Supporting information for

Targeted knockout of a novel chemokine-like gene increases anxiety and fear responses

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SI Materials and Methods

Animals. *Zebrafish:* Wild-type zebrafish (*Danio rerio*), and *Et(-1otpa::mmGFP)hd1* enhancer trap transgenic fish (1) were raised and maintained under standard condition at 28.5 ˚C. Embryos were fixed at specific stages as described previously (2). All experimental protocols and procedures were approved and conducted according to the approved guidelines and regulations of the Animal Ethics Committee of Chungnam National University).

Mouse: (Behavior) Animal care and experimental procedures followed the guidelines of the Institutional Animal Care and Use Committee of the Institute of Basic Science. Experiments were performed with male C57BL/6N mice (8-14 weeks of age). Mice were housed in groups of four or five with free access to food and water, under controlled temperature and light conditions (23 ℃, 12-h light: 12-h dark cycle). Experiments were performed during the light phase.

Mouse: (Brain slice electrophysiology) Adult male mouse Crh-IRES-Cre; Ai14 (TdTomato) were group housed in controlled temperature (20 ± 2 ºC) and lighting (12-h light, 12-h dark) conditions with ad libitum access to food and water. Mouse experimental procedures were approved by the University of Otago Animal Ethics Committee.

The golden fish project. After conducting a genetic screen, the mapping of chemically induced mutants is difficult due to high cost and labor. Insertional mutagenesis is an alternative approach to chemical mutagenesis which speeds up the identification of mutated genes (3, 4). Using this approach, we established a unique insertional mutagenesis system based on the Sleeping Beauty transposon (4). In addition to the green fluorescent protein (GFP) gene, we introduced a melanin-concentrating hormone (MCH) gene as a transgene reporter in our insertional mutagenesis. MCH was originally isolated from the pituitary gland of fish where it controls skin pigmentation. Enhanced expression of MCH in transgenic medaka fish

can induce melanosome aggregation and change body color into albino or “golden” (5). Although GFP reporter can be monitored under fluorescent microscopy in live zebrafish embryos, however, it is not easy to detect the signal which is expressed in small subsets of cells in a specific tissue in adult zebrafish. This lack of GFP fluorescent penetration in adult zebrafish could be overcome by using our dual reporter system; GFP plus MCH. After visual screening of mutants with GFP fluorescence and melanosome aggregation, mapping of the insertion site was performed using the DNA walking Speedup kit (Cat. K1052, Seegene, Seoul. Korea)

Isolation of *samdori* gene family. After screening and mapping of insertional mutants, we discovered a novel chemokine gene, named *samdori (sam)* which means the third son in Korean (Fig. S1). From the NCBI database we further identified the existence of total eight members of this *sam* gene family in the zebrafish genome. Since there were five *SAM* genes in mammals, we named them as *sam-1a, -1b, 2, 3a, -3b, 4, 5a,* and *5b,* respectively.

Cloning of zebrafish *samdori* gene family. The full-length cDNAs of zebrafish *sam-1a, 2, 3b, 4, 5a, 5b* genes were isolated using the 5’-, 3’-RACE (Rapid Amplification of cDNA ends) technique (FirstChoice RLMRACE Kit, Ambion). Primers used: *sam-1a*, 5’-CAGCCGGTGCTCCTGCCATGTCCTGGCTC-3’ for forward, 5’-GGAGCTCTTTGTTAGGTCCGGGGATGA-3’ for reverse; *sam-1b*, 5’-CGATACAGAGGAGTGTTGGGATGTCGTGG-3’ for forward, 5’-GCAGTGATTCTACCTCTGCTACAAGGA-3’ for reverse; *sam-2*, 5’-ACGCCGCTGAATGAACCGATTACCGG-3’ for forward, 5’-GAGCGAACGCACTGCTTTACACAC-3’ for reverse; *sam-3a*, 5’-CAGCTCAACGAGGGGGATGCGGGAGAGG-3’ for forward, 5’-GGTCCCAGACTATCGTGTGACCTTGGTC-3’ for reverse; *sam-3b*, 5’-

ACTACCGCGCCAACAGGATGCA-3' for forward, 5'-GTCCTGGGACAAAAGAGCTGA-3' for reverse; *sam-4*, 5'-GCTCGTTTAAAACAAACATCTGTCTTCAGGAC-3' for forward, 5'-GAACTGCCATGTTGTTGCTATCGTGTGACC-3' for reverse; *sam-5a*, 5'-GGAGATGTGGCGTGGATGCTGAAGGCAG-3' for forward, 5'-CCTCCCACTGTTAGGACACCGTTGTAGT-3' for reverse; *sam-5b*, 5'-ACGCGTCAGATCCTCGGAAAGATGCAGC-3' for forward, 5'-CCACTGGCGTCAGGATACCGTGGTTGT-3' for reverse.

Whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization and two-color *in situ* hybridization were performed as described previously with some modifications. RNA probes for *aoc1, agrp, c-fos, crhb, cxcr4b, dat, fam84b, gad1b, gad2, hcrt, lov, mch, npy, otx5, oxt (itnp), pet-1, sst1, th, tphR, vglut2a, vglut2b,* and *sam2* gene, were synthesized using SP6, T7 or T3 RNA polymerase. For two-color *in situ* hybridization, fluorescein-labeled riboprobes were synthesized as described previously (6, 7).

Immunohistochemistry. Whole-mount immunostaining was performed as described previously (8). Fixed embryos were permeabilized with 10 μg/ml Proteinase K for 30 min and re-fixed with 4% paraformaldehyde in PBST for 20 min. The embryos were incubated with the primary and secondary antibody overnight. Antibodies used were rabbit-anti-5HT (ImmunoStar; 1:100), and goat-anti-rabbit Alexa Flour 488 (LifeTechnologies; 1:500).

Paraffin and frozen section. After whole-mount *in situ* hybridization, embryos were dehydrated by a graded series of ethanol treatments. Dehydrated embryos were transferred to xylene and soaked in paraffin overnight. After embedding, sections were cut at 5 μm thickness (9). In the case of frozen section, stained tissues were washed with PBS and cryoprotected in 30% sucrose/PBS (wt/vol) overnight at 4 ˚C. Tissues were embedded in

frozen section compounds (FSS 22, Leica Microsystems) and cryosectioned to 12- 20 µm (10).

Generation of zebrafish *sam2* and mouse *Sam2* KO mutants. *Zebrafish*: Target specific ZFN vectors for *sam2* were designed and synthesized, as previously described (11). ZFN vectors were linearized with *Pvu*II and capped mRNAs were synthesized using T7 RNA polymerase. Two to three nanograms of ZFN mRNA was injected into one cell stage embryos. For identification and genotyping of *sam2* KO fish, we used the *T7 endonuclease 1* (*T7E1*; New England Biolabs) assay to detect heteroduplex formation after DNA denaturation and annealing. Primers used for *sam2*^{cnu1} genotyping were *sam2*^{cnu1} forward (5'-TCTACTGAGGAGTGGTGTGA-3') and *sam2*^{cnu1} reverse (5'-GGTCAGTTTCAGAGAGCTGG-3'), respectively. Primers used for *sam2*^{cnu2} genotyping were *sam2*^{cnu2} forward (5'-AGACCGTCAAGTGCTCCTGC-3') and *sam2*^{cnu2} reverse (5'-GGTCAGTTTCAGAGAGCTGG-3'), respectively.

Mouse: TALEN vectors were linearized with *PvuII* and capped mRNAs were synthesized using T7 RNA polymerase. The pair of TALEN mRNA was injected into the cytoplasm of fertilized eggs. TALEN-mediated *Sam2* F0 mice were screened by *T7E1* assay as we previously described (12). The genomic DNA was prepared from tail biopsies and amplified using TALEN target site primers. Founder line #15 (14 bp-deletion) was backcrossed with C57BL/6N and heterozygous breeding was set up to generate *Sam2* KO mice (*Sam2*$^{-/-}$). Primers used for genotyping were mouse *Sam2* forward (5'-GTGAGAAATTCAGTGTTCTGGG-3') and mouse *Sam2* reverse (5'-CCTGAAGACAGCTCTCTGCA-3'). Further, PCR products were digested with *BslI* restriction enzyme to confirm the deleted sequence in mutant mouse.

