



Supplementary Information for

Unraveling the hidden role of a uORF-encoded peptide as a kinase inhibitor of PKCs

Divya Ram Jayaram^{1†}, Sigal Frost^{1†}, Chanan Argov^{2#}, Vijayasteltar Belsamma Liju^{1#}, Nikhil Ponnor Anto^{1¶}, Amitha Muraleedharan^{1¶}, Assaf Ben-Ari¹, Rose Sinay¹, Ilan Smoly², Ofra Novoplansky¹, Noah Isakov¹, Debra Toiber³, Chen Keasar⁴, Moshe Elkabets¹, Esti Yeger-Lotem² and Etta Livneh^{1*}

Correspondence to: etta@bgu.ac.il

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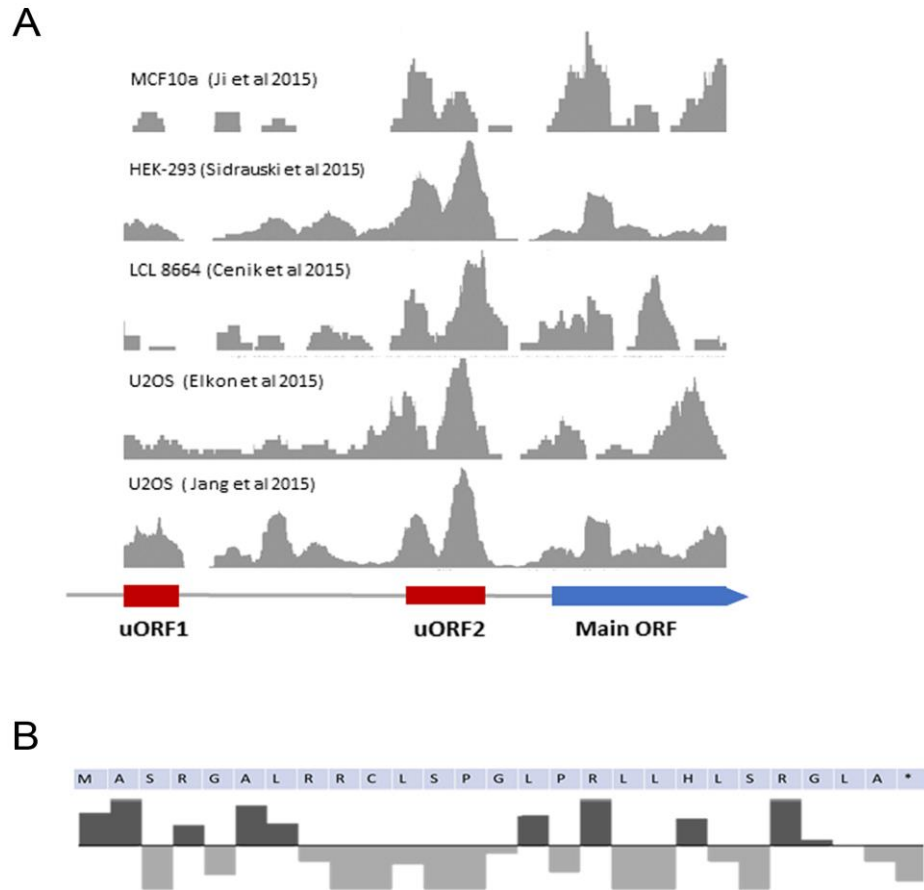


Fig. S1. Ribosome profiling reads across PKC η mRNA support translation of uORF2. (A) Ribosome profiling experiments in human cell lines show more reads in uORF2 compared to uORF1, and in most cell lines relative to PKC η main ORF. The cell lines portrayed were MCF10A(1), HEK293(2), LCL8664(3), U2OS(4, 5). (B) Codon-level conservation at uORF2 according to the PhyloCSF raw track on UCSC Genome Browser. 10/26 codons are conserved, including codons encoding the pseudo substrate site of the uORF2 peptide.

