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Original Article

Characterization of the N-terminally clipped histone H3 (ΔH3) from old chicken and rat liver

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Abstract: Site-specific proteolysis of the N- or C- terminus of histone tails has recently emerged as a novel form of irreversible post-translational modifications of histones. A proteolytically cleaved H3 product (named as ΔH3) has recently been reported in the liver of old chicken. In the present study, a comparison of the N-terminal amino acid sequences of the H3 and the ΔH3 revealed that the ΔH3 lacked 23 amino acids from the N-terminus. The proteolysis was observed only in histone H3 in the liver of old chicken, and it was absent in brain and erythrocytes. A ΔH3 like product was also observed in the liver of old rats. Further, the ΔH3 could be probed with H3 specific N-terminal pan acetylated antibody, suggesting that the chromatin domains containing the ΔH3 would be transcriptionally repressive. However, the ΔH3 could be probed with anti-H3K36me2 antibody (broadly a mark of transcriptionally permissive chromatin), suggesting that the ΔH3 though lacked major portion of the N-terminal region, still be important in determining the transcriptional status of the chromatin. Therefore, a comparison of the existing epigenetic marks on H3 and ΔH3 is also tabulated in the present study. To ascertain whether the cleavage is dependent on post-translational modifications of histone H3, bacterially expressed recombinant H3 was used as substrate for *in vitro* cleavage reaction. The analyses of the products revealed that the major cleaved product was different from that of the ΔH3, suggesting that the *in vivo* generation of ΔH3 requires histone H3 to be chromatin-bound.

Keywords: Histone H3, histone H3 proteolysis, ΔH3, epigenetic marks

Introduction

The N- and the C- terminal tails of core histones are subjected to a diverse array of reversible post-translational modifications. Some of the widely-studied modifications are methylation, acetylation, phosphorylation, ubiquitylation and ADP-ribosylation etc [1]. In addition to these reversible modifications, histones also undergo irreversible modifications by proteolysis of the N- or the C- terminal of histone tails, which also have been correlated to epigenetic regulation [2-9].

Proteolysis of the N- or the C- terminal histone tails have been reported in *Tetrahymena* [2], mammalian kidney cells (BHK cells) infected with Foot and Mouth Disease Virus (FMDV) [10, 11], cycad pollen [12], rat uterus [13], yeast [14, 15], mouse embryonic stem cells [16], human embryonic stem cells [17], calf thymus [18], monocytes [19] and chicken liver [20]. A recent attempt has been made to divide histone proteolysis into histone degradation and epigenetically inferential tail clipping [21].

Recently, our group has shown that chicken liver nuclear glutamate dehydrogenase (GDH) cleaves histone H3 *in vitro* [22]. By sequencing of the clipped H3 products, the *in vitro* proteolytic cleavage sites by GDH have been mapped at R2/T3, K4/Q5, R8/K9, G13/K14, K23/A24 and K27/S28 of histone H3 [22, 23]. Further, in the liver of old chicken, the histone H3 was N-terminally clipped *in vivo* and the product was named as ΔH3 [23, 24]. Another group has mapped the cleavage site at R26/K27 of histone H3 [24]. However, a careful analysis of the

above cleavage sites revealed that the mapped cleavage site (i.e., R26/K27) did not coincide with any of the *in vitro* cleavage sites by GDH [22]. Hence, it was essential to determine the exact site of cleavage on histone H3, and also to explore a possible fate of the cleaved product. In the present study, by comparing the N-terminal sequences of the H3 and the ΔH3 we identify that the ΔH3 lacks 23 amino acid residues from N-terminal region. Further, a ΔH3 like product was also observed in the liver of old rat while, it was absent in the erythrocytes and brain of the old chicken. To understand whether the cleavage would occur in a modification free histone H3, bacterially expressed histone was used to assess the cleavage in histones devoid of any post-translational modifications. The results suggested that the *in vivo* generation of the ΔH3 would require histone H3 to be chromatin-bound.

Materials & methods

Biological materials

White leg horn chicken, Wistar strain albino rats and Parks strain mice were used for the experiments. Rats and mice were of the age groups of ~18 weeks (termed as young) and ~108 weeks (termed as old). Tissues from young (~4 weeks) and old (~24 weeks) chicken were procured from slaughter house. Tissues from these organisms were collected following the guidelines of the departmental animal ethical committee and stored following standard methods [22], until used.

