Loss of ATRX leads to chromosome cohesion and congression defects

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Introduction

α Thalassemia/mental retardation X linked (ATRX) is a switch/sucrose nonfermenting-type ATPase localized at pericentromeric heterochromatin in mouse and human cells. Human ATRX mutations give rise to mental retardation syndromes characterized by developmental delay, facial dysmorphism, cognitive deficits, and microcephaly and the loss of ATRX in the mouse brain leads to reduced cortical size. We find that ATRX is required for normal mitotic progression in human cultured cells and in neuroprogenitors. Using live cell imaging, we show that the transition from prometaphase to metaphase is prolonged in ATRX-depleted cells and is accompanied by defective sister chromatid cohesion and congression at the metaphase plate. We also demonstrate that loss of ATRX in the embryonic mouse brain induces mitotic defects in neuroprogenitors in vivo with evidence of abnormal chromosome congression and segregation. These findings reveal that ATRX contributes to chromosome dynamics during mitosis and provide a possible cellular explanation for reduced cortical size and abnormal brain development associated with ATRX deficiency.

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α Thalassemia/mental retardation X linked (ATRX) is a chromatin remodeling enzyme implicated in early development of several organs, particularly the central nervous system. The ATRX protein contains conserved domains that suggest a role in the epigenetic regulation of chromatin structure and function, including a plant homeodomain-like zinc finger domain shared with de novo methyltransferases (DNMT3A/B and 3L) and a switch/sucrose nonfermenting family ATPase domain. ATRX has the ability to remodel chromatin and displays ATP-dependent translocase activity (Xue et al., 2003). It is highly enriched at pericentromeric heterochromatin (PCH) in mouse and human cells (McDowell et al., 1999) and associates directly with the chromoshadow domain (CSD) of heterochromatin protein 1α (HP1α; Lechner et al., 2005). It is also targeted to promyelocytic leukemia nuclear bodies by the C-terminal portion of the protein (Berube et al., 2007). Atrx loss of function in the mouse starting at cell stage 8 to 16 is embryonically lethal (Garrick et al., 2006) and conditional ablation of the full-length ATRX isoform in the mouse forebrain results in decreased cortical size at birth (Berube et al., 2005). Although ATRX has been proposed to regulate gene transcription, the protein appears to be hyperphosphorylated and highly enriched at condensed chromosomes during mitosis in human cells, which suggests an additional function during this stage of the cell cycle (Berube et al., 2000).

The faithful segregation of chromosomes during mitosis requires the physical linkage of sister chromatids from S phase until the onset of anaphase. The ring-shaped cohesin multiprotein complex is required for the maintenance of sister chromatid cohesion and plays a role in the proper separation and segregation of sisters during anaphase (Hirano, 2005). Cohesin at the chromosomal arms is released during prophase by the polo and aurora B kinases and the chromatin protein wings apart-like (Sumara et al., 2002; Gandhi et al., 2006; Kueng et al., 2006). Cohesion at PCH is protected by the Shugoshin family of proteins and prohibitin 2, and consequently persists until all the chromosomes are bi-oriented at the metaphase plate (McGuinness et al., 2005; Kitajima et al., 2006; Takata et al., 2007). Only then is the spindle checkpoint satisfied, resulting in the activation of the anaphase-promoting complex/cyclosome and subsequent cohesin cleavage by the thiol protease separase.
Stable loading of cohesin onto chromatin before DNA replication is mediated by the Sc2-Sc4 heterodimer in yeast (Ciosk et al., 2000; Watrin et al., 2006). Human Sc2, known as nipped-B–like (NIPBL [delangin]), mediates cohesin transfer onto chromatin during S phase and, like ATRX, associates with the CSD of HP1α (Lechner et al., 2005). Chromatin remodeling proteins have been implicated in chromosome cohesion. The Sth1 subunit of the yeast RSC chromatin remodeling complex has been shown to participate in cohesin loading on chromosomal arms but not at the centromere (Baetz et al., 2004; Huang et al., 2004). In human cells, the SNF2h/ISWI chromatin remodeling protein, a component of several remodeling complexes, was shown to participate in cohesin recruitment to specific sites on chromosome arms (Hakimi et al., 2002).

ATRX mutations in humans cause mental retardation and microcephaly (Gibbons et al., 1995; Picketts et al., 1996). Given that Atrx loss of function in the mouse forebrain results in reduced cortical mass (Berube et al., 2005) and that the protein becomes hyperphosphorylated at the onset of mitosis (Berube et al., 2000), we postulated that ATRX could have specific functions during mitosis. We now provide evidence that the switch/sucrose nonfermenting-like chromatin remodeling protein ATRX is required for normal chromosome congression, cohesion, and segregation in human cultured cells. Loss of ATRX in neuronal progenitors by Cre/loxP recombination in vivo also resulted in abnormal chromosome segregation, as indicated by the high incidence of micronuclei and dispersed chromosomes. Mitotic dysfunction could induce cell death in the developing brain and/or cause a reduction in symmetric cell divisions, therefore reducing the precursor pool. Either of these scenarios or a combination thereof would be predicted to reduce cortical size at birth.

