Expression and localization of androgen receptor-interacting protein-4 in the testis

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Domanskyi A, Zhang FP, Nurmi M, Palvimo JJ, Toppari J, Jänne OA. Expression and localization of androgen receptor-interacting protein-4 in the testis. Am J Physiol Endocrinol Metab 292: E513–E522, 2007. First published September 26, 2006; doi:10.1152/ajpendo.00287.2006.—Androgen receptor-interacting protein 4 (ARIP4) belongs to the SNF2 family of proteins involved in chromatin remodeling, DNA excision repair, and homologous recombination. It is a DNA-dependent ATPase, binds to DNA and mononucleosomes, and interacts with androgen receptor (AR) and modulates AR-dependent transactivation. We have examined in this study the expression and cellular localization of ARIP4 during postnatal development of mouse testis. ARIP4 was detected by immunohistochemistry in Sertoli cell nuclei at all ages studied, starting on day 3, and exhibited the highest expression level in adult mice. At the onset of spermatogenesis, ARIP4 expression became evident in spermatogonia, pachytene, and diplotene spermatocytes. Immunoreactive ARIP4 antigen was present in Leydig cell nuclei. In Sertoli cells ARIP4 was expressed in a stage-dependent manner, with high expression levels at stages II–VI and VII–VIII. ARIP4 expression patterns did not differ significantly in testes of wild-type, follicle-stimulating hormone receptor knockout mice, and luteinizing hormone receptor knockout mice. In testes of hypogonadal mice, ARIP4 was found mainly in interstitial cells and exhibited lower expression in Sertoli and germ cells. In vitro stimulation of rat seminiferous tubule segments with testosterone, FSH, or forskolin did not significantly change stage-specific levels of ARIP4 mRNA. Heterozygous ARIP4+/− mice were haploinsufficient and had reduced levels of Sertoli-cell specific androgen-regulated Rhox5 (also called Pem) mRNA. Collectively, ARIP4 is an AR coregulator in Sertoli cells in vivo, but the expression in the germ cells implies that it has also AR-independent functions in spermatogenesis.

coregulator; spermatogenesis; haploinsufficiency

NORMAL TESTIS DEVELOPMENT and spermatogenesis require coordinated actions of several peptide and steroid hormones, such as gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (13). GnRH stimulates synthesis and secretion of FSH and LH that interact with their cognate receptors in Sertoli cells and Leydig cells. FSH receptor (FSHR) is expressed in Sertoli cells (28), whereas luteinizing hormone receptor (LHR) is expressed primarily in Leydig cells (45). Upon LH stimulation, adult Leydig cells (ALC) produce testosterone that binds to the androgen receptor (AR) in Sertoli cells, Leydig cells, and peritubular myoid cells (3, 37, 38).

GnRH plays an important role in normal development of the male phenotype. Loss of GnRH signaling in hypogonadal (hpg) mice due to mutations in the GnRH gene results in undetectable levels of LH and FSH. As a result, gonads fail to develop postnatally and spermatogenesis is arrested at the pachytene spermatocyte stage (4, 23). Androgen signaling is indispensable for fetal sexual differentiation and spermatogenesis. AR knockout (ARKO) mice exhibit complete androgen insensitivity, and spermatogenesis in mice with Sertoli cell-selective knockout (SCARKO) cannot progress through meiosis (5, 7). The only indispensable function of LH is the regulation of testosterone synthesis in ALC (13). LHR knockout (LuRKO) mice are born phenotypically normal. However, testosterone levels in adult LuRKO mice are significantly decreased, and spermatogenesis is initially arrested at the round spermatid stage (46). In contrast to GnRH, testosterone, and LH, FSH is not indispensable for male fertility. FSHR knockout (FShRKO) male mice are fertile, although they have lower sperm counts than the corresponding wild-type mice (1).

