Supplementary materials

Methylation-dependent and -independent roles of EZH2 synergize in CDCA8 activation in prostate cancer

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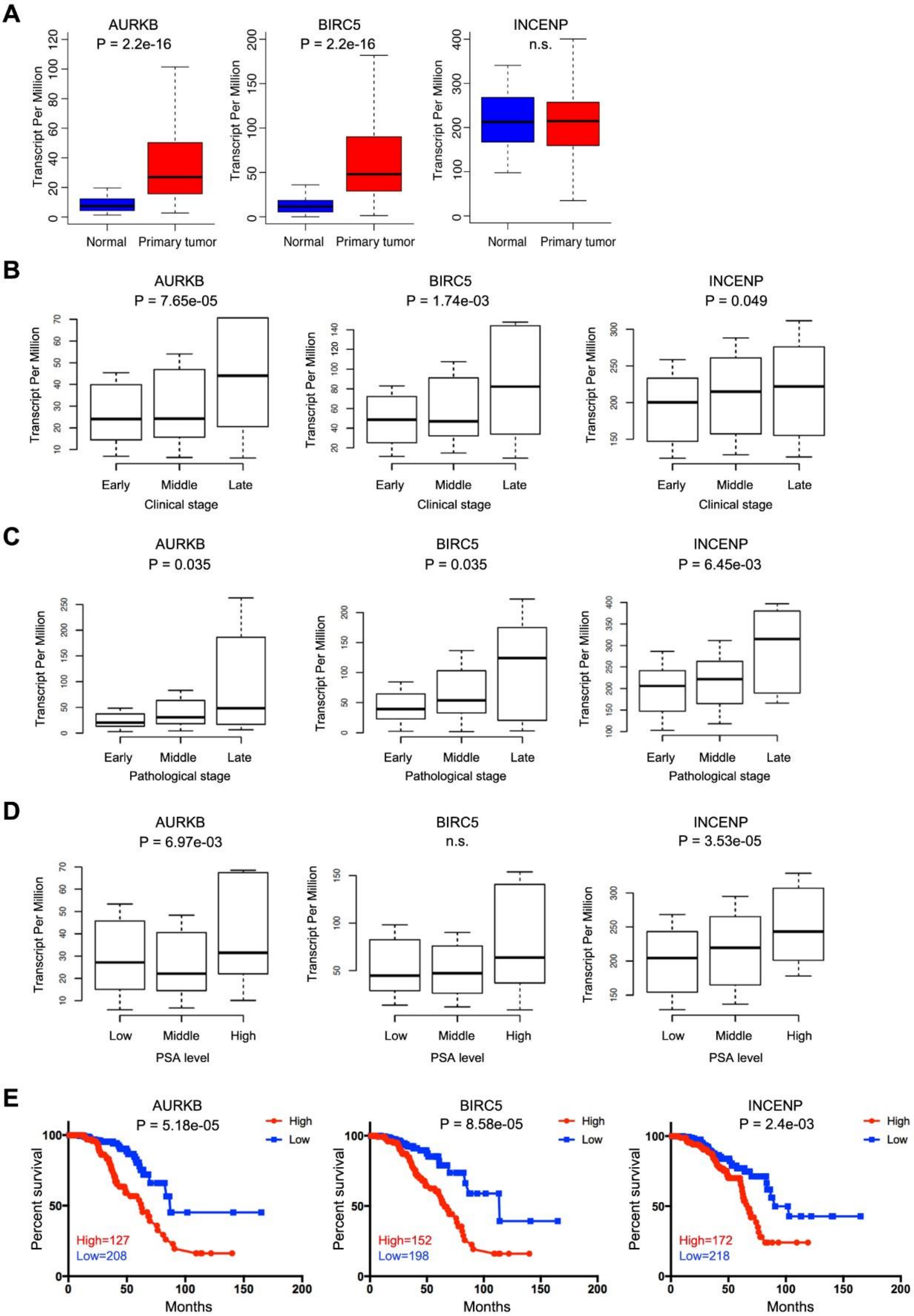