Open tank test, scototaxis test and thigmotaxis test in zebrafish. To measure anxiety-like behavior, male zebrafish siblings of

identical age and size (3 to 3.5 cm) were tested. Fish were placed into the novel open tank (15 cm × 15 cm × 25 cm; height × width × length) for 10 min and their movements were recorded with a video camera (13, 14). Open tank test was repeated eighteen times for control and twenty eight times for the *sam2*^{cnu1} allele. For the scototaxis test, we measured the preference of black vs. white compartments. Individual fish was placed into a tank coated with black and white plastic sheet and allowed to acclimate for 5 min and then monitored by video recording for 15 min (15, 16). Scototaxis test was repeated eighteen times for control and twenty four times for the *sam2*^{cnu1} allele. For the thigmotaxis, we placed six-male fish in the tank (15 cm × 15 cm × 25 cm; height × width × length) for 30 min and measured the time spent in the corner (one fifth of tank's left and right parts) and the center (rest of corner of the tank) every one minute. The experiment was repeated twelve times of both the control and *sam2*^{cnu1} allele. EthoVision XT7 (Noldus Info Tech, Wageningen, The Netherlands) was used to analyze fish behavior. To track a single fish, we selected the "difference" in steps of the detection setting of Ethovision (16).

Alarm substance assay (skin extract)

Alarm substance was freshly prepared by making 10-15 shallow lesions on both side of the zebrafish skin using a sharp razor. Seven fish were immersed in 50 ml of 20 mM Tris-Cl (pH 8.0) for 25 seconds and filtered using a 0.2 μm filter (Sartorius stedim, 16534). To test alarm response, five three-month-old fish were placed in tank containing 4 liters of aquarium water. After acclimated fish in the aquarium water for 30 minutes, gently added 10 ml of alarm substance into the surface of the water (17, 18).

Three-dimensional reconstruction of swim path and fish positions. Fish position (x and y coordinates from the dorsal view and z coordinates from frontal view) was recorded at 20 sec intervals for the early phase recording (5-8 min) and the late phase recording (13-16 min) time based on our preliminary data. Inter-fish distances or individual gaps (measured between

the focal fish and its shoal mates) were calculated using the (distance= $\sqrt{(x_a - x_0)^2 + (y_a - y_0)^2 + (z_a - z_0)^2}$) where x_0, y_0 and z_0 were x, y and z coordinates of the focal fish while x_a, y_a and z_a represent coordinates of shoal member at the defined time (19, 20). Meanwhile x, y and z coordinates of the core fish were considered as the zero point of in the shoal at the defined time. Cohesion experiment was repeated three times in the *sam2*^{cnu1} and twice in the *sam2*^{cnu2} allele. Both alleles showed similar results. For statistical analysis SigmaPlot (Version 10, SigmaPlot, Chicago, IL, USA) was used. The effect size (Cohen's *d*) was calculated for the social cohesion of control and KO fish at early and late phases.

Open field test in mice. The open field chamber consisted of a 40 cm (W) x 40 cm (L) x 40 cm (H) white acrylic box. Mice were placed in one of the four corners of the open field and allowed to explore the chamber for 30 min. The field was illuminated at 10 lux. Total distance moved for 30 min was recorded for each mouse. After the test, each mouse was returned to its home cage and the box was wiped clean using 70% ethanol and distilled water. Activity was monitored and analyzed on Ethovision 9.0 (Noldus Information Technologies, Leesburg, VA). Each behavior test was performed with one-week interval. Behavior results were obtained from at least three different batches of animals.

Elevated plus maze. Custom made mouse elevated plus maze was made of grey acrylic base and consisted of four arms (two cross-shaped arms (length: 30 cm, width: 5 cm, height from floor: 40 cm). Two arms (closed arms) were enclosed with 15-cm high walls and the other two (open arms) were not. The enclosed arms and the open arms faced each other on opposite sides. For the test, mice were placed in the center square and allowed to move freely for five minutes. Open arm entries were defined as the mouse having all four paws onto the open arm. Time spent in open arms (%; [time in open

arms] / [time in closed arms + time in open arms] x100) was analyzed to measure anxiety. Behavior was monitored and analyzed on Ethovision 9.0 (Noldus Information Technologies, Leesburg, VA).

Fear conditioning. Fear conditioning was performed as previously described with minor modifications (21). For the training, mice were placed in the fear conditioning chamber (context A) for 3 min, and then mice were received paired stimuli of tone (30 s, 86 dB, 3000 Hz) and a co-terminating shock (1 s, 0.7 mA). Mice underwent a total of four conditioned stimulus-unconditioned stimulus (CS-US) pairs separated by 120-sec intervals. Twenty-four hours after training, contextual fear memory was tested in the same chamber (context A) for 5 mins in the absence of the auditory stimulus and shock. After 3 hrs, cued fear memory was tested in a different context (context B: novel chamber, odor, floor, and visual cues). After 10 min of exploration time, the 30-sec auditory CS was delivered. The freezing of the mice was recorded and analyzed with FreezeFrame software (Actimetrics, Wilmette, IL).

RT-PCR analysis of mouse *Sam2* expression

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, mice were euthanized and brains were isolated and immediately frozen on dry ice. Frozen brains were stored at -80 °C prior to cryosectioning. The next day, the brains were cryosectioned at a thickness of 250 μm. Frozen sliced brain tissue was collected on cold slide glass and dissected under a microscope. Total RNAs from each brain tissue of wild-type mice were extracted using GeneAll Hybrid-R (GeneAll Biotechnology, Korea). cDNA was synthesized following manufacturer protocol (SuperScript IV VILO Master Mix, Invitrogen). Primers used for RT-PCR were *Sam2* forward (5'-TTCCAGATCGCAAAGGATGG-3') and *Sam2* reverse (5'-AGTCTTCAGGACCCAACGTT-3'), respectively. Primers used for the endogenous control were *Actb* forward (5'-CGGGCTGTATTCCCCTCCATCG-3') and *Actb* reverse (5'-

TGGTGAAGCTGTAGCCACGCTC-3'), respectively.

Preparation of highly purified SAM2 protein

Mammalian SAM2 (residues 31-134, except for the signal peptide) fused to the C-terminal of Protein G was cloned into the modified pX vector (22, 23). This recombinant protein was expressed by transient transfection using PEI in human embryonic kidney 293 EBNA1 (HEK 293E) cells. Transfected HEK 293E cells were grown in suspension for 72 hours at 33 °C. The cells were sonicated in buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl and 0.5 mM 1,4-Dithiothreitol. The SAM2 protein was prepared by on-column cleavage with PreScission protease. For high purity protein production, the eluted protein was further purified on HiTrap Q HP column (GE Healthcare) operated with a linear NaCl gradient (50-500 mM) with 20 mM Tris-HCl (pH 7.5) and 0.5 mM 1,4-Dithiothreitol, and on HiLoad 26/600 Superdex 75 pg gel filtration column (GE Healthcare) in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.5 mM 1,4-Dithiothreitol. The purity and molecular weight (~12 kDa) of the purified SAM2 protein was confirmed by 10% Tris-tricine-SDS-PAGE analysis, using the protein size marker (Xpert 2 Prestained Protein Marker, 7 to 240 kDa, GenDEPOT) (Fig. S12).

Brain slice electrophysiology. Acute 200 µm coronal brain slices containing the PVN were cut using a vibratome (VT 1200S) and left to recover in 30 ºC artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO3. 1.25 NaH2PO4, 2.5 CaCl2, 1.5 MgCl2 and 10 glucose, saturated in 95% CO_2/5% O_2. Whole-cell patch-clamp recordings were made from tdTomato-fluorescent CRH neurons visualized under an upright microscope (Olympus). Whole-cell patches were made using borosilicate glass electrodes (tip resistance: 2-5 MΩ) filled with an internal solution of (in mM): 130 KCl, 10 HEPES, 0.2 Na2-GTP, 2 Mg2-ATP, adjusted to pH 7.2 with KCl, and to 290 mosM with sucrose. Neurons were voltage clamped at -60 mV. Evoked currents (100 µA) were made in the surrounding PVN tissue using

a monopolar ACSF-filled glass stimulating electrode. Currents were recorded in the presence of CNQX (10 µM). Signals were acquired using a Multiclamp 700B amplifier (Molecular Devices) connected to a Digidata 1440a, and filtered at 2 kHz before digitizing at 10 kHz. Currents were analyzed using Clampfit (Molecular Devices) or MiniAnalysis (Synaptosoft).

Oligonucleotide-based aCGH. Microarray-based comparative genomic hybridization (aCGH) was performed using custom-designed, oligonucleotide-based whole-genome microarray, either 105K (for GC42855, SignatureChipOS version 1.1, manufactured by Agilent, Santa Clara, CA) or 135K (for GC48823, SignatureChipOS version 2.0, manufactured by Roche NimbleGen, Madison, WI), according to previously described methods (24, 25).

Fluorescence *in situ* hybridization (FISH) analysis. Deletions were confirmed and visualized via metaphase FISH analysis using either RP11-22B1 from 12q14.3 (GC42855) or RP11-76L1 from 12q14.1 (GC48823), according to previously described methods (26).