Results

ATRX-depleted HeLa cells exhibit unusual nuclear morphology and DNA bridging

The ATRX gene yields two major protein isoforms: a full-length 280-kD protein and a truncated form, ATRXt, of 180 kD (Garrick et al., 2004). Both ATRX protein isoforms exhibit exclusive nuclear localization and are highly enriched at PCH (Fig. 1, A and C). We induced transient ATRX depletion in HeLa cells by transfecting siRNAs. Cells were treated with two siRNA duplexes, siATRX1 and siATRX2, each designed to simultaneously silence the full-length and truncated ATRX proteins. Both duplexes caused substantial silencing of ATRX isoforms as assessed by immunostaining (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200706083/DC1), and this was confirmed by quantitative RT-PCR (Q-RT-PCR; Fig. S1 B) and Western blot analysis (Fig. 1 B). Coimmunofluorescence staining of centromeres using the calcinosis, Raynaud’s phenomenon, esophageal dismotility, sclerodactyly, and telangiectasia (CREST) antibody demonstrated that only minimal amounts of ATRX protein are detectable at PCH in siRNA-treated cells (Fig. 1 C). We observed that the nuclei of depleted cells appeared lobulated and that the cells showed evidence of intranuclear DNA bridges and poorly resolved chromatin masses during interphase (Fig. 1 D). We scored the number of abnormal nuclei at 48, 72, and 96 h after transfection and found an increased incidence of lobulated nuclei and intranuclear bridges (Fig. 1 E). Such abnormalities can be indicative of mitotic defects and prompted us to further investigate the function of ATRX in mitosis.

Prolonged prometaphase-to-metaphase transition upon down-regulation of ATRX

To investigate the kinetics of mitotic progression, we assessed the outcome of both transient and stable depletion of ATRX in HeLa cells that express histone H2B fused to GFP (HeLa-H2B-GFP [HG]). Stable clones were difficult to expand, which suggests that reduced ATRX expression imparts negative effects on cell division, proliferation, or viability. A negative impact on cell division was previously reported upon conditional ablation of the full-length ATRX protein in embryonic stem cells, although the cause was not determined (Garrick et al., 2006). We chose two stable cell lines for our analysis, designated shATRX1 and shATRX2, each expressing a short hairpin RNA that targets a distinct sequence within the ATRX transcript. Both stable clones expressed substantially reduced levels of ATRX RNA and protein as determined by Q-RT-PCR and Western blot analysis (Fig. 2 A).

Time-lapse videomicroscopy was used to evaluate the outcome of both transient and stable ATRX silencing. We generated videos of live cells (10 h) and from these measured the time required to complete each stage of mitosis (stable clone, n = 50; transient depletion, n ≥ 120). This analysis revealed that ATRX-depleted cells took longer than control cells to undergo mitosis (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200706083/DC1) because of an extended transition from prometaphase to fully aligned metaphase (Fig. 2 B; P < 0.001). The delays in prometaphase were underestimated because a subset of mitotic cells that were arrested at prometaphase or metaphase for the total duration of the observation period (10 h) were not included in this analysis. These chronically arrested prometaphase cells often became nonadherent and/or died, as indicated by membrane blebbing and highly condensed chromatin (Video 1). Collectively, these results show that ATRX is required for the normal transition from prometaphase to metaphase during mitosis.

ATRX depletion affects chromosome congression

The mitotic delay in ATRX-depleted cells occurred primarily between prometaphase and metaphase, a time when condensed chromosomes are congressing toward the metaphase plate. We noticed that metaphase plates in transiently depleted cells and in the shATRX1 and shATRX2 stable cell lines were often characterized by misaligned chromosomes or chromosomes that remained randomly distributed over the bipolar microtubule spindle (Fig. 2 C and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200706083/DC1), which suggests that the prometaphase-to-metaphase delay might be caused by impaired chromosome congression to the spindle equator. To evaluate the extent of this defect, we scored the number of metaphasic cells with misaligned chromosomes in control and ATRX-depleted live cell cultures. A greater proportion of metaphase plates had...
interferes with normal prometaphase-to-metaphase progression, causes impaired chromosome congression, and is sometimes accompanied by chromosome decondensation. Sister chromatid cohesion is compromised in ATRX-depleted cells Abnormal chromosomal congression could be caused by perturbed targeting of outer kinetochore proteins to the centromere, spindle defects, abnormal PCH, or reduced cohesion between sister chromatids. Congression defects have been reported upon depletion of the outer kinetochore proteins centromere-associated protein (CENP)-E and CENP-F (mitosin) that are essential for stable microtubule-kinetochore capture (Wood et al., 1997; Yao et al., 1997; McEwen et al., 2001; Tanudji et al., 2004; Yang et al., 2005). We examined the ability of CENP-E and CENP-F to localize to kinetochores in ATRX-depleted cells and found that both proteins localized normally at the kinetochore of metaphasic cells (Fig. 3A). The loss of factors that control PCH structure can also cause aberrant mitosis (Melcher et al., 2000; misaligned chromosomes in the shATRX1- and shATRX2-depleted clones compared with controls (Fig. 2, C and E). In addition, we followed stably (shATRX1, n = 100) and transiently (siATRX, n ≥ 120) depleted mitotic cells by videomicroscopy over a 10-h period and observed an approximately threefold (stable depletion) and fourfold (transient depletion) increase in the number of cells displaying congression defects in ATRX-depleted cells compared with control cells during that time period (Fig. 2D). In addition, metaphasic cells often appeared less condensed in ATRX-depleted cells and were sometimes observed to undergo decondensation/recondensation before anaphase onset by live imaging (Fig. 2E, middle; and Video 3). We further confirmed the general decondensed appearance of ATRX-depleted metaphasic chromosomes by staining chromosome spreads with DAPI. The frequency of spreads with a staining pattern indicative of more decondensed chromatin (wider chromosomes) was higher in cells transiently depleted of ATRX using siATRX1 and siATRX2 duplexes (Fig. 2F) than in control cells. These results demonstrate that transient as well as stable silencing of ATRX interferes with normal prometaphase-to-metaphase progression, causes impaired chromosome congression, and is sometimes accompanied by chromosome decondensation.

