

## RESEARCH ARTICLE SUMMARY

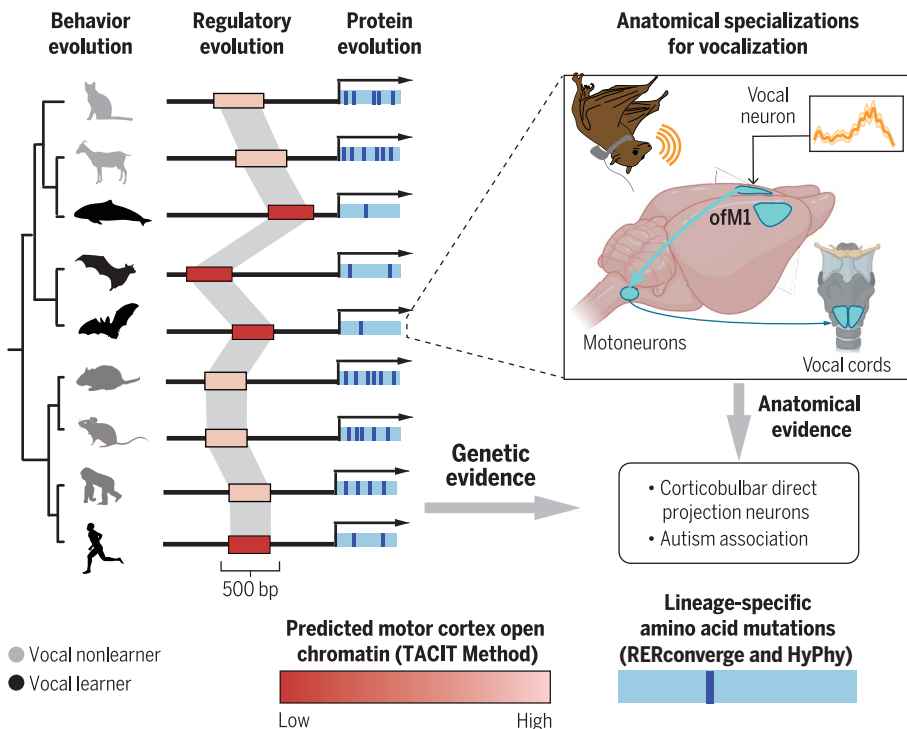
## ZONOMIA

## Vocal learning–associated convergent evolution in mammalian proteins and regulatory elements

Morgan E. Wirthlin<sup>†</sup>, Tobias A. Schmid<sup>†</sup>, Julie E. Elie<sup>†</sup>, Xiaomeng Zhang, Amanda Kowalczyk, Ruby Redlich, Varvara A. Shvareva, Ashley Rakuljic, Maria B. Ji, Ninad S. Bhat, Irene M. Kaplow, Daniel E. Schaffer, Alyssa J. Lawler, Andrew Z. Wang, BaDoi N. Phan, Siddharth Annaldasula, Ashley R. Brown, Tianyu Lu, Byung Kook Lim, Eiman Azim, Zoonomia Consortium, Nathan L. Clark, Wynn K. Meyer, Sergei L. Kosakovsky Pond, Maria Chikina, Michael M. Yartsev<sup>\*‡</sup>, Andreas R. Pfenning<sup>\*‡</sup>

**INTRODUCTION:** Vocal production learning (“vocal learning”), or the ability to modify vocalizations according to the social environment, forms the basis of human speech production. Among the Boreoeutherian mammals, this trait has evolved independently in four different lineages: humans, bats, cetaceans, and pinnipeds. In vertebrates, the evolution of vocal learning behavior has been associated with the evolution of brain anatomical features, including cortical long-range projection neurons (e.g., songbirds and humans). Moreover, neural circuits for the production of learned vocalization display convergent evolution in patterns of gene expression.

**RATIONALE:** Despite evidence for the convergent evolution of vocal learning at the behavioral, anatomical, and gene expression levels in vertebrates, the genetic underpinnings of vocal learning and human speech in mammals are poorly understood. New machine learning approaches and the newly sequenced mammalian genomes of the Zoonomia Consortium provide the foundation to rigorously study this question. The repeated evolution of vocal learning across mammals allows us to determine which parts of the genome are significantly associated with the behavior.



**Finding vocal learning–associated regions of the mammalian genome.** We compared the evolution of vocal learning behavior to the evolution of coding and noncoding elements of the genome, leveraging anatomical, electrophysiological, and epigenomic experiments in the Egyptian fruit bat orofacial motor cortex (ofM1). We show convergent evidence of the importance of long-range projection neurons and autism-associated gene networks.

**RESULTS:** First, we studied convergent evolution in protein-coding regions using the RERconverge and HyPhy methods to find 200 significantly associated genes. The genes that tend to be under higher constraint in vocal learning mammals are enriched for genes involved in human autism. However, the vast majority of genes are driven by signals from only one or two clades of vocal learning mammals, suggesting that a large component of the genetic basis for the trait may lie instead in the convergent evolution of regulatory elements. To explore that hypothesis, we performed an anatomical and functional characterization of the Egyptian fruit bat motor cortex. We identified a subregion of the motor cortex that is implicated in vocal production and directly projects to the motoneurons controlling the bat's larynx. This allowed us to profile candidate regulatory elements active in this vocalization-associated subregion of the motor cortex by measuring open chromatin. These open chromatin regions and 222 mammalian genomes of the Zoonomia Consortium served as input to the Tissue-Aware Conservation Inference Toolkit (TACIT) machine learning approach, which was applied to find 50 candidate regulatory elements whose predicted motor cortex open chromatin measurements across mammals are highly correlated with the presence of vocal learning behavior. Many of these open chromatin regions were near genes associated with autism, and they tended to overlap with open chromatin specific to the long-range projection neurons that have been implicated in the evolution of vocal learning.

**CONCLUSION:** Although it is impossible to know which parts of the genome evolved for human speech production, we are able to use the repeated evolution of a component of that behavior, vocal learning, to find significantly associated genes and noncoding regions. Our results demonstrate that the presence of vocal learning behavior in a given clade leads to weak selective pressure across a broad range of genes and stronger selective pressure across a smaller number of motor cortex noncoding regions. These genes and noncoding regions show an association with autism, which suggests that there are shared regulatory networks for vocal and social behavior that tend to adapt in similar ways when a lineage evolves vocal learning behavior. More broadly, our results suggest that the evolutionary history of selective pressures across a location in the genome can provide insight into how that region might influence human behavior. ■

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## Vocal learning–associated convergent evolution in mammalian proteins and regulatory elements

Morgan E. Wirthlin<sup>1,2†§</sup>, Tobias A. Schmid<sup>3†</sup>, Julie E. Elie<sup>3,4†</sup>, Xiaomeng Zhang<sup>1¶</sup>, Amanda Kowalczyk<sup>1,2</sup>, Ruby Redlich<sup>1</sup>, Varvara A. Shvareva<sup>5</sup>, Ashley Rakuljic<sup>5</sup>, Maria B. Ji<sup>6</sup>, Ninad S. Bhat<sup>5</sup>, Irene M. Kaplow<sup>1†</sup>, Daniel E. Schäffer<sup>1#</sup>, Alyssa J. Lawler<sup>2,7¶</sup>, Andrew Z. Wang<sup>1</sup>, BaDoi N. Phan<sup>1,2</sup>, Siddharth Annaldasula<sup>1</sup>, Ashley R. Brown<sup>1,2</sup>, Tianyu Lu<sup>1</sup>, Byung Kook Lim<sup>8</sup>, Eiman Azim<sup>9</sup>, Zoonomia Consortium<sup>#</sup>, Nathan L. Clark<sup>10</sup>, Wynn K. Meyer<sup>11</sup>, Sergei L. Kosakovsky Pond<sup>12</sup>, Maria Chikina<sup>13</sup>, Michael M. Yartsev<sup>3,4\*††</sup>, Andreas R. Pfenning<sup>1,2\*††</sup>

