Supporting Information

Abe et al. 10.1073/pnas.1413484112

SI Materials and Methods

Animal Care and Treatment. All experiments conformed to the guidelines of the National Institutes of Health (NIH) experimental procedures and were approved by the Animal Care and Use Committee of Kyoto University. The founder birds of our aviary were obtained from a local pet trader (Asada Choju Trading Company) and maintained by breeding in Kyoto University. Zebra finches used in this study were raised in our aviary and kept in rooms with a 14-h light, 10-h dark period. Food and water were given ad libitum throughout the rearing and the analysis. For the normal rearing, juvenile birds were kept with their parents in a mating cage (33 × 33 × 42 cm, visually concealed from other cages by white paper screens) until 60 dph and afterward kept with birds of similar age. For the rearing with experimentally controlled environments, juvenile birds just after the fledge state (25–32 dph, after they became able to feed themselves independently) were removed from their home cage and kept in a soundproof chamber until 140 dph, together with four to six age-matched birds of mixed genotypes and sexes. In the soundproof chamber, their cage was placed adjacent to a cage of a mature male bird (tutor) between which they could interact visually and auditorily. At 60 and 90 or 140 dph, male birds were isolated in another soundproof chamber alone; and their songs (undirected songs) were recorded for 2–3 d as described earlier (1). After the recording at 140 dph, these chamber-reared birds were moved to the aviary and kept in cages with normally reared birds with mixed genotypes. Chamber-reared birds were kept in the aviary for more than 4 wk before being subjected to the auditory conditioning test or the hearing threshold analysis. For song development analysis, all of the birds available at the time of the experiment were used and reported. Data from birds that died before 140 dph were excluded from analyses except for the survival curve analysis. In our tutoring condition, we did not observe a significant effect of cross-learning between the juveniles tutored together; WT birds reared only with WT birds, WT birds reared together with DN birds, and WT birds reared together with Actv birds (syllable similarity score at 140 dph; WT only, 77.8%, $n = 16$; WT with DN, 82.5%, $n = 8$, WT with Actv, 75.8%, $n = 7$; $P > 0.5$ in each pairwise comparison, Tukey's post hoc test). To obtain samples for histological and molecular biological studies, subjects were randomly selected from the available similar-aged birds of each genotype.

Generation of Transgenic Zebra Finch. To generate transgenic zebra finches, freshly laid eggs were collected from nests, and lentiviral vectors were microinjected around the central portion of the embryos as described earlier (2). The expression construct EGFP-zfCREB encodes a fusion of EGFP and zebra finch CREB (3) under the control of human synapsin promoter, –575 to –98 bp from the transcription start site of human *SYN1* (4). Although *SYN1* gene is not present in avian genome (5), being regulated by the endogenous gene transcription molecular system, this promoter is known to drive transgene expression specifically to all neurons, as previously tested in the in vivo and in vitro transfection experiments using viral vectors and also in transgenic studies (4, 6). A woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was added to enhance expression. Because the overexpression of WT CREB may function as an activator of CREB (7), we created two lines of transgenic birds having repressed (S119A) and enhanced (Y120F) function of CREB. Full-length CREB were PCR amplified from zebra finch cDNA by a primer set, 5′-GGTACCAGAAGATGACCATGGAATCTGGAGCA-3′ and 5′-GGTACCTTAATCTGATTTGTGGCAGTAA-3′. Mutant CREB gene was created by the primer set of 5′-GTCCAGTCGACCCGCCTACAGAAAA and 5′-GGTACCTTAATCTGATTTGTGGCAGTAA-3′ for CREB(S199A)-C-term, 5′-GTCCAGTCGACCCTCCTTCAGAAAA-3′ and 5′-GGTACCTTAATCTGATTTGTGGCAGTAA-3′ for CREB(Y120F)-C-term, each of which is concatenated with the zfCREB-N-term region, which was amplified with the primer set 5′-GGTACCAGAAGATGACCATGGAATCTGGAGCA-3′ and 5′-GGAGGGTCGACTGGACAGAATT-3′. All constructs were confirmed by sequencing. Viral titers [infectious units (ifu)] were determined by quantifying the genome-integrated transgene by a quantitative PCR analysis of the genomes, 2 d after transfecting the virus into HEK293T cells. A total of 150–300 nL of viral solution (titer, $5.0 \times 10^{9-11}$ ifu·mL^{-1}) was injected by a glass pipette, at four to six sites per embryo through a small hole in the egg shell. Special care was taken to keep the opening in the egg shell membrane as small as possible. After injection, virus-injected eggs were sealed with adhesive film and incubated at 37.5 °C in a humidified incubator until they hatched. We obtained hatched chicks from 10% to 25% of virus-injected eggs, depending on the transgene constructs. Hatched chicks were moved into the nests of foster parents that had similar aged chicks, and reared until 60 dph. Germ line transmission of transgene in each virus-injected bird was analyzed by performing PCR-mediated genotyping of the offspring that were produced by crossing with WT birds (G_1 generation). Transgene expression was further checked by RT-PCR and by immunostaining of brain sections of offspring for screening of transgenic lines expressing the transgenes. Only the lines showing the expression of transgenes in their brain were selected for further expansion of the colony. Germ line transmission and the expression of the transgenes (EGFP-CREB) in offspring was observed in 20 out of 1,473 virus-injected eggs. By crossing these 20 founder birds (11 for DN and 9 for Actv) with WT birds, a total of 116 DN and 103 Actv G_1 offspring were obtained and used in this study. Because the reproductive efficiency of G_1 offspring was low, we used G_1 offspring from multiple lines for the behavioral and histological analysis in this study.

PCR and Reverse Transcription. For genotyping, genomic DNA from each bird was purified from blood. PCR-based genotyping was conducted with the following primer sets: EGFP, 5′-TCAAGATCCGCCACAACATC-3′ and 5′-TCTCGTTGGGGTCTTTGCTC-3′; *GAPDH*, 5′-AGTGAAGGCTGCTGCTGATG-3′ and 5′-CGCATCAAAGGTGGAGGAA-3′; WPRE, 5′-CCGTTGTCAGGCAACGTG-3′ and 5′-AGCTGACAGGTGGTGGCAAT-3′. For reverse transcription, total RNA was isolated from the entire telencephalon of the right hemisphere from each bird (60 dph) using TRIzol reagent (Life Technologies) and reverse transcribed with a PrimeScript RT reagent kit with gDNA-eraser (Takara Bio) according to the manufacturer's instruction. For the lentivirus-based transcription reporter, a gene-specific primer mix targeted at WPRE (5′-CCACATAGCGTAAAAGGAGCAAC-3′, 5′-TGGTTGCTGTCTCTTTATGA-3′) and exogenous PEST sequence (5′-GCAAGCAGCAGGGTGTCTATC-3′, 5′-ACAGGGACAGCAGAAA-3′) was used in the reverse transcription that was conducted at 42 °C.

Quantitative real-time PCR (qPCR) was performed with a StepOnePlus system (Applied Biosystems) with SYBR Premix ExTaqII (Takara Bio). All qPCR was conducted at 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles of 95 °C for 15 s and 63.5 °C for 1 min, except for the qPCR of mRNA for the

lentivirus-based transcription reporter, which was conducted at 95 °C for 2 min, and then 49 cycles of 95 °C for 5 s and 65.0 °C for 30 s. The specificity of the reaction was verified by melting curve analysis and gel electrophoresis. Primer sets used for the qPCR are listed in Table S1.