Sister chromatid cohesion is compromised in ATRX-depleted cells Abnormal chromosomal congression could be caused by perturbed targeting of outer kinetochore proteins to the centromere, spindle defects, abnormal PCH, or reduced cohesion between sister chromatids. Congression defects have been reported upon depletion of the outer kinetochore proteins centromere-associated protein (CENP)-E and CENP-F (mitosin) that are essential for stable microtubule-kinetochore capture (Wood et al., 1997; Yao et al., 1997; McEwen et al., 2001; Tanudji et al., 2004; Yang et al., 2005). We examined the ability of CENP-E and CENP-F to localize to kinetochores in ATRX-depleted cells and found that both proteins localized normally at the kinetochore of metaphasic cells (Fig. 3A). The loss of factors that control PCH structure can also cause aberrant mitosis (Melcher et al., 2000;
http://www.jcb.org/cgi/content/full/jcb.200706083/DC1), which suggests that ATRX is not required for proper targeting of these proteins to PCH.

We next measured the distance between metaphasic sister kinetochores in ATRX-depleted cells using CREST immunostaining. Sister chromatid interkinetochore distances were significantly increased in both shATRX1- and shATRX2-aligned metaphases compared with control metaphases (Fig. 3 B, P < 0.05 by analysis of variance [ANOVA]). Increased distance between sister chromatids could reflect a more decondensed state of chromatin and perhaps fewer nucleosomes associated with Peters et al., 2001). Specific histone modifications that characterize PCH, including trimethylated H3K9 and H4K20 and monomethylated H3K27, were not visibly affected in ATRX-depleted cells (unpublished data). Trimethylation of Lys9 on histone H3 (H3K9me3) by the Suv39H1 methyltransferase recruits HP1α and forms a compact heterochromatic structure. HP1α in turn interacts with several chromatin factors, including NIPBL, ATRX, and chromatin assembly factor 1 (Lechner et al., 2005). However, we observed that nuclear HP1α as well as HP1β immunoreactivity were indistinguishable between transiently depleted and control cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200706083/DC1), which suggests that ATRX is not required for proper targeting of these proteins to PCH.

We next measured the distance between metaphasic sister kinetochores in ATRX-depleted cells using CREST immunostaining. Sister chromatid interkinetochore distances were significantly increased in both shATRX1- and shATRX2-aligned metaphases compared with control metaphases (Fig. 3 B, P < 0.05 by analysis of variance [ANOVA]). Increased distance between sister chromatids could reflect a more decondensed state of chromatin and perhaps fewer nucleosomes associated with

Figure 2. Stable and transient depletion of ATRX extends the transition to metaphase and induces congression defects. (A) HeLa-HG cells that stably express pSuper-shATRX1, pSuper-shATRX2, or pSuper [empty vector control] were generated and the level of ATRX depletion was measured by Q-RT-PCR (top) and Western blot analysis (bottom). Q-RT-PCR results were normalized to GAPDH expression and protein loading on the Western blot was controlled with α-tubulin. Error bars represent the standard deviation from triplicate samples. (B) Mitotic cells were followed in real time by videomicroscopy and the duration of prometaphase was measured for HG control and HG-shATRX1 stably depleted cells (n = 50 each) and also in transient transfections with siATRX1 (n > 120). Horizontal lines indicate the mean value of each dataset. (C) The fraction of metaphasic cells with misaligned chromosomes [inset] was evaluated in live cell cultures of HeLa-HG, HG-pSuper, HG-shATRX1, and HG-shATRX2 stable clones [n = 100 for each]. (D) Live mitotic cells were followed over a 10 h period and scored for congression defects in stable (n = 50) and transient experiments (n > 120). (E) Selected panels from live cell videomicroscopy experiments of control HeLa-HG cells and ATRX-depleted cells from nuclear envelope breakdown (NEBD) to anaphase onset (AO). ATRX-depleted mitotic cells often displayed misaligned chromosomes (arrowheads) that resolved before the onset of anaphase (middle), whereas, in some cases, anaphase was initiated despite the presence of misaligned chromosomes (bottom). A subset of metaphase chromosomes underwent cycles of general decondensation and recondensation (the asterisk indicates a decondensed metaphase plate). Numbers indicate minutes after NEBD. (F) Morphological assessment of chromosome decondensation in control and transiently depleted cells. Graph depicts the percentage of metaphase spreads that display a more decondensed appearance [far right]. Bars: (C) 5 μm; (E) 16 μm, (F) 20 μm.
pairs displaying loss of cohesion) was observed at low levels in both controls and depleted cells (Fig. 3, C and D), indicating that ATRX depletion results in reduced but not complete loss of sister chromatid cohesion.

