ARTICLES

Regulation of HP1–chromatin binding by histone H3 methylation and phosphorylation

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Tri-methylation of histone H3 lysine 9 is important for recruiting heterochromatin protein 1 (HP1) to discrete regions of the genome, thereby regulating gene expression, chromatin packaging and heterochromatin formation. Here we show that HP1α, -β, and -γ are released from chromatin during the M phase of the cell cycle, even though tri-methylation levels of histone H3 lysine 9 remain unchanged. However, the additional, transient modification of histone H3 by phosphorylation of serine 10 next to the more stable methyl-lysine 9 mark is sufficient to eject HP1 proteins from their binding sites. Inhibition or depletion of the mitotic kinase Aurora B, which phosphorylates serine 10 on histone H3, causes retention of HP1 proteins on mitotic chromosomes, suggesting that H3 serine 10 phosphorylation is necessary for the dissociation of HP1 from chromatin in M phase. These findings establish a regulatory mechanism of protein–protein interactions, through a combinatorial readout of two adjacent post-translational modifications: a stable methylation and a dynamic phosphorylation mark.

Genomic DNA within the eukaryotic nucleus is organized into distinct chromosomal domains[1,2]. Structural and functional changes in the organization and dynamics of such specialized chromatin areas are key to controlling genome function[3]. Cytologically defined domains of euchromatin and heterochromatin have been functionally linked to gene content and activity. Euchromatin generally keeps genes competent for transcription, whereas heterochromatin contains predominantly transcriptionally silent genes and includes specialized chromosome structures such as centromeres and telomeres[4,5].

Members of the heterochromatin protein 1 (HP1) family have important roles in heterochromatin organization[4,6]. The three isoforms of HP1 (-α, -β, and -γ) in higher eukaryotes have been associated with constitutive (that is, pericentric and telomeric) heterochromatin and some forms of facultative (that is, developmentally regulated) heterochromatin[5]. Although all HP1 isoforms are localized predominantly to pericentric heterochromatin, HP1β and -γ can also be found at euchromatic sites[7], where they are presumably involved in gene repression[5,6]. During mitosis, a fraction of HP1α stays associated with (peri-)centromeric chromosome regions[7–9], and the single HP1 homologue in *Schizosaccharomyces pombe*, Swi6, is required for proper chromosome segregation and cohesion of sister centromeres[10].

Recruitment of HP1 proteins to certain sites of the genome involves interactions with multiple components of chromatin. In particular, the methylation of histone H3 on lysine 9 (H3K9me) is important for bringing HP1 to distinct chromosomal areas[11–13], and both tri-methylated H3K9 (H3K9me3) and HP1 are thought to be crucial for establishing and maintaining domains of heterochromatin[14,15]. Indeed, an amino-terminal chromodomain found in all HP1 proteins specifically interacts with H3K9me *in vitro*[16–18], particularly in its di- and tri-methylated states[19,20]. Although binding of HP1 to H3K9me is fairly weak, the overlap of HP1 proteins and H3K9me3 at heterochromatic sites of different cell types validates the biological importance of this effector–mark interaction. Such an apparent increase in cellular binding strength may be due to the cooperative effects of HP1 dimerization and additional stabilizing interactions with other chromatin factors[5,11,13,21].

In agreement with the rather weak HP1–H3K9me3 interaction, a large fraction of the cellular HP1 molecules are not stably incorporated into heterochromatin, but instead show rapid on/off kinetics from their subnuclear target areas[9,22,23]. The dynamic properties of HP1 and other components of chromatin domains may be critical for creating an ever-changing—but overall steady—architectural framework within which nuclear processes can take place[21]. The mechanisms that control the dynamic interaction between HP1 and H3K9me3, however, have not been established.

Methylation–phosphorylation of histone H3 in M phase

As the bulk of mammalian HP1 proteins dissociate from chromatin during mitosis[7–9], we sought to investigate the role of the HP1–H3K9me3 interaction in the dynamic association of HP1 proteins with chromatin in the context of the cell cycle. As observed for the different HP1 isoforms in mammalian cell lines[7,12,18,24,25], we found that HP1β localized to dot-like structures in the cell nucleus of 10T1/2 cells at interphase (Fig. 1a). These dot-like structures contain constitutive pericentric heterochromatin and show strong staining with DAPI[21]. Consistent with the involvement of H3K9 methylation in HP1 recruitment, the H3K9me3 mark was found enriched in the same subnuclear areas in interphase cells. In contrast, this colocalization was not observed in M-phase cells, in which HP1β was diffusely distributed throughout the cell[7–9] while the H3K9me3 mark remained tightly localized to condensed chromosomes. Western blot (Fig. 1b) and quantitative mass spectrometric (Fig. 1d) analyses

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of histones isolated from cells arrested at M phase by nocodazole revealed that the overall level of H3K9me3 is not altered when cells enter mitosis. Similarly, the cellular protein levels of the different HP1 isoforms remained unchanged (Fig. 1b). These analyses indicate that HP1 dissociates from mitotic chromatin without loss of the 'recruiting' H3K9me3 mark, and suggest that the HP1–H3K9me3 interaction must be somehow interrupted as cells enter M phase.

