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Loss of Atrx Affects Trophoblast Development and the Pattern of X-Inactivation in Extraembryonic Tissues

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ATRX is an X-encoded member of the SNF2 family of ATPase/helicase proteins thought to regulate gene expression by modifying chromatin at target loci. Mutations in ATRX provided the first example of a human genetic disease associated with defects in such proteins. To better understand the role of ATRX in development and the associated abnormalities in the ATR-X (alpha thalassemia mental retardation, X-linked) syndrome, we conditionally inactivated the homolog in mice, Atrx, at the 8- to 16-cell stage of development. The protein, Atrx, was ubiquitously expressed, and male embryos null for Atrx implanted and gastrulated normally but did not survive beyond 9.5 days postcoitus due to a defect in formation of the extraembryonic trophoblast, one of the first terminally differentiated lineages in the developing embryo. Carrier female mice that inherit a maternal null allele should be affected, since the paternal X chromosome is normally inactivated in extraembryonic tissues. Surprisingly, however, some carrier females established a normal placenta and appeared to escape the usual pattern of imprinted X-inactivation in these tissues. Together these findings demonstrate an unexpected, specific, and essential role for Atrx in the development of the murine trophoblast and present an example of escape from imprinted X chromosome inactivation.


Introduction

ATR-X syndrome is a severe, nonprogressive form of X-linked mental retardation that is frequently associated with multiple congenital abnormalities [1]. It is usually associated with a mild form of α-thalassaemia, caused by reduced expression of structurally intact α-globin genes, and characterised by the presence of β-globin tetramers (haemoglobin H inclusion bodies) in peripheral red blood cells. Carrier females occasionally manifest haemoglobin H inclusions, but are otherwise intellectually and physically normal. Studies of X-chromosome inactivation in carrier females have demonstrated preferential inactivation of the chromosome bearing the abnormal allele in a variety of tissues [2], and this skewing of X-inactivation is thought to explain the mild phenotype observed in carriers. The ATR-X syndrome is caused by mutations in a gene (ATRX) that comprises 36 exons spanning 300 kb of genomic DNA at Chromosome Xq13.3 [3]. This gene encodes two dominant protein isoforms (Figure 1). As well as the full-length ATRX protein of ~280 kDa, which is encoded by a transcript of ~10 kb, we recently demonstrated that a truncated isoform called ATRXt (~200 kDa) is produced from a transcript of around 7 kb, which arises when intron 11 fails to be spliced from the primary transcript and an alternative intronic poly(A) signal is used [4]. The mouse homolog of the ATRX gene, Atrx, is also situated on the X chromosome, and also gives rise to full-length (Atrx, ~280 kDa) and truncated (Atrx, ~200 kDa) isoforms [4,5]. Disease-causing missense mutations are clustered in two regions of the gene: a PHD-like zinc finger domain and a SNF2-like ATPase domain (Figure 1) [6]. The former motif is thought to be involved in protein-protein interactions in chromatin [7], and the latter is a feature of chromatin-remodelling proteins, and the presence of disease-causing mutations indicates the functional importance of these domains. ATRX has been shown to remodel chromatin [8]. It also interacts with HP1 at heterochromatin [9] and is recruited to promyelocytic leukemia nuclear bodies via an interaction with Daxx [10]. Furthermore, disruption of ATRX leads to diverse changes in DNA methylation [11]. Nevertheless, the role ATRX plays in gene expression remains unclear.

The consistent core of clinical and haematological features observed in ATR-X patients suggests that, like the SWI2/SNF2 chromatin-remodelling protein, ATRX probably regulates transcription of a discrete set of target genes. However, although there are clearly others to be found, at present the...
Synopsis

ATRX belongs to a class of proteins that may modify how DNA is packaged into chromatin, altering the accessibility of other proteins in the nucleus to DNA. In this way, ATRX is thought to influence gene expression. Mutations in the ATRX gene, which is located on the female sex chromosome (X), provided the first example of a human disease (ATR-X syndrome) associated with defects in such proteins. Affected males (X(MUT)Y) have multiple developmental abnormalities in a wide variety of systems. Currently, it is not understood how proteins like ATRX influence cell biology. To address this question, the authors deleted the version of the gene in mice, *Atrx*. Although affected male mice (X(MUT)Y) started to develop normally, they died early in development because they failed to form a normal placenta. In the placenta, female mice normally inactivate the X chromosome that they inherit from their fathers (Xp), so if females inherit from their mother an X chromosome (Xm) that bears the abnormal copy of *Atrx* (X(MUT)Xp), one would predict that, like affected males, they would fail to form a normal placenta. The authors unexpectedly found this not to be so. They showed, instead, that in such females the normal, paternally derived *Atrx* gene is active. This study has therefore demonstrated an important facet of X-chromosome imprinting.

Results

Generation of ES Cells Lacking Full-Length Atrx

Like the human gene, the mouse *Atrx* gene is also X-linked, such that a direct disruption of the single *Atrx* allele in male ES cells would immediately give rise to the null state. No targeted clones were recovered after attempted homologous recombination in male E14TG2a ES cells using two different vectors. Affected male mice (X(MUT)Y) have multiple developmental abnormalities in a wide variety of systems. Currently, it is not understood how proteins like ATRX influence cell biology. To address this question, the authors deleted the version of the gene in mice, *Atrx*. Although affected male mice (X(MUT)Y) started to develop normally, they died early in development because they failed to form a normal placenta. In the placenta, female mice normally inactivate the X chromosome that they inherit from their fathers (Xp), so if females inherit from their mother an X chromosome (Xm) that bears the abnormal copy of *Atrx* (X(MUT)Xp), one would predict that, like affected males, they would fail to form a normal placenta. The authors unexpectedly found this not to be so. They showed, instead, that in such females the normal, paternally derived *Atrx* gene is active. This study has therefore demonstrated an important facet of X-chromosome imprinting.

α-globin genes remain the only confirmed targets for transcriptional regulation by ATRX. Little is currently known about the precise role of the ATRX protein during mammalian development. To investigate the role of this protein during mouse development, we generated a conditionally deleted allele of the *Atrx* gene in mouse embryonic stem (ES) cells, and used these cells to examine the effect of ablating expression of the full-length Atrx protein in ES cells and in mouse embryos.

