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ANDROGEN RECEPTOR-DEPENDENT AND INDEPENDENT FUNCTIONS OF ARIP4

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ACADEMIC DISSERTATION

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To the memory of my grandmother Maria

CONTENTS

ABSTRACT

Androgen receptor (AR) is necessary for normal male phenotype development and essential for spermatogenesis. AR is a classical steroid receptor mediating actions of male sex steroids – testosterone and 5α-dihydrotestosterone. Numerous coregulators interact with the receptor and regulate AR activity on target genes.

This study deals with the characterization of androgen receptor-interacting protein 4 (ARIP4). ARIP4 binds DNA, interacts with AR *in vitro* and in cultured yeast and mammalian cells, and modulates AR-dependent transactivation. ARIP4 is an active DNA-dependent ATPase, and this enzymatic activity is essential for the ability of ARIP4 to modulate AR function. On the basis of sequence homology in its ATPase domain, ARIP4 belongs to the SNF2 family of proteins involved in chromatin remodeling, DNA repair, and homologous recombination. Similar to its closest homologs ATRX and Rad54, ARIP4 does not seem to be a classical chromatin remodeling protein in that it does not appear to form large protein complexes *in vivo* or remodel mononucleosomes *in vitro*. However, ARIP4 is able to generate superhelical torsion on linear DNA fragments. ARIP4 is covalently modified by SUMO-1, and mutation of six potential SUMO attachment sites abolishes the ability of ARIP4 to bind DNA, hydrolyze ATP, and activate AR function. ARIP4 expression starts in early embryonic development. In mouse embryo ARIP4 is present mainly in the neural tube and limb buds. In adult mouse tissues ARIP4 expression is virtually ubiquitous. In mouse testis ARIP4 is expressed in the nuclei of Sertoli cells in a stage-dependent manner. ARIP4 is also present in the nuclei of Leydig cells, spermatogonia, pachytene and diplotene spermatocytes. Testicular expression pattern of ARIP4 does not differ significantly in wild-type, FSHRKO, and LuRKO mice. In the testis of *hpg* mice, ARIP4 is found mainly in interstitial cells and has very low, if any, expression in Sertoli and germ cells. Heterozygous *Arip4*$^{+/-}$ mice are fertile and appear normal; however, they are haploinsufficient with regard to androgen action in Sertoli cells. In contrast, *Arip4*$^{-/-}$ embryos are not viable. They have significantly reduced body size at E9.5 and die by E11.5. Compared to wild-type littermates, *Arip4*$^{-/-}$ embryos possess a higher percentage of apoptotic cells at E9.5 and E10.5. Fibroblasts derived from *Arip4*$^{-/-}$ embryos cease growing after 2-3 passages and exhibit a significantly increased apoptosis and decreased proliferation rate than cells from wild-type embryos. Our findings demonstrate that ARIP4 plays an essential role in mouse embryonic development. In addition, testicular expression and AR coregulatory activity of ARIP4 suggest a role of ARIP4-AR interaction in the somatic cells of the testis.

ORIGINAL PUBLICATIONS

The following original articles are referred to in the text by their Roman numeral:

I. **Rouleau N, Domans'kyi A, Reeben M, Moilanen AM, Havas K, Kang Z, Owen-Hughes T, Palvimo JJ, Jänne OA** (2002) Novel ATPase of SNF2-like protein family interacts with androgen receptor and modulates androgen-dependent transcription. Mol Biol Cell 13: 2106–2119

II. **Domanskyi A, Virtanen KT, Palvimo JJ, Jänne OA** (2006) Biochemical characterization of androgen receptor-interacting protein 4. Biochem J 393: 789–795

III. **Domanskyi A, Zhang FP, Nurmio M, Palvimo JJ, Toppari J, Jänne OA** (2006) Expression and localization of androgen receptor-interacting protein 4 (ARIP4) in the testis. Am J Physiol Endocrinol Metab, doi:10.1152/ajpendo.00287.2006, *in press*

IV. **Zhang FP, Domanskyi A, Palvimo JJ, Sariola H, Partanen J, Jänne OA** (2006) Androgen receptor-interacting protein 4 (ARIP4) is essential for mouse embryonic development and cell proliferation. *Submitted*

In addition, some unpublished data are presented.

ABBREVIATIONS

AF	activation function
AMH	anti-Müllerian hormone
ANPK	androgen receptor-interacting nuclear kinase
AR	androgen receptor
ARE	androgen response element
ARIP4	androgen receptor-interacting protein 4
ARKO	androgen receptor knockout
ATP	adenosine triphosphate
ATRX	α-thalassemia, mental retardation, X-linked
bp	base pair
cAMP	cyclic adenosine-3'-5'-monophosphate
CBP	cAMP responsive element binding (CREB)-binding protein
DBD	DNA-binding domain
Dhh	Desert hedgehog
DHT	5α-dihydrotestosterone
ds	double-stranded
E	embryonic day (day post conception)
EGFP	enhanced green fluorescent protein
Fgf9	fibroblast growth factor 9
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
FSHRKO	follicle-stimulating hormone receptor knockout
FXRβ	farnesoid receptor β
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
GST	glutathione S-transferase
H	hinge
HAT	histone acetylase
HDAC	histone deacetylase
hpg	hypogonadal
HSP	heat shock protein
kDa	kilodalton

LBD	ligand-binding domain
LH	luteinizing hormone
LuRKO	luteinizing hormone receptor knockout
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MMTV	mouse mammary tumor virus
NCoR	nuclear receptor corepressor
NLS	nuclear localization signal
NTD	amino-terminal domain
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
Pdgf-A	platelet-derived growth factor A
PGC	primordial germ cell
PIAS	protein inhibitor of activated STAT
PR	progesterone receptor
RT-PCR	reverse transcriptase – polymerase chain reaction
SCARKO	Sertoli cell-selective androgen receptor knockout
Sf1	steroidogenic factor 1
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SNURF	small nuclear really interesting new gene (RING) finger
Sox9	Sry-like high mobility group (HMG)-box 9
SRC	steroid receptor coactivator
Sry	sex-determining on Y chromosome
SUMO	small ubiquitin-related modifier
SWI/SNF	switch/sucrose non-fermenting
T	testosterone
TBP	TATA-binding protein
Tfm	testicular feminization
TRAP	thyroid hormone receptor-associated protein
Wnt4	wingless-related MMTV integration site 4
Wt1	Wilm's tumor 1
ZFR	zinc finger region

INTRODUCTION

The coregulatory proteins interact with AR and modulate its transcriptional activity on target genes. These coregulators regulate AR activity by multiple mechanisms, such as promoting chromatin remodeling, DNA binding, recruitment of general transcription factors associated with RNA polymerase II complex to androgen-responsive promoters (Heinlein and Chang 2002, Jänne et al. 2000, Kang et al. 2004). Targeting such coregulators to indirectly inactivate AR signaling in prostate tumors is a promising approach to control prostate cancer progression and enhance the response to androgen ablation therapy (Heinlein and Chang 2004, Scher and Sawyers 2005).

In a search for novel proteins interacting with AR the androgen receptor-interacting protein 4 (ARIP4) was identified. ARIP4 is an active DNA-dependent ATPase modulating AR-dependent transcription both *in vivo* and *in vitro*. ARIP4 expression in male germ cells implies that the protein has also AR-independent functions in spermatogenesis. Moreover, the study of *Arip4* knockout mouse model demonstrated the essential role of ARIP4 in mouse embryonic development.

REVIEW OF THE LITERATURE

1. Early development of mouse embryo

Mouse embryonic development is a relatively well-studied process (Hogan et al. 1994, Kaufman 1992) (Edinburgh Mouse Atlas Project, genex.hgu.mrc.ac.uk). During the early embryonic days, the fertilized egg undergoes significant changes as outlined in Table 1.

After about 4.5 days post-conception (embryonic day (E) 4.5) embryo implantation occurs. At this time the embryo contains three distinct tissue lineages – trophectoderm, primitive endoderm, and primitive ectoderm (epiblast). Primitive ectoderm and extra-embryonic ectoderm form the core of the egg cylinder that is covered by visceral endoderm, whereas parietal endoderm covers the inner surface of the mural trophectoderm. Secretions of parietal endoderm cells at about E5.0 form a thick basement membrane that serves as a major barrier between the maternal and fetal environments during the early embryonic stages. Parietal endoderm, trophoblast cells, and a basement membrane between them constitute the parietal yolk sac. Simultaneously, placenta development starts from the formation of the ectoplacental cone that becomes filled with maternal blood (Hogan et al. 1994).

Table 1. Features of the mouse embryo at early stages of development. Adapted from (Kaufman 1992, Kaufman and Bard 1999)

Principal features	Embryonic day
2-cell embryo	1.5
8-cell embryo (morula)	2.5
16-cell embryo	3.0
Blastocyst	4.0
Attaching blastocyst	4.5
Implantation. Early egg cylinder. Ectoplacental cone. Reichert's membrane	5.0
Egg cylinder differentiation. Proamniotic cavity	6.0
Amnion. First evidence of the allantois. Precursors of primordial germ cells (PGCs) in the extra-embryonic mesoderm	7.0
Neural plate development and neural folds formation	7.5
First somites. Neural folds begin to close. PGCs at the base of allantois	8.0–8.5
Axial rotation ("turning"). PGCs in the hindgut endoderm	8.5–9.0
Cephalic neural folds elevate and fuse. Rostral neuropore closes. Three primitive brain vesicles form. Urogenital ridges clearly seen	9.0–9.5
Caudal neuropore forms. Forelimb buds appear. PGCs in the gonadal components of the urogenital ridges	9.5–10.25
Caudal neuropore closes. Hindlimb and tail buds appear	10.25–10.75
Distinct genital ridges with PGCs	11.0

During E5.5–E7.5, the egg cylinder differentiates into embryonic and extra-embryonic regions, and a proamniotic cavity forms. Later, amnion and chorion divide the proamniotic cavity into amniotic, exocoelomic, and ectoplacental cavities. At E6.5, when the embryonic axis is already determined, the primitive ectoderm develops into embryonic endoderm, mesoderm, and ectoderm (Hogan et al. 1994, Kaufman and Bard 1999).

About one day later (E7.5), the neural system starts to develop. The neural plate forms in the rostral part of the embryonic axis, and early head folds elevate. Half a day later, the neural folds begin to fuse and three primary brain vesicles – forebrain, midbrain, and hindbrain – appear at E8.5–E9.0 (Kaufman and Bard 1999).

The heart and vascular system start to develop in parallel with the neural system at E7.5–E8.0. Primitive blood vessels appear at E8.0 simultaneously with the primitive heart tube that starts contracting around E8.5 (Conway et al. 2003). At this time embryonic and

extra-embryonic blood circulations fuse, and primitive nucleated red blood cells originating from the blood islands in visceral yolk sac appear in the embryonic vasculature.

Around day E8.0–E8.5, the mouse embryo undergoes axial rotation. During this process the embryo reverses the initial location of its germ layers and becomes completely surrounded by extra-embryonic membranes – amnion and visceral yolk sac (Hogan et al. 1994, Kaufman and Bard 1999).

By E9.0 the heart has relatively well-defined regions that later increase in volume, differentiate, and develop into components of the four heart chambers. At this time, the heart beats regularly and powerfully and the primitive circulation is established (Conway et al. 2003). Vascular system differentiates, and first evidences of asymmetry appear. The primitive liver forms at E10.5, rapidly enlarges and becomes the principal source of hemopoiesis at E11.0–E11.5 (Kaufman and Bard 1999).

Thus, at E11.5 the mouse embryo has quite advanced and actively developing nervous and vascular systems, starting the development of other organs.

2. Male sex determination

2.1 Gonadal morphology

Alkaline phosphatase-positive primordial germ cells (PGCs) can be detected in the extra-embryonic mesoderm of the posterior amniotic fold already at E7.0–E7.5 (Table 1) (Ginsburg et al. 1990, Hogan et al. 1994, Kaufman and Bard 1999). At E8.0, these cells migrate towards the base of allantois and then to the hindgut endoderm. Most germ cells get to the base of allantois by E8.5–E9.0 and continue to migrate towards the urogenital ridges (McLaren 2003). During migration germ cells undergo active mitosis. At E9.5, the urogenital ridges can be clearly seen extending from the mid-trunk to the mid-tail region (Hogan et al. 1994). At E10.0–E10.25, the lateral part of each urogenital ridge forms the mesonephros, and the medial part forms the gonads. About this time Wolffian (or mesonephric) ducts start to develop, whereas Müllerian (or paramesonephric) ducts form one day later, but regress in the males soon afterwards. At E11.5, the gonads contain large numbers of germ cells. At this time the gonads are indifferent, that is, their morphology does not reflect the sex of the embryo (Fig. 1) (Brennan and Capel 2004). Sertoli cell precursors start to differentiate in male gonads at E11.5. Increased proliferation of interstitial cells, migration of mesonephric cells, vascularization, and development of Leydig cells follow Sertoli cell differentiation (Brennan and Capel 2004).

At E12.5, the testicular cords extend and incorporate the germ cells and Sertoli cells, and fetal Leydig cells are present in the interstitial tissue (Fig. 1) (Brennan and Capel 2004, Karl and Capel 1998). Primitive seminiferous tubules form by E13.0, and germ cells continue to proliferate within these tubules until mitotic arrest at about E14.0 (Hogan et al. 1994). After birth, germ cells move to the periphery of the seminiferous tubules, so that on postnatal days 3–7 type A spermatogonia appear lying on the basement membrane (Brennan and Capel 2004, Hogan et al. 1994).

2.2 Molecular mechanisms of male sex determination

Three proteins – sex-determining on Y chromosome (Sry), Sry-like high mobility group (HMG)-box 9 (Sox9), and fibroblast growth factor 9 (Fgf9) – together with other growth factors and pro-testis proteins are actively involved in male sex determination (Fig. 1) (Ronfani and Bianchi 2004).

