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Supplemental Data

Somatic Mutations in *TSC1* and *TSC2* Cause Focal Cortical Dysplasia

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Supplemental figures

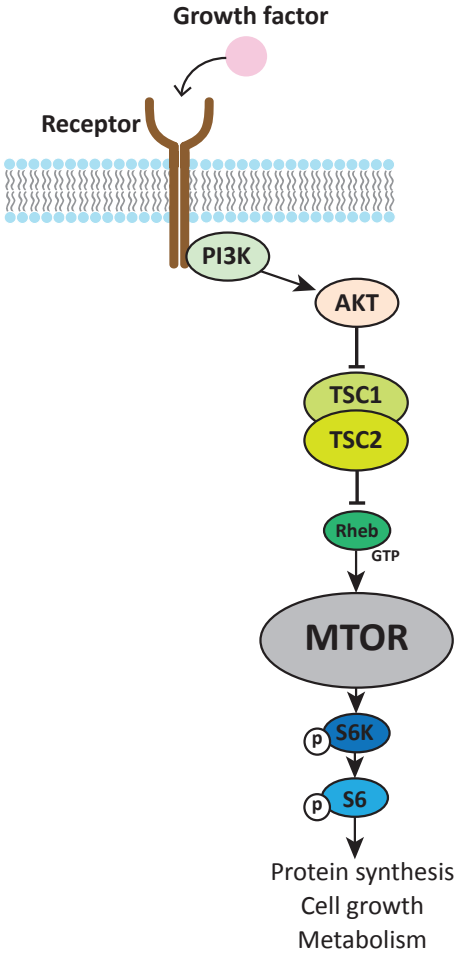


Figure S1. Schematic figure of the PI3K-AKT-TSC-mTOR pathway.

The PI3K-AKT-TSC-mTOR pathway, including downstream phosphorylated ribosomal protein S6 kinase (P-S6K) and phosphorylated ribosomal protein S6 (P-S6), is presented. The *PIK3*, *AKT* and *TSC* genes encode well-known regulators of the mTOR signaling. The TSC1/TSC2 complex regulates mTOR activity via hydrolysis of Rheb-GTP protein. P-S6K and P-S6 protein are the major readout of mTOR pathway activation in protein synthesis, cell growth and metabolism.

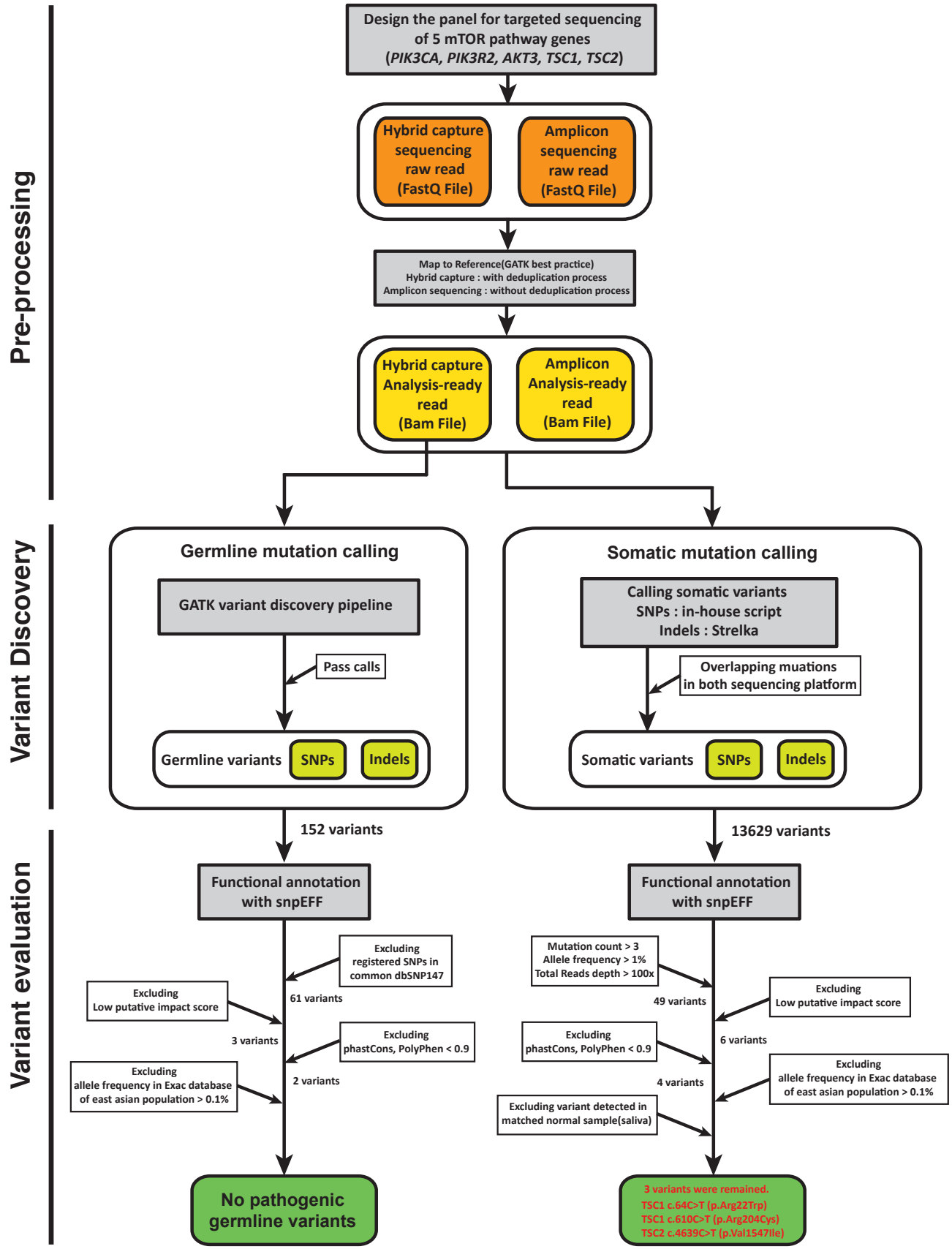
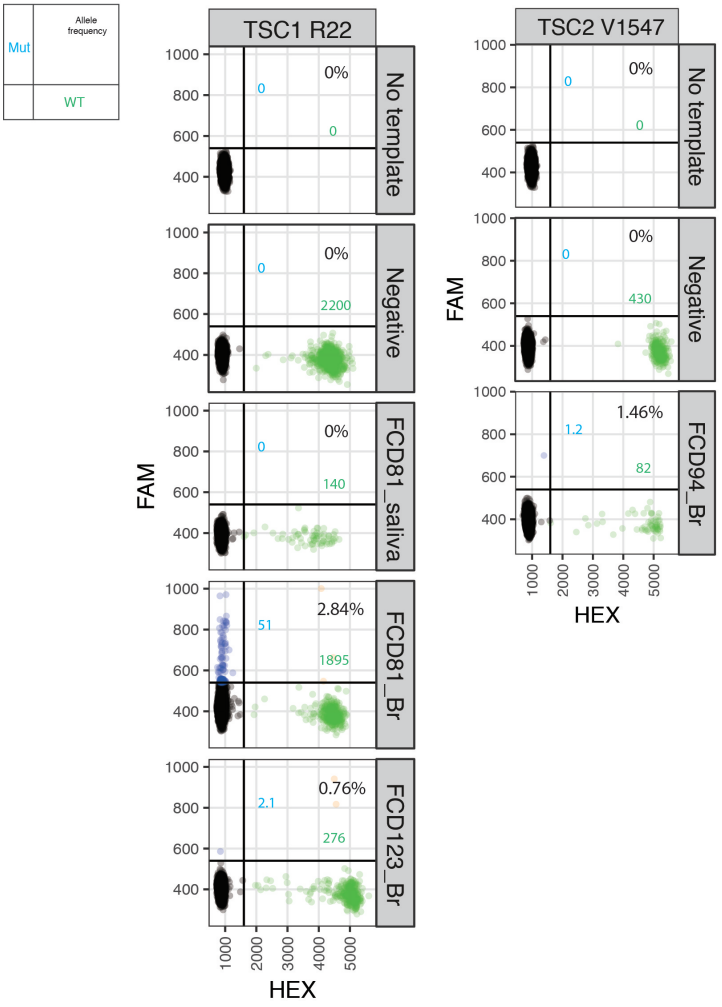

Pre-processing
Variant Discovery
Variant evaluation
Design the panel for targeted sequencing of 5 mTOR pathway genes (PIK3CA, PIK3R2, AKT3, TSC1, TSC2)
Hybrid capture sequencing raw read (FastQ File)
Amplicon sequencing raw read (FastQ File)
Map to Reference(GATK best practice)
Hybrid capture : with deduplication process
Amplicon sequencing : without deduplication process
Hybrid capture Analysis-ready read (Bam File)
Amplicon Analysis-ready read (Bam File)
Germline mutation calling
GATK variant discovery pipeline
Pass calls
Germline variants
SNPs
Indels
Somatic mutation calling
Calling somatic variants
SNPs : in-house script
Indels : Strelka
Overlapping muations in both sequencing platform
Somatic variants
SNPs
Indels
152 variants
13629 variants
Functional annotation with snpEFF
Functional annotation with snpEFF
Excluding registered SNPs in common dbSNP147
61 variants
Excluding Low putative impact score
3 variants
Excluding phastCons, PolyPhen < 0.9
2 variants
Excluding allele frequency in Exac database of east asian population > 0.1%
Mutation count > 3
Allele frequency > 1%
Total Reads depth > 100x
49 variants
Excluding Low putative impact score
6 variants
Excluding phastCons, PolyPhen < 0.9
4 variants
Excluding allele frequency in Exac database of east asian population > 0.1%
Excluding variant detected in matched normal sample(saliva)
No pathogenic germline variants
3 variants were remained.
TSC1 c.64C>T (p.Arg22Trp)
TSC1 c.610C>T (p.Arg204Cys)
TSC2 c.4639C>T (p.Val1547Ile)

Figure S2. Schematic diagram of the bioinformatics pipeline to analyze the targeted deep sequencing data from 5 mTOR pathway genes.

(A) Data for the raw reads data qualified with FastQC was mapped to their correct genomic sequence and analysis-ready reads were analyzed to identify germline and somatic mutations. Because the PCR-based sequencing contained many duplicated reads, we modified the GATK pipeline to exclude deduplication process for mapping the the raw amplicon reads. We used hybrid capture data for the germline calling process. We then excluded 1) registered mutations in the public database (common dbSNP147); 2) mutations with a putative low impact score; 3) mutations with a PolyPhen score <0.9 and phastCons score <0.9; and 4) mutations with allele frequency in ExAC database of east asian population > 0.1%. As a result, No pathogenic germline mutations were found. Somatic single nucleotide variations and indels were called using an in-house script and strelka, respectively. Analysis ready bam files were converted to pileup files using SAMtools (http://samtools.sourceforge.net) and called for mutation/reference count using customized python script. Next, we called the overlapping mutations from both sequencing platforms and annotated the mutation using the snpEFF program.[1] We then excluded 1) mutations with a mutant read <4, allele frequency <1% and total read depth < 100 ×-to remove erroneous calls; 2) mutations with a putative low impact score; 3) mutations with a PolyPhen score <0.9 and phastCons score <0.9; and 4) mutations with allele frequency in ExAC database of east asian population > 0.1%. 5) mutations detected in matched peripheral samples (e.g. saliva). As a result, three mutations were remained for functional validation.

