Table S1: Gene sequences used in this study. Related to Figure 1.

	Species (Common)	Species (Scientific)	GC-C Acession	NPR1	NPR2	Uroguanylin	Guanylin
Primates	Human	*Homo sapiens*	NM_004963.4	NM_000906.4	NM_003995.4	NP_009033.1	NP_291031.2
	Rhesus Macaque	*Macaca mulatta*	XM_001089601.4	XM_002808236.3	NM_001205340.1	XP_001087987.1	XP_001085421.1
	Pigtailed Macaque	*Macaca nemestrina*	XM_011741690.2	XM_011769501.2	XM_011748195.2	XP_011762016.1	XP_011762017.1
	Chimpanzee	*Pan troglodytes*	XM_528746.6	XM_016927544.2	XM_001168194.5	XP_524686.2	XP_001172840.1
	Orangutan	*Pongo abelii*	XM_002822972.2	XM_024245905.1	XM_024252690.1	XP_002811014.1	XP_002811013.1
	Baboon	*Papio anubis*	XM_021922068.1	XM_003892689.4	XM_003911520.4	XP_003891732.1	XP_003891733.1
	Night Monkey	*Aotus nancymaae*	XM_012447637.1	XM_012476152.2	XM_012463327.2	XP_012289925.1	XP_012289889.1
	Gorilla	*Gorilla gorilla gorilla*	XM_019037844.1	XM_019022930.1	XM_004048004.3	XP_004025613.1	XP_004025614.1
	Marmoset	*Callithrix jacchus*	XM_017976534.1	XM_017966300.1	XM_002806498.3	NA	XP_002750736.1
	Crab Eating Macaque	*Macaca fascicularis*	XM_005570213.2	XM_005541752.2	XM_005581348.2	XP_005543815.1	XP_005543814.1
	Gibbon	*Nomascus leucogenys*	XM_003265528.2	NA	XM_011939383.1	XP_003273395.1	XP_012366311.1
	Sooty Mangabey	*Cercocebus atys*	XM_012058487.1	XM_012076614.1	XM_012087083.1	XP_011934624.1	XP_011934623.1
	Drill	*Mandrillus leucophaeus*	XM_011990839.1	NA	XM_011980142.1	XP_011857110.1	XP_011857111.1
	Black and White Colobus	*Colobus angolensis palliatus*	XM_011954200.1	NA	XM_011939383.1	XP_011800278.1	XP_011800291.1
	Squirrel Monkey	*Saimiri boliviensis boliviensis*	XM_003934548.2	XM_003941876.2	XM_003943789.2	XP_003937037.1	XP_003937036.1
	Gold Snub Nosed Monkey	*Rhinopithecus roxellana*	XM_010368479.1	XM_010364757.1	XM_010376182.2	XP_010370113.1	XP_010370115.1
	African Green Monkey	*Chlorocebus sabaeus*	XM_007967738.1	XM_007977049.1	XM_007968782.1	XP_007977383.1	XP_007977382.1
	Mouse Lemur	*Microcebus murinus*	XM_012753729.2	XM_012791226.1	XM_012769991.2	XP_012620637.1	XP_012620638.1
	Tarsier	*Carlito syrichta*	XM_008071877.1	NA	XM_008063120.2	XP_008065362.1	XP_008065363.1
Bats	Pale spear-nosed bat	*Phyllostomus discolor*	XM_028532971.1		XM_028507867.1	XM_028512623.1	XM_028511197.1
	Big brown bat	*Eptesicus fuscus*	XM_008140435.2		XM_028146098.1	XM_008151290.2	XM_008151289.2
	Black flying fox	*Pteropus alecto*	XM_006912643.3		XM_006916002.2	XM_006903991.3	XM_006903992.3
	Vampire bat	*Desmodus rotundus*	XM_024575708.1		XM_024560188.1	XM_024554679.1	XM_024554672.1
	Little brown bat	*Myotis lucifugus*	XM_006084275.3		XM_014468054.2	XM_006087239.3	XM_006087238.3
