Histone proteolysis: A proposal for categorization into clipping and degradation

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We propose for the first time to divide histone proteolysis into histone degradation and the epigenetically connoted histone clipping. Our initial observation is that these two different classes are very hard to distinguish both experimentally and biologically, because they can both be mediated by the same enzymes. Since the first report decades ago, proteolysis has been found in a broad spectrum of eukaryotic organisms. However, the authors often not clearly distinguish or determine whether degradation or clipping was studied. Given the importance of histone modifications in epigenetic regulation we further elaborate on the different ways in which histone proteolysis could play a role in epigenetics. Finally, unanticipated histone proteolysis has probably left a mark on many studies of histones in the past. In conclusion, we emphasize the significance of reviving the study of histone proteolysis both from a biological and an experimental perspective.

Keywords: epigenetics; histone clipping; histone degradation; histone proteolysis

Introduction

Histone proteolysis has a long history of disregard

Proteolysis associated with nucleosomes was described about 50 years ago, even before histones received their current nomenclature [1]. Not surprisingly, many subsequent reports have alluded to the theoretical transcriptional implications of such enzymatic reaction because of the central role that histones play in DNA packaging and epigenetic regulation. While evidence of such epigenetic potential is only now gradually emerging, it is increasingly becoming clear that histones are also being degraded at much higher rates than was initially anticipated. Together this has created a very confusing amalgam of recent reports in which findings with often little biological coherence are continuously being cross-referenced.

A brief history of histone proteolysis shows that it has consistently been treated in stepmotherly fashion by the scientific community. As the first papers started to suggest that histone truncation might greatly impact transcription, a sudden surge of biochemical studies on nuclear histone-degrading enzymes occurred during the 70s and 80s (partially reviewed in [2]). While histone proteolysis nearly disappeared from the publication record during the 90s and early 2000s, a second wave of papers seemed to be upon us by around 2010, with the publication of the first evidence of the epigenetic potential of histone clipping in mouse and yeast [3, 4]. Strangely, however, this promise was again not fulfilled. At the very least it is surprising that especially in light of its function as a posttranslational modification (PTM) – which can sweep away en masse all other modifications [5] – histone clipping is not being picked up by the broader scientific community at a time when over 5,000 papers are published on histones every year. A first step in tackling this apparent lack of interest is to recognize the difficulty of studying proteolysis in a well-defined biological context.

Here, we have categorized earlier reports into different classes, each of which contributes to the biological picture in...
its own way (Table 1). In doing so, it became increasingly clear that even the main two classes, which we call here “histone degradation” and “histone clipping”, are far from straightforward to discriminate. In part this is due to the fact that (i) the same enzymes apparently can mediate both histone degradation and histone clipping, (ii) many reports may potentially have been based on in vitro side effects, blurring coherence of the biological context, (iii) many reports only briefly mention detecting histone fragments, but never investigated their origin, because it was outside the scope of the authors at the time, and (iv) even the most well-documented reports struggle to completely elucidate the biological significance of degradation and clipping, maybe in part because of redundancy.

Table 1. Structural overview of the different classes of histone proteolysis in 100 references

