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Bioorthogonal Chemistry for the Isolation and Study of Newly Synthesized Histones and Their Modifications

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Abstract

The nucleosome is an octamer containing DNA wrapped around one histone H3–H4 tetramer and two histone H2A–H2B dimers. Within the nucleosome, histones are decorated with post-translational modifications. Previous studies indicate that the H3–H4 tetramer is conserved during DNA replication, suggesting that old tetramers serve as a template for the modification of newly synthesized tetramers. Here, we present a method that merges bioorthogonal chemistry with mass spectrometry for the study of modifications on newly synthesized histones in mammalian cells. HeLa S3 cells are dually labeled with the methionine analog azidohomoalanine and heavy $^{13}C_6$,$^{15}N_4$ isotope labeled arginine. Heavy amino acid labeling marks newly synthesized histones while azidohomoalanine incorporation allows for their isolation using bioorthogonal ligation. Labeled mononucleosomes were covalently linked via a copper catalyzed reaction to a FLAG-GGR-alkyne peptide, immunoprecipitated, and subjected to mass spectrometry for quantitative modification analysis. Mononucleosomes containing new histones were successfully isolated using this approach. Additionally, the development of this method highlights the potential deleterious effects of azidohomoalanine labeling on protein PTMs and cell cycle progression, which should be considered for future studies utilizing bioorthogonal labeling strategies in mammalian cells.

Introduction

The nucleosome, the basic repeating unit of chromatin, consists of DNA wrapped around an octamer of core histone proteins, with two copies of each histone H2A, H2B, H3, and H4. Incorporation of histone variants and addition of covalent histone post-translational

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modifications (PTMs) alter nucleosome composition and provide multiple layers of regulation of chromatin function and structure.(1, 2) For over 50 years, research has focused on identifying and mapping histone PTMs.(2) The histone code hypothesis suggests that PTMs constitute a code that is read by effector proteins to mediate their function.(3) Histone PTMs are involved in numerous cellular processes including transcription, apoptosis, DNA damage, and cell-cycle regulation.(2)

Given their functional relevance, studying the mechanisms behind the establishment and maintenance of histone PTMs is an essential step toward understanding chromatin biology. Nucleosome assembly, histone deposition, and PTM patterns must be characterized to best understand these mechanisms. It is known that during DNA replication, nucleosomes are deposited on both daughter strands.(4) The origin of histones within these nucleosomes has been disputed;(4-6) however, both density gradient(5) and mass spectrometry (MS)(6) analyses of heavy labeled nucleosomes indicate that the canonical H3–H4 tetramer is conserved while H2A–H2B dimers can be exchanged. This suggests that some octamers are predominantly new and contain newly synthesized tetramers with a new and an old H2A–H2B dimer. Core tetramer conservation and deposition of predominantly new nucleosomes on both daughter strands suggests that PTM propagation could occur via an internucleosomal templating event, where the neighboring octamer serves as a template for PTM acquisition. In contrast, intranucleosomal templating would imply that tails within a nucleosome serve as templates and are symmetrically modified. The finding that symmetrical nucleosomes are not necessary for lysine methylation acquisition refutes this mechanism for some PTMs.(7) To determine the mechanisms of PTM propagation, it is necessary to develop methods that allow for predominantly new octamers to be distinguished from old neighboring ones and PTMs across both species to be quantitated. Bioorthogonal ligation methods such as CuAAC (copper-catalyzed azide–alkyne cycloaddition reactions) have been applied to the investigation of chromatin.(8-10) Pulse labeling with the azide containing noncanonical amino acid azidohomoalanine (AHA) followed by bioorthogonal ligation allows for the tagging, isolation, and quantitation of newly synthesized proteins.(11, 12) Since AHA is recognized by the endogenous tRNA synthetase for methionine and incorporates in a residue specific manner, any methionine containing protein can be labeled. Using this single label technology, the Henikoff group developed CATCH-IT (covalent attachment of tagged histones to capture and identify turnover) to isolate DNA from mononucleosomes for the study of nucleosome turnover.(13)

Stable isotope labeling by amino acids in cell culture (SILAC) strategies in combination with MS have been developed to study PTMs.(14-19) For example, methylation kinetics have been determined using $^{13}CD_3$-methionine.(14, 15) The downside of such a strategy is the inability to distinguish old versus newly synthesized histone in peptides lacking methionine. This distinction can be made by labeling synchronized cells with a heavy amino acid like arginine that incorporates throughout histone proteins.(19)

Bottom-up MS can provide quantitative information about many PTM sites on proteins within a single sample.(20) The low starting material requirements of bottom-up MS make it feasible to merge chromatin immunoprecipitation (ChIP) methodologies with quantitative PTM analysis of histone proteins from nucleosomes, a technique referred to as ChIP with

quantitative MS (ChIP-qMS).(21, 22) ChIP-qMS technologies improve upon bulk histone studies by focusing the genomic area of study and/or isolating intact nucleosomes. The problem with these studies is that they rely on PTM antibody specificity or on overexpression of epitope tagged chromatin-related proteins.

Within, we describe the development of BLETS-ChIP-qMS (bioorthogonal ligation of epitope tags with stable isotope labeling for chromatin immunoprecipitation and quantitative mass spectrometry), a bioorthogonal chemistry-based method to isolate octamers containing newly synthesized histones for quantitative histone PTM analysis. Briefly, the method (Figure 1) involves labeling synchronized cells with AHA and heavy arginine. Mononucleosomes are then prepared and epitope tagged via a CuAAC reaction to facilitate immunoprecipitaion of nucleosomes containing new histones. Histones are then subjected to MS for quantitative PTM analysis. Combining AHA labeling with SILAC allows for the isolation and differentiation of new histones from old histones without relying on protein overexpression or PTM antibody specificity. MS of isolated mononucleosomes facilitates quantitation of PTMs on both new and old histone. Our development of this methodology showed that considerations must be made when utilizing AHA labeling for the study of protein PTMs and chromatin biology given its effect on protein methylation acquisition and cell cycle dynamics.

