Supporting information for "Structural insight into the substrate scope of viperin and viperin-like enzymes from three domains of life"

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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 purchased from Cayman Chemical (Ann Arbor MI). The pDB1282 plasmid was a kind gift from Professor Squire Booker's lab (Pennsylvania State University, State

College, PA). UV-visible spectra were recorded on an Agilent Cary 60 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. DNA oligonucleotides were from Integrated DNA Technologies; nucleoside triphosphates (ATP, CTP, GTP and UTP) were Ultrapure solutions from GE Healthcare or, for MmVip experiments, from Sigma; deuterium containing nucleotides were from Cambridge Isotopes (San Diego CA); all other reagents were of the highest grade from either Sigma, Fisher, VWR, Takara Bio, or Acros Organics. All DNA vectors were sequence verified with dideoxy sequencing performed by Genewiz.

Cloning of the TvVip, NvVip, MlVip and MmVip genes. The *TvVip* (Uniprot ID: G9MQB8_HYPVG), NvVip (Uniprot ID: A7RNF3), SbVip (Uniprot ID: A9L1Z3), MlVip (Uniprot ID: J0S9F5), LmVip (UniParc ID: UPI0006E20534) MmVip (Uniprot ID: Q8CBB9) variants (H79A, Y302A, Y302F, S124A, N77A), and RnVip (Uniprot ID: O70600) variants (H77A and K317A) genes were commercially synthesized by IDT containing ligation independent cloning (LIC) sites that are complementary to the vector pNIC28-Bsa4. The synthesized genes 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 TvVip, NvVip and MmVip. It has been previously shown that removal of the N-terminal amphipathic domain is important for the production of soluble recombinant viperin from human, rat, and mouse(4-6) Based on this observation, Pred-Signal(7) suggested that only TvVip contained a signal peptide (residues 1-15) for cellular localization, which was removed. The remainder of our targeted proteins did not possess a predicted amphipathic sequence and NvVip, LmVip, SbVip and, MlVip were synthesized as full-length gene sequences for heterologous expression.

TvVip_NT: G9MQB8

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TvVip_NT: G9N0G3

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GTT GTA ACT CGC CTG AAC TAC GAA GAG GAT ATG AAT GAA CAC ATT GAT ACG TTG CAA
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TvVip_Coding: G9N0G3

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SbVip_Coding: A9L1Z3

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LmVip_NT

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MmVip (H79A)_NT
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MmVip (H79A)_Coding

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MmVip (Y302A)_NT

<u>5'-</u>

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MmVip (Y302A)_Coding

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MmVip (Y302F)_NT

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TCCTTTCTTATCCTAGATGAATATATGCGCTTTCTGAACTGTACCGGTGGCCGGAAGGACCCTTCC

AAGTCTATTCTGGATGTTGGCGTGGAAGAAGCAATAAAGTTCAGTGGATTTGATGAGAAGATGTTT
CTGAAGCGTGGCGGAAAGTATGTGTGGAGTAAAGCTGACCTGAAGCTGGACTGG*TAA*<u>*CAGTAAAG*</u>
<u>*GTGGATA-3'*</u>

MmVip (Y302F)_Coding
<u>*MHHHHHHSSGVDLGTENLYFQS*</u>MGKEQPQVRGELEETQETQEDGNSTQRTTPVSVNYHFTRQCNYK
CGFCFHTAKTSFVLPLEEAKRGLLLLKQAGLEKINFSGGEPFLQDRGEYLGKLVRFCKEELALPSVSIVS
NGSLIQERWFKDYGEYLDILAISCDSFDEQVNALIGRGQGKKNHVENLQKLRRWCRDYKVAFKINSVIN
RFNVDEDMNEHIKALSPVRWKVFQCLLIEGENSGEDALREAERFLISNEEFETFLERHKEVSCLVPESN
QKMKDSFLILDEYMRFLNCTGGRKDPSKSILDVGVEEAIKFSGFDEKMFLKRGGKYVWSKADLKLDW

MmVip (S124A)_NT
<u>5'-</u>
<u>TACTTCCAATCC</u>ATGGGGAAGGAACAGCCACAGGTCCGGGGTGAGCTGGAGGAGACCCAGGAGA
CCCAGGAAGATGGGAACAGCACTCAGCGCACAACCCCCGTGAGTGTCAACTACCACTTCACTCGT
CAGTGCAACTACAAATGTGGCTTCTGCTTCCACACAGCCAAGACATCCTTCGTGCTGCCCCTGGA
GGAGGCCAAGCGAGGACTGCTTCTGCTCAAACAGGCTGGTTTGGAGAAGATCAACTTTGCGGGA
GGAGAACCCTTCCTTCAGGACAGGGGTGAATACTTGGGCAAGCTTGTGAGATTCTGCAAGGAGGA
GCTAGCCCTGCCCTCTGTGAGCATAGTGAGCAATGGCAGCCTTATCCAGGAGAGATGGTTCAAGG
ACTATGGGGAGTATTTGGACATTCTTGCTATCTCCTGCGACAGCTTCGATGAGCAGGTTAATGCTC
TGATTGGCCGTGGTCAAGGAAAAAAGAACCACGTGGAAAACCTTCAAAAGCTGAGGAGGTGGTGC
AGGGATTACAAGGTGGCTTTCAAGATCAACTCTGTCATTAATCGCTTCAACGTGGACGAAGACATG
AATGAACACATCAAGGCCCTGAGCCCTGTGCGCTGGAAGGTTTTCCAGTGCCTCCTAATTGAGGG
TGAGAACTCAGGAGAAGATGCCCTGAGGGAAGCAGAAAGATTTCTTATAAGCAATGAAGAATTTGA
AACATTCTTGGAGCGTCACAAAGAGGTGTCCTGTTTGGTGCCTGAATCTAACCAGAAGATGAAAGA
CTCCTACCTTATCCTAGATGAATATATGCGCTTTCTGAACTGTACCGGTGGCCGGAAGGACCCTTC
CAAGTCTATTCTGGATGTTGGCGTGGAAGAAGCAATAAAGTTCAGTGGATTTGATGAGAAGATGTT
TCTGAAGCGTGGCGGAAAGTATGTGTGGAGTAAAGCTGACCTGAAGCTGGACTGG
TAA<u>*CAGTAAAGGTGGATA-3'*</u>

MmVip (S124A)_Coding
<u>*MHHHHHHSSGVDLGTENLYFQS*</u>MGKEQPQVRGELEETQETQEDGNSTQRTTPVSVNYHFTRQCNYK
CGFCFHTAKTSFVLPLEEAKRGLLLLKQAGLEKINFAGGEPFLQDRGEYLGKLVRFCKEELALPSVSIVS
NGSLIQERWFKDYGEYLDILAISCDSFDEQVNALIGRGQGKKNHVENLQKLRRWCRDYKVAFKINSVIN
RFNVDEDMNEHIKALSPVRWKVFQCLLIEGENSGEDALREAERFLISNEEFETFLERHKEVSCLVPESN
QKMKDSYLILDEYMRFLNCTGGRKDPSKSILDVGVEEAIKFSGFDEKMFLKRGGKYVWSKADLKLDW

MmVip (N77A)_NT
<u>5'-</u>
<u>TACTTCCAATCC</u>ATGGGGAAGGAACAGCCACAGGTCCGGGGTGAGCTGGAGGAGACCCAGGAGA
CCCAGGAAGATGGGAACAGCACTCAGCGCACAACCCCCGTGAGTGTCGCGTACCACTTCACTCG
TCAGTGCAACTACAAATGTGGCTTCTGCTTCCACACAGCCAAGACATCCTTCGTGCTGCCCCTGGA
GGAGGCCAAGCGAGGACTGCTTCTGCTCAAACAGGCTGGTTTGGAGAAGATCAACTTTTCTGGAG
GAGAACCCTTCCTTCAGGACAGGGGTGAATACTTGGGCAAGCTTGTGAGATTCTGCAAGGAGGAG
CTAGCCCTGCCCTCTGTGAGCATAGTGAGCAATGGCAGCCTTATCCAGGAGAGATGGTTCAAGGA
CTATGGGGAGTATTTGGACATTCTTGCTATCTCCTGCGACAGCTTCGATGAGCAGGTTAATGCTCT
GATTGGCCGTGGTCAAGGAAAAAAGAACCACGTGGAAAACCTTCAAAAGCTGAGGAGGTGGTGCA
GGGATTACAAGGTGGCTTTCAAGATCAACTCTGTCATTAATCGCTTCAACGTGGACGAAGACATGA
ATGAACACATCAAGGCCCTGAGCCCTGTGCGCTGGAAGGTTTTCCAGTGCCTCCTAATTGAGGGT
GAGAACTCAGGAGAAGATGCCCTGAGGGAAGCAGAAAGATTTCTTATAAGCAATGAAGAATTTGAA
ACATTCTTGGAGCGTCACAAAGAGGTGTCCTGTTTGGTGCCTGAATCTAACCAGAAGATGAAAGAC
TCCTACCTTATCCTAGATGAATATATGCGCTTTCTGAACTGTACCGGTGGCCGGAAGGACCCTTCC
AAGTCTATTCTGGATGTTGGCGTGGAAGAAGCAATAAAGTTCAGTGGATTTGATGAGAAGATGTTT
CTGAAGCGTGGCGGAAAGTATGTGTGGAGTAAAGCTGACCTGAAGCTGGACTGG***TAA<u>CAGTAAA</u>***
<u>GGTGGATA-3</u>'

MmVip (N77A)_Coding

*MHHHHHHSSGVDLGTENLYFQS*MGKEQPQVRGELEETQETQEDGNSTQRTTPVSVAYHFTRQCNYK
CGFCFHTAKTSFVLPLEEAKRGLLLLKQAGLEKINFSGGEPFLQDRGEYLGKLVRFCKEELALPSVSIVS
NGSLIQERWFKDYGEYLDILAISCDSFDEQVNALIGRGQGKKNHVENLQKLRRWCRDYKVAFKINSVIN
RFNVDEDMNEHIKALSPVRWKVFQCLLIEGENSGEDALREAERFLISNEEFETFLERHKEVSCLVPESN
QKMKDSYLILDEYMRFLNCTGGRKDPSKSILDVGVEEAIKFSGFDEKMFLKRGGKYVWSKADLKLDW

