Published in final edited form as:

Methods. 2015 November 15; 90: 8–20. doi:10.1016/j.ymeth.2015.06.008.

Characterization of histone post-translational modifications during virus infection using mass spectrometry-based proteomics

Katarzyna Kulej^{#1,2}, Daphne C. Avgousti^{#1,2}, Matthew D. Weitzman^{1,2}, and Benjamin A. Garcia³

¹ Division of Cancer Pathobiology, Children's Hospital of Philadelphia

² Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania

³ Epigenetics Program, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania

[#] These authors contributed equally to this work.

Abstract

Viruses are obligate intracellular parasites that necessarily rely on hijacking cellular resources to produce viral progeny. The success of viral infection requires manipulation of host chromatin in order to activate genes useful for production of viral proteins as well as suppress antiviral responses. Host chromatin manipulation on a global level is likely reliant on modulation of post-translational modifications (PTMs) on histone proteins. Mass spectrometry (MS) is a powerful tool to quantify and identify novel histone PTMs, beyond the limitations of site-specific antibodies. Here, we employ MS to investigate global changes in histone PTM relative abundance in human cells during infection with adenovirus. Our method reveals several changes in histone PTM patterns during infection. We propose that this method can be used to uncover global changes in histone PTM patterns that are universally modulated by viruses to take over the cell.

1. Introduction

Histone proteins are essential components of chromatin in eukaryotes. Post-translational modifications (PTMs) on histone proteins and histone variants directly affect chromatin structure [1-5], which modulates gene expression, DNA repair and cell duplication events such as mitosis and meiosis [6-8]. Histones assemble as octamers that are wrapped by DNA every ~147 base pairs constituting the repeating unit known as the nucleosome. Aberration in the normal catalysis and regulation of histone PTMs has been linked to the development of many diseases [9,10]. Because of this, the scientific community has increased focus on

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

characterizing histone PTMs, specifically in developing techniques that improve accuracy and throughput.

The role of histone PTMs during virus infection has only recently come into prominence with the recognition that viral manipulation of host cells necessarily hijacks chromatin signaling [11-13]. Many studies have focused on histone PTMs associated with viral genomes at various stages during infection [14-18] where others have investigated specific histone PTM changes on host genomes, largely through antibody-based approaches [19-21], or investigating the effect of specific viral proteins [22,23]. Previous work from the Garcia lab provided the first comprehensive quantitative analysis of global histone PTMs from cytomegalovirus infection [11]. We propose that mass spectrometry based methods can be used to compare global histone PTMs across multiple virus infections to provide a framework for identifying common mechanisms used by viruses to manipulate host chromatin. Here, we describe a detailed mass spectrometry based protocol to approach histone PTM characterization during adenovirus infection in primary fibroblast cells.

Mass spectrometry (MS)-based proteomics has been applied to histone analysis with success for over 15 years, and several strategies have been developed to identify and quantify not only single PTMs, but also their combinatorial patterns (reviewed in [24,25]). MS is mostly coupled to high performance nano liquid chromatography (nanoLC), as histone peptide separation assists in depth and confident characterization, though elution time can also be used to discriminate PTMs (see section 6). As with most proteomic analyses, histones are digested into short peptides to facilitate their separation and detection. The protocol we describe includes derivatization of lysine residue side chains with propionic anhydride, proteolytic digestion with trypsin and subsequent derivatization of peptide N-termini specifically to analyze histone PTM patterns during adenoviral infection. This protocol leads to generation of ArgC-like peptides, i.e. only cleaved after arginine residues. While such peptides could be obtained using the enzyme ArgC, the addition of propionic groups to the peptide increase its hydrophobicity improving LC retention. Histone peptides, like any other peptide, can be subjected to enrichment to facilitate detection of specific PTMs such as phosphorylation. Unlike methylation and acetylation, phosphorylation on histones is present in sub-stoichiometric amounts in most non-synchronized mammalian cells making proteomics analysis challenging. Therefore, in our protocol we applied the well-established titanium dioxide (TiO₂) procedure for enrichment of phosphopeptides [26-28]. A variety of quantitative proteomics methods may also be incorporated into this protocol, including label-free [29] or metabolic labeling (stable isotope labeling by amino acids in cell culture; SILAC) [30]. In general, the high mass accuracy and sensitivity of modern MS along with the possibility of integrating nanoLC-MS with tandem mass spectrometry (MS/MS) has dramatically increased the confidence in histone peptide identification and quantification, making MS the current technique of choice for histone PTM analysis.

2. Sample Preparation

The following protocols focus on histone isolation from human IMR90 fibroblast cells infected with adenovirus. However, different combinations of viruses and cells can be used. Histones may also be extracted from yeast, animal tissues or plant cells. For these types of

samples be sure to refer to well-established nuclei isolation procedures. After successful nuclear or chromatin isolation, our protocol can be universally used for purification and analysis of histones and their post-translational modifications (PTMs). The complete workflow described in this protocol is illustrated in the **Figure 1**.

2.1. Materials and Reagents

Technical Note. All the solutions and buffers should be prepared with ddH₂O (Milli-Q water, UHQ), analytical grade reagents and highest purity chemicals. Organic solutions should be prepared fresh or stored for no more than two weeks before performing the protocol to avoid changes in buffer composition. This note applies to all steps of nuclei isolation, histone extraction and purification, and MS analysis.

1. Cells: IMR90 lung fibroblast cells (ATCC).
2. Culture medium: Eagle's Modified Essential Medium (EMEM, VWR) supplemented with 1% penicillin-streptomycin (Gibco® Life Technologies) and 10% fetal bovine serum (FBS) (Gibco® Life Technologies) for complete media, or only 2% FBS for infection media.
3. CellStar® Tissue Culture Dishes 100mm × 20mm (or similar polystyrene sterile dishes).
4. Corning® Cell Lifter 19mm blade with 180mm handle (or similar cell scraper).
5. Corning® Cellgro Dulbecco's Phosphate-Buffered Saline (DBPS).
6. 0.25% Trypsin-EDTA (Gibco® Life Technologies).

2.2. Cell preparation and virus infection in culture

1. Thaw cryo-vial of IMR90 cells in water bath at 37 °C.
2. Resuspend cells in 5mL of pre-warmed complete EMEM media.
3. Spin cells at 500 × g for 2 minutes, aspirate supernatant.
4. Resuspend pellet in 2mL of warm EMEM and plate in 1 × 100mm dish with 8mL of media (10mL total).
5. Grow cells until confluent and split into 3 × 100mm dishes using trypsin.
6. When cells have reached 80-90% confluence (approximately $6-7 \times 10^6$ cells per 1 × 100mm dish), infect two of the three plates with wild type adenovirus type 5 at a multiplicity of infection (MOI) of 20. This means 20 infectious particles per cell depending on the unit of virus titer. The volume required can be calculated as follows: Volume of virus = [MOI (plaque forming units per cell) * number of cells] / [viral titer (plaque forming units per mL)]. For example, for an MOI of 20, with a viral titer of 4.33×10^{10} pfu/mL, a volume of 0.0046mL or 4.6µl would be needed for 10×10^6 cells. An MOI of 20 with adenovirus will result in all cells becoming infected.

Technical Note. For the procedure described, cells were not synchronized but maintained as a mixed population prior to infection. If desired, cells can be synchronized in G1 using standard methods for the cell type used, such as isoleucine deprivation, and released at the time of infection.

7. Add the appropriate amount of virus to a minimum volume of infection media, i.e. 3mL per 100mm dish. Aspirate media from the cells, wash once with DPBS (Gibco) then add media including virus to the two plates to be infected. For the remaining plate, aspirate media, wash and then add 3mL of infection media without virus; this will serve as a 'mock' infected control plate.
8. Incubate the three plates at 37 °C for 2 hours, rocking gently every 20 minutes to ensure efficient infection.
9. After two hours, add 7mL of complete media to each plate and keep at 37 °C. Optional: Virus-containing media can be aspirated and 10mL of complete media can be added instead of topping up.
10. At 24 hours post infection (hpi), counting from initial infection, harvest mock infected plate and one of the two infected plates by trypsin or scraping.
11. At 48 hpi, harvest the remaining plate by trypsin or scraping.

Technical Note. If using different viruses, adjust the MOI accordingly to the standard used in the literature to ensure that all cells become infected.

3. Cell nuclei isolation

3.1. Materials and Reagents

Technical Note. Reagents listed below have to be ice-cold prior to use.

1. Nuclei isolation buffer (NIB-250): 15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 250 mM sucrose.
2. Reducing agent (add fresh to NIB-250 buffer prior to use): 1 M dithiothreitol (DTT) in ddH₂O (1000×).
3. Protease inhibitors (add fresh to NIB-250 buffer prior to use): 200 mM AEBSF in ddH₂O (400×).
4. Phosphatase inhibitors (add fresh to NIB-250 buffer prior to use): 2.5 μM microcystin in 100% ethanol (500×).
5. Histone deacetylase inhibitors (HDAC inhibitor) (add fresh to NIB-250 buffer prior to use): 5 M sodium butyrate, made by titration of 5 M butyric acid using NaOH to pH 7.0 (500×).
6. Alternative detergent: 10% (v/v) NP-40 in ddH₂O.
7. Alternative storage buffer: NIB-250 buffer supplemented with 1mM DTT, 0.5mM AEBSF, 0.005μM microcystin, 10mM sodium butyrate, 5% glycerol.