Isolation of nuclei

The nuclei from liver and brain tissues of chicken and liver of rat and mouse were isolated as previously described [25] with minor modifications [26] and from chicken erythrocytes following the standard methods [22]. The purity of the nuclei was verified under a phase contrast microscope at each step of nuclei isolation. Nuclei were estimated following already established methods [27].

Isolation of histones

Histones were extracted from the purified nuclei by acid extraction method [28]. Extracted histones were quantified as previously described [27]. The histones were analysed on a high resolution SDS-18% PAGE as described [29] with minor modifications [30].

Western blotting

Western Blotting was performed following the standard protocol [31]. Anti-H3 (against full length H3), anti-acetylated anti-H3 (corresponding to N-terminal 20 amino acids with Kac, kind gifts from Dr. Sharon Y. Roth, M.D. Anderson Cancer Centre, USA) and anti-H3K36-me2 (Abcam, USA) were used as primary antibodies. Signals of the western blots were developed following standard methods [31].

N-terminal sequencing of H3 and ΔH3

Chicken liver total histones were separated on SDS-18% PAGE, transferred onto a PVDF membrane in modified transfer buffer (10% methanol, 95 mM glycine, 12.5 mM Tris base), and, stained with 0.5% Ponceau S. The H3 and the ΔH3 bands were excised out and sequenced commercially from Department of Chemistry, IIT Mumbai, India. The procedure used a gas phase automated protein sequencer from Shimadzu model PPSQ10. The first 6 amino acids sequences from the N-terminus for both H3 and ΔH3 were aligned manually to identify the cleavage site in H3.

Histone isolation from H1 depleted soluble chromatin of rat liver

H1 depleted soluble chromatin was prepared from nuclei of liver of old rat by standard method [32]. Histones were isolated from the soluble chromatin as reported earlier [32].

Purification of histone H3 expressed in bacteria

A recombinant *Xenopus* histone H3 cloned in pET 3a was received as a kind gift from Dr. Tony Kouzarides, University of Cambridge, UK. This plasmid was transformed and histone H3 was expressed in *E. coli* BL21 (DE3) pLys S strain following standard methods [22]. The histone H3 expressed in bacteria was purified following standard methods [22].

H3 cleavage assay

Chicken liver mitochondrial GDH was purified following standard methods [22]. The H3 cleavage assay was performed following an assay system developed by us [22]. Briefly, 2 μg of bacterially expressed histone H3 was incubated with the GDH in specific assay conditions. The reaction was stopped and the H3 cleavage was monitored by analysing these samples on SDS-