Perturbed Growth and Methylation Defects in Atrxnull ES Cells

*Atrx*null ES cells could be maintained in culture but were generally slower growing than *Atrx*+/− ES clones, and appeared to undergo higher rates of spontaneous differentiation. We investigated directly the effect of Atrx on ES cell growth by comparing Atrx+/− and Atrxnull ES cell clones in competition

Figure 1. Schematic Representation of the ATRX Isoforms

Shown at the top is the human *ATRX* cDNA. The boxes represent the 36 exons. The introns are not to scale. The alternative splicing of exons 6 and 7 is indicated. Shown underneath are the two *ATRX* protein isoforms. Full-length ATRX (~280 kDa) is encoded by the largest open reading frame. The positions of the principal features (the PHD-like domain and the seven SWI/SNF-like motifs) are indicated. Above full-length ATRX is shown the truncated ATRXt isoform (apparent molecular weight of ~200 kDa) that arises through the failure to splice intron 11 and the use of an intronic poly(A) signal. The intron-encoded region of ATRXt is indicated as a filled grey box. Locations of recombinant proteins (A2, FXN5P, and H-300) used to generate antibodies are shown. The scale bar represents 200 amino acids.

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cultures. Equal numbers of Atrx<sup>+</sup> (bearing either an Atrx<sup>WT</sup> or an Atrx<sup>flox</sup> allele) and Atrx<sup>−/−</sup> (bearing an Atrx<sup>Δ18Neoeo</sup> allele) ES cells were inoculated into cultures and the mixed cultures were passaged (1:3 split) every 2 d for 8–10 d. The relative abundance of the different alleles in the culture at each time point was analysed by Southern blotting (Figure 3A). The clone containing the Atrx<sup>−/−</sup> allele was rapidly outgrown by both Atrx<sup>WT</sup> ES cells and cells bearing the Atrx<sup>flox</sup> allele. In

Figure 2. Cre-Mediated Ablation of Full-Length Atrx Protein in ES Cells

(A) Strategy for targeted deletion of exon 18 of the Atrx gene. The top line shows the wild-type allele (Atrx<sup>WT</sup>) at the region surrounding exon 18. Below is shown the targeting vector and the targeted allele (Atrx<sup>flox</sup>) resulting from homologous recombination. The loxp target sites of the Cre recombinase are shown as black triangles, and the three possible recombination events that can be mediated by the Cre recombinase are indicated (labelled A, B, and C in the Atrx<sup>flox</sup> allele). At bottom is shown the Cre-recombined allele (Atrx<sup>Δ18Neoeo</sup>) (resulting from recombination event C) in which both exon 18 and the MC1Neo<sup>A</sup> selection cassette have been deleted. EcoRI (labelled E) and SacI (labelled S) sites present on the targeted 129 strain X chromosome are indicated. Black bars indicate the positions of the probes used in Southern blots.

(B) Southern blot analysis of EcoRI-digested DNA from either wild-type ES cells (E14) or targeted ES cell clones bearing the Atrx<sup>flox</sup> allele (1/F12 and 1/G11) hybridised with either the 20/27 (left blot) or Hae0.9 (right blot) probes. The EcoRI fragment of the Atrx<sup>WT</sup> allele (18.5 kb) has been replaced with the expected fragments of 11.2 kb (20/27 probe) or 8.5 kb (Hae0.9 probe).

(C) Southern blot analysis of SacI-digested DNA from either wild-type ES cells (E14) or targeted ES cell clones bearing the Atrx<sup>flox</sup> allele (1/F12 and 1/G11) or Cre-recombinant clones derived from these (1/F12B1F12 and 1/G11D5). The membrane was hybridised with the intron 17 probe indicated in (A). The expected bands of 6.2 (Atrx<sup>WT</sup>), 5.0 (Atrx<sup>flox</sup>), and 2.8 (Atrx<sup>Δ18Neoeo</sup>) kb were observed.

(D) Northern blot analysis of RNA from ES cells shown in (C). The membrane was hybridised first to a probe from exon 10 of the Atrx gene (top blot) and subsequently to a β-actin cDNA probe as loading control (bottom blot). The transcripts responsible for full-length Atrx (~10 kb) and the truncated Atrx isoforms (~7 kb) are indicated.

(E) Western blot analysis of whole-cell extracts from the clones shown in (C) using an anti-ATRX monoclonal antibody (23C, raised against peptide A2 of the human ATRX protein shown in Figure 1). The full-length and truncated Atrx isoforms are indicated.

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Figure 3. Growth and Methylation Defects in Atrxnull ES Cells

(A) Cultures were inoculated with equivalent numbers of ES cells bearing different Atrx alleles as indicated, and were serially passaged. After the indicated days of coculture, DNA extracted from a sample of cells was analysed by Southern blot to detect the Atrx alleles. DNA was digested with SpeI, and the membrane was hybridised with the 20/27 probe shown in Figure 2A. The expected sizes of the different alleles are indicated.

(B) Schematic diagram of the transcribed portion of the mouse rDNA repeat with the 18S, 5.8S, and 28S genes indicated. The positions of the limit-digesting enzymes BamH (labelled B) and EcoRI (labelled E) and the probes (RIB3 and RIB4) used in the Southern blots shown in (C) are indicated. Below are shown the locations of the methylation-sensitive enzymes (SmaI, PvuI, and MluI) whose methylation status has been analysed in the Southern blots shown in (C).

(C) DNA from Atrx-positive (Atrx\textsuperscript{WT}, bearing either an Atrx\textsuperscript{WT} or Atrx\textsuperscript{floxed} allele) or Atrxnull (bearing the Atrx\textsuperscript{Al\&\text{neo}} allele) ES cells and 7-d embryoid bodies were digested with the enzymes shown and analysed by Southern blotting using the probes indicated. Arrows indicate the fully methylated copies (cut by only the limit-digesting enzyme). Phosphorimagier quantitation of the blots are shown below. The y-axis shows the percentage of copies that are undigested by the methylation-sensitive enzyme as a percentage of the total signal from cut and uncut rDNA. Mean values are indicated by horizontal lines, and the significance of the differences between the Atrx-positive and Atrxnull populations are shown for each enzyme.

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a control competition experiment between different clones bearing functional Atrx alleles \((Atrx^{WT} \text{ and } Atrx^{flox})\), both clones continued to be equally represented after 8 d of cocultivation. Thus, although Atrxnull ES cells could be recovered and maintained in culture by a conditional targeting approach, these cocultivation experiments suggested that the absence of Atrx does negatively impact upon normal ES cell growth.