We next sought to analyse whether additional modifications occur in the vicinity of the K9me3 mark on the H3-tail during mitosis. We therefore purified H3 from HeLa cells arrested at M phase and used mass spectrometry to examine the modification pattern on the H3-tail[12,26]. This approach revealed the existence of a phospho-mark (ph)

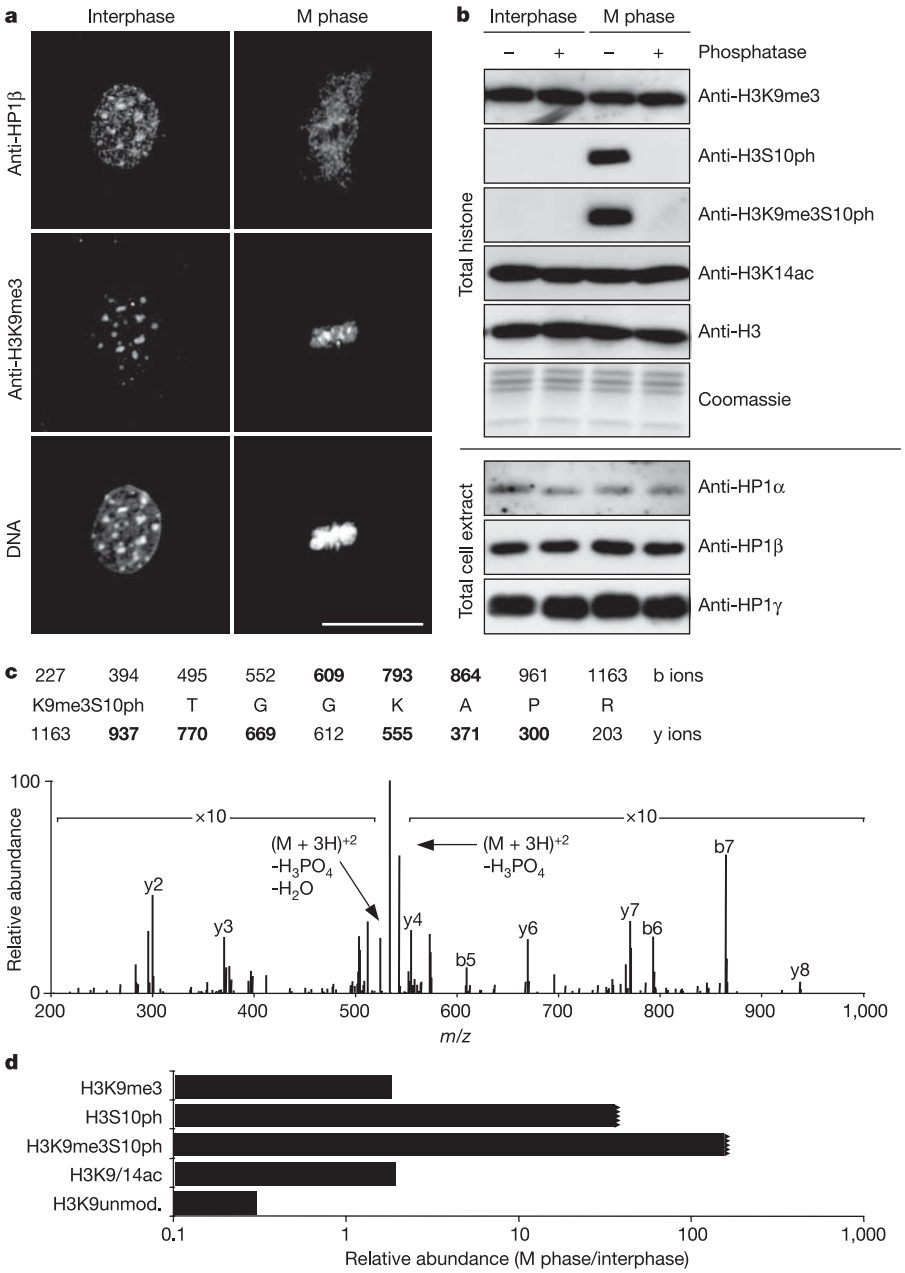
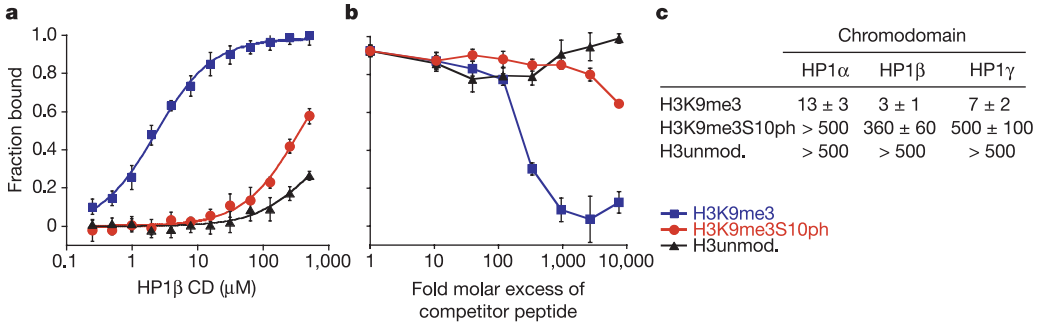


Figure 1 | Coordinate behaviour of HP1 and mitotic phosphorylation of H3S10 in the context of H3K9me3. **a**, Distribution of HP1β in10T1/2 fibroblast cells during interphase (left) and mitosis (right, metaphase stage shown), analysed by immunostaining. DNA was stained with DAPI. Scale bar, 10 μm. **b**, Western blot analysis of histones (top) and cell extracts (bottom) prepared from asynchronously growing (interphase) or nocodazole-arrested (M phase) HeLa cells after treatment with or without alkaline phosphatase. Gels stained with Coomassie were used as loading controls. See Supplementary Fig. S1 for characterization of the H3K9me3S10ph-specific antibody. **c**, Tandem mass spectrometry (MS/MS) spectrum recorded on doubly protonated $(M + 2H)^{2+}$ ions derived from residues 9–17 of histone H3 isolated from mitotically arrested HeLa cells. This spectrum confirms the presence of K9me3 and S10ph on the same H3-tail. The measured (582.3127 Da) and calculated (582.3115 Da) weights for the parent $(M+2H)^{2+}$ ions are within 2.2 p.p.m. Predicted masses for singly charged fragment ions of type b (acylium ions containing the N terminus) and type y (truncated peptides containing the C terminus) are shown above and below the peptide sequence; those observed in the spectra are bold. Abundances of fragments other than those corresponding to $(M+2H)^{2+}$–H_3P0_4 and $(M+2H)^{2+}$–(H_3P0_4 and H_2O) are amplified by a factor of 10. **d**, Relative abundance of H3 N-terminal peptides isolated from asynchronous (interphase) or nocodazole-arrested (M phase) HeLa cells as determined by mass spectrometry[12]. Acetylation marks on H3K9 and H3K14 cannot be discriminated by this method (H3K9/K14ac). H3unmod, unmodified H3-tail. Note that neither H3S10ph nor H3K9me3S10ph was detected in the asynchronous sample. Plotted minimal values for these entries used the detection limit observed in the analysis of the asynchronous sample (~0.01%).



