, and P-KXac; X = 9, 27, or 36). These findings showed all *Ks were compatible with the ribosomal peptide synthesis upon genetic code reprogramming, yielding the designated peptide as a main product.

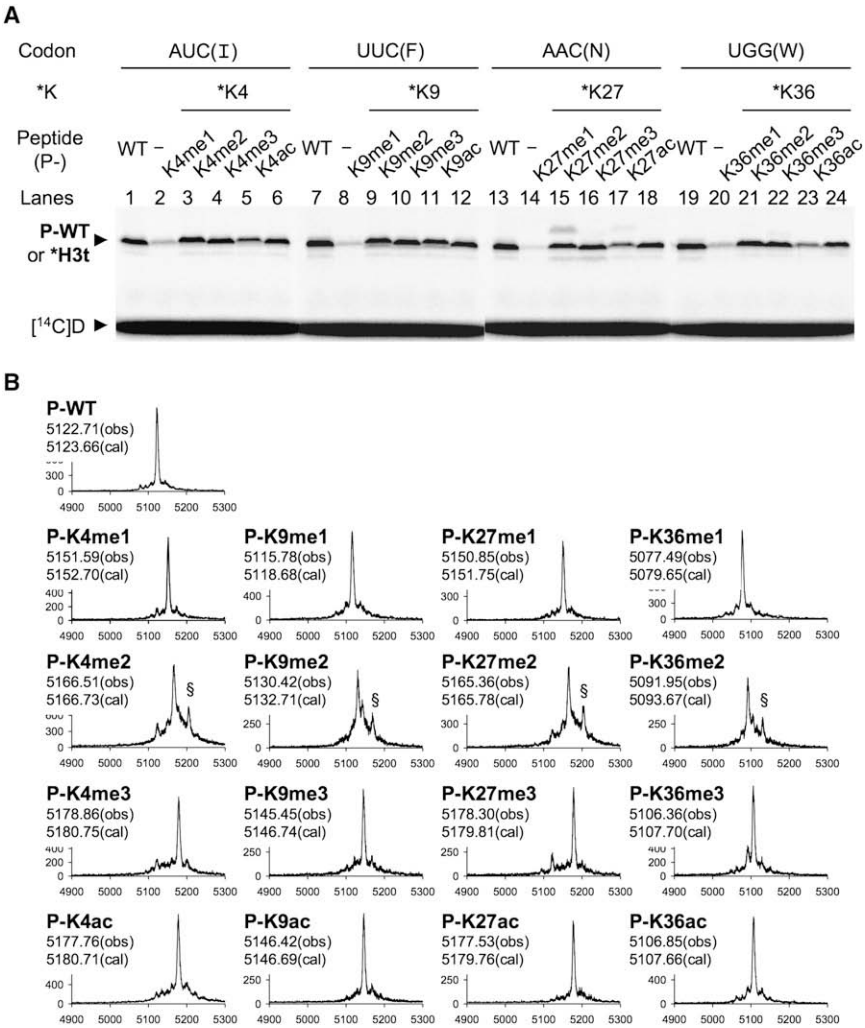

Figure 2. Single Incorporations of *Ks into H3t

(A) Analysis of expressed peptides containing a single *K substitution. Incorporation efficiencies were examined on the 15% SDS-tricine-PAGE using the wild-type peptide that has I4, F9, N27, and W36 as a control. Because the *w*PURE system used in this study did not contain any of these amino acids, a proper combination of amino acids were exogenously added to the translation; for example, F, N, and W were added to the translation for I codon suppressions. Unincorporated [^{14}C]-D is shown at the bottom of the gel.

(B) MALDI-TOF analysis of each *H3t peptide after anti-Flag antibody affinity purification. § indicates a peak of the potassium adduct. Note that an intense peak corresponding to the potassium adduct was present in the series of me2K-containing *H3t.

Interaction of HP1 with the Translated H3t Peptides Containing a Methylated Lysine at the Ninth Position

The next critical question was whether the translated H3t peptides containing the modified Ks (*H3t peptides) were able to exhibit the expected modification-specific interactions with effector proteins. To confirm this, we chose heterochromatin protein 1 (HP1), of which the chromodomain is known to interact with K9-modified *H3t peptides in a methylation-dependent manner (Jacobs et al., 2001; Lachner et al., 2001). Two well-established analytical methods for detecting protein-protein interactions, pull-down analysis and surface plasmon resonance (SPR), were employed to show the meK9-specific recruitment of the chromodomain of HP1 (chromoHP1) by the *H3t peptide.

