Defective survival of proliferating Sertoli cells and androgen receptor function in a mouse model of the ATR-X syndrome

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X-linked ATR-X (alpha thalassemia, mental retardation, X-linked) syndrome in males is characterized by mental retardation, facial dysmorphism, alpha thalassemia and urogenital abnormalities, including small testes. It is unclear how mutations in the chromatin-remodeling protein ATRX cause these highly specific clinical features, since ATRX is widely expressed during organ development. To investigate the mechanisms underlying the testicular defects observed in ATR-X syndrome, we generated ScAtrxKO (Sertoli cell Atrx knockout) mice with Atrx specifically inactivated in the supporting cell lineage (Sertoli cells) of the mouse testis. ScAtrxKO mice developed small testes and discontinuous tubules, due to prolonged G2/M phase and apoptosis of proliferating Sertoli cells during fetal life. Apoptosis might be a consequence of the cell cycle defect. We also found that the onset of spermatogenesis was delayed in postnatal mice, with a range of spermatogenesis defects evident in adult ScAtrxKO mice. ATRX and the androgen receptor (AR) physically interact in the testis and in the Sertoli cell line TM4 and co-operatively activate the promoter of Rhox5, an important direct AR target. We also demonstrate that ATRX directly binds to the Rhox5 promoter in TM4 cells. Finally, gene expression of Rhox5 and of another AR-dependent gene, SpinlW1, was reduced in ScAtrxKO testes. These data suggest that ATRX can directly enhance the expression of androgen-dependent genes through physical interaction with AR. Recruitment of ATRX by DNA sequence-specific transcription factors could be a general mechanism by which ATRX achieves tissue-specific transcriptional regulation which could explain the highly specific clinical features of ATR-X syndrome when ATRX is mutated.

INTRODUCTION

The ATR-X (alpha thalassemia, mental retardation, X-linked) syndrome is a severe developmental disorder affecting males caused by mutations in the ATRX gene (1,2). Despite ATRX being almost ubiquitously expressed during embryonic development, the highly specific symptoms in ATR-X syndrome patients suggest critical functions for ATRX in the brain (severe mental retardation), the blood system (mild alpha thalassemia) and in the testis (urogenital abnormalities) (3,4). Similarly in mice, conditional knockout studies point to a tissue-cell type-specific requirement for ATRX. Mice lacking ATRX in the forebrain show apoptosis of cortical neurons, leading to reduced forebrain size; however, loss of ATRX in chondrocytes has no effect on bone growth (5,6). ATRX is a large nuclear protein (280 kDa) with two highly conserved domains. The N-terminal ATRX-DMNT3L-DNMT3A (ADD) domain comprises a GATA-type zinc finger and a plant homeodomain (PHD) often found in chromatin-associated proteins (7). The C-terminal switch

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(SWI)/sucrose non-fermenting (SNF)-like ATPase domain displays nucleosome-remodeling activity in vitro (8,9). Nearly all ATR-X syndrome mutations fall within these two domains, highlighting their importance for ATRX protein function (10). SWI/SNF complexes often play important roles in regulation of gene expression. They can disrupt histone–DNA contacts and alter the accessibility of transcription factors to nucleosomal DNA (11,12). ATRX forms a chromatin-remodeling complex with the death domain-associated protein DAXX which is required for the localization of H3.3 at telomeres (8,13,14). Just recently, Law et al. (15) demonstrated that ATRX regulates the expression of genes such as the α-globin gene cluster by binding to G-rich tandem repeat sequences. However, it is not known how ATRX achieves tissue-specific gene regulation. There is emerging evidence that ATRX also plays crucial roles during the cell cycle of proliferating cells. In HeLa cells, ATRX is required for the transition from prophase to metaphase during mitosis (16), whereas in mouse embryonic stem (ES) cells, ATRX is recruited to the telomeres during S phase, and knockdown of ATRX in these cells causes a DNA damage response at telomeres (14).

The ATR-X syndrome is a disorder of sex development formerly known as an intersex condition (17). The abnormalities in sexual differentiation in ATR-X syndrome range widely, from complete male-to-female sex reversal in severe forms to ambiguous genitalia, small penis and hypospadias in mild forms (18). In the two ATR-X syndrome cases with gonadal histology reported, small poorly formed testes were described consisting largely of interstitial stroma and scattered seminiferous tubules with few or no germ cells inside (4,19). The etiology of these testicular abnormalities is not known, and current mouse models are not informative as complete Atrx knockout mice die in utero before sex determination (20).

In this study, we investigated the role of ATRX in testis development and function. We found that ATRX is strongly expressed in the supporting Sertoli cell lineage of the mouse testis. Sertoli cells have important functions in the fetal gonad for testis formation and testis cord elongation and in the adult testis to provide structural and nutritional support for germ cells during spermatogenesis (21–23). Through conditional inactivation of Atrx specifically in Sertoli cells, we show that ATRX plays an important role in fetal Sertoli cell proliferation and thus testis cord elongation, as well as in Sertoli cell-mediated regulation of spermatogenesis, where, as a co-factor of the androgen receptor (AR), ATRX stimulates the expression of essential genes such as Rhox5.

RESULTS

ATRX is expressed in Sertoli cells throughout mouse testicular development

In human and rat adult testes, ATRX is widely expressed (24). An immunohistochemical study of ATRX expression during mouse testicular development (C57BL6 mouse strain) revealed that ATRX protein is also apparent in all testicular cell types at embryonic day 14.5 (E14.5), with strongest expression in the Sertoli cells (Fig. 1A). By E17.5, ATRX protein is less apparent in germ cells, whereas it remains robustly expressed in the Sertoli cells at puberty (postnatal day 28; P28) and in adulthood (P70; Fig. 1A).

