Histone acetylation and subcellular localization of chromosomal protein BRD4 during mouse oocyte meiosis and mitosis†

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Most specific and general transcription factors (TFs) become dissociated from hypoacetylated mitotic chromosomes, which may contribute to transcriptional silencing during mitosis. Only some chromosomal proteins, such as bromodomain containing protein 4 (BRD4), have a potential to associate with mitotic chromosomes in a histone acetylation-dependent manner. It remains to be fully demonstrated whether similar displacement of nuclear factors takes place in meiotic oocytes whose chromosomes become globally deacetylated. To address this, we here examined the subcellular localization of BRD4 in conjunction with the acetylation status of histones in mouse oocytes. Immunofluorescence studies revealed that BRD4 preferentially localized to mitotic chromosomes in early embryos. In contrast, not only endogenous BRD4 but also exogenous BRD4 overexpressed by mRNA microinjection were displaced from meiotic chromosomes whose histones H3 and H4 were deacetylated. Treatment with trichostatin A (TSA), an inhibitor of histone deacetylases, induced histone hyperacetylation of meiotic chromosomes from which endogenous BRD4, however, remained dissociated. Finally, meiotic chromosomal localization of BRD4 could be achieved by BRD4 overexpression together with TSA-induced histone hyperacetylation. These results indicate that, unlike mitosis, histone acetylation is necessary but not sufficient for chromosomal localization of BRD4 during meiosis, suggesting that meiotic oocytes may have additional mechanism(s) for displacement of chromosomal proteins and TFs.

Key words: BRD4/bromodomain/histone acetylation/meiosis/oocyte

Introduction
During the proliferation of somatic cells, the information on active genes is transmitted precisely to daughter cells, thereby maintaining the lineage-specific programme of gene expression in the subsequent generations. In spite of the faithful transmission of genetic information, the whole genome becomes almost inert through the displacement of most specific and general transcription factors (TFs) from the chromosomes during mitosis (Hershkovitz and Riggs, 1995; Martinez-Balbas et al., 1995; Muchardt et al., 1996; Segil et al., 1996). To maintain the minimum information for lineage-specific gene programmes over generations, there may exist mechanism(s) called ‘cell memory’ by which certain epigenetic modifications remain as markers of cell identity in the chromosomes during mitosis (Jeppesen, 1997; Turner, 2000, 2002). Histone modifications including methylation, phosphorylation and acetylation have been proposed as candidate epigenetic markers of cell memory (Strahl and Allis, 2000; Turner, 2000, 2002).

It has been suggested that distinct histone acetylation patterns function as recognition codes for the recruitment of different TFs upon transcriptional activation (Agalioti et al., 2002; Turner, 2002). During mitosis, the activities of histone acetyltransferases and deacetylases are down-regulated (Kruhlak et al., 2001), and general hypoaetylation of core histones takes place together with the displacement of TFs from mitotic chromosomes, which correlates with the steep fall in transcriptional activities (Kruhlak et al., 2001). However, some lysine residues on histones H3 and H4 remain acetylated on mitotic chromosomes, which may serve as markers for transcriptional memory (Kruhlak et al., 2001; Valls et al., 2005). Transcription restarts at the end of telophase when chromatin-binding proteins and TFs are reloaded onto chromatin (Prasanth et al., 2003). Thus, histone acetylation has been proposed as an epigenetic marker for the propagation of genomic information from one cell generation to the next (Jeppesen, 1997; Turner, 2000; Jenuwein and Allis, 2001). In contrast to histone hypoacetylation during mitosis in somatic cells, meiotic chromosomes become globally deacetylated in oocytes (Kim et al., 2003). Meiosis-specific histone deacetylation is...
thought to contribute to transcriptional silencing and erasing of cell memory, which may be required for the reprogramming of the oocyte genome to allow the remarkable transformation from differenti-ated oocytes into the totipotent embryos of the next generation (Schultz et al., 1999; Kim et al., 2003). However, it remains to be demonstrated whether non-histone chromosomal proteins, as well as TFs, are displaced from meiotic chromosomes in mouse oocytes.

