Supporting Materials:

Aytes et al.

***ETV4* promotes metastasis in response to combined activation of PI3-kinase and Ras signaling in a mouse model of advanced prostate cancer**

Summary of Supporting Figures

Supporting Tables

Supporting Datasets (*uploaded separately*)

Supporting Figures and Legends

Figure S1: Supplementary histopathological analyses of mouse prostate cancer phenotypes

(A-F) Representative H&E images of sections from the ventral and dorsolateral prostate of mice of the indicated genotypes.

(G-V) Representative H&E images of sections from the anterior prostate of mice of the indicated genotypes. Images were captured on a whole slide scanner. The upper rows illustrate the low magnification (1-2X) configuration and the lower rows show a representative high magnification (20X) region. With the exception of the mouse indicated (corn oil), all other mice were induced with tamoxifen at 2 months of age. Following the classification system of Park 2002, pathological assessment was as follows: (G, H, K, L) within normal limits; (I, J, M, N) PIN 3 pattern with ducts filled with atypical cells. Panels O-V show the range of histopathology of *NPK* prostate tumors: (O, S): atypical epithelium (PIN III/IV) (P, T); more severe PIN (PIN IV) with invasion and areas of necrosis (Q, U); combination of atypical glandular and squamous epithelium; and (R, V) partially necrotic tumor mass, with a tumor composed of spindle cells.

Figure S1

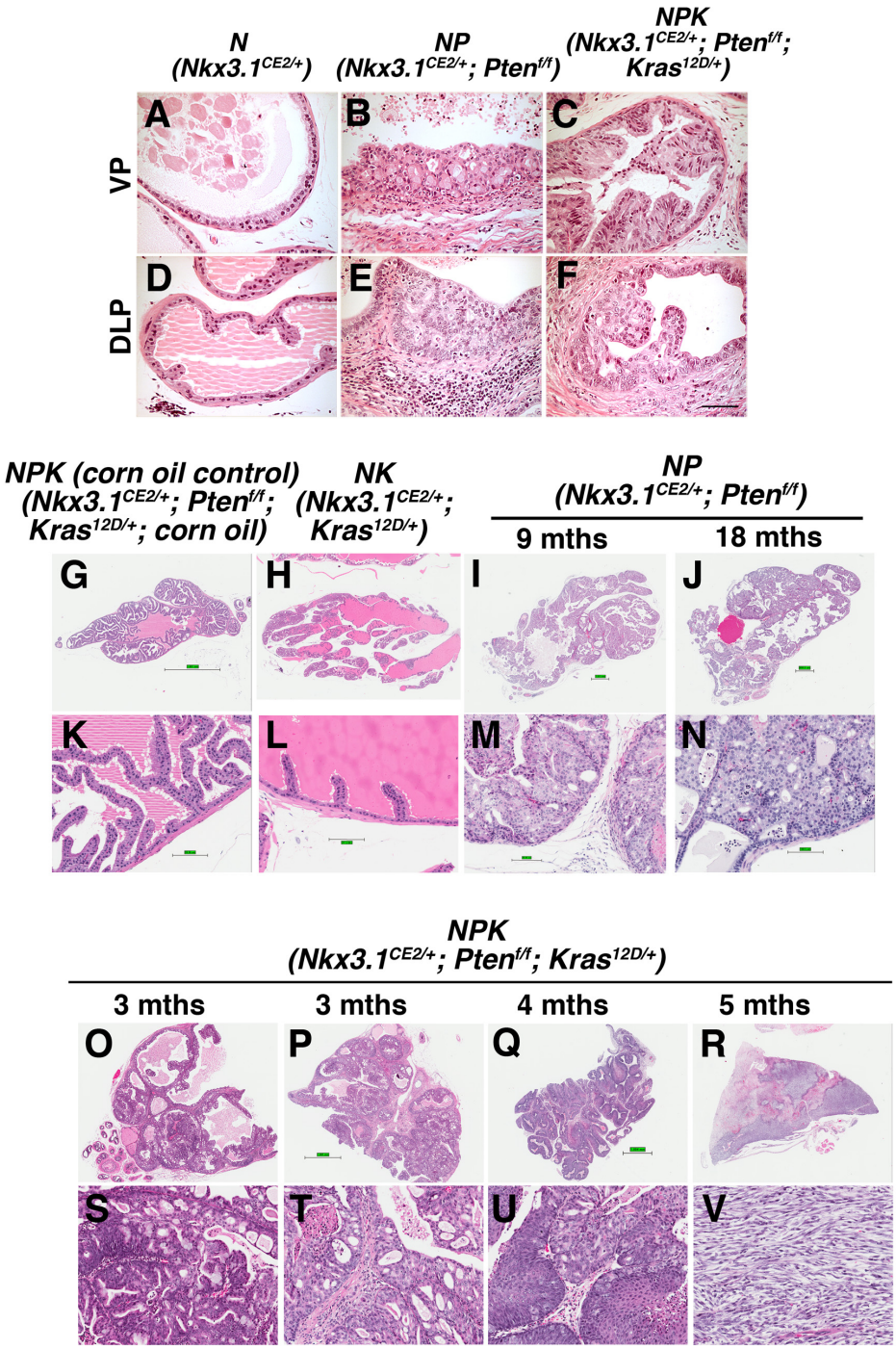
N
(Nkx3.1CE2/+)
NP
(Nkx3.1CE2/+; Ptenf/f)
NPK
(Nkx3.1CE2/+; Ptenf/f;
Kras12D/+)
VP
DLP
A
B
C
D
E
F
NPK (corn oil control)
(Nkx3.1CE2/+; Ptenf/f;
Kras12D/+; corn oil)
NK
(Nkx3.1CE2/+;
Kras12D/+)
NP
(Nkx3.1CE2/+; Ptenf/f)
9 mths
18 mths
G
H
I
J
K
L
M
N
NPK
(Nkx3.1CE2/+; Ptenf/f; Kras12D/+)
3 mths
3 mths
4 mths
5 mths
O
P
Q
R
S
T
U
V

Figure S2. Supplementary analyses of expression profiling of *NPK* vs *NP* prostate tumors

(A) Pathway analyses. Heat map of differentially-expressed genes between the *NPK* versus *NP* mouse prostate tumors (t-test *p*-value 0.001) showing genes from selected pathways that are significantly enriched (*p* value of 0.001 to $p = 0.004$).

