SUPPORTING ONLINE MATERIAL

Rearrangements of the *RAF* Kinase Pathway in Prostate Cancer, Gastric Cancer and Melanoma

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Materials and Methods

Cell lines and Tissues

NIH3T3, RWPE-1, and HEK293 cell lines used in this study were obtained from the American Type Culture Collection. Prostate cancer tissues were obtained from the University of Michigan tissue core and University of Michigan Rapid Autopsy Program which are part of the University of Michigan Prostate Cancer Specialized Program Of Research Excellence (S.P.O.R.E). Five different tissue microarrays containing a total of 512 samples with 90-100 samples each comprising samples for prostate cancer progression with benign prostate, PIN prostate, prostate tumors and warm autopsy samples. All the TMA's were used for the FISH evaluation of both *RAF1* and *BRAF*. Prostate tissues from the radical prostatectomy series at the University Hospital Ulm (Ulm, Germany) comprised of 149 cases of primary prostate cancer (n=136) and lymph node metastasis (n=13) from patients that underwent radical prostatectomy and lymph node dissection between 1989 and 2001 at Ulm University (Ulm, Germany) and were collected under an IRB approved protocol. The mean age is 64. The distribution of Gleason pattern is as follows: GS 6 (2%), GS 7 (24%), GS 8-10 (68%), GS N/A (6%). The pT staging distribution is as follows: pT2 (5%), pT3 (93%), pT N/A (2%). The PSA levels range from 0.6 - 262 ng ml^{-1} (median 23.75 ng ml^{-1}). 25 (18%) patients received androgen-deprivation therapy pre-operatively. A TMA was constructed using three 0.6 mm high density cancer tissue cores per case.

A small cohort of 59 transurethral resection of prostate (TURP) FFPE material was retrieved from McGill University Hospitals (Montreal, Canada) with approval from the local Institutional Review Boards. The patients had been treated with one or multiple therapeutic protocols (radiation therapy, brachytherapy and/or androgen deprivation therapy). The castration-resistant status was determined clinically based on PSA levels and disease progression under treatment. Tissue cores at diameters of 0.6 mm were obtained from areas containing high density tumor and subjected to tissue microarray (TMA) construction. Informed consent from all the patients and institutional review board approval were obtained from the respective institutions.

Real Time PCR validation

Quantitative PCR (QPCR) was performed using Power SYBR Green Mastermix (Applied Biosystems) on an Applied Biosystems StepOnePlus Real-Time PCR System. All oligonucleotide primers were obtained from Integrated DNA Technologies and are listed below. The *GAPDH*, primer was used as a control. All assays were performed and repeated twice and results were plotted as average fold change relative to *GAPDH*.

SLC45A3 F	5'-AGCCGCGCGCCTCGGCCA-3'
BRAF R	5'-ATCAGGAATCTCCCAATCATCACT-3'
SLC45A3 F	5'-GTACCAGCCCCACCCCTCTATCC-3'
SLC45A3 R	5'-TCAGTGGACAGGAAACGCACCATA-3'
BRAF EX8-Stop F	5'-GCCCCAAATTCTCACCAGTCCGTC-3'

BRAF EX8-Stop R	5’-TCAGTGGACAGGAAACGCACCA-3’
BRAF EX10-Stop F	5’-ATGAAACACTTGGTAGACGGGA-3’
BRAF EX10-Stop R	5’-TCAGTGGACAGGAAACGCACCA-3’
BRAF EX2 F	5’-AACATATAGAGGCCCTATTGGACA-3’
BRAF EX3 R	5’-AGAAGATGTAACGGTATCCATTG-3’
BRAF EX4 F	5’-GGAGTTACAGTCCGAGACAGTCTAA-3’
BRAF EX5 R	5’-CAGTAAGCCAGGAAATATCAGTGTC-3’
BRAF EX6 F	5’-AGCGTTGTAGTACAGAAGTTCCACT-3’
BRAF EX7 R	5’-AGATGTTAGGGCAGTCTCTGCTA-3’
BRAF EX8 F	5’-TGTGCATATAAACACAATAGAACCTG-3’
BRAF EX10 R	5’-TTCGATTCCTGTCTTCTGAGG-3’
BRAF EX11F	5’-AAAACACTTGGTAGACGGGACTC-3’
BRAF EX12R	5’-CTTGTAACTGCTGAGGTGTAGGTG-3’
BRAF EX13 F	5’-TTGTATCACCATCTCCATATCATTG-3’
BRAF EX14 R	5’-GGATGATTGACTTGGCGTGTA-3’
BRAF EX15 F	5’-CTACAGTGAAATCTCGATGGAGTG-3’
BRAF EX16 R	5’-TCATACAGAACAATTCCAAATGC-3’
BRAF EX17 F	5’-CGAGGATACCTGTCTCCAGAT-3’
BRAF EX18 R	5’-GATGCACTGCGGTGAATTTTT-3’
BRAF 3’UTR F	5’-AGTGAGAGAGTTCAGGAGAGTAGCA-3’
BRAF 3’UTR R	5’-AAGTATAAATTTTAGTTTGGGGAAAAA-3’
RAF1 EX5 F	5’-CATGAGCACTGTAGCACCAAA-3’
ESRP1 EX14 R	5’-AGCAGCTGTAGGGAAGTAGCC-3’
ESRP1 EX13 F	5’-GTACTACCCAGCAGGCACTCA-3’
RAF1 Ex6 R	5’-CTGGGACTCCACTATCACCAA-3’
RAF1 F	5’-ATGGAGCACATACAGGGAGCT-3’
ESRP1 R	5’-TTAAATACAAACCCATTCTTTGG-3’
ESRP1 F	5’-ATGACGGCCTCTCCGGATTA-3’
RAF1 R	5’-CTAGAAGACAGGCAGCCTCG-3’
DUSP6 F	5’-CCGCAGGAGCTATACGAGTC-3’
DUSP6 R	5’-CCTCGTCCTTGAGCTTCTTG-3’
SPRY2 F	5’-CCCCTCTGTCCAGATCCATA-3’
SPRY2 R	5’-CCCAAATCTTCCTTGCTCAG-3’
AGTRAP F	5’-ATCCCTTTGCAGTCCCAGA-3’
BRAF R	5’-CTGTGGAATTGGAATGGATTTT-3’
GAPDH F	5’-TGCACCACCAACTGCTTAGC-3’
GAPDH R	5’-GGCATGGACTGTGGTCATGAG-3’

Gene Expression Profiling

LNCaP and VCaP cells were starved for 48 hours and treated with 1 nM R1881 for 24 and 48 hours and RNA isolated from these cells were used for microarray analysis. Gene expression microarray profiling was performed using the Agilent Whole Human Genome Oligo Microarray according to the manufacturer's protocol.