8. Low-binding 1.5mL eppendorf tubes (Sorenson BioScience Inc., Utah, US), or Falcon 15mL conical tubes, depending on the cell pellet volume.

3.2. Procedure for cell nuclei isolation

Technical Note. Perform all steps at 4°C to minimize enzymatic activities that could interfere with histone PTMs.

1. For 0.1mL of cell pellet, prepare approximately 5mL of NIB-250 lysis buffer. To 5mL NIB-250 buffer, add 5 µl of 1 M DTT, 12.5 µl of 200 mM AEBSF, 10 µl of 2.5 µM microcystin and 10 µl of 5 M sodium butyrate.
2. Re-suspend cell pellet in 10:1 (v/v) ratio of NIB-250 buffer supplemented with inhibitors, and include 0.2% NP-40 (Nonidet P 40).L

Technical Note. If cell pellet is smaller than 100 µl transfer cells suspended in NIB-250 lysis buffer to 1.5mL eppendorf tubes and continue to homogenization step.

NP-40 is a mild, nonionic detergent effective in isolation of cytoplasmic proteins while preserving nuclear membranes when used at low concentrations.

3. Homogenize cells by gentle pipetting. Cells have to be homogenized very well, with no clumps left in solution.
4. Incubate homogenized cells on ice for 10–15 min. The cells will lyse and release nuclei.
5. Centrifuge homogenate at 1,000 $\times$ g for 5–10 min at 4 °C. The pellet contains mostly cell nuclei, while the supernatant contains cytoplasmic components. Slow speed is important at this step to avoid extensive lysing of the nuclei due to the centrifugal force.
6. Discard the supernatant and wash the nuclei pellet by gently re-suspending with 10:1 (v/v) NIB-250 buffer supplemented with inhibitors. At this step do not add NP-40. The detergent should be removed prior to histone extraction. Optional: If desired, the supernatant from this step can be used for RNA extraction and subsequent gene expression analysis.
7. Centrifuge nuclei suspension at 1,000 $\times$ g for 5 min at 4 °C and discard supernatant.
8. Repeat Steps 6-7 two to four times to ensure no NP-40 remains. Removal of NP-40 is evident when gentle pipetting during the washing step no longer forms bubbles.

Technical Note. After Step 8, the samples can be re-suspended in the minimum volume of NIB-250 buffer (about 50 µl, or the minimum volume to cover the nuclei pellet) supplemented with 1mM DTT, 0.5mM AEBSF, 0.005µM microcystin, 10mM sodium butyrate, 5% glycerol, and stored at –80 °C.

9. Continue to purification of histone proteins (section 4).

4. Purification of histone proteins

Histones are highly enriched in basic amino acid residues. This property facilitates their interaction with the phosphoric acid backbone of DNA. Therefore, histone extraction is based on the solubility of histones in acid (0.2 M H₂SO₄) followed by precipitation with highly concentrated TCA (33%).

Alternatively, high-salt extraction can be used to purify histones. This protocol is intrinsically milder, as it does not use strong acid. This preserves acid-labile PTMs (e.g phosphorylation of arginine, lysine and histidine residues) and may increase the yield and purity of extracted histones because TCA precipitation co-precipitates many other chromatin binding proteins. However, high-salt extraction leads to high salt content in the final sample, which is incompatible with nanoLC-MS/MS. Salt removal may generate inconsistent sample losses, making the high-salt extraction procedure not favorable in our workflow. Depending on the desired outcome, either extraction protocol can be used. For the high-salt histone extraction protocol, refer to *Shechter D. et al., 2007* [31] or *Rodriguez-Collazo P. et al., 2014* [32].

4.1. Materials and Reagents

Technical Note. Reagents listed below should be ice-cold prior to use.

1. Nuclei solubilization buffer: 0.2 M H₂SO₄ in ddH₂O.
2. Acid (TCA) precipitation buffer: 100% TCA (w/v) in ddH₂O.
3. Washing buffer 1: 100% (w/v) acetone, 0.1% HCl.
4. Washing buffer 2: 100% (w/v) acetone.
5. Histone solubilization buffer: ddH₂O or 50 mM ammonium bicarbonate (NH₄HCO₃), pH 8.0.
6. Low-binding 1.5mL eppendorf tubes (Sorenson BioScience Inc., Utah, US), or falcon 15mL conical tubes, depending on the cell pellet volume.
7. Glass Pasteur pipets.

4.1.1. Procedure for purification of histone proteins—Technical Note. Perform all steps at 4°C to minimize enzymatic activities that could potentially interfere with histone PTMs.

1. Re-suspend nuclei pellet with 5:1 (v/v) ratio of 0.2 M H₂SO₄ by gentle pipetting. Nuclei should be re-suspended very well, with no clumps left in solution. Alternatively, if some small nuclei aggregates remain, gentle vortexing can be used to create a homogenous nuclei suspension.

Technical Note. H₂SO₄ solution should be freshly made. Acid standing for long periods of time at room temperature can result in changes in pH that will affect the yield of the extraction.

2. Incubate the sample with constant rotation or gentle shaking for 2–4 h at 4 °C. For samples starting from a cell pellet larger than 500 µl, a 2 h extraction time is sufficient. Longer incubation is not recommended, as other contaminant basic proteins will co-extract. For a small sample size (<200 µl cell pellet), 4 h extraction provides a better yield.
3. Centrifuge sample at 3,400 $\times$ g for 5 min at 4 °C to remove nuclear debris.
4. Transfer the supernatant containing histone proteins into a new 1.5mL or 15mL tube, depending on the sample volume. Repeat Steps 3–4.
6. Add 100% TCA to the sample solution with a ratio of 1:3 (v/v), to obtain a final TCA concentration of 33%. This step will precipitate histones.

Technical Note. It is very important for TCA to be freshly made. TCA left at 4 °C for more than a month will not be as efficient in precipitating histones.

7. Let the mixture precipitate on ice for at least 1 h at 4 °C. Do not disturb the precipitation. For samples that start with small cell pellet volume (<200 µl), overnight precipitation is recommended.
8. Pellet histones by spinning at 3,400 $\times$ g for 5 min at 4 °C. Remove the supernatant by aspiration without touching the precipitated proteins. In particular, do not disturb the white layer condensed around the bottom or the sides of the tube, as these are the histones. The pellet in the very bottom of the tube contains mostly contaminant proteins or other biomolecules.
9. By using a glass Pasteur pipette rinse the tube with 100% acetone supplemented with 0.1% HCl to cover the precipitated proteins. Acetone is used to remove the remaining TCA from the solution without dissolving the protein pellet.

Technical Note. It is crucial to use glass Pasteur pipettes to transfer acetone as this organic solvent can dissolve plastic.

10. Centrifuge sample at 3,400 $\times$ g for 5 min at 4 °C and discard supernatant.
11. Repeat Steps 9-10 using 100% acetone without 0.1% HCl.
12. Dry pellet with air flow or by leaving the tube open on the bench. Acetone evaporates quickly.
13. Dissolve the histones with 50mM NH₄HCO₃, pH 8.0 in minimum volume. For pellets in a 1.5 mL eppendorf tube, 30-50 µl should be sufficient.

Technical Note. Histones generate a thin layer at the side of the eppendorf tube, while the white pellet at the bottom of the tube is mostly contaminants. This bottom pellet usually does not resuspend. Alternatively, resuspension can be facilitated by using water + 0.1% trifluoroacetic acid (TFA), but this should be used only if the aim is to separate intact histone variants using RP-HPLC (as described by [33]) (**Figure 2A**). Fractionation of intact histone variants by RP-HPLC ideally requires at least 100 µg of starting material.

14. Centrifuge sample at $3,400 \times g$ for 5 min at 4 °C and transfer the supernatant to a new 1.5 mL tube. Perform this step only for initial cell pellets higher than 200 μ L.

Technical Note. Purified histones can be stored in ddH₂O or 50mM NH₄HCO₃ at –80 °C. Optional: before freezing the sample collect a few μ L for quality control of histone extraction (section 4.2).

4.2. Quality control of histone extraction

Bradford protein assay or amino acid analysis (AAA) is recommended to measure the concentration of purified histones. Do not use techniques that rely on absorbance at 280 nm as histones do not contain many aromatic amino acid residues. To verify the purity of extracted histones prior to MS analysis, standard 1-dimensional SDS-PAGE may be applied (**Figure 2B**). Gradient gel (4-12%) or high percentage gels (12%-15%) are ideal for efficient separation of histone bands.

