

Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer

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High androgen receptor (AR) level in primary tumour predicts increased prostate cancer-specific mortality. However, the mechanisms that regulate AR function in prostate cancer are poorly known. We report here a new paradigm for the forkhead protein FoxA1 action in androgen signalling. Besides pioneering the AR pathway, FoxA1 depletion elicited extensive redistribution of AR-binding sites (ARBs) on LNCaP-1F5 cell chromatin that was commensurate with changes in androgen-dependent gene expression signature. We identified three distinct classes of ARBs and androgen-responsive genes: (i) independent of FoxA1, (ii) pioneered by FoxA1 and (iii) masked by FoxA1 and functional upon FoxA1 depletion. FoxA1 depletion also reprogrammed AR binding in VCaP cells, and glucocorticoid receptor binding and glucocorticoid-dependent signalling in LNCaP-1F5 cells. Importantly, FoxA1 protein level in primary prostate tumour had significant association to disease outcome; high FoxA1 level was associated with poor prognosis, whereas low FoxA1 level, even in the presence of high AR expression, predicted good prognosis. The role of FoxA1 in androgen signalling and prostate cancer is distinctly different from that in oestrogen signalling and breast cancer.

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Introduction

The androgen receptor (AR) mediates male sex steroid-dependent regulation of cell growth, differentiation and homeostasis (Heinlein and Chang, 2002). This receptor also plays an important role in both androgen-dependent and castration-resistant prostate cancer (Wang *et al.*, 2009). In the nucleus, AR binds to cognate DNA response elements to mediate cell- and tissue-specific regulation of target gene expression (Heinlein and Chang, 2002; Heemers and Tindall, 2007). To form a productive transcription complex on chromatin and to bring about diverse biological actions of androgens, AR needs to communicate with coregulatory proteins (coactivators and corepressors) and collaborating transcription factors (Shang *et al.*, 2002; Kang *et al.*, 2004; Wang *et al.*, 2005). Recent genome-wide studies have shown that nuclear receptors, such as oestrogen receptor (ER), glucocorticoid receptor (GR) and AR, bind to chromatin *in vivo* far away from transcription start sites of their target genes, which implies that distal enhancers are the primary receptor loading sites and suggests that the receptors utilize a distal regulatory mode of transcriptional control (Carroll *et al.*, 2005; John *et al.*, 2008; Lin *et al.*, 2009; Cheung and Kraus, 2010). However, the underlying mechanisms that guide the receptors to their distal chromatin sites to ensure that regulation of only the intended genes occurs are still elusive.

FoxA1/HNF-3 α , a winged-helix transcription factor, is a member of the forkhead family, and it plays a critical role in the growth and differentiation of variety of organs, such as prostate, breast, lung and bladder (Lee *et al.*, 2005; Friedman and Kaestner, 2006; Kaestner, 2010). In mouse prostate development, FoxA1 is required in both epithelial cell differentiation and ductal morphogenesis and patterning (Gao *et al.*, 2005). FoxA1 has been reported to be involved in AR-mediated transcriptional regulation of prostate genes, such as rat *probasin* and human *prostate-specific antigen (PSA)* genes (Gao *et al.*, 2003; Mirosevich *et al.*, 2006). FoxA1-binding sites are found in close proximity of AR-binding sites (ARBs) in regulatory regions of these genes, and AR and FoxA1 have been reported to interact through their DNA-binding domains (Gao *et al.*, 2003; Lee *et al.*, 2008).

FoxA proteins can behave as pioneer factors that engage chromatin before other transcription factors (Kaestner, 2010), and FoxA proteins are able to bind to nucleosomal DNA (Belikov *et al.*, 2009; Sekiya *et al.*, 2009). FoxA1 is a pioneer factor in the ER α -mediated transcriptional programme (Carroll *et al.*, 2005; Eeckhoutte *et al.*, 2006; Lupien *et al.*, 2008), and it also influences a subset of AR target genes (Gao *et al.*, 2003; Lupien *et al.*, 2008; Jia *et al.*, 2009). On the other hand, FoxA1 is capable of creating a compact chromatin structure through recruitment of corepressor complexes, such as the Groucho family of proteins (Sekiya and Zaret, 2007), and a large proportion of FoxA1-binding sites are located outside the active chromatin regions (Eeckhoutte *et al.*, 2009). In this work, we have used LNCaP-1F5 cells, derivatives of LNCaP

cells engineered to express the rat GR (Cleutjens *et al*, 1997) and chromatin immunoprecipitation (ChIP)-sequencing to delineate the genome-wide binding sites of AR and FoxA1—the AR and FoxA1 cistromes—in parental LNCaP-1F5 cells and in cells depleted of FoxA1 protein, in order to assess the role of FoxA1 in AR binding to chromatin and androgen-dependent transcription programme. To delineate the generality of these results, similar experiments on AR binding were conducted in VCaP cells, and on GR binding and glucocorticoid signalling in LNCaP-1F5 cells. Importantly, we also examined the predictive role that FoxA1 protein expression plays in prostate cancer progression, and our results show that, unlike in breast cancer (Badve *et al*, 2007; Albergharia *et al*, 2009), high FoxA1 protein level in primary prostate cancer specimens is associated with poor prognosis of the disease, whereas low FoxA1 level predicts good disease outcome, even in the presence of high AR expression.

Results

AR and FoxA1 cistromes in LNCaP-1F5 cells

Genome-wide distribution of ARBs on LNCaP-1F5 cell chromatin was analysed by using ChIP-seq after a 2-h exposure to 100 nM 5 α -dihydrotestosterone (DHT). The 2-h time point was selected on the basis of previous results on AR loading onto LNCaP cell chromatin (Kang *et al*, 2004; Wang *et al*, 2005; Thompson *et al*, 2006), showing that AR binding peaks by 2 h, and it stays relatively stable at least for the ensuing 12 h. Dose-response experiments indicated that 100 nM DHT was a saturating concentration and that a half-maximal AR loading onto chromatin was achieved by 1–3 nM DHT, as determined by using *PSA* and *TMPRSS2* enhancers as the binding regions (Supplementary Figure S1). By using the MACS algorithm (Zhang *et al*, 2008), a total of 8419 high-confidence ARBs (false discovery rate (FDR) <2%) were found under these conditions (Sahu *et al*, in preparation).

Motif over-representation analysis of the AR cistrome revealed that the FoxA1 motif was the most over-represented *cis*-element (ratio = 2.62, $P < 10^{-244}$) (Figure 1A; Supplementary Table S1). This result together with the importance of FoxA1 in ER α signalling prompted us to assess genome-wide FoxA1-binding sites and their relation to the ARBs. FoxA1 ChIP-seq on LNCaP-1F5 chromatin identified initially 23 420 FoxA1-binding sites (FDR <2%). The peaks called from MACS were used to construct a transcription factor association strength (TFAS) for each gene (Ouyang *et al*, 2009). This analysis identified 6215 ARBs (Supplementary Table S2) and 18 281 FoxA1-binding sites (Supplementary Table S3) that were mapped to the nearest RefSeq gene on the basis of peak intensity and the proximity to the gene within a \pm 100-kb window. Comparison of AR and FoxA1 cistromes revealed that a high proportion of ARBs (~71%) overlapped with FoxA1-binding sites (Figure 1B), with the median distance between the respective binding peaks being 43 nt (range, 0–653 nt; Supplementary Figure S2), suggesting a global role of FoxA1 in androgen signalling and providing credence to the pioneering role of FoxA1 in AR binding to chromatin (Wang *et al*, 2007; Lupien *et al*, 2008). With regard to the FoxA1 cistrome, however, only 24% of FoxA1-binding sites overlapped with ARBs. Examples of overlapping AR- and FoxA1-binding sites for some well-known AR target

genes (*KLK3*, *KLK2*, *DNM1L* and *TMPRSS2*) are depicted in Supplementary Figure S3.

Dual role of FoxA1 in AR binding to chromatin

To examine the genome-wide role of FoxA1 in androgen signalling in more detail, we depleted FoxA1 in LNCaP-1F5 cells using FoxA1 mRNA-specific siRNA. FoxA1 mRNA and protein levels decreased by ~80% upon the 72-h siRNA exposure (Figure 1C; Supplementary Figure S4A), at which time point the cells—treated with control or specific siRNAs—were exposed to 100 nM DHT for 2 h, followed by AR- and FoxA1-binding site identification with ChIP-seq. As expected on the basis of the data shown in Figure 1C, FoxA1 depletion did not result in a complete loss of FoxA1-binding sites; there were 4076 residual FoxA1 sites in the FoxA1-depleted cells (~17% of that in parental cells) (Supplementary Table S4). Intriguingly, FoxA1 depletion resulted in a marked increase (over 2.5-fold) in the number of ARBs, and the AR cistrome in FoxA1-depleted cells comprises 17 022 ARBs (FDR <2%) as opposed to 6215 ARBs in parental cells (Figure 1D; Supplementary Table S5). Comparison of the AR cistromes in parental and FoxA1-depleted cells (siFoxA1 cells) indicated that 57% of the original ARBs (3517 sites) in parental cells were unchanged in the depleted cells, whereas 43% of the parental cell ARBs (2698 sites) were lost upon FoxA1 depletion. Importantly, 13 505 completely new ARBs were found in siFoxA1 cells, that is, more than twice that of parental cells (Figure 1D). Only a small proportion (<10%) of these new ARBs are found in data sets published thus far on ARBs in different prostate cancer cell lines.

