miR-221 promotes the development of androgen independence in prostate cancer cells via down-regulation of HECTD2 and RAB1A

SUPPLEMENTAL INFORMATION

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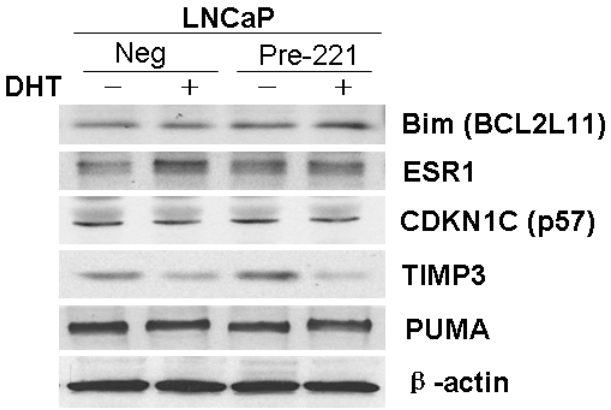
Supplemental Results

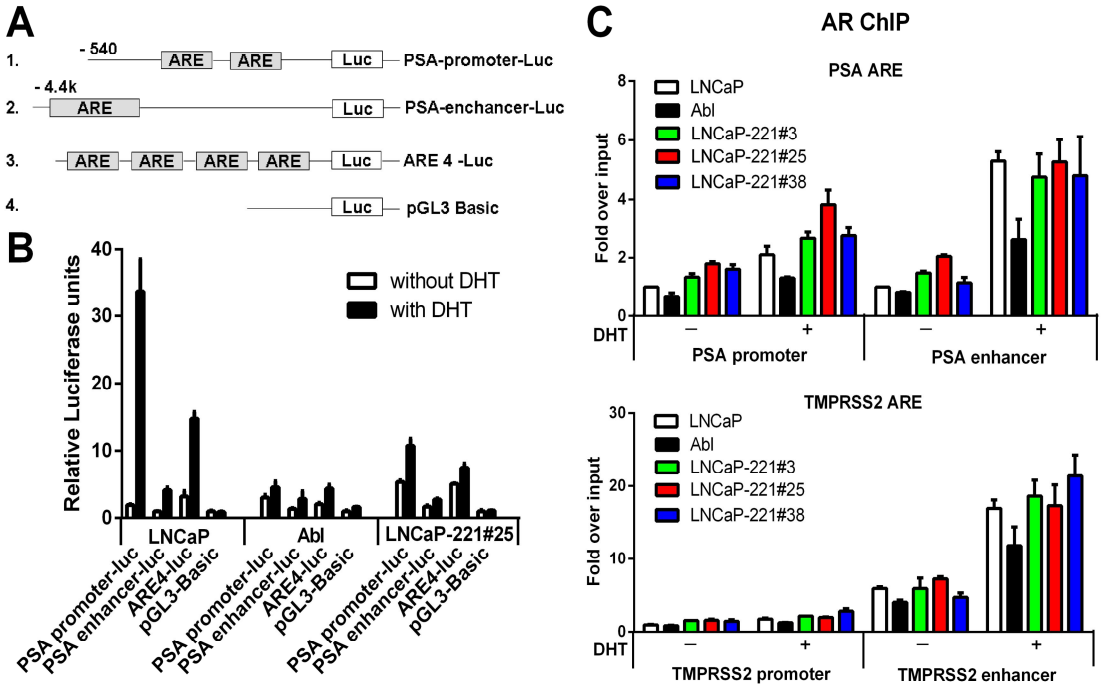
Figure S1. Validation of reported miR-221/-222 targets in LNCaP cells



Western blot of cell lysate from LNCaP cells. LNCaP cells were transiently transfected with 30 nM scramble negative RNA (Neg) or miR-221 precursors (Pre-221) for 24 hours and treated with ("+") or without ("-") DHT for another 24 hours.

