

NAR Breakthrough Article

Three-tiered role of the pioneer factor GATA2 in promoting androgen-dependent gene expression in prostate cancer

Dayong Wu^{1,†}, Benjamin Sunkel^{1,2,†}, Zhong Chen¹, Xiangtao Liu¹, Zhenqing Ye³, Qianjin Li¹, Cassandra Grenade¹, Jingdong Ke¹, Chunpeng Zhang¹, Hongyan Chen^{1,4}, Kenneth P. Nephew⁵, Tim H.-M. Huang³, Zhihua Liu⁴, Victor X. Jin³ and Qianben Wang^{1,2,*}

¹Department of Molecular and Cellular Biochemistry and the Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, OH 43210, USA, ²Ohio State Biochemistry Graduate Program, The Ohio State University, Columbus, OH 43210, USA, ³Department of Molecular Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA, ⁴State Key Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China and ⁵Medical Sciences Program and Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Bloomington, IN 47405, USA

Received August 13, 2013; Revised November 26, 2013; Accepted December 15, 2013

ABSTRACT

In prostate cancer, androgen receptor (AR) binding and androgen-responsive gene expression are defined by hormone-independent binding patterns of the pioneer factors FoxA1 and GATA2. Insufficient evidence of the mechanisms by which GATA2 contributes to this process precludes complete understanding of a key determinant of tissue-specific AR activity. Our observations suggest that GATA2 facilitates androgen-responsive gene expression by three distinct modes of action. By occupying novel binding sites within the AR gene locus, GATA2 positively regulates AR expression before and after androgen stimulation. Additionally, GATA2 engages AR target gene enhancers prior to hormone stimulation, producing an active and accessible chromatin environment via recruitment of the histone acetyltransferase p300. Finally, GATA2 functions in establishing and/or sustaining basal locus looping by recruiting the Mediator subunit MED1 in the absence of androgen. These mechanisms may contribute to the generally positive role of GATA2 in defining AR genome-wide binding patterns that determine androgen-responsive gene expression profiles. We also find that GATA2 and FoxA1

exhibit both independent and codependent co-occupancy of AR target gene enhancers. Identifying these determinants of AR transcriptional activity may provide a foundation for the development of future prostate cancer therapeutics that target pioneer factor function.

INTRODUCTION

Androgen signaling mediates diverse and complex functions throughout the body ranging from skeletal development and maintenance to spermatogenesis on ligand activation of the androgen receptor (AR) (1). A member of the nuclear hormone receptor family, AR mediates androgen-dependent gene expression following release from cytoplasmic heat-shock proteins, receptor phosphorylation and nuclear translocation whereupon AR homodimers bind to recognition sites within regulatory elements of target genes (2). This mechanism of hormone-dependent gene expression facilitates the fetal development of the prostate and regulates maintenance and normal function of the prostate secretory epithelium (1,3). In androgen-dependent prostate cancer (ADPC), androgen-stimulated AR function plays a vital role in the aberrant proliferation of epithelial cells, thus androgen deprivation therapy (ADT) precipitates marked disease regression (3,4). As is too often the case

*To whom correspondence should be addressed. Tel: +1 614 247 1609; Fax: +1 614 688 4181; Email: qianben.wang@osumc.edu

†These authors contributed equally to the paper as first authors.

however, reestablished AR activity leads to ADT resistance, marking the fatal progression to castration resistant prostate cancer (CRPC) (5–7). How tissue-, cell type-, and disease stage-specific patterns of androgen-stimulated gene expression are generated is an area of considerable research focus, and a complete picture of the activities determining this specificity is required for our understanding of androgen-driven prostate cancer progression and for the rational design of potent prostate cancer therapeutics.

In nuclear receptor (NR)-regulated gene transcription, pioneer factor function has been studied extensively, providing information on the manner in which tissue-specific, hormone-responsive gene expression is controlled (8–10). Pioneer factors are characterized by their ability to engage compact chromatin, initialize local chromatin decondensation and provide an environment amenable to the recruitment of transcription factors and activation of transcription (10). Their role in determining NR target gene expression was suggested on analysis of common DNA sequence motifs enriched within genome-wide NR binding sites identified using chromatin immunoprecipitation combined with tiled oligonucleotide microarrays (ChIP-on-chip) or high-throughput sequencing technology (ChIP-seq) (11–15). Two key pioneer factors emerged from complementary analyses in distinct hormone-related cancers, breast and prostate, driven by estrogen receptor (ER) and AR transcriptional activity, respectively. Significantly enriched within ER and AR binding sites were motifs recognized by the Forkhead box (FOX) and GATA pioneer transcription factor families (10,12,13,16,17).

FOX proteins, specifically the FOXA subclass, have attracted considerable attention for their capacity to impact local chromatin architecture, their essential role in priming for temporal patterns of gene expression observed in early organogenesis and their influence over tissue-specific patterns of NR target gene expression, of particular interest in hormone-related cancers (9,10,18,19). In particular, FoxA1, expressed alongside ER and AR in the developing and mature mammary and prostate ductal epithelia, respectively (20–23), has been shown to contribute significantly to maintaining the oncogenic functions of these nuclear receptors in both treatment-sensitive and -resistant breast and prostate cancers (11,16). While the role of FoxA1 in ER-positive breast cancers appears to be the positive regulation of ER-chromatin binding, resulting in canonical and noncanonical ligand-dependent ER target gene expression and breast cancer cell proliferation (11,24), a more complex relationship between AR and FoxA1 exists in prostate cancer. Initial studies described an essential role of FoxA1 in directing AR-chromatin binding for the activation of androgen-dependent gene expression (16,25). However, a complementary analysis later revealed that FoxA1 silencing resulted in the expected loss of many AR binding events as well the surprising gain of additional AR binding sites not observed in parental cells, suggesting both exclusionary and facilitative roles for FoxA1 in directing AR binding events and determining androgen-dependent gene expression (26,27).

While GATA family proteins have long since been identified as putative pioneer factors acting in concert with FOX proteins in establishing temporal gene expression patterns required for fetal liver development (28,29), progress in understanding GATA pioneer function in NR-driven cancers has been largely limited to ER activity. GATA3 expression patterns positively correlate with both ER and FoxA1 in the developing, mature and cancerous mammary epithelium, and are in fact necessary for determining hormone-responsive gene expression and breast cancer cell proliferation (30–34). As stated, prostate cancer-specific AR binding sites are also significantly enriched in GATA motifs, and GATA2, overexpressed in high-risk prostate cancer, has been shown to play an essential role in productive AR-chromatin binding resulting in androgen-responsive gene expression (12,16,35). Given existing evidence for the central role of GATA family members in defining transcription factor activity, the demonstrated function of GATA factors in facilitating hormone-dependent gene expression, and the dearth of information on the mechanism by which GATA proteins contribute functionally to NR-transcriptional regulation, further analyses are needed to elucidate the means by which GATA pioneer factors function in hormone-related cancers.

In the present work, we focus specifically on the role of GATA2 in recruiting AR to distal enhancers elements of androgen-responsive AR target genes in androgen-responsive prostate cancer cell lines. We provide evidence that GATA2 acts at multiple levels to contribute positively to AR transcriptional activity by enhancing AR expression itself, facilitating AR-enhancer binding by establishing an accessible local chromatin environment, and enhancing AR target gene expression through involvement in the formation and maintenance of regulatory chromatin loops between AR-bound distal enhancers and AR target gene promoters. These mechanisms may account for the generally positive role of GATA2 in defining global AR binding that regulates AR target gene expression. Additionally, we reveal a complex relationship between FoxA1 and GATA2 in mediating AR expression and in site-specific AR recruitment facilitating AR target gene expression.

MATERIALS AND METHODS

Cell culture

The prostate cancer cell line LNCaP was purchased from the American Type Culture Collection (ATCC), and C4-2B cells were purchased from ViroMed Laboratories. These cells were maintained in RPMI 1640 media (Invitrogen) supplemented with 10% FBS at 37°C in 5% CO₂. For individual experiments, the medium was replaced by phenol red-free RPMI 1640 medium containing 5% charcoal-stripped FBS. The cells were passaged in our laboratory for less than 6 months after resuscitation.

RNA interference

Control siRNA (siControl) and siRNAs targeting AR, GATA2, FoxA1 (ON TARGET plus™ siRNA) were

purchased from Dharmacon (Dharmacon, Lafayette, CO). siRNA transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours before transfection, LNCaP cells are seeded at a density of 5×10^5 cells/well for six well plates or 8×10^6 cells/150 mm dish. The cells are transfected with 40 nM siRNA as described in the manufacturer's protocol and maintained for 72 h in hormone-free medium. Then the cells are harvested or treated with R1881 for an additional 4 h before harvest. The sequences for siRNAs are listed in Supplementary Table S1.

Western blots

Western blot analyses were carried out as previously described (36). Briefly, LNCaP cells were collected and lysed in RIPA lysis buffer [1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, proteinase inhibitor cocktail (Roch)] for 20 min on ice, and the proteins were resolved on 10% SDS-polyacrylamide gels before being transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% milk powder (Bio-Rad) in $1 \times$ TBS containing 0.5% Tween-20 for 1 h, washed with TBS/Tween, and incubated with the specific antibodies for 2 h. Antibodies used are listed in Supplementary Table S2.

Quantitative real-time RT-PCR

Real-time reverse transcriptase PCR (qRT-PCR) was carried out as previously described (36). Total RNA was isolated from LNCaP cells with the RNeasy Mini kit (Qiagen, 74104). QRT-PCR was conducted on 2 μ g of RNA by using the MultiScribe Reverse Transcriptase and Power SYBR Green PCR Master Mix reagents (Applied Biosystems), according to the manufacturer's instructions. Each reaction was performed in triplicate. The primers used are listed in Supplementary Table S1.

ChIP and ChIP sequencing

ChIP was performed as previously described (37) with a few modifications. Briefly, LNCaP cells (1×10^7 cells/150-mm dish) were plated and grown in phenol red-free RPMI 1640 supplemented with 5% charcoal-stripped FBS for 3 days (Cells were treated with 1 nM R1881 for an additional 4 h as required). Cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Chromatin was then sonicated, diluted, and immunoprecipitated with specific antibodies (Supplementary Table S2) at 4°C overnight. Protein A-Sepharose beads were added and incubated for 1 h with rotation. The beads were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and finally two times with TE buffer. After being drained with a 27 G \times 8 in. needle (Becton Dickinson NJ), the beads are extracted two times with 60 μ l 1% SDS, 0.1 M NaHCO₃ by vortexing in a Thermomixer (Eppendorf) and pooled eluates were heated at 65°C for 16 h to reverse the cross-linking.

DNA fragments were purified with the QIAquick PCR purification kit (Qiagen 28104) and used as the template in quantitative PCR reactions. Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used, and the samples were amplified with the StepOnePlus Real Time PCR System (Applied Biosystems). The primers used in real-time PCR are listed in Supplementary Table S1. ChIP-seq library preparation was performed using the Truseq ChIP sample preparation kit (part# 15023092) with 10 ng of purified ChIP-DNA. The libraries were amplified using 15 PCR cycles, and sequencing was performed on the Illumina HiSeq 2500 platform at the Ohio State University Comprehensive Cancer Center (OSUCCC) sequencing core (50 bp read length, single-end), with four multiplexed samples per lane. ChIP-seq reads were aligned against the standard hg19 build of the human reference genome with Bowtie v1.0.0 (38), allowing at most two mismatches. Reads with more than one valid alignment were removed, leaving only uniquely mapped reads for further analysis. Peaks were called using MACS v1.4.2 (39) with *P*-value threshold 1.0e-8. Heatmap counts were generated using HOMER v4.3 (40) with bin size 50 bp. The raw ChIP-seq data have been submitted to the Gene Expression Omnibus (GEO) repository under the accession number GSE52725.

