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Genome restructuring in mouse embryos during reprogramming and early development

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Abstract

Although a growing number of studies investigates functional genome organization in somatic cell nuclei, it is largely unknown how mammalian genome organization is established during embryogenesis. To address this question, we investigated chromocenter formation and the peculiar arrangements of chromosome domains in early mouse embryos. At the one-cell stage, we observed characteristic arrangements of chromosomes and chromocenter components. Subsequently, starting with the burst of zygotic genome transcription major rearrangements led to the establishment of somatic type chromocenters with a defined spatio-temporal organization. These processes appeared to be completed at the blastocyst stage with the onset of cell differentiation. During the same developmental period, a fraction of pericentric heterochromatin that was late replicating in the first cycle underwent switches in replication timing, spatial organization and epigenetic marks. Cloning experiments revealed that the genome organization typical for more advanced stages was quickly reverted into the one-cell stage-specific form after nuclear transfer, supporting the idea that reprogramming associated genome remodeling in normal and cloned embryos is determined by cytoplasmic factors. Together, the results suggest that distinct but characteristic forms of nuclear genome organization are required for genome reprogramming in early embryos and for proper regulation of differential gene expression patterns at later stages.

Keywords: Nuclear organization; Embryo; Chromocenter; Replication; Reprogramming

Introduction

Recent results suggest that the spatial organization of genomes in the cell nucleus contributes to their functional regulation (Gasser, 2001; Misteli, 2004; Taddei et al., 2004). Concerning the spatial organization of mammalian genomes, particular attention has been paid to the spatial arrangements of chromosomes (Boyle et al., 2001; Cremer et al., 2003; Croft et al., 1999; Parada et al., 2002) and on the arrangements of centromeres and pericentric heterochromatin (Haaf and Schmid, 1989, 1991; Manuelidis and Borden, 1988; Solovei et al., 2004; Weierich et al., 2003). During interphase, pericentric heterochromatin from several chromosomes often forms clusters giving rise to so-called chromocenters (Haaf and Schmid, 1991). Chromocenters are especially prominent in mouse cells

Abbreviations: CENP, centromeric protein; Cy3-AP3-dUTP, 5-amino-propargyl-2′-deoxyuridine 5′-triphosphate coupled to Cy3 fluorescent dye; Dig-dUTP, digoxigenin-11-2′-deoxyuridine-5′-triphosphate; DimH3K9, histone H3 dimethylated at lysine 9; HP1, heterochromatin protein 1; NPB, nucleolar precursor body.

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(Hsu et al., 1971) and represent a characteristic feature of all different somatic mouse cell types investigated so far. They are typically brightly labeled by DNA-specific dyes like Hoechst and are surrounded by centromeres (Cerda et al., 1999; Guenatri et al., 2004).

Chromocenters represent major heterochromatic domains, and recent results shed light on the molecular nature of heterochromatin at chromocenters. It has been shown that histone methyltransferases homologous to Su(var)3-9 specifically methylate lysine 9 of histone H3 at chromocenters (Peters et al., 2001). These modified histone tails are bound by the α and β isoforms of heterochromatin protein 1 (HP1), and these epigenetic marks are crucial for maintenance and propagation of heterochromatin at chromocenters (Minc et al., 1999; Muchardt et al., 2002; Hayakawa et al., 2003; Gilbert et al., 2003; Maison and Almouzni, 2004; Cheutin et al., 2003).

Chromocenters appear to be important for regulating proper centromere function. In this regard, the association of different satellite repeats, which display a distinct replication timing, with different central and peripheral zones of chromocenters appears to be crucial (Guenatri et al., 2004). On the other hand, chromocenters seem to play an important role in gene regulation. Association of genes with chromocenters correlates with silencing. Indeed changes in these patterns of association have been observed during cell differentiation and with the transcriptional status of individual genes (Ayyanathan et al., 2003; Brown et al., 1997, 1999, 2001; Francastel et al., 1999; Schubeler et al., 2000; Skok et al., 2001). Although chromocenters appear to be important multifunctional domains with a defined spatio-temporal substructure in somatic cells, chromocenters have not been observed in early mouse embryos, and it was an open question when and how they become established during development and how this relates to the establishment of regulated nuclear functions.

It is well known that dramatic changes occur in nuclear architecture and chromatin structure at the beginning of development, in parallel to the establishment of regulated nuclear functions: (i) after sperm entry, the paternal chromatin decondenses and chromatin-bound proteins (such as protamines) are exchanged for histones (McLay and Clarke, 2003), (ii) during the first cell cycle, both genomes remain separated and undergo parent-of-origin differential modifications of epigenetic marks. The DNA of paternal genome is rapidly demethylated (Reik and Walter, 2001; Reik et al., 2001; Santos et al., 2002; Beaujean et al., 2004) and binds preferentially acetylated histone H4 (Adenot et al., 1997), whereas the DNA of the maternal genome retains its DNA methylation level and its capacity to bind di- and trimethylated histone H3 on lysine 9 (Arney et al., 2002; Cowell et al., 2002; Liu et al., 2004; Reik et al., 2003; Santos et al., 2005). Furthermore, nucleoli are inactive and unusual structures of compact fibrillar spheres termed Nucleolar Precursor Bodies (NPBs, see Chouinard, 1971; Flechon and Kopecny, 1998) are observed until the four/eight-cell stage. Interestingly, despite their differences in epigenetic marks and the fact that they remain separated in the same cytoplasm, each genome displays at the one-cell stage a similar spatio-temporal distribution of replication foci as observed in somatic cells (Bouniol-Baly et al., 1997; Ferreira and Carmo-Fonseca, 1997).

Concerning transcriptional regulation, it has been shown that the zygotic genome of the mouse is transcriptionally silent after fertilization, and that RNA polymerases II and I become sequentially active by the end of the one-cell and the end of the two-cell stage, respectively (Bouniol et al., 1995; Zatsepina et al., 2003; for review, see Latham, 1999). After an unspecific burst of transcription, a progressive requirement for specific transcription factors is established during the following cell cycles (Nothias et al., 1995). Cell differentiation associated with differential gene expression patterns is first observed at the blastocyst stage after about six cycles.

Although a growing number of studies investigates functional genome organization in somatic cell nuclei, virtually nothing is known on how functionally important domains like chromocenters become established during early embryonic development and how their establishment might be related to the mixing of the two genomes and the establishment of differential gene expression patterns. In addition, it is unclear how genomes might be reorganized during reprogramming at early embryogenesis and which factors might determine these processes. Here, we describe corresponding results obtained with early mouse embryos after natural fertilization or nuclear transfer.

Materials and methods

Mouse embryo collection and culture

Embryos were produced by natural fertilization of C57/CBA mice. Superovulation was induced by injection of pregnant mare serum gonadotropin (PMSG, Intervet, 5 UI) followed, 48 h later, by injection of human chorionic gonadotropin (hCG, Intervet, 5 UI). Female mice were then mated with C57/CBA males. Fertilization occurred at about 12 h after hCG injection which was used as reference point for embryonic development (hours post-hCG, i.e., hphCG, see Fig. 1). Fertilized eggs were collected at the one-cell stage from the *ampulla* with M2 medium (Sigma) after a brief treatment with 1 mg/ml of hyaluronidase in phosphate-buffered sodium (PBS, pH 7.5) to separate them from the surrounding follicular cells. Two-cell (32 hphCG, 40 hphCG, and 48 hphCG) and four-cell (60 hphCG) stages were grown in vivo and directly collected from the mice oviducts. Later stages (eight cells to blastocyst) were obtained by in vitro culture in M16 medium (Sigma) at 37°C in a humidified atmosphere enriched in 5% of CO_2. Similarly, experiments addressing the localization of late replicating DNA were performed with embryos collected at 24 hphCG and cultured in vitro after microinjection of the nucleotide analog. All experimental sets contained embryos from four or five different mice taking the relative asynchrony of fertilization into consideration. All experiments were repeated at least 3 to 4 times.

Nuclear transfer

C57/CBA female mice were superovulated with PMSG and hCG as described above. Oocytes were collected from oviducts 14 hphCG and washed in M2. Subsequently, they were incubated in M2 containing 5 μg/ml cytochalasin B and placed in a chamber on the stage of an inverted microscope (Nikon) equipped with micromanipulators (Nikon-Narishige MO-188). The chromatin spindle (visualized under differential interference contrast) was aspirated into the pipette as described by Zhou et al. (2001). Donor chromosomes were issued from ES cells (gift from Dr. Nagy, Toronto), previously cultured in DMEM and synchronized in metaphase, by gently aspirating them in and out of the injection pipette (outer diameter

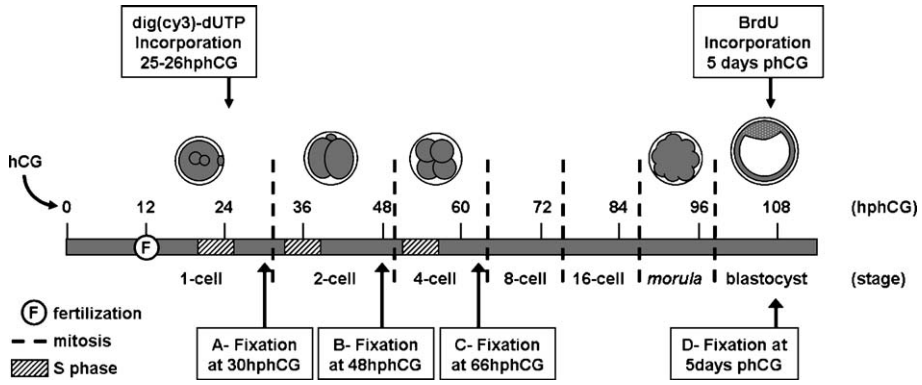

Fig. 1. Time scale. Embryos have been collected after stimulation of female mice with human chorionic gonadotropin (hCG, injection of hCG: time point 0). The scheme depicts the timing of subsequent embryonic development and of the experimental manipulations performed (hphCG: hours after injection of hCG).

