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Comprehensive multi-omic profiling of somatic mutations in malformations of cortical development

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68

69 **Abstract**

70 Malformations of cortical development (MCD) are neurological conditions displaying focal
71 disruption of cortical architecture and cellular organization arising during embryogenesis, largely
72 from somatic mosaic mutations, and causing intractable epilepsy. Identifying the genetic causes
73 of MCD has been a challenge, as mutations remain at low allelic fractions in brain tissue resected
74 to treat condition-related epilepsy. Here, we report a genetic landscape from 283 brain resections,
75 identifying 69 mutated genes through intensive profiling of somatic mutations, combining
76 whole-exome and targeted-amplicon sequencing with functional validation including *in utero*
77 electroporation of mice and single-nucleus RNA sequencing. Genotype-phenotype correlation
78 analysis elucidated specific MCD gene sets associating distinct pathophysiological and clinical
79 phenotypes. The unique single-cell level spatiotemporal expression patterns of mutated genes in
80 control and patient brains implicate critical roles in excitatory neurogenic pools during brain
81 development, and in promoting neuronal hyperexcitability after birth.

82

Introduction

MCDs are heterogeneous groups of neurodevelopmental disorders with localized malformation of cortical structures, often presenting with intractable epilepsy[1]. Major MCD subtypes include different classes of focal cortical dysplasia (FCD), hemimegalencephaly (HME), and tuberous sclerosis complex (TSC)[2]. The International League Against Epilepsy (ILAE) has classified FCD subtypes based on neuropathological features and cell types[3]. Fifty percent of patients with epileptic surgery due to refractory epilepsy show cortical dysplasia, and 50~75% of MCD patients become seizure-free after surgical resection, which has led to remarkable clinical benefits[4-6]. The abnormal histology of resected regions includes loss of cortical lamination, enlarged dysplastic neurons, or balloon cells, sometimes accompanied by other brain abnormalities. Similar to brain tumors, it can be difficult to predict pathology before surgery.

Again, like with brain tumors, genetic studies may offer insights into mechanisms. Somatic mTOR pathway gene mutations are frequently detected in HME and type II FCD foci[7,8]. Recently, small- or medium-size cohort studies (<130 cases) confirmed these results and correlated defects in neuronal migration, cell size, and neurophysiology[9-11]. Still, 30~70% of MCD cases remain genetically unsolved[9-11], suggesting other genes are yet to be discovered.

Detecting mutant alleles in resected bulk tissue from MCD patients is challenging because unlike in brain tumors, the mutant cells in MCD are probably not hyperproliferative, and thus variant allelic fraction (VAF) are low, often <5%, diluted by genomes of surrounding non-mutated cells[12]. Fortunately, new computational algorithms can help reduce false-positive and false-negative signals, even when no 'normal' paired sample is available for comparison[13-15]. Recent deep-learning technologies and state-of-the-art image-based artificial intelligence software such as DeepMosaic, trained on non-cancer mosaic variants have significantly improved accuracy[14]. The NIH-supported Brain Somatic Mosaicism Network (BSMN) established a 'Common pipeline', incorporating a 'best practice' workflow to reliably and reproducibly identify low VAF somatic variants[16]. With these advances, we assessed for mosaic variants that might point to gene networks beyond mTOR in MCD lesions. Our results offer insights into potentially druggable pathways in cases of incomplete resection or drug-resistant forms of MCD.

Results
Sequencing approach to identify causes of MCD
To perform a thorough genetic screening for somatic mutations in resected epileptic tissue, we formed the FCD Neurogenetics Consortium and from 22 separate international centers, enrolled 293 cases that met clinical and pathological criteria for FCD or HME. Our cohort included 30 HME cases, 80 type I-, 128 type II-, 31 type III-, and 12 unclassified-FCD cases. We included acute resected brains from 10 neurotypicals and 2 TSC cases for comparison (Fig. 1a, Supplementary Table 1). Patients with environmental causes, syndromic presentations such as TSC, inherited mutations, multifocal lesions, or tumors were excluded (Methods).

We used a three-phase genetic screening, each followed by filtering for likely causative mutations using published methods[17,18], and each followed by orthogonal targeted amplicon sequencing (TASeq) for intra-case validation and VAF quantification, normalized with controls (~5000 X, TASeq) (Fig. 1b, Extended Data Fig. 1). In Phase 1, we performed amplicon sequencing (AmpliSeq, ~1000 X) profiling the entire open reading frame of 87 genes ('MCD panel v1', Supplementary Table 2a) previously detected in FCD/HMEs or known PI3K-AKT3-mTOR interactors (Supplementary Table 2b). In Phase 2, 80 cases (75 unsolved cases plus 5

129 solved cases) from Phase 1 and additionally collected 54 cases, we performed unbiased deep
130 whole-exome sequencing (WES, ~300 X) on paired samples, where available, or unpaired
131 samples (i.e. brain plus blood/saliva vs. brain only). In Phase 3, from an additional subcohort of
132 96 new cases (86 MCD plus 10 neurotypical), we designed the 'MCD panel v2' (Supplementary
133 Table 2c) including known and novel genes detected in Phases 1 and 2 (Methods). We also re-
134 sequenced unsolved cases from Phase 2 (30 cases), expecting that the higher read depth afforded
135 by panel sequencing could provide greater sensitivity to detect low VAF mutations potentially
136 missed by WES. Phase 2 used BSMN best practice guidelines for mapping and variant calling[16]
137 (Extended Data Fig. 1b,c).

138 From Phases 1 to 3, 1181 candidate somatic single nucleotide variant (sSNV) calls were
139 identified (Supplementary Table 3a). Of these, 627 were excluded based on gnomAD allele
140 frequencies, dinucleotide repeats, homopolymers, and additional BSMN established criteria
141 (Methods)[19,20]. This yielded 554 candidate sSNVs, each assessed by TASeq, yielding 108
142 validated sSNVs (19.4% validation rate, Fig. 1c, Supplementary Table 3b), comparing similarly
143 to other BSMN effort validation rates in WGS[16,21]. The validation rate of candidate sSNV calls in
144 each phase was 12.1% (15/124), 20.9% (67/320), and 23.6% (26/110), respectively. The
145 measured square-root transformed VAFs between the AmpliSeq/WES and TASeq were
146 correlated as expected (Spearman ρ = 0.7725) (Fig. 1d). Of the 69 genes mutated in 76 patients,
147 60 were not previously implicated in MCD. Eight were recurrently mutated, including 6 known
148 MCD genes (*MTOR*, *PIK3CA*, *SLC35A2*, *TSC2*, *AKT3*, *BRAF*) as well as 2 novel candidates
149 (*ATP2A1*, *PPFIA4*) (Fig. 1e, Extended data Fig. 2a,b). There were also several genes mutated
150 that were recently linked to epilepsy-associated developmental lesions (*FGFR2*, *NIPBL*,
151 *NPRL3*)[22], one gene recently identified in FCD (*RHEB*[23]), and 57 genes we found mutated in a
152 single brain sample.

153 We estimate only ~7% of mutations identified are likely attributable to false discovery
154 during variant calling, based upon the background mutation rate in 75 BSMN neurotypical brain
155 samples, and published experience from the BSMN[16,24], processed with the same workflow (see
156 Methods). Thus, 93% of our candidate and known MCD mutations would not have been
157 identified in a size-matched neurotypical control cohort. We estimate the false negative rate of
158 phase 2 was 1.67%, assuming VAF rates comparable to what was detected (Methods). We also
159 calculated the probability of identifying the same gene mutated in two separate patients by
160 chance (Methods), taking into consideration the mutation rate, cohort size of each phase, gene
161 length, and panel size. *ATP2A1* and *PPFIA4*, the two novel recurrently mutated candidates, both
162 reached significance from our permutation analysis (p = 0.000127 for *ATP2A1* and p = 0.000258
163 for *PPFIA4,* Methods).

164 Most patients (80.52%, 62 cases) showed a single somatic mutation, but some showed two
165 somatic mutations (14.29%, 11 cases), and a few showed more than two mutations (5.19%, 4
166 cases). Interestingly, HME-4144 showed 11 different somatic mutations, all of which were
167 validated with TASeq. Although there are several possible explanations for HME-4144, we
168 suspect this reflects clonal expansion from a driver mutation, with the detection of multiple
169 passenger mutations, as reported in brain tumors[25].

170 Single-base mutational signatures (SBS) can describe potential mutational mechanisms in
171 human disease[26]. We found 60.2% of mutations were C>T, likely arising from mutation of the
172 methylated CpG dinucleotide DNA epigenetic mark[27] (Extended Data Fig. 3). Enrichment of
173 SBS1 and SBS5, clock-like mutational signatures suggest endogenous mutations arising during
174 corticogenesis DNA replication.

Functional dissection of the MCD genes

Interestingly, most validated genes were non-recurrently mutated in our cohort, suggesting substantial genetic heterogeneity in MCD. This nevertheless provided an opportunity to study converging functional gene networks. Thus, we performed Markov clustering with a STRING network generated from the putative MCD genes[28], as well as recently reported novel MCD candidates (*NAV2, EEF2, CASK, NF1, KRAS, PTPN11*)[22,29] (Fig. 2a). We identified four clusters, with cluster 1 ("mTOR pathway") showing the highest term enrichment to the mTOR/MAP kinase signaling, supporting prior results for Type II MCDs. Because MCD panel v1 included many mTOR pathway genes not identified as mutated in patients from WES, we repeated the analysis by excluding genes not identified *a priori* from WES and recovered the same clusters (Extended Data Fig. 4). Cluster 1 also highlighted newly identified genes *FGFR2, KLHL22, RRAGA, PPP2R5D, PIK3R3, EEF2, EIF4G1,* and *MAPK9*. Cluster 2 identified "Calcium Dynamics" and included genes *ATP2A1, RYR2, RYR3, PSEN2, TTN*, and *UTRN*. Cluster 3 was labeled "Synaptic Functions" and included genes *CASK, GRIN2C, and PPFIA4*. Cluster 4 was labeled "Gene Expression" and included intellectual disability genes, mostly involved in nuclear function, including *NUP214, PRR14, PCNT, NIPBL, SRCAP, ASH1L, TRIP12,* and *MED13* (Fig. 2b, Methods). We further performed ClueGO analysis and found enrichment in mTOR signaling, focal adhesion assembly, cardiac muscle cell contraction, and artery morphogenesis (Extended Data Fig. 5). ClueGO also displayed isolated gene ontology (GO) term clusters such as 'calcium ion import' and 'protein localization to synapse'. There were recurrently mutated genes in all four clusters, and while several of these clusters were not previously reported in MCDs, they were previously implicated in epilepsy, neurodevelopmental and neurodegenerative disease[30,31], suggesting functional overlap with MCDs.

Functional validation of selected genes in mouse brain

To investigate the roles of novel MCD genes and modules, we first revisited previous publications characterizing our novel MCD genes in the context of cortical development. In the 'gene expression' group in Fig. 2, *NIPBL*[32], *ZNF335*[33], and *ZNF423*[34] were already reported as key regulators of neuronal migration during murine cortical development, but not in somatic mosaicism or MCD contexts. To expand functional validation, we selected two potential canonical mTOR pathway mutations (*RRAGA* p.H226R, *KLHL22* p.R38Q)*,* and two genes not known as mTOR interactors (*GRIN2C* p.T529M, *RHOA* p.P75S), discovered in FCD-7967, 3560, 5157, and 3876 respectively. *RRAGA* encodes Ras-related GTP binding A (RAGA), a GTPase sensing amino acid and activating mTOR signaling. The mosaic p.H226R mutation occurs within the C-terminal 'roadblock' domain (CRD), which binds to the RAGB protein and is conserved throughout vertebrate evolution (Extended Data Fig. 2c) and thus could change binding affinity[35]. *KLHL22* encodes a CUL3 adaptor, determining E3 ubiquitin ligase specificity, and mediating degradation of DEPDC5, required for mTORC1 activation[36]. The KLHL22 *p.*R38Q variant in FCD-3560 is near the BTB (Broad-Complex, Tramtrack, and Bric- à-brac) domain that interacts with CUL3 (Extended Data Fig. 2d), suggesting the variant could enhance mTORC1 activity. *GRIN2C* encodes a subunit of the NMDA receptor regulating synaptic plasticity, memory, and cognition[37,38], the dysfunction of which is implicated in neurocognitive diseases [39,40]. *GRIN2C* p.T529M mutation is located in the S1 glutamate ligand-binding domain (S1 LBD) (Extended Data Fig. 2e). *GRIN2A* p.T531M mutation, an analog mutation of *GRIN2C*

220 p.T529M in our cohort, was previously reported in epilepsy-aphasia spectrum disorders, where it
221 increased NMDA receptors 'open-state' probability[40]. This suggests that the p.T529M mutation
222 activates the channel, likely in an mTOR-independent fashion. *RHOA* encodes RHOA protein, a
223 small GTPase, regulating cytoskeletal dynamics, cell migration, and cell cycle. RHOA p.P75S
224 mutation is located in the interdomain region between the second GTP/GDP binding domain and
225 Rho insert domain (Extended Data Fig. 2f). This mutation is implicated in skin cancer multiple
226 times in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (DB)[41]. Thus, all
227 mutations assessed here are likely gain-of-function and exert functional impact on cells in which
228 they are expressed.
229   To test this hypothesis, we introduced episomal expression vectors carrying mutant or
230 wildtype (WT) genes co-expressing enhanced green fluorescent protein (EGFP) into the dorsal
231 subventricular zone via electroporation at mouse embryonic day 14 (E14), then harvested tissue
232 at either E18 to assess migration, or at postnatal day 21 (P21) to assess cell size and phospho-S6
233 as a reporter of mTOR activity[42] (Fig. 3a). In E18 cortices, we found EGFP-positive cells
234 expressing mutant but not WT forms of *RRAGA* and *KLHL22* showed significant migration
235 defects of varying severity, whereas mutant *GRIN2C* showed no defect (Fig. 3b). These
236 migration defects in *RRAGA* and *KLHL22* mutant cells replicate major findings of MCD
237 disrupted cortical architecture. Notably, *RHOA* WT and mutant cells both showed significantly
238 disrupted neuronal migration and atypical cell clusters near the subventricular zone (SVZ)
239 (Extended Data Fig. 6a), likely due to the high level of expression. Nevertheless, some low GFP-
240 expressing cells, likely containing a small number of plasmid copies, showed evidence of
241 migration, whereas mutant cells, irrespective of the level of GFP, showed disrupted migration.
242 This indicates the *RHOA* mutation can contribute to the malformation of cortical development.
243   We next assessed cellular phenotype at P21 with samples available in both mice and the
244 corresponding patients and found enlarged cell body area in mutant forms of *KLHL22* and
245 *GRIN2C* compared with wildtype. In contrast, elevated levels of pS6 staining, described
246 previously in association with mTOR pathway mutations[6], was found only in mutant *KLHL22,*
247 but not in mutant *GRIN2C* mice (Fig. 3c). Interestingly, the *RRAGA* mutant cells showed
248 increased pS6 level but not enlarged cell bodies compared to wildtype. Since this case (FCD-
249 7967, type 2B) has additional *MTOR* mutation, the increase in cell body size of dysplastic cells is
250 likely to be induced by *MTOR* but not *RRAGA* mutation (Extended Data Fig. 6b).
251   To assess correlation with human samples, we assessed available archived neuropathological
252 tissue sections for histology and pS6 activity. Similar to our mouse models, we found patient
253 FCD-3560 carrying *KLHL22* p.R38Q showed enlarged neurons that co-stained for excess pS6,
254 whereas FCD-5157 carrying *GRIN2C* p.T529M showed only a slight increase in cell body size
255 and no evidence of excessive pS6 (Fig. 3d). While this analysis does not take into account the
256 genotype of individual cells, it suggests *KLHL22* but not *GRIN2C* mutations impact mTOR
257 signaling.
258