Sertoli cells express FSHR and AR and thus integrate androgen and FSH signaling. FSHR is expressed in rat Sertoli cells in a stage-dependent fashion, with the highest levels at stages XIII–II and lowest levels at stages VII–VIII (11, 38). FSH concentration increases steadily after birth, which promotes Sertoli cell proliferation and induces an increase in AR expression in Sertoli cells (13, 38). Similar to FSHR, AR is also expressed in a stage-dependent fashion in Sertoli cells, with the highest expression levels being detected at stages VII–VIII (3, 32, 37, 39). Rising concentration of FSH and testosterone at puberty and increased AR expression regulate the final maturation of Sertoli cells (32).

Upon hormone binding, AR translocates into the nucleus, where it regulates transcription of androgen-responsive genes. AR interacts with numerous coregulatory proteins that modulate its function and activity (12, 14, 17, 20, 21). One such coregulatory protein is androgen receptor-interacting protein 4 (ARIP4). ARIP4 is a nuclear protein that interacts with AR and modulates AR-dependent transactivation (29). ARIP4 also interacts with Dyrk1A, a serine/threonine kinase involved in neuronal development and in brain physiology (35). ARIP4 binds to DNA and mononucleosomes, and it exhibits ATPase activity that is dependent on double- and single-stranded DNA.

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ARIP4 shares in its ATPase domain the highest sequence similarity with the ATRX protein. ATRX protein mutations cause (α-thalassaemia mental retardation X-linked) mental retardation with α-thalassaemia and genital abnormalities, suggesting involvement of ATRX protein in sexual differentiation (40). ARIP4 mRNA is ubiquitously expressed in adult mouse tissues, with the highest levels being found in kidney, liver, and testis (29).

We have studied the role of ARIP4 in the regulation of testicular functions by analyzing its expression and cellular localization during the postnatal development of mouse testis. Since GnRH, FSH, LH, and testosterone are the major hormones regulating testicular functions, we asked whether disruption of hormonal signaling in hpg, FSHRKO, and LuRKO mice affects testicular expression pattern of ARIP4. These knockout animals have different testicular phenotypes. Hpg mice exhibit arrested spermatogenesis at the pachytene spermatocyte stage (4, 23), and spermatogenesis in LuRKO mice is initially arrested at the round spermatid stage (46), whereas FSHRKO mice are fertile (1). Therefore, the use of three different knockout models permitted us to compare AR and ARIP4 expression pattern in mice having testicular phenotypes of different severity. We have also analyzed the endocrine regulation of ARIP4 by studying its stage-dependent expression in vitro in cultured seminiferous tubule segments. The fact that ARIP4 is important for androgen action in vivo is demonstrated by the finding that heterozygous ARIP4+/− mice are haploinsufficient and have attenuated expression of the Sertoli cell-specific Rhox3 gene. Modulation of AR signaling in somatic cells and regulation of cell proliferation in germ cells emerge as two possible targets of ARIP4 function in the testis.

**MATERIALS AND METHODS**

**Experimental animals.** Sprague-Dawley rats at ~4 mo of age and C57BL mice were housed in a constant temperature (20°C) and light-dark cycle (lights on 0600–2000) with free access to food and water. Animals were killed by CO2 asphyxiation and neck dislocation, light-dark cycle (lights on 0600–2000) with free access to food and water. Animals were killed by CO2 asphyxiation and neck dislocation, and testes were removed for subsequent analysis. The University of Helsinki and the University of Turku Committees on Ethics of Animal Experimentation approved all animal experiments.