To investigate a possible cell-cycle defect in the absence of Atrx, we analysed the cell cycle distribution of bromodeoxyuridine (BrdU)-pulsed ES cells by flow cytometry (Figure S1A). Surprisingly, both Atrxnull ES cell clones exhibited a cell cycle profile that was indistinguishable from ES cells bearing a functional Atrx allele \((Atrx^{WT} \text{ or } Atrx^{flox})\). We also specifically quantitated the mitotic index within each population by flow cytometry after staining ES cells for phosphorylated (Ser10) histone H3, a specific marker of mitosis (Figure S1B) [13]. Consistent with the normal cell-cycle profile observed above, there was no depletion in the size of the mitotic population in the Atrxnull ES clones, despite their slow growth. Finally, we investigated whether the growth defect in the Atrxnull ES cells was due to an up-regulation of apoptosis by staining cells with Annexin V (Figure S2) and found that the proportion of apoptotic cells was not significantly affected by the absence of full-length Atrx. Thus, the growth defect observed in ES cells lacking Atrx is not due to a specific cell cycle block or significant induction of cell death. While the cause of the proliferative delay is not yet clear, since Atrxnull ES cells appear to undergo higher rates of spontaneous differentiation (unpublished data), it seems likely that the observed growth defect reflects the spontaneous transition from fast-cycling, undifferentiated ES cells into more slowly cycling, differentiated cell types in these cultures.

It has been shown that disease-causing mutations in the human ATRX gene give rise to changes in the normal pattern of DNA methylation at several repetitive sequences within the human genome [11]. Notably, the transcribed region of the ribosomal DNA (rDNA) repeat was found to be significantly hypomethylated in ATR-X patients relative to normal individuals. Using methylation-sensitive restriction enzymes, hypomethylated in ATR-X patients relative to normal ribosomal DNA (rDNA) repeat was found to be significantly

**Early Embryonic Lethality in Atrxnull Male Mice**

To investigate the role of the Atrx protein during mouse development, we initially established lines of mice bearing the Atrxnull allele. Two independently targeted Atrxnull ES cell clones with normal male karyotype were injected into C57BL6 blastocysts to produce chimaeric mice, which were then used to obtain germline transmission. Intercrosses between males hemizygous \((Atrx^{flox}/Y)\) and females heterozygous \((Atrx^{WT}/Atrx^{flox})\) for the floxed allele were also carried out to generate homozygous females \((Atrx^{flox}/Atrx^{flox})\). Males hemizygous and females heterozygous or homozygous for the Atrxnull allele were viable, appeared healthy, and bred normally, suggesting that, as expected, the Atrxnull allele was functionally normal. To generate Atrxnull mice by Cre-mediated recombination, the Atrxnull mice were crossed with mice harboring a transgene in which the Cre recombinase is expressed under the control of the regulatory elements of the mouse GATA-1 gene \((GATA1-\text{cre})\) [14]. Widespread expression of the GATA1-cre transgene has been demonstrated during early embryogenesis [14]. We more accurately defined the onset of GATA1-cre expression using a ROSA26 reporter strain, in which a β-galactosidase neo` fusion reporter gene is expressed only after Cre-mediated excision of loxP-flanked transcription and translation termination signals [14]. We found that the GATA-1 cre transgene was already active at the 16-cell morula stage of development (0.5 days postcoitus [dpc]) (Figure 4A).

To generate Atrxnull mice, heterozygous floxed females \((Atrx^{WT}/Atrx^{flox})\) were mated with hemizygous GATA-1cre transgenic males \((Atrx^{WT}/Y;GATA1-\text{cre}^{+/-})\). No Atrxnull males \((Atrx^{null}/Y;GATA1-\text{cre}^{+/-})\) were recovered at birth, indicating that the absence of Atrx results in embryonic lethality. This finding was unexpected, since human ATR-X patients clearly survive to adulthood (see Discussion). Embryos were dissected at 7.5, 8.5, and 9.5 dpc and genotyped by PCR analysis of DNA extracted from yolk sac or total embryo (Figure 4B and Protocol S1). Atrxnull males were present at expected mendelian ratios (~25%) at both 7.5 dpc and 8.5 dpc (Table 1). However, by 9.5 dpc, depletion was observed both in the number of Atrxnull males (7%) and in the total number of males recovered (31%). No Atrxnull males were recovered after 9.5 dpc. Thus the absence of Atrx gives rise to embryonic lethality in mice before 9.5 dpc.

To investigate the morphology of Atrxnull embryos prior to death, embryos from the above crosses were initially dissected in their decidua at 7.5 dpc, and paraffin sections were stained with haematoxylin (Figure 5A) or with an anti-ATRX antibody (Figure 5B–5E). Immunohistochemical staining revealed that Atrx was widely expressed in wild-type 7.5 dpc embryos (Figure 5B), Expression was highest in the embryonic region (Figure 5C) and the chorion (Figure 5D). Detectable but lower levels of expression were observed in the ectlaplacental cone (Figure 5D) and surrounding decidual tissue. We also observed very high levels of Atrx expression in trophoblast giant cells (TGCS) surrounding the Reichert’s membrane (Figure S5E). Within the large nuclei of these TGCS, the typical nuclear association of Atrx with blocks of pericentromeric heterochromatin [15] was clearly observable. Only background staining was seen in the corresponding Atrxnull embryonic tissues (Figure S5B–5D), while expression in the surrounding decidual tissue (of maternal origin) was normal and served as an antibody staining control (unpub-
Trophoblast Defect in Mice Lacking Atrx

Morphologically, 7.5 dpc Atrxnull embryos were dramatically reduced in size and appeared developmentally retarded relative to stage-matched wild-type embryos (Figure 5A and 5B). However, despite their reduced size, the general morphology and organisation of embryonic structures in Atrxnull conceptuses appeared grossly normal. The amnion and chorion were clearly present and the amniotic, exocoelomic, and ectoplacental cavities were distinguishable, as were all three embryonic germ layers (Figure 5A–5C). At 8.5 dpc, embryos were dissected free of decidua, and observed in whole mount. Individual conceptuses were genotyped by PCR using DNA isolated from yolk sac as described in Protocol S1. Consistent with observations at 7.5 dpc, the general morphology of the embryo proper of Atrxnull conceptuses also appeared grossly normal at 8.5 dpc. The head fold had clearly formed, and expression of the early mesoderm marker brachyury (T) [16] was detected in the primitive streak and emerging notochord by whole-mount in situ hybridisation (WMISH) (Figure 5F), indicating that Atrxnull embryos had gastrulated.

