Appendix

Section 1. Glossary

Gene. A transcriptional unit of the genome that gives rise to one or more RNA variants, including mRNA variants.

mRNA. A transcript that is made from a gene of the genome and that usually includes 5' and 3' UTR regions, a protein coding sequence, and a polyA tail at the 3' end.

mRNA Variants. Alternatively spliced, alternatively polyadenylated, alternatively initiated, sense, antisense, and RNA edited transcripts that most commonly arise from the same gene in the genome. However, mRNA variants could also arise from duplicated genes in the same animal that have small differences between the genes or the same gene in different animals that have been mutated in one animal relative to another. Distinguishing the source of a given variant usually requires the genomic sequence of one or more individuals.

cDNA. A complementary DNA copy of the mRNA.

Full-Length cDNA. A cDNA of the entire mRNA, including the 5' UTR, protein coding sequence, and 3' UTR. Because it is difficult to determine the start of the 5' UTR without genomic DNA and other experimental procedures, often the assessment of full-length cDNA is determined by whether there is a full protein coding sequence and the presence of a 5' UTR. This criterion was the one used in this study.

Partial cDNA. A cDNA that contains a partial sequence of the mRNA, often not including the 5' UTR and the beginning of the protein coding sequence, because of incomplete synthesis of the first-strand reaction in constructing cDNA libraries. This criterion was the one used in this study.

cDNA Cluster. A set of cDNAs with similar but sometimes not identical sequences where the differences, when present, can include cDNA variants, such as spliced and alternatively polyadenylated. A cluster theoretically represents one gene of the genome and its mRNA variants; variants can also be from duplicate genes of the genome with alternative sequences.

cDNA Subcluster. A set of cDNAs with nearly identical sequence. Any small nucleotide differences could be the result of either sequencing or cloning artifacts or SNPs among animals. A subcluster theoretically represents one mRNA of the transcriptome.

Regular cDNA Library. Library of cDNAs where the transcript population is representative of the mRNA population from which the library was made. Thus, high-, intermediate-, and low-abundance mRNAs will be present at similar relative amounts in a regular cDNA library.

Normalized cDNA Library. Library of cDNAs where the transcript population has been subtracted against itself, reducing redundancy of high-abundance mRNAs and increasing representation of low-abundance mRNAs such that all cDNAs are present at a similar frequency. Normalization saves time and cost in the isolation of unique transcripts when randomly picking cDNAs from the library.

Abundant cDNA Library. Library of cDNAs left over from normalization, where the transcript population is enriched for abundantly expressed genes.

Subtracted cDNA Library. Library of cDNAs where the transcript population of one sample has been subtracted from the transcript population of a second sample, leading to enrichment of transcripts specific to the second sample. Thus subtraction facilitates isolating regulated transcripts in the sample of interest but also increases the frequency of isolating those transcripts when randomly picking cDNAs from the subtracted library.

Transcriptome. Whereas the genome includes all genes and intergenic DNA of an organism, the transcriptome includes all RNAs synthesized in the lifetime of an organism, including protein coding, non-protein-coding, alternatively spliced, alternatively polyadenylated, alternatively initiated, sense, antisense, and RNA edited transcripts (1).

Protein Coding Sequences (cds). The cds, also known as the open reading frame (ORF), is the part of the cDNA that encodes the protein.

UTRs. The UTRs, or untranslated regions, are the parts of an mRNA that do not code for the protein. The UTRs are at the 5' and 3' ends of the mRNA and are called 5' UTR and 3' UTR, respectively.

Section 2. Supplemental Results

Supplemental results are shown in Figs. 9–19 and Tables 1–6 and as referenced in the main text. The figures are presented in the order of material as described in the main text. Below are additional descriptions of supplementary results:

2.1 Concordance of Microarray Data with *in Situ* Hybridization Data. The microarrays were initially analyzed as described in *Results* of the main text. After obtaining the set of 41 true- and false-positive transcripts (Table 5), we reanalyzed the microarray hybridization data using GeneSpring analysis software (Agilent Technologies, Palo Alto, CA) for concordance with the *in situ* data. First, we noted that in each array there was a dye bias in one direction or the other in the 3X SSC spots, which is normally not removed in microarray data processing. To remove this bias, for each array hybridization, we determined the average ratio difference for the 3X SCC spots and used that number to multiply all spot intensities of one dye (the one with the bias) such that the average ratio of the new 3X SCC values was 1. Then, we subtracted the background intensities of the area around each spot without DNA. We then filtered these values on the average intensities of the plasmid DNA spots +2 times the SD if and only if both dye intensities of each spot were equal to or below this threshold. The remaining spot intensities represent hybridization to DNA, and thus, this processing reduces statistical error. Next, we imported all processed data for each array into GeneSpring software and performed *t* tests on the log ratios for each spot across the 3 or 4 replicate arrays per vocal nucleus. To determine a *P* value cut-off, we noted that before filtering the 3X SCC and plasmid DNA spots, all had *P* values >0.2 (Fig. 14*D*), and many of the regulated genes had *P* values >0.2. Thus, we used this *P* value to assess the concordance of the array data with the *in situ* data (Table 5). When doing so, 55% of the 41 genes on the array were statistically concordant with the *in situ* data. The nonconcordance of the remaining genes indicates that although some replicate samples produced results of induced expression, this number of replicates is not sensitive enough for statistical analysis although it is for qualitative human examination. Some of the nonconcordance could be due to the possibility that the microarrays were hybridized with samples from animals that sang for 1 h, whereas the *in situ*s showed peak expression for some genes by 3 h.

2.2 Cross-Species Hybridizations. It is well known that protein coding regions, which are included in full-length cDNAs, are more highly conserved between different species than

noncoding regions. In this regard, full-length vs. partial cDNAs can affect the level of success for cross-species hybridizations. For example, the 3' end of the zebra finch FoxP1 cDNA does not cross-hybridize to FoxP1 mRNA in parrot brain, whereas a full-length zebra finch clone cross-hybridizes (Fig. 9) (2). To determine whether our zebra finch full-length cDNAs on the microarrays will cross-hybridize to other avian species, we examined and successfully cross-hybridized canary probes for brain sex differences (Fig. 18).