| Enzyme Classification | Enzyme | Substrate (specificity) | Organism | Tissue | References |
|-----------------------|--------|-------------------------|----------|--------|------------|
| Biologically unclassified | Early reports | Neutral serine protease, Trypsin-like | Mainly H1/H3 degradation | Rat/Calf Liver/thymus | [1, 2; 6–23] |
| | In vitro assays | Trypsin | H1>H3>H2A>H4 | Rat Liver/skin | [24–26] |
| | | Other enzymes | H3R26/H4K20/H4R19 | | [27] |
| | | Enzyme panel screening | E.g. Cathepsin D is “H2A-specific.” | | [28] |
| | | Direct expression of truncated histones | H3K27/H4K10/1820/H2A31-117/H2B24-122 | | [29–31] |
| Biologically classified | Developmental | Inhibited by Leupeptin and TLCK Proteasome (PA200) | Histones, not protamines | Trout/rat/ mouse | [32, 33] |
| | Embryogenesis | Sph – Cathepsin L | Degradation Protamines (H1/H2B SPK motif) | Yeast/mouse Sea Urchin Testis Embryo | [34] [35–41] |
| | Macronuclei | H3A21/H2B14/H4G13/H2A18/H1 proteolysis (H1uUyUy) | Tetrahymena | Macronuclear degradation | [42, 43] |
| | | Pathogen | EVO gene | Chlamydia | [44] |
| | Continuous | Cathepsin L | H3A21 (H3c-1 Ab) i - H3 C-term | Human | Cell lines | [45] |
| | | Proteasomal | Proteasome (PA200) | Degradation | Yeast/ mouse Testes | [34] |
| | Immunological | NET formation Induced apoptosis | Azurophilic enzymes GranzymeA | All histones Mainly H1 | Human Neutrophils Cells targeted by T-cells | [46–48] [49] |
| | Clipped histone expression | N-tail | H4J4-26/H3I4-30/ H2A46-40/H2B8-32 | Yeast | | [50–55] |
| | | | H4I4-26/H3I3-20/H3I1-28 | Yeast | | [56] |
| | | C-tail | H2AE121 | Human Embryonic kidney cell line | | [57, 58] |
| | | | H2AV114/H2AS122 | Human Embryonic kidney cell line | | [59] |
| | | H2AspV114 | H2Asp – neutrophil elastase | H2AV114 | Cali/human/ mouse Thymus/ haematopoietic cells | [60–73] |
| | | H2AspE11 | Neutral aspartate protease | H2AE91 | Chicken Liver | [19, 74–76] |
| H2A-specific | Buforins (AMP) | Pepsin/Cathepsin D | H2AS19/H2AY39 | Trout/ amphibia Mucosa | [77, 78] |
| H3-specific | Developmental | Micronuclei Pathogens | Protease 3C | H3T6 (H3F) H3L2D | Tetrahymena PeM/D HIV | Microculei HK6 cells T-cells | [79–83] [84–86] [87] |
| | | | H3 C-terminus H1 | H3A21-H3A31 | mengovirus Mouse/ human/ rat Mouse | Ehrlich ascites tumor ESC and hepatitis/myo genic Mammary gland | [88] [3, 89–92] [93] |
| | | | H3K23 | Yeast | Sporulation/ starvation | | [4, 94, 95] |
| | | | PRB1 | H3A21 | | | |
| | | Other | Glutamate dehydrogenase Legumen | H3R18/H3K23/ H3R26/H3K27 chH (12 kDa) | Quali/ chicken Human | Colorectal cancer | [96–99] [100] |


Histone proteolysis

The initial impetus for classification

An appreciable number of the reports on histone proteolysis mentioned here have recently been subsumed for the first time in a review [101]. However, this very informative review perpetuates the main message found in the discussion of many of these records: Histone proteolysis could play a very important role in epigenetics. While supporting such notion ourselves, our findings on both histone H2A and histone H3 proteolysis [70, 71, 92] did not straightforwardly corroborate earlier findings and hypotheses. This encouraged us to extrapolate the current epigenetically biased view by more clearly categorizing earlier reports into different functional classes of (biologically regulated) processes. In summary of the text, Table 1 structurally bundles the references in their respective categories. By no means is this a complete overview, as many papers only briefly mention a histone truncation event.

Histone degradation

Biologically unclassified degradation

Did early reports simply lack experimental precautionary measures?: Many of the early reports on histone proteolysis focused primarily on rat liver and calf thymus (partially reviewed in the introduction of [2]). They describe either total degradation or a susceptibility of histone H1 and histone H3 to a neutral serine protease with a trypsin-like specificity. Intensive effort was made to avoid cytoplasmic contamination and the proteolytical activity was consistently hard to detach from the chromatin. Furthermore, some reports incubated the proteases with substrates other than histones to validate their specificity. This “neutral protease” activity was later attributed to different enzyme activities in different tissues and animals [14, 20, 21]. At least three different proteases were pursued in search of the “neutral protease”, one of which cleaves a histone H3K23 [22] and one that was attributed to cytoplasmic contamination [19]. One report mentions a high molecular weight (HMW) “protease A” that could be converted into a low and an intermediate MW protease by high pH or NaCl concentrations [17, 23]. Most claims of epigenetic potential of these protease activities were made on the basis of their apparent chromatin association and the impact of DNA or nucleotides on enzyme activity. Yet, because no convincing evidence of true histone clipping was provided in these early reports they are categorized here.

