Supporting Information

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SI Materials and Methods

Cloning and DNA Constructs. Mouse cDNAs of Braf35 and iBraf were kindly provided by L. Sumoy (Center for Genomic Regulation, Barcelona, Spain) (1). The RanGAP1-C-ter expression construct, corresponding to the C terminus of RanGAP1, was derived from plasmid pET28RanGAP1-C2 (2) and was used as a control for sumoylation (3). Expression vectors for UBC9 and dnUBC9 (C93S) were previously described (4). GST-iBraf and GST-BHC80 constructions were derived from pGEX-4T-2 and pGEX-6P-3 (GE Healthcare), respectively. Full-length human BHC80 cDNA was obtained from pCMV-BHC80-delta4 kindly provided by Tadashi Baba (University of Tsukuba, Ibaraki, Japan) (5). Plasmids for in vitro transcription/translation reactions were constructed using pBlueScript SK(+). Further details about cloning strategies will be provided upon request. Knockdown plasmids were constructed in pSUPER (OligoEngine) using the manufacturer's protocol and the following siRNA sequences: for shcBRAF35, 5′-CCGCATAGCCAGTGAACAT-3′, and for shiBRAF, 5′-AGAAAGAAGCAGACACAAA-3′.

Transfection and Immunohistochemistry. Transient transfections of 293T and HeLa cells with expression plasmids were performed with FuGENE (Roche) and Lipofectamine 2000 (Invitrogen), respectively. Transfections of P19 cells were performed according to Farah et al. (6). For immunohistochemistry, P19 cells were grown on coverslips and transfected using Lipofectamine 2000 (Invitrogen). Three days posttransfection, cells were fixed for 10 min in 4% (wt/vol) formaldehyde/PBS, permeabilized for 5 min in 0.5% (vol/vol) Triton X-100/PBS, washed, and then blocked in 3% (wt/vol) BSA/PBS. Polyclonal TuJ1 (βIII-tubulin antibody) and anti-GFP antibodies were purchased from Abcam. Secondary antibodies used were TRITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) and Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes). Cells were examined under a motorized upright wide-field microscope (DM6000B; Leica). Confocal images were captured by a confocal Leica TCS SP5 microscope using an HCX PL APO Lambda blue 63× 1.4 oil objective at 22 °C. Image analysis was carried out using Leica and Adobe Photoshop software.

Coimmunoprecipitations. Whole-cell extracts from 293T cells were obtained by lysing the cells in immunoprecipitation (IP) buffer (50 mM Tris·HCl, pH 8, 150 mM NaCl, 1 mM EDTA, pH 8, 1% (vol/vol) Triton X-100, 1 mM PMSF, and protease inhibitors). The extracts were precleared for 3 h at 4 °C with protein A or protein G Sepharose beads (GE Healthcare) pre-equilibrated in the same buffer. The precleared extracts were then incubated overnight at 4 °C with 3 μg of the appropriate antibody. Rabbit or mouse purified IgG (Sigma-Aldrich) was used as a control. Immunocomplexes were purified by adding 30 μL of 50% (vol/vol) protein A or protein G Sepharose beads. Finally, after three washes with IP buffer, bound proteins were eluted by boiling the beads in Laemmli sample buffer containing 5% (vol/vol) β-mercaptoethanol, separated by SDS/PAGE, and visualized by Western blot with the appropriate antibodies using ECL Plus (GE Healthcare). See below for a list of antibodies.

ChIP Assays. ChIP assays were performed as previously described (7). The human RNA polymerase II polypeptide A (POLR2A) exon was used as a control for unspecific binding of genomic DNA to beads or IgGs. Quantification of immunoprecipitated DNA was performed by real-time PCR with the Applied Biosystems 7500 FAST Real-Time PCR System, using Applied Biosystems Power SYBR Green Master Mix. ChIP was quantified by using three real-time PCR determinations. Provided data are the average of at least three independent experiments.

In Vivo Sumoylation Assays and Purification of Endogenous Sumoylated Proteins. Sumoylation assays in cells were performed as described in García-Gutiérrez et al. (4). For Western blot of the in vivo sumoylation assays, cell extracts were prepared in 8 M urea, 10 mM Tris·HCl (pH 8.0), separated by SDS/PAGE, and visualized by Western blot with the appropriate antibody using ECL Plus (GE Healthcare).