Bromodomains are conserved protein modules that bind to acetyl-ated histones and are present in a number of chromatin-binding proteins, including histone acetyltransferases, general TFs and chromatin remodelling factors (Zeng and Zhou, 2002). BRD4 is a mamma-lian bromodomoid protein that belongs to the conserved bromodomain and extraterminal domain (BET) family (Dey et al., 2000) whose members carry two tandem bromodomains and an additional extraterminal domain (Florence and Faller, 2001). Previous analysis with fluorescence loss in photobleaching showed that BRD4 dynamically interacts with acetylated histones H3 and H4 in living interphase cells (Dey et al., 2003). A unique and notable feature of BRD4 is that it behaves as a non-histone chromosomal protein, being tightly associated with chromosomes during mitosis when many other nuclear factors are released into the cytoplasm (Dey et al., 2000). Similar to interphase cells, localization of BRD4 to mitotic chromosomes is attributed at least in part to its binding to acetylated histones.

In this study, taking advantage of the unique property of BRD4, we here examined the acetylation status of histones in conjunction with the subcellular localization of BRD4 to address whether meiotic oocytes displace chromosomal proteins as well as TFs from their chromosomes through regulation of histone acetylation and deacetylation. We here provide evidence suggesting that not only histone deacetylation but also additional mechanism(s) are required for the displacement of chromosomal proteins, including BRD4, during mouse oocyte meiosis.

Materials and methods

Preparation of mouse oocytes and embryos

Germinal vesicle (GV) stage oocytes were obtained from 4-week old female BDF1 mice (Sankyo, Tokyo, Japan) as the cumulus–oocyte complex (COC). Female mice were induced to ovulate with intraperitoneal injection of 5 IU pregnant mare’s serum gonadotropin (PMSG, Teikokuzoki, Tokyo, Japan) and were killed by cervical dislocation 48 h later. After removing the ovaries and trimming fat and mesentery, they were recovered in M2 medium (Sigma–Aldrich, St Louis, MO, USA) and were killed by cervical dislocation 48 h later. After removing the ovaries and trimming fat and mesentery, they were recovered in M2 medium (Sigma–Aldrich, St Louis, MO, USA) (Fulton and Whittingham, 1978) for cell manipulation under a Leica M8 stereoscopic microscope (Leica Microsystems, Heidelberger, Germany). After mechanical removal of COCs by a 29-gauge needle, GV stage oocytes were pooled together after dispersion and washed for removal of cumulus cells mechanically by pipetting with a thin pipette in M2 medium containing 0.1% hyaluronidase (Sigma–Alrich). Then, denuded spherical oocytes of uniform size containing intact GV stage oocytes were allocated to treatment groups and cultured in Toyoa Yokoyama Hoshi (TYH) medium (Mitsubishi Kagaku Iatron, Tokyo, Japan) (Toyoa et al., 1971) covered with mineral oil (Sigma–Alrich) at 37°C under 5% CO2 in humidified air for in vitro maturation. To prevent germinal vesicle breakdown (GVBD), GV stage oocytes were treated with 100 μg ml⁻¹ dibutyryl cyclic AMP (dbcAMP, Sigma–Alrich), a membrane permeable cyclic AMP analog (Wassarman et al., 1976), in combination with or without 50 ng ml⁻¹ trichostatin A (TSA, Sigma–Alrich), a potent histone deacetylase inhibitor (Yoshida et al., 1995). GVBD, metaphase I (MI) and metaphase II (MII) stage oocytes were collected 4, 9 and 17 h, respectively, after culture of GV stage oocytes in the absence of 100 μg ml⁻¹ dbcAMP in combination with or without 50 ng ml⁻¹ TSA. The fertilized embryos were prepared by in vitro fertilization (IVF). Spermatozoa were dissected from the cauda epididymides of 8-week-old male BDF1 mice (Sankyo), mixed slightly and cultured in TYH medium covered with mineral oil for more than 1 h at 37°C under 5% CO2 in humidified air for spermatozoal capacitation. MII stage oocytes were obtained from ovulation-induced 4-week-old female BDF1 mice with PMSG, as described above, followed 48 h later by human chorionic gonadotropin (hCG, Mochida, Tokyo, Japan). After 12 h of hCG injection, ovulation-induced mice were killed at different times and the oviductal ampul-laee were broken to release the COCs at the MII stage of in vivo ageing. MII stage oocytes were inseminated with capacitated spermatozoa (1.0 × 10⁶ cells ml⁻¹). After removing cumulus cells with 0.1% hyaluronidase, the emission of the second polar body and the formation of the pronu-clei were observed with a stereoscopic microscope after 5 h of IVF and culture in TYH medium. Embryos by the interphase of the 1-cell stage and the interphase and metaphase of the 2-cell stage were obtained 5, 30 and 36 h after insemination and cultured in modified Whitten’s medium (Mitsu-bishi Kagaku Iatron) (Beckmann and Day, 1995) in the presence or absence of 50 ng ml⁻¹ TSA, respectively. All animal studies were approved by our Institutional Review Board(s).

