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# Histone variant macroH2A1.2 is mono-ubiquitinated at its histone domain

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**Abstract**

Histone macroH2A1.2 (macroH2A) is an unusual histone H2A variant with a large non-histone macrodomain at its carboxyl terminal. MacroH2A1.2 is enriched in facultative heterochromatin, including inactivated X chromosomes in mammalian females and senescence-associated heterochromatin foci. We show here that a small population of macroH2A1.2 is mono-ubiquitinated in human HeLa cells. Mass spectrometry analysis revealed that the specific targeting sites for the mono-ubiquitination are Lys115 and Lys116 of the histone domain. A corresponding Lys119 conserved in histone H2A is also mono-ubiquitinated by Ring protein in the polycomb group complex. We suggest that the mono-ubiquitination of macroH2A1.2 and histone H2A has similar or synergistic implications, but that the multiple ubiquitination sites in macroH2A1.2 might confer a variety of functions upon macroH2A1.2 to modulate chromatin states.


*Keywords:* Histone macroH2A; Histone variants; Mono-ubiquitination; Histone modification; Facultative heterochromatin

The incorporation of histone variants into nucleosomes can influence events such as gene expression, DNA repair, and meiosis [1]. The core histone H2A (H2A) has the largest family of heteromorphous variants, including histones H2A.Z, H2A.X, H2A.Bbd, and macroH2A. This family displays chemical variability at the C-terminus, suggesting that the C-terminal region is involved in nucleosome stability and higher-order structures [1,2].

MacroH2A is an unusual histone variant. It has an N-terminal region with high sequence homology to H2A, but also contains a 25 kDa, non-histone macrodomain [3]. MacroH2A consists of three similar proteins: macroH2A1.1, macroH2A1.2, and macroH2A2 [3–5]. MacroH2A1.1 and macroH2A1.2 are produced from the same gene by alternative splicing [3,4]. MacroH2A2, encoded by a separate gene, has a similar but non-identical pattern of localization [5,6]. Although the exact functions of macroH2A remain unknown, the enrichment of macroH2A on the inactive X chromosome of mammalian females [5,7] and on specialized domains of transcriptionally silent senescence-associated heterochromatin foci in senescent cells [8] suggests some role for macroH2A in the formation of facultative heterochromatin. Indeed, biochemical data indicate that the presence of macroH2A interferes with the binding of transcription factors and severely impedes SWI/SNF nucleosome remodeling [9].

Histone modifications regulate dynamic conformational changes in chromatin structure, resulting in transcriptional activation or repression [10–12]. Enzymatic

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activities responsible for the mono-ubiquitination of histones H2A and H2B were recently discovered, suggesting roles in their mono-ubiquitination [13–15]. For instance, H2A is mono-ubiquitinated by Ring proteins in the polycomb repressive complex 1 (PRC1) [15,16], and this modification is involved in X chromosome inactivation in mammalian females [16,17].

In this study, we showed the presence of mono-ubiquitinated macroH2A1.2 in human cells, consistent with a recent report [18]. Furthermore, using mass spectrometry analysis, we demonstrated that mono-ubiquitin is conjugated at either Lys115 or Lys116 in the histone domain of macroH2A1.2. We suggest that the multiple ubiquitination sites in macroH2A1.2 potentially confer a variety of functions on macroH2A, such as multiple modifications in H3 and H4.

## Materials and methods

*cDNA synthesis and construction of expression vectors.* The cDNAs of macroH2A1.2 and ubiquitin B were synthesized by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA extracted from human HeLa cells. The final expression vectors, pMSCV*puro*-mH2A1.2-HA-FLAG, pMSCV*puro*-mH2A1.2-FLAG, pQCXIN-HA-Ub, and pQCXIN-3×HA-Ub, were constructed based on pMSCV*puro* and pQCXIN vectors (BD Bioscience). Details of their construction will be provided upon request.

*Production of retrovirus-infected HeLa cells.* All procedures were based on the protocols described at http://www.stanford.edu/group/nolan/protocols. Expression vectors were transfected into Phoenix Amphotrophic packaging cell lines (a gift from Dr. Nolan) using Lipofectamine (Invitrogen). Virus-infected cells were selected with puromycin or G418. All experiments were performed in stable cell lines.