For the purification of endogenous sumoylated proteins, whole-cell extracts from 10^7 His-tagged mature SUMO (His-HA-SUMO1GG) transfected cells were prepared using 500 μL of lysis buffer (0.2 M phosphate buffer, pH 8, 8 M urea). His-HA-SUMO1GG was purified using His-Select Nickel-Affinity Gel (Sigma-Aldrich) as indicated by the manufacturer. Purified proteins were eluted by boiling the beads in Laemmli sample buffer containing 5% (vol/vol) β-mercaptoethanol, separated by SDS/PAGE, and visualized by Western blot with the appropriate antibody using ECL Plus (GE Healthcare).

Antibodies. The antibodies used for immunoprecipitations and Western blot were mouse monoclonal anti-Flag M2 (Sigma-Aldrich), rat monoclonal anti-HA (Roche), mouse monoclonal anti-His (GE Healthcare), rabbit polyclonal anti-LSD1 (Abcam), mouse monoclonal anti-LSD1 (AOF2; Sigma-Aldrich), rabbit polyclonal anti-CoREST (Millipore), mouse monoclonal anti-HDAC2 (3F3; Millipore), mouse monoclonal anti-Braf35 (Abnova), and rabbit polyclonal anti-iBraf (Sigma-Aldrich). As secondary antibodies, goat anti–rat-HRP and anti–rabbit-HRP (Sigma-Aldrich) were used.

***In Ovo* Electroporation and Immunofluorescence.** Electroporation and preparation of the embryos for immunofluorescence were carried out as described previously (8). Eggs were incubated at 38 °C (Hamburger-Hamilton stage 13; HH13) for electroporation and embryos were recovered after 30 h (HH21). To evaluate the efficiency of electroporation, a GFP expression vector (pEGFP-N1; Clontech) was systematically coelectroporated at a concentration of 0.3 μg/μL. Other constructs were electroporated at a concentration of 1 μg/μL. The protocol for immunofluorescence has been described previously (9). Polyclonal TuJ1 (βIII-tubulin antibody) and anti-GFP antibodies were purchased from Abcam. Secondary antibodies used were TRITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) and Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes). Confocal images were captured by a confocal Leica TCS SP5 microscope using an HCX PL APO Lambda blue 20× 1.4 oil objective at 22 °C. Image analysis was carried out using Leica and Adobe Photoshop software.

Yeast Two-Hybrid Assay. Yeast two-hybrid screening was performed with the ProQuest Two-Hybrid System (Invitrogen) in the MaV203 strain, according to the manufacturer's protocol. We used a ProQuest two-hybrid, 8.5 d post coitum mouse embryo cDNA library cloned in the pPC86 vector (Invitrogen). Positive clones were identified by growth on selective medium lacking histidine, tryptophan, and leucine and with 25 and 50 mM 3-amino-1,2,4-triazole and characterized by sequencing. Direct interaction assays were performed by using yeast transformed with full-length iBraf fused to Gal4-BD, and the fragments obtained in the screening were fused to Gal4-AD. Mapping of the

interactions was performed by transformation with either full-length or smaller fragments of iBraf fused to Gal4-AD and the protein of interest fused to Gal4-BD.

Primers Used for RT-Quantitative PCR. ***SCN1A.*** Forward:. 5′-TACTCCTCCCCACACCAGTC-3′.

Reverse: 5′-GCGAAGTCGTTCTCAGATCC-3′.

SCN2A2. Forward: 5′-GAATGAGGGTTGTTGTAAATGC-3′.

Reverse: 5′-AGACAGATATCCAAGTCCTACG-3′.

SCN3A. Forward: 5′-TGCTCCCCTCATCAGTCTCT-3′.

Reverse: 5′-TATTGCGTCTTGGGGAAAAC-3′.

Human GAPDH. Forward: 5′-GAGTCAACGGATTTGGTCGT-3′.

Reverse: 5′-AATGAAGGGGTCATTGATGG-3′.

Mouse GAPDH. Forward: 5′-AACTTTGGCATTGTGGAAGG-3′.