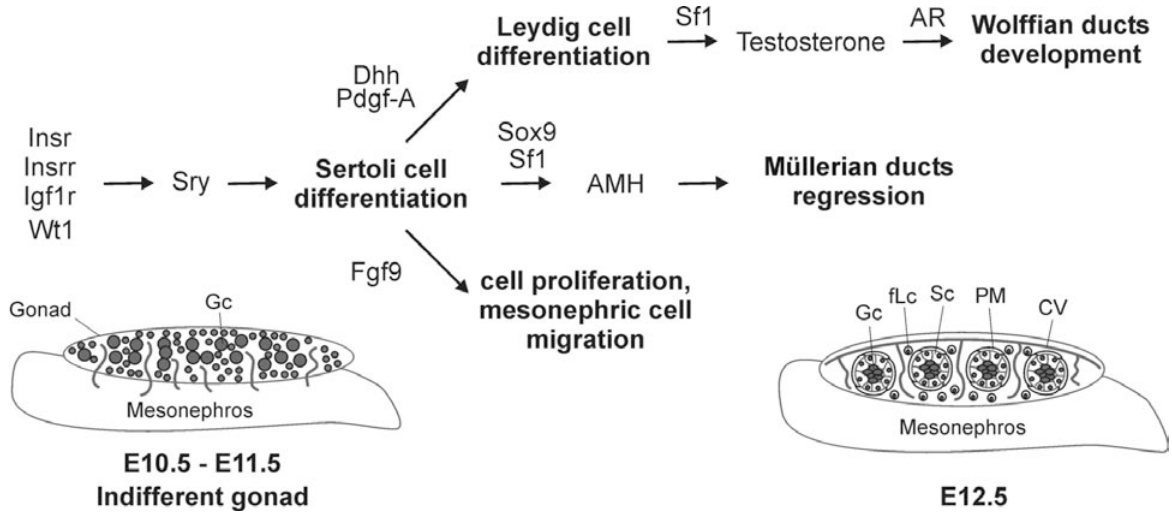

Fig. 1. Scheme of the male differentiation cascade and gonadal morphology. Gc, germ cell; Sc, Sertoli cell; fLc, fetal Leydig cell; PM, peritubular myoid cell; CV, coelomic vessel. Adapted from (Brennan and Capel 2004, Ronfani and Bianchi 2004).

Around E10.5, Wilm's tumor 1 protein (Wt1) along with signals from the insulin receptor family members (insulin receptor (Insr), insulin receptor-related receptor (Insrr), and insulin-like growth factor 1 receptor (Igf1r)) activate *Sry* expression in the genital ridges (Brennan and Capel 2004, Hogan et al. 1994, Park and Jameson 2005). Sry acts either as a classical transcription factor or as a chromatin remodeler to initiate Sertoli cell differentiation and male development (Brennan and Capel 2004, Ronfani and Bianchi 2004). At E11.5, the

differentiating precursors of Sertoli cells start expressing Sox9. Sox9 cooperates with orphan nuclear receptor steroidogenic factor 1 (Sf1) to activate the expression of the anti-Müllerian hormone (AMH) that promotes regression of Müllerian ducts (Ronfani and Bianchi 2004). In addition, Sox9 upregulates expression of *Fgf9* that is essential for proliferation of Sertoli cell precursors. A recently proposed model suggests the establishment of a feed-forward loop where Fgf9 promotes expansion of Sertoli cell precursors that secrete more Fgf9. This effect stabilizes Sox9 expression, and represses the ovary-promoting gene *Wnt4* (wingless-related MMTV integration site 4) (Kim et al. 2006). Wnt4 is essential for ovarian cell differentiation and function, and it may also be involved in early testis development (Jeays-Ward et al. 2004, Vainio et al. 1999). In addition to stimulating the proliferation of Sertoli cell precursors, Fgf9 promotes migration of mesonephric cells. Sprouty2 – a membrane-associated inhibitor of tyrosine kinase signaling – is involved in the regulation of migration (Chi et al. 2006). Differentiating Sertoli cells also express Desert hedgehog (Dhh) and platelet-derived growth factor A (Pdgf-A) that promote Leydig cell differentiation and Sf1 expression (Fig. 1) (O'Shaughnessy et al. 2005). In differentiating Leydig cells Sf1 activates expression of proteins involved in testosterone biosynthesis (Park and Jameson 2005). Testosterone acts through the androgen receptor expressed from E12.5–E13.0 onwards in the mesonephric mesenchyme around the differentiating Wolffian ducts (Cooke et al. 1991, Crocoll et al. 1998), and supports survival and differentiation of Wolffian ducts into the vas deferens, seminal vesicles, and epididymis. In females, Wnt4 signaling suppresses testosterone biosynthesis (Heikkilä et al. 2005), Wolffian ducts regress due to the absence of testosterone signaling, whereas Müllerian ducts persist due to the presence of Wnt4 signaling and the absence of AMH (Vainio et al. 1999).

The male sex determination mechanism was characterized using several knockout mouse models (Brennan and Capel 2004). Male mice with *Sry* deletion develop as fertile females (Lovell-Badge and Robertson 1990). Conditional knockout of *Sox9* in the developing gonads interferes with Sertoli cell differentiation and seminiferous tubule formation (Chaboissier et al. 2004). Most gonads in *Fgf9* knockout male mice are fully sex-reversed and do not express male markers downstream of *Sry* (Colvin et al. 2001). *Sf1* knockout mice lack adrenal glands and gonads (Luo et al. 1994, Sadovsky et al. 1995), whereas *Dhh* knockout male mice exhibit differentiation defects of fetal Leydig cells (Yao et al. 2002).

3. Cellular organization of adult testis

Morphologically, the adult testis is organized into seminiferous tubules separated by the interstitial space (Russell et al. 1990). Spermatogenesis takes place within the seminiferous tubules, where Sertoli cells and germ cells are located. Sertoli cells and germ cells are surrounded by peritubular myoid cells. The interstitial space contains blood and lymphatic vessels, and Leydig cells (Fig. 2).

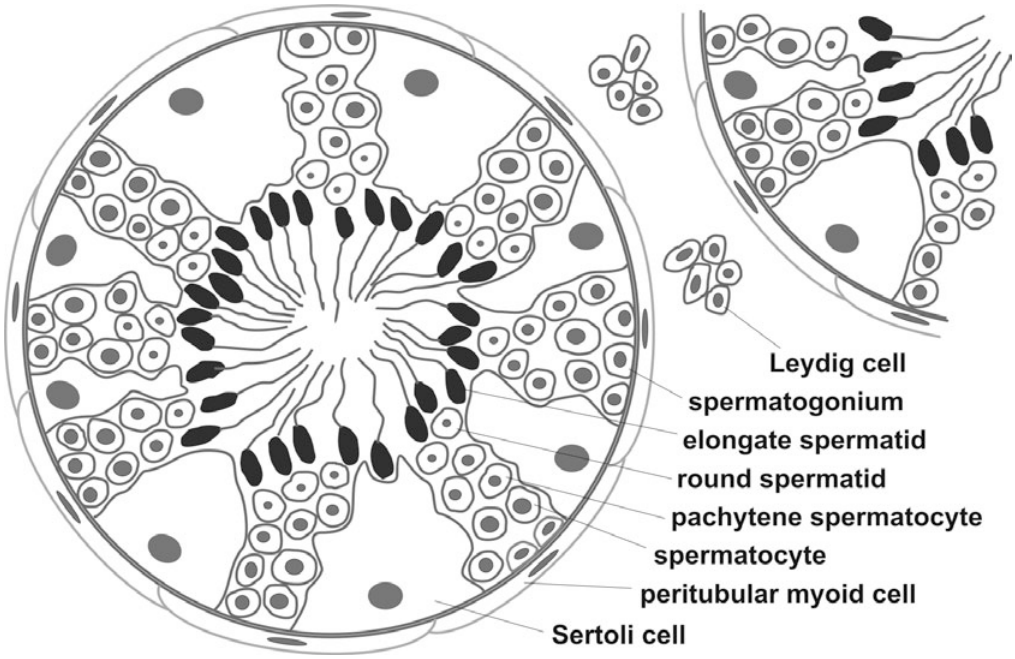

Fig. 2. Scheme of the cellular organization of the testis. Adapted from (Russell et al. 1990).

3.1 Sertoli cells

Sertoli cells support spermatogenesis by maintaining the integrity and compartmentalization of the seminiferous epithelium, delivering nutrients to the germ cells, and regulating the spermatogenic cycle (Russell et al. 1990). In neonatal testis, Sertoli cells are immature and able to proliferate. Sertoli cells undergo extensive proliferation, growth, and functional maturation during the postnatal period in order to support spermatogenesis after puberty. The maturation involves the loss of proliferative ability, formation of tight junctions, and changes in gene expression. Testosterone, but not FSH, is required for Sertoli cell proliferation in male mice during fetal and early neonatal life (Johnston et al. 2004). However, later in life thyroid hormone, androgens, follicle-stimulating hormone (FSH), and paracrine factors secreted by germ cells regulate and modulate Sertoli cell maturation (Sharpe

et al. 2003). FSH and thyroid hormone both induce AR expression and suppress AMH expression in maturating Sertoli cells (Arambepola et al. 1998, Holdcraft and Braun 2004, Tan et al. 2005b). The increase in concentrations of FSH and testosterone at puberty and increased expression of AR enable final maturation of Sertoli cells (Sharpe et al. 2003). Recent studies of Sertoli cell-selective *Ar* knockout (SCARKO) mice have shown that Sertoli cells proliferate and express maturation markers even in the absence of AR expression. However, SCARKO Sertoli cells are unable to support late meiotic and post-meiotic germ cells (Tan et al. 2005b). The presence of immature Sertoli cells in adult testis results in spermatogenesis disorders (Sharpe et al. 2003). Mature Sertoli cells express FSH receptor (FSHR) and AR, and thus integrate androgen and FSH signaling. FSH receptor is expressed in Sertoli cells in a stage-dependent fashion, having the highest levels at stages XIII–II and the lowest levels at stages VII–VIII (Heckert and Griswold 1991, Tan et al. 2005b). Similar to FSHR, AR is also expressed in a stage-dependent manner in Sertoli cells, with the highest expression levels detected at stages VII–VIII (Bremner et al. 1994, Sharpe et al. 2003, Suarez-Quian et al. 1999, Tan et al. 2005b). The stage-specific patterns of protein expression in Sertoli cells most probably result from the combination of hormone actions with signals coming from developing germ cells (Griswold 1995, Tan et al. 2005a).

3.2 Leydig cells

The main function of the Leydig cells is the production of testosterone from cholesterol. There are two generations of Leydig cells, arising in mammals in distinct periods of life.

The first generation develops during fetal life and is responsible for male sex determination. These cells differentiate from mesenchymal-like stem cells and start producing testosterone around E12.5. Neither the initial differentiation nor testosterone production in mouse fetal Leydig cells depend on luteinizing hormone (LH) signaling (O'Shaughnessy et al. 1998, Zhang et al. 2001). However, as noted before, the differentiation of fetal Leydig cells appears to depend on the Sertoli cell products Dhh and Pdgf-A (Fig. 1) (O'Shaughnessy et al. 2005). Fetal Leydig cells have no mitotic activity and gradually regress during late fetal period and after birth (Habert et al. 2001).

The second Leydig cell generation arises during puberty and supports spermatogenesis. The adult Leydig cells develop from undifferentiated mesenchymal-like cells that proliferate neonatally and differentiate into precursor Leydig cells. During puberty, these cells mature, start to express Leydig cell-specific markers, such as LH receptor, and

increase testosterone production (Habert et al. 2001). The first adult Leydig cells start to appear in the mouse around postnatal day 7, their number reaches adult levels around postnatal day 30 (O'Shaughnessy et al. 2002, O'Shaughnessy et al. 2005). Growth and maturation of the adult Leydig cell population depend on LH signaling (Ahtiainen et al. 2007, Huhtaniemi et al. 2006). In addition, androgens, acting either directly on the Leydig cells or through the peritubular myoid cells, are required for normal Leydig cell development (O'Shaughnessy et al. 2005, Zhang et al. 2004).

3.3 Peritubular myoid cells

Peritubular myoid cells originate from mesonephric cells that migrate to the developing gonads between E11.5–E13.5. Peritubular myoid cells express actin filaments and other cytoskeletal proteins such as myosin, desmin, and α-actinin, and provide structural integrity to the seminiferous tubule. Due to the ability to contract, peritubular myoid cells mediate the transport of spermatozoa and testicular fluid though seminiferous tubules. In addition, these cells take part in the regulation of spermatogenesis by expressing components of extra-cellular matrix and growth factors that affect Sertoli cell functions (Maekawa et al. 1996, Siu and Cheng 2004, Tan et al. 2005b, Verhoeven et al. 2000). Peritubular myoid cells might also mediate androgen action on Leydig cells (O'Shaughnessy et al. 2005). Male mice lacking *Ar* in peritubular myoid cells have reduced sperm counts, suggesting that functional AR in these cells is required to maintain normal function of Sertoli cells and peritubular myoid cells contractility (Zhang et al. 2006).

3.4 Germ cells

In the process of spermatogenesis, diploid stem cell spermatogonia develop into haploid spermatozoa. This development can be divided into three major phases – mitotic (proliferation) phase, meiotic phase, and spermiogenic (differentiation) phase (Fig. 3) (Russell et al. 1990).

During the mitotic phase, spermatogonial stem cells (A_s) located in the periphery of seminiferous tubules divide and give rise to either new A_s cells or to interconnected A-paired (A_{pr}) spermatogonia. Spermatogonial self-renewal and differentiation depend, at least in part, on glial cell-derived neurotrophic factor (GDNF) produced by Sertoli cells (Meng et al. 2000). Interestingly, when cultured under appropriate conditions, spermatogonial stem cells from neonatal or adult mouse testis can acquire properties of embryonic stem cells (Guan et al. 2006, Kanatsu-Shinohara et al. 2004). Progenitors of an A_{pr} cell connected by intercellular

bridges are called A-aligned (A_{al}) spermatogonia. A-aligned spermatogonia divide synchronously to form chains of 8, 16, and even 32 intercellular bridge-connected A_{al} cells. These cells differentiate into A_1 spermatogonia that undergo subsequent mitotic divisions and give rise to A_2, A_3, A_4, intermediate (In), and B spermatogonia (Fig. 3) (de Rooij 2001).