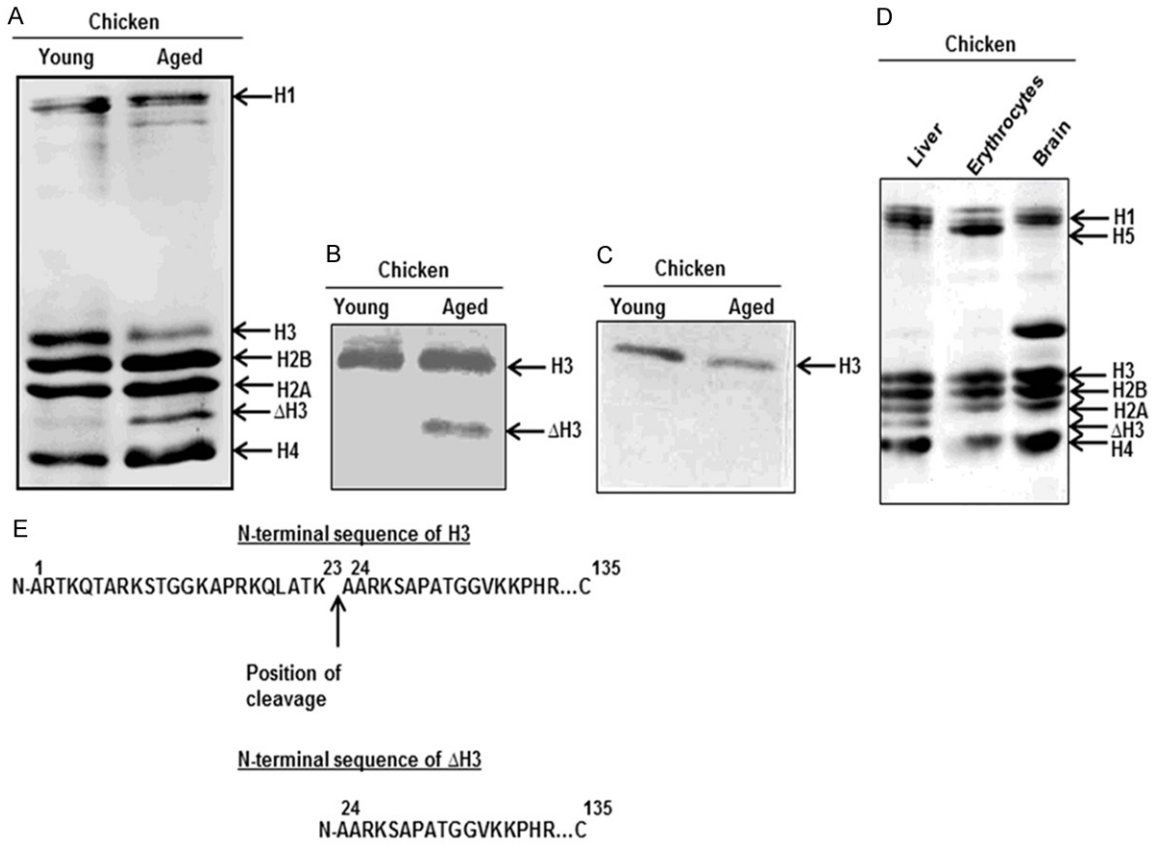

Figure 1. Observation of a N-terminally 23 amino acids cleaved H3 (ΔH3) in chicken liver. (A) Total histones were extracted from nuclei purified from liver of young and old chicken and analysed on SDS-PAGE. The novel additional band in old chicken that migrated between H2A and H4 was identified as ΔH3. (B) Resolved histones as described in (A) were transferred onto PVDF membrane and probed with anti-H3 antibody. (C) The histones resolved as described in (A) were transferred onto PVDF membrane and probed with anti-acetylated H3 antibody (against a peptide of H3 N-terminal region containing 20 amino acids with acetyl groups placed on Lys residues) by DAB reaction. (D) Total histones isolated from the nuclei of liver, erythrocytes and brain of old chicken were resolved on SDS-PAGE. (E) The H3 and ΔH3, in the total histone preparations from liver of old chicken liver were excised out and first 6 amino acids from N-terminus were sequenced. Upper panel: Full length sequence of histone H3. Lys-23 (K23) and Ala-24 (A24) represent the site of cleavage for generation of the ΔH3. Lower Panel: Sequence of the ΔH3.

18% PAGE and staining with coomassie brilliant blue R-250 [22].

Results

The ΔH3 is an N-terminally 23 amino acids cleaved product of H3

Total histones isolated from liver of young and old chicken were analysed on SDS-PAGE. A faster migrating extra band was observed in the histones isolated from liver of old chicken, with a corresponding reduction in H3 (**Figure 1A**). The extra band was immunoreactive to anti-H3 antibody (**Figure 1B**); however, it was not recognized by anti-acetylated H3 antibody (**Figure 1C**). All these findings suggested that the extra band was indeed the ΔH3, which has also been reported earlier [24]. It was also observed that the ΔH3 was absent in the histone preparations from brain and erythrocytes of old chicken (**Figure 1D**). The first 6 amino acids from N-terminus for the H3 and the ΔH3 were identified as 'ARTKQT' and 'AARKSA', respectively. Both the sequences were aligned manually to the available sequence of H3 to deuce the cleavage site. It revealed that the ΔH3 lacked 23 amino acids from the N-terminus, indicating that the cleavage site was at K23/A24 of H3 (**Figure 1E**).

A ΔH3 like product is also observed in liver of old rat

Histone preparations from liver of young as well as old rat were analysed to explore the presence of ΔH3 in organisms other than chicken. A