Reverse: 5′-GGATGCAGGGATGATGTTCT-3′.

TUBB3. Forward: 5′-TGGAGCGCATCAGCGTATAC-3′.

Reverse: 5′-GCCCTGGGCACATACTTGTG-3′.

Primers Used for ChIP-Quantitative PCR. ***SCN1A promoter.*** Forward: 5′-AGCAATTTCTTTACAGCCTAGTTTTCCTC-3′.

Reverse: 5′-CTGACAAGTGATTCCTGGCCAAGATAGT-3′.

RNA polymerase II polypeptide A (POLR2A) exon. Forward: 5′-TCTCCTTTGATGGCTCCTATGTC-3′.

Reverse: 5′-AATTCCACTGTTACCGTTTCCTCA-3′.

1. Sumoy L, et al. (2000) HMG20A and HMG20B map to human chromosomes 15q24 and 19p13.3 and constitute a distinct class of HMG-box genes with ubiquitous expression. *Cytogenet Cell Genet* 88(1-2):62–67.
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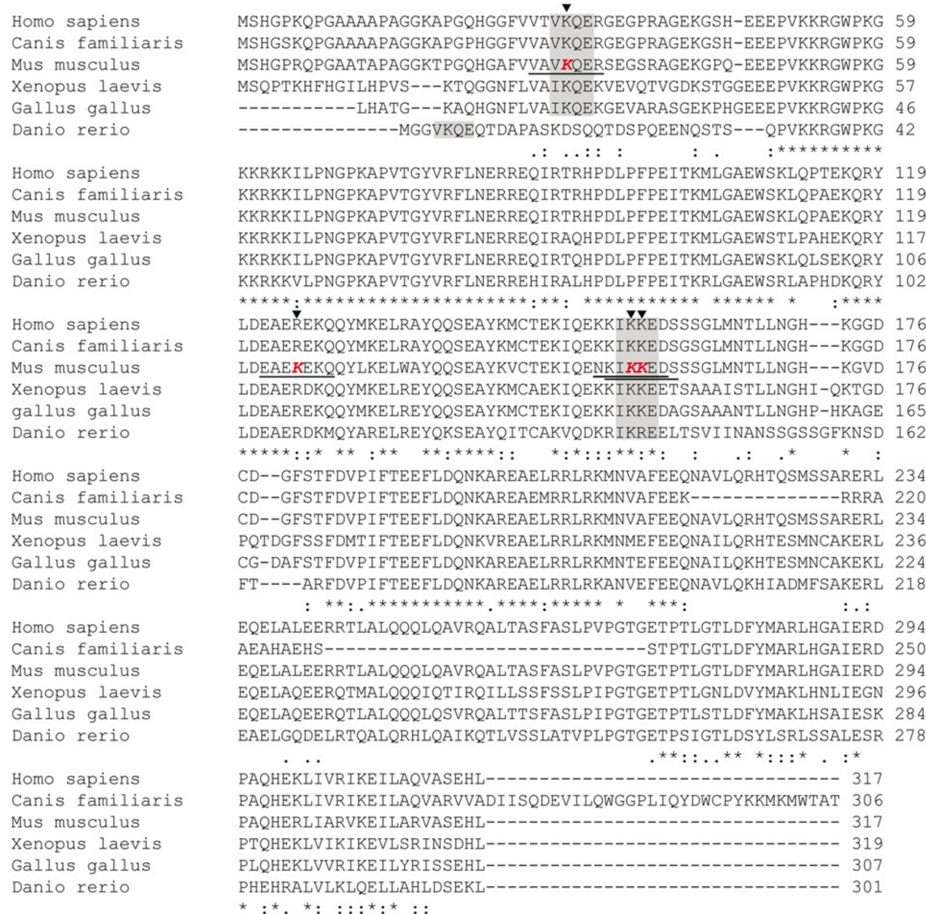

Fig. S1. Protein sequence alignment of Braf35 sequences from different organisms. Sumoylation sites as defined by the SUMOsp 2.0 program (1) are underlined in mouse sequences. The putative sumoylated residues of mouse sequences are highlighted in red. Sequence conservation of the sumoylation sites among different organisms is boxed in gray.