4.3. Propionic anhydride derivatization prior histone digestion

Proteomics has evolved into a high-throughput strategy due to the combination of online sample separation with MS detection. While separation and characterization of intact proteins is challenging to perform on a large-scale, analysis of shorter sequences (i.e. peptides) allows for high confident identification and quantification. Peptides are mostly generated using trypsin, a proteolytic enzyme that cleaves with high specificity and efficiency after lysine and arginine residues. Masses in the range of 600-2000 Da are in general easily ionized, and can be detected with high mass accuracy (<5 ppm) and resolution (> 60,000). Conversely, smaller masses such as peptides of 6 or fewer amino acids, are difficult to retain by chromatography. Histones are highly enriched in basic amino acid residues such as lysine and arginine, which would result in excessively short peptides if digested with trypsin without precautions. Thus, the protocol includes a step of lysine and peptide N-terminal chemical derivatization [34]. Propionic anhydride derivatization blocks the ϵ -amino groups of unmodified and monomethyl lysine residues, allowing trypsin to perform proteolysis only at the C-terminus of arginine residues. Derivatized lysine residues, contrary to unmodified ones, cannot exchange protons with the solution and thus the peptides are generally only doubly or triply charged, facilitating MS and MS/MS detection. Moreover, N-terminal derivatization increases peptide hydrophobicity and thus reversed-phase chromatographic retention and separation. Finally, as described during the preparation, N-terminal derivatization may be performed using isotopically labeled propionic anhydride (D10), which allows multiplexed analysis.

4.3.1. Materials and Reagents

1. Propionylation solution: 25% propionic anhydride (D0), 75% (w/v) 2-propanol.

Technical Note. Alternatively, 75% (w/v) 2-propanol may be replaced with 75% (w/v) acetonitrile.

2. Ammonium hydroxide (NH₄OH), 28% NH₃ in water.
3. Histone solubilization buffer: 50 mM NH₄HCO₃, pH 8.0.

4.3.2. Procedure for propionic anhydride derivatization of histones—**Technical**

Note. Steps 3-9 must be performed in a streamlined manner without any interruptions for maximum reaction efficiency. These steps should be performed in the fume hood. If any steps are interrupted, make new reagents for additional samples.

1. Dissolve 50-100 µg histone proteins in 20-30 µl of 50 mM NH_4HCO_3 , pH 8.0. If samples are in pure ddH_2O , add 100 mM NH_4HCO_3 to the final concentration of 50 mM. The final volume of histone suspension should not exceed 30-40 µl.

Technical Note. Smaller amount of histones may also be used. However, this may result in poorer final yields.

2. Dip a P10 pipette tip into the sample and spot 0.5-1 µl onto pH indicator strip to monitor the pH. This is sufficient to monitor the current pH roughly without sample loss. NH_4OH and glacial acetic acid can be used to adjust the pH to ~8.0. If the sample volumes are below 30 µl, particular care should be used in adjusting the pH. Do not add more than 1-2 µl of NH_4OH or glacial acetic acid at the time.
3. Prepare the propionylation reagent by mixing propionic anhydride with 2-propanol in the ratio 1:3 (v/v); e.g. for three samples that have the volume of 30 µl, mix 15 µl of propionic anhydride and 45 µl of 2-propanol. **This reagent must be made fresh every 3-4 samples.**
4. Rapidly add the propionylation reagent to the histone sample with a ratio of 1:2 (v/v); e.g. 15 µl propionylation reaction for 30 µl sample.
5. Immediately add NH_4OH to adjust the pH to ~8.0. Propionic anhydride reacting with the free amines of the peptides produces propionic acid that decreases pH. Usually, adding NH_4OH to the sample with a ratio of 1:5 (v/v) is sufficient to re-establish pH 8.0; e.g. 6 µl of NH_4OH to 30 µl of sample.

Technical Note. Carefully evaluate the amount of NH_4OH added, as a pH higher than 10.0 leads to labeling of other amino acid residues and can generate undesired byproducts.

6. Mix immediately by vortexing.
7. Check pH with the same procedure as Step 2.
8. Briefly centrifuge and incubate samples at 37 °C on a heat block or in a water bath for 15 min.
9. Repeat Steps 3-8 for all samples desired, always taking care not to process more than 3-4 samples per batch of propionylation reagent.
10. Dry samples down to 5–10 µl in a SpeedVac centrifuge. This evaporates unreacted propionic anhydride, 2-propanol, acetic acid and ammonia gas released from NH_4OH . If samples dry out completely, no significant sample losses occur.
11. Re-suspend or dilute samples with ddH_2O to achieve 30 µl of final volume.

12. Repeat Steps 2–10. A double round of histone propionylation ensures >95% of reaction completion.

Technical Note. Samples can be stored at -80°C as dry or reconstituted in ddH₂O.

13. Continue with proteolytic digestion with trypsin (section 4.4)

4.4. Proteolytic digestion with trypsin (in solution)

4.4.1. Materials and Reagents

1. Histone solubilization buffer: 50 mM NH₄HCO₃
2. Digestion solution: 2% trypsin (Sequencing Grade Modified Trypsin, Madison, WI, US).
3. Stop digestion solution: 100% (w/v) formic acid.

4.4.2. Procedure for trypsin digestion of purified histones

1. Re-suspend histones in 50 mM NH₄HCO₃ to achieve a concentration of 1 µg/µl or higher. More dilute samples lead to lower trypsin efficiency.
2. Verify that pH is about 8.0 using pH strips as in section 4.3.
3. Add trypsin to histone samples at a ratio of 1:20; e.g. 5 µg of trypsin for 100 µg of histones.

Technical Note. There is no need to repeat Bradford assay at this point, continue as if no loss has occurred.

4. Incubate at 37°C for 12 h or overnight.
5. Stop the digestion by adding 100% formic acid for a final concentration of 5%. Verify if the final pH is 2.0.
6. Dry down the sample to 5–10 µl in a SpeedVac centrifuge.

Technical Note. Sample can be stored at -80°C .

7. Continue with propionylation of histone peptides at N-termini after trypsin digestion (section 4.5).

4.5. Propionylation of histone peptides at N-termini after trypsin digestion

This section is to derivatize peptide N-termini. This procedure is not essential for most histone peptides, but it facilitates the nanoLC retention of the shortest ones (e.g. amino acids 3–8 in histone H3), as the propionyl group increases peptide hydrophobicity.

It is also possible to differentially derivatize the peptide N-termini of two samples with light and heavy propionic anhydride (Sigma-Aldrich; C/D/N isotopes). One sample can be modified with a D0 propionic anhydride (CH₃CH₂CO)₂O, while the other with a D10 propionic anhydride (CD₃CD₂CO)₂O. This leads to a delta mass between the light and heavy labeled peptides of +5.0 Da and multiplexing analysis can be performed. This delta mass is sufficient to discriminate the light and the heavy peptide at the full MS level.

Derivatization with heavy propionic anhydride can be performed at the lysine side chains as well (section 4.3.2). However, this leads to a different mass shift for each peptide, according to the number of lysine residues. The two samples should be mixed in equal amounts to obtain the least variation in ionization efficiency. The procedure to extract the area of heavy labeled peptides is the same as the light version.

4.5.1 Materials and Reagents (see section 4.3.1)

4.5.2. Procedure for propionylation of histone peptides at N-termini after trypsin digestion

1. Re-suspend samples in 30 μ l of 100 mM NH_4HCO_3 .
2. Repeat Steps 2–12 of section 4.3.2. If light and heavy anhydride will be used, perform propionylation with the light form in one sample, and with the heavy form in the other sample.
3. Re-suspend or dilute samples with 50-100 μ l ddH₂O + 0.1% TFA. If propionylation with light and heavy anhydride was performed, samples can now be mixed together.

Technical Note. Sample can be stored at -80°C .

4. If enrichment of phosphorylated peptides is desired, skip to TiO₂ batch mode enrichment of phosphopeptides (section 5). Otherwise, follow to desalting on Reversed-Phase (RP) Columns prior to nanoLC-MS/MS analysis (section 4.6).

4.6. Desalting/Concentration the Peptide Mixture on Reversed-Phase (RP) Columns

The protocol we describe above leads to the presence of salts in the sample at this stage of the preparation. Salts are detrimental for nanoLC-MS analysis. Firstly, ionized salts are injected into the mass spectrometer, contributing to suppression of the peptide signal and contamination of the instrument. Moreover, salts may form ionic adducts with peptides, reducing the signal intensity of the analyte without adducts. Overall, this prevents efficient identification and quantification of peptides. Desalting can be performed offline with Stage-tips or online when the nanoLC-MS setup consists of a two-column system. However, we recommend offline desalting, as it prevents eventual clogging of the nanoLC.

4.6.1. Materials and Reagents

1. POROS Oligo R3 reversed phase material (Applied Biosystems, Foster City, CA, US).
2. P200 and P1000 pipette tips.
3. 3M Empore C18 disk (3M Bioanalytical Technologies, St. Paul, MN, US).
4. Syringe for nanoLC loading (P/N 038250, N25/500-LC PKT 5, SGE, Ringwood, Victoria, Australia).
5. Plastic syringe of 1mL or 5mL (4606051V, B. Braun Medical Inc., US) to create the pressure of the micro-columns.

