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Dynamics and dispensability of variant-specific histone H1 Lys-26/Ser-27 and Thr-165 post-translational modifications

Running title: **Dynamic linker histone modifications**

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Abbreviations: ChIP, chromatin immunoprecipitation; PTMs, post-translational modifications; PKA, Protein Kinase A; CDK, Cyclin-dependent kinase; HA, hemagglutinin; GSEA, Gene Set Enrichment Analysis; WT, wild-type

ABSTRACT

The linker histone H1 in mammals, involved in DNA packaging into chromatin, is represented by a family of variants. H1 tails undergo post-translational modifications (PTMs) that can be detected by mass spectrometry. We developed antibodies to analyse several of these as yet unexplored PTMs including the combination of H1.4 K26 acetylation or trimethylation and S27 phosphorylation. H1.2-T165 phosphorylation was detected at S and G2/M phases of the cell cycle and was dispensable for chromatin binding and cell proliferation; while the H1.4-K26 residue was essential for proper cell cycle progression. We conclude that histone H1 PTMs are dynamic over the cell cycle and that the recognition of modified lysines may be affected by phosphorylation of adjacent residues.

*Highlights

HIGHLIGHTS

- Human histone H1.2 T165 is phosphorylated at S and G2/M phases of the cell cycle.
- H1.2 T165 phosphorylation marks highly proliferative cancer cells.
- H1.2 T165 is dispensable for cell proliferation and H1.2 binding to chromatin.
- H1.4 S27 is phosphorylated at G2/M whether adjacent K26 is modified or not.
- H1.4 K26 residue is required for cell proliferation and heterochromatin loading.

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1. INTRODUCTION

The nucleosome core, which consists of 146 bp of DNA wrapped around an octamer of core histones, is the fundamental subunit of chromatin. In addition, histone H1 protein binds to linker DNA between two nucleosomes helping stabilize the chromatin fiber. In mammals, the H1 family includes up to eleven H1 variants that are either ubiquitous or tissue-specific. The reason for this remains unclear, but both their evolutionary conservation and significant differences in their amino acid sequence suggest that they are not functionally equivalent [1,2]. Given its role in the formation of higher-order chromatin structures, H1 has been classically seen as a structural component related to chromatin compaction. However, in recent years, the view that H1 plays a more dynamic role in regulating gene expression is gaining strength in the field, mostly due to H1 knock-out or knock-down studies in several organisms [3-8].

Among H1 variants, there are seven somatic H1 histones in human cells: H1.1 to H1.5 are expressed in a replication-dependent manner, whereas H1.0 and H1X are replication-independent. H1.1 is restricted to certain tissues, H1.0 accumulates in terminally differentiated cells, and the rest are ubiquitously expressed. H1 histones are lysine-rich proteins presenting three domains: a short basic N-terminal tail, a highly conserved central globular domain and a long positively-charged C-terminal tail. The chromatin binding affinity and residence time is different between H1 subtypes due to differences mainly in the C-terminal tail, but also in the N-terminal tail [9-11].

Like core histones, linker histone H1 is post-translationally modified. According to the 'histone code' hypothesis, histone post-translational modifications (PTMs) provide tools for altering chromatin structure, either modifying electrostatic interactions DNA-histones or allowing recruitment of chromatin modifying enzymes [12]. With the development of mass spectrometry methods, some attempts to map H1 PTMs have been made in various species, showing that H1 is modified mainly by phosphorylation, but also by acetylation, methylation, ubiquitination, formylation and citrullination [13-19]. Certain H1 PTMs may modulate interactions with partners and could explain some reported specific functions of H1 variants. Only some of the aforementioned histone H1 PTMs have been further analyzed *in vivo*.

Phosphorylation is the most well characterized modification of H1 histone and is tightly coupled to the cell cycle. The level of H1 phosphorylation progressively increases during the S phase, reaches its maximum from the late G2 phase into mitosis, and decreases rapidly in the telophase. Serine and threonine residues in both N- and C-terminal tails are phosphorylated by cyclin-dependent kinases (CDKs) through the recognition of the (S/T)-P-X-(K/R) motif [13,20]. H1 phosphorylation in S phase promotes the DNA decondensation needed for replication, while phosphorylation in G2/M is involved in mitotic chromosome condensation. It has also been demonstrated that different H1.5 residues are phosphorylated at different cell cycle phases [21]. Furthermore, H1 is also phosphorylated by kinases other than CDKs. For instance, H1.4-S27, H1.4-S35 and H1.5-T10 phosphorylations are catalyzed by Aurora B kinase, protein kinase A (PKA), and glycogen synthase kinase-3 (GSK-3), respectively [22-24]. Interestingly, significantly higher levels of phosphorylated H1 have been observed in high-grade invasive bladder cancer cells compared with normal human bladder epithelial cells, in accordance with the greater proliferation capacity of cancer cells [25]. In agreement with the presence of H1 phosphorylation in the interphase nucleus, H1 phosphorylation is also involved in gene regulation. For instance, H1 is phosphorylated before being displaced from the mouse mammary tumor virus promoter upon hormone induction [26]. Moreover, site-specific phosphorylation of histone H1 facilitates transcription by RNA polymerase I and II and has been implicated in ribosome biogenesis and control of cell growth [27].

H1 phosphorylation, together with methylation and acetylation, is also involved in heterochromatin formation by regulating HP1 binding. Lysine residue K26 on H1.4 is known to be both methylated and acetylated. G9a histone lysine methyltransferase mediates mono- and di-methylation, whereas JMJD2 reverses this modification [28]. Thus, HP1 binds specifically to methylated H1.4-K26, but the interaction is regulated by a ‘phospho-switch’ on the adjacent H1.4-S27 phosphorylation sites, and this prevents HP1 binding [29]. Moreover, histone deacetylase SirT1 interacts with H1 and deacetylates H1.4 at K26, regulating the formation of repressive heterochromatin [30]. Interestingly, G9a and Glp1 are also able to methylate H1.2-K187, but this methylation, which is stable during the cell cycle, cannot recruit HP1 and it is not reversed by JMJD2D [31].

Moreover, Kamieniarz et al. showed that H1.4-K34 acetylation by GCN5 is associated with promoters of active genes and regulatory regions. This modification seems to positively regulate transcription, both by increasing H1 mobility and by recruiting transcription factors [32]. Finally, histone H1 is also PARylated as a result of poly ADP-ribose polymerase (PARP-1) activity mediating H1 displacement from promoters and, consequently, leading to chromatin remodeling and transcription activation. In fact, PARP-1 and H1 are mutually exclusive in active promoters [33-36].

These observations favor the view that specific modifications in linker H1 subtypes lead to them having specific functions in different processes. Thus, we decided to develop antibodies specifically recognizing some of the as yet uncharacterized H1 PTMs, among others: K17 acetylation, K26-S27 single and double modifications (acetylation or trimethylation, and phosphorylation), K46 ubiquitination and T165 phosphorylation, and we investigated the dynamics of these newly-described histone H1 PTMs. Recognition of all the modifications changed during the cell cycle due to the phosphorylation of Ser- or Thr-specific residues over the course of DNA replication and mitosis. As a consequence, the recognition intensity of neighboring acetylated or methylated Lys residues by specific antibodies decreased. We also investigated whether K26 and T165 residues were required for cell proliferation and proper loading of H1.4 and H1.2, respectively, onto chromatin. Expression of a K26A mutant of H1.4 affected cell proliferation, gene expression and H1.4 stabilization in heterochromatin. Instead, H1.2-T165A showed no differences with respect to wild-type (WT) H1.2. Finally, H1.2-T165 phosphorylation was found to be a marker for proliferation and cancer malignancy.

