

CELLULAR NEUROSCIENCE

Defective EV-mediated transport of SHH alters neural fate specification in EPM1 epilepsy

Andrea Forero^{1†}, Veronica Pravata^{1†}, Fabrizia Pipicelli², Elisa Frenna^{1,2,3,4}, Alessandro Soloperto², Marta Ianni^{1,5}, Natalia Abate^{1,5}, Francesco Di Matteo², Zagorka Bekjarova², Laura Canafoglia⁶, Francesca Ragona⁶, Giuseppina Maccarrone², Mariano Gonzalez Pisfil⁷, Christian Wahl-Schott⁸, Filippo M. Cernilogar^{9,10}, Matthias Eder², Rossella Di Giaimo^{1,2,5*}, Silvia Cappello^{1,2,11*}

The extracellular milieu, including extracellular vesicles (EVs), plays a pivotal role in brain development. In this study, we sought to elucidate the pathogenesis of progressive myoclonus epilepsy type 1 (EPM1), a disease caused by mutations in the *CSTB* gene, using cerebral organoids (COs) derived from patient cells. The results demonstrate that EPM1 COs display increased electrophysiological activity and disrupted excitatory/inhibitory (E/I) balance. Single-cell RNA sequencing analysis of ventral EPM1-COs revealed an abnormal specification of progenitor fate, with a shift toward dorsal neuron identities. We demonstrated that this misspecification is driven by a functional alteration of the ventral signaling niche, resulting from impaired EV dynamics and altered protein cargo. Mechanistically, we identified Sonic Hedgehog (SHH) as a direct physical interactor of *CSTB* and demonstrated that *CSTB* deficiency leads to reduced SHH content and secretion. Our findings establish *CSTB* as a safeguard of ventral patterning and identify the *CSTB*-SHH-EV axis as a potential therapeutic target for mitigating the E/I imbalance associated with EPM1.

INTRODUCTION

The extracellular space, encompassing the extracellular matrix (ECM), soluble molecules and extracellular vesicles (EVs), is critical for brain development, influencing cellular processes such as proliferation, differentiation and migration (1, 2). A clear example of its importance is observed during cortical development, where both excitatory projection neurons (ENs) and inhibitory interneurons (INs) rely on cues from the extracellular environment to reach their proper locations within the cerebral cortex. ENs migrate radially from the dorsal pallium into the cortical plate, while INs are generated in the ventral telencephalon and migrate tangentially to the neocortex (3–5). Their migration is directed by a complex network of extracellular signals, including molecules and proteins secreted into the extracellular space via EVs (1, 6, 7). Disruptions within the extracellular space can lead to significant neuronal dysfunction (8), which may interfere with proper neurodevelopment and contribute to a range of neurological conditions, including autism spectrum disorders and intellectual disabilities (9). Progressive myoclonus epilepsy type I (EPM1), while not directly classified as a neurodevelopmental disorder, shares common features of neuronal dysfunction often observed in these disorders. It is the most prevalent type of progressive myoclonus

epilepsy (PME), a group of inherited disorders marked by myoclonus, epilepsy, and progressive neurological decline (10). EPM1 is inherited in an autosomal recessive manner and is primarily caused by biallelic mutations in the *CSTB* gene, which encodes Cystatin-B, a ubiquitous 98-amino acid protein (11). The majority of patients with EPM1 (~90%) carry a 12-nucleotide repeat expansion mutation in the promoter region of the *CSTB* gene, leading to a notable reduction in *CSTB* transcript and protein levels, with levels reduced to less than 10% of normal (12). The clinical manifestations of EPM1 vary based on the genetic mutations. Patients with compound heterozygous mutations, involving the repeat expansion and a truncating null mutation, exhibit a severe form of the disease, characterized by earlier onset and accelerated progression (13, 14). Homozygous patients with two null mutations present the most severe form of neonatal-onset progressive encephalopathy (15, 16). These variations highlight the crucial role of *CSTB* in normal brain development. *CSTB* was initially identified as an inhibitor of cysteine proteases, essential for the regulation of cellular proteostasis by protecting against proteases leaking from lysosomes (17). Beyond this, *CSTB* protein has a proposed chaperone-like function (18, 19) and is also involved in various cellular processes, including modulating chromatin-associated proteolytic activity during neurogenesis (20), interacting with cytoplasmic proteins related to growth and differentiation (21), mTOR signaling (22), and autophagy (23, 24). *CSTB* also plays a substantial role in synaptic plasticity (25–28). In *CSTB* knockout (*Cstb*^{−/−}) mice, which serve as a model for EPM1, early gene expression alterations in the cerebellum and granule neurons have been associated with early synaptic changes and inflammation (29). These alterations suggest disrupted synaptic maturation, with a particular emphasis on GABAergic signaling. Impaired GABAergic inhibition has been documented in patients with EPM1 (30, 31), who also exhibit electroencephalogram abnormalities and progressive cerebral and cerebellar atrophy (29, 32–34). This underscores *CSTB*'s crucial role in maintaining neural function. Our previous research demonstrated that *CSTB* is not only essential for intracellular regulation but also secreted to instruct neighboring cells during neurogenesis (35).

¹Physiological Genomics, Biomedical Center (BMC), LMU Medizin, LMU Munich, Munich, Germany. ²Max Planck Institute of Psychiatry, Munich, Germany. ³International Max Planck Research School for Translational Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany. ⁴Graduate School of Systemic Neurosciences (GSN), Ludwig-Maximilians-University, Munich, Germany. ⁵Department of Biology, University of Naples Federico II, Naples, Italy. ⁶Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy. ⁷Core Facility Bioimaging and Walter-Brendel-Centre of Experimental Medicine, Biomedical Center, Ludwig Maximilian University, Munich, Germany. ⁸Division of Cardiovascular Physiology and Pathophysiology, Biomedical Center (BMC), LMU Medizin, LMU Munich, Munich, Germany. ⁹Division of Molecular Biology, Biomedical Center (BMC), LMU Medizin, LMU Munich, Munich, Germany. ¹⁰Department of Science and Technological Innovation, University of Piemonte Orientale, Alessandria, Italy. ¹¹Munich Cluster for Systems Neurology (SyNergy), Munich, Germany.

*Corresponding author. Email: digiaino@unina.it (R.D.G.); silvia.cappello@bmc.med.lmu.de (S.C.)

†These authors contributed equally to this work.

Functional CSTB levels influence the expansion of progenitor, and pathologically low CSTB leads to reduced recruitment of interneurons while driving premature differentiation of progenitors (35). Together, these findings highlight the multifaceted roles of CSTB in neural development and function.

Given that IN migration is altered in EPM1 and considering CSTB's role in extracellular signaling, we hypothesize that these alterations could extend beyond migration, potentially affecting interneuron development itself. Aberrant CSTB signaling may therefore lead to broader defects in IN maturation and function, contributing to the overall pathology of EPM1. In this study, we use human cerebral organoids (COs) generated from EPM1 patient lines (EPM1-COs) to model the disease. We show that EPM1-COs can recapitulate the pathologically altered electrophysiological properties observed in patients with EPM1. We then focus on ventrally patterned COs, which give rise to INs, and demonstrate that EPM1 ventral organoids exhibit altered patterning, leading to an impaired excitatory-inhibitory (E/I) balance. We propose that CSTB alters the extracellular environment through changes in the biogenesis and composition of EVs, particularly by modulating Sonic Hedgehog (SHH) levels within these vesicles. This altered SHH signaling via EVs is likely a critical factor in influencing neural differentiation and fate, thereby playing a notable role in the pathogenesis of EPM1.

RESULTS

Physiological alteration of neuronal activity in EPM1-COs is caused by changes in the neuronal fate

To investigate the mechanisms underlying the increased neuronal excitability and disrupted E/I balance observed in EPM1, we generated COs from two patient and two control induced pluripotent stem cell (iPSC) lines. Given that robust, spontaneously firing neuronal networks require extended maturation (36), we characterized organoids at late developmental stages (8 months to 1 year), hereafter referred to as “mature organoids.” Our findings revealed that mature EPM1-COs exhibit significantly higher electrophysiological activity compared to two control lines (CTRL-COs), as indicated by spike distribution and average spike number (Fig. 1, A to D, and fig. S1, A to E). Specifically, EPM1-COs showed a greater percentage of high-frequency spikes, defined as spikes with an interspike interval below 500 ms compared to CTRL-COs (Fig. 1D and fig. S1D). The complementary analysis of mature organoids confirmed this persistent E/I imbalance driven by a fundamental shift in neural cell fate (fig. S1, F to K), directly correlating the loss of inhibitory identity and aberrant excitatory specification with the observed functional hyperexcitability. This points to altered subtype maturation, consistent with the hypersynchronous firing recorded in these tissues.

The observed hyperactivity in EPM1-COs may result from an imbalance between excitatory and inhibitory neurons, which is a common feature in epileptic conditions (37). To investigate this possibility, we examined the differentiation fate of unpatterned neural progenitor cells (NPCs) derived from patient or control cells. After 8 weeks of differentiation, we found a significant increase in the number of excitatory marker-expressing neurons in EPM1 cultures, along with a corresponding decrease in inhibitory marker-expressing neurons compared to controls (Fig. 1, E to H, and fig. S2, A to H). These findings suggest that the hyperactivity observed in EPM1-COs could be driven by an imbalance in the production of neuronal subtypes, with a shift favoring excitatory over inhibitory neurons. To further

validate these results, we generated neurons derived from both ventral (vNeurons) and dorsal (dNeurons) progenitors, dissociated from 30-day-old patterned EPM1- and CTRL-COs. Dorsally regionalized COs mimic the cellular architecture of the dorsal telencephalon, resulting in an enrichment of excitatory neurons. In contrast, ventrally regionalized COs (vCOs) resemble the ganglionic eminences, which predominantly give rise to inhibitory interneuron populations (38). This approach allows us to directly compare the distinct neuronal subtypes influenced by regional patterning, providing insights into how EPM1 affects both excitatory and inhibitory specification. In line with the previous results, vNeurons from ventrally patterned EPM1 COs (EPM1-vCOs) exhibited a different proportion of GAD67-positive (inhibitory) and TBR1-positive (excitatory) neurons compared to controls (Fig. 1, I, J, L, and M, and fig. S2I). Specifically, ventral EPM1 progenitors yielded fewer GAD67-positive neurons and more TBR1-positive neurons than controls, while dorsally derived progenitors did not exhibit significant differences in neuronal specification when compared to control NPCs (Fig. 1, I to N, and fig. S2, I and J). To assess whether EPM1 vNeurons exhibited heightened neuronal activity driven by the altered differentiation of ventral progenitors, we used multielectrode array (MEA) analysis. For MEA recordings, we focused on the channel with the highest activity to record the spike activity and investigate potential alterations in the neuronal network properties. The analysis confirmed heightened neuronal activity in EPM1 patient-derived cultures after 10 weeks of differentiation (fig. S2, K to M). When combining dorsal control cells with ventral EPM1 cells, EPM1 vNeurons exhibited markedly higher activity than their control counterparts (fig. S2, N and O). These findings suggest that ventral NPCs from patients with EPM1 undergo an aberrant differentiation pathway, contributing to the altered electrophysiological properties observed in EPM1-COs.

Overall, these results suggest that the altered E/I balance and increased neuronal activity in EPM1-COs may be associated with the aberrant differentiation of ventral progenitors, leading to an imbalance that drives increased excitability in the neuronal network.

EPM1 ventral progenitors and neurons change cell identity

Building on our observations of altered neuronal activity and disrupted cell fate decisions in EPM1-derived cells, we sought to investigate early molecular mechanisms underlying these changes by analyzing the transcriptomic profiles of progenitors and their neuronal progeny. To identify the developmental origins of this hyperexcitability, we performed single-cell RNA sequencing (scRNA-seq) on 40-day-old vCOs derived from EPM1 (EPM1-vCOs) and control (vCOs) lines. To first ensure that our organoids maintained proper regional identity across genotypes, we performed immunohistochemical analysis for the forebrain marker FOXG1. The robust expression of FOXG1 at both day 30 and day 70 confirmed consistent telencephalic specification in both control and EPM1-vCOs, providing a stable regional background for our subsequent cell-fate analyses (fig. S3A). The selection of day 40 for single-cell analysis was based on the observation that CSTB protein levels peak during this period of active neurogenesis (35). Given that this stage represents a critical transition where the progenitor pool is being definitively patterned into specific neuronal identities, we hypothesized that CSTB serves as a safeguard for ventral cell fate. Consequently, its deficiency at this peak stage likely initiates the aberrant patterning of ventral progenitors, providing a molecular bridge between early protein loss and the long-term E/I imbalance observed in mature organoids. Following

