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Dihydrotestosterone activates AP-1 in LNCaP prostate cancer cells

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ABSTRACT

A cross-talk between androgen/androgen receptor signaling and the AP-1 transcription factor has been proposed. In this study, we asked whether activation of AP-1 modifies androgen-responsive gene transcription, and whether androgens effect AP-1-regulated gene transcription. We show that activation of AP-1 via expression of a constitutively active mutant of mitogen-activated/extracellular signal responsive kinase kinase (MEK) kinase-1 did not increase the activity of the androgen-responsive probasin promoter. Likewise, expression of a constitutively active mutant of the transcription factor c-Jun, which is a major constitutent of AP-1, did not increase the activity of the probasin promoter. In contrast, 5α-dihydrotestosterone (DHT) activated both the probasin promoter and the AP-1-regulated collagenase promoter in LNCaP prostate cancer cells. The AP-1 binding site within the collagenase promoter was identified as DHT-responsive element. In line with this, DHT increased the activities of the c-Jun promoter and the tumor necrosis factor alpha promoter, which both contain AP-1 binding sites. The signal transduction pathway coupling DHT stimulation with AP-1 activation required c-Jun, MAP kinases and androgen receptors, but was independent of transient receptor potential melastatin-8 (TRPM8) channels, proposed to function as ionotropic testosterone receptors. Expression of the GTPase activating protein RGS2 attenuated DHT-induced activation of AP-1, indicating that the DHT-induced signaling cascade involves G proteins.

1. Introduction

The androgens testosterone and 5α-dihydrotestosterone (DHT) regulate gene transcription via binding to the androgen receptor, a steroid hormone receptor that translocates into the nucleus following binding of the ligand. The activated androgen receptor binds in the nucleus to the androgen response element of androgen-regulated genes, encompassing the consensus sequence 5´-AGAACANNNTGTTCT-3´, and therefore enhances transcription of these genes. Androgen response elements were found in the proximal promoter regions of androgen-responsive genes, but also within distal enhancers, many kilobases upstream of the proximal promoter. Using the prostate cancer cell line LNCaP as model system, gene expression studies identified several hundred androgen-responsive genes (Jin et al., 2013). These genes are either directly controlled by androgens via binding of the androgen receptor to androgen response elements within the regulatory regions of these genes, or they are indirectly regulated by androgens via the stimulation of androgen-induced transcription factors distinct from the androgen receptor. Androgens are important in controlling growth and proliferation of the prostate gland and are particularly important for the progression of androgen-sensitive prostate cancer cells.

The transcription factor AP-1 (activator protein-1) is composed of basic region leucine zipper (bZIP) transcription factors of the Jun, Fos and ATF proteins. The activation of AP-1 is mediated by many signaling molecules such as neurotransmitterns, hormones, and growth factors. We have shown that stimulation of G protein-coupled receptors (Rössler et al., 2008; Thiel and Rössler, 2011; Thiel et al., 2012), transient receptor potential channels (Lesch et al., 2015; Thiel and Rössler, 2017; Backes et al., 2018), or L-type voltage gated Ca^{2+} channels (Langfermann et al., 2018) activates AP-1. In addition, AP-1 is activated in response to nutrients or plant-derived compounds such as resveratrol or hyperforin (Müller et al., 2012; Thiel and Rössler, 2014, 2017). The signaling molecules induce in the cell the activation of stimulus-responsive protein kinases, in particular mitogen-actived protein kinases ERK1/2, p38, and JNK that function as signal transducers (Whitmarsh and Davis, 1996). These kinases phosphorylate and activate AP-1-constituting transcription factors like c-Jun or c-Fos. AP-1 has been described to regulate cellular growth, and proliferation in a tissue-specific fashion. c-Jun-deficient mouse embryos die at mid-gestation (Johnson et al., 1993), suggesting that c-Jun is required for normal development. c-Jun is required for the proliferation of fibroblasts, as fibroblasts derived from c-Jun-deficient embryos show greatly reduced growth rates

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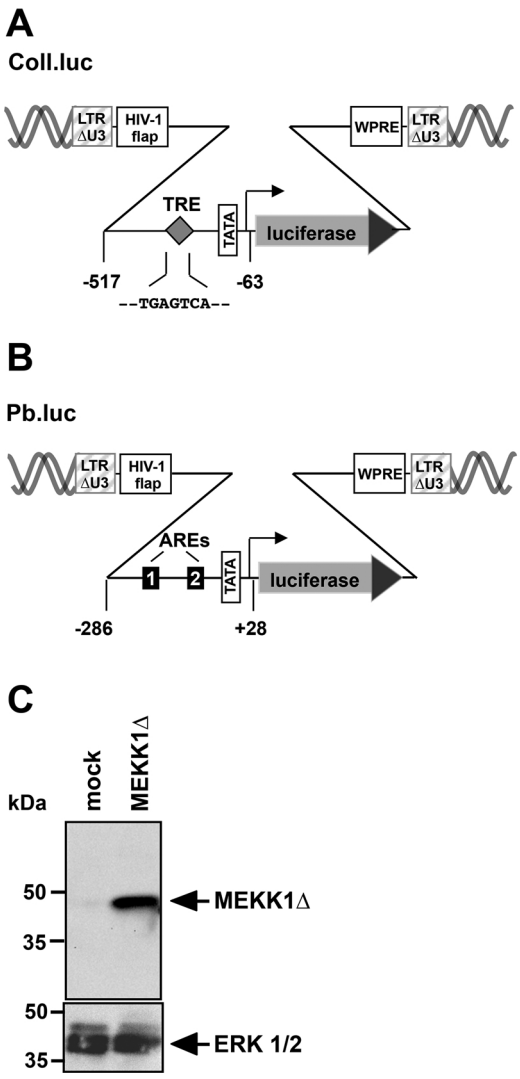

Fig. 1. Expression of a constitutively activate mutant of the protein kinase MEKK1 triggers the activation of AP-1, but has no effect on probasin promoter activity. (A) Provirus encoding a collagenase promoter/luciferase reporter gene (Coll.luc). TRE, TPA-responsive element. (B) Provirus encoding a probasin promoter/luciferase reporter gene (Pb.luc). ARE-1, ARE-2, androgen responsive elements. (C) Expression of MEKK1Δ in LNCaP cells. LNCaP cells were infected with a recombinant lentivirus encoding FLAG-tagged MEKK1Δ. As a control virus, we used in this study a lentivirus generated with the plasmid pFUW (mock). The Western blot was incubated with an antibody against the FLAG-tag. kDa, molecular-mass markers. As a loading control, we used an antibody that detected ERK1/2. (D, E) Cells contained either an integrated collagenase promoter/luciferase reporter gene (Coll.luc) (D) or a probasin promoter/luciferase reporter gene (Pb.luc) (E) and expressed MEKK1Δ as indicated. Following infection, cells were serum-starved for 24 h. We normalized luciferase activity to the protein concentration. Data shown are mean ± SD of four independent experiments performed in quadruplicate (∗∗∗, $P < 0.001$; n.s. not significant).

(Johnson et al., 1993). Primary c-Jun-deficient keratinocytes also have been shown to proliferate poorly (Zenz et al., 2003). Constitutively activated AP-1 is found in tumor cells derived from patients with classical Hodgkin´s disease and AP-1 supports proliferation of these lymphoma cells (Mathas et al., 2002). c-Jun is required for hepatocyte proliferation and liver regeneration (Behrens et al., 2002). AP-1 has also been described to regulate initiation, progression and recurrence of prostate cancer (Edwards et al., 2004; Ouyang et al., 2008).

Several investigators have described a cross-talk between androgen receptor and AP-1. c-Jun, an important AP-1 constituent, has been proposed to reduce the activity of androgen-responsive promoters (Sato et al., 1997; Hsu and Hu, 2013). In contrast, it has also been suggested that c-Jun increases androgen receptor-mediated transactivation (Bubulya et al., 1996), thus leading to enhanced transcription of androgen-regulated genes. In this study, we have analyzed the signaling pathways leading to androgen receptor or AP-1-regulated transcription. While signaling molecules that strongly increased AP-1 activity in prostate cancer cells failed to stimulate an androgen-responsive promoter, we observed that the androgen DHT activated both androgen-responsive and AP-1-responsive transcription units. The signal transduction pathway of DHT required MAP kinases as signal transducers, c-Jun as an activator of AP-1-regulated transcription and the androgen receptor. Expression experiments suggest that DHT signaling additionally includes the activation of G proteins to stimulate AP-1 in LNCaP prostate cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

LNCaP and 22Rv-1 prostate cancer cells were a kind gift of Mathias Montenarh and Claudia Götz from our Department. The cells were authenticated through RT-PCR analysis of TRPM8 mRNA, which serves as biomarker for prostate cancer cells (Fuessel et al., 2003). LNCaP cells were cultured in RPMI medium containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. HEK293 cells and HEK293-M8 cells were cultured as described (Bödding et al., 2007; Thiel et al., 2012; Thiel and Rössler, 2014). Stimulation with DHT (10 nM or 100 nM, Sigma-Aldrich # A8380), 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 10 ng/ml, Calbiochem # 524400-1), or icilin (10 µM, Santa Cruz # sc-201557) was performed for 24 h in RPMI medium containing 0.05% FBS.