Immunofluorescence

Oocytes, embryos and NIH3T3 cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min at 37°C and then permeabilized with 0.2% Triton X-100 PBS for 15 min at room temperature (RT). After blocking for non-specific staining with 5% (bovin serum albumin) BSA/PBS for 1 h at RT, the cells were incubated with 1:400 polyclonal rabbit anti-BRD4 antibody (Dey et al., 2000), 1:200 polyclonal rabbit anti-activating protein 2 (AP-2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 1:200 polyclonal rabbit antibodies against acetylated lysine 9 and 14 on histone H3 (AcH3/K9K14) or acetylated lysine 5 on histone H4 (AcH4/K5) (Upstate Biotechnology, Lake Placid, NY, USA) for 1 h at RT or overnight at 4°C. The antibodies that bound to the cells were probed with Cy2 or Cy3 anti-rabbit IgG (Jackson Immuno Research Laboratories, Bar Harbor, ME, USA) for further 1 h at RT. Counterstaining was conducted with 2 mg ml⁻¹ Hoechst 33342 (Sigma–Alrich) to visualize DNA by incubation for 20 min at RT. Finally, the cells were mounted on glass slides and viewed with a Leica DMIRE2 inverted microscope (Leica) using a 40× and 63× oil immersion objective. The captured images were processed using Adobe Photoshop CS2™ software (Adobe Systems, San Jose, CA, USA). The experiment was conducted three times with at least five oocytes and embryos per group.

Plasmid construction

EGFP-fused Brd4 fragments were excised from pc1-EGFP-Brd4 (Maruyama et al., 2002) and cloned into pTRE (Clontech Laboratories, Palo Alto, CA, USA) to generate pTRE-EGFP-Brd4. For mRNA microinjection experiments, pcDNA3-Brd4-EGFP and pcDNA3-EGFP were constructed as follows. The pN3-Brd4-EGFP (Maruyama et al., 2002) was digested with NotI and XhoI. pcDNA3 (Invitrogen, Carlsbad, CA, USA) was digested with ApaI. Digested fragments containing Brd4-EGFP and pcDNA3 were both treated with T4 DNA polymerase (Takara, Tokyo, Japan) to make blunt-ends and ligated for the construction of pcDNA3-Brd4-EGFP. The pN3-EGFP (Clontech) and pcDNA3 were both digested with NotI and XhoI and ligated for the construction of pcDNA3-EGFP.

Generation of stable transfectants capable of inducing EGFP-BRD4 under DOX control

Doxycycline (DOX, Sigma–Alrich)-inducible Brd4 expression was performed as described elsewhere (Maruyama et al., 2002). Along with the procedure, NIH3T3 stable transfectants (doxEGFP-BRD4) were generated following transfection of pTk-hygro and pTRE-EGFP-Brd4 into cells that had been transfected with pTet-On vector (Clontech).

Immunoblot

doxEGFP-BRD4 cells were first inoculated on 60 mm dishes at 4.0 × 10⁵ cells dish⁻¹ and cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma–Alrich) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic–antimycotic mixture (Gibco, Grand Island, NY, USA) at 37°C under 5% CO2 in humidified air. Once confluent, the cells were washed twice in PBS and maintained in DMEM with or without DOX (2 μg ml⁻¹).
to induce EGFP-BRD4. After 8 h of treatment, doxEGFP-BRD4 cells were washed twice in cold PBS and lysed with 200 μl RIPA buffer (20 mmol l⁻¹ Tris–HCl (pH 7.5), 150 mmol l⁻¹ NaCl, 1 mmol l⁻¹ EDTA, 1% Na-deoxycholate, 0.1% sodium deoxycholate (SDS), 1 mmol l⁻¹ Na₂VO₃, 50 mmol l⁻¹ NaF and 1 mmol l⁻¹ Na₂MoO₄) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). After three cycles of stirring for 15 s and chilling for 5 min at 4°C, the cell lysates were centrifuged at 17 000g for 10 min at 4°C and stored immediately at −80°C until electrophoresis. The protein concentration was measured using a DC protein assay kit (BioRad, Hercules, CA, USA).