These reports might be considered by many to be “outdated”. However, apart from confirming the most susceptible histone sequence stretches, they can also provide important insights into which tissues are specifically prone to enzymatic degradation.

In vitro histone truncation assays provide important structural insights: Primarily with a view to structural study, many groups have incubated chromatin, whole nucleosomes and separate histones with enzymes. Only a small selection is shown in Table 1. Although most of these reports have no direct biological significance, they have provided several crucial insights that should be taken into account when considering histone proteolysis:

1. The nucleosome retains its globular structure, even when the tails are removed [27]. Tails do play an essential role in the higher order solenoid formation of chromatin [24] and removing them from the nucleosome makes DNA increasingly susceptible to degradation by DNase enzymes [26].

2. Not surprisingly, the linker Histone H1 is the most susceptible to histone proteolysis, followed by H3, which has the largest N-tail protruding from the nucleosome. The order in which histones are attacked by enzymes has thus been recognized to be H1 > H3 > H2A > H4 > H2B, and some reports caution that for this reason it is hard to study H1 and H3 proteolysis in vivo [25]. Bohm et al. have even suggested that H3A21 truncation, described in more detail below under histone clipping, is the result of autolysis.

3. Truncated histones have also been recombinantly expressed for structural studies, leading to similar conclusions as those found by enzymatic treatment [29–31]. For example, a recent FRET study structurally reinterpreted increased susceptibility to DNAse degradation as a more “breathing” conformation of truncated nucleosomes [31].

These basic structural insights can significantly help in the search for new histone proteolytical events as well as in explaining earlier findings. Moreover, they can serve as a first framework for the potential biological consequences of a histone proteolysis event.

Biologically classified degradation

Histone degradation occurs predominantly during important developmental transitions: Increasingly it is becoming clear that the once presumed extremely stable histones, are in fact degraded during different developmental stages in diverse organisms. Here again, not many studies conclusively contribute to the elucidation of this process (for a review see [32]). Here, we have classified these developmentally related histone degradation events into four different groups:

1. During spermatogenesis, histones are largely replaced transiently by transition proteins and subsequently by protamines in postmeiotic cells [33]. These histones thus need to be degraded, a process that was initially described to be mediated by a serine-type enzyme that is specific for histones, does not degrade protamines, and is associated primarily with oligonucleosomes in mouse. To the best of our knowledge, this enzyme has not yet been annotated [32]. Apart from enzymatic degradation, the proteasome can also degrade histones during spermatogenesis. Some reports hint at a ubiquitin-mediated degradation, but it was shown recently that the proteasome can also degrade histones based on their acetylation status [34].

2. During early embryogenesis, protamines and the remaining sperm histones again need to be degraded. This was first attributed to a cystein protease named SpH in sea urchin, where this process has been studied most extensively [36]. This SpH enzyme was recently found to be a nuclear Cathepsin L (CATL) [39].
Histone degradation in earlier Eukaryotes has also been described during specific stages of development, such as macronucleus degradation in, for example, *Tetrahymena* [43]. Remarkably, the most abundant fragment of histone H3 is H3A21, which will be discussed in greater detail under histone clipping.

Some intracellular pathogens such as *Chlamydia* seem to be able to selectively degrade their own histones upon infection [44].

Taken together, developmental histone degradation can be expected to be an omnipresent phenomenon in eukaryotic organisms, especially during reproduction. The lack of studies focusing on these histone turnover events greatly hampers insight into its relation to histone clipping, for the latter process seems to be associated mainly with differentiation, which equally is an important developmental transition.