Vocal production learning (“vocal learning”) is a convergently evolved trait in vertebrates. To identify brain genomic elements associated with mammalian vocal learning, we integrated genomic, anatomical, and neurophysiological data from the Egyptian fruit bat (*Rousettus aegyptiacus*) with analyses of the genomes of 215 placental mammals. First, we identified a set of proteins evolving more slowly in vocal learners. Then, we discovered a vocal motor cortical region in the Egyptian fruit bat, an emergent vocal learner, and leveraged that knowledge to identify active cis-regulatory elements in the motor cortex of vocal learners. Machine learning methods applied to motor cortex open chromatin revealed 50 enhancers robustly associated with vocal learning whose activity tended to be lower in vocal learners. Our research implicates convergent losses of motor cortex regulatory elements in mammalian vocal learning evolution.

Vocal production learning (“vocal learning”), the ability of an organism to modify the acoustic properties of its vocalizations as a result of social experience, is an example of convergent evolution, having evolved independently within multiple lineages of birds and mammals, including humans, where it manifests as speech (Fig. 1A) (1, 2). Vocal learning has been extensively studied in songbirds, highlighting numerous shared behavioral features of birdsong and speech learning, such as a dependence on auditory input during a critical developmental period and a juvenile babbling phase of sensorimotor exploration prior to the maturation of the adult vocalizations (1). Convergence between song-learning birds and humans extends to neuroanatomical specializations, including direct corticobulbar projections from the vocal motor cortex analog to the hindbrain motoneurons controlling the vocal apparatus (3) and shared transcriptional specializations in analogous speech- and song-specialized brain regions (4). Thus, songbirds have become a premier model for exploring

the fundamental brain anatomical, molecular, and genomic features associated with vocal learning (1, 3). An expanding literature on vocal learning behavior across mammals suggests an underappreciated diversity in the phenotypic expression of vocal learning across the taxa traditionally thought to possess it (2, 5–8). Study of the diverse forms of mammalian vocal learning behaviors could broaden our understanding of the core molecular, anatomical, and physiological brain mechanisms of vocal learning and of the mechanisms underlying the convergent evolution of skilled motor behaviors more broadly.

We evaluated evidence of convergent genomic specializations shared among four lineages of vocal learning mammals that independently evolved this trait—bats, cetaceans, pinnipeds, and humans (Fig. 1A)—using whole-genome datasets and recently developed computational approaches. Specifically, we used protein-coding sequences from genomes generated by the Zoonomia Consortium (9, 10) and models of evolutionary rate convergence (11, 12) to

identify protein-coding regions repeatedly associated with the evolution of vocal learning across mammals. Although we found 200 protein-coding genes significantly associated with vocal learning, none of them showed strong evidence of selection in all four mammalian clades, and only five showed strong evidence of selection in three out of the four clades. Owing to individual lineages contributing disproportionately to many of the protein-coding results, we hypothesized that noncoding regulatory elements might also be under constraint for the evolution of vocal learning. We next profiled open chromatin, a proxy for regulatory element activity (13), in multiple brain regions and somatic tissues in the Egyptian fruit bat (*Rousettus aegyptiacus*), a mammal with robust vocal plasticity (14–16), to identify vocalization-associated regulatory genomic specializations. We accomplished this by combining anatomical tracing and electrophysiological recordings in vocalizing bats to identify a region of the motor cortex associated with vocal production. The vocalization-associated epigenomic data collected from this bat species, combined with hundreds of mammalian genomes (17, 18), their associated reference-free whole-genome alignments (19), and high-quality epigenomic data from the motor cortex of multiple additional mammalian species (20–22), provided the foundation to apply a machine learning approach, the Tissue-Aware Conservation Inference Toolkit (TACIT) (23). This approach allowed us to identify putative enhancers, distal regulatory elements that tend to be highly tissue specific, associated with the convergent evolution of vocal learning. We combined recently developed computational tools and neuroanatomical experiments in the Egyptian fruit bat and found evidence of convergent evolution in both protein-coding and noncoding DNA sequences.

## Results

## Convergent evolution in protein sequence associated with vocal learning behavior

To explore the possibility of shared genomic specializations associated with vocal learning, we first applied RERconverge (11) to recently released protein-coding alignments obtained for hundreds of mammals (10) to identify protein-coding genes whose relative rates of

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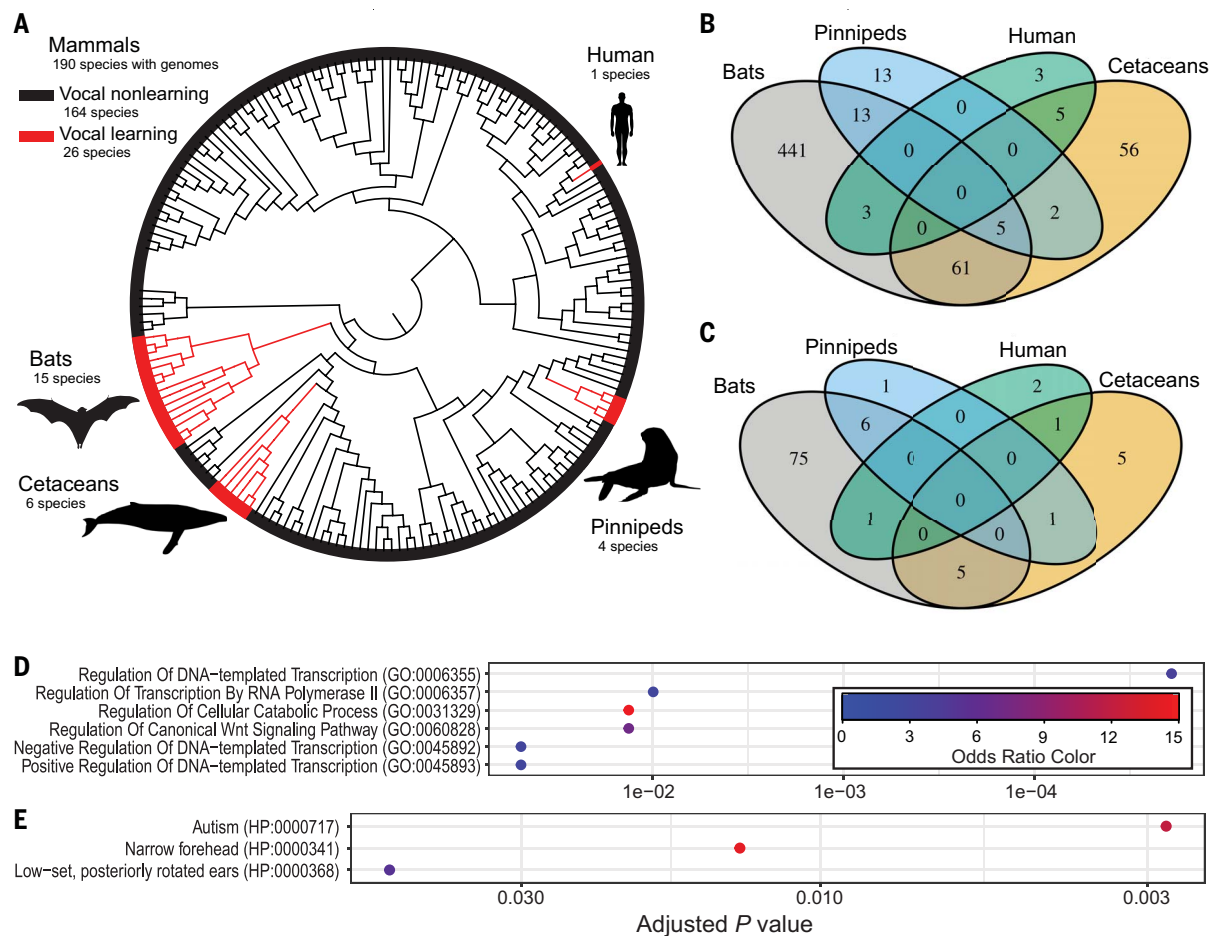
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**Fig. 1. Convergent changes in protein sequence associated with vocal learning evolution across 215 mammalian species.** (A) A cladogram of mammalian species whose genomes were analyzed in this study highlights the convergent evolution of vocal learning species (red) relative to vocal nonlearners (black). The phylogenetic tree used in our analyses was derived from (98). (B and C) Each of the genes implicated by RERconverge with lower (B) or higher (C) evolutionary rates in vocal learners is annotated based on whether it