Confirmation of SLC45A3-BRAF and ESRP1-RAF1 protein expression by Western Blotting

The *ESRP1-RAF1* fusion positive prostate cancer tissue and fusion negative tissues were homogenized in NP40 lysis buffer (50 mM Tris-HCl, 1% NP40, pH 7.4, Sigma, St. Louis, MO), and complete protease inhibitor mixture (Roche) and phosphatase inhibitor (EMD Bioscience). Unfortunately fresh frozen material for the *SLC45A3-BRAF* index case was not available for similar assay. For testing the expression and assess the molecular weight of the fusion protein in the fusion positive tissues, HEK293 cells were transfected with *SLC45A3-BRAF* and *ESRP1-RAF1* fusion constructs separately (cloned in pDEST40 expression vector - Invitrogen) and as well as vector control and the transfected cells were lysed in NP40 lysis buffer with protease inhibitor. Fifteen micrograms of each protein extract were boiled in sample buffer, separated by SDS-PAGE, and transferred onto Polyvinylidene Difluoride membrane (GE Healthcare). The membrane was incubated for one hour in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) and incubated overnight at 4ºC with anti-BRAF (Santa Cruz) and anti-RAF1 mouse monoclonal antibody (1:1000 in blocking buffer (BD Bioscience). Following three washes with TBS-T, the blot was incubated with horseradish peroxidase-conjugated secondary antibody and the signals visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare). Blot was re-probed with anti-β-actin mouse monoclonal (1:5000, Sigma) antibodies.

Foci Formation Assay

NIH3T3 cells (1.5 X 10^5) in 35-mm plastic dishes were transfected with 2 μg of DNA of the plasmid of interest. All the transfections were performed using Fugene 6 according to the manufacturer's protocol (Roche Applied Sciences). Plasmids for fusion transcripts *SLC45A3-BRAF*, *BRAF* Ex8-stop, and *BRAF* Ex10-stop and *BRAF* mutant V600E were used along with control plasmids (pDEST40 and pBABE respectively). Three days after transfection, cells were split into 140-mm dishes containing DMEM with 5% Calf Serum (Life Technologies). The cultures were fed every 3-4 days. After 3 weeks, the cells were stained with 0.2% crystal violet in 70% ethanol for the visualization of foci, and were counted on colony counter (Oxford Optronix, software v4.1, 2003). Foci counts were further confirmed manually.

***BRAF^{V600}* Mutation Detection by Pyrosequencing**

One to 2 μg of total RNA isolated from fresh frozen localized prostate cancer (n=229), metastatic prostate cancer (n=37) and benign prostate (n=8) tissue samples, and a panel of melanoma (34), gastric cancer (25) was converted into cDNA using Superscript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Biotinylated sequencing templates were generated by PCR amplification of a 375 bp fragment spanning the mutation in codon 600 (V600, Exon 15) of the *BRAF* gene using primers from PyroMark Q24 BRAF kit (Biotage-Qiagen) according to manufacturer's instructions. 10 μl of the biotinylated PCR products were immobilized on streptavidin coated Sepharose beads (Streptavidin Sepharose High Performance, GE Healthcare) using Pyromark Q24 Vacuum Prep Workstation, followed by removal of non-biotinylated strand by sodium hydroxide denaturation followed by wash in

neutralization buffer and 70% ethanol. The single stranded biotinylated templates were then mixed with 0.3 mM sequencing primer and 'sequencing by synthesis' was carried out through dispensation of the query nucleotide sequence using PyroMark Q24 platform, as described before. The nucleotide sequence ACAG**A/T**GAAA for codon 600 was analyzed and visualized by Pyromark Q24 1.0.10 software. A panel of 9 melanoma cell lines (SK-MEL-2, SK-MEL-5, SK-MEL-19, SK-MEL-28, SK-MEL-29, SK-MEL-103, G-361, Malme-3M, mel-1 with known mutation status was used to serve as assay standards.

NIH3T3-*SLC45A3-BRAF* or RWPE- *SLC45A3-BRAF* Xenograft Models

Four week old male Balb C nu/nu mice were purchased from Charles River, Inc. (Charles River Laboratory, Wilmington, MA). Stable polyclonal NIH3T3 cells or RWPE over-expressing fusion transcript *SLC45A3-BRAF* or vector pDEST40 or single clone (5×10^6 cells) were resuspended in 100µl of saline with 20% Matrigel (BD Biosciences). Cells were implanted subcutaneously into the left flank region of the mice. Mice were anesthetized using a cocktail of xylazine (80-120 mg kg^{-1} IP) and ketamine (10 mg kg^{-1} IP) for chemical restraint before implantation. Ten mice were included in each group. Growth in tumor volume was recorded weekly by using digital calipers and tumor volumes were calculated using the formula $(\pi/6)$ $(L \times W^2)$, where L = length of the tumor and W = width. All procedures involving mice were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan and conform to their relevant regulatory standards.