A number of ChIP-seq sites were validated by ChIP-qPCR for the three classes of ARBs (Supplementary Figure S5). The ChIP-qPCR data agreed very well with ChIP-seq results. Similar to LNCaP-1F5 cells, FoxA1 depletion resulted in a marked redistribution of ARBs in another prostate cancer cell line, the VCaP line, which contains a much higher number of ARBs than that in LNCaP-1F5 cells (Figure 2A and B). In VCaP cells, close to 32 000 new ARBs were found in siFoxA1 cells, and around 6000 ARBs were lost upon FoxA1 depletion. ChIP-qPCR validation for a number of FoxA1-independent and FoxA1-pioneered ARBs in VCaP cells is shown in Supplementary Figure S6. The ARBs in parental LNCaP-1F5 cells exhibited 87% overlap with those in parental VCaP cells. In siFoxA1 cells, the three ARB categories in LNCaP-1F5 cells overlapped with those in VCaP cells as follows: FoxA1-independent ARBs, 91%; FoxA1-pioneered ARBs, 10%; and FoxA1-depletion dependent new ARBs, 31%. The new ARBs in siFoxA1 VCaP cells validated in Figure 2C are also present in siFoxA1 LNCaP-1F5 cells.

FoxA1 defines three classes of ARBs and AR-regulated transcription programmes

FoxA1 depletion results defined three distinct classes of ARBs: (i) the sites that are independent of FoxA1, and FoxA1 depletion does not perturb them (two examples are shown in Figure 1E), (ii) the sites that require FoxA1 as a pioneer factor to recruit AR onto chromatin and that disappear upon FoxA1 depletion (Figure 1F) and (iii) the sites that are masked by FoxA1 and become available for AR binding upon FoxA1 depletion (Figure 1G). This last, previously unrecognized class includes two subgroups; first,

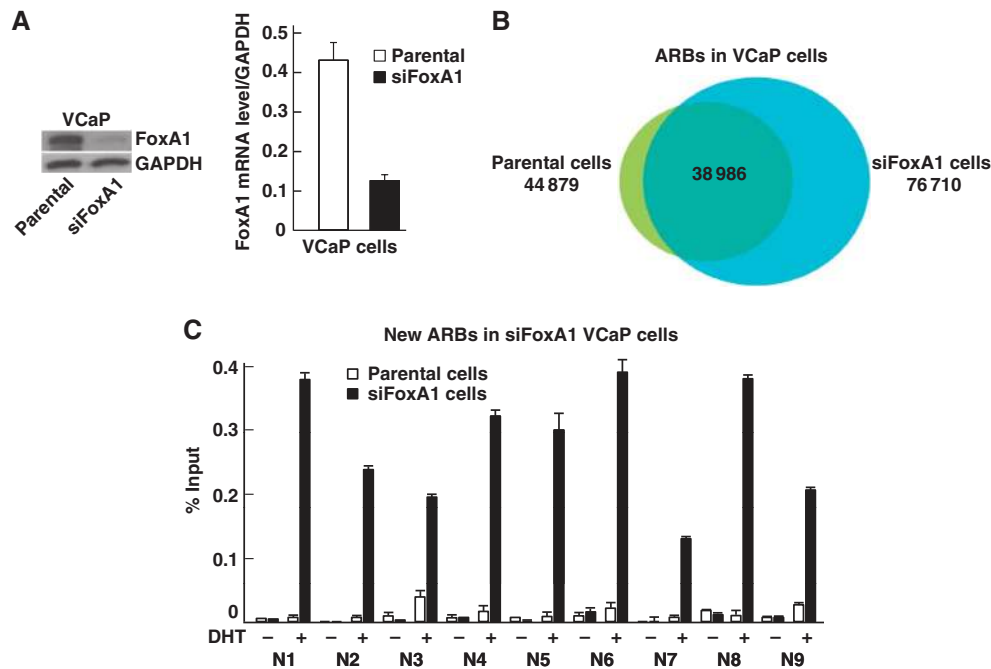


Figure 2 FoxA1 depletion in VCaP cells. (A) FoxA1 mRNA and protein levels. VCaP cells were treated for 72 h with siRNA specific for FoxA1 mRNA (siFoxA1) or control siRNA (parental). (B) Overlap of ARBs (FDR <2%) in parental and FoxA1-depleted VCaP cells. (C) Directed ChIP validation of new ARBs in parental (white bars) and FoxA1-depleted (solid bars) VCaP cells. The cells were exposed to 100 nM DHT (+) or vehicle (-) for 2 h prior to ChIP assays. Mean + s.e.m. values for duplicate samples are shown.

ined their distribution in relation to androgen-regulated genes. To this end, we compared all androgen-dependent and androgen-independent (stably expressed) genes to binding sites unique to AR, shared by AR and FoxA1, and unique to FoxA1 within ± 100 kb of the transcription start sites of the genes. AR and FoxA1 sites were significantly more enriched in parental cells among androgen-regulated genes (both up- and down-regulated) than among androgen-independent genes (Figure 3B). Importantly, the majority of androgen-regulated genes in these cells exhibited significant enrichment for binding sites shared by AR and FoxA1 over those unique to AR and, in each instance, the proportion of up-regulated genes exceeded that of down-regulated ones (Figure 3B). By contrast, in FoxA1-depleted cells, the majority of genes that were regulated by androgen exhibited enrichment of binding sites unique to AR, indicating that their regulation was independent of FoxA1 (Figure 3C). In siFoxA1 cells, a minor fraction of the genes was enriched for unique FoxA1 sites or for sites shared by AR and FoxA1, which is likely due to the fact that there were residual FoxA1 sites after siRNA exposure (see above). Thus, FoxA1 depletion relieves a marked repressive feature from chromatin that, in turn, permits emergence of new ARBs that are linked to expression of novel androgen-responsive genes.

FoxA1 depletion altered the transcription programme already in the absence of androgen (Figure 3D), and similar transcript numbers were up-regulated (188) and down-regulated (198) by FoxA1 depletion. Likewise, the androgen-induced transcription programmes were significantly different between parental and siFoxA1 cells (Figure 3D). The differentially expressed genes upon androgen exposure segregated into three classes (Figure 3E). The expression levels of genes unique to parental cells lost their androgen regulation in siFoxA1 cells concomitantly with the loss of ARBs,

whereas the genes whose ARBs were FoxA1-independent, maintained androgen responsiveness also in siFoxA1 cells. The new genes that were uniquely regulated in siFoxA1 cells by androgen upon the emergence of new ARBs in their regulatory regions were not regulated by DHT in parental cells.

Even though FoxA1 depletion did not affect androgen-mediated regulation of FoxA1-independent genes, such as *PSA*, *NFKBIA*, *SPDEF* and *NDRG1* (Supplementary Figure S8A), and the entire *kallikrein* cluster on chromosome 19 (Supplementary Figure S9), it influenced their basal expression levels; either up (*PSA* and *NFKBIA*) or down (*SPDEF* and *NDRG1*). It is currently not known whether these changes in basal expression relate to the changes in androgen sensitivity (see above). Nevertheless, the fold changes in response to androgen were not markedly different in parental and siFoxA1 cells. Basal expression levels of the genes that required FoxA1 pioneering were also altered, but importantly, their androgen regulation vanished upon FoxA1 depletion, as exemplified by *LPAR3*, *LRIG1*, *EXTL2* and *AFF3* genes (Supplementary Figure S8B). The genes that became androgen-regulated in FoxA1-depleted cells, such as *ETS2*, *EDN2*, *FOXO1* and *CITED1*, exhibited changes in their basal expression levels, and showed robust androgen induction only in siFoxA1 cells (Supplementary Figure S8C).

Pathway analysis using the WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt/>) pathway maps indicated that the three distinct AR-regulated transcription programmes in LNCaP-1F5 cells involved some shared, but to a great extent dissimilar biological processes. In particular, a large number of pathways regulated by DHT in siFoxA1 cells were not androgen-dependent among the pathways that were either independent of or pioneered by FoxA1 (Supplementary Table S6). Under FoxA1 depletion conditions, the new ARBs shared by LNCaP-1F5 and VCaP cells were linked to genes represent-