For calculations of relative expression values, each value was divided by the mean value of WT. For calculating how each gene expression has changed in the transgenic DN-CREB or Actv-CREB birds, $\log_2$ of the expression change against WT was calculated for each gene. The expression change shown in Fig. 2*D* indicates the absolute value of the $\log_2$ of relative expression values:

$$\text{Expression change to WT} = \left|\log_2\left(\frac{V_{TgN}}{V_{WT}}\right)\right|,$$

where V_{WT} is a mean expression value of WT and V_{TgN} is a mean expression value of either DN-CREB or Actv-CREB birds. Because the currently reported zebra finch genome often contains unsequenced regions particularly in promoter regions, and the information about the transcription initiation sites is limited, we performed transcription binding site analysis on the putative human homolog of such genes, estimated by the Unigene cluster (2013/03/01; www.ncbi.nlm.nih.gov/unigene/). The presence of cAMP-response elements in their promoters was queried in the CREB target database (8).

To analyze the integration loci of the transgenes, genomic DNA from each transgenic line was purified from brain using Wizard SV Genomic DNA Purification System (Promega). Purified genomic DNAs were digested with EcoRI. The digested products were self-ligated and conducted with a PCR using a primer set against exogenous 3′-LTR sequence (9) (5′-AGTAGTGTGTGCCCGTCTGT-3′ and 5′-TGAGGCTTAAGCAGTGGGTTC-3′). The PCR products were cloned into cloning vectors and sequenced. Sites of transgene integration were mapped by BLAT (10) using zebra finch genome assembly (WashU taeGut324/taeGut2).

Lentivirus-Based Transcription Reporter. For the lentivirus-mediated reporter assay, reporter genes were each expressed from bidirectional promoters (minimal promoter and *PGK1* promoter), insulated by a FII insulator. A constitutive human *PGK1* promoter expressed 2× FLAG-tagged Histone-2B for the infection reference. As a transcription factor-reporter control, destabilized nuclear GFP (green fluorescent protein fused with NLS and PEST sequences) was expressed under the control of a minimal promoter (from pGL4.29; Promega). For the CREB-reporter, CRE sequence (from pGL4.29; Promega) was inserted before the minimal promoter. The expression of each reporter mRNA was quantified by qPCR. The reporter activity was calculated by dividing the reporter mRNA (CREB-reporter or Control-reporter) by the reference mRNA (Flag-tagged histone). For the in vivo reporter activity analysis, the lentivirus particle (titer, $2.0 \times 10^{10–11}$ ifu·mL^{-1}) was injected into the striatum (0.30 μL at four sites; distance from the Y sinus: lateral, ±1,400 μm; rostral, 5,500 μm; depth, 2,750 and 2,250 μm at beak angle 65° and 70°) in adult (180–360 dph) males under ketamine/xylazine anesthesia. Each bird was injected with LV-CREB-reporter and LV-Control-reporter in the alternative hemisphere. After 2 wk of keeping the subjects in soundproof chambers together with mixed genotype, birds were euthanized, and the total RNA was collected from each hemisphere and quantified by qPCR. For the in vitro reporter activity analysis, HEK293T cells infected with LV-CREB-reporter or LV-Control-reporter lentivirus were stimulated with forskolin (100 μM; Wako Pure Chemical Industries) or vehicle (0.1% DMSO), 1 wk after the transfection. The mRNA was collected 14 h after the stimulation and quantified by qPCR.

Histological Analysis. Immunostaining was performed on free-floating cryosections (40 μm) of tissue perfused with 3% (wt/vol) paraformaldehyde solution as described earlier (1). Rabbit anti-phosphorylated CREB (S133; Abgent; AP3077a; 1:1,000), anti-GFP (Life Technologies; A6455; 1:800), and mouse anti-NeuN (EMD Millipore; MAB377; 1:750) were used. All sections were imaged with a fluorescence microscope (BZ-9000; Keyence). Images were acquired with a 10× objective and joined to show the whole section images. Immunostained signals were quantified using Image J (NIH) as described previously (1). For the quantification of nuclear volumes, every third section (50 μm) from the perfusion-fixed hemisphere from male birds was collected and subjected to immunostaining with NeuN antibody and 4′,6′-diamidino-2-phenylindole (DAPI) (Dojindo Molecular Technologies) staining, and was imaged using a 4× objective. Nuclei were outlined manually according to the stained image by an experimenter blind to the genotype, and the area was measured by using Bioanalyzer II (Keyence).

Song Similarity Analysis. At each developmental time point, birds were isolated in a soundproof chamber; and their songs (undirected songs) were recorded with a microphone (ECM8000; Behringer), digitized at 44.1 kHz, and digitally filtered at 0.7–14.5 kHz. The songs were analyzed with Sound Analysis Pro-2011 (11) (SAP2011, version 2011.103), using the similarity batch mode with the default setting except for the "minimal duration of similarity selection," which was set at 51 ms. Song similarities were calculated by two methods: syllable-based calculation and motif-based calculation. For the former, syllables, longer than 100 ms in duration and distinct from each other, were selected manually from each tutor's song and used as the template syllables. Each template syllable was compared with every syllable in the tutee's song, and the score of syllables that showed the highest value was adopted; then scores of multiple template syllables were averaged. For the motif-based analysis, a whole motif (four to six syllables) was used as a motif template. For each tutee's song, the whole-motif comparison was performed against the syllable or the motif templates, without defining the target syllable or motif; and similarity scores (percentage of imitation) were calculated. Ten bouts of songs, randomly selected from the recorded song corpus, were analyzed and averaged for each condition. For the acoustic comparison of calls, 10 bouts of long calls were collected from the recorded corpus and used as call templates. For the calculation of the similarity score of calls, each of 10 call-templates of a tutee's call (recorded in isolation at 140 dph) was scored against each 10 call-template of the tutor's and the values were averaged. Acoustic features of tutee's calls were calculated by SAP2011 using the 10 call-templates and averaged.

Behavioral Analysis. Behavioral analysis was done as described previously (1). Only male birds were used for the behavioral analysis. All behavioral analysis were done from 10:00 AM to 6:00 PM. For hearing threshold analysis, adult birds were isolated in a soundproof chamber for a day. In the next day, the birds were presented with increasing step of white-noise volume (45, 47, 65, and 71 dB; 30 s each) played through a speaker, and the behavioral reaction were video recorded. Call responses were counted off-line by an experimenter who was blind to the genotype of the subject. To compare the behavioral reaction among relatively silent and active individuals, normalization of behavioral reaction number was used. For normalization of the call behavior, the absolute values of the number compared with that for the silent period were divided by the call number in the silent period (30 s before the presentation of 45-dB noise).

