



Identification of histone H3 clipping activity in human embryonic stem cells



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Abstract Posttranslational histone modifications are essential features in epigenetic regulatory networks. One of these modifications has remained largely understudied: regulated histone proteolysis. In analogy to the histone H3 clipping during early mouse embryonic stem cell differentiation, we report for the first time that also in human embryonic stem cells this phenomenon takes place in the two different analyzed cell lines. Employing complementary techniques, different cleavage sites could be identified, namely A21, R26 and residue 31. The enzyme responsible for this cleavage is found to be a serine protease. The formation of cleaved H3 follows a considerably variable pattern, depending on the timeframe, culture conditions and culture media applied. Contrary to earlier findings on H3 clipping, our results disconnect the link between declining Oct4 expression and H3 cleavage.

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Introduction

In all eukaryotes, DNA is tightly associated with histone proteins in order to form chromatin, of which the fundamental subunit is the nucleosome (Luger et al., 1997). Each nucleosome consists of four different core histone types (H2A, H2B, H3 and H4), which have been very well evolutionarily conserved. Chromatin structure is essential for compaction of genomic DNA but also

represents a physical barrier to control DNA accessibility and gene expression.

In embryonic stem cells (ESC) the delicate balance of self-renewal and differentiation into specific lineages is determined by many lineage-restricted promoters that are associated with highly combinatorial posttranslational histone modification (PTM) patterns which may determine their selective priming of gene expression during lineage commitment. Together with DNA methylation, ATP-dependent chromatin remodeling, RNA interference, non-coding RNA and incorporation of histone variants, these properties form the “epigenetic signature” (Tollervey and Lunyak, 2012).

Not only ESC differentiation but also other biological contexts are characterized by a continuous interplay of

Abbreviations: PTM, posttranslational histone modification; ch3, clipped histone H3; NE, nuclear extract.

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installation and removal of histone PTMs. To accomplish the latter, several mechanisms can be at play. Apart from enzymatic elimination of modifications (Kouzarides, 2007; Tollervey and Lunyak, 2012) and histone exchange (Bernstein and Hake, 2006; Skene and Henikoff, 2013; Tollervey and Lunyak, 2012), also regulated proteolytic histone cleavage has been suggested to play such role (Duncan and Allis, 2011; Duncan et al., 2008).

Duncan et al. showed that H3 is proteolytically cleaved at its N-terminus during early differentiation of mouse ESC (mESC) and they provide evidence for the regulatory capacity of covalent modifications herein (Duncan et al., 2008). Cathepsin L was found to cleave histone H3, with alanine 21 being the primary site of cleavage (Adams-Cioaba et al., 2011; Duncan et al., 2008). This truncated H3 form is detected during the first days of both monolayer differentiation (with and without retinoic acid induction) and embryonic body (EB) formation. Similar clipping events of H3 associated with other cellular processes including viral infection (Falk et al., 1990; Tesar and Marquardt, 1990), aging (Gonzalo, 2010; Mahendra and Kanungo, 2000) and sporulation (Santos-Rosa et al., 2009) have also been reported. Additionally, H3 protease activity was also found in chicken liver and Tetrahymena micronuclei (Allis and Wiggins, 1984; Allis et al., 1980; Mandal et al., 2012, 2013). Although the molecular consequences of any histone clipping event are yet to be defined, these data seem to suggest an evolutionary conserved process.

Here, we show for the first time that histone H3 clipping also occurs in human ESC lines (hESC) in addition to mESC. Several cleavage sites were assigned and the clipping enzyme was characterized as a serine protease. The manifestation of this proteolytic event can theoretically have an impact on several levels such as pluripotency and differentiation. Our results indicate that H3 cleavage can indeed be accompanied by a loss of Oct4 expression, although more research will be needed to determine any causal effect.

Experimental procedures

Cell culture

Two human ES cell lines were used: the UGENT2 cell line, created in-house and the WA01 Oct4-eGFP knock-in reporter cell line, obtained from WiCell (Zwaka and Thomson, 2003). Both cell lines were cultured (5% O₂ and 5% CO₂ at 37 °C) on a feeder layer of Mitomycin C inactivated mouse embryonic fibroblasts (MEF). Cells were passaged every 4 to 6 days, using 1% collagenase type IV and glass beads. Culture medium consisted of knock-out DMEM supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 20% knock-out serum replacement, 4 ng/ml basic fibroblast growth factor (b-FGF), 100 u/ml penicillin and 100 µg/ml streptomycin.

Feeder-free cultures were maintained on a Vitronectin XF coating (Primorigen), in combination with Essential 8 medium (Life Technologies). Cultures were split every 2 to 3 days by means of EDTA-passaging.

Differentiation was induced by omitting b-FGF from the medium and by adding retinoic acid (RA) to the culture medium at a final concentration of 2 µM.

Fluorescence microscopy

Fluorescence microscopy images of eGFP expression (ex 485 nm, em 515 nm, exposure time 5000 ms) were acquired on an Axiovert 200M inverted fluorescence microscope equipped with the Axiovision multichannel fluorescence module and an AxioCam MRM camera (Carl Zeiss). Colonies (without auto-fluorescent medium) were screened at 10× magnification using a Carl Zeiss short distance Plan-Achromat® objective and visualized using Zeiss filter set no. 38 (BP 470/40, FT 495, BP 525/50). For larger colonies, several images were stitched by use of Photoshop CS4 on TIFF images (Adobe).

Acid histone extraction

Cells were isolated using 0.25% trypsin-EDTA. After a washing step, cells were resuspended in Triton extraction buffer (TEB; 0.5% Triton X 100 and 0.02% NaN₃ in PBS) at a cell density of 1 × 10⁷ cells/ml, and incubated for 10 min on ice. Subsequently the cells were centrifuged at 1500 rpm for 10 min at 4 °C, and resuspended in half the volume of TEB. After centrifuging again, the cells were resuspended in 0.2 N HCl at a cell density of 4 × 10⁷ cells/ml and incubated overnight at 4 °C, after which the supernatant containing the histones, was isolated. Protease inhibitors (Roche, 11836170001) were freshly added to each buffer for every experiment, unless stated otherwise. Protein concentration of the extracts was measured by BCA assay.

Direct boiling in Laemmli buffer

Direct boiling in SDS-loading dye for protein isolation was performed. Human ESC were directly boiled (3 min at 100 °C) after harvesting with trypsin, in 2× Laemmli buffer (4% SDS, 20% glycerol and 10% beta-mercaptoethanol in 50 mM Tris (pH 6.8)).

Gel electrophoresis and Western blotting

Dried samples were dissolved in 2× Laemmli buffer, separated on a 15% Tris-HCl gel (BioRad Laboratories) and tank-blotted on a nitrocellulose or a PVDF membrane. The membrane was incubated overnight at room temperature with the appropriate antibody. The following antibodies were purchased from commercial vendors: a C-terminus directed histone H3 Ab (1:1000; Abcam, ab10799), an N-terminus directed histone H3 Ab (1:1000; Merck Millipore, 05-499) and an antibody specific for the H3 N-terminus when cleaved after A21 (H3.cs1 Ab; 1:1000; Active Motif, 39573). All blots were detected by chemiluminescence using a Versadoc imaging system (BioRad Laboratories). Biotinylated histone H3 (as described below) was specifically visualized using HRP-conjugated avidin (45 min incubation; 1:100000; eBioscience, 18-4100-94). When needed, blots were stripped by incubating at 50 °C for 4 to 6 h in stripping buffer containing 2% SDS, 0.1 M betamercaptoethanol, 0.05 M Tris (pH 6.8).