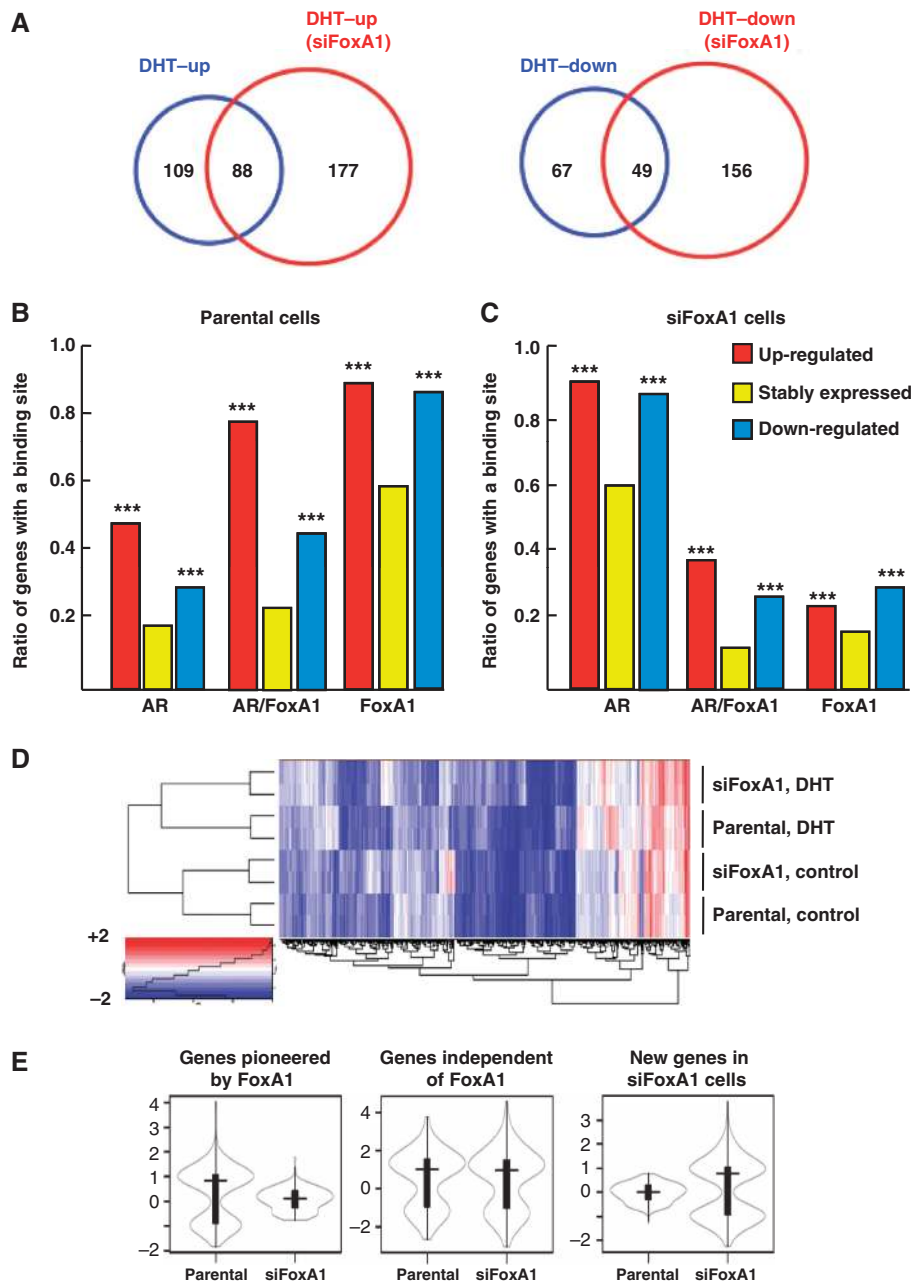


Figure 3 Dual role of FoxA1 in androgen-dependent transcription programme. (A) Venn diagram showing the numbers of androgen-regulated genes in parental and siFoxA1 cells. The cells were exposed to 100 nM DHT for 24 h, after which RNA was isolated for expression profiling by microarray using the cut-off of ≥ 1.7 -fold change. (B, C) Correlation between androgen-regulated (up- and down-regulated) and androgen-independent (stably expressed) genes and the incidence of binding sites unique to AR, shared by AR and FoxA1, and unique to FoxA1 within a window of ± 100 kb of TSSs of the genes in parental cells (B) and in siFoxA1 cells (C). ***Significantly different ($P < 0.001$) from stably expressed genes. (D) Unsupervised hierarchical clustering of androgen-regulated transcripts in parental and siFoxA1 cells before (Control) and after exposure to DHT. Heatmaps of biological duplicate samples are shown. (E) Violin plots (Hintze and Nelson, 1998) summarizing the changes in androgen-dependent transcripts in the three classes of genes defined by FoxA1.

ing a number of new pathways or gene ontology categories related to hormonal signalling and cell proliferation (Supplementary Tables S7 and S8).

Motif and cis-element analyses

The DNA sequence in itself may act as an allosteric ligand for nuclear receptors, thereby affecting both receptor confirmation and regulatory activity (Meijsing *et al*, 2009). *De novo* motif search and motif over-representation analyses identified canonical ARE and FoxA1 motifs as the top-scoring

cis-elements in the AR and FoxA1 cistromes of parental LNCaP-1F5 cells, respectively (Figure 4A and B). The ARBs independent of FoxA1 contained a top-scoring ARE very similar to that in parental cells (Figure 4C). Intriguingly, *de novo* motif search revealed a distinct top-scoring *cis*-element for the ARBs that were lost upon FoxA1 depletion (Figure 4D). This element may be considered as a tail-to-tail ARE spaced by four nucleotides, or alternatively, as compilation of an ARE half-site and a partial FoxA1 motif (*cf.*, Figure 4A and B). *De novo* motif search for an extended

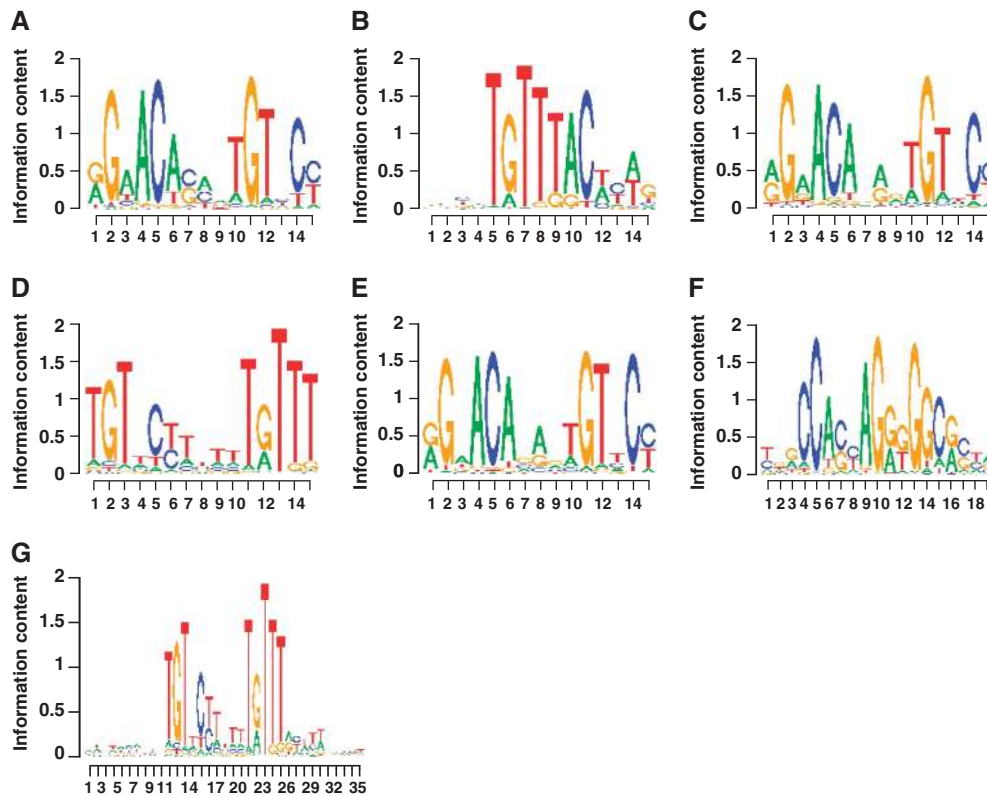


Figure 4 Top-scoring *cis*-elements by *de novo* motif search. (A) Top-scoring *cis*-element for the ARBs in parental cell AR cisrome. (B) Top-scoring *cis*-element for the FoxA1-binding sites in parental cell FoxA1 cisrome. (C) Top-scoring *cis*-element for the FoxA1-independent ARBs. (D) Top-scoring *cis*-element for ARBs that required FoxA1 pioneering. (E) Top-scoring *cis*-element for the new ARBs that appeared upon FoxA1 depletion. (F) CTCF as the over-represented *cis*-element in the unique FoxA1-binding sites in parental cells and the new ARBs in siFoxA1 cells. (G) Top-scoring *cis*-element for the FoxA1-pioneered ARBs identified by an extended *de novo* motif search, in which the number of nucleotides in the search was 35 instead of 15 as in (A–F).

35 nt sequence supported the latter possibility (Figure 4G). Importantly, this unique sequence was highly over-represented among the ARBs pioneered by FoxA1 and corresponded 26% of all these sites, but was absent in the two other ARB categories (Supplementary Table S9). The median spacing between AR- and FoxA1-binding sites in the category of lost ARBs was 41 nt (range, 0–454 nt; Supplementary Figure S2). And finally, the new ARBs that appeared in siFoxA1 cells exhibited a canonical ARE as the top-scoring by *de novo* motif (Figure 4E); these sites were masked in parental cells in a FoxA1-dependent fashion and thus not accessible to AR binding in these cells.

Besides the FoxA1 motif, additional *cis*-elements flanking the ARBs in parental cells included those for forkhead family members, such as FoxA2, and FoxF2, together with E2F1, GATA1 and STAT1 motifs (Supplementary Table S1). Of note, the three ARB categories defined by FoxA1 were enriched for different sets of flanking *cis*-regulatory elements (Supplementary Table S9). An important *cis*-element enriched in the unique FoxA1 cisrome, that is, the FoxA1-binding sites that did not overlap with ARBs in parental cells (Figure 1), was that for CTCF (ratio = 8.13, $P = 8.9 \times 10^{-82}$). This motif (Figure 4F) was also over-represented among the ARBs that appeared upon FoxA1 depletion (ratio = 2.8, $P = 1.32 \times 10^{-22}$). The new ARBs in siFoxA1 cells were not flanked by FoxA1 motifs; rather, other *cis*-elements were enriched in the proximity of these sites (Supplementary Table S9). Intriguingly, some transcription factors binding to these latter *cis*-elements

became androgen regulated in siFoxA1 cells, including members of the ETS family and FOXO1 (Supplementary Figure S8). It remains to be established whether they can substitute for FoxA1 in guiding AR binding to the intended genomic sites that subsequently reprogrammes androgen-regulated transcription in FoxA1-depleted cells.

