Mx1 and Mx2 key antiviral proteins are surprisingly lost in toothed whales

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Edited by Francisco J. Ayala, University of California, Irvine, CA, and approved May 14, 2015 (received for review January 28, 2015)

Viral outbreaks in dolphins and other *Delphinoidea* family members warrant investigation into the integrity of the cetacean immune system. The dynamin-like GTPase genes Myxovirus 1 (*Mx1*) and *Mx2* defend mammals against a broad range of viral infections. Loss of Mx1 function in human and mice enhances infectivity by multiple RNA and DNA viruses, including orthomyxoviruses (influenza A), paramyxoviruses (measles), and hepadnaviruses (hepatitis B), whereas loss of Mx2 function leads to decreased resistance to HIV-1 and other viruses. Here we show that both *Mx1* and *Mx2* have been rendered nonfunctional in *Odontoceti* cetaceans (toothed whales, including dolphins and orcas). We discovered multiple exon deletions, frameshift mutations, premature stop codons, and transcriptional evidence of decay in the coding sequence of both *Mx1* and *Mx2* in four species of *Odontocetes*. We trace the likely loss event for both proteins to soon after the divergence of *Odontocetes* and *Mystocetes* (baleen whales) ∼33–37 Mya. Our data raise intriguing questions as to what drove the loss of both *Mx1* and *Mx2* genes in the *Odontoceti* lineage, a double loss seen in none of 56 other mammalian genomes, and suggests a hitherto unappreciated fundamental genetic difference in the way these magnificent mammals respond to viral infections.

gene loss | *Mx* genes | cetaceans | immune system | toothed whales

Dolphins and other whales are subject to attack by a range of viruses similar to those that plague humans and other terrestrial mammals. Viruses known to infect cetaceans include cetacean morbillivirus (CMV), genital papillomaviruses, cetacean poxviruses (causing tattoo skin disease), and alpha- and gammaherpesviruses (1–6). Of these viruses, CMV has probably had the most significant effect on cetacean populations in modern times. Periodic CMV epizootics in bottlenose dolphins (*Tursiops truncatus*) have been documented for decades (7–9), and isolated CMV epizootics have also been reported in other *Delphinidae* family members, including pilot whales (*Globicephala* sp.) (10, 11). Most recently, from 2013 to 2014, the National Marine Fisheries Service (www.nmfs.noaa.gov) reported more than 1,000 bottlenose dolphin strandings along the eastern coast of the United States, with their death attributed to an emergent outbreak of CMV. In humans, Myxovirus genes (*Mx*) defend against a broad range of viruses, including measles, a morbillivirus group member (12, 13). Here we show that modern toothed whales (*Odontocetes*), including dolphins, killer whales, and sperm whales, have inherited defunct copies of both *Mx1* and *Mx2* genes, and propose that these gene losses may profoundly alter the way toothed whales respond to antiviral activity.

Myxovirus genes predate vertebrate origin, with most mammals harboring two *Mx* gene copies (14). Mx proteins are structurally similar to GTPase dynamins, which are involved in the endocytotic mechanics of all eukaryotes, and possess an N-terminal GTPase domain (G domain), a bundle signaling element, and a C-terminal stalk domain (Fig. 1). Mutations in the G domain (T103A, deletion 81–84, K83A, K83M) decrease GTPase activity and increase infectivity by orthomyxoviruses (specifically, thogoto virus and influenza A virus) (15, 16). A recent structural study of the Mx1 protein proposing a model for dynamin assembly identified specific residues within the stalk domain (I376, R408, M527, F602, K614, L617, L620) that are required for oligomerization and resultant antiviral activity (17). A strong signature of recurrent positive selection on the hypervariable L4 loop region containing amino acids 533–572 within the stalk domain (Fig. 1) has revealed that *Mx1* is engaged in an evolutionary arms race with rapidly evolving viral proteins (18, 19). Naturally occurring single amino acid changes in the Mx1 L4 loop of primates has led to differential infectivity by orthomyxoviruses (18). In addition, deleting residues 532–572 containing the L4 loop of human Mx1 was shown to impair colocalization with the viral proteins of La Crosse Virus in infected cells (17, 20), and a polymorphism V379I in human Mx1 has been recently suggested to predispose infants to severe respiratory syncytial viral disease (21). Wild-type mice with full-length Mx1 are resistant to influenza virus infection, whereas inbred strains harboring a large deletion (exons 8–10) or a premature stop codon (K389*) are highly susceptible to influenza infection (22). (Fig. 2*A* summarizes the known *Mx1* loss-of-function mutations). Finally, Mx1 has been shown to inhibit Hepatitis B virus replication (23). Although its structural mechanisms are less well understood, Mx2 is known to possess potent antiviral activity against HIV-1 (24–26) and other viruses (27, 28). Taken together, these data demonstrate that loss-of-function *Mx* mutations decrease antiviral activity and enable viral infection and replication in host mammalian cells.

