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Formation of facultative heterochromatin in the absence of HP1

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Facultative heterochromatin is a cytological manifestation of epigenetic mechanisms that regulate gene expression. Constitutive heterochromatin is marked by distinctive histone H3 methylation and the presence of HP1 proteins, but the chromatin modifications of facultative heterochromatin are less clear. We have examined histone modifications and HP1 in the facultative heterochromatin of nucleated erythrocytes and show that mouse and chicken erythrocytes have different mechanisms of heterochromatin formation. Mouse embryonic erythrocytes have abundant HP1, increased tri-methylation of H3 at K9 and loss of H3 tri-methylation at K27. In contrast, we show that HP1 proteins are lost during the differentiation of chicken erythrocytes, and that H3 tri-methylation at both K9 and K27 is reduced. This coincides with the appearance of the variant linker histone H5. HP1s are also absent from erythrocytes of *Xenopus* and zebrafish. Our data show that in the same cell lineage there are different mechanisms for forming facultative heterochromatin in vertebrates. To our knowledge, this is the first report of cell types that lack HP1s and that have gross changes in the levels of histone modifications.

Keywords: differentiation/erythrocyte/gene expression/heterochromatin/histone modification

Introduction

Chromatin regulates critical cellular processes such as transcription, DNA replication and repair. Although chromatin packaging at the level of the nucleosome is well understood, it is not clear how further levels of packaging occur. DNA binding dyes stain some chromatin domains within nuclei more intensely than others, differentiating chromatin into heterochromatin and euchromatin respectively. Euchromatin generally corresponds to active (or potentially active) gene regions, while heterochromatin is normally transcriptionally silent. Constitutive heterochromatin describes large segments of the genome, primarily arrays of tandemly repeated (satellite) sequences, which are packaged in a permanently inactive form that is thought to be more compact than euchromatin (Gilbert and Allan, 2001). In mammalian cells constitutive heterochromatin is principally found at centromeric and peri-centromeric regions.

The cytological division of chromatin into euchromatin and heterochromatin does not readily lend itself to a direct molecular definition. However, there are different chromatin-associated biochemical marks important in distinguishing the heterochromatic state from euchromatin. These include: DNA methylation, histone methylation and the absence of histone acetylation (reviewed in Richards and Elgin, 2002). In addition, heterochromatin protein 1 (HP1) proteins, a class of multifunctional chromatin-associated adapter proteins, are present at blocks of constitutive heterochromatin in diverse eukaryotes, where they are thought to be important for regulating heterochromatin-mediated silencing and chromosome structure (Ekwall *et al*., 1995; Kellum *et al*., 1995; Yamaguchi *et al*., 1998). There are three HP1 proteins (α, β and γ) in mammals. HP1α and β are concentrated at pericentric heterochromatin, although HP1β can also be seen at more diffuse nucleoplasmic sites, whereas HP1γ is predominantly localized in euchromatin (Minc *et al*., 2000; Nielsen *et al*., 2001). This is a similar range of distribution patterns to those reported for the three HP1 proteins in *Drosophila* (Smothers and Henikoff, 2001).

Analysis of HP1 structure reveals three functional domains; an N-terminal chromodomain (CD), a central hinge domain (HD) and a C-terminal chromoshadow domain (CSD). Dimerization and interaction of HP1s with other chromosomal proteins is thought to occur through the CSD (Brasher *et al*., 2000; Smothers and Henikoff, 2000). The CD binds to histone H3 methylated at K9 (metH3-K9) (Bannister *et al*., 2001; Lachner *et al*., 2001; Jacobs and Khorasanizadeh, 2002; Nielsen *et al*., 2002). HP1α may also bind to DNA and linker histones through the HD (Nielsen *et al*., 2001; Meehan *et al*., 2003). The HD may also be involved in targeting HP1 to heterochromatin through an RNA binding activity (Muchardt *et al*., 2002).

Facultative heterochromatin is defined as euchromatic regions that become packaged into a compact heterochromatic-like form in a developmentally regulated manner. Facultative heterochromatin is not characterized by repetitive sequences, so at the DNA sequence level it is entirely different from constitutive heterochromatin. However, facultative heterochromatin has many of the same molecular signatures as constitutive heterochromatin at the nucleosome level. Histone hypoacetylation and H3-K9 methylation occur during formation of the inactive X chromosome (Xi) in somatic cells of female mammals (Jeppesen and Turner, 1993; Peters *et al*., 2002) and the X chromosome that is silenced within the XY body in

spermatogenesis is also enriched in metH3-K9 (Cowell *et al*., 2002), but is not depleted in H4 acetylation (Armstrong *et al*., 1997). In insects, both the bithorax complex in *Drosophila* and the paternal heterochromatic chromosomes in male mealy bugs are also enriched in metH3-K9 (Cowell *et al*., 2002).

HP1β and γ, but not HP1α, also concentrate on the mammalian XY body during pachytene (Cowell *et al*., 2002; Metzler-Guillemain *et al*., 2003), and on the condensed chromosome set in mealy bugs (Cowell *et al*., 2002). The presence of HP1s at these types of facultative heterochromatin suggests a mechanism of formation similar to that of constitutive heterochromatin. However, HP1s do not accumulate on the Xi (Peters *et al*., 2002), suggesting there are HP1-independent pathways to the formation of facultative heterochromatin. Instead, blocks of facultative heterochromatin can be enriched in variant histones and non-histone proteins. For example, the mammalian inactive X of somatic and germinal cells contains variant histones macroH2A.1 and 2 (Costanzi and Pehrson, 1998; Hoyer-Fender *et al*., 2000; Chadwick and Willard, 2001), and Brca1 (Ganesan *et al*., 2002).

At the bithorax complex in *Drosophila*, polycomb group (PcG) proteins establish a repressed chromatin state in order to regulate the pattern of *Hox* gene expression during development (reviewed by Orlando, 2003). Some PcG proteins contain a chromodomain, similar to that found in HP1s, that has been shown to bind to tri-metH3-K27 (Cao *et al*., 2002; Czermin *et al*., 2002). Recent evidence has implicated PcG proteins and metH3-K27 in the initiation of mammalian X chromosome inactivation (Wang *et al*., 2001; Plath *et al*., 2003; Silva *et al*., 2003). Therefore, while the role of HP1s in formation of constitutive heterochromatin seems almost universal, there appear to be many routes to the formation of facultative heterochromatin.

The facultative heterochromatin formed in the nuclei of terminally differentiated erythrocytes of chicken has been used as a model system to study the developmentally regulated condensation and repression of chromatin (Weintraub, 1984). In chickens this correlates with the expression of the variant linker histone H5 that is able to condense the chromatin fibre (Bergman *et al*., 1988), and with the expression of a serpin-like protein called MENT (Grigoryev *et al*., 1999). *Xenopus* nucleated erythrocytes have the replacement linker histone $H1^0$, the accumulation of which also coincides with cessation of proliferation and the compaction of chromatin (Koutzamani *et al*., 2002), and fish erythrocytes contain similar, although less well characterized, replacement linker histones. Likewise mouse embryonic erythrocytes are nucleated and have condensed chromatin.