RnVip (H77A)_NT

*5'**TACTTCCAATCC***ATGGGTGAGCCCAAGGAGACTCAGGAGACCCACGAAGATCCAGGCAGTGCT
CAGCCCACAACCCCCGTTAGTGTCAACTACCACTTCACGCGTCAGTGCAACTACAAATGTGGCTTC
TGCTTCCACACGGCCAAGACATCCTTCGTGCTGCCCCTGGAGGAGGCCAAGCGAGGACTGCTTC
TGCTCAAACAGGCTGGTATGGAGAAGATCAACTTTTCAGGAGGAGAACCCTTCCTACAGGACAGG
GGTGAATACTTGGGCAAGCTCGTGAGATTCTGCAAGGAGGAGCTAGCCCTGCCCTCTGTGAGCAT
AGTGAGCAATGGCAGCCTTATCCGAGAGAGATGGTTCAAGGACTATGGAGACTATTTGGACATTCT
TGCTATCTCCTGTGACAGCTTTGATGAGCAGGTTAATGTTCTCATTGGTCGTGGCCAAGGGAAAAA
GAACCATGTGGAAAACCTTCAAAAGCTGCGGAAGTGGTGCAGGGATTACAAGGTGGCTTTCAAGA
TCAATTCCGTCATTAATCGCTTCAACGTGGACGAAGATATGAATGAGCACATCAAGGCGCTGAGCC
CTGTCCGCTGGAAGGTTTTCCAATGCCTCCTGATTGAGGGTGAGAACTCAGGAGAAGATGCCCTG
AGGGAAGCAGAAAGATTTCTTATAAGCAATGAAGAATTTGAAGCATTCTTACAGCGTCACAAAGAT
GTGTCCTGCTTGGTGCCTGAATCTAACCAGAAGATGAAAGACTCCTACCTTATCCTGGATGAATAT
ATGCGCTTTTTGAACTGTACCGGTGGCCGG**GCC**GACCCTTCCAGGTCCATCCTGGATGTTGGCGT
GGAGGAAGCGATCAAATTCAGTGGGTTTGATGAGAAGATGTTTCTGAAGCGCGGGGGGAAGTATG
TATGGAGTAAGGCTGACCTGAAGCTAGACTGG*TAACAGTAAAGGTGGATA-3*'

RnVip (H77A)_Coding

*MHHHHHHSSGVDLGTENLYFQS*MGEPKETQETHEDPGSAQPTTPVSVNYHFTRQCNYKCGFCFHTA
KTSFVLPLEEAKRGLLLLKQAGMEKINFSGGEPFLQDRGEYLGKLVRFCKEELALPSVSIVSNGSLIRER
WFKDYGDYLDILAISCDSFDEQVNVLIGRGQGKKNHVENLQKLRKWCRDYKVAFKINSVINRFNVDED
MNEHIKALSPVRWKVFQCLLIEGENSGEDALREAERFLISNEEFEAFLQRHKDVSCLVPESNQKMKDS
YLILDEYMRFLNCTGGRADPSRSILDVGVEEAIKFSGFDEKMFLKRGGKYVWSKADLKLDW

RnVip (K317A)_NT

*5'**TACTTCCAATCC***ATGGGTGAGCCCAAGGAGACTCAGGAGACCCACGAAGATCCAGGCAGTGCT
CAGCCCACAACCCCCGTTAGTGTCAACTACGCCTTCACGCGTCAGTGCAACTACAAATGTGGCTT
CTGCTTCCACACGGCCAAGACATCCTTCGTGCTGCCCCTGGAGGAGGCCAAGCGAGGACTGCTT
CTGCTCAAACAGGCTGGTATGGAGAAGATCAACTTTTCAGGAGGAGAACCCTTCCTACAGGACAG
GGGTGAATACTTGGGCAAGCTCGTGAGATTCTGCAAGGAGGAGCTAGCCCTGCCCTCTGTGAGC
ATAGTGAGCAATGGCAGCCTTATCCGAGAGAGATGGTTCAAGGACTATGGAGACTATTTGGACATT
CTTGCTATCTCCTGTGACAGCTTTGATGAGCAGGTTAATGTTCTCATTGGTCGTGGCCAAGGGAAA
AAGAACCATGTGGAAAACCTTCAAAAGCTGCGGAAGTGGTGCAGGGATTACAAGGTGGCTTTCAA
GATCAATTCCGTCATTAATCGCTTCAACGTGGACGAAGATATGAATGAGCACATCAAGGCGCTGAG
CCCTGTCCGCTGGAAGGTTTTCCAATGCCTCCTGATTGAGGGTGAGAACTCAGGAGAAGATGCCC
TGAGGGAAGCAGAAAGATTTCTTATAAGCAATGAAGAATTTGAAGCATTCTTACAGCGTCACAAAG
ATGTGTCCTGCTTGGTGCCTGAATCTAACCAGAAGATGAAAGACTCCTACCTTATCCTGGATGAAT
ATATGCGCTTTTTGAACTGTACCGGTGGCCGGAAGGACCCTTCCAGGTCCATCCTGGATGTTGGC
GTGGAGGAAGCGATCAAATTCAGTGGGTTTGATGAGAAGATGTTTCTGAAGCGCGGGGGGAAGTA
TGTATGGAGTAAGGCTGACCTGAAGCTAGACTGGTAACAGTAAAGGTGGATA*TAACAGTAAAGGT
GGATA-3*'

RnVip (K317A)_Coding

*MHHHHHHSSGVDLGTENLYFQS*MGEPKETQETHEDPGSAQPTTPVSVNYAFTRQCNYKCGFCFHTA
KTSFVLPLEEAKRGLLLLKQAGMEKINFSGGEPFLQDRGEYLGKLVRFCKEELALPSVSIVSNGSLIRER
WFKDYGDYLDILAISCDSFDEQVNVLIGRGQGKKNHVENLQKLRKWCRDYKVAFKINSVINRFNVDED

MNEHIKALSPVRWKVFQCLLIEGENSGEDALREAERFLISNEEFEAFLQRHKDVSCLVPESNQKMKDS
YLILDEYMRFLNCTGGRKDPSRSILDVGVEEAIKFSGFDEKMFLKRGGKYVWSKADLKLDW

Cloning of the wildtype Rattus norvegicus viperin gene was performed as described previously. (5)
RnVip:
*MHHHHHHSSGVDLGTENLYFQS*MGEPKETQETHEDPGSAQPTTPVSVNYHFTRQCNYKCGFCFHTAKTSFV
LPLEEAKRGLLLLKQAGMEKINFSGGEPFLQDRGEYLGKLVRFCKEELALPSVSIVSNGSLIRERWFKDYGDYL
DILAISCDSFDEQVNVLIGRGQGKKNHVENLQKLRKWCRDYKVAFKINSVINRFNVDEDMNEHIKALSPVRWKV
FQCLLIEGENSGEDALREAERFLISNEEFEAFLQRHKDVSCLVPESNQKMKDSYLILDEYMRFLNCTGGRKDPS
RSILDVGVEEAIKFSGFDEKMFLKRGGKYVWSKADLKLDW

Expression and purification of TvVip, NvVip, MlVip MmVip and RnVip. BL-21 (RIL) competent cells containing the helper plasmid pDB1282 were transformed with a viperin plasmid. This strategy allows for the expression of the viperin-like gene in the presence of the *isc* operon of FeS cluster assembly proteins (8). A single colony was used to inoculate an overnight culture of P-0.5G defined minimal media (9) with subsequent incubation at 37 °C. PA-5052, a defined medium for auto induction (9), 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 30 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 seconds on and 12 seconds off, for a total of 20 minutes 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 centrifugals filter unit (10,000 M.W. cut-off). The protein concentration was estimated using an extinction coefficient calculated from the amino acid sequence (Expasy ProtParam).

Expression and purification of MmVip and variants for mutagenesis experiments. Methods were the same as above but with the following modifications. BL-21 (non-RIL) competent cells were used containing the helper plasmid pDB1282. Iron and cysteine were added to final concentrations of 100 μM and 1.2 mM, respectively. MmVip (30 g) cell paste was resuspended in 30 mL lysis buffer. Cells were cooled in an ice bath while being subjected to cycles of 3 seconds on and 20 seconds off, for a total of 30 minutes of sonic disruption (80% output). Cell lysate was centrifuged for 1.5 hours at 10,000 × g at 4 °C.

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 an Agilent Technologies 6490 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). For MmVip variant experiments, assay mixtures were separated on an Agilent Technologies InfinityLab Poroshell 120 HILIC-Z (2.1 x 100 mm, 2.7 μm particle size, PEEK lined) and equilibrated in 10% buffer A (10 mM ammonium acetate buffered to pH 9, 5 μM methylenediphosphonic acid,

10% acetonitrile) and 90% buffer B (10 mM ammonium acetate buffered to pH 9, 5 μM methylenediphosphonic acid, 90% acetonitrile).

In vivo production of 3'-deoxy-3',4'-didehydro-CTP in HEK293-F cells: The human (Hs) viperin wild type, H78A, and K318A coding sequences were PCR amplified (template: accession number AF442151.1, Genscript) using PCR primers shown in **Table S1** and cloned into a modified version of pCDNA3.3 using InFusion HD (Clontech) to generate constructs expressing full-length Hs viperin.