10–15 μm) followed by microinjection into the cytoplasm of the enucleated oocytes.

The nuclear transfer embryos were activated by incubating them for 6 h in Ca^{2+}-free medium containing 10 mM Sr^{2+}. Embryos with visible nuclei, considered as activated, were transferred into Sr^{2+}-free M16 medium and cultured at 37°C in a humidified atmosphere enriched in 5% of CO_2.

Bovine embryo production

Bovine embryos were produced by in vitro fertilization (IVF). Cumulus–oocyte complexes from slaughtered cows were matured in M199 medium (Gibco-BRL) supplemented with 10% fetal calf serum, 10 μg/ml FSH-LH, and 1 μg/ml estradiol-17b for 24 h at 39°C in a humidified 5% CO_2 atmosphere. Cumulus-expanded oocytes were then inseminated in vitro with frozen/thawed bovine sperm. Modified Tyrode's medium was used for capacitation and fertilization. After thawing, motile spermatozoa were selected by swim-up, concentrated by centrifugation, resuspended to a final concentration of 10^6 cells/ml in the fertilization medium supplemented with 1% heparin, and incubated for 18–20 h at 39°C. After IVF, the cumulus cells were mechanically removed by vortexing and pipetting, and the denuded presumptive zygotes were cultured in B_2 medium (Laboratoire C.C.D., France) seeded with Vero cells. Embryos at one-cell, four-cell, early eight-cell, late eight-cell and blastocyst stages were fixed with 2% PFA in PBS for 30 min after 20 h, 42 h, 51 h, 69 h, and 8 days of culture respectively.

Labeling of the late replicating DNA in one-cell embryos

One-cell embryos were microinjected into the cytoplasm with 1 ± 0.5 pl of a solution containing 20 μM dig-dUTP (Roche) in 2 mM PIPES, pH 7.4, 140 mM KCl at 25.5–26.5 hphCG (dilution approximately 1:2000). Microinjections were performed on a Nikon inverted microscope combined with Narishige micromanipulators and an Eppendorf microinjector. After culture for 1 h (one-cell, 27 hphCG), 7 h (metaphase, 32 hphCG), 22 h (two-cell, 48 hphCG), 34 h (four-cell, 66 hphCG), or 5 days (blastocyst), the microinjected embryos were fixed and processed for indirect immunofluorescence. Control experiments showed that microinjection of dig-dUTP or Cy3-dUTP (see below) did not impair in vitro development to blastocyst (77% after microinjection, $n = 83$, versus 84% without microinjection, $n = 161$).

In vivo tracking of Cy3-labeled DNA

One-cell embryos were microinjected with 20 μM Cy3-AP3-dUTP (Amersham Bioscience) at 25.5–26.5 hphCG. Microinjection was performed as described above. After in vitro culture for 1 h, embryos were individually put into microdrops of M2 medium placed on a coverslip which was set in a chamber flood with mineral oil. Embryos were briefly imaged with a confocal microscope equipped with a heating stage, and sorted according to their replication pattern. All embryos exhibiting the same replication pattern were cultured in the same drop of M16 medium (37°C, 5% CO_2) and fixed at the late two-cell (48 hphCG) or at blastocyst (5 days phCG) stages.

Double pulse labeling

One-cell embryos were microinjected with 20 μM Cy3-AP3-dUTP at 25.5–26.5 hphCG, as described above. After culturing until the blastocyst stage, replicating DNA of cells that were in S phase was pulsed labeled by adding 50 μM BrdU (Sigma) to the medium for 10 min. Blastocysts were washed twice in M2 at 37°C, fixed, and processed for indirect immunofluorescence.

Immunofluorescence and mounting

Embryos were fixed with 2% paraformaldehyde (PFA) in PBS for 20 min at room temperature (RT) and permeabilized with 0.5% Triton X-100 (30 min, RT). For Br-DNA detection, DNA was denatured with 4N HCl (1 h, 37°C). For immunostaining, embryos were blocked with 3% bovine serum albumin (BSA) in PBS for 45 min. Incubation with the primary antibodies was performed overnight at 4°C. After two washes with 0.05% Tween-20 in PBS (30 min each), embryos were incubated with the secondary antibodies (1 h at RT) and rinsed again to remove excess of antibodies. Embryos were briefly post-fixed (2% PFA, 10 min, RT), and chromatin was counterstained with 4 μmg/ml Hoechst 33342 (Calbiochem) or 1 μM Sytox Green (Molecular Probes) for 15 min at RT.

For microscopy, embryos were deposited on slides and mounted under a coverslip with citifluor (Citifluor Products). Alternatively ("3D-preserved" setting), they were put into individual microdrops of PBS-BSA (3%) on a coverslip set in a chamber flood with mineral oil.

Antibodies

All antibodies were diluted as indicated in PBS-BSA (3%). The mouse monoclonal anti-HP1β antibody was obtained from Euromedex (clone 1MOD 1A9, 1:400). The centromeres were labeled with a human CREST antibody which recognizes both CENP-A and B (gift from Dr. Paul Kalitsis, Melbourne, 1:200). Dig-labeled DNA was detected with a sheep polyclonal anti-digoxigenin antibody (Roche, 1:200) and Br-labeled DNA with a mouse monoclonal anti-BrdU antibody (Becton Dickinson, 1:150). Histone H3 dimethylated at lysine 9 was detected with a rabbit polyclonal antibody raised against a branched peptide (see Results, gift from Dr. Thomas Jenuwein, Vienna, 1:500).

Microscopy and image analysis

Entire embryos were scanned with a distance between light optical sections ranging from 0.3 and 0.5 μm. Confocal microscopy was performed with a Zeiss LSM 510 confocal laser scanning microscope equipped with an oil immersion objective (Plan Apochromatic 63× n.a.1.4), and imaging was performed with the 488-, 535-, and 633-nm wavelengths of the lasers. Epifluorescence microscopy was performed with a Nikon inverted microscope (Eclipse TE 300) using a filter

wheel equipped with standard filters for FITC, rhodamine, and Hoechst emissions. Images were acquired through a water immersion objective (CFI Plan Apochromatic 60× WI n.a.1.20 WD 0.22) with a cooled CCD camera (Coolsnap HQ, Roper Scientific) driven by the Metamorph 6.0 software. Image stacks were deconvoluted based on a calculated theoretical point spread function (PSF). 3D recontructions of image stacks were performed using Amira software. Volume calculations were performed using Image J software. From "3D-preserved" blastocysts, all foci were defined and segmented by thresholding. The number of pixels above the threshold was counted in every optical section containing the signal (focus or cluster), and from these numbers, the volumes of foci and clusters were calculated.

Results

Centromeres and other components of chromocenters are associated with NPBs periphery at the one-cell stage

Chromocenters strongly enriched in HP1β and surrounded by centromeres (Fig. 2a) are a characteristic feature of all somatic cell types of the mouse investigated so far. In contrast, chromocenters have not been observed in cells of early mouse embryos. However, condensed DNA brightly stained by the DNA specific dye Hoechst, which in somatic cells accumulates within the chromocenters, is associated in one-cell embryos with the periphery of NPBs (Fig. 2c) (Debey et al., 1989). These suggest that chromocenter formation might involve major reorganizations of DNA and other components of chromocenters during development. In order to test this hypothesis, we investigated in early mouse embryos collected in vivo (for one-, two-, four-cell stages) or cultured in vitro (in the case of eight-cell stages and blastocysts) the nuclear organization of different components involved in the formation of chromocenters (Fig. 1).

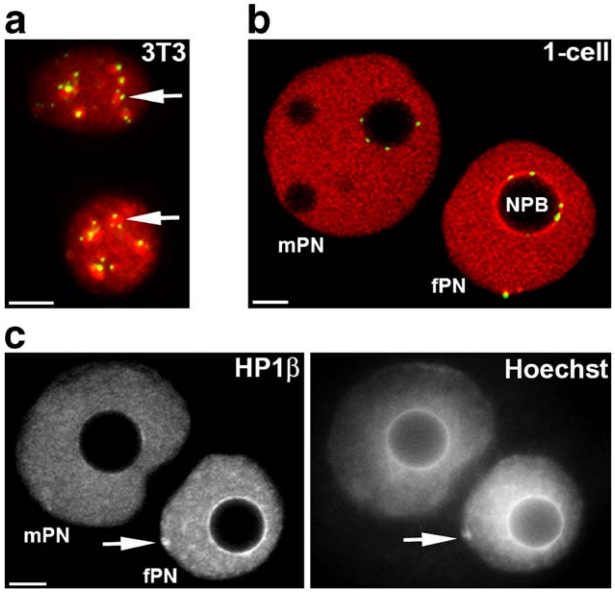

Fig. 2. Distribution of HP1β and centromeres in somatic and embryonic mouse cells. (a) NIH 3T3 nuclei (epifluorescence microscopy). Centromeres detected by immunostaining with an antibody against CENP A and B (green) are closely associated with chromocenters (arrows) enriched in HP1β (red). Scale bar: 3 μm. (b) Light-optical section of a one-cell embryo fixed at 25 hphCG. Centromeres (green) and HP1β (red) have been detected by immunostaining in the male (mPN) and female (fPN) pronucleus. NPBs appear unstained. Panel c shows another one-cell stage fixed at 25 hphCG: the DNA has been stained with Hoechst (right-hand image, epifluorescence microscopy) and HP1β has been detected by immunostaining (left-hand image, single light-optical section). The arrow indicates a nucleoplasmic "patch" of HP1β associated with brightly Hoechst stained DNA. Scale bars: 5 μm.