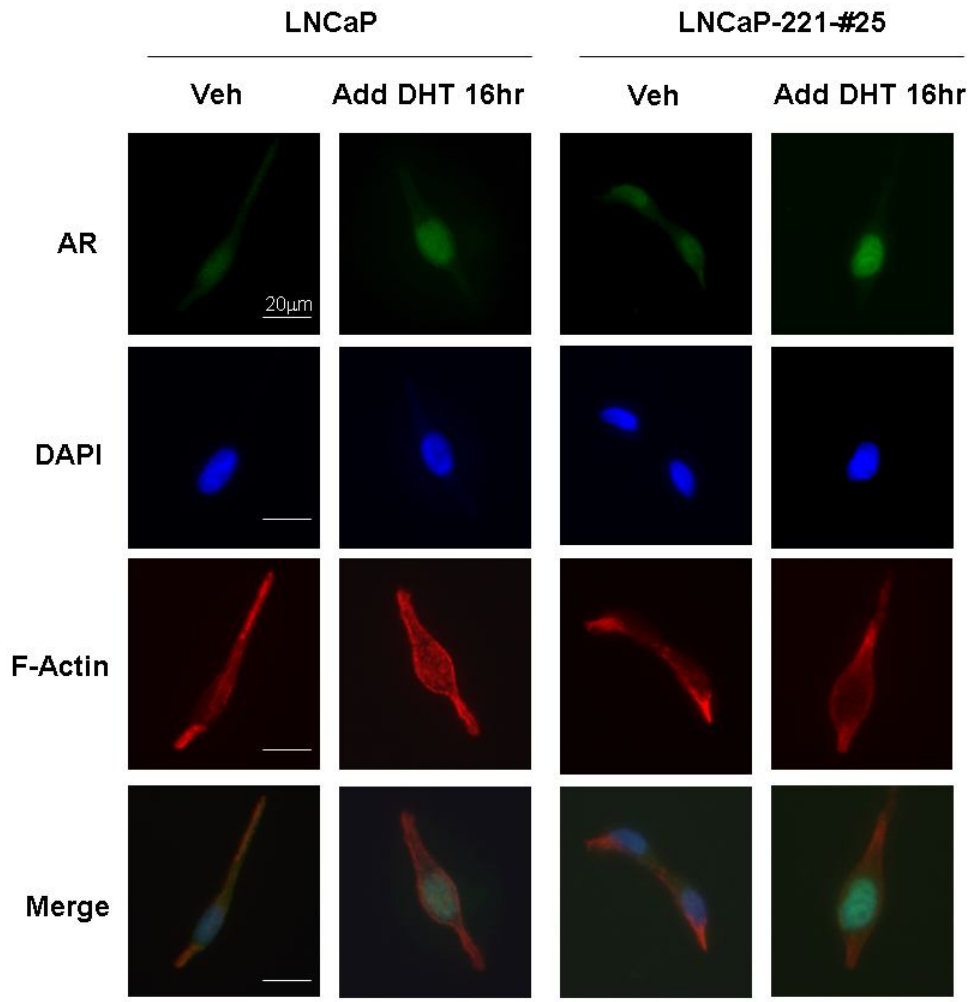
LNCaP cells are PTEN and c-kit negative cells.

Figure S2. MiR-221 expression influence AR activity but not AR occupancies on AREs



