Analysis of Histone Exchange during Chromatin Purification

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Abstract

Central to the study of chromosome biology are techniques that permit the purification of small chromatin sections for analysis of associated DNA and proteins, including histones. Chromatin purification protocols vary greatly in the extent of chemical cross-linking used to prevent protein dissociation/re-association during isolation. Particularly for genome-wide analyses, chromatin purification requires a balanced level of fixation that captures native protein-protein and protein/DNA interactions, yet leaving chromatin sections soluble and accessible to affinity reagents. We have applied a relative quantification methodology called I-DIRT (isotopic differentiation of interactions as random or targeted) for optimizing levels of chemical cross-linking for affinity purification of cognate chromatin sections. We show that fine-tuning of chemical cross-linking is necessary for isolation of chromatin sections when minimal histone/protein exchange is required.

Keywords: Cross-linking; Histone; Chromatin; Affinity Purification.

Abbreviations

I-DIRT, isotopic differentiation of interactions as random or targeted; MALDI, matrix-assisted laser desorption ionization; FA, formaldehyde; ChIP, chromatin immunoprecipitation; ChIP-chip, chromatin immunoprecipitation with DNA chip readout; ChIP-seq, chromatin immunoprecipitation with DNA sequencing readout.

1. Introduction

The eukaryotic genome is highly organized into transcriptionally active or repressive chromatin compartments, which consist of repeating octamers of histones called nucleosomes. Access to these regions may be epigenetically regulated in part by covalent post-translational modifications (PTMs) of histone proteins [1]. Histone PTMs are proposed to act as chemical flags that functionally partition chromatin through direct binding/targeting of protein complexes with distinct properties [2]. The field of chromatin biology employs technologies like ChIP (chromatin immunoprecipitation), affinity purification of protein/histone complexes for proteomic analysis, and more recent technology that allows for the purification of chromosome sections for proteomic analysis [3-5]. Central to each of these techniques is the purification of chromatin sections with cognate histones. To overcome the inherent exchange of histones and other proteins during the isolation of chromatin sections, investigators utilize in vivo chemical cross-linking with agents such as formaldehyde. However, a quantitative analysis of the level of protein exchange has not been reported. Additionally, the purification of a chromatin bound protein complex can be challenging as too much cross-linking renders the complex insoluble, while too little cross-linking does not trap less stable protein interactions [6].

Here we utilize an isotopic labeling approach with affinity purification to readily gauge levels of histone exchange in purified chromatin samples. The approach described is an application of our previously reported I-DIRT (isotopic differentiation of interactions as random or targeted) technology (Fig. 1) [7]. The fundamental basis of I-DIRT is the mixing
2. Material and methods

*Saccharomyces cerevisiae* HTB1::TAP-HIS3 BY4741 (Open Biosystems) cells were grown in isotopically light synthetic media, while an arginine auxotrophic strain (arg4::KAN BY4741, Open Biosystems) was grown in isotopically heavy synthetic media (13C$_6$ arginine, 80 mg/L, Cambridge Isotope Laboratories, CLM-2265). Synthetic media consisted of 6.7 g/L yeast nitrogen base without amino acids (Sigma), 2 g/L synthetic drop-out media minus lysine (US Biological), 80 mg/L lysine (Fisher) and 20% (w/v) glucose (Fisher). Both strains were grown to ~3 x 10$^7$ cells/mL at 30°C, cross-linked for 5 minutes with formaldehyde (0, 0.05, 0.25 or 1.25% formaldehyde (Sigma)), and quenched for 5 minutes with 125 mM glycine. Cells were harvested, frozen as pellets in liquid nitrogen, mixed 1:1 (isotopically light cells: heavy cells) by cell weight, and co-cryogenically lysed with a Retsch MM301 mixer mill. One gram of each lysate (equivalent to ~1.5 x 10$^8$ cells) was re-suspended in 5 mL of affinity purification buffer (20 mM HEPES pH 7.4, 300 mM NaCl, 0.1% tween-20, 2 mM MgCl$_2$ and 1% Sigma fungal protease inhibitors). Chromosomal DNA was sheared to ~800nt sections with a Bioruptor (Diagenode). The Bioruptor was set to 12 cycles of 30 seconds with sonication followed by 30 seconds without sonication, set to the “high” sonication option, and maintained at 4°C with a circulating water bath. The resulting lysates were clarified by centrifugation (2,500 x g) for 10 min. H2B-TAP was collected from the supernatants with 4 mg of IgG-coated Dynabeads (Invitrogen) for 4 hours at 4°C [6]. Beads were washed 5-times with affinity purification buffer and treated with 0.5 N ammonium hydroxide / 0.5 mM EDTA to elute proteins. Eluted proteins were lyophilized, re-suspended in a reducing SDS-PAGE loading buffer and heated at 90°C for 20 min (which provided for reversal of formaldehyde cross-links).