To determine whether HP1s and histone modifications play a role in these forms of facultative heterochromatin we have examined the expression of HP1 proteins and the presence of histone H3 K4, 9 and 27 methylation in mouse and chicken erythrocyte nuclei. Our data indicate that although centromeric heterochromatin is universally associated with tri-methylated histone H3 K9 and HP1 proteins, facultative heterochromatin is formed and maintained by different mechanisms. We find elevated levels of metH3-K9 and abundant HP1 in the nuclei of mouse erythrocytes, and an absence of metH3-K27. In contrast there is a total absence of HP1s from adult chicken, frog and fish nucleated erythrocytes, and decreased levels of metH3-K9. Hence there must be an HP1-independent pathway for the formation of heterochromatin during erythrocyte differentiation in these vertebrates. HP1 levels decrease during the differentiation of chicken embryonic erythrocytes as the levels of H5 increase, suggesting that H5 might replace the role of HP1s. To our knowledge, this is the first report of cell types that lack HP1s and that have gross changes in the levels of histone modifications.

Results

Chromatin association and localization of HP1 isoforms in mammalian and chicken cells

Differential localizations of HP1 isoforms in mouse cells have been reported (Minc *et al*., 2000; Nielsen *et al*., 2001). This suggests that despite their sequence similarities, HP1s α, β and γ interact with chromatin in different ways. To examine this we isolated the proteins bound to higher order chromatin fibres from mouse cells under physiological conditions (Gilbert and Allan, 2001). Immunoblotting using monoclonal antibodies specific to each HP1 isoform (Nielsen *et al*., 1999) demonstrates that most HP1α is stably associated with the chromatin fibres, while HP1β and γ are readily lost during purification (Figure 1A). This suggests that HP1β and γ have a lower affinity for the chromatin fibre than does HP1α, which is surprising given the similar exchange kinetics of all three HP1 isoforms at either euchromatin or heterochromatin measured by fluorescence recovery after photobleaching (Cheutin *et al*., 2003). HP1α has previously been shown to remain tightly bound to chromatin during salt extraction (Remboutsika *et al*., 1999).

We compared the nuclear distribution of the three HP1 isoforms in mouse and human tissue culture cells by immunofluorescence (Figure 1B and C). As reported previously (Minc *et al*., 2000; Nielsen *et al*., 2001), HP1α is concentrated at constitutive heterochromatin, indicated by DAPI bright regions, in mouse cells fixed with paraformaldehyde (pFa) (Figure 1B). Consistent with the strong biochemical association of HP1α with chromatin (Figure 1A), this staining pattern persists in cells fixed with methanol:acetic acid (data not shown). However, the diffuse nucleoplasmic or euchromatin-associated pool of HP1α is lost after extraction of mouse nuclei with methanol:acetic acid. This suggests that the mode of binding of HP1α in heterochromatin differs from that at euchromatic locations, and is consistent with the differing dynamics of HP1s at heterochromatic and euchromatic sites (Cheutin *et al*., 2003; Festenstein *et al*., 2003).

HP1β and γ are also detected at sites of heterochromatin, but there is also a large pool of these proteins more diffusely distributed throughout the nucleoplasm and HP1γ is predominantly found at euchromatic sites (Figure 1B).

The distribution of HP1 isoforms in human cells has not been well described. Compared with mouse cells, human cells have smaller blocks of constitutive heterochromatin that are not easily distinguished cytologically. Much constitutive heterochromatin in human cells is pericentromeric; therefore, we compared the distribution of HP1 isoforms with centromeric antigens detected with CREST