Conditional inactivation of Atrx in Sertoli cells leads to small testes and reduced tubule volume

During fetal and early postnatal testis development, proliferation of Sertoli cells is necessary for elongation of the testis cords, the future seminiferous tubules (22). On the basis of strong Sertoli cell expression of ATRX, we speculated that small testes and reduced tubule volume described in ATR-X syndrome patients could arise from Sertoli cell defects. Therefore, we inactivated Atrx specifically in the Sertoli cell lineage in mice from E13.5 using a conditional Cre-lox excision strategy. AMH-Cre/+ mice, which express Cre recombinase in Sertoli cells from E13.5 (25), were crossed with mice homozygous for a conditional (‘floxed’) knockout allele of Atrx (Atrxfloxflox mice) (5) to obtain hemizygous AMH-Cre/+; Atrx+/floxflox (hereafter Sertoli cell Atrx knockout, ScAtrxKO) mice (Fig. 1B). ATRX immunohistochemistry in E14.5 ScAtrxKO testes revealed that ~95% of Sertoli cells (>1000 Sertoli cells were counted per gonad, n = 3) lacked ATRX expression (Fig. 1Bi), demonstrating that ablation of the ATRX protein in Sertoli cells was efficient in ScAtrxKO mice. No ATRX inactivation was detected in other cell types of the testis including interstitial and germ cells, as expected (Fig. 1Bi). Inactivation of the Atrx gene in ScAtrxKO mice was confirmed by polymerase chain reaction (PCR) of genomic DNA isolated from whole testes (Supplementary Material, Fig. S1).

To investigate whether the loss of ATRX affects fetal testis development, we examined testicular architecture by fluorescence-based three-dimensional modeling (26). At E15.5, both Atrx+/floxflox (hereafter control) and ScAtrxKO testes displayed the typical wild-type arrangement of parallel, transverse testis cords close to the surface, with no measurable difference in testis volume or cord volume (Fig. 2A and Supplementary Material, Videos S1 and S2). By E17.5, the cords in the control testes elongated and buckled under the surface to invade the core of the gonad, whereas in ScAtrxKO testes, the cords remain parallel and close to the surface. Intriguingly, in ScAtrxKO testes, some testis cords were discontinuous and even isolated testis cord fragments were observed (red arrows in Fig. 2A and Supplementary Material, Videos S3 and S4; 5, 10 and 12 breakpoints in three gonads analyzed). No breakpoints were observed in control testes. Relative to controls, both testis volume and cord volume in ScAtrxKO mice were reduced to 65 and 49%, respectively, by E17.5 (Fig. 2A). Immunohistochemistry for AMH (anti-Müllerian hormone), a marker of immature Sertoli cells, confirmed that E17.5 ScAtrxKO testes contained fewer testis cords than control, particularly in the core region (Fig. 2B).

Testis weights of P70 adult ScAtrxKO mice were reduced to 20% of control values, whereas no difference in overall body weight was observed (Fig. 2C, Table 1). At P70, control testes had established a dense network of seminiferous tubules surrounded by interstitial cells, including Leydig (CYP17A1-positive) cells (Fig. 2C). In transverse sections of ScAtrxKO testes, the tubules were less densely packed, with the interstitial space increased. Though in sections the
number of Leydig cells appeared higher in ScAtrxKO than control testes (741 \pm 101 versus 221 \pm 34 Leydig cells/mm²; Fig. 2C), the total number of Leydig cells was comparable with control mice when accounting for the small testis size of ScAtrxKO mice.

**ScAtrxKO testes contain fewer Sertoli cells**

The reduced cord volume in ScAtrxKO testes suggested a reduction in the number of Sertoli cells. To examine this, we counted Sertoli cells at E15.5 and E17.5 marked by ATRX.
immunohistochemistry for AMH. At E15.5, ScAtrxKO testes showed a modest reduction (~20%) in the number of Sertoli cells when compared with control testes (Fig. 3A). By E17.5, in control testes, the number of Sertoli cells increases by 3.2-fold due to extensive proliferation. However, in E17.5 ScAtrxKO testes, no such increase was measured, and Sertoli cell numbers were 28% of control numbers (Fig. 3A). Examination of the testis cord structure in E17.5 ScAtrxKO testes revealed that the distribution of Sertoli cells around the cord periphery was unusually sparse (Fig. 3B), whereas the number of germ cells inside the cords was increased. However, the number of germ cells in the entire ScAtrxKO testes was in fact similar to the number in control testes due to the reduced cord volume of ScAtrxKO testes (Fig. 3B). Expression of laminin, a marker of the basal lamina, appeared normal in ScAtrxKO testes, suggesting that testis cord integrity was intact despite the reduced Sertoli cell numbers (Fig. 3B).

We also counted the number of Sertoli cells at P12 and P50 by immunohistochemistry for SOX9, a universal marker of fetal and adult Sertoli cells. The relative reduction in the number of Sertoli cells in ScAtrxKO testes (26 and 22% of control) was very similar to that observed at E17.5 (Supplementary Material, Fig. S2), indicating that the loss of Sertoli cells predominantly occurred during fetal life. However, although the proportion of Sertoli cells relative to total cell numbers in E17.5 ScAtrxKO testes was reduced to 35% of control (Supplementary Material, Fig. S2), at P12 and P50, it was partly restored to 75 and 82% of control, respectively. This is due to the fact that postnatal testes are increasingly populated by germ cells whose numbers are directly dependent on the number of supporting Sertoli cells (27).