*Preparation of the chromatin fraction and purification of C-terminal-tagged macroH2A1.2-containing nucleosomes.* Nuclei were isolated as described previously [19]. Isolated nuclei were extracted with buffer C (25 mM Hepes–KOH [pH 8.0]; 150 mM NaCl; 20% glycerol; 1.5 mM $MgCl_2$; and 1 mM EDTA) and resuspended at a concentration of $3 \times 10^8$ nuclei/mL in micrococcal nuclease (MNase) buffer (20 mM Tris–HCl [pH 7.5]; 150 mM NaCl; 2 mM $MgCl_2$; 1 mM $CaCl_2$; 5% glycerol; and 0.05% Triton X-100). MNase (Sigma) was added to the nuclei suspension to a final concentration of 6 U/mL. The reaction was terminated by the addition of EGTA/EDTA (5 mM final concentration) after incubation at 27 or 37 °C for 30 min. The supernatant will hereafter be referred to as the chromatin fraction.

Immuno-affinity purification of C-terminal-tagged macroH2A1.2-containing nucleosomes was performed with anti-FLAG monoclonal antibody (mAb)-conjugated beads (M2; Sigma) or anti-FLAG mAb and anti-hemagglutinin (HA) mAb-conjugated beads (12CA5; Roche) in a buffer containing 150 mM NaCl and 0.025% Tween 20, as described previously [20]. For mass spectrometry (Fig. 3), the FLAG eluates were dialyzed against buffer H500 (100 mM sodium phosphate [pH 5.2]; 500 mM NaCl) loaded onto hydroxyapatite (HAP; Sigma) and eluted with buffer H1000 (100 mM sodium phosphate [pH 5.2]; 1 M NaCl). The eluate was dialyzed against MNase buffer without $CaCl_2$ and concentrated by affinity purification with anti-HA-mAb-conjugated beads.

*Mass spectrometry.* Protein bands excised from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels were subjected to thin-gel digestion overnight at 37 °C with trypsin (Promega; Fig. 1) or endoproteinase LysC (LysC; Wako; Fig. 3). Molecular mass analysis of the peptides was performed by matrix-assisted laser desorption ionization–time-of-flight/mass spectrometry (MALDI-TOF/MS) using an Ultraflex TOF/TOF spectrometer (Bruker Daltonics). Peptides were identified by comparison of the molecular weights determined by MALDI-TOF/MS with the theoretical peptide masses of macroH2A and ubiquitin.

## Results

### *Affinity purification of macroH2A1.2-containing nucleosomes*

Epitope-tagged histones expressed in cultured cells have been used successfully to analyze the physiological roles and dynamics of the histones [20,21]. To understand the function of macroH2A-containing nucleosomes, we established human HeLa cell lines stably expressing C-terminal-tagged macroH2A1.2-HA-FLAG (macroH2A1.2-HA-FLAG) by retroviral transduction. To obtain a soluble chromatin fraction, we treated isolated nuclei intensively with MNase to digest about half the genomic DNA into a ~200 bp ladder of mono-nucleosome sizes (Fig. 1A). The macroH2A1.2-HA-FLAG-containing nucleosomes were purified from the chromatin fraction by sequential affinity purifications with anti-FLAG antibody and then with anti-HA antibody. As a control, we performed mock purification from untransduced HeLa cells, which did not express epitope-tagged proteins (Fig. 1B, lanes 1 and 3). Western blotting with anti-macroH2A1 antibody showed that expression of macroH2A1.2-HA-FLAG was slightly lower than that of endogenous mH2A and that macroH2A1.2-HA-FLAG was successfully concentrated by affinity purification (Fig. 1B, compare lanes 2 and 4). Interestingly, the anti-macroH2A1 antibody reacted to a slow-migrating band (arrow) in addition to endogenous macroH2A1 and macroH2A1.2-HA-FLAG (Fig. 1B).

Silver staining of the same HA eluates analyzed in Fig. 1B revealed that macroH2A1.2-HA-FLAG was coimmunoprecipitated with many proteins (Fig. 1C). Therefore, we excised several slow-migrating bands above macroH2A1.2-HA-FLAG and subjected them to thin-gel trypsin digestion for subsequent mass spectrometric analysis. The result showed that a slow-migrating band (Fig. 1C, arrow) included peptides derived from both macroH2A1.2-HA-FLAG and ubiquitin (data not shown). The estimated molecular mass of the band was approximately 50 kDa; the molecular masses of macroH2A1.2-HA-FLAG and ubiquitin are 43 and 8.4 kDa, respectively. Therefore, we inferred that macroH2A1.2 is mono-ubiquitinated in human HeLa cells.

