Supplementary Materials and Methods

Supplementary Figures S1-S3

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KD Sørensen *et al*:

"Prognostic significance of aberrantly silenced *ANPEP* expression in prostate cancer"

Supplementary Materials and Methods

RNA extraction, cDNA synthesis and quantitative RT-PCR.

Total RNA from cultured cells was isolated using the RNeasy MinElute Cleanup Kit (Qiagen). RNA samples with RIN>8.5 (determined on a 2100 Agilent Bioanalyzer) were used for further analysis. cDNA synthesis was performed with SuperScript II Reverse Transcriptase (Invitrogen) using oligo(dT) priming. ANPEP expression was measured using TaqMan Gene Expression Assay Hs00952642_m1 and TaqMan Universal PCR Master Mix on a real-time ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Ubiquitin C (UBC) was used for normalization (Abildgaard *et al,* 2012). All real-time PCRs were run in triplicates.

Bisulfite sequencing.

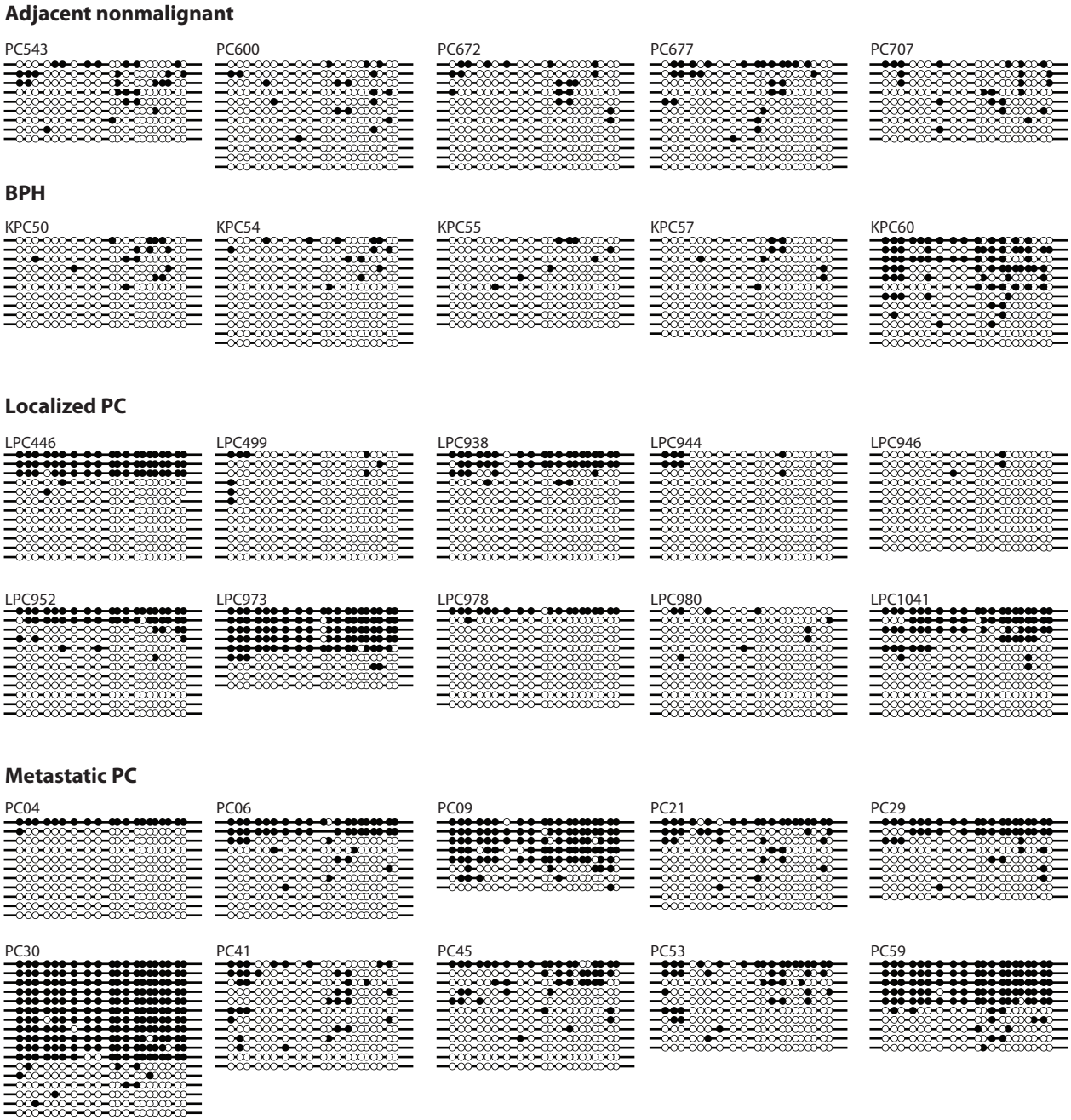
Genomic DNA from cell lines and carefully selected 20-µm sections of fresh frozen Tissue-tek embedded BPH, PC and AN prostate tissue samples was isolated using the PUREGENE DNA Purification Kit (Gentra Systems) with proteinase K treatment (100 units, 30 min, 55C). The EpiTect Bisulfite Kit (Qiagen) was used for conversion of genomic DNA. A 241-nt region of the converted *ANPEP* promoter was amplified with TEMPase Hot Start DNA Polymerase (Ampliqon) using primers 5'-GGTTTGGGATGTATTAGGTTTT-3' and 5'-TCCCAAATACCAAAAAAAT TAAATTA-3'. Amplicons were purified from agarose gels and subcloned into the pCR4-TOPO vector (Invitrogen). Several individual clones were sequenced for all samples.

