Protein Kinase A-mediated Serine 35 Phosphorylation Dissociates Histone H1.4 from Mitotic Chromosome*§

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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 a novel N-terminal phosphorylation of the major H1 variant H1.4 at serine 35 (H1.4S35ph), which accumulates at mitosis immediately after H3 phosphorylation at serine 10. Protein kinase A (PKA) was found to be a kinase for H1.4S35. Importantly, Ser-35-phosphorylated H1.4 dissociates from mitotic chromatin. Moreover, H1.4S35A substitution mutant cannot efficiently rescue the mitotic defect following H1.4 depletion, and inhibition of PKA activity increases the mitotic chromatin compaction depending on H1.4. Our results not only indicate that PKA-mediated H1.4S35 phosphorylation dissolves H1.4 from mitotic chromatin but also suggest that this phosphorylation is necessary for specific mitotic functions.

Eukaryotic DNA wrapped by core histones H2A/H2B/H3/H4 to form nucleosomes is further packed by linker histone H1. Ten H1 subtypes exist in human (1). Among them, H1.4 is one of the most abundant isoforms. 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, including phosphorylation (2).

H1 has long been recognized as a phosphoprotein, and its total phosphorylation level is cell cycle-dependent (3–6). In addition, H1 phosphorylation is linked to chromatin compaction and remodeling, heterochromatin formation, DNA replication, and transcription (6–8). Nevertheless, function of site-specific phosphorylation and the upstream kinases is just emerging. It was recently shown that H1.5 phosphorylated at Thr-10 by glycogen synthase kinase-3 binds to mitotic chromosome (9). Interphase phosphorylation of H1.4 at Ser-187 likely regulates transcription of ribosomal and steroid hormone-responsive genes (10).

In this study, we identified cAMP-dependent protein kinase A (PKA) as a kinase for mitotic phosphorylation of H1.4 at Ser-35, which resulted in a dissociation of H1.4 from the mitotic chromatin. The facts that the 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.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization, Antibodies, Inhibitors, and Drugs, and Plasmids are described in the supplemental data.

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 (11). Briefly, cells at 10% confluence in six-well plates were transfected with 20 nM of siRNA by Lipofectamine 2000 (Invitrogen) for 4 days. Stable knockdown of H1.4 in HeLa cells was accomplished by a lentivirus encoding shRNA against H1.4. HeLa cells at 10% confluence in six-well plates were transduced with lentivirus encoding scramble shRNA (Ctrl) or shRNA against H1.4 (shH14). Cells were sorted by EGFP fluorescence by FACS (BD Biosciences) to enrich the population of infected cells.

Western Blotting, Histone Extraction, and Fractionation—Western blotting was performed as described (12). 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 (13). Briefly, cells were harvested and incubated with 0.2 N H2SO4 for 30 min at 4 °C. After centrifugation, the supernatants were collected and added with TCA to precipitate the remaining proteins. The precipitants were washed with cold acetone and air-dried. The dried proteins were dissolved in distilled H2O, and the concentrations were determined. Fraction*

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The on-line version of this article (available at http://www.jbc.org) contains supplemental “Experimental Procedures,” Figs. 1–6, and additional references.

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The abbreviations used are: PKA, cAMP-dependent protein kinase A; NE, nuclear extract; NP, nuclear pellet; Ab, antibody.
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ation of nuclear extract and nuclear pellet was performed as described (14) with modifications. Briefly, cells were first incubated with hypotonic buffer (20 mM Tris, pH 8.0, 5 mM KCl, 2 mM MgCl₂, 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 MgCl₂, 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 volumes of NE and EP were applied to Western blot for analysis.

In Vitro Kinase Assay—The in vitro kinase assay was performed as described (15). Briefly, calf thymus H1 (14–155, Millipore) or core histones (10223565001, Roche Applied Science) was incubated with recombinant PKA catalytic subunit (P6000, New England Biolabs) 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, 10 mM MgCl₂, 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 MgCl₂, 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 volumes of NE and EP were applied to Western blot for analysis.

