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博士論文

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Doctoral dissertation

蛋白激脢 A 媒介之絲氨酸 35 的磷酸化導致連結組蛋白 H1.4 從有絲分裂中的染色質上游離之探討

Protein Kinase A-Mediated Serine 35 Phosphorylation Dissociates Histone H1.4 from Mitotic Chromosome

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Abstract in Chinese

中文摘要

真核細胞之 DNA 由核心組蛋白(Core histones)及連結組蛋白(Linker histone)纏繞成 Nucleosomes。組蛋白之轉譯後修飾(Posttranslational modification)構成組蛋白密碼(Histone code)，可調控細胞基因轉錄(Gene transcription) 。相對於核心組蛋白，連結組蛋白之轉譯後修飾及其生理意義並不清楚。本實驗室利用質譜儀鑑定蛋白質轉譯後修飾的策略鑑定出許多在連結組蛋白 H1.4 上尚未被鑑定的蛋白質轉譯後修飾，其中，我們發現其 N 端第 35 個思氨酸 （Serine 35）上具有一磷酸化位置，此磷酸化在有絲分裂時候會累積。進一步研究發現蛋白質磷酸激脢 A （PKA）為其主要的磷酸化酵素。思氨酸 35 磷酸化後的 H1.4 會減低原本 H1.4 和染色質連結的能力。我們進一步發現此氨基酸對於抑制 H1.4 基因表現後引發的有絲分裂缺陷是重要的。在抑制 PKA 活性的實驗中，我們亦發現 PKA 對於染色質在有絲分裂下的濃縮扮演相當的角色，而這樣的角色有部分是跟 H1.4 有關的。我們同時發現在思氨酸 35 鄰近區域有一離氨酸 （lysine 33）會被進行甲基化，SET7，一個組蛋白 H3 上第四個離氨酸的甲基脢亦會對 H1.4 進行甲基化。並且，我們也證明此兩個鄰近的蛋白質轉譯後修飾具有互相調控的機制存在。在此篇研究中，我們闡述了此一 H1.4 之 N 端 serine 35 磷酸化在細胞分裂的重要性，在發現其鄰近的離氨酸 33 之甲基化的同時也發現了此兩修飾相互調控的可能。因此，此篇研究提供了更進一步的訊息，作為將來明瞭連結組蛋白密碼之複雜功能之用。

關鍵字

連結組蛋白，蛋白質磷酸激脢 A，細胞週期，磷酸化，有絲分裂，染色質濃縮

Abstract

Global histone H1 phosphorylation correlates with cell cycle progression. However, the function of site-specific H1 variant phosphorylation remains unclear. Our mass spectrometry analysis revealed several new modifications on H1. Among them, a novel N-terminal phosphorylation of the major H1 variant H1.4 at serine 35 (H1.4S35ph) was found to accumulate at mitosis. Protein kinase A (PKA) was found to be a kinase for H1.4S35. Importantly, S35-phosphorylated H1.4 contains weaker binding affinity to mitotic chromatin. Moreover, H1.4S35A substitution mutant cannot efficiently rescue the mitotic defect following H1.4 depletion and inhibition PKA activity increases the mitotic chromatin compaction in a H1.4-dependent manner. In addition, an adjacent methylation on lysine 33 was also identified. We further demonstrated that SET7, a histone H3K4 methyltransferase, can methylate H1.4K33 both *in vivo* and *in vitro*. Finally, a crosstalk between H1.4S35ph and H1.4K33me was characterized. Our results not only indicate that PKA-mediated H1.4S35 phosphorylation interferes H1.4 binding affinity to mitotic chromatin, but also suggest that this phosphorylation is necessary for specific mitotic functions, where adjacent H1.4K33me might involve this regulation.

KEYWORDS

Linker histone, H1, H1.4, protein kinase A, cAMP-dependent protein kinase, PKA, Cell cycle, phosphorylation, mitosis, DNA compaction

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INTRODUCTION

INTRODUCTION

I. Linker histone H1 family

Histone H1 is the most complicated histone proteins because there are eleven H1 subtypes identified in the human genome (Happel and Doenecke, 2009), including H1.1, H1.2, H1.3, H1.4, H1.5, H1t, H1T2, H1oo, HILS1, H1x, and H1.0. They can be further classified according to the tissue specificity. Somatic subtypes, including H1.1, H1.2, H1.3, H1.4, and H1.5, locate in a cluster on chromosome 6 and express ubiquitously in somatic cells in a replication-dependent manner. There are four variants belong to germline-specific subtypes. H1t, H1T2, and HILS1 belong to a spermatogenesis subtype and H1oo is an oocyte-specific subtype. H1.0, a replacement subtype, locates solitarily on chromosome 22 and the expression is related to terminal differentiation (Zlatanova and Doenecke, 1994). H1x is also solitary and it locates on chromosome 3 and expresses ubiquitously. In addition to the somatic subtype, the expression of H1t is also replication-dependent while the rest of the tissue-specific variants are replication-independent. There are several nomenclatures of H1 variants existed. While the most used nomenclature is followed by Albig & Doenecke's method, a comparison of these nomenclatures is organized (Table 1).

II. Structure of histone H1 and DNA binding

Linker histone H1 is one of the major chromatin-bound proteins, of which the structural function related to DNA binding has been extensively studied. H1

consists of a well-conserved hydrophobic globular domain (GD) in the middle and less conserved short N- and longer C-terminal tails (NTD and CTD, respectively). Both NTD and CTD are very rich in lysine residues. Unlike core histones, H1 shows greater diversity among variants and also across species, predominantly in the lysine-rich NTD and CTD (Izzo et al., 2008). GD binds to DNA by two motif: a classical winged-helix motif and a domain on the opposite face clustered with basic residues (Ramakrishnan et al., 1993). The asymmetric binding of H1 on the linker DNA between two nucleosomes protects the chromosome from micrococcal nuclease digestion from 146 to 168 bp (Noll and Kornberg, 1977). Regardless of the similar ternary structure that H1 variants share, each variant exhibits different binding affinity to DNA, based on the diverse CTD.

III. The biological role of linker histone H1

1. Introduction

The major function of linker histone H1 is generally thought to facilitate the package of adjacent nucleosomes into higher order chromatin structure. This dramatic change of chromatin structure is a critical event for many biological functions. For instance, H1 is required for metaphase chromosome compaction (Maresca and Heald, 2006), implicated a role in germ layer determination (Flickinger, 2006), a target for viral proteins (Fassati et al., 2003; Konesky et al., 2006; Lesner et al., 2004; Trotman et al., 2001) and also involved in DNA methylation (Rupp and Becker, 2005), chromatin remodeling (Hill, 2001) and transcriptional regulation (Buttinelli et al., 1999). In addition, H1 also exerts its function by interaction with other proteins, such as p53, HMG1,

HP1, and PARP1. A summary of H1-interacting proteins and their functions are organized. (Table 2)

2. H1 and gene transcription

Histone H1 has been long considered as a general transcriptional repressor. To activate chromatin for transcription, the chromatin modifiers have to be recruited to the promoter region of genes targeted by transcriptional factors which usually contain the DNA sequence-specific binding domain (Kinyamu and Archer, 2004). The chromatin modifiers are believed to result in synergistic enhancement of factor-specific trans-activation by modifying both histone proteins and specific transcriptional factors. Posttranslational modification of histone proteins by histone modifiers constitutes the histone code which plays an important role in eukaryotic gene expression. In contrast to core histones H2A/H2B/H3/H4, little is known as regards H1 modification.

IV. Posttranslational modifications on linker histone H1

1. Introduction

Posttranslational modifications (PTMs) on proteins including phosphorylation, acetylation, methylation, ubiquitination, sumoylation, and neddylation have attracted more and more scientists, because PTMs have been regarded as a more fine-tune system for the regulation of biological events. Along with the improvement of the technology of mass spectrometry, more and more PTMs were identified. Almost all H1 family members consist of a conserved hydrophobic

globular domain and less conserved N- and C-terminal tails. Both tails can undergo a number of posttranslational modifications (PTM) including phosphorylation (Happel and Doenecke, 2009). Recently, a burst of articles have been published on the identification of PTMs on histone H1 in various species, including human, mouse, drosophila, and tetrahymena (Deterding et al., 2008; Garcia et al., 2004; Hergeth et al., 2011; Lu et al., 2009; Matafora et al., 2009; Pham and Sauer, 2000; Tweedie-Cullen et al., 2009; Wisniewski et al., 2007; Wood et al., 2009). A summary of PTMS on H1 is organized (Table 3).

2. Methylation of H1

To date, there are only two methylation sites on H1 being studied in its biological functions. First, lysine 26 of H1.4 can be di-/tri-methylated by Ezh2 (Kuzmichev et al., 2004) and mono-/di-methylated by G9a (Trojer et al., 2009) and this methylation was involved in gene repression (Kuzmichev et al., 2004). Later on the transcriptional inhibitory mechanism is proved to mediate the recruitment of the heterochromatin protein 1 (HP1) onto the target of silencing through interaction of the chromo domain of HP1 between H1.4K26me2 (Daujat et al., 2005). On the other hand, phosphorylation of H1.4S27 results in disruption of HP1-H1.4 interaction (Daujat et al., 2005), supporting the so-called “phospho switch” model proposed by David Allis. The demethylase responsible for removing H1.4K26me was also identified as JMJD2 (Trojer et al., 2009). Interestingly, while Ezh2 is previously thought to be the H3K27 methyltransferase, Danny Reinberg’s group showed that the substrate preference of Ezh2-containing PRC complex is due to the

incorporation of a different subunit, EED. Ezh2-containing PRC complex incorporated with EED3/4 prefer H3K27 as substrate, while Ezh2-containing PRC complex incorporated with EED1 prefer both H3K27 and H1K26 as substrates (Kuzmichev et al., 2004). On the contrary, Yi Zhang's group later argued that under different *in vitro* condition, Ezh2-containing PRC complex prefer H3 rather than H1 and addition of H1 can further stimulate H3K27 methylation (Martin et al., 2006). The second H1 methylation site was identified recently through a candidate research. Schneider's group demonstrated that G9a and its interacting methyltransferase Glp1 specifically methylate H1.4 at lysine 26 and H1.2 at lysine 187 *in vitro* (Weiss et al., 2010). JMJD2 specifically remove H1.4K26me2, but not H1.2K187me2. HP1 does not bind H1.2K187me2. Nevertheless, knowledge of H1 methylation accumulates slowly, while potential methylation sites other than K26 and K187 have only identified by proteomics approach with no further biological characterization (Wisniewski et al., 2007).

3. Acetylation of H1

Danny Reinberg's group identified a H1.4-interacting protein, SIRT1, which is a histone deacetylase. SIRT1 removes the acetylation on H1.4K26 and facilitate subsequent recruitment of H 1.4 to promoter, accompanied with a comcomitant reduction of H3K9 and H4K16 acetylation, and H3K79 methylation. The outcome of these multiple alternation on histone marks promote the formation of facultative heterochromatin of the very promoter region (Vaquero et al., 2004). On the other hand, proteomic approaches identified several sites other

than K26. Garcia et al only identified K62ac on H1.2, H1.3, and H1.4 (Garcia et al., 2004).On the other hand, Wisniewski et al found four acetylation sites : K52, K64, K85, and K97 (Wisniewski et al., 2007). Interesting, the acetylation sites on H1 identified so far all locate within the globular domain, which is responsible for the binding to DNA. It is reasonable to think that the neutrlization of positive charges on the globular domain by acetylations likely affects the DNA binding proterty of H1. Anothoer interesting evidence is that cells with faster division rate contains more acetylation and cells with lower division rate contains less acetylations, implying a relationship of H1 acetylation and cell proliferation (Wisniewski et al., 2007).

4. Ubiqitination of H1

Polyubiquitination of proteins have been linked to stablized proteins from 26S proteasome-mediated degradation (Thrower et al., 2000) and monoubiquitination has been linked to translocation signals (Terrell et al., 1998) and other biological functions Ubiquitination of histones on H2A and H2B have been known for more than 30 years and the functions have been extensively examined. H2A ubiquitination is generally associated with transcriptional silencing. On the contrary, H2B ubiquitination is associated with both transcriptional silencing and activating (Weake and Workman, 2008). In 2000, Pham and Sauer discovered that Drosophila $TAF_{II}250$ contains ubiquitination activity and histone H1 is its substrate. Ubiquitination of H1 is involved in transcriptional activation. The site of ubiquitination is not identified (Pham and Sauer, 2000). Later on, Wisniewski et al identified a specific

site on K46 of H1.2, H1.3 and H1.4 with ubiqutination in HeLa, but not MCF7 cells (Wisniewski et al., 2007). However, whether the human homologue of $TAF_{II}250$ is responsible for H1K46 ubiquitination is still unclear neither is the biological relevance of this ubiquitination site. So far no sumoylation or neddylation on H1 has been reported.

5. ADP-ribosylation of H1

ADP-ribosylation of H1 has only been found in Drosophila using proteomic appraoch. No biological function is characterized (Villar-Garea and Imhof, 2008).

6. Phosphorylation of H1

- **H1 is highly phosphorylated**

Phosphorylation of core histones has been known to be crucial for transcription, DNA repair and mitosis. A summary of functional phosphorylation on core histones is organized (Table 4). Besides core histones, H1 has also long been recognized as a phosphoprotein and its total phosphorylation level is cell cycle-dependent (Alexandrow and Hamlin, 2005; Pochron and Baserga, 1979; Roth and Allis, 1992; Wood et al., 2009). Phosphorylation of H1 was observed in relative early works. The Global phosphorylaiton level of H1 increases from G1, S, and G2/M phase, with a maximal level at late G2 (Roth and Allis, 1992). However, studied mainly focused on somatic H1 variants H1.2/3/4/5 showed that H1 is both phosphorylated in interphase as well as mitosis. The most well-characterized phosphorylation of H1.5 variant contains

three sites at interphase (Ser18, Ser173, and Ser189) with additional sites during mitosis (Thr11, Thr138, and Thr155). Note that all sites reside in a CDK consensus motif (S/T-P-X-Z, with X being a polar amino acid and Z a basic amino acid) except Thr11. In addition, Thr 11 and Ser18 locate at N-termial tail where others locate at C-terminal tail. As for H1.2/3/4, although are less abundant than C-terminal phosphorylation, N-terminal phosphorylation of H1.2/3/4 can still be detected in recent studies with the help of advanced mass spectrometic technology. H1.2 is found to be phosphorylated at Thr31, Ser36, Ser174, Thr146, and Thr154. H1.3 is found to be phosphorylated at Ser37, Ser189, Thr147, Thr155, and Thr180. H1.4 is found to be phosphorylated at Thr18, Ser172, and Ser187 (Sarg et al., 2006). Interestingly, among the above-mentioned sites, only Thr146/154 on H1.2 and Thr 147/155 on H1.3 reside in the CDK motif, implying additional kinases may participate in phosphorylation of H1 *in vivo.* Recently, several studies identified non-CDK kinases that phosphorylate H1 *in vivo*. Zhao et al demonstrated PKCε as a H1 kinase in vascular smooth muscle, although specific site of phosphorylation was not identified (Zhao et al., 2004). Talasz et al demonstrated G1 phase phosphorylation of H1.5S17, mitotic phosphorylation of H1.5S172 and H1.5T10, and colocalization of H1.5S172 with replication and transcription sites (Talasz et al., 2009). Using proteomic approach, Sarg et al demonstrated that during spermatogenesis, histone H1t is phosphorylated first at two nonconsensus motif SPKS sites Ser-140 and Ser-186 followed by two additional sites with CDK consensus motif Ser-177 and Thr-155 (Sarg

et al., 2009). O'Brien et al identified H1.1T152 as a substrate of p-TEFb,a comlex of CDK9 and cyclin T1, and demonstrated the involvment of H1.1T152 phosphorylation in HIV Tat transactivation on c-fos and Hsp70 genes (O'Brien et al., 2010). Schneider's group identified Aurora B as the well-known H1.4S27 kinase. They demonstrated that H1.4S27 phosphorylation is also cell cycle-dependnt. It accumulates at mitosis and S27E mutant increased the binding affinity of H1.4 by FRAP assay (Hergeth et al., 2011).

◆ **H1 phosphorylation is involved in many biological processes**

It was argued that the phosphorylation of H1 in Tetrahymena thermophila is a function of cell activity or growth rate (Gorovsky et al., 1974). Phosphorylation of H1 is related to cell cycle, replication, DNA decondensation, chromatin remodeling, as well as transcription (Alexandrow and Hamlin, 2005; Finn et al., 2008; Govin et al., 2010; Horn et al., 2002). Phosphorylation of H1 has been studied and shown critical in the formation of the heterochromatin (Hale et al., 2006). On the other hand, H1 has long to be thought as a general transcriptional repressor based on its ability to help pack higher order chromatin structure. However, more and more evidences suggest that it is not the case. Depletion of three H1 variants resulted in shortened nucleosomal repeat and, strikingly, minor altered gene expression with only 29 genes affected (Fan et al., 2005). Other H1 knockout work also implied that H1 variants influence different subset of gene expression (Alami et al., 2003). Using a site-directed mutagenesis method, Dou and his colleagues demonstrated that H1Ser-Glu mutant mimicing

phsophorylation status regulate the expression of specific genes (Dou et al., 1999). Moreover, Msx1 homeoprotein was found to cooperate with H1.4 in repression of myogenic gene expression (Hsu et al., 2004). Further evidence by Rosenlfeld's group emphasized the role of phosphorylated H1 in regulation of a specific gene promoter in response to certain stimuli (Zhu et al., 2011). Nevertheless, function of site-specific phosphorylation and the upstream kinases are just emerging. It was recently shown that H1.5 phosphorylated at T10 by glycogen synthase kinase-3 binds to mitotic chromosome (Happel et al., 2009). Interphase phosphorylation of H1.4 at S187 likely regulates transcription of ribosomal and steroid hormone-responsive genes (Zheng et al., 2010).

V. Protein kinase A (PKA)

1. Introduction

cAMP-dependent protein kinase, also known as protein kinase A (PKA), belongs to an important class of kinases, referred to as arginine-directed kinases or AGC-family kinases. These kinases share a substrate specificity characterized by arginine at position -3 relative to the phosphorylated serine or threonine. The consensus motif of the substrates for the PKA has been characterized as "RRXS/T". PKA has been known to be involved in various cellular processes (Beebe, 1994; Costanzo et al., 1999; Francis and Corbin, 1994). The most important upstream secondary messager for PKA activation is cyclic AMP (cAMP), which is generated from ATP by adenylate cyclases intracellularly

(Hanoune and Defer, 2001). Signallings transduced from extracullular stilmuli and making uses of cAMP as the secondary messanger are extremely important for cells because they govern a variety of cellular processes such as proliferation, differentiation, and apoptosis (Stork and Schmitt, 2002). PKA, taking the form of holoenzymes, are composed of two catalytic (C) subunits and two regulatory (R) subunits. PKA are also isozymes with two isoforms: PKA-I and PKA-II (Tasken et al., 1997). PKA isoforms differ in regulatory subunits. PKA-I contains regulatory subunit I (RIα or RIβ) and PKA-II contains regulatory subunits II (RIIα or RIIβ) (Tasken et al., 1993). The inactive holoenzymes are activated once two cAMP bind to the two R subunits and subsequently release the C subunits. The released C subunit is thus activated and can phosphorylate substrates both in the cytoplasm and in the nucleus (Tasken et al., 1997). PKA has a relatively broad range of substrates, with the concesus motif “RRAS/T”, where R indicates the basic amino acids and the last aminoacid can be either serine or threonine. The major and also the most wellknown substrate of PKA is the transcription factor of cAMP response element binding (CREB). CREB, bound on a CRE (TGACGTCA), recruits its coactivator CREB binding protein (CBP) upon PKA phosphorylation, leading to activation of CREB targets, which include by far about 4,000 genes (Mayr and Montminy, 2001). Besides CREB, many proteins have been found to be a target of PKA. A summary of PKA substrates is organized (Table 5)

2. PKA and mitotis

PKA has been known to be involved in normal centrosome division via

phosphorylation of centrin, a component of centrioles (Lutz et al., 2001). thus providing an evidence for a role of PKA in mitosis. In addition, PKA-II localizes at the centrosome via at least two PKA-anchoring proteins (AKAPs), AKAP350 (also called AKAP450 or CG-NAP (Goto et al., 1999; Schmidt et al., 1999; Witczak et al., 1999) and pericentrin (Diviani et al., 2000). In particular, pericentrin, as a component of centrosome, binds to γ-tubulin and dynein and regulate the spindle organization through recruitment of PKA onto centrosomes (Diviani et al., 2000).

