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Definition of a FoxA1 Cistrome that is Crucial for G1-S Phase Cell-Cycle Transit in Castration-Resistant Prostate Cancer

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Abstract

The enhancer pioneer transcription factor FoxA1 is a global mediator of steroid receptor (SR) action in hormone-dependent cancers. In castration-resistant prostate cancer (CRPC), FoxA1 acts as an androgen receptor co-factor to drive G2-M phase cell-cycle transit. Here we describe a mechanistically distinct SR-independent role for FoxA1 in driving G1-S phase cell-cycle transit in CRPC. By comparing FoxA1 binding sites in prostate cancer cell genomes, we defined a co-dependent set of FoxA1-MYBL2 and FoxA1-CREB1 binding sites within the regulatory regions of the Cyclin E2 and E2F1 genes that are critical for CRPC growth. Binding at these sites

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upregulate the Cyclin E2 and Cyclin A2 genes in CRPC but not in earlier stage androgen-dependent prostate cancer (ADPC), establishing a stage-specific role for this pathway in CRPC growth. Mechanistic investigations indicated that FoxA1, MYBL2 or CREB1 induction of histone H3 acetylation facilitated nucleosome disruption as the basis for co-dependent transcriptional activation and G1-S phase cell-cycle transit. Our findings establish FoxA1 as a pivotal driver of the cell-cycle in CRPC which promotes G1-S phase transit as well as G2-M phase transit through two distinct mechanisms.

Keywords

FoxA1; cistrome; cell-cycle G1-S progression; castration-resistant prostate cancer

Introduction

The FoxA subfamily of winged helix/forkhead box (Fox) transcription factors, which consists of three members, FoxA1, FoxA2 and FoxA3, have been found to play important roles in multiple stages of development, metabolism, differentiation and proliferation (1). FoxA proteins function as “pioneer factors” that engage chromatin before other transcription factors (2-5). Recent studies have further shown that FoxA1 functions as a pioneer factor for steroid hormone receptors (SR), including androgen receptor (AR) in prostate cancer cells and estrogen receptor (ER) in breast cancer cells, directing AR- and ER-regulated, hormone (androgen and estrogen)-responsive genes (6-8). These studies further suggested that FoxA1 acts upstream of AR and ER to regulate their target genes in hormone-dependent prostate and breast cancers.

In both androgen-dependent prostate cancer (ADPC) and fatal, castration-resistant prostate cancer (CRPC), AR expression and functionality has been well documented (9), but the receptor appears to play different roles in the two diseases. For example, in ADPC, AR functions primarily to promote G1-S cell-cycle progression, by transcriptional and/or post-transcriptional regulation of CDKN1A, CCND1 and CDKN1B (10). However, in CRPC, the primary function of AR appears to be regulation of G2-M transition. Thus, the receptor appears to be “reprogrammed” to direct transcriptional regulation of G2-M phase-specific genes, including *UBE2C* and *CDK1* (11) by mechanisms that are not well understood. Consistent with the critical role of FoxA1 in assisting SR binding, FoxA1 silencing in CRPC leads to decreased AR binding to enhancers of G2-M phase genes and lower gene expression levels (11). Hence, in CRPC, FoxA1 appears to be mainly involved in AR-regulated, G2-M cell-cycle progression. However, whether this pioneer factor can also function independently in CRPC, in addition to collaborating with AR, has not been investigated.

In the current study, we examined the role of FoxA1 in CRPC cell-cycle progression by silencing FoxA1 in unsynchronized CRPC cells. We found, that in the absence of FoxA1, CRPC experienced a G1-S block and, unexpectedly, not G2-M arrest. Our comprehensive integrated analysis of gene expression and FoxA1 cistrome data further revealed direct upregulation of *CCNE2* by FoxA1 binding sites specific to CRPC, as well as indirect

upregulation of *CCNA2* by E2F1. We further established a requirement for CREB1 and MYBL2 in CRPC-specific FoxA1 binding, through histone H3 acetylation-facilitated nucleosome disruption, resulting in upregulation of CCNE2 and CCNA2 expression and enhanced growth of CRPC. Collectively, these data indicate that FoxA1, together with CREB1 and MYBL2, drive G1-S progression in CRPC, which is distinct from the classic role of FoxA1 as an AR cofactor.

Materials and Methods

Cell lines

The ADPC cell line LNCaP was purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. The CRPC cell line abl was provided by Zoran Culig (Innsbruck Medical University, Austria). A second CRPC cell line CWR22Rv1 was provided by Steven P. Balk (Harvard Medical School, Boston, Massachusetts). LNCaP was authenticated by ATCC, and abl and CWR22Rv1 were authenticated as described previously (12, 13). All three cell lines were passaged in our laboratory for less than six months after resuscitation.

RNA interference

siRNA transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The sequences for siRNAs were listed in Supplementary Table S1.

Western blots

Western blots were performed as previously described (14). Antibodies used are listed in the Supplementary Table S2.

Cell proliferation assay

Cell proliferation was measured using the WST-1 kit (Roche, Indianapolis, IN) as previously described (15).

Fluorescence-activated cell sorting analysis (FACS)

Cells were collected, and DNA contents were analyzed by a FACS Calibur cell flow cytometer (Becton Dickinson Biosciences, San Diego, CA) as previously described (14, 15).

Real-time RT-PCR

Real-time RT-PCR was performed as previously described (14). Primers used are listed in Supplementary Table S1.

ChIP-on-chip assay, ChIP assay, and Re-ChIP assay

The ChIP-on-chip experiments were performed in biological triplicates as previously described (11). The raw data of FoxA1 ChIP-on-chip have been submitted to the Gene Expression Omnibus (GEO) repository under the accession number GSE26329. ChIP and Re-ChIP were performed as previously described (16). Antibodies for ChIP and Re-ChIP assays are listed in Supplementary Table S2.

Correlation of FoxA1 cistrome in LNCaP and abl cells with clinical ADPC and CRPC microarray data

A meta-analysis was performed using three clinical ADPC/CRPC gene expression microarray datasets (17-19) from Oncomine (20). The overexpressed and underexpressed genes in CRPC versus ADPC, and randomly selected genes were correlated with three FoxA1 binding groups. Details are available in the Supplementary Materials and Methods.

Formaldehyde-assisted isolation of regulatory elements (FAIRE)

FAIRE was performed as previously described (21, 22). All primer sequences are listed in Supplementary Table S1.

Motif finding in abl-specific FoxA1 binding regions

MDmodule (23) was used to perform a *de novo* search for motifs significantly enriched in abl-specific binding sites with LNCaP-specific binding regions as background. Motif length varied from 6 nt to 15 nt. Significantly enriched motif was annotated using STAMP (24).