shows a significant signature within the four vocal learning clades based on a Bayes factor  $\geq 5$  (18). (D and E) All significant gene ontology categories (adj.  $P < 0.10$ , EnrichR) are plotted for the 200 genes with conserved (D) and accelerated (E) selection in vocal learning clades on the basis of results from the RERconverge and HyPhy RELAX analyses. The points are colored by the odds ratio within the set of implicated genes relative to the genes outside of the set, which corresponds to the degree of enrichment within that set.

evolution differ between vocal learners and other mammals and those that may thus be under selection related to vocal learning (11, 24). We analyzed 16,209 high-quality protein-coding gene alignments across 215 boreoeutherian mammals, including 26 vocal learning species, 164 vocal nonlearners, and 25 species without confident annotations (Fig. 1A and data S1) (materials and methods). We found evidence for lower evolutionary rates in vocal learners compared with nonlearners in 804 genes and evidence for elevated evolutionary rates in 102 genes [ $\tau$  adjusted (adj.)  $P < 0.01$  and permutations (24) adj.  $P < 0.01$ ] (fig. S1 and data S2). To identify which specific clades were driving the differential rates of evolution, we applied a Bayes factor analysis that examined each clade individually for evidence of selection (18) (data S2). Despite the large number of significant associations based on RERconverge, we found no single protein-coding gene with

consistently lower or elevated evolutionary rates in all four vocal learning clades. Among the genes with reduced evolutionary rates in vocal learning species, we found only 5 out of 804 protein-coding genes with strong evidence of selective pressure in three out of the four vocal learning clades: *CENPC*, *CAISPERG*, *MGA*, *TREML2*, and *ZCWPW1* (Bayes factor  $> 5$ ) (Fig. 1, B and C). None of these proteins reached the threshold for selection in the human lineage (materials and methods), which could indicate different mechanisms of evolution in the laurasiatherian vocal learning clades relative to humans. Our results suggest that the vast majority of protein-coding genes that we identified are evolving much faster or much slower in one clade but are only weakly associated with vocal learning across the other lineages.

Among the most strongly associated genes were *CENPC* (fig. S2A) ( $\tau = -0.30$ ;  $\tau$  adj.  $P = 8.7 \times 10^{-6}$ ; permutations adj.  $P \leq 0.001$ ), and *GRM8*

(fig. S2B) ( $\tau = 0.26$ ;  $\tau$  adj.  $P = 3.5 \times 10^{-4}$ ; permutations adj.  $P \leq 0.001$ ). *GRM8* represents an especially promising candidate because it has previously been linked to anatomical specializations for vocal learning in songbirds (25) and is a known target of the speech-associated *FOXP2* transcription factor (TF) (26). In primates and rodents, both *GRM8* and *FOXP2* are markers of a rare class of medium spiny neurons implicated in motor control that co-express both *DRD1* and *DRD2* dopamine receptors (27–29). Our results support a model in which vocal learning behavior is only partially explained by differences in protein-coding gene evolutionary rates.

To further explore selection on these vocal learning-associated protein-coding genes, we applied an additional set of tools from the HyPhy package that compare nonsynonymous (dN) and synonymous (dS) substitution rates in the nucleotides of the amino acid sequence

(30). In this study, these sensitive evolutionary models of codon substitution formally compare selective regimes, modeled as dN/dS distributions, between branches annotated with the vocal learning phenotype and the rest of the phylogenetic tree (12, 30). The results were largely consistent with amino acid-level methods: the protein-coding genes with lower protein evolutionary rates in vocal learning clades also tended to be under higher constraint in vocal learning clades (Wilcoxon  $P = 2.5 \times 10^{-4}$ ), and the genes with higher evolutionary rates in vocal learning species showed evidence of accelerated evolution in these same clades (Wilcoxon  $P = 7.9 \times 10^{-9}$ ) (fig. S3 and data S3). To further explore the functional trends of protein-coding genes associated with the evolution of vocal learning, we focused on the set of proteins that showed consistent behavior between RERconverge and the HyPhy RELAX model (Benjamini-Hochberg false discovery rate  $q < 0.05$ ). This yielded a set of 126 proteins that were more slowly evolving in vocal learning clades and 74 with an elevated rate of evolutionary changes relative to other species. The complementary approaches of RERconverge and HyPhy RELAX identified a total of 200 vocal learning-associated genes, which suggests that this behavior, vocal learning, is having a substantial impact on protein evolutionary rates.

We further interrogated evolutionary pressures across the vocal learning-associated genes by looking for evidence of diversifying position selection using the HyPhy BUSTED-PH model. Evidence of diversifying positive selection was found in 6.3% (13 transcripts, 9 genes) of the set of genes with elevated rates of evolution in vocal learning species based on RERconverge and the HyPhy RELAX model. As expected, we identified much lower rates of diversifying positive selection in the gene with lower rates of evolution in vocal learning species (1.0%) and within the set of randomly chosen transcripts (2.0%). Among the nine genes that showed evidence of positive selection, eight have been associated with neurodevelopment (*CCDC136*, *KIDINS220*, *LRRN1*, *RSG5*, *CYLD*, *GABRA5*, *NETO2*, and *KIAA1109*) (31–38). The gene *CCDC136* has more directly been associated with multiple language-related phenotypes in humans (31, 39, 40). These results suggest that the vocal learning-associated genes across mammals may tend to play a role in human brain development and vocal behavior.

To more systematically explore the functional trends within these 126 and 74 protein-coding genes, we conducted a gene ontology analysis using EnrichR. Protein-coding genes with lower evolutionary rates in vocal learning species were associated with regulation of DNA-templated transcription ( $P = 1.10 \times 10^{-6}$ , adj.  $P = 1.9 \times 10^{-5}$ ), regulation of canonical Wnt signaling pathway ( $P = 5.1 \times 10^{-5}$ , adj.  $P = 0.013$ ), and the autism human phenotype ontology ( $P = 5.8 \times 10^{-6}$ , adj.

