LETTER

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A naturally occurring antiviral ribonucleotide encoded by the human genome

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Supplementary Information for "A naturally occurring antiviral ribonucleotide encoded by the human genome"

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Methods

Materials. Cysteine, L-tryptophan, 2-mercaptoethanol, L-(+)-arabinose, ferric chloride, and 5'-deoxyadenosine (5'dA) were purchased from Sigma Corp (St. Louis, MO). N-(2-hydroxyethyl)piperizine-N'-(2-ethanesulfonic acid) (HEPES) and imidazole were purchased from Fisher Scientific. Pre-poured sephacryl S-200 gel-filtration columns were purchased from GE Biosciences (Piscataway, NJ). All other buffers and chemicals were of the highest grade available. *S*-adenosyl-L-methionine was synthesized and purified as described previously[1]. *E. coli* flavodoxin and flavodoxin reductase, and the pDB1282 plasmid were kind gifts from S. Booker's lab (Pennsylvania State University, State College, PA). UV-visible spectra were recorded on an Agilent 8453 spectrophotometer (Santa Clara, CA). Sonic disruption of *E. coli* cell suspensions was carried out with a 550 sonic dismembrator from Fisher Scientific using a horn containing a 0.5 inch tip. RNA oligonucleotides were from Dharmacon, Inc; DNA oligonucleotides were from Integrated DNA Technologies; nucleoside triphosphates (ATP, CTP, GTP and UTP) were Ultrapure solutions from GE Healthcare; [α-^{32}P]-ATP (3000 Ci/mmol) was from Perkin Elmer; all other reagents were of the highest grade from either Sigma, Fisher or VWR. All DNA vectors were sequence verified with dideoxy sequencing performed by Genewiz.

Protein sequence for rVIP construct.

*MHHHHHHSSGVDLGTENLYFQS*MGEPKETQETHEDPGSAQPTTPVSVNYHFTRQCNYKCGF
CFHTAKTSFVLPLEEAKRGLLLLKQAGMEKINFSGGEPFLQDRGEYLGKLVRFCKEELALPSVSI
VSNGSLIRERWFKDYGDYLDILAISCDSFDEQVNVLIGRGQGKKNHVENLQKLRKWCRDYKVA
FKINSVINRFNVDEDMNEHIKALSPVRWKVFQCLLIEGENSGEDALREAERFLISNEEFEAFLQR
HKDVSCLVPESNQKMKDSYLILDEYMRFLNCTGGRKDPSRSILDVGVEEAIKFSGFDEKMFLKR
GGKYVWSKADLKLDW

Codon optimized gene sequence for hCMPK2.

5'tacttccaatccatgGCTCCCCCCCCGTCGTTTCGTGTTGGAGTTACCTGACTGCACGCTGGCACA
CTTCGCGCTTGGGGCTGATGCGCCCGGAGACGCAGACGCTCCCGACCCGCGTTTAGCGG
CGCTTTTGGGACCGCCTGAGCGCAGTTATTCATTATGCGTTCCTGTGACCCCAGATGCCG
GGTGTGGGGCCCGTGTTCGCGCTGCACGTTTGCATCAACGCTTGCTTCATCAATTGCGTC
GCGGGCCATTCCAGCGTTGCCAGTTGTTGCGTTTATTATGTTATTGCCCCGGCGGGCAAG

CCGGAGGAGCTCAACAGGGTTTTCTTTTACGTGACCCGCTTGATGACCCTGATACGCGCC
AGGCATTACTGGAGTTACTGGGCGCTTGCCAGGAAGCTCCCCGCCCACATTTGGGGGAAT
TTGAAGCAGATCCCCGCGGTCAGTTGTGGCAACGTCTTTGGGAGGTCCAGGACGGCCGC
CGTTTACAGGTCGGTTGTGCTCAGGTCGTCCCAGTCCCTGAACCGCCGCTGCACCCTGTC
GTACCAGATTTACCTTCATCAGTTGTGTTCCCGGACCGTGAGGCGGCTCGTGCGGTACTG
GAGGAATGTACATCTTTTATTCCAGAAGCACGCGCCGTTTTGGATTTAGTAGATCAGTGTC
CGAAACAGATCCAAAAGGGCAAGTTTCAGGTCGTGGCTATTGAAGGATTGGATGCAACCG
GTAAGACAACTGTTACTCAGAGCGTGGCTGACTCACTTAAAGCGGTGCTGCTGAAATCTCC
GCCTTCATGCATTGGTCAATGGCGTAAGATCTTCGATGATGAGCCGACTATTATCCGTCGT
GCGTTTTATTCACTGGGCAATTATATTGTCGCAAGCGAGATTGCTAAGGAGTCAGCTAAAT
CGCCAGTCATTGTTGATCGTTACTGGCATTCTACTGCAACCTACGCTATTGCAACCGAAGT
ATCGGGTGGCCTGCAGCATCTGCCACCAGCACACCACCCCGTTTACCAATGGCCGGAGGA
TTTACTGAAGCCCGATTTAATCCTGCTTTTAACCGTTTCTCCTGAAGAACGCCTGCAACGTC
TTCAAGGCCGTGGAATGGAGAAGACGCGCGAGGAGGCGGAGTTAGAAGCTAACTCCGTG
TTCCGTCAAAAAGTCGAGATGTCATATCAACGCATGGAAAACCCTGGTTGCCACGTGGTGG
ACGCTAGTCCCAGTCGTGAAAAGGTACTTCAGACGGTGCTTAGCCTTATTCAAAACAGCTT
TTCAGGTCCGTAACAGtaaaggtggata3'

Codon optimized gene sequence for NS5A.

5'tacttccaatccatgCCACAACTGCCTGGGATTCCCTTTGTGTCCTGCCAGCGCGGGTATAGGG
GGGTCTGGCGAGGAGACGGCATTATGCACACTCGCTGCCACTGTGGAGCTGAGATCACTG
GACATGTCAAAAACGGGACGATGAGGATCGTCGGTCCTAGGACCTGCAGGAACATGTGGA
GTGGGACGTTCCCCATTAACGCCTACACCACGGGCCCCTGTACTCCCCTTCCTGCGCCGA
ACTATAAGTTCGCGCTGTGGAGGGTGTCTGCAGAGGAATACGTGGAGATAAGGCGGGTGG
GGGACTTCCACTACGTATCGGGTATGACTACTGACAATCTTAAATGCCCGTGCCAGATCCC
ATCGCCCGAATTTTTCACAGAATTGGACGGGGTGCGCCTACACAGGTTTGCGCCCCCTTG
CAAGCCCTTGCTGCGGGAGGAGGTATCATTCAGAGTAGGACTCCACGAGTACCCGGTGG
GGTCGCAATTACCTTGCGAGCCCGAACCGGACGTAGCCGTGTTGACGTCCATGCTCACTG
ATCCCTCCtaacagtaaaggtggat3'

Cloning of the Rattus norvegicus viperin gene. The *Rattus norvegicus* viperin gene from the mRNA Best5 transcript (GI: 3135886) was commercially synthesized by GenScript (Piscataway, NJ) and placed into the commercially available pET23a+ vector. To generate a

plasmid for expression in *E. coli*, the above gene was truncated to amino acid residues 51-361 (this construct does not contain the N-terminal amphipathic ER localization sequence, but contains the remainder of the protein) with PCR amplification using the primers rVIP_Forward (5'-tacttccaatccATGGGTGAGCCCAAGGAGACTC-3') and rVIP_Reverse (5'-tatccacctttactgTTACCAGTCTAGCTTCAGGTCAGC-3'), which contain ligation independent cloning (LIC) sites (underlined) that are complementary to the vector pNIC28-Bsa4. The amplified gene and vector were ligated using standard LIC methods[2,3]. The resulting construct adds 22 additional amino acids to the N-terminus, which includes a 6x-His-tag and a TEV cleavage site (see below, underlined and italicized) and is denoted as rVIP.