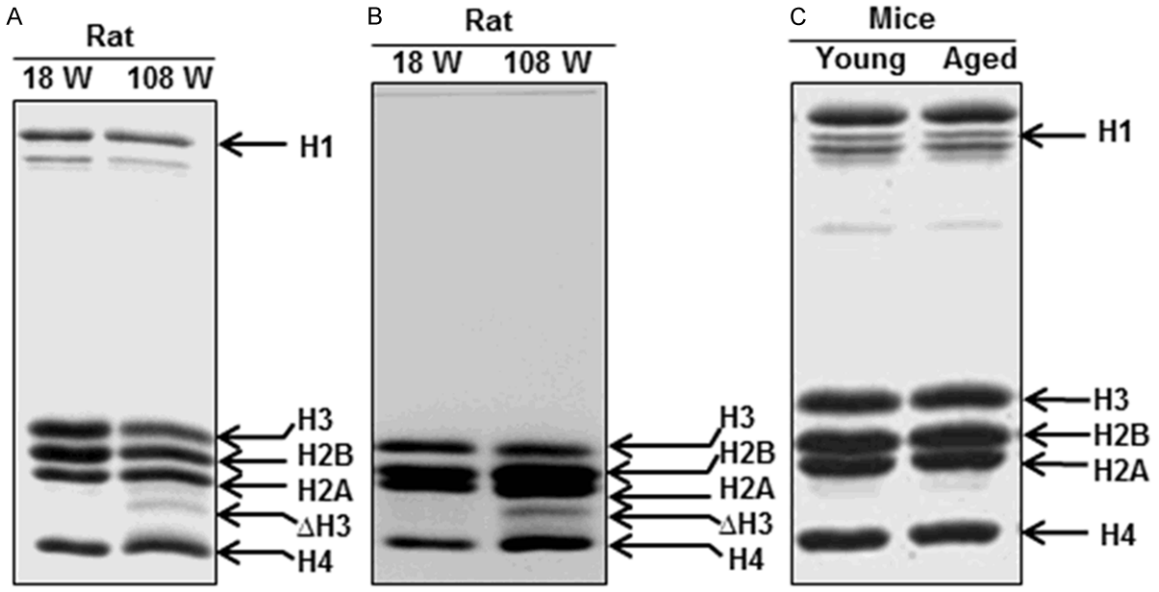

Figure 2. A ΔH3 like product is also generated in liver of old rat. A. Total histones were isolated from the nuclei of young (18 weeks) and old (108 weeks) rat liver and resolved on SDS-PAGE and stained with 0.1% coomassie blue. B. Histones prepared from H1 depleted soluble chromatin from the nuclei of liver of young and old rat were resolved on SDS-PAGE and stained as above. C. Total histones from liver of young and old mice were resolved on SDS-PAGE and stained as above.

Table 1. Status of various global histone modifications on H3 and ΔH3

Histone \ Mod	K4me	K4me2	K9me	K9me2	K9ac	K14ac	K23ac	K27ac	K27me2	K56ac
H3	+	+	+	+	+	+	+	+	+	+
ΔH3	-	-	-	-	-	-	-	+	+	-

Western blot analysis of global histone modification status of H3 and ΔH3 adapted and analysed from already published reports [24, 35].

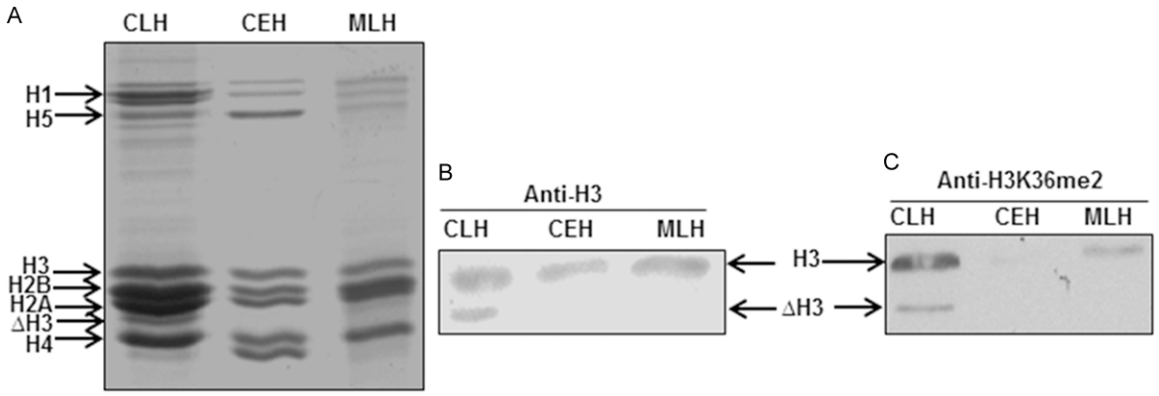

Figure 3. The ΔH3 immunoreacts to anti-H3K36me2 antibody similar to H3. (A) Total histones isolated from nuclei of liver of old chicken (CLH), erythrocytes of old chicken (CEH) and liver of old mice (MLH) were analysed by SDS-PAGE and stained with 0.1% coomassie blue. (B) Resolved histones as described in (A) were transferred on to a nitrocellulose membrane and probed with anti-H3 antibody and processed by enhanced chemiluminescence. (C) Resolved histones as described in (A) were transferred on to a nitrocellulose membrane and probed with an anti-H3 antibody specific for dimethylated Lys 36 (K36me2) antibody.

