Analysis of epigenetic modifications of chromatin at specific gene loci by native chromatin immunoprecipitation of nucleosomes isolated using hydroxyapatite chromatography

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Chromatin immunoprecipitation (ChIP) is routinely used to examine epigenetic modification of histones at specific genomic locations. However, covalent modifications of histone tails can serve as docking sites for chromatin regulatory factors. As such, association of these regulatory factors with chromatin could cause steric hindrance for antibody recognition, resulting in an underestimation of the relative enrichment of a given histone modification at specific loci. To overcome this problem, we have developed a native ChIP protocol to study covalent modification of histones that takes advantage of hydroxyapatite (HAP) chromatography to wash away chromatin-associated proteins before the immunoprecipitation of nucleosomes. This fast and simple procedure consists of five steps: nuclei isolation from cultured cells; fragmentation of chromatin using MNase; purification of nucleosomes using HAP; immunoprecipitation of modified nucleosomes; and qPCR analysis of DNA associated with modified histones. Nucleosomes prepared in this manner are free of contaminating proteins and permit an accurate evaluation of relative abundance of different covalent histone modifications at specific genomic loci. Completion of this protocol requires ~1.5 d.

INTRODUCTION

Epigenetic modification of chromatin plays an important role in establishing the many different cell types that share the same genome within an organism. To understand the role of epigenetics in regulating different cellular processes, ChIP is used extensively to evaluate the presence of different chromatin modifications at specific genomic locations. Chromatin states that are currently evaluated by ChIP include both post-translational modifications of histones[1–3] and methylation of DNA[4]. Conditions vary slightly amongst the many ChIP protocols currently in use, but these methods can be classified into one of two groups: (i) those that use chromatin isolated under native cellular conditions—native ChIP (N-ChIP)[1,2,5] or (ii) those that use chromatin isolated from cells where proteins and DNA have been covalently bound to each other—cross-linked ChIP (X-ChIP)[6,7]. The advantages of N-ChIP over X-ChIP have been described[2] and include predictability of antibody specificity; efficiency of precipitation; and ability to analyze DNA without PCR amplification. The main disadvantage of NChIP is that it is mostly limited to studying histone modifications, as proteins must be tightly associated with chromatin to be coprecipitated (M.B., unpublished observation). Thus, in most cases, it can be viewed that N-ChIP is the technique of choice to study post-translational modifications of histones or DNA, whereas X-ChIP is the method of choice for studying chromatin-associated factors (i.e., transcriptional activators/repressor, cofactors, DNA repair factors). This being said, it has been shown over the last few years that many covalently modified histone tails can act as docking sites for chromatin regulatory factors[8–10]—part of the histone code[11]. In this code, different covalent histone modifications are recognized and become stably associated to regulatory factors via specific interaction domains[12]. An example of such an interaction is the recruitment of TFIID to specific promoters through the interaction of TAF3 (via its PHD domain) with H3K4me3 (ref. 13). As such, there is potential for these chromatin regulatory factors to cause steric hindrance for antihistone antibodies, resulting in an underestimation of a particular modification's relative enrichment at a genomic location of interest. To overcome this problem, we have developed a modified N-ChIP protocol (HAP-ChIP) that removes cofactors that might cause steric hindrance before immunoprecipitation (see **Fig. 1**). The HAP-NChIP protocol reported here provides a method to alleviate the problem of steric hindrance caused by cofactors that mask antibody epitopes. However, the problem of steric hindrance caused by multiple covalent modifications on the same histone tail is not remedied by this technique. Overcoming this problem will require the development of new antibodies that recognize multiple covalent modifications in tandem. Although some antibodies recognizing multiple histone modifications do exist, a better understanding of the histone code is necessary to comprehend the interrelationship between these different covalent marks. Only then will we be able to begin to rationally design antibodies that will overcome the steric hindrance of multiple histone modifications and bring us closer to elucidating the mechanism by which combinations of these modifications regulate gene expression.

Standard N-ChIP protocols are performed on nonpurified nucleosomes extracted from nuclei[1,5]. Here, we have established an improved NChIP procedure that takes advantage of the high-affinity interaction between DNA and HAP[14] to obtain nucleosomes that are free of contaminating cofactors. Washes of 600 mM NaCl are commonly used to remove proteins (including histone H1) from chromatin in the preparation of purified histones by

HAP chromatography[15]. Purified histone octamers are then usually eluted from DNA using 2 M NaCl. In contrast, our protocol elutes nucleosomes by increasing the phosphate concentration in the buffer to 500 mM $NaPO_4$ at pH 7.2 in the presence of low NaCl concentrations. This procedure preserves the interaction of DNA with the core histones, resulting in a concentrated population of pure nucleosomes that can be diluted for immediate use in immunoprecipitation. This procedure requires significantly less time than those that isolate chromatin over a sucrose gradient[2] and has been optimized for analysis of genomic loci using qPCR.

The nucleosomal arrays obtained from this procedure, ranging from 1 to 3 nucleosomes, provide a genomic resolution for histone modifications of approximately 500 bp. This is ideal for qPCR analysis of genomic loci where the positioning of the nucleosomes has not yet been characterized. As the standard size of amplicons for qPCR range from 80 to 200 bp, the use of shorter nucleosomal arrays (i.e., mononucleosomes) greatly decreases the probability that a given primer/probe set will lie within the same mononucleosome. A second important advantage of our protocol is that nuclei are lysed using high concentrations of NaCl immediately following fragmentation by micrococcal nuclease, thereby resulting in an even representation of the different types of chromatin (euchromatin versus heterochromatin) in our preparation (see **Fig. 2**). This is in contrast to the passive diffusion of fragmented chromatin from the nucleus that is used in other N-ChIP protocols and could potentially result in the overrepresentation of regions of chromatin that are transcriptionally active. Our improved protocol was successfully used to examine the relative enrichment of trimethylation at lysine 4 of histone H3 (H3K4me3) within the 5′-end of genes activated during myogenesis in C2C12 cells[16]. In these studies, we demonstrated that H3K4me3 was greatly enriched at muscle-specific genes at a time point coinciding with their expression. Furthermore, we demonstrated that Ash2L-containing histone methyltransferase complexes were responsible for establishing this epigenetic mark and that this complex was targeted to specific promoters by a p38-dependent phosphorylation of Mef2d (ref. 16).

The protocol described below has been optimized for qPCR analysis of genomic loci, but could easily be adapted to the recently described methods of ChIP-microarrays[17,18] or ChIP-sequencing[19]. However, to maximize the potential of these powerful techniques, the user should modify conditions to increase the resolution of the ChIP by isolating mononucleosomes before immunoprecipitation. This could be accomplished by applying the nucleosomes eluted from the HAP column to a sucrose gradient before immunoprecipitation as described in the N-ChIP protocol developed by O'Neill and Turner[2]. Similarly, HAP-ChIP can be adapted to the recently described carrier ChIP protocol that permits ChIP on as few as 100 mammalian cells[3]. Although we have not yet tested the protocol on as few as 100 cells, we have successfully used it to examine the methylation status of several muscle-specific genes in as few as 50,000 primary mouse myoblasts (data not shown). Under these conditions, we observe enrichment of various histone modification at levels equal to or greater than those observed in conditions where we start with 10^7 C2C12 cells (data not shown). Finally, we believe that our ChIP protocol could be easily adapted to immunoprecipitation of methylated DNA (MeDIP) (ref. 4).

