

# Essential Role for Co-chaperone Fkbp52 but Not Fkbp51 in Androgen Receptor-mediated Signaling and Physiology\*

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Fkbp52 and Fkbp51 are tetratricopeptide repeat proteins found in steroid receptor complexes, and Fkbp51 is an androgen receptor (AR) target gene. Although *in vitro* studies suggest that Fkbp52 and Fkbp51 regulate hormone binding and/or subcellular trafficking of receptors, the roles of Fkbp52 and Fkbp51 *in vivo* have not been extensively investigated. Here, we evaluate their physiological roles in Fkbp52-deficient and Fkbp51-deficient mice. Fkbp52-deficient males developed defects in select reproductive organs (*e.g.* penile hypospadias and prostate dysgenesis but normal testis), pointing to a role for Fkbp52 in AR-mediated signaling and function. Surprisingly, ablation of Fkbp52 did not affect AR hormone binding or nuclear translocation *in vivo* and *in vitro*. Molecular studies in mouse embryonic fibroblast cells uncovered that Fkbp52 is critical to AR transcriptional activity. Interestingly, Fkbp51 expression was down-regulated in Fkbp52-deficient males but only in affected tissues, providing further evidence of tissue-specific loss of AR activity and suggesting that Fkbp51 is an AR target gene essential to penile and prostate development. However, Fkbp51-deficient mice were normal, showing no defects in AR-mediated reproductive function. Our work demonstrates that Fkbp52 but not Fkbp51 is essential to AR-mediated signaling and provides evidence for an unprecedented Fkbp52 function, direct control of steroid receptor transcriptional activity.

Androgen receptor (AR)<sup>3</sup> is a hormone-induced transcription factor that controls male sexual development and other important physiologies. Similar to other members of the

nuclear receptor family (1, 2), AR has three major functional domains: an N-terminal transactivation domain, a DNA-binding domain, and a C-terminal ligand-binding domain (3–5). Mutations found in each of these domains lead to a series of AR functional defects associated with androgen insensitivity syndrome (AIS) or partial AIS in humans (6, 7). The majority of AIS and partial AIS patients have developmental defects in the male reproductive system. Loss-of-function AR mutations in mice recapitulate many of the reproductive defects found in AIS patients. For example, the AR-deficient (androgen receptor knock-out; ARKO) mouse (8) and the *tfm* (testicular feminization mutant) mouse (9) both develop severe defects of testicular development and an overall lack of male sexual differentiation, including hypospadias and penile agenesis. The *tfm* male mouse demonstrates many female secondary structures, including vagina and teats (10).

Molecular regulation of AR function can be achieved at several levels, such as spatial-temporal expression of the receptor, modulation of ligand binding, cytoplasm to nucleus translocation, and DNA binding and transcriptional activities (11, 12). Prior to hormone binding, steroid receptors form large protein complexes containing the molecular chaperone heat shock protein 90 (Hsp90) as well as various co-chaperone tetratricopeptide repeat (TPR) proteins (13–15). These co-chaperones include Fkbp52 and Fkbp51 (FK506-binding protein 52 and 51, respectively), Cyp40 (cyclophilin 40), and PP5 (protein phosphatase 5). Fkbp52 and Fkbp51 are ubiquitously expressed proteins with peptidyl prolyl *cis/trans*-isomerase activity that is inhibited by the binding of FK506 immunosuppressant ligand (16–18). Each TPR protein enters into steroid receptor complexes through a direct and competitive binding at the C terminus of Hsp90 via its essential TPR domain (19–21). Although Fkbp52 and Fkbp51 share a similar domain structure, as well as 60% sequence identity and 75% similarity, they do differ in that Fkbp51 is missing a C-terminal calmodulin-binding domain.

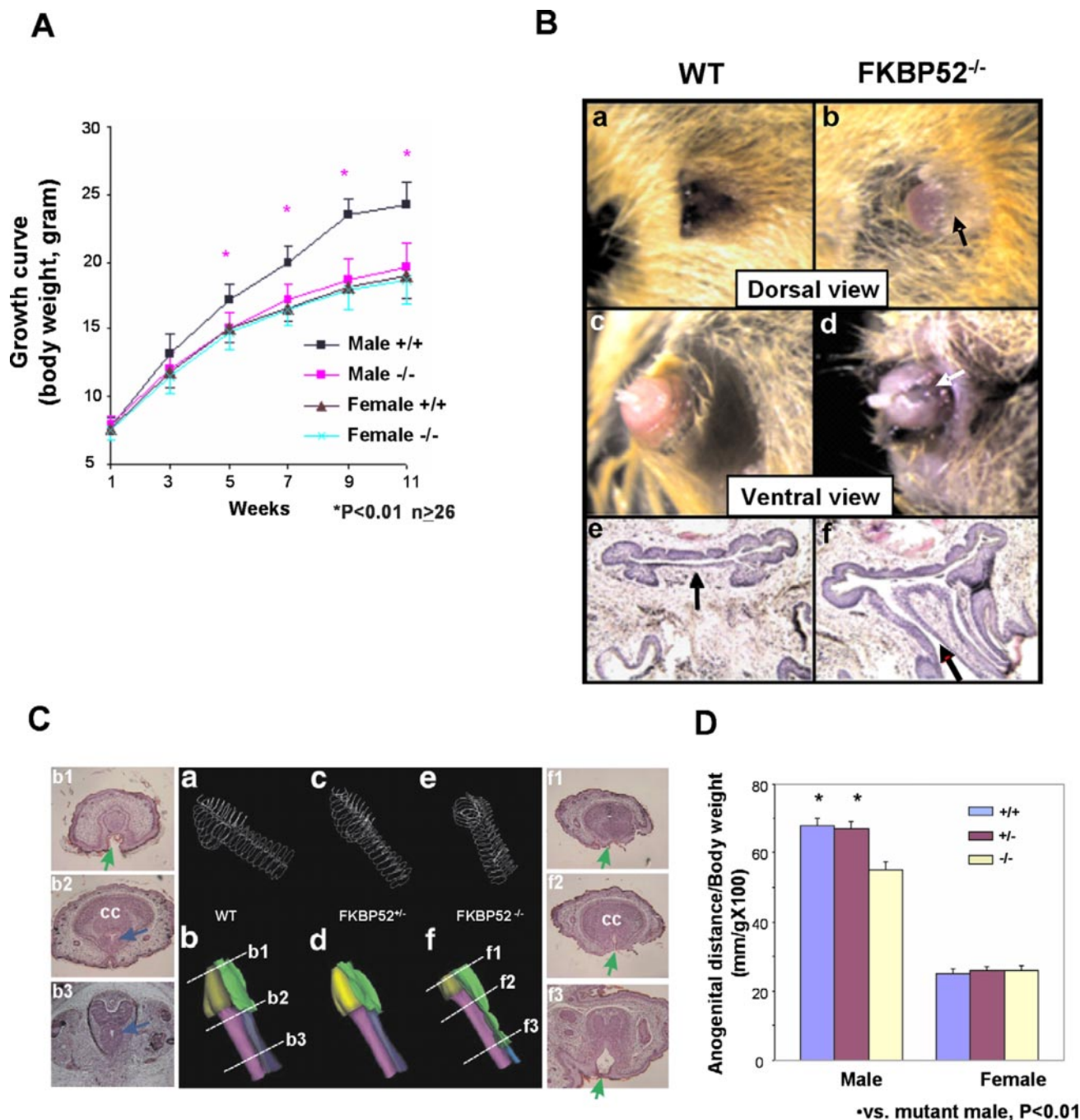
To date, most studies on TPR control of the steroid receptor (SR) action have been done using conventional molecular and cellular approaches and using the glucocorticoid (GR) and progesterone (PR) receptors as models. It has been shown that Fkbp52 is localized to both cytoplasm and nucleus but that the cytoplasmic fraction co-localizes with microtubules in a complex containing dynein (22, 23). For these reasons, it was pro-

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<sup>3</sup> The abbreviations used are: AR, androgen receptor; AIS, androgen insensitivity syndrome; ARKO, androgen receptor knock-out; TPR, tetratricopeptide repeat; SR, steroid receptor; GR, glucocorticoid receptor; PR, progesterone receptor; RT, reverse transcription; MEF, mouse embryo fibroblast; MMTV, murine mammary tumor virus; CAT, chloramphenicol acetyltransferase; PSA, prostate-specific antigen; DHT, dihydrotestosterone.



