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## Identification of novel TMPRSS2:ERG mechanisms in prostate cancer metastasis: involvement of MMP9 and PLXNA2

Anthony Turpin, Carine Delliaux, Pauline Parent, Hortense Chevalier, Carmen Escudero-Iriarte, Franck Bonardi, Nathalie Vanpouille, Anne Flourens, Jessica Querol, Aurélien Carnot, et al.

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1 **Title**

2 Fascin-1 expression is associated with neuroendocrine prostate cancer and directly suppressed  
3 by androgen receptor

4  
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## Abstract

**BACKGROUND:** Neuroendocrine prostate cancer (NEPC) is an aggressive form of prostate cancer, arising from resistance to androgen-deprivation therapies. However, the molecular mechanisms associated with NEPC development and invasiveness are still poorly understood. Here we investigated the expression and functional significance of Fascin-1 (FSCN1), a pro-metastasis actin-bundling protein associated with poor prognosis of several cancers, in neuroendocrine differentiation of prostate cancer.

**METHODS:** Differential expression analyses using Genome Expression Omnibus (GEO) database, clinical samples and cell lines were performed. Androgen or antagonist's cellular treatments and knockdown experiments were used to detect changes in cell morphology, molecular markers, migration properties, and *in vivo* tumour growth. Chromatin immunoprecipitation-sequencing (ChIP-Seq) data and ChIP assays were analysed to decipher androgen receptor (AR)-binding.

**RESULTS:** We demonstrated that *FSCN1* is upregulated during neuroendocrine differentiation of prostate cancer *in vitro*, leading to phenotypic changes and NEPC marker expression. In human prostate cancer samples, FSCN1 expression is restricted to NEPC tumours. We showed that the androgen-activated AR downregulates *FSCN1* expression and works as a transcriptional repressor to directly suppress *FSCN1* expression. AR antagonists alleviate this repression. In addition, FSCN1 silencing further impairs *in vivo* tumour growth.

**CONCLUSION:** Collectively, our findings identify *FSCN1* as an AR-repressed gene. Particularly, it is involved in NEPC aggressiveness. Our results provide the rationale for the future clinical development of FSCN1 inhibitors in NEPC patients.

## 65 BACKGROUND

66 Prostate cancer (PCa) is the most common cancer in males worldwide. Early detection of the  
67 disease and local therapies, including surgical and radiation treatments, have improved the  
68 overall survival of PCa patients [1]. However, most PCa mortalities are caused by the metastatic  
69 progression of the disease [2]. Because PCa is driven by androgen through the nuclear androgen  
70 receptor (AR), androgen-deprivation therapy (ADT) alone or combined with radiotherapy or  
71 chemotherapy has been used as routine treatment. However, despite the initial high response  
72 rate to ADT, nearly all patients evolve to castrate-resistant prostate cancer (CRPC) associated  
73 with poor prognosis [3,4]. Patients with CRPC develop resistance arising from multiple  
74 molecular mechanisms, such as increased androgen biosynthesis in the tumour  
75 microenvironment or through alterations of AR signalling, including AR mutations,  
76 constitutively active AR variants in the absence of ligands, AR gene amplifications, use of other  
77 signalling pathways or reliance on non-AR-mediated pathways [3,5,6]. Moreover, it has been  
78 shown that a small proportion of CRPC tumour cells acquire a neuroendocrine phenotype with  
79 neuronal marker expression, including chromogranin A (CHGA), synaptophysin (SYP) or  
80 enolase 2 (ENO2) [7,8].

81 Neuroendocrine prostate cancer (NEPC), rarely arising *de novo*, is a highly aggressive form of  
82 PCa with a proliferative area of the tumour mass and metastasis progression [9,10]. The origin  
83 of NEPC has been suggested to arise from the selection pressure exerted by ADT, inducing the  
84 neuroendocrine transdifferentiation of CRPC cells [11–13]. A hallmark of NEPC is the loss of  
85 AR signalling during neuroendocrine transdifferentiation, resulting in resistance to ADT [14].  
86 Therefore, with the introduction of AR-targeted therapies, such as enzalutamide, a second-  
87 generation non-steroidal AR inhibitor [15], the incidence of NEPC is expected to increase  
88 [16,17].

89 Interestingly, in our previous studies, we identified a series of genes involved in the metastasis  
90 progression [18–20]. Among the identified genes, we selected the *Fascin-1* (*FSCN1*) gene  
91 originally identified as a gene that encodes an actin-bundling protein known to stabilize  
92 filopodia and invadopodia by regulating the parallel bundling of actin filaments [21,22]. *FSCN1*  
93 is a key regulator of cell cytoskeleton remodelling, which drives cell adhesion, migration and  
94 invasion [23]. *FSCN1* is not expressed in most adult human epithelia, but high *FSCN1*  
95 expression has been associated with increased mortality in breast, ovarian, colorectal and  
96 pancreatic carcinomas and metastasis [24–27].

97 In PCa, FSCN1 expression has been reported in PCa stromal cells in tumours with high Gleason  
98 scores [28] and in tumour cells in a CRPC sample [29]. Given these intriguing reports, we,  
99 therefore, assessed *FSCN1* involvement in PCa progression. In this study, we report that the  
100 *FSCN1* gene is repressed by AR in androgen-dependent cell lines. Moreover, *FSCN1*  
101 expression, absent in primary PCa, is detected in NEPC tumours and may be involved in tumour  
102 progression. Our study identifies FSCN1 as a new marker of NEPC and a potential mediator of  
103 invasiveness.

## 104 **METHODS**

### 105 **Cell lines and treatments**

106 Human PCa cell lines VCaP, LNCaP, MDA-PCA-2b, DU145, NCI-H660 and PC3 were  
107 obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines  
108 were grown in ATCC-recommended media supplemented with 10% fetal bovine serum (FBS,  
109 Thermo Fisher Scientific, Waltham, MA, USA). PC3M-luc-C6 (PC3M) cell line was purchased  
110 from Caliper (Perkin Elmer, Hopkinton, MA, USA). The PC3c cell line was obtained from  
111 Edith Bonnelye and cultured as described in Fradet et al. [30]. PC3c clones, stably transfected  
112 with either an empty pcDNA3.1 vector or a *TMPRSS2:ERG* (T1E4) expression vector, were  
113 obtained as described previously [18]. All cells were subcultured every 3-4 days and maintained  
114 in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Cells were not used beyond 15 passages; they  
115 were tested for mycoplasma contamination with the MycoAlert™ mycoplasma detection kit  
116 every six months (Lonza, Basel, Switzerland). To activate the androgen receptor, VCaP and  
117 LNCaP cells were respectively treated with 100 nM and 10 nM of dihydrotestosterone (DHT,  
118 Sigma-Aldrich, St. Louis, MO, USA) for 2 or 16 h after being hormone-starved using charcoal-  
119 depleted serum containing the medium for 8 h. The activity of AR was inhibited with 50 μM  
120 of bicalutamide (BIC) (Sigma-Aldrich).