In-gel digest of propionylated histones

Vacuum-dried histones were propionylated as described (Zhang et al., 2013) and separated by gel electrophoresis. Sypro Ruby (Invitrogen) stained gel pieces were cut out and subsequent in-gel digestion was performed as described before (Glibert et al., 2014), with only a slight modification to the protocol: 25 mM ammonium bicarbonate buffer was used and the alkylating agent applied was iodoacetamide (100 mM). After peptide extraction out of the gel pieces, samples were vacuum-dried and a second round of propionylation was completed to propionylate the newly generated N-termini.

Flow cytometry

Oct4-eGFP levels were monitored after cell isolation and resuspension in flow buffer (1% BSA, 0.1% NaN₃ in PBS) with an FC500 (Beckman Coulter) using the CXP analysis software.

Reverse transcription — quantitative PCR

RNA isolation, cDNA preparation and RT-qPCR analysis were performed as described before (Vossaert et al., 2013). In short, cells were suspended in Trizol after isolation and stored at -80°C . After reverse transcription (SuperScript II kit, Invitrogen), qPCR analysis was performed for pluripotency gene *POU5F1* (Taqman assay Hs01895061_u1) with normalization to an optimized pool of 3 reference loci, namely *B2M* (RTPrimerDB ID #2), *RPL13A* (#6) and *AluSq* (FW: CATGGTGAAA CCGCTCTCTA-RE: GCCTCAGCTCCCGAGTAG) (all SYBR Green assays).

Histone biotinylation

One mg of purified histone H3, isolated from calf thymus (Roche), was biotinylated using the EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific, 21425), according to the manufacturer's conditions. After the biotinylation reaction, the excess of biotin-label was removed by addition of 5% hydroxylamine (HA)-solution in a concentration of 6 μL HA per 100 μL sample.

N-terminal sequence analysis

A histone extract taken one day after induction of differentiation and known to contain cH3 as validated by Western blotting, was blotted onto PVDF and proteins were visualized using Ponceau S staining. Subsequently the cleaved area was cut and subjected to Edman degradation, which was outsourced to Eurosequence (Groningen, The Netherlands) and performed according to standard procedures.

Mass spectrometry method

Propionylated peptides were dissolved in 0.1% formic acid in water (buffer A) and separated on a PepMap 100 (C18) column (I.D. 75 μm , length 25 cm, particle size 5 μm) by use of a U3000 LC-system (Dionex) at a flow rate of 300 nL/min. Elution was performed with 80% acetonitrile/0.1% formic

acid (buffer B) using a gradient of 10% to 60% buffer B in 60 min. A Q-TOF Premier mass spectrometer (Waters) was operated in the data-dependent mode, using a nano-ESI source with resolution of 10,000. Survey MS scans were acquired (m/z 425–1300) and up to 7 precursors (m/z 50–2300) with charge state 2+, 3+ or 4+ exceeding the signal threshold were isolated for fragmentation by collision induced dissociation, using the collision energy profile as suggested by the manufacturer. An inclusion list contained precursor m/z value 574.3 since preliminary experiments suggested that this is a cleavage site fragment.

Mass spectrometry data analysis

Database searching was performed against a custom-made database containing human histone sequences obtained from the National Center for Biotechnology (NCBI) database, using a Mascot 2.3 in house server (Matrix Science). Mass error tolerances for the precursor ions and its fragment ions were set at 0.35 Da and 0.45 Da respectively. Enzyme semi-specificity was set to Arg-C, allowing for up to two missed cleavage sites. Variable modifications included acetylation, dimethylation and propionylation on lysine (K), methylation and dimethylation on arginine (R) and oxidation of methionine. Lysine monomethylation was searched as the sum of propionylation and methylation since monomethylated lysine residues can still be propionylated. N-terminal propionylation and carbamidomethylation of cysteine residues were set as fixed modifications. Redundant peptides were filtered including only the highest scoring match under the highest scoring protein containing that match. Low confidence identifications were excluded using an expectancy value cut-off of 0.05. Nevertheless all of the used spectra were additionally manually validated using Mascot Distiller software (Matrix Science).

Nuclear extraction and protease inhibitor experiments

Nuclear extracts were prepared using the commercially available Episeeker Nuclear Extraction Kit (Abcam, ab113474), omitting protease inhibitors during extraction. Calf H3 (1 μg) and nuclear extract (0.5 μg) were incubated together in buffer (150 mM NaCl, 2 mM betamercaptoethanol, 0.1 mM EDTA, 10% glycerol and 25 mM Tris-HCl (pH 7.5)) at 37°C for 1 h, after which the assay was inactivated by incubation at 99°C for 10 min. All samples were analyzed by Western blotting.

For the inhibition assay the following commercially available inhibitors were used (Fig. 6B): 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich, 76307), E64 (Sigma-Aldrich, E3132), EDTA (Sigma-Aldrich, E5134), Bestatin (Sigma-Aldrich, B8385), Pepstatin A (Sigma-Aldrich, P5318), protease inhibitor cocktail tablets (Roche, 11836170001) and three specific cathepsin L inhibitors (I (219421), III (219427) and CAA0225 (219502), purchased from Calbiochem. Inhibitors were pre-incubated with the nuclear extract for 15 min at 37°C prior to addition of the H3 substrate.

Results

Feeder-Free cultured hESC show continuous histone H3 cleavage during differentiation

To verify whether the histone H3 N-tail is cleaved during human embryonic stem cell differentiation in analogy to mESC (Duncan et al., 2008), two hESC lines were monitored: an Oct4-eGFP reporter hESC cell line that expresses eGFP under the control of the Oct4 promoter and a non-reporter cell line UGENT2. Both cell lines were cultured in feeder-free conditions during 5 days after induction of differentiation by addition of 2 μ M retinoic acid (RA) to the culture medium, in the absence of b-FGF.

Cells were isolated every 24 h and histone extractions were prepared.

The differentiation status during the experiments was validated for both cell lines. Morphologically each cell line displayed clear traits of differentiation, resulting in more lengthened cells and loss of round colony shape (data not shown). To further validate the efficiency of differentiation, Oct4-eGFP reporter cells were directly monitored by both flow cytometry and fluorescence microscopy (Scheerlinck et al., unpublished results). Flow data showed a clear drop in eGFP signal through time (Fig. 1A), and microscopy imaging visualized a sustained but definite reduction in eGFP throughout the hESC colonies (Fig. 1B). To monitor loss of pluripotency in the conventional non-reporter stem cell line (UGENT2), RT-qPCR analysis of the pluripotency gene *POU5F1* encoding for the transcription factor Oct4, coordinately confirmed differentiation in these cells (Fig. 1C).

Samples were monitored for histone H3 cleavage by means of Western blotting analysis, using an H3 C-terminally directed antibody. For both cell lines, lower molecular weight bands of histone H3 were visualized at each time point throughout the differentiation experiment, indicating N-terminal cleavage of H3 (Fig. 1D).