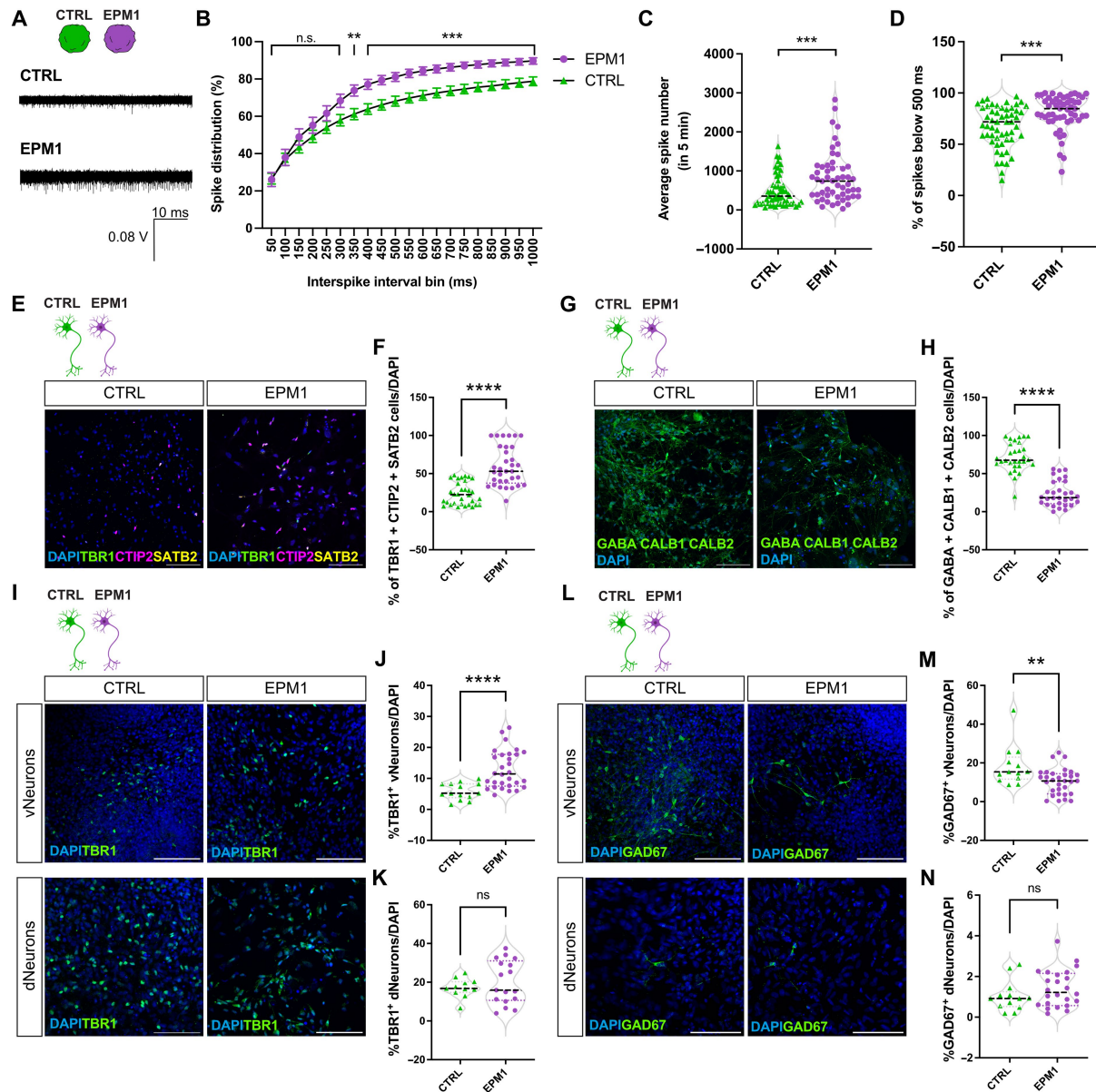


Fig. 1. Altered electrophysiological activity and neuronal composition in EPM1. All data represent pooled results from two control iPSC lines (CTRL.1 and CTRL.2) and two patient iPSC lines (EPM1.1 and EPM1.2). (A) Representative electrophysiological traces of CTRL and EPM1 COs. (B) Percentage of spike distribution at interspike interval bins ranging from 50 to 1000 ms for CTRL and EPM1 COs. $n = 53$ to 55 per condition. Statistical significance was determined using the Mann-Whitney test. $**P < 0.01$ and $***P < 0.001$. (C). Average spike number detected within 5 min for CTRL and EPM1 COs. $n = 53$ to 55 per condition. Statistical significance was determined using the Mann-Whitney test. $***P < 0.001$. (D). Percentage of spikes below 500 ms detected in CTRL and EPM1 COs. $n = 53$ to 55 per condition. Statistical significance was determined using the Mann-Whitney test. $***P < 0.001$. (E) Representative images of CTRL and EPM1 neurons immunostained for excitatory neuronal markers TBR1, CTIP2, and SATB2. DAPI (4',6-diamidino-2-phenylindole) staining is presented as a nuclear counterstain. Scale bars, 100 μm . Created in BioRender. A. Forero (2026); <https://BioRender.com/m6y4b1p>. (F) Quantification of the percentage of cells positive for TBR1/CTIP2/SATB2 in CTRL versus EPM1 neural cultures normalized by total DAPI nuclear staining. $n = 30$ per condition. Statistical significance was determined using the Mann-Whitney test, $****P < 0.0001$. (G) Representative images of CTRL and EPM1 neurons immunostained for inhibitory neuronal markers GABA/Calretinin (CALB2)/Calbindin (CALB1). DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μm . Created in BioRender. A. Forero (2026); <https://BioRender.com/m6y4b1p>. (H) Quantification of the percentage of cells positive for GABA/CALB2/CALB1 in CTRL versus EPM1 neural cultures. $n = 33$ per condition. Statistical significance was determined using the unpaired Student's t test, $****P < 0.0001$. (I) Representative images of TBR1-positive cells in CTRL and EPM1 neurons derived from ventral (vNeurons) and dorsal (dNeurons) progenitors. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μm . Created in BioRender. A. Forero (2026); <https://BioRender.com/m6y4b1p>. (J) Quantification of TBR1-positive cells/DAPI in CTRL versus EPM1 vNeurons. $n = 15$ to 30 per condition. Statistical significance was determined using the Mann-Whitney test. $****P < 0.0001$. (K) Quantification of TBR1-positive cells/DAPI in CTRL versus EPM1 dNeurons. $n = 11$ to 17 per condition. Statistical significance was determined using the unpaired Student's t test, n.s., not significant. (L) Representative images of GAD67-positive cells in CTRL and EPM1 neurons derived from ventral (vNeurons) and dorsal (dNeurons) progenitors. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μm . Created in BioRender. A. Forero (2026); <https://BioRender.com/m6y4b1p>. (M) Quantification of GAD67-positive cells/DAPI in CTRL versus EPM1 vNeurons. $n = 13$ to 31 per condition. Statistical significance was determined using the unpaired Student's t test, $**P < 0.01$. (N) Quantification of GAD67-positive cells/DAPI in CTRL versus EPM1 dNeurons. $n = 13$ to 24 per condition. Statistical significance was determined using the unpaired Student's t test. ms, milliseconds; min, minutes.

stringent quality control, we retained 10,972 high-quality cells across EPM1 and control ventral organoids. To visualize cellular heterogeneity, we integrated cells from vCOs (two control lines) and EPM1-vCOs (two patient lines), projecting them onto a two-dimensional (2D) Uniform Manifold Approximation and Projection (UMAP) space (Fig. 2A and fig. S3B). The resulting clusters encompassed a wide array of cell types, including neural crest cells, neural progenitors (e.g., forebrain and striatal radial glia), and mature neurons. This dataset enabled us to interrogate the transcriptional similarities and differences between progenitors and neurons from both control and EPM1 origins.

We focused initially on the diversity within the progenitor population. Progenitors expressed classical markers, including *SOX2*, *NES*, and *VIM* (fig. S3C), and were further subdivided into five distinct subclusters: “Progenitor,0” to “Progenitor,4” (Fig. 2B and fig. S3D). Notably, the frequencies of the Progenitor,0 and Progenitor,2 subclusters differed significantly between EPM1 and control cells (Fig. 2C and fig. S3E), with Progenitor,0 being predominantly enriched in control cells, while Progenitor,2 was overrepresented in EPM1 cells. Progenitor,0 was characterized by the expression of medial ganglionic eminence (MGE) markers such as *NKX2-1* and *SIX3*, whereas Progenitor,2 exhibited dorsal progenitor marker *PAX6* expression (Fig. 2, D and E). The Progenitor,1 and Progenitor,3 subclusters, meanwhile, displayed a combination of *MEIS2* and *PAX6*, markers commonly associated with the dorsal lateral ganglionic eminence (dLGE) (Fig. 2, D and E).

To rigorously quantify the transcriptomic divergence between genotypes, we performed replicate-aware pseudobulk differential expression analysis on the pooled progenitor population. This analysis results revealed a clear shift in the molecular landscape, highlighting the significant down-regulation of ventral determinants such as *NKX2-1* and *MEIS2* in EPM1 progenitors (fig. S3F). To further elucidate the functional impact of these changes, we conducted a Gene Ontology (GO) biological process overrepresentation analysis, summarized as a GO term network (fig. S3G). This analysis identified a systemic disruption of early patterning programs, with EPM1 cells showing an enrichment in terms related to dorsal forebrain development and excitatory lineage commitment, while control cells were enriched for pathways governing MGE identity and GABAergic interneuron specification.

To validate our scRNA-seq findings, we performed immunofluorescence analysis, which confirmed a reduction in *NKX2-1*⁺ progenitors and an increase in *MEIS2*⁺, *PAX6*⁺ (*PAX6*⁺/*ASCL1*[−]) and *EOMES*⁺ dorsal progenitors in EPM1-vCOs compared to control vCOs (Fig. 2F and fig. S3H). Immunostaining and scRNA-seq revealed also a significant down-regulation of the ventral patterning genes *GSX2* and *ASCL1* (fig. S3, I and J). Further validation through immunostaining of dissociated cells from EPM1-vCOs reinforced the predominance of *MEIS2*⁺ progenitors in the EPM1 condition (fig. S3K).

Next, we characterized the neuronal populations, which expressed common neuronal markers such as *DCX* and *MAP2* (fig. S4A). We identified three subclusters: Neurons,0, Neurons,1, and Neurons,2 (Fig. 3A and fig. S4B). EPM1-derived cells were enriched in the Neurons,0 subcluster, while control-derived cells were predominantly present in the Neurons,1 subcluster (Fig. 3B and fig. S4C). Consistent with the progenitor data, the Neurons,0 subcluster was enriched for excitatory neuron markers, including *THSD7A*, *CACNA2D1*, and *NEUROD1* (Fig. 3, C and D, and fig. S4E), while Neurons,1, primarily populated by control cells, expressed genes associated with

inhibitory neurons, such as *DLX1*, *DLX2*, and *GAD1* (Fig. 3, C and D, and fig. S4D). Immunofluorescence on 70-day-old vCOs revealed a higher proportion of ventricles containing *TBR1*⁺ and *SATB2*⁺ excitatory neurons in EPM1-vCOs compared to controls (Fig. 3E). However, among these marker-positive ventricles, the percentage of *TBR1*⁺ and *SATB2*⁺ cells remained unchanged (fig. S4D). This suggests that the number of ventricles with an altered cell identity, rather than the percentage of positive cells within a positive ventricle, drives altered neuronal production. The expression of these excitatory neuron markers is regulated by *NEUROG2* and the neurogenic transcription factor *EOMES* (Fig. 2F). In addition, consistent with the increased expression of dLGE progenitor markers—known to give rise to medium spiny neurons—we observed an enrichment of *DARPP32*⁺ striatal neurons in EPM1-vCOs by immunostaining (fig. S4F). Collectively, these findings indicate that EPM1-vCOs exhibit a fundamental failure in maintaining MGE identity. The loss of *GSX2* likely acts as a developmental switch, allowing progenitors and neurons to shift toward both dorsal (*TBR1*⁺) and LGE (*DARPP32*⁺) fates, thereby driving the observed E/I imbalance.

EPM1 cells exert a dominant non-cell-autonomous effect on control cells

On the basis of our previous findings that CSTB is secreted and can remodel the extracellular environment, thereby influencing surrounding cells (35), we investigated whether altered CSTB levels affect cell fate in a non-cell-autonomous manner. We generated mosaic vCOs (mvCOs) containing green fluorescent protein (GFP)-labeled CTRL cells (mvCTRL) and nonlabeled EPM1 cells (mvEPM1). The scRNA-seq was performed on fluorescence-activated cell sorting-sorted cells from 40-day-old mvCOs, and mvCTRL and mvEPM1 datasets were integrated to map cellular diversity (Fig. 4A and fig. S4G).

The scRNA-seq data revealed a trend toward reduced expression of the MGE progenitor marker *NKX2-1* and increased expression of LGE markers such as *MEIS2* in mvCOs compared to patterned vCOs (Figs. 4B and 2D, and fig. S4H). To compare these datasets more directly, we performed a joint integration of vCO and mvCO samples, which confirmed this transcriptomic shift (fig. S4I). In this integrated analysis, the reduction in MGE-identity was further supported by a similar downward trend in the expression of *ASCL1*, a key ventral ganglionic eminence marker, and *OLIG2*, a downstream target of *NKX2-1* signaling (fig. S4I).

However, because mosaic ventricles consist of partially intermixed CTRL and EPM1 cells, single-cell sequencing cannot reliably distinguish purely CTRL from EPM1 ventricles. We therefore complemented this exploratory analysis with immunofluorescence quantification, which revealed a significant reduction of *NKX2-1*⁺ and increase of *MEIS2*⁺ ventricles in mvCOs (Fig. 4, C to F). These results support that CSTB deficiency can influence neurogenesis through non-cell-autonomous mechanisms.

EPM1 EVs present altered biogenesis, dynamics, and composition

CSTB has been detected in the culture medium of COs, as previously reported (35), and, notably, has also been observed to be released via EVs (1, 28). To explore the extrinsic mechanisms underlying EPM1, we investigated how EVs could alter the extracellular environment and influence neighboring cells. We isolated a mixed population of small EVs, including both exosomes and small microvesicles, with an enrichment for vesicles ranging from 100 to 200 nm (fig. S5A), from

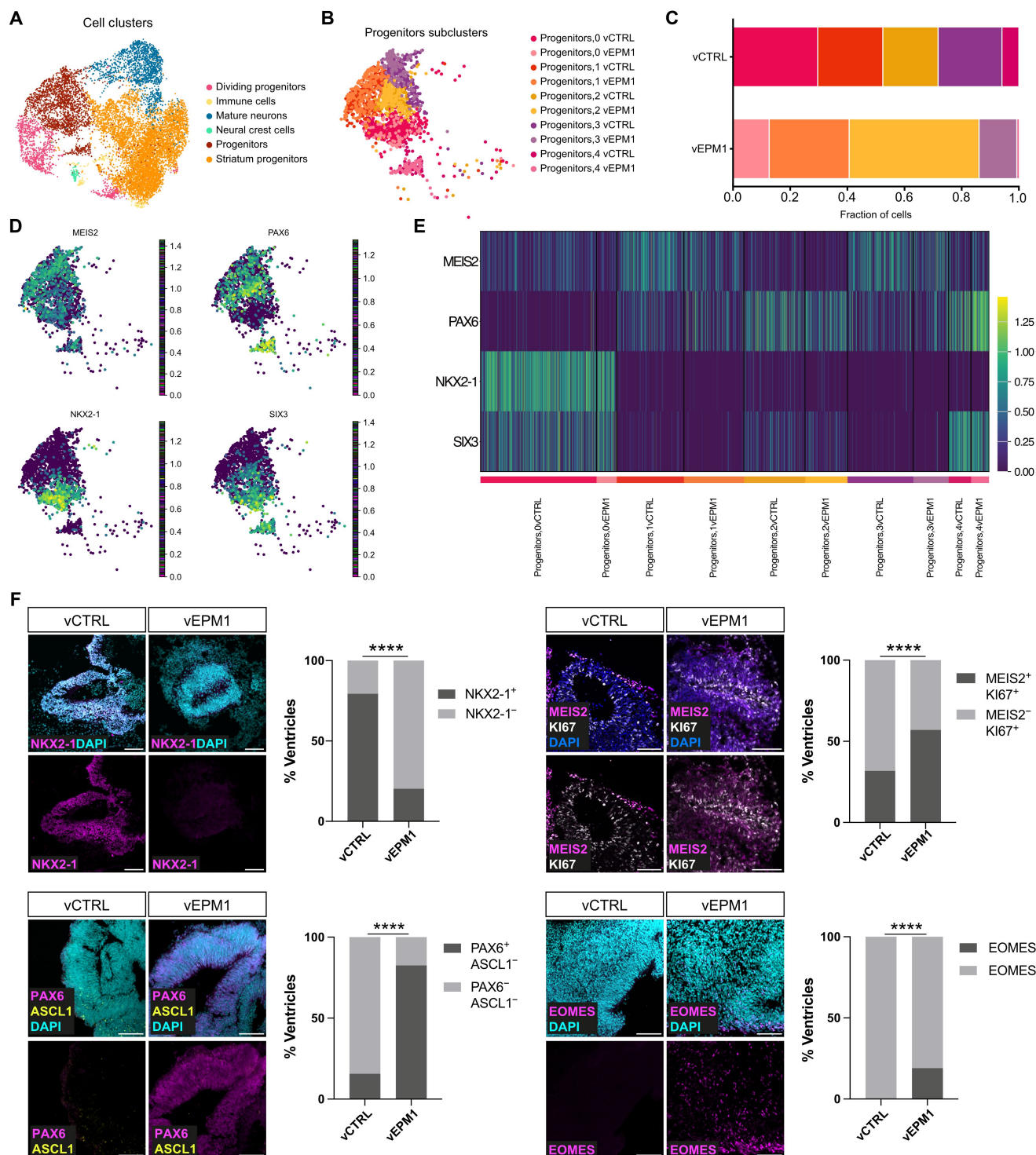


Fig. 2. Changes in progenitor identity in ventral EPM1 COs. All data represent pooled results from two control iPSC lines (CTRL.1 and CTRL.2) and two patient iPSC lines (EPM1.1 and EPM1.2). (A) UMAP visualization of scRNA-seq data from CTRL and EPM1 vCOs, clustered by cell types (dividing progenitors, immune cells, neurons, neural crest cells, progenitors, and striatum progenitors). (B) UMAP visualization of five subclusters within the progenitor clusters (Progenitors,0; Progenitors,1; Progenitors,2; Progenitors,3; and Progenitors,4). Lighter colors represent Progenitors derived from vEPM1, while darker colors indicate those from vCTRL. (C) Bar plot illustrating the fraction of vCTRL and vEPM1 cells in the five subclusters (Progenitors 0, 1, 2, 3, and 4) identified within the Progenitors cluster. (D) UMAP visualization showing spatial expression of *MEIS2*, *PAX6*, *NKX2-1*, and *SIX3* across vCTRL and vEPM1 cells in the Progenitor subclusters. (E) Heatmap showing the expression levels of *MEIS2*, *PAX6*, *NKX2-1*, and *SIX3* in vCTRL and vEPM1 cells composing the five Progenitors subclusters (Progenitors, 0; 1; 2; 3; and 4). (F) Representative images of CTRL and EPM1 vCOs immunostained for progenitor markers *NKX2-1*, *MEIS2/Ki67*, *PAX6/ASCL1*, and *EOMES* and quantifications of the percentage of ventricles positive for each of the corresponding immunostainings in CTRL versus EPM1 vCOs. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. $n = 9$ to 11. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$.