Results and Discussion

Heavy Arginine and AHA Mark New Histones

In order to identify and isolate nucleosomes containing newly synthesized histones, a dual labeling system that incorporated both an isotopically heavy arginine (Arg*) and a noncanonical amino acid was developed. Methionine analog AHA allows for isolation, while heavy arginine labeling allows for identification of newly synthesized histone. Arginine labeling was chosen because histones derivatized for bottom-up MS analysis contain a single C-terminal arginine residue on every peptide,(20) thus ensuring all histone peptides can be identified as new or old. Labeling of a peptide containing a single methionine and a single arginine residue can result in four possible outcomes: (1) “old” peptides containing a methionine and marked as old with a light arginine (Met, Arg), (2) “new” peptides containing a methionine and marked as new with a heavy arginine (Met, Arg*), (3) “new” peptides containing an AHA and marked as old with a light arginine (AHA, Arg), and (4) “new” peptides containing an AHA and marked as new with a heavy arginine (AHA, Arg*). All possible combinations are acceptable except for (AHA, Arg), which improperly marks a new peptide as old and would skew quantitation given that we monitor the new/old histone ratio by SILAC quantitation.

To test dual incorporation, labeling media was made in-house such that methionine was replaced with equal molar amounts of AHA and light arginine was replaced with heavy arginine. Labeling media was referred to by the percentage of AHA present, with the remainder being methionine; for example, 50% AHA media contains 50% AHA and 50% methionine. HeLa cells were labeled for 24 h with 100% AHA and Arg*. Histones were harvested and subjected to bottom-up MS analysis. Two methionine-containing peptides can

be easily detected in core histones—histone H3 (117–128) VTIMPKDIQLAR and histone H4 (79–92) KTVTAMDVVYALKR.

Quantification using area under the curve from the extracted ion chromatogram (XIC) for these peptides showed that the amount of dual heavy arginine and AHA incorporation in total histone was 21% on H3 (117–128), and 12% was labeled on H4 (79–92) (Figure 2, panel a). MS/MS fragmentation using collision-induced dissociation (CID) confirmed dual label incorporation with appropriate mass shifts for both heavy arginine (+10 Da) and AHA (–5 Da) in new peptides compared to old peptides (Figure 2, panel b).

Label incorporation needed to be maximized to ensure sufficient quantities of isolated histone for quantitative PTM analysis. The higher percentage of dual labeled (AHA,Arg*) compared to singly labeled (Met,Arg*) new peptides is beneficial for isolation because AHA facilitates immunoprecipitation. Additionally, AHA-containing peptides that were light labeled (AHA,Arg) account for <0.3% of both histone H3 and H4 (Figure 2, panel a). Heavy arginine incorporation occurred more efficiently than AHA incorporation, negating the concern of isolating histones that are improperly marked as old.

AHA Labeling Impacts Histone Synthesis and PTMs

Histone methyltransferases utilize S-adenosyl methionine (SAM) as the methyl donor. Since SAM is derived from methionine,(23) the complete substitution of methionine with AHA in labeling media could impede the establishment of normal histone methylation patterns. At the same time, the presence of methionine in labeling media could out-compete AHA for incorporation and prevent effective labeling. To investigate the proper balance, HeLa cells were labeled for 24 h with media containing heavy arginine and different percentages of AHA (Figure 3, panel a). Histones were acid extracted and subjected to bottom-up MS. The results demonstrate that labeling with media containing AHA at concentrations of 90% or greater allowed for adequate AHA incorporation. Increasing AHA concentration from 90% to 100% doubled AHA incorporation (Figure 3, panel b), demonstrating the importance of optimizing media conditions for efficient labeling.

The use of heavy arginine in these experiments allowed for differentiation of new and old histone and their respective PTMs. The expected ratio of old/new histone should be roughly 50:50 since the cells divide once during labeling and new histone is synthesized. We verified this to be true for cells labeled with 0% and 50% AHA and Arg* for 24 h. However, as AHA percentage increased, the percentage of old histone increased with ratios as high as 74:26 old/new on H3 (117–128) and 81:19 on H4 (79–92) for 100% AHA and Arg* labeled cells (Figure 3, panel b). This indicates that high AHA concentrations in the media impede new histone synthesis.

Histone PTM levels for single modifications for new, old, and total peptide were also quantified in these samples (Figure 4, Supporting Information Table 1a–c). As expected, high AHA percentages impeded acquisition of lysine methylation; however, reintroduction of up to 10% methionine had an ameliorating effect. On new peptides, lysine methylation levels were clearly dependent on the AHA concentration, with a steady decline in the relative abundance of di- and tri-methyl peptides on H3 (9–17) and H3 (27–40) as AHA

increases from 90 to 100% (Figure 4, panel a). This decrease also occurred on old histone; however, the decline was not as drastic (Figure 4, panel b). Due to a decrease in new histone synthesis at high AHA percentages, the calculated methylation levels on total histones from cells labeled under these conditions were skewed and appeared more similar to levels on old histone (Figure 4 panel b,c). The decrease in methylation was accompanied by an increase in the abundance of the unmodified form and occasionally the acetylated form of the peptide. Interestingly, there was an increase in di- and tri-methylated peptides in 50% AHA media as compared to the control as evidenced by H3K27me2. This implies that methionine depletion is not the only factor capable of impacting histone PTMs; the presence of AHA can also cause changes.

AHA Labeling Impedes Cell Cycle Progression

Methionine is an essential amino acid that is important for protein synthesis, DNA methylation, and protein methylation and can impact cellular growth.(23) Either when depleted or in excess, methionine has been found to arrest cancer cell lines at various stages of the cell cycle.(23, 24) To determine the effect of AHA labeling on cell cycle progression, a single pool of HeLa cells was synchronized to the G2/M phase using a thymidine-nocodazole block, released to the late G1 phase and then split and transferred into either 0% or 100% AHA media. Progression through S-phase was monitored using flow cytometry analysis (Figure 5, panel a). By 6 h post labeling, there was a noticeable delay with most 100% AHA labeled cells remaining in G1/early S phase while most 0% AHA labeled cells progressed into S phase (Figure 5, panel b). These results indicate that methionine depleted AHA labeling impeded normal cell cycle progression. The delay in normal cell cycle progression could explain the decrease in new histone synthesis; extended periods of labeling in media containing low methionine impeded cell division, thereby slowing the rate of new histone synthesis.

Overcoming the Impact of AHA Incorporation

Since we demonstrated that 100% AHA labeling causes a cell cycle delay, alternate labeling media were tested on synchronized cells to maximize AHA incorporation while allowing normal cell cycle progression. To monitor progression, cells were subjected to flow cytometry as above. To monitor global AHA incorporation, cells were subjected to CuAAC reactions with an Alexa-Flour 488 Alkyne (Supporting Information Figure 1, panel a). The fluorescence data show that very high AHA percentages (>90%) were needed for efficient label incorporation. Cells labeled with >90% AHA were able to progress to G2/M by 8 h, indicating a normal cell cycle progression relative to cells in 100% AHA (Supporting Information Figure 1, panel b–d). Taken together, these results indicate that labeling with >90% AHA was required to balance sufficient AHA incorporation with cell cycle progression.