Continuous histone H3 cleavage in the Oct4-eGFP reporter hESC line is not related to the Oct4 expression level

The results above differ substantially from the data reported for differentiating mESC, which display a pattern with upcoming cleaved H3 (cH3), reaching a maximum intensity after 4 days, and decreasing again towards the end of the experiment (Duncan et al., 2008). The continuous H3 clipping found in feeder-free cultured hESC could be best explained by assuming that the timeframe was not extended enough to demonstrate the disappearance of cH3. The cH3 pattern might also be influenced by the speed and heterogeneity of differentiation, as also shown by Duncan et al., where spontaneous and RA induced differentiation and embryonic body formation all lead to different cleavage patterns in terms of timescale. Therefore we repeated the experiment with the Oct4-eGFP reporter cell line, monitoring the hESC in feeder free culture for a longer period of time (14 days), and subjected them to three different methods of differentiation to vary the speed at which they lose stemness throughout this timeframe: (i) in their

undifferentiated status by culturing the cells in E8 medium, (ii) inducing spontaneous differentiation by omitting basic fibroblast growth factor (b-FGF) from the culture medium and (iii) enforcing directed differentiation by addition of RA. Morphological changes were observed using light microscopy (data not shown), global Oct4 expression and differentiation were monitored using flow cytometry. According to the flow plots shown in Fig. 2A, the three used culture conditions indeed differ greatly in their (non-) maintenance of stemness throughout the experiment. When analyzing the hESC, it is clear that E8 medium promoted the undifferentiated status in contrast to both differentiation methods which led to a decreased Oct4 expression. While spontaneous differentiation divided the culture system into two groups with loss of the Gaussian distribution in the flow histogram, RA induced a collective decrease in Oct4 expression of all cells resulting in a preserved Gaussian distribution shifting towards lower fluorescence. Also, differentiation induced by RA occurred much faster and a minimal Oct4 expression was reached after 12 days of differentiation while spontaneous differentiation continued at a slower pace and did not reach a minimum limit in Oct4 expression after 14 days of differentiation.

Despite the clear differences in Oct4 expression as described above, all time points in each culture condition appeared to display clear N-terminal H3 clipping when monitored with Western blotting (Fig. 2B).

H3 clipping in hESC is not an in vitro artifact

To exclude the possibility that this continuous histone H3 cleavage in a feeder-free culturing system is actually an in vitro artifact induced during extraction, we spiked biotinylated calf histone H3 during hESC histone extraction at day 1 of differentiation for subsequent avidin-HRP detection on Western blot. We initially confirmed that indeed the biotinylated histone H3 still is susceptible to proteolytical degradation (Supplemental Fig. 1). After spiking biotinylated calf H3 during extraction, a lower band was detected on the Western blot from the histone extracts only when using the C-terminal H3 antibody. This band was absent when immunoblotting was done using avidin-HRP, which specifically highlights the biotinylated fraction (Fig. 3A). This implies that the histone H3 cleavage was indeed already present during culturing and that the truncated form was not created during extraction. Moreover, when the extraction was performed both with and without protease inhibitors, no difference between these samples was found, confirming that no additional cleavage was induced during extraction.

In addition, the TEB/HCl extraction protocol was compared with direct boiling of the hESC after harvesting. Directly boiling in Laemmli buffer diminishes the steps of the extraction procedure and thus the steps in which cH3 can be artificially formed. For this experimental set-up, Oct4-eGFP reporter hESC were differentiated with 2 μ M RA for 24 h. After this the hESC were harvested and split into two to carry out both protocols simultaneously on the same starting material. As can be seen in Fig. 3B, both protocols result in the same Western blotting image containing cH3.