Formaldehyde-assisted isolation of regulatory elements

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was carried out as previously described (41). Briefly, formaldehyde was added directly to the cell culture medium at room temperature to a final concentration of 1% and incubated for 10 min. 1 M glycine was added to a final concentration of 125 mM for 5 min at room temperature to quench the formaldehyde. Cells were rinsed with phosphate buffered saline and collected in 1.5-ml tubes. After brief centrifugation, the cell pellets were resuspended in 400 μ l of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl at pH 8.0, 1 mM EDTA) and incubated on ice for 5 min. Samples were then sonicated for 7 min (30 s on/off cycles) using a Bioruptor (Diagenode) at the highest intensity. The cell lysates were centrifuged at 13 000 rpm for 10 min at 4°C to precipitate cellular debris, and the soluble chromatin was transferred to a new tube. After two rounds of phenol-chloroform extraction, the aqueous phase was combined and incubated at 65°C overnight to reverse cross-linking. DNA was purified with the Qiagen PCR purification Kit (Qiagen 28104) and eluted in 100 μ l TE buffer. Relative enrichment in the FAIRE-treated DNA was calculated with DNA from untreated cells serving as the control. All primer sequences are listed in Supplementary Table S1.

Quantitative chromosome conformation capture assay

Quantitative chromosome conformation capture (3C) qPCR assays were performed as described (16) with some modifications. Briefly, nuclei were first cross-linked with formaldehyde at a final concentration of 1% for 10 min, then digested with 400 units of Bgl II (NEB) and

ligated under extra-diluted conditions. After reversing the cross-linking, DNA was purified by phenol–chloroform extraction followed by ethanol precipitation. Real time PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems). The probe and primer sequences are listed in Supplementary Table S1. The data were normalized for primer efficiency differences using BAC RP11-197J19 that covers the ABCC4 locus. GADPH loading control was used to normalize DNA concentration. The interaction of two Bgl II sites in the GADPH locus was used for comparison between different 3C assays.

RESULTS

Genome-wide overlap of AR/GATA2/FoxA1 binding

To assess the global overlap of pioneer factor chromatin occupancy with hormone-stimulated AR-DNA binding, we began by performing ChIP-seq in the androgen-responsive prostate cancer cell line LNCaP. GATA2 and FoxA1 ChIP-seq assays were performed in the absence of hormone, while AR ChIP-seq was performed in the presence of hormone to provide a picture of the extent to which basal pioneer factor occupancy determines androgen-dependent AR binding. The four lowermost segments of Figure 1A display the density of aligned ChIP-seq reads for each factor within a 10-kb window centered over AR binding site locations. Notably, only 45% of all AR binding events occur in regions without prior pioneer factor occupancy, indicating that while additional factors likely facilitate AR genome-wide binding patterns, in this prostate cancer cell context, coordinate binding of GATA2, FoxA1 or both is a key feature of AR chromatin occupancy. That combinatorial pioneer factor activity is a critical determinant of AR distribution is suggested by the observation that 13 457 sites exhibit AR/GATA2/FoxA1 binding, while 15 342 and only 5036 AR binding sites overlap only with FoxA1 or GATA2, respectively (Figure 1A and B). Based on these findings, we went on to characterize potential mechanisms by which these pioneer factors, in particular the lesser-characterized GATA2, facilitate AR binding to DNA in advance of androgen-responsive gene expression.

GATA2 promotes androgen receptor expression

A previous study reported putative GATA2 binding sites within the *AR* gene promoter in LNCaP cells, suggesting a potential influence of GATA2 over AR-target gene expression via direct regulation of *AR* itself (35). Surprisingly, our GATA2 ChIP-seq and standard ChIP analyses in LNCaP cells did not reveal a significant enrichment of GATA2 binding within this previously described region, constituting the promoter of the minor AR(A) isoform (42) (Figures 2A and B). In contrast, two significant GATA2 binding peaks were observed within regulatory elements of the full-length AR(B) isoform (referred to henceforth simply as AR): one 4.6 kb upstream of the TSS, overlapping a FoxA1 binding site (Site 1), and the second within the AR promoter region (+600 bp, Site 2) (Figure 2A). While GATA2 binding to

the promoter of the minor AR(A) isoform was previously shown to depend on androgen stimulation (35), ChIP assays for GATA2 and FoxA1 in LNCaP cells revealed no such reliance on hormone stimulation, revealing that these pioneer factors engage *AR* regulatory elements prior to androgen induction of AR expression (Figure 2B).

To evaluate the relative contribution of each pioneer factor to the regulation of AR expression, we individually reduced GATA2 and FoxA1 expression by siRNA transfection and measured AR mRNA and protein levels before and after stimulation with the synthetic androgen R1881. siGATA2 transfection resulted in a significant reduction in AR mRNA levels prior to and following hormone stimulation (Figure 2C), concordant with the observed reduction in GATA2 recruitment to the *AR* promoter and upstream regulatory elements (Supplementary Figure S1A). Surprisingly, in hormone-depleted conditions, siFoxA1 transfection resulted in an increase in AR mRNA (Figure 2C) despite loss of FoxA1 binding to the upstream regulatory element (Supplementary Figure S1B). These results were reflected in the depletion of AR protein levels following siGATA2 transfection in the presence and absence of hormone and in the moderate accumulation of AR protein following siFoxA1 transfection in the absence of hormone (Figure 2D). Notably, while siFoxA1 transfection resulted in an accumulation of GATA2 protein in the absence of hormone, this observation does not initially explain the concomitant increase in AR protein level, as no significant increase in GATA2 binding to the upstream AR regulatory element was observed under these conditions (Supplementary Figure S1C).

A previous report from Cai *et al.* found that FoxA1-mediated binding of AR to a downstream repressor region establishes a negative feedback loop to control AR expression (43). We performed AR and FoxA1 ChIP using primers for the reported repressor element, and found that, consistent with previous results, hormone stimulation enhances occupancy of both factors to this region, enhancing the negative feedback loop. Following siFoxA1 transfection, basal AR occupancy of the repressor was significantly reduced in accordance with enhanced AR expression due to feedback loss. Hormone stimulation following FoxA1 knockdown was sufficient to enhance AR repressor binding and reestablish appropriate feedback signaling (Supplementary Figure S1D), explaining our observed expression patterns following pioneer factor knockdown. We also performed cycloheximide (CHX) chase assays in LNCaP cells transfected with Control-, GATA2-, or FoxA1-targeting siRNAs to rule out the impact of AR protein stabilization and/or destabilization on the observed pattern of AR accumulation, though only a marginal effect of pioneer factor silencing on AR half-life was observed (Supplementary Figure S1E). While additional factors must be considered to gain a full understanding of pioneer factor function in regulating *AR* expression, these results provide evidence that GATA2 is capable of binding AR regulatory elements independent of hormone status and is integral to both basal and androgen-stimulated AR expression patterns. In this way, GATA2 contributes positively to androgen-

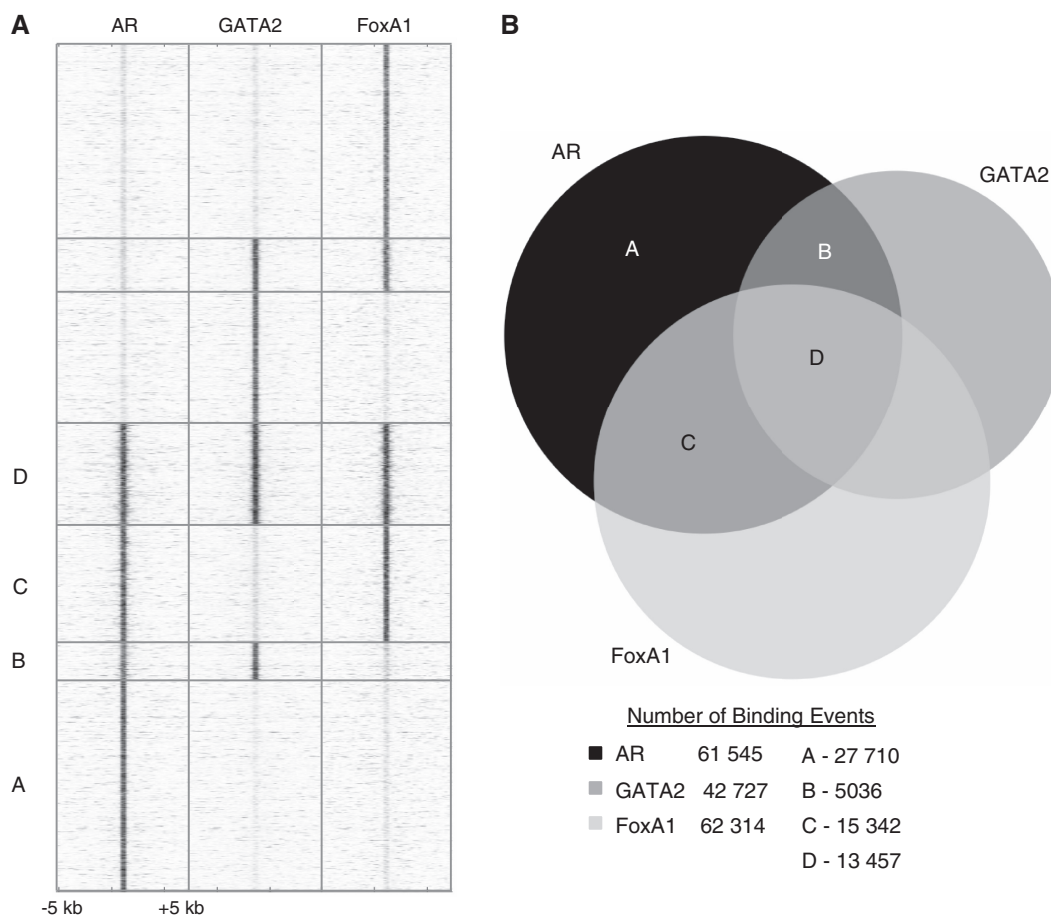


Figure 1. Genome-wide overlap of AR, GATA2, and FoxA1. (A) Heatmap displaying the density of aligned ChIP-seq reads for AR (left), GATA2 (center) and FoxA1 (right) within a 10-kb window centered over individual and shared factor binding sites. Lettered segments indicate factor occupancy in four classes of AR binding sites: A—AR only, B—AR/GATA2, C—AR/FoxA1 and D—AR/GATA2/FoxA1. (B) Venn diagram representing the proportions of shared factor binding sites relative to total factor binding sites. Lettered regions are consistent with panel (A), and binding site numbers are provided for each.

responsive AR target gene expression by direct up-regulation of *AR*.