Statistical Analysis. Analysis was performed on appropriate data sets to ascertain normal distribution by calculating Kurtosis and Skewness values. All values represent mean ± standard error of mean (S.E.M.). Statistical significance for the pair was analyzed by the Mann-Whitney *U* test and Student's *t* test (unpaired, two tailed, assuming unequal variance), unless otherwise indicated. Effect size measures for two independent groups by Cohen's *d*. ANOVA with Tukey's *post hoc* test were used for group comparisons. Statistical significance was depicted as follows n.s. (non-significant, $P > 0.05$), *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

Case presentation. Query of Signature's database of abnormalities revealed two overlapping microdeletions (GC42855 and GC48823) at 12q14.1. GC42855 is 6 years-old girl with postnatal failure to thrive (weight and height in the third percentile), developmental delay, large fontanel, clinodactyly, café au lait

spots, scoliosis, osteopenia, temper tantrums, and excessive fear for being alone and heights. GC48823 is 4 years-old girl with autism and seizures. A child 251128 has behavioral problems and intellectual disability and 287965 had global development delay. 288660 has ADHD, autism spectrum disorder, and generalized joint laxity. No additional follow-up clinical information was available for these five individuals.

290951 is a 6 year-old boy, assessed at the age of 5 in clinical genetics with a history of global mild developmental delay, severe speech and language delay, autism spectrum disorder, and significant family history of intellectual disability. He had poor sucking and reflux as baby, requiring speech pathology input for initial bottle feeding. He did not start walking or talking until around the age of 2. He also had quite significant reflux and a number of ‘blue’ episodes as a baby. Developmental problems were also picked up at preschool and he had a full developmental assessment in 2013 which diagnosed him with mild global developmental delay and autism. Griffiths Assessment at the age of 4 years 11 months showed mild developmental delay in overall 2 year 9 month to 3 year 3 month age range with:

- Locomotor : 3-3.5 year equivalent
- Social: 3-3.5 year
- Language: 3-3.5 year
- Hand eye coordination: 2.75-3.25 year
- Performance: 2.25-2.75 years
- Practical reasoning: 2.75-3.25 year
- Language: simple 4-5 word sentences

He was also found to have dysfunction in social participation, planning and ideas and sensory processing issues with touch avoidance and sensory overload. He had inconsistent unusual eye gaze, echolalia, tangential speech, social difficulties, lack of pretend play, meltdowns, restricted behaviour and sensory issues consistent with autism spectrum disorder. At the moment he still has problems with sleep disruption with difficulty initiating sleep and early waking. He also occasionally gets constipated. He has a good appetite and is otherwise healthy.

For his inattention at school, he was started on Ritalin (methylphenidate) and parents noticed significant difference in his attention, behaviour and socialisation. The school have also noticed the same. He is in kindergarten in a support class with five other children and the teachers are very pleased with how he has progressed after starting on stimulant medication. The family have also had significant problems with his behaviour. He does not follow commands well and has episodes where he will cry incessantly and have melt-downs. He also exhibits recurrent self-stimulating hand movement such as pill rolling, flapping as well as repetitive gustatory movements. They have never noticed any seizure activity, regression, or any other abnormal movements.

On examination at 5 years he weighs 17.7 kg (25^{th} centile), his height is 113 cm (50^{th} centile) and head circumference 49.7 cm (2^{nd}-50^{th} centile). He has quite a long face with a broad forehead, light blue eyes, very crowed teeth and a high arched palate. He has a long pronounced chin and also very long cupped ears with very fleshy ear lobes with a horizontal crease across the lobes. His cardiovascular examination was normal as were his genitalia. He has no birth marks. His measurements were:
Inner canthal 2.5 cm, outer canthal 8.5 cm, interpupillary 5.5 cm, palm length 6.8 cm, mid finger length 5 cm. Left hand had single palmar crease and ears were 6.5cm long. Multisequence multiplanar MRI study of the brain has been performed before and after contrast. The ventricles and cerebral sulci outline normally. There is no focal abnormality seen within the grey or white matter. There is no evidence of mesial temporal sclerosis or mass lesion is identified. No evidence of neuronal migrational disorder. No abnormal enhancement identified. The extra-cerebral spaces have a normal appearance. *MRI studies of the brain* had normal findings.

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Supplementary Video and Figures

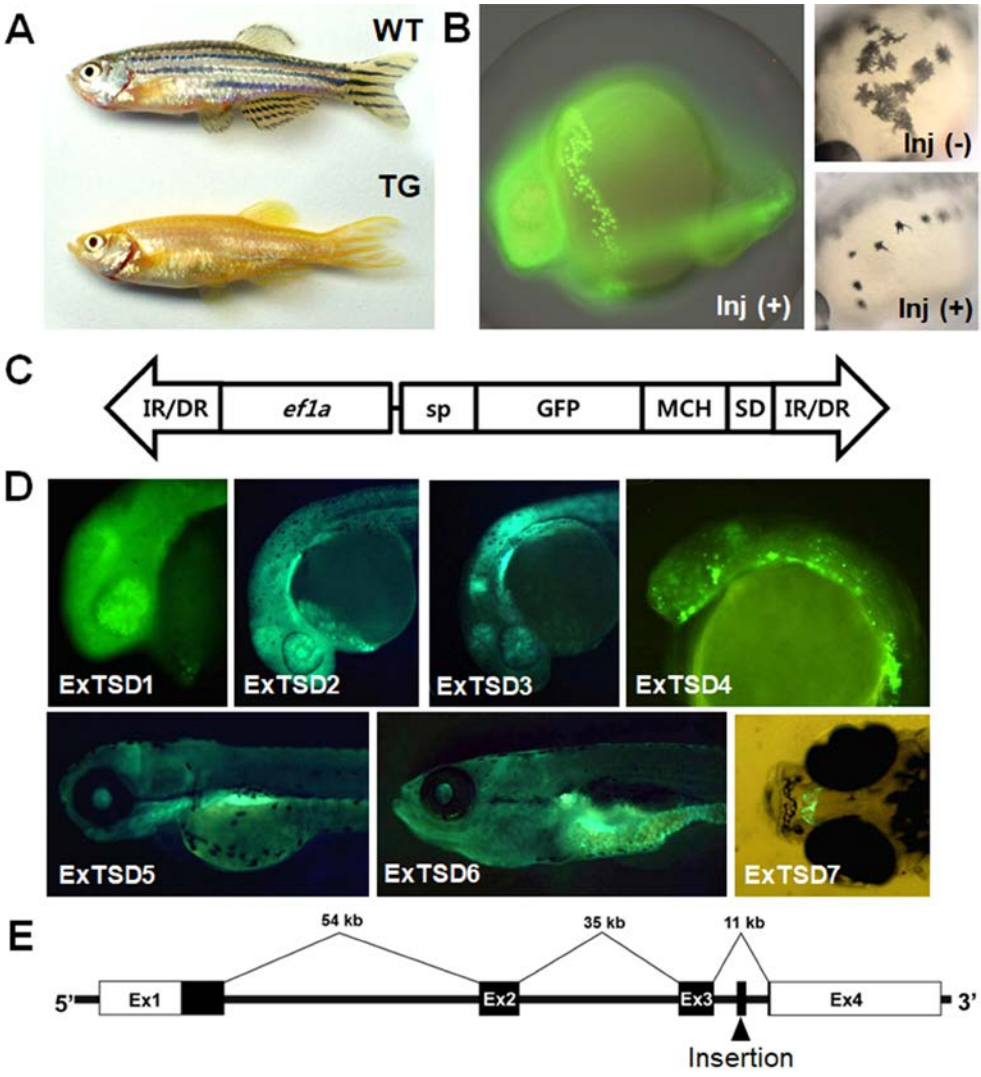

Figure S1. Dual reporter system used for insertional mutagenesis, the golden fish project. (**A**) Body color change in MCH-expressing transgenic zebrafish (TG, a *cmv:mch tg* line), compared to wild-type adult fish (WT). (**B**) Expression of dual reporter, GFP-MCH fusion protein, in *ef1a:gfp-mch*-injected zebrafish embryos. GFP fluorescence and melanosome aggregation are detectable in injected embryos, inj (+), compared to un-injected control embryo, inj (-). (**C**) Structure of a gene trap vector used in insertional mutagenesis. IR/DR; inverted repeat/direct repeat element; *ef1a*, *elongation factor 1 alpha* (*ef1a*) gene promoter; sp, signal peptide; GFP; green fluorescent protein; MCH, melanin-concentrating hormone; SD, splice donor. (**D**) Representative mutants showing GFP expression in various tissues (ExTSD1-7; images not to scale). (**E**) Mapping of the insertion site (arrow) in the intron 3 of a *samdori* gene on chromosome 4.