A template sequence that contained the UUC(F) codon at position 9 was translated to Flag-tagged H3t, *H3tK9ac, *H3tK9me1, *H3tK9me2, and *H3tK9me3 in the *w*PURE system, in the presence of the corresponding *K-tRNA$^{Asn\text{-}E2}_{GAA}$ (Figure 3A). As a negative control, the same template was translated in the absence of aminoacyl-tRNA$^{Asn\text{-}E2}_{GAA}$ in the same *w*PURE system. *H3t peptides were displayed on an agarose resin through covalently immobilized anti-Flag antibody, and the resulting resin was used directly as bait to pull down chromoHP1. The immobilized molecules were acid eluted from the resin and analyzed by immunoblot using anti-Flag and anti-polyhistidine polyclonal antibodies (Figure 3B). The *H3t peptides were detected only with the addition of charged tRNA$^{Asn\text{-}E2}_{GAA}$ (Figure 3B, lanes 2–6 versus lane 1), confirming that the *H3t peptide expression was solely the consequence of genetic code reprogramming. Significantly, chromoHP1 was detected only when *H3t containing meK9 was used as bait (Figure 3B, lanes 2 and 3 versus lanes 4–6; see also Figure S3 for product verification by mass analysis). We clearly show that *H3tK9me3 was able to pull down chromoHP1 the most effectively among the *H3t peptides. Our findings are in good agreement with an earlier report in which the affinity of 15-mer synthetic H3t peptides (Ala1–Ala15) containing K9me1, k9me2, and k9me3 were in the order of K9me1 < K9me2 < K9me3 (Fischle et al., 2003).

To quantitatively assess the affinity of *H3t peptides, we next performed SPR where the expressed *H3t peptides were immobilized on a sensor chip that was covalently coupled with an anti-Flag antibody. When 2 μM of chromoHP1 was flowed over the respective *H3t peptides on the chip, we reproducibly observed that the *H3tK9me3 exhibited a resonance unit at equilibrium (ΔReq) value 5–20 times higher than other *H3t peptides, indicating its strong affinity to chromoHP1 (Figure 3C). We then varied the concentrations of chromoHP1 using *H3tK9me3 as a binding partner to obtain the dissociation constant of 0.76 ± 0.05 μM (Figure 3C, inset). This value was slightly lower but similar to the reported values 2.4–4.0 μM using the aforementioned short H3t peptide with the K9me3 modification. Collectively, these findings show that the *H3t peptides prepared by genetic code reprogramming can be used to detect interactions with a known effector protein qualitatively and quantitatively.

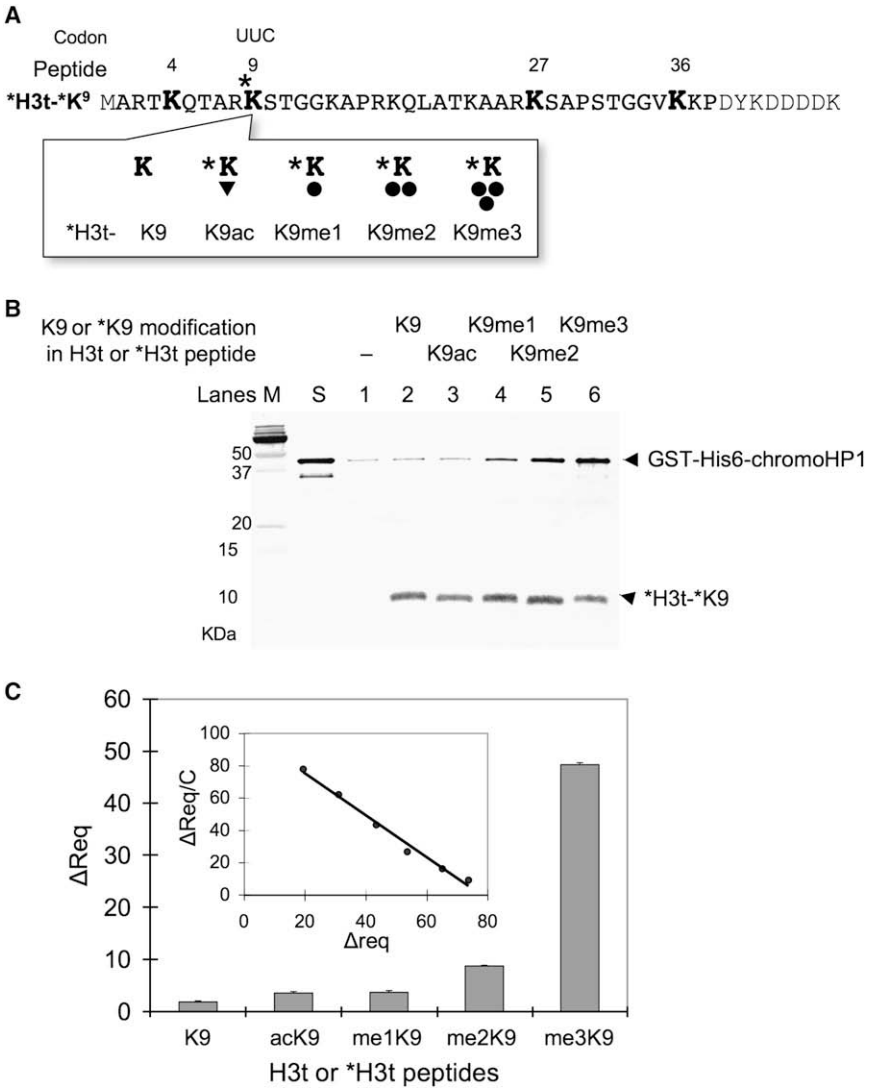

Figure 3. Modification-Specific Interaction of *H3t with the Chromodomain of HP1

(A) *H3t peptides used for the pull-down or the sensor chip for SPR analysis. Each peptide contains *K at position 9.