To investigate whether the reduced size of the Atrxnull embryos was due to an increase in apoptosis, we analysed sections of paraffin-embedded 7.5 dpc embryos by TdT-mediated dUTP nick end labeling (TUNEL) assay (Figure 6A). Very few apoptotic cells were detected in wild-type 7.5 dpc embryos. In Atrxnull embryos, a slight increase in the apoptotic population was evident. However, consistent with our observation of a grossly normal apoptotic index in Atrxnull ES cells (Figure S2), the apoptotic response observed in Atrxnull embryos was also not uniform, but was restricted to a low number of scattered TUNEL-positive cells. Since this small apoptotic response is unlikely to account fully for the dramatic size deficit observed in Atrxnull embryos, a possible proliferation defect was also investigated by immunohistochemical staining of 7.5 dpc embryo sections for the mitosis marker phosphorylated (Ser10) histone H3 [13]. Relative to the very high mitotic index observed in wild-type embryos, the proportion of mitotic cells observed in Atrxnull embryos at 7.5 dpc was dramatically reduced (Figure 6B). Taken

### Table 1. Distribution of Atrx Genotypes in Timed Matings

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<th>Group</th>
<th>Female</th>
<th>Male</th>
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<tr>
<td></td>
<td>Wild Type</td>
<td>Carrier&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Atrx&lt;sup&gt;WT/WT&lt;/sup&gt;; GATA1-Cre&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>Atrx&lt;sup&gt;WT/WT&lt;/sup&gt;; GATA1-Cre&lt;sup&gt;–/–&lt;/sup&gt;</td>
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<td></td>
<td>7.5 dpc, number observed (%)&lt;sup&gt;b&lt;/sup&gt; (n = 39)</td>
<td>5 (13%)</td>
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<tr>
<td></td>
<td>8.5 dpc, number observed (%)&lt;sup&gt;b&lt;/sup&gt; (n = 59)</td>
<td>14 (24%)</td>
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<tr>
<td></td>
<td>9.5 dpc, number observed (%)&lt;sup&gt;b&lt;/sup&gt; (n = 71)</td>
<td>31 (44%)</td>
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Crosses were carried out initially between Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup> females and Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup> males. Litters were dissected at the times shown and genotyped by PCR as described in Protocol S1. These crosses would be expected to yield wild-type females (Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup>), carrier females (Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup>), wild-type males (Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup>) and null males (Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup>) in a ratio of 1:1:1:1. Subsequently, breedings were also carried out between females carrying a single recombined allele (Atrx<sup>WT/WT; Atrx<sup>Cre–/–</sup>recombinant</sup>) and wild-type males (Atrx<sup>WT/Y</sup>B6; GATA1<sup>Cre–/–</sup>), which would be expected to yield the same four Atrx genotypes in a 1:1:1:1 ratio as shown above. The data from these two breedings have been combined in the table, but the same trends were observed when these two crosses were considered separately.

<sup>a</sup>The Atrx<sup>WT/WT</sup>B6; GATA1<sup>Cre–/–</sup> allele is a combination of the Atrx<sup>WT/WT</sup>B6 and the Atrx<sup>Cre–/–</sup> B6 alleles (resulting from recombination events B and C in Figure 2A). Both alleles were recovered in combination with the GATA1<sup>Cre</sup> transgene and both are null for full-length Atrx protein (unpublished data).

<sup>b</sup>ND indicates that the Atrx genotype could not be determined by PCR. Sex was determined as described in Figure 4B.

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together, these results suggest that the size deficit observed in Atrxnull embryos prior to lethality largely reflects a proliferative defect, with a minor but indirect contribution from increased apoptosis. Although a growth defect was also observed in Atrxnull ES cells (Figure 3A), in contrast to the Atrxnull embryos, the mitotic index of the ES cell population (as measured with the same antibody) was not depleted (Figure S1B). These observations suggest that the mitotic defect observed in embryos is unlikely to be a direct, cell-autonomous effect of the absence of Atrx, and is more likely to be a secondary effect resulting from the failure to develop a normal trophoblast (see below).

Trophectoderm Failure in Atrxnull Embryos

Whole-mount observation of 8.5 dpc embryos revealed that, in contrast to the basically normal although delayed morphology of the embryo itself, the extraembryonic tissues of Atrxnull conceptuses appeared highly disorganised. When embryos were removed from decidua, the surrounding trophoblast...
layer appeared dramatically reduced in Atrxnull embryos relative to wild-type littermates, and the underlying ectoplacental cone appeared reduced and abnormally shaped (Figure 7A). Vacated deciduas surrounding 8.5 dpc wild-type and Atrxnull embryos were bisected and analyzed by WMISH for expression of placental lactogen-1 (Pl-1), a marker of terminally differentiated TGCs. The number of Pl-1-expressing cells attached to the decidual wall after removal of the embryo is an indication of the density of trophoblast cells surrounding each implantation site [17]. We found that the population of Pl-1-expressing cells was depleted in the decidual implantation sites containing Atrx null embryos relative to those containing wild-type littermates (Figure 7B); this was also apparent at 7.5 dpc, as determined by immunohistochemical staining of paraffin sections of embryos (Figure 7C). A TGC deficiency in the absence of Atrx is consistent with the observation that Atrx is highly expressed in giant cells surrounding wild-type 7.5 dpc embryos (Figure 5E).

To investigate whether the trophoblast defect was restricted to the production of secondary TGCs (produced by diploid precursors in the ectoplacental cone and derived originally from the polar trophoderm overlying the inner cell mass of the blastocyst) or also affected the production of primary TGCs (resulting from differentiation of the mural trophoderm of the blastocyst), blastocysts (3.5 dpc) from crosses between AtrxWT/WT females and GATA1-Cre homozygous transgenic males (AtrxWT/Y;GATA1-Cre+/+) were cultured in vitro for 5 d to monitor outgrowth of the primary trophoblast. After 5 d, individual blastocyst cultures were scored for the extent of primary trophoblast outgrowth, and the Atrx genotype and sex of the blastocyst were determined by PCR. Most blastocysts hatched from the zona pellucida within 24 h, and trophoblast cells spreading out from the inner cell mass could usually be detected within 48 h of culture. No difference was observed in the rate or extent of trophoblast outgrowth over 5 d of culture between Atrxnull blastocysts (Atrxnull/Y, n = 6) and blastocysts bearing an AtrxWT allele (AtrxWT/WT, n = 6; AtrxWT/null, n = 6; AtrxWT/Y, n = 6) (examples shown in Figure 7D), suggesting that the defect specifically involves the secondary giant cell compartment. This is consistent with the observation that Atrxnull conceptuses implant successfully and survive to gastrulation. Taken together, these data suggest that loss of Atrx results in a defect in formation of the secondary trophoblast that is apparent from 7.5 dpc. Despite initiating normal organization in the embryo proper, Atrxnull conceptuses exhibit a proliferative defect by 7.5 dpc and die by around 9.5 dpc.
probably due to a nutritional deficit resulting from failure to develop a normal trophoblast.