2. EXPERIMENTAL PROCEDURES

2.1. Cell lines, mutagenesis and culture conditions

Experiments were performed in the breast cancer-derived T47D-MTVL cell line or HeLa cells, or its derivative cells stably expressing hemagglutinin (HA)-tagged H1 variants (H1-HA), or inducible shRNAs against H1 variants, described previously [7]. The mutagenesis of H1-HA was performed with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). T47D and HeLa cells were grown in RPMI 1640 medium and DMEM, respectively, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Colcemid (Sigma) was added at 50 ng/ml when indicated, and doxycycline (Sigma) was added at 2.5 mg/ml. Colon cancer cell lines HCT116, SW48, SW480, HCT15, LS147T, LS180 and Caco2 were grown in DMEM with 10% FBS (except for Caco2 for which 20% FBS was used).

2.2. Cell cycle and proliferation analysis

For cell cycle analysis, the cells were washed with cold PBS 1×, fixed in 70% ethanol and stained with a solution containing 3% Ribonuclease A (10 mg/ml, Sigma) and 3% solution A (38 mM sodium citrate, 500 µg/ml propidium iodide) in PBS 1×. Then, the samples were analyzed by fluorescence-activated cell sorting (FACS) using a FACS Calibur machine (BD Biosciences), CellQuest analysis software and the ModFit program. For cell proliferation analysis, the cells of interest expressing HA-tagged H1 (GFP-positive) were mixed 1:1 with parental cells (GFP-negative) and cultured. Every three days, the cells were split and the percentage of cells that were GFP-positive was measured by FACS.

2.3. Histone H1 extraction, gel electrophoresis and immunoblotting

Histone H1 was purified with a 5% perchloric acid solution for 1 hour at 4ºC. Soluble acid proteins were precipitated with 30% trichloroacetic acid overnight at 4ºC, washed twice with 0.5 ml of acetone and reconstituted in water. Protein concentration was determined with the Micro BCA protein assay (Pierce). The purified histones were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 hour, and incubated with primary antibodies overnight at 4ºC and with secondary antibodies conjugated to fluorescent dye (IRDye 680 goat anti-rabbit IgG, Li-Cor) for 1 hour at

room temperature. Then, the bands were visualized and quantified in an Odyssey Infrared Imaging System. All the polyclonal antibodies specifically recognizing human H1 PTMs that are described here were produced on request by Abcam or Millipore by rabbit immunizations with the peptides indicated in Table 1. Other antibodies used in this study, including histone H1 variant specific antibodies generated in our laboratory, are available from Abcam: H1.0 (ab11079), H1.2 (ab17677), H1.3 (ab24174), H1.5 (ab24175), H1-T18P (ab3595), H1.4-T146P (ab3596), EZH2 (ab51605), Suv39H (ab12405), and HA (ab9110). Total H1 antibody was from Millipore (05-457). HP1ß was from Euromedex (1MOD-1A9-AS).

2.4. Phosphatase treatment of H1 extracts

Purified H1 histones were incubated for 30 min at 30ºC with recombinant Lambda Protein Phosphatase (New England BioLabs) at 5 to 100 Units in a 50-µl reaction volume containing 50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5 and 1 mM $MnCl_2$. The reaction was stopped by heat inactivation after the addition of gel electrophoresis loading buffer and H1 was resolved in SDS-PAGE for immunoblotting.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP was performed according to the Upstate-Millipore standard protocol. Briefly, cells were fixed using 1% formaldehyde, harvested and sonicated to generate chromatin fragments between 200 and 500 bp. The sheared chromatin was immunoprecipitated overnight using 2 µg of antibody against HA. The immunocomplexes were recovered using a mix of protein A and protein G Sepharose, washed and eluted. The cross-linking was reversed at 65ºC overnight and immunoprecipitated DNA was recovered using the PCR Purification Kit from Qiagen. The presence of the genomic regions of interests was checked by real-time PCR, which was performed on ChIP and input DNA using EXPRESS SYBR GreenER qPCR SuperMix Universal (Invitrogen) and specific oligonucleotides in a Roche 480 LightCycler. ChIP values were corrected for their corresponding input chromatin sample. All oligonucleotide sequences used for the amplifications are available upon request.

2.6. Immunoprecipitation

Nuclear extracts were prepared by sonication of nuclei in 10 mM HEPES (pH 7.9), 2.25 μM $MgCl_2$, 20% (vol/vol) glycerol, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 M NaCl. Supernatant was dialyzed in BC200 buffer: 25 mM Tris (pH 7.9), 10% (vol/vol) glycerol, 0.2 mM EDTA, 200 mM KCl). For immunoprecipitations, nuclear extracts adjusted to equal protein concentrations were incubated for 2 h with antibodies. Protein A beads were then added to the mixes, which were further incubated for 1 h. Beads were collected by centrifugation and washed three times in BC200 buffer. Proteins were eluted in 2x SDS sample buffer at 80°C for 10 min.

2.7. Purification and infrared spectroscopy of human H1.2 and its mutants

HA-tagged H1.2 and its mutants, T165A and T165E, were cloned into pEV21a generating histidine tagging, and these vectors transformed into *E. coli* BL21 (DE3). Recombinant proteins were expressed and purified by metal-affinity chromatography as previously described [37]. Then, the buffer was exchanged and the recombinant proteins were digested with carboxypeptidase A (Type II-PMSF treated) in the conditions specified by the manufacturer (Sigma) to remove the His_6-tag. The proteins were further purified by hydroxyapatite chromatography on a CHT-II cartridge (Bio-Rad) as previously described [38]. Finally, the proteins were desalted by gel filtration through Sephadex G-25 (Amersham Biosciences) and lyophilized. FTIR spectra of human histone H1.2 and its mutants were measured at 5 mg/ml in 10 mM HEPES (pH 7.0) plus 140 mM NaCl in D_2O medium. Measurements were performed on an FT600 Bio-Rad spectrometer equipped with an MCT detector, using a demountable liquid cell with calcium fluoride windows and 50-μm spacers. Typically, 1000 scans were performed for each background and sample, and the spectra were obtained with a nominal resolution of 2 cm^{-1}, at 22ºC. DNA-protein complexes contained the appropriate amount of DNA for each protein/DNA ratio (w/w). The DNA contribution to the spectra of the complexes with the H1.2 species was subtracted as described elsewhere [39]. Data treatment and band decomposition of the original amide I' band were performed as previously described using GRAMS 9.0 software [40].

2.8. RNA extraction, reverse transcriptase PCR and microarrays

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions, and the cDNA was obtained from 100 ng of total RNA

using a SuperScript VILO cDNA synthesis Kit (Invitrogen). The real-time PCRs were performed using EXPRESS SYBR GreenER qPCR SuperMix Universal (Invitrogen) and specific oligonucleotides in a Roche 480 LightCycler. The procedures for microarray hybridization using an Illumina platform (GPL6883 Illumina HumanRef-8 v3.0 Expression BeadChip) and data analysis are described elsewhere [7]. Gene ontology analysis was performed using the Gene Set Enrichment Analysis (GSEA) program. Microarray data sets are available in the Gene Expression Omnibus (GEO) database under the accession number GSE55786.