(B) Pull-down analysis of *H3t interaction with chromoHP1. Pulled-down prey (chromoHP1) and the baits (*H3t peptides) were probed using anti-polyhistidine and anti-Flag antibodies, respectively. M, protein size marker; S, chromoHP1 (2% of input); 1, pull-down with the bait prepared from the translation in the absence of both F and aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$; 2–6, pull-down with *H3t-K9, -K9ac, -K9me1, -K9me2, and -K9me3, respectively.

(C) SPR analysis of *H3t interaction with chromoHP1. Respective peptides were immobilized on the sensor chip through preimmobilized anti-Flag tag antibody. Resonance units in the equilibrium with 2 μM of chromoHP1 were measured and normalized against the amount of *H3t bound to the chip. Mean values of triplicate measurements of two independent sets of experiments and the SD are shown. A scatchard plot of *H3tK9me3-chormoHP1 with varying concentrations of chromoHP1 is shown in the inset.

Synthesis of H3t Peptides by Multiple Incorporations of Modified Lysines

We next directed our investigation to multiple incorporations of *Ks into H3t. Using the template shown in Figure 1C, codons at positions 4, 9, 27, and 36, coding for I, F, N, and W, respectively, were reprogrammed to assign me1K, me3K, acK, and me2K, respectively. We then suppressed the reprogrammed codons using the designated *K-tRNA$^{Asn\text{-}E2}_{NNN}$ in the *w*PURE system with proper supplementation of the cognate amino acids. The template sequence was expressed in the *w*PURE system supplemented with all amino acids to yield P-WT as a positive control, and the same template was translated in the *w*PURE system to show no detectable background expression (Figure 4A, lanes 1 and 2). When the suppressions were performed using the corresponding *K-tRNA$^{Asn\text{-}E2}_{NNN}$, a clear band appeared in each experiment (Figure 4A, lanes 3–6). Increasing the number of suppressions resulted in a concomitant decrease in the overall expression level, yet even the quadruple suppression was achieved at an expression level of 37% to that of P-WT (Figure 4A, lane 6, P-Q versus lane 1, P-WT). Most importantly, MALDI-TOF analysis of each Flag-purified peptide showed that the observed major peak was consistent with the expected MW for the multiple suppressions (Figure 4A, P-D, P-T, and P-Q).

To perform the ribosomal synthesis of *H3t peptides, two approaches are applicable. One is to prepare different *H3t peptides from the same template using reprogrammed genetic tables with customized *K assignments for each *H3t peptide (Figure 4B). This is possible because the dFx technology enables us to readily prepare any desired *K-tRNA$^{Asn\text{-}E2}_{NNN}$ to create a new reprogrammed genetic table. The other approach is to use a unique genetic table (e.g., as shown in Figure 1D), assigning each respective *K and then expressing the desired *H3t peptides from different mRNA sequences. In this method, swapping the positions of reassigned codons to *K in mRNA dictates the positions of *K incorporation in each *H3t peptide (Figure 4C). To show the versatility of our methodology, we employed both approaches to synthesize *H3t peptides.

In the first approach, each reprogrammed codon was assigned to K, meK, me2K, me3K, or acK by charging the respective *K onto tRNA$^{Asn\text{-}E2}_{NNN}$ (Figure 4B). In this series of experiments, the incorporation of K at preselected positions was also performed by the suppression using K-tRNA$^{Asn\text{-}E2}_{NNN}$ like other *K incorporations (as indicated by the color-coded Ks in Figure 4B). Upon expression of each peptide by the respective reprogrammed genetic table, we confirmed the product by MALDI-TOF analysis (see *H3t-II, *H3t-III, and *H3t-IV). The observed MW of the major peak was consistent with the expected values for the respective peptides in all cases, indicating that the desired *H3t peptides were synthesized.

