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Title: Histone tail cleavage as a novel epigenetic regulatory mechanism for gene expression

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ABSTRACT

Chromatin is an intelligent building block that can express either external or internal needs through structural changes. To date, three methods to change chromatin structure and regulate gene expression have been well-documented: histone modification, histone exchange, and ATP-dependent chromatin remodeling. Recently, a growing body of literature has suggested that histone tail cleavage is related to various cellular processes including stem cell differentiation, osteoclast differentiation, granulocyte differentiation, mammary gland differentiation, viral infection, aging, and yeast sporulation. Although the underlying mechanisms suggesting how histone cleavage affects gene expression in view of chromatin structure are only beginning to be understood, it is clear that this process is a novel transcriptional epigenetic mechanism involving chromatin dynamics. In this review, we describe the functional properties of the known histone tail cleavage with its proteolytic enzymes, discuss how histone cleavage impacts gene expression, and present future directions for this area of study.

INTRODUCTION

In eukaryotic cells, the genomic DNA is packed in the nucleus via its compaction into chromatin. The fundamental unit of chromatin is called the nucleosome, in which approximately 147 base pairs of DNA are wrapped around an octamer of core histone. Histones are highly conserved basic proteins found in eukaryotes and even in some archaea (1-6). They are divided into five major families: H1, H2A, H2B, H3, and H4. Two copies of H2A, H2B, H3, and H4 histones form the core histone octamer, while the linker histone H1 binds to the nucleosome at the site where internucleosomal DNA enters and exits, further contributing to the structural and functional flexibility of chromatin (5, 7, 8).

Chromatin is a dynamic structure, existing either as heterochromatin or euchromatin. Heterochromatin is more compact and transcriptionally inactive since genes are not easily accessible by transcription machinery proteins. In contrast, euchromatin is a decondensed type of chromatin and is transcriptionally active (3, 9). To allow transcription machinery proteins access to compacted DNA, a process known as chromatin remodeling reorganizes the chromatin structure to expose or hide regions of DNA for transcriptional regulation (10). It has been reported that chromatin remodeling occurs in several ways including histone modifications, exchange of histone variants, and ATP-dependent chromatin remodeling (11).

The core histones contain two common domains: histone folding and histone tail. The N-terminal tail of all four core histones and the C-terminal tail of H2A and H2B protrude outside the nucleosome, which undergoes a variety of posttranslational modifications (PTMs) including methylation, acetylation, propionylation, butylation, crotonylation, 2-hydroxyisobutylation, malonylation, succinylation, formylation, ubiquitination, citrullination, phosphorylation, O-GlcNAcylation, and ADP ribosylation (12-22). Functional outcomes of

these histone modifications have been investigated. A well-established hypothesis suggests that histone modification may lead to alteration of contacts between different histones in adjacent nucleosomes or the interaction of histones with DNA. Notably, acetylation neutralizes the positive charge of the lysine and weakens the interaction between histone and DNA, resulting in loosening the chromatin structure (13, 23). Another well-characterized hypothesis of histone modification is that distinct patterns of histone modifications, or “histone codes”, provide signals for the recruitment of specific non-histone protein complexes including transcription factors or proteins that modify the chromatin structure (18, 24, 25). Diverse histone modifications within the chromatin act in a coordinated and orderly fashion to regulate gene transcription (26). The precise alteration of chromatin histone modification is regulated by both the chemical and physical changes of core histones. A typical mechanism of chemical modification is the selective enzymatic addition and elimination of specific histone modifications by histone-modifying enzymes (“writers” and “eraser”), which is a reversible chemical change (27, 28). A more extreme mechanism utilizes physical changes of nucleosome so that the ATP-dependent chromatin remodeler catalyzes the exchange of canonical histones with histone variants, resulting in the removal of all pre-existing PTMs (29). An intermediate mechanism between these two mechanisms is the regulated proteolysis of histone tail, which physically and irreversibly removes pre-existing multiple PTMs (30-33).