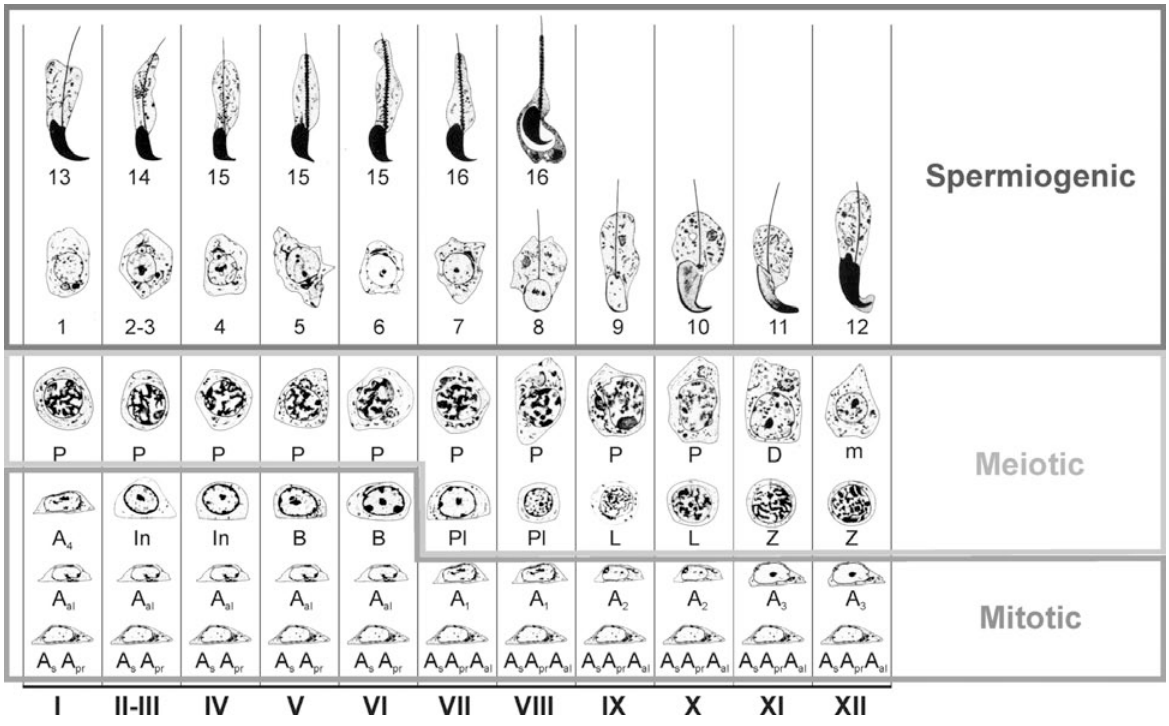

Fig. 3. Scheme of the spermatogenesis in the mouse seminiferous epithelium. Roman numerals indicate stages of the epithelial cycle. A_s, spermatogonial stem cells; A_p, A-paired spermatogonia; A_{al}, A-aligned spermatogonia; A_1–A_4, type A_1–A_4 spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; m, meiotic division; 1–16, step 1–16 spermatids. Adapted from (de Rooij 2001, Russell et al. 1990).

The last mitosis of B spermatogonia into preleptotene spermatocytes (Pl) marks the start of the meiotic phase of spermatogenesis. Preleptotene spermatocytes go through leptotene (L), zygotene (Z), pachytene (P), and diplotene (D) phases of the first meiotic prophase. The first meiotic division gives rise to secondary spermatocytes that rapidly undergo the second meiotic division to become haploid spermatids (Fig. 3).

Gradual differentiation (such as formation of spermatid head, acrosome, and flagellum) of spermatids into fully maturated spermatozoa constitutes the last, spermiogenic phase of spermatogenesis. Based on the spermatid morphology, the spermiogenic phase is

divided into several steps (16 steps in the mouse, 19 steps in the rat) (Fig. 3) (Russell et al. 1990).

Spermatogenesis in the mouse initiates shortly after birth. During the prepubertal period, the cellular composition of the testis changes reflecting the advancement of the first wave of spermatogenesis. Thus, up to postnatal day 6, the principal developing germ cells within the seminiferous tubule are spermatogonial stem cells, A-paired, and A-aligned spermatogonia. Meiosis initiates around postnatal day 10 with the appearance of preleptotene and leptotene spermatocytes. Zygotene spermatocytes appear two days later, whereas early pachytene spermatocytes can be seen around postnatal day 14. At about day 18, late pachytene spermatocytes are first observed, and two days later round spermatids occur in increasing numbers, marking the onset of spermiogenesis (Bellve et al. 1977).

4. The cycle of the seminiferous epithelium

The germ cells within the seminiferous tubules are organized in cell associations named spermatogenesis stages. Over time, each seminiferous tubule segment cycles through the ordered sequence of spermatogenesis stages. There are 12 stages in the mouse cycle and 14 stages in the rat cycle (Fig. 3, roman numerals). Particular stages have a constant cell composition. For example, in the mouse testis type A_4 spermatogonia, early pachytene spermatocytes, step 1 spermatids, and step 13 spermatids always group together and thus constitute stage I (Fig. 3) (de Rooij 2001, Russell et al. 1990). Spermatogonial stem cells, A-paired, and A-aligned spermatogonia are present at all spermatogenesis stages.

5. Hormonal regulation of spermatogenesis

Normal testis development and spermatogenesis require coordinated action of several peptide and steroid hormones, such as gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone (T) (Fig. 4) (Holdcraft and Braun 2004). Hypothalamic neurons produce a decapeptide GnRH and secrete it into the blood in a pulsatile manner. GnRH interacts with its receptor – a member of rhodopsin-like G protein-coupled transmembrane receptor family – that is expressed on secretory cells in anterior pituitary. These cells synthesize and secrete FSH and LH in response to GnRH stimulation (Fig. 4) (Burns and Matzuk 2002, Jennes and Conn 1994, Rispoli and Nett 2005, Shacham et al. 2001). Loss of GnRH signaling in hypogonadal (*hpg*) mice due to mutation in

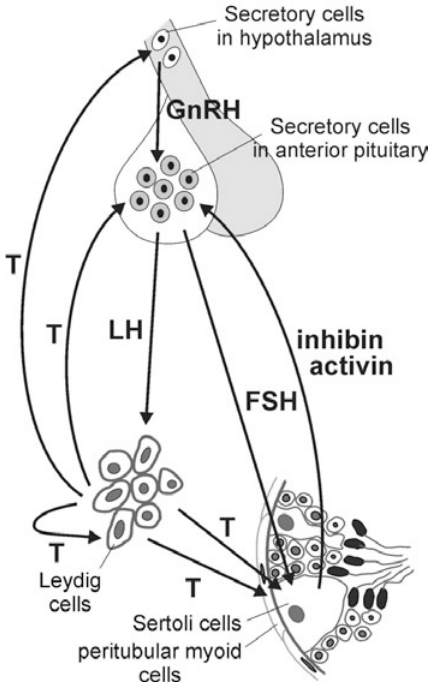

Fig. 4. Hormonal regulation of spermatogenesis. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone. Adapted from (Holdcraft and Braun 2004).

the *Gnrh* gene results in undetectable levels of LH and FSH. As a result, the gonads fail to develop postnatally and spermatogenesis is arrested at the pachytene spermatocyte stage (Cattanach et al. 1977, Mason et al. 1986).

FSH and LH are glycoprotein hormones belonging to the transforming growth factor β (TGFβ) superfamily. They share a common α-subunit, but their β-subunits are different (Holdcraft and Braun 2004). FSH and LH interact with the corresponding receptors on Sertoli and Leydig cells.

FSH receptor and LH receptor are G protein-coupled transmembrane receptors. FSHR is expressed on Sertoli cells (Rannikko et al. 1995, Tan et al. 2005b), and LHR is expressed on Leydig cells (Lei et al. 2001). In male rats, full-length FSHR mRNA is detected by RT-PCR in the testes from embryonic day 16.5 onwards, and FSH binding to testicular homogenates at embryonic day 17.5 detects functional FSHR on Sertoli cells (Rannikko et al. 1995). Sertoli cells respond to FSH stimulation by producing paracrine signals stimulating Leydig cells that, in turn, increase testosterone production (Lecerf et al. 1993). In rat testis, LHR mRNA expression, ligand binding, and LH-stimulated cAMP production appear at embryonic day 15.5. LHR expression is confined to fetal and adult Leydig cells (Zhang et al. 1994).

The only indispensable function of LH in mouse is the stimulation of growth and maturation of adult Leydig cells (Ahtiainen et al. 2007, Huhtaniemi et al. 2006). Luteinizing hormone receptor knockout (LuRKO) mice are born phenotypically normal having normal testosterone levels (Zhang et al. 2001). However, adult Leydig cells do not differentiate in LuRKO mice, and therefore, testosterone levels in adult LuRKO mice are significantly decreased and spermatogenesis is initially arrested at the round spermatid stage (Zhang et al. 2004). Interestingly, prepubertally initiated testosterone replacement therapy can rescue the

external male phenotype and restore full spermatogenesis, but not adult Leydig cell numbers, in male LuRKO mice (Pakarainen et al. 2005).

In contrast to GnRH, testosterone, and LH, FSH is dispensable for male fertility. Male *Fshr* knockout (FSHRKO) mice are fertile albeit having a reduced number of Sertoli cells and lower sperm counts than corresponding wild-type mice (Abel et al. 2000, Johnston et al. 2004).

Adult Leydig cells produce testosterone that binds to AR in Sertoli cells, Leydig cells, and peritubular myoid cells (Bremner et al. 1994, Suarez-Quian et al. 1999, Tan et al. 2005b). AR is also expressed in the brain, in particular, in hypothalamus and pituitary neurons (Crocoll et al. 1998, Simerly et al. 1990). Testosterone levels are regulated via an autocrine feedback on the Leydig cells and an endocrine feedback on GnRH- and LH-producing cells (Collins et al. 2003, Holdcraft and Braun 2004). Administration of exogenous testosterone leads to a marked slowing in GnRH pulse frequency in men and inhibits LH and FSH release by a direct effect on the pituitary. The regulation of GnRH release at the hypothalamus is also mediated in part by aromatization of testosterone to estradiol (Amory and Bremner 2003). In addition, Sertoli cells express inhibins and activins – members of the TGFβ superfamily – that, respectively, suppress and enhance FSH production (Fig. 4) (Burns and Matzuk 2002).

Androgen signaling is indispensable for the fetal sexual differentiation and spermatogenesis. *Ar* knockout (ARKO) mice exhibit complete androgen insensitivity syndrome, whereas the spermatogenesis in Sertoli cell-selective *Ar* knockout (SCARKO) mice cannot progress through meiosis (Chang et al. 2004, De Gendt et al. 2004).

6. The nuclear receptor superfamily

The nuclear receptor superfamily consists of proteins that transduce signals important for development, metabolic regulation, and reproduction. Analysis of the complete sequence of the human genome revealed the presence of 48 functional members of the nuclear receptor superfamily (Robinson-Rechavi et al. 2001). In addition to these 48 receptors, mice possess a functional farnesoid receptor β (FXRβ) and thus have 49 nuclear receptors (Escriva et al. 2004). A recent study surveyed mRNA expression patterns of all mouse nuclear receptors in different tissues revealing the relationship between expression patterns and functions of nuclear receptors (Bookout et al. 2006).

All nuclear receptors share a similar structural organization. They have a modular structure consisting of the amino-terminal domain (NTD or A/B), the DNA-binding domain

(DBD or C), the hinge region (or D domain), and the ligand-binding domain (LBD or E) (Fig. 5). Some nuclear receptors also contain a carboxyl-terminal F domain (McEwan 2004).

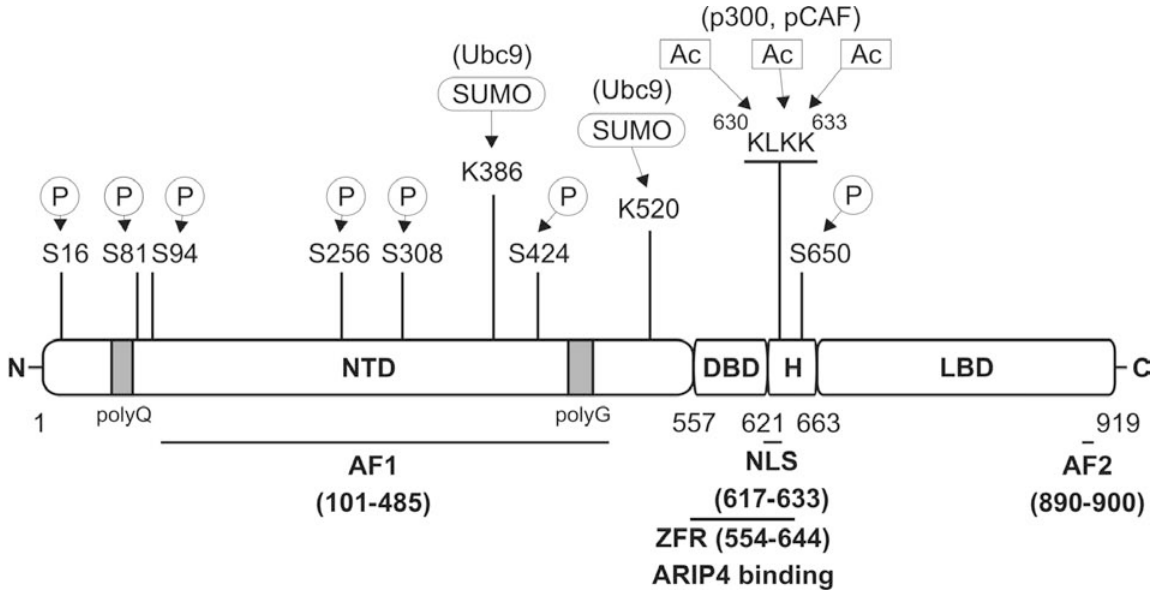

Fig. 5. Structure and covalent modifications of human androgen receptor protein. Shaded boxes correspond to polyglutamine and polyglycine tracts. NTD, N-terminal domain; DBD, DNA-binding domain; H, hinge region; LBD, ligand-binding domain; AF1, AF2, activation function 1, 2; NLS, nuclear localization signal; ZFR, zinc finger region; polyQ, polyglutamine tract (residues 58–78); polyG, polyglycine tract (residues 449–472); P, phosphorylation; Ac, acetylation; SUMO, sumoylation. Amino acid residues are numbered according to UniProt sequence P10275. Adapted from (Dehm and Tindall 2005).