**FIGURE 1. Morphological and histological analysis of *Fkbp52*-deficient males.** *A*, growth curves of wild-type and *Fkbp52*-deficient mice; *B*, comparison of adult wild-type and *Fkbp52*-deficient male external genitalia at dorsal (*a* and *b*) and ventral surfaces (*c* and *d*). The arrows (in *b* and *d*) indicate the underdeveloped foreskin and ectopic urethral opening at the ventral aspect compared with normal morphology in controls (*a* and *c*). Shown are histological sections of wild-type (*e*) and *Fkbp52*-deficient (*f*) male genitalia. A red arrow indicates the ectopic urethral opening at the ventral aspect compared with normal morphology in controls (black arrow in *e*). *C*, three-dimensional reconstruction of the mouse embryonic day 18.5 penises. Wire frame images of outer penile skin and urethra of the three *Fkbp52* genotypes are shown in *a*, *c*, and *e*. In *b*, *d*, and *f*, the skins had been artificially removed. The glans penis is colored in gold, whereas the urethral opening is green, corpus cavernosum (cc) is purple, and the closed urethra is blue. Note that the urethral opening persisted throughout the *Fkbp52*-deficient penile shaft, whereas normal controls only have a temporary urethral opening at the distal end. The side panels show representative sections of wild-type and mutant penises. The green arrows indicate open urethra, and blue arrows indicate closed urethra. *D*, comparison of anogenital distances in wild-type, heterozygous, and *Fkbp52*-deficient mice. The anogenital distance, normalized by the animal body weight, was significantly shorter in *Fkbp52*-deficient males compared with littermate wild-type and heterozygous males.

posed that *Fkbp52* serves as an adaptor between the GR·Hsp90 complex and the microtubule transport machinery (24). Indeed, various groups have shown that *Fkbp52* contributes to both the dynein interaction and hormone-induced translocation of GR (25–27). Meanwhile, a second line of inquiry has

provided evidence for reciprocal control of GR and PR hormone binding function by *Fkbp52* and *Fkbp51*, with *Fkbp52* causing potentiation and *Fkbp51* causing attenuation of this activity (15, 28, 29). These observations suggest a model in which differential incorporation of TPR proteins into SR com-

# Role for *Fkbp52* but Not *Fkbp51* in AR-mediated Signaling

**TABLE 1**  
Comparison of reproductive organs and functions in male mice

	Wild-type	Heterozygous	Homozygous
Body weight (g)	29.1 ± 3.1 (n = 32) <sup>a</sup>	28.8 ± 2.0 (n = 38) <sup>a</sup>	25.2 ± 2.4 (n = 32)
Testis/Body (mg/g)	7.00 ± 1.19 (n = 32)	7.10 ± 1.35 (n = 38)	7.71 ± 1.72 (n = 32)
Epididymis/Body (mg/g)	2.52 ± 0.44 (n = 32)	2.48 ± 0.38 (n = 33)	2.47 ± 0.39 (n = 28)
Seminal vesicular/Body (mg/g)	7.40 ± 1.31 (n = 32) <sup>a</sup>	7.63 ± 1.28 (n = 38) <sup>a</sup>	5.28 ± 1.58 (n = 32)
Penile/Body (mg/g)	0.77 ± 0.13 (n = 32) <sup>a</sup>	0.83 ± 0.13 (n = 36) <sup>a</sup>	0.56 ± 0.16 (n = 31)
Penile length (mm)	6.1 ± 0.16 (n = 5) <sup>a</sup>	6.1 ± 0.19 (n = 8) <sup>a</sup>	4.4 ± 0.33 (n = 8)
Sperm count (× 10 <sup>6</sup> /epididymis)	12.6 ± 2.9 (n = 10)	12.1 ± 1.3 (n = 10)	9.6 ± 2.9 (n = 10)
Sperm motility	69.0 ± 6.8 (n = 10) <sup>a</sup>	66.2 ± 2.5 (n = 10) <sup>a</sup>	47.7 ± 8.8 (n = 10)
Serum testosterone (ng/ml)	1.89 ± 0.85 (n = 17) <sup>a</sup>	ND <sup>b</sup>	3.62 ± 0.46 (n = 19)
Serum DHT (pg/ml)	175.4 ± 56.3 (n = 17) <sup>a</sup>	ND	264.3 ± 74.8 (n = 19)

<sup>a</sup> Versus homozygous (−/−), *p* < 0.01.

<sup>b</sup> ND, not determined.

plexes forms the basis for selective control of hormone binding and subcellular trafficking.

Although evidence for interaction of *Fkbp52* with AR is fairly common (21, 30, 31), reports of *Fkbp51* in AR complexes are limited (32). However, it has recently become clear that *Fkbp51* is a highly sensitive AR-regulated gene, at least in the prostate cancer cell line LnCAP, where androgen-induced expression of *Fkbp51* can be blocked by the Hsp90 inhibitor geldanamycin (32–34). These observations would suggest that *Fkbp52* and *Fkbp51* play essential and perhaps distinct roles in AR function, yet little is known on this matter. Interestingly, a recent report by Febbo *et al.* (32) suggests that overexpression of *Fkbp51* has a stimulatory effect on AR-mediated transcriptional activity rather than inhibition, as known for GR and PR. Thus, it is possible that *Fkbp52* and *Fkbp51* exert distinct and diverse effects on various members of the steroid receptor family.

With the above considerations in mind, we set out to generate mice genetically deficient in *Fkbp52* and *Fkbp51*. Although it was fully expected that alterations to GR-regulated physiology would be a prominent feature of *Fkbp52* loss, we were surprised to find no such effect. Instead, the primary phenotype of *Fkbp52* ablation was infertility in both male and female mice. Female *Fkbp52*-deficient mice were sterile due to a selective loss of activity by the PR-A isoform in the uterus, leading to a complete failure of implantation (35). Here, we report that *Fkbp52* is critical to male fertility by controlling AR-mediated signaling and physiology. Loss of *Fkbp52* resulted in aberrant penile development causing hypospadias, as well as prostate dysgenesis, while leaving other AR-regulated organs unaffected. Molecular studies showed that *Fkbp52* loss had a dramatic effect on AR-mediated gene expression that, surprisingly, was not due to altered AR hormone-binding and/or nuclear translocation functions. Analysis of *Fkbp51* as an AR target gene showed it to be down-regulated only in *Fkbp52*-deficient tissues exhibiting altered AR activity. By generating and analyzing *Fkbp51*-deficient mice, we were able to assess its direct contribution to AR activity *in vivo* and its role as a target gene in AR-mediated development of the penis and prostate. Interestingly, no alterations to male (or female) physiology were noted in the *Fkbp51*-deficient mice, showing that *Fkbp51* is not essential to AR signaling *in vivo*.