	Chromodomain		
	HP1α	HP1β	HP1γ
H3K9me3	13 ± 3	3 ± 1	7 ± 2
H3K9me3S10ph	> 500	360 ± 60	500 ± 100
H3unmod.	> 500	> 500	> 500

Figure 2 | Binding of HP1 to an H3K9me3 peptide is impaired by phosphorylation of H3S10. **a**, Binding of the HP1β chromodomain (CD) to the indicated H3-tail peptides was analysed by fluorescence polarization assays. Data show average ± s.d. for three independent experiments. **b**, Competition experiment between a fluorescein-labelled H3K9me3 peptide bound to the HP1β chromodomain (95% saturation) and the indicated unlabelled H3 peptides, analysed by fluorescence polarization. Data show average ± s.d. for three independent experiments. **c**, Dissociation constants (K_d in μM) for the interaction of the chromodomains of the different human HP1 isoforms with H3K9me and H3K9meS10ph peptides. Values represent averages ± s.d. from ≥3 independent experiments.

on Ser 10 (H3S10) next to K9me3 (Fig. 1c). Dual-mark combinations of H3K9me1S10ph and H3K9me2S10ph were also detected (data not shown). No phosphorylation on Ser 10, alone or in combination with K9me, could be found on H3 purified from cells at interphase. To further validate the existence of H3K9me3S10ph, we raised an antiserum that specifically recognizes this dual-mark combination (Supplementary Fig. S1). Western blot analysis of histones prepared from nocodazole-arrested cells indeed verified the occurrence of H3K9me3S10ph specifically in M phase chromatin (Fig. 1b). These data are consistent with H3S10ph being a 'mitotic mark' that first appears at pericentromeric heterochromatin in late G2 (refs 27, 28), and raise the possibility that the dual-mark combination of H3K9me3S10ph controls HP1–chromatin binding.

H3S10ph inhibits the HP1–H3K9me3 interaction

To investigate the effect of H3S10 phosphorylation on the HP1–H3K9me interaction, we analysed the binding of HP1 to dually modified H3K9me3S10ph peptides using fluorescence polarization measurements. In direct binding (Fig. 2a) and indirect competition experiments (Fig. 2b), we detected a significant loss in the affinity of HP1 chromodomains for a dually modified H3K9me3S10ph peptide, compared with the interaction between HP1 and a H3K9me3 peptide. Although the HP1 chromodomains reproducibly interacted better with the H3K9me3S10ph peptide than with the unmodified (unmethylated) control H3 peptide, we note that the affinity for the H3K9me3 mark was two order of magnitudes lower for all HP1 isoforms when the H3S10ph modification was present (Fig. 2c). These effects were not restricted to HP1chromodomains, as experiments using full-length recombinant proteins showed the same loss of binding (Supplementary Fig. S2a). Mutating the conserved glutamic acid residue within the H3-binding groove of HP1 that forms a hydrogen bond with H3S10 (ref. 19) confirmed the importance of this interaction for HP1–H3K9me binding (Supplementary Fig. S2). Our biophysical studies reveal a putative function for the dual-mark combination of K3K9me3S10ph in the inhibition of HP1 recruitment. The competition studies further suggest that binding of HP1 to the methylated H3-tail is fully reversible and highly dynamic, thereby supporting the rapid exchange of HP1 from heterochromatin[9,22,23].

Phosphorylation of H3S10 removes HP1 from H3K9me3

Although we detected dually modified H3K9me3S10ph in mitotic cells (Fig. 1), earlier studies have reported a reduced *in vitro* activity of the principal mitotic H3S10 kinase, Aurora B (ref. 29), on K9-methylated substrates compared with the unmodified H3-tail[30]. As Aurora B is activated by additional factors within the chromosomal passenger complex (CPC)[31–33], we analysed the native H3S10 kinase

