SUPPLEMENTAL METHODS

Post-mortem samples. 47 samples (13 autism, 35 control) were obtained from NICHD Brain and Tissue Bank for Developmental Disorders, and 43 samples (22 autism, 21 control) are from the Harvard Brain Tissue Resource Center. Tissue was excised from cerebellar vermis (31 samples), cerebellar hemisphere (16 samples), anterior cerebellum (30 samples), cerebellar lobule 6 (12 samples), and unspecified region of cerebellum (1 sample), and kept frozen. Demographic information for each sample is listed in the Supplemental Table 1. 29 autism and 49 control samples were included for statistical analysis.

RNA and DNA isolation from cerebellar tissue. Frozen tissue blocks were soaked in 10X volume of RNAlater®-ICE solution (Ambion) and kept at -80°C over night. After removing RNAlater®-ICE solution, 6X volume of cell disruption buffer (Ambion) containing RNasin® Ribonuclease Inhibitor (Promega) and Complete Protease Inhibitor Cocktail tablet (Roche) was added to the tissue. Tissue was homogenized using a hand-held homogenizer (Power Gen 35, Fisher Scientific) for ~30sec. Total RNA of >~200 bases in size was isolated from 350µl of the homogenate using *mir*Vana™PARIS™ kit (Ambion) following manufacturer's instruction. RNA integrity was assessed by following three methods. First, RNA concentration was measured using Biomate3 spectrophotemeter (Thermo Scientific). To examine the quality of RNA, 3µl of each sample was separated on 1% agarose gel in RNase-free condition. RNA Integrity Number (RIN) was then measured using 100-200ng of sample and RNA 6000 Nano kit on Bioanalyzer (Agilent) following manufacturer's instructions. 11 samples displayed RIN<3 and were removed from the analysis.

To isolate genomic DNA, 175µl of homogenate was treated with 0.5µg/µl ProteinaseK (Promega) in buffer containing 60mM Tris, pH8.0, 100mM EDTA, and 0.5% SDS at 55°C over night. Phenol:chloroform:isoamylalcohol (25:24:1) solution was then mixed to the digest. The mixture was centrifuged at14,000 rpm for 10min at 4°C and the upper aqueous layer was collected. The same volume of chloroform was then added and the same procedure was repeated. Collected upper layer was mixed with 1/10 volume of 3M sodium acetate, pH5.2 and one volume of 95% ethanol to precipitate genomic DNA. Precipitated DNA was washed with 70% EtOH and the air-dried pellet was dissolved in 10mM Tris, pH8.0 and 1mM EDTA.

Genotyping *rs1861972* and *rs1861973*. Each post-mortem sample was genotyped for *rs1861972* and *rs1861973* using Luminex® technology. Duplex PCR was performed to amplify the regions encompassing *rs1861972* and *rs1861973* using 80ng of genomic DNA, 0.17µM each of forward and reverse primers, 0.2mM of dNTP, 2.5mM of $MgCl_2$, and 0.13µl of AmpliTaq Gold® DNA polymerase (Applied Biosystems) in total of 20µl. Primer sequences are as follows: *rs1861972* forward CCTGCCTATGGGTTGCTTTA; *rs1861972* reverse GGTGATAAGAGGCTCAACCC; *rs1861973* forward CCCTTTCCCCATGGATAGC; *rs1861973* reverse CCAGACATGGGACTGCTTC. PCR cycling conditions are as follows: one cycle at 94°C for 10 min, 40 cycles at 94°C for 40 sec, 60°C for 30 sec, 60°C to 65°C for 1min, and 72°C for 2min, one cycle at 72°C for 10 min. Two microliter of the PCR product was used for ligation detection reaction with 0.01µM each of common primers (*rs1861972* common TGGCCTTGCCCCCTCTATCTTTAAACTACAAATCTAAC; *rs1861973* common CCAAAACCTGGGGCAGGCTATCTTTAAACTACAAATCTAAC) and allele specific primers for each polymorphism (*rs1861972*-A

AATCCTTTTTACTCAATTCAATCACACCACTCCCTGCCAA; *rs1861972*-G CAATTAACTACATACAATACATACCACCACTCCCTGCCAG; *rs1861973*-C CTTTTCAAATCAATACTCAACTTTCCTTACAGCGACCCTGC; *rs1861973*-T CTTTAATCCTTTATCACTTTATCACCTTACAGCGACCCTGT), 0.05µl of Taq DNA ligase (New England Biolabs) in total of 15µl. Conditions for ligation detection reaction are as follows: one cycle at 95°C for 1 min, 31 cycles at 95°C for 15 sec and 58°C for 2 min. Bead hybridization was performed by adding 50µl of mixture containing 0.8µl each of beads, 0.032µM of universal oligo, and hybridization buffer (3M tetramethylammonium chloride, 0.1% SDS, 50mM Tris-HCl, pH8.0, and 3mM EDTA, pH8.0) to the total of ligation detection reaction. Conditions for bead hybridization was as following: 95°C for 2 min, 70°C to 51°C for 1 min per 1°C decrement, 50°C to 38°C for 2 min per 1°C decrement, and 37°C for 20 min. 0.18µl of SAPE and 5.82µl of hybridization buffer was added to the hybridized mixture and incubated at 37°C for 40 min. Fluorescence was then detected on Luminex® 100™ flow cytometry machine. Fluorescence signal from both alleles was collected for each locus and the signal proportion of one allele over both alleles was plotted for each sample. Genotypes were assigned after clustering three genotype groups (two homozygotes and one heterozygote). Samples with ambiguous assignments were re-genotyped.

