Direct Cooperation Between Androgen Receptor and E2F1 Reveals a Common Regulation Mechanism for Androgen-Responsive Genes in Prostate Cells

D. M. Altintas, M. S. Shukla, D. Goutte-Gattat, D. Angelov, J. P. Rouault, S. Dimitrov, and Jacques Samarut

Institut de Génomique Fonctionnelle de Lyon (D.M.A., J.P.R., J.S.), Université de Lyon, Université Claude Bernard Lyon 1, Centre National de la Recherche Scientifique (CNRS), Institut National de la Recherche Agronomique, Ecole Normale Supérieure de Lyon, F-69346 Lyon Cedex 07, France; Hospices Civils de Lyon (J.S.), 69229 Lyon, France; Laboratoire de Biologie Moléculaire de la Cellule (M.S.S., D.A.), Université de Lyon, CNRS, Ecole Normale Supérieure de Lyon, F-69346 Lyon, France; and Université Joseph Fourier-Grenoble 1 (D.G.-G., S.D.), Institut National de la Santé et de la Recherche Médicale Institut Albert Bonniot Unité 823, 38042 Grenoble Cedex 9, France

We have studied the regulation of *ATAD2* gene expression by androgens in prostate cells. *ATAD2* is a coactivator of the androgen receptor (AR) and the MYC protein. We showed that *ATAD2* expression is directly regulated by AR via an AR binding sequence (ARBS) located in the distal enhancer of its regulatory region. The gene is also regulated by the E2F1 transcription factor. Using knockdown and chromatin immunoprecipitation technique approaches, we could demonstrate that AR and E2F1 functionally collaborate and physically interact between each other. From the analysis of chromatin conformation, we conclude that this cooperation results from a chromatin looping over the *ATAD2* promoter region between the ARBS and E2F1 binding site in an androgen-dependent manner. Furthermore, we could show that several genes overexpressed in prostate cancer and potentially involved in several aspects of tumor development have an ARBS and an E2F1 binding site in their regulatory regions and exhibit the same mechanism of regulation by both transcription factors as *ATAD2*. **(*Molecular Endocrinology* 26: 1531–1541, 2012)**

NURSA Molecule Pages[†]: Nuclear Receptors: AR; **Ligands:** R1881.

The androgen receptor (AR) is a ligand-dependent transcription factor. When activated by androgens, AR regulates gene transcription (1). Gene expression can be regulated by AR either directly (2, 3) or indirectly (4). Direct gene activation involves binding of AR to AR binding sequences (ARBS) on DNA, whereas indirect activation implies functional interaction with other transcription factors.

In men, AR is essential for sexual differentiation and prostate development. It is implicated in every step of prostate cancer, which is the most common cancer among men in Western countries (5, 6). Androgens activate cell proliferation at the very early stages of tumor development (7). Androgen suppression therapy is then used against prostate cancer development. At some advanced stages of tumor progression leading to metastasis, androgen suppression therapy becomes inefficient and tumor development becomes independent of androgens, which leads to the development of the castration-resistant prostate cancer (CRPC) (8, 9). In some cases, antiandrogen compounds can even activate cell proliferation (10).

Escape of tumor growth from control by androgens results from alterations in the function of the AR-signaling pathway. The molecular bases of these alterations are still poorly understood but might include hypersensitivity

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[†] Annotations provided by Nuclear Receptor Signaling Atlas (NURSA) Bioinformatics Resource. Molecule Pages can be accessed on the NURSA website at www.nursa.org.

Abbreviations: AR, Androgen receptor; ARBS, AR binding sequence; 3C, chromosome conformation capture; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; CRPC, castration-resistant prostate cancer; DBD, DNA-binding domain; DNase, deoxyribonuclease; ds, double stranded; E2FBS, E2F1 binding site; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mtARBS, mutant ARBS; q, quantitative; RB, retinoblastoma protein; si, small interfering; TSS, transcriptional start site; WT, wild type.

to androgens due to amplification of AR or AR cofactors, AR constitutive transcriptional activity due to mutations of the receptor or its cofactors, or alterations in intracellular signaling pathways that cooperate with AR.

Noteworthy, the AR and retinoblastoma protein (RB)/E2F1 pathways are in cross talk (11). Once RB is phosphorylated, it releases E2F1, which then transactivates its target genes and induces cell cycle progression through the G_1 to S phase (12). Inactivation or loss of RB has been shown to induce immortalization of prostate cells. Loss of integrity of the RB1 gene locus is overrepresented in CRPC and metastasic prostate cancer and is associated with a poor outcome (11, 13–16).

Hence, identifying downstream target genes of the RB/E2F1/AR axis should help in understanding the mechanisms of prostate carcinogenesis. In addition, it is likely that identifying some of these genes might provide helpful diagnostic and prognostic markers of prostate tumor development.

Our attention was recently attracted by the identification of the *ATAD2* gene, which encodes a cofactor of the MYC protein, a protein directly involved in cell proliferation. E2F1 protein regulates ATAD2 gene expression (17). Moreover, expression of this gene is activated by androgens, and the level of gene expression is increased in androgen-dependent and androgen-independent prostate tumors (18). We therefore investigated whether *ATAD2* gene might be a direct target of AR for its transcriptional regulation by androgens and how the RB/E2F1/AR pathway might regulate it. We first identified a functional ARBS within the ATAD2 gene distal enhancer region and showed that this site binds AR within the chromatin. We then analyzed the collaboration between E2F1 and AR in gene regulation in human prostate cancer cells and showed that E2F1 interacts with AR on the promoter of *ATAD2* through a chromatin loop to activate transcription when stimulated by androgens.

In addition, we analyzed chromatin immunoprecipitation (ChIP)-seq data from the literature to identify genes that might share the same regulation mechanism as *ATAD2*. In LNCaP cells, we found six other genes that show the same expression pattern as ATAD2. Their expression is stimulated by androgen treatment, and knockdown of E2F1 decreased their mRNA levels. Moreover, Re-ChIP experiments demonstrated that AR and E2F1 interact in their promoter region. This mechanism of regulation by the RB/E2F1/AR axis might, thus, be a general mechanism for several androgen-regulated genes in prostate cells.

