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RWDD1 interacts with the ligand binding domain of the androgen receptor and acts as a coactivator of androgen-dependent transactivation

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ABSTRACT

During embryogenesis, the development of the male genital is dependent on androgens. Their actions are mediated by the androgen receptor (AR), which functions as a transcription factor. To identify AR coregulators that support AR action during the critical time window of androgen-dependent development in the genital tubercle of male mice, we performed yeast two-hybrid screenings with cDNA libraries of genital tubercles from male mouse embryos using human AR as bait. RWD domain containing 1 (RWDD1) was identified as an AR-interacting protein from three independent libraries of the embryonic days E15, E16 and E17. The interaction between the AR and RWDD1 was confirmed in vitro and in vivo and the ligand binding domain of the AR was shown to be sufficient to mediate the interaction. RWDD1 enhanced AR-dependent transactivation in reporter assays with promoters of different complexity and in different cell lines. These results suggest that RWDD1 functions as a coactivator of androgen-dependent transcription.

1. Introduction

In mammalian embryos, androgens produced in the developing testis and their competent modes of action are required for the sexual development of male external genitalia. This is demonstrated in humans, as naturally occurring mutations affecting either androgen biosynthesis or androgen signaling through the androgen receptor (AR) lead to 46,XY disorders of sex development (DSD) with female or ambiguous external genitalia (Mendonca et al., 2010; Werner et al., 2010). The molecular mode of action of the androgen signaling cascade in sexual differentiation is still not completely understood to explain the phenotypic variability of patients with 46,XY DSD. Also, in some cases with androgen insensitivity syndrome no mutation in the AR could be detected and the molecular mechanism underlying the pathology remains to be solved (Deeb et al., 2005). Apart from the binding of appropriate androgens as ligands, AR signaling largely depends on the interaction with other proteins acting as coregulators. The role of AR coregulators in sexual differentiation and especially in 46,XY DSD is still elusive.

The AR is a nuclear receptor that acts as an androgen-dependent transcription factor. Like other members of the nuclear receptor family, e.g. the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), the AR shows a modular organization. It bears an N-terminal domain (NTD), a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Claessens et al., 2008). A short hinge region connects the DBD to the LBD. Without androgens, the AR resides in the cytosol in a complex with chaperones and cochaperones, e.g. heat shock protein 90 (Hsp90) and FK506 binding protein of 52 kDa (FKBP52) (Smith and Toft, 2008). Androgen binding leads to important conformational rearrangements in the LBD of the AR that result in the formation of a hydrophobic protein binding pocket, called activation function 2 (AF2). Since the AF2 mediates interaction with the ^{23}FQNLF^{27}-motif in the N-terminus of the AR, androgens induce an intramolecular N/C-terminal interaction in the nuclear receptor (Doesburg et al.,

Abbreviations: aa, amino acid; AF, activation function; AR, androgen receptor; CBP, CREB-binding protein; DBD, DNA-binding domain; DHT, dihydrotestosterone; E, embryonic day; GR, glucocorticoid receptor; GST, glutathione S-transferase; HRE, hormone response element; LBD, ligand-binding domain; mAR, murine androgen receptor; MR, mineralocorticoid receptor; NTD, N-terminal domain; RLU, relative luciferase units; RWDD1, RWD domain containing 1; SUMO, small ubiquitin-related modifier; SWI/SNF, mating type switching/sucrose nonfermenting; UBC9, ubiquitin carrier protein 9; TEV, tobacco etch virus.

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1997; He et al., 2000; Langley et al., 1995; Schaufele et al., 2005). Upon androgen binding, the AR translocates into the nucleus, dimerizes and binds to androgen response elements in target genes, thus driving androgen-dependent transcription. The NTD seems to be released from the LBD when the AR is bound to DNA (van Royen et al., 2007). This conformational change is probably necessary for the efficient association of certain coregulator proteins to the AF2 of the AR.

Coregulator proteins interact with the nuclear receptor and either promote or suppress transcription (coactivators and corepressors). A large number of AR coregulators have been identified to date, most of them act on several members of the nuclear receptor family (www.nursa.org). The AR LBD is very important for coregulator binding, as many coregulators bearing FxxLF and LxxLL motifs bind to the hydrophobic groove formed by the AF2 (He et al., 2002; van de Wijngaart et al., 2006, 2009; Zhou et al., 2002). Also coregulators interacting with the NTD, the hinge and the DBD have been described (Heemers and Tindall, 2007). Coactivators and corepressors regulate transcription by a diverse array of molecular functions and enzymatic activities (Bulynko and O'Malley, 2011). Some coregulators function already in the cytosol and affect androgen-dependent transcription in a rather indirect way, e.g. by keeping the AR in the correct conformation or by facilitating its translocation to the nucleus. Other AR coregulators fulfil their role after the translocation of the receptor into the nucleus, being part of the transcriptional complex on target genes. In the nucleus, coactivators recruit proteins of the general transcriptional machinery and exhibit activities that alter the chromatin structure to facilitate transcription. For example, members of the well-characterized p300/CBP proteins alter histone–DNA interactions via their histone acetyltransferase activity (Ogryzko et al., 1996), and proteins of the SWI/SNF complex unwrap chromatin in an ATP-dependent manner (Marshall et al., 2003).

Specificity of AR-dependent gene expression is thought to be controlled by the recruitment of certain sets of coregulators that might underlay tissue specific expression. Accordingly, the expression of androgen responsive genes in the male genital tubercle, the anlage of the external genitalia, is the result of specific AR coregulator complexes promoting or repressing AR-target gene transcription. Our aim was to identify coregulators of the AR that are expressed in the male mouse genital tubercle during the critical time window of androgen-dependent development in the embryo. Intra-uterine application experiments in mice with the anti-androgen flutamide have defined the time window of androgen-dependent masculinisation to embryonic days 15–17 (E15–E17) (Miyagawa et al., 2009). Therefore, we generated three cDNA libraries from male mouse genital tubercles of the developmental stages E15, E16 and E17. The libraries were used in independent yeast two-hybrid screens to identify proteins that associate with the ligand-activated C-terminal part of the AR. RWD domain containing 1 (RWDD1) was identified as an AR-interacting protein in all three screens. RWDD1 is a highly conserved and ubiquitously expressed protein of unknown function. It bears an RWD domain at the N-terminus, which is named after the type of proteins in which the domain was first identified; RING finger proteins, WD-domain containing proteins and DEAD-like helicases (Doerks et al., 2002). The RWD domain is supposed to function in protein interaction. In the present study we confirm the interaction of RWDD1 and the AR by GST pull-down assays and coimmunoprecipitations, and we define the LBD of the AR as sufficient for the interaction. By reporter assays in different cell lines we show that RWDD1 can activate AR-dependent transcription, suggesting that RWDD1 functions as a coactivator of the AR.