3. RESULTS

In order to study the dynamics of histone H1 PTMs in cultured human cells, we developed polyclonal antibodies specifically recognizing some of the H1 PTMs described elsewhere by mass spectrometry analysis [6,7] namely: K17 acetylation (K17-Acet), K26 acetylation / S27 phosphorylation (K26-Acet/S27-P), K26 trimethylation / S27 phosphorylation (K26-Met/S27-P), K46 ubiquitination (K46-Ub), and T165 phosphorylation (T165-P) (Table 1). All the antibodies were obtained by standard procedures and their specificities were evaluated by dot blot (see Supplementary data, S1).

We analyzed the dynamics of these H1 PTMs using the T47D breast cancer cell line either untreated or treated with Colcemid to induce cell cycle arrest in mitosis. Histone H1 proteins were then extracted and analyzed by Western blot (Fig. 1). The level of H1 acetylation on K17 decreased upon accumulation of cells in mitosis, while the adjacent CDK-mediated T18 phosphorylation increased. We established that the H1 variant specifically recognized by the K17-Acet antibody was H1.4 by immunoprecipitation of stably-expressed H1.4-HA followed by immunoblotting and by depletion of the endogenous H1.4 (see Supplementary data, S2A and B), in agreement with the peptide sequence used for generating this antibody. H1 ubiquitination on K46 also decreased during G2/M arrest and the signal almost disappeared upon knock-down of H1.5 indicating that it may be the variant harboring this modification in cultured T47D cells, despite the peptide used for rabbit immunization being common to four somatic H1 variants (Fig. 1) (see Supplementary data, S2C).

Then, we analyzed phosphorylation on T165, which increased upon Colcemid-induced mitosis arrest (Fig. 1). T165 phosphorylation was specific for the H1.2 variant as shown by both immunoprecipitation of stably expressed H1.2-HA (Fig. 2A and Supplementary data, S3A), and depletion of the endogenous H1.2 (Fig. 2B). T165-P of H1.2 at mitosis was also seen in HeLa cells (Fig. 2C). Moreover, the antibody was specific for the phosphorylated residue as the hybridization signal disappeared upon phosphatase treatment of purified H1 (Fig. 2D and Supplementary data, S3B).

Release of cultured cells from a double thymidine block revealed that H1.2 T165 phosphorylation occurred parallel to phosphorylation of H1.4 T146, a known CDK

target site (Fig. 3A). In HeLa cells, six hours after the double thymidine block release, most cells were in S phase, while T165 and T146 residues appeared to be phosphorylated and remained in this state when the cells moved to G2/M. In T47D cells, there was a low rate of transition to the S phase and the phosphorylation increased at eight hours coinciding with a peak in the number of cells in G2/M. This suggests that H1.2 T165 is phosphorylated during replication and mitosis, similar to other H1 residues targeted by CDKs. Nonetheless, T165 is not within a CDK recognition motif and its phosphorylation was unaffected by the CDK inhibitor roscovitine (see Supplementary data, S4). The KinasePhos web tool [41] predicted T165 to be in a Protein Kinase C (PKC) recognition motif but T165-P was not affected by the PKC inhibitor GF109203X either. Similarly, it was unaltered by inhibitors of other candidate kinases, namely Aurora, PI3K, pTEF-b and PKA, it remaining unclear which enzyme is responsible for the phosphorylation in this case (see Supplementary data, S4 and data not shown).

At this point, we have identified the presence of H1.2 T165 phosphorylation in breast (T47D) and cervical (HeLa) cancer cell lines. Next, we explored whether the phosphorylation of H1.2 T165 also occurred in several colon cancer cell lines and, if so, whether there were differences between cell lines derived from low- or high-grade cancers. T165-P was detected and was more intense in cells derived from high-grade cancers, similar to H1.4 T146-P (Fig. 3B). As high-grade cancer cells are more proliferative, this finding indicates that both modifications may serve as proliferation markers for cancer diagnosis. In addition, H1.3 was absent in the cell lines derived from higher-grade primary tumors, suggesting that not only PTMs but also H1 variants abundance may differ over the course of tumor progression.

In order to investigate the dispensability of T165 and its phosphorylation, this residue was substituted by Ala and Glu within a stably expressing hemagglutinin-tagged H1.2 (H1.2-HA) in T47D cells in order to inhibit or mimic this phosphorylation (H1.2-T165A and H1.2-T165E), respectively (see Supplementary data, S5A). Mutations of this T165 residue had no apparent effect on proliferation and the cell cycle, on H1.2 binding to chromatin (see Supplementary data, S5B-C), or on the global structure of the mutated proteins compared with the WT as judged by infrared spectroscopy on recombinant H1.2 in the presence or not of DNA (see Supplementary data, Table S1).

As predicted, the T165-P antibody was unable to recognize H1.2-T165A, but constitutively recognized H1.2-T165E (see Supplementary data, S5D). ChIP of H1.2-HA showed that this variant bound to active and repressed promoters, coding regions and satellite DNA with nearly no differences between the mutated and the WT forms of H1.2 (Fig. 4). Active genes (CDK2, JUN), but not repressed genes (NANOG), showed local H1.2 (WT or mutant) depletion at the transcription start sites (TSS) compared with the corresponding upstream promoter region, as described elsewhere [33,42].

Additionally, we designed several antibodies specifically recognizing the acetylated or trimethylated form of H1.4-K26, alone or in combination with H1.4-S27 phosphorylation. The antibodies recognizing the K26-3Met and K26-Acet alone cannot detect these modifications when the adjacent serine (S27) is phosphorylated, as shown by dot blot analysis (see Supplementary data, S1). Conversely, the antibodies K26-Met/S27-P and K26-Acet/S27-P can only detect K26 modifications in the presence of S27-P. Accordingly, Colcemid treatment, predicted to increase H1.4-S27 phosphorylation, resulted in a lower level of detection of K26-3Met and K26-Acet by antibodies raised against these single modifications, whereas there was a higher level of detection with the antibodies against double modifications, suggesting a switch from mono- to bi-modified H1.4 K26-S27 residues (Fig. 1).

Antibody binding decreased upon H1.4 knock-down in T47D and HeLa cells, as well as upon phosphatase treatment of purified H1, demonstrating its H1.4 variant and phospho-specificity (see Supplementary data, S6). The release of cultured T47D cells from a double thymidine block revealed that bi-modified K26-S27 increased at G2/M (8 h after release), as did single S27 phosphorylation, while single K26 trimethylation decreased (Fig.5A and Supplementary data, S7). Interestingly, in HeLa cells K26-Met/S27-P did not accumulate in S phase as occurred for H1.4 T146 and H1.2 T165 phosphorylation (Fig. 3A).