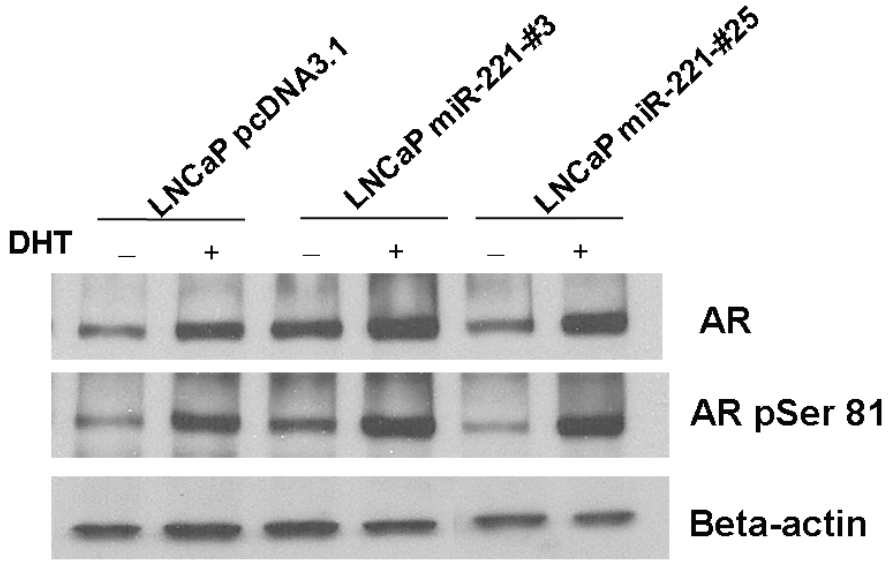
(A). Schematic diagrams for four different luciferase reporter constructs driven by different promoter elements (PSA-promoter-luc, PSA-enhancer-luc and ARE 4-luc and original empty vector-pGL3 Basic). ARE boxes indicate the approximate locations of AREs, Luc boxes indicate the luciferase gene. (B). Comparison of luciferase activities derived from PSA promoter-luc, PSA-enhancer-luc, ARE4-luc or empty pGL3 Basic in LNCaP, LNCaP-Abl and LNCaP-221#25. Cells were transfected with indicated luciferase constructs without (white bars) or with treatment of 10 nM DHT for 24 hours (black bars). Relative luciferase units were calculated by comparing with raw *Rennila* luciferase units from transfection efficiency control, pRL SV40. (C). ChIP analyses of AR recruitment to AREs on the PSA (upper panel) or TMPRSS2 (lower panel) promoter or enhancers in LNCaP, LNCaP-Abl and LNCaP-miR-221 over-expressing cell lines. ChIP assays were performed using the anti-AR antibody in cells treated without or with DHT. In B and C, each column represents the mean of three independent experiments ±SD.

Figure S3. AR subcellular localization is not affected in LNCaP miR-221 over-expressors