Quantitative Reverse Transcription PCR. First strand cDNA was synthesized from total RNA and quantified using Taqman® real-time qPCR. 3µg of RNA was treated with 2units of DNaseI (New England Biolabs) in a buffer containing 2.5mM $MgCl_2$, 0.5mM $CaCl_2$, and 10mM Tris-HCl, pH7.6 and RNasin® Ribonuclease Inhibitor (Promega) at 37°C for 30min. DNaseI was subsequently inactivated by heating at 75°C for 10min. First strand cDNA was then generated

using 1µg of DNaseI treated RNA and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instruction. Quantitative PCR was conducted using one twentieth of total cDNA and Taqman® probe sets for human *EN2* (Hs00171321_m1, fluorescent dye FAM labeled) and *GAPDH* internal control (4326317E, fluorescent dye VIC labeled) on ABI7900HT (Applied Biosystems). *EN2* level was normalized to endogenous *GAPDH* level by subtracting *GAPDH* Ct from *EN2* Ct (ΔCt). Average of normalized Ct (ΔCt) values was obtained from three replicates of qRT-PCR reaction. ΔΔCt method was used to calculate relative changes in expression levels. *HTR5A, INSIG1, CNPY1, RBM33,SHH, CUX1,* and *NFIB* levels were measured in the same manner using probe sets Hs00225153_m1, Hs01650979_m1, Hs01073160_m1, Hs00369593_m1, Hs00179843_m1, Hs00738848_m1, and Hs00232149_m1, respectively, where all probes are labeled with fluorescent dye FAM. After Taqman qRT-PCR one sample failed to amplify and was excluded from the analysis.

Over-expression, knock-down analysis and knockout analysis. For the *EN2* over-expression and knockdown analysis in human Embryonic Kidney 293T (HEK293T), the cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% Fetal Bovine Serum at 37 ºC under 5% CO_2. Cells were grown to ~85% confluency before human *EN2* protein coding region cloned into pCMV-Tag3B vector or the empty vector was transfected using Lipofectamine2000 (Invitrogen) following manufacturer's protocol. Cells were collected 24hrs after transfection and total RNA was isolated using TRIzol® reagent (Invitrogen) following manufacturer's protocol. Transient knock-down of *EN2* was generated as described previously (9). The single and double CUX1 and NFIB knock-downs have been reported previously (9). All cell lines were maintained as described above.

EN2 overexpression and knockdown was also conducted in PFSK-1 cells, which were maintained in RPMI-1 (Invitrogen), 10% Fetal Bovine Serum and 1x Penicillin/Streptomycin at 37C under 5% CO2. Lentivirus was utilized to stably transduce PFSK-1 with the *EN2* overexpression and knockdown constructs. To generate the virus, a second generation lentivirus system was used that included a lentivector (pWPI-CMV-EN2 for overexpression, pGIPZ hEN2 shRNA for knockdown) and packaging plasmids pMD2G and psPAX2. These constructs were co-transfected into HEK-293T cells, virus was harvested 48 hours post-transfection and concentrated using a Beckman ultracentrifuge for 1.5 hours at 25,000xg. Concentrated virus was used to transduce 400,000 cells in media supplemented with 8ug/ml of polybrene with an MOI>1. Fresh media was added 24 hours post-transduction and cells were collected 24 hours following media change. RNA was extracted using Ribozol (AMRESCO) following manufacturer's protocol and processed for qRT-PCR as previously described.

For over-expression, knock-down and knock-out analysis, flanking transcript levels were measured by Taqman® qRT-PCR as described earlier. The following primers were used: (*Gapdh*: F TGTTCCTACCCCCAATGTGTC, R GGAAGTTGCTGTTGAAGTCGCA; *Shh* F GGATGAGGAAAACACGGGAGCA, R TCATCCCAGCCCTCGGTCACT; *Cnpy1* F CAGCTAGATGGTTTGCCTCCTG, R TATGCTCCGAGGAAGGGAGACA; *Insig1* F GACGAGGTGATAGCCACCAT, R ACTTGTGTGGTTCTCCCAGG) and PCR conditions (95C 1' 1 cycle; 95C 30'', 55C 30", 72C 40" 45 cycles) primer sequence and PCR conditions. ΔΔCt method was used to measure gene expression differences compared to controls. To test for significance two-tailed, paired Student T-test was performed for 3-10 independent experiments using average ΔCt values

ISHs for Shh were performed as described [2]. The 3'UTR was PCR amplified (F CATTCTCATCAACCGGGTGC; R AAGGCAGTACAGAAGACGGC cloned and anti-sense and sense probes were generated. 60 micron transverse sections were generated, ISHs were performed on all sections and then the Shh expression domain was quantified by ImageJ for all sections in stage-matched embryos

SUPPLEMENTAL TABLES

Table S1. Demographic information of post-mortem samples.