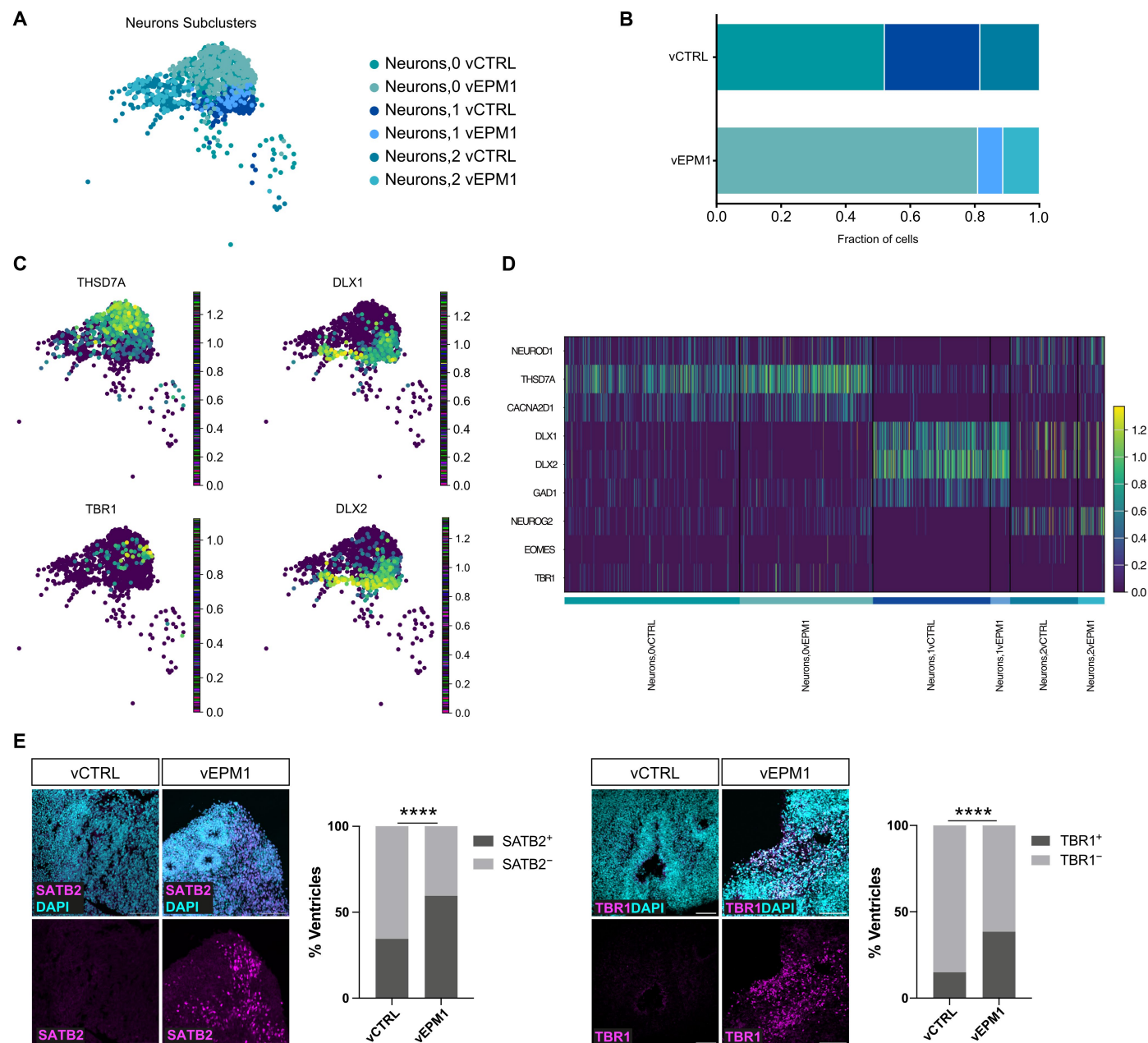


Fig. 3. Changes in neuron identity in ventral EPM1 COs. All data represent pooled results from two control iPSC lines (CTRL.1 and CTRL.2) and two patient iPSC lines (EPM1.1 and EPM1.2). (A) UMAP visualization of the three Neurons subclusters (Neurons,0; Neurons,1; and Neurons,2) identified within the neurons cluster. Lighter colors represent Neurons derived from vEPM1, while darker colors indicate those from vCTRL. (B) Bar plot illustrating the fraction of vCTRL and vEPM1 cells in each of the Neurons subclusters (Neurons,0; Neurons,1; and Neurons,2). (C) UMAP visualization highlighting the expression of excitatory neuronal markers (*THSD7A*), inhibitory neuronal markers (*DLX1* and *DLX2*), and dorsal telencephalon marker (*TBR1*). (D) Heatmap showing the expression levels of *NEUROD1*, *THSD7A*, *CACNA2D1*, *DLX1*, *DLX2*, *GAD1*, *NEUROG2*, *EOMES*, and *TBR1* in vCTRL and vEPM1 cells composing the neurons subclusters (Neurons,0; Neurons,1; and Neurons,2). (E) Representative images of CTRL and EPM1 vCOs immunostained for neuronal markers SATB2 and TBR1 and quantification of the percentage of ventricles positive for each of the corresponding immunostainings in CTRL versus EPM1 vCOs. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. $n = 9$ to 11. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$.

the culture medium of vCOs and EPM1-vCOs using differential ultracentrifugation (1, 39, 40). Immunoelectron microscopy confirmed the presence of CSTB within EVs, with some EVs also positive for the EV marker CD81 (Fig. 5A). CSTB abundance in EVs changes during CO development with two peaks at day 40 and at later stages of development together with other developmental markers such as RELN and

MAP2 [Fig. 5B (1)]. Moreover, CSTB overlaps with CST3 in EVs at later stages of development (fig. S5B), and interestingly, CST3 has a beneficial effect in mice lacking CSTB (41).

Given that CSTB is secreted in EVs and we identified non-cell-autonomous effects on E/I differentiation in mosaic vCOs, we hypothesized that EVs could be key players in this phenotype. To explore

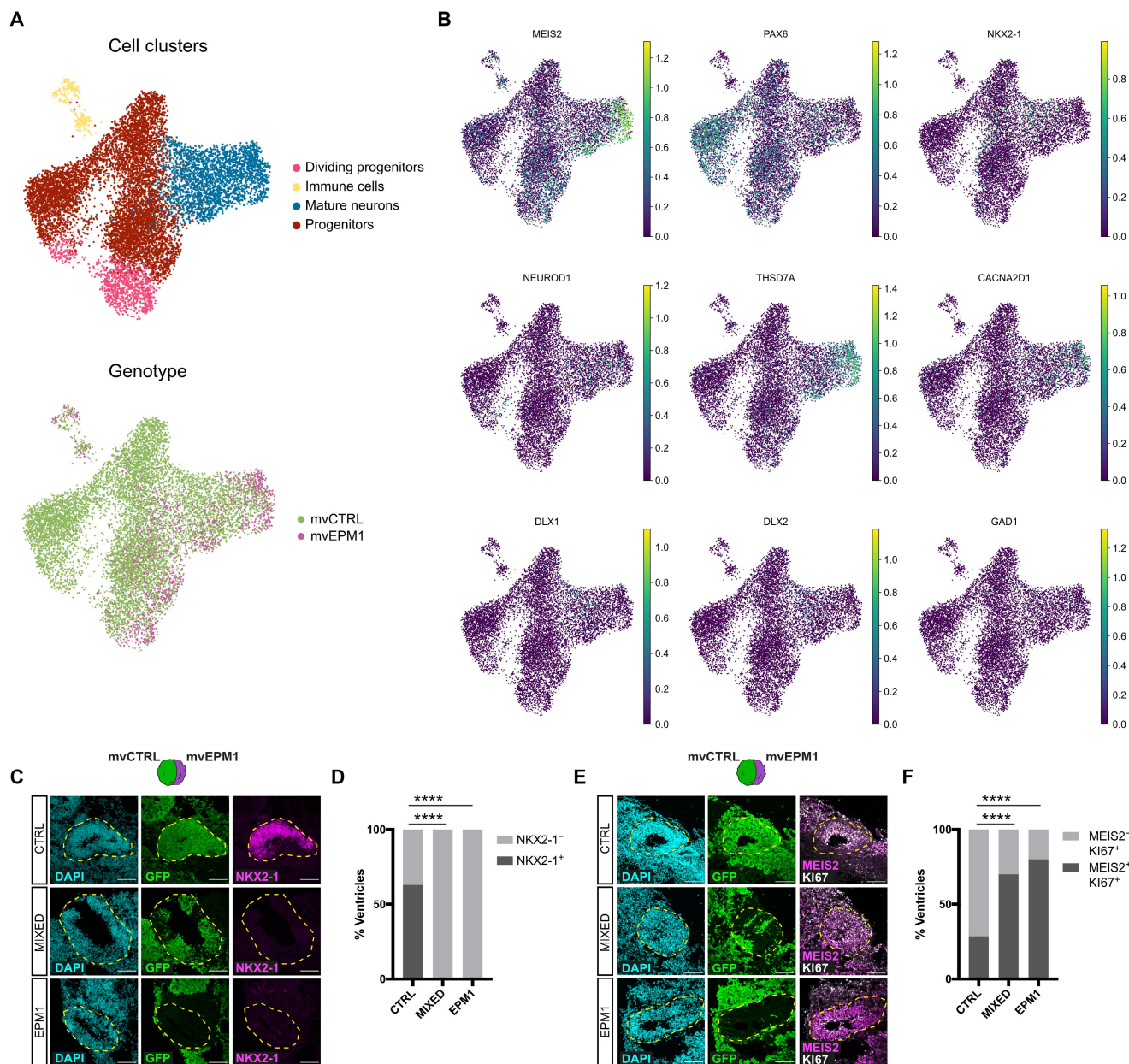


Fig. 4. Non-cell-autonomous effect of EPM1 cells in mosaic ventral COs. All data represent pooled results from two control iPSC lines (CTRL.1 and CTRL.2) and two patient iPSC lines (EPM1.1 and EPM1.2). **(A)** UMAP visualization of scRNA-seq data of mosaic CTRL (mvCOs) and EPM1 (mvEPM1) vCOs, clustered by cell types (top; dividing progenitors, immune cells, mature neurons, progenitors) and genotype (bottom; mvCTRL and mvEPM1). **(B)** UMAP visualization of the expression of progenitor markers (*MEIS2*, *PAX6*, and *NKX2-1*), excitatory neuronal (*NEUROD1*, *THSD7A*, and *CACNA2D1*), and inhibitory neuronal (*DLX1*, *DLX2*, and *GAD1*) markers. **(C)** Representative images of CTRL (GFP⁺), EPM1 (GFP⁻), and mixed (GFP⁺/GFP⁻) ventricles in mvCOs, immunostained for NKX2-1. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. **(D)** Quantification of the percentage of CTRL (GFP⁺), EPM1 (GFP⁻), and mixed (GFP⁺/GFP⁻) ventricles positive for NKX2-1. $n = 44$. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$. **(E)** Representative images of CTRL (GFP⁺), EPM1 (GFP⁻), and mixed (GFP⁺/GFP⁻) ventricles in mvCOs, immunostained for MEIS2 and Ki67. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. **(F)** Quantification of the percentage of CTRL (GFP⁺), EPM1 (GFP⁻), and mixed (GFP⁺/GFP⁻) ventricles positive for MEIS2 and Ki67. $n = 26$. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$.

this, we next aimed to investigate the biogenesis and secretion dynamics of EVs. We began by using nanoparticle tracking analysis (NTA), a real-time technique used to measure the size and concentration of nanoparticles, such as EVs, by tracking their Brownian motion. NTA was used to monitor EV secretion during the early stages of differentiation of NPCs into neurons. EPM1 young neurons (day 5

to day 12 of differentiation) secreted a lower amount of EVs with an average diameter of 115 to 165 nm compared to CTRL cells and showed a trend toward larger diameter vesicles (fig. S5C). These findings prompted us to further examine EV secretion dynamics under EPM1 conditions. To achieve this, we used live imaging of membrane fusion activity in both control and EPM1 neurons using total

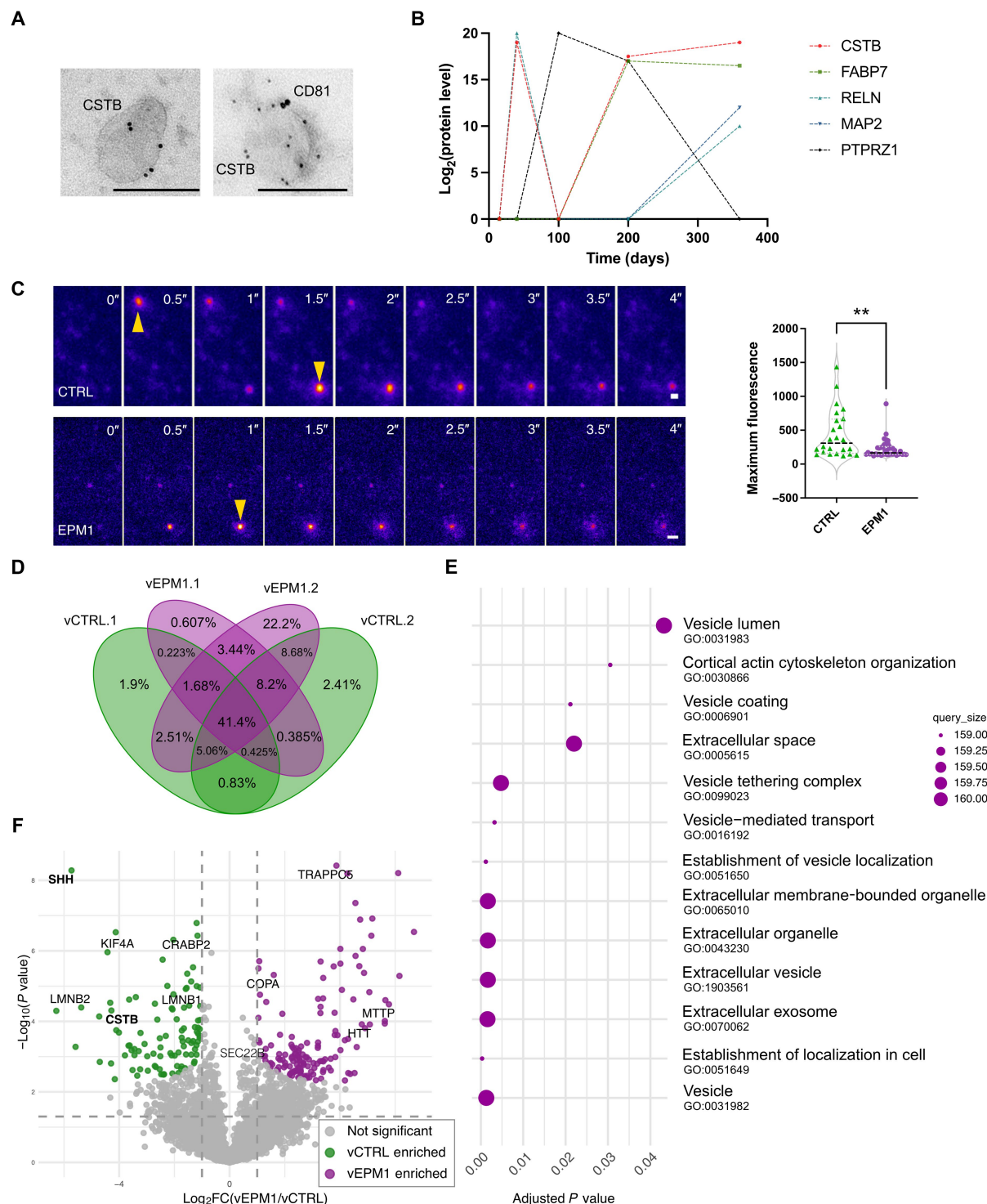


Fig. 5. Alterations in EV composition and dynamics in EPM1. All data represent pooled results from two control iPSC lines (CTRL1 and CTRL2) and two patient iPSC lines (EPM1.1 and EPM1.2). **(A)** Immuno-electron micrographs of CD81 (large dots) and CSTB (small dots) in EVs collected from COs. Scale bars, 1 μ m. **(B)** Protein expression levels of CSTB, FABP7, RELN, MAP2 and PTPRZ1 in EVs derived from COs at different developmental stages (expressed in days). **(C)** Representative time lapse images of MVB-PM fusion events (arrowheads) in CTRL and EPM1 neurons. Scale bars, 1 μ m. Quantification of the maximum fluorescence of the fusion events detected in CTRL and EPM1 neurons. Every dot refers to a single cell analyzed, $n = 29$ (CTRL), $n = 26$ (EPM1). Statistical significance was determined using the Mann-Whitney U test. $**P < 0.01$. **(D)** Venn diagram depicting the percentage of unique and common proteins detected in EVs from CTRL vCOs (green) and vEPM1-COs (purple). **(E)** GO enrichment analysis of the unique proteins detected in EPM1-vEVs. **(F)** Volcano plot of proteins enriched in CTRL-vEVs (vCTRL, green) versus EPM1-vEVs (vEPM1, purple).

internal reflection fluorescence (TIRF) microscopy. This was performed after overexpressing CD63-pHluorin, a tetraspanin-based pH-sensitive optical reporter that detects multivesicular body–plasma membrane (MVB-PM) fusion events (Fig. 5C and fig. S5D) (42). EPM1 neurons displayed a decreased number of fusion events per minute, indicating altered EV secretion in pathological conditions. After identifying differences in EV secretion, we next investigated whether the protein cargo of EVs differed between control and EPM1 conditions. Using mass spectrometry, we profiled the protein content of EVs from CTRL (vEVs) and EPM1 (EPM1-vEVs), detecting 2690 proteins for vEV1, 3362 for vEV2, 2805 for EPM1-vEV1, and 4654 for EPM1-vEV2. Consistent with previous studies (40), standard EV markers (CD9, CD63, CD81, CD82, PDCD6IP, and TSG101) were present in all samples (fig. S5E), while negative markers such as ALB (component of non-EV coisolated structures) and AGO (mainly found outside of EVs) (40) were not detectable. We compared proteins secreted by each control and patient line to identify EPM1-specific vEV signatures, finding that control and EPM1-vEVs shared a large proportion of proteins (41.4%) (Fig. 5D). However, 3.4% of the total detected proteins (170) were unique to EPM1-vEVs (Fig. 5D and table S1) and enriched for processes involved in cell trafficking from the ER to the extracellular space (Fig. 5E). Among the proteins unique to vEVs (0.8%, 41 proteins; table S1), no specific GO terms were identified. We then focused on differentially expressed proteins (DEPs) between vEVs and EPM1-vEVs (Fig. 5F and table S1). The GO term analysis of DEPs confirmed the accumulation of proteins involved in cell trafficking accumulated within EPM1-vEVs (fig. S5F). In contrast, proteins depleted in EPM1-vEVs were associated with mRNA processing, nuclear pore localization, mitotic spindle formation, cytoskeletal organization, and axogenesis, suggesting that CSTB deficiency leads to systemic pathological changes (fig. S5F). As expected, CSTB was significantly down-regulated in EPM1-vEVs. Notably, many proteins altered in EPM1-vEVs are encoded by genes associated with epilepsy and brain malformations (fig. S5, G and H) (36), highlighting the clinical relevance of the EV-mediated signaling disrupted in EPM1. Together, these data demonstrate that CSTB deficiency impairs the secretion machinery and release dynamics of EVs, prompting a deeper investigation into whether the protein composition of the remaining secreted EVs is also fundamentally altered.