SUPPLEMENTARY TABLE

Supplementary Table 1. Clinicopathological characteristics of the index cases in prostate, gastric cancer and melanoma with *BRAF* and *RAF1* gene rearrangement

a

	Sample ID	Age	Diagnosis	Gleason Score	CR	*ERG* rearrangement	*BRAF* rearrangement	*RAF1* rearrangement	Fusion
BRAF	PCA3	59	PCA	4+4	-	-	+	-	*SLC45A3-BRAF*
	PCA44	75	PCA	4+4	+	-	+	-	
	PCA45	NA	PCA	4+4	+	-	+	-	
	PCA46	89	PCA	5+4	+	-	+	-	
	MET37	63	MET	4+5	+	+	+	-	
	PCA47	62	PCA	4+3	-	-	5'del	-	
RAF1	PCA17	NA	PCA	3+4	-	-	-	+	*ESRP1-RAF1*
	PCA48	66	PCA	3+3	-	-	-	3'del	
	MET36	62	MET	NA	+	-	-	3' del	
	PCA49	NA	PCA	NA	-	-	-	3' del	

b

Sample ID	Age	Sex	Diagnosis	*BRAF* rearrangement	*RAF1* Rearrangement	Fusion
GCT15	61	F	Gastric adenocarcinoma	+	-	*AGTRAP-BRAF*
GC#10	52	F	Adenocarcinoma of GE junction	5' del	-	-
MEL23	58	M	Metastatic Melanoma	+	-	-
MEL24	88	F	Metastatic Melanoma	-	+	-

CR: Castration-resistant: PCA, clinically localized prostate cancer; MET, metastatic prostate cancer

SUPPLEMENTARY REFERENCES

[1] Maher, C. A. et al., Transcriptome sequencing to detect gene fusions in cancer. *Nature* 458 (7234), 97 (2009).

[2] Kleer, C. G. et al., EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 100 (20), 11606 (2003).

[3] Cao, Q. et al., Repression of E-cadherin by the polycomb group protein EZH2 in cancer. *Oncogene* 27 (58), 7274 (2008).