Proteins were resolved on 4-20% Novex Tris-Glycine gels (Invitrogen), visualized by colloidal Coomassie staining and the region of the gel containing core histones was excised as 2-mm bands for protein identification. Proteins were digested in-gel with 100 ng trypsin (Roche) and peptides were subjected to tandem mass spectrometric analysis with a coupled Eksigent NanoLC-2D and Thermo LTQ-Orbitrap mass spectrometer [6]. Briefly, peptides were eluted from a New Objective IntegraFrit column (10 cm, 50 µm ID) packed with 4 µm Phenomenex Jupiter Proteo resin over 50 minutes with a gradient of 0.1% acetonitrile / 0.1% formic acid to 75% acetonitrile / 0.1% formic acid (0.5 µL/min flow rate) and fragmented at 35% collision energy. Precursor ions were measured in the Orbitrap mass analyzer, while peptide fragmentation and fragment ion detection occurred in the linear ion trap. A Mascot (version 2.2.03) database search identified isotopically light and heavy arginine containing histone peptides. Database parameters were as follows: precursor ion tolerance 10 ppm, fragment ion tolerance 0.6 Da, fixed modification of carbamidomethyl on cysteine, variable modification of oxidation on methionine, and 2 missed cleavages possible with trypsin (see Supplemental Table 1 for a list of all...
proteins identified). Monoisotopic peak areas were extracted for each of the arginine containing histone peptides from the raw data files using Qual Browser version 2.0 (Thermo) and the percent isotopically light peptide was calculated (Supplemental Table 2). For each histone reported, multiple arginine-containing peptides were identified and the average percent isotopically light is reported.

3. Results and Discussion

We investigated the utility of in vivo chemical cross-linking with formaldehyde for preventing histone exchange during chromatin purification. Chemical cross-linking has been utilized to trap in vivo protein-protein and/or protein-DNA interactions for mass spectrometric analysis; however, extensive cross-linking can render the chromatin insoluble [6]. To identify the amount of chemical cross-linking required to prevent histone exchange during chromatin purification, the relative quantitative affinity purification strategy I-DIRT (isotopic differentiation of interactions as random or targeted) was utilized (Figure 1) [7]. Saccharomyces cerevisiae cells containing a TAP-tagged histone H2B were grown in isotopically light synthetic media, while an arginine auxotrophic strain (arg4::KAN) was grown in isotopically heavy synthetic media (13C6 arginine). Both cultures were independently cross-linked with formaldehyde; and harvested cells were mixed 1:1 (isotopically light cells: heavy cells) for cocryogenic lysis. Chromosomal DNA was sheared to ~800nt sections (Figure 2A). As the percentage of cross-linking increased the ability to shear genomic DNA decreased as shown in Figure 2A. Low levels of sheared DNA were detectable up to 1.25% formaldehyde cross-linking. The purification of these sheared chromosomal sections via H2B-TAP was followed by western blotting (Figure 2B). As observed for DNA shearing in Figure 2A, increasing amounts of formaldehyde cross-linking showed a decrease in the ability to purify sheared chromatin. A cross-linking level of 1.25% formaldehyde was found to be near the upper limit of chemical cross-linking that still provided for shearing and enriching chromatin sections.

Chromatin sections were next purified from ~1.5 x 1010 cells (1:1 mixture of light H2B-TAP and heavy non-tagged cells) and proteins co-purifying with H2B-TAP were resolved by SDS-PAGE (Figure 2C). H2B-TAP, H2B, H2A, H3 and H4 were visible by Coomassie staining and detected by mass spectrometry at each level of formaldehyde cross-linking tested. Proteins were digested in-gel with trypsin and peptides were subjected to tandem mass spectrometric analysis with a coupled Eksigent NanoLC-2D and Thermo LTQ-Orbitrap mass spectrometer [6]. A Mascot database search identified arginine containing histone peptides. Monoisotopic peak areas were extracted for each of the arginine containing histone peptides and the percent isotopically light peptide was calculated. The following numbers of arginine-containing histone peptides were used for the percent isotopically light calculation: histone H2B-TAP (100 peptides), H3 (169 peptides), H2A (45 peptides), and H4 (95 peptides). If

