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ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes

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Abstract

ATRX is a centromeric heterochromatin binding protein belonging to the SNF2 family of helicase/ATPases with chromatin remodeling activity. Mutations in the human ATRX gene result in X-linked α-thalassaemia with mental retardation (ATRX) syndrome and correlate with changes in methylation of repetitive DNA sequences. We show here that ATRX also functions to regulate key stages of meiosis in mouse oocytes. At the germinal vesicle (GV) stage, ATRX was found associated with the perinucleolar heterochromatin rim in transcriptionally quiescent oocytes. Phosphorylation of ATRX during meiotic maturation is dependent upon calcium calmodulin kinase (CamKII) activity. Meiotic resumption also coincides with deacetylation of histone H4 at lysine 5 (H4K5 Ac) while ATRX and histone H3 methylated on lysine 9 (H3K9) remained bound to the centromeres and interstitial regions of condensing chromosomes, respectively. Inhibition of histone deacetylases (HDACs) with trichostatin A (TSA) disrupted ATRX binding to the centromeres of hyperacetylated chromosomes resulting in abnormal chromosome alignments at metaphase II (MII). Similarly, while selective ablation of ATRX by antibody microinjection and RNA interference (RNAi) had no effect on the progression of meiosis, it had severe consequences for the alignment of chromosomes on the metaphase II spindle. These results suggest that genome-wide epigenetic modifications such as global histone deacetylation are essential for the binding of ATRX to centromeric heterochromatin. Moreover, centromeric ATRX is required for correct chromosome alignment and organization of a bipolar meiotic metaphase II spindle.

Keywords: Oogenesis; Chromatin remodeling; Histone deacetylation; Heterochromatin; Centromeres; Chromosome segregation; RNAi; Helicases

Introduction

The completion of meiosis in higher eukaryotes requires the segregation of homologous chromosomes during the first meiotic division, followed by subsequent segregation of sister chromatids to opposing poles of the meiotic spindle upon resumption of metaphase II. Although the mechanisms involved in the establishment of a haploid chromosome complement in mature gametes are not fully understood, accurate reduction in chromosome number depends on the specialized and distinct functions of centromeres at the metaphase I (MI) and metaphase II (MII) stages of meiosis (Nasmyth, 2002; Petronczki et al., 2003). For example, separation of homologous chromosomes at anaphase I occurs while sister chromatid cohesion is maintained at centromeric heterochromatin regions on the chromosomes (Bernard and Allshire, 2002; Eijpe et al., 2003; Jibak Lee et al., 2003; Petronczki et al., 2003). Centromeres are specialized heterochromatin domains with unique properties to bind large protein complexes that mediate attachment of chromosomes to microtubules at the meiotic spindle (Pluta et al., 1995). Importantly, alterations in chromosome segregation during meiosis constitute a major cause of aneuploidy in mammalian oocytes (Hassold and Hunt, 2001).

Centromeric heterochromatin regions in the eukaryotic genome have been long recognized cytologically as highly condensed, transcriptionally repressive domains present during the interphase stage of the cell cycle (Dillon and Festenstein, 2002). However, the molecular composition and

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mechanisms of heterochromatin formation are only beginning to be unraveled. Current evidence indicates that in addition to repetitive centromeric DNA sequences, a higher order chromatin structure provided by regularly spaced nucleosomes (the basic unit of chromatin formation) is essential for the binding of large protein complexes to heterochromatin regions in the nucleus (Dillon and Festenstein, 2002; Jenuwein and Allis, 2001; Moazed, 2001). Moreover, recent advances in our understanding of the role of histone acetylation and histone methylation in modulating higher order chromatin structure have contributed significantly to the initial dissection of the molecular components and mechanisms of heterochromatin formation in model organisms such as fission yeast and *Drosophila melanogaster* (Jenuwein and Allis, 2001; Moazed, 2001). Studies have since revealed some evolutionary conservation in mammals of the basic mechanisms of chromatin modifications in which binding of different histone proteins to the chromatin template, as well as their posttranslational modifications (i.e., histone acetylation and histone methylation), contributes to the establishment of critical epigenetic marks. For instance, methylation of histone H3 at lysine 9 (H3K9 methylation) provides a binding site for heterochromatin binding protein 1 (HP1) in human and mouse fibroblasts in an essential step for the nucleation of heterochromatin formation and proper chromosome segregation (Bannister et al., 2001; Lachner et al., 2001). Importantly, heterochromatin formation also occurs outside of centromeric regions (Dillon and Festenstein, 2002), and both centromeric and pericentromeric heterochromatin formation is required for the modulation of homologous chromosome interactions during male meiosis in mice (Peters et al., 2001) and for proper chromosome segregation in yeast (Bernard and Allshire, 2002; Bernard et al., 2001).

In spite of its essential role for the progression of meiosis, the mechanisms involved in centromeric and pericentromeric heterochromatin formation in mouse oocytes are unknown. More than 20 proteins have been shown to bind centromeric heterochromatin (Saffery et al., 2000) in human somatic cells; however, amongst these, one of the least understood groups is that of the chromatin remodeling complexes. ATRX is a member of the SWI/SNF family of helicases or ATPases with chromatin remodeling activity (Gibbons et al., 1997; Picketts et al., 1998). ATRX has been mapped to the long arm of the human X chromosome (Xq13.3) and contains an open reading frame encoding a 280 kDa protein with a plant homeodomain (PHD) region at the amino terminus responsible for interactions with HP1 and a helicase domain at the carboxyl terminal region (Picketts et al., 1998). Spontaneous mutations in the PHD region in humans induce α-thalassaemia, seizures, and in extreme cases, gonadal dysgenesis (ATRX syndrome) (Gibbons et al., 1997). Moreover, ATRX binds pericentric heterochromatin regions in mouse somatic cell lines and to the short arms of human acrocentric chromosomes (McDowell et al., 1999). The specific cellular function of ATRX is not known at present; however, ATRX associates with HP1α during mitosis (Bérubé et al., 2000) and with the SET domain of chromatin modifying proteins (Cardoso et al., 1998).

The patterns of ATRX expression and nuclear compartmentalization in mammalian oocytes are not known. Importantly, whether ATRX has a function in germ cell nuclear architecture and the progression of meiosis remains undetermined. This study was undertaken (I) to determine the mechanisms involved in regulating the binding of ATRX to centromeric heterochromatin in mammalian oocytes and (II) to test the hypothesis that the ATRX protein plays a role during female meiosis. Our results indicate that genome-wide epigenetic modifications such as global histone deacetylation at the onset of meiosis are essential for the binding of ATRX to centromeric heterochromatin in mouse chromosomes. Moreover, centromeric ATRX is required for the maintenance of a bipolar metaphase II spindle and for the establishment of proper chromosome alignment during meiosis.

Materials and methods

Oocyte collection and culture

Oocyte–cumulus cell complexes were obtained from 22-day old (C57BL/6J × SJL/J) F_1 mice that had been injected 48 h earlier with 5 IU pregnant mare serum gonadotropin (PMSG; National Hormone and Peptide Program, NIDDK). Oocytes were collected in 2.5 ml of TCM-199 medium (GIBCO, Life Technologies, Grand Island, NY) supplemented with 10 μl of Milrinone (Sigma Co., St Louis, MO) to prevent germinal vesicle (GV) breakdown. Cumulus cells were removed by repeated pipetting. Denuded oocytes were cultured in fresh TCM-199 supplemented with 10 μM Milrinone (Sigma) at 37°C under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 before their allocation to experimental groups. Trichostatin A (TSA; Biomol Research Lab. Inc., Plymouth Meeting, PA), a potent and specific inhibitor of histone deacetylases (HDACs) (Yoshida et al., 1995), was used at a concentration of 100 nM. KN-93 (Biomol), a specific inhibitor of calcium calmodulin kinase II (CamKII), was used at a concentration of 10 μM.

