

## 1 REPORT

2 **Threshold of somatic mosaicism leading to brain dysfunction**  
3 **with focal epilepsy**4 Jintae Kim,<sup>1</sup> Sang Min Park,<sup>1,2</sup> Hyun Yong Koh,<sup>3</sup> Ara Ko,<sup>1,4</sup> Hoon-Chul Kang,<sup>5</sup> Won Seok  
5 Chang,<sup>5</sup> Dong Seok Kim<sup>5</sup> and Jeong Ho Lee<sup>1,2</sup>6 **Abstract**

7 Somatic mosaicism in a fraction of brain cells causes neurodevelopmental disorders, including  
8 childhood intractable epilepsy. However, the threshold for somatic mosaicism leading to brain  
9 dysfunction is unknown. In this study, we induced various mosaic burdens in focal cortical dysplasia  
10 type II (FCD II) mice, featuring mTOR somatic mosaicism and spontaneous behavioral seizures. The  
11 mosaic burdens ranged from approximately 1,000 to 40,000 neurons expressing the mTOR mutant in  
12 the somatosensory (SSC) or medial prefrontal (PFC) cortex. Surprisingly, approximately 8,000 to 9,000  
13 neurons expressing the mTOR mutant, which are extrapolated to constitute 0.08-0.09% of total cells or  
14 roughly 0.04% of variant allele frequency (VAF) in the mouse hemicortex, were sufficient to trigger  
15 epileptic seizures. The mutational burden was correlated with seizure frequency and onset, with a higher  
16 tendency for electrographic inter-ictal spikes and beta- and gamma-frequency oscillations in FCD II  
17 mice exceeding the threshold. Moreover, mutation-negative FCD II patients in deep sequencing of their  
18 bulky brain tissues revealed somatic mosaicism of the mTOR pathway genes as low as 0.07% in  
19 resected brain tissues through ultra-deep targeted sequencing (up to 20 million reads). Thus, our study  
20 suggests that extremely low levels of somatic mosaicism can contribute to brain dysfunction.

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**Running title:** Threshold of mosaicism causing epilepsy

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neurodevelopment

## Introduction

Somatic mosaicism (or mutations) which can arise during development or during the aging process, are increasingly recognized as an important genetic causes of brain disorders such as focal epilepsy, autism, schizophrenia, and Alzheimer's disease.<sup>1</sup> While these conditions show widespread brain dysfunction, somatic mutations are found in a small fraction of brain cells such as neurons,<sup>2,3</sup> astrocytes,<sup>4</sup> oligodendrocytes,<sup>5</sup> and microglia,<sup>6</sup> even in specific brain areas. Although a single cell with harmful somatic mutations is unlikely to cause widespread brain dysfunction in those with non-neoplastic neurological diseases, the exact level of somatic mutation burden that disrupts the brain function has remained unexplored thus far.

Focal cortical dysplasia (FCD) is a focal malformation of cortical development and is the most common cause of intractable childhood epilepsy subjected to epilepsy surgery.<sup>7</sup> Among the various subtypes of FCD, FCD type II (FCDII) is the prominent type characterized by cortical malformation involving dyslamination and the presence of dysmorphic neurons<sup>8</sup>. This specific subtype of FCD is primarily

1 attributed to somatic mosaicism, which leads to the abnormal activation of mTOR kinase within the  
2 dysmorphic neurons in the affected focal cortical region.<sup>2</sup> We and other group have demonstrated that  
3 this somatic mosaicism often can be detected at levels as low as 1% of the mutational burden (or variant  
4 allele frequency, VAF) in brain tissues extracted during surgical procedures.<sup>3,9,10</sup>

5 Notably, such a low level of somatic mosaicism is also adequate to induce epileptic seizures in a mouse  
6 model of FCDII, created through the *in utero* electroporation of human mTOR mutant or CRISPR/Cas9  
7 expressing vectors.<sup>2,11,12</sup> However, it is essential to acknowledge the current limitations of sequencing  
8 technology that hinder the detection of low-level (e.g. less than 5%) pathogenic somatic mosaicism in  
9 patient's tissues.<sup>13</sup> Consequently, the precise threshold for somatic mutation that results in overall brain  
10 dysfunction remains uncertain.