2.3 Library Representations. We performed an analysis to determine whether library subtraction was effective at increasing the proportional representation of the 33 singing-regulated genes (Table 6). We tallied the library source for clones representing the 33 genes, using the total number of clones picked from each library, to generate observed percentage values versus expected percentage values, with the null hypothesis that the clones were distributed randomly among libraries. We then used a conservatively chosen threshold of 10% from the expected percentage when there were six or more clones of the same mRNA variant in the database. Relying on percentage values generated from fewer than six clones would bias the analysis across the six libraries examined. By using these criteria, 12 of the 33 genes met this cut-off (Table 6, red colored values). Of these, 10 were represented at a higher proportion among the subtracted libraries (Table 6, bolded values). Within the subtracted libraries, however, there were differences. For example, c-jun and Hsp70 were enriched in the adult singing–silent subtracted library (0062), whereas Hsp90α and JSC were enriched in the juvenile singing–silent and other juvenile subtracted libraries. For genes where the number of isolated clones was less than six, such as for *c-fos*, there was no apparent enrichment in the subtracted libraries, even when there were a large number of total clones picked from a library (>2,000). These findings suggest that subtraction was partly effective at isolating singing-regulated genes.

Section 3. Supplemental Materials and Methods

Supplemental methods are shown in Figs. 9–21 and as referenced in the main text. Fig. 19 shows an outline of our approach that is useful for following each of the major steps below, starting with prior knowledge for collecting brains of animals in specific brain states.

3.1 Species, Brain States, and Behaviors. We chose the zebra finch (*Taeniopygia guttata*) because it is the most commonly used songbird in neuroethology. Animals were bred at Duke University, Dokkyo University, Tokyo Medical and Dental University, or the City College of

New York. All animal procedures were approved in accordance with the Animal Care and Use Committees at the respective universities. We collected brains of 60 animals in 57 different brain states within 15 experimental categories (Table 1) to clone as much of the songbird brain transcriptome as possible. This large collection of brain states ensures expression of many RNAs of the brain transcriptome, a prerequisite before constructing cDNA libraries. The number of animals used is more than the number of states because of duplication of those in directed and undirected singing for different libraries (bird IDs 58–60 vs. 31 and 32). For constructing normalized libraries, it is not necessary to include replicate mRNA samples of a condition to clone the regulated mRNAs. They will potentially be cloned as long as they are expressed in the animal used for the library. Animal states are:

(IDs 1–4) Embryonic samples. Zebra finches take ≈13–15 days to develop *in ovo*. We collected brains under a dissecting microscope at embryonic days 10 and 15 (E10 and E15), with the goal of obtaining brain sex-determination genes and genes involved in embryonic neurogenesis. Breast muscle samples were used to determine sex by PCR of sex-specific genes (method below); one male and one female brain of each age (E10 and E15) were then taken for cDNA library construction.

(IDs 5–12) Developmental posthatch samples. After hatching, zebra finches take ≈18–23 days to fledge the nest. During this period, the size of the male vocal nuclei grow, whereas those of the female atrophy (3). We collected brains of animals at posthatch day 1 (PH1), when vocal nuclei have not yet been identified; PH5 and PH10, when vocal nuclei are readily identified; and PH15, when the vocal nuclei begin to show atrophy in females. Breast muscle tissue samples were used to determine sex by PCR analysis of sex-specific genes; one male and one female brain of each age were then taken for cDNA library construction.

(IDs 13–28) Vocal learning phases. Zebra finch vocal learning occurs in four overlapping developmental phases (4): (*i*) auditory acquisition of a tutor's songs from PH23–25 onward, (*ii*) subsong, which is akin to human infant babbling from ≈PH30–40, (*iii*) early to late phases of plastic song from ≈PH40–70, and (*iv*) adult crystallized song from ≈PH90 onward. For each of these phases, we collected brains of males (≈PH25, -35, -55, -70, and -85) that were in overnight silent conditions, followed by 2 h of silence in the morning or 2 h of hearing playbacks of songs and singing. For silent animals, the presence of subsong or plastic song was checked the day before sacrifice. For the singing animals, these song types were verified the day of sacrifice. The

silent animals will have genes that are down-regulated in the singing animals at each vocal learning phase. The singing animals will have genes that are induced between 0 and 2 h after singing at each vocal learning phase. Males at PH25 do not sing, and therefore, we have no singing group for this age. We also collected brains of females hearing playbacks for 2 h from the same ages as the males, because we surmised that females must learn to recognize the songs of males during development and this could be accompanied by changes in gene regulation different from those in males. All behavior was conducted in sound-isolation boxes and was video and audio taped for experimental verification and archival purposes.

(IDs 29–35 and 57) Sensory- and motor-regulated waves of gene expression. Hearing novel song (sensory stimulation) induces a minimum of two waves of gene expression in the auditory forebrain (5). These waves of mRNA syntheses are required for the formation of sensory and, perhaps, motor memories. To capture these mRNAs, we placed adult animals (>PH150) overnight into sound-isolation chambers, and, on the next day, we collected the brains of a male and female in silent conditions (0 min), a male hearing playbacks of song that is novel to him (one song bout per minute) where he sang undirected song in response for 0.5 h, another hearing playbacks of a new song every 0.5 h and singing for 2 h, another for 6 h, and a female hearing novel songs and responding with calls for 2 h. From the 0 time-point sample, we expect to clone cDNAs that are down-regulated by sensory and motor behaviors; from the 0.5-h sample, those up-regulated in the beginning of the first wave; from the 2-h sample, those at the end of the first wave; and from the 6-h sample, those at the end of the second wave. Playing a new song every 30 min ensured continued singing from the listening bird and theoretically ensured induction of overlapping waves of gene expression (in sensory and motor brain areas), in case there are other time windows where expression changes occur. In these waves of gene expression, *BDNF* is also induced and related to enhanced survival of new neurons in song nuclei (6); therefore, we increase the probability of cloning such genes in our libraries. Including brains from females ensured cloning of cDNAs that represent genes induced by hearing or vocalizing innate calls that may be different from those induced in males.

(IDs 31, 32, and 58–60) Social context. Singing in different social contexts results in different patterns of gene induction among vocal nuclei (7). Undirected singing causes high levels of *egr-1* gene expression throughout the vocal system, whereas directed singing to another bird causes moderate levels of *egr-1* gene expression in the lateral half of the anterior vocal pathway and in the motor pathway's robust nucleus of the arcopallium (RA). Thus, we collected the brains of

three birds singing directed song to a female for 30 min (IDs 32, 58, and 59). We performed these collections at different times for different libraries. We collected two additional birds singing undirected song for 30 min while alone in a sound box (ID 31) and while in a cage in an aviary (ID 60), and this difference in context results in differences in egr-1 protein expression in RA (8).

(ID 36) Aging. Zebra finch lifespan in captivity is usually 5–7 years. We collected the brain of a 3.5-year-old male after he had been singing for 30 min. We expect to clone some cDNAs representing genes associated with aging in the brain.