FoxA1 translates the H3K4me2 mark in a genome-wide fashion

FoxA1-binding sites have been predominantly found at genomic sites that are rich in dimethylated histone H3 lysine 4 (H3K4me2) marks and poor in dimethylated H3 lysine 9 (H3K9me2) marks (Lupien *et al*, 2008; He *et al*, 2010). H3K4me2 marks are associated with both enhancers and transcription start sites of genes (Barski *et al*, 2007; Heintzman *et al*, 2007). H3K4me2 ChIP-seq in both parental and FoxA1-depleted LNCaP-1F5 cells was conducted to examine whether the genome-wide distribution of H3K4me2 marks was altered by FoxA1 depletion and if yes, whether there was any correlation between these marks and the new ARBs in siFoxA1 cells. Distribution of the majority of H3K4me2-marked sites in parental cells was in agreement with that in a previous report (Lupien *et al*, 2008); FoxA1 translated this epigenetic mark and guided AR to bind to proper chromatin sites upon androgen exposure, in that ~70% of the sites shared by AR and FoxA1 possessed H3K4me2 marks. Of note, FoxA1 depletion did not affect the majority of the H3K4me2 marks, as illustrated by the two

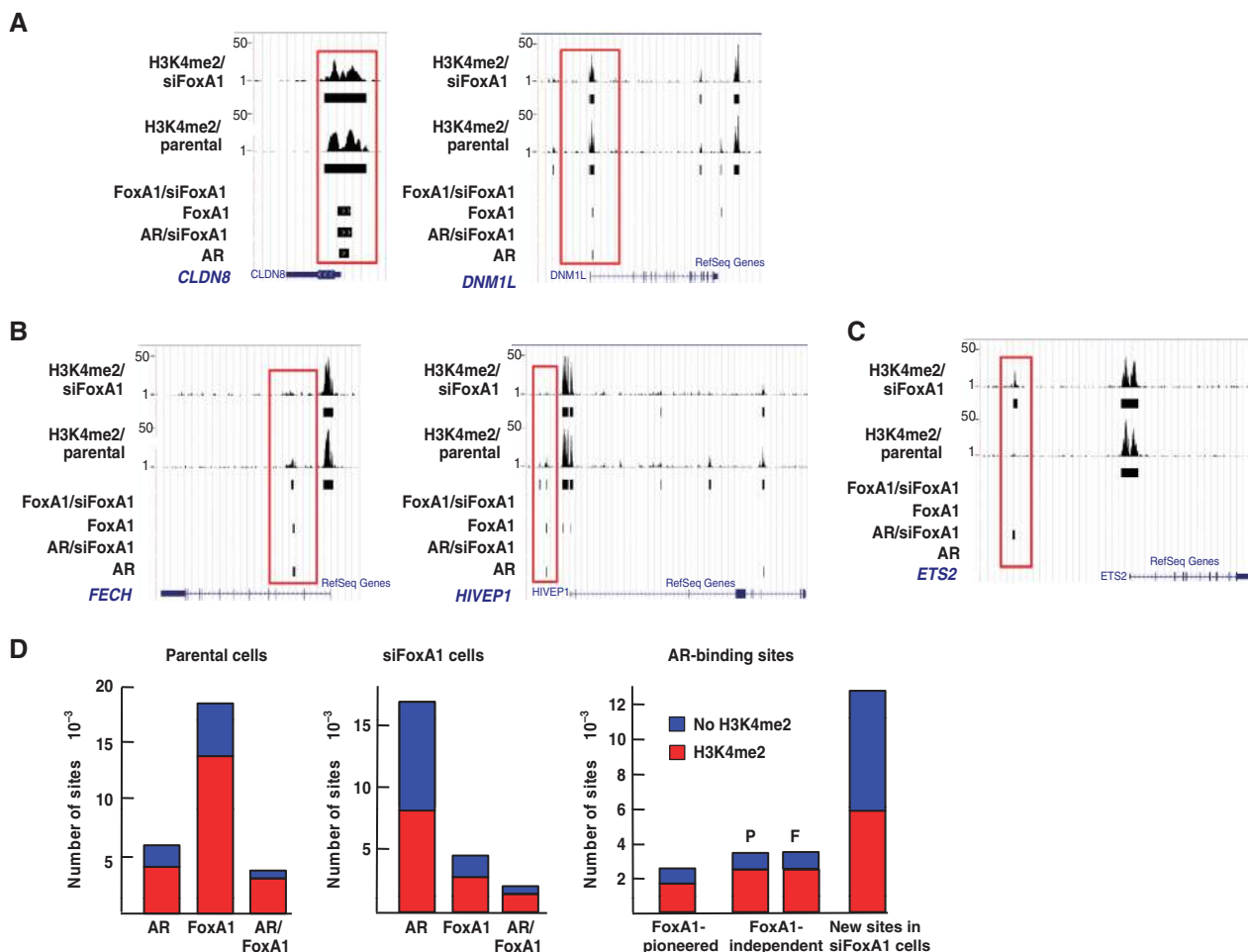


Figure 5 H3K4me2 marks in parental and FoxA1-depleted cells. (A) AR-/FoxA1-binding sites and H3K4me2 marks in parental and siFoxA1 cells at *CLDN8* and *DNM1L* loci. H3K4me2 marks are shown by raw tag counts and the solid black bar shows the binding sites after peak calling on the UCSC genome browser. (B) AR-/FoxA1-binding sites and H3K4me2 marks in parental and siFoxA1 cells at *FECH* and *HIVEP1* loci. (C) AR-/FoxA1-binding sites and H3K4me2 marks in parental and siFoxA1 cells at the *ETS2* locus. AR recruitment upon FoxA1 depletion occurs concomitantly with the appearance of new H3K4me2 marks. (D) Distribution of AR-/FoxA1-binding sites and H3K4me2 marks in parental cells (left panel), siFoxA1 cells (middle panel), and in the three classes of ARBs defined by FoxA1 (right panel). H3K4me2 marks for FoxA1-independent ARBs are shown separately for parental (P) and FoxA1-depleted cells (F).

examples in Figure 5A. *CLDN8* does not require FoxA1 to guide receptor binding, and AR can recognize H3K4me2 marks either on its own or through another collaborating factor. *DNM1L* is a FoxA1-pioneered locus that maintains H3K4me2 marks despite the absence of both FoxA1 and AR binding in siFoxA1 cells. In some instances, however, H3K4me2 marks disappeared upon FoxA1 depletion with a subsequent loss of both AR binding and androgen-dependent gene expression, as illustrated by *FECH* and *HIVEP1* loci in Figure 5B. In addition, FoxA1 depletion could also result in the appearance of new H3K4me2 marks that were occupied by new ARBs in siFoxA1 cells, as shown by the *ETS2* locus in Figure 5C.

As mentioned above, localization of most AR- and FoxA1-binding sites correlated with the presence of H3K4me2 marks in parental LNCaP-F15 cells (Figure 5D). Nevertheless, about one-third of the unique ARBs, one-fourth of FoxA1-binding sites, and one-fifth of shared AR/FoxA1 sites were without the H3K4me2 marks in parental cells (Figure 5D). For the three classes of ARBs defined by FoxA1, about 70% of the sites pioneered by FoxA1 and the sites independent of FoxA1

contained H3K4me2 marks. However, close to 50% of the ARBs in siFoxA1 cells did not have H3K4me2 marks, suggesting that other epigenetic marks and/or other collaborating transcription factors are required to guide AR to recognize these sites in the absence of FoxA1.

Correlation of AR and FoxA1 binding with DNaseI-hypersensitive sites

The nuclease accessible sites (DNaseI-hypersensitive (DHS) sites) are good indicators to distinguish between accessible and non-accessible chromatin sites that are known to be highly cell-specific and often dictated by the available compilation of chromatin remodellers and other associated factors (John *et al*, 2008; Boyle *et al*, 2008). Genome-wide mapping of DHS sites (Song and Crawford, 2010) was used to examine in LNCaP-1F5 cells whether FoxA1 depletion resulted in an open chromatin conformation that subsequently created new ARBs, or whether chromatin was already in an open conformation prior to FoxA1 depletion. The results showed that most of the AR- and FoxA1-binding sites associate with DHS sites in parental cells and that the majority of these sites