$P = 0.0028$ ) (Fig. 1, D and E, and data S4). The genes with accelerated evolutionary rates were not enriched for any pathways at an adjusted  $P < 0.05$  with at least five genes contributing. The enrichment of autism-associated genes among the set of genes with greater levels of conservation in vocal learners (*MECP2*, *RAD21*, *DYRK1A*, *SIMI*, *FSTSJ1*, *MEIS2*, and *FGFR1*) is particularly interesting given the prevalence of speech delay and early vocal production differences in human subjects with autism (41, 42) as well as the previous association between autism genes and the evolution of vocal behavior in birds and bats (43, 44). On the basis of the association with autism, we further explored the function of the vocal learning-conserved gene set in the context of early vocal production differences. Although only four human loci have been associated with differences in early vocal production, protein-coding genes overlapping two of these loci show higher levels of conservation in vocal learning clades (*INSC* and *DAPK3*) (45).

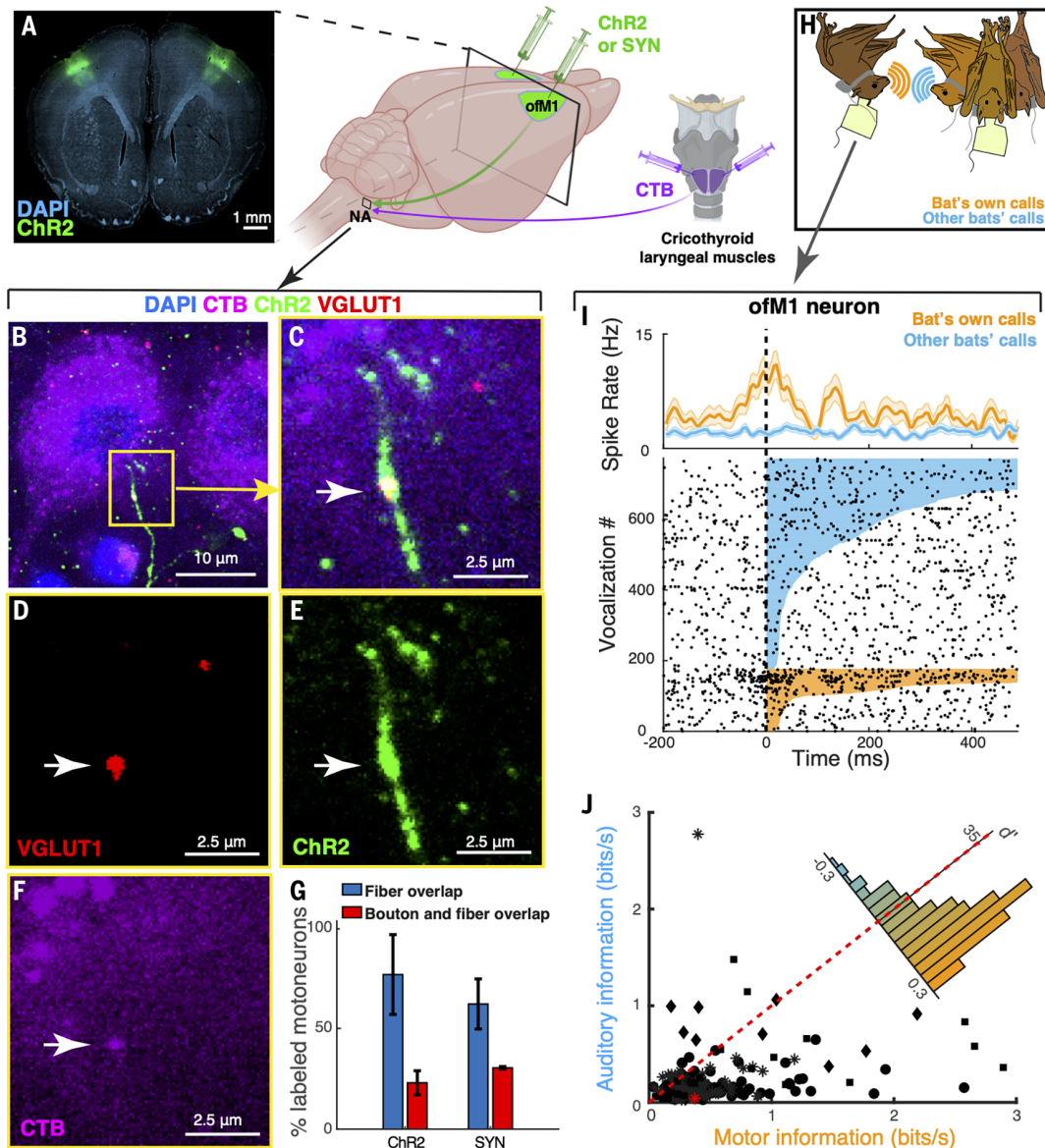
#### Identification of a vocal production region in Egyptian fruit bats

The enrichment of TFs in the set of vocal learning-associated proteins suggests that differences in gene regulation are likely to be a major factor in the evolution of vocal learning. Because gene regulation is often tissue specific, we sought to identify motor regions of the brain involved in vocal production and contrast their epigenomic profiles with that of those not involved in vocal production. We conducted this comparison in the Egyptian fruit bat, *R. aegyptiacus*, a mammalian species with robust vocal plasticity (16, 46) and with data on its motor cortex mapping (47). To identify a candidate region, we were guided by the hypothesis that fine vocal motor control, a key ability in vocal learning, may be associated with the anatomical specialization of the motor cortex (48–51). Previous work suggested that a cortical region controlling complex vocal behavior would be characterized by a direct, monosynaptic projection onto the motoneurons controlling the vocal source (in mammals, the larynx) (48–52). Such a direct connection has been observed robustly in humans (53–56) and vocal learning birds [songbirds, parrots, and hummingbirds (57–59)] but has not been reliably found in vocal nonlearning species such as chimpanzees (41) or mice (60).

We first determined whether a direct corticobulbar anatomical connection existed in *R. aegyptiacus*. Guided by cortical mapping experiments (47), we injected anterograde tracers into the part of the motor cortex that has been associated with orofacial motor control, the orofacial motor cortex (ofM1), and identified fluorescently labeled descending cortical fibers in the hindbrain region where the laryngeal motoneurons reside, the nucleus ambiguus

(NA) (Fig. 2A; fig. S4, A and B; and movie S1). To test the existence of a direct monosynaptic projection, we also specifically identified laryngeal motoneurons in the NA by retrogradely labeling them through bilateral muscular injection of cholera toxin B (CTB) into the cricothyroid muscles of the bat larynx (Fig. 2A). We validated the colocalization of descending cortical fibers and local synaptic boutons with laryngeal motoneurons by using two complementary labeling approaches: one relying on the immunostaining of synapses (vesicular glutamate transporter 1, VGLUT1) and one using viral labeling of synapses (synapsin or synaptophysin dual-label virus, SYN) (Fig. 2, B to F, and fig. S5). Across five bats, 79.2% of the retrogradely labeled motoneurons (61/77) colocalized with descending cortical fibers, and 26% of them (20/77) colocalized with both cortical fibers and synaptic boutons, pointing to the existence of a robust direct corticospinal projection to laryngeal motoneurons (Fig. 2G). This colocalization in the NA was consistent across the different techniques (Fig. 2G and fig. S5) and could not be found in any other brainstem motor nuclei, including the hypoglossal nucleus, which controls the tongue and neck muscles (fig. S4, C to E). We noted that the corticobulbar fibers crossed the midline anterior to the NA at the level of the facial nucleus, offering a direct contralateral path for the innervation of the NA (fig. S4F). These anatomical findings highlight the bat ofM1 as a possible candidate region associated with vocal production.

