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A novel role for the Aurora B kinase in epigenetic marking of silent chromatin in differentiated postmitotic cells

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Combinatorial modifications of the core histones have the potential to fine-tune the epigenetic regulation of chromatin states. The Aurora B kinase is responsible for generating the double histone H3 modification tri-methylated K9/phosphorylated S10 (H3K9me3/S10ph), which has been implicated in chromosome condensation during mitosis. In this study, we have identified a novel role for Aurora B in epigenetic marking of silent chromatin during cell differentiation. We find that phosphorylation of H3 S10 by Aurora B generates high levels of the double H3K9me3/S10ph modification in differentiated postmitotic cells and also results in delocalisation of HP1β away from heterochromatin in terminally differentiated plasma cells. Microarray analysis of the H3K9me3/S10ph modification shows a striking increase in the modification across repressed genes during differentiation of mesenchymal stem cells. Our results provide evidence that the Aurora B kinase has a role in marking silent chromatin independently of the cell cycle and suggest that targeting of Aurora B-mediated phosphorylation of H3 S10 to repressed genes could be a mechanism for epigenetic silencing of gene expression.

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Introduction

Establishing specific gene expression programmes during cell differentiation requires controlled activation and silencing of large numbers of genes. Once these patterns of expression or repression have been set up, they must be kept in place to maintain the identity of the differentiating cell. Chromatin is known to play a key role in these processes. It can exert its effects both at the local level, with single nucleosomes modulating access of factors to promoters and enhancers, and on a more global level through large-scale condensation of megabase regions or even entire chromosomes (Heard, 2005; Dillon, 2006). Chromatin-mediated silencing of gene expression is strongly influenced by epigenetic modifications of the core histones, which include methylation and acetylation of lysine residues and phosphorylation of serine residues. These modifications can directly affect chromatin packaging as well as influence the binding of chromatin proteins and transcription factors (reviewed in Turner, 2007).

The presence of epigenetic modifications can alter the structure of gene expression domains that include the gene and surrounding sequences, and when these modifications extend over larger regions, they facilitate packaging of the DNA into visibly condensed heterochromatin. Constitutive heterochromatin, which is present throughout the cell cycle, is relatively gene poor and enriched for repetitive satellite sequences. Facultative heterochromatin is quite different in that it is the result of a developmentally regulated condensation of gene-rich euchromatic regions, which are decondensed and transcriptionally active in some cell types. Some of the most dramatic examples of facultative heterochromatin are observed during the final stages of cell differentiation. Terminally differentiated cells that exhibit this type of widespread heterochromatinisation include rod photoreceptor cells (Araki *et al*, 1988), neutrophils (Olins and Olins, 2005), nucleated erythrocytes (Gilbert *et al*, 2003) and plasma cells (Schulze *et al*, 1984). It seems likely that formation of facultative heterochromatin during cell differentiation involves at least some of the silencing mechanisms that affect gene expression locally. This suggests that there may be a point during cell differentiation when these local gene-silencing events reach a critical mass that is sufficient to give rise to visibly condensed facultative heterochromatin.

Methylation of histone H3 lysine 9 (K9) has been shown to be involved in epigenetic silencing of euchromatic genes and also marks the constitutive heterochromatin that is found close to centromeres (Bannister *et al*, 2001; Nielsen *et al*, 2001; Ayyanathan *et al*, 2003; Hediger and Gasser, 2006). An important function of methylated H3K9 groups is to act as a recognition site for heterochromatin protein 1 (HP1), which binds to the methylated lysine via the chromodomain region

(Lachner *et al*, 2001). HP1 was originally identified as a modifier of position effect variegation in *Drosophila* and has been shown to associate strongly with constitutive heterochromatin. There is also evidence that it is recruited directly to some gene promoters where it participates in transcriptional silencing (Nielsen *et al*, 2001; Ogawa *et al*, 2002; Ayyanathan *et al*, 2003). Three HP1 proteins have been identified in mammals (α, β and γ). HP1α is mainly associated with constitutive heterochromatin, HP1β is present both on pericentric heterochromatin and euchromatin, whereas HP1γ is predominantly euchromatic (Minc *et al*, 1999).

Binding of HP1 to H3K9 has been shown to be affected by the presence of a phosphate group at serine 10 (S10ph). Phosphorylation of H3 S10 during G2/M has been found to prevent HP1β from binding to the adjacent H3K9me residue. As a result, HP1β is released from chromatin at the onset of mitosis (Fischle *et al*, 2005; Hirota *et al*, 2005). The kinase responsible for S10 phosphorylation is the Aurora B kinase, a component of the chromosomal passenger complex, which modulates chromosome structure and segregation at mitosis by promoting HP1 displacement from the chromosomes, and chromosome alignment and attachment to the microtubules of the mitotic spindle (Vader *et al*, 2006). The other subunits of the complex, INCENP, Survivin and Borealin, are non-enzymatic and are involved in regulating and targeting Aurora B to its substrates. In non-transformed cells, Aurora B has, until now, been considered to be highly cell-cycle regulated and to be involved primarily in protein phosphorylation during mitosis. The double histone H3 tri-methylated K9/phosphorylated S10 (H3K9me3/S10ph) modification generated by Aurora B is known to be widely distributed on mitotic chromosomes, and inhibition of S10 phosphorylation has been shown to interfere with chromosome condensation during mitosis (Hendzel *et al*, 1997; Van Hooser *et al*, 1998; Wei *et al*, 1998). The double modification has been proposed to be a marker of M phase (Fischle *et al*, 2005), but it has not been shown previously to be involved in modulating chromatin structure outside mitosis.

Here we show that in addition to its functions during mitosis, the Aurora B kinase mediates formation of the double H3K9me3/S10ph modification independently of the cell cycle during cell differentiation. In terminally differentiated postmitotic plasma cells, this results in displacement of HP1β from facultative heterochromatin. We also use microarray analysis to demonstrate the presence of domains of H3K9me3/S10ph at silent genes in differentiated cells. Our results suggest that binary modifications can play an important role in modulating the effects of specific histone modifications at different stages of development.

Results

Experimental systems

To identify epigenetic markers that are involved in long-term silencing of gene expression during cell differentiation, we initially screened facultative heterochromatin in terminally differentiated bone marrow plasma cells for the presence of candidate marks that might play a role in silencing. The results of this analysis revealed an unexpected correlation between the presence of visible heterochromatin and the binary K9me3/S10ph modification on histone H3. The double modification was detected by immunofluorescence (IF) using an antibody that specifically recognises these modifications when they are present in combination on the same histone H3 molecule (Figure 1A). The specificity of the antibody was confirmed by western blotting (Supplementary Figure S2), peptide ELISA and peptide competition, which showed that it did not crossreact with tri-methyl K9 (K9me3) alone, phospho-S10 (S10ph) alone, di-methyl K9/phospho-S10 (K9me2/S10ph) or tri-methyl K27/phospho-S28 (K27me3/S28ph) (Figure 1A). Analysis by IF using this antibody showed strong staining of facultative heterochromatin in 100% of bone marrow plasma cells. A particularly striking aspect of this result is the fact that plasma cells are postmitotic, whereas the double modification had previously been thought to be associated with the G2/M phase of the cell cycle and displacement of HP1 from mitotic chromosomes. This finding led us to perform a detailed analysis of the role of the double H3K9me3/S10ph modification in heterochromatin formation and epigenetic marking of silent genes during cell commitment and differentiation.

The model systems that were used for the analysis were differentiation of mesenchymal stem cells and terminal differentiation of B cells into plasma cells (Figure 1B and C). The multipotent mesenchymal stem cell line C3H10T1/2 can be induced to differentiate into osteocytes, chondrocytes and adipocytes and has been used extensively as an *in vitro* model for mesenchymal stem cell differentiation (Shea *et al*, 2003). In this study, the cells were differentiated into osteocytes by treatment with thyroxine and dexamethasone (Peister *et al*, 2004). Plasma cells were generated *in vitro* by differentiation of primary splenic B cells (see Materials and methods and Supplementary Figure S1). Terminal differentiation of B cells into plasma cells is accompanied by widespread gene silencing and formation of facultative heterochromatin as the cell becomes specialised for the production and secretion of large amounts of antibody (Underhill *et al*, 2003). The *in vitro* differentiation system (Supplementary Figure S1) offered the advantage of providing enough plasma cells for chromatin analysis.

