SUPPLEMENTAL MATERIALS

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The use of mice for this research approved by Nankai Animal Care and Use Committee and/or by the Review Board of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Plasmids and ES cell culture

The coding region of mouse Hdac6 gene was amplified by RT-PCR and inserted into pCAGIPuro (from Dr. Hitoshi Niwa) between XhoI and NotI sites. The resulting plasmid was the *Hdac6* overexpression vector DK26. Mouse ES cells were cultured in Dulbecco's modified eagle medium (DMEM, Mediatech), supplemented with 15% fetal bovine serum (FBS, Hyclone), 100 μM β-mercaptoethanol (Sigma), 2 mM L-glutamine, 50 units/ml penicillin/streptomycin, 0.1 mM MEM nonessential amino acid (Invitrogen), and 1,000 units/ml ESGRO (Chemicon). 20 μg of DK26 was electroporated into ES cells with the BioRad gene pulser II (the setting parameters were 200 Ω, 0.25 kV, 500 μFD). One day after electroporation, ES cells were selected with 1.25 μM puromycin for 7-10 days. Surviving colonies were then picked and cultured.

Quantitative RT-PCR

RNA was purified from ES cells or mouse tail tip with RNeasy purification kit (Qiagen). cDNA was synthesized with TransScript II First-Strand cDNA Synthesis SuperMix kit (transgen). Real-time PCR was performed to quantify the expression levels of genes. PCR amplification was carried out in 25 μl reaction volume, containing 12.5 μl of 2×Brilliant SYBR Green QPCR master mix (TOYOBO), and 150 nM of each primer. Real-time PCR was performed with BioRad real-time PCR machine. PCR cycle parameters were: 10 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 57 °C, and 30 sec at 72 °C, and then a dissociation curve of the amplified DNA was acquired. Results were normalized with β-actin. Primers for *Hdac6* were CATTGCTGCTTTCCTGCACATCCT and TCCAGGGACAGAATCAACTTGCCT. Primers for β-*actin* were CAGAAGGAGATTACTGCTCTGGCT and TACTCCTGCTTGCTGATCCACATC.

Production of transgenic *Hdac6* (*Hdac6*^{tg}) mouse

An *Hdac6* overexpression plasmid (DK26) was introduced into V6.5 or BF10 mouse ES cell lines by electroporation. Stable clones were selected by puromycin resistance. Hdac6^{tg} chimera mice were generated by injection of *Hdac6*^{tg} ES cells into 4-8 cell embryos (Huang et al., 2008). Through germline transmission, *Hdac6*^{tg} chimera mice gave birth to F1 mice which carried the *Hdac6* transgene. Mice were housed in a specific pathogen free (SPF) facility.

Genotyping PCR

Genomic DNA was isolated from ES cells or mouse tail tip with DNeasy Blood & Tissue Kit (Qiagen). PCR cycle parameters were: 2 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 57 °C, and 2 min at 72 °C, and then hold at 4°C. For mouse ES cells and chimera mice characterization, primer F2 (ATCTCAGCTGGCTTTGATGC) and primer R (TTAGTGTGAGTGGGGCATGTCCTC) were used. Primer F1 (ATGAGTCACTGCAACCTCTG) together with primer R was used for *Hdac6*^{tg} F1 mouse characterization.

Western blot analysis

ES cells were lysed in lysis buffer, and protein concentration measured using BCA Protein Assay Kit (Beyotime) to ensure equal loading. The samples were resolved by SDS-PAGE, followed by transferring onto a PVDF membrane (Millipore). The membrane was blocked for 60 min in TBS with 5% non-fat dry milk, and then probed with anti-Hdac6 antibody (Abcam), anti-actin antibody (Abcam), anti-acetylated tubulin and anti-α-tubulin (Sigma Aldrich). Bound primary antibodies were recognized by HRP–linked secondary antibodies (GE Healthcare). Immuno-reactivity was detected by ECL Plus (Beyotime) and Kodak light film.

