Stromal epigenetic alterations drive metabolic and neuroendocrine prostate cancer reprogramming

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Supplementary Methods

Methyl-MiniSeq™ Sequence alignments and data analysis. Sequence reads from bisulfite-treated EpiQuest libraries were identified using standard Illumina basecalling software and then analyzed using a Zymo Research proprietary analysis pipeline, which is written in Python and used Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/) to perform the alignment. Index files were constructed using the bismark_genome_preparation command and the entire reference genome. The non-directional parameter was applied while running Bismark. All other parameters were set to default. Filled-in nucleotides were trimmed off when doing methylation calling. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Fisher's exact test or t-test was performed for each CpG site which has at least five reads coverage, and promoter, gene body and CpG island annotations were added for each CpG included in the comparison. Reduced Representation Bisulfite Sequencing BaseClear (Netherlands) performed quality control, sequence processing and mapping of sequence reads as per their 'EpiQuest Genome-wide Basic' service package. Sequence depth was determined per CpG dinucleotide as the number of reads where methylation status could be determined and CpGs with a depth less than 10 reads in either condition were excluded. Methylation status of the remaining CpGs was calculated as the percentage of methyl-cytosine in total cytosine. Heat map was made using metaboanlyst (http://www.metaboanalyst.ca) software using top 25 genes analyzed by ANOVA.

Lentiviral Infections in mouse stromal cells. Lentivirus of the empty, GFP expressing vector, and Ras^{V12} gene were transduced in wild type mouse prostatic fibroblasts in the presence of 5.0 μg/mL polybrene (Sigma-Aldrich). After stable clones were selected under hygromycin (50 μg/mL) selection, Ras expression was verified by western blotting.

RNA expression level. All real-time PCR assays were carried out using three technical replicates and three independent cDNA syntheses on a 7500 Real-Time PCR System (Applied Biosystems). Data were normalized using an internal control gene, either GAPDH or β-actin. Relative quantification of expression levels was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used for qPCR are listed in Supplemental Table S5.

Western blots. Western blots performed with 4-12% SDS-polyacrylamide gels. Following electrophoresis gels were transferred to PVDF membrane (BioRad) in transfer buffer (25 mM Tris; 200 mM glycine; 20% methanol v/v). Membranes were blocked and subsequently incubated with primary and secondary antibodies in phosphate-buffered saline containing 0.1% Tween20 (Sigma) and 5% non-fat dry milk or bovine serum albumin for at least 1 hr each. Detection was performed using alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich). Antibody information are listed in Supplementary Table S5. Experiments were repeated in at least three independent experiments, and one of the representative blots was shown.

Active Ras pull-down assay. Ras activity was detected using a Ras Activation Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions using 500 µg protein for each condition. RAS activity was reflected by the amount of Ras-GTP pulled down by Raf1 RBD (Ras-binding domain) relative to total Ras expression by western blotting.

ATP assay. CWR22Rv1 and C4-2B cells ($1X10^5$) were plated into a 6 cm culture dish and allowed to adhere. After 24 hrs, the medium was replaced with conditioned media obtained from primary mouse wild type or Ras^{V12} prostatic fibroblasts as well as primary human NAF or CAF. Controls included vehicle (DMSO) and oligomycin (2 µM). After 72 hrs incubation with the conditioned media, the cells were lysed for measurement of intracellular ATP levels using an ATP determination kit (Thermo Fisher) according to the manufacturer's instructions. Luminescence was measured with Monolight 3010 luminometer (Pharmingen, San Diego, CA). All values were normalized to total protein and the cellular ATP level was expressed as nanomole per microgram of protein.

Metabolome analysis. CWR22Rv1 cells were grown in NAF or CAF conditioned media for 72 hrs. Cell pellets were flash frozen in liquid nitrogen for untargeted metabolic profiling of known and unknown metabolites using liquid chromatrography-mass spectrometry by Metabalon Inc. as previously described (1).

Measurements of glutamine. Glutamine was measured in blood plasma and conditioned media by using

glutamine detection kit (Abcam) following manufacturer protocol. Deproteinization step was performed in plasma samples prior to glutamine detection. The absorbance was measured at 450 nm using a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories).

TCGA analysis. Gene expression data were downloaded from the TCGA GDAC Firehose portal [Broad Institute TCGA Genome Data Analysis Center (2016), Firehose stddata__2016_01_28 run. Broad Institute of MIT and Harvard. doi:10.7908/ C11G0KM9]. For analyzing PHB expression, the normalized RSEM counts were used after log2 transformation. *RASAL3* transcriptomic sequencing status was retrieved from the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/).

Generation the Cas9-CRISPR RASAL3 gene deleted prostatic fibroblasts. We used lentiviral transduction to create stable Cas9 (CRISPR associated protein 9) expressing wild type mouse fibroblast clones. To generate the virus in 293T cells, we co-transfected lentiCas9-Blast (Addgene, 52962), pCMV-dR8.91 (Addgene, 8455) and pCMV-VSVG (Addgene, 8454) following the BioT (Bioland Scientific LLC) protocol. We selected a viral titer which gave us a 10% infection efficiency to ensure one Cas9 gene copy per cell. Briefly, virus was added to prostatic fibroblasts in suspension at different ratios including an uninfected control. Two days post infection, we selected Cas9 positive cells with blasticidin (5 μg/mL) while the cells were in suspension. Guide RNA (sequences: CTTGGAAAACCGGAGGCGAT) was transfected into Cas9 expressing fibroblasts and single cell clones selected for screening *Rasal3* deletion by western blotting.