Results

FoxA1 promotes CRPC cell growth by driving both G1-S and G2-M cell-cycle progression

Our previous findings that FoxA1 binding at the enhancer regions of several cell-cycle G2-M phase genes (e.g. *UBE2C* and *CDK1*) is required for overexpression of these genes in CRPC (11) prompted us to further examine the functional role of FoxA1 in CRPC. We first tested the effect of FoxA1 silencing on cell proliferation in abl, a CRPC cell line that closely models clinical CRPC (11, 12, 25). Short interfering RNAs (siRNAs) targeting FoxA1 (two independent siRNAs) were transfected into abl. Both siRNAs dramatically reduced cellular FoxA1 levels (Fig. 1A), and significantly decreased androgen-independent cell proliferation of abl (Fig. 1A). The inhibitory effect of FoxA1 silencing on cell proliferation was also observed in CWR22Rv1 (Supplementary Fig. S1A), another CRPC cell model (26). We next examined the effect of FoxA1 silencing on CRPC cell-cycle progression using a thymidine-nocodazole block in order to enrich abl cells at mitosis (15). Consistent with the role of FoxA1 in the upregulation of cell-cycle G2-M phase genes in CRPC cells (11), FoxA1 silencing caused a G2-M accumulation in the treated abl cells (Fig. 1B). However, FoxA1 knock down resulted in a G1-S block in unsynchronized abl and CWR22Rv1 cells (Fig. 1C and Supplementary Fig. S1B), suggesting that FoxA1 promotes both G1-S and G2-M progression in CRPC cells. As a major role of AR in CRPC is to upregulate G2-M phase gene expression and promote the G2-M transition (11), these data further indicate that the ability of FoxA1 to promote G1-S progression is an AR-*independent* phenomenon. In agreement with our previous findings (16), silencing of FoxA1 had no effect on dihydrotestosterone (DHT)-stimulated LNCaP G1-S progression and cell proliferation (Supplementary Fig. S1C and S1D). Taken together, these data suggest that FoxA1 is required for androgen-independent but not androgen-dependent growth of prostate cancer.

FoxA1 upregulates G1 phase genes *CCNE2* and *CCNA2* to enhance CRPC cell growth

To identify FoxA1-regulated genes that contribute to FoxA1-mediated G1-S transition in CRPC cells, abl cells were transfected with siFoxA1 and a control siRNA (siControl), cultured for 72 hours, and analyzed for expression of the G1 phase genes *CCNE2*, *CCNA2*, *CCNE1*, *CDK2*, *CDK4*, *CDK6*, *CCND1*, *CCND2*, *CCND3*, *CDKN1A*, and *CDKN1B* and *RB*. LNCaP cells transfected with these siRNAs and treated with DHT (10 nM) for 4 hours and 24 hours served as controls. Among the eleven genes examined by qRT-PCR, expression levels of *CCNE2*, *CCNA2* and *CDK2* were higher in abl cells transfected with siControl compared to similarly transfected, untreated or DHT-treated LNCaP (Fig. 2A), although *CCNE2*, *CCNA2* and *CDK2* expression levels were increased after DHT treatment of LNCaP cells (Fig. 2A) (27). Silencing of FoxA1 markedly decreased *CCNE2* and *CCNA2* but not *CDK2* expression in abl but not LNCaP cells (Fig. 2A). Importantly, re-analysis of gene expression profiles from three independent clinical studies (17-19) confirmed that expression of *CCNE2* and *CCNA2* was greater in cases of CRPC compared to ADPC (Fig. 2B). Western blot analysis demonstrated that CCNE2 and CCNA2 protein levels were also greater and FoxA1-dependent in abl than LNCaP cells, in the presence or absence of DHT (Fig. 2C). Consistent with previous reports showing that CCNE2 and CCNA2 binding and activation of CDK2 lead to RB phosphorylation (28, 29), FoxA1-enhanced expression of CCNE2 and CCNA2 protein increased FoxA1-dependent expression of phosphorylated CDK2 and phosphorylated RB in abl versus LNCaP (Fig. 2C). The inhibitory effect of FoxA1 depletion on mRNA and protein expression levels of CCNE2 but not CCNA2 was also observed in CWR22Rv1 cells (Supplementary Fig. S2A).

We next examined the functional role of CCNE2 and CCNA2 in ADPC and CRPC cell growth. In cell proliferation assays, CCNE2 or CCNA2 silencing markedly decreased abl and CWR22Rv1 growth (Fig. 2D and Supplementary Fig. S2B), suggesting that CCNE2 and CCNA2 play an essential role in CRPC cell proliferation. To test whether differences in expression of CCNE2 and CCNA2 were responsible for androgen-independent and androgen-dependent phenotypic differences between abl and LNCaP cells, we generated LNCaP cell lines stably expressing CCNE2 or CCNA2. Consistent with a previous report that overexpression of G1 cyclins was not sufficient to induce LNCaP growth in the absence of androgen (30), no effect of CCNE2 or CCNA2 overexpression on cell proliferation and G1-S progression was observed for vehicle-treated LNCaP cells (Fig. 2E and Supplementary Fig. S2C). However, while DHT treatment (1 nM or higher) increased growth of vector transfected LNCaP, a growth response in LNCaP cells overexpressing CCNE2 or CCNA2 was observed at a lower (0.1 nM) concentration of DHT (Fig. 2E), suggesting that CCNE2 and CCNA2 overexpression increases LNCaP androgen sensitivity.

A distinct FoxA1 CRPC cistrome regulates differentially expressed genes in clinical CRPC versus ADPC

In order to investigate the mechanism underlying FoxA1-regulated *CCNE2* and *CCNA2* gene expression, we mapped the FoxA1 cistromes in LNCaP and abl cells by combining chromatin immunoprecipitation with Affymetrix human whole genome tiling arrays (ChIP-on-chip). Using the MAT algorithm (31) with a p-value cut-off of ≤1E-4, we identified 14,965 and 18,110 FoxA1 binding sites in LNCaP and abl cells, respectively. Overlapping

analysis of FoxA1 binding in LNCaP and abl identified 14,248 common FoxA1 binding regions between the two cell lines, 717 LNCaP-specific FoxA1 binding sites and 3,862 abl-specific FoxA1 binding sites (Fig. 3A and Supplementary Fig. S3A). The ChIP-on-chip results were confirmed using direct ChIP for FoxA1 on a subset of common FoxA1 binding regions, LNCaP-specific FoxA1 binding regions, abl-specific FoxA1 binding regions and negative regions (Supplementary Fig. S3B). To determine the functional significance of common, LNCaP-specific and abl-specific FoxA1 binding sites, these three types of FoxA1 binding sites were correlated to gene expression profiles from three clinical studies on ADPC and CRPC cases (17-19). Interestingly, abl-specific FoxA1 binding sites were significantly enriched within 20 kb or 40 kb of the transcription start sites (TSS) of overexpressed (e.g. *CCNE2* [Fig. 2B]) and underexpressed genes (but more notably overexpressed genes) in CRPC versus ADPC, but not randomly selected genes (Fig. 3B and Supplementary Fig. S3C and S3D). By contrast, no obvious enrichment of LNCaP-specific FoxA1 binding sites and common FoxA1 binding sites was observed near overexpressed, underexpressed or randomly selected genes (Fig. 3B and Supplementary Fig. S3C and S3D). These results suggest that abl-specific FoxA1 binding sites may, in general, directly regulate differentially expressed genes in CRPC versus ADPC.