Cloning of human cytidine monophosphate-UMP-CMP-kinase 2 (CMPK2) and hepatitis C virus non-structural protein 5A (NS5A). For *E. coli* expression of hCMPK2 and HCV NS5A, the genes (below) with LIC overhangs (underlined) were synthesized by GenScript. hCMPK2 was codon optimized for *E. coli* expression. The gene fragments were sub-cloned as above.

Cloning of human hydroxylacyl-CoA dehydrogenase/mitochondrial tri-functional protein (HadHB) and human farnesyl diphosphate synthase (FPPS). The following plasmids from DNASU repository (DNASU.org) were used as templates for PCR amplification: HsCD00041710 for HadHB and HsCD00077552 for FPPS. The following primers were used to amplify the gene fragments corresponding to residues 1-419 for FPPS and residues 49-475 for HadHB

HadHB forward,

5'-TACTTCCAATCCATGAAACCCAATATAAGGAATGTTGTGGTGG-3', HadHB

and reverse,

5'-TATCCACCTTTACTGCTATTTTGGATAAGCTTCCACTATCATAGCATGG-'3)

FPPS forward,

5'-TACTTCCAATCCATGCCCCTGTCCCGCTGGTT-3'

and FPPS reverse

5'-TATCCACCTTTACTGCTACTTTCTCCGCTTGTAGATTTTGCG-3'). The amplified genes were cloned into the N-terminal TEV cleavable-hexa-histadine-and-maltose binding protein (MBP)-encoding plasmid pMCSG9 by LIC methods[2].

Expression and purification of rVIP. BL-21 (RIL) competent cells containing the helper plasmid pDB1282 were transformed with the rVIP plasmid. This strategy allows for the expression of rVIP in the presence of the *isc* operon of FeS cluster assembly proteins. A single colony was used to inoculate an overnight culture of P-0.5G defined minimal media[4] with subsequent incubation at 37 °C. PA-5052, a defined medium for auto induction[4], was inoculated (1:100 dilution) with the overnight starter culture, and grown to an OD_{600} of 0.3 before adding 0.2% L-arabinose to induce the expression of genes on pDB1282. Fe and cysteine were also added to final concentrations of 25 μM and 150 μM, respectively. After approximately five hours post inoculation, the temperature was lowered to 22 °C and the cells were allowed to grow for another 20 hours before being harvested by centrifugation at 10,000 × g. The cell pellets were flash frozen and stored in liquid N_2 until purification. All subsequent protein purification steps were carried out in a customized MBraun anaerobic glove box maintained at <0.1 ppm oxygen. rVIP (30 g) cell paste was resuspended in 100 mL lysis buffer containing 50 mM HEPES, pH 7.5, 300 mM KCl, 10% glycerol, 2 mM imidazole, and 10 mM 2-mercaptoethanol (BME). Cells were cooled in an ice bath while being subjected to cycles of 3 sec on and 12 sec off, for a total of 20 min of sonic disruption (80% output). The lysate was then centrifuged for 1 hour at 15,000 × *g* at 4 °C. The resulting supernatant was passed over a 5 mL Fast-Flow Ni-Sepharose column (GE Biosciences), equilibrated in lysis buffer, with AKTA Purifier FPLC. After loading, the column was washed with 20 column volumes of lysis buffer, and eluted with 2 column volumes of elution buffer (lysis buffer with 500 mM imidazole). The resulting eluent with high UV_{280} nm absorbance was collected and loaded onto a HiPrep 16/60 S-200 size-exclusion column equilibrated with 50 mM HEPES, pH 7.5, 150 mM KCl, 5% glycerol, and 5 mM DTT. The resulting fractions displaying brown color were pooled and concentrated by ultracentrifugation using an Amicon centrifugal filter unit (10,000 M.W. cut-off). The protein concentration was estimated using an extinction coefficient calculated from the amino acid sequence (1.19 g/L/AU).

Expression of *h*CMPK2, FPPS, HadHB and NS5A was performed as described above with the exception that the BL-21 (RIL) competent cells were transformed with the corresponding plasmids, but without pDB1282. The growths did not receive additional

arabinose, Fe, or cysteine. Protein purification steps were conducted as described above for rVIP with the exception that the final buffer for NS5A contained 20% glycerol, rather than 5%.

Analytical Size Exclusion Chromatography of rVIP with FPPS, NS5A and HadHB: The following manipulations were performed in an MBraun anaerobic chamber maintained at <0.1 ppm oxygen. 50 μL of 100 μM rVIP in a buffer containing 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM SAM, and 2 mM CTP was mixed with 50 μL of additional buffer or, 50 μM FPPS, NS5A or HadHB. Each sample was injected onto a Superdex 200 10/300GL size-exclusion column (GE Biosciences) pre-equilibrated with 50mM HEPES, pH 7.5, 150 mM KCl and 5% glycerol and then eluted within 1.5 column volumes of equilibration buffer. All traces showed no change in protein elution volume for both rVIP and the putative interacting partner. The lack of interaction may be due to the fact that these experiments do not use native protein constructs or that the proper conditions for interaction are not met under these non-physiological conditions (i.e. other interacting partners are required or some undefined metabolite).

General methods. High-performance liquid chromatography (HPLC) with detection by mass spectrometry (LC-MS) was conducted on an Agilent Technologies (Santa Clara, CA) 1200 system coupled with either an Agilent Technologies 6490 triple Quadrupole or a 6410 triple Quadrupole mass spectrometer. The system was operated with the associated MassHunter software package, which was also used for data collection and analysis. Assay mixtures were separated on an Agilent Technologies Agilent Eclipse Plus C18 RRHD (2.1 mm × 50 mm, 1.8 μm particle size) unless otherwise noted and equilibrated in either ion-pairing buffer for separating nucleotides (A: 100 mM triethylamine buffered to pH 7 with acetate, B: 100% methanol), or acidic buffer for resolving 5'dA (A: 0.1% formic acid, 5% methanol, B: 100% acetonitrile).

Substrate specificity assays. Reactions contained in a total volume of 100 μL: 50 μM rVIP, 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM SAM, 1 mM of appropriate nucleotides, with the

exception of UDP-glucose, CDP-choline, CDP-ethanolamine and polyinosinic/polycytidylic acid (Poly IC) which were each 10 mM, and 5 mM dithionite. Reaction mixtures lacking dithionite were incubated for 5 minutes at 37 °C, and a 10 µL aliquot was removed (t=0) and added to 10 µL of a solution containing 100 mM H_2SO_4 and 2 mM L-tryptophan (internal standard) to yield a final internal standard concentration of 1 mM. Reactions were initiated with dithionite and incubated for four minutes before being quenched in acid as described above. The samples were centrifuged at 16,000 x g to remove the precipitated protein and the supernatants were injected onto an Agilent Eclipse Plus C18 RRHD column equilibrated in 100 % buffer A (acidic buffer noted above). A gradient of 0-5% B was applied from 0-0.5 min, and 5-20% B from 0.5-2 min, and 20-50% B from 2-2.5 min before returning to 100% A from 2.5-3 min. The column was allowed to re-equilibrate for 1 min under initial conditions before subsequent sample injections. Detection of 5'dA (Supplementary Table 6) was performed using electrospray ionization in positive mode (ESI^+) with multiple reaction monitoring (MRM). Standard curves were generated with 5'dA purchased from Sigma-Aldrich.