GATA2 enhances AR target gene expression: complexity in combinatorial pioneer factor occupancy

While previous studies have demonstrated the importance of pioneer factors in androgen-stimulated AR target gene expression, rigorous analyses have been limited to the role of FoxA1 (16,26,27). As GATA motifs and indeed GATA2 binding events are significantly enriched alongside FOX motifs/FoxA1 binding events overlapping AR occupied regions, and as GATA2 knockdown or chemical inhibition is understood to result in reduction of AR target gene expression (12,44), we chose to systematically evaluate the interplay between GATA2 and FoxA1 in facilitating AR binding to androgen-responsive gene regulatory elements and the impact these factors have on androgen-dependent gene expression. As the vast majority of AR/GATA2 overlapping binding sites occur alongside FoxA1 (Figure 1), we focused our attention on genes apparently coregulated by these three factors. Referring once again to our ChIP-seq datasets, we

identified overlapping AR, GATA2 and FoxA1 binding events within regulatory elements of two androgen-responsive genes that provided informative cases of combinatorial pioneer factor activity: *ABCC4* and *ADPGK*.

ABCC4, which encodes a member of the multidrug resistance protein (MRP) subfamily of ATP-binding cassette transporter proteins, has been shown to exhibit androgen-dependent expression (45). Importantly, three independent studies have suggested that while *ABCC4* mRNA and protein expression is enhanced in primary prostate cancer compared with nonneoplastic prostate tissue, lost expression over time significantly correlates with metastatic disease progression, biochemical recurrence following androgen-deprivation therapy and high Gleason score (45–47). As such *ABCC4* is a clinically relevant androgen-responsive gene product with potential prognostic value in predicting the development of androgen independence in advanced prostate cancer. Intronic androgen-stimulated AR binding in the *ABCC4* locus (29.9 kb downstream of the TSS) was found to occur alongside hormone-independent GATA2 and FoxA1 binding sites (Figure 3A and B). Basal and androgen-stimulated AR recruitment to this site is significantly reduced following

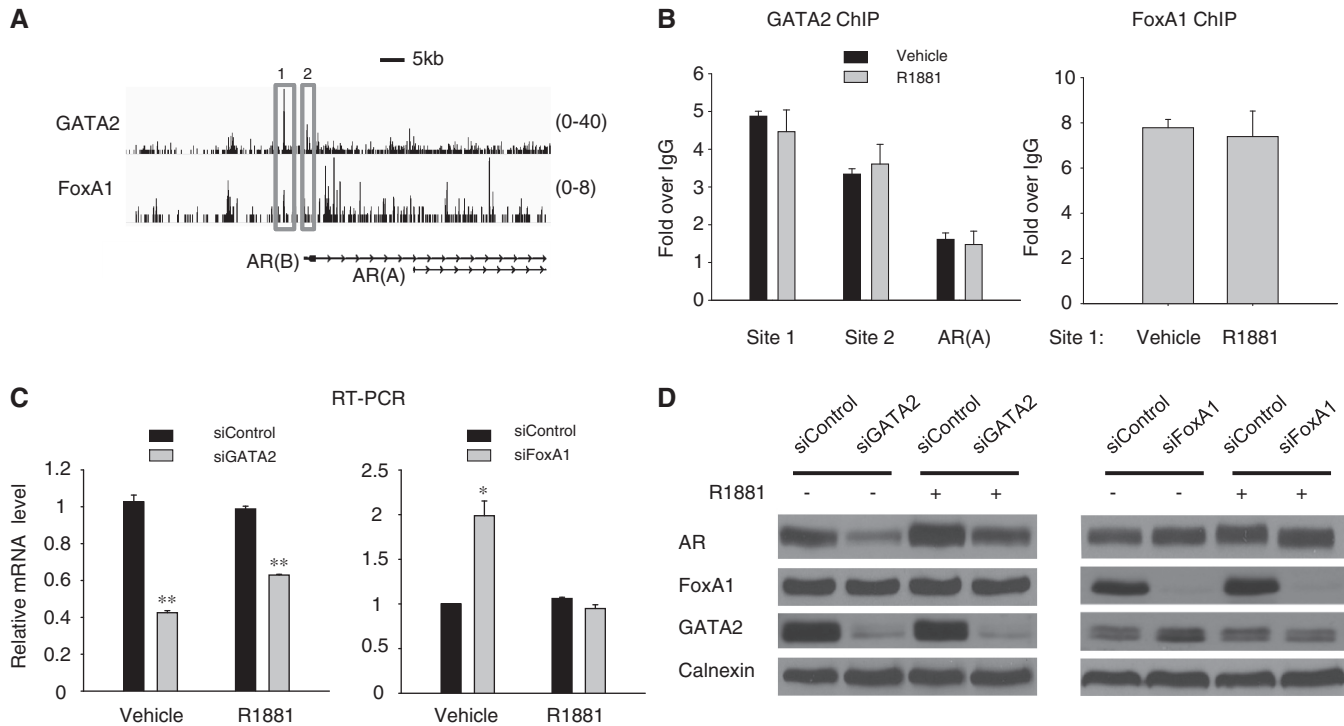


Figure 2. Novel GATA2 binding sites in the *AR* locus. (A) GATA2 and FoxA1 binding sites in the *AR* locus are shown in IGV. Previously reported GATA2 binding site is located immediately downstream of the AR(A) TSS. (B) LNCaP cells were grown for 72 h in the absence of hormone and were then treated with 1 nM R1881 or ethanol for 4 h before harvest. ChIP assays were performed using antibodies against GATA2 or FoxA1. (C) LNCaP cells were transfected with indicated siRNAs. After 72 h, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. Real-time RT-PCR was then performed using *AR* gene-specific primers. (D) LNCaP cells were transfected with siControl, siFoxA1 or siGATA2. After 72 h, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. Western blots were performed with indicated antibodies. * $P < 0.05$, ** $P < 0.01$. Results of ChIP and qRT-PCR assays reported as the mean of two to four replicates, with error bars representing the standard deviation.

siFoxA1 transfection (Supplementary Figure S2A), and following siGATA2 transfection (Supplementary Figure S2B), AR recruitment is reduced nearly to the level observed following RNA interference of AR itself, demonstrating that each factor plays a central role in AR recruitment at this site (Figure 3C). Consonantly, androgen-stimulated *ABCC4* mRNA expression in siGATA2 and siFoxA1 transfected cells was reduced nearly to siAR levels compared with control knockdown (Figure 3D). To assess the hierarchical relationship between AR, GATA2 and FoxA1 binding at this locus, we first characterized GATA2 and FoxA1 recruitment to the *ABCC4* regulatory element following siAR transfection. Neither FoxA1 nor GATA2 enrichment was affected by AR knockdown in the presence or absence of androgen, demonstrating that both factors engage this site prior to androgen stimulation and independently of AR (Figure 3E). In addition, FoxA1 recruitment was unaffected by siGATA2 transfection as was GATA2 recruitment following siFoxA1 transfection, revealing that in this instance, these pioneer factors function independently of one another in binding this regulatory element prior to and following hormone stimulation (Figure 3F).

ADP-dependent glucokinase (encoded by *ADPGK*) was recently reported as a novel androgen-dependent AR target gene with a likely role in defining cellular energy metabolism in LNCaP cells (48). While mechanistic studies of

ADPGK have not been conducted in a model of prostate cancer, a general role for the enzyme in supporting glycolytic energy production under hypoxic stress conditions characteristic of many solid tumors has been proposed, and in fact, *ADPGK* null H460 human lung cancer cells exhibited decreased clonogenic survival in hypoxic conditions compared with parental cells (49). Of potential relevance to prostate cancer is the recent finding that ADPGK activity can induce NF- κ B signaling in response to ROS production (50). The interplay between NF- κ B and AR in the progression of prostate cancer has been of considerable interest and one report suggests that NF- κ B2/p52 up-regulation may be a basis for resistance to the second-generation antiandrogen, enzalutamide (51). Within the *ADPGK* locus, robust AR, GATA2 and FoxA1 binding sites overlap 49.6 kb downstream of the TSS, at an intergenic regulatory element (Figure 4A). Here, androgen stimulation resulted not only in enhanced AR binding but also in increased FoxA1 and, to a lesser extent, GATA2 binding (Figure 4B). This is consistent with previous studies showing significant increases in pioneer factor binding at sites exhibiting adjacent AR and pioneer factor motifs following hormone treatment and likely reflects global changes in the chromatin landscape, allowing for enhanced factor occupancy, or stabilized pioneer factor occupancy owing to interactions with fully assembled DNA-binding transcriptional complexes (12). However, this

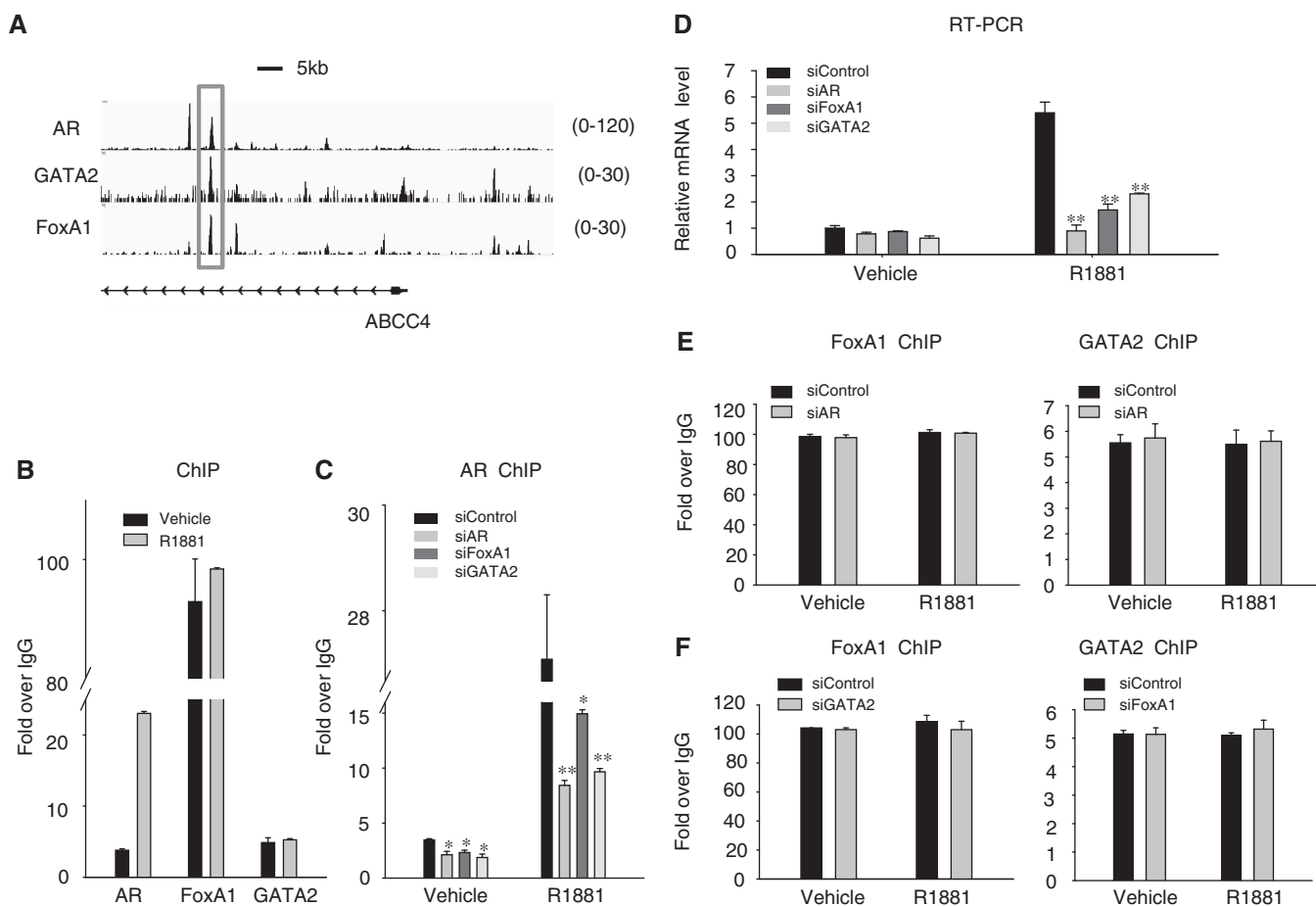


Figure 3. GATA2 regulates *ABCC4* expression. (A) AR, GATA2 and FoxA1 binding sites are shown in IGV. (B) LNCaP cells were grown for 72 h in the absence of hormone and were then treated with 1 nM R1881 or ethanol for 4 h before harvest. ChIP assays were performed using antibodies against AR, GATA2 or FoxA1. (C and D) LNCaP cells were transfected with siControl or factor-specific siRNA in the absence of hormone. After 72 h, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. (C) ChIP assays were conducted with AR-specific antibody, or (D) Real-time RT-PCR was performed using gene-specific primers. (E) LNCaP cells were transfected with siControl or siAR in the absence of hormone. Seventy-two hours after siRNA transfection, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. ChIP assays were performed with indicated antibodies. (F) LNCaP cells were transfected with siControl, siGATA2, or siFoxA1 in the absence of hormone. Seventy-two hours after siRNA transfection, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. ChIP assays were performed with indicated antibodies. * $P < 0.05$, ** $P < 0.01$ Results of ChIP and qRT-PCR assays reported as the mean of two to four replicates with error bars representing the standard deviation.