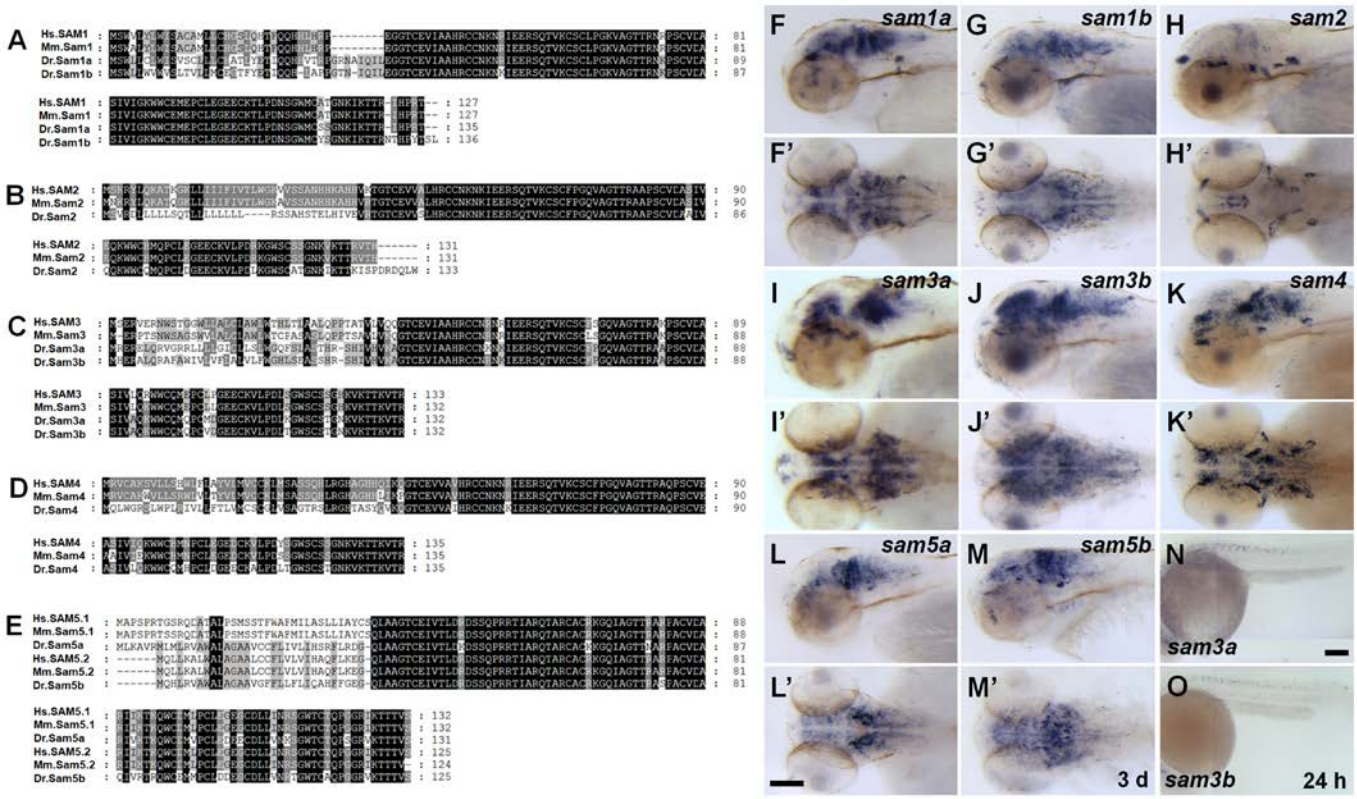

Figure. S2. Conserved synteny and CNS-specific expression of *samdori* gene family. (**A**) Alignments of zebrafish sam1a and sam1b amino acids with that of human SAM1 and mouse Sam1 (Genbank accession for human and mouse, NM_213609; NM_182808, respectively). (**B**) Alignments of zebrafish sam2 amino acids with human SAM2 and mouse Sam2 (NM_178539; NM_182807). (**C**) Alignments of zebrafish sam3a and sam3b amino acids with human SAM3 and mouse Sam3 (NM_182759; NM_183224). (**D**) Alignments of zebrafish sam4 amino acid with human SAM4 and mouse Sam4 (NM_001005527; NM_177233). (**E**) Alignments of zebrafish sam5a and sam5b amino acids with human SAM5.1, SAM5.2 and mouse Sam5.1, Sam5.2 (NM_001082967; *NM_015381; NM_001252310; NM*_134096). **(F-O)** Whole-mount *in situ* hybridization was performed to detect the expression of mRNAs. All *sam* gene family members are exclusively expressed in the brain. Three day-old (3d) embryos (**F-M'**). *sam3a and sam3b show*ed additional expression in the spinal cord at 24 h, lateral views (**N**, **O**). Lateral views (**F-M**). Dorsal views (**F'-M'**). Scale bar, 100 µm.

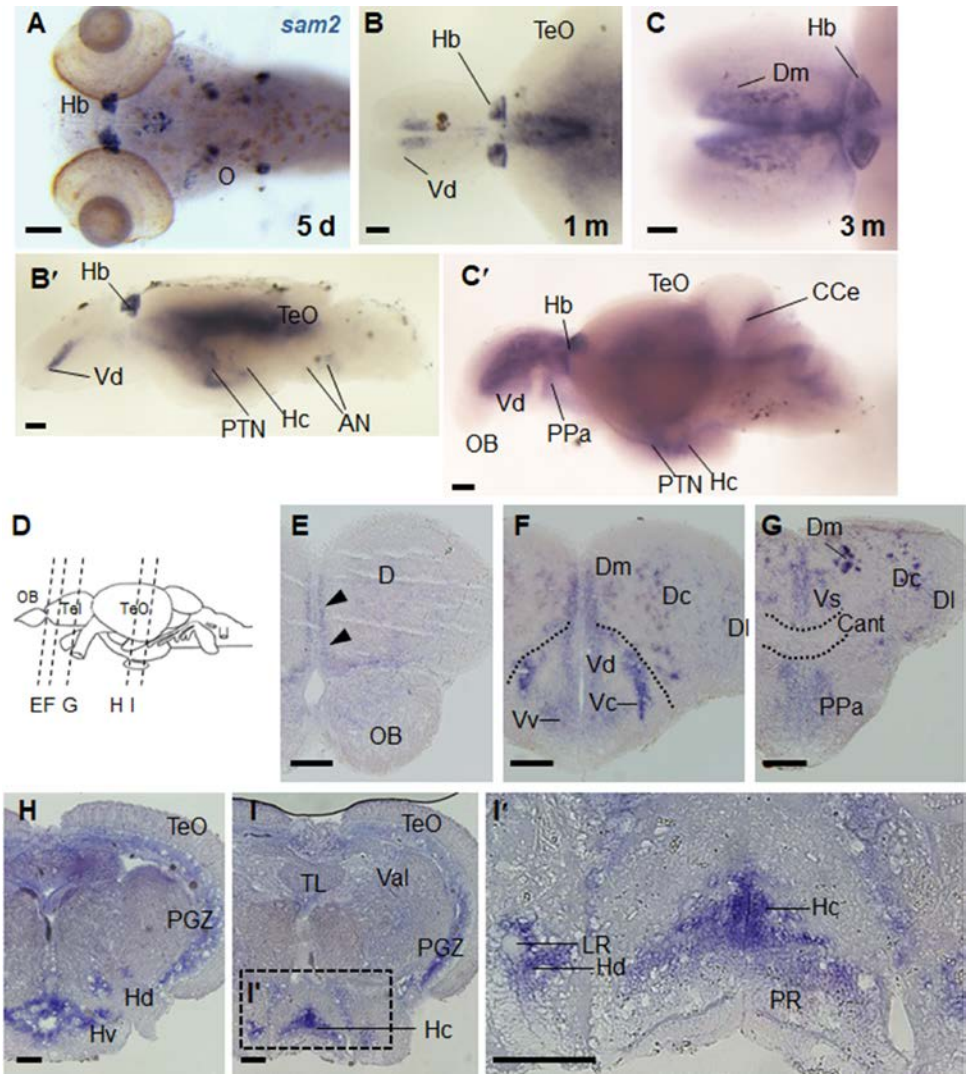

Figure. S3. Expression of *sam2* in the larval and adult brain. Whole-mount *in situ* hybridization of *sam2* in larva and adult (1- and 3-month; 1 m, 3 m) **(A)** *sam2*-expressing cells in 5 day-old larvae. Habenula (Hb) expression is already prominent at this stage. Expression in the otic neurons (O) is also seen. **(B, B')** At 1-month, expression of *sam2* in the brain. Dorsal telencephalic view **(B)** and lateral view **(B')**. **(C, C')** *sam2* expression in the adult brain. Dorsal telencephalic view **(C)** and lateral view **(C')**. **(E-I')** Serial sections of adult brain. Levels of the cross-sections are indicated in **(D)**. Scale bar, 100 µm. AN, auditory nerve; Cant, anterior commissure; CCe, corpus cerebelli; D, area dorsalis telencephlali; Dc, central zone of area dorsalis telencephali; Dl, lateral zone of area dorsalis telencephali; Dm, medial zone of area dorsalis telencephali; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; LR, lateral recess of diencephalic ventricle; OB, olfactory bulb; PGZ, periventricular gray zone; PPa, parvocelluar preoptic nucleus, anterior part; PR, posterior recess; PTN, posterior tuberal nucleus; TeO, optic tectum; TL, torus longitudinalis; Val, lateral division of vlvula cerebelli; Vc, central nucleus of area ventral telencephali; Vd, dorsal nucleus of area ventral telencephali; Vs, supracommissural nucleus of area ventral telencephali; Vv, ventral nucleus of area ventral telencephali.