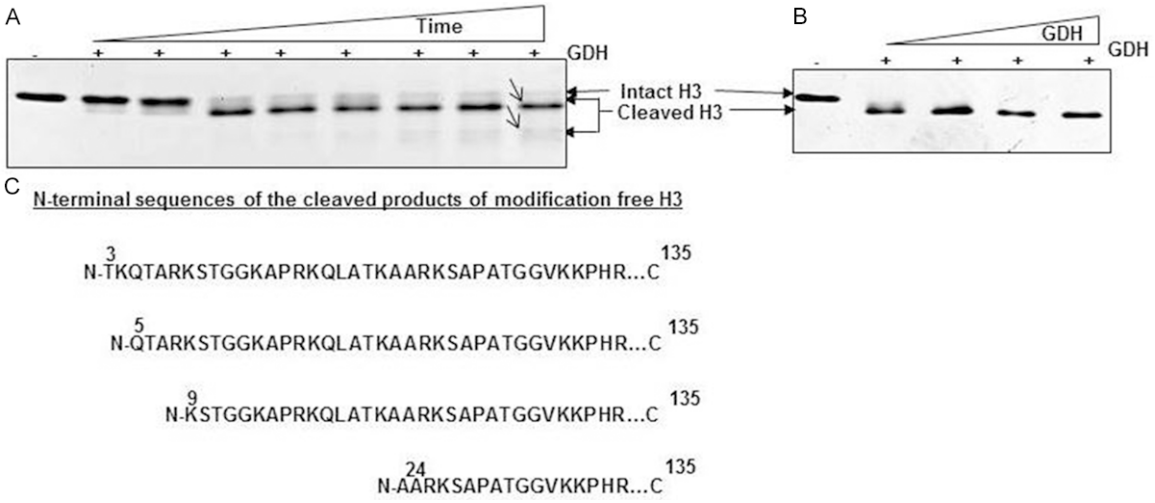

Figure 4. Assessment of cleavage in the post-translational modification free histone H3. The H3 cleavage assay was performed by incubating bacterially expressed histone H3 with GDH in the standard assay condition already developed and subsequently analysed on SDS-PAGE. A. 2 µg of bacterially expressed histones were incubated with 1 µg of chicken liver mitochondrial GDH for different time duration (5, 15, 30, 45, 60, 90, 120 and 180 min). B. 2 µg of bacterially expressed histones were incubated with different amount of GDH (0.1, 0.5, 1 and 2 µg) for 1 h. C. N-terminal sequences of the cleaved products of modification free H3.