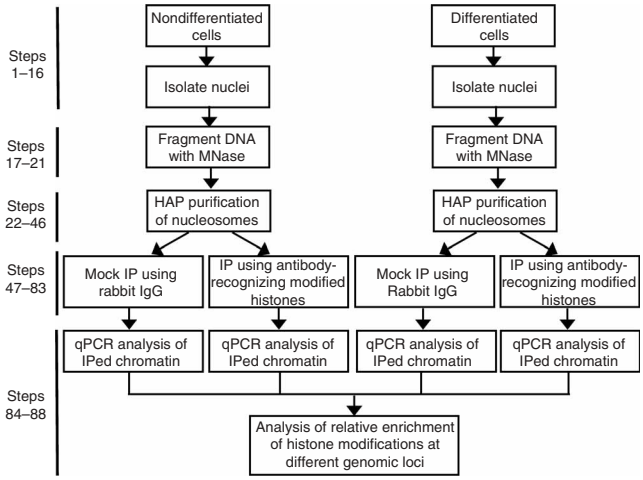

Figure 1 | Flowchart of the different steps of the HAP-NChIP protocol.

Experimental design

In the protocol below, we describe conditions for analyzing epigenetic modifications of histones at specific genomic loci. This approach is outlined schematically in **Figure 1**. Description of this procedure has been divided into five steps that take about 1.5 d to complete. These steps include the following: (i) isolation of nuclei (2 h); (ii) fractionation of chromatin using MNase (0.25 h); (iii) purification of nucleosomes using hydroxyapatite (0.5 h); (iv) immunoprecipitation of nucleosomes (20 h); and (v) real-time PCR analysis of DNA associated with modified histones (3 h). This is a very simple protocol to implement, but there are several variables to consider before starting.

Controls

Amount of chromatin input into the ChIP. When comparing two biological samples for epigenetic modifications, you should begin with similar amounts of material. There are several points where you can ensure that equivalent amounts of chromatin are present. Initially, you should start with roughly the same number of cells between the two biological samples (Step 1). Once nuclei are

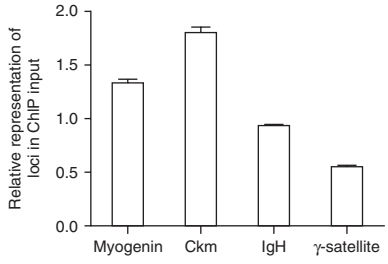

Figure 2 | Analysis of loci representation in chromatin eluted from HAP column. DNA isolated from either the chromatin fractions purified using HAP chromatography or C2C12 cell genomic DNA where subjected to qPCR using primers that recognize the indicated genomic loci. The absolute representation of loci lying in euchromatin (Ckm and Myogenin), facultative heterochromatin (IgH), and constitutive heterochromatin (γ satellite DNA) was determined by generating a standard curve of Ct values for each primer set using the genomic DNA and then determining the amount of genomic DNA that provides the same Ct as an aliquot of the HAP eluted chromatin. Average values of triplicate qPCRs are displayed with error bars corresponding to ±s.d.

isolated, measurement of nucleic acid content before MNase digestion (Step 16) then permits you to adjust the concentration of chromatin to compensate for any loss that may have occurred during the lysis steps. Nucleic acid content is again measured after elution from the HAP column (Step 60), and volumes can be adjusted to ensure that similar amounts of DNA are used in the immunoprecipitation. Finally, an aliquot of the elution from the HAP column (input for the immunoprecipitation) is retained (Step 60) for qPCR analysis such that all values obtained from the immunoprecipitated material can be corrected during the data analysis step (Step 88) for small variations in the amount of input material.

Specificity of immunoprecipitation. It is important to ensure that chromatin containing your locus of interest has not been immunoprecipitated nonspecifically. Ideally, this is accomplished by performing a mock immunoprecipitation in parallel using preimmune serum (available if you have prepared the antibody yourself). If preimmune serum is not available, IgG isolated from an animal of the same species from which the antibody was generated is a good alternative. This is prepared in parallel with your specific antibody in Step 52 (perform Steps 47–88 in parallel for both test and mock antibodies). In calculating the amount of specific chromatin that is immunoprecipitated with a given antibody (Step 88), it is strongly advised to subtract the amount of chromatin immunoprecipitated with the control antisera/IgG from that immunoprecipitated with the antibody recognizing the modification of interest (specific IP = antibody IP − control IgG IP). This will help maximize the signal-to-noise ratio. Note that, if multiple ChIPs are performed in parallel, and the antibodies used are generated in different species, it is necessary to carry out a mock immunoprecipitation using IgG from each of those species.

Control for pipetting error in qPCR. As qPCR requires extensive pipetting of small volumes, it is advised to perform all analysis of a biological sample in triplicate and present the data ± s.d.

MNase digestion

In this protocol, chromatin is fragmented using MNase, which preferentially cleaves DNA in the internucleosomal regions. We generally establish conditions that provide oligonucleosomes ranging from 1 to 3 nucleosomes in length when observed by ethidium bromide staining after agarose gel electrophoresis (**Fig. 3a**). Using these conditions, we have succeeded in extracting comparable amounts of euchromatin and heterochromatin from nuclei (**Fig. 2**). As immunoprecipitation of such a population of nucleosomes enriches for the longer chromatin fragments (longer nucleosomal arrays have more histones that have the potential to be immunoprecipitated), we estimate that this degree of fragmentation provides us with a resolution of ~500 bp. It should be noted that studies aimed at examining the modifications of histones within the constitutive heterochromatin may require higher concentrations of MNase to obtain similar degrees of resolution, as compacted DNA is more resistant to nuclease digestion. If there are concerns about the resolution of ChIPs at a locus of interest, the average length of the nucleosomal fragments containing a locus of interest can be determined by indirect end-labeling[20].

Size of chromatin fragments obtained following HAP chromatography

In this protocol, we have used hydroxyapatite chromatography to purify nucleosomal arrays of 1–3 nucleosomes. This technique provides lower resolution than N-ChIP protocols that use a sucrose gradient to isolate mononucleosomes after MNase digestion[2]. However, in developing conditions for these studies, our goal was to obtain the best possible resolution while maximizing probability of a successful outcome. We reasoned that, as qPCR amplicons are usually 80–200 bp in length and mononucleosomes are 147–200 bp in length, it can become problematic to design a primer set that will fall within a short fragment without previous detailed knowledge of the positioning of nucleosomes. Furthermore, several studies have demonstrated that nucleosomes shift upon activation of gene expression[21]. Thus, a primer set that is designed to work in a single nucleosome could provide erroneous results if one of the primers fails to bind to its target DNA sequence after gene activation. Thus, we believe that the 1–3 nucleosome resolution is best for qPCR-based analysis of N-ChIP experiments. On the other hand, mononucleosome resolution is more adapted to N-ChIP-microarray and N-ChIP-sequencing studies. Thus, for these latter studies, we recommend purification of nucleosomes by HAP chromatography with a further step of mononucleosome isolation by sedimentation on a 5–25% (wt/vol) sucrose gradient[2].

