

RESEARCH ARTICLE SUMMARY

AVIAN GENOMICS

Evidence for a single loss of mineralized teeth in the common avian ancestor

Robert W. Meredith,* Guojie Zhang, M. Thomas P. Gilbert, Erich D. Jarvis, Mark S. Springer*

INTRODUCTION: The absence of teeth or edentulism has evolved on multiple occasions within vertebrates, including birds, turtles, and a few groups of mammals (anteaters, baleen whales, and pangolins). There are also mammals with enamelless teeth (aardvarks, sloths, and armadillos). All toothless/enamelless vertebrates are descended from ancestors with enamel-capped teeth. In the case of birds, it is the roperod dinosaurs. Instead of teeth, modern birds use a horny beak (rhamphotheca) and part of their digestive tract (muscular gizzard) to grind up and process food. The fossil record of early birds is fragmentary, and it is unclear whether tooth loss evolved in the common ancestor of all modern birds or convergently in two or more independent lineages.

RATIONALE: Tooth formation in vertebrates is a complicated process that involves many different genes. Of these genes, six are essential for the proper formation of dentin (*DSPP*) and enamel (*AMTN*, *AMBN*, *ENAM*,

AMELX, and *MMP20*). We examined these six genes in the genomes of 48 bird species, which represent nearly all living bird orders, as well as the American alligator, a representative of Crocodylia (the closest living relatives of birds), for the presence of inactivating mutations that are shared by all 48 birds. The presence of such shared mutations in dentin and enamel-related genes would suggest a single loss of mineralized teeth in the common ancestor of all living birds. We also queried the genomes of additional toothless/enamelless vertebrates, including three turtles and four mammals, for inactivating mutations in these genes. For comparison, we looked at the genomes of mammalian taxa with enamel-capped teeth.

RESULTS: All edentulous vertebrate genomes that were examined are characterized by inactivating mutations in *DSPP*, *AMBN*, *AMELX*, *AMTN*, *ENAM*, and *MMP20*, rendering these genes nonfunctional. The dentin-related gene *DSPP* is

functional in vertebrates with enamelless teeth (sloth, aardvark, and armadillo). All six genes are functional in the American alligator and mammalian taxa with enamel-capped teeth. More important, 48 bird species share inactivating mutations in both dentin-related (*DSPP*) and enamel-related

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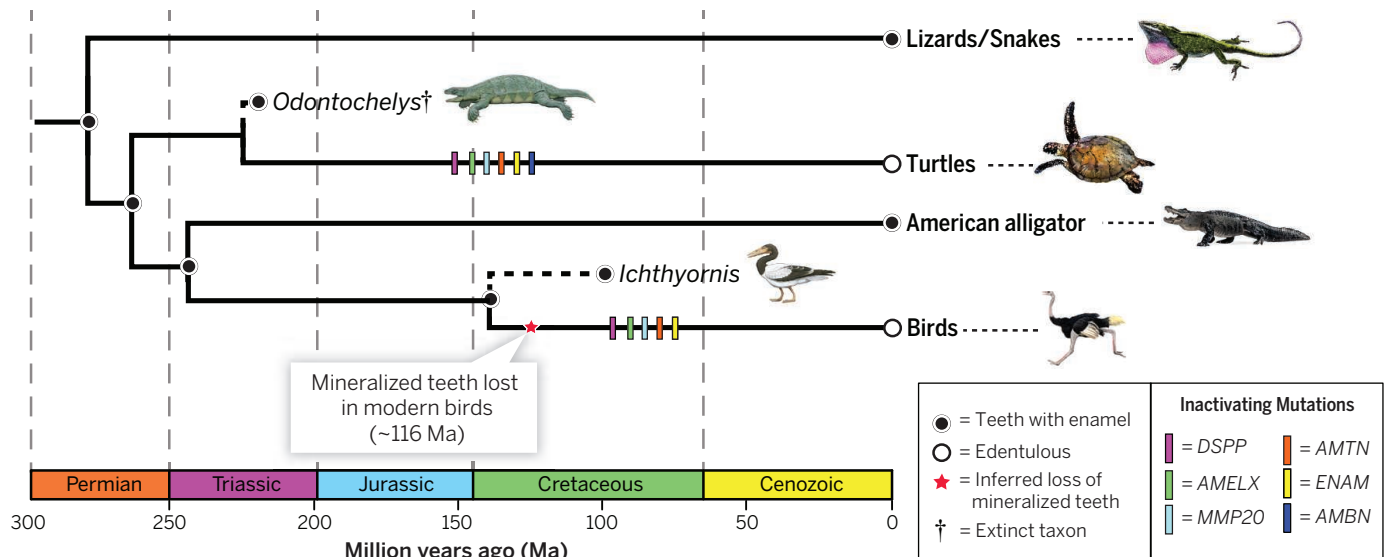
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genes (*ENAM*, *AMELX*, *AMTN*, and *MMP20*), indicating that the genetic machinery necessary for tooth formation was lost in the common ancestor of all modern

birds. Furthermore, the frameshift mutation rate in birds suggests that the outer enamel covering of teeth was lost about 116 million years ago.