## 12 **Materials and methods**

### 13 **Patient ascertainment**

14 This study included two patients with a diagnosis of mutation-negative FCDII in deep gene panel  
15 sequencing,<sup>14</sup> who had undergone epilepsy surgery at Severance Children's Hospital, Yonsei  
16 University College of Medicine. Patients underwent comprehensive evaluation with video-EEG  
17 monitoring, high resolution MRI, fluorodeoxyglucose-positron emission tomography (PET), and  
18 subtraction ictal single-photon emission computed tomography (SPECT) co-registered to MRI to  
19 localize anatomic lesions. All patients met the study entry criteria for FCDII.<sup>15</sup> The pathologic  
20 diagnosis of FCDII was assessed by an experienced neuropathologist and reconfirmed for this  
21 study according to the consensus classification by the International League Against Epilepsy  
22 Diagnostic Methods Commission.<sup>7</sup> This study was performed in accordance with protocols  
23 approved by Severance Hospital and the KAIST Institutional Review Board and Committee on  
24 Human Research, and all human tissues were obtained with informed consent.

## Mouse care and information

C57BL/6 mice (DBL, Korea) were used in the experiments without sex discrimination, and they were housed in isolated cages with free access to food and water, and maintained in a room with a constant temperature of 23°C on a 12-h light-dark cycle with lights off at 7:00 p.m. All of the mice used in this study were healthy with normal immune status and had not been involved in any previous test or drug treatment. All mouse experiments were approved by and performed in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of KAIST.

## Mouse modeling and in vivo phenotype analysis

Model was generated according to previous articles with minor modification.<sup>2,11</sup> Timed pregnant mice (E14.5) were used for generating somatic mosaicism of MTOR variant to one side of the hemicortex. For the behavioral seizure analysis, every mouse involved in this experiment underwent video seizure monitoring for ten consecutive weeks (postnatal day 21~84) to detect seizure onset and frequency. For the EEG analysis, every mouse involved in this experiment also underwent video-EEG monitoring in eight to nine weeks after being born, with data acquired and sampled to study electrographic abnormalities including interictal spikes and the EEG spectra density. Detailed information about *in utero* electroporation and the video and EEG monitoring and analysis conducted here is given in the **Supplementary materials** section.

## Imaging and three-dimensional reconstruction analysis

After *in vivo* monitoring, the mouse brain was perfused, fixed and sliced into 50~60 sections for the subtotal imaging of the whole cortex. After image acquisition, the MATLAB software AMaSiNe was used to reconstruct the three-dimensional brain, calculate the number of mutant cells and automatically annotate each mutant cell onto Allen's brain atlas.<sup>16</sup> Detailed information about the cell counting and volume estimation procedures is given in the **Supplementary materials**.

## Ultra-deep amplicon sequencing analysis

Genomic DNA was extracted from freshly frozen brain sections. Site-specific PCR amplification was done using specific primers to validate the identified mutations. (**Supplementary table 1**). After amplification, products were sequenced on a NovaSeq sequencer (Illumina, USA) to achieve a high depth level. FASTQ files were preprocessed with fastp<sup>17</sup> aligned to the reference genome of hg19 or mm10 and called for expected point mutations or indel read counts. Detailed information about the sample acquisition and library preparation is also described in the **Supplementary materials**

## Statistical analysis

All values in the figures are presented as the mean  $\pm$  SEM. Grouped results were analyzed with Student's t test and ANOVA where appropriate with GraphPad Prism7 (GraphPad Software). Non-linear fitting was done with the logistic curve fitting built-in function:

$$y = A + B / (1 + 10^{C(D-x)}) \quad (1)$$

## Results

### Threshold number of mutation-carrying neurons triggering epileptic seizures in the FCDII mouse model

To investigate the threshold of the somatic mosaicism disrupting brain function, we employed an FCDII mouse model that exhibits mTOR somatic mosaicism in neurons through *in utero* electroporation of the MTOR p.Leu2427Pro plasmid (**Fig 1A**).<sup>2</sup> These mice display spontaneous behavioral seizures during development.<sup>2</sup> By adjusting the amount of injected plasmid within the range of 0.1 to 3  $\mu$ g, we were able to generate a spectrum of mosaic burdens in the FCDII mice, ranging from approximately 1,000 to 40,000 neurons expressing the mTOR mutant, located in either the somatosensory (SSC) or medial prefrontal (PFC) cortex (**Supplementary Fig 1**).