To further corroborate the role of ofM1 in vocal control, we tested whether ongoing single-cell neural activity in this area was associated with vocal production. We performed wireless electrophysiological recordings from four bats engaged in free vocal interactions with peers (Fig. 2H). Vocalizations were identified and recorded by using wireless call detectors placed around the necks of the individual bats (materials and methods) (46). We found that about half of the recorded single units in ofM1 (115/237) showed a significant change in firing rates when the bats produced vocalizations compared with when they stayed quiet (fig. S6, A to C) [analysis of variance with a Poisson generalized linear model per cell;  $P$  value threshold = 0.001 (materials and methods)]. In 25% of ofM1 cells that were excited during vocal production (26/104), the change of activity could not be accounted for by jaw or tongue movements, indicating that these cells were engaged in the motor control of movements specific to vocal production (fig. S6D). Furthermore, many of the single units had a sustained increase of activity during production of vocalizations, but not during perception of vocalizations (Fig. 2I). To further assess this specific neural modulation during vocal motor production, we quantified the information between the time-varying



**Fig. 2. Identification of an anatomically specialized motor cortical region targeting laryngeal motoneurons in the Egyptian fruit bat.** (A) (Right) Schematic of anatomical tracing approaches. Retrograde tracer CTB (purple) was injected bilaterally into the cricothyroid muscles to label brainstem motoneurons in NA. Simultaneously, an anterograde viral tracer [channelrhodopsin-2 (ChR2) or SYN, green] was injected bilaterally into the ofM1 to label corticobulbar projections into NA. (Left) Example coronal section showing cortical injection sites with anterograde tracer (ChR2, green) and 4',6-diamidino-2-phenylindole (DAPI) labeling (cyan). (B to F) Laryngeal motoneurons in the NA identified by using a retrograde tracer (CTB, purple), cortical fibers labeled with ChR2 (green), corticobulbar synapses labeled with VGLUT1 (red), and DAPI (blue). (B) and (C) are overlaid images showing colocalization of fibers with a synaptic bouton on the retrograde-labeled cell (white arrow). (C) to (F) are magnifications of the yellow-boxed area in (B). (G) Percentage of laryngeal motoneurons labeled with CTB that are colocalized with cortical fibers (blue) or with both cortical fibers and synaptic boutons (red).

firing rate and the amplitude modulation of the vocalizations. This analysis confirmed that ofM1 neurons had significantly higher motor than auditory information (Fig. 2J) (likelihood-ratio

test on linear mixed-effects models,  $n = 219$ ,  $\chi^2 = 62.515$ ,  $df = 1$ ,  $P = 2.6645 \times 10^{-5}$ ; average  $d'$  change in information gain during motor production =  $0.15 \pm 0.13$ , corresponding to an

increase of  $0.286 \pm 0.035$  bits/s). Combined, the results of the anatomical and electrophysiological study defined ofM1 as a motor cortical area associated with vocal production in *R. aegyptiacus*.

Both tracing techniques qualitatively yielded similar results: ChR2,  $n = 51$  cells from three bats; SYN,  $n = 26$  cells from two bats. (H) Illustration of the experimental setup during which wireless electrophysiological recordings were conducted from the identified cortical region in freely behaving and vocalizing bats. (I) Spiking activity of an example ofM1 neuron aligned to the onset of vocalizations produced (bat's own calls, orange) or heard (other bats' calls, blue) by the bat subject. (Top) Time-varying mean firing rate and (bottom) corresponding raster plot. Colored lines in the raster plot show the duration of each vocalization. Note the increased firing rate during vocal production as compared with that of hearing. (J) Information (materials and methods) between the time-varying firing rate and the amplitude of produced ( $x$  axis) versus heard ( $y$  axis) vocalizations for 219 single units (marker shapes indicate bat ID,  $n = 4$  bats). The cell shown in (I) is highlighted in red. (Inset) The distribution of  $d'$  between motor and auditory information for the same cells. Note that the distribution is heavily skewed toward higher motor information rather than auditory information coded in the activity of the recorded neurons. Error bars are mean  $\pm$  SEM throughout the figure.

### Epigenomic specializations in the vocal production region of the Egyptian fruit bat motor cortex

We next sought to epigenomically profile candidate regulatory elements in vocal and nonvocal brain regions in *R. aegyptiacus* to identify vocal learning-associated regulatory genomic specializations. We generated a multi-tissue atlas of open chromatin data, indicative of regulatory activity, by performing assay for transposase-accessible chromatin sequencing (ATAC-seq) (61) across seven brain regions and three somatic tissues of *R. aegyptiacus* (materials and methods), including ofM1 (Fig. 3A). From a total set of 88,389 noncoding, non-promoter open chromatin regions (OCRs) in the primary motor cortex (M1), we identified 348 candidate enhancers with differential open chromatin between ofM1 and wing motor cortex (wM1) ( $P < 0.05$ ) (Fig. 3B and data S5) (materials and methods). Genes proximal to OCRs with differential open chromatin between ofM1 and wM1 were significantly enriched for functional association with neuronal projections and transcriptional regulation (data S6). These included OCRs near the genes of 51 known TFs, including *FOXP2*, a TF that has been extensively implicated in human speech and vocal learning (Fig. 3C) (62). Notably, genes

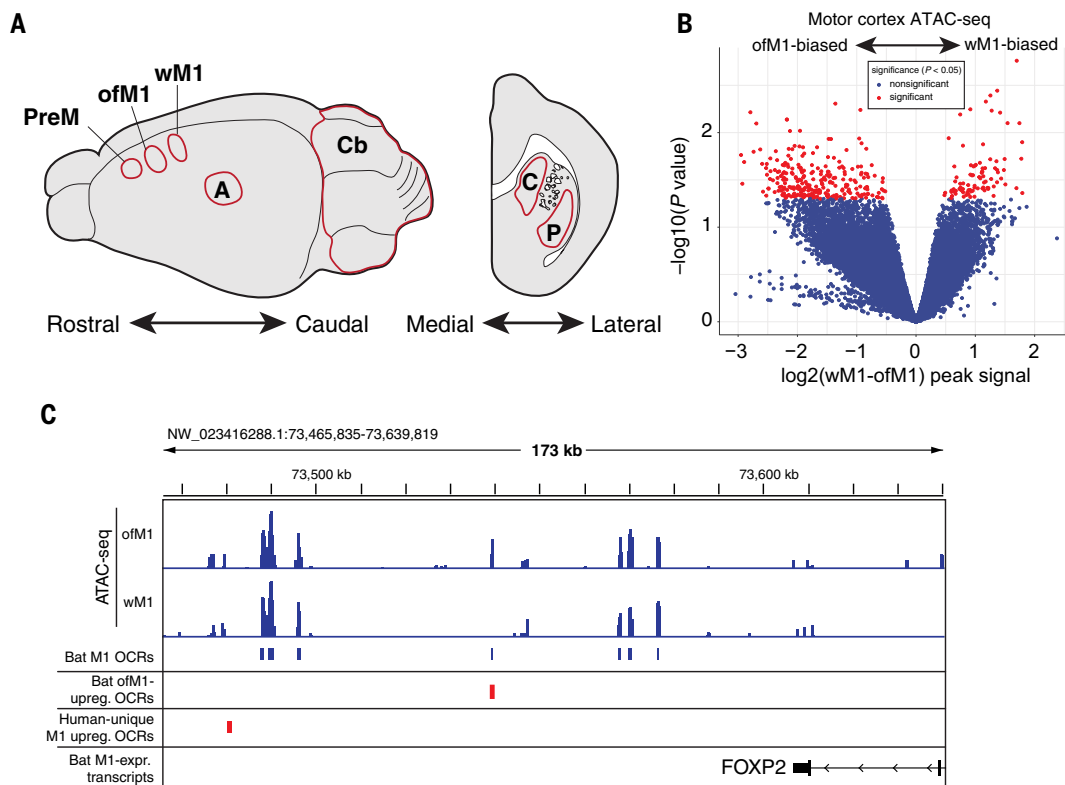
near OCRs differentially open between bat ofM1 and wM1, including genes that we had identified as being under convergent acceleration in vocal learners using the evolutionary approaches of RERconverge ( $n = 11$ ) and HyPhy RELAX analysis ( $n = 3$ ; *GATA3*, *LRRN1*, and *TNIP3*) (data S6). These specialized regions of open chromatin, coupled with an enrichment of TFs in the set of vocal learning-associated protein-coding genes, suggest that both cis and trans differences in gene regulation contribute to the evolution of vocal learning behaviors.