ATRX depletion transiently activates the spindle checkpoint

The mitotic delay incurred by ATRX depletion prompted us to survey the status of the spindle checkpoint proteins in ATRX-depleted cells. As such, the preanaphase mitotic delay might result from continued mitotic checkpoint signaling at misaligned chromosomes. Activation of the spindle checkpoint was confirmed by staining for the checkpoint proteins BubR1 and Bub1 at misaligned chromosomes. We observed that both proteins were enriched at a subset of kinetochores in ATRX-depleted metaphases, with a strong signal at misaligned chromosomes (Fig. 4 A). We further tested the robustness of the spindle checkpoint by analyzing the fate of cells after exposure to nocodazole, a microtubule-depolymerizing agent. Nocodazole-induced mitotic arrest was robust in both control and ATRX-depleted cells, demonstrating that ATRX is not required for spindle checkpoint activation (Fig. 4 B). We next selectively destabilized nonkinetochore fibers by cold treatment. The residual intact kinetochore fibers were visualized by α-tubulin staining. We observed that...
ATRX depleted cells were characterized by highly irregular arrays of kinetochore fibers (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200706083/DC1).

**Reduced levels of ATRX induce chromosome segregation defects**

Despite the activation of spindle checkpoint proteins, we observed a modest accumulation of mitotic cells in ATRX-depleted cultures by FACS analysis (Fig. S2 B), which suggests that checkpoint activation could not be maintained completely in all cells. As a result, an increased number of cells with lagging chromosomes at anaphase, telophase, and G1 (Fig. 4 D and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200706083/DC1) were observed, indicating a problem with chromosome segregation. A three- to sevenfold increase in the number of cells displaying interphase DNA bridges was observed 48–96 h after transfection with ATRX siRNA duplexes compared with control cells (Fig. 4 D and E). DNA bridges in mammalian cells produce micronuclei when the bridges resolve and reform as small membrane-bound DNA bodies (Hoffelder et al., 2004). The number of micronuclei increased 3.4-fold compared with control cells by 96 h after siRNA treatment (Fig. 4 D). Collectively, these data suggest that the spindle checkpoint control is compromised in a subset of cells, causing chromosome segregation errors characterized by lagging chromosomes, interphase DNA bridges, and the subsequent formation of micronuclei.

**Defective mitosis in vivo in ATRX null neuroprogenitors**

The Cre-mediated deletion of the Atrx gene in the developing mouse forebrain leads to a reduction in cortical and hippocampal size in newborn pups. In these mice, conditional deletion of the Atrx gene occurs in nearly 100% of cells in the developing forebrain starting at approximately embryonic day (E) 8.5 (Berube et al., 2005). The analysis of the mitotic defects by live cell video-microscopy in ATRX-depleted HeLa cells provided indicators of mitotic anomalies that could potentially occur in the Atrx null developing mouse brain, and evidence of such abnormalities would indicate that ATRX contributes to corticogenesis in part by participating in progenitor cell mitosis. The primary progenitor cells in the developing cortex are the neuroepithelial cells in the
mitotic neuroepithelial cells lining the ventricular space was evaluated. We observed a high occurrence of pyknotic nuclei, which is indicative of cells undergoing apoptosis in the vicinity of mitotic cells (arrow). We also observed a very high incidence of micronuclei or misaligned chromosomes (Fig. 5B). We quantified the occurrence of micronuclei/misaligned chromosomes by scoring both hemispheres in sections from four littermate Atrx null and control embryos and limited our analysis to the mitotic layer that lines the hippocampal hem, the hippocampal primordium, and the dorsal cortical neuroepithelium (Fig. 5C, between 1 and 2). This analysis revealed a significant increase in the number of micronuclei in Atrx null mice compared with controls (Fig. 5C), which suggests that Atrx loss of function in vivo in mouse neuroprogenitors causes mitotic defects that are similar to those observed by RNA interference in cultured human cells.