Western blot analysis

Groups of 300 denuded oocytes were washed in phosphate-buffered saline (PBS) supplemented with protease inhibitor cocktail (1 mM DTT, 1 mM pefabloc, 10 μg/ml leupeptin, aprotinin, and pepstatin; Sigma) and frozen at −70°C in 10 μl of Laemmli buffer (Laemmli, 1970). In order to remove phosphate groups, 1 IU protein phosphatase (PPA; Calbiochem, La Jolla, CA) was added to samples in phosphatase buffer (50 mM Tris–HCl, 5 mM DTT, 20 mM $MgCl_2$, and 100 μg/ml BSA). Samples of metaphase I or II oocytes were incubated at 30°C for 30 min. This reaction was subsequently terminated by the addition of an equal

volume of 2× Laemmli buffer followed by storage at −80 °C. Samples were thawed on ice then heated to 100 °C for 5 min. Proteins were separated by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS and transferred onto a hydrophobic polyvinylidene difluoride (PVDF) membrane (Amersham, Piscataway, NJ) for 1 h at 100 V. The membrane was blocked in PBT buffer (2% BSA in PBS with 0.2% Tween 20; PBT) for 1 h at room temperature, then incubated overnight with a goat anti-ATRX antibody (1:1000; Santa Cruz Biotechnology) at 4 °C. After several washes in PBT, the membrane was exposed to a horseradish peroxidase-conjugated rabbit anti-goat secondary antibody (Jackson Immuno research, West Chester, PA) for 1 h at room temperature. ATRX protein was visualized by a chemiluminescence reaction using the ECL+Plus system (Amersham).

Immunochemistry

To determine the subcellular localization of ATRX protein, oocytes at the GV stage were fixed with 2% paraformaldehyde for 15 min at room temperature then permeated with PBS supplemented with 0.2% Triton X for 10 min. Oocytes were washed and stored at 4 °C in PBS + 10% bovine serum. To detect ATRX after germinal vesicle breakdown, oocytes with condensing chromosomes were exposed to a hypotonic treatment of 1% sodium citrate for 12 min. Protein cross-linking was performed by incubation with 2% paraformaldehyde as described above. Oocytes were exposed to a 1:5 dilution of the 23c ATRX antibody, kindly provided by D. Higgs (McDowell et al., 1999), for 1 h at room temperature; after several washes in PBT buffer, oocytes were incubated for 1 h in a 1:1000 dilution of a Cy3-conjugated sheep anti-mouse IgG (Jackson Immuno research). Maturing oocytes were simultaneously stained with antibodies directed against either histone H4 acetylated on lysine 5 (H4K5 Ac; 1:400), histone H3 methylated on lysine 9 (H3K9 Meth; Upstate Biotechnology; 1:400), or histone H3 trimethylated on lysine 9 ($H3K9_{Me3}$ 1:5000; a generous gift from K. Muegge) and detected with a 1:1000 dilution of an Alexa-Fluor 488 anti-rabbit secondary antibody (Molecular Probes, Inc., Eugene, OR). Centromeric proteins Cenp-A, B, and C were detected with a 1:10 000 dilution of the CREST antibody (kindly provided by W. Earnshaw) followed by a 1:150 dilution of an Alexa-Fluor 488 goat anti-human antibody (Molecular Probes). DNA was counterstained with Hoechst 33258 (Sigma) and oocytes were mounted on Poly-Prep™ slides (Sigma). For analysis of the meiotic spindle, oocytes were fixed overnight in 4% paraformaldehyde then incubated with 3.8 μg/ml of a mouse monoclonal anti-β-tubulin (IgG; Sigma) antibody for 1 h and detected with 1.3 μg/ml of an FITC-conjugated anti-mouse IgG (Jackson Immuno research). Chromosomes were counterstained with 10 μg/ml propidium iodide (Sigma). Laser scanning confocal microscopy was performed on a Leica TCS-NT confocal microscope equipped with an air-cooled argon ion laser (568 nm excitation) system (Leica Microsystems, Exton, PA). Epifluorescence analysis was conducted using an ultraviolet light (340–380 nm excitation) and a rhodamine (515–560 nm) filter on a DMRX/E microscope (Leica Microsystems).

Antibody microinjection

Denuded oocytes were cultured under oil in 50 μl of TCM-199 medium supplemented with 10 μM Milrinone (Sigma) to maintain arrest at the GV stage. A mixture of the anti-ATRX antibodies 23c, 39f (McDowell et al., 1999), and a polyclonal antibody directed against the C-terminus of ATRX (Santa Cruz Biotech.) was microinjected into the cytoplasm of denuded oocytes. The polyclonal anti-ATRX antibody was injected at a final concentration of 0.2 μg/μl. The 23c and 39f antibodies were provided as tissue culture supernatants and co-injected as an equal volume (1:1:1) mixture or after a 1:2 dilution in TCM-199 medium. Control oocytes were microinjected with 1 μg/μl IgG1 (Sigma). Microinjected oocytes were then cultured in 500 μl of TCM-199 medium supplemented with 10 μM Milrinone for 24 h. Oocytes were released from prophase arrest after extensive rinsing in TCM-199 medium and allowed to resume meiosis following transfer to fresh TCM-199 medium for a period of 14 h. For the analysis of meiotic configurations, microinjected oocytes were fixed overnight in 4% paraformaldehyde before immunostaining of the meiotic spindle.

RNA interference after microinjection of double stranded or short interfering RNA

To determine the function of ATRX during meiosis, translation of the ATRX protein in mouse oocytes was specifically inhibited using an RNA interference (RNAi) approach. Double stranded (DS) RNAs were generated by modifications of previously described methods (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 1999). Sense and antisense sequences were transcribed in vitro from PCR products with the use of an in vitro ligated T7 RNA polymerase (Ambion Inc., Austin, TX). Primers were designed from a region of the plant homeodomain (PHD) of the mouse ATRX sequence and used as a template to generate double stranded RNAs. Equimolar quantities of sense and antisense RNA were annealed in vitro and exposed to RNAse A (Ambion) to remove single stranded RNA then subjected to phenol–chloroform extraction. Formation of double stranded RNA was confirmed by gel electrophoresis. Short interfering (esi) RNAs were generated by enzymatic cleavage of DS RNA with RNA polymerase III (Yang et al., 2002). Double stranded RNAs and esi RNAs were also generated from a completely unrelated sequence (Cyclophilin) to be used as the negative control.

Preovulatory oocytes were maintained at the GV stage by exposure to the phosphodiesterase inhibitor Milrinone (10 μM) and microinjected in the cytoplasm with a solution containing 0.2 μg/μl of a double stranded (DS) RNA or a

short interfering (esi) RNA against ATRX. Oocytes were released from prophase arrest 24 h after microinjection and allowed to undergo meiotic maturation in vitro for 14 h.

To confirm that microinjection of (DS) RNA specifically ablated ATRX protein without inducing nonspecific effects on the expression of other chromatin binding proteins, microinjected oocytes were simultaneously labeled with a mouse monoclonal antibody against ATRX and with a polyclonal antibody against histone H3 methylated at lysine 9 (H3K9). Control mock-injected oocytes were microinjected with culture medium alone (no RNAi). Furthermore, a nonrelated DS RNA generated against Cyclophilin (0.2μg/μl) was injected into a different group of oocytes to further validate the specificity of the RNAi approach.

Statistical analysis

Data are presented as the mean percentage of at least three independent experiments; variation among replicates is presented as the standard error of the mean (SEM). The percentage of oocytes exhibiting acetylated histones (H4K5 Ac), methylated histones (H3K9 meth), or ATRX staining, as well as the proportion of oocytes undergoing meiotic maturation after antibody microinjection or RNAi, were analyzed using arcsine transformed data and compared by analysis of variance (ANOVA) using StatView (StatView: SAS Institute Inc., Cary, NC). When a significant *F*-ratio was defined by ANOVA, groups were compared using the Fisher's PLSD post-hoc test. Differences were considered significant when ($P < 0.05$).

Results

ATRX *is localized at heterochromatin domains in the mammalian oocyte genome*

ATRX binds centromeric heterochromatin regions in human and mouse somatic cells (McDowell et al., 1999).

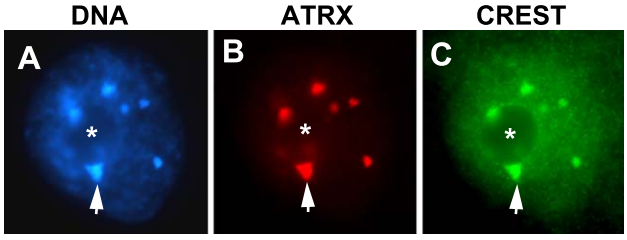

Fig. 1. Subnuclear localization of ATRX to heterochromatin domains in mouse oocytes. Fluorescent micrograph of mouse oocytes after staining with the DNA-specific fluorochrome Hoechst 33258 (blue; A). Immunochemical localization of the ATRX protein (red) revealed that ATRX preferentially localizes with sites of bright Hoechst fluorescence (B; arrow). Simultaneous labeling of centromeric proteins with the CREST antibody (green) confirmed that ATRX binds to centromeric heterochromatin domains in the oocyte genome (C; arrow). The position of the nucleolus is indicated by (*).