Results

Identification of a candidate ARBS in the *ATAD2* promoter

ATAD2 gene expression has previously been shown to be enhanced when prostatic LNCaP cells are treated with androgens (18). With this in mind, we aimed first at checking whether *ATAD2* might be a direct target gene of AR. To test this hypothesis, we analyzed *ATAD2* gene response to androgens in LNCaP cells in the presence of cycloheximide (CHX), a protein synthesis inhibitor.

Figure 1A shows that the increase in ATAD2 mRNA after R1881 was preserved in the presence of CHX. As expected, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was not affected by CHX or R1881 treatments (Fig. 1B). Similar results were obtained in another prostate cell line, RWPE-1-AR cells, expressing the AR (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). These data suggest that *ATAD2* gene activation might be directly controlled by androgens via a direct interaction of AR with an ARBS within the gene.

We then searched for putative ARBS in the *ATAD2* gene. We first scanned a potential regulatory region from −10 to +1 kb from the transcriptional start site (TSS) using NUBIScan software (19), an algorithm for the detection of nuclear receptor binding sites. Because the algorithm identified more than 50 putative binding sites for AR in this region with a significant score of more than 0.7 (Supplemental Fig. 2), we performed a second screening by comparing these sequences between species. The rationale was that if the *ATAD2* gene plays an important role in prostate development, its regulatory sequences might be conserved among several mammalian species. The sequence of this region in human was then compared with other species using d-code software to find conserved sequences. As shown in Supplemental Fig. 2, we could identify one potential ARBS of sequence AGAACAgagTGTACA in a very conserved region, at 9.2 kb upstream from the TSS in the human *ATAD2* gene. This upstream region containing this putative ARBS will then be referred to as distal enhancer region.

AR binds to the ARBS of the *ATAD2* distal enhancer region *in vitro*

To check the binding of AR to this putative ARBS, we first performed EMSA experiments with bacterially produced AR-DNA-binding domain (DBD) purified to homogeneity (see *Materials and Methods* and Supplemental Fig. 3). A 49-bp oligonucleotide from the *ATAD2* genomic sequence containing the potential ARBS was used as binding probe. As seen in Fig. 1C, increasing the amount of AR-DBD protein resulted in a clear band shift, indicating a strong binding to this DNA sequence (Fig. 1C, *left panel*). In contrast, in the presence of a mutated version of the ARBS, AR-DBD binding was very weak (Fig. 1C, *right panel*). Competition experiments with increasing amounts of cold oligonucleotide carrying the wild-type (WT) ARBS (WT oligo) strongly decreased the

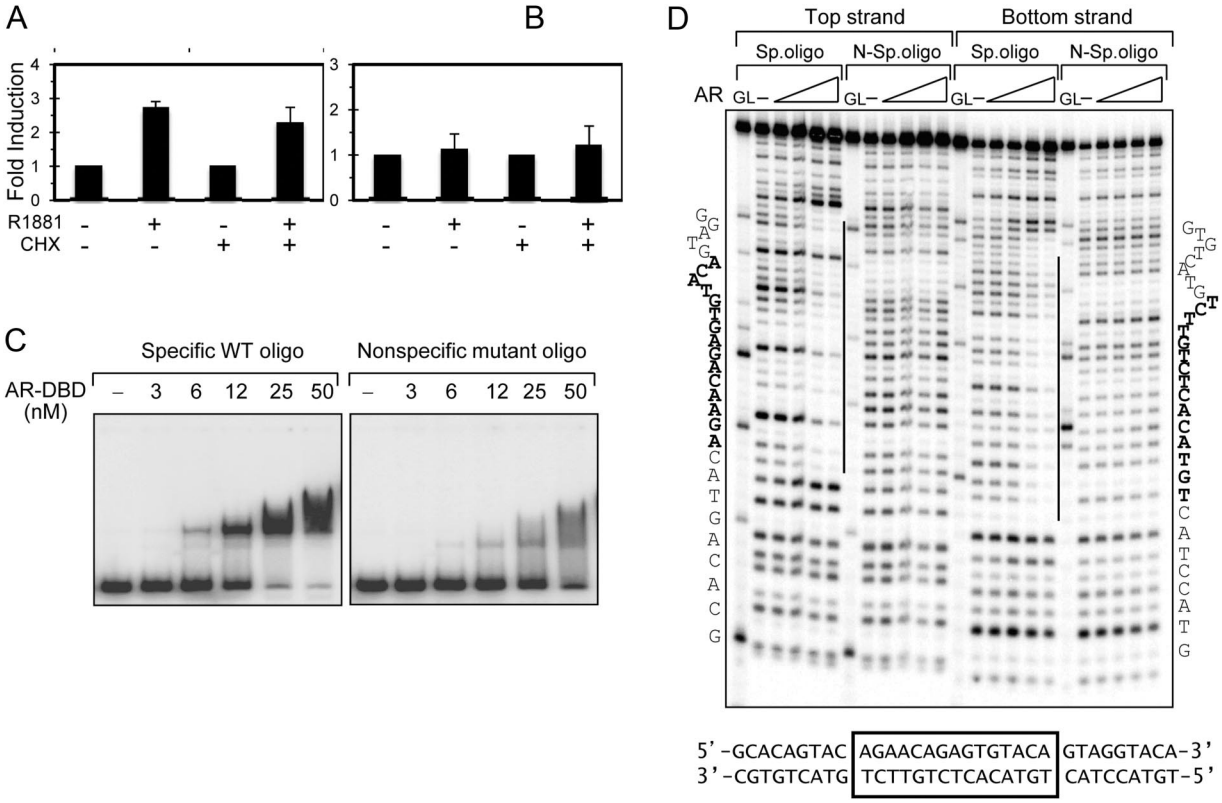

FIG. 1. Androgen-dependent *ATAD2* expression. Expression of ATAD2 mRNA. Total RNA was isolated from control and R1881-treated LNCaP cells, and the mRNA levels of *ATAD2* (A) and GAPDH (B) were quantified by RT-qPCR. Data represent means ± SD, n = 3. C, Binding assay for the AR-DBD protein on the ARBS of the ATAD2 promoter *in vitro*. EMSA were carried out on a *ATAD2* promoter oligonucleotide containing the ARBS (*left panel*) or the nonspecific mutant oligonucleotide (*right panel*). D, DNase I footprinting of the binding of AR-DBD on ATAD2-ARBS. Sp.oligo and N-Sp.oligo refer, respectively, to WT and mutant ATAD2-ARBS. GL− indicates a guanine ladder for the corresponding oligonucleotide-labeled strand obtained after UV-laser irradiation and formamidopyrimidine [fapy]-DNA glycosylase (FPG) treatment. Base sequences of the specific WT and mutant oligonucleotides are shown. The position of the AR-DBD footprint is denoted by *vertical lines*.