Figure 5. ATRX association with mitotic chromosomes and evidence of mitotic defects in ATRX-deficient neuroprogenitors in vivo. (A) ATRX staining of the cortex at E13.5. ATRX is highly expressed in all cells but is highest in the cortical hem (CH), which gives rise to hippocampal structures. ATRX is also expressed at increased levels in the differentiated neurons of developing cortical plate (CP). Mitotic cells that line the lateral ventricle are highly enriched for ATRX protein (arrows). Higher magnification of the cortical hem (right) demonstrates ATRX staining of mitotic chromosomes in cells that line the lateral ventricle (arrow). Punctate nuclear staining of ATRX in cycling cells of the ventricular zone (VZ) is characteristic of ATRX localization at PCH. (B) Cryosections obtained from control and ATRX null telencephalon were stained with DAPI to visualize mitotic chromosomes lining the lateral ventricle (LV) at E13.5. An increased incidence of micronuclei or dispersed chromosomes was detected in the vicinity of the mitotic layer (arrows) in the ATRX null embryonic brain. (C) The mean number of micronuclei or dispersed chromosomes were scored in the mitotic layer from point 1 to 2 as indicated by the white arrows (top) and are represented in the graph below (n = 4 for each control and ATRX null brain; P < 0.0001 by nonpaired t test). Error bars represent the standard deviation of counts from a total of 24 cortical hemisphere from a total of four brains. Cn, cortical neuroepithelium; Hh, hippocampal hem; Hp, hippocampal primordium. Bars: [A] 100 μm; [B] 40 μm; [C] 200 μm.

proliferative ventricular zone. Because of the fact that cells undergo cyclical migration within the ventricular zone, mitotic progenitors are always localized at the edge of the ventricular zone facing the lateral ventricle. Cortical sections from normal embryos at E13.5 were first examined for the pattern of ATRX protein expression and localization. ATRX immunoreactivity was indeed enriched at condensed chromatin of mitotic neuroprogenitors lining the lateral ventricle, which is consistent with a mitotic function (Fig. 5A, arrows). ATRX was also expressed in cycling cortical progenitors in a punctate nuclear pattern characteristic of PCH in mouse cells and in differentiated neurons, as has been found previously (McDowell et al., 1999; Berube et al., 2005). To evaluate the outcome of Atrx loss of function on mitotic cells in vivo during corticogenesis, brain sections from four different Atrx null and littermate controls were fixed and stained with DAPI to visualize nuclei and chromatin and the status of mitotic neuroepithelial cells lining the ventricular space was evaluated. We observed a high occurrence of pyknotic nuclei, which is indicative of cells undergoing apoptosis in the vicinity of mitotic cells, which confirms previous findings (Berube et al., 2005). We also observed a very high incidence of micronuclei or misaligned chromosomes (Fig. 5B). We quantified the occurrence of micronuclei/misaligned chromosomes by scoring both hemispheres in sections from four littermate Atrx null and control embryos and limited our analysis to the mitotic layer that lines the hippocampal hem, the hippocampal primordium, and the dorsal cortical neuroepithelium (Fig. 5C, between 1 and 2). This analysis revealed a significant increase in the number of micronuclei in Atrx null mice compared with controls (Fig. 5C), which suggests that Atrx loss of function in vivo in mouse neuroprogenitors causes mitotic defects that are similar to those observed by RNA interference in cultured human cells.
Discussion

It has been suggested that brain size is partly dictated by the correct expression of genes that control neuroprogenitor mitosis. Microcephalin (Alderton et al., 2006; Bartek, 2006), abnormal spindledle microcephaly associated (Bond et al., 2002; Fish et al., 2006), Cdk 5 regulatory-associated protein 2, and CENP-J (Bond et al., 2005) all have roles in assembling, maintaining, and orienting the mitotic spindle and mutations of these genes in humans causes primary microcephaly. Our data now suggest that the loss of both ATRX protein isoforms (full-length and truncated forms), whether by transient or stable depletion, in HeLa cells causes defective sister chromatid cohesion and chromosome congression at the metaphase plate and that loss of ATRX in the mouse forebrain also results in mitotic defects. Although ATRX is highly enriched at PCH, we did not detect abnormal targeting of CENP-E, which is thought to orient the mitotic spindle and mutations of these genes in humans causes primary microcephaly. ATRX function on chromatin dynamics and architecture during meiosis will be required to clarify these issues.

Several genes involved in chromosome cohesion cause multiple developmental abnormalities when mutations arise in humans. The best examples to date include the NIPBL (delangin), SMCA1, and SMCA3 genes mutated in Cornelia de Lange syndrome (CdLS) and the ESCO2 gene mutated in Robert’s syndrome (Krantz et al., 2004; Vega et al., 2005; Musio et al., 2006; Borck et al., 2007; Deardoff et al., 2007). All of these factors are components of the cohesion complex or regulators of cohesion loading or unloading. Syndromes caused by ATRX mutations display partial phenotypic overlap with CdLS, including cognitive delay, craniofacial dysmorphism, microcephaly, gastrointestinal defects, and problems with the genitourinary system, hearing, and ocular development. Another similarity between ATRX and NIPBL is a shared protein motif that confers the ability to associate with the HP1α CSD (Lechner et al., 2005).