2.2. Lentiviral gene transfer

The lentiviral transfer vectors pFUW-MKP-1, pFUWc-JunΔN, pFUW-C2/c-Jun, and pFUW-FLAG-MEKK1Δ have been described elsewhere (Bauer et al., 2007; Mayer et al., 2008; Spohn et al., 2010; Thiel and Rössler, 2014). An expression vector encoding HA-tagged androgen receptor was a kind gift of Scott M. Dehm from the University of Minnesota, USA (Chan et al., 2012). The coding region was excised with

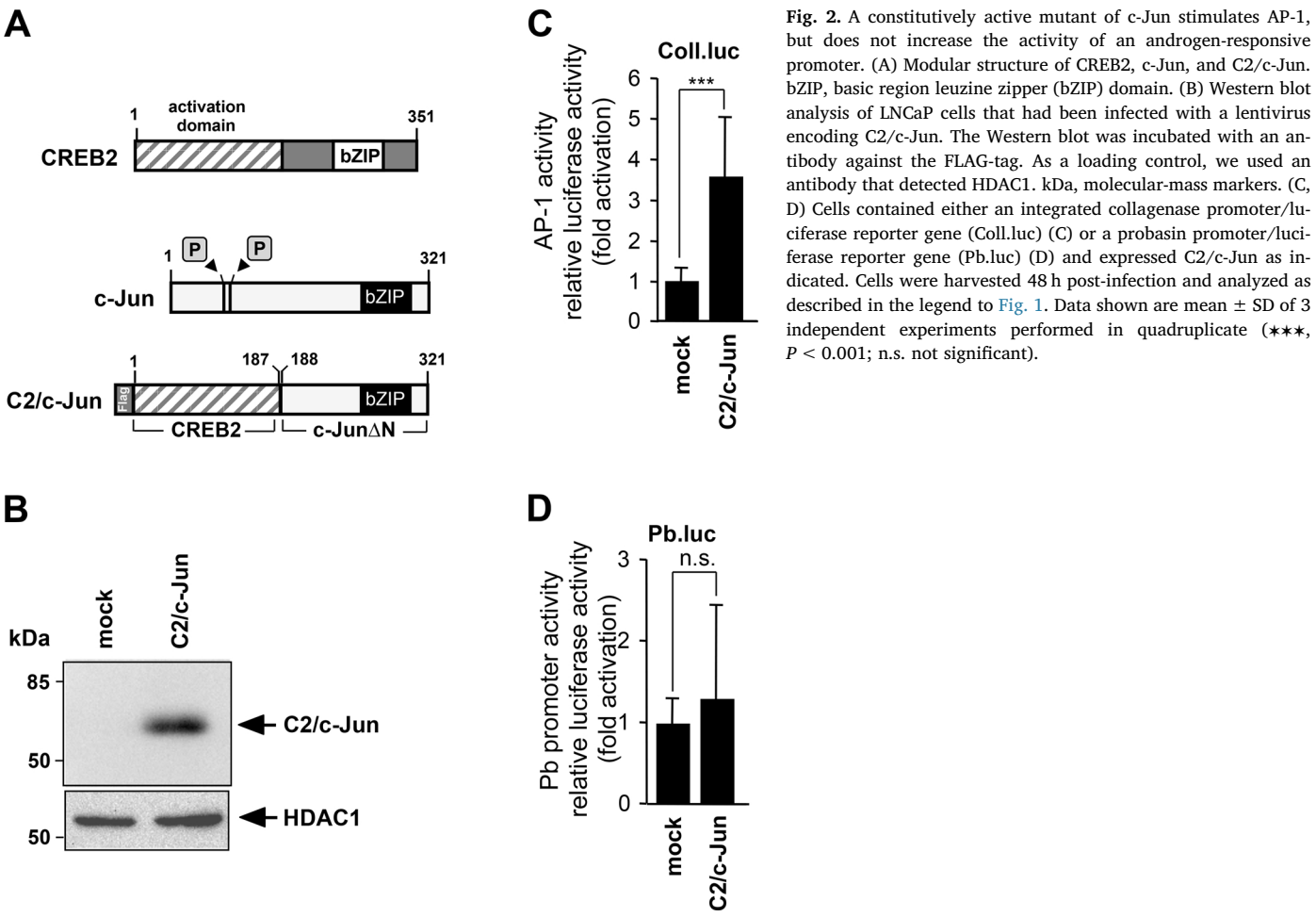

Fig. 2. A constitutively active mutant of c-Jun stimulates AP-1, but does not increase the activity of an androgen-responsive promoter. (A) Modular structure of CREB2, c-Jun, and C2/c-Jun. bZIP, basic region leuzine zipper (bZIP) domain. (B) Western blot analysis of LNCaP cells that had been infected with a lentivirus encoding C2/c-Jun. The Western blot was incubated with an antibody against the FLAG-tag. As a loading control, we used an antibody that detected HDAC1. kDa, molecular-mass markers. (C, D) Cells contained either an integrated collagenase promoter/luciferase reporter gene (Coll.luc) (C) or a probasin promoter/luciferase reporter gene (Pb.luc) (D) and expressed C2/c-Jun as indicated. Cells were harvested 48 h post-infection and analyzed as described in the legend to Fig. 1. Data shown are mean ± SD of 3 independent experiments performed in quadruplicate (∗∗∗, $P < 0.001$; n.s. not significant).

BamHI and cloned in the BamHI site of pFUW (Lois et al., 2002). The plasmid pcDNA3.1 + -HA-RGS2 was purchased from the UMR cDNA Resource Center, University of Missouri, USA. The plasmid was cut with PmeI. The fragment was cloned into the HpaI site of pFUW. The viral particles were produced as described (Keim et al., 2012; Rössler and Thiel, 2015).

2.3. Lentiviral expression of short hairpin RNAs (shRNAs)

The lentiviral vector pLentiLox3.7 (pLL3.7) was purchased from the American Type Culture Collection (Manassas, VA). The sequence used to knock down human androgen receptor expression has been described (Wang et al., 2009). The oligonucleotides for creating RNAi stem loops for pLL3.7 were designed as described (http://mcmanuslab.ucsf.edu/protocols/ll37stemloop_design.pdf).

2.4. Reporter assays

The lentiviral transfer vectors pFWColl.luc, pFWColl.lucΔTRE, pFWc-Jun.luc, pFWMMTV.luc, and pFWTNFα.luc have been described (Rössler et al., 2008; Müller et al., 2010, 2011; Thiel and Rössler, 2011, 2014, 2017). Plasmid pFWPb.luc was generated by inserting an NheI/Ecl136II fragment derived from the plasmid pGL3-Pb into a lentiviral transfer vector 5′ of the luciferase coding region.

2.5. RT-PCR

An RNeasy Mini Kit (# 74104, Qiagen, Hilden, Germany) was used to isolate total RNA from HEK293 cells, HEK293-M8 cells, LNCaP cells and HepG2 cells. Reverse transcription was performed with Revert Aid™ Reverse Transcriptase (# EP0441, ThermoFisher Scientific, Darmstadt, Germany, 200 U) and random hexamer primers (# SO142, ThermoFisher Scientific, Darmstadt, Germany). PCR was performed with Taq DNA Polymerase (# M0267S, New England Biolabs, Frankfurt, Germany, 1 U) and the TRPM8 specific primers, 5′-GATTT TCACCAATGACCGCCG′ and 5′-CCCCAGCAGCATTGATGTCG-3′, to amplify a 503 bp TRPM8 cDNA fragment. Control primers used to detect human GAPDH cDNA were 5`-TTCCAGCAGCGAGATCCCT-3` and 5`-CACCCATGACGAACATGGG-3`. The PCR products were visualized on an ethidium bromide-containing agarose gel.