(B) Real-time PCR analyses of selected differentially expressed genes between the *NPK* versus *NP* mouse prostate tumors with expression levels of mRNA represented as relative fold change. *p*-values compare the experimental to the control (wild-type prostate).

(C) Pathway analyses. Expression signatures from the *NPK* versus *NP* mouse prostate tumors were mapped to their human orthologs and annotated to biological processes using GSEA analyses. The total height of the curve indicates the extent of enrichment, with the normalized enrichment score (NES) and *p*-values indicated.

Figure S2

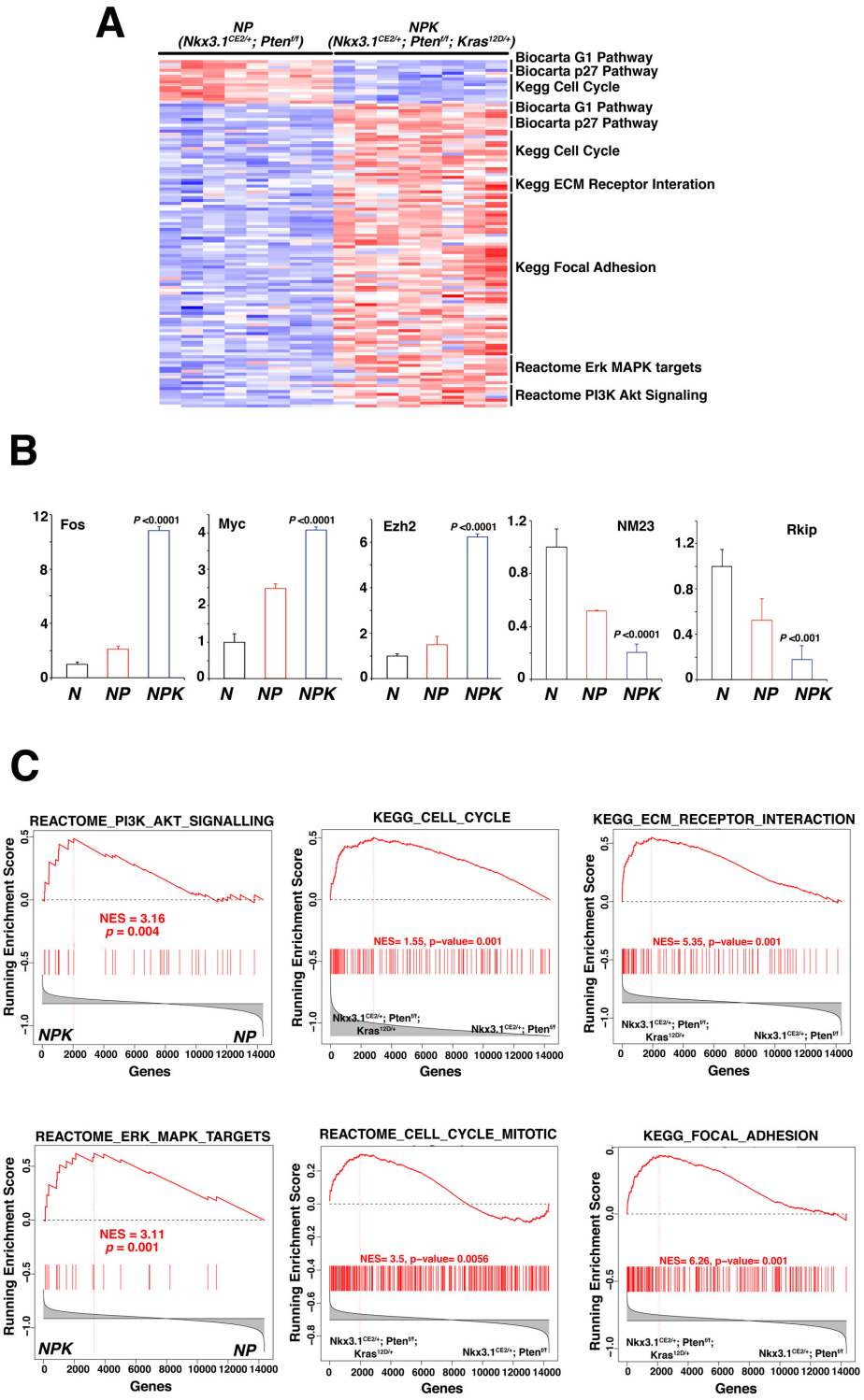
A
NP
(Nkx3.1CE2/+; Ptenf/f)
NPK
(Nkx3.1CE2/+; Ptenf/f; Kras12D/+)
Biocarta G1 Pathway
Biocarta p27 Pathway
Kegg Cell Cycle
Biocarta G1 Pathway
Biocarta p27 Pathway
Kegg Cell Cycle
Kegg ECM Receptor Interation
Kegg Focal Adhesion
Reactome Erk MAPK targets
Reactome PI3K Akt Signaling
B
Fos
P <0.0001
Myc
P <0.0001
Ezh2
P <0.0001
NM23
P <0.0001
Rkip
P <0.001
N
NP
NPK
C
REACTOME_PI3K_AKT_SIGNALLING
NES = 3.16
p = 0.004
KEGG_CELL_CYCLE
NES= 1.55, p-value= 0.001
KEGG_ECM_RECEPTOR_INTERACTION
NES= 5.35, p-value= 0.001
REACTOME_ERK_MAPK_TARGETS
NES = 3.11
p = 0.001
REACTOME_CELL_CYCLE_MITOTIC
NES= 3.5, p-value= 0.0056
KEGG_FOCAL_ADHESION
NES= 6.26, p-value= 0.001
Running Enrichment Score
Genes
NPK
NP