TABLE S1: PCR primers for HsVip wild type, H78A, and K318A

construct	forward primer (5'-3')	reverse primer (5'-3')
Hs viperin	TCGAACCCTTCTCGAGCCACCAT GTGGGTGCTTACACCTGC	TCCAGGCGCTGGATCCTACTTGTCGTCGTCGTCC TTGTAGTCCCAATCCAGCTTCAGATCAGC
H78A viperin	CTGAACTGTAGAAAGGGACGGGC GGACCCTTCCAAGTCCATCC	GGATGGACTTGGAAGGGTCCGCCCGTCCCTTTCT ACAGTTCAG
K318A viperin	CCCAACCAGCGTCAACTATGCCT TCACTCGCCAGTGCAACTAC	GTAGTTGCACTGGCGAGTGAAGGCATAGTTGACG CTGGTTGGG

Plasmids encoding Hs viperin or Hs viperin variants, 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 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 hour at 37 °C. Cells were grown in FreeStyle Expression Medium with gentle shaking and harvested at 24 hours post transfection. Cell count and viability were measured with an automated cell counter after staining with trypan blue (Countess, Life Technologies) and harvested by centrifugation at 400 × g for 3 minutes.

Metabolite extraction of HEK293 FreeStyle: 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, $^{13}C_9{}^{15}N_2$-3'-deoxy-3',4'-didehydro-UTP, $^{13}C_9{}^{15}N_3$-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 cells. The tubes were vortexed for 30 seconds and then incubated on ice for 10 minutes. The lysates were pelleted by centrifugation at 13,000 × g for 10 minutes 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 minutes, and 15-50% B from 3.25-3.75 minutes before returning to 100% A from 3.75-5 minutes. The column was allowed to re-equilibrate for 1 minute 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 (**Table S2**).

TABLE S2: Retention time and fragmentation products monitored by LC-MS (6490)

	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)
$^{13}C_9{}^{15}N_3$ 3'-deoxy-3',4'-didehydro-UTP	2.6	476 (380)	378.0 (8)	158.8 (10)
3'-deoxy-3',4'-didehydro-UTP	2.6	465 (380)	366.4 (8)	158.8 (10)

*Respective fragmentor voltages are in parenthesis

†Respective collision energies are in parenthesis

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 ($V_{cell} = 1.38 \times 10^{-12}$ 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 1 where V is equal to the volume of one cell).

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

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

In vitro production of 3'-deoxy-3',4'-didehydro-CTP and 3'-deoxy-3',4'-didehydro-UTP: A reaction (1 mL total volume) containing 300 µM NvVip or TvVip, 50 mM HEPES, pH 7.5, 150 mM KCl, 2 mM SAM, 2 mM or $^{13}C_9{}^{15}N_3$-CTP (NvVip) or $^{13}C_9{}^{15}N_2$-UTP (TvVip), and 5 mM dithionite was incubated for 2 hours at 37°C. These large-scale reactions typically produce between 1600 and 1800 nmoles of 3'-deoxy-3',4'-didehydro-*N*TP. After two hours the reaction was precipitated with 100mM 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 less than 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-*N*TP can be easily separated from starting material (CTP or UTP) and diphosphate moieties**.** The concentration of the purified product was determined by UV absorbance at 271nm or 262nm for ddhCTP and ddhUTP, respectively, using the extinction coefficient for CTP or UTP (13.1×10^3, 10.2×10^3 A_m). Verification of purity was performed using electrospray ionization in negative mode (ESI -) with an MS2-scan window from 150-600 *m/z*.

Substrate Specificity Assays.

General LC-MS procedures for ESI-: 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 minutes, and 0.8-20% B from 0.5-2 minutes, and then 20-50% B from 2-2.5 minutes before returning to 99.2% A from 2.5-3 minutes. The column was allowed to re-equilibrate for 1 minute under initial conditions before subsequent sample injections. This method allows for facile separation of the starting material (CTP or UTP) from product (3'-deoxy-3',4' -dideoxy-CTP, or 3'-deoxy-3',4' -dideoxy-UTP). 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. The mass to charge ratio (*m/z*) of unlabeled UTP is 483.1, and increases by one mass unit for each deuterium substitution. The *m/z* of the resulting ddhUTP is 465.1, which increases by one mass unit for each deuterium substitution. 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/UTP), 460-470 (ddhCTP/ddhUTP) and 245-255 (5'dA).

Nucleotide specificity: Reactions contained in a total volume of 100 µL: 50 µM *TvVip, NvVip or MmVip*, 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM SAM, 1 mM of appropriate nucleotides (ATP, GTP, CTP or UTP), 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 five 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 minutes, and 5-20% B from 0.5-2 minutes, and 20-50% B from 2-2.5 minutes before returning to

100% A from 2.5-3 minutes. The column was allowed to re-equilibrate for 1 minute under initial conditions before subsequent sample injections. Detection of 5'dA (**Tables S3, S4 and S5**) 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.

TABLE S3: Fragmentation products monitored by LC-MS

	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

TABLE S4: Retention time and products monitored by LC-MS

	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

TABLE S5: Products monitored by LC-MS

	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)
3'-deoxy-3',4'-didehydro-UTP	465 (380)	366.4 (6)	158.78 (10)

*Respective fragmentor voltages are in parenthesis
†Respective collision energies are in parenthesis

Competition assays with deuterium containing nucleotides: Reactions contained in a total volume of 100 µL: 50 µM *TvVip, NvVip or MmVip*, 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM SAM, 1 mM deuterium incorporated CTP or UTP (labeled CTP contains deuterium at the following position; (2'-^{2}H, 3'-^{2}H, 4'-^{2}H, 5'-$^2H_2$ and 5''-$^2H_2$-CTP, labeled UTP contains deuterium at the following position; (5-^{2}H, 3'-^{2}H, 4'-^{2}H, 5'-$^2H_2$ and 5''-$^2H_2$-UTP) in the presence of three natural abundance nucleotides (e.g. NvVip and MmVip reactions contain 1mM deuterium-CTP and 1mM of each UTP, ATP and GTP). Reaction mixtures were initiated with dithionite and incubated for 5 minutes and the resulting deuterium incorporated and natural abundance 5'dA was monitored as described above for LC-MS.

Competition assays with UDP-glucose and Isopentyl pyrophosphate (IPP): Reactions contained in a total volume of 100 µL: 50 µM *TvVip, NvVip or MmVip*, 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM SAM, 1 mM CTP (NvVip, MmVip) or UTP (TvVip) alone or in the presence of 10mM UDP-glucose or IPP. Reaction mixtures were initiated with dithionite and incubated for 4 minutes and the amount of 5'dA was monitored as described above for LC-MS.

Product formation assays with MmVip and MmVip variants
Reactions contained in a total volume of 150 µL (ddhCTP formation) or 60 µL (2',3'-deoxy-3',4'-didehydrocytidine triphosphate formation): 25 µM *MmVip or MmVip variant*, 50 mM HEPES pH 7.5, 150 mM KCl, 1 mM SAM, 1 mM of CTP, and 5 mM dithionite. For experiments with 2'-deoxycytidine-5'-triphosphate, 10 mM of nucleotide substrate was utilized. Reaction mixtures lacking dithionite were incubated for 5 minutes at room temperature, and an aliquot was removed (t=0) and added to a solution containing 100 mM H_2SO_4 and 200 µM L-tryptophan (internal standard) in a 1:1 ratio to yield a final internal standard concentration of 100 µM. Reactions were initiated

with dithionite and incubated at the indicated timepoints before being quenched in acid as described above. The samples were centrifuged at 4,000 x g to remove any precipitate and the supernatants were injected onto an Agilent InfinityLab Poroshell 120 HILIC-Z column equilibrated in 90% buffer B (10:90 water to acetonitrile) and 10% buffer A (90:10 water to acetonitrile). A gradient of 10-24% buffer A was applied from 0-1.5 minutes, 24-27% A from 1.5-5 minutes, and 27-40% B from 5-6 minutes, before returning to 10% A from 6.5-7.5 minutes. The column was allowed to re-equilibrate for 1 minute under initial conditions before subsequent sample injections. Detection of ddhCTP and the m/z corresponding to 2',3'-deoxy-3',4'-didehydrocytidine triphosphate (**Table S6**) was performed with electrospray ionization in negative mode (ESI^-) with multiple reaction monitoring (MRM).

TABLE S6: Retention time and products monitored by LC-MS for experiments with MmVip and MmVip variants

	Retention time	Parent Ion*	Product Ion 1†	Product Ion 2†
Tryptophan (IS)	2.0	203 (380)	115.9 (4)	141.8 (8)
3'-deoxy-3',4'-didehydro-CTP	5.5	464 (380)	365.4 (8)	158.6 (10)
2',3'-deoxy-3',4'-didehydro-CTP	4.4	448 (380)	349.9 (8)	158.5 (10)

*Respective fragmentor voltages are in parenthesis
†Respective collision energies are in parenthesis

2D-HSQC 1H/13C: 2D-^{1}H,^{13}C-HSQC 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 experiments on a dual ^{13}C,^{15}N-labeled sample. (See **Table S7 and Table S8** for full list of chemical shift data).