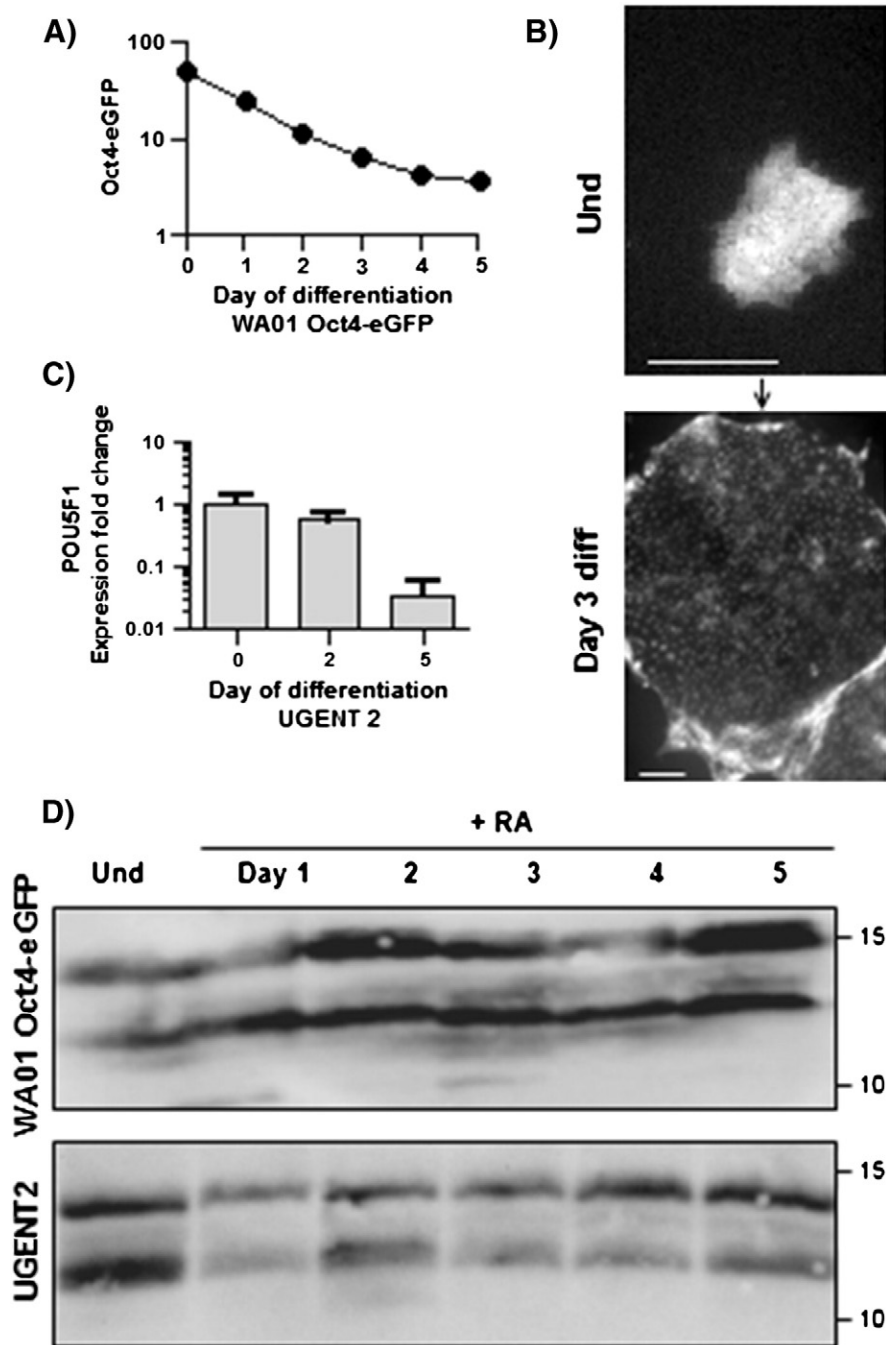


Figure 1 Histone H3 cleavage in human embryonic stem cells under feeder-free culture conditions. Human ESC samples were collected during five days after differentiation induction with retinoic acid. Differentiation was confirmed by [A] flow cytometry Oct4-eGFP analysis for the Oct4-eGFP cell line. In addition, [B] fluorescence microscopy data affirmed a significant drop in Oct4-eGFP signal when comparing an undifferentiated colony ('Und') and a colony after three days of differentiation (scale bars represent 200 μ m). Also RT-qPCR analysis [C] illustrated the decrease in *POU5F1* expression (encoding for Oct4) over time for the UGENT2 cell line. [D] Western blot analysis (2 μ g histone extract per sample) using a C-terminal H3 antibody reveals N-terminal H3 cleavage in both the Oct4-eGFP reporter (upper image) and UGENT2 cell line (lower image).

Similar histone H3 cleavage sites are found in mouse and human ESC

To verify if this H3 truncation event has any parallel to the clipping event reported in mESC, we next set out to identify the cleavage site(s).

Applying the antibody directed against the C-terminus of H3, cH3 is visualized, in some cases as multiple bands. As opposed to the C-terminal antibody, immunoblotting with an antibody directed against the N-terminal end of histone H3 detected no cH3 for both the Oct4-eGFP reporter and UGENT2 cell line (Figs. 4A and B). Only the band of intact

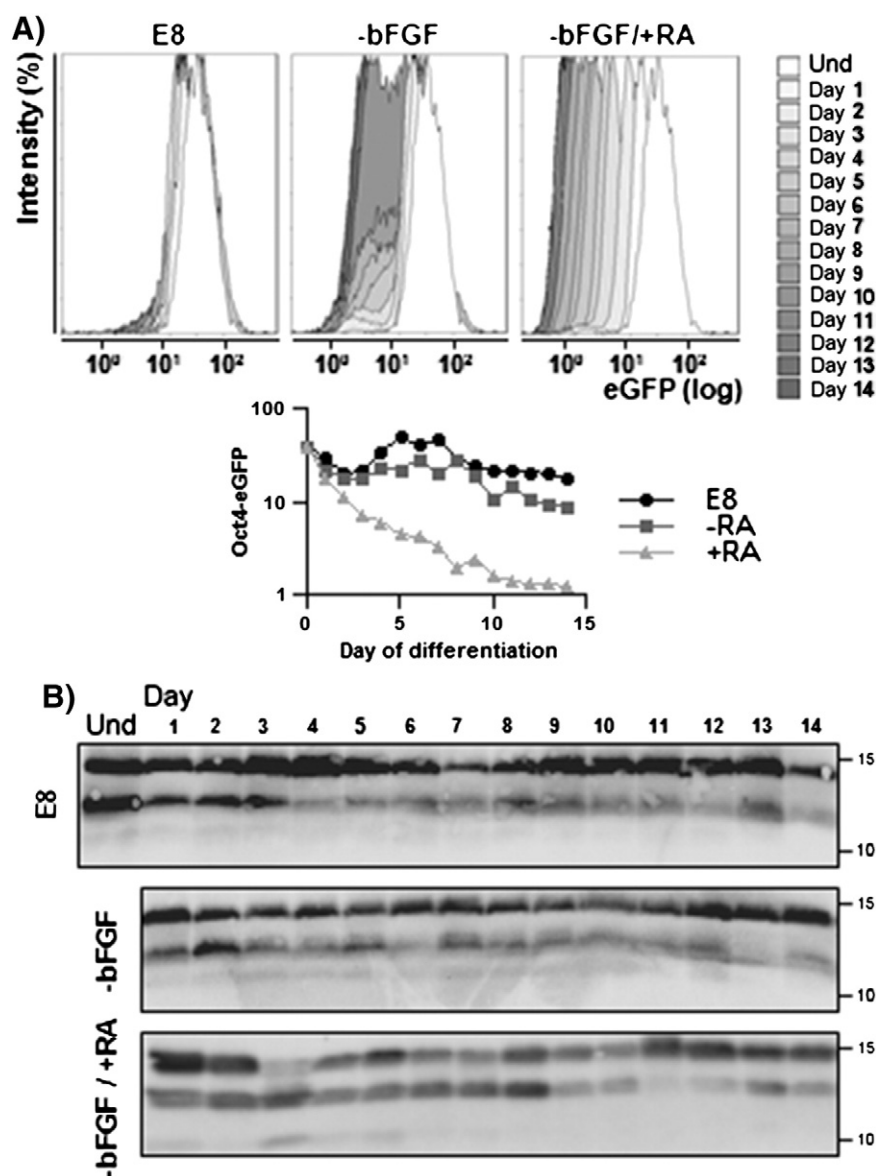


Figure 2 H3 cleavage in feeder-free cultured hESC under application of different culture media within an extended time frame. The Oct4-eGFP reporter cell line was cultured for 14 days in different conditions: (i) maintaining the pluripotent state in E8 medium, (ii) differentiating spontaneously (in the absence of b-FGF) and (iii) differentiating after RA induction. [A] Flow cytometry analysis displays a definite reduction in Oct4-eGFP levels under differentiation-allowing circumstances. The flow histogram plots show a variable shift in Oct4-eGFP signal under differentiating conditions: while the signal in RA-stimulated cells decreases collectively for the total population, the spontaneously differentiating cells are divided into two groups with different paces of differentiation. All data shown on the flow histogram depict data from the undifferentiated stage until day 14 of differentiation, with the most anterior graph representing the Oct4-eGFP signal from undifferentiated cells, and the graph most at the back illustrating day 14. [B] Western blot images (2 μ g samples, C-terminal H3 antibody) show continuous cH3 formation in hESC, regardless of the hESC culture condition.

histone H3 was seen, indicating N-terminal cleavage. When using the C-terminal antibody, the distance of the most intense cH3 band to the intact H3 form, implies a loss of approximately 3 kDa, which roughly corresponds to about 30 amino acids. Using the histone H3.cs1 antibody that was developed to specifically detect H3 truncated after A21 (Duncan et al., 2008), a weak signal was detected for samples of the Oct4-eGFP cell line, suggesting A21 as a possible target site also in hESC. Apart from the most prominent A21, Duncan et al. found additional histone H3

cleavage sites in mESC, so we pursued further investigation of the presence of any other possible cleavage site(s) in hESC. A specific mass spectrometry (MS) approach for histone analysis was therefore optimized which is subsequently described in more detail. A regular bottom-up approach using trypsin would cleave after each lysine (K) or arginine (R) (except when followed by a proline), resulting in unidentifiably small peptides because of the abundant presence of these basic amino acids in histones. We thus propionylated the histones prior to digestion, which

