Supplementary Information

Supplementary Materials and Methods

Immunohistochemistry

Standard immunohistochemistry techniques were used throughout this study. Primary antibodies were: anti-Ki67 (1:20; Vector Labs, #VP-K452), anti-BRDU (1/150; BD Bioscience, #347580), anti-Androgen Receptor (AR; 1/100; Labvision Neomarkers, #1358), anti-Cytokeratin (CK) 5 (1:1000; Covance, #PRB-160P), anti-Pan-cytokeratin (1/500; Cell Signalling Technology, #4545) anti-Vimentin (1/100; Cell Signalling Technology, #5741), anti-PTEN (1:100; Cell Signalling Technology, #9559), anti-phospho-AKT (Thr308; 1:100, Cell Signalling Technology, #5056), anti-phospho-AKT (Ser473; 1:100; Cell Signalling Technology, #4060 XP), anti-phospho-MAPK (phospho-ERK1/2; Thr202/Tyr204; 1:100; Cell Signalling Technology #4376), anti-β-catenin (1:100; BD Transduction Laboratories, #610154), anti-phospho-GSK3β (Ser9; 1:200, Cell Signalling Technology, #9336), anti-phospho-S6K (1:100; Ser240-244, Cell Signalling, #5364 XP), anti-MMP-7 (1/100; Santa Cruz, #12346 G022), anti-Cyclin-D1 (1/00; Cell Signalling Technology, #2978s), anti-p-MTOR (Ser2448; 1/100, Cell Signalling Technology #5536 XP), anti-p-MEK (Ser221; 1/75; Cell Signalling Technology #2338. Each staining run included a slide incubated with a non-specific IgG as a negative control.

Immunostaining was quantitated either by calculating of the percentage of positive cells in the number of total cells (Ki67, BrDU and Caspase-3 staining) or the "Quickscore" method was used [27]: the proportion of immune-positive (stained) cells within the tissue sections of three mice (three separate 10x magnification fields were taken for each mouse) was scored in the range 1–6, corresponding to proportions of 0–4% (1), 5–19% (2), 20–39% (3), 40– 59% (4), 60–79% (5), and 80–100% (6), respectively. The average intensity of staining is scored in the range 0–3: negative (no staining) = 0, weak = 1, intermediate = 2, and strong staining = 3. An overall score was then

calculated multiplying proportion and intensity staining.

Western blot analysis

Standard western blot techniques were used. Antibodies used were: anti-PTEN (1:1000, Cell Signalling #9559), anti-phospho AKT (Thr308; 1:100, Cell Signalling Technology, #5056), anti-phospho AKT (ser 473; 1:1000, Cell Signalling Technology, #4060 XP), anti-phospho-ERK1/2 (Thr202/Tyr204; 1:1000, Cell Signalling Technology, #4376), anti-β-catenin (1:1000; BD Transduction Laboratories, #610154), anti-phospho-S6K (1:100; Ser240-244, Cell Signalling, #5364 XP), p-70 S6K (1/1000; Cell Signalling Technology, 2708) anti-β-actin (1:5000; Sigma).

Tissue micro-array (TMA)

The Welsh Cancer Bank (www.walescancerbank.com) constructed a TMA including prostate cancer of varying grades and benign prostate tissue. There were 317 prostate samples (73 normal and 244 cancers). 220 samples were obtained from radical prostatectomy samples, 95 from core biopsy samples and 2 from transurethral resection of prostate (TURP) samples. There were 123 Gleason score (GS) 6 (3+3) samples, 69 GS 7 (47 GS 3+4 and 22 Gleason 4+3), 43 GS 8 (40 GS 4+4 and 3 GS 3+5), 5 GS 9 (4 GS 4+5 and 1 GS 5+4) and 4 GS 10 (5+5).

TMA scoring was performed using Quickscore as described above [27]. All samples were evaluated blinded from clinical details.

Supplementary Figure Legends

Supplementary Figure S1: Histological characterisation of invasive adenocarcinoma, reactive stroma and squamous metaplasia in $Catnb^{+/ex3}Pten^{fl/fl}$ K-$Ras^{+/V12}$ triple mutant prostate tumours. (A) Tumours contained frequent mitoses (black arrow), and occasional apoptotic bodies (red arrow). (B) Higher power magnification of hematoxylin and eosin stained tumours with widespread cribriform pattern (*), mitoses (black arrow), and occasional apoptotic bodies (red arrow). (C) When mice succumb to disease, diffuse invasion of epithelial cells into the stroma is seen (between dotted lines). (D) Invasive adenocarcinoma with spindle mesenchymal cells in the stroma (inset). (E) Pan-cytokeratin (pCK) IHC staining positive for epithelial cells in gland-like regions within the stroma (arrow). (F) Staining with the mesenchymal marker vimentin showing positive staining of invasive glands within the stroma (inset arrow), a characteristic of EMT. (G) Diffuse adenocarcinoma with areas of keratin formation adjacent to flattened nuclei (inset) at areas of squamous metaplasia. (H) Areas of squamous metaplasia stained avidly for basal marker CK5. Scale bars as indicated. Insets magnified two times.