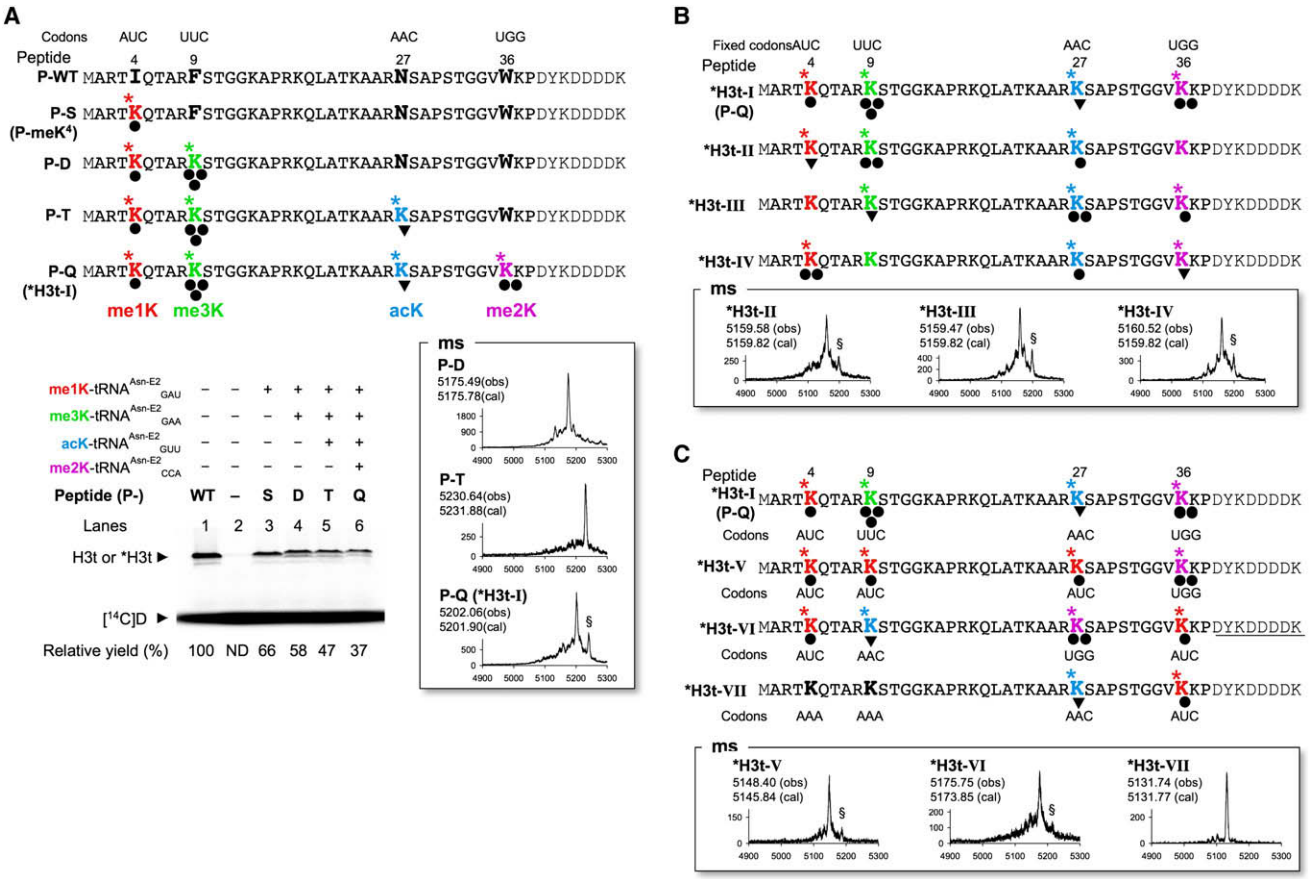

Figure 4. Multiple Incorporations of *Ks into H3t

(A) Demonstration of multiple incorporations of *Ks into H3t. Single to quadruple modifications can be coded in mRNA using the reassignment scheme shown in Figure 1C. Multiple incorporations were assessed quantitatively (left) and qualitatively (right) using 15% tricine-SDS-PAGE and MALDI-TOF, respectively.

(B) Multiple incorporations of *Ks into H3t peptides using codon reassignments using the single mRNA sequence.

(C) Multiple incorporations of *Ks into H3t peptides using codon arrangements in mRNA. § indicates a peak of the potassium adduct of me2K-containing *H3t peptides. The triangle and circle denote acetylation and methylation, respectively.

In the second approach, we prepared three mRNA templates encoding *H3t peptides (Figure 4C). Because the reprogrammed genetic codon table was fixed in this series of experiments (Figure 1D), each mRNA sequence dictated the identity and the position of *Ks incorporated, giving three different *H3t peptides with different MW. Unmodified K was assigned by the cognate AAA codon in mRNA (as indicated by black K in Figure 4C). The respective translated peptides were analyzed by MALDI-TOF, showing that the major peak of each peptide was consistent with the expected MW. Thus, this approach is also reliable for the synthesis of *H3t peptides with various combinations of K and *Ks at any designated positions.

Interplay among Lysine Modifications at Positions 9, 14, and 27 of H3t Peptides in Its binding to chromoHP1

HP1-histone H3 binding is mostly attributed to H3K9me, and, in many cases, the modification can be connected to transcriptional silencing or heterochromatin formation. However, it has also been shown that H3tK27me3 can recruit HP1 to H3t in vitro with a reduced affinity compared with that of an H3tK9me3 peptide (Fischle et al., 2003), even though the neighboring amino acids of K9 and K27 are similar (Figure 5A). However, acetylation at K14 has been generally conceived to be a transcription-facilitating epigenetic mark as opposed to K9me. Mass analyses of in vivo modifications of histone H3 revealed that these two epigenetically opposite modifications coexist in the same molecule of histone H3 (Bonenfant et al., 2007; Garcia et al., 2007; Thomas et al., 2006). Furthermore, a significant portion of histone H3 was shown to contain K9meK27me double modification or K9meK14acK27me triple modification (Garcia et al., 2007). In terms of the role of these modifications on the interaction between *H3t and HP1, it has been reported using synthetic *H3t peptides lacking the K27 modification that *H3tK9me3K14ac did not show an appreciable difference from *H3tK9me3 in qualitative pull-down experiments (Hirota et al., 2005; Mateescu et al., 2004). However, there might be interplay between the modifications of K9 and K14 along with K27 in the HP1-H3t interaction, which had not been investigated. To address the above question, we performed SPR experiments where *H3t peptides containing combinatorial modifications at the above K residues were tested for binding to chromoHP1.