On the basis of sequence similarities in the DBD and LBD domains, nuclear receptors can be divided into six subfamilies (Escriva et al. 2004). Along with estrogen, glucocorticoid, mineralocorticoid, progesterone receptors, and estrogen-receptor-related orphan receptors, the androgen receptor belongs to the steroid receptor subfamily (Beato and Klug 2000, Escriva et al. 2004).

The LBDs and especially the DBDs are conserved among the nuclear receptors, whereas the NTDs are variable both in amino acid sequence and in length (Kumar and Thompson 2003, Lavery and McEwan 2005). The AR NTD harbors an activation function 1 (AF1) (Fig. 5) containing two discrete transactivation units, Tau1 (residues 101–370, with the core region at residues 173–196) and Tau5 (residues 360–528) (Callewaert et al. 2006, Jenster et al. 1995). When artificially separated from the LBD, the AF1 region acts as a constitutively active hormone-independent transcription activator. The AF1 is very strong in AR, in contract to most other nuclear receptors (Palvimo et al. 1993, Rundlett et al. 1990).

Within the DBD, eight conserved cysteine residues coordinate two zinc ions and form two zinc finger structures arranged as perpendicular α-helixes. Nuclear receptors bind to DNA as homo- or heterodimers (with retinoid X receptor), or as monomers. The canonical receptor binding DNA sequences AGAACA or AGGTCA can be arranged as inverted repeats with a 3-nucleotide spacing, direct repeats with variable spacing, or as single elements (Escriva et al. 2004, McEwan 2004).

The flexible hinge region links the DBD to the LBD. A ligand-dependent bipartite nuclear localization signal (NLS) is located between the carboxyl-terminus of the DBD and the hinge domain (Fig. 5) (Zhou et al. 1994).

The LBDs of all nuclear receptors have a common overall three-dimensional structure, while the main structural differences are in the ligand-binding pocket (Gronemeyer et al. 2004). These differences dictate the ligand specificity of a given receptor. The AR LBD contains 11 α-helixes, that are numbered according to a canonical structure of nuclear receptor LBD (Wurtz et al. 1996), and a highly hydrophobic ligand-binding pocket (Matias et al. 2000, Pereira de Jesus-Tran et al. 2006, Sack et al. 2001). The AR LBD also comprises an activation function 2 (AF2) region (Fig. 5). Mutations within AF2 greatly reduce ligand-dependent transcriptional activation (Thompson et al. 2001).

7. Androgen receptor

AR is a classical steroid receptor mediating actions of male sex steroids – testosterone and 5α-dihydrotestosterone. AR is necessary for the normal male phenotype development and is essential for spermatogenesis. In mouse testis, AR protein is detected in Sertoli cells, Leydig cells, and peritubular myoid cells (Tan et al. 2005b).

Mouse *Ar* coding region spans about 167 kb on chromosome X and comprises 8 exons (Faber et al. 1991) (MGI database, www.informatics.jax.org, MGI:88064).

Mouse AR protein (UniProt database, www.ebi.uniprot.org, P19091) consists of 899 amino acid residues and shares 97% sequence homology with rat AR and 84% with human AR, having identical DBD and LBD sequences (Charest et al. 1991). Human AR (UniProt, P10275) (Fig. 5) contains two polymorphic homopolymeric amino acid stretches in its NTD: the polyglutamine tract, which is 11–37 residues long, and the polyglycine tract, which spans 12–29 residues (Gottlieb et al. 2004). A polyglutamine tract expansion of more than 38 residues causes hormone-dependent dysfunction and degeneration of motor neurons in males leading to spinobulbar muscular atrophy (SBMA, or Kennedy's disease) – an adult-onset

motor neuropathy causing progressive muscle wasting and weakness (Gottlieb et al. 2004, La Spada et al. 1991). Recently, a knock-in mouse model of this disease has been described, where an expanded polyglutamine tract (113 CAGs) from the human *AR* was introduced into the mouse *Ar* gene. Similar to Kennedy's disease patients, these mice developed hormone and glutamine length-dependent neuromuscular weakness, partial androgen insensitivity, and testicular pathologies (Yu et al. 2006a, Yu et al. 2006b).

In testicular feminized (*Tfm*) mice, a spontaneously occurring frameshift mutation in the AR NTD results in a premature termination of AR translation at amino acid residue 412 (Charest et al. 1991, He et al. 1991). The loss of AR activity in these mice leads to total androgen insensitivity (Bardin et al. 1973, Lyon and Hawkes 1970). Several other mouse models have been created, such as total (ARKO) or Sertoli cell-selective (SCARKO) *Ar* knockout (Chang et al. 2004, De Gendt et al. 2004, Sato et al. 2004, Yeh et al. 2002). Male *Tfm* and ARKO mice are externally feminized, having a blunt-end vagina and a clitoral-like phallus (Bardin et al. 1973, Sato et al. 2004, Yeh et al. 2002). *Tfm* and ARKO males lack seminal vesicles, vas deferens, epididymis, and prostate; however, they have small undescended testes where spermatogenesis is arrested at the pachytene spermatocyte stage (Blackburn et al. 1973, Yeh et al. 2002). In addition, ARKO males exhibit reduced heart-to-body weight ratio (Ikeda et al. 2005). Male-typical behaviors in *Tfm* and ARKO male mice are impaired whereas female-typical behaviors are absent (Lyon and Hawkes 1970, Sato et al. 2004). ARKO females appear normal, but have reduced fertility and defective folliculogenesis (Hu et al. 2004).

In humans, loss-of-function mutations in the *AR* gene result in androgen insensitivity disorders of different severity, whereas activation mutations frequently lead to development and progression of prostate cancer (Abate-Shen and Shen 2000, Hyytinen et al. 2002, Linja and Visakorpi 2004, Quigley et al. 1995, Thompson et al. 2003).

8. Androgen receptor in transcription control

Unliganded AR resides in the cytoplasm forming a complex with chaperones such as Hsp70, Hsp90, and FKBP52 that maintain AR in a conformation able to bind hormone (Fig. 6) (Cheung-Flynn et al. 2005, Gobinet et al. 2002). Due to its lipophilic nature, free testosterone can diffuse through the cellular membrane (Mendel 1989); however, when bound to sex hormone-binding globulin, testosterone may also be internalized through endocytosis (Hammes et al. 2005). Intracellular testosterone can bind AR directly, or it can be reduced to

5α-dihydrotestosterone (DHT) by 5α-reductase (5α-R) (Fig. 6). Upon hormone binding, the AR LBD changes its conformation so that helix 12 folds over the ligand-binding pocket (Gobinet et al. 2002). Interaction between AF2 region and FXXLF motif in the amino-terminus of AR further stabilize ligand-bound conformation (He et al. 2006, Heinlein and Chang 2002, Hsu et al. 2005). Binding of the ligand to AR triggers dissociation of chaperones, phosphorylation, exposure of two zinc fingers in the DBD, and unmasking of dimerization motifs and the NLS (Gobinet et al. 2002, Gottlieb et al. 2004). Importin-α recognizes the unmasked NLS and, together with importin-β, mediates translocation of liganded AR through the nuclear-pore complex (Black and Paschal 2004).

Upon nuclear translocation, AR recognizes and binds to androgen response elements (AREs) in DNA, and interacts with the basal transcription machinery. Numerous coregulatory proteins modulate AR activity (Gelmann 2002, Heinlein and Chang 2002, Jänne et al. 2000, Kang et al. 2004, Lee and Chang 2003, Lee et al. 2001, Lee and Kraus 2001, Lonard and O'Malley B 2006). Interestingly, different coregulators interact with agonist (T, DHT)-bound or antagonist (casodex)-bound AR (Kang et al. 2004, Shang et al. 2002).

Agonist-bound AR interacts with proteins of p160 family that includes histone acetyltransferases SRC-1 (also called NCoA1) and SRC-3 (also called p/CIP, ACTR, AIB1, RAC3, or TRAM1) (Aranda and Pascual 2001, Heinlein and Chang 2002). Together with the third p160 family member SRC-2 (also called TIF2, GRIP1, or NCoA2), these proteins interact with acetyltransferases CBP (CREB binding protein), p300 (highly related to CBP), and PCAF (p300/CBP-associated factor) (Fig. 6) (Black and Paschal 2004, Heinlein and Chang 2002, Shang et al. 2002). In addition, AR interacts with histone demethylases LSD1 and JHDM2A (Metzger et al. 2005, Yamane et al. 2006) and, through BAF57, with ATP-dependent chromatin remodeling complex SWI/SNF (Fig. 6) (Link et al. 2005). As we will discuss later, a core component of SWI/SNF complex is an SNF2-family ATPase – either BRM or BRG1. Interestingly, there seems to be a preference for BRM over BRG1 as the core ATPase for AR activity (Marshall et al. 2003). Chromatin remodeling by ATP-dependent remodeling complexes, histone demethylation by demethylases, and histone acetylation by the histone acetyltransferases collectively serve to loosen histone-DNA interactions and facilitate AR binding to AREs. Canonical ARE consists of two half-sites AGAACA arranged as inverted repeats with a 3-nucleotide spacing (Gobinet et al. 2002). However, recent chromatin immunoprecipitation experiments mapping AR binding sites on chromosomes 21 and 22 demonstrate that only 10% of binding sites contain these canonical AREs. In many cases the AR half-site was found in association with FoxA1, C/EBPA, GATA, or Oct1 binding motifs,

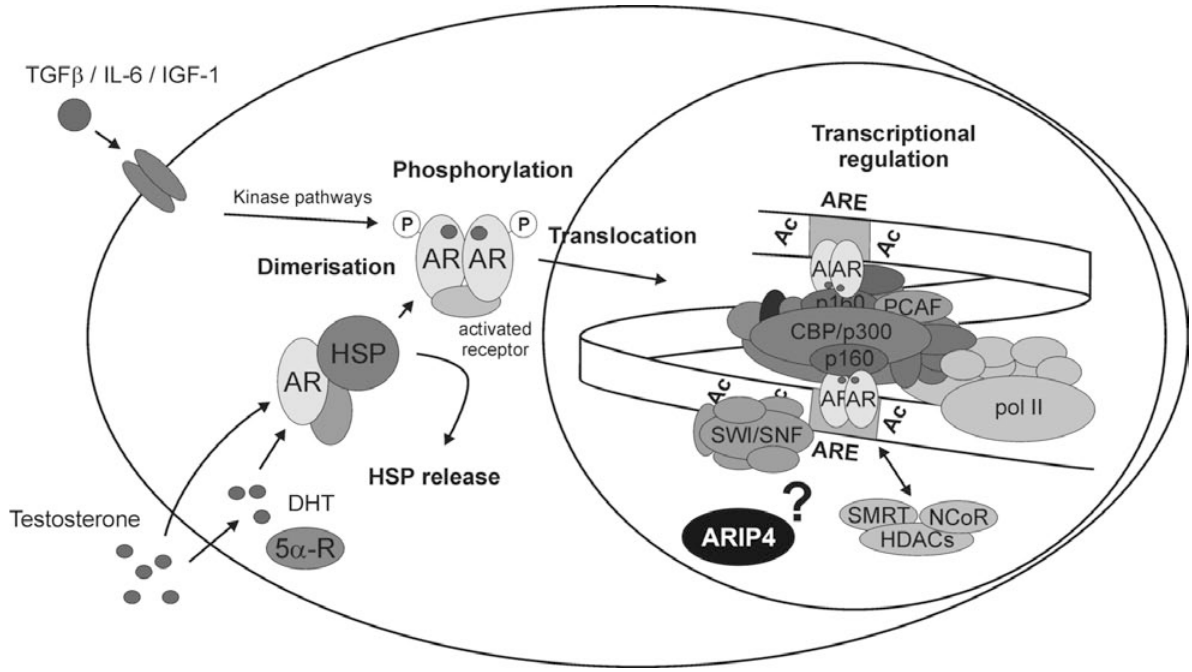

Fig. 6. Transcriptional regulation by androgen receptor. DHT, dihydrotestosterone; HSP, heat shock proteins; 5α-R, 5α-reductase; ARE, androgen response element; pol II, RNA polymerase II; HDACs, histone deacetylases; P, phosphorylation; Ac, acetylation. Adapted from (Dehm and Tindall 2005, Shang et al. 2002).

suggesting that AR collaborates with other DNA-binding transcription factors to regulate gene transcription (Qianben Wang and Olli A. Jänne, personal communications). AR binding to DNA changes the conformation of the NTD and influences its interaction with AR coregulators (Brodie and McEwan 2005). Thyroid hormone receptor-associated protein (TRAP)-Mediator complex interacts with AR and facilitates recruitment of RNA polymerase II (Wang et al. 2002). In addition, direct interactions with TATA-binding protein (TBP), TFIIB, and RNA polymerase II bridge AR complex to the basal transcriptional machinery (Fig. 6) (Black and Paschal 2004, Heinlein and Chang 2002, Shang et al. 2002). Direct interaction of AR amino-terminus with TFIIF further stabilizes the transcription activation complex (McEwan and Gustafsson 1997). Recent report shows that transcriptional repressors NCoR and SMRT can actively compete with coactivators for binding to agonist-bound AR and attenuate activation of AR target genes (Fig. 6) (Yoon and Wong 2006).