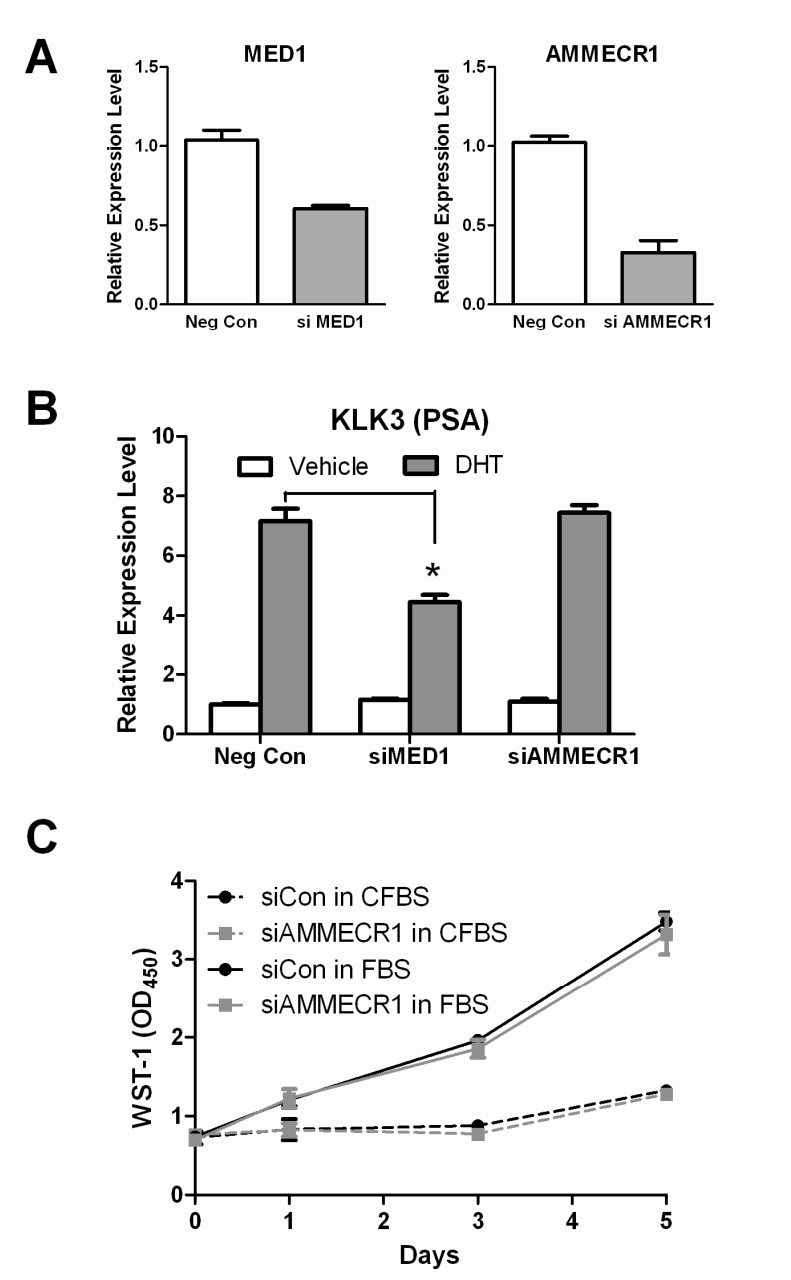
LNCaP (Left two columns) or LNCaP-miR-221 over-expressors (LNCaP-221-#25, Right two columns) were kept in hormone-depleted medium for 96 hrs before treated with (Add DHT) or without (Veh) 10 nM DHT for 16 hrs. Cells were then fixed and immunostained with AR (the first row), DAPI (the second row) or F-actin (the third row). Scale bars: 20 μM.

Figure S4. AR Ser81 site remains phorsphorylated in LNCaP miR-221 over-expressors



Protein expression of total AR, pAR at site Ser81 and loading control of beta-actin in LNCaP vector control cells (LNCaP pcDNA3.1), two miR-221 over-expressors (LNCaP miR-221-#3, LNCaP miR-222-#25). Total cell lysate were prepared from indicated cell lines after treated with ("+") or without ("-") DHT. Equal amount of proteins were subjected to Western blotting with the indicated antibodies.

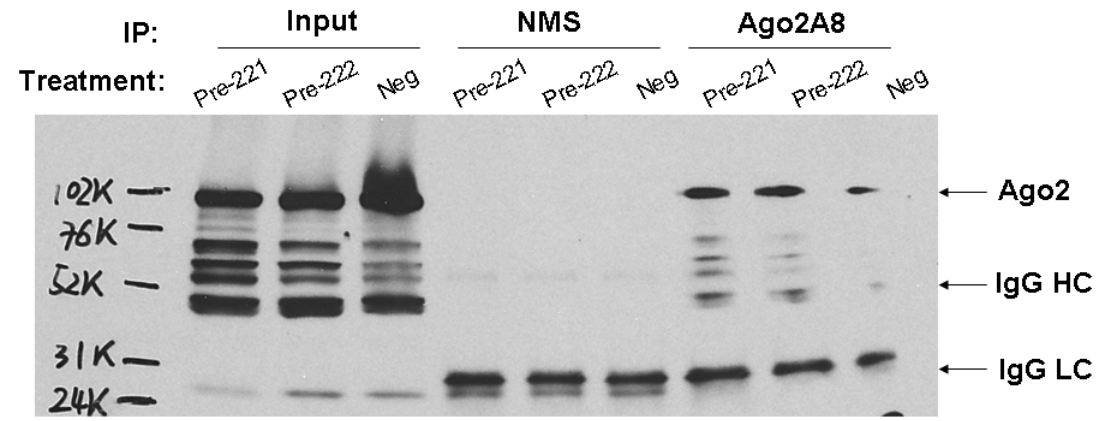
Figure S5. The expression level of AMMECR1 has no impact on KLK3 (PSA) transcription and androgen independent growth



