Supporting Information

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SI Materials and Methods

Plasmids, Cloning, and Mutagenesis. Flag-JMJD1A, myc-JMJD1A, and Flag-AR-FL in the pcDNA3 vector were described previously (1, 2). The AR minigene reporter plasmid was described previously (3), and mutations of the G-tracts (poly-G to poly-T) were performed with a Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's instructions. AR-V7 in the pQCXIN vector was provided by Charles Sawyers, Memorial Sloan Kettering Cancer Center, New York, and subcloned into a Flag-pcDNA3 or pLvx-IRES-zsGreen1 vector. The sequences of HNRNPF, HNRNPH, and U2AF65 were obtained by PCR with cDNA from Rv1 cells as a template and cloned into Flag-pcDNA3 vector. The targeting sequence of AR-V7 shRNA was described previously (4) and cloned into the pLKO.1 vector. All other shRNAs used in this study were purchased from Sigma–Aldrich. Primers used for cloning are the following. AR-V7 in Flag-pcDNA3: 5′-AATTGGATCCGAAGTGCAGTTAGGGCTGGGAAGGGTC-3′, 5′-AATTGAATTCTCAGGGTCTGGTCATTTTGAGATGCTTG-3′; AR-V7 in pLvx-IRES-zsGreen1: 5′-AATTGAATTCATGGACTACAAGGACGATGATGACAAGGAAGTGCAGTTAGGGCTGGGAAGGGTC-3, 5′-AATTGGATCCTCAGGGTCTGGTCATTTTGAGATGCTTG-3′; HNRNPF in Flag-pcDNA3: 5′-AATTGGATCCATGATGCTGGGCCCTGAGGGAGGTGAAG-3′, 5′- AATTCTCGAGCTAGTCATAGCCACCCATGCTGTTCTG-3′; HNRNPH in Flag-pcDNA3: 5′-AATTGGATCCATGATGTTGGGCACGGAAGGTGGAGAG-3′, 5′-AATTCTCGAGCTATGCAATGTTTGATTGAAAATCACTG-3′; U2AF65 in Flag-pcDNA3: 5′-AATTGGATCCTCGGACTTCGACGAGTTCGAGCGGCAG-3′, 5′-AATTGAATTCCTACCAGAAGTCCCGGCGGTGATAAGA-3′; AR-V7 shRNA in pLKO.1: 5′-CCGGAAGGCTAATGAGGTTTATTTTCTCGAGAAAATAAACCTCATTAGCCTTTTTTTG-3′, 5′-AATTCAAAAAAAGGCTAATGAGGTTTATTTTCTCGAGAAAATAAACCTCATTAGCCTT-3′.

Immunohistochemistry Staining. Sections of human prostate cancer TMA were used for immunohistochemistry staining of JMJD1A, AR-V7, or HNRNPF. Sections of Rv1 xenograft tumor were used for immunohistochemistry staining of Ki67 or active caspase-3. Antigen retrieval was performed using Dako target retrieval solution, followed by a 3% hydrogen peroxidase block for 30 min. Specimens were incubated with primary antibody diluted in Dako antibody diluent overnight at 4 °C. Slides were then washed three times with PBS/Tween-20 and incubated with Dako-labeled polymer-HRP (anti-rabbit or anti-mouse) for 1 h at room temperature (RT). Slides were then washed four times with PBS/Tween-20, developed with DAB, and counterstained with hematoxylin. TMA slides were scanned using a BLISS digital imaging system (Olympus Canada, Inc.). Images were viewed using digital Image Hub (SlidePath digital pathology solution). Two pathologists scored the samples based on the intensity of positive cells in a defined area of 1-mm cores. To quantify JMJD1A or AR-V7 staining, staining intensity was classified as follows: 0 (no staining), 1 (weak staining), 2 (medium staining), or 3 (high staining). Scores of 0 and 1 were defined as low expression, while scores of 2 and 3 were defined as high expression. The Mann–Whitney *U* test was used to assess the relationship between AR-V7 and JMJD1A. For Ki67 staining, the percentage of positively stained nuclei was calculated in 10 high-power fields. For active caspase-3 staining, the number of positively stained nuclei was counted in 10 high-power fields. The results were shown as means ± SD, and the ANOVA test was used for statistical analysis.