CONCLUSIONS: We postulate, on the basis of fossil and molecular evidence, a two-step scenario whereby tooth loss and beak development evolved together in the common ancestor of all modern birds. In the first stage, tooth loss and partial beak development commenced on the anterior portion of both the upper and lower jaws. The second stage involved concurrent progression of tooth loss and beak development from the anterior portion of both jaws to the back of the rostrum. We propose that this progression ultimately resulted in a complete horny beak that effectively replaced the teeth and may have contributed to the diversification of living birds. ■

The list of author affiliations is available in the full article online.
*Corresponding author. E-mail: meredithr@mail.montclair.edu (R.W.M.); mark.springer@ucr.edu (M.S.S.)
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Observed shared inactivating mutations in tooth formation. Related genes were mapped onto a time tree depicting evolutionary relationships and times of divergence between modern birds, the closely related extinct taxon *Ichthyornis*, and the American alligator. The hypothesized loss of mineralized teeth on the modern bird branch at 116 million years ago (Ma) is based on frameshift mutation rates.

Evidence for a single loss of mineralized teeth in the common avian ancestor

Robert W. Meredith,^{1*} Guojie Zhang,^{2,3} M. Thomas P. Gilbert,^{4,5} Erich D. Jarvis,⁶ Mark S. Springer^{7*}

Edentulism, the absence of teeth, has evolved convergently among vertebrates, including birds, turtles, and several lineages of mammals. Instead of teeth, modern birds (Neornithes) use a horny beak (rhamphotheca) and a muscular gizzard to acquire and process food. We performed comparative genomic analyses representing lineages of nearly all extant bird orders and recovered shared, inactivating mutations within genes expressed in both the enamel and dentin of teeth of other vertebrate species, indicating that the common ancestor of modern birds lacked mineralized teeth. We estimate that tooth loss, or at least the loss of enamel caps that provide the outer layer of mineralized teeth, occurred about 116 million years ago.

With ~10,000 species, birds are the most species-rich clade of edentulous (toothless) jawed vertebrates (1). All edentulous or enamelless amniote lineages are derived from ancestors that possessed enamel-capped teeth (Fig. 1). Edentulism also occurs in turtles and mammalian lineages (anteaters, baleen whales, and pangolins) (2, 3), with some mammals lacking tooth enamel (aardvarks and sloths) (4), but these clades are less diverse than birds and together comprise only ~385 species. Modern birds share a common ancestry with toothed, maniraptoran theropod dinosaurs (5). Instead of teeth, modern birds use two highly specialized structures, a rhamphotheca (horny beak) for food acquisition and, as part of the digestive tract, a muscular gizzard for food processing (5). These structures are proposed to have played a role in enabling birds to adapt to a multitude of previously unavailable ecospace and diversify relative to other edentulous vertebrates (5).

Throughout their evolutionary history (~170 million years ago (Ma)), independent tooth reduction and loss has occurred in multiple lineages of Avialae (living birds and all fossil theropods that are more closely related to Neornithes than to the dinosaurs *Dromaeosaurus albertensis* or *Troodon formosus*) (6). Minimally, there are six lineages of Avialae that show evidence of tooth

reduction, with four lineages exhibiting complete tooth loss. Neornithes are hypothesized to have become edentulous somewhere between 125 and 65.5 Ma (5, 7). Among avialae lineages exhibiting tooth reduction or loss, a rhamphotheca has also independently evolved (5, 7). The multiple independent iterations of tooth reduction and loss in Avialae suggest that ancestral odontogenetic pathways may have been predisposed to tooth reduction and loss in this clade (5).

Most phylogenetic analyses suggest that teeth were lost in the common ancestor of modern birds (8, 9), although dentate taxa from the Mesozoic (*Hesperornis* and *Ichthyornis*) have been recovered inside of crown Neornithes in some cladistic studies, implying that tooth loss could have instead occurred convergently among different members of this clade (10). The oldest fossils of putative neornithines are from the Cretaceous, but these fossils are fragmentary, lack complete jaw elements, and fail to resolve whether edentulism evolved in the common ancestor of all modern birds or convergently in at least two subclades (5).

Parallel tooth and enamel loss

The inactivation of enamel-related genes parallels the loss of enamel in the mammalian fossil record (4, 11–13). The presence of shared, inactivating mutations in dentin- and enamel-related genes could therefore be used as a molecular proxy to assess whether teeth were lost once or on multiple occasions in the common ancestor of living birds, as well as to estimate the timing of tooth loss. We queried the genomes of 48 living bird species that span the phylogeny of modern Aves (14), including representatives of the two major clades of birds (Palaeognathae and Neognathae) and nearly all extant bird orders, as well as a representative of Crocodylia [*Alligator mississippiensis* (American alligator)], which is the extant sister group of birds, by performing a comparative analysis of six genes that have dentin (*DSPP*) or enamel-related (*AMTN*, *AMBN*,

