Supporting Information

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SI Materials and Methods

Determination of Ethanol Levels. Ethanol concentration in the culture medium was determined by gas chromatography (Varian; 3400) using isopropanol as the internal standard. The concentration used in the human brain slice cultures (Fig. S7*A*) falls in which moderate effects on cortical development, such as local deficiency of neuronal migration, has been reported in rat cortical slices (1). Blood alcohol concentration (BAC) was examined on E14 pregnant CD-1 mice following the standard protocol. The peak of BAC (200–250 mg/dL) 30 min after each administration mimics the condition of daily binge drinking, and falls within the range of that used in our slice cultures (Fig. S7*B*).

RNA Purification. Total RNA was extracted from each human fetal cortical slice and mouse embryonic cerebral cortex using RNeasy Plus mini kit (Qiagen). Medial cortical regions (hippocampal primordium) and the ganglionic eminence were excluded. The quality of the total RNA was evaluated by Bioanalyzer RNA 6000 kit (Agilent) to include only the samples showing RNA integrity number (RIN) >9.0. Extracted human RNAs were combined so that each final sample include the total RNAs from cortical slices picked at a regular interval from frontal to occipital levels of both hemispheres, to minimize the variations of cortical regions among samples. To obtain enough amounts for hybridization, RNA samples from two human fetal brains were combined for each microarray analysis. Similarly, RNA samples from eight mouse embryonic brains (four embryos each from two dams) were mixed for each analysis.

Microarray Hybridization. Labeled cRNAs for hybridization were synthesized according to the protocol recommended by Affymetrix. Briefly, first-strand cDNA was synthesized from the isolated total RNA with an oligo(dT) primer containing the T7 promoter. After synthesis of the second strand, the cDNA was used as a template for in vitro transcription of cRNA using the Bioarray High-Yield RNA transcription labeling kit (Enzo Diagnostics). Biotin-labeled cRNA was purified, fragmented, and hybridized to the Affymetrix GeneChips (Mouse Genome 430 2.0, Human Genome U133 Plus 2.0 arrays) at Yale Neuroscience Microarray Center facility. A total of eight microarrays were used in this study (2 microarrays per experiment [duplicate analysis] × 2 conditions [control and ethanol-exposed] × 2 species [human and mouse]).

Biological Annotation Analysis. GO annotation analysis was performed using the Web-based National Institute of Allergy and Infectious Diseases DAVID. Each GO term was assigned from the GO database of biological process. Uncorrected $P < 0.05$ was used to evaluate overrepresentation of a given annotation in a gene list, in comparison with a background gene list. See the DAVID Web site for further details (http://david.abcc.ncifcrf.gov/). The tree of the biological processes was illustrated using GoSurfer. Enriched pathway analysis and functional network analysis were performed by using Panther and Ingenuity pathways analysis (Ingenuity Systems), respectively.

Flow Cytometric Analysis. The mouse cortex was dissected into small pieces in cold PBS and incubated with TripLE Select (Invitrogen) at 37 °C for 10 min. After dissociation, cells were fixed and permeabilized using Fix and Perm kit (Invitrogen) and proceeded to immunostaining. Anti-galactosidase (Abcam; 1:1,000), GFP (Invitrogen; 1:1,000), Satb2 (Abcam; 1:200), Cutl1 (SCBT; 1:200), Pou3f2 (SCBT; 1:50), Foxp2 (Abcam; 1:1,000), Bcl11b (Abcam; 1:500), Crym (Abcam; 1:500), Foxp1 (Abcam; 1:500), Lhx2 (SCBT; 1:50), Bhlhb5 (SCBT; 1:100), Nestin-APC conjugated (R&D Systems; 1:20) and Tuj1-FITC conjugated (Covance; 1:500) antibodies were used. FITC, APC, and PE conjugated secondary antibodies were used at 1:1,000 (Jackson Immunoresearch). For the detection of transcription factors, cells were pretreated with 100% methanol on ice for 10 min. The recording and analysis were performed using FACSCalibur (BD Biosciences) at the Yale cell sorter facility and FlowJo (TreeStar), respectively. Cell debris were sorted first according to forward scatter/side scatter (FSC/SSC) gating. Dead cells were labeled using LIVE/DEAD Fixable Dead Cell stain kit (Invitrogen) and eliminated from the analysis.