Results

To determine the state of the *Mx* genes in dolphins and other whales, we compared the genomic regions containing *Mx1* and

Significance

Everybody loves dolphins. And orcas. And *Mx* (Myxovirus) genes. *Mx* genes are important immune genes that help mammals fight many RNA and DNA viruses, including HIV, measles, and flu. We make a surprising discovery: dolphins, orcas, and likely all toothed whales lost both *Mx* genes soon after they diverged from baleen whales and ungulates, which preserve these important genes intact. Because both genes were likely lost simultaneously, we speculate that a viral outbreak exploiting the *Mx* genes may have forced the toothed whale's ancestor to sacrifice both. Because the *Mx* genes are so important, and because all 56 nontoothed whale sequenced mammals carry *Mx* genes, our discovery makes an important contribution to help preserve these magnificent mammals.

Author contributions: B.A.B., A.M., J.G.C., R.J., and G.B. designed research; B.A.B. performed research; B.A.B., A.M., J.G.C., R.J., and G.B. analyzed data; and B.A.B., A.M., J.G.C., R.J., and G.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1501844112/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1501844112

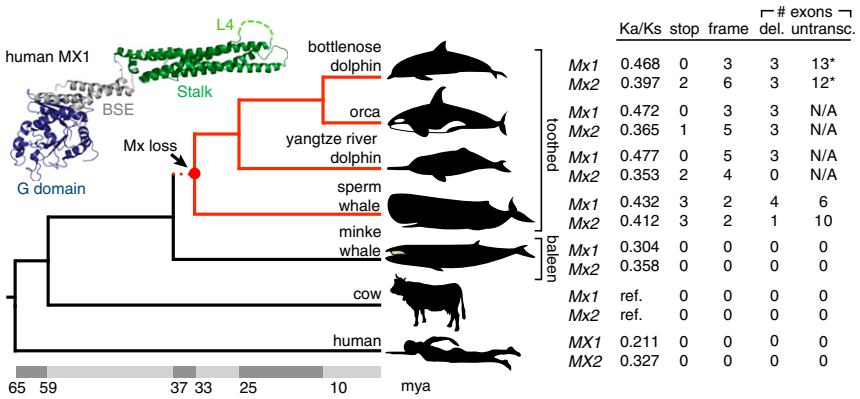

Fig. 1. Mx1 and Mx2 proteins were lost in toothed whales. (*Top Left*) The protein structure of human Mx1 is shown (PDB ID code 3SZR). The dotted line shows the predicted location of the L4 loop (17). BSE, bundle signaling element. (*Left*) Cladogram showing phylogentic relationship and approximate divergence times of five cetaceans relative to cow and human outgroups (29) (mya, millions of years ago). The proposed loss event of Mx1 and Mx2 is designated by a black arrow and a red dashed line. Solid red lines represent lineages with Mx1 and Mx2 loss. (*Right*) Columns: K_a/K_s ratio for *Mx1* and *Mx2* of each species relative to cow, the number of premature stop codons, frameshift mutations, deleted exons, and untranscribed exons of the *Mx* genes coding regions in all species. Asterisks indicate the transcription data are from a closely related Indo-Pacific humpback dolphin. N/A, not available. Note that the minke whale *Mx1* and *Mx2* show no signs of erosion.