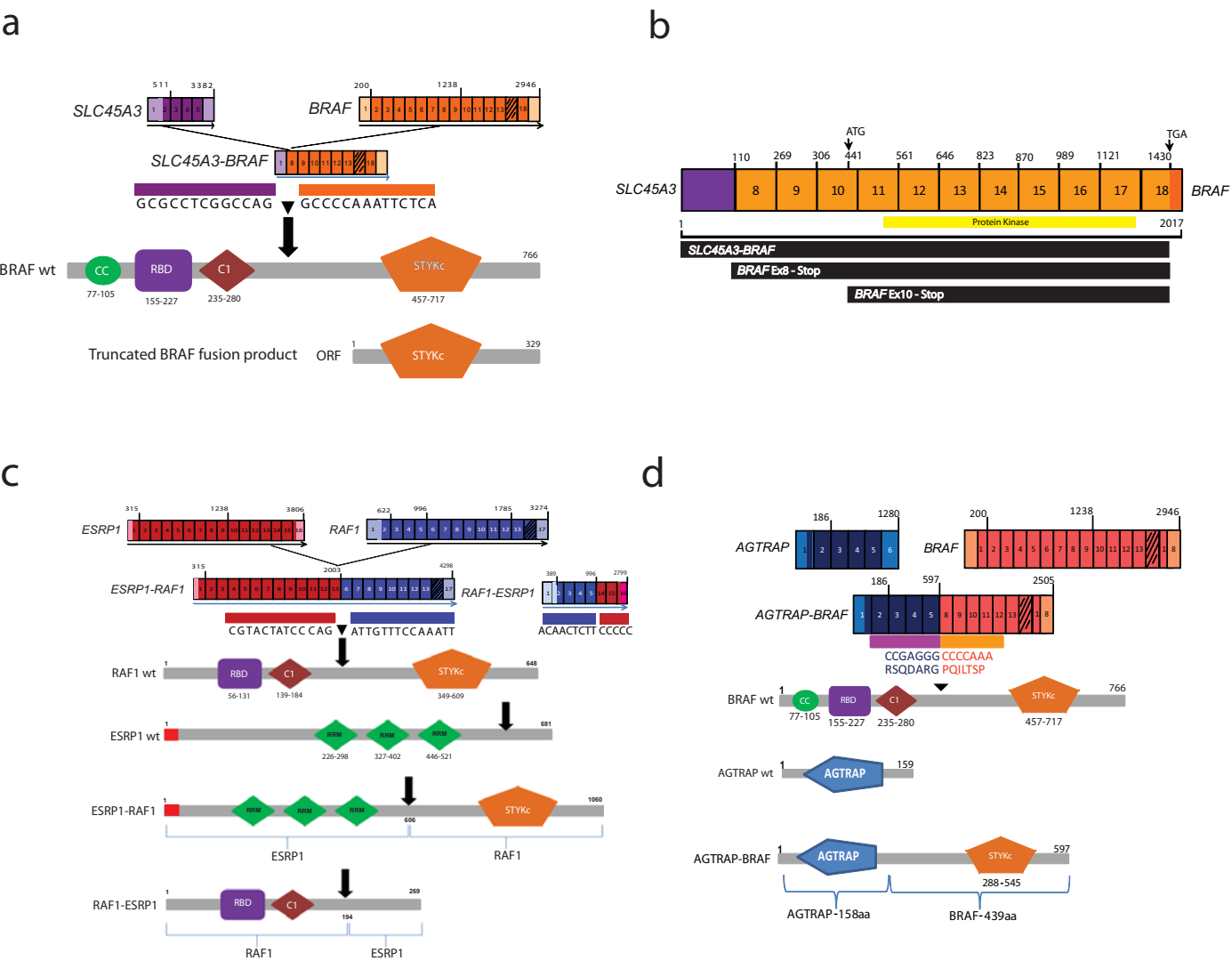

Figure S1 | Exon and protein domain structure of *BRAF* and *RAF1* wild type and fusion gene constructs. **a**, Schematic diagram showing the exon structure of wild type *SLC45A3* (purple) and *BRAF* (orange) and *SLC45A3-BRAF* fusion gene in the top panel. 5' and 3' untranslated exons are shown in light shaded colors. The protein domain structure of wild type BRAF (766aa) and truncated BRAF (329aa) are shown in the bottom panel. The first untranslated exon of *SLC45A3* fused with exon 8 of *BRAF* result in the loss of the N-terminus RAS binding domain (RBD) in BRAF and expression of a 329 aa long truncated BRAF protein retaining the entire functional kinase domain. **b.** Full length *SLC45A3-BRAF* fusion transcript (2017bp) containing the first un-translated exon of *SLC45A3* (purple box) and exon 8 to the last exon of *BRAF* (orange boxes) was cloned into pDEST40 vector. The predicted open reading frame (ORF) starts with the ATG (position 441) and stop codon (TGA-position 1430). The 990bp ORF forms a 37kDa truncated BRAF protein with intact protein kinase domain. 3' Un-translated region of *BRAF* gene is indicated as dark orange shade in the last exon. Horozontal bars indicate the three constructs (*SLC45A3-BRAF*, *BRAF* Ex8-Stop and *BRAF* Ex10-Stop) used in the functional validation experiments. **c**, Schematic diagram showing the exon structure of wild type *ESRP1* (red) and *RAF1* (blue) genes and *ESRP1-RAF1* (left) and *RAF1-ESRP1* (right) reciprocal fusion transcripts in the top panel. 5' and 3' untranslated exons are shown light shaded colors. The *ESRP1-RAF1* fusion produced by balanced reciprocal translocation result in the expression of two reciprocal fusion transcripts. The fusion between exon 13 of *ESRP1* with exon 6 of *RAF1* result in a 4298bp fusion transcript expressing a 1060aa fusion protein containing the RNA recognition motif of ESRP1and entire functional kinase domain of RAF1. The reciprocal fusion transcript produced by the fusion between exon 5 of *RAF1* and exon 14 of *ESRP1* result in a 2799bp transcript with an open reading frame coding for a 269 aa protein. Schematic diagram showing the exon structure of wild type *AGTRAP* (blue) and *BRAF* (orange) and *AGTRAP-BRAF* fusion gene in the top panel. 5' and 3' untranslated exons are shown in light shaded blue and orange colors. The protein domain structure of wild type BRAF (766aa) and AGTRAP (159aa) and the AGTRAP-BRAF fusion protein (597aa) are shown in the bottom panel. The breakpoint in *AGTRAP* located 6 nucleotides before the stop codon in the last exon was fused with exon 8 of *BRAF* resulting in an open reading frame (ORF) of 597aa producing an aproximately 70kDa AGTRAP-BRAF fusion protein.

Palanisamy, et al. Supplementary Figure 2

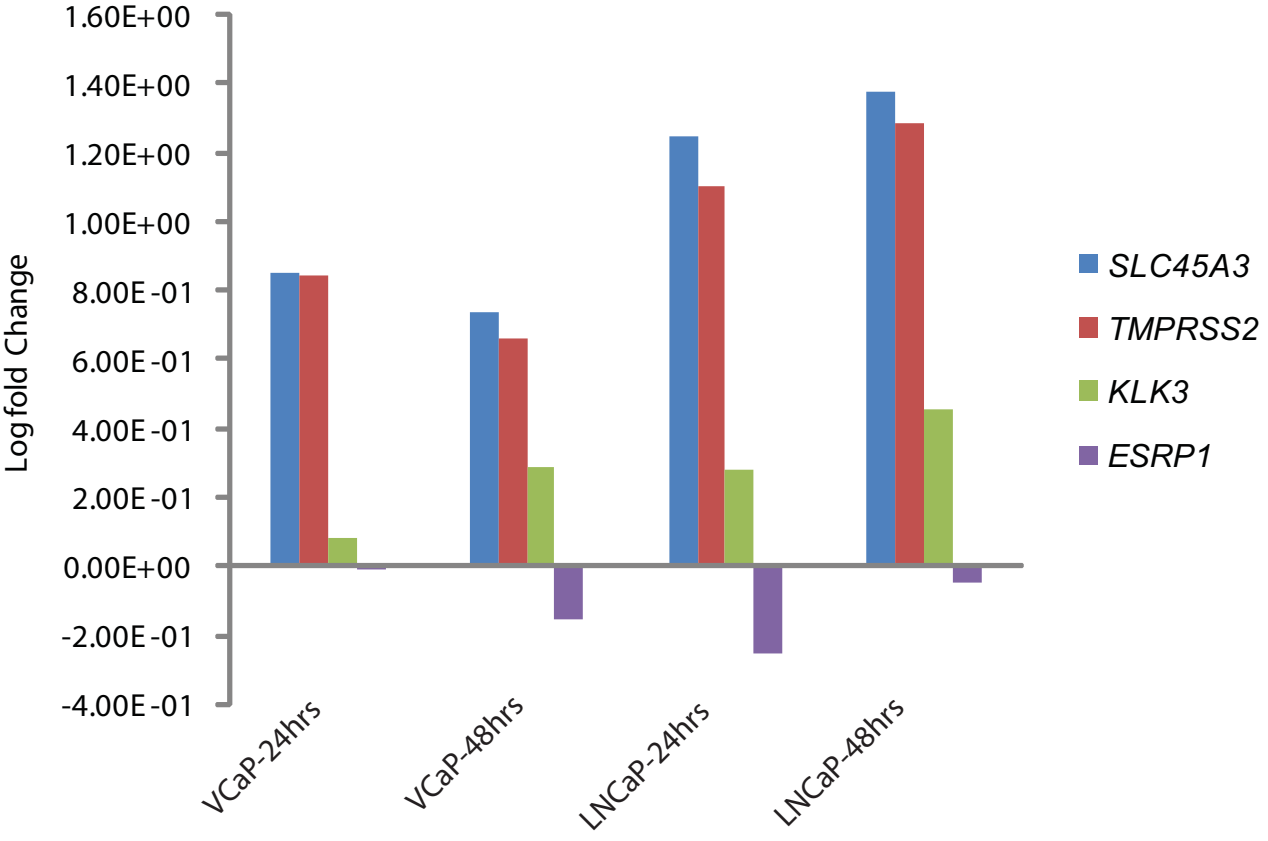

Figure S2 | *ESRP1*, the 5' fusion partner of *RAF1* is not regulated by androgen. VCaP and LNCaP cells treated with R1881for 24 hrs and 48hrs and gene expression monitored by Agilent microarrays.