On the other hand, Both PKA-I and PKA-II are known to binds to microtubules (Imaizumi-Scherrer et al., 2001; Vallee et al., 1981), suggesting the involvement of PKA in intracelluar transport, polarity, and cell division. Moreover, PKA was found to be a kinase for stathmin, a protein which is resposible for the stabilization of microtubules (Larsson et al., 1997), and phosphorylation of stathmin by PKA is required for normal assembly of the mitotic spindles (Gradin et al., 1998; Howell et al., 1999).

In addition to centrosome division and spindle organization, PKA also participates in metaphase-anaphase transition. The most important regulator involved for this process is anaphase-promoting complex or cyclosome (APC/C). APC/C is a ubiquitin E3 ligase and it targets several key molecules for protein degradation, leading to the transition from metaphase to anaphase. Activation of APC/C is shown to negatively regulated by PKA in eukaryotic organisms (Kotani et al., 1998; Yanagida et al., 1999).

Interestingly, It was reported that RIα localized at the center of the midbody during cytokinesis (Imaizumi-Scherrer et al., 2001). Although specific function of PKA in this compartment is still unclear. Several mitotic

kianses located at the midbody are also reported to be important for cytokinesis such as polo-like kinase, citron kinase, and aurora related kinase (Giet and Prigent, 1999; Glover et al., 1998; Madaule et al., 1998), implying a similar relationship of the functional localization of PKA at the midbody might also exist.

AIMS

AIMS

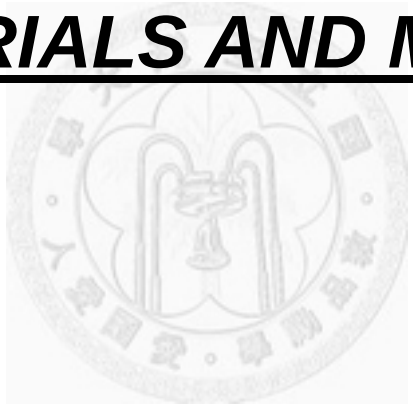
PTMs on histones have been proved to be important in various cellular functions happen near the chromatin, especially in transcriptional regulation, DNA repair, replication and cell division. While many PTMs on core histones have been extensively studied, PTMs on the linker histone H1s have not.

In this study, we try to characterize additional PTMs on H1 as well as their functionalities. The following questions need to be investigated:

1. What novel PTMs exist on the linker histone?
2. What enzymes mediate these modifications?
3. What are the functions of these PTMs?
4. Is there any interplay between these PTMs?

In this study, we identified cAMP-dependent protein kinase A (PKA) as a kinase for mitotic phosphorylation of H1.4 at serine 35 (S35), which resulted in a decrease of DNA binding affinity. The facts that H1.4S35A mutant fails to efficiently rescue the mitotic defect caused by H1.4 depletion and that PKA inhibition results in mitotic chromatin compaction further suggest an important role of the specific phosphorylation during mitosis. In addition, an adjacent methylation site, H1.4K33me, was also identified and the modification is mediated by SET7, a histone H3K4 methyltransferase. We found that loss of H1.4K33me facilitates the accumulation of H1.4S35ph in unsynchronized condition, implying a possible function of H1.4K33me to prevent aberrant phosphorylation of H1.4S35 during interphase.

MATERIALS AND METHODS



MATERIALS AND METHODS

1. Cell culture and synchronization

Human cervical adenocarcinoma cell line HeLa (ATCC, CCL-2) and HEK 293T/17 (ATCC, CRL-11268) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, Sigma), 100 U/ml penicillin, and 100 g/ml streptomycin (GIBCO) at 37°C with 5% CO2. Synchronization of HeLa cells at G1/S transition was achieved by double thymidine method (2 mM thymidine, T1895, Sigma) (Jackman and O'Connor, 2001). Synchronization of HeLa cells at metaphase was accomplished by nocodazole (400 ng/ml, M1404, Sigma) for 16 h. Release of synchronization was done by washing cells with warm PBS for 3 times and re-incubate cells with complete DMEM.

2. Antibodies, inhibitors and drugs

The antibodies (Abs) used for immunoblotting were H1 (sc-10806, Santa Cruz Biotechnology), H3 (ab1791, Abcam), H3 (phosphor S10) (06-570, Millipore), H1 (phosphor T146) (ab3596, Abcam), PKAα catalytic subunit (GTX104934, GeneTex), PKAβ (sc-904, Santa Cruz Biotechnology), β-tubulin (mab5562, Millipore), FLAG (F1804, Sigma), β-actin (mab501, Millipore), CDC2 (GTX108120, GeneTex), Lamin B (sc-2617, Santa Cruz Biotechnology), HA (MMS-101R, Covance). The Ab used for immunofluorescence was H1 (sc-8030, Santa Cruz Biotechnology). The kinase inhibitors used were H89 (2910, Tocris), and KN-62 (422706, Merck).

3. Generation of PTM-specific antibodies

Two short peptides from the amino-terminus of H1.4 were synthesized containing a single phosphorylated serine at position 35 for generation of rabbit anti-H1.4S35ph Abs (GAAKRKA$_{p}$**S**GPPVSEL) for anti-H1.4S35ph Ab and of rabbit anti-H1.4K33me1 Ab (SAGAAKR$_{me1}$**K**ASGPPVS) for anti-H1.4K33me1 Ab. With the addition of an artificial cysteine, the peptides were conjugated to maleimide-activated keyhole limpet hemocyanin (KLH). Rabbits were then immunized with injections of conjugated peptide in the presence of Freund's complete or incomplete adjuvant. Antibody purification from anti-sera was performed using affinity purification method. Briefly, anti-sera were collected and first loaded into a column containing unmodified peptides (GAAKRKA**S**GPPVSEL-C for anti-H1.4S35ph Ab or SAGAAKR**K**ASGPPVS-C for anti- H1.4K33me1 Ab) immobilized on iodoacetyl groups-containing Sulfolink Coupling Gel agarose beads (Pierce) using the sulfhydryl group on the C-terminal artificial cysteine. This step is to exclude site-specific Ab against unmodified peptides. The flow-through were further loaded in a column containing immobilized modified peptides (GAAKRKA$_{p}$**S**GPPVSEL-C or SAGAAKR$_{me1}$**K**ASGPPVS-C, respectively). This step is to capture PTM-specific Ab against modified peptides. Finally, the antibodies captured on the beads were eluted using low-pH glycine solution. The prepared affinity columns were used to first exclude site-specific Ab against unmodified peptides, then capture PTM-specific Ab against modified peptides. The eluted Abs were stored at -30°C in the presence of 50% glycerol. Rabbit anti-H1.4S35ph Ab #2 was in collaboration with GeneTex using

"AAKRKA$_{p}$**S**GPP" peptide as the antigen. This Ab can be available from GeneTex (GTX121669).

4. Plasmids

PKA regulatory subunit α1 (PRKAR1α), PKAα catalytic subunit, PKAβ catalytic subunit, aurora A, aurora B, aurora C, CAMKIIβ, CAMKIIγ, CAMKIIδ, CDC2, PLK1, and NEK2 for mammalian expression were cloned from HeLa cDNA into pcR3.1-FLAG (Invitrogen). H1.4 for mammalian expression was cloned from HeLa cDNA into pcDNA3-HA (Invitrogen) and pcDNA3.1/V5-HIS (Invitrogen). The plasmid encoding FLAG-PRKAR1α-DN was constructed by generating triple mutations (G201E, G235D, and R333D) from pcR3.1-FLAG-PRKAR1α using site-directed mutagenesis method as described (Ou et al., 2011). pcDNA3-HA-H1.4S35A and pcDNA3.1-H1.4S35A-V5HIS encoding the H1.4S35 phosphorylation mutant were also produced by site-directed mutagenesis. shRNA against H1.4 was designed according to the previous report (Sancho et al., 2008), targeting "GAAGAGCGCCAAGAAGACC" and cloned into pFUGW lentiviral vector as described (Su et al., 2009). H1.4 constructs resistant to the shRNA described above were also generated by site-directed mutagenesis (termed pcDNA3.1-rH1.4WT-V5HIS and pcDNA3.1-rH1.4S35A-V5HIS). According to the previous report (Sancho et al., 2008), the targeted sequence was changed to "GAAatctGCgAAGAAGACC" where the letters in lower case indicate the changes which did not change the amino acid sequence. The primers used for cloning are listed (Table 6).

5. Mass spectrometry

Histone H1 was separated on a 15% SDS-polyacrylamide gel followed by trypsin in-gel digestion. The digested fragments were analyzed using high resolution and high mass accuracy nanoflow LC-MS/MS on an LTQ-FT (Linear quadrupole ion trap-Fourier transform ion cyclotron resonance) mass spectrometer (Thermo Fisher Scientific).

6. Knockdown experiments by siRNA transfection or lentivirus transduction

Scramble siRNA (4390846, Ambion), siRNA against PKAα (s11066, Ambion) or PKAβ (s11069, Ambion) was used for transient knockdown of PKA catalytic subunits as described (Lee et al., 2010). Briefly, cells at 10% confluence in 6 well plates were transfected with 20 nM of siRNA by lipofectamine 2000 (Invitrogen) for 3 days. Stable knockdown of H1.4 in HeLa cells was accomplished by lentivirus encoding shRNA against H1.4. HeLa cells at 10% confluence in 6 well plates were transduced with lentivirus encoding scramble shRNA (Ctrli) or shRNA against H1.4. Cells were sorted by EGFP fluorescence by FACSAria (BD Biosciences) to enrich the population of infected cells.

7. Western blotting, histone extraction, and fractionation

Western blotting was performed as described (Hsu et al., 2004). For preparation of total cell lysates, cells were lysed in total cell lysis buffer (50 mM HEPES, pH 7.4, 5 mM EDTA, 1% Triton X-100) and incubated on ice for 10 min. Histone extraction was prepared as described (Shechter et al., 2007).

Briefly, cells were harvested and incubated with 0.2 N H_2SO_4 for 30 min at 4°C. After centrifugation, the supernatants were collected and added with trichloroacetic acid (TCA) to precipitate the remaining proteins. The precipitants were washed with cold acetone and air-dried. The dried proteins were dissolved in dH_2O and the concentrations were determined. Fractionation of nuclear extract and nuclear pellet was performed as described (Lee and Skalnik, 2002) with modifications. Briefly, cells were first incubated with hypotonic buffer (20 mM Tris, pH 8.0, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA) to obtain the nuclei. The nuclei were incubated with hypertonic buffer (50 mM Tris, pH 8.0, 420 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA) for 30 min on ice. After centrifugation, the supernatants were collected as nuclear extract (NE). The pellets were resuspended with the same volume of hypertonic buffer and incubated with Benzonase (E8263, Sigma) for 30 min at 37°C to dissolve the bulk chromatin. After centrifugation, the supernatants were collected as nuclear pellet (NP). Equal volume of NE and EP were applied to western blot for analysis.

8. *In vitro* kinase assay

The *in vitro* kinase assay was performed as described (Djouder et al., 2010). Briefly, Calf thymus H1 (14-155, Millipore) or core histones (10223565001, Roche) was incubated with recombinant PKAα catalytic subunit (P6000, NEB) or recombinant Aurora B kinase (325901, Merck) in the presence of ATP at 37°C for 1 h in kinase buffer (50 mM Tris-HCl and 10 mM $MgCl_2$). Western blot was then applied using H1.4S35ph Ab or H3S10ph Ab.

9. Immunofluorescence microscopy

Immunofluorescence staining was performed as described (Tu et al., 2008) with modifications. Briefly, cells seeded on serum-coated slides in a 12 well plate were fixed by 1% (v/v) formaldehyde in PBS for 15 min at room temperature. After fixation, cells were permeabilized with permeabilization buffer (0.01% Triton X-100-containing PBS) for 10 min and blocked in blocking buffer (0.01% Triton X-100-containing PBS, 3% bovine serum albumin) for 1h at room temperature. The primary and the fluorophore-conjugated secondary antibody were subsequently incubated for overnight and 1h, respectively, at 4°C, with three washes using permeabilization buffer. Cells were then incubated with 300 nM 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) for 15 min at room temperature. The cover slides were mounted by Gold-antifade mounting solution (P36934, Invitrogen) and sealed with nail polish. Fluorescence microscopy images were collected using an OLYMPUS IX71 fluorescence microscopy fitted with an UPlanFl 60x/N.A. 1.25 oil objective. The merge images were created using Adobe Photoshop CS.

10. Propidium iodide staining

To stain HeLa cells with propidium iodide for analysis of cellular DNA content, 5×10^5 HeLa cells were harvested and washed by PBS once on ice. After resuspending the cells in 100 μL of PBS, 900 μL of 70% ethanol was added drop by drop with continuing gentle vortex. The fixed cells were incubated and stored in -20 °C overnight. Next day, the cells were washed by PBS twice and wash buffer (PBS containing 0.1% Triton X-100) once and stained with

propidium iodide (5 μg/mL, Sigma) plus DNase-free RNase A (200 μg/mL) at 37°C for 15 min, protected from light. The stained cells were analyzed by BD FACSCanto^{TM} II (BD Biosceince).

11. Micrococcal nuclease (MNase) sensitivity assay

Mnase sensitivity assay was performed as previously described (Hashimoto et al., 2010) with modifications. In brief, nuclei were isolated using a hypotonic buffer (Tris-Cl pH 7.5, 10 mM NaCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 0.5 % NP-40) and treated with 0.2 U/μl of MNase for 15 min at 37°C. Reaction was terminated by addition of 5 mM of EDTA. DNA were then precipitated by ethanol in the presence of 0.3 M sodium acetate (pH 5.2) and dissolved in TE buffer. The purified DNA was then separated on a 1.5% TAE agarose gel pre-stained with SYBR Green I. Quantification analysis was done by Metamorph.

12. Generation of biotin-labeled probe, purification of oligonucleosomes from HeLa cells, and nucleosome reconstitution

The biotin-labeled DNA probe was designed and amplified as described (Suganuma et al., 2008) with modifications. Briefly, a 216 bp DNA fragment containing the 601 nucleosome positioning sequences was PCR amplified from pGEM-3Z-601, a gift kindly provided by Jerry Workman, with 5' biotin-labeled primers. The sequences of the primers are: 5'-CGACTGGCACCGGCAAGGT-3' (forward) and 5'-AGGGAATACACTACCTGGGATA-3' (reverse). The resulting DNA probes

were purified by gel extraction using gel extraction kit (Qiagen). Purification of oligonucleosomes from HeLa cells was performed as described (Juan et al., 1994). Purified oligonucleosomes were collected and stored at -80°C. Nucleosome reconstitution was performed by the octamer transfer method as described (Gutierrez et al., 2007). Briefly, the DNA probe was incubated with HeLa oligonucleosomes in buffer DR (10 mM Tris-Cl pH 7.4, 1 mM EDTA, 5 mM DTT and 0.5 mM PMSF) containing 1 M NaCl. NaCl concentration was further diluted to 0.8 M, 0.6 M, 0.4 M, 0.2 M by buffer DR. Final dilution to 0.1 M was made by buffer FDR (10 mM Tris-Cl pH 7.4, 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.1% NP-40, 20% glycerol and 200 μg/mL of BSA). Between each dilution, the sample was incubated at 37°C for 15 min. Reconstituted nucleosomes were subjected to electrophoresis mobility shift assay immediately.

13. Electrophoresis mobility shift assay (EMSA)

After the nucleosomes were formed on the biotin-labeled DNA probe, recombinant H1.4 purified from E coli was mixed with the nucleosomal DNA templates at 37°C for 30 min, followed by electrophoresis by a 5% native polyacrylamide gel in 0.5X TBE buffer at 100 V for 2 h. The DNA was then transferred onto Zeta-Probe GT genomic tested blotting membrane (BioRad) in 0.5X TBE buffer at 100 V for 1 h. DNA was further crosslinked on the blot by UV at 120 mJ/cm^2 (Ultraviolet crosslinker, UVP LLC). Detection of biotin-labeled DNA was performed by Chemiluminescent Nucleic Acid Detection kit (Thermo) accordingly.

14. Metaphase spread

Metaphase spread was performed as described (Padilla-Nash et al., 2007). Briefly, HeLa cells were gently suspended in 75 mM KCl and incubated at room temperature for 25 min. Cells were prefixed with one drop of fixation buffer (75% methanol and 25% acetic acid, vol/vol) and spun at 250 × g for 5 min. The supernatant was discarded and cells were smoothly washed with fixation buffer twice. Cells were then re-suspended in fixation buffer and one drop of cell suspension was added onto a glass slide. The slide was air dried at room temperature and subjected to immunofluorescence staining.

15. Quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR)

For analysis of mRNA expression level, RT-qPCR was performed. Briefly, cells were harvested with TRIzol reagent (Invitrogen) and total RNAs were purified. cDNA were synthesized by reverse transcriptase SuperScript II (Invitrogen). The cDNA were analyzed by real-time PCR using LightCycler® 480 SYBR Green I Master (04 887 352 001, Roche), normalized to S26 rRNA expression. The primers used for RT-qPCR are listed (Table 7).

16. Histone methylation assay

For histone methylation assay, purified bovine H1, bacterial-purified recombinant H1.4, or bovine core histones were used as substrates. Briefly, SET7 was incubated with substrates in histone methylation buffer (50 mM

Tris-Cl pH 8.0, 5 mM MgCl2, 4 mM DTT) at 30°C for 30 min.

17. *In vivo* methylation labeling

To perform *in vivo* methylation labeling, the cells are incubated in methionine-free medium containing 10% dialyzed FBS for 24 h. The medium was replaced by ^{3}H-SAM for another 24 h. The cells were then harvested and histones were extracted by acid extraction method. Histones were separated by electrophoresis on 15 % SDS-PAGE. The gel was dried and exposed to Hyperfilm™ ^{3}H (GE Amersham) for 3 weeks.

RESULTS

RESULTS

1. Mass spectrometry analysis identifies many novel PTMs on H1

To get more insight into how linker histone H1 function can be regulated by posttranslational modifications, we collaborated with Dr. Pang-Hung Hsu in Dr. Ming-Daw Tsai's lab in the Institute of Biological Chemistry, Academia Sinica for his specialty in mass spectrometry analysis. H1 were enriched by acid extraction from HeLa cells and analyzed by liquid chromatography (Agilent Technologies, CA) coupled with mass spectrometry (LTQ-FT, Thermo Scientific, Inc., MA). We also decided to analyze cells treated with nocodazole, a microtubule de-stabilizer that arrests cells at mitotic phase (Otaegui et al., 1994) because we expected a dramatic change of PTMs during the entry of mitosis and also because several serine or threonine phosphorylations on H1 have been known to occur during mitosis. Following this design, we successfully detected H1.4T17ph and H1.4S186ph, which have been identified as two of the mitosis-associated phosphorylation sites. In addition to the known PTMs, several novel modifications were also found in our system (Table 6) either responding to nocodazole or not. Most of the fragments detected were from somatic variants H1.2 and H1.4 rather than H1.3 or H1.5, probably because of their abundance. In our system, we detected several new modifications constantly in both non-treated and nocodazole-treated groups, including H1.2K16me1 (for monomethylation), H1.2K74me1/2 for mono/dimethylation), H1.2K109me3 (for trimethylation), H1.2K148me2, H1.3K75me2, H1.4K16ub (for ubiquitination), H1.4K20/21me2, H1.4K33me1/2, H1.4K45me3, H1.4K74me1/2, H1.4K109me3, H1.4K167ac (for acetylation),

and H1.4S186ph (for phosphorylation). We also detected new modifications only in non-treated group, including H1.2S1ph, H1.2K16me2, H1.2K20me2, H1.2K33me1/2, H1.2K33ac, H1.2K45me2, H1.2K45ac, H1.2K62ac, H1.2K89me2, H1.2K105me2, H1.2K116me1/2, H1.2K190me2, H1.4K20/21ub, and H1.5K33ac. In addition, modifications which only appeared in nocodazole-treated group included H1.2S101ph, H1.2T145ph, H1.4K16me1, H1.4T17ph, H1.4S35ph, H1.4S40ph, H1.4K62me1/2, H1.4K63ac, H1.4R78me1, H1.4S101ph, H1.4K138ac, and H1.4T145ph. The evidence showed here that so many novel PTMs still can be found gives us an intriguing hint that, indeed, linker histone is also under highly-controlled regulation at post-translational level by PTMs just like core histones.