The AHA labeling pulse length has to be optimized to ensure adequate AHA incorporation into histones as well as prevent cell cycle delays and aberrant methylation levels. To this end, cells were synchronized using a double thymidine block, split, released into 95% AHA and Arg* labeling media for either 4 or 6 h and then transferred into Arg*-only media until 12 h post release. Cells were collected together to ensure histone PTMs were compared

between cells at the same cell cycle phase and to give cells time to synthesize histone. Shorter labeling periods decreased AHA incorporation into histones to <1% on histone H3 and H4 (Supporting Information Table 2), illustrating the necessity of 95% AHA and Arg* media for synchronized pulse labeling experiments. Extending pulse length from 4 to 6 h resulted in a ~3-fold increase in incorporation, while maintaining new histone synthesis. As opposed to labeling for 24 h, synchronizing and pulse labeling ameliorated the impact of AHA labeling on new histone synthesis.

To observe the effect of pulse length on methylation acquisition, single histone modifications were quantified for these samples. For the majority of modifications like H3K9 methylation, levels were within standard deviations of each other (Figure 6 and Supporting Information Table 3a–c). However, H4K20me2 decreased on both new and old histone (Figure 6, panel a,b). H4K20 methylation patterns are linked to cell cycle progression,(15, 17) so these modifications may be particularly sensitive to perturbations caused by labeling. Despite this, as with new histone synthesis, synchronizing and pulse labeling cells alleviates the effect of AHA labeling on methylation acquisition for most modifications. This further highlights the need to optimize labeling conditions for these types of studies.

CuAAC Reactions and MS Detection of Triazoles

Our bioorthogonal ligation method relies on a CuAAC reaction, a 1,3-dipolar cycloaddition reaction between a terminal alkyne and an azide.(8) The alkyne peptide (FLAG-GGR-alkyne) consisted of a FLAG epitope, cleavable linker, and the alkyne containing propargylgylcine residue.(11) During CuAAC reactions, the azide of the AHA residue incorporated in histone becomes covalently linked to a FLAG-GGR-alkyne peptide via a 1,4-disubstituted 1,2,3-triazole ring (triazole). After histone digestion and derivatization, the FLAG-GGR is cleaved, leaving a propionylated triazole in place of the AHA residue. By monitoring the amount of triazole formation in optimization experiments, CuAAC reaction efficiency can be compared across experimental conditions and more efficient reaction conditions can be determined. Additionally, by comparing the amount of triazole formation in ChIP versus input, enrichment of methionine-containing histone peptides can be estimated. Relative quanitification of triazole-containing peptides using area under the curve measurements from nanoLC-MS/MS allows for such comparisons.

In order to quantify triazoles by MS, the mass shift of the triazole addition must be determined. To this end, FLAG-GGR-alkyne peptide and AHA residue alone were reacted to create a triazole containing peptide (Supporting Information Figure 2, panel a). After derivatization, nanoLC-MS/MS analysis confirmed the presence of propionyl groups on two lysine residues, the N-terminal amine and the y1 ion (Supporting Information Figure 2, panel b). Following trypsin digestion and another round of propionylation, a predominant ion with a mass of 369.19 Da was present in the MS spectra (Supporting Information Figure 2, panel b inset). This peak indicates that the peptide was efficiently cleaved at the C-terminal of the arginine residue to release the FLAG-GGR linker. Taken together, these results indicate that triazole formation resulted in a peptide mass shift of 168 Da and that it remained intact during MS analysis and CID MS/MS fragmentation.

Triazole formation on histone peptides results in a mass shift of 168 Da. Additionally, the triazole ring alters the chemical properties of the peptide and could induce a retention time shift. To determine elution order, AHA labeled histones were reacted with FLAG-GGR-alkyne and then digested, propionylated, and subjected to nanoLC-MS/MS analysis. Chromatographic peaks corresponding to triazole containing peptides showed that these peptides elute prior to methionine and AHA containing peptides. Identification of these peaks facilitates relative quantification of triazole formation (Supplemental Figure 3).

To maximize triazole formation, CuAAC reactions were performed on acid extracted histones, and triazole formation was monitored by MS or Western blots against the FLAG epitope. Optimization experiments demonstrated that copper I bromide (CuBr) is a more efficient catalyst than copper II sulfate ($CuSO_4$) (Supplementary Figure 4), which is consistent with previous findings.(25) Furthermore, addition of fresh CuBr/FLAG-GGR-alkyne during the reaction augmented triazole formation (data not shown). Performing two rounds of CuAAC reactions was previously reported to increase triazole formation on intact nucleosomes.(13) These findings were incorporated into our experiments on mononucleosomes.

ChIPs of CuAAC Reacted Mononucleosomes

Dual labeling and CuAAC reactions were combined to optimize ChIPs of reacted mononucleosomes (Figure 7, panel a). Mononucleosomes were prepared from asynchronous cells labeled for 24 h with 95% AHA, reacted with 1× or 3× FLAG-GGR-alkyne peptide, immunoprecipitated with anti-FLAG resin, eluted by competitive elution with FLAG peptide and subjected to Western blot or quantitative PTM analysis. Optimization of this method highlighted the need to remove both excess peptide and CuBr in TBTA prior to immunoprecipitation using EDTA chelation in combination with centrifugal columns (Figure 7, panel b).

Bottom-up MS of methionine-containing peptides showed enrichment was achieved with old/new ratios going from almost 83:17 old/new in input samples to up to 50:50 old/new in ChIPs (Supporting Information Table 4). Theoretically, any or all of the five methionines in core histones could facilitate immunoprecipitation. Up to 4-fold enrichment of triazoles at both H3Met120 and H4Met84 indicates that both of these residues facilitate immunoprecipitation. The two methionines in histone H2B are located in a single peptide that is not compatible with bottom-up MS analysis, making these methionines difficult to monitor; however, the almost 50:50 old/new ratio is consistent with immunoprecipitation of methionines from histone H2B as well. Since H2A–H2B dimers exchange,(14) old histone H3–H4 tetramers are probably isolated via new histone H2B present in exchanged dimers. Although these results are consistent with previous findings on octamer content, it should be noted that oligonucleosome contamination and biases in triazole formation could impact enrichment ratios and should be considered in future studies.