In a typical experiment, 8 μg of cell lysates were mixed with RIPA buffer plus 2 × 500 μl sample buffer and heated at 95°C for 5 min. The heated samples were separated on 6% SDS–polyacrylamide gel electrophoresis (PAGE) for 2 h at 125 V and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore, Bedford, MA, USA) for 2 h at 52 V using transfer buffer made by mixing 50 mmol Tris aminomethane, 40 mM glycine, 0.04% SDS and 20% methanol. The membrane was incubated overnight with a primary antibody against EGFP (1:10 000 dilution; Jackson Immuno Research Laboratories) in TBS-T containing 5% BSA (Sigma–Aldrich) for 2 h at RT to block non-specific binding sites. After three 10-min washes in TBS-T, the membranes were incubated overnight with a primary antibody against H3 or H4, 50 V using transfer buffer made by mixing 50 mmol Tris aminomethane, 40 mM glycine, 0.04% SDS and 20% methanol. The membrane was incubated in TBS-T [20 mmol Tris–HCl, 100 mmol NaCl (pH 7.6) and 0.1% Tween-20] containing 5% BSA (Sigma–Aldrich) for 1 h at RT to block non-specific binding sites. After three 10-min washes in TBS-T, the membranes were incubated overnight with a primary antibody against EGFP (1:10 000 dilution; Clontech) in TBS-T containing 1% BSA. After overnight incubation, membranes underwent three 10-min washes in TBS-T and were incubated for 1 h with a matching horse-radish peroxidase (HRP)-conjugated secondary antibody (1:10 000 dilution; Jackson Immuno Research Laboratories) in TBS-T containing 1% BSA. After three 10-min washes in TBS-T, bound antibodies were detected using an enhanced chemiluminescence (ECL) plus detection kit (Amersham Biosciences Co., Piscataway, NJ, USA) according to the manufacturer’s instructions and exposed to X-ray films (Eastman Kodak, Rochester, NY, USA).

**Time-lapse video microscopy of living cells**

Prior to microscopic analysis, doxEGFP-BRD4 cells were allowed to attach and spread on glass bottom 60-mm dishes at 4 × 10³ cells per dish and grown for 12 h in DMEM supplemented with 10% FCS and 1% antibiotic–antimycotic mixture at 37°C under 5% CO₂ and 95% air. Furthermore, to induce EGFP-BRD4, the cells were cultured for 12 h at 37°C in a CO₂ tissue culture incubator. After 12 h of treatment, doxEGFP-BRD4 cells during mitosis were placed under a time-lapse video microscope in a 37°C incubator. Images were captured using a Leica TCS-SP2 confocal laser scanning unit (Leica) attached to a DM IRE2 inverted microscope using a 63× oil immersion objective. Filters and lightpaths were controlled with a filter wheel and shutters. To minimize bleaching of fluorescence, EGFP-BRD4 fluorescence images were taken at 2 min intervals by recording for 1–4 s using a photometric digital camera controlled by Leica confocal software, version 2.5. Time-lapse series were subsequently converted to 8-bit images and assembled using Adobe Photoshop CS2™ software.

**Preparation and verification of mRNA for microinjection**

For the linearization of plasmids, pDNA3-EGFP was digested with EcoRV, and pDNA3-Brd4-EGFP was digested with NotI. These digested plasmids were subjected to proteinase K treatment to eliminate RNase by mixing 5 μg purified digested plasmids, 20 μl proteinase K, 2.5 μl of 10% SDS and incubating the mixture at 50°C for 30 min. Then, an in vitro transcription reaction was carried out using a mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocols. The constructed mRNA for EGFP and BRD4-EGFP were added with a poly(A) tail of at least 150 nucleotides in length to the 3’ termini of mRNA, using the Poly (A) Tailing Kit (Ambion) according to the manufacturer’s protocols. The addition of the poly(A) tail was confirmed by agarose-formaldehyde gel electrophoresis.