Micrococcal Nuclease Sensitivity Assay—The micrococcal nuclease sensitivity assay was performed as described (16) 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 1 h at room temperature. The primary and the fluorophore-conjugated secondary antibodies were subsequently incubated for overnight and 1 h, respectively, at 4 °C, with three washes using permeabilization buffer. Cells were then incubated with 300 nM DAPI (Sigma) for 15 min at room temperature. The cover slides were mounted with Prolong® 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 60× numerical aperture 1.25 oil objective. The merge images were created using Adobe Photoshop CS.

RESULTS

H1.4 Is Phosphorylated at Serine 35 during Mitosis—Post-translational modifications of H1 variants extracted from HeLa cells were analyzed by liquid chromatography (Agilent Technologies) coupled with mass spectrometry (LTQ-FT, Thermo Scientific, Inc.). A particular phosphorylated H1.4 fragment on Ser-35 (H1.4S35ph) was identified and found increased in response to nocodazole treatment (Fig. 1A). Note that cells treated with nocodazole arrest at mitotic phase (22). To study the identified H1.4 phosphorylation site, a specific Ab against H1.4S35ph was generated (see supplemental “Experimental Procedures”). The Ab was shown to be specific as it recognized only the H1.4 peptide containing Ser-35 phosphorylation, but not the same peptide without phosphorylation (Fig. 1B). Consistently, the H1.4S35ph Ab detected a signal from total lysates or histone extracts of HeLa cells with the size of H1 (~34 kDa).
in the presence of nocodazole (Fig. 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 Ser-35 phosphorylation (Fig. 1D, compare lanes 3–5 with lane 2). Pre-incubation of the Ab with non-phosphorylated H1 peptide (Fig. 1D, 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 diminished the H1.4S35ph signal. The Ab also detected an increased signal in normal mitotic HeLa cells obtained by mitotic shake-off (Fig. 1G). The identity of mitotic cells collected this way was confirmed by DAPI staining of cellular DNA (supplemental Fig. 1). We also show in supplemental Fig. 2 that the mitotic lung cancer cell H1299 collected by nocodazole treatment is also enriched in H1.4S35 phosphorylation, excluding the possibility that H1.4S35ph is only specific to HeLa.

Ser-35-phosphorylated H1.4 Accumulates at Mitosis Immediately after H3S10 Phosphorylation—Next, we investigated the temporal expression of the mitotic H1.4 Ser-35 phosphorylation. Cells were synchronized by nocodazole or double thymidine treatment and released for a specific time, followed by western to analyze the level of H1.4S35ph. As shown in Fig. 2A and supplemental Fig. 3, cells exiting from nocodazole-induced mitotic arrest displayed time-dependent decrease of H1.4S35ph as well as histone H3 phosphorylation at Ser-10 (H3S10ph) and H.4 phosphorylation at Thr-146 (H1.4T146ph), two mitotic markers. This indicates that H1.4S35ph is inversely correlated with the mitotic exit. A similar result was obtained using thymidine to arrest cells (Fig. 2B).
Cells exiting from thymidine-induced G1/S arrest caused H1.4S35ph accumulation peaking at 10 h after release and immediately after the appearance of H3S10ph. H3S10ph was induced from 8 to 11 h after release (Fig. 2A). These results indicate that H1.4S35ph phosphorylation is induced at a very specific time window of mitosis.

**PKA Phosphorylates H1.4 at Ser-35 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). Potential kinase candidates thus identified include PKA or 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 analyzed for their ability to phosphorylate H1.4S35ph. As shown in Fig. 3A, ectopic expression of PKAc alone caused H1.4S35ph accumulation. Other kinases, including Aurora kinases CAMKIIα, CAMKIIγ, CAMKIIδ, CDC2, NEK2, and PLK1, failed to do so.