Hydrogen atom abstraction specificity: Reactions of 100 µL total volume contained 100 µM rVIP, 50 mM HEPES pH 7.5, 150 mM KCl, 1 mM SAM, and 1 mM deuterium-labelled CTP (site-specifically labelled CTP contains deuterium at the following positions: 3'-^{2}H,4'-^{2}H, 5'-$^2H_2$,5-^{2}H-CTP or 2'-^{2}H-CTP,3'-^{2}H-CTP,4'-^{2}H-CTP or 5'-$^2H_2$-CTP). Reaction mixtures lacking dithionite were incubated at 37°C for 5 min. Reactions were initiated by the addition of 5 mM dithionite and incubated for 4 min before aliquots were removed and quenched as described above. The supernatants were collected after centrifugation at 15,000 rpm and 2 µL was directly injected onto an Agilent Eclipse Plus C18 RRHD column equilibrated in 99.2 % buffer A and 0.8% B. Buffer A (100 mM triethylamine buffered to pH 7 with acetate) is an ion-pairing buffer for separating nucleotides, while B is 100% methanol. A gradient of 0.8-5% B was applied from 0-0.5 min, and 0.8-20% B from 0.5-2 min, and then 20-50% B from 2-2.5 minutes before returning to 99.2% A from 2.5-3 min. The column was allowed to re-equilibrate for 1 min under initial conditions before subsequent sample injections. This method allows for facile separation of the starting material (CTP) from product (3'-deoxy-3',4' -dideoxy-CTP). The mass to charge ratio (*m/z*) of unlabeled CTP is 482.1, and

increases by one mass unit for each deuterium substitution. The *m/z* of the resulting ddhCTP is 464.1, which increases by one mass unit for each deuterium substitution. However, if deuterium abstraction occurs, the resulting product will have an *m/z* identical to that of the natural abundance product. Lastly, 5'dA has an *m/z* equal to 250.1 in ESI- and should increase by one mass unit if a deuterium is incorporated at the 5'-position (*m/*z=251.1). As such, detection of substrates and products was performed using electrospray ionization in negative mode (ESI^-) with an MS2-Scan window from 480-490 (CTP), 460-470 (ddhCTP) and 245-255 (5'dA).

Site-specific isotopically labeled CTP molecules: CTP isotopologues were synthesized from the respective deuterium-labeled ribose substrates (2-^{2}H, 3-^{2}H, 4-^{2}H, or 5,5'-$^2H_2$-D-ribose, Omicron Biochemicals, 98% ^{2}H enrichment), using enzyme-linked reactions.[5] 1.2 mM uracil, 20 mM phosphoenolpyruvate, 0.2 mM ATP, 0.1 mM GTP, 5 mM L-glutamine, in a buffer of 50mM potassium phosphate (pH 7.8), 10 mM $MgCl_2$, and 2 mM DTT were mixed with the appropriate deuterium-containing ribose substrates (1 mM). A total of eight enzymes were added (ribokinase, pyruvate kinase, adenylate kinase, PRPP synthetase, uracil phosphoribosyltransferase, UMP-CMP kinase, nucleotide diphosphate kinase, and CTP synthetase) to the mixture and incubated overnight at 37 °C. The resulting CTP product from each synthesis was purified from other nucleotides by HPLC using a Phenomenex 250 × 4.6 mm C-18 column in 8 mM tetrabutylammonium bisulfate, 50 mM KH_2PO_4 buffer (pH 6), and water/acetonitrile gradient. The pooled CTP isotopologues were dried on speedvac, dissolved in water, and desalted on HPLC (same column as above) using a solvent mixture of 75% water and 25% acetonitrile. After evaporating all solvent, the desalted CTPs were redissolved in water. The pure CTP concentration was determined by UV absorbance at 271 nm (9,000 $M^{-1}cm^{-1}$).

2'-deoxy-CTP vs deutero-CTP substrate specificity assays. Reactions of 100 µL total volume contained 100 µM rVIP, 50 mM HEPES pH 7.5, 150 mM KCl, 1 mM SAM, 1 mM deutero-CTP (3'-^{2}H,4'-^{2}H,5'-$^2H_2$,5-^{2}H-CTP), and unlabelled 1 mM 2'-deoxy-CTP. Reaction mixtures lacking dithionite were incubated at 37 °C for 5 min. Reactions were initiated by the

addition of 5 mM dithionite and incubated for 4 min before being quenched as described above, and treated as described above using ion-pairing buffer (triethylamine buffered to pH 7 with acetate). Detection of the resulting 5'dA was performed using electrospray ionization in negative mode (ESI^-) with an MS2-Scan window from 245-255 (5'dA).

Formation of 3'-deoxy-3',4'-didehydro-CTP and 5'dA by rVIP. Reactions of 100 µL total volume contained 100 µM rVIP, 50 mM HEPES pH 7.5, 150 mM KCl, 1 mM SAM, 2 mM CTP, and either 100 µM flavodoxin, 10 µM flavodoxin reductase, and 2 mM NADPH or 5 mM dithionite. Reaction mixtures lacking dithionite or NADPH were incubated at 37 °C for 5 min. Reactions were initiated by the addition of 5 mM dithionite or 2 mM NADPH and incubated for the indicated times before being quenched as described above. The samples were then treated as described above using ion-pairing buffer (triethylamine buffered to pH 7 with acetate) for nucleotide separation. Detection of substrates and products was performed using electrospray ionization in negative mode (ESI^-) with single-ion monitoring (SIM) (Supplementary Table 7). A product peak of 464 can be readily observed in negative mode using triethylammonium-acetate (TEAA) buffered to pH 7. This method allows for facile separation of the starting material (CTP) from product (3'-deoxy-3',4'-didehydro-CTP), while preserving the triphosphate. Standard curves were generated with 5'dA and CTP purchased from Sigma-Aldrich. A standard curve for 3'-deoxy-3',4'-didehydro-CTP was constructed using the purified compound (described below), and assuming an identical extinction coefficient to CTP at 271nm (9,000 M^{-1} cm^{-1}).

Steady-state kinetics: Reactions contained the following in a total volume of 100 µL: 0.5 µM rVIP, 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM SAM, 2.44 µM to 5000 µM CTP, and 5 mM dithionite. Reaction mixtures lacking dithionite were incubated at 37 °C for 5 min. Reactions were initiated by the addition of 5 mM dithionite and incubated for 5 min before an aliquot was removed and quenched by mixing with an equal volume of 100 mM H_2SO_4 with 2 mM tryptophan (IS). All assays were performed in triplicate. Detection of 5'dA was used as a proxy for formation of ddhCTP after a 1:1 correlation was established (Supplementary Table 6). Standard curves were generated with 5'dA purchased from

Sigma-Aldrich. V_{max} and K_m values were determined by fitting the data using a least-squares method with the standard Michaelis-Menton equation.

In vitro production of 3'-deoxy-3',4'-didehydro-CTP: A reaction (1 mL total volume) containing 300 µM rVIP 50 mM HEPES, pH 7.5, 150 mM KCl, 2 mM SAM, 2 mM CTP or $^{13}C_9{}^{15}N_3$-CTP, and 5 mM dithionite was incubated for 2 hr at 37 °C. These large-scale reactions typically produce between 1600 and 1800 nmoles of 3'-deoxy-3',4'-didehydro-CTP. After 2 hr the reaction was precipitated with 100 mM H_2SO_4 to remove the protein, and subjected to centrifugation at 15,000 × g. The supernatant was diluted 5-fold into ice-cold 10 mM ammonium bicarbonate buffer (pH 9.0) to achieve a final KCl concentration of <50 mM, then loaded onto a Mono Q 5/50 GL column (GE Biosciences) pre-equilibrated with 10 mM ammonium bicarbonate (pH 9.0). The column was washed with 20 mL of 10 mM ammonium bicarbonate (pH 9.0), and elution was performed using a linear elution gradient (200 mL) of 200 mM to 800 mM ammonium bicarbonate, pH 9, where 3'-deoxy-3',4'-didehydro-CTP can be easily separated from CTP. The concentration of the purified product was determined by UV absorbance at 271 nm using the extinction coefficient for CTP (listed above). Verification of purity was performed using electrospray ionization in negative mode (ESI -) with an MS2-scan window from 150-600 *m/z*.