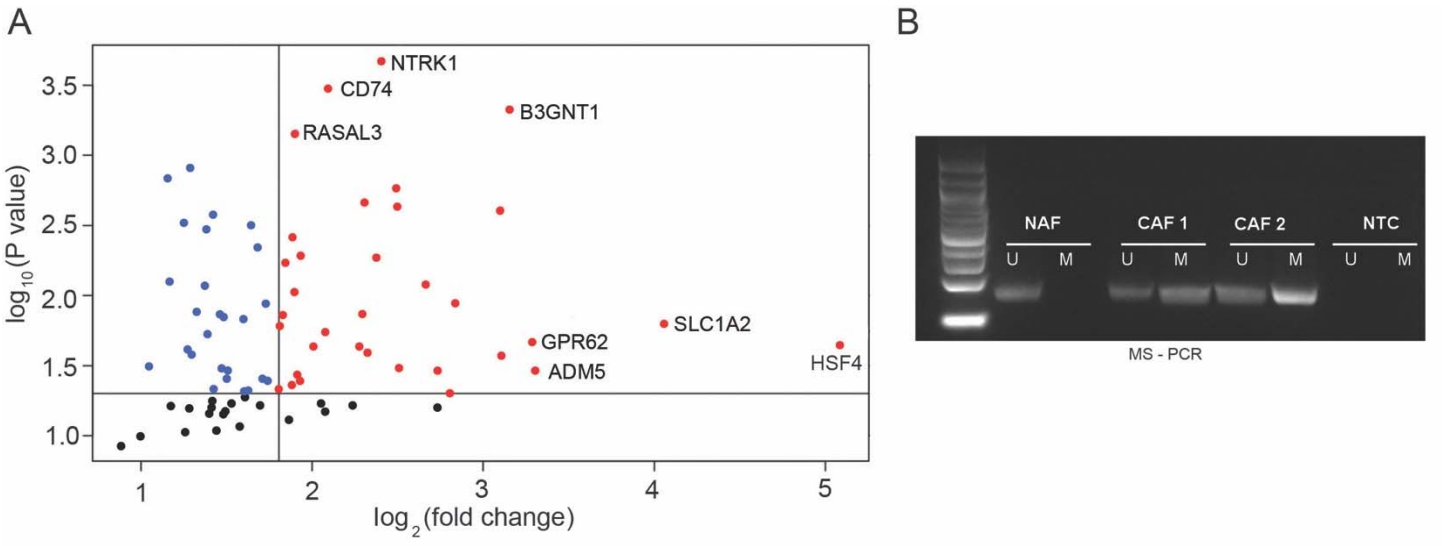
Tumor-stromal cell co-culturing assays: Transwell plates (6-well), with cell culture inserts of 6.5 mm in diameter and 8.0-μm pore size, were used. Fibroblasts and epithelial cells were seeded at (3:1) on the top (120,000) and Bottom (40,000) of the transwell apparatus. Before the experiment, the epithelial cells were grown in glutamine free RPMI media.

Viability assays. Cell viability assays were performed using Cas9-wild type and Rasal3-KO fibroblasts and CWR22RV1 cells. Briefly, cells were plated at 1 x 10^3 cells/well in a 96-well plate in complete medium (DMEM/RPMI with 10% FBS). After 24 hrs, cells were treated with varying concentrations of macropinocytosis

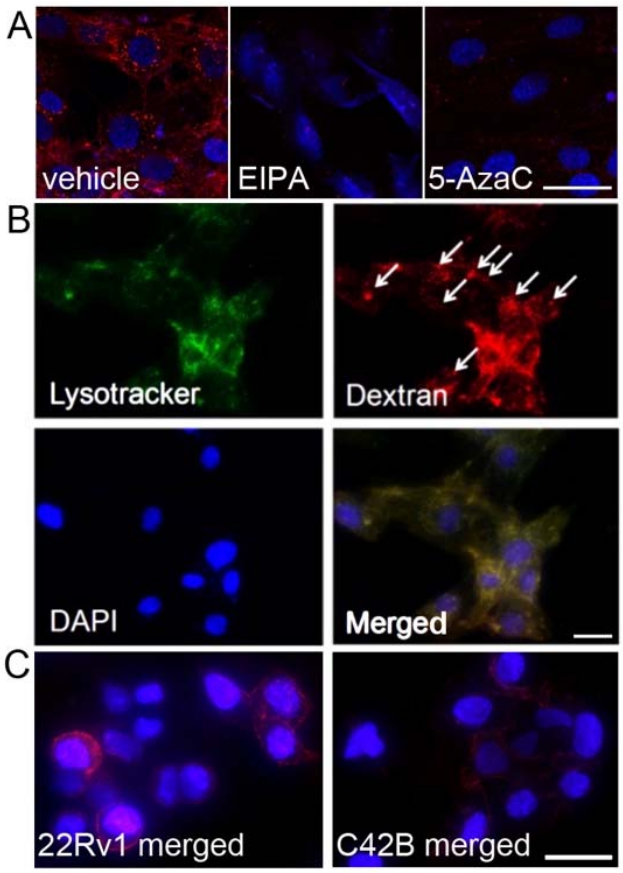
inhibitor, EIPA, at 10 μM and 75 μM. Cell viability was measured by the MTT viability kit (Promega). The absorbance of the MTT formazan was determined at 570 nm using ELISA reader.

Si-RNA transfection: *GLS* and *SLC1A5* gene silencing by small interfering RNA (pooled siRNA) targeted to GLS was purchased from Santa Cruz Biotechnology. Briefly, cells were transiently transfected with SLC1A5 or GLS-specific or scrambled (control) siRNA using lipofectamine reagent (Invitrogen, Carlsbad, CA) (2). qRT-PCR was used to validate *SLC1A5* or *GLS* knockdown. Transfected cells were treated with or without L-glutamine (2 mM) and mRNA levels of select human neuroendocrine genes measured using qRT-PCR.