Mx2 in cow (*Bos taurus*) and human to the full genomes of five cetaceans: minke whale (*Balaenoptera acutorostrata*), sperm whale (*Physeter macrocephalus*), Yangtze River dolphin (*Lipotes vexillifer*), bottlenose dolphin (*T. truncatus*), and killer whale (*Orcinus orca*). We identified exactly one alignment from either cow or human *Mx1* and *Mx2* to each of these species, suggesting that there have been no *Mx* duplication events in the cetacean lineage. We next found clear signs of gene erosion, such as multiple exon deletions, frameshift mutations, and premature stop codons in the coding sequences of both *Mx1* and *Mx2* genes in sperm whale, Yangtze River dolphin, killer whale, and bottlenose dolphin, but found none in the coding sequence of either gene in minke whale, cow, or human (Figs. 1 and 2). For example, *Mx1* loss was evidenced in bottlenose dolphin by: (*i*) a frameshifting deletion of exons 10–12 in the stalk domain, including the L4 loop; (*ii*) two additional frameshift mutations in exons 3 and 5 of the GTPase

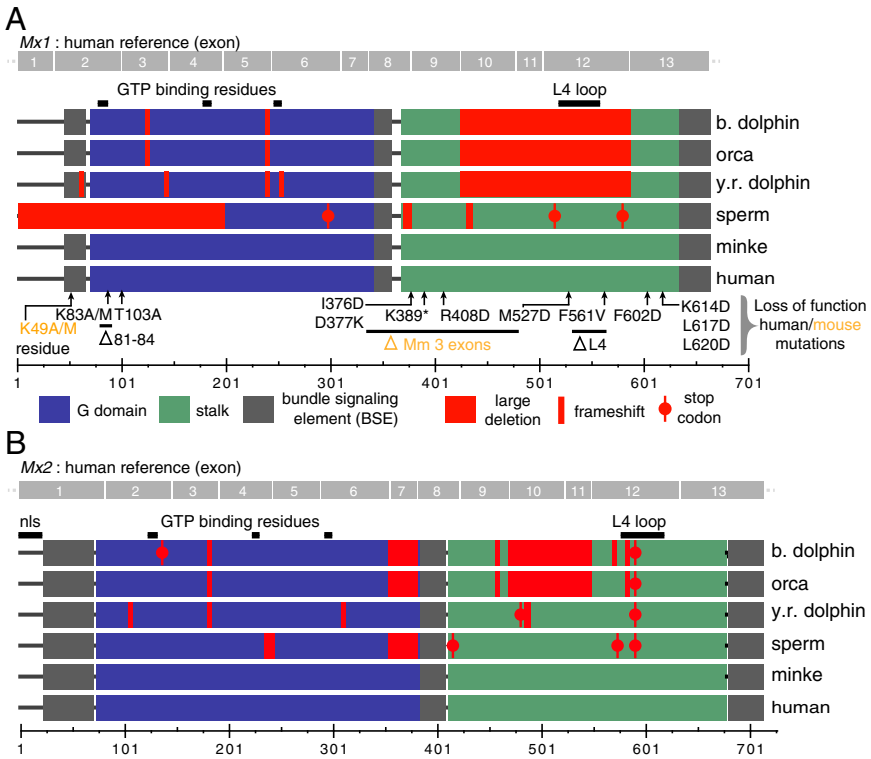

Fig. 2. *Mx1* and *Mx2* gene mutations in toothed whales. (*A*) Scaled schematic showing the mutations in *Mx1* for each species, referenced against human *Mx1*. The *x* axis denotes amino acid position. *Mx1* regions are annotated and color-coded to highlight Mx1 functional domains (colors match *Top Left*). Large deletions, frameshift mutations, and stop codons are shown in red. Mutations in human and mouse *Mx1* that result in loss of function are indicated (see text). (*B*) The same scaled schematic for *Mx2*.