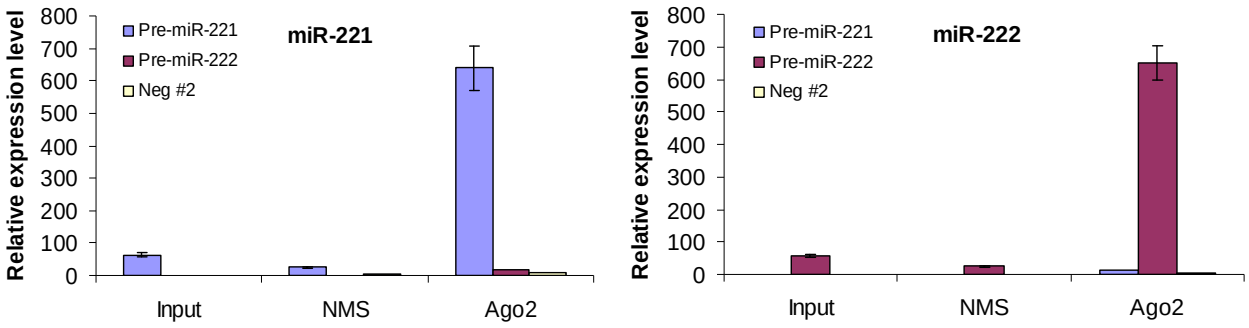
A: LNCaP cells were transiently transfected with 100 nM negative RNAi (Neg Con), MED1 RNAi (siMED1) or AMMECR1 RNAi (siAMMECR1) for 48 hours
B: KLK3 (PSA) mRNA levels were measured from LNCaP cells transfected with RNAis and treated with vehicle or DHT for another 24 hours.
C: Growth curves of LNCaP cells transient transfected with RNAis were determined by WST-1 in medium with regular serum (in FBS) or with charcoal treated serum (androgen-free, in CFBS).