A


Mut
Allele frequency
WT
TSC1 R22
TSC2 V1547
No template
Negative
FCD81_saliva
FCD81_Br
FCD123_Br
FCD94_Br
0%
0
2200
140
2.84%
51
1895
0.76%
2.1
276
1.46%
1.2
82
430
FAM
HEX
1000
2000
3000
4000
5000
400
600
800

B

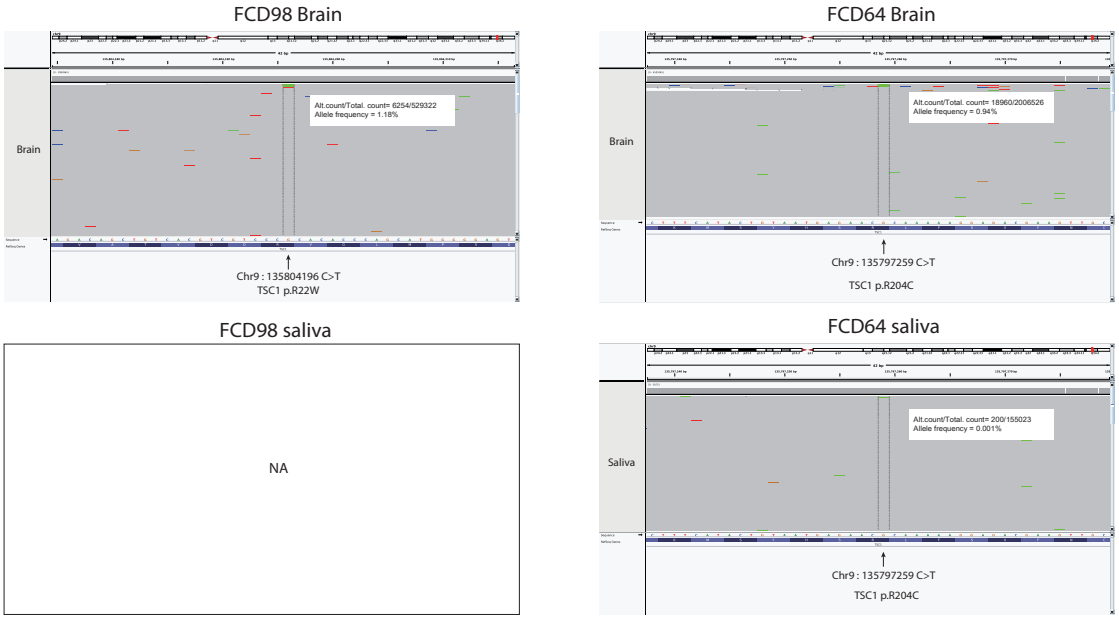

FCD98 Brain
Brain
Alt.count/Total. count= 6254/529322
Allele frequency = 1.18%
Chr9 : 135804196 C>T
TSC1 p.R22W
FCD64 Brain
Alt.count/Total. count= 18960/2006526
Allele frequency = 0.94%
Chr9 : 135797259 C>T
TSC1 p.R204C
FCD98 saliva
NA
FCD64 saliva
Saliva
Alt.count/Total. count= 200/155023
Allele frequency = 0.001%
Chr9 : 135797259 C>T
TSC1 p.R204C

Figure S3. Identified somatic mutations were confirmed by droplet digital PCR (ddPCR) and ultra-high depth amplicon sequencing.

(A) Mutant–specific (FAM, blue signal) and wild–type–specific (HEX, green signal) locked nucleic acid (LNA) probes targeting the site of TSC1 R22 and TSC2 V1547 were used. FAM and HEX signal threshold were determined using no DNA template group ('No template'). Genomic DNA extracted from saliva of healthy individual was used as negative control ('Negative'). Blue or green numbers shown in the plots represent the copy number of mutant or wild-type positive signal calculated by Quantasoft program, respectively. Mut, mutant, WT, wild–type. (B) Visualization of the identified brain somatic mutations in deep amplicon sequencing. Arrow indicate the target locus in integrated genomic view (collapsed mode) of aligned sequencing file. Observed allele frequency of deep sequencing are calculated by dividing altered allele count by total allele count. NA, not available, Alt, altered.

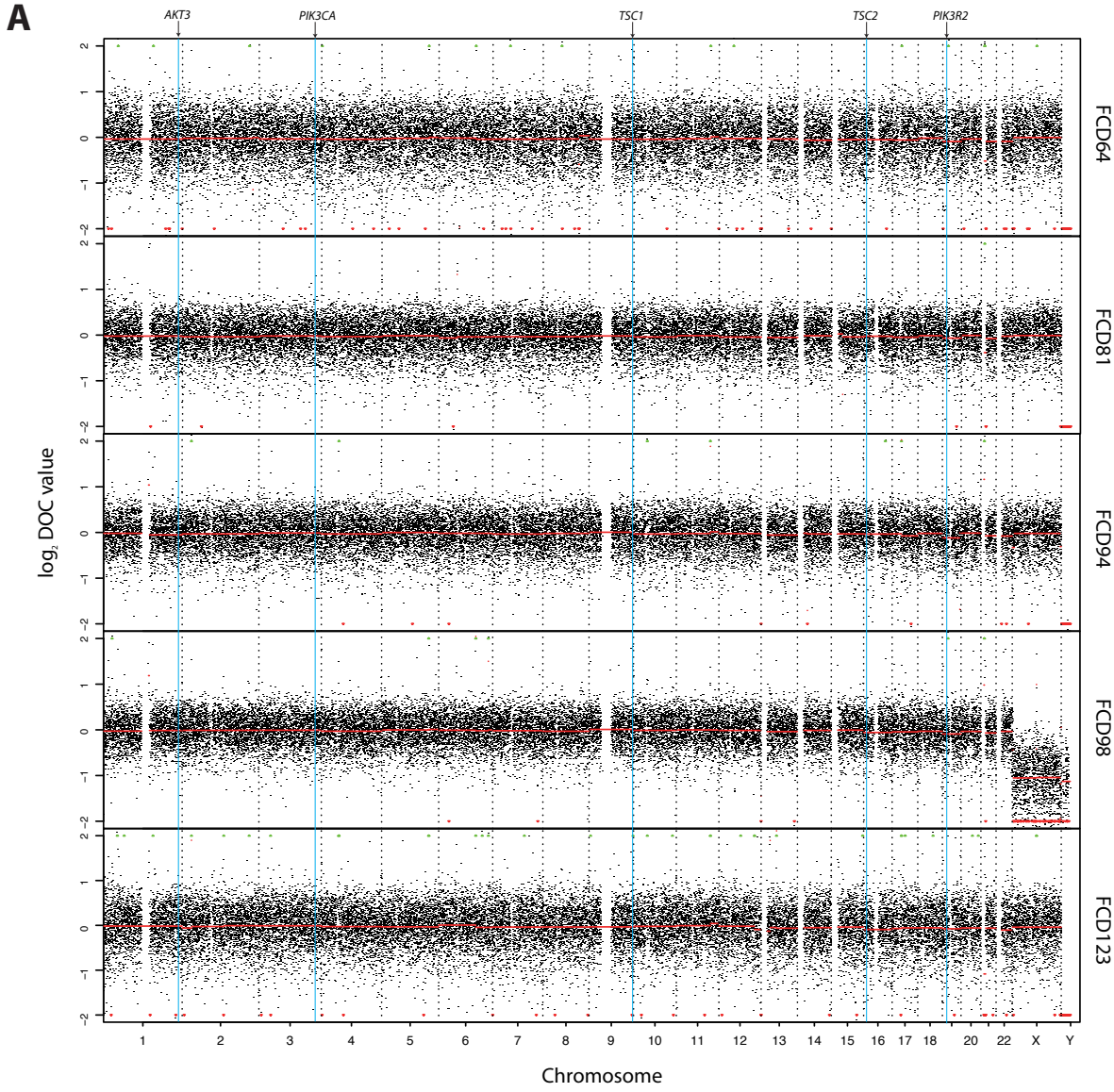

A
AKT3
PIK3CA
TSC1
TSC2
PIK3R2
FCD64
FCD81
FCD94
FCD98
FCD123
log2 DOC value
Chromosome
B
Segmentation value
-0.5
0
0.5
Log2 ratio
AKT3
PIK3CA
TSC1
TSC2
PIK3R2
FCD64
FCD81
FCD94
FCD98
FCD123

Figure S4. No germline CNVs were detected in the individuals carrying *TSC1* or *TSC2* somatic mutations. (A) To detect germline CNVs in 5 individuals carrying somatic mutation on *TSC1* or *TSC2*, targeted panel sequencing data of these individuals were analyzed using copywriteR.[2] We calculated the number of reads that map to genome-wide consecutive 100 kb windows (bins) to generate the depth of coverage (DOC). No definitive large CNV were detected in genome-wide scale. Blue lines indicate the target sites of gene panel including *AKT3, PIK3CA, TSC1, TSC2, PIK3CA*. Segmentation values depicted in red. (B) Segmentation values of 5 individuals on the site of target genes were represented as heatmap.