Genotype-phenotype correlations in MCD patients
260 To assess the phenotypic contributions of the MCD genes we found, we focused on 76 of our
261 'genetically solved' MCD cases, comparing detailed neuropathology, brain imaging, and clinical
262 course. We performed Pearson correlation followed by hierarchical clustering based upon ILAE
263 neuropathological diagnosis, compared with GO term-based curated genesets (Fig. 4,
264 Supplementary Table 3c) or with sSNVs in COSMIC DB because a subset of MCDs shows cell
265 over-proliferation, similar to cancer, in the lesion during cortical development. (Methods,

6

266   Supplementary Table 4). We found that FCD Type IIA and Type IIB, and HME were more
267   tightly clustered than FCD Type I or III (Fig. 4a), likely reflecting shared neuropathological
268   features that include large dysplastic neurons. As expected, FCD Type IIA, Type IIB, and HME
269   were positively associated with the mTOR pathway GO term and the presence of oncogenic
270   variants, FCD Type III, however, was associated with the MAPK pathway, consistent with
271   recent publications implicating *BRAF, FGFR2, NOD2,* and *MAPK9* in their etiology[43-45]. FCD
272   Type I showed strong positive correlations for glycosylation, consistent with recent findings of
273   somatic mutations in *SLC35A2* and *CANT1*[46,47].
274   We next investigated correlations between clinical phenotypes extracted from detailed
275   medical records including seizure type, neuropsychological examination, and positron emission
276   tomography (PET) metabolism, often used to help localize seizure focus[48,49]. Seizure frequency,
277   early age of onset, Engel score, and history of infantile spasms drove clinical clustering, likely
278   reflecting shared clinical features in the most challenging patients. Focusing on the correlations,
279   PET hypometabolism around the resected region, correlated positively with COSMIC DB entry,
280   and negatively with MAPK and ubiquitination (Fig. 4b), suggesting divergent metabolic
281   mechanisms. Abnormal neurological examination correlated positively with COSMIC DB entry
282   and negatively with Type I histology, which may reflect the effects of mutations on baseline
283   neurological function.

284

285   **MCD genes enriched in the excitatory neuronal lineage**
286   To infer the cell type in which MCD genes function, we mapped the net expression of the MCD
287   genes (i.e. eigengene) onto a published single-cell transcriptome dataset from the 2nd-trimester
288   human telencephalon, at a time when mutations probably arose[50] (Fig. 5a,b). This showed a
289   strong positive correlation of the net expression levels of the MCD geneset with dividing radial
290   glial cells (Pearson $r = 0.3655$, $p = 7.915 \times 10^{-121}$) and a moderate correlation in dividing
291   intermediate progenitor cells (IPCs; Pearson $r = 0.1527$, $p = 2.448 \times 10^{-21}$) and mature excitatory
292   neuron cells (Pearson $r = 0.1780$, $p = 1.557 \times 10^{-28}$). We found a lack of positive correlation with
293   inhibitory neuronal lineages including medial and central ganglionic eminences (MGE, CGE)
294   and mature interneuron clusters (Fig. 5c). We next performed deconvolution of the MCD geneset
295   into four module eigengenes, utilizing weighted co-expression network analysis (WGCNA),
296   which identified cell types classified as mature excitatory neurons (turquoise and blue),
297   microglia (brown), and unassigned (grey) (Fig. 5d). Quantification supported enrichment in
298   dividing radial glia, excitatory neurons, and microglia, the latter likely driven by MCD candidate
299   genes *IRF8* and *VSIG4* (Fig. 5e). Taken together, the expression of MCD genes is more enriched
300   in dorsal cortex neurogenic pools and implicated in the maturation of excitatory rather than
301   inhibitory neurogenic pools, as well as microglia.

302

303   **MCD gene expression is enriched in dysplastic cells**
304   We next performed single-nucleus RNA sequencing (snRNAseq) analysis in resected MCD
305   brain tissue. We reasoned that single-nucleus transcriptomes would be more revealing than bulk
306   transcriptomes, but the average VAF of ~6% in our MCD cohort meant that the vast majority of
307   sequenced cells would be genetically wild-type. We thus decided to focus snRNAseq on resected
308   cortex from patients with shared pathological MCD hallmarks across a range of VAFs. We
309   selected five resected brain samples, one from a patient with FCD (FCD-4512 *SLC24A2*
310   p.V631I, 0.6% VAF and *SRCAP* c.2630+1G>A, 1.64% VAF), two from patients with HME

311　(HME-4688 *PIK3CA* p.E545K, 25.1% VAF and HME-6593 *PIK3CA* p.H1047R, 13.1% VAF),
312　and two from patients with TSC meeting full diagnostic criteria. We also included brains from
313　four neurotypical cases for comparison. We sequenced a total of 33,206 nuclei (see Methods).
314　　　While the FCD brain snRNAseq data showed substantial overlapping cell clusters with
315　controls using UMAP clustering, HME and TSC brains showed distinct cell cluster distributions
316　(Fig. 6a,b). We found that very few HME cells matched expression patterns for typical brain
317　cells, even after standard normalization and scaling (Fig. 6b,c, see Methods). We characterized
318　transcriptomic profiles of each cluster based upon established marker gene expression (Fig. 6d)
319　as well as differentially expressed gene (DEG) analysis (Extended Data Fig. 7a, Supplementary
320　Table 5a) and weighted gene coexpression network analysis (WGCNA, Extended Data Fig. 7b,
321　Supplementary Table 5b) to assign likely cell types. We labeled these clusters according to their
322　closest relatives: 'Excitatory neuron-like (ExN-L)', 'astrocyte-like (Ast-L)', 'oligodendrocyte
323　progenitor cell-like (OPC-L) or 'oligodendrocyte-like (OD-L)'. Even with these categories, some
324　clusters remained undefined (U) (Fig. 6a, Extended Data Fig. 7c).
325　　　We noted that several of the HME clusters showed excessive expression of growth factor
326　receptor (GFR) gene families, specifically *FGFR1* in cluster OPC-L1/2 in HME, *FGFR2* in
327　cluster Ast-L1/3 and OD-L, *FGFR3* in Ast-L1/3, *EGFR* in Ast-L1/3 and OPC-L1/2, and
328　*PDGFRA* in cluster OPC-L1/2 (Fig. 6d). To identify the cell types expressing these genes, we
329　performed RNA in situ hybridization in HME brain sections followed by hematoxylin-eosin
330　staining. We found co-localization of these same *FGFR* family, *EGFR,* and *PDGFRA* transcripts
331　with dysplastic cells (Extended Data Fig. 8). Previous experiments indicate that it is most often
332　the dysplastic cells within HME and MCD that carry mosaic mutations[9], suggesting an effect of
333　these mutations on growth factor receptor expressions that correlates with dysplasia.
334　　　Next, we investigated the net expression patterns of genes mutated in MCD within this
335　snRNAseq dataset. The net expression of the MCD geneset was enriched in Ast-L1/3 and OD-L,
336　which were labeled as dysplastic cells (Fig. 6e). Interestingly, the individual mutated genes
337　displayed converging expression patterns resulting in four different eigengenes (Fig. 6f, grey,
338　turquoise, blue and brown), with gene members for each eigengene (Extended Data Fig. 9).
339　These show distinct enrichment patterns across cell types, implying that members of each
340　eigengene may be associated with the pathophysiology of the corresponding dysplastic cell type
341　in HMEs.
342　　　We next performed a pseudo-bulk DEG analysis by aggregating all cell types within the
343　same disease condition, comparing HME, TSC, and FCD with CTRL (Extended Data Fig. 10a,
344　Supplementary Table 5c). and detected 1735, 1017, and 981 differentially expressed genes in
345　HME, TSC, and FCD, respectively. Intriguingly, 21.33% (16/75) or 13.33% (10/75) of MCD
346　mutated genes in our list overlapped with DEGs of HME or TSC, but less overlapped with FCD
347　(7/75, 9.33%) likely due to low VAF. Permutation tests suggest that the overlaps in HME and
348　TSC are unlikely to have arisen by chance (see Methods). This suggests that many MCD-
349　mutated genes are misregulated in MCD-specific cell types.
350　　　Next, we performed cell-type specific DEG analysis comparing disease with CTRL group
351　to investigate pathophysiology within Ast, ExN, OPC, and OD lineages determined by single-
352　cell analysis (Extended Data Fig. 10b, Supplementary Table 5c). Interestingly, in the HME brain,
353　the Ast lineage, which has the largest upregulated DEGs across cell types, showed increased
354　expression of genes related to lipid biosynthesis or energy metabolism and postsynaptic
355　membrane. These same genes were also enriched in mutated mTOR-expressing sorted cell
356　populations in a rodent FCD model[51]. In the TSC Ast lineage, the genes related to calcium

357 dynamics and synaptic function were strongly upregulated compared to CTRL Ast, which was
358 also found in the ExN lineage (Supplementary Table 5d). In FCD ExN lineage compared to
359 CTRL ExNs, terms relevant to mTOR downstream pathways (ribosome complex-related terms),
360 calcium dynamics (calcium channel complex), and synapse (postsynapse-related terms) were
361 enriched. Moreover, at the individual gene level, many histone remodeling enzymes or
362 transcription factors participating in gene expression regulation were encountered within the
363 DEGs of FCD ExN (Supplementary Table 5c), consistent with the four enriched terms in Fig. 2.
364 Taken together, the predicted pathways and functions in MCD based on the gene ontology of 75
365 MCD genes are altered in Ast or ExN lineage in MCD, suggesting underlying pathogenic
366 mechanisms of MCDs.
367
Discussion
369 Our multi-omics study of the genetic landscape of MCD confirmed the important role of
370 mTOR/MAP kinase and glycosylation pathways, seen in about 60.5% of those with mutations.
371 Moreover, our results also suggest a role for biological processes including gene expression,
372 synaptic function, and calcium dynamics, which made up the other 39.5% of those with
373 mutations. While these pathways may be independent in mediating MCD pathophysiology, it is
374 possible that they may have some crosstalk [52-54].
375 Only 76 of 283 patients showed one or more putative somatic mutations as a likely cause
376 of MCD. There could be numerous causes for the relatively low solve rate in MCD, including the
377 potential to miss very low VAF (<0.5%) mutations and the contribution of complex mutations
378 that could be missed by our pipeline. Finally, although patients with environmental causes,
379 syndromic, or inherited causes were excluded from our cohort, these factors could still contribute
380 to MCD.
381 With our approach, we identified two recurrently-mutated genes not previously
382 implicated in MCD and validated 3 other genes found mutated in single patients from prior
383 studies. Confirming the remaining candidate and identifying further MCD candidate genes will
384 require larger MCD cohorts. Including novel MCD candidate genes emerging from 300X WES
385 into the 1000X Phase 3 AmpliSeq allowed both confirmation of mutations, a more accurate
386 estimate of VAF, and identification of additional patients with these genes mutated that would
387 have been perhaps missed with 300X WES. *In vivo* functional validation by modeling mutations
388 in developing mouse brains suggests many identified genes likely contribute to disease. Like
389 with de novo germline mutations discovered in autism, we suggest that there could be dozens if
390 not hundreds of additional MCD genes, based on the low number of recurrently mutated genes
391 [55].
392 The four gene networks, mTOR/MAP kinase, calcium dynamics, synapse, and gene
393 expression, play important roles both during neurogenesis and neuronal migration, as well as in
394 establishing neuronal excitability. For instance, calcium dynamics regulates cytoskeletal activity
395 and excitability[56,57]. Genotypic information also showed correlations with phenotype, for
396 instance, PET brain hypometabolism and abnormality in the neurological examination correlated
397 with the presence of a likely oncogenic variant within COSMIC DB.
398 We also characterized the expression patterns of MCD mutated genes in neurotypical and
399 MCD brains at single-cell resolution. The cell types most strongly expressing mutated genes
400 include dorsal forebrain radial glial progenitors and their daughter excitatory neurons, consistent
401 with the likely site of origin of somatic brain mutations[58]. The fact that mutated genes also
402 showed the strongest enrichment with these same clusters suggests that mutated genes can drive

gene expression in convergent pathways. The prior studies indicating that MCD dysplastic cells express markers for both glia and neurons[59] are consistent with our findings, with mutations driving critical roles predominantly in dividing radial glia, with profound effects on lineage and cellular dysplasia. The MCD genes in patient brains found in our study demonstrated critical roles during cortical development, significantly correlating with patient phenotypes. These findings could lead to new molecular classifications and diagnostic strategies for MCD, and ultimately to personalized therapies for epilepsy.

Author contributions

C.C., X.Y., Sa.B., St.B., and J.G.G. designed the study. C.C., S.M., and S.K. conducted functional validation. C.B., V.S., A.S.N., E.R., C.C., and G.H. coordinated the clinical database. X.Y., C.C., M.W.B., L.L.B., R.D.G., J.G., M.X., A.P.L.M., and K.N.J. organized, handled, and sequenced human samples. X.Y., C.C., T.B., Y.W., A.A., X.X., Z.L., and B.C. performed bioinformatics and data analysis. C.C. and K.I.V. performed the RNAscope experiment. C.D., H.W.P., C.A.B.G., S.H.K., H.K., H.U., M.P., A.S., C.A.H., D.D.L., C.A.G., M.D.S., S.S., M.N., D.D.G., K.I., Y.T., H.C., J.T., V.C., R.G., O.D., W.A.S., H.R.M., and G.W.M. provided resected brain tissues and clinical data from FCD patients. C.C., X.Y., and J.G.G. wrote the manuscript. All authors read and commented on the manuscript before submission.

Competing Interests Statement

The authors declare no competing interests.