2. H1.4 is phosphorylated at serine 35 during mitosis

Among these novel PTMs of H1, a particular phosphorylated H1.4 fragment on S35 (H1.4S35ph) was identified by MS/MS spectrum (Figure 1A) in response to nocodazole treatment and the amount of this modification is about 4 % of total H1.4. However, this only gave us a hint that the treatment increased the phosphorylation. This phosphorylation does not necessarily occur only in mitotic cells. To study this phosphorylation site in more detail, a site-specific antibody (Ab) against H1.4S35ph was generated (see Supplementary Experimental Procedures). We collaborated with Dr. Tora Laszlo in the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) from France and GeneTex Incorporation from Taiwan for their specialty to generate Abs (Ab #1 and Ab #2, respectively). The sequence of the peptide for anti-H1.4S35ph Ab #1 is “GAAKRKA$_{\mathbf{p}}$**S**GPPVSEL”, where the phospho-serine located in the

middle. The sequence of the peptide for anti-H1.4S35ph Ab #2 is "AAKRKA$_{p}$**S**GPP", where the phospho-serine located near the carboxyl terminus. Both Abs showed good specificity while Ab #2 contained greater titer. Therefore, anti-H1.4S35ph Ab #2 was used in all the following experiments. The Ab was shown to be specific as it recognized only the H1.4 peptide containing phosphorylated S35, but not the same peptide without phosphorylation (Figure 1B). Consistently, the H1.4S35ph Ab detected a signal from total lysates or histone extracts of HeLa cells with the size of H1 (around 34 kDa) in the presence of nocodazole (Figure 1C, lanes 2 and 4). Furthermore, the nocodazole-induced signal gradually disappeared upon pre-incubation of the H1.4S35ph Ab with increasing amounts of H1.4 peptide containing phosphorylated S35 (Figure 1D, compare lanes 4 to 6 to lane 2). Pre-incubation of the Ab with non-phosphorylated H1 peptide (lane 3) or an unrelated phosphorylated peptide (lane 7) did not have any effect. In addition, pre-incubation of the nitrocellulose membrane with alkaline phosphatase before adding Ab significantly reduced the H1.4S35ph signal (Figure 1E), indicating that the band recognized by H1.4S35ph Ab indeed was a phosphoprotein. We next demonstrated that the anti-H1.4S35ph Ab was specific to H1.4 variant as specifically knocking down H1.4 (arrow), but not H1.2 or H1.3 (Supplementary Figure 1) completely diminished the H1.4S35ph signal (Figure 1F). In addition, in order to test whether anti-H1.4S35ph Ab can only recognize H1.4 at phospho-serine 35, a reconstitution mutant H1.4S35A was generated and was overexpressed in 293T cells in the presence of nocodazole. Western blot showed that anti-H1.4S35ph Ab failed to recognize H1.4S35A in the presence of nocodazole (Supplementary Figure 2), indicating that anti-H1.4S35ph Ab specifically recognizes H1.4S35ph, but not other

phosphorylation sites on H1.4. Note that the overexpressed tagged H1.4 is bigger, resulting a mobility shift which can separated from endogenous H1.4. Moreover, the Ab also detected an increased signal in normal mitotic HeLa cells obtained by mitotic shake-off (Figure 1G). Successful isolation of mitotic cells by this way was confirmed by DAPI staining of the condensed cellular DNA (Supplementary Figure 3), showing that majority of the cells were at mitotic phase. We also showed that the mitotic lung cancer cells H1299 collected by nocodazole treatment were also enriched in H1.4S35 phosphorylation (Supplementary Figure 4), excluding the possibility that H1.4S35ph is only specific to human HeLa, 293T, or bovine thymus.

3. Parallel accumulation of H1.4S35 phosphorylation and H3S10 phosphorylation during mitosis

Next we investigated the temporal expression of the mitotic H1.4 S35 phosphorylation. Cells were synchronized by nocodazole or double thymidine treatment and released for certain durations, followed by western to analyze the level of H1.4S35ph. As shown in Figure 2A, cells exiting from nocodazole-induced mitotic arrest displayed time-dependent decrease of H1.4S35ph as well as two mitotic marker histone H3 phosphorylation at S10 (H3S10ph) and H1.4 phosphorylation at T146 (H1.4T146ph) (Supplementary Figure 5). This indicates that H1.4S35ph is inversely correlated with the mitotic exit. Similar result was obtained using thymidine to arrest cells (Figure 2B). Cells exiting from thymidine-induced G1/S arrest showed H1.4S35ph accumulation peaking at 10 h after release and the accumulation of H1.4S35ph was parallel with that of H3S10ph, which was induced from 8 to 10

h after release (Figure 2B). These results indicate that H1.4S35 phosphorylation is induced at a very specific time window of mitosis.

4. PKA phosphorylates H1.4 at S35 *in vivo*

To identify the kinase for H1.4S35ph, the amino acid sequence around the phosphorylation motif was analyzed using an online tool (PhosphoSitePlus, Cell Signaling Technology, http://www.phosphosite.org/homeAction.do). Several potential kinase candidates were predicted, include protein kinase A (PKA) and calmodulin kinase II (CaMKII), but not cyclin-dependent kinase. Given that H1.4S35ph is induced during mitosis, the corresponding kinase should be expressed or activated at the same time. Based on the above two reasons, related mitotic kinases were selected (Table 8) and analyzed for their ability to phosphorylate H1.4S35. As shown in Figure 3A, ectopic expression of PKAα alone caused H1.4S35ph accumulation. However, other kinases including aurora kinases, CAMKIIβ, CAMKIIγ, CAMKIIδ, CDC2, NEK2 and PLK1 failed to do so. Since overexpressing PKA catalytic subunit alone is sufficient to accumulate H1.4S35ph level, we suspect that PKA agonists such as forskolin, isoproterenol or 8-bromo-cAMP should also do the trick. This implies that H1.4S35ph might be PKA-dependent, but not absolutely mitosis-dependent. Therefore, we can only consider H1.4S35ph as a mitotic marker in certain situations. Consistently, the nocodazole-induced H1.4S35ph was greatly abolished by PKA inhibitor H89 (Figure 3B, compare lane 3 to lane 2) but not altered by CaMK inhibitor KN-62 (compare lane 4 to lane 2). In contrast, H89 had no effect on H3S10ph (Figure 3B), suggesting trivial effect of H89 on the entry of mitosis. Moreover, PKA knockdown diminished H1.4S35ph

but not H3S10ph after mitotic arrest by nocodazole (Figure 3C). Since depletion of PKA disrupted meiotic arrest in oocytes (Duncan et al., 2006), the effect of PKA knockdown on the decrease of H1.4S35ph level may not due to a decrease of mitotic population. H1.4S35ph was also reduced by overexpression of a dominant mutant of PKA regulatory subunit 1 α (PRKAR1A) after mitotic arrest by nocodazole (Figure 3D, compare lane 4 to lane 3). However, we can not achieve a 100% of reduction of H1.4S35ph by disrupting PKA activity, suggesting that there maybe other kinases or protein phosphatases also regulate H1.4S35ph during mitosis.

5. PKA directly phosphorylates H1.4 at S35 *in vitro*

The above results together suggested that PKA is a kinase for H1.4S35 phosphorylation. To prove that H1.4S35 is directly phosphorylated by PKA, an *in vitro* kinase assay was performed. Indeed, PKA directly phosphorylated H1.4 at S35 (Figure 4, lanes 9-12) and mass analysis showed that S35 was the major site for PKA (data not shown). In contrast, aurora B phosphorylated H3S10, but not H1.4S35 (Figure 4, lanes 4 to 8). However, due to the weak activity of aurora B proteins we got, the result needs further investigations (see discussion). Although PKA also catalyzed S10 phosphorylation of the purified H3 (Figure 4, lanes 9 and10), PKA depletion or inhibition did not reduce H3S10ph (Figure 3B, 3C, and 3D). Our experiments together demonstrated that PKA is the kinase for H1.4S35ph *in vitro* and *in vivo*.

6. S35-phosphorylated H1.4 decreases the binding affinity of H1.4 to mitotic chromatin

Next, we would like to study the relationship between H1-DNA interaction and H1.4S35 phosphorylation. To investigate how S35-phosphorylated H1.4 behaves, the level of H1.4S35ph in nuclear soluble extract (NE) and nuclear pellet (NP) was compared. NE contains the protein fraction enriched in proteins not or less bound to chromatin, where NP contains the protein fraction enriched in chromatin-bound proteins. The results showed that the nocodazole-induced H1.4S35ph only existed in NE, implying that the S35-phosphorylated H1.4 has reduced affinity towards the mitotic chromatin (Figure 5A, lane 2). In contrast, nocodazole-induced H3S10ph occurred only in NP (Figure 5A, lane 4). Semi-quantification of the ratio of H1.4S35ph and total H1 showed that the ratio of total H1 in NE (dissociated from chromatin) was increased from 48.4% to 53.4% upon nocodazole treatment and the level of total H1 in NP (associated with chromatin) was decreased from 51.5% to 46.5% by nocodazole (Figure 5B). Note that the anti-total H1 antibody used here can recognize both H1.2 and H1.4, and the amount of H1.4 in HeLa is about 50 % of total H1, implying that the result here may be underestimated. A greater effect would be expected if anti-H1.4 antibody were available. To test whether S35 phosphorylation indeed reduces H1.4 binding to chromatin, the effect of H1.4S35A was analyzed in the presence of nocodazole. Indeed, H1.4S35A (S35A) can not be extracted into NE fraction as efficiently as H1.4WT (WT) (Figure 5C). Semi-quantification showed that 63.6% of wild type H1.4 was located in NE. In contrast, only 37.0% of H1.4S35A appeared in NE

(Figure 5D). These results indicate that the phosphorylation mutant H1.4S35A is less sensitive to be extracted by salt from mitotic chromatin.

To investigate in more details if H1.4 phosphorylated at S35 binds less efficiently to chromatin, electrophoresis mobility shift assay (EMSA) was applied using a 216 bp nucleosomal DNA template containing the nucleosome positioning sequence, which in theory can only hold one nucleosome. As shown in Figure 5E, addition of E. coli-purified recombinant His_6-H1.4 caused a shift of mononucleosome, indicating the binding of His_6-H1.4 onto mononucleosomes (compare lane 4 to lane 2). Importantly, adding PKA to phosphorylate H1.4 *in vitro* reduced the level of the shifted band, suggesting that His_6-H1.4 phosphorylated at S35 by PKA binds less to mononucleosomes (compare lane 5 to lane 4). Quantitative analysis of the shifted bands showed a significant reduction of H1.4 binding to mononucleosome after phosphorylation by PKA (11.1 ± 10.0 %) compared to control (35.9 ± 15.7 %) (Figure 5F), with efficient phosphorylation of His_6-H1.4 at S35 by PKA *in vitro* (Figure 5G). Together we believe that S35-phosphorylated H1.4 contains reduced affinity towards mitotic chromatin and that H1.4S35ph is important for this reduction both *in vivo* and *in vitro*.

7. H1.4S35A substitution mutant cannot efficiently rescue the mitotic defect caused by H1.4 depletion

Since H1.4S35ph is specifically accumulated during mitosis, we wondered if PKA-mediated H1.4S35 phosphorylation plays a role in mitosis. First, we examined if H1.4 controls cell cycle progression. As shown in Supplementary Figure 6A, FACS analysis using propidium iodide staining indicates that the

decrease in the number of G2/M cells by knocking down H1.4 accompanied an increase of G1 population in unsynchronized cells. When cells were synchronized at G1/S and released for 9 h, a delay in both G1 and S phases was observed (Supplementary Figure 6B). In addition, we also found that H1.4-depleted cells grew slower (Supplementary Figure 7A) and were more likely to die (Supplementary Figure 7B). These evidences imply that H1.4-depleted cells have defects in entering both S and G2 phases and cell survival, leading to subsequent growth delay. The mitotic index measured by staining H3S10 phosphorylation confirmed that H1.4 depletion resulted in the decrease of mitotic cell population (Figure 6A). Second, we analyzed if H1.4S35ph contributes to H1.4 function in mitotic phase. To this, rH1.4WT or rH1.4S35A resistant to the shRNA-mediated translational inhibition was ectopically expressed in H1.4 knockdown cells for mitotic index analysis. Consistently, 9 h after release from G1/S transition block by double thymidine treatment, the number of H1.4-depleted cells entering mitosis was dramatically reduced (Figure 6B, shH1.4+vec). Importantly, ectopic expression of wild type H1.4 (shH1.4+rH1.4WT), but not H1.4S35A mutant (shH1.4+rH1.4S35A), rescued the defect, indicating that H1.4S35 phosphorylation is required for mitotic progression. Note that the protein levels of the ectopically expressed rH1.4WT and rH1.4S35A mutant in H1.4 KD cells are equal but a bit less than the endogenous level of H1.4 (Figure 6C, compare lanes 4 and 5 to 1 and 2). This might explain why we only obtained partial rescue effect (Figure 6B).

8. Inhibition of PKA activity increases the mitotic chromatin compaction in a H1.4-dependent manner

Since H1 is required for higher order chromatin structure and chromatin condensation occurs during mitosis, we suspected that H1.4S35 phosphorylation is important for proper mitotic chromatin compaction. Nocodazole-treated HeLa nuclei with or without PKA inhibitor H89 were subjected to micrococcal nuclease (MNase) digestion. As shown in Figure 7A, PKA inhibition increased mitotic chromatin compaction, as evident by the disappearance of low molecular weight MNase-digested fragments and the accumulation of high molecular weight MNase-resistant fragments (Figure 7A, compare lane 3 to 2; and Figure 7B). Importantly, the above effect was lost upon H1.4 depletion (Figure 7A, compare lane 5 to 4; and Figure 7C). To directly visualize chromosome compaction, mitotic chromosomes were analyzed by metaphase spread assay. As shown in Figure 8A, the chromosomes under H89 treatment exhibited a more compact structure, indicating that H89 likely induced chromatin condensation or blocked chromatin decondensation. Quantification of the mitotic cells showed that the percentage of the X-shaped chromosome in nocodazole-treated cells was greatly reduced in the presence of H89 (Figure 8B). This result is consistent with our observation by the nuclease sensitivity assay (Figure 7). The above results suggest that H1.4S35 phosphorylation likely contributes to the maintenance of optimal chromatin compaction during mitosis.

9. H1.4 is methylated at lysine 33

Among all other novel modifications on H1 we identified, we also detected an uncharacterized methylation site on lysine 33 of H1.4. The MS/MS spectrum (Figure 9A) confirmed the existence of H1.4K33 methylation. We found that about 25 % of total H1 in the calf thymus is methylated at lysine 33 by quantitative mass spectrometry analysis (Figure 9B). Moreover, we also identified this modification in human cervical carcinoma cell line HeLa. To investigate this modification in more detail, a site-specific antibody against human H1.4K33me1 (anti-H1.4K33me1 Ab) was generated using the antigen peptide "SAGAAKR**meK**ASGPPVS" by the help of Dr. Tora Laszlo. The dot blot showed good specificity to K33me1 peptide (K33me1), but not unmodified K33 peptides (K33) (Figure 9C). Moreover, Overexpression of H1.4K33R-V5HIS mutant could not be recognized by anti-H1.4K33me1 Ab while Overexpression of H1.4WT-V5HIS could (Figure 9D), indicating that anti-H1.4K33me1 Ab specifically recognizes H1.4K33me1, but not other modified/unmodified regions on H1.4.

10. SET7 directly methylates H1.4K33me1 *in vivo* and *in vitro*

Our lab previously found that H1 can be methylated by SET7, a H3K4 monomethyltransferase *in vitro* (unpublished data, our lab). However, whether SET7-mediated methylation on H1 happens on K33, the well-known K26 or other unidentified lysine is still unclear. Mass spectrometry analysis revealed that the major site on H1 methylated by SET7 *in vitro* is indeed on K33 (data not shown). Using *in vitro* methylation assay with free recombinant H1.4, we

found a dramatic increase of H1.4K33me1 signal of the anti-H1.4K33me1 antibody, implying good efficiency of SET7 toward H1.4K33 (Figure 10A). Moreover, methylation assay using reconstituted H1.4-containing oligonucleosomes (Supplementary Figure 8) showed that nucleosome-associated H1.4 was a better substrate than free H1.4 (Figure 10B) To further investigate whether SET7 can methylate H1.4 *in vivo*, we labeled cells with ^{3}H-S-adenosyl-methionine in the presence of SET7 and/or H1.4 overexpression and collected the histones for autoradiography. The result showed that overexpression of SET7 increases the methylation level of both exogenous and endogenous H1.4 (Figure 10C). On the other hand, overexpressed wild type, but not catalytic mutant SET7 immunoprecipitated from cells methylated H1.4 on K33 and H3 on K4, but not H1.4K25 (Figure 10D). To further characterize the result from Figure 10C, cells overexpressed with SET7 were analyzed by anti-H1.4K33me1 antibody. The result showed that overexpression of SET7 increases the level of H1.4K33me1 (Figure 10E), implying that SET7 is sufficient for H1.4K33me1. However, Knockdown of SET7 altered the level of H1K33 monomethylation only in trivial (Figure 10F), suggesting a redundant effect. Other methyltransferases might also participate in H1.4K33 methylation.

11. Loss of H1.4K33me1 enhances H1.4S35ph accumulation

Since H1.4K33me1 is adjacent to H1.4S35ph, it is reasonable to hypothesize that there should be a crosstalk between these two PTM sites. Interestingly, western blot showed the level of H1.4S35ph on H1.4K33R mutant dramatically increased in an unsynchronized condition, implying arginine mutation on K33

cause S35 phosphorylation, regardless of the necessity for cells to enter into mitosis (Figure 11A, lane 3). Moreover, using quantitative mass spectrometry analysis, overexpressed H1.4K33R, but not H1.4K25R mutant indeed showed increased level of H1.4S35ph also in an unsynchronized condition (Figure 11B), suggesting that H1.4K33me1 might be required for prevent non-mitotic H1.4S35 phosphorylation.

DISCUSSION

DISCUSSION

In the current study, we have demonstrated that histone H1.4 is phosphorylated at S35 by PKA during mitosis (Figures 1, 2, 3, and 4) and that this phosphorylation decreases the DNA binding affinity of H1.4 (Figure 5). H1.4S35ph is important for both normal mitosis progression (Figure 6) and normal condensation of mitotic chromosome (Figure 7 and 8).

Since majority of H1 exerts its functions in the context of chromatin and that phosphorylation of a protein generally causes a change on the net charge of the protein, which in turn might alter the ionic interaction between protein/protein or protein/DNA, the relationship between H1-DNA interaction and H1 phosphorylation has been studied from 90s. Phosphorylation is generally thought to decrease the affinity of H1 to chromatin due to a change of net charge (Baatout and Derradji, 2006). Earlier H1-DNA interaction studies in sea urchin by measuring the dissociation of H1 from DNA-cellulose *in vitro* suggested that H1 when highly-phosphorylated at amino-terminus has a reduced affinity towards DNA, but not that at carboxyl-terminus (Hill et al., 1990). On the contrary, Heald's group demonstrated that phosphorylation of Xenopus somatic H1 variant B4 by cdk1 facilitates B4 association from chromatin (Freedman and Heald, 2010). Due to this discrepancy, we speculate that the effect of H1 phosphorylation on H1-DNA interaction should be site- or variant-specific. In this study, we demonstrated a site-specific effect of S35 phosphorylation on H1.4-DNA binding and normal cell cycling. Our data are consistent with a model proposed by David Allis that phosphorylation of linker histone H1 tails promotes chromatin decondensation (Roth and Allis, 1992)

and supported by Heald's group using a GFP-fused H1.2 mutant with all 5 cdk1/2 sites mutating to aniline (M1-5) to perform fluorescence recovery after photobleaching (FRAP). While expression of M1-5 resulted in G1 accumulation (Hale et al., 2006), they found that M1-5 recovered more slowly in the presence of cdk2 activity at S phase, at the time the cells need relaxation of chromatin (Contreras et al., 2003). Also consistent with this report is that increased H1 phosphorylation results in chromatin relaxation (Herrera et al., 1996).