### 121 **Transfection of constructs or siRNA in PC3, LNCaP and VCaP cells**

122 Predesigned and pooled small interfering RNAs (siRNAs) (siFSCN1: ON-TARGETplus  
123 human FSCN1 siRNA) and the control siRNA (ON-TARGETplus Non-targeting Pool  
124 D001810-10) were obtained from GE Dharmacon (Lafayette, CO, USA). Cells were transfected  
125 with siRNA (50 nM) using the Lipofectamine 2000 reagent (Thermo Fisher Scientific)  
126 according to the manufacturer's instructions. Gene-knockdown or overexpression effects were

127 evaluated 72 h after transfection. Transient overexpression experiments involved cell  
128 transfection with the AR expression plasmid (PSG5-AR) (4 µg) or with the empty vector  
129 (PSG5) using the Lipofectamine 2000 reagent.

### 130 **Generating stable FSCN1 knockdown cell lines**

131 PC3M prostate cells were maintained using modified Eagle's medium (MEM) (Biowest,  
132 L0440-500) supplemented with 1mM Sodium Pyruvate (Fisher scientific, 11530396), 1%  
133 penicillin-streptomycin (Gibco; 15140122) and 10% Fetal Bovine Serum (FBS) (Gibco;  
134 10270106) at 37°C in 5% CO<sub>2</sub>. PC3M cells were transduced with lentivirus containing either  
135 an irrelevant short hairpin (shScramble) (Sigma-Aldrich SHC002V) or two different shFSCN1  
136 (Sigma-Aldrich, TCR000012342, and TRCN0000123039) and selected with puromycin  
137 1µg/mL (Fisher Bioreagents, BP2956-100).

138 For lentiviral production, HEK293T cells were transfected by adding a mixture of 150mM  
139 NaCl, DNA (50% of the indicated shRNA vector, 10% pCMV-VSVG, 30% pMDLg/pRRE,  
140 and 10% pRSV rev) and polyethyleneimine polymer (Polysciences Inc; 23966-1), which was  
141 previously incubated 15 min at room temperature. After 24h, the transfection medium was  
142 removed and changed to a fresh medium in which viruses were produced. The virus-containing  
143 medium was then collected and filtered with a 0.45 µm filter unit (Merck Millipore; 051338) at  
144 24 and 48 hours. The virus-containing medium was used to infect PC3M cells, as mentioned  
145 above.

### 146 **Cell growth and migration assays**

147 For MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, 1,000 PC3M  
148 cells expressing shScramble, shFSCN1.2, or shFSCN1.3 were seeded in a well of a 96-well  
149 plate, and cell growth for 7 days. Cells were incubated with 0.3 mg of MTT (Panreac Apllichem,  
150 A2231) per mL of culture medium without FBS for 3 h at 37 °C. Subsequently, the MTT-  
151 containing medium was removed, and cells were incubated with isopropanol. The absorbance  
152 was measured at 565nm with infinite M2000 Pro (Tecan), and data were acquired using Tecan  
153 i-control 1.11 software.

154 For migration assay, 20,000 cells of PC3M shScramble, shFSCN1.2 or shFSCN1.3 was seeded  
155 in MEM without FBS in transwell filter chambers (Corning, 3422) in triplicate. One hour after  
156 the seeding, MEM with 10% FBS was added to the lower chamber as a chemoattractant, and  
157 cells were kept at 37 °C for 24 h. After removing non-migrating cells from the upper surface of

158 the membrane, migrating cells were fixed with paraformaldehyde 4% for 15 min, and nuclei  
159 were stained with 0.25 µg/mL of PBS-DAPI (4',6-diamidino-2-phenylindole). Finally, nuclei  
160 were counted using an epifluorescence microscope and ImageJ software.

### 161 **Tumour xenografts**

162 To assess tumour growth capacity,  $3 \times 10^6$  cells of either PC3M shScramble, shFSCN1.2 or  
163 shFSCN1.3 were subcutaneously implanted per flank in NOD-SCID mice. Tumour growth and  
164 mice weight were measured twice per week. Tumour volumes were calculated using the  
165 formula:  $V = (\text{length} \times \text{width}^2) / 2$ . According to institutional guidelines, mice were euthanized  
166 using CO<sub>2</sub> inhalation once the experiment reached the endpoint, and tumours were surgically  
167 extracted and individually weighted. All animal procedures were approved by the Ethics  
168 Committee of Animal Experimentation (CEEAA) at the Vall d'Hebron Institute of Research and  
169 by the Catalan Government.

### 170 **RNA extraction and gene expression analysis**

171 Total RNA was extracted from cells using the NucleoSpin RNA Plus kit (Macherey-Nagel,  
172 Düren, Germany) according to the manufacturer's instructions. For retrotranscription, 1 µg of  
173 total RNA was used to generate cDNA using the High-Capacity RNA-to-cDNA kit (Applied  
174 Biosystems, Foster City, CA, USA). Real-time quantitative PCRs (qPCRs) were performed  
175 using the Power SYBR™ Green PCR Master kit (Applied Biosystems, Foster City, CA, USA)  
176 on a Stratagene Mx3005P qPCR System (Stratagene, La Jolla, CA, USA) according to the  
177 manufacturer's instructions. The relative expression levels of individual genes were calculated  
178 using the  $-2\Delta\Delta\text{CT}$  method. All reactions were normalised to human *GAPDH* or *L32* genes and  
179 ran in triplicate. Optimal primer specificity and efficiency were validated according to the  
180 Mx3005P qPCR system user's guide. The primers used in this study can be found in  
181 [Supplementary Table S1](#).