Specific FoxA1 binding sites directly upregulate *CCNE2* in CRPC cells

Based on strong correlation between global FoxA1 binding and differential gene expression profiles, we next investigated regulation of *CCNE2* by FoxA1 in CRPC-abl and ADPC-LNCaP cells. ChIP-on-chip analysis identified an abl-specific FoxA1 binding site at the CCNE2 promoter region, three abl-specific, FoxA1 binding sites located -24.0 kb, -9.8 kb and +23.5 kb away from the TSS of *CCNE2*, and a common FoxA1 binding site (defined as MAT-score ≥3.72 in both cell lines; see the Supplementary Materials and Methods) 14.3 kb downstream of TSS of *CCNE2* (Fig. 4A). The common site displayed stronger FoxA1 binding in abl (MAT-score 9.87) compared to LNCaP (MAT-score 4.37). No overlap was seen between these abl-specific FoxA1 binding sites and AR binding regions in abl cells (11) (data not shown). Direct ChIP analysis demonstrated higher FoxA1 occupancy at the CCNE2 promoter region and the four putative CCNE2 enhancer regions in abl versus LNCaP cells (Fig. 4B). To further characterize the CCNE2 promoter and the putative CCNE2 enhancer regions, ChIP assays were performed in LNCaP and abl cells using antibodies against an enhancer histone mark H3K4 mono-methylation (H3K4me1) (32), phosphorylated RNA polymerase II at serine 5 (p-Pol II), and transcription coactivators CREB binding protein (CBP) and Mediator 1 (MED1). Enrichment of H3K4me1 was observed at the four putative CCNE2 enhancer regions compared with the CCNE2 promoter region in abl, and the level of H3K4me1 was higher at putative CCNE2 enhancers 1, 2 and 4 in abl compared to LNCaP (Fig. 4C), indicating that these four distal FoxA1 binding regions function as enhancers in abl cells. Greater p-Pol II level and increased CBP and MED1 binding at the CCNE2 enhancer and promoter regions in abl versus LNCaP cells (Fig. 4C) further supported the hypothesis that these FoxA1 binding sites may play more important transcriptional regulatory roles in abl than in LNCaP cells. In addition, H3K4me1 levels were higher, and greater recruitment of FoxA1, p-Pol II, CBP and MED1 at UBE2C and/or CDK1 enhancers was observed in abl versus LNCaP cells (Supplementary Fig. S4A), in agreement with our previous findings (11).

As transcriptionally active *cis*-regulatory elements always reside within nucleosome-depleted regions (NDRs) (33) and the FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) technique has been used to successfully identify NDRs (21, 22), we utilized FAIRE to analyze the local chromatin structure of the FoxA1 binding regions. LNCaP and abl cells were transfected with siControl or siFoxA1 followed by FAIRE. In siControl transfected cells, a higher FAIRE signal was observed at the CCNE2 enhancer and promoter regions in abl cells than in LNCaP cells (Fig. 4D), and importantly, FoxA1 silencing decreased FAIRE enrichment at the CCNE2 enhancer and promoter regions only in abl cells (Fig. 4D). Taken together, these data strongly indicate that increased FoxA1 binding induces nucleosome depletion at the CCNE2 enhancer and promoter regions. We obtained essentially similar results at the UBE2C and CDK1 enhancers (Supplementary Fig. S4B).

CRPC-specific FoxA1 binding sites upregulate *CCNA2* via *E2F1*

Although, in general, FoxA1 binding was significantly enriched near overexpressed genes in CRPC compared with ADPC (Fig. 3B), we were unable to identify, using the ChIP-on-chip technique, abl-specific FoxA1 binding sites near *CCNA2* (Fig. 5A), indicative of an indirect mechanism of FoxA1-mediated *CCNA2* upregulation in abl cells (Fig. 2A). As previous studies in other systems demonstrated direct regulation of *CCNA2* by E2F1 transcription factor binding to the CCNA2 promoter region (34, 35), and our FoxA1 ChIP-on-chip and direct FoxA1 ChIP analyses identified and confirmed two putative E2F1 enhancers reside 15.8 kb and 33.4 kb downstream of the TSS of *E2F1* (Fig. 5A and 5B), we hypothesized that FoxA1 may regulate CCNA2 expression through a direct upregulation of *E2F1*. As expected, increased levels of H3K4me1 and p-Pol II, higher occupancy of CBP and MED1, and higher FAIRE enrichment at E2F1 enhancers, were observed in abl compared to LNCaP cells (Fig. 5C and 5D), resulting in FoxA1-dependent, increased mRNA and protein levels of E2F1 in abl versus LNCaP cells in the absence of androgen (Fig. 5E). Similar to CCNE2 regulatory regions, no AR binding was observed at these two E2F1 enhancers in abl cells (data not shown). We next performed ChIP to examine whether E2F1 directly regulates *CCNA2* in abl cells, and expression of CCNA2 mRNA following E2F1 silencing was also examined in LNCaP and abl cells. As shown in Figure 5F and 5G, E2F1 binding at the CCNA2 promoter was increased, and E2F1-dependent, CCNA2 mRNA expression was greater in abl versus LNCaP in the absence of DHT, demonstrating that *CCNA2* is a direct E2F1 target gene in abl but not LNCaP cells. Thus, FoxA1, through a direct transcriptional regulation of *E2F1*, indirectly upregulates (induces) CCNA2 expression in abl cells.

Recruitment of MYBL2 and CREB1 leads to FoxA1 binding and CRPC-specific target gene expression through histone acetylation-facilitated nucleosome disruption

We next investigated the mechanisms responsible for abl-specific FoxA1 binding resulting in abl-specific FoxA1 target gene expression. Given that previous studies have reported that cooperation among transcription factors can result in altered chromatin binding activity (7, 11, 16), we hypothesized that this may be the case for FoxA1 binding in abl but not in LNCaP cells. Thus, to examine whether other transcription factors may affect FoxA1 binding, we performed a *de novo* transcription factor motif search within the abl- and LNCaP-specific FoxA1 binding regions. As expected, Forkhead motifs were significantly

enriched within both abl- and LNCaP-specific FoxA1 binding sites compared with the whole genome background (abl HyperGeometric p-values (36): 5.7E-27 for abl and 1.0E-27 for LNCaP). Interestingly, MYB and CREB motifs were significantly enriched within abl-specific FoxA1 binding regions compared with LNCaP-specific FoxA1 binding regions (HyperGeometric p-values: 5.9E-20 for MYB and 1.1E-15 for CREB) (Fig. 6A), indicating that transcription factors recognizing MYB and CREB motifs may play a "cooperative role" in FoxA1 binding and abl-specific FoxA1 target gene regulation. As ubiquitous expression of MYBL2 (within the MYB family) and CREB1 (within the CREB family) has been reported (37, 38), we examined expression and chromatin binding of MYBL2 and CREB1 in abl and LNCaP cells. MYBL2 and CREB1 protein levels were higher in abl versus LNCaP cells (Fig. 6B), and ChIP assays showed greater recruitment of MYBL2 and CREB1 to regulatory regions of the abl-specific, FoxA1 target genes *CCNE2*, *E2F1*, *UBE2C* and *CDK1* (Fig. 6C and Supplementary Fig. S5A) in abl versus LNCaP cells.