Based on our results, H1.4S35 phosphorylation is likely essential for specific mitotic functions. First, H1.4S35 phosphorylation may play a role in controlling mitotic entry or transition from telophase to cytokinesis as H1.4S35ph was sharply increased to a maximal level at metaphase with the concomitant increase of H3S10 phosphorylation and was diminished after cells exist from mitosis (Figure 2). The positive role of H1.4S35 phosphorylation in mitotic entry was further supported by the finding that adding back the non-phosphorylation mutant H1.4S35A could not efficiently rescue the mitotic defect in H1.4-depleted cells (Figure 6B). Although we found that stable H1.4-depleted cells have defects on cell growth and survival, the effect of H1.4S35 phosphorylation on mitosis progression is specific, as demonstrated by the previous rescue experiment. Design transient knockdown experiment should ameliorate the long-term defect by establishing bicistronic system that contains inducible knockdown H1.4 together with inducible overexpression of H1.4WT or H1.4S35A.

Second, H1.4S35 phosphorylation seems to be necessary for maintaining proper mitotic chromatin structure (Figure 7 and Figure 8). It has been reported by other groups that phosphorylation of H1 helps H1 dissociation from promoter regions of specific genes (Dou et al., 1999), which in turn allows transcription factor binding (Lee and Archer, 1998) and chromatin remodeling (Horn et al., 2002). The accessibility of transcription factors and remodeling factors to the promoter region is most likely established by certain degree of chromatin decondensation. If the hypothesis is correct, we expect to see S35-phosphorylated H1.4 falls off from a certain subset of genes necessary to be turned on during mitosis. This is an interesting possibility waiting for investigation in the future.

Besides the demonstration of the functional significance of H1.4S35ph, we also demonstrated the role of the corresponding kinase, PKA, on this H1.4S35ph-related function. PKA has been known to be activated after entry of mitosis (Kotani et al., 1998) and regulates spindle formation (Gradin et al., 1998), CDC2/cyclin B activity (Han and Conti, 2006), and anaphase promoting complex (APC) activity (Kotani et al., 1998). Our results further revealed that PKA may alter the behavior of H1.4 (Figure 5) and regulate mitosis through phosphorylating H1.4S35 (Figure 6, 7, and 8). On the other hand, due to the weak activity of aurora B kinase in our *in vitro* kinase assay system (Figure 4), we can not rule out that aurora kinase may also contribute to H1.4S35 phosphorylation. However, in agreement with our conclusion, a recent report from Schneider's group (Hergeth et al., 2011) demonstrated that aurora B phosphorylates H1.4 at serine 27 and mutation of serine 27 to alanine completely abolishes the aurora B-mediated H1.4 phosphorylation, indicating

that S27 is the only site at H1.4 to be phosphorylated by aurora B during mitosis when aurora B is activated (Fu et al., 2007). In addition, although we have measure that the major site on H1.4 for PKA is serine 35 by quantitative mass spectrometry analysis, a negative experiment is clearer to exclude the existence of other phosphorylations by in vitro PKA kinase assay using ^{32}P-γ-ATP as phosphate donor and autoradiography.

In order to further study the temporal-spatial behavior of H1.4S35ph, immunofluorescence microscopy is an important approach to study the intracellular localization of H1.4S35ph using anti-H1.4S35ph Ab. Unfortunately, the specificity of this Ab on the immunostaining experiment still awaits confirming by using either H1.4-depleted or PKA-depleted cells. Nevertheless, based on our preliminary immunofluorescence data, we found that S35-phosphorylated H1.4 was accumulated and excluded from chromatin from prophase to telophase, and disappeared during cytokinesis (Supplementary Figure 10), which is consistent with our biochemical results. Serial optical sections acquired from fluorescence confocal microscopy further showed that the distribution of S35-phosphorylated H1.4 is indeed outside of mitotic chromatin (Supplementary Figure 12). In contrast, H3S10ph showed a totally distinct pattern (Supplementary Figure 13), which colocalized with DAPI signals. The above results clearly indicate that S35-phosphorylated H1.4 specifically dissociated from mitotic chromatin. At prophase and metaphase, H1.4S35ph was not only distributed in chromatin-free nuclear plasma but also enriched in two dots likely representing the centrosomes (Mahoney et al., 2006) (Supplementary Figure 11). This finding suggests that the dissociated S35-phosphorylated H1.4 may have a role in microtubule organization and

mitotic spindle formation, two known functions of centrosomes (Doxsey et al., 2005). Although we can not rule out that this phenomenon is false-positive due to the possible problems on the specificity, these possibilities await further investigation. Alternatively, we tried to visualize the effect of H1.4S35 phosphorylation on the H1.4 distribution by using EGFP-fused H1.4 proteins overexpressed in cells. Unfortunately, we did not find obvious extra-chromosomal distribution of EGFP-H1.4WT at either interphase or distinct mitotic stages. On the other hand, we found no obvious effect of S35A or S35E mutant on the localization of H1.4 either (Supplementary Figure 14). Based on this data, we can only say that phosphorylation-mimicking mutant S35E is not sufficient for H1.4 to dissociate from chromatin. One might logically reckon that this process should need extra phosphorylations (for example, those five Cdk sites) together for the maximal ability of H1.4 to dissociate. Due to the small amount of H1.4 being phosphorylated at S35 (our quantitative mass analysis), it's also possible that the amount of dissociated EGFP-H1.4 is too small to be detected. The definite solution for this issue still relies on the strict control to demonstrate the capability of the anti-H1.4S35ph Ab.

Base on the consensus motif of PKA substrates, we aligned the H1 sequences between several species (Supplementary Figure 9). Interestingly, the consensus motif of PKA substrates "RRXS/T)" is conserved in human, mouse, rat, bovine, frog, and fly, but not C. elegans. However, the probability of being phosphorylated by PKA for the conserved motif among human, mouse, rat, and bovine ("RKXS", 47%) is almost three times higher than that of the conserved motif among frog and fly ("KKXS", 16%) (Shabb, 2001). This

observation implies that the regulation of cell division involving H1.4S35ph may be more readily operatable in higher eukaryotes than in lower eukaryotes.

On the other hand, there is another PKA motif found on H1.4 at T141 ("KKAT").This potential PKA-mediated phosphorylation might interfere our result by either being crossreacted by anti-H1.4S35ph Ab or by complicating the PKA inhibition experiment. Thus, we demonstrated, using western blot on nocodazole-treated H1.4S35A mutant that the anti-H1.4S35ph Ab can only recognize nocodazole-treated H1.4WT, but not nocodazole-treated H1.4S35A (Supplementary Figure 2), indicating that the specificity of anti-H1.4S35ph Ab is solely against H1.4 on phospho-serine 35. In addition, the T141 phosphorylation is not detected in all the H1 proteomic researches, implying that T141 phosphorylation, if there is any, is very trivial.

In addition to H1.4S35ph, an adjacent lysine methylation site, K33, was also identified next to serine 35 during mass spectrometry analysis (Figure 9A). We proved that this H1K33 methylation both exist in bovine and human (Figure 9B and 9D) and an H1.4K33me1-specific antibody was generated (Figure 9C and 9D). We further identified SET7, a previous known histone H3K4 methyltransferase, is able to methylate human H1.4K33me1 both *in vivo* and *in vitro* (Figure 10), although a redundant effect was also found by the inefficient reduction of H1.4K33me1 after SET7 KD (Figure 10F). Furthermore, we proposed interplay between H1.4K33me1 and H1.4S35ph. We found that loss of H1.4K33me1 facilitates accumulation of H1.4S35ph at unsynchronized condition (Figure 11). However, more delicate experiments will dissect this hypothesis more clearly by using pre-methylated H1.4 to perform *in vitro* PKA

kinase assay using ^{32}P-γ-ATP.

To further elucidate another way around the crosstalk between H1.4K33me and H1.4S35ph, we also investigate if H1.4S35ph affects H1.4K33me. To do this, we performed *in vitro* methylation assay using recombinant H1.4WT, K33R, or S35E mutant as substrates. The result showed that H1.4S35E is a poor substrate for SET7 (Supplementary Figure 10A). In addition, *in vitro* peptide methylation assay coupled with quantitative MALDI analysis was also performed with the addition of human pappilomavirus (HPV) E7 protein, which was previously found to have the ability to enhance SET7-mediated H1 methylation *in vitro* (unpublished data in our lab). The result showed that S35E substituted peptide [hH1.4 (28-39) S35E] can not be efficiently methylated by SET7 *in vitro*, while the wild-type peptide [hH1.4 (28-39)] can (Supplementary Figure 10B). In addition, H1.4S35E, the mutant that mimics phosphorylation, showed less H1.4K33me1 level, while H1.4WT and H1.4S35A showed comparable level of H1.4K33me1 (Figure 11A, compare lane 5 to lane 2 and 3), which is consistent with the *in vitro* methylation result.

In conclusion, we provide a novel role for H1.4 to actively participate in mitosis through PKA-mediated S35 phosphorylation. In addition, we demonstrate a crosstalk between H1.4S35ph and an adjacent K33 methylation, which might regulate the timely phosphorylation of H1.4S35ph at appropriate cell cycle stage for normal cell division.

ABBREVIATION

ABBREVIATION

PKA, Protein kinase A; H1.4S35ph, serine 35 phosphorylation on H1.4; NE, nuclear extract; NP, nuclear pellet; Ab, antibody; MNase, micrococcal nuclease; shRNA, short hairpin RNA;

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TABLES

Tabel 1. Nomenclatures of H1 variants

	Official symbol	Albig & Doenecke[§]	Ohe & Iwai[¶]	Official full name	Alias
Replication-dependent	HIST1H1A	H1.1			
	HIST1H1B	H1.5	H1a		
	HIST1H1C	H1.2	H1d		
	HIST1H1D	H1.3	H1c		
	HIST1H1E	H1.4	H1b		
	HIST1H1T	H1t			
Replication-independent	H1F0			H1 member 0	H1.0
	H1FNT			H1 testis-specific	H1T2
	H1F00			H1 oocyte-specific	H1oo
	H1FX			H1 member X	H1x

[§] (Albig et al., 1993); [¶] (Ohe et al., 1986; Ohe et al., 1989)

Table 2. H1-interacting partners

Variant	H1-interacting proteins	Approaches	Functions	References
	HuSirT1	In vivo pull-down	Facilitate HDAC activity on H4K16ac thereby help gene shot down	(Vaquero et al., 2004)
	Prothymosin α	IP	Sequester H1 from chromatin thereby activates genes for proliferation	(Karetsou et al., 1998; Papamarcaki and Tsolas, 1994)
	HPV E1	Far-Westernl; IP; affinity purification	Sequester H1 from chromatin thereby help DNA replication	(Swindle and Engler, 1998)
	Lysyl oxidase (LOX)	Affinity purification	Deaminate lysines on H1 thus alter chromatin structure similar to acetylation	(Giampuzzi et al., 2003)
	CaM kinase II (CaMKII)	Enzymatic inhibition assay	CaMKII activity is inhibited by H1 via calmodulin	(Rasmussen and Garen, 1993)
	Nucleolin	Blotting assay	Sequester H1 from chromatin	(Erard et al., 1988)
H1.4	Msx1	IP; in vivo pull-down	Mediates the assembly of repressive chromatin on MyoD gene	(Lee et al., 2004)
	Protein kinase Cε (PKCε)	Blotting assay; Kinase activity assay	Recruit PKCε to specific promoters	(Zhao et al., 2004)
H1.4	Heterochromatin protein 1 (HP1)	Pull-down; affinity columns in vitro	Recruit HP1 to condensed chromatin	(Daujat et al., 2005; Nielsen et al., 2001)
	HMG1	Fluorescence spectroscopy	HMG1 and H1 competes binding to the linker DNA, thus affects chromatin structure	(Kohlstaedt and Cole, 1994)
H1.1	Barrier to autointegration factor (BAF)	Blotting assay; microtiter binding assay; IP	Help virus integrates into open, active chromatin	(Montes de Oca et al., 2005)
H1.2	p53	In vitro pull-down; IP	Repress p53-depedent transcription	(Kim et al., 2008)
	Ribosomal proteins	IP; ChIP; fluorescence co-localization; blotting assay	Coordinates between chromatin structure, transcription, and translation	(Ni et al., 2006)
	Poly ADP-ribose polymerase 1 (PARP1)	FLAG pull-down; IP; fluorescence	Involved in chromatin condensation	(Kraus, 2008)
	Nuclear autoantigenic sperm protein (NASP)	EMSA; affinity purification; surface Plasmon resonance (SPR)	Serves as a H1 chaperone which deposit H1 onto nucleosome arrays	(Finn et al., 2008; Wang et al., 2008)
	DFF40	EMSA; affinity purification	Activation of chromatin fragmentation for apoptosis	(Liu et al., 1999; Widlak et al., 2005)

Modified from (McBryant et al., 2010)

Table 3. Post-translational modifications on linker histones

Modification	Variant	Site	Function	Note	Reference
Acetylation	H1.2	K62	ND		(Garcia et al., 2004)
	H1.3	K62	ND		
	H1.4	K26	ND		
	H1.4	K62, K64, K85, K97	ND		(Wisniewski et al., 2007)
Methylation	H1.4	K26	TS Repression		(Kuzmichev et al., 2004)
		K33	ND		Chu, et al, unpublished data
Phosphorylation	H1.2	T31, S36, S174	ND	Non-cdk motif	(Sarg et al., 2006)
		T146, T154	ND	Cdk motif	
	H1.3	S37, S189, T180	ND	Non-cdk motif	
		T147, T155	ND	Cdk motif	
	H1.4	T18, S172, S187	ND	Non-cdk motif	
		S27	Mitosis	AuroraB	(Hergeth et al., 2011)
		S35	Mitosis	PKA	(Chu et al., 2011)
	H1.5	T11	Mitosis	Non-cdk motif	(Sarg et al., 2006)
		S18, T138, T155, S173, S189	Mitosis	Cdk motif	
		S17	ND	G1 phase	(Talasz et al., 2009)
		T10, S172	ND	Mitosis	
	H1t	S140, S186	ND	Non-cdk motif	(Sarg et al., 2009)
		T155, S177	ND	Cdk motif	
	H1.1	T152	TS		(O'Brien et al., 2010)

ND, not determined, TS, transcription; M, mitosis

Table 4. Phosphorylations on core histones

Histone	Site	Kinase	Phosphatase	Function	Reference
H3	T3	Haspin	PP1	Mitosis	(Dai et al., 2005; Qian et al., 2011)
	T6	DLK, Chk1	ND	TS	(Metzger et al., 2010)
	S10	MSK1, MSK2, IKK-α, PIM1, Aurora B	PP1	TS, mitosis	(Crosio et al., 2002; Drobic et al., 2010; Hendzel et al., 1997; Yamamoto et al., 2003; Zippo et al., 2007)
	T11	PKCβ1 Cdk1	PP1γ	TS, mitosis	(Houben et al., 2005; Metzger et al., 2008; Shimada et al., 2010; Shimada et al., 2008)
	S28	MSK1, MSK2, Aurora B	PP1	TS, mitosis	(Drobic et al., 2010; Goto et al., 1999)
	Y41	JAK2	ND	TS	(Dawson et al., 2009)
	T45	PKCδ	ND	Apoptosis, DNA repair,	(Baker et al., 2010; Hurd et al., 2009)
	T118		ND	Chromatin remodelling	(North et al., 2011)
H4	S1	CK2	ND	TS repression, DNA repair, Mitosis	(Barber et al., 2004; Cheung et al., 2005; Govin et al., 2010)
	H18	His H4 kinase	ND	ND	(Attwood et al., 2010)
	S47	PAK2	ND	Replication	(Kang et al., 2011)
H2A	H2A.1 S1	Aurora B, RSK2, MSK1	ND	Mitosis, TS repression	(Barber et al., 2004; Zhang et al., 2004)
	H2A.X S16	RSK2	ND	Cell transformation	(Zhu et al., 2011)
	H2A.X S139	ATM, ATR, DNA-PK RSK2	PP1, PP2A, PP4, Wip1	DNA repair, Mitosis, apoptosis	(McManus and Hendzel, 2005; Sedelnikova et al., 2003; Zhu et al., 2011)
	H2A.X Y142	WSTF	EYA1/3	DNA repair	(Cook et al., 2009; Xiao et al., 2009)
	MacroH2A T128	ND	ND	Chromatin condensation	(Chu et al., 2006)
	MacroH2A S137	Cdks (in vitro)	ND	Chromatin condensation, Senescence	(Bernstein et al., 2008; Zhang et al., 2005)
H2B	S14	Mst1, PKCδ	ND	Apoptosis, DNA repair	(Ajiro et al., 2010; Mecklenbrauker et al., 2004)
	S32	RSK2	ND	Cell transfromation	(Lau et al., 2011)

ND, not determined, TS, transcription Modified from (Perez-Cadahia et al., 2010)

Table 5. Published PKA substrates

Substrates	Accession No.	Sites	Reference
5-Lipoxygenase	P09917	Ser523	(Luo et al., 2004)
Bad (Mouse only)	Q61337	Ser155	(Tan et al., 2000)
CREB	P16220	Ser133	(Montminy and Bilezikjian, 1987)
Engrailed-2	NP_001418	Ser276	(Hjerrild et al., 2004)
ER81	P50549	Ser191, Ser216, Ser334	(Wu and Janknecht, 2002)
GPRK1	Q15835	Ser21	(Horner et al., 2005)
GPRK7	Q8WTQ7	Ser23, Ser36	(Horner et al., 2005)
H1.4 (Linker Histone H1.4)	NP_005312	Ser35	(Chu et al., 2011)
hIK1	NP_002241	Ser312, Thr327, Ser 332, and Thr348	(Gerlach et al., 2000)
HMG-14	P05114	Ser6	(Prymakowska-Bosak et al., 2001)
IKCa1	NP_002241	Ser 312, Thr 327, Ser 332, Thr 348	(Gerlach et al., 2000)
L-type Ca2+ channel (C)	Q01815	Ser 1928	(Davare et al., 2000)
Lactase-phlorizin hydrolase preproprotein (LPH)	NP_002290	Ser 1916	(Keller et al., 1995)
Moesin-ezrin-radizin-like protein (MERLIN)	NP_861969	Ser 518	(Alfthan et al., 2004)
Myosin-binding protein C,	Q14896	Ser 265, Ser 300, Thr 274	(Mohamed et al., 1998)
MYPT1	O14974	Ser695	(Wooldridge et al., 2004)
NDRG1 (RTP)	Q92597		(Agarwala et al., 2000)
NF-kappaB p65 subunit (RelA)	Q04206	Ser276	(Zhong et al., 1997)
NIPP-1 (Nuclear inhibitor of protein phosphatase1)	Q12972	Ser178, Ser199	(Vulsteke et al., 1997)
Phosducin	P20941	Ser73	(Reig et al., 1990)
PP Inhibitor 1	Q13522	Thr35	(Endo et al., 1996)
PP1 3A	Q00756	Ser48, Ser67 in rabbit	(Hubbard and Cohen, 1989)
Rap-1 small GTP binding protein	P62834	Ser180	(Lerosey et al., 1991)
RhoA	NP_058082	Ser 188	(Dong et al., 1998)
RIM1 alpha	Q9JIR4 (rat)	Ser413, Ser1600	(Lonart et al., 2003)
Tau	AAC04279	Ser262, Ser320, Ser324, Ser356	(Schneider et al., 1999)
Transition Protein 2 (TP2)	NP_058753 (rat)	Thr101, Ser109	(Meetei et al., 2002)
VASP (vasodilator-stimulated phosphoprotein)	NP_003361	Ser 157, Ser 239, Thr 278	(Li et al., 2003)
Vimentin	P08670	Ser39, Ser51	(Tsujimura et al., 1994)
ZNF239 / MOK-2	Q16600	Ser46	(Harper et al., 2009)

Modified from http://www.kinasource.co.uk

Table 6. PTMs on H1 isotypes identified by LTQ-FT/MS/MS in HeLa cells

Isotypes	Sites	PTMs	Non-treated	Nocodazole-treated
H1.2	S1	P	+	
	K16	Me1	+	+
		Me2	+	
	K20	Me2	+	
	K33	Me1	+	
		Me2	+	
		Ac	+	
	K45	Me2	+	
		Ac	+	
	K62	Ac	+	
	K74	Me1	+	+
		Me2	+	+
	K89	Me2	+	
	S101	P		+
	K105	Me2	+	
	K109	Me3	+	+
	K116	Me1	+	
		Me2	+	
	T145	P		+
	K148	Me2	+	+
	K190	Ac	+	
H1.3	K75	Me2	+	+
H1.4	K16	Me1		+
		Ubiq	+	+
	T17	P		+
	K20/21	Ubiq	+	
	K20/21	Me2	+	+
	S35	P		+
	S40	P		+
	K33	Me1/2	+	+
	K45	Me3	+	+
	K62	Me1		+
		Me3		+
	K63	Ac		+
	K74	Me1/2	+	+
	R78	Me1		+
	S101	P		+
	K109	Me3	+	+
	K138	Ac		+
	T145	P		+
	K167	Ac	+	+
	S186	P	+	+
H1.5	K33	Ac	+	

P, phosphorylation; Me1, monomethylation; Me2, dimethylation;
Ac, acetylation; Ubiq, Ubiquitination

Table 7. List of candidate kinases for H1S35 phosphorylation during mitosis.