observation does raise the question of whether androgen-stimulated increases in FoxA1 and GATA2 are a result of pioneer factor-like AR activity, as has been reported for the glucocorticoid receptor (GR), a related hormone-inducible transcription factor (52,53). AR knockdown revealed no impact on GATA2 or FoxA1 occupancy in the absence of hormone, indicating that these factors bind independently of AR at this location before androgen treatment. Yet in the presence of hormone, enhanced AR binding and transcription complex assembly likely stabilizes pioneer factor occupancy, as GATA2 and FoxA1 binding levels following hormone treatment were diminished by siAR transfection (Figure 4C and D). That GATA2 and FoxA1 act to recruit AR to this regulatory element was demonstrated by AR ChIP following siGATA2 or siFoxA1 transfection, as basal and androgen-stimulated AR enrichment was inhibited by knockdown of these pioneer factors (Figure 4E and Supplementary Figures S3A and B). Basal and androgen-stimulated expression of

ADPGK was also significantly inhibited by knockdown of AR, GATA2 or FoxA1, indicating that all three factors are essential for transcriptional activation at this site (Figure 4F). The mechanism behind this strong dependence was readily explained by assessing the interplay between GATA2 and FoxA1 binding to this region. siFoxA1 transfection resulted in a significant loss of GATA2 enrichment before and after hormone treatment, and similar results were obtained for FoxA1 enrichment following siGATA2 transfection (Figure 4G and H). These results indicate that androgen-stimulated AR binding to this regulatory element is facilitated following the codependent binding of GATA2 and FoxA1 to the site, and that loss of either of these pioneer factors results in lost binding of the complementary pioneer factor, binding of AR and expression of *ADPGK* in this context.

To further support these findings, we performed a series of experiments in an additional androgen-responsive prostate cancer cell line C4-2B. Our results suggest that

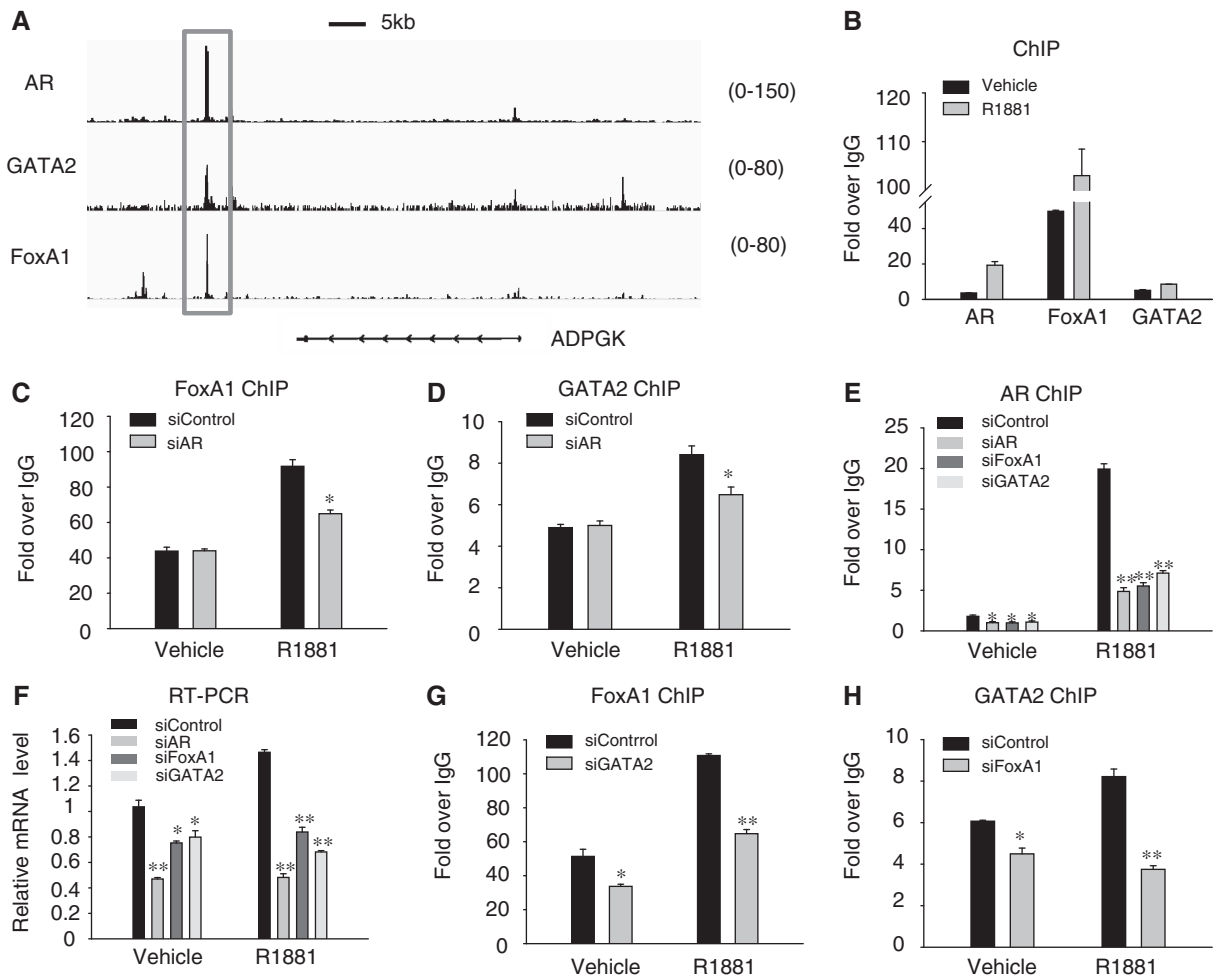


Figure 4. GATA2 regulates *ADPGK* expression. (A) AR, GATA2 and FoxA1 binding sites are shown in IGV. (B) LNCaP cells were grown for 72 h in the absence of hormone and were then treated with 1 nM R1881 or ethanol for 4 h before harvest. ChIP assays were performed using antibodies against AR, GATA2 or FoxA1. (C and D) LNCaP cells were transfected with siControl or siAR in the absence of hormone. After 72 h, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. ChIP assays were conducted with indicated antibodies. (E and F) LNCaP cells were transfected with siControl or factor-specific siRNA in the absence of hormone. After 72 h, cells were treated with 1 nM R1881 or ethanol for an additional 4 h before (E) ChIP assays were conducted with AR-specific antibody, or (F) Real-time RT-PCR was performed using gene-specific primers. (G and H) LNCaP cells were transfected with siControl, siGATA2, or siFoxA1 in the absence of hormone. 72 h after siRNA transfection, cells were treated with 1 nM R1881 or ethanol for an additional 4 h. ChIP assays were performed with indicated antibodies. * $P < 0.05$, ** $P < 0.01$. Results of ChIP and qRT-PCR assays reported as the mean of two to four replicates, with error bars representing the standard deviation.

elements of this mechanism of AR target gene coregulation by GATA2 and FoxA1 are conserved across these two systems. A high degree of concordance was observed for patterns of hormone-independent pioneer factor occupancy of *AR* regulatory elements and regulation of *AR* expression (Supplementary Figure S4A and C). Similarly, hormone-independent pioneer factor occupancy was observed in advance of androgen-stimulated AR binding to the *ABCC4* and *ADPGK* regulatory regions, and loss of either pioneer factor significantly abrogated subsequent AR binding (Supplementary Figure S4D and G). Despite the agreement between *ADPGK* expression changes following AR, GATA2 and FoxA1 knockdown, siFoxA1 transfection of C4-2B cells actually induced *ABCC4* expression while AR and GATA2 knockdown resulted in reduced *ABCC4* expression (Supplementary Figure S4H and I), revealing cell line differences affecting context-specific

pioneer factor function. This initial investigation of a small subset of androgen-responsive genes reveals two important characteristics of pioneer factor function in AR transcriptional activation. First, the results support a model that GATA2 serves a generally positive role in mediating androgen-stimulated binding of AR to target gene regulatory elements subsequent to direct regulation of AR. Second, GATA2 and FoxA1 exhibit two distinct models of combinatorial binding to AR target gene regulatory elements: independent and codependent.

GATA2 establishes accessible chromatin signature in regulatory elements

A well-established role for pioneer factors is in facilitating local chromatin reorganization, allowing for improved accessibility for additional DNA-binding transcription factors (10). We asked whether GATA2 might act in this way to enhance AR binding to distal gene regulatory

elements, thus allowing for androgen-dependent AR target gene expression. Our histone mark ChIP-seq data (Z.C. and Q.W., unpublished data) provided a picture of the chromatin environment within the vicinity of shared AR/GATA2/FoxA1 binding sites within AR target gene loci of interest, illustrating that these sites are enriched with active enhancer-specific histone modifications (histone H3 lysine 4 mono- and dimethylation, and lysine 27 acetylation—H3K4me1, H3K4me2 and H3K27ac, respectively) and void of the canonically repressive histone mark, histone H3 lysine 27 trimethylation (H3K27me3) and the promoter-specific histone H3 lysine 4 trimethylation (H3K4me3) (Figure 5A and B) (54).

We confirmed the ChIP-seq results for the *ABCC4* and *ADPGK* regulatory elements, demonstrating that these sites exhibit typical enhancer characteristics. Relative to two control regions (GATA2 binding sites enriched in H3K27me3), androgen-independent levels of H3K4me1 and H3K4me2 were significantly enriched, while H3K4me3 was only modestly enriched (Figure 5C). Additionally, H3K27ac, an indicator of active chromatin, was enriched within *ABCC4* and *ADPGK* regulatory elements prior to androgen stimulation, compared to control loci (Figure 5D). Finally, we confirmed that these sites are depleted of the repressive H3K27me3, which showed robust enrichment within the control regions (Figure 5E). These results suggest that a relatively active chromatin environment can be found at the regulatory elements of androgen-dependent genes prior to hormone stimulation. In addition to an active chromatin signature, the *ABCC4* and *ADPGK* regulatory loci also exhibit enhanced accessibility as measured by FAIRE (41), indicating relatively high dissociation of DNA from nucleosomes within these regions (Figure 5F). That androgen-independent pioneer factor occupancy of these loci correlates with this active and accessible chromatin signature supports the established role for these factors in priming AR target genes for transcriptional responsiveness to hormone stimulation.