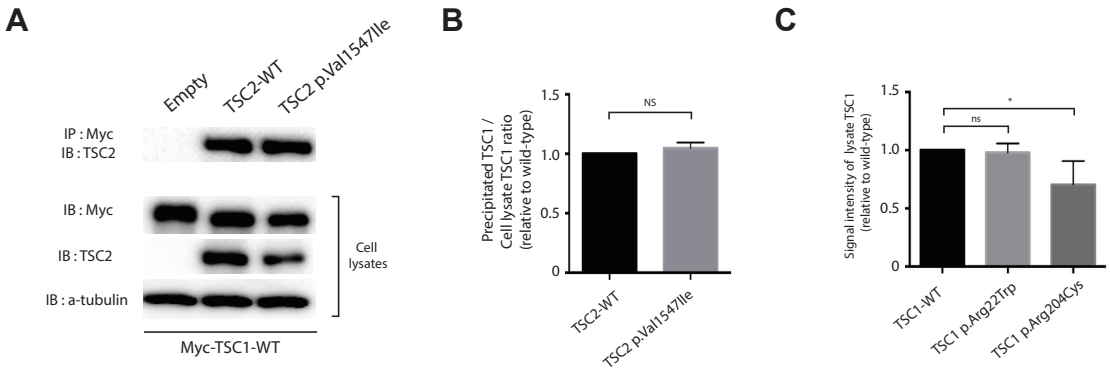
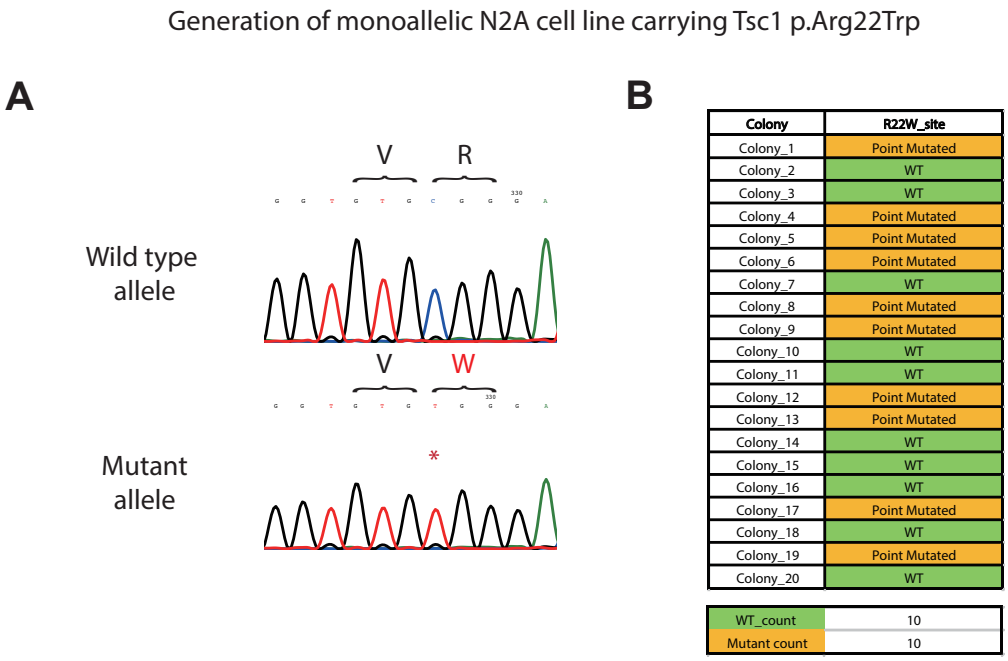


Figure S5. Immunoprecipitation assay of mutant TSC2 protein and wild type TSC1 protein and quantification of the expression of wild type and mutant TSC1 protein in **Figure 2D.**

(A) HEK293T cells were transiently co-transfected with myc-tagged wild-type TSC1 and flag-tagged wild-type TSC2 or mutant TSC2. Lysates were immunoprecipitated with anti-myc antibody and immunoblotted with anti-TSC2 antibody. (B) Quantification of the TSC1 blotting intensity immunoprecipitated with TSC2 antibody. Data are the mean ± SEM (n=4 per group). Student's t-test, compared with wild-type. NS, no significant difference. (C) Quantification of the expression of wild type and mutant TSC1 protein in **Figure 2D**. Data are the mean ± SEM (n=4 per group). **P*<0.05 compared with wild type, Student's t-test. WT, wild type; ns, not significant.

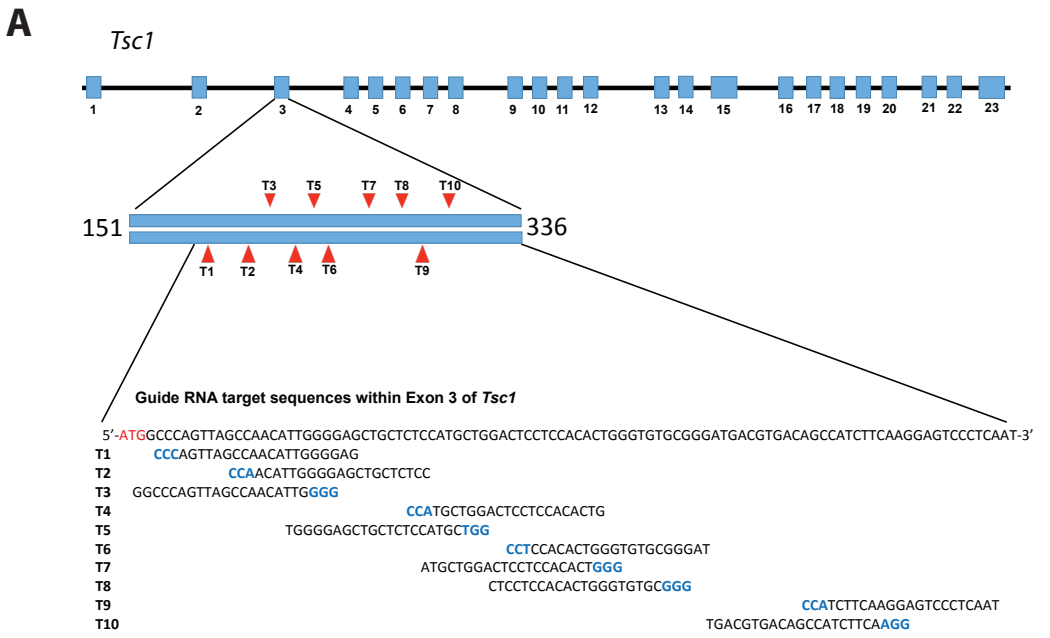


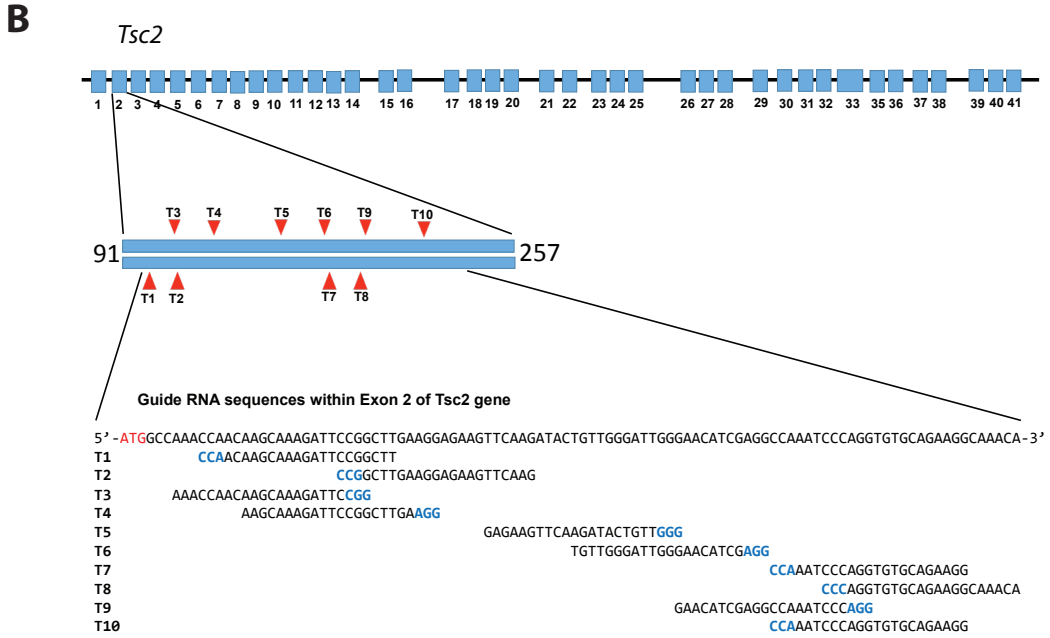
Colony	R22W_site
Colony_1	Point Mutated
Colony_2	WT
Colony_3	WT
Colony_4	Point Mutated
Colony_5	Point Mutated
Colony_6	Point Mutated
Colony_7	WT
Colony_8	Point Mutated
Colony_9	Point Mutated
Colony_10	WT
Colony_11	WT
Colony_12	Point Mutated
Colony_13	Point Mutated
Colony_14	WT
Colony_15	WT
Colony_16	WT
Colony_17	Point Mutated
Colony_18	WT
Colony_19	Point Mutated
Colony_20	WT

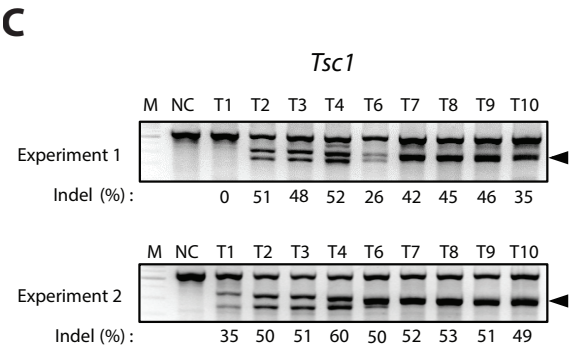
WT_count	10
Mutant count	10

Figure S6. Generation of Neuro2A cell line carrying monoallelic TSC1 p.Arg22Trp mutation.

(A) The Sanger sequencing of TSC1 Arg22 site from wild-type and mutant colony, which were sub-cloned from Neuro2A cell line with monoallelic TSC1 p.Arg22Trp mutation. The single point mutation (red asterisk) was generated. It resulted in single amino acid substitution observed in individuals with FCD. V, Valine; R, Arginine; W, Tryptophan. (B) The subcloning and subsequent colony sequencing result of TSC1 p.Arg22 site. The number of wild-type and mutant colony was counted. The result shows that the established Neuro2A clone contain monoallelic TSC1 p.Arg22Trp mutation (heterozygous mutation).