Primer and probe design

Although many algorithms exist to design qPCR primers, we generally use the PrimerQuest algorithm that is freely available on the Integrated DNA technologies (IDT) website (http://www.idtdna.com/Scitools/Applications/Primerquest/). We prefer the hydrolysis probe technology for qPCR using a probe labeled with 6-carboxyfluorescein (6-FAM) at the 5′-end and Black Hole Quencher 1 (BHQ1) at the 3′-end. In designing a primer/probe set, we usually identify ~500 bp of DNA sequence centered over a DNA element of interest. This DNA sequence is then used for a basic search that identifies possible PCR primers/probe pairs for

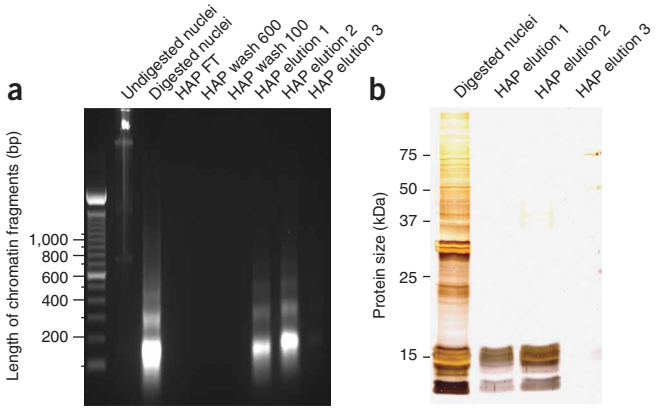

Figure 3 | Analysis of nucleosomes isolated by hydroxyapatite chromatography. (**a**) Nucleosomal arrays isolated from C2C12 nuclei treated with micrococcal nuclease were subjected to purification using hydroxyapatite. Fractions obtained from Steps 16 (undigested nuclei), 21 (digested nuclei), 26 (HAP FT), 28 (HAP wash 600), 31 (HAP wash 100) and 34 and 35 (HAP elution 1–3) were deproteinized and separated on a 1.2% agarose gel in Tris-glycine buffer. (**b**) A 6 µl aliquot of the fractions obtained in Steps 21 (digested nuclei) and 34 and 35 (HAP elutions 1–3) were diluted to 8 µl with SDS-PAGE gel-loading buffer and boiled for 5 min. Proteins were then separated on a 12% SDS-PAGE gel and visualized using silver stain.

real-time PCR analysis. Amplicons obtained from your primer sets should range from 80 to 200 bp. To ensure specificity of your primers/probe sets, identified sequences should be subject to a blast search to confirm that they recognize a unique sequence within the genome.

Expected results

Successful completion of this protocol will provide the user with information regarding whether a specific histone post-translational modification exists at nucleosomes present within a specific genomic location. However, enrichment values are not absolute, and must be compared relative to other genomic locations immunoprecipitated with the same antibody. In addition, relative levels of two different modifications at a single genomic position cannot be directly measured by this technique. Thus, to obtain meaningful biological information from these studies, comparisons must be performed by examining multiple genomic locations (spatial) and/or different time points during a biological process (temporal).

MATERIALS

REAGENTS

- Trizma base (Sigma, cat. no. T6066)
- HEPES potassium salt (HEPES K^+) (Sigma, cat. no. H0527)
- Glycerol (Sigma, cat. no. G7757)
- Sodium chloride (NaCl) (Sigma, cat. no. S3014)
- Potassium chloride (KCl) (Sigma, cat. no. P9541)
- Sucrose (Sigma/Fluka, cat. no. 84097)
- Magnesium chloride ($MgCl_2 \cdot 6H_2O$) (Sigma, cat. no. M2670)
- Calcium chloride ($CaCl_2 \cdot 2H_2O$) (Sigma, cat. no. C3306)
- NP-40 substitute (Sigma/Fluka, cat. no. 74385) ! **CAUTION** Toxic, wear gloves when handling.
- Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Sigma, cat. no. E3889)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (Sigma, cat. no. E5134)
- Sodium phosphate monobasic (NaH_2PO_4) (Sigma, cat. no. S8282)
- Sodium phosphate dibasic (Na_2HPO_4) (Sigma, cat. no. S7907)
- Phenylmethanesulfonyl fluoride (PMSF) (Sigma, cat. no. P7626) ! **CAUTION** Toxic, wear gloves when handling.
- DL-dithiothreitol (DTT) (Sigma, cat. no. D9779)
- Sodium dodecyl sulfate (SDS) (Sigma, cat. no. L4390) ! **CAUTION** Toxic, wear gloves and mask when weighing powder and gloves when handling solution.
- β-Glycerophosphate (Sigma/Fluka, cat. no. 50020)
- Phosphate-buffered saline (PBS) (Sigma, cat. no. P3813)
- Salmon sperm DNA (Sigma, cat. no. D1626)
- Albumin from chicken egg whites (Sigma, cat. no. A5503)
- Trichostatin A (Sigma, cat. no. T8552)
- RNase A (Sigma, cat. no. R4642)
- Proteinase K (Sigma, cat. no. P4850)
- Sodium acetate (NaAc) (Sigma, cat. no. S2889)
- EtOH (99%) ! **CAUTION** Flammable, keep away from open flame.
- Agarose (Invitrogen, cat. no. 15510-027)
- Ethidium bromide (Sigma, cat. no. E1510)
- Glycogen (Fermentas, cat. no. R0561)
- Phenol/chloroform/isoamyl alcohol (Sigma, cat. no. P2069) ! **CAUTION** Toxic, wear gloves when handling.
- Chloroform/isoamyl alcohol (Sigma, cat. no. C0549) ! **CAUTION** Toxic, wear gloves when handling.
- Micrococcal nuclease (Sigma, cat. no. N5386)
- DNA ladder (100 bp) (Invitrogen, cat. no. 15628-050)
- Macro-prep ceramic hydroxyapatite type I, 20 μm (Bio-Rad, cat. no. 157-0020)
- Micro spin columns (Nest Group, cat. no. SEM0000)
- Dynabeads protein A (Dynal, cat. no. 100.02)
- Dynabeads protein G (Dynal, cat. no. 100.04)
- Siliconized Eppendorf tubes (VWR, cat. no. 20170-650)
- PCR tubes (0.1 ml) and caps for RotorGene 6000 (Corbett, cat. no. 3001-002)
- AmpliTaq gold with GeneAmp 10X PCR buffer II kit (Applied Biosystems, cat. no. N808-0249)
- dATP, 100 mM solution (Invitrogen, cat. no. 55082)
- dCTP, 100 mM Solution (Invitrogen, cat. no. 55083)
- dGTP, 100 mM Solution (Invitrogen, cat. no. 55084)
- dTTP, 100 mM Solution (Invitrogen, cat. no. 55085)
- Primers and hydrolysis probes set (custom order from IDT)

EQUIPMENT

- Scientific Industries Vortex-Genie (or equivalent)
- Eppendorf biophotometer (or equivalent)
- Eppendorf 5424 microcentrifuge (or equivalent)
- Dynal MPC-S magnetic bead concentrator (or equivalent)
- Barnstead Thermolyne Labquake rotator (or equivalent)
- Corbett RotorGene 6000 real-time PCR machine (or equivalent)
- Eppendorf Vacufuge concentrator (or equivalent)
- Eppendorf thermomixer (or equivalent)
- Beckman-Coulter Allegra X15R tabletop centrifuge (or equivalent)
- Alpha Imager HP gel documentation system (or equivalent)
- Bio-Rad PowerPac basic power supply (or equivalent)
- Bio-Rad Mini-Sub Cell GT (or equivalent)

REAGENT SETUP

PBS Dissolve contents of one pouch in 1 liter of water. Autoclave and store at 4 °C for up to 6 months.

NP-40 substitute (10% vol/vol) 10 ml of NP-40 substitute; make up to 100 ml with water. Store at room temperature (RT; 22 °C) for up to 6 months.

SDS (10% wt/vol) 10 g of SDS; make up to 100 ml with water. ! **CAUTION** Store at RT for up to 6 months.

PMSF (200 mM) 1.74 g of PMSF; make up to 50 ml with EtOH. ! **CAUTION** Store at RT for up to 1 month.

DTT (1 M) 3.09 g of DTT; make up to 20 ml with 10 mM sodium acetate, pH 5.2. Filter-sterilize, aliquot and store at −20 °C for years.

