

## Dynamic chromatin modifications characterise the first cell cycle in mouse embryos

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### Abstract

On fertilisation, gametes undergo epigenetic reorganisation and re-establish totipotency. Here, we investigate links between chromatin remodelling and asymmetric maintenance of DNA methylation in the early mouse embryo. Using antibodies for lysine specific H3 methylation reveals that the male pronucleus is negative for di- and trimethyl H3-K9 yet the female is positive for these residues. However, the male is positive for monomethyl H3-K9 and H3-K27 and these signals increase during pronuclear maturation. Non-histone chromatin proteins of the Polycomb group are found in the paternal compartment as early as sperm decondensation. However, trimethyl H3-K27 is not observed in the male until the completion of DNA replication. Heterochromatin protein 1 beta (HP1 $\beta$ ) is abundant in the male pronucleus, despite the absence of di- and trimethyl H3-K9, and co-localises with monomethyl H3-K9. Recent evidence identifies monomethyl H3-K9 as the preferred substrate of Suvar39h, the histone methyl transferase (HMT) responsible for heterochromatic H3-K9 trimethylation. The association of HP1 $\beta$  with monomethyl H3-K9 may assist in preventing further modification of H3-K9. Association of dimethylation but not trimethylation of H3-K9 with DNA methylation, in the female pronucleus, suggests a mechanistically significant link. These differences begin to provide a chromatin based explanation for paternal-specific active DNA demethylation and maternal specific protection in the mouse.

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### Introduction

On fertilisation, highly specialised gametes undergo remodelling, restoring totipotency and reforming the diploid zygote. This event brings together the meiotically arrested MII oocyte and the fully matured sperm with chromatin organised in protamine based toroidal structures (Balhorn et al., 2000; Braun, 2001). Despite the similar genetic content of the gametes, differences in epigenetic information reinforce their distinctive nature.

During the first cell cycle, remodelling of maternal and paternal components re-establishes transcriptional activation of zygotic gene expression (Latham and Schultz, 2001; Ram and Schultz, 1993). Epigenetic alterations are central to achieving this end, involving chromatin and DNA methylation changes. DNA methylation is reprogrammed during development influencing genome stability and gene regulation, especially differential DNA methylation associated with imprinted gene expression (Reik and Walter, 2001). However, DNA methylation does not influence transcription in isolation; rather, it exerts its influence through interactions with other epigenetic features of chromatin (Bird, 2002; Li, 2002) which are competent to create stable and heritable changes associated with cellular differentiation and development.

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Epigenetic regulation of gene silencing includes non-histone chromosomal proteins such as the polycomb gene group (Pc-G)/trithorax (trx) complexes (Lund and van Lohuizen, 2004; Orlando, 2003; Otte and Kwaks, 2003) and modifications of the amino-terminal tails of core histones (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000). Covalent modifications of acetylation, methylation, phosphorylation and ubiquitination at key residues in nucleosomal histones create sufficient unique configurations at all single copy genes prompting the idea that superimposed on the genetic code is an epigenetic marking system represented by a 'histone code' (Jenuwein and Allis, 2001). Recent studies in *Neurospora* and *Arabidopsis* have led to the suggestion that DNA methylation and histone methylation are inter-related, although whether this can be extended to mammals has not yet been firmly established (Espada et al., 2004; Fuks et al., 2003; Jackson et al., 2002; Lehnertz et al., 2003; Tamaru and Selker, 2001; Tamaru et al., 2003).

In early mouse embryos, DNA methylation is reprogrammed genome-wide (Reik et al., 2001). Within 1 h of fertilisation, paternal-specific active demethylation is initiated and establishes a parental asymmetry in the early embryo (Santos et al., 2002). The identity of this activity remains elusive, as is the nature of the mechanism that protects the female pronucleus from a similar fate. The significance of this reprogramming is unclear at present, but it is probably involved in the resetting of gametic epigenetic patterns necessary to achieve transcriptional competence during early development (Dean et al., 2003).

We have been interested in the nature of chromatin organisation, one that is permissive for paternal specific active demethylation and another that confers resistance to the female pronucleus in the milieu of the oocyte cytoplasm. In the context of the role epigenetic marking may have in establishing heritable memory systems, the immediate post fertilisation changes may well dictate long range outcomes for embryonic growth and development. Particular emphasis has been placed on the status of the modification of the lysine residues 9 and 27 of histone H3 and the Pc-G group proteins Eed/Ezh2. The organisation of heterochromatin, distribution of epitopes and the intensity of the signals are highly specific in the zygote and differ between the respective pronuclei. These differences may reflect developmentally significant states of chromatin modification uniquely associated to remodelling of the gametes. These results point to a possible role for epigenetic modifications, including both DNA methylation and chromatin, interacting to reinforce parent-of-origin based information essential for maintaining imprinting. The reconciliation of these two mechanistically inter-related systems may provide key insights into the fundamental processes necessary to achieve full term development on somatic nuclear transfer.

## Material and methods

### *Collection of mouse oocytes*

To provide a detailed characterisation of the dynamic chromatin modifications that occur on fertilisation, precise timing of progression of pronuclei was made possible by fertilising them *in vitro*. IVF and/or natural matings fertilised oocytes used throughout this study were derived from a cross of (C57BL/6J X CBA/Ca) F<sub>1</sub> females, hereafter referred to as B6CBAF<sub>1</sub>. Sperm was obtained from B6CBAF<sub>1</sub> males. Unfertilised oocytes used in IVF were collected from B6CBA F<sub>1</sub> mice approximately 14 h post human chorionic gonadotropin (hCG) injection. IVF generation of fertilised oocytes was that described in Summers et al. (1995) with the exception that sperm capacitation was carried out in modified KSOM (Lawitts and Biggers, 1993) containing 5.56 mM glucose, initially in the absence of BSA, and then in the presence of 15 mg/ml embryo tested BSA (Sigma).

### *BrdU labelling*

Fertilised oocytes were pulse-labelled *in vitro* with 5-bromo-deoxyuridine (BrdU) in order to evaluate time of replication. The zygotes were incubated in M16 medium (Sigma) supplemented with 100 µM BrdU (Sigma) for 3 h at 37°C in 5% CO<sub>2</sub> in air. After incubation, the zygotes were fixed for 15 min in 4% paraformaldehyde in PBS and permeabilised with 0.2% Triton X-100 in PBS for 1 h at room temperature.

### *Immunofluorescence staining*

#### *Characterisation of antibodies*

Highly specific antibodies raised to the mono-, di- and trimethylated states of H3-K9 and mono- and trimethylated H3-K27 were used (Lehnertz et al., 2003; Peters et al., 2003) and were characterised on mouse foetal fibroblasts grown on coverslips (Supplementary Fig. 1).