Palanisamy, et al. Supplementary Figure 3

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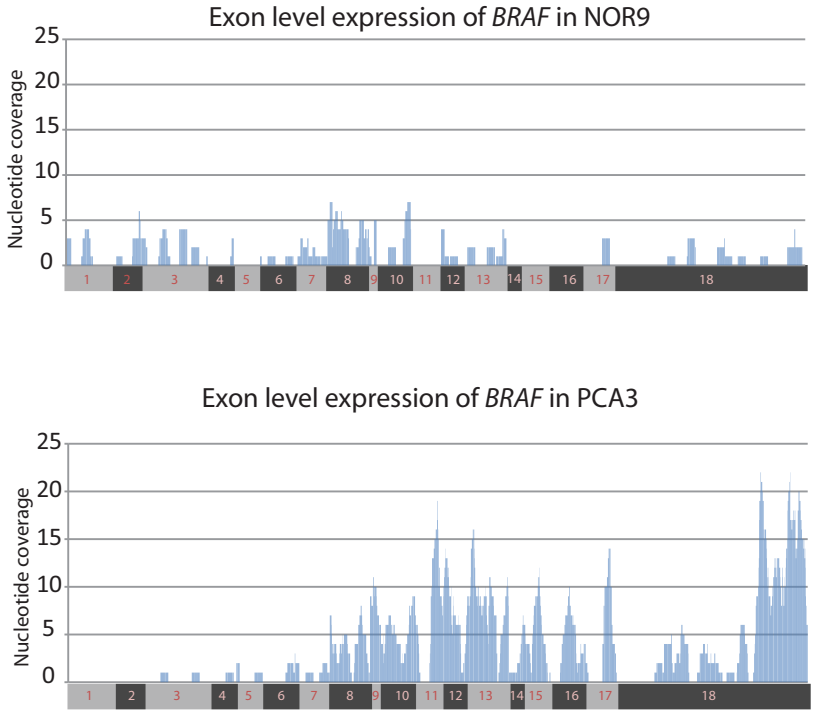

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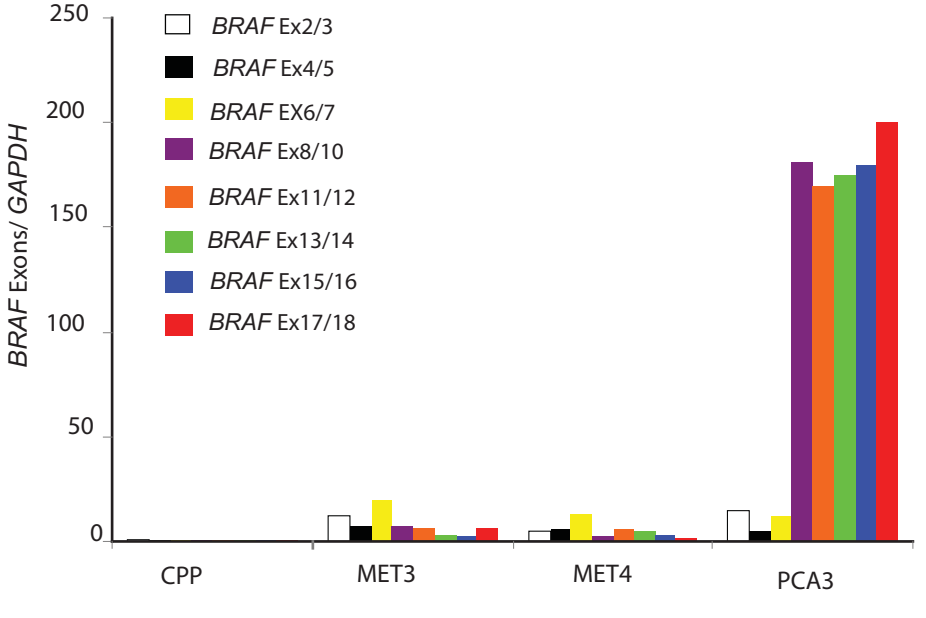

Figure S3 | RNA-seq exon coverage and qRT-PCR validation of *BRAF* exons in normal, metastatic prostate samples and index case (PCA3). **a**. Exons are shown at the bottom in alternating shades of grey. Blue bars highlight the nucleotide coverage across the exons. **b**. qRT-PCR using exon spanning primers showing high level expression of BRAF exons 8-18 relative to the exons 1-7 in PCA3.