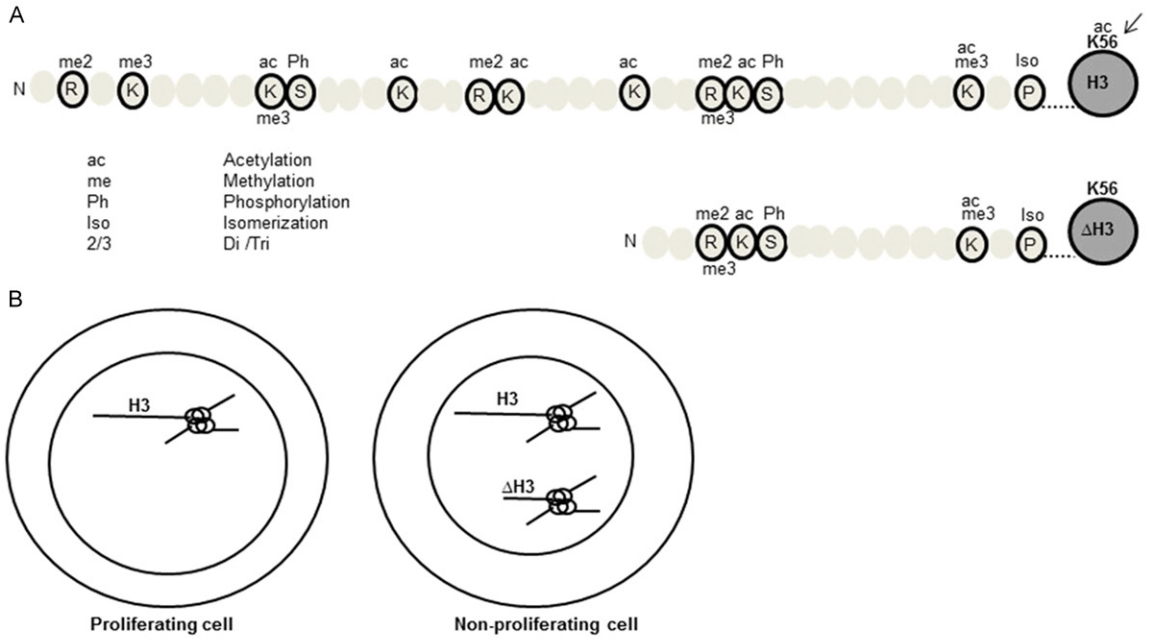

Figure 5. A plausible model to depict the distribution of ΔH3. A. Post-translational modification map of histone H3 and ΔH3. Different modifications are represented by corresponding abbreviations. In general, the activation marks are on the upper side of the peptide and the repressive marks are on the lower side of the peptide. 'Arrow' indicates selective presence of K56ac mark in H3. B. A plausible model representing the distribution of H3 and ΔH3 in the chicken liver.

ΔH3 like product was also found in the histones isolated from liver of old rat, while it was absent in that of the young (**Figure 2A**). It was next sought to investigate, whether the ΔH3 like product was chromatin bound in the liver of old rat. Soluble chromatin was prepared from nuclei of liver of young and old rat. Histones were isolated from these soluble chromatin

fractions and were analysed on SDS-PAGE. A ΔH3 like product was also observed in the histones isolated from these soluble chromatin fractions (**Figure 2B**). This result further confirmed that the ΔH3 like product was a part of the chromatin and was not produced due to some cytoplasmic contamination during isolation. Surprisingly, a ΔH3 like product was absent in histones isolated from liver of old mice (**Figure 2C**). It would be interesting to investigate histone H3 proteolysis in other organisms, to understand its physiological role.

Evaluation of epigenetic marks on the ΔH3

There have been evidences supporting the role of N-terminal tail clipping of H3 in epigenetic reprogramming [21]. The downstream effect of the proteolysis can be broadly categorized as either formation of transcriptionally permissive or repressive chromatin domains. Since the ΔH3 lacked 23 amino acids from N-terminus, it was intended to evaluate the differences in the epigenetic marks on H3 and ΔH3. A summary of the comparative list of existing modifications on H3 and the ΔH3 is represented in **Table 1**. From the table, it is evident that the ΔH3 was immunoreactive to anti-H3K27ac antibody, which is a mark of transcriptionally active chromatin [33]. It was thus suggested that the ΔH3 might not be a part of repressive chromatin. Therefore, presence of any other epigenetic mark(s) for transcriptionally permissive chromatin (such as H3K36me2) was explored in ΔH3. Since the chicken erythrocyte is classically known to possess transcriptionally repressed chromatin [34], chicken erythrocyte histones were used in the present study as histones possessing repressive marks. Total histones from liver and erythrocytes of old chicken and liver of old mice were isolated and resolved on SDS-PAGE (**Figure 3A**) and probed with anti-H3 (**Figure 3B**) and anti-H3K36me2 antibodies (**Figure 3C**). The ΔH3 was immunoreactive to anti-H3K36me2 antibody, suggesting that it might form a part of transcriptionally permissive chromatin. This observation was in conformity with the other permissive modification, such as H3K27ac on ΔH3 (**Table 1**). Though it was difficult to establish a precursor product relation by comparing the anti-H3K36me2 signals of H3 and ΔH3; an increased signal of the same in ΔH3 compared to the H3 of chicken erythrocytes suggested that ΔH3 might be a part of transcriptionally permissive chromatin domains.

Assessment of cleavage in post-translational modification free histone H3

It was intended to evaluate whether post-translational modifications in H3 were essential for its cleavage. As a bacterially expressed histone H3 would be devoid of any post-translational modification; it was used as a substrate to study the H3 cleavage. Bacterially expressed histone H3 was incubated with chicken liver mitochondrial GDH (demonstrated earlier to have H3 specific protease activity) for increasing time durations (**Figure 4A**) or with increasing amount of GDH (**Figure 4B**). The results indicated that the major cleavage of the bacterially expressed histone H3 occurred at the 8th amino acid residue (**Figure 4C**), and which was different from the chromatin bound H3, where, the cleavage site was only specific to 23rd amino acid residue.

