Histone Monoubiquitylation Position Determines Specificity and Direction of Enzymatic Cross-talk with Histone Methyltransferases Dot1L and PRC2*§

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Background: “Designer” nucleosomes are key tools to investigate functions of histone modifications.

Results: Histone H2Aub inhibits PRC2 methylation of histone H3 Lys-27 but not Dot1L methylation of H3 Lys-79.

Conclusion: The position of nucleosome monoubiquitylation affects the specificity and direction of cross-talk with histone methyltransferases.

Significance: Cross-talk between histone modifications and chromatin enzymatic activities may be an important mechanism for generating distinct biological outputs.

It is well established that chromatin is a destination for signal transduction, affecting many DNA-templated processes. Histone proteins in particular are extensively post-translationally modified. We are interested in how the complex repertoire of histone modifications is coordinately regulated to generate meaningful combinations of “marks” at physiologically relevant genomic locations. One important mechanism is “cross-talk” between pre-existing histone post-translational modifications and enzymes that subsequently add or remove modifications on chromatin. Here, we use chemically defined “designer” nucleosomes to investigate novel enzymatic cross-talk relationships between the most abundant histone ubiquitylation sites, H2AK119ub and H2BK120ub, and two important histone methyltransferases, Dot1L and PRC2. Although the presence of H2Bub in nucleosomes greatly stimulated Dot1L methylation of H3K79, we found that H2Aub did not influence Dot1L activity. In contrast, we show that H2Aub inhibited PRC2 methylation of H3K27, but H2Bub did not influence PRC2 activity. Taken together, these results highlight how the position of nucleosome monoubiquitylation affects the specificity and direction of cross-talk with enzymatic activities on chromatin.

Chromatin is the physiologically relevant form of eukaryotic genomes and thereby the biological substrate for DNA-templated processes. Histone proteins are extensively post-translationally modified. We are interested in how the complex repertoire of histone modifications is coordinately regulated to generate meaningful combinations of “marks” at physiologically relevant genomic locations. One important mechanism is “cross-talk” between pre-existing histone post-translational modifications and enzymes that subsequently add or remove modifications on chromatin. Here, we use chemically defined “designer” nucleosomes to investigate novel enzymatic cross-talk relationships between the most abundant histone ubiquitylation sites, H2AK119ub and H2BK120ub, and two important histone methyltransferases, Dot1L and PRC2. Although the presence of H2Bub in nucleosomes greatly stimulated Dot1L methylation of H3K79, we found that H2Aub did not influence Dot1L activity. In contrast, we show that H2Aub inhibited PRC2 methylation of H3K27, but H2Bub did not influence PRC2 activity. Taken together, these results highlight how the position of nucleosome monoubiquitylation affects the specificity and direction of cross-talk with enzymatic activities on chromatin.

2 The abbreviations used are: PTM, post-translational modification; MTase, methyltransferase; Ub-SH, ubiquitin-aminoethanethiol.
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a4(K119C), H2B.k, and H2B.k(K120C) were expressed as His6-tagged fusion proteins in recombinant human histones H3.2(C110A), H4, H2A.a4, H2A.a4(K119C), H2B.k, and H2B.k(K120C) were expressed as His6-tagged fusion proteins in H3K4me in vitro and in vivo was recently identified.

Despite the remarkable progress in understanding the mechanistic role of H2Bub in transcriptional regulation (17–20), the function of the predominant ubiquitylated species in metazoan chromatin, H2A monoubiquitylated at Lys-119 (H2Aub), remains less well understood. Studies of H2Aub function have been slowed in part by its restriction mostly to metazoans (unlike H2Bub, which is present in yeast), where histone genetics is not tractable. However, the discovery of the major E3 ligase for H2Aub in mammalian systems, Ring1B, a member of the PRC1 (Polycomb repressive complex 1) family (21), opened the door to studies implicating H2Aub in developmentally regulated transcriptional repression. Recently, using nucleosomes reconstituted with endogenous H2A or H2Aub, Nakagawa et al. (22) showed that deubiquitylation of H2Aub allowed for efficient methylation of H3K4 in vitro. Our specialized nucleosomal reagents allowed us to test whether H2Aub also functions antagonistically to H2Bub in cross-talk with another MTase involved in active transcription, Dot1L. Additionally, given the established direct enzymatic cross-talk between H2Bub and MTases implicated in transcriptional repression, particular PRC2. Using chemically defined mononucleosomes, we tested whether PRC2-mediated H3K27 methylation activity is sensitive to the presence of H2Aub or H2Bub in nucleosomal substrates.