domain; and (*iii*) a K_a/K_s (nonsynonymous to synonymous substitution rate) using cow as a reference over twice as high as the K_a/K_s from cow to human. *Mx2* loss was evidenced in bottlenose dolphin by: (*i*) two frameshifting deletions spanning exons 7 and 10–11; (*ii*) three additional frameshift mutations in exons 3, 9, and 12; and (*iii*) two premature stop codons, one in the L4 loop at residue 593 and another in exon 2 at residue 139, either of which would trigger nonsense-mediated decay. Similarly, sperm whale, Yangtze River dolphin, and killer whale all had at least four inactivating mutations in each *Mx* gene (Figs. 1 and 2). We can safely rule out any sequencing artifacts because all cetacean assemblies used were sequenced to at least 30× depth of coverage, multiple loss-of-function mutations are shared across species at base pair resolution, and multiple inactivating mutations are found in each species' gene, each supported by multiple reads (Fig. S1 and Table S1). To rule out alignment errors, we found individual sequencing reads that spanned each of the large deletions for each species. These reads confirmed the precise edge of each deletion by mapping to intronic DNA flanking the lost exon-spanning region (Fig. 3*A* and Fig. S1). Verification of frameshifts and premature stop codons was done by inspecting a multiple alignment of each exon (Fig. 3*B*). Our comparative genomic analyses thus suggest that both *Mx1* and *Mx2* are eroding in all toothed whales (*Odontocetes*), whereas the *Mx* genes of baleen whales (*Mysticetes*) are intact.

To examine whether any transcripts were emanating from the *Mx* gene loci in cetaceans, we obtained RNA-seq data from toothed whales (sperm whale and Indo-Pacific humpback dolphin, *Sousa chinensis*), a baleen whale (minke whale), and closely related ungulates (cow and sheep). Each RNA-seq set was mapped to its respective genome, with the exception of mapping the Indo-Pacific humpback dolphin to the genome of its close relative the bottlenose dolphin (*Methods*). From this mapping, we observed complete coverage of the *Mx1* and *Mx2* coding sequences by RNA-seq traces in the ungulates and baleen whale, and low and very partial coverage of both genes in the toothed whales (parts of *Mx1* exons 5–10 and 13, and parts of *Mx2* exons 1–3 in sperm whale, and only a part of *Mx2* exon 1 in Indo-Pacific humpback dolphin) (Fig. 1 and Fig. S2). Because the sperm whale and bottlenose dolphin genome themselves are missing some *Mx1* and *Mx2* exons, we also constructed full *Mx1* and *Mx2* gene models by "baiting" the missing toothed whale exons with minke whale sequence. In mapping the toothed whales' RNA-seq traces to these gene models (*Methods*), not a single trace mapped to the bait exons, further validating that the deletions we identified in toothed whales are real. These data suggest that full-length *Mx1* and *Mx2* genes are transcribed in baleen whales, but that neither Mx1 nor Mx2 proteins are made in toothed whales.

We next sought to date the most likely evolutionary time frame for *Mx1* and *Mx2* inactivation. Our comparative genomic and transcriptomic analyses suggest that both *Mx1* and *Mx2* are retained functional in minke whale, but lost their function in toothed whales (*Odontocetes*) after the divergence from baleen whales (*Mystocetes*) (Fig. 1). We identified candidates for inactivating mutations in both *Mx* genes that could have originated in the most common ancestor of toothed whales. For *Mx1*, a large deletion spanning exons 10–12 is shared between bottlenose dolphin, orca, and Yangtze River dolphin, yet this region is still present in sperm whale. However, the sperm whale genome contains a frameshift mutation in exon 10 and two premature stop codons in exon 12, which according to experimental data from mice and human would likely result in *Mx1* loss-of-function (Fig. 2*A* and Table S1) (17, 22). Any of these three events are thus candidates for ancestral mutations leading *Mx1* to be lost in *Odontocetes*. In the L4 loop of *Mx2*, we observed a conserved premature stop codon in the four species of *Odontocetes* that would lead to nonsense-mediated decay of this gene (Figs. 2*B* and 3*B*). By parsimony, these observations place the estimated loss-of-function of both *Mx1* and *Mx2* genes in the common ancestor of toothed whales, after the split from baleen whales ~33–37 Mya (29).