The translation of EGFP and Brd4-EGFP mRNA were confirmed by in vitro translation assays using TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI, USA) according to the manufacturer’s protocols. As in vitro translation reaction, mixtures of purified 1 μg EGFP or Brd4-EGFP mRNA, 20 μl of reticulocyte lysate, 10 μg of RNase A and 50 μCl of L-[³⁵S] methionine (GE Healthcare Bio-Sciences, Piscatway, NJ, USA) were incubated at 30°C for 90 min. One microlitre of the incubated mixture was separated by 18% and 6% SDS–PAGE gels for the detection of EGFP and BRD4-EGFP, respectively, and the bands were visualized by autoradiography.

**Microinjection of mRNA**

The linearized EGFP or Brd4-EGFP mRNA in PBS were microinjected directly into the cytoplasm of fully grown mouse blastomere stage oocytes at a concentration of 1.5 μg l⁻¹ using NWO-202 micromanipulators and IM-300 microinjectors (Narishige, Tokyo, Japan). In each experiment, oocytes were injected within 45 min after oocyte collection. The microinjected GV stage oocytes were treated and collected in a similar way as described above. The experiment was conducted three times, with at least five embryos per group.

**Results**

**Histone acetylation and subcellular localization of BRD4 during mitosis in fibroblast NIH3T3 cells**

We first examined the acetylation status of histones H3 and H4 in conjunction with the cellular localization of BRD4 in mitotic NIH3T3 cells. Indirect immunofluorescence using specific antibodies against AcH3/K9K14, AcH4/K5 and BRD4 revealed that BRD4 formed a faint, diffuse coat over mitotic chromosomes (Figure 1C) whose H3/K9K14 was acetylated (Figure 1A) but H4/K5 was deacetylated.

[Figure 1. Effects of trichostatin A (TSA) on the acetylation status of histones H3 and H4 and the subcellular localization of bromodomain containing protein a (BRD4) in mitotic NIH3T3 cells. NIH3T3 cells were treated without (A–C) or with TSA (D–F) for 12 h and immunostained with antibodies against AcH3/K9K14 (A and D), AcH4/K5 (B and E) or BRD4 (C and F) that were visualized by Cy2- (A, B, D and E) or Cy3- (C and F) labelled secondary antibodies. DNA was stained with Hoechst 33342. Bars (A–F), 5 μm.]
These patterns of histone acetylation were consistent with a previous report (Kruhlak et al., 2001). BRD4 has been originally reported to tightly associate with mitotic chromosomes in P19 embryonal carcinoma cells (Dey et al., 2000); however, subsequent studies demonstrated that the chromosomal localization of BRD4 depends on the acetylation status of histones, and therefore its localization patterns are variable among cell lines (Dey et al., 2003; McPhillips et al., 2005). Treatment with TSA increased the levels of AcH3/K9K14 (Figure 1D) and AcH4/K5 (Figure 1E) concomitant with an increase in the amount of chromosomally bound BRD4 (Figure 1F), which was in agreement with the previous report (Dey et al., 2003). Peptide-binding assays show that BRD4 preferentially binds to acetylated H3/K9K14 and H4/K5 peptides (Dey et al., 2003), which consistently accounts for the spatio-temporal behaviours of BRD4 in NIH3T3 cells as presented here. Thus, the chromosomal localization of BRD4 was highly dependent on the level of histones H3 and H4 acetylation in mitosis.