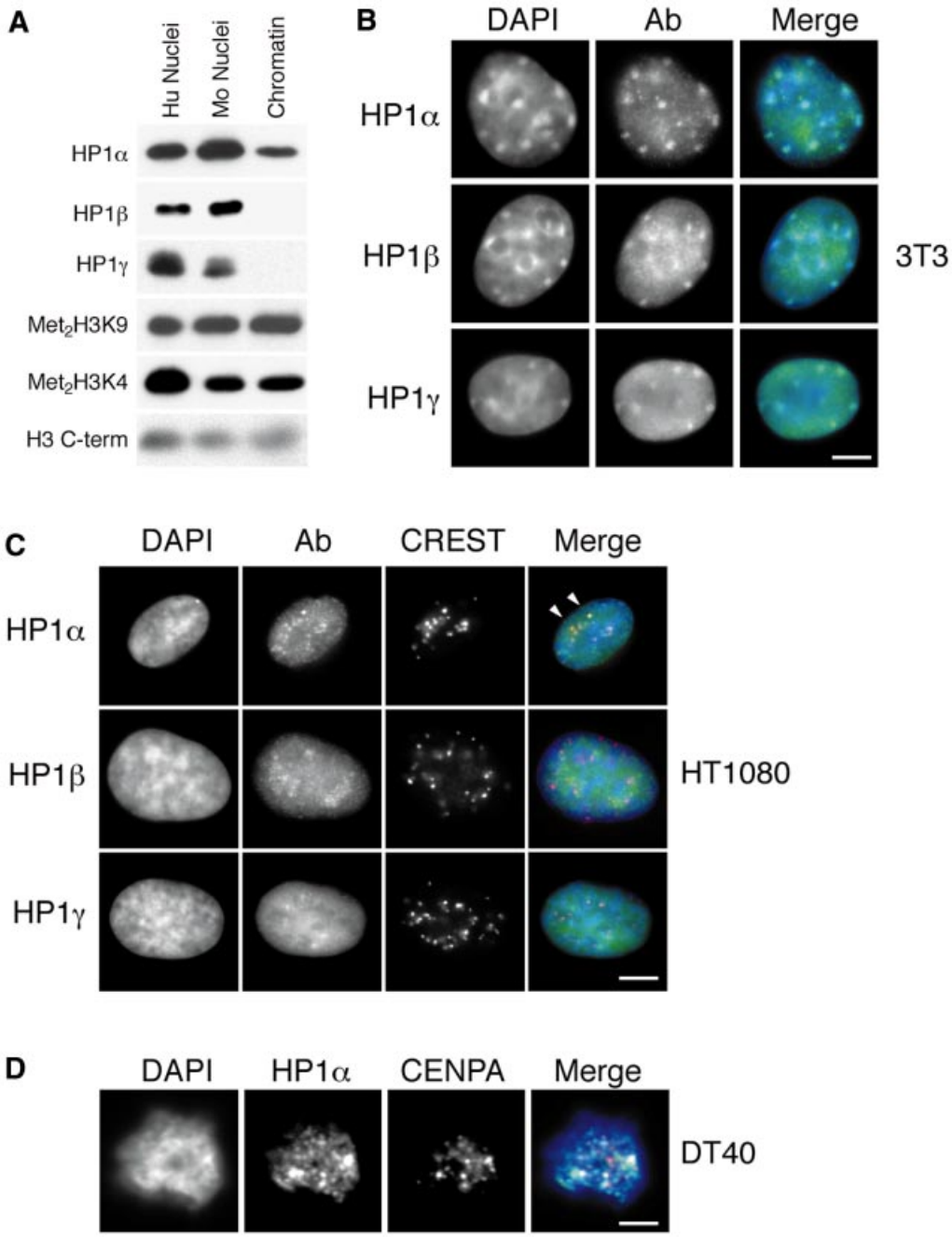

Fig. 1. Distribution of HP1 isoforms in human, mouse and chicken cells. (**A**) Immunoblotting of proteins isolated from whole human (hu) or mouse (mo) nuclei, or from mouse chromatin using antisera that detect HP1s α, β and γ, di-methyl H3-K9 (met$_2$H3-K9), and di-methyl H3-K4 (met$_2$H3-K4). An antibody that recognizes H3 is used as a loading control. (**B**) Immunofluorescence of mouse 3T3 nuclei fixed with pFa using antibodies that detect HP1s α, β and γ (green). DNA was counterstained with DAPI (blue) to highlight the foci of heterochromatin. (**C**) Co-immunostaining of human HT1080 cells using antibodies that detect HP1s α, β and γ (green) and with CREST antiserum, which detects centromeric antigens (red). DNA was counterstained with DAPI (blue). Arrowheads indicate where foci of HP1α and CREST staining are coincident. (**D**) Co-immunostaining of chicken DT40 cells with antibody recognizing HP1α (green) and CENP-A (red). Scale bars, 5 μm.

antisera. HP1α shows a concentration at centromeric sites in human cells, coincident with foci of CREST staining (arrowheads in Figure 1C). No concentration of HP1β or γ could be detected at centromeric domains (detected by CREST) and there are clear non-centromeric foci of these HP1 isoforms in human cells (Figure 1C).

On chicken chromosomes, blocks of constitutive heterochromatin are found at centromeric and telomeric regions of the macrochromosomes, at centromeric regions of the microchromosomes, and also on the Z and W sex chromosomes (Schmid *et al*., 1989). In chicken DT40 cells the most prominent foci of HP1α staining are close to centromeres (detected by antibody recognizing the centromere protein CENP-A) (Figure 1D).

metH3-K9 and HP1 distribution in mammalian and chicken nuclei

Binding of HP1 proteins to metH3-K9 is important for their recruitment to chromatin (Bannister *et al*., 2001; Lachner *et al*., 2001; Jacobs and Khorasanizadeh, 2002;

Nielsen *et al*., 2002). We assessed how the distribution of HP1s compares with that of metH3-K9 using two different antibodies. The first was raised against a linear peptide, recognizes di-methylH3-K9 (met_2H3-K9) and detects facultative heterochromatin of the Xi (Boggs *et al*., 2002). met_2H3-K9 is distributed diffusely in mouse and human nuclei with no concentration at the murine heterochromatic foci detected by DAPI staining (Figure 2A), nor the centromeric domains detected by CREST sera in male human cells (Figure 2B). The second antibody was raised against a branched di-methylH3-K9 peptide and is thought to predominantly recognize tri-methylH3-K9 (met_3H3-K9) (Peters *et al*., 2001). Antigens detected by this antibody are concentrated at mouse pericentric heterochromatin (Figure 2A) and foci in human nuclei, some of which are coincident with CREST staining (arrows in Figure 2B). Other foci do not correspond to centromeric regions detected by CREST (arrowheads in Figure 2B) and may represent other blocks of heterochromatin in the human genome. met_3H3-K27 has recently been associated with PcG protein recruitment in the X-inactivation process (Silva *et al*., 2003). Western blot analysis indicated that male mouse 3T3 cells have this modification (in the absence of Xi) (Figure 6A), prompting us to study its distribution pattern (Figure 2A). As for met_2H3-K9, it is nuclear diffuse and does not appear to be associated with any specific cellular structures.

To assess the correspondence between met_3H3-K9 and HP1α distribution, we carried out double staining on mouse and human nuclei. While there is a strong correspondence between met_3H3-K9 and HP1 staining at the foci of pericentric heterochromatin detected by DAPI staining of mouse cells, there is little coincidence between the two antibody staining patterns outside of these regions in mouse and human cells (Figure 2C). This suggests that HP1α and met_3H3-K9 are not interdependent outside of centromeric heterochromatin. Likewise, HP1α and me_2H3-K9 are not coincident in human, mouse and chicken nuclei (Supplementary figure 1, available at *The EMBO Journal* Online).

In chicken DT40 cells, foci of met_3H3-K9 and HP1α staining are coincident, resembling the pattern seen in mouse cells (Figure 2C).

HP1 and H3 methylation in the compact facultative heterochromatin of vertebrate erythrocytes

Whilst a relationship between HP1 proteins and the formation of constitutive heterochromatin is well established, it is not clear what role HP1s may have in the formation of compact facultative heterochromatin. Chromatin compaction and a widespread silencing of gene expression occur during the terminal differentation of erythrocytes (Rowley and Radcliffe, 1988). The genome-wide compaction of chromatin within nucleated erythrocytes is apparent in the intense DAPI-staining and reduced nuclear volume of mouse and chicken erythrocytes in comparison with mouse liver and chicken pre-B cells (Figure 3). The chromatin condensation within *Xenopus* and zebrafish erythrocyte nuclei is less apparent.

To establish whether there is a role for HP1s and histone modifications in the chromatin condensation in chicken erythrocytes, we analysed chromatin from DT40 (pre-B) chicken cells and from nucleated adult chicken erythrocytes by western blot. Similar levels of H3 dimethylated at K9 (Figure 4A) were found in the chromatin of mouse 3T3 cells, chicken DT40 cells and chicken erythrocytes. In contrast, we found decreased levels of met_3H3-K9 (antisera raised against a linear tri-methylated H3-K9 peptide) in chicken erythrocyte chromatin in comparison with DT40 chromatin (Figure 4A and B). This suggested that there might also be reduced levels of HP1 proteins in chicken erythrocytes. In fact, we were unable to detect any HP1 isoforms in adult chicken erythrocytes (Figure 4A and B) by western blotting. Recently, it has been shown that chromodomain containing PcG proteins can bind to met_3H3-K27 (Cao *et al*., 2002; Czermin *et al*., 2002), leading to the possibility that heterochromatin formation in chicken erythrocytes is mediated by this H3 modification and binding of a chicken PcG protein. However, western blotting with an antibody against met_3H3-K27 showed that levels of this histone modification are very low in chicken erythrocytes (Figure 4A and B).