Escape from Imprinted Inactivation of the Paternally Inherited Atrx<sup>WT</sup> Allele in Extraembryonic Tissues of Carrier Female Mice

Female mice carrying an Atrx<sup>WT/null</sup> allele (Atrx<sup>WT/null</sup>; GATA1-cre<sup>/+</sup>) were detected at 9.5 dpc (Table 1) and recovered at birth (unpublished data), although at both time points the number of carrier females was lower than that of wild-type (Atrx<sup>WT/WT</sup>; GATA1-cre<sup>/+</sup>) females, suggesting that a proportion of carrier female embryos died in utero. Surviving adult carrier female mice were not phenotypically normal and exhibited mild behavioural abnormalities, although some could reproduce. For all Atrx<sup>WT/null</sup> carrier female embryos presented in Table 1, the Atrx<sup>WT</sup> allele was paternally derived, while the Atrx<sup>null</sup> allele was maternally derived. In the mouse, X chromosome inactivation is subject to parental imprinting in the trophectoderm and primitive endoderm lineages that give rise to the extraembryonic tissues, resulting in inactivation of the paternal X chromosome (Xp) [18]. In contrast, in tissues of the embryo proper (derived from the inner cell mass of the blastocyst) X-inactivation is random [19]. Thus, in the extraembryonic compartment of Atrx<sup>WT/null</sup> carrier females, normal imprinted X-inactivation would be expected to result in silencing of the paternally derived Atrx<sup>WT</sup> allele, leaving only the Atrx<sup>null</sup> allele on the active maternal X (Xm) and thereby render the extraembryonic tissues null for full-length Atrx protein. However, the absence of Atrx in the extraembryonic compartment is lethal in Atrx<sup>null/Y</sup> males. This suggested the possibility of an escape from imprinted inactivation of the paternally derived Atrx<sup>WT</sup> allele in the extraembryonic compartment of a proportion of carrier female embryos.

To investigate further, we crossed homozygous Atrx<sup>lox/lox</sup> females and homozygous GATA1-cre transgenic males (Atrx<sup>WT</sup>; Y; GATA1-cre<sup>+/+</sup>), which would be expected to yield only Atrx<sup>null</sup> males (Atrx<sup>null</sup>; Y; GATA1-cre<sup>+/+</sup>) and carrier females (Atrx<sup>WT/null</sup>; GATA1-cre<sup>+/+</sup>). In these carrier females, the Atrx<sup>WT</sup> allele is paternally inherited. Embryos were dissected in their decidua at 7.5 dpc, and paraffin sections were stained with anti-Atrx antibody, along with sections from a wild-type 7.5 dpc embryo for comparison (Figure 8). As described above, Atrx expression was detected in every cell in the epiblast (embryo proper) region of wild-type 7.5 dpc embryos (Figure 8B). In contrast, the epiblast region of carrier female embryos was composed of a mosaic of small clusters of Atrx-positive cells (in which the Atrx<sup>null</sup> allele on Xp had been inactivated) and Atrx-negative cells (in which the Atrx<sup>WT</sup> allele alone on Xp had been inactivated), indicating that the Atrx gene was subject to normal random X-inactivation in the epiblast. Remarkably, clear Atrx expression could also be detected in the extraembryonic tissues of carrier females, as shown in the extraembryonic-derived chorionic ectoderm (Figure 8C). Atrx expression could be detected in almost all cells of the chorionic ectoderm. Atrx expression was also clearly detected in other extraembryonic structures, including TGGs (unpublished data). Escape from silencing of an Xp-inherited Atrx<sup>WT</sup> allele was also observed in the chorionic ectoderm of carrier females at 8.5 dpc (Figure S4). Thus, although random X-inactivation occurs normally within the epiblast, the Atrx<sup>WT</sup> allele (inherited on the Xp chromosome) escaped the normal imprinted X-inactivation in the extraembryonic compartment of some carrier females.

Discussion

We investigated the role of the Atrx protein in mouse development. By using a conditional knockout approach, we ablated the full-length Atrx protein first in ES cells and embryoid bodies, and then in developing mouse embryos.

Atrx in ES Cells

Atrx<sup>null</sup> ES cells could not be recovered by direct targeting and were eventually generated by adopting a conditional targeting approach. This is consistent with our observation that Atrx is highly expressed in ES cells, and that the absence of full-length Atrx imparts a growth disadvantage relative to cells bearing a functional Atrx allele. At present, the cause of the proliferative delay in Atrx<sup>null</sup> ES cells is not known.
Interestingly, we demonstrated that apoptosis is not significantly up-regulated in ES cells lacking Atrx and is only mildly elevated in Atrx\textsuperscript{null} 7.5 dpc mouse embryos. In contrast, it was recently shown that the loss of Atrx markedly increased the apoptotic population in the differentiating cells of the embryonic cortex and postnatal hippocampus, when Atrx expression was ablated in the developing mouse forebrain using the Atrx\textsubscript{floxB} allele described here [20]. The human ATRX protein has been shown to associate in a complex with Daxx [8], a protein that has been implicated in multiple pathways for the regulation of apoptosis [21]. It is possible that disruption of the mouse Atrx-Daxx complex (by ablation of the Atrx protein) could have triggered a universal proapoptotic response. However, our observations in ES cells demonstrate that the induction of apoptosis is not an automatic response triggered by the removal of Atrx in all cell types, and suggest that the inappropriate apoptosis observed in the Atrx-mutant forebrain may reflect a requirement for Atrx during terminal differentiation.

### An Unexpected Role for Atrx in Development of the Mouse Trophoblast

We show that Atrx\textsuperscript{null} male mice are not viable and embryos die by around 9.5 dpc. Before death, Atrx\textsuperscript{null} embryos exhibit a markedly reduced mitotic index, suggesting a proliferative defect. Although the embryonic compartment of the conceptus appears initially normal, by 7.5 dpc Atrx\textsuperscript{null} embryos display abnormalities in development of the trophoblast, including a depletion in the population of TGCs surrounding the conceptus and a reduction in the size of the ectoplacental cone, which contains the diploid giant cell precursors [22]. TGCs are highly differentiated, postmitotic cells that ultimately form an epithelial layer at the periphery of the placenta, which interfaces with the maternal cells of the decidua [23]. These highly invasive cells are important for mediating initial invasion of the uterine tissue, but are also involved in remodelling the maternal decidua after implantation and in secreting hormones that regulate fetal and maternal growth [24]. Since Atrx\textsuperscript{null} embryos appear to implant normally, lethality is likely to arise due to a failure of TGC function later during development.