## MATERIALS AND METHODS

**Generation of *Fkbp52*-deficient Mice and *Fkbp51*-deficient Mice**—The generation of *Fkbp52*-deficient mice was described in a previous report (35). To generate *Fkbp51*-deficient mice, a promoter-trapped ES cell line RRC236 containing an insertional mutation in the mouse *Fkbp51* gene was identified and obtained from BayGenomics (36). The gene trap vector (pGT1Lxf) contains a splice acceptor sequence upstream of the reporter gene *β-geo*. Using genomic PCR, Southern blot, and sequencing analyses, we confirmed a single genomic insertion at intron 4 of the mouse *Fkbp51* gene. The chimeric male mice were generated from RRC236 ES cell line and were further bred to C57BL/6J females to generate F1 offspring. Genotypes were determined by Southern blot analysis. Northern blot, quantitative RT-PCR, and Western blot analyses confirmed that there was no *Fkbp51* leaky expression in *Fkbp51*-deficient mice. The SC11518 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to detect *Fkbp51* by Western blotting. *Fkbp52*/*Fkbp51* compound mutants were generated by interbreeding heterozygous *Fkbp52* and *Fkbp51* mutant mice. We also used PCR analysis of the *sry* gene to determine genders of embryos. All animal experiments were carried out using a protocol approved by the Indiana University School of Medicine Institutional Animal Care and Research Advisory Committee.

**Morphology, Histology, and Immunohistochemistry**—We examined the morphology and histology of multiple tissues and organs from male mice and age-matched littermate control mice. Embryos were harvested from timed mating females by cesarean section. Isolated embryos and tissue samples were fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned (6 μm) and stained with hematoxylin and eosin. Three-dimensional reconstruction was carried out as previously described (37). Immunohistological analysis was carried out as previously described (38). Antibody against AR (SC7305) was from Santa Cruz Biotechnology.

**Gel Electrophoresis and Western Blotting**—Samples were resolved on denaturing SDS gels (39). Transfer of samples to Immobilon-P® membranes and immunoblotting were performed as previously described (26). The SC7305 (Santa Cruz Biotechnology) antibody against AR was used to probe for receptor, whereas various antibodies were used to probe for *Fkbp52* (SC1803; Santa Cruz Biotechnology), *Fkbp51* (SC11518; Santa Cruz Biotechnology), Cyp40 (PA3-022; Affin-

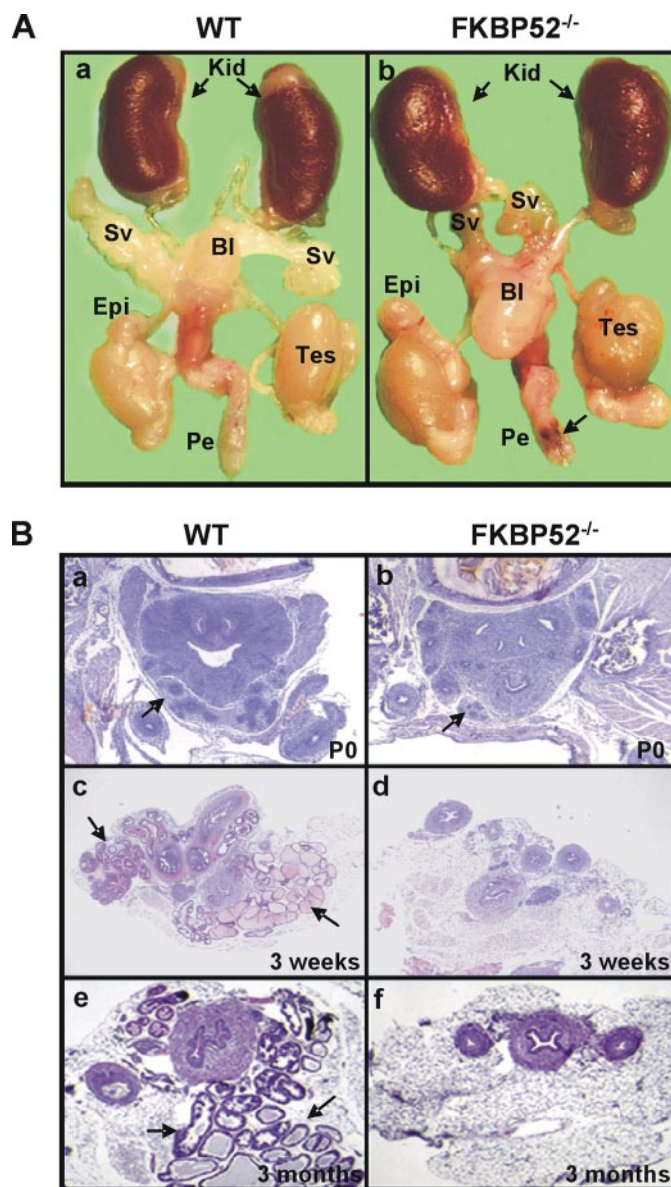
ity BioReagents), and PP5 (gift from Dr. Michael Chinkers) (40). The blots were then incubated with appropriate peroxidase-conjugated counter antibodies, followed by detection of bands by enhanced chemiluminescence.

**Serum Hormone Measurement**—Afternoon blood samples were drawn (between 1:00 and 2:00 p.m.) from the right ventricle of adult mice (2–3 months of age). Serum levels of testosterone and DHT (dihydrotestosterone) were analyzed by using radioimmuno assay kits (DSL-4000 for testosterone, DSL-9600 for DHT; Diagnostic Systems Laboratories, Inc., Webster, TX). Steroid measurements were performed at the Endocrinology Core Laboratory of the Indiana University School of Medicine.

**Reporter Gene Assays in Mouse Embryonic Fibroblast Cells**—Mouse embryonic fibroblasts (MEFs) were isolated from wild-type and *Fkbp52*-deficient embryonic day 13.5 embryos. MEF cells were cultured in DMEM with 15% FBS until confluence. To generate immortalized MEF cells, primary cells were transfected with SV40. Pooled transformed cells were maintained in DMEM supplemented with 10% FBS. Both wild-type and *Fkbp52*-deficient MEF cells maintain normal fibroblast morphology with high proliferative activity. MEF cells were either transiently or permanently transfected with expression vectors for human AR (kind gift of Lirim Shemshedini). The MMTV-CAT reporter and PSA (prostate-specific antigen)-luciferase reporter (kind gift of Marianne Sadar) constructs were used to assay for AR activity (41, 42). CAT enzyme activity was measured as described by Nordeen *et al.* (43), using [<sup>3</sup>H]acetyl-CoA as substrate, whereas luciferase activity was measured using a commercial kit (Promega). All values were normalized for transfection efficiency by co-transfection with a cytomegalovirus-driven galactosidase reporter.

**AR Hormone Binding Assay**—Wild-type and *Fkbp52*-deficient MEF cells were transiently transfected with human AR expression plasmid (pSG5). Forty-eight hours following transfection, cell pellets were washed twice with phosphate-buffered saline and resuspended in ice-cold homogenization buffer (10 mM HEPES, 1.5 mM EDTA, and 10 mM sodium molybdate, pH 7.4) with protease inhibitors, followed by Dounce homogenization and centrifugation for 10 min at 16,000 × *g*. Supernatants (cytosol fraction) were used for the binding assay without freezing. In a typical binding assay, 150 μl of cytosol (~2.0 mg/ml) were incubated with 10.0 nM [<sup>3</sup>H]mibolerone, (70.0 Ci/mmol) for 20 h on ice. Mibolerone is a synthetic high affinity AR ligand. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled mibolerone (10 μM). Protein-bound radioactivity was isolated using 1% dextran-coated charcoal in 10 mM HEPES buffer (pH 7.4). Specific binding was normalized for AR expression as determined by Western blot and expressed as disintegrations/min/milligram of cytosol protein.