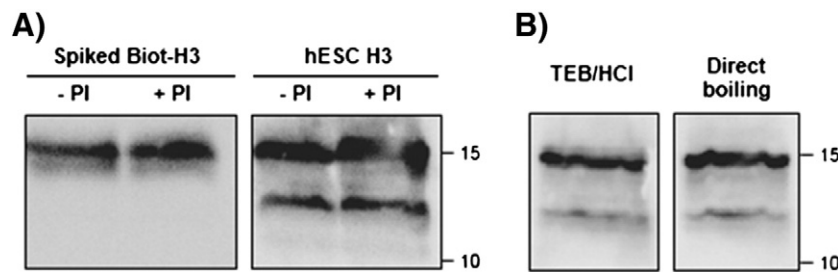


Figure 3 Histone H3 cleavage is not an in vitro artifact. [A] Comparison of spiked biotinylated H3 ('Biot-H3') with the H3 content extracted from hESC shows that only the latter undergoes truncation. As both immunoblotting images demonstrate, the use of protease inhibitors (–PI versus +PI) during the extraction procedure does not influence the outcome. Per lane 2 μ g sample was loaded. [B] Illustrates the comparison of two different extraction protocols: TEB/HCl extraction (left, 2 μ g sample) versus direct boiling in Laemmli buffer (right, 5 μ l loaded). Western blotting analysis shows no difference in cH3 content between those protocols.

modifies all free primary amine groups (the N-termini and the ϵ -amino group of unmodified and monomethylated K). Of note, when the N-terminus or a lysine residue is covered by an endogenous modification other than monomethylation, this residue cannot be propionylated but is also blocked from proteolytical digestion by that modification itself. Consequently, trypsin now only cleaves C-terminal to arginine residues mimicking an Arg-C digestion for histone H3, resulting in larger, identifiable peptides (Garcia et al., 2007). By post-digestion propionylation all newly generated N-termini are subsequently being propionylated as well. The specific cleavage sites present in cultured hESC can then be detected as peptides containing a non-arginine C-terminus or which start N-terminally with an amino acid not neighboring an arginine. Spectra corresponding

to such so-called semi-Arg-C peptides were additionally manually validated.

Hence we separated propionylated histone extractions of the feeder-free cultured Oct4-eGFP reporter cell line at four different time points after RA induction (D1, D2, D3 and D6) on SDS-PAGE (Fig. 4C). This way, multiple molecular weight bands could be cut out for subsequent in-gel digestion using trypsin. After a second round of propionylation, samples were analyzed by RPLC and ESI-MS/MS. Of all identified histone H3 peptides, 100% ended C-terminally with R and 94.4% had an N-terminus adjoining an R, confirming successful propionylation.

First, to confirm the N-terminal histone H3 cleavage by MS we monitored the presence or absence of an N-terminal

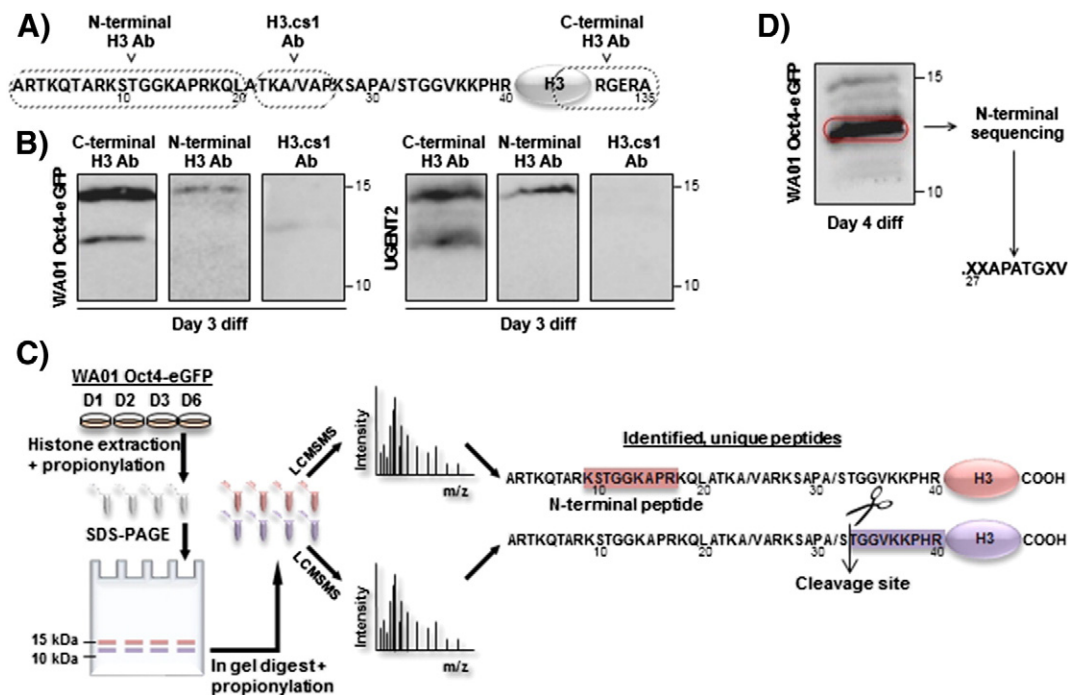


Figure 4 Elucidation of the cleavage sites. The application of differentially directed antibodies for immunoblotting (antibody epitopes displayed in [A]) illustrates that the cleavage is situated N-terminally, and proposes alanine21 as one of the possible cleavage sites [B]. [C] Mass spectrometry analysis after propionylation of different hESC samples (Day 1, day 2, day 3 and day 6 of differentiation) also reveals residue 31 as a site of cleavage. [D] An additional site, arginine26, was annotated by Edman degradation.

histone H3 peptide in the intact H3 and the cH3 gel bands. The peptide K₁₉STGGKAPR starting after R₁₈ covers the N-terminus of histone H3 and was only identified in the H3 bands and not the cH3 (Fig. 4C), in contrast to the globular histone H3 peptides and the C-terminal V₁₁₇TIMPKDIQLAR which were present in both. Two differentially modified semi-Arg-C peptides both started at residue 32 which N-terminally does not flank an R: T₃₂GGVK_{prop}K_{prop}PHR and T₃₂GGVK_{prop}K_{me-prop}PHR (Supplemental Figs. 2 and 3). Since these spectra correspond to the peptide generated by cleavage after amino acid 31 and this residue itself is not represented in the spectrum, it is impossible to define whether amino acid 31 is an alanine or serine, coming from the H3.1/H3.2 or the H3.3 isoform respectively. At each of the four different time points A₃₁ or S₃₁ clipping was identified in the gel pieces containing cH3, but not intact H3. Of note, using this mass spectrometry method no cleavage at an arginine residue can be detected, since the protocol itself introduces clipping after arginine during digestion. Indeed, by applying Edman degradation, we also assigned R26 as a cleavage site (Fig. 4D). Since the amino acid at position 31 is identified by Edman degradation as an alanine this cleaved form is derived from the H3.1 and/or H3.2 isoforms.

In conclusion, both Western blotting and mass spectrometry confirmed N-terminal cleavage with an intact C-terminus, and the latter assigned amino acid 31 as a cleavage site for histone H3 without isoform specification. N-terminal sequence analysis added R26 as a cleavage site, at least for the H3.1 and/or H3.2 isoforms.