The fidelity of staining was compared between a commercially supplied antibody against dimethylated H3-K9 (Upstate) and a second antibody raised against 2x aa5–15 of H3 with dimethylated K9 (see Supplementary Fig. 2) (Peters et al., 2003). Fertilised oocytes were stained for 1 h at 1:500 dilution of both diK9 antibodies and detected with goat α-rabbit IgG-Alexa 594 (Molecular Probes) as secondary antibody.

Initial characterisations of the antibodies raised against a branched form of dimethylated H3-K9 (see Supplementary Fig. 1: branched) suggested the potential for cross-reactivity with other methylated lysine epitopes, significantly lysine 27 (K27). This suspicion was later confirmed and is reported herein (Peters et al., 2003). Therefore, to establish a base line for comparison, IVF generated embryos were stained with an antibody raised against trimethylated K27. The results indicate that despite the potential for cross reactivity

of the branched antibody with K27 none is observed (Supplementary Fig. 3).

#### Staining of fertilised oocytes

Fertilised oocytes generated by IVF and natural matings were washed in PBS, fixed for 15 min in 4% paraformaldehyde in PBS and permeabilised with 0.2% Triton X-100 in PBS for 30 min at room temperature. Fixed materials were blocked in 0.05% Tween-20 in PBS containing 1% BSA overnight at 4°C prior to the application of primary antibodies. For the experiments described in Fig. 4B, Triton X-100 was replaced in all solutions with Saponin (S-4521, Sigma) at incremental concentrations between 0.1% and 3%. Samples were incubated either for 1 h at room temperature or overnight at 4°C with immunoaffinity-purified rabbit IgG antibodies (all diluted 1:500) against mono-( $\alpha$ -2x-mono-methH3-K9), di-( $\alpha$ -2x-di-methH3-K9) (Peters et al., 2003) and tri-( $\alpha$ -2x-tri-methH3-K9) (Lehnertz et al., 2003) methylated histone H3 lysine 9;  $\alpha$ -4x-dimethH3-K9 antibody raised against a branched peptide (Peters et al., 2001) and mono-( $\alpha$ -2x-mono-methH3-K27) or trimethylated ( $\alpha$ -2x-tri-methH3-K27) histone H3 lysine 27 (Peters et al., 2003). In order to establish continuity of results, we have compared the staining pattern of the previously used  $\alpha$ -dimethyl-H3-K9 (Upstate 07-212) and the new  $\alpha$ -2x-di-methH3-K9 (Peters et al., 2003) both diluted 1:500 (Supplementary Fig. 2). Antibodies to some of the Pc-G proteins were also used in this study:  $\alpha$ -Eed mouse monoclonal and  $\alpha$ -Ezh2 rabbit polyclonal (Sewalt et al., 1998) diluted 1:200; mouse  $\alpha$ -HP1 $\beta$  (Chemicon, clone MAB3448);  $\alpha$ -acH3-K9 (Upstate 06-942) diluted 1:500 all incubated 1 h at room temperature. Detection was achieved using either goat  $\alpha$ -rabbit IgG-Alexa 488 (Molecular Probes) or goat  $\alpha$ -rabbit IgG-Alexa 594 (Molecular Probes) as secondary antibodies. Double antibody staining was accomplished by sequential incubation of the relevant primary and secondary antibodies. DNA was stained with DAPI (1 $\mu$ g/ml) and all samples were mounted in 50% glycerol in PBS (Sigma).

Simultaneous antibody staining of chromatin components and DNA methylation (5MeC) was performed as previously described (Santos et al., 2003) with modifications. Briefly, after permeabilisation with 0.2% Triton X-100, the embryos were treated with 4 N HCl for 10 min at room temperature and washed and blocked overnight; simultaneous incubation with both primary antibodies followed by simultaneous secondary detection was used. DNA denaturation conditions have been optimised such that the double staining results are indistinguishable from single epitope detection of the chromatin proteins. The same protocol was used for simultaneous BrdU (DAKO clone Bu20a-FITC conjugated antibody) and tri-methH3-K27 staining.

#### Digital imaging microscopy and analysis

Observations were made using an Olympus BX41 epifluorescence microscope. Grey-scale images were

recorded digitally with a high-resolution CCD camera (F-View) with the AnalySIS 3.2 image analysis software (SIS GmbH) using separate Band-Pass filter sets for Alexa Fluor 488, Alexa Fluor 594 and DAPI. The same software package was used to obtain image intensity profiles (grey-value intensity of designated row across the image) of HP1 $\beta$  stained fertilised oocytes as portrayed in Fig. 4B and to quantify the pixel intensity in the perinucleolar heterochromatic rings of both male and female pronuclei. This analysis was performed in 20 fertilised oocytes (PN<sub>3</sub>–PN<sub>5</sub>) and the integral values were used to calculate female versus male intensity ratios. Grey-scale images were pseudo-coloured after capture and merged using Adobe Photoshop 6.0 software.

Confocal images were obtained using a Leica TCS SP1 system (488 and 568 nm excitation lines; 500–560 and 600–660 nm capture) with the exception of images in Fig. 3C that were obtained using a Zeiss LSM 510 META.

Co-localisation software (ImageJ 1.32-<http://rsb.info.nih.gov>) was applied to grey-scale digital images of 5  $\mu$ m confocal sections (Supplementary Fig. 4). This software highlights the co-localised points (appearing white

Table 1  
Asymmetric chromatin features in the one-cell mouse embryo

Compartment (PN stage) <sup>a</sup>	Epigenetic characteristic	Comments
m, p (PN <sub>0</sub> )	monomethylated H3-K9	Increasing signal in $\delta$ ; continuous staining in $\delta$ pronucleus at perinucleolar ring
m (PN <sub>0</sub> ), p (PN <sub>5</sub> )	dimethylated H3-K9	Uniform punctate distribution; continuous perinucleolar ring (overnight incubation)
m (PN <sub>0</sub> ), p (–)	trimethylated H3-K9	Intense discontinuous perinucleolar rings
m (PN <sub>0</sub> ), p (–)	branched configuration (heterochromatin specific)	Intense discontinuous perinucleolar rings
m (PN <sub>0</sub> ), p (PN <sub>1</sub> )	acetylated H3-K9	Uniform punctate distribution
m (PN <sub>0</sub> ), p (PN <sub>1</sub> )	HP1 $\beta$	Intense discontinuous perinucleolar rings; staining much greater in $\delta$ ; discontinuous in $\delta$
m, p (PN <sub>0</sub> )	Eed/Ezh2	Punctate with weak perinucleolar rings from PN <sub>4</sub>
m, p (PN <sub>0</sub> )	monomethylated H3-K27	Intense discontinuous perinucleolar rings; staining much greater in $\delta$ ; continuous perinucleolar rings in $\delta$
m (PN <sub>0</sub> ), p (PN <sub>3</sub> /PN <sub>4</sub> )	trimethylated H3-K27	Punctate in $\delta$ ; perinucleolar ring in $\delta$ ; co-localises with Eed/Ezh2; post-replication
m (PN <sub>0</sub> ), p (PN <sub>0</sub> –PN <sub>3</sub> )	DNA methylation	Paternal specific active demethylation; pericentromeric CpG CH <sub>3</sub> remains in $\delta$

m—maternal; p—paternal.