binding of the labeled oligonucleotide (Supplemental Fig. 4, *left panel*). Under the same conditions, addition of the oligonucleotide carrying a mutated ARBS had a much weaker effect (Supplemental Fig. 4, *right panel*).

To further better define the binding domain of AR-DBD to the ARBS, we next performed a deoxyribonuclease (DNase) I footprinting assay. The binding reaction was carried out using increasing amounts of AR-DBD protein, similarly to Fig. 1C. The data in Fig. 1D clearly show that AR-DBD binds specifically to the WT-ARBS sequence on the forward and reverse strands. No specific binding was observed on mutated ARBS.

Taken together, these data demonstrate that the potential ARBS identified within the distal enhancer region of *ATAD2* gene indeed interacts strongly and specifically with AR *in vitro* and suggest that it could be a good candidate as a functional ARBS *in vivo*.

AR binds to the ARBS of the *ATAD2* distal enhancer region and mediates the response to androgens *in vivo*

To ascertain whether the ARBS identified within the *ATAD2* enhancer region is functional in cells *in vivo*, we performed ChIP using formaldehyde cross-linked LNCaP cells. Figure 2A shows a diagram of the *ATAD2* regulatory region where A2 is the region containing the putative ARBS located in the distal enhancer region, at −9.2 kb from the transcription start site. A1 and A3 are regions located 2 kb upstream and downstream, respectively, of A2. E2 is the region containing the E2F1 binding site (E2FBS) previously described, located in the proximal promoter, at −272 pb from the TSS (17). E1 and E3 are regions located 2 kb upstream and downstream of E2.

ChIP experiments carried out with an anti-AR antibody showed a strong enrichment of DNA on the ARBS in the presence of R1881 (Fig. 2B). As expected, no binding was observed in the A1 and A3 control regions. Identical data were obtained in the RWPE-1-AR cells (Supplemental Fig. 5).

Altogether, these data suggest that AR binds specifically to the identified ARBS both *in vitro* and *in vivo* and might mediate androgen regulation.

Cooperation between E2F1 and AR in the regulation of *ATAD2* expression

ATAD2 is functionally regulated by E2F1 through an E2FBS located within the proximal promoter region 272

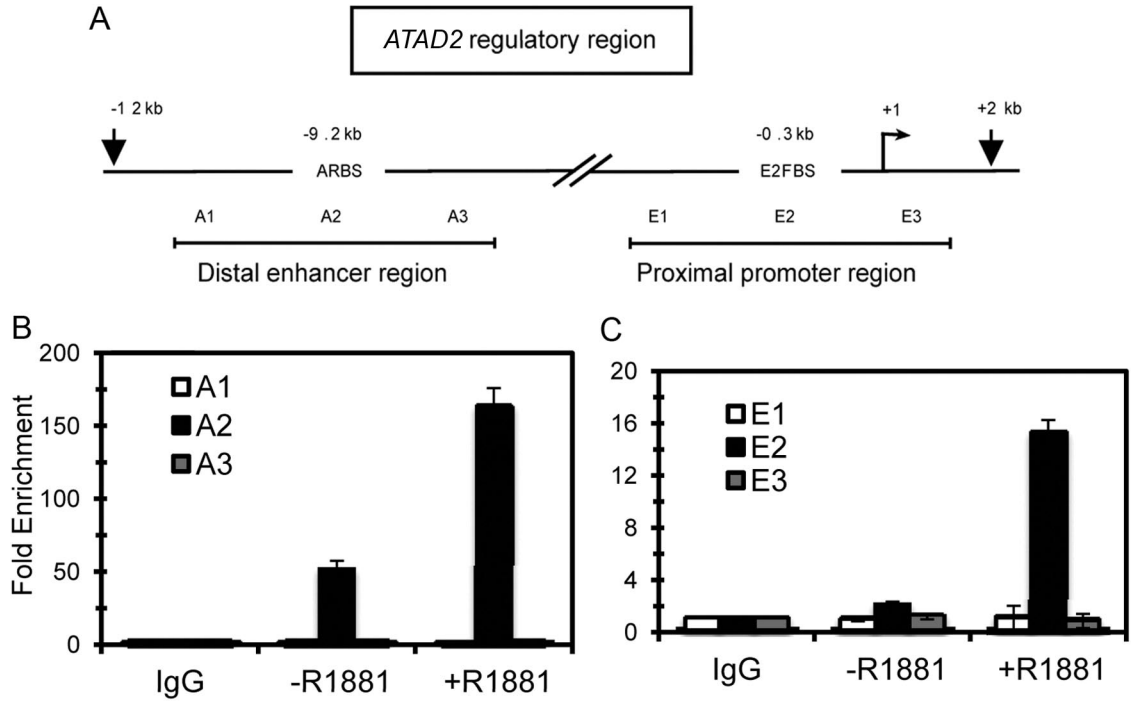

FIG. 2. Study of the enhancer promoter. ChIP were performed on LNCaP cells treated with R1881 or untreated after cross-linking with formaldehyde. A, Schematic representation of the ATAD2 promoter. A2 and E2 represent the respective specific binding sites of AR and E2F. E1, E3, A1, and A3 are regions located 2 kb upstream and downstream of the specific binding sites of E2F and AR, respectively, and were used as controls for the binding specificity. B and C, ChIP assays on LNCaP cells using an anti-AR antibody and detection of binding on sites on the distal enhancer (B) and proximal (C) promoter regions. Values are the means of three independent experiments. *Error bars* indicate SD.

bp upstream of the TSS (17). We therefore wondered whether a molecular link between AR and E2F1 could exist that would explain the elevated levels of *ATAD2* observed in prostate cancer. We first analyzed in LNCaP cells by coimmunoprecipitation experiments whether AR and E2F1 could physically interact to each other. As shown in Fig. 3A, we could observe that indeed both transcription factors bind to each other and that this interaction was not dependent upon androgen stimulation.