### Convergent evolution in candidate enhancer sequences associated with vocal learning behavior

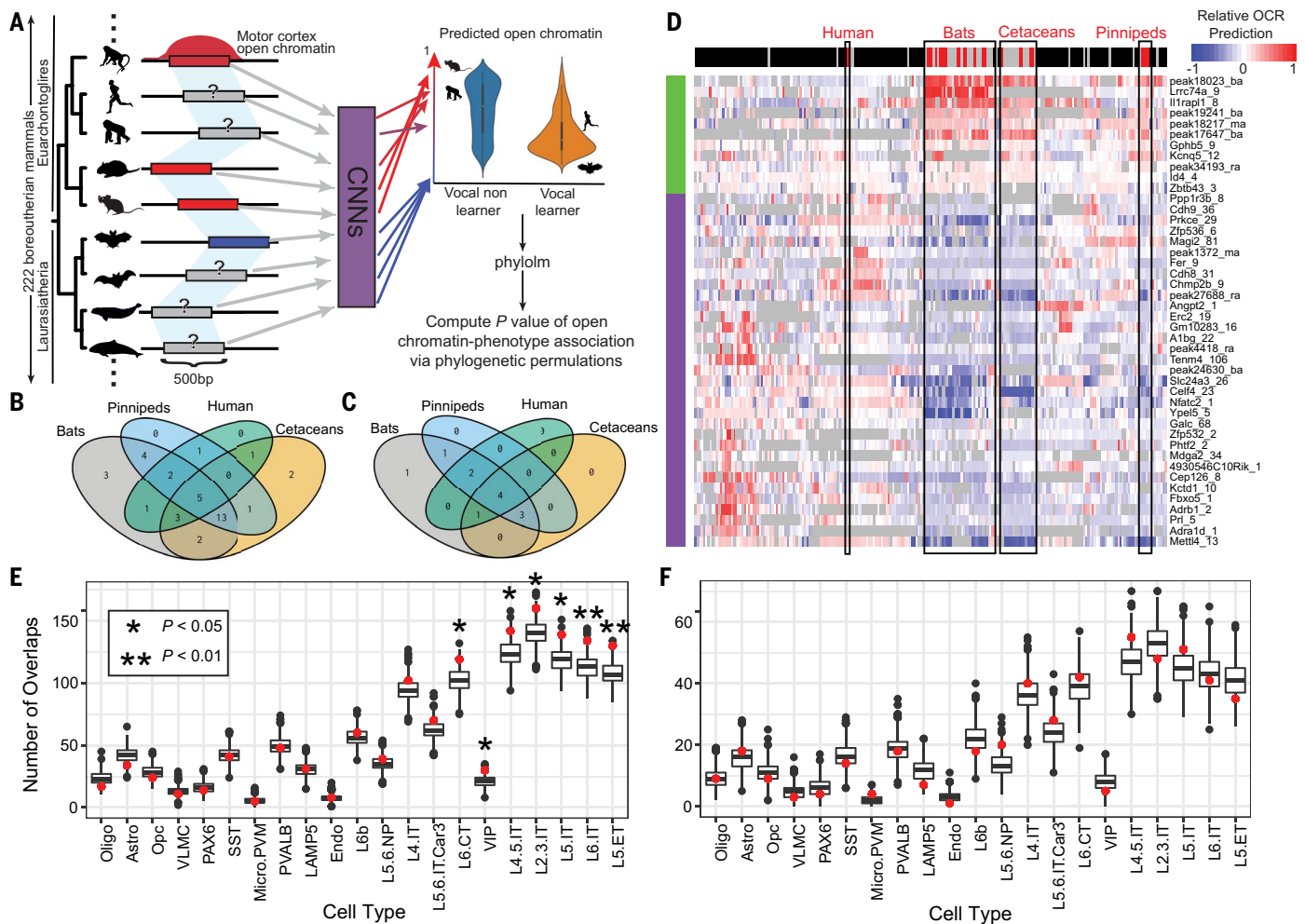
Because there is accumulating evidence that cis-regulatory differences in enhancer regions are driving the evolution of complex traits (63–65), we sought to identify OCRs whose tissue- and cell type-specificity would be shared across species of vocal learners. Detecting cis-regulatory element differences associated with trait evolution is challenging because many enhancers can preserve the same regulatory function even when the underlying genome sequence is highly divergent, and many cis-regulatory elements have tissue-specific activity (66–68). Thus, methods for convergent evolution

that rely on the alignment of individual nucleotides between species (11, 69, 70) are likely to miss a substantial proportion of key candidate enhancers.

We therefore sought to extend our search for cis-regulatory elements whose evolution is associated with vocal learning behavior using a recently developed machine learning approach, TACIT (23). Given that it is infeasible to map the brains and collect motor cortex tissue from each vocal learning and closely related non-learning species, the TACIT approach uses machine learning models (23) to predict motor cortex open chromatin across orthologous regions of the genome (66–68). TACIT then associates predictions with vocal learning in a way that corrects for phylogenetic relationships (Fig. 4A). We used the predictions from convolutional neural networks (CNNs) trained by using DNA sequence-based M1 open chromatin data obtained in this study for *R. aegyptiacus* with ATAC-seq and collected earlier for the mouse (21), rat, and macaque (20) to predict motor cortex open chromatin across 222 mammalian genomes (materials and methods) (23). Given that parvalbumin has been shown to be a shared marker of brain areas critical for vocal learning in songbirds and humans (4), we also used CNNs trained



**Fig. 3. Differential open chromatin in bat ofM1 relative to wM1.** (A) Open chromatin was profiled from seven dissected brain regions of the Egyptian fruit bat. Premotor vocal area, PreM; auditory cortex, A; cerebellum, Cb; caudate, C; putamen, P. (B) Volcano plot of ATAC-seq OCRs with differential activity between ofM1 and wM1 of the Egyptian fruit bat. (C) Genome browser showing ofM1 and wM1 ATAC-seq traces at the 3' end of the *FOXP2* locus. Reproducible M1 open chromatin regions (OCRs) are indicated in blue, with a differentially active OCR in ofM1 relative to wM1 indicated in red.