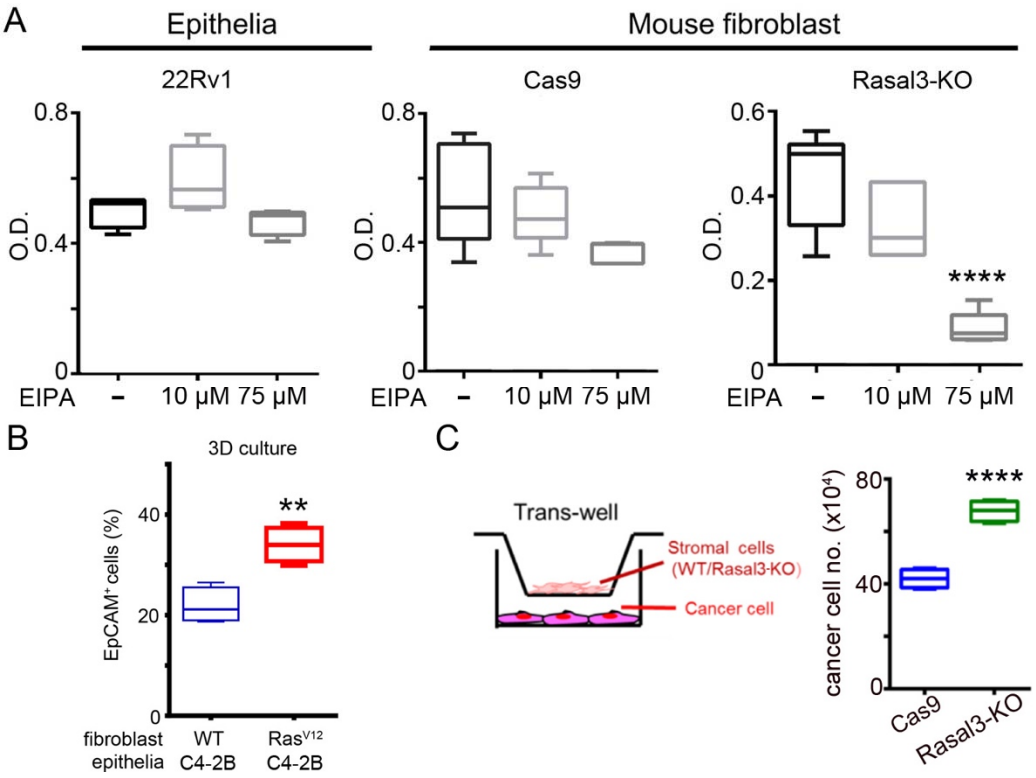
Supplementary Figures (1-7)



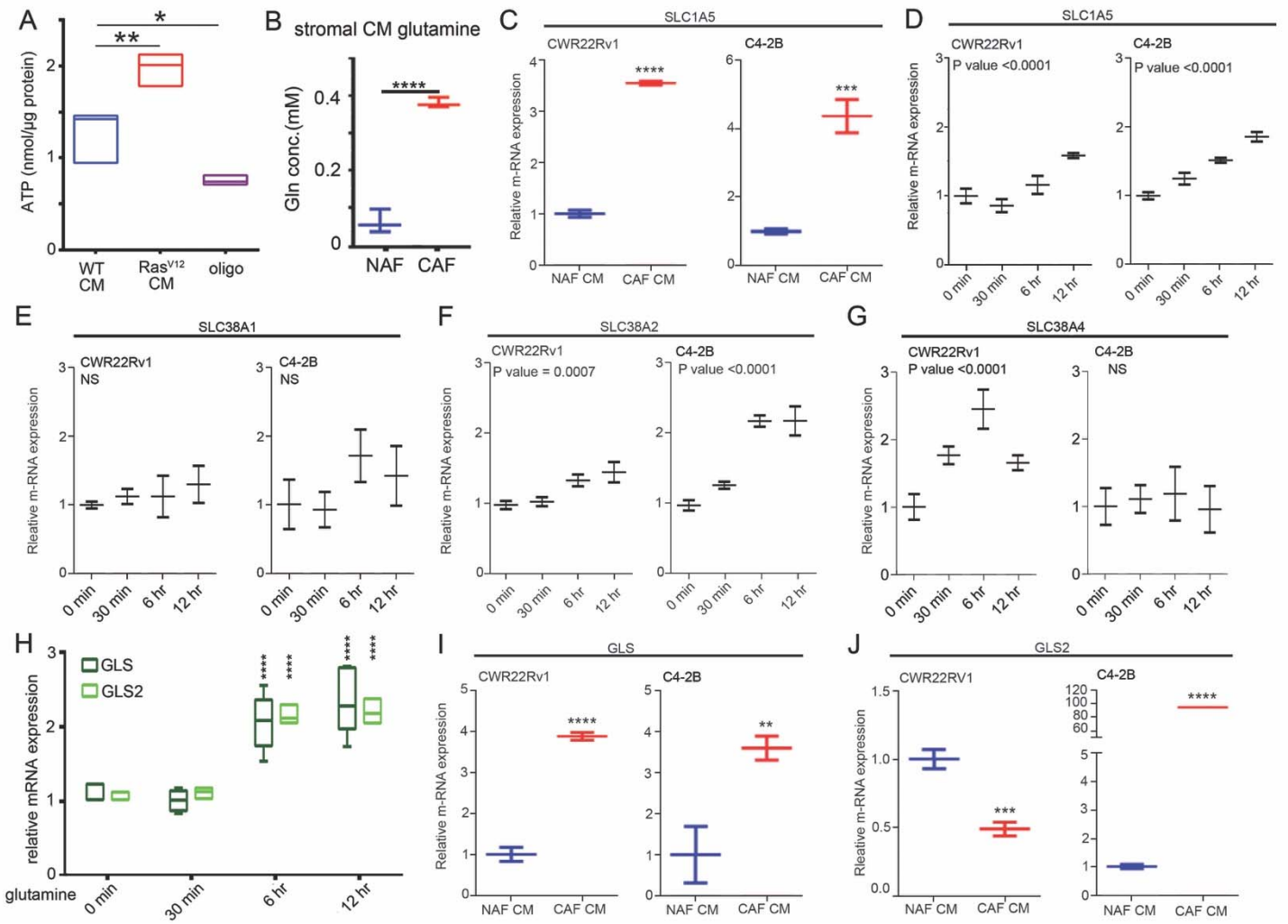
Supplementary Figure 1. Methylome analysis revealed differential RASAL3 promoter methylation. (**A)** Volcano plot of methylated genes from the heat map shown in Figure 1A was generated with a fold-change cutoff of 1.5 and a P-value cutoff of 0.05. (**B)** Representative image of methylation-specific PCR (MS-PCR) for exon 2 of the *RASAL3* gene in primary human NAF and CAF. Each experiment was repeated 3 times.



