

Phylogenetic Distribution of CMP-Neu5Ac Hydroxylase (CMAH), the Enzyme Synthetizing the Proinflammatory Human Xenoantigen Neu5Gc

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Abstract

The enzyme CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) is responsible for the synthesis of *N*-glycolylneuraminic acid (Neu5Gc), a sialic acid present on the cell surface proteins of most deuterostomes. The *CMAH* gene is thought to be present in most deuterostomes, but it has been inactivated in a number of lineages, including humans. The inability of humans to synthesize Neu5Gc has had several evolutionary and biomedical implications. Remarkably, Neu5Gc is a xenoantigen for humans, and consumption of Neu5Gc-containing foods, such as red meats, may promote inflammation, arthritis, and cancer. Likewise, xenotransplantation of organs producing Neu5Gc can result in inflammation and organ rejection. Therefore, knowing what animal species contain a functional *CMAH* gene, and are thus capable of endogenous Neu5Gc synthesis, has potentially far-reaching implications. In addition to humans, other lineages are known, or suspected, to have lost *CMAH*; however, to date reports of absent and pseudogenetic *CMAH* genes are restricted to a handful of species. Here, we analyze all available genomic data for nondeuterostomes, and 322 deuterostome genomes, to ascertain the phylogenetic distribution of *CMAH*. Among nondeuterostomes, we found *CMAH* homologs in two green algae and a few prokaryotes. Within deuterostomes, putatively functional *CMAH* homologs are present in 184 of the studied genomes, and a total of 31 independent gene losses/pseudogenization events were inferred. Our work produces a list of animals inferred to be free from endogenous Neu5Gc based on the absence of *CMAH* homologs and are thus potential candidates for human consumption, xenotransplantation research, and model organisms for investigation of human diseases.

Key words: Neu5Gc, CMAH, pseudogene.

Introduction

Sialic acids are a family of more than 50 nine-carbon sugars that are typically found at the terminal ends of *N*-glycans, *O*-glycans, and glycosphingolipids that are secreted or attached to the cell membrane. They are involved in recognition processes, frequently serving as ligands for receptor-mediated interactions that enable intercellular or host–pathogen recognition. Sialic acids may also function as a class of “self-associated molecular patterns” (SAMPs), whose presence/absence in a species serves as a signal to modulate innate immune responses (Varki 2011). Sialic acids are found predominantly in deuterostomes (the group including chordates, hemichordates, and echinoderms), being uncommon in other organisms (Warren 1963; Corfield and Schauer 1982; Staudacher et al. 1999; Angata and Varki 2002; Schauer 2004).

The most common sialic acids are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc).

The enzyme cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (*CMAH*) catalyzes the synthesis of Neu5Gc by hydroxylation of Neu5Ac (Schauer et al. 1968; Schoop et al. 1969; Schauer 1970). *CMAH*^{−/−} mice lack Neu5Gc, indicating that *CMAH* is the only enzyme capable of synthesizing Neu5Gc (Hedlund et al. 2007; Bergfeld et al. 2012). This enzyme and/or its encoding gene has been found in many chordates, in echinoderms and in a hemichordate (Kawano et al. 1995; Martensen et al. 2001; Varki 2009; Bergfeld et al. 2012; Ikeda et al. 2012). In nondeuterostomes, homologous *CMAH* sequences have recently been detected only in two green algae and in a few prokaryotes, with phylogenetic analyses suggesting a horizontal gene transfer event from green algae to deuterostomes (Simakov et al. 2015).

Consistently, Neu5Gc has been reported in a variety of deuterostomes including echinoderms, fish, amphibians, and the majority of mammals studied so far (see exceptions

below) (Muralikrishna et al. 1992; Klein et al. 1997; Tangvoranuntakul et al. 2003; Schauer et al. 2009; Samraj et al. 2015). It is generally thought that nondeuterostomes cannot synthesize Neu5Gc, even though some can incorporate it from the environment (Schauer et al. 1983).

Some deuterostomes, including humans, have undergone inactivation or loss of the gene *CMAH*, and therefore have lost the ability of synthesizing Neu5Gc. In an ancestor of humans, an Alu-mediated deletion removed a region of the genome encompassing a 92-bp exon of *CMAH* (Irie and Suzuki 1998; Hayakawa et al. 2001; Chou et al. 2002). Deletion of this exon resulted in a frameshift mutation, as a result of which the human protein is only 72 amino acids long and nonfunctional (the full ancestral *CMAH* protein was 590 amino acids long). Therefore, human tissues exhibit very low levels of Neu5Gc (Muchmore et al. 1998; Tangvoranuntakul et al. 2003; Diaz et al. 2009), which are probably the result of incorporation from animal foods. It has been estimated that this inactivation took place 2.5–3 Ma (Chou et al. 2002) and was fixed rapidly in the population, probably with the intervention of positive selection (Hayakawa et al. 2006). Inactivation of *CMAH* may have affected human biology in multiple ways (for a comprehensive review, see Varki 2009; Okerblom and Varki 2017). First, it may have freed our ancestors from pathogens that require attaching to Neu5Gc for infection, such as *Plasmodium reichenowi* (responsible for malaria in chimpanzees and gorillas; Martin et al. 2005), *E. coli* K99 (Kyogashima et al. 1989), transmissible gastroenteritis coronavirus (Schwegmann-Wessels and Herrler 2006), and simian virus 40 (Campanero-Rhodes et al. 2007). Nonetheless, inactivation of *CMAH* probably made humans susceptible to pathogens preferentially recognizing Neu5Ac, such as *Plasmodium falciparum* (Martin et al. 2005) and *Streptococcus pneumoniae* (Hentrich et al. 2016). Remarkably, *P. falciparum* emerged from *P. reichenowi* after inactivation of *CMAH* in humans (Rich et al. 2009; Varki and Gagneux 2009). Second, the *CMAH* pseudogene may have been driven to fixation via sexual selection. In the time in which the presence of a functional *CMAH* gene was polymorphic in the ancestral hominin population, anti-Neu5Gc antibodies in the reproductive tract of Neu5Gc-negative females may have targeted Neu5Gc-containing sperm or fetal tissues, thus reducing reproductive compatibility (Ghaderi et al. 2011). Third, loss of Neu5Gc may have unchained a series of changes in human sialic acid biology and its controlling genes. Out of the <60 genes known to be involved in sialic acid biology, at least 10 have undergone human-specific changes, some of which have been linked directly to Neu5Gc loss (Altheide et al. 2006; Varki and Varki 2007; Varki 2009). Fourth, the phenotypes of *CMAH*^{-/-} mice suggest that loss of Neu5Gc may have contributed to a number of human-specific diseases (Hedlund et al. 2007; Chandrasekharan et al. 2010; Kavalier et al. 2011).