**Quantitative RT-PCR**—Total RNAs were isolated from mouse tissues or cells using TRIzol (Invitrogen). First strand cDNAs were synthesized by the iScript cDNA synthesis kit (Bio-Rad) using 1 μg of RNA as template according to the manufacturer's instructions. Real time PCR was performed using iCycler iQ (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). The relative expression was normalized to glyceraldehyde-3-phosphate dehydrogenase. The sequences of specific primers are listed as follows: human AR, 5'-TGGAAGCCATTGAGCCAGGTGTAG-3' and 5'-CGTCC-



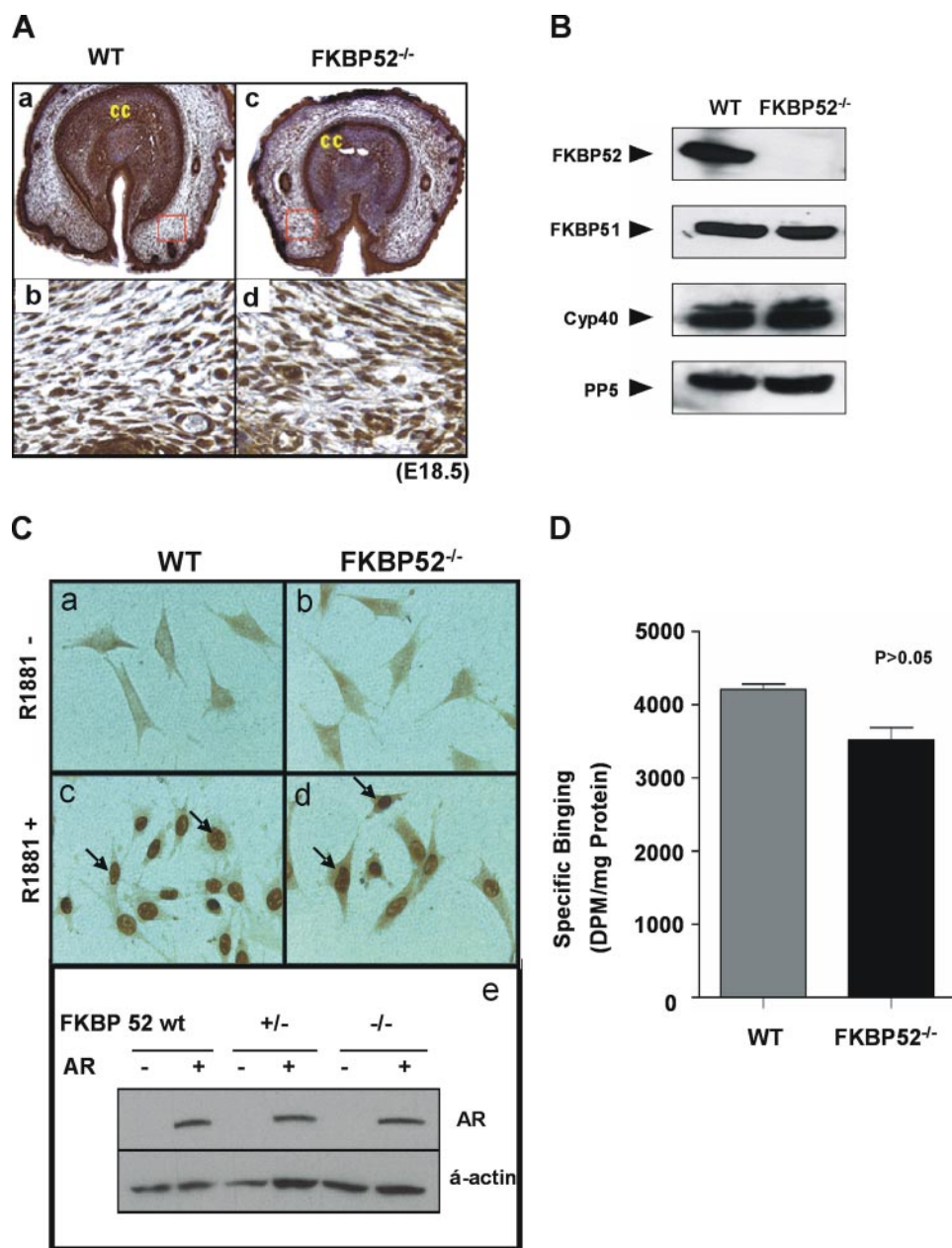
**FIGURE 2.** *A*, morphological comparison of male internal reproductive organs in wild-type (*WT*) (*a*) and *Fkbp52*-deficient adult mice (*b*). *Fkbp52*-deficient males have overall normal testis (*Tes*) formation and normal epididymis (*Epi*) but significantly smaller seminal vesicles (*SV*). *Kid*, kidney; *Bl*, bladder; *Pe*, penis. The *black arrow* indicates the urethral opening. *B*, histological analysis of prostate gland development in *Fkbp52*-deficient and age-matched littermate control mice at birth (*P0*) to 3 months old. Prostate glands are initially formed during embryonic development in both wild-type (*a*) and *Fkbp52*-deficient mutant (*b*) but lack further growth after the birth (*d*) and eventually become dysgenic in *Fkbp52*-deficient adult males (*f*) when compared with littermate wild-type mice (*c* and *e*). Prostate glands are indicated by *black arrows*.

ACGTGTAAGTTGCGGAAG-3'; *Fkbp52*, 5'-GCCTCTCGAA-GGAGTGGACATCAG-3' and 5'-CGGTCCAGACTGGAGT-CAAACCTTG-3'; *Fkbp51*, 5'-TGCAGATCTCCATGTGCCA-GAGG-3' and 5'-GCTCCTTCTACAGCCTTCTTGCTCC-3'; glyceraldehyde-3-phosphate dehydrogenase, 5'-TCCTGGTAT-GACAATGAATACGGC-3' and 5'-TCTTGCTCAGTGT-CCTTGCTGG-3'.

## RESULTS

**Reduced Fertility in *Fkbp52*-deficient Males**—*Fkbp52*-deficient mice were generated as previously described (35).

## Role for *Fkbp52* but Not *Fkbp51* in AR-mediated Signaling



**FIGURE 3. Analysis of *Fkbp52* deficiency on AR nuclear translocation and hormone binding.** *A*, immunohistochemical staining shows that AR is highly expressed in all cell types of wild-type (WT) (*a*) and *Fkbp52*-deficient genital tubercles (embryonic day 18.5) (*c*) and that AR nuclear localization is not altered in *Fkbp52*-deficient mutants (*d*) compared with wild-type controls (*b*). *B*, ablation of *Fkbp52* has no compensatory effect on expression of *Fkbp51*, *Cyp40*, and *PP5* in *Fkbp52*-deficient MEF cells. *C*, using AR-stably transfected MEF cells to determine the AR nuclear translocation activity in *Fkbp52*-deficient cells. Without hormone (R1881) treatment (*a* and *b*), ARs are mainly localized in cytoplasm. Upon R1881 treatment, ARs in both wild-type (*c*) and *Fkbp52*-deficient (*d*) MEF cells translocate to nuclei in a similar fashion. The overall AR expression levels in AR-transfected cells are comparable among these cell lines, as evaluated by Western blot analysis (*e*). Genotypes of the cells are as indicated. *D*, measurement of AR hormone-binding capacity in cytosols from AR transfected wild-type and *Fkbp52*-deficient MEF cells using [<sup>3</sup>H]mibolerone. The results shown are the means  $\pm$  S.E. of two independent experiments, each performed in triplicate. No significant effect of *Fkbp52* ablation is seen on AR hormone-binding function.

*Fkbp52*-deficient mice were viable. However, mutant males showed a rate of growth equivalent to that of wild type females (Fig. 1A). By breeding to wild type female mice, we found that *Fkbp52*-deficient males had greatly reduced fertility. Only 5% of *Fkbp52*-deficient males were able to plug females. Analysis of plugged females showed significantly smaller litters from *Fkbp52*-deficient males (mean =  $2.1 \pm 0.9$ ;  $n = 7$ ) compared

with wild type males (mean =  $6.8 \pm 1.6$ ,  $n = 16$ ,  $p < 0.001$ ). *Fkbp52*-deficient females were sterile due to implantation failure. Detailed analysis of *Fkbp52*-deficient females and the role of *Fkbp52* in regulating progesterone receptor function at the uterus was reported elsewhere (35).