When grown on MEF hESC cleave H3 in an identical temporal window compared to mESC

Since mESC differentiation showed various temporal clipping patterns depending on the differentiation protocol applied, we further extended the different culture conditions to also include the more classical culture system on a MEF feeder layer. Although these MEF are mitotically inactivated prior to cell culturing, cH3 formation derived from MEF cannot be excluded. Thus, RA was first added to a culture consisting only of a confluent layer of MEF, without hESC present. No cleavage was detected by immunoblotting (Supplemental Fig. 4).

When RA was added to hESC cultured on MEF, both morphological assessment of the differentiated status, and flow cytometry as well as RT-qPCR confirmed a decrease in Oct4 level as differentiation proceeded for the Oct4-eGFP reporter and UGENT2 cell line respectively (Figs. 5A and B). Of note, as seen by fluorescence microscopy within one colony patches or 'islets' of undifferentiated cells became apparent, indicating heterogeneous stemness within colonies (Fig. 5C). Remarkably, this Oct4 expression pattern is clearly different from what was seen when growing hESC feeder-free, which showed a diffuse expression pattern in the abovementioned experiments.

Surprisingly, when analyzing these histone extracts with Western blotting, a more 'mouse-like' pattern of H3 cleavage becomes visible: on day 2 of differentiation cH3 appears, attains a maximum on day 4 and fades significantly on the last day of the experiment. This pattern was visualized for both

cell lines, again confirming the cleavage capability of both the reporter and non-reporter cell line (Fig. 5D).

Also here the N-terminal nature of the H3 cleavage was double-checked using the N-terminally directed antibody, which visualized only the band of intact histone H3. In addition, a clear signal was detected using the H3.cs1 antibody for the UGENT2 sample, once more pointing out A21 as a possible cleavage site (Fig. 5E).

Histone H3 clipping activity in hESC is exerted by a serine protease

In order to identify the clipping enzyme, we conducted different calf H3 incubation assays, whether or not with the application of various protease inhibitors. To this end, we prepared a nuclear extract (NE) from Oct4-eGFP hESC for which histone H3 cleavage was confirmed in the corresponding histone extract, and validated it to be capable of calf H3 proteolysis. Endogenous H3 was not detectable in the amount of NE used in these experiments.

In a first experiment we compared the incubation of H3 with hESC NE after immunoblotting with the three different antibodies described before. This confirmed that the cleavage pattern induced by the NE is similar to what was seen in the histone extract of Oct4-eGFP hESC: the cleavage is exclusively of N-terminal nature and there is no detection of A21 as a possible cleavage site (Fig. 6A).

In the following inhibition assay, several inhibitors were included (Fig. 6B), comprising both general inhibitors for a certain protease enzyme class and specific inhibitors for cathepsin L, in parallel to the mESC results, where cathepsin L was identified as the responsible clipping enzyme. Western blotting analysis pointed out that only AEBSF, a serine protease inhibitor, could establish inhibition of the cleavage, whereas all other inhibitors could not demonstrate any notable effect on the incubation. Also the protease inhibitor cocktail was able to inhibit the proteolytic activity on histone H3. The specific inhibitors for cathepsin L were not able to diminish the cleavage (Fig. 6C).

Discussion

Histone clipping was reported for the first time even before these proteins received their current nomenclature (Johns, 1964; Phillips and Johns, 1959). Surprisingly however, these truncation events are still greatly understudied. Yet, from a practical point of view, techniques such as chromatin-immunoprecipitation run the risk of not detecting the substantial influence that histone clipping might have on the their outcome. All techniques employing specific antibodies to the H3 N-terminus in general or to modifications thereof in particular, will fail in case of H3 proteolytic cleavage. Also mass spectrometry-based (quantitative) comparisons of e.g. H3K4 and H3K27 methylations devaluate when dealing with samples where clipped histones are present.

Histone clipping has been reported in several distinct biological systems (Allis and Wiggins, 1984; Allis et al., 1980; Falk et al., 1990; Gonzalo, 2010; Mahendra and Kanungo, 2000; Mandal et al., 2012, 2013; Tesar and Marquardt, 1990), entailing different biological settings and thus potential roles of this PTM. However, only recently it was