Figure Legends
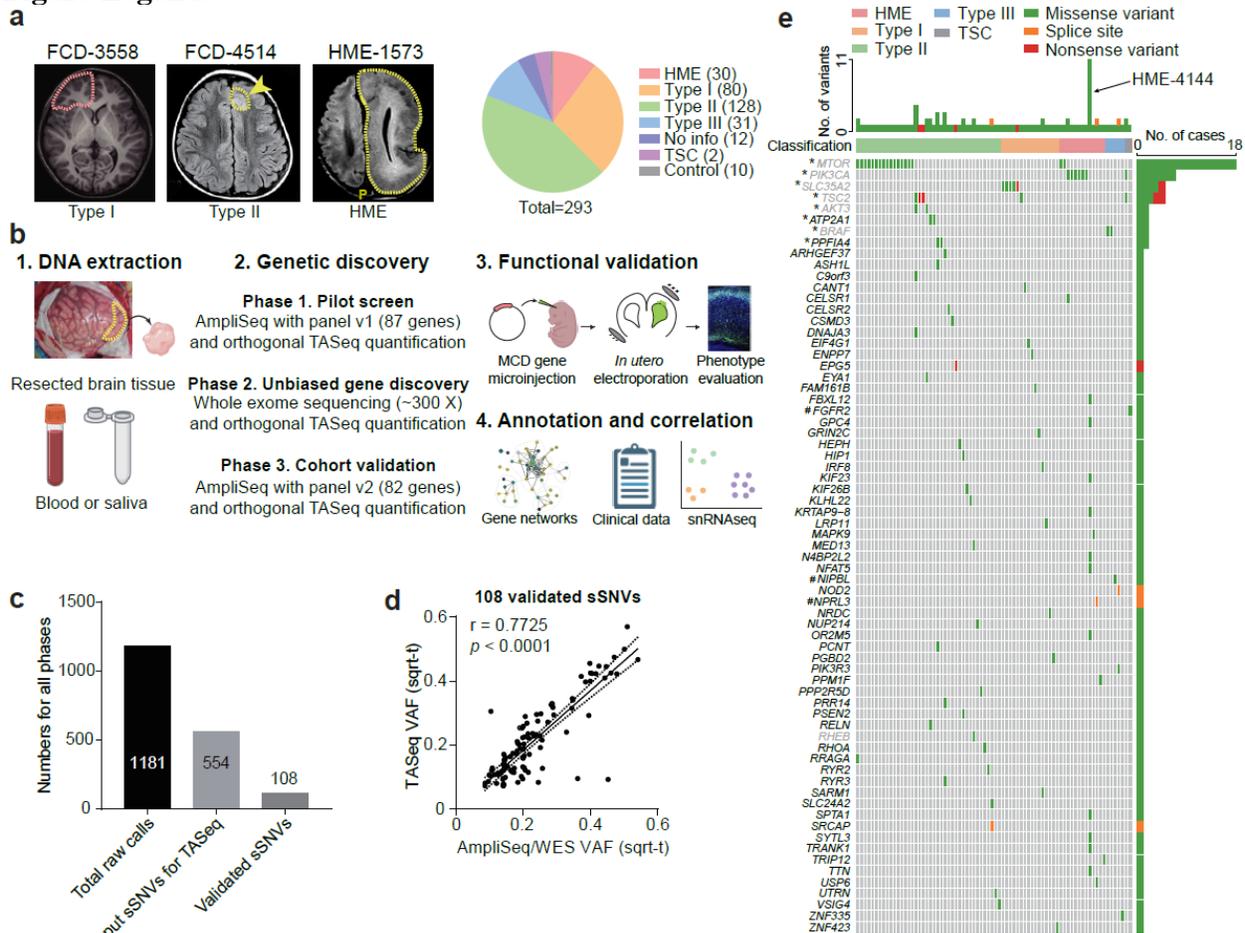
Figure 1. Comprehensive genetic profiling and validation of somatic variants in 283 MCD patients. (a) Representative MRI image of FCD-3558 (FCD type I), FCD-4514 (FCD type II), HME-1573, and composition MCD cohort. Yellow arrow and dash: affected brain regions. FCD-3558, MRI was non-lesional, the epileptic focus was mapped to the right frontal lobe (red dashed line) and resected. (b) Three-phase comprehensive genetic MCD profiling workflow, followed by quantification/validation of each variant with target amplicon sequencing (TASeq). Phase 1] 1000 ×pilot screening of DNA with an 87-gene mTOR-related panel. Phase 2] 300 ×whole-exome sequencing (WES) with best-practice somatic variant discovery for novel candidate genes. Phase 3] Cohort-level validation with an updated, high-confidence TASeq gene set arising from Phases 1 and 2. A subset of somatic mutations was functionally validated in mice. Annotation and correlation with gene networks, clinical data, and single-single-nucleus RNAseq. (c) 1181 sSNV calls were detected from all three phases, yielding 108 validated sSNVs. (d) Correlation between square-root transformed (sqrt-t) AmpliSeq/WES variant allele fraction (VAF) and TASeq VAF. Solid line: Simple linear regression (goodness of fit). Dashed lines: 95% confidence band. Spearman correlation coefficient ρ and corresponding two-tailed *t*-test *p*-value. (e) Oncoplot of 69 genes from 108 validated sSNVs. Top: most patients had one gene mutated, a few patients had more than one gene mutated, and patient HME-4144 had 11 different validated genes mutated. Grey: genes recurrently mutated in previous HME/FCD cohorts. *: genes recurrently mutated in our cohort. #: genes non-recurrently mutated but complementary to other cohorts of epilepsy-associated developmental lesions.
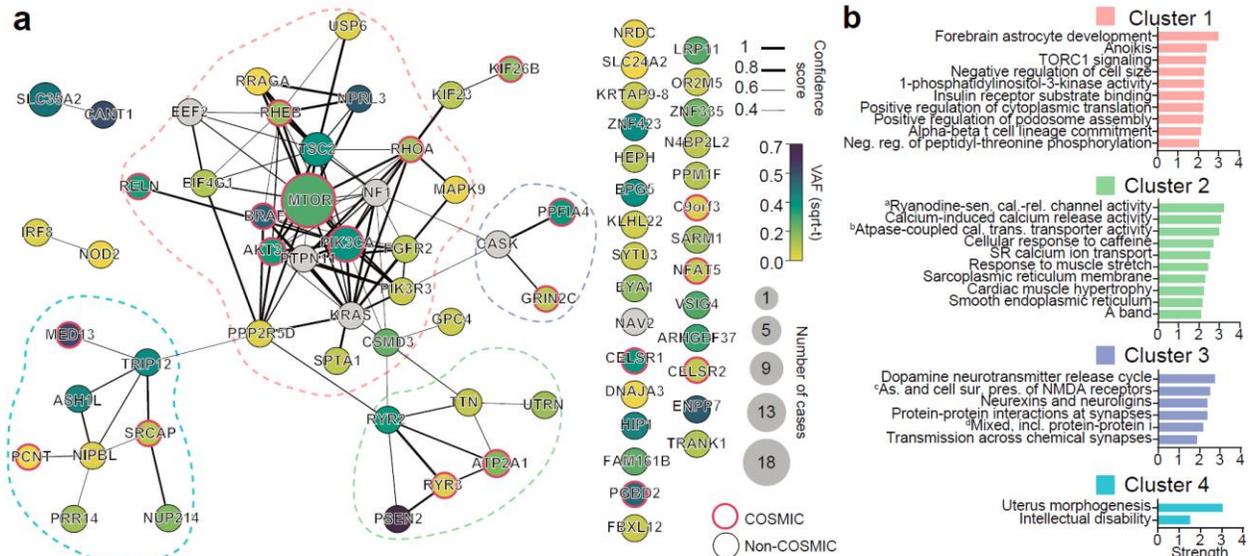
Figure 2. Genes mutated in MCD highlight four major gene networks. (a) STRING DB pathway analysis of the 69 MCD discovered genes and six novel genes (a total of 75 genes) from recent publications identify MTOR/MAP kinase pathway (pink, Cluster 1), Calcium dynamics (green, Cluster 2), Synapse (purple, Cluster 3), Gene expression (blue, Cluster 4). Edge thickness: STRING confidence score. Node size and color: square root transformed (sqrt-t) number of patients carrying a given mutation and average VAF across all patients, respectively. Non-clustered orphan genes were listed at right. Red border: variant reported in COSMIC DB. (b) Gene Ontology (GO) analysis results confirm the functions of compositions in each network. Top GO terms or KEGG pathways based on strength. Strength calculated by STRING.
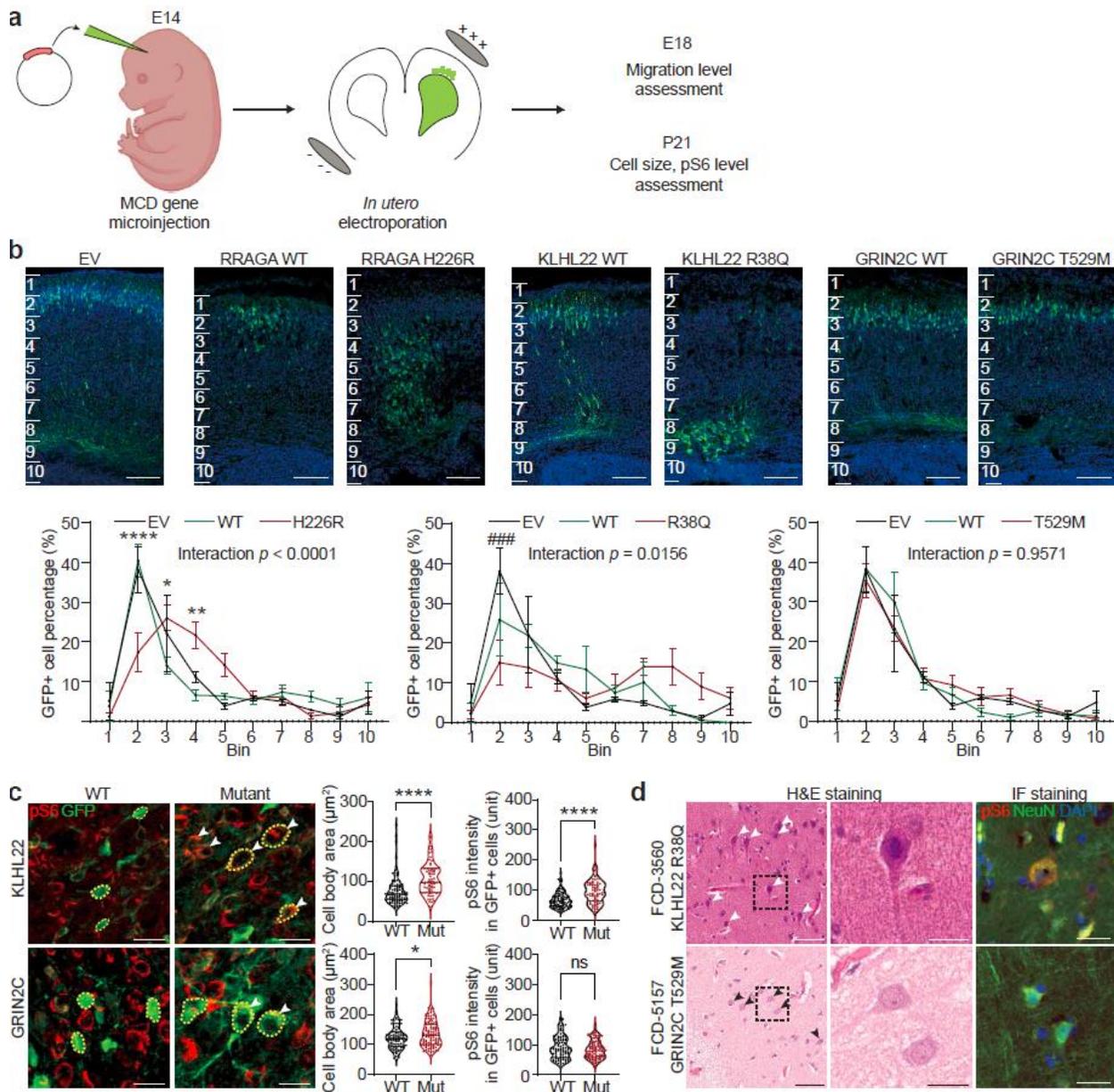
Figure 3. Selected novel MCD somatic variants show functional defects in embryonic mouse brain and patient samples. (a) Validation of candidate mosaic variants in mice. (b) Two different mutations in novel FCD type II genes, *RRAGA* H226R and *KLHL22* R38Q, but not a novel FCD type I gene, *GRIN2C*, disrupt cellular radial migration from the subventricular zone (SVZ). Below: two-way ANOVA and Sidak multiple comparisons with *p*-values of interaction between genotype and bin factor. * or # indicates a *p*-value in comparison between WT and mutant group, or EV and mutant group respectively. Ten bins from the surface of the cortex (top) to SVZ (bottom). Scale bar: 100 μm. Mean ±SEM (standard error mean). n=3, 3, 6, 3, 6, 4, 3 biologically independent animals for EV, RRAGA WT, RRAGA H226R, KLHL22 WT, KLHL22 R38Q, GRIN2C WT, and GRIN2C T529M, respectively. (c) Immunofluorescence in postnatal day 21 mouse cortices for KLHL22 and GRIN2C wild-type (WT) or mutant isoform. Neurons expressing mutant KLHL22 and GRIN2C recapitulate histological phenotypes shown in (d), with enlarged cell bodies (white arrow) compared to WT isoforms (WT control), whereas

13

only neurons expressing KLHL22 but not GRIN2C mutant isoform display increased pS6 levels compared to control. Yellow dashed lines: examples of cell body size quantification. Two-tailed Mann-Whitney *U*-test. Dashed lines and dotted lines in the violin plots indicate median and quartiles, respectively. Scale bar: 20 µm. n=105 cells (3 mice), 70(3), 95(3), 107(3) for KLHL22 WT, KLHL22 R38Q, GRIN2C WT, and GRIN2C T529M, respectively. (d) H&E and phospho-S6 (pS6) staining of the resected brain from FCD-3560 and FCD-5157. One representative H&E or IF staining is shown for each patient case. The box area is zoomed in the middle image. Arrows: dysplastic cells. Right: Immunofluorescence (IF) for pS6 and NeuN. Note that dysplastic pS6-positive neurons with increased pS6 levels are present in FCD-3560 but not in FCD-5157. Scale bar: 60 µm on the left, 20 µm on the middle and right. ****$p < 0.0001$; *$p < 0.05$; ns, non-significant. ###$p < 0.001$. EV: empty vector.