In contrast to histone modification or histone exchange, the mechanism and the biological role of histone tail cleavage have not been fully understood. Although the presence of a proteolytic enzyme in histone preparations of calf thymus was observed in 1959 (34), and truncated histones were found in calf thymus and *Tetrahymena* in 1970s (35, 36), few publications were reported until the early 2000s. However, the regulated cleavage of histone has become an active and interesting topic of epigenetic regulation since the late 2000s.

Various enzymes responsible for histone clipping and the biological significance associated with histone cleavage have been studied as shown in Table 1. We discuss the identified enzymes, the regulation, and the biological significance of histone cleavage. Moreover, we propose putative mechanisms that suggest how histone cleavage modulates gene expression.

Histone H2A and H2B cleavage

In 1976, a protease associated with purified calf thymus chromatin was found to act specifically upon histone H2A, yielding a C-terminal truncated H2A. This fragment was derived from the removal of fifteen amino acids from the C-terminal H2A, of which Val114 was the new C-terminal residue (36). The responsible enzyme was further investigated and named the H2A specific protease (H2Asp) (37,38). The truncation of H2A was also observed in both myeloid and lymphatic leukemia cells (39-41) and retinoic acid-induced differentiation of THP-1 promonocytes into macrophages (42,43). Later, it was argued that H2Asp might be neutrophil elastase (44). Neutrophil elastase is a neutrophil-specific protease required for neutrophil extracellular trap (NET) formation. It has been reported that, upon reactive oxygen species production, the neutrophil elastase translocates from azurophilic granules into the nucleus and degrades specific histones, leading to chromatin decondensation (45). However, the biological significance of the C-terminal clipped H2A in NET formation remains unclear.

Volger *et al.* showed that cells expressing C-terminally truncated H2A (amino acids 1-114) were vulnerable to cellular stress, implying a pivotal role of this tail in cellular homeostasis (46). They then demonstrated that the H2A C-terminal tail is important in nucleosome stability and mobility *in vivo* and *in vitro*. Furthermore, they showed that this C-terminal H2A tail is implicated in regulating chromatin remodeling process by ISWI-type remodelers and functions as a recognition module for histone H1. These results suggest that H2A C-terminal

tail (amino acids 115-129) stabilizes the nucleosomal core particle, while mediating the protein interactions regulating chromatin dynamics.

In 1993, a second H2A specific protease was found in a chicken liver nuclear extract (47). It was argued that by using a protease inhibition assay, this H2Asp is an aspartic acid-like protease. The H2Asp generated a single clipped H2A product (H2AGlu91) in the *in vitro* cleavage assay. Interestingly, it was found that the expression and the activity of H2Asp were only detected in liver nuclear extract (tissue-specific). However, the function of the C-terminal deleted H2A and the characterization of the responsible protease remains elusive.

Recently, an enzyme for an H2B tail cleavage was reported by Pejler et al. They demonstrated that tryptase clipped off the N-terminal tails of histone H2B as well as histone H3 (48, 49). Mast cells contain a high content of cytoplasmic secretory granules densely packed with bioactive substances, including a mast cell specific protease, tryptase, which performs pro-inflammatory functions. Unexpectedly, they revealed that the tryptase relocates from granules into the nucleus during the cell death process and cleaves core histones. The tryptase-dependent truncation of Histone H2B and H3 also occurred in mast cell differentiation. They also found that the tryptase is mostly localized to the heterochromatin, and chromatin fraction from tryptase deficient cells is more resistant to micrococcal nuclease than that from wild type. This implies that tryptase may control the chromatin structure by promoting the formation of euchromatin over heterochromatin. In their subsequent study (49), however, they demonstrated that the absence of tryptase leads to the age-dependent accumulation of H2BK5ac, which was associated with an extensive upregulation of markers of non-mast cell lineages. These results suggest that tryptase can positively or negatively modulate gene expression depending on the chromatin state of the mast cells.

Histone H3 cleavage

The clipping of Histone H3 has been investigated more intensively than that of other histones. Histone H3 has numerous sites that are susceptible to several proteases (33) and the N-terminal cleavage of H3 has been reported in many cellular processes, including mouse embryonic stem cell (ESC) differentiation, viral infection, aging, yeast sporulation, senescence, DNA damaging stress, and osteoclastogenesis (50-56). Additionally, H3 protease activity has been found in *Tetrahymena* micronuclei, avian liver tissues, human ESCs, and mouse mast cells (48, 54, 57-59).

