Supplemental materials for

Evolutionary Conservation of human ketodeoxynonulosonic acid production is independent of sialoglycan biosynthesis

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Supplemental Materials and Methods

Detection of free α-ketoacids including sialic acids and by LC-MS. Analyses of α-ketoacids including Kdn, Neu5Ac, Neu5Gc (Supplemental Table 1) were done using LTQ-Orbitrap Discovery (Thermo Scientific) mass spectrometry attached to Ultimate3000 HPLC system (Thermo-Dionex) after tagging with DMB. The profiling of α-ketoacids was done using Phalanx C18 column (150mm x 1.0mm, 5µm) from Higgins Analytical. A step-gradient of solvents at a flow rate of 50 µL was used to get optimal separation between the sugar moieties. 5% aqueous methanol containing 0.1% formic acid was used as Solvent-A and mixture of 5% methanol, 45% water and 50% acetonitrile containing 0.1% formic acid was used as Solvent-B; the details of gradient is as follows. For 0-5 min mixture of 83% of solvent-A and 17% of solvent-B; for 5.1-10min 80% of solvent-A and 20% of solvent-B; for 10.1 to 15min 75% of solvent-A and 25% of solvent-B was used; for 15.1-20 min 70% of solvent-A and 30% of solvent-B is used and finally from 20.1-25min 50% solvent-A and 50% solvent-B was used. The mass spectral data was obtained in positive mode.

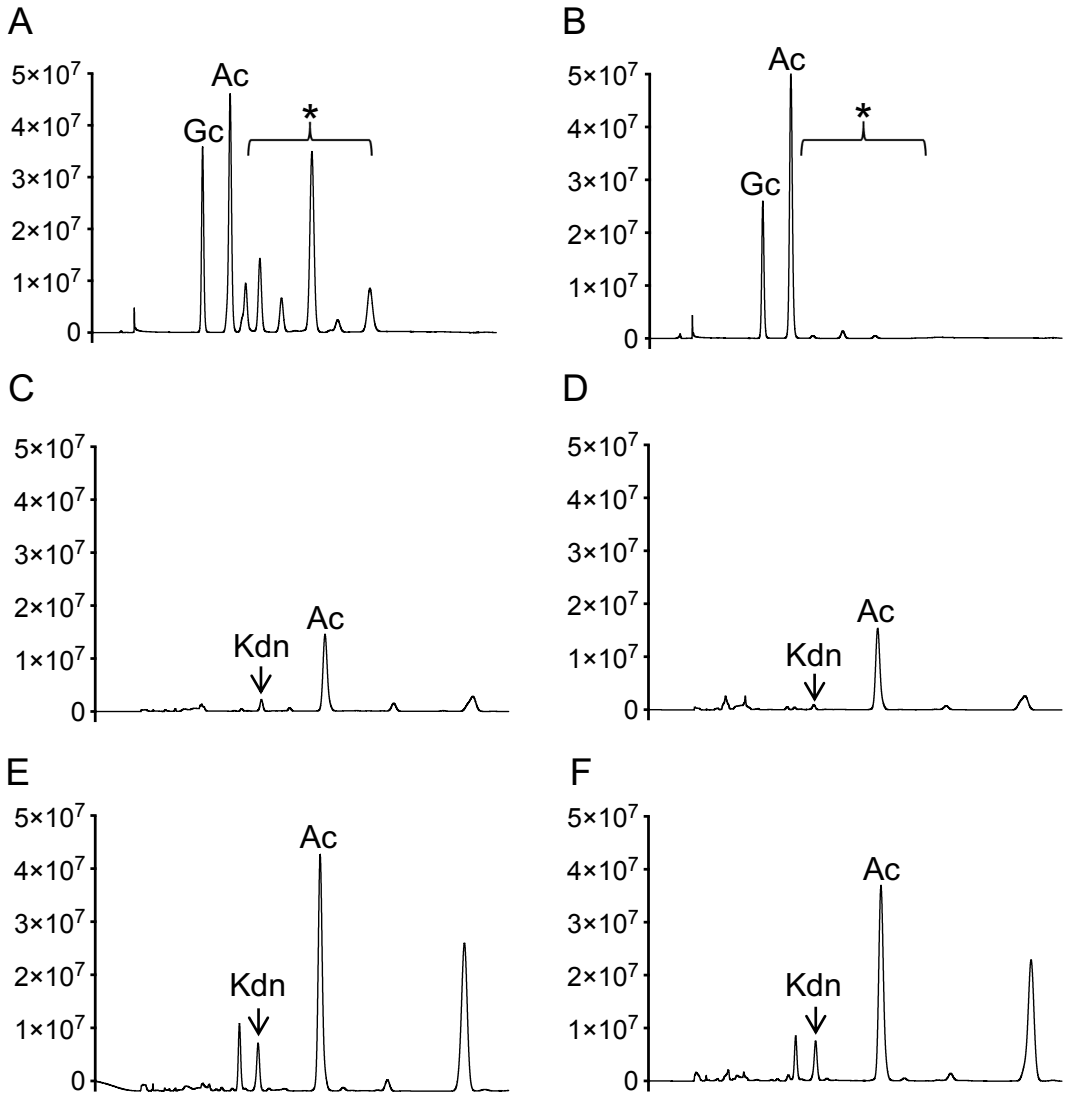
Sequence alignments, tests for selection and phylogenetic tree. Evolutionary analyses of NANS were conducted in MEGA X (Kumar S, Stecher G, Li M, Knyaz C, and Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549). Information about sequences are given in Supplemental Table 8. Multiple alignment based on NANS nucleotide sequences was performed using MUSCLE with UPGMA algorithm.