For the auditory conditioning, five zebra finch undirected songs were selected from our zebra finch song corpus. Because the songs selected in this study were recorded >3 y before the birth of

the subjects, each subject bird had never been exposed to exactly the same songs before the conditioning experiments. For the presentation of the control song stimulus, two bouts of songs were played for 10 s followed by 2 min, 50 s of silence. For the presentation of the conditioned song stimulus (CS), two bouts of songs were played for 10 s followed by 50 s of silence and then two bouts of a crow's call [unconditioned stimulus (US)]. The crow's call was used because our initial study revealed that they can reliably elicit freezing behavior in the subject birds even after repeated presentation. After the presentation of US, 6 min of silence followed before the presentation of the next songs. For one training block (TB), each of five song stimuli (including the CS) was randomly ordered and played a total of three times, and the numbers of call responses were averaged for each song stimulus. Total 59.9 s of five conspecific songs and 12.5 s of crow's call was presented in one training block. For the experiment with another set of songs, the result of which is shown in Fig. S6, 27.9 s of five conspecific songs and 3.20 s of crow's calls was presented in one training block. Each TB were separated with 20–30 min of silent intervals. A total of eight training blocks were performed over 2 successive days. The first and the last block of each day were video recorded and designated as TB1, TB4 (day 1) and TB5, TB8 (day 2). The orderings of songs were randomly determined, and the experimenter was blind to which songs had been played. Call responses were counted off-line by an experimenter who was blind to the genotype of the subject. To compare the behavioral reaction among relatively silent and active phase of subjects, normalization of behavioral reaction number was used. For normalization of the call behavior to the CS, the number of call responses during the 1-min period before the CS presentation was subtracted from the number of call responses of the 1-min period after (and during) the CS, and divided by the sum of the values before and after. For the US, the number of calls during the 1-min period before the CS presentation was subtracted from the number of call responses of the 1-min period after (and during) the US, and divided by the sum of the values. Statistical analysis was performed using the paired t test on raw values before and after the stimulus presentation (without normalization).

For the histological analysis of CREB phosphorylation during the auditory conditioning, WT male birds were auditory conditioned as described above. After three rounds of training blocks, before the start of TB4, 230–260 min after the beginning of the conditioning, the subjects were anesthetized and immediately perfused with fixative. Before the birds were killed, the subject had been repeatedly exposed to 108 song bouts of five conspecific songs. The experimental time course of the conditioning experiment overlaps with some of the previous reports that observed habituation to the repeatedly presented stimuli in respect to the neuronal spiking (12), the expression of immediately early genes (13), and the activation of MAPK-signaling (14). During the conditioning, we did not restrain the subject nor omit the subject executing certain behavior from analysis.

For the behavioral analysis of drug-injected birds, drugs were dissolved in PBS and stereotaxically injected bilaterally into the striatum (0.35 μL at four sites; distance from the Y sinus: lateral, ±1,400 μm; rostral, 5,500 μm; depth, 2,750 and 2,250 μm at beak angle 65° and 70°) of adult (180–360 dph) males under ketamine/xylazine anesthesia. For the identification of the injection site, Alexa 488-conjugated cholera toxin B (Invitrogen) was injected along with the drug. STO609 was purchased from EMD Millipore and used at 20 μM in final 0.4% DMSO. After the recovery (~2 h) from anesthesia, birds were conducted with auditory conditioning sessions. To identify the extent of diffusion of STO609 (M_r, 374.35) in the drug injection experiments, we injected DAPI (M_r, 277.32) similarly to WT birds and histologically estimated the area of drug diffusion. We estimated that drugs diffuse within a radius of 673.8 ± 58.7 μm (mean ± SD; $n = 6$) from the injection locus during the 4- to 6-h period. We cannot rule out the possibility that the area outside the drug-injected striatum may have contributed to some of the effect in the auditory conditioning.

Statistical Analysis. All experiments were performed with a minimum of three independent biological replicates. The n values in this study represent biological replicates. Sample sizes were chosen according to standard practice in the field and to previous analysis (1). Significance level of $P = 0.05$ was used to reject the null hypothesis. Statistical analysis was performed using PRISM 6.03 software (GraphPad).

1. Abe K, Watanabe D (2011) Songbirds possess the spontaneous ability to discriminate syntactic rules. *Nat Neurosci* 14(8):1067–1074.
2. Agate RJ, Scott BB, Haripal B, Lois C, Nottebohm F (2009) Transgenic songbirds offer an opportunity to develop a genetic model for vocal learning. *Proc Natl Acad Sci USA* 106(42):17963–17967.
3. Sakaguchi H, Wada K, Maekawa M, Watsuji T, Hagiwara M (1999) Song-induced phosphorylation of cAMP response element-binding protein in the songbird brain. *J Neurosci* 19(10):3973–3981.
4. Scott BB, Lois C (2005) Generation of tissue-specific transgenic birds with lentiviral vectors. *Proc Natl Acad Sci USA* 102(45):16443–16447.
5. Warren WC, et al. (2010) The genome of a songbird. *Nature* 464(7289):757–762.
6. Seidl AH, et al. (2013) Transgenic quail as a model for research in the avian nervous system: A comparative study of the auditory brainstem. *J Comp Neurol* 521(1):5–23.
7. Han J-H, et al. (2007) Neuronal competition and selection during memory formation. *Science* 316(5823):457–460.
8. Zhang X, et al. (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc Natl Acad Sci USA* 102(12):4459–4464.
9. Li J, et al. (1999) Leukaemia disease genes: Large-scale cloning and pathway predictions. *Nat Genet* 23(3):348–353.
10. Kent WJ (2002) BLAT—the BLAST-like alignment tool. *Genome Res* 12(4):656–664.
11. Tchernichovski O, Nottebohm F, Ho CE, Pesaran B, Mitra PP (2000) A procedure for an automated measurement of song similarity. *Anim Behav* 59(6):1167–1176.
12. Chew SJ, Mello C, Nottebohm F, Jarvis E, Vicario DS (1995) Decrements in auditory responses to a repeated conspecific song are long-lasting and require two periods of protein synthesis in the songbird forebrain. *Proc Natl Acad Sci USA* 92(8):3406–3410.
13. Mello CV, Clayton DF (1994) Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. *J Neurosci* 14(11 Pt 1):6652–6666.
14. Cheng H-Y, Clayton DF (2004) Activation and habituation of extracellular signal-regulated kinase phosphorylation in zebra finch auditory forebrain during song presentation. *J Neurosci* 24(34):7503–7513.