Lentiviral Vector Packaging and Transduction of Prostate Cancer Cells. Lentiviral vector encoding shRNAs or AR-V7 was packaged in 293T cells by calcium phosphate transfection. The supernatants that contained lentiviral particles was collected 48 h after transfection. Prostate cancer cells were then transduced with the supernatant in the presence of polybrene (8 μg/mL) for 24 h before replacement with fresh growth media. Cells were analyzed at 48 or 72 h posttransduction.

Rv1 Cell Xenograft Model. Eight-week-old male NSG mice were randomly divided into three groups ($n = 20$ per group). Rv1 cells (1×10^6 cells per 100 μL of 1:1 PBS/Matrigel) were injected s.c. into the flanks of the mice. After 1 wk, half of the mice in each group were castrated and the other half were sham-castrated. For the castration procedure, we anesthetized the mice with pentobarbital (60 mg/kg of body weight, i.p.) and used buprenorphine (0.1 mg/kg of body weight, s.c.) as a postoperative analgesic. All animals remained viable after the procedure and recovered readily. We collected xenograft tumors 2 wk later and measured tumor weights.

Two-Dimensional Colony Formation Assay. Rv1 cells (5×10^3 cells per well) were seed into six-well plates in duplicate. After 3 wk, cells were fixed in 3.5% paraformaldehyde and stained with 0.2% crystal violet. Cell colony images were taken under a dissection microscope with a camera. The number of cell colonies (>100 μm in diameter) was determined in 10 high-power fields.

Immunoprecipitation and Western Blotting. For immunoprecipitation, cells were harvested in lysis buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM sodium orthovanadate, and a 1× protease inhibitor mixture. To immunoprecipitate Flag-tagged proteins, lysates were incubated with M2 beads (Sigma–Aldrich) overnight, beads were washed three times, and precipitated proteins were eluted with SDS loading buffer. For whole-cell lysates, cells were harvested using radioimmunoprecipitation assay buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, and 1× protease inhibitor mixture]. Lysates were subjected to SDS/PAGE, and proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences), which were then probed with primary antibodies followed by a secondary antibody conjugated to fluorescent dye. Blots were imaged using an Odyssey detecting system (LI-COR Biotechnology).

Identification of Proteins Coprecipitated with JMJD1A by Liquid Chromatography-MS Analysis. Flag-tagged JMJD1A or empty control lentiviral vector was transduced into Rv1 cells in five 15-cm dishes. Immunoprecipitation of Flag-JMJD1A was performed as described above. The precipitated proteins were eluted with 3× flag peptides. The eluted samples were subject to in-solution trypsin digestion, followed by liquid chromatography-MS analysis and a database search (Burnham Institute), to determine identities of the coprecipitated proteins.

qRT-PCR Analysis. Total RNA from cells was prepared using a Total RNA Miniprep Kit (Sigma–Aldrich), followed by digestion with DNase I. cDNA was synthesized using random hexamers for SYBR Green qPCR analysis. Primers for peptidylprolyl isomerase