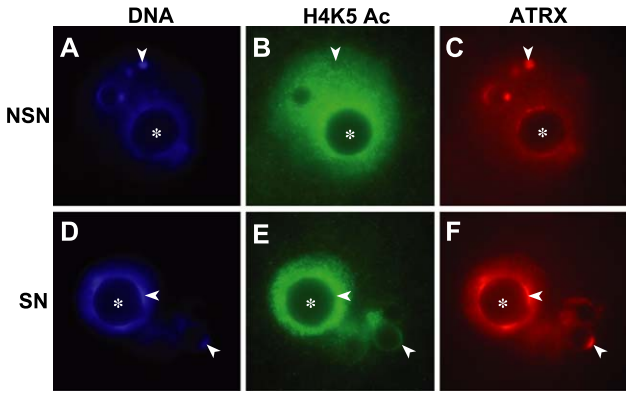

Fig. 2. Association of ATRX with nucleolar heterochromatin and persistence of histone H4 acetylation in the germinal vesicle (GV) of preovulatory oocytes. Epifluorescence microscopy of representative GV stage mouse oocytes presenting the non-surrounded nucleolar (NSN) configuration (A–C) or the surrounded nucleolar (SN) configuration (D–F). Oocytes were simultaneously stained with Hoechst 33258 (blue; A, D), with an antibody that specifically recognizes histone H4 acetylated at lysine 5 (H4K5 Ac; green, B, E) and with a mouse monoclonal antibody against ATRX (red; C, F). Heterochromatin regions in the oocyte genome showing bright Hoechst fluorescence and ATRX staining are indicated (arrowheads). The position of the nucleolus is indicated by (*).

However, whether ATRX associates with heterochromatin domains in mammalian oocytes is not known. Experiments were thus conducted to determine the subnuclear localization of ATRX in fully grown mouse oocytes. Immunocytochemical studies revealed that the ATRX protein (red) is preferentially associated with sites of bright Hoechst fluorescence (blue) in the oocyte genome (Figs. 1A, B; arrows). Importantly, simultaneous immunostaining of the centromeric proteins Cenp-A, B, and C (green) with the CREST antibody (Pluta et al., 1995) confirmed that ATRX binds centromeric heterochromatin domains in the germinal vesicle (Figs. 1B, C; arrows), although some faint ATRX staining could still be detected in the nucleoplasm. These results indicate that ATRX is a component of centromeric heterochromatin domains in the germinal vesicle.

The mouse oocyte genome undergoes dynamic modifications in chromatin structure. For example, growing oocytes exhibit a decondensed chromatin configuration termed non-surrounded nucleolus (NSN). In contrast, chromatin is found condensed in close apposition with the nucleolus in a configuration termed surrounded nucleolus (SN) in transcriptionally quiescent, preovulatory oocytes (BouniolBaly et al., 1999; De La Fuente and Eppig, 2001; Debey et al., 1993; Mattson and Albertini, 1990; Wickramasinghe and Albertini, 1992). Importantly, in somatic cells, histone acetylation is associated with the maintenance of a decondensed, transcriptionally permissive chromatin structure (Grunstein, 1997; Turner, 2000). However, whether changes in histone acetylation occur during the transition into the SN configuration in mouse oocytes is not clear. Acetylation of histone H4 at lysine

5 (H4K5 Ac) is a reliable marker of the fully acetylated status of histone H4 (Kruhlak et al., 2001); thus, experiments were conducted to determine whether changes in H4K5 Ac staining occur in transcriptionally repressed oocytes showing the SN configuration. In oocytes presenting the NSN configuration, heterochromatin regions exhibit a characteristic bright staining pattern after exposure to the DNA fluorochrome Hoechst 33258 (Blue; Fig. 2A, arrowhead). Immunochemical detection of histone H4 acetylated at lysine 5 (H4K5 Ac) revealed high levels of histone acetylation (green) in the nucleoplasm of NSN oocytes except for heterochromatin regions, in which the H4K5 Ac signal was considerably reduced (Fig. 2B, arrowhead). This antibody exclusively detects histone H4 acetylated on lysine 5 and shows no cross-reactivity with deacetylated histone H4 (data not shown). In contrast, ATRX was found predominantly associated with heterochromatin regions corresponding to bright Hoechst staining foci (Fig. 2C, arrowhead), although faint nucleoplasmic staining could also be detected. In oocytes presenting the SN configuration, chromatin is condensed and distributed around the nucleolus (Fig. 2D) including the small nucleoli observed in a small percentage of oocytes (lower arrowhead). In spite of this chromatin condensation, histone H4 remained fully acetylated as indicated by the presence of H4K5 Ac staining in oocytes with the SN configuration (Fig. 2E). Moreover, ATRX was found in close apposition with the perinucleolar region in oocytes with the SN configuration (Fig. 2F, arrowheads).

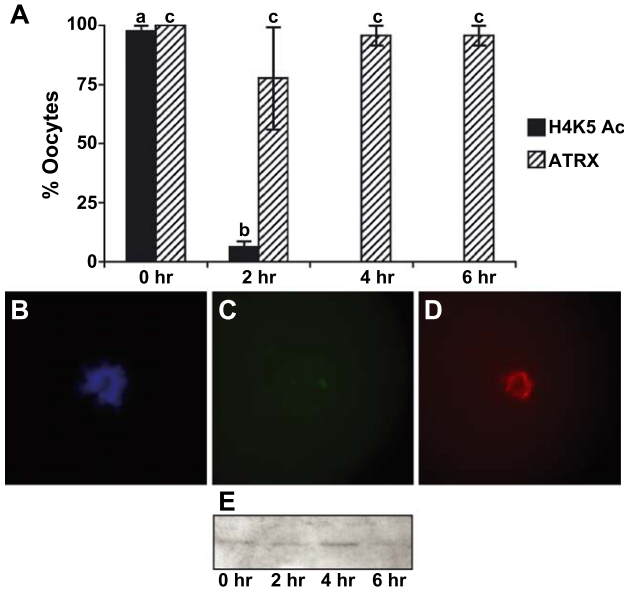

Fig. 3. Chromosomal localization of ATRX and global histone deacetylation associated with meiotic resumption. The patterns of acetylated histone H4 (H4K5 Ac) and ATRX localization were determined in preovulatory oocytes at the GV stage (0 h) or after meiotic maturation at times indicated (A). The proportion of oocytes presenting H4K5 Ac staining is dramatically reduced ($P < 0.0001$) upon resumption of meiosis (2 h). Chromosomes were stained with Hoechst 33258 (B). Histone H4K5 Ac is no longer associated with condensing chromosomes at later stages of meiosis and is only detected as diffuse cytoplasmic staining (green; C). In contrast, ATRX remains associated with chromosomes throughout meiosis (red; D). No changes were detected in the electrophoretic mobility of ATRX protein during the first 6 h of meiotic maturation (E). Data are presented as the mean ± SEM of four independent experiments.

Chromosomal ATRX *localization and global histone deacetylation upon meiotic resumption*

The patterns of histone H4 acetylation (H4K5 Ac) and ATRX staining were then analyzed simultaneously in groups of oocytes up to 6 h after the onset of meiosis in vitro. The proportions of oocytes presenting H4K5 Ac and/or ATRX staining at different time points evaluated are illustrated in Fig. 3A; the values represent results of three independent experimental replicates. Consistent with our previous experiment, the majority of preovulatory oocytes (97.5%) at the GV stage (0 h; $n = 165$) present a high level of acetylated histone H4 (H4K5 Ac) in the nucleoplasm as well as ATRX staining (100%) in association with heterochromatin domains (Fig. 3A). Interestingly, coincident with the resumption of meiosis (2 h; $n = 147$), a wave of global histone deacetylation takes place in the oocyte genome, and H4K5 Ac staining is no longer detectable on condensing chromosomes. Instead, only diffuse staining is observed in the cytoplasm of maturing oocytes (Figs. 3B, C). This was reflected in a significant ($P < 0.0001$) decrease in the proportion of oocytes (6.2%) presenting H4K5 Ac staining associated with chromosomes (Fig. 3A). In contrast, ATRX remained associated with chromosomes in a punctuate pattern resembling staining of centromeric heterochromatin (Fig. 3D). This pattern was consistently observed at 2 h (78%), 4 h (95.7%; $n = 131$), and 6 h (95.4%; $n = 158$) after meiotic resumption. Western blot analysis of the ATRX protein over a similar period of meiotic maturation revealed a single band of the expected molecular weight (280 kDa) in oocytes at the GV stage (0 h) and after the onset of meiosis (2–6 h). No differences were observed in the relative levels or the electrophoretic mobility of ATRX protein (Fig. 3E) or in the levels of H4K5 Ac at these time points (data not shown). These results suggest that global histone deacetylation occurs in the chromosomes of mouse oocytes upon meiotic resumption. In addition, ATRX remains associated with focal sites on condensing chromosomes even at a time when major chromatin modifications are taking place during meiosis.