TABLE S7: 2D-HSQC NMR chemical shifts from current study

	ddhCTP		**ddhUTP**		**CTP**		**UTP**	
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	6.28	95.4	5.99	91.0	6.28	96.6
C2'	4.91	81.3	4.93	82.1	4.33	73.8	4.41	72.6
C3'	5.46	104.6	5.35	105.4	4.27	77.6	4.36	76.5
C4'	ND[a]	ND[a]	ND[a]	ND[a]	4.16	85.4	4.22	86.1
C5'	4.68	62.8	4.62	62.1	4.26	67.2	4.28	67.7
C5	6.04	99.2	5.81	106.2	6.14	99.3	5.93	106.1
C6	7.46	143.7	7.48	143.6	8.10	144.1	7.89	143.9

[a] ND = Not detected

TABLE S8: 2D-HSQC NMR chemical shifts from Gizzi et al., 2018

	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] Gizzi et al., 2018
[b] Reference(10)
[c] HMDB database

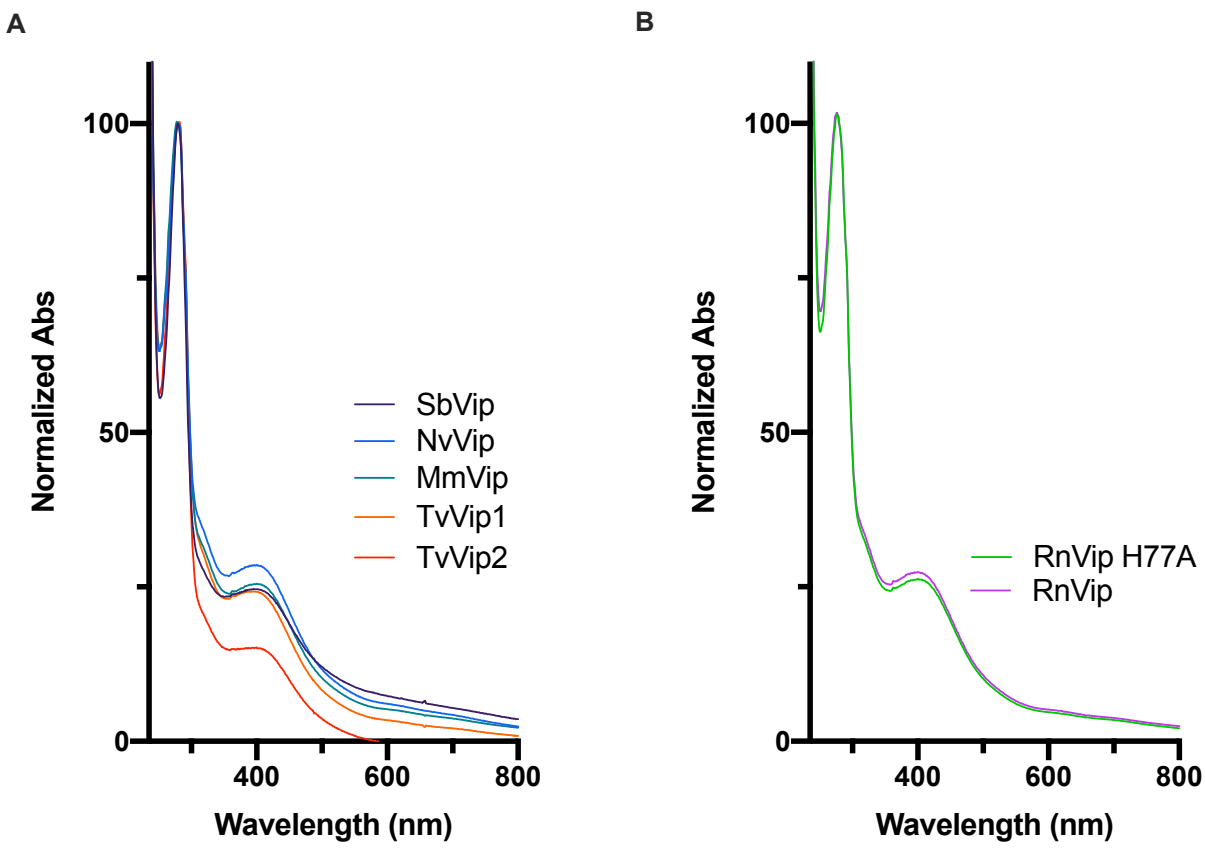
Crystallization of NvVip and TvVip: Diffraction-quality crystals were obtained by sitting-drop vapor diffusion at 20 °C in an anaerobic chamber maintained at <0.1 ppm oxygen (MBraun). Both NvVip and TvVip were treated with TEV protease to remove the N-terminal hexahistidine tag before screening. Drops of 0.4 µL of NvVip [10 mg/mL in 10 mM HEPES (pH 7.5), 1 mM SAM, 1 mM CTP] were mixed with 0.4 µL of precipitant (0.1 M Tris-HCl, pH 7.0, 20% (w/v) PEG 2000 MME) and equilibrated against a solution of 0.5 M LiCl. Drops of 0.4 µL of TvVip [10 mg/mL in 10 mM HEPES (pH 7.5), 1 mM SAM, 1 mM UTP] were mixed with 0.4 µL of precipitant (0.1 M Tris-HCl, pH 8.5, 0.2 M NaCl, 25% (w/v) PEG 2000 MME) and equilibrated against a solution of 0.5 M LiCl. Crystals were mounted in nylon loops and flash cooled in liquid nitrogen, inside the anaerobic chamber, and stored in liquid nitrogen prior to data collection. Diffraction data were collected at NSLSII (Brookhaven National Lab, Brookhaven, NY) on beamline 17-ID-2 (FMX). All diffraction data were integrated and scaled using the HKL3000 suite or XDS and aimless. Diffraction from the NvVip crystals was consistent with the orthorhombic space group $P2_12_12_1$ that extended to a resolution of 1.42 Å with unit cell parameters consisting of a=36.87 Å b=50.53 Å c=137.74 Å (a=b=c=90°) with one molecule per asymmetric unit. Diffraction from the TvVip crystals was consistent with the trigonal space group $P3_1$, with unit cell parameters a=b=85.94 Å, c=111.72 Å, with 3 molecule per asymmetric unit. Phases were determined by molecular replacement using the previously solved murine viperin structure (PDB: 5VSL). Subsequent rounds of automated model building were performed by AutoBuild in PHENIX (11), interspersed with manual model building and refinement using Coot (12), phenix.refine (11), and Refmac5 (13). All figures were produced using PyMOL (Shrodinger, LLC). The final model consists of residues 18-298, containing 4 iron ions, 4 sulfide ions. Data collection and refinement statistics are shown in **Table S9.**

Table S9. Data collection and refinement statistics

	Nvvip (7N7H)	TvVip (7N7I)
Data processing		
Wavelength (Å)	0.9793	0.9793
Space group	$P2_12_12_1$	$P3_1$
Cell dimensions		
α, β, γ (°)	90, 90, 90	90, 90, 120
a, *b*, *c* (Å)	36.87 50.53 137.74	85.94 85.94 111.71
Resolution (Å)¶	28.75 – 1.42 (1.47 – 1.42)	19.82 – 3.2 (3.31 – 3.2)
Redundancy¶	12.9 (10.6)	10.6 (9.7)
Completeness (%)¶	99.95 (99.84)	99.45 (96.18)
<I /σI>¶	18.01 (3.16)	8.46 (1.2)
R_{merge}¶	0.105 (0.559)	0.35 (1.1)
$R_{measure}$¶	0.110 (0.588)	0.11(1.12)
$CC_{1/2}$¶	0.998 (0.807)	0.976 (0.60)
Wilson *B*-factor (Å^2)	13.70	49.5
Refinement		
No. reflections	637162 (51403)	15107 (1437)
R_{work} / R_{free}	0.1514/0.1718	0.2169/0.2483
No. atoms	2745	6933
Protein	292	6822
Fe^{3+}, S^{2-}	4,4	12, 12
Water	337	
B-factors (Å^2)		
Protein	18.89	42.94
Ligand/ion	11.01	45.72
Water	30.79	
R.m.s. deviations		
Bond lengths (Å)	0.021	0.003
Bond angles (°)	1.77	0.67
Ramachandran analysis		
Favored (%)	95.82	96.12
Allowed (%)	4.18	3.88
Outliers (%)	0.0	0.0

[1]Data were collected at NSLSII beamline 17-ID-2.
¶Highest resolution shell is shown in parenthesis.

Supplemental Figures

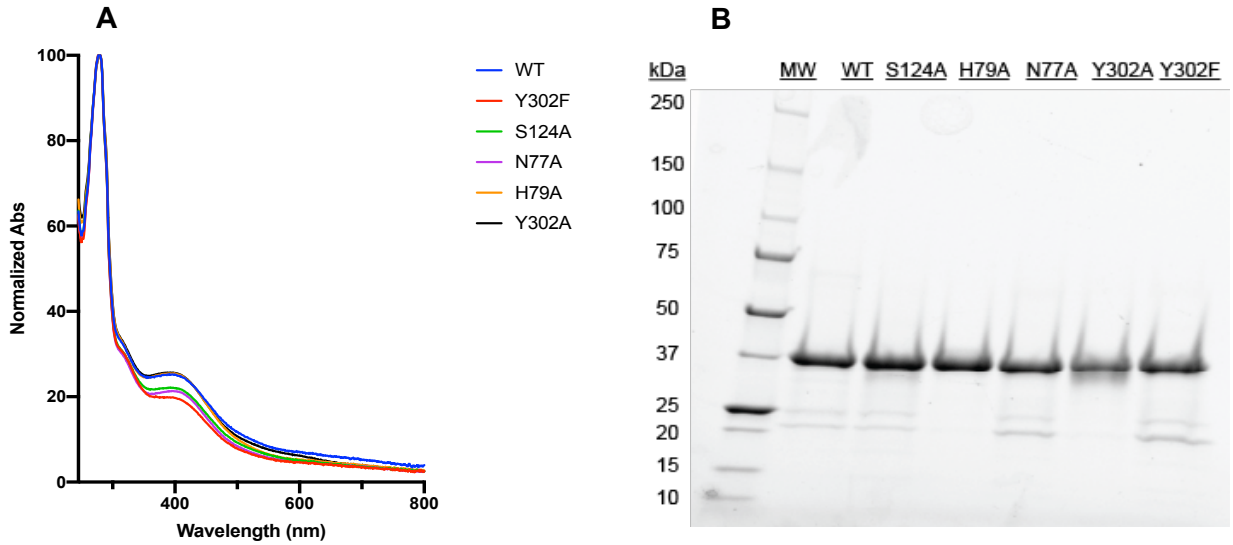


C

FeS Cluster Occupancy

	SbVip	NvVip	MmVip	TvVip1	TvVip2	RnVip WT	RnVip H77A
Protein concentration (mM) @280 (calculated using protein seq)	1.55	1.46	3.73	1.51	0.80	2.32	2.42
FeS concentration (mM) @400nm ($14000\ M^{-1}\ cm^{-1}$)	1.09	1.28	3.36	1.21	0.46	2.06	2.26
Protein/FeS ratio (280/400)	1.42	1.14	1.11	1.25	1.74	1.13	1.07

Figure S1: Purified viperin-like enzymes. UV-visible spectra of **A,** SbVip, NvVip, MmVip, TvVip and TvVip2, and **B**, RnVip WT and RnVip H77A proteins. All absorbances are normalized to 100 percent at A280. **C**, The A280/A400 ratios of all viperin and viperin-like enzymes from this study calculated by dividing the protein concentration (extinction coefficient from ProtParam, ExPasy.org) by the FeS cluster concentration (calculated at 14000 $M^{-1}\ cm^{-1}$)(1). Concentrations above are shown in mM and reflect the high solubility and stability of each protein.