qRT-PCR. The cDNA was synthesized using SuperScript First-Strand synthesis system for RT-PCR with random hexamer primers (Invitrogen). GAPDH levels were detected by Taqman GAPDH control reagents and used for normalization. Thermocycling was carried out with Applied Biosystems 7900 and monitored by SYBR Green I dye detection. The primer designs (5′ > 3′) for mouse are: Hsp70, ggccagggctggattact, gcaaccaccatgcaagatta; Fn1, cggagagagtgcccctacta, cgatattggtgaatcgcaga; Hsp40, aagcgcgagattttcgac, cttcctccactggggctac; Fos, gggacagcctttcctactacc, gatctgcgcaaaagtcctgt, gatctgcgcaaaagtcctgt; ApoE, gaccctggaggctaaggact, agagccttcatcttcgcaat; CDKN1A, ttgccagcagaataaaaggtg, tttgctcctgtgcggaac; Gadd45b, ctgcctcctggtcacgaa, ttgcctctgctctcttcaca; Vegfa, aaaaacgaaagcgcaagaaa, tttctccgctctgaacaagg; Eomes, accggcaccaaactgaga, aagctcaagaaaggaaacatgc; Fbxw7, cagacttgtcgatactggagaattt, gatgtgcaacggttcatcaat; Fezf2, gcaaaggctttcaccaaaaa, gcatgtggaaggtcagattg; Satb1, aggagtgccccctttcac, tgctgctgagacatttgcat; Dab1, acttcaagtagctgtgaaaaccag, cttcgctgcgatcctgac; and Satb2, tttagccagctggtggagac, cacctccctagcttgattattcc.

The primer designs for human are: HSP70, ggagtcctacgccttcaaca, ccagcaccttcttcttgtcg; FN1, gcgagagtgcccctactaca, gttggtgaatcgcaggtca; HSP40, ggcctacgacgtgctcag, gtgtagctgaaagaggtaccattg; FOS, ctaccactcacccgcagact, aggtccgtgcagaagtcct; APOE, ggatccttgagtcctactcagc, tcttcctgcctgtgattgg; CDKN1A, cgaagtcagttccttgtggag, catgggttctgacggacat; GADD45b, cattgtctcctggtcacgaa, taggggacccactggttgt; VEGFA, tgcccgctgctgtctaat, tctccgctctgagcaagg; EOMES, gtggggaggtcgaggttc, tgttctggaggtccatggtag; FBXW7, gaactccagtagtattgtggacctg, catggtccaactttcttttcattt; FEZF2, caaaggctttcaccaaaaagg, gggtgtgcatatggaaggtt; SATB1, aatggcattgctgtctctagg, actttccaacctggattagcc; DAB1, cggaggatgagtcgagagg, gtcatggagagaagccaagg; and SATB2, ccagagcacattagccaaaga, tgtgctatttacaatggatgaaatc.

Immunoblot and Immunohistochemistry. Protein samples from mouse cortices and cultured human cortical slices were harvested and used for immunoblotting using a standard protocol. The antibodies used are polyclonal anti-Hsp70 (a kind gift from Dr. Nakai, Yamaguchi University, Japan, 1:500) and anti-Dab1 (E-19, SCBT; 1:300). The analysis of band intensity was performed using a standard protocol. Immunohistochemistry was performed using a standard protocol with the following primary antibodies: polyclonal anti-Tbr2 (Millipore; 1:500), anti-Neuropilin1 (R&D Systems; 1:100), and the antibodies used for the flow cytometric analysis. The nuclei were counterstained with DAPI. Detection of β-galactosidase activities was performed using a standard protocol.

Quantification and Statistics Used in Wet Experiments. All statistical data are presented as mean ± SEM. P values were calculated from multiple experiments (at least $n = 3$ for each experimental condition).

1. Mooney SM, Miller MW (2003) Ethanol-induced neuronal death in organotypic cultures of rat cerebral cortex. *Brain Res Dev Brain Res* 147:135–141.