Detection of Man-6P in human serum and urine by HPAEC-PAD. The frozen serum and urine samples were thawed and centrifuged at 3,000 rpm for 5 min at 4°C. Samples were divided into two 500 µL aliquots and one of them was added Man-6P (0.2 µg total per sample). Then the samples were filtered by Microcon-3 filter at 14,000 X g for 20 min at 4°C. Next, the flow through was passed over 1mL column packed with Dowex cation exchange resin (AG 50W-X8, 200-400 mesh). Then the resin was washed with 1 mL ultrapure water and the flow through was collected for Man-6P analysis using HPAEC-PAD (Dionex ICS-3000). Carbopac PA-1 column (Dionex CarboPac PA1 column 4 mm x 250 mm, 4µm, with a 4 mm x 50 mm Guard) was used for

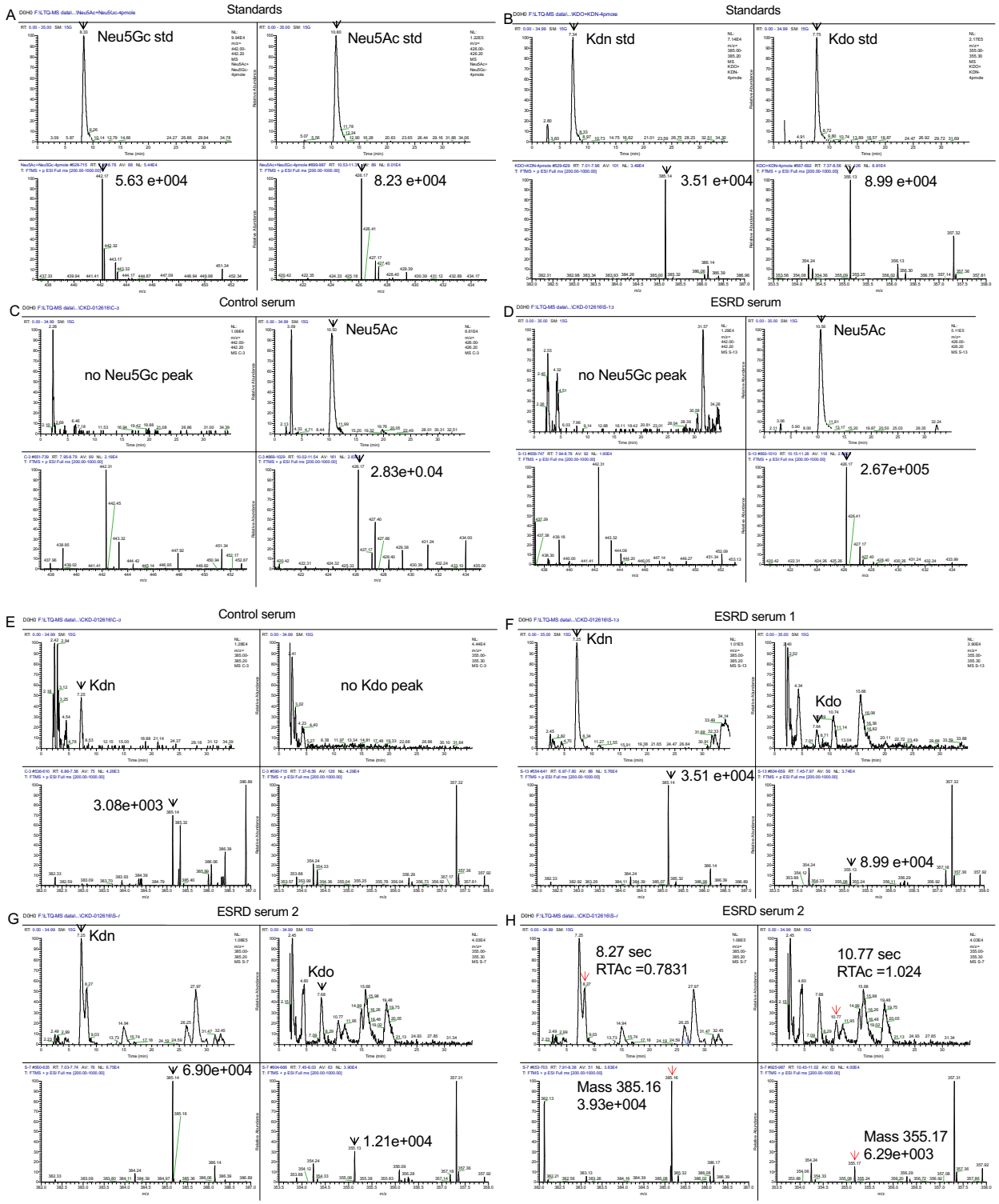
monosaccharide profiling with 100 mM NaOH and 250 mM NaOAc as gradient mixture. Quantification was done by comparing with the known quantity of standard monosaccharides purchased from Sigma.