6. RP loading buffer: 0.1% TFA.
7. RP elution buffer 1: 60% ACN, 0.1% TFA.
8. RP elution buffer 2: 75% ACN, 0.1% TFA.

4.6.2. Protocol for desalting/concentration the peptide mixture on reversed-phase (RP) columns

1. By using a P1000 pipette tip, cut a disk of C18 material from a 3 M Empore™ Solid Phase Extraction Disk C18 and deposit this minidisk to the bottom of a P200 pipette tip. You can push the minidisk out of the P1000 tip by using a fused silica capillary. Ensure that the disk is securely wedged in the bottom of the tip. Alternatively, use Syringe for nanoLC loading to create a small plug of C18 membrane material.
2. Pack 1-2 cm column with R3 material slurry in 100% ACN by applying air pressure using a syringe.

Technical Note. Use 2 cm column if sample contains more than 10 µg of material.

3. Equilibrate the column with 50 - 80 µl of 0.1% TFA.
4. Load the acidified sample (pH< 2.0) into the column and make a gently air pressure. It is important to perform this step slowly. If the sample has dried, add 80 µl of 0.1% TFA to reconstitute the sample prior to loading. Collect the flow through (FT).
5. Optional: pass the FT through the micro-column a second time to increase the binding of peptides.
6. Wash the column with 80 µl of 0.1% TFA.
7. Elute the peptides into a new low-binding eppendorf tube with 80 µl of 60% ACN/0.1% TFA.
8. Repeat step 6 using 50 µl of 75% ACN/0.1% TFA
9. Lyophilize sample using SpeedVac. Make sure samples are completely dried as the presence of ACN may prevent column binding during nanoLC-MS analysis.

5. Titanium Dioxide (TiO₂) Batch Mode Enrichment of Phosphopeptides (optional)

Histone phosphorylation mainly occurs on serine, threonine and tyrosine residues. Phosphorylation of these amino acid residues is known to be acid-stable (O-phosphorylation). Titanium Dioxide (TiO₂) enrichment is based on the selective interaction of water-soluble phosphates with porous TiO₂ microspheres via binding to the TiO₂ surface. Phosphopeptides bind to the TiO₂ beads under acidic conditions and elute under alkaline conditions. Thus, extraction (H₂SO₄) and precipitation (TCA) of phosphorylated histone proteins in acidic conditions followed by TiO₂ pull-down protocol is an excellent method

for enrichment and identification of O-phosphorylated type of histone peptides. The TiO₂ enrichment protocol was adapted from *K. Engholm-Keller. et al., 2012 [35]*.

5.1. Materials and Reagents

1. Titanium dioxide (TiO₂) beads (Titansphere, 5 µm, GL Sciences Inc., Japan).
2. Low-binding 1.5mL eppendorf tubes (Sorenson BioScience Inc., Utah, US).
3. TiO₂ loading buffer: 80% ACN, 5% trifluoroacetic acid (TFA) and 1 M glycolic acid.
4. TiO₂ washing buffer 1: 80% ACN, 1% TFA.
5. TiO₂ washing buffer 2: 10% ACN, 0.1% TFA.
6. TiO₂ elution buffer: 1.5% ammonium hydroxide (pH 11.3), always prepare fresh solution.
7. 3M Empore C8 disk (3M Bioanalytical Technologies, St. Paul, MN, US).

5.2. Procedure for enrichment for phosphorylated peptides using Titanium Dioxide (TiO₂)

1. Transfer 1.5 mg of TiO₂ beads (for 250 µg of starting material) to a new low-binding eppendorf tube.

Technical Note. To reduce nonspecific binding to the TiO₂ resin, it is important to adjust the amount of TiO₂ beads to the amount of sample. The optimal quantity is 0.6 mg TiO₂ beads per 100 µg peptide solution [35].

2. Dilute the histone peptide sample at least 10 times in TiO₂ loading buffer (v/v) and transfer to the eppendorf tube with the TiO₂ beads. Alternatively, adjust the sample volume to achieve the proper loading buffer concentration, for example, for 100 µl sample add 50 µl water, 50 µl of 100% TFA, 800 µl of 100% ACN and 76 mg of glycolic acid.

Technical Note. If the sample has been lyophilized, it is crucial to reconstitute the sample in a small volume of 0.1% TFA. The TiO₂ loading buffer should be added slowly to avoid peptide precipitation.

3. Incubate the sample in the vortex shaker for 10 min at RT. Centrifuge to pellet the beads using a tabletop centrifuge for 15 sec.
4. Transfer the supernatant to a fresh low-binding 1.5mL eppendorf tube containing half of the amount of the TiO₂ beads used in the first round. Repeat the incubation as described in step 3 to increase the yield of phosphorylated peptides.
5. Save the supernatant (TiO₂-FT). This can be used to analyze non-phosphorylated peptides.
6. Pool the TiO₂ beads from the two incubations using 100 µl of TiO₂ loading buffer and transfer to a new low-binding 1.5mL eppendorf tube. This step is

performed to avoid contamination with unmodified/unphosphorylated peptides that may bind to the eppendorf tube surface.

7. Vortex the sample for 10 sec and then pellet the beads using a tabletop centrifuge. Remove supernatant and pool with the TiO₂-FT.
7. Add 70-100 µl of TiO₂ washing buffer 1, vortex for 15 sec and centrifuge to pellet the beads. This step is performed to remove the contaminant hydrophobic non-modified peptides.
8. Repeat step 7 using 50-100 µl of TiO₂ washing buffer 2 in order to remove the hydrophilic unmodified peptides. Washing buffer 2 is used to remove peptides that bind to TiO₂ exploiting its hydrophilic properties.
9. Dry the beads for 10 min in the SpeedVac centrifuge or leave the tube open on the bench.

Technical Note. It is very important to dry the TiO₂ beads. If some liquid remains, it is necessary to check and adjust the pH to 11.3 in the next step.

10. Elute the phosphopeptides by adding 100-150 µl of TiO₂ elution buffer to the beads. Vortex and incubate the solution in the vortex shaker for 10 min to allow efficient elution.
11. Centrifuge the solution for 1 min and pass the supernatant over a filter (C8 stage tip), plugged into a P200 tip as described in section 4.6.2, to avoid the presence of TiO₂ beads in the solution. Collect the flow through into a new low-binding 1.5mL eppendorf tube.
12. Wash the TiO₂ beads by shaking at RT with 30 µl of elution buffer for 5 min. Then pass the eluate over the C8 filter and pool with the other TiO₂ eluate. To recover any peptide that bound to the C8 filter add 5 µl of 30% ACN to the C8 stage tip, pass this solution through and combine with the TiO₂ eluates.
13. Lyophilize the eluted peptides. Any remnant of ammonia can interfere with the subsequent steps.
14. Additionally lyophilize the TiO₂-FT. In the case of a low amount of material, the TiO₂-FT can be used to analyze non-phosphorylated histone peptides.

5.3. Desalting/Concentration of the TiO₂-FT on HLB/RP Cartridge

The FT from the first TiO₂ may be used for further analysis of other histone PTMs such as acetylation or methylation. The choice between POROS Oligo R3 or Oasis HLB (Hydrophilic Lipophilic Balanced) cartridge depends on the quantity and quality of material. For peptide samples with quantity higher than or equal to 500 µg, the Oasis HLB cartridge is often the best choice. It is recommended to desalt the FT from TiO₂ beads due to the presence of glycolic acid in the sample, which may interfere with further nanoLC-MS/MS analysis.

5.3.1. Materials and Reagents

1. 100 % methanol.
2. 100% acetonitrile (ACN).
3. RP loading buffer: 0.1% TFA.
4. RP elution buffer 1: 60% ACN, 0.1% TFA.
5. RP elution buffer 2: 75% ACN, 0.1% TFA.
6. Oasis HLB cartridges (Waters, Milford, MA, US).
7. Plastic syringe of 1mL or 5mL (4606051V, B. Braun Medical Inc., US) to create the pressure of the Oasis HLB cartridges

5.3.2. Procedure for desalting/concentration of the TiO₂-FT on HLB/RP Cartridge

1. Add 0.1% TFA to the lyophilized TiO₂-FT sample to achieve approximately 1mL of final volume and adjust the pH to 2.0.
3. Activate the HLB cartridge with 1mL of 100% methanol followed by 1mL of 100% ACN.
4. Equilibrate the cartridge twice with 2 mL of 0.1% TFA.
5. Load the sample onto the HLB cartridge slowly and collect the FT.
6. Pass the FT through the same HLB cartridge a second time.
6. Wash the cartridge twice with 2mL of 0.1% TFA.
7. Elute the peptides in a new low-binding eppendorf tube with 1mL of 60% ACN/ 0.1% TFA and lyophilize the sample.
8. Reconstitute the sample in 100 µl of 0.1% TFA.
9. Take an aliquot for AAA to determine peptide concentration, optionally (section 4.2).