First, the distributions of HP1β and of the centromere specific proteins CENP A and B were assessed by immunostaining. In one-cell embryos (25 hphCG, Fig. 1, n = 50), HP1β was diffusely distributed in the nucleoplasm of male (mPN) and female (fPN) pronuclei (Figs. 2b and c). In mPN, no specific accumulation of HP1β was observed. In contrast, in fPN HP1β accumulated at the peripheries of NPBs and colocalized with brightly Hoechst stained DNA (Figs. 2b and c). In addition, occasional nucleoplasmic "patches" of brightly Hoechst stained DNA were observed in fPN, which also colocalized with accumulations of HP1β (Figs. 2b and c, arrows).

Immunostaining of the centromeres with the antibody against CENP A/B proteins resulted in a pattern of brightly stained dots (Figs. 2a and b). These dots were associated almost exclusively with the periphery of NPBs in both PNs (Fig. 2b). However, while in fPN 100% of NPBs were associated with centromeres, only about 60% of the NPBs were associated with centromeres in mPN (such NPBs devoided of centromeres in the mPN are shown in Fig. 2b). Occasionally, centromeres were found in the nucleoplasm (2 on average in fPN and 1 on average in mPN, n = 24 embryos). These nucleoplasmic centromeres however were associated with a nucleoplasmic accumulation of HP1β in 86% of the cases in fPN (Fig. 2b, arrow).

Nuclear organization of pericentric DNA at the one-cell stage

To define more precisely the nature of the DNA associated with NPBs periphery at the one-cell stage, we used replicational pulse labeling during the first S phase. This first S phase takes place between 20 hphCG and 26 hphCG (Fig. 1), with an asynchrony between male and female PNs (Bouniol-Baly et al., 1997; Worrad et al., 1994; Ferreira and Carmo-Fonseca, 1997). It is known that replicational pulse labeling of mPN and fPN during the first embryonic S phase results in a spatio-temporal sequence of labeling patterns similar to those observed in somatic cells (Bouniol-Baly et al., 1997; Ferreira and Carmo-Fonseca, 1997; O'Keefe et al., 1992).

As expected, the most frequent pattern (70%, n = 64) of dig-dUTP incorporation after microinjection between 25 and 26 hphCG was the late pattern (Bouniol-Baly et al., 1997; Ferreira and Carmo-Fonseca, 1997): intense labeling of substantial parts of the NPBs periphery in fPN and, in both PN, less intensely labeled sites in the nucleoplasm and at the nuclear peripheries (Fig. 3a, embryos fixed at 27 hphCG). Control experiments showed that microinjection of 20 μM dig-dUTP or 20 μM Cy3-dUTP (see below) did not impair in vitro development to blastocyst (77% after microinjection, n = 83, versus 84% without microinjection, n = 161).

To map the dig-labeled DNA on mitotic chromosomes, sets of labeled embryos were fixed at 30 hphCG when most embryos

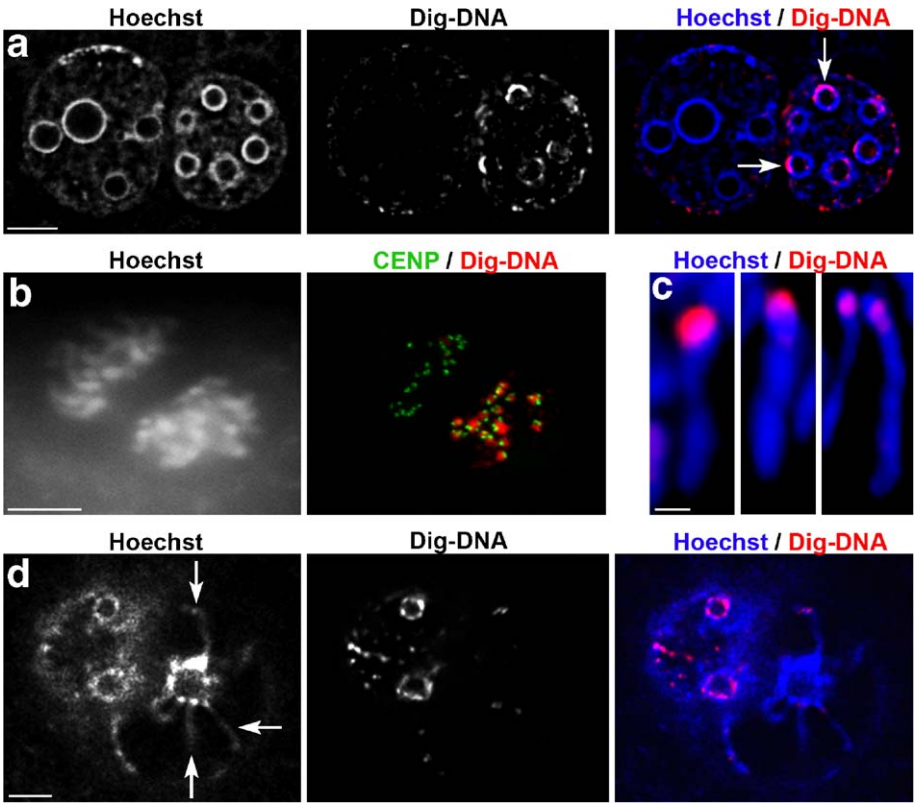

Fig. 3. Arrangements of late replicating DNA and chromosomes in one-cell embryos. (a) One-cell embryo microinjected with dig-dUTP at 25 hphCG and fixed at 27 hphCG (Hoechst counterstain: blue). Due to the asynchronous replication timing of the PNs dig-dUTP (red) was predominantly incorporated into the fPN, where it mainly concentrated at the NPBs periphery (arrows). Some weakly labeled sites are also observed at the PNs periphery. Scale bar: 10 μm. (b) Labeling pattern on mitotic chromosomes (dig-dUTP microinjection at 25 hphCG, fixation at 30 hphCG). DNA counterstained with Hoechst was imaged by epifluorescence microscopy, while the other panel shows a single confocal section (dig-DNA: red; centromeres: green). Scale bar: 10 μm. Panel c shows additional examples of dig-dUTP (red) labeled mitotic chromosomes from the fPN (Hoechst counterstain: blue). Scale bar: 1.5 μm. (d) One-cell embryo at prophase after dig-dUTP (red) microinjection (microinjection at 25 hphCG, fixation at 29 hphCG) and Hoechst counterstaining (blue). Mainly the NPBs periphery of the fPN is labeled. The mPN shows the "cartwheel" organization of chromosomes, with the centromeres attached to the NPB periphery, while the rest of the chromosomal domains stretch out towards the nuclear periphery (marked by arrows). Scale bar: 10 μm. The images in panels a, c, and d were obtained in the "3D-preserved" setting by epifluorescence microscopy combined with deconvolution.

progress through the first mitosis (Fig. 1). Coimmunostaining of metaphases for dig-DNA and CENP A/B revealed that chromosomes derived from fPN displayed intensely dig-labeled pericentric heterochromatin (Figs. 3b and c). Different labeling patterns could be distinguished: labeling could extend over the whole pericentric region (Fig. 3c, left), or only over its terminal part (Fig. 3c, middle), or yet over a more internal band of the pericentric region (Fig. 3c, right). This suggests differences among the pericentric sequences with regards to their replication timing. In addition, some non-pericentric sites were relatively weakly labeled on both sets of chromosomes, which probably correspond to the minor nucleoplasmic sites labeled in both PNs.

In embryos that were in prophase at the time of fixation, condensing chromosomes displayed a characteristic arrangement (see also Debey et al., 1989) with the labeled end attached to the NPB periphery, while the rest of the chromosomes reached out towards the nuclear periphery, as in a "cartwheel" (Fig. 3d). The fact that centromeres and late replicating pericentric DNA are associated with the NPB periphery during the whole interphase of the first cell cycle suggests a characteristic radial organization of chromosomal territories at that stage.

The establishment of pro-chromocenters is associated with the onset of transcription

Next we assessed the nuclear organization of the different chromocenters components at the subsequent stages. First, we concentrated on centromeres and HP1β. During the second cell cycle, the nuclear distribution of both HP1β and centromeres changed dramatically. At the early two-cell stage (32 hphCG, Fig. 4 upper panel, $n = 32$), HP1β was diffusely distributed in the nucleoplasm but was also enriched at the periphery of some NPBs. However, only 29% of NPBs were associated with HP1β accumulations that partially covered NPBs periphery (Fig. 4). In parallel, prominent accumulations of HP1β appeared in the nucleoplasm. At the late two-cell stage (48 hphCG, $n = 45$), the number of NPBs associated with HP1β further decreased, while the number of nucleoplasmic accumulations of HP1β increased (Fig. 4 upper panels).

