SUPPLEMENTARY FIGURES AND LEGENDS



Fig. S1. Helical wheel analysis of the N-terminal amino acid sequence of 2'-PDE. Helical wheel representation of residues 7-24 of 2'-PDE showing the amphipathic nature of the region. The chemical properties of each residue are shown by yellow (hydrophobic residues), blue (basic residues), green (hydrophilic residues) and purple (acidic residues).



Fig. S2. 2'-PDE co-localizes with mitochondria in HEK293 cells. (A)-(C). Immuno-fluorescence microscopy images of a HEK293 cell transiently expressing the 2'-PDE₆₁₅-His protein. 36 hours post-transfection, cells were probed with an anti-His-tag antibody (Rockland) and stained with a FITC-conjugated secondary antibody (green). Mitchondria were visualized by treating live cells with Mitotracker (red). Images are merged in panel C. Scale bars; 10 μ m.

<u>2'-PDE₆₁₅-His</u>					
10	20	30	40	50	
MW <u>R</u> LPGA <u>R</u> AA	L <u>R</u> VI <u>R</u> TAVE <u>K</u>	LS <u>R</u> AEAGSQT	AAGAME <u>R</u> AVV	<u>R</u> CVPSEP <u>K</u> LS	
60	70	80	90	100	
LSFALADGSH	<u>K</u> NM <u>QR</u> DQSEP	lg <u>r</u> vls <u>r</u> iat	NAL <u>K</u> GHA <u>K</u> AA	AA <u>KK</u> S <u>RKSRP</u>	
110	120	130	140	150	
NASGGAACSG	PGPEPAVFCE	PVVK LYYREE	AVAEDVLNVD	AWQDGAVLQI	
160	170	180	190	200	
GDV <u>k</u> Y <u>k</u> VE <u>R</u> N	PPAFTELQLP	RYIMAGFPVC	PKLSLEFGDP	ASSLFRWYKE	
210	220	230	240	250	
A <u>K</u> PGAAEPEV	GVPSSLSPSS	PSSSWTETDV	EE <u>R</u> VYTPSNA	DIGL <u>R</u> L <u>K</u> LHC	
260	270	280	290	300	
TPGDGQ <u>R</u> FGH	SRELESVCVV	EAGPGTCTFD	HRHLYT <u>KK</u> VT	EDALIRTVSY	
310	320	330	340	350	
NILADTYAQT	FESPTULYPY				
	BEORIVEITI	CAPYALELDY	RONLIOKELT	GYNADVICLQ	
360	370	CAPYALELDY 380	RONLIOKELT	GYNADVICLQ	
360 EVD <u>R</u> AVFSDS	370 LVPALEAFGL	CAPYALELDY 380 EGVFRIKQHE	RONLIOKELT 390 GLATFY <u>RK</u> SK	GYNADVICLO 400 FSLLSQHDIS	
360 EVD <u>R</u> AVFSDS 410	370 LVPALEAFGL 420	CAPYALELDY 380 EGVFRIKQHE 430	RONLIOKELT 390 GLATFYRKSK 440	GYNADVICLQ 400 FSLLSQHDIS 450	
360 EVD <u>R</u> AVFSDS 410 FYEALESDPL	370 LVPALEAFGL 420 HKELLEKLVL	CAPYALELDY 380 EGVFRIKOHE 430 YPSAQEKVLQ	RONLIOKELT 390 GLATFYRKSK 440 RSSVLQVSVL	GYNADVICLQ 400 FSLLSQHDIS 450 QSTKDSSKRI	
360 EVD <u>RAVFSDS</u> 410 FYEALESDPL 510	370 100000000000000000000000000000000000	CAPYALELDY 380 EGVFRIKOHE 430 YPSAQEKVLQ 530	<u>R</u> QNLIQKELT 390 GLATFY <u>RKSK</u> 440 <u>RSSVLQVSVL</u> 540	GYNADVICLQ 400 FSLLSQHDIS 450 QSTKDSSKRI 550	
360 EVD <u>RAVFSDS</u> 410 FYEALESDPL 510 PSTGMYHFVI	370 LVPALEAFGL 420 HKELLEKLVL 520 NGSIPEDHED	CAPYALEIDY 380 BGVFR KQHE 430 YPSAQEKVLQ 530 WASNGEEERC	RONLIOKELT 390 GLATFYRKSK 440 RSSVLQVSVL 540 NMSLTHFFKL	GYNADVICLQ 400 FSLLSQHDIS 450 QSTKDSSKRI 550 KSACGEPAYT	
360 EVDRAVFSDS 410 FYEALESDPL 510 PSTGMYHFVI 560	370 100 100 100 100 100 100 100 1	2380 2677 278 278 278 278 278 278 278 278 278 2	<u>RONLIOKELT</u> 390 GLATFY <u>RKSK</u> 440 <u>RSSVLQVSVL</u> 540 NMSLTHFF <u>K</u> L	GYNADVICLQ 400 FSLLSQHDIS 450 QSTKDSSKRI 550 KSACGEPAYT 600	
360 EVDRAVFSDS 410 FYEALESDPL 510 PSTGMYHFVI 560 NYVGGFHGCL	370 LVPALEAFGL 420 HKELLEKLVI NGSIPEDHED 570 DYIFIDLNAL	CAPYALEDY 380 EGVFR 1 YPSAQEKVLQ 430 WASNGEEERC 530 WASNGEEERC 580 EVEQVIPLPS 530	RONLIQKELT 390 GLATFYRKSK 440 RSSVLQVSVL 540 NMSLTHFFKL 590 HEEVTTHQAL	GYNADVICLQ 400 FSLLSQHDIS 450 QSTKDSSKRI 550 KSACGEPAYT 600 PSVSHPSDHI	
360 EVDRAVFSDS 410 FYEALESDPL 510 PSTGMYHFVI 560 NYVGGFHGCL 610	370 1VPALEAFGL 420 HKELLEKLVL 520 NGSIPEDHED 570 DYIFIDLNAL	CAPYALEIDY 380 EGVFRIKOHE 430 YPSAQEKVLQ 530 WASNGEEERC 580 EVEQVIPLPS	RONLIQKELT 390 GLATFYRKSK 440 ESSVLQVSVL 540 NMSLTHFFKL 590 HEEVTTHQAL	GYNADVICLQ 400 FSLLSQHDIS QSTEDSSKEI 550 KSACGEPAYT 600 PSVSHPSDHI	