**RNA extraction.** Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany) or TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Testes from FSHRKO and hpg mice used for RNA extraction were kind gifts of Drs. Margaret Abel and Harry Charlton (University of Oxford). Generation of LuRKO mice has been described previously (47). Testes from mice 5, 10, 20, and 200 days old were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, dehydrated, and embedded in paraffin using an automated tissue processor. Sections of 5-μm thickness were mounted onto SuperFrost Plus slides (Menzel), dewaxed, and rehydrated. To block endogenous peroxidase activity, slides were incubated in 3% hydrogen peroxide in methanol. Slides were boiled for 10 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval, washed in Tris-buffered saline (TBS), and blocked in TBS containing 1% BSA (Sigma-Aldrich, St. Louis, MO) and 3% normal goat serum (Vector Laboratories, Burlingame, CA). Slides were incubated with primary antibody (anti-ARIP4 K7991a1 at 1:400 dilution, anti-AR at 1:200, or nonimmune rabbit serum at 1:200) overnight at 4°C. Immunodetection was performed with VECTASTAIN ABC and peroxidase DAB substrate kits (Vector Laboratories) according to the manufacturer’s instructions. Slides were dehydrated in ascending ethanol series and mounted in Permount mounting medium (Fisher Chemicals, Fair Lawn, NJ).

**Microdissection and hormone stimulation.** Rat seminiferous tubules were dissected into 5-mm long stage-specific segments under a stereomicroscope by the transillumination-assisted microdissection method (19, 42). For the hormone stimulation, three segments corresponding to each stage range were pooled together and cultured for 2 h in DMEM/Ham’s F-12 medium (1:1) supplemented with 15 mM HEPES, 1.25 g/l sodium bicarbonate, 10 mg/l gentamycin sulfate, 60 mg/g penicillin, and 1 g/l BSA at 34°C in a humidified atmosphere containing 5% CO2 (25). The segments were subsequently incubated in the above-mentioned culture medium supplemented with 0.1 mM 3-isobutyl-1-methylxanthine (Mix, Sigma-Aldrich) in the presence or absence of 1 μM testosterone, 10 ng/ml FSH (Sigma-Aldrich), or 10 μM forskolin (Sigma-Aldrich) for 4, 8, or 16 h.

**Quantitative RT-PCR.** The cDNA was synthesized using random hexamer primers with Super-Script III first-strand synthesis kit for RT-PCR (Invitrogen). Two to four independent samples were used for each data point, and duplicates of each sample were analyzed by quantitative RT-PCR. For genomic DNA contamination control, samples with no added reverse transcriptase enzyme were included. Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics) in a 20-μl volume with 3.4 mM MgCl2, 1 μM forward and reverse primers (primer sequences are listed in Table 1), and the LightCycler-DNA Master SYBR Green I mix (Roche) according to the manufacturer’s instructions. PCR protocol included initial 10-s denaturation (95°C) followed by 45 cycles.
of 10-s denaturation (95°C), 5-s annealing (65°C, 57°C for GAPDH), 10-s extension (72°C), and 5-s SYBR Green I signal measuring (80°C, 82°C for GAPDH, 86°C for AR). To detect possible unspecific amplification, DNA melting step was included after completion of PCR cycles. The resulting curves were quantified using LightCycler analysis software according to the manufacturer’s instructions.

RESULTS

Developmental expression of ARIP4 in the testis. The amount of ARIP4 mRNA in the mouse testis during postnatal development was analyzed by Northern blotting. ARIP4 mRNA level was low in newborns but increased on days 5 and 10, reaching the peak on day 20 (Fig. 1, A and B). In situ hybridization of testis sections from 7-mo-old mice detected high levels of ARIP4 mRNA mainly in pachytene and diplo-tene spermatocytes at stages X and XI (Fig. 1, C and D). Control hybridization with the sense probe detected no specific signal (data not shown). It should be noted that high mRNA levels in pachytene spermatocytes do not necessarily correspond to high protein amounts (18). To analyze the pattern of ARIP4 protein expression, we utilized immunostaining with anti-ARIP4 antibodies.