2. Materials and methods

2.1. Plasmids

The human AR expression plasmid pSVAR0 (Brinkmann et al., 1989) was a kind gift of A.O. Brinkmann (Rotterdam, the Netherlands). The yeast expression plasmids used as bait for the yeast two-hybrid screens or for retesting selected clones were obtained by subcloning fragments of the human AR from pSVAR0 into pGBKT7 (Clontech, Mountain View, CA, USA) downstream of the *GAL4* DNA-binding domain. pGBK_AR555, pGBK_AR618 and pGBK_AR645 encoded fusion proteins of the *Gal4* DNA-binding domain and amino acids 555–920 (DBD–hinge–LBD), 618–920 (hinge–LBD) or 645–920 (LBD) of human AR, respectively (Fig. 1B). For the RWDD1 expression plasmid the cDNA encoding human full-length *RWDD1* was amplified from human reference mRNA (Stratagene, La Jolla, CA, USA) and cloned into pcDNA3.1(+) (Life technologies, Darmstadt, Germany). The bacterial expression plasmid pGST–RWDD1 encoded a fusion protein of GST and full length human RWDD1 and was obtained by subcloning *RWDD1* into pGEX-4T.1 (GE Healthcare, Little Chalfont, UK).

The plasmid pT7-ARpolyA for *AR* in vitro translation was constructed by ligating the T7 promoter as paired oligos (T7promoter.NheI.s: CTAGCTAATACGACTCACTATAGGGA and T7promoter.NheI.a: CTAGTCCCTATAGTGAGTCGTATTAG) into the NheI site of pSVAR0, upstream of the *AR* coding region. A polyA-stretch of 30 adenine residues was cloned into the BamHI-site downstream of the *AR*. For the construction of the plasmids p3xFLAG-AR.LBD and p3xFLAG-AR.NTD coding for aa 645–920 or aa 1–555 of the human AR, respectively, the *AR* fragments were amplified from pSVAR0 and cloned into pcDNA3.1(+) containing an N-terminal 3xFLAG-tag upstream and a polyA stretch downstream of the cloning site. The GR expression construct was obtained by subcloning the human *GR* full-length cDNA clone IRAUp969B0272D (Imagene, Berlin, Germany) into pcDNA3.1(+). The luciferase reporter construct containing the proximal promoter of the mouse *Rhox5* gene was a kind gift of F. Claessens (Leuven, Belgium) (Zuccarello et al., 2008). The $(HRE)_2$TATA luciferase reporter plasmid was a kind gift of G. Jenster (Rotterdam, The Netherlands). For the probasin reporter construct a 454 bp fragment of the rat probasin promoter was amplified from rat DNA using the primer pair PB-426-KpnI (TGGTACCAAAGCTTC CACA AGTGCATTT) and PB-26-HindIII (AAGCTTCTGTAGGTATCTG GACCTCACTG). The KpnI/HindIII promoter fragment in the $(HRE)_2$-TATA reporter plasmid was then replaced by the probasin promoter fragment. The plasmid pFR-Luc containing five tandem repeats of yeast *GAL4* binding sites and a firefly luciferase reporter was purchased from Stratagene (La Jolla, CA, USA). The Renilla luciferase plasmid phRG-TK was purchased from Promega (Madison, WI, USA). Chimeric GAL4-DBD expression constructs were cloned into pCMV-BD (Stratagene). For pBD-AR.NTD the DNA coding for the AR N-terminus (aa 1–537) was amplified from pSVAR0. For the cloning of *RWDD1* constructs fused to the GAL4-DBD full length ORF of *RWDD1* or the respective domains were amplified from pRWDD1. The expression plasmids pBD-RWDD1 (1–119), and pBD-RWDD1 (97–243) express the N-terminal RWD domain (aa 1–119) or the C-terminal 147 residues of RWDD1, respectively. pAR_L713F has been described before (Holterhus et al., 2000). Further details of the plasmid constructs can be obtained upon request.

2.2. Antibodies

Western blot analyses were done with the following antibodies: rabbit anti-RWDD1 (17036-1-AP) and anti-DRG2 (14743-1-AP)

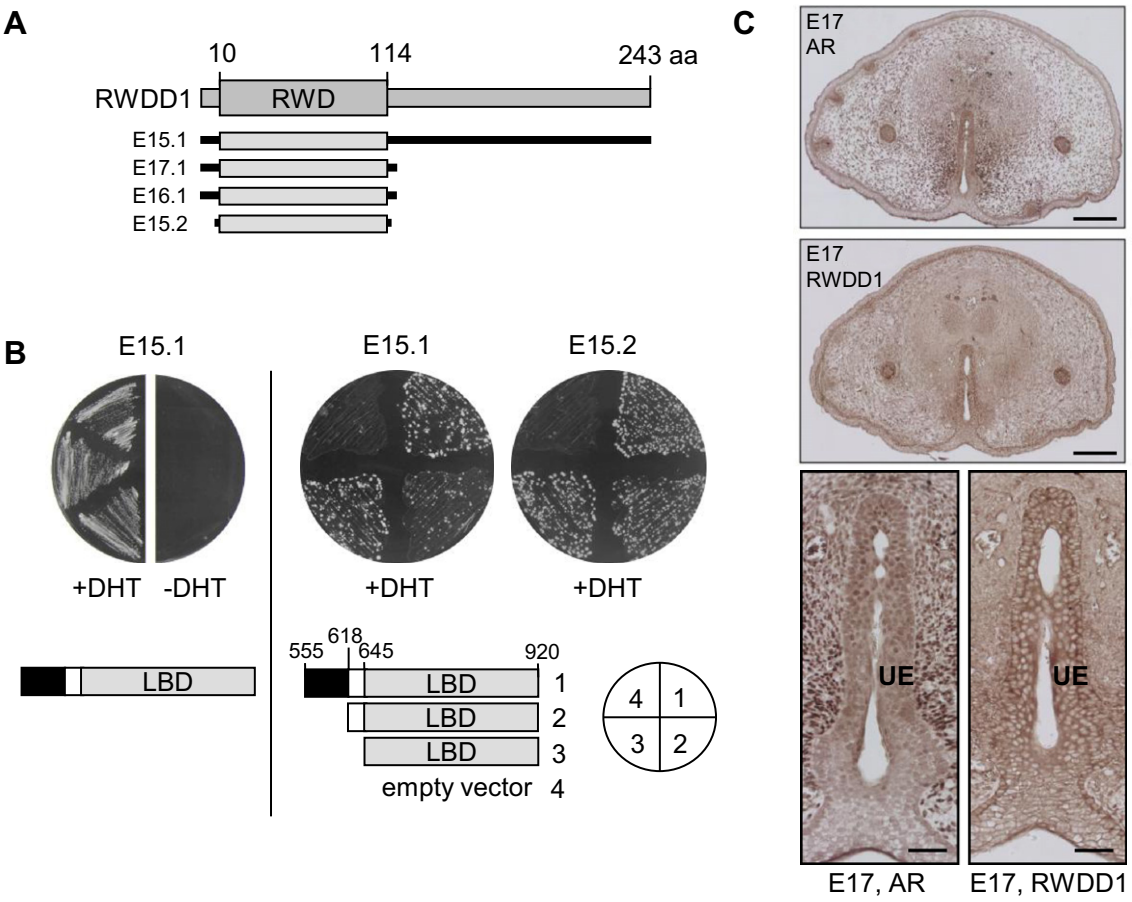

Fig. 1. Rwdd1 interacts with the LBD of the AR in a yeast two-hybrid system and is coexpressed with the AR in the genital tubercle of male mouse embryos. (A) Schematic representation of RWDD1 and of four *Rwdd1* cDNA clones of different length isolated by yeast two-hybrid screenings with a C-terminal construct of human AR as bait in the presence of 100 nM DHT. Three independent screenings with cDNA libraries of genital tubercles extracted from E15, E16 or E17 male mouse embryos were performed. All clones contained the RWD domain of RWDD1. (B) Yeast two-hybrid assay with RWDD1 encoding clones. One of the full-length clones encoding RWDD1 (E15.1) and the smallest clone (E15.2) containing only the RWD domain were retransformed into the yeast strain AH109 together with an AR bait and yeast cells were grown on selective medium with and without 100 nM DHT. Growth of yeast cells occurs only in the presence of hormone (left panel). Retransformation with the bait used for the screening (bait 1), shorter AR baits (bait 2 and 3) or the empty vector (4) as negative control revealed that the AR-LBD is sufficient for interaction (right panel). In the schematic representation the different domains of the AR baits, the DBD (black), the hinge region (white) and the LBD (gray) are indicated. (C) Immunohistochemical stainings of transverse sections of E17 genital tubercle from male mouse embryos. Transverse sections were stained with an AR or RWDD1 antibody. The lower pictures show an enlarged image section of the urethra. Scale bars = 200 μm (upper images) or 50 μm (image sections). UE = urethral epithelium.