Individual ID[a]	Diagnosis	Age	Sex	PMI[b]	RIN[c]	Ethnicity	Cause of Death	*rs1861972*	*rs1861973*
UMB4671	Autism - confirmed by ADI-R	4.5	F	13	3.2	African American	Multiple injuries	AA	CC
UMB1638	Autism - confirmed by ADI-R	20.8	F	50	3.8	English-White	Seizure disorder	AA	CC
UMB4231	Autism - Suspected	8.8	M	12	4.6	African American	Drowning	AA	CC
AN19511	Autism - confirmed by ADI-R	8	M	22.16	4.6	English-White	Cancer (sarcoma)	AA	CC
AN08873	Autism - confirmed by ADI-R	5	M	25.5	4.9	English-White	Asphyxia due to drowning	AA	CC
AN09714	Autism - confirmed by ADI-R	60	M		5	Unknown	Pancreatic Cancer	AA	CC
AN11989	Autism - confirmed by ADI-R	30	M	16.06	5.4	Northern European White	Congestive Heart Failure	AA	CC
AN01227	Autism - Suspected	82	M	24.67	5.5	English-White	Arteriosclerotic Cardiovascular Disease	AA	CC
UMB5176	Autism - confirmed by ADI-R	22.5	M	18	6.4	African American	Subdural hemorrhage	AA	CC
UMB4721	Autism - confirmed by ADI-R	8.8	M	16	6.5	African American	Drowning	AA	CC
UMB5027	Autism - Suspected	38	M	26	7	African American	Obstruction of bowel due to adhesion	AA	CC
AN06420	Autism - confirmed by ADI-R	39	M	13.95	7.2	English-White	Cardiac Tamponade	AA	CC
AN13872	Autism - confirmed by ADI-R	5	F	32.73	7.2	Asian	Drowning	AA	CC
AN00493	Autism - confirmed by ADI-R	27	M	8.3	3.2	English-White	Drowning	AG	CT
AN08792	Autism - confirmed by ADI-R	30	M	20.33	3.2	English-White	Gastrointestinal bleeding	AG	CT
AN17777	Autism - confirmed by ADI-R	49	F	16.33	3.6	Unknown	Cardiopulmonary Arrest	AG	CT
AN16115	Autism - confirmed by ADI-R	11	F	12.88	4.6	English-White	Drowning	AG	CT
UMB4899	Autism - confirmed by ADI-R	14.3	M	9	4.9	English-White	Drowning	AG	CT
AN01570	Autism - confirmed by ADI-R	18	F	6.75	5.2	English-White	Seizure	AG	CT

AN09730	Autism - confirmed by ADI-R	22	M	25	5.5	English-White	Aspiration, seizure	AG	CT
UMB4999	Autism - Suspected	20.8	M	14	6	English-White	Cardiac arrthymia	AG	CT
AN04682	Autism - Suspected	15	M	23.23	6.8	Unknown	Asphyxia, hanging	AG	CT
UMB5115	Autism - confirmed by ADI-R	46.4	M	29	6.8	English-White	Complications of pseudomyxoma peridonci	AG	CT
AN08166	Autism - confirmed by ADI-R	29	M	43.25	7.8	Eastern Mediterranean	Seizure Suspected	AG	CT
UMB5144	Autism - confirmed by ADI-R	7.2	M	3	8.1	Caucasian	Cancer	AG	CT
AN12457	Autism - confirmed by ADI-R	29	F	17.83	4.2	English-White	Seizure Disorder	GG	TT
AN16641	Autism - confirmed by ADI-R	9	M	27	4.6	English-White	Seizure disorder	GG	TT
AN03632	Autism - Suspected	49	F	21.08	5.2	Unknown	Cancer	GG	TT
AN00764	Autism - confirmed by ADI-R	20	M	23.66	6.5	English-White	Auto Trauma	GG	TT
AN03217	Control	19	M	18.58	3.1	English-White	Pneumonia	AA	CC
AN12137	Control	31	M	32.92	3.8	Unknown	Asphyxia	AA	CC
UMB818	Control	27.2	M	10	4.6	Caucasian	Multiple injuries	AA	CC
UMB4543	Control	28.7	M	13	5.1	Caucasian	Multiple injuries	AA	CC
UMB1499	Control	4.5	F	21	5.3	Asian	Lymphocytic myocarditis	AA	CC
AN10679	Control	41	F	14	5.5	Unknown		AA	CC
AN17868	Control	46	M	18.78	5.5	Unknown	Heart Attack	AA	CC
AN11184	Control	64	M	27.68	5.5	Unknown	Cardiac Arrest	AA	CC
UMB1860	Control	8	M	5	5.6	Caucasian	Cardiac arrhythmia	AA	CC
UMB1080	Control	16.5	M	21	5.6	African American	Cardiac arrythmia, seizure	AA	CC
UMB1706	Control	8.6	F	20	5.7	African American	Rejection of cardiac allograft transplantation	AA	CC
UMB5173	Control	10.8	F	10	6	Caucasian	Asthma	AA	CC
AN02583	Control	68	M	16.58	6.6	Unknown	Ruptured Aortic Aneurysm	AA	CC