Fig. S3. Identification of 2'-PDE in the 2'-PDE₆₁₅-His-specific mitochondrial preparation from COS-7 cells. The presence of 2'-PDE was determined by LC-MS/MS of tryptic peptides derived from all proteins in the mitochondrial sample. In the listed protein sequence identified peptides are in red, and dark boxes underneath show the detected y-ions (upper red boxes) and b-ions (lower red boxes). Arg(R)/Lys(K) residues are underscored to indicate tryptic clevage sites.



Fig. S4. Gel-analysis following off-gel fractionation of a HEK293 mitochondrial preparation. Subsequent to off-gel isoelectric focusing in the pH interval 3-10, the protein composition within collected samples (1-12) was analyzed by Coomassie blue staining of the gel-electrophoresed fractions. Fraction 6, in which two 2'-PDE-specific peptides was later identified is marked in red. The M lane contains marker proteins of different molecular weights. 20 µl of each protein sample were applied to the gel.

	292	323	+ 356
Human 2'-PDE Mouse 2'-PDE Human CNOT6 Mouse CCR4 Human ANGEL1 Mouse ANGEL1 Human NOC Mouse NOC	KKVTEDALIRT <mark>VSYNILA</mark> KKVTEDSFIRTVSYNILA DRTRPTALFSVMCYNVLC DRTRPTALFSVMCYNVLC AGDGPQFQFTIMSYNILA AGDGPQFQFTTMSYNILA DCPSTHPPIRVMQWNILA DCSSSHSPIRVMQWNILA	DTYAQTEFSRTVLYPYCAPYALELDYRQNLIQKELTG) DTYAQTEFSRTVLYPYCAPYALELDYRQNLIQKELTG) DKYATRQLYGYCPSWALNWDYRKKAIIQEILS(DKYATRQLYGYCPSWALNWDYRKKAIIQEILS(QDLMQQSSELYLHCHPDILNWNYRFVNLMQEFQHW QDLMQQSSELYLHCHPDILNWNYRFANLMQEFQHW QALGEGKDNFVQCPVEALKWEERKCLILEEILA) QALGEGKDNFVQCPVEALKWEERKCLILEEILA)	NADVICLQEVDRAVFSDSL 361 'NADLICLQEVDRAVFSDSL 360 NADIVSLQEVETEQYSFF 250 'NADIISLQEVETEQYSFF 250 IDPDILCLQEVQEDHYWEQL 308 IDPDILCLQEVQEDHYWEQL 305 'QPDILCLQEVDHYFDTF 203 'QPDILCLQEVDHYFDTF 203
	367	386	418
Human 2'-PDE Mouse 2'-PDE Human CNOT6 Mouse CCR4 Human ANGEL1 Mouse ANGEL1 Human NOC Mouse NOC	VPALEAFGLEGVFRIKOH VPALEAFGLEGVFRIKOH LVELKERGYNGFFSPKSR LVELKERGYNGFFSPKSR EPSLRMMGFTCFYKRRTG QPLLSRLGYQGTFFPKPW QPLLSRLGYQGTFFPKPW	EGLATFYRKSKFSLLSQHDISFYE- EGLATFYRKSKFSLLSQHDISFYE- ARTMSEQERKHVDGCAIFFKTEKFTLVQKHTVEFNQL ARTMSEQERKHVDGCAIFFKTEKFTLVQKHTVEFNQL CKTDGCAVCYKPTRFRLLCASPVEYFRO CKTDGCAVCYKPTRFRLLCASPVEYFRO SCKTDGCALFFLQNRFKLVNSANIRLTAM SPCLDVEHNNGPDGCALFFLQNRFKLISSTNIRLTAM	LESDPLHKELLEKLVLYPS 423 ILKSDPLHKELLEKLALNPL 422 MANSEGSEAMLNRVMTKDN 325 ILELLNRDNV 364 ILKT 262 ILKT 260