To examine how these altered levels of HP1s and histone modifications are regulated during development we analysed the chromatin from 10-day embryonic chicken erythrocytes by western blot and Coomassie-stained gel (Figure 4C). During erythrocyte maturation the level of H5 increases (Ruiz-Carrillo *et al*., 1974) while the level of HP1α decreases. In some adult chicken erythrocyte preparations trace amounts of HP1α are present due to white-cell contamination.

We also detected no HP1α or γ in pycnotic chromatin from frog and fish nucleated erythrocytes (Figure 5A) (we were unable to detect *Xenopus* or zebrafish HP1β isoforms using available antibodies). HP1α was also undetectable in adult chicken erythrocytes by immunofluorescence (Figure 5B), but the distribution of met_3H3-K9 in chicken erythrocytes and *Xenopus* erythrocytes is similar to that in other somatic cells (e.g. DT40 chicken cells in Figure 1C). This indicates that the distribution of this histone modification is unaffected by the absence of HP1α and suggests that chromatin condensation in non-mammalian vertebrate erythrocytes occurs without HP1s. Moreover, HP1s must also have been lost from the constitutive heterochromatin in these cells (Figures 1 and 5), and so cannot be essential for their survival (Filesi *et al*., 2002). met_3H3-K27 in chicken erythrocytes was present at low levels by western blot (Figure 4A) and has a diffuse distribution pattern by immunofluorescence (Figure 5B). These nuclei were obtained from female chicken cells that carry ZW sex chromosomes and so would not exhibit any dosage compensation, unlike male cells, which carry ZZ sex chromosomes. Therefore, male cells might inactivate one chromosome (McQueen *et al*., 2001) possibly by tri-methylation at H3-K27 (Figure 6C).

HP1 and H3 methylation in mouse nucleated embryonic erythrocytes

To assess whether the loss of HP1s from erythrocytes is restricted to avian and fish species or whether it also occurs during mammalian erythrocyte differentiation, we examined nucleated erythrocytes from early mouse embryos. During mouse embryogenesis non-enucleated erythrocytes express embryonic haemoglobins, before the switch to expression of foetal haemoglobins at ~12 days post-coitum (d.p.c.). The chromatin in erythrocytes from 12.5 d.p.c.

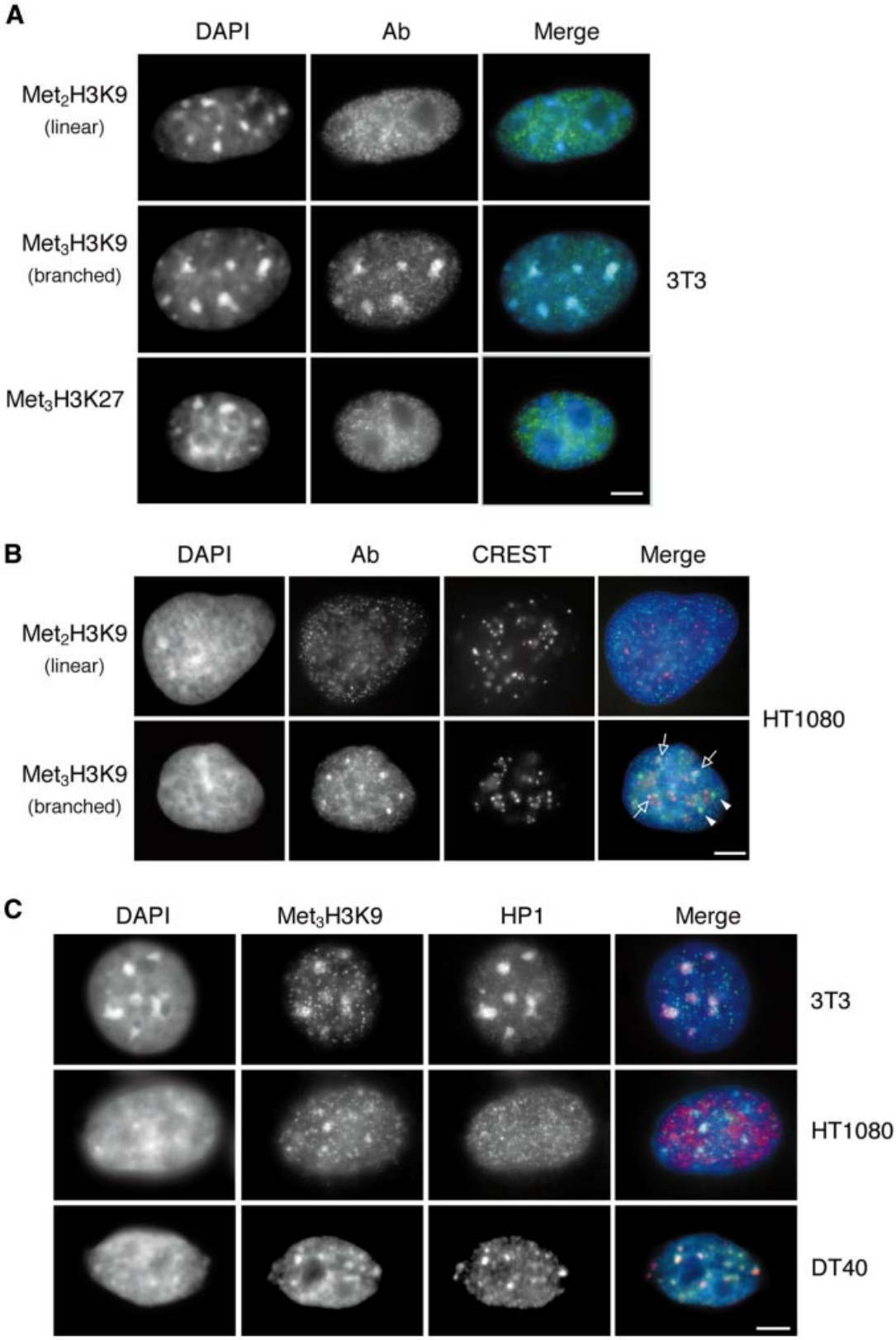

Fig. 2. Distribution of metH3-K9 in human, mouse and chicken cells. (**A**) Immunostaining of mouse 3T3 with antisera (green) raised against a linear peptide dimethylated at K9 of H3 (met_2H3-K9 linear; Boggs *et al*., 2002), or against a branched di-methylated peptide (met_3H3-K9 branched, but which detects tri-methylated H3-K9; Peters *et al*., 2001) or against a linear peptide tri-methylated at K27 (Silva *et al*., 2003). DNA was counterstained with DAPI (blue). (**B**) Co-immunostaining of human HT1080 cells with metH3-K9 linear and branched antibodies (green) and CREST serum (red). DNA was counterstained with DAPI (blue). Arrows indicate sites of co-staining. Arrow heads indicate foci of met_3H3-K9 that do not correspond to centromeres detected by CREST. (**C**) Co-localization of HP1α with tri-methyl H3-K9 in mouse 3T3, human HT1080 and chicken DT40 cells. Scale bars, 5 μm.