In 1980, Allis *et al.* reported that micronuclei of *Tetrahymena* contained two electrophoretically distinct forms of histone H3; the fast migrating form (H3^F) and the slow migrating form (H3^S). The fast migrating form, H3^F was derived from H3^S by a physiologically regulated, proteolytic processing event in the condition of cell growth and/or division, but not in non-growth and starvation, indicating that H3 proteolytic cleavage may occur regularly each generation at a specific point in the cell cycle (60).

Several studies have shown the effect of viral infection on the integrity of histones. It was reported that infection of BHK cells with foot-and-mouth disease virus (FMDV) caused the loss of histone H3 and the simultaneous formation of a new chromatin-associate protein (Pi) which migrates between histones H2A and H4 on SDS-polyacrylamide gels (52). Subsequently, Falk *et al.* showed that Pi is the truncated H3 form lacking the 20 N-terminal residues by proteolytic cleavage during infection (61). Also, they found that the transition of histone H3 into Pi was catalyzed by the FMDV 3C protease, which had been known to be related in the proteolytic processing of the viral polyprotein into the mature gene products. Another group reported that FMDV protease 3C inhibits gene transcription and its inhibitory

mechanism might involve the truncation of histone H3 (62). These results suggested that the FMDV virus controls the host cell's transcription by modulating histone H3 N-terminal cleavage.

Cathepsin L, a lysosomal protease, is a member of the papain-like super family of cysteine protease. Cathepsin L plays an important role in extracellular matrix degradation related to cancer, bone remodeling, and cardiovascular disease, and functions as an immune modulator via the generation of peptide antigens for MHC class II presentation (63, 64). Although Cathepsin L is mainly localized within lysosome, it is also found in the nucleus, and the functions of Cathepsin L in the nucleus have been reported (65-68). In 2008, Duncan *et al.* demonstrated that Cathepsin L proteolytically processes histone H3 during mouse ESC differentiation (51), in the first report presenting the implication of histone cleavage in the alteration of epigenetic signatures upon differentiation. Histone H3 was cleaved at the N-terminal tail during ESC differentiation, with alanine 21 being the primary site of cleavage, while Cathepsin L was identified as a protease responsible for H3 N-terminal tail clipping. They also suggested that this cleavage activity might be modulated by covalent modifications such as H3K18ac or H3K27me2 via *in vitro* cleavage assays. Moreover, this removal of histone H3 N-terminal tail inhibited the ability of CBX27 to bind to H3K27 methylation, indicating the histone H3 cleavage by Cathepsin L could lead to significant downstream effects. The biological significances of the Cathepsin L-mediated H3 cleavage during ESC differentiation remain elusive.

The proteolytic processing of histone H3 tail by Cathepsin L was also observed in models of oncogene-induced and replicative senescence (50). It was found that unlike ESC differentiation, H3.3 is preferentially cleaved over H3.1 during cellular senescence. RNA-sequencing studies revealed that the overexpression of the cleaved H3.3 in fibroblasts

results in transcriptional downregulation of cell cycle genes, with significant overlap of genes that lose H3K4me3 during cellular senescence. It was suggested that H3.3 tail cleavage plays a key role in silencing the transcription of cell cycle-promoting genes by removing H3K4me3.

In 2009, Santos-Rosa *et al.* used a yeast model and detected the endopeptidase activity of nuclei preparations toward histone H3, which was low in cells growing exponentially but increased in cells grown into stationary phase or shifted to sporulation (55). They found that the H3 endopeptidase is a serine protease clipping histone H3 after alanine 21, but were unable to further define a specific protease among the 24 serine proteases of *S. cerevisiae*. They also showed that loss of the H3 N-terminal tail correlates with the induction of gene expression that precedes H3 displacement. To test if the H3 tail-cleavage is prerequisite for gene induction, Santos-Rosa *et al.* used a yeast strain having the mutation in the histone H3 cleavage site which is unable to cleave the H3 tail. This mutant showed impaired expression of genes activated on the stationary phase and sporulation, indicating that the cleavage of the H3 tail at gene promoters contributes to the proper induction of gene expression. After 5 years, it was reported the vacuolar proteinase B (Prb1) has a cleavage activity toward histone H3 N-terminus in both whole cell extracts and nuclear extracts of *S. cerevisiae*. They demonstrated that the purified Prb1 can cleave the H3 N terminus between Lys23 and Ala24 *in vitro* (69).