	Large flying fox	*Pteropus vampyrus*	XM_011375981.2		XM_023524003.1	XM_011377936.2	XM_011377934.2
	Great roundleaf bat	*Hipposideros armiger*	XM_019643276.1		XM_019663310.1	XM_019643726.1	XM_019643727.1
	Natal long-fingered bat	*Miniopterus natalensis*	XM_016197509.1		XM_016222895.1	XM_016205154.1	XM_016205152.1
	Egyptian fruit bat	*Rousettus aegyptiacus*	XM_016140541.1		XM_011381514.2	XM_016139967.1	XM_016139966.1
	David's myotis	*Myotis davidii*	XM_006773282.2		XM_006758620.2	XM_006762574.2	XM_006762575.1
	Brandt's bat	*Myotis brandtii*	XM_005866793.2		XM_014531524.1	XM_005866051.1	XM_005866052.2
	Chinese rufous horseshoe bat	*Rhinolophus Sinicus*	(Sequence recovered from BLAST)		NA		
Caniformia	California sea lion	*Zalophus californianus*	XM_027591714.1		XM_027614789.1		
	Grizzly bear	*Ursus arctos horribilis*	XM_026502374.1		XM_026506245.1		
	Red fox	*Vulpes vulpes*	XM_025995541.1		XM_026013266.1		
	Northern fur seal	*Callorhinus ursinus*	XM_025879900.1		XM_025852888.1		
	Dingo	*Canis lupus dingo*	XM_025455488.1		XM_025432462.1		
	Dog	*Canis lupus familiaris*	XM_022411076.1		NA		
	Hawaiian monk seal	*Neomonachus schauinslandi*	XM_021690455.1		XM_021691775.1		
	Giant panda	*Ailuropoda melanoleuca*	XM_011223360.2		XM_002918884.3		
	Ferret	*Mustela putorius furo*	XM_013057870.1		XM_004747571.2		
	Pacific walrus	*Odobenus rosmarus divergens*	XM_004406992.2		XM_004392264.2		
	Polar bear	*Ursus maritimus*	XM_008707020.1		XM_008695194.1		
Bovids	Cow	*Bos taurus*	NM_174547.2		NM_174126.2		
	Zebu	*Bos indicus*	XM_019961517.1		XM_019966181.1		
	Yak	*Bos mutus*	XM_005903614.2		XM_005900781.2		
	Bison	*Bison bison bison*	XM_010858720.1		XM_010846069.1		
	Water buffalo	*Bubalus bubalis*	XM_006055135.2		XM_006064377.2		
	Goat	*Capra hircus*	XM_018048449.1		NM_001285674.1		
	Sheep	*Ovis aries*	XM_027967845.1		XM_004004267.4		
	Mouflon	*Ovis aries musimon*	XM_012150903.1		NA		
Myomorpha	House mouse	*Mus musculus*	NM_001127318.1		NM_173788.4		
	Ryukyu mouse	*Mus caroli*	XM_021165238.1		XM_021159499.2		
	Gairdner's shrewmouse	*Mus pahari*	XM_021190835.1		XM_021222144.2		
	Norway Rat	*Rattus norvegicus*	NM_013170.1		NM_053838.1		
	Mongolian gerbil	*Meriones unguiculatus*	XM_021644291.1		XM_021658450.1		
	Prairie vole	*Microtus ochrogaster*	XM_005364517.2		XM_026786619.1		
	Deer Mouse	*Peromyscus maniculatus bairdii*	XM_006976515.1		XM_006986011.1		
	Chinese hamster	*Cricetulus griseus*	XM_027429909.1		XM_027403228.1		
	Golden hamster	*Mesocricetus auratus*	XM_005072889.3		XM_013119692.2		
	Lesser Egyptian jerboa	*Jaculus jaculus*	XM_004671850.1		XM_004658329.2		