Adenovirus infection of ES cell clones and flow cytometry

ES cells were plated on a 12 well plate a day before infection (1×10^5 cells/well). After 24 hours, cells were infected with the replication-deficient recombinant human adenovirus type 5 (dE1/E3) expressing eGFP (Ad-GFP). Ad-GFP virus stock was diluted as 1×10^8, 1×10^7, and 1×10^6 infectious unit/ml (ifu/ml) with ES cell culture medium. For infection of cells, old medium was aspirated from the 12 well plates and 500 μl of medium containing virus was added to the plate. After 36 hours, cells were collected and GFP-positive cells were counted by flow cytometry.

Virus infection test of transgenic mice

Virus

Highly pathogenic avian Influenza A (H5N1) (A/Duck/Shanghai/13/2001(H5N1)) virus stock was cryopreserved in liquid nitrogen container by seed virus department of Harbin Veterinary Research Institute. Virus stock was thawed by wet ice bath and diluted by phosphate buffered saline for inoculation.

Animals and challenge experiments

Studies with highly pathogenic H5N1 avian influenza viruses were conducted in a biosecurity level 3+ laboratory approved by the Chinese Ministry of Agriculture. Sex & age-matched transgenic mice (5 week or 11-17 week old) and their wild-type littermates were housed in isolator cages ventilated under negative pressure with HEPA-filtered air. Specifically, 18 TG mice (5 week old) and their 16 wild-type littermate in the infected group were monitored daily for morbidity, as measured by weight loss, and mortality for 12 d postinoculation. TG mice (11-17 week old, n=12)

