



# Mosaic Matters: A Transcriptomic Window into FCDII Epileptogenesis

## Cell-type-informed genotyping of mosaic focal epilepsies reveals cell-autonomous and non-cell-autonomous disease-associated transcriptional programs

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While it is widely accepted that somatic variants that activate the PI3K-mTOR pathway are a major cause of drug-resistant focal epilepsy, typically associated with focal cortical dysplasia (FCD) type 2, understanding the mechanism of epileptogenesis requires identifying genotype-associated changes at the single-cell level, which is technically challenging with existing methods. Here, we performed single-nucleus RNA-sequencing (snRNA-seq) of 18 FCD type 2 samples removed surgically for treatment of drug-resistant epilepsy, and 17 non-FCD control samples, and analyzed additional published data comprising >400,000 single nuclei. We also performed simultaneous single-nucleus genotyping and gene expression analysis using two independent approaches: 1) a method that we called genotyping of transcriptomes enhanced with nanopore sequencing (GO-TEN) that combines targeted cDNA long-read sequencing with snRNA-seq, 2) ResolveOME snRNA-seq and DNA genotyping. snRNA-seq showed similar cell identities and proportions between cases and controls, suggesting that mosaic pathogenic variants in PI3K-mTOR pathway genes in FCD exert their effect by disrupting transcription in conserved cell types. GO-TEN and ResolveOME analyses confirmed that pathogenic variant-carrying cells have well-differentiated neuronal or glial identities, with enrichment of variants in cells of the neuroectodermal lineage, pointing to cortical neural progenitors as possible loci of somatic mutation. Within FCD type 2 lesions, we identified upregulation of PI3K-mTOR signaling and related pathways in variant-carrying neurons, downregulation of these pathways in non-variant-carrying neurons, as well as associated changes in microglial activation, cellular metabolism, synaptic homeostasis, and neuronal connectivity, all potentially contributing to epileptogenesis. These genotype-specific changes in mosaic lesions highlight potential disease mechanisms and therapeutic targets.

## Commentary

Focal cortical dysplasia type II (FCDII) is a malformation of cortical development that is highly associated with drug-resistant epilepsy requiring neurosurgical treatment in children.<sup>1</sup> FCDII is caused by postzygotic somatic variants in PI3K-mTOR pathway genes, leading to focal mosaic lesions containing both variant-carrying and non-variant-carrying cells.<sup>2</sup> The PI3K-mTOR pathway regulates numerous cellular functions, including cell growth, protein synthesis, and metabolism, and all known FCDII-associated variants hyperactivate mTOR signaling.<sup>2</sup> Histopathologically, FCDII lesions are characterized by loss of cortical laminar organization and presence of dysmorphic neurons (enlarged, misoriented neurons with abnormal morphology and mTOR hyperactivation) and balloon cells (immature, uni- or multi-nucleated cells co-expressing neuronal and glial markers). Despite

major advances in understanding the genetic and molecular basis of FCDII over the past decade, the mechanisms underlying epilepsy development remain unclear. From a genetic perspective, understanding when and where the somatic variants arise, and how they shape cell identities and cell type-specific transcriptional programs in mosaic human brains, could help illuminate epileptogenic processes in FCDII. Addressing these questions requires advanced genetic approaches such as parallel DNA and RNA analyses from single nuclei, which remain technically challenging.

In the present study, Bizzotto *et al* combined single-nucleus transcriptomics with cell-type-resolved genotyping to interrogate the cellular origins of the pathogenic variants and how they impact transcriptional programs in variant-carrying and non-variant-carrying cells in FCDII lesions.<sup>3</sup> The authors performed single-nucleus RNA-sequencing (snRNA-seq) analyses





on 22 FCDII surgical samples, 21 neurotypical cortex controls, and 4 non-lesional epilepsy controls – the latter is critical for distinguishing FCDII-specific alterations from global epilepsy-related changes. Variants in PI3K-mTOR pathway genes, including *PIK3CA*, *MTOR*, *TSC1*, *AKT3*, and *DEPDC5*, were present in 15 FCDII samples. Cell-type-resolved, genotype-informed transcriptional analyses were performed using two approaches: GO-TEN, a novel method developed by the authors combining targeted cDNA long-read sequencing with snRNA-seq, and ResolveOME, an established parallel snRNA-seq and DNA genotyping workflow.