A
Tsc1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
T3 T5 T7 T8 T10
151
336
T1 T2 T4 T6 T9
Guide RNA target sequences within Exon 3 of Tsc1
5'-ATGGCCCAGTTAGCCAACATTGGGGAGCTGCTCTCCATGCTGGACTCCTCCACACTGGGTGTGCGGGATGACGTGACAGCCATCTTCAAGGAGTCCCTCAAT-3'
T1 CCCAGTTAGCCAACATTGGGGAG
T2 CCAACATTGGGGAGCTGCTCTCC
T3 GGCCCAGTTAGCCAACATTGGGG
T4 CCATGCTGGACTCCTCCACACTG
T5 TGGGGAGCTGCTCTCCATGCTGG
T6 CCTCCACACTGGGTGTGCGGGAT
T7 ATGCTGGACTCCTCCACACTGGG
T8 CTCCTCCACACTGGGTGTGCGGG
T9 CCATCTTCAAGGAGTCCCTCAAT
T10 TGACGTGACAGCCATCTTCAAGG


B
Tsc2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 35 36 37 38 39 40 41
T3 T4 T5 T6 T9 T10
91
257
T1 T2 T7 T8
Guide RNA sequences within Exon 2 of Tsc2 gene
5'-ATGGCCAAACCAACAAGCAAAGATTCCGGCTTGAAGGAGAAGTTCAAGATACTGTTGGGATTGGGAACATCGAGGCCAAATCCCAGGTGTGCAGAAGGCAAACA-3'
T1 CCAACAAGCAAAGATTCCGGCTT
T2 CCGGCTTGAAGGAGAAGTTCAAG
T3 AAACCAACAAGCAAAGATTCCGG
T4 AAGCAAAGATTCCGGCTTGAAGG
T5 GAGAAGTTCAAGATACTGTTGGG
T6 TGTTGGGATTGGGAACATCGAGG
T7 CCAAATCCCAGGTGTGCAGAAGG
T8 CCCAGGTGTGCAGAAGGCAAACA
T9 GAACATCGAGGCCAAATCCCAGG
T10 CCAAATCCCAGGTGTGCAGAAGG


C
Tsc1
M NC T1 T2 T3 T4 T6 T7 T8 T9 T10
Experiment 1
Indel (%): 0 51 48 52 26 42 45 46 35
M NC T1 T2 T3 T4 T6 T7 T8 T9 T10
Experiment 2
Indel (%): 35 50 51 60 50 52 53 51 49

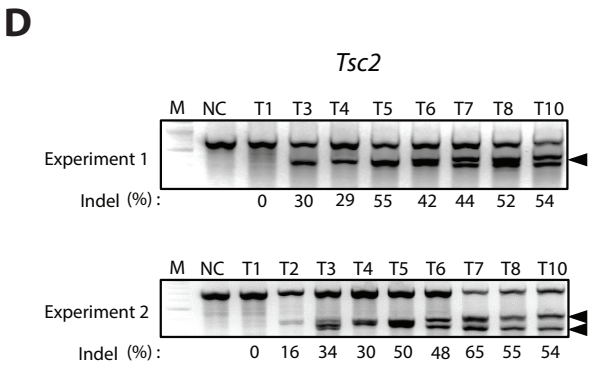
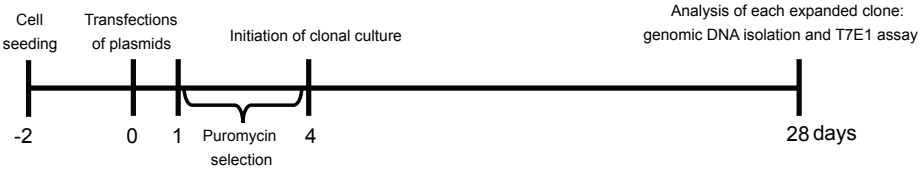

D
Tsc2
M NC T1 T3 T4 T5 T6 T7 T8 T10
Experiment 1
Indel (%): 0 30 29 55 42 44 52 54
M NC T1 T2 T3 T4 T5 T6 T7 T8 T10
Experiment 2
Indel (%): 0 16 34 30 50 48 65 55 54

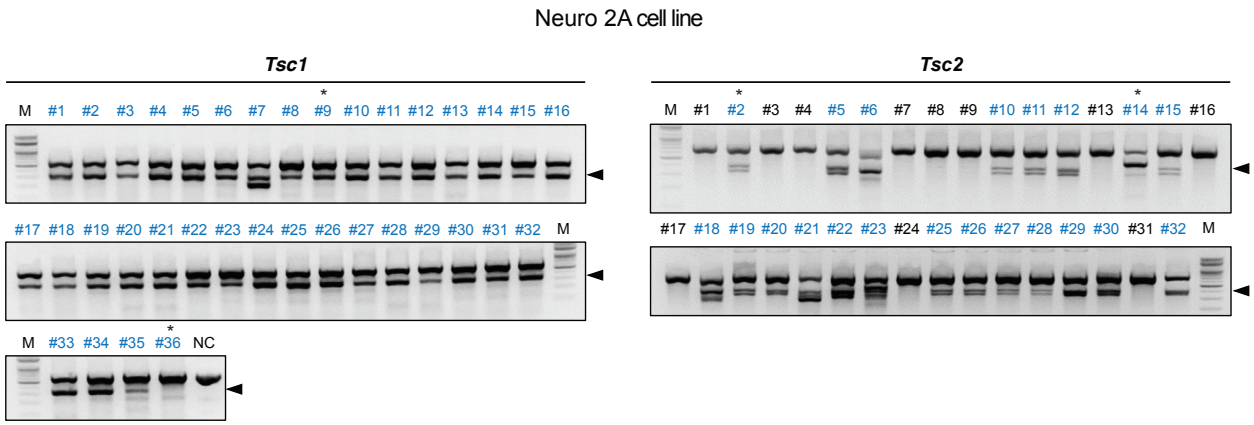
Figure S7. Selection of sgRNAs targeting the mouse *Tsc1* and *Tsc2* genes.

(A-B) Schematic of the Cas9-targeting sites in the mouse *Tsc1* (A) and *Tsc2* (B) locus with the designed sgRNAs (T1-T10) targeting sequences in exon 3 of *Tsc1* (A) or exon 2 of *Tsc2* (B). Blue boxes represent exons. Red arrowheads indicate the position of the sgRNA targeting top (T1, T2, T4, T6, and T9 for *Tsc1*; T1, T2, T7 and T8 for *Tsc2*) and bottom (T3, T5, T7, T8, and T10 for *Tsc1*; T3, T4, T5, T6, T9 and T10 for *Tsc2*) strands. PAM sequences are indicated in bold blue fonts. (C-D) The cleavage efficiency of sgRNAs targeting *Tsc1* (C) and *Tsc2* (D) were determined using T7E1 assays in Neuro2A cells following transfection with plasmids encoding Cas9 and sgRNAs. The results of two independent experiments are shown. The size markers (M) are shown on the left. Arrowheads indicate the expected positions of the DNA bands cleaved by T7E1. The numbers at the bottom of the gel indicate mutation percentages measured by band intensities. Untransfected cells served as the negative control (NC).

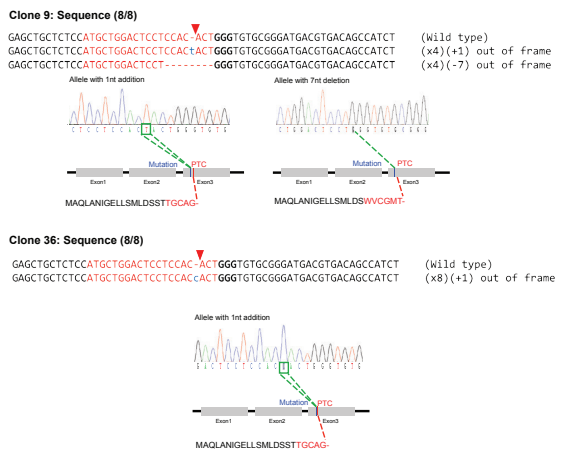
A


Cell seeding
Transfections of plasmids
Initiation of clonal culture
Analysis of each expanded clone: genomic DNA isolation and T7E1 assay
-2
0
1
Puromycin selection
4
28 days

B


Neuro 2A cell line
Tsc1
Tsc2
M #1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13 #14 #15 #16
#17 #18 #19 #20 #21 #22 #23 #24 #25 #26 #27 #28 #29 #30 #31 #32 M
M #33 #34 #35 #36 NC

C


Tsc1 -knockout clones from Neuro 2A cells
Clone 9: Sequence (8/8)
GAGCTGCTCTCCATGCTGGACTCCTCCAC-ACTGGGTGTGCGGGATGACGTGACAGCCATCT (Wild type)
GAGCTGCTCTCCATGCTGGACTCCTCCACtACTGGGTGTGCGGGATGACGTGACAGCCATCT (x4)(+1) out of frame
GAGCTGCTCTCCATGCTGGACTCCT--------GGGTGTGCGGGATGACGTGACAGCCATCT (x4)(-7) out of frame
Allele with 1nt addition
Allele with 7nt deletion
Mutation
PTC
MAQLANIGELLSMLDSSTTGCAG-
MAQLANIGELLSMLDSWVCGMT-
Clone 36: Sequence (8/8)
GAGCTGCTCTCCATGCTGGACTCCTCCAC-ACTGGGTGTGCGGGATGACGTGACAGCCATCT (Wild type)
GAGCTGCTCTCCATGCTGGACTCCTCCACcACTGGGTGTGCGGGATGACGTGACAGCCATCT (x8)(+1) out of frame
Allele with 1nt addition
MAQLANIGELLSMLDSSTTGCAG-

D

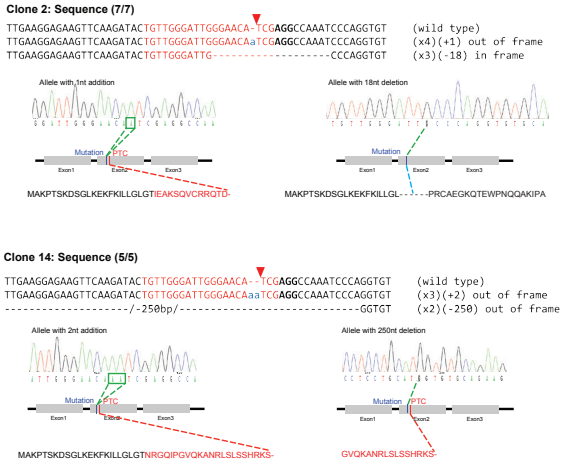

Tsc2 -knockout clones from Neuro 2A cells
Clone 2: Sequence (7/7)
TTGAAGGAGAAGTTCAAGATACTGTTGGGATTGGGAACA-TCGAGGCCAAATCCCAGGTGT (wild type)
TTGAAGGAGAAGTTCAAGATACTGTTGGGATTGGGAACAaTCGAGGCCAAATCCCAGGTGT (x4)(+1) out of frame
TTGAAGGAGAAGTTCAAGATACTGTTGGGATTG----------CCCAGGTGT (x3)(-18) in frame
Allele with 1nt addition
Allele with 18nt deletion
Mutation
PTC
MAKPTSKDSGLKEKFKILLGLGTIEAKSQVCRRQTD-
MAKPTSKDSGLKEKFKILLGL---PRCAEGKQTEWPNQQAKIPA
Clone 14: Sequence (5/5)
TTGAAGGAGAAGTTCAAGATACTGTTGGGATTGGGAACA--TCGAGGCCAAATCCCAGGTGT (wild type)
TTGAAGGAGAAGTTCAAGATACTGTTGGGATTGGGAACAaaTCGAGGCCAAATCCCAGGTGT (x3)(+2) out of frame
----------/-250bp/----------GGTGT (x2)(-250) out of frame
Allele with 2nt addition
Allele with 250nt deletion
Mutation
PTC
MAKPTSKDSGLKEKFKILLGLGTNRGQIPGVQKANRLSLSSHRKS-
GVQKANRLSLSSHRKS-

Figure S8. Generation of *Tsc1*- and *Tsc2*-mutated Neuro2A cell lines.