Inactivation of *CMAH* in humans meant that Neu5Gc became a foreign antigen. Neu5Gc from animal foods

(predominantly red meats and milk products; Tangvoranuntakul et al. 2003; Samraj et al. 2015) is incorporated into the glycoproteins of human tissues (Tangvoranuntakul et al. 2003; Bardor et al. 2005; Banda et al. 2012), where it is thought to elicit an immune response that may result in chronic inflammation, rheumatism, and cancer (Varki and Varki 2007). This may explain, at least in part, the link between red meat consumption and cancer (Rose et al. 1986; Giovannucci et al. 1993; Fraser 1999; Tavani et al. 2000; Willett 2000; Linseisen et al. 2002; Bosetti et al. 2004; Zhang and Kesteloot 2005; Tseng et al. 2015). In support of this hypothesis, Neu5Gc often concentrates in human tumors and sites of inflammation (Malykh et al. 2001; Tangvoranuntakul et al. 2003; Diaz et al. 2009), and *CMAH*^{-/-} mice develop systemic inflammation and a high frequency of cancer when fed with bioavailable Neu5Gc (Samraj et al. 2015).

In the context of animal-based xenotransplantation, tissues from animals expressing Neu5Gc have been shown to cause human recipients to develop antibodies against Neu5Gc, triggering inflammation and contributing to delayed tissue rejection (Salama et al. 2015; Hurh et al. 2016). Neu5Gc has been found in most tissues of pigs such as heart, kidney, liver, pancreas (Bouhours and Bouhours 1988; Bouhours et al. 1996; Diswall et al. 2010), and in adult pig pancreatic islets (Komoda et al. 2004), raising concerns over their application in xenotransplantation.

In addition to humans, *CMAH* has also been reported or suggested to be inactivated or lost in other animal lineages independently. New World monkeys underwent inactivation of *CMAH* ~30 Ma due to inversion of exons 4–13 and loss of exons 4–8 and 10–13. This may explain why they are susceptible to certain human pathogens, such as *P. falciparum* (Springer et al. 2014). In the ferret genome, the first nine exons of the gene have been lost, and PCR analyses did not detect conserved portions of the gene in a number of pinnipeds and musteloids, indicating that *CMAH* was inactivated in an ancestor of pinnipeds and musteloids (Ng et al. 2014). Sequence similarity searches against the genomes of chicken and zebra finch did not detect any *CMAH* homolog (Schauer et al. 2009) and southern-blot analysis did not detect expression of the gene in chicken liver (Kawano et al. 1995). Consistently, Neu5Gc has been shown to be rare in birds and reptiles (Fujii et al. 1982; Schauer and Kamerling 1997; Ito et al. 2000; Schauer et al. 2009). These observations led to the hypothesis that the *CMAH* gene may have been lost in an ancestor of Sauropsida (reptiles and birds) (Schauer et al. 2009). According to this hypothesis, the low amounts of Neu5Gc detected in some reptiles and birds may have been incorporated from the diet. Analysis of the platypus genome did not reveal any *CMAH* homolog, and Neu5Gc was not detected in platypus muscles or liver (Schauer et al. 2009), or in the milk of the Australian spiny anteater

echidna (Kamerling et al. 1982), suggesting that *CMAH* was also lost in an ancestor of extant monotremes.

In cats, blood types A, B, and AB are determined by the presence or absence of Neu5Gc on certain erythrocyte glycolipids, and the absence of Neu5Gc in type B cats might be due to mutations in the 5'-UTR or the protein-coding regions of *CMAH* (Bighignoli et al. 2007; Omi et al. 2016). Depending on their geographical origin, dog breeds express Neu5Gc (Northern China, Korea, and Southern Japan) or not (Europe, Hokkaido dog from North of Japan) (Yasue et al. 1978; Hashimoto et al. 1984). The molecular basis of why certain dog breeds lack Neu5Gc is yet to be elucidated.

Given the biological and biomedical relevance of Neu5Gc, it is important to know what animals have the ability to synthesize it. Knowing the exact lineages in which *CMAH* has been inactivated or lost will allow scientists to identify lineages that may have experimented changes similar to those experienced by humans, and will have implications for human nutrition and xenotransplantation. However, to date all studies of *CMAH* evolution have been restricted to specific organisms or groups of organisms. Here, we conduct the first comprehensive analysis of the evolution of *CMAH*. For that purpose, we analyzed all available genomic data in the National Center for Biotechnology Information (NCBI) databases, and the genomes of 323 deuterostomes. We found that the gene has been lost or inactivated at least 31 times during deuterostome evolution.

Materials and Methods

Genomic Data Set and Determination of *CMAH* Presence/Absence

We used all genomic data available from the NCBI Genome database to determine the occurrence of *CMAH* in nondeuterostomes. We then focused on the 321 deuterostome genomes and 2 echinoderm *CMAH* mRNA sequences available in the NCBI Genome database as of January 2017 (NCBI Coordinators 2016). An initial screening for the presence or absence of the *CMAH* gene was done using sequence similarity searches (BLASTP and TBLASTN searches) using the chimpanzee *CMAH* protein sequence as query. If BLASTP searches failed for a given organism (which depend on *CMAH* proteins being annotated), TBLASTN searches were conducted (which can detect unannotated sequences). All BLAST searches were performed using an *E*-value cut-off of 10^{-10} (Altschul et al. 1990). All genomes, depending on the level of assembly, were either screened in the NCBI RefSeq genomic database (non-redundant, well-annotated reference sequence database) or in the NCBI WGS database (genome assemblies of incomplete genomes with or without annotation).

Gene Annotation and Curation

Gene annotations, particularly for nonmodel organisms, are known to be subjected to high rates of error (Devos and

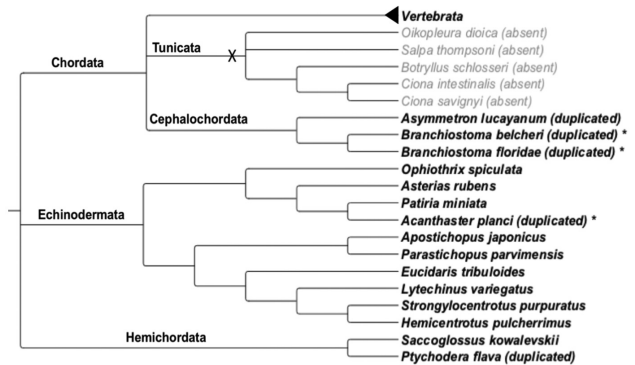


Fig. 1.—Presence/absence of the gene *CMAH* in nonvertebrates. Species in which gene is absent or inactivated are marked in gray. “X” symbols represent inferred gene loss events. *One of the duplicates is a pseudogene.