C

FeS Cluster Occupancy

	MmVip WT	MmVip Y302F	MmVip S124A	MmVip N77A	MmVip H79A	MmVip Y302A
Protein concentration (mM) @280 (calculated using protein seq)	0.74	0.76	1.05	1.21	1.27	1.58
FeS concentration (mM) @400nm (14000 M^{-1} cm^{-1})	0.55	0.46	0.76	0.79	0.86	1.08
Protein/FeS ratio (280/400)	1.35	1.65	1.38	1.53	1.47	1.46

Figure S2: Purified MmVip and variants. UV-visible spectra of **A**, MmVip WT, MmVip Y302F, MmVip S124A, MmVip N77A, MmVip H79A, and MmVip Y302A proteins. All absorbances are normalized to 100 percent at A280. **B,** The purification products of MmVip WT and variants from this study. **C,** The A280/A400 ratios of all viperin and viperin-like enzymes from this study calculated by dividing the protein concentration (extinction coefficient from ProtParam, ExPasy.org) by the FeS concentration (calculated at 14000 M^{-1} cm^{-1})(1). Concentrations above are shown in mM and reflect the high solubility and stability of each protein.

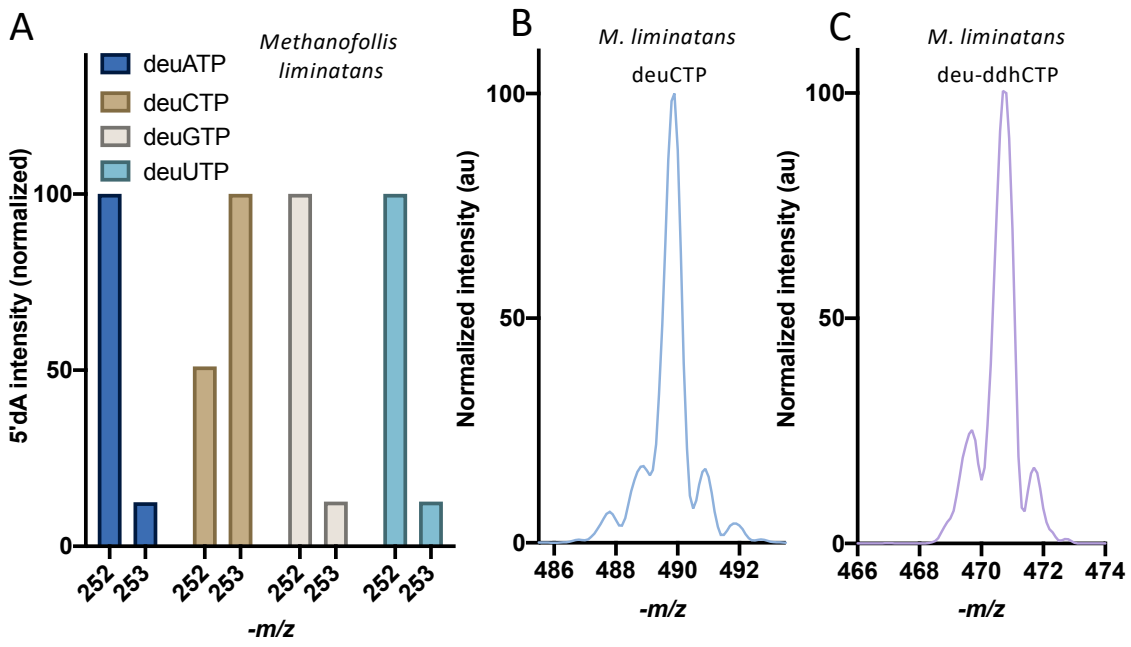


Figure S3. Nucleotide selectivity of MlVip. A, 50 μM of MlVip was mixed individually with four deuterium labeled ribonucleotides (1 mM) and 1 mM SAM and the resulting 5'dA was monitored using ESI+ and the natural abundance 5'dA (m/z 252) and deuterium incorporated 5'dA (m/z 253) plotted. The specificity of MlVip for CTP is noticeably higher than for the other three ribonucleotides. 50 μM of MlVip was then mixed with 1 mM deuterium incorporated CTP **B**, (-m/z = 489) and 1mM SAM to look for deuterium containing ddhCTP product **C**. A peak corresponding to -19 mass units (-m/z = 471) less than the starting deuCTP can be observed; deuterium incorporated ddhCTP has highly similar isotopic distribution to the deuterium containing starting material.

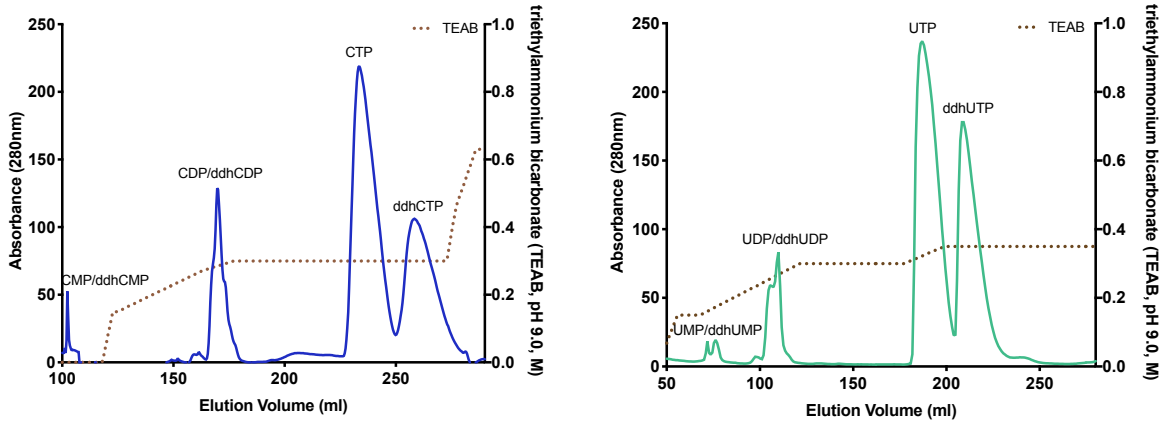


Figure S4: Purification of ddhCTP and ddUTP using cation exchange. Purification of ddhCTP and ddhUTP was achieved using a MonoQ (GE life sciences) column equilibrated in 0.05 M triethylammonium bicarbonate pH 9, with an elution gradient (dashed line) from 0.05 M to 0.8 M (see methods for elution profile).

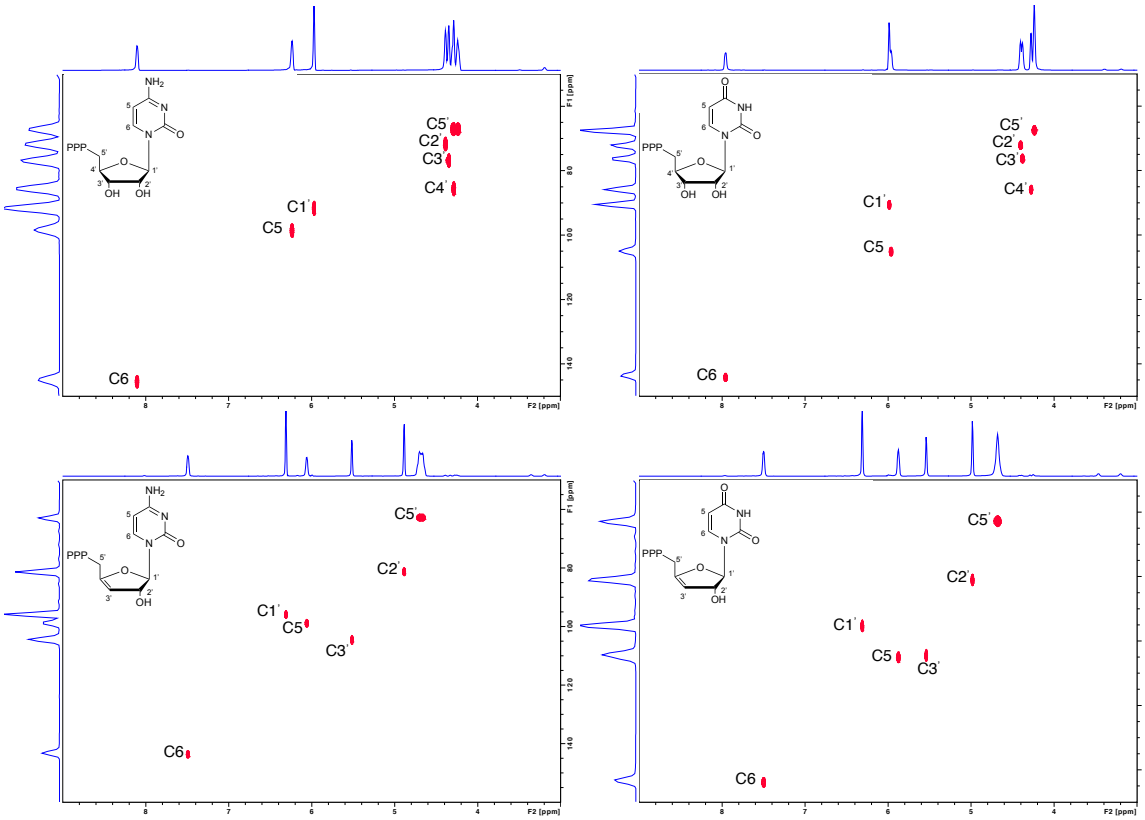


Figure S5. NMR characterization of ddhCTP and ddhUTP. 2D-HSQC NMR spectra collected on purified CTP (upper left), UTP (upper right), 3'deoxy-3',4'-didehydro-cytidine-triphosphate (lower left), 3'deoxy-3',4'-didehydro-uridine-triphosphate (lower right) in D_2O.