In vivo production of 3'-deoxy-3',4'-didehydro-CTP in HEK293-F cells: The human viperin coding sequence was PCR amplified (template: accession number AF442151.1, Genscript) using PCR primers shown in Supplementary Table 3 and cloned into a modified version of pCDNA3.3 using InFusion HD (Clontech) to generate constructs expressing full-length human viperin (hVIP) with either an N- or C-terminal FLAG tag or truncated hVIP (N-terminal 1-42 residues deleted) with a C-terminal FLAG tag. hCMPK2 was PCR amplified from accession number BC132821 (human MGC collection) and cloned into a modified version of pCDNA3.3 containing a C-terminal triple-strep-tag using InFusion HD to generate two full-length constructs: untagged or with a C-terminal triple-strep tag.

Plasmids encoding hVIP alone, hCMPK2 alone, or both hVIP and hCMPK2 were transfected into HEK293-Freestyle (HEK293-F) cells using PEI. 500 ng of total plasmid DNA (250 ng of

each plasmid of interest, where 250 ng of a control plasmid (pcDNA3.3 containing the gene for the fluorescent protein mCherry) was used for hVIP alone or hCMPK2 alone) was mixed with 2 μg PEI, incubated for 10 minutes at room temperature, and then added to 1ml of cells (1 × 10^6 cells/ml) and incubated for 1 hr at 37 °C. Cells were grown in FreeStyle Expression Medium with gentle shaking and harvested at 16, 24, 48, and 72 hr post-transfection. Cell count and viability were measured with an automated cell counter after staining with tryptan blue (Countess, Life Technologies, Supplementary Table 4) and harvested by centrifugation at 400 × g for 3 min.

In vivo production of 3'-deoxy-3',4'-didehydro-CTP in RAW264.7 cells: RAW 264.7 cells were obtained from ATCC laboratories and expanded using standard techniques before aliquots were removed, supplemented with 10% DMSO and stored using slow freeze methods. Cell aliquots were thawed and maintained in DMEM/10% FBS before each experiment. RAW 264.7 cells were grown to ~80-90% confluence, and passaged by treating with 10mM EDTA and plated into fresh medium every 2-3 days (1:10). For induction of viperin, approximately 3.5 × 10^6 Raw264.7 cells were seeded into a 6 well tissue culture plate in complete medium (DMEM/10%FBS) with 4 hr of incubation at 37 °C to allow them to attach to the surface. The cells were then washed twice with PBS before being stimulated with mouse IFN-α (fresh aliquot from -80) dissolved in 2 ml of serum free medium. The cells were then incubated for 18 hr at 37 °C. Mouse IFN-α was purchased from Invitrogen (Thermo Fisher Scientific, 14-8312-80) with a specific activity of 10 U/ng. Concentrations of 0, 100, 500 or 2500 U/ml (or 0, 10, 50, 250, 500 ng/ml) were used for cell stimulation. After 18 h, 100 μL of culture media was tested for nitric oxide production using Griess reagent. Cells were washed with PBS and frozen on dry ice before lysis and extraction as described below.

Metabolite extraction of HEK293 FreeStyle and RAW264.7 cells: An ice-cold mixture (500 μL) of extraction solvent containing acetonitrile/methanol/water (40:40:20 + 0.1M formic acid) and internal standards at known concentrations ($^{13}C_9{}^{15}N_3$-3'-deoxy-3',4'-didehydro-CTP, $^{13}C_9{}^{15}N_3$-CTP, $^{13}C_{10}{}^{15}N_5$-ATP, $^{13}C_{10}{}^{15}N_5$ –GTP, and $^{13}C_9{}^{15}N_2$-UTP) were added to pelleted HEK293-F or RAW264.7 cells. The tubes were vortexed for 30 seconds and then incubated

on ice for 10 min. The lysates were pelleted by centrifugation at 13,000 x g for 10 min at 4 °C. The supernatant was transferred to fresh tubes and dried under vacuum. Each sample was resuspended in 30 µL of HPLC buffer A (TEAA pH 7, described above) and injected onto an Agilent Eclipse Plus C18 RRHD column equilibrated in 100% buffer A. The column was held at 0% B from 0-1.5 minutes followed by a gradient of 0-15% B from 1.5-3.25 min, and 15-50% B from 3.25-3.75 min before returning to 100% A from 3.75-5 min. The column was allowed to re-equilibrate for 1 min under initial conditions before subsequent sample injections. Detection of products was performed by two separate injections using electrospray ionization in negative mode (ESI^-) with the following MRM methods (Supplemental Tables 8 and 9). Calculation of intracellular concentrations of metabolites (C_{cell}) was performed according to equation 1, where L is the peak area of the unlabeled (light) metabolite, and H is the peak area of the $^{13}C^{15}N$-(heavy) internal standard in the extraction solution. S is the concentration of the $^{13}C^{15}N$ standard and V_{sample} is the final volume of the sample after extraction, lyophilization and resuspension. N_{count} is the number of cells in the sample (typically 1×10^6 to 4×10^6) and V_{cell} is the volume of one cell. For HEK293-F cells, the volume (V_{cell} = 1.38×10^{-12} L) was calculated assuming an average diameter of 13.9 µm (data is from invitrogen specifications sheet for Human embryonic kidney cells 293)[9] and a perfect sphere (according to equation 2 where V is equal to the volume of one cell). For RAW264.7 cells, the volume (V_{cell} = 0.697×10^{-12} L) was calculated assuming an average diameter of 11 µm (data is from Nexcelom specifications sheet for Raw 264.7 macrophages)[10] and a perfect sphere (according to equation 2 where V is equal to the volume of one cell) [6,7]. However, these volumes are an approximate value due to shape heterogeneity in the RAW cell population.

$$C_{cell} = \frac{L}{H} \text{ x } S \text{ x } \frac{V_{sample}}{N_{count} \, x \, V_{cell}} \quad (1)$$

$$V_{cell} = \frac{4}{3}\pi r^3 \quad (2)$$

Statistical Analysis of the effect of CMPK2 on ddhCTP production: These statistical analyses aimed to test whether the concentration of ddhCTP in cells expressing hVIP differed

significantly from cells only, as well as when both hVIP and hCMPK2 were co-expressed. Statistical significance between groups at each time point was tested using a 2-way analysis of variance (ANOVA) testing the effects of group, time, and their interaction on the concentration of ddhCTP. A post-hoc analysis (Tukey's multiple comparisons test) was used to test differences among grouped sample means (hVIP alone, hVIP + hCMPK2, Cells only) at each time point (16, 24, 48 and 72 hr). We also aimed to test whether nucleotide concentrations between groups (hVIP alone, hVIP with hCMPK2 and cells only) at each time differed significantly. We performed a 2-way analysis of variance (ANOVA) testing the effects of group, time, and their interaction on concentration of nucleotides.

Statistical Analysis of the effect of ddhC on ZIKV titer: These statistical analyses aimed to test whether ddhC concentration is a significant determinant of variation in ZIKV titer. Statistical significance between the $\log_{10}$ transformed ZIKV titers at each time point was tested using an ANOVA to determine the effects of ddhC concentration, time, and their interaction on ZIKV titer. A post-hoc analysis (Dunnet's multiple comparison test) was used to test differences among ZIKV titers at each ddhC concentration (i.e., 0 vs 0.1 mM, 0 vs 0.5 mM, 0 vs 1 mM) at each time point (24, 48 and 72 hr) for each MOI (0.1, 1.0).