from ProteinTech (Chicago, IL, USA), mouse anti-AR (F39.4.1.) from BioGenex (San Ramon, CA, USA), rabbit anti-AR (N-20) from Santa Cruz (Santa Cruz, CA, USA), rabbit anti-GAPDH (ab9485) from Abcam (Cambridge, UK), rabbit anti-SRC1 (SP5308P) from Acris Antibodies (Herford, Germany), horseradish peroxidise-coupled anti-mouse IgG from GE Healthcare (Little Chalfont, UK), rabbit anti-actin (ab9485), mouse anti-FLAG M2 and horseradish peroxidise-coupled anti-rabbit IgG from Sigma (Taufkirchen, Germany).

2.3. Cell culture and transfections

HeLa and Cos1 cells were maintained in Dulbecco's minimal essential medium (DMEM) with the nutrient mix F-12 (Sigma, Taufkirchen, Germany) supplemented with 10% fetal calf serum (PAA Laboratories, Coelbe, Germany) at 37 °C. LNCaP cells were grown in DMEM with 5% fetal calf serum at 37 °C. The PC1 cell line was a gift from M. Orgebin-Crist and is a mouse epididymal cell line immortalized with SV40 large T-antigen (Araki et al., 2002). P17 is a cell line derived from PC1 cells stably expressing N-TAP-mAR in addition to the endogenous wt-mAR (Mooslehner et al., 2012). N-TAP-mAR is a mouse AR construct containing an amino-terminal 3xFLAG-tag and a His-tag separated by a tobacco etch virus cleavage site (N-TAP-tag, amino-terminal tandem affinity purification tag). PC1 and P17 cells were cultured in Iscove modified Dulbecco medium (IMDM), 0.1 mM nonessential amino acids, 1 mM Na-pyruvate and 10% fetal calf serum at 33 °C in the presence of 5 nM mibolerone. The medium of P17 cells contained additionally 750 mM G418 for selection of stable transformants expressing N-TAP-mAR. All media were phenol-red free. Charcoal stripped fetal calf serum was used for experiments with androgens or dexamethasone. All cell lines were maintained in a 5% CO_2/95% air atmosphere.

HeLa and Cos1 cells were transfected with FuGene HD transfection reagent (Roche, Mannheim, Germany).

2.4. Whole cell extracts

For whole cell extracts cells were washed with PBS, lysed in buffer A (40 mM Tris pH 7.4, 1 mM EDTA, 10% Glycerol, 10 mM DTT, 1% Triton, 0.5% sodium deoxycholate, 0.08% SDS, protease inhibitors and phosphatase inhibitors (Complete Protease Inhibitor Cocktail and PhosSTOP, respectively; Roche, Mannheim, Germany) and homogenized using QiaShredder columns (Qiagen, Hilden, Germany).

2.5. Yeast two-hybrid library construction and screening

The library construction and yeast two-hybrid screens were performed using the Matchmaker library construction and screening kit (Clontech, Mountain View, CA, USA). For the preparation of cDNA libraries, genital tubercle tissue was dissected from CD1 mouse embryos of the developmental stages E15, E16 or E17. Sex determination of the mouse embryos was performed by Y chromosome-specific PCR on DNA from tail tissue using the primers Zfy1_s (GACTAGACATGTCTTAACATCTGTCC) and Zfy1_a (CCTATTGCATG GACAGCAGCTTATG) (Wang et al., 2001). 7–9 male genital tubercles were combined and the RNA was extracted using the RNA plus Mini kit (Qiagen, Hilden, Germany). The quality and the quantity of the RNA were controlled using an Agilent RNA 6000 kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). 0.8–1.4 μg of male genital tubercle RNA was used for the first strand synthesis of the cDNA. All subsequent steps of the library construction were done according to the Matchmaker kit instructions. For screening, the yeast strain AH109 containing pGBK_AR555 was cotransformed with the cDNA library and the SmaI-linearized vector pGADT7-Rec and selected on medium supplemented with 100 nM DHT and lacking adenine, histidine, leucine and tryptophan. Colonies were isolated after 3–5 days of incubation at 30 °C. The plasmids were recovered and the inserts of pGADt7-Rec were identified by sequencing. To verify the results, plasmids isolated by the screening were transformed into *Escherichia coli* DH5α, purified and retransformed by the Li-Acetate method (Ito et al., 1983) into yeast strain AH109 together with pGBK_AR555, pGBK_AR618, pGBK_AR645, or pGBKT7 as negative control. Colonies were selected as described before and images were taken after 4 days.

2.6. GST pull-down assays

For the purification of GST–RWDD1 and GST, *E. coli* BL21 (DE3) cells transformed with pGST–RWDD1 or pGEX-4T-1 were grown in LB-medium, 50 mg/l Ampicillin at 37 °C and induced with 0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{600} 0.7–0.8 for 3 h at 25 °C. Bacteria were lysed by sonication in PBS–0.5%Tween, 0.5 mM PMSF, supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) and the extract was cleared by centrifugation for 15 min at 12,000*g*. GST–protein was bound to glutathione-Sepharose (GE-Healthcare, Little Chalfont, UK) for 1 h at 4 °C, recovered on Poly-Prep chromatography columns (Bio-Rad, München, Germany), washed several times with PBS–0.5% Tween, 0.5 mM PMSF, protease inhibitors, and finally taken up in binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 0.5 mM PMSF, protease inhibitors, 1 mg/ml BSA). GST and GST–RWDD1 bound to glutathione-Sepharose were quantified by SDS–PAGE and Coomassie-staining and adjusted to similar concentrations with empty beads in binding buffer.

To test in vitro interactions, full length AR, 3xFLAG-AR.LBD or 3xFLAG-AR.NTD (plasmids pT7-ARpolyA, p3xFLAG-AR.LBD or p3xFLAG-AR.NTD) were transcribed and translated in vitro using the TNT Quick Coupled Transcription/Translation system (Promega, Madison, WI, USA). In vitro translated AR, 3xFLAG-AR.LBD or 3xFLAG-AR.NTD was added to the GST protein-covered beads in binding buffer with or without 100 nm DHT, and incubated for 3 h at 4 °C. The beads were washed in the presence of DHT or vehicle, twice with binding buffer and three times in binding buffer without BSA and finally boiled in SDS sample buffer. The binding reactions were analyzed by SDS–PAGE, Ponceau S staining and Western blotting.