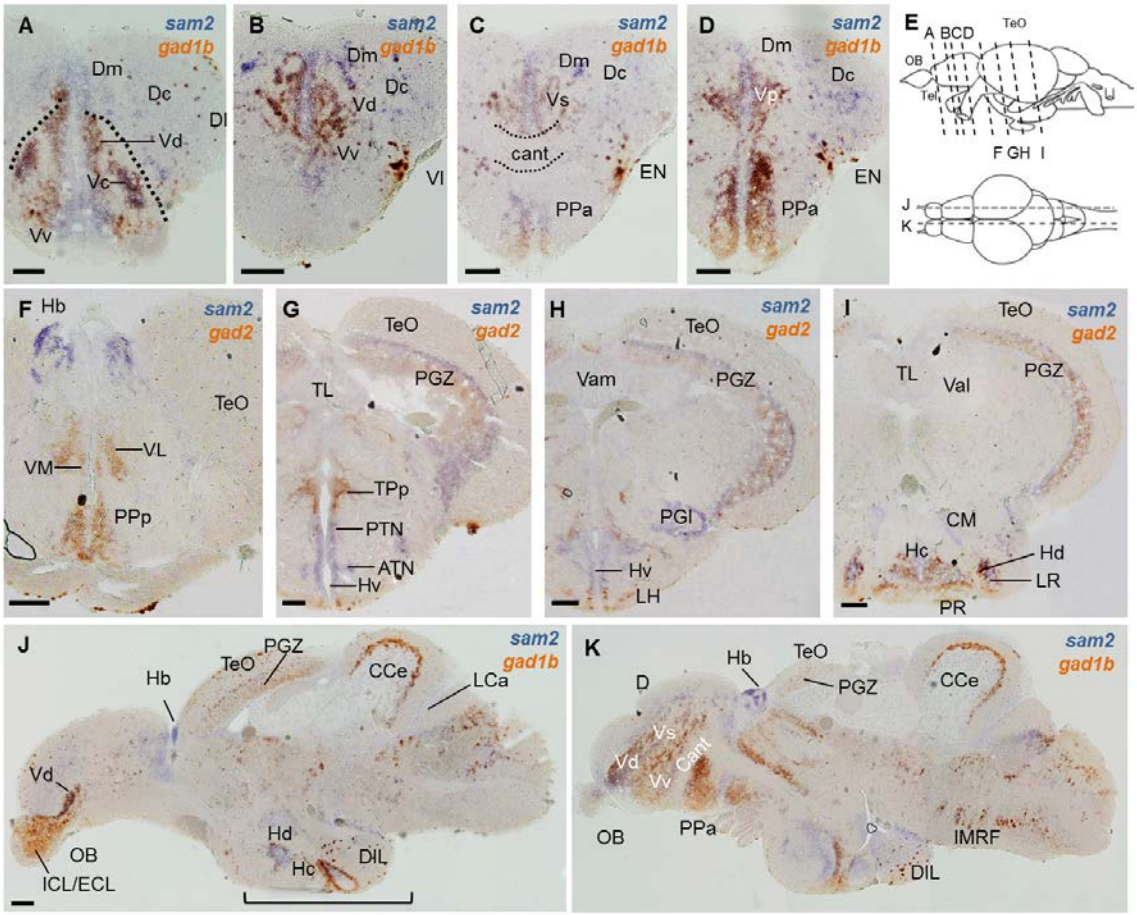

Figure. S4. Two color *in situ* hybridization of *sam2* with *gad1b* or *gad2* in adult brain. (A-D) Cross section of telencehpalic region. **(F-I)** Cross section of diencephalic area. **(J, K)** Sagittal section of whole brain. Expression region of *sam2* is partially overlapped by that of *gad1b* in several brain regions, including telencephalon and hypothalamus. Levels of the sections (top, cross section; bottom, sagittal section) are indicated in **(E)**. Scale bar, 100 μm. ATN, anterior tuberal nucleus; Cant, anterior commissure; CCe, corpus cerebelli; CM, corpus mamillare; D, area dorsalis telencephlali; Dc, central zone of area dorsalis telencephali; DIL, diffuse nucleus of the inferior lobe; Dl, lateral zone of area dorsalis telencephali; Dm, medial zone of area dorsalis telencephali; ECL, external cellular layer of olfactory bulb; EN, entopedunclular nucleus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; ICL, internal cellular layer of olfactory bulb; IMRF, intermediate reticular formation; LCa, caudal lobe of the cerebellum; LH, lateral hypothalamic nucleus; LR, lateral recess of diencephalic ventricle; OB, olfactory bulb; PGl, lateral preglomerular nucleus; PGZ, periventricular gray zone; PPa, parvocelluar preoptic nucleus, anterior part; PPp, parvocellular preoptic nucleus, posterior part; PR, posterior recess; PTN, posterior tuberal nucleus; TL, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum; Val, lateral division of vlvula cerebelli; Vam, medial division of valvula cerebelli; Vc, central nucleus of area ventral telencephali; Vd, dorsal nucleus of area ventral telencephali; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; Vp, Ventral pallium; Vs, supracommissural nucleus of area ventral telencephali; Vv, ventral nucleus of area ventral telencephali.