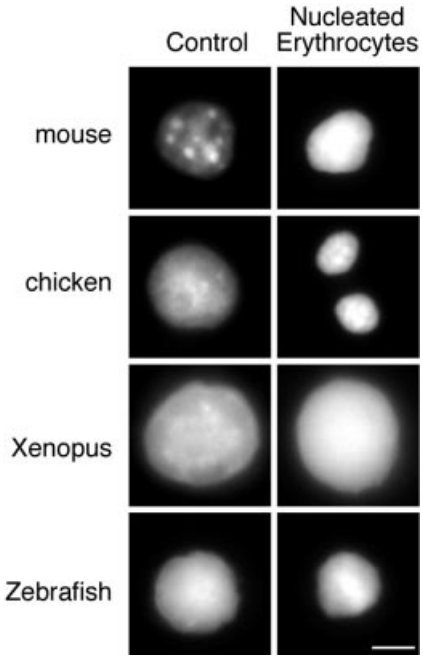

Fig. 3. Chromatin condensation in vertebrate nucleated erythrocytes. DAPI staining of mouse, chicken, *Xenopus* and zebrafish nucleated erythrocytes (right hand panel) contrasted with the staining pattern of nuclei from control tissues (mouse, *Xenopus* and zebrafish liver) and chicken DT40 cells. Scale bar, 5 μm.

mouse embryos appears to be highly compacted compared with that in mouse foetal liver cells (Figure 3). However, western blotting shows that in contrast to non-mammalian vertebrate erythrocytes, mouse embryonic erythrocytes have elevated levels of HP1α compared with liver cells (Figure 6A). This is accompanied by increased levels of met_3H3-K9 and a dramatic loss of tri-methylation at H3-K27 (Figure 6A). met_3H3-K27 has been shown to be present during X inactivation (Silva *et al*., 2003). In male 3T3 cells it has a nuclear diffuse distribution pattern (Figure 2A), whilst in female mouse C127 epithelial cells it is nuclear diffuse with a very clear accumulation on the inactive X chromosome coincident with *Xist* staining (Figure 6C). However, by immunofluorescence we were unable to detect met_3H3-K27 staining in mouse erythrocytes from embryos (confirmed to be female by X chromosome painting), and we were also unable to detect *Xist* by RNA fluorescence *in situ* hybridization (FISH) (Figure 6C). This suggests that facultative heterochromatin formation in murine embryonic erythrocytes may be mediated by HP1, and that PcG family members are unlikely to be involved.

The presence of elevated levels of HP1α and met_3H3-K9 in mouse erythrocytes suggests that there might be a spreading of HP1 from the normal sites of constitutive heterochromatin (Figure 2C) to a more global distribution across the genome. However, immunofluorescence indicates that HP1α is still predominantly localized to blocks of heterochromatin in mouse erythrocytes whilst the distribution of met_3H3-K9 is more diffuse (Figure 6B). Thus, a redistribution of HP1α to sites throughout the genome is unlikely to be solely responsible for the formation of facultative heterochromatin in these cells.

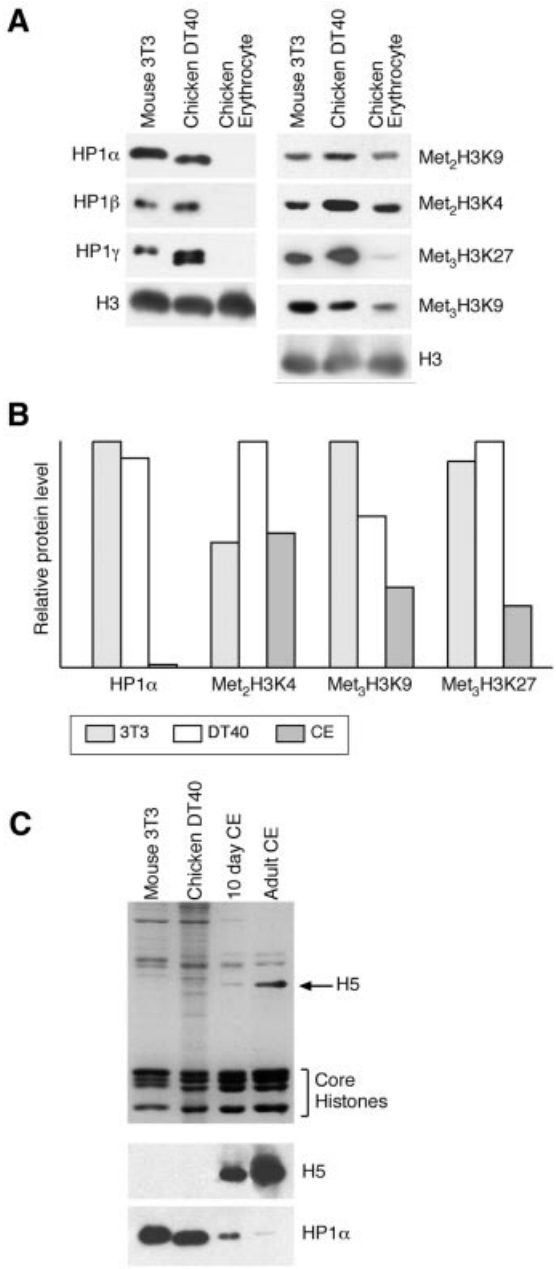

Fig. 4. Loss of HP1s from chicken erythrocytes. (**A**) Western blot of mouse 3T3, chicken DT40 and adult chicken erythrocyte nuclear proteins with antibodies detecting HP1 isoforms, di- (met_2H3-K9) and tri- (met_3H3-K9) methylated H3 K9, and H3 methylated at K4 and K27. Antibody detecting H3 is used as a control. (**B**) Quantification of HP1α, met_2H3-K4, met_3H3-K9 and met_3H3-K27 levels in mouse, DT40 and chicken erythrocyte nuclei. Signals were normalized with respect to total histone H3. (**C**) Coomassie-stained gel of mouse 3T3, chicken DT40, chicken day 10 embryonic erythrocytes and adult chicken erythrocytes. Histone H5 is indicated by an arrow. Western blots of equivalent samples probed with antibodies against H5 and HP1α are shown below.