For GST pull-downs with LNCaP total cell extracts or nuclear extracts, cells were incubated with 1 nM R1881 or vehicle for 1 h and trypsinized. All subsequent steps were done in the presence of R1881 or vehicle. For pull-downs with total cell extracts, the cells were washed in PBS and lysed in IP buffer (25 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5 mM PMSF, protease inhibitors), 0.5% NP-40 for 30 min on ice. The extracts were sonicated 10 times for 10 s, cleared by centrifugation at 20,000*g* for 20 min and diluted 1:1 with IP buffer. Finally, the extracts were adjusted to 1 μg protein/μl with IP buffer, 0.25% NP-40. For GST pull-downs with LNCaP nuclear extracts, cells were incubated for 1 h with 1 nM R1881, trypsinized and washed in PBS. Nuclear extracts were prepared according to the Nuclear Complex Co-IP Kit (Active Motif, Carlsbad, CA, USA) manual with minor deviations. Briefly, after lysis of the cells in hypotonic buffer, the nuclei were collected by centrifugation at 15,000*g* for 30 s, washed two times in hypotonic buffer without detergent and digested in complete lysis buffer with micrococcal nuclease for 1 h at 4 °C. After enzymatic shearing, the nuclear extracts were sonicated 10 times for 10 s, cleared by centrifugation at 20,000*g* for 5 min and finally diluted in IP buffer, 0.25% NP-40 supplemented with 1 nM R1881. GST–proteins bound to glutathione-Sepharose were incubated for 2.5 h with the total cell extract or nuclear extract of LNCaP cells. Finally, the beads were washed four times with IP buffer, 0.25% NP-40 and boiled in loading buffer. The binding reactions were analyzed by SDS–PAGE, Ponceau S staining and Western blotting.

2.7. Immunoprecipitations

Immunoprecipitations with crude nuclear extracts of PC1 and P17 cells were done as described in (Mooslehner et al., 2012).

2.8. Immunohistochemistry

Paraffin-embedded 5 μm sections of E17 CD1 mouse embryos fixed in 4% paraformaldehyde/PBS were deparaffinized and rehydrated with EZ-Dewax and Optimax Wash Buffer (BioGenex, San Ramon, CA, USA) following the supplier's instructions. For antigen retrieval, the sections were incubated for 30 min at 97 °C in 10 mM Tris pH 9, 1 mM EDTA, 0.05% Tween 20. After washing with TBS-Triton (20 mM Tris pH 7.6, 140 mM NaCl, 0.025% Triton), the sections were incubated in 10% normal goat serum (Abcam, Cambridge, UK), 1% BSA, TBS for 2 h at room temperature. The first antibody, rabbit anti-RWDD1 (1:200, ProteinTech; 17036-1-AP) or rabbit anti-AR (1:100, Dianova; DLN-12143), was applied in 1% BSA/TBS over night at 4 °C. After two washes with TBS-Triton, the sections were incubated with biotinylated anti-rabbit IgG and antigen detection was performed using the Vectastain ABC-Kit (Vector Laboratories, Burlingame, CA, USA) and Pierce Metal Enhanced DAB substrate (Thermo Scientific, Rockford, USA) following the suppliers instructions. After dehydration with increasing concentrations of ethanol, the samples were mounted in organic mounting medium (Thermo Scientific) and visualized using a Keyence BZ-9000 microscope (Keyence, Osaka, Japan).

Dissection of mouse embryo tissue samples was performed in compliance with the German laws and was approved by the ministry of agriculture and environment of Schleswig–Holstein as project 32/A05/08. All studies of the EuroDSD project were approved by the ethics committee of the University of Lübeck (reference 08-081).

2.9. Reporter assays

For cotransactivation assays, HeLa or Cos1 were seeded at 80,000 cells per well in 24-well plates in medium supplemented with 10% charcoal stripped fetal calf serum and transiently transfected after 24 h. Per well, cells were transfected with 200 ng of the respective luciferase reporter plasmid (*Rhox5*, $(HRE)_2TATA$, or probasin), 30 ng of the expression plasmid of the nuclear receptor (AR, AR_L713F, or GR), 75 ng of the RWDD1 expression plasmid or

the empty vector pcDNA3.1(+), 5 ng of the constitutive Renilla luciferase expression plasmid phRG-TK and 0.65 μl Fugene HD (Promega, Madison, WI, USA). Five hours after transfection, cells were incubated for 18 h with either 10 nM DHT, 1 nM dexamethasone or vehicle, respectively. Firefly and Renilla luciferase activities were detected using the Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA) and a LUCY 3 Luminometer (Anthos, Krefeld, Germany). The activity of the Renilla luciferase was used to normalize for transfection efficiency. All transfections were performed in triplicates and in at least three independent experiments.

To test the transactivation activity of RWDD1, HeLa or Cos1 cells in 24-well plates were transfected accordingly, with 100 ng pBD-RWDD1, pBD-RWDD1 (1–119), pBD-RWDD1 (97–243), pBD-AR.NTD or pBD, together with 200 ng of the reporter plasmid pFR-Luc and 5 ng of phRG-TK per well. All subsequent steps were done like for the cotransactivation experiments.

3. Results

3.1. Identification of RWDD1 as an AR-interacting protein in the male genital tubercle

To search for proteins that interact with the AR in the male genital tubercle, we performed three independent yeast two-hybrid screens with cDNA libraries generated from male genital tubercles of mouse embryos at the developmental stages E15, E16, or E17, respectively. An AR fragment that contained the DBD, the hinge region and the LBD of human AR (amino acids 555–920) was used as bait, and screening was performed in the presence of 100 nM DHT. Human and mouse AR are almost identical within the region of the bait and differ only in eight amino acids in a short 16 amino acid stretch of the hinge–LBD boundary. The functionality of the yeast two-hybrid screens was confirmed for each library by the recovery of already known AR coregulators, e.g. protein inhibitor of activated STAT 1 (PIAS1) and steroid receptor coactivator 1 (SRC1) (Bevan et al., 1999; Nishida and Yasuda, 2002). From all three cDNA libraries nine independent *Rwdd1* clones were isolated. These clones showed clear growth on selective medium only in the presence of DHT. Six of the *Rwdd1* clones identified from the yeast two-hybrid screens contained full-length *Rwdd1*. Three clones resulted from internal priming in an adenosine-rich stretch within the coding region and mainly spanned the N-terminal RWD domain (Fig. 1A). Using the yeast two-hybrid system, one of the full-length clones (E15.1) and the shortest clone containing only the RWD domain (E15.2) were tested for binding to AR baits of different length in the presence of DHT. Both *Rwdd1* clones showed growth not only in combination with the AR bait used in the yeast two-hybrid screen (pGBK_AR555) (Fig. 1B, bait 1), but also with shorter AR baits containing only the hinge region and the LBD (bait 2) or the LBD alone (bait 3). Without AR bait no growth of the yeast cells was observed.

Immunohistochemical staining confirmed the presence of RWDD1 protein in the male genital tubercle of E17 mouse embryos (Fig. 1C). RWDD1 was ubiquitously expressed in the anlage of the external genitalia, but appeared especially enriched in the cytosol of the urethral epithelium. Cells of the urethral epithelium also expressed the AR strongly (Fig. 1C) as it has been previously described by others (Miyagawa et al., 2009).