(A) Schematic representation illustrating the process used to generate clones carrying the *Tsc1*- and *Tsc2*-mutation. Clonal culture of Neuro2A cells was initiated 4 days after transfection with plasmids encoding Cas9 and sgRNAs targeting *Tsc1* and *Tsc2*. Transfected cells were enriched by puromycin treatment for 3 days. Genomic DNA from each clone was analyzed 24 days after the initiation of the clonal culture. (B) T7E1-based clonal analysis. The genomic DNA isolated from each clone was subjected to the T7E1 assay. Arrowheads indicate the expected position of the DNA bands cleaved by T7E1. Untransfected cells served as the negative control (NC). Clones that were successfully cleaved by T7E1 are indicated in blue font. M, size markers. (C-D) The *Tsc1* gene sequences from Neuro2A clones containing heterozygous (clone #9) and homozygous (clone #36) biallelic mutations in exon 3 (C). The *Tsc2* gene sequences from Neuro2A clones containing heterozygous biallelic mutations in exon 2 (D). The sgRNA recognition sites and protospacer adjacent motif are shown in red and bold font, respectively. Deleted bases are indicated by dashes, and inserted bases are shown in a small blue font. The number of occurrences is shown in parentheses (for example, × 4 and × 8 indicate the number of each sequence). The number of deleted and inserted bases is described in the parentheses on the right. The sequence and sequencing chromatogram for each allele are shown. The locus of each mutation and the premature termination codon (PTC) generated by the mutation are depicted in schematics of the *Tsc1* or *Tsc2*. Red arrowheads indicate the Cas9 cleavage sites. Clones that contain frame-shifting biallelic mutations without wild type alleles (clone 36 as TSC1 knockout cells and clone 14 as TSC2 knockout cells) were used in the subsequent studies as *Tsc1*- or *Tsc2*-knockout cells.

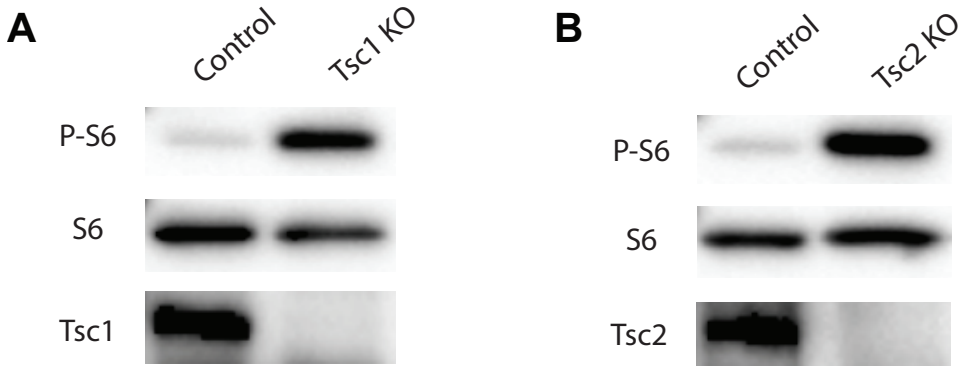


Figure S9. Hyperactivation of the mTOR pathway in *Tsc1*- and *Tsc2*-mutated Neuro2A cells.

(A-B) 2. Neuro2A cells without Cas9 and sgRNA served as the control parental cells. TSC1 and TSC2 were not detected, suggesting each gene were knocked out using the CRIPSR-Cas9 system; the level of phosphorylated S6 increased robustly in both stable cell lines, indicating that mTOR pathway was hyperactivated by disrupting the TSC complex.

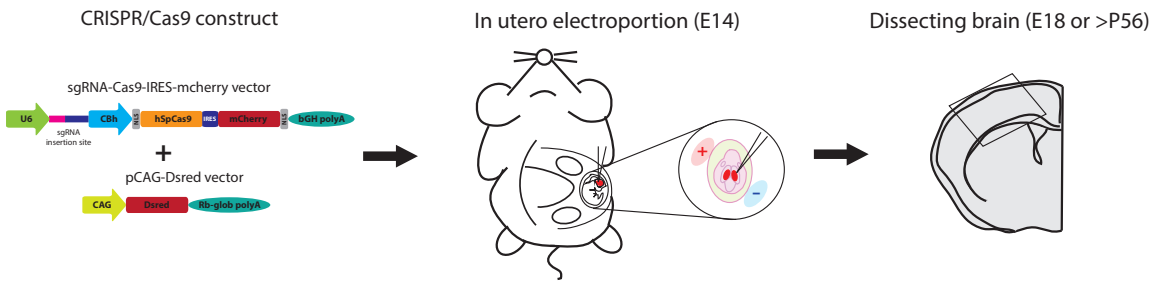


Figure S10. Schematic showing the procedure of *in utero* electroporation at E14 followed by analysis of the brain coronal sections at E18 or after P56.

We electroporated *Tsc1* or *Tsc2* targeted CRISPR-Cas9 constructs (sgRNA-Cas9-IRES-mCherry vector) with a dsRed reporter vector (pCAG-dsRed) into the developing mouse brain at embryonic day (E) 14. Then, we dissected the brain at E18 or postnatal day (P) >56 for subsequent analysis such as migration analysis, colocalization analysis and cell size analysis.

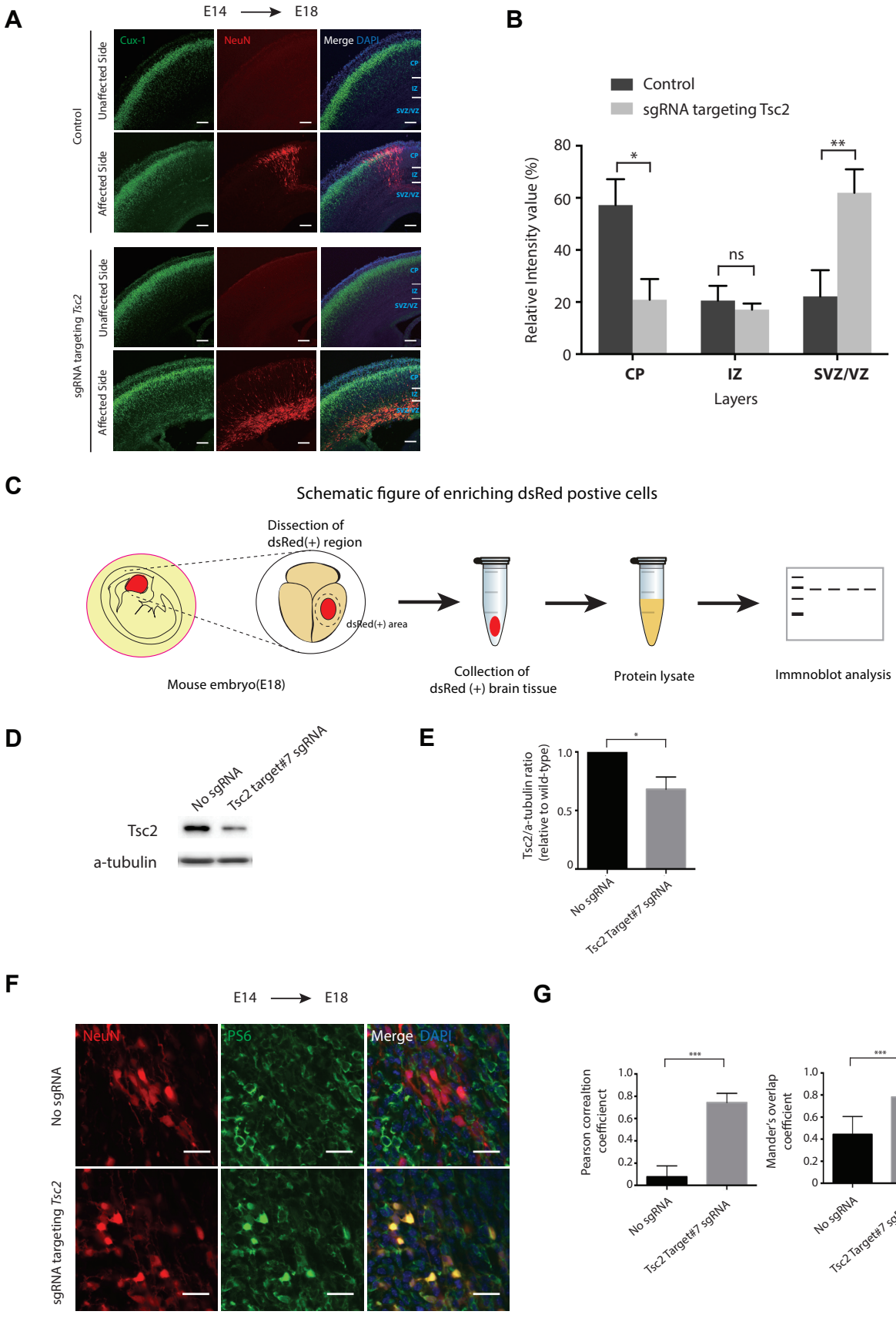

A
E14 ⟶ E18
Control
Unaffected Side
Affected Side
sgRNA targeting Tsc2
Cux-1
NeuN
Merge DAPI
CP
IZ
SVZ/VZ
B
Control
sgRNA targeting Tsc2
Relative Intensity value (%)
80
60
40
20
0
*
ns
**
CP
IZ
SVZ/VZ
Layers
C
Schematic figure of enriching dsRed postive cells
Dissection of
dsRed(+) region
dsRed(+) area
Mouse embryo(E18)
Collection of
dsRed (+) brain tissue
Protein lysate
Immnoblot analysis
D
No sgRNA
Tsc2 target#7 sgRNA
Tsc2
a-tubulin
E
Tsc2/a-tubulin ratio
(relative to wild-type)
1.0
0.5
0
*
No sgRNA
Tsc2 Target#7 sgRNA
F
E14 ⟶ E18
No sgRNA
sgRNA targeting Tsc2
NeuN
PS6
Merge DAPI
G
Pearson correaltion
coefficienct
Mander's overlap
coefficient
1.0
0.8
0.6
0.4
0.2
0
***
No sgRNA
Tsc2 Target#7 sgRNA

Figure S11. The CRISPR construct targeting *Tsc2* is sufficient to disrupt neuronal migration and significantly reduce the expression level of TSC2 protein during the early brain developmental period.