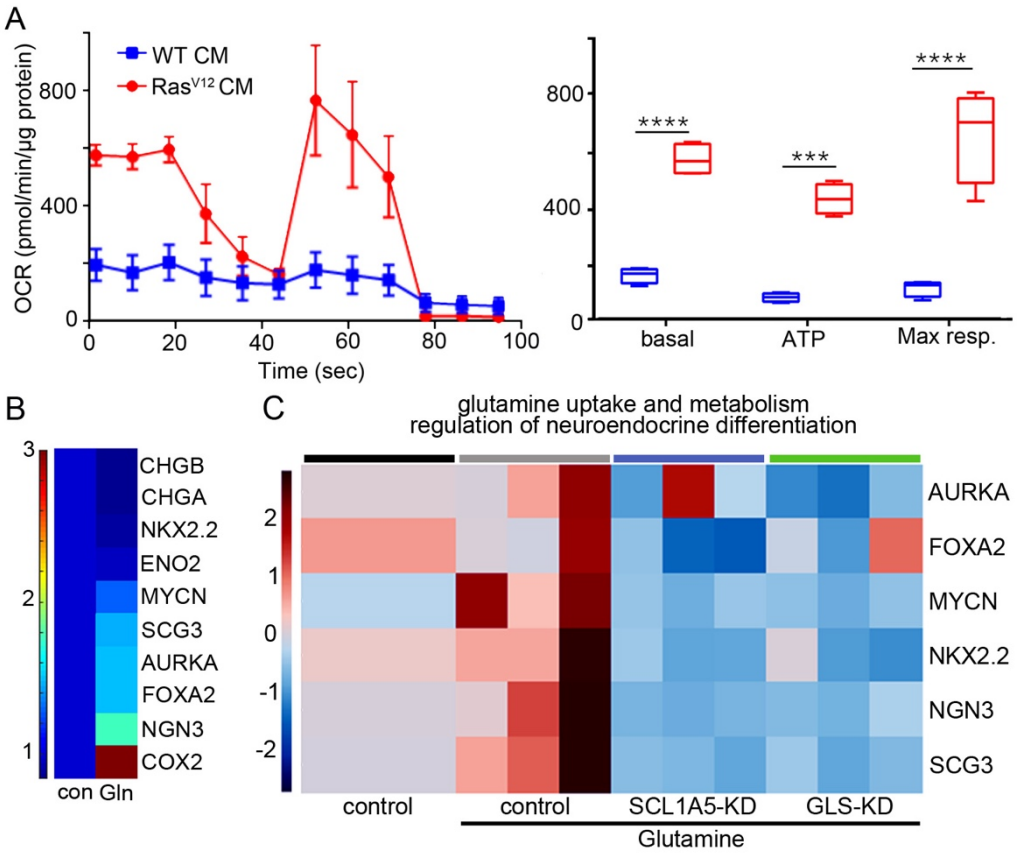
Supplementary Figure 2. Macropinocytosis of prostatic CAF and epithelia. (A) Primary CAF were treated with DNA de-methylating agent, 5-Aza DC (5 μM), or macropinocytosis inhibitor, EIPA (25 μM). Macropinocytosis was detected by fluorescent TMR-dextran labeling (red). (**B)** Acidification of macropinosomes were monitored by co-localization of Lysotracker (green) with TMR-Dextran suggesting fusion of macropinosomes with lysosomes. Images show representative confocal microscopic images. (**C)** TMR-dextran staining of PCa epithelia (CWR22Rv1 and C4-2B) suggest little to no uptake of the dye in macropinocytic vesicles. DAPI staining (blue) identifies nuclei. Each experiment was repeated 3 times. Scale bars: 30 μm.



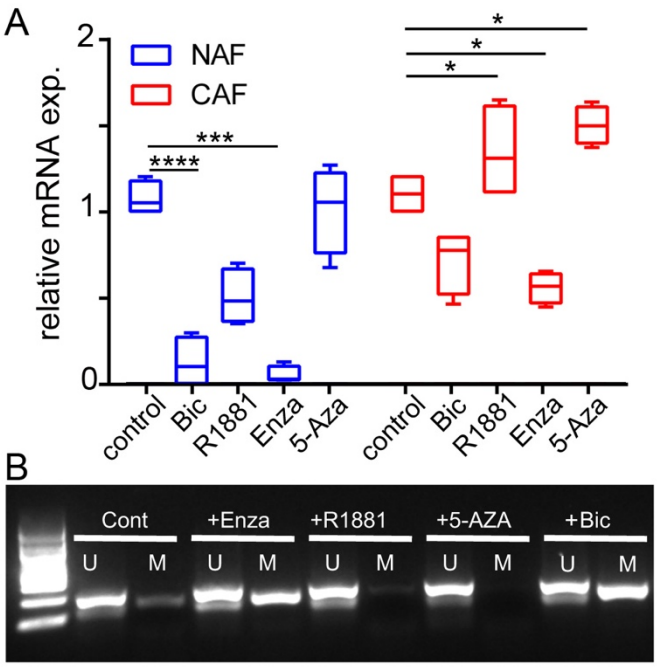
Supplemental Figure 3. Fibroblastic macropinocytosis is regulated by RASAL3 and Ras signaling. (A) The viability of CWR22Rv1 and mouse prostatic fibroblasts (Cas9 control and Rasal3-KO) was measured by MTT assay following 72 hrs incubation with macropinocytotic inhibitor, EIPA. The bar graphs represent the absorbance of the MTT formazan determined at 570 nm from a single experiment representative of a total 3 independent experiments, each performed in quadruplicate. Statistical significance was determined by one-way ANOVA. **(B)** Quantitation of EpCAM-positive epithelial cells were achieved by FACS of 3D co-cultures of human C4-2B epithelia and wild type (WT) or Ras^{V12} mouse fibroblasts. Statistical significance was determined by unpaired t test. **(C)** Stromal fibroblasts (Cas9 or Rasal3-KO) cultured with CWR22Rv1 epithelia using a standard transwell apparatus. Co-culture of CWR22Rv1 cells with Rasal3-KO stromal fibroblast for 48 hrs resulted in increased cell proliferation compared to that with Cas9 (control) fibroblasts, as assessed by trypan blue cell counting. Epithelial and stromal cells grown in glutamine free either DMEMF12 or RPMI media. are expressed as mean ± SD of 3 independent experiments. Statistical significance was determined by two-tailed student's t test. $^{**}P < 0.01$, $^{****}P < 0.0001$.