**Continuous degradation is mediated by lysosomes and the proteasome:** Apart from specific developmental stages, histones are also being replaced during normal cell growth at much higher rates than was previously assumed [102]. To our knowledge, only little is thus far known about what happens to these evicted histones, but at least two different pathways by which subsequent histone degradation could be mediated are becoming evident:

1. **Lysosome-mediated processing**, for example, such as found in cytoplasmic chromatin fragments (CCF) that are budded off the nucleus and degraded in the autophagy pathway, a process that seems to be increased by senescence [45]. Maybe not surprisingly, an important part of the degradation is mediated by CATL, and the specific H3A21 fragment mentioned under histone clipping is particularly abundant in these CCF.

2. **Proteasome-mediated degradation:** Just as for the process of histone degradation during spermatogenesis, acetylation also precedes histone eviction at double stranded DNA breaks [34].

Continuous degradation might be the hardest process to experimentally isolate from epigenetic histone clipping, as there is no experimental setting where it is known to be absent, and it will prove very hard to isolate every stage of CCF formation from histones in chromatin.

**Histone degradation also plays a role in immunology:** Recent reports on histone proteolysis events in samples from blood seem to suggest such truncated species might predominantly have an immunological origin.

1. Neutrophil extracellular traps (NET) have evolved in neutrophils and other cells as a way to eliminate invading pathogens [46]. These NETs are generated via a specific form of cell death in which chromatin decondenses and binds to granular and cytoplasmic antimicrobial proteins such as myeloperoxidase and neutrophil elastase (NE). These structures are then released into the milieu. The entangled enzymes proteolyze histones in NETs at very specific sites, generating specific band patterns on Western blot [47].

Of note, the most abundant lower band of histone H2A has a similar molecular weight to that of the H2AV114 fragment discussed under histone clipping. Of note, selective translocation of active NE to the nucleus is a condition sine qua non to initiate NET formation [47], and is regulated at least in part by histone citrullination [48].

2 In vitro and after cell loading with perforin, GranzymeA completely degrades histone H1 and cleaves core histones into ~16-kDa fragments [49]. Histone digestion provides a mechanism for unfolding compacted chromatin and facilitating endogenous DNase access to DNA during T cell and natural killer cell granule-mediated apoptosis.

Blood cells might thus prove to be a very hard target in which to find the potential role of histone clipping in myeloid cell differentiation as described below.

**Histone clipping**

We have separately classified histone clipping as the category comprising very specific enzymatic cleavage events that have been regarded to be of potential epigenetic importance. The amino-terminal tail of the histones protrudes from the nucleosome and can become modified by many different PTMs. With the onset of the use of mass spectrometry in this field, a new wave of PTM discovery is currently ongoing [103]. A striking example of this was the discovery of a staggering 67 new histone marks in 2011 [104]. It is thus surprising that histone clipping as a potential epigenetic modification continues to escape the attention of the broader scientific community, while it too can be identified by mass spectrometry [3, 70, 71, 92].

**Direct expression of clipped histone forms in cells does not compromise viability**

Apart from the recombinantly expressed truncated histone forms that have contributed greatly to the understanding of the structural role of tails in nucleosome formation, specific truncated histone forms have also been directly expressed in living cells. These studies have provided important biological insights into how these truncated forms might exert their epigenetical influence and into how “stretchable” these truncation events are in terms of cell viability.

1. **N-tail truncation:** All core histones have been expressed in yeast without their respective N-tails [50–52, 54]. It was thus shown that all four N-termini are dispensable for viability in yeast. The sequence near the helical core of each histone seems to play an important role in the repression of basal transcription [55].

2. **C-tail truncation:** To our knowledge, only the H2A C-tail has been expressed in a truncated form in cells, as this is the only histone to have a truly protruding C-tail from the nucleosome [57, 59]. At all three different locations expressed in human cell lines (V114, E211, and S122) it was found that both chromatin compaction and transcription were impacted by this truncation event. Of note, the H2AV114 form is also generated by NE-mediated H2A clipping.
discussed below. For completeness, we here also mention that nickel(II) treatment induces a H2A C-tail truncation as well, making this another potential model to study the molecular outcome of tail truncation in living cells [58].