**Table 1.** Body, testis and seminal vesicle weight and anogenital distance of control and ScAtrxKO mice

<table>
<thead>
<tr>
<th>Group</th>
<th>P70 body weight (g)</th>
<th>P70 Testis weight (mg)</th>
<th>P70 seminal vesicle (mg)</th>
<th>P70 anogenital distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.9 ± 0.2 (n = 4)</td>
<td>95 ± 8 (n = 4)</td>
<td>124 ± 15 (n = 3)</td>
<td>XY 16 ± 0.4, XX 7 ± 0.2 (n = 4)</td>
</tr>
<tr>
<td>ScAtrxKO</td>
<td>24.8 ± 1.1 (n = 4)</td>
<td>19 ± 2 (n = 4)***</td>
<td>23 ± 4 (n = 3)**</td>
<td>XY 15 ± 0.4 (n = 4)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for the number of mice indicated (n).

**P < 0.01.

***P < 0.001 versus control.

**Figure 3.** Analysis of Sertoli cell number and testis cord structure in ScAtrxKO testes. (A) Average number of Sertoli and germ cells in control and ScAtrxKO testes (n = 3). Values are the mean ± SEM. **P < 0.01. (B) Double-immunofluorescence staining for the Sertoli cell marker GATA4 (green, nuclear), and the basal lamina marker laminin (red, extracellular) and immunofluorescence staining for TRA98, a germ cell marker, in E17.5 control and ScAtrxKO testes. Dashed lines mark the basal lamina of the testis cords. Scale bars are 20 μm.

Sertoli cells in ScAtrxKO testes show prolonged G2/M phase and undergo apoptosis

The reduced number of Sertoli cells in E17.5 ScAtrxKO testes could be the result of cell death and/or decreased proliferation. First, we examined fetal ScAtrxKO testes for co-expression of AMH and cleaved CASP3, a marker of apoptosis. At E15.5 and at E17.5 in particular, ScAtrxKO testis cords showed a marked increase in the number of CASP3-positive Sertoli cells when compared with littermate controls (8-fold at E15.5 and 40-fold at E17.5; Fig. 4). Similar results were obtained by performing terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Supplementary Material, Fig. S3A). Moreover, 4′,6-diamidino-2-phenylindole (DAPI) staining of fetal ScAtrxKO testis sections revealed that CASP3-positive Sertoli cells had small fragmented nuclei (Supplementary Material, Fig. S3B). These data strongly suggest that ATRX-negative Sertoli cells are undergoing apoptosis. In contrast to fetal stages, there was no rise in Sertoli cell apoptosis in adult ScAtrxKO testes at P70 (Fig. 4A). Consistent with this finding, testis weight reduction in 1-year-old ScAtrxKO mice (83%) was very similar to the reduction in P70 ScAtrxKO mice (80%; Table 1), indicating that testis weight loss was not progressive during adult life. To examine fetal Sertoli cell proliferation in ScAtrxKO mice, we performed double immunohistochemistry for AMH and phospho-histone H3 (PH3), a cell cycle marker of late G2 phase and mitosis (28). Relative to control, the number of PH3-positive Sertoli cells in E16.5 ScAtrxKO testes was increased by 38% (Fig. 5A). Such an increase could be caused by increased proliferation. Alternatively, since the knockdown of ATRX in the HeLa cell line resulted in...
prolonged mitosis with associated cell death (16), it was possible that the increased number of PH3-positive Sertoli cells in ScAtrxKO testes was the result of an extended G2/M phase. If the latter is true, the proportion of cells found in the other phases of the cycle should be relatively decreased. To test this, pregnant Atrx<sup>lox/lox</sup> (E16.5; crossed with AMH-Cre mice) were injected with 5-bromo-2′ deoxyuridine (BrdU) which labels cells undergoing DNA synthesis (S phase). Double immunohistochemistry for BrdU and AMH revealed that the number of BrdU-positive Sertoli cells was reduced by 13% in ScAtrxKO testes when compared with control (Fig. 5B). The PH3 staining and BrdU labeling data combined indicate that in ScAtrxKO testes the G2/M phase of Sertoli cells is prolonged, leading to an extension of the cell cycle such that fewer cells are proliferating. This cell cycle defect might underlie the increased Sertoli cell death observed in ScAtrxKO testes. We also explored the possibility that Sertoli cell apoptosis in ScAtrxKO testes might due to pre-mature maturation of Sertoli cells which normally occurs at post-natal stages. As shown in Figure 4A and Supplementary Material, Figure S3B, apoptotic ATRX-negative Sertoli cells express the immature Sertoli cell marker AMH and do not express the mature Sertoli cell marker GATA1 (Supplementary Material, Fig. S4), indicating that ATRX-negative Sertoli cells do not undergo maturation during fetal stages.

Taken together, our results suggest that small testes and reduced tubule volume in ScAtrxKO mice are a consequence of defective Sertoli cell proliferation with associated Sertoli cell apoptosis.

**Figure 4. ScAtrxKO testes show increased Sertoli cell apoptosis.** (A) Double-immunofluorescence staining for the Sertoli cell marker AMH (red, cytoplasmic) and cleaved CASP3 (green, nuclear), a marker of apoptosis, in E15.5 control and ScAtrxKO testes. The arrows point to cleaved CASP3-positive apoptotic cells in the ScAtrxKO testis cords. (B) Average percentage of CASP3-positive Sertoli cells in control and ScAtrxKO testes at E15.5, E17.5 and P70. To determine the percentage of CASP3-positive Sertoli cells in each testis, all Sertoli cells were counted in three or more testis sections (>1000 Sertoli cells). Values are the mean ± SEM (n = 4). ∗P < 0.05; ***P < 0.001. Scale bars are 20 μm.