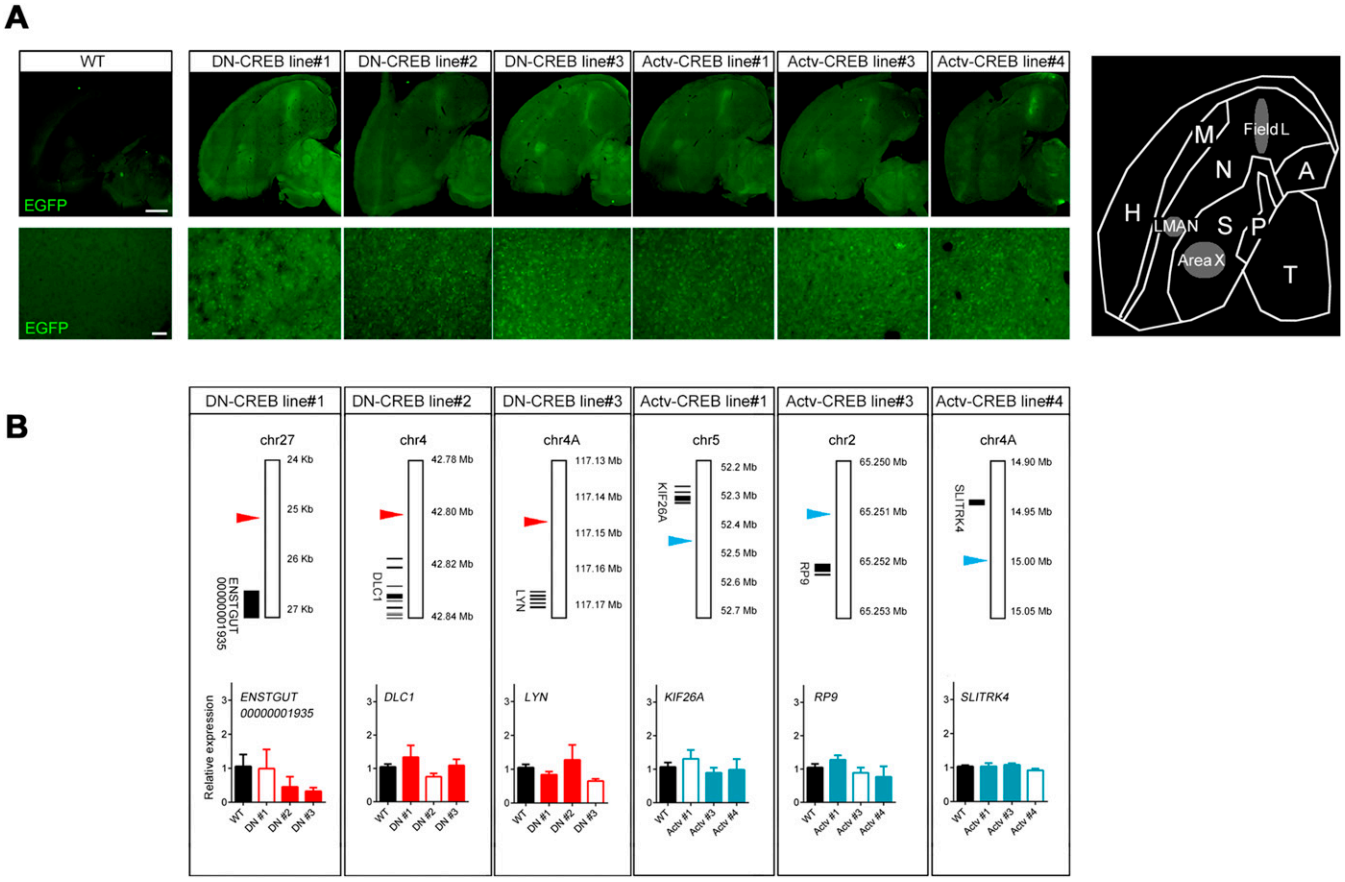

Fig. S1. Expression of transgenes in the individual lines of the transgenic finches. (*A*) Images of immunostained sagittal brain sections from WT (*Left*) and each three line of G_1-transgenic DN-CREB (*Middle*) and Actv-CREB (*Right*) birds. Upper row, low-magnification images (scale bars, 500 μm); lower row, high-magnification image of nidopallium immunostained with EGFP (scale bars, 50 μm). Far right, anatomical profiles. A, arcopallium; H, hyperpallium; M, mesopallium; N, nidopallium; P, pallidum; S, striatum; T, thalamus. (*B*) Loci of chromosome integration (red or blue triangle) of transgene in each line identified by inverse PCR analysis (7) (*SI Materials and Methods*). The expression of the nearest transcripts of each integration locus was analyzed by quantitative RT-PCR and normalized to the expression level of WT birds. Bar graphs indicate mean ± SEM.