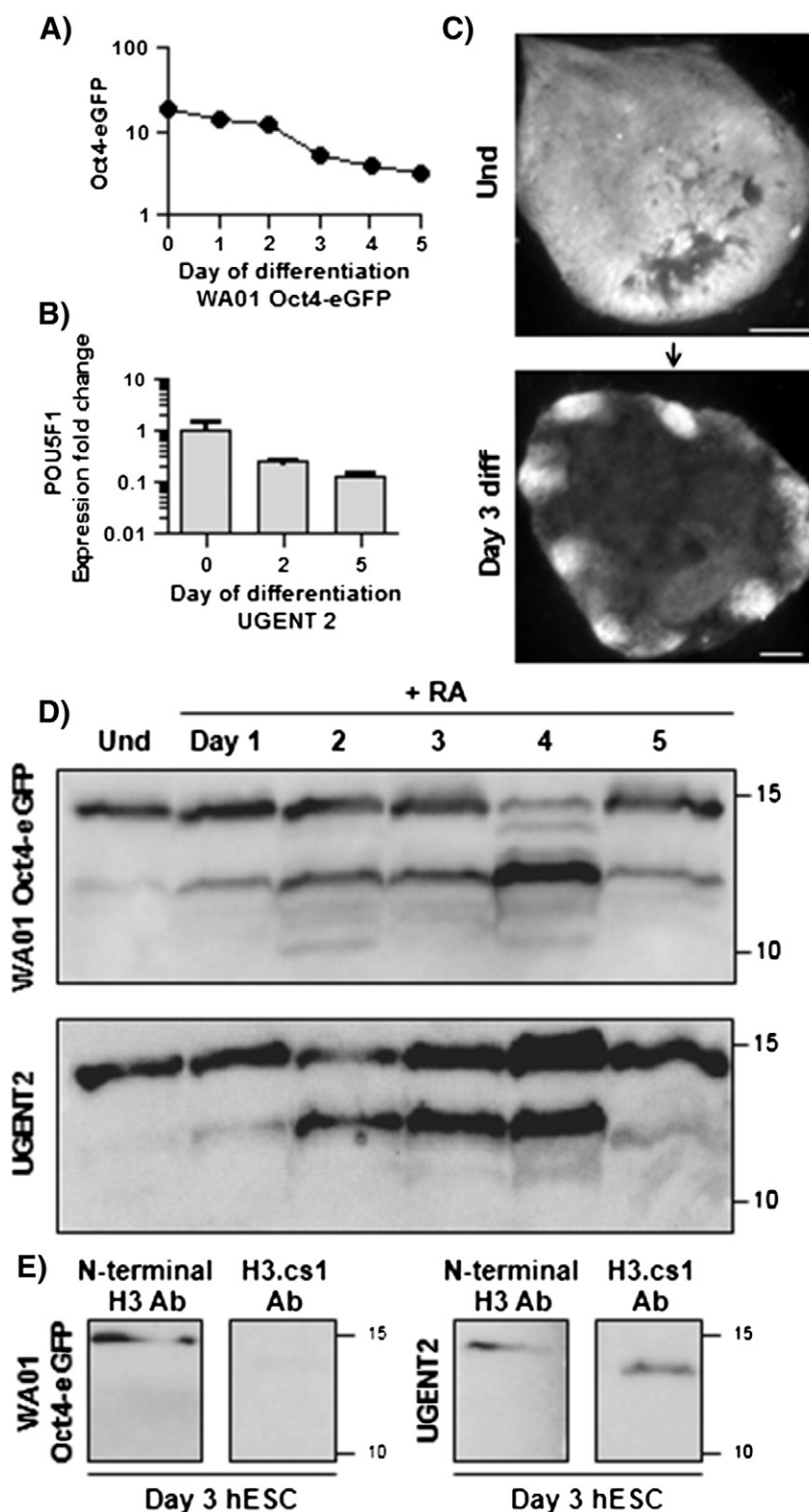


Figure 5 A different H3 cleavage pattern is seen in MEF cultured hESC. Aside from the application of feeder-free culture, additional differentiation experiments with hESC cultured on a MEF feeder layer were conducted. The differentiated status is again confirmed by flow cytometry [A] and RT-qPCR [B]. [C] Fluorescence microscopy analysis indicates differentiation heterogeneity among colonies, as within each colony several 'islets' of concentrated Oct4-eGFP expression was seen (scale bars represent 200 μ m). [D] Western blotting analysis reveals that when hESC are cultured on MEF, a cleavage pattern is shown that resembles more to the mESC results. [E] Also, the N-terminal orientation of the cleavage and A21 as a possible cleavage site is confirmed by application of the N-terminal and H3.cs1 antibody respectively.

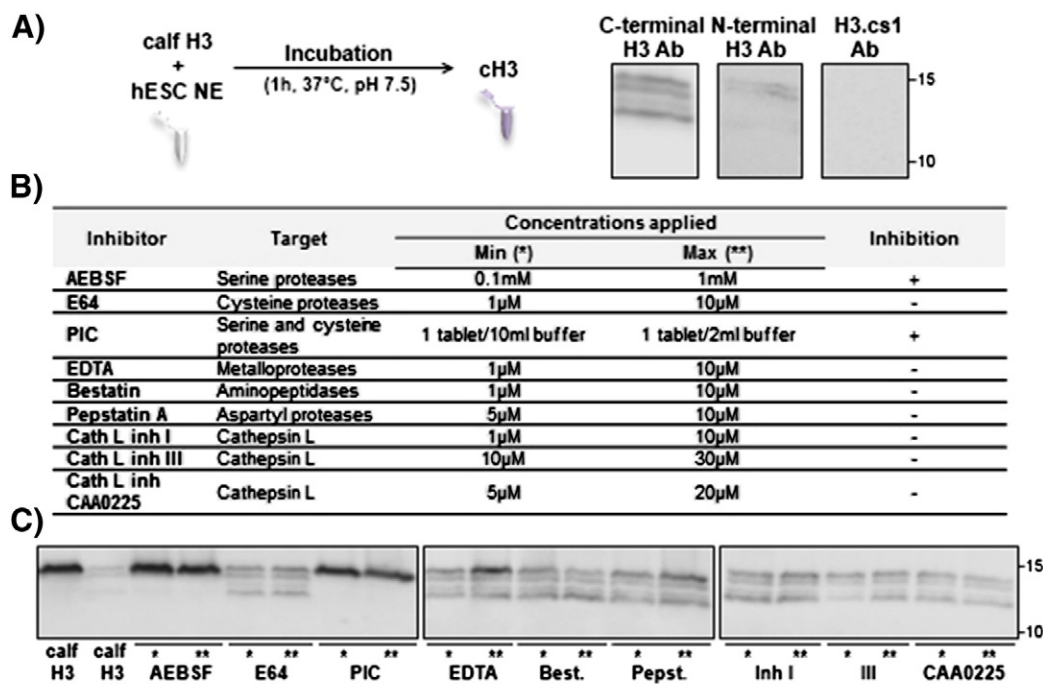


Figure 6 The histone H3 clipping activity in hESC is exerted by a serine protease. [A] In order to investigate the responsible cleavage enzyme, incubation assays were set up in which 1 μg calf histone H3 was incubated with 0.5 μg hESC nuclear extract (NE). The nuclear extract was prepared from the Oct4-eGFP cell line and contains N-terminal histone H3 cleavage activity, as was confirmed in the corresponding histone extracts (data not shown). Immunoblotting analysis of the incubation product displays several clipped H3 (cH3) fragments, with confirmed N-terminal origin. A21 could not be confirmed as a cleavage site (H3.cs1 Ab). The list of all inhibitors included, with their targets, the applied concentrations and their outcome in the inhibition assay is displayed in panel [B]. [C] Western blotting analysis points out that only when using a serine protease inhibitor the cleavage activity of the nuclear extract can be inhibited. The latter is confirmed by the additional inhibitory effect of the protease inhibitor cocktail, targeting mainly serine and cysteine proteases.

admitted into the epigenetic landscape with the discovery of transient histone H3 clipping in differentiating mESC (Duncan et al., 2008) and sporulating yeast (Santos-Rosa et al., 2009). The evolutionarily conserved sequence of histone proteins has been suggested to underlie the surprising occurrence of A21 clipping in both mouse and yeast, and we thus set out to verify whether the H3 clipping that accompanies mESC differentiation also takes place during hESC differentiation.

Duncan and colleagues monitored cH3 levels under several differentiation protocols in mESC, namely spontaneous monolayer differentiation after withdrawal of LIF, induced differentiation with RA and embryonic body formation. Depending on the protocol applied, a different cH3 pattern was visualized. Where RA induction leads to an undulating pattern of upcoming and decreasing H3 clipping centered around days 2 and 3 of differentiation, embryonic body formation displayed a faster migrating H3 band which peaked between day 8 and 12 but did not disappear completely after 14 days.

Here, we report that indeed histone H3 N-terminal clipping occurs in differentiating hESC and that its temporal appearance is equally influenced by the culture conditions. In feeder-free conditions, the two hESC lines tested (UGENT2 and WA01 Oct4-eGFP) show a continuous cH3 pattern after RA induced differentiation, resembling the results of EB formation in mESC. In contrast, when switching

to culturing hESC on a feeder layer of MEF, both these cell lines obtain a pattern of upcoming cleavage appearing on day 1, reaching a maximum intensity at day 4 and fading again at the last day of the experiment, similar to the temporal pattern described for RA induced mESC differentiation. Together, this suggests that within both human and mouse, the specific control over histone H3 cleavage during differentiation is profoundly influenced by experimental culture conditions applied. Although the cleavage event in mESC and hESC is appreciably similar, caution should be taken when functionally comparing these events, not in the least because hESC resemble more to mouse epiblast stem cells than regular mouse embryonic stem cells. The latter are considered to represent a more homogeneous and naïve pluripotent state compared to hESC, which are designated to be in a heterogeneous and primed pluripotent condition and thus probably reflect a more developed state (Gafni et al., 2013; Nichols and Smith, 2009; O'Leary et al., 2012). In contrast to Duncan et al., we could not find any clear correlation between the progression of differentiation and histone H3 clipping. The pluripotency status was monitored in this report by following Oct4 levels, i.e. specifically with an Oct4-eGFP reporter cell line. When monitoring three different differentiation methodologies, H3 cleavage seemed to be unaffected, while the Oct4 status was found to be influenced in the predicted way: a decrease in expression following (induced) differentiation. Considering

the open and highly dynamic state of pluripotent hESC chromatin, this might not be such a surprise (Tollervey and Lunyak, 2012). In line with this, on a Sypro Ruby stained SDS PAGE gel we also observed some reduction in H4 band intensity at some time points (data not shown). Whether this indeed is a clipping event remains to be determined. Of note, when Duncan et al. inhibited cathepsin L, H3 clipping was abrogated while Oct4 expression still decreased, adding yet another argument against a direct link between Oct4 expression levels and histone clipping.