(ID 37) Sleep. A number of electrophysiological and molecular changes occur during sleep in mammals and in songbirds. These changes include (*i*) reinduction of immediate early genes after REM sleep and a day of rich environmental experience (9) and (*ii*) after replay of singing-like electrophysiological activity (10). To capture cDNAs representing genes regulated in some of these states, we collected the brain of a male that had been sleeping for 2 h in the dark after a day of hearing and singing in the presence of other birds singing. We surmised that 2 h is sufficient time to induce genes in song nuclei due to song replay of electrophysiological activity and also that darkness will induce expression of circadian clock-regulated genes (11).

(IDs 38–40) Kainate-induced seizures. Kainate is an agonist for kainate and other (at high concentrations) glutamate receptors. When injected at high levels, kainate causes epileptic seizures (12). Seizures induce expression of genes involved in signal transduction and brain repair (13). Three male zebra finches were given an i.p. injection of 10 mg/kg of body weight kainate in saline. Seizures were observed for ≈0.5 h, and brains were taken at 0.5, 2, and 6 h. These time points are expected to capture the waves of gene induction after activation of glutamate receptors.

(IDs 41–42) Apomorphine. Apomorphine is an agonist of dopamine receptors (both D1 and D2). Through these receptors, apomorphine induces expression of a large set of genes in mammalian striatum (14). Two males were given an i.p. injection of 1 mg/kg of body weight apomorphine in saline. Brains were taken at 0.5 and 2 h, respectively.

(ID 43) Deafening. Deafening prevents vocal learning, and it causes already learned song to deteriorate (15, 16). We collected the brain of an adult animal that had been deafened at PH15. Thus, the bird did not have the opportunity for vocal learning. The animal was alone in a sound-isolation chamber 2 h before collection.

(IDs 44–47) Social isolation. Social isolation (acoustic and visual) also prevents normal vocal learning and extends the critical period for vocal learning (17). Four males were socially isolated at PH24–25 before the critical period of vocal learning, followed by four different experiences: one male was placed in the aviary as an adult at PH120, remained there to interact with other birds for 2 years, and is, thus, expected to have long-term affects of social isolation; another was placed in the aviary at PH120 and taken several weeks thereafter for short-term affects; another was kept isolated up to PH67 and then heard 30 min of song playbacks for the first time to induce genes involved in auditory acquisition for vocal learning, with a delayed critical period; and, finally, another was kept isolated up to PH67 without any social experiences to clone genes that are aberrantly regulated by social isolation.

(48–56) Rapid vocal learning. We used a previously described rapid vocal learning paradigm (18) to further ensure that we obtain cDNAs of genes activated during vocal learning. Juvenile males (n = 8) were housed with only their mothers until the age of 30 days. Thereafter, they were housed singly in sound-isolation chambers. At PH38–57 days old (day 0 of the experiment), a lever switch with a red disk attached was placed next to a plastic model bird. When the young juvenile spontaneously pecked the disk, a tutor song (we called SAMBA) was played from a speaker housed inside the model bird. Only 25 s total of song that day (day 0) was allowed, regardless of the number of times the disk was pecked. No more song playbacks were given on any other day. The birds were then killed at different times of song imitation (Fig. 10): day 1 of training and then singing but no signs of vocal change (bird ID 51); day 1 of training (9:45 a.m.), inserting a stop in a harmonic stack, cleaving the sound into two syllables in the hour before sacrifice at 3 p.m. (ID 48); day 2 after training, showing a typical posttraining elevation of subsong pitch over 5 h before sacrifice (ID 54); day 3 after training, evening, showing a second phase of pitch elevation with mean pitch increasing by 7 p.m. (ID 50); day 3, afternoon, showing insertion of short-duration high notes in vocal output (ID 55); day 4, morning, 1 h after singing began, showing recovery of song structure from overnight deterioration (ID 49); day 7, afternoon, showing stereotyped syllable structure and syntax, singing within the last 30 min (ID 52); and day 8, midday, producing syllables and overall motif structure clearly matching the song model, SAMBA (ID 53). We included a control bird who had access to the training keys, but was given no playback on day 1 and did not show signs of change in its isolate song that day or the next few days (ID 56). This bird was killed after it had been singing isolate song for several hours.

3.2 PCR Protocol for Sex Determination. Embryonic and juvenile animals do not have sex plumage differences and thus their sex was determined by PCR of genomic size differences for the chromo-helicase-DNA-binding protein (CHD) on the W (male) and Z (female) chromosomes (19), with minor modifications. To isolate genomic DNA for PCR, breast muscle tissue or blood was lysed in 100 μl of digestion buffer (10 mM Tris·HCl (pH 8.0)/150 mM NaCl/10 mM EDTA/0.1% SDS) with 2 μg of Proteinase K (Qiagen, Valencia, CA) at 65°C overnight, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated in ethanol. PCR primers were: 5'-TTGCCAAGGATGAGAAACTG-3' and 5'-TCTTCTCCTCCTACTGTGTT-3'. The primers bind both CHD-W and CHD-Z. PCR was performed at 94°C for 5 min for 1 cycle, followed by 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for 32 cycles. Males have one band at ≈1,000 bp from CHD-W, and females have two bands, one at ≈1,000 bp from CHD-W and the other 267 bp from CHD-Z.

3.3 Construction of Full-Length cDNA Libraries. From each brain, total RNA was isolated by using the Trizol method (GIBCO-BRL, Carlsbad, CA), followed by mRNA isolation using standard kits (Qiagen). All samples, except for those used to generate our initial libraries (#0031, 0033, 0052, 0054, 0055, and 0056; Table 2) and our embryonic libraries (0057), were treated with DNase (Promega, Madison, WI). We did not treat the embryonic samples with DNase because further processing of the small mRNA amounts recovered from these small brains would have resulted in RNA concentrations too low for library construction. We kept the embryonic samples separate from the 50-mix library (0058) to prevent diluting the concentration of potential transcript variants of developmentally regulated genes. No size selection was performed, and thus, RNAs of all sizes are cloned.