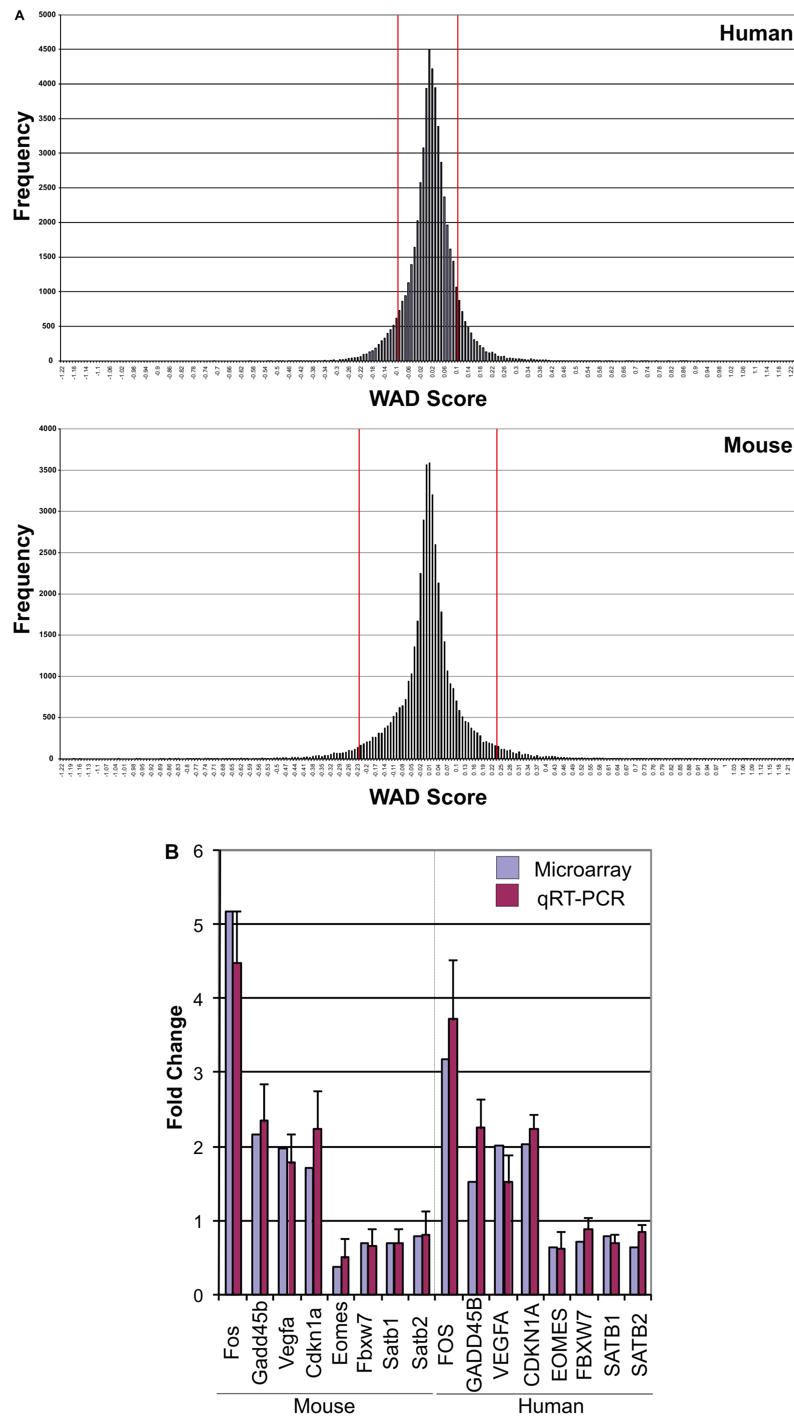

Fig. S1. Histogram of probe set frequency and qRT-PCR validation. (*A*) WAD cutoff and frequency of probe sets. Histograms show the frequency of probe sets corresponding to the WAD score. The vertical red lines demarcate the WAD cutoff points. (*B*) Validation of the microarray data by qRT-PCR. The qRT-PCR values (red columns) indicate the mean ± SD of the fold change of indicated gene expression by mFAE compared with PBS exposure (control) ($P < 0.05$ for all genes by *t* test). Each experiment was performed using RNA samples of mouse and human cortices separately extracted from six embryos from three dams (two embryos per dam) and eight cultured slices from four human embryonic brains (two slices each per brain), respectively. Microarray data (blue columns) show the mean fold change values.