6. Identification and relative quantification of histone PTMs - nanoLC-MS/MS analysis

Histone peptides are extensively modified and therefore unusual in proteomics analysis. Because of this, histone peptides are not ideal for traditional database searching. Enabling many dynamic PTMs in the software search results in a large number of possible candidates, which may lead to an increase in the false discovery rate estimation, and thus in either low sensitivity of the identification or even false positives. If using this approach, database searching engines equipped with a PTM localization score should be used as the large number of modifiable sites on histone peptides as well as incomplete fragmentation easily leads to erroneous allocation of a modification position. High mass accuracy and previous knowledge about retention time assists quantification, which is performed by extracting the ion chromatogram (**Figure 3A**). High mass accuracy is particularly important as some

histone modifications, such as trimethylation (42.047 Da) and acetylation (42.011 Da), are nearly isobaric. Moreover, such peptides can be present in a variety of completely isobaric forms; e.g. the peptide of histone H3 aa 9-17 can be modified at H3K9ac and H3K14ac. This demonstrates that the intact mass of the peptide is not always sufficient to discriminate two peptides. Because of this, we recommend targeted MS/MS analysis for peptides that are known to have isobaric forms (**Figure 3B**) or adopt data-independent acquisition methods [36]. In this section we describe an approach for histone PTM analysis of samples obtained after section 4.6 (before TiO₂ enrichment of phosphorylated peptides). The same protocol can be applied to the samples enriched on TiO₂ beads and TiO₂-FT.

6.1. nanoLC- MS Analysis and Data search

1. Prepare the nanoLC using C18 chromatography, possibly using nanoflows to increase the sensitivity of the analysis. Use a gradient of 30-60 min from 0 to 30% buffer B (Buffer A: 0.1% formic acid, Buffer B: 95% can, 0.1% formic acid).
2. Program the MS acquisition method using high resolution at least for the Full MS event. Include targeted MS/MS for species with isobaric forms. Include at least 3-4 data-dependent MS/MS events at each scan cycle, as they may be necessary to identify the correct MS peak of the peptide of interest.
3. Perform nanoLC-MS analysis. Aside from the list of targeted masses of the isobaric peptides, all other MS settings should be kept as traditional settings for proteomics analysis.
4. Acquired data should be analyzed either manually or using automated software (we recommend Skyline [37]) to extract the ion chromatogram. The same peptide can be present in differently modified forms. All peptide masses should be calculated prior to extracting the ion chromatogram. As lysine residues were derivatized using propionic anhydride, no lysine residue should be unmodified. Considering only the most common PTMs a lysine residue could be either acetylated (42.011 Da), trimethylated (42.047 Da), dimethylated (28.031 Da), unmodified (56.026 Da, corresponding to the mass of the propionylation) or monomethylated (70.042 Da, corresponding to the mass of the monomethylation + propionylation) (**Figure 3A**).

Technical Note. Lysine acetylation can be distinguished from trimethylation by taking hydrophobicity into account. Acetylation is more hydrophobic than trimethylation, therefore, the acetylated peptide has higher retention time and elutes later than the trimethylated form. The unmodified form of the same peptide elutes last due to the propionyl group added to the lysine. In summary, the order of hydrophobicity for a peptide with one modifiable lysine is di- and trimethylated < acetylated < unmodified (propionylated) < monomethylated (propionylated).

5. In the case of isobaric peptides, an additional layer of information is required: the MS/MS spectrum. All peptides that carry more than one site modifiable by the same PTM should be considered as potentially isobaric. For instance, the peptide of histone H3 KSTGGKAPR (aa 9-17) can carry an acetylation on both K9 and K14 residues; the mass of the peptide + 1 acetyl group should be included in the list of the targeted peptides. Once the extracted ion

chromatogram of the given precursor mass is extracted, the ratio of the unique fragment ions for each of the two species (K9ac and K14ac) should be calculated. This ratio should be used to divide the peak area generated by the precursor MS scan between the two species (**Figure 3B**).

6. Once all the peak areas have been extracted, calculate the relative abundance of each PTM by calculating the sum of all different modified forms of a histone peptide (100%), and divide the area of the particular peptide by the total histone peptide areas.

7. Data Analysis

The relative percentage of a histone PTM, calculated as described in the previous section, is a good indication of the actual PTM occupancy on the histone protein. However, due to biases in ionization efficiency, some PTMs may be over- or underestimated by MS compared to their actual abundances [38,39]. Because of this, we recommend using the obtained relative abundance to compare the changes of a PTM between sample conditions, rather than estimating its stoichiometry as compared to other modified forms. We performed an analysis of histones isolated from human IMR90 fibroblast cells infected with adenovirus. The dataset described was acquired using a nanoLC-MS setup consisting on an EasyLC (Thermo Scientific) coupled with an Orbitrap Fusion or Q Exactive MS (Thermo Scientific). The EasyLC was equipped with a picofrit nanocolumn directly interfaced with the MS using an NSI source (nano electrospray). Data were visualized in Thermo Xcalibur Qual Browser and peptide quantification was extracted using our in-house software EpiProfile [40]. Results are reported in **Table 1**. Analysis of multiple conditions can be performed using plots such as bar plots (**Figure 4A** and **5A-B**), which clearly highlight the relative abundance of individual peptides in different conditions. Alternatively, volcano plots (**Figure 4B**) can be used which include the statistical *p*-value in the comparison, assisting in the interpretation of which changes are statistically significant. Finally, heatmaps (**Figure 4C**) are also particularly useful when multiple conditions are analyzed as they represent the trend of a given peptide across the conditions and allow for clustering of common trends. We represent examples of such graphs using the results obtained from our analysis (**Table 1**).

8. Insight into histone phosphorylation sites

To date, a large number of phosphorylated residues on histones have been reported, but additional phosphorylation sites are still being identified. Known functions of histone phosphorylation take place during processes such as mitosis, meiosis, transcription, chromatin remodeling, cellular response to DNA damage, DNA repair, or apoptosis. The abundance of specific histone phosphorylation marks may vary dramatically between interphase and mitosis during the cell cycle [41]. Moreover, it is likely that individual histone phosphorylation marks show different half-lives [42]. The labile nature of phospho-sites and the relatively low abundance of most histone phosphorylation marks make them difficult to detect by MS. In the above protocol we performed TiO₂ chromatography to enrich phosphorylated histone peptides from a low amount of starting material (for ~50 µg of purified histones). **Table 2** shows examples of identified histone phosphorylation sites

altered upon adenoviral infection. Our analysis revealed phosphorylation sites mostly at the N-terminal region of histones. Single phospho-histone marks we detected include H1.2S35ph, H3.3S28ph, or H3S10/T11ph, which are involved in processes such as chromatin compaction and remodeling, heterochromatin formation, transcription or the DNA damage response. Phosphorylation of H3S10, T11 and S28 has been shown to be clearly associated with H3 acetylation, strongly implicating these modifications in transcription activation [43]. In our study, a number of phospho-marks were seen in combination with neighboring lysine acetylation and/or methylation such as H3K9me1S10phK14ac, H3K9me2S10phK14ac or H3K9me3S10phK14ac, which supports the hypothesis of crosstalk between histone marks. Both, H3K9me2S10phK14ac and H3K9me3S10phK14ac showed a slight increase (~1.08-1.3 fold change) from 0 to 24hpi, and subsequently decrease (~2.4- 33.2 fold change) from 24 to 48hpi. These phosphorylation changes are consistent with a shift into S-phase, a known effect of adenoviral infection [44-46], compared to mock infected cells. In addition, H3K9me3S10ph and H3K9me2S10ph were decreased more than two-fold upon viral infection. In contrast, H1.2S35ph showed a steady increase (5-fold change) from 0 to 48hpi. Histone H1 subtypes are post-translationally modified, primarily by phosphorylation at multiple sites. The significance of this modification is unclear, but is believed to reduce the affinity of histone H1 for chromatin [47,48]. Indeed, Chu CS. *et al.*, reported that histone H1.4 phosphorylation at S35, a variant of H1.2, seems to be necessary for maintaining proper mitotic chromatin structure such as chromatin decondensation [49]. Viruses have been shown to increase nuclear size and result in chromatin decondensation [50,51], these effects are consistent with the changes in H1 phosphorylation induced by adenovirus we observe here.

9. Discussion

Our results show a number of changes in histone modifications over the course of adenovirus infection. We also find that some histone modifications that do not change significantly during infection, such as most acetylation marks on histone H4 (**Figure 4A**). We find combinations of modifications that are decreased at 24hpi and then increased at 48hpi, such as H3K9me2 and 3 (**Figure 4, Table 1**). Hypermethylated H3K9 is known to characterize condensed heterochromatin, and thus gene inactivation [52]. Conversely, we also find combinations of marks that are increased at 24hpi and then decreased at 48hpi, such as H3.3K36me2 and H3.3K27meK36me2 (**Figure 4, Table 1**). K36me2 is a histone mark that occupies gene bodies of active genes. The final decrease of this PTM would be in agreement with the increase of H3K9me3, as these two marks are known to have opposite functions. Overall, these fluctuations likely reflect dynamic changes to the chromatin state affected by many viral proteins such as E1A, discussed below, or other viral proteins that localize to cellular chromatin.