2.6. Western blot

Nuclear extracts and whole cell extracts were prepared as described (Kaufmann and Thiel, 2002). The following antibodies were used: anti-c-Jun (Santa Cruz, Heidelberg, Germany, # sc-1694), anti-HDAC1 (Santa Cruz, Heidelberg, Germany, # sc-81598), anti-ERK1/2 (Santa Cruz, Heidelberg, Germany, # sc-153), HA.11 (clone 16B12, Covance, BioLegend # MMS-101 P, Fell, Germany), and M2 (Sigma-Aldrich, Steinheim, Germany, # F3165). Enhanced chemiluminescence was used to visualize the immunoreactive bands (Spohn et al., 2010; Mayer et al., 2011).

2.7. Statistics

Statistical analyses of the data were done as described (Müller et al., 2011), based on at least three independent experiments performed in quadruplicate.

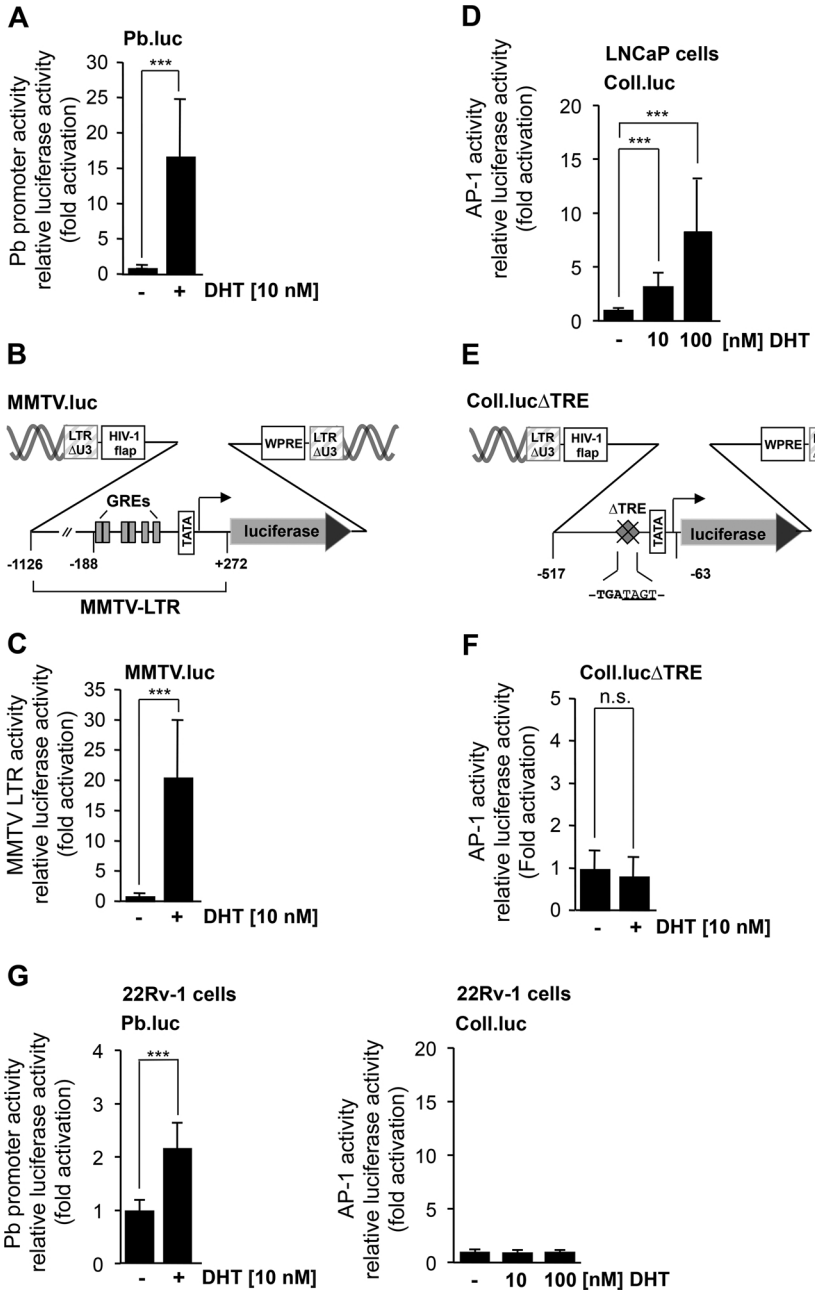

Fig. 3. DHT stimulation increases steroid- and AP-1-responsive reporter gene transcription. LNCaP cells containing either the Pb.luc reporter gene (A), a reporter gene under the control of the glucocorticoid and androgen-responsive MMTV LTR (B, C), the Coll.luc reporter gene (D), or a collagenase promoter/luciferase reporter gene with a mutated TRE (Coll.lucΔTRE) were analyzed (E, F). The integrated proviruses encoding MMTV.luc and Coll.lucΔTRE are shown in (B) and (E); GRE, glucocorticoid response element. The cells were serum-starved for 24 h and then stimulated with DHT (10 nM or 100 nM) for 24 h. Cell extracts were analyzed as described in the legend to Fig. 1. Data shown are mean ± SD of seven (A, C, D, 10 nM DHT), four (D, 100 nM DHT) or 3 (F) independent experiments performed in quadruplicate (✶✶✶, $P < 0.001$; n.s. not significant). (G) 22Rv-1 prostate cancer cells containing either an integrated probasin promoter/luciferase reporter gene (Pb.luc) or a collagenase promoter/luciferase reporter gene (Coll.luc) were stimulated with DHT (10 or 100 nM) for 24 h as indicated. Cell extracts were analyzed as described in the legend to Fig. 1. Data shown are mean ± SD of three independent experiments performed in quadruplicate (✶✶✶, $P < 0.001$; n.s. not significant).

3. Results

3.1. Activation of c-Jun N-terminal protein kinase (JNK) increases AP-1 activity in LNCaP cells, but does not change the activity of the probasin promoter

We analyzed the impact of JNK activation in regulating AP-1 and testosterone-responsive promoters. As a sensor to measure AP-1 activity, a collagenase promoter-controlled luciferase reporter gene (Coll.luc) was used (Rössler et al., 2008; Müller et al., 2011). The collagenase promoter contains a well characterized AP-1 binding site, known as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE). To measure testosterone-regulated gene transcription, we analyzed the activity of the probasin promoter which contains two binding sites for the androgen receptor (Rennie et al., 1993). We used lentiviral gene transfer to integrate the reporter genes into the genome of LNCaP cells. Thus, the reporter genes were embedded into a nucleosomal structure of the chromatin. A schematic depiction of the integrated proviruses containing either the Coll.luc reporter gene or the probasin promoter/luciferase reporter gene (Pb.luc) is shown in Fig. 1A and B. An activator of AP-1 is the MAP3 kinase mitogen-activated/extracellular signal responsive kinase kinase (MEK) kinase-1 (MEKK1) that induces the activation of JNK. LNCaP cells were infected with a lentivirus encoding a truncated, constitutively active form of MEKK1, MEKK1Δ. The expression of FLAG-tagged MEKK1Δ in LNCaP cells was verified by Western blotting (Fig. 1C). The MEKK1Δ-mediated activation of JNK triggered a strong stimulation of AP-1 (Fig. 1D), while the probasin promoter was not activated (Fig. 1E).

3.2. Expression of a constitutively active mutant of c-Jun increases AP-1 activity in LNCaP cells, but has no effect on probasin promoter activity

Originally, AP-1 was described as a heterodimer of c-Jun and c-Fos (Chiu et al., 1988). Many AP-1 complexes contain c-Jun. The activity of c-Jun is regulated by phosphorylation of serine residues 63 and 73 by JNK (Dérijard et al., 1994; Morton et al., 2003). Overexpression of c-