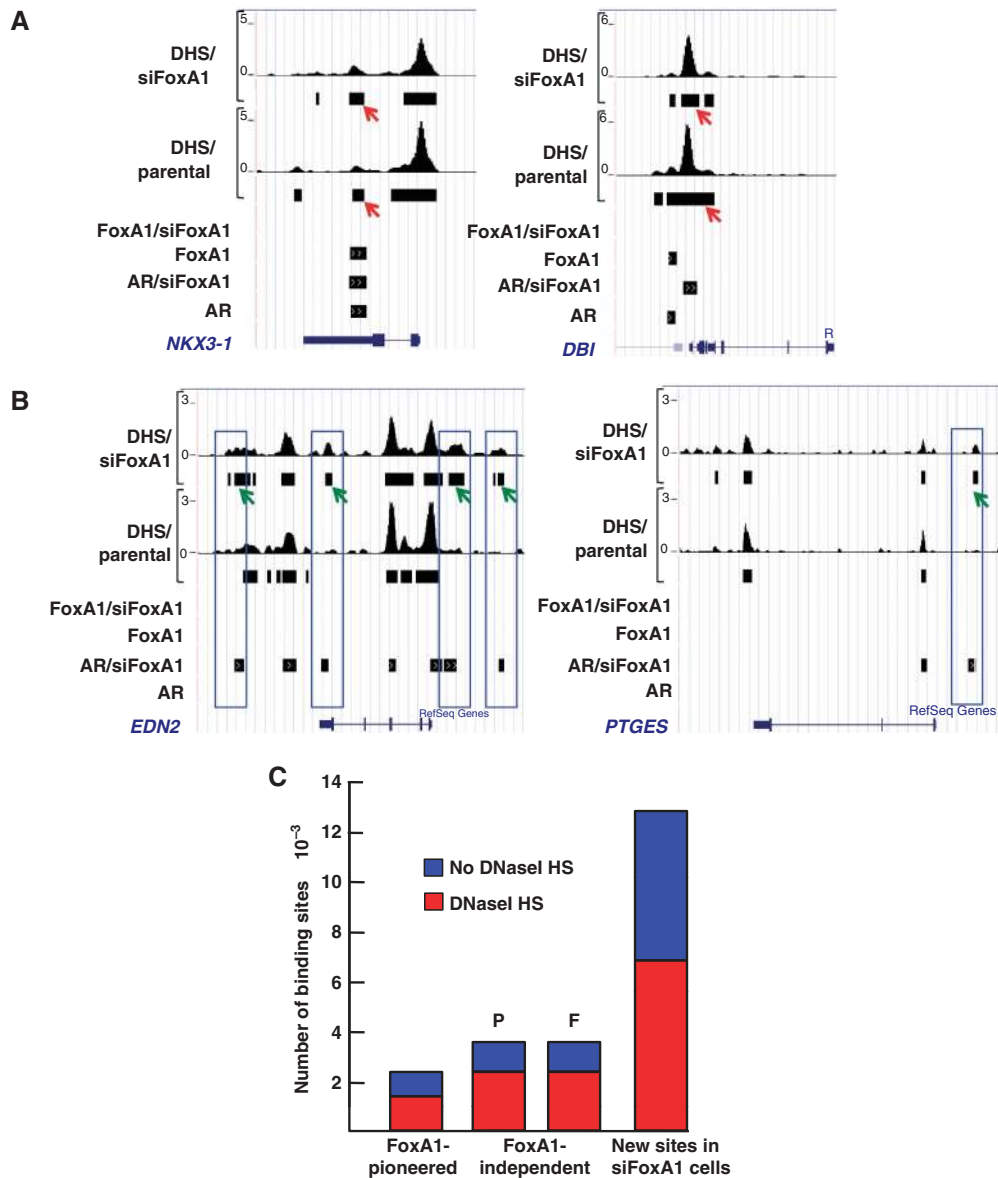


Figure 6 Comparison of DHS sites in parental and FoxA1-depleted LNCaP-1F5 cells. (A) DHS sites together with AR- and FoxA1-binding sites in parental and siFoxA1 cells at the *NKX3-1* and *DBI* loci. The constitutively open sites are shown marked by red arrows. (B) DHS sites together with AR- and FoxA1-binding sites in parental and siFoxA1 cells at the *EDN2* and *PTGES* loci. The *de novo* DHS sites are marked by green arrows. *EDN2* and *PTGES* genes are androgen-regulated only in siFoxA1 cells. (C) Proportion of the DHS sites in the three ARB classes defined by FoxA1. DHS sites for FoxA1-independent ARBs are shown separately for parental (P) and FoxA1-depleted cells (F).

(~70%) were constitutively open already prior to FoxA1 and AR binding to chromatin. Examples of this arrangement are depicted in Figure 6A for *NKX3-1* and *DBI* loci. However, FoxA1 depletion also affected chromatin conformation in a number of instances, as illustrated by the appearance of new DHS sites concomitantly with the appearance of new ARBs, which is exemplified by *EDN2* and *PTGES* loci in Figure 6B. These new ARBs occupying *de novo* DHS sites comprises 16% of all the ARBs mapped in siFoxA1 cells, and they may be formed through recruitment of chromatin modifying proteins to these loci in an AR-dependent manner.

Comparison of genome-wide colocalization of DHS sites with the ARBs in the three categories defined by FoxA1 revealed that approximately the same proportion of the DHS sites—two-thirds—overlapped with ARBs that were either FoxA1-independent or pioneered by FoxA1 (Figure 6C).

However, similar to the H3K4me2 marks, only one-half in the new ARBs in siFoxA1 cells overlapped with DHS sites (*cf.*, Figures 5D and 6C).

FoxA1 depletion elicits redistribution of GR-binding sites and reprogrammes glucocorticoid signalling

LNCaP-1F5 cells have been engineered to express the rat GR (Cleutjens *et al*, 1997) and, therefore, provided an opportunity to examine in a single cell line whether reprogramming of androgen signalling by FoxA1 depletion in LNCaP-1F5 or VCaP cells was unique to AR. This issue is of particular significance in view of a recent report showing that FoxA1 depletion abrogates almost all genome-wide ER-binding sites and blunts concomitantly oestrogen-dependent signalling in breast cancer cells (Hurtado *et al*, 2011). ChIP-seq analyses revealed the presence of 5971 GR-binding sites (GRBs) in

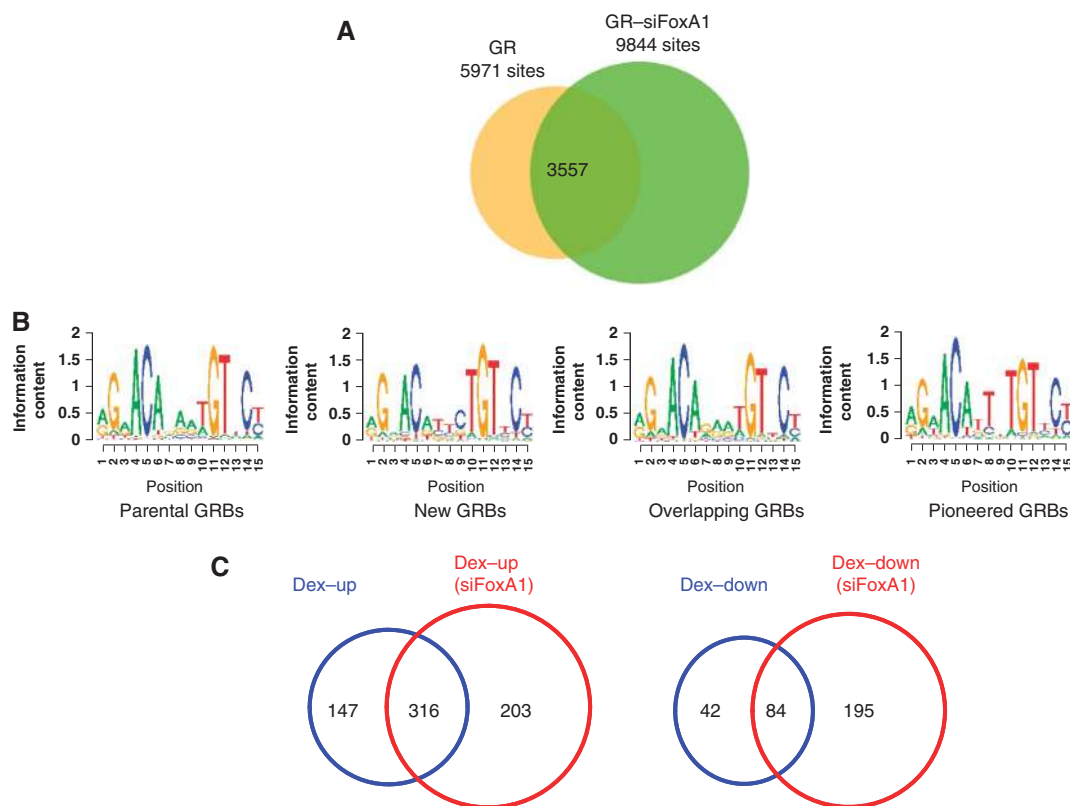


Figure 7 FoxA1 depletion in LNCaP-1F5 cells brings about redistribution of GR binding and commensurate changes in GR-dependent transcription programme. **(A)** Overlap analysis of GRBs (FDR < 2%) in parental (GR) and FoxA1-depleted cells (GR-siFoxA1). **(B)** Top-scoring *cis*-elements, as identified by *de novo* motif search in different subclasses of GRBs. **(C)** The number of genes up- and down-regulated by Dex (Dex-up and Dex-down, respectively) exposure in parental and FoxA1-depleted (siFoxA1) LNCaP-1F5 cells.

parental LNCaP-1F5 cells and 9844 GRBs in siFoxA1 cells after a 2-h dexamethasone (Dex) exposure (Figure 7A). Redistribution of GRBs upon FoxA1 depletion showed tripartite classification very similar to that of ARBs, in that 60% of the GRBs in parental cells were maintained and 40% were lost upon FoxA1 depletion, and a large number of completely new GRBs (6287 sites) emerged in siFoxA1 cells (Figure 7A). When the top-scoring *cis*-elements were analysed by *de novo* motif search, similar elements were found in the three subclasses and, unlike the situation with ARBs, the GRBs that were lost upon FoxA1 depletion did not possess a clearly distinct *cis*-element (Figure 7B). Despite their very similar top-scoring *cis*-elements, the two receptors did not bind to identical chromosomal loci, as only one-fifth of the new ARBs overlapped with the new GRBs in siFoxA1 cells.