Altered EV-mediated SHH delivery underlies non-cell-autonomous specification defects

To understand the molecular basis of the extrinsic defects in EPM1, we focused on SHH that was one of the most significant depleted cargos in EPM1-vEVs and emerged as a promising candidate for explaining the specification defects (Fig. 5F). Given that SHH signaling establishes the ventral gradient necessary for MGE identity (43–46), we hypothesized that the altered EV composition directly affects progenitor patterning. Since CSTB forms complexes with various proteins, we investigated whether it physically interacts with SHH. The co-immunoprecipitation (Co-IP) of CSTB in vCO extracts identified SHH as a direct interactor, alongside the ESCRT-associated protein CHMP1A that specifically helps the package of SHH in EV for proper release and signaling (47) (Fig. 6A). This suggests that CSTB may participate into the sorting of SHH into secretory vesicles. GO analysis of CSTB interactors further confirmed an enrichment in terms related to “Extracellular Exosome” and the “SHH pathway” (Fig. 6B). To identify whether SHH signaling was altered in EPM1, we performed qPCR analysis of key targets in vNPCs. Downstream targets

such as *GLI1*, *NKX2-1*, and *OLIG2*—previously identified as down-regulated in our scRNA-seq data—were indeed reduced in EPM1 vNPCs (Fig. 6C). The quantification of intra- and extracellular SHH levels revealed that EPM1 cells contain less SHH intracellularly compared to CTRL cells, thereby reducing its secretion into the extracellular milieu (Fig. 6D and fig. S5I). To confirm the causal role of CSTB in this process, we overexpressed CSTB in EPM1 vNPCs, which successfully increased the release of SHH into the medium, demonstrating that CSTB directly mediates SHH secretion and availability (Fig. 6E and fig. S5J).

We then hypothesized that the observed differences in EV composition could potentially explain the altered differentiation outcomes in progenitor identity. To test this hypothesis, we treated NPCs with CTRL vEVs or EPM1-vEVs for 12 hours and analyzed their transcriptome through bulk RNA-seq (Fig. 6F and table S1). Compared to untreated NPCs and vEVs, EPM1-vEVs induced several transcriptional changes, confirming that the altered protein cargo of EPM1-vEVs can indeed influence NPC transcripts quickly. Although the short-term exposure to EVs did not result in significant effects on the SHH pathway, we identified several changes associated with cell trafficking and the regulation of WNT and bone morphogenetic protein signaling pathways (Fig. 6F). Then, we sought to determine whether a longer exposure could affect the ventral identity markers. Treating control vNPCs with EPM1-vEVs for 2 weeks was sufficient to reduce the expression of SHH downstream targets (Fig. 6G). Conversely, treating EPM1 vNPCs with control vEVs did not result in a significant rescue within this timeframe, suggesting that 2 weeks may be insufficient to revert the established pathological state or that the cellular machinery in EPM1 progenitors is partially desensitized (fig. S5K). A clear evidence for the impact of the defective secretion of SHH and its non-cell-autonomous role in EPM1, came from our mosaic organoid analysis. The pseudobulk RNA analysis of vCOs showed *NKX2-1* enriched in vCTRL progenitors, while *WNT3/3A* were enriched in vEPM1 progenitors (Fig. 6H and table S1). In mosaic organoids (mvCOs), *NKX2-1* was no longer differentially expressed between genotypes, indicating that the presence of neighboring control cells—and potentially their secreted SHH—helped stabilize the MGE identity in EPM1 cells (Fig. 6I and table S1). To test this directly, we treated vEPM1 organoids for 2 weeks with exogenous SHH. Excitingly, this chronic treatment reduced the abundance of *MEIS2*⁺ and *PAX6*⁺ ventricles while significantly restoring *NKX2-1*⁺ ventricular identity (Fig. 6, J to L). Furthermore, in line with the rescue observed at the progenitor level, an increase in *GAD67*⁺ and *CALB2*⁺ inhibitory neurons was also achieved following exogenous SHH treatment (fig. S5, L to O). Overall, these data identify SHH as a critical mediator of the EPM1 phenotype and suggest that CSTB safeguards ventral patterning by facilitating the vesicular delivery of SHH to the extracellular environment.

DISCUSSION

In this study, we aimed to explore the mechanisms through which both CSTB deficiency drives EPM1 disease progression. Using brain organoids derived from iPSCs from patients with EPM1 harboring mutations in the *CSTB* gene, we successfully recapitulated impaired electrophysiological activity, highlighting defects in the E/I balance that likely contributes to the onset of epilepsy and other neurological symptoms. While studies have documented reduced GABAergic inhibition and cortical thinning in patients with EPM1 and *CSTB*

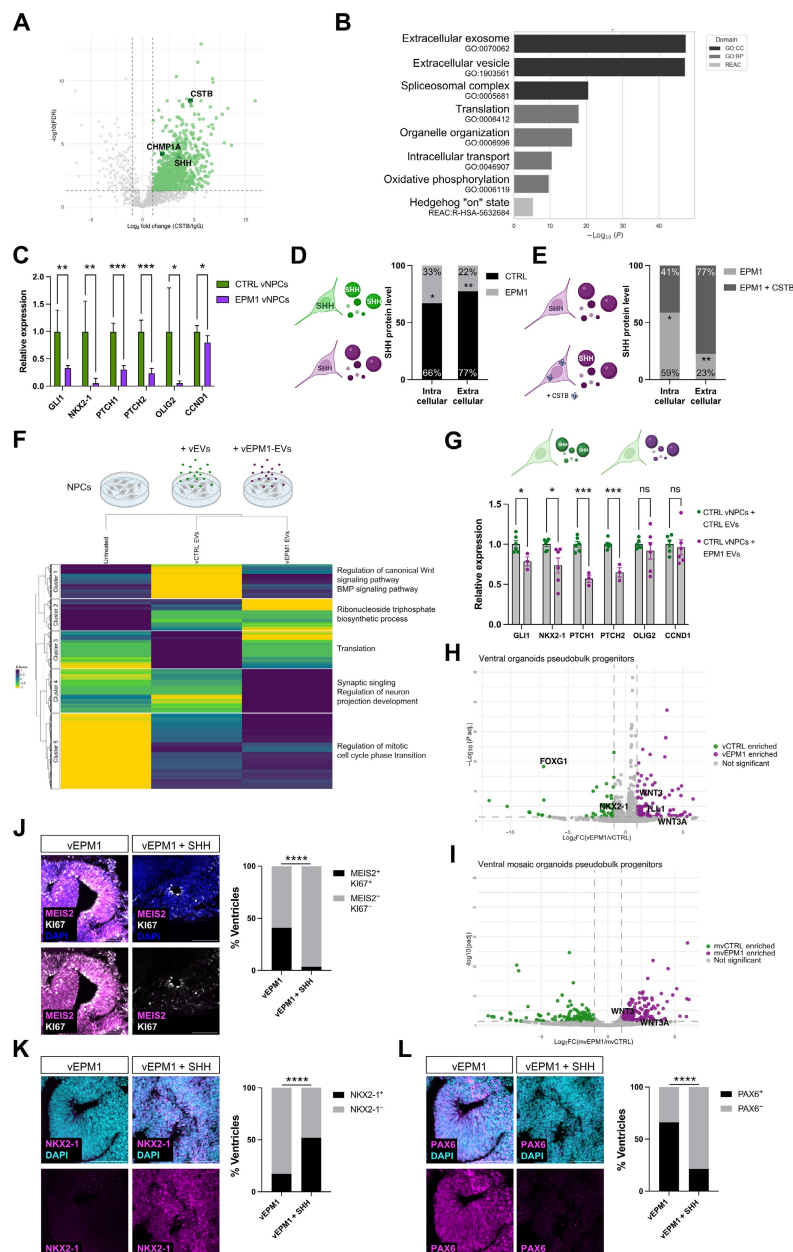


Fig. 6. Defects in EV-mediated SHH transport in EPM1. All data represent pooled results from two control iPSC lines (CTRL.1 and CTRL.2) and two patient iPSC lines (EPM1.1 and EPM1.2). **(A)** Volcano plot showing proteins significantly enriched (green) in CSTB co-IP compared to control (IgG). **(B)** GO enrichment analysis of significantly enriched proteins in CSTB co-IP compared to control (IgG). **(C)** Reverse transcription qPCR (RT-qPCR) analysis of SHH pathway target genes (*GLI1*, *NKX2-1*, *PTCH1*, *PTCH2*, *OLIG2*, and *CCND1*) in CTRL and EPM1 vNPCs. **(D)** Quantification of intracellular and extracellular SHH protein levels in CTRL versus EPM1 vNPCs. Scheme created in BioRender (<https://BioRender.com/mj7rl71>). **(E)** Quantification of intracellular and extracellular SHH protein levels in EPM1 versus CSTB-overexpressing EPM1 vNPCs. Scheme created in BioRender (<https://BioRender.com/mj7rl71>). **(F)** Clustered heatmap showing the expression levels of the differentially regulated genes in NPCs following the acute treatment of vEVs, vEPM1-EVs, or no treatment. GO terms enriched in each cluster are listed on the right. Scheme created in BioRender (<https://BioRender.com/tpd7euk>). **(G)** RT-qPCR analysis of SHH pathway target genes (*GLI1*, *NKX2-1*, *PTCH1*, *PTCH2*, *OLIG2*, and *CCND1*) in CTRL vNPCs following treatment (1 week) with CTRL-EVs versus EPM1-EVs. Scheme created in BioRender (<https://BioRender.com/mj7rl71>). **(H)** Volcano plot showing pseudobulk analysis for the progenitor cluster of vCOs. Genes enriched in vCTRL are highlighted in green, and genes enriched in vEPM1 are highlighted in purple. **(I)** Volcano plot showing pseudobulk analysis for the Progenitor cluster of scRNA-seq data from mosaic vCOs. Genes enriched in mvCTRL are highlighted in green, and genes enriched in mvEPM1 are highlighted in purple. **(J)** Representative images of MEIS2 and Ki67 immunostaining in vEPM1 and SHH-treated vEPM1 COs and quantification of the percentage of positive ventricles for each condition. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. $n = 9$ to 11. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$. **(K)** Representative images of NKX2-1 immunostaining in vEPM1 and SHH-treated vEPM1 COs and quantification of the percentage of positive ventricles for each condition. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. $n = 9$ to 11. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$. **(L)** Representative images of PAX6 immunostaining in vEPM1 and SHH-treated vEPM1 COs and quantification of the percentage of positive ventricles for each condition. DAPI staining is presented as a nuclear counterstain. Scale bars, 100 μ m. $n = 9$ to 11. Statistical significance was determined using an exact binomial test. **** $P < 0.0001$.

knockout mouse models (30, 31), our findings uncover a much earlier developmental origin: a pathological misspecification of progenitor cell fate. Here we showed that low levels of CSTB in EPM1 result in intrinsic effects such as progenitor cell misspecification, and extrinsic effects, mediated by non-cell-autonomous cues conveyed by EVs, which influence the neighboring cells. We propose that reduced GABAergic signaling depends on the pathological misspecification of progenitor cell fate, which led to an altered neuronal output characterized by an unexpected shift toward dorsal cortex and dLGE identities. Cell type-specific alterations in the ventral fate of progenitor cells during early development leading to fewer GABAergic neurons have also been described in other neurodegenerative disorders such as Huntington's disease (48). Our observation of an aberrant shift toward dorsal and LGE identities at the expense of MGE-derived interneurons provides a neurodevelopmental framework for the subcortical involvement reported in patients with EPM1. While gross anatomical malformations are not typically visible on clinical magnetic resonance imaging, advanced 3D texture analysis has revealed significant microstructural alterations in the putamen and thalamus of patients with EPM1 (49). This is further supported by voxel-based morphometry showing progressive volume loss in these regions (50) and histopathological evidence of striatal neuronal degeneration (51). The expansion of DARPP32⁺ striatal-like neurons and the loss of ventral identity we report likely represent the developmental origins of these later-stage subcortical deficits. These common pathological features underscore the importance of investigating aspects of neurodevelopment in diseases not directly classified as a neurodevelopmental disorders. Our study builds on these findings by showing that these alterations arise from an intrinsic reduction of CSTB protein levels and are manifested through extrinsic influences, particularly EVs, which can modulate the neurogenic niche.

The unique protein content of EPM1-vEVs suggests a link between altered trafficking mechanisms and disease pathology. In EPM1 EVs, we identified many components of the TRAPP complex, which regulates vesicle tethering and fusion (52). Disruptions in TRAPP function may impair EV production and release, affecting cell communication in the developing brain, leading to defects in the delivery of proteins and lipids necessary for membrane biogenesis and vesicle formation. To further support our data, we demonstrated that EPM1 cells release fewer CD63⁺ EVs, which supports the disruption in the biogenesis and release of EVs. This failure in the secretory machinery results in a functional collapse of the ventral signaling niche, primarily due to the loss of EV-mediated SHH delivery.

Mechanistically, we identified that CSTB physically interacts with SHH, potentially facilitating its sorting into the secretory pathway. In EPM1, the reduction of extracellular SHH leads to the down-regulation of canonical targets such as GLI1 and the master ventral regulator GSX2. In the developing telencephalon, GSX2 is essential for repressing dorsal programs; its loss effectively removes the “molecular brake,” allowing for the ectopic expression of PAX6 and the subsequent expansion of TBR1⁺ excitatory neurons within nominally ventral territory. This is consistent with the established sensitivity of human neural tissue to SHH gradients (53) and aligns with recent findings that pathological EVs can act as potent morphogen carriers that dictate dorso-ventral patterning (7), who reported that pathological EVs secreted by vCOs with mutations in the ECM component LGALS3BP gene influence progenitor cell fate and dorso/ventral patterning, further highlighting the role of EVs in cell fate specification during brain development. Crucially, we demonstrated that this

specification defect is reversible. The exogenous supplementation of SHH was sufficient to rescue the ventral identity of EPM1 progenitors, restoring NKX2-1 expression and suppressing the aberrant PAX6 program. This rescue not only establishes a direct causal link between SHH signaling deficiency and EPM1 cell fate specification but also identifies the SHH pathway as a potential therapeutic target. By identifying CSTB as a safeguard of the ventral niche, our study reveals a complex interplay between protein trafficking, morphogen delivery, and lineage commitment (fig. S6). These findings provide a new perspective on EPM1, shifting the focus toward early developmental interventions aimed at modulating the E/I balance before the onset of irreversible neurological decline.

MATERIALS AND METHODS

IPSCs culture

iPSCs were previously reprogrammed from two control lines of fibroblasts (54) and peripheral blood mononuclear cells origin and from two EPM1 patient lines (35). iPSCs were cultured on Matrigel (Corning) coated plates (Thermo Fisher Scientific, Waltham, MA, USA) in mTesR1 basic medium supplemented with 1× mTesR1 supplement (STEMCELL Technologies, Vancouver, Canada) at 37°C and 5% CO₂ and ambient oxygen level. Passaging was done using accutase (STEMCELL Technologies) treatment.

Generation of labelled iPSC lines

The GFP-labeled iPSC lines were generated using the piggyBac transposase (1 µg) and PB-GFP (1 µg) nucleofection (55). Single cells of iPSCs were transfected with the Amaxa Nucleofector 2b (program B-016). Red fluorescent protein-positive colonies were picked and cultured on Matrigel (Corning/VWR International, 354234)-coated plates in mTeSR1 basic medium (STEMCELL Technologies, 85850) supplemented with 1× mTeSR1 supplement (STEMCELL Technologies, 85850) at 37°C and 5% CO₂.