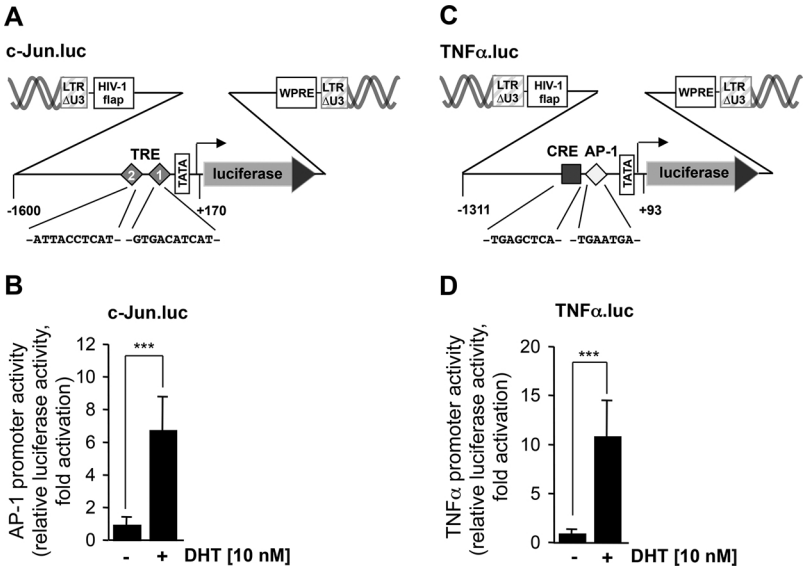

Fig. 4. DHT stimulation increases c-Jun and TNFα promoter activities in LNCaP cells. (A, C) proviruses encoding either a c-Jun promoter/luciferase reporter gene (c-Jun.luc) (A) or a TNFα promoter/luciferase reporter genes (TNFα.luc) (C). The locations and sequences of the AP-1 binding sites (TRE) within the c-Jun promoter are depicted in (A). The locations and sequences of the CRE and the AP-1 binding sites of the TNFα promoter are depicted in (C). (B, D) Elevated c-Jun and TNFα promoter activities in DHT-stimulated LNCaP cells. LNCaP cells containing either an integrated c-Jun promoter/luciferase reporter gene (c-Jun.luc) (B), or a TNFα promoter/luciferase reporter gene (TNFα.luc) were analyzed (D). The cells were incubated in medium with 0.05% serum for 24 h and then stimulated with DHT (10 nM) for 24 h. Cell extracts were analyzed as described in the legend to Fig. 1. Data shown are mean ± SD of three independent experiments performed in quadruplicate (✶✶✶, $P < 0.001$).

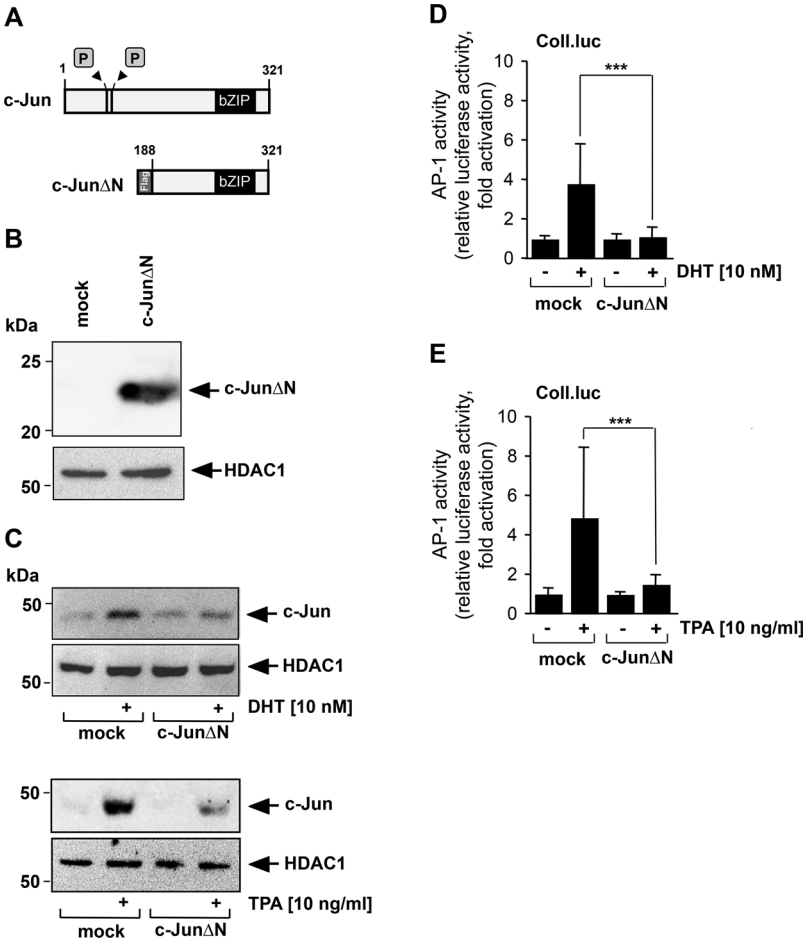

Fig. 5. A dominant-negative mutant of c-Jun blocks DHT and phorbol ester-induced biosynthesis of c-Jun and activation of AP-1 in LNCaP cells. (A) Modular structure of c-Jun and c-JunΔN. bZIP, basic region leucine zipper domain; P, phosphorylation sites. (B) Expression of c-JunΔN in LNCaP cells infected with an c-JunΔN-expressing lentivirus. The Western blot was incubated with an antibody against the FLAG-tag. As a loading control, we used an antibody that detected HDAC1. kDa, molecular-mass markers. (C) Expression of c-JunΔN attenuates c-Jun expression in DHT and TPA-stimulated LNCaP cells. Cells expressed c-JunΔN as indicated. Cells were cultured for 24 h in medium containing 0.05% serum and then stimulated with either DHT (10 nM) or TPA (10 ng/ml) as indicated. Nuclear proteins were analyzed by Western blotting with an anti-c-Jun antibody. As a loading control, an anti-HDAC1 antibody was used. (D, E) Expression of c-JunΔN attenuates DHT and TPA-induced activation of AP-1. LNCaP cells containing an integrated Coll.luc reporter gene were analyzed. Cells expressing c-JunΔN are indicated. Cells maintained in serum-reduced medium were stimulated with DHT (10 nM) (D) or TPA (10 ng/ml) (E) for 24 h. Cell extracts were analyzed as described in the legend to Fig. 1. Data shown are mean ± SD of four (D) or five (E) independent experiments performed in quadruplicate (✶✶✶, $P < 0.001$).

Jun without a simultaneous activation of JNK results in the expression of a biologically inactive protein (Al Sarraj et al., 2005). We therefore expressed a constitutively active c-Jun mutant, C2/c-Jun, consisting of the bZIP domain of c-Jun, and the transcriptional activation domain of CREB2 (Fig. 2A). The expression of C2/c-Jun in LNCaP cells that were infected with a lentivirus encoding the c-Jun mutant, was verified by Western blotting (Fig. 2B). Next, LNCaP cells were infected with a lentivirus encoding C2/c-Jun and with a lentivirus containing either the Coll.luc (Fig. 2C) or the Pb.luc (Fig. 2D) reporter gene. Expression of C2/c-Jun increased the AP-1 activity in LNCaP cells, while the activity of the probasin promoter was not changed in C2/c-Jun-expressing LNCaP cells. Together with the previous data, we conclude that the androgen-responsive probasin promoter is not regulated by either JNK or c-Jun. In contrast, elevated AP-1 activity was measured following either stimulation of JNK or expression of a constitutively active c-Jun molecule.

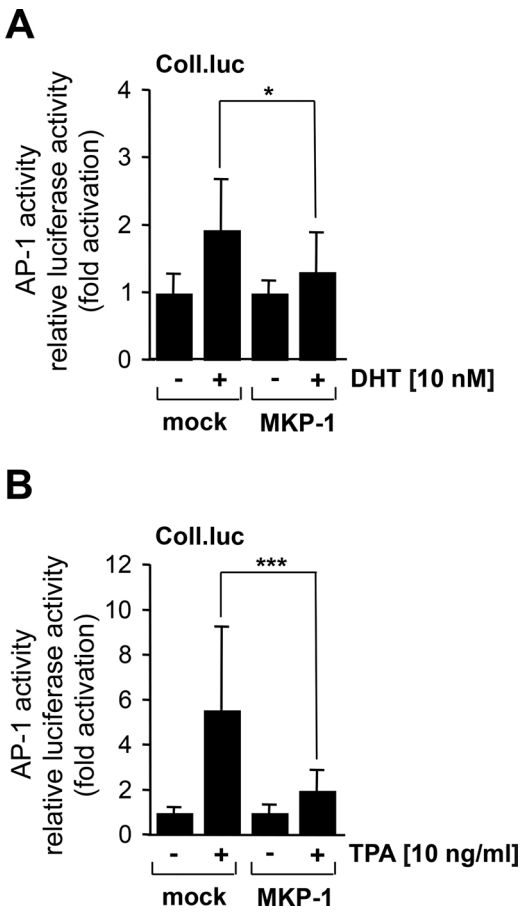

Fig. 6. DHT-induced upregulation of AP-1 is regulated by MAP kinases. LNCaP containing an integrated Coll.luc reporter gene were analyzed. The expression of MKP-1 is indicated. Serum-starved cells were stimulated with DHT (10 nM) (A) or TPA (10 ng/mg) (B) for 24 h. Data shown are mean ± SD of three (A) or five (B) independent experiments performed in quadruplicate (∗, $P < 0.05$; ∗∗∗, $P < 0.001$).