In agreement with RNA blot data, ARIP4 immunostaining became evident in Sertoli cell nuclei starting on day 5 (Fig. 2, A and B). Sertoli cells in seminiferous cords were stained much stronger than interstitial cells. We observed similar ARIP4 expression on day 10 (Fig. 2, C and D). On day 20, Sertoli cell nuclei possessed stage-specific ARIP4 staining. In addition, nuclei of pachytene spermatocytes were ARIP4 positive, with increased staining of XY bodies (Fig. 2, F, inset, arrowheads). ARIP4 immunostaining was also evident in the nuclei of Leydig cells. In adult testis (Fig. 2, G and H), Sertoli cell nuclei had strong ARIP4 immunostaining at stages II–VI and even stronger ARIP4 immunostaining at stages VII–VIII, but weaker ARIP4 immunostaining at stages IX–XII. Leydig cell nuclei became clearly ARIP4-positive in adult testis (ALC; Fig. 2, G and H). Weak staining was detected in differentiating spermatogonia. Pachytene spermatocyte nuclei at stages II–V were stained relatively strongly, with the XY bodies being ARIP4

Table 1. Primers for quantitative RT-PCR

<table>
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<tr>
<th>mRNA</th>
<th>Primers</th>
<th>Reference</th>
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<tbody>
<tr>
<td>ARIP4</td>
<td>TTAGGCACAGGCGAAAGGATGCC</td>
<td>PrimerBank (13507688a1) (44)</td>
</tr>
<tr>
<td></td>
<td>CTCCTGGATCTGCGGAGTAGCTTTCG</td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>AAGGACGCTTGTGAATTTGGGAC</td>
<td>PrimerBank (34)</td>
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<tr>
<td></td>
<td>TCCAGACATCTTCAAGCAGAC</td>
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</tr>
<tr>
<td>Rhox5</td>
<td>GGCAGCGATCATCATCTGGAACA</td>
<td>PrimerBank (48)</td>
</tr>
<tr>
<td></td>
<td>CAAATCTCAGCTGCGAGCGAAAA</td>
<td></td>
</tr>
<tr>
<td>Mouse AR</td>
<td>CTGGGAGAGGCTTCTACCCAC</td>
<td>PrimerBank (7304901a1) (44)</td>
</tr>
<tr>
<td></td>
<td>GGGTCTATGGTACGCGGCCTCG</td>
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</tr>
<tr>
<td>Eppin</td>
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<td>PrimerBank (8)</td>
</tr>
<tr>
<td></td>
<td>TGGGATCTGAGGAGAGCGCTCCTG</td>
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</tr>
<tr>
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<td>PrimerBank (31980789a1) (44)</td>
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<td></td>
<td>CTGCAGCTCTGCTGCTATCTGG</td>
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<tr>
<td>Rat ARIP4</td>
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<td></td>
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<tr>
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<td>Rat Rhox5</td>
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ARIP4, androgen receptor-interacting protein 4; AR, androgen receptor; FSHR, follicle-stimulating hormone receptor.
ARIP4 expression in testes of mice aged 5 (A and B), 10 (C and D), and 20 days (E and F) and adult mice (G and H). ARIP4 is evident in the nuclei of Sertoli cells at all ages studied, reaching the highest expression level in adult mice. In adult testes, Sertoli cell nuclei have strong ARIP4 immunostaining at stages II–V and VII–VIII. With the onset of spermatogenesis, ARIP4 is expressed in spermatogonia, pachytene, and diplotene spermatocytes in a stage-dependent manner. XY bodies in the nuclei of pachytene spermatocytes are strongly stained (F and H, insets). ARIP4 is also present in the nuclei of adult-type Leydig cells. Representative fetal Leydig cells (FLC), adult Leydig cells (ALC), Sertoli cells (Se), peritubular myoid cells (P), mesenchymal cells (M), spermatogonia (sg), pachytene (psc) and diplotene spermatocytes (dsc), and XY bodies (arrowheads) are indicated. Roman numerals indicate stages of the seminiferous epithelial cycle. Bar, 50 μm.