ENAM, *AMELX*, and *MMP20*) patterns of gene expression. Of the hundreds of genes associated with tooth development, most are pleiotropic and perform essential functions outside of tooth development (15). By contrast, the aforementioned six genes have been hypothesized to be tooth-specific based on mutagenesis studies in mice, inactivation of these genes in one or more edentulous/enamelless vertebrate species, and natural genetic variation in humans that causes nonsyndromic amelogenesis imperfecta, dentinogenesis imperfecta, and dentin dysplasia (11–13, 16–20) (table S1). At the same time, other evidence indicates that these six genes are pleiotropic and are expressed outside of tooth development (21–23) (table S1). In addition to querying bird genomes, we also queried the genomes of additional edentulous/enamelless vertebrates, including three cryptodiran turtles [*Chrysemys picta bellii* (Western painted turtle), *Chelonia mydas* (green sea turtle), and *Pelodiscus sinensis* (Chinese soft-shelled turtle)] and four mammals [*Dasyurus novemcinctus* (nine-banded armadillo), *Choloepus hoffmanni* (Hoffmann's two-toed sloth), *Oryzteropus afer* (aardvark), and *Manis pentadactyla* (Chinese pangolin)] for signatures of molecular decay in these genes. As a control, we queried the genomes of additional mammalian taxa with enamel-capped teeth [*Canis lupus familiaris* (domestic dog), *Loxodonta africana* (African bush elephant), and *Physeter macrocephalus* (giant sperm whale)].

AMTN, *AMBN*, *ENAM*, *AMELX*, *MMP20*, and *DSPP* were recovered from the genomes of living birds, albeit with numerous inactivating mutations that render these genes nonfunctional (figs. S1 to S8 and tables S2 and S5 to S10). However, the synteny of these six tooth-related genes is strongly conserved across species. The only exceptions occur in *Meleagris gallopavo* (turkey), where *ENAM* exon 9 is found on both chromosome 1 and Z (13), and in the *Pterocles gutturalis* (sandgrouse), where there appear to be multiple copies of exon 5 (four copies), exon 6 (three copies), and exon 10 (two copies) of *MMP20* scattered across five contigs (fig. S7). Similar to other vertebrate genomes, including human and crocodile, we found that *AMBN* is located between *AMTN* and *ENAM* in the secretory calcium-binding phosphoprotein (*SCPP*) gene cluster (24) (Fig. 2), *AMELX* is found within the *ARHGAP6* gene (Fig. 2), *DSPP* is located within the *SIBLING* gene cluster between *SPARCL1* and *DMPI* (24) (Fig. 2), and *MMP20* is clustered with other matrix metalloproteinase proteins between *MMP27* and *MMP7* (25) (Fig. 2 and fig. S8). These synteny results provide unequivocal evidence that the avian pseudogenes for *AMBN*, *AMELX*, *AMTN*, *DSPP*, *ENAM*, and *MMP20* are strictly orthologous with functional copies of these genes in vertebrates with enamel-capped teeth.

Loss of dentin and enamel genes

The remnants of *DSPP*, *AMBN*, *AMELX*, *AMTN*, *ENAM*, and *MMP20* genes in all bird genomes are characterized by exonic deletions and/or other inactivating mutations, including exon deletions, frameshift mutations, stop codons, and

¹Department of Biology and Molecular Biology, Montclair State University, Montclair, NJ 07043, USA. ²China National GeneBank, Beijing Genomics Institute-Shenzhen, Shenzhen, 518083, China. ³Centre for Social Evolution, Department of Biology, Universitetsparken 15, University of Copenhagen, DK-2100 Copenhagen, Denmark. ⁴Center for GeoGenetics, Natural History Museum of Denmark, University of Copenhagen Øster Voldgade 5-7, 1350 Copenhagen, Denmark. ⁵Trace and Environmental DNA Laboratory, Department of Environment and Agriculture, Curtin University, Perth, Western Australia 6102, Australia. ⁶Department of Neurobiology, Howard Hughes Medical Institute and Duke University Medical Center, Durham, NC 27710, USA. ⁷Department of Biology, University of California, Riverside, CA 92521, USA.

*Corresponding author. E-mail: meredith@mail.montclair.edu (R.W.M.); mark.springer@ucr.edu (M.S.S.)