Because of the known link between the presence of the double modification and mitosis, the cell division status of the cells was determined before and after differentiation by measurement of bromodeoxyuridine (BrdU) incorporation (Figure 1D). The activated B cells were clearly cycling (75% $BrdU^+$), whereas the plasma cells were almost entirely postmitotic (3% $BrdU^+$). The undifferentiated C3H10T1/2 mesenchymal stem cells were also actively cycling (89% $BrdU^+$) and became largely postmitotic after differentiation (8% $BrdU^+$).

The histone H3K9me3/S10ph double modification becomes enriched on chromatin of differentiated postmitotic cells

The mesenchymal stem-cell and B-cell differentiation systems were used to analyse the distribution of the double modification before and after cell differentiation and exit from the cell cycle. Immunostaining was carried out using the anti-H3K9me3/S10ph antibody. Staining of the undifferentiated mesenchymal stem cells showed a strong punctate staining for the double modification in 23% of the cells. The punctuate staining coincided with the DAPI-dense pericentromeric heterochromatin (Figure 2A). The larger size of these cells

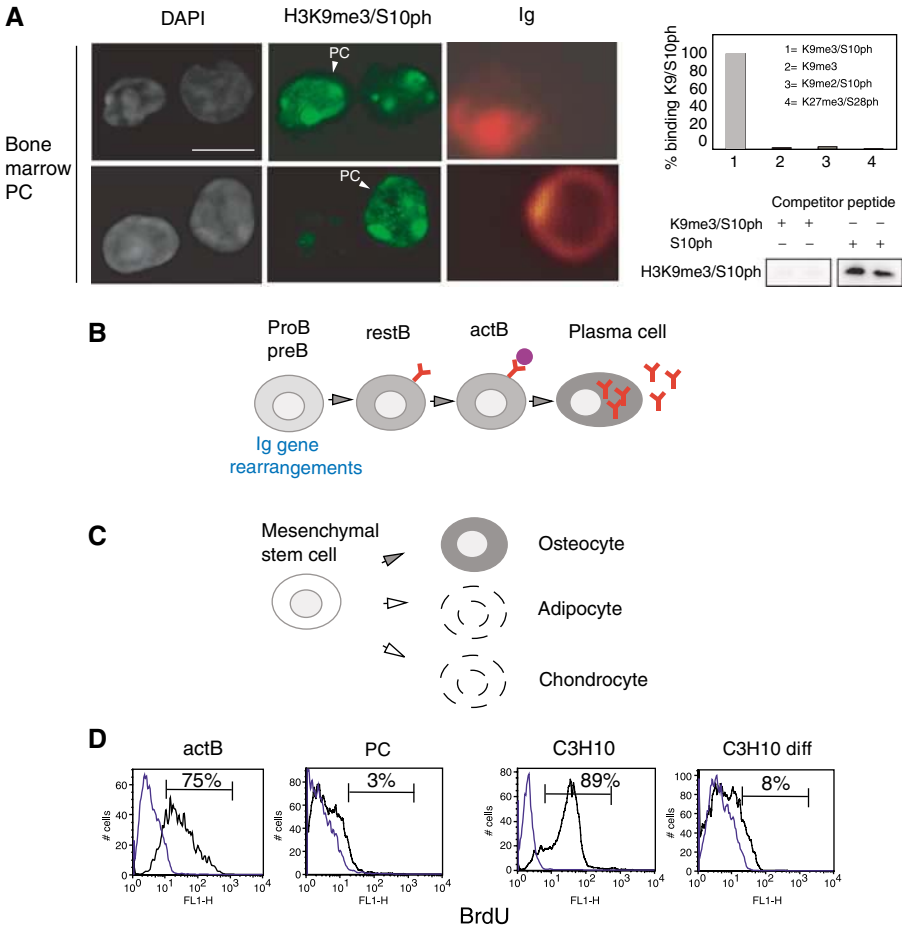


Figure 1 Increased levels of H3K9me3/S10ph in plasma cell chromatin. (**A**) Immunofluorescence (IF) analysis of the distribution of H3K9me3/S10ph in bone marrow plasma cells (PC). Plasma cells (indicated by arrows) were identified by high levels of cytoplasmic immunoglobulins (Igs). Scale bar = 10 μm. Histograms represent a peptide ELISA assay showing that the α-H3K9me3/S10ph antibody has high affinity for a peptide spanning aa 5–13 of histone H3 containing K9me3 and S10ph (1) and has negligible crossreactivity with peptides of the same region with either K9me3 alone (2) or K9me2/S10ph (3) or a peptide corresponding to aa 24–33 of H3 containing K27me3/S28ph (4). Western blotting with the α-H3K9me3/S10ph antibody shows that a peptide corresponding to aa 5–13 of histone H3 containing S10ph alone does not compete for binding to the epitope in a peptide competition assay. (**B**) Scheme showing the stages of B-cell development. Rearrangement of heavy- and light-chain Ig genes at the pro- and pre-B-cell stages gives rise to resting B cells, which are activated by encounter with antigen and differentiate into postmitotic antibody-secreting plasma cells. ProB, preB = progenitor and precursor B cells; rest B = resting B cells; act B = activated B cells. (**C**) Cell lineages originating from differentiation of C3H10T1/2 mesenchymal stem cells. (**D**) Bromodeoxyuridine (BrdU) incorporation in activated B cells, plasma cells and mesenchymal cells. The cells were incubated in medium with 10 μM BrdU. Incorporation of BrdU was measured by staining with FITC-conjugated α-BrdU antibody (Beckton and Dickinson) and FACS analysis. Staining for BrdU in the cell populations (black) is compared to background levels of autofluorescence in unstained cells (blue).

identified them as being in G2 and this was confirmed by FACS analysis of the level of the double modification in cells that were gated according to DNA content (Figure 2D). The results of the immunostaining and FACS are in agreement with the observation by Fischle *et al* (2005) of a global increase in H3K9me3/S10ph that coincides with the onset of G2/M. The staining was much lower in the remaining cells and many of the cells showed little signal. The variation in the signal that was observed in the dull cells is likely to reflect progression through the different stages of the cell cycle. A quite different result was obtained when the cells were induced to differentiate along the osteogenic pathway. The differentiated postmitotic cells showed staining in the nuclei of all the cells analysed (Figure 2A). The staining in the differentiated cells was found at the pericentromeric heterochromatin and also as a more diffuse signal that appeared to extend over much of the rest of the chromatin.

In the activated B-cell cultures, 27% of the cells showed a strong punctate staining for the double modification. Once again, the larger size of the positive cells and the FACS analysis identified them as being in G2 (Figure 2B and E). A smaller proportion (16%) showed an intermediate staining of the pericentromeric heterochromatin, but the majority of the cells (56%) were negative for the double modification. The differentiated plasma cells showed a very strong nuclear staining in approximately 60% of the cells, despite the fact that these cells were clearly postmitotic. In the positively staining cells, the signal for H3K9me3/S10ph was particularly intense on the large masses of DAPI-dense facultative heterochromatin, but was also present as a more diffuse staining on the rest of the chromatin (Figure 2B). The observation that around 40% of the cells were negative for the double modification could be related to the fact that these are short-lived plasma cells in which a proportion of the cells