While the mechanisms by which FoxA1 influences chromatin accessibility is understood to derive from its structural similarity to the linker histone H1, allowing FoxA1 to displace H1 and disrupt heterochromatin (18,19), the impact of GATA2 on chromatin structure and histone modification status in AR signaling has been suggested through correlative studies, though not thoroughly described (44,55). Having observed an enrichment of the active histone mark H3K27ac, we asked what impact GATA2 and FoxA1 have on the recruitment of histone acetyltransferases (HATs) to the *ABCC4* and *ADPGK* loci prior to hormone stimulation. We focused on p300, as this histone-modifying enzyme showed the most robust enrichment within these sites prior to androgen treatment (Supplementary Figure S5A and B) (56).

p300 enrichment was significantly influenced by pioneer factor status, as siGATA2 transfection resulted in decreased p300 recruitment to both gene regulatory elements in the absence of hormone, while siFoxA1 transfection only significantly decreased p300 levels at the *ADPGK* locus (Figure 5G). Additionally, levels of H3K27ac and patterns of chromatin accessibility at each

gene locus mimicked p300 recruitment (Figure 5H and I). To our surprise knockdown of either pioneer factor resulted in a significant loss in H3K4me1/2 at both the *ABCC4* and *ADPGK* loci (Supplementary Figures S5E–H). While a similar observation was made previously on FoxA1 depletion (26,27), this is a novel observation for GATA2. Though the mechanism is not immediately apparent, pioneer factor occupancy likely affects recruitment of H3K4me1/2-specific histone methyltransferases and/or demethylases. Interestingly, trends in hormone-independent GATA2-mediated recruitment of p300 and establishment of active, accessible chromatin closely resemble trends in androgen-stimulated AR binding (Figures 3C and 4E), suggesting a central role for GATA2 in providing an amenable environment for AR binding subsequent to hormone stimulation. That FoxA1-independent GATA2 occupancy of the *ABCC4* locus is sufficient for the recruitment of p300 and the establishment of active, accessible chromatin, suggests that this is an important mechanism by which GATA2 functions upstream of AR to mediate androgen-responsive gene expression. Together, these results demonstrate that GATA2, with variable input from FoxA1, plays a general role in enhancing androgen-stimulated AR-mediated expression of these target genes by engaging gene regulatory elements in the absence of hormone and recruiting histone-modifying enzymes that activate local chromatin. In this way, androgen-dependent gene loci are primed for expression following hormone stimulation.

GATA2 facilitates regulatory chromatin loop formation

A prominent feature of AR binding is the tendency toward occupancy outside of proximal promoter regions of its target genes. To facilitate transcription, long-distance interactions form between AR-bound distal enhancers and androgen-responsive gene promoters, requiring a host of intermediary protein–protein interactions involving the Mediator coregulatory complex (12,16,57,58). While it is understood that pioneer factor residence on regulatory DNA elements persists beyond the priming period and into periods of active transcription (10), their role in facilitating and maintaining activating chromatin loops is not well established. A previous study showed that FoxA1 is partially responsible for establishing regulatory chromatin loops in castration resistant prostate cancer cell lines (37), suggesting that this pioneer factor is able to recruit requisite loop-forming activities including the Mediator subunit MED1. We asked whether GATA2 and FoxA1 are required in regulatory loop formation for androgen-dependent AR target gene expression by performing 3C assays (59) at the *ABCC4* locus in LNCaP cells.

We assessed the interaction between the downstream *ABCC4* regulatory element, bound by AR, GATA2, and FoxA1, with the promoter region. The downstream site exhibited enrichment of MED1 prior to hormone treatment, reflected by the relatively high cross-linking frequency observed in the absence of hormone. Following androgen treatment, MED1 occupancy was enhanced, congruent with increased cross-linking frequency

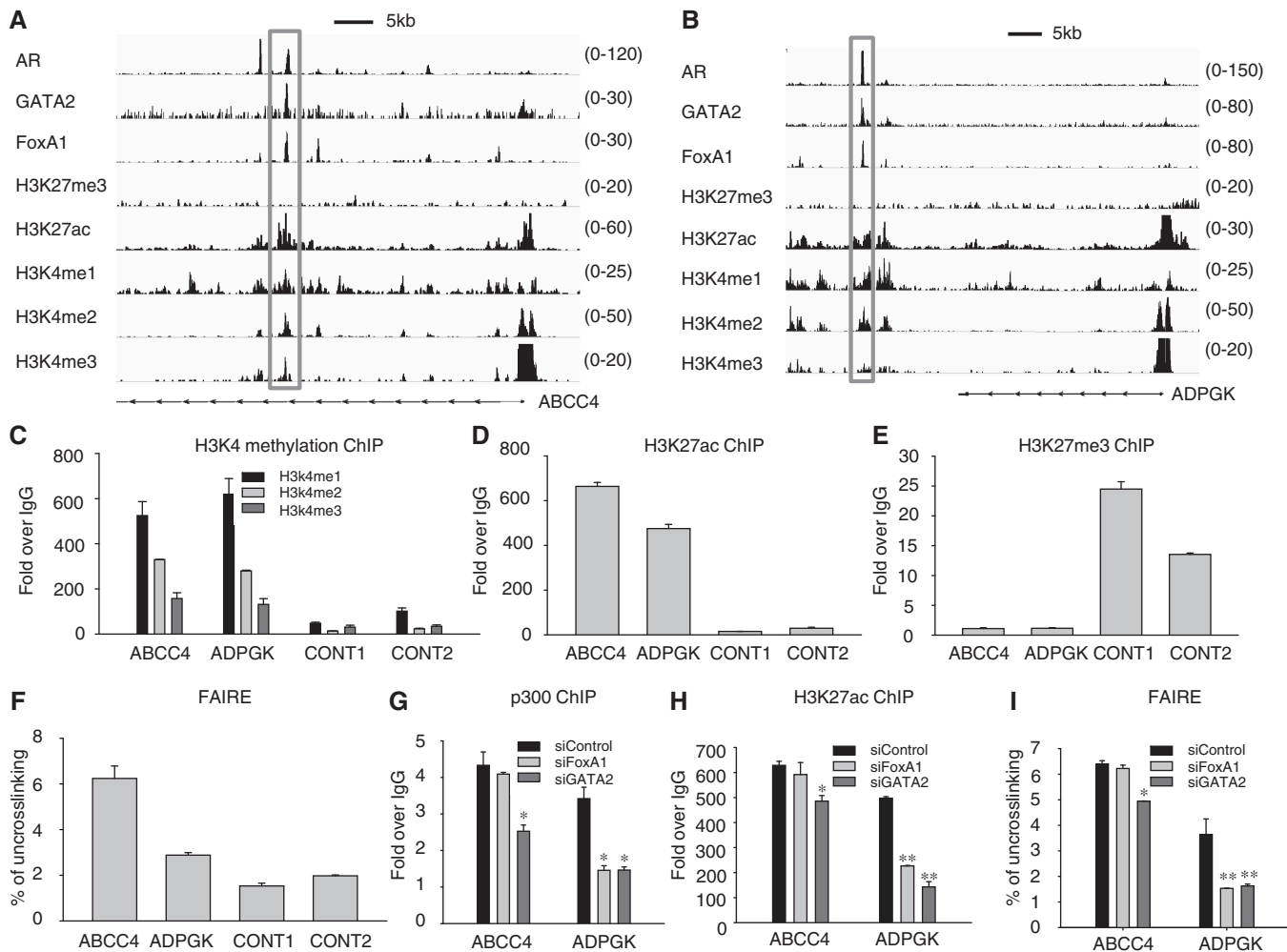


Figure 5. The effect of GATA2 on chromatin environment. (A and B) AR, GATA2 and FoxA1 binding sites at the *ABCC4* and *ADPGK* loci are shown in IGV along with epigenetic marks—H3K27me3, H3K27ac, H3K4me1, H3K4me2 and H3K4me3. (C–E) LNCaP cells were grown for 72 h in the absence of hormone. ChIP assays were performed using specified antibodies. (F) LNCaP cells were grown in the absence of hormone for 72 h, and FAIRE-qPCR was performed using locus-specific primers. (G–I) LNCaP cells were transfected with siControl, siGATA2, or siFoxA1 in the absence of hormone. After 72 h, (G and H) ChIP assays were performed using specified antibodies or (I) FAIRE-qPCR was performed using locus-specific primers. * $P < 0.05$, ** $P < 0.01$. Results of ChIP and FAIRE-qPCR assays reported as the mean of two to four replicates, with error bars representing the standard deviation.

indicating stronger long-distance interaction between this site and the *ABCC4* promoter (Figure 6A and Supplementary Figure S5A). Importantly, siGATA2 and siFoxA1 transfection resulted in significant decreases in cross-linking frequency in the absence of hormone (Figure 6B), suggesting that these pioneer factors play important roles in facilitating basal chromatin loop formation, which may combine with basal active chromatin signatures and accessibility to prime *ABCC4* for androgen-stimulated expression.

To assess the mechanism by which these pioneer factors contribute to chromatin loop formation and maintenance, we performed MED1 ChIP at the *ABCC4* enhancer following siControl, siGATA2 and siFoxA1 transfection. Our results support previous findings that FoxA1 functions in the recruitment of MED1 to distal regulatory elements (37), as FoxA1 knockdown precipitated a significant reduction in MED1 occupancy. Importantly,

siGATA2 transfection produced similar results, revealing that GATA2 also functions in the basal recruitment of chromatin loop-forming activities (Figure 6C). As previous work has shown that phosphorylation of MED1 is required for locus looping and AR target gene expression (37), we performed p-MED1 ChIP to show that both FoxA1 and GATA2 are central to the recruitment of this activated MED1 form (Figure 6D). It is worth recalling that GATA2 and FoxA1 bind independently of one another at the downstream *ABCC4* regulatory element (Figure 3F); thus, decreases in MED1, p-MED1 and cross-linking frequencies are the result of independent pioneer factor knockdown and reflect the individual impact of each pioneer factor on loop formation and maintenance. MED1 and p-MED1 recruitment to the *ADPGK* locus following siFoxA1 and siGATA2 transfection mimicked results at the *ABCC4* locus (Supplementary Figure S5C and D), though codependent binding of