Considering the reported role of the H1.4-K26 residue in recruiting HP1 proteins, we then decided to mutate this residue and compare the growth of T47D cell lines stably expressing HA-tagged H1.4 WT with K26A. The H1.4-K26A mutation led to a decrease in cell proliferation compared with H1.4-WT expression, possibly due to G2/M arrest (Fig. 5B and C and Supplementary data, S8). In chromatin extracts, we

verified that both H1.4-WT and K26A are equally well incorporated into chromatin (Fig. 5D). Interestingly, the presence of H1.4-K26A in chromatin was associated with lower levels of H1.4 partners which are known to bind this residue, i.e., HP1ß and EZH2 (Fig. 5D and Supplementary data, S9). Furthermore, the H1.4-K26A mutant was less well stabilized in heterochromatin satellite regions, as shown by ChIP (Fig. 5E and Supplementary data, S10).

In order to investigate whether the potential modifications of H1.4-K26 play a role in gene expression control, we compared global gene expression of H1.4-WT and K26A over-expressing cell lines using microarrays. The expression of both proteins produced both up- and down-regulation of some genes compared with levels in control cells. Most genes deregulated upon H1.4-WT expression were also affected in K26A expressing cells, but around 19% depended on K26 integrity (Fig. 5F and Supplementary data, Table S2). Interestingly, K26A expression deregulated approximately twice as many genes as H1.4-WT, maybe due to the proliferation defect in K26A cells. In relation to this, ontology analysis of genes differentially expressed in H1.4-WT and K26A cells using the GSEA program revealed changes in biological functions related to the cell cycle and DNA metabolism (see Supplementary data, Table S3). Further evidence that H1.4 levels may influence gene expression came from the observation that some of the genes up- or down-regulated upon H1.4 overexpression were deregulated in the opposite sense upon H1.4 knockdown, mainly genes repressed by H1.4 such as CDC20B or ROPN1L (see Supplementary data, S11).

To test if the genes found to be differentially expressed upon H1.4 expression that depended on K26 are normally enriched/depleted for H1.4 we took our ChIP-seq data on H1.4-HA distribution [42] and look at the number of enriched and depleted islands on those genes compared to random samples of the same number of genes (see Supplementary data, S12). Although up-regulated genes presented more H1.4 (and other H1 variants, data not shown) enrichment islands and less depletion islands than random genes, *p*-values indicated that this was not significant. Similarly, down-regulated genes presented non-significant reduced numbers of enrichment islands, but average number of depletion islands. In conclusion, no evidences yet point towards the notion that the subset of genes deregulated upon depletion or overexpression of a particular H1 variant are specifically enriched on such variant.

4. DISCUSSION

Depletion experiments of individual H1 variants in cultured cells have shown that distinct small subsets of genes are regulated by each variant and different phenotypes are produced [7]. Histone variant-specific PTMs could account for differential effects of the accumulation or depletion of distinct variants. Moreover, PTMs may be dynamic, adapting to cell cycle progression or responding to external stimuli or to the activation of a variety of signaling pathways. One of the consequences of the H1 variant PTMs may be an alteration of the interaction between histones and DNA, or between histones and chromatin-associated proteins.

Post-translational modifications of core histones, in particular H3 and H4, have been extensively studied and are the basis for the so-called 'histone code' involved in the regulation of genome functions including gene expression control. By contrast, little is known about linker histone PTMs. Few modifications, mostly CDK-dependent Ser and Thr phosphorylations, were investigated before two reports described the identification of new histone H1 PTMs by mass spectrometry analysis [13,14]. The historical difficulty of obtaining good antibodies against H1 variants or their PTMs has left uninvestigated the occurrence and functional relevance of such PTMs.

We have developed and tested polyclonal antibodies against several H1 PTMs including phosphorylation, acetylation, trimethylation and monoubiquitination, and also antibodies against double modifications (Lys-acetyl/Ser-phospho or Lys-trimethyl/Ser-phospho). Here we show the results with the antibodies that are able to detect the occurrence of H1 PTMs in a breast cancer cell line. In addition to the PTMs described above, we also tested antibodies raised against the following modifications present in several H1 variants: K34, K46, K85 and K90 acetylations, K90 formylation, and the double modification K34-acet/S36-phospho (see Supplementary data, S13). Those raised against acetylated Lys were specific for the modified peptide and reacted with H1 extracted from cells in Western blot experiments, but overall were not further characterized due to their low reactivity.

We have focused on the dynamics, histone variant specificity and dispensability of two modified residues: H1.2 T165 phosphorylation and H1.4 K26 acetylation or

trimethylation, and its interplay with phosphorylation of its neighbor S27. We show that phosphorylation of these amino acids occurs during the G2/M phase of the cell cycle. In fact, H1.4-S27 phosphorylation by Aurora B over the course of mitosis has been previously reported [24]. While the global increase in H1 phosphorylation during mitosis was expected, the decrease in K46-ubiquitination is more surprising and needs further investigation. Less K26 acetylation and trimethylation were also detected when cells accumulated in mitosis, presumably due to S27 phosphorylation of existing K26-unmodified, acetylated or trimethylated histone H1.4, as the level of detection with the double-modification antibodies was higher. This indicates that the double modifications exist in vivo at the same histone tail at the same time, and that anti K26-acet or K26-trimet antibodies are unable to recognize these modifications when a phosphate group is added to the neighboring Ser. Similarly, as proposed by the phospho-switch model, a protein binding to modified K26 may be released when S27 is phosphorylated [29].

Interestingly, T165-P started to accumulate in S phase, as previously reported for the CDK-dependent phosphorylation of histone H1 at (S/T)-P-X-(K/R) motifs, suggesting that this modification may have a more complex role than chromosome compaction during mitosis. H1 phosphorylation over the course of DNA replication has been implicated in chromatin decompaction. Our attempts to identify the kinase responsible for T165 phosphorylation have so far been unsuccessful. It would be interesting to determine which as yet unknown signaling pathways regulate this newly described phosphorylation on histone H1.2, as well as its biological relevance. One possibility could be that multiple kinases target H1.2 T165 redundantly. We have shown that T165-P is dispensable for cell cycle progression and H1.2 binding to chromatin. This suggests that T165-P may be additive to the CDK-dependent phosphorylation of other Ser and Thr residues and by itself have limited structural consequences, instead of representing a highly specific regulatory binding site for a specific factor. Alternatively, T165-P may be dispensable for the cell type analyzed here grown under cell culture conditions or even completely non-functional, but may be more relevant under other conditions.

On the other hand, here we suggest that K26 modifications are required for proper cell proliferation and stabilization of heterochromatin. The mutation of the H1.4-K26 residue has a significant effect on the cell cycle and gene expression, probably due to a defect in the recruitment of histone H1 partners to chromatin. Expression of H1.4-K26A

induced both up- and down-regulation of gene expression compared with H1.4-WT expression, consistent with both active and repressive effects of H1.4-K26 PTMs. Very few genes presented altered expression in opposite senses whether H1.4 was overexpressed or knocked down, and we do not believe that this is due to specific binding of H1.4 to these promoters, as we have previously reported that all gene promoters are bound by any H1 variant quite indistinctly [42]. We have shown here that a subset of genes differentially expressed upon H1.4 overexpression do not contain significant enrichment of this variant. Despite ubiquitous binding of H1 variants to promoters, differential interaction with partners, probably mediated by variant-specific PTMs, may explain specific participation of H1 variants to gene expression.