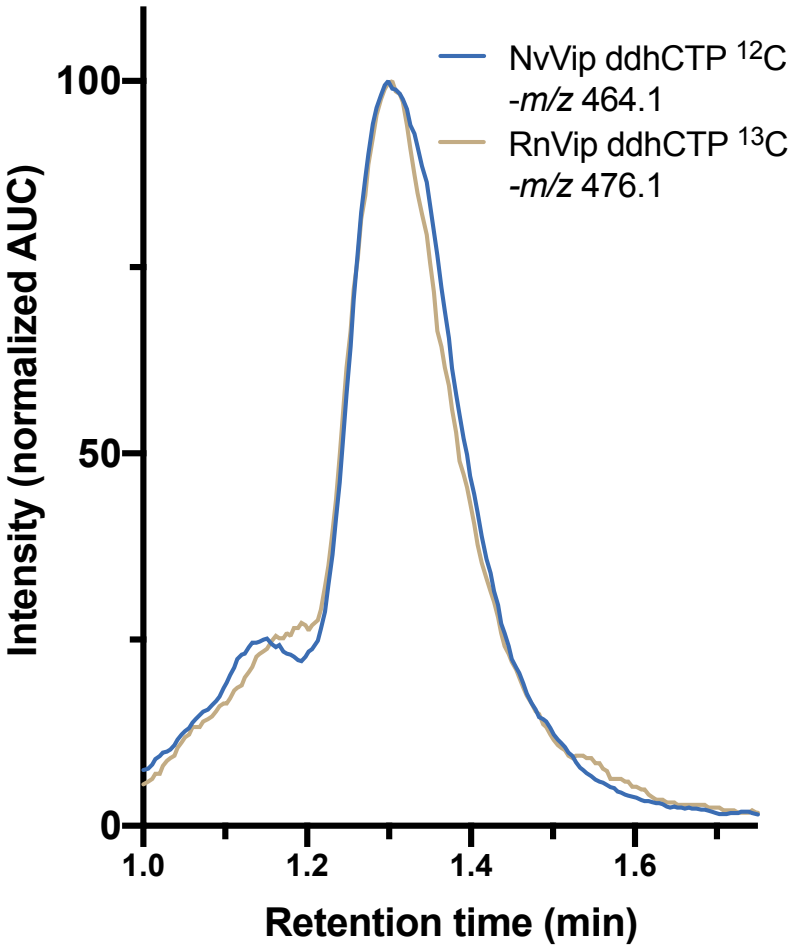


Figure S6: Comparison of reaction products, ddhCTP, from RnVip and NvVip. High-performance liquid chromatography analysis showing the co-injection of purified ddhCTP from a reaction of RnVip containing $^{13}C^{15}N$-labeled CTP and purified ddhCTP from a reaction of NvVip containing unlabeled CTP.

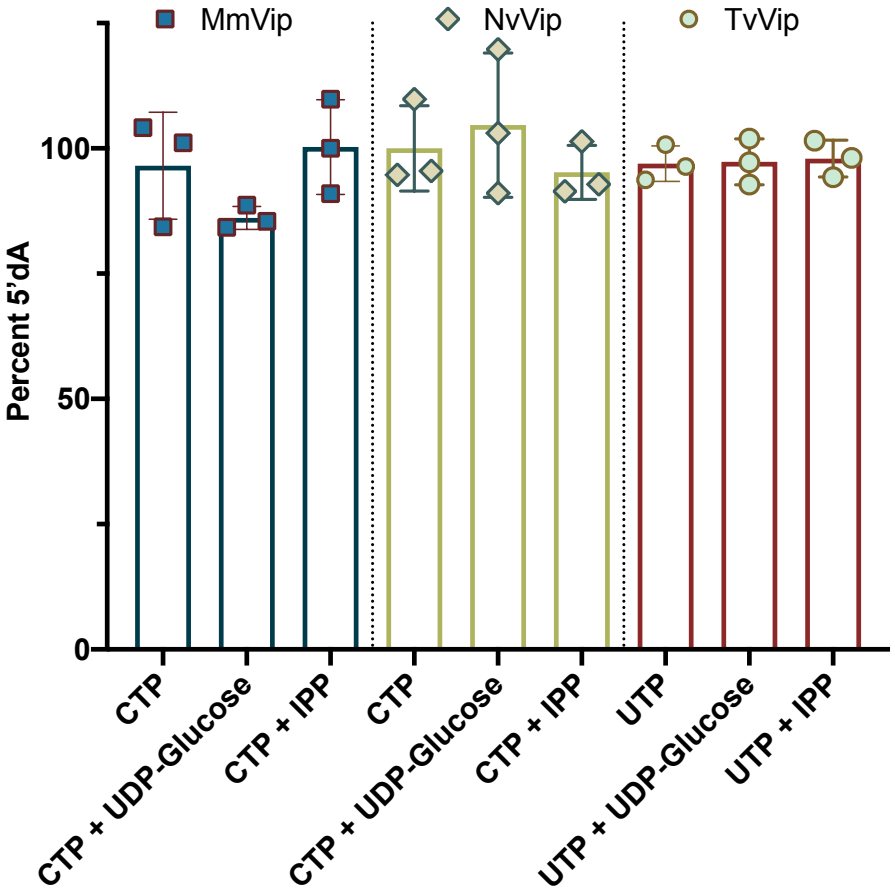


Figure S7: Nucleotide selectivity of SbVip, LmVip, and MlVip. Competition assays for the indicated viperin homolog (50 μM) were performed by mixing 2mM SAM and 1 mM of their respective NTP with 10 mM UDP-glucose or 10 mM isopentyl-pyrophosphate (IPP). The normalized 5'dA production from a 5-min time point is plotted. ANOVA analysis indicates there is no significant difference in 5'dA production in the presence of UDP-glucose or IPP. Data are mean ± s.d. from three independent experiments.

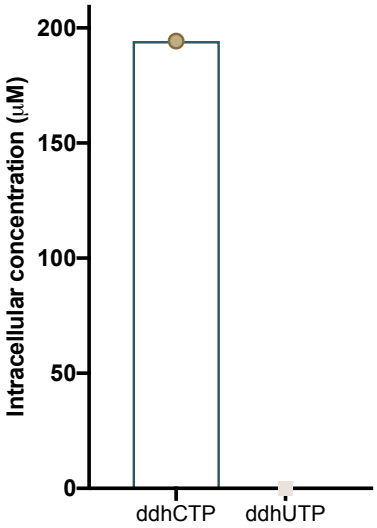


Figure S8: Intracellular concentration of ddhCTP and ddhUTP. HEK293T cells expressing HsVip produces 970-fold higher intracellular concentrations of ddhCTP than ddhUTP.