The viability of cells that express truncated histone forms not only argues in favor of the plausibility of clipping actually occurring in vivo; it also is a first and very important reminder of the potential pitfalls in studying histone proteolysis from an epigenetic perspective. As these tails can be discarded genome-wide, while they can also be modified by such a complex network of PTMs, the most obvious mechanism by which such cells survive seems to be through redundancy of regulation, which would be in line with the evolutionary conservation of histones in all eukaryotic organisms.

**H2A-specific clipping still lacks proof of epigenetic significance**

So far, only H3 N-tail- and H2A C-tail-specific histone clipping have been described in considerable detail. Figure 1 summarizes these clipping events in their interplay with the Polycomb group (PcG) proteins. Historically, H2A specific proteolysis was described first, and we thus commence by summarizing the reports hereon.

1 In 1976 a paper was published describing for the first time a chromatin-bound proteolytic activity in calf thymus with unique specificity, for histone H2A [64]. Under high ionic strength a pentadecapeptide is cleaved from the C-tail of histone H2A isolated from thymus, making valine 114 (V114) its new carboxy-terminal residue. In the following 10 years this enzyme was further studied and was named the “H2A specific protease” (H2Asp) [60, 62, 63, 72, 73]. This fragment was also found in histone extracts from several myeloid and lymphatic leukemia cells [67, 69]. More recently the epigenetic potential of this clipping event gained interest when it was found that retinoic acid (RA)-induced differentiation of THP-1 promonocytes into macrophages is briefly accompanied by histone H2AV114 clipping [65, 71]. While pursuing this cleavage in our samples of Chronic Lymphatic Leukemia B-cells, we have recently found strong evidence that the H2Asp actually might be neutrophil elastase (NE) [70, 71]. Up until now all references made to the H2AV114 fragment were epigenetically inspired, because such truncation also removes the K119 ubiquitylination site (Fig. 1). However, with NE now part of the story, experimentally uncoupling such epigenetic promise from, for example, NET formation will most probably prove very challenging.

2 Very recently, a second “H2A-specific protease” was described in chicken liver that clips H2A near its globular domain, at H2AE91 [74]. To the best of our knowledge, the identity of this enzyme is still unknown. However, based on the classification of previous reports on avian cells, CATD (light chain) surfaces as a potential candidate [19, 28, 74–76]. Of note, if this link is correct, at least one earlier report has attributed this specific enzyme activity to cytoplasmic proteolysis to generate anti-microbial peptides (AMP) called buforins (reviewed in [78]). These buforins have also been detected in gastric fluid of pigs, cattle and humans. When NET were first described, the authors also referred to the antimicrobial properties of histones and their derived peptides [46], but to the best of our knowledge these have not been extensively studied to date.

3 A completely different kind of histone H2A clipping has been described in the context of host defense. Some amphibians and fish are able to cleave off N-terminal parts of histone H2A by pepsin- and cathepsin D mediated proteolysis to generate anti-microbial peptides (AMP) called buforins (reviewed in [78]). These buforins have also been detected in gastric fluid of pigs, cattle and humans. When NET were first described, the authors also referred to the antimicrobial properties of histones and their derived peptides [46], but to the best of our knowledge these have not been extensively studied to date.

Despite its long history, H2A-specific clipping is nothing near a coherent biological framework. Many challenges remain, especially now that one of the H2A-specific proteases turns out to be NE. Experimentally resolving H2A clipping during myeloid differentiation from NET formation will require stringent controls.

**H3-specific clipping: The only epigenetic footing?**

Compared to histone H2A, H3 has been studied more extensively as an epigenetic template in general. In the same
way the role of histone H3 clipping as a PTM has been more elaborately studied.

1 Two electrophoretically distinct forms of histone H3 were described in 1980 to be selectively present in micronuclei of *Tetrahymena thermophila*, the faster species of which was generated by the removal of six amino acids from the N-terminal tail [79, 80]. This modification was later speculated to be a demethylation mechanism [83], and it has since been regarded to be a different modification from the above-mentioned degradation of histones in the transcriptionally active macronucleus of *T. thermophila* [43].