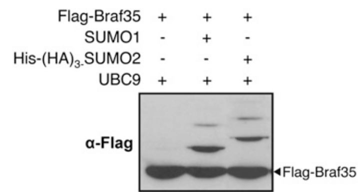

Fig. S2. BRAF35 is modified by SUMO2. 293T cells were transfected with expression vectors for the indicated proteins. Flag-Braf35 was detected by Western blot using anti-Flag antibodies.

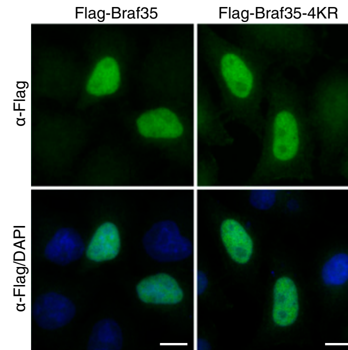

Fig. S3. Nuclear localization of Braf35 is not altered in the Braf35-4KR mutant. HeLa cells were transfected with plasmids expressing Flag-Braf35 or Flag-Braf35-4KR. Distribution of expressed proteins was determined by immunofluorescence using anti-Flag antibodies. Nuclei were counterstained with DAPI. (Scale bars, 10 μm.)

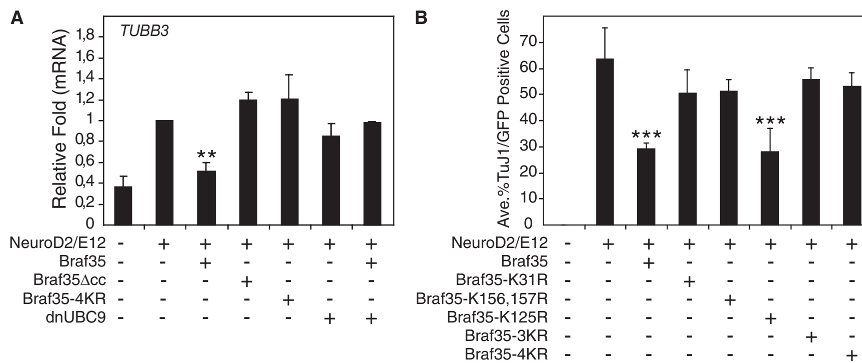

Fig. S4. Braf35-dependent inhibition of neuronal differentiation in P19 cells. (*A*) Quantification of differentiation of P19 cells by RT-quantitative (q)PCR of *TUBB3* mRNA. P19 cells were transfected with a GFP expression vector and with expression vectors encoding the indicated proteins or empty vector. Three days after transfection, total mRNA was isolated and levels of transcript of βIII-tubulin gene (*TUBB3*) were determined by RT-qPCR. Data are the average of three independent experiments ±SD. (*B*) Contribution of different sumoylation sites to Braf35-dependent inhibition of neuronal differentiation in P19 cells. P19 cells were transfected with expression vectors for the indicated proteins or empty vector together with GFP expression vector. Three days after transfection, cells were analyzed by TuJ1 immunostaining and GFP expression. TuJ1-positive cells were scored as a percentage of GFP-positive transfected cells. The result of transfection with Braf35-4KR is again included for comparison. Data are the average of three independent experiments ±SD. $^{**}P < 0.05$, $^{***}P < 0.01$ by ANOVA analysis, compared with cells transfected only with NeuroD2 and E12 expression vectors.

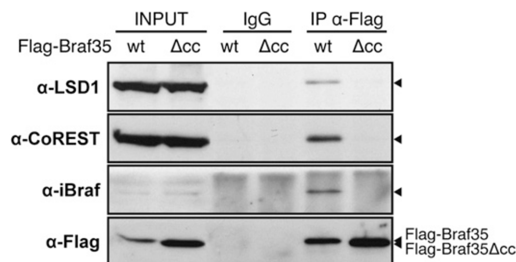

Fig. S5. Braf35Δcc mutant does not interact with the LSD1–CoREST complex. 293T cells were transfected with 4 μg of a plasmid encoding the wild type or mutant version (Δcc) of Flag-Braf35. Thirty-six hours after transfection, Flag-Braf35 was immunoprecipitated from cell extracts with anti-Flag antibodies or with rabbit purified IgG as a control. The immunoprecipitated proteins and 3% of the input extract were subjected to SDS/PAGE and analyzed by Western blot with anti-LSD1 (input), anti-CoREST, anti-iBraf, and anti-Flag antibodies.