Quantitative methylation-specific PCR (MethyLight).

Using gDNA Eliminator columns from the miReasy FFPE Kit (Qiagen), genomic DNA was extracted from 1.5-mm punch biopsies from a subset of the FFPE tissue blocks used to generate the

RP cohort TMA. All biopsies were taken in close proximity to the cores represented on the TMA. DNA was bisulfite converted using the EZ-96 Gold kit (Zymo Research). A methylation-specific MethyLight assay was designed for the *ANPEP* promoter: primers 5‘-TTTTTGTCGTCGTAG TTCG-3’ and 5’-GAATACACAAAACTCCCTACG-3’; probe FAM-5’-GGGAGGGGTTTAGAG TTTCGTT-3’-BHQ1. For normalization and test of input DNA quality and quantity, a CpG-free region of *MYOD1* was analyzed in parallel using primers 5’-CCAACTCCAAATCCCCTCTCTAT-3’ plus 5’-TGGTTTTTTTAGGGAGTAAGTTTGTT-3’ and probe FAM-5’-TCCCTTCCTATTCC TAAATCCAACCTAAATACCTCC-3’-BHQ1. Primers and probes were purchased from Eurofins MWG Operon (Ebersberg, Germany). MethyLight reactions were run in triplicates in 384-well plates on an ABI 7900 Real-time PCR System using TaqMan Universal PCR Master Mix without AmpErase UNG (Applied Biosystems). MethyLight analyses included multiple water blanks as well as bisulfite-converted and non-converted methylated and unmethylated control DNA (CpGenome Universal Methylated and Unmethylated DNA, respectively; Millipore). A serial dilution of methylated control DNA was used for standard curve construction. In total, 248 samples (16 AN, 15 BPH, 183 RP/LPC, 24 MPC and 10 CRPC) with Ct values below 35 for *MYOD1* were included in the final analysis. The relative *ANPEP* promoter methylation level was determined as the *ANPEP/MYOD1* ratio.