We next examined whether MYBL2 and CREB1 affect FoxA1 binding and p-Pol II loading at regulatory regions of abl-specific FoxA1 target genes. LNCaP and abl cells were transfected with siRNAs targeting MYBL2 or CREB1, and Western blot analyses and ChIP assays were performed using an anti-FoxA1 antibody. No effect of MYBL2 or CREB1 silencing on FoxA1 protein expression levels was observed; however, silencing of either transcription factors decreased FoxA1 binding at the enhancers and promoters of CCNE2 and E2F1, as well as the UBE2C enhancers and the CDK1 enhancer in abl but not LNCaP cells (Fig. 6D and Supplementary Fig. S5B, S5C). In addition to decreased FoxA1 binding, ChIP analyses further demonstrated that MYBL2 and CREB1 silencing reduced p-Pol II levels on most enhancers and promoters of abl-specific FoxA1 target genes, similar to the effect of FoxA1 silencing itself (Supplementary Fig. S5D). Collectively, these results demonstrate that MYBL2 and CREB1 significantly alter FoxA1 binding and p-Pol II loading on chromatin.

To reveal the hierarchical relationship among MYBL2, CREB1 and FoxA1 binding, we silenced FoxA1 and examined MYBL2 and CREB1 binding on chromatin. ChIP analyses showed that FoxA1 silencing decreased MYBL2 and CREB1 recruitment to most regulatory sites of *CCNE2*, *E2F1*, *UBE2C* and *CDK1* in abl but not LNCaP cells (Fig. 6E and Supplementary Fig. S6A). Serial ChIP (re-ChIP) analyses of FoxA1/MYBL2 and FoxA1/CREB1 further demonstrated a stronger FoxA1-MYBL2 or -CREB1 interaction on abl-specific FoxA1 target gene loci in abl versus LNCaP cells (Supplementary Fig. S6B). These data suggest that FoxA1-MYBL2 or -CREB1 co-dependently bind to the same regulatory elements of abl-specific FoxA1 target genes.

To further investigate the molecular mechanisms for the co-dependent binding of FoxA1-MYBL2 and -CREB1 on chromatin, we examined the effect of their silencing on histone acetylation. Altered chromatin structure can greatly influence transcription factor access to chromatin, and histone acetylation has been shown to have profound effects on chromatin architecture leading to a decrease in inter-nucleosomal interaction (33, 39). LNCaP and abl cells were transfected with siRNAs targeting FoxA1, MYBL2 or CREB1, and ChIP assays were performed using an antibody against acetylated histone H3 (AcH3). Silencing of FoxA1, MYBL2 or CREB1 reduced AcH3 levels at regulatory sites of abl-specific FoxA1

target genes in abl but not LNCaP cells, and the level of AcH3 reduction was similar to CBP (a potent histone acetyltransferase [HAT]; positive control) silencing (Fig. 6F and Supplementary Fig. S6C), suggesting that these three transcription factors, possibly via recruitment of CBP and other HATs, can significantly induce histone acetylation, leading to nucleosome disruption at FoxA1 target gene regulatory regions specifically in abl cells (Fig. 4D, 5D and 6G and Supplementary Fig. S4B and S6D).

Finally, we examined the effect of MYBL2 and CREB1 silencing on abl-specific FoxA1 target gene expression. Expression of *CCNE2*, *E2F1*, *CCNA2*, *UBE2C* and *CDK1* after MYBL2 or CREB1 silencing in LNCaP and abl cells were assessed by qRT-PCR. Although silencing of MYBL2 and/or CREB1 decreased CCNE2, E2F1, CCNA2, UBE2C and CDK1 mRNA levels in LNCaP cells to some extent, in abl cells, knocking down of these two transcription factors greatly reduced mRNA expression of these abl-specific FoxA1 target genes (Fig. 6H and Supplementary Fig. S6E). As anticipated, slower growth of siMYBL2- or siCREB1-transfected CRPC cells was observed versus siControl (Fig. 6I and Supplementary Fig. S6F), presumably due to decreased expression of the five essential cell-cycle genes, indicating that MYBL2 and CREB1 are critical for abl-specific FoxA1 target gene expression and CRPC cell growth.

DISCUSSION

In the present study, we found that in addition to its known role as an AR collaborator in regulating CRPC-specific, AR target G2-M gene transcription and thus a driver of G2-M cell-cycle progression (11) (Fig. 1 and Supplementary Fig. S4-S6), FoxA1 directs CRPC G1-S cell-cycle progression through direct regulation of *CCNE2*, and indirect regulation of *CCNA2* via *E2F1* (Fig. 2, 4 and 5). These findings establish a previously undescribed yet essential role for FoxA1 as a master cell-cycle regulator, required for both G1-S and G2-M progression in CRPC, whereas AR is mainly involved in promoting cell-cycle G2-M but not G1-S transition in CRPC (Fig. 7) (11, 40).

The findings that an abl-specific FoxA1 cistrome promotes both G1-S and G2-M cell-cycle progression, through transcriptional regulation of non-AR target genes (*CCNE2* and *E2F1*) (Fig. 4 and 5) and AR target genes (*UBE2C* and *CDK1*) (Supplementary Fig. S4) (11), raise the question, what are the mechanisms controlling differential FoxA1 binding in abl and LNCaP cells? Although it has been established that H3K4me1 and H3K4 dimethylation (H3K4me2) levels determine differential FoxA1 binding in different cell types (6, 11) (Fig. 4C and 5C), whether differential expression and binding of other transcription factors affect FoxA1 binding is unknown. By employing an integrated computational and experimental approach, we identified transcription factors MYBL2 and CREB1 as potential regulators of abl-specific FoxA1 binding. Furthermore, by combining ChIP, siRNA-ChIP and re-ChIP analyses, we demonstrated co-dependent FoxA1/MYBL2 and FoxA1/CREB1 binding to the same DNA fragments in regulatory regions of abl-specific FoxA1 target genes *CCNE2*, *E2F1*, *UBE2C* and *CDK1* (Fig. 6 and Supplementary Fig. S5 and S6). This co-dependent chromatin binding is determined by the nonredundant function of FoxA1, MYBL2 and CREB1 in inducing histone H3 acetylation and thus facilitating nucleosome depletion at abl-specific FoxA1 binding regions (Fig. 6F and 6G and Supplementary Fig. S6C and S6D). Our

findings are consistent with previous *in vitro* findings that activator-dependent histone H3 acetylation leads to an open chromatin structure through disruption of both inter- and intrafiber internucleosome interactions (41, 42). Thus, although histone modifications and nucleosome depletion are not required for FoxA1 to open *in vitro*-reconstituted condensed chromatin (4), our findings suggest that *in vivo* FoxA1 binding requires both active histone H3K4 methylation and other collaborating transcription factors capable of inducing histone acetylation and/or nucleosome disruption.