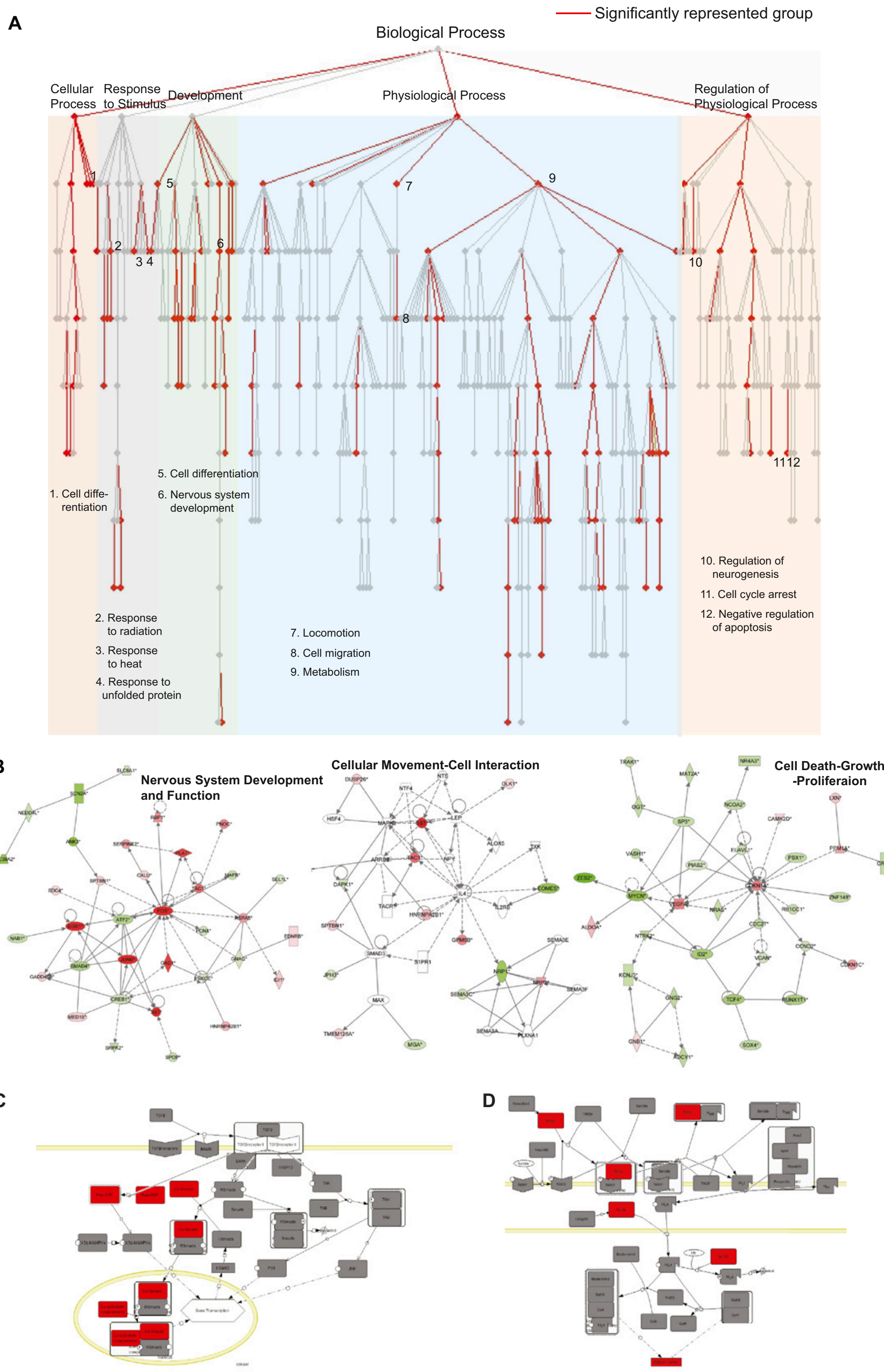

Fig. S2. Biological processes enriched in both human and mouse mFAE models. (*A*) Biological processes enriched in both human and mouse mFAE models were identified and visualized using GoSurfer. In the hierarchical tree, the nodes represent individual GO biological process categories and the branches indicate the relationships between each biological category. The GO categories containing more than three genes are shown as nodes. Significantly altered processes (uncorrected $P < 0.05$) are highlighted in red. The numbers listed below the hierarchical tree refer to the selected GO category names as biologically interesting/relevant for understanding the mFAE phenotypes. (*B*) Functional network analysis of enriched genes. Functional network analysis of the enriched genes in the mFAE model. The modules identified to be most significantly altered by mFAE are shown. Nodes were assigned to each gene. Red and green nodes represent the up- and down-regulated genes, respectively. The shapes of the nodes indicate protein functions: rhombus, enzyme; ellipse, transcriptional

Legend Continued on following page

regulator; circle, other functions. Solid and broken lines indicate direct and indirect interactions, respectively. Each arrow from node *X* to *Y* indicates that *X* acts on *Y*. (*C* and *D*) Genes affected by mFAE in TGF-β and Notch signaling pathways. TGF-β (*C*) and Notch (*D*) signaling pathways were visualized and the affected genes by mFAE were highlighted in red using Panther. The legend for pathway illustration is available on the Panther Web site (http://www.pantherdb.org/).

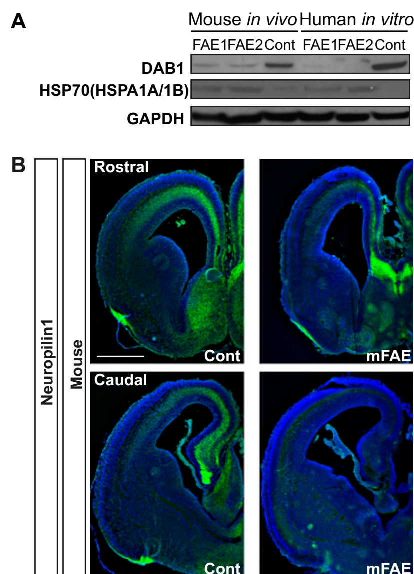

Fig. S3. Validation of the altered gene expression by immunoblot and immunohistochemistry. (*A*) Immunoblots of HSP70 and DAB1, showing their increase and decrease, respectively, in both mouse and human mFAE models. (*B*) Reduction of Neuropilin1 immunohistological labeling (green) in both the rostral (*Upper*) and caudal (*Lower*) regions of the mouse cortex by mFAE. n = 8/8 embryos from four dams. (Scale bar, 2 mm.)