Regular full-length cDNA libraries were constructed by using the method developed by Carninci (20), normalized libraries by the method of Carninci *et al.* (21), and subtracted libraries by the method of Hirozane-Kishikawa *et al.* (22), all using the 5'-cap-trapper approach (20) with the following modification: first-strand cDNA synthesis was performed in individual reactions for the brain mRNA of each bird, using oligo-dT_{16}-3' primers with unique sequence IDs (Table 1). For normalized libraries, the first-strand reactions were then pooled (0.3 μg each) in various combinations, their second strands synthesized, and the resultant cDNAs processed without further protocol modification. For subtracted libraries, first, regular cDNA libraries were made of specific brain samples (Table 2). Second, these were converted to single-strand sense and antisense strands with the subtracted driver antisense in 12.2- to 74.1-fold excess; we focused on

enriching for genes related to (*i*) male brain (0061), (*ii*) adult singing (0062), (*iii*) rapid vocal learning (0063), (*iv*) juvenile brain (0064), and (*v*) juvenile singing (0065, Table 2). For all library types, the basic 3' primer for first-strand syntheses was 5'-GAGAGAGAGAGGATCCACAC-CHANGE-T_{16}VN-3'; the underlined GGATCC sequence is the BamHI restriction enzyme site; the sequences further 5' are cleaved off during cloning; the underlined text "CHANGE" represents the six nucleotides that are changed to serve as unique 3' primer ID tags for each animal (Table 1); the V = A,G,C and *n* = A,G,C,T redundancies help anchor the oligo-dT_{16} of the primer to the beginning of the polyA tail of the mRNAs. The first-strand reactions yield more than enough material to make one library and thus provide products for the various regular, normalized, and subtracted libraries (Table 1).

The 5' primers used for second-strand synthesis had unique sequence IDs to tag all zebra finch libraries made by using the RIKEN method, and these were

GN5: 5'-AGAGAGAGAGCTCGAGCTCTACACAGGTGACACACTAGAACCAGNNNNN-3'

N6: 5'-AGAGAGAGAGCTCGAGCTCTACACAGGTGACACACTAGAACCANNNNNN-3'

PN:3'**NH2***-TCTCTCTCTCGAGCTCGAGATGTGTCCACTGTGTGATCTTGGT-P-5'

Second up: 5'-AGAGAGAGAGCTCGAGCTCTACACAGGTGACACACTAGAACCA-3'

The GN5 and N6 primers were preannealed to the PN primer to make double-stranded linkers. The 5 and 6 degenerate Ns at the 3' end of the GN5 and N6 primers, respectively, were used as overhangs, which bind the 3' ends of the first-strand cDNAs. After binding, the second strand was synthesized. The underlined sequence "GAGCTC" is the XhoI restriction site used for cloning; the sequences further 5' were cleaved from the final insert during cloning. The XhoI- and BamHI-restricted cDNAs were cloned into the SalI (sharing the same overhang sequence with XhoI) and BamHI site of the λ-Full-Length-Cloning vector 1E (λ-FLC-I-E), replacing the E stuffer (23) (Fig. 20). To prevent restriction digest of inserts when cloning in the vector, the cDNA syntheses reactions include methylated nucleotides.

To produce the male-enriched library #0061, cDNAs from the silent adult male library #0066 were subtracted with those from the adult female library #0067. Because the female also heard

song, subtracted library #0061 is expected to contain cDNAs enriched for genes down-regulated by sensory stimulation. To produce singing behavior-enriched library #0062, cDNAs from the male 2-h singing library #0068 were subtracted with those of the adult male silent library #0066. To produce rapid vocal learning-enriched library #0062, cDNAs from library #0069 of a tutored juvenile whose songs were rapidly changing were subtracted with those from #0070 of a nontutored juvenile bird whose song was not changing. To produce vocal learning-enriched library #0064 that would have cDNAs induced by singing, cDNAs from juvenile library #0069 were subtracted with those of the silent adult male library #0066. To produce a vocal learning-enriched library #0065 that would have cDNAs induced during learning independent of singing, cDNAs from juvenile singing library #0069 were subtracted with those of the adult male singing library #0068.

3.4 Bacterial Stocks and Plasmid DNA Preparations. From the library stocks, plasmid transformants were plated out at a density of 1,000–2,000 colonies per 150-mm LB agar plates supplemented with 100 μg/ml ampicillin. Colonies were hand picked with sterile micropipette tips into 1.5 ml of LB media supplemented with 100 μg/ml ampicillin in 2-ml-capacity 96-well plates (USA Scientific, Ocala, FL). After 6–8 h of growth, a 100-μl aliquot was transferred into a storage plate at a final concentration of 15% sterile glycerol and frozen at –80°C as a glycerol stock. The rest of the sample was grown for another 4–6 h, for a total growth time of 12–14 h. We found that >12-h growth made the bacteria susceptible to losing ≈20% of the clones; reincubation from frozen stocks of >12-h initial growth resulted in a higher number, ≈30%, of bacteria to lose clones (Fig. 21). Loss of clones occurred either by bacterial lysis or clumping. Making a frozen glycerol stock of the 6- to 8-h-growth sample prevented the cDNA-loss problems in subsequent reinoculations (Fig. 21). We believe this problem was due to an unknown property of the full-length clones in the bacteria, because the same *Escherichia coli* strain transformed with cDNA from other libraries without a high proportion of full-length clones did not have this problem (data not shown). The 12- to 14-h-growth samples were used for plasmid DNA preparation.

Plasmid DNA was isolated in a 96-well format by using a modified alkaline lysis method of Birnboim and Doly (24) for ≈2/3 of the clones. After the addition of 100 μl of solution I [50 mM glucose/25 mM Tris·HCl (pH 8.0)/10 mM EDTA], 200 μl of solution II (0.2 N NaOH/1% SDS), and 150 μl of solution III (3 M sodium acetate/11.5% acetic acid), and then 100 μl of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The plates were sealed, vortexed, and spun at 500 × *g* for 5 min with a Marathon 21000R Centrifuge (Thermo Electron, Waltham, MA).

Approximately 350 μl of supernatant was transferred to a fresh 2-ml 96-well plate and precipitated at –80°C for 15 min or overnight with 800 μl of 100% ethanol. The plates were then spun at 500 × *g* for 30 min, washed, and spun with 1 ml of 70% ethanol, twice. Ethanol was removed, DNA dried, and 50 μl of sterile distilled H_2O (dH_2O)was added. For the other 1/3 of the clones, plasmid DNA was prepared at SeqWright (Houston, TX; www.seqwright.com). From the resuspended DNA preps, 1–2 μl (0.1–0.3 μg) was used for DNA sequencing.