**Selective Reproductive Defects in Adult *Fkbp52*-deficient Males**—The most striking morphological defect observed in adult *Fkbp52*-deficient males was the presence of hypospadias with 100% penetrance in the external penile genitalia (Fig. 1B). Similar to hypospadias in humans, the foreskin was underdeveloped, and the penile glans and anterior portion of tubercle were exposed in the majority of *Fkbp52*-deficient mutant males, compared with the normal male genital tubercle that was surrounded and covered by foreskin (compare *a* and *b*). All *Fkbp52*-deficient males showed an ectopic opening on the ventral side of the penis (*c* and *d*), which was confirmed by histological staining (*e* and *f*). Three-dimensional reconstruction of serial histological sections of embryonic genital tubercles confirmed the hypospadias phenotype to be a developmental defect resulting from failure to form urethral seams (Fig. 1C). These defects mimic the typical clinical features of hypospadias patients. In addition, the length and weight of *Fkbp52*-deficient penises were significantly less when compared with littermate controls (Table 1). The anogenital distance was shortened in *Fkbp52*-deficient males (3–10 weeks of age) (Fig. 1D), a feminization phenotype similar to that seen in ARKO and testicular feminization (*tfm*) mice, albeit to a lesser degree (8, 10).

Interestingly, primary sex organs appeared to be unaffected by *Fkbp52* loss. *Fkbp52*-deficient males developed normal size of testes and epididymis (Table 1) and had normal scrotal sacs and inguinal canals (data not shown). Histological analysis confirmed seminiferous tubules, spermatogenesis, Sertoli cells, and Leydig cells all to be normal in the *Fkbp52* mutant testis (data not shown). Sperm motility rates were slightly lower in mutant males compared with wild type and heterozygous littermates (Table 1). In contrast, the majority of reproductive tissues affected in *Fkbp52*-deficient

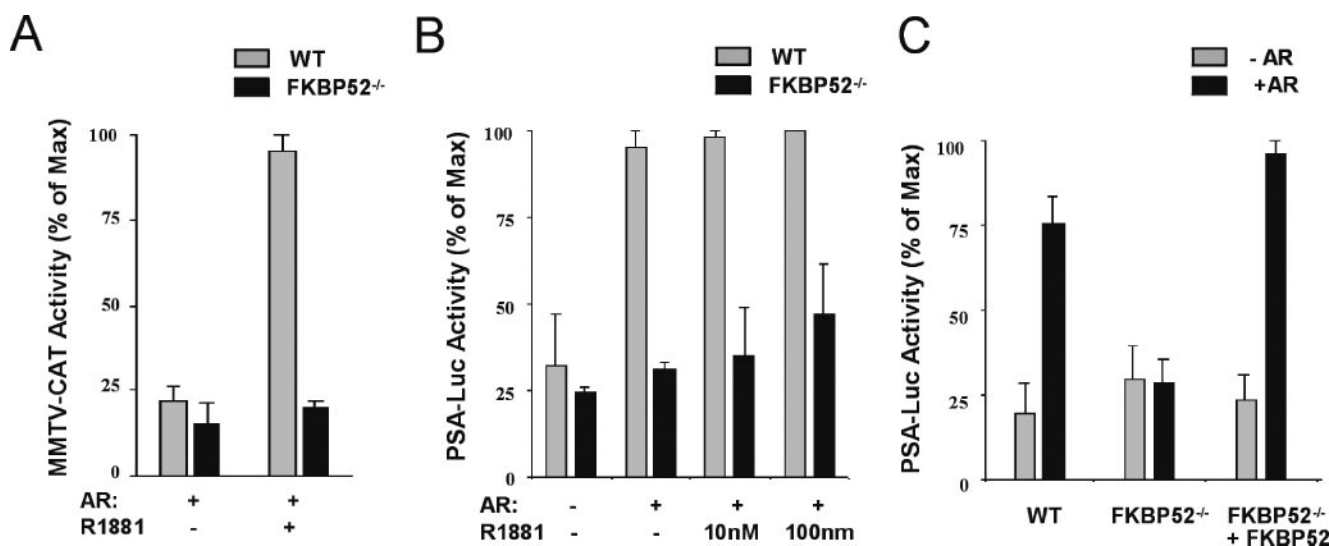


FIGURE 4. Analysis of AR transcriptional activity in Fkbp52-deficient MEF cells. *A*, transcriptional enhancement activity by hormone at MMTV-CAT is inhibited in Fkbp52-deficient cells. *B*, transcriptional enhancement activity at the PSA-luciferase reporter is also inhibited. *C*, analysis of AR transcription activity in Fkbp52-deficient MEF cells with reintroduction of human Fkbp52. Values represent the means  $\pm$  S.E. of four independent experiments.

males were the secondary sex organs. Seminal vesicles (SV) were present but significantly smaller in Fkbp52-deficient mice (Table 1 and Fig. 2*A*). Prostate glands were initially formed through the embryonic developmental process but lacked further growth at puberty and eventually became dysgenic in Fkbp52-deficient adult males (Fig. 2*B*). This phenomenon is another example of compromised AR-mediated function due to Fkbp52 deficiency, since AR activity is required for the formation of mature prostate glands (44).

Since secondary sex characteristics are determined by steroidal sex hormones, we measured serum testosterone and DHT levels in Fkbp52-deficient males. Levels of each hormone were slightly but significantly elevated in Fkbp52-deficient adult males (Table 1), demonstrating that lack of androgen production is not the cause of the abnormal phenotypes seen in Fkbp52-deficient males.

**Normal Androgen Receptor Expression, Hormone Binding, and Nuclear Translocation in Fkbp52-deficient Mice**—Because Fkbp52-deficient males showed growth curves similar to females and had selective defects in reproductive organs, we reasoned that altered AR signaling might be the underlying cause of the defects. To test whether Fkbp52 ablation led to reduced levels of AR, we performed Northern blot and Western blot analyses. In both penis and testis, Fkbp52 loss had no effect on AR mRNA and protein levels (data not shown). Thus, Fkbp52 is not essential to AR expression or AR stability. To assess possible impairment of AR nuclear translocation, immunohistochemical staining using antibody against AR was performed on genital tubercles isolated from embryos at embryonic day 18.5 (Fig. 3*A*). In agreement with our Western blot data, overall staining of AR was equal in both wild type and homozygous embryonic tubercles. In wild type tubercle cells, AR was primarily located in the nucleus (Fig. 3*A, b*), presumably due to activation by circulating testosterone. Because reports for GR have shown a role for Fkbp52 in nuclear translocation (26, 45), we were surprised to see nuclear localization of AR in Fkbp52-deficient tubercle cells (Fig. 3*A, d*). To confirm this

behavior, we analyzed AR nuclear translocation in Fkbp52-deficient and wild type MEF cells stably transfected with AR. Fig. 3*B* shows the complete absence of Fkbp52 in Fkbp52-deficient MEF cells and that loss of Fkbp52 has no compensatory effect on expression of Fkbp51, Cyp40, and PP5 in these cells. Prior to treatment with androgen agonist R1881, AR was primarily located in the cytoplasm of both wild type and Fkbp52-deficient MEF cells (Fig. 3*C, a and b*). After R1881 treatment, AR in both cell lines moved to the nucleus efficiently (Fig. 3*C, c and d*). It therefore appears that AR nuclear translocation is not dependent on the presence of Fkbp52. Consistent with this finding, AR hormone-binding activity in Fkbp52-deficient MEF cells was also normal (Fig. 3*D*). Thus, the principal role for Fkbp52 in AR-mediated signaling must be a novel effect downstream of the hormone binding and nuclear translocation events.