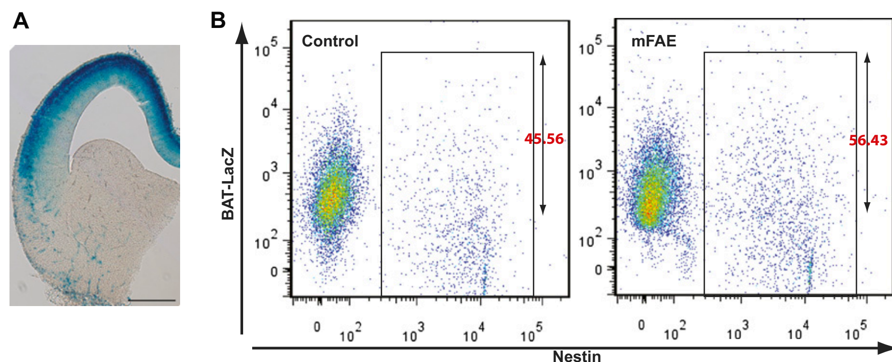

Fig. S4. Increase of Wnt signaling activity by mFAE in the cortex. (*A*) Representative image showing the basal level of Wnt reporter expression in the cortex of E16 BAT-LacZ transgenic mouse (without ethanol exposure). (Scale bar, 2 mm.) (*B*) Flow cytometric analysis of Wnt reporter expressing cells. BAT-LacZ reporter transgenic embryos were subjected to ethanol, and the numbers of $LacZ^+$ and $Nestin^+$ cortical cells were analyzed. The numbers in red indicate the percentage of $LacZ^+$ cells (in the range shown by two-headed arrows) in the total $Nestin^+$ cells (within the boxed area). Compared with the control condition, 23 ± 4.2% (mean ± SEM) increase of $LacZ^+$ cells in total $Nestin^+$ cells was observed by mFAE (n = 7 for each condition, $P < 0.05$, t test). Similar increase was observed in another Wnt reporter transgenic mouse line, FOS-LacZ (n = 6 each).