### 182 **Western blotting and ChIP**

183 Cell proteins were extracted, and separated in 4 to 10% SDS-PAGE. After migration, samples  
184 were transferred by electrophoresis to PVDF membranes (Transfert-blot Turbo Transfer  
185 system, Bio-Rad, Hercules, CA, USA). Membranes were incubated with blocking buffer for 3  
186 h, then with the primary antibody overnight. Membranes were incubated with secondary HRP-  
187 conjugated antibodies for 1 h. After washing, membranes were revealed using Clarity™

188 Western ECL substrate (Bio-Rad). Antibodies used were as follows: anti-FSCN1 (ab126772,  
189 Abcam Epitomics, Cambridge, UK), anti-AR (sc-816, N-20 Santa Cruz Biotech, Dallas, TX,  
190 USA), anti-vinculin (Invitrogen VIN-01, Thermo Fisher Scientific), anti-GAPDH (sc-32233  
191 Santa Cruz Biotech or (ab128915, Abcam) and anti- $\beta$ -actin (Clone AC-15, Sigma-Aldrich).

192 Chromatin immunoprecipitation (ChIP) assays were performed as described [20] using either  
193 a polyclonal rabbit anti-AR antibody (AR sc-7305, Santa Cruz Biotech) or a control rabbit IgG  
194 antibody (Rabbit DA1E Ab IgG XP®, Cell Signaling Technology, Danvers, Massachusetts,  
195 USA). The genomic DNA was purified using NucleoSpin Clean-up columns (Macherey-Nagel)  
196 according to the manufacturer's instructions. Immunoprecipitated genomic DNA was analysed  
197 using qPCR, and the primers used in this study are given in [Supplementary Table S2](#).

### 198 **Human prostate cancer samples**

199 Human PCa samples (n=52) were obtained from the local tumour tissue bank (Tumorotheque  
200 Alliance Cancer, Lille, France) after approval by the internal review board (CSTMT-042,  
201 27/07/2009). As described by Tian et al. [18], these tumour tissue samples, which had been  
202 extracted and subsequently frozen, originated from radical prostatectomies or transurethral  
203 prostatic resections performed at Lille University Hospitals (CHU de Lille). In addition, four  
204 NEPC tissues were collected and sectioned to analyse the expression of *FSCN1* (Supplementary  
205 Table S3). All patients were informed, and consent was obtained by the referring physician.

### 206 **Bioinformatic analysis**

207 To analyse *FSCN1* and *KLK3* mRNA expression levels in normal and adjacent prostate tissues  
208 or primary and metastatic prostate cancer samples, expression microarray data were  
209 downloaded from the GEO database with accession numbers GSE35988, GSE6919, GSE8511,  
210 GSE3325.

211 To determine the recruitment of AR on the promoters of *FSCN1* and *KLK3*, we analysed  
212 publicly available ChIP-seq data (GSE55062, GSE43791, GSE40050, GSE62442, GSE61838,  
213 GSE32356, GSE56288) for AR binding in untreated, DHT-, BIC- or ENZ-treated PCa cells  
214 and in patient prostate normal and tumour samples. AR binding was then visualised on the  
215 UCSC browser (<http://www.genome.ucsc.edu>) [31]. Tracks from the human ENCODE project  
216 for H3K27ac and H3K4me1 marks, often found near active enhancer elements, and H3K4me3  
217 effects, often found near active promoters well as DNase I hypersensitivity peak clusters, have



218 been added. The same process was done to determine the recruitment of FOXA1, along with  
219 AR, on the promoters of *FSCN1* and *KLK3*, using publicly available ChIP-seq data (GSE94682,  
220 GSE83860, GSE58428, GSE56046, GSE161948).

221 To visualise the enhancer-associated histone modification H3K27-acetylated (H3K27ac) at the  
222 *FSCN1*, *KLK3* and *CHGA* genes, H3K27ac profiles of five representative prostate cancer  
223 adenocarcinoma (PRAD) and five neuroendocrine prostate cancer (NEPC) patient-derived  
224 xenografts (PDXs) were extracted from Baca et al. 2021 [32].

## 225 **Immunohistochemistry and immunofluorescence**

226 Immunohistochemistry of human tissue sections was performed using anti-FSCN1 at 1:100  
227 (ab49815 Abcam and FCN01 55K-2 ThermoFisher). Sections were incubated with secondary  
228 HRP-conjugated antibodies. Counterstaining was performed using Mayer's hematoxylin  
229 (Merck, Darmstadt, Germany).

230 Immunofluorescence assays were performed as described by Tian et al. [18]. The antibodies  
231 used in this study were anti-FSCN1 at 1:100 and Alexa Fluor™ 488-conjugated anti-rabbit IgG  
232 (Invitrogen, dilution 1:500). Images were acquired using an LSM 710 confocal microscopy  
233 system and ZEN 2010 software (Carl Zeiss, Oberkochen, Germany).

## 234 **Fluorescent gelatine degradation assay**

235 Poly-D-lysine-coated coverslips were washed with PBS and fixed with 0.5% glutaraldehyde  
236 for 15 min. Then, the coverslips were inverted on a 100 µl drop of gelatin conjugated with FITC  
237 (fluorescein, G13187 Invitrogen, Thermo Fisher Scientific) and incubated for 10 min in the  
238 dark. After washing with PBS, the residual reactive groups were quenched with 5 mg/ml  
239 sodium borohydride for 10 min and washed with PBS. Cells were plated in 24-well plates  
240 containing a coverslip coated with a fluorescent gelatin matrix and incubated at 37°C. After 3  
241 h, cells were fixed with 4% formaldehyde, permeabilised with 1% BSA with 10% normal goat  
242 serum, 0.3 mM glycine and 0.1% TBS-Tween. Then, cells were labelled for filamentous actin  
243 with Alexa Fluor™ 555 phalloidin (8953S, Cell Signaling Technology) and Hoechst (H33258,  
244 Sigma-Aldrich). Confocal images were acquired using the Olympus FV500 confocal laser  
245 scanning microscope and FluoView software (Olympus). Sites of matrix degradation were  
246 visible as dark areas (spots) in the bright green fluorescent gelatin matrix.

247

## 248 **Statistical analysis and image processing**

249 Statistical analyses were performed using the GraphPad Prism software (San Diego, CA, USA).  
250 The statistical methods used in this study are indicated in the corresponding figure legends. All  
251 image processing was carried out using Image J.