Interestingly, antagonist-bound AR is loaded onto promoter of the androgen-responsive *PSA* gene approximately as efficiently as the agonist-bound receptor (Kang et al. 2002). Antagonist-bound AR interacts with corepressors NCoR and SMRT that form

complexes with histone deacetylases mediating chromatin condensation and repression of target gene transcription (Kang et al. 2004, Yoon and Wong 2006). In response to proinflammatory cytokines, such as interleukin 1β, NCoR complex can be dismissed from antagonist-bound AR leaving the receptor free to interact with coactivators and activate transcription of target genes (Baek et al. 2006, Zhu et al. 2006).

The mechanism outlined above is by no means complete, as numerous other AR coregulators have been identified. A steadily growing database of putative nuclear receptor coregulators – the Nuclear Receptor Signaling Atlas (NURSA) – is available online (www.nursa.com), as well as the list of AR coregulators (androgendb.mcgill.ca/ARinteract.pdf). These coregulators modulate AR-dependent regulation either by interacting with DNA-bound AR (type I coregulators) or by stabilizing ligand-bound receptor conformation, influence AR protein stability and subcellular distribution (type II coregulators) (Heinlein and Chang 2002). AR, its coregulators, and components of the RNA polymerase II complex are also subject to different covalent modifications including sumoylation, acetylation, and phosphorylation (Fig. 5) (Fu et al. 2000, Gioeli et al. 2002, Poukka et al. 2000). These modifications are mediated by SUMO-1 ligases such as the PIAS family proteins, acetyltransferases p300, CBP, PCAF, androgen receptor-interacting nuclear kinase (ANPK), MAPK family kinases, and other coregulators (Dehm and Tindall 2005, Fu et al. 2000, Heinlein and Chang 2002, Kotaja et al. 2002, Moilanen et al. 1998a). SUMO-1 ligases PIAS1 and ARIP3/PIASxα enhance sumoylation of AR and repress AR-dependent transcription (Nishida and Yasuda 2002, Wu and Mo 2007). Recently, a small carboxyl-terminal domain phosphatase 2 (SCP2) was found to interact with AR, possibly acting as a "molecular brake" in AR-dependent transcription (Thompson et al. 2006). Covalent modifications influence AR transactivation and provide a mechanism for cross-talk between androgen and growth factor signaling (Dehm and Tindall 2005, Heinlein and Chang 2002, Heinlein and Chang 2004). Finally, some nuclear receptor coregulators are involved in splicing and thus can spatially and temporally coordinate transcription and RNA maturation (Auboeuf et al. 2005).

AR-dependent gene regulation is a very dynamic process. AR is highly mobile in the nucleus, and agonist- and antagonist-bound receptors have distinct nuclear localization patterns (Black and Paschal 2004, Karvonen et al. 2002). Chromatin immunoprecipitation assays have shown that AR, coregulators, and RNA polymerase II are recruited to promoters and enhancers of AR target genes (such as *PSA* and *KLK2*) in transient and cyclic fashion (Kang et al. 2004, Kang et al. 2002, Shang et al. 2002, Thompson et al. 2006). This dynamic

pattern appears to be critical for AR-dependent transcriptional regulation and depends on the proteasome function (Dennis and O'Malley 2005). Evidence that AR interacts with E3 ubiquitin ligase SNURF (also called RNP4) (Häkli et al. 2004, Moilanen et al. 1998b) and is subject to ubiquitination (Tian et al. 2006) further supports the importance of the proteasome function in AR-dependent transactivation.

9. The SNF2 protein family

9.1 Overview of the SNF2 family

Yeast sucrose non-fermenting protein 2 (SNF2) – the founding member of the SNF2 protein family – was first described in a study analyzing *Saccharomyces cerevisiae* mutants having defects in sucrose or raffinose fermentation (Neigeborn and Carlson 1984). The characteristic feature of the SNF2 family is the presence of a highly conserved domain of about 400 amino acid residues that is involved in ATP binding and hydrolysis (Eisen et al. 1995). Bioinformatic analyses have revealed the presence of 17 and 21 SNF2 family members in *S. cerevisae* and *Caenorhabditis elegans* genomes, and 29 SNF2 family members in the human genome (Chervitz et al. 1998, Linder et al. 2004).

Further amino acid sequence analysis of the ATPase domains divides the human SNF2 family into two major groups (Fig. 7) (Flaus et al. 2006, Linder et al. 2004). The first group includes proteins, such as BRM (also called SNF2α or SMARCA2), BRG1 (also called SNF2β or SMARCA4), SNF2H (also called SMARCA5), chromodomain (CHD) proteins, and INOC1 (also called INO80) that utilize the energy of ATP hydrolysis to loosen the contacts between DNA and histone octamers. Parameters of ATPase reaction and functional differences among these proteins have been relatively well characterized (Aalfs et al. 2001, Boyer et al. 2000, Fan et al. 2003). These proteins serve as the core ATPase components of large multiprotein ATP-dependent chromatin remodeling complexes, such as SWI/SNF, ISWI, NuRD, and INO80 (Jones et al. 2000, Kingston and Narlikar 1999, Roberts and Orkin 2004, Shen et al. 2000, Vignali et al. 2000) that facilitate or inhibit DNA access for transcriptional factors and/or histone-modifying enzymes (Lusser and Kadonaga 2003) and serve as coregulators for numerous transcription factors, including nuclear receptors (Belandia and Parker 2003, Lemon and Freedman 1999). Human BRM protein can also associate with several components of the spliceosome and regulate inclusion of alternative exons in the mRNA of several genes. Interestingly, the chromatin-remodeling activity of BRM is not required for its action on splicing (Batsche et al. 2006). The first group of the

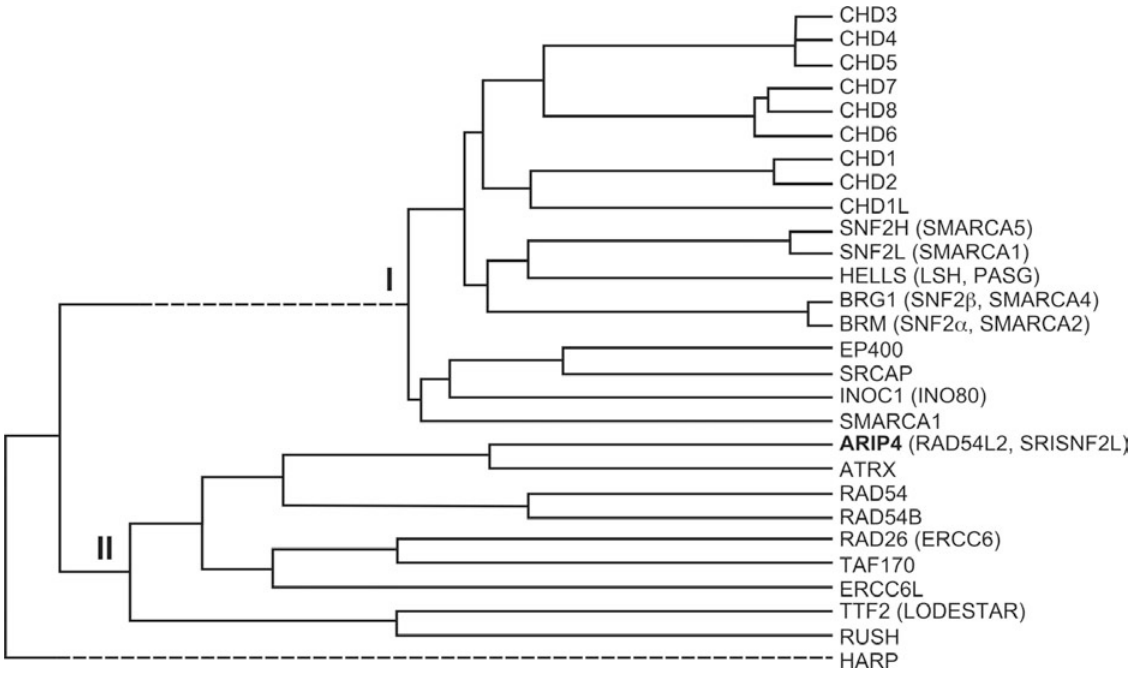

Fig. 7. Overview of the human SNF2 protein family. The tree results from the alignment of amino acid sequences of the SNF2 ATPase domains. Adapted from (Linder et al. 2004).

SNF2 family also includes HELLS (also called LSH or PASG) – an important factor in determining DNA methylation that is crucial for heterochromatin organization (Dennis et al. 2001).

The second major group of the SNF2 family includes proteins, such as the α-thalassemia mental retardation X-linked protein (ATRX), RAD54, RAD26 (also called ERCC6), and TTF2 (also called LODESTAR), that are involved in DNA excision repair, homologous recombination, or termination of transcription (Allard et al. 2004, Bugreev et al. 2006, Kanaar et al. 1996, Liu et al. 1998, van der Horst et al. 1997). In the presence of RAD51, RAD54 displays an ATP-dependent chromatin remodeling activity (Alexeev et al. 2003, Alexiadis and Kadonaga 2002). Similarly, an ATP-dependent chromatin remodeling complex consisting of ATRX and death domain-associated protein (DAXX) have been described (Tang et al. 2004a, Xue et al. 2003). However, both ATRX and RAD54 are not active chromatin remodelers, and their complexes differ from SWI/SNF and NuRD in mononucleosome remodeling activities (Xue et al. 2003). Instead of being active chromatin remodeler, RAD54 catalyzes D-loop formation during recombination and promotes homologous DNA pairing and branch migration of Holliday junctions during DNA repair process (Bugreev et al. 2006, Jaskelioff et al. 2003, Petukhova et al. 1998). ATPase domain of ARIP4 has a high sequence similarity with the ATPase domain of ATRX protein (Fig. 7)

(Linder et al. 2004). Based on the ATPase domain homology, ARIP4 together with RAD54 and ATRX comprise RAD54-like subfamily of SNF2 protein family (Flaus et al. 2006).

Thus, the two groups of the SNF2 family proteins reflect functional differences between the SNF2 family members. The proteins belonging to the first group are "true" ATP-dependent chromatin remodelers involved in transcriptional regulation, whereas the proteins from the second group, despite exhibiting some chromatin remodeling activity, are generally involved in DNA repair and homologous recombination (Linder et al. 2004).

9.2 The role of SNF2-family proteins in apoptosis and cell proliferation

Apoptosis is an essential process required for embryonic development and maintenance of normal cellular homeostasis. Numerous pathological conditions, such as cancer, autoimmunity, or degenerative disorders, can arise as a result of defects in apoptosis (Adams 2003, Danial and Korsmeyer 2004).

Caspases (cysteine-containing proteases that cleave aspartic acid-containing protein motifs) play a central role in apoptosis by cleaving several hundred cellular substrates (Thornberry and Lazebnik 1998). Caspases are produced as inactive zymogens that are activated in response to apoptotic stimuli, such as activation of cell death receptors on the cell surface or various forms of stress (intracellular damage or lack of growth factor stimulation). After apoptosis activation, upstream (initiator) caspases (caspase-8 in death receptor pathway or caspase-9 in stress pathway) undergo dimerization and autocatalytic cleavage. Activated initiator caspases cleave and activate the downstream (effector) caspases, such as caspase-3 and caspase-7, that initiate general proteolysis (Adams 2003).

The central role in the control of apoptosis belongs to the proteins of Bcl-2 family that includes several pro-apoptotic (such as Bax, Bad, and Bak) and pro-survival (such as Bcl-2 and Bcl-x_L) factors (Adams 2003, Shibue and Taniguchi 2006). The pro-survival factor Bcl-2 prevents Bax/Bak activation under normal conditions. In response to apoptotic stimuli, phosphorylated Bad binds Bcl-2 and prevents it from interacting with Bax/Bak. In turn, Bax and Bak promote permeabilization of the outer mitochondrial membrane leading to the cytosolic release of other pro-apoptotic proteins such as cytochrome *c*, Smac/DIABLO and HtrA2/Omi, that both initiate and promote caspase activation (Shibue and Taniguchi 2006). Other apoptosis control factors include inhibitors of apoptosis (IAPs) that directly bind and inhibit caspase-3, -7, and -9 (Danial and Korsmeyer 2004).

Several reports suggest the involvement of the SNF2 family proteins in regulation of apoptosis and cell proliferation. Embryos lacking functional *Brg1* or *Snf2h* die before the

implantation stage, some time between E3.5 and E6.5, due to defects in cell proliferation (Bultman et al. 2000, Stopka and Skoultchi 2003). In contrast, *Brm* knockout mice develop normally, probably due to the compensation provided by Brg1. However, fibroblasts derived from *Brm* null mice exhibit proliferation defects (Reyes et al. 1998). Mice lacking *Hells* exhibit abnormal lymphoid development and renal defects, and die within a few hours after birth (Geiman et al. 2001). ATRX-Daxx complex appears to be involved in apoptosis regulation and in mammalian sex differentiation (Tang et al. 2004a, Tang et al. 2004b, Xue et al. 2003). Deletion of the *Atrx* gene causes early embryonic lethality and conditional knockout of *Atrx* in forebrain results in marked increase in neuronal apoptosis, indicating that ATRX is a critical mediator of cell survival during early development and neuronal differentiation (Berube et al. 2005). In contrast, mice lacking *Rad54* appear normal despite reduced resistance to ionizing radiation and reduced homologous recombination in *Rad54*$^{-/-}$ ES cells (Essers et al. 1997).

AIMS OF THE STUDY

The subject of this research project is the androgen receptor-interacting protein 4 (ARIP4; the official mouse gene name is Rad54 like 2 (*Rad54l2*); also called Srisnf2l). At the time this project was initiated, there was evidence that ARIP4 interacts with AR in yeast and mammalian two-hybrid assays and acts as an AR coregulator in reporter gene assays. Analysis of ARIP4 sequence revealed a region of high similarity with the ATPase domain of SNF2 family of chromatin remodeling proteins. Northern blot analysis indicated that ARIP4 mRNA is virtually ubiquitous in adult rat and mouse tissues.