Supplementary Figure 1. Dysregulation of CPC members other than CDCA8 in PCa

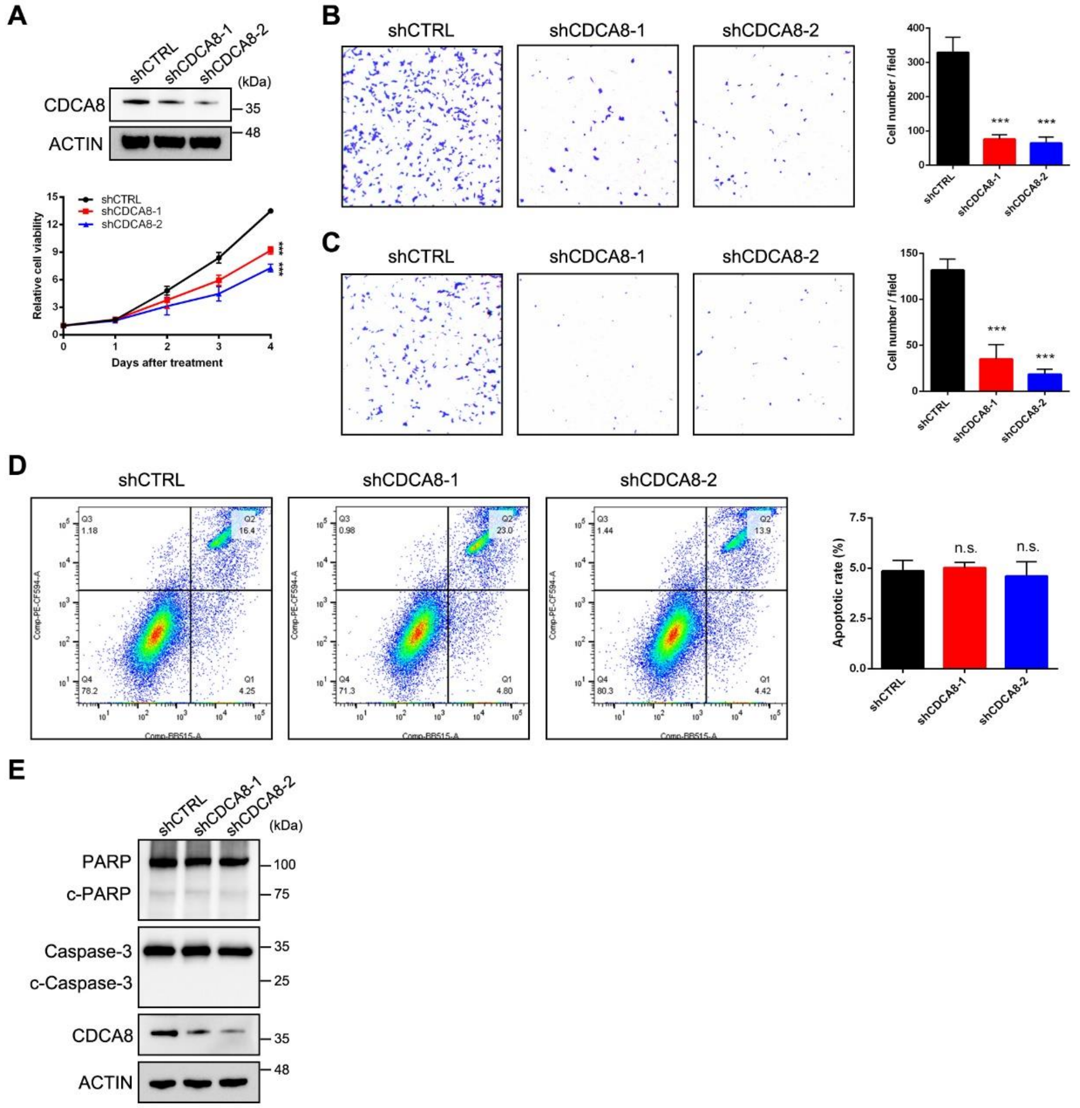
(A) Box plots showing the mRNA levels of AURKB, BIRC5 and INCENP in normal (n=52) and PCa (n=497) specimens using data from TCGA.

(B) Box plots showing the mRNA levels of AURKB, BIRC5 and INCENP in PCa patients with different clinical stages ($n_{(early)}$=177, $n_{(middle)}$=173, $n_{(late)}$=53) using data from TCGA.

(C) Box plots showing the mRNA levels of AURKB, BIRC5 and INCENP in PCa patients with different pathological stages ($n_{(early)}$=186, $n_{(middle)}$=293, $n_{(late)}$=10) using data from TCGA.