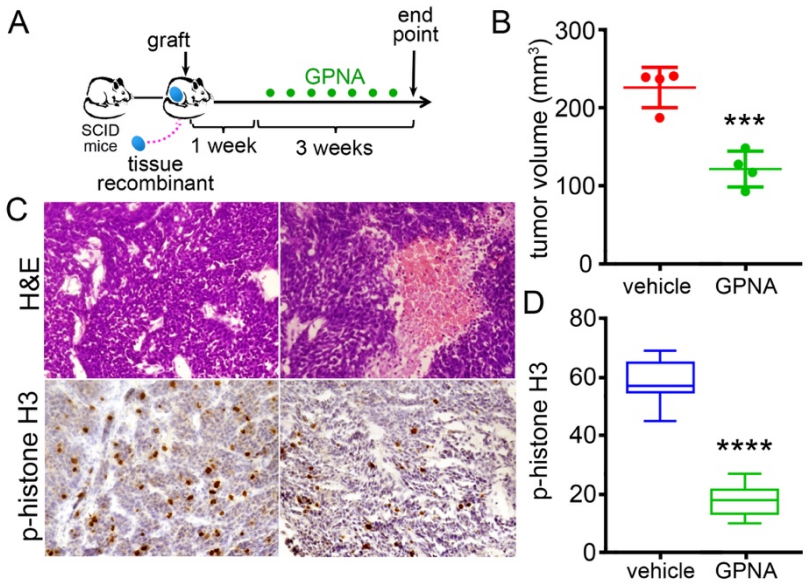
Supplementary Figure 4. Stromal glutamine uptake and metabolism by CWR22Rv1 and C4-2B prostatic epithelia. (A) ATP was measured in C4-2B cells after incubation with conditioned media (CM) from mouse wild type (WT) or Ras^{V12} mouse prostatic fibroblasts or oligomycin. Data are represented as mean ± SD, with n=3-4 per group, analyzed by one-way ANOVA with multiple comparison. **(B)** Glutamine concentrations were measured in the conditioned media derived from NAF and CAF cultured for 72 hrs. Data were obtained from 3 independent measurements, and each experiment was performed in quadruplicate. Statistical significance was determined by two-tailed student's t test. **(C**) Relative gene expression levels of *SLC1A5*, in CWR22Rv1 and C4-2B cells were measured following incubation with NAF or CAF conditioned media (CM) for 72 hrs. Statistical significance was determined by one-way ANOVA, n = 3–5 per group. **(D-G)** Relative gene expression levels of glutamine transporters (*SLC1A5*, *SLC38A1*, *SLC38A2* and *SLC38A4*) were measured in CWR22Rv1 or C4-2B cells treated with glutamine (2 mM) at indicated time points. Data represent the mean ± SD, Statistical significance was determined by two-tailed student's t test.**(H)** Relative gene expression levels of glutaminase isoforms (*GLS* and *GLS2*) in C4-2B cells were measured after treating with glutamine (2 mM) at indicated time points. Data represent the mean ± SD was evaluated by one-way ANOVA followed by sidak multiple comparison. n = 3–5 per group. **(I-J)** Relative gene expression levels of GLS and GLS2, in CWR22Rv1 and C4-2B cells were measured following incubation with NAF-CM or CAF-CM for 72 hrs. and stromal cells grown in glutamine free either DMEMF12 or RPMI media. Statistical significance was determined by one-way ANOVA, n = 3–5 per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and **** *P* < 0.0001. NS, not significant.



Supplemental Figure 5. Stromal glutamine supports the energy needs and mediates neuroendocrine differentiation of PCa epithelia. (A) Oxygen consumption rate (OCR) trace for WT-CM and Ras^{V12}-CM-cultured C4-2B cells, measured using a Seahorse Bioscience XF24 Extracellular Flux Analyzer. The injection order of oligomycin, FCCP, and rotenone along with antamycin resulted in a profile, normalized to protein concentration (n=4). Data shown of single experiment representative of a total 3 independent experiments, each performed in quadruplicate. The mean and S.D. of the three independent OCR experiments are represented by the bar graph highlighting basal, ATP-linked and maximal respiration elevated by Ras^{V12}-CM treated C4-2B cells including, compared to WT-CM conditioned media. Statistical significance was determined by two-tailed student's t test. ***P < 0.001, and ****P < 0.0001. **(B)** Heatmap summarizing the qPCR results comparing the C4-2B cell expression of neuroendocrine marker genes following treatment with L-glutamine (2 mM). Two-way ANOVA analysis indicated the overall P value with F-test P < 0.0001 (n = 3). **(C)** Following transfection with either GLS or SLC1A5 siRNA or scrambled control siRNA, CWR22Rv1 cells were incubated with 2 mM L-glutamine for measurement of mRNA levels of select neuroendocrine genes, assessed by qRT-PCR. Results are the average of at least three independent experiments. (B-C) Epithelial and stromal cells grown in glutamine free either DMEMF12 or RPMI media. Statistical significance is summarized in Supplementary Table 5.



Supplemental Figure 6. *RASAL3* expression and promoter methylation is androgen dependent. (**A**) Quantitative rtPCR analysis of *RASAL3* in NAF and CAF was performed following 5 day treatment with bicalutamide (Bic, 10^{-5} M), enzalutamide (Enza, 10^{-5} M), R1881 (10^{-9} M), or 5-Azacytidine (5-Aza, 5 mmol/L). *RASAL3* expression was normalized GAPDH expression. Data represent the mean ± SD. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001, by two-way ANOVA followed by multiple comparison. Results are the average of at least three independent experiments. (**B**) MS-PCR of the *RASAL3* exon 2 methylation status in CAF after treatment with enzalutamide, R1881, 5-Azacytidine and bicalutamide was performed. The PCR products in the lanes marked "U" indicate unmethylated templates for *RASAL3* gene, whereas the products in the lanes marked "M" indicate methylated templates. Data are representative of three independent experiments.



Supplemental Figure 7. GPNA treatment of intact mice with tissue recombinant xenografts of PCa epithelia and CAF. (**A**) Schematic diagram of the experimental design of control or GPNA treatment of mice xenografted with tissue recombinants in the sub-renal capsule consisted of CWR22Rv1 (2 x 10^5 cells) and CAF (6 x 10^5 cells). (**B**) Quantitated tumor volumes were analyzed by two-tailed t test (n=4). (**C**) H&E and phosphorylated histone H3 (p-histone H3) immunohistochemical staining of PCa xenografts indicate regulation by glutamine uptake antagonist, GPNA. Experiment was performed 3 separate times. Representative image of 3 independent experiments is shown. (**D**) Quantification of the frequencies of p-histone H3-positive cells per 20x field are represented as a mean ± SD was evaluated by two tailed Student's t test. ***$P < 0.001$, ****$P < 0.0001$.