2 In a completely different biological setting, foot-and-mouth disease virus expresses the so-called protease 3C (P3C) in host cells, which mediates clipping of host histone H3 at leucine 20 [84–86, 105]. It is thus tempting to speculate that this virus might have evolved a mechanism to manipulate the epigenetic tools of its host.

3 In 2008 enzymatic truncation of histones resurfaced: The H3 N-terminus is clipped from A21 to K27 by CATL in differentiating mouse embryonic stem cells (mESC) [3]. Recently, we too found comparable clipping events from A21 to A/S1 in differentiating of human ESC, but our data point towards a serine protease activity [92], and we emphasize the influence of the culture conditions used. The report on mESC was actually the first ever to specifically investigate the potential epigenetic implications of such clipping events. The authors found that cleaved H3 showed altered affinity for Cbx7, another epigenetic mediator. Also, certain modifications inhibited the clipping at H3A21 when synthetic peptide substrates with different modifications were tested. Importantly, a later in vitro enzyme-substrate study on similar H3-derivates peptide substrates has put the impact of PTMs on CATL clipping efficiency into perspective [89]. This apparent contradicting importance of PTMs on the tail is most probably due to the effect that PTMs have on the quaternary structure of the nucleosome and the surrounding DNA. Now we also classify the additional H3 band seen on western blot in *Drosophila* polytenes here because the authors of this manuscript interpreted it as a differentiation-related event [106].

4 Almost simultaneously with the publication of the CATL mediated H3 truncation in differentiating mESC, a similar histone clipping event mediated by a serine protease was found in sporulating yeast cells [4]. Santos-Rosas et al. extended the epigenetic implications by showing that H3 truncation precedes H3 eviction from induced promoters and that abrogation of H3 tail clipping impairs gene induction. The occurrence of a proteolytic fragment of H3 beginning at amino acid 23 in yeast cells (actually reported 15 years earlier) might also be functionally related to this clipping event [94]. Despite the fascinating epigenetic implications and the parallel to the differentiating mESC it took five years for the first yeast candidate enzyme to appear in literature: Cerevisin (PRB1 or vacuolar protease B) is now at least considered to be capable of H3 clipping in starving yeast [95].

5 A surprising H3 N-tail specificity for the H3R18-K27 sequence stretch was reported recently for glutamate dehydrogenase (GDH) found in chicken liver tissue [97, 98]. When browsing in earlier reports it is tempting to speculate that the histone H3 truncation seen in senescent quail liver much earlier might be due to the same enzymatic interaction [96, 99]. Whether the C-terminal H3 proteolysis in hepatocytes from mice deprived of spermidine should be categorized here is even more speculative, however [107].

6 Nuclear localized cysteine protease legumain (LGMN) has emerged as a new enzyme to be able to clip the histone H3 N-terminal tail. In human colorectal cancer cell lines legumain probably clips the same sequence stretch that is truncated by CATL [100].

7 The latest addition to the list of histone H3 clipping enzymes is CATD, which migrates to the nucleus to clip H3.3 between K23 and R24 in involuting mammary glands [93].

Based on the overview of histone H3 clipping events, we agree with the notion that H3 clipping could well represent a common feature of differentiation [90]. Taken together, these papers for the first time are starting to provide increasingly strong evidence for epigenetic regulation of histone clipping (reviewed in [5, 108]), but evidence is still not conclusive. Uncoupling it from continuous histone degradation, which is mediated by the same enzymes, will probably be the biggest challenge.

**Can histone clipping still be brushed aside?**

The lack of corroborating evidence for transcriptional implications of histone clipping calls for prudence. When we first came across the H2AV114 clipping event in leukemia samples [71] we started to pursue this truncation in a context of potential epigenetic effects, based on the literature at hand. However, the finding that the "H2A-specific protease" actually is NE for the first time suggested that histone degradation explains the presence of H2AV114 more accurately [70]. Still, the fact that NE can be active in the nucleus, can be expressed by non-myeloid cell types and can even be actively taken up by others [109–111], emphasizes the importance of elucidating its nuclear biology. Similarly, the interaction of GDH, CATL, LGMN, P3C, PRB1, and CATD with histones should be studied in light of this broader perspective.