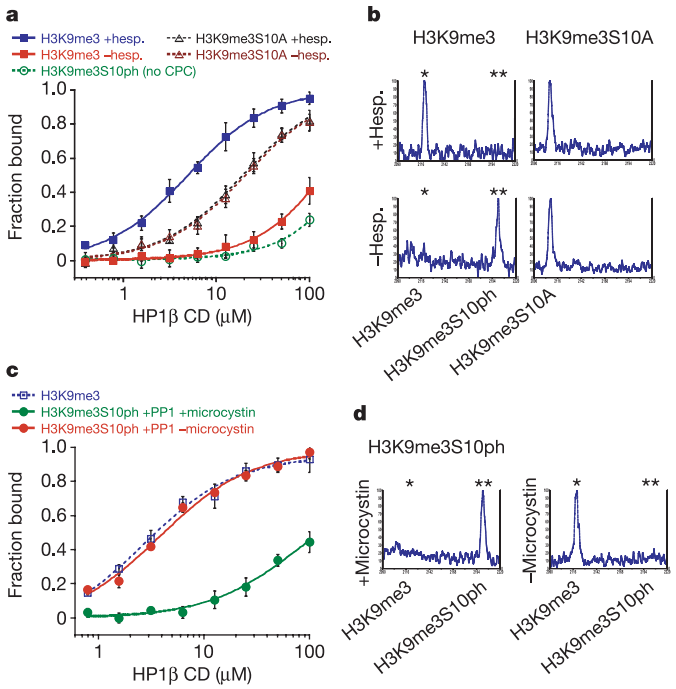


Figure 3 | Reversible phosphorylation of H3S10 disrupts the HP1–H3K9me3 interaction. **a**, Loss of HP1 binding to H3K9me3 by phosphorylation of H3S10. Interaction of the HP1β chromodomain (CD) with H3K9me3 and H3K9me3S10A peptides after phosphorylation by CPC in the presence (+hesp.) or absence (−hesp.) of hesperadin, measured by fluorescence polarization ($n = 3$; error bars show s.d.). **b**, Substrate histone peptides from the phosphorylation reactions in **a** containing 50 μM HP1β chromodomain were analysed by MALDI-TOF mass spectrometry. Left panels show loss of H3K9me3 signal (*) over the course of the reaction, and appearance of H3K9me3S10ph peptide (**). Right panels show control H3K9me3S10A reaction. **c**, Gain of HP1 binding to a H3K9me3S10ph peptide after dephosphorylation of H3S10. Interaction of the HP1β chromodomain with a fluorescein-labelled H3K9me3S10ph peptide after dephosphorylation by phosphatase PP1 in the presence or absence of microcystin LR, measured by fluorescence polarization ($n = 3$; error bars show s.d.). **d**, Substrate histone peptides from the dephosphorylation reactions in **c** containing 50 μM HP1β chromodomain were analysed by MALDI-TOF mass spectrometry. Loss of H3K9me3S10ph signal (**) over the course of the reaction, and appearance of the H3K9me3 peptide (*) are shown.

complex on modified H3-tail peptides. The relative ability of Aurora B to phosphorylate H3S10 when K9 is methylated to various degrees is very similar to that measured with the unmodified substrate (Supplementary Fig. S3).

We then tested whether enzymatic phosphorylation of H3S10 could eject HP1 bound to H3K9me3. Preliminary experiments using the CPC kinase complex indicated that an H3K9me3 peptide could be phosphorylated even in the presence of a large molar excess of HP1 chromodomain (Supplementary Fig. S4). Interaction of a fluorescein-labelled H3K9me3 peptide with increasing amounts of HP1β chromodomain after CPC kinase reaction was recorded using fluorescence polarization measurements. Similar to the reduced interaction between HP1 and the dually modified H3K9me3S10ph peptide (Fig. 2), we detected a significant loss of HP1β chromodomain binding to the methylated H3 peptide after the phosphorylation reaction. However, this change in binding was not observed when Aurora B was inhibited by the small molecule hesperadin[34,35] (Fig. 3a) or when a H3K9me3 peptide containing a Ser 10 to Ala 10 substitution (S10A, which shows about fivefold reduced interaction with the HP1 chromodomain) was used[19]. Mass spectrometric analysis of the H3-tails after the kinase reaction verified phosphorylation of the H3K9me3 peptide, but not the control H3K9me3S10A peptide, confirming the specificity of CPC-mediated phosphorylation at the H3S10 site (Fig. 3b). Notably, the HP1β chromodomain was neither phosphorylated nor degraded over the course of the reaction, and similar results were obtained with the chromodomains of HP1α and HP1γ as well as full-length HP1β protein (Supplementary Fig. S4). In a reverse reaction sequence, we detected a gain-of-binding of the HP1β chromodomain to an H3K9me3S10ph peptide in the presence of protein phosphatase 1 (PP1), an enzyme known to dephosphorylate H3S10ph (ref. 28) (Fig. 3c, d). This gain in binding was sensitive to the phosphatase inhibitor microcystin LR. On the basis of these observations, we reason that transient (reversible) phosphorylation of H3S10 during mitosis might control the dynamic interaction between HP1 and H3K9me3.

H3K9me3S10ph concurs with mitotic HP1 relocation

Next, we examined the effect of H3S10 phosphorylation on the association of HP1 with chromatin *in vivo* and investigated the role of dually modified H3K9me3S10ph in the release of HP1 from chromatin at the onset of mitosis. We stained asynchronously growing cultures of 10T1/2 cells with H3K9me3S10ph-specific antiserum and analysed when this dual-mark occurred during the cell cycle (Fig. 4). As expected from our western blot and mass spectrometric analyses of H3 (Fig. 1), the dually modified H3 epitope was not observed in interphase cells, which showed a dot-like subnuclear distribution of HP1β (Fig. 4, interphase). H3K9me3S10ph was only detected in cells that had lost this characteristic HP1β localization pattern and showed diffuse HP1β nuclear staining. There, the dual-mark combination was restricted to the dot-like areas showing strong staining with DAPI (Fig. 4, G2). Upon chromosome condensation (Fig. 4, metaphase), HP1β was found diffusely distributed throughout the cell, and little overlap with the anti-H3K9me3S10ph and DAPI staining was detected. Only as cells exited mitosis and phosphorylation of H3S10 disappeared did we observe re-association of HP1β with chromatin. Similar observations were made in HeLa cells and for the -α and -γ isoforms of HP1 (data not shown). In agreement with the dot-like appearance of the anti-H3K9me3S10ph immunostaining at the onset of mitosis, we detected this dual-mark combination enriched at the centromeric and pericentromeric regions with a more spotted appearance on the chromosome arms of metaphase

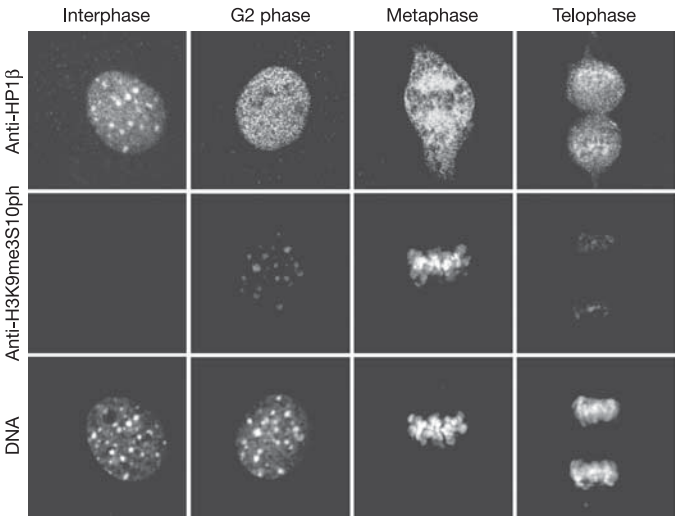


Figure 4 | Temporal occurrence of the dual K9me3S10ph epitope on H3 coincides with dissociation of HP1 from mitotic chromatin. 10T1/2 cells were stained with the indicated primary antibodies. DNA was stained with DAPI. Columns represent cells at the indicated stages of the cell cycle. Scale bar, 10 μm.