Discussion

Initial progress in understanding heterochromatin structure came from the analysis of constitutive heterochromatin, for example, in screens for suppressors of position effect variegation in flies. HP1 was identified in this way, and HP1 proteins have now been shown to be important in the

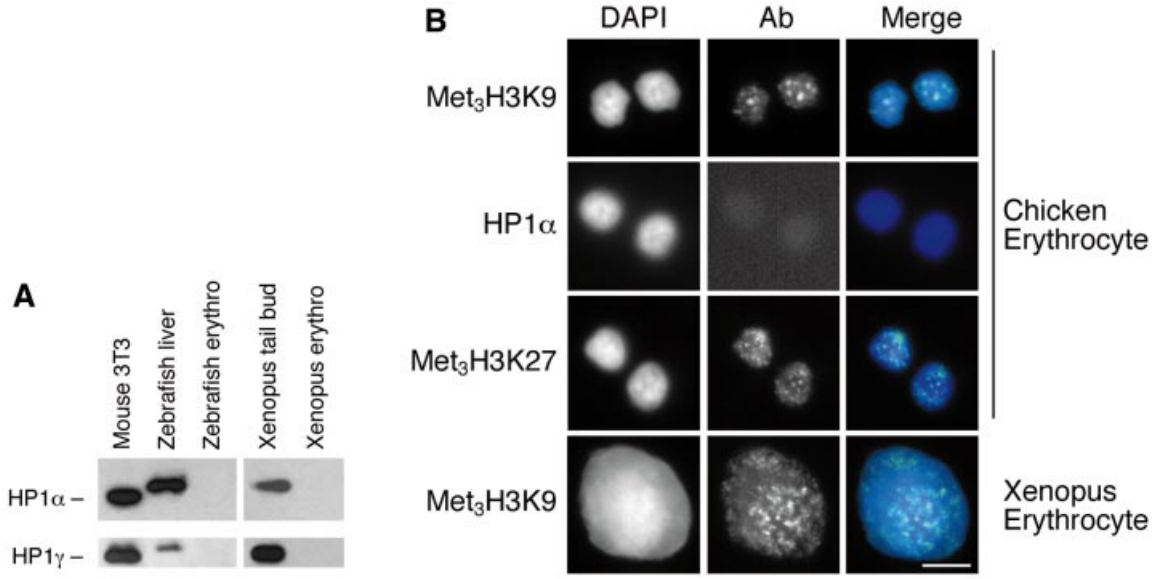

Fig. 5. Absence of HP1α from chicken, fish and *Xenopus* erythrocytes. (**A**) Western blot of proteins from fish liver and nucleated erythrocytes, and from *Xenopus* tail bud tadpole nuclei and erythrocytes, using antibodies raised against *Xenopus* HP1α and HP1γ. HP1β isoforms could not be detected in *Xenopus* or zebrafish cells using available antibodies. (**B**) Immunostaining of chicken and *Xenopus* nuclei with met$_3$H3-K9 or met$_3$H3-K27 antisera, or HP1α antibody (green) and DNA (blue). Scale bar, 5 μm.

formation and propagation of constitutive heterochromatin structure in many organisms. HP1 proteins have been implicated in some forms of facultative heterochromatin (XY body and mealy bug chromosome condensation), but not in others (somatic X inactivation) (Cowell *et al*., 2002; Peters *et al*., 2002; Metzler-Guillemain *et al*., 2003). In addition, differently methylated forms of H3 have been seen within facultative heterochromatin. SET domain-containing PcG group proteins in *Drosophila* have been shown to be H3 K27 histone methyltransferases (HMTases). The chromodomain of other PcG proteins bind this methylated form of H3 (Cao *et al*., 2002; Czermin *et al*., 2002). However, the bithorax complex that is repressed by PcG complexes and the condensed chromosome set in mealy bugs, are also enriched in meH3-K9 (Cowell *et al*., 2002). H3 becomes transiently tri-methylated at K27 during the initiation of X inactivation in embryonic stem cells (Plath *et al*., 2003; Silva *et al*., 2003). This mark may be supplemented by metH3-K9 at later stages of inactivation (Boggs *et al*., 2002), although we have clearly shown there is a concentration of met$_3$H3-K27 on the inactive X in terminally differentiated somatic mouse cells (Figure 6C). Therefore, there appear to be many mechanisms through which facultative heterochromatin is formed, and there is a need to study this in a variety of systems.

To try to clarify the histone modifications and role of HP1s in the formation of facultative heterochromatin, we used nucleated erythrocytes as a model system. Chicken, frog and fish erythrocytes are normally nucleated, in contrast to adult mammalian erythrocytes, which are enucleated late in differentiation. However, during embryogenesis, mammalian erythrocytes remain nucleated, but during their differentiation there is a dramatic increase in chromatin condensation and a decrease in transcriptional activity (Rowley and Radcliffe, 1988; Grigoryev *et al*., 1999) (Figure 3).

Abundant HP1 and metH3-K9, but an absence of metH3-K27, in mouse erythrocytes

Analysis of the nucleosome modifications in embryonic erythrocyte chromatin by western blot indicates that there are elevated levels of met$_3$H3-K9 in these cells (in comparison with liver and 3T3 cells) (Figure 6A) and, in contrast, we are unable to detect any tri-methylation of H3-K27 in erythrocytes (Figure 6A and C). Hence, we think it unlikely that PcG proteins are responsible for the chromatin compaction and gene silencing in mouse embryonic erythrocytes. Furthermore, we were unable to detect *Xist* RNA on the inactive X chromosome. This suggests that the mechanism of global facultative heterochatin formation in these cells is able to obviate the need for both *Xist* and met$_3$H3-K27. However, coincident with the increased levels of met$_3$H3-K9 in mouse erythrocytes we also detect enhanced levels of HP1α (Figure 6A), suggesting that HP1 could have a role in formation of facultative heterochromatin in these cells. It is suggested that HP1 spreads across heterochromatin through binding to histone H3 methylated at K9 (Bannister *et al*., 2001; Lachner *et al*., 2001; Partridge *et al*., 2002). Furthermore, it has been suggested that HP1 (or its orthologue) is required for the spread of met$_3$H3-K9 itself (Hall *et al*., 2002). Whilst we see some diffuse re-distribution of met$_3$H3-K9 in mouse erythrocyte nuclei, HP1α is still predominantly concentrated at the foci of constitutive pericentric heterochromatin (Figure 6B). This is consistent with the observation that, in fission yeast, met$_3$H3-K9 can spread from ectopic sites (via the action of the HMTase clr4) in the absence of the HP1 homologue swi6 (Partridge *et al*., 2002).