**Histone acetylation and subcellular localization of BRD4 in mouse oocytes and early embryos**

We then examined the acetylated status of histones H3 and H4 together with the intracellular distribution of BRD4 in mouse oocytes and early embryos. Indirect immunofluorescence using specific antibodies against AcH3/K9K14, AcH4/K5 and BRD4 revealed that both H3/K9K14 and H4/K5 became acetylated and BRD4 was preferentially localized in the nucleus of GV stage oocytes (Figure 2A, H and O). Also, BRD4 was present in the nucleus of 1-cell and 2-cell stage embryos (Figure 2S and T), and in the condensed chromosomes of 2-cell metaphase stage embryos (Figure 2U), all of whose H3/K9K14 and H4/K5 were acetylated (Figure 2E–G and L–N). In contrast, H3/K9K14 and H4/K5 became deacetylated, and BRD4 was dissociated from condensed chromosomes and dispersed into the cytoplasm in GVBD (Figure 2B, I and P), MI (Figure 2C, J and Q) and MII stage oocytes (Figure 2D, K and R). The histone acetylation and deacetylation patterns presented here are consistent with the previous results, except for H4/K5, which has been reported to be deacetylated during mitosis in early embryos (Kim et al., 2003). Wee et al. (2006) have recently demonstrated that H4/K5 become acetylated during mitosis in bovine IVF embryos being consistent with our present data.

As BRD4 binds to acetylated histones H3 and H4, it is reasonable to speculate that the dissociation of BRD4 from meiotic chromosomes may be attributable to the deacetylation of histones H3 and H4 during meiosis. To test this, we examined whether TSA-induced histone hyperacetylation can localize BRD4 to meiotic chromosomes. As shown in Figure 3A, E–H and L–N, treatment with TSA provoked comparable or increasing levels of histone H3/K9K14 and H4/K5 acetylation in comparison with non-treated oocytes and early embryos (Figure 2A, E–H and L–N). Consistent with this finding, immunofluorescence signals for BRD4 were comparable or more prominent in TSA-treated GV oocytes, 1-cell, 2-cell and 2-cell metaphase embryos (Figure 3O and S–U), as compared with non-treated oocytes and embryos (Figure 2O and S–U). Notably, meiotic chromosomes in oocytes treated with TSA were strongly immunoreactive with specific antibodies against AcH3/K9K14 (Figure 3B–D) and AcH4/K5 (Figure 3I–K), indicating that treatment with TSA successfully induced histone hyperacetylation in meiotic oocytes. However, in spite of the histone hyperacetylation (Figure 3B–D and I–K), BRD4 was dissociated from condensed chromosomes and dispersed into the cytoplasm during meiosis (Figure 3P–R), indicating that...
Histone acetylation is not the sole determinant of chromosomal localization of BRD4 in meiotic oocytes.

Subcellular localization of AP-2 in mitotic NIH3T3 cells and meiotic oocytes

Besides some chromosomal proteins including BRD4, at least two TFs, p67SRF (Gauthier-Rouviere et al., 1991) and AP-2 (Martinez-Balbas et al., 1995) were found to remain associated with the condensed metaphase chromosomes. The AP-2 TF family is required for multiple aspects of mouse embryo development (Eckert et al., 2005), most of whose isoforms are expressed in dynamic patterns before and after fertilization of the oocyte (Winger et al., 2006). We, therefore, examined the subcellular localization of AP-2 in mitotic NIH3T3 cells and meiotic oocytes. As shown in Figure 4A, AP-2 remained preferentially associated with mitotic chromosomes, being consistent with the previous report (Martinez-Balbas et al., 1995). In contrast, AP-2 was dispersed in the cytoplasm at MI and MII stages (Figure 4B). Thus, meiotic oocytes are able to dissociate not only BRD4 but also AP-2, a mitotic chromosome-associated TF, from meiotic condensed chromosomes.

Subcellular localization of EGFP-BRD4 during mitosis in living NIH3T3 cells

Displacement of BRD4 from meiotic chromosomes raises another possibility that there may be unknown BRD4-binding factor(s) that inhibit the association of BRD4 with meiotic chromosomes, presumably by competing out BRD4. Indeed, BRD4 is known to interact with several proteins including signal-induced proliferation-associated protein 1 (SPA-1), replication factor C (RFC-140, the largest subunit), positive transcription elongation factor in transcription b (P-TEFB) and bovine papillomavirus E2 protein (Maruyama et al., 2002; Farina et al., 2004; Jang et al., 2005; Yang et al., 2005). In support of this hypothesis, besides histone hyperacetylation by TSA, overexpression of BRD4 also leads to the efficient and tight association of BRD4 to chromatin (Dey et al., 2003). To address the spatio-temporal behaviours of overexpressed BRD4, we generated NIH3T3 stable clones in which the expression of EGFP-BRD4 was induced by DOX (Figure 5A). Time-lapse video imaging of the stable clone treated with DOX revealed that EGFP-BRD4 was entirely present on fully condensed mitotic chromosomes (Figure 6B), which is in agreement with the previous report that exogenous BRD4 is also exclusively associated with mitotic chromosomes (Dey et al., 2000, 2003).