Consistently, the nocodazole-induced H1.4S35ph was greatly abolished by PKA inhibitor H89 (Fig. 3B, compare lane 3 with lane 2) but not altered by CaMK inhibitor KN-62 (compare lane 4 with lane 2). In contrast, H89 had no effect on H3S10ph (Fig. 3B). Moreover, PKA knockdown diminished H1.4S35ph but not H3S10ph (Fig. 3C). H1.4S35ph was also reduced upon expression of a dominant mutant of PKA regulatory subunit 1α (PRKAR1α-DN) (Fig. 3D, compare lane 4 with lane 3).

**PKA Directly Phosphorylates H1.4 at Ser-35 in Vitro**—The above results together suggest that PKA is a kinase for H1.4S35ph. To prove that H1.4S35ph is directly phosphorylated by PKA, an in vitro kinase assay was performed. Indeed, PKA directly phosphorylated H1.4 at Ser-35 (Fig. 4, lanes 1–4, 9–12), and Ser-35 appeared to be the major site for PKA, as evident by mass analysis (data not shown). In contrast, Aurora B phosphorylated H3S10, but not H1.4S35ph (Fig. 4, lanes 4–8). Although PKA also induced Ser-10 phosphorylation of the purified H3 (Fig. 4, lanes 9 and 10), PKA depletion or inhibition did not reduce H3S10ph (Fig. 3, B–D). Therefore, our experiments together demonstrate that PKA is the kinase phosphorylating H1.4 at Ser-35 in vitro and in vivo.

Ser-35-phosphorylated H1.4 Dissociates from Mitotic Chromatin—Earlier peptide-DNA interaction assays suggested that H1 when phosphorylated has a reduced affinity toward DNA (23). To investigate whether Ser-35-phosphorylated H1.4 behaves similarly, the level of H1.4S35ph in NE and NP was compared. NP contains chromatin-bound proteins. The results showed that the nocodazole-induced H1.4S35ph only existed in NE, implying that the Ser-35-phosphorylated H1.4 has reduced affinity toward the mitotic chromatin (Fig. 5A, lane 2). In contrast, nocodazole-mediated H3S10ph occurred in NP (Fig. 5A, lane 4). In agreement with the finding that Ser-35-phosphorylated H1.4 in mitotic fraction bound less efficiently to chromatin, the level of total H1 disassociated from chromatin (enriched in NE) was increased from 48.4 to 53.4% upon nocodazole treatment, and consistently the level of total H1 associated with chromatin (enriched in NP) was decreased from 51.5 to 46.5% by nocodazole (quantification shown in Fig. 5A, right panel). To test whether Ser-35 phosphorylation indeed reduces H1.4 binding to chromatin, the effect of the substitution mutant of H1.4 with Ser-35 replaced by alanine (H1.4S35A) was analyzed upon nocodazole treatment. Again, 63.6% of wild type H1.4 was located in NE. In contrast, only 37.0% of H1.4S35A appeared in NE (Fig. 5B). These results indicate that the phosphorylation mutant H1.4S35A has a stronger preference to associate with the mitotic chromatin.

To further study the temporal-spatial behavior of H1.4S35ph, immunostaining was performed with cells fixed at different mitotic phases (Fig. 5C). It was found that Ser-35-phosphorylated H1.4 was increased and excluded from chromatin from prophase to telophase, and disappeared during cytokinesis (Fig. 5C). In contrast, H3S10ph showed a totally distinct pattern (supplemental Fig. 4). The above results clearly indicate that Ser-35-phosphorylated H1.4 specifically dissociated from mitotic chromatin.

To further investigate whether H1.4 phosphorylated at Ser-35 binds less efficiently to chromatin, EMSA was applied using a 216-bp nucleosomal DNA template, which, in theory, can only hold one nucleosome. As shown in Fig. 5D, addition of H1.4–H1.4 caused a shift of mononucleosome, indicating the binding of H1.4–H1.4 onto mononucleosomes (left panel, compare lane 4 with lane 2). Importantly, adding PKA to phosphorylate H1.4 in vitro reduced the level of the shifted band, suggesting that H1.4–H1.4 phosphorylated at Ser-35 by PKA binds less to mononucleosomes (Fig. 5D, left panel, compare lane 5 with lane 4). Quantitative analysis of the shifted bands showed a significant reduction of H1.4 binding to mononucleosome after phosphorylation by PKA (Fig. 5D, middle panel, 11.1 ± 10.0%) compared with control (Fig. 5D, middle panel, 35.9 ± 15.7%). The right panel in Fig. 5D shows the efficient phosphorylation of H1.4–H1.4 at Ser-35 by PKA in vitro. Together, we
FIGURE 3. PKA mediates 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 Ser-35 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 blotting using Abs against the indicated proteins. Ponceau S staining is presented as the loading control.
believe that Ser-35-phosphorylated H1.4 contains reduced affinity toward mitotic chromatin and that H1.4S35ph is important for this reduction both in vivo and in vitro.