^{13}Carbon-^{13}Carbon-COSY, 2D-HSQC 1H/13C, 1D-H and 1D-^{31}P NMR: 2D-^{1}H,^{13}C-HSQC and 2D-^{13}C,^{13}C-CT-COSY NMR spectra were recorded on a Bruker AVANCE HD III 600 MHz NMR spectrometer operated with TopSpin 3.2 software. The sample was dissolved in D_2O and all NMR measurements were carried out at 25 °C. Proton and carbon chemical shifts are based on DSS as an internal standard. The assignment of proton and carbon signals was performed using 2D-^{1}H,^{13}C-HSQC and 2D-^{13}C,^{13}C-CT-COSY experiments on a natural abundance sample or dual ^{13}C,^{15}N- labeled sample. 1D-^{31}P NMR measurements were conducted on a Bruker 300MHz spectrometer operated with TopSpin 3.2 software using a natural abundance sample containing 1mM purified 3'-deoxy-3',4'-didehydro-CTP dissolved in 100% D_2O. ^{13}C-^{13}C COSY NMR on the uniformly labeled CTP-derived viperin-catalyzed product unambiguously defined its carbon-carbon connectivity, which demonstrated that the carbon backbone was unchanged from the CTP starting material (Extended Data Fig. 2a). Comparison of the ^{1}H-^{13}C 2D HSQC chemical shift data of the

natural abundance viperin product with those of CTP demonstrated the complete loss of the 4' proton signal at 4.27 ppm. In addition, the ^{13}C-^{13}C COSY indicates that the C4' carbon resonance shifted from 85.4 ppm to 161.2 after conversion of CTP to the new product (see Supplemental Table 1 for full list of chemical shift data; Extended Data Fig. 2). The ^{1}H-^{13}C 2D-HSQC NMR spectra derived from synthetic 3'-deoxy-3',4'-didehydro-cytidine nucleoside (ddhC)[8] and the natural abundance viperin product exhibited nearly complete overlap, with the exception of the 5'-ribose position, which we attribute to the presence of the triphosphate moiety (Supplemental Table 1; Extended Data Fig. 2). The ^{31}P NMR spectrum of the viperin product exhibited three resonances at -9.5 (doublet, α), –19.5 (triplet, β), and –3.9 (doublet, γ) ppm, which are consistent with a linear triphosphate functionality and almost identical to the spectrum of a CTP standard (Extended Data Fig. 2b).

hCMPK2 assays: Reactions of 100 µL total volume contained 5 µM of hCMPK2, 50 mM HEPES pH 7.5, 100 mM KCl, 1 mM ATP or GTP, and either 1 mM CMP, UMP, CDP, UDP or a 10/50/40 mixture of ddhCMP/ddhCDP/ddhCTP. The ddhCMP/ddhCDP/ddhCTP mixture was generated by incubating 1.2 mM ddhCTP at 25 °C for 72 hr and quantified using UV absorbance assuming the extinction coefficient for all three compounds are identical to CTP (see above). Reaction mixtures lacking ATP or GTP were incubated at 22 °C for 5 min. Reactions were initiated by the addition of 1 mM ATP or GTP and incubated for 1 min before being quenched as described above for rVIP assays. Detection of CTP, UTP and ddhCTP was conducted using electrospray ionization in negative mode (ESI^-) with MRM (Supplemental Table 2).

Construction of DV, WNV and ZIKV RdRp bacterial expression plasmids: The DV and ZIKV RdRp (NS5 gene) was cloned into the pET26Ub-CHIS bacterial expression plasmid using a similar procedure as described for WNV RdRp (NS5 gene).[9] This system allows for the production of ubiquitin fusion proteins containing a carboxy-terminal hexahistidine tag that are then co and/or post-translationally processed by the ubiquitin protease, co-expressed from a second plasmid, pUBPS[10]. Briefly, the DV RdRp coding region was amplified using the DV type 2 strain cDNA (NC_001474.2) as template (kindly provided by

E. Harris, UC Berkley School of Public Health), oligonucleotides (5'-TGGTCCTGCGTCTCCGCGGTGGAGGAACTGGCAACATAGGAGAG-3') and (5'-GGTGACCAGAGGATCCCCACAGGACTCCTGCCTCTTC-3') and Deep Vent DNA polymerase (NEB). The ZIKV RdRp coding region was amplified using a synthetic NS5 gene construct as template (synthesized by GenScript) based upon the NS5 amino acid sequence of the Zika virus strain BeH815744 (AMA12087.1), oligonucleotides (5'-TGGTCCTGCGTCTCCGCGGTGGAGGTGGCGGTACCGGCGAAACCCTGGGC-3') and (5'-GGTGACCAGAGGATCCCAGAACGCCCGGGGTGCT-3'). The PCR products of DV and ZIKV RdRp were gel purified and cloned into the pET26Ub-CHIS plasmid using SacII and BamHI sites. The final constructs (pET26Ub-DV-RdRp-CHIS and pET26Ub-ZIKV-RdRp-CHIS) were confirmed by sequencing at the Pennsylvania State University's Nucleic Acid Facility.

Construction of HCV RdRp bacterial expression plasmid: The HCV RdRp (NS5B gene) was cloned into the pET22 plasmid containing a C-terminal hexahistidine tag. The region that codes for the final 21 C-terminal amino acids were removed to improve solubility. Briefly, the HCV RdRp coding region was amplified using the Con1 replicon as template[11], oligonucleotides (5'-GCGGAATTCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGAGCA TGTCCTACACA-3') and (5'-GCGGAATTCCTCGAGCTATTAATGGTGGTGATGGTGGTGACCAGAGGATCCCCG GGGTCGGGCACG-3') and Deep Vent DNA polymerase (NEB). The PCR product was gel purified and cloned into the pET22 plasmid using XbaI and XhoI sites. The final construct (pET22-HCV-NS5B-Delta21CT-CHIS) was confirmed by sequencing at the Pennsylvania State University's Nucleic Acid Facility.

Construction of HRV-C and PV RdRp bacterial expression plasmids: The HRV-C RdRp (3D gene) was cloned into the pSUMO bacterial expression plasmid using a similar procedure as described for PV RdRp (3D gene)[9]. This system allows for the production of SUMO fusion proteins containing an amino-terminal hexahistidine tag fused to SUMO that can be purified by Ni-NTA chromatography and subsequently processed by the SUMO protease, Ulp1. Briefly, the HRV-C RdRp coding region was amplified using the HRV-C15 cDNA

(Accession# GU219984) as template (provided by A. Palmenberg, University of Wisconsin-Madison), oligonucleotides (5'-GAACAGATTGGAGGTGGTGAGATCACTGCCAAACAT-3') and (5'-CCGCAAGCTTGTCGACTTACTATATGAATTGATCTATCCATTGCCT-3') and Deep Vent DNA polymerase (NEB). The PCR product of HRV-C RdRp was gel purified and cloned into the pSUMO plasmid using BsaI and SalI sites. The final construct (pSUMO-HRV-C RdRp) was confirmed by sequencing at the Pennsylvania State University's Nucleic Acid Facility.

Expression and purification of DV, WNV, ZIKV, HCV, HRV-C and PV RdRps. DV, ZIKV and HCV RdRps were expressed and purified using the same procedure reported for WNV RdRp[12]. Briefly, expression was performed at 15 °C by auto-induction, cells harvested, lysed by French press, subjected to PEI precipitation followed by AMS04 precipitation, Ni-NTA chromatography, gel filtration and the protein concentrated using Vivaspin concentrators. HRV-C RdRp was expressed and purified using the same procedure reported for PV RdRp[13,14]. Briefly, expression was performed at 25 °C by auto-induction, cells harvested, lysed by French Press, subjected to PEI precipitation followed by AMS04 precipitation, Ni-NTA chromatography, cleavage by Ulp1, phosphocellulose chromatography, gel filtration and the protein concentrated using Vivaspin concentrators.