and their 10 wild-type littermate in the infected group were operated as same as above. During the experiment, animals had free access to food and water. In this investigation, the mice were lightly anesthetized with Avertin (Sigma-Aldrich) and then inoculated intranasally (i.n.) 60 µl for 5 week old mice or 80 µl for 11-17 week old mice with 0.8 LD50 or 1.1 LD50 of virus diluted in sterile phosphate buffered saline (PBS). Mock-infected control animals were inoculated intranasally (60 µl) with sterile PBS. Mice were monitored daily for weight changes and signs of disease for 14 days.

Virus titration

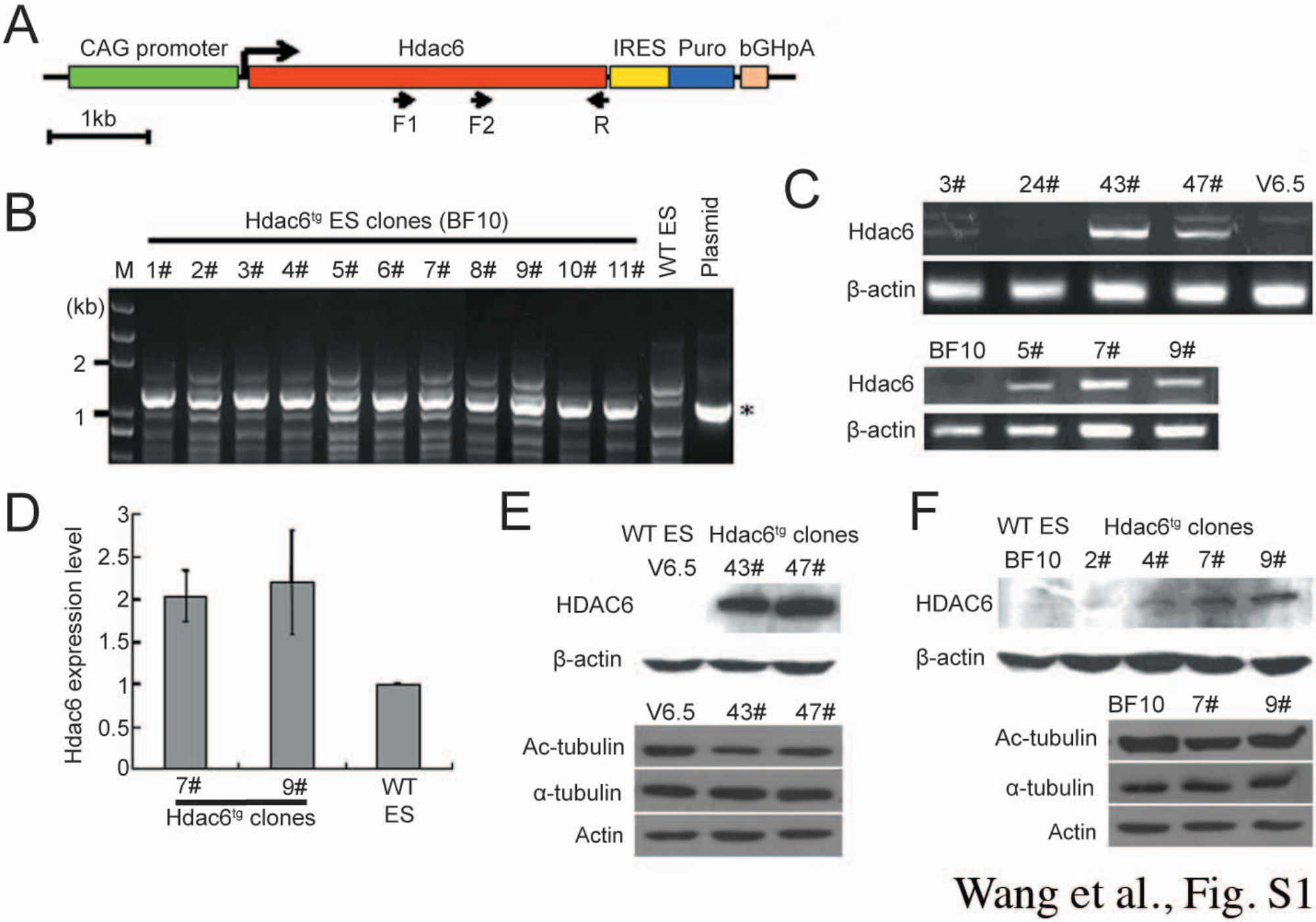
Mice were humanely put sleep by standard method of cervical dislocation. Right lobes of lung and upper part of trachea were accurately measured to assure the same starting amount for titration, then the samples were put in viral maintaining solution and immediately frozen in liquid nitrogen, based on standard protocols. Samples were collected and homogenized in cold phosphate-buffered saline on days3, 6, 9 postinoculation. Clarified homogenates were titrated for viral infectivity in embryonated chicken eggs from initial dilutions of 1:10. Viral titers were expressed as mean log10 EID50 per milliliter (EID50: fifty percent egg infectious dose).