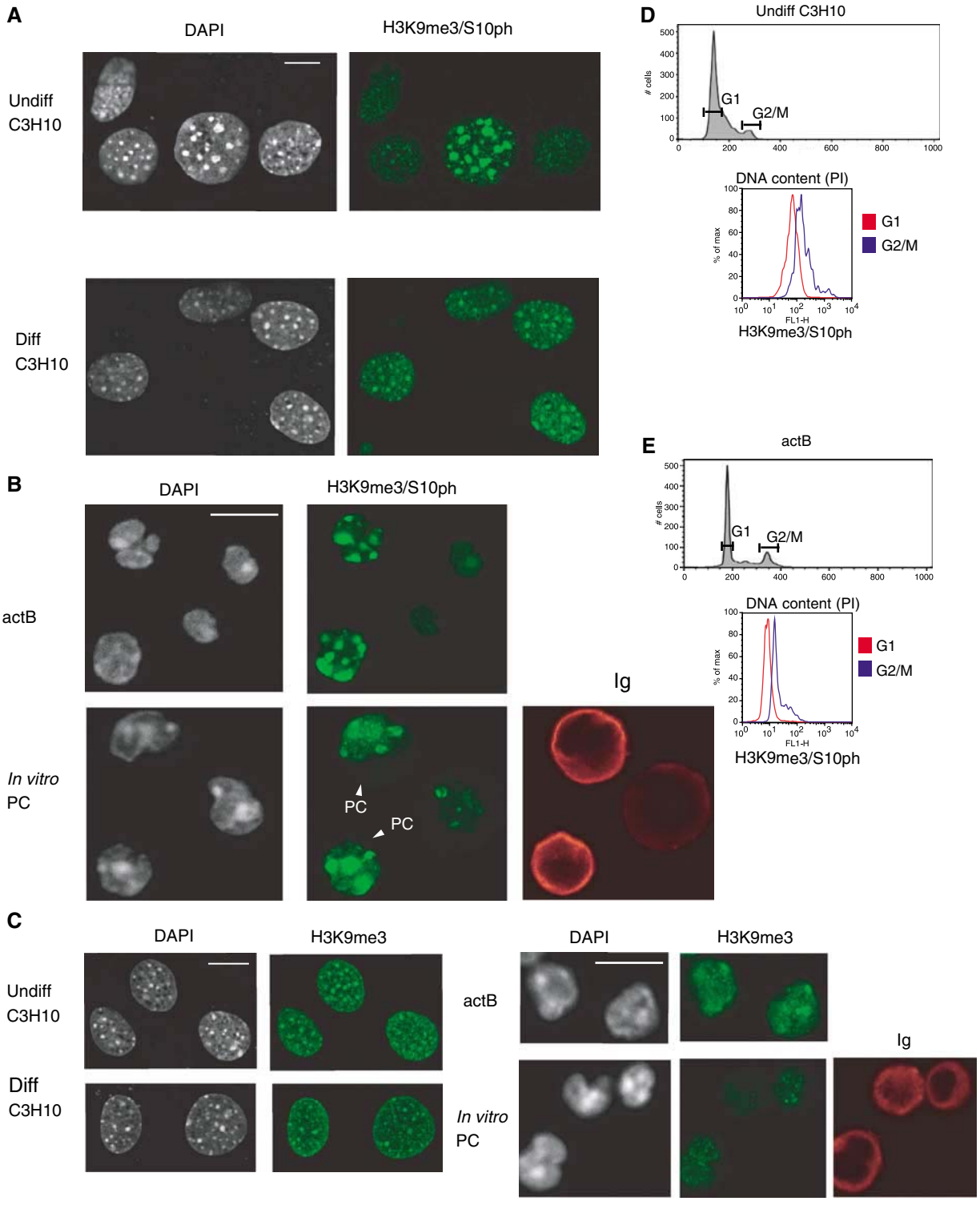


Figure 2 Distribution of the histone H3K9me3/S10ph double modification during cell differentiation. (**A**) H3K9me3/S10ph staining before and after differentiation of C3H10T1/2 mesenchymal stem cells. (**B**) H3 me3K9/pS10 staining in activated B cells and in plasma cells differentiated *in vitro* from splenic B cells. Plasma cells are indicated by arrows. (**C**) Staining for H3K9me3 in mesenchymal, activated B and plasma cells. (**D**, **E**) FACS analysis was used to show that the brightly stained cells observed by immunofluorescent staining were predominantly G2/M. Cells were gated into G1 and G2/M populations by propidium iodide (PI) staining for DNA content, and staining with α-H3K9me3/S10ph was measured in these populations.

are likely to have chromatin that is already undergoing pre-apoptotic changes. It should be noted that bone marrow plasma cells, which have been shown to be long-lived (Calame, 2006), showed a strong staining for the double modification in all the cells analysed (Figure 1A).

The staining pattern for H3K9me3/S10ph was compared with the pattern obtained using an antibody that was specific for H3K9me3 alone (Figure 2C). There was no difference in the level and distribution of the single modification between undifferentiated and differentiated mesenchymal stem cells.

The differentiated plasma cells gave a lower H3K9me3 signal compared to activated B cells. This may reflect the very high levels of the double H3K9me3/S10ph modification that were observed in plasma cells.

Analysis of the double and single modifications by western blotting gave results that broadly supported the conclusions from the immunostaining. In particular, the plasma cells showed a much higher global level of the double modification than activated B cells (Figure 3A). The modification was completely absent from resting B cells. Significant levels of the double modification were also present in differentiated postmitotic C3H10T1/2 cells (Figure 3A). The levels were somewhat lower than the level in undifferentiated cells, but this was likely to reflect the fact that these cells were cycling and therefore included significant numbers of G2/M cells. The levels of the single H3K9me3 and H3S10ph modifications detected by western blotting showed only small variations between differentiated and undifferentiated cells (Figure 3A and B). The lower level of the H3K9me3 single modification that was observed by immunostaining of plasma cells compared with the level measured by western blotting (Figures 2C and 3B) could be due to reduced accessibility of the modification to intracellular antibody staining, suggesting that it may be located within the heterochromatin mass.

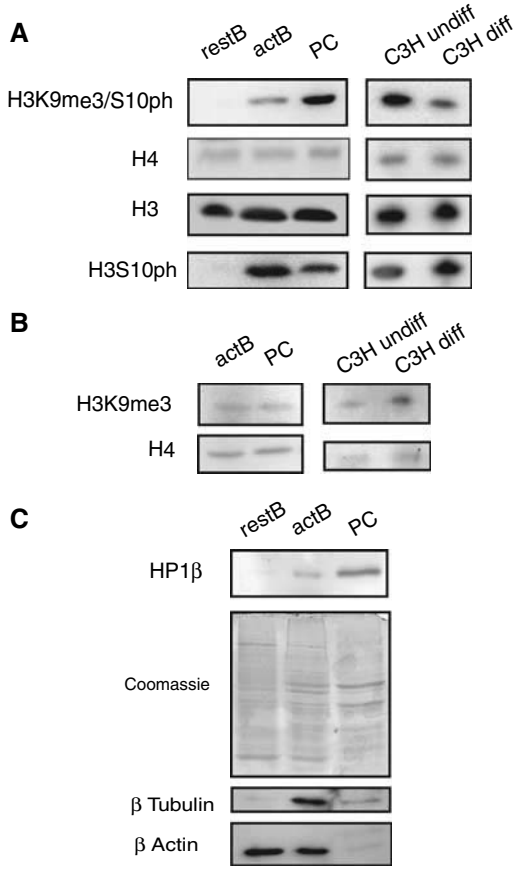


Figure 3 Global levels of H3K9me3/S10ph following cell differentiation. Western blotting of acid extracts from nuclei of resting B cells (rest B), activated B cells (act B), plasma cells (PC) and undifferentiated (C3H undiff) and differentiated (C3H diff) mesenchymal cells using (**A**) α-H3K9me3/S10ph and α-H3S10ph and (**B**) α-H3K9me3. The levels of histone H4 and H3 were used as a loading control. (**C**) Whole-cell extracts from purified resting B, activated B and plasma cells were analysed by western blotting using an α-HP1β antibody. The Coomassie-stained gel and western blots for β-tubulin and β-actin are shown as loading controls.

Aurora B kinase is responsible for generating the double histone H3K9me3/S10ph modification in non-cycling differentiated cells

We next tested whether the histone H3K9me3/S10ph mark in plasma cells and differentiated mesenchymal stem cells correlates with the presence of the Aurora B kinase, which is known to be responsible for generating the phospho-S10 moiety of the double modification during mitosis (Fischle *et al*, 2005). Immunostaining of postmitotic plasma cells showed intense staining for Aurora B, which coincided with the large DAPI-dense regions of facultative heterochromatin (Figure 4B). More diffuse staining was also observed on the rest of the chromatin. This pattern correlates closely with the distribution of the H3K9me3/S10ph staining (Figure 2B). The proportion of plasma cells that stained positively for Aurora B (54%) was similar to the proportion that was positive for the double modification. Aurora B was also present in 100% of differentiated mesenchymal cells where it was concentrated into dot-like nuclear foci that did not coincide with constitutive pericentromeric heterochromatin (Figure 4A). Activated B cells and undifferentiated mesenchymal stem cells both gave a pattern of Aurora B staining that was similar to the H3K9me3/S10ph distribution with staining of the pericentromeric heterochromatin observed in the G2 cells and largely absent from G1 cells (Figure 4A and B). This distribution was confirmed by FACS analysis of the levels of Aurora B in G1 and G2/M cells (Figure 4C and D).