Valencia 2001; Tu et al. 2012). For each *CMAH* homolog, the nucleotide sequence of the coding region (CDS) was translated in silico and aligned with the chimpanzee sequence using ProbCons, version 1.12 (Do et al. 2005). The resulting protein sequence alignment was used to guide the alignment of the CDSs. The CDS alignments were visualized using BioEdit, version 7.0.0 (Hall 1999). Where possible, all gene annotation errors were fixed manually. Erroneous and extra exons (not showing significant similarity to the chimpanzee sequence) were removed. Missing exons (present in the chimpanzee sequence but not in the species of interest) were searched for in the genome using the chimpanzee sequence as query in TBLASTN or BLASTN searches. If the missing exon could not be detected, a careful analysis was conducted to determine whether the exon had been lost or it was part of an unsequenced region. Given the small size of the coding segment of exon 1 (only eight nucleotides), no attempt was made to annotate this exon.

Where possible, signatures of pseudogenization (premature stop codons, frameshift mutations, and exon losses) were verified by visualizing the original chromatograms in the Sequence Read Archive (SRA) database. *CMAH* sequences were only considered pseudogenes if at least one pseudogenization signature was confirmed.

Phylogenetic Analysis

The trees represented in figures 1–4 were derived from the NCBI Taxonomy database (Sayers et al. 2009) using PhyloT (Letunic and Bork 2007). Relevant polytonies observed in the orders Caniformia and Chiroptera were resolved based on prior phylogenetic analyses (Tsagkogeorga et al. 2013; van Valkenburgh et al. 2014; Lei and Dong 2016). The trees were visualized using the TreeGraph software (Stöver and Müller 2010).

Phylogenetic analyses were used to better characterize certain gene duplication events in fish and nonvertebrate

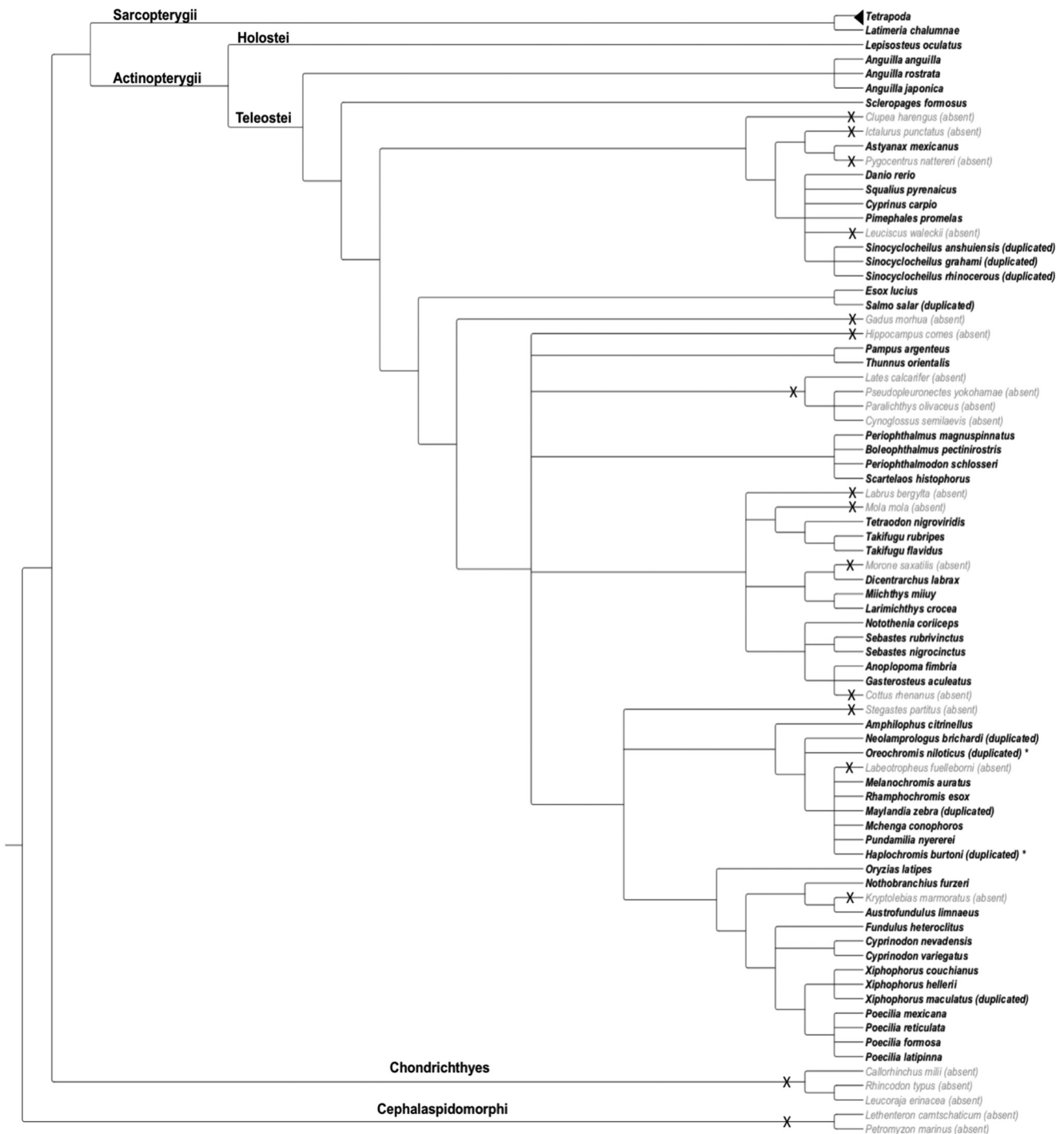


FIG. 2.—Presence/absence of the gene *CMAH* in fish. Species in which gene is absent or inactivated are marked in gray. “X” symbols represent inferred gene loss events. *One of the duplicates is a pseudogene.

deuterostomes and to discard lateral gene transfer. These analyses were conducted using IQ-Tree (Nguyen et al. 2015) with default parameters. Only sequences covering at least 20% of the length of the gene were included in our phylogenetic analyses.