Subcellular localization of BRD4-EGFP during meiosis in mouse oocytes

We hypothesized that overexpression of BRD4 would overcome the possible sequestration of BRD4 and thereby facilitate its localization to meiotic chromosomes, as observed in mitotic chromosomes (Figure 5). To test this, we first prepared mRNA for EGFP or BRD4-EGFP followed by the addition of a long poly(A) tail (Figure 6A) and overexpressed them by microinjection into the cytoplasm of mouse GV stage oocytes. Figure 6B shows that these poly(A) + mRNA were successfully translated in vitro. As shown in Figure 6C, EGFP alone was distributed diffusely in the nucleus and cytosol in GV stage oocytes. In contrast, BRD4-EGFP was exclusively located in the nucleus (Figure 6Da). However, BRD4-EGFP was dissociated from condensed chromosomes during meiosis, as determined by confocal microscopy (Figure 6Db–d), which was similar to EGFP alone (Figure 6C, lower two panels). Thus, either histone hyperacetylation or overexpression of BRD4 alone is insufficient to localize BRD4 to meiotic chromosomes.

Finally, to overcome the resistance of BRD4 to meiotic chromosomal localization, the GV stage oocytes overexpressing BRD4-EGFP by mRNA microinjection were co-incubated with TSA and collected at the GVBD, MI and MII stages. Confocal microscopy of those TSA-treated living oocytes revealed that BRD4-EGFP was predominantly located in the nucleus at the GV stage (Figure 6De) and that it became tightly associated with condensed chromosomes during meiosis (Figure 6Df–h). We also performed immunofluorescence using an anti-BRD4 antibody followed by incubation with a Cy3
secondary antibody. As shown in Figure 6E, EGFP fluorescence signals (green) were consistent with immunofluorescence signals for BRD4 (red), indicating that EGFP fluorescence was derived from the exogenously introduced BRD4-EGFP. Notably, meiotic condensed chromosomes preferentially displayed immunofluorescence signals for BRD4 in TSA-treated, but not untreated, oocytes overexpressing BRD4-EGFP (Figure 6E). Thus, BRD4 overexpression in combination with TSA treatment was required for meiotic chromosomal localization of BRD4.

Discussion

The biological significance of meiosis-specific histone deacetylation remains elusive. Epigenetic regulation of chromatin configuration and gene expression by histone acetylation (Jepessen, 1997; Turner, 2000; Jenuwein and Allis, 2001) supports the idea that meiosis-specific histone deacetylation may contribute to transcriptional silencing and the erasing of cell memory, which may be required to ensure genome remodelling and reprogramming for the transformation from differentiated oocytes into totipotent zygotes (Li, 2002; Kim et al., 2003; Sarmento et al., 2004). On the contrary, Rybouchkin et al. (2006) have demonstrated that meiotic deacetylation of somatic histones is not important for further development of nuclear transfer embryos, casting doubts on the assumptions that meiotic deacetylation has reprogramming significance. This may not, however, eliminate the possible biological significance of histone deacetylation in the reprogramming during mouse oocyte meiosis, because the natural reprogramming events are not equivalent to those that take place in nuclear transfer embryos. Recently, De La Fuente et al. (2004) have reported that exposure to TSA during meiotic maturation induces...
abnormal chromosome alignment in mouse MII stage oocytes. Furthermore, Akiyama et al. (2006) have demonstrated that inadequate histone deacetylation during oocyte meiosis frequently causes aneuploidy and embryo death in mice. Given the possible role of BRD4 in transmission of cell memory during mitosis (Dey et al., 2003; Loyola and Almouzni, 2004) and its displacement from meiotic chromosomes as presented here, it is possible that meiotic deacetylation may at least in part contribute to the erasing of cell memory and genome reprogramming. Elucidation of biological relevance of meiotic deacetylation awaits further studies.