<sup>a</sup> Pronuclear stages as outlined in Santos et al. (2002).

in the RGB) of two 8-bit images (threshold of channels 100; ratio setting value 50%).

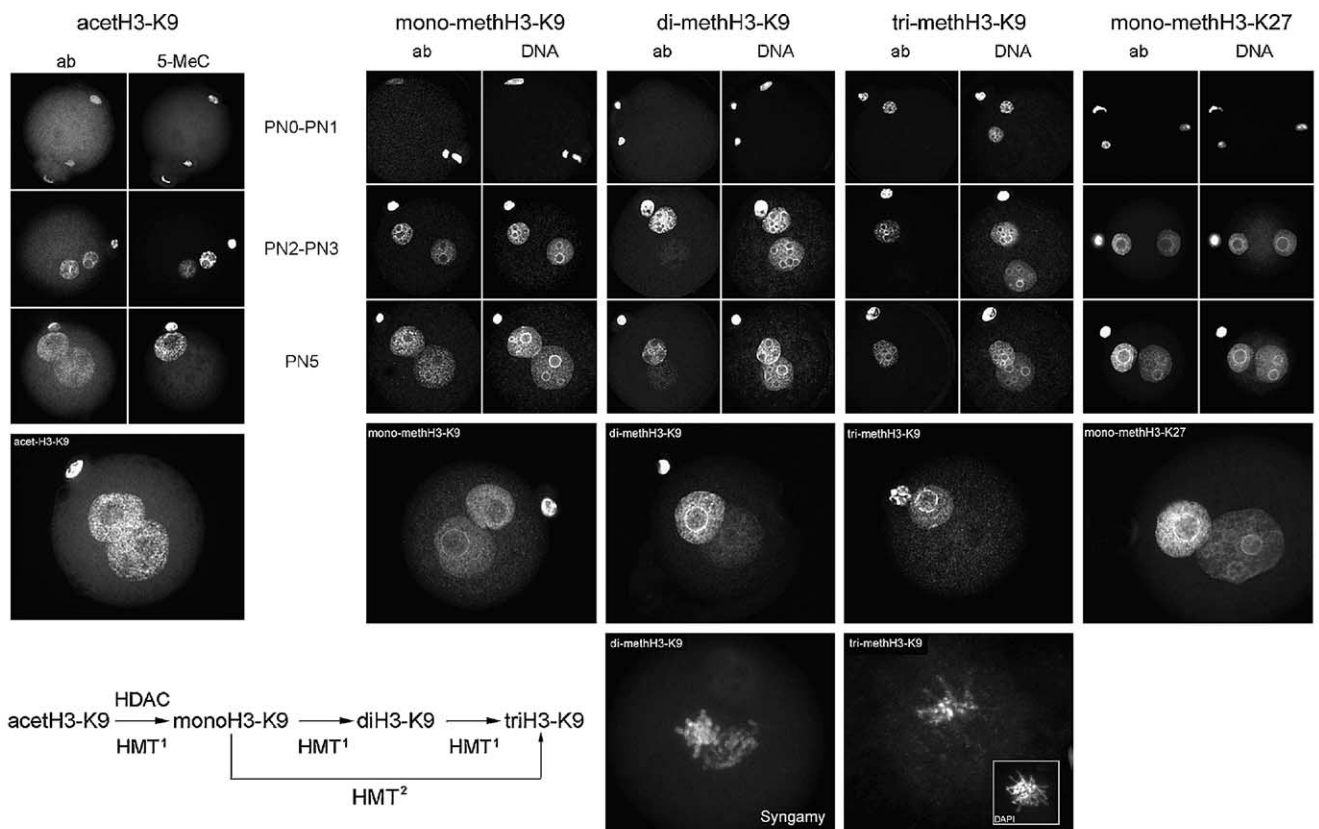
## Results

Epigenetic marking by DNA methylation and histone modification has been reported in a number of model systems (Fuks et al., 2003; Jackson et al., 2002; Tamaru and Selker, 2001; Tamaru et al., 2003). A vast body of literature indicates that chromatin modifications dictate transcriptional states (Adenot et al., 1997; Santos-Rosa et al., 2002; Schubeler et al., 2004; Zhang and Reinberg, 2001; reviewed in Fischle et al., 2003a). These modifications may in turn rely on DNA methylation to formalise configurations and establish heritable memory patterns essential for cell differentiation and development (Bachman et al., 2003; Jaenisch

and Bird, 2003). Therefore, we were interested in examining the potential relationship of DNA methylation to the organisation of chromatin components in the early mouse embryo.

To investigate these epigenetic marks in context, we devised a method of antibody staining whereby the DNA methylation status and histone modification, i.e. methylation, could be evaluated in the same embryo (see Materials and methods). Natural mating and in vitro fertilised (IVF) oocytes were collected and analysed for a selection of chromatin modifications thought to operate in concert with DNA methylation. Particular emphasis has been placed on the lysine residues of histone H3, largely owing to positioning of the amino terminal tail in the major groove of the DNA helix (Luger et al., 1997).