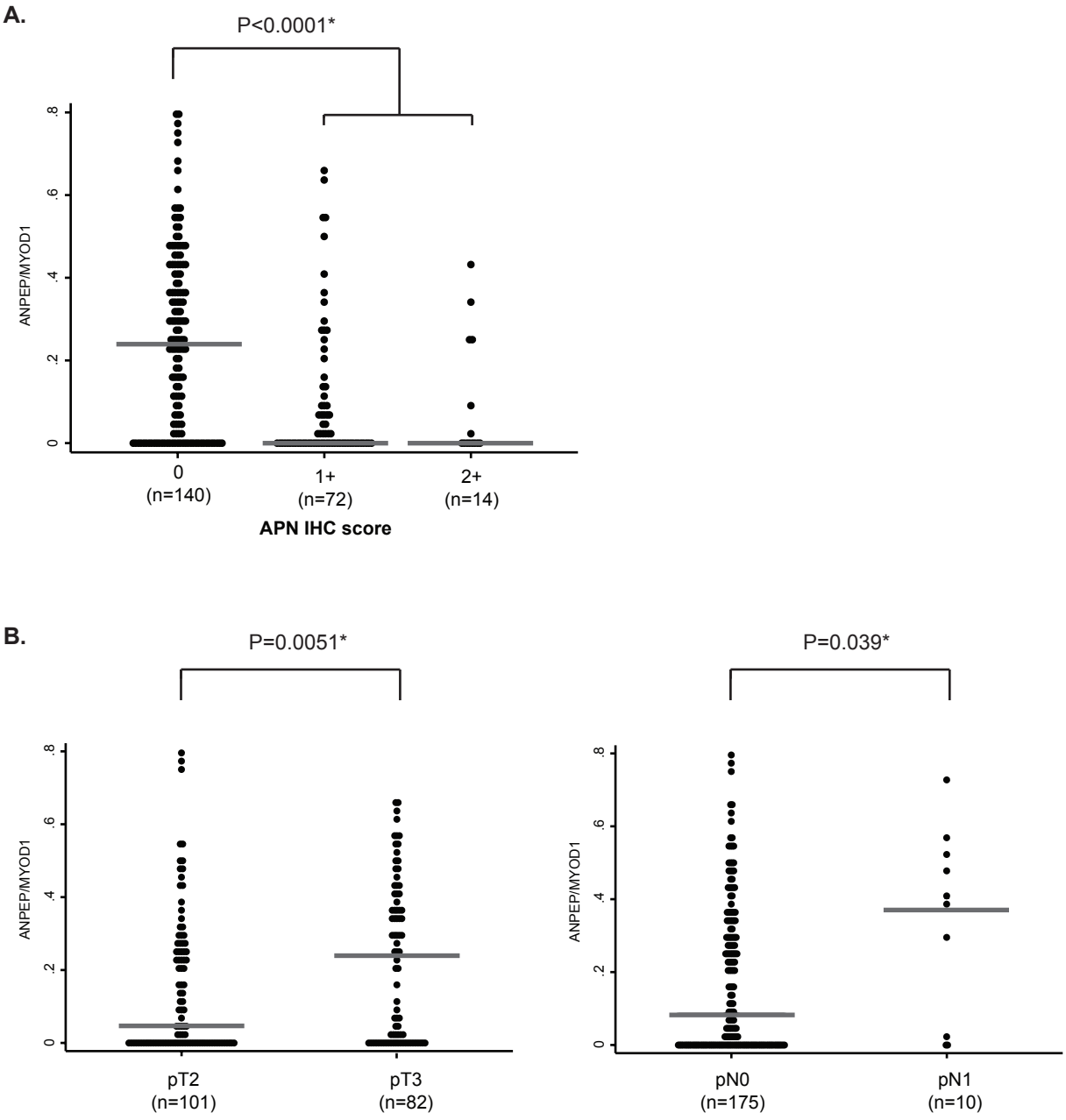
Supplementary Figure S1



Supplementary Figure S1: *ANPEP* methylation patterns in adjacent nonmalignant (n=5), BPH (n=5), and prostate cancer tissue samples from patients with localized (n=10) or metastatic PC (n=10), as determined by bisulfite sequencing. All tissue samples were macrodissected. Open and closed circles, unmethylated and methylated CpGs, respectively. Each row represents one clone.