Absence of HP1s and reduced H3 methylation of K9 and K27 in chicken erythrocytes

To establish whether similar changes in histone methylation and HP1 are present in the erythrocytes of non-

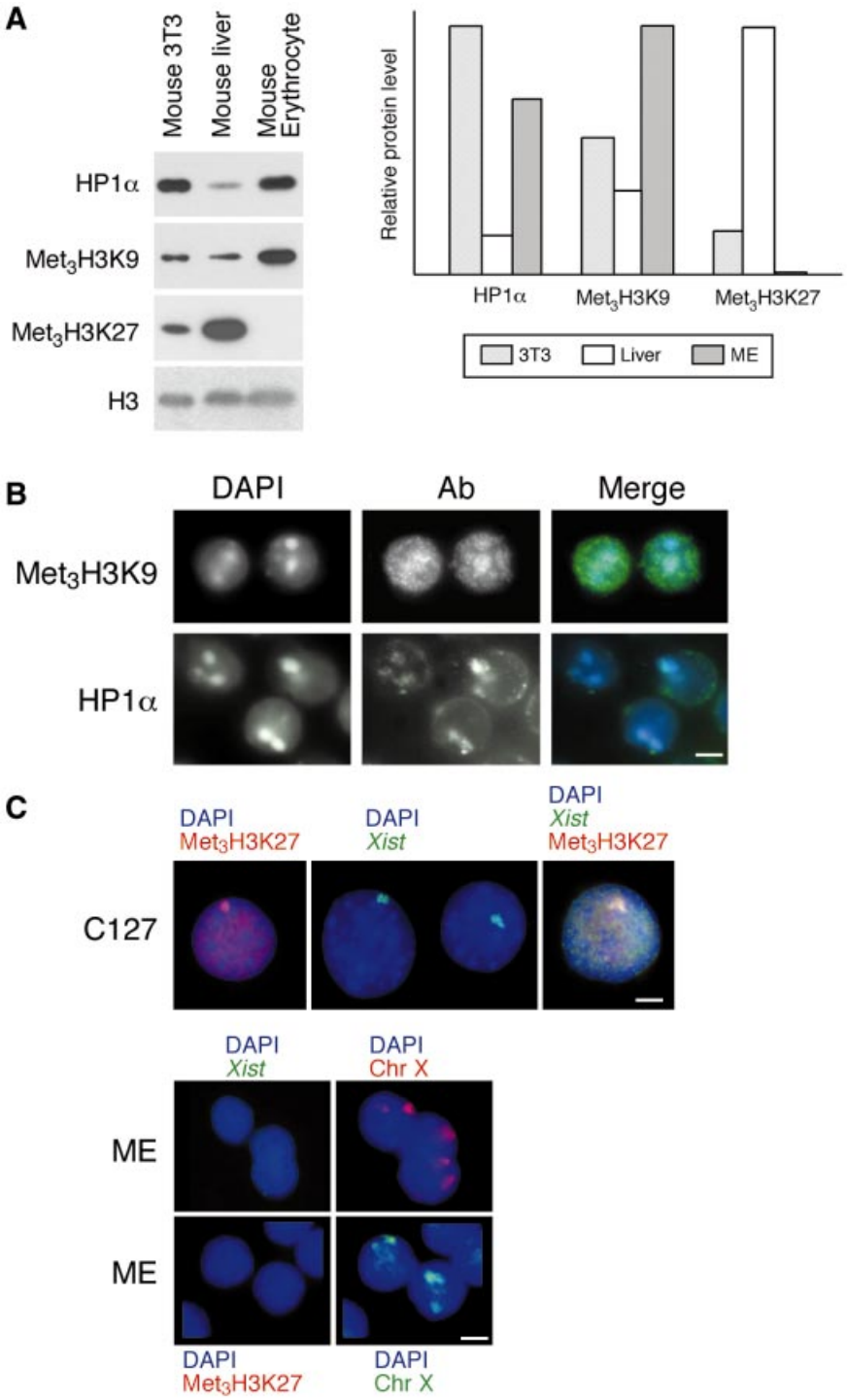

Fig. 6. HP1α and methylated H3 in mouse erythrocytes. (**A**) Western blot of proteins from mouse 3T3, liver and embryonic erythrocyte nuclei using antibodies detecting HP1α, met$_3$H3-K9 and met$_3$H3-K27. H3 antibody is used as a loading control. The graph to the right shows the quantification of these blots. (**B**) Immunostaining of HP1α and met$_3$H3-K9 (green) in mouse embryonic nucleated erythrocytes. DNA was counterstained with DAPI (blue). (**C**) Female C127 cells: immunostaining for met$_3$H3-K27 (red), RNA FISH for *Xist* (green) and double immunostaining for met$_3$H3-K27 (red) and RNA FISH for *Xist* (green). Mouse embryonic nucleated erythrocytes: RNA FISH for *Xist* (green) followed by DNA FISH for X chromosome (red), and immunostaining for met$_3$H3-K27 (red) followed by DNA FISH for X chromosome (green). Scale bar, 5 μm.

mammalian vertebrates, we analysed nuclei of chicken erythrocytes. DAPI staining reveals the chromatin in these small nuclei to be more compact than that in control (DT40) chicken cells (Figure 3), consistent with electron micrographs of chromatin in avian erythrocytes (Ruiz-Carrillo *et al*., 1974; Rowley and Radcliffe, 1988). By western blot, levels of H3 tri-methylated at both K9 and K27 are reduced in chicken erythrocytes (Figure 4) and, most strikingly, levels of all three HP1 isoforms are undetectable in adult chicken erythrocytes, as confirmed by immunofluorescence (Figure 5B). We also could not detect HP1α or γ in erythrocytes from *Xenopus* or

zebrafish (Figure 5). Hence, we suggest that there can be no role for HP1 in the formation of compact facultative heterochromatin in erythrocytes of these vertebrates. Levels of di-methylated H3-K9 have been analysed by chromatin immunoprecipitation at the β-globin locus of chicken erythrocytes (Litt *et al*., 2001). High levels of met_2H3-K9 were found over compact chromatin outside of the globin locus and over developmentally inactive globin genes. It was proposed that an insulator acts to prevent the spread of metH3-K9 and accompanying silencing proteins, such as HP1, into the globin locus (Litt *et al*., 2001). However, the absence of HP1s from chicken erythrocytes suggests that this is not necessary, although we cannot rule out a role for other chromo-box proteins that could bind metH3-K9. For example, fission yeast Chp1 also binds to metH3-K9 (Partridge *et al*., 2002).

Recently, it was suggested that HP1 function is essential for cell survival in mammalian cells, since injection of HP1 antibodies into cells resulted in cell death (Filesi *et al*., 2002). Our analyses show that HP1s are not essential for cell survival in some vertebrate erythrocytes, and moreover indicates that the expression of HP1 proteins is developmentally regulated. It will be important to analyse the presence of HP1 isoforms in other differentiated cell types.

Specialized proteins to mediate chromatin condensation in erythrocytes

Our data suggest that HP1 is not involved in the chromatin condensation of chicken, frog and fish erythrocytes. Instead, other specialized chromatin-associated proteins may be recruited to perform this role in erythrocyte differentiation. Currently, there are two such candidate proteins. The serpin-like protein MENT is able to induce large-scale chromatin condensation *in vitro* and when ectopically expressed (Grigoryev *et al*., 1999). Its accumulation during avian erythrocyte differentiation strongly correlates with the extent of chromatin condensation (Grigoryev and Woodcock, 1993). The mechanisms of recruitment and action of MENT are unknown, but it is an abundant basic protein that associates with polynucleosomes (Grigoryev *et al*., 1999).