Discussion

The loss of both *Mx* genes in Odontocetes is highly surprising, as we found an intact *Mx* ortholog in every 1 of 56 other mammalian genomes (*Methods*), and as the *Mx* genes increase fitness through antiviral activity in mice and humans, and also in cows (30, 31) and pigs (32), more closely related to whales. It is compelling to consider what events drove *Mx* loss in this clade of highly social marine mammals. Although other forces and scenarios cannot be ruled out (33), an intriguing hypothesis is that the loss enabled the survival of Odontoceti ancestors from a virus that took advantage of Mx function, possibly through direct interaction with the Mx proteins [e.g., human individuals and cells that inactivate the *CCR5* gene are resistant to HIV-1 infection (34)], or through mimicry of the Mx proteins (35). Because herpes simplex virus 1 (HSV-1) has been shown to enhance infection by inducing an alternative splice variant of *Mx1* omitting exons 10–12, and because alphaherpesviruses closely related to HSV-1 still infect dolphins today, an ancestor of HSV-1 is one possible candidate pathogen for this theory (2, 3, 36). It has also been shown that the IFN response, including induction of the *Mx* genes, contributes to the persistency of some measles virus infections (37). Ancient loss of Mx proteins may have (therefore) been beneficial to the Odontoceti concestor. Given their wide range of antiviral activities in closely related mammals, and because a similar double loss is not observed in any of 56 other mammalian whole genomes, it is tempting to speculate that mutations elsewhere in toothed whale genomes have arisen to compensate for this dramatic loss. Future functional experiments to understand how the loss of Mx1 and Mx2 function impact cellular antiviral defense strategies in dolphins, orcas, and other

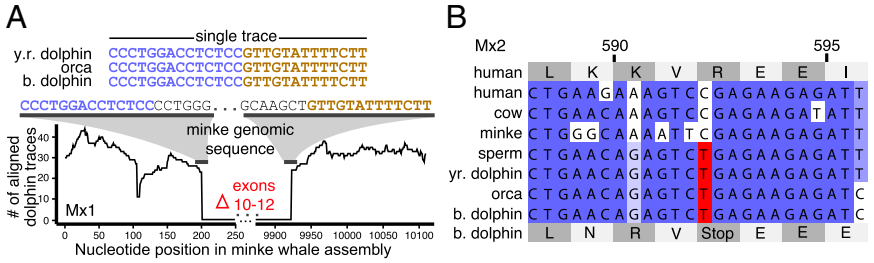

Fig. 3. Multiple read and multiple species support for observed mutations. (*A*) Validation of the *Mx1* large deletion spanning exons 10–12 and covering the L4 loop. Single sequencing traces for bottlenose dolphin and orca, and a genomic scaffold for Yangtze River dolphin map precisely to intronic DNA flanking minke whale exons 10–12, with intervening DNA deleted. (*B*) Multiple alignment of five cetaceans, cow, and human Mx2 showing a conserved stop codon (red T) within the L4 loop of sperm whale, Yangtze River dolphin, bottlenose dolphin, and orca. Amino acid sequences for human and bottlenose dolphin are shown.

toothed whales, especially compared with their baleen next of kin, will likely aid in these magnificent species' preservation (38, 39).