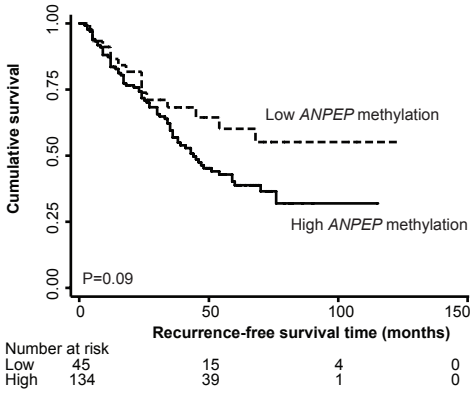
The bisulfite sequencing results suggested that there was considerable variation in the ratio of cancer cell DNA (hypermethylated clones) to nonmalignant cell DNA (unmethylated clones) in the PC samples (e.g. compare PC04 and PC09). This is consistent with variable cancer cell content after macrodissection. Hence, it was not meaningful to simply compare average methylation levels (number of methylated CpG sites/total number of CpG sites analyzed) between these samples. Instead, to evaluate the presence of aberrant hypermethylation in a given sample, we used the maximum methylation level detected for each sample, i.e. the percentage of methylated CpG sites in the most heavily methylated clone/sequence (see Figure 2B).

Supplementary Figure S2



Supplementary Figure S2: *A*, Dot plot showing significantly higher *ANPEP/MYOD1* promoter methylation (as determined by MethyLight analysis) in APN negative samples (IHC score = 0) than in APN positive samples (IHC score 1+ and 2+). *B*, *ANPEP* promoter methylation is significantly higher in pT3 compared to pT2 tumors (left) and in pN1 compared to pN0 tumor (right). The number of patients in each group are given in brackets. Grey horizontal bars indicate median *ANPEP/MYOD1* methylation levels in each group. P values for Mann-Whitney U tests are given at the top. Significant P-values are marked by an asterix.

Supplementary Figure S3



Supplementary Figure S3. Kaplan-Meier plots of RFS for the RP cohort based on ANPEP/MYOD1 methylation levels (lower quartile vs. all others). P value for two-sided log-rank test is given.

Supplementary Table S1: *Overview of patient samples on RP and CT cohort TMAs*

TMA / Specimen type	**Total number of cores analyzed**	**Number of cores with APN score determined** [a]
RP cohort TMA	386	313
PC (RP)	*267*	*236*
MPC	*29*	*26*
CRPC	*12*	*12*
LNM	*18*	*13*
AN	*30*	*7*
BPH	*30*	*19*
CT cohort TMA	111	95
PC (TURP)	*111*	*95*

[a] APN immunohistochemistry scores could not be determined for all patients due to absence of epithelial cells in the cores on the TMAs or, in a few cases, lost cores.

Supplementary Table S2:
Clinicopathological characteristics of PC patient cohorts, including patient subsets with/without APN immunohistochemistry data.