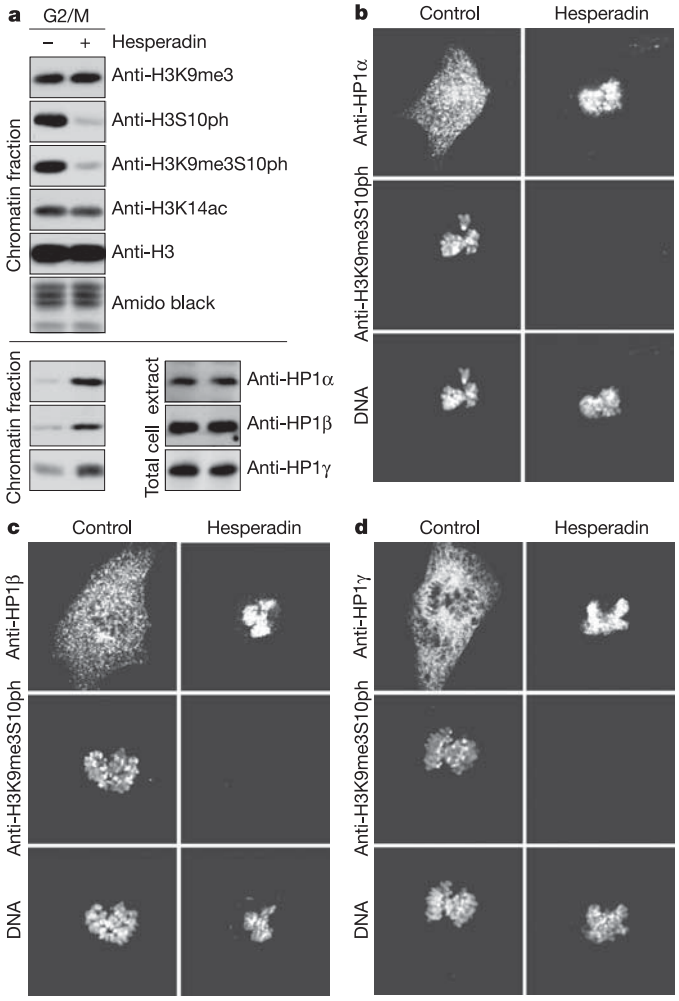


Figure 5 | Inhibition of Aurora B kinase results in retention of HP1 on M-phase chromatin. a, HeLa cells synchronized at the G2/M transition were treated with 200 nM of the Aurora B inhibitor hesperadin (+) or with vehicle (−). Total cellular chromatin or whole-cell extracts were analysed by western blot. Blots stained with amido black were used as loading controls. **b**–**d**, Immunofluorescence staining of 10T1/2 cells at M phase, with or without treatment with 200 nM hesperadin, using anti-HP1α (**b**), anti-HP1β (**c**) and anti-HP1γ (**d**) primary antibodies. DNA was stained with DAPI. Scale bars, 10 μm.

chromosome spreads (Supplementary Fig. S5). Together, our immunofluorescence analyses link the dissociation of HP1 from condensed chromatin in M phase to the temporal and local occurrence of the dual-mark combination of H3K9me3S10ph.

Lack of H3S10ph retains HP1 on mitotic chromosomes

To examine whether phosphorylation of H3S10 is causally linked to the release of HP1 from chromatin at the onset of mitosis, we analysed the mitotic behaviour of HP1 when Aurora B is inhibited. Treatment of G2/M-synchronized HeLa cells with hesperadin resulted in the loss of the mitotic H3S10ph mark[35] and, as predicted, the elimination of the dual H3K9me3S10ph epitope (Fig. 5a). In contrast, no change in the global levels of H3K9me3 and acetylated Lys 14 on H3 (H3K14ac) were observed, and the overall protein levels of HP1α, -β and -γ were not affected. Similar effects of hesperadin on the phosphorylation of H3S10 and the loss of the dual-mark combination of H3K9me3S10ph were observed in 10T1/2 cells (Supplementary Fig. S6).

We then analysed the effect of inhibiting mitotic H3S10 phosphorylation on HP1 distribution and localization in M-phase cells. As observed in other cell types[7–9], HP1α is partly retained on M-phase chromosomes and shows an otherwise diffuse distribution throughout 10T1/2 cells at stages when chromatin is condensed. In contrast, HP1β and -γ, are largely absent from compacted M-phase chromosomes in these cells (Fig. 5b–d, control). Inhibition of Aurora B kinase activity by hesperadin, as revealed by the absence of anti-H3K9me3S10ph immunostaining, resulted in dramatic changes in the localization of all HP1 isoforms in M-phase cells (Fig. 5b–d, hesperadin): HP1α, -β, and -γ remained localized to condensed chromatin in the absence of mitotic H3S10 phosphorylation. Hesperadin treatment did not affect the distribution of HP1 in interphase cells (Supplementary Fig. S6). Similar results were obtained with ectopically expressed HP1–green fluorescent protein (GFP) fusion proteins in HEp-2 cells and after knockdown of Aurora B by RNA interference (RNAi) (Supplementary Figs S7, S8). Furthermore, biochemical fractionation of chromatin isolated from hesperadin-treated HeLa cells verified the increased association of HP1α, -β and -γ with mitotic chromatin when H3S10 phosphorylation is inhibited (Fig. 5a). Together, these experiments indicate that phosphorylation of H3S10 by Aurora B is necessary for the dissociation of HP1 from mitotic chromatin.