ATRX now joins other chromatin remodeling complexes implicated in cohesion, such as RSC and Snf2h/NURD, either by loading cohesion during DNA replication or recruiting the cohesion complex to specific chromosomal regions (Hakimi et al., 2002; Baetz et al., 2004; Huang et al., 2004; Huang and Laurent, 2004). Although the cohesion complex is required for normal chromatid cohesion during mitosis, it also has a function in facilitating long-range enhancer–promoter interactions. The developmental abnormalities in CdLS and the ATRX syndromes could therefore be explained by the specific deregulation of gene expression at loci that rely on long-range enhancer interactions mediated by the cohesion complex and its regulators. An important focus of research will therefore be to identify such target genes and determine how chromosome cohesion might control their expression.

Materials and methods

Cell culture and transfection

HeLa (American Type Culture Collection) and HG cells (a gift from G.M. Wahl, Salk Institute for Biological Studies, La Jolla, CA) were grown at 37°C with 5% CO2 in DME supplemented with 10% FBS (Sigma-Aldrich). HG growth media was supplemented with 2 μg/ml blastocidin S HCl (Invitrogen) to maintain transgene expression. For siRNA transfection, cells were seeded 24 h before transfection using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The final siRNA concentration used in all experiments was 8 nM. In HeLa cells, the transfection efficiency was 90–95% based on immunofluorescence detection of the ATRX protein isoforms. For population synchronization at G1/S, transfected cells were incubated with 10 μM hydroxyurea and released after 16 h by removal of the drug.

RNA interference

The synthetic oligonucleotides siATRX1 (5'-GAGGAAACUCUACUGUAUUAU3'), siATRX2 (5'-GCGAGAAAGUCUAAAGAUU3'), and siATRX1 scrambled (5'-GAAUUGACUGAUUCUACAUU3') were obtained from Dharmacon. The control (nontargeting) duplex was obtained from Sigma-Aldrich. siRNA duplexes were transfected with Lipofectamine 2000 according to the manufacturer’s instructions. Mock-transfected samples were treated similarly but without the addition of siRNA.

Western blot analysis

Cells were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0, 0.5% deoxycholic acid, 0.1% SDS, 0.2 mM PMSF, 0.5 mM NaF, 0.1 mM NaVO4, and 1 protease inhibitor cocktail tablet [Complete mini, EDTA-free; Roche]) for 5 min on ice. Extracts were sonicated and quantified using the DC protein assay (Bio-Rad Laboratories). 20 μg of protein was resolved on 6 or 12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were probed with rabbit α-ATRX H300 (Santa Cruz Biotechnology, Inc.), mouse α-ACTR H300 (Santa Cruz Biotechnology, Inc.), mouse α-ATRX 391 (a gift of D.J. Picketts, Ottawa Health Research Institute, Ottawa, Canada; and D. Higgs, University of Oxford, UK), followed by the appropriate horseradish peroxidase–conjugated secondary antibody (1:10,000, GE Healthcare). After washing, the membrane was incubated in ECL before exposure to x-ray film. The membrane was reprobed with mouse α-α-tubulin (1:40,000, Sigma-Aldrich) as a loading control.

Q-RT-PCR analysis

Total RNA was isolated using the RNeasy mini kit (QiAGEN). First-strand cDNA was synthesized from 3 μg of total RNA using random primers and a reverse transcription cocktail containing 5x first strand buffer, 100 mM DTT, 25 mM dNTPs, RNA guard (GE Healthcare), and Superscript RT (Invitrogen). PCR reactions were performed on a continuous fluorescence detector (Chromod4; MJ Research) in the presence of iQ SYBR green supermix (Bio-Rad Laboratories) and analyzed with OptiGene 3 and Genex software (Bio-Rad Laboratories) using the standard curve corresponding threshold method of quantification. Samples were ampliﬁ ed as follows: 95°C for 30 s, 55°C for 30 s, 0.1 mM Na3VO4, and 1 protease inhibitor cocktail tablet [Complete mini, EDTA-free; Roche]) for 5 min on ice. Extracts were sonicated and quantified using the DC protein assay (Bio-Rad Laboratories). 20 μg of protein was resolved on 6 or 12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were probed with rabbit α-ATRX H300 (Santa Cruz Biotechnology, Inc.), mouse α-ACTR H300 (Santa Cruz Biotechnology, Inc.), mouse α-ATRX 391 (a gift of D.J. Picketts, Ottawa Health Research Institute, Ottawa, Canada; and D. Higgs, University of Oxford, UK), followed by the appropriate horseradish peroxidase–conjugated secondary antibody (1:10,000, GE Healthcare). After washing, the membrane was incubated in ECL before exposure to x-ray film. The membrane was reprobed with mouse α-α-tubulin (1:40,000, Sigma-Aldrich) as a loading control.

Flow cytometry

Exponentially growing cells were harvested, washed with calcium/magnesium-free (CMF) PBS [137 mM NaCl, 2.7 mM KCl, 1.5 mM potassium phosphate monobasic, and 12 mM sodium phosphate dibasic] three times and 1.5 × 10⁶ cells were fixed dropwise with 90% ethanol and stored at -4°C for at least 12 h. To detect DNA content, cells were washed and stained with propidium iodide/RNase staining solution (Sigma-Aldrich), and 2% BSA in CMF PBS for 30 min at room temperature followed by overnight incubation at 4°C. Cell populations were analyzed by flow cytometry on an EPICS XL-MCL instrument (Beckman-Coulter). Data analysis to determine the proportion of cells in each phase of the cell cycle was performed using the Expo 32 software package (Beckman Coulter).