(D) Box plots showing the mRNA levels of AURKB, BIRC5 and INCENP in PCa patients with different PSA levels ($n_{(low)}$=181, $n_{(middle)}$=209, $n_{(high)}$=48) using data from TCGA.

(E) The association between AURKB, BIRC5 or INCENP expression and Disease-free survival time of PCa patients was analyzed by Kaplan-Meier analysis using data from TCGA.



Supplementary Figure 2. CDCA8 maintains the aggressiveness of PCa cells

(A) Cell viability assay was used to assess the proliferative capacity of control and CDCA8-deficient PC-3 cells. The knockdown efficiency of CDCA8 was validated by western blot.

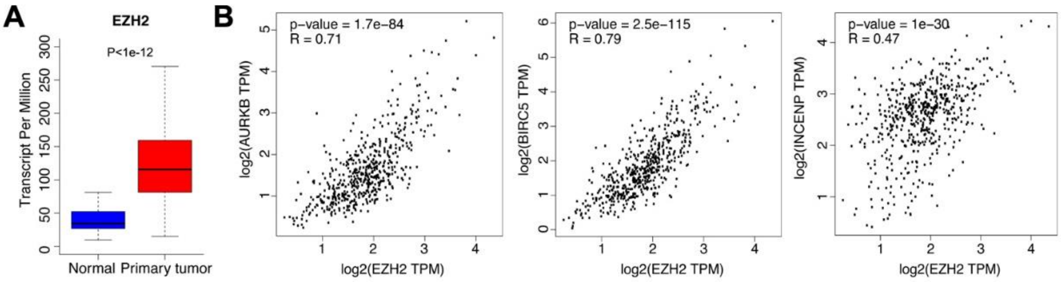
(B) Boyden chamber migration assay was performed to determine the migratory capability of PC-3 cells after CDCA8 depletion. Graph showing the number of migrated cells in the lower surface of filter at 24 h. Data represent Mean ± SD from n=5 random fields per filter.

(C) Boyden chamber invasion assay was performed to determine the invasive capability of PC-3 cells after CDCA8 depletion. Graph showing the number of migrated cells passing through Matrigel at 24 h. Data represent Mean ± SD from n=5 random fields per filter.

(D) Flow cytometry assays were performed to analyze the apoptotic rate in control and CDCA8-deficient C4-2 cells. Graph showing the percentage of apoptotic cells at each group.

(E) Western blot analysis of the protein levels of full-length and cleaved PARP or Caspase-3 in C4-2 cells upon CDCA8 knockdown.