Simultaneously, an increasing number of centromeres relocated from the periphery of NPBs (68% on average associated with NPBs at 32 hphCG, Fig. 4) to the nucleoplasm (42% on average associated with NPBs at 48 hphCG, Fig. 4). In the nucleoplasm, centromeres were always associated with

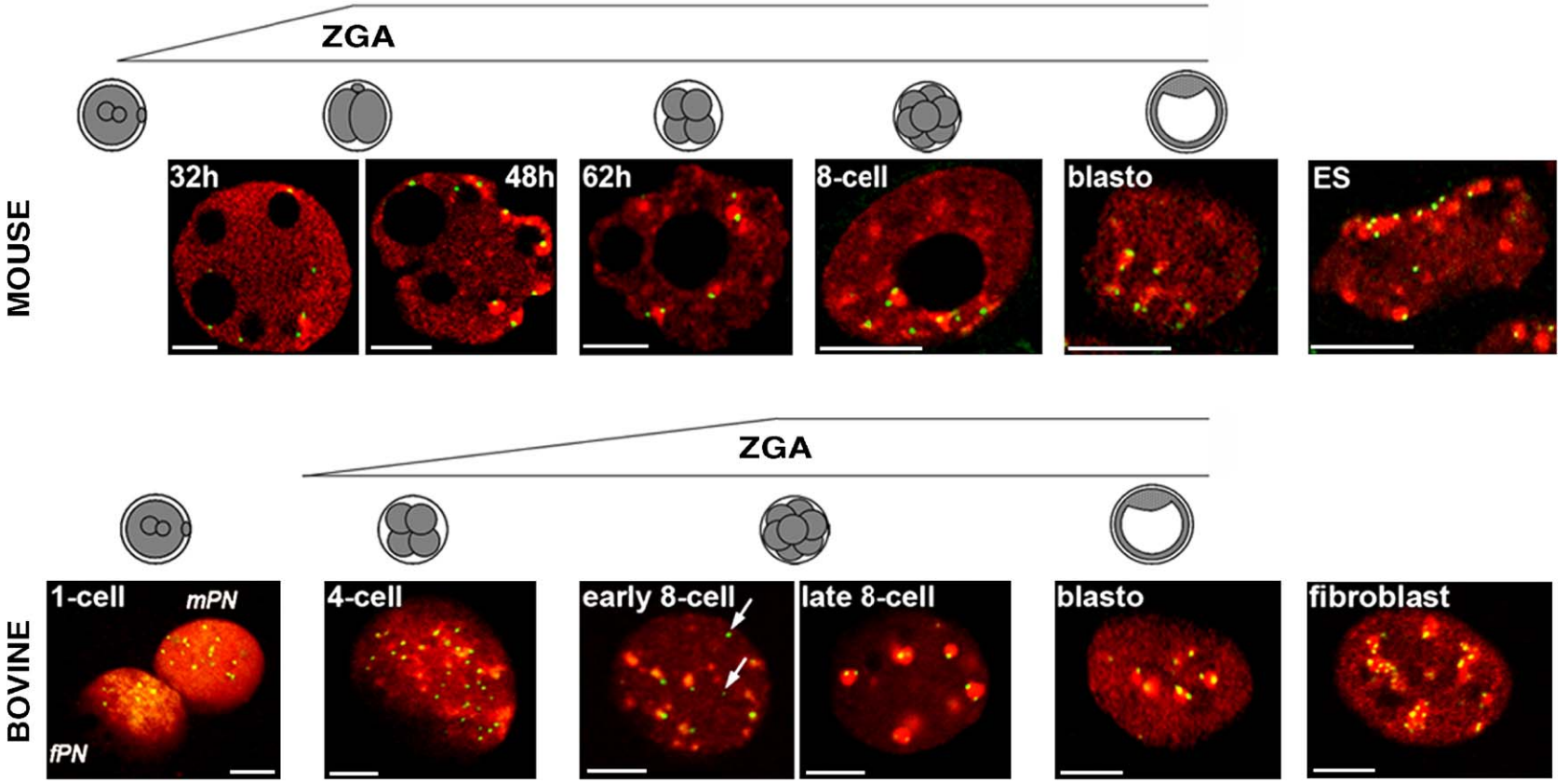

Fig. 4. Chromocenter formation at early embryonic stages is coincident with zygotic genome activation in mouse and bovine. The upper panels show single light-optical sections of nuclei from early two-cell (32 hphCG), late two-cell (48 hphCG), four-cell (62 hphCG), eight-cell and blastocyst stages (inner cell mass cell) and an ES cell in mouse, all labeled with antibodies against HP1β (red) and centromeres (green). The lower panels show the distribution of HP1β (red) and centromeres (green) in bovine nuclei from one-cell, four-cell, early eight-cell, late eight-cell and blastocyst stages (inner cell mass cell), plus a fibroblast (single light-optical sections). Scale bars: 5 μm.

HP1β accumulations. This suggests that important steps in the formation of chromocenters occur during the second cell cycle: (1) accumulation of HP1β at discrete sites in the nucleoplasm after dissociation from NPBs peripheries and (2) relocation of centromeres from the periphery of NPBs to the sites of HP1β accumulation.

Remarkably, these profound reorganizations took place within only one cell cycle and appeared to be completed at the four-cell stage, where HP1β was not associated anymore with NPBs, but was found enriched at discrete nucleoplasmic sites (62 hphCG, Fig. 4 upper panels, $n = 48$). In parallel, only 10% of centromeres remained located at NPBs periphery, whereas the rest always associated with local accumulations of HP1β. As the local accumulations of HP1β associated with centromeres strongly resembled chromocenters, we called them pro-chromocenters. At the eight-cell stage (when NPBs are turning into true nucleoli; Zatsepina et al., 2003, $n = 18$), as well as in blastocyst (inner cell mass and trophectoderm, $n = 34$) and embryonic stem (ES) cells ($n = 98$), centromeres were associated with local accumulations of HP1β (Fig. 4 upper panel). Together, these results suggest that the most dramatic nuclear rearrangements associated with chromocenter formation take place mainly during the second cell cycle.

As this period coincides in the mouse with the major burst of zygotic transcription termed ZGA (zygotic genome activation), we wondered whether there might be any functional link between these two events. We thus performed similar experiments with bovine embryos, where ZGA occurs at the eight-cell stage, i.e., later than in the mouse (for review, see Latham, 1999). From one- to four-cell stages, no chromocenter-related organization of HP1β and centromeres was observed (Fig. 4 lower panels, $n = 14$ and 15 for one-cell and four-cell stages respectively). The formation of local accumulations of HP1β associated with centromeres started at the beginning of the eight-cell stage and was complete at the end of this cycle (Fig. 4 lower panels, $n = 32$): indeed, the number of pro-chromocenters clearly increased during the eight-cell cycle, whereas the number of single centromeres that were not associated with any HP1β accumulation decreased. Chromocenters were also clearly visible in blastocyst cells (from both inner cell mass and trophectoderm, $n = 8$) and in differentiated cells such as fibroblasts (Fig. 4 lower panels). This striking coincidence between pro-chromocenter formation and ZGA in mouse as well as in bovine embryos strongly suggests a functional link between these two events.

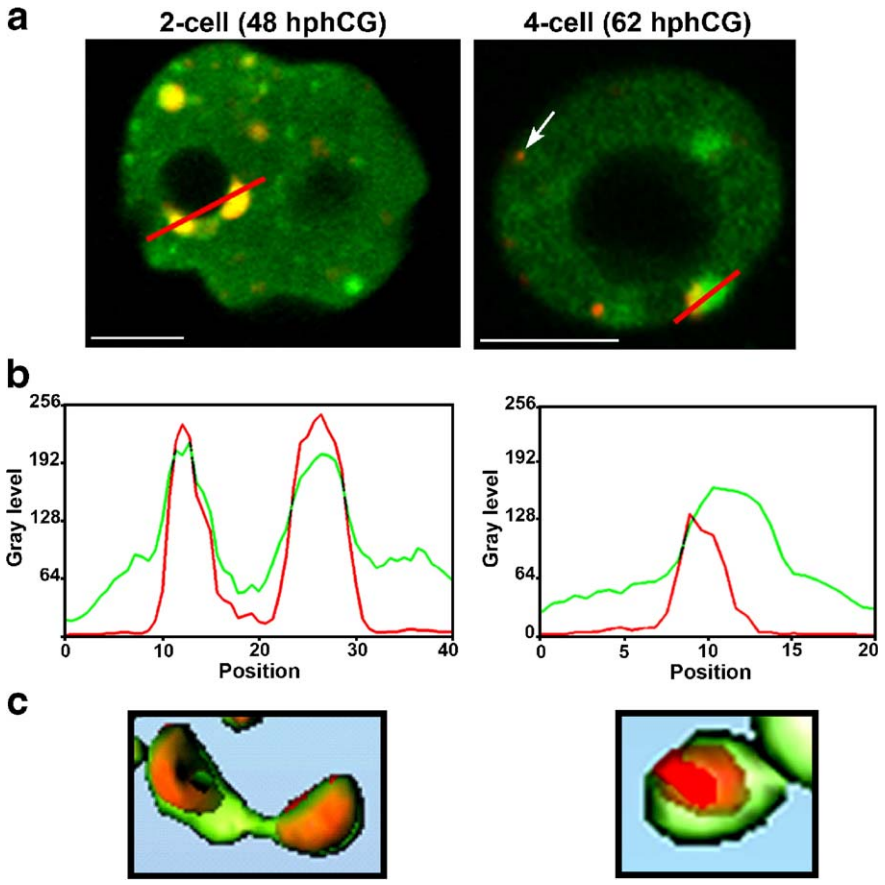

Fig. 5. DNA replicating late at the one-cell stage is organized into pro-chromocenters at two-cell and four-cell stages. (a) The panels (single light-optical sections) show nuclei from late two-cell (left, 48 hphCG) and four-cell (right, 62 hphCG) stages. DNA was labeled with dig-dUTP during late S phase of the one-cell stage (red). HP1β was detected by immunostaining (green). Dig-labeled DNA and HP1β colocalize within huge and intensely labeled pro-chromocenters (colocalization of red and green signals appears orange-yellow). Scale bars: 10 μm. (b) Fluorescence intensity profiles (HP1β, green; dig-DNA, red) along the red lines drawn on the pictures in a). (c) 3D reconstructions of the double-labeled chromocenters underlined in panel a.