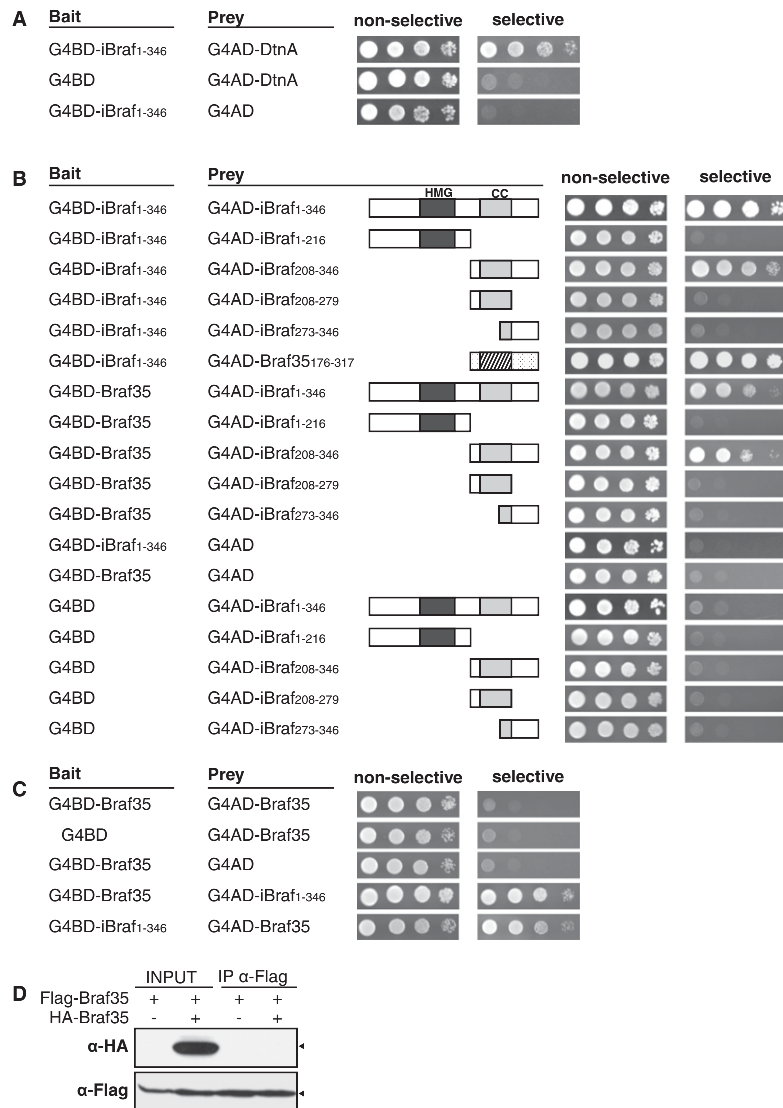

Fig. S6. Analysis of iBraf and Braf35 interactions. (*A*) Interaction of iBraf and α-Dystrobrevin (Dtna) by yeast two-hybrid analysis. (*B*) Homo- and heterodimerization of iBraf and analysis of the region of iBraf involved in the interaction by yeast two-hybrid analysis. A schematic representation of proteins used as prey is shown. CC, coiled-coil domain; HMG, high-mobility group domain. (*C*) Braf35 does not homodimerize by yeast two-hybrid analysis. (*A*–*C*) For yeast two-hybrid experiments, GAL4-DNA–binding domain (G4BD) and GAL4-activation domain (G4AD) fusion proteins were coexpressed in the yeast strain MaV203. The ability of yeast cells to grow in nonselective medium [synthetic defined (SD) medium lacking tryptophan and leucine] is indicative of the presence of both bait and prey plasmids. Positive interactions are indicated by the growth of yeast cells on selective medium (SD medium lacking tryptophan, leucine, and histidine supplemented with 50 mM 3-amino-1,2,4-triazole). All of the constructs were tested for autoactivation in the presence of the partner plasmid without a cloned insert. (*D*) Braf35 does not form homodimers in human cells. 293T cells were transfected with plasmids encoding Flag-Braf35 or HA-Braf35 as indicated (+) or empty vector (–). Thirty-six hours after transfection, Flag-Braf35 was immunoprecipitated from whole-cell extracts with anti-Flag M2 affinity gel (Sigma-Aldrich). Immunoprecipitated proteins and 3% of input extract were subjected to SDS/PAGE and analyzed by immunobloting with anti-HA or anti-Flag antibodies.