Purifying Selection Analysis

The *Xenopus tropicalis* and *Xenopus laevis* *CMAH* sequences are annotated as pseudogenes in the NCBI RefSeq database. We aligned the two CDSs and estimated the nonsynonymous to synonymous divergence ratio, d_N/d_S , using the codeml

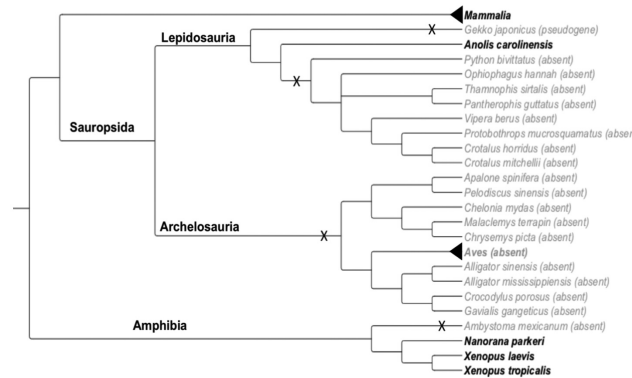


FIG. 3.—Presence/absence of the gene *CMAH* in amphibians, birds, and reptiles. Species in which gene is absent or inactivated are marked in gray. “X” symbols represent inferred gene loss events.

program of the PAML package, version 4.4 (Yang 2007). We tested whether this d_N/d_S ratio was significantly different from 1 using a likelihood ratio test. Twice the difference of the log-likelihoods of both models (M0 with a free d_N/d_S ratio vs. M0 with a fixed d_N/d_S of 1) was assumed to follow a χ^2 distribution with one degree of freedom.

Results and Discussion

CMAH in Nondeuterostomes

We first used the chimpanzee *CMAH* protein sequence (Chou et al. 1998) as query in a BLASTP search against the NCBI nr database, excluding all sequences from deuterostomes. The first two hits corresponded to the green algae *Ostreococcus tauri* and *Micromonas commoda*, and 60 prokaryotes, including representatives of proteobacteria, firmicutes, cyanobacteria, actinobacteria, nitrospirae, FCB group (Fibrobacteres, Chlorobi, and Bacteroidetes), and archaeobacteria (supplementary table S1, Supplementary Material online). No hits were detected in any other group, including nondeuterostome animals. The distribution of *CMAH* homologs in nondeuterostomes is equivalent to that observed by Simakov et al. (2015), who proposed that the *CMAH* gene could have been passed onto the deuterostome lineage by green algae through horizontal gene transfer.

CMAH in Nonvertebrate Deuterostomes

We next focused our analyses on the 322 deuterostome genomes available from the NCBI Genome database, including those for 8 echinoderms, 2 hemichordates, 3 cephalochordates, 5 urochordates (tunicates), and 304 vertebrates (table 1). These represent almost completely sequenced genomes. The *CMAH* mRNA sequence for another two echinoderm species were obtained from the NCBI nr database: *Asterias rubens* (ID: AJ308602.1) (Martensen et al. 2001) and *Hemicentrotus pulcherrimus* (ID: AB699316.1). In each

genome, gene similarity searches (BLASTP and/or TBLASTN searches using the chimpanzee *CMAH* protein as query) were used to identify *CMAH* homologs. Where necessary, gene annotations were curated manually to resolve the intron/exon structure, and to determine signatures of inactivation/pseudogenization (missing exons, frameshift mutations, and premature stop codons).

CMAH homologs were detected in all of the studied echinoderms, hemichordates, and cephalochordates; however, no *CMAH* orthologs (active or inactive) were detected in urochordates. These results indicate that *CMAH* was present in the ancestor of deuterostomes, and it was lost in an ancestor of urochordates. Figure 1 summarizes the distribution of *CMAH* homologs across nonvertebrate deuterostomes, and supplementary table S2, Supplementary Material online, contains a detailed description of all found genes. Supplementary data set S1, Supplementary Material online, is a multiple sequence alignment of representative nonvertebrate deuterostome sequences.

We found putatively functional copies in all ten echinoderm species studied. *Acanthaster planci* (crown-of-thorns starfish) has two copies: one putatively functional and another putatively inactivated due to a premature stop codon in coding exon 10 (table 2). Our observations are consistent with prior works that have reported the presence of the *CMAH* enzyme, its encoding gene, or Neu5Gc in all echinoderms studied so far. The enzyme was purified from gonads of *Asterias rubens* (common star fish), *Ctenodiscus crispatus* (mud star), *Strongylocentrotus pallidus* (pale sea urchin), and a species of *Holothuria* (a sea cucumber) (Gollub and Shaw 2003). The *Asterias rubens* *CMAH* cDNA was subsequently cloned and sequenced (Martensen et al. 2001), revealing a highly conserved *CMAH* coding sequence. Neu5Gc was detected in whole body extracts of *Ophioderma brevispina* (a brittle star), *Nemaster rubiginosa* (sea lily), and *Sclerodactyla briareus* (a sea cucumber) (Warren 1963; Sumi et al. 2001) and in the egg jelly coat of *Paracentrotus lividus* (a sea urchin) (Yesilyurt et al. 2015).

Our searches identified *CMAH* homologs in the two hemichordates studied, *Ptychodera flava* (yellow acorn worm) and *Saccoglossus kowalevskii* (acorn worm). These sequences were also noted by Simakov et al. (2015).

We identified *CMAH* homologous sequences in all the studied cephalochordates, including one copy in *Asymmetron lucayanum* (Bahama lancelet), five in *Branchiostoma belcheri* (Chinese amphioxus), and seven in *Branchiostoma floridae* (Floridan amphioxus). One of the *B. belcheri* copies had a premature stop codon in coding exon 6, and one of the *B. floridae* copies had a premature stop codon in coding exon 13. Our results are in agreement with a previous report of Neu5Gc in *B. belcheri* (belcher’s lancelet) (Guérardel et al. 2012).