ᵃGene expression regulation
ᵇAbnormal neuropsychological examination
ᶜAbnormal neurological examination
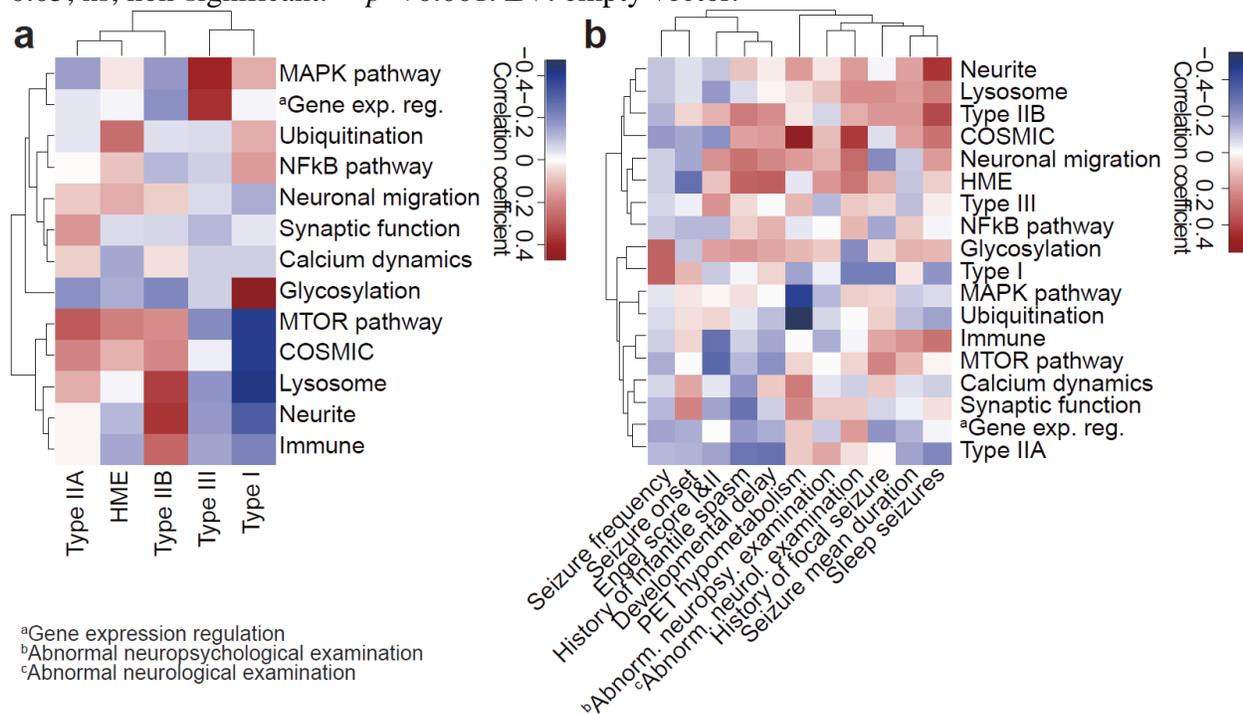
Figure 4. Clinical phenotypic outcomes correlate with genotype-based classifications in MCD. (a) Correlation heatmap for classification based on genetic information (y-axis) vs. International League Against Epilepsy (ILAE) classification based on histology (x-axis) using Pearson correlation. Shade: the value of the Phi coefficient. Note that Type IIA and HME are enriched with mTOR and Ubiquitination genes, while Type I is enriched in Glycosylation and depleted in MTOR and COSMIC genes. HME: hemimegalencephaly. (b) Correlation between classification based on genetic information and various clinical phenotypes. Shade: the value of Phi (binary data) or Pearson (continuous) correlation coefficient. For example, positron emission tomography (PET) hypometabolism is enriched in COSMIC genes and depleted in the MAPK pathway, whereas abnormal neurological examination is enriched in COSMIC genes. Raw data are in Supplementary Table 4.
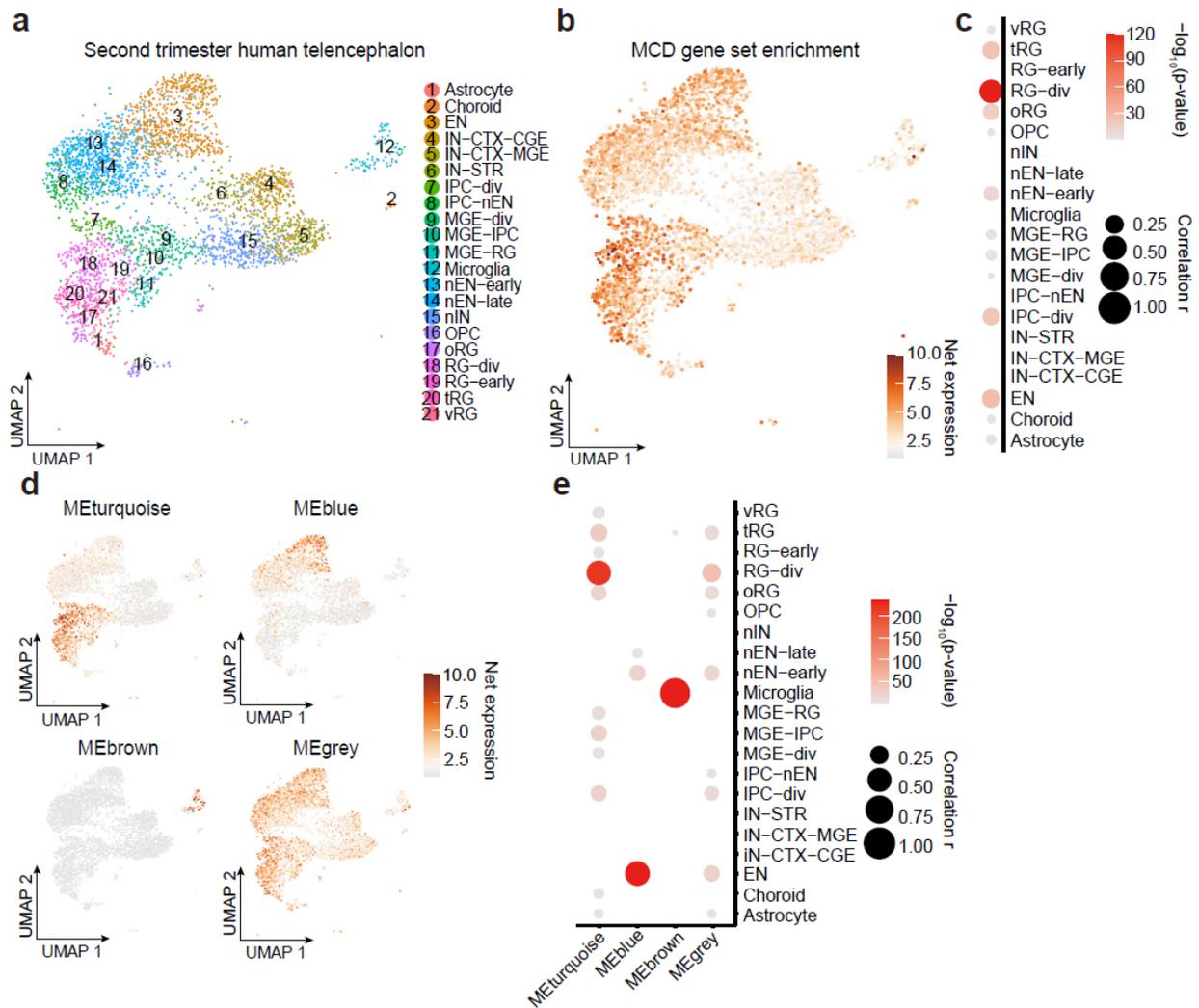
Figure 5. Single-nucleus transcriptomes reveal MCD gene enrichment in radial glia and excitatory neurons in the developing human cortex. (a) Uniform Manifold Approximation and Projection (UMAP) for single-nucleus transcriptome in 2nd-trimester fetal human telencephalon from a public dataset[50]. (b) UMAP enrichment patterns of an eigengene using MCD genes. Note enrichment for excitatory neurons and radial glia (dark brown). vRG: vertical radial glia, tRG: truncated radial glia, RG-div: dividing radial glia, oRG: outer radial glia, EN: excitatory neuron, nEN: newborn excitatory neuron, IPC: intermediate progenitor cell, STR: striatum, IN: interneuron, CTX: cortex, MGE: medial ganglionic eminence, CGE: central ganglionic eminence. (c) Quantification of enrichment of (b) based on cell types, showing enrichment for RG-div. (d) Four eigengenes decomposed from (b). (e) Quantification of enrichment of (d) based on cell types showing enrichment in dividing radial glia, microglia, and inhibitory cortical neurons from the medial ganglionic eminence (MGE). Net expression: Relative and scaled net expression level of a given eigengene generated by a defined gene list. The size and color of the dot plots in (c) and (e) indicate Pearson correlation coefficient r and corresponding non-adjusted asymptotic *p*-value derived from a two-sided Student's *t*-test, respectively.

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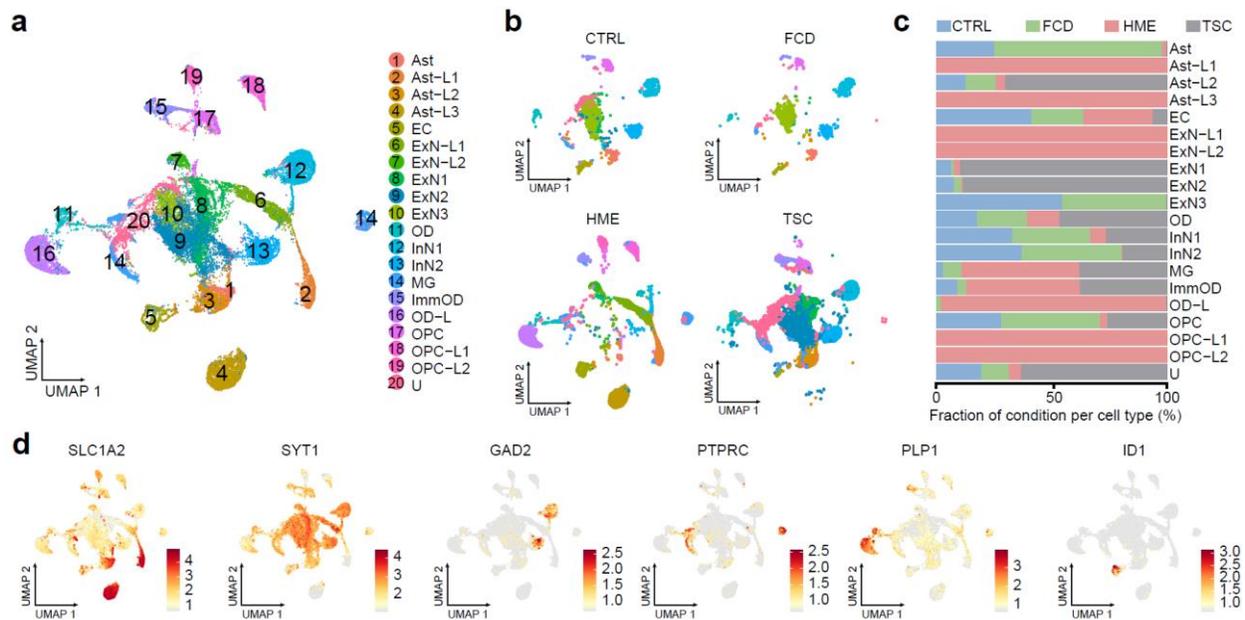
Figure 6. Single-nucleus transcriptomes showed MCD gene expression enriched in MCD-specific cell types. (a) UMAP for the 33,206 single-nucleus transcriptomes from cortical resections, revealing disrupted cell clusters, especially for HME and TSC brain, but only mild disruption in FCD brain. Cell type classification. Ast: astrocyte, EC: endothelial cells, ExN: excitatory neuron, ImmOD: immature oligodendrocyte, InN: inhibitory neuron, MG: microglia, OD: oligodendrocyte, OPC: Oligodendrocyte precursor cells, U: unidentified. (b) UMAP is split by disease condition. (c) The proportion of disease conditions for each cell type. (d) Expression pattern of selected marker genes in normal human cortex vs. several atypical markers related to brain cancer or Alzheimer's disease. Color scale: Average expression level. (e) Eigengene with all 75 MCD genes and quantification of enrichment based on cell types. (f) four genes decomposed from (e) and quantification of results. Net expression: Relative and scaled net expression level of a given eigengene generated by a defined gene list. The size and color of the dot plots in (e) and (f) are Pearson correlation coefficient r and corresponding non-adjusted asymptotic p-value derived from a two-sided Student's t-test, respectively.

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Methods

The study protocol was approved by the UC San Diego IRB (#140028). Informed consent was obtained from all participants or their legal guardians at the time of enrollment. All work with mice was performed in accordance with UCSD IACUC protocol S15113.

Overview of the FCD cohort

This study is a multi-center international collaboration. We recruited a cohort of 283 individuals from the 'FCD Neurogenetics Consortium' (see the member list). These individuals were diagnosed with FCD type I, II, III, HME, or TSC and underwent surgical resection to treat drug-resistant epilepsy between 2013 and 2021. Any cases that underwent surgical resection due to environmental factors, for example, stroke, or acute trauma, were excluded. For each individual, resected brain tissue was collected, along with paired blood or saliva samples and parental samples, where available. Clinical history, pre- and post-operative brain imaging, histopathology, ILAE classification according to the surgical tissue pathology report, and Engel surgical outcome score (at least two years after surgery) were collected, when available.

DNA extraction

Pulverized cortical samples (~0.3 g) were homogenized with a Pellet Pestle Motor (Kimble, #749540-0000) or Handheld Homogenizer Motor (Fisherbrand, #150) depending on the size of the tissue, and resuspended with 450 μL RLT buffer (Qiagen, #40724) in a 1.5 ml microcentrifuge tube (USA Scientific, #1615-5500). Homogenates were then vortexed for 1 minute and incubated at 70°C for 30 minutes. 50 μl Bond-Breaker TCEP solution (Thermo Scientific, #77720) and 120 mg stainless steel beads with 0.2 mm diameter (Next Advance, #SSB02) were added, and cellular disruption was performed for 5 minutes on a DisruptorGenie (Scientific industries). The supernatant was transferred to a DNA Mini Column from an AllPrep DNA/RNA Mini Kit (Qiagen, #80204) and centrifuged at 8500 xg for 30 seconds. The column was then washed with Buffer AW1 (kit-supplied), centrifuged at 8500 xg for 30 seconds and washed again with Buffer AW2 (kit-supplied), and then centrifuged at full speed for 2 minutes. The DNA was eluted two times with 50 μl of pre-heated (70°C) EB (kit-supplied) through centrifugation at 8,500 xg for 1 minute.

AmpliSeq and WES sequencing for somatic mutation candidates

AmpliSeq and whole-exome sequencing (WES) were used at different phases to perform the genetic screening within available samples from the cohort. Customized AmpliSeq DNA panels for Illumina (Illumina, #20020495) were used for Massive Parallel Amplicon Sequencing[21]. 87 or 82 genes related to the mTOR pathway or curated based on the results of Phase 1 and 2, respectively, were subjected to the AmpliSeq design system; a list of designed genes is provided in Supplementary Table 2a-c. Two pools were designed for tiling the capture region. Genomic DNA from extracted tissue was diluted to 5 ng/uL in low TE provided in AmpliSeq Library PLUS (384 Reactions) kit (Illumina, #20019103). AmpliSeq was carried out following the manufacturer's protocol (document #1000000036408v07). For amplification, 14 cycles each with 8 minutes were used. After amplification and FUPA treatment, libraries were barcoded with AmpliSeq CD Indexes (Illumina, #20031676) and pooled with similar molecular numbers based on measurements made with a Qubit dsDNA High Sensitivity kit (Thermo Fisher Scientific,

703 #Q32854) and a plate reader (Eppendorf, PlateReader AF2200). The pooled libraries were
704 subjected to Illumina NovaSeq 6000 platform for PE150 sequencing. The AmpliSeq design in
705 'Phase 1' is under design ID IAA7610, and the AmpliSeq design in 'Phase 3' is under design ID
706 IAA26010.
707   Genomic DNA (~ 1.0 μg) was prepared for whole-exome sequencing, and libraries were
708 captured using the Agilent SureSelect XT Human All Exon v.5 or Nextera DNA Exome kits.
709 Then, 100, 125, or 150 bp paired-end reads (median insert size ~ 210 bp) were generated using
710 the Illumina HiSeq X 2500 platform. The sequencing experiments were designed to yield three
711 datasets of ~ 100X coverage on each sample, with a coverage goal of 300X from the brain and
712 100X from blood/saliva.