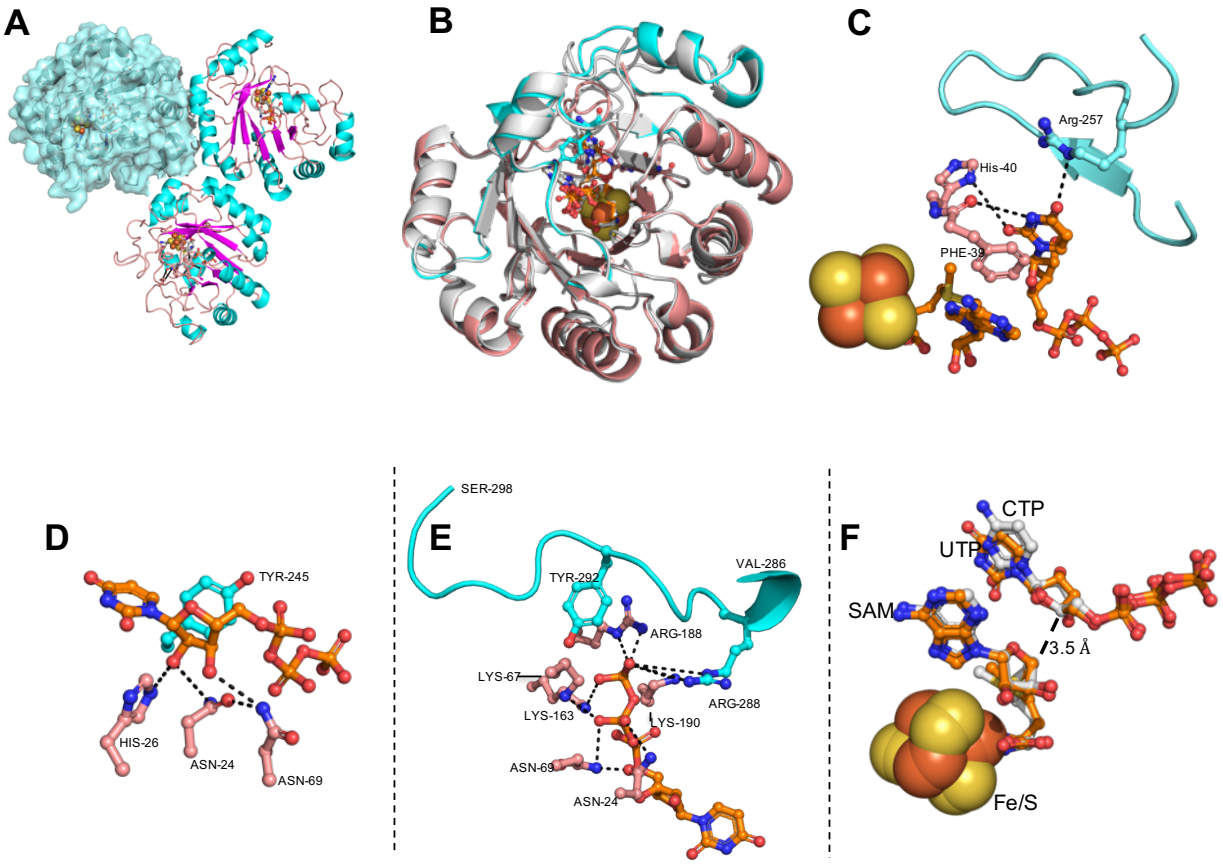


Figure S9: Structural comparison of viperin-like enzymes. A, Three molecules of TvVip make up the asymmetric unit, shown as a space filling model for one monomer. The canonical partial $(\beta/\alpha)_6$ TIM-barrel, helices colored pink and supporting alpha-helices in teal. **B,** Comparison of TvVip (teal) with the NvVip (grey) and MmVip (pink) show modest sequence similarity (45% and 54%, respectively), but high structural similarity (0.63 Å and 0.73 Å with NvVip (over 240 Cα-atoms) and MmVip (219 Cα-atoms). **C,** β-8 loop from TvVip containing Arg 257 (NvVip Lys 249, MmVip Lys 319) that coordinates the uridine base. **D,** The 2' and 3' hydroxyl of the ribose of UTP hydrogen bond with His-26, Asn-24, and Asn-69 (NvVip His-9, Asn-7 and Asn-52), while a base stacking interaction occurs with Tyr-245 (NvVip Tyr-232). **E,** There are a number of hydrogen bonding contacts formed between TvVip and the phosphate tail of UTP. The C-terminal tail of viperin acts as a proteinaceous cap that contains the substrate within the catalytic site. **F,** CTP and UTP are oriented in the active site of NvVip and TvVip, respectively, with high structural overlap.

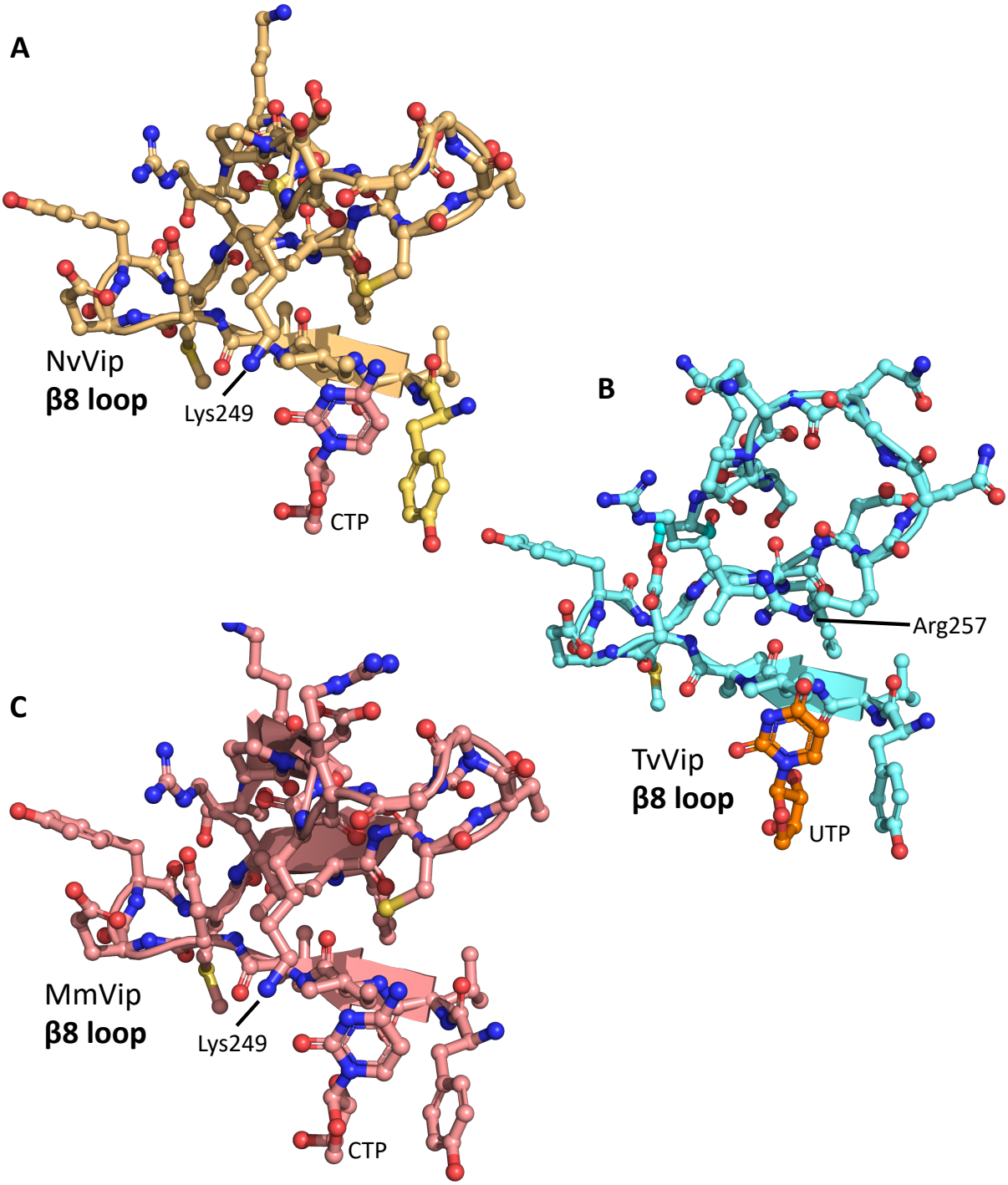


Figure S10: Structural comparison of the β-8 loop from viperin-like enzymes. β-8 loop from **A,** NvVip **B,** TvVip, and **C,** MmVip that coordinates the cytidine (NvVip, Lys 249; and MmVip, Lys 319) or uridine base (TvVip, Arg 257).

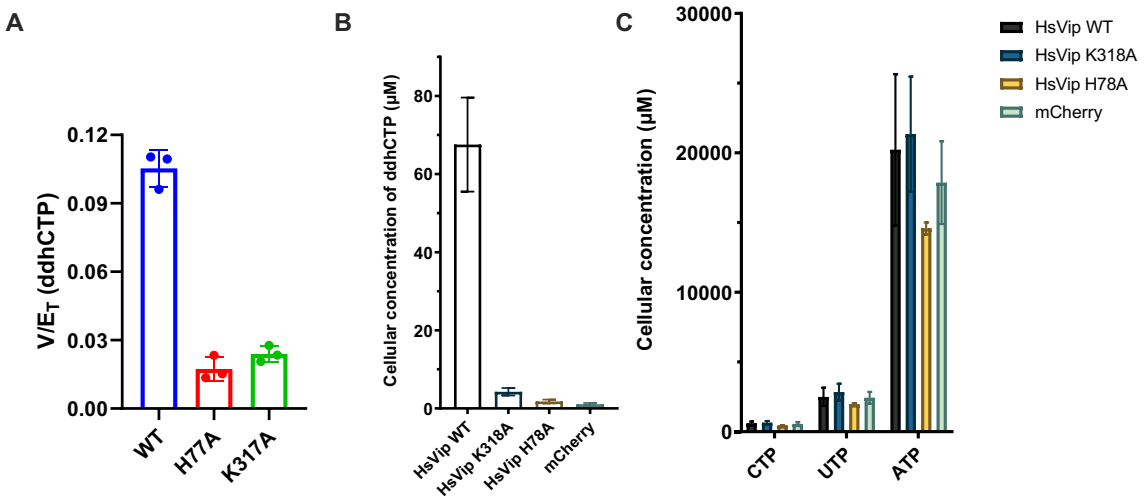


Figure S11: H77A and K317A protein variants reduce ddhCTP production. A, 25 μM of RnVip (blue, WT), RnVip H77A variant (red) or RnVip K317A variant (green) were each mixed with 1 mM CTP and 1 mM SAM and quenched at indicated time points (H77A, P = 0.00009; K317A, 0.00009). The resulting ddhCTP was quantified using ESI-. **B,** Cells expressing wild type human viperin (wt), K318A variant (K317 equivalent in RnVip), H78A variant (H77 equivalent in RnVip), or mCherry empty vector were analyzed for ddhCTP formation. Cells expressing viperin variant enzymes (K318A, P = 0.00082; or H78A, P = 0.0007) produce markedly less ddhCTP versus wild type viperin. **C,** Nucleotide concentrations of cells expressing wild type human viperin (wt), K318A variant, H78A variant, or mCherry empty vector. Data are mean ± s.d. from three biologically independent samples; P values for **A** and **B** were calculated using unpaired t-tests.