Cell fractionation and sodium borohydride treatment. Cells and media were collected after incubation with mannose. The media was centrifugated and 1.5 mL aliquot was passed filtered through a Microcon-10 filter and lyophilized. Cells were harvested with 10mM EDTA and washed with PBS; suspension cells were directly washed with PBS. Hereon all fractions are kept on ice. The cell pellets were lysed using ice cold water which had been titrated to pH 7.5 with 1M NH_4OH (for preservation of CMP-glycans) and sonicated with two 15 second bursts, with 15 second rest in-between. The homogenates were centrifuged at 100,000 g for 1 hour. The supernatant was collected, adjusted to 87.5% ethanol and centrifuged at 20,000 g for 10 min. The resulting supernatant was diluted to 10 % ethanol (using pH 7.5 water), frozen and lyophilized; this fraction was called FSF (Free-Sugar Fraction) containing Neu5Ac, Kdn, CMP-Neu5Ac, CMP-Kdn. The resulting pellet was called SPF (Soluble Protein Fraction) and was stored at -80˚C. The cell pellet was washed once with 3 mL cold water pH 7.5, sonicated, and centrifugation at 100,000 g for 1 hour. The membrane pellet was suspended in 200 µL water pH 7.5. Sodium borohydride treatment was performed on 25 µL of the FSF and media (after lyophilization, the dried material was dissolved in 200 µL ice-cold water). The sample was mixed with 25 µL of 0.5 M $NaBH_4$, incubated at 37˚C for 15 min, and quenched with 25 µL 5 M acetic acid, for 5 min at 4˚C and 10 min at37˚C. Control samples which were not subjected to borohydride treatment, had 25 µL of water added, 25 µL 5 M acetic acid, and incubated on ice for the first 20 min, and finished with 10 min incubation at 37˚C. The pH of each reaction was monitored.

Supplemental Figures and Tables



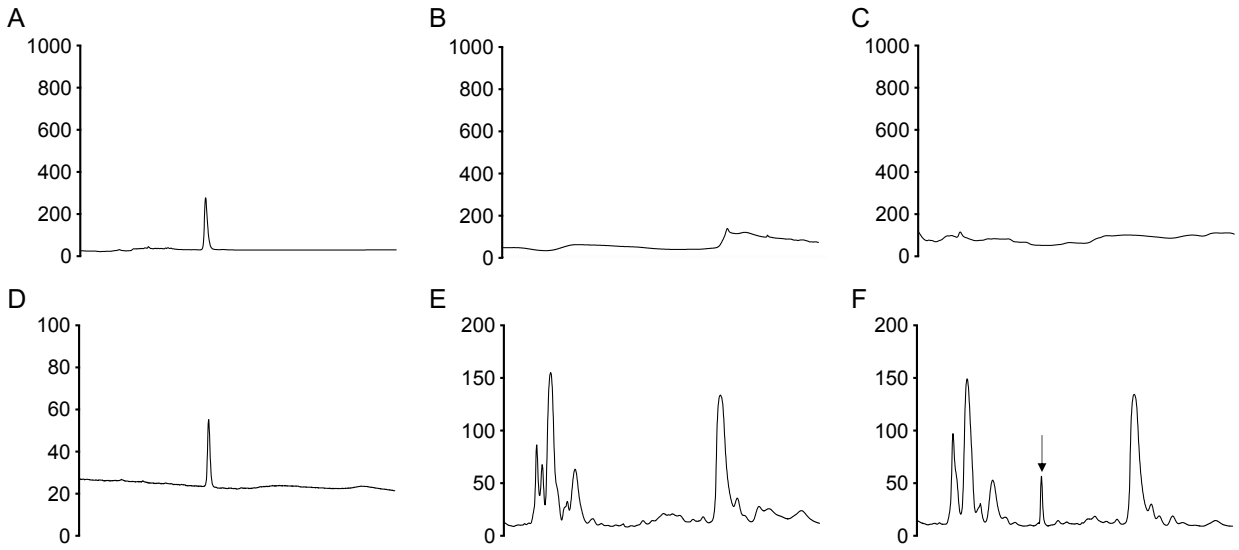
Supplemental Figure 1. HPLC analyses for free and CMP-sialic acid via de *O*-acetylation. (A) Bovine submaxillary mucin (BSM) was used as a standard for O-acetyl groups of sialic acids (Sias) and (B) the de-O-acetylation treatment. O-acetyl groups and solubilizing groups such as Neu5Gc7Ac, Neu5,7Ac2 (*) can be removed with NaOH treatment at 37˚C for 30 min. Control (C, D) and ESRD (E, F) serum samples did not show any significant peak shift (0-50 min in HPLC run) after NaOH treatment (D and F), suggesting that there was no O-acetyl form accumulation. Kdn, Neu5Ac (Ac), Neu5Gc (Gc) were annotated. Y-axis showed peak counts in HPLC run.



Supplemental Figure 2. LC/MS analyses for free α-ketoacids including sialic acids after DMB-tagging.