H1.4S35A Substitution Mutant Cannot Efficiently Rescue Mitotic Defect following H1.4 Depletion—Next, we tested whether PKA-mediated H1.4S35 phosphorylation has an effect on mitosis. First, we examined whether H1.4 controls cell cycle progression. As shown in supplemental Fig. 5A, FACS analysis 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 (supplemental Fig. 5B). This implies that H1.4-depleted cells might have defects in entering both S and G2 phases.

FIGURE 5. Ser-35-phosphorylated H1.4 dissociates from the mitotic chromatin. A, Ser-35-phosphorylated H1.4 binds less to chromatin. NE and NP from HeLa cells with or without nocodazole treatment were subjected to Western blotting. B, 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. A and B, quantification of total H1 or HA-H1.4 by Multi Gauge (version 3.0) was shown in the right panels. C, H1.4S35ph does not colocalize with mitotic chromatin. HeLa cells were subjected to immunofluorescent microscopy analysis using H1.4S35ph Ab. The representative images of cells at different cell cycle stages were shown. Scale bar, 20 μm. Note that 100% of cells in each group displayed the same pattern. The number of cells counted in each group is as follows: interphase, >10 000; prophase, >110; prometaphase, >55; metaphase, >400; anaphase, >55; telophase, >230; and cytokinesis, >230. D, H1.4 phosphorylation by PKA reduces H1.4 binding to nucleosomes. Left panel, EMSA assays were performed with DNA probe alone (lane 1) or in the presence of indicated proteins (lanes 2–5). Middle panel, the H1-shifted bands (indicated by an asterisk) in lanes 4 and 5 were quantified by ImageJ software. Error bars represent S.D. from three independent experiments. *, p < 0.05. Right panel, Western blotting control of in vitro kinase assays of lanes 4 and 5 in left panel.
The mitotic index by staining H3S10 phosphorylation confirmed H1.4 depletion resulted in the decrease of mitotic cell population (Fig. 6A). Second, we analyzed whether H1.4S35ph contributes to H1.4 function in mitotic phase. To this, wild type H1.4 or H1.4S35A 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 (Fig. 6B, shH1.4+svec). Importantly, ectopic expression of wild type H1.4 (shH1.4+H1.4WT), but not H1.4S35A mutant (shH1.4+H1.4S35A), rescued the defect, indicating that H1.4S35 phosphorylation contributes to mitotic progression. Note that the protein levels of the ectopically expressed wild type H1.4 and S35A mutant in H1.4 knockdown cells are equal but a bit less than the endogenous level of H1.4 (Fig. 6C, compare lanes 4 and 5 with lanes 1 and 2). This may explain why the rescue of the phenotype by wild type H1.4 was incomplete (Fig. 6B).

Inhibition of PKA Activity Increases Mitotic Chromatin Compaction Depending on H1.4—Finally, we evaluated whether H1.4S35 phosphorylation is necessary for proper mitotic chromatin compaction. Nocodazole-treated HeLa nuclei with or without PKA inhibitor H89 were subjected to micrococcal nuclease digestion. As shown in Fig. 7A, PKA inhibition increased mitotic chromatin compaction, as evident by the disappearance of low molecular weight micrococcal nuclease-digested fragments and the accumulation of high molecular weight products (Fig. 7A, compare lane 3 with 2; and Fig. 7B). Importantly, the above effect was lost upon H1.4 depletion (Fig. 7A, compare lane 5 with 4; and Fig. 7C). To directly visualize chromosome compaction, mitotic chromosomes were analyzed by metaphase spread assay. As shown in Fig. 7, D and E, the percentage of the X-shaped chromosome in nocodazole-treated cells was greatly reduced when the cells also received the PKA inhibitor H89. The chromosomes under this condition exhibited a more compact structure, indicating that H89 likely induced chromatin condensation or blocked chromatin decondensation. This result is consistent with what we observed by using the nuclease sensitivity assay shown in Fig. 7A. The above results suggest that H1.4S35 phosphorylation likely contributes to the maintenance of optimal chromatin compaction during mitosis.