Generation of unpatterned human organoids

Unpatterned human organoids were generated according to (56, 57) and as previously described in (35). COs were generated starting from 9000 single iPS cells per well. Organoids were kept in 10-cm dishes on a shaker at 37°C and 5% CO₂ and ambient oxygen level with medium changes every 3 to 4 days.

Generation of patterned human organoids

Patterned human organoids were generated according to (38). Embryoid bodies (EBs) generated from iPSCs were patterned to have ventral and dorsal identity. During the neuronal induction step, EBs were treated individually with SAG (1:10,000) (Millipore, 566660) + IWP-2 (1:2,000) (Sigma-Aldrich, I0536) for inducing ventral identity and with cyclopamine A (1:500) (Calbiochem, 239803) for inducing dorsal identity. After this point, the generation of organoids followed methods according to (56, 57). Organoids were kept in 10-cm dishes on a shaker at 37°C and 5% CO₂ and ambient oxygen level with medium changes every 3 to 4 days.

EPM1-vCOs treatment with SHH

EPM1-vCOs were treated with or without SHH (200 ng/ml; R&D Systems, 1845-SH-100/CF) resuspended in sterile phosphate-buffered saline (PBS) according to Fleck *et al.* (58) from day 11 (i.e., immediately after the neural induction phase) until day 30 at every change of medium

every 3 to 4 days. On day 30, organoids were fixed and embedded for immunostainings.

Generation of mosaic human organoids (vmCO)

iPSCs from GFP-labeled iPSC control line and from EPM1 iPSCs were dissociated into single cells using Accutase (Sigma-Aldrich, A6964), mixed together in a ratio of 30% GFP-control cells:70% EPM1 cells, and transferred in a total of approximately 9000 cells to one well of an ultralow-attachment 96-well plate (Corning). The protocols continued as described in the “Generation of patterned human organoids” section.

NPC and neuron generation and culture

NPCs were generated and maintained as previously described (59, 60). NPCs were generated from two control iPSC lines, which generated a ratio of 60% neurons and 40% astrocytes in accordance with this protocol, providing electrophysiologically mature neurons in a more physiological environment. Neural differentiation was conducted as previously described (61). All the cells were kept in an incubator at 37°C and 5% CO₂ and ambient oxygen level with medium changes every 2 to 3 days.

EV collection and analysis

Analysis was performed as previously described in (1). Briefly, in accordance with Théry *et al.* (40) and Forero *et al.* (1), EVs were collected from conditioned media from COs and 2D cultured cells by the following steps: centrifugation at 300g for 10 min, supernatant centrifugation at 2000g for 10 min at 4°C, supernatant centrifugation at 10,000g for 30 min at 4°C, supernatant centrifugation at 100,000g for 90 min at 4°C in a fixed-angle rotor (TH865, Thermo Fisher Scientific), followed by pellet wash with 1× PBS and centrifugation at 100,000g for 90 min at 4°C. Alternatively, the miRCURY Exosome Cell/Urine/CSF Kit (QIAGEN, 76743) was used to isolate EVs from conditioned medium according to the manufacturer's instructions. For NPCs, EVs were collected from three independent cultures of control NPCs. Neuronal EVs were collected from three independent neuronal differentiation cultures. Similarly, astrocyte EVs were collected from three independent cultures of astrocytes. For COs, EVs were collected from conditioned media of 20 to 30 different COs in culture.

For the NTA, fresh, unfrozen EV suspensions were diluted in PBS and analyzed using a Particle Metrix ZetaView PMX110-Z NTA (Particle Metrix GmbH, Inning am Ammersee, Germany) equipped with a 520-nm laser. For each measurement, samples were introduced manually, the temperature was set to 24°C, and two cycles were performed by scanning at 11 discrete positions in the cell channel and recording 60 frames per position (video setting: high). The following recommended parameters were used for the measurement: Sensitivity: 80.0; Shutter: 70; Frame rate: 30; Minimum brightness: 20; Minimum area: 5; Maximum area: 1000; Maximum brightness: 255; Tracking radius: 2; Minimum trace length: 15; nm/class: 5; Classes/decade: 64.

After capture, the videos were analyzed for particle size and concentration using the ZetaView Software 8.05.12 SP1.

For immune-electron microscopy, aliquots of EV suspensions were analyzed by I. Miinalainen at Biocenter Oulu / EM laboratory, Finland (62). Vesicles were deposited on Formvar carbon-coated, glow-discharged grids and incubated in a blocking serum containing 1% bovine serum albumin (BSA) in PBS. CD81, CSTB primary

antibodies, and secondary gold conjugates (Zymed, San Francisco, CA, USA) were diluted in 1% BSA in PBS. The blocking efficiency was controlled by performing the labelling procedure in the absence of primary antibody.

Dissociation of ventral and dorsal organoids for 2D cultures

One-month old ventral and dorsal d-COs and v-COs generated from control and EPM1 iPSCs were dissociated as previously described (35), with some modifications. Briefly COs were dissociated to single cells using Accutase (Sigma-Aldrich, A6964). Single cells were then plated onto poly-L-ornithine (10 µg/ml) (Sigma-Aldrich, P4957)/laminin (10 µg/ml) (Sigma-Aldrich, L2020)-coated coverslips with some modifications in wells of 24-well plates (Corning). Cells coming from a pool of two to three organoids were plated in 12 wells of 24-well plates with NPCs medium (NPC medium) (61).

vNPC exposure to EVs from vCOs

CTRL- and EPM1-vCOs were dissociated on day 20 and 20,000 cells per well were plated in a 24-well plate with NPC medium + fibroblast growth factor 2 (FGF2) (20 ng/ml). From day 2 postplating (dpp) to 6dpp, half of the medium was replaced with fresh differentiation medium containing vitamin A (61); from 8 to 13dpp, cells were kept only in differentiation media + vitamin A. On 4, 8, and 11dpp, CTRL and EPM1 NPCs were treated with CTRL-EVs and EPM1-EVs. EVs were collected from 24 donor organoids (day 30 to day 37) for each genotype, and each EV suspension was used to treat 24 wells of cells. On 13dpp, cells were either fixed for immunohistochemistry or collected for quantitative polymerase chain reaction (qPCR).

EPM1-vNPCs nucleofection

EPM1-vNPCs dissociated from EPM1-vCOs were plated (20000 cells per well) in a 24-well plate with NPC medium + FGF2 (20 ng/ml), and when cells reached 70 to 90% confluency, they were transfected with Lipofectamine 3000 reagents according to manufacturer (Thermo Fisher Scientific). A total of 500 ng of total DNA was used for each well. Plasmid used for transfection where pEGFP-C1 (35) and pcDNA3.1-humanCSTB (GeneScript).

Immunohistochemistry

COs were fixed using 4% paraformaldehyde for 1 hour at 4°C, cryopreserved with 30% sucrose, embedded in OCT compound mounting medium for cryotomy (VWR chemicals, 361603E), and stored at −20°C. Sections of organoids (16 µm) were prepared using a cryotome. NPCs and neurons were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by three times 5-min washing with 1× PBS. Next, cells were blocked against unspecific binding and permeabilized in blocking buffer (10% normal goat serum and 0.02% Triton X-100 in 1× PBS) for 1 hour. Primary antibodies diluted in blocking buffer were then added to the cells in the dilutions specified below and incubated overnight. On the second day, cells were washed five times for 5 min each in PBS with 0.1% Tween (PBS-T) and then incubated for 2 hours in secondary antibodies raised against the host animal of the primary antibody. Secondary antibodies were diluted in blocking buffer and the dilutions used are listed below (Table 1). DAPI (4',6-diamidino-2-phenylindole) was added as a nuclear counterstaining. Last, cells were washed three times with PBS-T and mounted on object slides with Fluoromount-G (Thermo Fisher Scientific, 00-4958-02).

Table 1. Immunostaining antibodies.				
Primary antibodies				
Antibody	Host	Company	Catalog no.	Dilution
TBR1	Rabbit	Abcam	Ab31940	1:500
CTIP2	Rat	Abcam	Ab18465	1:500
SATB2	Mouse	Abcam	Ab51502	1:500
GABA	Rabbit	Sigma	A2052	1:500
CALB1	Rabbit	Swant	CB-38a	1:1000
CALB2	Rabbit	Swant	CR7697	1:1000
GAD67	Mouse	Millipore	MAB5406	1:1000
NKX2-1	Mouse	Merk	MAB5460	1:500
MEIS1/2/3	Mouse	Santa Cruz Biotechnology	SC 101850	1:300
KI67	Rabbit	Abcam	AB15580	1:500
GFP	Chicken	Rockland	600-901-215	1:500
ASCL1	Mouse	Santa Cruz Biotechnology	sc-374104	1:200
EOMES	Sheep	R&D Systems	AF6166	1:200
FOXG1	Rabbit	Abcam	AB182559	1:300
GSX2	Rabbit	Abcam	AB229239	1:200
DARPP32	Rabbit	Abcam	AB40801	1:500
PAX6	Rabbit	BioLegend	PRB-278p	1:250
Secondary antibodies (confocal microscopy)				
Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L)	Goat	Thermo Fisher Scientific	A11008	1:500
Alexa Fluor 488 Goat Anti-Chicken IgG (H+L)	Goat	Thermo Fisher Scientific	A11039	1:500
Alexa Fluor 546 Goat Anti-Mouse IgG (H + L)	Goat	Thermo Fisher Scientific	A21143	1:500
Alexa Fluor 647 Goat Anti-Mouse IgG (H+L)	Goat	Thermo Fisher Scientific	A21242	1:500
Alexa Fluor 647 Goat Anti-Rabbit IgG (H+L)	Goat	Thermo Fisher Scientific	A21244	1:500
Alexa Fluor 546 Goat Anti-Rat IgG (H+L)	Goat	Thermo Fisher Scientific	A11081	1:500

Imaging

Confocal stack images were obtained using a Leica SP8 confocal microscope based on a DMI8 stand (Leica Microsystems, Wetzlar, Germany), equipped with 20×/0.75 (oil), 40×/1.10 (water), and 63×/1.30 (glyc) objectives. Images were then processed using ImageJ (63–65).

For the live imaging of neuron exocytosis, young neurons (1 to 2 weeks) from two control NPC lines and two patient NPC lines were transfected with the plasmid pCMV-Sport6-CD63-pHluorin [a gift from D. Pegtel (Addgene plasmid # 130901; <http://n2t.net/addgene:130901>; RRID:Addgene_130901] using the Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, USA) as instructed in the protocol. Forty-eight hours following the transfection, cells were imaged using a Leica TIRF system and a 100×/1.47 numerical aperture objective as previously described (42). The videos obtained were analyzed using the AMvBE (Analyzer of Multivesicular Body Exocytosis) macro previously developed (42) for ImageJ (64).

Silicon probe recordings in COs

For recordings of spontaneous spike activity in COs, we followed Di Matteo *et al.* (36). An 8-month-old CO was glued with a tiny drop (0.25 µl) of Histoacryl (B. Braun) on a polypropylene mesh that was mounted on a circular plastic frame. Afterward, the mCO was incubated at 37°C for at least 30 min in carbogen gas (95% O₂/5% CO₂)–saturated artificial cerebrospinal fluid (ACSF) consisting of (in mM): 121 NaCl, 4.2 KCl, 29 NaHCO₃, 0.45 NaH₂PO₄, 20 glucose, 0.5 Na₂HPO₄, 1.1 CaCl₂, and 1 MgSO₄. Subsequently, the CO was transferred with the holding device to the recording chamber and superfused with warm (37°C) carbogenated ACSF at a flow rate of 2.5 ml/min. For additional stabilization, a custom-made anchor was gently placed on the top of the hCO. Recordings were performed using 16-channel probes (Cambridge Neurotech, ASSY-1 E1), which were connected to a ME2100 system (Multichannel Systems). The headstage was mounted on a PatchStar micromanipulator (Scientifica). Recording data were high-pass filtered at 100 Hz, low-pass filtered at 4 kHz, digitized at 20 kHz, and transferred via a MCS IFB

interface board (Multichannel Systems) to a personal computer. Data were stored using the software Multichannel Experimenter (Multichannel Systems). Visually guided insertion of the silicon probe into COs was conducted using a SliceScope microscope (Scientifica) equipped with a 2.5× objective. Once neuronal activity has been detected, the probe was allowed to stabilize in the tissue for 5 min, and afterward, the activity was recorded for 5 min. For each condition, independent recording areas were used as biological replicates. Recordings were performed in at least three independent areas per CO, keeping a distance of at least 400 μm between two adjacent areas. Independent COs, each from at least two different batches, were analyzed. Sample sizes were $n = 27$ for Ctrl#1, $n = 28$ for Ctrl#2, $n = 28$ for EPM1.1, and $n = 25$ for EPM1.2.

MEA recordings

After 30 days in culture, dorsal and ventral organoids from every genotype were dissociated to single cells as previously described (35). The dorsal and ventral cell suspensions with the same genotype were then mixed at a 50:50 ratio and plated into the 24-well MEA plate (Multichannel System) previously coated with poly-L-ornithine/laminin. Each well contained 12 gold electrodes (30- μm electrode diameter, 300- μm electrode spacing). The neuronal differentiation was sustained for the following 7 weeks by supplementing the complete Neurobasal medium with BDNF (20 ng/ml), glial cell line-derived neurotrophic factor (20 ng/ml), 1 μM cyclic adenosine 3',5'-monophosphate, 200 μM ascorbic acid, and laminin (2 $\mu\text{g}/\text{ml}$).

The recording of spontaneous spike activity was conducted 8 weeks after dissociation using the Multiwell-MEA-System (Multichannel Systems). Five-minute recordings were performed at 37°C under a humidified gas atmosphere (95% O_2 /5% CO_2). Data were high-pass filtered at 1 Hz, low-pass filtered at 3.5 kHz, digitized at 20 kHz, and stored with the Multiwell Screen software (Multichannel Systems). For each condition, independent wells were analyzed as biological replicates. At least three independent wells for each of the three different batches were analyzed. Analysis was conducted with the Multiwell Analyzer software (Multichannel Systems). For each recording, the channel with the highest activity was used for the analysis.

Sample sizes in homo-genotype condition were $n = 12$ for Ctrl#1, $n = 12$ for EPM1.1, and $n = 12$ for EPM1.2.

Sample size in hetero-genotype condition were $n = 5$ for Ctrl#1, $n = 5$ for EPM1.1, and $n = 5$ for EPM1.2.