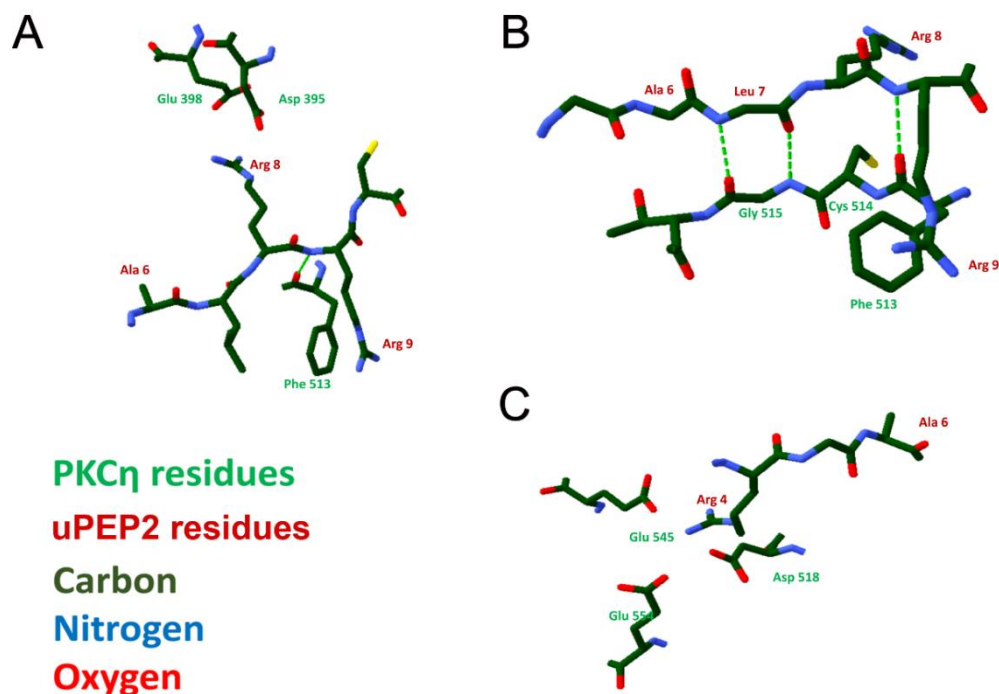


Fig. S2. Suggested interactions between PKC η and uPEP2. The molecular model of the uPEP2/PKC η complex suggests several interactions that may be responsible for the affinity and specificity of the peptide. Amino acids marked in green or magenta belong to PKC η or uPEP2, respectively. Atom color code: Dark green – carbon, red – oxygen, blue – nitrogen. A dashed green line depicts a hydrogen bond. (A) Putative specific interactions of arginine residues 8 and 9 of uPEP2. Arg 9 faces the solvent and pi-stacks with Phe 513 of PKC. Arg 8 faces the protein and its positively charged guanidino group interacts with two negatively charged residues of PKC, Glu 398 and Asp 395. (B) Beta-sheet residues and hydrogen bonds between PKC η and uPEP2 strands. The side-chain of Leu7 is hidden for clarity. (C) Putative electrostatic interactions of the positively charged Arg4 of uPEP2 with the negatively charged Asp 518, Glu 545, and Glu 554 of PKC η .

efficient inhibition of PKC η catalytic activity. Autoradiograph and immunoblots showing the effect of uPEP2 full-length peptide in inhibiting the kinase activity of PKC η compared to shorter peptides lacking N-terminal 1-9 residues (uPEP2₍₁₀₋₂₆₎) and the internal η PS peptide. (B) uPEP2 inhibits the kinase activity of novel PKCs compared to classical and atypical PKCs. Kinase activity assays of different PKC isoforms were performed in the absence or presence of uPEP2 and MBP as a substrate. ³²P-MBP and MBP protein band signals were quantified using the Image Lab software, and the relative amounts of ³²P-MBP/MBP are presented in bar diagrams. Anti-HA detecting overexpressed PKC expression, and MBP protein bands were used as loading controls. IgL, Ig light chain.