Supplementary Figure S2: Percentage of invasive adenocarcinoma. Assessed at 100 days (n=3) as a function of genotype. Triple mutant mice had a greater percentage of invasive adenocarcinoma compared to all double mutant mice at 100 days. There was no invasive adenocarcinoma seen in WT, all single mutants and $Catnb^{+/ex3}K$-$Ras^{+/V12}$ mice at 100 days. Error bars = median with range, Mann-Whitney U: *$p<0.05$, **$p<0.01$).

Supplementary Figure S3: Rate of growth of prostate tumours. Dry prostate gland weights (n=3) were recorded and plotted against time to assess the rate of growth of tumours. $Catnb^{+/ex3}Pten^{fl/fl}$ K-$Ras^{+/V12}$ triple mutant prostate tumours had a faster rate of growth compared

with double mutants (*Pten$^{fl/fl}$ K-Ras$^{+/V12}$*, *Catnb$^{+/ex3}$K-Ras$^{+/V12}$*, *Catnb$^{+/ex3}$Pten$^{fl/fl}$*) and single mutants (*Catnb$^{+/ex3}$, Pten$^{fl/fl}$, K-Ras$^{+/V12}$*; difference in dry prostate weight of *Catnb$^{+/ex3}$Pten$^{fl/fl}$ K-Ras$^{+/V12}$* mice vs *Catnb$^{+/ex3}$Pten$^{fl/fl}$* mice at 100 days significant to P<0.05, unpaired two-tailed t-test). Similarly, double mutants had a greater rate of growth compared to single mutant mice and single mutants (other than *K-Ras$^{+/V12}$*) had a greater rate of growth than WT mice. Medians and ranges plotted.

Supplementary Figure S4: Proliferation of prostatic lesions by Ki67 and BRDU staining. (A,B) Percentage (%) Ki67 staining at (A) 100 days and (B) Endpoint (500 days or when sick). (C) Percentage BRDU positivity at endpoint. Error bars = mean with SD. For clarity, significance of staining in triple mutant vs double mutant cohorts only is presented on the graphs (NS, not significant;* P<0.05; ** P<0.01; *** P<0.001; unpaired two-tailed t-test on Log10 transformed data, n=3). Green bars, single allele mice. Blues bars, double alleles. Red bar, triple allele. Full statistical comparisons of all genotypes are shown in the tables below. (D) Wild-type prostatic epithelium demonstrates minimal proliferation when stained for Ki67. (E) Areas of mPIN have tufting of epithelium into the gland (*), which showed avid nuclear staining for Ki67. (F) Similarly, areas of focal invasion displayed strong Ki67 staining at the basement membrane and particularly at the site of invasion into the stroma (^). (G) Diffuse invasive adenocarcinoma had widespread Ki67 staining, as the normal glandular architecture is lost. (H) Negative BRDU staining in wild-type prostate epithelium. (I) Positive BRDU staining in diffuse adenocarcinoma of the prostate. Scale bars as indicated.

Supplementary Figure S5: Rate of apoptosis according to percentage (%) of Caspase-3 positivity at endpoint (500 days or when sick). WT and *K-Ras$^{+/V12}$* mutant mice demonstrated no Caspase-3 positivity. Caspase-3 positivity was low across all other genotypes with no significant

difference in levels. Green bars, single allele mice. Blues bars, double alleles. Red bar, triple allele. Error bars = mean with 95% CI.

Supplementary Figure S6: Immunohistochemistry for p-GSKβ^{Ser9} for all mouse models.
Immunohistochemistry was performed on cohorts of mice (n=4) at the endpoint of the experiment (500 days or when sick) using antibodies against p-GSKβ^{Ser9}. WT (A), *Pten$^{fl/fl}$* (B), *Catnb$^{+/ex3}$* (C), *K-Ras$^{+/V12}$* (D) and *Catnb$^{+/ex3}$K-Ras$^{+/V12}$* (E) demonstrated minimal staining. *Pten$^{fl/fl}$ K-Ras$^{+/V12}$* (F) and *Catnb$^{+/ex3}$Pten$^{fl/fl}$* (G) tumours demonstrated heterogeneous positive staining for p-GSKβ^{Ser9}. *Catnb$^{+/ex3}$Pten$^{fl/fl}$K-Ras$^{+/V12}$* (H) tumours stained avidly for p-GSKβ^{Ser9}. Bars = 200 μm. Insets magnified two times. (I) Quickscore quantification (mean with 95% CI) of staining for p-GSKβ^{Ser9}. Green bars, single allele mice. Blues bars, double alleles. Red bar, triple allele. Significance of staining in triple mutant vs double mutant cohorts only is presented on the graphs (NS, not significant** P<0.01; *** P<0.001; unpaired two-tailed t-test, n=4). Full statistical comparisons of all genotypes are shown in the accompanying table.