**ScAtrxKO mice show delayed spermatogenesis and a wide range of seminiferous tubule defects**

In normal P12 testes, the germinal epithelium of the seminiferous tubules thickens, germ cells are entering the first wave of meiosis, and a central lumen has formed (Fig. 6A). At P21, many spermatocytes (Sp in Fig. 6B) have completed meiosis to become round spermatids (Rs in Fig. 6B), which subsequently elongate and develop into mature sperm (S in Fig. 6C). However, in ScAtrxKO testes at P12, the seminiferous tubules had a thinner germinal epithelium and no lumen was evident at this stage (Fig. 6A). At P21, a lumen had formed within most tubules of ScAtrxKO testes; however, the germ cells had not yet developed into round spermatids (Fig. 6B), suggesting a delay in spermatogenesis in ScAtrxKO mice. By P70, in adult ScAtrxKO testes, spermatogenesis appeared normal in the majority of seminiferous tubules (Fig. 6Cii). However, 10–30% of tubules (n = 5 testes) showed a wide variety of spermatogenesis defects including tubules with germ cell development arrested at the round spermatid stage (Fig. 6Ciii) or at meiosis (Fig. 6Civ), tubules with severe germ cell hypoplasia, and Sertoli cell only (SCO) tubules (Fig. 6Cv and Supplementary Material, Fig. S5). Through analysis of consecutive testis sections, we found that the latter two tubule types were often discontinuous, with reduced diameters close to their breakpoints (Supplementary Material, Fig. S5). However, due to the highly convoluted nature of the adult testis, it was not possible to determine whether all these tubules were discontinuous.

Since ScAtrxKO testes display spermatogenesis defects and are significantly smaller than control testes, we assessed the fertility of 5-month-old ScAtrxKO mice. Of four ScAtrxKO mice analyzed, three produced litter sizes comparable with control male mice (7.5 versus 7.2 embryos per litter) (Table 2). However, the ScAtrxKO mouse with the lowest testis weight produced only 1.2 embryos per litter on average. These data show that the majority of ScAtrxKO male mice show normal fertility despite having small testes and can produce functional sperm. This is consistent with previous observations in mice in which ATRX interacts with the AR to activate Rhox5 gene transcription

**ATRX and AR proteins interact and co-operatively activate Rhox5 gene transcription**

Mature Sertoli cells provide structural and nutritional support for germ cells during spermatogenesis (21). We investigated whether the underlying cause of the spermatogenesis defects in ScAtrxKO testes could be failure of Sertoli cell maturation, a process normally accompanied by a switch from AMH to GATA-1 expression (22). However, AMH/GATA-1 co-immunohistochemistry revealed that this switch had occurred normally in P14 ScAtrxKO mice (Supplementary Material, Fig. S6) and so does not directly explain the observed phenotype.

Recently, ATRX was identified as 1 of 309 potential direct AR-binding partners by an in vitro T7 phage display screen (Supplementary data in 30). Protein interaction was found...
between the ligand-binding domain of AR (507–919 amino acids) and the 75 amino acid region of the ATRX protein (amino acids 1259–1335). AR, which is activated by testosterone and dihydrotestosterone, is a nuclear receptor and binds as a transcription factor to androgen response elements in target gene promoters (31). Apart from other...
tissues such as prostate and external genitalia, AR is also expressed in the Sertoli cells of the testis where it is important for their functional maturation; specifically Sertoli cell AR is required for the completion of meiosis and spermatid development into mature sperm (32–34). ATRX might be an important co-factor of AR in Sertoli cells. First, we asked whether ATRX and AR interact in vivo. Co-immunoprecipitation experiments in the Sertoli cell line TM4 (35) and in P50 and P12 (data not shown) wild-type testes showed that AR was co-immunoprecipitated by an ATRX-specific (Fig. 7A). Conversely, ATRX was co-immunoprecipitated using an AR-specific antibody.

Rhox5 is the only well-characterized direct target gene of AR in Sertoli cells and plays an important role during spermatogenesis (31,36,37). RHOX5 is a transcription factor exclusively expressed in Sertoli cells that regulates the expression of genes including those encoding proteins controlling cell cycle regulation, apoptosis, metabolism and cell–cell interactions (38). Targeted deletion of Rhox5 in male mice causes germ cell apoptosis and a reduction in sperm production, sperm motility and fertility (36). Rhox5 is thus considered as a pivotal mediator of AR action in the testis. To investigate whether ATRX can modulate AR-dependent transactivation of Rhox5, co-transfections were performed in TM4 cells using the proximal 655 bp promoter of Rhox5 (Rhox5Pp) (31,37) fused to a luciferase reporter construct. Upon testosterone stimulation (100 nM) and the addition of the Ar expression construct, a 55-fold increase in Rhox5Pp activity was observed, whereas the addition of the Ar and Atrx expression constructs together resulted in a 141-fold activation (2.6-fold increase; Fig. 7B). The addition of the Atrx expression construct alone did not affect reporter activity.