A (PPIA) served as an internal control. Duplicate or triplicate samples were used for qPCR analysis, and independent experiments were repeated at least three times. Data were presented as means ± SD (n = 3). Primers for qPCR analysis of human gene transcripts were as follows: PPIA: 5′-GACCCAACACAAATGGTTC-3′, 5′-AGTCAGCAATGGTGATCTTC-3′; JMJD1A: 5′-CAGGAGCTCCACATCAGGTT-3′, 5′-TGCATCTTTCACTGCATGGT-3′; AR-FL: 5′-CAGTGGATGGGCTGAAAAAT-3′, 5′-GGAGCTTGGTGAGCTGGTAG-3′; AR-V7: 5′-GCAATTGCAAGCATCTCAAA-3′, 5′-CAACCCCAACGTCAAAGTCT-3′; AR-V1: 5′-CCATCTTGTCGTCTTCGGAAATGTTATGAAGC-3′, 5′-CTGTTGTGGATGAGCAGCTGAGAGTCT-3′; NKX3.1: 5′-ACTTGGGGTCTTATCTGTTGGA-3′, 5′-CTCGATCACCTGAGTGTGGG-3′; FKBP5: 5′-CATCAAGGCATGGGACATTGG-3′, 5′-TCGAGGGAATTTTAGGGAGACT-3′; SREBF1: 5′-GCCCCTGTAACGACCACTG-3′, 5′-CAGCGAGTCTGCCTTGATG-3′; KLK3: 5′-CACCTGCTCGGGTGATTCTG-3′, 5′-CCACTTCCGGTAATGCACCA-3; SLC45A3: 5-CCTTCACGCTGTTTTACACGG-3′, 5′-CGCCTTCATCATAGTGTCTCC-3′; STEAP2: 5′-GGTCACTGTAGGTGTGATTGG-3′, 5′-ACCACATGATAGCCGCATCTAA-3′; STEAP1: 5′-CCCTTCTACTGGGCACAATACA-3′, 5′-GCATGGCAGGAATAGTATGCTTT-3′; NDRG1: 5′-CCAACAAAGACCACTCTCCTC-3′, 5′-CCATGCCCTGCACGAAGTA-3′; HNRNPF: 5′-AATTGTGCCAAACGGGATCAC-3′, 5′-GTGTTTCCCTAGAGCCTTCTCA-3′; c-Myc: 5′-GGCTCCTGGCAAAAGGTCA-3′, 5′-AGTTGTGCTGATGTGTGGAGA-3′; SYF2: 5′-GGAAAAACCCTGATCTGGGATT-3′, 5′-TCTCCGGCTATATTTGTCTCGT-3′; SRSF7: 5′-CGGTACGGAGGAGAAACCAAG-3′, 5′-AGCCACAAATCACCTTTCCATC-3′; SREK1: 5′-GCCTGGTGCAGGATTGCTT-3′, 5′-TGCTGGTATAGCTCCCAAACT-3′; PHF5A: 5′-TGGTGTTGCCATCGGAAGAC-3′, 5′-CAGGGACGCACATAGGAGTC-3′; RAVER2: 5′-CTGAGCAACCGCAGGAAAATC-3′, 5′-ACATCTGAATTGCGTTCTGGG-3′; ESRP1: 5′-GCCAAGCTAGGCTCGGATG-3′, 5′-CAGTCCTCCGTCAGTTCCAAC-3′; RBFOX2: 5′-GACGCAATGGTTCAGCCTTTT-3′, 5′-GCGTACTTCCGTAGAGTGTCAG-3′; PTBP1: 5′-AGCGCGTGAAGATCCTGTTC-3′, 5′-CAGGGGTGAGTTGCCGTAG-3′; YBX1: 5′-GGGGACAAGAAGGTCATCGC-3′, 5′-CGAAGGTACTTCCTGGGGTTA-3′.

AR Minigene Reporter Assay. The AR-null PC3 cells in 12-well plates were transfected with AR minigene reporter (200 ng) using Lipofectamine 2000 (Invitrogen) in triplicate. After 72 h, RNAs were collected for qRT-PCR analysis with the following primers to evaluate AR-V7 or AR-FL splicing: AR-V7 splicing: 5′-CAGGGATGACTCTGGGAGAA-3′, 5′-GCCCTCTAGAGCCCTCATTT-3′; AR-FL splicing: 5′-TCTTGTCGTCTTCGGAAATGT-3′, 5′-AAGCCTCTCCTTCCTCCTGTA-3′.