We have proposed that phosphorylation of H1 residues such as T165 in H1.2 and T146 in H1.4 may be considered proliferation markers and, accordingly, mark tumor cells with the highest rate of division, regularly associated with the highest level of malignancy. It is likely that cell cycle-dependent phosphorylation of certain Ser or Thr residues in the tails of H1 variants is of major importance in determining the architecture of chromatin during cell proliferation and differentiation. Alterations in the regulation of H1 phosphorylation may be involved in the genetic dysregulation that is fundamental to carcinogenesis. Aberrant chromatin structure may contribute to malignant transformation and tumor formation. Understanding the role of different H1 variants and their modification profiles may be relevant to many aspects of cell survival, proliferation, apoptosis, differentiation and transformation.

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Appendix A. Supplementary data

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FIGURE LEGENDS

Figure 1. Histone H1 PTMs are dynamic over the cell cycle. Histone H1 was extracted from T47D cells treated or not with Colcemid (50 ng/ml) overnight, resolved in SDS-PAGE, and immunoblotted with antibodies specifically recognizing human H1.0 or histone H1 PTMs.

Figure 2. The T165-P antibody is specific for H1.2. (A) Immunoblot of H1.2-T165-P after H1.2 immunoprecipitation. H1.2-HA was immunoprecipitated with an anti-HA antibody, or IgG as a control, from nuclear extracts of cells stably expressing H1.2-HA, and blotted with the indicated antibodies. (B) Immunoblot of H1.2-T165-P after H1.2 depletion. T47D derivative cells stably infected with a lentiviral inducible system for the expression of shRNA against H1.2 (H1.2 KD) were treated for 6 days with doxycycline or left untreated. Colcemid was added 4 hours prior to H1 extraction where indicated. H1 extracts were analyzed by immunoblot against H1.2-T165-P, H1.2 or H1.5 as a loading control. (C) HeLa cells harboring the inducible shRNA against H1.2 or a control random shRNA were treated and analyzed as in (B). Coomassie staining of H1 PAGE is shown as a loading control. (D) Immunoblot of H1.2-T165-P after phosphatase treatment. H1 was extracted from T47D cells treated or not with colcemid overnight, incubated with increasing concentrations of Lambda Protein Phosphatase for 30 min and immunobloted with H1.2-T165-P antibody. Coomassie staining and H1.0 immunoblot are shown as loading controls.

Figure 3. H1.2 T165-P occurs at S and G2/M phases of the cell cycle and marks highly proliferative cancer cells. (A) Analysis of H1 phosphorylation after release from double-thymidine block. HeLa or T47D cells were arrested at the beginning of the S phase using a double thymidine block. After release for different times, cells were harvested to perform cell cycle analysis by fluorescence-activated cell sorting (FACS) after propidium iodide staining, and H1 extraction. Immunoblot was performed with the indicated H1 phospho-specific antibodies. H1.2 blotting and Coomassie staining were used as loading controls. Graphs with band density quantification are shown in Supplementary data, S7C. The upper graphs represent the percentage of cells in G1, S and G2/M phases as established by FACS. (B) H1.2 T165-P in colon cancer cell lines at different stages of malignancy. H1 was extracted from seven different colon cancer cell

lines growing exponentially and immunoblotted against the indicated antibodies. Colon cancer cell lines are ordered by origin from high- to low-grade malignancy and proliferation rate. HCT116 (1; grade D), SW48 (2; grade IV), SW480 (3; grade III-IV), HCT15 (4; grade C), LS147T (5; grade B), LS180 (6; grade B), and Caco2 (7; grade I). Grades A to D (Dukes' classification), or I to IV, from low to high malignancy, are indicated. Coomassie staining was used as loading control; notice that LS180 was overloaded. Relative band density quantification for each antibody respect to H1.2 (Pearson's R correlation coefficient between H1.2 and Coomassie was 0.95) and relative to the maximal value is shown on the right panels.

Figure 4. The T165 residue is dispensable for proper H1.2 loading onto chromatin. Cross-linked chromatin from T47D cells stably expressing H1.2-HA WT, T165A or T165E was used for ChIP experiments with an anti-HA antibody or IgG as control. The precipitated DNA fragments were used for qPCR with primers for the indicated regions of CDK2, JUN and NANOG genes (upstream promoter, TSS and within the coding region), and for the repetitive satellite regions SAT2 and SATa. CDK2 and JUN represent active genes in T47D cells, while NANOG is repressed. The coding region of NANOG was not assessed. Amplification of input DNA (representing 1% of immunoprecipitated DNA) was used for normalization. The means and SDs are shown for a representative experiment quantified in triplicate. Significance between H1 mutant data sets was tested using the Student's *t*-test (* $p\text{-value}<0.05$; ** $p\text{-value}<0.01$; ns, no significance).

Figure 5. The H1.4-K26 residue is required for proper cell proliferation and stabilization in heterochromatin. (A) H1.4 S27-P occurs at the G2/M phase of the cell cycle. T47D cells were arrested at the beginning of S phase using a double thymidine block. Upon release at different times, cells were harvested to perform cell cycle analysis by FACS after propidium iodide staining, and H1 extraction. Immunoblot was performed with the indicated H1.4 antibodies. H1.0 blotting was performed as a loading control. The upper graphs represent the percentage of cells in G1, S and G2/M phases as established by FACS. Graph with band density quantification is shown in Supplementary data, S7B. (B) Expression of H1.4-K26A impairs cell proliferation. Both H1.4-HA WT and K26A stably expressing cell lines (GFP-positive) were mixed 1:1 with parental T47D cells (GFP-negative). Every three days, cells were split and the

percentage of GFP-positive cells measured by fluorescence-activated cell sorting. Data are expressed as the ratio of GFP-positive versus GFP-negative cells over time and correspond to a representative experiment performed in duplicate. (C) K26A cells accumulate in G2/M. Cell cycle profile after propidium iodide (PI) staining of H1.4-HA WT and K26A expressing cells, represented as the percentage of cells in G1, S and G2/M. Data correspond to a representative experiment performed in duplicate. The means and SDs for four independent experiments are shown. Significance was tested using the Student's *t*-test (* p-value<0.05; ** p-value<0.01). (D) Chromatin content in H1.4-HA WT or K26A stably expressing cells. Chromatin was extracted from cells stably expressing H1.4-HA WT or K26A and analyzed by immunoblot with the indicated antibodies. Bands were quantified and the ratio between K26A and WT is indicated. (E) Detection of histone H1.4-HA WT or K26A at heterochromatic regions. Cross-linked chromatin from T47D cells stably expressing H1.4-HA WT or K26A was used for ChIP experiments with an anti-HA antibody. The precipitated DNA fragments were exposed to qPCR with primers for the indicated regions. Amplification of input DNA (representing 1% of immunoprecipitated DNA) was used for normalization. The means and SDs are shown from a representative experiment quantified in triplicate. Significance was tested using the Student's *t*-test (** p-value<0.01). (F) Expression of H1.4-HA WT and K26A deregulates common and unique genes. Venn diagrams showing the number of genes up- or down-regulated with a fold change (FC) ≥1.4 in H1.4-HA WT or K26A expressing cells compared to control cells established by Illumina microarray hybridization.

Table 1. Antibodies recognizing histone H1 post-translational modifications.