To examine whether ATRX binds to the Rhox5 promoter in vivo, we performed a chromatin immunoprecipitation (ChIP) assay. An anti-ATRX antibody was used to immunoprecipitate chromatin from testosterone-treated TM4 cells and TM4 cells transiently co-transfected with ATRX and AR expression constructs. To assess the efficiency of the ATRX ChIP, we used the Aff3 gene (a putative transcriptional activator involved in lymphoid development) which is enriched by ATRX ChIP in mouse ES cells (15), whereas Gapdh was used as a negative control. The Rhox5 promoter was analyzed using two primer sets which span the promoter region of Rhox5 (Fig. 7C). In both untransfected and transfected TM4 cells, Aff3 enrichment was detected in the ATRX pulldowns, whereas the Gapdh promoter showed no enrichment (Fig. 7D). The Rhox5 promoter was not enriched in untransfected TM4 cells. However, in transfected TM4 cells, the Rhox5 promoter was found to be enriched in the ATRX pulldown by both primer sets. For all genes tested, no enrichment was detected in the non-specific immunoglobulin (Ig) G pulldown (Fig. 7D).

In summary, our data suggest that ATRX can directly enhance the transcription of Rhox5 by acting in a complex with the AR protein.

ATRX regulates Rhox5 gene expression in vivo

Finally, we wanted to test whether ATRX is important for the transcription of Rhox5 in vivo. We measured the mRNA levels of Rhox5, Ar and Sox9 (an AR-independent gene and marker of Sertoli cell identity) in whole testes using quantitative RT–PCR (qRT–PCR) at P12, when spermatogenesis has commenced, and at P50 in adult testes. The qRT–PCR data of ScAtrxKO testes were adjusted to account for the reduced Sertoli cell proportion relative to total cells as mentioned.

Table 2. Assessment of fertility of control and ScAtrxKO mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Average number of embryos (number of litters)</th>
<th>Testis weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1)</td>
<td>6.8 (n = 5)</td>
<td>108</td>
</tr>
<tr>
<td>Control (2)</td>
<td>8.5 (n = 5)</td>
<td>135</td>
</tr>
<tr>
<td>Control (3)</td>
<td>7.5 (n = 5)</td>
<td>159</td>
</tr>
<tr>
<td>Control (4)</td>
<td>7.2 (n = 5)</td>
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</tr>
<tr>
<td>ScAtrxKO (1)</td>
<td>9.0 (n = 5)</td>
<td>22</td>
</tr>
<tr>
<td>ScAtrxKO (2)</td>
<td>7.0 (n = 5)</td>
<td>30</td>
</tr>
<tr>
<td>ScAtrxKO (3)</td>
<td>7.4 (n = 5)</td>
<td>28</td>
</tr>
<tr>
<td>ScAtrxKO (4)</td>
<td>1.2 (n = 7)</td>
<td>18</td>
</tr>
</tbody>
</table>

Figure 7. ATRX and AR proteins interact and co-operatively activate Rhox5 gene transcription. (A) ATRX and AR were immunoprecipitated from TM4 nuclear extracts and P50 testes extracts and analyzed by western blotting with the indicated antibodies. Control reactions were done with IgG. (B) TM4 cells were transfected with Rhox5 promoter-containing LUC plasmid. Cells were co-transfected with AR expression plasmid, pCAGG–ATRX plasmid or empty vectors in the presence or the absence of T. The values are normalized to transfection efficiency and expressed relative to that of basal Rhox5–LUC in the absence of AR and T. Values are the mean ± SEM (n = 3). *P < 0.05 versus AR-mediated Rhox5 activity in the presence of T. (C) Schematic of the Rhox5 promoter with the vertical lines representing AR-binding sites. The two amplified ChIP fragments are indicated. TSS, transcriptional start site. (D) Untransfected and transfected (with 10 µg of pCAGG–ATRX and pSG5–AR) TM4 cells were treated with 100 nM testosterone as indicated. Formaldehyde-cross-linked DNA was immunoprecipitated using an ATRX antibody or rabbit IgG, as a negative control. Precipitated DNA fragments (ChIP) and DNA from lysate before immunoprecipitation (5% input) were subjected to PCR with primers covering the Rhox5 promoter region from −327 to −8 (Rhox5-1) and −456 to −345 (Rhox5-2, middle panels). Primers to Gapdh were used (upper panel) as a negative control and primers to the Aff3 gene to assess the ATRX ChIP efficiency (lower panel). As a negative control precipitated DNA fragments from untransfected TM4 cells were also subjected to ChIP which showed no enrichment at the Rhox5 regions. ATRX ChIP experiments were undertaken three times.
taken together, our data indicate that ATRX in postnatal and adult Sertoli cells regulates the expression of AR-dependent genes, such as Rhox5, a gene whose Sertoli cell expression facilitates spermatogenesis (36). ATRX appears to have a more important role in the regulation of AR-dependent genes during the first wave of spermatogenesis than in adulthood.

**DISCUSSION**

**ATRX is important for fetal Sertoli cell proliferation and survival**

In this study, we show that ATRX plays an important role in the proliferation and survival of fetal Sertoli cells and, in turn, for elongation of fetal testis cords. Following Atrx inactivation at E14.5, loss of Sertoli cells in ScAtrxKO testes occurred rapidly between E15.5 and E17.5, a period when Sertoli cells normally proliferate extensively, resulting in reduced testis cord volume and small testes. Fetal ScAtrxKO testes developed discontinuous testis cords, which may be the result of local depletion of Sertoli cells. Discontinuous tubules were also observed in adult ScAtrxKO testes, which contained few or no germ cells (SCO tubules). We speculate that the discontinuous testis cords established during fetal life persist into adulthood, but fail to support local germ cell populations.