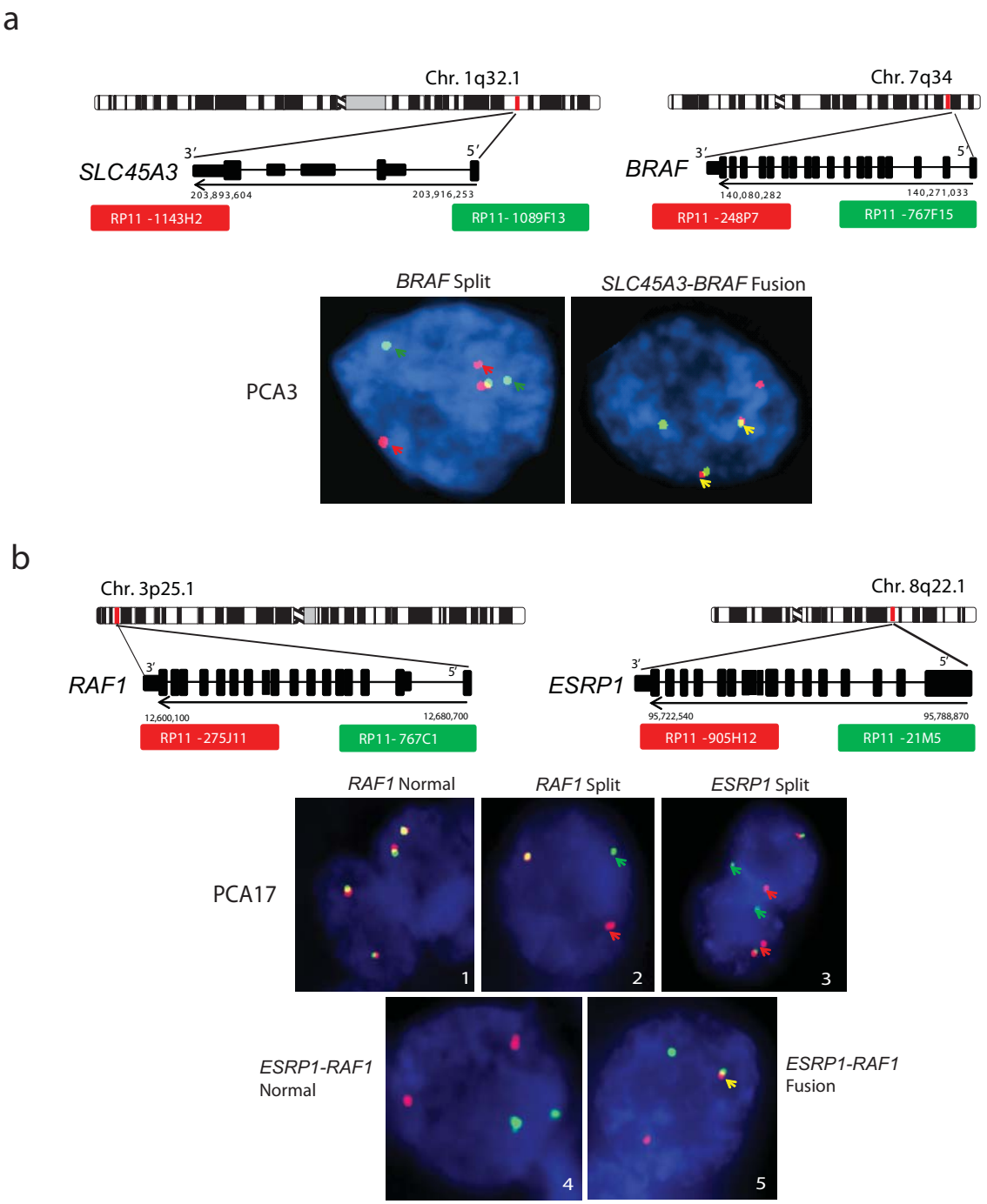

Figure S4 | Genomic organization and FISH validation of *BRAF* and *RAF1* gene rearrangement. **a**, Schematic diagrams showing the genomic location of *SLC45A3* (left) and *BRAF* (right) genes on chromosome1q32.1 and 7q34 respectively. The green and red rectangles with BAC clone identification numbers indicate the 5' (green) and 3' (red) clones used in the FISH analysis on PCA3 (bottom panel). *BRAF* split probe show two copies of rearranged chromosomes (green and red arrows) and *SLC45A3* 5'-*BRAF* 3' fusion probes show two copies of fusion signals (yellow arrows). **b**, Schematic diagrams showing the genomic location of *ESRP1* (right) and *RAF1* (left) genes on chromosome 8q22.1 and 3p25.1 respectively. The green and red rectangles with BAC clone identification numbers indicate the 5' (green) and 3' (red) clones used in the FISH analysis on PCA17 (bottom panel). *RAF1* split probe show two colocalizing signal in the normal cells (1) rearranged signal pattern in tumor cells (2) (red and green arrows). *ESRP1* split probe show rearrangement in the tumor cells (3) (green and red arrows). 5' *ESRP1* probe and 3' *RAF1* probes show separate green and red signal in normal cells (4) and one fusion signal in tumor cells (5).