RNA Pull-Down. RNA pull-down experiments were performed as previously reported (3). Briefly, biotinylated RNA oligos (Sigma–Aldrich) were immobilized onto streptavidin beads (Pierce) in binding buffer [20 mM Hepes-KOH (pH 7.9), 80 mM potassium glutamate, 0.1 mM EDTA, 1 mM DTT, 20% glycerol] at 4 °C for 2 h. Flag-tagged HNRNPF, JMJD1A, or U2AF65 was transfected into 293T cells and purified with anti-Flag M2 beads. Immobilized RNA oligos were incubated with purified Flag-HNRNPF, Flag-JMJD1A, and/or Flag-U2AF65 in binding buffer containing 30 U/mL RNase OUT and 15 μg/mL yeast tRNA at 4 °C for 2 h. Streptavidin beads were washed three times with binding buffer and once with washing buffer [20 mM Hepes-KOH (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 75 mM KCl, 20% glycerol]. The bound proteins were eluted with SDS loading buffer and analyzed by Western blotting. The RNA oligo sequences were as follows: WT oligo: 5′-rUrUrCrArUrArCrUrArGrArArArArArUrUrCrCrGrGrGrUrUrGrGrCrArArUrUrGrCrArArGrCrArUrC-3′; mutant oligo: 5′-rUrUrCrArUrArCrUrArGrArArArArArUrUrCrCrUrUrUrUrUrGrGrCrArArUrUrGrCrArArGrCrArUrC-3′.

RIP Assay. A RIP assay was performed as previously reported (3, 5), with minor modifications. Cell nuclei were extracted with buffer A [10 mM Hepes (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor mixtures, 50 U/mL RNase OUT]. Nuclear extracts were solubilized in RIP lysis buffer [100 mM KCl, 5 mM MgCl2, 10 mM Hepes (pH 7.4), 0.5% Nonidet P-40, 1 mM DTT, protease and phosphatase inhibitor mixtures, 100 U/mL RNase OUT] and precleared with 5 μg of control IgG and Protein A/G agarose beads (Santa Cruz Biotechnology). They were then used for immunoprecipitation with 5 μg of antibodies (control IgG, JMJD1A, HNRNPF, or U2AF65) overnight at 4 °C, followed by incubation with Protein A/G agarose beads for 4 h at 4 °C. The beads were washed with buffer I [100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.4), 1 mM DTT] three times and then with buffer II [100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.4), 1 mM DTT, 1 M urea] three times. The beads were incubated with DNase I (10 units) for 15 min at 37 °C, followed by 30 μg of proteinase K treatment for 30 min at 50 °C. Precipitated RNAs were extracted by TRIzol and used for cDNA synthesis and qRT-PCR analysis. PCR primers used were as follows: P1 region: 5′-ACCTCCCCAACTTTACATGCT-3′, 5′-CAGGGTCTGGTCATTTTGAGA-3′; P2 region: 5′-CTGTGACCAGGGAGAATGGT-3′, 5′-CTGGAAGCCTCTCCTTCCTC-3′. Data were calculated as a percentage of input and plotted as fold changes over control IgG. The RIP assay was performed in triplicate and repeated in three independent experiments. Results were presented as means ± SD (n = 3).

ChIP Assay. Cells were cross-linked using 1% formaldehyde for 10 min at RT, and then quenched with 5 M glycine. Cell nuclei were extracted as described in the RIP assay and sonicated to obtain 500-bp chromatin fragments. One hundred micrograms of chromatin was incubated with 5 μg of antibodies overnight at 4 °C, followed by incubation with 30 μL of Protein A/G agarose beads for 4 h. After four washes, cross-linking was reversed and DNA was purified using spin columns and subjected to qPCR analysis. PCR primers used were as follows: P1 region: 5′-ACCTCCCCAACTTTACATGCT-3′, 5′-CAGGGTCTGGTCATTTTGAGA-3′; P2 region: 5′-CTGTGACCAGGGAGAATGGT-3′, 5′-CTGGAAGCCTCTCCTTCCTC-3′. Data were calculated as a percentage of input and presented as means ± SD (n = 3).