Supplementary Tables (1-6)

Supplementary Table 1. List of the top 200 genes from methylome sequencing (RRBS) data. Provided as an EXCEL file.

Supplementary Table 2. Statistical analysis for Figure 4F.

Gene ID	Ras^{V12} vs. WT (*P* Value)
AURKA	0.301221545
CHGA	0.115214879
CHGB	0.042668993
ENO2	0.002282061
FOXA2	0.000591958
NKX2.2	0.011728483
NGN3	0.000630702

Supplementary Table 3. Statistical analysis for Figure 4G.

GENE ID	f.value	*P* value	FDR
MYCN	11.593	0.0027751	0.016651
SCG3	9.1245	0.005825	0.017475
NGN3	5.9942	0.019191	0.036413
NKX2.2	5.4784	0.024275	0.036413

Supplementary Table 4. Statistical analysis for Figure 5C.

GENE ID	f.value	*P* value	FDR
MYCN	254.43	2.84E-08	2.84E-07
COX2	146.23	2.51E-07	1.26E-06
CHG B	67.39	5.09E-06	1.70E-05
NGN3.3	17.855	0.00066417	0.00166
CHG A	13.293	0.0017849	0.00357
AURKA	11.317	0.0029958	0.004993
NKX 2.2	7.7214	0.0095231	0.013604
SCG	6.9703	0.012727	0.015908
FOXA2	6.4649	0.015661	0.017401
MYCN	254.43	2.84E-08	2.84E-07

Supplementary Table 5. Statistical analysis for Supplemental Figure 5C.

GENE ID	Glutamine vs. Control (*P* Value)	Glut+ Rapamacyin vs Control (*P* Value)
CHGA	0.01	1.47E-06
FOXA2	0.01	0.004050679
SCG3	0.008	0.000159964
ENO2	0.006	0.224168157
NGN3	0.08	0.2504569
MYCN	0.05	0.259416618
NKX2.2	0.01	0.004050679
AURKA	0.004	0.000273876

Supplementary Table 6. Details of reagents, antibodies and primers used in study. Provided as an EXCEL file.

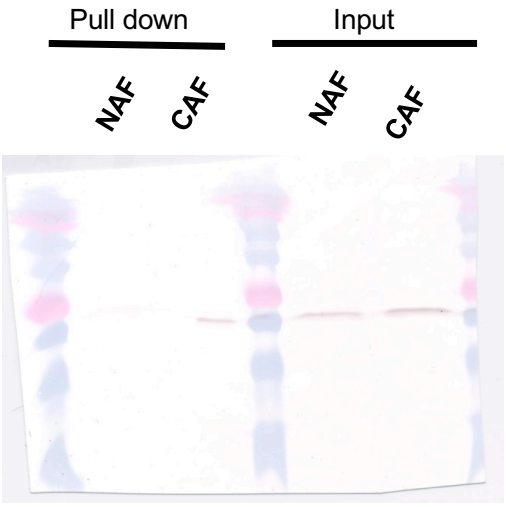
Supplementary Material References

1. Evans AM, DeHaven CD, Barrett T, Mitchell M, and Milgram E. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem.* 2009;81(16):6656-67.
2. Haldar S, Dru C, Mishra R, Tripathi M, Duong F, Angara B, Fernandez A, Arditi M, and Bhowmick NA. Histone deacetylase inhibitors mediate DNA damage repair in ameliorating hemorrhagic cystitis. *Sci Rep.* 2016;6(39257.

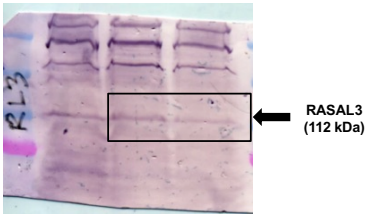
Full unedited gel for figure 2

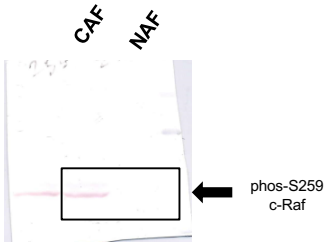
Figure 2a raw Western blot

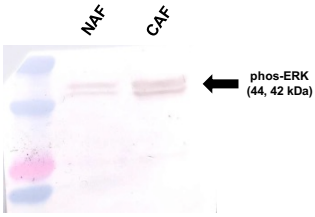
Ras activity

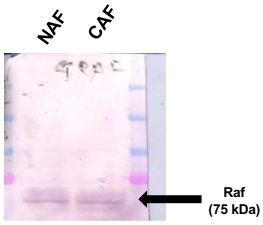


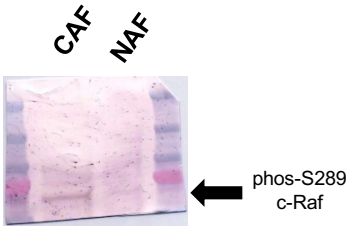
Western blots











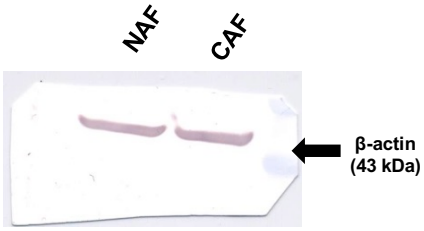
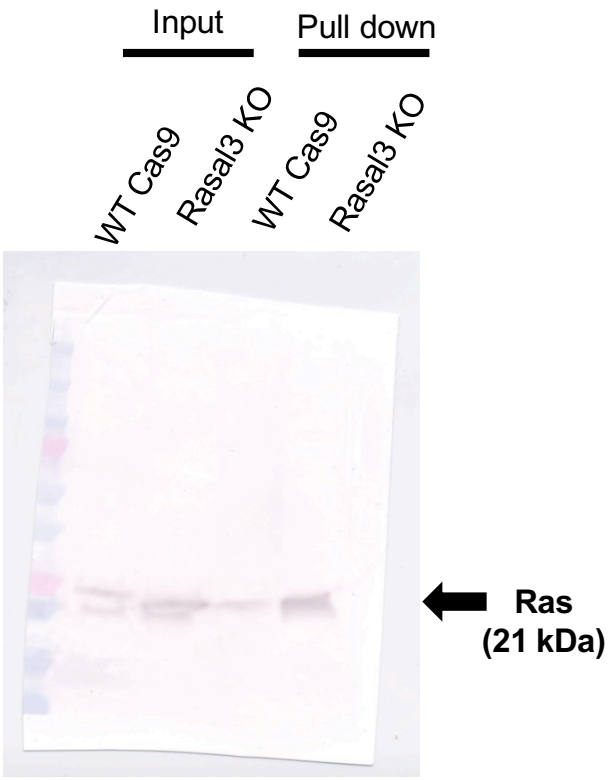