(A) *In utero* electroporation of the CRISPR-Cas9 construct targeting *Tsc2* (sgRNA-Cas9-IRES-mCherry vector) disrupts neuronal migration in the developing mouse neocortex. The images show coronal sections of mouse brains at E18 electroporated with the CRISPR construct. Electroporated mice without sgRNA expression served as the control. Scale bar, 100 μm. (B) The bar charts show the relative fluorescence intensities reflecting the distribution of electroporated cells within the cortex. Data are the mean ± SEM (n=5-6 per group). **P*<0.05 and ***P*<0.01 compared with control, two-way ANOVA with Bonferroni's multiple comparison test. CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular and ventricular zone (C) Schematic figure of enriching dsRed-positive cells in brain tissue. We resected dsRed-positive region of embryonic brain with scalpel and extracted protein lysates for subsequent immunoblot analysis. (D) Immunoblotting for TSC2 and α-tubulin in the embryonic brain lysate with enriched dsRed-positive cells. The TSC2 level was significantly decreased, suggesting that target gene was properly knockout. (E) Quantification of the blotting intensity. Data are the mean ± SEM (n=3 per group). **P*<0.05, Student's t-test. (F) The CRISPR construct targeting *Tsc2* induces the increase of mTOR activity in the developing mouse neocortex. Phosphorylation of S6 protein is significantly increased in dsRed-positive cells carrying CRISPR construct targeting *Tsc2*. (G) Co-localization analysis is performed using Pearson's correalation coefficient and Mander's overlap coefficient. Data are the mean ± SEM (n=6~7 per group). ****P*<0.001, Student's t-test., Scale bar, 20 μm.

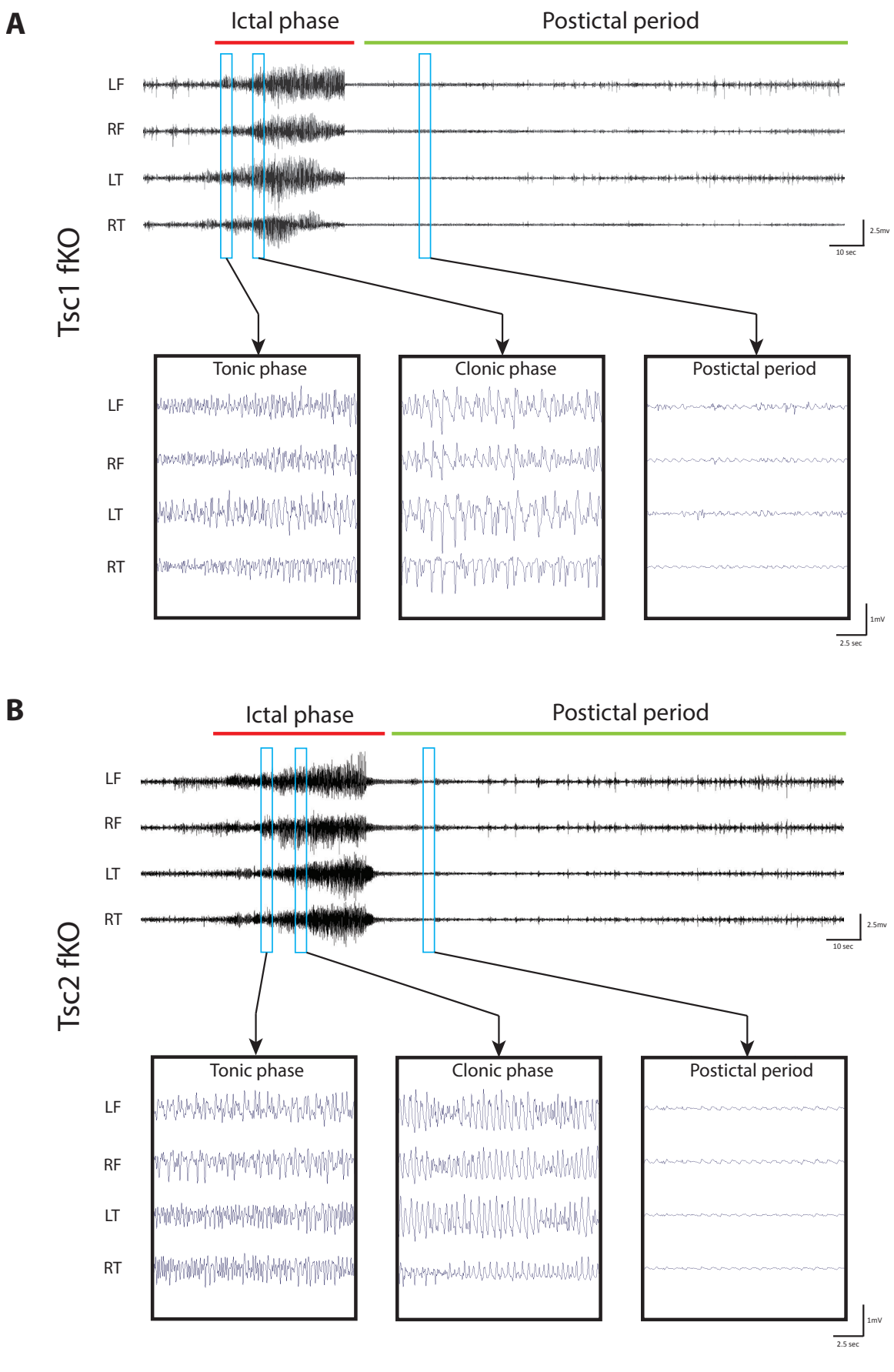

A
Ictal phase
Postictal period
LF
RF
LT
RT
2.5mv
10 sec
Tsc1 fKO
Tonic phase
Clonic phase
Postictal period
LF
RF
LT
RT
1mV
2.5 sec
B
Ictal phase
Postictal period
LF
RF
LT
RT
2.5mv
10 sec
Tsc2 fKO
Tonic phase
Clonic phase
Postictal period
LF
RF
LT
RT
1mV
2.5 sec

Figure S12. EEG wave pattern analysis of the ictal phase and postictal period.

(A-B) The *Tsc1* and *Tsc2* fKO mice showed generalized tonic-clonic seizures consisting of 2 phases (tonic, clonic), followed by postictal attenuation. During the tonic phase, a low amplitude and fast spike were observed with all electrodes, and these waves evolved to exhibit relatively high amplitudes with a spike-wave pattern during te clonic phase. In the postictal period, the wave pattern displayed a diffused attenuation of amplitude. The wave patterns for the EEG signals were recorded from 4 epidural electrodes located on the left frontal lobe (LF), right frontal lobe (RF), left temporal lobe (LT), and right temporal lobe (RT).

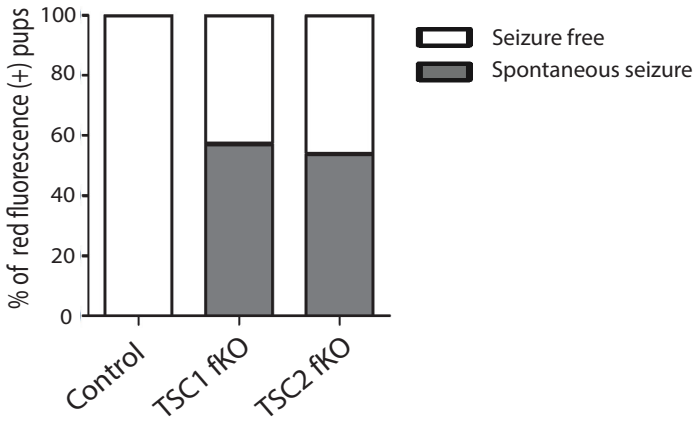


Figure S13. The percentage of mice showing spontaneous seizure after in utero application of the CRISPR-Cas9 system.

Mice showing spontaneous seizures were counted by video-EEG recording (12h per d for > 2 d). n= 6-14 per group.

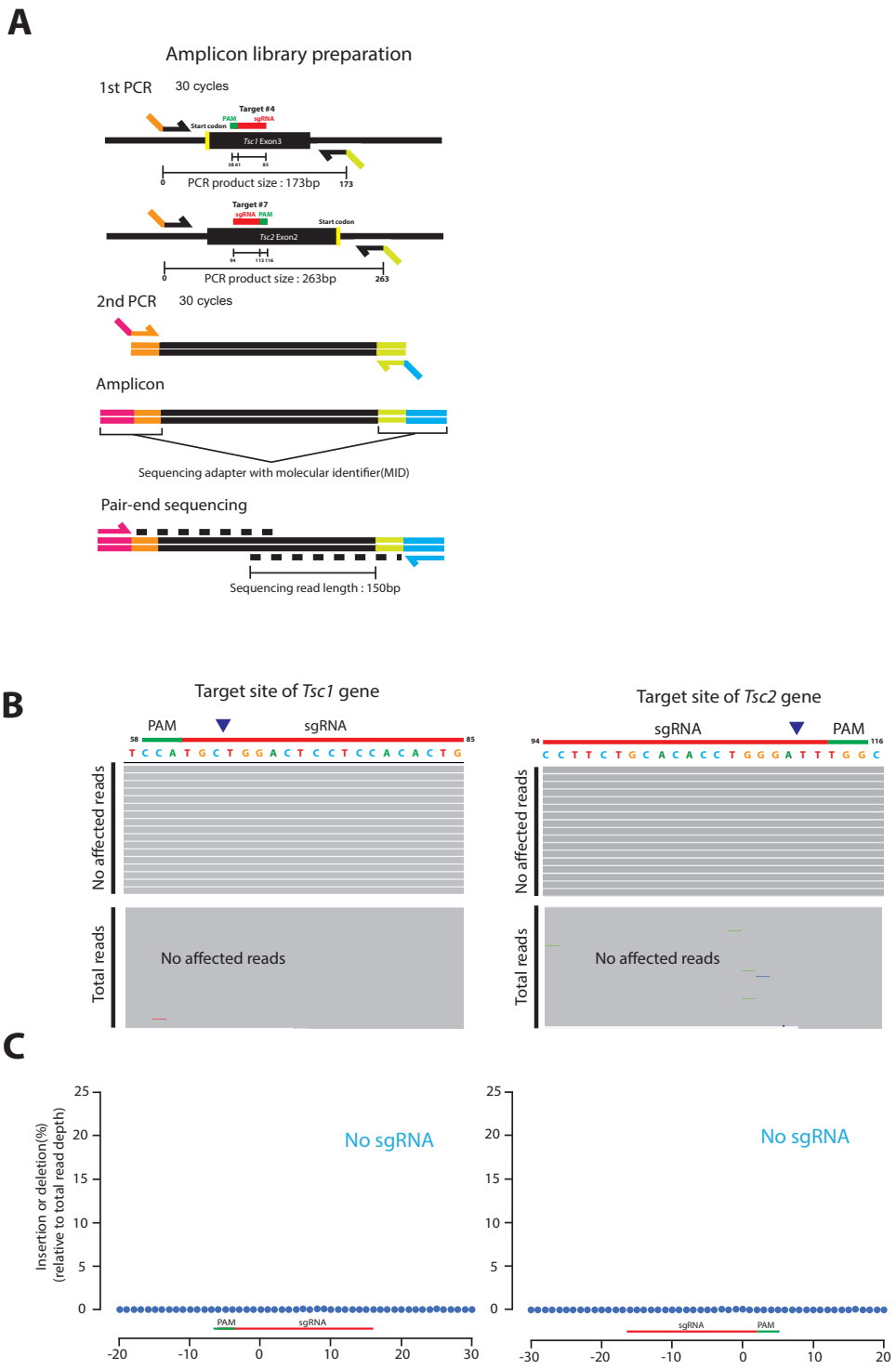

A
Amplicon library preparation
1st PCR
30 cycles
Target #4
PAM
sgRNA
Start codon
Tsc1 Exon3
58 61
85
0
PCR product size : 173bp
173
Target #7
sgRNA PAM
Start codon
Tsc2 Exon2
94
113 116
0
PCR product size : 263bp
263
2nd PCR
30 cycles
Amplicon
Sequencing adapter with molecular identifier(MID)
Pair-end sequencing
Sequencing read length : 150bp
B
Target site of Tsc1 gene
PAM
sgRNA
58
85
T C C A T G C T G G A C T C C T C C A C A C T G
No affected reads
Total reads
No affected reads
Target site of Tsc2 gene
sgRNA
PAM
94
116
C C T T C T G C A C A C C T G G G A T T T G G C
No affected reads
Total reads
No affected reads
C
Insertion or deletion(%)
(relative to total read depth)
25
20
15
10
5
0
No sgRNA
PAM
sgRNA
-20
-10
0
10
20
30
No sgRNA
sgRNA
PAM
-30
-20
-10
0
10
20