Indirect immunofluorescence microscopy

For immunofluorescence detection, cells were fixed with 3:1 ethanol/methanol up to 4 d after control or siRNA transfection and incubated with the following.
primary antibodies: α-ATRX H300 (1:200; Santa Cruz Biotechnology, Inc.), α-ATRX 39f (1:20; provided by D.J. Picketts), α-ubulin (1:1,500; Sigma-Aldrich), α-phosphohistone H3(S10) (1:200; Millipore), human α-CREST (1:10,000; W. Brinkley, Baylor College of Medicine, Houston, TX), α-BubR1 (1:200; BD Biosciences), α-ub1 (1:1,000; a gift from S. Taylor, University of Manchester, Manchester, UK), α-CENPF (1:400; Santa Cruz Biotechnol- ogy, Inc.), α-CENPF [a gift from S. Taylor], α-Holo (1:200; Millipore), and α-H1B (1:200; Millipore). Secondary antibodies included goat α-rabbit Alexa 594 (1:1,500), donkey α-rabbit Alexa 488 (1:1,500), goat α-mouse Alexa 488 (1:1,500), goat α-mouse Alexa 594 (1:1,500), and goat α-human Alexa 647 (1:1,500; Invitrogen). Coverslips were mounted with Vectashield H-1000 (Vector Laboratories).

**Generation of ATRX-depleted stable clones**

Pairs of sense 60-mer oligonucleotides corresponding to the 19-mer siATRX1 and siATRX2 siRNA target sequences and their reverse complement (underlined sequences) were designed that contained 5’ BglII and 3’ HindIII restriction sites to facilitate cloning (Integrated DNA Technologies, Inc.). Sequences (underlined sequences) were designed that contained 5’ GAATTTCTCTG TTTTTA-3', and shATRX2 (sense), 5’ GATCCCC GCAGAGAAATTCCTAAAGA TTCAAGAGATCTTTAGGAATTTCGTCCTTTTTA-3’, and shATRX2 (sense), 5’ GATCCCC GCAGAGAAATTCCTAAAGA TTCAAGAGATCTTTAGGAATTTCGTCCTTTTTA-3’.

The oligonucleotides were annealed to complimentary antisense 60-mer oligonucleotides in buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, and 1 mM EDTA at 90°C for 4 min followed by 70°C for 10 min, and then step cooled to 37°C for 20 min using a thermocycler. Exponentially growing HG were transfected with empty pSuper for 10 min, and then step cooled to 37 °C for 20 min using a thermocycler. Secondary antibodies included goat α-rabbit Alexa 594 (1:1,500), donkey α-rabbit Alexa 488 (1:1,500), goat α-mouse Alexa 488 (1:1,500), goat α-mouse Alexa 594 (1:1,500), and goat α-human Alexa 647 (1:1,500; Invitrogen). Coverslips were mounted with Vectashield H-1000 (Vector Laboratories).

**Time-lapse and live cell microscopy**

Hela-HG, HG+Super, HG>shATRX1, or HG>shATRX2 cells were plated on 35-mm glass-bottom tissue culture dishes (MatTek) in DME with 10% FBS. After 24 h, the media was replaced with CO2-independent media (Invitrogen) supplemented with 10% FBS and 4 mM l-glutamine (Sigma-Aldrich). For transient experiments, cells were plated and transfected and scored 48–72 h after transfection. Cells were imaged using an automated inverted microscope (DMI 6000b; Leica) equipped with a live cell stage-mounted environment chamber [New Eclose] to maintain the cells at 37°C during imaging. Phase-contrast and fluorescence [GFP] images were captured every 3 min for 10 h using OpenLab Software automation (5.0; PerkinElmer).

To measure mitotic duration, cells that initiated mitosis (determined by nuclear envelope breakdown) and reentered G1 (nuclear decondensation) within the time frame of the experiment (10 h) were analyzed [n > 50]. This analysis did not include cells that were arrested at prometaphase or that died during the timeframe of the experiment. To measure the frequency of unaligned chromosomes at metaphase, cells were seeded in 35-mm culture wells (BD Biosciences) in DME with 10% FBS and at least 500 metaphase cells were scored per sample. For mitotic spindle checkpoint arrest, Hela-HG or HG>shATRX1 cells were treated with 100 ng/ml nocodazole for 16 h. Rounded anaphase cells and adherent interphase cells were quantified using live cell microscopy (n > 1000).