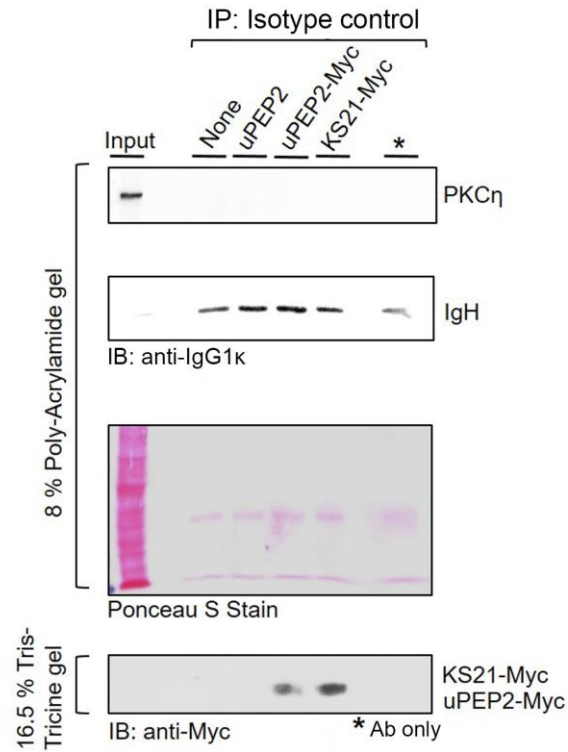


Fig. S4. IgG control antibody does not bind PKC η . HEK-293T lysates overexpressing PKC η isoform were subjected to immunoprecipitation using Protein A/G beads pre-adsorbed with anti-mouse IgG1 κ antibody and Myc-tagged peptides (uPEP2, uPEP2-Myc, or the non-relevant control peptide KS21-Myc).

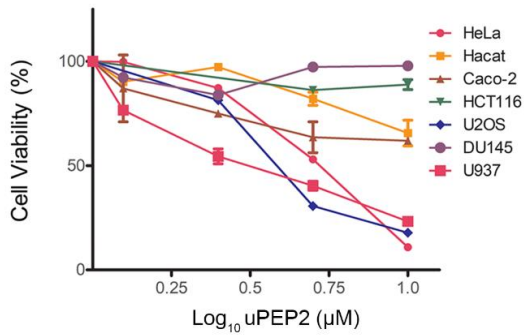
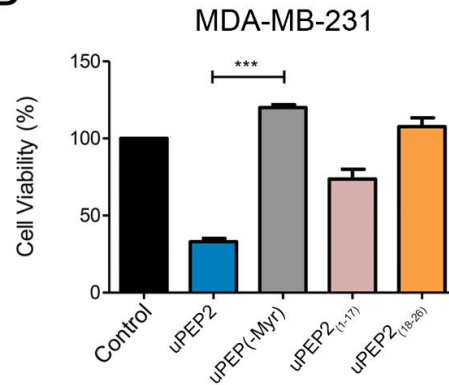
A**B**

Fig. S5. uPEP2 inhibits cell proliferation of various cancer cells. (A) uPEP2 inhibited cell viability of HeLa (cervix), U2OS (osteosarcoma) and U937 (lymphoma), whereas DU145 (prostate) and HCT116 (colon) were not affected. (B) The control peptides uPEP2(-Myr) (incapable of entering the cells), uPEP2₍₁₋₁₇₎ and uPEP2₍₁₈₋₂₆₎ (deletion mutants) were less effective in suppressing MDA-MB-231 proliferation compared to full-length uPEP2. P-values calculated using one-way ANOVA. ***P ≤ 0.001 and ns ≥ 0.05