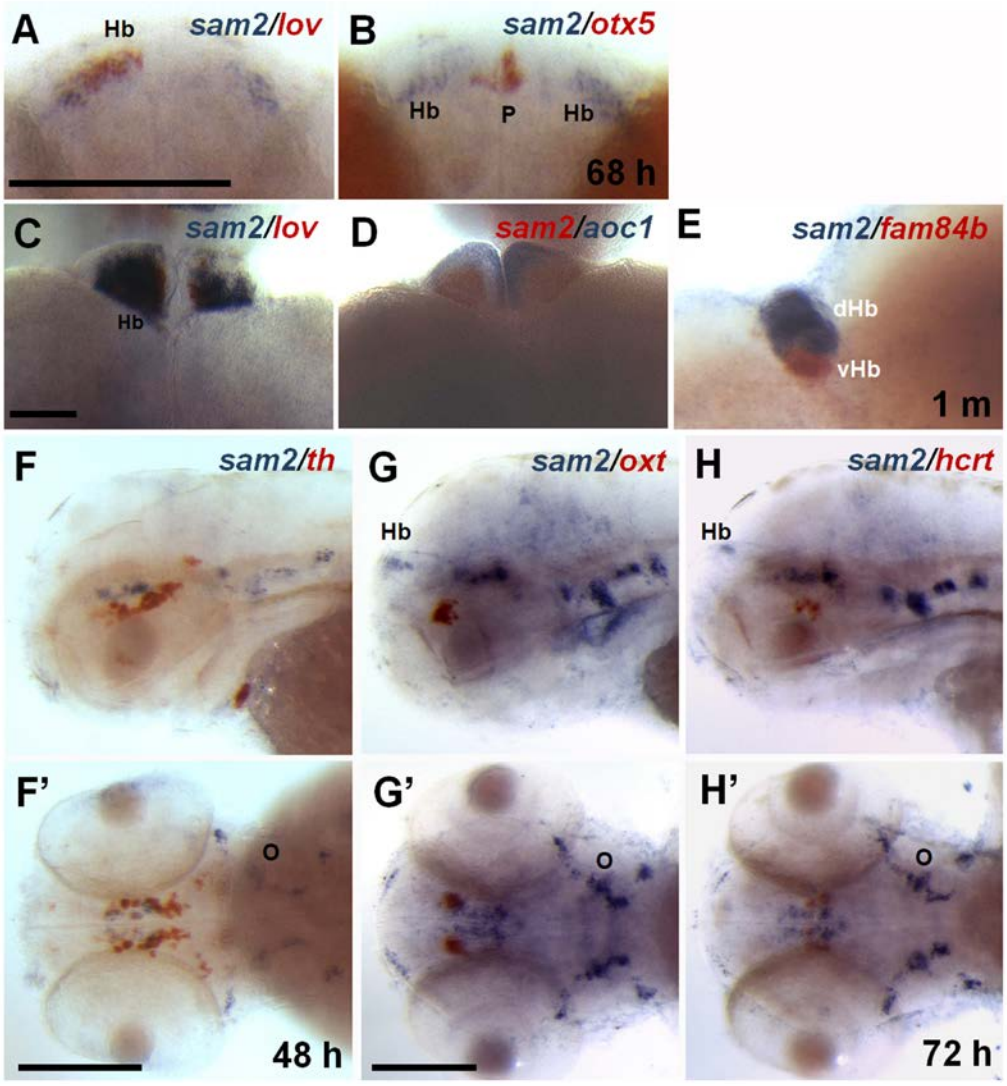

Figure. S5. Characterization of *sam2*-expressing cell populations in larval zebrafish. Two-color whole-mount *in situ* hybridization was performed to identify *sam2*-positive cells, comparing with known habenula (Hb) markers: *lov* (*kctd12.1*), dorsal Hb (dHb); *aoc1*, ventral Hb (vHb); *fam84b*, both dHb and vHb marker; *otx5*, pineal organ (p) marker. (**A**, **B**) 68 hour-old larvae. Dorsal view. (**C**-**E**) Adult brain. Dorsal view, except for lateral view in (**E**). Note the specific expression of *sam2* in the dorsal Hb. Other than Hb, *sam2*-expressing cells do not overlap with markers of dopaminergic neurons (*th at 48h*; **F**, **F'**), oxytocinergic neurons (*oxt* at 72h; **G**, **G'**), and hypocretinergic neurons (*hcrt* at 72h; **H**, **H'**). **F**-**H**, lateral view; **F'**-**H'**, dorsal view. Scale bar, 200 µm.

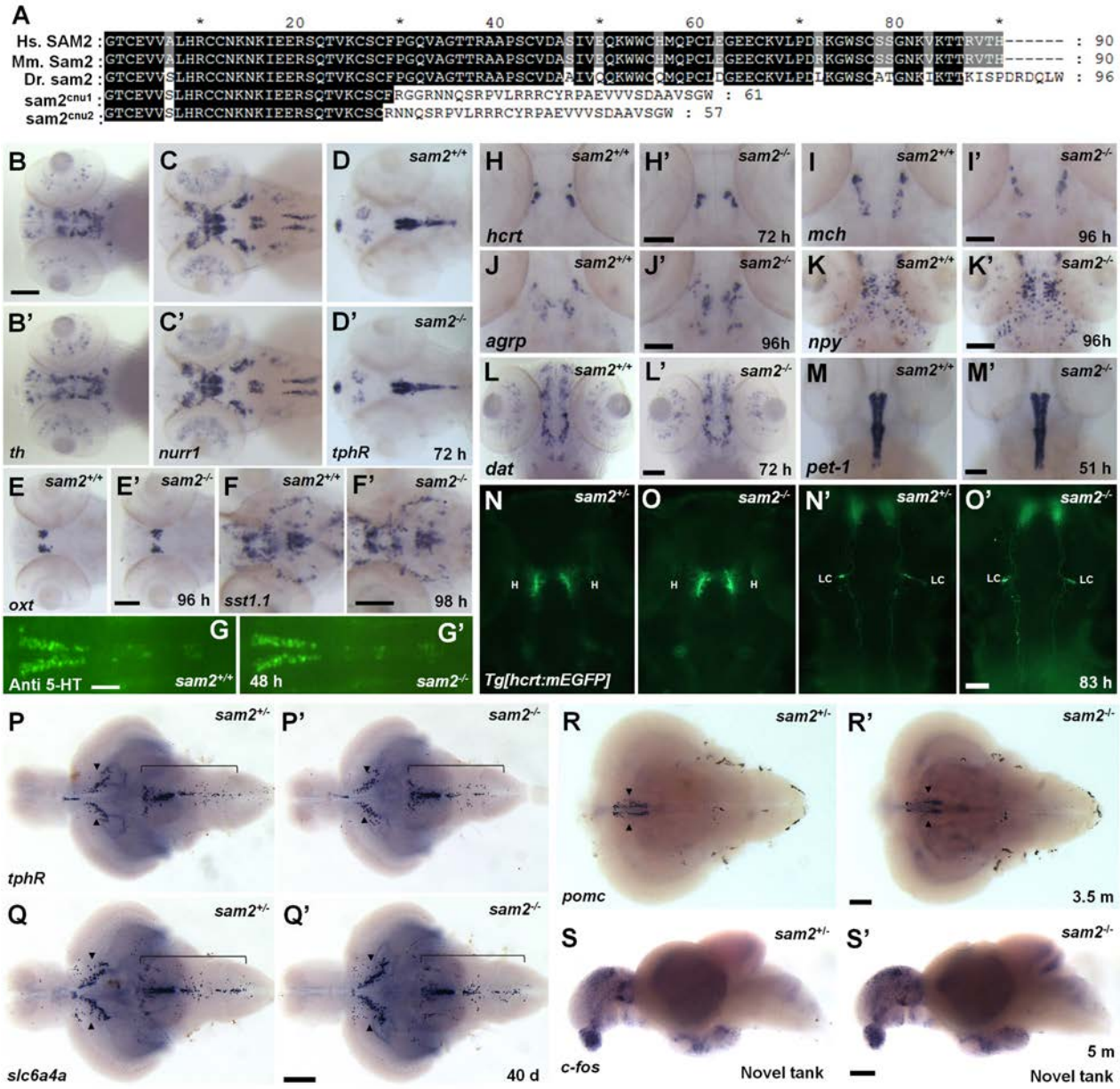

Figure. S6. Normal expressions of various neuronal markers in *sam2*^{cnu1} KO fish. (**A**) Alignment of human, mouse, and zebrafish Sam2 peptides. Putative amino acid sequences of mutant forms are also aligned. Dopaminergic neuron (*th*, **B-B'**; *nurr1*, **C-C'**; *dat*, **L-L'**), serotonergic neuron (*tphR*, **D-D'**; 5-HT, **G-G'**; *pet-1*, **M-M'**), oxytocinergic neuron (*oxt*, **E-E'**), and IPN (*sst1*, **F-F'**) markers. Additional neural markers: *hcrt* (**H-H'**), *mch* (**I-I'**), *agrp* (**J-J'**), and *npy* (**K-K'**). (**N-O'**) Normal hypothalamus (H) - locus coeruleus (LC) axonal connections in *sam2*^{cnu1} KO fish as judged by *Tg[hcrt:EGFP]* transgene expression. Dorsal views, anterior to the top. Scale bar, 100 μm. (**P-R'**) Serotonergic neuron (*tphR*, *slc6a4a*) and adrenocorticotropic hormone precursor *pomc* marker in adult brain. Arrowheads indicate the ventral posterior tuberculum (**P-Q'**) and the nucleus lateralis tuberis (**R**, **R'**). Bracket defines the raphe (**P-Q'**). (**S-S'**) *c-fos* expression in the novel tank assay. Scale bar, 500 μm.

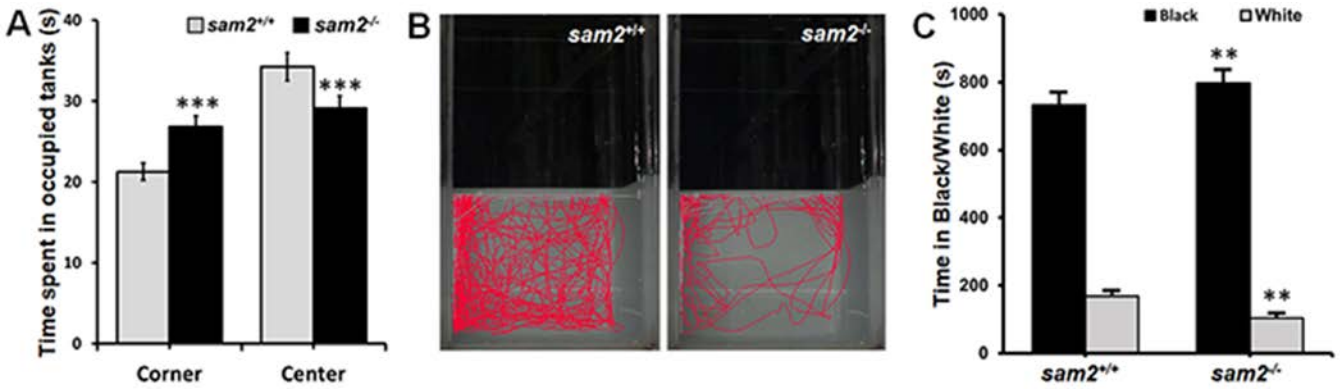

Figure. S7. *sam2*^{cnu1} KO fish show anxiety behavior in open tank and scototaxis tests. (**A**) In the open tank, thigmotaxis was measured by comparing the mean time spent in the tank's corner (one fifth of tank's left and right part) vs. its center (rest of corner of the tank) every 1 min interval for 30 min (*sam2*$^{+/+}$, n = 12; *sam2*$^{-/-}$, n = 12; Corner, Cohen's d = 0.93, Mann-Whitney U = 908, P = 0.00001; Center, Cohen's d = 0.81, Mann-Whitney U = 982, P = 0.00001). (**B**) Scototaxis test. Movement tracking of control and *sam2* KO fish in the black/white tank. Red lines indicate the locomotion of fishes recorded only in the white arena. (**C**) Total time in black or white arena (second, s) (*sam2*$^{+/+}$, n = 18; *sam2*$^{-/-}$, n = 24; Black arena, Cohen's d = 1.07, Mann-Whitney U = 96, P = 0.0083, White arena, Cohen's d = 0.76, Mann-Whitney U = 95.5, P = 0.008).