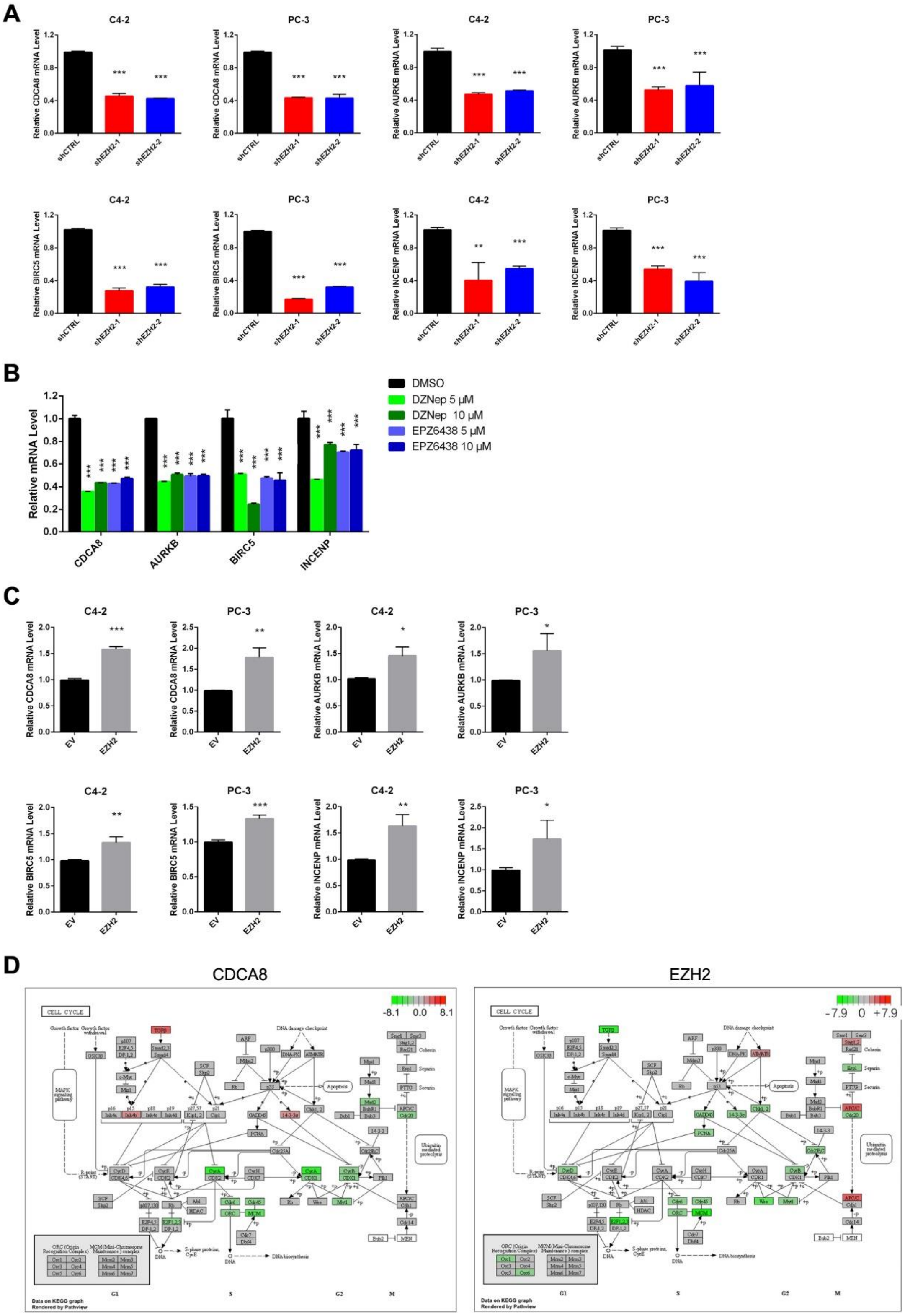
*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ is based on the student's t-test unless otherwise stated. Values are mean ± SD of three independent experiments.



Supplementary Figure 3. CPC members other than CDCA8 are positively correlated with EZH2 in PCa

(A) Box plot showing the mRNA level of EZH2 in normal (n=52) and PCa (n=497) specimens using data from TCGA.

(B) Scatter plots showing the relationship between AURKB/BIRC5/INCENP and EZH2 expressions using data from TCGA, with Spearman correlation coefficient (R) and P values as indicated. TPM, transcript per million.



Supplementary Figure 4. EZH2 regulates transcription of CDCA8 and other CPC members

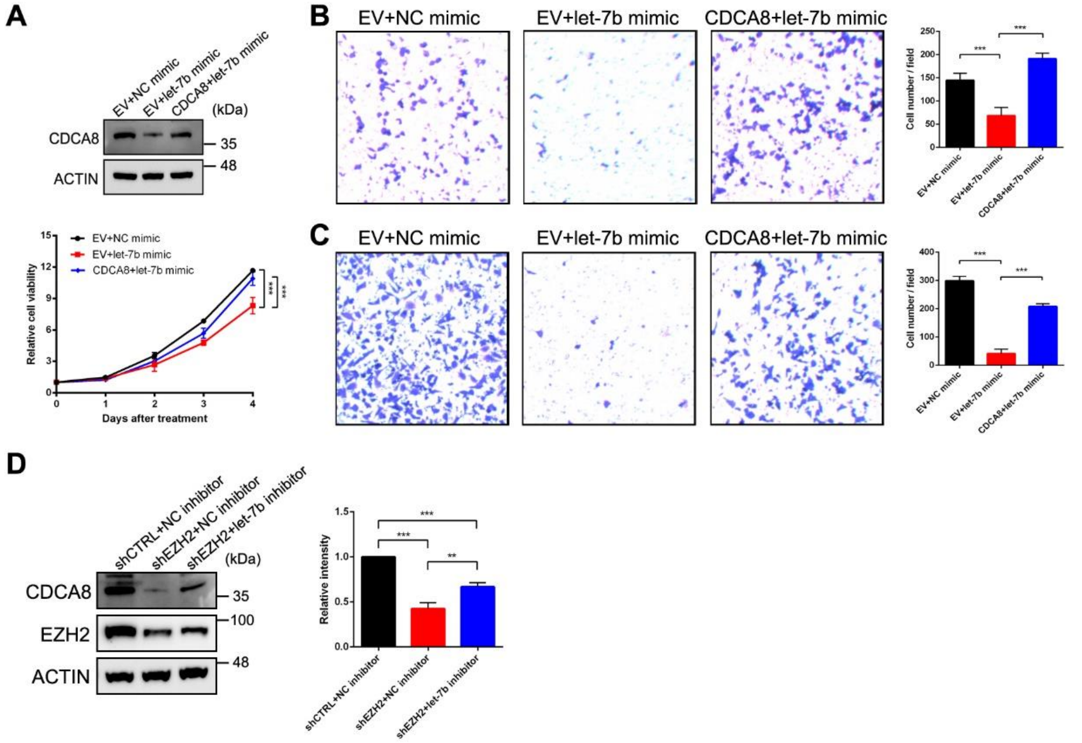
(A) RT-qPCR analysis of the mRNA levels of CDCA8 and other CPC members in PCa cell lines upon EZH2 knockdown.