A detailed evaluation of the staining and distribution of the signal indicated very precise and distinctive patterns for



HMT<sup>1</sup> ≡ G9a, ESET/SETDB1

HMT<sup>2</sup> ≡ Suv39h

HMT<sup>3</sup> ≡ Undefined

acetH3-K27  $\xrightarrow[\text{HMT}^3]{\text{HDAC}}$  monoH3-K27

Fig. 1. Dynamic chromatin organisation during the first cell cycle. In vitro fertilised oocytes were collected and the chromatin status of lysine 9 and 27 was assessed using indirect epifluorescence. Maternal and paternal chromatin both stained for acetylated H3-K9 (acetH3-K9), which remains throughout the first cell cycle. During this early phase, mono- but not di- or trimethylated H3-K9 is observed in the male pronucleus. For acetylation to give way to methylated modifications demonstrates the presence of both histone deacetylase (HDAC) and histone methyl transferase (HMT) in the sperm nucleus. Dimethylated H3-K9 is first detected post replication in the male pronucleus (PN<sub>5</sub>) while triK9 is not observed in the male during the first cell cycle. The presence of monoK27 in the early decondensing sperm suggests that a further HMT must also be present. On overnight incubation of primary antibody, the distinctive spatial organisation and relative quantitative distribution of the various epitopes are observed (last panel in each series). Representative images of metaphase chromosomes observed at syngamy are included for di and triK9. Dimethylated H3-K9 but not triK9 is observed in the paternal set of chromosomes. The biochemical pathways for K9 and K27 methylation are outlined below.

each of the methylation modifications of K9 and K27 (Table 1). On decondensation, the sperm nucleus acquired H3-K9 acetylated histones and stained positive for mono-methylated H3-K9 (monoK9) and K27 (monoK27) (Fig. 1). During this time, paternal DNA methylation is specifically lost (Santos et al., 2002). Remarkably, in contrast, no di-(diK9) or trimethylated H3-K9 (triK9) (including the heterochromatin specific branched configuration) signal was observed in decondensing sperm (Fig. 1; di-, trimethK9). However, anaphase chromosomes of maternal origin stained very intensely for DNA methylation and all H3-K9 methylation modifications (Fig. 1; PN<sub>1</sub>). Intense staining in perinucleolar regions confirm that di- and triK9 and monoK27 are associated to pericentromeric and centromeric satellites (Arney et al., 2002; Dillon and Festenstein, 2002). Immediately on fertilisation, monoK27 was observed in both the metaphase chromosomes of the oocyte and the decondensing sperm nucleus. Co-incident staining with DAPI indicates an association with the AT rich satellites (Kapuscinski, 1995; Tanious et al., 1992) that comprise the perinucleolar rings seen in both pronuclei prior to syngamy.

The presence of monoK9 but not di- or triK9 reinforces the notion that several independent and specific activities are required for the transition between active acetylated residues and their methylated counterparts associated to transcriptional silencing and formation of constitutive heterochromatin. During the phase (up to 6 h post fertilisation) when paternal specific active DNA demethylation occurs (Mayer et al., 2000; Oswald et al., 2000; Santos et al., 2002), the male pronucleus has not yet acquired di- or triK9 modifications. Several hours later, following full maturation of the pronuclei, di-, but not tri-K9, is first detectable in the male. On condensation of chromosomes at syngamy, this is particularly noticeable. Despite the higher density chromatin organisation, tri-K9 remains undetectable in paternal chromosomes (Fig. 1; syngamy). In contrast, double labelling for di- or triK9 and 5MeC indicated that these marks are abundant in the female pronucleus. There was apparent co-localisation between diK9 and DNA methylation and no apparent association with triK9, even in pericentromeric regions (Fig. 2). This was supported by image analysis using software specific for this application (Supplementary Fig. 4). This confirms a correlation between DNA and histone H3-K9 methylation, which may be functionally significant in centromeric heterochromatin. Thus, the timing of the disappearance of DNA methylation suggests that demethylation proceeds in, and is perhaps permitted by, the absence of genome-wide H3-K9 methylation in the male. In turn, diK9 modification may fully confer protection from demethylation in the female.

During the earliest stages of decondensation and the completion of meiosis, only the maternal chromatin is positive for triK27 reinforcing an early asymmetry between the two pronuclei (Fig. 3A). However, triK27 is first detected in the male by late PN<sub>3</sub> or early PN<sub>4</sub>. By the time