3.2. RWDD1 interacts with the AR in vitro and in vivo and the LBD is sufficient to mediate this interaction

To verify the interaction between RWDD1 and the AR as indicated by the yeast two-hybrid system, we performed GST pull-down assays with GST-tagged human RWDD1 recombinantly expressed and purified from *E. coli*. GST–RWDD1 efficiently precipitated the AR from total cell extract of LNCaP cells in the presence or absence of androgen (Fig. 2A). In control experiments with GST alone no precipitation of the AR could be detected, demonstrating specificity of the observed RWDD1–AR interaction. In parallel experiments we tested the interaction of RWDD1 with nuclear AR of LNCaP cells stimulated with 1 nM R1881. Subsequently, the pull-down with GST–RWDD1 showed specific interaction also with AR from the nuclear fraction (Fig. 2A). To test for a direct physical interaction between RWDD1 and the AR, next, we performed GST pull-down assays with in vitro translated AR. In vitro translated full length AR was specifically precipitated by GST–RWDD1 (Fig. 2B), and no difference in binding could be observed in experiments performed in parallel in the presence or absence of 100 nM DHT. We also examined whether the LBD of the AR is sufficient for the interaction with RWDD1, as already suggested by the results of the yeast two-hybrid system. GST–RWDD1 was tested for binding of an in vitro translated 3xFLAG-tagged construct of the AR-LBD. In the absence of hormone, the 3xFLAG-tagged AR-LBD was efficiently pulled down by GST–RWDD1 (Fig. 2C), while a 3xFLAG-tagged construct of the AR-NTD was not showing any interaction with GST–RWDD1. These results indicated that RWDD1 directly interacts with the LBD of the AR.

Next, we analyzed the association of RWDD1 and mouse AR (mAR) in vivo. The mouse epididymal cell line P17 stably expresses N-TAP-mAR in addition to the wild type mAR (Mooslehner et al., 2012). N-TAP-mAR consists of mouse AR and an N-terminal 3xFLAG-tag and His-tag separated by a tobacco etch virus (TEV) protease cleavage site. For immunoprecipitation experiments we used the N-TAP-mAR expressing cell line and the original PC1 cell line expressing only wild-type AR as negative control.

In the presence of 5 nM mibolerone, N-TAP-mAR was precipitated from crude nuclear extracts with anti-FLAG magnetic beads. The N-TAP-mAR and associated proteins were released from the beads by TEV protease cleavage. Western blot analysis of the released protein complex demonstrated the association of RWDD1 and N-TAP-mAR in P17 cells (Fig. 2D). As expected, N-TAP-mAR was also precipitating the wild type form of the mAR. This indicated that the N-terminal tag was not hindering mAR dimer formation. In control experiments with the PC1 cell line, no RWDD1 was released from anti-FLAG magnetic beads, demonstrating that RWDD1 in the P17 cell line was precipitated by the N-TAP-mAR.

3.3. RWDD1 enhances AR-dependent transactivation

To demonstrate the functional significance of the observed interaction between the AR and RWDD1, we transiently cotransfected HeLa cells and Cos1 cells with a luciferase reporter plasmid bearing the mouse *Rhox5* proximal promoter with two androgen response elements, an AR expression plasmid and an expression plasmid of human RWDD1 and analyzed the luciferase activity. In both cell types, overexpression of RWDD1 caused a clear 3–4-fold enhancement of AR-dependent transactivation in the presence of 10 nM DHT (Fig. 3A). In contrast, no effect on reporter gene expression could be observed in the absence of AR ligand. Western blot analysis of the HeLa cell extracts showed that RWDD1 overexpression led to a twofold upregulation of AR protein but had only a marginal effect on DRG2, the known binding partner of RWDD1. No effect on other proteins such as the AR coregulator SRC1, actin and GAPDH was observed. In Cos1 cells, AR protein expression was unaffected by the transient overexpression of Rwdd1, indicating that Rwdd1 does not enhance AR dependent transactivation just by augmenting AR protein expression.

To assess whether RWDD1 has a general effect on AR-dependent transcription, we analyzed the effect of RWDD1