1. Fan L, et al. (2016) Regulation of c-Myc expression by the histone demethylase JMJD1A is essential for prostate cancer cell growth and survival. *Oncogene* 35:2441–2452.
2. Qi J, et al. (2013) The E3 ubiquitin ligase Siah2 contributes to castration-resistant prostate cancer by regulation of androgen receptor transcriptional activity. *Cancer Cell* 23:332–346, and erratum (2013) 23:853.
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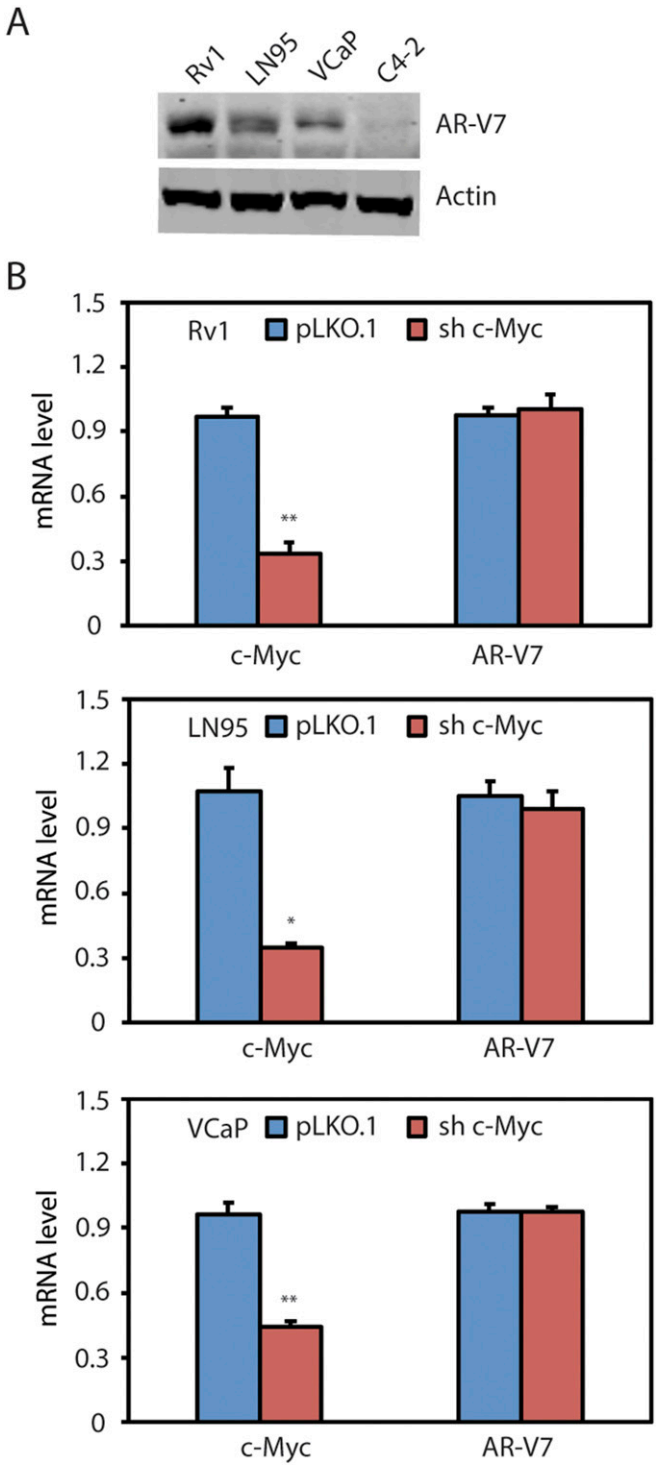

Fig. S1. (*A*) Western blot showing the level of AR-V7 protein in the indicated prostate cancer cells. (*B*) Indicated prostate cancer cells were transduced with pLKO.1 control or c-Myc shRNA. Forty-eight hours posttransduction, RNAs were collected and analyzed by qRT-PCR for c-Myc or AR-V7. The comparison with statistical significance is indicated with an asterisk (**$P < 0.01$, *$P < 0.05$; *t* test).