EXPERIMENTAL PROCEDURES

Recombinant Histone Expression and Purification—Recombinant human histones H3.2(C110A), H4, H2A.a4, H2A.a4(K119C), H2B.k, and H2B.k(K120C) were expressed as His6-tagged fusion proteins in Escherichia coli and purified as described previously (23). β-Mercaptoethanol-H2A(K119C) and β-mercaptoethanol-H2B(K120C) adducts formed during purification and tobacco etch virus/ribonuclease 3C cleavage steps were reduced with 20 mM tris(2-carboxyethyl)phosphine at pH 8.5 and purified by reverse-phase HPLC before use in H2AssUb and H2BssUb ligation reactions. Careful removal of reducing agents by extensive dialysis and/or HPLC is crucial for all histone preparations because the eventual disulfide bonds in H2AssUb and H2BssUb are extremely sensitive to reducing agents (see Fig. 2D). Recombinant Xenopus histones were prepared as described previously (15).

H2AssUb and H2BssUb Preparation—Disulfide-directed ubiquitylation of human histones H2A(K119C) and H2B(K120C) (see Fig. 1C) was performed as described previously (24) with the following alteration. Here, the ubiquitin-amineoethanethiol (Ub-SH) adduct, rather than the histone cysteine sulphydryl, was activated with 2,2′-dithiobis(5-nitropyridine). Briefly, an asymmetric disulfide between the C-terminal sulphydryl of Ub-SH and 2,2′-dithiobis(5-nitropyridine) was generated by incubation of 2,2′-dithiobis(5-nitropyridine) and Ub-SH in a 1:3 mixture of acetic acid and water. After 18–24 h at 25 °C, the reaction was dialyzed against water to precipitate unreacted 2,2′-dithiobis(5-nitropyridine), and the Ub-SH-5-nitro-2-pyridinesulfenyl product was purified by C18 reverse-phase HPLC. Purified Ub-SH-5-nitro-2-pyridinesulfenyl was mixed at 1:31 molar ratio with histone (either H2A(K119C) or H2B(K120C)) in a small volume of 6 M guanidine HCl and 1 M HEPES (pH 6.9), and the reaction was stopped with 0.1% TFA after 10 min. The resulting H2AssUb and H2BssUb were purified by C18 reverse-phase HPLC. Note that Invitrogen NuPAGE precast gels reduce the disulfide bond between Ub-SH and histone H2A(K119C) and H2B(K120C), but Bio-Rad TGX gels leave the disulfide intact.

H2Bub/H2Aub(G76A) Preparation—Xenopus H2Bub(G76A) was prepared by semisynthetic expressed protein ligation (see Fig. 1C) as described previously (25). Xenopus H2Aub(G76A) was prepared using an adapted semisynthetic expressed protein ligation strategy which will be described in detail elsewhere.

Octamer Preparation—Octamers were assembled by combining the appropriate purified histones at ~2 mg/ml in denaturing buffer (50 mM MOPS (pH 7), 6 M guanidine HCl, 10% glycerol, and 5 mM EDTA) and dialyzed overnight into high salt buffer (10 mM MOPS (pH 7), 2 mM NaCl, 10% glycerol, and 0.5 mM EDTA). Folded octamers were purified from soluble material by FPLC with Superdex 200 (GE Healthcare) and stored at −20 °C in 50% glycerol. Xenopus H2Bub(G76A) octamers were prepared as described previously (15).

Nucleosome Positioning Sequence Preparation and Mononucleosome Assembly—DNA fragments for mononucleosome assemblies were prepared by EcoRV digest of 32×153-bp tandem repeats of the 601 positioning sequence (26) cloned into pUC19 (a kind gift from Kyle Chiang). Plasmid backbone fragments were precipitated with 7.5% PEG 6000 at 500 mM NaCl, and soluble 153-bp fragments were purified with several phenol/chloroform extractions and ethanol-precipitated. Mononucleosomes were prepared by gradient dialysis as described previously (23), followed by dialysis into low salt buffer (10 mM Tris (pH 7.5), 10% M NaCl, 1 mM EDTA, and 10% glycerol). Assembly quality was assessed by native 5% PAGE and ethidium bromide staining.

3 J. Kim, J.-A. Kim, C. D. Allis, R. G. Roeder et al., personal communication.

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Dot1L MTase Assays—FLAG-tagged human Dot1L was purified from a baculovirus expression system as described previously (15, 25). Typical assays combined 4.1 pmol of mononucleosomes, 0.14 pmol of FLAG-tagged human Dot1L, and 15 pmol of S-[3H]adenosyl-l-methionine (1 μCi of [3H]) in 12 μl of reaction buffer (20 mM Tris (pH 8.0), 10 mM NaCl, 5 mM MgCl2, and 1 mM EDTA) at 30 °C. Activity was assessed by fluorography and/or scintillation counting.