Secondly, we wanted to ascertain the functional implication of both E2F1 and AR transcription factors in the regulation of *ATAD2* gene expression. We then transfected LNCaP cells with specific small interfering (si)RNA aimed at knocking down the respective transcription factors. siE2F1 and siAR transfection showed the very efficient and specific knockdown of E2F1 and AR, respectively, at both mRNA and protein levels (Supplemental Fig. 6, A and B). As expected, ATAD2 mRNA levels were up-regulated in the presence of R1881 in LNCaP cells transfected with scrambled siRNA. In contrast, siAR or siE2F1 completely abolished androgen activation. The basal level of *ATAD2* gene expression in the absence of R1881 was greatly diminished in siE2F1-transfected LNCaP cells. In contrast, this basal level was not affected in scrambled siRNA- or siAR-transfected LNCaP cells. Thus, E2F1, in contrast to AR, might then control the basal expression level of *ATAD2*. Level of GAPDH mRNA used as a negative control was not affected by these treatments. Identical data were obtained in the RWPE-1-AR cells (see Supplemental Materials and Methods and Supplemental Fig. 6C), indicating that the cooperation between AR and E2F1 in regulating ATAD2 gene expression is a general phenomenon in prostate cells.

Next, we wanted to analyze the direct role of the ARBS in the transcriptional control of *ATAD2* gene expression. A 1.5-kb section of the distal *ATAD2* enhancer region containing either the WT-ARBS or a mutant ARBS (mtARBS) construct was inserted upstream of the luciferase reporter gene (Supplemental Fig. 7A). These constructs were transfected into LNCaP cells. The data presented in Supplemental Fig. 7B show that stimulation with R1881 enhanced the luciferase activity produced from the expression vector containing the WT-ARBS. Under the same conditions, the mtARBS did not mediate any significant increase in activity. The ARBS sequence of the distal enhancer region of *ATAD2* does therefore confer androgen-dependent regulation.

To study the functional importance of the proximal promoter in the regulation of *ATAD2* gene expression, we have designed deletion mutants of 100 bp within this region and analyzed their activity in expression vectors upon transient transfection. Structures of the expression