Figure S3: Analyses of the EMT phenotype in *NPK* mice

(A-C) Immunofluorescence images of E-Cadherin showing reduced staining in the *NPK* tumors. Scale bars represent 100 μm. (D, E) Real-time PCR analyses of EMT markers with expression levels of mRNA represented as relative fold change. Panel D compares the *NPK* to the *NP* and *N* prostate/prostate tumors; *p*-values compare *NPK* tumors to the control (*N,* wild-type prostate). Panel E compares the 3-month to the 2 or 1-month *NPK* prostate tumors; *p*-values compare the 3-month *NPK* tumors to the 1-month *NPK* tumors.

Figure S3

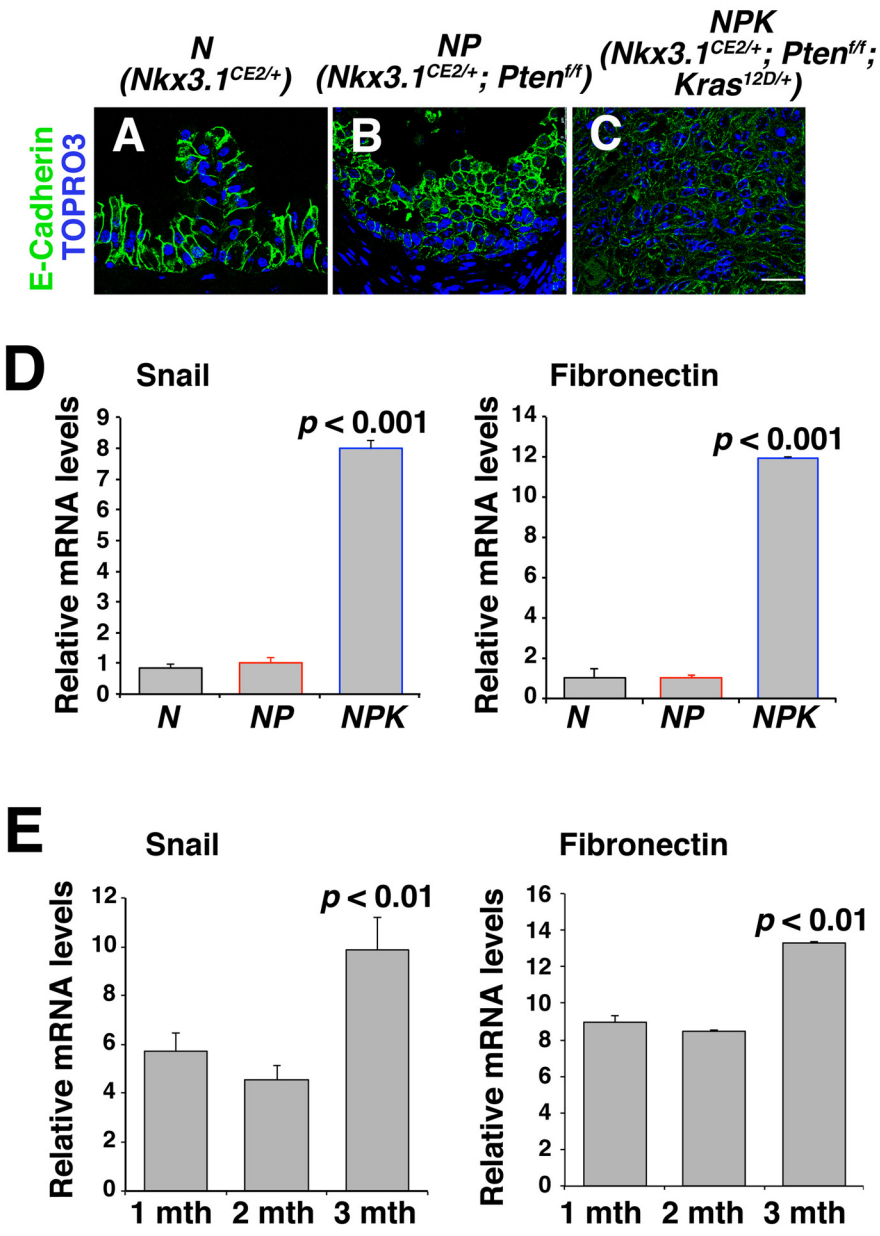
N
(Nkx3.1CE2/+)
NP
(Nkx3.1CE2/+; Ptenf/f)
NPK
(Nkx3.1CE2/+; Ptenf/f; Kras12D/+)
A
B
C
E-Cadherin
TOPRO3
D
Snail
p < 0.001
Relative mRNA levels
N
NP
NPK
Fibronectin
p < 0.001
Relative mRNA levels
N
NP
NPK
E
Snail
p < 0.01
Relative mRNA levels
1 mth
2 mth
3 mth
Fibronectin
p < 0.01
Relative mRNA levels
1 mth
2 mth
3 mth

Figure S4: Supplementary phenotypic analyses of metastases in the *NPK* mice

(A-K) Additional analyses of metastasis as shown in main Figure 4.

(A-C) Whole slide scans of lymph nodes of mice of the indicated genotype.

(D-K) Analyses of lymph node metastases in *NPK* mice. Representative images from lumbar lymph nodes from mice of the indicated genotype showing H&E staining (E, D) or IHC staining for the relevant markers (F-K). Note that the tumor cells engulf the lymph node and are found throughout the tissue interspersed with the inflammatory cells.