PRC2 MTase Assays—Recombinant PRC2 complex (Ezh2, FLAG-Eed3, Suz12, and RbAP48) was purified using a baculovirus expression system as described previously (27). Typical assays combined 200 ng of nucleosomal DNA, 20 ng of Ezh2 in PRC2 complex, and 15 pmol of S-[3H]adenosyl-l-methionine (1 μCi of [3H]) in 12 μl of reaction buffer (20 mM Tris (pH 8.0), 10 mM NaCl, 2.5 mM MgCl2, 1 mM EDTA, and 0.5 mM DTT) at 30 °C. Activity was assessed by fluorography and/or scintillation counting.

Antibodies—The antibodies used were anti-H2A (Millipore 07-146), anti-H2B (Proteintech), anti-H2AK119ub (22), and anti-H2BK120ub (Cell Signaling Technology 5546).

RESULTS

Design and Characterization of Designer Mononucleosomes—The inherent heterogeneity of chromatin purified from endogenous sources has historically obstructed efforts to establish direct enzymatic cross-talk relationships between histone PTMs. Ideal substrates for cross-talk studies require robust and specific installation of modifications on otherwise unmodified histones (28). Recently, several groups have developed chemical strategies for the site-specific installation of histone PTMs, including monoubiquitylation (29). These chemically defined modified histones can be combined with histones expressed recombinantly in E. coli, devoid of PTMs, to reconstitute so-called designer nucleosomes.

We utilized two complementary methods to quantitatively install monoubiquitin at position 119 in H2A and position 120 in H2B (Fig. 1C): disulfide-mediated (ssUb) (24) and expressed protein ligation (native) (25). These approaches are more specific than in vitro enzymatic ubiquitylation and generate histone-ubiquitin linkages that differ minimally from the native linkage (24, 25). Furthermore, H2BssUb and native H2Bub function indistinguishably from H2Bub with the native linkage in Dot1L MTase stimulation (24, 25). For our studies, unmodified and ubiquitylated histones were assembled into designer octamers of six types: human unmodified, H2AssUb, and H2BssUb and Xenopus unmodified, H2Bub(G76A), and H2Aub(G76A) (Fig. 2, A and B).

Relative to other prevalent histone PTMs, monoubiquitylation is an extremely bulky modification (itself being ~60% of the molecular weight of histones H2A and H2B). For our enzymatic cross-talk studies, we sought to eliminate the possibility that ubiquitylation-dependent changes in array conformation could alter the accessibility of a target lysine for subsequent methylation. Therefore, we reconstituted our octamers into designer mononucleosomes using a 153-bp sequence containing the 601 strong positioning sequence (26). The structural homogeneity of assembled mononucleosomes was assessed by native PAGE electrophoresis and ethidium bromide staining (Fig. 2C).

Anticipating that the disulfide linkage in our ssUb nucleosomes could present challenges for biochemical studies given the frequent necessity of reducing conditions for enzymatic activity, we performed titrations with DTT to ascertain whether low concentrations of reducing agent would leave the histone-ubiquitin disulfide bond appreciably intact. However, the mobility of H2AssUb and H2BssUb generated by disulfide-mediated ubiquitylation at position 119 in H2A and position 120 in H2B. Right, native refers to Xenopus laevis (xl) H2Aub(G76A) and H2Bub(G76A) generated by semisynthetic expressed protein ligation.

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H2Bub, but Not H2Aub, Participates in Cross-talk with Dot1L MTase for H3K79—Monoubiquitylation of H2BK120 is a potent stimulator of Dot1L-mediated methylation of H3K79 (15). Interestingly, monoubiquitylation at several other positions on the nucleosome axial surface, and even on the radial edge, also dramatically stimulated Dot1L MTase activity (Fig.
Taken together, these studies reveal significant positional plasticity in the histone ubiquitylation site for Dot1L MTase stimulation. The location of H2AK119ub, on the opposite end of the nucleosome axial face, and its 10-fold greater abundance than H2BK120ub in vivo (33, 34) make H2Aub an interesting test case for Dot1L plasticity to stimulation by histone ubiquitylation.