PTM	H1 variant (PTM)[1]	Immunizing peptide	H1 variant (peptide)[2]	Source	Catalog no.	Addit. PTMs[3]
K17 acetyl	2, 3, 4, 5	AAPAPAE(ac**K**)**T**P	4	Millipore	NA	T18ph
K26 acetyl	4	AR(ac**K**)**S**AGAAKR**K**	4	Millipore	NA	S27ph, K34ac
K26 trimethyl	4	VKKKAR(3me**K**)**S**AGAA	4	Abcam	NA	S27ph
S27 phospho	4	VKKKAR**K**(p**S**)AGAA	4	Abcam	NA	K26me/ac
K26 acetyl / S27 phospho	4	AR(ac**K**)(p**S**)AGAAKR**K**	4	Millipore	06-1372	K34ac
K26 trimethyl / S27 phospho	4	VKKKAR(3me**K**)(p**S**)AGAA	4	Abcam	ab33231	–
K46 ubiquitin	2, 3, 4	IT(ub**K**)AVAAS**K**	2, 3, 4, 5	Millipore	NA	K52ac
T165 phospho	2	AA(p**T**)VT**K**KVAK	2	Millipore	06-1370	K168ac

[1] H1 variants where the indicated PTM has been described in the Garcia et al. or Wisniewski et al. reports.

[2] H1 variants where the designed immunizing peptides share 100% sequence identity.

[3] Residues present in the immunizing peptides that can receive additional PTMs according to the reports indicated above.

NA, not currently available.

Figure
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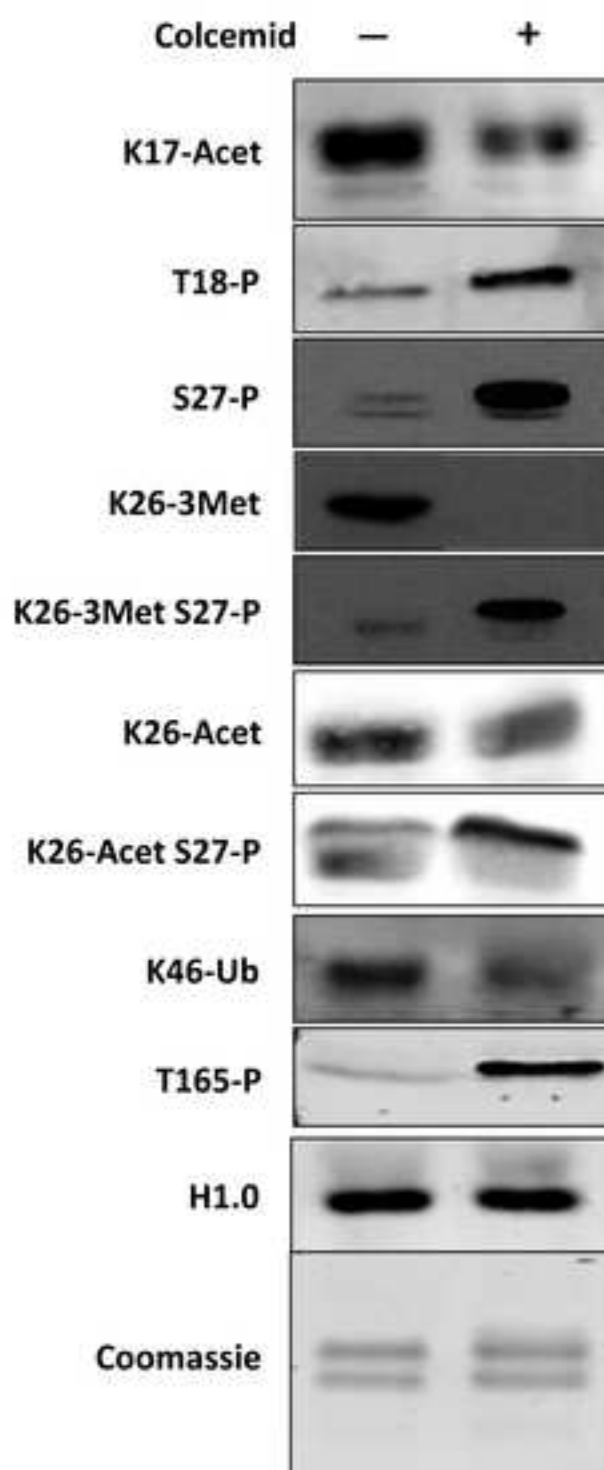

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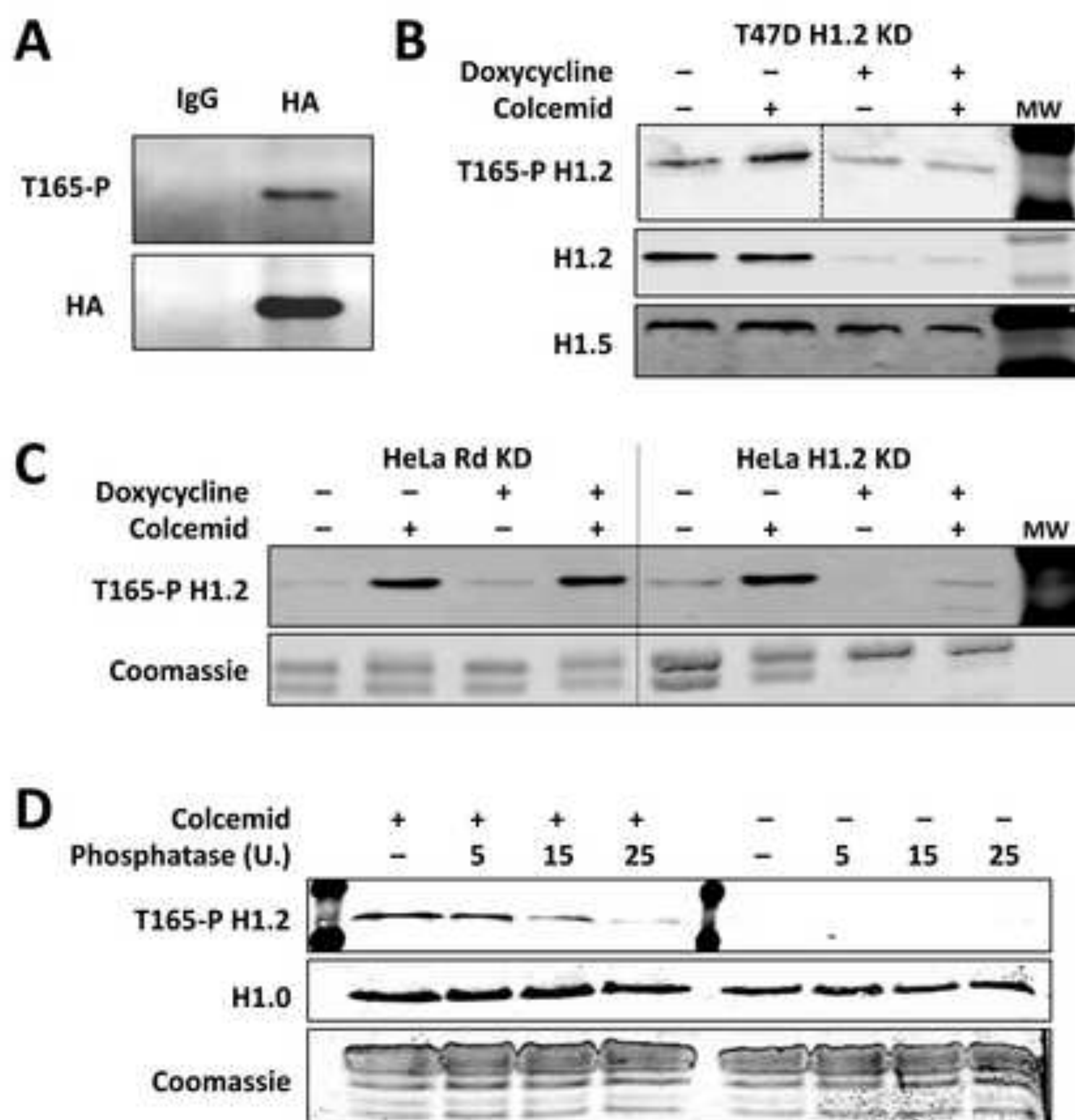

