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Review Article

Decoding the *trans*-histone crosstalk: Methods to analyze H2B ubiquitination, H3 methylation and their regulatory factors

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ABSTRACT

Regulation of histone H3 lysine 4 and 79 methylation by histone H2B lysine 123 monoubiquitination is an evolutionarily conserved *trans*-histone crosstalk mechanism, which demonstrates a functional role for histone ubiquitination within the cell. The regulatory enzymes, factors and processes involved in the establishment and dynamic modulation of these modifications and their genome-wide distribution patterns have been determined in many model systems. Rapid progress in understanding this *trans*-histone crosstalk has been made using the standard experimental tools of chromatin biology in budding yeast (*Saccharomyces cerevisiae*), a highly tractable model organism. Here, we provide a set of modified and refined experimental procedures that can be used to gain further insights into the underlying mechanisms that govern this crosstalk in budding yeast. Importantly, the improved procedures and their underlying principles can also be applied to other model organisms. Methods presented here provide a rapid and efficient means to prepare enriched protein extracts to better preserve and assess the steady state levels of histones, non-histone proteins and their modifications. Improved chromatin immunoprecipitation and double immunoprecipitation protocols are provided to measure the occupancy and distribution of proteins and their modified forms at specific chromatin regions or loci. A quick and easy method to measure overall protein abundance and changes in protein–protein and protein–DNA interactions on native chromatin is also described.

1. Introduction

In a eukaryotic nucleus, DNA is wrapped around an octamer of basic histone proteins (H2A, H2B, H3 and H4) to form nucleosomes, the building blocks of chromatin. An important regulatory mechanism that governs overall chromatin structure for factor access is the covalent posttranslational modifications of histones. Histones are extensively adorned with a wide variety of modifications, including acetylation, phosphorylation, methylation, ubiquitination and sumoylation [1,2]. Methylation of lysine residues (K) in histones can be present as a mono- (me1), di- (me2) or trimethylated (me3) form, further diversifying the function and complexity of this modification. While histone modifications serve as "marks" for cellular processes [2,3] and are recognized by factors with specific interaction modules [1,4], their regulation is highly dynamic with the presence of different types and families of enzymes that can either add or remove them [5]. Additionally, these enzymes, which might exist as components of multi-protein complexes, are themselves regulated by both endogenous and exogenous cues. Another interesting facet to the regulation of histone modifications is that one modification can regulate another within the same histone (in *cis*) or on different histones (in *trans*), a phenomenon termed the "histone crosstalk" [6–8]. Regulation of H3K4 and -K79 methylation by H2BK123 monoubiquitination (H2Bub1) during transcription is a well-studied example of a *trans*-histone modification crosstalk.

This *trans*-histone regulatory circuit is evolutionarily conserved, as H2Bub1 is important for H3K4 and -K79 methylation in most eukaryotes [9]. However, several valuable characteristics, such as the short life cycle, powerful genetics, ease of genome manipulation and being easily amenable to biochemical and high-throughput genome-wide studies, have made budding yeast (*Saccharomyces cerevisiae*) as the model system of choice to identify and extensively investigate the regulatory pathways, factors and enzymes involved in mediating this *trans*-histone crosstalk. Rad6, the E2 ubiquitin-conjugating enzyme and Bre1, the E3 ubiquitin ligase are required to conjugate ubiquitin to K123 present in the H2B C-terminal helix [10,11]. Ubp8 and Ubp10 are the deubiquitinases involved in the removal of this conjugated ubiquitin to maintain the total H2Bub1 levels in the cell [9]. Set1-COMPASS, a multi-protein complex consisting of the methyltransferase (Set1) and seven subunits (Swd1, Swd2, Swd3, Bre2, Sdc1, Spp1 and Shg1), catalyzes all forms of H3K4 methylation (H3K4me). Dot1, a non-SET domain methyltransferase, catalyzes

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all forms of H3K79 methylation (H3K79me) [1,11]. A role for H2Bub1 in modulating the enzymatic functions of these methyltransferases by affecting their ability to catalyze the different forms of H3K4 and K79 methylation is now well established [9,11]: H2Bub1 is required for H3K4me2, H3K4me3 and H3K79me3. Additionally, H3K4me1 and H3K79me2 are severely reduced in the absence of H2Bub1.

To explain the mechanism of *trans*-histone crosstalk, it was proposed that the ubiquitin conjugated onto H2B might act as a "bridge" to directly recruit the methyltransferases [12]. Since Set1 and Dot1 associate with chromatin even in the absence of H2Bub1 [13,14], their recruitment does not appear to be the basic mechanism by which H2Bub1 participates in the crosstalk. Two studies have alluded to Swd2, a Set1-COMPASS subunit, as a key link in this crosstalk [15,16], but their conflicting findings and conclusions have left the regulation of methyltransferase functions by H2Bub1 as an open question. H2Bub1 has also been proposed to act as a "wedge" to open-up the chromatin and allow access to the enzymes [12,17]. However, contrary to its supposed role in opening up chromatin, using chromatin immunoprecipitation assays (ChIP) and salt-dependent nucleosome disruption assays, we recently showed that H2Bub1 stabilizes the nucleosome by preventing the constant H2A-H2B eviction [18]. This finding addresses a longstanding question in chromatin biology as to whether conjugation of bulky ubiquitin moiety onto histones affects nucleosome structure. Further, it has provided a new working model for the *trans*-histone crosstalk: addition of ubiquitin onto H2B acts as a "glue" to hold the nucleosome together and provides a stable platform for the prolonged chromatin association of Set1-COMPASS and Dot1 to promote their processive methylation. However, understanding this *trans*-histone crosstalk is an ongoing saga that is far from completion.