**Compromised AR Transcriptional Activity in Fkbp52(-/-) MEF Cells**—To determine if Fkbp52 controlled AR transcriptional activity, we measured AR activity at two heterologous reporter genes. When the AR transcriptional activity was measured using the MMTV-CAT reporter, a dramatic loss of ligand-induced AR activity was seen in the Fkbp52-deficient cells (Fig. 4*A*). To confirm this result, we used another AR-specific promoter, PSA-luciferase. Interestingly, in some cell systems, this promoter is known to have androgen-independent AR activity (42, 46). This was the case in the MEF cells, since AR activity did not increase with the addition of hormone (Fig. 4*B*). More importantly, however, this activity was also dependent on Fkbp52, since the PSA promoter activity was completely inhibited in Fkbp52-deficient MEF cells. To confirm that loss of AR activity was due to Fkbp52 rather than a nonspecific property in the Fkbp52-deficient MEF cells, we reintroduced Fkbp52 into the Fkbp52-deficient MEF cells. Fig. 4*C* shows complete reactivation of PSA promoter activity following Fkbp52 reexpression. As a whole, these data show Fkbp52 to be critical to AR transcriptional activity.

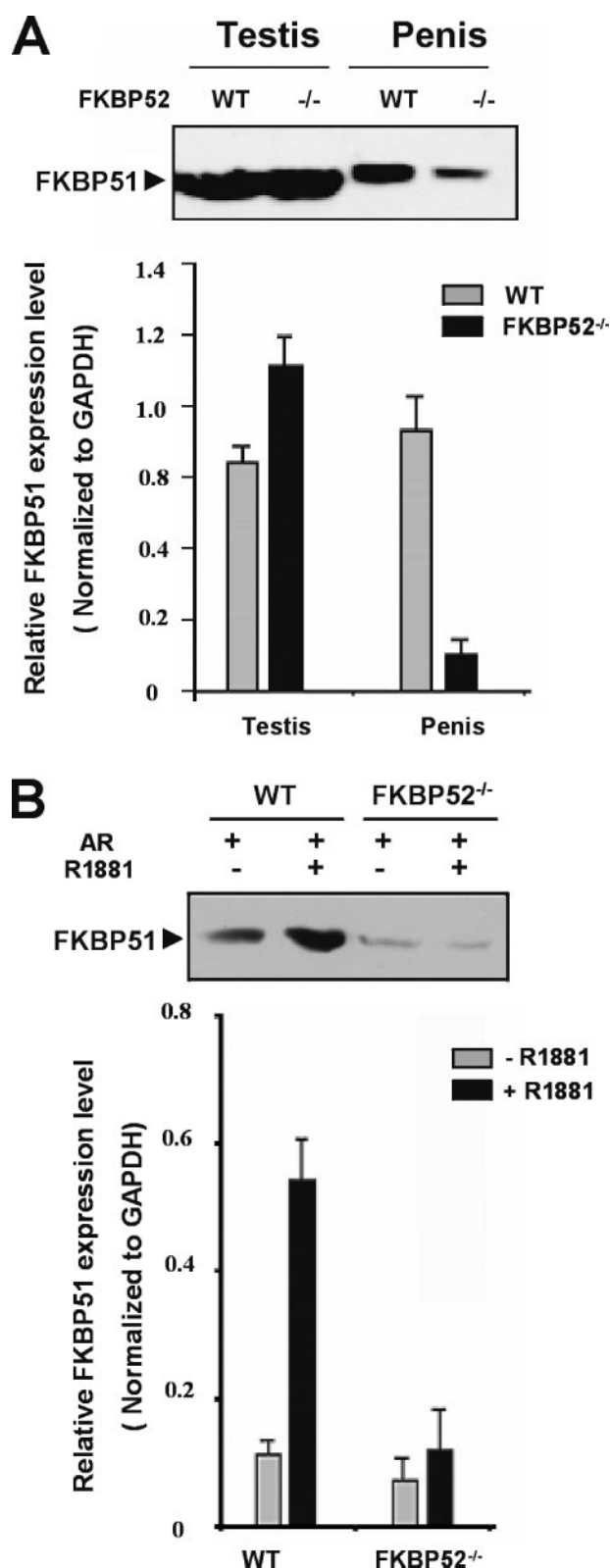
**Tissue-selective Loss of AR Activity in Fkbp52-deficient Males**—The results of Fig. 4 suggest that Fkbp52 is essential to AR activity and provide the basis for the aberrant development seen in

## Role for Fkbp52 but Not Fkbp51 in AR-mediated Signaling

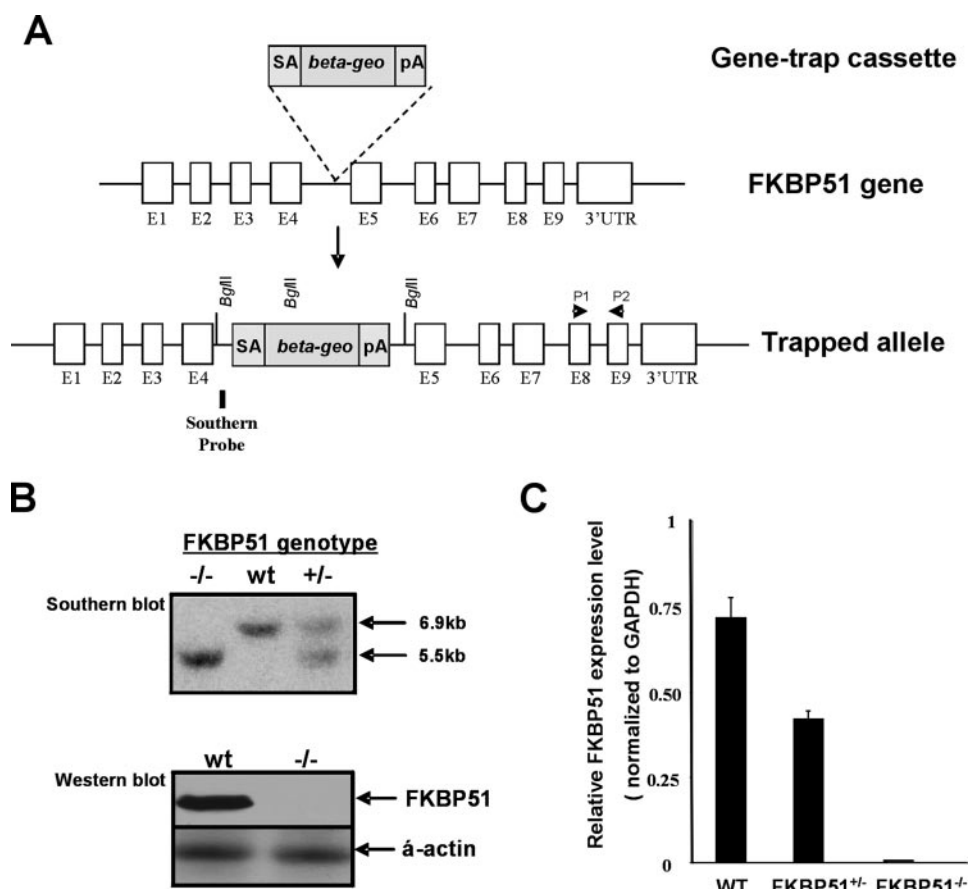
the prostate and penile tissues of Fkbp52-deficient mice. However, Fkbp52 cannot be exerting a global effect on AR activity, since Fkbp52-deficient males show normal testicular and epididymis development, two organs that are severely affected in ARKO mouse models (8, 47). Thus, a major unanswered question is the mechanism by which AR of the testes, for example, gets around the loss of Fkbp52. One possibility is that other members of the SRA-TPR family compensate for Fkbp52, at least on a tissue-specific basis. To test, we compared patterns of TPR protein expression in testicular and penile tissues in both wild type and Fkbp52-deficient animals. Analysis by quantitative RT-PCR showed no major effect of Fkbp52 loss on Cyp40 or PP5 expression in both testes and penis (data not shown), suggesting that compensatory up-regulation is not a likely mechanism for normal AR activity in the testes of Fkbp52-deficient animals. Interestingly, a dramatic down-regulation of Fkbp51 expression by quantitative RT-PCR was observed in the penis of Fkbp52-deficient animals but not in the testis (Fig. 5A). This tissue selectivity was confirmed by Western blotting for Fkbp51 protein (Fig. 5A, top). It was not surprising that Fkbp51 expression was down-regulated in the Fkbp52-deficient penis, since it has been shown that Fkbp51 expression is controlled by AR (32, 48). To further confirm this finding, we analyzed androgen-induced Fkbp51 expression in Fkbp52-deficient MEF cells stably transfected with AR. Consistent with the down-regulation of Fkbp51 in the penis, quantitative RT-PCR analysis revealed that Fkbp51 expression levels were significantly lower in Fkbp52-deficient MEF cells when compared with control MEF cells after R1881 treatments (Fig. 5B). Western blot further confirmed this result (Fig. 5B, top). Together, these results appear to indicate that AR activity is indeed reduced in the Fkbp52-deficient penis and MEF cells and that Fkbp51 expression is regulated by AR activity.