### *Detection of mono-ubiquitination of macroH2A1.2*

We then performed Western blotting with various anti-ubiquitin antibodies, but the many trials we con-

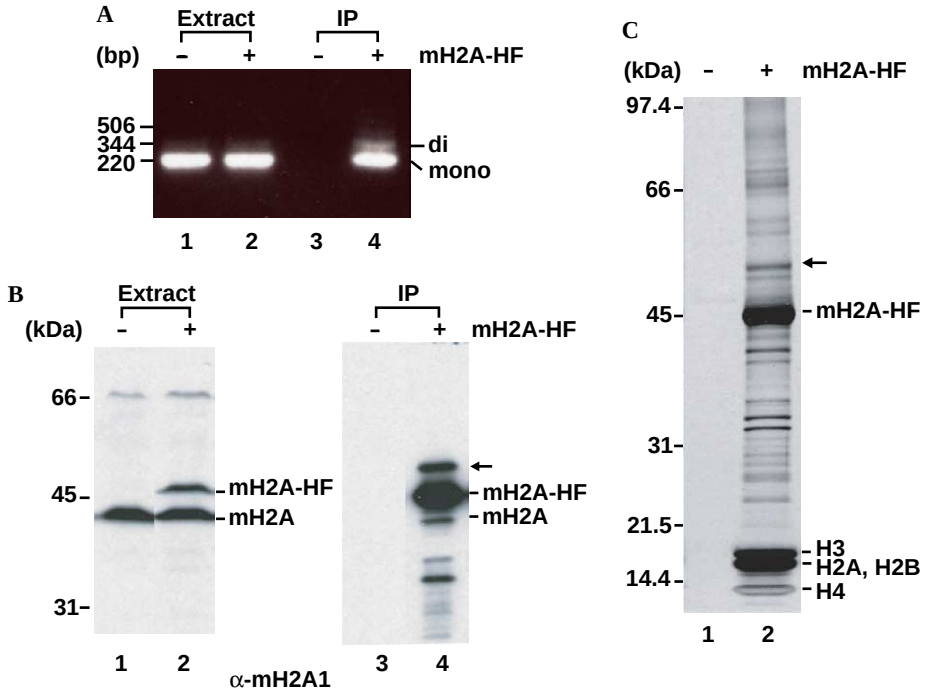


Fig. 1. Purification of macroH2A1.2-containing nucleosomes. Human HeLa cell lines expressing macroH2A1.2-HA-FLAG were obtained by retroviral transduction (Materials and methods). Isolated nuclei of the cells were digested with MNase and centrifuged to obtain the soluble chromatin fraction. MacroH2A1.2-HA-FLAG-containing nucleosomes were purified from the chromatin fraction by a series of affinity purifications with anti-FLAG antibody and anti-HA antibody. (A) Analysis of the DNA extracted from the chromatin fraction (Extract, lanes 1 and 2) and the affinity-purified nucleosome fractions (IP, lanes 3 and 4) of untransduced human HeLa cells (−) and HeLa cells expressing macroH2A1.2-HA-FLAG (mH2A-HF) (+). DNA size marker is shown on the left and the DNA bands corresponding to mono- and di-nucleosomes are shown on the right. (B) A Western blot (15% polyacrylamide gel) of the chromatin fraction (Extract, lanes 1 and 2) and the affinity-purified nucleosome fractions (IP, lanes 3 and 4) of untransduced human HeLa cells (−) or HeLa cells expressing macroH2A1.2-HA-FLAG (+) was probed with anti-mH2A1 antibody (Upstate). MacroH2A1.2-HA-FLAG (mH2A-HF) and endogenous macroH2A1 (mH2A) are indicated. The slow-migrating band is indicated by an arrow. Protein size markers are shown on the left. (C) Silver staining of the affinity-purified macroH2A1.2-HA-FLAG fraction (+, lane 2) and mock-fraction (−, lane 1) (on a 4–20% polyacrylamide gradient gel; Wako). The slow-migrating band including macroH2A-HA-FLAG and ubiquitin is indicated by an arrow. MacroH2A1.2-HA-FLAG and major histones determined by mass spectrometry are shown on the right. Protein sizes are shown on the left.