We did not find *CMAH* homologs in any of the five urochordate genomes studied, indicating that *CMAH* was lost in

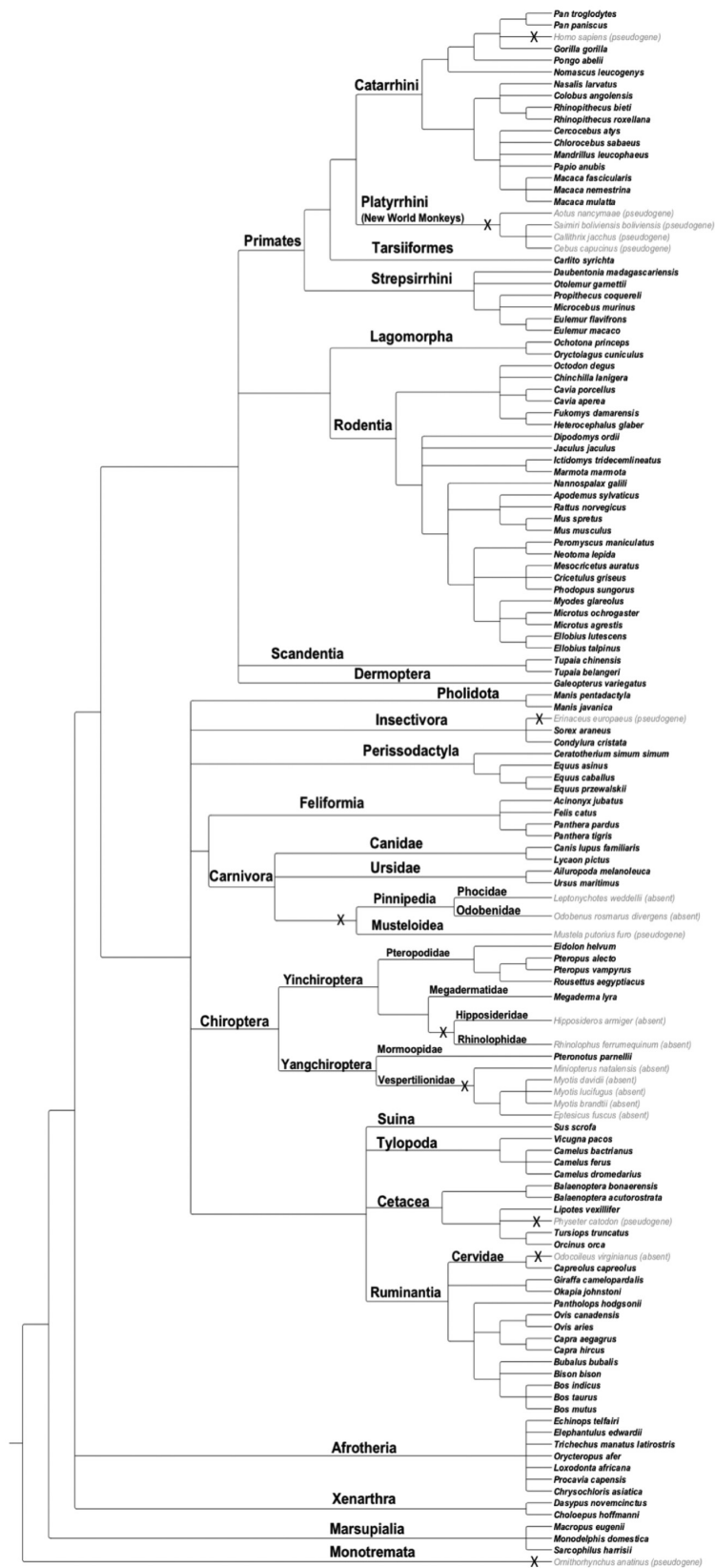


Fig. 4.—Presence/absence of the gene *CMAH* in mammals. Species in which gene is absent or inactivated are marked in gray. “X” symbols represent inferred gene loss events.

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an ancestor of urochordates. These results are in agreement with prior observations that tunicates are devoid from sialic acids (Warren 1963).

CMAH in Fish

Several groups have independently reported nonsignificant levels (<2% of the sialic acid fraction) of Neu5Gc in the meat of different fish species belonging to the classes Actinopterygii (including tilapia, yellowfin tuna, mahi mahi, swordfish, rainbow trout and sardines, crucian carp, grass carp, golden pomphret, and European seabass) and Chondrichthyes (including the thresher shark) (Chen et al. 2014; Samraj et al. 2015). The roe of salmon and whitefish,

in contrast, exhibit high Neu5Gc concentrations (Samraj et al. 2015). Our sequence similarity searches against 77 fish genomes belonging to classes Cephalaspidomorphi (lampreys, $n=2$), Chondrichthyes (cartilaginous fishes, $n=3$), Sarcopterygii (lobe-finned fishes, $n=1$), and Actinopterygii (ray-finned fishes, $n=71$) show that the gene is present in classes Sarcopterygii (1 species) and Actinopterygii (71 species). A total of 16 gene loss events were inferred to have occurred in the fish lineages, including complete gene losses at the ancestors of Cephalaspidomorphi and Chondrichthyes (fig. 2).

Gene duplicates were observed in *Sinocyclocheilus anshuiensis*, *Sinocyclocheilus grahmi*, and *Sinocyclocheilus rhinoceros*, and our phylogenetic analyses indicate a duplication in a common ancestor. In addition, species-specific CMAH gene duplicates were observed in the genomes of *Maylandia zebra* (zebra mbuna), *Neolamprologus brichardi* (princess cichlid), *Salmo salar* (Atlantic salmon), *Xiphophorus maculatus* (southern platyfish), *Haplochromis burtoni* (Burton’s mouthbrooder), and *Oreochromis niloticus* (Nile tilapia). The last two species having premature stop codon in coding exons 8 and 5, respectively, in their duplicate copies (table 2).

Table 1

Number of Deuterostome Genomes Covered in This Study

Group	Number of Genomes Available	Number of Genomes with Putatively Functional CMAH	Number of Genomes with CMAH Absent or Pseudogene
Echinoderms	10 ^a	10 ^a	0
Cephalochordates	3	3	0
Hemichordates	2	2	0
Urochordates	5	0	5
Fish	78	55	23
Amphibia	4	3	1
Reptiles	19	1	18
Birds	73	0	73
Mammals	129	110	19
Total	323	184	139

^aIncludes two unsequenced genomes with available CMAH mRNA sequences.