(L-S) Additional analyses of the lineage-tracing study shown in main Figure 6. (L-O) Histological phenotype of *NPK-YFP* prostate tumors from mice induced with tamoxifen for the indicated times. (P-S) Representative confocal images of lymph nodes from *NPK-YFP* mice analyzed at the indicated time-points following tamoxifen induction. Images are shown for YFP (green) co-stained with CK8 (red) and visualized with DAPI (blue).

Figure S4

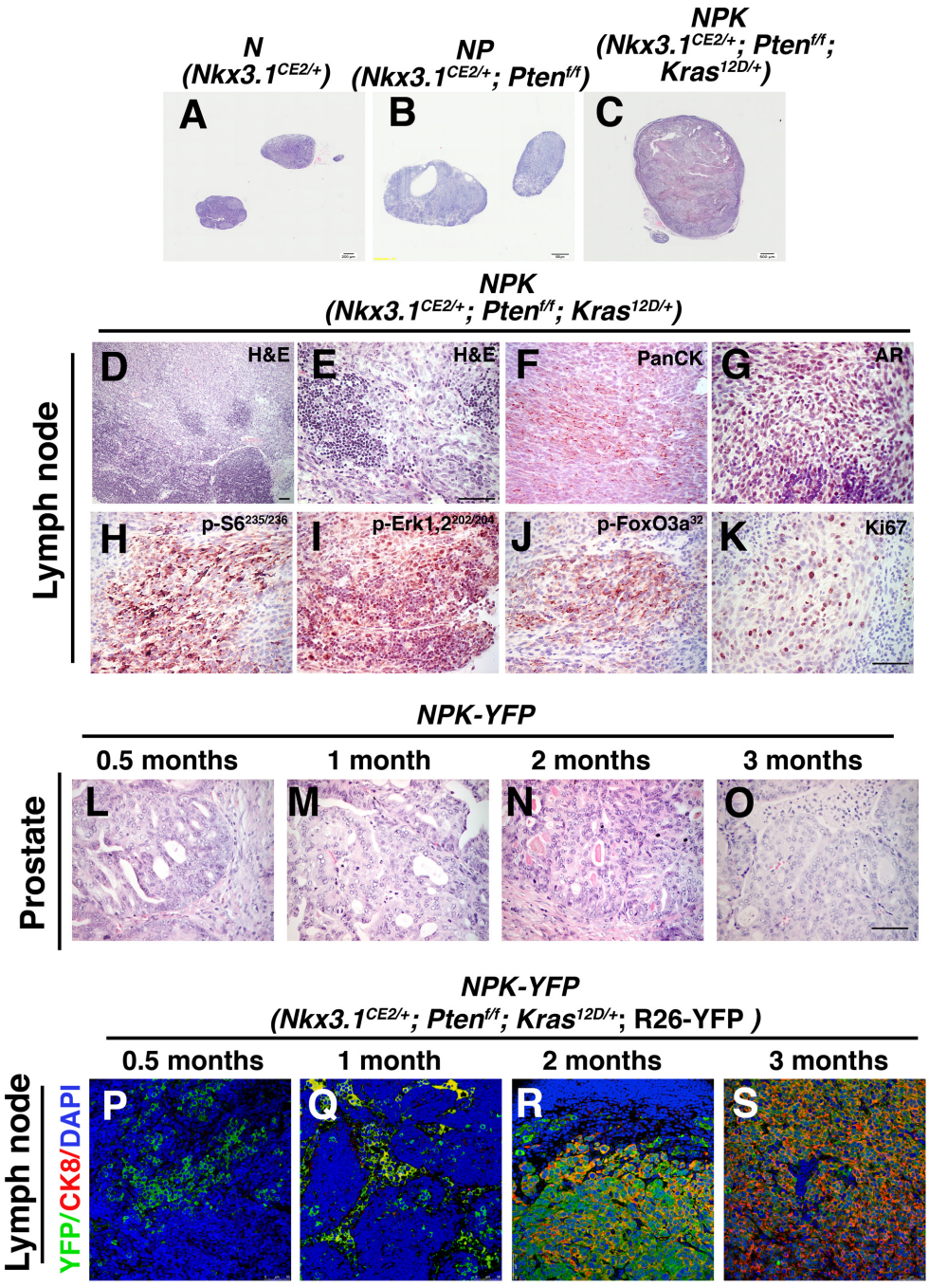
N
(Nkx3.1CE2/+)
NP
(Nkx3.1CE2/+; Ptenf/f)
NPK
(Nkx3.1CE2/+; Ptenf/f; Kras12D/+)
A
B
C
NPK
(Nkx3.1CE2/+; Ptenf/f; Kras12D/+)
Lymph node
D H&E
E H&E
F PanCK
G AR
H p-S6235/236
I p-Erk1,2202/204
J p-FoxO3a32
K Ki67
NPK-YFP
0.5 months
1 month
2 months
3 months
Prostate
L
M
N
O
NPK-YFP
(Nkx3.1CE2/+; Ptenf/f; Kras12D/+; R26-YFP)
0.5 months
1 month
2 months
3 months
Lymph node
YFP/CK8/DAPI
P
Q
R
S

Figure S5: Molecular analyses of *ETS* gene expression and activation in prostate cancer

(A) GSEA analyses showing the lack of enrichment of human *ERG* target genes in reference signatures comparing *NPK* versus *NP* prostate tumors *(left)* as well as in 3-months versus 1-month *NPK* prostate tumors (*right*). Shown are the enrichment scores (NES) and *p*-values, which are not significant in either case. (B) Real-time PCR analyses showing the relative mRNA expression levels for *Erg, Etv1, Etv4,* and *Etv5* in *N, NP* and *NPK* prostate/prostate tumors; *p* values compare the experimental to the control (*N,* normal prostate). Only *Etv4* and *Etv5* are significant. (C) Correlation of the expression levels of the indicated *ETS* gene, *ERG*, *ETV1,* or *ETV5*, with PI3-kinase and Ras signaling in human prostate cancer in the Taylor dataset (Taylor et al, 2010) showing that none are correlated with PI3-kinase and Ras signaling. Shown are both Spearman Rho and Kendall z correlation coefficients. The color key indicates relative expression levels of ETS genes and PI3-k and Ras pathways.