Our data suggest that G2/M phase is prolonged in ATRX-negative Sertoli cells leading to extension of the cell cycle as evidenced by a 38% increase in PH3-positive (G2/M phase) and a modest decrease (13%) in BrdU-positive (S phase) Sertoli cells in ScAtrxKO testes. We speculate that Sertoli cell apoptosis might be a consequence of this cell cycle defect. Consistent with this interpretation, it has previously been reported that ATRX-depleted cells die as a consequence of mitotic defects (16). Knockdown of ATRX in cultured HeLa cells leads to defects in chromosome cohesion and congression, resulting in a prolonged transition from prometaphase to metaphase, and a proportion of cells were chronically arrested in mitosis and underwent apoptosis. Also from this study, analysis of the ATRX null forebrain revealed a high occurrence of dying cells in the vicinity of mitotic cortical progenitors (16).

Though these data imply similar roles for ATRX in mitotic Sertoli cells in vivo and cultured HeLa cells, there might be differences as to when ATRX is first required. In the nucleus, PH3 starts to be expressed in late G2 phase at pericentromeric heterochromatin (speckled expression) at the onset of chromosome condensation, spreads throughout the condensing chromosomes and is complete just prior to the formation of prophase chromosomes (28) (see also Fig. 5A). In control testes, ~95% of PH3-positive Sertoli cells displayed a pre-prophase pericentromeric heterochromatin expression pattern (arrowed in Fig. 5A). In ScAtrxKO testes, the number of Sertoli cells with pericentromeric PH3 expression was increased by 38%. These data indicate that the cell cycle in ATRX-negative Sertoli cells might already be prolonged at late G2 and/or early prophase, suggestive of a role for ATRX during chromosome condensation. Similar to our observation in ATRX-negative Sertoli cells, inhibition of phosphorylation of histone H3 leads to cell arrest in late G2 phase,

**Table 3.** Testosterone and FSH levels in control and ScAtrxKO mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum testosterone (ng/ml)</th>
<th>FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.06 ± 0.35 (n = 7)</td>
<td>11.6 ± 0.58 (n = 7)</td>
</tr>
<tr>
<td>ScAtrxKO</td>
<td>1.48 ± 0.43 (n = 7)</td>
<td>14.0 ± 0.65 (n = 7)</td>
</tr>
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Values are mean ± SEM for the number of mice indicated (n). *P < 0.05 versus control.

above. The graphs for the non-adjusted qRT–PCR data can be found in Supplementary Material, Figure S7. At both stages, expression of Sox9 and Ar was unchanged. In contrast, Rhox5 expression was significantly down-regulated at P12 and at P50 by 9- and 2.5-fold, respectively (Fig. 8). We also measured the expression of Spinlw1, another Sertoli cellspecific AR-dependent gene. At P12, Spinlw1 expression was significantly down-regulated by 2.5-fold. Though not significant, at P50, expression of Spinlw1 was down-regulated by 41%. There still remained the possibility that reduced testosterone levels might contribute to the reduced expression levels of Rhox5. However, in ScAtrxKO mice, the levels of serum testosterone and the weight of the seminal vesicle, whose growth is androgen dependent, were similar to controls (Tables 1 and 3), suggesting that the spermatogenesis defects are not caused by insufficient testosterone levels. In contrast, FSH levels were significantly elevated in ScAtrxKO mice by 21% when compared with control mice (Table 3). This is consistent with dysfunction of Sertoli cells which control FSH secretion. Similarly, in ARKO mice, FSH levels were increased by 34% (33).
demonstrating that PH3 is required during the initiation of chromosome condensation and entry into mitosis (39). Intriguingly, ATRX and PH3 co-localize at pericentromeric heterochromatin and ATRX can directly interact with histone H3.3 (14). It will thus be interesting to test in the future whether ATRX is involved in the phosphorylation of histone H3 at pericentromeric heterochromatin.

**ATRX modulates AR-dependent gene expression in spermatogenesis**

Here, we provide evidence that ATRX plays an important role in spermatogenesis modulating the expression of AR-dependent genes in Sertoli cells as an AR co-factor since (i) ScAtrxKO testes show a delay in spermatogenesis, (ii) two AR-dependent genes, Rhox5 and Spinβ1, are down-regulated in ScAtrxKO testes, (iii) ATRX and AR proteins interact both in TM4 cells and in the testis, (iv) in the presence of AR, ATRX can increase Rhox5 promoter activity in TM4 cells and (v) ATRX occupies the Rhox5 promoter in TM4 cells. Several AR co-regulators have been identified as members of the SWI/SNF family of chromatin-remodeling proteins (40). One, the chromatin-remodeling factor ARIP4, also interacts with AR to regulate the androgen-dependent gene expression in Sertoli cells. Heterozygous ARIP4 knockout mice (homozygous ARIP4 knockout mice die during early embryogenesis) show a modest but significant reduction (≏20%) in Rhox5 expression levels (41,42).

Adult ScAtrxKO testes showed a wide range of spermatogenesis defects including tubules with spermatogenic arrest at meiosis and at the round spermatid stage, whereas the majority of tubules displayed normal spermatogenesis. Although there is some phenotypic overlap with the Sertoli cell-specific AR knockout mice (ARKO mice; meiotic arrest) and mice with a hypomorphic AR in Sertoli cells (arrest at the round spermatid stage) (32–34), it is unlikely that the abnormalities in ScAtrxKO mice are solely caused by reduced AR activity. ScAtrxKO testes contain ‘SCO’ tubules and tubules with spermatogenic arrest at early meiosis, severe phenotypes which have not been observed in ARKO mice. It is thus possible that the spermatogenesis defects in adult ScAtrxKO mice are in part the result of the discontinuous tubule network. The relatively mild spermatogenesis phenotype in ScAtrxKO testes when compared with ARKO mice can be explained by the fact that AR-dependent genes are still expressed to some extent in ScAtrxKO testes, particularly at adult stages. Therefore, ATRX is an important but not essential AR co-regulator in Sertoli cells.