UMB 1226	Control	23.2	M	21	6.8	Caucasian	Drowning	AA	CC
AN08677	Control	38	M	25.47	6.8	Unknown		AA	CC
UMB 1846	Control	20.6	F	9	6.9	Caucasian	Multiple injuries	AA	CC
UMB 3	Control	5.4	M	17	7	Caucasian	Drowning	AA	CC
UMB 4670	Control	4.6	M	17	7	Caucasian	Commotio cordis	AA	CC
UMB 1710	Control	26.1	F	12	7.1	Caucasian	Cardian temponade	AA	CC
UMB 4722	Control	14.5	M	16	7.1	Caucasian	Multiple injuries	AA	CC
UMB 1708	Control	8.1	F	20	7.1	African American	Compressional asphyxia and multiple injuries	AA	CC
UMB 1790	Control	13.7	M	18	7.2	Caucasian	Multiple injuries	AA	CC
UMB 1284	Control	3.3	F	11	7.5	African American	Drowning	AA	CC
UMB 1670	Control	13.3	M	5	7.6	Caucasian	Asphyxia by hanging	AA	CC
UMB 5180	Control	1.7	M	25	7.8	Caucasian	Drowning	AA	CC
UMB 1793	Control	11.7	M	19	8.1	African American	Drowning	AA	CC
AN02456	Control	4	F	17.02	5.1	Unknown	Acute Bronchopneumonia After Tonsillectomy	AG	CT
AN17425	Control	16	M	26.16	3.2	Unknown	Heart Attack	AG	CT
AN12552	Control	56	M	23.61	4.4	African American	Multiple Injuries	AG	CT
AN15566	Control	32	F	28.92	4.9	Unknown		AG	CT
AN12240	Control	51	M	4.75	5.4	English-White	Heart Attack	AG	CT
UMB 1185	Control	4.7	M	17	5.4	Caucasian	Drowning	AG	CT
UMB 1864	Control	2.5	F	8	5.7	Caucasian	Laryngitis, and bronchiolitis	AG	CT
AN01410	Control	41	M	27.17	5.8	Unknown		AG	CT
AN15622	Control	30	M	14.83	6.1	English-White	Asphyxia	AG	CT
UMB 1297	Control	15.2	M	16	6.5	African American	Multiple injuries	AG	CT

UMB 1582	Control	42.1	M	24	6.6	Caucasian	Cardiac arrythmia	AG	CT
UMB 1541	Control	20.6	F	19	6.7	Caucasian	Head injury	AG	CT
UMB 1407	Control	9.1	F	20	6.9	African American	Asthma	AG	CT
AN04432	Control	22	M	24.3	6.9	English-White	Central Hepatic Laceration	AG	CT
AN13295	Control	56	M	22.12	7	Unknown		AG	CT
UMB 1674	Control	8.9	M	36	7.1	Caucasian	Hyperthermia and drowning	AG	CT
UMB 1136	Control	33.99	F	19	7.3	Caucasian	Arteriosclerotic cardiovascular disease	AG	CT
UMB 4590	Control	20.5	M	19	7.6	Caucasian	Dialated cardiomyopathy	AG	CT
AN10833	Control	22	M	21.47	7.6	Unknown		AG	CT
UMB 1714	Control	12.4	M	22	4.4	African American	cardiac arrthymia	GG	TT
AN17344	Control	47	M	23	5.3	English-White	Unknown	GG	TT
UMB 4787	Control	12.9	M	15	7.4	African American	Asthma	GG	TT
AN14771	Control	30	M	23	8.2	English-White	Cardiac arrhythmia	GG	TT

a- Anonymous ID for each sample
b- Post-mortem interval
c- RNA integrity number

Table S2. Age and Sex distribution of Autism and control groups.

Age group (years)	Autism Male	Control Male	Autism Female	Control Female
0-6	2	4	3	3
7-12	6	6	2	4
13-18	2	8	1	-
19-35	8	11	2	6
36-50	4	5	2	1
51-65	3	5	-	-
66-82	1	1	-	-

Table S3. Distribution of individuals for affection status and *rs1861972-rs1861973* genotype[a]

	Autism	Control
AC/AC	13	26
AC/GT	12	19
GT/GT	4	4
Sum	29	49

[a]-The distribution is shown for 78 individuals included in statistical analysis.

Table S4. Covariate analysis of *EN2* levels for final model[a]

Covariate	P-value[c]
Age	**0.0174**
PMI	**0.0224**
RIN	**<.0001**
Affection	0.1144
Genotype	0.0677
Affection*Genotype[b]	**0.0006**

[a]- The full model includes all 10 covariates. Insignificant covariates were removed sequentially to obtain the final model.
[b]- Interaction between affection status and genotype
[c]- Type 3 tests of fixed effects. Probability for each covariate is adjusted after considering effects of all significant covariates. Significant values (5% cut-off) are in bold.

Table S5. *CUX1* levels comparisons based on genotypes or affection status

Comparison	Least squared mean[a]	Standard errors[b]	P-value[c]	Fold change[d]
AC/AC vs. AC/GT	-0.1184	0.1079	0.5188	1.0855
AC/AC vs. GT/GT	0.2630	0.2415	0.5240	0.8334
AC/GT vs. GT/GT	0.3814	0.2504	0.2864	0.7677
Autism vs. Control	0.08475	0.1258	0.5029	0.9429

[a]- Least squared means were calculated from ΔΔCt values (ΔCt^{autism}-$\Delta Ct^{control}$ or $\Delta Ct^{1st\ genotype}$-$\Delta Ct^{2nd\ genotype}$) after adjusting for significant covariates

[b]- Standard error for estimated difference

[c]- Tukey-Kramer test was performed considering all the significant covariates. No significant difference was observed (5% cut-off).

[d]- Fold change of each first group versus the second is calculated as $2^{-(least\ squared\ mean)}$.