(B) RT-qPCR analysis of the mRNA levels of CDCA8 and other CPC members in C4-2 cells after treatment of EZH2 inhibitors as indicated.

(C) RT-qPCR analysis of the mRNA levels of CDCA8 and other CPC members in PCa cell lines upon EZH2 overexpression.

(D) KEGG graphs showing the dysregulated genes in cell cycle pathway upon CDCA8 knockdown or EZH2 knockdown in C4-2 cells.

*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ is based on the student's t-test unless otherwise stated. Values are mean ± SD of three independent experiments.



Supplementary Figure 5. Repression of let-7b is partially responsible for the EZH2-mediated CDCA8 upregulation in PCa

(A) Cell viability assay showing that overexpression of CDCA8 could rescue the decreased proliferation rate in C4-2 cells treated with let-7b mimics. The protein level of CDCA8 in each group was detected by western blot. EV, empty vector.

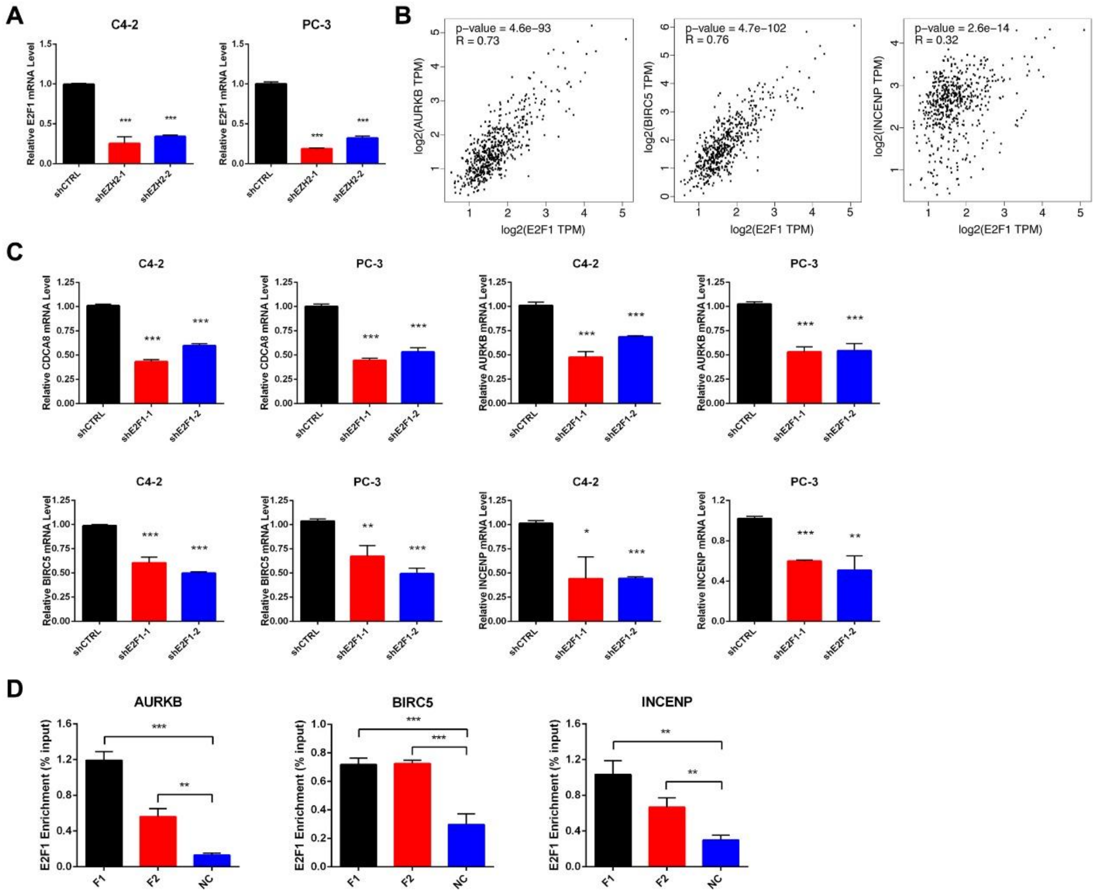
(B) Boyden chamber migration assay showing that overexpression of CDCA8 could rescue the decreased migration rate in C4-2 cells treated with let-7b mimics. Graph showing the number of migrated cells in the lower surface of filter at 24 h. Data represent Mean ± SD from n=5 random fields per filter.