**DISCUSSION**

In the current study, we demonstrate that histone H1.4 was phosphorylated at Ser-35 by PKA during mitosis (Figs. 1–4) and that this phosphorylation dissociated H1.4 from the mitotic chromatin (Fig. 5). Although it is generally believed that phosphorylation decreases H1 affinity for DNA, this assumption is not true in all cases. For example, phosphorylation of *Xenopus* somatic H1 variant B4 by cyclin-dependent kinase 1 increases B4 association with chromatin (24). Thus, the effect of H1 phosphorylation is likely site- or variant-specific.

H1.4S35 phosphorylation is likely essential for specific mitotic functions. First, H1.4S35 phosphorylation may control mitotic entry or transition from telophase to cytokinesis as H1.4S35ph was sharply increased at prophase and decreased in cytokinesis (Fig. 5C). 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 decrease in number of mitotic cells following H1.4 depletion (Fig. 6B). Second, H1.4S35 phosphorylation seems to be necessary for maintaining proper mitotic chromatin structure (Fig. 7). It has been reported by others that phosphorylation of H1 helps H1 dissociation from promoter regions of specific genes (25), which in turn allows transcription factor binding (26) and chromatin remodeling (7). The accessibility of transcription factors and remodeling factors to the pro-
moter region is most likely established by certain degree of chromatin decondensation. If the hypothesis is correct, we expect to see Ser-35-phosphorylated H1.4 falls off from genes necessary to be turned on during mitosis. This possibility is currently under investigation. Third, 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 (Fig. 5C) (27). The unique finding suggests that Ser-35-phosphorylated H1.4 dissociated from chromatin may have an active role in microtubule organization and mitotic spindle formation, known functions of centrosomes (28). These possibilities await further investigation.

Our results suggest that PKA contributes to mitotic regulation, which is consistent with previous reports in which PKA activity peaks at mitosis (29) and regulates spindle formation (30), CDC2/cyclin B activity (31), and anaphase-promoting complex activity (29). In contrast, Aurora family kinases, although being shown to have a major function in mitosis (32), do not phosphorylate H1.4 at Ser-35 (Figs. 3 and 4). In agreement with our result, a recent report by Hergeth et al. (33) demonstrates 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 Ser-27 is the only site at H1.4 to be phosphorylated by Aurora B. Given that serine 35 of H1.4 is conserved among vertebrates, but not non-vertebrates (supplemental Fig. 6), the regulation of cell division involving H1.4S35ph may only exist in higher eukaryotes. In summary, we provide a novel role for H1.4 to actively participate in mitosis through PKA-mediated Ser-35 phosphorylation.

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REFERENCES


FIGURE 7. PKA inhibitor H89 induces the mitotic chromatin compaction depending on H1.4. A, HeLa cells stably transduced with lentivirus encoding scramble shRNA or shRNA against H1.4 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 a micrococcal nuclease sensitivity assay. The intensity of micrococcal nuclease-digested nucleosomal DNA fragments was quantified by Metamorph software as a percentage of total intensity of each lane (B and C). Mono, di, and tri indicate mononucleosome, dinucleosome, and trinucleosome. D, 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 immunofluorescent microscopy. Scale bar, 20 mm. E, quantitative analysis of the frequency of X-shape formation in D. The number of cells observed in each group are 67 for nocodazole (Noco) and 46 for nocodazole+H89 (Noco+H89).
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