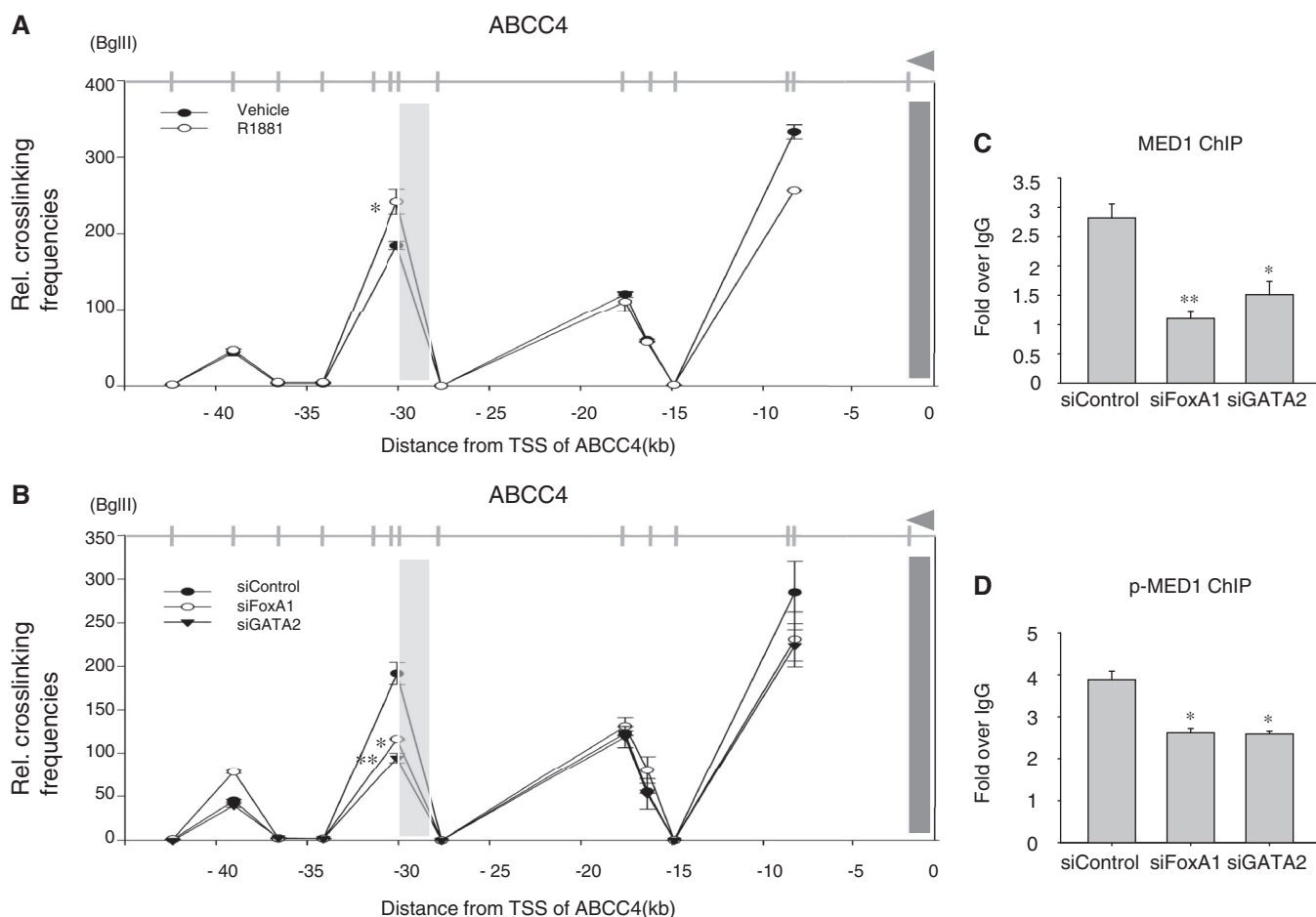


Figure 6. 3C analysis of interaction between GATA2 binding site and *ABCC4* promoter region. (A) LNCaP cells were grown for 72 h in the absence of hormone and were then treated with 1 nM R1881 or ethanol for an additional 4 h before cell harvest. 3C assays were performed using locus-specific primers. (B) LNCaP cells were transfected with siControl, siFoxA1 or siGATA2 and grown in the absence of hormone for 72 h. 3C assays were performed using locus-specific primers. (C and D) LNCaP cells were transfected with siControl, siGATA2 or siFoxA1 in the absence of hormone. After 72 h, ChIP assays were performed using specified antibodies. * $P < 0.05$, ** $P < 0.01$. Results of ChIP and 3C assays reported as the mean of two to four replicates, with error bars representing the standard deviation.

FoxA1 and GATA2 at this site prevents our evaluating the independent contribution of each pioneer factor in this instance.

GATA2 supports genome-wide AR binding

Having identified three prominent mechanisms by which GATA2 contributes to androgen-responsive AR target gene expression, which suggests a generally positive role for this pioneer factor in recruiting AR to target gene loci subsequent to activation of chromatin within regulatory DNA elements, we sought to evaluate the prevalence of this activity in determining AR binding patterns genome-wide. To address this question, we performed AR ChIP-seq following hormone stimulation in LNCaP cells transfected with Control- or GATA2-targeting siRNA molecules. 49 082 (79.5%) of the AR binding sites identified in siControl conditions were lost following GATA2 knockdown (Figure 7A). Among the 12 529 (20.2%) AR binding sites that were maintained following siGATA2 transfection, a significant loss in ChIP-seq read density within these regions was observed. The loss and

decrease of AR binding after GATA2 silencing presumably resulted from the combined loss of AR expression (Figure 2C and D) and GATA2-established active and accessible chromatin structures at these locations. Corroborating our single gene analyses, AR binding within the *ABCC4* and *ADPGK* loci was severely reduced (Figure 7B). Additional, indirect effects on AR binding are also likely, as the nearly 80% loss in binding significantly outnumbers the direct overlap of the AR and GATA2 cistromes (Figure 1A and B), though these effects cannot be distinguished from the upstream effect on AR expression. In stark contrast to the tremendous gain in AR binding sites previously reported following FoxA1 knockdown in prostate cancer cell lines (26,27), only 131 AR binding sites were unique to siGATA2 conditions. These siGATA2-specific AR binding sites fall into two categories: (i) direct GATA2 exclusion of AR binding and (ii) trans-repression of AR binding from a distance. Notably only 19 sites exhibited a gain in AR binding at sites occupied by GATA2 in parental cells, indicating that >85% of gained AR binding events are

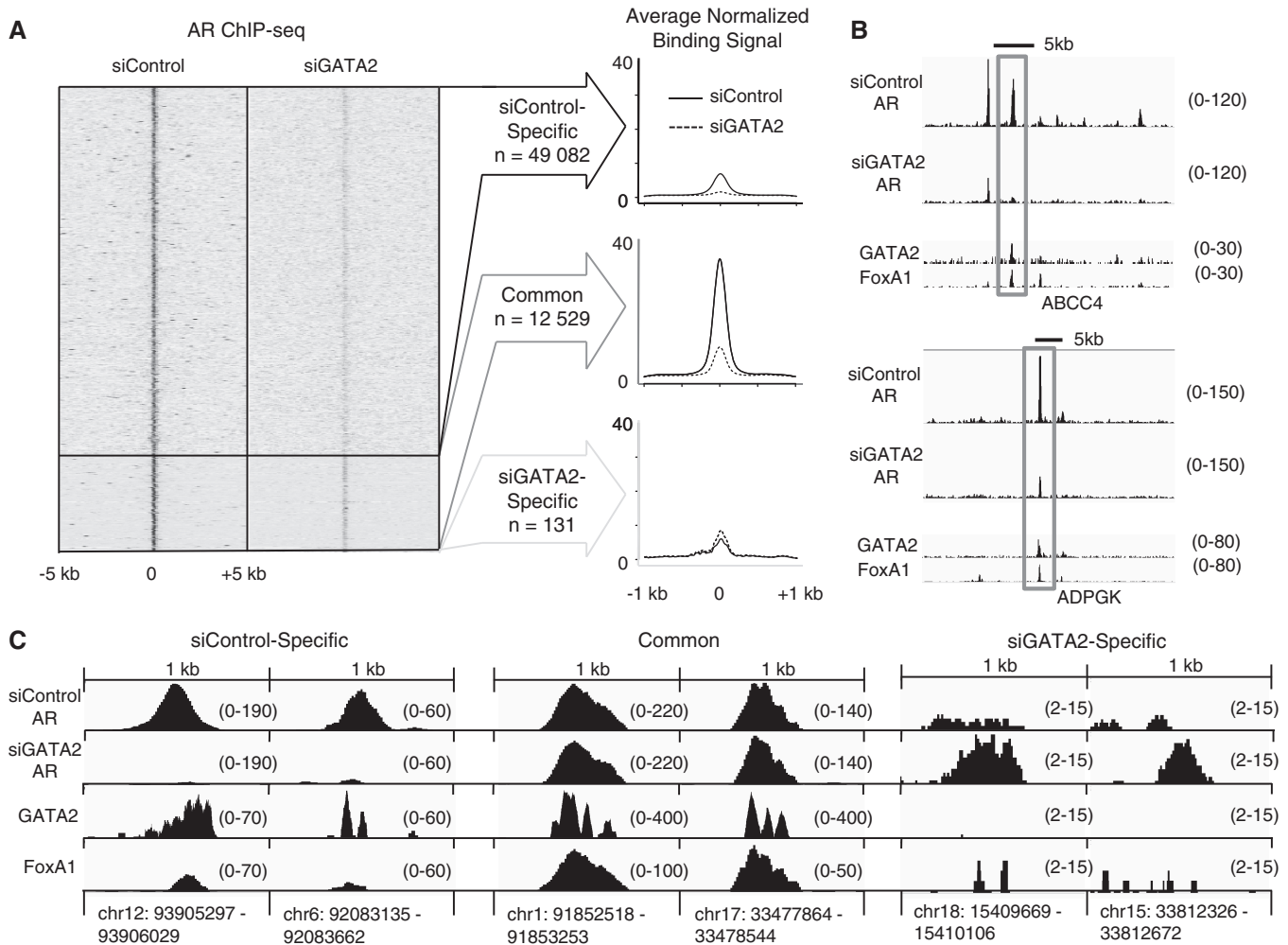


Figure 7. Global impact of GATA2 on AR binding patterns. (A) Aligned reads heatmaps from AR ChIP-seq assays performed in LNCaP cells transfected with Control- (left) or GATA2-targeting (right) siRNA within a 10 kb window centered over AR peaks. Binding site totals are provided for each category, as are the average signal plots. (B) IGV images displaying significantly reduced AR binding within the *ABCC4* (top) and *ADPGK* (bottom) loci. (C) IGV images of examples of each category of AR binding site. Chromosomal coordinates are provided.

the result of relief of indirect or trans-repressive GATA2 effects. Figure 7C gives examples of each of the three categories of AR binding sites. These results further substantiate our claim that through its important roles in up-regulating *AR* expression and recruiting AR to activated chromatin, GATA2 serves a generally, if not exclusively, positive role in defining AR genome-wide binding patterns that determine androgen-responsive gene expression profiles.

DISCUSSION

In the present study, we sought to close the gap in knowledge between mechanisms by which prominent pioneer factors, GATA2 and FoxA1, contribute to AR-mediated gene expression in androgen-responsive prostate cancer cells. While FoxA1 has been a major focus in previous studies of AR activity, limited evidence has been provided to illustrate a mode of action for GATA2, though its contribution to AR target gene expression has

long been appreciated. Our initial genome-wide analysis provided compelling evidence that GATA2 plays a pivotal role alongside FoxA1 in defining androgen-stimulated AR binding patterns and encouraged an in-depth investigation into the mechanisms used by GATA2 both in dictating AR binding and in modulating AR target gene expression (Figure 1). We showed that GATA2 operates first by directly regulating expression of *AR* itself via occupying novel upstream and promoter regulatory elements identified by ChIP-seq. While previous work from B—hm *et al.* reported direct regulation of *AR* by hormone-stimulated GATA2 binding at the promoter of the minor AR(A) isoform (35), we observed hormone-independent occupancy of GATA2 within regulatory elements of the major AR(B) isoform. As GATA2 knockdown resulted in significant loss of AR expression before and after androgen treatment, we suggest that GATA2 mediates both basal and hormone-stimulated expression of full-length AR from these binding sites (Figure 2).