	429	457	486
Human 2'-PDE Mouse 2'-PDE Human CNOT6 Mouse CCR4 Human ANGEL1 Mouse ANGEL1 Human NOC Mouse NOC	AQEKVLQRSSVLQVS AQEKVLQRSSVLQIS IGVAVLLELRKESIEMPS IGVAVLLELRKESIEMPS GLVLLLQPLVPEGLGQVS GLVLLLQPLVPEGLGQVS NQVAIAQTLECKESGRQ- NQVAIAQTLECKESGRQ-	VLQSTKDSSKRICVANTHLYWHPKGGYIRLIQMAVALA VLQSTTDSSKKICVANTHLYWHPKGGYIRLIQMEVALV GKPHLGTEKQLILVANAHMHWDPEYSDVKLVQTMMFLS GKPHLGTEKQLILVANAHMHWDPEYSDVKLVQTMMFLS VAPLCVANTHILYNPRRGDVKLAQMAILLA VAPFCIAVTHLKARTGWERFRSAQGCDLLQ FCIAVTHLKARTGWERFRSAQGCDLLQ	HIRHVSCDLYPG 488 'HIRHVSRDLYPG 487 'EVKNIIDKASRNLKSSVLG 400 'EVKNIIDKASRSLKSSVLG 400 'EVDKVARLSDG 423 'EVDKVARLSDG 420 NLQNITQG 314 NLQNITQG 312
	490	521	542
Human 2'-PDE Mouse 2'-PDE Human CNOT6 Mouse CCR4 Human ANGEL1 Mouse ANGEL1 Human NOC Mouse NOC	IPVIFCGDFNSTPS IPVIFCGDFNSTPS EFGTIPLVLCADLNSLPD ECGTIPLVLCADLNSLPD SHCPIILCGDLNSVPD SHCPIILCGDLNSVPD AKIPLIVCGDFNAEPT AKIPLIVCGDFNAEPT	TGMYHFVINGSIPEDHEDWASNGEER TGMYHFVISGSIAEDHEDWASNGEER SGVVEYLSTGGVETNHKDFKELRYNESLTNFSCHGKNG SGVVEYLSTGGVETNHKDFKELRYNESLTNFSCNGKNG SPLYNFIRDGELQYHGMPAWKVSGQEI SELYNFIRDGELQYNGMPAWKVSGQEI 'EEVYKHFASSSLNLNSAYKLLSADG	RCNMSLTHFFKLKS-AC 544 RCSMPLSHCFKLKS-AC 543 TTNGRITHGFKLQS-AYES 474 MTNGRITHGFKLKS-AYEN 474 PFSHQLYQRKLQAPLWPSSL 485 QSEPPYT 362 QSEPPYT 360
	550 *	580	*
Human 2'-PDE Mouse 2'-PDE Human CNOT6 Mouse CCR4 Human ANGEL1 Mouse ANGEL1 Human NOC Mouse NOC	GEPAYTNYVGGFHGOLDY GEPAYTNYVGGFHGOLDY GLMPYTNYTFDFKGIIDY GLMPYTNYTFDFKGIIDY GIT-(488-600)-TVDY GIT-(485-597)-TVDY TWKIRTSGECRHTLDY TWKIRTSGECRHTLDY	TFIDLNALEVEQVI-PLPSHEEVTTH-QALPSVSHPST TFIDLNTLEVEQVI-PLPSHEEVTTH-QALPSVSHPST TFYSKPQLNTLGILGPLDHHWLVENNISGCPHPLIPST TFYSKPQLNTLAILGPLDHHWLVENNISGCPHPLIPST TFFSAESCENGNR(616-645)NGLPNPFCSST TFFSAESCENENR(613-642)NGLPNPFYSST TWYSKHALNVRSALDLLTEEQIGPNRLPSFNYPST TWYSRHALSVTSALDLLTEEQIGPNRLPSFHYPST	HIALVCDLKWK 609 OHIALVCDLKWK 608 OHFSLFAQLELLLPFLP 557 OHFSLFAQLELLLPFLP 557 OHLCLLASFGMEVTAP 670 OHLSLVCDFSFTEESDGLS 431 OHLSLVCDFSFNEEPHELF 329