3.3. DHT activates transcription of androgen-, glucocorticoid-, and AP-1 responsive reporter genes in LNCaP prostate cancer cells

Next, we assessed the effect of the androgen DHT on steroid- and AP-1-regulated promoters. Fig. 3A shows that the activity of the androgen-responsive probasin promoter is strongly increased in DHT-treated LNCaP cells. Transcription of a reporter gene under the control of the MMTV LTR, which has been frequently used as androgen-responsive transcription unit (Bubulya et al., 1996; Hsu and Hu, 2013), was also increased as a result of DHT stimulation (Fig. 3B, C). Furthermore, DHT stimulation activated transcription of the AP-1-responsive Coll.luc reporter gene in a dose-dependent manner (Fig. 3D). To verify that the AP-1 site was responsible for this effect, we mutated the AP-1 sequence 5´-TGAGTCA-3´ of the collagenase promoter to 5`-TGATAGT-3´ (Fig. 3E), leading to an inactivation of the TRE. Transcription of a reporter gene, controlled by the mutated collagenase promoter, was not more activated following stimulation of the cells with DHT (Fig. 3F). These data indicate that DHT requires the AP-1 site to stimulate reporter gene transcription in LNCaP cells. Thus, the binding site for AP-1 functions as a DHT-responsive element. DHT also stimulated transcription of a probasin promoter-controlled reporter gene in 22Rv-1 prostate cancer cells. However, in this cell line, DHT did not stimulate AP-1 (Fig. 3G).

3.4. DHT stimulation activates the AP-1-regulated c-Jun and TNFα promoters in LNCaP cells

To confirm the previous results that DHT-stimulation activated AP-1 we analyzed the activities of the c-Jun and TNFα promoters in DHT-stimulated LNCaP cells. There are two AP-1 binding sites within the c-Jun promoter (jun1TRE, jun2TRE) (Fig. 4A). The TNFα promoter contains a cAMP response element (CRE), encompassing the sequence 5´-TGAGCTCA-3´ (Tsai et al., 1996) (Fig. 4C). This sequence is very similar to the TRE sequence 5´-TGAGTCA-3´. The CRE is a critical regulatory element for stimulus-induced TNFα gene transcription (Falvo et al., 2010). AP-1 has been shown to bind to both the c-Jun and the TNFα gene (van Dam et al., 1993; Bauer et al., 2007). Fig. 4B and D shows that DHT strongly stimulated transcription of reporter genes that were controlled by either the c-Jun or the TNFα promoter.

3.5. DHT-induced activation of AP-1 requires c-Jun

Many AP-1 transcription factor complexes contain c-Jun or a c-Jun homologous protein. We therefore assessed the importance of c-Jun in the regulation of AP-1 in DHT-stimulated LNCaP cells. We expressed c-JunΔN, a truncated, dominant-negative mutant of c-Jun. We have documented the biological activity of this mutant in several studies (Thiel and Rössler, 2011, 2014, 2017; Thiel et al., 2012; Lesch et al., 2015). As shown in Fig. 5A, the c-Jun mutant lacks the transcriptional activation domain and is therefore not able to activate transcription. The mutant, however, retains the bZIP domain and can therefore heterodimerize with wild-type c-Jun, thus forming inactive heterodimers that block cognate AP-1 DNA binding sites. The expression of c-JunΔN was verified in infected LNCaP cells via Western blot analysis (Fig. 5B). Expression of c-JunΔN significantly reduced the biosynthesis of c-Jun in DHT-stimulated LNCaP cells (Fig. 5C). As a control, we show that expression of c-JunΔN also interfered with the upregulation of c-Jun biosynthesis in phorbol ester (TPA)-stimulated LNCaP cells (Fig. 5C). Fig. 5D and E shows that expression of c-JunΔN in LNCaP cells significantly reduced the AP-1 activity following stimulation of the cells with either DHT (Fig. 5D) or TPA (Fig. 5E).

3.6. DHT-induced activation of AP-1 in LNCaP cells requires MAP kinases

Stimulation of MAP kinases, in particular JNK and p38, increases the cellular AP-1 activity, as shown in Fig. 1 and reported by us and others (Whitmarsh and Davis, 1996; Lesch et al., 2015). We therefore speculated whether MAP kinases might be necessary for the DHT-induced activation of AP-1 in LNCaP cells. We expressed MAP kinase phosphatase-1 (MKP-1) in LNCaP cells, an enzyme that catalyzes the dephosphorylation of the MAP kinases ERK1/2, p38 and JNK (Shapiro and Ahn, 1998). It is particularly active in dephosphorylating JNK and p38 (Shapiro and Ahn, 1998; Chi et al., 2006). Fig. 6A shows that expression of MKP-1 significantly reduced the DHT-induced activation of AP-1. Likewise, MKP-1 expression reduced the AP-1 activity in TPA-treated LNCaP cells (Fig. 6B). Thus, activated, nuclear MAP kinases are necessary for the activation of AP-1 in DHT-treated LNCaP cells.

3.7. DHT-induced activation of AP-1 in LNCaP cells requires androgen receptors

LNCaP cells are highly sensitive to DHT stimulation. We asked whether the androgen receptor is necessary for DHT to activate AP-1. To investigate whether DHT-induced activation of AP-1 requires expression of the androgen receptor we expressed an androgen receptor-specific small hairpin (sh) RNA. To verify the biological activity of the androgen receptor-specific shRNA, we switched to a heterologous expression system, HEK293 cells. The cells were infected with a recombinant lentivirus, encoding HA-tagged androgen receptor (Fig. 7A). Cells were also infected with a lentivirus containing the Pb.luc reporter

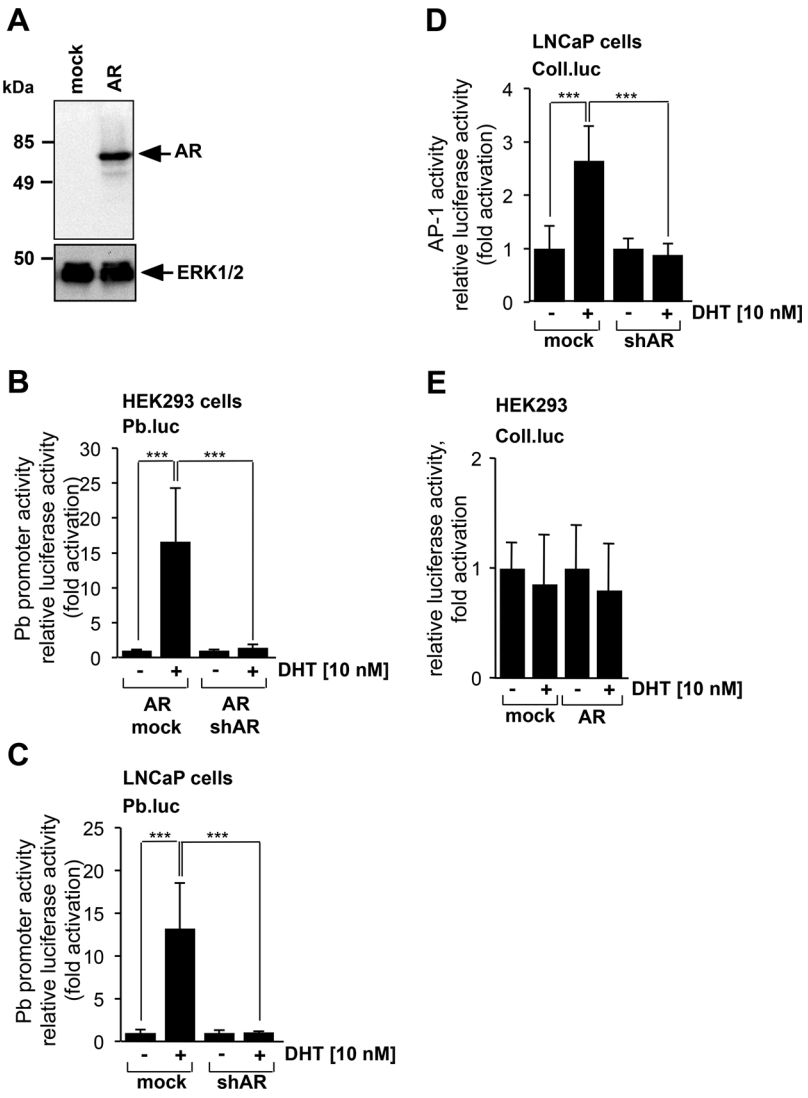

Fig. 7. DHT-induced activation of AP-1 requires the expression of androgen receptors. (A) Expression of the HA-tagged androgen receptor (AR) in HEK293 cells. The cells were infected with recombinant lentivirus encoding HA-tagged androgen receptor. The Western blot was incubated with an antibody against the HA-tag. As a loading control, we used an antibody that detected ERK1/2. kDa, molecular-mass markers. (B) HEK293 cells containing an integrated Pb.luc reporter gene were analyzed. The cells were infected with a lentivirus encoding the androgen receptor as indicated (AR). In addition, we infected the cells with a lentivirus that encoded for an androgen receptor-specific shRNA (shAR). As a control, we used a lentivirus generated with plasmid pLL3.7 (mock). Serum-starved cells were stimulated with DHT (10 nM, 24 h), harvested and analyzed. Data shown are mean ± SD of 3 independent experiments performed in quadruplicate (✶✶✶, $P < 0.001$). (C, D) LNCaP cells containing an integrated reporter gene under the control of either the probasin promoter (C) or the collagenase promoter (D) were infectd with a lentivirus that encoded for an androgen receptor-specific shRNA. Serum-starved cells were stimulated with DHT (10 nM, 24 h), harvested and analyzed. Data shown are mean ± SD of 3 independent experiments performed in quadruplicate (✶✶✶, $P < 0.001$). (E) HEK293 cells containing an integrated Coll.luc reporter gene were infected with a lentivirus encoding the androgen receptor (AR). Serum-starved cells were stimulated with DHT (10 nM, 24 h), harvested and analyzed. Data shown are mean ± SD of 3 independent experiments performed in quadruplicate (n.s., not significant).