DV, WNV, ZIKV and HCV RdRp-catalyzed nucleotide incorporation assays. To assemble DV, WNV, ZIKV and HCV RdRp elongation-competent complexes, 0.5 or 2 μM purified DV, WNV, ZIKV or HCV RdRp was mixed with 10 μM pGGC RNA primer, 1 μM RNA template (5'-UUUAGCUCUUCCUCUUUGCC-3'), 20 μM ATP, 20 μM GTP and 0.1 μCi/μL [α-32P]-ATP for 30 min at 30 °C in 50 mM HEPES pH 7.5, 20 mM NaCl, 5 mM MgCl2 and 10 mM 2-mercaptoethanol. For single nucleotide incorporation assays, elongation complexes were assembled, reactions were initiated with either 10 μM CTP, 3'-dCTP or ddhCTP nucleoside triphosphate substrate and allowed to proceed for various amounts of time before being quenched with 50 mM EDTA. For chain termination experiments, reactions were allowed to proceed for an additional 1 min in the presence of the next correct nucleotide substrate (10 μM UTP) before being quenched. To evaluate the ability of ddhCTP or 3'-dCTP to inhibit RdRp elongation, elongation complexes were assembled, reactions were initiated with the addition of UTP and CTP and varying concentrations of either ddhCTP or 3'-dCTP and

allowed to proceed for 10 min before being quenched with 50 mM EDTA. Products were resolved from substrates by denaturing PAGE. DV and WNV RdRp was diluted immediately prior to use in enzyme dilution buffer, which consisted of 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol and 20% glycerol. The volume of enzyme added to any reaction was always less than or equal to one-tenth the total volume.

HRV-C and PV RdRp-catalyzed nucleotide incorporation assays: Reactions were performed essentially as described in detail in[15,16]. Elongation complexes were assembled by incubating 2 μM purified HRV-C or PV RdRp with 1 μM (0.5 μM duplex) ^{32}P-labeled-sym/sub-AUGC RNA primer-template (5'-CGAUGGGCCC-3') for 3 min in the presence of UTP and ATP. Reactions were initiated with either 10 μM CTP, 3'-dCTP or ddhCTP nucleoside triphosphate substrate and allowed to proceed for various amounts of time before being quenched with 50 mM EDTA. For chain termination experiments, reactions were allowed to proceed for an additional 1 min the presence of the next correct nucleotide substrate (10 μM GTP) before being quenched. To evaluate the ability of ddhCTP or 3'-dCTP to inhibit HRV-C RdRp elongation, purified HRV-C RdRp was mixed with RNA primer-template in the absence of nucleotides to assemble complexes, reactions were then initiated with the addition of UTP, ATP, CTP, GTP and varying concentrations of either ddhCTP or 3'-dCTP and allowed to proceed for 10 min before being quenched with 50 mM EDTA. Products were resolved from substrates by denaturing PAGE. All reactions were performed at 30 °C in 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol and 5 mM $MgCl_2$. HRV-C and PV RdRp was diluted immediately prior to use in enzyme dilution buffer, which consisted of 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol and 20% glycerol. The volume of enzyme added to any reaction was always less than or equal to one-tenth the total volume.

Denaturing PAGE analysis of polymerase-catalyzed reaction products. Two volumes of loading buffer, 10 μL, (90% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol) was added to 5 μL of quenched reaction mixtures and heated to 90 °C for 2-5 min prior to loading 5 μL on a denaturing 23% polyacrylamide gel containing 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and 7 M urea. Formamide gels contained an

additional 20% formamide. Electrophoresis was performed in 1x TBE at 90 W. Gels were visualized by using a PhosphorImager (GE) and quantified by using ImageQuant TL software (GE).

In vivo conversion of ddhC to ddhCTP by HEK293-F and Vero cells. HEK293-F cells were grown in FreeStyle Expression Medium at an initial concentration of 1 x 10^6 cells/mL and supplemented with either 0 or 1 mM ddhC. HEK293-F cells were shaken at 50 rpm for 24, 48, and 72 hr. At indicated time points, cells were counted and viability was measured with an automated cell counter after staining with tryptan blue (Countess, Life Technologies) before being harvested by centrifugation at 400 × g for 3 min. Adherent Vero cells (ATCC® CCL-81™) were seeded into 3 mL of DMEM/2%FBS in a 6-well tissue culture plate at a concentration of 250,000 cells per well with either 0 or 1 mM ddhC before incubation at 37 °C for 24 hr. Because of the large volume used in this experiment and limited amount of ddhC, we performed time dependent conversion of ddhC to ddhCTP at 0.3 mM. Again, 250,000 cells were used for the 24 hr sample and 150,000 were used for both the 48 and 72 hr samples. After the designated amount of incubation, the cells were released from the plate by treatment with trypsin for 5 min at 37 °C. The cells were resuspended in DMEM media and counted as described above. The cells were then pelleted at 400 × g for 3 min. Both Vero and HEK293-F cells were lysed and analyzed as described above for the determination of intracellular concentration of ddhCTP. The volume of a Vero cell was estimated as 1.38 × 10^{-12} L.

Inhibition of Zika virus release with ddhC inhibitor. Zika virus strains (MR 766 (Uganda; 1947), PRVABC59 (Puerto Rico; 2015) and R103451 (Honduras; 2015) and were obtained from BEI resources (https://www.beiresources.org) and were passaged once on C6/36 cells to generate virus stocks. Vero cells (American Type Culture Collection, ATCC, Bethesda, MD) were maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen) at 37 °C with 5% CO_2. A. albopictus C6/36 (C6/36) cells were grown in Minimum Essential Media (MEM)(Invitrogen) containing 10% FBS and 1% penicillin/streptomycin and 25 mM

HEPES buffer (Gibco™ Life technologies) at 30 °C with 5% CO_2. Vero cells were grown to confluency on 96 well plates and were treated with different concentrations of ddhC (0, 0.1, and 1 mM) for 24 h. After this 24 h period, media over cells were removed and cells were infected at a multiplicity of infection (MOI) of 0.1 or 1 with fresh media containing the original concentrations of ddhC (0, 0.1, and 1 mM) and one of three strains of ZIKV; African strain MR766 (Uganda 1947), PRVABC59 (Puerto Rico; 2015) and R103451 (Honduras; 2016, GenBank: KX262887). The cells were infected with virus for 3 hr before the inoculum was removed and cells were treated with fresh media containing the original concentrations of ddhC (0, 0.1, and 1 mM) without virus. At 24, 48 and 72 h.p.i., samples were collected for viral titres and media over the cells were again replaced with fresh media containing the original concentrations of ddhC. The viral titres at 24, 48 and 72 h.p.i. were determined using the plaque assay. Data are mean ± s.d. from three biologically independent samples, *P* values from a two-way ANOVA with Dunnett's post hoc analysis (Supplementary Table 5).

Vero cell cytotoxicity assay. Confluent Vero cells grown on 96-well plates were treated with ddhC at various concentrations (0.0, 0.1, 0.5 and 1.0 mM in triplicate) for 96 hr. Media over cells were replaced at every 24 hr with fresh media supplemented with additional ddhC. At indicated time points, cells were trypsinized for 2 min with 20 µl 0.25% trypsin (Gibco™ Life technologies) at 37 °C for 2 min. Cells were resuspended in 80 µl DMEM with 10% FBS. 10 µl of cells were mixed with 10 µl of 4% trypan blue solution and the number of viable cells were counted using a hemacytometer.

Data availability. All data supporting the conclusions of the paper are available on reasonable request from the authors.