## 252 **RESULTS**

### 253 ***FSCN1* expression in PCa cells is suppressed by androgen receptor**

254 Using an AR-independent cell line (PC3c derived from the PC3 cell line), in a previous study  
255 which aimed to define the role of the ERG fusion (T1E4), the most frequent chromosomal  
256 rearrangement in PCa (>50%), we identified a series of genes potentially involved in cell  
257 migration and invasion [18]. Among these genes, we showed that *FSCN1* is highly expressed  
258 in PC3c clones, which stably expresses the ERG-T1E4 fusion. This association suggested that  
259 *FSCN1* expression was regulated by the fusion ([Supplementary Fig. S1a](#)). However, when we  
260 examined *FSCN1* expression in a series of human primary prostate cancer (n=52), fusion-  
261 positive (n=32) or fusion-negative (n=20) and normal prostate tissues (n=20), we observed that  
262 no significant differences between these groups of samples ([Supplementary Fig. S1b](#)) and no  
263 correlation with the ERG transcription factor status. Therefore, ERG fusion is not the key  
264 regulator of *FSCN1* in PCa.

265 We then explored *FSCN1* expression in various PCa cell lines ([Fig. 1a](#)). Interestingly, except  
266 in the PC3c cell line, which differs from PC3 in its strong osteomimicry properties, *FSCN1*  
267 turned out to be highly expressed in cell lines that are negative for AR expression ([Fig. 1a, b](#)),  
268 such as PC3, PC3M and DU145, but not in any of the androgen-dependent cell lines such as  
269 LNCaP, MDA-PCa-2b and VCaP, suggesting that AR plays a role in the regulation of *FSCN1*  
270 expression in PCa cells. To confirm the androgen inhibition effect of *FSCN1* expression, we  
271 performed RT-qPCR and Western blot analyses of AR-positive VCaP cells treated with either  
272 1 to 1000 nM of the AR activator DHT alone ([Fig. 1c](#)), or in combination with 50  $\mu$ M of BIC,  
273 an AR antagonist ([Fig. 1e](#)). We used *KLK3* gene encoding PSA (Prostate Specific Antigen),  
274 known to be an AR-activated target gene, as a positive control, and successfully showed that  
275 DHT treatment is associated with a decrease in *FSCN1* expression at the mRNA level. Using  
276 WB analyses, we demonstrated that the expression of *FSCN1* decreased upon DHT treatment

277 (Fig.1d and f), and this inhibition was partially recovered by treatment with BIC. Our data  
278 showed that androgens inhibit *FSCN1* expression through AR signalling.

### 279 ***FSCN1* is upregulated in NEPC**

280 To gain insight into the potential role of *FSCN1* in PCa, we investigated the *FSCN1* expression  
281 in normal, primary and metastatic PCa cohorts using published datasets (Fig. 2). We analyzed  
282 *FSCN1* expression in published transcriptomic datasets from four independent PCa cohorts  
283 [33–36]. First, we observed an inverse correlation between *FSCN1* and *KLK3* expression,  
284 suggesting that a decrease of AR activity, corresponding to a low *KLK3* expression, is  
285 associated with an increased expression of *FSCN1* (Fig 2a). Second, as shown in Fig. 2b,  
286 compared with normal or primary PCa samples, *FSCN1* expression levels were significantly  
287 higher in metastatic PCa samples. This difference in expression suggests its potential role in  
288 metastasis.

289 To further test the *in vivo* validity of the gene expression correlations from the PCa datasets,  
290 immunohistochemical assays of FSCN1, using two independent antibodies (Fig. 3a and  
291 Supplementary Fig. S2), were used and showed that its expression was only detectable in blood  
292 vessels, either in primary prostate tumours or in lymph node and bone metastasis, samples were  
293 already described in [18,20]. Therefore, in accordance with our *in vitro* study in which we  
294 observed that AR-negative cell lines express *FSCN1* and the analysis of the PCa datasets, we  
295 hypothesized that *FSCN1* expression is specifically associated with NEPC. In Fig. 3b, using  
296 NEPC samples that were positive for neuroendocrine markers (Supplementary Table S3), such  
297 as CHGA and SYP, we detected the FSCN1 protein in NEPC cancer cells. By contrast, FSCN1  
298 was undetectable in any adenocarcinoma studied, as exemplified in Fig. 3a, b. Taken together,  
299 these results suggest that FSCN1 is enriched in NEPC.

### 300 **Androgen deprivation triggers *FSCN1* expression in androgen-sensitive cells**

301 Lineage plasticity in PCa has been linked to neuroendocrine differentiation involving  
302 transdifferentiation of prostate adenocarcinoma cells. The underlying mechanisms have been  
303 studied using androgen deprivation cell models [37]. To further demonstrate that *FSCN1*  
304 expression is associated with an NEPC phenotype, we took advantage of a cell model consisting  
305 of an androgen-depleted culture of LNCaP and VCaP cell lines [37]. Using a phenol-red-free  
306 medium and a charcoal-depleted serum to remove androgens, we mimicked androgen

307 deprivation in culture (Fig. 4a). Compared with unstripped serum conditions (LNCaP or VCaP),  
308 cells cultured for 14 to 21 days in androgen-deprivation conditions (LNCaP-NE or VCaP-NE)  
309 gradually developed elongated cytoplasmic protrusions, neurite-like structures, consistent with  
310 previous reports (Fig. 4b) [37]. Importantly, LNCaP-NE and VCaP-NE cells showed increased  
311 expression of NEPC markers, such as *CHGA*, *ENO2*, *SYP*, and the NE regulators, the *neural*  
312 *POU-domain transcription factor BRN2*, and the *SRY-related HMG-box gene 2 (SOX2)*, and  
313 showed a strong decrease in *KLK3* (Fig. 4c,e and Supplementary Fig. S3a, b). Measured after  
314 14 days of androgen-deprivation culture, LNCaP-NE and VCaP-NE showed a remarkable  
315 increase in *FSCN1* expression at the transcript and protein levels (Fig. 4d,f). Because *FSCN1*  
316 has been described as an important protein for invadopodia formation [38,39], which are crucial  
317 for tumour cell invasion in cancer progression, we evaluated the ability of LNCaP cells to form  
318 invadopodia under androgen-deprivation conditions (Supplementary Fig. S3c, d). The presence  
319 of invadopodia was confirmed by culturing cells on top of a fluorescently conjugated matrix  
320 (fluorescein gelatin), staining cells for F-actin and examining colocalization between F-actin  
321 puncta and degradation of fluorescent gelatin (black regions) [38]. After 6h, matrix degradation  
322 was identified with the appearance of fluorescence-negative areas (black holes in  
323 Supplementary Fig. S3c, d), F-actin and nucleus labelling, using phalloidin and Hoechst dyes,  
324 respectively, revealed LNCaP invadopodia formation in androgen-deprivation culture  
325 conditions. Using antibodies against *FSCN1*, we detected the *FSCN1* protein -as expected- in  
326 the matrix degradation area corresponding to the invadopodia [22].