Histone H3 cleavage is not *in vitro* generated during the experiment as visualized here by the use of biotinylated histone H3 during the extraction procedure. Also, protease inhibitors did not seem to influence the cH3 intensity, whether or not they are present. Moreover, both TEB/HCl extraction and direct boiling in SDS PAGE Laemmli yields the same results, further supporting the endogenous formation of cH3. The fact that MEF themselves do not display any clipping, but can completely change the temporal appearance of cH3 in differentiating hESC, further argues in favor of a biologically regulated process. Of note, we also verified the occurrence of H3 clipping in a human, terminally differentiated cell line (Burkitt's lymphoma Raji cells) and these cells did not form any clipped histone H3 (data not shown).

Many different histone H3 cleavage sites have been reported in the past, even within one study. In differentiating mESC (Duncan et al., 2008), A21 is the primary site of cleavage, though multiple other sites were also found at T22, K23, A24, R26 and K27. By the use of Western blotting, Edman degradation and mass spectrometry respectively, we confirmed two of those cleavage sites in hESC, namely A21 and R26, and assigned residue 31 as an additional new cleavage site in hESC. Unfortunately, with the techniques applied, no definite distinction could be made between the H3.1/H3.2 and H3.3 isoform cleavage respectively. Nevertheless, we expect H3.1 or H3.2 to be the cleaved isoform, since A31 is followed by threonine just as A21, which can thus be suspected to be susceptible to a similar enzyme activity.

It is worth noting that despite the many hurdles that need to be overcome when using mass spectrometry for the analysis of histone H3, several arguments add up to the likelihood of this newly identified cleavage site at residue 31. First, the annotated peptide was N-terminally propionylated, indicating no in-source decay causing this fragment to appear. Second, elution time patterns of the precursor and its cleaved form are distinct, further arguing against in-source formation of the cleaved fragment out of the intact precursor. Third, by first separating the histones with SDS-PAGE, the location of annotated histone H3 and its cleaved form in the gel allows the projection of the peptide data back onto the precursor proteins: the N-terminally cleaved fragment could only be annotated in lower MW (cH3) gel bands, in contrast to the N-terminal peptide itself which was found only in the highest MW (intact H3) fraction. Finally, all spectra corresponding to a cleaved peptide were manually validated by an expert before taken into account. On the other hand, more cleavage sites are expected to be present which cannot be identified by MS, as seen for the A21 and R26 sites detected by a specific antibody and Edman degradation respectively. This can be explained by the

limited possibilities of MS to annotate small peptides with high reliability. When a cleavage site is found to be close to an arginine, which is cleaved during digestion, the resulting peptides will consist of too little amino acids to be identified reliably. As a result not all possible cleavage sites can theoretically be identified by MS but their existence should not be disregarded.

Only few of the published histone clipping reports actually also categorize the protease responsible for this event (reviewed in Azad and Tomar (2014)). Duncan et al. (2008) assigned the cleavage to cathepsin L, a lysosomal cysteine protease, while glutamate dehydrogenase has been brought forward by Mandal et al. (2013). Santos-Rosa et al. (2009) who originally published the yeast clipping event, could only categorize the enzyme as a serine protease. It was just recently that Xue et al. (2014) found that the vacuolar protein Prb1 (Cerevisin) is required for the N-terminal H3 clipping in *Saccharomyces cerevisiae*. Finally, the foot-and-mouth disease virus expresses the so-called protease 3C, a cysteine protease, in the host cells which mediates clipping of host histone H3 at leucine 20 (Falk et al., 1990; Tesar and Marquardt, 1990).

The incubation assays performed here with the application of different inhibitors point out that the clipping enzyme is a serine protease, since apart from AEBSF none of the inhibitors for other protease classes could establish any effect on the incubation. This was confirmed by the fact that the protease inhibitor cocktail, which inhibits mainly serine and cysteine proteases, was also able to inactivate the clipping activity. We also included three specific inhibitors for cathepsin L, in analogy to the clipping in mESC, but no decrease in cleavage was seen due to these inhibitors. However, caution should be taken in the search for the responsible enzyme. As also others already suggested (Duncan et al., 2008; Santos-Rosa et al., 2009), enzyme redundancy and overlapping functions could impede its identification.

Despite the great epigenetic promise of such radical PTM, histone clipping, and more specifically its biological potential and the mechanisms by which it could exert its transcriptional effects, remain surprisingly understudied. Since the two landmark discoveries of H3 clipping in mouse and yeast in 2008 and 2009 respectively, four major mechanisms have been formulated by which histone proteolysis can influence gene expression programs (Duncan and Allis, 2011).

First, in yeast, a direct regulatory role of gene expression has been attributed to the removal of the N-tail and its repressive marks at promoter regions (Santos-Rosa et al., 2009). As such, histone H3 cleavage clears repressive marks massively, hence allowing for gene expression activation. Second, cH3 might provide a new binding site for protein complexes that could not be bound before clipping, thus fulfilling an active role in protein recruitment. On the other hand, also a passive regulatory role should be considered if other proteins are no longer able to bind the shortened histone H3 (Duncan and Allis, 2011; Santos-Rosa et al., 2009). Third, Santos-Rosa et al. (2009) also proposed nucleosome eviction and histone replacement as another framework in which clipping might regulate gene expression, as they report that H3 clipping precedes nucleosome eviction and subsequent gene induction. Finally, the N-terminal peptide itself might

establish its translation regulation by binding its own mRNA (Duncan and Allis, 2011). Which of these mechanisms, if any, is at play in (differentiating) hESC remains to be elucidated.

In short, based on previous findings and theoretical background, H3 clipping could be involved in several processes linked to gene expression control and differentiation. But the question still remains as to whether H3 proteolysis correlates directly with or causes such processes. Further studies will hopefully help elucidate the role of this new epigenetic mark in hESC and their differentiation process.

Conclusions

As a member of the epigenetic network, regulated histone proteolysis has been occasionally described earlier in diverse biological settings, yet being largely understudied. Histone H3 clipping and histone proteolysis in general might skew experimental findings substantially, both from a biological and a technical point of view. In this report we show for the first time that this posttranslational modification is also present in human embryonic stem cells, and is mediated by a serine protease. The temporal pattern of cleaved H3 is highly dependent on the culture protocol applied, as seen for both cell lines used in this report. Although in first instance we too detected the clipping upon early stem cell differentiation, we found that the clipping process is not necessarily accompanied by a decrease in Oct4 expression, as also undifferentiated hESC can contain cleaved H3 fragments. Thus, more research is needed to fully elucidate the potential biological role(s) of histone H3 cleavage.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2014.05.002>.

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