Thus, although we recognize that reflecting on the clipping functionality of all these different enzymes could be considered somewhat preliminary, we briefly extend here the review by Duncan and Allis [112] in which they summarize different mechanisms that could be at play when CATL engages in regulated histone clipping. Here we broaden this view to all candidate histone clipper by trying to answer the caption question from different points of view.

**The enzymes’ point of view: Different enzymes appear in similar biological stories**

One comparison in the literature frames CATL and NE together in a specifically appealing epigenetic picture: Retinoic acid (RA)-induced differentiation of THP-1 promonocytes into
macrophages is briefly accompanied by histone H2AV\textsubscript{114} clipping [65, 71], just as H3 clipping by CATL is induced while mESC and hESC are differentiated with RA [3, 92]. Although in our hands both these events showed considerable amounts of variability, their combined suggestive importance led us to mine the literature on these enzymes for their reported role in cell cycle regulation and especially differentiation. Indeed both enzymes can process and hyperactivate the same transcription factor CDP/CUX during G1/S transition [113–116]. While CATL has been classified into the so-called “differentiation module” in an in silico gene co-expression network derived from expression analysis of differentiating ESC [117], mutations in ELA2, the gene encoding NE, cause a neutrophil differentiation disease called cyclic neutropenia [118, 119]. Also, a hitherto undefined substrate of NE in the nucleus promotes leukemogenesis in an acute myeloid leukemia mouse model [120]. While the substrate in all these reports was never considered to be the histones themselves, active enzymes in the nucleus, including GDH, LGMN, P3C, and PRB1, might very well clip histone tails and thus impact the cell cycle or regulate differentiation in this way.

**The substrates’ point of view: Clipping could impact transcription in many ways**

**Clipping occurs mainly at the epigenetic hub of nucleosomes**

The N-terminus of H3 and the C-terminus of H2A protrude from the nucleosome at the entry and exit points of the DNA [121] (Fig. 1). Histone H2A is the only core histone that contains an additional flexible C-terminal extension besides the N-terminal tail. These tails are thus very important substrates in epigenetics, and target to a plethora of different epigenetic regulators. One such example is the PcG of proteins, which specifically and sequentially targets the H3 N-tail and the H2A C-tail in what is called the hierarchical recruitment model leading to transcriptional inhibition [122]. This repressive signal is counterbalanced by the activating Trithorax (Trx) Proteins that trimethylate H3K4, a process best known in the context of bivalent gene regulation during differentiation. One tempting model for epigenetic regulation by histone clipping would thus be the direct proteolytical interference with the hierarchical recruitment model or any other PTM cascade centered around the histone tails.

**Clipping influences chromatin compaction**

Apart from directly interfering with other PTMs, the impact of histone clipping on chromatin compaction can be considered a separate but complementary mechanism for explaining potential transcriptional consequences. In vitro both enzyme incubation essays and direct expression of truncated histones have hinted at increased “breathability” of chromatin when tails are lacking. However, only by expressing truncated histone forms in cells was it convincingly illustrated that indeed chromatin dynamics and expression patterns could both be influenced by histone clipping events, without viability being compromised.

**Clipping might precede histone eviction**

As mentioned above, histone degradation and histone clipping might be very hard to distinguish in some cases. This is best illustrated when considering histone eviction, where histones are either degraded or re-inserted into the chromatin after transcription. To the best of our knowledge Santos-Rosa [4] provided the only evidence of a histone-clipping event preceding histone eviction for gene transcription. They consider both an active and a passive role of H3 truncation in eviction: Either by occluding repressors of this process or by recruiting a protein complex necessary for eviction.