ducted were unsuccessful (data not shown). Consequently, we set up a more sensitive system to detect mono-ubiquitination of macroH2A1.2 by establishing human HeLa cell lines in which C-terminal-tagged macroH2A1.2-FLAG (macroH2A1.2-FLAG) and/or N-terminal-tagged HA-ubiquitin (HA-ubiquitin or 3× HA-ubiquitin was expressed; in 3× HA-ubiquitin, three units of HA peptides were arranged in a head-to-tail fashion at the C-terminal of ubiquitin). First, whole cell extracts prepared from each cell line were subjected to Western blotting and probed with anti-HA antibody to confirm the incorporation of HA-ubiquitin or 3× HA-ubiquitin into proteins (Fig. 2A). Characteristic smear signals were observed only when HA-ubiquitin or 3× HA-ubiquitin was expressed (Fig. 2A, lanes 3–6), supporting that HA-ubiquitin or 3× HA-ubiquitin in the HeLa cells was successfully conjugated to proteins. To determine whether macroH2A1.2-FLAG was ubiquitinated with HA-ubiquitin or 3× HA-ubiquitin, macroH2A1.2-FLAG-containing nucleosome fractions were purified from the chromatin fractions of each cell line by affinity purification with anti-FLAG antibody. They were then subjected to Western blotting and probed with anti-HA antibody. Shifted bands were detected only when both macroH2A1.2-FLAG and HA-ubiquitins were co-expressed (Fig. 2B, black arrows in lanes 4 and 6). The sizes of the shifts were in accordance with the lengths of monomeric HA-ubiquitin and 3× HA-ubiquitin. These data strongly suggested that macroH2A1.2-FLAG is covalently modified by mono-ubiquitin in human HeLa cells.

### *Identification of lysine residues targeted for mono-ubiquitination in macroH2A1.2*

Next, we undertook to confirm the existence of mono-ubiquitinated macroH2A1.2 and determine its mono-ubiquitination sites. For these purposes, we further purified macroH2A1.2-HA-FLAG from the chromatin fractions used in Fig. 1, with an HAP column (Materials and methods). Coomassie brilliant blue staining showed that macroH2A1.2-HA-FLAG was co-puri-

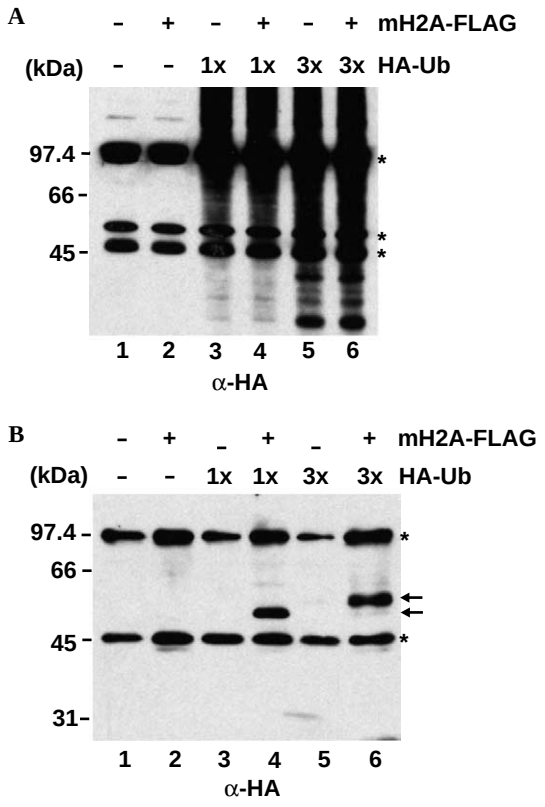


Fig. 2. Detection of mono-ubiquitination in macroH2A1.2. Human HeLa cell lines expressing C-terminal-tagged macroH2A1.2-FLAG and/or N-terminal-tagged HA-ubiquitin were established by retroviral transduction. MacroH2A1.2-FLAG-containing nucleosomes were purified from the chromatin fractions by affinity purification with anti-FLAG antibody. (A) Whole cell extracts or (B) nucleosome fractions from human HeLa cells expressing macroH2A1.2-FLAG (mH2A-FLAG; lanes 2, 4, and 6), HA-ubiquitin (1×; lanes 3 and 4), and/or 3× HA-ubiquitin (3×; lanes 5 and 6) as indicated were subjected to Western blotting and probed with anti-HA antibody. The black arrows in (B) indicate the expected bands of mono-ubiquitinated macroH2A1.2-FLAG, while the asterisks indicate non-specific bands that cross-react with the anti-HA antibody. The expression levels of macroH2A1.2-FLAG were almost equal and the macroH2A1.2-FLAG was equally well purified for each cell line (data not shown). Protein size markers are shown on the left.