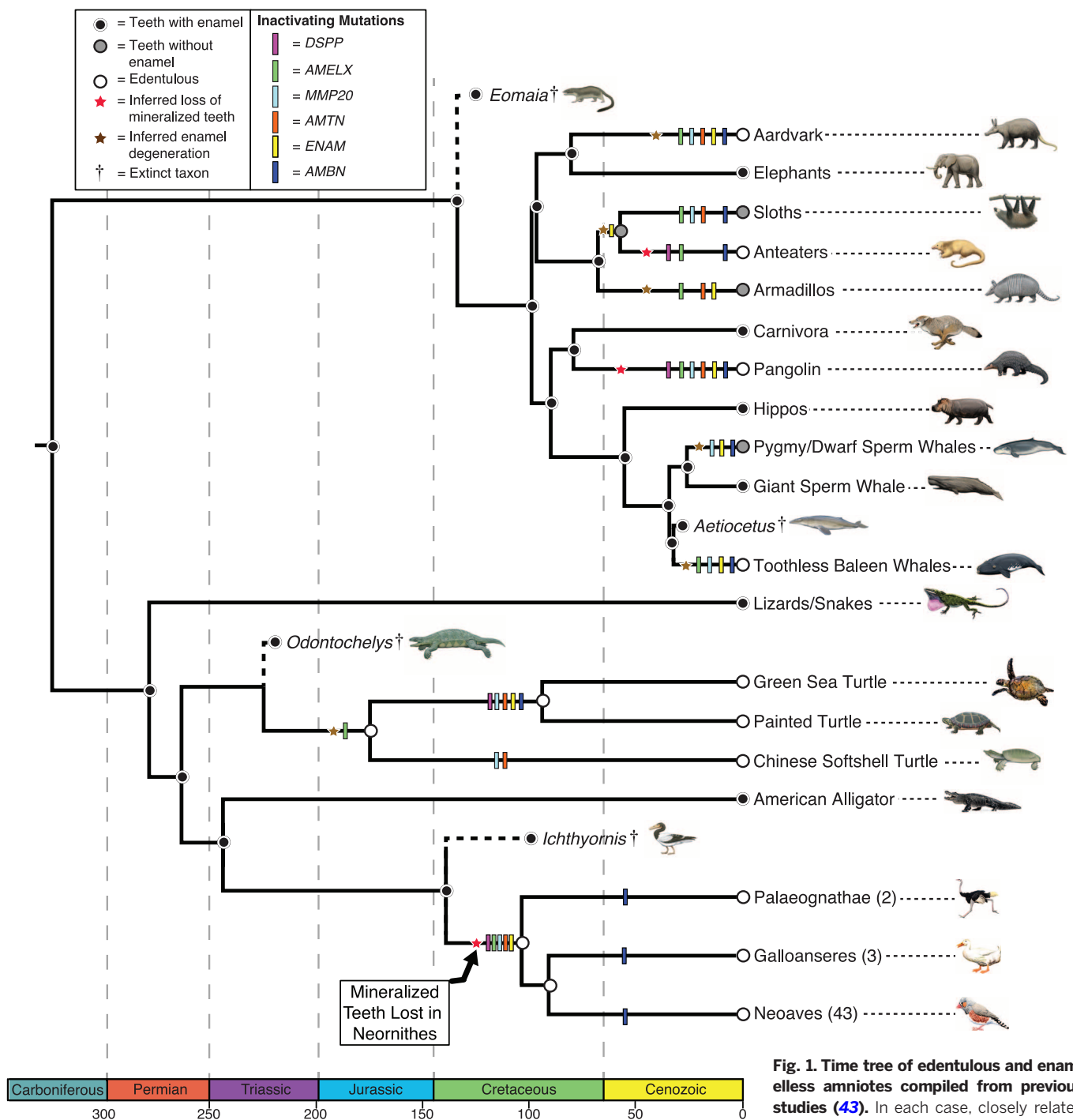


Fig. 1. Time tree of edentulous and enamelless amniotes compiled from previous studies (43). In each case, closely related extinct (*Odontochelys*, *Ichthyornis*, *Eomaia*, and *Aetiocetus*) and extant dentate taxa are also shown. Estimates for the timing of enamel loss in mammals are from fossils, phylogenetics, molecular clocks, frameshift mutations, and/or ratio of nonsynonymous to synonymous substitutions (dN/dS) (4, 12). Convergent enamel loss within Xenarthra is inferred for *Pilosa* (sloths and anteaters) and multiple armadillo lineages (4). Information on the enam- and dentin-related genes for pygmy/dwarf sperm whales, anteaters, and baleen whales are from previous studies (4, 11, 12, 16, 44). Hypothesized loss of mineralized teeth on the stem neornithine branch is assumed on the basis of indel substitution rates (27, 43). Numbers in brackets indicate the number of bird genomes analyzed for that particular group. Ghost branches are arbitrarily shown as representing ~5 million years, except for *Ichthyornis*, where the ghost branch was extended to accommodate the depiction of inactivating mutations on the stem Neornithes branch.

splice-site mutations (figs. S1 to S8; tables S2 and S5 to S10; and nexus files 21 to 26). Importantly, all 48 neornithine species share inactivating mutations in both dentin-related (*DSPP*) and enamel-related genes (*ENAM*, *AMELX*, *AMTN*, and *MMP20*) (Fig. 1, Table 1, and tables S5 and S7 to S10). This pattern of shared inactivating mutations indicates that the genetic machinery for tooth formation, including both dentin and enamel, was lost on the branch leading to all extant birds. Additional frameshift mutations in these genes have continued to accumulate in crown lineages and are diagnostic of Palaeognathae, Neognathae, and more nested lineages within these clades (Table 1). The remnants of