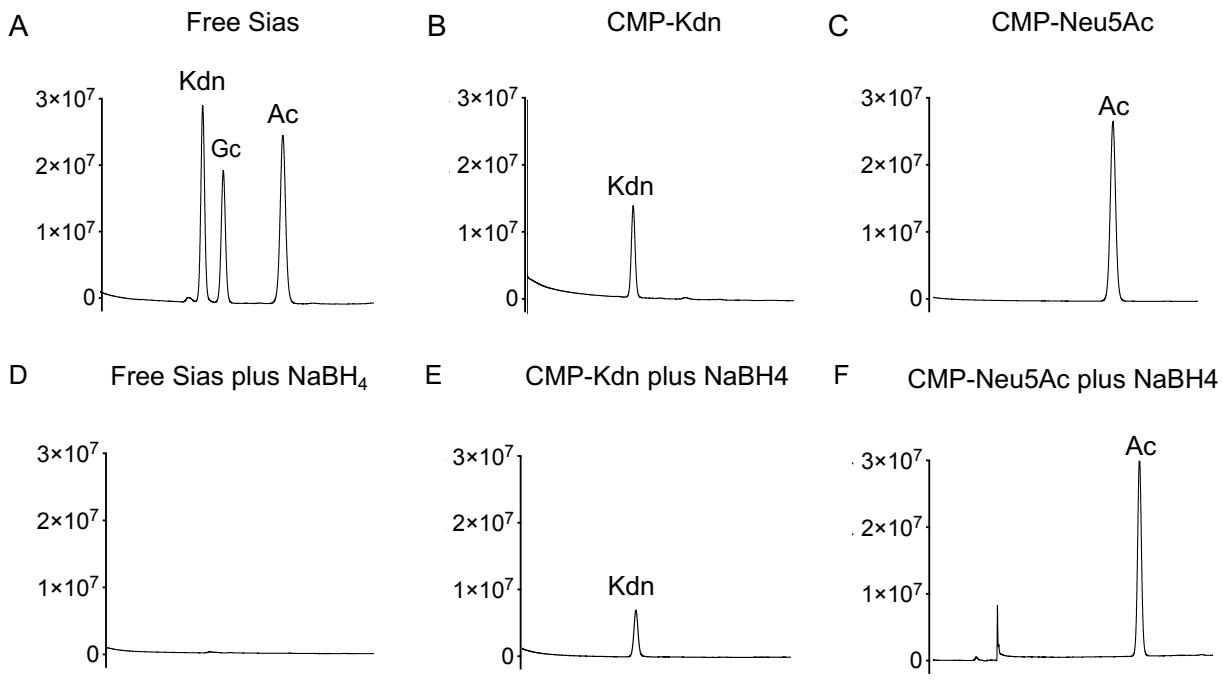
Peak analyses of standards (std) for (A) Neu5Gc and Neu5Ac, (B) Kdn and Kdo in LC-MS. Neu5Gc and Neu5Ac peaks in (C) control and, (D) end-stage of renal disease (ESRD) patient

serum. Kdn and Kdo peaks in (E) control and, (F), (G) ESRD. (H) Examples of unknown peaks that were found or significantly accumulated only in ESRD (red arrows). The same data are presented for (G) and (H) with different regions highlighted.



Supplemental Figure 3. HPAEC-PAD Analyses for Mannose-6P.

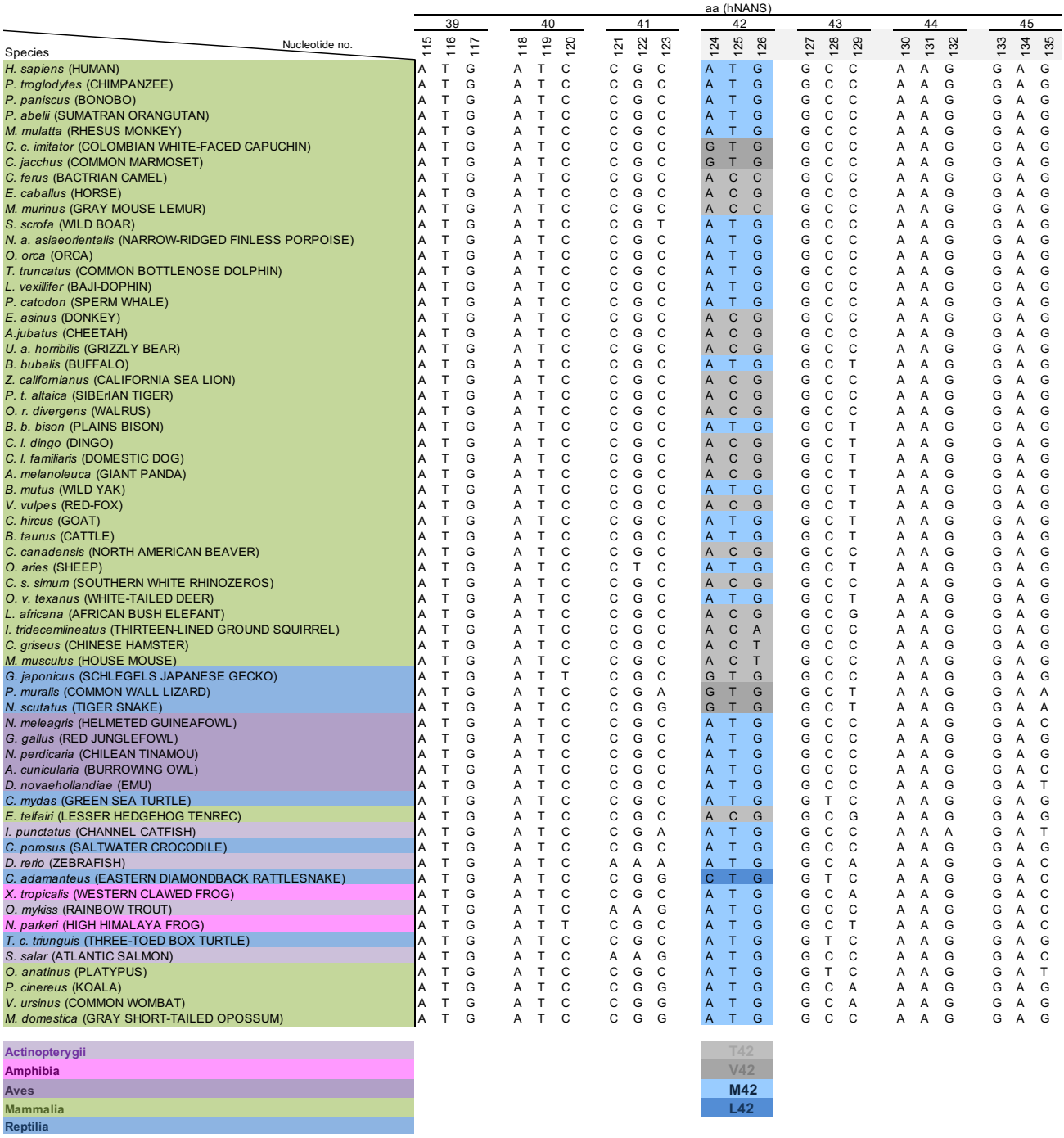
HPAEC-PAD peaks for (A) 0.2 μg (0.8 nmol) Man-6P standard for serum analysis; (B) without and (C) with external 0.2 μg Man-6P adding to the serum sample 1 hour after mannose ingestion when the serum mannose peak comes (Figure 3A). (D) Peaks for 0.2 μg Man-6P standard for urine analysis (E) without and (F) with external 0.2 μg Man-6P addition to the urine collected 4 hours after mannose ingestion when urine mannose peak comes. Man-6P is detected only in the spiked urine sample (arrow) (F), suggesting that Man-6P could not exist in human serum and urine, as well. Y-axis showed peak counts in HPAEC-PAD run.