Table S2: Likelihood ratio tests for positive selection in mammalian GC-C and NPR genes. Related to Figure 1.

Group	Gene	# species	BUSTED p-value	PAML M1-M2 p-value	PAML M7-M8 p-value	PAML M0 dN/dS	PAML M8 tree length	Sites BEB > 95%
Primates	GC-C	19	0.001	0.0004	1.53E-05	0.243	1.067	6
Primates	GC-C (extracellular)	19	1.77E-04	0.0023	0.0012	0.435	1.595	6
Primates	GC-C (Intracellular)	19	1.000	1.0000	0.6974	0.084	0.703	0
Primates	NPR1	14	1.000	1.0000	0.0624	0.066	0.561	1
Primates	NPR2	19	1.000	0.5576	0.9996	0.043	0.399	0
Bats	GC-C	12	<1.0E-18	1.69E-19	7.27E-21	0.429	1.774	35
Bats	GC-C (extracellular)	12	2.22E-16	5.99E-24	7.65E-25	0.954	2.626	41
Bats	GC-C (Intracellular)	12	0.1	1.0000	0.8561	0.128	1.158	0
Bats	NPR2	11	0.994	1.0000	0.9999	0.042	0.582	0
Myomorpha	GC-C	10	2.36E-06	1.0000	1.60E-05	0.133	1.877	5
Myomorpha	NPR2	10	1.000	1.0000	0.9971	0.013	0.880	0
Caniformia	GC-C	11	0.065	0.9926	0.9052	0.189	0.543	0
Bovids	GC-C	8	1.000	0.2218	0.2017	0.117	0.172	1

Table S3: Positively selected amino acid positions in primate and bat GC-C identified by PAML. Related to Figure 1.

	Sites with dN/dS > 1 *	Probability
Primate GC-C	S21	0.909
	F22	**0.966**
	S23	0.908
	Q25	**0.975**
	V224	**0.983**
	G251	0.939
	Q279	**0.965**
	R312	0.923
	T317	0.93
	D364	**0.99**
	W372	0.927
	Y406	**0.985**
	S419	0.928
	R688	0.915
Bat GC-C	**I 21**	**0.99**
	W 22	**0.981**
	T 23	**0.997**
	S 24	**0.996**
	P 25	**0.977**
	S 27	**0.997**
	H 31	**0.975**
	I 34	**0.971**
	Q 48	**0.995**
	E 52	**0.997**
	E 60	**0.966**
	R 66	**0.964**
	E 67	**0.996**
	I 70	**0.989**
	N 71	0.917
	S 72	0.901
	S 79	0.901
	S 81	**0.955**
	Y 84	0.909
	P 88	**0.998**
	V 93	0.929
	T 111	**0.98**
	K 114	**0.986**
	A 177	**0.99**
	S 186	0.913
	N219	0.941
	N 221	**0.992**
	K 230	0.944
	H 239	0.913
	N 240	0.944
	T 251	**0.989**
	T 254	0.937
	S 256	**0.998**
	I 257	0.914
	Q 282	0.929
	S 300	0.927
	T 304	**0.972**
	I 308	**0.977**
	S 309	0.915
	M 318	**0.951**
	D 345	0.933
	L 349	**0.989**
	E 402	**0.992**
	V 404	**0.996**
	E 406	**0.985**
	A 407	**0.952**
	T 410	**0.99**
	N 419	0.942
	N 423	0.932
	A 425	0.937
	E 427	**0.993**
	R 428	**0.958**
	N 744	0.944
	R 993	0.94

*Posterior probabilities >90% by PAML Model 8 Bayes Emperical Bayes (BEB) analysis, probabilities >95% are bolded
amino acididentities are shown for *H. sapiens* and *M. lucifugus* in primates and bats, respectively

Table S4: GC-C dose-response values and statistics. Related to Figure 2.