Figure S14. The process used for deep amplicon sequencing of CRISPR-Cas9 targeted *Tsc1* and *Tsc2* sites in microdissected cells.

(A) Schematic figure showing the amplicon sequencing process. A two-step PCR protocol was used to amplify amplicons from genomic DNA that contained a molecular identifier (MID) surrounding the predicted cutting site of Cas9. Paired-end sequencing of the amplicon libraries was performed. (B) Integrative Genomic Viewer showing the deep sequenced *Tsc1* and *Tsc2* sites in isolated dsRed- positive neurons from CRISPR-Cas9 electroporated mice without sgRNA expression (control mice). The arrowhead indicates the theoretical cutting site of Cas9. PAM, protospacer adjacent motif. (C) The frequency (relative to total reads) and position of indels are shown. The '0' position indicates the theoretical cutting site of Cas9 guided by sgRNA.

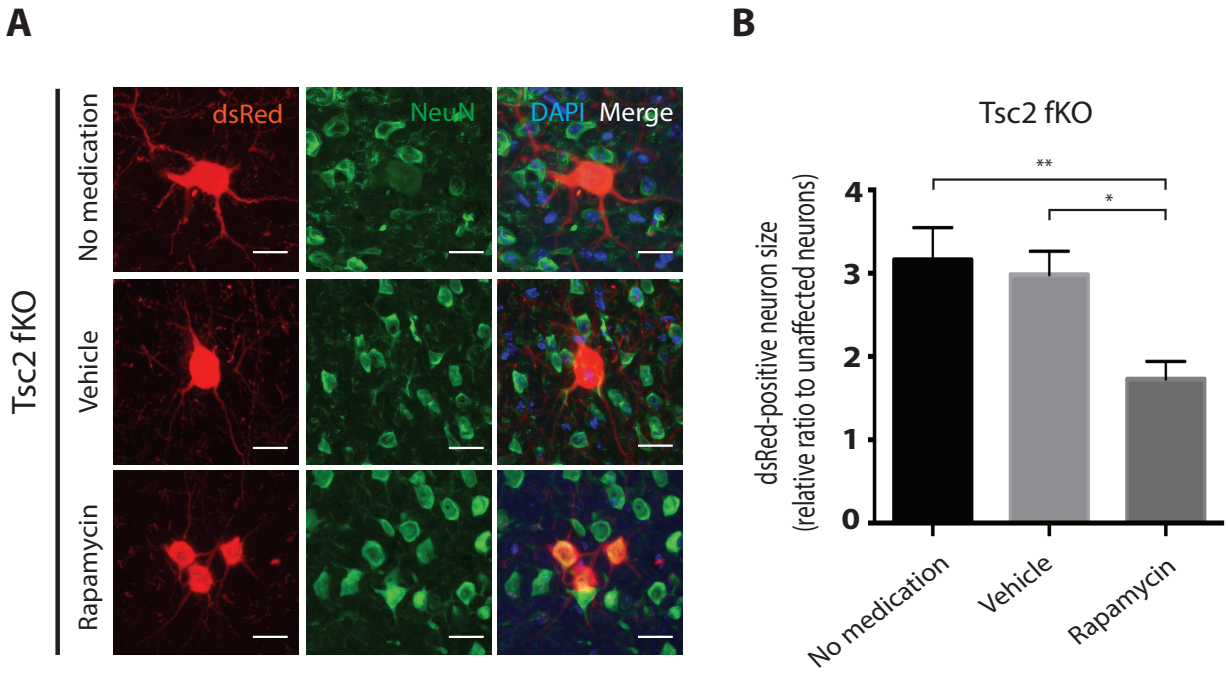


Figure S15. Cytomegalic neurons induced by CRISPR construct targeting *Tsc2* are rescued by rapamycin treatment.

(A) Co-immunostaining for NeuN and DAPI. The soma size of dsRed positive neurons was measured. (B) Bar chart of neuronal size in each group in affected cortical regions. The increased soma size was rescued by rapamycin treatment. Data are the mean ± SEM (n=32-35 per group). **P*<0.05 and ***P*<0.01, compared with dsRed-negative neurons, one-way ANOVA with Bonferroni's post-test. Scale bars, 20 μm.

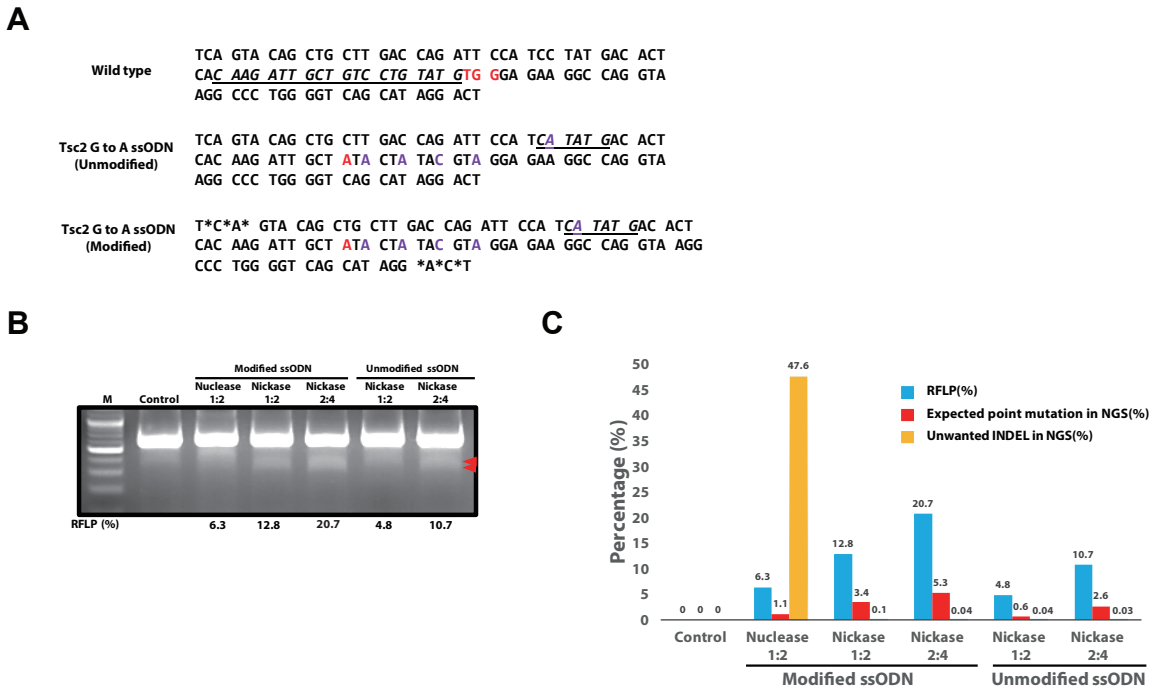


Figure S16. Comparison of homology-directed repair (HDR) efficiency of modified and unmodified ssODN with Nuclease and Nickase for *Tsc2* c.4576G>A (p.Val1526Ile) using RFLP assay and deep sequencing.

(A) Schematic sequence of the 102-mer ssODN's. Wild-type sequence showing guide sequence (italic and underlined letters) and PAM (red and bold letters). 102-mer unmodified and modified ssODN's which includes several silent mutations which are represented in bold purple letters and point mutation is represented in bold red letter. A NdeI restriction enzyme site was incorporated near to guide sequence, is represented in italic and underlined letters, which is absent at the wild type sequence. In 102-mer phosphorothioate modified ssODN which is represented in asterisk at both sides of 5' and 3' end of the ssODN. (B) RFLP-based analysis in Neuro2A cells, the genomic DNA isolated from different transfected conditions of modified and unmodified ssODN with Nuclease and Nickase pooled cells was subjected to the PCR. PCR amplicons were digested with NdeI in an RFLP assay to detect sequences that resulted from homology-directed repair. Arrowheads indicate the expected position of DNA bands cleaved by NdeI digestion. Untransfected cells were used as the negative control. (C) Deep sequencing (NGS) analysis for transfected Neuro2A cells, the graph showing the efficiency of each experimental set.

The set of Nickase & modified ssODN (2:4 ratio) showed the highest percentage of expected point mutation without unwanted indel.

Supplemental tables

Table S1. Molecular genetic information of FCDII individuals enrolled in this study

See the attached excel file Table_S1.xlsx.