Recent studies into the function of adenoviral small E1A showed that this protein is sufficient to causes changes in histone modifications, such as a decrease in H3K18ac and an increase in all three methylation states of H3K79 [22]. In contrast, we find that wild-type adenovirus infection leads to overall increase in H3K18ac, a decrease in H3K79me2, but an increase in H3K79me1 over the two time points tested (**Figure 4, Table 2**). Wild-type adenovirus expresses many proteins, in addition to E1A, which may regulate multiple

cellular chromatin factors resulting in these differences. Moreover, the methylation state of H3K79 may be dependent on other PTMs in the local chromatin region. Recent work from the Garcia lab found that knockdown of the H3K79 methyltransferase DOT1L did not affect adenoviral titers [11], suggesting methylation of this mark is not essential for progeny production. It will be interesting to determine the fluctuations in histone PTM patterns produced upon infection with mutant viruses lacking individual viral proteins.

Many viruses, including adenovirus, are known to arrest cells in S phase of the cell cycle to facilitate viral replication [44,53-56]. Consistent with this, we find a decrease in H3S10 phosphorylation at 24 and 48 hpi in any combination with other histone marks (**Table 2**). Interestingly, we find a dramatic decrease in H4K20 methylation during infection (**Figure 4, Table 2**). This mark has been linked to genome stability [57] and may reflect fundamental changes to cellular chromatin that allow viral hijacking. Further studies will address the role of the enzymes responsible for this mark during adenoviral infection.

In summary, this protocol describes an efficient method for identifying and characterizing histone PTM patterns in infected cells. We provide a method for isolating, modifying and analyzing histone PTMs as well as enriching phosphorylated histone peptides and suggest formats for data analysis.

Acknowledgments

We thank Simone Sidoli for valuable insight and members of the Garcia and Weitzman labs for assistance. This work was supported in part by a Pilot Grant from the Institute for Immunology of the University of Pennsylvania to MDW and BAG, as well as NIH grants to MDW (CA 0970930) and BAG (AI118891 and GM110174). DA is supported in part by T32 CA 115299.

References cited

1. Huang H, Sabari BR, Garcia BA, Allis CD, Zhao Y. SnapShot: histone modifications. *Cell*. 2014; 159:458–458. e1. doi:10.1016/j.cell.2014.09.037. [PubMed: 25303536]
2. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007; 128:693–705. doi:10.1016/j.cell.2007.02.005. [PubMed: 17320507]
3. Shilatifard A. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. *Annu. Rev. Biochem.* 2006; 75:243–269. doi:10.1146/annurev.biochem.75.103004.142422. [PubMed: 16756492]
4. Smith E, Shilatifard A. The chromatin signaling pathway: diverse mechanisms of recruitment of histone-modifying enzymes and varied biological outcomes. *Molecular Cell*. 2010; 40:689–701. doi:10.1016/j.molcel.2010.11.031. [PubMed: 21145479]
5. Rando OJ, Chang HY. Genome-wide views of chromatin structure. *Annu. Rev. Biochem.* 2009; 78:245–271. doi:10.1146/annurev.biochem.78.071107.134639. [PubMed: 19317649]
6. Corpet A, Almouzni G. A histone code for the DNA damage response in mammalian cells? *Embo J*. 2009; 28:1828–1830. doi:10.1038/emboj.2009.180. [PubMed: 19587679]
7. Polo SE, Jackson SP. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev*. 2011; 25:409–433. doi:10.1101/gad.2021311. [PubMed: 21363960]
8. Xu D, Bai J, Duan Q, Costa M, Dai W. Covalent modifications of histones during mitosis and meiosis. *Cell Cycle*. 2009; 8:3688–3694. [PubMed: 19855177]
9. Portela A, Esteller M. Epigenetic modifications and human disease. *Nat. Biotechnol.* 2010; 28:1057–1068. doi:10.1038/nbt.1685. [PubMed: 20944598]

10. Chi P, Allis CD, Wang GG. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. *Nat. Rev. Cancer*. 2010; 10:457–469. doi:10.1038/nrc2876. [PubMed: 20574448]
11. O'Connor CM, Dimaggio PA, Shenk T, Garcia BA. Quantitative proteomic discovery of dynamic epigenome changes that control human cytomegalovirus (HCMV) infection. *Molecular & Cellular Proteomics*. 2014; 13:2399–2410. doi:10.1074/mcp.M114.039792. [PubMed: 24987098]
12. Terhune SS, Moorman NJ, Cristea IM, Savaryn JP, Cuevas-Bennett C, Rout MP, et al. Human cytomegalovirus UL29/28 protein interacts with components of the NuRD complex which promote accumulation of immediate-early RNA. *PLoS Pathog*. 2010; 6:e1000965. doi:10.1371/journal.ppat.1000965. [PubMed: 20585571]
13. Britton L-MP, Sova P, Belisle S, Liu S, Chan EY, Katze MG, et al. A proteomic glimpse into the initial global epigenetic changes during HIV infection. *Proteomics*. 2014; 14:2226–2230. doi: 10.1002/pmic.201400116. [PubMed: 25116026]
14. Komatsu T, Nagata K. Replication-uncoupled histone deposition during adenovirus DNA replication. *Journal of Virology*. 2012; 86:6701–6711. doi:10.1128/JVI.00380-12. [PubMed: 22496236]
15. Knipe DM, Lieberman PM, Jung JU, McBride AA, Morris KV, Ott M, et al. Snapshots: chromatin control of viral infection. *Virology*. 2013; 435:141–156. doi:10.1016/j.virol.2012.09.023. [PubMed: 23217624]
16. Wong CM, McFall ER, Burns JK, Parks RJ. The role of chromatin in adenoviral vector function. *Viruses*. 2013; 5:1500–1515. doi:10.3390/v5061500. [PubMed: 23771241]
17. Placek BJ, Huang J, Kent JR, Dorsey J, Rice L, Fraser NW, et al. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. *Journal of Virology*. 2009; 83:1416–1421. doi:10.1128/JVI.01276-08. [PubMed: 19004946]
18. Lieberman PM. Keeping it quiet: chromatin control of gammaherpesvirus latency. *Nature Reviews Microbiology*. 2013; 11:863–875. doi:10.1038/nrmicro3135. [PubMed: 24192651]
19. Fonseca GJ, Thillainadesan G, Yousef AF, Ablack JN, Mossman KL, Torchia J, et al. Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell Host and Microbe*. 2012; 11:597–606. doi:10.1016/j.chom.2012.05.005. [PubMed: 22704620]
20. Fonseca GJ, Cohen MJ, Nichols AC, Barrett JW, Mymryk JS. Viral retasking of hBrel/RNF20 to recruit hPaf1 for transcriptional activation. *PLoS Pathog*. 2013; 9:e1003411. doi:10.1371/journal.ppat.1003411. [PubMed: 23785282]
21. Chaurushiya MS, Lilley CE, Aslanian A, Meisenhelder J, Scott DC, Landry S, et al. Viral E3 ubiquitin ligase-mediated degradation of a cellular E3: viral mimicry of a cellular phosphorylation mark targets the RNF8 FHA domain. *Molecular Cell*. 2012; 46:79–90. doi:10.1016/j.molcel.2012.02.004. [PubMed: 22405594]
22. Horwitz GA, Zhang K, McBrien MA, Grunstein M, Kurdistani SK, Berk AJ. Adenovirus small e1a alters global patterns of histone modification. *Science*. 2008; 321:1084–1085. doi:10.1126/science.1155544. [PubMed: 18719283]
23. Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ, Kurdistani SK. Epigenetic reprogramming by adenovirus e1a. *Science*. 2008; 321:1086–1088. doi:10.1126/science.1155546. [PubMed: 18719284]
24. Young NL, Dimaggio PA, Garcia BA. The significance, development and progress of high-throughput combinatorial histone code analysis. *Cell. Mol. Life Sci*. 2010; 67:3983–4000. doi: 10.1007/s00018-010-0475-7. [PubMed: 20683756]
25. Sidoli S, Cheng L, Jensen ON. Proteomics in chromatin biology and epigenetics: Elucidation of post-translational modifications of histone proteins by mass spectrometry. *J Proteomics*. 2012; 75:3419–3433. doi:10.1016/j.jprot.2011.12.029. [PubMed: 22234360]
26. Pinkse MWH, Uitto PM, Hilhorst MJ, Ooms B, Heck AJR. Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal. Chem*. 2004; 76:3935–3943. doi:10.1021/ac0498617. [PubMed: 15253627]