**Fig. 4. Vocal learning-associated convergent evolution in motor cortex open chromatin regions implicates specific neuron subtypes.** (A) Overview of the TACIT (23) approach to vocal learning. OCRs were identified in the motor cortex (M1). Measured open chromatin from the M1 (four species) was used to train CNNs to predict M1 open chromatin from sequence alone. Red bars and corresponding arrows indicate the presence of a peak, whereas the blue bars represent the absence. The same OCRs were then mapped across 222 mammalian genomes (left), and the identified sequences were used as input to the CNNs to predict open chromatin activity. TACIT identified OCRs whose predicted open chromatin across species was significantly associated with those species' vocal learning status. (B and C) The four-way Venn diagrams represent the number of OCRs implicated by TACIT (both M1 and PV+) as displaying low (B) or high (C) activity in each of the vocal learning clades based on a *t* test. (D) The heatmap visualizes specific open chromatin regions along the

to predict cell type-specific open chromatin by using ATAC-seq data from mouse and human M1 parvalbumin-positive neurons (M1-PV+) (22, 23, 71). We identified regions whose predicted open chromatin was consistently lower or higher in vocal learners relative to vocal nonlearners by using phylogenetic logistic regression (72, 73) with phylogenetic permutations (24) [permutations adj.  $P < 0.1$ ; materials and methods, (23)] (Fig. 4A). We identified 33 open chromatin regions from our M1 CNN models that had lower predicted open chroma-

tin in vocal learning species and 11 that had higher predicted open chromatin in vocal learning species (Fig. 4D, table S1, and data S7). From the M1-PV+ predictions, we identified five candidate enhancers that had lower predicted activity in vocal learning species and one candidate enhancer that had higher predicted regulatory activity in vocal learning species. (table S2 and data S7). Unlike the protein-coding genes, most vocal learning-associated enhancers showed evidence of higher or lower activity in at least three out of the four vocal learning clades (Fig. 4,

rows (predicted higher in vocal learners, green; predicted lower in vocal learners, purple) across 222 mammals in the columns (vocal learners, red; vocal nonlearners, black; insufficient or conflicting evidence, gray). The color in each cell corresponds to the z-scored predicted open chromatin, with low open chromatin in blue, mean open chromatin in white, and high open chromatin in red. (E and F) Plots comparing vocal learning-associated open chromatin regions with those from cortical mouse cell types. For open chromatin regions predicted to be significantly less (E) or more (F) open in vocal learning species ( $P < 0.05$ ), the red point indicates the number of overlapping regions (*y* axis) across mouse cortical cell types (*x* axis). The bar plot shows the distribution across 1000 permutations of the peaks implicated by TACIT. The notches extend  $1.58 \times \text{IQR} / \sqrt{n}$  (IQR, interquartile range), which gives a confidence interval of roughly 95%. Cell types were ordered based on their enrichment measured by permutation for the peaks lower in vocal learning species.

B to D, and data S8). Consistent with the finding that convergent vocal learning-associated gene regulation is primarily repressive (4), we found that most candidate enhancers ( $n = 38/50$  OCRs, 76%) had lower predicted open chromatin activity in vocal learning relative to vocal non-learning mammals (fig. S7).

To interpret potential functions of the vocal learning-associated candidate enhancers, we annotated the nearest genes in the mouse (data S9). In many cases, the genes closest to these putative enhancers have been associated with

significant developmental delay or complete absence of speech when disrupted in humans (tables S1 and S2). Four of the OCRs identified by the M1 model were proximal to genes—*GALC*, *TCF4*, *TSHZ3*, and *ZNF536*—that were also near OCRs with differential activity between bat ofM1 and wM1 (data S6). Two of the vocal learning-associated M1 OCRs were proximal to genes *DAAMI* and *VIP*, which were previously shown to have convergent gene expression between humans and song-learning birds (4). To further explore the function of the vocal learning-associated OCRs in the motor cortex, we annotated their cell-type specificity using publicly available mouse BICCN data (74). The cell type most enriched for OCRs predicted to be lower in vocal learning species was the layer V extratelencephalic (ET) neuron, which has previously been implicated in vocal learning (75), but strong enrichments were also found for other cortical excitatory neurons (Fig. 4, E and F, and data S10).

Among genes near vocal learning-associated OCRs, the *DACT1* (TACIT adj.  $P = 0.0014$ ; RERconverge  $\tau$  adj.  $P < 0.0001$ ) and *CELF4* (TACIT adj.  $P < 0.023$ ; RERconverge  $\tau$  adj.  $P < 0.0034$ ) proteins also displayed significantly lower relative evolutionary rates in vocal learners. Despite the lack of direct evidence in the literature for its role in speech production, *CELF4* has been associated with autism in the human population (76), and its function in layer V pyramidal neurons has been linked with seizures in mice (77).

Multiple M1-PV+ interneuron OCRs associated with vocal learning are near genes previously associated with autism. For example, an OCR that is negatively associated with vocal learning evolution is in an intron of the gene *CCSER1*, which has nonsense mutations implicated in autism (78) and is in a locus associated with musical beat synchronization (79). An OCR that is positively associated with the evolution of vocal learning is in an intron of the gene *CNTNAP4*, whose deletions and copy number variation in humans and mice have been implicated in neurological disorders, including autism in humans (80, 81). To test whether these associations would have been identified by chance, we tested whether vocal learning-associated OCRs tended to be near genes associated with autism. We found that the M1-PV+ OCRs with human orthologs near genes associated with autism (82) tended to be more significantly associated with vocal learning evolution than other OCRs with human orthologs (Wilcoxon  $P = 0.0071$ ).

## Discussion

Convergent evolution of vocal learning has been associated with convergent evolution at the neuroanatomical level: Cortical motor regions driving vocal production in humans and songbirds [human motor cortex and songbird

robust nucleus of the arcopallium (RA)] show increased connectivity with the brainstem and striatum (3). These same motor regions also show convergent evolution in patterns of gene expression, with commonly decreased gene expression found in both song-learning birds and humans (4). In this study, we investigated convergent evolution of vocal learning in mammals, both at the anatomical and the genetic levels. Firstly, we found a direct motor corticobulbar connection from a cortical region implicated in vocal production in a vocal learning bat. Secondly, we revealed widespread evidence of convergent evolution across vocal learning mammals in protein-coding sequences and candidate regulatory enhancers.

Our parallel study of both coding and noncoding regions linked with the vocal learning trait identified many protein-coding genes (200) and a smaller number of noncoding regions (50), distal sites of open chromatin that are associated with vocal learning. Although a larger number of significant protein-coding genes were identified, the vast majority of these were primarily driven by strong evidence in one of the vocal learning clades and only weak evidence in the other three. By contrast, most significant noncoding regions showed robust evidence of convergent selective pressure in at least three out of the four clades. The larger number of identified proteins relative to open chromatin regions could have been due to better statistical power from being able to directly model nucleotide evolution in protein-coding sequences, which tend to be more stable than regulatory elements across species (11, 12). However, only 5/200 of the significantly associated protein-coding genes showed robust evidence of differential rates of evolution in at least three of four vocal learning clades. Out of these 200 proteins, many were neurodevelopmental transcription factors, which are among the most highly conserved genes in mammals (83) and thus likely to play roles in a broad range of contexts that could constrain their evolution. It is also possible that some of the identified proteins could be associated with other convergent traits that correlate with vocal learning across mammals, including echolocation (bats and cetaceans), marine adaptations (cetaceans and pinnipeds), or increased longevity (bats, cetaceans, and humans).