To determine the number, location, and volumes of neurons expressing the mutant mTOR, we analyzed coronal slice images annotated to Allen's brain atlas using AMaSiNe<sup>16</sup> which reconstructs and calculates each neuron's location within the brain's standard coordinate framework (**Fig 1B**). Our analysis revealed a strong correlation between the number of mutant neurons and the volume of the area where these mutant neurons were distributed. Interestingly, behavioral seizures were only observed when there were more than 8,666 mutant neurons in the SSC, spanning an area nearly 10 mm<sup>3</sup> in size in the cortex (**Figs 1C and D**). Similarly, in the PFC, epilepsy development required at least 9,650 mutant neurons (**Figs 1E and F**). Considering that the isocortex in one hemisphere of the mouse consists of nearly ten million cells,<sup>18</sup> our finding suggests that a mere 0.08~0.09% of mutated cells or roughly 0.04% of variant allele frequency (VAF) in the mouse hemicortex, are sufficient to trigger epileptic seizures, disrupting the overall brain function.

## **Relationship between the level of mosaicism and the severity of epileptic seizures**

Next, we investigated whether the number of mutant neurons above the threshold was correlated with the seizure frequency and onset. We identified behavioral seizures by monitoring rearing, falling, and tonic-clonic seizures (Racine score 3-5) through 24-hour video surveillance from P21 to P90 (**Supplementary Fig 2**). Our findings revealed a non-linear sigmoidal relationship between seizure frequency and the number of mutant neurons in the somatosensory cortex (**Fig. 2A**). Furthermore, we observed an inverse correlation between seizure onset and the number of mutant neurons (**Fig. 2B**). A similar pattern was observed in the prefrontal cortex model, with a different plateau for the average seizure frequency (**Figs. 2C and D**).

For electrographic abnormalities, we conducted an EEG analysis and found a higher interictal spike frequency in the SSC of the FCDII mice exceeding the threshold number of mutant neurons (**Figs. 2E and F**). High-frequency oscillations are known to contribute significantly to hyperactive neural field bursts.<sup>19,20</sup> Consistent with this, we observed increased beta and gamma EEG spectral band-power levels during the night (12 PM-1 AM) when the number of mutant neurons exceeded the threshold (**Figs. 2E and G, Supplementary Fig 3**). Taken together, our findings indicate that electrographic abnormalities prominently appear as the number of mTOR mutant neurons is exceeding the threshold and that the

burden of mTOR somatic mosaicism in the cortex is positively correlated with the severity of epileptic seizures.

### **Threshold of somatic mosaicism leading to epileptic seizures under a physiological condition**

Somatic mutations originating from neural stem cells affect both neurons and glial cells during brain development.<sup>21</sup> Notably, prior research identified somatic mutations in MTOR in neurons and glial cells of FCDII patients.<sup>3</sup> In the FCDII model expressing the mosaic mTOR mutant, *in utero* electroporation of mutant plasmids mainly led to the expression of the mutant mTOR protein in neurons,<sup>2</sup> which may underestimate the burden of somatic mosaicism actually present in FCDII patients. To address this concern, we utilized another FCDII mouse model with somatic mosaicism of Tsc2, which leads to mosaic hyperactivation of mTOR kinase in both neural and glial lineages by the CRISPR-Cas9 plasmid (**Fig 3A**).<sup>11</sup> Subsequently, we assessed the presence of behavioral seizures and measured the mutational burden of somatic mosaicism in the affected cortical regions. Given the expected low level of mutational burden in somatic mosaicism across different cell types, we performed ultra-high depth targeted amplicon sequencing with over ten million read depth at the Tsc2 sgRNA target site to measure low-level mutational burden (**Fig 3B**).

As a result, we established FCDII models with Tsc2 somatic mutations, exhibiting various VAFs of indel reads ranging from 0.55% to 1.59%, corresponding to the same level of mutated cells in the resected brain cortex. Interestingly, behavioral seizures were only observed when the percentage of mutated cells in the resected cortex exceeded 1.07% (**Fig 3C**). Therefore, considering the nature of brain somatic mosaicism, where somatic mutations arise from neural stem cells present in both neurons and glial cells during development, our findings suggest that as few as 1% of mutated cells can be sufficient to trigger epileptic seizures and brain dysfunction in FCDII patients.

### **Presence of extremely low-level somatic mosaicism in mutation-negative FCDII**

Finally, we investigated whether such a low level of somatic mosaicism is indeed present in FCDII patients who could not be genetically diagnosed through current deep-sequencing technologies such as deep whole exome or gene panel sequencing with several hundreds to thousands of read depths.