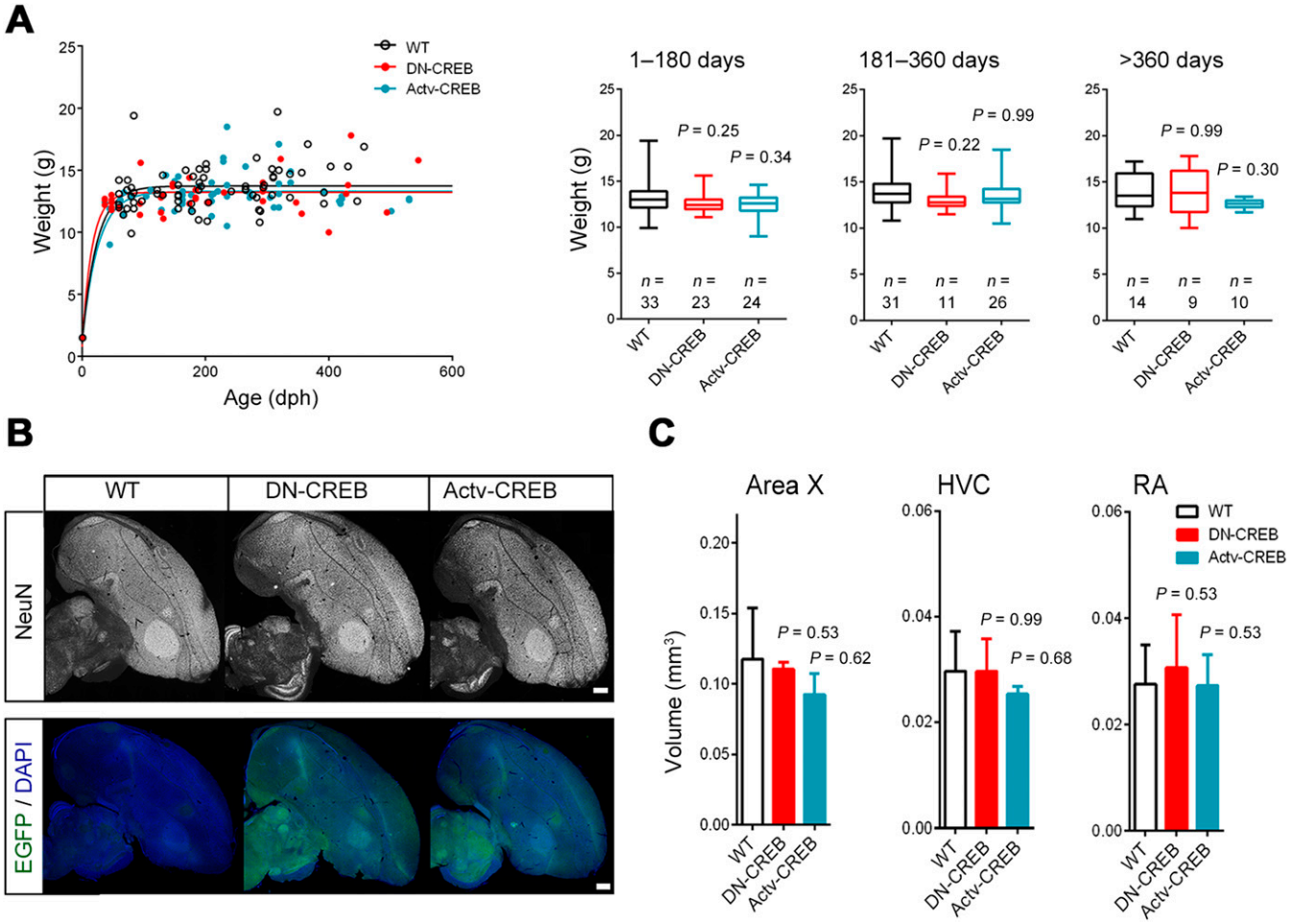

Fig. S2. Basal phenotype of the transgenic finches. (*A*) Scatter plot of body weight. Data from male G_1-transgenic birds are shown. Graphs at the *Right* show box-and-whisker plots of the average body weight of three age groups: 1–180 d (*Left*), 181–360 d (*Middle*), and >360 d (*Right*). *P* values (as determined by Bonferroni–Dunn's test) against WT in each age group are indicated. No significant differences were observed (Kruskal–Wallis test; 1–180 d, $P = 0.23$; 181–360 d, $P = 0.28$; >360 d, $P = 0.31$). (*B*) Images of immunostained sagittal brain sections from WT (*Left*) and G_1-transgenic DN-CREB (*Middle*) and Actv-CREB (*Right*) birds. Upper row, NeuN; lower row, EGFP and DAPI staining. (Scale bars, 500 μm.) (*C*) Average volumes of hemispheric Area X (*Left*), HVC (*Middle*), and robust nucleus of the arcopallium (RA) (*Right*) from WT ($n = 3$), DN ($n = 3$), and Actv ($n = 3$) G_1-TgN birds, aged 190–250 dph. Bar graph shows mean ± SD. *P* values (as determined by Dunnett's test) against WT for each genotype are shown. No significant difference was observed for any nucleus.

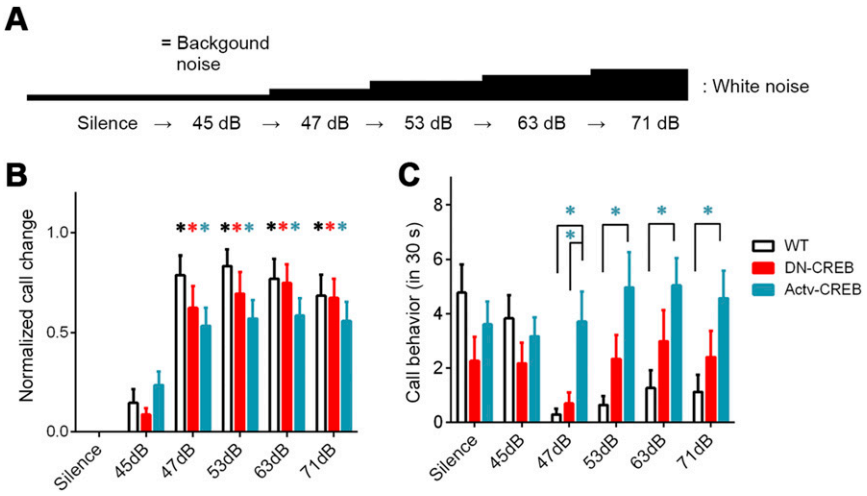

Fig. S3. Hearing threshold was not affected in transgenic birds. (*A*) Schematic of the hearing threshold analysis. Adult male birds (>140 dph; WT, $n = 14$; DN, $n = 14$; Actv, $n = 15$) were presented with increasing volumes of white noise (random noise with uniform spectral density, 45 to 71 dB, 30 s each), and the number of call behaviors was counted. The background noise under these experimental conditions was ∼45 dB. (*B*) Normalized differences in call reaction number. The number of calls during each period was normalized to that in the silent period. *$P < 0.05$, Dunnett's test compared with 45-dB data of each genotype. (*C*) Raw numbers for call behavior in each period (30 s). The birds that increased the call behavior in response to 47-dB sound: 0 of 14 in WT, 3 of 14 in DN-TgN, and 8 of 14 in Actv-TgN. A significant difference in the behavioral reaction was observed in Actv-CREB TgN birds. *$P < 0.05$, Tukey's post hoc test [two-way ANOVA: genotype factor, $F_{(2,240)} = 12.14$, $P < 0.001$]. Bar graphs indicate mean ± SEM.

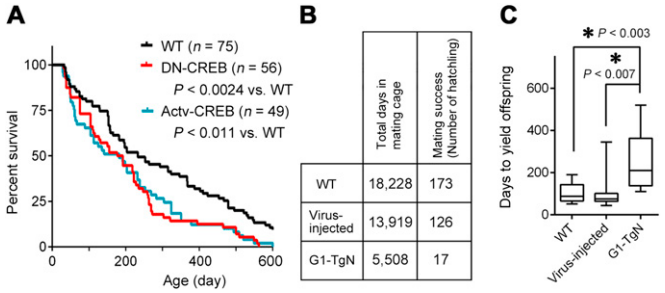

Fig. S4. Reduced survival ratio and mating performance in the transgenic birds. (*A*) Survival ratio curve of G_1 offspring and WT. Summarized data of both sexes are shown. All birds were reared under a song-training experimental paradigm from 30 to 140 dph, and afterward kept in an aviary with mixed genotype birds of similar age. Significant differences were observed between WT vs. DN, and WT vs. Actv (Mantel–Cox log-rank test). (*B*) Mating performance analysis. The total number of days in the mating cage and the number of occasions when offspring were produced by WT (41 pairs), virus-injected birds (birds hatched from the virus-injected egg, with and without germ line transmission confirmation, 25 pairs), and G_1-TgN birds (15 pairs) are listed. Birds of both sexes were mated with WT birds. Significant differences were observed in WT vs. G_1-TgN, and virus-injected vs. G_1-TgN ($P < 0.0001$ and $P < 0.0001$, respectively; χ^2 test). (*C*) Number of days required to yield offspring. The total days in the mating cage were divided by the number of occasions of egg hatching. Only the pairs that succeeded to produce offspring are used for the calculation. One-way ANOVA, $P < 0.0033$. The *P* values calculated from Tukey's post hoc analysis are shown. Boxes and whiskers show the respective median and 25th to 75th percentiles and 10th to 90th percentiles.