Expression of ARIP4 and AR in testis of adult wild-type and ARIP4<sup>+/−</sup> mice. Targeted disruption of mouse ARIP4 gene results in early embryonic lethality (Zhang FP, Domanskyi A, Palvimo JJ, Sariola H, Partanen J, Jänne OA, unpublished observations). Heterozygous ARIP4 animals, however, are fertile and appear normal. To study testicular phenotype of ARIP4 heterozygotes, we compared expression patterns of ARIP4 and AR in testes from wild-type and ARIP4<sup>+/−</sup> mice (Fig. 3). ARIP4<sup>+/−</sup> testis had normal histology (data not shown). Expression of ARIP4 antigen in testes from ARIP4<sup>+/−</sup> mice was positive (Fig. 2H, inset, arrowheads). ARIP4 expression in pachytene spermatocytes was still detectable at stages VII and VIII but became weaker from stage IX onward (Fig. 2, G and H). Leptotene and zygote spermatocytes also exhibited some immunostaining. In general, weak ARIP4 immunostaining in spermatocytes was detectable throughout the development. Early round spermatids appeared faintly stained, whereas all other spermatids were negative. No specific signal was detected on control slides stained with nonimmune rabbit serum (data not shown).
qualitatively similar to that of wild-type testes, with positive Leydig and Sertoli cell nuclei, spermatogonia, pachytene, and diplotene spermatocytes (Fig. 3, A, C, and E). In ARIP4+/− and in wild-type testes, AR was detected in Leydig, Sertoli, and peritubular myoid cells (Fig. 3, B, D, and F). Sertoli cell nuclei had a strong ARIP4 immunostaining at stages II–VI and VII–VIII and a strong AR immunostaining at stages VII–VIII. Thus the disruption of one ARIP4 gene allele in ARIP4+/− mice did not influence either testicular histology or expression pattern of ARIP4 and AR.

Expression of ARIP4 and AR in testis of adult wild-type, FSHRKO, LuRKO, and hpg mice. To get more insight into mechanism(s) governing the stage-specific expression of ARIP4 and AR in Sertoli cells, we compared the amounts of these proteins in testis from 4-mo-old wild-type, FSHRKO, LuRKO, and hpg mice. Loss of FSH, LH, or GnRH signaling in the corresponding knockout animals results in testicular phenotypes of different severity. In wild-type mouse testis, both ARIP4 and AR were detected in the nuclei of Sertoli and Leydig cells (Fig. 4, A and B). In Sertoli cells, both ARIP4 and AR expression were clearly stage dependent. ARIP4 expression in Sertoli cells was high at stages II–VI and, similar to AR, peaked at stages VII–VIII. In addition, we detected AR in peritubular myoid cells, but not in germ cells (Fig. 4B). ARIP4 was clearly detectable in spermatogonia, pachytene, and diplotene spermatocytes, but not in peritubular myoid cells (Fig. 4A).

ARIP4 expression pattern in the testis of FSHRKO (Fig. 4C) and LuRKO (Fig. 4E) mice did not differ significantly from that of wild-type mice (Fig. 4A). In both FSHRKO and LuRKO testis, interstitial cells and Sertoli cells as well as spermatogonia and spermatocytes were ARIP4 positive and round spermatids were negative (Fig. 4, C and E). FSHRKO testis exhibited stage-specific ARIP4 expression with higher levels at stages II–V and lower levels at stage XI (Fig. 4C). Spermatogenesis in LuRKO mice is initially arrested at the round spermatid stage, and elongated spermatids are rarely detected in testis sections from older mice (46). Absence of elongated spermatids in LuRKO testis makes it difficult to stage seminiferous tubule sections. However, we detected higher ARIP4...
expression in Sertoli cells and spermatocytes at stages I–VII, and lower expression at stage XII (Fig. 4E). AR was detectable in interstitial, Sertoli, and peritubular myoid cells in FSHRKO and LuRKO testis sections. Similar to wild-type testis, Sertoli cell nuclei of FSHRKO and LuRKO testes exhibited stage-specific AR expression (Fig. 4, B, D, and F).