HP1 binds to M-phase chromosomes lacking H3S10ph

To further investigate the molecular mechanism by which HP1 dissociates from chromosomes in M phase and to avoid the experimental limitations of tissue culture systems, we turned to *Xenopus* egg extracts, from which metaphase chromosomes can be purified in a well-controlled manner[36]. Consistent with the results in 10T1/2 and HeLa cells, we observed the dual-mark combination of H3K9me3S10ph (Supplementary Fig. S9) and the dissociation of *Xenopus* HP1α (xHP1α)–GFP from chromosomes upon entry into M phase (data not shown). To examine whether this dissociation is caused by phosphorylation of H3S10 by Aurora B, we monitored the chromosome-binding activity of xHP1 in egg extracts depleted of the Aurora B-containing CPC complex (ΔCPC)[36], in which chromosomal H3S10ph was greatly decreased but the level of H3K9me3 was not affected (Supplementary Fig. S10). Indeed, using anti-xHP1α antibodies (Supplementary Fig. S11), we detected increased binding of endogenous xHP1α to metaphase chromosomes assembled in ΔCPC extracts compared with control extracts (Fig. 6a). To quantify this response, metaphase chromosomes were purified from extracts incubated with [^{35}S]-labelled xHP1α, -β and -γ. At least sevenfold more xHP1 protein was associated with metaphase chromosomes assembled in ΔCPC extracts compared with control extracts (Fig. 6b).

We next sought to verify that this aberrant association of HP1 with M-phase chromosomes in ΔCPC extracts is dependent on chromodomain–H3K9me3 interaction. First, we examined the effect caused by mutating one of the three aromatic residues essential in HP1 chromodomains for binding H3K9me *in vitro*[19,37] (see Supplementary Fig. S2). In agreement with the importance of the HP1 chromodomain in the chromosome-binding activity of xHP1α, the

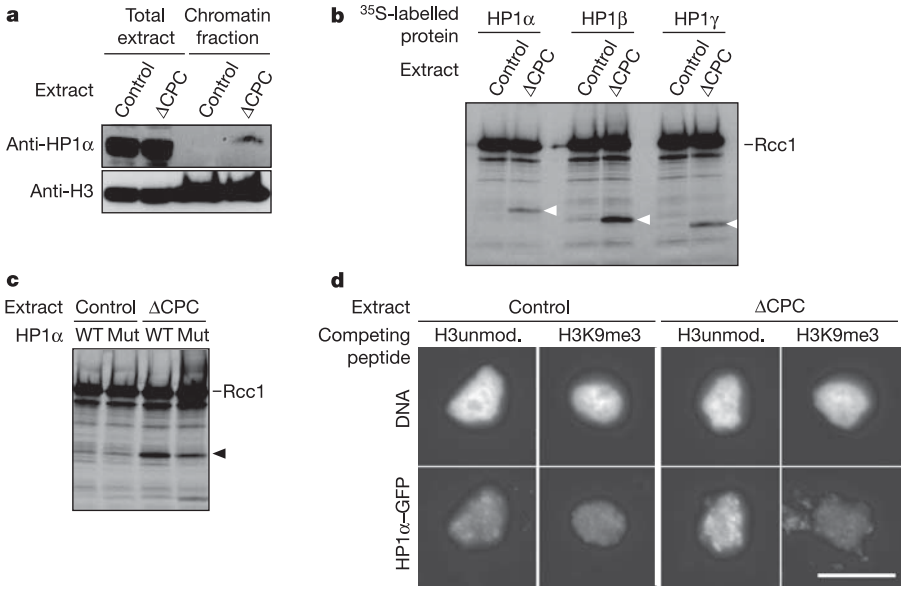


Figure 6 | HP1 binds more strongly to M-phase chromosomes in the absence of Aurora B. a, Western blot of control or ΔCPC *Xenopus* egg extracts (total extract) and purified metaphase chromosomes assembled in these extracts (chromatin fraction) using anti-xHP1α and anti-H3 antibodies. **b**, Autoradiography of proteins bound to purified metaphase chromosomes assembled in control or ΔCPC *Xenopus* egg extracts containing [^{35}S]-labelled Rcc1 (loading control) and xHP1α, -β or -γ (arrowheads). **c**, Autoradiography of proteins bound to purified metaphase chromosomes assembled in control or ΔCPC extracts containing [^{35}S]-labelled Rcc1 (loading control) and xHP1α (WT) or chromodomain mutant xHP1αW57A (mut). Arrowhead indicates [^{35}S]-labelled xHP1α. **d**, Binding of xHP1α–GFP to metaphase chromosomes assembled in ΔCPC extract is competed by an H3K9me3 peptide, but not an unmodified control peptide. DNA was stained with Hoechst 33258 and HP1α–GFP was visualized by immunostaining using an anti-GFP antibody. Scale bar, 10 μm.

amount of xHP1αW57A on metaphase chromosomes purified from ΔCPC extracts was reduced to approximately 20–30% of that seen with the wild-type protein (Fig. 6c). Second, we investigated whether binding of xHP1 to metaphase chromosomes in ΔCPC extracts could be inhibited by a peptide that competes with the chromodomain–H3K9me3 interaction. Although neither an unmodified nor a K9me3 H3-tail peptide had an effect on the chromosomal binding of xHP1α–GFP in control extracts (Fig. 6d, left), the addition of an H3K9me3 peptide, but not an unmodified control peptide, to ΔCPC extracts significantly reduced the chromosomal binding of xHP1α–GFP (~50%, $P < 0.0001$, $n = 50$ in each of two independent experiments) (Fig. 6d, right). These results imply that HP1 proteins can only bind to metaphase chromosomes in the absence of H3S10 phosphorylation, and that this association depends on chromodomain–H3K9me3 interaction.