Tris, pH 7.4 (10 mM) 121.2 mg of Trizma base, pH to 7.4 with HCl; make up to 100 ml with water. Autoclave and store at RT for up to 6 months.

TE 606 mg of Trizma base (50 mM final), 37 mg of EDTA (1 mM final); adjust pH to 8.0 with HCl and make up to 100 ml with water. Autoclave and store at RT for up to 6 months.

STE (0.1 M) 606 mg of Trizma base (50 mM final), 37 mg of EDTA (1 mM final), 584 mg of NaCl (100 mM final); make up to 100 ml with water. Autoclave and store at RT for up to 6 months.

Proteinase K (10 mg ml^{-1}) 100 mg of proteinase K; make up to 10 ml with sterile TE, pH 8. Aliquot and store at −20 °C for several years.

Sodium acetate, pH 5.2 (3 M) 24.61 g of sodium acetate; adjust pH to 5.2 with glacial acetic acid and make up to 100 ml with water. Autoclave and store at RT for up to 6 months.

Albumin (10 mg ml^{-1}) 100 mg of ovalbumin; make up to 10 ml with ChIP buffer 1. Filter-sterilize and store at 4 °C for up to 1 month.

Trichostatin A (1 mM) 1 mg of trichostatin A; make up to 3.3 ml with ethanol. Aliquot and store at −20 °C for several years.

NaCl (5 M) 29.22 g of NaCl; make up to 100 ml with water. Autoclave and store at RT for several years.

Na_2HPO_4 (1 M) 141.96 g of Na_2HPO_4; make up to 1 liter with water. Autoclave and store at RT for several years.

NaH_2PO_4 (1 M) 119.98 g of NaH_2PO_4; make up to 1 liter with water. Autoclave and store at RT for several years.

Buffer N 182 mg of Trizma base (15 mM final), 87 mg of NaCl (15 mM final), 447 mg of KCl (60 mM final), 8.56 g of sucrose (250 mM final), 102 mg of $MgCl_2 \cdot 6H_2O$ (5 mM final) and 15 mg of $CaCl_2 \cdot 2H_2O$ (1 mM final); adjust pH to 7.5 with hydrochloric acid and make up to 100 ml with water. Filter-sterilize and store at 4 °C for up to 1 week. At the time of use, add trichostatin A, DTT, β-glycerophosphate and PMSF to a final concentration of 1 μM, 1 mM, 10 mM and 200 μM, respectively.

2× lysis buffer 182 mg of Trizma base (15 mM final), 87 mg of NaCl (15 mM final), 447 mg of KCl (60 mM final), 8.56 g of sucrose (250 mM final), 102 mg of $MgCl_2 \cdot 6H_2O$ (5 mM final), 15 mg of $CaCl_2 \cdot 2H_2O$ (1 mM final) and 6 ml

of 10% (vol/vol) NP-40 substitute (0.6% vol/vol final); adjust pH to 7.5 with hydrochloric acid and make up to 100 ml with water. Filter-sterilize and store at 4 °C for up to 1 week. At the time of use, add trichostatin A, DTT, β-glycerophosphate and PMSF to a final concentration of 1 μM, 1 mM, 10 mM, and 200 μM, respectively.

EX50 2.76 g of HEPES K^+, 2.92 g of NaCl, 304 mg of $MgCl_2 \cdot 6H_2O$, 190 mg of EGTA, 3.06 g of β-glycerophosphate and 100 ml of glycerol. Adjust pH to 7.6 with hydrochloric acid and make up to 1 liter with water. Autoclave and store at 4 °C for up to 6 months. At the time of use, add DTT and PMSF to a final concentration of 1 mM and 200 μM, respectively.

MNase (1 U μl^{-1}) Dissolve 500 units of MNase in 500 μl of EX50 buffer. Aliquot and store at −80 °C for up to 1 year.

10× MNase stop buffer 4.18 g of EGTA (110 mM final) and 4.09 g of EDTA (110 mM final); make up to 100 ml with water. Autoclave and store at RT for up to 6 months.

HAP buffer 1 684 μl of 1 M Na_2HPO_4, 316 μl of 1 M Na_2HPO_4 (5 mM $NaPO_4$, pH 7.2 final), 7.01 g of NaCl (600 mM final) and 400 μl of 0.5 M EDTA (1 mM final); make up to 200 ml with water. Autoclave and store at 4 °C for up to 6 months. At the time of use, add trichostatin A, β-glycerophosphate and PMSF to a final concentration of 1 μM, 10 mM and 200 μM, respectively.

HAP buffer 2 684 μl of 1 M Na_2HPO_4, 316 μl of 1 M Na_2HPO_4 (5 mM $NaPO_4$, pH 7.2 final), 1.17 g of NaCl (100 mM final) and 400 μl of 0.5 M EDTA (1 mM final); make up to 200 ml with water. Autoclave and store at 4 °C for up to 6 months. At the time of use, add PMSF to a final concentration of 200 μM.

HAP elution buffer 68 ml of 1 M Na_2HPO_4, 32 ml of 1 M Na_2HPO_4 (500 mM $NaPO_4$, pH 7.2 final), 1.17 g of NaCl (100 mM final) and 400 μl of 0.5 M EDTA (1 mM final); make up to 200 ml with water. Autoclave and store at 4 °C for up to 6 months. At the time of use, add PMSF to a final concentration of 200 μM.

10× Tris-glycine buffer 288 g of glycine and 60.6 g of Trizma base; make up to 2 liters with water. Store at RT for up to a year.

ChIP buffer 1 303 mg of Trizma base (25 mM final), 102 mg of $MgCl_2 \cdot 6H_2O$ (5 mM final), 746 mg of KCl (100 mM final), 10 ml of glycerol (10% vol/vol final), 1 ml of 10% (vol/vol) NP-40 substitute (0.1% vol/vol final); make up to 100 ml with water. Autoclave and store at 4 °C for up to 6 months. At the time of use, add PMSF to a final concentration of 200 μM.

ChIP buffer 2 303 mg of Trizma base (25 mM final), 102 mg of $MgCl_2 \cdot 6H_2O$ (5 mM final), 2.24 g of KCl (300 mM final), 10 ml of glycerol (10% vol/vol final), 1 ml of 10% (vol/vol) NP-40 substitute (0.1% vol/vol final); make up to 100 ml with water. Autoclave and store at 4 °C for up to 6 months. At the time of use, add PMSF to a final concentration of 200 μM.

ChIP elution buffer 606 mg of Trizma base (50 mM final), 37 mg of EDTA (1 mM final), 10 ml of 10% (wt/vol) SDS (1% wt/vol final); make up to 100 ml with water. Filter-sterilize and store at 4 °C for up to 1 month.

Sheared salmon sperm DNA (10 mg ml^{-1}) 1 g of salmon sperm DNA; make up to 100 ml with 0.4 M NaOH and stir overnight in a beaker. Place beaker on a hot plate, and bring to a boil. Continue to boil for 45 min. Cool on ice and neutralize with glacial acetic acid to pH 7. Spin out any debris in benchtop centrifuge at 3,725*g* at 4 °C for 30 min. Recover supernatant and add 2 volumes of EtOH to precipitate DNA. Spin in benchtop centrifuge at 3,725*g* for 30 min. Recover pellet and dissolve in TE, pH 7.4, at a concentration of 10 mg ml^{-1}. Aliquot and store at −20 °C for several years.

PROCEDURE

Isolation of nuclei ● TIMING 2 h

1| This step can be performed using option A or option B depending on whether you are starting with adherent cells or cells grown in suspension.

(A) Adherent cells

(i) Starting with ~10^7 cells, wash cells twice with 10 ml of PBS and aspirate off the solution.