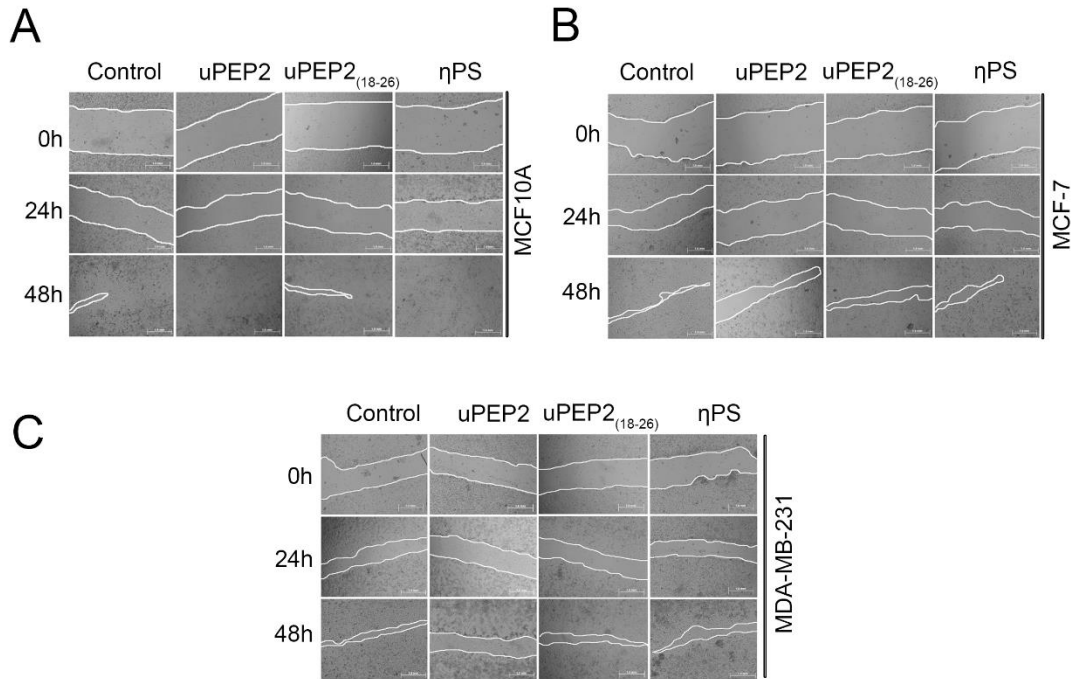


Fig. S6. uPEP2 inhibits wound closure of MCF7 and MDA-MB-231 cells but not of non-transformed MCF10A. Scratch assays were carried out in the presence of 5 μ M uPEP2, uPEP2₍₁₈₋₂₆₎ or η PS as described in Materials and Methods. Images were taken at indicated time points (0, 24 h & 48 h), and wound areas were normalized to time 0 (Scale bar-1mm). Data shown represents at least three independent experiments.

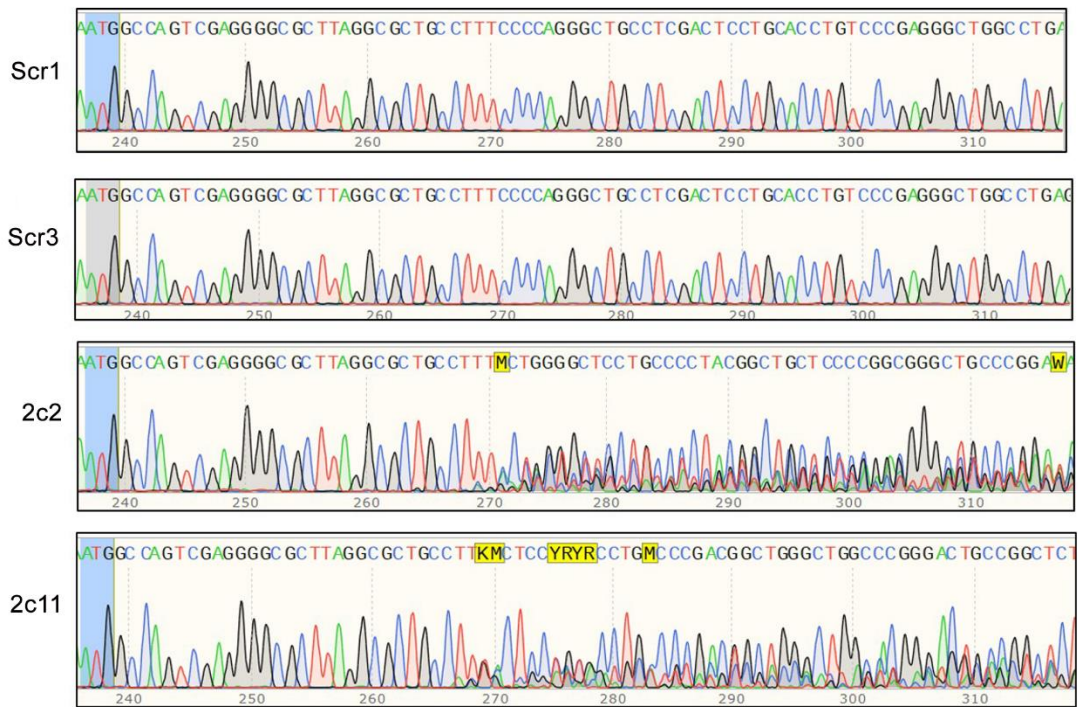


Fig. S7. Sequencing data of CRISPR/Cas9 edited uORF2 clones. Sanger sequencing chromatograms showing the genomic analysis of MCF-7 CRISPR/Cas9 clones (scrambled and uORF2-mutated) using primers (**Forward:** 5'-TTGGAAGGGACGGTCGG-3'; **Reverse:** 5'-GTTAGCGCAAAACTCCTCGT-3') flanking the DNA loci targeted by the uORF2 sgRNA (5'-CACCGTTAGGCGCTGCCTTTCCCCA-3'). Scrambled clones: Scr1 and Scr3; Mutated clones: 2c2 and 2c11.