Interestingly, the expression of MYBL2 and CREB1 is not only increased in abl compared with LNCaP (Fig. 6B), but, more importantly, in clinical cases of CRPC versus ADPC (43, 44). By contrast, strong FoxA1 protein expression persists in early- and late-phase of prostate cancer (Fig. 1A) (45, 46), suggesting that increased expression and binding of MYBL2 and CREB1 during prostate cancer progression alter FoxA1 genomic binding, leading to CRPC-specific upregulation of critical G1-S and G2-M cell-cycle genes by FoxA1 (Fig. 7). Therefore, MYBL2 and CREB1 may serve as new therapeutic targets for CRPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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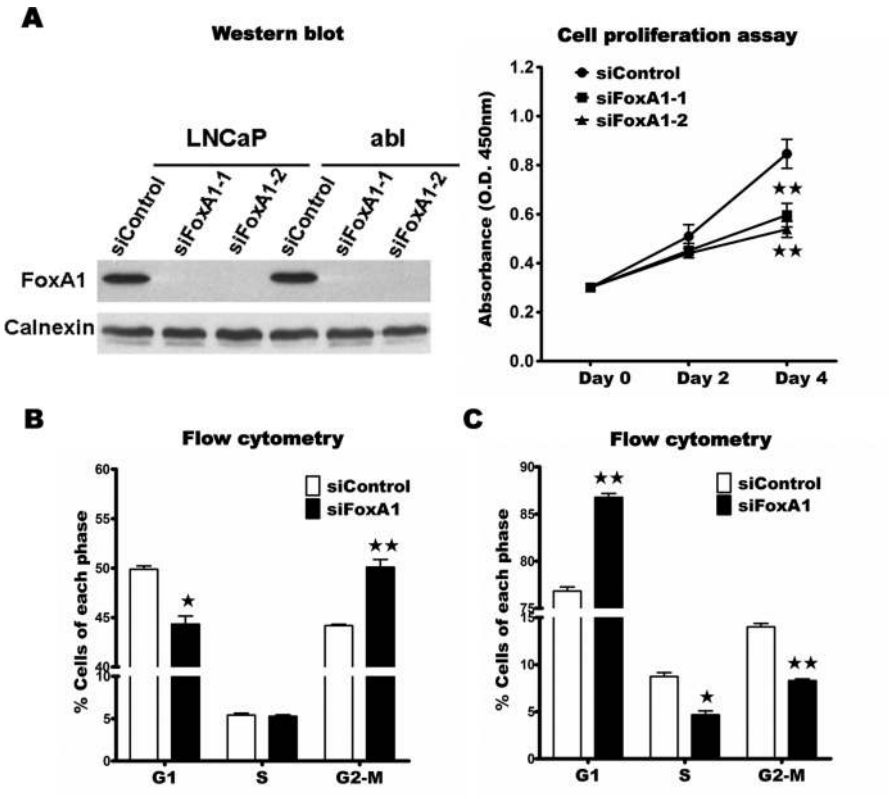

Figure 1.

FoxA1 silencing decreases CRPC cell growth by blocking both G2-M and G1-S cell-cycle progression. A, abl cell proliferation was measured after siRNAs transfection using the WST-1 assay. ** $p < 0.01$. B, abl cells were transfected with siRNAs and synchronized in mitosis by using a thymidine-nocodazole block, and cells were analyzed by flow cytometry. * $p < 0.05$, ** $p < 0.01$. C, Seventy-two hours after siRNAs transfection, abl cells were analyzed by flow cytometry. * $p < 0.05$, ** $p < 0.01$.

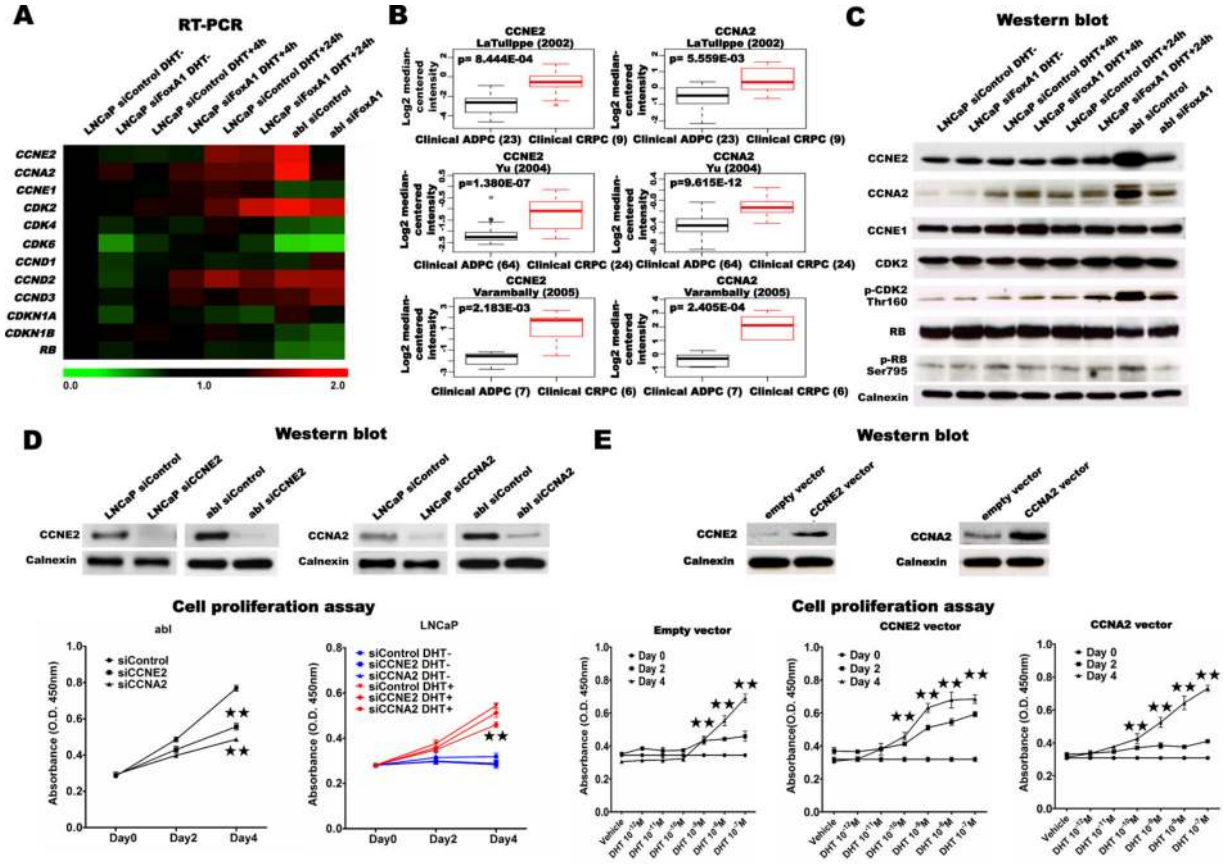

Figure 2.