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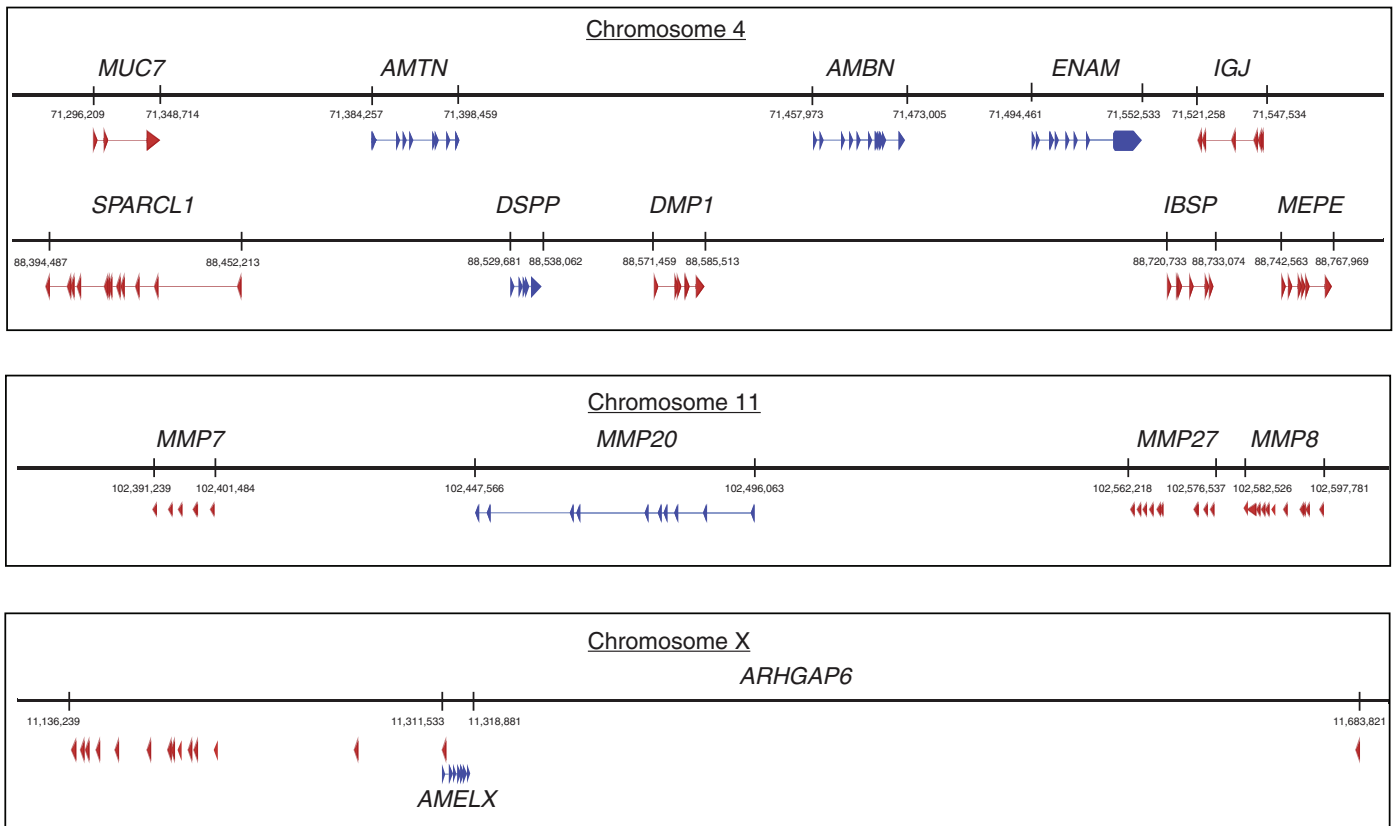


Fig. 2. Schematic representation of enamel- and dentin-related genes (blue) and flanking genes (red) from their positions in *Homo* (Ensembl 67), one of the most accurately annotated vertebrate genomes. Exons are indicated by arrows. Details of likely inactivating mutations identified in all enamelless and edentulous species are provided in tables S4 to S21.

Table 1. Total number of shared inactivating mutations in enamel- and dentin-related genes within birds.

	<i>ENAM</i>	<i>AMBN</i>	<i>AMTN</i>	<i>AMELX</i>	<i>MMP20</i>	<i>DSPP</i>
Neornithes	4	0	1	1	1	4
Palaeognathae	13	3	1	0	2	30
Neognathae	2	0	2	1	2	0
Galloanserae	1	1	1	2	0	1
Neoaves	4	0	2	0	3	4

these genes in extant birds are in advanced states of molecular decay, and we cataloged more than 1300 independent exon deletions and frameshift mutations in these six genes in crown Aves (tables S2 and S5 to S10).

Most of the *DSPP* gene has been lost from the genomes of neognath taxa, whereas relicts of this gene can be found in the two palaeognaths (ostrich and tinamou). Of these, *Struthio camelus* (ostrich) *DSPP* is more complete and retains portions of exon 1 (noncoding) and all five protein-coding exons (2 to 6) (figs. S5 and S6). *Tinamus guttatus* (tinamou), in turn, retains fragments of exons 5 and 6, as well as introns 1, 2, 4, and 5. Sire *et al.* (26) reported the presence of *DSPP* exon 2 in *Gallus gallus* (chicken), but we did not find any remnants of exonic regions of *DSPP* in chicken or other members of Galloanserae. Neoaves species retain ~1.5 kb of intron 4, fragments of exon 5 that

preserve frameshift mutations that are shared with paleognaths, and portions of the noncoding region of exon 6. The region separating *SPARCL1* and *DMP1* is truncated in birds (5 to 37 kb) relative to American alligator (45 kb). The diminutive size of this region was noted in *Gallus* (26), and this pattern is now documented in all newly sequenced bird genomes.