Supplemental Figure 4. DMB-HPLC analyses for free and CMP-activated form of sialic acids.

DMB-HPLC peaks for (A) free Sia 100 pmoles standards (Kdn, Neu5Gc (Gc) and Neu5Ac (Ac)); (B) CMP-Kdn; (C) CMP-Neu5Ac. (D) Free form of Kdn, Neu5Gc and Neu5Ac are sensitive to $NaBH_4$ treatment, but (E) CMP-Kdn and (F) CMP-Neu5Ac are resistant to $NaBH_4$.

Y-axis showed peak counts in HPLC run.



Supplemental Figure 5. Part of a multiple alignment of NANS nucleotide sequences

Codons specifying Methionine (M) and Leucine (L) at position 42 in human NANS (giving rise to NANS with Kdn synthase activity), are shaded in light and dark blue, respectively, while Threonine (T) and Valine (V) are shaded in light and dark grey. Species are shaded according to

classes: Actinopterygii (light purple), Amphibia (pink), Aves (dark purple) Mammalia (green), Reptilia (blue).

Supplemental Table 1. Standards used for Mass Spectrometry Analysis.

α-ketoacids	Abbreviation	Mass	Source
2-keto-3-deoxy-D-glycero-D-galacto-nononic acid	Kdn	385.14	Commercial
3-deoxy-D-manno-oct-2-ulosonic acid	Kdo	355.13	Commercial
N-glycolylneuraminic acid	Neu5Gc	442.16	Commercial
5,7-diamino-3,5,7, 9-tetradeoxy-D-glycero-D-galacto-non-2-ulosonic (legionaminic) acid	Leg	473	Commercial
7-O-acetyl-N-glycolylneuraminic acid	Neu5Gc7Ac	484.17	*BSM
N-acetylneuraminic acid	Neu5Ac	426.17	Commercial
8-O-acetyl-N-glyconeuraminic acid	Neu5Gc8Ac	484.17	BSM
5,7- diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic (pseudaminic) acid	Pse	451	Commercial
7-O-acetyl-N-acetylneuraminic acid	Neu5,7Ac2	468.18	BSM
9-O-acetyl-N-glyconeuraminic acid	Neu5Gc9Ac	484.17	BSM
8-O-acetyl-N-acetylneuraminic acid	Neu5,8Ac2	468.18	BSM
9-O-acetyl-N-acetylneuraminic acid	Neu5,9Ac2	468.18	BSM
8,9-di-O-acetyl-N-acetylneuraminic acid	Neu5,8,9Ac3	510	BSM
7,9-di-O-acetyl-N-acetylneuraminic acid	Neu5,7,9Ac3	510	BSM

Each standard for α-ketoacids including sialic acids is available in commercial inventories including *bovine submaxillary mucin (BSM).

Kdn relative to Neu5Ac increased in various mammalian cells after mannose feeding.

Supplemental Table 2. Kdn relative to Neu5Ac in cytosol.

Cell line	Mannose concentration					
	0 mM	1 mM	3 mM	5 mM	10 mM	15 mM
HEK293A	0.24	0.27	0.31	0.31	0.38	0.42
HUVEC	0.07	0.22	0.22	1.01	3.82	3.02
BJAB K88	1.47	2.19	3.66	4.87	8.84	14.23
BJAB K20	5.18	10.39	49.12	28.84	26.91	90.62

Supplemental Table 3. Kdn relative to Neu5Ac in growth media.

Cell line	Mannose concentration		
	0 mM	5 mM	15 mM
HEK293A	0.127	0.526	1.418
HUVEC	1.128	1.130	1.624
BJAB K88	0.121	0.125	0.239
BJAB K20	0.149	0.453	1.780
PMI WT	0.098	0.416	0.604
PMI KO[#]	0.527	1.502	1.181
HK2	0.815	3.386	5.085

[#]The corresponding amounts of mannose for PMI KO cells were 25 μM, 250 μM and 1 mM.