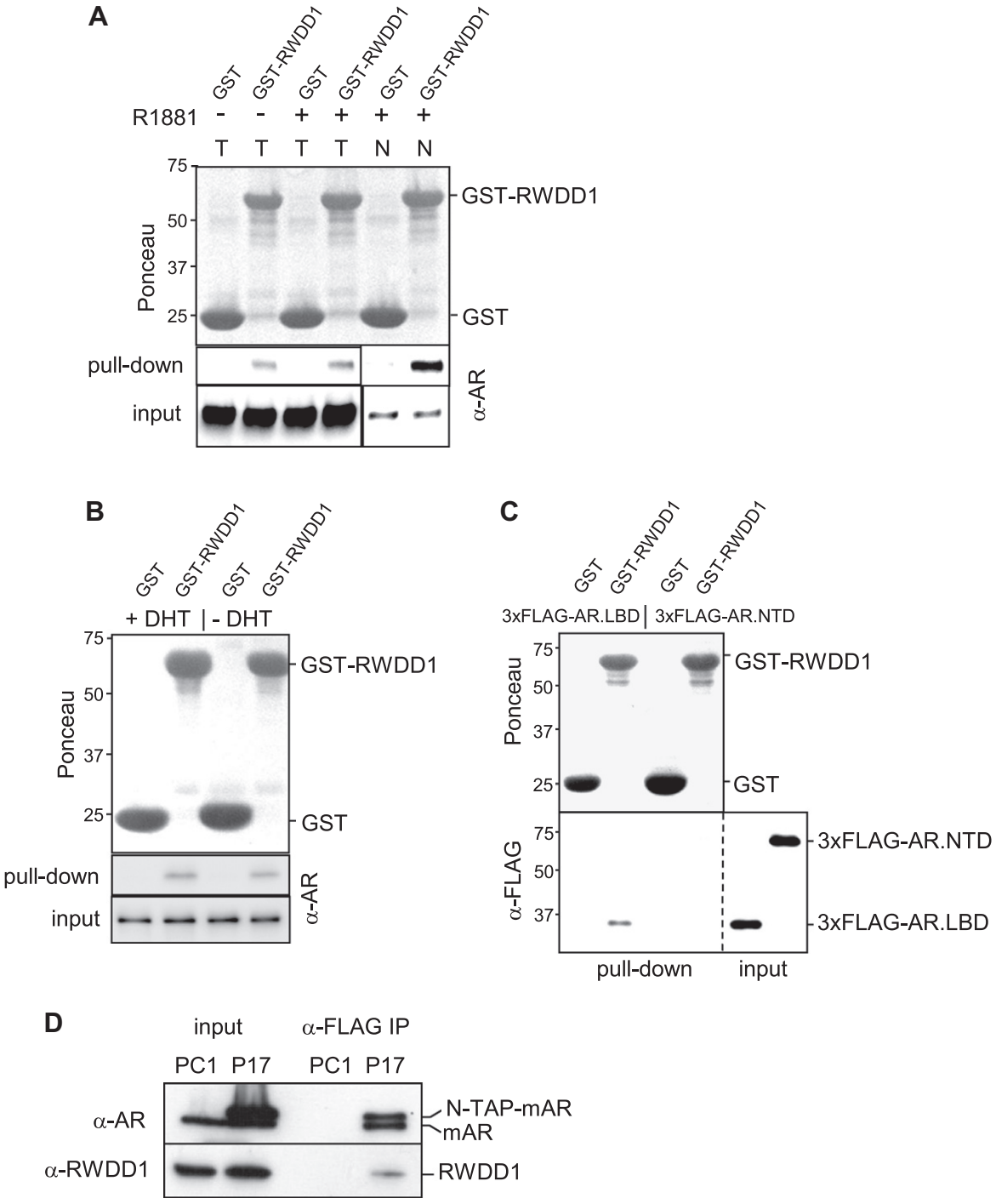

Fig. 2. RWDD1 interacts with the AR in vitro and in vivo. (A) RWDD1 forms a complex with the AR of total cell extracts or nuclear extracts of LNCaP cells. Total cell extracts (T) or nuclear extracts (N) of LNCaP cells, which were cultured for 1 h with 1 nM R1881 (+) or vehicle (−), were incubated with GST or GST–RWDD1 bound to glutathione-Sepharose beads. Precipitated proteins were analyzed by Western-blot using an anti-AR antibody. Thirty percent of the pull-down and 0.2% of the input is shown. The Ponceau S staining shows the GST and GST–RWDD1 protein in the pull-down. (B) Direct binding of RWDD1 to the AR in vitro. Full-length AR was in vitro translated and precipitated with GST or GST–RWDD1 bound to glutathione-Sepharose beads in the presence or absence of 100 nM DHT. Precipitated proteins were analyzed as in (A). 30% of the pull-down and 0.1% of the input is shown. (C) The LBD of the AR is sufficient for RWDD1 binding. A 3xFLAG-tagged construct of the AR-LBD and a 3xFLAG-tagged construct of the AR-NTD were in vitro translated and precipitated with GST or GST–RWDD1 bound to glutathione-Sepharose beads. Precipitated proteins were analyzed by Western blot using an anti-FLAG antibody. Thirty percent of the pull-down and 0.1% of the input is shown. (D) RWDD1 interacts with N-TAP-mAR in a mouse epididymal cell line. N-terminal FLAG-tagged mouse AR (N-TAP-mAR) was precipitated from crude nuclear extracts of P17 cells in the presence of 5 nM mibolerone with anti-FLAG antibody bound to magnetic beads. The precipitated proteins were analyzed using anti-AR and anti-RWDD1 antibodies. As a negative control extracts from PC1 cells expressing only wild type mAR were used in parallel. 4% of the immunoprecipitated protein (a-FLAG IP) and 0.03% of the input is shown.

overexpression on a minimal promoter with two hormone response elements ($(HRE)_2$-TATA) and on a rat probasin promoter by corresponding transient reporter assays in Cos1 and HeLa cells. In all assays, RWDD1 clearly enhanced AR transcriptional activation more than 3.5-fold in a DHT-dependent manner, but had no significant effect in the absence of DHT (Fig. 3B).