Figure S5

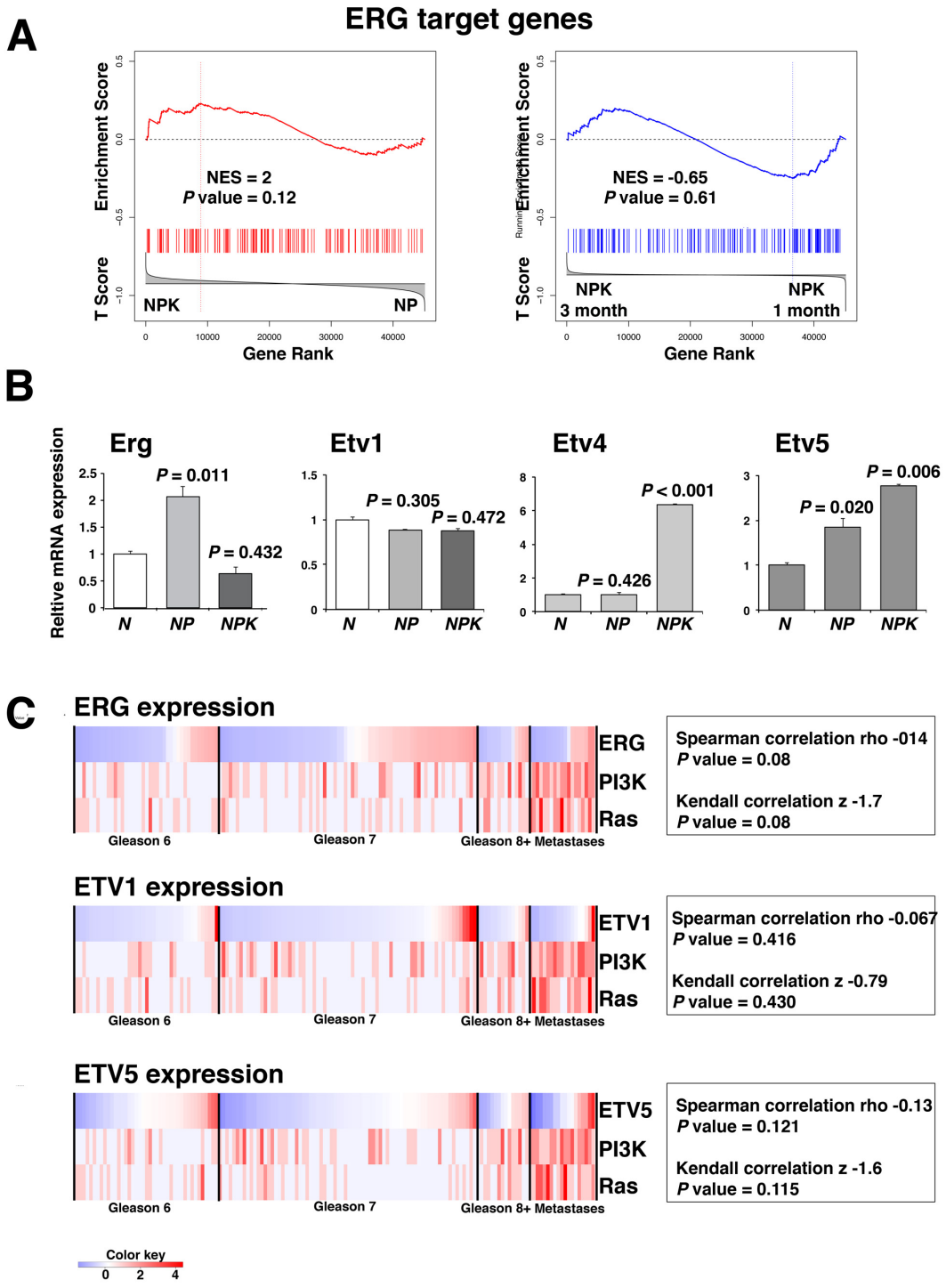
A
ERG target genes
Enrichment Score
T Score
NES = 2
P value = 0.12
NPK
NP
Gene Rank
NES = -0.65
P value = 0.61
NPK
3 month
NPK
1 month
Gene Rank
B
Reltive mRNA expression
Erg
P = 0.011
P = 0.432
N
NP
NPK
Etv1
P = 0.305
P = 0.472
Etv4
P < 0.001
P = 0.426
Etv5
P = 0.006
P = 0.020
C
ERG expression
ERG
PI3K
Ras
Gleason 6
Gleason 7
Gleason 8+ Metastases
Spearman correlation rho -014
P value = 0.08
Kendall correlation z -1.7
P value = 0.08
ETV1 expression
ETV1
Spearman correlation rho -0.067
P value = 0.416
Kendall correlation z -0.79
P value = 0.430
ETV5 expression
ETV5
Spearman correlation rho -0.13
P value = 0.121
Kendall correlation z -1.6
P value = 0.115
Color key
0 2 4

Figure S6: Correlation of *ETV4* target gene activity with *PI3-k* and *Ras* pathway activation in human prostate cancer metastases

Correlation of *ETV4* target gene activity with PI3-kinase and Ras signaling in human prostate cancer metastases from the Taylor dataset (Taylor et al, 2010) showing correlation particularly in bone and spine. The color key indicates relative expression levels of the pathways and activity levels of ETV4.