Supplemental Table 4. *Total conjugated Kdn relative to Neu5Ac in total cell lysate with NaOH and $NaBH_4$.

Cell line	Mannose concentration		
	0 mM	5 mM	15 mM
HEK293A	0.000	0.000	0.000
HUVEC	0.001	0.002	0.003
BJAB K88	0.002	0.011	0.024
BJAB K20	0.013	0.054	0.165
PMI WT	0.000	0.003	0.004
PMI KO[#]	0.003	0.004	0.003

*Free sialic acids (Sias) and CMP-Sias were destroyed by $NaBH_4$ treatment.
It showed total cell conjugated form of Sias.
[#]The corresponding amounts of mannose for PMI KO cells were 25 μM, 250 μM and 1 mM.

Supplemental Table 5. Kdn relative to Neu5Ac in membrane fraction.

Cell line	Mannose concentration	
	0 mM	15 mM
HEK293A	0	0.0020
BJAB K88	0	0.064
BJAB K20	0.0042	0.11
PMI WT	0	0.0014
PMI KO[#]	0.012	0.0005
HK2	0	0.0035

[#]The corresponding amounts of mannose for PMI KO cells were 25 μM and 1 mM.

Supplemental Table 6. Kdn relative to Neu5Ac in membrane fraction of HEK293A with fish *Cmas* transfection.

vector	Mannose concentration	
	0 mM	15 mM
non-transfected	0	0.0014
empty vector	0	0.0019
*Cmas1	0.0043	0.0163
*Cmas2	0.0076	0.0323

*Two types of zebrafish CMP-sialic acid synthetases (Cmas) with differential substrate affinity were transiently expressed in HEK293A cells, and non-transfected and empty vector transfected cells were set as controls. Cells were harvested 36 hours post mannose feeding (0 or 15 mM) and membrane fractions were prepared to analyze glycosidically-conjugated form of sialic acids by DMB-HPLC analysis (Kdn relative to Neu5Ac).

Supplemental Table 7. Identity and homology of human and other vertebrate NANS proteins.

Species	Accession number (Protein)	Identity	Homology	Class
Acinonyx jubatus	XP_014934957.1	96%	98%	Mammalia
Ailuropoda melanoleuca	XP_002914976.1	96%	98%	Mammalia
Athene cunicularia	XP_026721911.1	84%	92%	Aves
Bison bison bison	XP_010845354.1	97%	99%	Mammalia
Bos mutus	XP_005906043.1	97%	99%	Mammalia
Bos taurus	NP_001039947.1	97%	99%	Mammalia
Bubalus bubalis	XP_006063515.1	97%	99%	Mammalia
Callithrix jacchus	XP_008998209.1	98%	99%	Mammalia
Camelus ferus	XP_006187837.1	97%	98%	Mammalia
Canis lupus dingo	XP_025287783.1	96%	98%	Mammalia
Canis lupus familiaris	XP_538746.2	96%	98%	Mammalia
Capra hircus	XP_005683962.2	97%	99%	Mammalia
Castor canadensis	XP_020036388.1	97%	98%	Mammalia
Cebus capucinus imitator	XP_017383327.1	98%	99%	Mammalia
Ceratotherium simum simum	XP_004423294.1	96%	98%	Mammalia
Chelonia mydas	XP_007066359.1	85%	93%	Reptilia
Cricetulus griseus	XP_003500141.1	95%	98%	Mammalia
Crocodylus porosu	XP_019410688.1	88%	93%	Reptilia
Crotalus adamanteus	AFJ51454.1	84%	94%	Reptilia
Danio rerio	NP_996660.1	79%	92%	Actinopterygii
Dromaius novaehollandiae	XP_025955160.1	85%	94%	Aves
Echinops telfairi	XP_004711046.1	92%	97%	Mammalia
Equus asinus	XP_014715223.1	96%	98%	Mammalia
Equus caballus	XP_023485493.1	96%	98%	Mammalia
Gallus gallus	NP_001007976.1	87%	94%	Aves
Gekko japonicus	XP_015281630.1	84%	94%	Reptilia
Homo sapiens	NP_061819.2	100%	100%	Mammalia
Ictalurus punctatus	NP_001187692.1	78%	91%	Actinopterygii
Ictidomys tridecemlineatus	XP_005326366.1	96%	98%	Mammalia
Lipotes vexillifer	XP_007461720.1	97%	98%	Mammalia
Loxodonta africana	XP_003407624.1	95%	98%	Mammalia
Macaca mulatta	EHH23940.1	99%	99%	Mammalia