Loss of Fkbp51 expression in the aberrant penile tissue of Fkbp52-deficient males raises the possibility that Fkbp51 is either directly involved in normal penile development or indirectly involved in further AR actions necessary for development. Support for the latter possibility comes from Magee *et al.* (48), who showed recently that Fkbp51 is a positive regulator of AR transcriptional activity in the prostate cancer cell line LnCAP. Thus, AR control of Fkbp51 expression may form a positive feedback loop designed to maximize AR activity, perhaps in a developmental or tissue-specific fashion. Such a mechanism may, therefore, be responsible for the tissue-selective abnormalities seen in Fkbp52-deficient males.

**Generation and Characterization of Fkbp51-deficient and Compound Fkbp52/Fkbp51-deficient Mice**—To determine the contribution of Fkbp51 to AR signaling and male reproduction, we generated Fkbp51-deficient mice by using the BayGenomic Gene Trapping Resource (36). We confirmed a single insertion site in intron 4 in the mouse Fkbp51 gene (Fig. 6A). The trapped allele yielded an Fkbp51- $\beta$ geo fusion protein that lacked peptidyl-prolyl *cis/trans*-isomerase-like domain and all three TPR domains that are known to be critical to Fkbp51 function (49). Therefore, this gene-trapped allele was an Fkbp51 null allele. Northern, Western, and PCR analyses further confirmed the absence of leaky Fkbp51 expression in the mutant mouse strain (Fig. 6, B and C). Fkbp51 heterozygous mutant mice were fertile



**FIGURE 5. Analyses of Fkbp51 expression in the testis, penis, and MEF cells.** A, Western blot (upper panel) and quantitative RT-PCR analysis of Fkbp51 in testis and penis of wild-type (WT) and Fkbp52-deficient males. In Fkbp52-deficient penile tissues, Fkbp51 is dramatically down-regulated. B, Western blot (upper panel) and quantitative RT-PCR analysis of Fkbp51 in AR stably transfected wild-type and Fkbp52-deficient MEF cells with and without R1881 treatment (0.1 nM). In Fkbp52-deficient MEF cells, the induction of Fkbp51 expression by hormone treatment is significantly reduced. Values represent the means  $\pm$  S.E. of four independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



**FIGURE 6. Generation of *Fkbp51*-deficient mice.** *A*, genomic structure of the mouse *Fkbp51* gene, gene trap vector, and *Fkbp51* mutant allele. Southern blot and Western blot (*B*) and quantitative RT-PCR analyses (*C*) confirm the *Fkbp51* mutant allele to be null. For Southern blot, the genomic DNA was digested by *Bgl*III (New England Biolabs). The probe indicated in *A* reveals a 6.9-kb fragment from wild-type (*wt*) allele and a 5.5-kb fragment from *Fkbp51* mutant allele. Primers for quantitative RT-PCR analysis are indicated by a pair of triangles in *A*. UTR, untranslated region.

and were intercrossed to generate *Fkbp51*-deficient mice. Surprisingly, *Fkbp51*-deficient mice (male and female) appeared to have normal growth and fertility. We carefully analyzed the male reproductive system. *Fkbp51*-deficient males had normal formation of external genital tubercle, prostate, and the other male reproductive tissues (Fig. 7). *Fkbp52* expression also remained normal in all of the *Fkbp51*-deficient tissues and cells (data not shown). Taken together, these findings show that *Fkbp51* is not essential to AR-regulated physiology. We can also conclude that development of hypospadias and prostate dysgenesis in the *Fkbp52*-deficient males is not attributable to the loss of AR-mediated *Fkbp51* expression.

The above data suggest that *Fkbp52* and *Fkbp51* are not functionally redundant with respect to androgen control of male reproduction. However, given the high sequence homology between these two proteins (50), it remained possible that they shared a common function with respect to other SRs or, indeed, unknown client proteins. To address this question, we generated a series of compound *Fkbp52*/*Fkbp51* mutant mice. *Fkbp52*/*Fkbp51* double heterozygous mice were normal and were intercrossed to generate *Fkbp52*<sup>+/-</sup>/*Fkbp51*<sup>-/-</sup>, *Fkbp52*<sup>-/-</sup>/*Fkbp51*<sup>+/-</sup>, and *Fkbp52*<sup>-/-</sup>/*Fkbp51*<sup>-/-</sup> mice. *Fkbp52*<sup>+/-</sup>/*Fkbp51*<sup>-/-</sup> males were fertile and normal, whereas *Fkbp52*<sup>-/-</sup>/*Fkbp51*<sup>+/-</sup> males developed hypospadias similar

to *Fkbp52*-deficient males (data not shown). However, we were not able to obtain *Fkbp52*<sup>-/-</sup>/*Fkbp51*<sup>-/-</sup> mice at birth (data not shown), suggesting that compound *Fkbp52*/*Fkbp51*-deficient animals die *in utero*. Timed pregnancy studies further indicated that *Fkbp52*/*Fkbp51*-deficient mice died at an early embryonic stage (before embryonic day 7.5) that predates the start of the sex differentiation (detailed analyses of *Fkbp52*/*Fkbp51*-deficient mice will be reported elsewhere). These findings suggest that, although *Fkbp52* appears to be the major co-chaperone in regulating AR transcriptional activity, *Fkbp52* and *Fkbp51* are functionally redundant with respect to an early cellular function that is critical to embryonic development.

In summary, our current study demonstrates that *Fkbp52*, but not *Fkbp51*, is essential to AR-mediated signaling and physiology. *Fkbp52* is not required for AR to bind androgen or for its hormone-induced nuclear translocation. Instead, *Fkbp52* appears to play a novel yet critical role in AR transcriptional activity.

## DISCUSSION

In recent years, the complexity of molecular regulation of steroid receptor function has become apparent, with most investigators focusing on the mechanism by which co-regulatory proteins control tissue- and ligand-specific transcription activity by SRs (51–53). However, the discovery that inactive SRs exist as heterogeneous complexes, based on TPR protein content (54), suggests that the early stages of SR signaling may also contribute to diversity of action. To address this question, we have generated both *Fkbp52*-deficient and *Fkbp51*-deficient mice. In a prior report (35), we showed that the *Fkbp52*-deficient female is sterile due to a selective attenuation of some PR-regulated physiologies, in particular the uterine receptivity to implantation. In the current work, we show a similar selectivity of *Fkbp52* action in the male. Male *Fkbp52*-deficient mice showed dysgenic prostate and seminal vesicle development and penile hypospadias yet had apparently normal development and function of other AR-regulated tissues, such as the testes. Interestingly, loss of *Fkbp52* had no obvious effect on GR-mediated physiology. Meanwhile, loss of *Fkbp51* appears to be neutral with respect to AR, PR, and GR actions *in vivo*. Viewed as a whole, we believe that these observations usher in a new concept in which TPR proteins serve as agents for tissue- and receptor-specific control of steroidal actions.