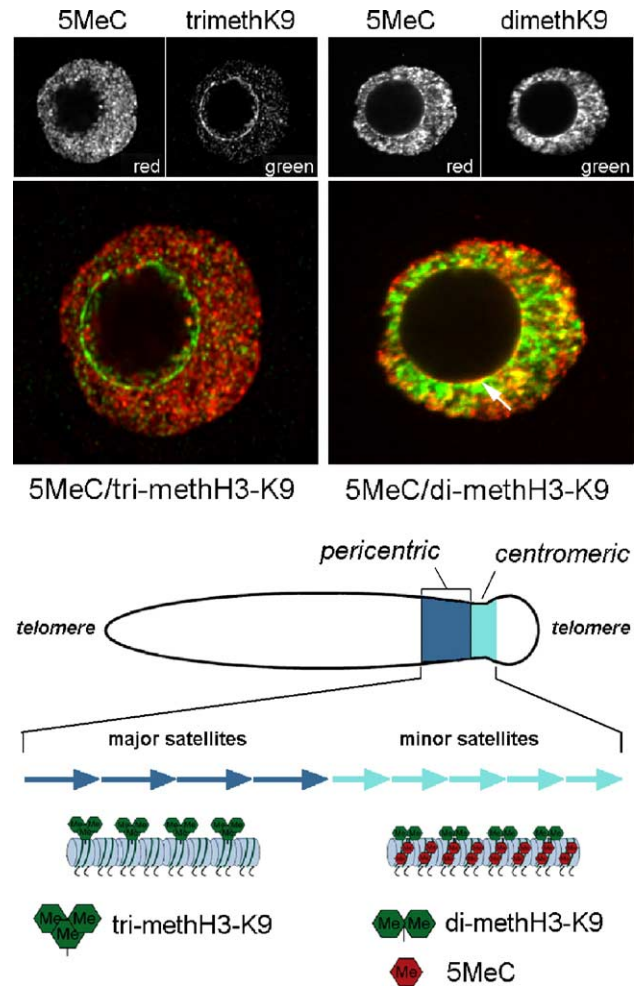
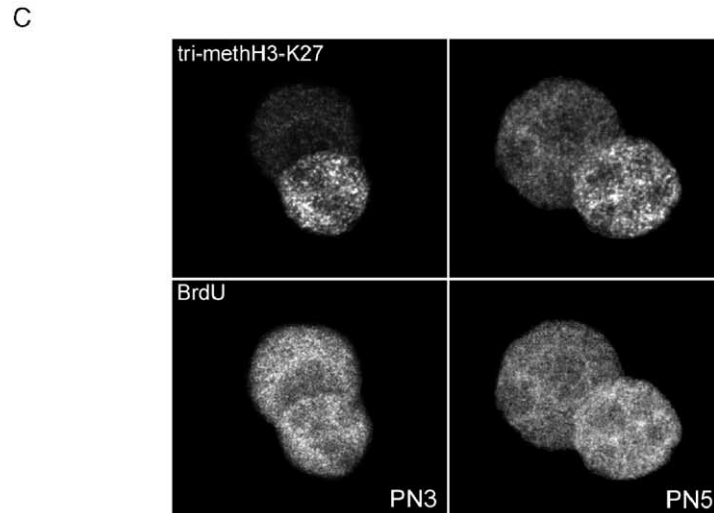
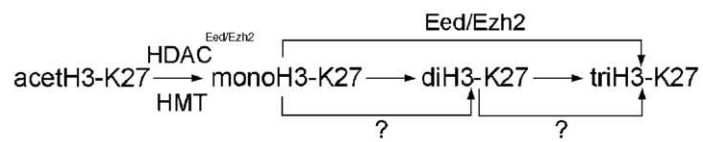
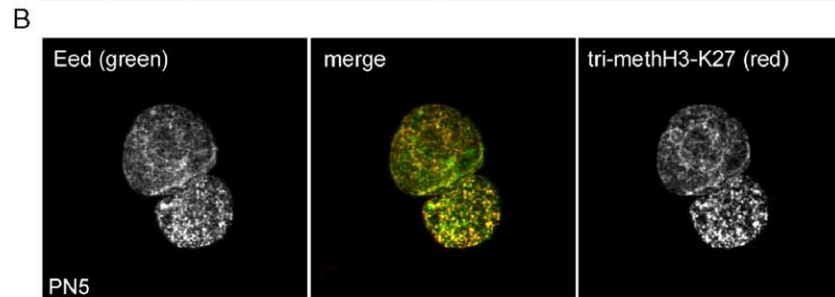
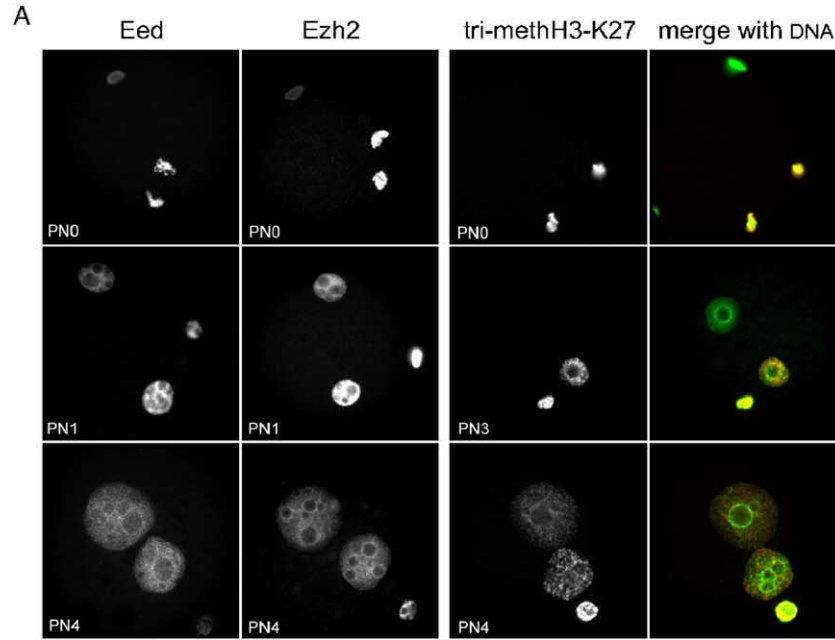


Fig. 2. Epigenetic marks in maternal heterochromatin. To investigate the potential interaction of DNA methylation and histone modifications in the fertilised zygote, PN<sub>2</sub> pronuclear staged oocytes were simultaneously labelled with antibodies to 5MeC and di- or trimethylated H3-K9 and evaluated using a Leica confocal microscope. TriK9 methylation is observed as a discontinuous perinucleolar ring (green). Merged channels recording triK9 (green) modification and DNA methylation (red) indicate clear areas of either one or the other but not co-localisation. Large heterochromatic foci seen closer to the nuclear periphery are likely to represent telomeres highly enriched for triK9. Dimethylated K9 forms an intense ring around the nucleolus (green) that coincides with the minor centromeric satellites that comprise, in part, maternal heterochromatin. This is apparent only on overnight incubation. Double staining of diK9 (green) and DNA methylation (red) showed areas of apparent co-localisation, namely in the heterochromatic rings (arrow). Co-localisation software, Image J 1.32 confirms these observations (Supplementary Fig. 4). These associations are in keeping with results obtained in ES cells depicted here in schematic form (Peters et al., 2003).

the mature pronuclei have formed (8–10 h post fertilisation; Santos et al., 2002), the male is also positive for this modification albeit less intensely than the female (Fig. 3B). This time point corresponds to the onset of DNA replication (Fig. 3C). The punctate distribution of triK27 suggests a non heterochromatic localisation especially as there is no concentration of signal in the centromeric regions that ordinarily occupy a perinucleolar position.

To further elaborate on the parental asymmetry observed for K27, we investigated the status of Eed and Ezh2 during these earliest stages of the first cell cycle. Polycomb (Pc) proteins are known to be associated with heritable epigenetic

regulation of gene expression in development. Eed/Ezh2 form complexes that methylate K27 via the SET domain containing histone methyl transferase (HMT) function associated to Ezh2 (reviewed in [Cao and Zhang, 2004](#)).



The MII oocyte is positive for both proteins but it is not seen in the male pronucleus until PN<sub>1</sub>. Despite the presence of Eed/Ezh2 and monoK27 in the male pronucleus, it does not become positive for triK27 until PN<sub>3</sub>, after replication has occurred (Figs. 3A, C). Co-localisation of Eed, Ezh2 and K27 reinforces their functional association (Fig. 3B; Eed/triK27) although the patterns of distribution are markedly different between the male and female pronuclei.

The pattern of DNA and histone H3-K9 methylation suggests that other components associated with silent chromatin configurations may also be present in one but not in both of the mature pronuclei in the fertilised oocyte. We have tested this hypothesis using an antibody to HP1 $\beta$ , a chromatin protein known to bind to methylated lysine 9. Double staining indicates that HP1 $\beta$  is coincident with monoK9 (male) and triK9 (female), but not diK9 in heterochromatic regions (Fig. 4A). This result supports suggestions of a functional association of HP1 binding with the spreading of histone methylation in heterochromatin (Bannister et al., 2001; Lachner et al., 2001). Recent results have indicated that HP1 $\beta$  has differential affinity (in descending order) for tri-, di- and monomethylated K9 (Fischle et al., 2003b). Despite the absence of the target in the male pronucleus, both pronuclei were found to contain HP1 $\beta$ . In an attempt to understand this unexpected outcome, fertilised embryos were subjected to a more stringent regime of permeabilisation and washing in order to determine whether the HP1 $\beta$  was equally tightly bound in the two pronuclei. Embryos were processed with varying concentrations of saponin (0.1%–3%) and stained for HP1 $\beta$ . On processing with saponin (1%), qualitative and quantitative differences in the HP1 $\beta$  signal become apparent (Fig. 4B). The much diminished staining intensity in perinucleolar rings of the male pronucleus (Fig. 4B; intensity profile) is more consistent with expectations based on the known binding substrates for HP1 $\beta$  (Cheutin et al., 2003; Festenstein et al., 2003; Fischle et al., 2003b). Quantification of perinucleolar staining of HP1 $\beta$  using AnalySIS 3.2 indicated an average signal ratio of female to male of  $1.6 \pm 0.25$  (n=20 zygotes). Similarly, the sharp outline in the female pronucleus suggests association with pericentromeric satellites known to be rich in triK9 modified chromatin (Lehnertz et al., 2003; Peters et al., 2003). However, the high definition and intense outline in the male reinforce the binding partnership of HP1 $\beta$  to heterochromatin, likely at monoK9 residues.