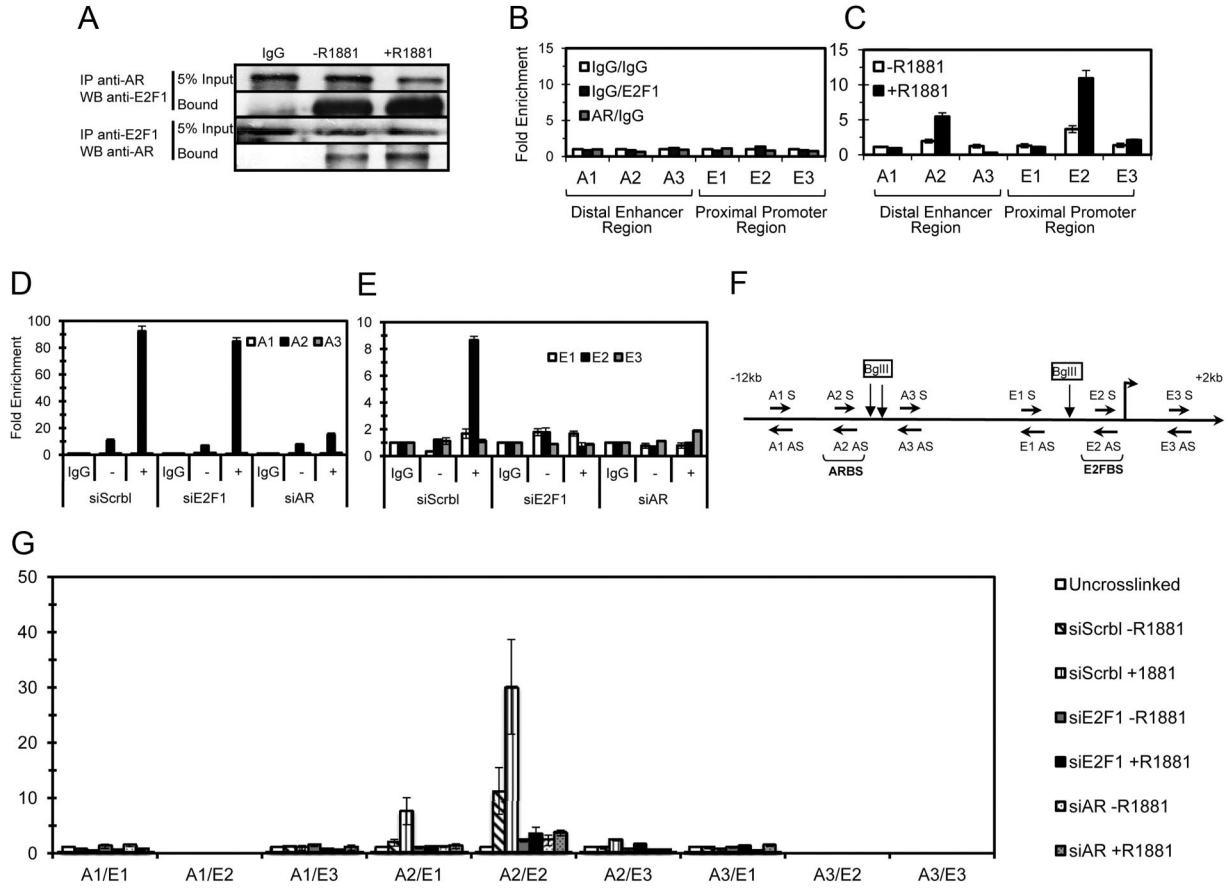

FIG. 3. Cooperation between E2F1 and AR. A, Coimmunoprecipitation was realized on LNCaP cells with AR antibodies (*upper panel*) or with E2F1 antibodies (*lower panel*). B and C, Re-ChIP were performed on exponentially growing LnCaP cells 28 after cross-linking. Chromatin was precipitated first with an anti-AR antibody and then with an anti-E2F1 antibody. Control experiments were performed using IgG in place of the respective anti-AR and anti-E2F1 antibodies (B). D and E, E2F1 is essential for AR recruitment on the proximal promoter. ChIP experiment on LNCaP cells transfected with scrambled siRNA, siE2F1, or siAR. qPCR was carried out on the distal enhancer (D) and the proximal promoter regions of ATAD2 (E). F, Design of the 3C experiment with the localization of the *Bgl*II sites on the ATAD2 promoter and the primers used for the experiment. H, Loop between distal enhancer and proximal promoter of ATAD2. 3C was performed in LNCaP cells. Cells were transfected either with scrambled siRNA, siE2F1, or siAR and treated with R1881 or ethanol as vehicle. Results are fold enrichment on noncross-linked cells. *Error bars* indicate SD.

vectors are depicted in Supplemental Fig. 8A. Luciferase experiments show that E1, E2, and E3 constructs displayed a high luciferase activity, which was completely abolished in siE2F1-transfected LNCaP cells, suggesting that this region confers E2F1-dependent luciferase activity (Supplemental Fig. 8, B–D). In contrast vector E4 showed no signal (Supplemental Fig. 8D), confirming that the E2FBS located at 272 bp upstream of the TSS is responsible for the E2F1-dependent transcriptional activation.

All these data suggest that AR and E2F1 are two major transcription factors for the control of *ATAD2* gene expression and that ARBS and E2FBS are fundamental binding sequences to mediate, respectively, androgen- and E2F1-dependent regulation. We then searched for a possible molecular cooperation between AR and E2F1 in the ATAD2 regulatory region context in LNCaP cells.

We first performed ChIP with an anti-AR antibody after formaldehyde cross-linking. The quantitative (q)PCR data presented in Fig. 2, B and C, show a strong enrichment in regions A2 and E2, which correspond, respectively, to the ARBS and the E2FBS. Thus, AR seemed to be recruited both on the ARBS and on the E2FBS when LNCaP cells were stimulated with R1881. As expected, no AR binding was observed in the A1, A3, E1, and E3 control regions (Fig. 2, B and C). We then wanted to explain how AR could bind to the E2FBS. One possibility might be the indirect recruitment of AR to the E2FBS via another protein. Noteworthy, interaction between AR and E2F proteins had been already reported on one gene expressed in prostate cells (20).

We therefore hypothesized that AR binds to the E2FBS through its chromatin-dependent association with E2F1. To test this hypothesis, we performed a Re-ChIP experiment (Fig. 3, B and C). LNCaP cell chromatin was first immunoprecipitated with an anti-AR antibody, followed by immunoprecipitation with an E2F1 antibody. As ex-

pected, no significant enrichment was observed with nonspecific antibodies used as controls (Fig. 3B). Figure 3C shows enrichment of the A2 and E2 regions of the *ATAD2* proximal promoter after immunoprecipitation with the two specific antibodies only when LNCaP cells were treated with R1881. As expected, no enrichment was observed on the control A1, A3, E1, and E3 regions. These results suggest that, within the chromatin, AR and E2F1 interact with each other on the E2FBS and the ARBS of the *ATAD2* gene in response to androgen stimulation. These results, together with data presented above, indicate that both transcription factors physically cooperate in the androgen regulation of the *ATAD2* gene.

To further validate the hypothesis that AR recruitment on the E2FBS was E2F1 dependent, we first down-regulated E2F1 expression in LNCaP cells and then performed a ChIP experiment with an anti-AR antibody. Figure 3D shows no difference in the recruitment of AR on the ARBS in siE2F1-transfected LNCaP cells compared with scrambled siRNA-transfected cells. As expected, AR was recruited to the E2FBS in scrambled siRNA-transfected cells (Fig. 3E). In contrast, in siE2F1-transfected cells, we observed an 8-fold decrease in AR recruitment on the E2FBS (Fig. 3E). As expected, in siAR-transfected cells, AR recruitment was strongly decreased on the ARBS and on the E2FBS. These data clearly demonstrate that, in the context of chromatin, AR binding to the E2FBS, but not to the ARBS, was dependent on the presence of E2F1.

The physical interaction of AR and E2F1 in the regulatory region of the *ATAD2* gene might result from the formation of a DNA loop between the ARBS and the E2FBS within the chromatin. To check this model, we designed a chromosome conformation capture (3C) experiment (21). The 3C protocol consists in cross-linking DNA-protein and protein-protein complexes with formaldehyde and digesting chromatin with appropriate restriction enzymes. If a chromatin loop exists, remote DNA regions become relatively proximal to each other, and a qPCR analysis on the digested DNA can identify such rearrangements. Figure 3F presents the positions of the primers and those of the two *Bgl*II restriction sites used to analyze the structural rearrangements within the *ATAD2* regulatory region. As shown in Fig. 3G, in formaldehyde-cross-linked cells treated with R1881, qPCR analyzes revealed a strong enrichment of fragments fused between sites A2 containing the ARBS and E2 containing the E2FBS, compared with noncross-linked cells. This enrichment was not observed neither in siE2F1-transfected cells, in siAR-transfected cells, nor in cells not stimulated with androgens. Other tested regions from the *ATAD2* regulatory region gave negative results.

We could then conclude that a DNA loop was formed between the ARBS and the E2FBS of the regulatory region in the presence of AR and E2F1 only when the cells were treated with R1881.