Western blotting analysis also showed that Aurora B is present in postmitotic plasma cells and differentiated mesenchymal cells (Figure 4E). The analysis of undifferentiated mesenchymal stem cells was also carried out on cells that were sorted into G1 and G2/M populations and showed that the level of Aurora B is higher in differentiated cells than in undifferentiated G1 cells (Figure 4E). These results indicate that Aurora B persists in differentiated postmitotic cells and provide support for the idea that it is involved in the epigenetic regulation of cell differentiation.

To directly test whether Aurora B is responsible for generating the double modification during differentiation, *in vitro*-differentiated plasma cells were treated with hesperadin, which specifically inhibits Aurora B activity (Hauf *et al*, 2003). Inhibition of the kinase resulted in a drastic reduction in the amount of H3K9me3/S10ph modification that was observed by western blotting (Figure 4F). This leads us to conclude that phosphorylation of H3 S10 by Aurora B is responsible for increasing the level of double H3K9me3/S10ph modification in postmitotic plasma cells.

Phosphorylation of histone H3 S10 blocks binding of HP1β to di- and tri-methylated K9

The presence of phosphorylated S10 has the potential to affect binding of proteins that recognise the adjacent methylated K9. One possible candidate for such an effect is HP1. Phosphorylation of S10 has been shown to modulate the binding of HP1 proteins to methylated histone H3K9, although different effects have been reported and there have been conflicting results for binding of different HP1 family members to di- and tri-methyl H3K9 (Mateescu *et al*, 2004; Fischle *et al*, 2005). We used surface plasmon

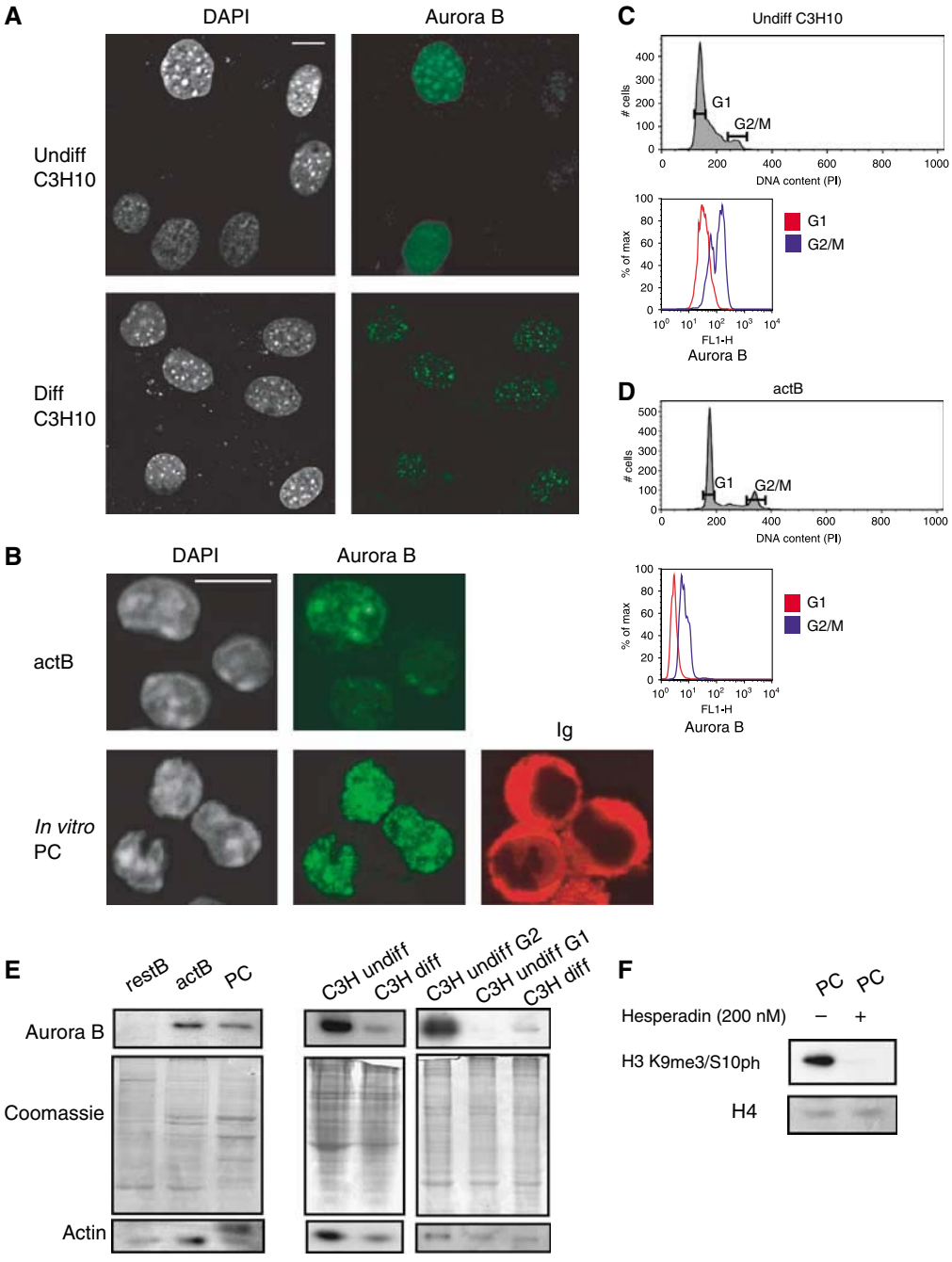


Figure 4 Aurora B persists in the nucleus following differentiation and its kinase activity is required for generation of the double H3K9me3/S10ph mark in differentiated postmitotic cells. (**A**) Distribution of Aurora B in undifferentiated and differentiated C3H10T1/2 cells. (**B**) Staining of Aurora B in activated B and plasma cells. (**C**, **D**) FACS analysis was used to show that the brightly stained cells observed by immunofluorescent staining were predominantly G2/M. Cells were gated into G1 and G2/M as in Figure 2 and staining with α-Aurora B was measured. (**E**) Whole-cell extracts from purified resting B, activated B and plasma cells and undifferentiated and differentiated C3H10T1/2 cells were analysed by western blotting using an α-Aurora B antibody. Aurora B levels were also measured in undifferentiated C3H10T1/2 cells that had been FACS sorted into G1 and G2/M populations (right panel; see Materials and methods for details). (**F**) Western blot with α-H3K9me3/S10ph of acid-extracted proteins from plasma cells cultured for 1 h with 200 nM Aurora B inhibitor hesperadin or in medium alone.

resonance (SPR) to examine the effect of S10 phosphorylation on the *in vitro* binding of HP1β to di- and tri-methyl H3K9. The SPR assay made use of a recombinant 6 × His-HP1β protein and H3 peptides containing covalent modifications. Phosphorylation of S10 markedly decreased the interaction of HP1β with both K9me3 and K9me2 peptides (Figure 5A). This result supports the idea that S10 phosphorylation forms part of a binary switch that regulates binding of HP1β to chromatin.