FoxA1 promotes CRPC growth through upregulation of CCNE2 and CCNA2. A, Seventy-two hours after siRNAs transfection into LNCaP in the absence (−) or presence (+) of DHT for 4 hours and 24 hours, and abl cells in the absence of DHT, total RNA was isolated and amplified by real-time RT-PCR. The mRNA expression data are summarized in heat maps. The color scale represents relative mRNA expression level. B, Boxplots show that CCNE2 and CCNA2 are overexpressed in CRPC cases (red) compared with ADPC cases (black). Number of patient is indicated in brackets. C, Western blots analyses were performed using the indicated antibodies. D, abl cells was transfected with siRNAs in the absence of DHT, and LNCaP cells were transfected with siRNAs in the absence (−) or presence (+) of 10 nM DHT. The cell proliferation was measured using the WST-1 assay. ** $p < 0.01$. E, LNCaP cells stably expressing pcDNA3.1-CCNE2, pcDNA3.1-CCNA2 or the empty vector were cultured in hormone-depleted medium and treated DHT from 10^{-12} M to 10^{-7} M. The cell proliferation was measured using the WST-1 assay. ** $p < 0.01$ as compared with Day 0.

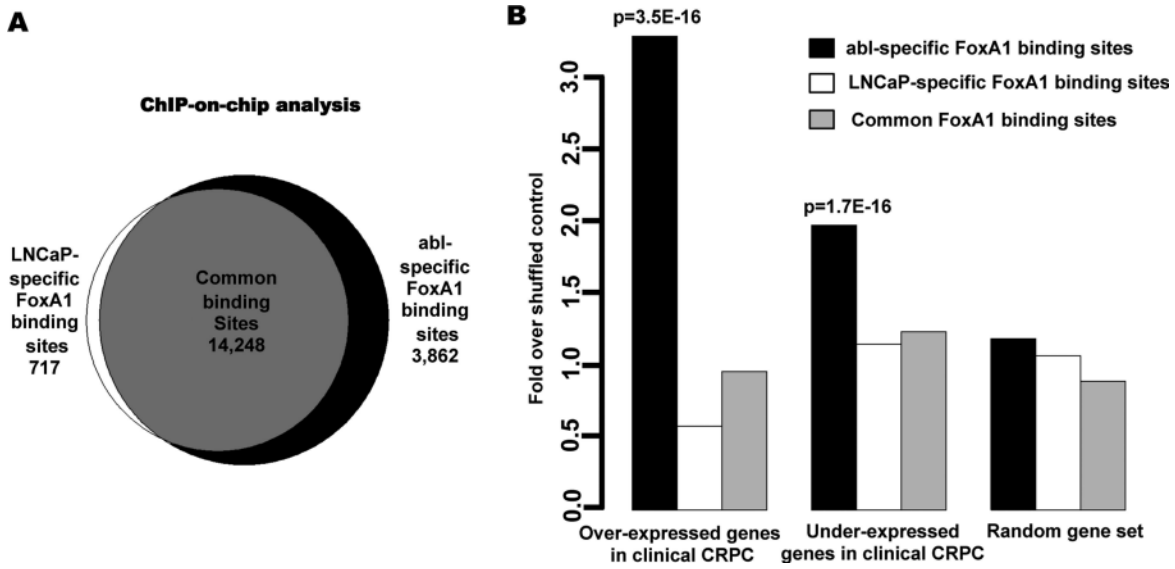

Figure 3.

Correlation of CRPC cell-specific FoxA1 cistrome with clinical CRPC/ADPC gene expression datasets. A, Overlap analysis showing the number of FoxA1-binding sites specific to LNCaP or abl or shared between the two cell lines (p-value ≤1E-4). B, Correlation between cell type-specific FoxA1-binding sites and differential expressed genes in clinical CRPC versus ADPC. FoxA1 binding sites are divided into three groups: abl-specific (black), LNCaP-specific (white) and common binding sites between the two cell lines (grey). Three gene lists are presented as separated stacked bars along X-axis. P-values upon each bar show the significance level of association between FoxA1 binding and genes having FoxA1 binding sites with 20 kb of the TSS.

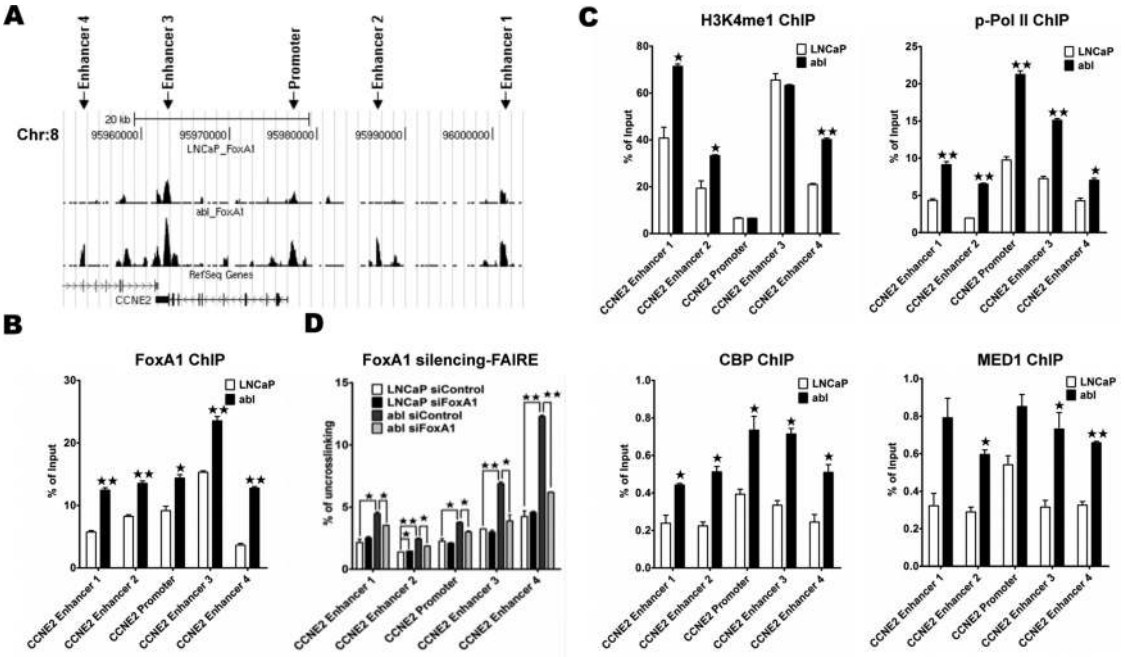

Figure 4.