Dot1L MTase activity was undiminished under nonreducing conditions (Fig. 3B), which allowed the use of ssUb nucleosomes as substrates for in vitro MTase cross-talk assays. As
reported previously (24), Dot1L methylation of H3 is greatly stimulated by nucleosomal H2BssUb. In striking contrast, the incorporation of H2AssUb into an otherwise unmodified nucleosome had no appreciable impact on Dot1L-mediated methylation (Fig. 3C). On the basis of these experiments, we conclude that plasticity in monoubiquitin stimulation of Dot1L does not extend to the most abundant ubiquitylated histone in metazoans, H2AK119ub. In addition to deepening our understanding of Dot1L MTase regulation in vitro, this result supports the view that differential functions of H2Aub and H2Bub exist in vivo with respect to Dot1L and likely other chromatin-remodeling activities.

PRC2, MTase for H3K27, Is Modestly Inhibited by H2Aub but Is Unaffected by H2Bub in Mononucleosomal Substrates—Previous studies have shown several MTases involved in active transcription to be remarkably sensitive to monoubiquitylated nucleosomal substrates (15, 16, 32). However, to our knowledge, there are no reports testing transcriptionally repressive MTases in direct cross-talk experiments with histone ubiquitylation. For a transcriptionally repressive MTase, we chose to investigate the regulation of PRC2, the MTase for H3K27, because of its important role in cellular differentiation, stem cell identity, X-inactivation, cell cycle regulation, and cancer (35).

PRC2 target genes have been mapped in a variety of cellular contexts, and in many cases, these genes can be either methylated at H3K27, H3K4, or both depending on the particular cellular/developmental context (36, 37). In fact, the balance between H3K4me and H3K27me at gene promoters may be an important mechanism of transcriptional regulation (38, 39). Others have shown that the activity of MTases for H3K4 are stimulated or repressed by H2Bub and H2Aub, respectively (16, 22). Given the opposing function of H3K4me and H3K27me in transcriptional regulation, we wondered whether H2Aub and H2Bub also directly and antagonistically regulate PRC2-mediated methylation of H3K27.

As reducing agents were required for PRC2 activity (Fig. 4A), we turned to native ubiquitylated mononucleosomes for our enzymatic cross-talk studies. Unlike Set1 and Dot1L MTases, PRC2 MTase activity was unaffected by the presence of H2Bub in nucleosomal substrates (Fig. 4B and supplemental Fig. 1). However, H2Aub modestly but reproduc-
ibly inhibited PRC2 MTase activity (Fig. 4C). This was an unexpected result because both H3K27me and H2Aub are transduced by PRC2 and PRC1, respectively, with well-established roles in gene repression. However, several recent publications provide evidence that the functional link between H2Aub and transcriptional repression is not as clear as was once thought (40–42), and these will be discussed further below.

**DISCUSSION**

Cross-talk between histone PTMs may have evolved as a way to amplify, buffer, and integrate diverse signals impinging on chromatin. Elucidating which PTMs “talk” to each other, which do not, and via what mechanisms is critical in our quest to understand cellular memory, development, and disease. To study direct enzymatic cross-talk relationships between PTMs, the use of highly defined in vitro chromatin templates is critical (28). Here, we utilized chemically defined monoubiquitylated histones to generate a set of mononucleosomal substrates for MTase assays to investigate novel functions of histone ubiquitylation and to shed light on the plasticity/specificity of cross-talk with histone MTases (Fig. 5).

In landmark papers, H2Bub was shown to be an in vivo requisite for methylation of H3K79 by Dot1 (9, 11). Careful subsequent studies using nucleosomes with site-specifically installed monoubiquitin revealed that H2BK120ub directly stimulates Dot1 MTase activity (15). Interestingly, H2BK120ub is not unique in its ability to cross-talk with Dot1L-mediated H3K79 methylation. Ubiquitylation at several other sites on the nucleosome axial face and on the radial edge can also stimulate Dot1L activity (24, 32). From these data, it is clear that Dot1L stimulation is relatively plastic with regard to the position of histone monoubiquitylation. Using a disulfide-mediated approach, we installed monoubiquitin at position 119 of H2A to test whether H2Aub, the most abundant ubiquitylated species in metazoan chromatin, could influence Dot1L MTase activity on mononucleosomes. Our results clearly show that the observed plasticity of the ubiquitylation site for Dot1L stimulation in vitro does not extend to H2Aub (Fig. 3C). Thus, even though H2Aub is a relatively abundant mark in mammalian systems, it seems likely that its biological function is not linked to H3K79 methylation and downstream events.

**FIGURE 5. Summary of enzymatic cross-talk relationships between histone monoubiquitylation and methylation.** Left, histone monoubiquitylation sites known to directly influence MTase activity directed toward the indicated histone methylation sites (top) are indicated. Inside, the relevant MTase and the type of cross-talk observed are indicated. †, the ubiquitylated histone directly stimulates the indicated MTase; ⊘, inhibition; ©, no detected stimulation or inhibition. The results presented here and those published by others (see text) are colored in blue and black, respectively.