Aurora B-dependent release of HP1β from heterochromatin in plasma cells

We next set out to investigate whether the presence of the double H3K9me3/S10ph modification affects the *in vivo* binding of HP1β to chromatin during cell differentiation. Immunostaining was used to determine whether HP1β is associated with the facultative heterochromatin of plasma cells (Figure 5C). In activated B cells, HP1β showed the expected localisation to pericentromeric heterochromatin

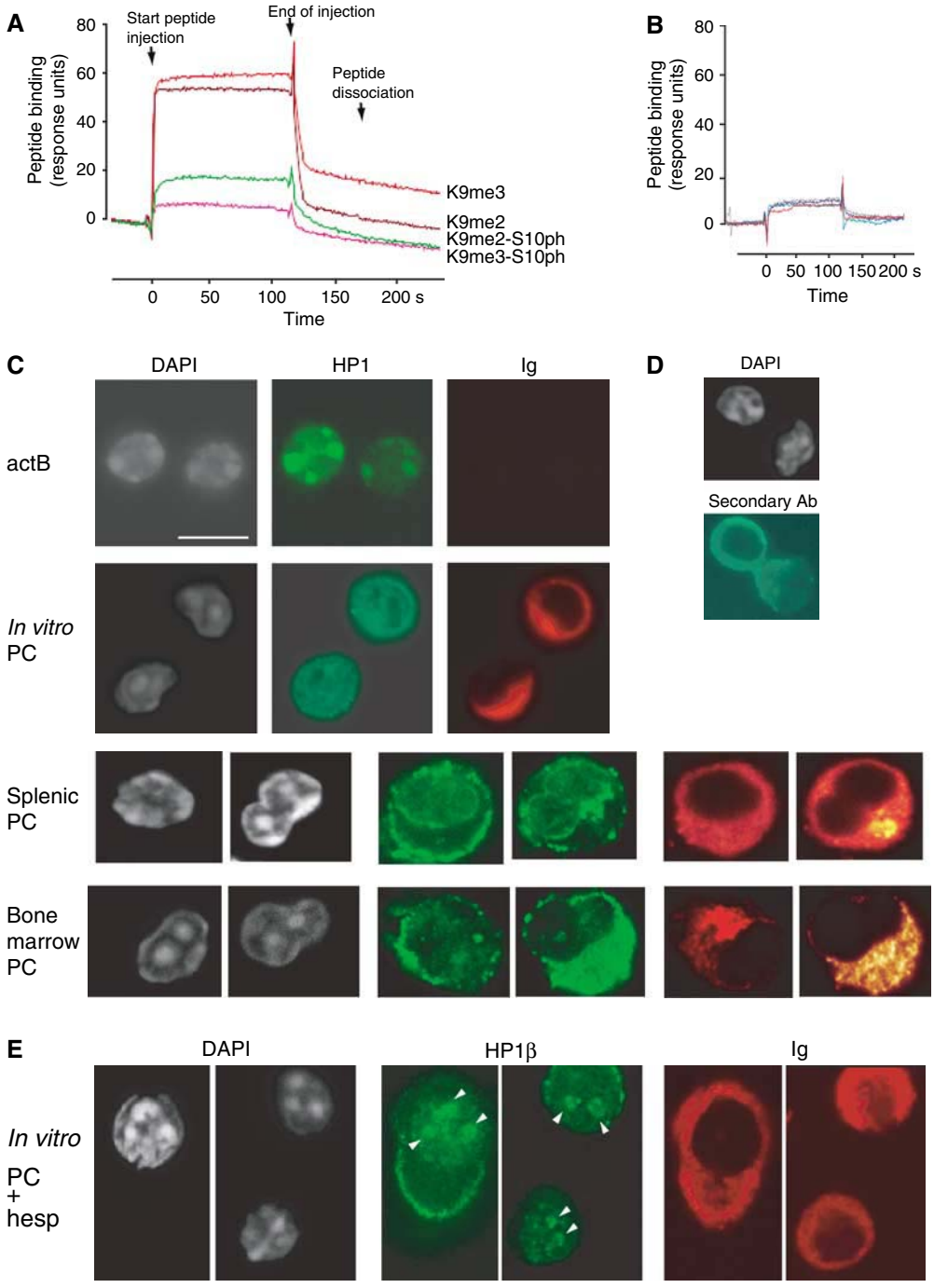


Figure 5 HP1β is relocated away from heterochromatin in plasma cells. (**A**) Surface plasmon resonance (SPR) analysis of binding of His-HP1β to peptides corresponding to aa 1–15 of histone H3 with the indicated modifications. Peptides (50 μM) were injected over His-HP1β immobilised on a Biacore flow cell. The presence of phosphorylated S10 reduces the affinity of HP1β for peptides containing both K9me3 and K9me2. (**B**) Nonspecific binding of the peptides to the chip when injected over a flow cell control lacking HP1β. (**C**) Distribution of HP1β relative to DAPI-intense regions in activated B and plasma cells. The analysis was carried out on plasma cells that were either differentiated *in vitro* (*in vitro* PC), isolated from the spleen of LPS-immunised mice (splenic PC) or isolated from the bone marrow of ovalbumin-immunised mice (bone marrow PC). (**D**) Staining of plasma cells with the Alexa 488 α-rat secondary antibody alone showed some crossreactivity with the mouse immunoglobulin (Ig) in the cytoplasm but not the nucleus. (**E**) Incubation of plasma cells in a medium containing 200 nM hesperadin for 1.5 h results in relocalisation of HP1β to heterochromatin (indicated by arrows) in 29% of the cells.

clusters. However, when the immunostaining was carried out on *in vitro*-differentiated plasma cells, HP1β was depleted from the heterochromatic regions and was present more abundantly in regions of the nucleus that stained less brightly with DAPI. The cytoplasmic HP1β staining in plasma cells was mainly due to crossreactivity of the secondary anti-rat antibody with mouse immunoglobulins (Igs) (Figure 5D). Western blot analysis showed that HP1β is still present in plasma cells, thereby excluding the possibility that the absence of heterochromatin staining is due to loss of HP1β from the cell (Figure 3C).

To test whether the results obtained with the *in vitro*-differentiated cells reflected the distribution of HP1 in plasma cells *in vivo*, we assessed HP1β localisation in the nuclei of

short- and long-lived plasma cells isolated *ex vivo*. The short-lived plasma cells were isolated from spleens of LPS-immunised mice and the long-lived plasma cells were obtained from the bone marrow of mice that had been immunised with ovalbumin (see Materials and methods). Interestingly, the two types of cells gave different results. The short-lived splenic plasma cells had a similar HP1β distribution to the *in vitro*-differentiated cells, whereas in the long-lived bone marrow cells, the localisation of HP1β to the heterochromatin was restored (Figure 5C). Analysis of the subnuclear localisation of HP1β in differentiated C3H10T1/2 cells showed that it is not visibly delocalised from heterochromatin (data not shown). This can be explained by the lower levels of the double H3K9me3/S10ph modification in these cells compared with plasma cells.

To establish whether Aurora B activity is responsible for HP1β release from heterochromatin in short-lived plasma cells, we analysed the effect of the Aurora B inhibitor hesperadin on HP1β distribution in plasma cell nuclei. Incubation of *in vitro*-differentiated plasma cells with hesperadin for 1.5 h resulted in relocalisation of HP1β to the heterochromatic foci in 29% of the cells (Figure 5E), compared with 1% of untreated cells. This result demonstrates that Aurora B is involved in modulating the interaction of HP1β with chromatin outside mitosis. The comparison between different types of plasma cells also suggests that the binding of HP1β to heterochromatin is part of a complex phenomenon, with other factors coming into play during the transition from short- to long-lived plasma cells.

Domains of H3K9me3/S10ph form at repressed genes in differentiated cells

The association of the double H3K9me3/S10ph modification with facultative heterochromatin in plasma cells suggests that it could be an epigenetic marker for gene silencing. To address this question directly, a custom oligonucleotide tiling microarray was used to analyse the modification at the level of individual genes and to relate it to transcriptional activity. The microarray covered a 2-Mb gene-rich region of mouse chromosome 3, with an average resolution of 100 bp (Figure 6). The 67 genes in the region have a variety of expression patterns ranging from ubiquitous to highly tissue specific. Expression of all of the genes was measured in C3H10T1/2 cells before and after differentiation by quantitative real-time PCR (data not shown).

Chromatin from undifferentiated and differentiated C3H10T1/2 cells was precipitated using the α-H3K9me3/S10ph and α-H3K9me3 antibodies and the DNA was hybridised to the microarray. Inspection of the pattern obtained for H3K9me3/S10ph revealed a strong enrichment for the double modification on repressed genes in differentiated cells (defined by average positive $\log_2$ IP/input ratio) (Figure 6, yellow shadowed domains). In contrast, a generally negative $\log_2$ ratio for K9me3/S10ph was found at transcriptionally active genes (purple shadowed domains). A striking feature of the pattern of enrichment of the double modification was that it appears to define domains that can extend across several adjacent repressed genes (Figures 6 and 7). The pattern is dependent on differentiation, as enrichment for H3K9me3/S10ph on repressed genes was not observed in undifferentiated C3H10T1/2 cells (Figures 6 and 7, light-blue trace).