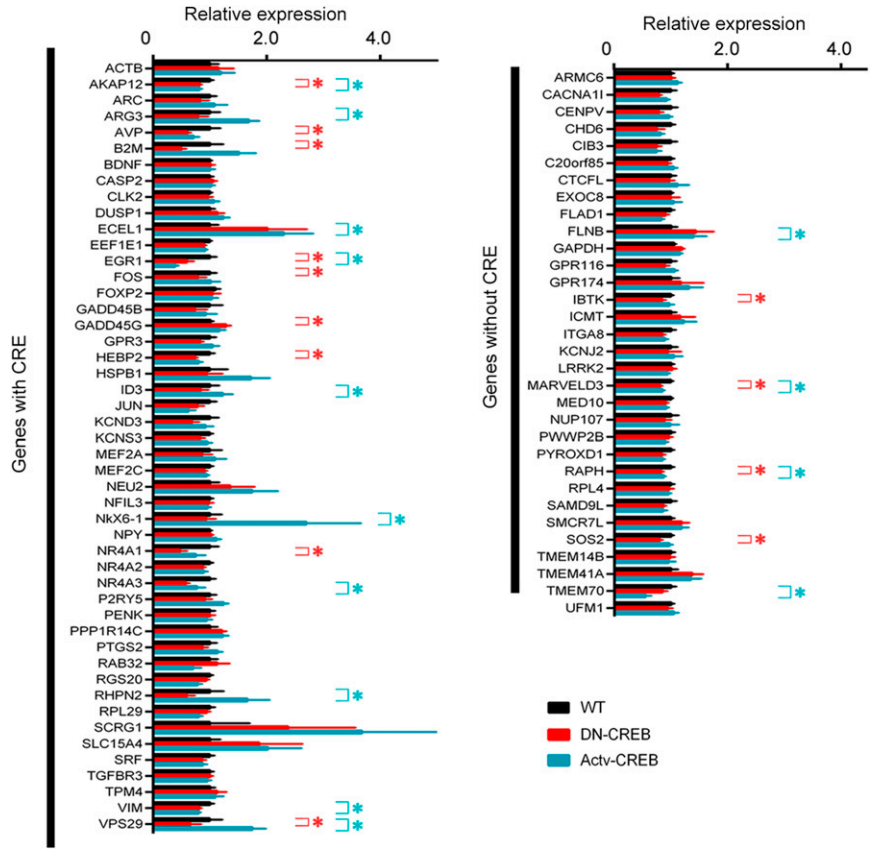

Fig. S5. Gene expression profiles of the transgenic finches. Quantitative real-time PCR analysis of mRNAs collected from brains of 60-dph birds ($n = 11$ birds for WT, DN, Actv). The bar graph shows the relative amount of expression normalized to the expression in WT birds. Genes are grouped according to whether the human homolog contains a CRE sequence in its promoter region (8). Bar graphs indicate mean ± SEM. $*P < 0.05$, Dunnett's test.

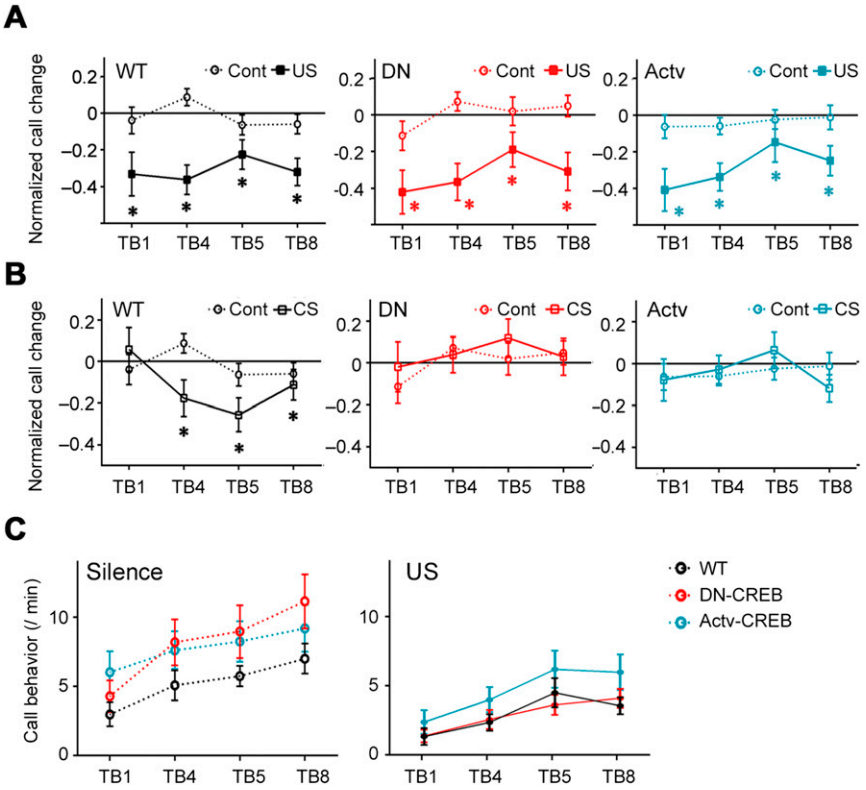

Fig. S6. Deficits in auditory-memory formation in transgenic zebra finches. (*A* and *B*) Fear-conditioning test using a different set of songs used in the experiment shown in Fig. 3. Behavioral reactions against control song stimulus (Cont) (dotted lines) are shown along with the reactions against unconditioned stimulus (US) (solid lines; *A*) and conditioned song stimulus (CS) (solid lines; *B*). Changes in call behavior number after the presentation of stimuli (Cont, US, and CS) are normalized and are shown for each genotype (WT, *Left*; DN, *Middle*; Actv, *Right*). Mean ± SEM are shown. Asterisks indicate a significant difference in the call response before and after the presentation of each stimulus, $P < 0.05$; Student's paired *t* test; WT, $n = 25$; DN, $n = 25$; Actv, $n = 25$. (*C*) Actual number of call behaviors before (Silence, dotted lines; *Left*) and after the presentation of US (solid lines; *Right*).