3.5 DNA Sequencing Requirements. We attempted to sequence up to ≈0.9 kb of the 5' and 3' ends of each clone. We found that standard procedures of PCR-DNA sequencing at Duke University or at various companies resulted in many failed reactions (>40%). This failure was due to high GC content at the 5' end of many of the full-length songbird cDNA clones; the reactions worked from the primer sequence to the junction between the vector and insert and failed thereafter. We theorize that the high GC content in the 5' end (and sometimes AT in the 3' end) allows folding of the cDNA, causing *Taq*DNA polymerase to stall and fall off. To solve this problem, we found that a proprietary protocol performed by SeqWright worked well, as well as a protocol at the Duke University DNA Cancer Sequencing Facility that we modified. This protocol required 5% DMSO in the sequencing reactions to melt secondary structure, ABI BigDyeTM version 1.1 terminator sequencing chemistry, and the following PCR cycle conditions: 95°C denaturing for 10 s, 50°C annealing for 5 s, and 60°C extension for 2 min repeated 25 times with AmpliTaq DNA Polymerase. The sequencing reactions were cleaned with Performa DTR 96-well plates (Edge BioSystems, Gaithersburg, MD). However, the sequencing reads were further improved by making fresh clean-up plates. To make the plates, we added Sephadex G-50 resin (Amersham Pharmacia Biosciences, Piscataway, NJ) to Whatman 96-well 350-μl hydrophilic PVDF plates (Whatman, Clifton, NJ) using a 96-well dispenser. The hydrated filter plates were spun on an empty 96-well microtiter plate at 910 × *g* (2,270 rpm on CR 422) for 5 min to remove excess water. The PCR sequencing reactions were then transferred to the corresponding Sephadex columns on top of the rinsed 96-well plates in the same orientation. The 96-well plates were stabilized with a MicroAmp Splash Free Base (Applied Biosystems, Foster City, CA). Plates were centrifuged for 5 min at 910 × *g*, sealed, and stored at –20°C. Later, an aliquot was loaded on the ABI3700 sequencer. The Sephadex filter plates were reused after being rinsed several times with water to remove all Sephadex. The 5' primer we used for sequencing was Fwd M13-21 or pFLC-Fwd1 (Fig. 20), and the 3' primer was RevM13 or pFLC-Rev (5'-GCGGATAACAATTTCACACAGGA-3').

3.6 Construction of a Songbird Transcriptome cDNA Database: www.songbirdtranscriptome.net. We used a Tamino XML (eXtensible Markup Language) database format (Software AG, Darmstadt, Germany, http://www.softwareag.com), an Apache web server, Apache Tomcat, MySQL and the Mind Electric's GLUE web service engine. The database has four levels of organization: (*i*) sequence read pages that contain the raw and processed individual sequences of each clone; (*ii*) clone pages that contain the reconstructed sequences from individual reads, the BLAST matches to other databases, and clone annotations; (*iii*) subcluster pages that contain nearly identical clones with associated annotations; and (*iv*) cluster pages that contain the subclusters with associated annotations (Fig. 12). Our machine processing strategy consists of six major steps, with values at each step determined empirically by user experts (authors of this report) in DNA sequence analyses.

3.6.1. Trimming. First, the untrimmed sequences and the chromatograms were placed as links on the sequence read pages, allowing all users to see the raw ABI sequence data for their own independent analyses. Then the ABI trace output files were run through phred to decode the traces, produce DNA sequence and quality data, and run through cross_match to clip vector sequences from the 3' and 5' ends (25, 26). We located 5' and/or 3' primer sequences at each end using a custom-written Java program, stored the information, and trimmed the primer sequence off. We left the polyA on the 3' sequence. We then trimmed off poor-quality sequences at the nonprimer sequenced ends in a moving average of 75 bps. Each base in the sequence was assigned a new phred average value calculated from the average of itself and 37 bases on either side. We then deposited the reverse compliment of the 3' read and the original orientation of the 5' read on the read pages for each clone. When the sequence read quality was too low for the primers or vector sequences to be found, we manually trimmed these clones.

3.6.2. Generating a Clone Sequence. We used phrap to generate clone consensus sequences from the trimmed 5' and/or 3' reads. The output was decoded by another custom-written Java program, which adds sequences at the clone level and assigns direction (3', 5'). To keep 5' and 3' partially sequenced clones together, all clone sequences that had nonoverlapping 5' and 3' reads were pasted together with 50 Ns. Thus, after quality trimming, the amount of total sequence in the database for any particular clone, at the time of publication of this report, will not be >1.8 kb (2 × 0.9 kb each sequence read). For genes that make mRNAs 1.8 kb or shorter, we potentially will have the full sequence in the database. Clone consensus sequences were deposited on the clone

page as a fasta file, with an associated graphical view of the phred quality scores (Fig. 12*E*). All clone sequences were deposited in GenBank: the EST database for partially 5'- and/or 3'-end sequenced clones (9,386 clones; accessions nos. DV570610–DV584230 for individual sequenced ends) and the nr database for fully sequenced clones (4,308 clones; accession nos. DQ213062–DQ217370).

3.6.3. Generating Subclusters. To group clones into subclusters, all quality trimmed clone sequences were examined by a BLAST (27) search against each other. All matches of a certain length (>100 bp) and identity (BLAST hsp bit score >40, 95% identity across 95% of the sequence) were grouped together by our Java program. The clone with the most amount of DNA sequenced was used as the sequence for the subcluster page. We did not generate a consensus sequence from clones of a subcluster because there may still be small differences between clones that make them distinct, such as RNA edited sequences. This clustering process allows for clones with small differences in length (<30 bp) of the 5' or 3' ends or with SNPs to be brought together into the same subcluster. We did this because, without genomic sequence and genomic sequence of different individuals, we do not know whether the SNPs are really SNPs or sequencing errors and whether the different sizes of the 3' or 5' ends are because of incomplete cloning or different start sites. Subcluster alignments were manually verified by using DIALIGN (28) (http://bibiserv.techfak.uni-bielefeld.de/dialign) by user experts and, when necessary, were manually reorganized. Users can also create multiple alignments by the DIALIGN user interface.

3.6.4. Generating Clusters. To group subclusters into clusters, the representative subcluster sequences (clone with the greatest amount of DNA sequenced) were BLAST searched against each other. All matches meeting a certain length (minimum of 150 bp) and percent identity (hsp bit score >40, 95%) of at least one hsp hit were grouped. No consensus sequence was generated at the cluster level because the cluster is expected to consist of mRNA variants. Cluster alignments were manually verified by using DIALIG, and, when necessary, were manually reorganized. Some subclusters from different genes were brought together in a cluster because one of the clones was chimeric (presumably a cloning artifact), containing at its 5' end the sequence of one gene and at its 3' end the sequence of the other gene. These subclusters were manually split into two new clusters and the chimera left as a cluster of its own.