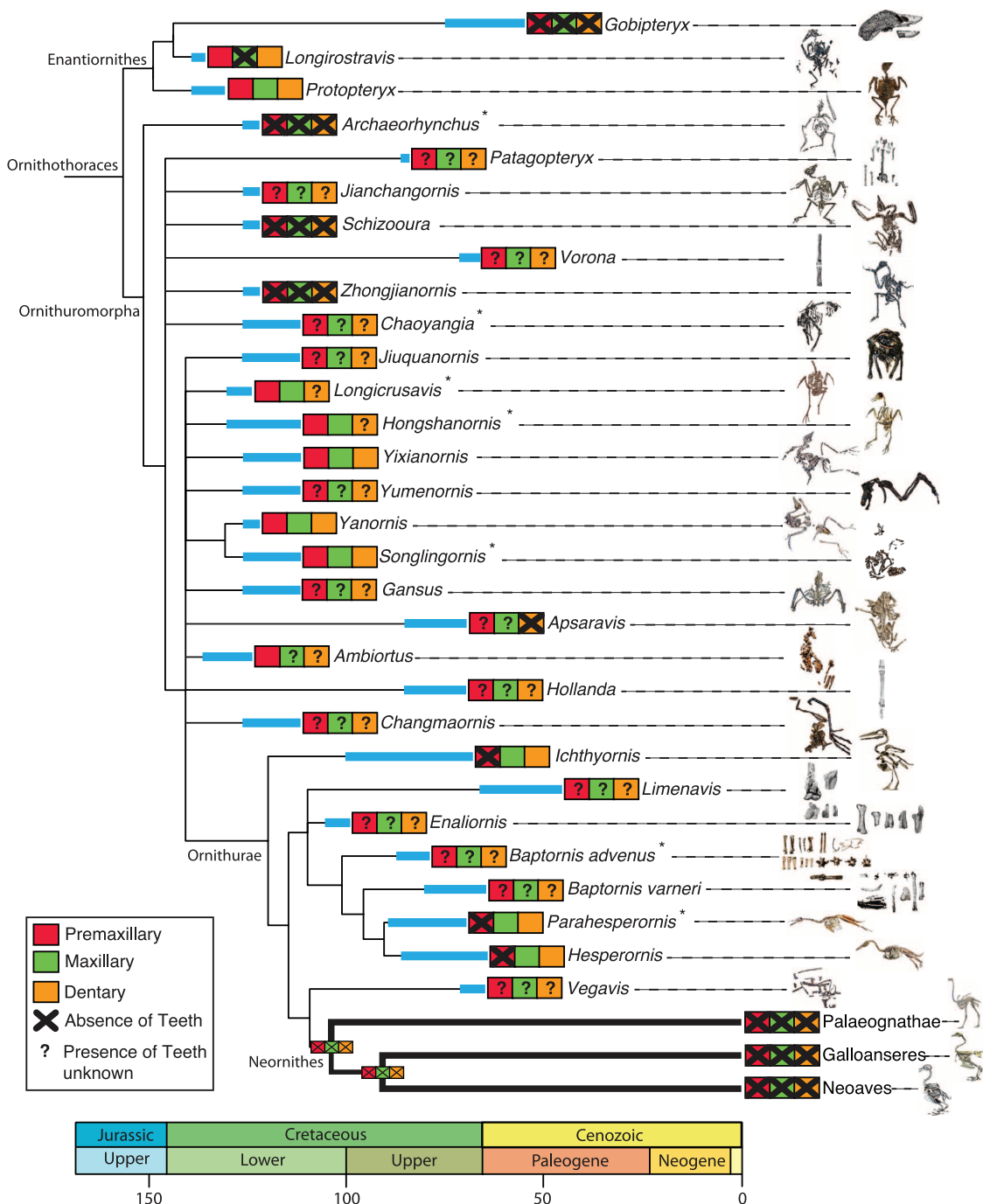
Remnants of *AMBN*, *AMELX*, *AMTN*, *ENAM*, and *MMP20* are more complete than *DSPP* (figs. S1 to S8 and tables S5 to S10), but in some cases whole exons or multiple-exon blocks have been excised from one or more genomes. For example, a block of *ENAM* containing exons 2 to 8 has been deleted in *Calypte anna* (Anna's hummingbird) (fig. S3 and table S2), and exon 1 of *MMP20* is absent from the genomes of living birds (figs. S7 and S8 and tables S2 and S10). *MMP20* is closely syntenic with other matrix metalloproteinase (*MMP*) genes in gnathostomes

with sequenced genomes (25). In American alligator, *MMP20* is located between *MMP27* and *MMP7*, and the region separating *MMP27* and *MMP20* is ~41 kb (fig. S8). By contrast, this region is reduced by almost 30 kb across a range of diverse bird genomes, including both Palaeognathae and Neognathae, which suggests that exon 1 was excised from the genome in the common ancestor of extant birds (fig. S8). In addition to the loss of exon 1, exon 3 is present only in *Anas platyrhynchos domestica* (domestic duck) (figs. S7 and S8 and table S2), which suggests multiple independent losses of this exon in birds.