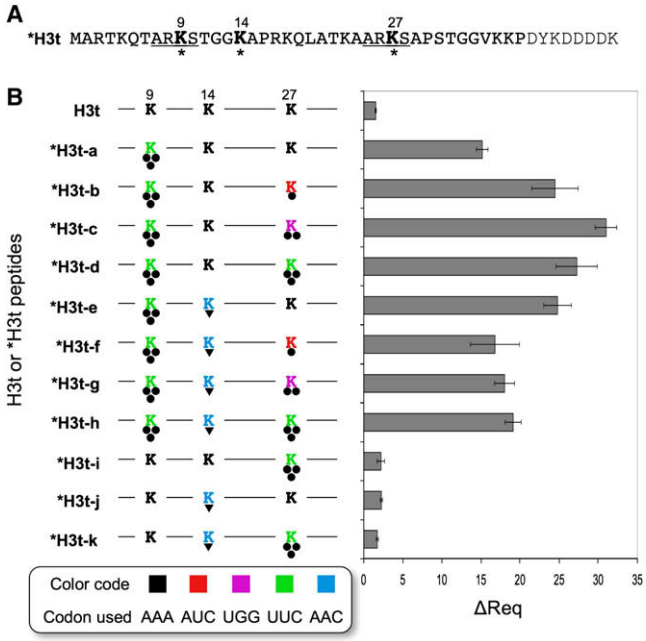

Figure 5. Interplay among Lysine Modifications at Positions 9, 14, and 27 in chromoHP1-*H3t Binding

(A) The sequence of *H3t peptides used. Positions 9, 14, and 27 contained modified Ks in various combinations. The consensus sequences in the *K9 and *K27 regions are underlined.

(B) SPR analysis of chromoHP1 interaction with *H3t peptides modified at positions 9, 14, and 27 in combinatorial manners. Modifications are summarized in the left panel. *Ks were incorporated into the peptide sequence using the codon assignments shown in color codes in the bottom left panel. The right panel shows resonance units observed in the equilibrium with 0.25 μM of chromoHP1 normalized against the amount of *H3t bound to the chip. Mean values and SD are shown in each bar, which were generated from triplicate measurements of two independent sets of experiment (*H3t-a∼h) or a single set of experiment (H3t and *H3t-i∼k). The triangle and circle denote acetylation and methylation, respectively.

We prepared a series of *H3t peptides with a total of 10 combinations of the methyl and acetyl modifications at positions 9, 14, and 27 (Figure 5A, *H3t-b–k; see also Figure S4 for the product verification by mass analyses) using the reprogrammed codons shown in Figure 1C (the summary of the reprogrammed codons used was also shown in the bottom panel of Figure 5B). The concentration of chromoHP1 in the mobile phase was kept at 0.25 μM to prevent saturation binding, where the amount of bound chromoHP1 (ΔReq) was expected to be proportional to the affinity of respective peptides. This let us assess the effect of modifications on chromoHP1-*H3t binding semiquantitatively. As shown earlier in Figure 3, K9me3 modification promoted the chromoHP1-*H3t binding (H3t versus *H3t-a, Figure 5B). K9me3K27me double modification resulted in elevated binding over K9me3 modification (*H3t-b∼d versus *H3t-a), though K27me3 alone (*H3t-i) did not show an appreciable positive effect on chromoHP1 binding compared with H3t. However, the K14ac modification on *H3tK9me3 (*H3t-a→*H3t-e) increased the binding to a similar level observed in *H3tK9me3K27me1 (*H3t-e versus *H3t-b), whereas the same modification on *H3tK9me3K27me1 (*H3t-b→*H3t-f) suppressed chromoHP1 binding down to the level of *H3tK9me3 (*H3t-f versus *H3t-a). Once K14ac modification was placed in *H3tK9me3-K14acK27me, further methylation(s) on K27 did not affect the affinity (*H3t-f→*H3t-g or *H3t-h). Finally, neither K14ac modification alone on H3t (*H3t-j) nor K27me3 modification on *H3tK27me3 (*H3t-k) showed a significant elevation in chromoHP1 affinity from that of H3t.

DISCUSSION

In this report, we show the preparation of *H3t with multiple modifications using genetic code reprogramming and the functional use of these peptides in elucidating histone modification-effector binding relationships. Because of the biological significance of histone modifications and our insufficient knowledge of their roles in epigenetic control, it has been of great interest to develop methods to prepare histones or histone tails containing *Ks. One such method is to use enzymes that are known to modify histones (Kouzarides, 2007). This method allows for the preparation of not only modified histone tails but also the full-length histones, which represents the most significant advantage of this method. However, many of such enzymes would modify multiple sites in the target histones with incomplete modifications, leaving certain heterogeneities in the modified histones (Winter et al., 2008). This may impose difficulties in investigating specific interactions of histones or histone tails with effector proteins in certain cases. An elegant alternative approach was recently reported where a cysteine residue was introduced to H3 and then chemically modified with 2-halo-ethylamine derivatives to install a *K analog (Simon et al., 2007). The virtue of this method is its simplicity for the synthesis of the full-length *H3 analogs, whereas an inherent limitation is that only a single site modification or multiple but homogeneous *K modifications (albeit this has yet to be demonstrated) is possible; therefore it is not applicable to addressing questions regarding the importance of combinatorial modifications at the arbitrary K residues.