	Radical prostatectomy cohort (n=267)	**Patients with APN score (n=236)**	**Patients without APN score (n=31) [a]**	**Conservative treatment cohort (n=111)**	**Patients with APN score (n=95)**	**Patients without APN score (n=16) [a]**
Median age, years (range)	62 (46-72)	62 (46-72)	61 (53-71)	75 (55-95)	76 (55-95)	74 (61-83)
Gleason score, n (%)						
4-6	111 (41.6)	88 (37.3)	23 (74.2)	37 (33.3)	34 (35.8)	3 (18.8)
7	128 (47.9)	122 (51.7)	6 (19.4)	20 (18.0)	16 (16.8)	4 (25.0)
8-10	28 (10.5)	26 (11.0)	2 (6.5)	53 (47.7)	44 (46.3)	9 (56.3)
Unknown	-	-	-	1 (0.5)	1 (1.1)	0 (0)
T stage [b], n (%)						
T1	-	-	-	91 (82.0)	77 (81.1)	14 (87.5)
T2	159 (59.6)	133 (56.4)	26 (83.9)	20 (18.0)	18 (18.9)	2 (12.5)
T3-4	108 (40.5)	103 (43.6)	5 (16.1)	0 (0)	0 (0)	0 (0)
PSA at diagnosis, n (%)						
< 10 ng/mL	73 (27.3)	63 (26.7)	10 (32.3)	-	-	-
≥ 10 ng/mL	193 (72.3)	172 (72.9)	21 (67.7)	-	-	-
Unknown	1 (0.4)	1 (0.4)	0 (0)	111 (100)	95 (100)	16 (100)
Nodal status, n (%)						
pN0	249 (93.3)	222 (94.1)	27 (87.1)	-	-	-
pN1	7 (2.6)	7 (3.0)	0 (0)	-	-	-
Unknown	11 (4.1)	7 (3.0)	4 (12.9)	111 (100)	95 (100)	16 (100)
Metastasis status [c], n (%)						
M0	267 (100)	236 (100)	31 (100)	111 (100)	95 (100)	16 (100)
M1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Surgical margin status, n (%)						
Negative	185 (69.3)	157 (66.5)	28 (90.3)	*NA*	*NA*	*NA*
Positive	78 (29.2)	75 (31.8)	3 (9.7)			
Unknown	4 (1.5)	4 (1.7)	0 (0)			
Early endocrine treatment, n (%)						
No	267 (100)	236 (100)	31 (100)	68 (61.3)	58 (61.1)	10 (62.5)
Yes	0 (0)	0 (0)	0 (0)	43 (38.7)	37 (38.9)	6 (37.5)
APN IHC, n (%)						
Score 0	130 (48.7)	130 (55.1)	0 (0)	69 (62.2)	69 (72.6)	-
Score 1+	79 (29.6)	79 (33.5)	0 (0)	22 (19.8)	22 (23.2)	-
Score 2+	27 (10.1)	27 (11.4)	0 (0)	4 (3.6)	4 (4.2)	-
Not determined	31 (11.6)	0 (0)	31 (100)	16 (14.4)	0 (0)	16 (100)
Median follow-up, months (range)	53 (1-131)	53 (1-131)	55 (12-119)	61 (1-180)	61 (1-180)	65 (16-135)

[a] APN immunohistochemistry scores could not be determined for these patients due to absence of epithelial cells in the cores on the TMAs or, in a few cases, lost cores.
[b] Pathological for RP cohort, clinical for CT cohort
[c] M0 includes patients without suspicion of metastases at bone scan or x-ray examination as well as patients clinically regarded as having organ-confined disease without objective verification. M1 includes patients with metastases verified by bone scan or x-ray examination as well as patient with manifest clinical symptoms of metastases but without objective verification.
NA, not applicable

Supplementary Table S3: *Association between APN immunoreactivity and clinicopathological parameters in the RP and CT cohort*

Radical prostatectomy	**APN IHC score**		**p-value**[a]
	Neg (0)	**Pos (1+/2+)**	
Gleason score			
5-6	46	42	0.095 [b]
7	74	48	
8-10	10	16	
T stage [c]			
pT2	66	67	0.065
pT3	64	39	
PSA			
< 10 ng/mL	36	27	0.77
≥ 10 ng/mL	94	78	
Margin status			
Negative	83	74	0.33
Positive	45	30	
VEGF score [d]			
Low (Score=0-2)	74	69	0.22
High (Score=3-5)	53	35	

Conservative treatment	**APN IHC score**		**p-value**[a]
	Neg (0)	**Pos (1+/2+)**	
Gleason score			
4-6	20	14	0.087 [b]
7	13	3	
8-10	35	9	
T stage [c]			
cT1	54	23	0.38
cT2	15	3	
Endocrine treatment			
No	42	16	1.0
Yes	27	10	
Microvessel density[e]			
Low	41	19	0.24
High	28	7	
VEGF score [e]			
Low (Score=0-2)	89	27	0.018
High (Score=3)	48	4	

[a] Determined by 2-sided Fisher's exact test, unless stated otherwise.

[b] Pearson's Chi-square test, 2-sided.

[c] Pathological for RP cohort, clinical for CT cohort.

[d] Data from (Vergis *et al*, 2008). VEGF scoring was based on the percentage of VEGF positive PC cells present in 0.6-mm cores on the RP cohort TMA. MVD was not determined.

[e] Data from (Borre *et al*, 1998). MVD density and VEGF score were determined in PC specimens (TURP) using one representative section. The most intensively stained "hotspot" was scored for each patient. VEGF scoring was based on the intensity of staining in PC cells.