Mandal *et al.* reported that a cysteine protease activity in chicken liver is specific for histone H3 (57). Later, they revealed via mass spectrometric analysis that this protease is glutamate dehydrogenase (58). Glutamate dehydrogenase was found to bind to four core histones but cleaving only the histone H3 tail in its free and chromatin context. Additionally, the truncation of H3 was observed in quail liver during the aging process, although the

responsible enzyme was not identified (54,70).

In the involuting mammary gland, the nuclear translocation of Cathepsin D caused the proteolytic cleavage of H3 at its amino terminal tail (71). They showed that H3 was preferentially cleaved by Cathepsin D between lysine 23 and alanine 24. Interestingly, they found that the nuclear translocation of Cathepsin D was mediated by its post-translational modification, tyrosine nitration.

Recently, our group found that matrix metalloproteinase 9 (MMP-9) is the principal protease of the histone H3 N-terminal tail during osteoclast differentiation (53). It has been shown that MMPs are a family of zinc-dependent endopeptidases that remodel the pericellular space primarily through the cleavage of extracellular matrix proteins. Although MMP-9 was known as a secretory protein, we detected the nuclear accumulation of MMP-9 during osteoclastogenesis through biochemical studies (74). We found that MMP-9 specifically cleaved H3K18-Q19 *in vitro* and *in vivo*. Furthermore, our results also showed that MMP-9 enzymatic activity toward H3 N-terminal tail is significantly facilitated by H3K18 acetylation and that p300/CBP is the histone acetyl transferases responsible for H3K18ac observed in osteoclast cells. Importantly, our findings indicated that MMP-9 was necessary for H3 tail cleavage near transcription start sites or gene bodies of a set of osteoclastogenic genes and concurrent gene activation during osteoclast differentiation, suggesting that H3 N-terminal proteolysis induces gene activation.

Shen *et al.* demonstrated that JMJD5 mediates histone H3 N-tail proteolytic cleavage at the carboxyl side of H3K9me1 under stress conditions such as a DNA damage response (56).

JMJD5, a Jumonji C (JmjC) domain-containing protein, was originally known as histone H3

lysine 36 dimethylation (H3K36me₂), responsible for gene expression. In A549 cells, under cell stress conditions, JMJD5 bound to gene promoters and cleaved H3K9me₁, suggesting JMJD5-mediated histone cleavage may be responsible for gene transcription regulation.

As previously mentioned, mast cell tryptase functioned as a clippase of histone H3 as well as H2B and, in aging mast cells, the partial loss of tryptase expression was accompanied by a decrease of H3 and H2B clipping (48, 49). While tryptase-deficiency did not affect the posttranslational modifications of histone H3, it caused an increase of H2BK5ac. Therefore, the biological significance of histone H3 clipping in mast cells still needs to be determined.

Other histones cleavage

A limited number of studies have reported the conversion of histone H1 or H4 into a smaller form via a regulated proteolysis. Nevertheless, the degradation of histone H1 or H4 has been shown in several biological processes including the macronuclear degradation of *Tetrahymena* and staurosporine-induced cell death (75-77). Interestingly, it was shown that histone H1 of *Tetrahymena* micronuclei was processed into smaller forms. *Tetrahymena* contains two different nuclei: a macronucleus and a micronucleus. Micronucleus-specific histone H1 differs from macronuclear H1. Micronucleus-specific histone H1 was larger and is proteolytically processed into different components (α , β , γ and δ). This proteolytic processing of H1 histone was regulated in the pathway of micronuclear differentiation (75). Lee et al. demonstrated that histone H4 is cleaved by granzyme A under apoptotic process in B-lymphocytes (76). Granzyme A is a serine protease in the cytotoxic granules found in natural killer cells and cytotoxic T cells. They suggested that Granzyme A-mediated H4 tail cleavage contributes to the disintegration of the chromatin architecture during the cell death process (76).