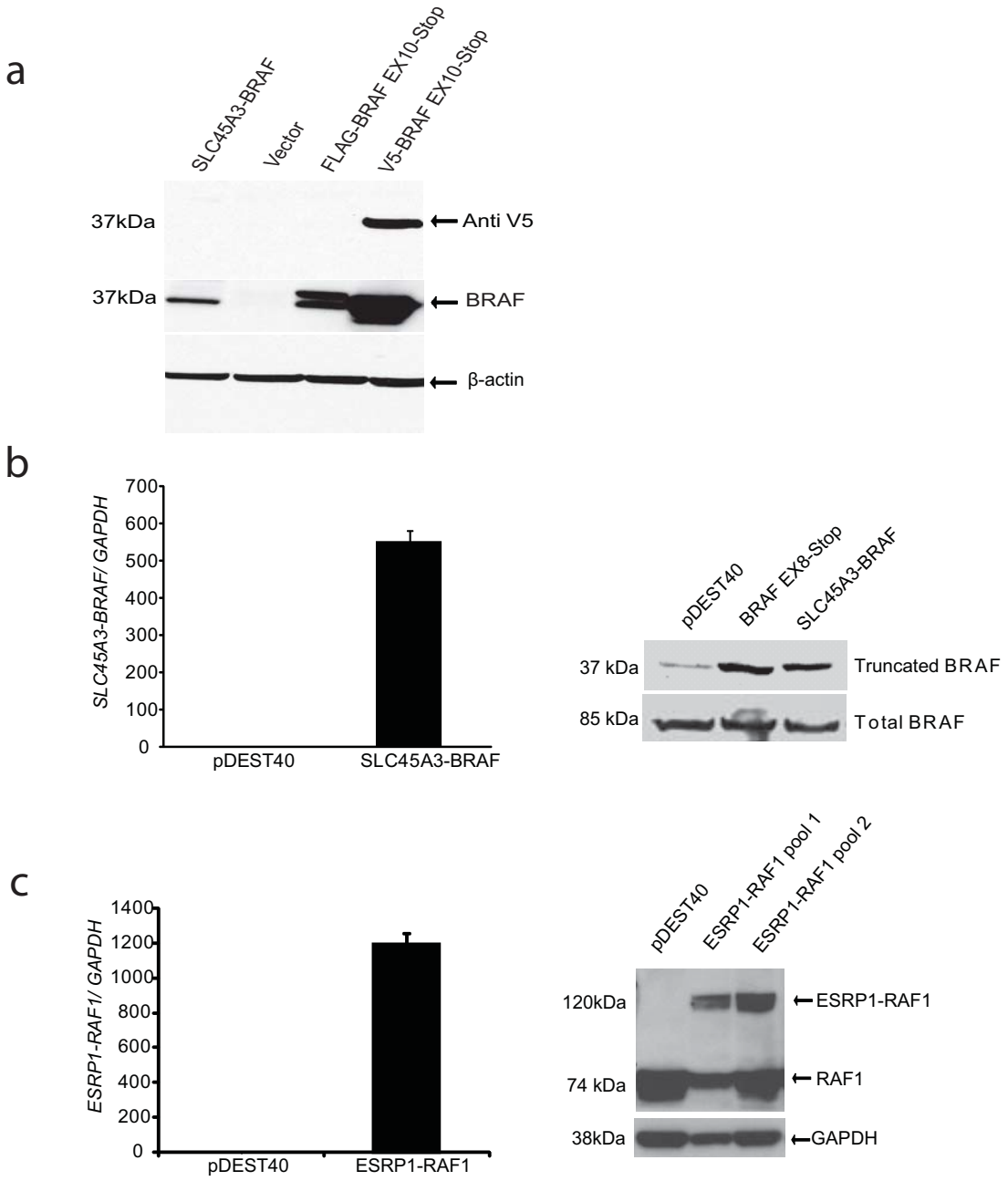

Figure S5 | Validation of expression constructs by qRT-PCR and western blot analysis. a. *SLC45A3-BRAF* expression constructs with N-terminus Flag tag and C-terminus V5 tag were transfected in HEK293 cells. Western blots generated using cell lysates were hybridized with antiV5 and BRAF antibodies. The double bands with BRAF antibody indicates the two different protein products produced by alternate start codons. **b.** Stable expression of *BRAF* EX8-Stop and *SLC45A3-BRAF* fusion construct in RWPE cells was validated by qRT-PCR and western blot analysis using BRAF specific antibody. Fusion specific primer from exon 1 of *SLC45A3* and exon 10 of *BRAF* was used in the qRT-PCR validation (left panel). In both the constructs by immunoblot analysis, the expected 37kDa protein was detected along with the endogenous 85kDa total BRAF protein (right panel). **c.**Stable expression of *ESRP1-RAF1* fusion construct in RWPE cells was validated by qRT-PCR (left panel) and western blot analysis (right panel) using RAF1 specific antibody. Fusion gene specific primers were used in the qRT-PCR validation. The expected 120kDa fusion protein was detected along with 74kDa endogenous RAF1 protein. **c.**

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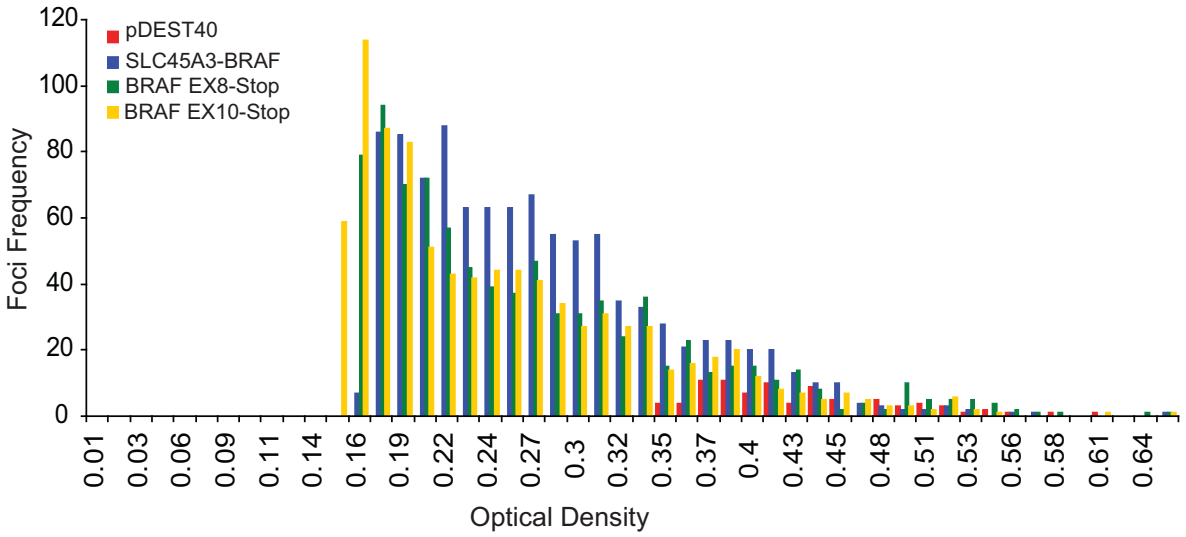