Discussion

Although histone H3S10ph is widely seen as a hallmark of mitosis, the function of this modification during M phase has been enigmatic[28]. Our data suggest that phosphorylation of H3S10 by Aurora B disrupts the chromodomain–H3K9me3 interaction (Figs 2 and 3), which is important for HP1 recruitment to chromatin during interphase[11,13]. This disruption causes a net shift in the dynamic HP1–chromatin binding equilibrium towards the unbound state[7,9,38]. In this reaction sequence, dephosphorylation of H3S10 at the end of mitosis[28] re-establishes the overall association of HP1 with chromatin. We propose that this binary 'methyl/phos switching' permits dynamic control of the HP1–H3K9me interaction[39].

Intriguingly, the mechanism for HP1 release from M-phase chromatin does not involve a temporary loss of H3K9me3 (Fig. 1), but instead requires a combination of this unchanging mark and the dynamic H3S10ph modification that is only transiently added to chromatin during mitosis. We reason that stable transmission of the heterochromatin-defining H3K9me3 mark is needed to accurately convey, from one cell generation to the next, which regions of the genome are supposed to be permanently silenced. If removal of HP1 from M-phase chromatin were accomplished by H3K9me3-erasing demethylase activities, the epigenetic information underlying this mark- and effector-system would have to be accurately re-established at the end of every cell cycle.

In addition to H3S10 phosphorylation, other mechanisms might be involved in the mitotic release of HP1 from chromatin. These might include further modifications of the H3-tail (for example by acetylation on K14, ref. 25), HP1 proteins and/or their interaction partners[6]. Nevertheless, inhibition (Fig. 5), knockdown (Supplementary Fig. S8) or depletion (Fig. 6) of Aurora B is sufficient to cause aberrant interaction of all HP1 isoforms with mitotic, condensed chromatin. Although we cannot exclude the possibility that HP1 proteins themselves might be *in vivo* targets of Aurora B kinase activity (for example, we reproducibly observed increased association of the xHP1αW57A mutant protein with metaphase chromosomes assembled in ΔCPC extracts, see Fig. 6c), it is known that the phosphorylation level of HP1β and -γ does not increase during mitosis[7]. As phosphorylation of an H3K9me3 peptide is sufficient to dissociate HP1 from this site *in vitro* (Figs 2, 3), we conclude that Aurora B-mediated phosphorylation of H3S10 must be the central event in mitotic release of HP1 from chromatin.

Notably, a fraction of HP1α, but not HP1β or -γ, remains associated with the (peri-)centromeric chromosome region[9] (Fig. 5), where it performs important functions for centromere cohesion and kinetochore formation[10,40] and might be required to identify and define this specialized area of heterochromatin throughout the cell cycle. This mitotic retention of HP1α at centromeres depends on a carboxy-terminal region of the protein, but is independent of the chromodomain[8]. We therefore suggest that 'methyl/phos switching' uniformly disrupts HP1–chromatin interaction but that mechanisms other than chromodomain–H3K9me3 interaction are responsible for the lingering HP1α association with pericentromeric regions.

What is the function of HP1 dissociation from chromatin during M phase? It is tempting to speculate that removal of HP1 is important for allowing access by factors necessary for mediating proper chromatin condensation and faithful chromosome segregation during mitosis. Indeed, inhibition of Aurora B in vertebrate cells results in defects in chromosome alignment, segregation, chromatin-induced spindle assembly and cytokinesis[29,35,36,41,42]. Furthermore, mutation of H3S10 causes faulty chromosome segregation in *Tetrahymena* and *S. pombe*, organisms that rely on HP1 and H3K9me3 for the establishment and maintenance of heterochromatin[43,44], but not in *Saccharomyces cerevisiae*, an organism that lacks this silencing system[45]. Interestingly, most histone phosphorylation sites are rapidly phosphorylated early in M phase[46]. It remains to be seen whether these bursts in histone phosphorylation are directly involved in the release of proteins bound to interphase chromatin, which might need to be removed to ensure faithful progression through mitosis. It is conceivable that similar 'methyl/phos switches' play critical roles in governing other histone–non-histone or even non-histone–non-histone interactions.

METHODS

In vitro protein binding assays were performed as previously described[47]. Details of recombinant protein expression and purification, peptides, and kinase reaction conditions can be found in Supplementary Methods. Western blotting and immunofluorescence analyses were done according to standard protocols (see Supplementary Table S1 for antibodies and dilutions). Anti-xINCENP antibodies[36] were used to deplete all components of the CPC from meiotic metaphase II-arrested *Xenopus laevis* egg extracts[34]. Replicated metaphase chromosomes were assembled and purified as previously decribed[36]. Details of association and competition experiments can be found in Supplementary Methods.