In contrast, the highest levels of the single H3K9me3 modification are observed in undifferentiated cells, with peaks of enrichment present on active and silent genes (Figure 6, light-green trace). When the cells were differentiated, it was no longer possible to detect significant levels of the single modification (Figure 6, dark-green trace) and this was mirrored by the increase in the level of the double modification. This result suggests that H3K9me3 becomes widely modified by the presence of S10ph during cell differentiation, with the double H3K9me3/S10ph modification forming domains across silent genes.

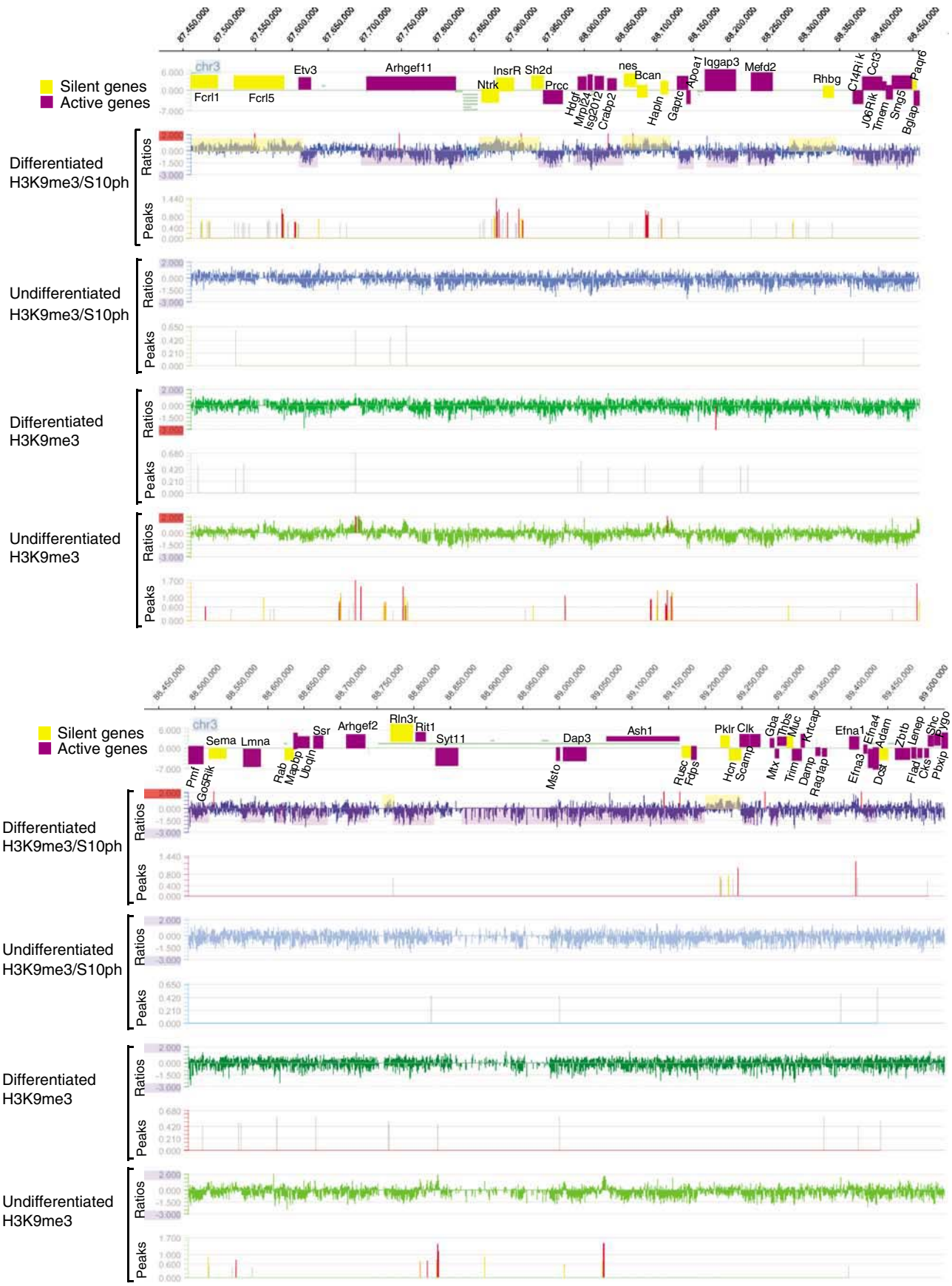
Conventional chromatin immunoprecipitation (ChIP) using quantitative real-time PCR was also carried out on activated B and plasma cells and was compared with the results from undifferentiated and differentiated mesenchymal cells. The results show increased levels of the double modification at silent genes in plasma cells compared with activated B cells (Supplementary Figure S3). Taken together, the data from the microarray and the PCR–ChIP analysis indicate that the H3K9me3/S10ph modification becomes progressively enriched at repressed genes as cells differentiate and suggest that it forms part of a mechanism for epigenetic marking of silent chromatin in differentiated postmitotic cells.

Discussion

A novel function for the Aurora B kinase in differentiated postmitotic cells

The results of this study add a new dimension to the biology of the Aurora B kinase by showing that it can act as a regulator of the epigenetic status of differentiated postmitotic cells. Previous work had shown that Aurora B is tightly regulated in cycling cells and phosphorylates proteins that are critical for the transit through mitosis (Carmena and Earnshaw, 2003). Aurora B expression is upregulated during the transition from S to G2, peaks in G2/M and is minimal in interphase (Terada *et al*, 1998). The observation that

Figure 6 ChIP-on-chip analysis shows enrichment for H3K9me3/S10ph on repressed gene domains in differentiated C3H10T1/2 cells. The tiling microarray covers a 2-Mb region of mouse chromosome 3 containing 67 genes with different expression patterns (yellow = silent gene; purple = active gene). All the genes coloured in yellow are silent in both undifferentiated and differentiated cells with the exception of nestin, which is expressed in mesenchymal stem cells and silenced during differentiation. Domains of H3K9me3/S10ph enrichment, corresponding to $\log_2$ ratios above 0, are shadowed in yellow while domains of depletion (negative $\log_2$ ratios) are shadowed in purple. Data are represented in the upper tracing as the scaled $\log_2$ ratio (pull-down/input) of the hybridisation signal for DNA immunoprecipitated using the indicated antibody (dark-blue tracing = H3K9me3/S10ph in differentiated C3H10T1/2; light-blue tracing = H3K9me3/S10ph in undifferentiated C3H10T1/2; dark green = H3K9me3 in differentiated C3H10T1/2; light green = H3K9me3 in undifferentiated C3H10T1/2). The distribution of peaks of enrichment is shown in the panel below each tracing. The peaks are color-coded (red = high probability peak, false discovery rate (FDR) $\leqslant 0.05$; yellow = intermediate probability peak, FDR > 0.1 and $\leqslant 0.2$; grey = low probability peak, FDR > 0.2) and were obtained using the NimbleGen default algorithm described in Materials and methods and in http://www.nimblegen.com/products/lit/nimblescan2.3users-guide.pdf. Validation of the microarray analysis by real-time PCR is described in Supplementary Figure S4.