27. Kuroda I, Shintani Y, Motokawa M, Abe S, Furuno M. Phosphopeptide-selective column-switching RP-HPLC with a titania precolumn. *Anal Sci*. 2004; 20:1313–1319. [PubMed: 15478342]
28. Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jørgensen TJD. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell Proteomics*. 2005; 4:873–886. doi:10.1074/mcp.T500007-MCP200. [PubMed: 15858219]
29. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: a critical review. *Anal Bioanal Chem*. 2007; 389:1017–1031. doi:10.1007/s00216-007-1486-6. [PubMed: 17668192]
30. Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell Proteomics*. 2002; 1:376–386. [PubMed: 12118079]
31. Shechter D, Dormann HL, Allis CD, Hake SB. Extraction, purification and analysis of histones. *Nature Protocols*. 2007; 2:1445–1457. doi:10.1038/nprot.2007.202. [PubMed: 17545981]
32. Rodriguez-Collazo P, Leuba S, Karahanian E, Zlatanova J. Salt-urea, sulfopropyl-sepharose, and covalent chromatography methods for histone isolation and fractionation. *Methods Mol. Biol*. 2014; 1094:295–307. doi:10.1007/978-1-62703-706-8_23. [PubMed: 24162997]
33. Lin S, Garcia BA. Examining histone posttranslational modification patterns by high-resolution mass spectrometry. *Meth. Enzymol*. 2012; 512:3–28. doi:10.1016/B978-0-12-391940-3.00001-9. [PubMed: 22910200]
34. Garcia BA, Mollah S, Ueberheide BM, Busby SA, Muratore TL, Shabanowitz J, et al. Chemical derivatization of histones for facilitated analysis by mass spectrometry. *Nature Protocols*. 2007; 2:933–938. doi:10.1038/nprot.2007.106. [PubMed: 17446892]
35. Engholm-Keller K, Birck P, Størling J, Pociot F, Mandrup-Poulsen T, Larsen MR. TiSH--a robust and sensitive global phosphoproteomics strategy employing a combination of TiO₂, SIMAC, and HILIC. *J Proteomics*. 2012; 75:5749–5761. doi:10.1016/j.jprot.2012.08.007. [PubMed: 22906719]
36. Sidoli S, Lin S, Xiong L, Bhanu NV, Karch KR, Johansen E, et al. SWATH Analysis for Characterization and Quantification of Histone Post-translational Modifications. *Molecular & Cellular Proteomics*. 2015 doi:10.1074/mcp.O114.046102.
37. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010; 26:966–968. doi:10.1093/bioinformatics/btq054. [PubMed: 20147306]
38. Lin S, Wein S, Gonzales-Cope M, Otte GL, Yuan Z-F, Afjehi-Sadat L, et al. Stable-isotope-labeled histone peptide library for histone post-translational modification and variant quantification by mass spectrometry. *Molecular & Cellular Proteomics*. 2014; 13:2450–2466. doi:10.1074/mcp.O113.036459. [PubMed: 25000943]
39. Sidoli S, Lin S, Karch KR, Garcia BA. Bottom-Up and Middle-Down Proteomics Have Comparable Accuracies in Defining Histone Post-Translational Modification Relative Abundance and Stoichiometry. *Anal. Chem*. 2015 doi:10.1021/acs.analchem.5b00072.
40. Yuan Z-F, Lin S, Molden RC, Cao X-J, Bhanu NV, Wang X, et al. EpiProfile quantifies histone modifications by extracting retention time and intensity in high-resolution mass spectra. *Molecular & Cellular Proteomics*. 2015 doi:10.1074/mcp.M114.046011.
41. Fischle W, Tseng BS, Dormann HL, Ueberheide BM, Garcia BA, Shabanowitz J, et al. Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature*. 2005; 438:1116–1122. doi:10.1038/nature04219. [PubMed: 16222246]
42. Sawicka A, Seiser C. Sensing core histone phosphorylation - a matter of perfect timing. *Biochim. Biophys. Acta*. 2014; 1839:711–718. doi:10.1016/j.bbagr.2014.04.013. [PubMed: 24747175]
43. Rossetto D, Avvakumov N, Côté J. Histone phosphorylation: a chromatin modification involved in diverse nuclear events. *Epigenetics*. 2012; 7:1098–1108. doi:10.4161/epi.21975. [PubMed: 22948226]
44. Cheetham BF, Bellett AJ. A biochemical investigation of the adenovirus-induced G1 to S phase progression: thymidine kinase, ornithine decarboxylase, and inhibitors of polyamine biosynthesis. *J. Cell. Physiol*. 1982; 110:114–122. doi:10.1002/jcp.1041100203. [PubMed: 7068769]

45. Grand RJ, Ibrahim AP, Taylor AM, Milner AE, Gregory CD, Gallimore PH, et al. Human cells arrest in S phase in response to adenovirus 12 E1A. *Virology*. 1998; 244:330–342. doi:10.1006/viro.1998.9102. [PubMed: 9601504]
46. Berk AJ. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene*. 2005; 24:7673–7685. doi:10.1038/sj.onc.1209040. [PubMed: 16299528]
47. Roth SY, Allis CD. Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends in Biochemical Sciences*. 1992; 17:93–98. [PubMed: 1412698]
48. Happel N, Doenecke D. Histone H1 and its isoforms: contribution to chromatin structure and function. *Gene*. 2009; 431:1–12. doi:10.1016/j.gene.2008.11.003. [PubMed: 19059319]
49. Chu C-S, Hsu P-H, Lo P-W, Scheer E, Tora L, Tsai H-J, et al. Protein kinase A-mediated serine 35 phosphorylation dissociates histone H1.4 from mitotic chromosome. *Journal of Biological Chemistry*. 2011; 286:35843–35851. doi:10.1074/jbc.M111.228064. [PubMed: 21852232]
50. Simpson-Holley M, Colgrove RC, Nalepa G, Harper JW, Knipe DM. Identification and functional evaluation of cellular and viral factors involved in the alteration of nuclear architecture during herpes simplex virus 1 infection. *Journal of Virology*. 2005; 79:12840–12851. doi:10.1128/JVI.79.20.12840-12851.2005. [PubMed: 16188986]
51. Lee S-B, Lee C-F, Ou DS-C, Dulal K, Chang L-H, Ma C-H, et al. Host-viral effects of chromatin assembly factor 1 interaction with HCMV IE2. *Nature Publishing Group*. 2011; 21:1230–1247. doi:10.1038/cr.2011.53.
52. Huang H, Lin S, Garcia BA, Zhao Y. Quantitative proteomic analysis of histone modifications. *Chem. Rev*. 2015; 115:2376–2418. doi:10.1021/cr500491u. [PubMed: 25688442]
53. Bain M, Sinclair J. The S phase of the cell cycle and its perturbation by human cytomegalovirus. *Rev. Med. Virol*. 2007; 17:423–434. doi:10.1002/rmv.551. [PubMed: 17676653]
54. Li G, Park HU, Liang D, Zhao RY. Cell cycle G2/M arrest through an S phase-dependent mechanism by HIV-1 viral protein R. *Retrovirology*. 2010; 7:59. doi:10.1186/1742-4690-7-59. [PubMed: 20609246]
55. Kudoh A, Daikoku T, Sugaya Y, Isomura H, Fujita M, Kiyono T, et al. Inhibition of S-phase cyclin-dependent kinase activity blocks expression of Epstein-Barr virus immediate-early and early genes, preventing viral lytic replication. *Journal of Virology*. 2004; 78:104–115. [PubMed: 14671092]
56. Knipe, DM.; Howley, P. *Fields Virology*. Lippincott Williams & Wilkins; 2013.
57. Jørgensen S, Schotta G, Sørensen CS. Histone H4 lysine 20 methylation: key player in epigenetic regulation of genomic integrity. *Nucleic Acids Research*. 2013; 41:2797–2806. doi:10.1093/nar/gkt012. [PubMed: 23345616]

Highlights

- We investigate histone post-translational modifications during adenovirus infection.
- We describe a detailed method for infection, histone enrichment, and mass spectrometry analysis.
- We provide alternative approaches where relevant for enrichment or analysis.
- Adenovirus infection leads to increases in some histone marks and decreases in others.
- We describe histone phosphopeptide enrichment using titanium dioxide (TiO₂) chromatography.

Figure 1. Workflow for analysis of changes in histone PTMs upon viral infection

(1) Cells are grown in normal media or media supplemented with “light” and “heavy” forms of amino acids for SILAC labeling. (2-3) Cells are infected with the virus of choice and subsequently harvested at different time points of infection. (4-5) After cell harvesting, intact nuclei are extracted, followed by acid precipitation (TCA) (or high-salt extraction) of nuclear proteins. (6) The yield of histone extraction can be verified by using protein quantification methods such as Bradford or AAA; the purity of the sample can be analyzed by SDS-PAGE gel electrophoresis. (7) When the histone amount is sufficient (100-200 µg), it is possible to fractionate the different histone variants by RP-HPLC. (8-9) The different fractions or the crude histone extract (in the case of low sample amounts) are propionylated and subsequently digested with trypsin. (10) A second round of propionylation is directed to the N-terminus of tryptic peptides. This step facilitates the nanoLC-MS/MS identification of very short peptides by increasing their hydrophobicity. (11) PTMs such as phosphorylation occur more rarely in histones in comparison with acetylation or methylation. Thus, performance of the TiO₂ protocol at this step may facilitate identification of phosphorylated sites at serine, threonine and tyrosine residues. (12) Removal of salt (and glycolic acid in the case of TiO₂ enrichment) prior to nanoLC-MS/MS analysis may be performed on R3 RP-columns or Oasis HLB cartridge depending of quantity and quality of sample. (13) Identification of histone PTMs by nanoLC-MS/MS. (14) Analysis of the MS data. Steps outlined with a red dotted line are optional.