(ii) Apply 4 ml of trypsin to cells and incubate for 5 min at 37 °C.

(iii) Add 6 ml of culture medium to the plate and collect the cells by pipetting up and down for a total of three times.

(iv) Transfer cell suspension to a 15-ml tube and spin at 524*g* for 5 min in a tabletop centrifuge (4 °C).

(v) Aspirate supernatant and discard.

(B) Suspension cells

(i) Transfer ~10^7 cells to a 15-ml tube and spin at 524*g* for 5 min in a tabletop centrifuge (4 °C).

(ii) Aspirate supernatant and discard.

2| Resuspend cells in 10 ml of ice-cold PBS containing 200 μM PMSF, 1 μM trichostatin A and 10 mM β-glycerophosphate. Try to obtain a single cell suspension (no visible clumps). Spin at 524*g* for 5 min in a tabletop centrifuge (4 °C). Aspirate supernatant and discard. Repeat this wash once more.

▲ CRITICAL STEP It is usually sufficient to look at the cells with the naked eye to determine whether there are any visible clumps present. If unsure, an aliquot of the cells can be examined under a microscope. If unable to disrupt the clumps, a short pulse of sonication can be attempted to separate aggregates.

3| Resuspend cells in 5 ml of ice-cold buffer N to obtain a single cell suspension (no visible clumps). Spin at 524*g* for 5 min in a tabletop centrifuge (4 °C). Aspirate supernatant. Repeat once more.

4| Resuspend cells in 1 ml of ice-cold buffer N by pipetting up and down using a P-1000.

▲ CRITICAL STEP Single cell suspensions must be obtained at this point (i.e., no clumps visible to the naked eye). If this is not obtained, lysis of the cells will not be homogeneous. If clumping of the cells occurs, try subjecting cell suspension to a short pulse of sonication. If this does not work, prepare new buffer N and start procedure once again.

5| Once single cell suspensions are obtained, vortex at low speed (i.e., Vortex-Genie speed 3 on a scale of 10) and simultaneously add dropwise 1 ml of ice-cold 2× lysis buffer.

6| Incubate on ice for 10 min to complete cell lysis.

7| Spin at 524*g* for 5 min in a tabletop centrifuge (4 °C). Remove supernatant by aspiration and discard.

▲ CRITICAL STEP At this point, the nuclear pellet should be white (as opposed to off-white/beige for the cellular pellet). A final concentration of 0.3% (vol/vol) NP-40 is sufficient to break open the different cell lines we have tested. However, some cell

lines may be resistant to this concentration of detergent. If your pellet does not become white, resuspend the pellet in buffer N and examine an aliquot under a microscope. If the majority of the cells are not lysed, prepare a new 2× lysis buffer with a slightly higher concentration of NP40 and repeat Steps 5–7.
? TROUBLESHOOTING

8| Resuspend nuclei in 500 µl of ice-cold buffer N by pipetting up and down using a P-1000.

9| Transfer nuclei to a siliconized Eppendorf tube and keep on ice.

10| Add a second 500 µl aliquot of ice-cold buffer N to the 15-ml tube (from Step 8) to wash out any remaining nuclei.

11| Combine with the first 500 µl of nuclei (from Step 9). The volume is now 1 ml.

12| Spin nuclei at 600*g* for 5 min in a microcentrifuge at 4 °C. Remove supernatant by aspiration and discard.

13| Resuspend nuclei again in 1 ml of buffer N by pipetting up and down using a P-1000.
▲ CRITICAL STEP Again try to obtain single nuclei suspensions (no visible clumps).

14| Spin nuclei at 600*g* for 5 min in a microcentrifuge at 4 °C. Remove supernatant by aspiration and discard.

15| Resuspend nuclei in 100 µl of buffer N.

16| Determine the relative nucleic acid content of the sample by spectrophotometry. This can be done by taking 4 µl of the nuclei suspension and placing in 400 µl of 2 M NaCl. Make sure to blank the spectrophotometer using 4 µl of buffer N in 400 µl of 2 M NaCl. Read the OD at 260 nm using a quartz cuvette. The nucleic acid concentration will be $OD_{260} \times 50$ ng $\mu l^{-1} \times 100$ (dilution factor) (see **Box 1** for an example).
▲ CRITICAL STEP The desired concentration is ~ 1 µg μl^{-1}. If the concentration is greater than 1 µg μl^{-1}, dilute the sample using buffer N. If the concentration is too low (less than 0.8 µg μl^{-1}), centrifuge the nuclei as described in Step 14 and resuspend in a smaller volume.

Fragmentation of chromatin using micrococcal nuclease (MNase) ● TIMING 15 min

17| Dilute the stock solution of MNase (1 U μl^{-1}) stock 1 in 20 using EX50 buffer to a final concentration of 0.05 U μl^{-1}.
▲ CRITICAL STEP Use a filtered tip to pipette concentrated MNase so that the pipette does not become contaminated with the nuclease for future experiments.

18| Add appropriate amount of MNase (see below) to 100 µl of single nuclei suspension.
▲ CRITICAL STEP The concentration of MNase to use in your digestion has to be determined empirically. We usually find good results using 50 milliunits per 40 µg of nucleic acid.
▲ CRITICAL STEP Ensure that nuclei are not clumped before adding MNase; if nuclei clump after resuspension, access of the MNase to chromatin will not be even, which might result in some DNA being of high molecular weight and a loss of resolution in your ChIP studies.

19| Incubate for 10 min in a waterbath at 37 °C.

20| Stop the reaction by adding 11 µl (1/10th volume) of MNase stop buffer, and place on ice.

BOX 1 | EXAMPLE OF SPECTROPHOTOMETER READINGS THAT CAN BE EXPECTED WHEN MEASURING NUCLEIC ACID CONCENTRATION IN NUCLEI

To determine the nucleic acid concentration, 4 µl of resuspended nuclei was diluted in 400 µl of 2 M NaCl (1 in 100 dilution). Nucleic acid content is then estimated based on the molar extinction coefficient for DNA (50 ng μl^{-1}).

$A_{260}/A_{280} = 1.58$
$A_{260}/A_{230} = 1.12$

$A_{230} = 0.204$
$A_{260} = 0.227$
$A_{280} = 0.144$
$A_{320} = 0.031$

Concentration of DNA $= OD_{260} \times$ dilution factor $\times$ 50 ng μl^{-1}
$= 0.227 \times 100 \times 50$ ng μl^{-1}
$= 1135$ ng μl^{-1}

21| Take a 5 µl aliquot of sample to visualize nucleosomal arrays on an agarose gel as described in Steps 36–45 (this sample corresponds to the digested nuclei in **Fig. 3a**).
▲ CRITICAL STEP Nucleosomes should be run on a gel to determine the average nucleosome length, as this is what determines the resolution of your ChIP studies.

Purification of the nucleosomes using hydroxyapatite ● TIMING **30 min**

22| Lyse nuclei by adding 15 µl of 5 M NaCl to the 111 µl of MNase digested sample (from Step 20).

23| Add 200 µl of HAP buffer 1 as well as 66 mg of hydroxyapatite resin per 100 µg of DNA, vortex thoroughly and incubate on a rotator for 10 min at 4 °C.

24| Transfer the chromatin/hydroxyapatite slurry to a Microspin column.

25| Place the Microspin column in an Eppendorf tube. Spin in microfuge for 1 min at 600*g* (4 °C).
? TROUBLESHOOTING

26| Keep a 5 µl aliquot of the column flow-through (FT) to visualize nucleosomal arrays on an agarose gel as described in Steps 36–45 (this sample corresponds to the HAP FT in **Fig. 3a**). The remaining flow-through can be discarded.