Proteomic analysis

Sample preparation for mass spectrometry

Analysis was performed as previously described in (1). Briefly, purified EVs, collected from the conditioned media of 20 to 30 COs in culture, and IVs, isolated from a pool of five to seven COs, were lysed in radioimmunoprecipitation assay buffer [150 mM NaCl, 50 mM Tris (pH 8), 0.1% DOC, 0.1% SDS, and 0.1% NP-40]. A 10 μg of protein for each sample was subjected to the modified FASP protocol (66). Briefly, the protein extract was loaded onto the centrifugal filter CO10 kDa (Merck Millipore, Darmstadt, Germany), and detergent was removed by washing five times with 8 M urea (Merck, Darmstadt, Germany) and 50 mM Tris (Sigma-Aldrich, USA) buffer. Proteins were reduced by adding 5 mM dithiothreitol (DTT) (Bio-Rad, Canada) at 37°C for 1 hour in the dark. To remove the excess of DTT, the protein sample was washed three times with 8 M urea and 50 mM Tris. Subsequently, protein thiol groups were blocked with 10 mM

iodoacetamide (Sigma-Aldrich, USA) at room temperature (RT) for 45 min. Before proceeding with the enzymatic digestion, urea was removed by washing the protein suspension three times with 50 mM ammonium bicarbonate (Sigma-Aldrich, Spain). Proteins were digested first by Lys-C (Promega, USA) at RT for 2 hours and then by trypsin (Premium Grade, MS Approved, SERVA, Heidelberg, Germany) at RT, overnight, and both enzymes were added at an enzyme-protein ratio of 1:50 (w/w). Peptides were recovered by centrifugation followed by two additional washes with 50 mM ammonium bicarbonate and 0.5 M NaCl (Sigma-Aldrich, Switzerland). The two filtrates were combined, and the recovered peptides were lyophilized under vacuum. Dried tryptic peptides were desalted using C18-tips (Thermo Fisher Scientific, Pierce, USA), following the manufacture instructions. Briefly, the peptides dissolved in 0.1% (v/v) formic acid (Thermo Fisher Scientific, USA) were loaded onto the C18-tip and washed 10 times with 0.1% (v/v) formic acid; subsequently, the peptides were eluted by 95% (v/v) acetonitrile (Merck, Darmstadt, Germany) and 0.1% (v/v) formic acid. The desalted peptides were lyophilized under vacuum. The purified peptides were reconstituted in 0.1% (v/v) formic acid for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

MS data acquisition

Desalted peptides were loaded onto a 25-cm, 75- μm ID C18 column with integrated nanospray emitter (Odyssey/Aurora, ionoptics, Melbourne) via the autosampler of the Thermo Easy-nLC 1000 (Thermo Fisher Scientific) at 60°C. Eluting peptides were directly sprayed onto the timsTOF Pro (Bruker Daltonics). Peptides were loaded in buffer A [0.1% (v/v) formic acid] at 400 nl/min, and the percentage of buffer B (80% acetonitrile, 0.1% formic acid) was ramped from 5 to 25% over 90 min, followed by a ramp to 35% over 30 min then 58% over the next 5 min, 95% over the next 5 min, and maintained at 95% for another 5 min. Data acquisition on the timsTOF Pro was performed using timsControl. The mass spectrometer was operated in data-dependent PASEF mode with one survey TIMS-MS and 10 PASEF MS/MS scans per acquisition cycle. Analysis was performed in a mass scan range from 100 to 1700 mass/charge ratio (m/z) and an ion mobility range from $1/K_0 = 0.85$ to $1.30 \text{ V} \cdot \text{s} \cdot \text{cm}^{-2}$ using equal ion accumulation and ramp time in the dual TIMS analyzer of 100 ms each at a spectra rate of 9.43 Hz. Suitable precursor ions for MS/MS analysis were isolated in a window of 2 Th for $m/z < 700$ and 3 Th for $m/z > 700$ by rapidly switching the quadrupole position in sync with the elution of precursors from the TIMS device. The collision energy was lowered as a function of ion mobility, starting from 45 eV for $1/K_0 = 1.3 \text{ V} \cdot \text{s} \cdot \text{cm}^{-2}$ to 27 eV for $0.85 \text{ V} \cdot \text{s} \cdot \text{cm}^{-2}$. Collision energies were interpolated linear between these two $1/K_0$ values and kept constant above or below these base points. Singly charged precursor ions were excluded with a polygon filter mask and further m/z , and ion mobility information was used for dynamic exclusion to avoid resequencing of precursors that reached a target value of 14,500 arbitrary unit. The ion mobility dimension was calibrated linearly using three ions from the Agilent ESI LC/MS tuning mix (m/z , $1/K_0$: 622.0289, 0.9848 $\text{V} \cdot \text{s} \cdot \text{cm}^{-2}$; 922.0097 $\text{V} \cdot \text{s} \cdot \text{cm}^{-2}$, 1.1895 $\text{V} \cdot \text{s} \cdot \text{cm}^{-2}$; 1221.9906 $\text{V} \cdot \text{s} \cdot \text{cm}^{-2}$, 1.3820 $\text{V} \cdot \text{s} \cdot \text{cm}^{-2}$).

Raw data analysis of MS measurements

Raw data were processed using the MaxQuant computational platform (version 1.6.17.0) (67) with standard settings applied for ion mobility data (68). Shortly, the peak list was searched against the Uniprot database of Human database (75069 entries, downloaded in

July 2020) with an allowed precursor mass deviation of 10 parts per million (ppm) and an allowed fragment mass deviation of 20 ppm. MaxQuant by default enables individual peptide mass tolerances, which was used in the search. Cysteine carbamidomethylation was set as static modification, and methionine oxidation, deamidation and N-terminal acetylation as variable modifications. The match-between-run option was enabled, and proteins were quantified across samples using the label-free quantification algorithm in MaxQuant generating label-free quantification (LFQ) intensities. The MS proteomics data have been deposited to the ProteomeXchange Consortium (69) via the PRIDE (70) partner repository with the dataset identifier PXD028438.

Bioinformatic analysis of EV proteomics

For the proteomic characterization in EVs, 5489 proteins were quantified. Proteins that were detected with more than 1 peptide and consistently detected in two of the three technical replicates per each condition were retained. Downstream analysis was performed using Perseus (<https://maxquant.net/perseus/>). The LFQs values were $\log_2$ -transformed. Missing values were replaced from normal distribution using the Perseus Imputation function (shift of 1.8 units of the SD and a width of 0.3). Analysis of differentially expressed proteins (DEP) was performed on the imputed data using *t* test in the volcano plot function of Perseus. Proteins with false discovery rate (FDR)-corrected *q* value <0.05 and $\log_2$ fold change values ($\log_2\text{FC}$) ≥ 1 and ≤ -1 were considered as differentially expressed.

Co-IP analysis

The co-IP was performed using the Pierce Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, catalog no. 88805) according to the manufacturer's instructions. Ventral cortical organoids are generated from two independent control iPSC lines. For each line, two biological replicates were prepared by pooling two organoids per replicate at day 40 of differentiation. Organoids were lysed in the kit lysis buffer [25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol (pH 7.4)] supplemented with 2× protease and phosphatase inhibitor cocktail (Sigma-Aldrich). For each condition, 7 μg of anti-CSTB antibody (Proteintech, catalog no. 10823-1-AP) was covalently crosslinked to Protein A/G magnetic beads using disuccinimidyl suberate to minimize antibody coelution. Equal amounts of clarified organoid lysates were incubated with antibody-coupled beads for 1 hour at room temperature under gentle rotation. After extensive washing, bound proteins were eluted with 110 μl of low-pH elution buffer (pH 2.0), immediately neutralized to pH 8.5, and subjected to LC-MS/MS analysis. Each IP sample was analyzed in triplicates by mass spectrometry (technical replicates). In parallel, control co-IPs were performed using species- and isotype-matched normal immunoglobulin G (IgG) crosslinked to Protein A/G beads, and proteins recovered in IgG control IPs were used to identify and subtract nonspecific background from CSTB interactors. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (70) partner repository with the dataset identifier PXD072599.

Bioinformatic analysis of CSTB interactome

For the CSTB interactome analysis, 2769 proteins were quantified. Proteins detected with more than one unique peptide and consistently identified in at least two of the three technical replicates per condition were retained. Downstream analysis was performed using Perseus (<https://maxquant.net/perseus/>). The LFQs values were $\log_2$ -

transformed. Missing values were imputed from a normal distribution using the Perseus Imputation function (down shift = 1.8 SDs; width = 0.3). To identify robust CSTB interactors, proteins that were common to both biological replicates in each control iPSC line were first determined. The resulting protein lists from the two control lines were intersected to obtain the set of proteins reproducibly detected across biological replicates. In parallel, proteins identified in IgG control co-IPs were used to determine nonspecific background. All proteins detected in IgG control samples were removed from the CSTB interactor list. The remaining CSTB-specific proteins (present in both biological replicates and absent from IgG controls) were considered high-confidence CSTB interactors and were subsequently visualized and used for downstream enrichment analyses. Functional enrichment analysis was carried out using g:Profiler (<https://biit.cs.ut.ee/gprofiler/>). GO biological process, cellular component, and molecular function categories were assessed, and significance was determined using the g:SCS algorithm for multiple testing correction (adjusted *P* < 0.05). The top enriched terms were visualized using bar plots.

RNA extraction, cDNA synthesis, and real-time qPCR

Ventral NPCs obtained by dissociation of vCOs and treated with EVs for 2 weeks were lysed in QIAzol Lysis Reagent (QIAGEN, 79306). RNA was extracted using the RNA Clean & Concentrator Kit (Zymo Research, R1015), and cDNA was synthesized using SuperScript III reverse transcriptase (Thermo Fisher Scientific, 18080-044) with Random primers (Invitrogen, 48190-11) according to the manufacturer's protocol. Subsequently, qPCR was performed in triplicates on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) (Roche) using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25742).

The primers sequences were as follows:

GAPDH (forward: 5'-AATCCCATCACCATCTTCCAGGA-3'; reverse: 5'-TGGACTCCACGACGTACTCAG-3'),

GLI1 (forward: 5'-AGCCTTCAGCAATGCCAGTGAC-3'; reverse: 5'-GTCAGGACCATGCACTGTCTTG-3'),

PTCH1 (forward: 5'-GCTGCACTACTTCAGAGACTGG-3'; reverse: 5'-CACCAGGAGTTTGTAGGCAAGG-3'),

PTCH2 (forward: 5'-GCACTATTACCGCAACTGGCTAC-3'; reverse: 5'-TCTCCAGTCTGGATGAGCAGCT-3'),

TTF-1 (NKX2-1) (forward: 5'-CAGGACACCATGAGGAACAGCG-3'; reverse: 5'-GCCATGTTCTTGCTCACGTCCC-3'), and

OLIG2 (forward: 5'-ATGCACGACCTCAACATCGCCA-3'; reverse: 5'-ACCAGTCGCTTCATCTCCTCCA-3').

The qPCR was done with initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Relative expression was calculated using the $\Delta\Delta\text{Cp}$ method.

scRNA-seq sample preparation

Sample preparation for scRNA-seq was performed as in (7). Briefly, single-cell dissociation was performed on five 40 days old-patterned spheroids randomly selected for each pattern condition. Single cells were dissociated using StemPro Accutase Cell Dissociation Reagent (Life Technologies), filtered through 30 and 20 μm filters (Miltenyi Biotec), and cleaned of debris using a Percoll (Sigma-Aldrich, P1644) gradient. Single cells were resuspended in ice-cold PBS supplemented with 0.04% BSA at a concentration of 1000 cells/ μl . Single cells were loaded onto a Chromium Next GEM Single Cell 3' chip (Chromium Next GEM Chip G Single Cell Kit, 16 rxns 10XGenomics

PN-1000127) with the Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1 (Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, 4 rxns 10xGenomics PN-1000128), and cDNA libraries were generated with the Single Index Kit T Set A, 96 rxns (10x Genomics PN-1000213) according to the manufacturer's instructions. Libraries were sequenced using Illumina NovaSeq6000 in 28/8/91-base pair (bp) mode (SP flowcell), and quality control and UMI counting were performed by the Max-Planck für molekulare Genetik (Germany).

scRNA-seq data analysis

Raw sequencing data were processed using Cell Ranger v6.0.0 (10x Genomics), a software suite for analyzing scRNA-seq data. The count function within Cell Ranger was used for read alignment, quantification of gene expression, and generation of a gene-cell matrix. The analysis was conducted on a high-performance computing cluster, using 250 GB of memory and 32 CPU cores on a single node. The human reference genome, GRCh38 version 1.2.0, was used for reads alignment and transcriptome generation, with intronic reads included to capture nascent transcripts and secondary alignments excluded.

Quality control (QC) and preprocessing of scRNA-seq data were performed using Scanpy (71). Cells with low quality, such as those with a high proportion of mitochondrial reads or an abnormally low number of genes expressed, were removed to ensure the retention of high-quality data for further analysis.

To remove low-quality cells from the dataset (Table 2), an outlier detection strategy was implemented on the basis of key quality metrics. Cells were filtered using thresholds based on the median and median absolute deviation (MAD) of these metrics. Specifically, outliers were defined as cells where any of the following conditions were met: the total counts per cell (log1p_total_counts) deviated more than five MADs from the median, the number of genes expressed per cell (log1p_n_genes_by_counts) deviated more than five MADs from the median, or the percentage of counts in the top 20 most expressed genes (pct_counts_in_top_20_genes) deviated more than five MADs from the median.

In addition, mitochondrial content was used to further identify and remove low-quality cells. Cells were considered outliers if the percentage of mitochondrial gene expression (pct_counts_mt) deviated more than three MADs from the median or exceeded a fixed threshold of 8%.

To identify and remove potential doublets from the dataset, the scDblFinder algorithm was used within the R environment,

integrated into the Python workflow using rpy2. The raw count matrix was transferred from the Scanpy object into the R environment, where the scDblFinder package was used to classify cells as doublets or singlets. Specifically, the SingleCellExperiment framework was used to store the expression data, and doublets were identified using scDblFinder with a random seed set for reproducibility.

Following this process, each cell was assigned a doublet score and a classification as either a doublet or singlet. Cells classified as doublets were excluded from the dataset before proceeding with downstream analyses, ensuring that only singlet cells were retained for further clustering and gene expression analysis.

Normalization was performed using the scanr method to account for variations in library size. Size factors were computed for each cell using the computeSumFactors function, leveraging preliminary clustering to improve accuracy. The count matrix was then divided by these size factors, and a log1p transformation was applied to the normalized data. This scanr-normalized data were stored in the adata.layers for downstream analyses. Highly variable genes were selected for downstream analysis using Scanpy's highly_variable_genes function. Principal components analysis (PCA) was applied to reduce the dimensionality of the dataset, and the top 30 principal components were used for neighborhood graph construction. To cluster the cells, the Leiden algorithm (72) was used using a resolution of 1. The neighborhood graph was constructed using the pp.neighbors function, and clustering was performed using tl.leiden. To visualize the clusters, UMAP was performed with the tl.umap function in Scanpy. Clusters were visualized in 2D space, and marker genes were used to assign cell types to the clusters. Differential gene expression analysis was performed between clusters using the Wilcoxon rank-sum test to identify potential marker genes for each cell population. Cell types were also annotated using CellTypist. The pretrained model developing_Human_Brain.pkl was applied for automated cell type prediction, as described by Xu *et al.* (73) and Domínguez Conde *et al.* (74). This automated annotation was run in parallel with marker gene-based manual annotation to ensure accuracy and consistency in cell type identification. Cells identified as fibroblasts through the automated annotation with CellTypist were excluded from the dataset to focus on other relevant cell populations. We retained a total of 10,972 cells for the ventral organoid experiments and 10,965 cells for the mosaic experiment. These high-quality cells were used for all subsequent analyses. Following this, batch effects were corrected, and datasets were integrated using Scanorama to ensure a harmonized view across different samples.

Table 2. scRNA-seq QC metrics. QC, quality control.				
Condition	Cell line/replicate	Cells loaded	Cells after QC	Cells after non-neuro cell types
Ventral	vCTRL 1	5,021	3,184	2,899
	vCTRL 2	9,115	5,515	5,369
	vEPM1 1	5,485	3,045	2,351
	vEPM1 2	5,285	3,307	353
Mosaic	mvCTRL 1	29,155	14,683	8,218
	mvCTRL 2	11,620	7,502	1,415
	mvEPM1 1	19,900	17,960	22
	mvEPM1 2	10,364	7,143	1,310

The integration was performed to account for technical variations while preserving the biological variance of interest.

Pseudobulk RNA-seq analysis was performed by aggregating the counts of cells belonging to specific cell types into pseudo-replicates. Cells annotated as progenitors were selected, and counts from these cells were summed across pseudo-replicates. The resulting pseudobulk data were analyzed using the DESeq2 pipeline via the pyDESeq2 package. The differential expression analysis was conducted between different conditions, and the results were visualized using volcano plots to identify significantly up- and down-regulated genes. Specific gene sets of interest, such as those involved in the Wnt signaling pathway and dorsoventral patterning, were highlighted in the analysis.

All analyses were performed using Python version 3.9.18. The following packages were used for the analysis: Scanpy version 1.9.6, Scanorama version 1.7.4, NumPy version 1.26.2, Pandas version 2.1.1, and Matplotlib version 3.7.1

Bulk RNA-seq

RNA-seq was performed on 10 ng of total RNA collected from three independent wells of NPCs from a 24-well plate. NPCs were not treated with EVs or treated for 12 hours with EVs collected by ultracentrifugation from 25 ml of conditioned medium collected from 28 to 37 days in culture COs (control ventral, EPM1 ventral). NPCs were lysed in 1 ml of TRIzol (QIAGEN) per well, and RNA was isolated using the RNA Clean & Concentrator Kit (Zymo Research) including the digestion of remaining genomic DNA according to producer's guidelines. RNA was further processed according to (75). Briefly, cDNA synthesis was performed with SMART-Seq v4 Ultra Low Input RNA Kit (Clontech catalog no. 634888) according to the manufacturer's instruction. cDNA was fragmented to an average size of 200 to 500 bp in a Covaris S220 device (5 min; 4°C; PP 175; DF 10; CB 200). Fragmented cDNA was used as input for library preparation with MicroPlex Library Preparation Kit v2 (Diagenode, catalog no. C05010012) and processed according to the manufacturer's instruction. Libraries were quality controlled by Qubit and Agilent DNA Bioanalyzer analysis. Deep sequencing was performed on a HiSeq 1500 system according to the standard Illumina protocol for 50-bp paired-end reads with v3 sequencing reagents.