fied with additional slow-migrating bands (arrow and asterisk in Fig. 3A). The bands were excised from the gel and thin-gel digested with LysC for mass spectrometry analysis. As shown in Fig. 3B, one of the slow-migrating bands (Fig. 3A, arrow) contained the peptide derived from macroH2A1.2-HA-FLAG (black arrows) and ubiquitin (white arrows). Importantly, two extra peptides were identified with molecular masses equal to the estimated molecular mass of the fused peptides of macroH2A1.2 and ubiquitin (Fig. 3B, asterisks 1 and 2): carboxyl-terminal ubiquitin attached to either Lys115 or Lys116 of macroH2A1.2, as shown in Fig. 3C. These peptides were not detected in the macroH2A1.2-HA-FLAG band (data not shown). Therefore, we concluded that the slow-migrating band in Fig. 3A was ubiquitinated macroH2A1.2-HA-FLAG and, judging from the molecular size of the shift, mono-ubiquitin (8.4 kDa) was conjugated specifically to either Lys115 or Lys116 of the histone domain.

## Discussion

In this study, we have shown the existence of mono-ubiquitinated macroH2A1.2 in human HeLa cell lines, consistent with a recent report that macroH2A1 binds to SPOP, which has a BTB domain [22,23], and that CULLIN3/SPOP conjugates mono-ubiquitin to macroH2A1 [18]. Furthermore, we have demonstrated that macroH2A1.2 is mono-ubiquitinated at either Lys115 or Lys116 in its histone domain, although it remains unclear whether CULLIN3/SPOP is responsible for the mono-ubiquitination of macroH2A1 at both Lys115 and Lys116. These two adjacent lysine residues are conserved in the major histones H2A and other H2A variants including macroH2A2, H2A.X, and H2A.Z (Fig. 3D). However, only the second lysine in H2A, Lys119, is mono-ubiquitinated by Ring proteins in PRC1 [15,16]. It would be interesting to know whether H2A mono-ubiquitinated at Lys119, macroH2A mono-ubiquitinated at Lys115, and macroH2A mono-ubiquitinated at Lys116 have any distinct functions.

Recently, the mono-ubiquitination of H2A and macroH2A has shown to be associated with X chromosome inactivation in mammalian females [16–18]. Ubiquitinated H2A was enriched in inactive X chromosomes with the Ring proteins in PRC1 [16,17]. The depletion of CULLIN3/SPOP, required for the mono-ubiquitination of macroH2A1, resulted in the dissociation of macroH2A1 from the inactive X chromosomes and the partial reactivation of the inactive X chromosome [18]. However, the exact functions of mono-ubiquitination in these histones H2A remain unknown. The C-terminus of H2A, which includes Lys119, and probably the C-terminus of macroH2A, which includes Lys115 and Lys116, project along the surface of the nucleosome [2]. It has also been reported that mono-ubiquitinated H2A enhances the association of the linker histone H1 (H1) with nucleosomes [24]. Although it has been suggested that H1 and macroH2A reside in mutually exclusive chromatin fractions with distinct, but redundant, functions [25], mono-ubiquitinated macroH2A might recruit H1 into the nucleosome to compact the chromatin further. Alternatively, mono-ubiquitination of macroH2A might influence the functions of its macrodomain. Recent observations have shown that the macrodomains are high-affinity ADP-ribose binding modules [26–28], suggesting that the macrodomain of macroH2A is involved in the metabolism of ADP-ribose and ADP-ribose-mediated chromatin modulation. Whether mono-ubiquitination of macroH2A affects