In order to determine whether ATRX binds to centromeric heterochromatin throughout meiosis, oocytes at the germinal vesicle stage and at metaphase I (10 h after the onset of meiosis) were immunostained with a monoclonal antibody against ATRX as described above; in this experiment, however, oocytes were simultaneously labeled with a monoclonal antibody against histone H3 methylated at lysine 9 (H3K9). Consistent with our previous

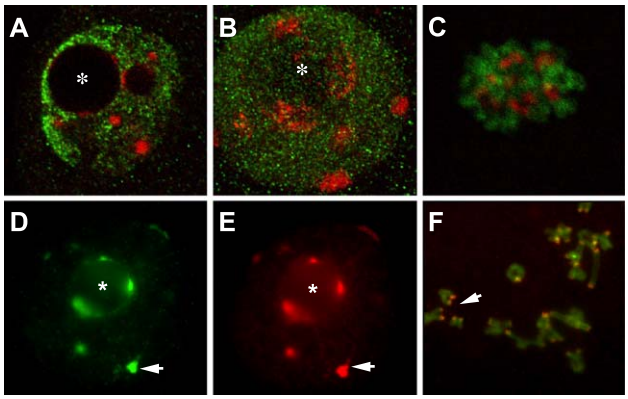

Fig. 4. ATRX binds to centromeric heterochromatin during meiosis. Laser scanning confocal microscopy of preovulatory oocytes at the GV stage (A, B) and metaphase I of meiosis (C). Single scan of a GV stage oocyte presenting diffuse nucleoplasmic staining of histone H3 methylated at lysine 9 (H3K9) as detected with a linear antibody shown in green, localization of ATRX protein is shown in red (A). Serial reconstruction on the *Z* axis for the same oocyte (B). H3K9 methylation persists on the interstitial regions of condensing chromosomes throughout meiosis, whereas ATRX is localized exclusively with centromeric heterochromatin regions (C). Immunostaining with the trimethylated $H3K9_{Me3}$ antibody (green) specifically detects heterochromatin domains in the GV (D; arrow). ATRX (red) is preferentially co-localized with centromeric heterochromatin (E; arrow) and exclusively co-localized with the centromeres of condensed chromosomes (F; arrow), whereas $H3K9_{Me3}$ staining (green) spreads throughout condensed chromosomes. The nucleolus is indicated by (*).

results, laser scanning confocal microscopy revealed ATRX staining at heterochromatin regions in oocytes at the GV stage (Figs. 4A, B; shown in red). Histone H3K9 methylation (Figs. 4A, B; shown in green) was detected in a diffuse pattern throughout the nucleoplasm of GV stage oocytes including those regions stained with ATRX. However, in contrast with the histone deacetylation event observed in chromosomes upon meiotic resumption, histone H3K9 methylation was consistently detected at interstitial regions of condensed chromosomes throughout meiosis (Fig. 4C; green). Interestingly, ATRX staining was consistently found exclusively associated with centromeric heterochromatin regions in meiotic chromosomes (Fig. 4C; red).

Use of a trimethylated antibody against $H3K9_{Me3}$ that exclusively stains centromeric heterochromatin (Peters et al., 2001) revealed several centromeric heterochromatin domains in the germinal vesicle (Fig. 4D; green) and confirmed that ATRX (red) preferentially co-localized with centromeric heterochromatin domains (Fig. 4E; arrow). Interestingly, upon chromosome condensation, $H3K9_{Me3}$ methylation is redistributed throughout the entire length of the chromosomes, whereas ATRX was observed exclusively localized to the centromeres of meiotic chromosomes (Fig. 4F; arrow). ATRX was also found to co-localize with centromeric proteins in condensed chromosomes after immunostaining with the CREST antibody (data not shown).

Calcium calmodulin kinase II-dependent phosphorylation of ATRX *in MI and MII stage oocytes*

Western blot analysis with the ATRX antibody against the C-terminus of the protein revealed a single band of 280 kDa in oocytes at the GV stage. However, analysis of maturing oocytes revealed a slower migrating band that consistently appeared as a doublet in oocytes at the metaphase I (MI; 10 h) and metaphase II (MII; 14 h) stages (Fig. 5A). To determine whether the slow migrating forms result from a phosphorylation event, taking place during meiosis, protein extracts obtained from MII stage oocytes were treated with a protein phosphatase (PPA) to remove phosphate groups that might be present. Similar results were obtained for three independent experimental replicates, and a representative example is shown in Fig. 5A. Treatment of oocyte extracts with PPA increased the mobility of ATRX protein detected in MII stage oocytes, demonstrating that the slow migrating doublet is due to phosphorylation of the ATRX protein at metaphase II (Fig. 5A; MII PPA). However, a doublet rather than single band was consistently detected even after PPA treatment. Similar results were obtained with oocytes at the MI stage (data not shown). Previous studies of *Drosophila* chromosomes indicate that phosphorylation of the heterochromatin binding protein (HP1)

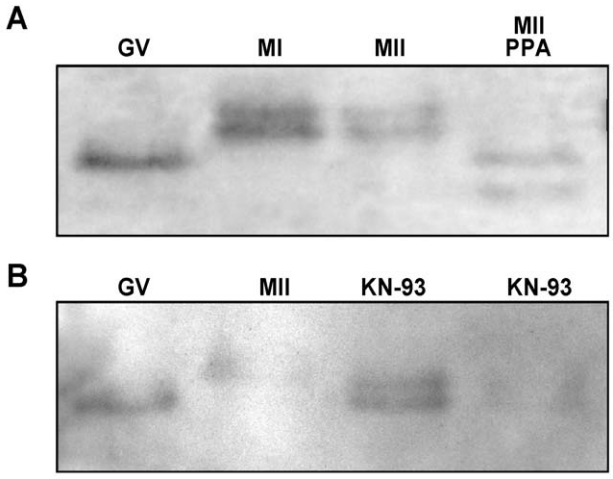

Fig. 5. Phosphorylation of ATRX protein during meiosis is mediated by the calcium calmodulin kinase (CamKII) pathway. Western blot analysis of ATRX protein in oocytes at the germinal vesicle (GV) stage, at metaphase I (MI) 10 h after in vitro maturation, or at the metaphase II (MII) stage after 14 h of meiotic maturation. ATRX is detected as a single band of 280 kDa at the GV stage; however, a doublet with slower mobility is detected in oocytes at MI and MII stage (A). Treatment of oocyte protein extracts with protein phosphatase (MII PPA) increased the mobility of ATRX to that observed at the GV stage (A). Treatment of oocytes with the calcium calmodulin kinase II (CamKII) inhibitor KN-93 (10 μM) during the progression of meiosis prevented phosphorylation of ATRX protein in oocytes at the MI (Lane 3) and MII (Lane 4) stage (B). This experiment was repeated three times with identical results and a representative example is shown.

is under control of the casein kinase pathway (Zhao and Eissenberg, 1999). However, treatment of maturing oocytes with the casein kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) had no effect on the phosphorylation of ATRX during meiosis (data not shown). To determine whether ATRX phosphorylation is under the control of an alternative signaling pathway, oocytes were treated with KN-93, a specific inhibitor of the calcium calmodulin kinase II (CamKII) pathway. Treatment of mouse oocytes with KN-93 interferes with meiotic resumption (Su and Eppig, 2002); therefore, in the present study, denuded oocytes were cultured for 2 h and allowed to undergo germinal vesicle breakdown before exposure to 10 μM KN-93 for 10 h. No changes in the electrophoretic mobility of ATRX occur immediately after germinal vesicle breakdown (Fig. 3E), thus ensuring that oocytes were exposed to KN-93 after germinal vesicle breakdown but before ATRX phosphorylation. Consistent with previous experiments, control, nontreated oocytes at the GV stage or the metaphase (MII) stage yielded a single fast migrating band or a slow migrating doublet form of ATRX, respectively (Fig. 5B). However, exposure of maturing oocytes to the calcium calmodulin kinase II (CamKII) inhibitor KN-93 prevented phosphorylation of ATRX at both MI and MII stages (Fig. 5B).