Sertoli cells in hpg testes develop atypically, and their nuclei are often centrally located, similar to 10-day-old wild-type testes (Ref. 27; also compare Fig. 2C with Fig. 4G). In the testis of hpg mice, ARIP4 was detected mainly in interstitial cells and, to a lower extent, in Sertoli cells and germ cells (Fig. 4G). ARIP4 expression in Sertoli and germ cells in hpg testis
was much lower than that in 10-day-old wild-type testis (Figs. 2C and 4G). However, this may be due to different tissue fixation methods used (PFA for wild type and Bouin’s solution for \( hpg \)). AR was mainly expressed in peritubular myoid cells of \( hpg \) testis but was also present in interstitial and Sertoli cells (Fig. 4H). Thus, the disruption of FSH and LH signaling in FSHRKO and LuRKO mice does not change the patterns of ARIP4 and AR expression.

**ARIP4 mRNA levels in rat seminiferous tubules in response to hormone stimulation.** To analyze potential hormonal factors responsible for the stage-specific expression of ARIP4 in adult testis, we measured relative amounts of ARIP4 (rat ortholog, GenBank accession no. XM_343471) and AR mRNA in rat seminiferous tubules at different stages of spermatogenesis. The level of rat ARIP4 mRNA peaked at stages II–VI and was significantly \((P < 0.05)\) lower at stages XIII–I (Fig. 5A). AR mRNA level at stages II–VI was also high; however, the highest level of AR mRNA was detected at stages VII–VIII (Fig. 5B).

We cultured stage-specific segments of seminiferous tubules from adult rat testes for 4, 8, or 16 h in the presence of a phosphodiesterase inhibitor (MIX) and 1 \( \mu M \) testosterone, 10 ng/ml FSH, or 10 \( \mu M \) forskolin. Neither testosterone nor FSH or forskolin significantly changed ARIP4 mRNA levels compared with untreated samples (Fig. 5, C and D). As a positive control, a 16-h testosterone treatment resulted in a 40–60% increase in Rhox5 mRNA level at stages VII–VIII (data not shown).

**Levels of ARIP4, AR, FSHR, Rhox5, and Eppin mRNA in testes of wild-type and mutant mice.** As shown above, the disruption of one ARIP4 gene allele did not qualitatively change the expression pattern of ARIP4 and AR proteins in the testis. To look for quantitative changes in gene expression, we compared levels of mRNAs encoding ARIP4, AR, FSHR, and androgen-dependent Rhox5 and Eppin (also called Spinlw1) in testes from 15-mo-old wild-type and \( \text{ARIP4}^{+/+} \) mice. Expression of Rhox5 in the testis is Sertoli cell specific and androgen-dependent (22). Eppin (epididymal protease inhibitor) is expressed in testis and epididymis, and its expression pattern resembles that of the Rhox5 gene (8, 36). Both Rhox5 and Eppin genes are strongly downregulated in testes of SCARKO mice (8) and, therefore, can serve as good natural indicators even of subtle changes in AR-dependent gene transcription. ARIP4 mRNA level in testes from \( \text{ARIP4}^{+/+} \) animals was reduced to 40–45% of that from wild-type controls (Fig. 6A). Immunoblot analysis of testis extracts detected a corresponding decrease in ARIP4 protein expression (data not shown). Tissue levels of AR and FSHR mRNA from wild-type and \( \text{ARIP4}^{+/+} \) mice were not significantly different, suggesting that Sertoli cell function is normal in \( \text{ARIP4}^{+/+} \) testes (Fig. 6A). However, Rhox5 mRNA level in \( \text{ARIP4}^{+/+} \) testes was significantly reduced \((P = 0.03, n = 4)\) to \(~80\%\) of that in wild-type controls (Fig. 6A). Eppin mRNA levels were reduced slightly, but not significantly, in testis of 15-mo-old \( \text{ARIP4}^{+/+} \) mice (Fig. 6A); however, the reduction was significant \((P = 0.03)\) in 3-mo-old \( \text{ARIP4}^{+/+} \) mice (data not shown). Collectively, these results indicate that \( \text{ARIP4}^{+/+} \) mice are haploinsufficient with regard to AR-specific gene expression in Sertoli cells and support the notion that ARIP4 is required for androgen-dependent regulation of at least some genes in vivo.