327 Next, we examined whether the expression of *FSCN1* is associated with NE marker expression  
328 during the emergence of NE phenotype in LNCaP and VCaP cells. As shown in Fig. 4g,  
329 *siFSCN1* were transfected on day 1 after androgen-deprivation culture (*i.e.* at the beginning of  
330 NE differentiation induction), and then RNA analyses were performed on day 4 post-NE  
331 induction. In Fig. 4h-i, we showed that knocking-down *FSCN1* expression by 60% in LNCaP  
332 and 90% in VCaP, reduced NE marker expression compared with the siControl. Moreover, we  
333 also found the expression of *FSCN1*, at mRNA and protein levels, in NCI-H660 cells, a unique  
334 and typical NEPC cell line (Supplementary Fig. S4).

335 Taken together, these results suggested a potential role of *FSCN1* in neuroendocrine  
336 transdifferentiation, and *FSCN1* may be a new NEPC marker.

337

### 338 **FSCN1 knockdown reduced tumour growth of AR-negative cells**

339 It has been shown that FSCN1 expression correlates with poor clinical outcomes and shorter  
340 survival across different cancer types [40]. Thus, we next studied the role of FSCN1 in AR-  
341 negative PCa cells. Among the possible cellular model, NCI-H660 is a cell line derived from  
342 human NEPC. However, this PCa cell line shows extremely slow growth kinetics, limiting its  
343 utilization in downstream functional analysis. We choose the PC3M cells, derived from PC3,  
344 which express *FSCN1*, as shown in [Supplementary Fig. S4](#). Moreover, these cells are AR-  
345 independent (AR- and PSA-) with characteristics of prostatic small cell neuroendocrine  
346 carcinoma [41,42]. We established PC3M cell lines expressing short hairpin RNA control  
347 (shScramble), or short hairpin RNA targeting specifically *FSCN1* (shFSCN1.2 and shFSCN1.3)  
348 ([Fig. 5a](#)). Of note, shFSCN1.2 and shFSCN1.3 inhibited the expression FSCN1 efficiently ([Fig.](#)  
349 [5a](#)). Importantly, *FSCN1* knockdown significantly decreased proliferation and cell migration  
350 of PC3M cells *in vitro* ([Fig. 5b, c](#)). We next assessed the functional impact of *FSCN1*  
351 knockdown on tumour growth *in vivo*. PC3M cells expressing either shScramble, shFSCN1.2  
352 or shFSCN1.3 were subcutaneously implanted into NOD-SCID mice. We could find that  
353 compared to shControl tumours, the tumour growth of shFSCN1.2 and shFSCN1.3 was delayed  
354 ([Fig. 5d](#)). In addition, tumours formed by PC3M cells expressing shFSCN1.2 and shFSCN1.3  
355 are significantly smaller than those formed with PC3M cells expression shScramble ([Fig. 5e,](#)  
356 [f](#)). Taken together, these *in vitro* and *in vivo* studies suggest that *FSCN1* knockdown could  
357 inhibit the proliferation and migration of neuroendocrine-like cells, leading to tumour growth  
358 delay.

### 359 **AR binds directly to the FSCN1 regulatory sequence**

360 Given that the expression of *FSCN1* was found in AR-negative PCa cells ([Fig. 1a](#)), we  
361 examined whether the *FSCN1* gene expression was downregulated by a direct AR-mediated  
362 mechanism in PCa cells. First, we took advantage of an AR-negative cell line, the PC3c-T1E4  
363 model, which highly expresses the *FSCN1* gene, to transiently overexpress AR and measure  
364 *FSCN1* expression variations ([Supplementary Fig. S1a, c](#)). Using transient transfection of an  
365 AR expression vector, we showed that AR expression significantly decreased *FSCN1*  
366 expression at the transcriptional levels ([Supplementary Fig. S1c](#)). Then, we hypothesised that  
367 AR could transcriptionally repress *FSCN1* in AR-positive VCaP and LNCaP cells by directly  
368 binding to the androgen response element (ARE) on the *FSCN1* gene. Because AR has been  
369 described both as a transcriptional activator and repressor [43], we looked for putative AREs

370 in the *FSCNI* regulatory regions. We used transcription factor prediction software for  
371 transcription factor binding site identification (HOMER) (Fig. 6a) and analysed publicly  
372 available ChIP-sequencing datasets (ChIP-seq) for AR binding to identify and localise AR  
373 binding 6,500 bp upstream of the *FSCNI* gene. Using datasets obtained with normal prostate  
374 tissues, prostate tumour samples and androgen-dependent cell lines such as VCaP and LNCaP  
375 cells (Fig. 6b, c and Supplementary Fig. S5), we identified a unique and major AR-binding  
376 peak in tumours and cell lines corresponding to AREs, located 6500 bp upstream of the *FSCNI*  
377 transcriptional start site. The *KLK3* gene, known to be regulated by AR through its direct and  
378 specific DNA binding, was used as a control in this analysis (Fig. 6c and Supplementary Fig.  
379 S6).

380 To test whether AR directly binds to the identified AREs of the *FSCNI* gene, we performed  
381 ChIP-qPCR assays with VCaP cells, untreated or DHT-stimulated for 16 h. Using five  
382 amplification fragments covering the potential AR-binding regions (Fig. 6b), we found that AR  
383 binding was enriched in three DNA fragments corresponding to potential AREs (Fig. 6d). The  
384 promoter of the *KLK3* gene was used as a positive control (Fig. 6d). These data are consistent  
385 with a mechanism (Fig. 6e) whereby AR acts as a direct transcriptional repressor of *FSCNI*  
386 expression, potentially associated with corepressors, and therefore, therapies based on androgen  
387 deprivation may reduce *FSCNI* transcriptional repression, hence resulting in its expression.