ChIPs Enrich Unmodified and Acetylated Forms

To observe PTM acquistion, ChIPs were performed on mononucleosomes from synchronized cells. HeLa cells were synchronized using a double thymidine block, released

into 95% AHA and Arg* media for 6 h, transferred into 0% AHA media for 2 h, and harvested. Mononucleosomes were prepared, reacted, cleaned, immunoprecipitated, eluted, and subjected to bottom-up MS. Methionine-containing peptides were quantified to monitor enrichment (Supporting Information Table 5). Triazole levels were below detection in part due to decreased AHA incorporation in pulse labeled synchronized cells compared to 24 h asynchronous cells. AHA,Arg* peptides and Met,Arg* peptides were used to calculate the percentage of new peptides. Old/new ratios of H3 (117–128) for ChIPs (66.9:33.1) compared to input (78.4:21.6) demonstrated partial enrichment.

PTMs on heavy arginine labeled peptides were quantified since these peptides come from isolated new H3/H4 tetramers. Normalization to both new and old input peptides showed enrichment of specific PTM patterns on new histones (Figure 8, Supporting Information Table 6). The largest changes were seen for unmodified or acetylated peptides, with a 3-fold enrichment of H3K9/K14ac on new tetramers. This is consistent with the findings that acetylation occurs rapidly.(18, 26) The slower establishment of methylation on new histone was expected given previous findings. Studies of histone methylation during the cell cycle indicate that, although gradually acquired, H3K9me3 requires progression to G1 to equilibrate and that levels remain low on new histone.(15, 16, 19) The presence of H3K9me3 on the isolated new histone at low levels compared to input was consistent with those studies. Methylation on H4K20 meanwhile is cell cycle stage specific with mono-, di-, and tri-methyls occurring stepwise beyond S-phase,(17) thus explaining the lack of higher methylation states on new histone. Taken together, our findings suggest the successful isolation of mononucleosomes containing newly synthesized and modified histones.

BLETS-ChIP-qMS has the potential to address larger questions about histone biology. Utilizing this approach on oligonucleosome species could address questions pertaining to PTM propagation. Quantitating PTM patterns on nucleosome species containing new histones alongside their neighbors at different times during the cell cycle would determine the timing of PTM acquisition and if PTM patterns on predominantly new nucleosomes mimic those on predominantly old nucleosomes, as hypothesized by the intranucleosomal templating model. Changes in chromatin structure and histone PTMs have been observed during various cell perturbations, such as DNA damage.(2) BLETS-ChIP-qMS could facilitate isolation of nucleosomes in these different cellular processes as well. MS can then be used to identify and quantify histone PTMs and to determine octamer content. Since histone variants can have distinct biological roles,(1) identifying which histone variants are present in the octamer is relevant to understanding chromatin biology. In addition to providing insight into the use of bioorthogonal chemistry to study protein PTMs, this work provides a method that can be adapted to address fundamental questions about histones and their PTMs.

Methods

Cell Culture and Medium

HeLa S3 mammalian cells were maintained at 37 °C and 5% CO_2 in spinner flasks in minimum essential medium Joklik (Sigma-Aldrich), 10% (v/v) newborn calf serum (HyClone), penicillin, streptomycin, and 1% (v/v) GlutaMAX (Invitrogen). For labeling

experiments, HeLa cells were spun, washed with phosphate buffered saline (PBS), and released into labeling medium. Labeling medium was made in-house to match the specifications of Joklik except for the replacement of methionine with equal molar amounts of AHA (Anaspec) and replacement of light arginine ($^{12}C_6$,$^{14}N_4$) with heavy l-arginine ($^{13}C_6$,$^{15}N_4$)-HCl (Cambridge Isotopes Laboratories, Inc.). It was supplemented with 200 mg mL^{-1} proline (Sigma-Aldrich), 10% (v/v) dialyzed fetal bovine serum (Gemini Biosciences), penicillin, streptomycin, and 1% (v/v) GlutaMAX (Invitrogen). Cells were harvested, pelleted at 600 rcf at 4°, washed with PBS, and stored at –80 °C.

Histone Acid Extraction and Derivatization

HeLa cell pellets were resuspended in nuclei isolation buffer (NIB: 15 mM Tris-HCl, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM sucrose, pH 7.5, 0.5 mM AEBSF, 10 mM sodium butyrate, 2.5 μM microcystein, 1 mM DTT) with 0.3% (v/v) NP-40 at a 10:1 buffer/packed cell volume ratio, incubated on ice for 5 min, washed 2× with NIB buffer, and spun at 3000 rpm. The nuclei pellet was resuspended in 0.4 N H_2SO_4 at a 5:1 buffer/nuclei pellet volume, rotated at 4 °C for 2 h, and spun at 3000 rpm for 5 min at 4 °C. Histones in the supernatant were acid extracted with 100% trichloroacetic acid (TCA) at a 1:5 ratio on ice, spun at 3000 rpm for 5 min at 4 °C, washed with 0.1% (v/v) HCl in cold acetone, washed with acetone, and dried.

Acid extracted histones (100 μg) were resuspended in water, brought to pH ~ 8 with ammonium hydroxide (NaOH), and derivatized using propionic anhydride.(21) For ChIPs, samples were dried down, boiled at 65 °C for 10 min, and derivatized. Briefly, propionic anhydride reagent was prepared fresh by dilution of propionic anhydride (Sigma-Aldrich) in isopropanol (Sigma-Aldrich) at a 1:3 volumetric ratio. Reagent was added 2× to histone at a 2:1 volumetric ratio, brought to a pH of ~8 with NaOH, reacted at 37 °C for 15 min, and dried to <35 μL. Histone was diluted in 100 mM pH 8 ammonium bicarbonate (ABC) and trypsin digested (Promega) at a 20:1 histone/enzyme ratio for 6 h at 37 °C. Reactions were quenched with acetic acid and dried to <10 μL. Subesequent propionic anhydride derivatization of the reagent was carried out to propionylate N-terminal amines. Reactions were desalted on C18 stage-tips, lyophilized to <5 μL, and diluted in buffer A in preparation for MS analysis.