Cooperation between AR and E2F1 is a general mechanism for several androgen-regulated genes in prostate cells

We next wanted to check whether the regulation of the *ATAD2* gene expression by AR and E2F1 might be generalized to other genes regulated by androgens in prostate cells, particularly to those overexpressed in prostate cancer. To check this hypothesis, we first compared two GEO datasets from AR ChIP-seq data and E2F1 ChIP-seq data (Fig. 4A) to identify candidate genes regulated by both AR and E2F1 (22, 23). We compared this analysis to microarray data for genes overexpressed in prostate cancer (24). This analysis revealed that among the 94 genes overexpressed in an adenocarcinoma specimen, 12 had both an ARBS and an E2FBS. qRT-PCR experiments in LNCaP cells showed that out of these 12 genes, six displayed the same expression pattern as that of *ATAD2* (Fig. 4B and Supplemental Table 1). Indeed, their mRNA levels were up-regulated in the presence of R1881, and siAR or siE2F1 completely abolished their androgen regulation. *GAPDH* was used as a negative control. As expected, an E2F1-dependent but AR-independent gene, *Cyclin D1*, was down-regulated only in siE2F1-transfected LNCaP cells (Supplemental Fig. 9), demonstrating that not all E2F1-dependent genes might be regulated by androgens.

We next performed a Re-ChIP experiment on the promoters of these six E2F1 and AR target genes. Figure 4C shows that AR and E2F1 were recruited on both the ARBS and the E2FBS of each of these six genes in the presence of R1881. Importantly, genes that did not have ARBS and E2FBS in their promoter like the GAPDH gene, or genes that had ARBS and E2FBS but did not follow the same expression pattern as *ATAD2* in Fig. 4B like PBX1 and TANK genes, did not show any enrichment in Re-ChIP experiments. These results strongly suggest that all these six genes share the same mechanism of regulation of expression as *ATAD2* and that AR and E2F1 interaction in the chromatin context has an active role in their transcriptional regulation. The functional cooperation and physical interaction of AR and E2F1 in terms of regulation of gene expression might then be a widely shared mechanism among genes regulated by androgens in prostate cells.

Discussion

The identification of the entire signaling pathway mediated by androgens in prostate cells is a prerequisite for

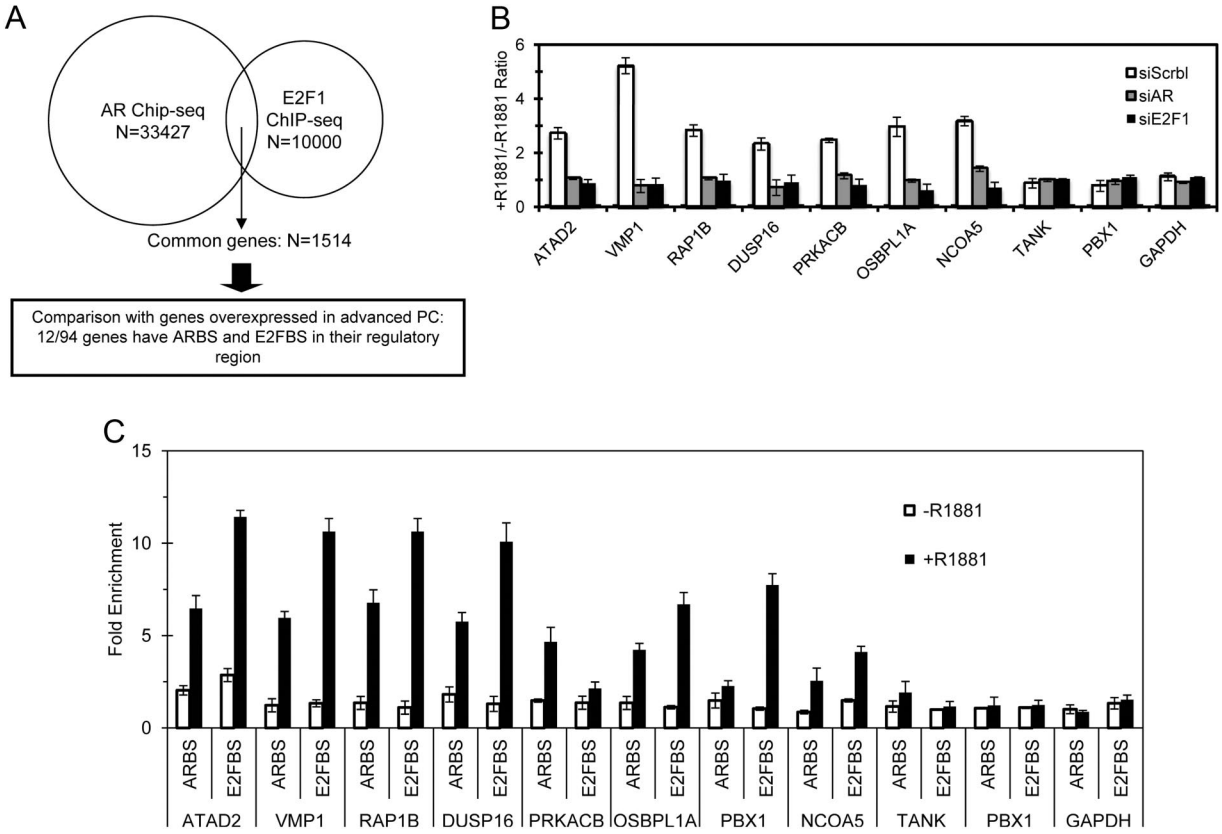

FIG. 4. A, Venn diagram comparing the GEO datasets. AR ChIP-seq data (GSM686926) and E2F1 ChIP-seq data (GSM558469) were compared in order to identify genes the expression of which might be regulated by AR and E2F1. The result of this analysis was compared with microarray data performed on an adenocarcinoma specimen (GSD2171) to focus on genes expressed in prostate cancer. B, Expression profile of genes identified using the analysis described in A in LNCaP cells transfected with scrambled siRNA, siAR, or siE2F1. Results show the ratio between R1881-treated and nontreated cells. Results are means ± SD. C, Re-ChIP on LNCaP cells treated or not with R1881 as explained in Fig. 3B. qPCR was carried out on the ARBS and E2FBS of each gene. Results are fold enrichment on control IgG.