Bulk RNA-seq analysis

Paired end reads were aligned to the human genome version GRCh38 using STAR v2.6.1d (76) with default options "--runThreadN 32 --quantMode TranscriptomeSAM GeneCounts --outSAMtype BAM SortedByCoordinate". Reads-per-gene counts were imported in R v4.1.0. Expression data from Bulk RNA-seq samples were loaded on Rstudio, focusing on transcript per million values. Data normalization was performed by calculating z-scores across expression values, facilitating comparability between samples, and rows with missing values were excluded to ensure data integrity. Hierarchical clustering was used to uncover patterns in expression profiles. Clusters were visualized on a heatmap, with both rows (genes) and columns (samples) grouped based on similarity. Gene clusters were extracted, and a final list of genes within each cluster was compiled and exported for further analyses. The R packages ComplexHeatmap (77), circlize (78), colorspace (79), GetoptLong (https://cran.r-project.org/web/packages/GetoptLong/), and ggpattern (https://github.com/coolbutuseless/ggpattern) were used for heatmap visualization, clustering, and customization. Dendsort (80) refined hierarchical clustering, while RColorBrewer (https://cran.r-project.org/web/packages/RColorBrewer/) and colorspace (79) managed color gradients. Pheatmap (https://

cran.r-project.org/web/packages/pheatmap/) enabled targeted gene display and write.table exported clustered gene lists.

Enrichment analysis

GO term analysis of differentially expressed proteins was tested using the FUMA algorithm (81) by inserting the DE protein lists into the GENE2FUNC software (FDR < 0.05) (https://fuma.ctglab.nl/) or with STRING (https://string-db.org).

Supplementary Materials

The PDF file includes:

Legend for table S1
Figs. S1 to S6

Other Supplementary Material for this manuscript includes the following:

Table S1

REFERENCES

1. A. Forero, F. Pipicelli, S. Moser, N. Baumann, C. Grätz, M. G. Pisfil, M. W. Pfaffl, B. Pütz, P. Kielkowski, F. M. Cernilogar, G. Maccarrone, R. D. Giaimo, S. Cappello, Extracellular vesicle-mediated trafficking of molecular cues during human brain development. *Cell Rep.* **43**, 114755 (2024).
2. K. R. Long, W. B. Huttner, The role of the extracellular matrix in neural progenitor cell proliferation and cortical folding during human neocortex development. *Front. Cell. Neurosci.* **15**, 804649 (2021).
3. O. Marín, J. L. Rubenstein, A. long, remarkable journey: Tangential migration in the telencephalon. *Nat. Rev. Neurosci.* **2**, 780–790 (2001).
4. C. G. Silva, E. Peyre, L. Nguyen, Cell migration promotes dynamic cellular interactions to control cerebral cortex morphogenesis. *Nat. Rev. Neurosci.* **20**, 318–329 (2019).
5. H. Wichterle, J. M. Garcia-Verdugo, D. G. Herrera, A. Alvarez-Buylla, Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat. Neurosci.* **2**, 461–466 (1999).
6. S. Amin, V. Borrell, The Extracellular Matrix in the Evolution of Cortical Development and Folding. *Front. Cell Dev. Biol.* **8**, 604448 (2020).
7. F. Pipicelli, N. Baumann, R. D. Giaimo, A. Forero-Echeverry, C. Kyrousi, R. Bonrath, G. Maccarrone, D. Jabaudon, S. Cappello, Non-cell-autonomous regulation of interneuron specification mediated by extracellular vesicles. *Sci. Adv.* **9**, eadd8164 (2023).
8. K. R. Long, W. B. Huttner, How the extracellular matrix shapes neural development. *Open Biol.* **9**, 180216 (2019).
9. E. Klingler, F. Francis, D. Jabaudon, S. Cappello, Mapping the molecular and cellular complexity of cortical malformations. *Science* **371**, (2021).
10. S. F. Berkovic, F. Andermann, S. Carpenter, L. S. Wolfe, Progressive myoclonus epilepsies: Specific causes and diagnosis. *N. Engl. J. Med.* **315**, 296–305 (1986).
11. M. D. Lalioti, H. S. Scott, C. Buresi, C. Rossier, A. Bottani, M. A. Morris, A. Malafosse, S. E. Antonarakis, Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy. *Nature* **386**, 847–851 (1997).
12. T. Joensuu, M. Kuronen, K. Alakurtti, S. Tegelberg, P. Hakala, A. Aalto, L. Huopaniemi, N. Aula, R. Michellucci, K. Eriksson, A.-E. Lehesjoki, Cystatin B: Mutation detection, alternative splicing and expression in progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1) patients. *Eur. J. Hum. Genet.* **15**, 185–193 (2007).
13. L. Canafoglia, E. Gennaro, G. Capovilla, G. Gobbi, A. Boni, F. Beccaria, M. Viri, R. Michelucci, P. Agazzi, S. Assereto, D. A. Coviello, M. Di Stefano, D. Rossi Sebastiano, S. Franceschetti, F. Zara, Electroclinical presentation and genotype-phenotype relationships in patients with Unverricht-Lundborg disease carrying compound heterozygous *CSTB* point and indel mutations. *Epilepsia* **53**, 2120–2127 (2012).
14. P. Koskenkorva, J. Hyppönen, M. Äikiä, E. Mervaala, T. Kiviranta, K. Eriksson, A.-E. Lehesjoki, R. Vanninen, R. Kälviäinen, Severe Phenotype in Unverricht-Lundborg Disease (EPM1) Patients Compound Heterozygous for the Dodecamer Repeat Expansion and the c.202C>T Mutation in the *CSTB* Gene. *Neurodegener. Dis.* **8**, 515–522 (2011).
15. G. M. S. Mancini, R. Schot, M. C. Y. De Wit, R. F. De Co, R. Oostenbrink, K. Bindels-de Heus, L. P. V. Berger, M. H. Lequin, F. A. T. De Vries, M. Wilke, M. A. van Slegtenhorst, *CSTB* null mutation associated with microcephaly, early developmental delay, and severe dyskinesia. *Neurology* **86**, 877–878 (2016).
16. A. O'Brien, C. R. Marshall, S. Blaser, P. N. Ray, G. Yoon, Severe neurodegeneration, progressive cerebral volume loss and diffuse hypomyelination associated with a homozygous frameshift mutation in *CSTB*. *Eur. J. Hum. Genet.* **25**, 775–778 (2017).
17. V. Turk, W. Bode, The cystatins: Protein inhibitors of cysteine proteinases. *FEBS Lett.* **285**, 213–219 (1991).

18. A. Rispoli, E. Cipollini, S. Catania, R. Di Giaimo, G. Pulice, S. Van Houte, F. Sparla, F. Dal Piaz, D. Roncarati, P. Trost, M. Melli, Insights in progressive myoclonus epilepsy: HSP70 promotes cystatin B polymerization. *Biochim. Biophys. Acta* **1834**, 2591–2599 (2013).
19. E. Žerovnik, Human stefin B: From its structure, folding, and aggregation to its function in health and disease. *Front. Mol. Neurosci.* **15**, 1009976 (2022).
20. E. Daura, S. Tegelberg, P. Hakala, A.-E. Lehesjoki, T. Joensuu, Cystatin B deficiency results in sustained histone H3 tail cleavage in postnatal mouse brain mediated by increased chromatin-associated cathepsin L activity. *Front. Mol. Neurosci.* **15**, 1069122 (2022).
21. R. Di Giaimo, New insights into the molecular basis of progressive myoclonus epilepsy: A multiprotein complex with cystatin B. *Hum. Mol. Genet.* **11**, 2941–2950 (2002).
22. M. Trstenjak-Prebenda, M. Biasizzo, K. Dolinar, S. Pirkmajer, B. Turk, V. Brault, Y. Herault, N. Kopitar-Jerala, Stefins Inhibit NLRP3 Inflammasome Activation via AMPK/mTOR signalling. *Cells* **12**, 2731 (2023).
23. M. Polajnar, T. Zavašnik-Bergant, K. Škerget, M. Vizovišek, R. Vidmar, M. Fonovič, N. Kopitar-Jerala, U. Petrovič, S. Navarro, S. Ventura, E. Žerovnik, Human stefin B Role in cell's response to misfolded proteins and autophagy. *PLOS ONE* **9**, e102500 (2014).
24. E. Žerovnik, Molecular and cellular processes underlying Unverricht-Lundborg disease—prospects for early interventions and a cure. *Explor. Neurosci.* **3**, 295–308 (2024).
25. R. Di Giaimo, E. Penna, A. Pizzella, R. Cirillo, C. Perrone-Capano, M. Crispino, Cross talk at the cytoskeleton–plasma membrane interface: Impact on neuronal morphology and functions. *Int. J. Mol. Sci.* **21**, 9133 (2020).
26. K. Gorski, C. B. Jackson, T. A. Nyman, V. Rezov, B. J. Battersby, A.-E. Lehesjoki, Progressive mitochondrial dysfunction in cerebellar synaptosomes of cystatin B-deficient mice. *Front. Mol. Neurosci.* **16**, 1175851 (2023).
27. E. Penna, A. Cerciello, A. Chambery, R. Russo, F. M. Cernilogar, E. M. Pedone, C. Perrone-Capano, S. Cappello, R. Di Giaimo, M. Crispino, Cystatin B Involvement in Synapse Physiology of Rodent Brains and Human Cerebral Organoids. *Front. Mol. Neurosci.* **12**, 195 (2019).
28. A. Pizzella, E. Penna, N. Abate, E. Frenna, L. Canafoglia, F. Ragona, R. Russo, A. Chambery, C. Perrone-Capano, S. Cappello, M. Crispino, R. Di Giaimo, Pathological deficit of Cystatin B Impairs synaptic plasticity in EPM1 human cerebral organoids. *Mol. Neurobiol.* **61**, 4318–4334 (2024).
29. T. Joensuu, S. Tegelberg, E. Reinmaa, M. Segerstråle, P. Hakala, H. Pehkonen, E. R. Korpi, J. Tynnelä, T. Taira, I. Hovatta, O. Kopra, A.-E. Lehesjoki, Gene expression alterations in the cerebellum and granule neurons of Cstb^{−/−} mouse are associated with early synaptic changes and inflammation. *PLOS ONE* **9**, e89321 (2014).
30. A. Buzzi, M. Chikhladze, C. Falcicchia, B. Paradiso, G. Lanza, M. Soukupova, M. Marti, M. Morari, S. Franceschetti, M. Simonato, Loss of cortical GABA terminals in Unverricht-Lundborg disease. *Neurobiol. Dis.* **47**, 216–224 (2012).
31. K. Silvennoinen, L. Säisänen, J. Hyppönen, S. M. Rissanen, P. A. Karjalainen, S. D'Ambrosio, D. Jimenez-Jimenez, S. Zagaglia, J. C. Rothwell, S. Balestrini, S. M. Sisodiya, P. Julkunen, E. Mervala, R. Kälviäinen, Short- and long-interval intracortical inhibition in EPM1 is related to genotype. *Epilepsia* **64**, 208–217 (2023).
32. L. Canafoglia, C. Ciano, F. Panzica, V. Scafoli, C. Zucca, P. Agazzi, E. Visani, G. Avanzini, S. Franceschetti, Sensorimotor cortex excitability in Unverricht-Lundborg disease and Lafora body disease. *Neurology* **63**, 2309–2315 (2004).
33. M. Kyllerman, K. Sommerfelt, A. Hedstrom, G. Wennergren, D. Holmgren, Clinical and Neurophysiological Development of Unverricht-Lundborg Disease in Four Swedish Siblings. *Epilepsia* **32**, 900–909 (1991).
34. S. Singh, R. H. Hämläinen, The Roles of Cystatin B in the Brain and Pathophysiological Mechanisms of Progressive Myoclonic Epilepsy Type 1. *Cells* **13**, 170 (2024).
35. F. Di Matteo, F. Pipicelli, C. Kyrousi, I. Tovecci, E. Penna, M. Crispino, A. Chambery, R. Russo, A. C. Ayo-Martin, M. Giordano, A. Hoffmann, E. Cusani, L. Canafoglia, M. Götz, R. Di Giaimo, S. Cappello, Cystatin B is essential for proliferation and interneuron migration in individuals with EPM1 epilepsy. *EMBO Mol. Med.* **12**, e11419 (2020).
36. F. Di Matteo, R. Bonrath, V. Pravata, H. Schmidt, A. C. Ayo Martin, R. Di Giaimo, D. Menegaz, S. Riesenberger, F. M. S. de Vrij, G. Maccarrone, M. Holzapfel, T. Straub, S. A. Kushner, S. P. Robertson, M. Eder, S. Cappello, Neuronal hyperactivity in neurons derived from individuals with gray matter heterotopia. *Nat. Commun.* **16**, 1737 (2025).
37. V. S. Sohal, J. L. R. Rubenstein, Excitation-inhibition balance as a framework for investigating mechanisms in neuropsychiatric disorders. *Mol. Psychiatry* **24**, 1248–1257 (2019).
38. J. A. Bagley, D. Reumann, S. Bian, J. Lévi-Strauss, J. A. Knoblich, Fused cerebral organoids model interactions between brain regions. *Nat. Methods* **14**, 743–751 (2017).
39. M. Mathieu, L. Martin-Jaular, G. Lavieu, C. Théry, Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol.* **21**, 9–17 (2019).
40. C. Théry, K. W. Witwer, E. Aikawa, M. J. Alcaraz, J. D. Anderson, R. Andriantsitohaina, A. Antoniou, T. Arab, F. Archer, G. K. Atkin-Smith, D. C. Ayre, J.-M. Bach, D. Bachurski, H. Baharvand, L. Balaj, S. Baldacchino, N. N. Bauer, A. A. Baxter, M. Bebawy, C. Beckham, A. B. Zavec, A. Benmoussa, A. C. Berardi, P. Bergese, E. Bielska, C. Blenkiron, S. Bobis-Wozowicz, E. Boilard, W. Boireau, A. Bongiovanni, F. E. Borràs, S. Bosch, C. M. Boulanger, X. Breakefield, A. M. Breglio, M. A. Brennan, D. R. Brigstock, A. Brisson, M. L. Broekman, J. F. Bromberg, P. Bryl-Górecka, S. Buch, A. H. Buck, D. Burger, S. Busatto, D. Buschmann, B. Bussolati, E. I. Buzás, J. B. Byrd, G. Camussi, D. R. Carter, S. Caruso, L. W. Chamley, Y.-T. Chang, C. Chen, S. Chen, L. Cheng, A. R. Chin, A. Clayton, S. P. Clerici, A. Cocks, E. Cocucci, R. J. Coffey, A. Cordeiro-da-Silva, Y. Couch, F. A. Coumans, B. Coyle, R. Crescitelli, M. N. Criado, C. D'Souza-Schorey, S. Das, A. D. Chaudhuri, P. de Candia, E. F. De Santana, O. De Wever, H. A. Del Portillo, T. Demaret, S. Deville, A. Devitt, B. Dhondt, D. D. Vizio, L. C. Dieterich, V. Dolo, A. P. D. Rubio, M. Dominici, M. R. Dourado, T. A. Driedonks, F. V. Duarte, H. M. Duncan, R. M. Eichenberger, K. Ekström, S. E. Andaloussi, C. Elie-Caille, U. Erdbrügger, J. M. Falcón-Pérez, F. Fatima, J. E. Fish, M. Flores-Bellver, A. Forsén, A. Frelet-Barrand, F. Fricke, G. Fuhrmann, S. Gabrielsson, A. Gámez-Valero, C. Gardiner, K. Gärtner, R. Gaudin, Y. S. Gho, B. Giebel, C. Gilbert, M. Gimona, I. Giusti, D. C. Goberdhan, A. Görgens, S. M. Gorski, D. W. Greening, J. C. Gross, A. Gualerzi, G. N. Gupta, D. Gustafson, A. Handberg, R. A. Haraszi, P. Harrison, H. Hegyesi, A. Hendrix, A. F. Hill, F. H. Hochberg, K. F. Hoffmann, B. Holder, H. Holthofer, B. Hosseinkhani, G. Hu, Y. Huang, V. Huber, S. Hunt, A. G.-E. Ibrahim, T. Ikezu, J. M. Inal, M. Isin, A. Ivanova, H. K. Jackson, S. Jacobsen, S. M. Jay, M. Jayachandran, G. Jenster, L. Jiang, S. M. Johnson, J. C. Jones, A. Jong, T. Jovanovic-Talisman, S. Jung, R. Kalluri, S.-I. Kano, S. Kaur, Y. Kawamura, E. T. Keller, D. Khamari, E. Khomyakova, A. Khvorova, P. Kierulff, K. P. Kim, T. Kislinger, M. Klingeborn, D. J. Klinke II, M. Kornek, M. M. Kosanović, A. F. Kovács, E.-M. Krämer-Albers, S. Krasemann, M. Krause, I. V. Kurochkin, G. D. Kusuma, S. Kuypers, S. Laitinen, S. M. Langevin, L. R. Languino, J. Lannigan, C. Lässer, L. C. Laurent, G. Lavieu, E. Lázaro-Ibáñez, S. L. Lay, M.-S. Lee, Y. X. F. Lee, D. S. Lemos, M. Lenassi, A. Leszczynska, I. T. Li, K. Liao, S. F. Libregts, E. Ligeti, R. Lim, S. K. Lim, A. Liné, K. Linemannstons, A. Llorente, C. A. Lombard, J. M. Lorenowicz, A. M. Löhrich, J. Lötvall, J. Lovett, M. C. Lowry, X. Loyer, Q. Lu, B. Lukomska, T. L. Lunavat, S. L. Maas, H. Malhi, A. Marcilla, J. Mariani, J. Mariscal, E. S. Martens-Uzunova, L. Martin-Jaular, M. C. Martinez, V. R. Martins, M. Mathieu, S. Mathivanan, M. Maugeri, L. K. McGinnis, M. J. McVey, D. G. Meckes Jr., K. L. Meehan, I. Mertens, V. R. Minciacci, A. Möller, M. M. Jørgensen, A. Morales-Kastresana, J. Morhayim, F. Mullier, M. Muraca, L. Musante, V. Mussack, D. C. Muth, K. H. Myburgh, T. Najrana, M. Nawaz, I. Nazarenko, P. Nejsun, C. Neri, T. Neri, R. Nieuwland, L. Nimrichter, J. P. Nolan, E. N. Nolte-t Hoen, N. N. Hooten, L. O'Driscoll, T. O'Grady, A. O'Loghlen, T. Ochiya, M. Olivier, A. Ortiz, L. A. Ortiz, X. Osteoetxea, O. Østergaard, M. Ostrowski, J. Park, D. M. Pegtel, H. Peinado, F. Perut, M. W. Pfaffl, D. G. Phinney, B. C. Pieters, R. C. Pink, D. S. Pisetsky, E. P. von Strandmann, I. Polakovicova, I. K. Poon, B. H. Powell, I. Prada, L. Pulliam, P. Quesenberry, A. Radeghieri, R. L. Raffai, S. Raimondo, J. Rak, M. I. Ramirez, G. Raposo, M. S. Rayyan, N. Regev-Rudzki, F. L. Ricklefs, P. D. Robbins, D. D. Roberts, S. C. Rodriguez, E. Rohde, S. Rome, K. M. Rouschop, A. Rugghetti, A. E. Russell, P. Saá, S. Sahoo, E. Salas-Huenuleo, C. Sánchez, J. A. Saugstad, M. J. Saul, R. M. Schifferers, R. Schneider, T. H. Schøyen, A. Scott, E. Shahaj, S. Sharma, O. Shatnyeva, F. Shekari, G. V. Shelke, A. K. Shetty, K. Shiba, P. R.-M. Siljander, A. M. Silva, A. Skowronek, A. M. Snyder II, R. P. Soares, B. W. Sódar, C. Soekmadji, J. Sotillo, P. D. Stahl, W. Stoorvogel, S. L. Stott, E. F. Strasser, S. Swift, H. Tahara, M. Tewari, K. Timms, S. Tiwari, R. Tixeira, M. Tkach, W. S. Toh, R. Tomasini, A. C. Torrecillas, J. P. Tosar, V. Toxavidis, L. Urbanelli, P. Vader, B. W. van Balkom, S. G. van der Grein, J. Van Deun, M. J. van Herwijnen, K. Van Keuren-Jensen, G. van Niel, M. E. van Royen, A. J. van Wijnen, M. H. Vasconcelos, I. J. Vechetti Jr., T. D. Veit, L. J. Vella, É. Velot, F. J. Verweij, B. Vestad, J. L. Viñas, T. Visnovitz, K. V. Vukman, J. Wahlgren, D. C. Watson, M. H. Wauben, A. Weaver, J. P. Webber, V. Weber, A. M. Wehman, D. J. Weiss, J. A. Welsh, S. Wendt, A. M. Wheelock, Z. Wiener, L. Witte, J. Wolfram, A. Xagorari, P. Xander, J. Xu, X. Yan, M. Yáñez-Mó, H. Yin, Y. Yuana, V. Zappulli, J. Zarubova, V. Žekas, J.-Y. Zhang, Z. Zhao, L. Zheng, A. R. Zheutlin, A. M. Zickler, P. Zimmermann, A. M. Zivkovic, D. Zocco, E. K. Zuba-Surma, Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **8**, 1535750 (2018).
41. G. Kaur, P. Mohan, M. Pawlik, S. DeRosa, J. Fajiculy, S. Che, A. Grubb, S. D. Ginsberg, R. A. Nixon, E. Levy, Cystatin C rescues degenerating neurons in a cystatin B-knockout mouse model of progressive myoclonus epilepsy. *Am. J. Pathol.* **177**, 2256–2267 (2010).
42. F. J. Verweij, M. P. Bebelman, C. R. Jimenez, J. J. Garcia-Vallejo, H. Janssen, J. Neefjes, J. C. Knol, R. De Goeij-de Haas, S. R. Piersma, S. R. Baglio, M. Verhage, J. M. Middeldorp, A. Zomer, J. van Rheenen, M. G. Coppolino, I. Hurbain, G. Raposo, M. J. Smit, R. F. G. Toonen, G. Van Niel, D. M. Pegtel, Quantifying exosome secretion from single cells reveals a modulatory role for GPCR signaling. *J. Cell Biol.* **217**, 1129–1142 (2018).
43. J. Ericson, J. Muhr, M. Placzek, T. Lints, T. M. Jessell, T. Edlund, Sonic hedgehog induces the differentiation of ventral forebrain neurons: A common signal for ventral patterning within the neural tube. *Cell* **81**, 747–756 (1995).
44. L. Puelles, J. L. R. Rubenstein, Forebrain gene expression domains and the evolving prosomeric model. *Trends Neurosci.* **26**, 469–476 (2003).
45. M. Rallu, J. G. Corbin, G. Fishell, Parsing the prosencephalon. *Nat. Rev. Neurosci.* **3**, 943–951 (2002).