Bottom-up nanoLC-MS/MS Analysis

Samples were separated on a 75 μm inner diameter fused silica microcapillary packed in-house with 5 μm Magic C18 AQ resin (Michrom Bioresources). Separation was performed with either an Easy-nanoLC (Thermo) or an Eksigent nanoLC-Ultra using a flow rate of 0.25 μL/min. Samples were eluted using a two step gradient: 2%–30% buffer B [buffer A, 0.1% formic acid; buffer B, 95% acetonitrile (ACN) in 0.1% formic acid] in 35 min and 30–98% buffer B in 20 min. For asynchronous labeling tests and ChIPs, the HPLC was coupled to either an LTQ-Orbitrap XL or an LTQ-Orbitrap-Velos. The MS method consisted of three segments starting with a full MS scan of *m/z* 110–2000 with a resolution of 100 000 (labeling tests) or a full MS scan of *m/z* 290–1450 with a resolution of 60 000 (ChIPs). The first segment had a full MS scan followed by 12 data dependent CID MS/MS scans. The second segment had a full MS scan followed by five targeted CID MS/MS scans of histone

H3 and H4 species and six data dependent CID MS/MS scans. The third segment had a full MS scan followed by 12 data dependent CID MS/MS scans. For cell cycle labeling tests, the HPLC was coupled to a Q-Exactive (Thermo). The MS method consisted of a full scan of *m/z* 290–1600 with a resolution of 100 000 followed by five MS/MS scans. Histone PTMs and labeled peptides were quantified using area under the curve in the XIC. Total histone was calculated by summing the areas of old and new histone.

Cell Cycle Synchronization

HeLa S3 cells were synchronized using either a nocodazole block or a double thymidine block as described.(27) Briefly, for a double thymidine block, HeLa cells were diluted to a confluency of 2×10^5, treated with 2.5 mM thymidine (Sigma-Aldrich) for 19 h, released into fresh medium for 8 h, treated with 2.5 mM thymidine for 19 h, and released into fresh normal or labeling medium. For ChIP experiments, cells were released into 95% AHA and Arg* labeling media for 6 h and transferred to 0% AHA media for 2 h prior to harvesting. For a nocodazole block, HeLas were diluted to a confluency of 2×10^5, treated with 2 mM thymidine (Sigma-Aldrich) for 19 h, released into normal media for 3 h, treated with 50 mM nocodazole for 12 h, released into normal media for 6 h, spun, washed with PBS, and released into labeling media. Cells were harvested by spinning at 600 rcf for 5 min, washed 2× with PBS, and stored at –80 °C.

Flow Cytometry Analysis

For cell cycle flow cytometry, approximately 1×10^6 cells were spun at 1000 rpm, washed in PBS, and resuspended in cold ethanol and PBS for fixation. Fixed cells were washed with 2× PBS and then stained in PBS with 0.08 mg mL^{-1} PI (Calbiochem) and 0.02 mg mL^{-1} RNase (Roche) at 25 °C in the dark for 1 h. Analysis was performed at the Princeton University Flow Cytometry Core Facility.

For AHA detection, cells were fixed with 4% (v/v) paraformaldehyde for 20 min, washed with PBS, permeabilized with 0.2% (v/v) Triton-X 100 for 10 min, and rinsed with 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween-20 in PBS. CuAAC reaction cocktail contained 10 μL of 100 mM $CuSO_4$, 50 μL of 1 M Tris at a pH of 8.5, 2.5 μL of Alexa-Flour 488 Alkyne, 50 μL of 1 M ascorbic acid, and 387.5 μL of water. Cells were resuspended in 100 μL of BSA with Tween-20, mixed with reaction cocktail, incubated for 30 min in the dark, washed with PBS, and analyzed.

Nucleosome Preparation

HeLa cell pellets were resuspended in NIB-250 with NP-40 at a 10:1 buffer volume/packed cell volume ratio, incubated on ice for 5 min, and spun at 3000 rpm to pellet nuclei. The nuclear pellet was washed 1× with a 10× volume of NIB-600 (NIB-250 as above with 600 mM NaCl), 1× with a 10× volume of NIB-250, and 2× with a 10× volume ratio of Hepes-50 buffer (20 mM Hepes, 50 mM NaCl, 5% (v/v) glycerol, 0.5 mM AEBSF, 10 mM sodium butyrate). The chromatin was resuspended in MNase Digestion Buffer; MNase was added and digestion proceeded at 37 °C for 45 min with fresh MNase added mid-digestion to ensure predominately mononucleosomes. Digestion was stopped by placing the reaction on

ice and was confirmed by running a small amount of chromatin or extracted DNA on a 2% agarose gel.

CuAAC Reactions

First, 1× FLAG-GGR-alkyne (DYKDDDDKGGR-PPG) and 3× FLAG-GGR-alkyne (DYKDHDGDYKDHDIDYKDDDDKGGR-PPG) peptides were synthesized and amidated at the C-termini (Genscript). Histones or mononucleosomes were brought to a pH of ~8 with solid ABC and mixed with $CuSO_4$ (100 μM $CuSO_4$, 2 mM ascorbic acid, 200 μM TBTA) or CuBr (20 mM CuBr was dissolved in 50 mM TBTA, and CuBr/TBTA was added to a final concentration of 200 μM TBTA). After the addition of either 25 μM 1× FLAG-GGR-Alkyne or 12 μM 3× FLAG-GGR-alkyne, protein was reacted at 25 °C on a rotator for 4 h. For CuBr reactions, fresh CuBr/TBTA/FLAG-GGR-alkyne was added at three equal time intervals. Reactions were subjected to Western blot or MS analysis.

For Western blot analysis, samples were run directly on an 18% acrylamide Tris-Glycine gel in 1× SDS buffer (0.25 M Tris, 1.92 M glycine, 1% (w/v) SDS). Proteins were transferred to 0.2 μm PVDF (Millipore) in transfer buffer (0.25 M Tris, 1.92 M glycine, 20% (v/v) MeOH). The membrane was blocked with Tris buffered saline with Tween-20 (TBS-T; 0.2 M Tris, 150 mM NaCl and 0.1% (v/v) Tween-20), incubated in mouse anti-FLAG M2 antibody (Sigma) diluted 1:1000 in TBS-T and 5% (w/v) milk, washed with TBS-T, incubated with antimouse horseradish peroxidase conjugated antibody (Jackson ImmunoResearch), washed, treated with enhanced chemilumnescence reagent (GE Healthcare Life Sciences), and exposed.