Fig. S5. Sequence alignment of the nuclease domains from known EEP nuclease proteins. Human (*Homo sapiens*) and mouse (*Mus musculus*) 2'-PDE sequences aligned with known deadenylase enzymes belonging to the CCR4, ANGEL, and Nocturnin families. Strictly conserved residues are coloured in red and partly conserved residues in blue. The five critical catalytic residues in the EEP enzymes are conserved amongst all families and marked with asterisks (*) while preserved motifs around these residues are indicated by black boxes. The alignment was done using the MultiAlin multiple alignment tool (74).



Fig. S6. Deletion of the mTP in 2'-PDE renders enzyme catalysis unchanged. (A). 2'-5' oligoadenylate degradation assay with bacterial lysates expressing 2'-PDE₆₁₅-His or 2'-PDE Δ mTP₆₀₀-His using a 2'-5' oligoadenylate tetramer, ApApApA, as substrate. EV (empty vector), a negative control lysate obtained from bacteria transformed with empty pTriEx-3 Neo vector. Reactions were performed in triplicate (mean/s.e.m.) using 10 µg of total protein. (B). Immunoblotting against the 2'-PDE-specific lysates from (A) using an anti-His antibody (Genscript) and 20 µg of total protein. The EV lysate (also from (A)) and a bacterial lysate expressing the OAS1-p42 protein were employed as negative and positive controls, respectively. Arrows mark the position of indicated proteins. The asterisk (*) shows an unspecific band.





Fig. S7. Chromatographic analyses of the 3'-5' exoribonuclease activity of 2'-PDE. Chromatograms showing the composition of the resulting sample after a 20 minutes incubation of 2'-PDE Δ mTP₆₀₀-His with 0.5 mM oligo-A, ApApApA (A), or oligo-C, CpCpCpC (B), substrates. The ratios of ~1:3 between the nucleoside (adenosine/cytidine) and the 5'-monophosphate nucleotide (AMP/CMP) are indicated. Chromatographic elution profiles were obtained by MonoQ Sepharose using 256 nm as the absorbance wavelength. Brown curves represent the experimental salt gradient.