Table S2. *Clinical findings related to TSC under the guidance of surveillance recommendations for suspected TSC

Individuals	[a]Genetics	[b]Brain	[c]Kidney	[d]Lung	[e]Skin	[f]Teeth	[g]Heart	[h]Eye
FCD64	No findings	No findings	No findings	No findings	No findings	No findings	No findings	No findings
FCD81	No findings	No findings	No findings	No findings	No findings	No findings	No findings	No findings
FCD94	No findings	No findings	No findings	No findings	No findings	No findings	No findings	No findings
FCD98	No findings	No findings	No findings	No findings	No findings	No findings	No findings	No findings
FCD123	No findings	No findings	No findings	No findings	No findings	No findings	No findings	No findings

*Tuberous Sclerosis Compex Surveilance[3].
[a]Findings for germline TSC1/2 mutations
[b]Findings for cortical tuber, subependymal nodule, subependymal gian cell astrocytoma or cerebral white matter radial migration lines
[c]Findings for renal angiomyolipoma or multiple renal cysts
[d]Findings for lymphangiomyomatosis
[e]Findings for facial angiofibromas, forehead plague, ungual fibroma, hypomelanotic macules, shagreen patch or “Confetti” skin lesions
[f]Findings for multiple pits in dental enamel, gingival fibromas
[g]Finding for cardiac rhabdomyoma
[h]Findings for multiple retinal nodular hamartomas or retinal achromic patch

Table S3. Summary of mutation calls in saliva samples from individuals carrying mutation in this study

Patient ID	Gene	Nucleotide changes	Protein changes	Hybrid capture sequencing				
				Tissue	Platform	% Mutant allele	Ref. count	Mut. Count
FCD064	TSC1	c.610C>T	p.Arg204Cys	Saliva	hybrid Capture	0.12	2605	3
FCD081	TSC1	c.64C>T	p.Arg22Trp	Saliva	hybrid Capture	0.00	122	0
FCD094	TSC2	c.4639G>A	p.Val1547Ile	Saliva	hybrid Capture	0.00	239	0
FCD098	TSC1	c.64C>T	p.Arg22Trp	Saliva	hybrid Capture	NA	NA	NA
FCD123	TSC1	c.64C>T	p.Arg22Trp	Saliva	hybrid Capture	0.44	2709	12
Patient ID	**Gene**	**Nucleotide changes**	**Protein changes**	**PCR amplicon Sequencing**				
				Tissue	**Platform**	**% Mutant allele**	**Ref. count**	**Mut. Count**
FCD064	TSC1	c.610C>T	p.Arg204Cys	Saliva	Amplicon	NA	NA	NA
FCD081	TSC1	c.64C>T	p.Arg22Trp	Saliva	Amplicon	NA	NA	NA
FCD094	TSC2	c.4639G>A	p.Val1547Ile	Saliva	Amplicon	NA	NA	NA
FCD098	TSC1	c.64C>T	p.Arg22Trp	Saliva	Amplicon	NA	NA	NA
FCD123	TSC1	c.64C>T	p.Arg22Trp	Saliva	Amplicon	NA	NA	NA

The following abbreviation is used: Ref, reference, Mut, mutation

Table S4. Summary of mutation calls in brain samples from individuals carrying mutation in this study

Patient ID	Replication	Gene	Nucleotide changes	Protein changes	Hybrid capture sequencing				
					Tissue	Platform	% Mutant allele	Ref. count	Mut. Count
FCD064	Biological,Crossplatform	TSC1	c.610C>T	p.Arg204Cys	Brain FFPE	hybrid Capture	1.75	225	4
FCD081	Biological,Crossplatform	TSC1	c.64C>T	p.Arg22Trp	Brain FFPE	hybrid Capture	2.81	173	5
FCD094	Biological,Crossplatform	TSC2	c.4639G>A	p.Val1547Ile	Brain FFPE	hybrid Capture	1.19	414	5
FCD098	Biological,Crossplatform	TSC1	c.64C>T	p.Arg22Trp	Brain FFPE	hybrid Capture	2.52	155	4
FCD123	Biological,Crossplatform	TSC1	c.64C>T	p.Arg22Trp	Brain FFPE	hybrid Capture	2.21	264	6
Patient ID	**Replication**	**Gene**	**Nucleotide changes**	**Protein changes**	**PCR amplicon Sequencing**				
					Tissue	**Platform**	**% Mutant allele**	**Ref. count**	**Mut. Count**
FCD064	Biological,Crossplatform	TSC1	c.610C>T	p.Arg204Cys	Brain FFPE	Amplicon	1.00	2375.00	24.00
FCD081	Biological,Crossplatform	TSC1	c.64C>T	p.Arg22Trp	Brain FFPE	Amplicon	2.00	441	9
FCD094	Biological,Crossplatform	TSC2	c.4639G>A	p.Val1547Ile	Brain FFPE	Amplicon	1.55	254.00	4.00
FCD098	Biological,Crossplatform	TSC1	c.64C>T	p.Arg22Trp	Brain FFPE	Amplicon	1.98	1038	21
FCD123	Biological,Crossplatform	TSC1	c.64C>T	p.Arg22Trp	Brain FFPE	Amplicon	1.37	794	11

The following abbreviation is used: Ref, reference, Mut, mutation

Table S5. Summary of mutation calls from all tested patients in this study

See the attached excel file Table_S5.xlsx.

Table S6. Functional annotations for detected missense mutations

Patients	Detected Gene	dbSNP(common)	Nucleotide change	Amino acid change	polyPhen	Grantham	PhastCons	GERP	SIFT
FCD64	TSC1	none	c.610C>T	p.Arg204Cys	1	180	1	6.08	0(damaging)
FCD81	TSC1	none	c.64C>T	p.Arg22Trp	0.999	101	0.928	3.21	0.04(damaging)
FCD94	TSC2	none	c.4639G>A	p.Val1547Ile	0.998	112	0.978	4.45	0.04(damaging)
FCD98	TSC1	none	c.64C>T	p.Arg22Trp	0.999	101	0.928	3.21	0.04(damaging)
FCD123	TSC1	none	c.64C>T	p.Arg22Trp	0.999	101	0.928	3.21	0.04(damaging)

Table S7. List of oligonucleotides used for sgRNA construction

Gene	Targets	Direction	Sequence (5' to 3')
Tsc1	**T1**	FP	CCGGCTCCCCAATGTTGGCTAACT
		RP	AAACAGTTAGCCAACATTGGGGAG
	T2	FP	CCGGGGAGAGCAGCTCCCCAATGT
		RP	AAACACATTGGGGAGCTGCTCTCC
	T3	FP	CCGGGGCCCAGTTAGCCAACATTG
		RP	AAACCAATGTTGGCTAACTGGGCC
	T4	FP	CCGGCAGTGTGGAGGAGTCCAGCA
		RP	AAACTGCTGGACTCCTCCACACTG
	T5	FP	CCGGTGGGGAGCTGCTCTCCATGC
		RP	AAACGCATGGAGAGCAGCTCCCCA
	T6	FP	CCGGATCCCGCACACCCAGTGTGG
		RP	AAACCCACACTGGGTGTGCGGGAT
	T7	FP	CCGGATGCTGGACTCCTCCACACT
		RP	AAACAGTGTGGAGGAGTCCAGCAT
	T8	FP	CCGGCTCCTCCACACTGGGTGTGC
		RP	AAACGCACACCCAGTGTGGAGGAG
	T9	FP	CCGGATTGAGGGACTCCTTGAAGA
		RP	AAACTCTTCAAGGAGTCCCTCAAT
	T10	FP	CCGGTGACGTGACAGCCATCTTCA
		RP	AAACTGAAGATGGCTGTCACGTCA
Tsc2	**T1**	FP	CCGGAAGCCGGAATCTTTGCTTGT
		RP	AAACACAAGCAAAGATTCCGGCTT
	T2	FP	CCGGCTTGAACTTCTCCTTCAAGC
		RP	AAACGCTTGAAGGAGAAGTTCAAG
	T3	FP	CCGGAAACCAACAAGCAAAGATTC
		RP	AAACGAATCTTTGCTTGTTGGTTT
	T4	FP	CCGGAAGCAAAGATTCCGGCTTGA
		RP	AAACTCAAGCCGGAATCTTTGCTT
	T5	FP	CCGGGAGAAGTTCAAGATACTGTT
		RP	AAACAACAGTATCTTGAACTTCTC
	T6	FP	CCGGTGTTGGGATTGGGAACATCG
		RP	AAACCGATGTTCCCAATCCCAACA
	T7	FP	CCGGCCTTCTGCACACCTGGGATT
		RP	AAACAATCCCAGGTGTGCAGAAGG
	T8	FP	CCGGTGTTTGCCTTCTGCACACCT
		RP	AAACAGGTGTGCAGAAGGCAAACA
	T9	FP	CCGGGAACATCGAGGCCAAATCCC
		RP	AAACGGGATTTGGCCTCGATGTTC
	T10	FP	CCGGCCAAATCCCAGGTGTGCAGA
		RP	AAACTCTGCACACCTGGGATTTGG

FP: forward primer, RP: Reverse primer

Table S8. The sequence of primers used in the T7E1 assay

Gene	Direction	Sequence (5' to 3')
Tsc1	FP	GCTCTTCCCACTTGAGCCATT
	RP	CAACACCATGCCTGACTGGA
	FP1	AAGCAGAGAGAACGACTCCCA
	RP1	TCTGTTCACAGCAAGAAAACCC
Tsc2	FP	GGTGAGGGGAATTGGTCGTT
	RP	GGAGGACCCAGTCTCACTACT
	FP1	TTCAAGCCCTCCATTGCGAA
	RP1	CGTGCTGAGAGAGTACAGGG

FP: forward primer, RP: Reverse primer

Table S9. The sequence of oligonucleotides used in targeted deep sequencing

Primer		Sequence(5'-3')
Primers 1 for RFLP assay	FP	GGTCCTTGGCCGTAGAGTTT
	RP	CCGGCATTAATAACGGTGGC
Primers 2 for RFLP assay	FP	CAGCTCTACCATTCGCCCTT
	RP	ACCCTCTGTCCTGCCACTTA
Region-specific primers for deep sequencing	FP	ACACTCTTTCCCTACACGACGCTCTTCCGATCT CTGTCTGGGTTGGTGGCTG
	RP	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTCTGACCTACTGCTGAGCC
Primers for Illumina indexing	FP	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC (8bp barcode) ACA CTC TTT CCC TAC ACG AC
	RP	CAA GCA GAA GAC GGC ATA CGA GAT (8bp barcode) GTG ACT GGA GTT CAG ACG TGT

FP: forward primer, RP: Reverse primer

Table S10. The sequence of primers and probes used in ddPCR

Target	Category	Sequence (5' to 3')
TSC1 **R22**	Forward primer	GTGGCTCTAAAGTCAATCTCTTCT
	Reverse primer	CTTCTTGCCATGCTGGACT
	Probe targeting G allele	/5HEX/T+G+C+GGG+A+CGA/3IABkFQ/
	Probe targeting A allele	/56-FAM/T+G+T+GGGA+C+GA/3IABkFQ/
TSC2 **V1547**	Forward primer	CCTCGACCAGATCCCATCATA
	Reverse primer	CCCTGTCCAGGCACCTA
	Probe targeting G allele	/5HEX/C+AGG+A+C+GGC+GA/3IABkFQ/
	Probe targeting A allele	/56-FAM/AC+AGG+A+T+GGC+GA/3IABkFQ/

+ : locked nucleic acid (LNA)

Supplemental References

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3. Northrup, H., Krueger, D.A., International Tuberous Sclerosis Complex Consensus Group (2013). Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 Iinternational Tuberous Sclerosis Complex Consensus Conference. pp. 243–254.