The specific aims of this project are:

- To study ARIP4 interaction with AR in physiological conditions
- To investigate the role of ARIP4 as a possible AR-specific chromatin remodeler
- To characterize the biochemical properties of ARIP4
- To elucidate AR-dependent and AR-independent functions of ARIP4 by studying *Arip4* knockout mice
- To analyze ARIP4 expression and regulation during postnatal testis development and spermatogenesis

METHODS

Detailed descriptions of the materials and methods used in this study can be found in the original publications (I–IV) as shown in Table 2.

Table 2. Methods used in this study

Method	Original publication
Plasmid construction and recombinant DNA technology	I, II
Bacterial, insect, and mammalian cell culture	I, II
Transfections and reporter gene assays	I, II
SDS-PAGE and immunoblotting	I, II
Immunoprecipitation	I, II
Production of recombinant proteins in bacterial, mammalian, and insect cells	I, II
ATPase activity assays	I, II
In vitro assays of protein-protein and protein-DNA interactions	I, II
Nucleic acid purification	I, II, III, IV
Histone octamer purification and nucleosome reconstitution	II
Immunohistochemistry	III, IV
Microarray data analysis	IV
In situ hybridization and Northern blotting	III
Quantitative RT-PCR	III, IV
Embryo whole-mount X-gal staining	IV
Isolation and culture of mouse embryonic fibroblasts	IV
Flow cytometry and cell proliferation analysis	IV

RESULTS AND DISCUSSION

1. ARIP4 is an active DNA-dependent ATPase of the SNF2 family (I, II, III)

In order to identify novel coregulators interacting with the DBD of AR, a yeast two-hybrid system was utilized. A fusion between LexA protein and the zinc finger region (ZFR; residues 554–644, Fig. 5) of human AR served as a bait to screen an E10.5 mouse embryo cDNA library fused to the VP16 activation domain. The positive clones were tested against several control plasmids to eliminate nonspecific interactions, and the remaining true positive clones were sequenced. This assay identified several potential AR coregulators, including SNURF, ANPK, and ARIP3/PIASxα (Moilanen et al. 1998a, Moilanen et al. 1998b, Moilanen et al. 1999). Some positive clones contained cDNA fragments of a previously uncharacterized protein that was named androgen receptor-interacting protein 4 (ARIP4). Subsequently, screening of an E11.5 mouse embryo cDNA library with a radiolabeled ARIP4 cDNA fragment corresponding to amino acid residues 91–230 revealed a full-length ARIP4 cDNA.

The complete mouse genome sequence (Waterston et al. 2002) greatly simplified the analysis of *Arip4* gene structure. The *Arip4* coding region spans about 97 kb on chromosome 9 and contains 22 exons (MGI database, www.informatics.jax.org, MGI:1933196). ARIP4 mRNA is approximately 10 kb in size and is relatively ubiquitously expressed in rat and mouse tissues, with the highest expression levels in liver, kidney, and testis.

ARIP4 is 1466 amino acid residues long protein that contains a region with sequence homology to the ATPase domain of the SNF2 protein family (residues 300–880, Fig. 9, 10). On the basis of its ATPase domain sequence, ARIP4 classifies into the second group of the SNF2 protein family with ATRX being its closest homolog in human and mouse (Fig. 7) (Linder et al. 2004). In contrast, the amino- and carboxyl-terminal regions of ARIP4 have no similarity to any currently known conserved protein domains. ARIP4 protein has orthologs in rat (98% sequence homology), human (96%), and dog (95%), but not in yeast.

Available data indicate that human *ARIP4* (UniGene database, Hs.105399) is ubiquitously expressed in adult human tissues. ARIP4 ESTs are also found in embryo, fetus, and juvenile (less than 17 years old) humans, but, interestingly, not in neonates and infants (less than 3 years old). In addition, *ARIP4* expression is detected in numerous cancers, including retinoblastoma, kidney, germ cell, and ovarian tumors (www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.105399). ARIP4 mRNA

levels are increased in advanced stages of prostate cancer (Linja et al. 2004). However, the role of ARIP4 in cancer progression remains to be elucidated. Yeast two-hybrid screens identified mouse ARIP4 protein as interaction partner for a dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (Dyrk1A) (Sitz et al. 2004) and carboxyl-terminal binding protein 1 (CtBP1) (Qinghong Zhang, personal communication). Apart from these data and our results presented in this work, not much is known about ARIP4 and its orthologs.

Analysis of ARIP4 protein sequence for the presence of short conserved motifs identified three bipartite nuclear localization signals, several sumoylation and phosphorylation sites, and three LXXLL motifs known to mediate interactions with nuclear receptors (summarized in Fig. 10) (Balla et al. 2006, Heery et al. 1997).

The sequence of ARIP4 suggests that there is a DNA-dependent ATPase activity in the protein. Indeed, purified ARIP4 binds ATP and has intrinsic ATPase activity that depends on the presence of double- or single-stranded DNA in the reaction mixture. Mutations within the putative catalytic centre (Lys-310 to Ala (K310A), and Asp-462/Glu-463 to Ala/Ala (DE462AA)) result in loss of the ATPase activity. In contrast, all other ARIP4 point mutants

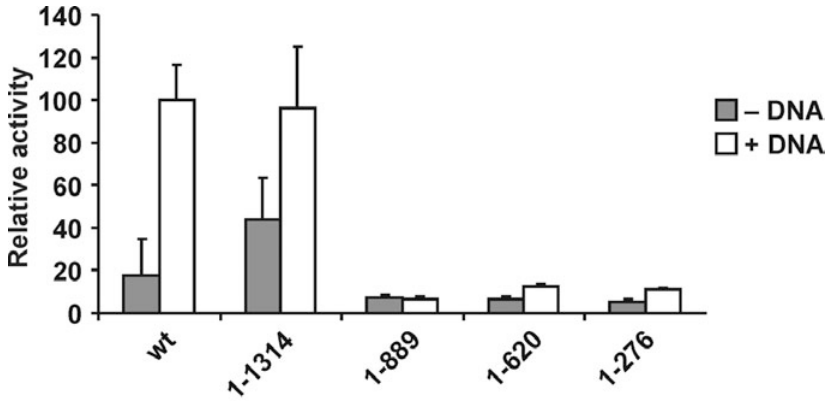

Fig. 8. ATPase activity of ARIP4 deletion mutants. Wild-type ARIP4 or indicated mutants were produced in COS-1 cells. ATPase activity in the presence or in the absence of 80 nM double-stranded DNA was assayed as described in (II). ATPase reaction was stopped after 60 min incubation at 30°C. One-microliter samples were spotted onto a poly(ethyleneimine) thin-layer plate that was developed in 1 M LiCl/1 M formic acid to resolve [^{32}P]P_i from [γ-^{32}P]ATP. The plate was subjected to autoradiography. The amount of released [^{32}P]P_i was quantified by scanning autoradiograms using Kodak Image station 440CF (Eastman Kodak, Rochester, NY). The values are shown relative to that of wild-type ARIP4 in the presence of double-stranded DNA (=100). Each bar represents the mean ± standard deviation values for three independent experiments.

except sumoylation-deficient ARIP4(6K→6R) have ATPase activities comparable to wild-type ARIP4 (Table 3). Carboxyl-terminal truncation of ARIP4 (ARIP4(1–1314)) does not affect enzymatic activity whereas ARIP4(1–889), despite having an intact ATPase domain, loses the ability to hydrolyze ATP. Similarly, ARIP4(1–620) and ARIP4(1–276) cannot hydrolyze ATP (Fig. 8). As described below, ARIP4(6K→6R), ARIP4(1–889), and shorter amino-terminal ARIP4 fragments cannot bind DNA in EMSA experiments (Fig. 9 and Table 3). Thus, the ability to bind DNA appears to be crucial for the ATPase activity of ARIP4.

By titrating the concentration of one reaction component and monitoring ADP release rate in a fluorometric ATPase assay, we estimated the kinetic parameters of ATP hydrolysis catalyzed by ARIP4. Estimated K_m for dsDNA and K_m for ATP are similar to those of BRG1 and SNF2h proteins (Aalfs et al. 2001). However, the catalytic activity of wild-type ARIP4 (being 600 ± 100 nmol ADP released per minute per milligram ARIP4 or approximately 120 molecules of ATP hydrolyzed by protein molecule per minute) is some 10 times lower than that of other ATP-dependent chromatin remodeling enzymes, that typically hydrolyze about 1000 ATP molecules per minute (Peterson 2000).

2. ARIP4 interacts with AR and binds to DNA (I, II)

The initial yeast two-hybrid screen identified an AR interaction domain residing within residues 91–230 of ARIP4. In yeast, the ARIP4 fragment spanning residues 86–227 (ARIP4(86–227)) interacts with the AR ZFR. However, deletion of 20 hinge region residues from the AR ZFR markedly weakens the ARIP4-AR interaction. The ARIP4(86–227) fragment also exhibits a weak interaction with the AR hinge-LBD region (residues 624–919). Both in yeast and in mammalian two-hybrid assays, ARIP4(86–227) exhibits strong testosterone-dependent interaction with full-length AR. However, when coexpressed with AR in mammalian cells, ARIP4(1–620) but not ARIP4(1–276) coimmunoprecipitates with the receptor. Interestingly, both ARIP4(278–1466) and ARIP4(278–1314) are capable of binding to AR ZFR in GST pull-down experiments (Fig. 9), possibly interacting with AR through LXXLL motifs that are known to mediate nuclear receptor binding (Heery et al. 1997). Finally, the interaction of ARIP4 with AR does not depend on ARIP4 ATPase activity or ability to bind DNA, as both ATPase-deficient mutant ARIP4-K310A and DNA binding-deficient mutant ARIP4(1–620) can recognize AR in coimmunoprecipitation experiments. Taken together, these results suggest a complex nature of ARIP4 – AR interaction that apparently involves ARIP4 region (86–227) and LXXLL motifs (Fig. 10).

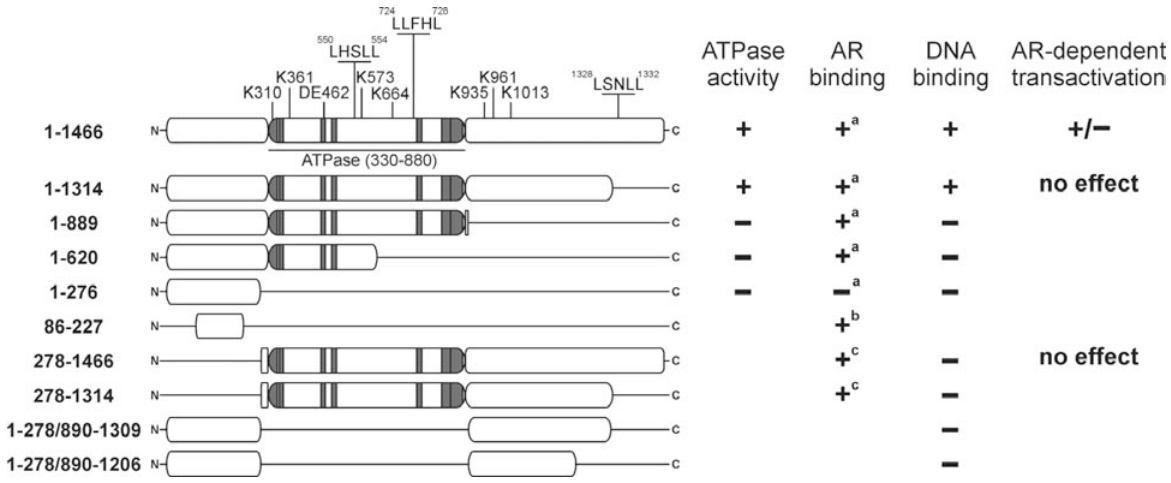

Fig. 9. ARIP4 deletion mutants and summary of their properties. ATPase domain, LXXLL motifs, and residues mutated to study ARIP4 ATPase activity (Lys-310, Asp-462/Glu-463) and sumoylation (Lys-361, Lys-573, Lys-664, Lys-935, Lys-961, and Lys-1013) are shown. Shaded boxes correspond to conserved motifs in the SNF2 ATPase domain. AR binding was assayed by coimmunoprecipitation ([a]), yeast two-hybrid ([b]), or GST pull-down ([c]). As marked by +/–, wild-type ARIP4 enhances AR activity on the minimal (ARE_4-tk) promoter and attenuates AR activity on a more complex (probasin) promoter.

Table 3. ARIP4 point mutants and summary of their properties

Mutant	**ATPase activity**	**DNA binding**	**AR dependent transactivation** (ARE_4-tk)	**Sumoylation**
Wild-type	+	+	+	+
K310A	–	+	repression	+
DE462AA	–		repression	
K573R	+		+	+
K664R	+		+	+
K935R	+	+	+	+
K1013R	+	+	+	+
KK361/961RR	+		+	+
KK573/964RR	+		+	+
4K→4R	+		+	+
5K→5R	+	+	+	reduced
6K→6R	–	–	no effect	–

Wild-type ARIP4, ARIP4(1–1314), and all tested point mutants except ARIP4(6K→6R) can bind to a 32-bp DNA fragment in EMSA experiments, whereas

ARIP4(6K→6R), ARIP4(1–889), and shorter amino-terminal ARIP4 fragments fail to bind DNA (Fig. 9 and Table 3). These results may suggest that the ARIP4 DNA binding domain localizes within residues 890–1314. We could not, however, directly demonstrate DNA binding of ARIP4(890–1316) or any other ARIP4 amino-terminal deletion mutant, including the mutants having amino-terminal region of ARIP4 (residues 1–278) fused to putative DNA binding region (residues 890–1206 or 890–1309). Thus, the presence of the ATPase domain (residues 300–880) within ARIP4 seems to be important for the correct folding of the DNA-binding domain. The combination of lysine mutations in ARIP4(6K→6R) may result in misfolding of the ARIP4 DNA binding domain, as three of the six lysine residues mutated in ARIP4(6K→6R) (Lys-935, Lys-961, and Lys-1013) are located within the putative DNA binding region.