Supplementary Table 1. 2D HSQC NMR chemical shifts for ddhC, ddhCTP and CTP

	ddhCTP[a] (rVIP product)		3'-deoxy-3',4'-didehydro-cytidine[a]		Petrova et al., 2010 3'-deoxy-3',4'-didehydro-cytidine[b]		CTP[c]	
Position	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift
C1'	6.31	96.2	6.27	96.4	6.28	96.6	5.99	92.0
C2'	4.91	81.3	4.93	81.4	4.94	81.6	4.33	76.8
C3'	5.51	104.6	5.35	103	5.35	103.3	4.51	74.6
C4'		161.2		163.9			4.26	85.4
C5'	4.68	62.8	4.29/4.26	58.9	4.28	59.1	4.26	67.2
C2		156.8		159.9				160.5
C4		167.1		169				168.9
C5	6.04	99.2	5.99	99.1	6.00	99.4	6.14	99.3
C6	7.46	143.7	7.38	143.6	7.39	143.9	7.96	144.1

[a] Current Study

[b] Reference[8]

[c] HMDB database

Supplementary Table 2. Retention time and products monitored by LC-MS (6410) for quantification of nucleotides hCMPK2 substrate activity assays

	Parent Ion*	Product Ion 1†	Product Ion 2†
Tryptophan (IS)	203 (90)	115.9 (4)	
UTP	482.6 (135)	384.7 (6)	158.5 (10)
CTP	481.6 (135)	383.7 (6)	158.5 (10)
3'-deoxy-3',4'-didehydro-CTP	464 (380)	365.4 (6)	158.6 (10)

*Respective fragmentor voltages are in parenthesis

†Respective collision energies are in parenthesis

Supplementary Table 3. Primers used to clone hVIP or hCMPK2 into pCDNA3.3 for expression in HEK293F cells

construct	forward primer (5'-3')	reverse primer (5'-3')
FLAG- hVIP	TCGAACCCTTCTCGAGCCACCATGGACTACAAGGACGACGACGACAAGTGGGTGCTTACACCTGC	TCCAGGCGCTGGATCCTACCAATCCAGCTTCAGATCAGC
hVIP-FLAG	TCGAACCCTTCTCGAGCCACCATGTGGGTGCTTACACCTGC	TCCAGGCGCTGGATCCTACTTGTCGTCGTCGTCCTTGTAGTCCCAATCCAGCTTCAGATCAGC
(Δ1-42) hVIP FLAG	TCGAACCCTTCTCGAGCCACCATGGCTACCAAGAGGAGAAAGCAGC	TCCAGGCGCTGGATCCTACTTGTCGTCGTCGTCCTTGTAGTCCCAATCCAGCTTCAGATCAGC
hCMPK2	TCGAACCCTTCTCGAGCCACCATGGCTCCGCCGCGCC	TCCAGGCGCTGGATCCTACGGTCCACTAAAACTATTCTGGATTAGGC
hCMPK2-3xstrep	TCGAACCCTTCTCGAGCCACCATGGCTCCGCCGCGCC	TCCAGGCGCTGGATCCGGTCCACTAAAACTATTCTGGATTAGC

Supplementary Table 4. Cell count and viability for the HEK293F expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only

time post transfection (hours)	16hr		24hr		48hr		72hr	
transfection	cells ($x10^6$)	viability (%)	cells ($x10^6$)	viability (%)	cells ($x10^6$)	viability (%)	cells ($x10^6$)	viability (%)
FLAG-hVIP + control plasmid	1.7	97	2.3	79	4.2	86	3.0	69
FLAG-hVIP + hCMPK2	1.5	96	1.8	86	3.1	92	3.8	86
hCMPK2 + control plasmid	1.5	98	2.7	89	3.7	96	3.8	91
FLAG-hVIP + hCMPK2-3xstrep	1.4	99	2.2	92	3.6	94	3.8	95
control plasmid	1.8	99	2.0	92	4.8	96	3.2	94

Supplementary Table 5. Fold reduction in ZIKV titer in the presence of ddhC*

	Time hpi[+]		
Virus (MOI 0.1)	**24 h**	**48 h**	**72 h**
Uganda (MR 766)	89.4	49.2	204.4
Puerto Rico (PRVABC59)	14.0	5.7	5.2
Honduras (R103451)	134.4	21.0	19.3
	Time hpi		
Virus (MOI 1.0)	**24 h**	**48 h**	**72 h**
Uganda (MR 766)	56.7	46.7	5.7
Puerto Rico (PRVABC59)	22.5	9.9	5.3
Honduras (R103451)	53.0	9.3	7.8

*Values represent the average fold difference in ZIKV titer between 0mM and 1mM ddhC treatment (n=3 biological replicates)

[+]Hours post infection

Supplementary Table 6. Retention time and fragmentation products monitored by LC-MS for quantitation of 5'dA for Km and nucleotide specificity assays

	Retention Time	Parent Ion*	Product Ion1†	Product Ion 2†
Tryptophan (IS)		188 (130)	146.1 (10)	118 (21)
5'dA		252.1 (130)	136 (13)	119 (60)

*Respective fragmentor voltages are in parenthesis

†Respective collision energies are in parenthesis

Supplementary Table 7. Retention time and products monitored by LC-MS for quantification of 5'dA, CTP, and ddhCTP

	Retention Time	Parent Ion*
Tryptophan (IS)	2.3	202 (250)
5'dA	2.8	250 (90)
CTP	1.7	482 (135)
3'-deoxy-3',4'-didehydro-CTP	1.8	464 (135)

*Respective fragmentor voltages are in parenthesis

Supplementary Table 8. Retention time and fragmentation products monitored by LC-MS (6490) for quantification of ddhCTP

	Retention Time	Parent Ion*	Product Ion1†	Product Ion 2†
$^{13}C_9{}^{15}N_3$ 3'-deoxy-3',4'-didehydro-CTP	2.1	476 (380)	377.5 (8)	158.6 (10)
3'-deoxy-3',4'-didehydro-CTP	2.1	464 (380)	365.4 (8)	158.6 (10)

*Respective fragmentor voltages are in parenthesis

†Respective collision energies are in parenthesis

Supplementary Table 9. Retention time and fragmentation products monitored by LC-MS (6490) for quantification of nucleotides for HEK293F and RAW264.7 metabolomic analyses

	Retention Time	Parent Ion*	Product Ion1†	Product Ion 2†
$^{13}C_{10}{}^{15}N_5$-ATP	3.2	520.9 (380)	422.8 (20)	158.5 (20)
$^{13}C_{10}{}^{15}N_5$-GTP	2.8	538.0 (380)	440.0 (12)	158.5 (12)
$^{13}C_9{}^{15}N_3$-CTP	2.0	494 (380)	395.3 (6)	158.5 (10)
$^{13}C_9{}^{15}N_2$-UTP	2.7	484.9 (380)	386.9 (10)	158.5 (10)
ATP	3.2	505.9 (380)	407.8 (20)	158.5 (20)
CDP	1.1	401.9 (380)	383.6 (6)	158.5 (6)
CTP	2.0	481.6 (380)	383.7 (6)	158.6 (10)
GTP	2.8	521.4 (380)	423.6 (12)	158.5 (12)
UDP	1.6	402.8 (380)	384.8 (12)	158.5 (12)
UTP	2.7	482.6 (380)	384.7 (10)	158.5 (10)

*Respective fragmentor voltages are in parenthesis

†Respective collision energies are in parenthesis

Supplementary Table 10. Two-way ANOVA analysis of ddhCTP in HEK293F cells expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only*