Supplementary Figure S7: Immunohistochemistry for p-ERK 1/2 for all mouse models.
Immunohistochemistry was performed on cohorts of mice (n=4) at the endpoint of the experiment (500 days or when sick) using antibodies against p-ERK 1/2. WT samples demonstrated diffuse nuclear staining (A). *Pten$^{fl/fl}$* (B) *and Catnb$^{+/ex3}$* (C) single tumours demonstrated weak heterogeneous staining. *K-Ras$^{+/V12}$* samples had mild staining for p-ERK 1/2 (D). *Catnb$^{+/ex3}$K-Ras$^{+/V12}$* tumours had focal staining for p-ERK 1/2 (E). *Pten$^{fl/fl}$ K-Ras$^{+/V12}$* tumours had strong p-ERK 1/2 (F) staining. *Catnb$^{+/ex3}$Pten$^{fl/fl}$* tumours demonstrated heterogenous positive staining for p-ERK 1/2 (G). *Catnb$^{+/ex3}$Pten$^{fl/fl}$K-Ras$^{+/V12}$* tumours stained avidly for p-ERK 1/2 (H). Bars = 200 μm. Insets magnified two times. (I) Quickscore quantification (mean with 95% CI) of staining for p-ERK 1/2. White bar, WT mice. Green bars, single allele mice. Blues bars, double alleles. Red bar,

triple allele. Significance of staining in triple mutant vs double mutant cohorts only is presented on the graphs (NS, not significant** P<0.01; *** P<0.001; unpaired two-tailed t-test, n=4). Full statistical comparisons of all genotypes are shown in the accompanying table.

Supplementary Figure S8: Biochemical analysis of signalling pathways in prostate tissue of single (*Catnb*$^{+/ex3}$, *K-Ras*$^{+/V12}$ and *Pten*$^{fl/fl}$) mutant and WT mice. (A) Western-blot analysis for markers of activation of PI3K, WNT, MAPK and mTOR signalling pathways. Protein was extracted from fresh frozen prostate tissue from 100-day old mice for each cohort (n=3). The following antibodies were used: PI3K pathway markers, PTEN, p-AKT^{Thr308}, p-AKT^{Ser473} and total AKT; WNT pathway marker, β-catenin; MAPK pathway marker, pERK1/2; mTOR pathway markers, pS6$^{240/244}$. β-actin was used as a loading reference. (B-F) Relative quantitation of p-AKT^{Thr308} (B), pAKT^{Ser473} (C), β-catenin (D), p-ERK1/2 (E) and p-S6$^{240/244}$ (F). Error bars = mean with 95% CI, *p<0.05, **p<0.01, unpaired two-tailed t test, n=3.

Supplementary Figure S9: Complex interactions between PI3K, Wnt and MAPK pathways resulting in aberrant mTORC1 signalling. Loss or mutation in the PTEN (phosphatase and tensin homolog) gene prevents dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), resulting in aberrant phosphoinositide 3-kinase (PI3K) and AKT signalling. Subsequent activated AKT results in loss of the inhibitory effect of Tuberous Sclerosis Complex (TSC) 1/2 causing increased mTORC1 (mechanistic target of rapamycin complex 1) and ribosomal protein S6K (S6K) signalling. WNT pathway activation results in inhibition of glycogen synthase kinase 3 (GSK-3), preventing it from activating TSC1/2 and thereby blocking its inhibitory effect on mTORC1. Inhibition of GSK-3 also prevents β-catenin phosphorylation and inhibits its degradation, thereby triggering canonical WNT signalling and indirectly inhibiting TSC1/2 via c-MYC. Stepwise phosphorylation of the RAS-RAF-MEK-ERK cascade in the mitogen-activated

protein kinase (MAPK) pathway results in mTORC1 upregulation through inhibition of TSC1/2 via ERK and downstream ribosomal S6 kinase (RSK). Negative feedback or inhibitory signals (dotted arrows) occur at multiple levels controlling mTORC1 activity. Androgen deprivation therapy (ADT) results in increased AKT and subsequent mTORC1 signalling; rapamycin binds and inhibits mTORC1.

Supplementary Table S1: Percentages of animals with nodal metastasis.

Supplementary Table S2: P-values for complete statistical analysis of Quickscore quantitation of β-catenin (membrane and nuclear), pAKT^{Thr308} and pS6$^{240\text{-}244}$. Unpaired t tests, n = 3.