Despite recent updates in the classification and diagnostic criteria for FCDII,<sup>8</sup> for which somatic mosaicism in mTOR pathway genes is now considered as a major genetic cause, approximately 40% of FCDII patients remain genetically unexplained.<sup>3,9</sup> In cases where deep whole exome or gene panel sequencing did not identify somatic mosaicism in mTOR pathway genes in bulk brain tissues, we recently discovered that such mutations were detectable in Fluorescence-Activated Cell Sorting (FACS) enriched cells with mTOR hyperactivation and increased phosphorylated S6 (P-S6).<sup>14</sup> To determine the precise mutational burden in these FCDII patients, we obtained multiple brain tissue samples from two FCDII individuals (FCD213 and FCD167) (**Fig 3D and Supplementary table 2**).

In these patients, enriched P-S6 cells exhibited the AKT3 c.49G>A (p.Glu17Lys) mutation in FCD213 and the MTOR c.6644C>T (p.Ser2215Phe) mutation in FCD167.<sup>14</sup> We conducted ultra-high-depth amplicon sequencing with up to ~19 million read depth in the resected bulk tissues and found VAFs of somatic mosaicism ranging from 0.07% to 0.60%, depending on the sampling site. The average VAF of somatic mutations was 0.25% in FCD213 and 0.50% in FCD167 in the brain. Compared to the error rates of G>A and C>T substitutions at all reference sequences in ultra-deep amplicons sequencing data of bulky brain tissues (**Table 1**) as well as the error rates of G>A and C>T substitution at the same mutation site in matched blood sequencing data (**Supplementary table 3**), VAFs of AKT3 c.49G>A (p.E17K) in FCD213 and MTOR c.6644C>T (p.S2215F) in FCD167 significantly exceeded the error rates, thereby showing that confirmation of under 1% of variant allele frequency could be achieved using ultra high depth sequencing over million read depths.

Given that somatic mutations are typically present in one allele of somatic cells, these mutational burdens correspond to 0.5% of mutated cells in FCD213 and 1% in FCD167. Remarkably, following epilepsy surgery, both patients showed significant level of recovery, underscoring that even this extremely low level of mutation was epileptogenic in these cases. In conclusion, our results demonstrate that even less than 0.5% of somatic mosaicism burden is sufficient to cause widespread brain dysfunction in FCDII patients(**Table 1**).

## Discussion

Here, we show that exceeding a specific threshold of somatic mosaicism leads to widespread brain dysfunction, including epileptic seizures in both mouse models and patients. In the FCDII mouse model, approximately 8,000-9,000 mutant neurons in the cortex, accounting for approximately 0.1% of mouse



isocortical cells,<sup>18</sup> are sufficient to disrupt normal brain function and cause epileptic seizures and electrographic abnormalities. In FCDII patients, even less than 0.5% of the somatic mosaicism burden found in focal epileptogenic tissue is enough to cause widespread brain dysfunction including epileptic seizures.

In this study, we conducted amplicon sequencing with a read depth of more than several million reads to validate the extremely low-level somatic mutations, ranging from 0.07 to 0.60% of the variant allele frequency (VAF), with background error rates of 0.011-0.027% of VAF in our patients. These mutations had been previously detected in deep sequencing of FACS-sorted pS6-positive cells.<sup>14</sup> Unfortunately, typical deep whole-exome sequencing (WES) or panel sequencing of bulk brain tissues is unlikely to achieve such a high level of read depth and detect ultra-low-level somatic mutations with <0.5% of VAF in an unbiased manner. Therefore, if bulk brain tissues of FCDII patients are negative for deep panel sequencing targeting FCDII-relevant genes with >1,000X read depth, as recommended by the International League Against Epilepsy (ILAE),<sup>8</sup> FACS-sorted pS6-positive cells could be subjected to deep panel sequencing followed by ultra-deep targeted amplicon sequencing to validate true ultra-low-level somatic mutations.<sup>14</sup>

In FCD II patients, the extent of brain lesions is known to be correlated with the VAFs of somatic mosaicism.<sup>10</sup> However, the relationship between the mutational burden of somatic mosaicism and the severity of epileptic seizure remains unclear. This study demonstrates that as the mutational burden increases, the epileptic seizure phenotype does not linearly correlate with the mutational burden but follows a sigmoidal pattern, indicating a sudden state transition to daily epileptic seizures above a certain threshold. This may explain why clinical symptoms may not accurately reflect the level of the variant allele frequency, as the severity plateaus within the detectable range of somatic mosaicism.