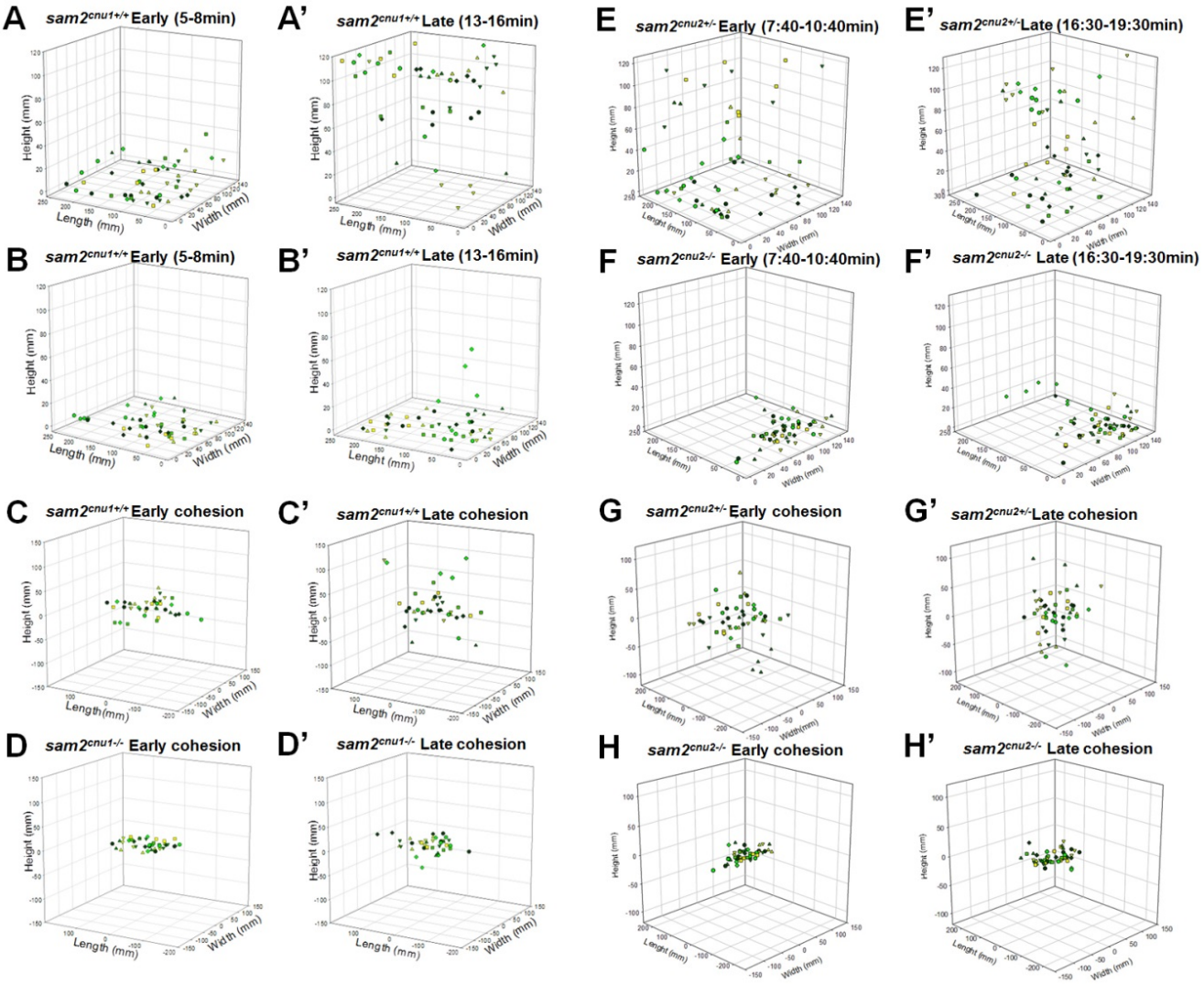

Figure. S8. Increased anxiety (A-B' and E-F') and social cohesion (C-D' and G-H') in two different *sam2* KO fish lines (*sam2*^{cnu1} and *sam2*^{cnu2}). Three-dimensional reconstructions of video trackings before habituation (early) and after habituation (late) to the new environment. Five or six fish were placed as a group in a novel tank. Different color spots indicate the actual position of individual fish in 20-second intervals of video recording (10 points for each fish). (**C-D'**, **G-H'**) Increased social cohesion in *sam2* KO fish. The focal fish is positioned in the center of the space and the relative position of its shoal members are indicated by different color spots. We measured the distance between the focal fish and one of its shoal members before habituation (early) and after habituation (late) to the new environment (*sam2*$^{cnu1+/+}$and *sam2*$^{cnu1-/-}$, n = 5; *sam2*$^{cnu2+/+}$ and *sam2*$^{cnu2-/-}$, n = 6).

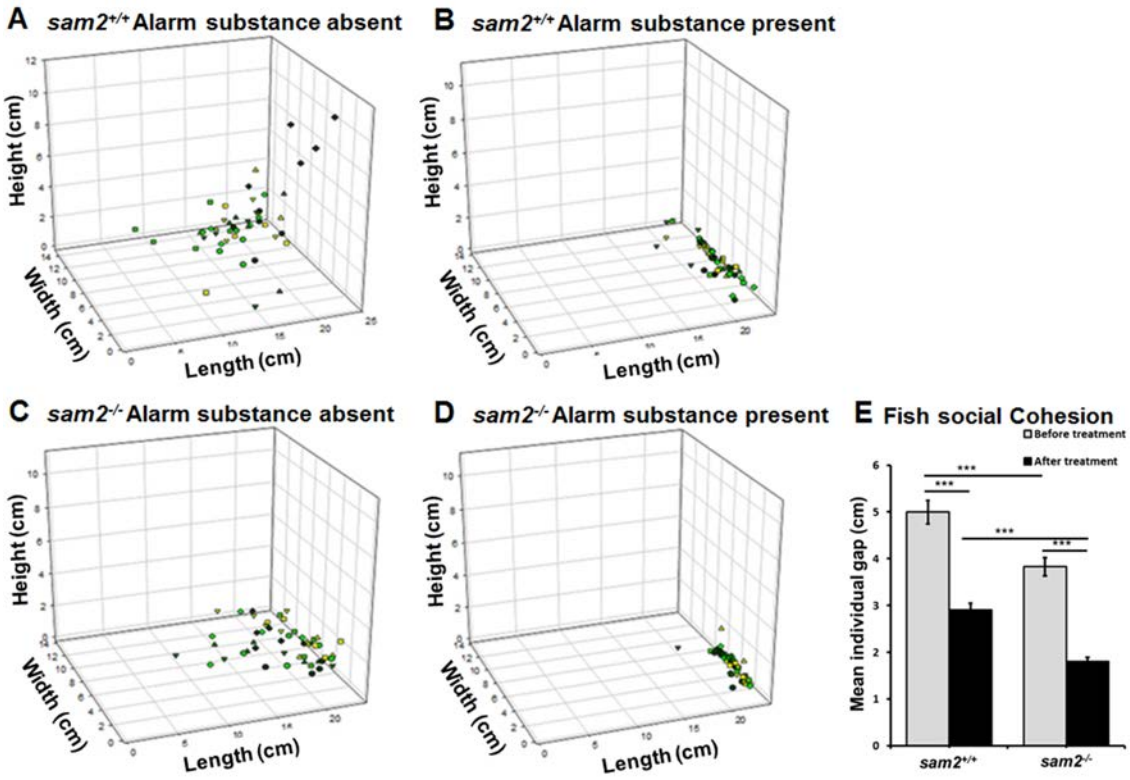

Figure. S9. Increased social cohesion of *sam2*^{cnu1} KO fish under fear-inducing condition. Five adult male fish were kept in a novel tank for 30 min and then treated with alarm substance. (**A**-**D**) Temporal three-dimensional reconstructions of video tracking before and 15 min after treatment of alarm substance. Different color spots indicate the actual positions of individual fish determined at 20-second intervals during 3-min-video recording (10 points for each fish). (**E**) Measurement of soc ial cohesion as the mean individual gap. ***P < 0.001; Student's t-test.