Global histone deacetylation at the onset of meiosis is required for continuous binding of ATRX *to centromeric heterochromatin*

To determine whether the wave of histone deacetylation observed upon meiotic resumption is of functional significance for the association of ATRX with centromeric domains, oocytes were allowed to undergo meiotic maturation in the presence or absence of trichostatin A (TSA), a potent inhibitor of histone deacetylases (HDACs). Denuded oocytes at the GV stage (n = 126) were treated with TSA (100 nM) for 8 h (TSA 8 h), rinsed in fresh media, and then allowed to undergo meiotic maturation in vitro for 14 h. Oocytes were also exposed to TSA for 8 h at the GV stage and during meiotic maturation (TSA 8 h + M; n = 184) or during the maturation period exclusively (TSA M; n = 169). Control oocytes (n = 147) were cultured in the presence of 0.01% dimethyl sulfoxide (DMSO), which was used as a vehicle to dissolve TSA. The proportions of maturing oocytes presenting acetylated histone H4 (H4K5 Ac) and ATRX staining at centromeric heterochromatin in three experimental replicates are illustrated in Fig. 6A. Consistent with our previous experiments, histone H4 acetylation (H4K5 Ac) was absent from the chromosomes of control MII oocytes, and only cytoplasmic staining was observed (Fig. 6C; green). At this stage, ATRX was consistently found associated with centromeric heterochromatin foci in 99.3% of the oocytes (Fig. 6D; red). Faint chromosomal H4K5 Ac was detected in only 23% of

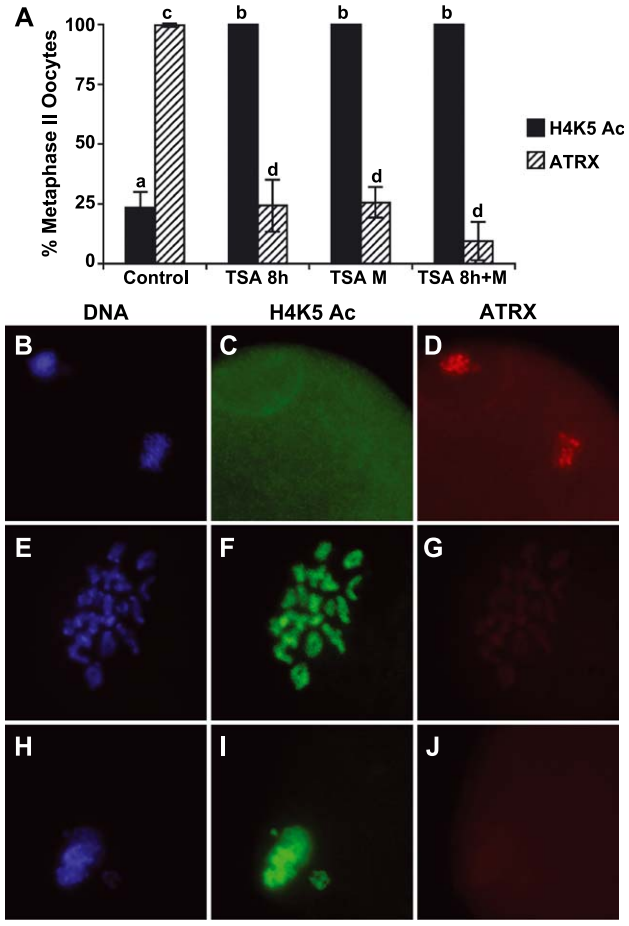

Fig. 6. Inhibition of histone deacetylation at the onset of meiosis disrupts the binding of ATRX to centromeric heterochromatin (A). Proportion of metaphase II oocytes showing histone H4 lysine 5 acetylation (H4K5 Ac; green) and localization of ATRX to centromeric heterochromatin (red) under different experimental conditions. Control metaphase II oocytes show deacetylated chromosomes (B, C) and binding of ATRX to centromeric heterochromatin (D). Inhibition of histone deacetylases with trichostatin A (TSA) induced chromosome hyperacetylation (E–F) and significantly reduced ($P < 0.0001$) the binding of ATRX to centromeric domains (G). Metaphase II stage oocytes with hyperacetylated chromosomes present abnormal chromosome alignments to the meiotic spindle (H–J). Oocytes were exposed to TSA (100 nM) at the GV stage (TSA 8 h), during meiotic maturation (TSA M), or at the GV stage and during meiotic progression (TSA 8 h + M). Data are presented as the mean ± SEM of four independent experiments.

control oocytes at the MII stage (Fig. 6A). Importantly, inhibition of HDACs with TSA during meiotic progression induced a state of hyperacetylation in condensed MI stage chromosomes as determined by intense H4K5 Ac staining (Figs. 6E, F; green) in 100% of oocytes ($P < 0.0001$). Moreover, ATRX was no longer found associated with centromeric heterochromatin domains in meiotic chromosomes. ATRX was found associated with centromeric heterochromatin in only 24% of oocytes treated at the GV stage (TSA 8 h), in 25% of oocytes treated during meiotic maturation exclusively (TSA M), and in 9.3% of oocytes treated with TSA both at the GV stage and during

meiotic progression (TSA 8 h + M), a significant decrease ($P < 0.0001$) compared with that in control oocytes (Fig. 6G). Similar effects were observed in the majority of MII stage oocytes, in which some hyperacetylated chromosomes were consistently found completely separated from the metaphase plate (Figs. 6H, I) and with no ATRX staining at centromeric heterochromatin (Fig. 6J). These results suggest that epigenetic modifications, such as global histone deacetylation, during meiosis are essential to maintain the association of heterochromatin binding proteins such as ATRX with centromeric domains in the chromosome.

Microinjection of ATRX *antibodies disrupts chromosome alignment at MII*

To gain some insight into the possible function of ATRX during meiotic progression in mouse oocytes, a mixture of antibodies directed against different regions of the ATRX protein were microinjected into the cytoplasm of GV stage oocytes. Groups of oocytes were microinjected with a 1:1:1 ($n = 119$) or 1:2; 1:2; 1:2 ($n = 78$) mixture of the 23c, 39f, and the anti-ATRX, C-terminus domain antibodies or with an equivalent volume of a nonimmune IgG1 ($n = 98$) as a negative control. Furthermore, an additional group of uninjected control oocytes ($n = 96$) was also included. Following microinjection, oocytes were maintained arrested at the GV stage for 24 h and subsequently allowed to resume meiosis. The proportion of oocytes that reached different stages of meiosis in four independent experimental replicates is illustrated in Fig. 7A. No differences were observed in the proportion of oocytes that reached the MII stage (84–92%) after microinjection with ATRX antibodies or IgG (92.1%) compared to uninjected control oocytes (93.4%). However, analysis of meiotic spindle configurations at the MII stage revealed a significant increase ($P < 0.0001$) in the proportion of oocytes with abnormal chromosome alignments on the meiotic spindle after injection of a (1:2; 42.1%) or a (1:1; 47.2%) mixture of ATRX antibodies compared with control IgG (10.5%) or noninjected oocytes (4.2%) (Fig. 7B). Control and IgG injected oocytes presented a typical barrel-shaped bipolar MII spindle with chromosomes tightly aligned at the equatorial region (Fig. 7C). In contrast, microinjection of ATRX antibodies resulted in abnormal meiotic configurations in which the MII spindle was no longer bipolar and appeared as a rosette-like configuration with chromosomes loosely attached at peripheral regions of the microtubular network (Fig. 7D). Furthermore, additional configurations were commonly observed in which chromosomes were found loosely attached to a disorganized meiotic spindle (Fig. 7E). These results indicate that microinjection of ATRX antibodies has no effect on the progression of maturing oocytes to the MII stage. However, meiotic spindle organization is severely compromised after immunoneutralization of ATRX as indicated by a significant increase in the proportion of oocytes with misaligned chromosomes.