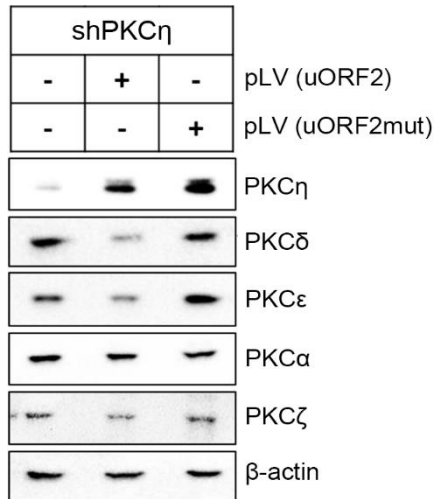


Fig. S8. Translation of uORF2 suppresses the expression of novel PKCs. PKC η -knockdown cells, shPKC η (sh3-3) (6, 7), were transduced with pLV(uORF2) and pLV(uORF2mut) expression plasmids as described in Materials and Methods. Positive cells expressing these plasmids were selected using puromycin (5 μ g/ml) and geneticin (G418; 0.2 mg/ml). Cell lysates were separated on 10% polyacrylamide gels and immunoblotted using antibodies directed against indicated PKC isoforms and β -actin (as loading control). Data shown represents at least three independent experiments.

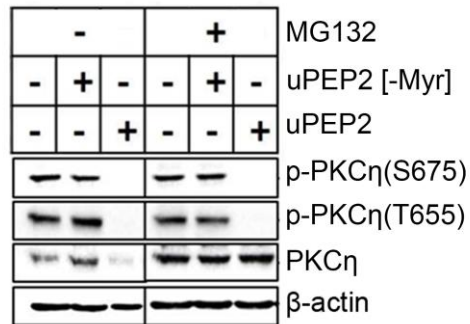
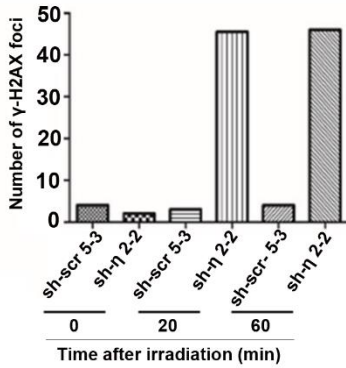


Fig. S9. The phosphorylation of PKC η priming sites, Ser675 and Thr655, is reduced by uPEP2 treatment of MCF-7 cells. MCF-7 cells were incubated in the presence/absence of the proteasome inhibitor MG132 (10 μ M) in serum-free DMEM for 1 h, followed by the addition of the peptides uPEP2[-Myr] and uPEP2 (10 μ M) for 4 h. Cells were then lysed as described in Materials and Methods and samples were separated on 10% polyacrylamide gels and immunoblotted using PKC η (p-S675), PKC η (p-T655) and PKC η specific antibodies. β -actin was used as a marker for equal protein loading .

A

Induction of γ -H2AX foci following exposure to X-ray



B

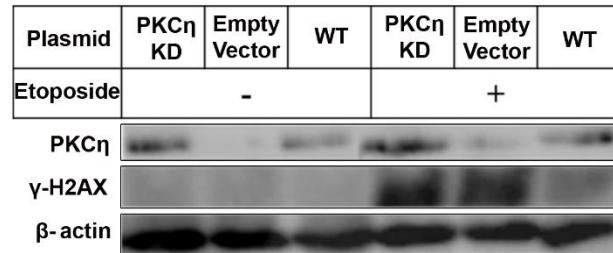


Fig. S10. The phosphorylation of γ H2AX is suppressed by PKC η . (A) MCF-7 clones expressing shPKC η (sh2-2) or scrambled control cells (scr 5-3) described in (7) were treated with X-ray (200 rad) for the indicated time points. The numbers of γ H2AX foci were detected using high-throughput cell microscopy. (B) The kinase activity of PKC η is required for the suppression of γ H2AX phosphorylation. PKC η -knockdown cells, shPKC η (sh2-2), were transfected with WT-PKC η cDNA, the kinase-dead PKC η -KD plasmid, and the control empty vector pHACE. The cells were treated with etoposide (50 μ M) and γ H2AX phosphorylation was detected using immunoblot analysis. β -actin was used as a marker for equal protein loading.