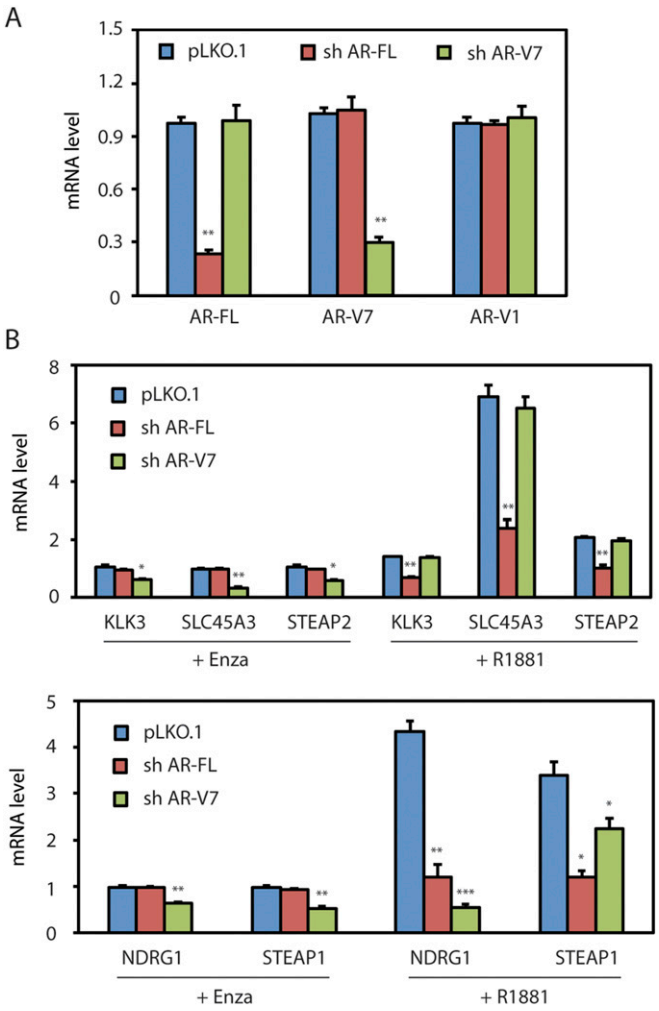

Fig. S2. (*A*) Rv1 cells (pLKO.1, AR-FL–knockdown, or AR-V7–knockdown) were analyzed by qRT-PCR for AR-FL, AR-7, or AR-V1. (*B*) Rv1 cells (pLKO.1, AR-FL–knockdown, or AR-V7–knockdown) were maintained in growth media that contained 5% charcoal-stripped FBS, supplemented with enzalutamide (Enza; 5 μM) or R1881 (1 nM). After 24 h, RNAs were collected and analyzed by qRT-PCR for transcripts of the indicated AR target genes. The comparison with statistical significance is indicated with an asterisk (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; ANOVA).