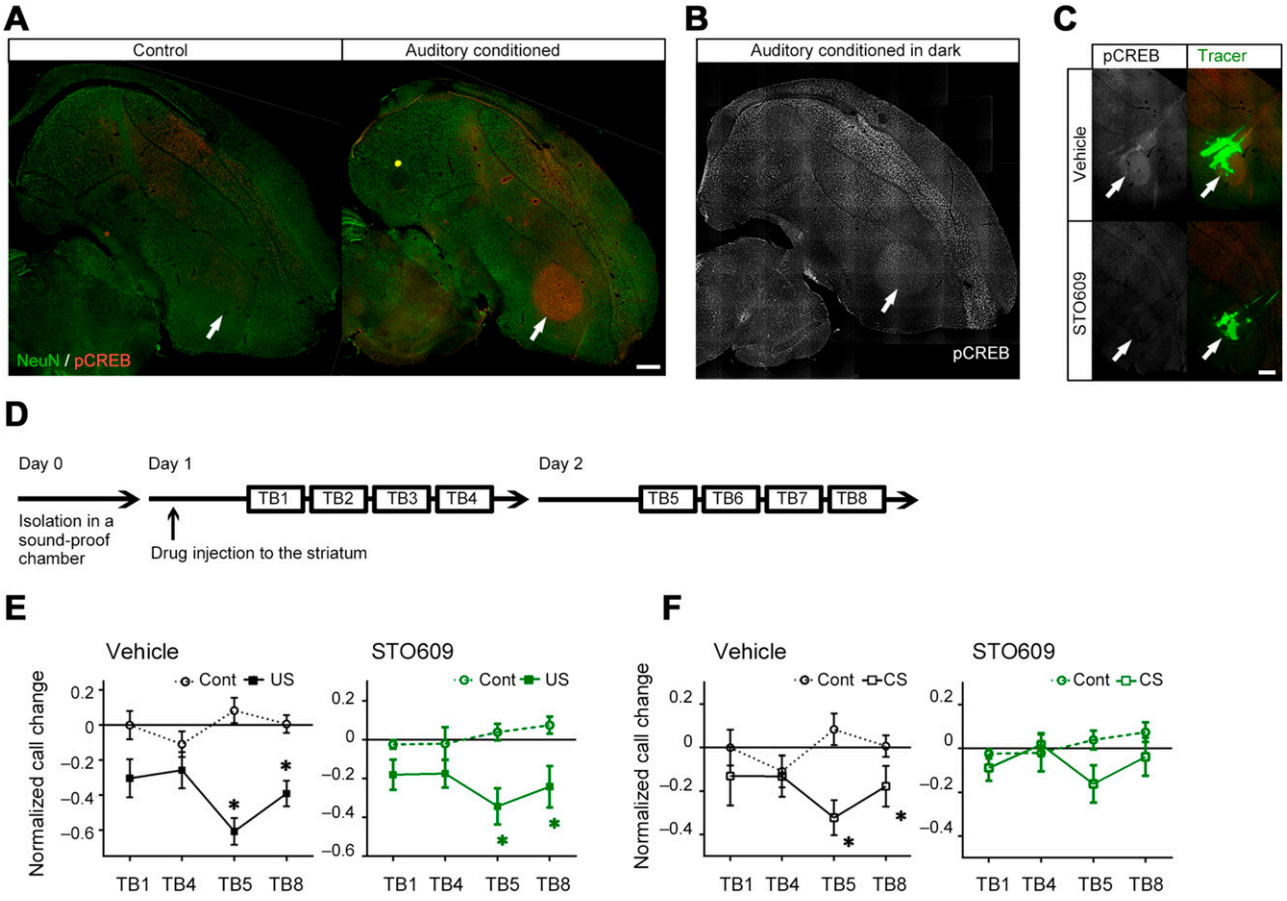

Fig. S7. CREB activation in basal ganglia is involved in the formation of memory. (*A*) Immunostained sagittal brain sections showing signals of phosphorylated CREB (pCREB) (red) and NeuN (green). Adult birds were subjected to auditory conditioning (Auditory conditioned) or kept in silence (Control). WT birds were subjected to the auditory conditioning as shown in Fig. 3*A*, and at the beginning of TB4, subjects were killed. (*B*) A section from birds subjected to auditory conditioning in a dark chamber. Arrows indicate the Area X. (*C*) Sections from birds injected with vehicle or STO609 along with Alexa 488-conjugated cholera toxin B (tracer) and then auditory conditioned. Arrows indicate the injection sites of the drug or vehicle. (Scale bars, 500 μm.) (*D*) Schematics of the drug injection and auditory conditioning experiment. (*E* and *F*) Results of the auditory conditioning experiments. Behavioral reactions against control song stimulus (Cont) (dotted lines) and unconditioned stimulus (US) (solid lines; *E*) or conditioned song stimulus (CS) (solid lines; *F*) are shown. Changes in call behavior number after the presentation of stimuli (Cont, US, and CS) are normalized and shown for each treatment (vehicle, *Left*; STO609, *Right*). Mean ± SEM are shown. Asterisks indicate the significant differences in call response before and after the stimulus presentation, $P < 0.05$; Student's paired *t* test. Vehicle, $n = 24$; STO609, $n = 24$.

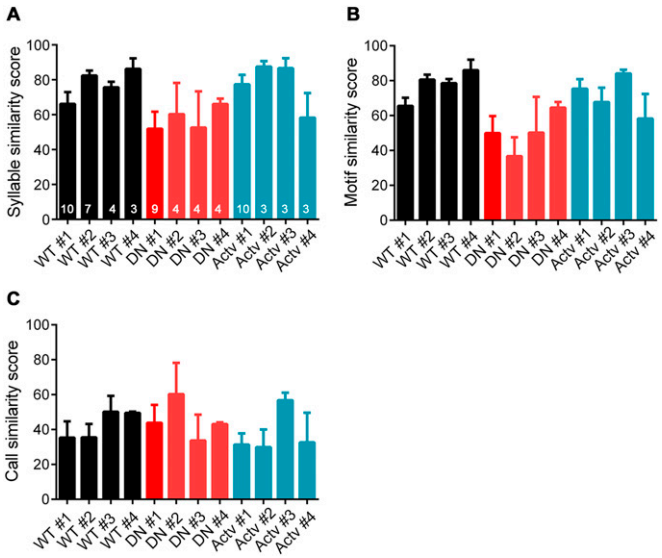

Fig. S8. Song and call development in the individual lines of the transgenic finches. (*A*) Similarity score of tutee's songs at 140 dph, calculated from the similarity of each syllable. Tutees are grouped according to transgenic or WT lines. Each line is derived from common biological parents. (*B*) Similarity score of tutee's songs at 140 dph, calculated from the similarity of motif. (*C*) Similarity score of tutee's call at 140 dph. Bar graphs indicate mean ± SEM. The numbers of WT and transgenic birds analyzed are indicated in the bar graph in *A*.