Glucocorticoid-dependent transcription programmes in parental and FoxA1-depleted LNCaP-1F5 cells revealed a pattern similar to that of androgen-mediated programmes, in that redistribution of GRBs in siFoxA1 cells was commensurate with changes in GR-dependent gene expression profiles, in both genes up- and down-regulated by Dex (Figure 7C). Importantly, only a small proportion (~15%) of androgen-regulated new transcripts in siFoxA1 cells were also regulated by glucocorticoid in the same cells.

Collectively, our results on both androgen and glucocorticoid signalling indicate that binding of their cognate receptors to chromatin is a mobile event and significantly regulated by another DNA-binding transcription factor, FoxA1. As a consequence, the steroid, the receptor and the *cis*-element are

necessary but not sufficient in most instances to guide AR and GR to the appropriate chromosomal locations, in order to initiate the intended hormonal signalling.

FoxA1 expression in prostate cancer and its relationship to disease outcome

Increased nuclear AR protein expression in either diagnostic biopsy and/or radical prostatectomy specimens is associated with a reduced time to prostate cancer-specific mortality (Donovan *et al*, 2008, 2009). This finding was verified in the patient cohort examined in this work (Supplementary Figure 10A). AR amplification and overexpression are also major features of castration-resistant prostate cancers (Visakorpi *et al*, 1995; Chen *et al*, 2004), and retinoblastoma tumour suppressor controls prostate cancer progression through modulation of AR expression (Sharma *et al*, 2010). These previous results, high expression of FoxA1 mRNA in normal and malignant prostate (Supplementary Figure S10B), and the ability of FoxA1 protein to reprogramme androgen signalling prompted us to investigate FoxA1 protein expression in primary tumours of 350 prostate cancer patients who underwent radical prostatectomy and had clinical follow-up for 11.3–25.0 years (Supplementary Tables S10–S12).

Almost all primary prostate cancer specimens expressed FoxA1 protein, and the antigen staining was predominantly nuclear (Figure 8A). FoxA1 staining intensity exhibited significant positive correlation with that of AR (Figure 8B). When FoxA1 expression was graded according to the staining intensity, 24 samples (6.9%) were negative or weakly

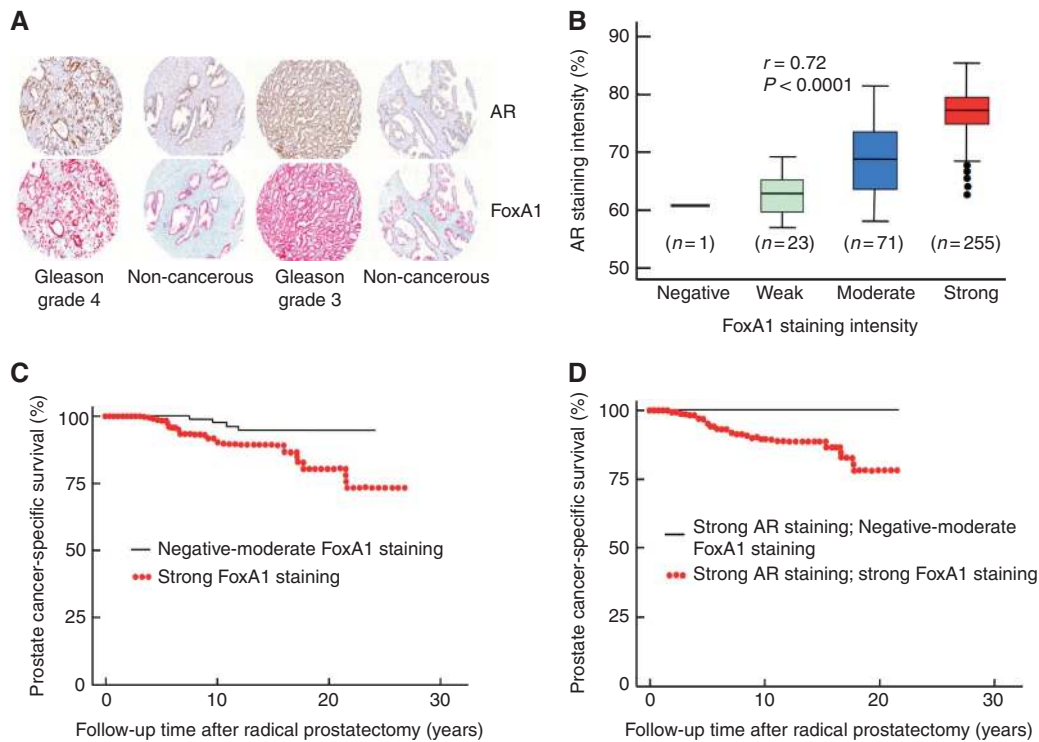


Figure 8 FoxA1 protein expression in prostate cancer tissue specimens and prostate cancer-specific survival. **(A)** Representative FoxA1 and AR staining patterns in prostate cancer and adjacent non-cancerous tissues of two patients. **(B)** Correlation between FoxA1 and AR staining intensity in the prostate cancer patient cohort ($n = 350$). **(C)** Disease-specific survival of 350 patients with prostate cancer according to the intensity of FoxA1 staining in the primary tumour. $\chi^2 = 4.36$. $P = 0.04$ (log-rank test). **(D)** Disease-specific survival of patients with high AR staining classified according to FoxA1 staining in the same specimen; either negative-moderate ($n = 18$) or high ($n = 222$). $\chi^2 = 2.57$. $P = 0.10$ (log-rank test).

staining, 71 samples (20.3%) contained moderate and 255 (72.9%) strong FoxA1 staining. FoxA1 staining intensity was significantly weaker in the adjacent non-cancerous than in the cancerous tissue, as illustrated by the two examples in Figure 8A ($P < 0.01$ for the entire material). Importantly, FoxA1 staining in primary prostate cancer specimens showed a significant relationship to disease outcome ($P < 0.05$), with a strong nuclear FoxA1 staining being associated with poor prognosis, that is, a reduced time to prostate cancer-specific mortality (Figure 8C). The hazard ratio was 2.89 (95% confidence interval 1.02–8.21). There was also a significant positive association of FoxA1 staining intensity and the percentage of FoxA1-stained nuclei to the Gleason grade of the tumours (Supplementary Table S12). Of note, low FoxA1 protein expression was associated with good prognosis, even in the presence of high AR protein expression that, in and of itself (see above), spells poor disease outcome (Figure 8D). This latter finding agrees with our data from cell line experiments, indicating that the level of FoxA1 plays an important role in AR pathway reprogramming.

In prostate cancer, high FoxA1 protein expression-dependent signalling appears to maintain a transcription programme with oncogenic potential. This may occur, at least in part, through active AR signalling that is supported by high FoxA1 expression; the support disappears upon FoxA1 depletion. Low FoxA1 expression would, in turn, be connected with reprogrammed AR pathway that involves activation of tumour suppressors or attenuation of AR-dependent oncogenic signalling. Potential examples of this dual role of FoxA1 are shown in Figure 9. In the case of *CCND3*, *FOXO3*, *PIM1*, *ACSL3*, *MYC*, *FOXO1* and *DAB2IP* loci (Figure 9A–G), FoxA1

depletion resulted in emergence of new ARBs; as a consequence, androgen regulation of the encoded mRNAs in LNCaP-1F5 cells was altered in a fashion that is consistent with activation of tumour suppression or attenuation of oncogenic potential. In the case of *CDK6*, loss of AR binding upon FoxA1 depletion blunted androgen-dependent expression of *CDK6* mRNA (Figure 9H). Comparison of FOXO1 and FOXO3 nuclear staining in 10 low-moderate FoxA1/high AR spots and 30 high FoxA1/high AR spots on three primary prostate cancer tissue microarray (TMA) slides revealed significant association of low-moderate FoxA1 protein level with stronger FOXO1 and FOXO3 staining intensity (Supplementary Table S13), thus being in concert with the results shown in Figure 9, in that FoxA1 depletion in LNCaP-1F5 cells brings about up-regulation of FOXO1 and FOXO3 mRNA accumulation.