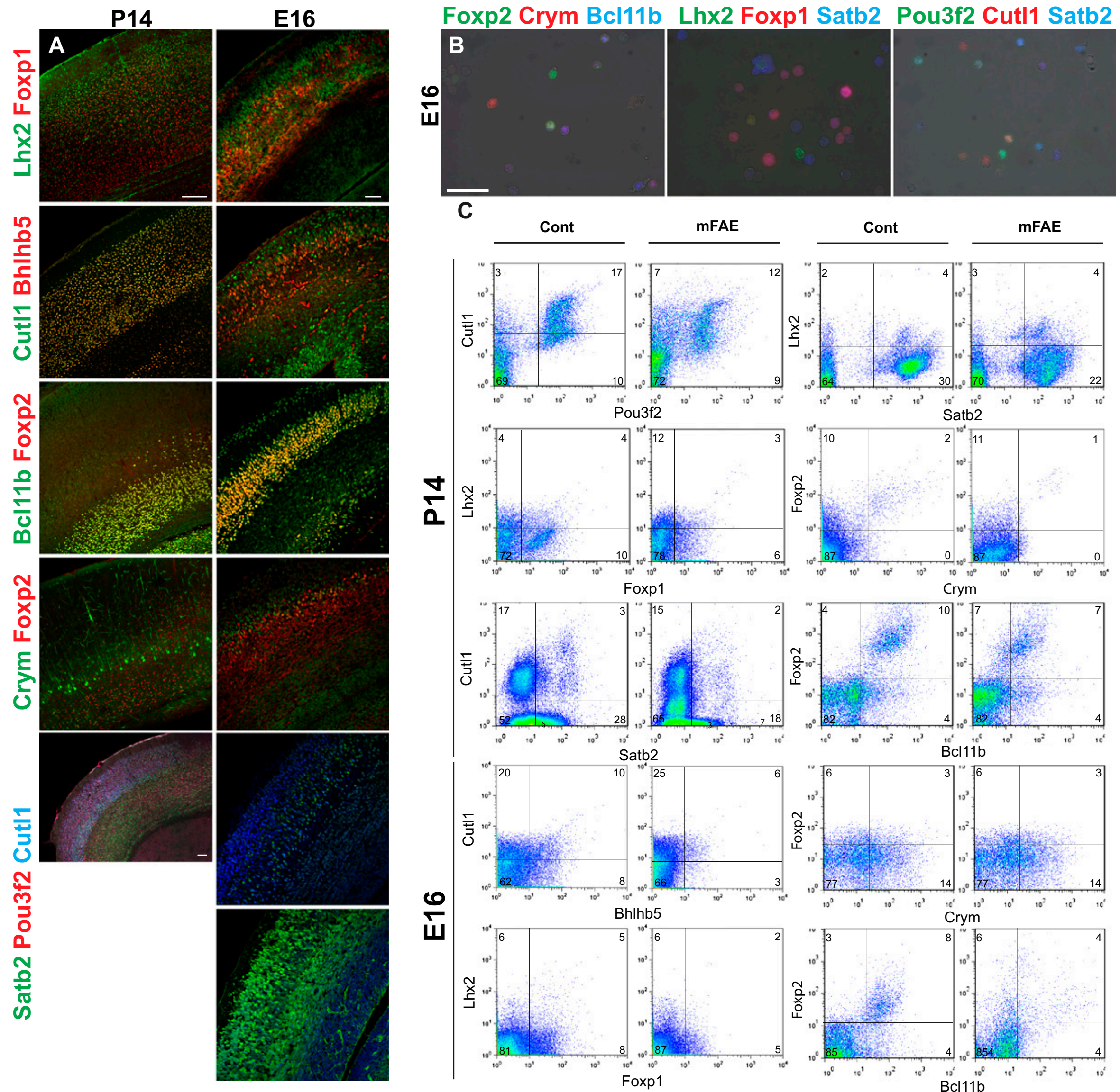

Fig. S5. Flow cytometric analysis of the cells expressing subtype markers in the control and ethanol-exposed mouse cortex. (*A*) Immunohistochemistry for neuronal subtype markers used for flow cytometric analysis. Immunohistochemistry on cortical sections of P14 and E16 mice from PBS-administered dams shows specific labeling of neuronal subtypes expressing the indicated markers in combination. [Scale bars, 0.2 (P14) and 0.05 (E16) mm.] (*B*) Immunocytochemistry for neuronal subtype markers used for flow cytometric analysis. Immunocytochemistry of cortical cells collected from control (PBS)-exposed E16 mice, showing specific labeling of neuronal subtypes expressing indicated markers in combination. Images were taken before flow cytometric analysis. (Scale bar, 0.05 mm.) (*C*) The analysis was performed on P14 and E16 cerebral cortices for the indicated markers. Decrease of the cells that express the enriched genes (*x* axis) within those expressing nonenriched genes (*y* axis) is evident in the cortex of ethanol-exposed embryos. The numbers at the four corners of the plots indicate the percentage of cells within each subdivision.