Triazole Ring Characterization

For mass shift analyses, 200 μg of FLAG-GGR-alkyne and 50 μg of AHA residue were mixed and brought to a pH of 8 with ABC, and CuAAC was reacted for 2–4 h. For peak characterization, acid extracted histone was CuAAC reacted as above. Samples were derivatized with propionic anhydride or derivatized with propionic anhydride and trypsin digested. The samples were stage tipped and separated using a 60 min gradient [buffer A, 0.1 M acetic acid; buffer B, 95% acetonitrile (ACN) 0.1 M acetic acid]. The HPLC was coupled to an LTQ-Orbitrap-XL. The MS method consisted of a single segment with a full MS scan of *m/z* 150–2000 and a resolution of 60 000. The full scan was followed by seven data dependent CID MS/MS scans.

Chromatin Immunoprecipitation

An equal volume of Hepes-50 with 20 mM EDTA was added to CuAAC reactions. They were then rotated at 25 °C for 5 min, concentrated on 30 kDa Amicon Ultra 0.5 mL centrifugal columns (Millipore) at 14 000 rpm, washed 3× with Hepes-50 with 20 mM EDTA, and washed 3× with Hepes-50. Protein was resuspended in Hepes-50 and anti-FLAG M2 agarose (Sigma-Aldrich), rotated at 4 °C for 8–12 h, washed with Hepes-50, and competitively eluted with FLAG peptide (Sigma-Aldrich). Eluate was subjected to MS or Western blot analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Abbreviations

ABC	ammonium bicarbonate
ac	acetyl
ACN	acetonitrile
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AHA	azidohomoalanine
Arg*	isotopically heavy arginine
BONCAT	bioorthogonal noncanonical amino acid tagging
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CATCH-IT	covalent attachment of tagged histones to capture and identify turnover
ChIP	chromatin immunoprecipitation
ChIP-qMS	chromatin immunoprecipitation with quantitative mass spectrometry
CID	collision induced dissociation
CuAAC	copper-catalyzed azide–alkyne cycloaddition reaction
CuBr	copper(I) bromide
$CuSO_4$	copper(II) bromide
Da	Dalton
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
H_2SO_4	sulfuric acid
HCl	hydrogen chloride
HPLC	high-performance liquid chromatography
KCl	potassium chloride
LTQ	linear triple quadruple
M	methionine

m/z	mass/charge
me	methyl
MeOH	methanol
$MgCl_2$	magnesium chloride
MS	mass spectrometry
NaOH	sodium hydroxide
NIB	nuclei isolation buffer
PI	propidium iodide
pr	propionylation
PBS	phosphate buffered saline
PTM	post translational modification
SAM	*S*-adenosyl methionine
SE	standard extraction
SILAC	stable isotope labeling by amino acids in cell culture
TBS-T	Tris buffered saline with Tween-20
TBTA	Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
TCA	trichloric acetic acid
TRI	triazole
Un	unmodified
XIC	extracted ion chromatogram

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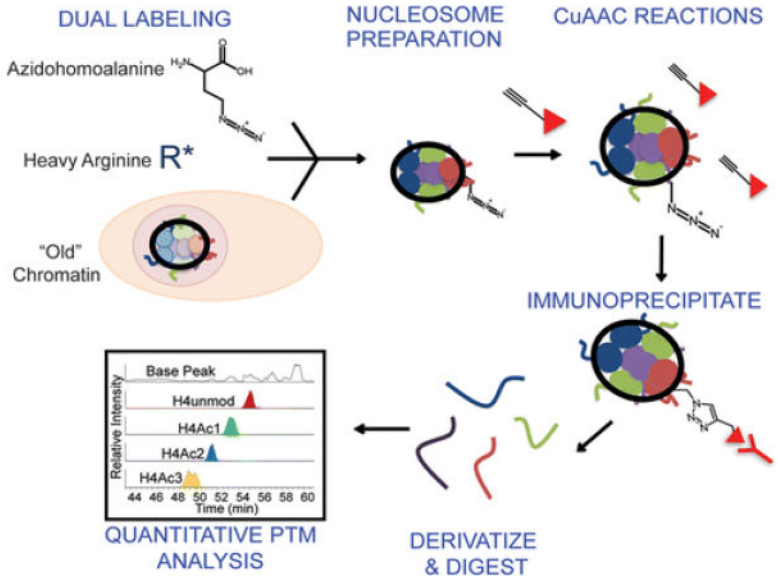

Figure 1.

Overview of the bioorthogonal chemistry-based method for studying histone PTMs. Cells are labeled with methionine analog azidohomoalanine (AHA) and heavy arginine (R*). Mononucleosomes prepared from labeled cells are ligated to an epitope tag via a cycloaddition reaction, immunoprecipitated, and separated. Histones are subjected to quantitative histone PTM analysis using nanoLC-MS/MS.