(C) Boyden chamber invasion assay showing that overexpression of CDCA8 could rescue the decreased invasion rate in C4-2 cells treated with let-7b mimics. Graph showing the number of migrated cells passing through Matrigel at 24 h. Data represent Mean ± SD from n=5 random fields per filter.

(D) Rescue assay showing that treatment of let-7b inhibitor could not fully restore the downregulation of CDCA8 in EZH2-deficient PC-3 cells, as measured by western blot. Graph represents the relative CDCA8 protein level in each group.

*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ is based on the student's t-test unless otherwise

stated. Values are mean ± SD of three independent experiments.



Supplementary Figure 6. E2F1 activates transcription of CPC members other than CDCA8 in PCa

(A) RT-qPCR analysis of the mRNA level of E2F1 in PCa cell lines upon EZH2 knockdown.

(B) Scatter plot showing the relationship between AURKB/BIRC5/INCENP and E2F1 expressions using data from TCGA, with Spearman correlation coefficient (R) and P value as indicated.

(C) RT-qPCR analysis of the mRNA levels of all CPC members in PCa cell lines upon E2F1 knockdown.

(D) ChIP-qPCR assay to monitor the enrichment of E2F1 at the promoter region of AURKB/BIRC5/INCENP in C4-2 cells. Two pairs of primers (F1 and F2) were used to amplify fragments inside AURKB/BIRC5/INCENP promoter region while another pair of primers (NC) targeting nearby region was used as negative control.

Supplementary Table 1. PCa TMA IHC data revealed correlation between expressions of CDCA8 and EZH2

Pearson r Correlation	
CDCA8 vs. EZH2	
Pearson r	
r	0.8234
95% confidence interval	0.7569 to 0.8730
R squared	0.6779
P value	
P (two-tailed)	< 0.0001
P value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	124

Supplementary Table 4. Antibodies used in this study

Target	Source	Application (Dilution)
CDCA8	Abcam, Cat#ab74473	IHC (1:50), WB (1:1000)
AURKB	Sigma, Cat#MABE627	WB (1:1000)
BIRC5	Cell signaling, Cat#2808	WB (1:1000)
INCENP	Abcam, Cat#ab12183	WB (1:1000)
EZH2	Cell signaling, Cat#5246	IHC (1:50), WB (1:1000), ChIP-qPCR (1:100)
E2F1	Cell signaling, Cat#3742	WB (1:1000), ChIP-qPCR (1:100)
ACTIN	Cell signaling, Cat#3700	WB (1:1000)
GAPDH	Cell signaling, Cat#5174	WB (1:1000)
Histone H3	Cell signaling, Cat#4499	WB (1:1000)
H3K27me3	Cell signaling, Cat#9733	WB (1:1000), ChIP-qPCR (1:100)
LC3A/B	Cell signaling, Cat#12741	WB (1:1000)
p62/SQSTM1	Cell signaling, Cat#88588	WB (1:1000)
PARP	Cell signaling, Cat# 9532	WB (1:1000)
Caspase-3	Cell signaling, Cat#9662	WB (1:1000)