388 Among the transcription factors known to contribute to the AR transcription activation or  
389 repression properties, we sought to examine the potential role of the FOXA1 (forkhead box A1)  
390 transcription factor, which has been shown to play a key role in AR-mediated gene regulation.  
391 FOXA1 is known to interact with AR and co-occupy the chromatin binding [44]. To further  
392 investigate the potential role of FOXA1 in *FSCNI* gene transcriptional repression, we analysed  
393 public ChIP-seq datasets for FOXA1 binding in androgen-dependent VCaP and LNCaP cells  
394 (Fig. 6c and Supplementary Fig. S7). We identified a FOXA1 binding element in the same area  
395 as the identified AREs within the *FSCNI* gene. To further confirm FOXA1 binding to the  
396 *FSCNI* gene regulation region, we performed CHIP-qPCR experiments on VCaP cells cultured  
397 with and without a charcoal-stripped medium. Results showed enrichment in FOXA1 binding  
398 sites at ARE fragments when a regular medium was used (Fig. 6f, left panel). The *KLK3*  
399 promoter was used as the positive control (Fig. 6c and Supplementary Fig. S8). In contrast, both  
400 AR and FOXA1 binding were drastically reduced in a hormone-depleted medium (Fig. 6f, right

401 panel). These results suggest that at least AR and FOXA1 regulate *FSCN1* expression in an  
402 androgen-dependent mechanism.

403 Finally, to assess the enhancer activity of the *FSCN1* gene in NEPC, we took advantage of the  
404 genome-wide H3K27 acetylation analyses performed by Baca et al. [32], in NEPC and prostate  
405 adenocarcinoma (PRAD) LuCap patient-derived xenografts (PDXs). In [Supplementary Fig. S9](#),  
406 the *FSCN1* gene shows an increase in H3K27 acetylation in NEPC compared with PRAD  
407 samples, suggesting increased enhancer activity in NEPC. As controls, the *CHGA* and *KLK3*  
408 genes, specifically expressed in NEPC and PRAD, showed respectively an increase and a  
409 decrease in H3K27 acetylation, as expected. This pattern suggests that *FSCN1* gene may be  
410 regulated by a neuroendocrine transcriptional program.

## 411 **DISCUSSION**

412 NEPC is a highly aggressive form of PCa that is increasing in incidence in association with the  
413 development of resistance to AR pathway inhibitors [3,45]. However, the molecular  
414 mechanisms associated with NEPC development and invasiveness are still poorly understood.  
415 The present study uncovers a previously unknown role for the FSCN1 protein in NEPC. FSCN1  
416 is an actin-bundling protein that plays a key role in actin stability in the invadopodia [39,46].  
417 By increasing membrane protrusions (filopodia and invadopodia), facilitating focal adhesion  
418 turnover and regulating nuclear organisation, FSCN1 promotes the physical translocation of  
419 metastatic cancer cells [46]. Importantly, FSCN1 overexpression has been strongly associated  
420 with poor prognosis and metastatic progression across different cancer types [24–27,46]. Here,  
421 we showed that, in addition to being significantly and specifically overexpressed in clinical  
422 NEPC samples, FSCN1 knockdown expression, particularly at the beginning of cell culture in  
423 androgen-deprivation conditions, was able to decrease the expression of NE markers  
424 significantly. This suggests that FSCN1 plays an important role in the NE marker expression  
425 and phenotype. Moreover, FSCN1 knockdown further migration cell behaviour, as assessed by  
426 cell migration assays *in vitro*. Therefore, our study connects NE differentiation with  
427 invadopodia formation. *In vivo*, FSCN1 knockdown inhibits tumour growth in mice. This  
428 finding, together with the observations of its effect on NE markers expression suggests that  
429 FSCN1 might act as an important mediator in NEPC development.

430 NEPC can emerge after therapeutic pressure through a process of tumour cell  
431 transdifferentiation from preexisting adenocarcinoma and very rarely *de novo* [47]. Indeed, the



432 improved potency and specificity of ADT have led to an increased prevalence of NEPC [48,49].  
433 Because NEPC features androgen deprivation and because the loss of AR expression has been  
434 described to enable NE differentiation in androgen-dependent cell lines, a question arises as to  
435 whether AR is able to repress the expression of NEPC genes and *FSCNI* in particular. Here,  
436 we provide compelling evidence that *FSCNI* is a direct androgen-repressed gene. AR is a  
437 hormone transcription factor, best known as an activator. It is associated with coactivators and  
438 chromatin modifiers to induce transcription of AR target genes, which are defined by direct AR  
439 binding to AREs at their regulatory genomic regions [50].

440 In our study, analyses of publicly available ChIP-seq datasets and ChIP-qPCR experiments  
441 revealed a direct AR binding in androgen-dependent conditions, where *FSCNI* expression is  
442 repressed. The AR-binding sites were identified 6.5 kb downstream from the transcription start  
443 site, and our results suggest that, in androgen-dependent conditions, AR acts as an *FSCNI* gene  
444 repressor, possibly with corepressors. In contrast, a decrease or absence of AR binding was  
445 observed in androgen-deprivation conditions. One of the possible AR corepressors may be  
446 FOXA1, a pioneering factor which makes chromatin accessible to AR, known to facilitate AR  
447 recruitment to target gene AREs [51]. Moreover, AR is known to bind to enhancer regions  
448 rather than to the promoter, recruiting FOXA1, thus activating enhancers [52]. Based on these  
449 reports, we took advantage of recently published ChIP-seq-FOXA1 datasets, to identify a peak  
450 in ChIP-seq-FOXA1 located in the same AR binding genomic region and confirmed that  
451 FOXA1 binds the same DNA fragments as AR in androgen-dependent conditions. Therefore,  
452 FOXA1 may act as an AR corepressor and thus downregulates *FSCNI* gene expression. Finally,  
453 because the H3K27ac mark has been reported to be a reliable predictor of enhancer activity, we  
454 showed that the H3K27ac profile of the *FSCNI* gene in at least five NEPC samples revealed  
455 strong activity, similarly to *CHGA*, an NE marker. In contrast, in five primary tumours (i.e.  
456 PRAD), only *KLK3* revealed strong H3K27ac activity.