Epigenetic regulatory mechanism by histone cleavage

The proteolytic cleavage of histone is an interesting and active topic of epigenetic modifications. Despite the increasing number of studies that demonstrate novel enzymes and cellular processes related to histone cleavages, the mechanism by which cleaved histones regulate gene expressions still needs to be explained. Here, we discuss recent findings, focusing on how histone cleavage impacts gene expression (Figure 1).

1. Influence on chromatin compaction

Eukaryotic DNA is packed into chromatin, which enables the genomic DNA to fit in the limited space of the nucleus. However, chromatin limits the accessibility of transcription factors and RNA polymerase to gene promoters. Thus, chromatin conformation plays an important role in gene expression (55).

Tailless nucleosomes have been used to investigate the role of histone tails (78, 79). Using tailless nucleosomes treated with trypsin to cleave off the core histone tail domains, it was reported that the histone tails participate in inter- or intra-nucleosomal interactions and play a significant role in regulating the accessibility of genomic DNA (79). Also, nucleosomes containing truncated H3 and/or truncated H4 were utilized to clarify the role of flexible tails of H3 and H4 histones. The N-terminal regions of histone H3 and H4 were implicated in the intranucleosomal interactions by restricting the DNA breathing motion and compacting the nucleosome (78). Therefore, histone tail truncations by specific protease might increase DNA accessibility and generate open conformation of nucleosomes, leading to gene activation.

2. Linking to histone eviction

Histones are evicted at many promoters during gene activation to allow access to the

transcription machinery (80,81). It was also suggested that the entire nucleosome is at least sometimes disassembled and reassembled during the process of RNA polymerase passage (80).

As mentioned previously, Santos-Rosa *et al.* identified a histone H3 endopeptidase in *S.cerevisiae*, which cleaves the H3 N-terminal tail at the promoter sites of genes following the induction of transcription (55). Notably, H3 clipping precedes the process of histone eviction, resulting in the localized clearing of repressive signals during the induction of gene expression. They suggested the possible role of a clipped H3 to mark the nucleosomes that are to be displaced prior to gene induction. Taken together with previous studies, histone tail cleavage may induce histone eviction, allowing easy access of transcription machinery to DNA during gene activation.

3. Removal of PTMs and interacting proteins

Diverse PTMs are found primarily on the tails of histones that extend distinctively from the nucleosome core, which is sensitive to proteases. To date, two characterized models have been used to consider the outcome of histone tail modifications. The first model suggests that histone tail modifications induce changes in the physical properties of the nucleosome that reduces inter- or intra-nucleosomal contacts, producing a more relaxed chromatin fiber. Of all known modifications, histone acetylation via the neutralization of the positive charge of lysine, and histone phosphorylation via the addition of negative charge, may cause decondensation of the chromatin structure (13). The second model suggests that modifications function as recognition marks to mediate the recruitment of effector proteins. Numerous chromatin-associated factors have been shown to specifically interact with modified histones through many distinct domains such as bromodomain, PHD finger, chromodomain, Tudor domain, PWWP domain, and MBT domain [62-64]. These histone tail

modifications are modulated by specific enzymes that add or remove modifications, respectively. Although specific modifications can be removed by specific enzymes, histone clipping may be an efficient way to erase multiple modifications of histone tails concurrently. That is, histone tail cleavage could cause the radical removal of multiple modifications, thereby blocking the recruitment of effector proteins or other PTM cascades on histone tails.

Perspectives

Although a growing body of studies has revealed histone tail cleavages in diverse cellular processes and identified specific proteases responsible for histone cleavages, these findings have raised many questions.