Table S1. PCR primers used in this study

Gene	Forward primer	Reverse primer
ACTB	5′-ctcgctggagaagagctacga-3′	5′-aggagggctggaagatgga-3′
AGR3	5′-attctacactagccttgtccctcct-3′	5′-tttcgtctccccaccctct-3′
AKAP12	5′-gctggtagatgcagttgctgtt-3′	5′-tcttcttcttctcttgcctgctc-3′
ARC	5′-ccacgtcccaactgaaaacc-3′	5′-cttccaaacattcatctccctctt-3′
ARMC6	5′-cagttccagtcccaaggtgt-3′	5′-aggatttgatgcttttgcctct-3′
B2M	5′-gtttcttcagagtcatttgacaagc-3′	5′-ccatcataccaccaaggctaca-3′
BDNF	5′-cttctacgagaccaaatgcaacc-3′	5′-cgccagccaactctctttt-3′
CACANA1L	5′-gagttggcctcagtcccttct-3′	5′-ccccgttctttgtggttgtt-3′
CASP2	5′-taggcaaatcaagcaacgagaa-3′	5′-ggtagatagccaggaaacaggtaaa-3′
CENPV	5′-atgggaggaagcagtgagga-3′	5′-accaagcatcacccaggaaa-3′
CLK2	5′-atgctggctgatcgcttctt-3′	5′-tgtagcccaactcatccacttct-3′
CHD6	5′-gctctacagtcccagtctccataac-3′	5′-tcatcctcttcttcctcctcatct-3′
CIB3	5′-cagcggatagcagaggtgttc-3′	5′-tcggggagccatttcactt-3′
CREB1	5′-atcgaagaagaaaagtctgaagagg-3′	5′-tgctcctccttgggtgatg-3′
CREB1(5′UTR)	5′-gcaaacacctggcatgaaaa-3′	5′-agtccattactcttggtgaatctgg-3′
CTCFL	5′-agccaagttatcaagtcgtaggtgt-3′	5′-cttgttccctgtatgtgcgttc-3′
C11orf58	5′-agaacatactggccgccttg-3′	5′-tccacctgcctcttcttgct-3′
C20orf85	5′-agctgcaaagcccaagctac-3′	5′-acctgacctccagccaataaac-3′
DLC1	5′-gacagcaagaggtggtcaagg-3′	5′-gaggaggagtttctggggatg-3′
DUSP1	5′-tgtgaagaacgagggaggaag-3′	5′-agataagcgaggcagatggtg-3′
ECEL1	5′-ggccgtagtcttctacctcacc-3′	5′-gggctttctgctccacattc-3′
EEF1E1	5′-tcaaccacattcagcattaccc-3′	5′-cacacctcattccaacacaaca-3′
EGR1	5′-aggcagaaggacaagaaagtgg-3′	5′-gatggaggaagggtaggatgtagtt-3′
ENSTGUT 00000005059	5′-gattcggcctacagctccttc-3′	5′-gatggacacgggctcttca-3′
EXOC8	5′-catgttcaaactgctcatgttcc-3′	5′-cacctccagccactccttct-3′
FLAD1	5′-agctctttgtcccctactgtatcct-3′	5′-ctgggttcttctgtgtgttggt-3′
FLNB	5′-aatcagcagggaagggaaaag-3′	5′-ccaaagcggacataaatcacatag-3′
FOS	5′-caggcggagattgctaacc-3′	5′-cacaactcctcgggcatctt-3′
FOXP2	5′-gctgtgaaagcgtttgtgaaga-3′	5′-gttggtgttgtgggagtttgag-3′
GADD45B	5′-tgggtgccttacgtgtgtct-3′	5′-tggggattgtggcttgg-3′
GADD45G	5′-cccgaatgaagaaggttgga-3′	5′-ctcaggcagggtgatagttgg-3′
GAPDH	5′-agtgaaggctgctgctgatg-3′	5′-cgcatcaaaggtggaggaa-3′
GPR3	5′-tgcagatctgtcgcatcgt-3′	5′-ggttgagcagggagttggag-3′
GPR116	5′-agatacacaacgccacagaaaca-3′	5′-caatacaacaggacagccgaag-3′
GPR174	5′-atgcagcaccaggaggataaa-3′	5′-tcacgaacccccaccaactc-3′
HEBP2	5′-agctctctttgtccggtcctt-3′	5′-gaatggttggcctctgtttctt-3′
HSPB1	5′-aggagaagcaggacgagca-3′	5′-agcattccatcgggagaca-3′
H2B-flag	5′-ggcgaactcgcgaaaca-3′	5′-cagcaggggatttgggatag-3′
IBTK	5′-ttgggtattcttcctcctccttc-3′	5′-cactccaatcaccattctacctttc-3′
ICMT	5′-gccacttcggatggtatatgtg-3′	5′-tgcgagggttgttgatgg-3′
ID3	5′-tcggagcagagtttagccatc-3′	5′-gcagtcgttcatatcgtagagca-3′
ITGA8	5′-ttggcagtgcaatcacacac-3′	5′-ctttccctctcctgtcttctcct-3′
JUN	5′-tacggatacaataacgccaaggt-3′	5′-tgcggcttcaggttgct-3′
KCND3	5′-taagggttttcagggttttcagg-3′	5′-gccatagtgagggaaaagaggag-3′
KCNJ2	5′-tcatcaacgtgggcgaga-3′	5′-caaccaaaacacacagccaaa-3′
KCNS3	5′-tcacgttatggaagagctttgtgt-3′	5′-ccttcctctttccgttcttgg-3′
LRRK2	5′-tcaaccaagacagaccattcca-3′	5′-aacccaccatcacccaagag-3′
MARVELD3	5′-gtctcatgctgggagtcgatac-3′	5′-tatgggtatgcttttcttgctcttc-3′
MED10	5′-gagtgtctggagcgagctttg-3′	5′-ttctgggaacacctttgtcagtt-3′
MEF2A	5′-ccagtgggaaatgggtttgt-3′	5′-gtcgggtttgcggttgtt-3′
MEF2C	5′-cccatcagccatctcaacaa-3′	5′-tgccaaccagtcacagaacc-3′
MYBL1	5′-tgggaggcagttgtttatgg-3′	5′-ttggtggcagtgtatgtgct-3′
NEU2	5′-cctacagacccaaagcgaaaag-3′	5′-aagaggcaggagaacaaaggtg-3′
NFIL3	5′-gcaagttaaagtggaagcaatgg-3′	5′-gaaccgagaaaggagtgtgagaa-3′
NPY	5′-ttgagggaaagcacagaaaaca-3′	5′-agaggtggggagtatgaactgaaa-3′
NR4A1	5′-atccccagtgccaccaa-3′	5′-tccaccgagtcctccttctc-3′
NR4A2	5′-gctgaatcgccccaactatt-3′	5′-ggcaccaaatcctccagttt-3′
NR4A3	5′-gatgccaatactgccggttc-3′	5′-agccgacctcttctcccttt-3′
NUP107	5′-ggatcttgatcctttgggatatg-3′	5′-ttctgttggggtcttttgtttg-3′
NKX6-1	5′-gccagcagattttcgctct-3′	5′-atcgtcctcctcctcgttctc-3′
P2RY5	5′-cggaacaatacggaacaaagg-3′	5′-ggcacaaagcagaagcagaa-3′
PENK	5′-aggccaaagagctgcaaaag-3′	5′-ggagaatggagtcggcaaag-3′
PLEKHA2	5′-gtaagatcacggtgccaaagg-3′	5′-ggttgatggagatgggtgtgt-3′

Table S1. Cont.

Gene	Forward primer	Reverse primer
PPP1R14C	5′-atcaaagagctgctaactcggataa-3′	5′-gtattcaaagtcccatttcccttg-3′
PTGS2	5′-cactgctgcccgataccttc-3′	5′-cctcatctgcctgctttggt-3′
PWWP2B	5′-aggacgaggaggcgtttgt-3′	5′-ggggttcagcgtgttcttg-3′
PYROXD1	5′-agcagctgaattcctccttcc-3′	5′-cctttctcctcacttccttcca-3′
RAB32	5′-ggtggctttgttggatggtt-3′	5′-tggtttccccacatcattctc-3′
RAPH1	5′-gttacttccttcttcgggcttct-3′	5′-tgggtgccttgtatttgttcc-3′
RGS20	5′-ctgctgcttttgttggtgct-3′	5′-ttctccttgctctctcttcttcttg-3′
RHPN2	5′-ggttatcagttgccaggacaca-3′	5′-agggagttttagtggggctttc-3′
RPL4	5′-ttacgccatctgctctgctc-3′	5′-ggttttcttgtagccctcaacct-3′
SAMD9L	5′-ggcggaagcacaatagcac-3′	5′-tcataatctctaagggcaagcaca-3′
SCRG1	5′-aaagtggaagggagtgaacgag-3′	5′-cagcagcacagagaccatcc-3′
SLC15A4	5′-ctccaacgacaccaactcca-3′	5′-gcaaaggcagagcacatcac-3′
SLITRK4	5′-cggggatagtagcaggcaaa-3′	5′-aaacacaaggaactggacaagga-3′
SMCR7L	5′-atgctggctgatcgcttctt-3′	5′-tgtagcccaactcatccacttct-3′
SOS2	5′-cctcatgctgatcccagtctatc-3′	5′-cactccctgtcctcttcatcct-3′
SRF	5′-caacgggacagtgctgaaga-3′	5′-ctggattgcctggactgga-3′
UFM1	5′-ctgcgattactcggcctacac-3′	5′-ccttatcctgtctcccaccaac-3′
tbGFP-PEST	5′-atcaccggcaccctgaa-3′	5′-ggtagaagccgtagcccatc-3′
TGFBR3	5′-caccagccagaggatgacag-3′	5′-ttgggagggagaaaggaaca-3′
TMEM14B	5′-cttggattgcagatgatggaaa-3′	5′-aaaagacaacgcacaggtagca-3′
TMEM41A	5′-caccctctgctacctgctctct-3′	5′-cctgttctcttctaccttcccttg-3′
TMEM70	5′-tgtaaagattccagacatcaccaag-3′	5′-aggatttgggaaaagcatagga-3′
TPM4	5′-actgatgctgaaggggaggt-3′	5′-cgctctcatctgctgctttct-3′
VIM	5′-cgaaattgccacctacagaaaac-3′	5′-ctctcaggttcaaagcagcaaa-3′
VPS29	5′-ctcatttcaggacacacacacaaa-3′	5′-tgatgttgttctctaaggcatggt-3′

PCR primer sets used in the quantitative PCR analysis of mRNAs.