Discussion

The present work shows several novel features in the AR-chromatin interaction. First, AR binding to chromatin is remarkably fluid and highly dependent on the forkhead protein FoxA1. Second, FoxA1 plays a dual role in regulating the accessibility of AR to chromatin; it serves as a pioneer factor for a subset of sites but importantly, it also creates—perhaps via recruitment of corepressor complexes (Sekiya and Zaret, 2007; Eeckhoutte *et al*, 2009)—an environment on chromatin that precludes AR binding to its cognate *cis*-elements. These latter sites become accessible to AR binding upon FoxA1 depletion. Third, there is a subset of ARBs that is independent of FoxA1 and may collaborate with or be pio-

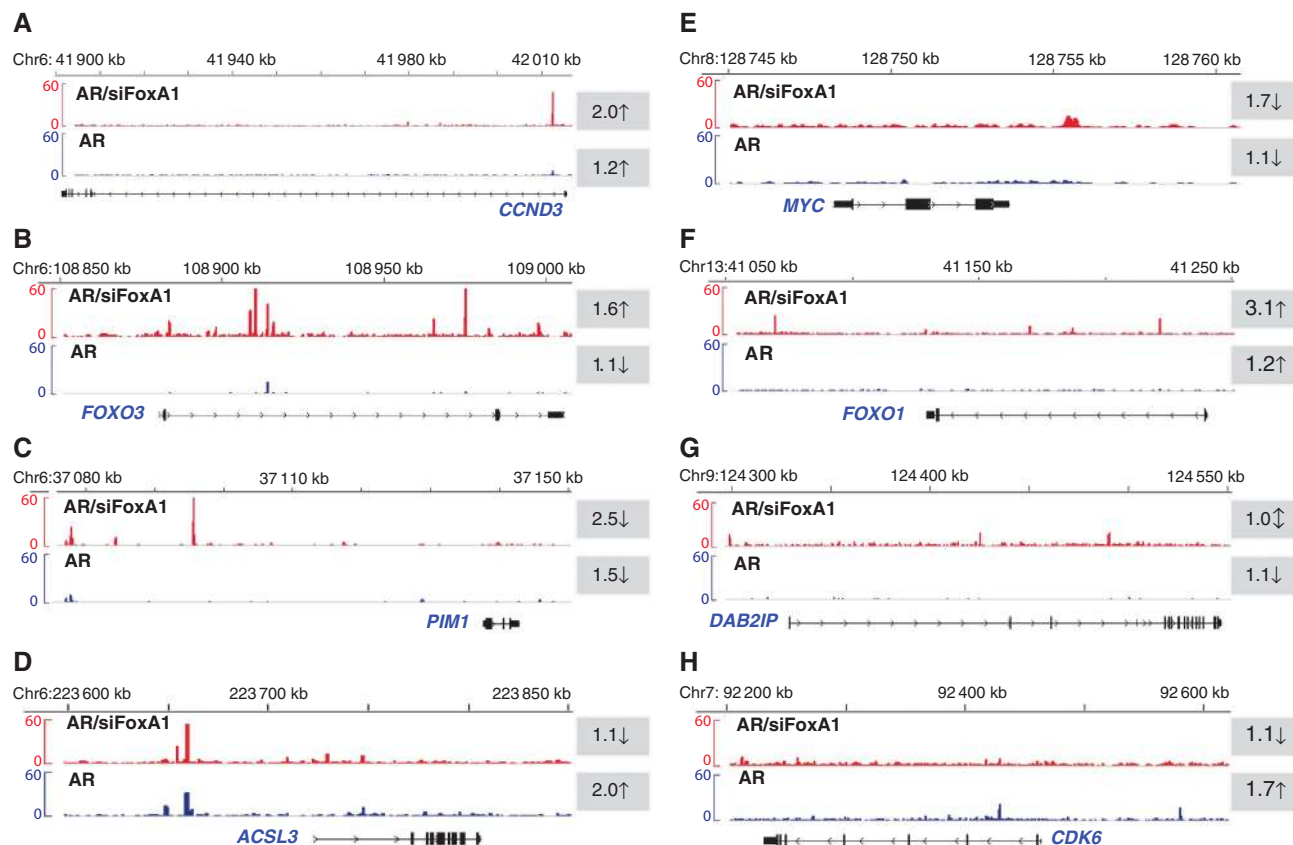


Figure 9 Examples of prostate cancer-associated genes with altered ARBs and expression profiles upon FoxA1 depletion. Distribution of ARBs in parental (blue) and siFoxA1 (red) cells in eight loci known to encode genes that are involved in prostate cancer development and/or progression. The shaded numbers with arrows (↑, up-regulation; ↓, down-regulation; ⇕, no effect) refer to fold induction of the encoded mRNA after a 24-h exposure to 100 nM DHT in LNCaP-1F5 cells, as determined by the microarray experiments.

needed by other DNA-binding transcription factors. They are present in parental cells and by this means FoxA1-independent; however, depletion of FoxA1 leads, at least in some instances, to augmented AR binding and increased androgen sensitivity. Fourth, redistribution of ARBs upon FoxA1 depletion correlates remarkably well with changes in androgen-dependent transcription programme, in that FoxA1-dependent loss of an ARB signifies blunted androgen responsiveness, and the appearance of a new ARB upon FoxA1 depletion is linked in many instances to the emergence of androgen responsiveness of a nearby gene. Fifth, the subset of ARBs that require FoxA1 pioneering utilizes a *cis*-element different from the canonical ARE. Importantly, these novel features in the AR-chromatin interaction are also true for GR binding to chromatin and glucocorticoid-dependent transcription programme upon FoxA1 depletion.

In the case of both AR and GR signalling, it was remarkable that FoxA1 depletion resulted in the appearance of a large number of new ARBs or GRBs which was linked to androgen or glucocorticoid regulation of nearby genes. Intriguingly, FoxA1 regulates ER binding and oestrogen signalling in breast cancer cells (Hurtado *et al*, 2011) in a fashion very different from that of AR and GR in prostate cancer cells. The fact that FoxA1 can prevent steroid receptor binding to specific chromatin sites, and that this property is reversible, has not been previously recognized in the nuclear receptor field. Of note, we did not knockout FoxA1 protein completely in our experi-

ments; rather, the level was depleted by ~80%. FoxA1 null mice exhibit neonatal mortality with a complex phenotype, including persistent hypoglycemia, progressive starvation, wasting and hypotriglyceridemia (Shih *et al*, 1999), and FoxA1 expression is critical for growth and differentiation of variety of organs, such as prostate, breast, lung and bladder (Kaestner, 2010). In addition, it has been reported that there is FoxA1 haploinsufficiency in mammary gland development (Bernardo *et al*, 2010). In view of these results, we envision that FoxA1 expression level—not only its presence or absence—plays an important regulatory role during development, in order to establish a proper chromatin environment for temporal and tissue- and cell-specific recruitment of steroid receptors, such as AR and GR, which, in turn, activate or attenuate the intended transcription programmes.

There was a considerable overlap between AR and FoxA1 cistromes in parental cells, and over 70% of the ARBs were shared by FoxA1-binding sites. However, FoxA1 pioneered only a subset of ARBs. That the FoxA1-pioneered ARBs possessed a unique *cis*-element different from canonical ARE-like motifs present in other ARBs suggests that this particular element plays an important role in the establishment of FoxA1-pioneered ARBs, which is supported by the finding that the unique *cis*-element was not present among the other ARB classes, in either parental or siFoxA1-depleted cells. FoxA1 binding to the unique *cis*-element may be the requisite initial event, and the adjacent ARE half-site of the

element is recognized by the AR, which is stabilized by the interaction of the two proteins (Gao *et al*, 2003; Wang *et al*, 2007). We cannot, however, exclude the possibility that AR binds to chromatin at these loci indirectly through FoxA1. Multiple overlapping AR- and FoxA1-binding sites were independent of the pioneering function of FoxA1. Some well-known AR target genes, such as *PSA* and some other *kallikrein* cluster genes, *NKX3-1* and *TMPRSS2* (Supplementary Figures S2 and S5) have ARBs of this category. In these cases, FoxA1 may bind to chromatin via AR or alternatively, chromatin structure has been modified by factors other than FoxA1, and chromatin is therefore available for independent AR and FoxA1 binding. Some ARBs or androgen-regulated genes of this class were not totally FoxA1-independent, in that FoxA1 depletion resulted in increased AR binding and enhanced androgen sensitivity over that in parental LNCaP-1F5 cells. This phenomenon could be due to competition between AR and FoxA1 for binding to their respective *cis*-elements that overlap at these sites.

The signals that determine whether FoxA1 binding to chromatin leads to its pioneering function or to formation of a compact chromatin structure are currently unknown. Recently, the histone mark H3K4me2 was shown to be instrumental in guiding FoxA1 to correct chromatin sites (Lupien *et al*, 2008), and our genome-wide results on androgen signalling support this result in most, but not in all cases. On the other hand, FoxA1 can also bind to unique sites on reconstituted chromatin *in vitro* without any specific histone modifications (Cirillo *et al*, 2002; Cirillo and Zaret, 2007) or on reconstituted nucleosomes in *Xenopus* oocytes *in vivo* (Belikov *et al*, 2009). Our genome-wide results in parental cells are in agreement with both situations, in that the majority of genomic sites possessed H3K4me2 marks together with AR- and FoxA1-binding sites, but there are also examples of sites in which FoxA1 depletion altered H3K4me2 marks and AR binding.

Three-fourths of FoxA1-binding sites did not overlap with ARBs in parental cells, and these sites along with the new ARBs in siFoxA1 cells were enriched for the CTCF *cis*-element. The CTCF insulator proteins (Fu *et al*, 2008; Zhang *et al*, 2010) appear to be important in recruiting FoxA1 to bind to these loci, and the DNA-bound FoxA1 is subsequently the signal to recruit the Groucho family of proteins to the same loci, in order to generate compact chromatin structure (Cirillo *et al*, 2002; Sekiya and Zaret, 2007) not available for AR binding. Genome-wide DHS assays showed that most of the ARBs in both parental and FoxA1-depleted cells were located at constitutively open chromatin loci, a result that is in agreement with the data demonstrating that GR binding in a mammary tumour cell line occurs mainly to pre-existing DHS sites (John *et al*, 2008). However, our results also showed that, in about one-sixth of the cases in siFoxA1 cells, the emergence of a new ARB was concomitant with the presence of a new DHS site. Whether or not these particular DHS sites are formed through AR-dependent recruitment of chromatin-remodelling enzymes, such as the Swi/Snf proteins, remains to be established.