	Human		Orangutan		*M. lucifugus*		*P. vampyrus**	
Agonist	**Log EC50**	**Maximum**	**Log EC50**	**Maxiumum**	**Log EC50**	**Maximum**	**Log EC50**	**Maximum**
STp	-6.345 ± 0.113	142.7 ± 8.437	-6.382 ± 0.196	372.1 ± 38.36	-7.028 ± 0.200	598.1 ± 48.08	-6.925 ± 0.183	646.4 ± 54.4
STh	-7.143 ± 0.127	117.5 ± 6.387	-5.82 ± 0.098	250.1 ± 15.650	-5.711 ± 0.116	560.5 ± 43.75	-5.528 ± 0.118	325.7 ± 26.77
Y-ST	-4.537 ± 0.635	279.9 ± 170.6	-5.126 ± 0.066	195.7 ± 12.67	-5.311 ± 7.455	434.2 ± 20.07	N/A	N/A
V-ST	-5.676 ± 0.139	131.2 ± 11.37	-5.736 ± 0.101	103.2 ± 5.663	-5.079 ± 0.1037	304.4 ± 33.31	-5.251 ± 0.061	351.0 ± 22.45
P value (Dunnett's T3 multiple comparison test)								
STp vs. STh	**0.0001**	0.1184	0.078	**0.0306**	**<0.0001**	0.9926	**<0.0001**	**<0.0001**
STp vs. Y-ST	0.0519	0.9587	**<0.0001**	**0.0005**	>0.9999	**0.0168**	N/A	N/A
STp vs. V-ST	**0.0029**	0.9584	**0.0306**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
STh vs. Y-ST	**0.0023**	0.9128	**<0.0001**	0.0517	>0.9999	0.0656	N/A	N/A
STh vs. V-ST	**<0.0001**	0.8718	0.9914	**<0.0001**	**0.0007**	**<0.0001**	0.1176	0.8501
Y-ST vs. V-ST	0.4165	0.9408	**<0.0001**	**<0.0001**	>0.9999	**0.0087**	N/A	N/A