FoxA1 directly regulates *CCNE2* in CRPC cells. A, FoxA1 binding sites in LNCaP and abl cells relative to the *CCNE2* gene are shown using the UCSC genome browser format. B-C, ChIP assays were performed in LNCaP and abl cells in the absence of androgen using an anti-FoxA1 antibody (B), or antibodies against H3K4me1, p-Pol II, CBP or MED1 (C). * $p < 0.05$, ** $p < 0.01$. D, LNCaP and abl cells were transfected with siRNAs in the absence of androgen. FAIRE-qPCR was performed 72 hours posttransfection. * $p < 0.05$, ** $p < 0.01$.

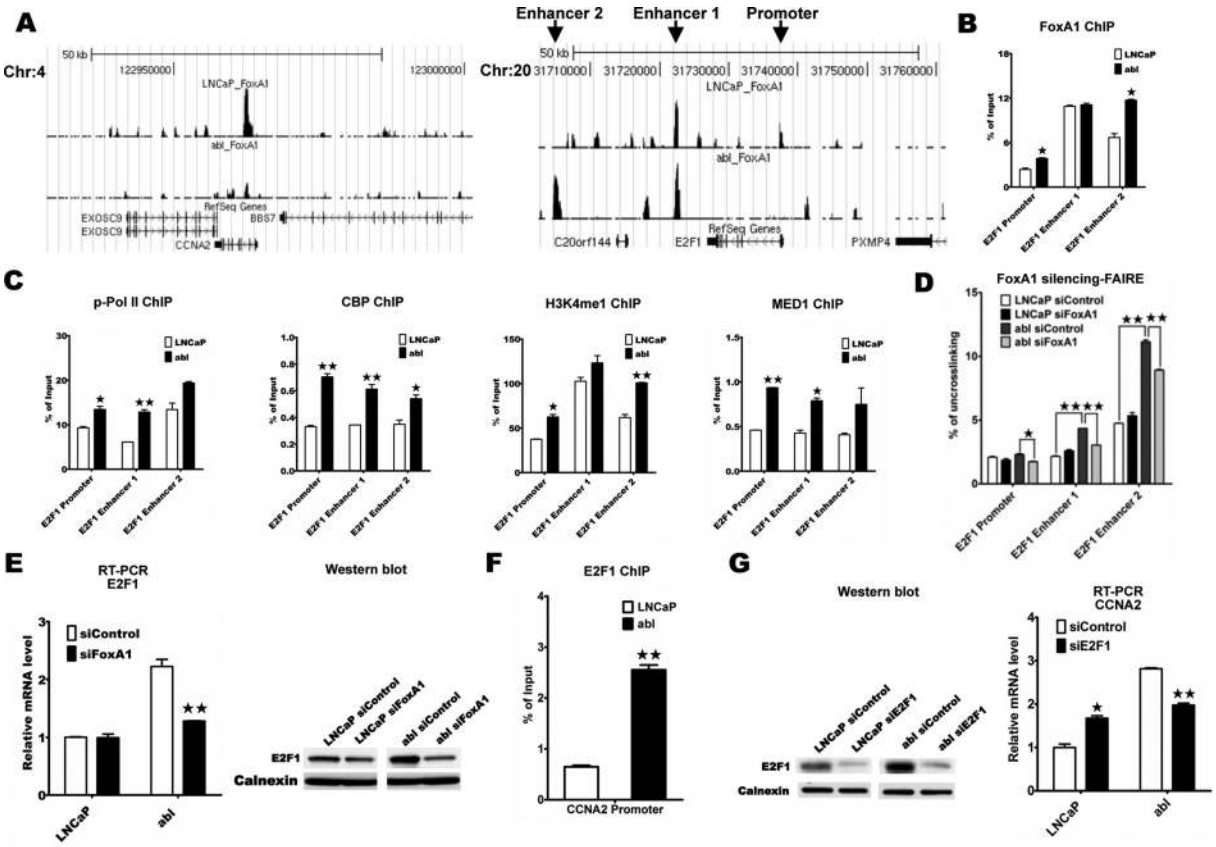

Figure 5.

FoxA1 regulates *CCNA2* via direct regulation of *E2F1*. A, FoxA1 binding sites in LNCaP and abl cells relative to the *CCNA2* and *E2F1* genes were shown using the UCSC genome browser format. B-C, ChIP assays were performed in LNCaP and abl cells in the absence of androgen using an anti-FoxA1 antibody (B), or antibodies against p-Pol II, CBP, H3K4me1 or MED1 (C). * $p < 0.05$, ** $p < 0.01$. D, LNCaP and abl cells were transfected with siRNAs in the absence of androgen. FAIRE-qPCR was performed 72 hours posttransfection. * $p < 0.05$, ** $p < 0.01$. E, LNCaP and abl cells were transfected with siRNAs in the absence of androgen. Real-time RT-PCR and Western blots were performed 72 hours after siRNAs transfection. ** $p < 0.01$. F, ChIP assays were performed using an antibody against E2F1 in untreated LNCaP and abl cells. ** $p < 0.01$. G, LNCaP and abl cells were transfected with siRNAs in the absence of androgen, and real-time RT PCR was performed. * $p < 0.05$, ** $p < 0.01$.