**Implications for the ATR-X syndrome**

Our data suggest that small testes and reduced tubule volume also reported in ATR-X syndrome patients (4,19) arose by apoptosis of proliferating Sertoli cells during fetal life, due to cell cycle defects at G2/M phase. In addition, reduced Sertoli cell proliferation as a consequence of prolonged G2/M phase might also contribute to these clinical findings. Sertoli cell apoptosis with consequent formation of discontinuous tubules could also explain the presence of SCO tubules in ATR-X patients. However, the extent of germ cell loss in ScAtrxKO mice was considerably milder than that reported in ATR-X patients. Inactivation of Atrx at an earlier time point in Sertoli cells may result in an earlier onset of apoptosis and thus in a larger proportion of germ cell-deficient tubules. Alternatively, since human ATRX is also expressed in germ cells (24), it is possible that the loss of germ cells in ATR-X patients could be due to the loss of cell autonomous functions of ATRX in this cell type. In contrast to ATR-X syndrome patients where ambiguous or female genitalia are present in 10% of cases (18), the male external genitalia in ScAtrxKO mice appeared to develop normally (anogenital distances were similar to control males, Table 1) which is consistent with the normal testosterone levels observed in these mice. It is tempting to speculate that ATRX might also act as an important AR co-factor in the developing external genitalia and that loss of ATRX function in this organ directly causes the external genitalia abnormalities.

Recently, it has been shown that ATRX can alter the expression of genes including the α-globin gene cluster by binding to G-rich tandem repeats and that the number of repeats correlated with the extent of gene expression changes in ATR-X syndrome patients (15). However, it is not known how ATRX, a widely expressed protein, achieves tissue-specific target gene expression. Moreover, in 35% of human and mouse ATRX-bound target genes, no tandem repeats are found. We also did not detect any tandem repeats in the 655-bp Rhox5 promoter sequence by using the program Tandem Repeats Finder (43). Here, we provide evidence showing that ATRX can enhance the transcription of Rhox5 through physical interaction with the DNA sequence-specific transcriptional activator, AR. Recruitment of ATRX by tissue-specific transcription factors to target genes could be a more general mechanism by which ATRX controls transcriptional processes which could explain how ATRX achieves tissue-specific target gene expression and why the ATR-X syndrome has highly specific clinical features.

**MATERIALS AND METHODS**

**Generation and genotyping of ScAtrxKO mice**

*ATRX*^lox/lox^ mice (5) were crossed with the *AMH-Cre/+* mouse line (25) to generate hemizygous *AMH-Cre/+;ATRX^lox/Y* (ScAtrxKO) male mice. For genotyping, genomic DNA was isolated from tail tissue (postnatal mice) or yolk sac (embryos). Primers and PCR conditions for the *Cre* (44) and *ATRX*^lox^ alleles (20) were used as described previously. All procedures involving mice were approved by the Animal Ethics Committee of Monash University, Australia.

**Fertility tests**

We assessed the fertility of 5-month-old control *Atrx*^lox/−^ and ScAtrxKO mice by mating one male with two C57BL/6 female mice for 1 week. Female mice were checked for vaginal plugs each morning, and litter sizes were determined at E9.5. This procedure was repeated until at least five females have successfully mated.
Histology and immunofluorescence

Testes were fixed, processed and sectioned as described previously (45). For histological analyses, paraffin sections were stained with hematoxylin and eosin (HE staining). For indirect immunofluorescence staining, frozen sections were incubated overnight with the following primary antibodies. Anti-AMH goat polyclonal (1:200; Santa Cruz, sc-6886), anti-ATRX rabbit polyclonal (1:200; Santa Cruz, sc-15408), anti-TRA98 rat monoclonal (1:200; Abcam, ab82527), anti-phospho-histone H3 rabbit polyclonal (1:200; Millipore, 06-570), cleaved CASP3 rabbit polyclonal (1:200; Cell Signaling, 9661), anti-GATA1 rat monoclonal (1:200; Santa Cruz, sc-266), anti-CYP17A1 goat polyclonal (1:800; Santa Cruz, sc-46081), anti-laminin rabbit polyclonal (1:200; Sigma, L9393), anti-GATA4 goat polyclonal (1:200; Santa Cruz, sc-1237) and anti-BrdU mouse monoclonal (1:200, Sigma, B2531). After washing for 30 min in phosphate-buffered saline, primary antibodies were detected by incubating sections for 1 h with Alexa dye-linked secondary antibodies (1000; Molecular Probes). Cell nuclei were visualized with DAPI (Invitrogen) at a concentration of 0.6 μg/ml in Dako Fluorescent Mounting Medium. Images were captured using fluorescence microscopy (Olympus Corp., NY, USA) or confocal microscopy (Olympus Corp.).

BrdU incorporation assay

For the BrdU incorporation, pregnant mice (E16.5) were injected intraperitoneally with BrdU (Sigma) at 0.1 mg/g body weight 2 h prior to harvest and embryos were processed as described previously (46).

TUNEL assay

TUNEL assay was performed using the ApopTaq® Peroxidase in situ apoptosis detection kit S7100 (Chemicon) according to the manufacturer`s instructions with minor modifications. Sections were counterstained with hematoxylin to visualize testis cords.

Testosterone and FSH measurements

Eight-week-old Atrxflox/Y (control) and ScAtrxKO mice were killed, and 1 ml of blood was taken by cardiac puncture. The serum was collected by centrifugation for 15 min at 3000g and stored at −20°C until assayed. Serum testosterone levels were measured by using the Testosterone direct RIA kit (Beckman Coulter). Serum FSH levels were measured using a double-antibody radioimmunoassay. Anti-rFSH-S-11 was used as a primary antibody, rFSH-I-9 as tracer and mouse FSH-RP1 as standard.