## Discussion

Epigenetic reprogramming is an integral process in the re-establishment of totipotency after fertilisation (Reik et al., 2001). Little is known about the chromatin alterations that accompany the immediate post-fertilisation period in terms of epigenetic changes although genome-wide changes in DNA methylation have been documented (Reik and Walter, 2001). Chromatin remodelling is a multi-stage process with the newly fertilised oocyte in transition between gametic chromatin (retaining meiotic features), and zygotic or embryonic chromatin (strictly mitotic) which will shortly become transcriptionally competent. The result is that both male and female pronuclei are remodelled in the same environment producing ‘opposing’ epigenetic outcomes. DNA methylation differentials are initially maximised, while chromatin organisation is progressively modified to become more functionally equivalent. The reconstitution of the diploid zygote is achieved with the remaining parental differences in epigenetic marks residing predominantly in heterochromatic regions (Figs. 1 and 4) and in imprinted genes.

Here, we report on a comprehensive investigation into chromatin dynamics that characterise the first cell cycle. A key finding is that de novo histone methylation from monomethyl to trimethyl K27 and particularly K9 in the male pronucleus is delayed despite the presence of the appropriate histone methyltransferases (Fig. 5). The delay may be caused by binding of HP1 $\beta$  or other proteins to methylated lysine residues and may have important roles in epigenetic modification of the two parental genomes and the initiation of embryonic transcription.

There are major epigenetic differences between gametes at fertilisation. Mature gametes have highly methylated DNA, yet shortly after fertilisation, the male pronucleus is rapidly and specifically demethylated by an active process (in the absence of DNA replication) (Mayer et al., 2000; Oswald et al., 2000; Santos et al., 2002) while there is a small but significant increase in the DNA methylation content of the female pronucleus (Oswald et al., 2000). Previous studies have shown that there are high levels of histone H3 di- and triK9 and K27 methylation in the female pronucleus, which are absent from the male (Arney et al., 2002; Cowell et al., 2002; Erhardt et al., 2003; Liu et al., 2004; Reik et al., 2003), suggesting the possibility that histone methylation protects the DNA in the female

Fig. 3. Chromatin proteins of the Polycomb group are differentially present in the newly fertilised mouse oocyte. The remodelling of the sperm also involves the incorporation of non-histone chromatin proteins from the Polycomb group of transcriptional repressors. (A) A low but detectable Eed/Ezh2 signal is present in the decondensed sperm. Despite the presence of the SET domain containing Ezh2, the only HMT competent to trimethylate H3-K27, K27 methylation does not occur until post replication (PN<sub>4</sub>). (B) By this stage, Eed/Ezh2 is represented in approximately equal proportion in both the male and female pronucleus as is triK27. Co-localisation at higher magnification indicates that the distribution and intensity of Eed and triK27 are differential between the male and female pronucleus. These results suggest that, although both parental compartments include Polycomb components and their substrate, the organisation remains different. The exact progression from acetylated to trimethylated K27 is not known. The presumed progression of modification is outlined below. (C) To establish the timing and progression of the appearance of triK27, fertilised oocytes were incubated in the presence of bromodeoxyuridine for 3 h prior to fixation and double staining with triK9. These results clearly indicate that the first appearance of triK27 in the male pronucleus occurs post replication by BrdU detection; thereafter, the intensity of triK27 increases.