Figure 2. Purification of formaldehyde cross-linked chromatin. (A) DNA shearing as a function of formaldehyde (FA) cross-linking. DNA was isolated from formaldehyde treated H2B-TAP cells, resolved by electrophoresis and visualized by ethidium bromide staining. (B) Affinity purification of chromatin was monitored by western blotting for H2B-TAP. P, lysate pellet; S1, pre-purification supernatant; S2, post-purification supernatant; AP, affinity purified. (C) Sheared chromatin was affinity purified then histones were resolved by SDS-PAGE, visualized by Coomassie staining and excised for mass spectrometric analysis/identification.
an arginine-containing histone peptide was identified more than once in the analysis, then each spectrum was used in the calculation of percent isotopically light peptides. Representative mass spectra for arginine containing peptides from H2B-TAP and H4 are shown in Figure 3A & B. Reported and averaged together in Figure 3C are both unmodified and post-translational modification containing histone peptides from triplicate experiments. Levels of light peptides near 100% reflect minimal exchange of a protein during chromatin purification, while levels near 50% reflect rapid exchange. In Figure 3C, we show the average of ten non-specifically associating proteins, which are often observed as contaminants in affinity purifications (Rp59b, L39B, Ssb1, Rp52, Ssc1, Act1, Enol, Hsc82, Ssa2, Fba1). The percent isotopically light for these non-specific proteins was approximately 50-60% reflecting rapid exchange of these non-specifically associating proteins with the purified chromatin. Arginine containing peptides from H2B-TAP were exclusively light because the tagged protein was only expressed in the isotopically light culture (Figure 3A & C). Histone H2A showed minimal exchange with H2B-TAP, which indicates that the H2B/H2A interaction is stable with or without cross-linking. However, histone H3 and H4 showed a similar level of exchange (~10-20%) from 0.05% to 0.25% formaldehyde cross-linking (Figure 3C). This level of H3/H4 exchange would significantly alter the purity of chromatin sections isolated for experimental analyses such as chromatin immuno-precipitation. The similar level of exchange of H3 and H4 likely reflects the preferential affinity of these histones, which form the tetrameric core of the nucleosome. At 1.25% cross-linking, the histone H3 and H4 showed no exchange. These results indicate that 1.25% formaldehyde cross-linking is necessary to eliminate any histone exchange during chromatin purification. It is noted that the 1.25% formaldehyde is specific for yeast synthetic media, as other medias will require different levels of cross-linking in accordance to their amine or cross-linking moiety content. Thus, at 1.25% formaldehyde cross-linking in yeast synthetic media, soluble chromatin sections can be generated by sonication and isolated by affinity purification without histone exchange.

We present the application of I-DIRT technology for determining the level of histone dissociation/re-association during purification of chromatin. The technique will be broadly applicable for demonstrating purification of cognate chromatin sections in different cellular growth media and with various in vivo chemical cross-linking reagents. Determining the optimal level of in vivo chemical cross-linking is needed to find the balance between solubilizing the chromatin and preventing histone exchange. This greater emphasizes the need to optimize the level of in vivo cross-linking as one desires to prevent exchange while maximizing purification.

4. Future perspectives

Currently, the localization of proteins and histone PTMs on chromosomes is largely studied with techniques like ChiP-chip and more recently ChiP-seq. These technologies

Figure 3. In vivo chemical cross-linking prevents histone exchange of cross-linked chromatin. Shown are representative mass spectra collected with an Orbitrap mass analyzer for doubly charged peptides from histone H2B-TAP (A) and histone H4 (B). The percent isotopically light peptide (%L) is indicated. (C) Percentage isotopically light arginine containing histone peptides are reported as a function of formaldehyde (FA) cross-linking. The standard error from triplicate experiments is shown. Levels approaching ~100% indicate minimal exchange, while those at ~50% reflect rapid exchange. The average of ten rapidly exchanging / non-specific proteins is shown (non-specific).
are quite powerful for the high resolution localization of a given protein or modified histone; however, they lack the ability to simultaneously identify all proteins bound and the combinatorial nature of the modified histones at a given chromosomal region. As the field matures, technology development will move toward the isolation of sections of chromatin for mass spectrometric analysis of cognate histones and bound proteins. Recent examples of this are the isolation of telomeric and origin of replication chromatin for mass spectrometric analyses [5,11]. The technique we report here will play a significant role in these types of studies as one would need to ensure that the purified chromatin is representative of the in vivo setting.

5. Supplementary material
Supplementary material regarding this manuscript is online available in the web page of JIOMICS.

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References