Finally, we have found no nucleotide sequence preference for the DNA binding of ARIP4, as ARIP4 interacted with all DNA oligonucleotides tested by EMSA, and poly(dI-dC)•(dI-dC) efficiently competes with DNA for ARIP4 binding. DNA binding does not depend on ARIP4 ATPase activity, as ATPase-deficient mutant ARIP4-K310A binds DNA similarly to wild-type ARIP4.

3. ARIP4 is an AR coregulator (I, II, III)

To study the effect of ARIP4 on AR-dependent transactivation, we performed several transient transfection assays in mammalian cells using reporter constructs driven by different promoters. Coexpression of wild-type ARIP4 with AR results in a two- to three-fold increase in AR transcriptional activity on a minimal (ARE_4-tk) promoter. This effect appears to be AR-specific, as ARIP4 fails to activate glucocorticoid receptor (GR) or progesterone receptor (PR) function on the same promoter. However, ARIP4 is capable of enhancing both AR- and GR-dependent transactivation of the MMTV promoter-driven reporter gene (Sitz et al. 2004). Interestingly, the carboxyl-terminal truncation mutant ARIP4(1–1314), despite retaining wild-type ATPase activity and ability to bind AR and DNA, is significantly less active in AR coactivation. Apparently, ARIP4 carboxyl-terminal region mediates interactions with other coactivators. Indeed, Dyrk1A – a serine/threonine kinase involved in neuronal development and mental retardation in Down syndrome patients – interacts with residues 1078–1466 of ARIP4 (Fig. 10). ARIP4 cooperates with Dyrk1A to modulate both AR- and GR-mediated transactivation in adult mouse brain, however, this effect is not mediated by phosphorylation of ARIP4 by Dyrk1A (Sitz et al. 2004). ARIP4 and Dyrk1A are coexpressed and colocalized

in neurons of the hippocampus (Sitz et al. 2004), where AR is also expressed (Kerr et al. 1995). The hippocampus plays a critical role in learning and memory, and it is the most clearly affected region of the brain in Down syndrome patients (Nadel 2003). Thus, by modulating AR activity in hippocampal neurons, ARIP4 may play a role in physiological processes in mammalian brain. Interestingly, mutations in a close ARIP4 homolog, ATRX, cause ATRX syndrome with mental retardation (Gibbons et al. 1995).

ATPase-deficient mutants of ARIP4 are unable to act as AR coactivators. Instead, they behave as *trans*-dominant negative regulators of AR function, probably by sequestering AR interaction partners. In contrast, mutations of potential sumoylation sites do not significantly affect ARIP4 coactivator activity. However, as expected, the ATPase- and sumoylation-deficient mutant ARIP4(6K→6K) unable to bind DNA also loses its ability to act as an AR coactivator (Table 3).

Surprisingly, ARIP4 fails to activate AR function on the more complex AR-dependent probasin promoter in transient transfection assay. In fact, wild-type ARIP4 and ATPase-deficient mutants attenuate AR-dependent transactivation. Thus, in transient transfection assays ARIP4 behaves as AR coactivator on a minimal promoter and as a modest AR corepressor on a more complex probasin promoter. This is not a totally unexpected behavior for a SNF2 family protein, as proteins of this family are known to act as promoter-specific transcriptional activators or repressors (Kingston and Narlikar 1999, Tyler and Kadonaga 1999). For example, the yeast SWI/SNF complex exhibits promoter specificity by down-regulating some genes while up-regulating other ones (Holstege et al. 1998).

Under physiological conditions, gene transcription occurs in the context of chromatin. Therefore, an assay using cells that have a stably integrated ("chromatinized") reporter gene approximates much better AR-dependent transactivation *in vivo*. A recent report shows that, in contrast to results from transient transfection assays, N/C interaction-defective AR mutants are inactive in regulating transcription of stably integrated reporter genes (Li et al. 2006). If we hypothesize that ARIP4 alters chromatin organization, ARIP4 coregulator properties must be studied on the chromatin-assembled reporter gene. Therefore, to investigate the properties of ARIP4 as an AR coregulator, we studied the effects of ectopic ARIP4 expression in a prostate cancer cell line that has been stably transfected with probasin promoter-driven reporter gene. In this system, wild-type ARIP4 elicits about a 1.5-fold increase in AR-dependent transactivation, whereas the ATPase-deficient mutants have no effect on AR function.

Additional evidence for the role of ARIP4 as an AR coregulator comes from heterozygous *Arip4*$^{+/-}$ mice. In *Arip4*$^{+/-}$ testis the mRNA level of a Sertoli cell-specific androgen-dependent protein Rhox5 (also called Pem) (Lindsey and Wilkinson 1996) is reduced to approximately 80% of that in wild-type controls. Similarly, mRNA level of Eppin (epididymal protease inhibitor) (Denolet et al. 2006, Sivashanmugam et al. 2003) is slightly reduced in *Arip4*$^{+/-}$ testis.

Taken together, these results have indicated that ARIP4 can function as an AR coregulator both in transient transfection assays and, more importantly, in assays using cells that have a stably integrated reporter gene. Moreover, we demonstrate that ARIP4 is required for androgen-dependent regulation of at least some genes *in vivo*.

4. ARIP4 binds, but does not remodel, reconstituted mononucleosomes (I, II)

What are the molecular mechanisms of ARIP4 action and, in particular, how does ARIP4 use the energy generated as a consequence of ATP hydrolysis? Several SNF2 family members use ATP hydrolysis energy to elicit superhelical torsion within linear DNA fragments. This is suggested to be the primary biomechanical activity shared by all ATP-dependent chromatin remodeling proteins (Havas et al. 2000). We have demonstrated that wild-type ARIP4, but not ATPase-deficient mutant ARIP4-K310A, can also generate a negative supercoiling within a linear DNA molecule.

To test whether ARIP4 exhibits mononucleosome remodeling activity, we utilized the restriction enzyme accessibility (REA) assay. For this assay, we used purified histone octamers to reconstitute mononucleosomes on ^{32}P-labeled DNA fragments bearing a nucleosome positioning sequence and a *Pst* I restriction site. REA assays with the ATPase-dependent chromatin remodelers BRG1 and SNF2h (Fan et al. 2003) and our REA assay with yeast RSC (Remodels the Structure of Chromatin) complex clearly demonstrate that the restriction enzyme can gain access to its recognition sequence only in the presence of chromatin remodeling activity. Should ARIP4 be a chromatin remodeler, *Pst* I would digest DNA at the cognate recognition site in the presence of ARIP4 and ATP. Under our experimental conditions, *Pst* I could not digest either intact mononucleosomes or those preincubated with ARIP4. However, wild-type ARIP4 binds to the reconstituted mononucleosomes and this binding is not sequence-specific, as unlabeled poly(dI-dC)•(dI-dC) competes efficiently with mononucleosomes for ARIP4 complex formation. Thus, our results

demonstrate that ARIP4 is not a classical chromatin remodeling protein. However, we cannot rule out the possibility that the presence of some specific histone modifications is required for ARIP4 to remodel successfully mononucleosomes. In addition, some other unknown ARIP4 interaction partners, absent in our *in vitro* assay, may be needed for the chromatin remodeling activity of ARIP4.

5. ARIP4 is sumoylated (II)

The ATPases of the SNF2 family frequently serve as core components of relatively stable large protein complexes (Asturias et al. 2002, Lusser and Kadonaga 2003, Smith et al. 2003, Vignali et al. 2000). In an attempt to find ARIP4 interaction partners, we performed gel filtration and coimmunoprecipitation experiments. Despite many different conditions tested, we failed to find ARIP4 interaction partners in HeLa nuclear extracts. Thus, under our experimental conditions, ARIP4 does not form stable high-molecular-mass protein assemblies. ARIP4 seems to interact with its protein partners only weakly and/or transiently in such a manner that, at any given time, only a small fraction of the ARIP4 pool is involved in protein-protein interactions.

A yeast two-hybrid screen using full-length wild-type ARIP4 as a bait led to the identification of only two biologically meaningful interacting partners for ARIP4, namely the small ubiquitin-related modifying protein (SUMO-1) and Ubc9. Ubc9 serves as an E2 conjugating enzyme that, together with an E1 activating enzyme Aos1/Uba2 and several E3 ligase enzymes, catalyzes sumoylation – a covalent attachment of SUMO-1 to lysine residue(s) of target proteins. Sumoylation can modulate transcriptional regulation, intracellular protein localization, DNA repair, and protein-protein interactions (Gill 2004, Muller et al. 2004, Seeler and Dejean 2003). ARIP4 contains one consensus site for SUMO-1 attachment at Lys-664 and several other consensus-like sequences, and it is indeed modified by SUMO-1 *in vivo*. A recent report suggests that ARIP4 can also be modified by SUMO-2 (Rosendorff et al. 2006). Interestingly, a lysine-to-arginine mutation at consensus Lys-664 does not significantly change the ARIP4 sumoylation pattern. It is known that mutation of the consensus sumoylation site can promote SUMO attachment to other lysine residues in the protein (Hoege et al. 2002). Mutations of five other sumoylation consensus-like sites at Lys-361, Lys-573, Lys-935, Lys-961, and Lys-1013 (Fig. 10) alter, but not abolish, ARIP4 sumoylation, and do not significantly affect ARIP4 DNA binding and AR coactivator activity (Table 3). Only combining mutations of these six lysine residues (6K→6R) results in a

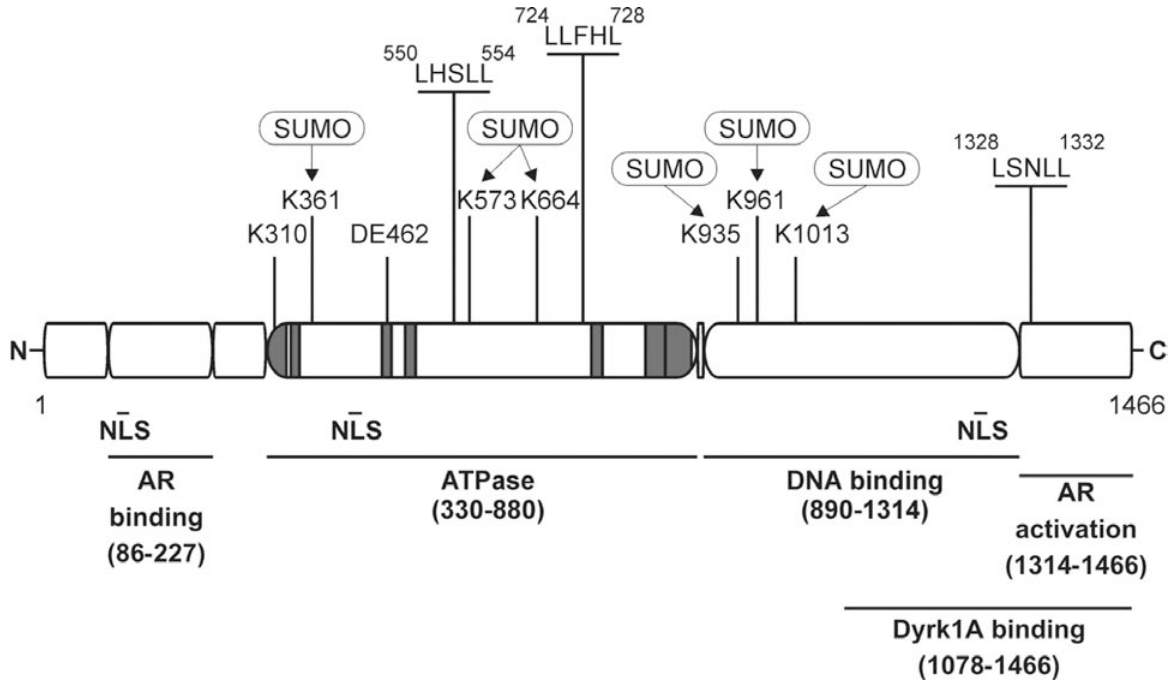

Fig. 10. ARIP4 functional regions and sumoylation sites. Shaded boxes correspond to conserved motifs in the SNF2 ATPase domain. SUMO, sumoylation; NLS, nuclear localization signal (residues 98–114, 412–428, and 1254–1271). Amino acid residues are numbered according to RefSeq sequence NP_109655.

substantial decrease in ARIP4 sumoylation. As mentioned earlier, ARIP4(6K→6R) is incapable of DNA binding. As a result, ARIP4(6K→6R) loses its ATPase activity and cannot enhance AR-dependent transactivation in transient transfection assay (Table 3). It remains to be established whether the defect in DNA binding is a direct consequence of sumoylation deficiency or if it is due to inappropriate protein folding.

6. ARIP4 is expressed in mouse embryo (IV)

To study the physiological role of ARIP4, we generated *Arip4*$^{+/-}$ mice using embryonic stem cells with a trapped *Arip4* allele available from the German Gene Trap Consortium (tikus.gsf.de). The gene trap vector was designed in such a fashion that after genomic integration the endogenous *Arip4* promoter regulates expression of the *lacZ* gene. Therefore, heterozygous and homozygous embryos express β-galactosidase (β-gal) instead of ARIP4. Thus, whole-mount staining of these embryos with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) can reveal the ARIP4 expression pattern.

Available data indicate that ARIP4 mRNA is detectable in mouse embryo already at the 2-cell stage (MGI database, www.informatics.jax.org). We have demonstrated the presence of ARIP4 mRNA in ES cells by Northern blotting. Our whole-mount X-gal staining and immunostaining with affinity-purified rabbit polyclonal anti-ARIP4 antibodies show that ARIP4 protein is mainly expressed in the developing neural tube and limb buds during E8.5–E12.5. Starting from E14.5, ARIP4 expression becomes virtually ubiquitous.

7. ARIP4 is expressed in postnatal mouse testes (III)

Androgen signaling through AR is one of the crucial events in testicular development. To understand further the role of ARIP4 as an AR coregulator, we analyzed ARIP4 expression in the mouse testis during postnatal development.