Microcebus murinus	XP_012624488.1	97%	99%	Mammalia
Monodelphis domestica	XP_001364128.1	87%	94%	Mammalia (Methateria)
Mus musculus	NP_444409.1	95%	97%	Mammalia
Nanorana parkeri	XP_018407945.1	76%	89%	Amphibia
Neophocaena asiaeorientalis asiaeorientalis	XP_024590225.1	97%	98%	Mammalia
Notechis scutatus	XP_026528761.1	84%	94%	Reptilia
Nothoprocta perdicaria	XP_025893877.1	84%	91%	Aves
Numida meleagris	XP_021236216.1	87%	94%	Aves
Odobenus rosmar divergens	XP_004392385.1	95%	98%	Mammalia
Odocoileus virginianus texanus	XP_020761234.1	96%	99%	Mammalia
Oncorhynchus mykiss	XP_021429201.1	79%	91%	Actinopterygii
Orcinus orca	XP_004271519.1	96%	98%	Mammalia
Ornithorhynchus anatinus	XP_001506765.1	89%	95%	Mammalia (Monotremata)
Ovis aries	XP_004005320.3	97%	99%	Mammalia
Pan paniscus	XP_008972309.1	99%	99%	Mammalia
Pan troglodytes	XP_024201562.1	100%	100%	Mammalia
Panthera tigris altaica	XP_007089351.1	96%	98%	Mammalia
Phascolarctos cinereus	XP_020860268.1	90%	94%	Mammalia (Methateria)
Physeter catodon	XP_023982297.1	97%	98%	Mammalia
Podarcis muralis	XP_028568080.1	85%	94%	Reptilia
Pongo abelii	XP_002820068.1	99%	99%	Mammalia
Salmo salar	ACI33492.1	79%	91%	Actinopterygii
Sus scrofa	NP_001172068.1	97%	99%	Mammalia
Terrapene carolina triunguis	XP_024062240.2	85%	93%	Reptilia
Tursiops truncatus	XP_004322851.1	97%	98%	Mammalia
Ursus arctos horribilis	XP_026361945.1	95%	98%	Mammalia
Vombatus ursinus	XP_027714871.1	89%	94%	Mammalia (Methateria)
Vulpes vulpes	XP_025868953.1	96%	98%	Mammalia
Xenopus tropicalis	XP_002935918.1	76%	90%	Amphibia
Zalophus californianus	XP_027470724.1	95%	98%	Mammalia

Identity (%) and homology (%) of vertebrate NANS protein sequences to H. sapiens NANS

NCBI Reference Sequence Numbers are given and species are shaded according to classes: Actinopterygii, Amphibia, Aves, Mammalia, Reptilia.

Supplementary Table 8. Eukaryotic NANS sequences

Species	Class	Order	Family	Accession number (Protein)	Accession number (mRNA)	Common name
Acinonyx jubatus	Mammalia	Carnivora	Felidae	XP_014934957.1	XM_015079471.2	Cheetah
Ailuropoda melanoleuca	Mammalia	Carnivora	Ursidae	XP_002914976.1	XM_002914930.3	Giant panda
Athene cunicularia	Aves	Strigiformes	Strigidae	XP_026721911.1	XM_026866110.1	Burrowing owl
Bison bison bison	Mammalia	Artiodactyla	Bovidae	XP_010845354.1	XM_010847052.1	Plains bison
Bos mutus	Mammalia	Artiodactyla	Bovidae	XP_005906043.1	XM_005905981.2	Wild yak
Bos taurus	Mammalia	Artiodactyla	Bovidae	NP_001039947.1	NM_001046482.1	Cattle
Bubalus bubalis	Mammalia	Artiodactyla	Bovidae	XP_006063515.1	XM_006063453.2	Buffalo
Callithrix jacchus	Mammalia	Primates	Callitrichidae	XP_008998209.1	XM_008999961.1	Common marmoset
Camelus ferus	Mammalia	Artiodactyla	Camelidae	XP_006187837.1	XM_006187775.2	Bactrian Camel
Canis lupus dingo	Mammalia	Carnivora	Canidae	XP_025287783.1	XM_025431998.1	Dingo
Canis lupus familiaris	Mammalia	Carnivora	Canidae	XP_538746.2	XM_538746.6	Domestic dog
Capra hircus	Mammalia	Artiodactyla	Bovidae	XP_005683962.2	XM_005683905.3	Goat
Castor canadensis	Mammalia	Rodentia	Castoridae	XP_020036388.1	XM_020180799.1	North American beaver
Cebus capucinus imitator	Mammalia	Primates	Cebidae	XP_017383327.1	XM_017527838.1	Colombian white-faced capuchin
Ceratotherium simum simum	Mammalia	Perissodactyla	Rhinocerotidae	XP_004423294.1	XM_004423237.2	Southern white rhinozeros
Chelonia mydas	Reptilia	Testudines	Cheloniidae	XP_007066359.1	XM_007066297.2	Green sea turtle
Cricetulus griseus	Mammalia	Rodentia	Cricetidae	XP_003500141.1	XM_027403145.1	Chinese hamster
Crocodylus porosus	Reptilia	Crocodilia	Crocodylidae	XP_019410688.1	XM_019555143.1	Saltwater crocodile
Crotalus adamanteus	Reptilia	Squamata	Viperidae	AFJ51454.1	JU175930.1	Eastern diamondback rattlesnake