ER-binding sites and oestrogen-dependent signalling in breast cancer cells vanish upon FoxA1 depletion (Hurtado *et al*, 2011), and high FoxA1 expression in breast cancer predicts favourable prognosis of the disease (Badve *et al*, 2007; Albergaria *et al*, 2009). These results on ER pathways

are in sharp contrast to our data on AR and GR pathways in a prostate cancer cell line, and the role of FoxA1 expression in prostate cancer tissue. Patients with high FoxA1 protein-expressing primary tumours had significantly higher prostate cancer-specific mortality than those with moderate or low FoxA1 levels, suggesting that high FoxA1 protein expression regulates signalling pathways important in prostate cancer progression, maybe in metastasis as well. In the absence of this signalling, or through activation of other events by low FoxA1 protein expression, prostate cancer does not seem able to progress. Consistent with this latter possibility is the up-regulation of FOXO family members, such as *FOXO1* and *FOXO3*, known to act as tumour suppressors, and down-regulation of oncogenes such as *PIM1* and *c-Myc* in siFoxA1 cells (Figure 8; Supplementary Figure S8). FOXO1 mediates PTEN suppression (Ma *et al*, 2009), inhibits AR signalling in prostate cancer, and loss of *FOXO1* by chromosomal deletion (13q14) promotes castration-resistant prostate cancer (Dong *et al*, 2006). Moreover, prostate cancer patients with FOXO1 protein positive tumours have been shown to have higher cancer-specific survival rates than those with FOXO1 negative tumours (Nakajima *et al*, 2011). Transcriptional down-regulation of *FOXO3* correlates inversely with increasing tumour grade in prostate cancer (Shukla *et al*, 2009). Down-regulation of *PIM1* and *c-Myc* in siFoxA1 cells should also be a beneficial feature, in that *PIM1* promotes *c-Myc* transcriptional activity and prostate cancer cell tumourigenicity (Kim *et al*, 2010), and *c-Myc* is known to repress FOXO3-dependent transcriptional targets (Chandramohan *et al*, 2008).

Interestingly, a recent risk SNP block analysis (Lu *et al*, 2011) revealed two annotation sets that were significantly enriched in prostate cancer, one for ARBs and the other one for FoxA1-binding sites, defined in previous ChIP-on-chip studies (Carroll *et al*, 2005; Wang *et al*, 2007), adding to the evidence that FoxA1 plays an important role in the pathogenesis of prostate cancer. Further studies are warranted to explore in detail the mechanisms and down-stream events by which FoxA1 modulates androgen signalling and regulates progression of prostate cancer. And finally, prostate-specific targeting of FoxA1 would potentially offer a novel therapeutic modality for prostate cancer.

While this work was under consideration for publication, similar findings on the role of FoxA1 in reprogramming of the AR pathway in a related prostate cancer cell line (LNCaP cells) was published (Wang *et al*, 2011).

Materials and methods

Cell culture

LNCaP-1F5 cell line (Cleutjens *et al*, 1997) was a kind gift from Dr Jan Trapman (Erasmus Medical Center). The presence of rat GR in LNCaP-1F5 cells does not alter the growth response of these cells to androgen from that of the parental LNCaP cells (Cleutjens *et al*, 1997). Likewise, the profile of androgen-regulated transcripts is very similar in LNCaP and LNCaP-1F5 cells (Sahu *et al*, manuscript in preparation).

FoxA1 depletion by RNA interference

Cells were cultured in RPMI-1640 containing 10% charcoal-stripped FBS to 60% confluence and then transfected with control siRNA or siRNA targeting FoxA1 mRNA (ON-TARGETplus™ SMARTpool siRNA, Dharmacon, Thermo Scientific) with Dharmafect-3 transfection reagent according to the manufacturer's instructions. The cells were incubated for 72 h and then exposed to steroid (DHT or Dex)

for 2 h (ChIP-seq) or for 24 h (gene expression profiling). The siRNA sequences are shown in Supplementary Table S14.

Chromatin immunoprecipitation

The antibodies used in the present work for ChIP assays were from the following sources: AR (Kang *et al*, 2004), FoxA1 (ab23738, Abcam), H3K4me2 (07-030, Millipore), CTCF (ab70303, Abcam), GR (Widen *et al*, 2000), normal rabbit IgG (sc-2027, Santa Cruz) and normal mouse IgG (sc-2025, Santa Cruz). The cells were exposed to DHT (100 nM) or Dex (100 nM) for 2 h prior to ChIP. Cells were fixed in 1% formaldehyde (Merck KGaA) for 10 min at room temperature and washed twice with ice-cold PBS. The cell suspension was centrifuged and the pellet resuspended for lysis in 400 μ l RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in 1 \times PBS) containing 2 \times protease inhibitors (Roche). The chromatin was sonicated to an average fragment size of 100–500 bp using Bioruptor UCD-300-TO (Diagenode), after which the samples were centrifuged at 15 000 r.p.m. for 15 min and the supernatant saved.

ChIP-sequencing

ChIP samples were processed according to Illumina's instructions, and DNA library was sequenced using Illumina Genome Analyzer II at the Biomedicum Functional Genomics core facility. Aligned read numbers are listed in Supplementary Table S15. The peak calling was carried out using the MACS algorithm (Zhang *et al*, 2008) in which binding sites are determined by comparing the density of reads at a genomic location in the antibody-specific ChIP-seq experiment to a local Poisson background density model fit to background reads that were control IgG ChIP-seq reads. MACS estimates a FDR by swapping the signal and background ChIP-seq data sets and repeating the peak-calling process to give an estimate of statistical significance. Using an FDR of <2%, the peaks called from MACS were used to map the binding site to the nearest RefSeq gene by building a TFAS. TFAS was built by computing the weighted sum of the corresponding ChIP-seq signal strength, where the weights reflect the proximity of the signal to the gene (Ouyang *et al*, 2009).

Gene expression profiling

LNCaP-1F5 cells were cultured in RPMI-1640 medium supplemented with 10% charcoal-stripped FBS for 4 days prior to siRNA transfection. Seventy-two hours after transfection, the cells (both parental and siFoxA1 cells) were exposed to 100 nM DHT or 100 nM Dex for 24 h. Total RNA was isolated using RNAeasy kit (Qiagen). RNA samples from two biological duplicates were hybridized to Illumina HumanHT-12 v3 Expression BeadChip Kits at the Biomedicum Functional Genomics core facility. The data analysis was performed by using Anduril software (Ovaska *et al*, 2010) together with 'R' (<http://www.r-project.org/>) and Bioconductor 'lumi' package (<http://www.bioconductor.org>). Raw intensity values were quantile normalized separately for the AR and GR experiments. The mean value of sample replicates was used to calculate differentially expressed genes. Fold changes of ≤ 1.7 and ≥ 1.7 were set as the cutoff values.

ChIP-seq and gene expression microarray data have been deposited in the Gene Expression Omnibus database with accession number GSE30624.

DNAaseI-hypersensitive site sequencing

DHS sites sequencing (DHS-seq) was performed essentially as described by Song and Crawford (2010). Additional details are described in Supplementary data.

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Real-time PCR

Quantitative RT-PCR was performed using SYBR green Mastermix (Roche). cDNA synthesis was carried out from 2 μ g total RNA using Transcriptor High Fidelity cDNA synthesis kit (Roche). Primers are listed in Supplementary Table S16.

Immunoblotting

Immunoblot analyses were performed using anti-AR (Kang *et al*, 2004), anti-FoxA1 (ab23738, Abcam) and anti-GAPDH (sc-47724, Santa Cruz) antibodies.

Prostate cancer patients

The use of the tissue specimens and patient information was approved by the Institutional Review Board of the Helsinki University Central Hospital. The median follow-up time of the 350 patients was 13.3 years (range, 11.33–25.0 years). Patient cohort details are in Supplementary Tables S10–S12.

TMA construction

TMA were constructed from formalin-fixed paraffin-embedded blocks of 350 patients who underwent prostatectomy at the Helsinki University Central Hospital. To account for tumour heterogeneity, two TMA cores were from the most dominant Gleason grade area and one from the second most dominant Gleason grade area. One core of each patient contained an adjacent non-cancerous area.

Immunohistochemistry

Freshly cut TMA sections were mounted on electrically charged glass slides (SuperFrost® Plus, Menzel-Gläser) that were stained with the Benchmark XT system (Ventana Medical Systems) using a biotin-free multimer-based detection system (ultraView™ Universal Red, Ventana). Antigen staining was carried out using anti-FoxA1 (ab23738, Abcam) or anti-AR antibody (NCL-AR-318, Immuno Diagnostics), and staining intensity was evaluated without prior knowledge of the Gleason grades, as described in detail in Supplementary data. Anti-FOXO1 antibody (HPA001252) was from Sigma-Aldrich and anti-FOXO3 antibody (9467) from Cell Signaling.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: The overall study was conceived and designed by BS and OAJ. The experiments were performed by BS with the help of TM, JL, ML, JK and TV in immunohistochemistry. BS, ML, KO, TM, ML and SH analysed the data. AR, AS, J-PT and SN collected the clinical data and prepared TMAs. BS and OAJ wrote the paper with substantial contributions from OK and SH.

Conflict of interest

The authors declare that they have no conflict of interest.

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