Inactivating mutations are also present in these dentin- and enamel-related genes in the three turtle species with genome sequences, including inactivating mutations in *AMELX* shared by the three turtle species we examined (figs. S11 and S12 and tables S17 to S22). However, the genomic record of the six tooth-related genes in turtles is largely incomplete, and additional shared inactivating mutations may have been lost by subsequent, more inclusive deletions. For example, exons 5 to 7 of *ENAM* are missing from all three turtle species (fig. S11), but one or more of these exons may have been deleted in the common ancestor of these taxa followed by subsequent overprinting (e.g., additional deletions) in *Chrysemys* and *Pelodiscus* (fig. S11 and table S3). These difficulties in reconstructing frameshift mutations in tooth-related genes in

Fig. 3. Time tree of representative ornithurans, indicating multiple iterations of tooth loss or reduction.

Ornithothoraces phylogeny from Wang et al. (45). Divergence times for crown Aves (thick black lines) are calculated on the basis of Jarvis et al. (14). Stratigraphic ages for fossil species (thick blue lines) are from the Paleobiology Database (www.paleobiology.org) and original descriptions when available (43). Ghost branches (thin black lines) extend ~5 million years beyond the oldest fossil in each clade or the immediately adjacent crownward node. Distribution or reduction of teeth across Ornithothoraces is from original descriptions/redescriptions (43). Asterisks indicate codings for the presence or absence of teeth that differ from original descriptions and/or the most recent review of tooth loss (5).



the early evolutionary history of turtles are not unexpected given that tooth loss in Testudines is at least as old as the Upper Jurassic (~145.5 to 161.2 Ma) (Fig. 1) (13). The antiquity of tooth loss in turtles may therefore have provided an extended window for tooth gene degradation in this clade (13).

Among enamelless mammals with genome sequences (sloth, armadillo, and aardvark), the *DSPP* gene remains intact, whereas *MMP20*, *ENAM*, *AMTN*, *AMELX*, and *AMBN* genes have been inactivated, with the exception of *AMBN* and *MMP20* in armadillo (Fig. 1, figs. S9 and S10, and tables S11 to S16) (13). There are no inacti-

vating mutations shared by the two xenarthrans (sloth and armadillo), although sloths share frameshift mutations in the *ENAM* gene with anteaters (4). The enamel-related *MMP20*, *ENAM*, *AMTN*, *AMELX*, and *AMBN* genes have also been inactivated in the edentulous Chinese pangolin, and all of the protein-coding exons have been excised from the dentin-related *DSPP* gene in this species (Fig. 1 and tables S11 to S14 and S16) (13). The functionality of *DSPP* (pygmy/dwarf sperm whales and baleen whales), *MMP20* (anteaters), *AMTN* (anteaters, pygmy/dwarf sperm whales, and baleen whales), and *AMBN* (anteaters) are currently unknown. We also did not find evi-

dence for molecular decay of tooth genes in the genomes of any of the nonavian toothed saurosid [Alligator, and *Anolis carolinensis* (Carolina anole)] or mammals (elephant, dog, and sperm whale) (Fig. 1) that were included in our study. These results confirm that inactivation of tooth genes has occurred convergently in birds, turtles, and multiple mammalian lineages.

Dating of mutations in genes associated with tooth loss in birds

The occurrence of shared inactivating mutations in both dentin- and enamel-related genes suggests that teeth were lost on the stem neornithine

branch, which extends from 244.2 to 101.6 Ma (14). On the basis of the rate at which frameshift mutations accumulate (27) in neutrally evolving, tooth-related pseudogenes in crown Neornithes and the avian time tree of the 48 species in (14), we estimate that the outer enamel covering of teeth was lost around 116 Ma (95% confidence interval = 105.7 to 128.3 Ma) (see the supplementary materials) in the common ancestor of all modern birds. The remnants of *DSPP* in modern birds are too incomplete to date dentin loss, but if the loss of *DSPP* is a molecular proxy for edentulism then tooth loss in birds occurred no later than 101.6 Ma on the basis of patterns of shared mutations in *DSPP* in modern birds. The closest relatives of crown Neornithes belong to Ichthyorniformes (e.g., *Guilddavis*, *Iaceornis*, and *Ichthyornis*) and Hesperorniformes (e.g., *Baptornis* and *Hesperornis*) (7, 28) and range from the late Cenomanian (*Ichthyornis*, ~93.5 Ma) to within 300,000 years of the Cretaceous-Paleogene (K/Pg) boundary (29) at 66 Ma when modern birds are thought to have undergone a rapid radiation of species (14). The presence or absence of teeth cannot be determined for most ichthyorniform and hesperorniform taxa owing to incomplete skeletal remains, but *Ichthyornis* and *Hesperornis* fossils possess both dentary and maxillary teeth (28). Our genomic results support the exclusion of these taxa, as well as all dentate bird fossils, from crown Neornithes. Instead, they suggest that members of Ichthyorniformes and Hesperorniformes were dentate contemporaries of edentulous crown birds for most of the Late Cretaceous. Other avian contemporaries of crown Aves in the Late Cretaceous included both toothed and edentulous birds belonging to the now extinct clade Entantionithes (“opposite birds”) (Fig. 2).