gene. In addition, cells were infected with a lentivirus encoding an androgen receptor-specific shRNA. As a control, we infected the HEK293 cells with a lentivirus that was generated with the lentiviral transfer vector pLL3.7 (mock). Fig. 7B shows that transcription controlled by the probasin promoter was strongly stimulated in HEK293 cells that expressed androgen receptors and that were stimulated with DHT. In contrast, no transcriptional stimulation was observed when the androgen receptor-specific shRNA was expressed. These data show that the androgen receptor-specific shRNA is functional, confirming previous results (Wang et al., 2009). Next, we analyzed LNCaP cells. Fig. 7C shows that the DHT-induced activation of the probasin promoter was completely blocked in the presence of androgen receptor-specific shRNAs, indicating that DHT-induced activation of the probasin promoter requires the expression of the androgen receptor. We repeated the experiment using the AP-1-responsive reporter gene, Coll.luc. Fig. 7D shows that DHT stimulation increased the cellular AP-1 activity. However, in the presence of androgen receptor-specific shRNAs, the induction of AP-1 was attenuated. These results indicates that DHT-induced activation of AP-1 in LNCaP cells requires the presence of the androgen receptor.

In HEK293 cells, no activation of AP-1 was observed following expression of the androgen receptor and stimulation with DHT (Fig. 7E). Similar results were obtained in the analysis of SH-SY5Y neuroblastoma cells (data not shown). Thus, although the androgen receptor is necessary in LNCaP cells for the signaling pathway connecting DHT stimulation with the activation of AP-1, in HEK293 cells and SH-SY5Y neuroblastoma cells other not yet identified components, in addition to the androgen receptor, are required to stimulate AP-1 with DHT.

3.8. DHT activates AP-1 in LNCaP cells independently of TRPM8 channels

Prostate cancer cells highly express TRPM8 channels, that mediates cold sensation in the peripheral nervous system. Recently, TRPM8 channels have been proposed to function as androgen receptors (Asuthkar et al., 2015a, 2015b). Stimulation of TRP channels TRPC6, TRPM3 and TRPV1 results in the activation of AP-1 (Müller et al., 2011; Lesch et al., 2015; Thiel and Rössler, 2017; Backes et al., 2018), suggesting that DHT may target TRPM8 channels in LNCaP cell, leading to the stimulation of AP-1. Fig. 8A confirms that TRPM8 is expressed in LNCaP cells, but not in HEK293 or HepG2 hepatoma cells. As a positive control, HEK293-M8 cells were analyzed that expressed human TRPM8 channels (Bödding et al., 2007). We stimulated LNCaP cells with the super-cooling agent icilin, a well characterized ligand for TRPM8, and measured AP-1 activity using the Coll.luc reporter gene. Fig. 8B shows that icilin stimulation did not increase the AP-1 activity in LNCaP cells, indicating that TRPM8 signaling is not connected with AP-1 activation in these cells.

As a control, we stimulated HEK293-M8 cells with icilin. Fig. 9A shows that activation of TRPM8 triggered a strong activation of AP-1 in these cells which was not observed in the parental HEK293 cell line that

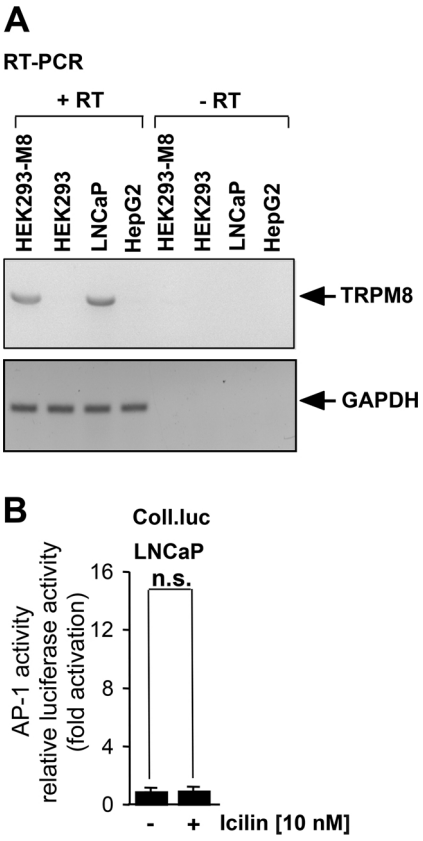

Fig. 8. DHT-induced activation of AP-1 in LNCaP cells does not require TRPM8 channels.
(A) Expression of TRPM8 in HEK293-M8 cells, HEK293 cells, LNCaP cells, and HepG2 cells. Expression of TRPM8 was monitored via RT-PCR. As a loading control, the concentration of GAPDH mRNA was determined. (B) LNCaP cells containing an integrated Coll.luc reporter gene were analyzed. Serum-starved cells were stimulated with icilin (10 μM) for 24 h. Data shown are mean ± SD of three independent experiments performed in quadruplicate.

did not express TRPM8. Given the fact that TRPM8 channels are functional in HEK293-M8 cells, we assessed whether TRPM8 functions as an androgen receptor in HEK293-M8 cells as proposed (Asuthkar et al., 2015a, 2015b). Fig. 9B shows that stimulation of HEK293-M8 cells with DHT did not trigger an activation of AP-1.

Stimulation of either TRPM3 or TRPC6 channels has been shown to activate interleukin-8 (IL-8) gene transcription via AP-1 (Rubil et al., 2018). The IL-8 gene functions in this context as a delayed response gene of AP-1. Fig. 9C and D shows that stimulation of TRPM8 channels with icilin in HEK293-M8 cells strongly increased IL-8 promoter activity. Furthermore, the AP-1 binding site within the IL-8 gene promoter was identified as the icilin-responsive element (Fig. 9E). We conclude that stimulation of TRPM8 channels leads to an activation of AP-1 and transcription of the AP-1-regulated IL-8 gene in HEK293-M8 cells. However, icilin did not activate AP-1 in TRPM8-expressing LNCaP cells. Thus, TRPM8 channels are not responsible for effect of DHT on the cellular AP-1 activity in these cells.

3.9. Expression of regulator of G protein signaling-2 attenuates AP-1 activation in DHT-stimulated LNCaP cells

Next, we speculated whether DHT activates a G protein-coupled receptor to exert its effect on AP-1. We expressed regulator of G protein signaling-2 (RGS2) in LNCaP cells (Fig. 10A) and measured the AP-1 activity with the Coll.luc reporter gene. Fig. 10B shows that expression of RGS2 interfered with the DHT-induced activation of AP-1. These result indicate that the DHT-induced signal cascade involves the activation of a G protein. Overexpression of RGS2 did not change the basal AP-1 activity in unstimulated LNCaP cells (Fig. 10C).

4. Discussion

The objective of this study was to investigate a postulated cross-talk between androgen signaling and AP-1 signaling in prostate cancer cells. It has been reported that c-Jun either inhibits the activity of androgen-responsive promoters (Sato et al., 1997; Hsu and Hu, 2013) or increases androgen receptor-mediated transactivation (Bubulya et al., 1996). These experiments were designed in two steps, first, an androgen was applied to stimulate androgen-regulated transcription, and second, AP-1 was activated either by stimulating the cells with the phorbol ester TPA, or by overexpressing wild-type c-Jun and/or c-Fos (Sato et al., 1997; Hsu and Hu, 2013). Thus, the question whether induction of AP-1 activates androgen-responsive transcription units, or whether an androgen stimulates AP-1 was not addressed in these studies. Furthermore, TPA is a strong activator of PKC, leading to the activation of AP-1, but also to a strong stimulation of other transcription factors such es Egr-1 and NF-κB (Bauer et al., 2005; Thiel et al., 2018). Therefore, the effects of TPA cannot be solely attributed to the activation of AP-1. Moreover, expression of c-Jun without a simultaneous activation of JNK results in the biosynthesis of a biologically inactive protein (Al Sarraj et al., 2005). In addition, transfection of μg amounts of a c-Jun expression vector (Bubulya et al., 1996; Tillman et al., 1998; Wise et al., 1998), controlled by a strong viral promoter/enhancer, triggers the biosynthesis of highly, non-physiological concentrations of c-Jun, that may have artefactually changed the activity, either positively or negatively, of androgen-responsive reporter genes.