A somatic mosaicism burden of less than 0.5% is sufficient to trigger widespread brain dysfunction such as epileptic seizures in FCD II patients, as noted above. One potential mechanistic explanation for this low-level threshold is that dysmorphic neurons expressing mTOR mutants in FCD II induce hyperexcitability in nearby non-mutant neurons through non-cell autonomous mechanisms, resulting in focal epileptic seizures.<sup>22</sup> Nevertheless, further studies at the molecular level are needed to understand how this threshold level of mutant carrying neurons can produce widespread abnormal excitability to distant regions, eventually disrupting the whole brain. Moreover, FCD patients show additional neuropsychiatric symptoms including intellectual abnormalities and autistic symptoms.<sup>3</sup> Further studies

would also be needed to find how these few cells could disrupt the circuit-level changes in brain physiology.

Currently used sequencing technologies and analysis algorithms have limitations if used to detect cases of low-level somatic mosaicism, such as a VAF of less than 1%, due to sequencing errors, noises, and artifacts from library preparation.<sup>23</sup> Although the threshold of somatic mosaicism dysregulating brain functions can vary depending on the cell-type, region, and disease phenotype<sup>9,24</sup>, our study emphasizes the need to develop technologies and tools capable of detecting extremely low-level somatic mosaicism in various patients with different types of neurodevelopmental disorders. To address this, the world's largest research initiative on somatic mosaicism, 'Somatic Mosaicism across Human Tissues (SMaHT)<sup>25</sup>' has recently been launched to accelerate the development of technologies and tools capable of discovering somatic mosaicism in the brain.

In summary, our study provides direct evidence of the extremely low-level threshold of somatic mosaicism leading to widespread brain dysfunction in both mice and humans.

## Data availability

Data related to the findings of this study are available within this article and in the **Supplementary materials**. Raw data are available from the author upon reasonable request.

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## Competing interests

J.H.L is a co-founder and the chief scientific officer of SoVarGen.

## Supplementary material

Supplementary material is available at *Brain* online.

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## Figure legends

**Figure 1 Threshold number of mutant neurons in the cortex triggering behavioral seizures.** (A) Schematic figure of the generation of FCDII mouse model with mTOR somatic mosaicism, followed by video seizure monitoring. *In utero* (E14.5) electroporation of MTOR p.Leu2427Pro plasmid induced somatic mosaicism of mutant mTOR overexpression in the mouse brain cortex with epileptic behavioral seizures. Onset and seizure frequency were monitored via video. (B) Schematic figure of the 3D reconstruction of mutant mTOR expressing cells using AMaSiNe. Approximately 60 coronal slices with a thickness of 100um were normalized to Allen's brain atlas database and were utilized to measure cell numbers and volumes in a reconstructed 3D brain. Reconstructed cell coordinates were further binned into 200um cubes to describe the mutant-bearing region. (C) Mutant cells and the volume of carrying region were linearly correlated in the somatosensory cortex. (Linear regression  $R=0.975$ ,  $n=34$ . X : no seizure, open circle: seizure) (D) Example of number and volume of mutant cells with below the threshold (up) and above the threshold (down) in the somatosensory cortex. The axis follows Allen's brain atlas data. (Scale bar x: 500um, y: 1mm) (E) Linear correlation found between mutant cells and the volume of the carrying region in the prefrontal cortex. (Linear regression.  $R=0.925$ ,  $n=25$ . X: no seizure, open circle: seizure) (F) Example of mutant cell carrying area below the threshold (up) and above the threshold (down) in the prefrontal cortex.

**Figure 2 Relationship between the number of mutant neurons, behavioral seizures, and electrographic abnormalities.** (A) A non-linear sigmoidal relationship between mutant cell number and epileptic seizure frequency was found in somatosensory cortex model. (B) A negative linear relationship between mutant cells and epileptic seizure onset was found in a somatosensory cortex model. (C) Relationship between the cell number and seizure frequency in prefrontal cortex model. (D) Relationship between the cell number and seizure onset in prefrontal cortex model. (E) Schematic figure of EEG monitoring in the MTOR model (somatosensory cortex) (F) Differences in the spike frequency below ( $<8,000$ ) and above ( $>8,000$ ) the threshold mutational burden (Unpaired Student t test with

Welch's correction, \*  $p < 0.05$ , Low; n = 8, High; n=11) (G) The relative spectral density during the night-time (24:00~01:00) band-power was calculated in 1-50Hz spectrum. The relative spectral density showed differences in the beta (12~20Hz) and gamma (20~50Hz) spectra during night. (Unpaired Student t test with Welch's correction, \*  $p < 0.05$ , \*\*  $p < 0.01$ )