457 Finally, NEPC is mostly defined as a subset of CRPC with a loss of AR expression or activity.  
458 Clinically, patients present low PSA levels with high metastatic burden in soft tissues [53].  
459 Since AR is best known as a transcription activator, efforts have been focused on AR-activated  
460 genes and ADT is the standard of care for PCa patients. However, the mechanisms by which  
461 the AR directly influences the induction of the NEPC phenotype and the expression of specific  
462 NE markers may involve key AR-regulated genes, and particularly AR-repressed genes, which  
463 could be re-expressed in absence of AR or AR-pathways [43]. For example, AR have been



464 shown to directly repress transcription of a master neural transcription factor BRN2 and the  
465 reprogramming transcription factor SOX2, both playing a significant role in the the progression  
466 of PCa to NEPC [54,55]. Therefore *FSCN1* gene could be part of the AR-repressed genes, such  
467 as *BRN2*, *SOX2*, *CCND1* (*Cyclin-D1*), *c-MET* (Hepatocyte growth/scatter factor receptor),  
468 which are re-expressed in the aggressive AR-independent form of the disease and involved in  
469 emergence or maintenance of the NEPC phenotype. Since *FSCN1* overexpression has been  
470 correlated with poor clinical outcomes and shorter survival across different cancer types,  
471 *FSCN1* has been suggested as a therapeutic target for blocking migration, invasion and  
472 metastasis, encouraging *FSCN1* inhibitors identification [56]. In addition, recent findings  
473 suggest that *FSCN1* may be a useful druggable target for controlling adrenocortical carcinoma,  
474 a rare disease [57]. Currently, some of these *FSCN1* inhibitors, which block the actin-bundling  
475 activity of *FSCN1*, are in clinical development in early phase trials (NCT03199586,  
476 NCT05023486).

477 In conclusion, given that NEPC has a poor prognosis and very limited therapeutic options, there  
478 is an urgent need to control NE lineage transdifferentiation and to develop novel therapeutic  
479 approaches that can extend the clinical response to ADT. Interestingly, AR-repressed genes  
480 represent promising markers and targets for diagnosis and therapeutic interventions in NEPC  
481 progression. Our data suggest that *FSCN1* may be considered as a new actor (i.e. mediator or  
482 “facilitator”), restricted to NEPC. Although *FSCN1* promotes the progression of many human  
483 cancers, it has not yet been approved as a biomarker in clinical practice. Therefore, *FSCN1* now  
484 needs to be studied in a well-designed prospective study to assess its independent value as a  
485 new biomarker. Targeting *FSCN1* might be a promising approach to treat NEPC.

486

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647

#### 648 **AUTHOR CONTRIBUTIONS**

649 MDC, TVT, AT and CD designed the study. AT, CD, PP, HC, CEI, JQ, NV and AF performed  
650 the experiments, acquired and analysed the results. AT, CD, TVT, PP, HC and MDC analysed  
651 and interpreted the data (e.g., statistical analysis, biostatistics, computational analysis). HT, FB,  
652 CD and NH performed bioinformatic analysis. AT, CD, AF, AC, XL, TL, AV and JO  
653 contributed to patient tissue collection, provided clinical information, and performed  
654 histological analyses and image acquisitions. MDC, TVT, AT, CD and FB contributed to the  
655 figure's design. MDC, TVT, AT, CD and YdL wrote the manuscript with the input of all  
656 authors. TVT provided funding. MDC supervised the project and provided funding. All authors  
657 discussed the results and commented on the manuscript.

658

#### 659 **ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

660 PCA tissue samples were obtained from Tumorothèque Alliance Cancer Lille, Lille University  
661 Hospitals (CHU of Lille) and Centre Oscar Lambret after approval by the internal review board  
662 (CSTMT-042, 27/07/2009). All patients were informed, and consent was obtained by the  
663 referring physician.

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#### 665 **CONSENT TO PUBLISH**

666 The authors consent to publish the paper.

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#### 668 **COMPETING INTERESTS**

669 The authors declare no competing interest.

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671 **Figure legends**

672 **Fig. 1 The *FSCN1* gene is downregulated by the androgen receptor in VCaP cells.** **a** RT-  
673 PCR evaluating expression levels of *FSCN1* (right panel) from multiple cell lines (PC3c, PC3,  
674 PC3M-Luc, DU145, LNCaP, MDA-PCa-2b, VCaP) in which androgen receptor (*AR*)  
675 expression (left panel) was measured in parallel. Data were normalized to *FSCN1* and *AR* levels  
676 in PC3c cells. **b** Western blot validating expression of AR and FSCN1 proteins in cell lines.  
677 GAPDH was used as a loading control. **c** RT-PCR showing *FSCN1* expression in VCaP cells  
678 (right panel) according to AR activation in the presence of increasing doses (1 to 1000 nM) of  
679 dihydrotestosterone (DHT). Prostate-specific antigen (*KLK3*) expression (left panel) was a  
680 positive control of AR activation in the same experiment. Error bars indicate n=3, mean +/- s.d.  
681 Statistical significance was determined by one-way ANOVA with a Dunnett multiple  
682 comparison test. **d** Western blot validating expression of FSCN1 in VCaP cells.  $\beta$ -actin was  
683 used as a loading control. **e** RT-PCR showing *FSCN1* expression in VCaP cells (right panel)  
684 according to AR activation or repression respectively in the presence of increasing doses (1 to  
685 1000 nM) of dihydrotestosterone (DHT) administered in the presence or the absence of 50  $\mu$ M  
686 bicalutamide (BIC). Prostate-specific antigen (*KLK3*) expression (left panel) was a positive  
687 control of AR activation or repression in the same experiment. Error bars indicate, mean +/-  
688 s.d. Statistical significance was determined by one-way ANOVA with a Dunnett multiple  
689 comparison test (n=3). **f** Western blot validating expression of *FSCN1* in VCaP cells.  $\beta$ -actin  
690 was used as a loading control. RT-PCR experiments were normalized to human *L32* genes and  
691 run in triplicate.

692 **Fig. 2 Expression of *FSCN1* in normal and cancerous human prostate.** **a** Scatter plots  
693 showing correlation of relative gene expression between *FSCN1* and *KLK3* in gene expression  
694 datasets GSE35988, GSE6919, GSE8511 and GSE3325. Pearson's test was used to determine  
695 the correlation coefficient and statistical significance. **b** Comparison of *FSCN1* expression  
696 profiles in normal prostate (Normal or Adjacent), primary prostate cancer (Primary PCa) and  
697 in metastatic prostate cancer (Metastatic PCa or CRPC) cells using gene expression datasets  
698 GSE35988, GSE6919, GSE8511, GSE3325. Statistical significance was determined by one-  
699 way analysis of variance (ANOVA) with a Tukey multiple comparison test.