In this study, we asked whether activation of AP-1 activates androgen-responsive genes, and whether androgens may activate AP-1-regulated genes. To measure transcription, we used reporter genes, controlled by either the androgen-responsive probasin promoter or the AP-1-regulated collagenase promoter. The reporter genes were implanted into the chromatin of LNCaP cells. By this means, we ensured that the reporter genes were packed into an ordered nucleosomal structure which is typical for the eukaryotic chromatin. To activate AP-1 via JNK, we expressed a constitutively active mutant of MEKK1 in LNCaP cells, controlled by the human UbC promoter. To directly activate AP-1, we expressed a constitutively active mutant of c-Jun in LNCaP cells. The results showed that the androgen-responsive transcription unit was not stimulated in LNCaP cells following stimulation of JNK and activation of AP-1. Thus, activation of AP-1 had no impact on androgen-responsive gene transcription. In contrast, this study showed that the androgen DHT stimulated AP-1 in LNCaP prostate cancer cells in a concentration-dependent manner. Furthermore, mutational analysis revealed that the classical AP-1 site of the collagenase promoter functioned as DHT-response element. These data were corroborated by experiments showing that the activities of the AP-1 regulated c-Jun and TNFα promoters were increased in DHT-regulated LNCaP cells. In addition, the biosynthesis of c-Jun was upregulated in DHT-treated LNCaP cells.

Androgen-responsive genes such as the probasin gene or the PSA gene are known since many years. Their expression level is significantly increased following stimulation with androgens. Using novel high-throughput genomic technologies, many more androgen-responsive genes have been detected, mostly using LNCaP cells as a model system (Jin et al., 2013). Many androgen-responsive genes are directly controlled by androgens via binding of the androgen receptor to androgen response elements within the regulatory region of the genes. The androgen receptor binding sites can be located more than 100 kilobases upstream of the proximal promoter region (Jin et al., 2013). In addition, there are androgen responsive genes that are indirectly regulated by androgens. This indirect regulation is based on the activation of transcription factors by androgen-activated androgen receptors that subsequently activate their target genes that lack androgen response

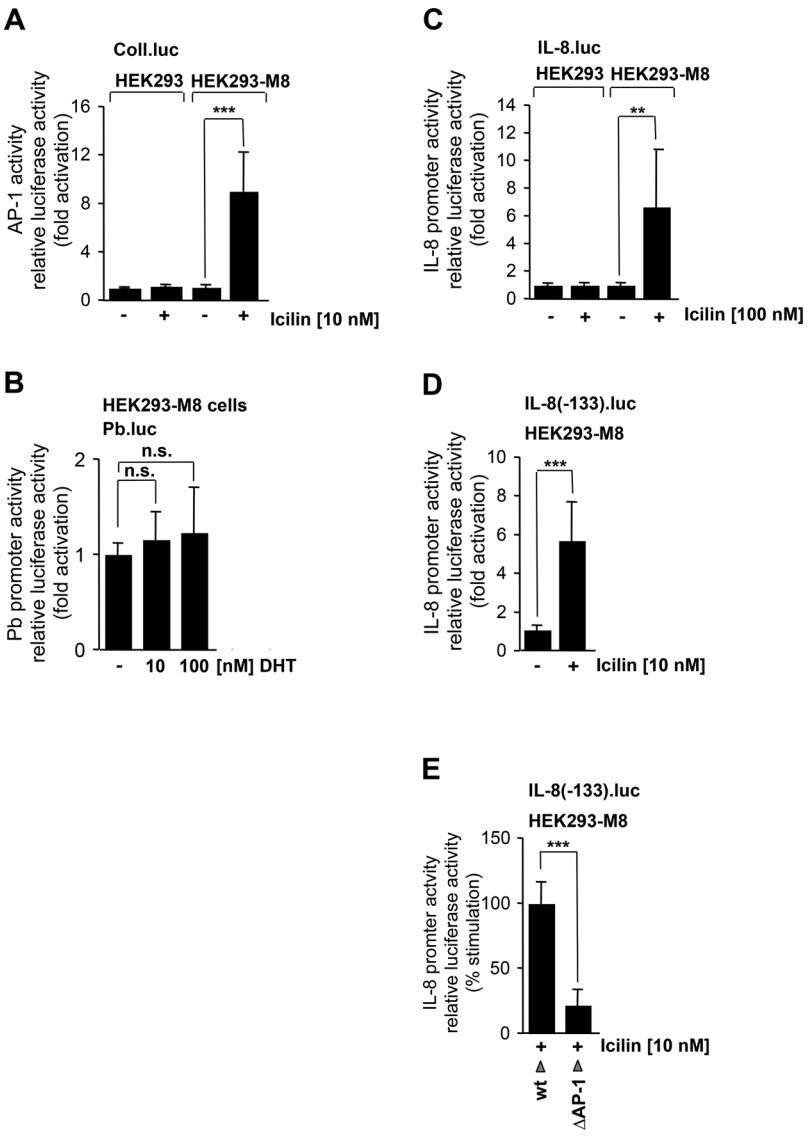

Fig. 9. Stimulation of TRPM8 channels with DHT leads to an activation of AP-1 in HEK293-M8 cells. (A) HEK293 cells (left) and HEK293-M8 cells (right) containing an integrated Coll.luc reporter gene were analyzed. Serum-starved cells were stimulated with icilin (10 μM) for 24 h. Data shown are mean ± SD of three independent experiments performed in quadruplicate (∗∗∗, $P < 0.001$). (B) DHT does not function as a ligand for TRPM8 in HEK293-M8 cells. HEK293-M8 cells containing an integrated probasin promoter/luciferase reporter gene (Pb.luc) were stimulated with DHT (10 and 100 nM) for 24 h. Data shown are mean ± SD of three independent experiments performed in quadruplicate (n.s., not significant). (C) HEK293 cells (left) and HEK293-M8 cells (right) containing an integrated luciferase reporter gene under the control of the human interleukin-8 (IL-8) promoter (sequence from -1481 to +44) were analyzed. Serum-starved cells were stimulated with icilin (10 μM) for 24 h. Data shown are mean ± SD of five independent experiments performed in quadruplicate (∗∗, $P < 0.01$). (D) HEK293-M8 cells containing an integrated reporter gene (IL-8(-133).luc) under the control of a truncated human IL-8 promoter (sequence from -133 to + 44) were analyzed. Serum-starved cells were stimulated with icilin (10 μM) for 24 h. Data shown are mean ± SD of three independent experiments performed in quadruplicate (∗∗∗, $P < 0.001$). (E) HEK293-M8 cells containing either the IL-8(-133).luc reporter gene or a reporter gene containing a mutated AP-1 binding site (ΔAP-1) within the IL-8 promoter were analyzed. The cells were stimulated with icilin (10 μM) for 24 h. Data shown are mean ± SD of three independent experiments performed in quadruplicate (∗∗∗, $P < 0.001$).

elements. Using chromatin-embedded reporter genes, we showed here for the first time that DHT stimulation activates the transcription factor AP-1 in LNCaP prostate cancer cells. These data explain why DHT stimulation activates genes such as the c-Jun gene or the TNFα gene, both lacking androgen response elements in their regulatory region.

Many signaling molecules that activate AP-1 use MAP kinases as signal transducers, in particular JNK or ERK1/2. Overexpression experiments involving MKP-1 revealed that DHT-induced activation of AP-1 in LNCaP prostate cancer cells required MAP kinase activity as well. MKP-1 has been shown to impair AP-1-regulated gene transcription in other cellular systems (Thiel et al., 2012; Thiel and Rössler, 2014, 2017; Backes et al., 2018). Thus, DHT employs a signaling pathway also used by G protein-coupled receptors or TRP channels to activate AP-1. Our results fit very well with the observation that stimulation of LNCaP cells with DHT activates the MAP kinase extracellular signal-regulated protein kinase ERK1/2 (Sen et al., 2010; Kawanami et al., 2018).