713

714 **Somatic variant calling from AmpliSeq and WES**
715 Reads were aligned to GRCh37 using BWA (version 3.7.16a), sorted per each read group with
716 SAMtools (version 1.7 for WES, version 1.9 for AmpliSeq), and merged into a single BAM file
717 with Sambamba (version 0.6.7). The merged BAM files were marked for duplicate reads using
718 Picard (v2.12.1 for WES, v2.18.27 for AmpliSeq), duplicated reads were not removed for
719 AmpliSeq because of the nature of the method. Then, we performed indel realignment and base
720 quality recalibration using GATK (v3.7.0), resulting in the final uniformed processed BAM files.
721   Both tissue-specific and tissue-shared mosaic variants were called from the AmpliSeq
722 and WES sequencing data. AmpliSeq and WES variants were called according to the availability
723 of the control tissue. Brain- and blood/saliva-specific variants were called using MuTect2
724 (GATK v3.8.1 for WES, v4.0.4 for AmpliSeq) paired mode and Strelka2 (v2.9.2) somatic
725 mode[60]; the BAM files from the brain sample (combined and non-combined from independent
726 sequencing libraries) and blood/saliva samples were treated as "tumor-normal" and "normal-
727 tumor" pairs separately and cross-compared between each other. Variants called by both callers
728 were listed. Mosaic variants shared between the brain and blood/saliva samples were called using
729 the single mode of MosaicHunter[15] by either combining all brain replicates or calling each
730 separate sample. Variants that passed all the MosaicHunter filters also were listed. Somatic
731 variants from WES data were further called by GATK (v3.7.0) haplotypecaller with ploidy
732 parameter set to 50, followed by a series of heuristic filters described as the best-practice by the
733 Brain somatic mosaicism network[16], tissue-shared variants were called by the combination of
734 MuTect2[61] (GATK v3.8.1) single-mode and DeepMosaic[14].
735   A union of different pipelines was selected to get maximum sensitivity. Mosaic
736 candidates from the combined lists were refined using the following criteria: (i) the variant had
737 more than 3 reads for the alternative allele; (ii) the variant was not present in UCSC repeat
738 masker or segmental duplications; (iii) the variant was at least 2 bp away from a homopolymeric
739 tract; and (iv) the variant exhibited a gnomAD allele frequency lower than 0.001. Variants that
740 exist in the 1000 genome project (phase 3) also were excluded from the analysis. Variants from
741 both exome data sources were tested and a combination of tissue-specific mosaic variants and
742 tissue-shared mosaic variants were collected and the credible interval of VAFs was calculated
743 using a Bayesian-based method described previously[62]. To filter for candidate MCD disease-
744 causing variants, we further filtered out synonymous variants in coding regions, variants with
745 CADD Phred score < 25, and candidates that fell out of coding regions and were not predicted to
746 affect splicing, annotated by ANNOVAR and BEDtools (version 2.27.1), the annotation scripts
747 were provided on GitHub (https://github.com/shishenyxx/MCD_mosaic).

21

False discovery estimation

To calculate the false discovery of random variants detected in normal samples, we incorporated 75 normal control samples (71 brains and 4 other organs) previously sequenced with 250-300X WGS, which should provide similar sensitivity as our exomes, the deep WGS were generated by efforts from the NIMH Brain Somatic Mosaicism Consortium[16], from controls[21], and from our recent mutation detection pipeline[24]. Variants were filtered based on the identical criteria as described in the above data analysis part, with >0.005 VAF, all on exonic regions defined by NCBI, and CADD score >25. While 13 variants remain positive from this pipeline from the 75 samples (0.17 per control), 306 candidate variants were determined in our 134 MCD exomes (2.28 per MCD case), which lead to an estimated 7.59% per sample false discovery rate (Supplementary Table 6).

False negative rate estimation for phase 2 relative to phase 1

Only FCD type II cases fulfilling the clinical criteria were considered for this calculation to control the comparison conditions between phase I and phase II. Of the 67 type II patients who underwent Phase 1 AmpliSeq with the 87 mTOR genes, 9 cases were positively validated with mTOR mutations (9/67, 13.43%). Of the 17 novel type IIA/B patients in WES that did not undergo Phase 1 AmpliSeq, 2 were detected with positively validate mTOR mutations on the 87 genes (2/17, 11.76%). Thus, we conclude that our false-negative rate for Phase 2 WES relative to the Phase 1 AmpliSeq is 13.43% - 11.76% = 1.67%.

Estimation of probability of observing recurrency in mutated genes in MCD

Based on the previously established estimation[63,64], we simulated the number of detected sSNVs and their recurrence. In phases 2 and 3 of our genetic discovery, we positively validated 67 and 26 sSNVs from 134 and 126 brains, respectively. Assuming the same mosaic mutation rate and cohort size, we would be expecting the same number $(67 + 26)$ of positively detected sSNVs. We permuted 10,000 times for 67 deleterious sSNVs from the 19909 human coding genes, plus 26 deleterious sSNVs from the 59 genes in phase 3, and estimated the distribution of the same gene being hit more than once. After correcting for gene length (average length of human coding genes: 66645.9, the average length of the 59 genes in phase 3: 158929.7, correlated relative to the average length[65]), from the permutation analysis, the probability of observing more than hits is $p = 0.000127$ for *ATP2A1* and $p = 0.000258$ for *PPFIA4*. The probability of observing more than one hit on each of the 59 genes is provided, respectively in Supplementary Table 7.

Orthogonal validation and quantification of mosaic mutations with targeted amplicon sequencing

Targeted amplicon sequencing (TASeq) with Illumina TruSeq was performed with a coverage goal of >1000X for 554 candidate variants detected by computational pipelines described above for both AmpliSeq and WES, to experimentally validate the mosaic candidates before functional assessment. PCR products for sequencing were designed with a target length of 160-190 bp with primers being at least 60 bp away from the base of interest. Primers were designed using the command-line tool of Primer3[66,67] with a Python (v3.7.3) wrapper[17,18] (Supplementary Table 8). PCR was performed according to standard procedures using GoTaq Colorless Master Mix (Promega, M7832) on sperm, blood, and an unrelated control. Amplicons were enzymatically

789 cleaned with ExoI (NEB, M0293S) and SAP (NEB, M0371S) treatment. Following
790 normalization with the Qubit HS Kit (ThermFisher Scientific, Q33231), amplification products
791 were processed according to the manufacturer's protocol with AMPure XP beads (Beckman
792 Coulter, A63882) at a ratio of 1.2x. Library preparation was performed according to the
793 manufacturer's protocol using a Kapa Hyper Prep Kit (Kapa Biosystems, KK8501) and barcoded
794 independently with unique dual indexes (IDT for Illumina, 20022370). The libraries were
795 sequenced on Illumina HiSeq 4000 or NovaSeq 6000 platform with 100 bp paired-end reads.
796 Reads from TASeq were aligned to GRCh37 with BWA (version 3.7.16a), sorted, realigned, and
797 recalibrated with SAMtools (version 1.9), Picard (version 2.18.27), and GATK v3.8.1. Candidate
798 variants were annotated with the same ANNOVAR and BEDtools (version 2.27.1) scripts also
799 provided on GitHub (https://github.com/shishenyxx/MCD_mosaic) and exact binomial
800 confidence intervals were calculated for the same variants in the target sample as well as normal
801 controls. Variants detected from AmpliSeq and/or WES are considered to be positively validated
802 in a given tissue by TASeq if 1) the 95% lower binomial confidence interval is higher than
803 0.5% ; 2) the 95% higher binomial confidence interval is lower than 40%; 3) the 95% lower
804 binomial confidence interval of the negative control is below 0.5%.

805

806 **Oncoplot generation**
807 Oncoplot in Fig. 1e was generated using maftools (v2.6.05) R library.

808

809 **Mutational signature analysis**
810 Mutational signature analysis was performed using a web-based somatic mutation analysis
811 toolkit (Mutalisk)[68]. PCAWG SigProfiler full screening model was used.

812

813 **STRING analysis**
814 STRING analysis was performed by STRING v11[28]. A total of 75 MCD genes (69 novel and
815 known genes from our cohort and 6 novel genes from two other recent MCD cohort studies)
816 were loaded as input and MCL clustering was performed. The terms in Gene Ontology (GO),
817 KEGG pathways, and the top 10 terms GO or KEGG pathways were shown in Fig. 2b. If there
818 are less than 10 terms for those terms (such as clusters 3 and 4 in Fig. 2), we included all the
819 terms in GO or KEGG pathways, Local network cluster (STRING), Reactome pathways, and
820 Disease-gene associations (DISEASES) to show the enriched terms. Visualization was
821 performed by Cytoscape v3.9.

822

823 **ClueGO analysis**
824 Visualization of the functionally grouped biological terms was performed by ClueGO v2.5 [69], a
825 Cytoscape plug-in. A total of 75 MCD genes from Fig. 2 were loaded and GO terms in the
826 'Biological Process' category were used for visualization. Terms with a $p < 0.01$, a minimum
827 count of 3, and an enrichment factor > 1.5, are grouped into clusters based on membership
828 similarities.

829

830 **Animals**
831 Pregnant Crl:CD1(ICR) mice (E14) for mouse modeling were purchased from Charles River
832 Laboratory. All mice used were maintained under standard group housing laboratory conditions

833 with temperatures of 18~23℃, 40~60% humidity, 12 hours of light/dark cycle, and free access to
834 food and water. The age and number of mice used for each experiment are detailed in the figure
835 legends. The sex of the embryos or subject mice used was not tested.

836

837 **DNA constructs**
838 *RRAGA*, *KLHL22*, and *RHOA* ORF regions were amplified from the hORFeome library and
839 inserted into the pCIG2 (pCAG-IRES-GFP) vector. *GRIN2C* ORF region was purchased from
840 DNASU Plasmid Repository at Arizona State University Biodesign Institute. Gibson Assembly
841 Cloning Kit (E5510S, New England Biolabs) was used for joining point mutation-carrying gene
842 fragments (amplified by primers below) and linearlized pCIG2 vector (digested by XhoI and
843 XmaI). The mutation was confirmed by Sanger sequencing.
844 The sequence information of primers used to amplify mutation-carrying DNA fragments is in
845 Supplementary Table 8.

846

847 **In utero electroporation**
848 In utero electroporation was performed as described[70] with endotoxin-free plasmids (0.5–1 μg)
849 plus 0.1% Fast Green (Sigma, catalog no. 7252) injected into a single lateral ventricle in E14.5
850 embryos then electroporated with BTX ECM830 instrument using pulses of 45 V for 50 ms with
851 455-ms intervals were used.

852

853 **Mouse brain sectioning**
854 The brain was fixed by submersion or perfusion with 4% paraformaldehyde (PFA) for E18.5 or
855 P21 mice respectively, cryoprotected in 30% sucrose for 48 hrs, embedded in Tissue-Tek A,
856 sectioned at 20 um (CryoStar NX70, Thermo Fisher Scientific), mounted onto SuperFrost Plus
857 slides, and dried on a 50 ℃ heating block before staining.

858

859 **Immunofluorescence staining and imaging**
860 A section was rehydrated and washed by 1X PBS for 10 min 3 times, permeabilized in PBST
861 (0.3% Triton X-100 in 1X PBS) for 10 min, and blocked by blocking solution (5% normal BSA
862 in 1X PBS) for 2 hrs in room temperature. Sections were stained with diluted primary antibodies
863 in the blocking solution overnight at 4 ℃. The next day, the sections were washed with PBST for
864 5 min three times and stained with secondary antibodies in blocking solution for 2 hrs in RT.
865 Blocking solution was dropped off from the slides and nuclei staining with DAPI solution
866 (0.1ug/ml of DAPI in PBST) was performed for 15 min. The slides were mounted with DAKO
867 fluorescent mount solution (catalog no. S3023). Zeiss 880 Airyscan Confocal is used for imaging
868 according to the manufacturer's instructions.

869

870 **Antibodies**
871 phospho-S6 (1:800 dilution, catalog no. 5364S ;Cell Signaling, AB_10694233), NeuN (1:100,
872 MAB377X; Sigma-Aldrich, AB_2149209), GFP (1:500, catalog no. GFP-1020, Aves Labs,
873 AB_10000240), Alexa Fluor Goat 488 chicken IgY (H+L) (1:1,000 dilution, catalog no. A-
874 11039, AB_2534096), Alexa Fluor 594 donkey anti-rabbit lgG (H+L) (1:1,000, catalog no.
875 R37119, AB_2556547).

Genotype-phenotype association

The functional modules to be tested were selected based on the enriched GO terms. A given
known and candidate MCD gene was assigned as a member to one or multiple modules based on
GO terms related to the given gene (results summarized in Supplementary Table 3c).
Subsequently, a given patient became a member of one (or multiple) functional module(s) if the
genes detected in that patient were assigned to that (those) functional module(s). To associate
likely oncogenic sSNVs with clinical phenotypes, the cases carrying a (or multiple) sSNV(s)
listed in COSMIC DB were labeled as 'COSMIC'. All available clinical information on the
patient was collected and harmonized using ILAE terms (summarized in Supplementary Table
4). Pearson correlation coefficients were calculated by cor.test() function in R. The value of
correlation coefficients was displayed as colors in the heatmap of Fig. 4 using r-gplots (v3.1.1)
package. If two groups with binary values were used for calculation, Phi coefficient was used.

Single-nucleus RNA sequencing

A fresh-frozen brain tissue (~50 mg) was placed into a glass dounce homogenizer containing 1
ml cold lysis buffer (0.05% (v/v) NP-40, 10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl) and
dounce 10 times with a loose pestle and following 10 times with a tight pestle. The homogenate
was incubated for 10 min in RT. 9 ml of wash buffer (1% BSA in 1X PBS) was added to the
homogenate and filtered by a 30 um cell strainer. The strained homogenate was spun down in
500 g to remove the supernatant. The pellet was resuspended with 5 ml of wash buffer. Straining
and spinning down steps were performed once more, and the pellet was resuspended into 500 ul
of wash buffer. 10 ul of nuclei resuspension was mixed with counting solution (0.02% Tween 20,
0.1ug/ml DAPI, 1% BSA in 1X PBS) and nuclei density was measured by manual nuclei
counting using DAPI signal. The resuspension was diluted by wash buffer to make the desired
concentration (800~1000 nuclei/ul). Maximum 2 samples were pooled together targeting 10000
nuclei per reaction. Gel beads emulsion (GEM) generation, cDNA, and sequencing library
constructions were performed in accordance with instructions in the Chromium Single Cell 3'
Reagent Kits User Guide (v3.1). A library pool was sequenced with 800 million read pairs using
NovaSeq 6000. Age and sex information in specimens used in Fig. 6: 5-year-old (yo) male for
CTRL-8352, 3 yo female for CTRL-8353, 4 month-old female for HME-4688, 3 yo male for
HME-6593 and 1 yo female for TSC-4258. The information for FCD-4512 is in Supplementary
Table 4.