CMAH in Amphibians

There are four amphibian genomes available in the NCBI Genome database, including three frogs and one salamander (table 1). We found putatively functional CMAH homologs in all three frogs, *X. tropicalis* (Western clawed frog), *X. laevis* (African clawed frog), and *Nanorana parkeri* (high Himalaya frog). However, no CMAH homolog was detected in the

Table 2

CMAH Pseudogenes Identified in This Study

Species	Common Name	Status	Type	Position ^a
<i>Homo sapiens</i>	Human	Known	Deletion of coding exon 3	74–103
<i>Aotus nancymaae</i>	Ma’s night monkey [New World monkey]	Known	Deletion of coding exon 3–15	74–605
<i>Cebus capucinus</i>	White-faced sapajou [New World monkey]	Known	Deletion of coding exon 3–15	74–605
<i>Saimiri boliviensis boliviensis</i>	Bolivian squirrel monkey [New World monkey]	Known	Deletion of coding exon 3–15	74–564
<i>Callithrix jacchus</i>	White-tufted-ear marmoset [New World monkey]	Known	Deletion of coding exon 3–15	74–564
<i>Ornithorhynchus anatinus</i>	Platypus	Known	PSC in coding exon 5	154
<i>Mustela putorius furo</i>	Domestic ferret	Known	PSC in coding exon 11	444
<i>Physeter catodon</i>	Sperm whale	Unknown	Deletion of coding exon 5	143–205
<i>Erinaceus europaeus</i>	Western European hedgehog	Unknown	PSC in coding exon 13	530, 556
<i>Gekko japonicus</i>	Japanese gecko	Unknown	PSC in coding exon 4	141
<i>Oreochromis niloticus</i> (copy 2)	Nile tilapia	Unknown	FSM in coding exon 4 and 5	122, 152
<i>Oreochromis niloticus</i> (copy 2)	Nile tilapia	Unknown	PSC in coding exon 5	177
<i>Haplochromis burtoni</i> (copy 2)	Burton’s mouthbrooder	Unknown	PSC in coding exon 8	305
<i>Branchiostoma belcheri</i> (copy 2)	Chinese amphioxus	Unknown	PSC in coding exon 6	213
<i>Branchiostoma floridae</i> (copy 3)	Floridan amphioxus	Unknown	PSC in coding exon 13	539, 545
<i>Acanthaster planci</i> (copy 2)	Crown-of-thorns starfish	Unknown	PSC in coding exon 10	425

PSC, premature stop codon; FSM, frameshift mutation.

^aPosition relative to chimpanzee protein.

salamander *Ambystoma mexicanum* (axolotl). These results suggest that the ancestor of amphibians had a functional *CMAH*, and that it was lost in an ancestor of salamanders (fig. 3). [Supplementary data set S2, Supplementary Material online](#), is a multiple sequence alignment including these sequences.

Previous studies did not detect Neu5Gc in the liver of the frog *Rana esculata* (Schauer et al. 1980) or in the brain gangliosides of *X. laevis* (Rizzo et al. 2002). It should be noted, nonetheless, that Neu5Gc is usually not detected in vertebrate brain, where it appears to have adverse effects (Naito-Matsui et al. 2017). The red blood cells of the salamander *Amphiuma means* also have been reported to contain only Neu5Ac (Pape et al. 1975). However, Neu5Gc has been reported in the oviductal mucins of fire-bellied toads (*Bombina bombina* and *Bombina variegata*) and the alpine newt (*Triturus alpestris*) (Schauer et al. 2009). In addition, *CMAH* has been identified as one of the key genes downregulated during oocyte maturation in *X. laevis* (Gohin et al. 2010).

The *CMAH* genes of *X. tropicalis* and *X. laevis* have been classified as pseudogenes in the NCBI RefSeq database (Gene IDs: 100216283, 379989). However, this has not been validated by any study so far. To gain insight into the functionality of these genes, we calculated the nonsynonymous to synonymous divergence ratio (d_N/d_S). This value was significantly < 1 ($d_N/d_S = 0.142$, $2\Delta\ell = 109.03$, $P = 1.60 \times 10^{-25}$), indicating strong purifying selection, and thus strongly suggesting functionality of the *CMAH* enzyme in *Xenopus*. Therefore, we suspect that the genes have been erroneously annotated as pseudogenes by the automatic gene annotation pipelines.

Our results suggest that *X. laevis* and other frogs previously reported to possess only Neu5Ac contain a *CMAH* enzyme with all its conserved domains. However, it is inconclusive at this point whether frogs, like fish, exhibit nonsignificant levels of Neu5Gc in most of their tissues despite having an intact *CMAH* coding sequence.

CMAH in Reptiles and Birds

Sequence similarity searches against the chicken and zebra finch genomes revealed no *CMAH* homologs (Schauer et al. 2009). In agreement, Schauer et al. (2009) reported the absence of Neu5Gc in several species of birds (chicken, duck, turkey, goose, ostrich, emu, scarlet macaw, budgerigar, swallow, and oriental swiftlet) and nonbird reptiles (green iguana, agama, green basilisk hatchling, anaconda, hundred pace viper, Taiwan stings snake, Taiwan beauty snake, crocodile, and Amboina box turtle). This led to the suggestion that *CMAH* may have been lost in an ancestor of Sauropsida (reptiles and birds). Neu5Gc was found in the gastrointestinal tract of ducks (Ito et al. 2000), the eggs of the budgerigar, and in both egg and tissues of an adult green basilisk (Schauer et al. 2009). However, the source of Neu5Gc in these species is yet

to be established, with diet being a likely origin (Schauer et al. 2009).

We queried the genomes of 73 birds and 19 nonbird reptiles for *CMAH* homologs (table 1). Surprisingly, we found homologs in the two lizards included in our data set, *Anolis carolinensis* (green anole lizard), and *Gekko japonicus* (Japanese gecko), but not in any snake, turtle, crocodylian, or bird. These results contradict the hypothesis that *CMAH* may have been lost in an ancestor of Sauropsida (Schauer et al. 2009). Instead, our results indicate that the gene was present in the most recent common ancestor of Sauropsida, and that it was lost at least twice during the evolution of the group: in the snake lineage, and in an ancestor of turtles, crocodylians, and birds (fig. 3). The *A. carolinensis* *CMAH* sequence appears to be functional. The *G. japonicus* sequence, however, contains a premature stop codon at the end of coding exon 4. This indicates a recent inactivation of *CMAH* in an ancestor of *G. japonicus* (table 2).

Our results indicate that the Neu5Gc observed in the duck gastrointestinal tract and in budgerigar eggs is not endogenous, strongly supporting the hypothesis that it may have been incorporated from the diet. However, the Neu5Gc observed in the eggs and adult tissues of the green basilisk (a lizard) might be endogenous, as our observations indicate the presence of *CMAH* in lizards.