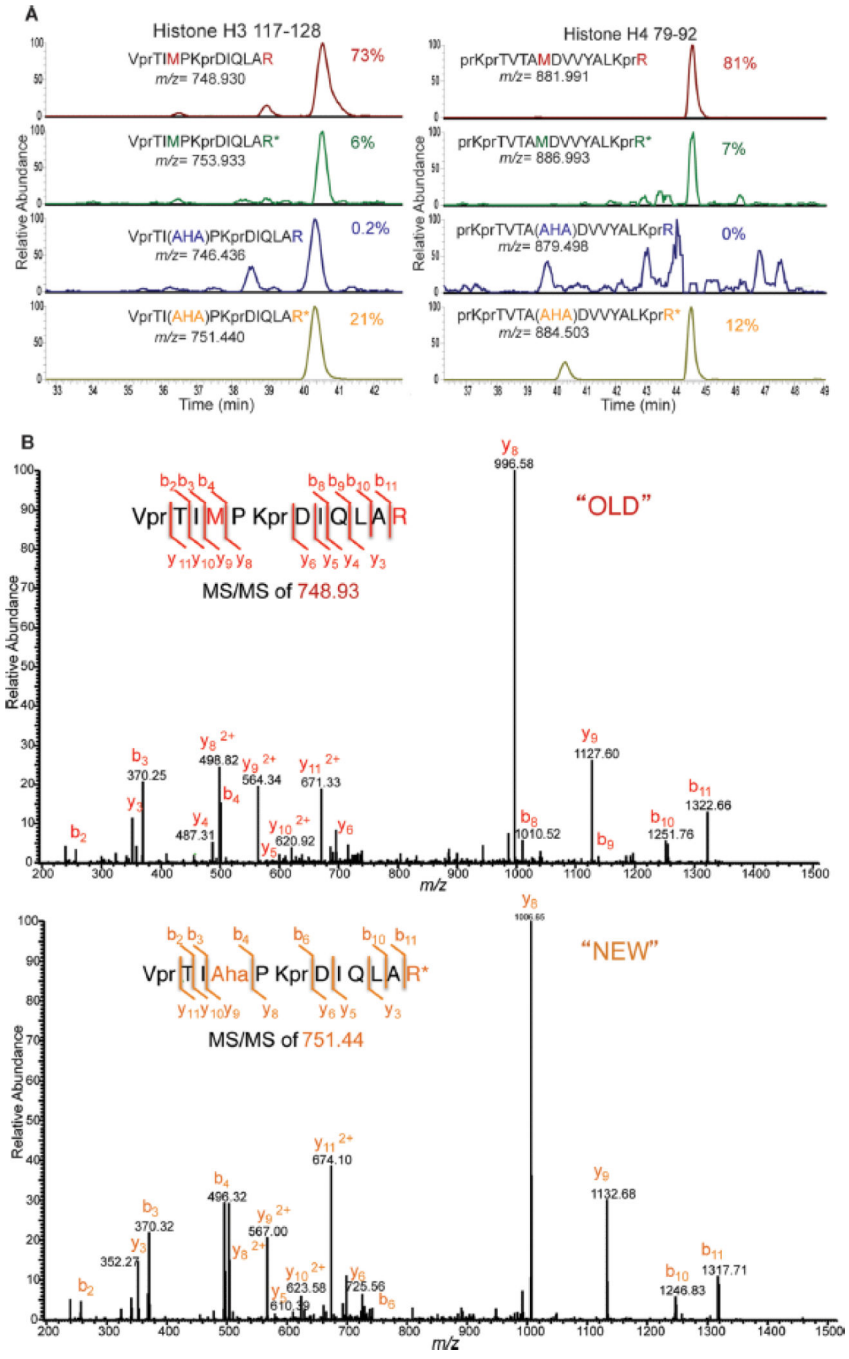

Figure 2.

Dual label incorporation in methionine-containing peptides. (a) XIC of histone H3 (117–128) VTIMPKDIQLAR and histone H4 (79–92) KTVTAMDVVYALKR show four possible species resulting from labeling. (b) Confirmation of dual label incorporation by CID MS/MS of histone H3 (117–128) with old VTIMPKDIQLAR and new VTI(AHA)PKDIQLAR* peptides. (AHA) = azidohomoalanine, R* = heavy arginine, and Pr = propionylation.

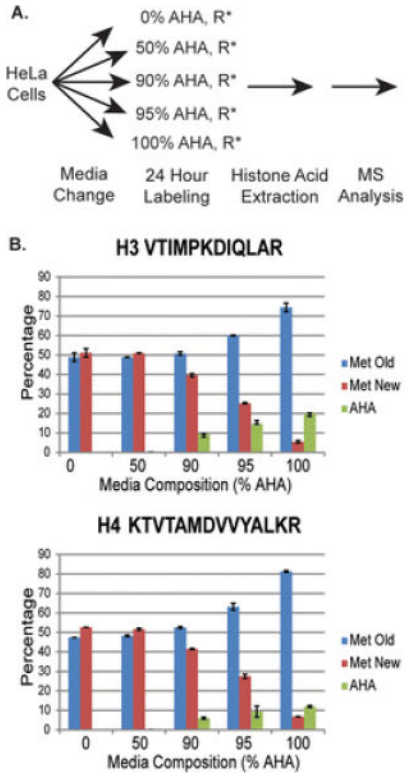

Figure 3.

AHA incorporation in dually labeled asynchronous cells. (a) Asynchronous HeLa cells were split into Arg*- and AHA-containing medias, labeled for 24 h and analyzed by nanoLC-MS/MS. (b) AHA incorporation at H3M120 in H3 (117–128) and H4M84 in H4 (79–92). Mean ± SE, $n = 3$.

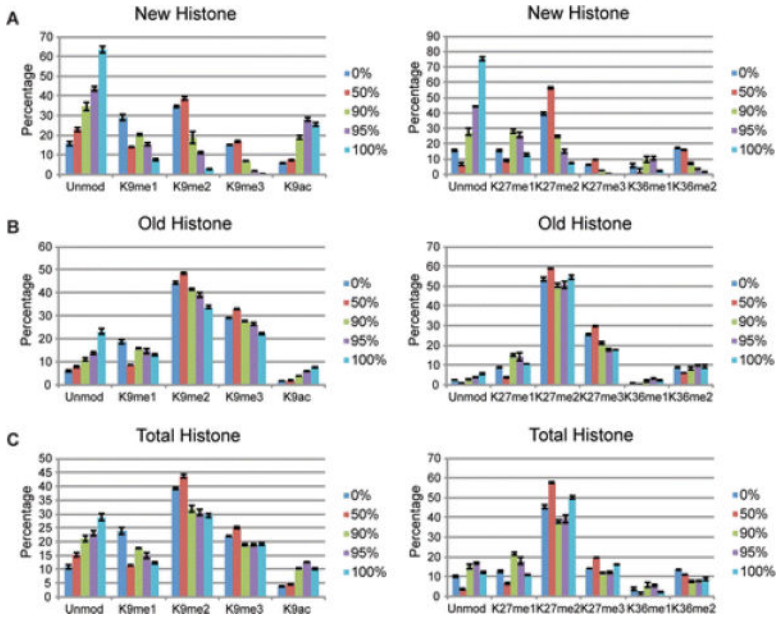

Figure 4.

Effect of AHA labeling on histone methylation patterns for H3 (9–17) and H3 (27–40) in dual labeled asynchronous cells. Percentage of single modifications on new (a), old (b), and total histone (c). Mean ± SE; $n = 3$.