Categories	Families	Members	Inhibitors
Mitotic kinases	The Cdk family	Cdk1 (Cdc2)	Roscovitine
	The Polo family	Plk1	Wortmannin
	The Aurora family	Aurora-A	ZM447439 (1000 nM)
		Aurora-B	ZM447439 (50 nM)
		Aurora-C	ZM447439 (250 nM)
	The NIMA family	Nek2	N/A
	Mitotic check point	Bub1	N/A.
		Bub1B	N/A.
		TTK	N/A
Motif-orientated candidates	The PKA family	PKA	H89
	The CAMKII family	CAMKII	KN-62

N/A: not available

Table 8. List of primers for cloning

Experiment	Gene	Primer	Sequence (5' - 3')	Target vector
Mammalian expression	Aurora A	Forward	ATAGGATCCATGGACCGATCTAAAGAAAA	pcR3.1-FLAG
		Reverse	ATAGGATCCAGACTGTTTGCTAGCTGATT	
	Aurora B	Forward	ATAGGATCCATGGCCCAGAAGGAGAACTC	pcR3.1-FLAG
		Reverse	ATAGGATCCGGCGACAGATTGAAGGGCAG	
	Aurora C	Forward	ATAGGATCCATGAGCTCCCCCAGAGCTGT	pcR3.1-FLAG
		Reverse	ATAGGATCCGGAAGCCATCTGAGCACAGG	
	CAMKIIβ	Forward	ATAGGATCCATGGCCACCACGGTGACCTGCA	pcR3.1-FLAG
		Reverse	ATAGGATCCCTGCAGCGGGGCCACAGGCG	
	CAMKIIδ	Forward	ATAGGATCCATGGCTTCGACCACAACCTGCA	pcR3.1-FLAG
		Reverse	ATAGGATCCGATGTTTTGCCACAAAGAGG	
	CAMKIIγ	Forward	ATAGGATCCATGGCCACCACCGCCACC	pcR3.1-FLAG
		Reverse	ATAGGATCCCTGCAGCGGTGCGGCAGG	
	CDC2	Forward	ATAGGATCCATGGAAGATTATACCAAAATAGAGA	pcR3.1-FLAG
		Reverse	ATAGGATCCCATCTTCTTAATCTGATTGTCCAAA	
	PKAα	Forward	ATAGGATCCATGGGCAACGCCGCCGCC	pcR3.1-FLAG
		Reverse	ATAGGATCCGGCAAGGAGTTTTCTGAGTTT	
	PKAβ	Forward	ATAGGATCCATGGCAGCTTATAGAGAA	pcR3.1-FLAG
		Reverse	ATAGGATCCAAATTCACCAAATTCTTT	
	PRKAR1α	Forward	ATAGGATCCATGGAGTCTGGCAGTACC	pcR3.1-FLAG
		Reverse	ATAGGATCCGACAGACAGTGACACAAA	
	PLK1	Forward	ATAGAATTCATGAGTGCTGCAGTGACTGC	pcR3.1-FLAG
		Reverse	ATAGAATTCGGAGGCCTTGAGACGGTTGC	
	NEK	Forward	ATAGGATCCATGCCTTCCCGGGCTGAGGACTAT	pcR3.1-FLAG
		Reverse	ATAGGATCCGCGCATGCCCAGGATCTGTCT	
	H1.4	Forward	AGATCTATGTCCGAGACTGCGCCTGCC	pcDNA3.1-V5 HIS
		Reverse	GGATCCCTTTTTCTTGGCTGCCGC	
	H1.4	Forward	CG GGATCC A TCCGAGACTGCGCCTGCC	pcDNA3-HA
		Reverse	G GAATTC CTACTTTTTCTTGGCTGCCG	
Bacterial expression	H1.4	Forward	CACCATGTCCGAGACTGCGCCTGCCGC	pET100
		Reverse	GGATCCTACTTTTTCTTGGCTGCCGC	
Site-directed mutagenesis	H1.4S35A	Forward	AGCGCAAAGCGGCTGGGCCCCCG	
		Reverse	CGGGGGCCCAGCCGCTTTGCGCT	
	H1.4S35E	Forward	GCCAAGCGCAAAGCGGAGGGGCCCCCGGTGTCC	
		Reverse	GGACACCGGGGGCCCCTCCGCTTTGCGCTTGGC	
	H1.4K33R	Forward	GTGCGGCCAAGCGCCGCGCGTCTGGGCCCCC	
		Reverse	GGGGGCCCAGACGCGCGGCGCTTGGCCGCAC	
	PRKAR1α-DN-G602A	Forward	GGGAAGGAGGGAGCTTTGAAGAACTTGCTTTGATTTA	
		Reverse	TAAATCAAAGCAAGTTCTTCAAAGCTCCCTCCTTCC	
	PRKAR1α-DN-G974A	Forward	GGGGCCTTCTGATTATTTTGATGAAATTGCACTACTGATGA	
		Reverse	TCATCAGTAGTGCAATTTCATCAAAATAATCAGAAGGCCCC	
	PRKAR1α-DN-G988A	Forward	GCACTACTGATGAATCATCCTCGTGCTGCCAC	
		Reverse	GTGGCAGCACGAGGATGATTCATCAGTAGTGC	

Table 9. List of primers for RT-qPCR

Gene	Direction	Sequence (5’ - 3’)
H1.2	Forward	AACAAGAAGGCAGCCTC
	Reverse	CTTCGCTTTCTTCGGTG
H1.3	Forward	TGGCTCCTTCAAACTCAACA
	Reverse	GCTTCTTTACCTTCTTAGGAGTC
H1.4	Forward	GAACAACAGCCGCATCA
	Reverse	CCTTCTTGGGCTTCTTCG
PKAα	Forward	TTCTCACACCTACGGCG
	Reverse	TAGCCCTGCTGGTCAAT
PKAβ	Forward	GATAGTGCTAACATTCGAGTACC
	Reverse	TACTCTGGAGTTCCACATAATGTC

FIGURES

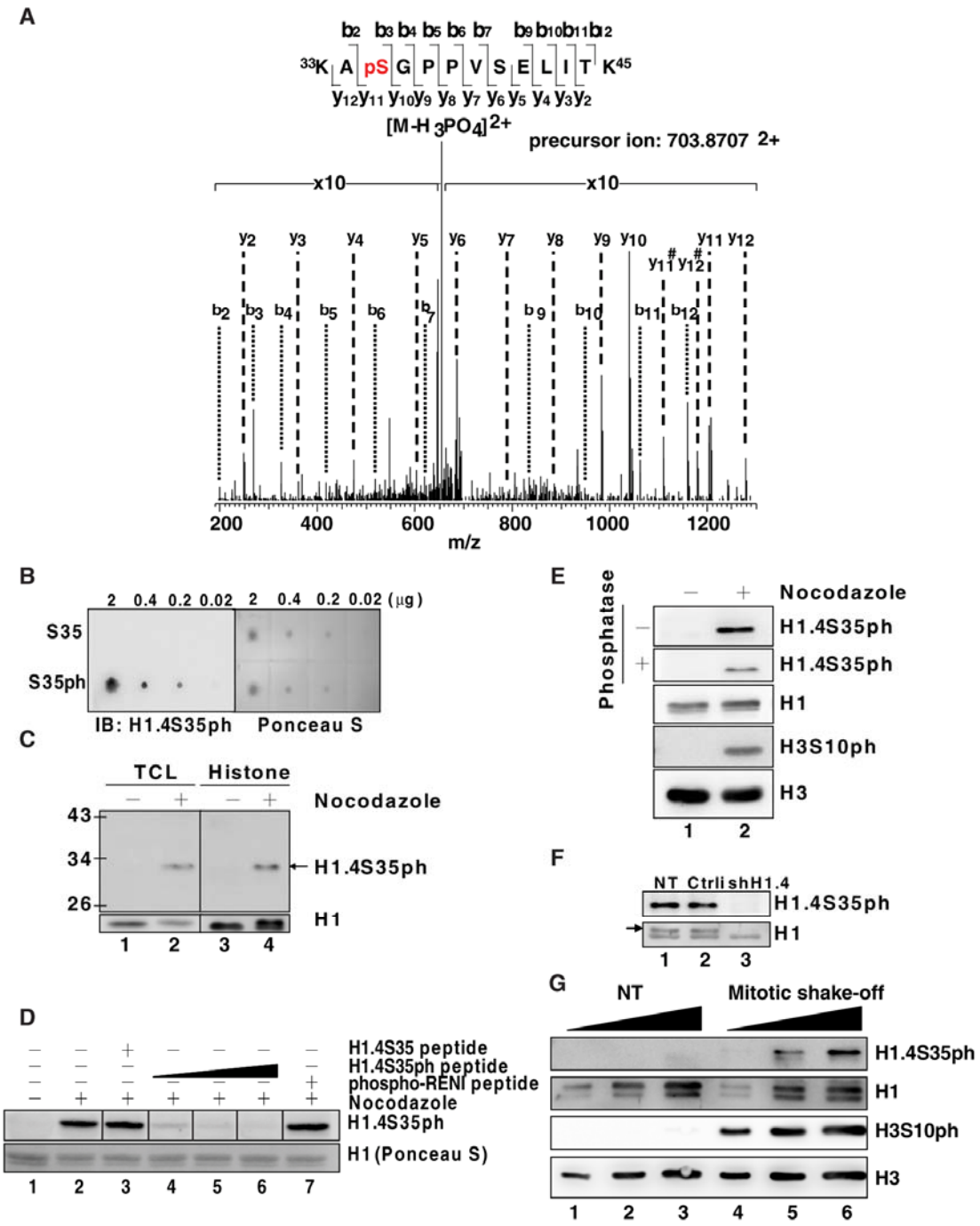

A
B
C
D
E
F
G
Nocodazole
H1.4S35ph
H1
H3S10ph
H3
Phosphatase
NT
Mitotic shake-off
IB: H1.4S35ph
Ponceau S
TCL
Histone
H1.4S35 peptide
H1.4S35ph peptide
phospho-RENI peptide
H1 (Ponceau S)
precursor ion: 703.8707 2+
m/z

Figure 1. H1.4S35 phosphorylation accumulates at mitosis. (A) The spectra of 33KApSGPPVSELITK45 fragment by LTQ-FT MS/MS analysis. Histone extracts from nocodazole-treated HeLa cells were separated by 15% SDS-PAGE. H1-sized bands were excised and subjected to in-gel trypsin digestion. The digested fragments were applied to high accurate LTQ-FT MS/MS analysis. **(B)** H1.4S35ph Ab recognizes H1.4 peptide containing phosphorylated Ser-35. Indicated amounts of Ser-35-phosphorylated (S35ph) or non-phosphorylated (Ser-35) H1.4 peptides were dotted on nitrocellulose membrane, followed by incubation with H1.4S35ph Ab (left panel) or Ponceau S (right panel). **(C)** H1.4S35ph Ab recognizes the endogenous H1. Total cell lysates (TCL) or histone extracts from HeLa were subjected to western blotting using indicated Ab. **(D)** H1.4S35ph signals can be competed by H1.4 peptide with phosphorylated Ser-35. Histone extracts from HeLa cells treated with nocodazole for 16 h were subjected to western blotting using H1.4S35ph Ab. The Ab was pre-incubated with 5% nonfat milk only (lane 2), unmodified H1.4 (amino acids 28–42) peptide (lane 3), increasing amounts of Ser-35-phosphorylated H1.4 (amino acids 28–42) peptide (lanes 4–6), or an unrelated phosphorylated Rad60-Esc2p-Nip45 peptide (lane 7). **(E)** the H1.4S35ph signal is reduced by alkaline phosphatase. Histone extracts from mock-treated or nocodazole-treated HeLa cells were subjected to SDS-PAGE separation and transferred onto membrane. The membrane with or without alkaline phosphatase treatment was then incubated with indicated Ab. **(F)** H1.4S35ph Ab specifically recognizes H1.4. Histone extract from mock-treated (NT) HeLa cells with no shRNA treatment (lane 1) or with lentiviral transduction of scramble (Ctrli, lane 2) or shH1.4 (shH1.4, lane 3) shRNA were subjected to western blotting using indicated Abs. The arrow indicates H1.4, whereas the lower band indicates H1.2. **(G)** Mitotic cells collected from mitotic shake-off are enriched in H1.4S35ph. HeLa cells cultured in T75 flasks were shaken, and the detached cells were collected.

Histones were extracted, and western blotting was performed using Abs against the indicated proteins. Three different amounts of histone were loaded: 0.5 μg (lanes 1 and 4); 1 μg (lanes 2 and 5); and 2 μg (lanes 3 and 6). NT indicates cells without shaking.

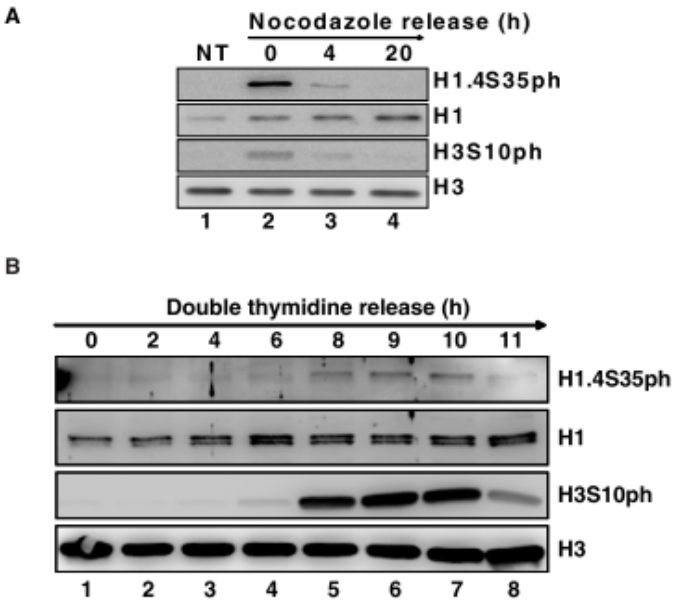


Figure 2. H1.4S35ph accumulation parallels to H3S10 phosphorylation and decreases at mitotic exit. Histones from HeLa cells released from nocodazole **(A)** or double thymidine block **(B)** for indicated time were subjected to western using indicated Abs. NT, mock-treated.

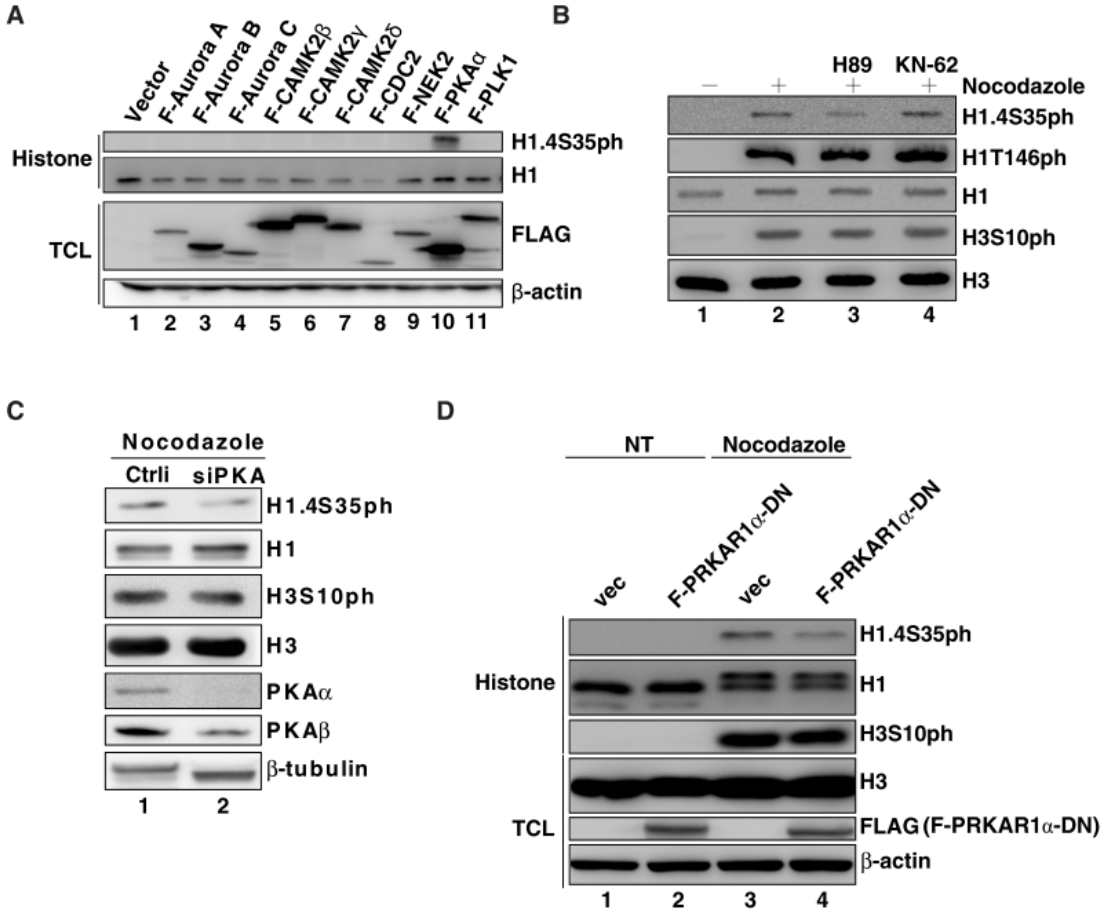

A
Vector
F-Aurora A
F-Aurora B
F-Aurora C
F-CAMK2β
F-CAMK2γ
F-CAMK2δ
F-CDC2
F-NEK2
F-PKAα
F-PLK1
Histone
H1.4S35ph
H1
TCL
FLAG
β-actin
1 2 3 4 5 6 7 8 9 10 11
B
H89
KN-62
Nocodazole
H1.4S35ph
H1T146ph
H1
H3S10ph
H3
1 2 3 4
C
Nocodazole
Ctrli
siPKA
H1.4S35ph
H1
H3S10ph
H3
PKAα
PKAβ
β-tubulin
1 2
D
NT
Nocodazole
vec
F-PRKAR1α-DN
vec
F-PRKAR1α-DN
Histone
H1.4S35ph
H1
H3S10ph
H3
TCL
FLAG (F-PRKAR1α-DN)
β-actin
1 2 3 4

Figure 3. PKA mediates the mitotic H1.4S35 phosphorylation *in vivo*. (A) PKA overexpression induces H1.4S35 phosphorylation. 293T cells were transfected with indicated kinases for 24 h and subjected to western blotting using indicated Ab. **(B)** PKA inhibition reduces H1.4S35 phosphorylation. HeLa cells incubated with nocodazole (400 ng/ml) for 16 h were treated with kinase inhibitor H89 or KN-62 for 2 h, followed by western blotting using Abs against the indicated proteins. **(C)** PKA knockdown down-regulates H1.4S35 phosphorylation. HeLa cells depleted with PKAα and PKAβ by siRNA for 2 days were incubated with nocodazole for 16 h and subjected to western blotting. **(D)** Expression of the dominant-negative PKA regulatory subunit inhibits H1.4S35 phosphorylation. HeLa cells with or without expressing the dominant-negative PKA regulatory subunit (F-PRKAR1α-DN) were incubated with nocodazole for 16 h and subjected to western blotting using Abs against the indicated proteins. vec, vector; NT, mock-treated; TCL, total cell lysate.