understanding the molecular basis of prostate cancer and more specifically the escape of prostate tumor cells from androgen regulation. Although many published studies have reported genes whose expression might be modified by androgen stimulation of prostate cells (25–31), very few true target genes of the AR in these cells have been identified so far. Moreover, prostate tumorigenesis is a multistep mechanism, which involves, in most cases, cross talk between multiple signaling pathways. Among these, the interaction between the AR and RB/E2F pathways was shown to be crucial in CRPC. Indeed, AR pathway alterations and loss of the *RB1* gene locus are very frequent in CRPC (11, 32, 33). In addition, in prostate cancer cells, loss of the *RB1* gene locus induces up-regulation of AR mRNA levels and enhanced AR activity in its target genes (11). Importantly, in CRPC samples, Sharma *et al.* (11) observed a correlation between AR mRNA and E2F1 mRNA levels but not with E2F3 mRNA level. Thus, E2F1 might be the major actor in the RB/E2F/AR axis. However the precise mechanisms of interaction between the AR and E2F pathways remain to be discovered.

Because E2F1 regulates the transcription of genes required for the cell cycle and because AR is essential for the growth of prostate cancer cells, we hypothesized that common target genes of both E2F1 and AR might play important roles in prostate tumorigenesis (12, 13, 26, 34). We were interested in the *ATAD2* gene because its expression had previously been shown to be enhanced by androgens in prostate cells (18). We have identified within the distal enhancer region of *ATAD2* a putative ARBS. Interestingly, this ARBS is nearly identical to that of the glucocorticoid-responsive elements, for which AR binding has been demonstrated (35). Moreover, it was revealed to bind to AR *in vitro* and *in vivo*. The *ATAD2* gene had previously been shown to be transcriptionally regulated by the E2F transcription factor (17). The E2FBS is located in the proximal promoter region of the gene. Although regulation of *ATAD2* gene expression by E2F1 proteins in prostate cells has never been reported, we show here that E2F1 binds directly to this binding site in prostate cells and that this binding is independent of the presence of androgens. We also show that AR binds in-

directly to this same site, and ChIP analyses suggested that AR and E2F1 might interact between each other on the E2FBS within the chromatin. Indeed, 3C and Re-ChIP experiments showed that the two transcription factors interact through a DNA loop formed between ARBS and E2FBS in an androgen- and E2F1-dependent manner. The role of E2F1 is essential in the androgen activation of *ATAD2*, because down-regulating the E2F1 expression with specific siRNA abrogated the response of ATAD2 to androgens. Importantly, siE2F1 also lowered the basal level of expression of *ATAD2*, whereas siAR did not. Because E2F1 can bind to the ATAD2 proximal promoter region without androgens, its role might be to induce basal level of expression of *ATAD2*. AR induces *ATAD2* expression in the presence of androgens in collaboration with E2F1, like in an enhanceosome (36). Our work clearly shows that interaction between AR and E2F1 within the chromatin context is necessary to activate *ATAD2* gene transcription in response to androgens in prostate cells.

Next, we compared AR ChIP-seq and E2F1-ChIP-seq data, and we extended our analysis to other genes expressed in prostate cancer samples (22–24). Among 94 genes overrepresented in Huang *et al*. (24) dataset, 12 had an ARBS and an E2FBS. We showed that, in LNCaP cells, six of these genes responded to androgens, and this activation was abolished by siE2F1 or siAR. In addition, Re-ChIP data showed that AR and E2F1 interact with each other in the chromatin of all these six genes. Using this approach, we then identified six new direct AR target genes. We anticipated that a chromatin conformation recalling the looping between ARBS and E2FBS described on ATAD2 gene might also control the regulation of these genes by androgens.

Identification of this dual AR/E2F1 regulatory pathway opens up new ways to understand gene deregulation in prostate cancer. Indeed, in RB-deficient prostate tumors, E2F1 becomes fully active to cooperate with AR and to enhance expression of target genes. Then, the mechanism we propose can help identifying potentially new crucial targets of the AR/RB/E2F axis. Interestingly, several of the new target genes we identified to be regulated through this pathway are involved in cell proliferation, apoptosis, and tumor development. For example, *ATAD2* exhibits antiapoptotic activity in prostate and breast cancer cells (18, 37), and the protein is a partner of MYC oncoprotein. RAP1B is overexpressed in gliomas and is implicated in angiogenesis (38, 39). DUSP16 regulates c-Jun N-terminal kinase signaling and apoptosis (40, 41). VMP1 interacts with USP9x and is involved in the authophagy pathway to prevent pancreatic cells from apoptosis (42). PRKACB phosphorylates and inhibits the proapoptotic BAD protein (43), and finally, NCOA5, a nuclear receptor coactivator, has been shown to regulate the transcription of the *c-Myc* gene (44).

Identification of these target genes and of their common regulation through a specific AR/RB/E2F axis might then open up new ways for therapeutic targeting strategies. Indeed, deciphering intimate molecular mechanisms of interaction between E2F1 and AR within the chromatin of target genes will provide new approaches for abrogating functional cooperation between both transcription factors, particularly in CRPC.

Materials and Methods

Plasmid constructs

To analyze the ATAD2 distal enhancer region, 1.5 kb of the gene promoter region (NC000008.10 regions from 124418705 to 124417179) were amplified by PCR using the following primers: ggtacccatctgttgtgttctggaattttg and ctcgagccctgtttccaaatcagatcaca. The pGL3-ARE promoter-driven reporter construct was made by inserting this product into the pGL3-Promoter Vector (Promega, Madison, WI). The ARE site was mutated using the Stratagene QuikChange II XL Site-Directed Mutagenesis kit (200523; Stratagene, La Jolla, CA). The pGL3-ARE vector was amplified with the following primers: cctagataaagaagcacagtactttgcctagcccaaagtatgc and gcatactttgggctaggcaaagtactgtgcttctttatctagg; 1 kb of ATAD2 proximal promoter region containing E2FBS was amplified by PCR using following primers: ggtaacccttaactaaacgatggcactct and aagcttgagatccagctccaggcgctcg (E1 fragment). Deletion mutants were obtained by PCR by shortening the E1 fragment each time by 100 pb using following primers: ggtaacagtcaaaacctgtcaaaggccaa for E2, ggtaacgggaagcagcaagcaggtgtcaa for E3, and ggtaacagaagtcagcgttttccccagga for E4. PCR products carried *Kpn*I/*Hin*dIII sticky ends and were ligated to a pGL2 vector (Promega).