Figure S6. MiR-221/-222 anti-AGO RIP-ChIP was validated in LNCaP cells

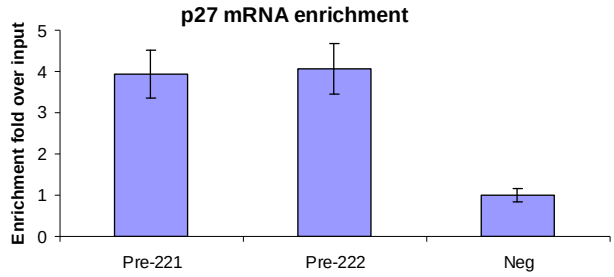
A



B

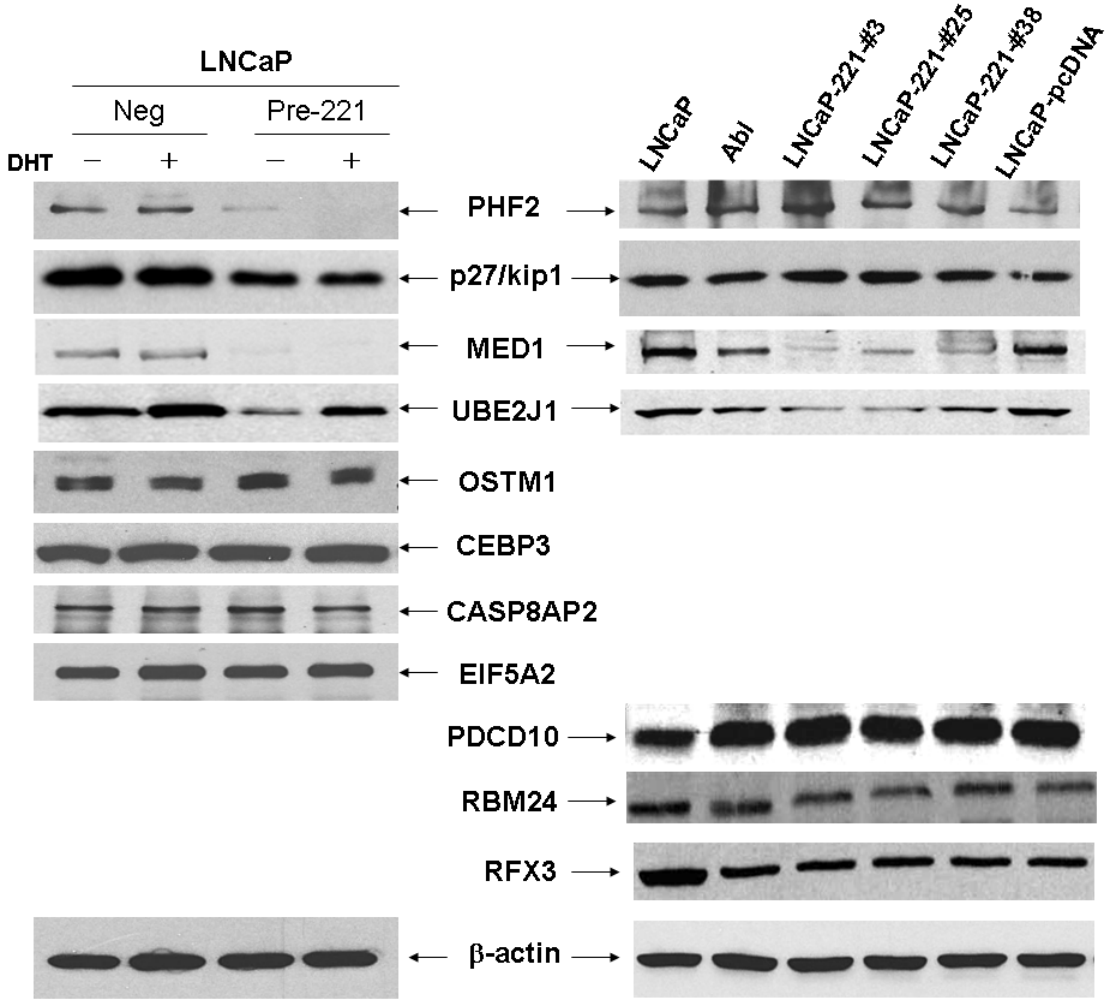


C



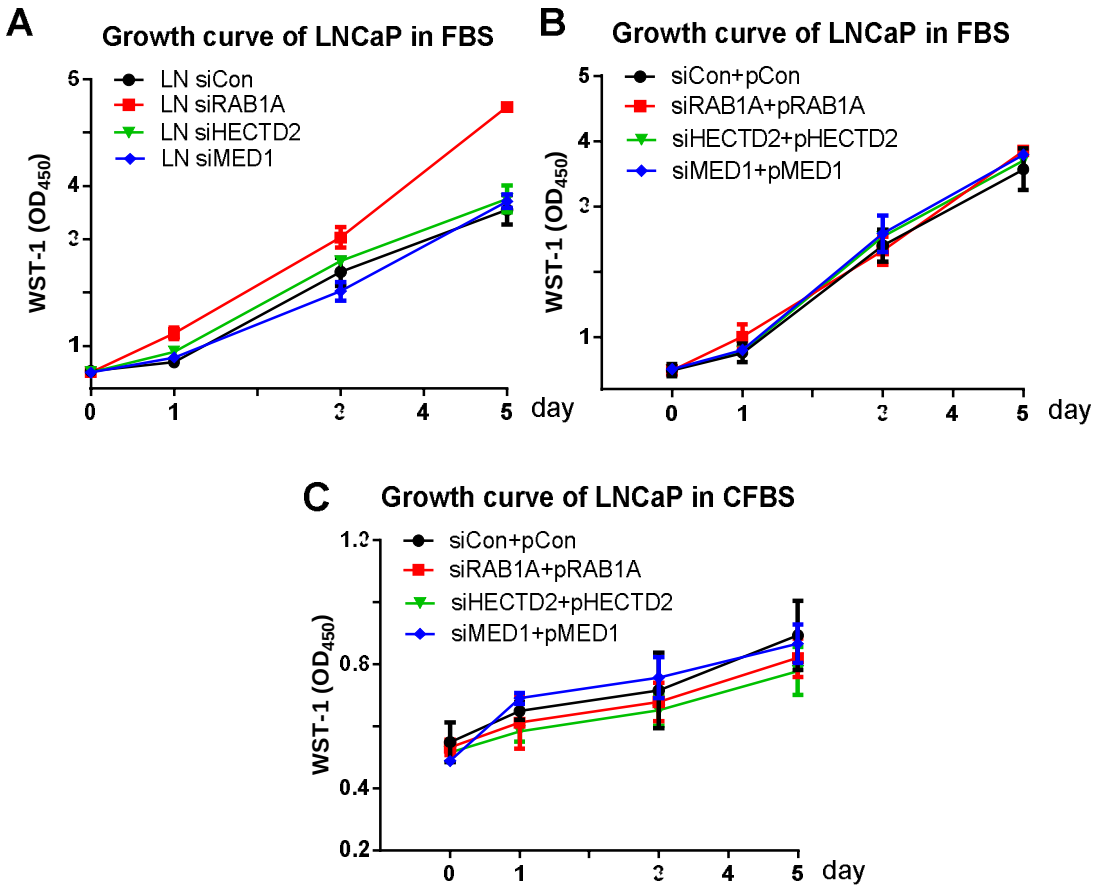
A Western blots analysis of co-IPed products using anti-AGO antibody. Co-IPs were performed on cells transfected with precursors of miR-221, miR-222 or negative scramble RNA control. As expected, AGO proteins were co-IPed with on anti-AGO (2A8), but not with non-immunized mouse serum (NMS). **B** Quantification of qPCR shows that miR-221 or miR-222 were highly enriched only in coIPed RNA from cells transfected with miR-221 or miR-222 precursors. **C** Quantification of qPCR shows that mRNA of known target of miR-221/-222, p27/kip1 can be significantly enriched in AGO RIP-chIP.

Figure S7. Validation of putative targets identified by anti-AGO RIP -ChIP



Left: Western blot of cell lysate from LNCaPs. LNCaP cells were transiently transfected with 30 nM scramble negative RNA (Neg) or miR-221 precursors (Pre-221) for 24 hours and treated with ("+") or without ("-") DHT for another 24 hours
Right: Western blot of cell lysate from LNCaP, LNCaP-Abl, LNCaP miR-221 over-expressors (LNCaP-221-#3, #25 and #38) and vector control (LNCaP-pcDNA).
Equal amounts of proteins were subjected to Western blotting with the indicated antibodies.

Figure S8. Rescue Experiment of LNCaP and LNCaP-miR-221cells



A. Growth Curve of LNCaP cells transfecting with siRNAs of RAB1A (red lines with squares), HECTD2 (green lines with triangles) or MED1 (red lines with diamonds) in regular medium (FBS). **B** and **C.** Growth curve of LNCaP cells co-transfecting with siRNAs and expression vectors in FBS (**B**) or in androgen-free medium (CFBS) (**C**). RAB1A siRNA and RAB1A expression vector, (siRAB1A+pRAB1A green line with squares); HECTD2 RNAi and HECTD2 expression vector, (siHECTD2+pHECTD2 red line with triangles); MED1 RNAi and MED1 expression vector (siMED1+pMED1, blue line with diamonds); or siRNA control and expression vector control (siCon+pCon, black line) over a 5-day time course. Each dots with bars, Mean of three replicates ± SD.