Discussion

While there are many reports on specific proteolysis of histone H3 [6, 21]; Cathepsin L and GDH are the only two H3 specific proteases characterized till date [16, 23]. In the *in vitro* reaction, chicken liver GDH cleaves histone H3 at multiple sites spanning in the N-terminal region. The results of the present study indicate that the identified *in vivo* cleavage site in histone H3 (K23/A24), coincided with one of such *in vitro* cleavage sites by GDH. It proposes a strong argument for the *in vivo* role of GDH in clipping H3 and generating the ΔH3. The above hypothesis is also supported by the observation that in old chicken, there in increased histone H3 cleavage and also increased nuclear localization of GDH [35]. On the contrary, the increased nuclear localization was not observed in chicken brain tissue where the ΔH3 was absent [35]. Further, GDH is reported to be excluded from the nuclei and localize in the cytoplasm and mitochondria of *Drosophila* [36]. We also failed to detect a ΔH3 like product in the histones from *Drosophila* (Tiwari and Purohit, unpublished observation). All these observations support the above hypothesized role of GDH in H3 clipping *in vivo.* However, the confirmation of the same anticipates further investigations, which are underway.

The cleavage of H3 in the liver tissue, though found in other systems also, is not a ubiquitous phenomenon. A ΔH3 like product was also

observed in rat liver (present study) and Japanese quail [37]. On the other hand, a ΔH3 like product was not found in the liver of old mice (present study), and in bovine and *Calottes* liver, and HeLa cells (data not shown).

Evaluation of epigenetic signatures on the ΔH3 revealed that it lacked most of the acetylation sites in comparison to H3. However, the ΔH3 possessed both activation marks (K27ac, K36me2) and repression marks (K27me2). The above proposed chromatin dynamicity is also well supported by the predominant role of H3K36me2 in transcriptional activation and minor role in repression in various organisms [38-42]. Also, from the present study, an increased K36me2 signal in the ΔH3 as compared to chicken erythrocyte histone H3, suggests that the ΔH3 containing chromatin domains might not exist in a transcriptionally repressed state. Furthermore, it has been reported that the ΔH3 could also be probed in comparable intensities in S1, S2 and P fractions in MNase digested nuclei from liver of old chicken, suggesting that the ΔH3 might form a part of transcriptionally active chromatin [35]. In summary, we suggest that similar to mouse embryonic stem cell differentiation and yeast models [14, 16], the ΔH3 generation also leads to formation of a more dynamic chromatin domain.

A careful comparison of the acetylation marks on H3 and ΔH3 (**Table 1**) indicates that in contrast to H3, the ΔH3 lacked K56ac mark (which peaks at S-phase) [43]. The absence of K56ac mark in ΔH3 was probably due to the lack of N-terminal binding site for Rtt 109 (a H3K56 acetyl transferase) [35]. The absence of H3-K56ac mark has been reported to direct chromatin in an assembled state [44]. In addition, it limits the entry of the cell into S phase. Hence, we propose that such an epigenetically connoted tail clipping might lead to selective localization of the ΔH3 (**Figure 5**).

A comparison of the *in vivo* H3 cleavage and the *in vitro* H3 cleavage reveal that the predominant H3 product in the *in vitro* reaction lacked 8 amino acids from the N-terminus due to cleavage at 8th amino acid residue. On the contrary, in case of ΔH3, there was a cleavage at 23rd amino acid residue of H3, produced *in vivo*. These findings suggested that the specific cleavage to produce ΔH3 was probably favoured when H3 remained in a chromatin bound state.

Collectively, the results presented here demonstrate that the ΔH3 is 23 amino acids clipped product of H3. The ΔH3 was found to be present in the histone preparations from liver of old chicken and it was absent in brain and erythrocytes. A ΔH3 like product was also observed in liver of old rat. However, detailed investigations need to be done in various organisms to understand the physiological role of histone H3 proteolysis. We hypothesize that the selective epigenetic marks on H3 and ΔH3 might be responsible for differential regulation of chromatin domains. Also, the conformation of the N-terminal tail in chromatin bound histone H3 might determine its specific cleavage.

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Disclosure of conflict of interest

None.

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