Embryonic lethality in mice in the absence of Atrx was a surprising finding, as there had been no suggestion of foetal loss in the human ATR-X syndrome. It is possible that the role of Atrx in the trophoblast is specific to mice and that ATRX has no role or is redundant in the human trophoblast. Indeed, the birth weight of babies with ATR-X syndrome is usually normal. An alternative explanation for the unexpectedly severe phenotype we observed in mice is that the Atrx\textsubscript{A18Aneo} deletion generated by Cre recombination completely ablates full-length Atrx protein (Figure 2E). In contrast, all disease-causing mutations characterised in human ATR-X pedigrees appear to give rise to hypomorphic alleles. Some full-length ATRX protein is detected in cases predicted to have truncating mutations (RJG, unpublished data), and residual ATPase activity in ATRX immunoprecipitates can be detected in Epstein-Barr virus-transformed lymphocytes of all human patients analysed to date (A. Argentaro and M. Mitson, unpublished data). The failure to observe a truly null ATRX allele among human patients strongly suggests that, as in the mouse, the complete absence of ATRX protein is incompatible with human fetal survival.

While this study has revealed an unexpected role for Atrx in the murine trophoblast, as a result of the early lethality observed in Atrx\textsuperscript{null} embryos it is not possible to rule out other roles for Atrx at later developmental stages in tissues of the embryo proper. Indeed, we show that Atrx is highly expressed throughout the entire developing embryo at 7.5 dpc (Figure 5B), and it is likely that Atrx function will turn out to be important for other differentiating tissues. Tetraploid aggregation experiments (in which mutant embryos are rescued with wild-type extraembryonic tissues) might shed more light on the role of Atrx during later mouse development, but these issues can be more subtly dissected by combining the conditional Atrx\textsubscript{floxB} allele that we have generated with different tissue-specific Cre transgenes. As mentioned above, this approach has already revealed a critical role for Atrx during neuronal differentiation in adult mice [20]. Further evidence that Atrx is also required at later stages of mouse development is provided by the observed dramatic skewing against Atrx-negative cells in some somatic tissues of carrier female mice, whose tissues initially comprise a mosaic of Atrx-positive and Atrx-negative cells as a result of random X-inactivation (M. Muers, personal communication).

Atrx joins an expanding list of mouse genes for which targeted disruption results in peri-implantation lethality as a result of trophoblast or placental abnormalities (reviewed in [25]). Comparison with other phenotypes might provide some insight into the role of Atrx in trophoblast development. Atrx-mutant embryos progress further than embryos nullizygous for factors involved in the initial specification of trophoblast stem cells (such as Cdx2) or in stem cell maintenance and proliferation (such as Eomes). Cdx2-mutant embryos fail to implant and die between 3.5 and 5.5 dpc [26], while Eomes-mutant blastocysts implant into the uterus, but arrest soon after implantation without forming organised embryonic or extraembryonic structures [27]. In contrast, Atrx-mutant embryos implant successfully and establish organised embryonic structures by 7.5 dpc. The Atrx-mutant phenotype closely resembles that observed in mice nullizygous for the basic helix-loop-helix transcription factor Hand1. Hand1-mutant conceptuses arrest at around 7.5 dpc and display a normal embryonic compartment, but, like Atrx-mutant embryos, ablation of Hand1 causes a reduction in the size of the ectoplacental cone and density of TGCs [28]. As with Atrx mutants, only arrested or resorbed Hand1-mutant conceptuses were recovered after 8.5 dpc. Also like Atrx, disruption of Hand1 specifically affects secondary giant cell formation, and primary trophoblast outgrowths from blastocysts appeared normal. Hand1 is required for terminal differentiation of secondary TGCs, and in its absence trophoblast cells arrest at a precursor stage in the ectoplacental cone [17,28]. Given the similarity of the Atrx- and Hand1-mutant phenotypes and the likelihood that Atrx acts as a transcriptional regulator by modifying chromatin structure, it will be of interest to determine whether Atrx is itself a regulator of Hand1 expression, or alternatively whether it acts as a coregulator of one or more of the downstream transcriptional targets of Hand1. It is noteworthy that, in the brain-specific Atrx knockout mice, the defect was observed in terminally differentiating neurons [20]. The secondary TGCs affected in the universal Atrx knockout reported here represent one of the first terminally differentiated tissues in the developing mouse, and this may point to the requirement for Atrx in the
high-level expression of some tissue-specific genes during the final stages of differentiation. Interestingly, the α-globin genes, the only confirmed transcriptional targets of regulation by human ATRX, are also highly expressed specifically during terminal differentiation within the erythroid lineage.

**Atrx Escapes Imprinted X-Inactivation in Extraembryonic Tissues of Carrier Female Mice**

Another surprising finding of this study is that, in carrier female embryos, a paternally inherited Atrx\(^{WT}\) allele appears to escape the process of imprinted X-inactivation, which ordinarily silences the Xp chromosome in the extraembryonic compartment of female murine tissues [18]. Silencing of the Atrx\(^{WT}\) allele on Xp should render these females null for Atrx in the extraembryonic tissues, since the normally active Xm chromosome carries the Atrx\(^{Xm}\) allele. Although not phenotypically normal, some Atrx carrier females developed to term and went on to reproduce. Thus, the failure to correctly silence the paternally derived Atrx\(^{WT}\) allele in the extraembryonic tissues of carrier females is consistent with our observations that in Atrx\(^{null}\) males, the Atrx protein plays an essential role in the development of the trophoblast and is necessary for survival in utero in the mouse.

The survival of Atrx carrier females contrasts with the phenotypes seen in carriers of mutations of other murine X-linked genes known to be essential in the extraembryonic compartment. For example, targeted disruption of the dyskerin (Dkc1), glucose 6-phosphate dehydrogenase (G6PD), and choroideremia (Chm) genes cause embryonic lethality in null male embryos through defects of the extraembryonic-derived tissues [29–31]. Female mice carrying mutations of these genes on the maternally inherited X chromosome also die in utero, whereas females that inherit the mutation on the Xp chromosome survive. Thus, unlike Atrx, these genes and/or their effects on cell growth are unable to circumvent the processes that ultimately cause all cells in the extraembryonic tissues to express only the maternally derived X chromosome. How might expression of the paternal Atrx\(^{WT}\) allele be maintained in the extraembryonic tissues of the Atrx carrier females?

One possibility is that, like some other X-linked genes, silencing of the Atrx gene on Xp is incomplete, such that there is always a low-level, leaky output of Atrx from a normally inactivated Xp chromosome in extraembryonic tissues. However, it was recently demonstrated that the paternal Atrx (called Xnp) allele is completely silenced in a normal mouse trophoblast stem cell line [32], suggesting that Atrx does not normally escape imprinted X-inactivation in the extraembryonic tissues of wild-type females. Thus, the expression of the Xp-linked Atrx\(^{WT}\) allele that we observed is unique to female carriers of the Atrx\(^{null}\) allele.