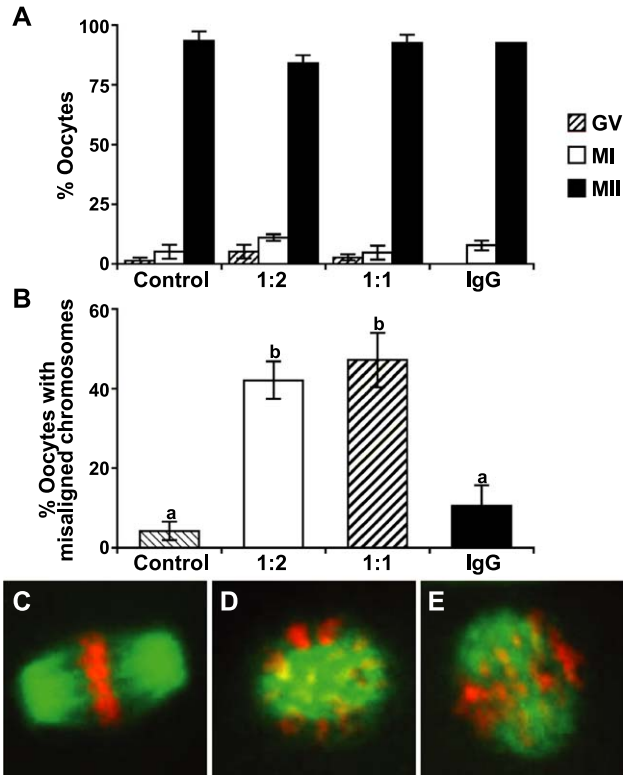

Fig. 7. Microinjection of ATRX antibodies disrupts chromosome alignment at the metaphase II stage. Proportion of oocytes at different stages of meiosis after microinjection of an equal volume (1:1:1) mixture of antibodies (23c, 39f, and polyclonal anti-ATRX) against different amino termini of the ATRX protein or after injection of a 1:2 dilution of mixed antibodies. Control oocytes were injected with nonimmune IgG (A). The majority of control noninjected oocytes or oocytes microinjected with a nonimmune IgG exhibited a barrel-shaped bipolar spindle (green) with chromosomes (red) tightly aligned at the equatorial region (C). Microinjection of ATRX antibodies increased the proportion ($P < 0.0001$) of oocytes presenting improper chromosome alignment on the metaphase II spindle (B). Although the spindle in the middle panel (D) corresponds to a metaphase II oocyte, it is no longer bipolar and chromosomes are only loosely attached peripherally to the microtubular network (the polar body in this oocyte was out of the focal plane). Chromosomes were also found misaligned within the spindle microtubules (E).

Specific ablation of ATRX *with RNAi disrupts chromosome alignment to the MII spindle*

To further test the hypothesis that ATRX plays a role during meiosis, the mRNA transcript encoding the ATRX protein in mouse oocytes was specifically ablated using an RNA interference (RNAi) approach. Oocytes were microinjected in the cytoplasm with double stranded (DS) RNA or a short interfering (esi) double stranded RNA. Oocytes were released from prophase arrest 24 h after microinjection and allowed to undergo meiotic maturation in vitro. The proportion of oocytes with centromeric ATRX staining and

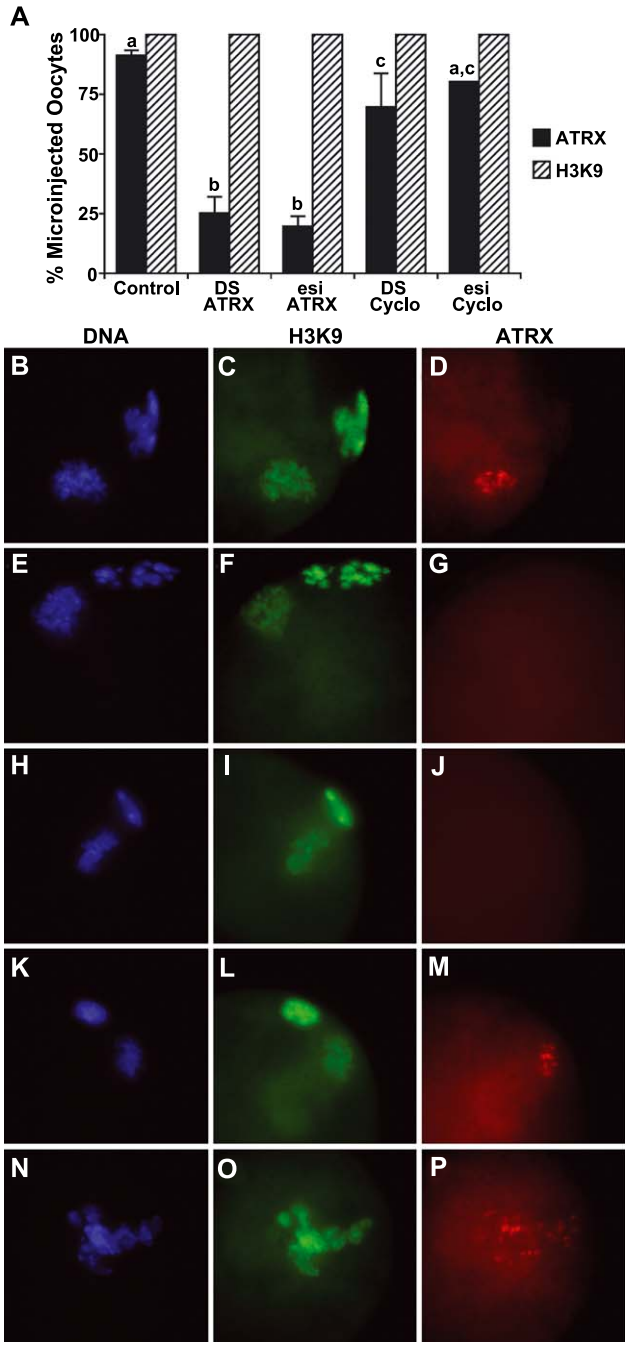

Fig. 8. Selective ablation of ATRX by RNA interference (RNAi). Proportion of metaphase II oocytes presenting histone H3 lysine 9 methylation (H3K9) and localization of ATRX to centromeric heterochromatin (A). Control patterns are shown for the chromosomes (B, blue), H3K9 (C, green), and ATRX (D, red). Microinjection of double stranded (DS ATRX) and short interfering (esi ATRX), specifically abrogated ATRX staining without affecting H3K9 methylation in condensed chromosomes (E–G) and (H–J), respectively. No significant effects were observed with control nonrelated (Cyclophilin) double stranded (DS) RNAs (K–M) or with esi RNAs against Cyclophilin (N–P). Oocytes were microinjected at the GV stage and allowed to undergo meiotic maturation for a period of 14 h. Data are presented as the mean ± SEM of four independent experiments.

H3K9 methylation in condensed chromosomes in three independent experimental replicates is illustrated in (Fig. 8A). Consistent with our previous experiments, >90% of control MII stage oocytes (n = 67) exhibit H3K9 staining (Fig. 8C) at condensed chromosomes and focal sites of ATRX labeling at centromeric heterochromatin regions (Fig. 8D). Microinjection of double stranded RNA (n = 69) generated from the ATRX sequence (DS ATRX) had no effect on the proportion of oocytes stained with H3K9 (Fig. 8A). Moreover, the levels of H3K9 staining in this group were similar to those observed in control oocytes microinjected with medium alone (Fig. 8F). In stark contrast, microinjection of DS ATRX significantly reduced the proportion ($P < 0.0001$) of oocytes with ATRX labeling, and showed only minimal cytoplasmic background fluorescence (Fig. 8G). Similarly, microinjection with short interfering, double stranded RNA (esi) from the ATRX sequence (n =

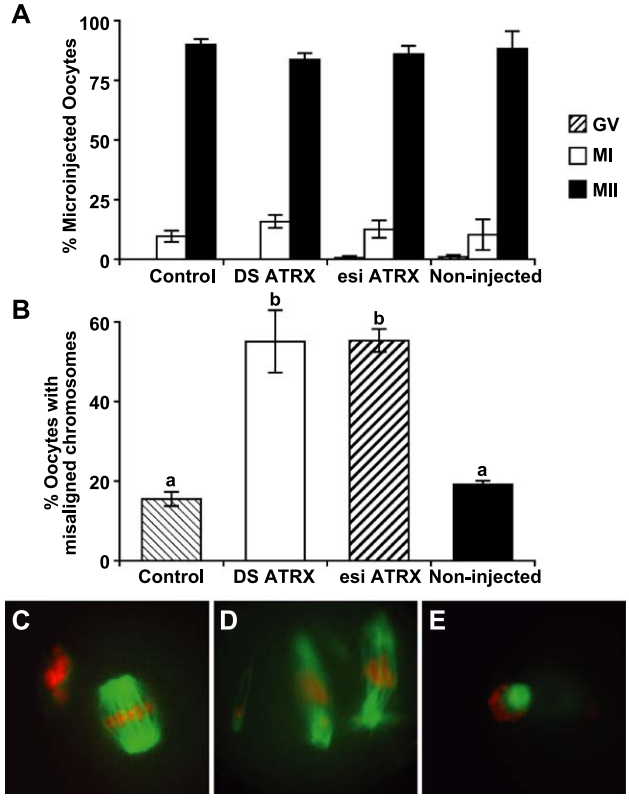

Fig. 9. Selective ablation of ATRX by RNA interference (RNAi) disrupts proper chromosome alignment in metaphase II stage mouse oocytes (A). Proportion of microinjected oocytes at the germinal vesicle (GV), metaphase I (MI), or metaphase II (MII) stage after 14 h of in vitro maturation. Inhibition of ATRX by double stranded (DS ATRX) and short interfering (esi ATRX) had no effect on the progression of meiosis. However, microinjection of DS ATRX or esi ATRX significantly increased ($P < 0.05$) the proportion of oocytes with abnormal chromosome alignments on the metaphase II spindle (B–E). Control oocyte shown in (C). Chromosomes were counterstained with propidium iodide and are shown in red; microtubules were stained with anti β-tubulin and are shown in green. Data are presented as the mean ± SEM of four independent experiments.