**Figure 3 Extremely low level of somatic mosaicism under a physiological condition.** (A) Tsc2 CRISPR editing *in utero* electroporation inducing somatic mutation of neuron and glial cells. (B) Multiclonal indels were observed in ultra-high depth amplicon sequencing data of bulky brain tissues carrying Tsc2 somatic mosaicism. (C) Percentage of sequencing reads with indels per total reads in Tsc2 mosaic mutation mice with or without seizures. Non-targeting indels were excluded. (D) Post-operative MRI of two FCD type II patients (FCD213 and FCD167), who were somatic mutation-negative for deep sequencing of their bulky brain tissues<sup>14</sup>. In both patients, somatic mutations in AKT3 or MTOR were detected at low levels in the deep sequencing of FACS enriched pS6(+) cells. In parallel, multiple samples of bulky brain tissues without FACS enrichment were subjected to ultra-high depth amplicon sequencing with up to 20 million read depths.

1 Table I Ultra deep amplicon sequencing of mutation-negative FCDII patients and their surgical outcomes

Surgical outcomes of FCD213 & FCD167					
Patients	Seizure frequency(/day) Before surgery		Seizure frequency(/day) 6 months after surgery		Seizure frequency(/day) 3 years after surgery
FCD213	26		0		0.03
FCD167	20		0		0.01
FCD213 : AKT3 c.49G>A (p.E17K)					
	Total reads	Altered reads	Variant allele frequency (%)	Error rate (Mean± SD %)	P value
FCD213 – i)	3,468,782	8,587	0.248	0.027 ± 0.012	<0.001
FCD213 – ii)	4,046,167	10,937	0.270	0.015 ± 0.005	<0.001
FCD213 – iii)	4,973,972	3,676	0.074	0.016 ± 0.006	<0.001
FCD213 – iv)	4,437,599	16,393	0.370	0.016 ± 0.005	<0.001
FCD167 : MTOR c.6644C>T (p.S2215F)					
	Total reads	Altered reads	Variant allele frequency (%)	Error rate (Mean ± SD %)	P value
FCD167 – i)	16,238,939	55,742	0.343	0.014 ± 0.010	<0.001
FCD167 – ii)	17,378,072	105,270	0.606	0.011 ± 0.006	<0.001
FCD167 – iii)	18,653,180	105,058	0.557	0.011 ± 0.006	<0.001