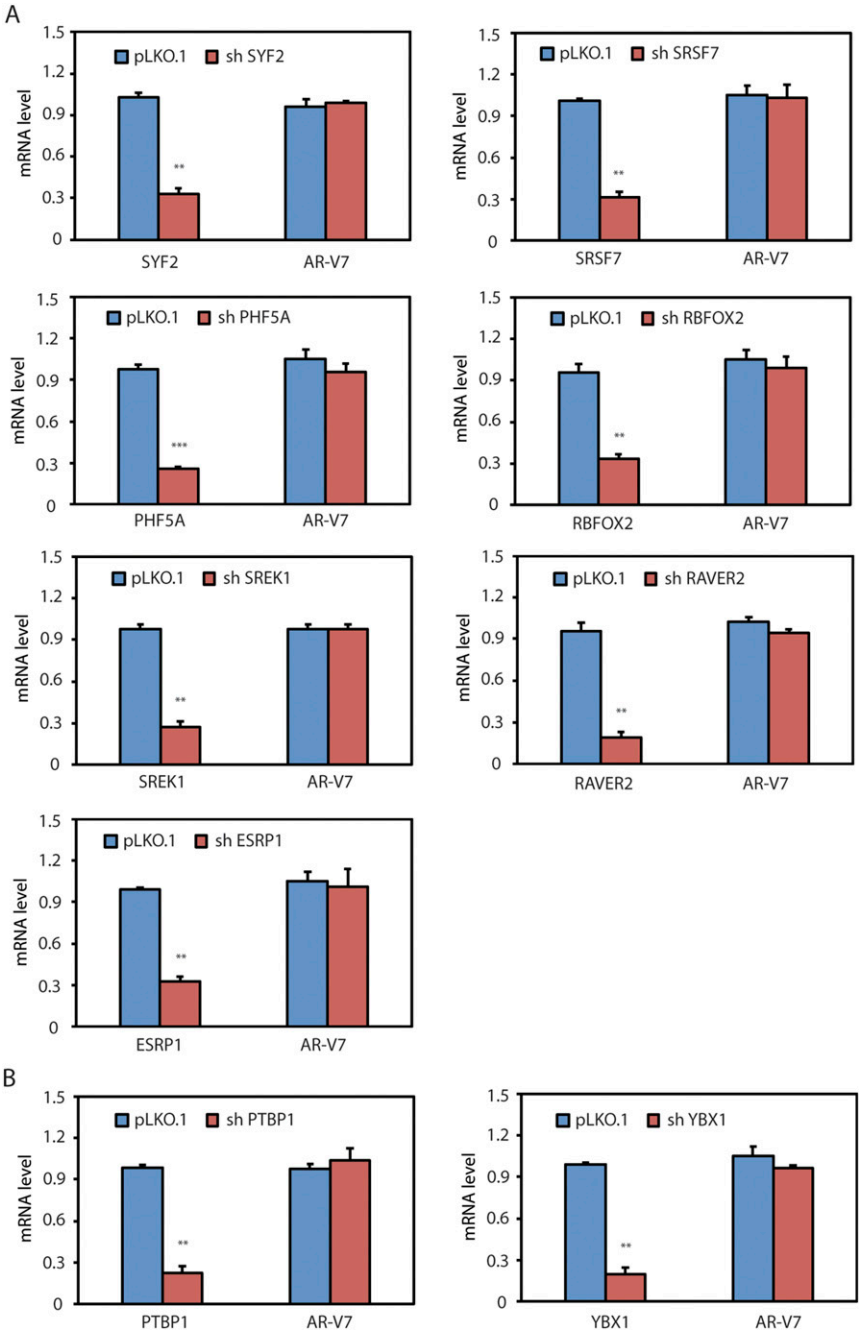

Fig. S3. Rv1 cells were transduced with pLKO.1 or shRNAs of the indicated splicing regulators. Forty-eight hours posttransduction, RNAs were collected and analyzed by qRT-PCR for the indicated gene or AR-V7. *A* and *B* are the results of qRT-PCR analysis on the splicing regulators identified by profiling array and mass spectrometry, respectively. The comparison with statistical significance is indicated with an asterisk (***$P < 0.001$, **$P < 0.01$; *t* test).