Single-nucleus RNAseq bioinformatics pipeline

Fastq files from single-nucleus libraries were processed through Cell Ranger (v6.0.2) analysis
pipeline with –include-introns option and hg19 reference genome. Pooled library was
demultiplexed and singlets were taken by demuxlet (v1.0). Seurat (v4.0.5) package was used to
handle single nuclei data objects. Nuclei passed a control filter (number of genes > 500, number
of reads >1000, percentage of mitochondrial gene < 10%) was used for downstream analysis.
Protein coding genes were used for further downstream analysis. Data were normalized and
scaled with the most variable 3000 features using the 'SCTransform' functions. Dimensionality
reduction by PCA and UMAP embedding was performed using runPCA and runUMAP
functions. Clustering was performed by FindNeighbors and FindClusters functions. Cell type
identification was performed using known cell type markers expressed in the brain including

921 excitatory (RORB, CUX2, SATB2), inhibitory neuron (*GAD1*, *GAD2*), astrocyte (*SLC1A2*,
922 *SLC1A3*), oligodendrocyte (*MOBP*, *PLP1*), immature oligodendrocyte (*BCAS1*),
923 oligodendrocyte precursor cell (*PDGFRA*), microglia (*PTPRC*), and endothelial cell markers
924 (*CLDN5*, *ID1*) as well as using positive markers found by FindAllMarkers function with 3000
925 most variable features in scaled data. DEG analysis was performed by 'FindMarkers' function in
926 Seurat v4.0 with all genes available in the assay. The genes with adjusted p-value < 0.01 were
927 taken and listed in Supplementary Table 5c. The final visualization of various snRNAseq data
928 was performed by ggplot2 (v3.3.5) and matplotlib (v3.5.0).

929

930 **Weighted gene co-expression network analysis**
931 'r-wgcna' package (v1.69) was used for WGCNA according to instructions[71]. Briefly, a
932 similarity matrix was generated based on Pearson's correlation coefficient value among the top
933 3000 variable features in single-nucleus transcriptome data, which was used to calculate the
934 subsequently signed type of network adjacency matrix. Next, the topological overlap matrix
935 (TOM) and the corresponding dissimilarity (1-TOM) value were generated from the adjacency
936 matrix. Finally gene modules were generated by 'cutreeDynamic' function with 'tree' method,
937 minAbsSplitHeight = 0.9 and minClusterSize = 30 option. Similar gene modules were merged by
938 'mergeCloseModules' function with cutHeight = 0.25.

939

940 **Correlation between cell type and eigengene expression**
941 Pearson correlation coefficient r (>0, negative values were not presented) between a given cell
942 type and net expression levels of a given geneset and Student asymptotic p-value for the
943 correlation were plotted as the colour and size of dots in dot plots, respectively.

944

945 **RNAscope**
946 We used published methods and purchased target probes (FGFR1 (catalog no. 310071-C2),
947 FGFR2 (311171), FGFR3 (310791), EGFR (310061-C4), PDGFRA(604481-C2)) for genes of
948 interest containing an 18-25 base region complementary to the target, as spacer sequencing, and
949 a 14 base Z-tail sequence[72], including RNA pol III positive control and random sequence
950 negative control, following the manufacturer recommendations (Advanced Cell Diagnostics,
951 Hayward, CA). Images were acquired on a Leica STED Sp8 with a Falcon microscope.

952

953 **Permutation analysis for the enrichment of MCD genes**
954 To test the enrichment of differentially expressed MCD genes in RNA sequencing against a
955 random distribution, we designed a permutation analysis. All human genes used in the single-cell
956 RNA-seq analysis (n=19909) were randomly shuffled 10,000 times and the same number of
957 genes as described in the differential expression analysis (Extended Data Fig. 10a) was selected
958 for each shuffle. The number of overlaps between each shuffle and the MCD genes was
959 compared and the number of overlaps was used as the outcome and a null distribution was
960 generated from the 10,000 shuffles. All 75 positively validated MCD genes are confirmed to be
961 existing in the initial gene list. After 10,000 permutations, the permutation p-value was
962 calculated with numbers observed to overlap.

963

964 **Statistics and Reproducibility**

26

965 Statistical analyses were performed by R or Prism 8 (GraphPad Software). Two-way ANOVA
966 and Sidak multiple comparisons were performed in Fig. 3b and Extended Data Fig. 6a with p-
967 values of interaction between genotype and bin factor. Mann-Whitney U-test was performed for
968 Fig. 3c and Extended Data Fig. 6b. The images used in Fig. 3d and Extended Data Fig. 8 were
969 taken from patient-derived tissue slices which are unique and not biologically reproducible.
970 Detailed statistical information is described in Supplementary Table 9.
971

Data availability

973 WES and AmpliSeq data are deployed on NIMH Data Archive under study number 1484
974 "Comprehensive multi-omic profiling of somatic mutations in malformations of cortical
975 development" and SRA under accession number PRJNA821916: "Comprehensive multi-omic
976 profiling of somatic mutations in malformations of cortical development". The BSMN
977 neurotypical brain data are available at NIMH Data Archive (NDA
978 study 644, 792 and 919, https://nda.nih.gov/study.html?tab=result&id=644,
979 https://nda.nih.gov/study.html?tab=result&id=792 and
980 https://nda.nih.gov/study.html?tab=result&id=919) and SRA: PRJNA736951. The raw and
981 processed snRNAseq dataset was deposited in Gene Expression Omnibus (GEO) under
982 accession number GSE218022.
983 gnomAD: https://gnomad.broadinstitute.org/
984 COSMIC: https://cancer.sanger.ac.uk/cosmic/
985 STRING: https://string-db.org/
986 Single-cell RNA-seq data from developing cortex (Nowakowski et al., 2017):
987 https://cells.ucsc.edu/?ds=cortex-dev
988 GRCh37 genome accession number: PRJNA31257
989

Code availability

991 Code to generate the figures and analyze the data are publically available on GitHub[73]
992 (https://github.com/shishenyxx/MCD_mosaic).
993

Method-only references

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Brain Somatic Mosaicism Network

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a

Phase I

80 cases
(75 unsolved +
5 solved)

63 cases
solved or
QNS

54 new
cases

Total=143
13 (9.09 %) solved

Number of case
■ HME (12)
■ Type I (44)
■ Type II (67)
■ Type III (16)
■ No info (4)

Phase II

38 cases
(8 solved +
30 unsolved)

96 cases
solved or
QNS

86 new
cases +
10 ctrls +
2 cases*

Total=134
44 (32.84 %) solved

■ HME (26)
■ Type I (40)
■ Type II (56)
■ Type III (12)
■ No info (0)

Phase III

Total=126 + 10 ctrls
24 (19.05 %) solved

■ HME (2)
■ Type I (38)
■ Type II (63)
■ Type III (12)
■ No info (9)
■ TSC (2)
■ Control (10)

b

Paired samples (brain + saliva/blood)

Sequencing (AmpliSeq, WES)

MuTect2 (SM)

MuTect2 (PM) ∩ Strelka2 | MosaicHunter | BSMN Common pipeline (WES only) | DeepMosaic (WES only)

Tissue-shared/tissue-specific mosaic variant list

Validation/tissue testing through TASseq

c

Unpaired samples (brain only)

Sequencing (AmpliSeq, WES)

MuTect2 (SM)

MuTect2 (SM) | MosaicHunter | BSMN Common pipeline (WES only) | DeepMosaic (WES only)

Tissue-shared/tissue-specific mosaic variant list

Validation/tissue testing through TASseq

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1099 Extended Data Fig. 1   Workflow of genetic discovery and bioinformatic pipeline to detect sSNVs in the
1100 MCD cohort.   (a) Workflow chart describing the flow of cases for each phase of genetic discovery.
1101 QNS: quantity not sufficient. 2 cases labeled by a star are sequenced in phase 1 but not phase 2. (b) The
1102 pipeline for paired samples. Notably, the dashed square indicates that the sharing variants between
1103 MuTect2 paired mode and Strelka2 were used for the downstream analysis. BSMN common pipeline and

1104   DeepMosaic were used only for WES datasets. The DeepMosaic input variants were generated by

1105   MuTect2 single mode. (c) The pipeline for unpaired samples. The pipeline is similar except that MuTect2

1106   single mode without Strelka2 is used. PM: paired mode, SM: single mode.

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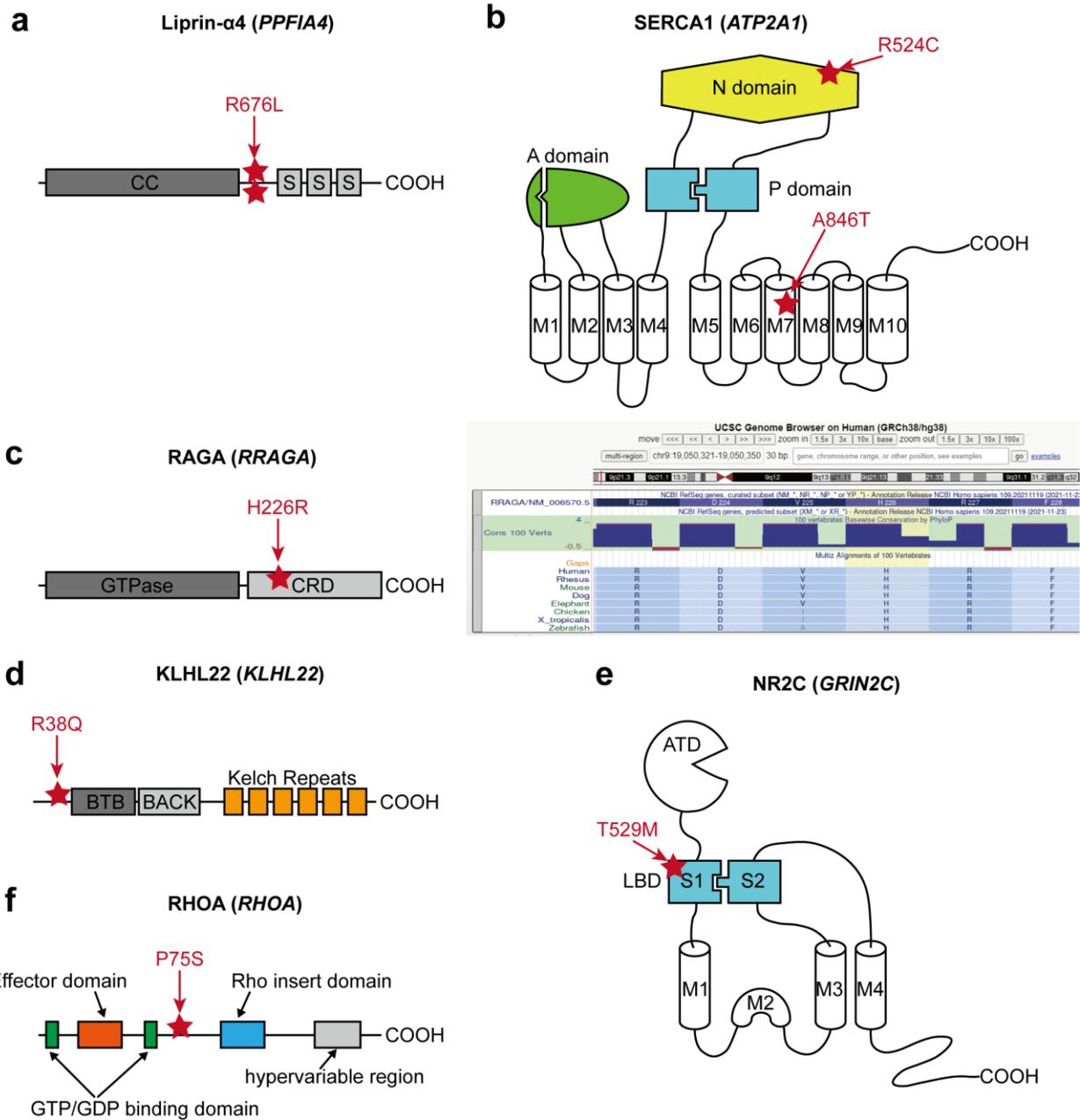
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Extended Data Fig. 2   The locations of the selected MCD variants.   (a) The location of two recurrent SNV calls is at the same position between the coiled-coil domain (CC) and the first SAM domain (S) of Liprin-α4. (b) Two different variants in SERCA1. p.R524C mutation is at the nucleotide ATP-binding (N) domain, whereas the pA846T variant is in the 7th transmembrane (M7) domain. A: Actuator domain, P: Phosphorylation domain, M: Transmembrane domain. (c) Left: The location of p.H226R variant in RAGA protein. GTPase: GTPase domain, CRD: C-terminal roadblock domain. Right: UCSC genome browser screenshot describing that p.H226 is a conserved site across all vertebrates. (d) The location of the p.R38Q variant in the N-terminal region before the BTB (Broad-Complex, Tramtrack, and Bric-à-brac) domain of KLHL22. (e) A variant in the S1 domain of NR2C. S1 and S2 together make the ligand-

1119   binding domain (LBD), the target of glutamate. ATD: Amino-terminal domain. (f) RHOA p.P75S variant

1120   in the interdomain space between the second GTP/GDP binding domain and Rho insert domain.

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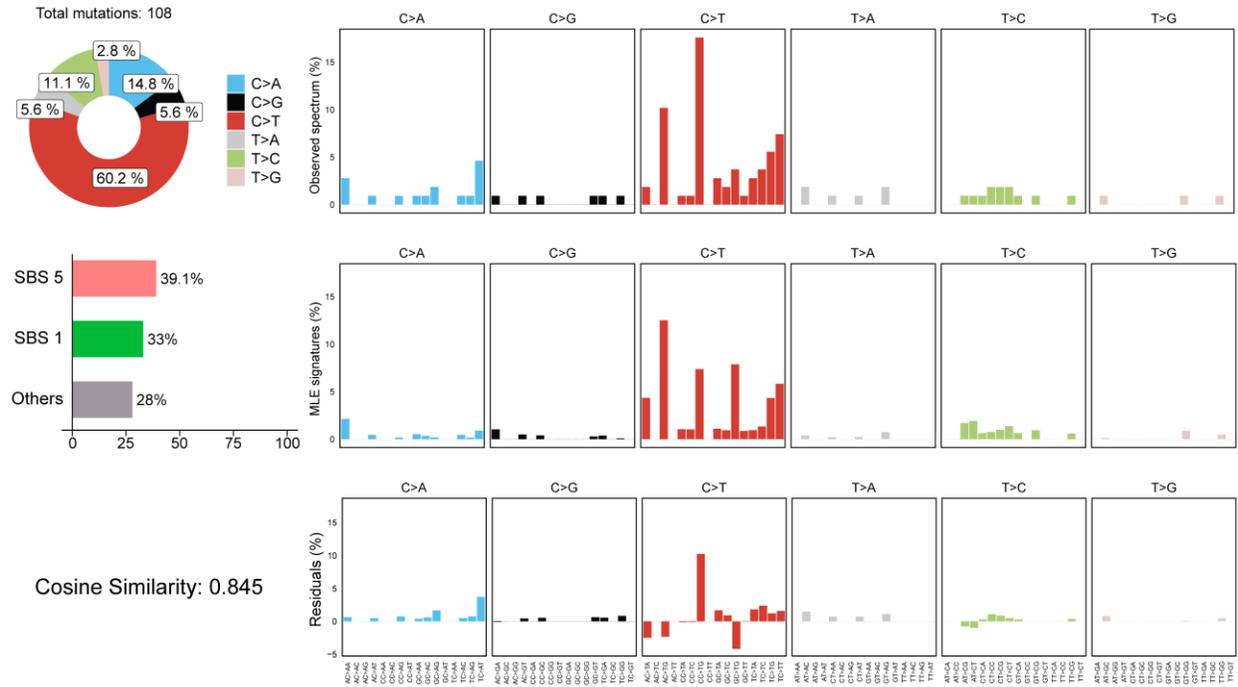
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1125 Extended Data Fig. 3   Mutational signature analysis shows cell-division-related clock-like signatures in
1126 the MCD cohort.   SBS5 (39.1%) and SBS1 (33%) revealed by Mutalisk are clock-like mutational
1127 signatures. SBS1 especially correlates with stem cell division and mitosis.