Most studies of the function of H2Bub in metazoans have focused on its role in transcriptional activation (43). Although the steady-state H2Bub level correlates well with the most highly expressed genes genome-wide (44), *in vivo* depletion of RNF20 (E3 ligase for H2BK120) and near abolishment of H2Bub revealed a subset of genes repressed by RNF20 (45). Surprisingly, genes repressed by RNF20 were found to be associated with higher levels of H2Bub than genes activated by RNF20. These data raise the possibility that H2Bub may be...
involved in transcriptional repression in some chromatin contexts.

Given that H2Bub dramatically and directly stimulates the evolutionarily unrelated MTases Dot1 and Set1, we wondered whether the ability of H2Bub to stimulate MTases is restricted to MTases involved in active transcription (e.g. H3K4me and H3K79me) or if it extends to MTases involved in transcriptional repression (such as PRC2). In vitro PRC2 MTase assays revealed no cross-talk, in either a stimulatory or inhibitory direction, between H2Bub and PRC2-mediated H3K27me (Fig. 4B). Based on these results, it is unlikely that putative H2Bub-mediated transcriptional repression, as observed in genomewide studies (45), is caused by direct stimulation of PRC2 activity. Going forward, it will be interesting to understand at the molecular/structural level how H2Bub selectivity stimulates MTases Dot1L and Set1 but not PRC2.

Although H2Aub is typically understood to cooperate with PRC2-mediated H3K27 methylation in transcriptional repression, we found that H2Aub produced a modest but reproducible inhibition of PRC2 activity in vitro (Fig. 4C). One more trivial explanation for our finding might be that the relatively close proximity of H2AK119ub to H3K27 in the nucleosome (Fig. 1B) may simply sterically hinder PRC2 MTase activity. Although this possibility cannot be ruled out by our studies, recently published data from several groups suggest that the widely accepted link between H2Aub and transcriptional repression may not be so straightforward (40–42).

Eskeland et al. (42) showed that the H2A E3 ligase activity of Ring1B is dispensable for transcriptional repression of Polycomb target genes and for long-range chromatin compaction of Hox gene clusters in vivo. In light of our in vitro results, it would be of interest to know whether cells with catalytically null Ring1B have attenuated or otherwise misregulated activation of Hox genes during differentiation.

Scheuermann et al. (41) identified a novel Polycomb repressive complex in Dro sophila with H2A deubiquitinase activity, PR-DUB. Interestingly, they found that H2A deubiquitinase activity is required for proper transcriptional repression of Polycomb target genes in vivo. Furthermore, repression of Polycomb target genes is even more severely compromised if dRing, the E3 ligase for H2Aub, and PR-DUB are simultaneously depleted. Therefore, at least in Dro sophila, complexes with directly opposing enzymatic activities on H2A synergistically regulate transcriptional repression. These results suggest that H2Aub in different genomic locations may have opposite influences on transcription or that dynamic cycles of H2A ubiquitylation and deubiquitylation may be required for proper gene repression. We look forward to studies that will investigate how the spatial, temporal, and kinetic control of H2Aub turnover on chromatin contributes to gene regulation. Such studies would greatly inform future in vitro studies using designer chromatin to more closely mimic the dynamics of histone ubiquitylation in vivo.

Finally, Richly et al. (40) identified ZRF1 as an H2Aub-binding protein involved in differentiation-dependent transcriptional activation of Polycomb target genes. If reduced PRC2 activity on Polycomb group-repressed genes is an important early step in pushing the chromatin state toward one more permissive to transcription, then our in vitro cross-talk data may provide another layer of mechanistic explanation for H2Aub involvement in transcriptional derepression.

Although our studies represent a significant advance in the use of designer nucleosomes containing H2Aub versus H2Bub, important uncertainties remain that will need to be addressed in future experiments. In particular, the role of ubiquitin turnover (e.g. due to the cyclic action of ubiquitin ligases and deubiquitinases) is not addressed in our system, nor are potential differences in spatial and temporal control of H2Aub and H3K27me in transcriptional regulation.

We look forward to subsequent studies that will use designer chromatin to explore enzymatic cross-talk relationships between H2Aub and H2Bub with not only MTases but also with other histone-modifying activities. More generally, we expect further study of histone PTM cross-talk relationships to be a fertile approach to understand how combinations of PTMs are coordinately regulated to maintain and affect dynamic changes in chromatin state, such as those that accompany gene expression changes resulting from developmental or environmental signal transduction.

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