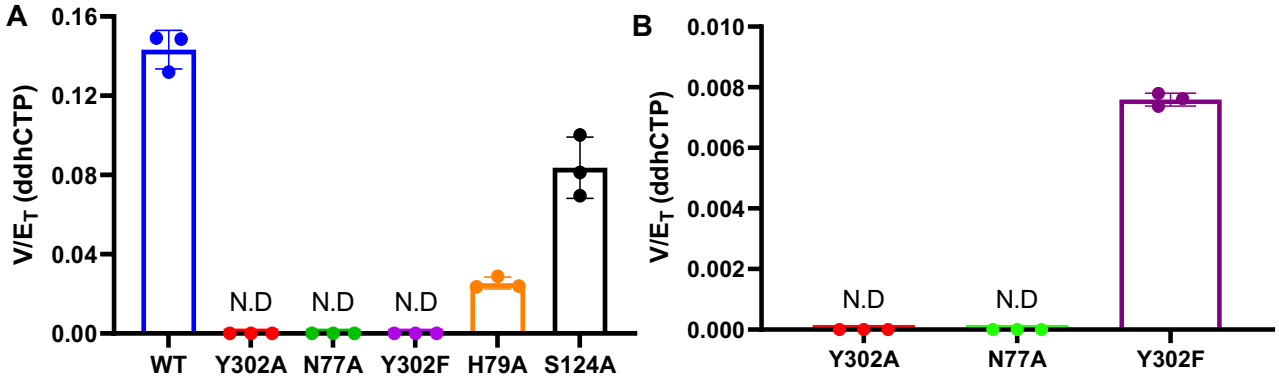


Figure S12. Y302A, N77A, Y302F, H79A, and S124A variants reduce ddhCTP formation. 25 μM of MmVip (blue, WT), MnVip Y302A variant (red), MmVip N77A variant (green), MmVip Y302F variant (purple), MmVip H79A variant (orange), and MmVip S124A variant (black), were each mixed with 1 mM CTP and 1 mM SAM and quenched after (**A**) 5 minutes or (**B**) 60 minutes. The resulting ddhCTP was quantified using ESI- and displayed as reaction velocity over total enzyme concentration. No product formation was detected for MmVip variants Y302A, N77A, and Y302F after 5 minutes or, Y302A or N77A after 60 minutes. Product formation was detected for the Y302F variant after 60 minutes. MmVip variants H79A, S124A, and Y302F produce significantly less ddhCTP than the wild type (H79A, P = 0.00004; S124A, P = 0.005; Y302F, P = 0.00002). Data are mean ± s.d. from three independent samples; P values were calculated using unpaired t-tests. (N.D = not detected)

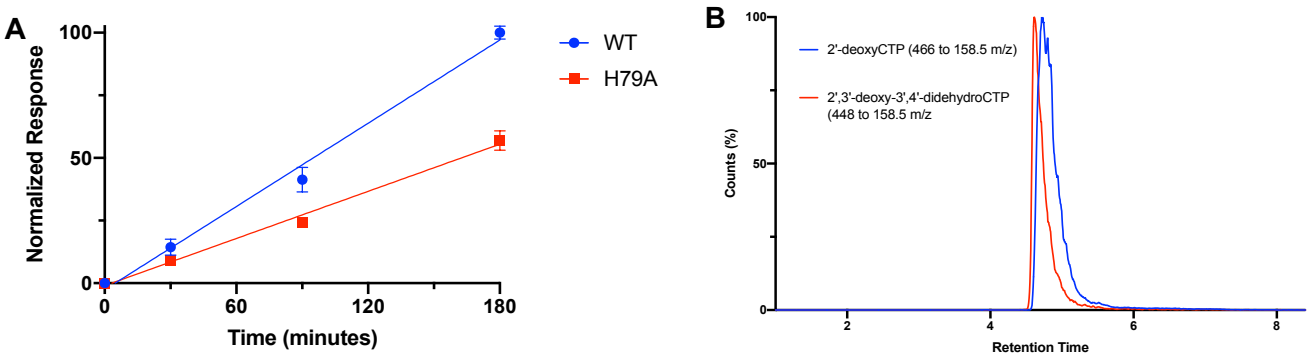


Figure S13. MmVip WT and H79A variant can catalyze the formation of 2',3'-deoxy-3',4'-didehydroCTP. A, 25 μM of MmVip (blue, WT) and MmVip H79A variant, were each mixed with 10 mM 2'-deoxyCTP and 1mM SAM and quenched at the indicated timepoints. The resulting 2',3'-deoxy-3',4'-didehydroCTP was quantified using ESI- and displayed as a ratio of the internal standard normalized to the highest value for wildtype MmVip. **B**, The retention times for 2'-deoxyCTP and 2',3'-deoxy-3',4'-didehydroCTP.

A

```
tr|G9MQB8|G9MQB8_HYPVG      MKDNQEAAAFGGRVRTGQVPVSVNYHFSRKCNKECLFCFHTATTSHV---EKPENA----      53
tr|A9L1Z3|A9L1Z3_SHEB9      MSTQNSSAENSTSSLVNVDELVINWHITEACNYNCSYCFAKWGKPKELHRSLPEIERFLD      60
                            *. ::.:*  .    ..   : :*:*::. ** :* :** .  . :    . **

tr|G9MQB8|G9MQB8_HYPVG      ------KRGLTLL-KQ--AGMKKINFAGGEPFLYPKFLGEMIDFCKETLQLESVSIVTNG      104
tr|A9L1Z3|A9L1Z3_SHEB9      NLSEYFIQGFHPLKKELGYESVRLNFAGGEPMMLGSTFFIALMLAKQ--KGFKTSVITNG      118
                                   :*:  * *:      ::*******::  . :   : :.*:  :  ..*::***

tr|G9MQB8|G9MQB8_HYPVG      SLVKEQFLQKHGRNIDILAVSCDSFNEATNIKIGRGSGDNVQ----KL-YEIGS-WCQKY      158
tr|A9L1Z3|A9L1Z3_SHEB9      HYLINSRLEFPKNVLDMVGISFDSQDLNTRVKIGRSDRKGNSLSVEELKTAIGNLVSTQK      178
                              : :. *:   . :*::.:* ** :  *.:****.. .. .    :*   **.  . :

tr|G9MQB8|G9MQB8_HYPVG      DIKFKLNTVVNKFNHLEDMNDHLNALQPFRWKCFQVLIVTGENDSDKTLRNAHSLTISDD      218
tr|A9L1Z3|A9L1Z3_SHEB9      GIKTKINTVVNSLNCEEDFSELITELKPFKWKVLQAMPYG-----------DDELLISRD      227
                            .** *:*****.:*  **:.: :. *:**:** :*.:             ..* ** *

                                                  TvVip β-8 loop
tr|G9MQB8|G9MQB8_HYPVG      EFDRFCERHSSQT-CLVPEPNRLMAKSYLILDEYMRFLDRNGQQ----PSKSILEVGVQQ      273
tr|A9L1Z3|A9L1Z3_SHEB9      KFDNFVATHSGIGLPIFAESNSTMTESYLMIDPKGRFYQNSSNGSGYVYSESINLCGVEN      287
                            :**.*   **.    :. * *  *::***::*   ** :...:      *:**   **::

tr|G9MQB8|G9MQB8_HYPVG      ALQAVFWDEEAFVERGGIYDWNKSSCSSDSKDLEW    308
tr|A9L1Z3|A9L1Z3_SHEB9      ALVQIEFNPIVFSSRYRKVDVDV-------VEL--    313
                            **  : ::  .* .*    * :         :*
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B

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tr|G9N0G3|G9N0G3_HYPVG      MLLALALISLVAVVLVLAFVPSSIVRQQTKGQIPVSVNYFFTRKCNKSCGFCFHTAKTSH      60
tr|G9MQB8|G9MQB8_HYPVG      --------------MKDNQEAAAFGGRVRTGQVPVSVNYHFSRKCNKECLFCFHTATTSH      46
                                         :      :::  :  .**:******.*:*****.* ******.***

tr|G9N0G3|G9N0G3_HYPVG      MEDISRAKRGLQLLQRAGMKKINFAGGEPFLYPKFLGELVDFCKEDLHLESVSIITNGSL      120
tr|G9MQB8|G9MQB8_HYPVG      VEKPENAKRGLTLLKQAGMKKINFAGGEPFLYPKFLGEMIDFCKETLQLESVSIVTNGSL      106
                            :*. ..***** **::**********************::***** *:******:*****

tr|G9N0G3|G9N0G3_HYPVG      VREEWVRKHAKNIDILACSCDSFDENMNIEIGRGTGNQVEILYRIAKWCRKNEIKFKLNT      180
tr|G9MQB8|G9MQB8_HYPVG      VKEQFLQKHGRNIDILAVSCDSFNEATNIKIGRGSGDNVQKLYEIGSWCQKYDIKFKLNT      166
                            *:*::::**.:****** *****:*  **:****:*::*: **.*..**:* :*******

tr|G9N0G3|G9N0G3_HYPVG      VVTRLNYEEDMNEHIDTLQPFRWKVFQVLIVEGENDSEKTLRDARRFTISDKQFEVFCSK      240
tr|G9MQB8|G9MQB8_HYPVG      VVNKFNHLEDMNDHLNALQPFRWKCFQVLIVTGENDSDKTLRNAHSLTISDDEFDRFCER      226
                            **.::*: ****:*:::******* ****** *****:****:*: :****.:*: **.:

tr|G9N0G3|G9N0G3_HYPVG      HRHHKSFVAEPNRLMASSYLLVDEYMRFIDKDGNKLTKSILDVGVEAAMKEIKWDVDAFQ      300
tr|G9MQB8|G9MQB8_HYPVG      HSSQTCLVPEPNRLMAKSYLILDEYMRFLDRNGQQPSKSILEVGVQQALQAVFWDEEAFV      286
                            *  :..:* *******.***::******:*::*:: :****:***: *:: : ** :**

tr|G9N0G3|G9N0G3_HYPVG      ERGGVYEWTKENEQVEACSTGMSEKLVW  328
tr|G9MQB8|G9MQB8_HYPVG      ERGGIYDWNKSSCSS------DSKDLEW  308
                            ****:*:*.*.. .        *:.* *
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Figure S14. Sequence alignments of four viperin-like enzymes. A, sequence alignment of TvVip (Uniport ID: G9MQB8_HYPVG) and SbVip (Uniprot ID: A9L1Z3_SHEB9). These two enzymes selectively catalyze the transformation of UTP to ddhUTP (the viperin motif is underlined and bolded). While the crystal structure of TvVip with UTP in the catalytic site shows that an arginine is used to coordinate the ketone oxygen of the pyrimidine base (the β-8 loop is italicized, and the coordinating Arg is highlighted), it remains unclear which corresponding residue(s) are used by the SbVip protein to select for UTP. **B**, sequence alignment of two viperin-like sequences found in *T. virens* (Uniprot ID: G9N0G3, G9MQB8 (TvVip used in this report)). Unlike all of the characterized proteins in this report that use a histidine (above His26) to support catalysis, a phenylalanine substituted in G9N0G3 renders this protein unable to perform any ddh-synthase activity. (the viperin and viperin-like motif is underlined and bolded, note the substitution from His to Phe in G9N0G3 vs G9MQB8).

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