Figure S6

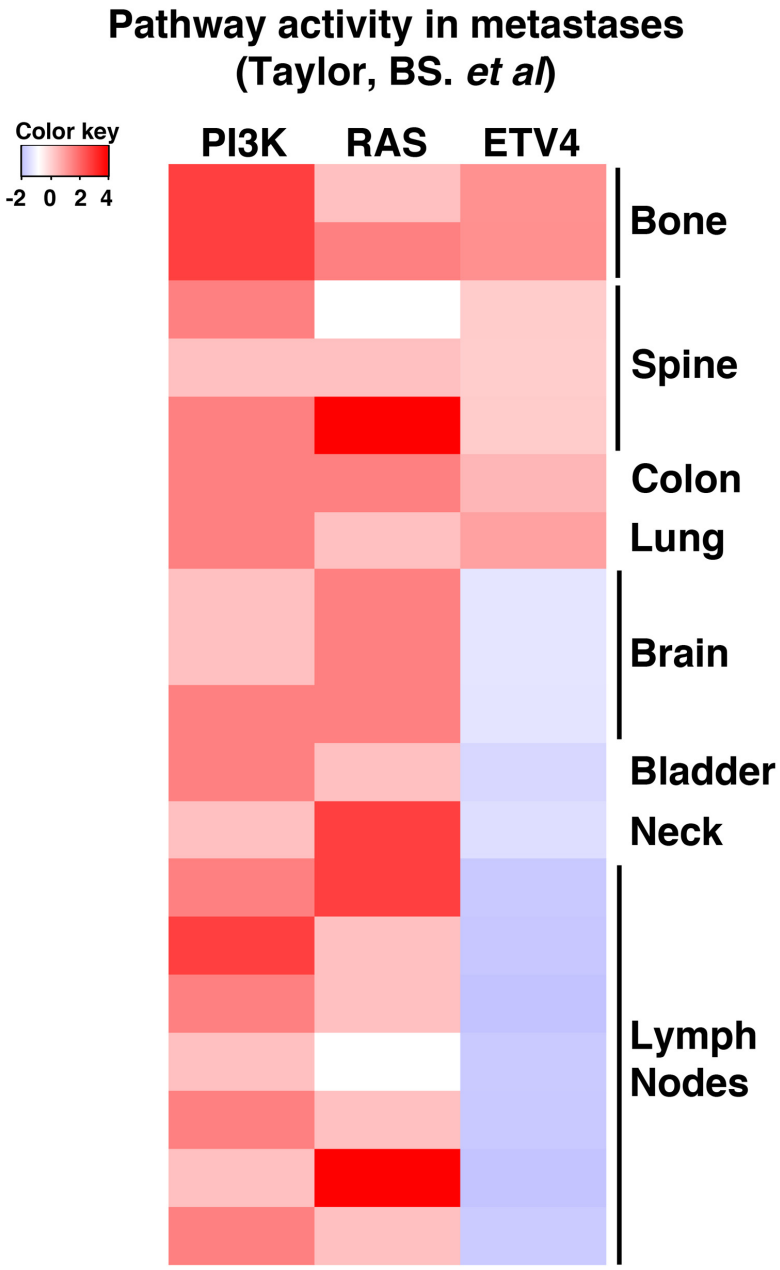
Pathway activity in metastases
(Taylor, BS. et al)
Color key
-2 0 2 4
PI3K
RAS
ETV4
Bone
Spine
Colon
Lung
Brain
Bladder
Neck
Lymph Nodes

Table S1: Summary of phenotypic analyses of mouse prostate tumors

Group	Genotype	N	Age	Phenotype	50% Survival	Weight (gms)	% KI67 cells	Metastases			
								Lymph node	Lung	Liver	DTCs
Control group 1: N (w/ Tamoxifen)	Nkx3.1$^{CreERT2/+}$; Pten$^{+/+}$; Kras$^{+/+}$	12	8	Within normal limits	> 24 months	0.1932 ± 0.20	ND	0/5	0/5	ND	ND
Control group 2: NPK (w/ Corn Oil)	Nkx3.1$^{CreERT2/+}$; Pten$^{flox/flox}$; Kras$^{12D/+}$	12	4	Within normal limits	> 24 months	0.0346 ± 0.001	3%	0/5	0/5	0/5	0/5
NK	Nkx3.1$^{CreERT2/+}$; Pten$^{+/+}$; Kras$^{12D/+}$	6	8	Within normal limits	> 24 months	0.3057 ± 0.24	ND	ND	ND	ND	ND
NP	Nkx3.1$^{CreERT2/+}$; Pten$^{flox/flox}$; Kras$^{+/+}$	18	9-12	PIN III and PIN IV, with microinvasive adenocarcinoma and squamous papilloma	~ 20 months	0.4501± 0.18	14% p<0.0001	0/5	0/10	ND	1/12
		10	18+	PIN III and PIN IV with microinvasive/invasive adenocarcinoma and squamous papilloma, squamound metaplasia.		2.9731 ± 3.9	ND	0/5	0/6[f]	ND	0/6
NPK	Nkx3.1$^{CreERT2/+}$; Pten$^{flox/flox}$; Kras$^{12D/+}$	30	2-5	Microinvasive/invasive adenocarcinoma with adenomatous, squamous, and spindle cell components	101 days p<0.0001	4.748 ± 4.0 p<0.0001	38% p<0.0005	5/5	15/15	4/5	18/20

Legend: **N** is the total number of mice analyzed. **Age** refers to the time of analyses **after induction with tamoxifen** (or treatment with corn oil); mice were induced at 2 months of age. **Phenotype** refers to the pathological description of this histological phenotype according to the classification of Park et al. 2002; specific examples of histological phenotypes are shown in Figure S1. **Survival** refers either to the age at which 50% of the mice succumb to the disease. **Tissue metastases** were scored by analyses of H&E and with two IHC markers (AR and Pan-Cytokeratin). **Disseminated tumor cells** (DTCs) were evaluated from bone marrow using a PCR based assay. All *P* values compare the experimental group (as indicated) to the control group.