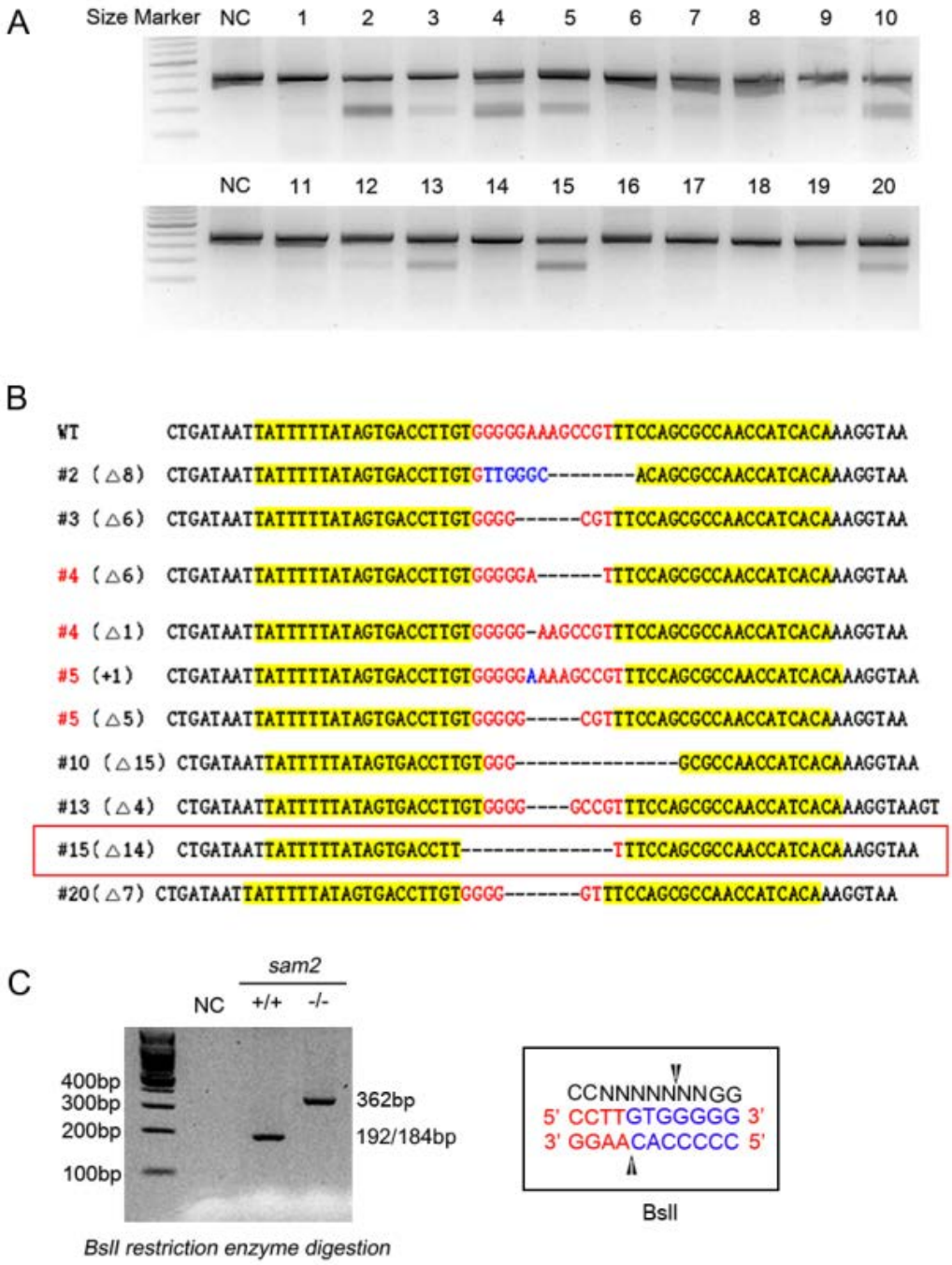

Figure. S10. Generation of TALEN-mediated *Sam2* KO mice (**A**) Founders of *Sam2* KO mice were identified with T7E1 assay. (**B**) Sequence alignments of various *Sam2* KO alleles. Selected *Sam2* mutant allele marked by a red box contains a 14 bp-deletion which causes a frameshift leading to a premature stop codon. (**C**) Genotyping results with PCR and *BslI* restriction enzyme digestion. Due to the 14 bp-deletion, mutant allele was not digested with *BslI*. Wild (*Sam2*$^{+/+}$): 184 and 192 bp; Knockout (*Sam2*$^{-/-}$): 362 bp. *BslI* enzyme digestion sequence is shown in the box. Sequence in blue was deleted in *Sam2* mutant allele.

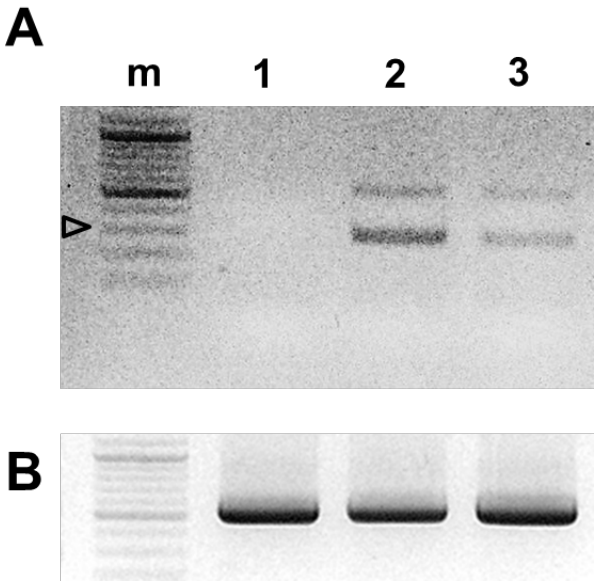

Figure. S11. RT-PCR analysis of *Sam2* expression in the mouse brain. (**A**) For reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was isolated from the dissected tissues after cryosection of frozen mouse brain. *Sam2* was highly expressed in the hippocampus and habenula but was hardly detected in the paraventricular nucleus (PVN). m, size markers; 1, PVN; 2, hippocampus; 3, habenula. Arrow head indicates 300 bp size marker. (**B**) *Beta-actin* (*Actb*) was used as an endogenous control.

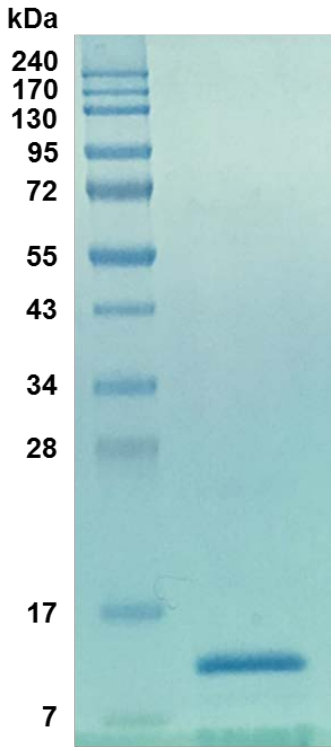

Figure. S12. SDS-PAGE gel analysis of purified SAM2 protein. For high purity protein production, the eluted protein after on-column cleavage was further purified on HiTrap Q HP column. The purity and molecular weight (~12 kDa) of SAM2 protein was confirmed by 10% SDS-PAGE analysis, using the protein size marker.

Supplementary Table 1. Summary of expression pattern of *sam2* in the adult zebrafish.

	sam2	Homologous region in tetrapod telencephalon
Dm	++	pallial amygdala
Dc	scattered	cortex
Dl	-	hippocampus
Dp	-	not applicable
Vd	++	striatum
Vc	++	not applicable
Vv	++	striatum and/or strially-derived septum
Vs	+	ventral part of central amygdala
PTN	+	ventral tegmental area
PPa	+	paraventricular nucleus (PVN)
PPp	+	paraventricular nucleus (PVN)
Hb	++(dorsal)	medial habenula
PGZ	+	
Hc	++	hypothalamus
Hd	++	hypothalamus
Hv	++	hypothalamus
CCe	+	
LCa	-	
EG	-	

Abbreviations: Dm, medial zone of area dorsalis telencephali; Dc, central zone of area dorsalis telencephali; Dl, lateral zone of area dorsalis telencephali; Dp, posterior zone of are dorsalis telencephali; Vd, dorsal nucleus of area ventral telencephali; Vc, central nucleus of area ventral telencephali; Vv, ventral nucleus of area ventral telencephali; Vs, supracommissural nucleus of area ventral telencephali; PTN, posterior tuberal nucleus; PPa, parvocelluar preoptic nucleus, anterior part; PPp, parvocellular preoptic nucleus, posterior part; Hb, Habenula; PGZ, periventricular gray zone; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; CCe, corpus cerebelli; LCa, caudal lobe of the cerebellum; EG, eminentia granularis.