* P. vampyrus Y-ST dose/response parameters outside of tested range

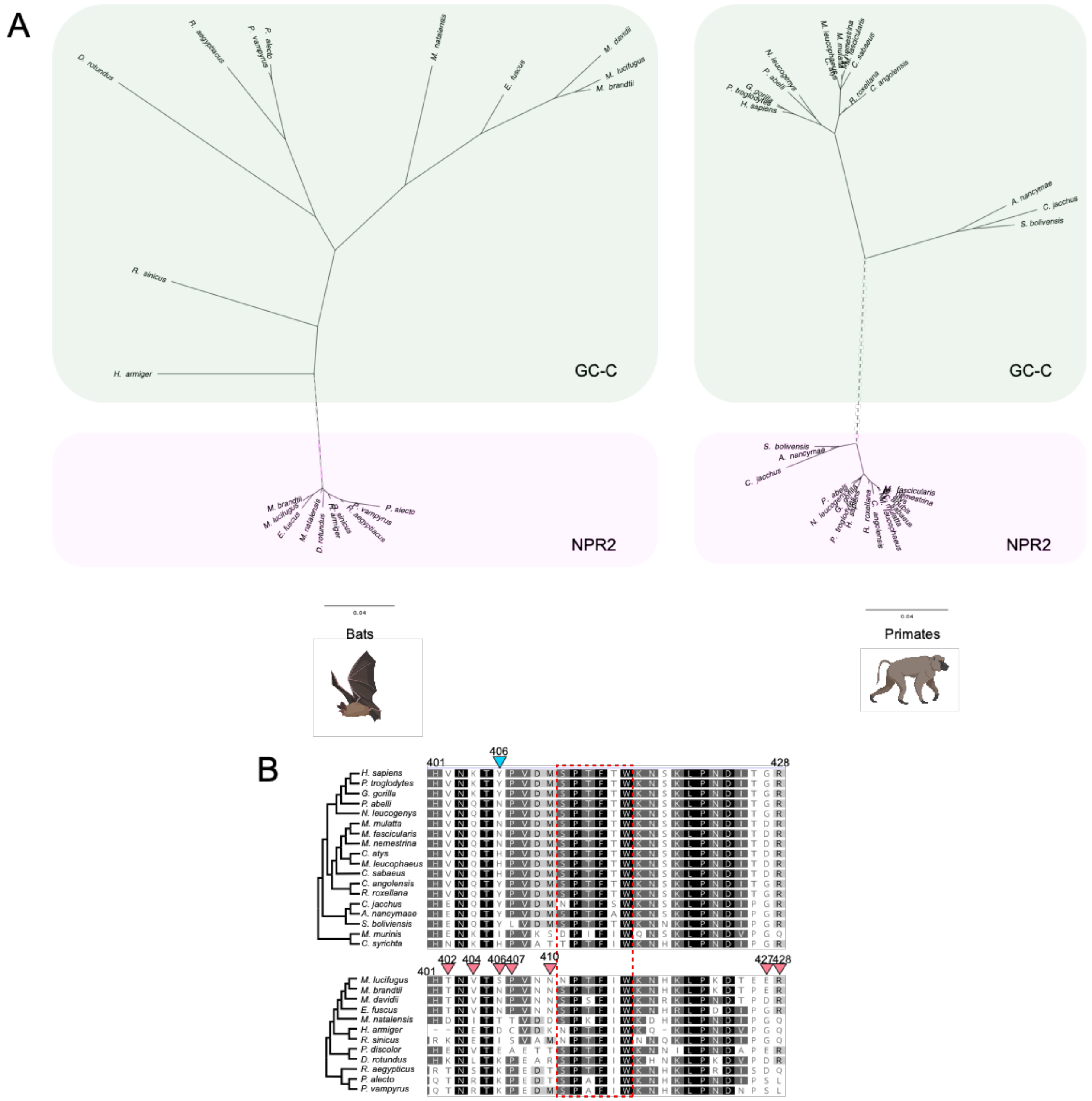

Figure S1: Evolutionary analysis of GC-C. Related to Figure 1. (A) Amino acid alignments were generated for the ligand-binding domains of GC-C and NPR2 in primates and bats. Trees were generated using PHYML with the Le Gascuel amino acid substitution model. Branches connecting NPR2 and GC-C trees are artificially collapsed (dashed line). (B) GC-C amino acid alignments are shown from primates (top) and bats (bottom) from amino acid 400-428. A proposed ligand-binding pocket is highlighted (red dashed box). Amino acid positions with significantly elevated dN/dS ratios are highlighted with blue (primates) or red (bats) triangles (PAML >95% posterior probability).