Table S6. *NFIB* levels comparisons based on genotypes or affection status

Comparison	Least squared mean[a]	Standard errors[b]	P-value[c]	Fold change[d]
AC/AC vs. AC/GT	-0.1004	0.1165	0.6661	1.0721
AC/AC vs. GT/GT	0.3379	0.2544	0.3843	0.7912
AC/GT vs. GT/GT	0.4383	0.2579	0.2125	0.7380
Autism vs. Control	0.01094	0.1277	0.9320	0.9924

[a]- Least squared means were calculated from ΔΔCt values (ΔCt^{autism}-$\Delta Ct^{control}$ or $\Delta Ct^{1st\ genotype}$-$\Delta Ct^{2nd\ genotype}$) after adjusting for significant covariates

[b]- Standard error for estimated difference

[c]- Tukey-Kramer test was performed considering all the significant covariates. No significant difference was observed (5% cut-off).

[d]- Fold change of each first group versus the second is calculated as $2^{-(least\ squared\ mean)}$.

Table S7. Covariate analysis of *SHH* levels for final model[a]

Covariate	P-value[b]
RIN	**0.0009**
Affection	**0.0327**
Genotype	**0.0391**

[a]- The full model includes all 10 covariates. Insignificant covariates were removed sequentially to obtain the final model.
[b]- Type 3 tests of fixed effects. Probability for each covariate is adjusted after considering effects of all significant covariates. Significant values (5% cut-off) are in bold.

Table S8. Covariate analysis of *INSIG1* levels for final model[a]

Effect	P-value[b]
Age	0.0564
Sex	**0.0100**
PMI	**0.0002**
RIN	**<.0001**
Affection	**0.0352**
Genotype	0.5873

[a]- The full model includes all 10 covariates. Insignificant covariates were removed sequentially to obtain the final model.
[b]- Type 3 tests of fixed effects. Probability for each covariate is adjusted after considering effects of all significant covariates. Significant values (5% cut-off) are in bold.

Table S9. Covariate analysis of *CNPY1* levels for final model[a]

Covariate	P-value[b]
RIN	**<.0001**
Affection	0.6627
Genotype	**0.0007**

[a]- The full model includes all 10 covariates. Insignificant covariates were removed sequentially to obtain the final model.
[b]- Type 3 tests of fixed effects. Probability for each covariate is adjusted after considering effects of all significant covariates. Significant values (5% cut-off) are in bold.

Table S10. *SHH* levels comparisons based on genotypes or affection status

Comparison	Least squared mean[a]	Standard errors[b]	P-value[c]	Fold change[d]
AC/AC vs. AC/GT	0.3735	0.1435	**0.0297**	0.7719
AC/AC vs. GT/GT	0.1430	0.2936	0.8777	0.9056
AC/GT vs. GT/GT	-0.2306	0.3021	0.7266	1.1733
Autism vs. Control	-0.3290	0.1512	**0.0327**	1.2561

[a]- Least squared means were calculated from ΔΔCt values (ΔCt^{autism}-$\Delta Ct^{control}$ or $\Delta Ct^{1st\ genotype}$-$\Delta Ct^{2nd\ genotype}$) after adjusting for significant covariates

[b]- Standard error for estimated difference

[c]- Tukey-Kramer test was performed considering all the significant covariates. Significant values (5% cut-off) are in bold.

[d]- Fold change of each first group versus the second is calculated as $2^{-(least\ squared\ mean)}$.

Table S11. *INSIG1* levels comparisons based on genotypes or affection status

Comparison	Least squared mean[a]	Standard errors[b]	P-value[c]	Fold change[d]
AC/AC vs. AC/GT	-0.05403	0.08223	0.7890	1.0381
AC/AC vs. GT/GT	-0.1703	0.1815	0.6184	1.1253
AC/GT vs. GT/GT	-0.1162	0.1799	0.7952	1.0839
Autism vs. Control	0.1960	0.09114	**0.0352**	0.8730

[a]- Least squared means were calculated from ΔΔCt values (ΔCt^{autism}-$\Delta Ct^{control}$ or $\Delta Ct^{1st\ genotype}$-$\Delta Ct^{2nd\ genotype}$) after adjusting for significant covariates

[b]- Standard error for estimated difference

[c]- Tukey-Kramer test was performed considering all the significant covariates. Significant values (5% cut-off) are in bold.

[d]- Fold change of each first group versus the second is calculated as $2^{-(least\ squared\ mean)}$.

Table S12. *CNPY1* levels comparisons based on genotypes or affection status

Comparison	Least squared mean[a]	Standard errors[b]	P-value[c]	Fold change[d]
AC/AC vs. AC/GT	-0.06545	0.1684	0.9202	1.0464
AC/AC vs. GT/GT	0.5877	0.1758	**0.0037**	0.6654
AC/GT vs. GT/GT	0.6531	0.1781	**0.0013**	0.6359
Autism vs. Control	-0.07337	0.1675	0.6627	1.0522

[a]- Least squared means were calculated from ΔΔCt values (ΔCt^{autism}-$\Delta Ct^{control}$ or $\Delta Ct^{1st\ genotype}$-$\Delta Ct^{2nd\ genotype}$) after adjusting for significant covariates

[b]- Standard error for estimated difference

[c]- Tukey-Kramer test was performed considering all the significant covariates. Significant values (5% cut-off) are in bold.

[d]- Fold change of each first group versus the second is calculated as $2^{-(least\ squared\ mean)}$.