Figure 2. Acid extraction and RP-HPLC purification of histones and histone variants
(A) Reverse-phase high performance liquid chromatography (RP-HPLC) of histone extract using acid extraction shows peaks corresponding to core histones. Multiple peaks corresponding to histone variants are apparent here. This method can be used to generate purified samples of histone variants for PTM analysis. (B) SDS-PAGE analysis of histones extracted using acid extraction method.

Figure 3. nanoLC-MS and nanoLC-MS/MS chromatograms of histone peptides

(A) Extracted ion chromatogram of the peptide aa 20-23 of histone H4 in its modified forms. The monomethylated form is the one with the highest mass and retention time because both N-terminal peptide and monomethylated lysine residues are propionylated. Di- and trimethylated lysine residues cannot be propionylated, and thus have masses smaller than the unmodified peptide. (B) On the top, LC-MS chromatogram of the histone H3 peptide aa 9-17 modified with one acetyl group. Since both H3K9ac and H3K14ac have the same mass, and thus highly similar chemical properties, the two peptides are almost completely

undistinguishable when observing their MS extracted chromatogram. By extracting the MS/MS fragment ion chromatogram, it is possible to distinguish between the two species, as most fragments are unique for one of the two species (middle and bottom chromatogram).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Histone H3 and H4 PTM changes 24 and 48 hours post infection (hpi)

(A) Bar plot representing the relative abundance of histone H4 most abundant acetylation patterns on K5, K8, K12 and K16. * Represents histone marks significantly different compared to MOCK, calculated by a two-tailed homoscedastic t-test with a p-value <5%. (B) Volcano plot showing the fold change and the statistical significance of histone peptide changes between MOCK and 24 hpi and (C) between MOCK and 48 hpi. Peptides that are statistically different between the two conditions are shown with a green glow. Statistical difference was calculated using a two-tail homoscedastic t-test based on three technical replicates. (D) Heatmap of all quantified peptides, representing the changes in trend between

the three conditions. Peptides with common trends (arbitrary threshold) are marked with common colors on the tree.

Figure 5. Histone H1 and H2A PTM changes 24 and 48 hours post infection

(A-B) Bar plot representing the histone H1 and H2A PTMs significantly altered in relative abundance compared to uninfected cells (MOCK). * Represents histone marks significantly different compared to MOCK, calculated by a two-tailed homoscedastic t-test with a p-value <5% based on three technical replicates.

Table 1

Relative abundance of histone H3 and H4 peptides quantified in MOCK, 24 and 48 hours post infection.

Marks	MOCK	24hpi	48hpi	Marks	MOCK	24hpi	48hpi
H3K4me1	19.07%	15.36%	17.32%	H3.3K27ac	0.12%	0.06%	0.04%
H3K4me2	0.06%	0.06%	0.65%	H3.3K27me1K36me1	0.44%	0.40%	32.21%
H3K4me3	0.07%	0.07%	0.12%	H3.3K27me2K36me1	13.97%	8.94%	14.91%
H3K4ac	0.00%	0.06%	0.02%	H3.3K27me1K36me2	0.00%	15.55%	13.94%
H3K9me1	23.08%	22.21%	21.99%	H3.3K27me2K36me2	0.05%	19.95%	0.06%
H3K9me2	2.96%	0.06%	6.71%	H3.3K27me3K36me1	3.80%	1.93%	2.38%
H3K9me3	8.19%	0.03%	6.88%	H3.3K27me1K36me3	0.00%	4.35%	3.93%
H3K9ac	2.16%	1.34%	1.04%	H3.3K27me3K36me3	0.00%	0.06%	0.00%
H3K9me1K14ac	9.95%	16.62%	13.45%	H3.3K27me3K36me2	0.00%	3.78%	1.00%
H3K9me2K14ac	5.90%	1.51%	9.80%	H3.3K27acK36me1	0.00%	0.01%	0.76%
H3K9me3K14ac	2.43%	4.52%	5.91%	H3.3K27acK36me2	11.03%	0.11%	0.63%
H3K9acK14ac	0.12%	0.13%	0.38%	H3.3K27acK36me3	12.39%	2.75%	4.64%
H3K14ac	20.77%	23.24%	17.36%	H3.3K36me1	0.55%	0.48%	0.12%
H3K23me1	0.02%	0.01%	0.02%	H3.3K36me2	10.72%	21.88%	8.87%
H3K23ac	12.24%	10.81%	19.25%	H3K56ac	0.40%	1.47%	0.20%
H3K18me1	0.02%	0.04%	0.14%	H3K79me1	9.78%	10.71%	18.04%
H3K18ac	3.69%	5.17%	11.73%	H3K79me2	17.33%	18.52%	0.49%
H3K18acK23ac	2.00%	1.30%	0.08%	H3K79me3	0.00%	0.02%	0.00%
H3K36me1	1.05%	0.49%	0.13%	H3K79ac	0.06%	0.11%	0.02%
H3K36me2	0.20%	0.20%	2.71%	H3K122ac	0.01%	0.00%	0.00%
H3K27me1	1.21%	0.63%	0.19%	H4K5ac	0.87%	0.59%	0.73%
H3K27me2	10.20%	6.26%	2.63%	H4K5acK8ac	0.52%	0.42%	0.36%
H3K27me3	19.66%	15.05%	7.96%	H4K5acK8acK12ac	0.29%	0.24%	0.31%
H3K27ac	0.16%	0.03%	0.64%	H4K5acK8acK12acK16ac	0.26%	0.23%	0.75%
H3K27me1K36me1	2.26%	1.66%	32.51%	H4K5acK8acK16ac	0.22%	0.19%	0.28%
H3K27me1K36me2	2.07%	8.12%	7.25%	H4K5acK12ac	0.73%	0.37%	0.47%
H3K27me1K36me3	0.94%	4.64%	2.41%	H4K5acK12acK16ac	0.23%	0.18%	0.20%
H3K27me2K36me1	12.42%	8.12%	8.45%	H4K5acK16ac	0.48%	0.45%	0.34%
H3K27me2K36me2	7.68%	5.77%	12.27%	H4K8ac	1.71%	1.26%	1.28%
H3K27me3K36me1	4.87%	4.64%	2.33%	H4K8acK12ac	0.67%	0.54%	0.48%
H3K27me3K36me2	2.11%	4.38%	3.29%	H4K8acK12acK16ac	0.41%	0.40%	0.63%
H3K27me3K36me3	0.04%	0.10%	0.07%	H4K8acK16ac	2.48%	2.05%	2.01%
H3K27acK36me1	0.00%	0.00%	0.01%	H4K12ac	2.10%	1.99%	2.22%
H3K27acK36me2	2.35%	1.02%	0.58%	H4K12acK16ac	2.44%	2.26%	2.22%
H3K27acK36me3	25.57%	32.28%	13.23%	H4K16ac	21.34%	19.14%	16.64%
H3.3K27me1	0.54%	0.73%	0.16%	H4K20me1	58.47%	59.49%	10.76%
H3.3K27me2	32.25%	7.82%	6.85%	H4K20me2	4.95%	11.92%	8.45%
H3.3K27me3	10.96%	7.32%	7.92%	H4K20me3	0.00%	0.01%	0.00%

Table 2
Examples of phosphorylated histone peptides enriched on TiO₂ beads and their relative abundance quantified in MOCK, 24 and 48 hours post infection

Highly enriched phospho-marks are in bold. The percentage refers to the abundance of the phosphorylated peptide as compared to the detected peptides in the enriched sample. Thus, such percentage does not reflect the actual occupancy of the phosphorylation on the histone protein, but the normalized abundance as compared to the detectable peptides.

Marks	MOCK	24hpi	48hpi	Marks	MOCK	24hpi	48hpi
H1.4K25me1S26ph	0.61%	0.00%	3.55%	H3T11ph	0.19%	0.00%	0.00%
H1.2S35ph	9.20%	16.98%	49.46%	H3K9me1S10phK14ac	0.47%	0.00%	0.00%
H1.5T38ph	0.00%	0.09%	4.62%	H3K9me2S10phK14ac	14.68%	15.95%	6.53%
H2B1AS79ph	98.23%	12.08%	99.65%	H3K9me3S10phK14ac	24.62%	33.16%	0.00%
H3S10ph	0.68%	0.10%	0.00%	H3K27me2S28ph	0.00%	0.03%	0.41%
H3K9me1S10ph	0.00%	0.00%	0.23%	H3K27me3S28ph	0.05%	0.17%	0.91%
H3K9me2S10ph	6.60%	3.46%	1.01%	H3.3S28ph	1.99%	0.27%	0.44%
H3K9me3S10ph	27.55%	24.56%	0.45%	H3.3K27me1S28ph	0.03%	0.08%	0.03%