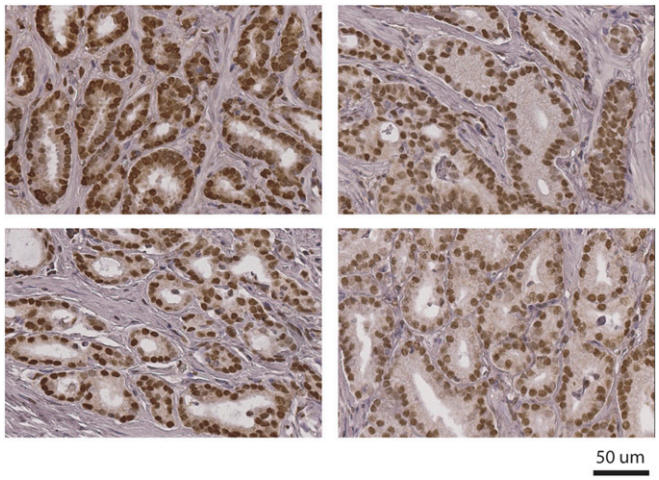

Fig. S4. Immunohistochemistry staining of HNRNPF was performed on a prostate cancer TMA containing 36 tumor samples of Gleason grades 3–5. The staining was developed with DAB (brown) and counterstained with hematoxylin (blue). Shown are representative images of HNRNPF staining on four samples of prostate cancer tissue.

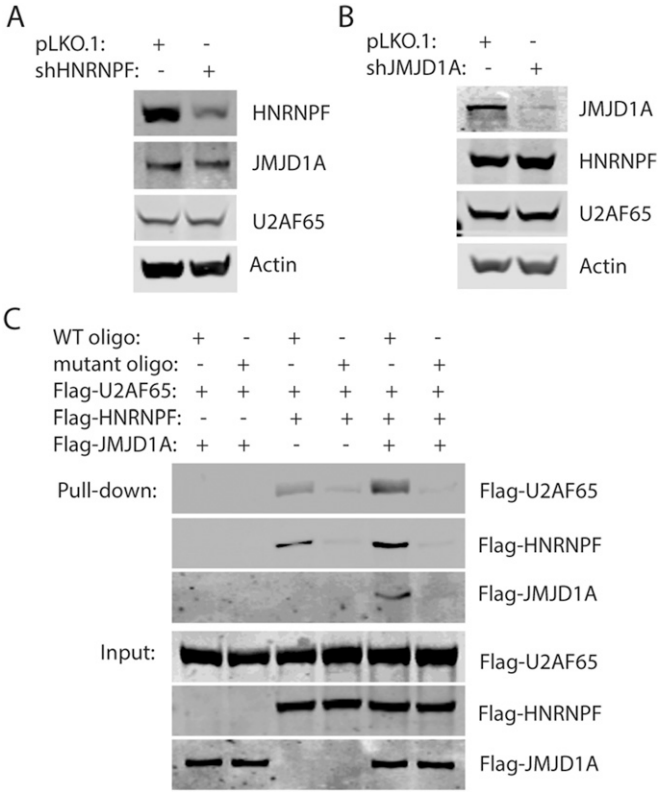

Fig. S5. (*A*) Rv1 cells (pLKO.1 or HNRNPF-knockdown) were analyzed by Western blotting for the indicated proteins. (*B*) Rv1 cells (pLKO.1 or JMJD1A-knockdown) were analyzed by Western blotting for the indicated proteins. (*C*) Purified Flag-U2AF65 was incubated with RNA oligo-conjugated beads (WT or G-tract mutant) in the presence of purified Flag-HNRNPF and/or Flag-JMJD1A. The bound proteins were analyzed by Western blotting with anti-Flag antibodies.

Table S1. Unique proteins in the Flag-JMJD1A immunoprecipitation (IP) assay after subtracting the common proteins identified in both control IP and Flag-JMJD1A IP assays

Table S1

Table S2. Common proteins identified between control immunoprecipitation (IP) and Flag-JMJD1A IP assays, which represent the nonspecific proteins bound to the M2 beads

Table S2