**Fixed chromosome spreads**

Hela cells were transiently transfected with no siRNA duplex (mock), siATRX1 scrambled (nonspecific control), siATRX1 or siATRX2. 70 h after transfection, and cloned cells were analyzed by shake off and individual cells were treated with 100 ng/ml KaryoMAX Colcemid (Invitrogen) for 2 h. Mitotic cells were then isolated and incubated in hypotonic solution (75 mM KCl) for 20 min followed by fixation in Carnoy’s Fix (3:1 methanol/acetic acid) and stored at −20°C. Fixed cells were dropped onto Superfrost Plus glass microscope slides (Thermo Fisher Scientific) and air dried. DNA was counterstained with 100 ng/ml DAPI and 2 μg/ml propidium iodide in Vectashield H-1000. For z-stack imaging, 0.4-μm interval z stacks were captured and deconvolved using iterative restoration with Velocity imaging software (4.0; PerkinElmer).

**Measure of interkinetochore distances**

To measure interkinetochore distance, mitotic Hela-HG, HG+Super, HG>shATRX1, and HG>shATRX2 stable cells were fixed in 3:1 ethanol/methanol and the kinetochores were stained using the human CREST antibody (1:10,000) and imaged using a 63x 1.4 NA oil immersion objective (Leica). Z stacks were captured at 0.4-μm z intervals spanning 20 μm. Kinetochores pairs were located from the outer edges of the CREST signal and the distance was measured using Velocity software. Only kinetochores pairs in single optical sections that were aligned at the metaphase plate were used to measure interkinetochore distances (n ≥ 100). Statistical differences were assessed by ANOVA. Significant differences in mean interkinetochore distance were assessed by ANOVA and a Tukey’s multiple comparison post hoc test. Differences were considered significant when P < 0.05. Statistical analysis was performed using GraphPad Prism (4.02; GraphPad Software, Inc.).

**Kinetochore microtubule assay**

Hela-HG, HG+Super, HG>shATRX1, or HG>shATRX2 cells were seeded in 35-mm dishes (Corning) in 2 ml DME with 10% FBS on 22-mm glass coverslips (VWR). After 48 h, the growth medium was replaced with ice-cold growth medium and the cells were incubated on ice for 10 min. The cells were rinsed twice with ice-cold PHEM buffer (60 mM piperazone ethanesulfonic acid, 45 mM Heps, 10 mM EGTA, and 2 mM MgCl2; pH 6.9), permeabilized with cold 0.5% Triton X-100 in PHEM buffer for 3 min, and fixed in 3:1 paraformaldehyde in PHEM at 15 min. The coverslips were rinsed with PHEM and processed for immunofluorescence staining and microscopy.

**Analysis of mitotic cells in the developing telencephalon**

The generation of ATRX-loxP mice was described previously [obtained from D. Higgs and R. Gibbons, University of Oxford; Berube et al., 2005; Garrick et al., 2006]. Male embryos conditionally deficient for Atrx were obtained by crossing homozygous Atrx-loxP females to heterozygous Foxg1Cre knock-in male mice and embryonic yolk sac DNA from E13.5 embryos was geno- typed by PCR as described previously [Berube et al., 2005]. Midday of the day of vaginal plug discovery was considered to be E0.5. At scheduled time points, pregnant females were anesthetized with CO2 and killed by cervical dislocation. Embryos and postnatal brains were fixed in 4% paraformaldehyde/PBS overnight at 4°C, sunk in 30% sucrose in PBS, and embedded in 15% sucrose and 50% optimal cutting temperature compound (Sakura). Tissue sections were cut at 10-μm thickness and mounted on SuperFrost Plus slides, air dried at room temperature, and stored at −80°C. For immunofluorescence staining, sections were thawed, rehydrated in PBS for 10 min, counterstained with DAPI, and mounted in Vectashield. Micronuclei and misaligned chromosomes from the mitotic layer lining the lateral ventricle were scored from both hemispheres of brain sections (n = 3) in four litter-matched control and ATRX null embryos using fluorescence microscopy. Analysis was restricted to the mitotic layer that lines the hippocampal hem, hippocampal primordium, and the dorsal cortical neuroepithelium (indicated by the area between 1 and 2 in Fig. 5 C).

**Details of image acquisition and processing**

All samples processed for microscopy were imaged using a DMI 6000b automated inverted microscope. If images were captured using a 63x 1.4 NA oil immersion lens, and 1.25 NA oil immersion lens (Leica), or a 5x dry objective (Leica). For oil immersion microscopy, we used immersion oil with a refractive index of 1.518 (Leica). All images were captured at ambient temperature except for image capture of live cells and time-lapse experiments, which were performed at 37°C. Experiments used different combinations of DAPI, goat anti–rabbit Alexa 594, goat anti–mouse Alexa 594, donkey anti–rabbit Alexa 488, goat anti–mouse Alexa 488, and goat anti–human 594 secondary antibodies. Digital microscopy images were captured with a digital camera (ORCA-ER; Hamamatsu). Openlab imaging software was used for manual and automated image capture and processing was performed using Velocity. All deconvolution was performed using iterative restoration set with a confidence limit of 95%.

**Online supplemental material**

Fig. S1 shows the validation of ATRX depletion in Hela cells. Fig. S2 shows prolonged mitotic time in ATRX-depleted cells. Fig. S3 shows H1B staining in ATRX-depleted cells. Fig. S4 shows abnormal spindle morphology in ATRX-depleted cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200706083/DJC1.

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