Zaret and Carroll described a role for pioneer factors in “establishing competence for gene expression” by engaging gene regulatory elements in advance of transcription factors, thereby priming genes for responsiveness to appropriate stimuli (10). FoxA1 facilitates NR-mediated gene expression by engaging compact chromatin, displacing H1 to generate localized accessibility, and also serves to recruit architectural components of transcription regulatory chromatin loops (18,37). These roles have been shown in various systems to contribute to NR target gene expression, but whether GATA2 similarly primes for ligand-inducible AR target gene expression has not been fully demonstrated. By investigating the contribution of GATA2 to androgen-responsive AR target gene expression, we observed GATA2 binding in advance of hormone treatment and AR binding, allowing for androgen-stimulated AR recruitment to target gene regulatory loci and gene expression (Figures 3 and 4). Within our subset of AR target genes, we observe a positive impact of GATA2 on androgen-dependent gene expression, though it is reasonable to suspect that a repressive model of target gene coregulation may be adopted in a site-specific manner. Additionally, we have provided evidence that, like the documented ability of FoxA1 to preclude AR binding at certain genomic locations (26,27), a small class of GATA2 binding sites appear exclusionary of AR occupancy and thus also play a negative role in preventing AR transactivation in these regions (Figure 7). However, this subset is negligible in comparison with the FoxA1 study, and only a minor fraction of these sites appear to be the result of direct exclusion of AR by GATA2 occupancy. Future analysis of the repressive mechanisms of GATA2 and/or FoxA1 in determining AR-mediated gene expression profiles is therefore of great interest.

Our analysis has provided some insight into the mechanisms by which GATA2 contributes to AR target gene expression. Previous studies have suggested a notable correlation between GATA2 and p300 recruitment to AR target gene loci: a model corroborated by concurrent loss in binding of the pioneer factor and histone modifier following treatment with a natural compound (44). Additionally, it has been demonstrated in model systems that GATA2 DNA-binding and transactivation potential rely in part on acetylation by p300, providing evidence of their direct interaction (60). We have extended these findings to show that GATA2 has a central role in recruiting p300 to target gene loci, whereupon this HAT activates chromatin via acetylation of H3K27, generating local chromatin accessibility prior to hormone stimulation. Within the *ABCC4* gene locus, we can observe the individual contribution of pioneer factors to chromatin activation, and we see that GATA2 but not FoxA1 knockdown results in lost p300 recruitment, diminished H3K27ac levels and chromatin compaction relative to siControl transfection (Figure 5). Thus, we demonstrate that GATA2 establishes active and accessible AR target gene enhancers, facilitating AR binding and subsequent gene expression.

We also show that GATA2, via recruitment of the Mediator coregulatory complex subunit, MED1, can

establish and/or maintain basal regulatory chromatin loops between AR-bound distal enhancers and androgen-responsive gene promoters. Again, at the *ABCC4* locus, we find that knockdown of either FoxA1 or GATA2 independently results in significant loss of MED1/p-MED1 occupancy, which destabilizes chromatin looping prior to androgen stimulation (Figure 6). This function has been previously demonstrated for FoxA1 (37), yet the contribution of GATA2 to chromatin loop formation has been more elusive. Occupancy of the GATA family member GATA1 along with its coactivator FOG1 at the β -globin locus has been shown to correlate with loop formation (61), providing the most definitive evidence to suggest a direct involvement of GATA factors in establishing and/or maintaining chromatin loops. While additional factors may be identified that can stabilize MED1-enhancer occupancy and basal chromatin loop formation, we suggest that androgen-independent GATA2 occupancy of AR target gene enhancers is a critical determinant of MED1-mediated locus looping in preparation for hormone-stimulated gene expression. Previous studies have implied significant roles for various transcription factors and coactivators in loop formation (58,61–64), and our current findings suggest that GATA2 plays a central role in these processes via recruitment of p300, allowing for basal chromatin remodeling, and MED1, facilitating basal locus looping.

Our genome-wide analysis of the impact of GATA2 silencing on AR distribution reflects two key functions of GATA2 in these prostate cancer cells. First, GATA2 knockdown significantly reduces AR protein levels and, subsequently, AR ChIP-seq signal intensity across the genome. Second, GATA2 silencing results in the loss of active chromatin signatures in regions primed for androgen-stimulated AR binding in parental cells, thus global ChIP-seq enrichment is diminished. While the comprehensive impact of GATA2 loss on transcription-regulatory chromatin looping remains in question, we have provided evidence that the loss of this critical GATA2 function, in combination with the dramatic impact on AR binding patterns, will broadly influence AR target gene expression.

Our findings support and further contribute to the growing body of knowledge of GATA family pioneer transcription factors that facilitate nuclear receptor-mediated, hormone-responsive gene transcription. Demonstrating that GATA2 is capable of facilitating open and active chromatin prior to hormone stimulation is concordant with previous observations that nucleosome-depleted regions (NDRs) in AR target gene enhancers overlap GATA2 binding sites that appear to be necessary for NDR maintenance in the absence of androgen (65). Additionally, the ER-collaborating pioneer factor, GATA3, important for breast cancer gene expression profiles, exhibits binding patterns that correlate with genome-wide ER-anchored chromatin loops associated with estrogen-responsive gene transcription (62,66). Further analysis is required to reveal whether or not GATA2 shares common functionality with GATA3, which appears capable of activating and/or silencing local chromatin in a site-specific manner

leading to enhanced and/or inhibited chromatin loop formation (66).

In summary, we have provided evidence that GATA2 contributes positively to androgen-dependent AR target gene expression by three distinct mechanisms. First, through binding regulatory elements of the *AR* locus, GATA2 directly upregulates basal and hormone stimulated *AR* expression. Secondly, by recruiting the histone acetyltransferase p300, GATA2 facilitates basal AR target gene enhancer activity and promotes chromatin accessibility, allowing for subsequent androgen-stimulated AR binding and target gene expression. Finally, GATA2 recruits MED1/p-MED1 to AR target gene enhancers prior to hormone stimulation, leading to basal chromatin loop formation/maintenance at androgen-responsive gene loci. These findings encourage future focus on elucidating additional means by which GATA2 contributes to AR-mediated gene expression to further our understanding of how this pioneer transcription factor influences AR binding patterns and downstream target gene expression in prostate cancer. Revealing critical determinants of AR activity offers an important opportunity to direct new therapeutic strategies at upstream components of the androgen signaling axis. As current prostate cancer therapeutic modalities too often fail in preventing biochemical recurrence in advanced cases, focusing future treatments towards activities of factors like GATA2, correlated with advanced prostate cancer (35), may prove a potent strategy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

National Institutes of Health [R01CA151979 to Q.W.; U54CA113001 to Q.W., T.H.-M.H., K.P.N.; T32CA009338 to C.G.]; Department of Defense grant [W81XWH-12-1-0615 to Q.W.]; 2011 V Foundation V Scholar Award (to Q.W.); The Ohio State University and Howard Hughes Medical Institute MED into GRAD Scholars Program Fellowship (to B.S.). Funding for open access charge: National Institutes of Health [R01CA151979, U54CA113001].

Conflict of interest statement. None declared.

REFERENCES

- De Gendt,K. and Verhoeven,G. (2012) Tissue- and cell-specific functions of the androgen receptor revealed through conditional knockout models in mice. *Mol. Cell. Endocrinol.*, **352**, 13–25.
- Lonergan,P.E. and Tindall,D.J. (2011) Androgen receptor signaling in prostate cancer development and progression. *J. Carcinog.*, **10**, 20.
- Heinlein,C.A. and Chang,C. (2004) Androgen receptor in prostate cancer. *Endocr. Rev.*, **25**, 276–308.
- Denmeade,S.R. and Isaacs,J.T. (2002) A history of prostate cancer treatment. *Nat. Rev. Cancer*, **2**, 389–396.
- Feldman,B.J. and Feldman,D. (2001) The development of androgen-independent prostate cancer. *Nat. Rev. Cancer*, **1**, 34–45.
- Pienta,K.J. and Bradley,D. (2006) Mechanisms underlying the development of androgen-independent prostate cancer. *Clin. Cancer Res.*, **12**, 1665–1671.
- Knudsen,K.E. and Penning,T.M. (2010) Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer. *Trends Endocrinol. Metabol.*, **21**, 315–324.
- Jozwik,K.M. and Carroll,J.S. (2012) Pioneer factors in hormone-dependent cancers. *Nat. Rev. Cancer*, **12**, 381–385.
- Augello,M.A., Hickey,T.E. and Knudsen,K.E. (2011) FOXA1: master of steroid receptor function in cancer. *EMBO J.*, **30**, 3885–3894.
- Zaret,K.S. and Carroll,J.S. (2011) Pioneer transcription factors: establishing competence for gene expression. *Genes Dev.*, **25**, 2227–2241.
- Carroll,J.S., Liu,X.S., Brodsky,A.S., Li,W., Meyer,C.A., Szary,A.J., Eeckhoute,J., Shao,W., Hestermann,E.V., Geistlinger,T.R. *et al.* (2005) Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell*, **122**, 33–43.
- Wang,Q., Li,W., Liu,X.S., Carroll,J.S., Janne,O.A., Keeton,E.K., Chinnaiyan,A.M., Pienta,K.J. and Brown,M. (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol. Cell*, **27**, 380–392.
- Jia,L., Berman,B.P., Jariwala,U., Yan,X., Cogan,J.P., Walters,A., Chen,T., Buchanan,G., Frenkel,B. and Coetzee,G.A. (2008) Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. *PLoS One*, **3**, e3645.
- John,S., Sabo,P.J., Thurman,R.E., Sung,M.H., Biddie,S.C., Johnson,T.A., Hager,G.L. and Stamatoyannopoulos,J.A. (2011) Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nat. Genet.*, **43**, 264–268.
- Lin,C.Y., Vega,V.B., Thomsen,J.S., Zhang,T., Kong,S.L., Xie,M., Chiu,K.P., Lipovich,L., Barnett,D.H., Stossi,F. *et al.* (2007) Whole-genome cartography of estrogen receptor alpha binding sites. *PLoS Genet.*, **3**, e87.
- Wang,Q., Li,W., Zhang,Y., Yuan,X., Xu,K., Yu,J., Chen,Z., Beroukhim,R., Wang,H., Lupien,M. *et al.* (2009) Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell*, **138**, 245–256.
- Yu,J., Yu,J., Mani,R.S., Cao,Q., Brenner,C.J., Cao,X., Wang,X., Wu,L., Li,J., Hu,M. *et al.* (2010) An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. *Cancer Cell*, **17**, 443–454.
- Cirillo,L.A., McPherson,C.E., Bossard,P., Stevens,K., Cherian,S., Shim,E.Y., Clark,K.L., Burley,S.K. and Zaret,K.S. (1998) Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. *EMBO J.*, **17**, 244–254.
- Cirillo,L.A., Lin,F.R., Cuesta,I., Friedman,D., Jarnik,M. and Zaret,K.S. (2002) Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol. Cell*, **9**, 279–289.
- Bernardo,G.M., Lozada,K.L., Miedler,J.D., Harburg,G., Hewitt,S.C., Mosley,J.D., Godwin,A.K., Korach,K.S., Visvader,J.E., Kaestner,K.H. *et al.* (2010) FOXA1 is an essential determinant of ERalpha expression and mammary ductal morphogenesis. *Development*, **137**, 2045–2054.
- Gao,N., Ishii,K., Mirosevich,J., Kuwajima,S., Oppenheimer,S.R., Roberts,R.L., Jiang,M., Yu,X., Shappell,S.B., Caprioli,R.M. *et al.* (2005) Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. *Development*, **132**, 3431–3443.
- Mirosevich,J., Gao,N. and Matusik,R.J. (2005) Expression of Foxa transcription factors in the developing and adult murine prostate. *Prostate*, **62**, 339–352.
- Mirosevich,J., Gao,N., Gupta,A., Shappell,S.B., Jove,R. and Matusik,R.J. (2006) Expression and role of Foxa proteins in prostate cancer. *Prostate*, **66**, 1013–1028.
- Hurtado,A., Holmes,K.A., Ross-Innes,C.S., Schmidt,D. and Carroll,J.S. (2011) FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat. Genet.*, **43**, 27–33.