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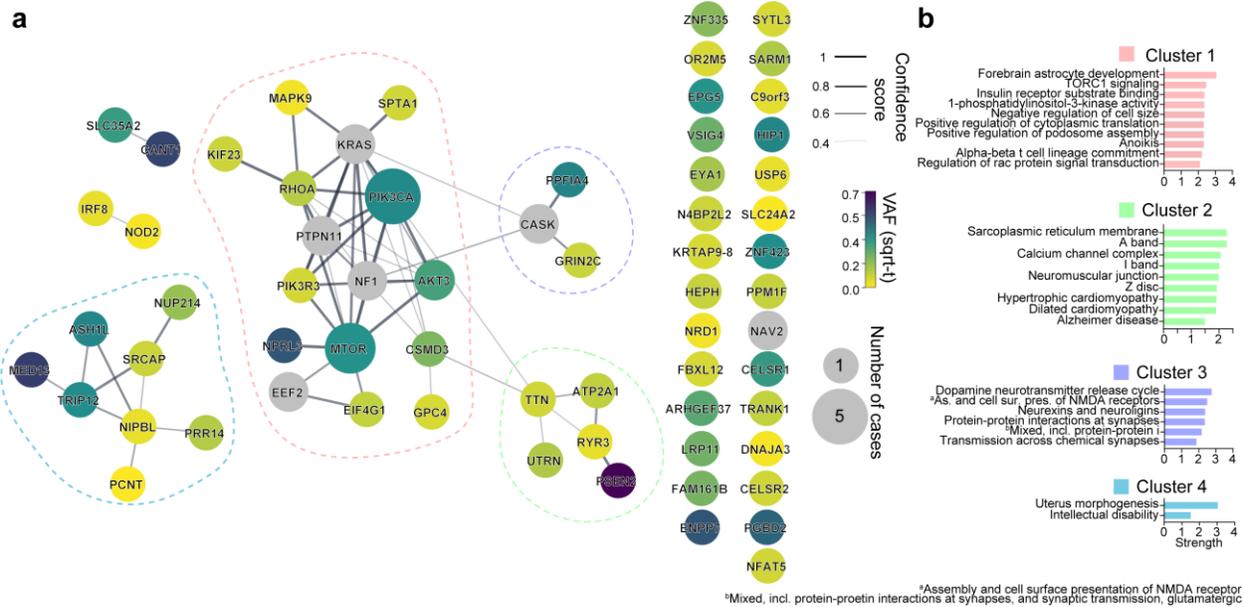
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Extended Data Fig. 4   Four major gene networks were reconstructed from the WES dataset.   (a)
STRING DB pathway analysis of the 59 MCD discovered genes and six novel genes (a total of 65 genes)
from recent publications identifies MTOR/MAP kinase pathway (pink, Cluster 1), Calcium dynamics
(green, Cluster 2), Synapse (purple, Cluster 3), Gene expression (blue, Cluster 4). Edge thickness:
confidence score calculated by STRING. Size and color of a node: square root transformed (sqrt-t)
number of patients carrying a given mutation and average VAF across all patients, respectively. Non-
clustered orphan genes are listed on the right. (b) Gene Ontology (GO) analysis results confirmed the
functions of compositions in each network. Top GO terms or KEGG pathways. Strength calculation and
cluster generation were performed by STRING.

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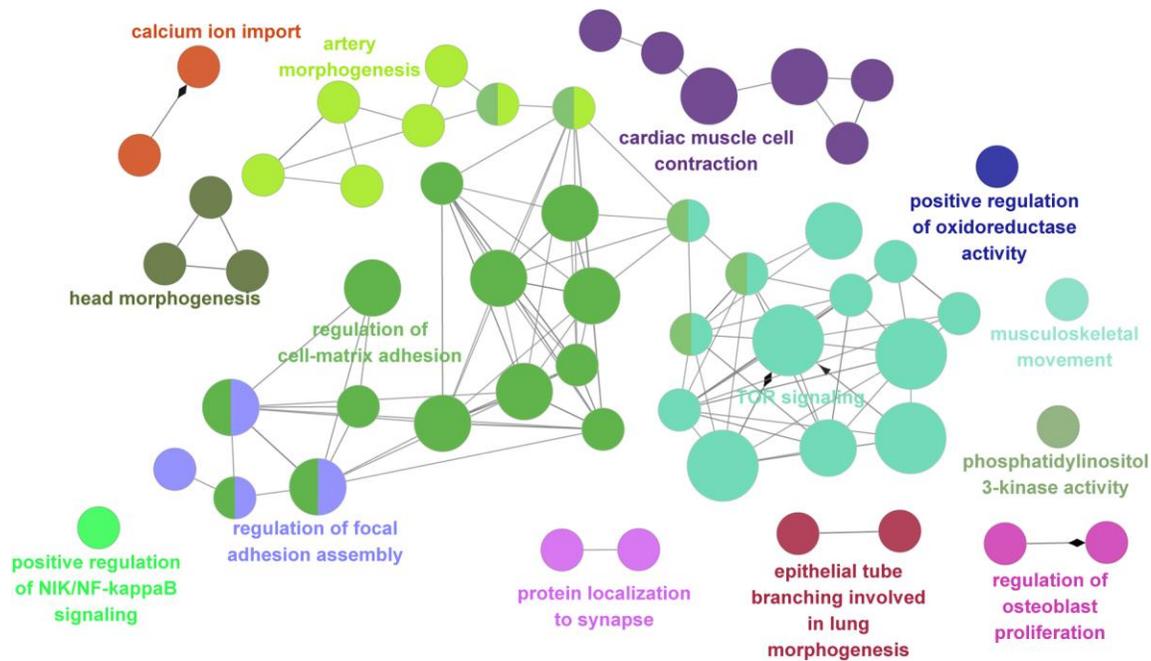
1143

1144 Extended Data Fig. 5 ClueGO analysis using the MCD genes result identifies the biological processes
1145 and molecular pathways. The main cluster is related to TOR signaling, regulation of cell-matrix
1146 adhesion, regulation of focal adhesion assembly, and artery morphogenesis. Notably, there are also
1147 isolated clusters that were not covered in previous studies, for example, cardiac muscle cell contraction,
1148 calcium ion import, and protein localization to the synapse. Corrected p-value with Bonferroni step down
1149 was reflected in node size (two-sided hypergeometric test, Large: $p < 0.0005$, medium: $p < 0.005$, small: p
1150 < 0.05). All p-values are in Supplementary Table 9.

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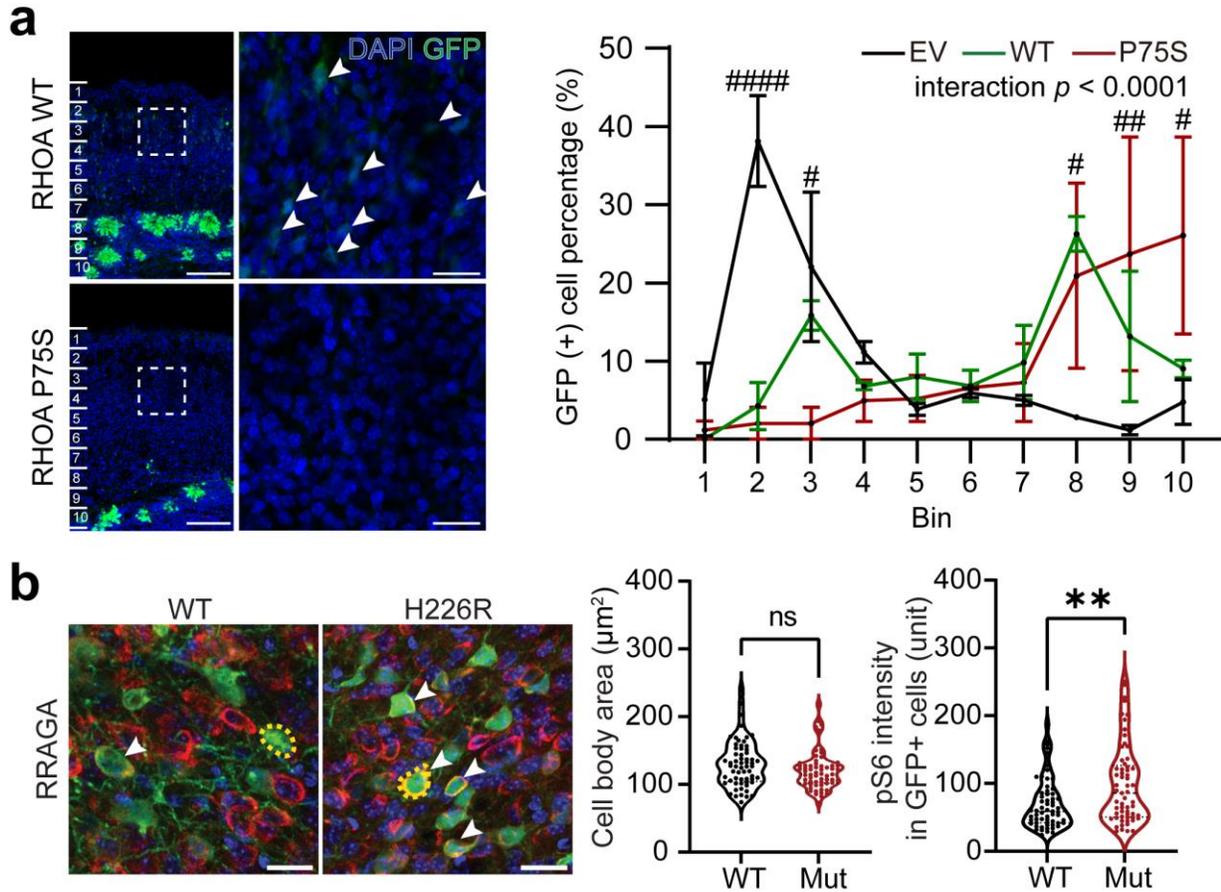
1152

Extended Data Fig. 6   Additional functional analyses for new RRAGA and RHOA mutations.   (a) Over-expression of RHOA WT and P75S mutant form in cortical neurogenic pool induce both significant defects in migration. Notably, some portion of WT form-expressing cells migrate to the superior cortical area (white arrow), whereas mutant form-expressing cells did not show any migrating cells at all. The dashed square area is magnified to the right side images. Scale bar: 100 μm and 20 μm for left and right images, respectively. Right, Quantification of the migration level. EV data was exported from Fig. 3b. Two-way ANOVA and Sidak multiple comparisons with p-values of interaction between genotype and bin factor. Ten bins from the surface of the cortex (top) to SVZ (bottom). n=3, 3, 2 biologically independent mice for EV, RHOA WT and RHOA P75S, respectively. Mean ± SEM. (b) Immunofluorescence in postnatal day 21 mouse cortices for RRAGA wild-type (WT) or mutant isoform. Yellow dashed lines: examples of cell body size quantification. Dashed lines and dotted lines in the violin plots indicate median and quartiles, respectively. Two-tailed Mann-Whitney U-test. Scale bar: 20 μm. n=61 cells (3 mice), 61 (2) for RRAGA WT and RRAGA H226R, respectively * or # indicates a p-value in comparison between WT and mutant group, or EV and mutant group respectively. ####$p < 0.0001$; ##$p < 0.01$; #$p < 0.05$; **$p < 0.01$; ns, non-significant.

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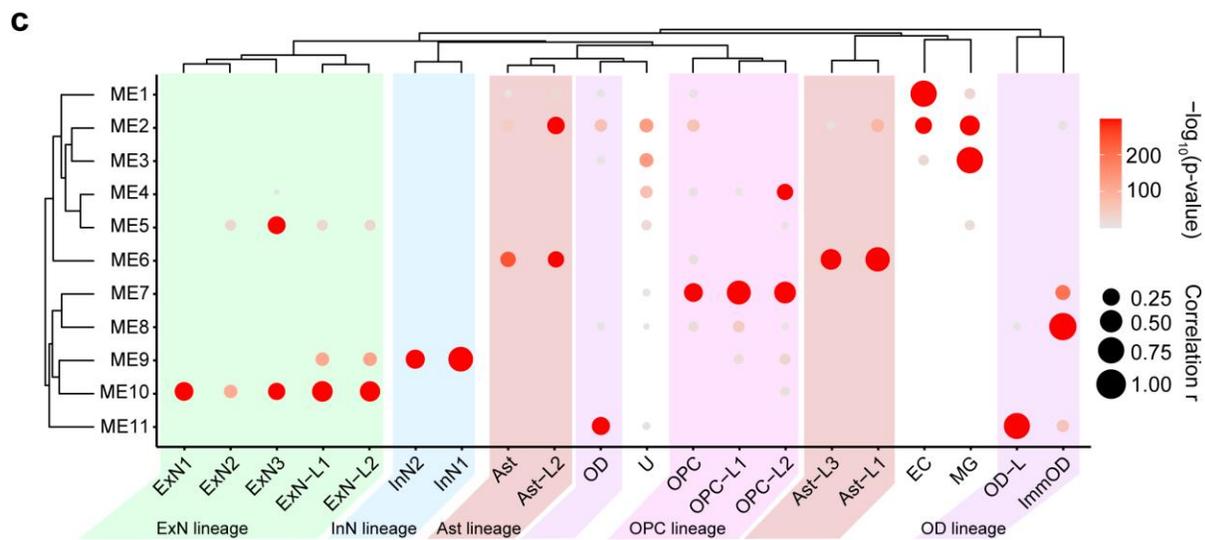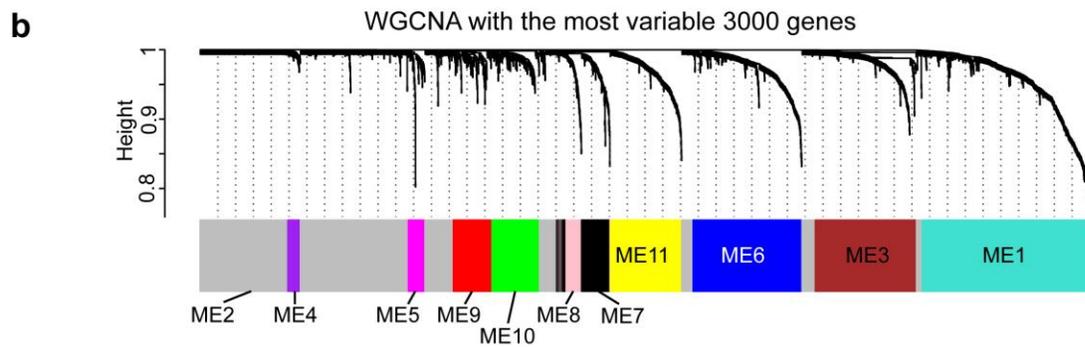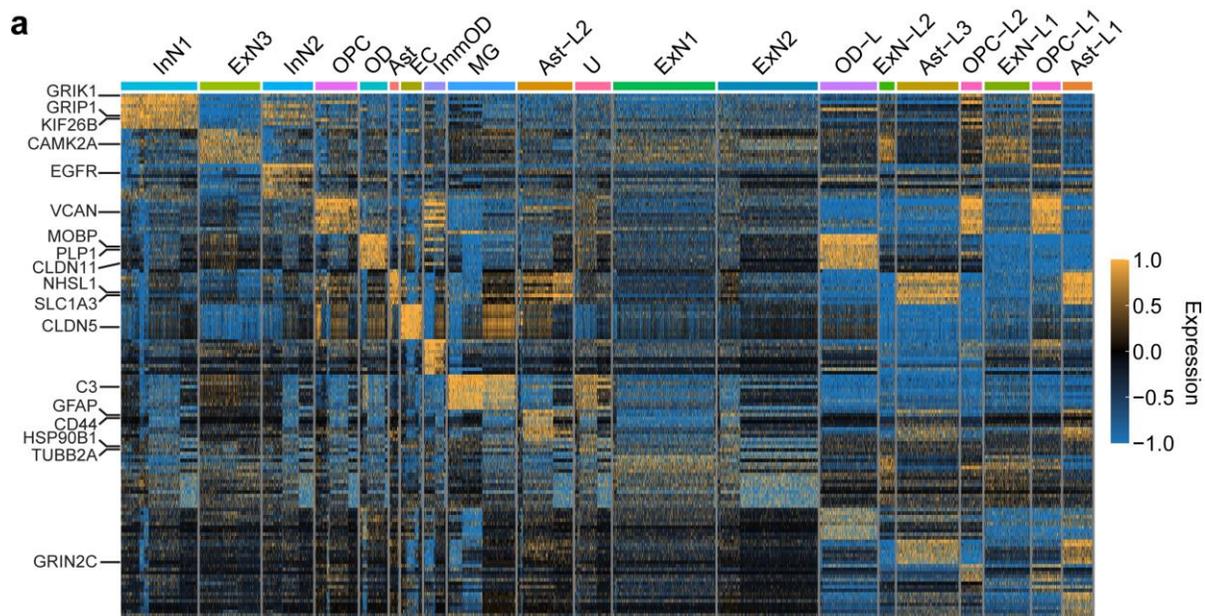
Extended Data Fig. 7   Cell-type identification by DEGs and WGCNA in the MCD snRNAseq dataset. (a) DEG analysis using FindAllMarker function in Seurat v4 package. The top 10 genes for each cluster were presented. Some notable marker genes are presented on the left side. Color: scaled gene expression level. (b) Description of WGCNA. The most variable 3000 genes were used for generating six