Table S2. Quantification of metastatic/disseminated tumor cells in lungs and bone marrow of *NPK* mice

NPK-YFP mice (corn oil control) 4 months after delivery of corn oil											
Mouse	Cellular characteristics					Proliferation			Disseminated tumor cells (qPCR)		
	Total cells counted	YFP expressing cells		CK8 expressing cells		Total YFP+	YFP+ Ki67+	% YFP+Ki67+	Ct	ΔCt[§]	2e-ΔΔCt[#]
		Total YFP+	% YFP+	Total YFP+/CK8+	% YFP+ that are CK8+						
#1	987	0	0	0	0	0	0	0	NV	NV	1
#2	424	0	0	0	0	0	0	0	NV	NV	1
#3	795	0	0	0	0	0	0	0	NV	NV	1
#4	685	0	0	0	0	0	0	0	NV	NV	1
Total	**2891**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**

NP-YFP mice (6 Months after tamoxifen induction)											
Mouse	Cellular characteristics					Proliferation			Disseminated tumor cells qPCR		
	Total cells counted	YFP expressing cells		CK8 expressing cells		Total YFP+	YFP+ Ki67+	% YFP+Ki67+	Ct	ΔCt	2e-ΔΔCt
		Total YFP+	% YFP+	Total CK8+/ YFP+	% YFP+ that are CK8+						
#1	569	0	0	0	0	0	0	0	25.52	-3.24	88.06958
#2	489	0	0	0	0	0	0	0	25.52	-3.24	88.06958
#3	238	0	0	0	0	0	0	0	25.81	-2.95	72.03229
#4	468	0	0	0	0	0	0	0	25.71	-3.05	77.20229
#5	394	0	0	0	0	0	0	0	ND	ND	ND
Total	**2158**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**25.64**	**-3.12**	**81.34343**

NPK-YFP mice (0.5 Months after tamoxifen induction)											
Mouse	Cellular characteristics					Proliferation			Disseminated tumor cells (qPCR)		
	Total cells counted	YFP expressing cells		CK8 expressing cells		Total YFP+	YFP+ Ki67+	% YFP+Ki67+	Ct	ΔCt	2e-ΔΔCt
		Total YFP+	% YFP+	Total CK8+/ YFP+	% YFP+ that are CK8+						
#1	575	0	0	0	0	0	0	0	27.61	-0.05	9.65028
#2	239	0	0	0	0	0	0	0	27.45	-0.21	10.78214
#3	394	0	0	0	0	0	0	0	27.65	-0.01	9.38639
#4	459	0	0	0	0	0	0	0	27.85	0.19	8.17133
#5	578	0	0	0	0	0	0	0	27.59	-0.12	19.51564
Total	**2245**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**27.63**	**-0.04**	**9.49754**

NPK-YFP mice (1 Months after tamoxifen induction)												
Mouse	Cellular characteristics					Proliferation			Disseminated tumor cells (qPCR)			
	Total cells counted	YFP expressing cells		CK8 expressing cells		Total YFP+	YFP+ Ki67+	% YFP+Ki67+	Ct	ΔCt	2e-ΔΔCt	
		Total YFP+	% YFP+	Total CK8+/ YFP+	% YFP+ that are CK8+							
#1	1215	12	0.98777	7	58.3333	7	0	0	26.36	-2.1575	41.58732	
#2	1715	14	0.8163	2	14.2857	2	0	0	26.76	-1.7575	31.51729	
#3	1527	9	0.5894	1	11.1111	1	0	0	27.06	-1.4575	25.6	
#4	958	25	2.6096	6	24.0000	6	0	0	26.92	-1.5975	28.20877	
#5	850	5	0.5882	0	0.0000	5	0	0	ND	ND	ND	
Total	**6265**	**65**	**16**	**1.0375**	**24.6154**	**21**	**0**	**0**	**26.775**	**-1.7425**	**31.72834**	

NPK-YFP mice (2 Months after tamoxifen induction)											
Mouse	Cellular characteristics					Proliferation			Disseminated tumor cells (qPCR)		
	Total cells counted	YFP expressing cells		CK8 expressing cells		Total YFP+	YFP+ Ki67+	% YFP+Ki67+	Ct	ΔCt	2e-ΔΔCt
		Total YFP+	% YFP+	Total CK8+/ YFP+	% YFP+ that are CK8+						
#1	1450	110	7.5862	75	68.1818	110	6	5.4545	25.27	-9.39	67.13090
#2	1398	15	1.0730	8	53.3333	15	2	13.3333	25.46	-9.20	58.84733
#3	1243	19	1.5286	12	63.1579	19	2	10.5263	25.59	-9.07	53.77654
#4	978	27	2.7607	16	59.2593	27	2	7.4074	25.52	-9.14	56.45014
#5	2026	230	11.3524	210	91.3043	230	9	3.9130	25.54	-9.29	67.91
Total	**7095**	**401**	**5.6519**	**321**	**80.0499**	**401**	**21**	**5.2369**	**25.476**	**-9.21**	**60.82298**

NPK-YFP mice (3 Months after tamoxifen induction)											
Mouse	Cellular characteristics					Proliferation			Disseminated tumor cells (qPCR)		
	Total cells counted	YFP expressing cells		CK8 expressing cells		Total YFP+	YFP+ Ki67+	% YFP+Ki67+	Ct	ΔCt	2e-ΔΔCt
		Total YFP+	% YFP+	Total CK8+/YFP+	% YFP+ that are CK8+						
#1	1298	473	36.4407	459	97.0402	694	171	24.6398	24	-4.52	213.86727
#2	625	163	26.0800	154	94.4785	997	158	15.8475	23.96	-4.56	219.8798
#3	2025	728	35.9506	695	95.4670	897	259	28.8740	23.9	-4.62	229.21726
#4	1325	325	24.5283	307	94.4615	162	31	19.1358	23.49	-5.03	304.55772
#5	1527	498	32.6130	454	91.1647	-	-	-	23.51	-5.01	300.36479
Total	**6800**	**2187**	**32.1618**	**2069**	**94.6045**	**2750**	**619**	**22.5091**	**23.772**	**-4.80**	**263.5049**