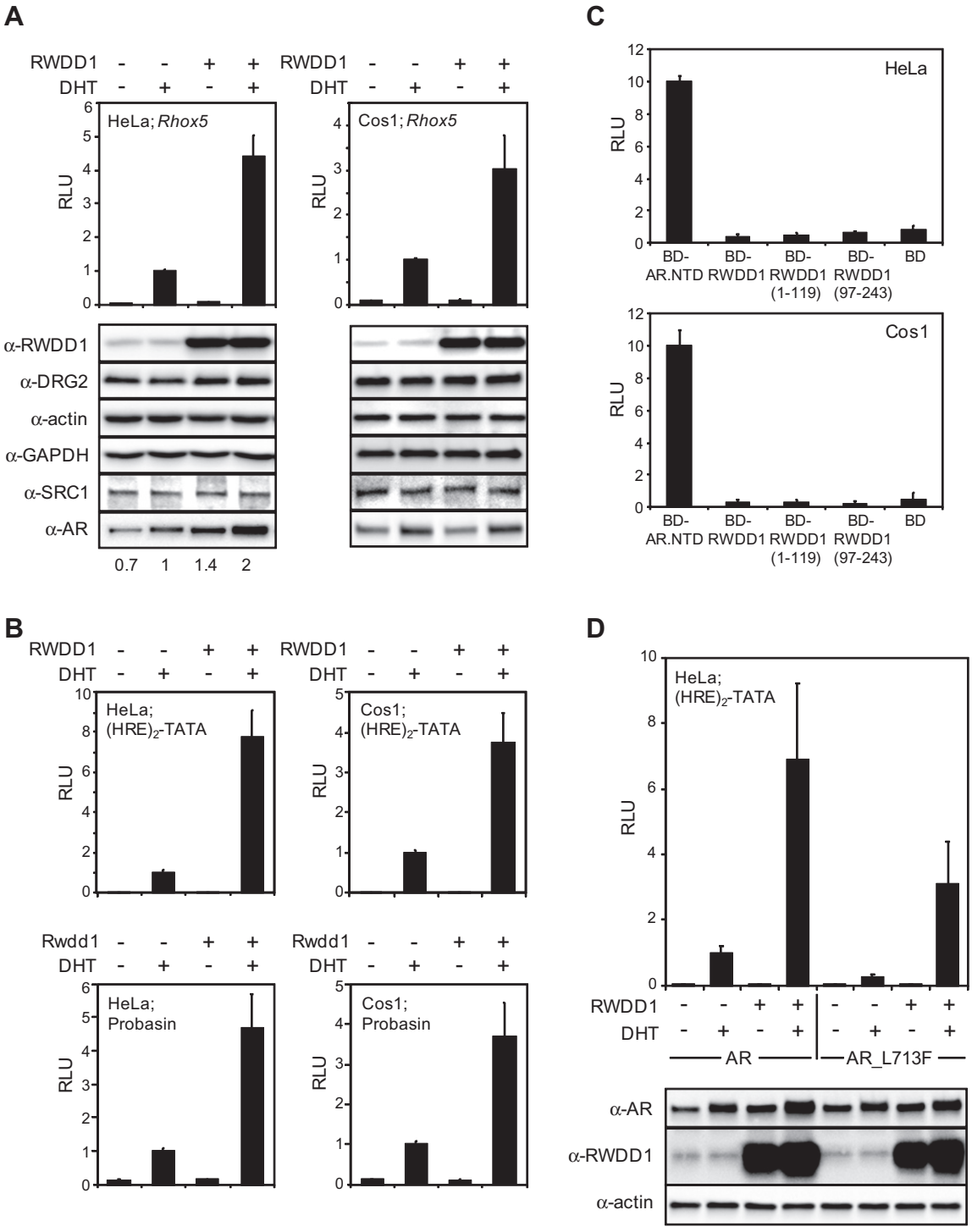

Fig. 3. RWDD1 enhances AR-dependent transactivation. (A) HeLa or Cos1 cells were transfected with an expression plasmid of human AR, a firefly luciferase reporter with the mouse *Rhox5* proximal promoter, and with RWDD1 expression plasmid or the empty vector. A plasmid expressing Renilla luciferase was cotransfected for normalization of transfection efficiency. Transfected cells were cultured for 18 h in the presence (+) or absence (−) of 10 nM DHT. The luciferase activity of cell lysates was measured and relative light units (RLU) were calculated as the ratio of firefly luciferase to Renilla luciferase. The value of cells cultured in the presence of DHT without RWDD1 overexpression was defined as 1. Error bars present the standard deviation. Protein extracts of cotransfected cells were also analyzed by Western blot with the indicated antibodies (lower panel). Numbers indicate the relative expression level of AR protein normalized to actin, AR protein from cells cultured in the presence of DHT without RWDD1 overexpression was defined as 1. Immunoblotted protein was quantified with a CCD camera. (B) HeLa or Cos1 cells were transfected with the AR expression plasmid, a firefly luciferase reporter with two hormone response elements ($(HRE)_2$-TATA) or a rat probasin promoter, the RWDD1 expression plasmid or the empty vector, and a plasmid expressing Renilla luciferase as in (A). Error bars present the standard deviation. (C) HeLa and Cos1 cells were cotransfected with a firefly luciferase reporter plasmid bearing five Gal4 binding sites and an expression plasmid of the Gal4-DBD alone (BD), the Gal4-DBD fused to either RWDD1 (BD-RWDD1), RWDD1 fragments BD-RWDD1 (1–119), BD-RWDD1 (97–243) or the NTD of the AR (BD-AR.NTD) as positive control. A plasmid expressing Renilla luciferase was cotransfected for normalization of transfection efficiency. Twenty-three hours after transfection the luciferase activity of cell lysates was measured and relative light units (RLU) were calculated as the ratio of firefly luciferase to Renilla luciferase. The value of cells expressing BD-AR.NTD was defined as 10. Error bars present the standard deviation. (D) HeLa cells were transfected with an expression plasmid of wild type AR or the L713F AR mutant, a firefly luciferase reporter with two hormone response elements ($(HRE)_2$-TATA), the RWDD1 expression plasmid or the empty vector and a Renilla luciferase plasmid and analyzed as in (A).

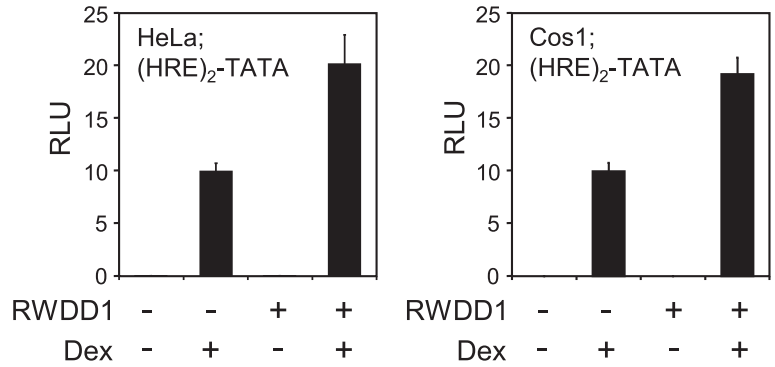

Fig. 4. RWDD1 enhances GR-dependent transactivation. HeLa or Cos1 cells were transfected with an expression plasmid of human GR, a firefly luciferase reporter with two hormone response elements ($(HRE)_2$-TATA), and with RWDD1 expression plasmid or the empty vector. A plasmid expressing Renilla luciferase was cotransfected for normalization of transfection efficiency. Transfected cells were cultured for 18 h in the presence (+) or absence (−) of 1 nM dexamethasone. The luciferase activity of cell lysates was measured using a dual luciferase assay and relative light units (RLU) were calculated as the ratio of firefly luciferase to Renilla luciferase. The value of cells cultured in the presence of dexamethasone and without RWDD1 overexpression was defined as 10. Error bars present the standard deviation.

3.4. RWDD1 has no intrinsic activation function

We examined whether RWDD1 possess an intrinsic activation function that may contribute to the enhanced AR-dependent transactivation in the reporter assays. For this analysis, we fused full-length RWDD1 to the Gal4-DBD and compared its transactivation capacity with the Gal4-DBD alone on a Gal4 responsive promoter. As positive control the AR-NTD, which is known to bear a strong androgen-independent activation function, was fused to the Gal4-DBD and analyzed in parallel experiments. While the AR-NTD showed the expected activation of the reporter gene in HeLa and Cos1 cells, in both cell lines neither the full-length RWDD1 fusion protein nor two fusion proteins expressing the N-terminal RWD domain or the C-terminal 147 amino acids of RWDD1 induced a significant reporter gene expression (Fig. 3C). The results clearly demonstrated that RWDD1 has no intrinsic activation function and indicated that the observed effects of RWDD1 overexpression in the AR transactivation assays were mediated via an AR activation function. These observations were in agreement with an AR coactivator function of RWDD1.

3.5. RWDD1 enhances AR-dependent transactivation independent of a functional AF2

Many AR coregulators mediate their function via the AF2 in the LBD of the AR. Since our previous experiments indicated that RWDD1 interacts with the AR-LBD, we addressed the question of whether AR coactivation by RWDD1 depends on a functional AF2. We compared the effect of RWDD1 on AR-dependent transcription in cotransactivation experiments with wild type AR and an AR mutant containing a lysine-to-phenylalanine amino acid exchange at position 713 (L713F) in the LBD. Leucine 713 is located at the base of the coactivator binding groove and directly participates in binding of FxxLF motifs of coactivators (Hur et al., 2004). AR L713F is a natural mutation associated with partial androgen insensitivity syndrome (PAIS) (Holterhus et al., 2000). The mutation disrupts the N/C-terminal interaction in the AR, which is mediated by binding of the N-terminal ^{23}FQNLF^{27}-motif to the AF2, indicating that the AF2 is severely affected (He et al., 2006). In transactivation experiments, the AR L713F mutation led to a reduced transcription of the reporter gene in comparison to wild type AR (Werner et al., 2006), but overexpression of RWDD1 strongly enhanced the transactivation capacity of the mutant (Fig. 3D). These results suggest that RWDD1 exerts AR coactivator function by a mechanism independent of the AF2.

3.6. RWDD1 enhances GR-dependent transactivation

To test whether RWDD1 has a coactivator function specific to the AR or whether it can also promote the activity of other nuclear receptors, we performed reporter assays for GR-dependent transactivation. HeLa and Cos1 cells were cotransfected with human GR and RWDD1 expression plasmids and transcription of a luciferase reporter gene driven by a minimal promoter with two hormone response elements was analyzed in the presence or absence of dexamethasone. Overexpression of RWDD1 clearly activated GR-dependent transactivation (Fig. 4). These data suggest that RWDD1 exhibits a coactivator function not only for AR-mediated transcription, but also for transcription via the GR.