27| Add 200 µl of HAP buffer 1 to Eppendorf tube that contained the chromatin/hydroxyapatite slurry in Step 23 to rinse it out, and then transfer onto the same Microspin column.

28| Spin column again in microfuge for 1 min at 600*g* (4 °C). Keep a 5 µl aliquot of wash to visualize nucleosomal arrays on an agarose gel as described in Steps 36–45 (this sample corresponds to the HAP wash 600 in **Fig. 3a**), and discard the remaining wash.

29| Repeat wash (Steps 27 and 28) three more times with 200 µl of HAP buffer 2 (four washes in total). Discard washes.

30| Add 200 µl of HAP buffer 2 to the same column.

31| Spin column again in microfuge for 1 min at 600*g* (4 °C). Keep a 5 µl aliquot of wash to visualize nucleosomal arrays on an agarose gel as described in Steps 36–45 (this sample corresponds to the HAP wash 100 in **Fig. 3a**), and discard the remaining wash.

32| Repeat wash (Steps 30 and 31) three more times with 200 µl of HAP buffer 2 (four washes in total). Discard washes.

33| Add 100 µl of HAP elution buffer to the same column. Transfer column to a fresh Eppendorf tube.

34| Spin column again in microfuge for 1 min at 600*g* (4 °C). Keep the elution on ice, and take a 5 µl aliquot for visualization of nucleosomal arrays on an agarose gel as described in Steps 36–45 (this sample corresponds to the elution 1 in **Fig. 3a**).

35| Repeat Steps 33 and 34 two more times by adding 100 µl of HAP elution buffer each time and using a fresh Eppendorf tube for each elution (to give three elutions in total). Keep the elutions on ice, and take a 5 µl aliquot of each for visualization of nucleosomal arrays on an agarose gel as described in Steps 36–45 (these samples correspond to the elution 2 and elution 3 in **Fig. 3a**).

36| Dilute 5 µl aliquots saved from Steps 21, 26, 28, 31, 34 and 35 to 200 µl with ChIP elution buffer, add 1 µl of proteinase K (10 mg ml^{-1}) and incubate for 30 min at 42 °C.

37| Add 200 µl of phenol/chloroform, and spin for 5 min at 9,300*g* in microcentrifuge (at RT).

38| Transfer the aqueous (top) layer to a second tube containing 200 µl of phenol/chloroform/isoamyl alcohol, vortex and spin for 5 min at 9,300*g*.

39| Transfer 180 µl of aqueous (top) layer to fresh tube. Add 20 µl of 3 M sodium acetate, 1 µl of glycogen and 500 µl of EtOH. Vortex and place at −80 °C for 30 min.

40| Spin for 15 min at 15,800*g* at 4 °C. Remove the supernatant by aspiration and discard.

41| Add 500 µl of 70% EtOH to the pellet, vortex and spin at 15,800*g* for 5 min at 4 °C. Remove the supernatant by aspiration and discard.

42| Dry pellet for 5 min in a speed vac without heat (or air-dry for 15 min).

43| Redissolve pellet in 5 µl of 0.1 M STE. Add 1 µl of 6× loading dye.

44| Run DNA samples on a 1.2% agarose in 1× Tris-glycine gel at a constant rate of 5–7 V cm^{-1}. Remember to run a 100-bp molecular weight marker alongside the digested chromatin.

45| Stain gel with ethidium bromide, and visualize using an ultraviolet transilluminator, or gel documentation system. A sample gel is shown in **Figure 3a**.
▲ CRITICAL STEP Most of the chromatin should appear in elutions 1 and 2, with a small amount in elution 3 (<5%). We generally pool elutions 1 and 2 (see Step 46) for proceeding to Step 60.
? TROUBLESHOOTING

46| Pool elutions 1 and 2 (200 µl total after pooling).

Immunoprecipitation of modified nucleosomes ● TIMING **20 h**
47| Vortex the Dynabeads protein A thoroughly to resuspend before pipetting. For each immunoprecipitation, pipette 20 µl of Dynabeads into a siliconized Eppendorf tube.
▲ CRITICAL STEP Do not forget to prepare mock antibody for immunoprecipitation in parallel to control for nonspecific interactions (see Experimental design section for further details).
▲ CRITICAL STEP Dynabeads protein G should be used instead of Dynabeads protein A if the antibody of interest is a monoclonal antibody of the mouse IgG1 or IgG3 subtypes.

48| Place the tube on the magnet for 1 min, and then pipette off (discard) the supernatant.

49| Remove the tube from the magnet, add 1 ml of ChIP buffer 1 and vortex briefly.

50| Place the tube on the magnet for 1 min and then pipette off (discard) the supernatant.

51| Repeat the washing steps (Steps 49 and 50) once with 1 ml of ChIP buffer 1, and discard the supernatant.

52| In a separate tube, dilute the required amount of antibody (~5 µg) in ChIP buffer 1 to obtain 100 µl final volume.

53| Transfer diluted antibody obtained in Step 52 to the washed beads obtained in Step 51.

54| Agitate for 1 h at 1,400 r.p.m. in an Eppendorf thermomixer (22 °C).

55| Place the tube on a magnet for 1 min and then pipette out the supernatant.
▲ CRITICAL STEP Supernatant still contains a significant amount of free antibody and can be saved for future western blotting.

56| Remove the tube from the magnet, add 1 ml of ChIP buffer 1 and vortex briefly.

57| Place the tube on the magnet for 1 min, and then pipette off (discard) the supernatant.

58| Repeat the wash with ChIP buffer 1 (Steps 56 and 57) once. Keep the beads on ice in the presence of ChIP buffer 1 until ready to proceed with Step 60.
▲ CRITICAL STEP In the interest of time, Steps 47–58 (binding antibody to beads) can be performed before (or during) the isolation of nuclei (Steps 1–21).

59| Place the tube on the magnet for 1 min and then pipette off (discard) the supernatant.

60| Dilute 180 µl of chromatin (180 µl of the 200 µl obtained in Step 46) with 2 ml of ice-cold ChIP buffer 1 to reduce $NaPO_4$ concentration. Add 2 µl of sheared salmon sperm DNA (10 µg ml^{-1} final) and 200 µl of 10 µg $µl^{-1}$ ovalbumin (1 µg $µl^{-1}$ final).
▲ CRITICAL STEP This is enough chromatin for four different antibodies.
▲ CRITICAL STEP Remember to keep 50 µl of the 2 ml of diluted chromatin to use as the input control during qPCR analysis on immunoprecipitated DNA (see 'Experimental design' for further details). This 50 µl sample should be further diluted to 400 µl final volume using 350 µl of ChIP elution buffer and subjected to the same purification as the immunoprecipitated DNA (outlined in Steps 75–83).
▲ CRITICAL STEP If multiple chromatin samples are being studied in parallel, ensure that the amount of chromatin used in each immunoprecipitation is similar by measuring OD_{260}. Take 4 µl of the HAP elution (Step 46) and place in 400 µl of 2 M NaCl. Make sure to blank the spectrophotometer using 4 µl of HAP elution buffer in 400 µl of 2 M NaCl. Read the OD at 260 nm using a quartz cuvette. The nucleic acid concentration will be OD_{260} × 50 ng $µl^{-1}$ × 100 (dilution factor). Adjust volumes with HAP elution buffer as necessary to ensure that approximately the same amount of chromatin is used in each immunoprecipitation.

61| Transfer 500 µl (~10 µg) of chromatin (from Step 60) into 20 µl of beads that have been previously coated with the antibody of your choice (see Steps 47–59). Vortex briefly.
▲ **CRITICAL STEP** Do not use too much chromatin, as the antibody should not be limiting in a ChIP study. If the concentration of the chromatin is greater than 20 µg ml^{-1}, dilute to 20 µg ml^{-1} with ChIP buffer 1.