Source of Variation	% of total variation	P value	P value summary	Significant	
Interaction	22.13	<0.0001	****	Yes	
Time	19.62	<0.0001	****	Yes	
Column Factor	45.66	0.0019	**	Yes	
Subjects (matching)	6.418	0.0284	*	Yes	
ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Interaction	76585	6	12764	F (6,18)=10.71	P<0.0001
Time	67885	3	22628	F (3,18) = 19.1	P<0.0001
Column Factor	158004	2	79002	F (2, 6) = 21.54	P<0.0018
Subjects (matching)	22211	6	3702	F (6,18) = 3.115	P<0.0284
Residual	21388	18	1188		

SS = sum-of-squares

DF = degrees of freedom

MS = mean squares

*n=3 biological replicates

Supplementary Table 11. Raw ddhCTP concentration data used in statistical analysis of post-hoc Tukey test from HEK293F cells expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only*

Post hoc test details **ddhCTP**	Mean 1	Mean 2	Mean Diff.	SE of Diff.	N1	N2	q	DF
16 hours post transfection								
hVIP Alone vs. hVIP + hCMPK2	74.66	93.56	-18.9	34.8	3	3	0.7679	24
hVIP Alone vs. Cells Only	74.66	0.05171	74.61	34.8	3	3	3.032	24
hVIP + hCMPK2 vs. Cells Only	93.56	0.05171	93.5	34.8	3	3	3.8	24
24 hours post transfection								
hVIP Alone vs. hVIP + hCMPK2	113.8	164.9	-51.09	34.8	3	3	2.076	24
hVIP Alone vs. Cells Only	113.8	0.3925	113.4	34.8	3	3	4.61	24
hVIP + hCMPK2 vs. Cells Only	164.9	0.3925	164.5	34.8	3	3	6.686	24
48 hours post transfection								
hVIP Alone vs. hVIP + hCMPK2	103.8	332.1	-228.3	34.8	3	3	9.276	24
hVIP Alone vs. Cells Only	103.8	0.5112	103.3	34.8	3	3	4.199	24
hVIP + hCMPK2 vs. Cells Only	332.1	0.5112	331.6	34.8	3	3	13.47	24
72 hours post transfection								
hVIP Alone vs. hVIP + hCMPK2	26.32	62.43	-36.12	34.8	3	3	1.468	24
hVIP Alone vs. Cells Only	26.32	0	26.32	34.8	3	3	1.07	24
hVIP + hCMPK2 vs. Cells Only	62.43	0	62.43	34.8	3	3	2.537	24

q = studentized range distribution

DF = degrees of freedom

SE = Standard Error

*n=3 biological replicates

Supplementary Table 12. Results from post-hoc Tukey test of HEK293F cells expressing FLAG-hVIP, FLAG- hVIP and hCMPK2, or cells only*

Number of families	4
Number of comparisons per family	3
Alpha	0.05

Tukey multiple comparisons test	Mean Diff	95% CI	Significant?	Summary	Adjusted P Value
16 hours post transfection					
hVIP Alone vs. hVIP + hCMPK2	-18.9	-105.8 to 68.01	No	ns	0.851
hVIP alone vs Cells Only	74.61	-12.3 to 161.5	No	ns	0.1021
hVIP + hCMPK2 vs Cells only	93.5	6.596 to 180.4	Yes	*	0.0333
24 hours post transfection					
hVIP Alone vs. hVIP + hCMPK2	-51.09	-138 to 35.82	No	ns	0.3236
hVIP alone vs Cells Only	113.4	26.52 to 200.3	Yes	**	0.009
hVIP + hCMPK2 vs Cells only	164.5	77.61 to 251.4	Yes	***	0.0002
48 hours post transfection					
hVIP Alone vs. hVIP + hCMPK2	-228.3	-315.2 to -141.4	Yes	****	<0.0001
hVIP alone vs Cells Only	103.3	16.41 to 190.2	Yes	*	0.0177
hVIP + hCMPK2 vs Cells only	331.6	244.7 to 418.5	Yes	****	<0.0001
72hours post transfection					
hVIP Alone vs. hVIP + hCMPK2	-36.12	-123 to 50.79	No	ns	0.5609
hVIP alone vs Cells Only	26.32	-60.59 to 113.2	No	ns	0.7328
hVIP + hCMPK2 vs Cells only	62.43	-24.47 to 149.3	no	ns	0.1929

ns = no significance

Mean Diff = mean difference

*n=3 biological replicates

Supplemenary Table 13. Two-way ANOVA analysis of ATP concentrations in HEK293F cells expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only*

Alpha 0.05

Source of Variation	% of total variation	P value	P value summary	Significant
Interaction	5.61	0.8592	ns	No
Time	7.67	0.3615	ns	No
Column Factor	35.37	0.0129	*	No
Subjects (matching)	10.82	0.5819	ns	No

ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Interaction	30285803	6	5047634	F (6,18)=0.4153	P=0.8592
Time	41386665	3	13795555	F (3,18) = 1.135	P=0.3615
Column Factor	190815719	2	95407859	F (2, 6) = 9.801	P=0.0129
Subjects (matching)	58407429	6	9734572	F (6,18) =0.8009	P=0.5819
Residual	218786299	18	12154794		

SS = sum-of-squares

DF = degrees of freedom

MS = mean squares

*n=3 biological replicates

Supplementary Table 14. Two-way ANOVA analysis of GTP concentrations in HEK293F cells expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only*

Alpha 0.05

Source of Variation	% of total variation	P value	P value summary	Significant
Interaction	13.45	0.2753	ns	No
Time	16.04	0.0443	*	Yes
Column Factor	19.59	0.1452	ns	No
Subjects (matching)	21.7	0.0878	ns	No

ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Interaction	19680399	6	3280067	F (6, 18) = 1.38	P=0.2753
Time	23483033	3	7827678	F (3, 18) = 3.294	P=0.0443
Column Factor	28665542	2	14332771	F (2, 6) = 2.708	P=0.1452
Subjects (matching)	31757642	6	5292940	F (6, 18) = 2.227	P=0.0878
Residual	42772786	18	2376266		

SS = sum-of-squares

DF = degrees of freedom

MS = mean squares

*n=3 biological replicates

Supplenentary Table 15. Two-way ANOVA analysis of UTP concentrations in HEK293F cells expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only*

Alpha 0.05

Source of Variation	% of total variation	P value	P value summary	Significant
Interaction	4.994	0.5300	ns	No
Time	49.33	<0.0001	****	Yes
Column Factor	20.6	0.0220	*	Yes
Subjects (matching)	8.015	0.2646	ns	No

ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Interaction	8087791	6	1347965	F (6, 18) = 0.8784	P=0.5300
Time	79887736	3	26629245	F (3, 18) = 17.35	P<0.0001
Column Factor	33364758	2	16682379	F (2, 6) = 7.712	P=0.0220
Subjects (matching)	12979341	6	2163224	F (6, 18) = 1.41	P=0.2646
Residual	27622164	18	1534565		

SS = sum-of-squares

DF = degrees of freedom

MS = mean squares

*n=3 biological replicates

Supplementary Table 16. Two-way ANOVA analysis of CTP concentrations in HEK293F cells expressing FLAG- hVIP, FLAG- hVIP and hCMPK2, or cells only*

Alpha 0.05

Source of Variation	% of total variation	P value	P value summary	Significant
Interaction	15.48	0.0658	ns	No
Time	38.28	0.0001	***	Yes
Column Factor	18.56	0.0325	*	Yes
Subjects (matching)	8.697	0.2774	ns	No

ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Interaction	666614	6	111102	F (6, 18) = 2.447	P=0.0658
Time	1648202	3	549401	F (3, 18) = 12.1	P=0.0001
Column Factor	799004	2	399502	F (2, 6) = 6.401	P=0.0325
Subjects (matching)	374451	6	62408	F (6, 18) = 1.375	P=0.2774
Residual	817161	18	45398		

SS = sum-of-squares

DF = degrees of freedom

MS = mean squares

*n=3 biological replicates

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