Figure 2d raw Western blot

Ras activity



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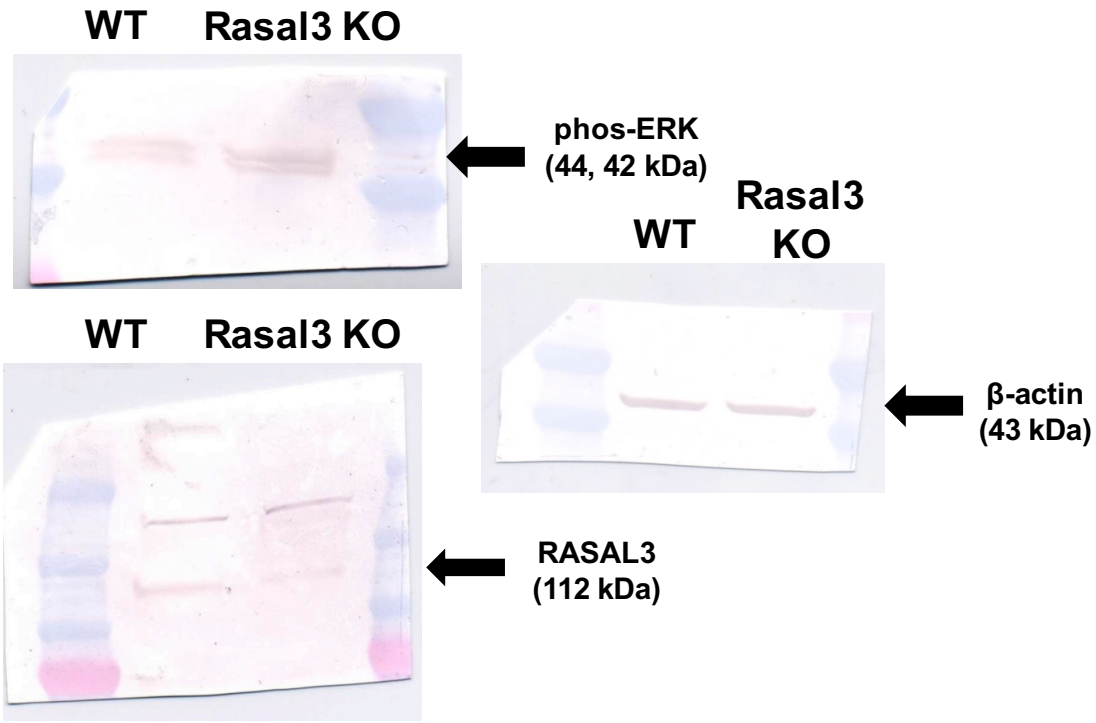
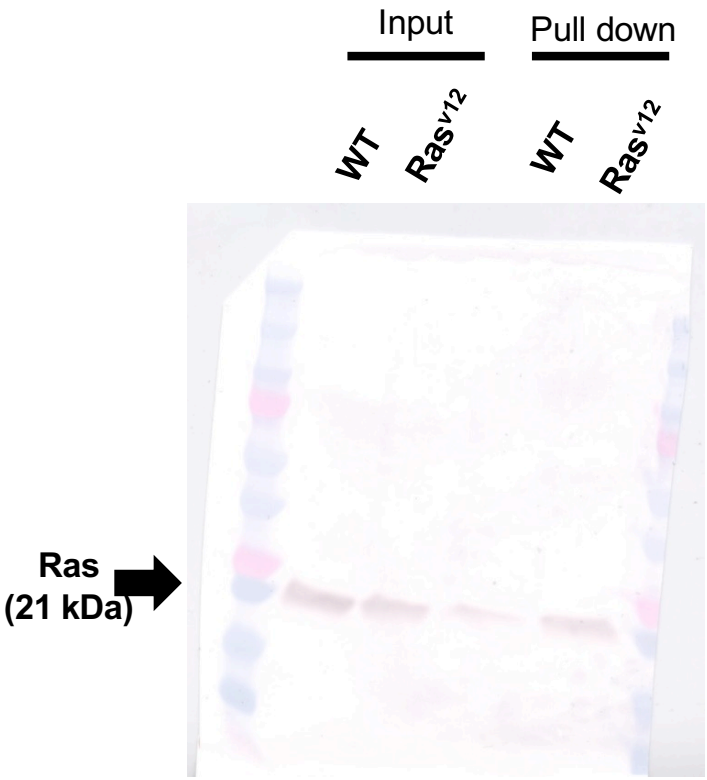


Figure 2f raw Western blot

Ras activity



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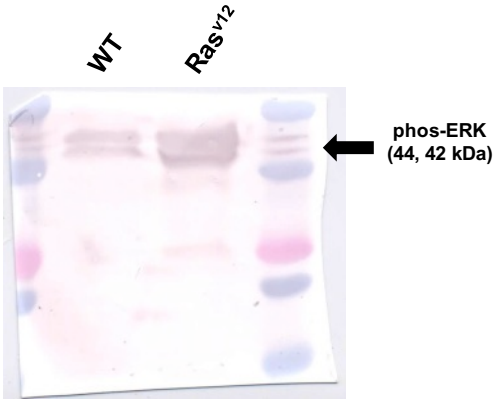