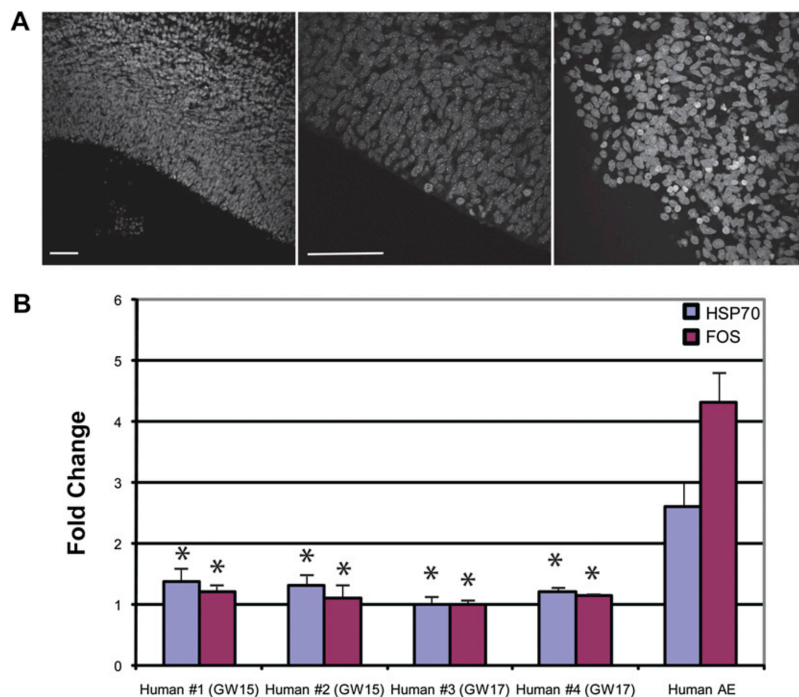

Fig. S6. Assessment of the preservation state of human cortical tissues before ethanol-exposure experiments. (*A*) Nuclear staining with DAPI on human cortical samples. The sample used for the study (*Left* and *Center* at lower and higher magnifications, respectively) shows very few pyknotic nuclei, whereas the excluded sample (*Right* at higher magnification) includes many pyknotic nuclei. Irregular nuclear morphology and alignment of cells are also evident. (Scale bars, 0.2 mm.) (*B*) qRT-PCR of *HSP70* and *FOS*, showing low expression profiles of these genes in the four cortical samples (nos. 1–4) used in this study compared with their levels in the alcohol-exposed cortex (AE). The values are the mean ± SEM of three experiments performed in triplicate. The mean values of sample 3 was set to 1.0. $^{*}P < 0.05$ by Mann-Whitney *u* test compared with AE.