Danio rerio	Actinopterygii	Cypriniformes	Cyprinidae	NP_996660.1	NM_206829.1	Zebrafish
Dromaius novohollandiae	Aves	Casuariiformes	Casuariidae	XP_025955160.1	XM_026099375.1	Emu
Echinops telfairi	Mammalia	Afrosoricida	Tenrecidae	XP_004711046.1	XM_004710989.1	Lesser hedgehog tenrec
Equus asinus	Mammalia	Perissodactyla	Equidae	XP_014715223.1	XM_014859737.1	Donkey
Equus caballus	Mammalia	Perissodactyla	Equidae	XP_023485493.1	XM_023629725.1	Horse
Gallus gallus	Aves	Galliformes	Phasianidae	NP_001007976.1	NM_001007975.1	Red junglefowl
Gekko japonicus	Reptilia	Squamata	Gekkonidae	XP_015281630.1	XM_015426144.	Schlegel's Japanese gecko
Homo sapiens	Mammalia	Primates	Hominidae	NP_061819.2	NM_018946.4	Human
Ictalurus punctatus	Actinopterygii	Siluriformes	Ictaluridae	NP_001187692.1	NM_001200763.2	Channel catfish
Ictidomys tridecemlineatus	Mammalia	Rodentia	Sciuridae	XP_005326366.1	XM_005326309.2	Thirteen-lined ground squirrel
Lipotes vexillifer	Mammalia	Artiodactyla	Lipotidae	XP_007461720.1	XM_007461658.1	Baji-dolphin
Loxodonta africana	Mammalia	Proboscidea	Elephantidae	XP_003407624.1 [	XM_003407576.2	African bush elephant
Macaca mulatta	Mammalia	Primates	Cercopithecidae	EHH23940.1	XM_015117229.2	Rhesus macaque
Microcebus murinus	Mammalia	Primates	Cheirogaleidae	XP_012624488.1	XM_012769034.1	Gray mouse lemur
Monodelphis domestica	Mammalia	Didelphimorphia	Didelphidae	XP_001364128.1	XM_001364091.4	Gray short-tailed opossum
Mus musculus	Mammalia	Rodentia	Muridae	NP_444409.1	NM_053179.3	House mouse
Nanorana parkeri	Amphibia	Anura	Dicroglossidae	XP_018407945.1	XM_018552443.1	High Himalaya frog
Neophocaena asiaeorientalis asiaeorientalis	Mammalia	Artiodactyla	Phocoenidae	XP_024590225.1	XM_024734457.	Narrow-ridged finless porpoise

Notechis scutatus	Reptilia	Squamata	Elapidae	XP_026528761.1	XM_026672976.1	Tiger snake
Nothoprocta perdicaria	Aves	Tinamiformes	Tinamidae	XP_025893877.1	XM_026038092.	Chilean tinamou
Numida melagris	Aves	Galliformes	Numididae	XP_021236216.1	XM_021380541.1	Helmeted guineafowl
Odobenus rosmarus divergens	Mammalia	Carnivora	Odobenidae	XP_004392385.1	XM_004392328.1	Walrus
Odocoileus virginianus texanus	Mammalia	Artiodactyla	Cervidae	XP_020761234.1	XM_020905575.1	White-tailed deer
Oncorhynchus mykiss	Actinopterygii	Salmoniformes	Salmonidae	XP_021429201.1	GBTD011617 35.1	Rainbow trout
Orcinus orca	Mammalia	Artiodactyla	Delphinidae	XP_004271519.1	XM_004271471.1	Orca
Ornithorhynchus anatinus	Mammalia	Monotremata	Ornithorhynchidae	XP_001506765	XM_001506715.5	Platypus
Ovis aries	Mammalia	Artiodactyla	Bovidae	XP_004005320.3	XM_004005271.3	Sheep
Pan paniscus	Mammalia	Primates	Hominidae	XP_008972309.1	XM_008974061.2	Bonobo
Pan troglodytes	Mammalia	Primates	Hominidae	XP_024201562.1	XM_024345794.1	Chimpanzee
Panthera tigris altaica	Mammalia	Carnivora	Felidae	XP_007089351.1	XM_007089289.2	Siberian tiger
Phasolarctos cinereus	Mammalia	Diprotodontia	Phascolarctidae	XP_020860268.1	XM_021004609.1	Koala
Physeter catodon	Mammalia	Artiodactyla	Physeteridae	XP_023982297.1	XM_024126529.2	Sperm whale
Podarcis muralis	Reptilia	Squamata	Lacertidae	XP_028568080.1	XM_028712247.1	Common wall lizard
Pongo abelii	Mammalia	Primates	Hominidae	XP_002820068.1	XM_002820022.4	Sumatran orangutan
Salmo salar	Actinopterygii	Salmoniformes	Salmonidae	ACI33492.1	BT045230.1	Atlantic salmon
Sus scrofa	Mammalia	Artiodactyla	Suidae	NP_001172068.1	NM_001185139.1	Wild boar
Terrapene carolina triunguis	Reptilia	Testudines	Emydidae	XP_024062240.2	XM_026646379.2	Three-toed box turtle
Tursiops truncatus	Mammalia	Artiodactyla	Delphinidae	XP_004322851.1	XM_004322803.2	Common bottlenose dolphin
Ursus arctos horribilis	Mammalia	Carnivora	Ursidae	XP_026361945.1	XM_026506160.1	Grizzly bear
Vulpes vulpes	Mammalia	Carnivora	Canidae	XP_025868953.1	XM_026013168.1	Red-fox
Vombatus ursinus	Mammalia	Diprotodontia	Vombatidae	XP_027714871.1	XM_027859070.1	Common wombat

Xenopus tropicalis	Amphibia	Anura	Pipidae	XP_002935918.1	XM_002935872.4	Western clawed frog
Zalophus californianus	Mammalia	Carnivora	Otariidae	XP_027470724.1	XM_027614923.	California sea lion

Vertebrate NANS sequences: Taxonomy, common names and accession numbers Taxonomy and common names of vertebrates included in phylogenetic analyses (Figure 5) are shown with species shaded according to classes: Actinopterygii (light purple), Amphibia (pink), Aves (dark purple) Mammalia (green), Reptilia (blue). NCBI Reference Sequence Numbers of NANS protein and mRNA sequences.