Chemically synthesized 20- to 30-amino-acid-long peptides containing *K at a certain specific sites has been widely and successfully used to elucidate the molecular interactions taking place on histones (Fischle et al., 2003; Jacobs et al., 2001; Lachner et al., 2001; Matthews et al., 2007; Meehan et al., 2003). Such short peptides can also be ligated to the C-terminal fragment of H3 or H4 to construct the full-length histones (Fischle et al., 2003; Shogren-Knaak et al., 2003, 2006). However, to elucidate the role of sparsely located modifications, the length of greater than 40 amino acids that span the tail would be preferable. This task is not easy to achieve using chemical synthesis, particularly if multiple *Ks with various kinds were necessary to be placed in the sequence.

Comparatively, ribosomal synthesis can be a practical and reliable method for the preparation of relatively long peptides with versatility. However, for the preparation of *H3t with multiple modifications, two prerequisites must be met: (i) the ribosome should use *Ks and (ii) the positions of these amino acids should be coded for in the mRNA precisely. Because all *Ks, including the bulkiest me3K, were incorporated into the peptide efficiently (Figure 2), the first prerequisite was easily met. The second prerequisite was solved by the use of genetic code reprogramming combined with the *w*PURE and flexizyme systems. This is, to the best of our knowledge, the first report concerning the ribosomal incorporation of *Ks with various degrees of methyl

modifications into a peptide chain. Combining these two findings, we could prepare a variety of *H3t peptides with four kinds of modifications at four different positions 4, 9, 27, and 36 in the desired combinations (Figure 4). The 47-mer Flag-tagged *H3t peptides with sparsely located such modifications were easily prepared using our approach.

Taking advantage of the fact that any kind of peptide tag can be appended to *H3t by expressing mRNA that encodes such a tag at the N or C terminus, the crude *H3t-Flag peptides from the translation can be directly immobilized on an anti-Flag antibody-coated resin or sensor chip; conveniently, the immobilized peptides can be used to confirm K9 modification-specific binding of *H3t to chromoHP1 (Figure 3). The quantitative analysis of the interaction between *H3tK9me3 and chromoHP1 using SPR showed tight binding with a dissociation constant of 0.76 μM. This value is slightly lower than a previously reported value (2.5–4.0 μM) using a short synthetic *H3tK9me3 peptide consisting of A1–A15 (Fischle et al., 2003; Jacobs and Khorasanizadeh, 2002). Although the exact reason for a decrease in the dissociation constant of our *H3tK9me3 is unclear, it is possible that the full-length *H3tK9me3 might act differently from the shorter peptide for the binding to chromoHP1. Nonetheless, the resin-bound *H3t could be used to pull down chromoHP1 (Figure 3), demonstrating the feasibility of our method for an application that allows us to pull down various and possibly novel effector proteins from cell lysates.

Finally, we have shown the use of our methodology in unveiling the possible interplay among the modifications at K9, K14, and K27. Resonance unit at equilibrium (ΔReq) of *H3t-chromoHP1 binding increased by an approximately 2-fold when the second modification of K27me was added to *H3tK9me3 (Figure 5, *H3t-a→*H3t-c). K27me3 modification alone on H3t did not positively influence binding under the conditions used here (Figure 5, *H3t-i). Despite that the neighboring residues of K9me3 and K27me3 are similar (see underlines in Figure 5A), the X-ray structure of the complex of *H3tK9me3 with chromoHP1 has revealed that T6 in the K9me3 region plays a critical role in fortifying their interaction, resulting in lower affinity of chromoHP1 to K27me3 (Fischle et al., 2003). However, a 2-fold enhancement in binding observed for *H3tK9me3K27me2 (Figure 5, *H3t-c) suggests that methylation(s) of K27 may contribute to the binding once chromoHP1 is recruited to the site of K9me3. Moreover, K14ac modification in *H3tK9me3K27me3 cancels the observed enhancement in affinity (Figure 5, *H3t-d→*H3t-h), exhibiting the binding ability similar to *H3tK9me3 (Figure 5, *H3t-a). These observations allow us to propose a model where methylation(s) on K27 retards the dissociation of chromoHP1 from the peptide by "shuttling" chromoHP1 between the K9me3 region and the K27me3 region of H3; when K14ac modification is added, the shuttling is disrupted, presumably by a conformational change of the peptide, neutralizing the enhancement by K27me3. However, this shuttling model fails to explain another observation made for the K14ac modification on *H3tK9me3 that enhances binding to chromoHP1 (Figure 5, *H3t-a→*H3t-e). Because *H3tK14ac does not bind to chromoHP1 (Figure 5, *H3t-j), this effect is clearly cooperative with the K9me3 modification. We therefore propose an alternative model that K14ac or K27me3 modification alone on *H3tK9me3 "locks" the peptide conformation with a favorable state for binding to chromoHP1. However, the simultaneous modifications in both sites unlock the favorable conformation, thereby canceling the enhancement. More biochemical and structural studies are required to reveal the molecular mechanism of the above two models in the future.