Table S1. Comparsion of down-regulated genes in LNCaP-Abl or LNCaP-miR-221 cells and bioinformatics predicted putative targets

Symbol	Decreased Fold in Abl cells*	Decreased Fold in miR-221 cells*	TargetScan	PicTar	miRanda
AMMECR1	**-2.08**	**-4.60**	√		√
BCHE	-160.08	-8.37			√
CDK8	-3.24	-3.40			√
SH3BGRL	-5.52	-11.03			√
EFNB2	-28.97	-6.40			√
RAB11B	-2.78	-19.10			√
MMP16	-4.11	-4.71			√
SLITRK3	-22.25	-17.09			√

* The two right columns show mRNA expression level of indicated genes compared with LNCaP cells. The left three columns show whether these mRNAs were predicted as targets of miR-221 by "TargetScan", "PicTar", or "miRanda" (Lewis et al. 2005; Krek et al. 2005; Enright et al. 2003).

Supplemental Materials and Methods
Table S2
ChIP primers

PSA promoter+ (Jia et al., 2003)	CCTAGATGAAGTCTCCATGAGCTACA
PSA promoter- (Jia et al., 2003)	GGGAGGGAGAGCTAGCACTTG
PSA enhancer + (Wang et al., 2005)	TGGGACAACTTGCAAACCTG
PSA enhancer- (Wang et al., 2005)	CCAGAGTAGGTCTGTTTTCAATCCA
TMPRSS2 promoter + (Wang et al., 2007)	CTGAGCCCCCACAATTGC
TMPRSS2 promoter - (Wang et al., 2007)	GGTGGGACACACCTCAGCC
TMPRSS2 enhancer + (Wang et al., 2007)	TGGTCCTGGATGATAAAAAAAGTTT
TMPRSS2 enhancer + (Wang et al., 2007)	GACATACGCCCCACAACAGA

PCR Primers used to clone miR-221:

miR-221-sense	CGAGATCTGTGAGAATTACTTGCAAGCTG
miR-221-antisense	CCGCTCGAGCATTGGTGAGACAGCCAATG

PCR primers used to clone 3'UTR to pMIR-REPORT miRNA Expression Reporter Vector (Ambion, Inc. Austin, TX)

RAB1A 3'UTR forward	TTAGACTAGTGCCTCCATCCTTTTCTCACA
RAB1A 3'UTR reverse	ATGGAAGCTTTTCATAGATGGAAAATTGAA

siRNA sequences (Dharmacon ON-TARGET plus SMARTpool siRNA)

siHECTD2	(1) CAAUUUGCCUUGAUGUUAG
	(2) GGGAUUAAUGCUAAAUUUG
	(3) CUGUUAGCCCGAAGAAAGA
	(4) AAACAGAAGUUCACCUGCA
siRAB1A	(1) CAGCAUGAAUCCCGAAUAU
	(2) GUAGAACAGUCUUUCAUGA
	(3) GGAAACCAGUGCUAAGAAU
	(4) UGAGAAGUCCAAUGUUAAA
siAMMECR1	(1) GGAUACAAAGCUCCGAUUA
	(2) GACCAUAGACUCCUUAUUG
	(3) GACCAGGUAUCGUAGUGAA
	(4) GUACAUGGCAUUAGAAUAG

Expression Vectors:
Myc-DDK-tagged ORF clone of HECTD2 (Origene, RC207254)
Myc-DDK-tagged ORF clone of RAB1A (Origene, RC201640)

3'UTR Reporter Vectors for miRNA Target Validation:
3' UTR clone of HECTD2 (Origene, SC218664)

AR phosphorylation detection: antibody against pAR-Ser81 was purchased from Millipore