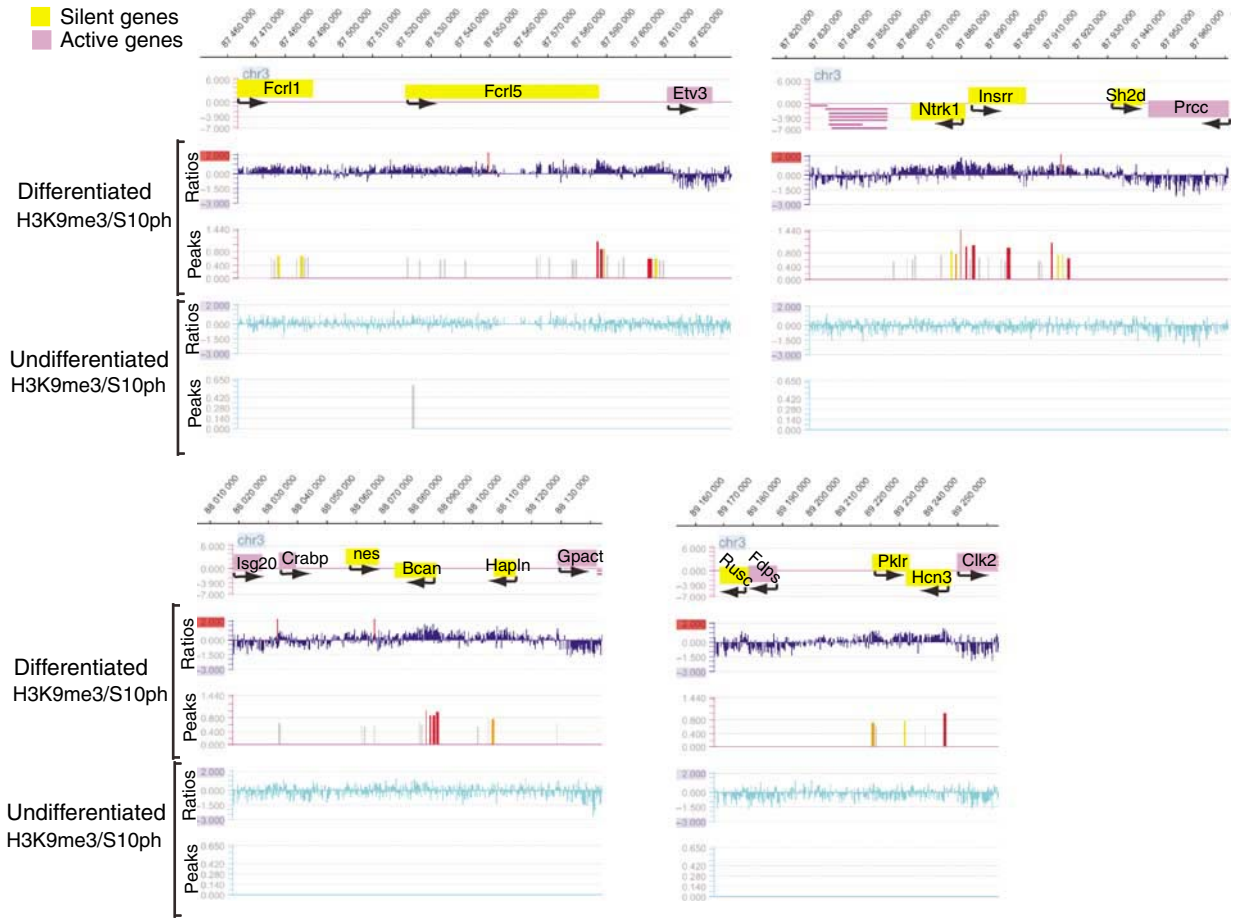


Figure 7 Close-up view of the repressed gene domains within the 2-Mb region. Horizontal arrows indicate the direction of transcription (yellow = silent gene; purple = active gene).

deregulation of Aurora B expression is associated with uncontrolled cell division in many types of cancer has reinforced the perception that the Aurora B kinase is primarily involved in cell-cycle regulation (Tatsuka *et al*, 1998). However, our results provide evidence that Aurora B also has important functions in non-dividing cells.

In dividing cells, Aurora B is known to be responsible for a transient peak of histone H3S10 phosphorylation, which generates the double H3K9me3/S10ph modification at the G2/M phase of the cell cycle. This leads to the release of HP1β and to chromatin changes necessary for chromosome segregation (Fischle *et al*, 2005; Hirota *et al*, 2005). Our study demonstrates that the double H3K9me3/S10ph modification is not restricted to mitotic cells, but is also found at high levels in differentiated postmitotic cells and that its presence depends on the activity of Aurora B.

A particularly striking feature of plasma cell differentiation is the formation of large clusters of facultative heterochromatin as the nucleus shrinks and the cell specialises to become an antibody factory. The double H3K9me3/S10ph modification is strongly associated with these heterochromatic regions. It is interesting that plasma cells show no detectable increase in the levels of H3K9me3 without the accompanying S10ph despite there being a substantial increase in the amount of heterochromatin in these cells compared with activated B cells. This suggests that the presence of S10ph modification has an important role in modulating the epigenetic marking of facultative heterochromatin by H3K9me3 during cell differentiation. Previous studies have implicated H3 S10 phosphorylation in gene activation (reviewed in Johansen and Johansen, 2006). Our results suggest that the combination of S10 phosphorylation with K9 methylation can reverse this effect and turn the modification into a silencing mark.

The double histone H3K9me3/S10ph modification is a marker for gene silencing during differentiation

Microarray and conventional ChIP analysis of the distribution of the double H3K9me3/S10ph modification revealed a developmentally regulated association of the modification with gene silencing. Differentiation of mesenchymal stem cells is accompanied by the formation of domains of enrichment for the double modification that can extend across several adjacent silent genes. There is a corresponding decrease in $\log_2$ ratios and in the number of enrichment peaks observed for the single H3K9me3 modification, which suggests that it is converted into the double modification by addition of the phosphate group at S10. The highest levels of the double modification were present in terminally differentiated plasma cells, as measured by IF, western blotting and ChIP of individual genes. These results suggest that the complexity of epigenetic regulation can be increased during cell differentiation, as the readout from single modifications is subject to progressive modulation by the addition of new modifica-

tions to the same histone molecule. One effect would be to influence the binding of chromatin proteins, either by creating new binding sites or by preventing proteins from binding to existing sites.

Aurora B generates a binary methyl/phospho switch that modulates HP1 binding to chromatin during differentiation

A key observation in this study is the finding that HP1β is displaced from facultative heterochromatin in short-lived plasma cells and that this displacement is reversed by inhibition of Aurora B. HP1 is a major component of constitutive heterochromatin, which is conserved in a wide range of organisms from fission yeast through to mammals. It binds to methylated H3K9 (Jacobs and Khorasanizadeh, 2002; Nielsen *et al*, 2002) and recruits the Su(Var)39 histone methyltransferase (Schotta *et al*, 2002), thereby promoting further H3K9 methylation and reinforcing the process of heterochromatinisation. HP1 also recruits histone deacetylases, cohesin and the CAF1 chromatin assembly complex (reviewed in Maison and Almouzni, 2004; Hediger and Gasser, 2006).

Although HP1 has been thought to be associated mainly with gene silencing, recent studies have shown that it is associated with transcriptionally active chromatin puffs on *Drosophila* polytene chromosomes (Piacentini *et al*, 2003) and that it binds within the transcribed regions of a number of active *Drosophila* genes (de Wit *et al*, 2007). Together with studies that have revealed the presence of methylated H3K9 within active transcription units (Vakoc *et al*, 2005), these results point to a dual role for HP1 in activating and silencing transcription. This conclusion has received further support from studies in *Schizosaccharomyces pombe* showing that Swi6/HP1 recruits the chromatin opening protein Epe1, which in turn makes heterochromatin more accessible to transcription by RNA polymerase II, a necessary precursor to RNAi-mediated heterochromatinisation (Zofall and Grewal, 2006). Displacement of HP1 from chromatin would, therefore, be expected to have complex effects on activation and silencing of transcription.

These results raise the possibility that displacement of HP1 could be important for long-term silencing of gene expression in differentiated cells. A second effect of displacing HP1 could be to allow binding of other, as yet, unidentified chromatin proteins that would recognise the double modification and would be involved in maintaining transcriptional silencing and formation of facultative heterochromatin. This idea is supported by the observation that HP1 is absent from facultative heterochromatin in chicken erythrocytes (Gilbert *et al*, 2003). However, the absence of HP1 from chicken erythrocyte heterochromatin appears to be due to complete loss of HP1 from the nucleus rather than a histone modification displacing HP1 from chromatin.

Our data indicate that the single H3K9me3 modification is converted by the Aurora B kinase into a double H3K9me3/S10ph modification on a proportion of nucleosomes at repressed genes. The double H3K9me3/S10ph modification would also generate a novel binding site that has the potential to be recognised by other proteins that participate in the formation of a permanent repressive structure.

A key requirement for generating the binary methyl/phospho switch in differentiated postmitotic cells is the uncoupling of Aurora B kinase regulation from the cell cycle. This is likely to involve association of the kinase with a different set of proteins as cells exit from the cell cycle. Identification of the factors that lead to this change in Aurora B function will be an important area of future research.