Regardless of the exact mechanism, in human cells contradictory epigenetic marks of K9me3 and K14ac do coexist in the same molecule of histone H3, and this double modification occasionally accompanies the third modification of K27me (Garcia et al., 2007). Also, K14ac was shown to be necessary together with phosphorylation (ph) at S10 to eject HP1 from histone H3K9me, during the G2/M phase of the human cell cycle (Mateescu et al., 2004); in other cases, S10ph alone was enough to break that binding (Fischle et al., 2005; Hirota et al., 2005). Thus, it can be speculated at this point that the effect of K14ac on HP1 binding to histone H3 would be K27me dependent and specific for the cell cycle and/or the position on the chromatin. Even though the crosstalk among K9me, S10ph, and K14ac modifications in histone H3 have been investigated using short peptides, our work suggests that the crosstalk of these modifications in the context with the K27 modification would be worthy to reinvestigate in a more extensive manner.

SIGNIFICANCE

Posttranslational methylation of lysines in histones poses significant impacts on several cellular functions, thus constituting one of important epigenetic marks. The effect of lysine methylation is dependent on the position and the degree (mono-, di-, and trimethylation) of modification, and can be augmented or nullified by (an)other modifications in the same and/or neighboring histone (Kouzarides, 2007). Because over 150 different combinations of lysine methylation and acetylation are known in histone H3 (Garcia et al., 2007), and such modifications are mostly found in the unstructured N terminus of histone H3 (H3t), it is required to prepare peptides with posttranslational modifications of which positions span the entire region of H3t. We here showed that the ribosome could use methylated lysine (KXme, X denotes the number of position) efficiently (regardless of its methylation state) as well as acetylated lysine (KXac). Upon genetic code reprogramming, we could prepare H3t peptides with lysine methylation and acetylation at designated sites in a combinatorial manner. Using this method, we also unveiled possible crosstalk among K9me, K14ac, and K27me of H3t upon binding to chromodomain of heterochromatin protein 1; K9me3 had a positive effect on binding, K27me augmented the positive effect, and the third modification, K14ac, nullified the augmenting effect of K27me only. Similarly, K14ac augmented K9me3-induced binding, and K27me nullified the effect of K14ac on binding. Being rapid and simple, this method would help to decipher the effect of combinatorial modifications in histones, and it can be extended to the investigations of other classes of peptides, such as p53, which contains posttranslational modifications, including lysine methylation (Chuikov et al., 2004).

EXPERIMENTAL PROCEDURES

Preparation of the Template DNA

A DNA sequence of the human H3.1 was referred to for the PCR preparation of the H3t peptide template with the addition of a C-terminal Flag tag. Briefly, K9F

(5′-CAGAC TGCCC GCTTC TCGAC CGGTG GTAAA GCA-3′) was annealed to Linker (5′-GCGAG CGGCT TTTGT AGCCA GTTGC TTCCT GGGTG CTTTA CC ACC GG-3′) and extended by Taq DNA polymerase. The extension mixture was diluted 20 times into the PCR mixture and amplified using K4I (5′-AAGAA GGAGA TATAC ATATG GCTCG TACAA TCCAG ACTGC CCGC-3′) and K27N (5′-CACCC CTCCA GTAGA GGGCG CACTG TTGCG AGCGG CTTTT GT-3′) as the 5′ and 3′ primer respectively. Similarly, the product was further extended twice using 5′T7 (5′-GTAAT ACGAC TCACT ATAGG GTTTA ACTTT AAGAA GG AGA TATAC AT-3′) and K36W (5′-CTTGT CGTCA TCGTC TTTGT AGTCA GGT TT CCACA CCCCT CCAGT AGA-3′) as the first primer pair, and 5′T7 and 3′FLAG (5′-CGAAG CTTAC TTGTC GTCAT CGTCT TTGTA-3′) as the second primer pair.

Syntheses of *K-DBEs

N^{α}-Boc-N^{ε}-methyl-L-lysine was synthesized as an N^{ε}-Boc derivative from N^{α}-Boc-L-lysine by (i) benzylation using benzaldehyde and $NaBH_4$, (ii) methylation using formaldehyde and $NaBH_3CN$, (iii) debenzylation with hydrogenation using Pd/C, and (iv) protection of the ε-methylamine with Boc group using Boc anhydride according to the reported procedure (Andruszkiewicz, 1988). Similarly, N^{α}-Boc-N^{ε},N^{ε}-dimethyl-L-lysine was synthesized from N^{α}-Boc-L-lysine using formaldehyde and $NaBH_4$ for the reductive amination. N^{α}-Boc-N^{ε},N^{ε},N^{ε}-trimethyl-L-lysine was synthesized using methyl iodide as a methylating reagent to quaternize the ε-amine of N^{α}-Boc-L-lysine according to the reported procedure (Chen and Benoiton, 1976).

Carboxylic acids of N^{α}-Boc-L-lysine derivatives were activated to 3,5-dinitrobenzyl esters, and finally, N^{α}-protecting groups were removed as described previously (Murakami et al., 2006).

Aminoacylation of $tRNA^{Asn\text{-}E2}$ with Amino Acids

$tRNA^{Asn\text{-}E2}$ and flexizyme dFx were prepared by the runoff transcription of appropriate templates as described previously (Murakami et al., 2006). Acceptor stem sequences of tRNA were changed from authentic *E. coli* $tRNA^{Asn}$ to enhance transcription (T1G) and the orthogonality toward aminoacyl-tRNA synthetases (C2G). Aminoacylation was accomplished by incubating heat-denatured/renatured tRNA with equimolar of flexizyme dFx in the presence of 0.1 M HEPES-KOH (pH 7.5), 0.6 M $MgCl_2$, and 5 mM *K-DBE for 3 hr on ice. Reactions were quenched by acidifying the reaction with NaOAc (pH 5.2), and aminoacylated tRNAs were recovered by repeated ethanol precipitations.