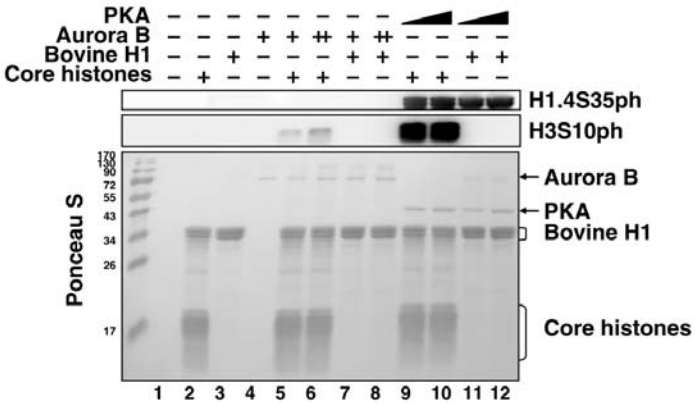


Figure 4. PKA phosphorylates H1.4 at S35 *in vitro*. *In vitro* kinase assays were performed with indicated recombinant proteins in the presence of bovine H1 or bovine core histones followed by western using Abs against the indicated proteins. Ponceau S staining is presented as the loading control.

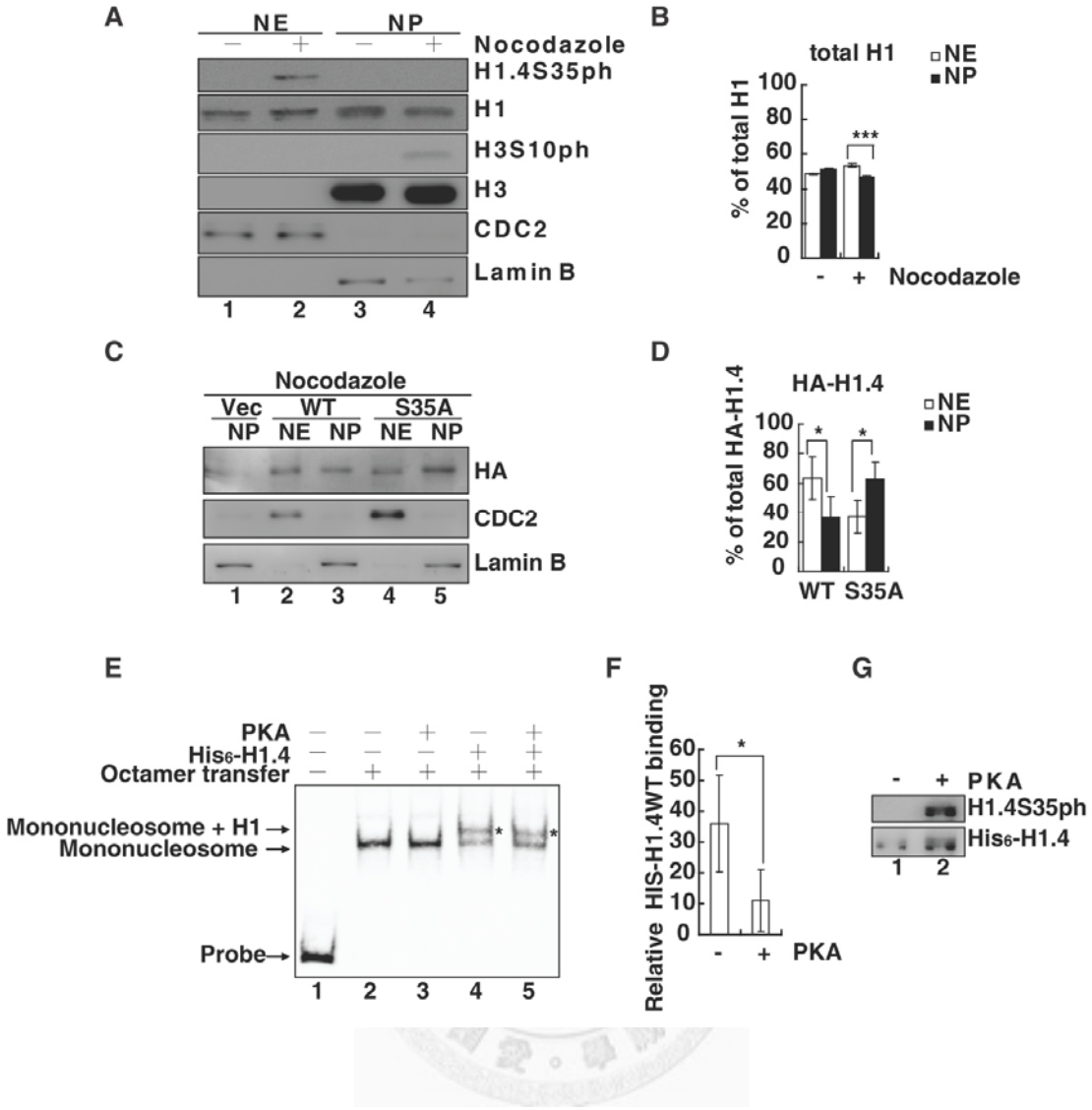

A
NE
NP
Nocodazole
H1.4S35ph
H1
H3S10ph
H3
CDC2
Lamin B
1 2 3 4
B
total H1
NE
NP
% of total H1
***
Nocodazole
C
Nocodazole
Vec
WT
S35A
NP NE NP NE NP
HA
CDC2
Lamin B
1 2 3 4 5
D
HA-H1.4
% of total HA-H1.4
WT S35A
E
PKA
His6-H1.4
Octamer transfer
Mononucleosome + H1
Mononucleosome
Probe
1 2 3 4 5
F
Relative HIS-H1.4WT binding
PKA
G
PKA
H1.4S35ph
His6-H1.4
1 2

Figure 5. S35-phosphorylation decreases DNA binding affinity of H1.4. (A) S35-phosphorylated H1.4 binds less to chromatin. Nuclear extracts (NE) and pellets (NP) from HeLa cells with or without nocodazole treatment were subjected to western. **(C)** Serine 35 is required for dissociation of H1.4 from mitotic chromatin. Similar experiments to (A) were applied except that HeLa cells overexpressing wild type H1.4 or the substitution mutant H1.4S35A were used. Quantification of total H1 for (A) or HA-H1.4 for (C) by Multi Gauge V3.0 was shown in **(B)** and **(D)**, respectively. **(E)** H1.4 phosphorylation by PKA reduces H1.4 binding to nucleosomes. EMSA assays were performed with DNA probe alone (lane 1) or in the presence of indicated proteins (lanes 2 to 5). **(F)** The H1-shifted bands (indicated by an asterisk) in lanes 4 and 5 in (E) were quantified by ImageJ software. Error bars represent standard deviations from 3 independent experiments. *$p < 0.05$. **(G)** Western control of *in vitro* kinase assays of lanes 4 and 5 in (E).

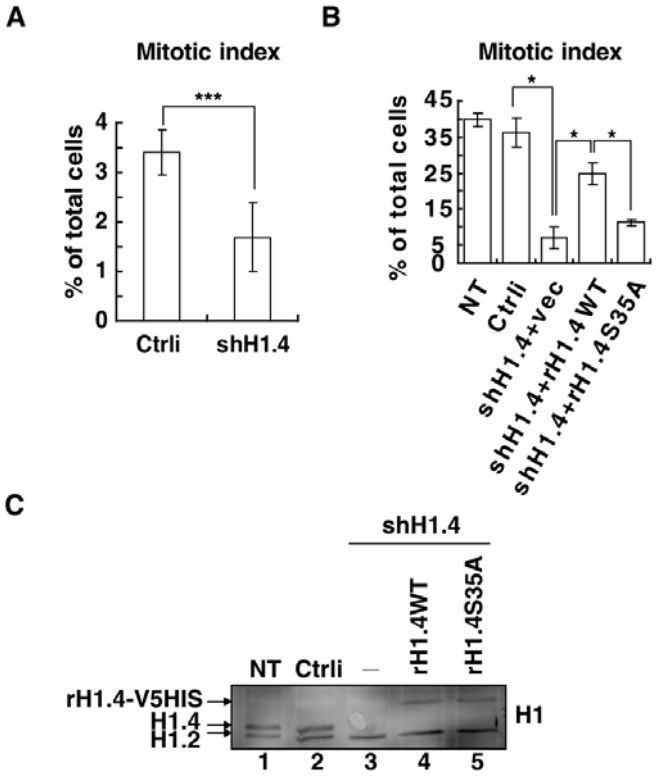


Figure 6. H1.4S35A substitution mutant fails to rescue the mitotic defect following H1.4 depletion. (A) H1.4 depletion decreases mitotic population in unsynchronized HeLa cells. **(B)** H1.4S35A substitution mutant fails to rescue the mitotic defect following H1.4 depletion. HeLa cells were mock-treated (NT) or stably transduced with scramble shRNA (Ctrli) or shRNA against H1.4 (shH1.4). Cells with shH1.4 were then transfected with pcDNA3.1/V5-HIS vector encoding wild type H1.4 (shH1.4+H1.4WT) or H1.4S35A (shH1.4+H1.4S35A). All cells were synchronized by double thymidine treatment and released for 9 h, followed by fixation with formaldehyde and DAPI staining. Cells at interphase or mitosis were counted based on the pattern of DAPI staining. The number of cells counted in each group is NT: 306, Ctrli: 421, shH1.4+vec: 548, shH1.4+H1.4WT: 410, and shH1.4+H1.4S35A: 404. **(C)** Western control of the ectopically expressed H1.4WT and H1.4S35A. Error bars in (A) and (B) represent standard deviations from at least 3 independent experiments. *p < 0.05; ***p < 0.001. NT, mock-treated.

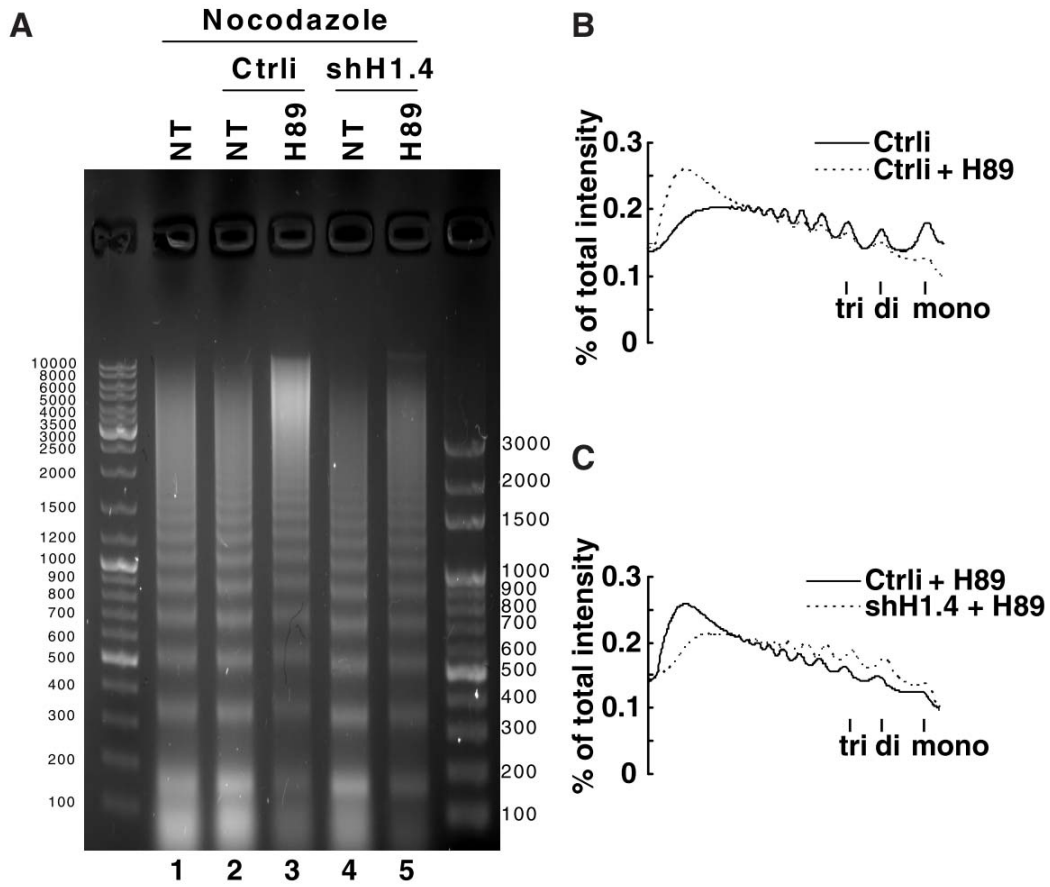


Figure 7. PKA inhibitor H89 induces the mitotic chromatin compaction depending on H1.4. (A) HeLa cells stably transduced with lentivirus encoding scramble shRNA (Ctrli) or shRNA against H1.4 (shH1.4) were treated with dimethyl sulfoxide (NT) or PKA inhibitor H89 for 2 h after 14 h of nocodazole treatment. Nuclei were then isolated and subjected to micrococcal nuclease sensitivity assay. The intensity of MNase-digested nucleosomal DNA fragments was quantified by Metamorph software as a percentage of total intensity of each lane **(B and C)**. Mono, di, tri indicate mononucleosome, dinucleosome, and trinucleosome.

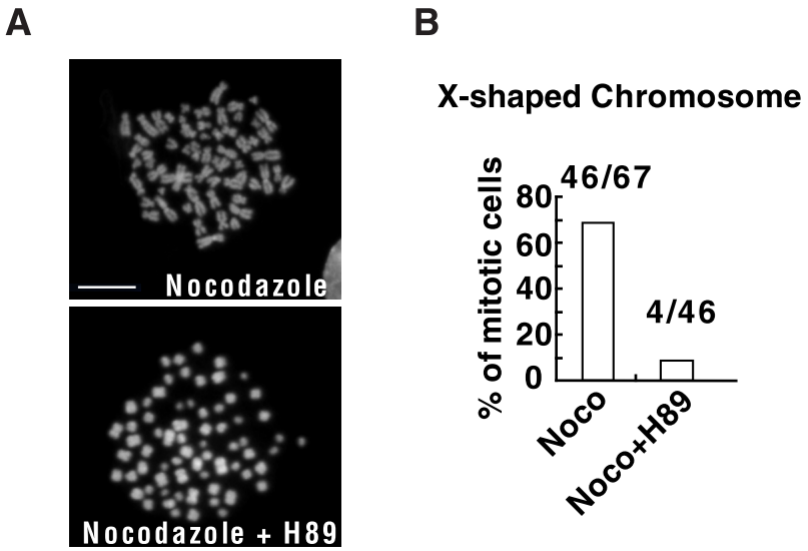


Figure 8. PKA inhibitor H89 induces the mitotic chromosome condensation. (A) H89 induces chromosome condensation in nocodazole-treated cells. Metaphase spread from HeLa cells treated with or without H89 in the presence of nocodazole were counterstained with DAPI and observed by immunofluorescence microscopy. Scale bar, 20 μm. **(B)** Quantitative analysis of the frequency of X-shape formation in 7D. The number of cells observed in each group is “nocodazole” (noco): 67, “nocodazole + H89” (noco + H89): 46.

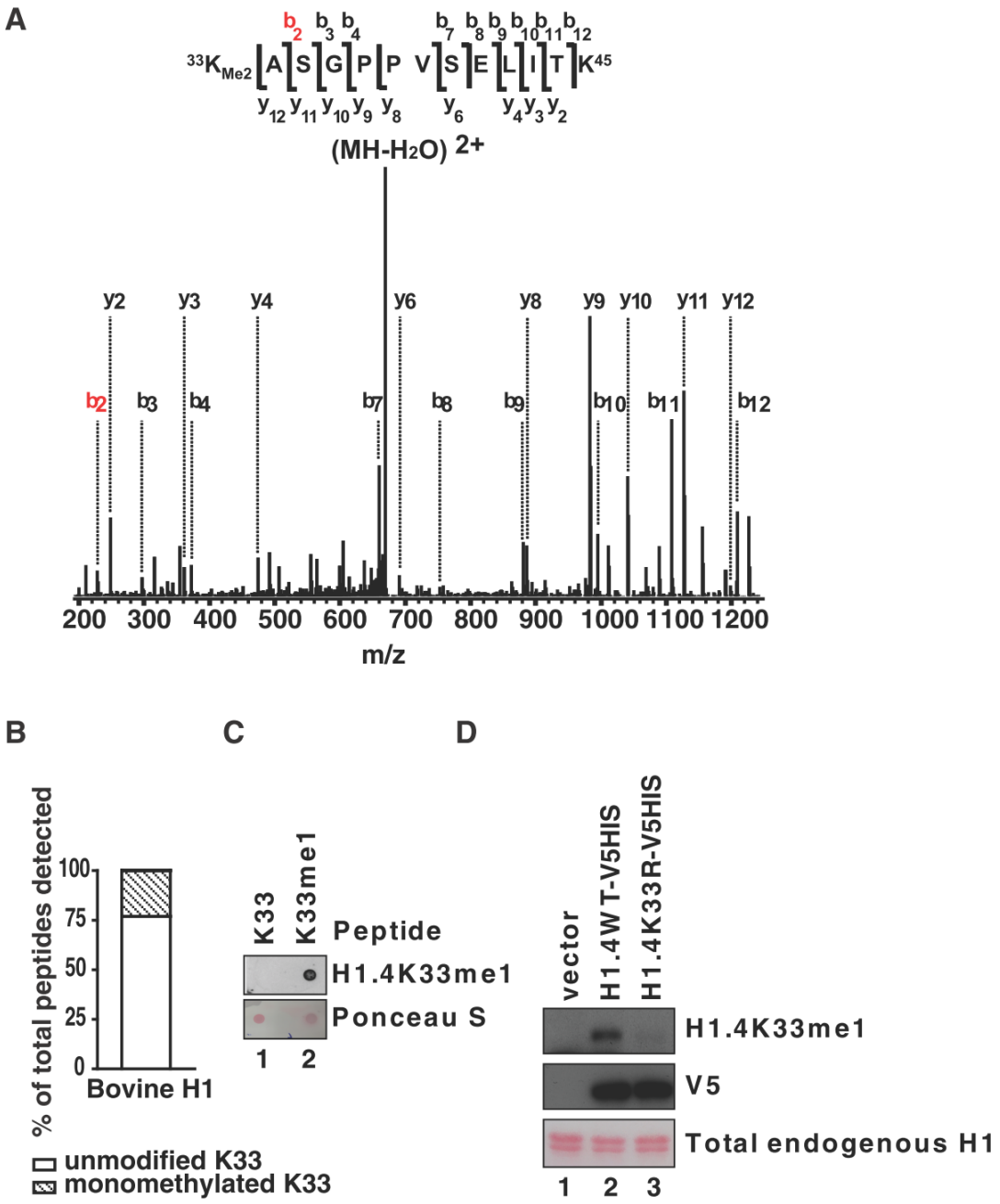

A
b2 b3 b4 b7 b8 b9 b10 b11 b12
33KMe2 A S G P P V S E L I T K45
y12 y11 y10 y9 y8 y6 y4 y3 y2
(MH-H2O) 2+
y2 y3 y4 y6 y8 y9 y10 y11 y12
b2 b3 b4 b7 b8 b9 b10 b11 b12
200 300 400 500 600 700 800 900 1000 1100 1200
m/z
B
% of total peptides detected
100
75
50
25
0
Bovine H1
unmodified K33
monomethylated K33
C
K33
K33me1
Peptide
H1.4K33me1
Ponceau S
1 2
D
vector
H1.4WT-V5HIS
H1.4K33R-V5HIS
H1.4K33me1
V5
Total endogenous H1
1 2 3

Figure 9. Lysine 33 on histone H1.4 is methylated. (A) A representative MS/MS spectrum of lysine 33 methylated H1.4 peptide on purified bovine histone H1 by LTQ-FT/MS/MS analysis. **(B)** High level of K33 methylation in bovine histone H1 by quantitative mass spectrometry analysis. **(C)** Dot blot analysis for H1.4K33me1 antibody shows good specificity towards H1.4K33me1 peptides (K33me1), but not unmodified H1.4 peptide (K33). **(D)** H1K33me1 antibody can not recognized H1.4K33R mutant in histone extract from 293T cells. Dot blot and western blot analysis were performed using indicated antibodies. Ponceau S stainings indicate loading control.