2  
3  
4



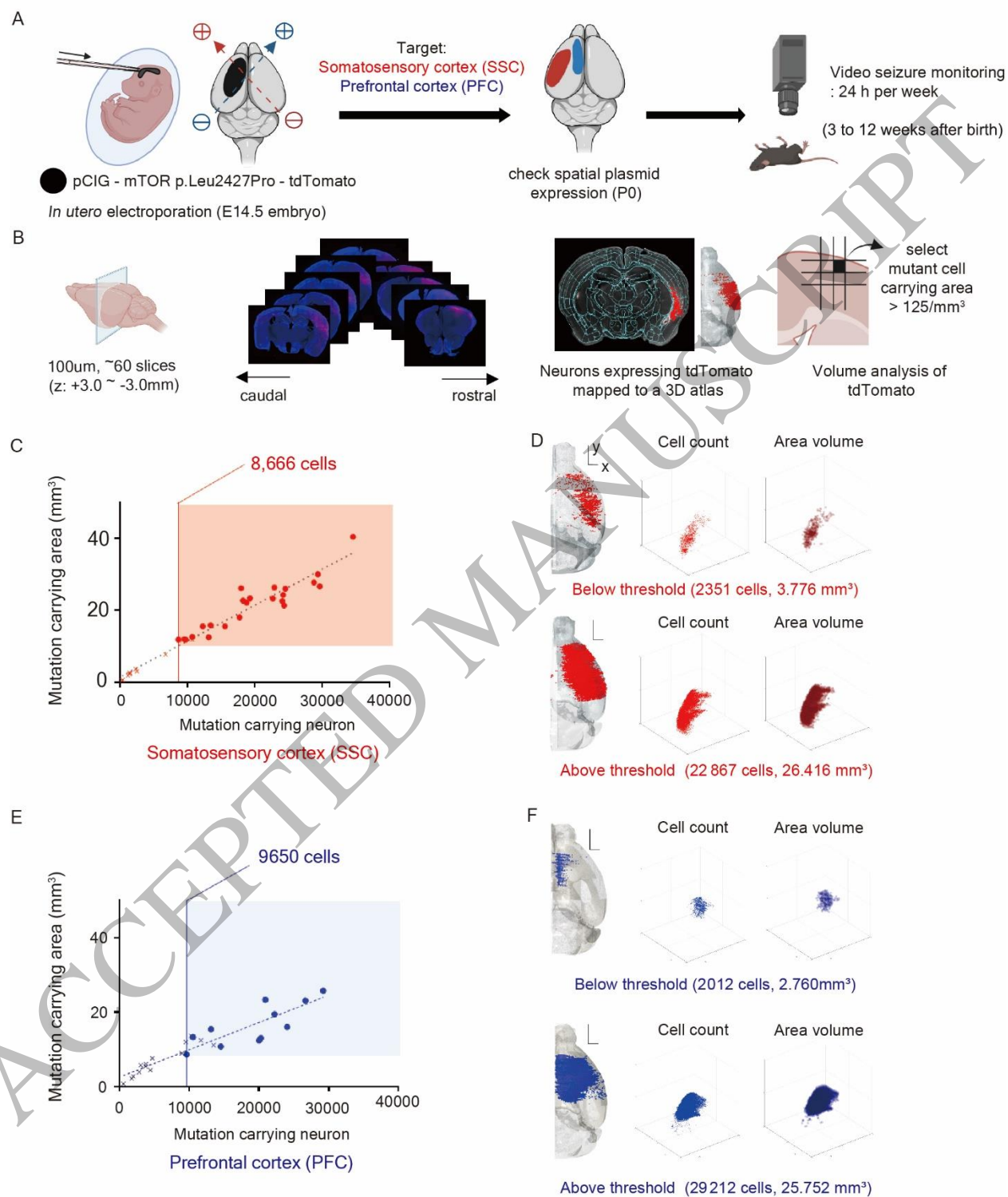


Figure 1  
165x208 mm (x DPI)