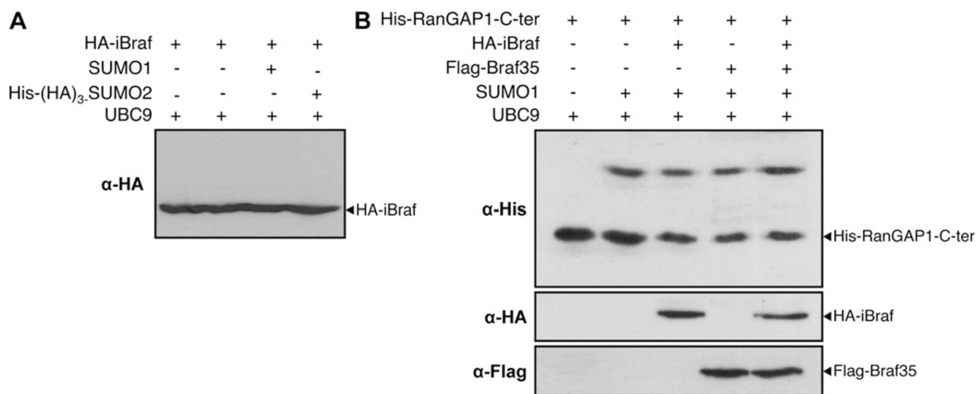

Fig. S7. iBraf is not modified by SUMO and is not a general inhibitor of sumoylation. (*A*) iBraf is not modified by SUMO1 or SUMO2. 293T cells were transfected with expression vectors for the indicated proteins. HA-iBraf was detected by Western blot using anti-HA antibodies. (*B*) iBraf is not a general inhibitor of sumoylation. 293T cells were transfected with expression vectors for the indicated proteins. His-RanGAP1-C-ter, HA-iBraf, and Flag-Braf35 were detected by Western blot using anti-His, anti-HA, or anti-Flag antibodies, respectively. (*Lower*) Inputs of the indicated proteins.

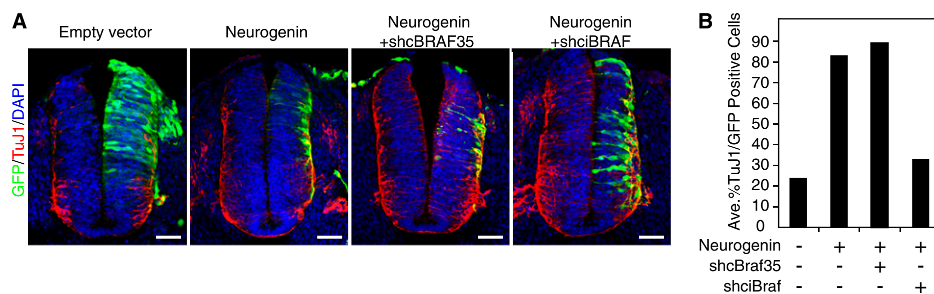

Fig. S8. Knockdown of chicken iBRAF (ciBRAF) impairs neuronal differentiation. (*A*) The neural tube of chicken embryos was electroporated with constructs expressing GFP, Neurogenin2, and shRNAs against chicken *BRAF35* (shcBRAF35) or chicken *iBRAF* (shciBRAF). GFP was used to monitor electroporation. Thirty hours postelectroporation, embryos were immunostained for TuJ1 and DAPI. (Scale bars, 50 μm.) Data are the average of three independent experiments. (*B*) Quantification of data presented in *A* as a percentage of TuJ1-positive cells per total number of GFP-positive cells.