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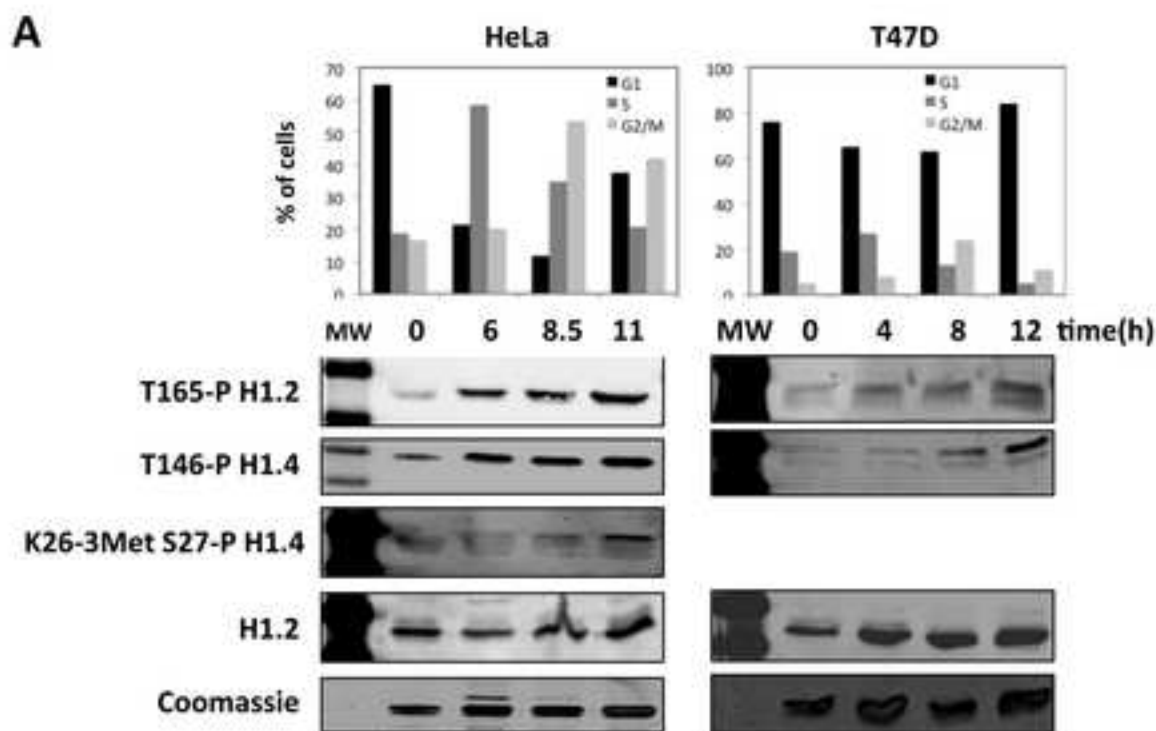