Nuclear organization of dig-labeled DNA at later stages of mouse embryos

As chromocenters represent the association of pericentric regions of several chromosomes, we next focused on the nuclear distribution of pericentric DNA from two-cell to blastocyst stage. In a first set of experiments, embryos were microinjected with dig-dUTP at 25–26 hphCG during late S phase of the one-cell stage as described above and cultured until 48 hphCG (late two-cell stage). As shown in Fig. 5a, most of the dig-labeled DNA colocalized at late two-cell stage with local accumulations of HP1β. Colocalization was confirmed by

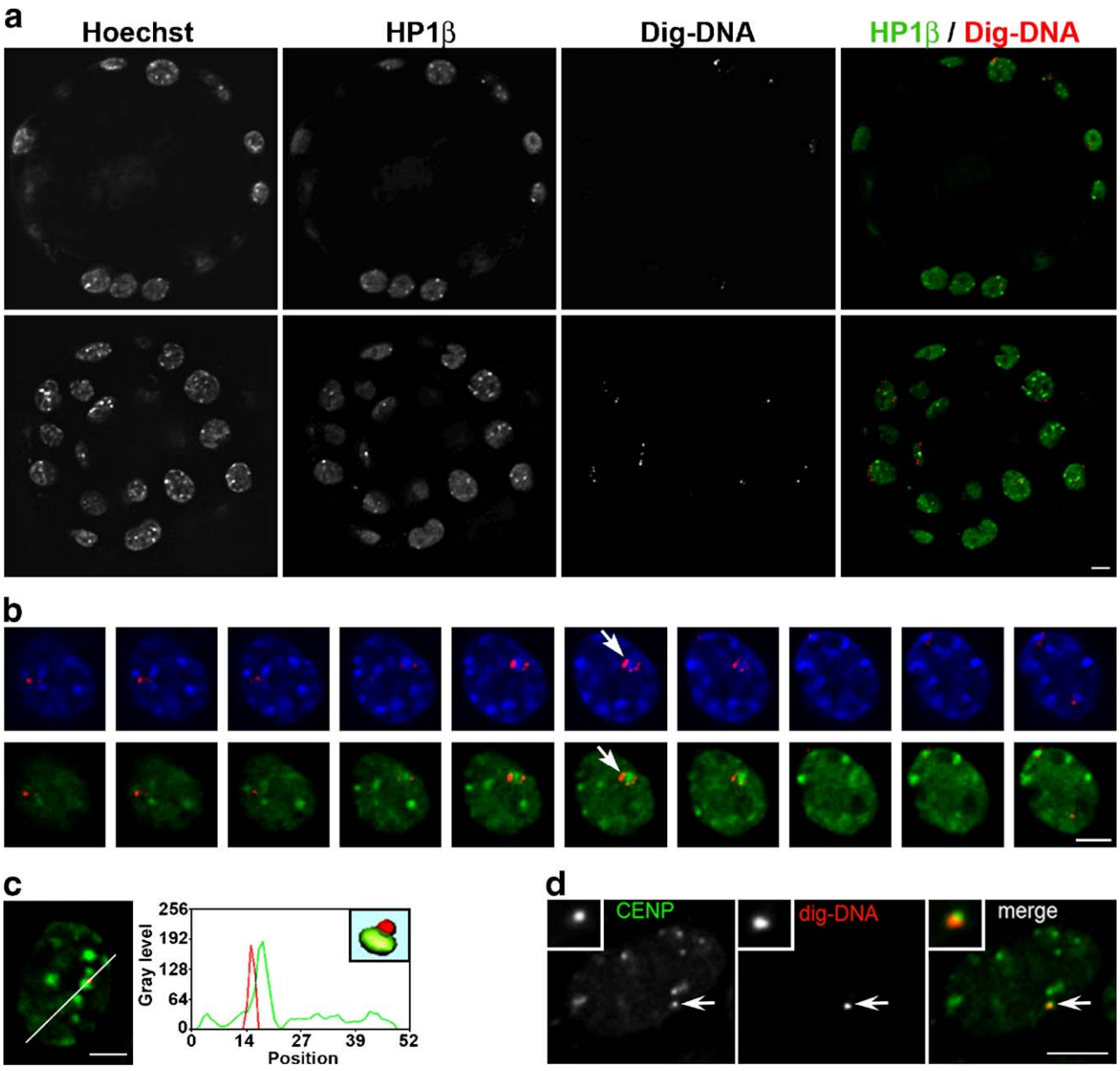

Fig. 6. Nuclear localization of dig-labeled DNA at the blastocyst stage. (a) Embryos were labeled with dig-dUTP during late S phase of the one-cell stage and fixed 5 days later at the blastocyst stage. Two different planes (top: trophectoderm; below: inner cell mass [left-hand] and trophectoderm [right-hand]) of a blastocyst are shown and the Hoechst counterstaining as well as the HP1β and dig-dUTP immunostainings are displayed. Merges of HP1β (green) and dig-dUTP (red) immunostainings are shown on the right-hand panels. Images were obtained in the "3D-preserved" setting by epifluorescence microscopy with deconvolution. Scale bar: 5 μm. (b) Trophectoderm nucleus shown in detail. Each row represents a series of consecutive light-optical sections ($\Delta z = 0.5$ μm). The merge of the dig-dUTP signal (red) with the Hoechst counterstain (blue) is shown at the top, while the merge of the dig-dUTP signal with the HP1β immunostaining (green) is shown below. Dig-dUTP labeled DNA is associated with the periphery of a chromocenter (arrow). Scale bars: 2.5 μm. (c) The left-hand panel shows a single plane (epifluorescence microscopy with deconvolution) of another blastocyst nucleus counterstained with Hoechst (green) and containing dig-labeled DNA (red). Dig-labeled DNA appears associated with the periphery of a chromocenter. The left-hand panel shows a fluorescence intensity profile along the white line drawn on the right-hand panel, and the inset shows a 3D reconstruction of the double-labeled chromocenter. (d) The panels (single light-optical sections) depict a double labeled blastocyst nucleus (CENP: green, dig-DNA: red). Centromeres are close to, but not colocalized with dig-labeled DNA. An enlargement of a centromere juxtaposed to dig-DNA (arrows) is shown in the insets. Scale bars: 2.5 μm.

fluorescence intensity profiles (Fig. 5b, left) (similar results were obtained in 82% of the cases, 32 pro-chromocenters from 7 blastomeres were analyzed, n = 26 embryos) and 3D reconstructions showing that dig-DNA was completely integrated into pro-chromocenters (Fig. 5c, left). These experiments were repeated 7 times, and the specific patterns described above were found in 80% of the two-cell embryos. In addition, we observed accumulations of HP1β that did not colocalize with labeled DNA and some foci of labeled DNA that did not colocalize with accumulations of HP1β. This DNA might correspond to the occasionally labeled non-pericentric chromosomal bands seen on late one-cell embryos after microinjection (Fig. 3). In essence, these findings show that the pericentric DNA dissociates from NPBs periphery and becomes assembled into pro-chromocenters already at the two-cell stage.

Similar experiments with embryos fixed at the four-cell stage (62 hphCG,) showed that dig-DNA labeled at 25–26 hphCG colocalized also with pro-chromocenters (Fig. 5, right-hand panels, n = 23 embryos). However, the fluorescence intensity profiles and 3D reconstructions revealed that dig-DNA often did not colocalize with the whole pro-chromocenter, but only with parts of it (Figs. 5b and c, right). This difference with the two-cell stage can be explained by the fact that labeled and unlabeled chromatids segregate randomly during the second mitosis (Taylor, 1984; Zink et al., 1998). Thus, different chromosomes with labeled and unlabeled heterochromatin most likely contributed to one pro-chromocenter at the four-cell stage. On the other hand, the strong dig-DNA/HP1β colocalization observed at the two-cell stage suggests that often, female (labeled heterochromatin, Fig. 3) and male (unlabeled heterochromatin) chromosomes contributed to separate pro-chromocenter at this stage and is consistent with previous results showing that the male and female genomes do not mix at least until the four-cell stage (Odartchenko and Keneklis, 1973; Mayer et al., 2000).

Next, we asked how this dig-dUTP DNA was organized at the blastocyst stage when the first cell differentiation between the inner cell mass and the trophectoderm occurs (Fig. 6). Twenty-two blastocysts of about 70 nuclei each were analyzed. On average, 37 nuclei within each blastocysts harbored dig-labeled DNA (note that originally labeled chromosome domains are distributed among a growing number of cells during development). Labeled DNA always occupied discrete foci in blastocyst nuclei and displayed a fluorescence intensity comparable to the one observed during the first cycle. Previous studies in somatic cells showed that replication-labeled DNA foci are equivalent to replication foci and represent stable structural/functional chromosome units (Jackson and Pombo, 1998; Ma et al., 1998; Sadoni et al., 2004). Our results thus indicate that chromosomes are organized into these stable units already during the first embryonic cell cycle.

Blastocyst nuclei, from both trophectoderm and inner cell mass, contained either single foci or clusters of foci, and we found on average two labeled regions (2 single foci, 2 clusters, or 1 single focus and 1 cluster) per labeled nucleus (Supplemental data, Table S1). Few foci localized in regions of lower Hoechst staining, mostly at the nuclear periphery. In most cases, 57% of the single foci and 70% of the clusters, the labeled regions were associated with HP1β accumulations that colocalized with Hoechst brightly stained DNA (Fig. 6b, supplemental data, Table S1). Surprisingly, those foci were not incorporated within HP1β accumulations as observed in two- and four-cell embryos but were strictly associated with their peripheries (Figs. 6b and c). As the centric subdomains are located in somatic cells at the periphery of chromocenters (Guenatri et al., 2004), we wondered whether labeled DNA could represent these domains. However, double labeling of dig-DNA and CENP A/B showed that labeled DNA and centromeres occupied neighboring but distinct subcompartments (Fig. 6d, observed in 89% of the cases, n = 44 nuclei analyzed from 4 different blastocysts).