62| Rotate tubes overnight at 4 °C using a Labquake rotator.
■ **PAUSE POINT** Chromatin should incubate with antibody for 6–20 h.

63| Place the tube on the magnet for 1 min and pipette off (discard) the supernatant.

64| Remove the tube from the magnet, add 1 ml of ice-cold ChIP buffer 2 to the beads and vortex briefly.

65| Rotate in Labquake rotator for 10 min at 4 °C.

66| Place the tube on the magnet for 1 min and then pipette off (discard) the supernatant.

67| Repeat the washing steps (Steps 64–66) twice with ice-cold ChIP buffer 2 and discard the supernatants.

68| Add 1 ml of ice-cold ChIP buffer 1 to the beads and vortex briefly.

69| Place the tube on the magnet for 1 min and then pipette off (discard) the supernatant.

70| Add 1 ml of ice-cold TE buffer to the beads and vortex briefly.

71| Place the tube on the magnet for 1 min and then pipette off (discard) the supernatant.

72| Add 200 µl of ChIP elution buffer to beads. Mix for 10 min at 1,400 r.p.m. using the Eppendorf thermomixer (65 °C).

73| Place on the magnet for 1 min, pipette out the supernatant into a fresh siliconized Eppendorf tube.

74| Repeat elution steps (Steps 72 and 73) once with 200 µl of ChIP elution buffer and combine both supernatants. The final elution volume is now 400 µl.

75| In the elution tube, add 17 µl of 5 M NaCl (0.2 M final) and 2 µl of RNase A (DNase-free). Incubate at 65 °C for 45 min.

76| Add 9 µl of 0.5 M EDTA (10 mM final concentration) and 2 µl of 10 mg ml^{-1} proteinase K (50 µg ml^{-1} final concentration). Incubate at 42 °C for 2h.

77| Add 400 µl of phenol/chloroform/isoamyl alcohol, vortex and spin for 5 min at 9,300*g* in microcentrifuge (at RT).

78| Transfer the aqueous (top) layer to a second Eppendorf tube containing 400 µl of chloroform/isoamyl alcohol, vortex and spin for 5 min at 9,300*g* (at RT).

79| Transfer 360 µl of aqueous (top) layer to fresh Eppendorf tube. Add 40 µl of 3 M sodium acetate, 1 µl of glycogen and 1 ml of EtOH. Place at −80 °C for 30 min.

TABLE 1 | Set up of master mix for qPCR analysis.

Reagent	Final concentration in Rx	Amount (µl) in 1 Rx	Master mix for 72 samples (No. of samples + 3 = 72) (µl)
H_2O	NA	8.65	623
10× Buffer	1	2.50	180
$MgCl_2$ 25 mM	5.5 mM	5.50	396
dA 10mM	200 µM	0.50	36
dC 10mM	200 µM	0.50	36
dG 10mM	200 µM	0.50	36
dT 20mM	400 µM	0.50	36
Forward primer 10 pmol $µl^{-1}$	200 nM	0.50	36
Reverse primer 10 pmol $µl^{-1}$	200 nM	0.50	36
TaqMan probe 10 pmol $µl^{-1}$	100 nM	0.25	18
AmpliTaq Gold 5 U $µl^{-1}$	0.5 U	0.10	7.2
Total		20.00	1,440
DNA	2–100 ng	5.00	None

80| Spin at 15,800*g* for 15 min at 4 °C. Remove the supernatant by aspiration and discard.

81| Add 500 µl of 70% EtOH to the pellet, vortex and spin at 15,800*g* for 5 min at 4 °C. Remove the supernatant by aspiration and discard.

82| Dry pellet for 5 min in a speed vac without heat (or air-dry for 15 min).

83| Redissolve pellet in 200 µl of 10 mM Tris, pH 7.4. This amount of precipitated DNA should be sufficient to analyze up to 20 genomic locations in triplicate.
■ **PAUSE POINT** Purified DNA can be stored at −20 °C for 6 months or at −80 °C for several years.

qPCR analysis of DNA associated with modified histones
● **TIMING 3 h**
84| Primers and probes can be designed against specific DNA sequences using the PrimerQuest algorithm available on the IDT website (see Experimental design for further details).

85| Set up qPCR by preparing enough master mix for your number of samples plus a little extra to control for pipetting error. A sample master mix for 69 samples is provided in **Table 1**. One master mix must be prepared for each hydrolysis probe/primer sets (i.e., for each genomic position you wish to study) and is pipetted into the PCR tubes.
▲ **CRITICAL STEP** Pipette carefully because small differences could result in large error bars.

86| An aliquot (5 µl) of the elutions from the immunoprecipitation (obtained in Step 83) is then pipetted into the tubes containing the master mix. Each elution should be analyzed in triplicate.

87| A series of qPCR should also be performed on a serial dilution of genomic DNA (50 ng to 16 pg) using each hydrolysis probe/primer set to prepare a standard curve. Genomic DNA is prepared from the cell line of interest using standard procedures[22]. A sample experiment setup using two biological samples (A and B), studied using three different antibodies that are analyzed with a single hydrolysis probe/primer set, is provided in **Table 2**.
▲ **CRITICAL STEP** Pipette carefully because small differences could result in large error bars.

TABLE 2 | Example of qPCR analysis of three different epigenetic modifications at a specific gene locus both before and after differentiation.

Tube	Sample	Tube	Sample
1	Input 0 h (A)	37	H3K4me3 0 h (A)
2	Input 0 h (A)	38	H3K4me3 0 h (A)
3	Input 0 h (A)	39	H3K4me3 0 h (A)
4	Input 0 h (B)	40	H3K4me3 0 h (B)
5	Input 0 h (B)	41	H3K4me3 0 h (B)
6	Input 0 h (B)	42	H3K4me3 0 h (B)
7	Input 48 h (A)	43	H3K4me3 48 h (A)
8	Input 48 h (A)	44	H3K4me3 48 h (A)
9	Input 48 h (A)	45	H3K4me3 48 h (A)
10	Input 48 h (B)	46	H3K4me3 48 h (B)
11	Input 48 h (B)	47	H3K4me3 48 h (B)
12	Input 48 h (B)	48	H3K4me3 48 h (B)
13	Mock 0 h (A)	49	H4ac 0 h (A)
14	Mock 0 h (A)	50	H4ac 0 h (A)
15	Mock 0 h (A)	51	H4ac 0 h (A)
16	Mock 0 h (B)	52	H4ac 0 h (B)
17	Mock 0 h (B)	53	H4ac 0 h (B)
18	Mock 0 h (B)	54	H4ac 0 h (B)
19	Mock 48 h (A)	55	H4ac 48 h (A)
20	Mock 48 h (A)	56	H4ac 48 h (A)
21	Mock 48 h (A)	57	H4ac 48 h (A)
22	Mock 48 h (B)	58	H4ac 48 h (B)
23	Mock 48 h (B)	59	H4ac 48 h (B)
24	Mock 48 h (B)	60	H4ac 48 h (B)
25	H3K4me2 0 h (A)	61	No template control
26	H3K4me2 0 h (A)	62	No template control
27	H3K4me2 0 h (A)	63	No template control
28	H3K4me2 0 h (B)	64	Standard mouse DNA 50 ng
29	H3K4me2 0 h (B)	65	Standard mouse DNA 10 ng
30	H3K4me2 0 h (B)	66	Standard mouse DNA 2 ng
31	H3K4me2 48 h (A)	67	Standard mouse DNA 400 pg
32	H3K4me2 48 h (A)	68	Standard mouse DNA 80 pg
33	H3K4me2 48 h (A)	69	Standard mouse DNA 16 pg
34	H3K4me2 48 h (B)	70	
35	H3K4me2 48 h (B)	71	
36	H3K4me2 48 h (B)	72	

ChIP was prepared on nucleosomes isolated from C2C12 cells under growth (A) or differentiation (B) conditions. Biological samples are analyzed in triplicate for 3 test antibodies (H3K4me2, H3K4me3 and H4ac) and a mock immunoprecipitation. A serial dilution of mouse genomic DNA is used to establish relative quantitation of the immunoprecipitated DNA.