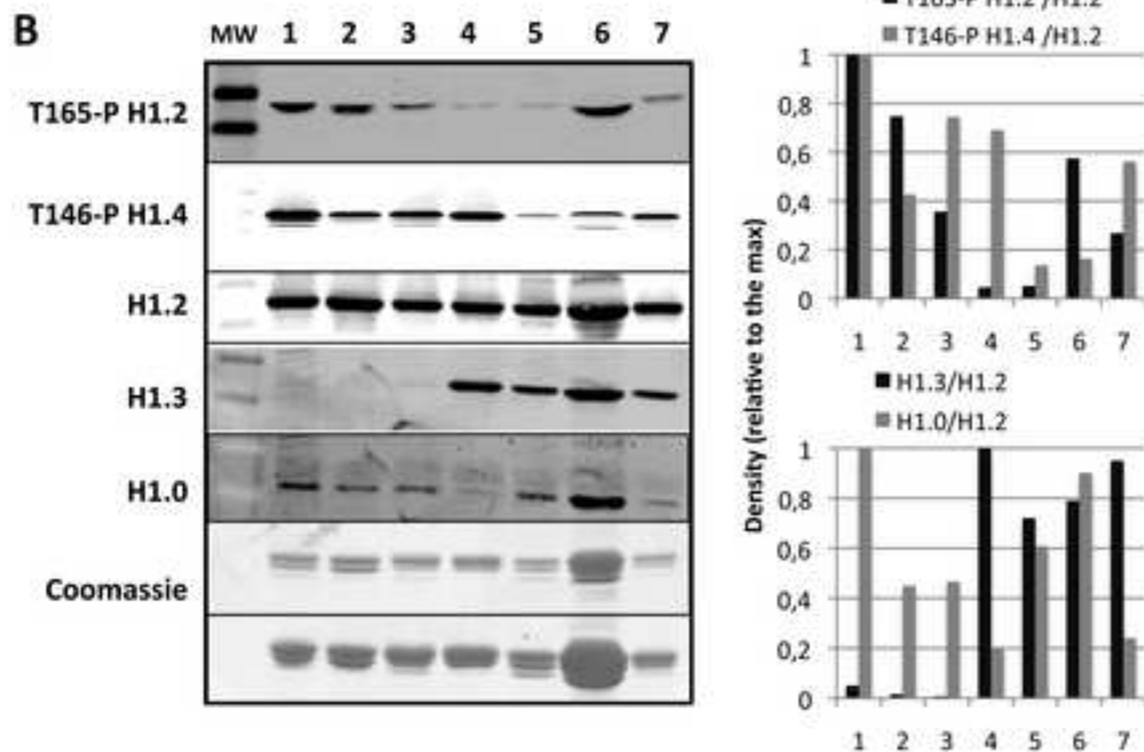

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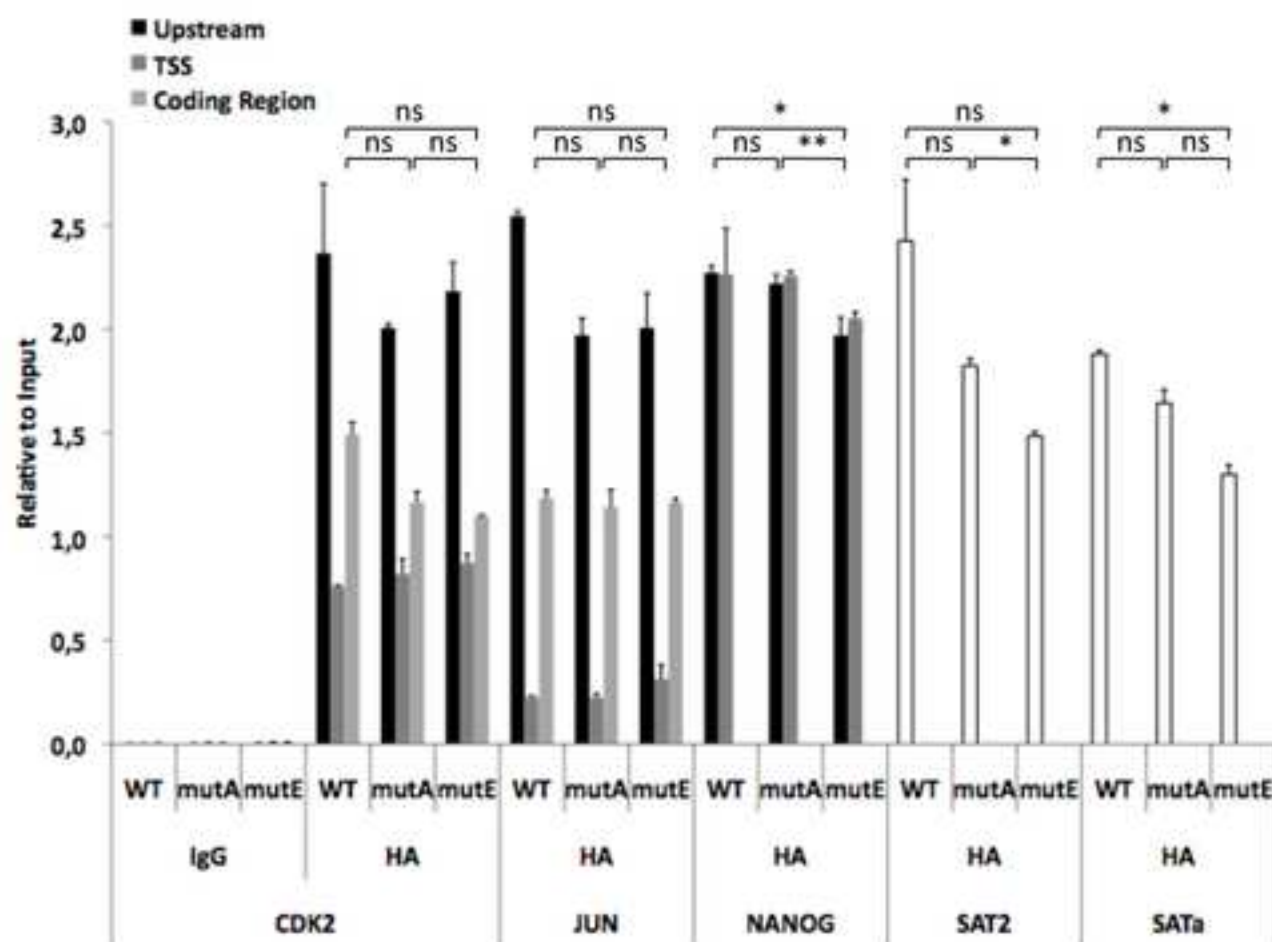

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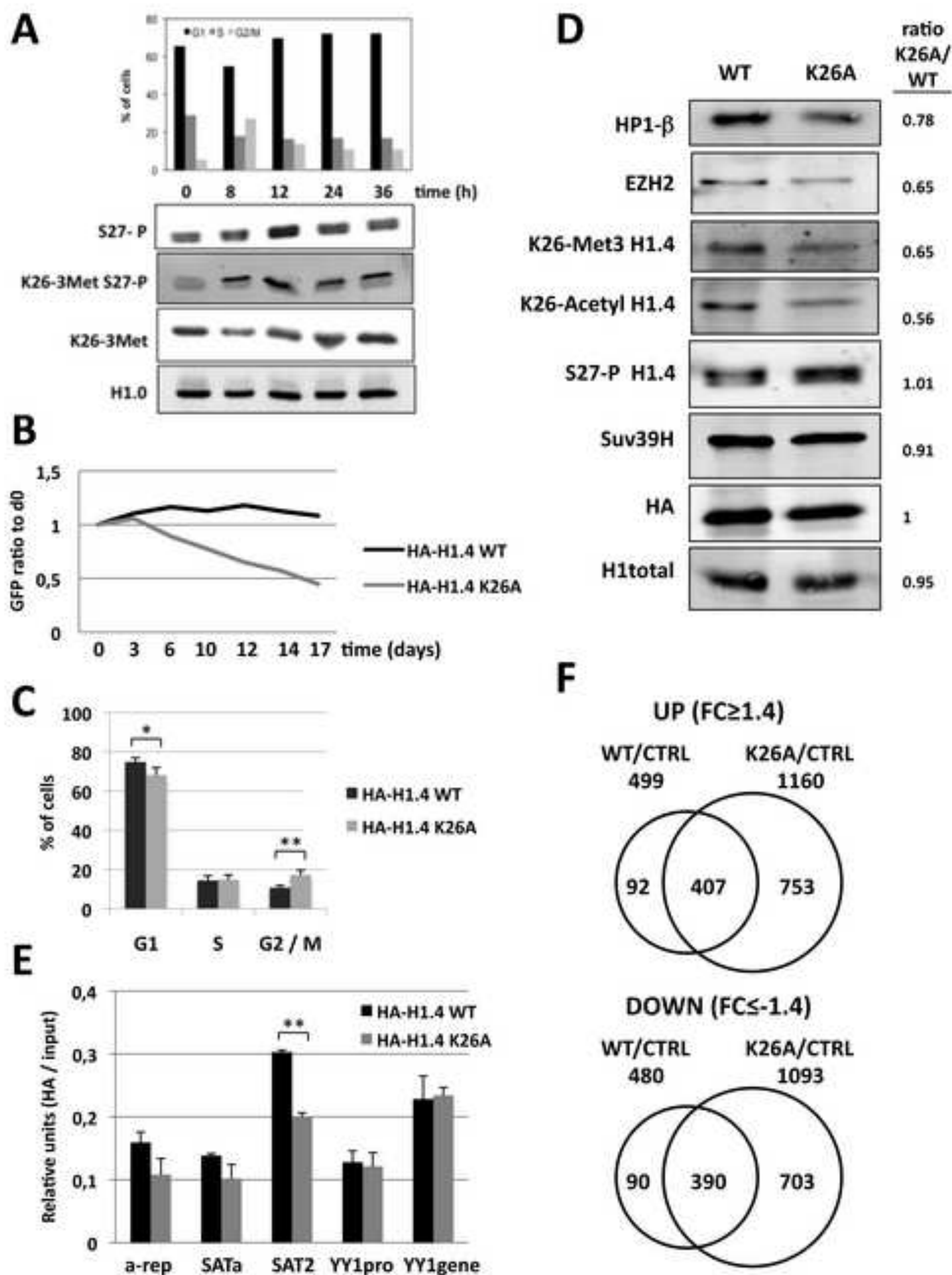

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