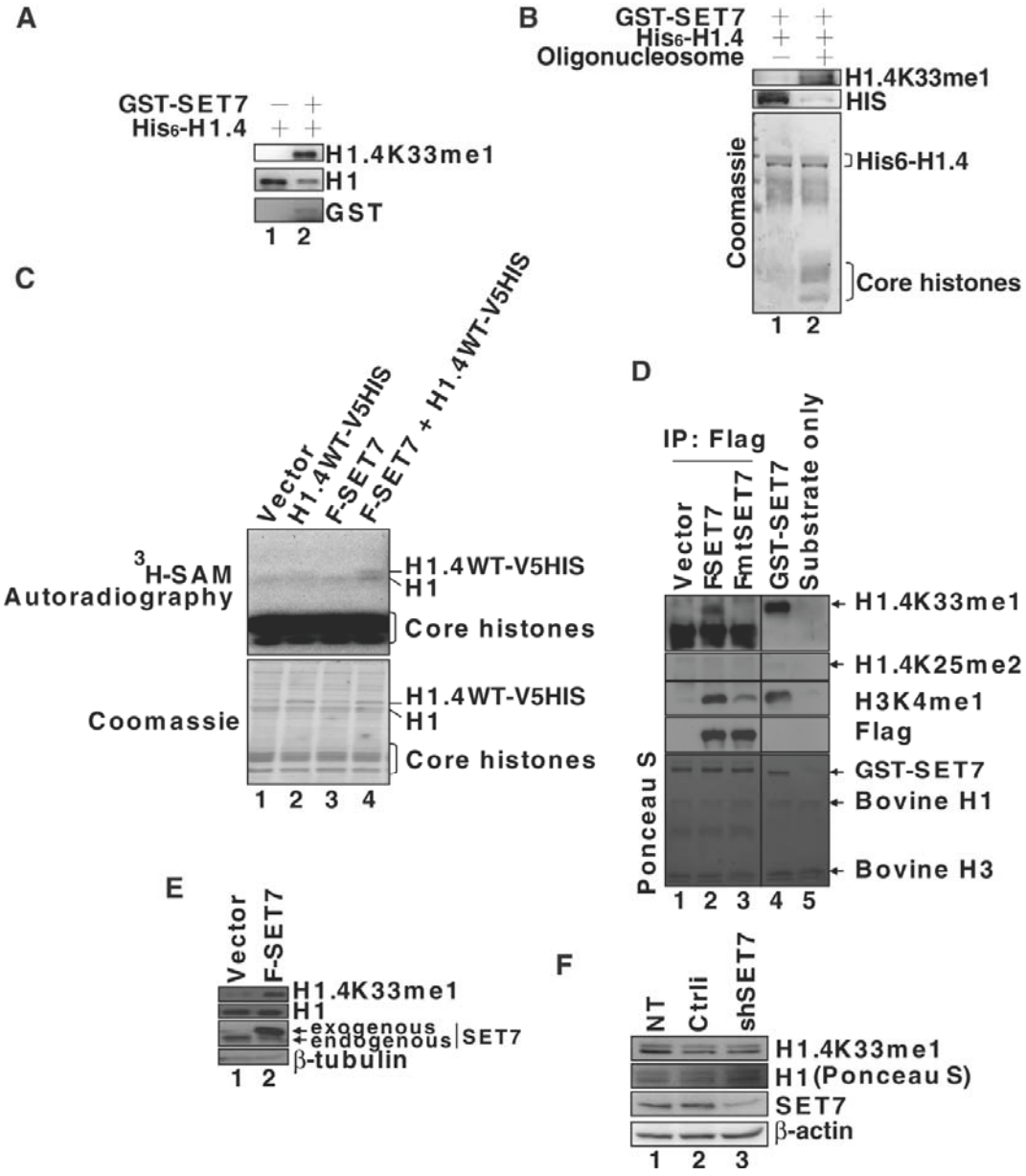

A
GST-SET7
His6-H1.4
H1.4K33me1
H1
GST
1 2
B
GST-SET7
His6-H1.4
Oligonucleosome
H1.4K33me1
HIS
Coomassie
His6-H1.4
Core histones
1 2
C
Vector
H1.4WT-V5HIS
F-SET7
F-SET7 + H1.4WT-V5HIS
3H-SAM
Autoradiography
H1.4WT-V5HIS
H1
Core histones
Coomassie
H1.4WT-V5HIS
H1
Core histones
1 2 3 4
D
IP: Flag
Vector
FSET7
FmtSET7
GST-SET7
Substrate only
H1.4K33me1
H1.4K25me2
H3K4me1
Flag
GST-SET7
Bovine H1
Bovine H3
Ponceau S
1 2 3 4 5
E
Vector
F-SET7
H1.4K33me1
H1
+exogenous
+endogenous
SET7
β-tubulin
1 2
F
NT
Ctrli
shSET7
H1.4K33me1
H1 (Ponceau S)
SET7
β-actin
1 2 3

Figure 10. SET7 directly methylates H1.4K33me1 *in vivo* and *in vitro.* (A) *In vitro* histone methylation assay using bacterial-expressed recombinant human H1.4 (His_6-H1.4) as substrates showed H1.4K33 can be methylated by recombinant SET7. **(B)** *In vitro* histone methylation assay using bacterial-expressed recombinant human H1.4 (His_6-H1.4) reconstituted on native oligonucleosomes from HeLa as substrates showed nucleosome-bound H1.4K33 can be a better substrate of recombinant SET7. **(C)** Overexpression of SET7 increases methylation level of H1 by in vivo labeling of ^{3}H-S-adenosyl-methionine. H1.4WT-V5HIS and/or Flag-SET7 (F-SET7) were overexpressed in H1299 cells in the presence of ^{3}H-S-adenosyl-methionine. The nuclei were isolated and separated by 15% SDS-PAGE. The gel was then dried and exposed to X-ray film. **(D)** SET7 immunoprecipitated from cell lysate methylates H1.4K33me1 and H3K4me1, but not H1.4K25me2. F-SET7 or F-mtSET7 was overexpressed in 293T cells and immunoprecipitated with anti-flag antibody. The immunoprecipitated proteins were applied to histone methylation assay. **(E)** Overexpression of SET7 increases H1K33me1 level. F-SET7 was overexpressed and histones were extracted for western blot. **(F)** Knockdown of SET7 only partially decreases H1.4K33me1 level. SET7 was knockdown by lentiviral-vector-based shRNA delivery. After 72 hours of transduction, histones were extracted for western blot. Coomassie blue and Ponceau S stainings indicate loading control.

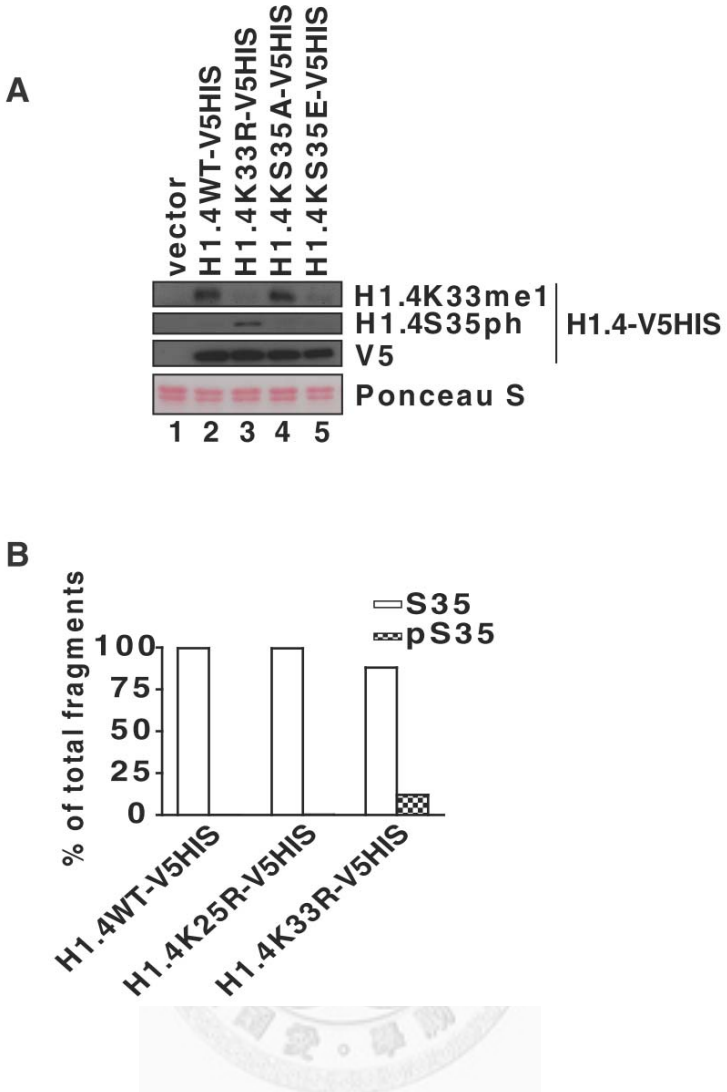
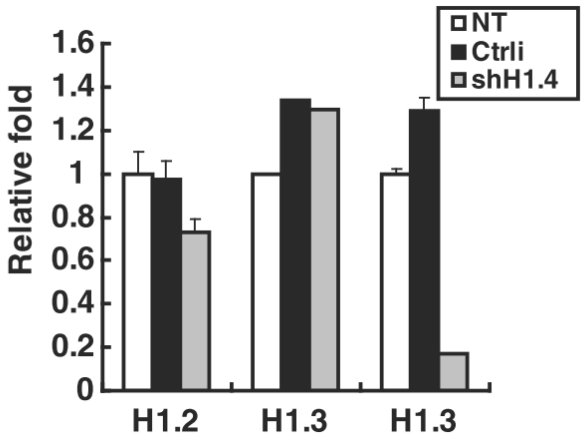
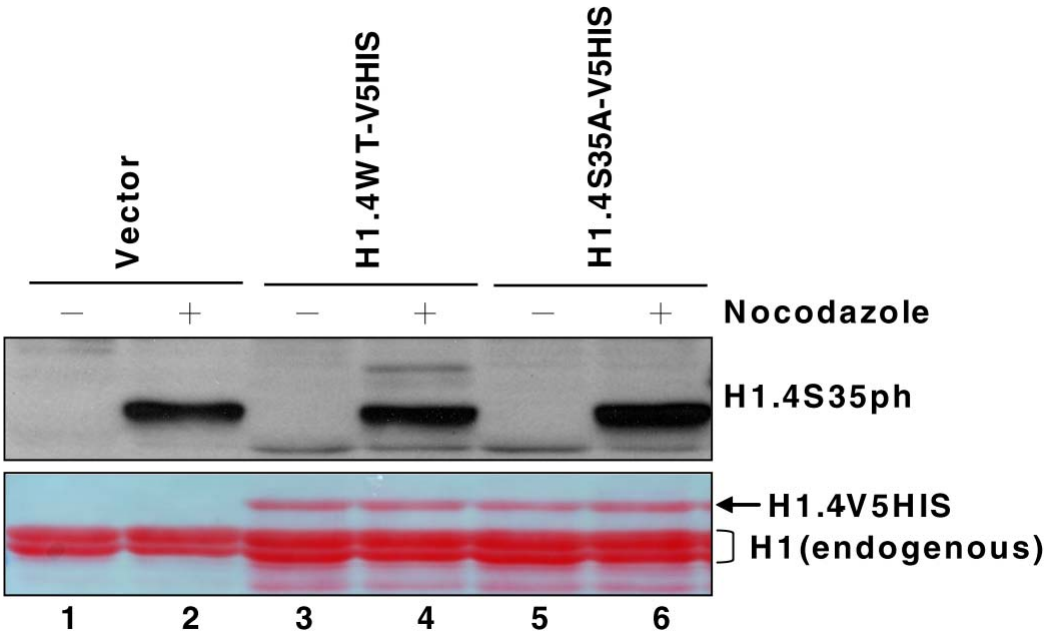


Figure 11. Loss of H1.4K33me1 enhances H1.4S35ph. (A) H1.4K33R mutant contains higher level of H1.4S35ph in unsynchronized condition. 293T cells were overexpressed with various H1.4 mutants including wild type H1.4 (H1.4WT-V5HIS), H1.4 with K33R substitution (H1.4K33R-V5HIS), H1.4 with S35A substitution (H1.4S35A-V5HIS), or H1.4 with S35E substitution (H1.4S35E-V5HIS) and histones were extracted for western blot. **(B)** H1.4K33R mutant accumulates more H1.4S35ph compared to wild-type and H1.4K25R mutant. 293T cells were overexpressed with H1.4WT-V5HIS, H1.4K25R-V5HIS, or H1.4K33R-V5HIS and histones were extracted for electrophoresis followed by trypsin in-gel digestion and LTQ-FT MS/MS analysis. Ponceau S staining indicates loading control.

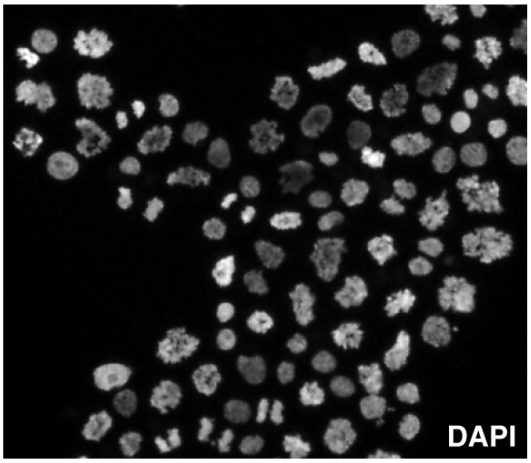
SUPPLEMENTARY FIGURES



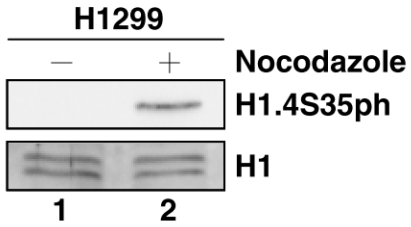
<u>Supplementary Figure 1.</u> Lentiviral-based knockdown specifically depletes H1.4 in HeLa cells. HeLa cells were transduced with lentiviral vector containing shRNA sequence against H1.4. RNA was extracted and cDNA was prepared by reverse transcription. Real-time PCR was performed and expression level was compared with β-actin.



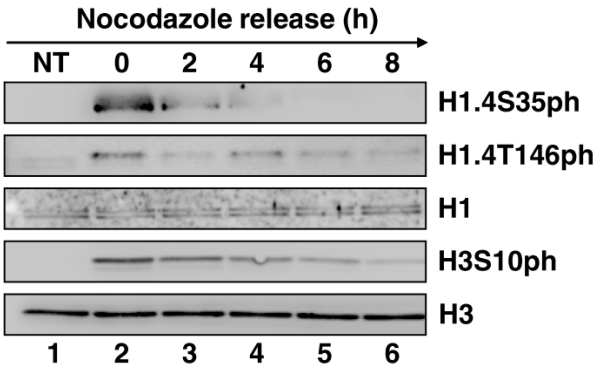
Supplementary Figure 2. Anti-H1.4S35ph antibody specifically recognizes phosphor-serine 35, but not other phosphorylation sites on H1.4. HeLa cells were transfected with empty vector (vector), H1.4WT (H1.4WT-V5HIS), or H1.4S35A (H1.4S35A-V5HIS) for 24 h and treated with nocodazole for further 16 h. Histones were extracted for western blot. Lower panel indicates Ponceau S staining as loading controls.



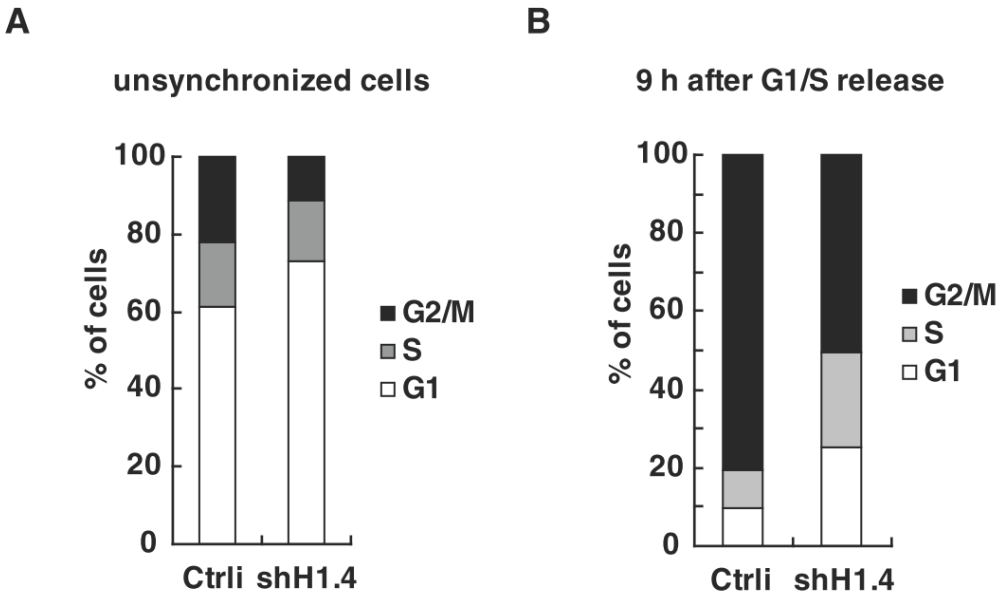
Supplementary Figure 3. Successful enrichment of mitotic cells from mitotic shake-off. HeLa cultured in T75 flasks were shaken and the detached cells were collected and centrifuged onto a glass slide by Cytospin. Immunofluorescence staining was performed using DAPI.