In particular, it would be interesting to determine the genes that are regulated by histone cleavage. Our integrated genome-wide analysis of ChIP-seq and RNA-seq data revealed that MMP-9 cleaved histone tails in approximately 8% of all protein-coding genes during osteoclastogenesis and many osteoclast-related genes were regulated (53). Additionally, JMJD5-mediated H3 tail clipping affected cell cycle-related gene transcription (56). However, the genes that are directly affected by protease-specific histone cleavage still need to be investigated.

It is also important to determine how proteases localize at specific genes under specific conditions. It is suggested that specific histone modifications could recruit histone proteases. We observed that H3K27me1 is necessary for the localization and function of MMP-9 at osteoclastogenic genes as a protease catalyzing H3 tail proteolysis (unpublished data). Alternatively, a crosstalk or interaction between proteases and other histone modifying enzymes might exist that enables histone proteases to localize at specific sites (30).

Finally, a way of controlling protease activity needs to be investigated. Protease can be post-translationally regulated. For example, the nuclear translocation of Cathepsin D was regulated by its post-translational modification, tyrosine nitration (71). Also, histone tail modifications affect the cleavage activity. It has been suggested that Cathepsin L cleavage activity might be modulated by covalent modifications such as H3K18ac or H3K27me2 (51). Moreover, CBP/p300-mediated acetylation of H3K18 facilitated MMP-9-dependent H3 proteolysis (53). Nonetheless, it will be interesting to determine the upstream effectors that stimulate or inhibit protease activity in signaling pathways.

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Figure legends

Fig.1. Schematic model showing the functional roles of histone cleavage in gene expression.

(A) Influence of genomic DNA or chromatin compaction on accessibility. Histone tail cleavages by protease might generate open chromatin structures and increase DNA accessibility for transcription factors, thereby contributing to gene activation. (B) Induction of histone eviction. The truncated histone by protease is unstable in the nucleosome. Histone tail cleavage may induce histone eviction, allowing easy access of transcription factors to DNA elements during gene activation. (C) Removal of PTMs and interacting proteins. Histone tail cleavage may lead to the radical removal of multiple modifications, resulting in blockage of the recruitment of effector proteins ("Reader" protein) or other PTM cascades on histone tails.