3.6.6. Annotation. We inserted into the read, clone, subcluster, and cluster pages primer annotation information. This included species, tissue source, animal source, age, sex, subject ID,

behavioral condition of animal, and library source of clone. BLAST hits (BLAST hsp >20, >150-bp match) were used to automatically annotate the songbird clones with gene names. This process was done by BLAST searches of all clones against public annotated databases: nt NCBI 7/5/2004 build (http://www.ncbi.nlm.nih.gov/BLAST; using blastn and blastx), chicken genome (29) EnsEMBL 6/2004 assembly (http://www.ensembl.org/Gallus_gallus/index.html), chicken transcriptome BBSRC 05/04/2004 cDNAs (http://www.chick.umist.ac.uk), chicken gene EST, Tigr 05/24/2004 build (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species = g_gallus), LocusLink NCBI 7/7/2004 data dump, UniGene NCBI 7/7/2004 data dump, HomoloGene NCBI build 36, Taxonomy NCBI 7/7/2004 data dump (http://www.ncbi.nih.gov), and Gene Ontology GO Consortium 7/2004 release (http://www.geneontology.org/GO.doc.shtml). BLAST hits against chromosomal sequences were filtered as well as hits below a bit-score cut-off 40, and match of <100 bp. We then used a custom-written Java program called UgoBLAST (UniGene–Gene Ontology–BLAST) to insert UniGene names into the clone pages, Gene Ontology terms into the clone pages, and LocusLink links (30, 31). We also performed a BLAST search of the clones against the nonannotated zebra finch ESTIMA database (http://titan.biotec.uiuc.edu/songbird; minus our clones from this study) but did not use the hits for gene name annotation. Annotations began at the clone level and were inherited upwards toward the cluster root. All annotations were manually verified and, when necessary, were renamed or corrected. The zebra finch cDNAs were preferably given the identical name of the homologous chicken, mammalian, or other species gene in the NCBI "Gene" database when the DNA or protein identity was 70–100% or 75–100%, respectively, or prefixed with "similar to" when the DNA or protein identity was 55–69% or 60–74% respectively. Qualitative judgments were also made. The database is publicly accessible at http://.songbirdtranscriptome.net.

3.7 Generating and Hybridizing Microarrays. To generate PCR products to print on microarray slides, the following protocol produced sufficient product from full-length cDNA clones: a 100-μl reaction with the flanking M13 forward and reverse primers (Fig. 20) at 2 μM final concentration each, buffer containing at final concentrations 16 mM ammonium sulfate, 20 mM Tris·HCl, pH 8.9, 150 ng/ml BSA, 2.5 μM $MgCl_2$, 250 μM equal mix of dGTP, dCTP, dATP, dTTP, 1.3% DMSO, 1.4 M Betaine, and a 1-μl 1/5 dilution of a laboratory-prepared *Taq* polymerase (31). For PCR amplification, the following parameters were used on a MJ Research PTC-225 Peltier Thermal Cycler (Waltham): 98°C 5 min, 54°C for 1 min, 72°C for 4 min for 1 cycle, followed by 98°C for 40 sec, 54°C for 1 min, and 72°C for 3 min for 35 cycles, with a final extension at 72°C for 5 min. Reactions without BSA, Betaine, or DMSO typical of standard PCR

protocols resulted in failure of amplification of many clones, presumably because of secondary structure from the high 5' UTR GC content. The BSA and other PCR reagents required removal by Arrayit 96-well 96100 PCR purification kit (TeleChem International, Sunnyvale, CA), because we found that the BSA clogged the microarrayer pin heads. The PCR products were resuspended in 3X SCC (at 0.1–0.5 μg of DNA per microliter) and printed on Ultra-gap microarray slides (Corning, Corning, NY) at the Duke University Medical Center Microarray Facility by using a GeneMachine OmniGrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI). Slides were stored in sealed boxes at room temperature.

The microarrays we generated for this study contained 18,433 spots. These were of cDNAs that we picked until December of 2004 and included all clones whether or not the sequence or PCR reaction worked: 5,952 clones from library #0058; 3,264 from #0061; 672 from #0062; 3,168 from #0063; 2,208 from #0064; and 2,592 from #0065 (17,856 total). Some clones (384) were spotted twice. The array also included 48 spots of immediate early gene PCR products spotted at different concentrations (15–250 ng/μl) generated for a related project (K.W., H.H., and E.D.J., unpublished work), 8 spots each of glutamate receptors GluR1, GluR5, NR1, NR2A, and NR2B (32), CREB, and G3PDH, also at different concentrations, 2 spots of the plasmid pFLC-I used to construct the cDNA libraries, 6 spots each of plasmids pCR3 and pME used to clone gene products of the glutamate receptors and the other above-mentioned genes, 8 spots each of polyA and yeast tRNA at different concentrations, and 50 spots of 3× SCC. The microarray data can be found at the National Institutes of Health Neuroscience microarray site http://arrayconsortium.tgen.org and GEO database http://www.ncbi.nlm.nih.gov/geo accession no. GPL3621, following the MIAMI-compliant format. Songbird cDNA arrays are available for other investigators through the Duke Neurosciences Microarray Center at http://arrayconsortium.tgen.org.

When we prepared microarray probes (using an oligo-dT primer) from whole forebrains of singing and silent males and hybridized them to our microarray slides, not many genes showed regulation in song nuclei by singing, whereas others (e.g., *egr-1* and *c-fos*) were visibly different in the microarray analysis (Fig. 14*Ab*). This finding suggested that the whole-brain mRNA was masking some song-nuclei signals. Thus, we repeated the singing experiment, perfused the animals with 4% paraformaldehyde in PBS, dissected the brains, postfixed them with 4% paraformaldehyde in PBS at 4°C for 3 h, and then placed them in 20% sucrose/0.4% paraformaldehyde in PBS overnight, sectioned 120-μm sagittal slices of the right hemisphere on a

freezing microtome at –20°C, and, under a dissecting microscope, dissected vocal nuclei (Fig. 14*Ba*) using custom-made punch biopsy tools; the left hemisphere was cut in 12-μm sections and was used for *in situ* hybridization verification of *egr-1* induction. For the dissected vocal nuclei, RNA was released from fixed proteins by digesting the punch biopsies with proteinase K (in buffer: 10 mM Tris·HCl, pH 8.0/150 mM NaCl/10 mM EDTA/0.1% SDS) at 60°C for 2 h and RNA isolated by TRIZOL extraction (GIBCO-BRL, Life Technologies). From these RNAs, the cDNA probes were too low in concentration to yield sufficient signal on the microarrays (data not shown). Thus, we performed linear amplification of the RNA by a protocol we developed (below), which, when made into Cy3 and Cy5 probes, yielded strong microarray signals and more singing-induced (red) or singing-repressed (green) differences on the microarrays compared with probes from the entire brain (Fig. 14 *A* and *B*).