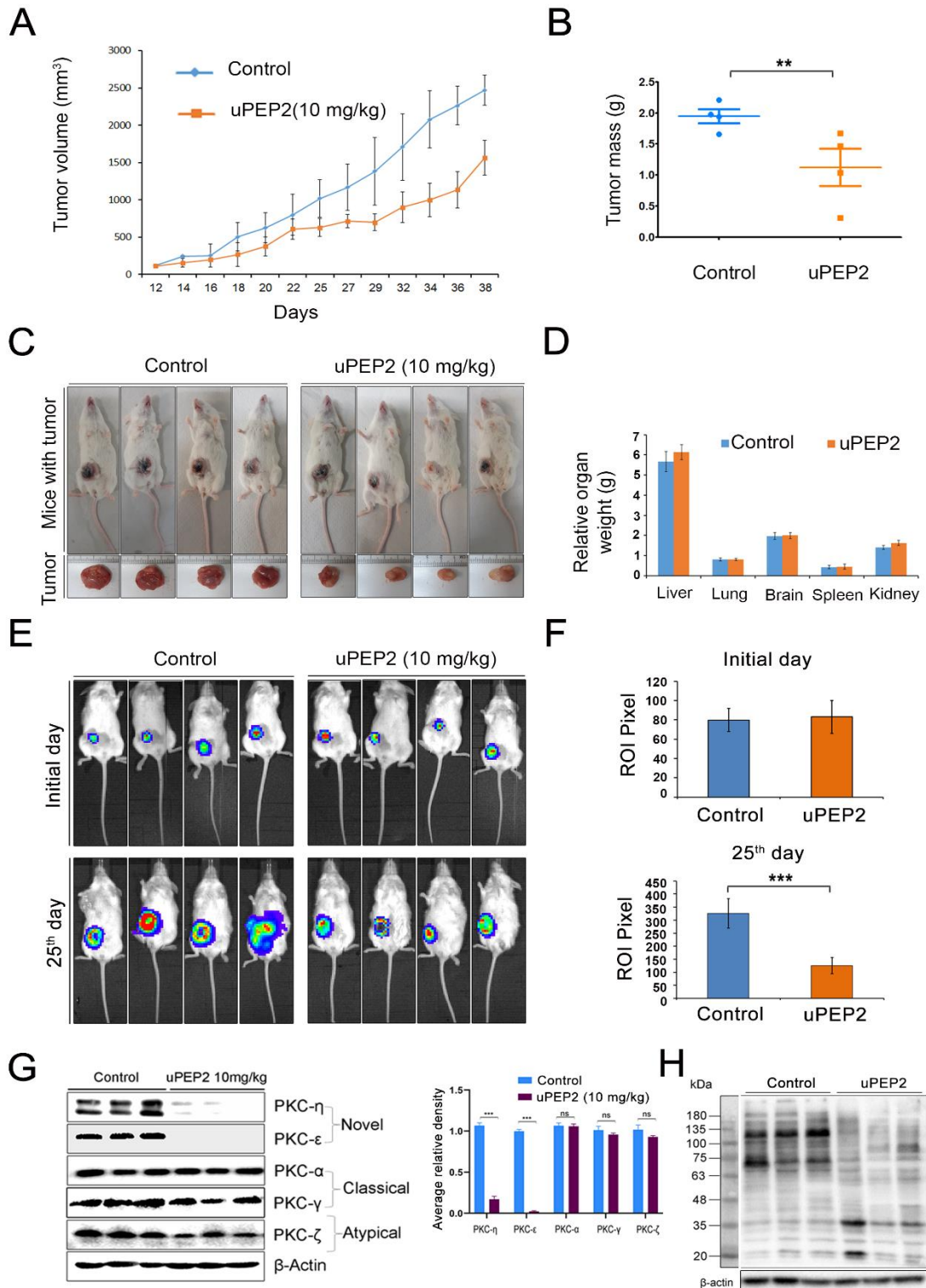


Fig. S11. uPEP2 inhibits the growth of human triple-negative breast cancer MDA-MB-231 xenografts in NOD-SCID mice. (A) Graph showing uPEP2 and Control treated