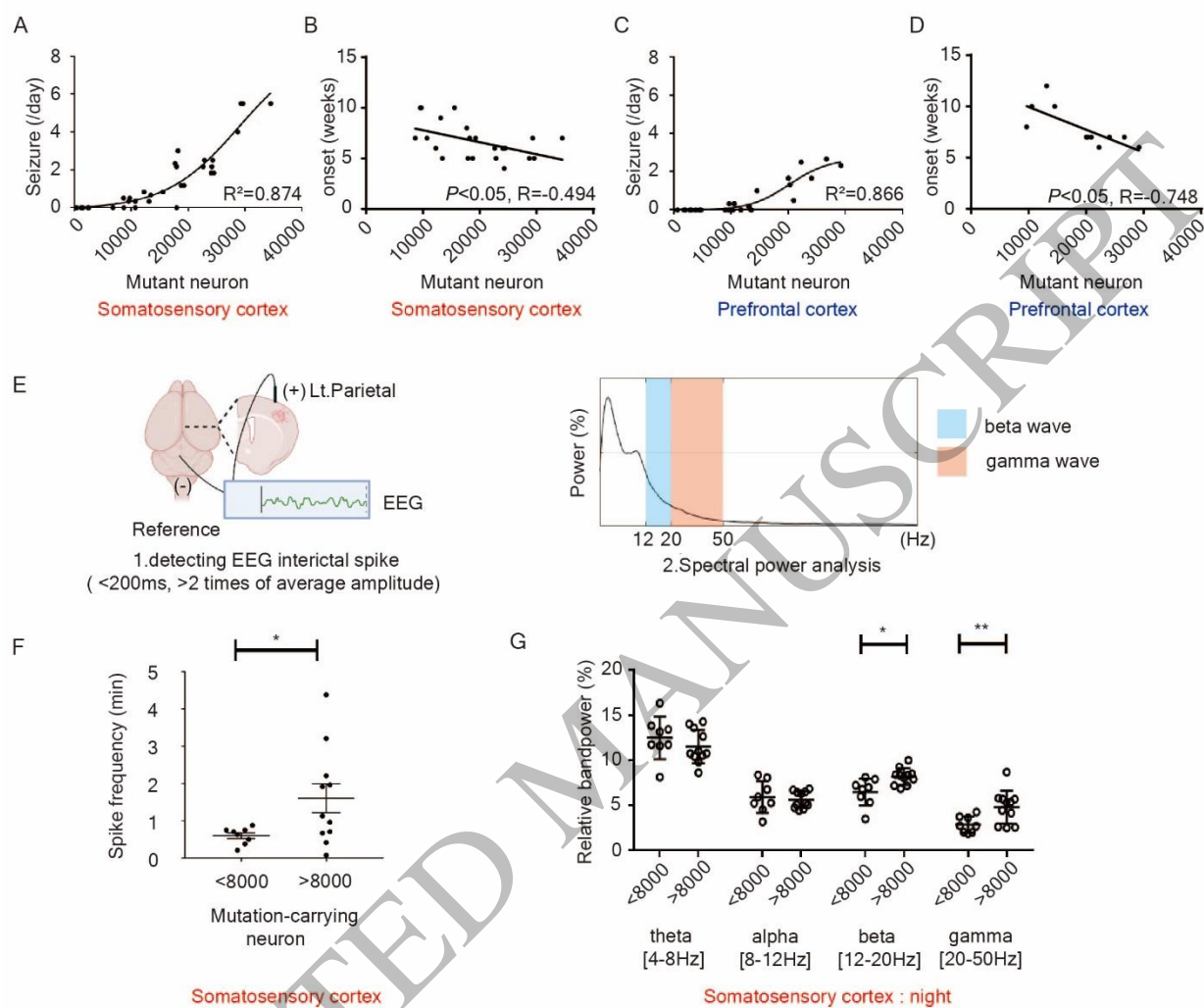


Figure 2  
165x140 mm (x DPI)

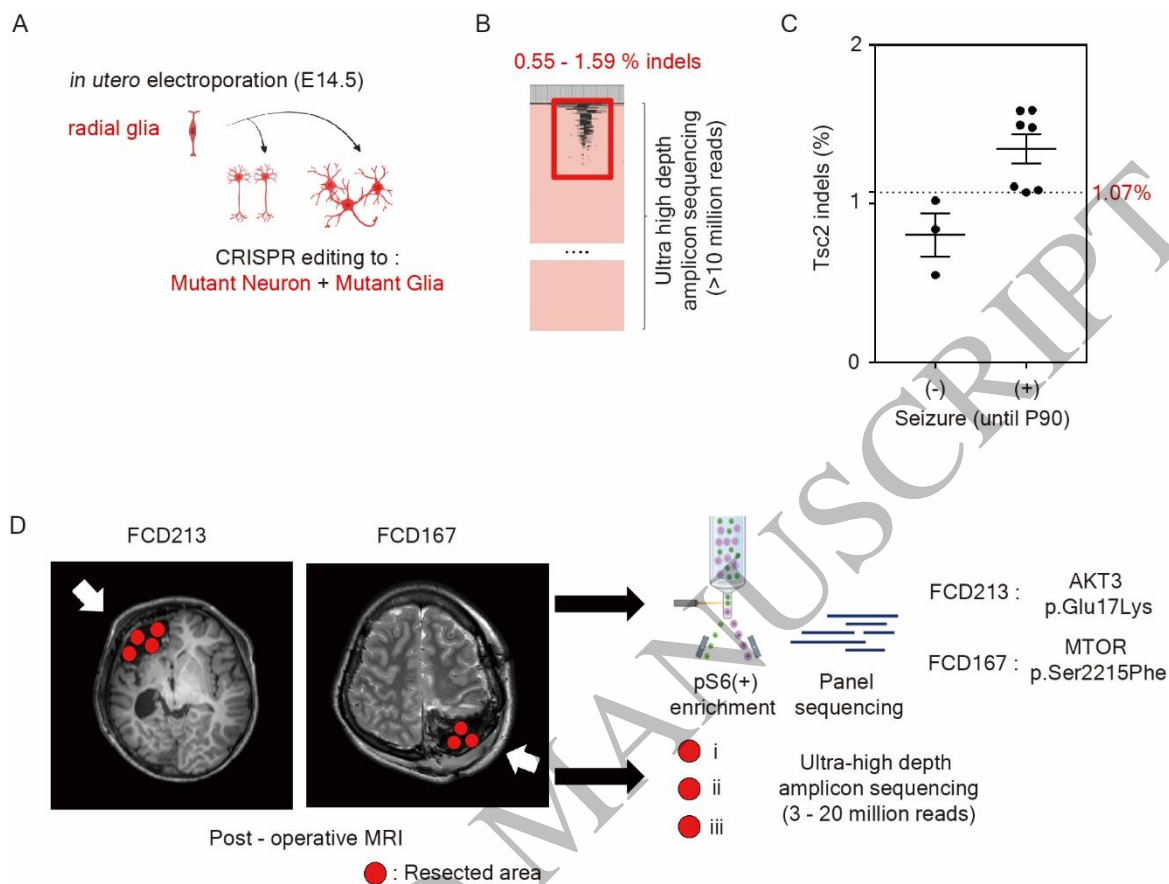


Figure 3  
153x115 mm (x DPI)