Together, these data show that further rearrangements at pro-chromocenters take place between the four-cell and the blastocyst stage, leading to changes in the spatial organization of associated DNA. In addition, cell differentiation does not seem to influence these processes as no differences were observed between cells belonging to the trophectoderm and the inner cell mass.

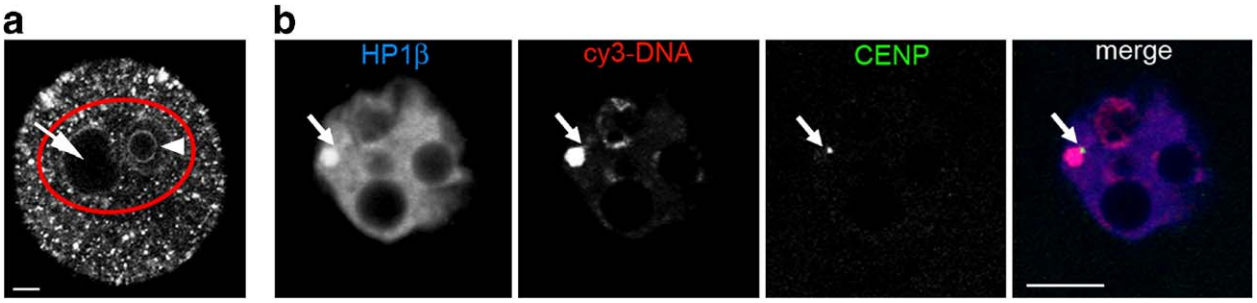

Fig. 7. In vivo tracking of labeled DNA. (a) Light-optical section of a living one-cell embryo microinjected with Cy3-dUTP at 25.5 hphCG and imaged at 27 hphCG. Note the abundance of cytoplasmic vesicles labeled with Cy3-dUTP. The pronuclear region is encircled by a red line. The mPN (arrow) is largely unlabeled whereas in the fPN labeled DNA is concentrated at the NPB periphery (arrowhead). (b) The panels show single light-optical sections at 48 hphCG of an embryo that displayed at 27 hphCG the same labeling pattern as in panel a. The nuclear patterns of HP1β, Cy3-labeled DNA, and centromeres are shown and the corresponding merge is displayed on the right (HP1β: blue; Cy3-DNA: red; CENP: green; colocalization of red and blue: pink-violet). Scale bars: 10 μm.

Development-dependent distribution of replication-labeled DNA is confirmed by in vivo tracking

Embryos from a given mouse can be relatively asynchronous (Bouniol-Baly et al., 1997). In order to exclude that the different spatial organizations of dig-labeled DNA observed at different developmental stages could be due to differences in the initial labeling patterns, we used Cy3-dUTP labeling, which allows to track in vivo the labeled DNA during development. One-cell embryos were microinjected with Cy3-dUTP (25–26 hphCG) and, after 1 h in vitro culture, were quickly examined by confocal microscopy. As in somatic cells, cytoplasmic vesicles accumulated Cy3-dUTP (Sadoni et al., 2004; Zink et al., 2003), but the replication patterns of the two PNs could nevertheless be precisely assessed (Fig. 7a). We found that the labeling patterns observed in living embryos after Cy3-dUTP microinjection were similar to those observed after dig-dUTP microinjection and immunostaining: 68% of the 41 embryos labeled by Cy3-dUTP (Fig. 7a) and 70% of the 64 embryos labeled by dig-dUTP presented an intense labeling at the NPBs periphery in the fPN (arrowhead) while the mPN (arrow) was largely unlabeled.

Embryos displaying late replication patterns of similar stages were put for in vitro culture into the same drop of culture medium (embryos do not develop individually in vitro). Subsequently, embryos were fixed either at 48 hphCG or at the blastocyst stage and HP1β as well as centromeres were detected by immunostaining. Fig. 7b shows a nucleus of a late two-cell embryo, which displayed at the one-cell stage the

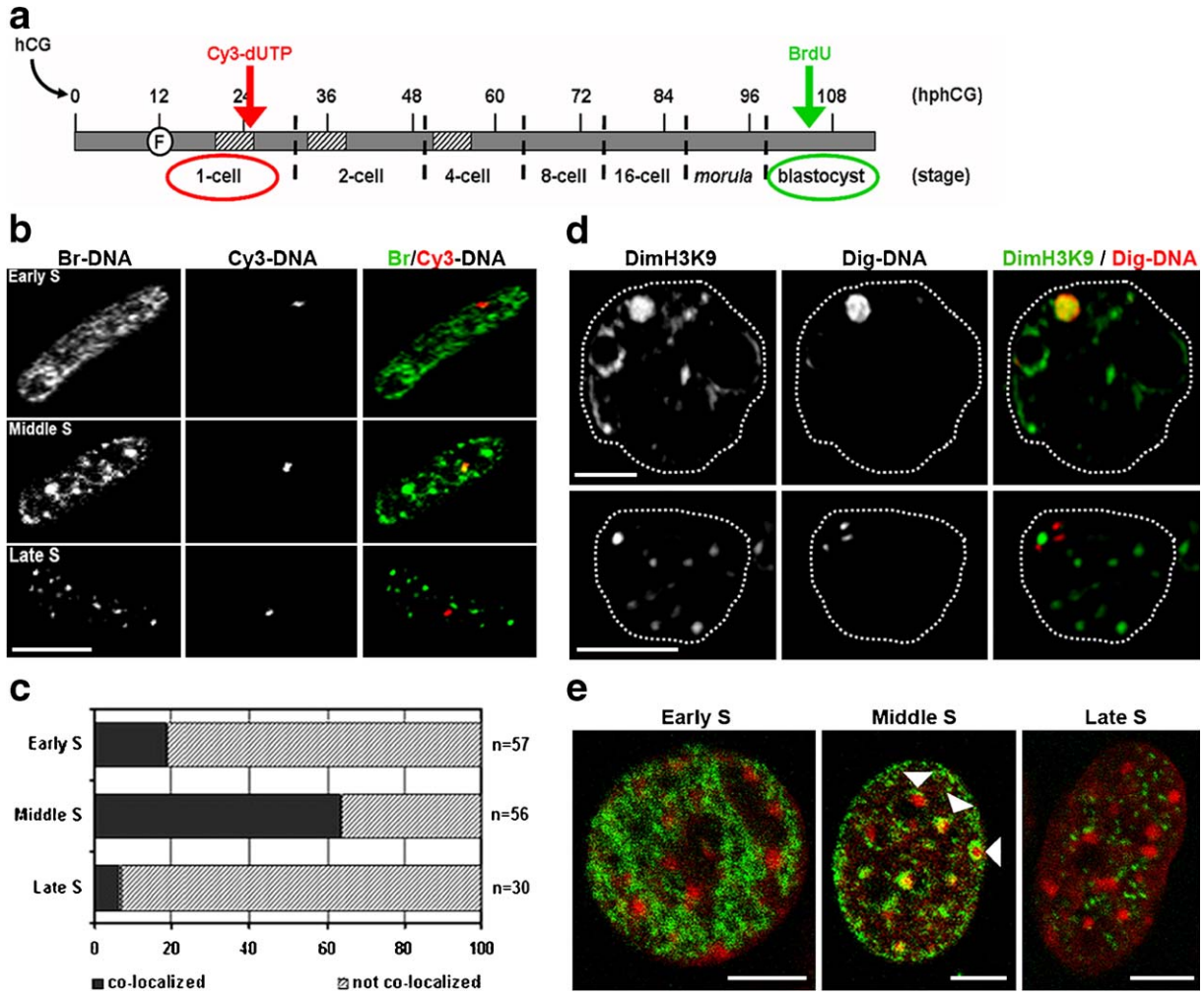

Fig. 8. Chromatin structure of chromocenters and replication timing of associated DNA. (a) Experimental procedure. DNA replicating late during the one-cell stage was labeled by microinjection of Cy3-dUTP (red) at 26 hphCG. Cy3-labeled embryos were cultured for 5 days in vitro until the blastocyst stage. Blastocysts were then pulse-labeled with BrdU (green) and fixed. (b) Codetection of Cy3-labeled DNA (red) and BrdU-labeled DNA (green) in three blastocyst nuclei. BrdU-labeling patterns are similar to replication labeling patterns observed in somatic cells (see panel e) and correspond to defined temporal stages of S phase as indicated on the images. Colocalization (yellow) of red and green signals is observed during mid S phase. Scale bar: 5 μm. (c) Average fractions of Cy3-labeled foci colocalizing (black) or not colocalizing (hatched) with BrdU-labeled DNA at the different stages of S phase. "*n*" indicates the number of nuclei analyzed in each case. (d) DNA was labeled during late S phase in one-cell embryos with dig-dUTP. DimH3K9 (green on the merged image, "branched" antibody) and dig-DNA (red on the merged image, colocalization of red and green appears orange–yellow) were detected by immunostaining after fixation at the late two-cell stage (48 hphCG, upper panels) or at the blastocyst stage (lower panels). Single nuclei from each stage are shown, the nuclear periphery is underlined by a white discontinuous line. Scale bars: 5 μm. (e) Single light-optical sections of NIH 3T3 nuclei counterstained with DAPI (red, note brightly stained chromocenters) and pulse-labeled with Cy3-dUTP (green). Cy3-labeling patterns typical for early (left), mid (middle), and late (right) S phase is shown. Mid replicating DNA is associated with the periphery of chromocenters (arrowheads).

labeling pattern shown in Fig. 7a. The distribution of Cy3-DNA was similar to that observed in dig-dUTP labeled embryos: Cy3-DNA colocalized with intensely labeled accumulations of HP1β and centromeres were associated with the peripheries of these accumulations. In addition, as observed after dig-dUTP labeling, some discrete less brightly Cy3-labeled sites were present that did not colocalize with any HP1β accumulations. At the blastocyst stage, Cy3-DNA was found at the periphery of the HP1β-labeled chromocenters, but not inside (data not shown). These findings confirmed previous observations with dig-labeled DNA and show that the observed stage-specific differences in the nuclear organization of labeled DNA were not due to differences in the initial labeling patterns.