SUPPLEMENTAL FIGURES

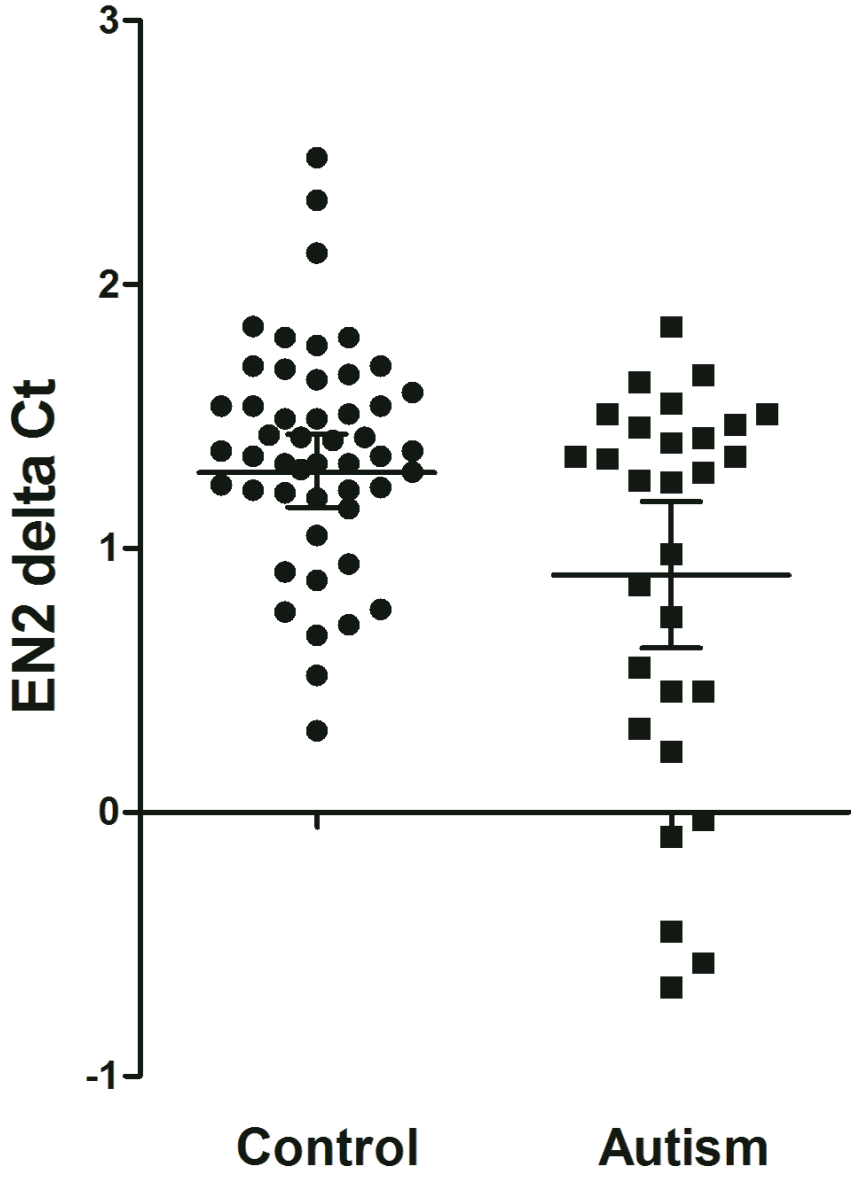

Figure S1. Distribution of *EN2* levels in control and affected cerebellar post-mortem samples

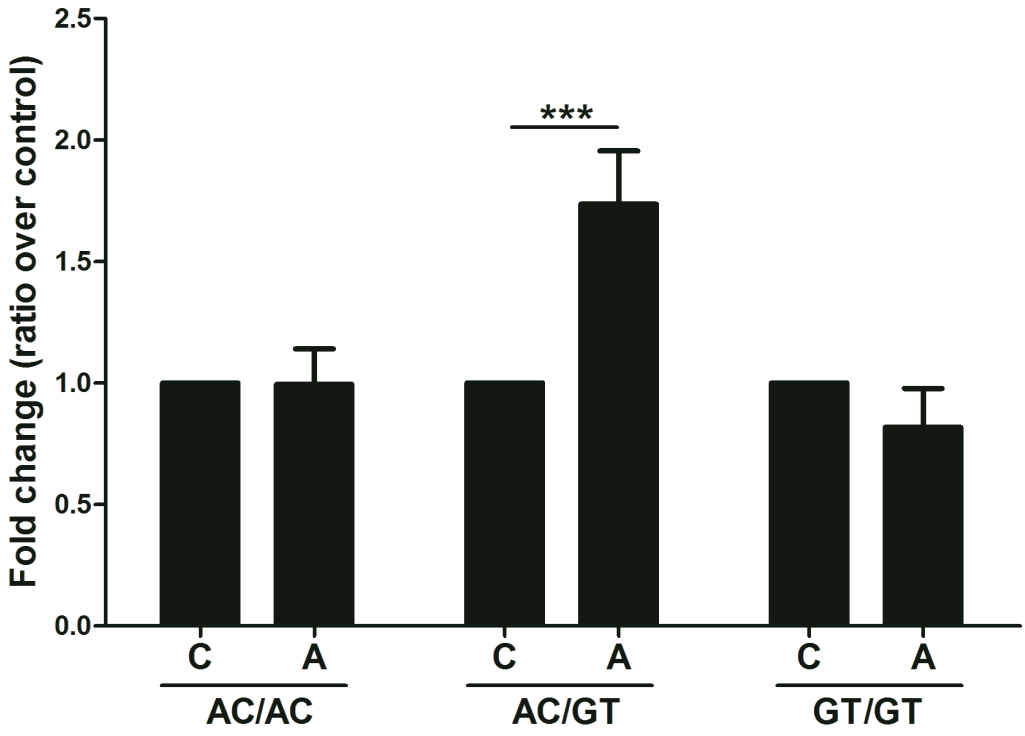

Figure S2. ***EN2*** **levels are elevated in affected individuals with an A-C/G-T genotype**