1174   co-expression module eigengenes (ME1 to ME11). The members of each ME are described in

1175   Supplementary Table 5b. (c) Enrichment of module eigengenes in cell type clusters. Atypical clusters

1176   showing similar patterns with a normal cell cluster were classified as the same lineage. We identified 5

1177   different lineages (Ast, OD, ExN, InN, OPC) coded as different colors. Notably, Ast-L1/2/3 and OPC-

1178   L1/2 show excessively increased expression of ME6 or ME7, Ast or OPC signature ME, respectively.

1179   OPC-L2 shows upregulation of ME4, related to the cell cycle, implying that HME has many over-

1180   proliferating OPC-L cells. Excitatory neuronal lineage typically expresses ME5 and ME10, but ExN-L1/2

1181   also shows increased expression of ME9, a signature of inhibitory neurons, compared to ExN1/2/3. OD-L

1182   cells are classified as OD lineage because they express excessive ME11, a signature to OD. U cluster,

1183   dominant in TSC, does not show a clear signature. The size and color of the dot plot are the Pearson

1184   correlation coefficient and corresponding non-adjusted asymptotic p-value derived from a two-sided

1185   Student's t-test, respectively.

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a

H&E   RNAscope
                                 DAPI FGFR3 PDGFRA

b

H&E   RNAscope
                                 DAPI FGFR2 FGFR1 EGFR

100 um
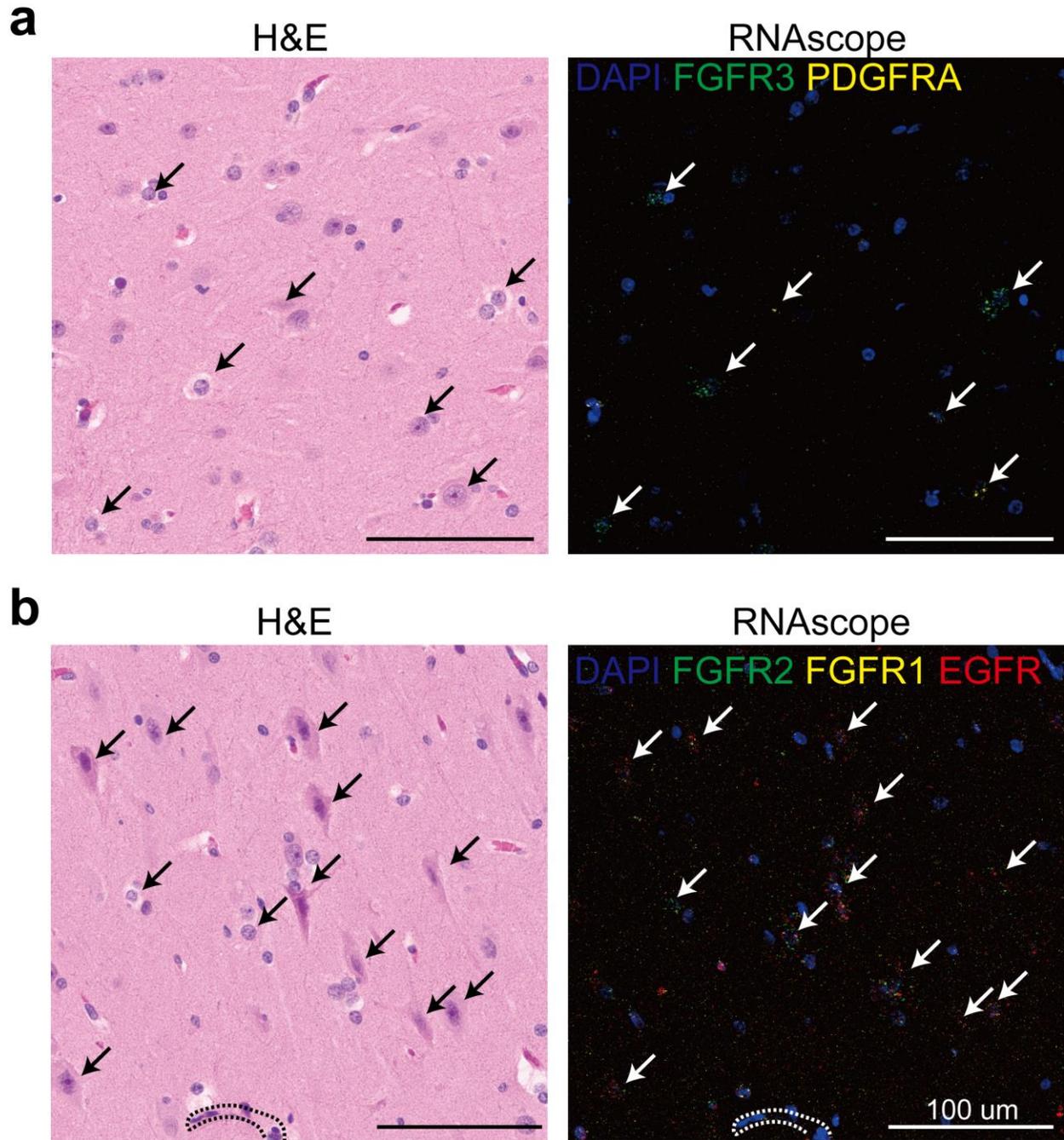
Extended Data Fig. 8   The validation of the snRNAseq result from HME-6593 shows that MCD dominant clusters are highly correlated with dysplastic cells in MCD.   (a-b) H&E (left) and RNAscope (right) for genes expressed highly in MCD brain (FGFR2, FGFR1, EGFR, top) or (FGFR3, PDGFRA bottom). Dashed lines: blood vessels. White/black arrows: dysplastic cells. One representative section is shown for each probe combination.
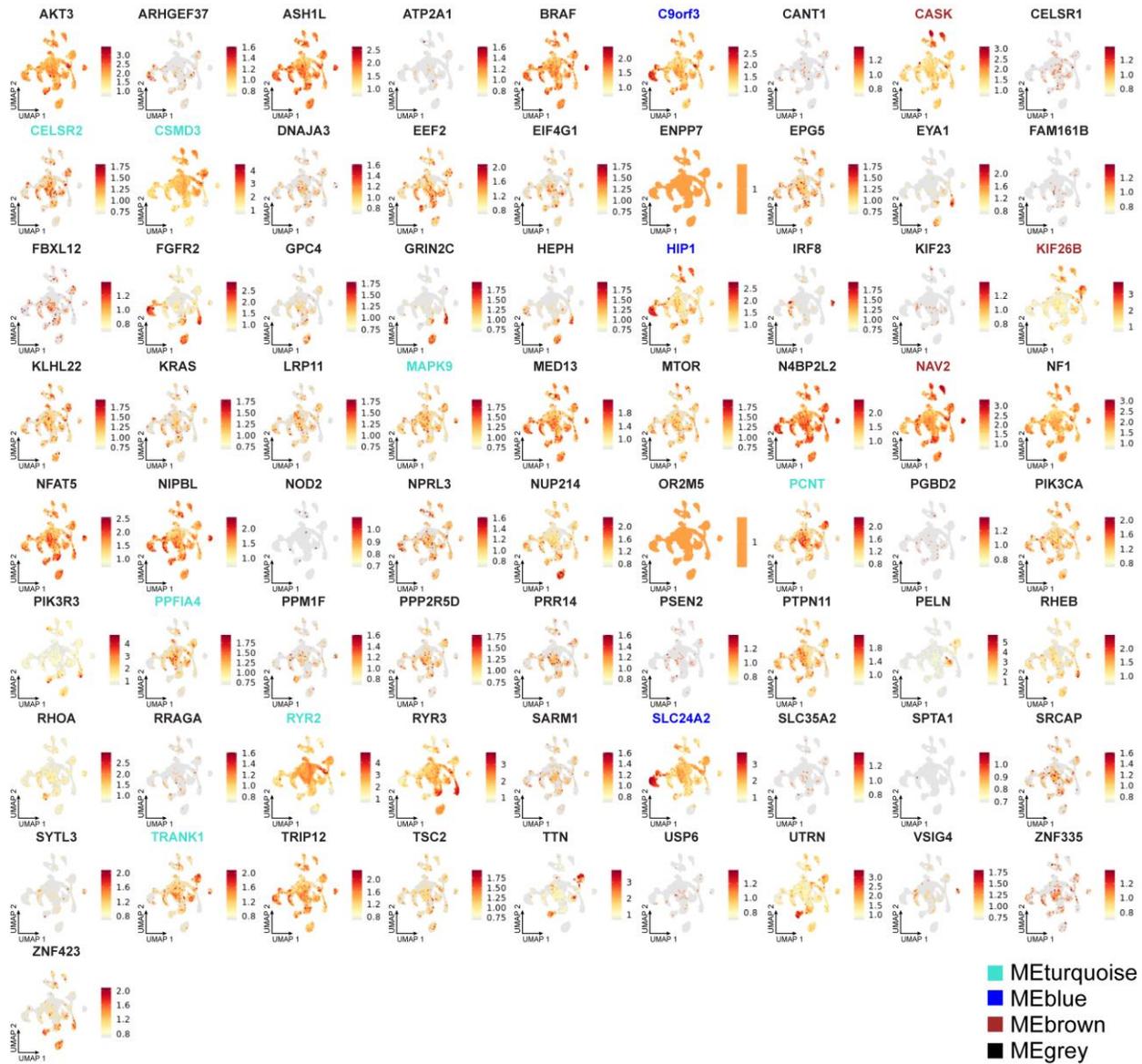
Extended Data Fig. 9   Expression patterns of individual MCD genes in the MCD snRNAseq dataset.
The gene members of each eigen module shown in Fig. 6d were colored according to the name of a given eigengene.

a

HME vs CTRL

59 | 16 | 1719

p = 0.0006

FGFR2 GRIN2C PIK3R3
CASK RYR3 HIP1 SLC24A2
LRP11 RYR2 TRANK1 PCNT
PTPN11 MAPK9 CSMD3 RELN TTN

TSC vs CTRL

65 | 10 | 1007

p = 0.0037

RYR2 RYR3
LRP11 PTPN11 NFAT5 HIP1
SLC24A2 KIF26B UTRN TTN

FCD vs CTRL

68 | 7 | 974

p = 0.0768

NAV2 ASH1L KIF26B
LRP11 SLC24A2 NFAT5 CSMD3

b

CTRL   HME

HME vs CTRL in Ast lineage
- acyl-CoA desaturase activity
- Farnesyl diphosphate metabolic process
- Glutamate catabolic process
- Regulation of cholesterol biosynthetic process
- Alpha-linolenic acid metabolic process
- Fatty acid biosynthesis
- Pyramidal neuron differentiation
- Terpenoid backbone biosynthesis
- Long-chain fatty-acyl-coa biosynthetic process
- Regulation of postsynaptic membrane organization

2.0 1.5 1.0 0.5 0.0

CTRL   TSC

TSC vs CTRL in Ast lineage
- Ryanodine-sensitive calcium-release channel activity
- Calcium-induced calcium release activity
- Kainate selective glutamate receptor activity
- Kainate selective glutamate receptor complex
- Ionotropic glutamate receptor activity
- Ionotropic glutamate receptor complex
- Sodium channel complex
- Tran.-gated ion channel activity ... postsynaptic membrane potential[a]
- Sodium channel activity
- Integral component of postsynaptic specialization membrane

2.5 2.0 1.5 1.0 0.5 0.0

CTRL   FCD

FCD vs CTRL in ExN lineage
- Calcium channel complex
- SRP-dependent cotranslational protein targeting to membrane
- Cytosolic ribosome
- Establishment of protein localization to endoplasmic reticulum
- Nuclear-transcribed mrna catabolic process, nonsense-mediated decay
- Regulation of mrna splicing, via spliceosome
- Viral transcription
- Large ribosomal subunit
- Postsynaptic specialization membrane
- Postsynaptic density membrane

1.5 1.0 0.5 0.0
Strength

[a]Transmitter-gated ion channel activity involved in regulation of postsynaptic membrane potential

Extended Data Fig. 10   Functional implication of MCD genes in MCD snRNAseq dataset.   (a) The 75 MCD genes overlap with DEGs of MCDs in contrast to controls. p-values derived from permutation tests (10,000 times) show a chance to show this overlap in a random sampling of DEGs from 19909 protein-coding genes used in these DEGs. Red or blue coloring of gene names indicates upregulated or downregulated DEGs in MCDs compared to CTRLs, respectively. HME and TSC show significant overlap with the MCD genes whereas an FCD case with low VAF did not, which is probably because of low VAF. One-sided permutation test. (b) Cell-lineage specific DEGs compared to the according to normal cell lineage of CTRL represent alterations of mTOR downstream pathways, calcium dynamics, and synaptic functions across. Red dots in UMAPs indicate the cells that participated in the comparison. Top 10 enriched GO or KEGG terms representing lineage-specific DEGs.