Global cell type analyses showed no novel cell types or shifts in cell type composition in FCDII lesions compared to control cases. The absence of distinct cell clusters corresponding to dysplastic neurons and/or balloon cells is somewhat surprising given their apparent morphological and functional abnormalities. Bizzotto *et al* postulated that these results could be due to the rarity of these cells in tissue, or loss of these cells during sample preparations or data filtering due to their atypical properties. Notably, these findings were replicated even when the analysis was restricted to samples with a high abundance of variant-carrying cells, performed with an alternative data integration method, and conducted without standard filtering. Thus, the study provides compelling evidence that the variants do not generate new, disease-associated cell identities. Rather, the variants seem to disrupt function within conserved cell types.

Cell-type-informed genotyping of FCDII lesions carrying *PIK3CA* and *MTOR* somatic variants confirmed that variant-carrying cells have well-differentiated cell identities and are restricted to cells of the neuroectodermal lineage, including cortical excitatory neurons, interneurons, oligodendrocytes, and astrocytes. Variants were particularly enriched in excitatory neuron subtypes, supporting the prevailing view that most variants arise in dorsal telencephalic radial glia giving rise to cortical excitatory neurons and glia.<sup>4–6</sup> The presence of variants in interneurons, canonically derived from the ganglionic eminences, is unexpected yet corroborates prior reports.<sup>4,7</sup> One explanation for this is that some interneurons might have a dorsal telencephalic origin, a theory that is supported by recent studies demonstrating human cortical progenitor cells can produce interneurons.<sup>8,9</sup> Alternatively, these variants may arise even earlier, in a forebrain founder progenitor common to both neuronal subtypes. This study found no evidence for variants in mesoderm-derived microglia, contrary to a previous report.<sup>7</sup> The reasons for this discrepancy are unknown and need further investigation.

Importantly, comparisons between variant-carrying and non-variant-carrying neurons revealed several genotype-specific transcriptomic alterations. Variant-carrying neurons showed upregulation of PI3K-mTOR signaling and pathways related to cell growth and metabolism. Notably, these same pathways were globally downregulated in non-variant-carrying neurons within FCDII lesions. Variant-carrying neurons also showed upregulation of inflammatory and immune activation pathways, as well as pathways associated with synapses and epilepsy. CellChat analyses of intercellular communication

networks further identified a global decrease in neuronal connectivity stability in FCDII lesions compared to controls, with more pronounced alterations in variant-carrying neurons. Increased microglial immune activation was also observed in FCDII lesions compared to both neurotypical controls and non-lesional epilepsy samples, suggesting this is an FCDII-specific rather than a global epilepsy-related effect.

These transcriptomic findings support a model in which mosaic somatic variants drive widespread neural circuit dysfunction by disrupting gene expression profiles in variant-carrying cells and surrounding non-variant-carrying cells. These observations corroborate the current consensus of a multifaceted mechanism of epileptogenesis involving both cell-autonomous and non-cell-autonomous disease-associated processes in FCDII. Notably, while the present study identifies several interesting genotype-specific transcriptional alterations, the functional significance of these changes – whether they causally contribute to epileptogenic mechanisms, serve as compensatory responses, or represent disease epiphenomena – critically remains to be established. Additionally, the cellular heterogeneity and histological complexity of mosaic FCDII lesions raise the possibility that gene expression profiles may be further influenced by the spatial positioning of specific cells, an aspect not captured by this study. Spatial transcriptomics approaches to profile dysmorphic neurons and balloon cells have been employed in recent studies,<sup>7,10</sup> and broader applications incorporating single-cell spatial analyses of variant-carrying cells and surrounding cell types will be valuable. Beyond transcriptional analyses, parallel investigations into translational, post-translational, and epigenetic modifications would yield a more complete understanding of disease pathogenesis. Single-cell and spatially resolved human multiomics analyses, alongside functional studies in animal and cellular models, are therefore essential next steps for establishing the biological relevance of variant-induced molecular and functional changes and elucidating epileptogenic mechanisms in FCDII.

From a clinical perspective, one important unresolved question is whether distinct PI3K-mTOR variants drive epileptogenesis through divergent mechanisms across the human FCDII spectrum. In the present study, pooling of multiple variants in the genotype-informed snRNA-seq analyses increases sensitivity and statistical power but inherently obscures variant-specific effects. Future investigations delineating transcriptomic alterations driven by individual variants could yield valuable insights into variant-specific disease mechanisms, which have important implications for developing targeted therapeutics for epilepsy.

In summary, this study offers important insights into the molecular genetics of FCDII, highlighting the complex, multifaceted transcriptional landscape underlying this disorder. While the functional impacts of the transcriptional changes remain to be established, comprehensive transcriptomic analyses in human FCDII tissue, as performed by Bizzotto *et al*, lay the groundwork for mechanistic investigations, guide the development and refinement of experimental models, and

ultimately, help accelerate progress towards meaningful therapeutic advances for individuals with FCDII-associated epilepsy.

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