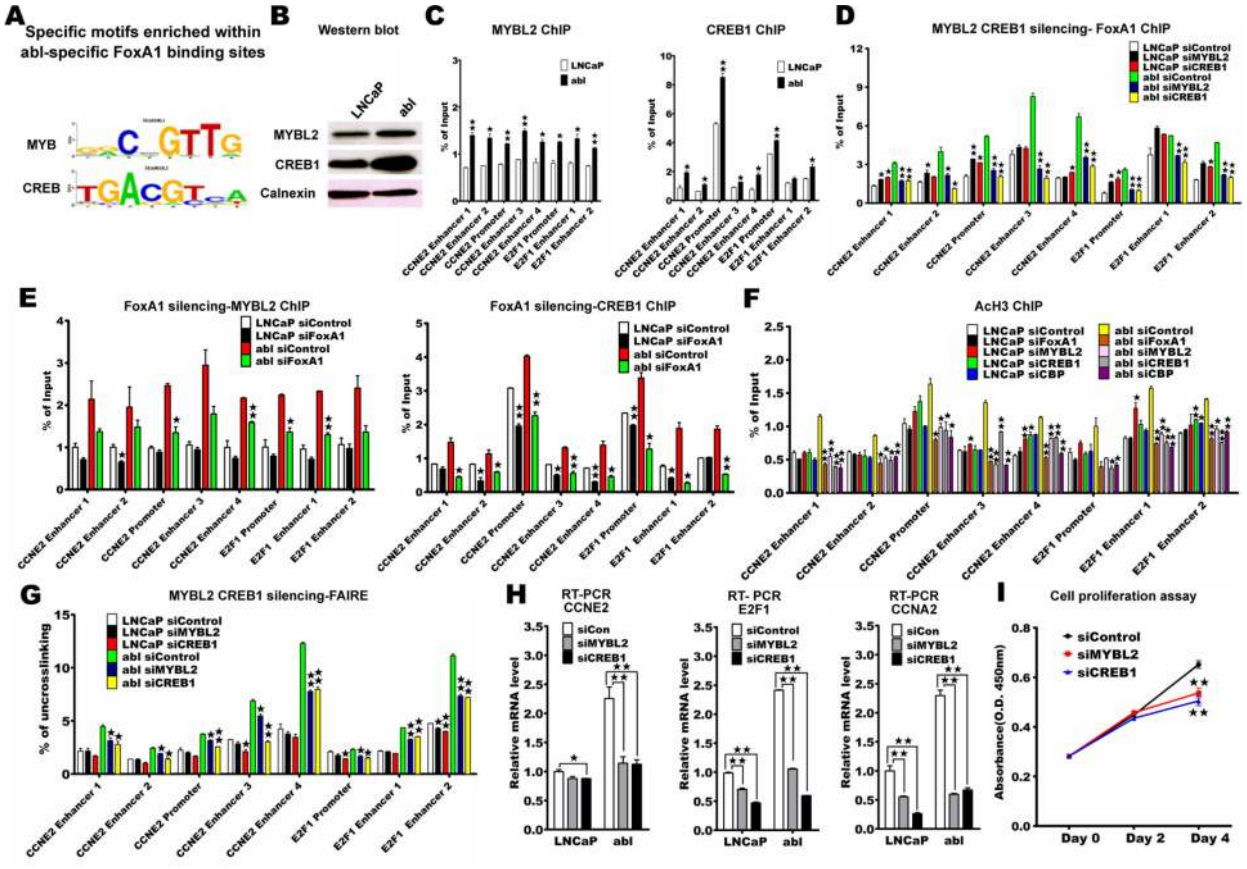

Figure 6.

MYBL2 and CREB1 cooperate with FoxA1 to regulate *CCNE2* and *E2F1*. A, *De novo* motif search revealed that MYB and CREB motifs are enriched within abl-specific FoxA1 binding sites. B, Western blots analyses were performed on untreated LNCaP and abl cell lysates using indicated antibodies. C, ChIP assays were performed using antibodies against MYBL2 or CREB1 in LNCaP and abl cells in the absence of androgen. * $p < 0.05$, ** $p < 0.01$. D-F, LNCaP and abl cells were transfected with siRNAs in the absence of androgen and ChIP assays were performed using an anti-FoxA1 antibody (D), or antibodies against MYBL2 or CREB1 (E), or an anti-AcH3 antibody (F). * $p < 0.05$, ** $p < 0.01$ as compared with LNCaP or abl siControl. G, LNCaP and abl cells were transfected with siRNAs in the absence of androgen and FAIRE-qPCR experiments were performed. * $p < 0.05$, ** $p < 0.01$ as compared with LNCaP or abl siControl. H, LNCaP and abl cells were transfected with siRNAs in the absence of androgen, and real-time RT-PCR was performed using gene-specific primers. * $p < 0.05$, ** $p < 0.01$. I, abl cells were transfected with siRNAs in the absence of androgen, and the cell proliferation was measured using the WST-1 assay. ** $p < 0.01$.

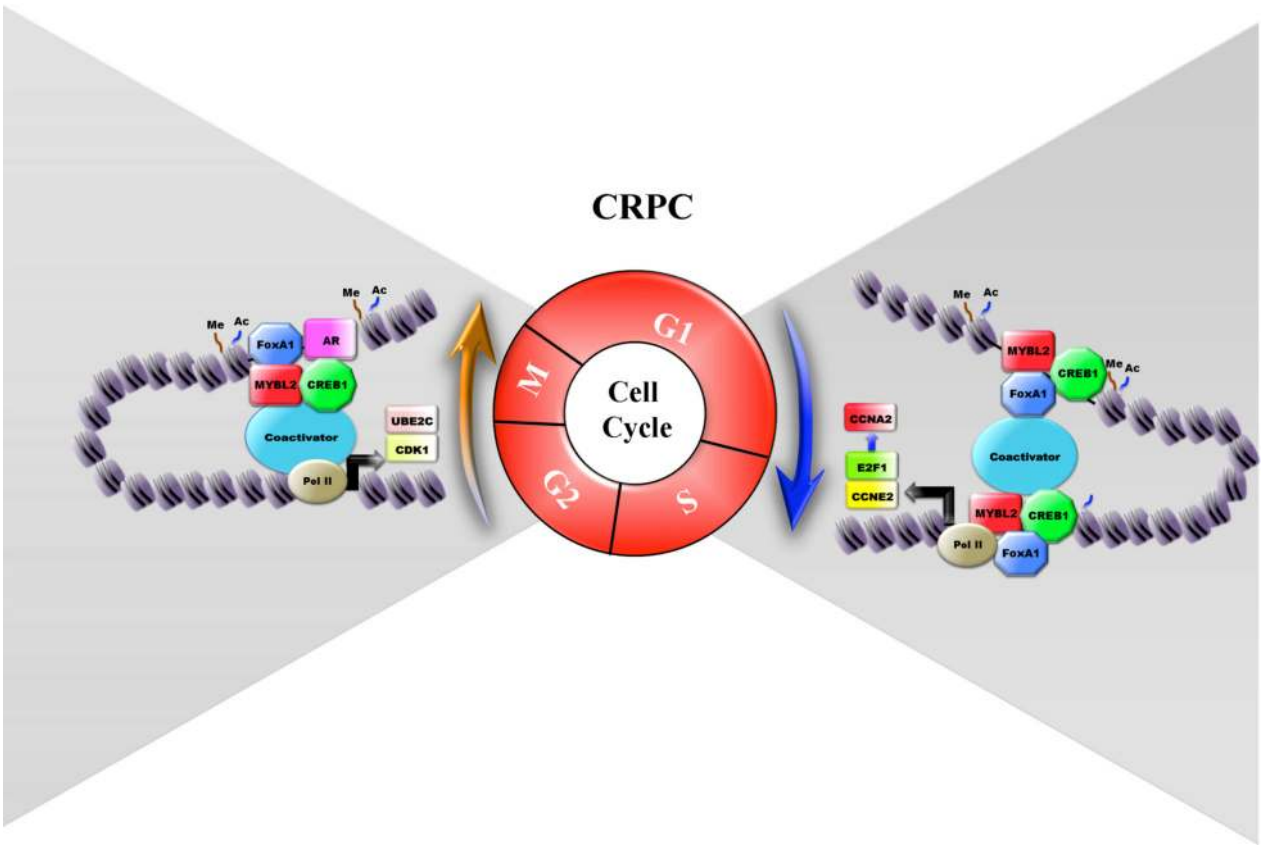

Figure 7.

A model for cell-cycle regulation in CRPC. FoxA1 is a master cell-cycle regulator in CRPC. FoxA1, together with MYBL2 and CREB1, regulates cell-cycle G1-S progression through transcriptional regulation of *CCNE2* and *E2F1*. FoxA1 also collaborates with AR to regulate cell-cycle G2-M transition through direct regulation of *UBE2C* and *CDK1*.