Methods

Genomic Analysis. We obtained the following genome assemblies: human assembly GRCh37 (40), cow (*B. taurus*) assembly Btau_4.6.1 (41), sheep (*Ovis aries*) genome assembly Oar_v3.143 (42), minke whale (*B. acutorostrata*) assembly BalAcu1.0 (43), sperm whale assembly Physeter_macrocephalus-2.0.2 (a gift of Wesley Warren, Genome Institute at Washington University School of Medicine in St. Louis, St. Louis), Yangtze River dolphin assembly Lipotes_vexillifer_v1 (44), killer whale assembly Oorc_1.1 (Baylor College of Medicine, Houston), and bottlenose dolphin (*T. truncatus*) assembly Ttru_1.4 (45). The genomic locations of the *Mx1* and *Mx2* coding sequences in cow and human were obtained from Ensembl (www.ensembl.org/index.html). Pairwise chained BLASTZ alignments from human and cow to the remaining species were obtained using doBlastZChainNet.pl from the University of California, Santa Cruz (UCSC) Genome Browser executables package (46, 47), with a minimum chain score of 20,000 and the linear gap penalty set to "medium." The output alignments indicated a unique map from the genomic region containing *Mx1* and *Mx2* in both human and cow to each of these five cetacean assemblies. We estimated the K_a/K_s ratio of both genes based on these alignments using the KaKs_Calculator software with default settings for the MLWL method (48). Raw sequencing read data were obtained for sperm whale, killer whale, and bottlenose dolphin from the National Center for Biotechnology (NCBI) Sequence Read Archive (SRA) (www.ncbi.nlm.nih.gov/sra). Reads from the cetacean species were aligned to the minke whale assembly using the NCBI Blast website (blast.ncbi.nlm.nih.gov/Blast.cgi) with the blastn algorithm, with 20,000 target sequences, the "Low complexity regions" filter disabled, and an alignment score threshold of 80. Deletions were validated in sperm whale, killer whale, and bottlenose dolphin by finding at least three sequencing reads that aligned with at least 15 bases on either side of the deleted region. We converted the pairwise alignments for each exon to a multiple alignment using Muscle v3.8.31 with a strict gap opening penalty of −800 (49). Frameshift mutations and stop codons were validated by manually inspecting multiple alignments of each exon across human, cow, and the five cetaceans. Furthermore, frameshift mutations and stop codons occurring only in sperm whale, killer whale, or bottlenose dolphin were validated by finding at least three raw sequencing reads containing the mutation.

We used pairwise chained alignments provided by the UCSC Genome browser (genome.ucsc.edu) to map the genomic region containing *Mx1* and *Mx2* in human to 54 noncetacean mammalian genomes. In 53 species, an *Mx* gene ortholog was found with 11–13 coding exons intact, free of any detectable premature stop codons or frameshifts. In a single species we had to resort to NCBI blast (blast.ncbi.nlm.nih.gov/Blast.cgi) on the human *Mx1* coding sequence and a newer tarsier assembly Tarsius_syrichta-2.0.1, to find a suitable alignment, as the older assembly currently in UCSC, Tarsyr1.0 (45) is lower quality in this region.

Transcriptomic Analysis. RNA-seq datasets were obtained from the SRA for: cow (leukocytes, SRR924543, 3.2Gb, Illumina Genome Analyzer IIx), Indo-Pacific humpback dolphin (leukocytes, ERX282316, 4.3Gb, Illumina HiSEq. 2000), sheep (skin, SRP018731, 20.2Gb, Illumina HiSEq. 2000), sperm whale (skin, SRP016870, 33.8Gb, Illumina HiSEq. 2000), minke whale (heart tissue, SRR918701, 8.6Gb, Illumina HiSEq. 2000) Gb, 10^9 bases. RNA-seq traces were mapped to corresponding references genomes using STAR (50) with the default settings, except for the maximum intron size and the maximum distance between aligned mates both set to 30,000 bases. Indo-Pacific humpback dolphin reads were mapped to the bottlenose dolphin reference genome (Ttru_1.4). Coverage was computed by first finding RNA-seq traces with sequence similarity to the corresponding genomic coding sequences using Blast, then aligning these traces to the genome of the respective species using STAR, and then finally counting the number of traces that map to each base of the genomic coding sequence. Toothed whale RNA-seq traces were also aligned to baited coding sequences (see text) using the NCBI Blast website "search SRA by experiment" mode with the blastn algorithm parameterized as above. For each trace, we accepted only the mapping having the highest alignment score considering all alignments to either of the baited *Mx1* or *Mx2* coding sequences.

ACKNOWLEDGMENTS. We thank Wesley Warren and the Genome Institute at Washington University School of Medicine for the sperm whale genome assembly, the Baylor College of Medicine for the killer whale genome assembly, and the scientific community at large for large-scale sequence data availability. This work was supported by a Stanford Center for Computational, Evolutionary, and Human Genomics postdoctoral fellowship (to A.M.); a Stanford School of Medicine postdoctoral Dean's fellowship (to A.M.); a PhRMA Foundation Informatics postdoctoral fellowship (to J.G.C.); the Packard Foundation (G.B.); a Microsoft Faculty fellowship (to G.B.); and National Institutes of Health Grant U01MH105949 (to G.B.).

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