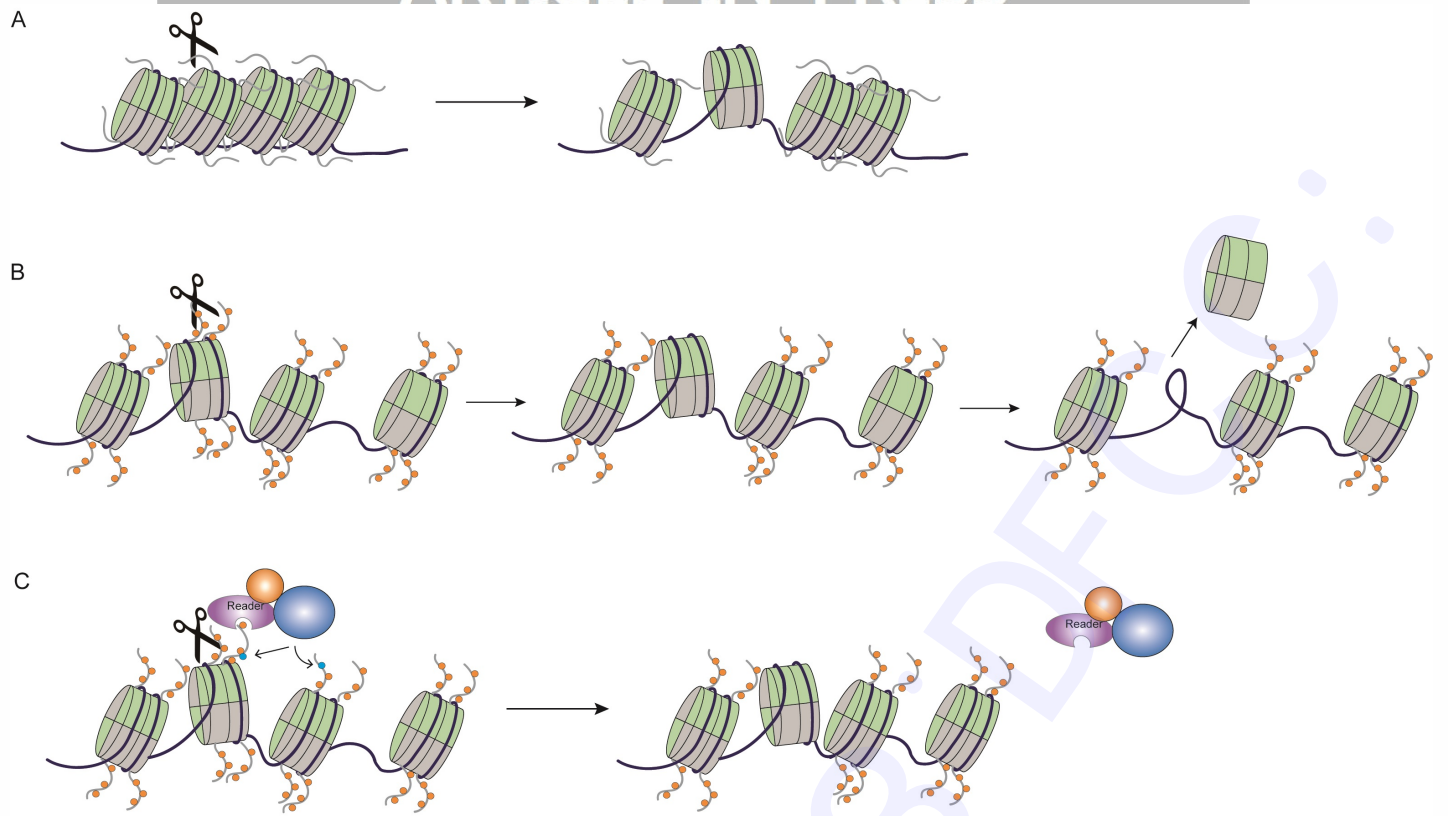


Fig. 1.

Table 1. List of histone proteases identified and their roles

Histone	Protease	Cleavage site(s)	Model	Biological significance of activity	Reference
H2A	Histone H2A specific protease (H2Asp)	Asn90-Asp91	Chicken liver extract	?	47
	Neutrophil Elastase	Val114-Leu115	Neutrophil	NET(neutrophil extracellular trap) formation	44
	H2A protease	Val114-Leu115	Calf thymus	?	38
H2B	Tryptase	?	Mouse mast cells	Mast cell differentiation	48, 49
H3	MMP-9	Lys18-Gln19	Bone marrow macrophages	Osteoclastogenesis	53
	Cathepsin L	Ala21-Thr22, Thr22-Lys23, Lys23-Ala24, Ala24-Ala25, Arg26-Lys27, Lys27-Ser28	Mouse ESCs	ESC differentiation	51
	Human ESC protease	Ala21-Thr22, Arg26-Lys27, Ala31-Thr32	Human ESCs	ESC differentiation	50
	Glutamate dehydrogenase	Lys23-Ala24, Lys27-Ser28	Chicken liver extracts	?	58
	Yeast endopeptidase	Ala21-Thr22	<i>S. cerevisiae</i>	Induced under nutrient deprivation and sporulation	55
	Vacuolar protease B (Prb1)	Lys23-Ala24	<i>S. cerevisiae</i>	?	69
	Foot-and-mouth disease virus 3C protease (FMDV 3C protease)	Leu20-Ala21	Infected BHK-21 cells	Host cell transcription shut off	61
	JMJD5	Lys9-Ser10	Lung cancer cells	Induced under DNA damage	56
	?	?	Tetrahymena micronuclei	?	60
	Cathepsin D	Lys23-Ala24	Mouse mammary gland	Involution mammary gland	71
	Tryptase		Mouse mast cells	Mast cell differentiation	48, 49
H4	Granzyme A	?	B-lymphoid	Staurosporine-induced cell death	76
Histone H1	?	H1 processed into three different components (α , β , γ).	Tetrahymena micronuclei	Cell division	75

Fig. 2.