Linear amplification: 0.2–0.5 μg of total RNA from vocal nuclei was mixed with 50 ng of oligo-dT_{24}-T7 primer (Ambion, Austin, TX) in a total volume of 2.5 μl, heated to 70°C for 5 min, spun, and chilled on ice for 1 min. First-strand synthesis was accomplished by the method of Carninci *et al.* (21), with 1× GC buffer (Panvera, Carlsbad, CA), 0.8 M sorbitol/trehalose, 0.4 mM dNTPs preheated to 42°C, and 300 units of SuperScript II DNA polymerase (Invitrogen). The reaction was placed in a PCR machine, incubated at 42°C for 40 min, 50°C for 10 min, 56°C for 10 min, and 70°C for 10 min, and kept on ice after completion. This first-strand cDNA was added to a second-strand cDNA-synthesis reaction in a total volume of 90 μl with a buffer containing 40 mM Tris·HCl, pH 6.9, 180 mM KCl, 9.2 mM $MgCl_2$, 20 mM NH_4SO_4, 0.3 mM nicotine dinucleotide, 194 nM dNTPs (Panvera), 3.8 nM DTT (Promega), 10 units of T4 DNA Ligase (Statagene, La Jolla, CA), 22.75 units of DNA Polymerase I (Panvera), and 1 unit of RNase H (Invitrogen) and incubated at 16°C for 2 h. After second-strand synthesis, 8.5 units of ExTaq Polymerase (Panvera), 25 units of Apligase (thermostable DNA ligase; Epicentre Biotechnologies, Madison, WI), and 3.0 units of Hybridase (thermostable RNase H; Epicentre Biotechnologies) were added and incubated at 65°C for 30 min. Free RNA was then degraded with 17.5 units of RNaseOne Ribonuclease (Promega) and 9 mM EDTA at 37°C for 30 min; proteins were then degraded with 10 μg of proteinase K (Qiagen) at 45°C for 30 min. Linear acrylamide (0.3 μg; Ambion) was added and extracted with a 100-μl equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). To ≈90 μl of supernatant, 1/10 of 3 M ammonium acetate was added and precipitated with 100% ethanol. The pellet was dissolved in 70 μl of dH_2O, further purified by means of the MicroBio-spin 6 column (Bio-Rad, Hercules, CA), and vacuum

dried. The double-stranded cDNA was dissolved in 16 μl of dH_2O and converted to linear-amplified RNA with T7 polymerase by using the Ambion T7 Mega Script kit (Ambion) and 50 ng of oligo-dT_{24}-T7 primer. The amplified linear RNA (aRNA, antisense strand) was precipitated and dissolved in 42 μl of dH_2O. Then 3–4 μg of aRNA was converted to fluorescently labeled, sense strand cDNA with dUTP amino-allyl dyes and random 6-mer primers. RNA was hydrolyzed, and the animo-allyl-labeled cDNA was coupled with N-hydroxysuccinimidyl-esterified Cy3 or Cy5 dyes, by using the Monoreactive Dye Pack (Amersham Pharmacia Biosciences).

We hybridized the probes to the slides using procedures described in the Cold Spring Harbor Laboratory Molecular Cloning Manual (33). The Cy3- and Cy5-labeled cDNAs of individual control and singing samples were then mixed, purified by using QIAquick PCR purification kit (Qiagen), and dried by evaporation. The mixed Cy3- and Cy5-labeled sense cDNAs were resuspended in hybridization buffer, denatured, and hybridized to microarray slides for 12–16 h at 42°C by using Arrayit hybridization cassettes (TeleChem International). After washes, the slides were scanned on a GenePix 4000A microarray scanner (Axon Instruments/Molecular Devices, Sunnyvale, CA). To determine the presence of DNA on spots of the microarray glass slides, a separate microarray slide was labeled with TOPO3 dye, which stains DNA. TOPO3-negative spots were removed from the array analyses. Also eliminated from analyses were spots with both silent AND singing mean signal intensities < mean ±2 times the SD of the Cy3 AND Cy5 background outside each spot. For the remaining spots, mean background signal was subtracted and candidate singing regulated clones chosen (criteria described in *Results*) for *in situ* hybridization verification.

3.8 Semihigh-Throughput *in Situ* Hybridizations and Quantitative Analysis. We tested several different *in situ* hybridization procedures and developed a method that yields the strongest hybridization signal and, thus, the shortest time of signal development (32). This protocol allows semihigh-throughput hybridization with at least 180 slides simultaneously in slide racks (30 or 60 spaces each) by using an oil bath to seal the coverslipped slides (34, 35). We collected brains of birds after 0 (silent overnight, $n = 4$), 0.5 ($n = 5$), 1 ($n = 4$), and 3 ($n = 4$) h of undirected singing, froze them sagittally in molds with Tissue-Tek on top of a dry ice ethanol bath, and cut and mounted serial sagittal 12-μm sections from the midline to the lateral end of song nuclei high vocal center (HVC) and RA. Then, we conducted a qualitative assessment of *in situ* quality in at least $n = 1$ animal per group, and, once satisfied, we repeated the experiment with all animals.

^{35}S-labeled riboprobes were made from T7 (sense) and T3 (antisense) promoter sites of clones (Fig. 20), by using T7 and T3 RNA polymerases (Promega). Frozen sections were fixed in 3% paraformaldehyde in PBS (pH 7.4), acetylated, dehydrated in an ascending ethanol series, and air-dried. The following hybridization solution gave a stable and strong signal: 50% formamide, 10% dextran sulfate, 1× Denhardt's, 12 mM EDTA (pH 8.0), 10 mM Tris·HCl (pH 8.0), 30 mM NaCl, 0.5 μg/μl yeast tRNA, 1 μg/μl polyA, and 10 mM DTT. The dextran sulfate was critical to generate strong hybridization signals. One hundred microliters of hybridization solution with ^{35}S-labeled probe (1×10^6 cpm per slide) was applied to each slide, coverslipped, and hybridized at 65°C overnight under mineral oil. The mineral oil was removed by two chloroform washes and excess probe removed by washing in 2× SSPE and 0.1% β-mercaptoethanol at room temperature for 1 h, 2× SSPE, 50% formamide, and 0.1% β-mercaptoethanol at 65°C for 1 h, and 0.1× SSPE twice at 65°C for 30 min each. Slides were dehydrated in an ascending ethanol series (70%, 95%, and 100% for 2 min each), and exposed to β-max hyperfilm (Kodak, Rochester, NY) for 2–3 days. The slides were then dipped in NTB-2 (Kodak) emulsion for 5 days to 3 weeks (depending on signal abundance), developed, sections counterstained with cresyl violet, and then coverslipped.