We show for the first time that BRD4 is displaced from chromosomes during meiosis in mouse oocytes. The spatio-temporal behaviour of BET-type bromodomain proteins during meiotic cell division has not been reported, except for BDF1 (Chua and Roeder, 1995), a yeast homologue of BRD2 (Florence and Faller, 2001). BDF1 is known to be associated with both mitotic and meiotic chromosomes (Chua and Roeder, 1995) and to preferentially bind to acetylated histone H4 (Matangkasombut and Buratowski, 2003). In budding yeast, meiotic-specific genes are transcriptionally repressed by active deacetylation of their histones in the mitotic cell cycle (Honigberg and Purnapatre, 2003; Kassir et al., 2003). During the transition from mitosis to meiosis, meiotic signals such as glucose and nitrogen depletion recruit histone acetyltransferases, including Gcn5, to the relevant promoters, induce histone acetylation and thereby relieve transcriptional repression (Honigberg and Purnapatre, 2003; Kassir et al., 2003). Thus, it is reasonable to postulate that the association of BDF1 with meiotic chromosomes can be attributable to histone acetylation induced by Gcn5 or other histone acetyltransferases. In contrast to budding yeast, histone deacetylase 1 tightly associates with meiotic chromosomes in mouse oocytes, presumably participating in global histone deacetylation (Kim et al., 2003). The displacement of BRD4 from deacetylated meiotic chromosomes, therefore, seems to be consistent with the current paradigm that BET-type bromodomain proteins, including BRD4, associate with chromatin in a histone acetylation-dependent manner.

In somatic cells, overexpression of BRD4 and/or histone hyperacetylation enhances the association of BRD4 with chromatin as shown here and described elsewhere (Dey et al., 2003). Our microinjection experiments, however, revealed that overexpressed BRD4-Ey GFP in mouse oocytes failed to bind to chromosomes during meiosis, which further substantiates the importance of histone acetylation for the chromosomal localization of BRD4. Nevertheless, histone hyperacetylation induced by TSA did not enforce the localization of endogenous BRD4 to meiotic chromosomes. These findings collectively suggest that histone acetylation may not be the sole determinant of chromosomal localization of BRD4 and that the meiosis-specific events other than global histone deacetylation may be required for the interaction between chromosomal proteins such as BRD4 and condensed chromosomes. In a series of attempts to enforce the meiotic chromosomal localization of BRD4, we finally succeeded in the placement of BRD4 onto meiotic chromosomes by means of BRD4 overexpression together with TSA-induced histone hyperacetylation. Such considerable resistance raises a possibility that, in addition to meiosis-specific global histone deacetylation, there may be mechanism(s) by which meiotic oocytes may have factor(s) to sequester chromatin-binding factors such as BRD4 away from chromosomes during meiosis. Indeed, one or both bromodomains of BRD4 function as the binding site not only to the acetylated histones but also to several cellular proteins, including SPA-1, RFC-140, P-TEFb and bovine papillomavirus E2 protein (Maruyama et al., 2002; Farina et al., 2004; You et al., 2004; Jang et al., 2005; Yang et al., 2005). It is, therefore, conceivable that unidentified factor(s) that can bind to BRD4 through its bromodomain(s) during meiosis may compete with the acetylated histones, entrap BRD4 and thereby inhibit the chromosomal interaction of BRD4. In this context, we postulate that overexpression of BRD4 enough to compete out the BRD4-binding factor(s) together with sufficient levels of histone hyperacetylation may allow BRD4 to localize to the meiotic chromosomes. Alternatively, it is conceivable that a possible post-translational modification of BRD4 such as (de)phosphorylation may contribute to the transient displacement during meiosis.

In summary, we demonstrate that histones H3 and H4 become deacetylated concomitantly with the displacement of mitotic chromosomal protein BRD4 from chromosomes during mouse oocyte meiosis. Although BRD4 binds to mitotic chromosomes in dose-dependent and histone acetylation-dependent manner in somatic cells, neither overexpression nor histone hyperacetylation could localize BRD4 to the meiotic chromosomes. Both are required for the meiotic chromosomal localization of BRD4. These results indicate that, unlike mitosis, histone acetylation is necessary but not sufficient for chromosomal localization of BRD4 during meiosis, suggesting that meiotic oocytes may have additional mechanism(s) for displacement of mitotic chromosomal proteins from condensed chromosomes.

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