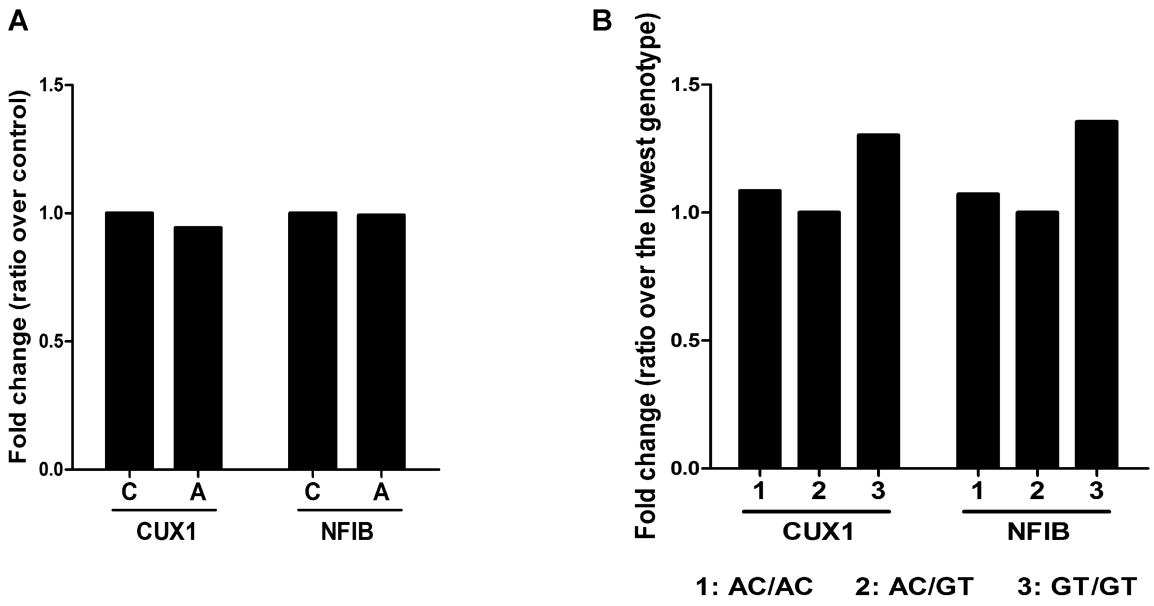

Figure S3. ***CUX1*** **and** ***NFIB*** **levels are not correlated with affection status or** ***rs1861972-rs1861973*** **genotype.**