Discussion

Previous studies have documented that *DSPP*, *AMBN*, *AMELX*, *AMTN*, *ENAM*, and *MMP20* are expressed outside of tooth development (21–23) (table S1). Our findings of widespread and convergent pseudogenization of these six genes across diverse edentulous and enamelless vertebrates provides evidence that these genes are tooth-specific with respect to their essential functions that are maintained by natural selection. Viewed from another perspective, these six genes are pleiotropic with the caveat that their pleiotropic roles outside of tooth development are non-obligatory. *DSPP*, which is involved in dentin formation, has been independently inactivated in three lineages of edentulous amniotes (birds, turtles, and Chinese pangolin). Similarly, *AMELX*, *AMBN*, *AMTN*, *ENAM*, and *MMP20* are critical for enamel formation (table S1) and have been independently inactivated in birds, turtles, pangolins, sloths, armadillos (except *MMP20* and *AMBN*), and aardvarks. Anteaters, baleen whales, pygmy/dwarf sperm whales, and narwhals also show evidence of inactivation of enamel-related genes, although sequence coverage for these genes is limited to one or a few partial exons of *AMBN*, *AMELX*, *ENAM*, and *MMP20* (4, 11, 12). If these genes played critical, essential roles out-

side of enamel or dentin development, we would instead expect them to be maintained by natural selection in edentulous and enamelless lineages. It is possible that redundancy in the transcriptome may have compensated for their non-tooth-specific functions in edentulous and enamelless lineages. For example, *MMP20*'s primary role is in enamel formation, but it is also expressed in developing dentin and together with *MMP2* cleaves *DSPP* in dentin formation (30). The *MMP2*-*MMP20* redundancy in *DSPP* cleavage (30) may have facilitated inactivation of *MMP20* once it was released from performing its function in enamel formation in enamelless taxa that nevertheless retain dentin-based teeth such as aardvark and sloth.

It is also possible that tooth-related genes are incidentally expressed outside of tooth development via gene expression neighborhoods (31, 32). Genes such as *AMBN*, *AMTN*, and *ENAM* occur in the *SCPP* gene cluster (24) that also includes bone-related genes and salivary proteins, and incidental coexpression may be expected to occur in some of these tissues. Expression studies provide support for the expression of these tooth-related genes in other biomineralized tissues (22, 33, 34) (table S1). Similarly, *MMP20* is located in one of the *MMP* gene clusters with several other matrix metalloproteinase genes, and its coexpression in nondental tissues (e.g., lung and large intestine) (21, 35, 36) with other *MMPs* may be attributed, in part, to gene expression neighborhoods.

The possibility of functional splice site variants also merits attention. Given that multiple splice site variants are associated with Amelogenin, the gene *AMELX* (37) may remain intact if inactivating mutations are confined to a subset of *AMELX* exons. However, our data identified widely distributed inactivating mutations in *AMELX* across all of the protein-coding exons of this gene, which suggests that no bird has viable splice site variants (table S9) (13).

Edentulism and the development of a complete rhamphotheca are integral features of modern birds. Beak development, in turn, is associated with the expression of an assemblage of beta-keratin genes that differs from the sets of beta-keratin genes associated with other epidermal appendages, including scales, claws, feathers, and the egg tooth (38). Expression studies suggest that inactivation of *BMP4* in the oral epithelium of chick embryos leads to early-stage odontogenic arrest (39). Moreover, *BMP4* expression levels in beak mesenchyme are correlated with beak morphology (40). The inactivation of tooth genes does not provide direct evidence for a complete rhamphotheca, but fossil-based evidence (e.g., *Hesperornis* and *Ichthyornis*) for the replacement of premaxillary teeth by a partial rhamphotheca in the first stage of rostral remodeling suggests that tooth loss and rhamphotheca development are tightly linked, as may be expected if keratinization of epithelial cells impeded tooth formation (41).

We postulate, on the basis of fossil and molecular evidence, a two-step scenario whereby tooth

loss and rhamphotheca development evolved in concert with each other on the stem neornithine branch. We propose that the first stage included tooth loss on the premaxilla and the development of a partial rhamphotheca on the premaxilla and anterior mandible (5, 42). Direct evidence for this incipient stage in the remaking of the avian rostrum derives from the fossil record (5, 42). Importantly, the evolution of a partial rhamphotheca may have been a prerequisite for complete edentulism so that the latter was adaptively viable (5). The second stage involved concurrent rostrocaudal progression of tooth loss and rhamphotheca development from the already modified anterior rostrum (5) (Fig. 2). We propose that this progression ultimately resulted in a complete rhamphotheca that effectively replaced the teeth and contributed to the diversification of living birds. (Fig. 3)

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SUPPLEMENTARY MATERIALS

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