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1. Felsenfeld, G. & Groudine, M. Controlling the double helix. *Nature* **421,** 448–453 (2003).
2. Khorasanizadeh, S. The nucleosome: from genomic organization to genomic regulation. *Cell* **116,** 259–272 (2004).
3. Grewal, S. I. & Moazed, D. Heterochromatin and epigenetic control of gene expression. *Science* **301,** 798–802 (2003).
4. Grewal, S. I. & Elgin, S. C. Heterochromatin: new possibilities for the inheritance of structure. *Curr. Opin. Genet. Dev.* **12,** 178–187 (2002).
5. Li, Y., Kirschmann, D. A. & Wallrath, L. L. Does heterochromatin protein 1 always follow code? *Proc. Natl Acad. Sci. USA* **99** (suppl.), 16462–16469 (2002).
6. Eissenberg, J. C. & Elgin, S. C. The HP1 protein family: getting a grip on chromatin. *Curr. Opin. Genet. Dev.* **10,** 204–210 (2000).
7. Minc, E., Allory, Y., Worman, H. J., Courvalin, J. C. & Buendia, B. Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* **108,** 220–234 (1999).
8. Hayakawa, T., Haraguchi, T., Masumoto, H. & Hiraoka, Y. Cell cycle behaviour of human HP1 subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. *J. Cell Sci.* **116,** 3327–3338 (2003).
9. Schmiedeberg, L., Weisshart, K., Diekmann, S., Meyer Zu Hoerste, G. & Hemmerich, P. High- and low-mobility populations of HP1 in heterochromatin of mammalian cells. *Mol. Biol. Cell* **15,** 2819–2833 (2004).
10. Pidoux, A. L. & Allshire, R. C. Kinetochore and heterochromatin domains of the fission yeast centromere. *Chromosome Res.* **12,** 521–534 (2004).
11. Stewart, M. D., Li, J. & Wong, J. Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol. Cell. Biol.* **25,** 2525–2538 (2005).
12. Peters, A. H. *et al.* Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol. Cell* **12,** 1577–1589 (2003).
13. Thiru, A. *et al.* Structural basis of HP1/PXVXL motif peptide interactions and HP1 localisation to heterochromatin. *EMBO J.* **23,** 489–499 (2004).
14. Sims, R. J. III, Nishioka, K. & Reinberg, D. Histone lysine methylation: a signature for chromatin function. *Trends Genet.* **19,** 629–639 (2003).
15. Schotta, G., Lachner, M., Peters, A. H. & Jenuwein, T. The indexing potential of histone lysine methylation. *Novartis Found. Symp.* **259,** 22–37 (2004).
16. Bannister, A. J. *et al.* Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410,** 120–124 (2001).
17. Jacobs, S. A. *et al.* Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *EMBO J.* **20,** 5232–5241 (2001).

18. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410,** 116–120 (2001).
19. Jacobs, S. A. & Khorasanizadeh, S. Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science* **295,** 2080–2083 (2002).
20. Fischle, W. *et al.* Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev.* **17,** 1870–1881 (2003).
21. Maison, C. & Almouzni, G. HP1 and the dynamics of heterochromatin maintenance. *Nature Rev. Mol. Cell Biol.* **5,** 296–304 (2004).
22. Cheutin, T. *et al.* Maintenance of stable heterochromatin domains by dynamic HP1 binding. *Science* **299,** 721–725 (2003).
23. Festenstein, R. *et al.* Modulation of heterochromatin protein 1 dynamics in primary mammalian cells. *Science* **299,** 719–721 (2003).
24. Maison, C. *et al.* Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. *Nature Genet.* **30,** 329–334 (2002).
25. Mateescu, B., England, P., Halgand, F., Yaniv, M. & Muchardt, C. Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. *EMBO Rep.* **5,** 490–496 (2004).
26. Syka, J. E. *et al.* Novel linear quadrupole ion trap/FT mass spectrometer: performance characterization and use in the comparative analysis of histone H3 post-translational modifications. *J. Proteome Res.* **3,** 621–626 (2004).
27. Hendzel, M. J. *et al.* Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106,** 348–360 (1997).
28. Prigent, C. & Dimitrov, S. Phosphorylation of serine 10 in histone H3, what for? *J. Cell Sci.* **116,** 3677–3685 (2003).
29. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406,** 593–599 (2000).
30. Yasui, Y. *et al.* Autophosphorylation of a newly identified site of Aurora-B is indispensable for cytokinesis. *J. Biol. Chem.* **279,** 12997–13003 (2004).
31. Honda, R., Korner, R. & Nigg, E. A. Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. *Mol. Biol. Cell* **14,** 3325–3341 (2003).
32. Chen, J. *et al.* Survivin enhances Aurora-B kinase activity and localizes Aurora-B in human cells. *J. Biol. Chem.* **278,** 486–490 (2003).
33. Murray, A. W. Cell cycle extracts. *Methods Cell Biol.* **36,** 581–605 (1991).
34. Andrews, P. D., Knatko, E., Moore, W. J. & Swedlow, J. R. Mitotic mechanics: the auroras come into view. *Curr. Opin. Cell Biol.* **15,** 672–683 (2003).
35. Hauf, S. *et al.* The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* **161,** 281–294 (2003).
36. Sampath, S. C. *et al.* The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. *Cell* **118,** 187–202 (2004).
37. Nielsen, P. R. *et al.* Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature* **416,** 103–107 (2002).
38. Sugimoto, K., Tasaka, H. & Dotsu, M. Molecular behaviour in living mitotic cells of human centromere heterochromatin protein HPLα ectopically expressed as a fusion to red fluorescent protein. *Cell Struct. Funct.* **26,** 705–718 (2001).
39. Fischle, W., Wang, Y. & Allis, C. D. Binary switches and modification cassettes in histone biology and beyond. *Nature* **425,** 475–479 (2003).
40. Obuse, C. *et al.* A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. *Nature Cell Biol.* **6,** 1135–1141 (2004).
41. Ditchfield, C. *et al.* Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *J. Cell Biol.* **161,** 267–280 (2003).
42. Lampson, M. A., Renduchitala, K., Khodjakov, A. & Kapoor, T. M. Correcting improper chromosome–spindle attachments during cell division. *Nature Cell Biol.* **6,** 232–237 (2004).
43. Mellone, B. G. *et al.* Centromere silencing and function in fission yeast is governed by the amino terminus of histone H3. *Curr. Biol.* **13,** 1748–1757 (2003).
44. Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A. & Allis, C. D. Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* **97,** 99–109 (1999).
45. Hsu, J. Y. *et al.* Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell* **102,** 279–291 (2000).
46. Dai, J., Sultan, S., Taylor, S. S. & Higgins, J. M. The kinase haspin is required for mitotic histone H3 Thr 3 phosphorylation and normal metaphase chromosome alignment. *Genes Dev.* **19,** 472–488 (2005).
47. Jacobs, S. A., Fischle, W. & Khorasanizadeh, S. Assays for the determination of structure and dynamics of the interaction of the chromodomain with histone peptides. *Methods Enzymol.* **376,** 131–148 (2004).

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