ARIP4 mRNA levels are low in testes from newborn mice, but become progressively abundant towards puberty and continue to be high through adulthood. ARIP4 protein is present in the nuclei of Sertoli cells at all ages studied. Starting from day 20, Sertoli cell nuclei possess stage-specific ARIP4 staining. In addition, Leydig cell nuclei become ARIP4-positive. We also detected ARIP4 in germ cells, namely in spermatogonia, pachytene and diplotene spermatocytes. In adult testis Sertoli cell nuclei have very strong ARIP4 immunostaining at stages II–VI and VII–VIII, and weaker at stages IX–XII. Pachytene spermatocyte nuclei at stages I–V are stained relatively strongly, with the XY-bodies being ARIP4-positive. The presence of ARIP4 in XY-bodies is not unexpected, since ARIP4 is sumoylated, and SUMO-1 is known to localize to XY-bodies in pachytene spermatocytes (Vigodner and Morris 2005). ARIP4 expression in pachytene spermatocytes is still detectable at stage VII, but becomes weaker from stage IX onwards. Leptotene and zygotene spermatocytes also exhibit some immunostaining. In general, weak ARIP4 immunostaining in spermatocytes is detectable throughout development. Early round spermatids appear faintly stained, whereas all other spermatids are negative.

We turned to heterozygous *Arip4*$^{+/-}$ mice to study how the reduction in ARIP4 levels affects the testicular phenotype. Despite having reduced ARIP4 mRNA and protein levels, *Arip4*$^{+/-}$ mice are fertile, appear normal, and have normal testicular histology. The expression pattern of ARIP4 antigen in testes from *Arip4*$^{+/-}$ mice is similar to that of wild-type testes, with positive Leydig and Sertoli cell nuclei, spermatogonia, pachytene and diplotene spermatocytes. Sertoli cell nuclei have 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 does not qualitatively change either testicular histology, ARIP4 or AR expression patterns. However, we detected quantitative changes in mRNA levels of androgen-dependent Rhox5 and Eppin proteins (Denolet et al. 2006, Lindsey and Wilkinson 1996, Sivashanmugam et al. 2003), indicating that *Arip4*$^{+/-}$ mice are haploinsufficient with regard to AR-specific gene expression in Sertoli cells.

8. Expression of ARIP4 in testis of adult wild-type, FSHRKO, LuRKO, and *hpg* mice (III)

Stage-dependent expression of ARIP4 in Sertoli cell nuclei closely resembles that of AR. ARIP4 expression is high at stages II–VI and, similar to AR, peaks at stages VII–VIII. The mechanisms governing the stage-dependent protein expression in Sertoli cells are poorly understood. To investigate how the disruption of hormonal signaling in testis influences ARIP4 and AR expression, we studied the testes from LuRKO, FSHRKO, and *hpg* mice.

In adult LuRKO mice, the testicular testosterone concentration is reduced by 97% in comparison to wild-type mice (Zhang et al. 2004). AR and FSHR mRNA levels are significantly higher in the testes of LuRKO mice than in wild-type testis, whereas ARIP4 mRNA levels in LuRKO testes do not differ from wild-type. However, neither *Lhr* nor *Fshr* knockout qualitatively affect testicular distribution and stage-specific expression of ARIP4 and AR proteins.

Sertoli cells in *hpg* testes develop atypically, and their morphology resembles that of 10-day-old wild-type testes (Myers et al. 2005). In the testis of *hpg* mice, ARIP4 protein is detected mainly in interstitial cells, whereas ARIP4 expression in Sertoli and germ cells in *hpg* testis is much lower then that in 10-day-old wild-type testis. At present, we can only speculate whether the reduced ARIP4 protein expression in Sertoli and germ cells is linked to spermatogenesis arrest in *hpg* testis.

Thus, disruption in testosterone, FSH, or LH signaling has a limited, if any, influence on the pattern of ARIP4 and AR expression in the testis. Accordingly, *in vitro* treatments of seminiferous tubule segments with testosterone, FSH, or forskolin for 4, 8, or 16 h do not significantly change ARIP4 mRNA levels.

9. Targeted disruption of mouse *Arip4* gene results in embryonic lethality (IV)

Genotyping of the progeny of *Arip4*$^{+/-}$ intercrosses demonstrates the expected Mendelian frequencies of both wild-type and heterozygote offspring. However, the homozygote pups are totally absent, indicating that *Arip4*$^{-/-}$ embryos are not viable. Most *Arip4*$^{-/-}$ embryos appear to develop normally up to E8.5, but no homozygous embryos are recovered from E11.5 onwards. The body size of *Arip4*$^{-/-}$ embryos is significantly reduced compared to that of wild-type littermates at E9.5 and E10.5. Histological analyses do not reveal any obvious developmental defects. However, almost all tissues of *Arip4*$^{-/-}$ embryos at E10.5 are proportionally smaller than those of wild-type embryos. The yolk sac and placenta of *Arip4*$^{-/-}$ embryos appear normal, and thus the embryonic lethality is unlikely due to the failure of embryonic blood circulation.

ARIP4 coimmunoprecipitates with transcriptional corepressor CtBP1 (Fig. 11). Mice lacking *Ctbp1* are viable, however, knockout of the highly homologous *Ctbp2* results in embryonic lethality at E10.5. *Ctbp2* null embryos are smaller and have defects in heart morphogenesis and in neural system development (Chinnadurai 2003, Hildebrand and Soriano 2002). Similarly, knockout of ARIP4-interacting *Dyrk1a* results in embryonic lethality between E10.5 and E13.5. *Dyrk1a*$^{-/-}$ embryos exhibit significant growth retardation and defects in morphology of the heart, liver primordium, branchial arches, and brain vesicles (Fotaki et al. 2002). At E9.5 CtBP1, CtBP2, Dyrk1A, and ARIP4 are expressed in developing neural tubes (Fotaki et al. 2002, Hildebrand and Soriano 2002). Interestingly, AR is also present in developing nervous system, but its expression occurs later during embryonic development (Kerr et al. 1995, Young and Chang 1998). The obvious phenotypic

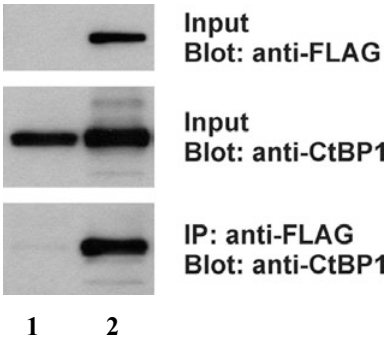

Fig. 11. ARIP4 interacts with CtBP1. COS-1 cells were transiently transfected with 100 ng of pcDNA-CtBP1 together with 700 ng pFLAG-CMV2 (lane 1) or pFLAG-ARIP4wt (lane 2). Immunoprecipitation and immunoblotting were performed as described in (II) using anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO) and anti-CtBP1 antibody (a gift from Dr. Qinghong Zhang, Oregon Health Sciences University, Portland, OR).

similarity among *Ctbp2*$^{-/-}$, *Dyrk1a*$^{-/-}$, and *Arip4*$^{-/-}$ embryos suggests that ARIP4-CtBP and ARIP4-Dyrk1A interactions may play important roles during early neural development.

10. ARIP4 influences apoptosis and cell proliferation (IV)

To further understand the molecular mechanisms causing embryonic lethality, we studied cell proliferation and apoptosis in E9.5 and E10.5 embryos. TUNEL staining indicated that at E9.5 and E10.5 *Arip4*$^{-/-}$ embryos have a higher proportion of apoptotic cells, especially in the neural tube. The neural tube of *Arip4*$^{-/-}$ embryos at E9.5 and E10.5 has a reduced proportion of proliferating phospho-histone H3-positive cells. Mouse embryonic fibroblasts (MEFs) derived from *Arip4* null embryos at E9.5 also exhibit impaired proliferation and increased apoptosis. These cells stop growing after 2–3 passages and have significantly lower growth rate than cells from wild-type littermates.

Microarray analysis of total mRNA expression profiles in E9.5 *Arip4*$^{-/-}$ and wild-type embryonic fibroblasts revealed that the expression of several apoptosis- and cell proliferation-related genes is deregulated in *Arip4*$^{-/-}$ MEFs. Deletion of ARIP4 also affects the expression of several genes involved in apoptosis and cell death, DNA replication and repair, neurogenesis, and development. Using quantitative real-time PCR we have confirmed the microarray results for several key genes involved in apoptosis, development, and cell growth and maintenance. Obviously, not all of these genes have to be direct targets of ARIP4. Observed deregulation of some genes can be a secondary effect due to general proliferation defects in E9.5 *Arip4*$^{-/-}$ fibroblasts. In order to detect the more subtle effects of ARIP4 deletion on gene expression patterns, embryonic fibroblasts derived from embryos at earlier developmental stages or *Arip4* null embryonic stem cells will have to be analyzed. Nevertheless, our results clearly demonstrate that the loss of ARIP4 affects gene expression pattern and proliferation of embryonic fibroblasts.

These results suggest that ARIP4 is involved in the regulation of apoptosis and cell proliferation. As we mentioned earlier, several other members of SNF2 family, including ATRX – an ARIP4 homolog, have been implicated in these processes (Tang et al. 2004a, Tang et al. 2004b, Xue et al. 2003). We could not detect AR mRNA in MEFs at E9.5. Therefore, ARIP4 functions in apoptosis and cell proliferation do not seem to be related to its activity as AR coregulator. We suggest that ARIP4 acts as a coregulator for other factors expressed at early stages of embryonic development. ARIP4 may influence cell proliferation through its interaction with Dyrk1A. *Dyrk1a*$^{-/-}$ embryos have a reduced number of

differentiating neuronal cells suggesting that this protein is involved in neuroblast proliferation (Fotaki et al. 2002). In addition, similar to other SNF2 family members, such as Rad54, ARIP4 may be involved in homologous recombination and DNA repair processes.

11. Future directions

Our current results suggest that ARIP4 plays a dual role in embryonic development and adult life. At E8.5–E10.5, ARIP4 is mainly expressed in the neural tube and limb buds, being involved in the processes of cell proliferation and apoptosis (IV). At later embryonic stages, ARIP4 becomes ubiquitously expressed and may modulate AR functions in male sex determination and differentiation. ARIP4 expression continues after birth. We have thoroughly followed postnatal expression of ARIP4 in the testis (III) and also demonstrated the ubiquitous expression of ARIP4 mRNA in adult mouse tissues (I). ARIP4 appears to modulate AR activity in somatic cells of the testis (III) and in hippocampal neurons (Sitz et al. 2004). Levels of ARIP4 mRNA are increased in advanced stages of prostate cancer and, therefore, ARIP4 may also be involved in prostate cancer progression (Linja et al. 2004). Finally, ARIP4 expression in AR-negative germ cells in adult testis implies that the protein has AR-independent functions in adult mouse (III).

Future studies need to be focused on analyzing the molecular mechanisms underlying ARIP4 action. Detectable amounts of ARIP4 protein are present in testicular teratoma-derived F9 cell line and in kidney-derived 293T cells (our unpublished results). The effects of small interfering RNA (siRNA)-mediated ARIP4 knockdown in these ARIP4-expressing cell lines may reveal new ARIP4 functions. Possible roles of ARIP4 in DNA repair and homologous recombination can be addressed by studying the response of ARIP4-depleted cells to DNA-damaging agents and/or ionizing radiation. Genome-wide comparison of gene expression patterns in wild-type and ARIP4-depleted cell lines, as well as *Arip4* null fibroblasts or embryonic stem cells, will lead to identification of new ARIP4 target genes. Potential ARIP4 target genes can also be identified by genome-wide mapping of ARIP4 binding sites using a combination of chromatin immunoprecipitation with microarrays (ChIP on chip) method. Recently, this method was successfully applied to map estrogen receptor binding sites on chromosomes 21 and 22 (Carroll et al. 2005).

The early embryonic lethality of *Arip4*$^{-/-}$ embryos precludes phenotypic analysis of *Arip4* null adult mice. However, recently the original gene trap method has been modified to allow creation of conditional trapped alleles (Schnutgen et al. 2003, Schnutgen et al. 2005).

Embryonic stem cells having conditional trapped *Arip4* are available from the German Gene Trap Consortium (tikus.gsf.de). Generation of mice with conditional mutations in the *Arip4* gene, especially testis- and brain-specific *Arip4* knockout mice, will undoubtedly reveal new fascinating details of ARIP4 function.

CONCLUSIONS

ARIP4 functions as AR coregulator *in vitro* and *in vivo* (I, II, III). We have characterized the biochemical properties of ARIP4 (I, II) and the expression and regulation of ARIP4 in mouse testis during postnatal development (III). We suggest that the ARIP4-AR interaction plays a role in postnatal testis development and spermatogenesis. In addition, our results clearly demonstrate that ARIP4 has an essential role in embryonic development and cell proliferation (IV). In summary, this study demonstrates that:

- ARIP4 is a nuclear protein that interacts with AR and modulates AR-dependent transactivation.
- ARIP4 binds to DNA and mononucleosomes, and it exhibits an ATPase activity that is dependent on double- and single-stranded DNA. However, in contrast to some other members of the SNF2 family, ARIP4 is not an active ATP-dependent mononucleosome remodeler.
- ATPase activity and the ability to bind DNA are critical for the coregulatory function of ARIP4.
- During embryonic development, ARIP4 is mainly expressed in the neural tube and limb buds. ARIP4 mRNA is ubiquitous in adult mouse tissues, with the highest levels found in liver, kidney, and testis.
- In testis, ARIP4 is mainly expressed in Sertoli cells, Leydig cells, spermatogonia, pachytene and diplotene spermatocytes. In Sertoli cells, ARIP4 protein expression is high at stages II–VI and VII–VIII.
- Heterozygous *Arip4*$^{+/-}$ mice appear normal, but are haploinsufficient with regard to AR-specific gene expression in Sertoli cells.
- Homozygous *Arip4*$^{-/-}$ embryos show severely retarded growth at E10.5, die by E11.5, and are completely resorbed at E12.5. The embryonic lethality during early organogenesis is associated with increased apoptosis and impaired cell proliferation.

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