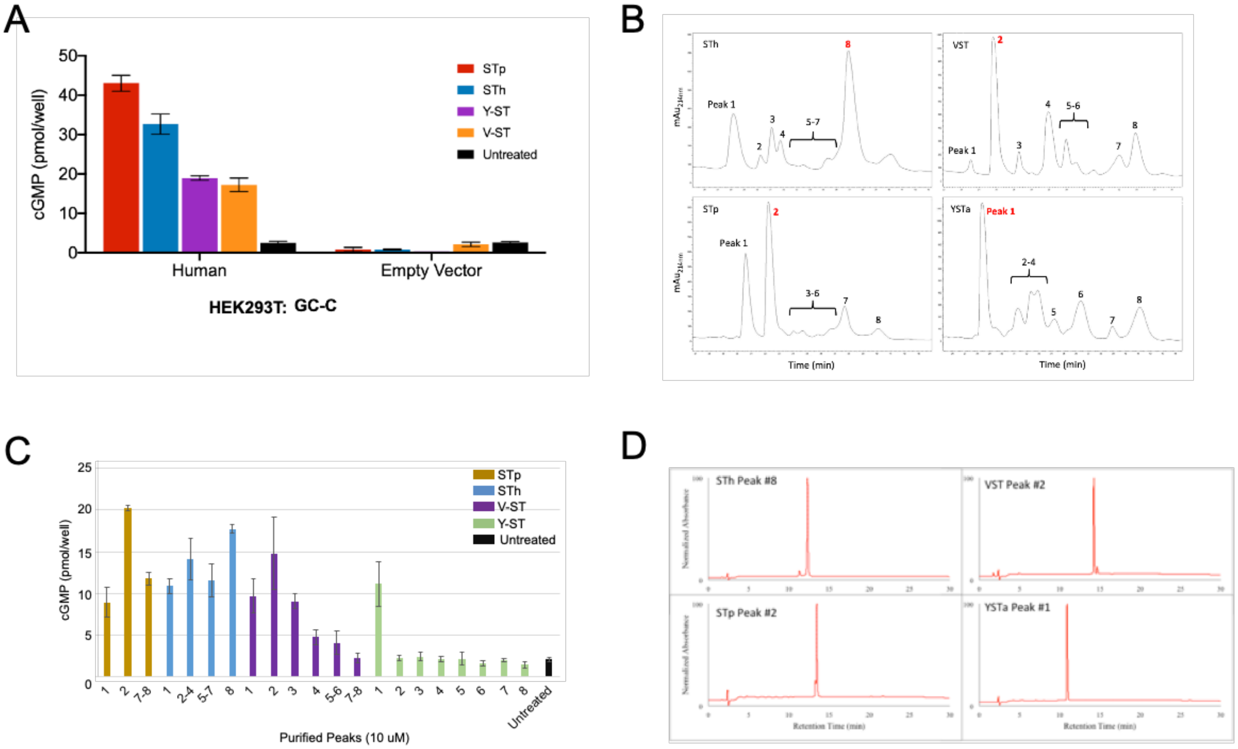

Figure S2: Synthesis and functional verification of STa variants. Related to Figure 2. (A) HEK293T cells transduced with lentivirus carrying Human GC-C or and empty vector were treated with the indicated peptides at a concentration of 5μM followed by measurement of intracellular cGMP by ELISA. (B) HPLC traces of each synthesized peptide shows evidence of ~8 peaks following air oxidation. Each peak was then collected for downstream analysis. (C) HEK293T cells expressing human GC-C were stimulated with the major purification products to identify the most potent fractions by measuring intracellular cGMP. For each peptide, the predominant oxidation product represented the most potent GC-C ligand. Some peaks were unable to be purified in isolation. (D) Final HPLC purification of toxin peptides used for analysis.

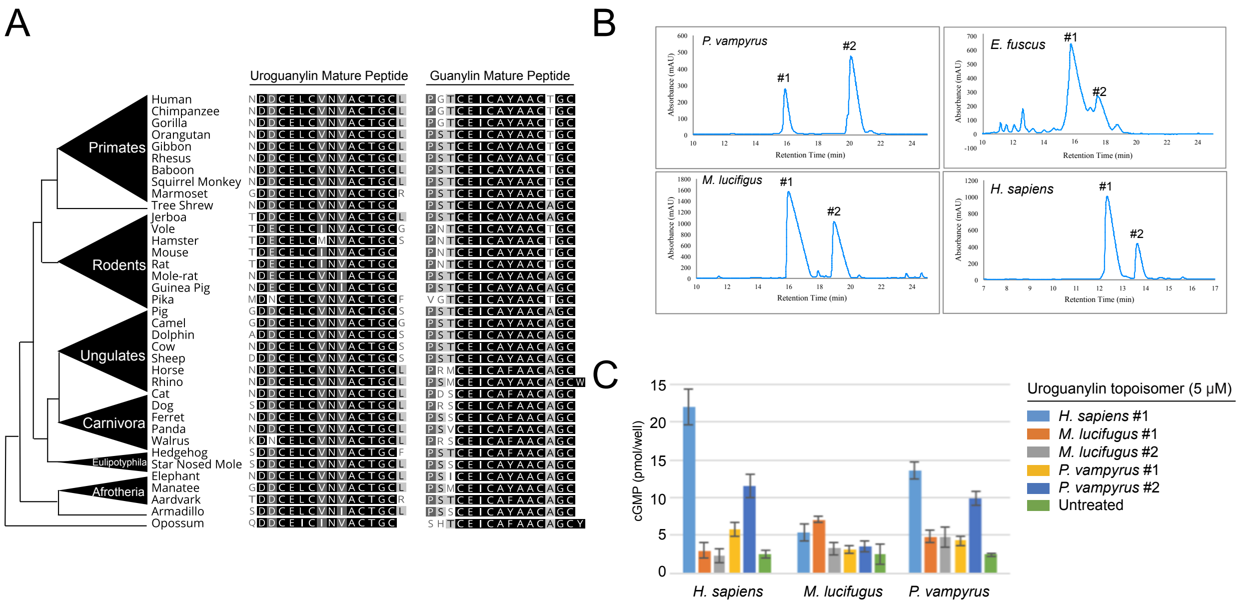

Figure S3: Evolutionary and functional comparison of uroguanylin. Related to Figure 4. (A) Guanylin and Uroguanylin secreted peptide sequences were downloaded from genbank for the indicated species and aligned using clustalW (B) HPLC traces of synthesized uroguanylin from the indicated species. Two distinct topoisomers were observed following oxidation of synthesized uroguanylin from each species. (C) cGMP generation assays in HEK293T cells expressing GC-C from the indicated species were performed to identify the most active topoisomer. Human isomer #1 is the known active peak. *E. fuscus* peak #1, *M. lucifugus* peak #1, and *P. vampyrus* peak #2 were used in in experimental assays.