Perhaps a more likely explanation for this phenomenon stems from experimental observations suggesting that imprinted X-inactivation is not imposed on all precursors of the mouse extraembryonic tissues: A subpopulation of cells may escape this process and make a random “choice” of which X chromosome will be inactivated. On average, 50% of the cells in this randomly inactivating subpopulation would be expected to maintain an active Xp chromosome. In support of this hypothesis, it has been demonstrated that expression of paternally transmitted X-linked lacZ [33,34] and GFP [35] transgenes failed to be silenced in a small subpopulation of extraembryonic cells. Further, it has been shown that in a subpopulation of extraembryonic cells, it is the Xm rather than the Xp that undergoes late replication, a molecular correlate of the inactive state [18,36]. Although initially small and quickly diluted in normal embryos, the cellular subpopulation that inactivates the Xm chromosome could rapidly expand to replace the normally imprinted cells in extraembryonic lineages if the normal silencing of Xp compromises cell growth or differentiation. Interestingly, it has been suggested that the size of the population that initially escapes imprinting may range widely (from 0% to 30%), even between genetically identical embryos [37], and this may account for the variable phenotype observed among females bearing Xm-linked mutant alleles of genes essential for normal extraembryonic development [38]. Put simply, carrier females bearing a small initial population of escaping cells would be more severely affected than those bearing a larger population. This could explain why we have observed significant phenotypic variation among Atrx carrier females, with some carriers dying in utero by 9.5 dpc (Table 1) and others developing to term.

Another possible mechanism is that inactivation of the paternal X proceeds normally in all cells, but subsequently the Atrx gene within individual cells is reactivated. Alternatively, in the absence of Atrx, the paternal allele may partially escape the normal process of silencing. In both of these cases, other genes on the paternal X chromosome must be inactivated and remain so, since blocking inactivation of the entire Xp chromosome causes embryonic lethality due to biallelic expression of X-linked genes in the trophoblast [39].

**Summary**

ATR-X syndrome is the first human genetic disease known to be caused by mutations in a chromatin remodelling factor. At present we do not know how ATRX influences gene expression or what effect it has on cell behaviour. Nevertheless, we have previously noted that none of the natural mutations causing ATR-X syndrome are nulls, which suggests that it plays a critical role in normal development. Results of conditional inactivation of Atrx in the developing mouse forebrain, based on the Atrx\(^{lox}\) allele described here, shows that Atrx exerts a major effect on terminally differentiating neurons. Conditional inactivation of Atrx in other tissues is underway. Here we have shown that animal-wide disruption of the Atrx gene causes a severe embryonic-lethal phenotype, revealing an essential role for Atrx in the formation of the murine trophoblast. In addition, Atrx appears to escape imprinted X-chromosome inactivation in the extraembryonic tissues of some carrier female mice.

**Materials and Methods**

**Generation of ES cells bearing the Atrx\(^{lox}\) allele.** Briefly, the targeting vector (shown in Figure 2A) places a loxP site within intron 18 and a loxP-flanked MC1neoA selection cassette in intron 17 of the Atrx gene. A detailed description of the targeting construct is provided in [20]. Linearised plasmid (150 μg) was electroporated into 1 × 10⁶ E14TG2a ES cells, and colonies resistant to G418 and ganciclovir were isolated. Homologous targeting events were identified by Southern blot of EcoRI-digested DNA and hybridisation with a 5′ probe (generated with primers PPS1.20 and PPS1.27) and a 3′ probe (a 0.9-kb HaeIII fragment) as shown in Figure 2A and 2B. DNA from correctly targeted clones was also digested with SacI and analysed by Southern blot and hybridisation with a probe from...
within intron 17 (a PCR product generated with primers PPS1.15 and Xnp46) to confirm that the loxP site within intron 18 had been included within the crossed-over region (Figure 2A and 2C). Sequences of primers are shown in Table S1.

Crossed recombination and characterization of Atrx<sup>−/−</sup> ES cells and embryoid bodies. ES cell clones bearing the Atrx<sup>loxops</sup> allele (1 × 10<sup>7</sup> cells) were transiently transfected with 50 μg of uncut Cre expression plasmid (pCAGGS-Cre-IRESPuro) [40]. Following transfection, cells were plated at a range of clonal densities in complete medium without G418, and subclones were expanded and analysed for the presence of a recombinant locus initially by PCR, to detect deletion of the MClnepA cassette, and then by Southern blot and hybridisation with the intron 17 probe described above (Figure 2A and 2C). Northern blots were carried out according to standard techniques using 20 μg of total RNA isolated using TRI Reagent (Sigma-Aldrich, St. Louis, Missouri, United States). The blot was hybridised with a probe from within exon 10 of the Atrx gene (generated with primers Mxnp4 and Mxnp28 [Table S1]). After it was stripped, the stripped film was rehybridised with a 106 bp A allele probe (Clontech, Palo Alto, California, United States). Protein extraction and detection of Atrx by Western blotting was performed as described previously [4], using the mouse monoclonal anti-ATRX antibody 23C [15]. Analyses of cell cycle and apoptosis are described in Protocol S1. Methylation of rDNA was analysed in DNA from ES cell clones or from embryoid bodies recovered after 7 d of in vitro differentiation as described previously [41]. Genomic DNA was digested with methylation-sensitive restriction enzymes as described and analysed by Southern blotting. The RIB3 and RIB4 probes (which were complementary to the rDNA, non-repetitive, rDNA repeat) have been described previously [11]. Oligonucleotide probes to detect Line 1 and Sine B1 repeats have been described previously [42]. The minor satellite probe was a 27-mer oligonucleotide (mCENT2). The major satellite probe was a 27-mer oligonucleotide (Dp271). The IAP probe was an 8-400 bp PCR product (primers 14A and 13K) amplified from an IAP inserted into the mouse agouti gene [43] and was a gift from Peter Warnecke and Tim Bestor. The PCR product included the entire 5′ LTR of the IAP. All oligonucleotide sequences are shown in Table S1.