Chromatin, which replicates late at the one-cell stage switches replication timing and chromatin structure by the blastocyst stage

So far, our results indicated that labeled DNA associated with chromocenters underwent structural rearrangements and modifications of its association to chromocenters between the four-cell and the blastocyst stage. We wondered whether this spatial reorganization was associated with changes of other features. First, we addressed possible changes in replication timing.

Therefore, one-cell embryos, first labeled with Cy3-dUTP at 25–26 hphCG and cultured until the blastocyst stage, were then pulse-labeled with BrdU and fixed directly afterwards (Fig. 8a). Nuclei of 10 blastocysts positive for both Cy3 and BrdU were characterized according to their BrdU pattern: early, mid-, and late S phase (Fig. 8b; from the extensive literature on replication labeling patterns: Leonhardt et al., 2000; Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Sadoni et al., 1999, 2004; Panning and Gilbert, 2005). The colocalization of Cy3- and BrdU-labeled DNA was then analyzed (Figs. 8b and c). Cy3-DNA colocalized with BrdU-DNA mainly during mid S phase (64%, arrow in Fig. 8b), while only minor fractions of labeled DNA colocalized at early (19%) or late (7%) S phase. These results indicate that the Cy3-DNA, which replicated late during the first cell cycle, switched its replication timing and replicated mainly during mid-S phase at the blastocyst stage.

In order to analyze whether this switch in replication timing correlated with remodeling of chromatin structure, we performed immunostaining with an antibody raised against a branched peptide presenting four "fingers" of the K9-dimethylated TARKST consensus sequence of the H3 amino-terminus

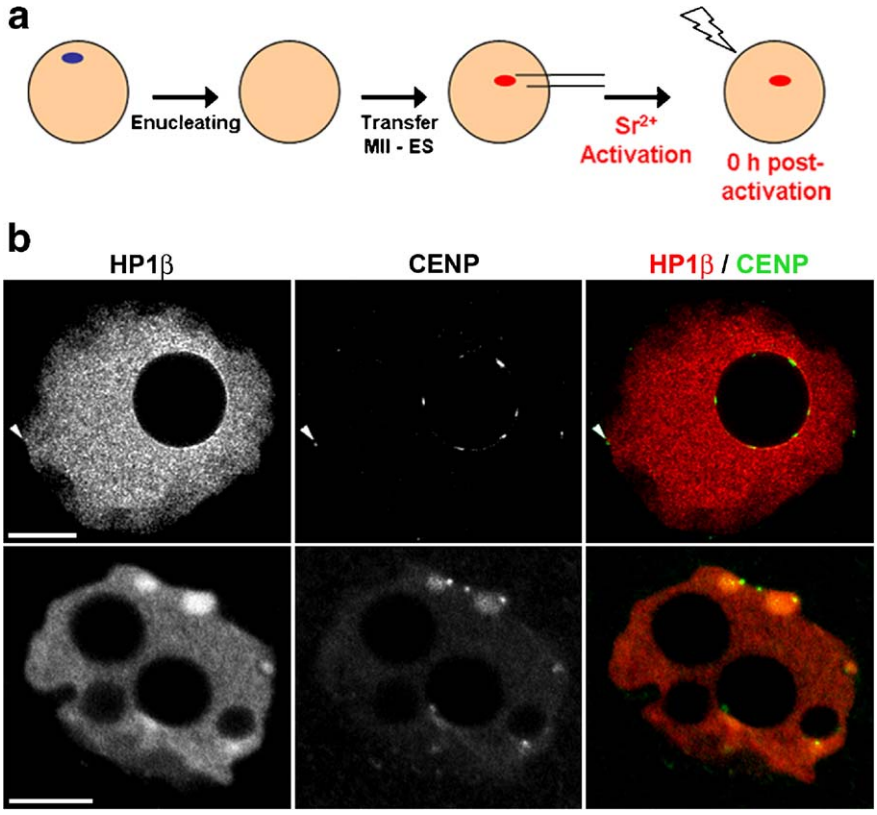

Fig. 9. Distribution of HP1β and centromeres after nuclear transfer. (a) Nuclear transfer procedure. Oocytes were enucleated (metaphase II chromosomes are shown in blue) and donor chromosomes (red) from ES cells arrested in metaphase (M ES) were injected into the oocyte cytoplasm (beige). After nuclear transfer, embryos were activated by exposure to Sr^{2+} (lightning, time point 0) and subsequently cultured in vitro. Embryos were fixed at late one-cell (10 h post-activation) and late two-cell (33 h post-activation) stages. (b) The images show light-optical sections of single nuclei from cloned embryos after double labeling of HP1β (red on the merged image) and centromeres (green on the merged image). At the late one-cell stage (10 h post-activation, upper panels), most of the centromeres were associated with the periphery of NPBs. Occasionally, some centromeres localized at the nuclear periphery (arrowhead). HP1β was diffusely distributed in the nucleoplasm and enriched at the NPBs periphery. At the late two-cell stage (33 h post-activation, lower panels), centromeres are associated with pro-chromocenters enriched in HP1β. Scale bars: 5 μm.

("branched" antibody, Maison et al., 2002; Peters et al., 2001), which specifically labels chromocenters (Guenatri et al., 2004; Maison et al., 2002). DNA labeled with dig-dUTP in one-cell embryos was heavily stained with this antibody at the two-cell (Fig. 8d, upper panels) but not at the blastocyst stage (Fig. 8d, lower panels). This shows that the chromocenter-specific chromatin structure recognized by the "branched" antibody was already established at this early stage. It also implies that chromatin replicating late during the first cell cycle switched its higher order structure in conjunction with the switch in replication timing and spatial rearrangements.

We confirmed that in blastocyst nuclei (Figs. 8b and c), as in somatic nuclei, mid-replicating DNA was associated with the periphery of chromocenters (Fig. 8e, arrowheads). Thus, at least with regard to this feature, chromocenters of blastocysts and somatic cells display a similar spatio-temporal organization.

The organization of pericentric DNA and the genesis of pro-chromocenters are embryo-specific features determined by ooplasmic factors

To demonstrate that the particular genome organizations observed at the one- and two-cell stages in mouse are embryo-specific patterns, we performed nuclear transfer experiments followed by similar HP1β and CENP immunostaining. Oocytes from superovulated mice were enucleated and metaphasic chromosomes from ES cells were transferred into the oocyte cytoplasm (Fig. 9a). It should be noted that ES cells derived from the inner cell mass of blastocysts display chromocenters as shown above (Fig. 4). After nuclear transfer and activation, cloned embryos were cultured in vitro and fixed at one-cell (10 h post-activation, equivalent to 22 hphCG, $n = 75$) and two-cell (33 h post-activation, equivalent to 45 hphCG, $n = 28$) stages. While a typical "pronuclear type" NPB appeared in all the clones after activation (Fig. 9b) (Borsuk et al., 1996), HP1β and most of the centromeres accumulated at the NPB periphery in 85% of the cases, similarly to normal one-cell stage embryos. This suggests that the nuclear structure was rapidly remodeled into the "cartwheel" organization. In all the two-cell stage embryos analyzed, HP1β and the centromeres became reorganized into pro-chromocenters as in normal two-cell embryos (Fig. 9b, bottom). These results show that the establishment of the one-cell stage-specific organization and the subsequent reorganization of the genome observed during normal embryogenesis are recapitulated after cloning, under the influence of oocyte cytoplasmic factors.

Discussion

In the present paper, we show various restructuring movements of chromatin during the first steps of embryonic development. This is, to our knowledge, the first study addressing the formation of chromocenters and the spatial organization of chromosomal domains in early mouse embryos, in relation to the establishment of transcriptional activity.

The results reveal that one-cell mouse embryos display during interphase a transient "cartwheel" organization characterized by the association of centromeric and pericentric regions to the NPBs periphery, while the remaining chromosomal domains stretch out towards the nuclear periphery.

This radial arrangement appears identical for the maternal and paternal genomes, despite differences in DNA methylation, histone modifications (Liu et al., 2004; Santos et al., 2005), and HP1β binding capacities of the pericentric regions. The lack of HP1β binding to paternal chromatin is consistent with the lack of trimethylated H3K9 in the mPN already observed by other authors (Arney et al., 2002; Liu et al., 2004; Santos et al., 2005) but contrasts with the faint accumulation of HP1β observed by Santos et al. (2005) around the male NPB. This difference to our present results may be due to differences in immunostaining procedures.

Nuclear transfer experiments also demonstrated that the more advanced form of genome organization present in ES cells can be quickly disrupted and reverted, under the influence of still unknown ooplasmic factors, into a typical embryonic organization, recalling the "cartwheel". Reorganizations were observed with regard to the formation of NPBs, the absence of chromocenters, the homogenous distribution of HP1β, and the radial organization of chromosomal domains. However, the fact that only 3.1% of the embryos reconstructed with ES metaphases develop to term suggest that the observed nuclear remodeling is not sufficient, and that more subtle modifications of the somatic genome are required to support full development (Zhou et al., 2001). Further experiments are now under way to characterize more precisely nuclear remodeling after nuclear transfer.