MDA-MB-231 xenograft tumors in mammary fat pads of NOD-SCID mice at different time points. Each value represents the mean volume (Mean \pm SEM) of tumors obtained from four mice. (B) Mean tumor mass of uPEP2 and control treated mice (Mean \pm SEM). (C) Images showing significant reduction of breast tumor mass by uPEP2. (D) uPEP2 has no adverse effects on relative organ weights (Liver, Lungs, Spleen, Kidney, and Brain) of breast tumor-bearing NOD SCID mice. (E) Live bioluminescent images of NOD-SCID tumor-bearing mice at initial and final days of uPEP2 treatment. (F) Quantification of the bioluminescent intensity of mice tumors shown in (E) at Regions of Interest (ROI) in pixels on Initial day (upper panel) and after 25 days of treatment (lower panel). Results are shown as Mean \pm SEM (n=4). P-values were calculated with one-way ANOVA. ***P-values \leq 0.001, **P-values \leq 0.01, *P-values \leq 0.05 and ns-values \geq 0.05. (G) uPEP2 down-regulates expression of novel PKCs in orthotopic xenograft breast cancer tumors in NOD-SCID mice. Dissected tumors were lysed and subjected to immunoblot analysis as described in Materials and Methods. Comparative quantification of novel, classical and atypical PKC protein expressions are represented in the bar diagram. (H) Phosphorylation of PKC substrates is reduced in uPEP2 treated breast tumor samples. Western blot analysis of cell extracts prepared from Control and uPEP2-treated tumors were immunoblotted with an antibody detecting the phosphor-(Ser) substrates of PKCs. P-values calculated using one-way ANOVA. ***P \leq 0.001, **P \leq 0.01, *P \leq 0.05 and ns \geq 0.05.

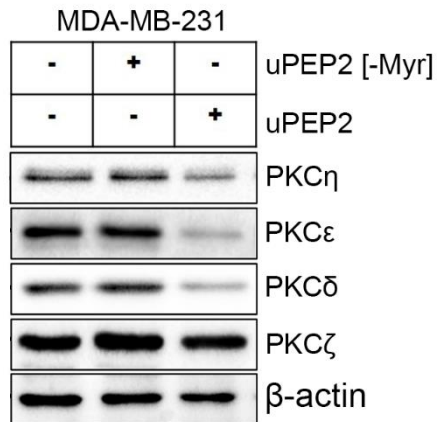


Fig. S12. Protein levels of novel PKC's are selectively downregulated in cultured MDA-MB-231 upon uPEP2 treatment. MDA-MB-231 cells, grown in a 100 mm dish to sub-confluence, were incubated in the absence/presence of uPEP2 or the control peptide uPEP2[-Myr] for 4 h (uPEP2[-Myr] peptide cannot enter cells). Cell extracts prepared from these cells were separated by 10% polyacrylamide gels and immunoblotted with the indicated anti-PKC antibodies. β -actin was used as a marker for equal protein loading.

Organism	uORF2 sequences	uPEP2 (translated)	RefSeq ID	Uorf2 location (nc)	Main ORF location (nc)	mRNA status
Human	ATGGCCAGTCGAGGGGCGCTTAGGCGCTGCCTT TCCCAGGGCTGCCTCGACTCCTGCACCTGTCC CGAGGGCTGGCCTGA	MASRGALRRCLSP GLPRLHLRGLA	NM_0062 55.4	512- 592	660-2711	verified
Gorilla	ATGGCCAGTCGAGGGGCGCTTGGGCGCTGCCTT TCCCAGGGCTGCCTCGACTCCTGCAGCTGTCC CGAGGGCTGGCCTGA	MASRGALGRCLSP GLPRLQLSRGLA	XM_0040 55266.2	512- 592	660-2711	predicted
Elephant	ATGGCGGGTCGAGGGGGTCTTGGGCGCTGCTTT TCCCAGGAGCTGCCTCCACTCCTGCGACTACCG CGAGGGCTGGCCTGA	MAGRGGGLGRCFSP PELPLLRLPRGLA	XM_0235 46623.1	236- 316	390-2483	predicted
Dog	ATGACCAGTGGAGGGGGGCTTGGGCGCTGCTTT TCCCAGGAGCTGCCTCCGCTCCGGCGGCTGCCG CGAGGGCTGGCCTGA	MTSGGGLGRCFSP ELRPLRRLPRGLA	XM_5478 44.6	230- 310	377-2428	predicted
Mouse	ATGGCCGGTCGAGGGGGCTCGGGCGCTGCTTC TTCCCAGAGCTGCCTCCACGCCGTGGCAGCGG AGAGGACTGCCCTGA	MAGRRLGRCFSP ELPPRPWQRRGLP	NM_0013 13977.1	220-297	804-2516	verified
Rat	ATGGCCGGTCGAGGGGGCTTGGGTGCTGCTTC TCCCAGGAGCTGCCTCCACGAGCGTGGCTGCCG AGAGGACTGCCCTGA	MAGRRLGCCFS RELPPRAWLRRGL P	NM_0310 85.2	40- 117	188-2239	verified

Table S1. uORF2 sequences in various mammalian species.