**Potential other outcomes of clipping**

By directly expressing several N-terminally truncated forms of H3, Psathas et al. [56] show that the removal of the H3 N-tail also interferes with intratail regulatory mechanisms. Adding yet another layer of complexity, the liberated N-terminal tail peptide of histone H3 may also directly bind the H3 mRNA [123]. In this way, H3 could regulate its own translation.

**The PTM’s point of view: Removing a histone tail might not be so drastic**

If indeed it is an important transcriptional regulator, histone clipping is arguably a very drastic PTM. Shortly after the discovery of H3 N-tail processing in both mouse and yeast, Osley [5] for the first time questioned how these histones are replaced. Only in 2010 it was shown that nucleosomes at regulatory elements in *Drosophila* S2 cells were reconstituted from new histones multiple times during one cell cycle with some regions showing histone turnover rates as fast as 1h [102]. The authors suggest that epigenetic information could be based on regulated nucleosome turnover, inasmuch as histone PTM and secondary effector proteins collectively dictate the intrinsic stability of a given nucleosome as well as its propensity to be remodeled. In this view, clipping a histone tail is not so different from any other PTM.

**Histone proteolysis calls for experimental precautionary measures**

Many more histone proteolysis events have most probably been encountered in the past but remained outside the scope of the authors at the time. One very remarkable recent case shows additional histone H3 bands in HIV latency-infected cell lines (NCHA1 and NCHA2) [87]. These bands are strongly reminiscent of the H3 clipping patterns as described earlier during foot-and-mouth disease virus infection, and could be interpreted as supporting evidence for a viral hijacking of the histone epigenome of the host [84–86, 105]. But until such findings are made the focus of separate research, this tempting hypothesis will remain untested.
CAT as well as NE were initially described in cytoplasmic vesicles as proteinases that degrade protein substrates with broad specificity and that both have well-defined elastolytic and collagenolytic activity [109, 124–127]. PRB in yeast also has long been associated mainly with vacuolar degradation [128], and GDH is known mainly as a metabolic enzyme. Only over the past decade was convincing evidence found for these enzymes as to their ability to migrate to the nucleus where they maintain enzymatic activity [95, 98, 111, 113, 114, 116, 120, 129]. It is important always to keep an open mind towards alternative functions of known proteins, as is beautifully reviewed in [130].

While this review focuses predominantly on the biological picture of histone proteolysis, it is equally important to realize that any experimental approach that targets histone tails and their modifications is prone to the effects of both in vitro and in vivo histone proteolysis. This is where the above-mentioned “early reports” could greatly contribute to awareness of the extent of efforts that might be needed to avoid involuntary histone proteolysis. As we and others have described before, inhibiting these enzymatic reactions can prove surprisingly difficult, especially if they strongly associate with chromatin [70, 131]. Epigenetic screening techniques such as antibody detection of modifications or immunoprecipitation of modified histone tails are entirely blind to these effects. If one only considers the intensity of the cleaved fragment of histone H3 in differentiating ESC from both mouse and human it is fair to state that up to half of all histone H3 can be clipped at certain time points in ESC differentiation [3, 92]. According to our knowledge, researchers have rarely specifically taken care to avoid this technical pitfall [132].

Conclusions

The urgency of a better understanding of histone proteolysis is patent. However, the difficulties of specifying the in vivo versus in vitro origin of this PTM, the fact that the same enzymes mediate both histone degradation and clipping, and the complexity and redundancy of the histone code, all contribute to the surprising shortage of reports on the biology of this potentially far-reaching PTM. This review for the first time suggests that future reports on histone proteolysis should be more explicitly classified into “histone degradation” and “histone clipping”.

One technique that holds the promise of detecting histone fragments more readily is (top-down) mass spectrometry, where all proteoforms in the sample can theoretically be monitored simultaneously [133]. But if push comes to shove, isolating the histones in the form that they were present inside the chromatin of the living cell is the true challenge for the field of histone epigenetics. Only by avoiding isolation all together can the presence of and the correlation between histone clipping and degradation be studied in vivo. One way this could be attained is by directly staining truncated histone in fixed cells in time-lapse experiments.

For now, trying to understand histone proteolysis still is a fuzzy art.

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