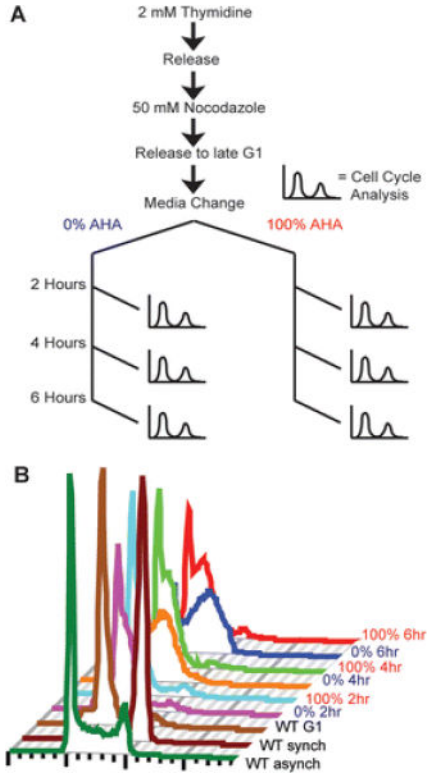

Figure 5.

Effect of AHA labeling on cell cycle progression. (a) Thymidine-nocodazole blocked HeLa cells were released to late G1/early S phase, split into 0% (blue) or 100% (red) AHA media, and subjected to flow cytometry analysis. (b) Progression of 0% AHA and 100% AHA labeled cells at 2 h intervals post G1. Asynch = asynchronous control. Synch = synchronized to G2/M.

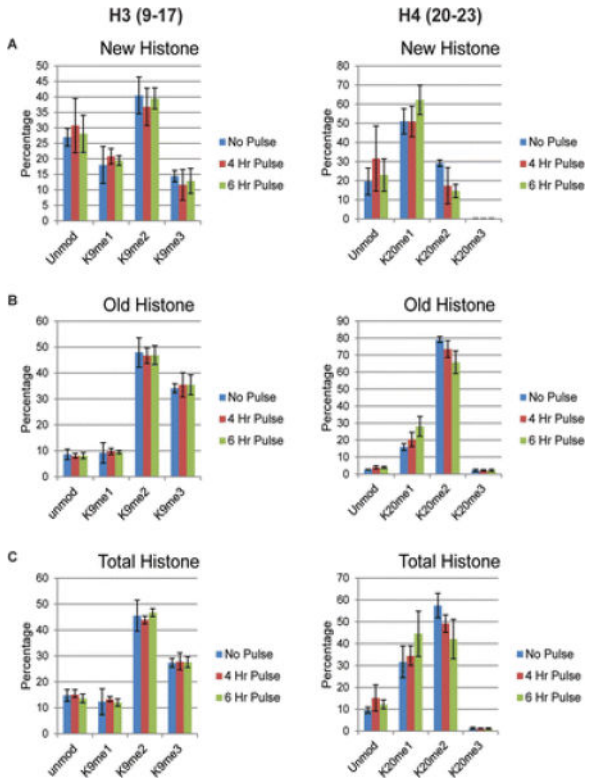

Figure 6.

Effect of AHA pulse labeling on histone methylation patterns in dual labeled synchronous cells. Percentage of single modifications on new (a), old (b), and total (c) histone for H3 (9–17) and H4 (20–23). Mean ± SE; $n = 4$.

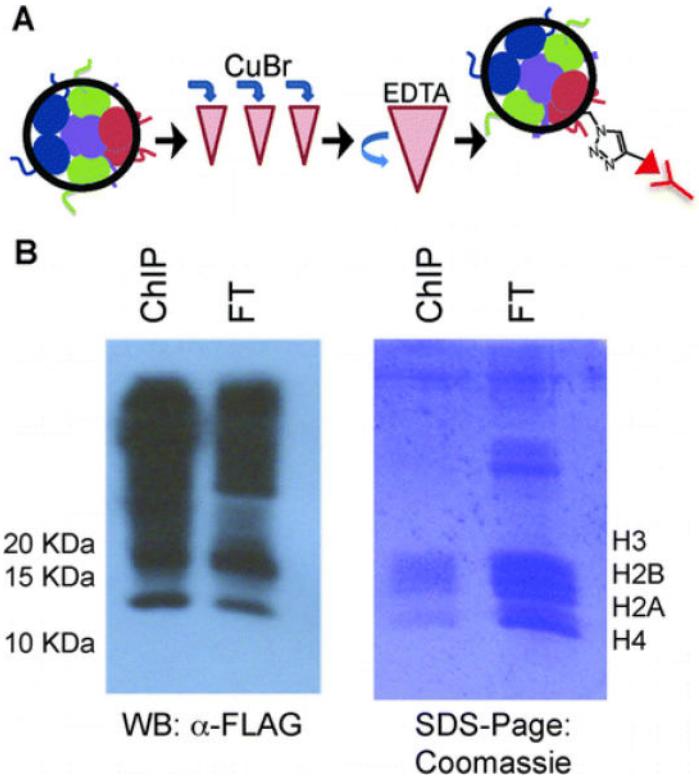

Figure 7.

ChIPs of CuAAC reacted nucleosomes. (a) AHA labeled nucleosomes are reacted, cleaned using EDTA and spin columns, and immunoprecipitated. (b) Western blot analysis of ChIPs using anti-FLAG antibodies (left). 100% ChIP and FT were loaded. FT = Flowthrough.

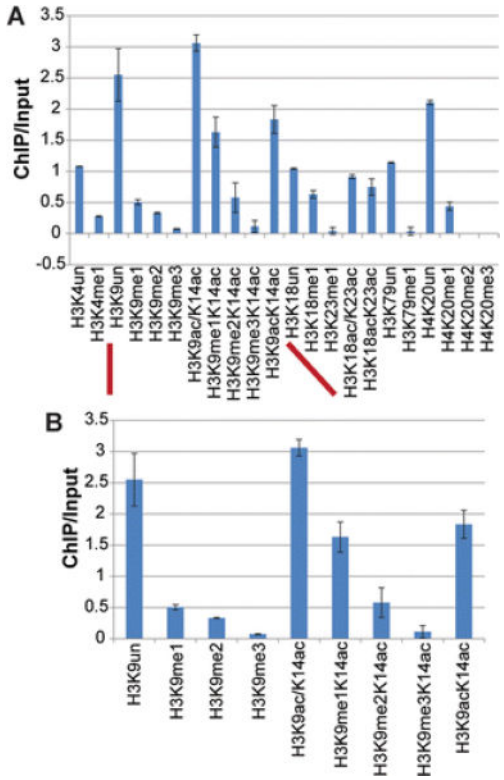

Figure 8.

Histone PTMs from ChIPs of CuAAC reacted mononucleosomes from synchronized cells. New peptides in ChIPs were normalized to total peptide in input. (a) Quantifiable modifications; (b) H3 (9–17) peptide mean ± SE $n = 3$.