(#1)	5'-CCCTAACTCCGCCCAGTTCC'3(forward) 5'-GGCGTCTTCCCAGGTGGCTTTACCA-3' (reverse)
(#2)	5'-TGGTAAAGCCACCTGGGAAGACGCC-3'(forward) 5'-GGACTCTGGCACAAAATCGT-3' (reverse)
(#3)	5'-GCTAGAGCTCGGGCGCGCAGGGC-3'(forward) 5'-AAAGCCTAGGCGCTCTCCCCACTCGG-3' (reverse).
(#4)	5'-GCTAGAGCTCGGGCGCGCAGGGC-3'(forward) 5'-AAAGCCTAGGCAGCCCTCGGGACAGGTGCAG-3' (reverse).

Table S2. Primers for DNA constructs.

pLV(uORF2)	pLV[Exp]-EGFP:T2A:Puro-CBh>{uORF+PKCeta}(VB210108-1118fkc) https://en.vectorbuilder.com/vector/VB210108-1118fkc.html
pLV(uORF2mut)	pLV[Exp]-EGFP:T2A:Puro-CBh>{uORF2mutated+PKCeta}(VB210108-1125kbt) https://en.vectorbuilder.com/vector/VB210108-1125kbt.html

Table S3. Expression Plasmids pLV(uORF2) and pLV(uORF2mut).

Antibody	Catalogue No:	Vendor
Luciferase	L2164	Sigma-Aldrich
GFP	sc-9996	Santa Cruz Biotechnology
β -actin	691001	ICN Biomedicals Inc
HA	MMS-101R	BioLegend
FLAG	14793	Cell Signaling Technology
PKC ϵ	610085	BD Biosciences
PKC α	610108	BD Biosciences
PKC θ	610090	BD Biosciences
PKC δ	610398	BD Biosciences
PKC η	ab179524	Abcam
PKC ζ	ab108970	Abcam
PKC γ	ab71558	Abcam

MBP	SMI-99P	BioLegend
DNA damage antibody sampler kit	9947T	Cell Signaling Technology
PKC η (phospho T655)	ab5798	Abcam
PKC η (phospho S675)	Custom made	PhosphoSolutions
PKC η	NBP2-38711	Novus Biologicals
Ki-67	ab15580	Abcam
Phospho-(Ser) PKC substrate antibody	2261	Cell Signaling Technology
rabbit IgG	ab172730	Abcam
Mouse IgG1 kappa	12-4714-42	eBioscience, ThermoFisher Scientific
anti-uPEP2	740/2	Rabbit-polyclonal antibody developed in our lab against the last 17 amino acids of uPEP2 sequence
affinity-purified anti-Myc	clone 9E10	Mouse monoclonal antibody developed in the lab of Prof. Noah Isakov, Ben-Gurion University of the Negev

Table S4. Antibodies used in this study.

References

1. Z. Ji, R. Song, A. Regev, K. Struhl, Many lncRNAs, 5'UTRs, and pseudogenes are translated and some are likely to express functional proteins. *Elife* **4**, e08890 (2015).
2. C. Sidrauski, A. M. McGeachy, N. T. Ingolia, P. Walter, The small molecule ISRIB reverses the effects of eIF2 α phosphorylation on translation and stress granule assembly. *Elife* **4** (2015).
3. C. Cenik *et al.*, Integrative analysis of RNA, translation, and protein levels reveals distinct regulatory variation across humans. *Genome research* **25**, 1610-1621 (2015).
4. R. Elkon *et al.*, Myc coordinates transcription and translation to enhance transformation and suppress invasiveness. *EMBO Rep* **16**, 1723-1736 (2015).

5. C. Jang, N. F. Lahens, J. B. Hogenesch, A. Sehgal, Ribosome profiling reveals an important role for translational control in circadian gene expression. *Genome research* **25**, 1836-1847 (2015).
6. H. Raveh-Amit *et al.*, Protein kinase Ceta activates NF-kappaB in response to camptothecin-induced DNA damage. *Biochem Biophys Res Commun* **412**, 313-317 (2011).
7. U. Zurgil *et al.*, PKC η promotes senescence induced by oxidative stress and chemotherapy. *Cell Death & Disease* **5**, e1531; doi:1510.1038/cddis.2014.1481 (2014).