50) had no effect on the proportion of oocytes presenting H3K9 staining in condensed chromosomes (Fig. 8I), whereas staining for ATRX was abrogated in the majority (>80%) of microinjected oocytes (Fig. 8J). Microinjection of a completely unrelated double stranded RNA sequence (DS Cyclo; $n = 57$) had no effect on H3K9 staining (Fig. 8L), and no effects on ATRX protein expression were detected (Fig. 8M). Similarly, microinjection of a 30 nucleotide short interfering (esi) RNA (esi Cyclo; $n = 51$) had no effect on the ATRX protein as demonstrated by simultaneous detection of H3K9 methylation (Fig. 8O) and focal sites of ATRX staining in centromeric heterochromatin (Fig. 8P). These results indicate that RNAi is an effective strategy for the selective ablation of ATRX.

To determine the potential function of ATRX during meiotic progression, preovulatory oocytes were microinjected with double stranded RNA (DS ATRX) or short interfering RNA (esi ATRX) at the GV stage and allowed to resume meiosis in vitro as above. The proportions of oocytes reaching different stages of meiosis in three independent experimental replicates under various treatment conditions are shown in (Fig. 9A). No significant differences were observed in the proportion of oocytes (83–90%) that reached the MII stage compared with uninjected control (88%) oocytes. However, analysis of chromosome and spindle configurations revealed a significant increase ($P = 0.003$) in the proportion of MII oocytes with abnormal chromosome alignments on the meiotic spindle (Fig. 9B). Control oocytes microinjected with maturation medium alone ($n = 60$) exhibited a typical barrel-shaped meiotic spindle with chromosomes tightly aligned at the equatorial region (Fig. 9C). In contrast, a high proportion of oocytes (55%; $n = 59$) microinjected with DS ATRX (Fig. 9D) or esi ATRX (55.3%, $n = 77$) presented aberrant chromosome configurations with improper alignment on spindle microtubules (Fig. 9E). These results suggest that centromeric ATRX is essential for proper chromosome alignment on the MII spindle.

Discussion

Accurate segregation of homologous chromosomes and of sister chromatids during mammalian meiosis is crucial for the subsequent transmission of a euploid chromosome complement to the developing conceptus (Hassold and Hunt, 2001). Chromosome segregation depends on the establishment of physical and biochemical interactions between spindle microtubules and specialized chromosomal regions localized at centromeric heterochromatin (Dobie et al., 1999; Karpen and Allshire, 1997; Pluta et al., 1995; Sullivan, 2001). In this study, we provide evidence indicating that ATRX, a member of the SNF2 family of helicase/ATPases with chromatin remodeling activity, is present at heterochromatin regions of the mouse oocyte genome. Results indicate that global or genome-wide histone deacetylation occurs upon meiotic resumption in mouse oocytes and that global histone deacetylation is necessary to maintain the association of the ATRX protein to centromeric heterochromatin domains in condensed chromosomes. In contrast with the highly dynamic changes in histone acetylation taking place upon germinal vesicle breakdown, the levels of histone H3 methylated at lysine 9 (H3K9) remained constant in the maternal genome during meiosis. Moreover, Western blot analysis revealed that ATRX is phosphorylated after 10–14 h of in vitro maturation coincident with oocytes reaching the MI and MII stage, respectively. Pharmacological inhibition of the calcium calmodulin kinase (CamKII) pathway with KN-93 prevented the appearance of the slow migrating forms of ATRX indicating that this phosphorylation event is mediated by the CamKII pathway in mouse oocytes. Importantly, functional ablation of ATRX protein by antibody microinjection and RNAi indicates that ATRX plays an essential role in the organization of proper chromosome alignment on the meiotic MII spindle.

Global histone deacetylation during meiosis is essential for binding of ATRX *to centromeric heterochromatin domains*

Localization of ATRX to centromeric heterochromatin has been previously demonstrated in human and mouse somatic cell lines (McDowell et al., 1999). However, the role of ATRX in nuclear compartmentalization and heterochromatin formation during meiosis is not known. The mammalian genome is subject to a series of dramatic changes in chromatin structure and function during gametogenesis (Debey et al., 1993; Mattson and Albertini, 1990; Patterton and Wolffe, 1996; Sassone-Corsi, 2002; Wickramasinghe and Albertini, 1992). Although the mechanisms involved in such dynamic chromatin modifications are not fully understood, analysis of component molecules of chromatin remodeling complexes, such as ATRX, in mouse oocytes provides a unique model to determine the contribution of heterochromatin binding proteins to germ cell nuclear architecture. Immunochemical analysis of growing oocytes at the GV stage revealed that ATRX is co-localized predominantly with centromeric proteins detected by the CREST antibody and with focal sites of bright Hoechst fluorescence known to correspond to heterochromatin regions (Peters et al., 2001; Pluta et al., 1995). However, diffuse ATRX staining could also be detected, albeit at lower levels, in the nucleoplasm of oocytes with decondensed chromatin presenting the non-surrounded nucleolus (NSN) configuration. Moreover, analysis of preovulatory oocytes with the surrounded nucleolus (SN) configuration, in which chromatin is condensed, indicate that ATRX is localized in close apposition to the perinucleolar heterochromatin rim. These findings are consistent with a predominant association of ATRX with heterochromatin domains during prophase I of meiosis. The molecular composition and mechanisms leading to the formation of

the perinucleolar heterochromatin rim in mouse oocytes with the SN configuration are not clear. However, our results indicate that ATRX is present, along with additional nucleolar components such as the upstream binding factor (UBF), RNA polymerase I subunits, and the splicing factor B23 (Zatsepina et al., 2000), in the perinucleolar heterochromatin rim of preovulatory mouse oocytes. Interestingly, a direct interaction between ATRX and UBF has also been demonstrated in human metaphase chromosomes wherein ATRX has been recently shown to associate with the short arms of acrocentric chromosomes carrying ribosomal DNA sequences (Gibbons et al., 2000).

Laser scanning confocal microscopy revealed that acetylated histone H4 was found in a diffuse nucleoplasmic staining pattern irrespective of oocyte chromatin configuration, except for heterochromatin regions, where its expression was only faintly detected. These observations are consistent with the net deacetylated status of heterochromatin domains in cells at the G2 stage of the cell cycle (Grunstein, 1997; Taddei et al., 1999). Interestingly, upon germinal vesicle breakdown, a wave of global histone deacetylation takes place at which time ATRX is bound exclusively to the centromeric heterochromatin regions of condensing chromosomes. Histone acetylation is generally associated with decondensed, transcriptionally competent chromatin regions, whereas histone deacetylation is largely associated with condensed chromatin. However, until now, most studies on histone deacetylation have been based on the analysis of promoter regions or alternative regulatory sequences of single copy genes (Grunstein, 1997; Turner, 2000). Our results indicate that global or genome-wide histone modifications are also of significant functional relevance during meiosis. Consistent with this hypothesis, recent studies indicate that chromatin modifications in yeast occur not only at specific promoter regions or at regulatory elements of single copy genes, but also throughout large sections of the genome (Berger and Felsenfeld, 2001; Katan-Khaykovich and Struhl, 2002; Vogelauer et al., 2000). Moreover, global deacetylation of histone H3 at lysines 9 and 14 during mitosis and lysine 12 of histone H4 in mouse oocytes has also been described recently (Kim et al., 2003; Kruhlak et al., 2001). Our results extend these observations and demonstrate that global histone deacetylation at the onset of meiosis is of functional significance for the binding of ATRX to centromeric heterochromatin.