CMAH in Mammals

The NCBI Genome database contains the genomes of 1 monotreme, 3 marsupials, and 126 placental mammals (table 1). We found putatively functional *CMAH* homologs in the genomes of all 3 marsupials and in 110 of the studied placental genomes, but none in the platypus (a monotreme). Our analyses suggest that at least ten *CMAH* gene loss or inactivation events occurred during mammalian evolution (mapped in fig. 4). Four of these events have been described previously, including one in the human lineage (Chou et al. 1998), one in an ancestor of New World monkeys (Springer et al. 2014), one in an ancestor of pinnipeds and musteloids (Ng et al. 2014), and one in an ancestor of platypus. The other five events are described here for the first time, including one in an ancestor of the sperm whale (*Physeter catodon*), two events in bats, one in an ancestor of the white-tailed deer (*Odocoileus virginianus*), and another in an ancestor of the European hedgehog (*Erinaceus europaeus*) (fig. 4). [Supplementary data set S3, Supplementary Material online](#), is a multiple sequence alignment of all mammalian vertebrates.

Neu5Gc is known to be present in most placental mammals studied so far (Davies et al. 2012) and has been reported in kangaroo (Schauer et al. 2009). In contrast, among monotremes Neu5Gc could not be detected in liver and muscle tissues of the platypus (*Ornithorhynchus anatinus*) (Schauer et al. 2009), nor in the milk of the spiny anteater echidna

(*Tachyglossus aculeatus*) (Kamerling et al. 1982). Schauer et al. (2009), did not find any *CMAH* homolog in the genome of platypus using BLAST searches. However, our analyses revealed a *CMAH* pseudogene in the platypus genome. Using TBLASTN searches we could retrieve eight of the protein-coding exons (the chimpanzee *CMAH* has 15 protein-coding exons). Exon 5 harbors a premature stop codon, which may explain the lack of Neu5Gc in this species (table 2).

Schauer et al. (2009) identified a *CMAH* homolog in the genome of the marsupial *Monodelphis domestica* (gray short-tailed opossum). Consistent with this observation, we found *CMAH* homologous sequences in the genomes of gray short-tailed opossum, tammar wallaby, and Tasmanian devil.

An inactivation of *CMAH* is known to have occurred in a human ancestor, ~2.5–3 Ma (Hayakawa et al. 2001; Chou et al. 2002). An Alu insertion resulted in a deletion of a genomic region encompassing coding exon 3, a 92-bp exon that codes for part of the Rieske catalytic domain. Our genomic analyses also found all the protein coding exons of the human gene except exon 3 (relative to chimp coding sequence).

The presence of inactive *CMAH* sequences, and the absence of Neu5Gc, was also reported in a number of New World monkeys, indicating that *CMAH* pseudogenized in an ancestor of New World monkeys (Springer et al. 2014). In agreement with these findings, we found a deletion spanning coding exons 3–15 in all available New World monkey genomes, including those of *Saimiri boliviensis boliviensis* (Bolivian squirrel monkey), *Cebus capucinus* (white-faced sapajou), *Callithrix jacchus* (marmoset), and *Aotus nancymae* (Nancy Ma's night monkey).

Ng et al. (2014) investigated the ferret genome and performed PCR analyses on another 14 species of musteloids and 2 species of pinnipeds. In all species investigated, they found a large deletion of nine protein-coding exons in the *CMAH* gene, suggesting a pseudogenization event in a common ancestor of musteloids and pinnipeds. In agreement with this hypothesis, we found no putatively functional *CMAH* genes in any of the musteloid and pinniped species investigated: The ferret (*Mustela putorius furo*) genome contains a disrupted *CMAH* gene, and the genomes of the Weddell seal (*Leptonychotes weddellii*) and the walrus (*Odobenus rosmarus divergens*) lack *CMAH* homologs.

We found a putatively functional *CMAH* coding sequence in the dog genome, consistent with prior results from Ng et al. (2014), who showed the presence of conserved *CMAH* coding exons 3, 5, 8, 11, and 12 in dogs. The dog genome assemblies available from the NCBI Genome database correspond to three breeds of European ancestry (boxer, poodle, and beagle). Dogs of Western ancestry seem to lack significant levels Neu5Gc, but Western dog breed cells exhibit low levels of canine *CMAH* transcripts and protein (Löfling et al. 2013), consistent with our observations.

Bats (order Chiroptera) are commonly classified into suborders Yangchiroptera and Yinchiroptera (Teeling et al. 2005; Tsagkogeorga et al. 2013; Lei and Dong 2016). Although one study showed that the milk of island flying fox, *Pteropus hypomelanus* (Pteropodidae, Yinchiroptera) contains Neu5Gc (Senda et al. 2011), no studies have reported the levels of Neu5Gc in other bat species. The NCBI Genome database contains the genomes of seven Yinchiroptera and six Yangchiroptera species. We found *CMAH* sequences in the families Pteropodidae and Megadermatidae of Yinchiroptera and in the family Mormoopidae of Yangchiroptera. In contrast, *CMAH* gene could not be detected in two species belonging to Rhinolophidae and Hipposideridae (Yinchiroptera) and in all five species belonging to Vespertilionidae (Yangchiroptera). Thus, the *CMAH* gene has undergone independent gene losses in both Yinchiroptera and Yangchiroptera (fig. 4).

The NCBI Genome database contains the genomes of 14 members of the taxon Ruminantia (ten bovids, two giraffids, and two cervids). All bovid and giraffid species represented in the database contain a putatively functional *CMAH* gene, consistent with the high levels of Neu5Gc reported in beef (Samraj et al. 2015). Among cervids, *CMAH* was present in the European roe deer (*Capreolus capreolus*), but not in the white-tailed deer (*O. virginianus*), indicating that the gene was lost specifically in the *O. virginianus* lineage. Of note, gangliosides isolated from antlers of sika deer (*Cervus nippon*) have been shown to contain both Neu5Ac and Neu5Gc (Jhon et al. 1999).

Our study involved six cetacean genomes, which exhibited a putatively functional *CMAH* gene, with the exception of sperm whale (*P. catodon*), in which exon 5 (relative to the chimpanzee *CMAH*) was missing. Inspection of the scaffold including exons 4 and 6 did not reveal any unsequenced region, and BLAST searches against the *P. catodon* genomic data revealed no sequences similar to exon 5. These observations indicate that *CMAH* might have pseudogenized in an ancestor of the sperm whale. Terabayashi et al. (1992) analyzed brain gangliosides of ten cetacean species, reporting the presence of low levels of Neu5Gc in only three species: sperm whale, Dall's porpoise, and killer whale. These results are in contrast with the fact that vertebrates generally do not express Neu5Gc in the brain, where it is believed to have adverse effects (Naito-Matsui et al. 2017). In addition, these species are carnivorous, thus raising the possibility that the observed Neu5Gc might have a dietary origin.