In light of the fact that ARIP4 acts as an AR coregulator in vitro (29) and in vivo, it was pertinent to examine how testosterone depletion affected ARIP4 mRNA levels. In adult LuRKO mice, the content of testosterone in the testis is reduced by 97% compared with wild-type mice (46). We measured ARIP4, AR, FSHR, Rhox5, and Eppin mRNA levels in total RNA isolated from testes of 4-mo-old wild-type and LuRKO mice. As expected, very low levels of Rhox5 mRNA and reduced levels of Eppin mRNA were detectable in testes of LuRKO mice. Interestingly, AR and FSHR mRNA levels were significantly \((P < 0.05)\) higher in testes of LuRKO mice than in wild-type testis (Fig. 6B). Likewise, elevated AR and FSHR mRNA levels were found in testes of \( hpg \) mice (Fig. 6C).
mouse testis sections and quantitative RT-PCR of rat seminiferous tubule segments indicated that ARIP4 mRNA levels varied according to the stage of the seminiferous epithelial cycle. Immunostaining of testis sections with affinity-purified anti-ARIP4 antibody confirmed stage-specific distribution of ARIP4. Immunoreactive ARIP4 antigen was present in the nuclei of Sertoli cells at all ages studied, and ALC were clearly ARIP4 positive. We also detected ARIP4 in spermatogonia, pachytene, and diplote spermatocytes. The presence of ARIP4 in XY bodies is not unexpected, since ARIP4 is sumoylated (9), and small ubiquitin-related modifier-1 is known to localize to XY bodies in pachytene spermatocytes (43). We were particularly interested in the stage-dependent expression of ARIP4 in Sertoli cells that closely resembles AR expression pattern. We confirmed here the previous results showing that AR mRNA levels are highest at stages VII–VIII (41). Expression of AR protein in rat testis is highest at stages VI–VIII and lowest at stages IX–XIV (39). In our experiments, both ARIP4 and AR proteins were detected in the nuclei of Leydig and Sertoli cells. The highest levels of ARIP4 and AR proteins in Sertoli cell nuclei were found at stages VII–VIII. ARIP4 protein expression was also high at stages II–VI.

The mechanisms governing the cyclic activity of Sertoli cells are not fully understood. Most likely, the combination of hormone actions with signals coming from developing germ cells regulates the stage-specific protein expression in Sertoli cells (10). In this work, we analyzed several knockout mouse models to study the effects of disrupted hormone signaling on ARIP4 and AR expression in the testes. Male LuRKO mice have severely impaired testosterone production in adult testes and significantly increased levels of LH and FSH in serum (46, 47). ARIP4 mRNA levels in testes from LuRKO mice were the same as those in wild-type testes, in striking contrast to Rhox5 mRNA. Rhox5 is a Sertoli cell-specific, androgen-dependent protein (22) serving as a positive control for disrupted androgen signaling. In agreement with a recent publication reporting androgen-dependent regulation of Eppin mRNA (8), we found reduced Eppin mRNA level in testes from LuRKO mice. We also observed an increase in AR and FSHR mRNA expression in LuRKO and hpg testes. Spermatogenesis in hpg mice is arrested at the pachytene spermatocyte stage (4, 23), and in LuRKO mice spermatogenesis is initially arrested at the round spermatid stage (46). Therefore, different cellular composition of wild-type, LuRKO, and hpg testes could account for some of the differences in the normalized mRNA values. However, LuRKO testes have also reduced numbers of AR-positive interstitial cells. Given that different cellular composition of wild-type, LuRKO, and hpg testes did not affect ARIP4 mRNA levels, it is unlikely that altered cellular environment accounts for such a high increase in AR mRNA level. It is known that androgen withdrawal results in upregulation of AR mRNA accumulation in rat prostate (31). In a similar fashion, reduced levels of testicular testosterone in adult LuRKO, hpg, and FSHRKO mice (2, 33, 46) can potentially be responsible for increased AR mRNA levels in the testes of these mutant mice. FSH upregulates FSHR mRNA in immature Sertoli cells (6), and thus increased serum FSH in LuRKO mice could account for the observed increase in testicular FSHR mRNA accumulation. It remains to be elucidated whether or not the reduced concentration of circulating FSH is responsible for increased FSHR mRNA levels in hpg testis. Interestingly, ARIP4 mRNA
content was slightly, but significantly, reduced in FSHRKO testis, possibly reflecting either the reduced number of Sertoli cells in testes of FSHRKO mice (15) or the importance of FSH signaling in the regulation of ARIP4 gene expression.