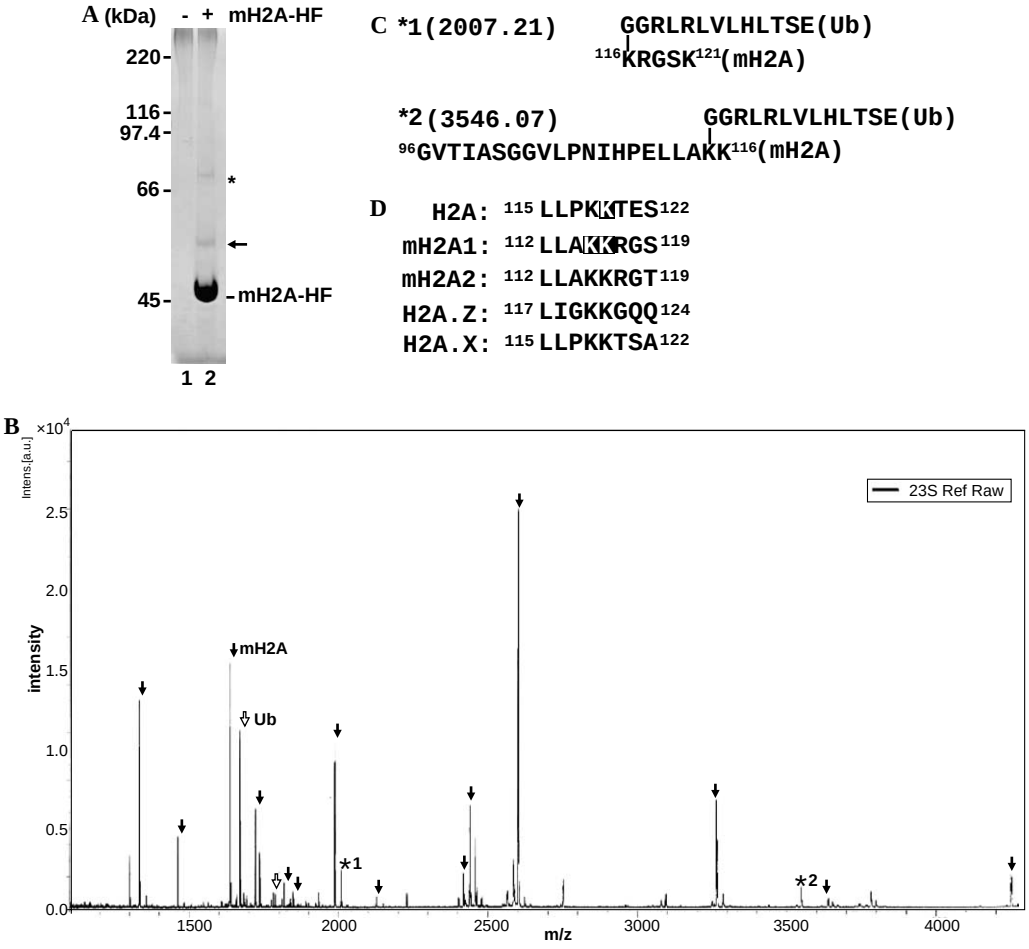


Fig. 3. Determination of the sites targeted for mono-ubiquitination in macroH2A1.2. For mass spectrometry analysis, macroH2A1.2-HA-FLAG was further purified from chromatin fraction in combination with affinity purifications and an HAP column (Materials and methods). (A) Coomassie brilliant blue staining of macroH2A1.2-HA-FLAG (mH2A-HF) purified from the transduced HeLa cells (+, lane 2) and the corresponding fractions from the non-transduced HeLa cells (−, lane 1) are shown (on a 12.5% polyacrylamide gel). An arrow and an asterisk indicate the slow-migrating bands of macroH2A1.2-HA-FLAG and Hsp70, respectively. (B) Mass spectrometric analysis of the slow-migrating band (arrow in A) thin-gel digested with LysC revealed the presence of two extra peptide peaks (∗1 and ∗2) in addition to the peptide peaks from macroH2A1.2-HA-FLAG (mH2A, black arrows) and ubiquitin (Ub, white arrows). The molecular masses of the extra peaks were equal to the estimated molecular mass of the fused peptides: carboxyl-terminal ubiquitin attached to either Lys115 (∗2) or Lys116 (∗1) of macroH2A1.2. (C) The amino acid sequences of the two fused peptides (∗1 and ∗2 in B) and the *m*/*z* values are shown. (D) The surrounding amino acids of H2A, H2A1, H2A2, H2A.Z, and H2A.X are aligned with the determined ubiquitination sites marked by black boxes.

these predicted functions of the macrodomain is an important question.

Mono-ubiquitination is thought to function as a signal for receptor internalization and DNA repair, probably via protein–protein interactions [29]. MacroH2A1.2 mono-ubiquitinated at Lys115 or Lys116 might be recognized by different effectors, fulfilling distinct roles, as is the case in the acetylation and methylation of histones H3 and H4 [10–12]. Recently, Abbott et al. [25] reported that macroH2A was poly-ADP-ribosylated. The identification of different modifications of macroH2A (ADP-ribosylation [25], mono-ubiquitination [18], and mono-ubiquitination at different sites, e.g., Lys115 or Lys116) may enhance our understanding of macroH2A-mediated chromatin formation.

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