The mechanisms regulating the specific localization of ATRX to centromeric domains are not clear. However, it has been suggested that histone modifications taking place on a genome-wide scale are important to reduce the nonspecific binding of heterochromatin proteins to chromosomal regions other than centromeric heterochromatin (van Leeuwen and Gottschling, 2002). In this study, induction of histone hyperacetylation with TSA did not prevent meiotic chromosome condensation. However, the presence of hyperacetylated histones in chromosomes interfered with the specific localization of ATRX to centromeric heterochromatin resulting in abnormal chromosome attachments with the meiotic spindle. Long-term exposure (> 5 days) of human fibroblasts (Taddei et al., 2001) or yeast cells (Ekwall et al., 1997; Grewal et al., 1998) to TSA also disrupts the binding of the different subunits of heterochromatin binding protein 1 (HP1) (Taddei et al., 2001) or the transcriptional co-repressor KRAB-associated-protein 1 (KAP-1) to centromeres of mitotic chromosomes (Matsuda et al., 2001). Collectively, these results suggest that epigenetic modifications such as global histone deacetylation may be essential during the progression of both mitosis and meiosis for the specific recruitment of heterochromatin proteins to centromeric domains in condensed chromosomes. Furthermore, our studies indicate that meiosis is particularly susceptible to disruptions in histone deacetylation as exposure to TSA for only 8 h was sufficient to prevent binding of ATRX to heterochromatin domains in mouse oocytes.

The CamKII pathway mediates phosphorylation of the ATRX *protein during meiosis*

The serine residues of ATRX are subject to phosphorylation in human mitotic cells (Bérubé et al., 2000). However, whether ATRX is subject to posttranslational modifications during meiosis has not been determined previously. Moreover, the cell signaling pathway(s) involved in ATRX phosphorylation are not known. Analysis of posttranslational modifications and subcellular localization of ATRX during meiotic progression revealed that ATRX is exclusively bound to the centromeres of condensing chromosomes shortly after germinal vesicle breakdown. However, the phosphorylated forms of ATRX cannot be detected until 10–14 h of meiotic maturation, in oocytes at the MI and MII stages, respectively. These results indicate that in contrast with other chromatin binding proteins, such as the human homologue of *Drosophila* suppression of position effect variegation SUV39H1 and HP1, whose centromeric heterochromatin localization is dependent upon protein phosphorylation (Aagaard et al., 2000; Zhao and Eissenberg, 1999), the binding of ATRX to centromeric domains precedes and therefore is not dependent on its phosphorylation in mouse oocytes. Our results also contrast with studies of ATRX in human somatic cell lines in which localization of ATRX to condensed chromatin during mitosis has been suggested to be phosphorylation-dependent (Bérubé et al., 2000). Species-specific differences in heterochromatin protein content and organization may account for this discrepancy. For example, comparison of HP1 protein content and posttranslational modifications in HeLa cells and mouse 3T3 fibroblasts revealed that different isoforms of HP1 segregate to different nuclear compartments and exhibit different patterns of cell cycle-related phosphorylation in these two cell lines (Minc et al., 1999).

The nature of the two ATRX isoforms that appear in MI and MII of meiosis is not known. It is conceivable that these two ATRX isoforms correspond to phosphorylation at two

different amino acid residues, or that they are products of an alternatively spliced transcript in mouse oocytes. Experiments are in progress to distinguish between these possibilities. Inhibition of the calcium calmodulin kinase (CamKII) pathway by KN-93 prevented the phosphorylation of ATRX at both MI and MII. These results provide, for the first time, biochemical evidence indicating that phosphorylation of the ATRX protein is under the control of the CamKII pathway during mammalian meiosis. This is in contrast with HP1 phosphorylation, which in *Drosophila* cells is under the control of the casein kinase pathway (Zhao and Eissenberg, 1999). These results suggest that phosphorylation of different heterochromatin binding proteins is under the control of distinct cell signaling pathways that may be of relevance for the spatiotemporal regulation of centromere and spindle microtubule interactions.

Binding of ATRX *to centromeric heterochromatin is important for the alignment of chromosomes to the meiotic MII spindle*

Localization of ATRX to centromeric domains during meiosis suggested a possible functional role in chromosome architecture and/or segregation. Functional ablation of ATRX by RNAi and antibody microinjection during meiotic progression produced similar results. Abrogation of ATRX function had no effect on the progression of maturing oocytes to the MII stage. However, meiotic spindle organization was severely compromised as indicated by a significant increase in the proportion of oocytes exhibiting chromosomes with abnormal alignment on the spindle microtubules. The most common configuration observed presented groups of chromosomes establishing a loose attachment to peripheral regions of the meiotic MII spindle. However, in extreme cases, spindle bipolarity was completely lost and chromosomes were arranged in a rosette-like configuration resembling a prometaphase stage, although such oocytes always exhibited a set of homologous chromosomes extruded into the polar body. Although these studies do not rule out the possibility that chromosome misalignment in response to ATRX ablation also occurs at MI, the high proportion of oocytes reaching MII after ATRX depletion argues against this possibility and suggests that the function of ATRX in mediating chromosome alignment is predominantly at the MII stage in mouse oocytes. Cohesion between sister chromatids at pericentromeric heterochromatin regions is crucial for chromosome alignment on the MII spindle (Bernard et al., 2001; Nasmyth, 2002; Petronczki et al., 2003; Tanaka et al., 2000). It is thus possible that interference with ATRX function decreases the efficiency of centromeric heterochromatin in mediating proper chromosome alignment exclusively at the metaphase II stage. In support of this hypothesis, Nonaka et al. (2002) recently demonstrated that cohesin complexes are enriched at centromeric heterochromatin of sister chromatids by a direct physical association with the heterochromatin protein Swi6 and its mouse homologue, HP1. This establishes a direct interaction among centromeric heterochromatin protein composition, centromere cohesion, and chromosome segregation (Bernard et al., 2001; Nonaka et al., 2002). Our results provide the first evidence indicating that in addition to its role as a putative transcriptional regulator (Gibbons et al., 1997, 2000; McDowell et al., 1999), ATRX also functions during key stages of meiosis in mouse oocytes.

Our experiments have also revealed that different post-translational modifications occur on the core histones H3 and H4 during meiotic progression. For example, in contrast with the rapid histone deacetylation event observed upon germinal vesicle breakdown, histone methylation persists through global changes in chromatin modifications during meiosis. These results are consistent with a role of histone methylation in providing the chromosomal environment necessary for the recruitment of heterochromatin binding proteins such as HP1 to centromeric domains (Bannister et al., 2001; Dillon and Festenstein, 2002; Lachner et al., 2001; Rea et al., 2000). Analysis of protein–protein interactions by yeast-two hybrid and co-immunoprecipitation studies indicates that ATRX physically interacts with HP1 in somatic cells (Bérubé et al., 2000; Le Douarin et al., 1996). It is thus conceivable that functional ablation of ATRX disrupts higher order structure at centromeric heterochromatin or alternative aspects of chromosome architecture that are essential for the establishment of a functional centromere. In support of this hypothesis, additional studies suggest that HP1 is involved in mediating chromosome–microtubule interactions (Ainsztein et al., 1998), as functional ablation of HP1 in *Drosophila* embryos disrupts heterochromatin condensation resulting in abnormal chromosome segregation (Kellum and Alberts, 1995).

Centromeric domains exhibit unique properties to nucleate kinetochore-associated proteins and mediate the poleward segregation of chromosomes (Sullivan, 2001). Although most studies have focused on the analysis of kinetochore-binding proteins, a growing body of evidence suggests that pericentric heterochromatin is also essential to maintain centromere structure and function (Bernard et al., 2001; Dobie et al., 1999; Jibak Lee et al., 2003; Melcher et al., 2000; Peters et al., 2001). Importantly, the large protein complexes present at centromeric domains are also subject to epigenetic modification (Ekwall et al., 1997; Grewal et al., 1998; Henikoff et al., 2001; Wiens and Sorger, 1998). Our results indicate that global or genome-wide histone deacetylation is essential for the binding of ATRX to centromeric domains during mammalian meiosis and provides functional evidence indicating that ATRX, a centromeric heterochromatin protein, regulates key stages of meiosis in that it plays an important role in establishing proper chromosome alignment and a bipolar meiotic spindle at metaphase II in mouse oocytes. Epigenetic modifications such as histone deacetylation during meiosis may thus be essential for recruitment of heterochromatin binding proteins involved in centromere structure and function.

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