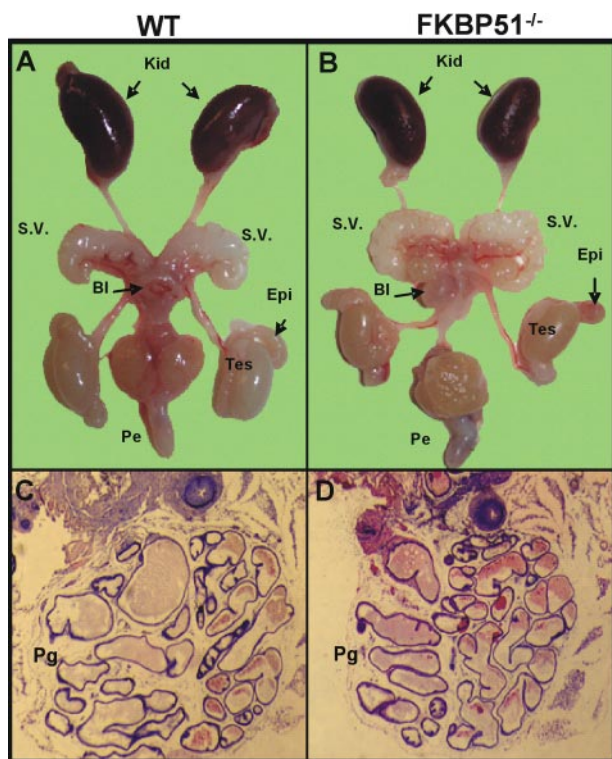


FIGURE 7. Morphological comparison of male internal reproductive organs in wild-type (A) and Fkbp51-deficient adult mice (B). Fkbp51-deficient males have overall normal testis (Tes) formation and normal epididymis (Epi) normal seminal vesicles (S.V.). Kid, kidney; Bl, bladder; Pe, penis. C and D, histological analysis of prostate glands in adult Fkbp51-deficient and age-matched littermate control mice. Fkbp51 mutant adult males have normal prostate glands (Pg). WT, wild type.

Clearly, a major unanswered question that derives from our work is why loss Fkbp52 or Fkbp51 appears to leave the *in vivo* actions of GR unaffected. Because the GR knock-out mouse is a perinatal lethal (55), we expected loss of Fkbp52 or Fkbp51 to have a similar phenotype if either of these proteins exerted an essential and global effect on GR actions. A possible explanation for this lack is the stress nature of cortisol secretion in which the main function of activated GR is to attenuate overactivity by “first responder” stress pathways, such as inflammation (56, 57). Thus, a defect of GR signaling in the Fkbp52-deficient animals may only be seen following a prior stress event, such as inflammatory challenge. Another explanation may lie with the concept of functional redundancy within the family of TPRs that regulate SR action. For example, it is possible that Fkbp51 compensates for loss of Fkbp52, with Fkbp52 reciprocating for loss of Fkbp51. With this in mind, we expected to see ablation of GR function in the double knock-out Fkbp52/Fkbp51 animals. Unexpectedly, the compound mutant turned out to be an embryonic lethal, suggesting that Fkbp52 and Fkbp51 are functionally redundant for an unknown factor(s), perhaps an orphan receptor, essential to early development. The Fkbp52/Fkbp51 compound mutant will now be an important reagent for identifying this unique developmental function and signal pathway. Of course, it is also possible that either PP5 or Cyp40 compensates for Fkbp52 and Fkbp51 in GR signaling. Because PP5 and Cyp40 are both known to interact with the motor protein dynein (25), either protein may provide

at least the mechanism by which GR and AR (see below) translocate to the nucleus. We have previously shown PP5 to be found in the GR heterocomplexes of L cells and that this interaction increases when Fkbp52 and Fkbp51 are removed from GR/HSP90 using FK506 (58). Thus, one of our future goals is to analyze the role of PP5 in SR signaling by generating appropriate PP5 mutant mice.

In addition to the lack of abnormal GR-related phenotypes, other unexpected results were found. For example, although Fkbp52 is clearly essential to AR activity in the MEF cells, no overt defect of testicular development was observed in Fkbp52-deficient males. Since studies in the ARKO mouse have shown AR activity to be essential for testis development (8, 47), our results must mean that AR activity in the testis is not dependent on Fkbp52, although this tissue expressed more Fkbp52 than penis or prostate (data not shown). Thus, we speculated that another TPR, perhaps Fkbp51, might fill this role in the testis. In the course of analyzing TPR expression levels in the tissues of wild type and Fkbp52-deficient mice, we found a dramatic down-regulation of Fkbp51 in Fkbp52-deficient penile tissue but not in the Fkbp52-deficient testis. Because Fkbp51 is a known target gene of AR action (29, 32, 33), this result was further evidence of compromised AR activity in the penis but not the testis. Moreover, since there is at least one report that Fkbp51 overexpression in prostate cells enhances AR activity (32), the interesting possibility was raised that a genetic interplay may exist between Fkbp52 and Fkbp51, perhaps for the purpose of regulating the tissue-selective functions of AR. However, by generating and analyzing Fkbp51-deficient mice, we have ruled out this possibility. It is now clear that Fkbp51 is not a target gene essential to AR control of prostate or penis development, nor is it as important as Fkbp52 to the intrinsic regulation of AR signaling *in vivo*. In short, down-regulation of Fkbp51 is probably just a simple reflection of compromised AR function.

An important question that arises from our study is the mechanism by which Fkbp52 regulates AR transcriptional activity. Our results in the MEF cells show that AR transactivity is severely compromised in the absence of Fkbp52, yet AR in these cells exhibited normal hormone binding and nuclear translocation. Thus, the principal role of Fkbp52 must be to control either the DNA recognition or transactivation functions of AR, perhaps by controlling recruitment of co-activators. Interestingly, we obtained similar results for the PR of Fkbp52-deficient females: loss of transactivity but normal hormone-binding function (35). Although Cyp40 or PP5 may account for the normal hormone-binding and translocation activities of these receptors, it is clear that Fkbp52 exerts an unexpected function on both AR and PR that, for the moment, leaves only room for speculation. The most likely mechanism is a lasting, down-stream effect of Fkbp52 peptidyl prolyl *cis/trans*-isomerase activity on the AF-1 or AF-2 domains of AR that is necessary for co-activator recruitment. Such a mechanism would be unprecedented for a TPR protein and will be the focus of future studies.

Our results have some similarities to those of Smith and co-workers (59), who independently made Fkbp52-deficient mice and reported infertility due to dysgenic development of

the penis and prostate. Our work extends upon their observations and provides more systematic and thorough analyses at the histological and molecular levels. Most notably, we demonstrate a critical role for Fkbp52 in regulating AR transcriptional activity and by assessing the contribution of Fkbp51 to the Fkbp52-deficient defect. More importantly, we herein provide the first report of Fkbp51-deficient animals, allowing us to conclude that Fkbp51 does not play an obvious or significant role in AR signaling *in vivo*, despite cellular and molecular evidence to the contrary. Finally, our studies have uncovered a novel and potentially unique function for Fkbp52 in the regulation steroid receptor signaling. This discovery has important clinical implications, such as the identification of a new pathogenetic pathway that may explain AIS in humans.

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