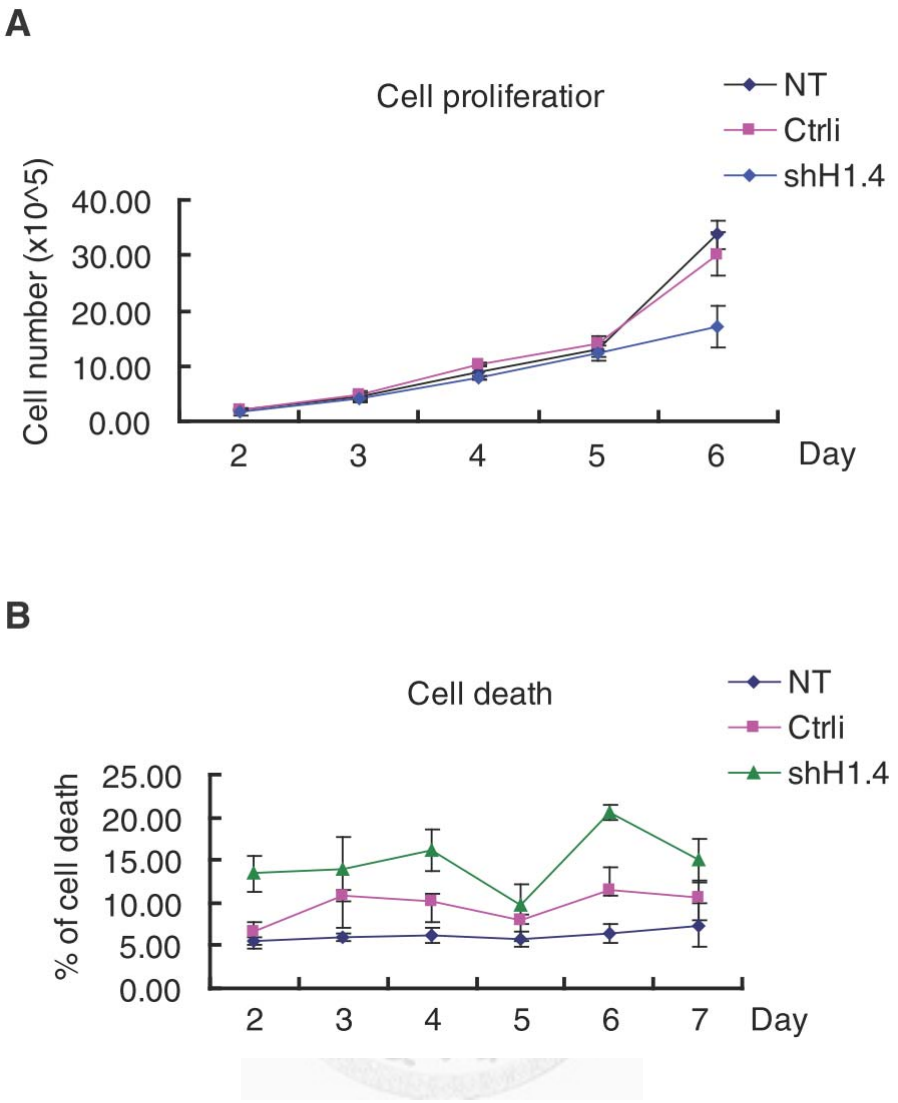
Supplementary Figure 4. Accumulation of H1.4S35ph in nocodazole-treated H1299 cells. H1299 cells were treated with nocodazole for 16 h, followed by histone extraction for western.



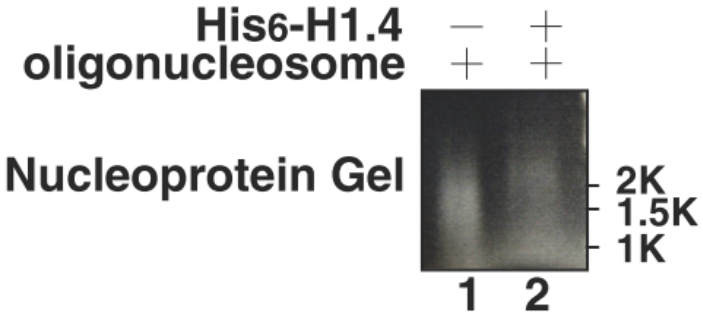
Supplementary Figure 5. Time course analysis of H1.4S35ph in cell cycle progression after release from nocodazole treatment. HeLa cells were treated with nocodazole for 16 h, released for indicated time, and followed by histone extraction for western using indicated antibodies.



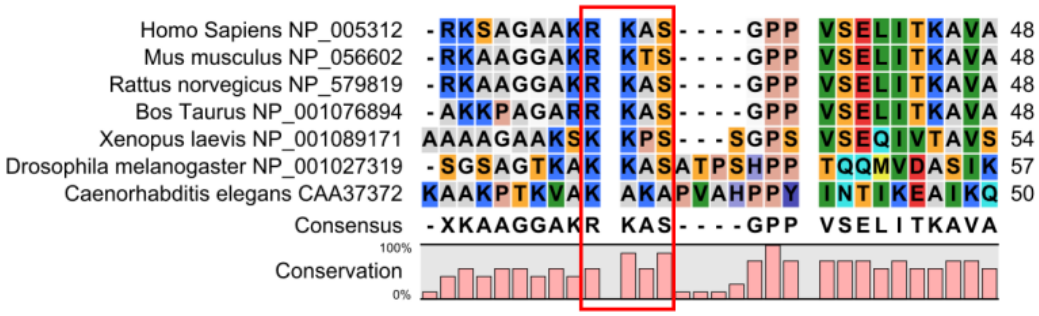
Supplementary Figure 6. Cell cycle analysis of H1.4 knockdown cells. Unsynchronized cells **(A)** or cells synchronized by double thymidine and released for 9 h **(B)** were stained by propidium iodide, followed by FACS analysis.



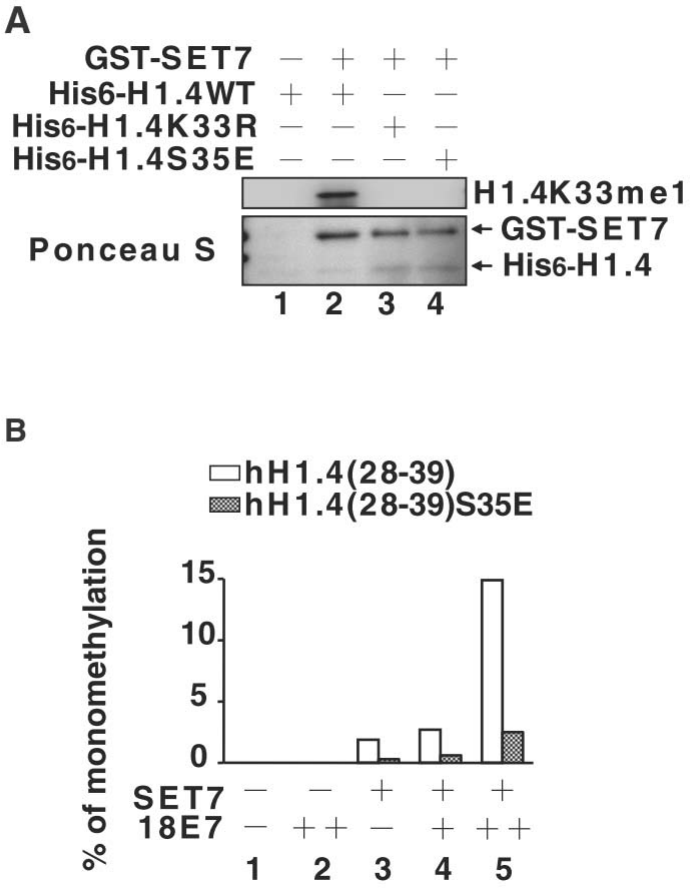
Supplementary Figure 7. Stable depletion of H1.4 results in growth delay and cell death. HeLa stably infected with shH1.4-lentivirus were enriched by GFP sorting using FACS. Proliferation rate **(A)** and cell death **(B)** at indicated days were measured by CASY TT cell analyzer (Innovatis)



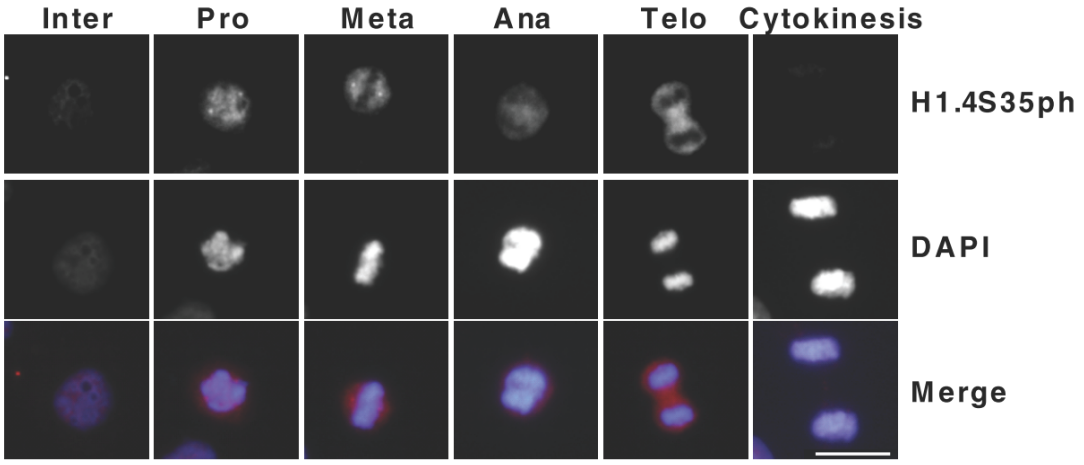
Supplementary Figure 8. H1.4 reconstitutes onto native oligonucleosomes. Recombinant H1.4 was incubated with native oligonucleosomes purified from HeLa nuclei using salt dilution method. The reconstituted H1.4-containing oligonucleosomes are subjected to electrophoresis using nucleoprotein gel prestained with Healthview SYBR DNA dye.



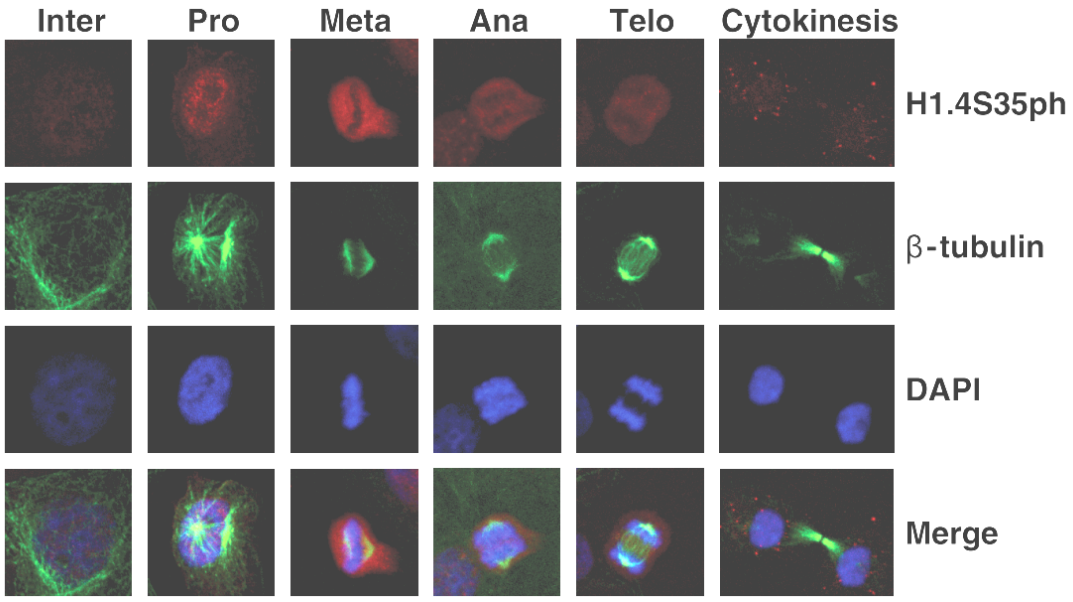
Supplementary Figure 9. Sequence alignment of the partial N-terminal region of H1.4 among species. The alignment was performed by CLC sequence viewer software within the region of human H1.4 (23-48). Red square indicates the PKA motif region with the consensus sequence “RRXS/T”.



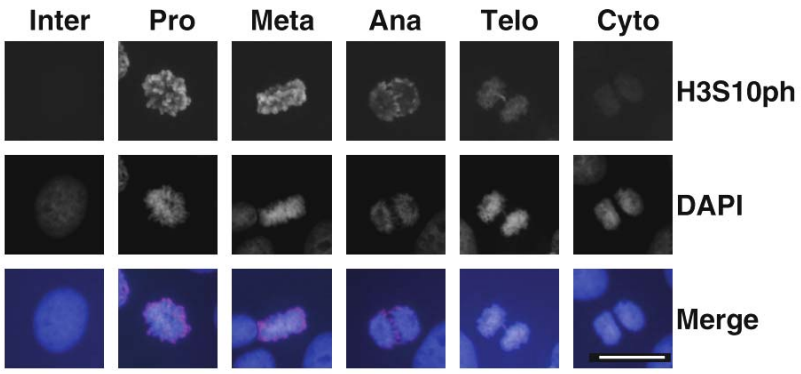
Supplementary Figure 10. H1.4S35ph impairs H1.4K33 methylation *in vitro*. (A) Recombinant His_6-H1.4S35E mutant protein mimicking phosphorylation is not a good substrate for SET7. *In vitro* histone methylation assay was performed using recombinant His_6-H1.4 proteins as substrates in the absence or presence of SET7. **(B)** S35E peptide is not a good substrate for SET7. *In vitro* histone methylation assay was performed using peptide containing a.a. 28 to 39 of H1.4 [H1.4(28-39)] or the same peptide with S35E substitution [H1.4(28-30)] as substrates in the absence or presence of SET7 and the viral oncoprotein E7 of the human pappilomavirus type 18. Ponceau S staining indicates loading control.



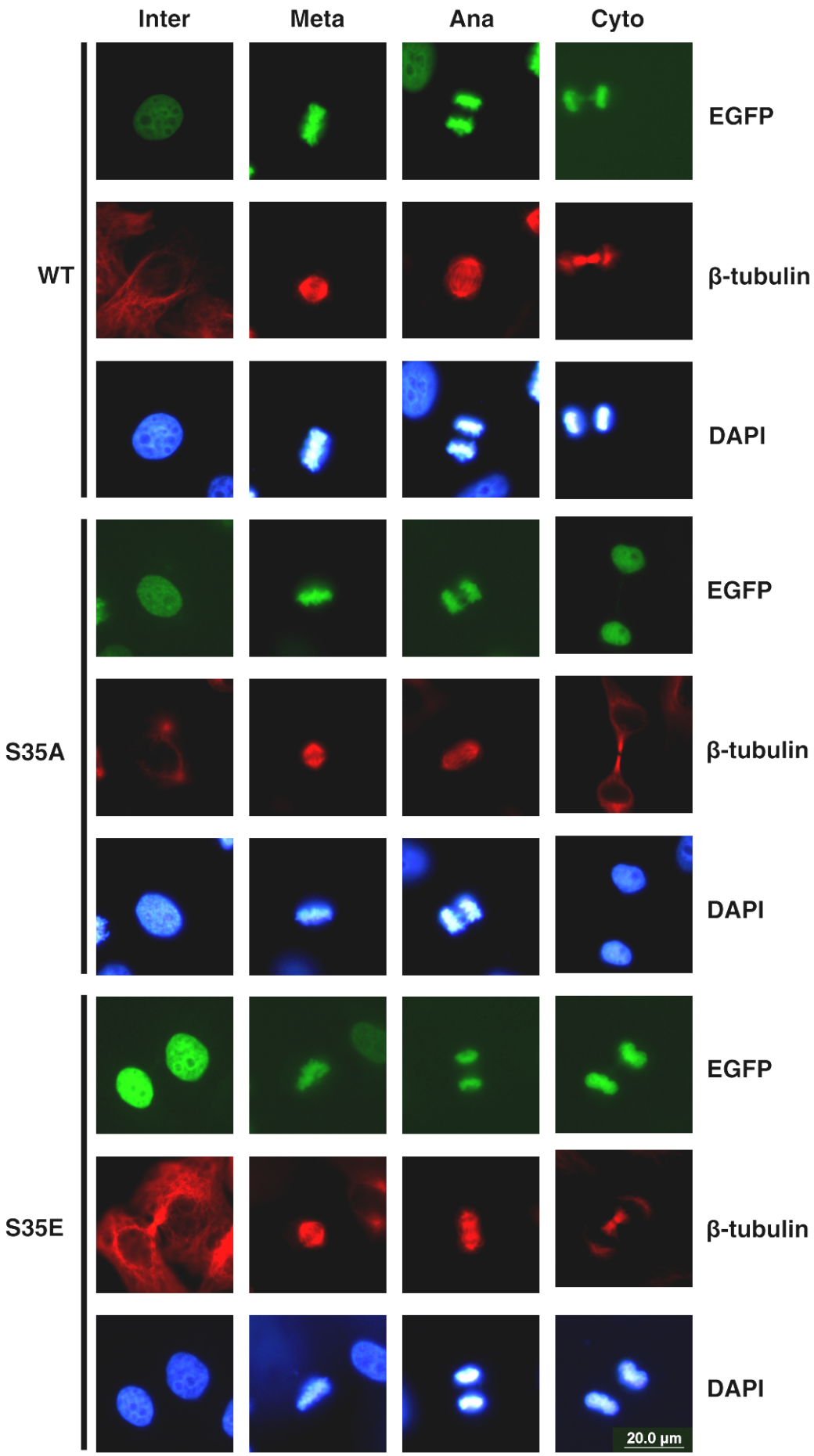
<u>Supplementary Figure 11.</u> Immunofluorescence microscopy showed exclusion of H1.4S35ph from mitotic chromatin. HeLa cells were subjected to immunofluorescence microscopy analysis using H1.4S35ph antibody (Red) and counterstained with DAPI (Blue). Merge represents 2-color overlay. The representative images of cells at different cell cycle stages were shown. Inter: interphase; Pro: prophase; Meta: metaphase; Ana: anaphase; Telo: telophase; Cyto: cytokinesis.



Supplementary Figure 12. Serial optical sections acquired from fluorescence confocal microscopy showed exclusion of H1.4S35ph from mitotic chromatin. HeLa cells were subjected to confocal immunofluorescence microscopy analysis using H1.4S35ph antibody (Red), β-tubulin antibody (Green), and counterstained with DAPI (Blue). Merge represents 3-color overlay. The representative images of cells at different cell cycle stages were shown. Inter: interphase; Pro: prophase; Meta: metaphase; Ana: anaphase; Telo: telophase; Cyto: cytokinesis.



Supplementary Figure 13. Analysis of intracellular localization of H3S10 phosphorylation. HeLa cells were subjected to immunofluorescence microscopy analysis using H3S10ph antibody (Red) and counterstained with DAPI (Blue). The representative images of cells at different cell cycle stages were shown. Scale bar, 20 m. Inter: interphase; Pro: prophase; Meta: metaphase; Ana: anaphase; Telo: telophase; Cyto: cytokinesis.


Inter
Meta
Ana
Cyto
WT
S35A
S35E
EGFP
β-tubulin
DAPI
EGFP
β-tubulin
DAPI
EGFP
β-tubulin
DAPI
20.0 µm

Supplementary Figure 14. Immunofluorescence microscopy of overexpressed EGFP-fused H1.4WT, H1.4S35A, or H1.4S35E mutant mainly colocalizes with mitotic chromatin. HeLa cells were subjected to immunofluorescence microscopy analysis using β-tubulin antibody (Red), counterstained with DAPI (Blue) and EGFP-H1.4 signals (Green). The representative images of cells at different cell cycle stages were shown. Inter: interphase; Meta: metaphase; Ana: anaphase; Cyto: cytokinesis.

LIST OF PUBLICATIONS

1. Chiang AN, Wu HL, Yeh HI, **Chu CS**, Lin HC, Lee WC, 2006, “Antioxidant effects of black rice extract through the induction of superoxide dismutase and catalase activities.” *LIPIDS,* 41 (8), 797-803 (SCI)

2. Ou DS, Lee SB*, **Chu CS***, Chang LH, Chung BC, Juan LJ, 2011, “Transcriptional activation of endoplasmic reticulum chaperone Glucose-regulated protein 78 (GRP78) by HCMV IE1-72 protein.” *CELL RESEARCH*, 21 (4), 642-653 (SCI) * Equally contributed

3. **Chu CS**, Hsu PH, Lo PW, Scheer E, Tora L, Tsai HJ, Tsai MD, Juan LJ, 2011, “Protein kinase A-mediated serine 35 phosphorylation dissociates histone H1.4 from mitotic chromosome.” *JOURNAL BIOLOGICAL CHEMISTRY*, Oct 14;286(41):35843-51. (SCI)
(NSC-96-2311-B-001-029 and NSC-99-2320-B-001-017)