Cell culture and transfection experiments

LNCaP cells were cultured as described previously (45). For transfection experiments, cells were plated in 96-well plates and cotransfected with either 0.75 μg of pGL3, pGL3-ARE, pGL3-mt, pGL2, pGL2-E1, pGL2-E2, pGL2-E3, or pGL2-E4 and either 7.5 pmol scramble siRNA or siE2F1 is used. For normalization with the β-galactosidase system, 0.125 μg of a strong cytomegalovirus promoter-containing vector was transfected. Transfections were carried out using Hiperfect (QIAGEN, Valencia, CA) reagent following the manufacturer's instructions. Cells were treated with 0.1 nM R1881, a synthetic androgen, for 36 h.

RWPE-1 and RWPE-1-AR cells were described previously (46).

Reverse transcription qPCR

RNA was extracted from cells using TRIzol reagent (MRC-Gene TR118) following the manufacturer's instructions. One microgram of this RNA was retrotranscribed (Superscript III 18080–044; Invitrogen, Carlsbad, CA). The cDNA obtained was amplified by qPCR using the QuantiTect SYBR Green PCR

kit (204145; QIAGEN). Primers used for qPCR are listed in Supplemental Table 2.

Western blotting

Cell lysates were prepared as described previously (46). Membranes were incubated with a polyclonal anti-E2F1 antibody (sc-193; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a polyclonal anti-AR antibody (PG-21; Millipore, Bedford, MA) and secondary antibodies coupled to peroxidase (antimouse IgG horseradish peroxidase W402B and antirabbit IgG horseradish peroxidase W401B; Promega). Equal loading was checked with a monoclonal antimouse antibody (sc-130301; Santa Cruz Biotechnology, Inc.).

Electrophoretic mobility shift assay

ATAD2-ARBS oligos were 5′ ^{32}-P end-labeled with T4 polynucleotidyl tranferase (Fermentas, Amherst, NY) and hybridized with the respective complimentary oligos to obtain ATAD2-ARBS double-stranded (ds)DNA. The oligo sequences used were: specific WT oligo, GATAAAGAAGCACAGTACAGAACAGAGTGTACAGTATGGTACATTTGTG and nonspecific mutant oligo, GATAAAGAAGCACAGTACTTTGCCTAGCCCAAAGTATGGTACATTTGTG. The AR-DBD domain (amino acids 524–648) fused to a 6xHis tag was expressed in *Escherichia coli* cells transformed using a pET-3 expression vector (211521; Stratagene). Recombinant protein was purified on Nickel-NTA agarose (30230; QIAGEN), following the manufacturer's instructions (Supplemental Fig. 3).

Binding between AR-DBD and DNA was performed in a 15-μl reaction volume containing 10 mM Tris (pH7.4), 100 mg/ml BSA, 50 mM NaCl, 2 mM $MgCl_2$, 0.01% Nonidet P-40, 1 mM dithiothreitol, and 5% glycerol (binding buffer). Briefly, 25 fmol labeled dsATAD2-ARBS oligonucleotides was incubated with increasing amounts of AR-DBD protein (see Fig. 2 for details) at room temperature for 10 min in the binding buffer, as described above. Reaction products were resolved by 5% native PAGE in 0.25× Tris-borate EDTA; gels were dried, exposed, and visualized on a phosphorimager (Fuji-FLA5100; Fuji, Tokyo, Japan).

For the competition experiments, AR-DBD binding was performed on the specific 5′ ^{32}-P end labeled ATAD2-ARBS dsDNA, as described above. Unlabeled dsDNA containing specific WT-ARBS or nonspecific mutant sequence was added to the reaction in increasing amounts and incubated for another 10 min at room temperature.

DNase I footprinting

Protein binding reactions on the 5′ ^{32}-P dsDNA fragments were performed as described above. DNase I (0.1 U; Invitrogen) was added to each reaction and incubated at room temperature for 2.5 min; 100 μl of Stop buffer (0.1% sodium dodecyl sulfate and 20 mM EDTA) were added to arrest the DNase I digestion. DNA was phenol:chloroform extracted, ethanol precipitated, and resolved on a 13% denaturing urea PAGE.

Coimmunoprecipitation

Nuclear extracts from 20 million LNCaP cells were prepared as it was previously described (46), and lysates were incubated with either anti-AR (PG-21; Millipore) or anti-E2F1 (sc-193; Santa Cruz Biotechnology, Inc.) antibodies. See Fig. 3A for details.

ChIP and Re-ChIP

ChIP and Re-ChIP experiments were carried out as described earlier (46). Primer sequences used for ChIP experiments are listed in Supplemental Table 3.

Chromosome conformation capture

3C experiments were carried out as previously described (21). The design of the experiment can be found in Fig. 3F. Primer sequences are listed in Supplemental Table 3.

Knockdown of gene expression using siRNA

Ten thousand LNCaP cells were transfected with either 20 μM E2F1 siRNA (SI00300083), AR siRNA (SI02757258), or nontargeting pool (D-001810-10; Dharmacon, Lafayette, CO) in the presence of 4 μl of HiPerFect Transfection Reagent (QIAGEN) for 48 h, following the manufacturer's instructions. RNA was collected, and target gene activation was determined by RT-qPCR. For ChIP experiments, 20 million cells were used for transfection, following the manufacturer's instructions.

Data analysis

AR ChIP-seq data (GSM686926) and E2F1 ChIP-seq data (GSM558469) were compared using Microsoft Access. The resulting table was compared with microarray data from prostate cancer samples (GSD2171) using Microsoft Access.

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Address all correspondence and requests for reprints to: Jacques Samarut, Institut de Génomique Fonctionnelle de Lyon, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, F-69346 Lyon Cedex 07, France. E-mail: jacques.samarut@ens-lyon.fr.

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