To identify the DHT receptor responsible for the activation of AP-1, we assessed the importance of the androgen receptor. Expression experiments of an androgen receptor-specific shRNA revealed that androgen receptors are required for the stimulation of AP-1 in DHT-treated LNCaP cells. We currently do not know the exact position of the androgen receptor in the signaling pathway leading to the activation of AP-1 in DHT-stimulated LNCaP cells. In line with our results, it has also been shown that activation of the protein kinase ERK1/2 in DHT-stimulated LNCaP cells requires the presence of the androgen receptor (Sen et al., 2010).

In contrast to LNCaP cells, expression of the androgen receptor in HEK293 and SH-SY5Y cells was not sufficient to activate AP-1, while the androgen-responsive probasin promoter was highly activated in DHT-stimulated HEK293 cells expressing the androgen receptor. The androgen receptor interacts with many other proteins in prostate cancer cells, establishing an “AR-interactome”, that contains more than 350 proteins that modulate androgen signaling (Hsiao et al., 2015). Thus, HEK293 and SH-SY5Y probably lack one or more proteins that are necessary to connect DHT stimulation with the activation of AP-1.

Next, we tested the TRPM8 channel as putative DHT receptor in LNCaP cells, because TRPM8 has been proposed to function as an ionotropic testosterone receptor in the prostate (Asuthkar et al., 2015a, 2015b). Although we confirmed that TRPM8 was expressed in LNCaP cells, AP-1 was not activated in icilin-stimulated LNCaP cells. This is in agreement with earlier observations that stimulation of LNCaP cells with icilin or menthol did not change the intracellular Ca^{2+} concentration (Bödding et al., 2007; Asuthkar et al., 2015c). In contrast, stimulation of HEK293-M8 cells which expressed human TRPM8 channels, with icilin strongly activated AP-1 and stimulated IL-8 gene

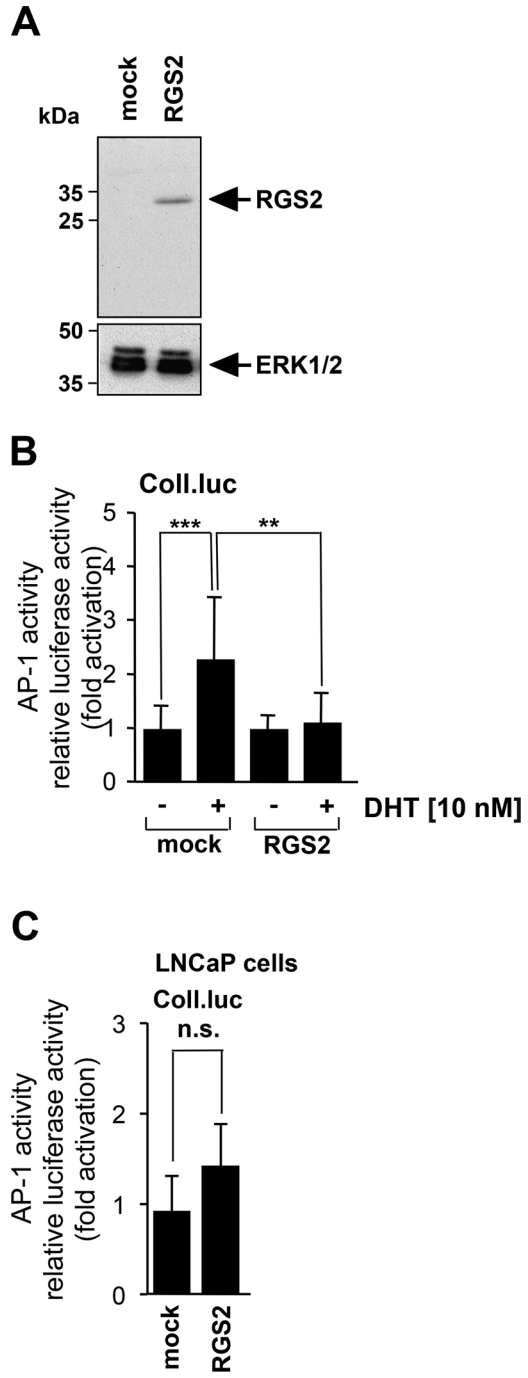

Fig. 10. DHT-induced activation of AP-1 in LNCaP cells is attenuated by expression of RGS2. (A) Expression of RGS2 in LNCaP cells infected with a lentivirus encoding RGS2. The Western blot was incubated with an antibody against the HA-tag. As a loading control, we used an antibody that detected ERK1/2. kDa, molecular-mass markers. (B) LNCaP cells containing a Coll.luc reporter gene were analyzed. Expression of RGS2 is indicated. Serum-starved cells were stimulated with DHT (10 nM) for 24 h. Data shown are mean ± SD of three independent experiments performed in quadruplicate (∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$). (C) Expression of RGS2 does not change basal AP-1 activity in LNCaP cells. LNCaP cells containing a Coll.luc reporter gene were either mock infected or infected with a lentivirus encoding RGS2. Serum-starved cells were harvested 48 h later. Data shown are mean ± SD of three independent experiments performed in quadruplicate (n.s., not significant).

transcription involving the AP-1 binding site within the proximal IL-8 promoter. Thus, stimulation of TRPM8 channels with icilin induced an activation of AP-1 as previously described for the stimulation of TRPM3, TRPC6, and TRPV1 channels (Müller et al., 2011; Lesch et al., 2015; Thiel and Rössler, 2017; Backes et al., 2018). The unresponsiveness of LNCaP cells to icilin can be explained by the fact that these cells express TRPM8 channels in the membrane of the endoplasmic reticulum, and not at the plasma membrane (Thebault et al., 2005). Thus, TRPM8 is not the DHT receptor leading to an activation of AP-1. Interestingly, we could not activate gene transcription in HEK293-M8 cells using DHT as a ligand. These results indicates that DHT does not function as a TRPM8 ligand in HEK293-M8 cells as proposed (Asuthkar et al., 2015a, 2015b). The statement that testosterone activates TRPM8 in primary human prostate cells was based on Ca^{2+} imaging and whole-cell patch clamp experiments (Asuthkar et al., 2015a). We used a transcriptional assay as an indication for TRPM8 activation. This strategy implies that the influx of Ca^{2+} ions through the TRPM8 channel, induced by TRPM8 ligands, is sufficient to induce an intracellular signaling cascade to the nucleus that finally leads to a change in gene transcription. Our results suggests that a rise in the intracellular Ca^{2+} concentration following testosterone stimulation is not sufficient to induce gene transcription.

The signaling pathway described in this study that leads to an activation of AP-1 involved an activation of MAP kinases and of c-Jun. Similary, stimulation of G protein-coupled receptors induces an activation of AP-1 (Thiel et al., 2012; Kaufmann et al., 2013; Thiel and Rössler, 2017). Expression experiments revealed that expression of the GTPase activating protein RGS2, known to block signaling via the G protein Gαq, attenuated the activation of AP-1 in DHT-stimulated LNCaP cells. These data suggest that a G protein-coupled receptor functions as the DHT receptor responsible for the signaling cascade that finally leads to an activation of AP-1. This suggestion is in agreement with a study showing that non-classical testosterone signaling in a spermatogenic cell line is mediated by a G protein coupled receptor and the subsequent activation of ERK1/2 (Shihan et al., 2014). Furthermore, androgens have been shown to increase the intracellular Ca^{2+} concentration in LNCaP cells via activation of a G protein-coupled receptor (Sun et al., 2006).

In summary, we have shown that DHT activates AP-1 in LNCaP prostate cancer cells, involving MAP kinases, c-Jun, the androgen receptor and G proteins. Thus, DHT stimulation activates directly, e.g. via binding to the androgen receptor, androgen receptor-regulated genes, and, via an intracellular signaling cascade, AP-1-controlled genes. Various studies have connected AP-1 activity with cell growth and proliferation, e.g. for Hodgkin lymphoma cells, keratinocytes, and hepatocytes (Behrens et al., 2002; Mathas et al., 2002; Zenz et al., 2003). In prostate cancer tissues, an increased concentration of c-Jun and phosphorylated c-Jun has been detected. c-Jun has been identified as a marker of high-risk prostate cancer. The fact that overexpression of c-Jun and its dimerization partner c-Fos leads to proliferation of prostate cancer cell lines (Edwards et al., 2004; Ouyang et al., 2008) suggests that DHT-induced stimulation supports the growth and proliferation of prostate cancer cells by activating AP-1. It will be necessary to identify the delayed response genes of AP-1 in prostate cancer cells whose gene products are involved in the regulation of proliferation and cellular growth.

Conflict of interest

The authors declare that they have no conflict of interest.

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