Translation

The PURE system (Shimizu et al., 2001) was purchased from Post Genome Institute Company, Ltd. (Tokyo, Japan) and was used according to the manufacturer's guide. The *w*PURE system contained all the aminoacyl-tRNA synthetases, but contained only 13 amino acids (C, E, F, H, I, N, and W were withdrawn, where C, cysteine; E, glutamate; H, histidine). When necessary, one or more of these amino acids were added to the translation mixture. Usually, a 3∼5 μl scale reaction with the addition of 0.2 mM of amino acids of choice was used for analysis. [^{14}C]D was added to the reaction for the gel analysis of the product. Each aminoacylated $tRNA^{Asn\text{-}E2}$ was added to a final concentration of 50 μM as a mixture with the flexizyme dFx: so that the total concentration of exogenous RNAs in the translation was 100 μM.

Analyses of the Ribosomally Synthesized H3t

*H3t peptides from the 5 μl scale translation reaction were immobilized on anti-Flag-M2 agarose (Sigma-Aldrich; St. Louis, MO) by incubating 1 hr in TBS (50 mM 0.1 M HEPES-KOH, 150 mM NaCl [pH 8.0]) with rotation at room temperature. After washing the resin with TBS briefly, peptides were eluted from the resin with 0.2% TFA by incubating 30 min at room temperature. Peptides in the eluate were bound to C18 resin (ZipTip; Millipore; Billerica, MA), washed and desalted with 0.1% TFA, and eluted directly onto the MALDI target plate with saturated matrix (*R*)-cyano-4-hydroxycinnamic acid (Bruker Daltonics; Billerica, MA) in the 1:1 mixture of acetonitrile and 0.2% TFA. Average molecular masses were recorded using AutoflexII® (Bruker) in a linear positive mode. The instrument was calibrated externally with peptide and protein standards (Bruker).

Preparation of chromoHP1

The chromodomain (residues 17–76) of *Drosophila* HP1 (SWISS-PROT accession code P05205) was subcloned into *Nco* I/BamHI sites of pET42a vector (Novagen; Madison, WI) and expressed in *E. coli* strain BL21(DE3) with an N-terminal GST and hexa-His tags. The chromodomain alone was shown to be active enough to form a complex with synthetic H3t peptide (Jacobs and Khorasanizadeh, 2002). chromoHP1 in the form of N-terminal GST-His6 fusion was purified by Talon metal affinity resins (Clontech; Mountain View, CA) and dialyzed into binding buffer (20 mM imidazole, 25 mM NaCl, 2 mM DTT [pH 7.6]).

Pull-Down Analysis

A 15 μl scale translation reaction was primed with the template encoding H3t shown in Figure 4A and one of K- or *K-$tRNA^{Asn\text{-}E2}_{GAA}$ in the absence of phenylalanine in the translation system. A translation without aminoacyl-$tRNA^{Asn\text{-}E2}_{GAA}$ was prepared as a negative control for the pull-down analysis. *H3ts from the translation reaction were immobilized on 2.5 μl of the anti-Flag-M2 agarose by incubating 1 hr in TBS with rotation at room temperature. After brief washing with TBS, the resin was blocked with 3% BSA in TBS (1 hr at room temperature with rotation), washed again with TBS, and the resin immersed in 3 μl of 25 μM chromoHP1 in binding buffer for 1 hr at room temperature. The resin was washed twice with washing buffer (20 mM imidazole, 150 mM NaCl, 0.05% Tween20 [pH 7.5]) and once again with low Tris buffer (4 mM Tris-HCl, 10 mM NaCl [pH 8.0]). Elution was performed twice (10 min) using 0.1 M Gly-HCl (pH 3.0), and combined eluates were dried under reduced pressure and analyzed by western blot. Both anti-polyhistidine (Sigma) and anti-Flag tag antibodies (Sigma) were used in the same blot to visualize chromoHP1 and *H3t peptides, respectively.

In Vitro Binding Analysis by SPR

SPR assays were performed on a Biacore 2000. The instrument was maintained at 15°C, and the flow rate was 20 μl/min throughout the assay. Anti-Flag M2 antibody (Sigma) was diluted to 45 μg/ml in 10 mM sodium acetate (pH 5.0) (GE Healthcare; Buckinghamshire, England) and covalently immobilized on a sensor chip CM5 (GE Healthcare) using the Amine Coupling Kit (GE Healthcare). Translation reactions were diluted 100 times in HBS-EP (GE Healthcare) and captured on anti-Flag tag antibody-coated flow cell for 2 min. Then, varying concentrations of chromoHP1 (specified in figure legends) were injected and allowed to interact with antibody-bound *H3t for 2 min, resonance units in equilibrium were normalized by captured amounts, and molecular weights (ΔReq) were evaluated. Ten millimolars Glycine-HCl (pH 2.0; GE Healthcare) was used to regenerate the antibody-coated sensor chip.

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/11/1166/DC1/.

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