25. Lupien, M. and Brown, M. (2009) Cistromics of hormone-dependent cancer. *Endocr. Relat. Cancer*, **16**, 381–389.
26. Sahu, B., Laakso, M., Ovaska, K., Mirtti, T., Lundin, J., Rannikko, A., Sankila, A., Turunen, J.P., Lundin, M., Konsti, J. *et al.* (2011) Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer. *EMBO J.*, **30**, 3962–3976.
27. Wang, D., Garcia-Bassets, I., Benner, C., Li, W., Su, X., Zhou, Y., Qiu, J., Liu, W., Kaikkonen, M.U., Ohgi, K.A. *et al.* (2011) Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. *Nature*, **474**, 390–394.
28. Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R. and Zaret, K.S. (1996) Hepatic specification of the gut endoderm *in vitro*: cell signaling and transcriptional control. *Genes Dev.*, **10**, 1670–1682.
29. Bossard, P. and Zaret, K.S. (1998) GATA transcription factors as potentiators of gut endoderm differentiation. *Development*, **125**, 4909–4917.
30. Asselin-Labat, M.L., Sutherland, K.D., Barker, H., Thomas, R., Shackleton, M., Forrest, N.C., Hartley, L., Robb, L., Grosveld, F.G., van der Wees, J. *et al.* (2007) Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat. Cell Biol.*, **9**, 201–209.
31. Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A. *et al.* (2000) Molecular portraits of human breast tumours. *Nature*, **406**, 747–752.
32. Yamaguchi, N., Ito, E., Azuma, S., Honma, R., Yanagisawa, Y., Nishikawa, A., Kawamura, M., Imai, J., Tatsuta, K., Inoue, J. *et al.* (2008) FoxA1 as a lineage-specific oncogene in luminal type breast cancer. *Biochem. Biophys. Res. Commun.*, **365**, 711–717.
33. Kouros-Mehr, H., Slorach, E.M., Sternlicht, M.D. and Werb, Z. (2006) GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell*, **127**, 1041–1055.
34. Eeckhoutte, J., Keeton, E.K., Lupien, M., Krum, S.A., Carroll, J.S. and Brown, M. (2007) Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. *Cancer Res.*, **67**, 6477–6483.
35. Bohm, M., Locke, W.J., Sutherland, R.L., Kench, J.G. and Henshall, S.M. (2009) A role for GATA-2 in transition to an aggressive phenotype in prostate cancer through modulation of key androgen-regulated genes. *Oncogene*, **28**, 3847–3856.
36. Zhang, C., Wang, L., Wu, D., Chen, H., Chen, Z., Thomas-Ahner, J.M., Zynger, D.L., Eeckhoutte, J., Yu, J., Luo, J. *et al.* (2011) Definition of a FoxA1 Cistrome that is crucial for G1 to S-phase cell-cycle transit in castration-resistant prostate cancer. *Cancer Res.*, **71**, 6738–6748.
37. Chen, Z., Zhang, C., Wu, D., Chen, H., Rorick, A., Zhang, X. and Wang, Q. (2011) Phospho-MED1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth. *EMBO J.*, **30**, 2405–2419.
38. Langmead, B., Trapnell, C., Pop, M. and Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.*, **10**, R25.
39. Zhang, Y., Liu, T., Meyer, C.A., Eeckhoutte, J., Johnson, D.S., Bernstein, B.E., Nussbaum, C., Myers, R.M., Brown, M., Li, W. *et al.* (2008) Model-based analysis of ChIP-Seq (MACS). *Genome Biol.*, **9**, R137.
40. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H. and Glass, C.K. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell*, **38**, 576–589.
41. Giresi, P.G., Kim, J., McDaniell, R.M., Iyer, V.R. and Lieb, J.D. (2007) FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res.*, **17**, 877–885.
42. Zeng, R., Liu, Z., Sun, Y. and Xu, C. (2012) Differential expression and function of AR isoforms in prostate cancer. *Oncol. Rep.*, **27**, 492–498.
43. Cai, C., He, H.H., Chen, S., Coleman, I., Wang, H., Fang, Z., Chen, S., Nelson, P.S., Liu, X.S., Brown, M. *et al.* (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. *Cancer Cell*, **20**, 457–471.
44. Shah, S., Prasad, S. and Knudsen, K.E. (2012) Targeting pioneering factor and hormone receptor cooperative pathways to suppress tumor progression. *Cancer Res.*, **72**, 1248–1259.
45. Ho, L.L., Kench, J.G., Handelsman, D.J., Scheffer, G.L., Stricker, P.D., Grygiel, J.G., Sutherland, R.L., Henshall, S.M., Allen, J.D. and Horvath, L.G. (2008) Androgen regulation of multidrug resistance-associated protein 4 (MRP4/ABCC4) in prostate cancer. *Prostate*, **68**, 1421–1429.
46. Cai, C., Omwancha, J., Hsieh, C.L. and Shemshedini, L. (2007) Androgen induces expression of the multidrug resistance protein gene MRP4 in prostate cancer cells. *Prostate Cancer Prostatic Dis.*, **10**, 39–45.
47. Montani, M., Hermanns, T., Muntener, M., Wild, P., Sulser, T. and Kristiansen, G. (2013) Multidrug resistance protein 4 (MRP4) expression in prostate cancer is associated with androgen signaling and decreases with tumor progression. *Virchows Arch.*, **462**, 437–443.
48. Massie, C.E., Lynch, A., Ramos-Montoya, A., Boren, J., Stark, R., Fazli, L., Warren, A., Scott, H., Madhu, B., Sharma, N. *et al.* (2011) The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. *EMBO J.*, **30**, 2719–2733.
49. Richter, S., Morrison, S., Connor, T., Su, J., Print, C.G., Ronimus, R.S., McGee, S.L. and Wilson, W.R. (2013) Zinc finger nuclease mediated knockout of ADP-dependent glucokinase in cancer cell lines: effects on cell survival and mitochondrial oxidative metabolism. *PLoS One*, **8**, e65267.
50. Kaminski, M.M., Sauer, S.W., Kaminski, M., Opp, S., Ruppert, T., Grigaravicius, P., Grudnik, P., Grone, H.J., Krammer, P.H. and Gulow, K. (2012) T cell activation is driven by an ADP-dependent glucokinase linking enhanced glycolysis with mitochondrial reactive oxygen species generation. *Cell Rep.*, **2**, 1300–1315.
51. Nadiminty, N., Tummala, R., Liu, C., Yang, J., Lou, W., Evans, C.P. and Gao, A.C. (2013) NF-kappaB2/p52 induces resistance to enzalutamide in prostate cancer: role of androgen receptor and its variants. *Mol. Cancer Ther.*, **12**, 1629–1637.
52. Perlmann, T. and Wrangé, O. (1988) Specific glucocorticoid receptor binding to DNA reconstituted in a nucleosome. *EMBO J.*, **7**, 3073–3079.
53. Voss, T.C., Schiltz, R.L., Sung, M.H., Yen, P.M., Stamatoyannopoulos, J.A., Biddie, S.C., Johnson, T.A., Miranda, T.B., John, S. and Hager, G.L. (2011) Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism. *Cell*, **146**, 544–554.
54. Calo, E. and Wysocka, J. (2013) Modification of enhancer chromatin: what, how, and why? *Mol. Cell*, **49**, 825–837.
55. Seo, W.Y., Jeong, B.C., Yu, E.J., Kim, H.J., Kim, S.H., Lim, J.E., Kwon, G.Y., Lee, H.M. and Kim, J.H. (2013) CCAR1 promotes chromatin loading of androgen receptor (AR) transcription complex by stabilizing the association between AR and GATA2. *Nucleic Acids Res.*, **41**, 8526–8536.
56. Jin, Q., Yu, L.R., Wang, L., Zhang, Z., Kasper, L.H., Lee, J.E., Wang, C., Brindle, P.K., Dent, S.Y. and Ge, K. (2011) Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation. *EMBO J.*, **30**, 249–262.
57. Wu, D., Zhang, C., Shen, Y., Nephew, K.P. and Wang, Q. (2011) Androgen receptor-driven chromatin looping in prostate cancer. *Trends Endocrinol. Metabol.*, **22**, 474–480.
58. Wang, Q., Carroll, J.S. and Brown, M. (2005) Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol. Cell*, **19**, 631–642.
59. Hagege, H., Klous, P., Braem, C., Splinter, E., Dekker, J., Cathala, G., de Laat, W. and Forne, T. (2007) Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat. Protoc.*, **2**, 1722–1733.
60. Hayakawa, F., Towatari, M., Ozawa, Y., Tomita, A., Privalsky, M.L. and Saito, H. (2004) Functional regulation of GATA-2 by acetylation. *J. Leukoc. Biol.*, **75**, 529–540.
61. Vakoc, C.R., Letting, D.L., Gheldof, N., Sawado, T., Bender, M.A., Groudine, M., Weiss, M.J., Dekker, J. and Blobel, G.A. (2005)

- Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol. Cell*, **17**, 453–462.
62. Fullwood, M.J., Liu, M.H., Pan, Y.F., Liu, J., Xu, H., Mohamed, Y.B., Orlov, Y.L., Velkov, S., Ho, A., Mei, P.H. *et al.* (2009) An oestrogen-receptor-alpha-bound human chromatin interactome. *Nature*, **462**, 58–64.
63. Hu, Q., Kwon, Y.S., Nunez, E., Cardamone, M.D., Hutt, K.R., Ohgi, K.A., Garcia-Bassets, I., Rose, D.W., Glass, C.K., Rosenfeld, M.G. *et al.* (2008) Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. *Proc. Natl Acad. Sci. USA*, **105**, 19199–19204.
64. Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S. *et al.* (2010) Mediator and cohesin connect gene expression and chromatin architecture. *Nature*, **467**, 430–435.
65. Andreu-Vieyra, C., Lai, J., Berman, B.P., Frenkel, B., Jia, L., Jones, P.A. and Coetzee, G.A. (2011) Dynamic nucleosome-depleted regions at androgen receptor enhancers in the absence of ligand in prostate cancer cells. *Mol. Cell. Biol.*, **31**, 4648–4662.
66. Theodorou, V., Stark, R., Menon, S. and Carroll, J.S. (2013) GATA3 acts upstream of FOXA1 in mediating ESR1 binding by shaping enhancer accessibility. *Genome Res.*, **23**, 12–22.