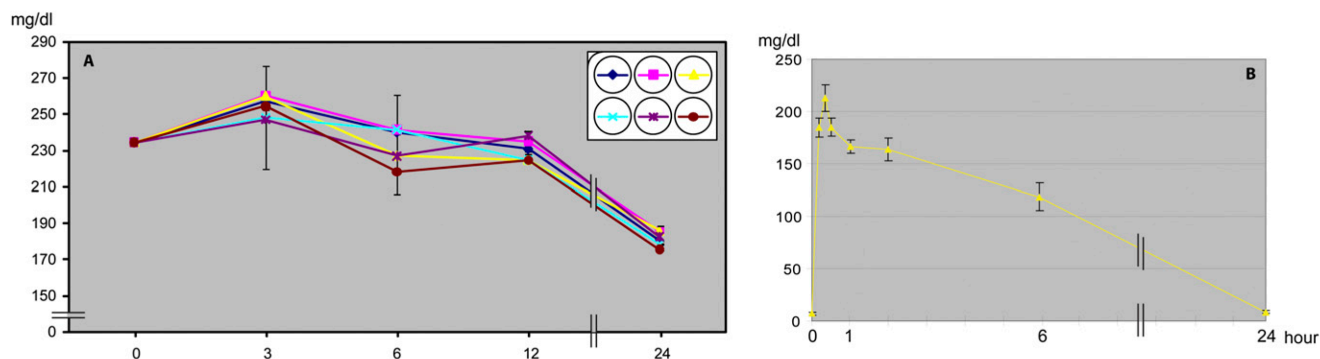

Fig. S7. Ethanol concentration in the culture medium and BAC profile in pregnant mice. (*A*) Ethanol concentration was examined at multiple time points in the indicated wells of 6-well plates ($n = X$). The *x* axis represents time (hour) after ethanol administration and the *y* axis represents the ethanol concentration (mg/dL) in the medium. No significant difference between wells was observed (P = NS by repeated measures ANOVA and Kolmogorov–Smirnov test). (*B*) BAC was examined at multiple time points before/after the ethanol injection at E14. The *x* axis represents time (hour) after ethanol administration and the *y* axis represents the BAC (mg/dL). The peak of BAC on the second and third days was slightly higher than that on the first day, consistent with the previous observation in rat (1).

1. Kotkoskie LA, Norton S (1990) Acute response of the fetal telencephalon to short-term maternal exposure to ethanol in the rat. *Acta Neuropathol* 79:513–519.

Table S1. Comparison of the number of differentially expressed genes with different WAD cutoff scores

Model	Stage	Exposure	No. of genes screened by various WAD cutoff							
			WAD025	WAD023	WAD02	WAD01	WAD-025	WAD-023	WAD-02	WAD-01
mFAE (mouse)	E14–17	i.p. daily	1,179	1,441	1,969	5,669	1,066	1,318	1,870	5,508
Restraint	E16–18	30 min daily	80	106	168	1,558	28	34	64	694
eFAE (GSE 9545)	E8	3 h	85	127	232	1,997	217	269	411	2,065
eFAE (GSE 1074)	E8–10	48 h	1,810	2,166	2,735	6,327	527	657	962	3,541
Dlx1/2 KO (GEO 1084)	E14	N/A	180	220	379	2,600	175	233	328	1,909
Dlx1/2 KO (GEO 8311)	E10	N/A	56	79	96	492	37	45	84	505
Rbpj cKO	E17	N/A	458	598	841	3,246	596	694	884	2,195

Comparison was made with the datasets of embryonic cortices from *Rbpj* conditional knockout mice and wild-type dams exposed to restraint stress as well as the datasets of eFAE (early midgestation fetal alcohol exposure, GSE9545, 1074) and *Dlx1/2* conditional knockout mice (GEO 1084, 8311).

Dataset S1. List of the up- and down-regulated probe sets obtained by WAD statistics

[Dataset S1 (XLS)]