4. Discussion

In male and female embryos, a cascade of developmental genes induces the initial outgrowth of the anlage of the external genitalia, the genital tubercle. Several genes involved in this unisexual process have been identified (Yamada et al., 2006). Upon testicular production of androgens in the male embryo, the sex dimorphic development of the genital tubercle is initiated. The early androgen-dependent development of the genital tubercle shows striking morphological similarity in mice and humans, despite differences at later stages, like the formation of a penile bone in mice (Yamada et al., 2003). The mouse has been extensively used as a model system to analyse male genital development and malformations like hypospadias (Li et al., 2006; Miyagawa et al., 2009, 2011). However, so far very little information exists about the molecular processes that occur in the male genital tubercle tissue during androgen-dependent outgrowth and masculinisation. To get new insights into these processes, we aimed to identify AR coregulators that function in the genital tubercle of male mouse embryos during the first androgen-dependent changes in embryonic development. RWDD1 was identified as an AR binding partner in three independent yeast two-hybrid screens, using cDNA-libraries of male genital tubercles of the developmental stages E15, E16 and E17. By reporter assays in different cell lines with AR-dependent promoters of different complexity we showed that RWDD1 functions as a coactivator of the AR. Previously, first indications that RWDD1 is involved in AR-dependent transactivation have been described by Kang et al. (2008b). They showed a small effect of RWDD1 on AR-dependent transactivation in reporter assays with the thymic epithelial cell line 427.1. A reason for the limited effect of Rwdd1 on AR function in 427.1 cells, compared to our results in Cos1 and HeLa cells, might be a high expression level of endogenous

RWDD1 in the 427.1 cell line. Limited overexpression of RWDD1, which could also be due to the method of transfection, might have led to a limited enhancement of reporter expression in the 427.1 cell line. In addition, the V5-tagged form of RWDD1 used in the transactivation assay by Kang et al. could have had reduced coactivation activity in comparison to wild type RWDD1 used in our experiments. By fluorescence resonance energy transfer (FRET) Kang et al. were unable to detect any interaction between RWDD1 and the AR (Kang et al., 2008b). The C-terminal GFP-tag of RWDD1 might have interfered with the interaction in this assay. By immunoprecipitations and GST pull-down assays we could confirm our results from the yeast two-hybrid system and detect an interaction between RWDD1 and the AR. Our in vitro binding assays also show that the LBD of the AR is sufficient for the interaction with RWDD1. The reporter assays with the L713F AR mutant suggest that RWDD1 binds the LBD outside of the AF2. This is in agreement with the fact that RWDD1 possess no LxxLL or FxxLF motif by which other coregulators typically interact with the AF2 (He and Wilson, 2003). The results of the yeast two-hybrid system indicate that the RWD domain of RWDD1 alone can mediate the binding to the AR LBD. Consistently, RWD domains are supposed to function in protein interaction (Doerks et al., 2002). However, in GST pull-downs we could not detect an interaction between the RWD domain alone and the AR (data not shown), indicating that in vivo also the C-terminus of RWDD1 may participate in efficient AR binding.

So far, RWDD1 has been mainly studied as a binding partner of developmentally regulated GTP-binding protein 2 (DRG2). A central region of RWDD1 adjacent to the N-terminal RWD domain has been shown to be important for the interaction with DRG2 and has been termed DFRP domain, since in this context RWDD1 was called DRG family regulatory protein 2 (DFRP2) (Ishikawa et al., 2005). Although RWDD1 and DRG2 seem to form a stable complex that is conserved from yeast to humans, the function of this complex remains poorly understood. In *Saccharomyces cerevisiae*, genetic interactions indicate that the yeast homologs of RWDD1 and DRG2, Gir2 and Rbg2, might fulfil a redundant function with Slh1, a putative RNA helicase involved in translation (Daugeron et al., 2011). However, a rather indirect participation of the RWDD1–DRG2 complex in translation, e.g. by regulating the post-translational modification and activity of important translational factors, would still explain the genetic interaction. Further investigations are necessary to decide whether RWDD1 functions as an AR coactivator independent of DRG2. The expression of DRG2 was mainly unaffected in the reporter assays. Therefore, an AR coactivator function of RWDD1 independent of its function in the RWDD1–DRG2 complex appears more likely. Experimental data and bioinformatic analysis suggest that RWDD1 is an intrinsically unstructured protein (Kang et al., 2008a). Unstructured proteins are supposed to be more flexible in mediating protein interactions and can typically interact with different proteins and more binding partners than would be predicted by their size (Tantos et al., 2012). Therefore an association of RWDD1 to discrete protein complexes and the participation in different molecular pathways would not be surprising.

Our results show that RWDD1 enhances not only AR-dependent but also GR-dependent transactivation. Since the LBDs of different steroid hormone receptors are highly homologous, most coregulators that bind the LBD interact with different members of the steroid receptor family. It remains to be answered by which molecular mechanism RWDD1 affects nuclear receptor transactivation. The growing knowledge about RWD domains might help to get an idea concerning this question. The structure of the RWD domain of the mouse GCN2 protein has been resolved and shown to have homology to E2 ubiquitin ligases and the SUMO conjugase UBC9 (Nameki et al., 2004). Although this most likely reflects a common ancestor, RWD domains have no enzymatic activity and most of the residues on the surface of the structure vary from the homologous ubiquitin and SUMO ligases. Since there is also a lot of sequence variation between the RWD domains of different proteins, their binding partners might be very different. However, during the last years RWD domains of mammalian proteins have been implicated in ubiquitin- and SUMO-dependent processes. The small protein RWD-containing sumoylation enhancer (RSUME) stimulates protein sumoylation via UBC9 and the RWD domain has been shown to be essential for this function (Carbia-Nagashima et al., 2007). FANCL, the catalytic subunit of the Fanconi Anemia core complex stimulates monoubiquitination of the substrate FANCD2 and bears a double-RWD (DRWD) domain which seems to be responsible for substrate binding (Alpi et al., 2008; Cole et al., 2010). Due to the described molecular functions of RWD domain-containing proteins, it appears tempting to speculate that RWDD1 functions as AR coactivator by participating in an ubiquitin- or SUMO-mediated process. Ubiquitination and sumoylation processes occur not only in the nucleus but also in the cytoplasm where RWDD1 is localized (Geiss-Friedlander and Melchior, 2007; Gioeli and Paschal, 2012). Although the steady-state localization of androgen-activated AR is mainly nuclear, the receptor shuttles between the compartments and can also be modified in the cytosol (Kesler et al., 2007). RWDD1 might affect post-transcriptional modifications of the AR that alter protein stability, as RWDD1 overexpression led to an upregulation of AR protein expression in HeLa cells (Fig. 3A). However, in Cos1 cells RWDD1 activated androgen-dependent transactivation without an effect on AR protein expression. The different effects of RWDD1 overexpression on AR protein observed in Cos1 and HeLa cells might reflect cell-line specific expression levels of proteins, which function together with RWDD1 to alter AR protein expression. The lower expression of a molecular partner could limit the effect of Rwdd1 overexpression in Cos1 cells.

However, the results in Cos1 cells indicate that RWDD1 activates AR function not only by enhancing AR protein expression. The data suggest that RWDD1 participates in a pathway that affects AR protein expression but leads also to another molecular effect on AR-dependent transcription. This supports the hypothesis that RWDD1 participates in posttranslational modifications. Protein modifications like sumoylation, ubiquitination and phosphorylation have been shown to influence simultaneously protein stability and protein interactions (Geiss-Friedlander and Melchior, 2007; Reddy et al., 2006; Ward and Weigel, 2009).

In summary, our data describe a new role for RWDD1 as AR coactivator. We show a clear effect of RWDD1 on androgen-dependent transactivation in cellular assays with different cells and promoters. Our yeast two hybrid results, immunoprecipitations and in vitro protein binding studies strongly suggest that RWDD1 interacts directly with the AR and indicate that the LBD of the AR is sufficient for the interaction. Further studies are required to analyze the molecular mechanism of RWDD1 in androgen-dependent transactivation and its role in genital development.

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