Generation of mice, crossed mice, and mutant mice. Targeted Atrx<sup>loxops</sup> ES cell clones were injected into C57BL/6 blastocysts and transferred into 2.5 dpc pseudopregnant recipients by standard techniques using 20 μg of total RNA isolated using TRI Reagent (Sigma-Aldrich, St. Louis, Missouri, United States). The blot was hybridised with a probe from within exon 10 of the Atrx gene (generated with primers Mxnp4 and Mxnp28 [Table S1]). After it was stripped, the stripped film was rehybridised with a 106 bp A allele probe (Clontech, Palo Alto, California, United States). Protein extraction and detection of Atrx by Western blotting was performed as described previously [4], using the mouse monoclonal anti-ATRX antibody 23C [15]. Analyses of cell cycle and apoptosis are described in Protocol S1. Methylation of rDNA was analysed in DNA from ES cell clones or from embryoid bodies recovered after 7 d of in vitro differentiation as described previously [41]. Genomic DNA was digested with methylation-sensitive restriction enzymes as described and analysed by Southern blotting. The RIB3 and RIB4 probes (which were complementary to the rDNA, non-repetitive, rDNA repeat) have been described previously [11]. Oligonucleotide probes to detect Line 1 and Sine B1 repeats have been described previously [42]. The minor satellite probe was a 27-mer oligonucleotide (mCENT2). The major satellite probe was a 27-mer oligonucleotide (Dp271). The IAP probe was an 8-400 bp PCR product (primers 14A and 13K) amplified from an IAP inserted into the mouse agouti gene [43] and was a gift from Peter Warnecke and Tim Bestor. The PCR product included the entire 5′ LTR of the IAP. All oligonucleotide sequences are shown in Table S1.

Immunohistochemistry, in situ hybridisation, and TUNEL assay. 7.5 dpc decidual swellings were dissected away from maternal tissue and fixed in 4% paraformaldehyde/PBS overnight at 4°C. After embryos were washed thoroughly in PBS, they were dehydrated through an ethanol series and xylene, embedded in paraffin, and sectioned at 5 μm. Sections were processed for immunohistochemistry using the ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, California, United States) according to the manufacturer's instructions. Sections were stained with rabbit polyclonal antibodies against ATRX (embryo proper) of a carrier female (Atrx<sup>WT/null</sup>) and Atrx<sup>−/−</sup> mice were crossed with GATA1-Cre transgenic mice as described in the text. Recombinant alleles were detected by Southern blotting as described above or by PCR as described in Protocol S1.

Supporting Information

Figure S1. Cell Cycle Analysis of Atrx-Positive and Atrx<sup>−/−</sup> ES Cells (A) Representative FACS profiles of BrdU-pulsed ES cells bearing either functional (Atrx<sup>+</sup> or Atrx<sup>−/−</sup>) or null (Atrx<sup>Alu<sub>Khom</sub></sup>) Atrx alleles showing BrdU-IFTG, (y-axis, logarithmic scale) against propidium iodide (x-axis, linear scale). The gated populations show cells in G1 (P<sup>+</sup>), BrdU-negative (R1), S (BrdU-positive) (R2), and G2/M (P<sup>y</sup>, BrdU-negative) (R3) phases of the cell cycle. Below is shown the quantitation of gated populations, indicating the percentage of cells in G1, S, and G2/M cell-cycle phases in each culture. (B) FACS profiles of ES cells bearing either functional (Atrx<sup>WT</sup> or Atrx<sup>Alu<sub>Khom</sub></sup>) or null (Atrx<sup>Alu<sub>Khom</sub></sup>) Atrx alleles stained for the mitosis marker phosphorylated (Ser10) histone H3 (FITC, y-axis, logarithmic scale) against propidium iodide (x-axis, linear scale). The size of the mitotic population (phosphoH3S10-positive, P<sup>y</sup> (gate R3)) is indicated for each profile. The ES cell clone analysed in each trace is indicated.

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Figure S2. Analysis of Apoptosis in Atrx-Positive and Atrx<sup>−/−</sup> ES Cells FACS analysis of ES cells bearing different Atrx alleles as shown, after staining for Annexin V (FITC, y-axis, logarithmic scale) and propidium iodide (x-axis, logarithmic scale). The size of the early apoptotic (Annexin-pos/BrdU-pos-P<sup>y</sup>), late apoptotic or necrotic (Annexin-pos, PI<sup>y</sup>) populations is indicated for each genotype.

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Figure S3. Normal DNA Methylatation at Mouse Repetitive Elements in Atrx<sup>WT</sup> ES Cells DNA from Atrx-Positive (bearing either an Atrx<sup>WT</sup> or Atrx<sup>Alu<sub>Khom</sub></sup> allele) or Atrx<sup>−/−</sup> (bearing the Atrx<sup>Alu<sub>Khom</sub></sup> allele) ES cells was digested with either a Cpg-methylation-sensitive enzyme HpaII (H) or its methylatation-insensitive isoschizomerMspI (M) as indicated, and digested DNA was analysed by Southern blotting. Membranes were hybridised with probes specific for the mouse Line 1 (A), Sine B1 (B), minor satellite (C), and IAP (D) repeat elements. No significant loss of CpG-methylation was observed at any of these repetitive elements in Atrx<sup>WT</sup> ES cells. As a positive control for loss of methylation, DNA from ES cells lacking either the Dnmt1 (Dnmt1<sup>−/−</sup>) or both the Dnmt3a and Dnmt3b (Dnmt3a<sup>−/−</sup>/Dnmt3b<sup>−/−</sup>) DNA methyltransferases was included in the analysis of Line 1 and Sine B1 repeats. Loss of CpG-methylation was clearly observed at these repetitive elements in these cell lines.

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Figure S4. Escape from Imprinted Inactivation in 8.5 dpc Carrier Female Embryo. A cross was carried out between a carrier female (Atrx<sup>WT/null</sup>) and wild-type male (Atrx<sup>WT/Y</sup>), and embryos were dissected in their decidua at 8.5 dpc. Any carrier female progeny of this cross will carry an Atrx<sup>WT</sup> allele on the Xp chromosome and an Atrx<sup>−/−</sup> allele on the Xm chromosome. Transverse paraffin sections were stained with the anti-ATRX antibody (H-300).

(A) High-magnification image (400X) of the neural fold region (nf) embryo proper of a carrier female (Atrx<sup>WT/null</sup>) embryo. This tissue is clearly comprised of a mosaic of Atrx-positive and Atrx-negative cells due to random X-inactivation. This section was counterstained with haematoxylin.

(B) High-magnification image (400X) of the same embryo depicted in (A), showing the extraembryonic derived-chorionic ectoderm (ce) and visceral endoderm (ve), two tissues that undergo imprinted X-inactivation. Although Atrx is poorly expressed in the visceral endoderm (even in wild-type females unpublished data), strong expression of Atrx can be seen in the chorionic ectoderm, indicating that the paternally-derived Atrx<sup>WT</sup> allele had escaped inactivation in this tissue.

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Protocol S1. Supplementary Methods

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Table S1. Primers Used in This Study

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Author contributions. DG, AJHS, WGW, DRH, and RJJG conceived and designed the experiments. DG, JA, RA, and RJJG performed the experiments. DG, RA, WGW, DRH, and RJJG analysed the data. LD contributed reagents/materials/analysis tools. LD provided technical assistance. DG wrote the paper.

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