Quantitative RT–PCR

Total RNA was extracted from gonads at appropriate developmental stages using the RNeasy Mini Kit (Qiagen), including on-column DNaseI treatment. For cDNA synthesis, 750 ng of total RNA was used with SuperScript III (Invitrogen) and random primers (Promega) as per the manufacturer’s instructions. cDNA samples were diluted 1:4 and 0.2 μl used in each 5 μl of qRT–PCR, containing SYBR green PCR master-mix (Applied Biosystems) and 150 nm each forward and reverse primers. Transcript levels were analyzed on an ABI7900 Sequence Detector System essentially as described previously, including verification of primer specificities and efficiencies (47). Relative gene expression was calculated using the ΔCt method with Sdha as normalizing gene. Primer sequences were as follows (F, forward primer; R, reverse primer):

\[
\text{Sdha: (F)} \quad \text{TGTTCAGTTCCACCCCAACA, (R)} \quad \text{TCTCCACGA CACCCCTCTGT}
\]

\[
\text{Ar: (F)} \quad \text{CCAGTCCAATTGTGCAA, (R)} \quad \text{TCTCTGGT ACTGTCCAACACG}
\]

\[
\text{Soy9: (F)} \quad \text{AGTACCGCCATCTGGACAC, (R)} \quad \text{TACTTGTA ATCGGGTGGGTCT}
\]

\[
\text{Rhox5: (F)} \quad \text{AGGCAGAGGAGAAATTTAATGGA, (R)} \quad \text{GCCACTATCCTGTCGCCATCA}
\]

\[
\text{Spinlw1: (F)} \quad \text{GCTTCTGCTCAAAGCTCTGTG, (R)} \quad \text{TTGCA GTGCTCAAAGTGTCTTC}
\]

Three-dimensional modeling

Tissue samples were processed, imaged and modeled as described in Combès et al. (26) with the following changes: serial optical sections were taken every 10 μm, samples were imaged on 10× and/or 20× objectives and equilibrated using a pixel size conversion factor to enable comparison. Gonad and cord volumes were calculated from three independent models for each age and sample group using the ‘imodinfo’ function within the Imod program (48). Computed volumes were consistent with manual approximations of gonad volume (area of midline optical section × depth of sample).

Determination of Sertoli, Leydig and germ cell numbers

To determine the percentage of Sertoli and germ cells in control and ScAtrxKO fetal testes, all DAPI-positive nuclei (>10 000 cells per testis were counted), all Sertoli cells (AMH-positive cells) and all germ cells (TRA98-positive) were counted in three or more testis sections 40 μm apart (n = 3 testes). Subsequently, total cell numbers were estimated using the total gonad volume as predicted by the three-dimensional models (Fig. 2). To determine the percentage of Sertoli cells in P12/P50 control and ScAtrxKO testes, all DAPI-positive nuclei (>10 000 cells per testis were counted) and Sertoli cells (SOX9-positive cells) were counted in four fields in each of three sections 200 μm apart (n = 3 testes). Subsequently, the reduction in Sertoli cell numbers in P12 and P50 ScAtrxKO testes relative to control testes was estimated using the weight of these testes. To determine the number of Leydig cells in P70 control and ScAtrxKO testes, all CYP17A1-positive Leydig cells (>2000 Leydig cells per testis were counted) were counted in four fields in each of three sections 100 μm apart (n = 3 testes).
Cell culture, transfection and luciferase assays

TM4 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 50 mg/ml of both penicillin and streptomycin. All cell culture reagents were obtained from Invitrogen Technologies. TM4 cells were transfected with Fugene 6 reagent following the manufacturer’s instruction. Cells (~1 x 10^5) were seeded in 12-well culture plates and transfected with 100 ng of AR expression plasmid, 250 ng of pCAGG-ATRX plasmid or empty vectors. Upon transfection, media were supplemented with 10% charcoal-stripped bovine serum and treated with 100 nM testosterone in all dishes. At 48 h post-transfection, the culture medium was removed, cell lysate collected and luciferase reporter activity was measured using the Promega Dual-Luciferase reporter system according to the manufacturer’s instructions (Promega).

Co-immunoprecipitation

Nuclear extracts from the TM4 cell line were obtained using a modified radio-immunoprecipitation assay buffer technique (49). Testes nuclear extracts from C57BL6 wild-type mice were isolated as described (50). Immunoprecipitation of ATRX was performed as described previously (49). The immunoprecipitates were resolved on a 3–8% Tris–acetate gel (Invitrogen). Western blotting analysis was carried out using monoclonal ATRX antibody (39f from Richard Gibbons, University of Oxford) and anti-AR (Santa Cruz, sc-52309).

ChiP assay

TM4 cells were seeded with ~5 x 10^4 per 10 cm dish. A total of four dishes were untransfected, whereas the other four dishes were each co-transfected with 10 µg of pCAGG-ATRX and 10 µg of pSG5-AR using Fugene 6 reagent following the manufacturer’s instructions. Media were supplemented with 10% charcoal-stripped bovine serum and cells treated with 100 nm testosterone or vehicle alone (ethanol at a concentration of 0.02%). At 48 h post-transfection, the culture medium was removed, cell lysate collected and luciferase reporter activity was measured using the Promega Dual-Luciferase reporter system according to the manufacturer’s instructions (Promega).

Statistical analysis

Statistical significance was determined by two-tailed t-test analyses.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