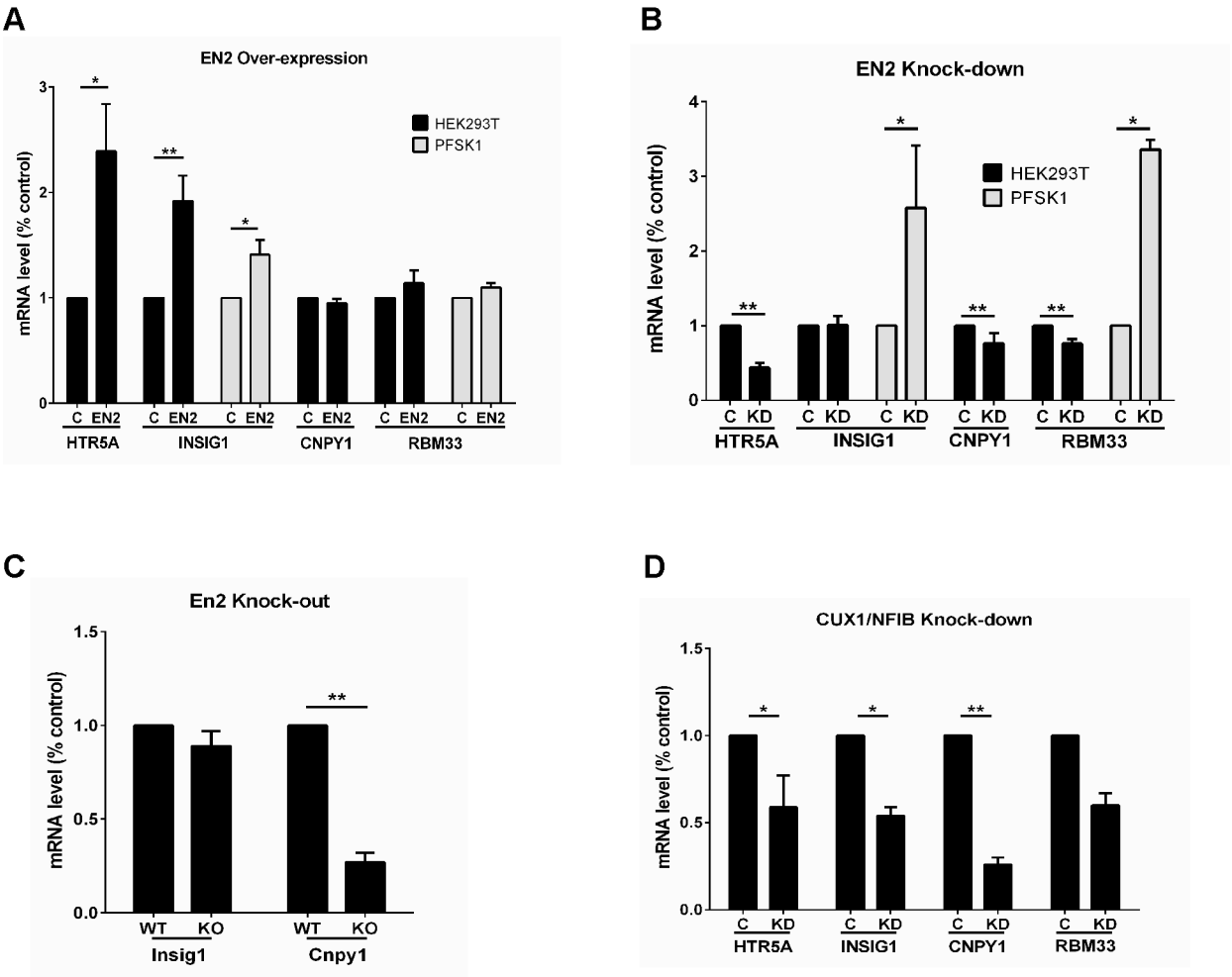

Figure S4. Over-expression, knock-down and knock-out of *EN2* affects *HTR5A*, *INSIG1*, *CNPY1* and *RBM33* mRNA levels in an inconsistent way

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Distribution of *EN2* levels in control and affected cerebellar post-mortem samples

The distribution of normalized *EN2* mRNA levels (DCt) is shown for 29 control and 49 affected cerebellar post-mortem sample. The solid line illustrates the geometric mean and error bars denote 95% confidence intervals. Lower DCt values correspond to higher expression levels

Figure S2. *EN2* levels are elevated in affected individuals with an A-C/G-T genotype

Because of the interaction between genotype and affection status, *EN2* levels were compared between genotypes (A-C/A-C, A-C/G-T, G-T/G-T) in control and autism groups separately. In each group *EN2* levels are normalized to 1 for the control group and presented as fold change for the affected individuals. Fold difference was calculated based on ΔΔCt values. Type 3 tests of fixed effects, *P<.05, **P<.01, ***P<.001, ****P<.0001. A – affected, C – control, AC/AC – individuals homozygous for the *rs1861972-rs1861973* A-C haplotype, GT/GT – individuals homozygous for the G-T haplotype, AC/GT – individuals heterozygous for the A-C/G-T haplotype.

Figure S3. *CUX1* and *NFIB* levels are not correlated with affection status or *rs1861972-rs1861973* genotype.

mRNA levels of *CUX1* and *NFIB* were measured using Taqman qRT-PCR. No interaction between affection status and genotype was observed so comparisions between autism and control group were

made regardless of genotype (A) and between the three genotypes regardless of affection status (B). (A) Transcript levels are presented as fold change in autism versus control groups. Fold difference was calculated using the formula $2^{-(\text{least squared mean})}$. No significance was observed by Type 3 tests of fixed effects. C – control, A – Autism group. (B) Each gene level is normalized to 1 for the genotype with the lowest quantity (AC/GT for both *CUX1* and *NFIB*). Levels for other genotypes are presented as fold change. Fold difference was calculated using the formula $2^{-(\text{least squared mean})}$. No significance was observed by Type 3 tests of fixed effects.

Figure S4. Over-expression, knock-down and knock-out of *EN2* affects *HTR5A*, *INSIG1*, *CNPY1* and *RBM33* mRNA levels in an inconsistent way

To investigate if *EN2* regulates *HTR5A*, *INSIG1*, *CNPY1* and *RBM33* expression, over-expression, knock-down and knock-out analysis were performed. (A) For over-expression analysis human *EN2* cDNA (EN2) or empty pCMV-Tag3B vectors (C) were transfected transiently into HEK293T (black bars) and PFSK1 (gray bars) cells. All flanking genes are expressed in HEK293T cells while only *INSIG1* and *RBM33* are expressed in PFSK1 cells. mRNA levels were measured by Taqman qRT-PCR and normalized to GAPDH. Relative mRNA levels are presented as fold difference of *EN2* over-expression versus control condition. qRTPCR was performed in triplicate and average ΔCt values were used for statistical analyses. N=8-9, (B) *EN2* knock-down (KD) was achieved by transfecting shRNAmir constructs (Open Biosystems) into HEK293T cells (black bars) and PFSK1 (gray bars) cells. A nonsilencing construct was used as a control (C). mRNA levels were measured as described above. N=4-10. (C) Ventral E10.5 mid-hindbrain junction was dissected from $En2^{+/+}$ and $^{ko/ko}$ littermates. Only *Insig1* and *Cnpy1* are expressed at this age, and mRNA levels were measured by SYBR Green qRT-PCR as described. N=11 (D) Both CUX1 and NFIB bind the *EN2* A-C haplotype and mediate its

transcriptional activator function. To investigate the effect of CUX1 and NFIB on the flanking gene levels, stable double knock-downs (KD) were established in HEK293T cells and analyzed. A non-silencing control cell line (C) was also generated. mRNA levels for each gene were measured using Taqman qRT-PCR N=3. Student T-test, two-tailed, paired, *P<.05, **P<.01, ***P<.001.