By contrast, 33/50 vocal learning-associated candidate enhancers had differential predicted open chromatin in at least three of four vocal learning clades; this independent convergence of gene regulatory function suggests that these enhancers may be critical for the evolution of vocal learning. Enhancers tend to have functions that are much more context- and tissue-specific (68), making them less functionally constrained than protein-coding genes, which could perhaps allow more flexibility for an individual enhancer to evolve a new role for a specific trait such as

vocal learning. Our results suggest that the evolution of mammalian vocal learning is largely driven by changes to the noncoding regulatory elements that orchestrate gene expression rather than by changes to the protein-coding genes themselves.

Despite the different methodologies applied to identify convergent evolution in coding and noncoding regions, both protein coding- and regulatory element-focused approaches implicated gene functional pathways associated with human autism. In our protein-coding analyses, genes with lower evolutionary rates based on both RERconverge and HyPhy RELAX were enriched for autism function. Likewise, in our analyses of regulatory evolution, multiple autism-linked genes were near human orthologs of the vocal learning-associated M1-PV OCRs. In humans, autism is often associated with speech delays and differences in social behavior, both of which could be related to the evolutionary trait of vocal learning ability (84). Broadly, this could be evidence that genomic loci associated with a complex trait across mammals may also be associated with variations in related traits within the human population.

The bulk motor cortex OCRs with lower predicted open chromatin in vocal learners showed the strongest tendency to overlap with OCRs in layer V ET neurons, which form long-range projections to the hindbrain motoneurons that control vocal production (Fig. 4E). These results are consistent with our previous findings that showed decreases in the expression of axon guidance genes in the motor cortex of vocal learning species (4). Among other functions, the layer V ET neurons implicated by TACIT create the corticospinal projections that have been hypothesized as an anatomical landmark of vocal learners (2, 3, 48, 49, 56, 85, 86). Furthermore, the neuroethological and anatomical experiments we conducted in *R. Aegyptiacus* provide evidence that corticospinal projection neurons are present in the motor cortex of the bat species and that this motor cortical region participates in vocal production. Thus, consistent with previous literature, our results support a model in which the loss of regulatory element activity in the motor cortex influences axon guidance properties of long-range projection neurons, which allow more robust connectivity between the cortex and the brainstem of vocal learning mammals (4, 85, 87). Alternatively, these genetic differences could relate to potential differences in the density of disynaptic connections that have been associated with skilled motor behavior, including vocalization in nonhuman primates (88, 89). Notably, these long-range projection neurons have also been associated with predisposition to autism (90).

## Materials and methods summary

To find vocal learning-associated convergent evolution in protein-coding sequences

of the mammalian genome, we began with amino acid-level multiple sequence alignments produced by the Zoonomia consortium (10). Those served as input to two classes of methods, RERconverge (11) and HyPhy (30). RERconverge with an additional permutations correction for phylogenetic structure (24) was used to find protein-coding sequences whose evolutionary rates were associated with the presence or absence of vocal learning. HyPhy RELAX was used to find protein-coding sequences that were evolving more slowly, neutrally, or faster in vocal learning species. In addition, the HyPhy BUSTED-PH method (12) was applied to find evidence of diversifying positive selection. The gene ontology analysis was performed on the intersection of the RERconverge and HyPhy results using EnrichR (91). To control for false positives across all methods, Benjamini-Hochberg false discovery rate correction (92) was applied.

To examine the existence of a direct monosynaptic projection in a vocal learning mammal, the corticobulbar projections in Egyptian fruit bats were mapped by tracing the projections from the orofacial motor cortex and from the cricothyroid muscles of the vocal cords. Performing immunohistochemistry in the brainstem revealed that synaptic boutons of cortical projection neurons overlapped with retrogradely-labeled motoneurons, confirming the existence of a direct monosynaptic projection. The role of the orofacial motor cortex during vocal production was then validated by quantifying the information between the vocalization amplitude and single cortical neuron activity measured wirelessly in vivo while the bats produced and listened to vocalizations.

To create an atlas of open chromatin regions in the bat MI, several brain regions, including wMI and ofMI, were separately dissected. The samples were cryopreserved, then the nuclei were isolated, and subsequently ATAC-Seq was performed to measure open chromatin. The open chromatin regions from this experiment were combined with previously published experiments in macaque, rat (23), and mouse (21) to create an atlas of cross-species motor cortex open chromatin.

To find vocal learning-associated convergent evolution in noncoding regions of the Boreoeutherian mammalian genome, the TACIT machine learning approach, was applied. Orthologous regions across genomes were found by combining the CACTUS whole-genome multiple sequence alignment (19), halLiftOver (93), and HALPER (94). Phyloglm (72) was then used to associate predicted motor cortex and parvalbumin-positive inhibitory interneuron open chromatin with binary annotations of vocal learning behavior. Phylogenetic permutations were applied to correct for phylogenetic tree structure and Benjamini-Hochberg to correct for multiple hypothesis testing. To identify potential trends in the cell-

type specificity of the implicated regions, permutations on the regions of the genome that were predicted to have significantly higher or lower open chromatin in vocal learning species were conducted.

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**Data and materials availability:** Egyptian fruit bat ATAC-seq data, including raw .fasta files, .bigWig files of genome-aligned reads, processed open chromatin .narrowpeak files, and extensive sample metadata, have been made available at GEO under accession ID GSE187366. Publicly available ATAC-seq data were obtained from GEO GSE159815 and GSE161374. Single-nucleus open chromatin was downloaded from the NEMO archive: [https://data.nemoarchive.org/biccn/grant/u19\\_cemba/cemba/epigenome/snccell/ATACseq/mouse/](https://data.nemoarchive.org/biccn/grant/u19_cemba/cemba/epigenome/snccell/ATACseq/mouse/). The code for the electrophysiology and functional components of the project are available here: <https://github.com/NeuroBatLab/LoggerDataProcessing/>; <https://github.com/NeuroBatLab/SoundAnalysisBats/>; <https://github.com/NeuroBatLab/LoggerDataProcessing/>; and [https://github.com/julielie/Kilosort2\\_Tetrode/configFiles/configFile16.m](https://github.com/julielie/Kilosort2_Tetrode/configFiles/configFile16.m). The code for the convergent evolution analysis can be found at <https://github.com/pfenninglab/TACIT>, <https://github.com/veg/hyphy>, and <https://github.com/veg/hyphy-analyses/tree/master/BUSTED-PH>. The histological and electrophysiology source data from this study are available on FigShare at <https://doi.org/10.6084/m9.figshare.25180430> (95) The code for conducting and visualizing the RERconverge analysis can be found here: <https://zenodo.org/doi/10.5281/zenodo.10641176> (96). The code for conducting and visualizing the TACIT analysis can be found here: <https://zenodo.org/doi/10.5281/zenodo.5952292> (97). **License information:** Copyright © 2024 the authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original US government works. <https://www.science.org/about/science-licenses-journal-article-reuse>

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## SUPPLEMENTARY MATERIALS

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## Vocal learning–associated convergent evolution in mammalian proteins and regulatory elements

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### Editor's summary

The ability to perform complex behaviors, including human speech, is encoded in the billions of nucleotides that make up the genome, the result of millions of years of evolutionary optimization. Although speech itself is uniquely human, vocal learning, the ability to modify vocal output as a result of experience, has evolved independently in multiple mammals, including many bats. Wirthlin *et al.* combined experiments in the bat brain with an artificial intelligence–based analysis of more than 200 genomes and found hundreds of genomic regions that are associated with vocal learning. Their results implicate networks of genes related to human autism. —Di Jiang

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