In avian erythrocytes some H1 is replaced with the variant linker histone H5 (Ruiz-Carrillo *et al*., 1974). When injected into mammalian cells, H5 can block both transcription and DNA replication (Bergman *et al*., 1988), and there are two DNA binding sites in the H5 globular domain necessary for binding to nucleosomes (Duggan and Thomas, 2000). The function of linker histones, and their localization with respect to nucleosomes, is poorly understood (Widom, 1998), and the consequences of the presence of the H5 variant on chromatin structure are yet to be determined. However, since we have observed that the decrease in HP1s in chicken erythrocytes during development parallels the increasing levels of H5 (Figure 4C), we speculate that H5 could replace the role of HP1 in chromatin compaction.

Since globin gene expression in chicken and mouse erythrocytes is used as a model system for the study of chromatin structure and gene expression, it is important to understand the molecular basis for the chromatin compaction and gene silencing that occur in these cell types. Examination of histone modifications and HP1 isoforms in these cell types is a first step along this road, and our data show that the conclusions drawn in one species, e.g. chicken, cannot readily be extrapolated to mammals. It will also be important to examine the presence of HP1 proteins and histone modifications in other differentiated cell types.

Materials and methods

Cell lines and tissues

Male mouse NIH 3T3 fibroblasts, female mouse C127 epithelial cells and human HT1080 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS). DT40 cells were cultured in RPMI supplemented with 10% FCS and 1% chicken serum. Mouse embryo blood was obtained from 12.5 d.p.c. embryos. Adult chicken blood was isolated from freshly slaughtered female leghorn chickens. Embryonic chicken blood was prepared from 10-day embryos. Zebrafish blood and liver and *Xenopus* blood were collected from freshly culled animals. Mouse liver was obtained from culled adult mice and *Xenopus* nuclei were obtained from tail bud tadpoles.

Preparation of nuclei and chromatin

Erythrocyte and tissue culture cell nuclei were prepared by a modification of the method of Gilbert and Allan (2001). The cells were washed in phosphate-buffered saline (PBS) and resuspended in nuclear buffer A (85 mM KCl, 10 mM Tris–HCl, 0.2 mM spermidine, 0.2 mM EDTA, 160 mM sucrose, 250 μM phenylmethylsulfonyl fluoride) on ice. An equal volume of nuclear buffer B (nuclear buffer A supplemented with an appropriate amount of NP-40) was added. The nuclei were pelleted in a benchtop centrifuge (2000 *g*, 4 min, 4°C) and washed into nuclear buffer A. The concentration of the nuclei was determined by measuring the A_{260}. Mouse and zebrafish liver nuclei were prepared using the above procedure except the cells were first homogenized in nuclear buffer A. Chromatin was prepared as described previously (Gilbert and Allan, 2001). To isolate chromatin-associated proteins the chromatin was fractionated on a sucrose gradient. Peak fractions were dialysed against TE and the proteins were precipitated using acetone and resuspended in SDS loading buffer.

Antibodies

The antibodies used were: mAb HP1α, β and γ [Chemicon; western blotting (wb) 1:1000, immunofluorescence (immuno) 1:500; Nielsen *et al*., 1999]; mAb histone H5 (from Michael Bustin; wb 1:1000); rabbit polyclonals against xHP1α (affinity purified; wb 1:100) and γ (from Richard Meehan; wb 1:1000); met_2H3-K9 (Upstate; wb 1:1000, immuno 1:500; Boggs *et al*., 2002), met_2H3-K4 (Upstate; wb 1:1000, immuno 1:500), met_3H3-K9 (wb 1:2000, immuno 1:500), met_3H3-K27 (wb 1:1000, immuno 1:100; Silva *et al*., 2003), 4× met_2H3-K9 (immuno 1:500; Peters *et al*., 2001), C-terminal H3 (wb 1:10 000; Verreault *et al*., 1996); and CENP-A (from Vinciane Regnier).

Western blotting

Nuclei were suspended in SDS loading buffer, fractionated by SDS–PAGE and transferred to a nylon membrane by semi-dry blotting. The membranes were probed with antibodies using standard techniques and detected by enhanced chemiluminescence.

Immunofluorescence

Mouse NIH 3T3, C127 and human HT1080 were grown on slides and fixed using 4% pFa in PBS. The cells were permeabilized using Triton X-100 in PBS and were sequentially incubated with the primary and secondary antibodies (Jackson Laboratories). All other cells and nuclei were cytospun onto slides and were pFa-fixed and processed as above.

FISH

RNA FISH. Cells were grown on slides and fixed using 4% pFA in PBS. The cells were permeabilized using Triton X-100 in PBS and stored in 70% ethanol at 4°C. The slides were dehydrated using 70, 90, 100% ethanol and air dried. One hundred and fifty nanograms of digoxigenin (DIG)-labelled *Xist* probe (pGPT16; Duthie *et al*., 1999), 20 μg of yeast tRNA and 5 μg of sonicated salmon sperm DNA were precipitated together and resuspended in 15 μl of hybridization mix (50% deionized formamide, 10% dextran sulphate, 1% Tween-20 in 2× SSC) and incubated under a sealed coverslip overnight at 37°C. Slides were washed

for 3 min in 50% formamide/2× SSC at room temperature, for 3 min in 50% formamide/2× SSC at 37°C, and for 3 min in 2× SSC at room temperature and 4× SSC 0.1% Tween-20. The *Xist* signal was detected using one layer of FITC-conjugated anti-DIG (raised in sheep) and one layer of FITC-conjugated anti-sheep. Slides were mounted in Vectashield with 1 μg/ml DAPI. For immuno-RNA FISH the cells were subsequently incubated with primary and secondary antibodies.

DNA FISH. Cells were grown on slides and fixed using 4% pFA in PBS. The cells were permeabilized using Triton X-100 in PBS and stored in 70% ethanol. The slides were washed briefly in 2× SSC and incubated with 100 μg/ml RNaseA for 1 h at 37°C. The slides were dehydrated using 70, 90, 100% ethanol and air dried. Slides were denatured in 70% formamide/2× SSC at 70°C for 90 s, transferred to ice-cold 70% ethanol, and then into 90 and 100% ethanol and air dried. Fifteen microlitres of biotin-labelled X-chromosome paint (Cambio) were denatured at 70°C, re-annealed at 37°C for 15 min and hybridized on the slide under a sealed coverslip overnight at 37°C. The slides were washed 4× 3 min in 2× SSC at 45°C, 4× 3 min in 0.1× SSC at 60°C and then 4× SSC 0.1% Tween-20 at room temperature. Biotinylated probes were detected using Texas Red-conjugated avidin, followed by biotinylated anti-avidin and a final layer of Texas Red-conjugated avidin. Slides were mounted in Vectashield with 1 μg/ml DAPI.

For RNA/DNA FISH or immuno-DNA FISH the slides were first processed for either RNA FISH or immuno. Coordinates of cells were taken and the slide was then processed for DNA FISH.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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