Legend: For quantification of immunofluorescence, total cell number were determine by counting DAPI-stained nuclei; YFP+ cells were determined by counting GFP-stained cells; CK8+ cells were determined by counting CK8-stained cells.
For quantification of disseminated tumor cells, the deleted *Pten*$^{f/f}$ allele (recombined only in tumor cells) was quantified by qPCR and the fold of enrichment to *Gapdh* calculated using the relative ΔΔCt method.
§ ΔCt refers to the difference between the qPCR cycle data for the *Pten*$^{f/f}$ allele and the *Gapdh* normalization control.
# $2e^{-\Delta\Delta Ct}$ refer to fold of enrichment of the *Pten*$^{floxed/floxed}$ versus *Gapdh.*
NV refers to no value data-point
ND refers to not determined.

Table S3: List of the oligonucleotides used in this study

Purpose and name	**Sequence**	
Genotyping Allele	**Forward**	**Reverse**
Nkx3.1$^{CreERT2/+}$	CAGATGGCGCGGCAACACC	GCGCGGTCTGGCAGTAAAAAC
Pten$^{flox/flox}$	ACTCAAGGCAGGGATGAGC	GTCATCTTCACTTAGCCATTGG
Kras$^{LSL-G12D/+}$	CCTTTACAAGCGCACGCAGACTGTAGA	GTCGACAAGCTCATGCGGGTG
R26R$^{LSL-YFP/YFP}$	Wild type allele -GCGAAGAGTTTGTCCTCAACC	AAAGTCGCTCTGAGTTGTTAT
	Mutant allele - GGAGCGGGAGAAATGGATATG	
Real Time qPCR	**Forward**	**Reverse**
Snail	CACACGCTGCCTTGTGTCT	GGTCAGCAAAAGCACGGTT
Fibronectin	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG
c-Fos	CGGGTTTCAACGCCGACTA	TTGGCACTAGAGACGGACAGA
Ezh2	AGCACAAGTCATCCCGTTAAAG	AATTCTGTTGTAAGGGCGACC
c-Myc	ATGCCCCTCAACGTGAACTTC	GTCGCAGATGAAATAGGGCTG
Rkip	CCAGCAGCATTTCATGGGAC	TGGTGCCACTCCCTGAATTTG
NM23	AGGAGCACTACACTGACCTGA	GGTTGGTCTCTCCAAGCATCA
Etv1	GCAAGTGCCTTACGTGGTCA	GCTTCAGCAAGCCATGTTTCTT
Etv4	TGGAGAGCAGTGCCTTTACTC	TTGATGGCGATTTGTCTGGGG
Etv5	TCAGTCTGATAACTTGGTGCTTC	GGCTTCCTATCGTAGGCACAA
Erg	ACCTCACCCCTCAGTCCAAA	TGGTCGGTCCCAGGATCTG
Pten$^{flox/flox}$ (deleted)	ACTCAAGGCAGGGATGAGC	GCTTGATATCGAATTCCTGCAGC
Gapdh	CTAGAGAGCTGACAGTGGGTAT	AGACGACCAATGCGTCCAAA
sRNA	**Clone ID**	**Mature antisense**
Etv4 shRNA#1	V2LMM_256957	TTAAGGTCTGGCATAAGAG
Etv4 shRNA#2	V3LMM_515851	TAGCAGCTGAGTCTGGCCA
Scramble shRNA	RHS4346	-

Table S4: List of antibodies for used in this study						
Antibody	**Company**	**Catalog #**	**Type**	**Use and dilution**		
				IF	**IHC**	**Western**
Androgen Receptor	Santa Cruz	SC-816	Rabbit		1:500	
Cytokeratin 5	Covance	PRB-160P	Rabbit	1:500		
Cytokeratin 8	Covance	MMS-162P	Mouse	1:500		
Cytokeratin 8	Abcam	ab14053	Chicken	1:1000		
E-Cadherin	BD Pharminogen	610181	Mouse	1:3000		
Ki67	Novacastra	NCL-Ki67p	Rabbit		1:2000	
Ki67	DakoCytomation	#M7249	Rat	1:500		
GFP	Roche	11 814 460	Mouse	1:1000		
Pan Cytokeratin	Dako	Z0622	Rabbit		1:200	
Phospho Akt (ser473)	Cell Signaling	3787	Rabbit		1:50	
Phospho Akt (ser473)	Cell Signaling	4060	Rabbit			1:1000
Total Akt	Cell Signaling	9272	Rabbit			1:1000
Phospho p44/42 MAPK (Erk1/2) (thr202/tyr204)	Cell Signaling	4376	Rabbit		1:100	
Phospho p44/42 MAPK (Erk1/2) (thr202/tyr204)	Cell Signaling	9101	Rabbit			1:1000
Total p44/42 MAPK (Erk1/2)	Cell Signaling	9102	Rabbit			1:1000
Phospho S6 Ribosomal Protein (ser235/236)	Cell Signaling	2211	Rabbit		1:100	1:1000
Total S6 Ribosomal Protein	Cell Signaling	2217	Rabbit			1:1000
Phospho-FoxO3a (ser318/321)	Cell Signaling	9465	Rabbit			1:1000
ETV4	Lilfespan Biosciences	LS-B1527	Rabbit		1:200	1:1000