Supplemental Figure Legend

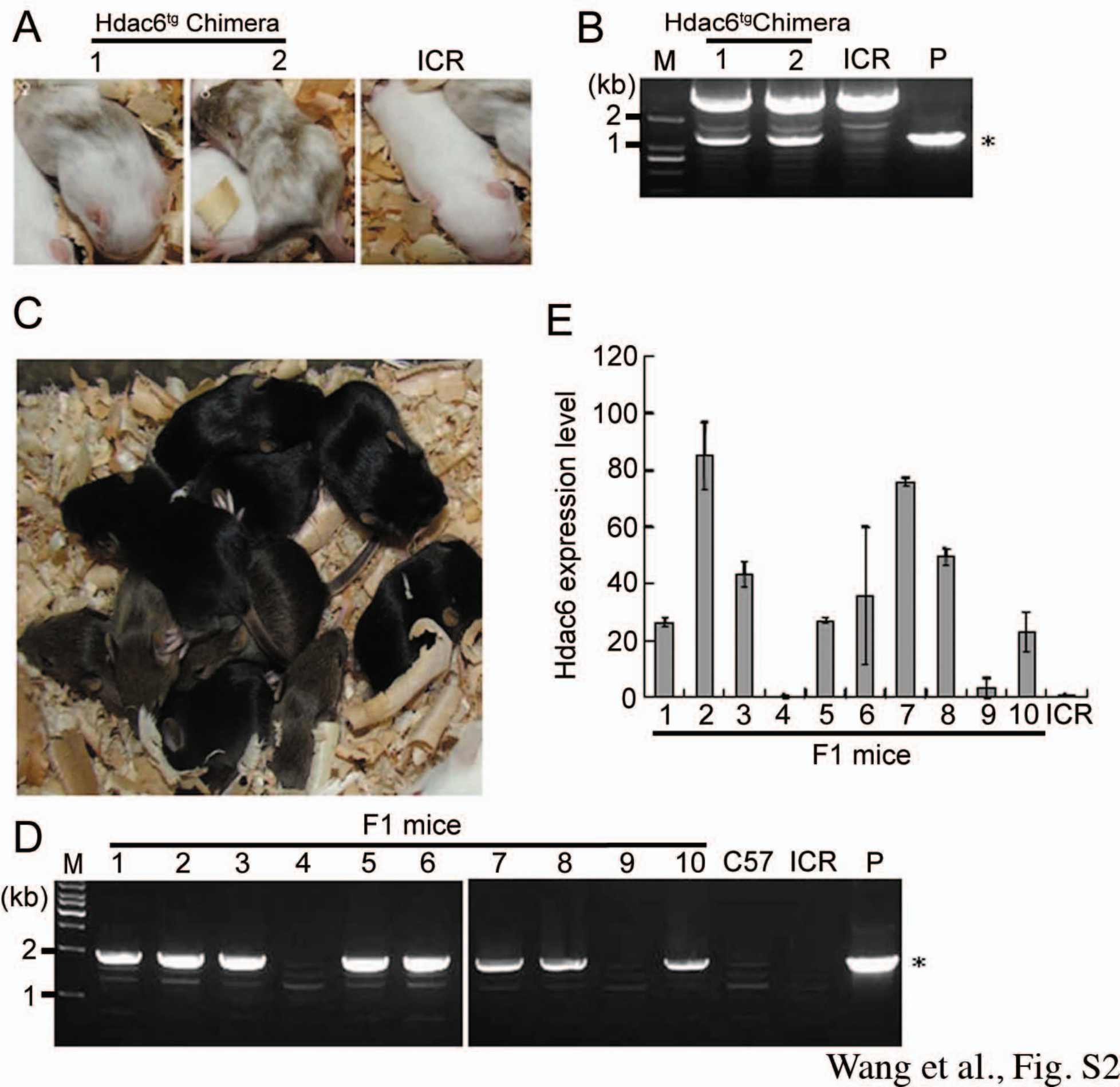
FIG. S1. Characterization of *Hdac6*^{tg} ES cells. (A) Schematic illustration of the *Hdac6* overexpression vector DK26. The expression of the *Hdac6*-IRES-*Puro* cassette was driven by the chicken *β-Actin* (CAG) promoter. Arrows mark PCR primers for the identification of transgenic ES cells and mice. Primers F2 and R were used for characterization of transgenic ES cells and chimera mice, whereas primers F1 and R for genotyping F1 transgenic mice. (B) PCR detected the integration of the *Hdac6* transgene in ES clones. Band at 1.2 kb DNA (highlighted with an asterisk) was amplified in positive control in which plasmid DK26 served as DNA template, while the band was absent in negative control with BF10 ES cell genomic DNA as DNA template. (C) Expression of *Hdac6* in selected clones from two independent ES cell lines (V6.5 and BF10) by RT-PCR. (D) Relative expression levels of *Hdac6* in ^{Hdac6tg} BF10 ES clone #7 and #9 by quantitative real-time PCR. (E) Protein levels of Hdac6 and α-tubulin acetylation in V6.5 WT and transgenic ES cell clones by Western blot analysis. (F) Protein levels of Hdac6 and α-tubulin acetylation in BF10 WT and transgenic ES cell clones by Western blot analysis.

FIG. S2. Production and characterization of *Hdac6*^{tg} mice. (A) *Hdac6*^{tg} chimera mice generated by 4-8 cell embryo injection. (B) PCR analysis confirmed the presence of transgenic *Hdac6* gene in chimera mice. (C) Germline transmitted transgenic Hdac6 mice through mating of a male *Hdac6*^{tg} chimera mouse and a female ICR mouse. All F1 mice had either black or grey coat color, indicating they were derived from

Hdac6^{tg} ES cells (with B6/C3H genetic background). (D) Genotyping of F1 mice. 8 F1 mice out of 10 harbored *Hdac6* transgene. (E) *Hdac6* mRNA expression in F1 mice measured by quantitative RT-PCR. Consistent with genotyping result, F1 mice with *Hdac6* transgene showed elevated expression levels of *Hdac6*.



Wang et al., Fig. S1



Wang et al., Fig. S2