To perform quantitative time-course analysis, x-ray film images were digitized with a Spot III camera and a dissecting microscope, and pixel intensities in vocal nuclei were measured with Photoshop (Adobe Systems, Mountain View, CA) histogram tools. From these values, the background levels on the film were subtracted. We then determined time-course expression profiles in a two-step process. First, ANOVAs with Fisher's post hoc test were performed for each gene in each vocal nucleus to determine whether there were statistical differences between the 0 time point and the 0.5-, 1-, and 3-h groups of singing animals. From this analysis, we obtained the peak time of average singing-regulated expression. Second, we used the ANOVAs to determine whether there were statistically significant or not significant differences in the 3-h time period relative to the peak time period. From the two steps combined, we obtained the six classes of statistically significant expression patterns.

For a control gene that is known to be regulated by singing, we cloned a zebra finch *egr-1* fragment of 1,100 bp using a 5' primer 5'-TGTGACCGGAGGCTTTCACGAT-3' and 3' primer 5'-AAACTTCTGCCACATGTGAGTGT-3', into the pGEM-T Easy vector (Promega); the antisense riboprobe was made with T7 RNA polymerase.

3.9 Immunohistochemistry and Western Blots. For immunohistochemistry, after silent or undirected singing conditions for 1–2 h, birds were anesthetized with a nembutal sodium solution (100 μl of 10 μg/μl), perfused with PBS and then 4% paraformaldehyde in PBS, and postfixed with 4% paraformaldehyde in PBS at 4°C for 3 h and then in 20% sucrose in PBS overnight. Sagittal 30-μm sections were cut on a freezing microtome and floated in PBS. Collected sections were then washed three times with PBS and incubated for 1–2 h in PBS containing 0.4% Triton X-100, 4% normal horse serum, and 1% BSA and then in fresh solution of the same composition overnight with the primary antibodies: anti-Egr1 (Zenk) polyclonal (sc-189, 1:300 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti c-Jun polyclonal (sc-45, 1:300 dilution; Santa Cruz Biotechnology), anti-Met Enkephalin polyclonal (cat# 20065, 1:300 dilution; ImmunoStar, Hudson, WI), anti-β-Actin monoclonal (A5316, 1:500 dilution; Sigma, St. Louis, MO), anti-Hu (1:500 dilution; Molecular Probes, Eugene, OR), or anti-glial fibrillary acidic protein (GFAP) (1:500 dilution; Chemicon, Temecula, CA) antibodies. After three PBS washes, secondary Cy3 (red) conjugated anti-rabbit or mouse antibodies (1:500 dilution; Jackson ImmunoResearch, West Grove, PA) were applied for 1–2 h, followed by three PBS washes. Brain sections were mounted onto glass slides and coverslipped with Vectashield's DAPI (stains nuclei blue) solution (Vector Laboratories, Burlingame, CA).

For Western blots, total brains of adult male zebra finches were homogenated in cold PBS, and the lysates were separated by 10% SDS/PAGE, transferred to a nitrocellulose membrane, and blocked with 5% skim milk in PBS for 1 h. Membranes were then incubated with the anti c-Jun (1:500 dilution), Met Enkephalin (1:400 dilution), or β-Actin (1:1,000 dilution) antibodies. Membranes were then reacted with an HRP-conjugated anti-rabbit or -mouse secondary antibodies (1:500 dilution; Zymed, San Francisco, CA), and binding was detected on x-ray films by using the ECL detection system (Amersham Pharmacia Bioscience).

3.10 Viral Vector Expression. We tested the efficiency of three lentiviral vectors containing different mammalian promoters attached to eGFP: (*i*) EF1α promoter, obtained from Tranzyme (Durham, NC); (*ii*) CMV promoter obtained from Larry Katz and Marguerita Klein of Duke University, modified from Lois *et al.* (36), and (*iii*) Ubiquitin C (Ubi C) promoter obtained from Pavel Osten of the Max Planck Institute, also modified from Lois *et al.* (36). Separately, we inserted ORFs of target genes attached with a FLAG tag into pFUGW (Fig. 17*A*) (36). The FLAG-tag sequence was fused with each gene at the 5' end by using a two-round nested PCR. For the first-round PCR, the first forward primer was 5'-

AG/GAT/GAC/GAC/GAT/AAG/**ATG**+[18≈21 bp 5'of gene of interest]-3' (the AG/GAT/GAC/GAC/GAT/AAG/ is part of FLAG tag ORF; the **ATG** is the start site; "/" separates codons). The first reverse primer was 5'-ATTT**GAATTC**+[reverse Stop site]+[18≈21 bp 3' of gene of interest]-3' (the **GAATTC** is the EcoRI site). The first-round PCR was performed at 95°C for 4 min for 1 cycle, followed by 94°C for 40 sec, 53°C for 30 sec, and 72°C at 1 kb/1 min for 13 cycles. The product was purified by a PCR purification kit (Qiagen) and used as a second-round PCR template.

For the second-round PCR, the second forward primer was 5'-AATA**GGATCC**CGCCACCATG/GAT/TAC/AAG/GAT/GAC/GAC-3' (the **GGATCC** is the BamHI site and ATG/GAT/TAC/AAG/GAT/GAC/GAC is part of the FLAG tag). The second reverse primer is the same as first reverse primer. The second-round PCR was performed at 95°C for 4 min for 1 cycle, followed by 94°C for 40 sec, 45°C for 30 sec, 72°C at 1 kb/1min for 5 cycles, and further followed by 94°C for 40 sec, 55°C for 30 sec, and 72°C at 1 kb/1 min for 13 cycles. The product was purified by a PCR purification kit (Qiagen), subcloned into the pGEM-easy vector (Promega), and sequenced to verify that no PCR mutations occurred. The FLAG-tagged ORF of the target gene was then digested with BamHI and EcoRI and ligated into BamHI/EcoRI-digested pFUGW (Fig. 17*A*).

Lentivirus production, concentration, and titer determination were done as described (36), with a minor modification of a shorter incubation period (48 h) after transfection into HEK293T cells. For viral transfection into songbird brains, isoflurane-anesthetized male zebra finches at PH120–140 were injected with 1×10^6 to 1×10^7pfu/μl titer (1.0–1.5 μl) into HVC and the lateral magnocellular nucleus of the anterior nidopalium (LMAN) in the nidopallidum and/or AreaX in the striatum. Injection was done with a Hamilton syringe, 25-gauge needle, at a slow rate of ≈1 ml/10 min. Expression of eGFP and FLAG-tagged Gadd45β were checked at 2 days to 3 months after injection by fluorescence microscopy, *in situ* hybridization, and immunohistochemistry (Fig. 17 *B*–*E*). The anti-FLAG M2 antibody (Sigma) was used at a 1:300 dilution.

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