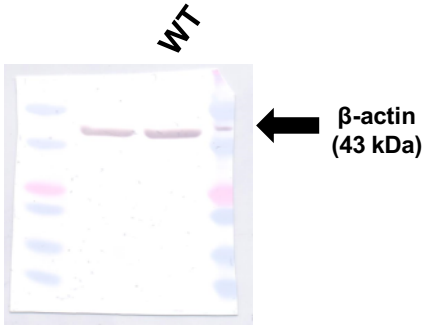

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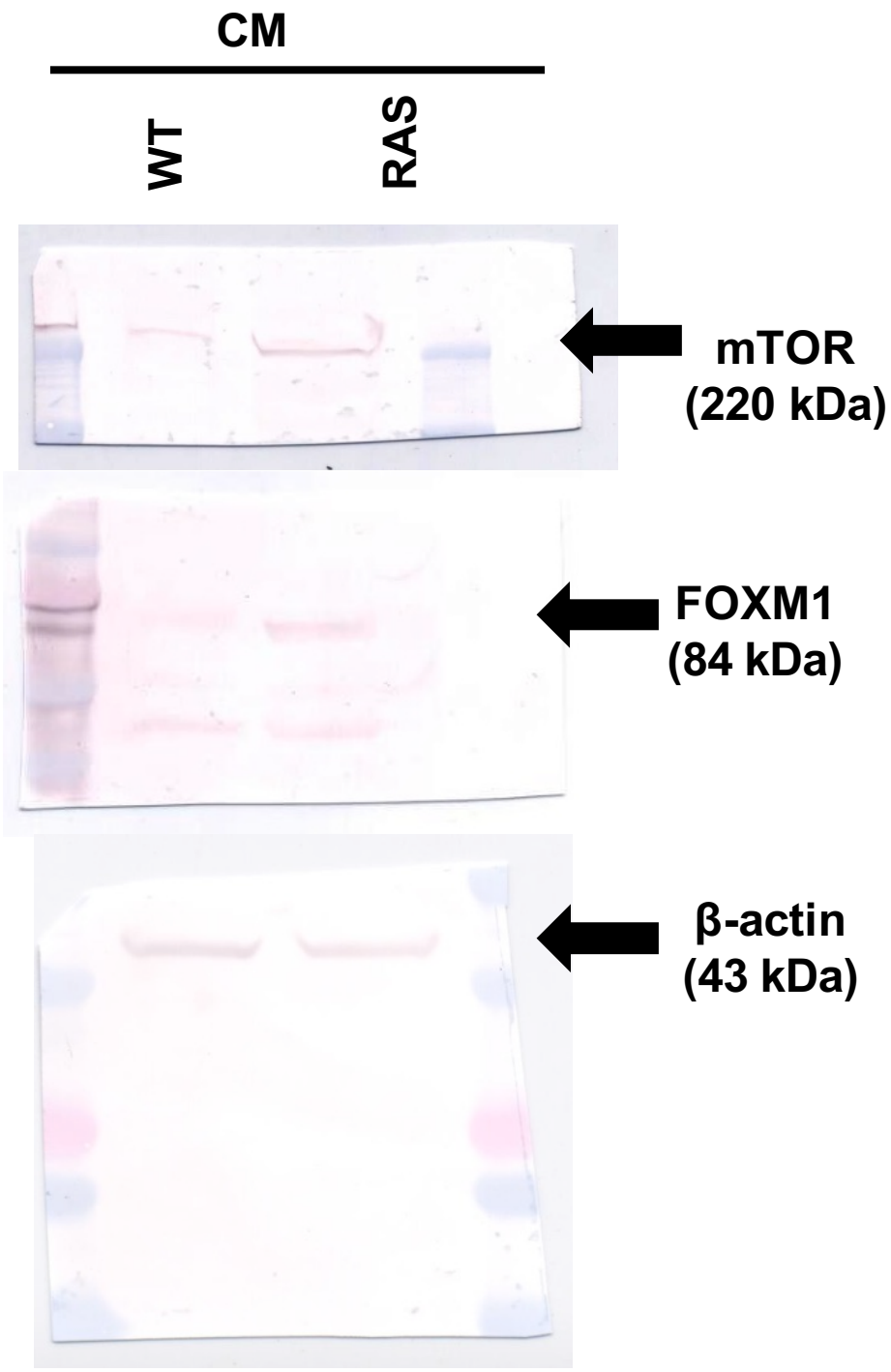

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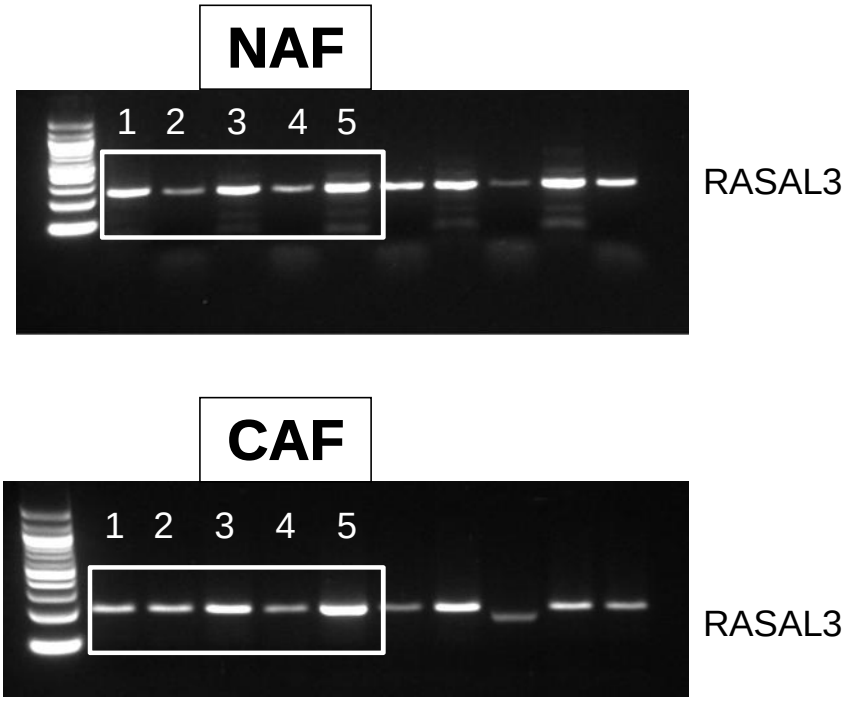
3. **R1881**
4. **Enzalutamide**
5. **5-Azacytidine**



1. control
2. Bicalutamide
3. R1881
4. Enzalutamide
5. 5-Azacytidine