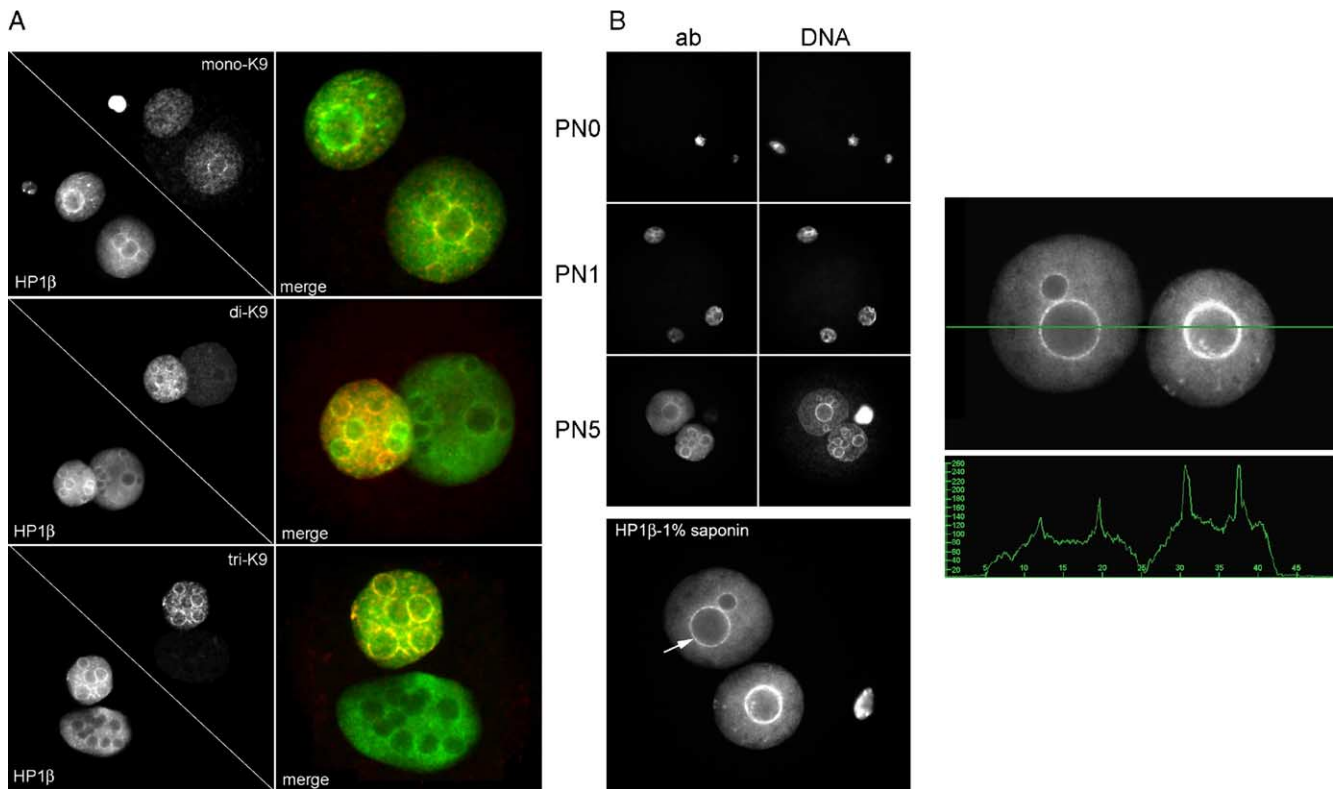


Fig. 4. Methylation status of H3-K9 influences association of HP1 $\beta$  in the male pronucleus. Double labelling of IVF generated early staged fertilised oocytes with mono-, di- and trimethylated H3-K9 and HP1 $\beta$  identifies differential binding of modified lysine residues occurring in the male and female pronucleus. (A) On merging of the two signal channels, co-localisation of monoK9 and HP1 $\beta$  in heterochromatin is obvious in the male pronucleus but absent from the female (upper panel). In contrast, trimethylated K9 co-localises to perinucleolar rings in the female (middle and lower panel) on merge (yellow rings). (B) HP1 $\beta$  is present at similar levels of intensity within 1 h of decondensation in the developing male pronucleus. In fully matured and replicated pronuclei, there is differential intensity of signal that is associated with the presence of di- or triK9. Extensive washing in 1% saponin highlights the quantitative and qualitative interactions in the male and female pronucleus. Note the breadth and brightness of the signal in the female pronucleus compared to the male (arrow). To provide a quantitative assessment of this difference, 20 similarly staged fertilised oocytes were evaluated using Analysis 3.2 software. An example for the embryo shown is included (right hand panel) illustrating the horizontal intensity profile.

pronucleus from demethylation and raising the question of when and how the paternal chromatin acquires these modifications.

Polycomb group proteins have been described to function in heritable epigenetic regulation of gene expression. In particular, the Eed/Ezh2 complex is significant since it has HMT activity on H3-K27. The very early appearance of both proteins in the decondensed sperm nucleus coincident with methylated K27 (Fig. 3A) is consistent with the proposed HMT function of the SET domain of Ezh2. Erhardt et al. demonstrated that this relationship holds true in the early fertilised embryo as conditional deletion of Ezh2 impairs asymmetric distribution of Eed between pronuclei and resulted in the absence of triK27. Following histone assembly in the decondensing sperm nucleus, K27 needs to be deacetylated, perhaps involving the Eed/Ezh2 remodelling complexes known to include HDACs (Tie et al., 2001; van der Vlag and Otte, 1999). It is not yet clear whether K27 is monomethylated by Ezh2 or by an as yet unknown HMT. The acquisition of K27 trimethylation coincides with the onset of DNA replication (Fig. 3C), suggesting that there is a transition in

the paternal pronucleus to a significantly different chromatin configuration. This delayed trimethylation despite the presence of monoK27 and Ezh2 is remarkable. It is possible that Ezh2 requires another signal, perhaps H3-K9 modification, in order to complete K27 trimethylation. Similar interactions have been reported recently where the presence of K9 trimethylation influenced the trimethylation of H3K20 (Schotta et al., 2004). Nevertheless, the presence of Polycomb group proteins in the male pronucleus provides a basis for the establishment of paternal epigenetic marks immediately on sperm remodelling, which may be heritable thereafter (Pickard et al., 2001).

An even more delayed progression from mono- to trimethylation is observed for K9. As with K27, immediately on decondensation, the sperm nucleus becomes positive for monoK9, requiring prior action of HDACs to modify the newly assembled acetylated nucleosomes (Verreault, 2000), and the presence of unknown HMTs that catalyze K9 monomethylation. Detailed examination of the distribution and signal intensity of monoK9 in the fertilised oocyte indicates that monomethylation is replication inde-