Although disruption of LH signaling in LuRKO mice resulted in upregulation of AR and FSHR (but not ARIP4) mRNA, LHR knockout did not affect qualitatively testicular distribution of ARIP4 and AR proteins. Likewise, the loss of FSH signaling in FSHRKO mice failed to have an obvious effect on the expression pattern of ARIP4 and AR proteins. Sertoli cells in hpg mice develop abnormally (27), and we found ARIP4 expression pattern in hpg testes to be similar to that in testes from 10-day-old mice. Reduced ARIP4 expression in Sertoli and germ cells and its possible link to arrest in spermatogenesis in hpg testis require further studies. In testes of all mutant mice studied, AR distribution was qualitatively similar, with the AR protein being detected in interstitial (Leydig) cells, Sertoli cells, and peritubular myoid cells.

Several studies have utilized microarray analysis to evaluate the effects of androgen or FSH on gene expression in rat and mouse testes (8, 24, 30, 48) and report no changes in ARIP4 or AR mRNA levels. However, these studies did not address different stages of seminiferous epithelial cycle. We dissected seminiferous tubule segments corresponding to different stages of the epithelial cycle and cultured them in the presence of testosterone, FSH, or forskolin. Testosterone, FSH, or forskolin treatments for 4, 8, or 16 h did not significantly change ARIP4 mRNA levels. Combining this result with similar distribution of ARIP4 antigen in testis sections from wild-type, FSHRKO, LuRKO, and hpg mice, we conclude that testosterone, FSH, or LH has limited, if any, influence on ARIP4 expression in the testis.

We (29) have reported previously that ARIP4 is an AR coregulator in vitro. We studied in this work ARIP4 functions in vivo by analyzing testes from heterozygous ARIP4+/− mice. ARIP4 knockout results in early embryonic lethality and our preliminary results suggest that ARIP4 is involved in the regulation of cell proliferation (Zhang FP, Domanskyi A, Palvimo JJ, Sariola H, Partanen J, Jänne OA, unpublished observations). ARIP4+/− mice are fertile, appear normal, and have normal distribution of ARIP4 and AR proteins in the testes. However, we found that ARIP4+/− mice were haploinsufficient with regard to androgen action in Sertoli cells in that these animals have reduced levels of androgen-dependent Rhox5 mRNA. Thus, at least in Sertoli cells, where ARIP4 is expressed together with AR, ARIP4 appears to be important for androgen action acting as an AR coregulator in vivo. However, we cannot formally rule out the possibility that ARIP4 regulates Rhox5 gene expression indirectly by affecting pathway(s) other than AR signaling. We are currently analyzing global effects of ARIP4 knockout on gene expression (Zhang FP, Domanskyi A, Palvimo JJ, Sariola H, Partanen J, Jänne OA, unpublished observations).

In summary, our results suggest that ARIP4 is an in vivo AR coregulator in the Sertoli cells of the testis. It remains to be elucidated whether this applies to other somatic cells of testis and/or to other androgen-regulated tissues. ARIP4 expression in the germ cells implies that it also has AR-independent functions. We have recently suggested involvement of ARIP4 in DNA recombination and chromosomal segregation (9), and these ARIP4 functions may potentially be among the several important processes that are involved in normal spermatogenesis.

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