46. Q. Xu, C. P. Wonders, S. A. Anderson, Sonic hedgehog maintains the identity of cortical interneuron progenitors in the ventral telencephalon. *Development* **132**, 4987–4998 (2005).
47. M. E. Coulter, C. M. Dorobantu, G. A. Lodewijk, F. Delalande, S. Cianferani, V. S. Ganesh, R. S. Smith, E. T. Lim, C. S. Xu, S. Pang, E. T. Wong, H. G. W. Lidow, M. L. Calicchio, E. Yang, D. M. Gonzalez, T. M. Schlaeger, G. H. Mochida, H. Hess, W.-C. A. Lee, M. K. Lehtinen, T. Kirchhausen, D. Haussler, F. M. J. Jacobs, R. Gaudin, C. A. Walsh, The ESCRT-III protein CHMP1A mediates secretion of sonic hedgehog on a distinctive subtype of extracellular vesicles. *Cell Rep.* **24**, 973–986.e8 (2018).
48. M. Galimberti, M. R. Nucera, V. D. Bocchi, P. Conforti, E. Vezzoli, M. Cereda, C. Maffezzini, R. Iennaco, A. Scolzi, A. Falqui, C. Cordiglieri, M. Cremona, I. Espuny-Camacho, A. Faedo, D. P. Felsenfeld, T. F. Vogt, V. Ranzani, C. Zuccato, D. Besusso, E. Cattaneo, Huntington's disease cellular phenotypes are rescued non-cell autonomously by healthy cells in mosaic telencephalic organoids. *Nat. Commun.* **15**, 6534 (2024).
49. S. Suoranta, K. Holli-Helenius, P. Koskenkorva, E. Niskanen, M. Kõnönen, M. Äikiä, H. Eskola, R. Kälviäinen, R. Vanninen, 3D Texture analysis reveals imperceptible mri textural alterations in the thalamus and putamen in progressive myoclonic epilepsy type 1, EPM1. *PLOS ONE* **8**, e69905 (2013).
50. P. Koskenkorva, E. Niskanen, J. Hyppönen, M. Kõnönen, E. Mervaala, H. Soininen, R. Kälviäinen, R. Vanninen, Sensorimotor, visual, and auditory cortical atrophy in Unverricht-Lundborg disease mapped with cortical thickness analysis. *AJNR Am. J. Neuroradiol.* **33**, 878–883 (2012).
51. P. Shannan, L. A. Pennacchio, M. K. Houseweart, B. A. Minassian, R. M. Myers, Neuropathological changes in a mouse model of progressive myoclonus epilepsy: Cystatin B deficiency and Unverricht-Lundborg disease. *J. Neuropathol. Exp. Neurol.* **61**, 1085–1091 (2002).
52. S. Zhao, C. M. Li, X. M. Luo, G. K. Y. Siu, W. J. Gan, L. Zhang, W. K. K. Wu, H. C. Chan, S. Yu, Mammalian TRAPPIII complex positively modulates the recruitment of Sec13/31 onto COPII vesicles. *Sci. Rep.* **7**, 43207 (2017).
53. G. Y. Cederquist, J. J. Asciolla, J. Tchieu, R. M. Walsh, D. Cornacchia, M. D. Resh, L. Studer, Specification of positional identity in forebrain organoids. *Nat. Biotechnol.* **37**, 436–444 (2019).
54. J. Klaus, S. Kanton, C. Kyrousi, A. C. Ayo-Martin, R. D. Giaimo, S. Riesenberger, A. C. O'Neill, J. G. Camp, C. Tocco, M. Santel, E. Rusha, M. Drukker, M. Schroeder, M. Götz, S. P. Robertson, B. Treutlein, S. Cappello, Altered neuronal migratory trajectories in human cerebral organoids derived from individuals with neuronal heterotopia. *Nat. Med.* **25**, 561–568 (2019).
55. F. Chen, J. LoTurco, A method for stable transgenesis of radial glia lineage in rat neocortex by piggyBac mediated transposition. *J. Neurosci. Methods* **207**, 172–180 (2012).
56. M. A. Lancaster, M. Renner, C.-A. Martin, D. Wenzel, L. S. Bicknell, M. E. Hurler, T. Homfray, J. M. Penninger, A. P. Jackson, J. A. Knoblich, Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).
57. M. A. Lancaster, J. A. Knoblich, Generation of cerebral organoids from human pluripotent stem cells. *Nat. Protoc.* **9**, 2329–2340 (2014).
58. J. S. Fleck, S. M. J. Jansen, D. Wollny, F. Zenk, M. Seimiya, A. Jain, R. Okamoto, M. Santel, Z. He, J. G. Camp, B. Treutlein, Inferring and perturbing cell fate regulomes in human brain organoids. *Nature* **621**, 365–372 (2023).
59. P. Reinhardt, M. Glatz, K. Hemmer, Y. Tsytyura, C. S. Thiel, S. Höing, S. Moritz, J. A. Parga, L. Wagner, J. M. Bruder, G. Wu, B. Schmid, A. Röpke, J. Klingauf, J. C. Schwamborn, T. Gasser, H. R. Schöler, J. Streneckert, Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling. *PLOS ONE* **8**, e59252 (2013).
60. A. Zink, P. Lisowski, A. Prigione, Generation of Human iPSC-derived Neural Progenitor Cells (NPCs) as Drug Discovery Model for Neurological and Mitochondrial Disorders. *Bio Protoc.* **11**, e3939 (2021).
61. N. Gunhanlar, G. Shpak, M. Van Der Kroeg, L. A. Gouty-Colomer, S. T. Munshi, B. Lendemeijer, M. Ghazvini, C. Dupont, W. J. G. Hoogendijk, J. Gribnau, F. M. S. De Vrij, S. A. Kushner, A simplified protocol for differentiation of electrophysiologically mature neuronal networks from human induced pluripotent stem cells. *Mol. Psychiatry* **23**, 1336–1344 (2018).
62. J. Van Deun, Q. Roux, S. Deville, T. Van Acker, P. Rappu, I. Miinalainen, J. Heino, F. Vanhaeck, B. G. De Geest, O. De Wever, A. Hendrix, Feasibility of Mechanical Extrusion to Coat Nanoparticles with Extracellular Vesicle Membranes. *Cells* **9**, 1797 (2020).
63. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: An open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
64. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
65. M. D. Abramoff, P. J. Magalhães, S. J. Ram, Image processing with ImageJ. *Biophotonics Int.* **11**, 36–42 (2004).
66. J. R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis. *Nat. Methods* **6**, 359–362 (2009).
67. S. Tyanova, T. Temu, J. Cox, The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat. Protoc.* **11**, 2301–2319 (2016).
68. N. Prianchnikov, H. Koch, S. Koch, M. Lubeck, R. Heilig, S. Brehmer, R. Fischer, J. Cox, MaxQuant software for ion mobility enhanced shotgun proteomics. *Mol. Cell. Proteomics* **19**, 1058–1069 (2020).
69. E. W. Deutsch, N. Bandeira, V. Sharma, Y. Perez-Riverol, J. J. Carver, D. J. Kundu, D. García-Seisdedos, A. F. Jarnuczak, S. Hewapathirana, B. S. Pullman, J. Wertz, Z. Sun, S. Kawano, S. Okuda, Y. Watanabe, H. Hermjakob, B. MacLean, M. J. MacCoss, Y. Zhu, Y. Ishihama, J. A. Vizcaino, The ProteomeXchange consortium in 2020: Enabling 'big data' approaches in proteomics. *Nucleic Acids Res.* **48**, D1145–D1152 (2020).
70. Y. Perez-Riverol, A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D. J. Kundu, I. Inguganti, J. Griss, G. Mayer, M. Eisenacher, E. Pérez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, S. Yilmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A. F. Jarnuczak, T. Ternent, A. Brazma, J. A. Vizcaino, The PRIDE database and related tools and resources in 2019: Improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).
71. F. A. Wolf, P. Angerer, F. J. Theis, SCANPY: Large-scale single-cell gene expression data analysis. *Genome Biol.* **19**, 15 (2018).
72. V. A. Traag, L. Waltman, N. J. Van Eck, From Louvain to Leiden: Guaranteeing well-connected communities. *Sci. Rep.* **9**, 5233 (2019).
73. C. Xu, M. Prete, S. Webb, L. Jardine, B. J. Stewart, R. Hoo, P. He, K. B. Meyer, S. A. Teichmann, Automatic cell-type harmonization and integration across Human Cell Atlas datasets. *Cell* **186**, 5876–5891.e20 (2023).
74. C. Domínguez Conde, C. Xu, L. B. Jarvis, D. B. Rainbow, S. B. Wells, T. Gomes, S. K. Howlett, O. Suchanek, K. Polanski, H. W. King, L. Mamanova, N. Huang, P. A. Szabo, L. Richardson, L. Bolt, E. S. Fasouli, K. T. Mahbubani, M. Prete, L. Tuck, N. Richoz, Z. K. Tuong, L. Campos, H. S. Mousa, E. J. Needham, S. Pritchard, T. Li, R. Elmentaite, J. Park, E. Rahmani, D. Chen, D. K. Menon, O. A. Bayraktar, L. K. James, K. B. Meyer, N. Yosef, M. R. Clatworthy, P. A. Sims, D. L. Farber, K. Saeb-Parsy, J. L. Jones, S. A. Teichmann, Cross-tissue immune cell analysis reveals tissue-specific features in humans. *Science* **376**, eabl5197 (2022).
75. F. M. Cernilogar, S. Hasenöder, Z. Wang, K. Scheibner, I. Bartscher, M. Sterr, P. Smialowski, S. Groh, I. M. Evenroed, G. D. Gilfillan, H. Lickert, G. Schotta, Pre-marked chromatin and transcription factor co-binding shape the pioneering activity of Foxa2. *Nucleic Acids Res.* **47**, 9069–9086 (2019).
76. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T. R. Gingeras, STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
77. Z. Gu, R. Eils, M. Schlesner, Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**, 2847–2849 (2016).
78. Z. Gu, L. Gu, R. Eils, M. Schlesner, B. Brors, *circize* implements and enhances circular visualization in R. *Bioinformatics* **30**, 2811–2812 (2014).
79. A. Zeileis, K. Hornik, P. Murrell, Escaping RGBland: Selecting colors for statistical graphics. *Comput. Stat. Data Anal.* **53**, 3259–3270 (2009).
80. R. Sakai, R. Winand, T. Verbeiren, A. V. Moere, J. Aerts, dendsort: Modular leaf ordering methods for dendrogram representations in R. *F1000Res* **3**, 177 (2014).
81. K. Watanabe, E. Taskesen, A. Van Bochoven, D. Posthumus, Functional mapping and annotation of genetic associations with FUMA. *Nat. Commun.* **8**, 1826 (2017).

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Defective EV-mediated transport of SHH alters neural fate specification in EPM1 epilepsy

Andrea Forero, Veronica Pravata, Fabrizia Pipicelli, Elisa Frenna, Alessandro Soloperto, Marta Ianni, Natalia Abate, Francesco Di Matteo, Zagorka Bekjarova, Laura Canafoglia, Francesca Ragona, Giuseppina Maccarrone, Mariano Gonzalez Pisfil, Christian Wahl-Schott, Filippo M. Cernilogar, Matthias Eder, Rossella Di Giaimo, and Silvia Cappello

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