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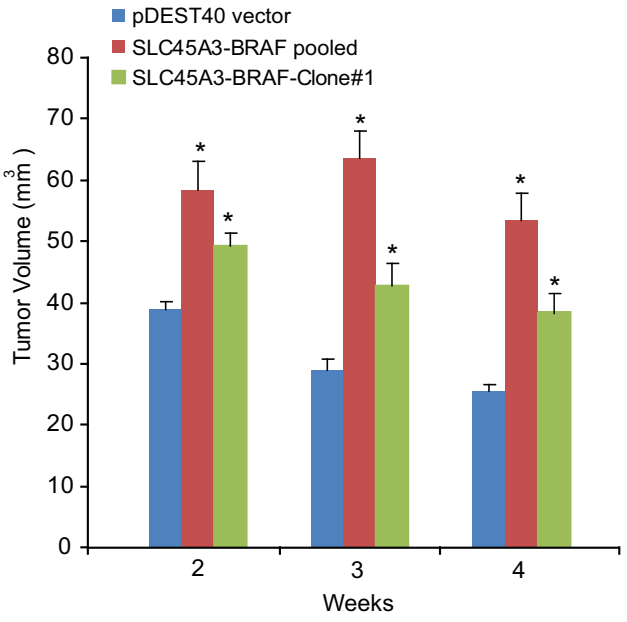

Figure S6 | a, Comparison of the foci frequencies of NIH3T3 cells expressing fusion transcript *SLC45A3-BRAF*, *BRAF* Ex8-Stop and *BRAF* Ex10-Stop and pDEST40 vector. Foci densities of NIH3T3 cells expressing fusion transcripts *SLC45A3-BRAF, BRAF* Ex8-stop, *BRAF* Ex10-stop and vector control (pDEST40) were evaluated using colony counter (Oxford Optronix Ltd., Oxford UK, software v4.1, 2003). Values for minimum colony radius and maximum colony radius were set at 0.10 mm and 2.75 mm respectively, while minimum colony density was fixed at 0.15 optical densities (OD). Bar diagram show frequencies of foci on y-axis falling on the range (0.01 to 0.65 OD) of optical densities on x-axis. **b, Stable RWPE cells over-expressing *SLC45A3-BRAF* form small tumors in Balb C nu/nu mice.** Stable RWPE cells (5x10) over-expressing *SLC45A3-BRAF* pooled or single clone were implanted sub-cutaneously in male immunodeficient mice. Tumor volume was monitored weekly. **P* <0.05 significant difference.

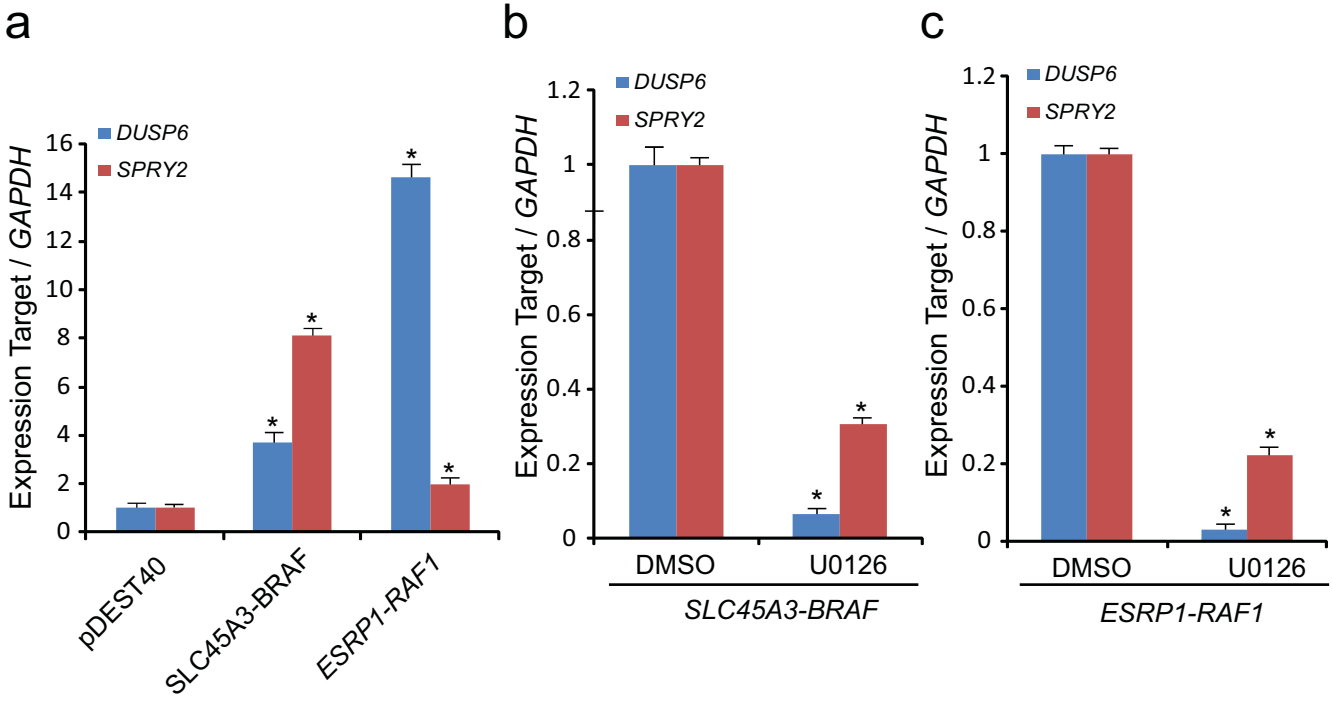

Figure S7 | Down-regulation of genes involved in the MEK pathway after U0126 treatment.
a, Stable RWPE cells expressing *SLC45A3-BRAF* or *ESRP1-RAF1* showed increase in *DUSP6* or *SPRY2* mRNA expression as compared to pDEST40 vector. **b, c,** MEK inhibitor (U0126, 10μM) treatment for 2 hours in Keratinocyte-spplement free media significantly decreases expression of these genes in RWPE cells expressing *SLC45A3-BRAF* or *ESRP1-RAF1*. Gene expression was normalized with vehicle treated samples. **P* <0.05 significant difference.