Materials and methods

Antibodies

The following antibodies were used in western blot analysis, ChIP and IF: α-histone H3 (Abcam ab1791), α-H4 (Upstate 07-108), α-H3K9me3/S10ph (Abcam ab5819) (peptide ELISA to confirm the specificity of this antibody was carried out as described previously; Chow *et al*, 2005), α-H3K9me3 (Upstate 07-442), α-Aurora B (Abcam ab2254), α-HP1β (anti-M31 rat monoclonal MCA1946; Serotec), α-mouse IgM-Texas red (Vector) and α-mouse IgG-Texas red (vector), α-β-tubulin (Santa Cruz Biotechnologies) and α-actin (Sigma). Control antibodies for ChIP were nonspecific rabbit IgG (Santa Cruz Biotechnologies) and α-FLAG antibody (Sigma). Secondary antibodies for western blot studies were α-rabbit IgG-horseradish peroxidase-conjugated antibody (Sigma) and α-rabbit IgG-alkaline phosphatase-conjugated antibody (Sigma).

Cell cultures

Resting B-cell isolation and B-cell activation were carried out as described previously (Sabbattini *et al*, 2001). The activated B cells were positively selected with biotinylated anti-CD19 antibody (BD Biosciences Pharmingen) conjugated to streptavidin beads (CELLection Dynabeads; Dynal) and released from the beads before further analysis.

C3H10T1/2 cells were expanded in complete expansion medium, DMEM medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax, 2 mM non-essential amino acids (Sigma) and 2 mM sodium pyruvate. Osteogenic differentiation was induced as described in Peister *et al* (2004) by culturing the C3H10T1/2 cells in IMDM supplemented with 10% FCS, 10% HS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax, 20 mM β-glycerolphosphate, 50 ng/ml thyroxine, 1 nM dexamethasone and 0.5 μM ascorbic acid (all from Sigma) for 3 weeks. To confirm differentiation, an aliquot of the cells was fixed for 2 min with 1% formaldehyde and then stained for phosphatase activity using OPD (Dako) as a chromogenic substrate. Real-time PCR was used to confirm expression of the osteogenic markers alkaline phosphatase and osteocalcin. For BrdU incorporation studies, C3H10T1/2 cells were incubated in media with 10 μM BrdU for 2 h.

In vitro differentiation and selection of plasma cells

Spleens from 6- to 10-week-old CBAB6F1 mice were disaggregated and the single-cell suspension was centrifuged on a Ficoll cushion to remove erythrocytes and cultured for 7 days in RPMI supplemented with 15% FCS, 50 μM 2-mercaptoethanol, 50 μg/ml gentamicin, 25 μg/ml LPS, 5 ng/ml IL-5, 5 ng/ml IL-6 and 10 ng/ml IL-10. On day 7, the cells were again centrifuged on a Ficoll cushion to remove dead cells and the cells obtained from the interface were blocked with 10% goat serum and 5 μg/ml anti-FC receptor γ III/II (BD Biosciences Pharmingen). The cells were then incubated with streptavidin beads (CELLection Dynabeads; Dynal) conjugated with biotinylated α-syndecan-1 antibody (BD Biosciences Pharmingen). Following magnetic selection, the cells were washed and released from the beads by digestion with DNaseI and incubated with α-B220-conjugated magnetic beads (Dynal) to deplete the $B220^+$ cells. After selection, the cultures were typically $>97\%$ α-syndecan-$1^+/>98\%$ $B220^-$. For plasma cell isolation *ex vivo* from bone marrow, mice were immunised with ovalbumin, followed by a second immunisation after 3 weeks. Bone marrow was collected 3 days after the second immunisation. Plasma cells were isolated from spleen 7 days after immunisation with LPS. Plasma cell-enriched cell preparations from bone marrow and spleen were obtained by centrifugation on a Ficoll cushion followed by positive selection with α-syndecan-1 antibody conjugated to magnetic beads. For BrdU incorporation assays, activated B and plasma cells were incubated in media with 10 μM BrdU for 6 h.

IF microscopy

IF analysis was carried out as described previously (Mak *et al*, 2002). Secondary antibodies used for fluorescent detection were Alexa 488 anti-rabbit and Alexa 488 anti-rat (both from Molecular Probes). Samples were mounted in Vectashield supplemented with DAPI (10 μg/ml) and images were collected by confocal microscopy using a TCS-SP1 microscope (Leica Microsystems) and Metamorph 4.0 software.

ChIP

ChIP analysis of unfixed chromatin or formaldehyde-fixed chromatin was carried out as described previously (Szutorisz *et al*, 2005). For plasma cells, the ChIP was carried out in the presence of SL2 *Drosophila* cells according to the carrier ChIP protocol described previously (O'Neill *et al*, 2006). Unfixed chromatin (150 μg) was immunoprecipitated with the following antibodies: 20 μl of anti-diacetylated histone H3 (Upstate 06-599), 100 μl α-H3K9me3/S10ph (Abcam ab5819), 20 μl α-H3K9me3 (Upstate) and as control 37 μl nonspecific rabbit IgG (Santa Cruz Biotechnology). For the fixed chromatin method, 500 μg of fixed chromatin was immunoprecipitated and the same ratio of chromatin to antibody was used that was described for the unfixed chromatin procedure. The input and immunoprecipitated samples were phenol/chloroform-extracted and resuspended in 100 μl of TE. For quantitation, 2 μl of the input and the immunoprecipitated DNA (IP) samples were amplified by real-time PCR using SYBR–green mix (Bio-Rad) and a DNA Engine Opticon system (MJ Research Inc.). PCRs were carried out in duplicate. Primer sequences can be obtained on request.

Microarray analysis

Microarrays were generated by NimbleGen Systems by tiling 50-bp oligonucleotides across a region of 2 Mb of mouse chromosome 13 (Chr3: 87462375–89534891) with 100-bp resolution. High-frequency repeats were excluded by repeat masking. DNA (4 μg) from immunoprecipitation and input was amplified using the GenomePlex WGA kit (Sigma). After amplification, the DNA was tested for enrichment at control loci and compared to the unamplified DNA by real-time PCR. The amplified input and pull-down DNA were labelled with Cy3 and Cy5 by random priming and hybridised to the microarray by NimbleGen. Two immunoprecipitations were performed for each antibody. Input and pull-down signal intensities, scaled $\log_2$ ratios and enrichment peaks were provided by NimbleGen. Enrichment peaks were calculated using a permutation-based algorithm (see NimbleScan User's Guide at http://www.nimblegen.com/products/lit/nimblescan2.3usersguide.pdf, default algorithm). This estimates the false discovery rate (FDR) for each peak, which is equal to the probability of finding a peak of comparable significance by chance. Significant peaks are characterised by low FDRs and correspond to four or more probes within a 500-bp sliding window with signals above the cutoff value (cutoff values are a percentage ranging from 90 to 15% of a hypothetical $\log_2$ ratio maximum, which is the mean + 6(standard deviation)).

In vitro binding analysis by SPR

The assays were performed on a Biacore X instrument (Biacore, Milton Keynes, UK) with Biacore HBS-EP buffer (10 mM Hepes pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20) at a flow rate of 10 μl/min. Anti-6 × His monoclonal antibody (Abcam; 6500 response units, RUs) and control anti-RNA polII monoclonal antibody (kind gift of Laszlo Tora; 6700 RU) were immobilised using the Amine Coupling Kit (Biacore) on flow cells 1 and 2, respectively, of a CM5 sensor chip. 6 × His-HP1β (300 nM) was injected simultaneously over flow channels 1 and 2 followed by injection over both flow channels of 50 μM solutions of peptides for 2 min. After the injection, the dissociation of the complex was followed for 180 s. Non-phosphorylated peptides were also injected at lower concentrations for comparability to phosphorylated peptide binding. The difference between the increase in RU between flow channels 1 and 2 was used to measure the specific binding of the peptides to His-HP1β. At least two injections were carried out for each peptide. Data were analysed using Bioevaluation 3.2 software (Biacore).

FACS sorting of G1 and G2/M cells

Live logarithmically growing undifferentiated C3H10T1/2 cells (2×10^7) were stained for 30 min at 37°C/5% CO_2 with the DNA-intercalating dye Hoechst 33342 (10 μg/ml). The cells were sorted by FACS into G1 and G2/M fractions on the basis of DNA content. Sorting was carried out with a FACS Vantage SE sorter (Becton Dickinson).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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