During subsequent stages, centromeric and pericentric regions are rapidly reorganized in association with the formation of chromocenters, which proceeds through two major steps summarized in Fig. 10. The first step involves large-scale rearrangements of HP1β, centromeres, and pericentric heterochromatin. These dramatic rearrangements occur in the mouse during the two-cell stage and lead to the formation of pro-chromocenters (interestingly, these reorganization events occur in parallel with the onset of zygotic transcription both in mouse and bovine embryos). Between the four-cell and the blastocyst stages, further spatial rearrangements of pericentric chromatin lead, in conjunction with switches in replication timing and chromatin structure, to the establishment of "mature" chromocenters with a defined spatio-temporal organization.

The establishment of a defined chromocenter substructure seems to occur simultaneously with the establishment of specifically regulated transcription patterns (for review, see Nothias et al., 1995) and the onset of differential gene expression, first occurring at the blastocyst stage (Palmieri et al., 1994; Kirchhof et al., 2000; Williams et al., 2002). Furthermore, recent genome-wide studies of gene activity in preimplantation mouse embryos revealed two major phases, one corresponding to the zygotic genome activation (one to two cells), and the second between the four-cell and blastocyst stages (Wang et al., 2004; Hamatani et al., 2004). It seems therefore worth noting that these two waves of transcription parallel the two major phases that we observe here in the dynamics of chromocenter formation.

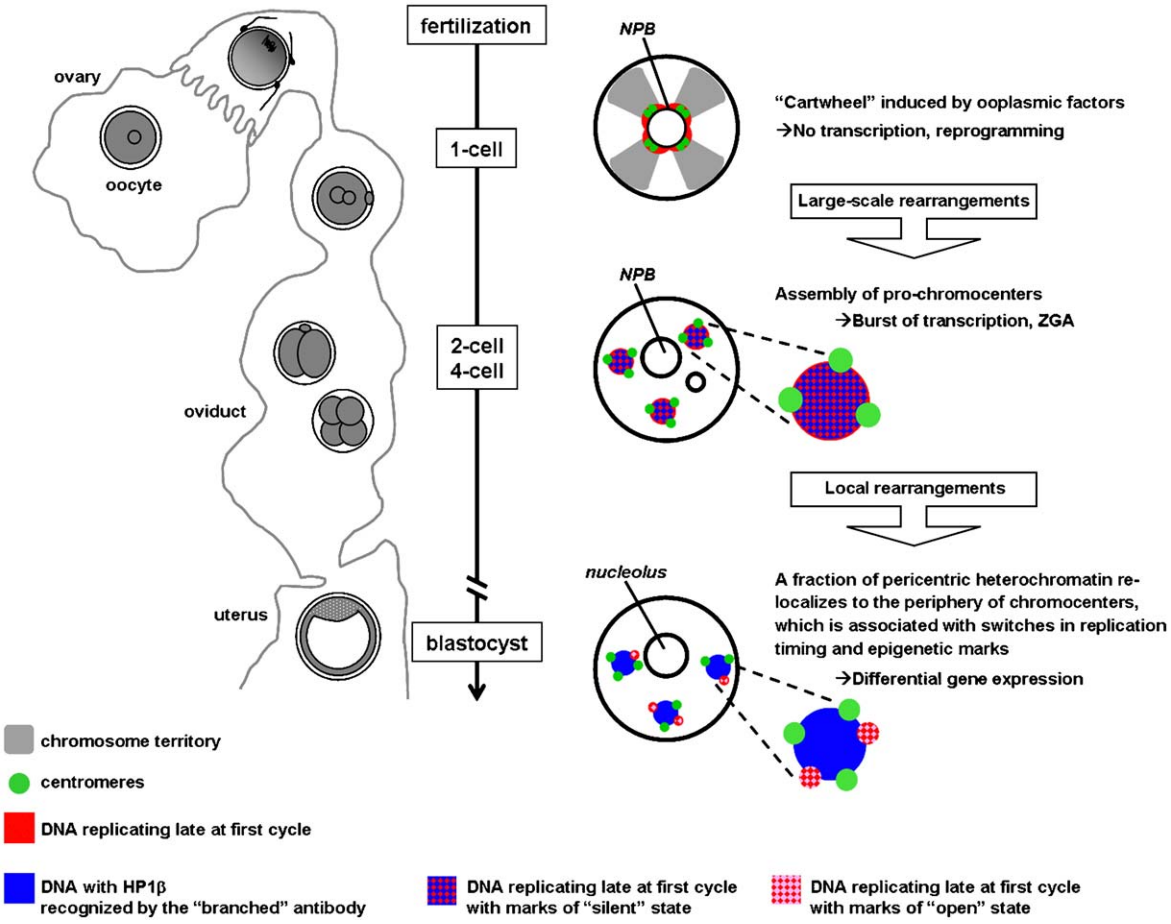

Fig. 10. Summary and model. The figure illustrates interphasic chromosomal domains arrangements and chromocenter formation (single nuclei drawn in the middle) during mouse embryonic development (schematized on the left). Both normal and cloned embryos proceed through the "cartwheel" organization during genome reprogramming. In two-cell embryos, pro-chromocenter formation then parallels the major burst of transcription. Finally, chromocenters with a defined spatio-temporal organization are formed by the blastocyst stage. The model proposes that such chromocenters are maintained from the blastocyst stage onwards and are important for proper regulation of differential gene expression patterns.

As the establishment of "mature" chromocenters displaying a defined spatio-temporal organization correlates with the onset of the regulated zygotic transcription, they are probably required for proper regulation of differential gene expression. This view is supported by the finding that in different somatic cell types and during cell differentiation individual genes differentially associate in accordance with their individual state of activity with chromocenters and other defined chromatin domains displaying a characteristic nuclear organization (Brown et al., 1997, 1999, 2001; Francastel et al., 1999; Schubeler et al., 2000; Zink et al., 2004). Thus, chromocenters and other defined heterochromatic and euchromatic compartments displaying a defined spatial organization might provide a basic framework in the nucleus, within which individual genes become positioned for proper regulation.

In this work, we used a novel approach, already applied in somatic cells (Sadoni et al., 2004; Zink et al., 1998), to covalently label chromatin at the one-cell stage and follow its dynamics during in vitro culture. The incorporation of dig-dUTP or Cy3-dUTP at late S phase of the first cell cycle allowed us to specifically mark pericentric DNA, essentially in the female genome. During the subsequent steps of development labeled pericentric chromatin underwent various changes which are summarized in Fig. 10: (i) at the two/four-cell stage, it was assembled into pro-chromocenters and presented epigenetic marks characteristic of silenced chromatin, i.e., HP1β association, H3K9 methylation and late replication (data not shown); (ii) at the blastocyst stage, it was segregated from chromocenters and presented a reverse profile of epigenetic marks, i.e., dissociation from HP1β, a chromatin structure not recognized anymore by the "branched" antibody, and earlier replication (essentially at mid S phase, see Fig. 8).

Ferreira and Carmo-Fonseca (1997) already noticed a lengthening of the time necessary to replicate the chromatin at the nuclear periphery between one-cell and *morula* stages, thus suggesting a structural remodeling of this region. Here, we show a change in the timing of replication for a well-defined portion of the genome that correlates with important spatial reorganizations of the genome during this period of development.

The associated inversion of epigenetic marks, from a "silent" to an "open" state is striking. Recent studies on *Drosophila* described the inclusion of developmentally regulated genes in the pericentric regions (Greil et al.,

2003). We hypothesize that a similar situation might exist in mouse genome: such genes, first silent and located in a highly repressive environment inside the pro-chromocenters at the two/four-cell stage, could then be activable owing to their segregation at the periphery of chromocenters where a more permissive structure would improve their accessibility to the transcription machinery. This type of regulation would not exclude additional mechanisms, such as differential gene recruitment as described above (Brown et al., 1997, 1999; Francastel et al., 2001).

It is worth noting that during the same developmental period, the paternal X-chromosome becomes first inactivated in all cells and then reactivated during blastocyst formation in the cells allocated to the inner cell mass (Mak et al., 2004; Okamoto et al., 2004). In parallel, precisely programmed changes in histones modifications patterns occur. Thus, another major heterochromatic compartment undergoes switches in epigenetic marks by the blastocyst stage, suggesting that this period is crucial for the formation of defined heterochromatic domains, which are stably maintained afterwards.

Our results also shed light on the substructure of chromocenters. At the four-cell stage particularly, chromocenters are composed of labeled and unlabeled pericentric regions due to the random segregation of the labeled DNA during the successive cell divisions. This suggests that the different pericentric regions engaged in a chromocenter are not mixed but remain spatially separated in distinct subcompartments, as proposed in a recent study investigating somatic cells (Guenatri et al., 2004). Guenatri et al. also showed that minor (centric) and major (pericentric) satellites form spatially distinct subdomains of chromocenters, which display a different replication timing. Here, we observed in the "mature" chromocenters of blastocysts a similar spatial organization of centric (CENP binding, peripheral position) and pericentric (HP1β binding, central position) heterochromatin. In addition, we found that the fraction of pericentric DNA which was late replicating at one-cell stage and segregated to the chromocenter periphery at the blastocyst stage may represent another compartment of chromocenters that differs from centromeres (Fig. 6d) and from the peripheral mid-replicated DNA observed by Quivy et al. (2004) since it is not enriched in HP1β.

Altogether, our results reveal two distinct forms of nuclear reorganization during preimplantation life that are apparently related to major changes in zygotic transcriptional regulation: (1) large-scale dynamics of chromosome territories and chromocenter compartments correlated with ZGA, (2) more subtle movements of pericentric heterochromatin associated with switches in epigenetic marks and replication timing that correlate with the onset of differential gene expression. These different remodeling events may play a fundamental role in reprogramming and in the correct establishment of embryonic activities.

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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.2006.01.009.

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