700 **Fig. 3 FSCN1 is highly expressed in human NEPC cells.** **a** IHC staining was performed on  
701 normal prostate and primary PCa (PCa 1 and PCa 2), using two distinct antibodies against  
702 FSCN1 (Abcam-IM20 and Thermo-55K1). BV: blood vessels, NG: Normal glands, T: Tumour.

703 Scale bar = 50  $\mu$ m. **b** Representative images showing hematoxylin-eosin-saffron (HES) and  
704 immunohistochemical staining for FSCN1, CHGA and SYP proteins in adenocarcinoma and  
705 four NEPC tumour samples (NEPC-1 to -4).

706 **Fig. 4 Androgen deprivation induces a neuroendocrine-like phenotype and triggers**  
707 **FSCN1 expression.** **a** Schematic representation of NE transdifferentiation using LNCaP and  
708 VCaP cells cultured with the charcoal-stripped medium in androgen-deprivation conditions for  
709 14 days (NE), compared with LNCaP and VCaP cultured with the normal medium in androgen-  
710 sensitive conditions (Control). **b** Representative phase-contrast microscopic images of the  
711 morphology of LNCaP and VCaP cells cultured for 14 days in normal medium (LNCaP and  
712 VCaP) or cultured in charcoal-stripped medium (NE). Arrowheads indicate neurite-like cell  
713 structures. **c, e** LNCaP (**c**) and VCaP (**e**) cells were cultured for 14 days in a charcoal-stripped  
714 medium to induce a neuroendocrine-like phenotype (NE). RT-PCR showing expression of  
715 neuroendocrine markers *Chromogranin-A (CHGA)* and *Enolase-2 (ENO2)*. *KLK3* expression  
716 is a positive control of androgen activity. Error bars indicate n=3, mean +/- s.d. Statistical  
717 significance was determined by a two-tailed unpaired Student *t*-test. Western blot validation of  
718 CHGA and NSE protein expression in NE cells and PSA protein expression in Control cells.  
719 GAPDH was used as a loading control. **d, f** RT-PCR evaluating expression levels of *FSCN1* in  
720 LNCaP (**d**) and in VCaP (**f**), respectively. Error bars indicate n=3, mean +/- s.d. Statistical  
721 significance was determined by a two-tailed unpaired Student *t*-test. Western blot validation of  
722 FSCN1 expression cells.  $\beta$ -actin was used as a loading control. **g** Schematic representation of  
723 the timeline for siRNA transfection of LNCaP and VCaP cells in androgen-deprivation culture  
724 conditions. **h, i** RT-PCR evaluating expression levels of *FSCN1*, *CHGA*, *ENO2* and  
725 *Synaptophysin (SYP)* in LNCaP cells (**h**) and VCaP cells (**i**) with si*FSCN1* in comparison with  
726 siControl. Error bars indicate n=3, mean +/- s.d. Statistical significance was determined by a  
727 two-tailed unpaired Student *t*-test.

728 **Fig. 5 FSCN1 loss affects prostate cancer cell growth *in vitro* and *in vivo*.** **a** FSCN1 protein  
729 expression was assessed by Western blotting in PC3M cells expressing shScramble,  
730 shFSCN1.2, and shFSCN1.3, respectively. GAPDH was used as a loading control. **b** Cell  
731 proliferation of PC3M cells expressing shScramble, shFSCN1.2, and shFSCN1.3 was assessed  
732 by MTT assay. **c** Cell migration of PC3M cells expressing shScramble, shFSCN1.2, and  
733 shFSCN1.3 was assessed by Transwell assay. Relative cell numbers of cells per field are shown.  
734 **d** PC3M cells expressing shScramble, shFSCN1.2, or shFSCN1.3 were implanted

735 subcutaneously, and tumour growth was assessed by biweekly measurements. **e** Tumour weight  
736 of PC3M tumours expressing shScramble, shFSCN1.2, or shFSCN1.3 at the endpoint. **f** Images  
737 of excised tumours at the endpoint.

738 **Fig. 6 AR protein binds to cis-elements of the *FSCN1* gene.** **a** Schema showing the AR-  
739 binding motif (ARE) obtained from JASPAR database. **b** Genomic location of the potential  
740 AREs on the *FSCN1* gene (TSS: transcription start site). Location of the qPCR-amplified  
741 fragments (a to e) overlapping potential AREs in an 800 bp domain (little black boxes). **c** UCSC  
742 genome browser representations showing AR and FOXA1 binding events on *FSCN1* and *KLK3*  
743 loci in VCaP cells. ChIP-seq profiles indicate AR enrichment (pink and red) and FOXA1  
744 enrichment (orange) on *FSCN1* and *KLK3* gene loci in the vehicle (GSM1328945 in pink),  
745 DHT-stimulated (GSM1328952 in red) or R1881-stimulated (GSM1354839 in orange) VCaP  
746 cells. **d** ChIP-qPCR assay confirmation of AR binding on the *FSCN1* regulatory region in DHT-  
747 stimulated VCaP cells (16 hrs), compared with untreated cells, using either anti-AR or anti-IgG  
748 antibodies. The results are expressed in % of input for each fragment a to e. *KLK3* promoter  
749 (Prom *KLK3*) was used as a positive control for the DHT stimulation experiment, and an  
750 irrelevant region (Control Region) was used as a negative control. Experiments were performed  
751 with n=3 biologically independent samples. Data were presented as mean +/- s.d. Statistical  
752 significance was determined by a two-tailed unpaired Student *t*-test. **e** Schema illustrating AR  
753 signalling-mediated regulation of *FSCN1* gene transcription in prostate cancer. AR: Androgen  
754 receptor, DHT: Dihydrotestosterone, FOXA1: forkhead box A1, ADT: Androgen-deprivation  
755 therapy/AR antagonists, RNA PolIII: RNA polymerase, TF: transcription factors. **f** ChIP-qPCR  
756 assays of FOXA1 binding on the *FSCN1* regulatory region a to e in VCaP cells cultured in  
757 androgen-sensitive (left panel) or androgen-deprivation (right panel) conditions using either  
758 anti-FOXA1 or anti-IgG antibodies. The results are presented in % of input. The *KLK3*  
759 promoter (Prom *KLK3*) was used as a positive control for the DHT-stimulation experiment. An  
760 irrelevant region (Control region) was used as a negative control. Experiments were performed  
761 with n=3 biologically independent samples. Data were presented as mean +/- s.d. Statistical  
762 significance was determined by a two-tailed unpaired Student *t*-test.