88| To calculate the relative enrichment of the genomic region during your immunoprecipitation, subtract the value obtained in your mock ChIP from the value obtained from your test antibody ChIP. This value can then be divided by the value obtained for your input material to determine the relative enrichment of a histone modification at a single genomic location.
? TROUBLESHOOTING

● **TIMING**
Steps 1–16, isolation of nuclei: ~2 h
Steps 17–21, fragmentation of chromatin using MNase: ~15 min
Steps 22–46, purification of nucleosomes using hydroxyapatite: ~30 min (if the purification is to be examined by agarose gel electrophoresis before proceeding to the immunoprecipitation, this step will take approximately 3 h)
Steps 47–83, immunoprecipitation of modified nucleosomes: ~20 h, including an overnight incubation of 14 h, which creates a natural pause point. (Preparation of the antibody for immunoprecipitation (Steps 47–62) will take ~2 h and must be

completed before the overnight incubation. To save time, this can be done concurrently with Steps 1–21 or during the agarose gel electrophoresis step (Step 44). On day 2, washing of the antibody and elution will require approximately 4–6 h depending on the number of different antibodies studied in parallel.)
Steps 84–88, analysis of DNA associated with modified histones: ~3 h for each different qPCR study

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	Cells do not lyse in presence of NP-40	Cells are resistant to 0.3% NP-40	Try making a new 2× lysis buffer containing greater than 0.6% NP-40
		NP-40 solution is too old	Prepare master mix afresh
25	Some HAP resin is found in the flow-through after centrifugation of the column	A column containing a frit at the bottom has not been used or frit is of poor quality	Add a small amount of siliconized glasswool to the bottom of the column before adding HAP/chromatin slurry
45	Chromatin appears only as high molecular weight fragments after MNase digestion	MNase concentration is too low, or inactive	Dilute MNase afresh (Step 17)
		$CaCl_2$ concentration of buffer is too high or too low	Prepare buffer N afresh
	Chromatin appears as a mixture of short nucleosomal arrays and higher molecular weight fragments after MNase digestion	A single nuclei suspension was not attained before MNase digestion	Expose cells to a short burst of sonication to break up cells. If this does not work, start over with a new batch of cells and ensure that cells/nuclei do not clump
	Nucleosomes are detected in the flow-through of the HAP column	The ratio HAP/chromatin is too low	Increase the amount of HAP resin (Step 23)
		The pH of the buffers is not correct	Ensure that the pH of the HAP buffer is 7.2
	Nucleosomes are detected in the wash fractions of the HAP purification	The pH of the buffers is not correct	Ensure that the pH of the HAP buffers is 7.2
88	No signal is observed after qPCR	The master mix is not made correctly	Prepare master mix afresh
		Your set of primers/probe is not efficient	Choose another set of primers/probes
	A high background/low enrichment is observed after qPCR	The ratio of beads/antibody over chromatin is too low and the antibody is saturated	Decrease the amount of chromatin to make sure your antibody is not saturated (Step 61)

ANTICIPATED RESULTS

The procedure described above generates ~200 µg of nucleic acid for MNase digestion from ~10^7 mammalian cells. Conditions for digesting the chromatin with MNase should be determined empirically to obtain a mixture of nucleosomal arrays consisting mainly of mononucleosomes (150–175 bp), but laddering up to at least trinucleosomes should be visible in UV light after treatment with ethidium bromide (see **Fig. 3a**, digested nuclei). This will provide a resolution of ~500 bp in ChIP analysis. Lysis of the nuclei in the presence of high salt leads to an efficient extraction of the chromatin that binds to the HAP resin to form a thick paste. Once transferred to the column and subjected to centrifugation, no chromatin should be released in the column flow-through. If chromatin is detected in the flow-through, it is likely that you have not used the proper ratio of HAP resin to nucleic acid content. Similarly, no chromatin should be present in the wash fractions. In contrast, chromatin should be released within the first two elution steps (see **Fig. 3a**). The chromatin eluted from the HAP column should be largely devoid of contaminating proteins that were present in the extracted chromatin before HAP binding (see **Fig. 3b**). As shown by silver-stained SDS–polyacrylamide gel electrophoresis (SDS-PAGE), only four protein species between 12 and 18 kDa, which represent histones H3, H2B, H2A and H4 in descending order, are detected in the elution fractions. Furthermore, genomic

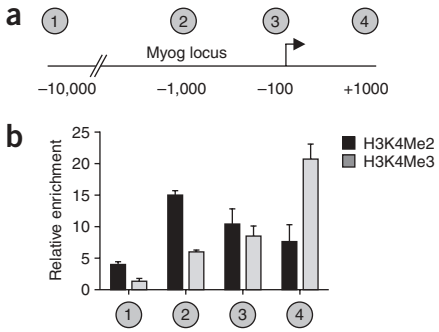

Figure 4 | Distribution of H3K4me2 and H3K4me3 across the Myog locus. (**a**) Schematic representation of the Myog gene locus. The numbers 1 through 4 represent the position of primer sets used in the ChIP studies. (**b**) Native ChIP analysis was used to measure relative enrichment of H3K4me2 and H3K4me3 at various locations along the Myog locus in differentiating (48 h) C2C12 cells as described above. Resolution of the native ChIP was ~500 bp after micrococcal nuclease digestion. Relative enrichment is expressed as the signal observed on the Myog gene with respect to that observed at the inactive IgH gene. Average values of duplicate qPCRs are displayed with error bars corresponding to ±s.d. Each experiment was performed at least twice independently[16]. Reproduced with permission from *Nature Structural and Molecular Biology*.

DNA should be representative of all types of chromatin, including euchromatin, facultative heterochromatin and constitutive heterochromatin (see **Fig. 2**).

Figure 4 exemplifies an application of this protocol to the study of dimethylation and trimethylation of H3K4 across the myogenin locus. In differentiating myoblasts, when the myogenin gene is actively transcribed, we observe that the promoter region is preferentially marked by dimethylation at histone H3 lysine 4 (H3K4me2), whereas the transcribed region of the gene is marked by trimethylation at histone H3 lysine 4 (H3K4me3). Using these antibodies, we observe a 1.5- to 2-fold greater enrichment of histone modifications compared to X-ChIPs performed using the same primer/probe sets (data not shown). However, the fold enrichment observed in HAP-NChIP compared to X-ChIP depends on the antibody used and can be 20- to 50-fold greater for some antibodies (data not shown). We believe that this difference likely reflects the degree to which the modification is sterically hindered under X-ChIP conditions, as well as the differential ability of antibodies to recognize their target in X-linked conditions. Indeed, the anti-H3K4me2 and anti-H3K4me3 antibodies have been used quite extensively in the literature to localize these marks of active genes and as such appear quite efficient in X-ChIP conditions. Finally, when assessing a ChIP study, you must keep in mind the quality of the antibodies with respect to their specificity (i.e., how many fold greater is their affinity for the modified histone versus unmodified histone). Thus, it should be remembered that quality of the ChIP analysis is very dependent upon the quality of the antibody that is used for immunoprecipitation.

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