Several questions pertaining to the mechanism of nucleosome stabilization by H2Bub1 and the chromatin association of Set1-COMPASS and Dot1 remain to be explored. The basic patch in H4N-terminal plays a role in a novel *trans*-histone pathway by controlling the chromatin binding and functions of Dot1 [19]. However, the question as to how H2Bub1 controls Dot1 function remains unanswered. While a "docking site" for Dot1 on chromatin via the H4 tail region is now known, how Set1-COMPASS associates with chromatin and how this multi-subunit protein complex is assembled on chromatin remain unknown. We recently found that residues R119 and T122 in the H2B C-terminal helix interact with Spp1, a Set1-COMPASS subunit, and they modulate the chromatin association, integrity and overall stability of Set1-COMPASS independent of H2Bub1 [20]. Importantly, we have uncovered a "docking" surface for only Set1-COMPASS, since mutations in R119 and T122 do not affect the functions of Dot1; thereby, revealing an uncoupling of the H2Bub1-mediated co-regulation of H3K4 and -K79 methylation. Therefore, a simple model that can be proposed for the *trans*-histone crosstalk between H2Bub1 and H3K4 methylation is as follows: H2Bub1 stabilizes the nucleosome by preventing H2A-H2B eviction. In turn, this leads to the retention of a "docking site" for Set1-COMPASS present in H2B on chromatin, culminating in increased complex integrity and stability of Set1-COMPASS needed for high levels of processive H3K4 methylation. While considerable effort has been invested in understanding how the methyltransferases associate with chromatin, the binding of Rad6/Bre1 and Ubp8/Ubp10 to chromatin remains poorly understood and needs further investigation.

In this report, we provide detailed procedures used in our previous studies to address some of the questions mentioned above. An improved method is provided to assess the steady state levels of histones and any other proteins in total cell extracts or nuclear extracts isolated under native or denaturing condition (Sections 2.1.1 and 2.1.2). A quick and easy method to isolate and detect H2Bub1, a highly labile modification, employing a simple boiling procedure is described in Section 2.1.3. To measure changes in the chromatin association of histones, histone modifying enzymes and their regulatory factors, two different assays are described in Section 2.2. The chromatin association assay measures global changes in protein levels on chromatin obtained from isolated nuclei (Section 2.2.1). On the other hand, local changes in the occupancy and distribution of histones, histone modifications and factors on genes or in different regions of a gene can be assessed employing the ChIP assay (Section 2.2.2.1). A procedure to evaluate changes in the distribution and occupancy of H2Bub1 using chromatin-double immunoprecipitation is provided in Section 2.2.2.2.

2. Methods

2.1. Assessment of global H3K4 and -K79 methylation and H2Bub1 by Western blotting

2.1.1. Preparation of yeast whole cell extracts by bead-beat procedure

1. Cultures grown overnight are used to re-inoculate fresh 50 ml medium at a starting OD_{600} 0.2 and grown to a final OD_{600} 0.8–1.2. Cells ($30–40 \times 10^7$) are harvested by centrifugation at 3000 rpm for 2 min and washed once with 1 ml water while transferring to a 2.0 ml screw-cap tube.
2. Cells are collected by centrifugation at 3000 rpm for 2 min and the water is completely discarded. The pellets are stored at −80 °C until further use.
3. The cells are thawed by vigorous vortexing in the presence of 400 μl SWBNG buffer [10 mM Tris.Cl, pH 7.4, 300 mM Sorbitol, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 0.1% Igepal-30 (NP40), protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A)] or SUME buffer (10 mM MOPS, pH 6.8, 0.5% SDS, 8 M Urea, 10 mM EDTA, protease inhibitors) [21].
   Technical Notes:
   a. SWBNG buffer is used to isolate proteins under non-denaturing or native condition and SUME buffer is used to isolate proteins under denaturing conditions.
   b. A variety of buffers can be used to effectively isolate proteins under native conditions as reported previously [22]. In SWBNG buffer, the inclusion of a detergent (NP-40) helps to better solubilize proteins and addition of glycerol prevents protein aggregation.
   c. A low initial NaCl concentration (100 mM) is maintained to prevent excessive sample heating during the bead-beating process.
4. Acid-washed glass beads (Sigma) are added to the top of the cell suspension. Cells are lysed by agitation four times for 30 s each in a Mini-beadbeater (Biospec) set at "homogenize" mode. The lysates are cooled on ice for 30 s between successive agitations.
   Technical Note: Cooling on ice should be avoided for samples prepared in SUME buffer to prevent precipitation of SDS.
5. A hole is punched at the bottom of the 2 ml screw-cap tube using an 18-guage needle. The lysate is collected by brief centrifugation into a 15 ml collection tube fitted with an adaptor, which is made by cutting the top part of a 5 ml syringe.
6. The lysate is resuspended by brief vortexing and an aliquot (300 μl) is transferred to a labeled 1.5 ml microfuge tube. 26 μl 5 M NaCl is added to the tube to obtain a final NaCl concentration of 500 mM. The lysate is vortexed vigorously for 30–40 s.
   Technical Note: Increased NaCl concentration to 500 mM aids in disrupting the nucleosome and solubilization of histones [18,23].