Our data set included three genomes of the group Insectivora. Two of these genomes, *Sorex araneus* (common shrew) and *Condylura cristata* (star-nosed mole) contained a putatively functional *CMAH* gene. In contrast the *CMAH* sequence of *E. europaeus* (common hedgehog) contained a premature stop codon in coding exon 12.

Mammalian species in which we found putatively functional *CMAH* genes include a number of species in which

Neu5Gc has previously been described, including pig (Malykh et al. 1998), sheep (Koizumi et al. 1988), cow, horse, elephant, dolphin, chimpanzee, macaque, mouse, rat, and rabbit (Davies et al. 2012).

Evolution of the *CMAH* Gene Structure

The chimpanzee *CMAH* coding sequence (CDS) consists of 15 exons. The Rieske iron–sulphur domain, which contains the active site of the *CMAH* enzyme, is sequenced by exons 2–4 (position 43–345 bp in the chimp CDS). The structure of the gene is generally well conserved across deuterostomes, with the following exceptions. First, echinoderms and hemichordates exhibit an extra intron that interrupts exon 11, at a position that is equivalent to position 1329 of the chimpanzee CDS (see [supplementary data set S3, Supplementary Material](#) online). Second, all studied fish, except *Latimeria chalumnae* (coelacanth) and *Lepisosteus oculatus* (spotted gar), exhibit an extra intron that interrupts exon 6 (after nucleotide 702 of the chimpanzee coding sequence). This suggests an intron insertion in teleost fishes. Third, the *CMAH* gene of *A. carolinensis* lacks the intron between exons 6 and 7 (after nucleotide 768 of the chimpanzee coding sequence).

Potential Implication of Our Findings

We have characterized the phylogenetic distribution of the *CMAH* gene. Among nondeuterostomes, the gene is present in two green algae and in a handful of bacteria and archaea. Within deuterostomes, potentially functional *CMAH* homologs are present in 184 of the 323 genomes studied. Mapping the presence and absences of putatively functional *CMAH* homologs onto the deuterostome phylogeny allowed us to infer a total of 31 independent gene loss or pseudogenization events ([figs. 1–4](#)). Our inferred gene trees ([supplementary figs. S1–S3, Supplementary Material](#) online) do not contradict the species tree ([figs. 1–4](#)), indicating that lateral gene transfer does not account for the observed phylogenetic distribution. A few of these events had already been described, including those in the human (Chou et al. 1998) and platypus (Schauer et al. 2009) lineages, an ancestor of New World monkeys (Springer et al. 2014), and an ancestor of pinnipeds and musteloids (Ng et al. 2014). The other 27 events represent new discoveries. At one point, our results contradict a prior hypothesis. Based on the observations that Neu5Gc is rare in reptiles and birds, and that *CMAH* is absent in birds, Schauer et al. (2009) suggested that *CMAH* may have been lost in an ancestor of Sauropsida. Our analyses, however, identified a putatively functional *CMAH* sequence in the green anole lizard *A. carolinensis*, implying that the most recent common ancestor of Sauropsida had a functional *CMAH* gene, which was then lost both in the snake lineage and in an ancestor of turtles, crocodylians, and birds ([fig. 3](#)). The fact that *CMAH* was lost so many times during the evolution of deuterostomes strongly suggests that the

gene is not essential. However, given the relevance of Neu5Gc (e.g., as part of the ancestral SAMP; Varki 2011), its loss probably needs to be compensated by adjustments in sialic acid biology.

Due to the incompleteness of all available deuterostome genome assemblies (Chain et al. 2009), 41 of the coding sequences of putatively functional *CMAH* genes identified in our study have some unsequenced fraction (typically, one coding exon is unsequenced; [supplementary table S2](#) and data sets S1–S3, [Supplementary Material](#) online). It is possible that some of these unsequenced regions may contain pseudogenization signatures (premature stop codons or frameshift mutations). In addition, our study has not considered the *CMAH* promoter. Therefore, it is possible that some of the *CMAH* homologs classified as “putatively functional” in our study might actually be pseudogenes. Finally, for most species only one genome is available, making it impossible to detect polymorphic variants of the *CMAH* gene.

We expect that animals with a putatively functional *CMAH* gene should be able to synthesize endogenous Neu5Gc, whereas those with pseudogenic or absent *CMAH* homologs should not. Given the toxicity of Neu5Gc for humans, determining what animals lack the capability of synthesizing this sialic acid is key from the point of view of human nutrition and xenotransplantation research. Prior studies have quantified Neu5Gc levels in the tissues of many animals. It should be noted, however, that Neu5Gc can be incorporated from the diet (Tangvoranuntakul et al. 2003), which means that finding Neu5Gc in the tissues of a certain organism does not imply that the organism can synthesize Neu5Gc endogenously. We have produced a list of species that lack a functional *CMAH* gene, and that should be free from Neu5Gc if fed with a Neu5Gc-free diet. These species are thus interesting candidates for human consumption and/or xenotransplantation. Our list includes, for instance, all poultry, 23 species of fish and the white-tailed deer.

Loss of Neu5Gc triggered a cascade of changes in the sialic acid biology of humans, with several evolutionary and biomedical consequences (for review, see Okerblom and Varki 2017). The animals lacking a functional *CMAH* gene identified in this study may have undergone similar changes, making them ideal model organisms for the study of human sialic acid biology and its related diseases. For instance, the altered sialic acid profile of humans makes us susceptible to pathogens using Neu5Ac for binding and recognition of the host, including *P. falciparum* (Martin et al. 2005), *S. pneumoniae* (Hentrich et al. 2016), and Influenza type A virus (Rogers and Paulson 1983). Ferrets, which lack a functional *CMAH* gene (Ng et al. 2014), are used as model organisms to study the transmission mechanisms of human-adapted influenza-A virus strains (Ng et al. 2014). Likewise, New World monkeys, which also lack a functional *CMAH* gene, have been proposed as model organisms for the study of the effects of anti-Neu5Gc antibodies in xenotransplantation

(Salama et al. 2015). In addition, species lacking a functional *CMAH* gene are potential reservoirs for Neu5Ac-binding human pathogens (Chothe et al. 2017). For instance, bats are asymptomatic hosts to viruses like Hantaviruses (Guo et al. 2013), which might lack the antigenic Neu5Gc on their viral envelopes. Our study very significantly expands the list of animals with these characteristics.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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