Supplementary Table 5. Primers used in this study

Name	Sequence (5´to 3´)	Application
GAPDH	F: GGAGCGAGATCCCTCCAAAAT	RT-qPCR
	R: GGCTGTTGTCATACTTCTCATGG	
CDCA8	F: GAAGGGCAGTAGTCGGGTG	RT-qPCR
	R: TCACGGTCGAAGTCTTTCAGA	
AURKB	F: CAGTGGGACACCCGACATC	RT-qPCR
	R: GTACACGTTTCCAAACTTGCC	
BIRC5	F: AGGACCACCGCATCTCTACAT	RT-qPCR
	R: AAGTCTGGCTCGTTCTCAGTG	
INCENP	F: AAGCTCATGGAGTTTCTCTGC	RT-qPCR
	R: CGTCTCTTCTTCCGTCGGTTC	
E2F1	F: ACGCTATGAGACCTCACTGAA	RT-qPCR
	R: TCCTGGGTCAACCCCTCAAG	
let-7b promoter -F1	F: CCCCAGGAAGGTGGTAGCC	ChIP-qPCR
	R: GGACAGAGTGTAGCATGAGGATGA	
let-7b promoter -F2	F: GTGACAGCGTCGCAAAATG	ChIP-qPCR
	R: GCAGGAAACCACCAACCAG	
let-7b promoter -NC	F: ACGGGGCAGCCACCAA	ChIP-qPCR
	R: TGAGGGAGGACGGAAGGAC	
CDCA8 promoter -F1	F: AGCAGAATCCTACAGCCGACC	ChIP-qPCR
	R: GCGAACTATACAAACTACTACTCCCG	
CDCA8 promoter -F1	F: CGCATTGGGCGGAAGA	ChIP-qPCR
	R: CCCGAGACAAGGGCTGAG	
CDCA8 promoter -NC	F: CAAGACCCGTGTGCGAGC	ChIP-qPCR
	R: TGTTGATTACATCAGGGAGCGT	
AURKB promoter -F1	F: TCAGAGGGTCCGTTGGGC	ChIP-qPCR
	R: CCGTGAGAAGCAGAGAAAAAGAG	
AURKB promoter	F: CTAAACTGGAAGCCAAGCGTG	ChIP-qPCR

-F2	R: TCCAAGGCACTGCTACTCTCC	
AURKB promoter -NC	F: TGCCTCCCAGGTTCAAGC	ChIP-qPCR
	R: GCCAACACAGTGAAACCCC	
BIRC5 promoter -F1	F: ACTCAAGTGATGCTCCTGCCT	ChIP-qPCR
	R: CGACTGCTTTCAAAGAACGC	
BIRC5 promoter -F2	F: AGGACTTACTGTTGGTGGGACG	ChIP-qPCR
	R: TGACTGCACGACCTGGGTTT	
BIRC5 promoter -NC	F: TGGCAGCCCCACACAGA	ChIP-qPCR
	R: AAGGAAAGCATGAGCACGACT	
INCENP promoter -F1	F: TTGGTTGGGAACTGTGGACTT	ChIP-qPCR
	R: GGGGTCTGGGGCTTTCTC	
INCENP promoter -F2	F: GTGTTTTCTCTTGCTCAATGCTT	ChIP-qPCR
	R: CCTCCCACAACCCTCTCCC	
INCENP promoter -NC	F: CCTCATCTGGCGATTGTGTC	ChIP-qPCR
	R: TGTAAAGCAAGTAGGTTGGGTCA	
E2F1 promoter -F1	F: CGCCGTTGTTCCCGTCA	ChIP-qPCR
	R: CGCCGCTGCCTGCAAAGT	
E2F1 promoter -F2	F: GCCTATGTTCCGGTGTCCC	ChIP-qPCR
	R: GCGGCGGTTCCTATTGG	
E2F1 promoter -NC	F: AGACGGGGAGCATCACAGG	ChIP-qPCR
	R: CCCTCATCCCTCACCACAGA	