## Dynamic chromatin modifications during the formation of the male pronucleus

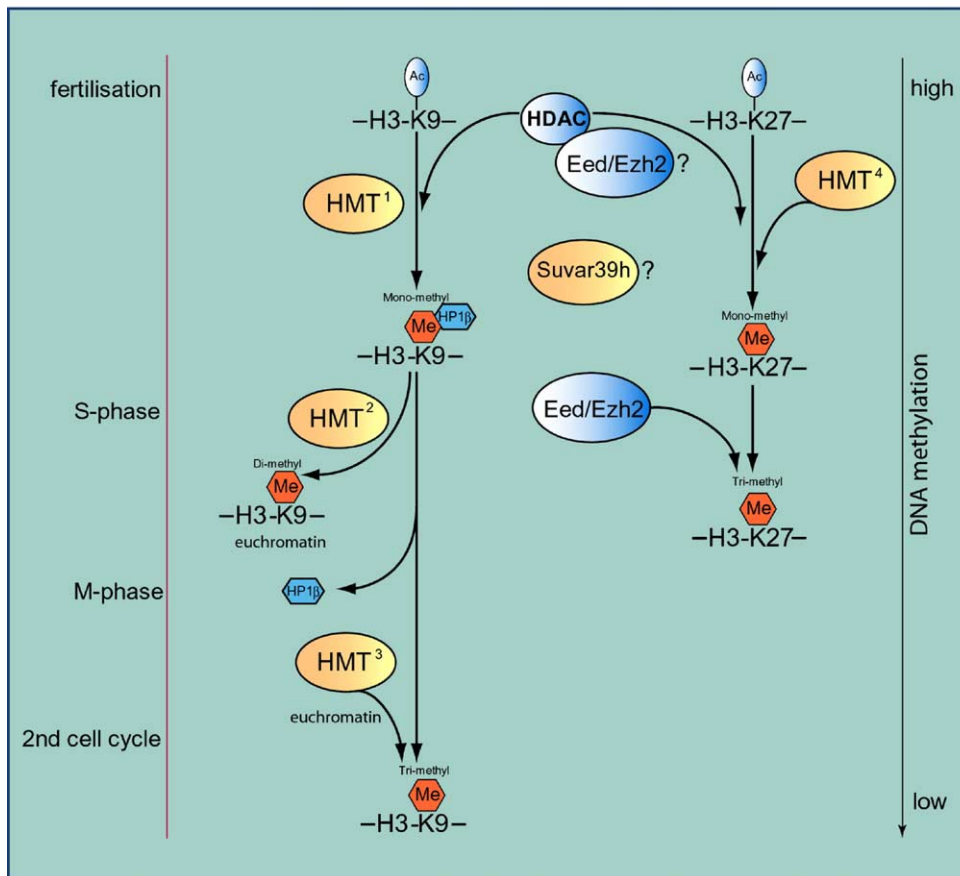


Fig. 5. Epigenetic alterations in the male pronucleus during the first cell cycle. DNA methylation and chromatin modification and organisation are central to epigenetic reprogramming in mammals. Paternal specific active demethylation may be a permissive event associated with the organisation of chromatin that results on remodelling the sperm nucleus. The newly incorporated acetylated histones must be altered by histone deacetylases (HDACs) and histone methyltransferases (HMTs), consistent with the detection of monomethylated K9 and K27 of histone H3. Although the identity of the monoK9 HMT is not known (HMT<sup>1</sup>), G9a and ESET/SET DB1 are possible candidates. Despite the early detection of Eed/Ezh2, there are no reports of K27 monomethylase activity directly associated with them (HMT<sup>4</sup>). As Eed/Ezh2 is frequently found in a larger remodelling complex in association with histone deacetylases, this may provide the source of HDACs necessary to initiate the transition to the methylated state. In silico mining of oocyte and fertilised oocyte libraries identifies the presence of Suvar 39h2 mRNA. This raises an interesting possibility that Suvar 39h is present, but it does not trimethylate K9 until active DNA demethylation is completed (thereby ensuring the early transcriptional burst from the male pronucleus in G1). In the male, the presence of monoK9 in conjunction with Hp1β may afford protection and ensure the maintenance of the essential centromeric DNA methylation thought to be associated with chromosome stability. Activities associated with euchromatin, evident in the male pronucleus, include a dimethylating HMT (HMT<sup>2</sup>) and yet another activity that restores trimethylation to paternal alleles (HMT<sup>3</sup>). Whether there are four distinctive HMTs involved in these reactions is not absolutely certain at this time.

pendent and is concentrated around the nucleoli where the pericentromeric satellites associated to heterochromatin reside. This pattern is completely different from that in somatic cells, where monoK9 does not associate overtly with heterochromatin (Supplementary Fig. 1). MonoK9 is also found in heterochromatic structures in Suvar 39h double null ES cells (Peters et al., 2003; Schotta et al., 2004), suggesting that the structure and organisation of heterochromatin are fluid or plastic and may be quite different in the pluripotent embryo as compared to differentiated cells.

The transition to K9 trimethylation, which is dependent on Suvar 39h, is only found after the two-cell stage (data not shown). Perhaps unexpected was that HP1β is found in the male pronucleus (from PN<sub>2</sub>, i.e. within 2–4 h post

fertilisation), but not apparently associated with any particular nuclear structures. In keeping with the expectation of the preferred binding association, HP1β co-localises with heterochromatic trimethylK9 in the female (Fig. 4; Arney et al., 2002). Stringent washing removes some but not the entire signal and reveals an association of HP1β with monoK9. This may mark regions that are destined to become subsequently modified and identified as heterochromatic. A recent report identified an HP1β association with RNA in the presence of H3-K9 methylation (Maison et al., 2002). However, results from Meehan et al. (2003) have suggested that HP1 may bind directly to DNA via its hinge region. This suggests that localisation of HP1 in vivo may not depend exclusively on H3-K9 methylation.

These observations suggest a model in which the differential modifications of lysine residues in the male and female pronuclei are regulated by the absence or presence of specific HMTs, and more importantly by the availability of their substrates (Fig. 5). The presence of monomethylation discounts previous models which proposed that the differential chromatin structure observed could be attributed to the absence or exclusion of HDACs in the male pronucleus (Adenot et al., 1997). On the basis that Eed/Ezh2 is present from a very early stage, we propose that the HDACs associated with the Eed/Ezh2 complex are available, and perhaps together with other HDACs, deacetylate both K9 and K27 of H3. Suvar 39h2 mRNA is present in oocyte and fertilised oocyte libraries (UniGene Clusters Mm.9244 and Mm.128273 at <http://www.ncbi.nlm.nih.gov/UniGene> on 6 July, 2004), suggesting that this HMT is also present at this early stage. It is remarkable that Suvar 39h does not use the monoK9, its preferred substrate (Peters et al., 2003), to produce triK9 in the male pronucleus. It is tempting to speculate that, in the unique environment of the zygote, there is a significant interaction between Hp1 $\beta$  and monoK9 (hence, the co-localisation we observe) that results in inability of Suvar 39h to convert mono to trimethyl. Indeed, recent results support the idea that delayed K9 methylation is caused by inhibitory factors rather than lack of enzymatic activity (Liu et al., 2004). Similarly, Eed/Ezh2 does not further modify monoK27 to triK27 in the same compartment. This mechanism of delayed histone methylation may be critical to ensure that rapid and efficient DNA demethylation of the male pronucleus proceeds unhindered. The detection of triK27 in advance of triK9 suggests that K9 may have a more prominent role in protecting from demethylation. Indeed, in the male pronucleus, monoK9 is localised in the pericentromeric regions which are the only visible structures that do not lose 5MeC (Supplementary Fig. 5; Santos et al., 2002). In the female pronucleus, co-localisation occurs between 5MeC and diK9 but not triK9 (Fig. 2; Supplementary Fig. 4). This association is most evident in perinucleolar rings known to contain satellite sequences (Arney et al., 2002). The co-localisation with diK9 suggests that these signals correspond to the minor satellites (Peters et al., 2003).

In general terms, the substantial and rapid remodelling of sperm epigenetic modifications may be a prerequisite to ensure the transcriptional burst that takes place at the end of the first cell cycle (Latham et al., 1992) and prior to the major zygotic gene activation at the two-cell stage (Sawicki et al., 1981). Some of the remodelling events may also play a role in reprogramming of paternal imprints and in post-zygotic epigenetic modification (Reik et al., 2001). Our observations lend further support to the concept that methylation of H3-K9 may protect against demethylation of DNA during the very early stages of chromatin remodelling and pronuclear formation (Arney et al., 2002; Cowell et al., 2002; Santos et al., 2002).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2005.01.025](https://doi.org/10.1016/j.ydbio.2005.01.025).

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