Human cerebral organoids reveal progenitor pathology in EML1-linked cortical malformation

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Abstract

Malformations of human cortical development (MCD) can cause severe disabilities. The lack of human-specific models hampers our understanding of the molecular underpinnings of the intricate processes leading to MCD. Here, we use cerebral organoids derived from patients and genome edited-induced pluripotent stem cells to address pathophysiological changes associated with a complex MCD caused by mutations in the echinoderm microtubule-associated protein-like 1 (EML1) gene. EML1-deficient organoids display ectopic neural rosettes at the basal side of the ventricular zone areas and clusters of heterotopic neurons. Single-cell RNA sequencing shows an upregulation of basal radial glial (RG) markers and human-specific extracellular matrix components in the ectopic cell population. Gene ontology and molecular analyses suggest that ectopic progenitor cells originate from perturbed apical RG cell behavior and yes-associated protein 1 (YAP1)-triggered expansion. Our data highlight a progenitor origin of EML1 mutation-induced MCD and provide new mechanistic insight into the human disease pathology.

Keywords EML1; malformation of human cortical development; cerebral organoids; perturbed progenitor cells; YAP1 signaling

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

Human cortical expansion and lamination relies on the intrinsic organization and behavior of RG cells and precise neuronal migration (Lui et al, 2011). Disruption of these processes can cause MCD including microcephaly (small brain), megalencephaly (large brain), lissencephaly (loss of gyriﬁcations), polymicrogyria (numerous small gyriﬁcations), and heterotopia (abnormally positioned neurons) (Klingler et al, 2021). These abnormalities can coexist in complex forms. For example, individuals carrying a mutation in the EML1 gene (coding for a microtubule-associated protein (Richards et al, 2015)) exhibit megalencephaly with a polymicrogyria-like cortex above a ribbon-like subcortical heterotopia (rl-SH) in the region of the former outer subventricular zone (SVZ), resembling a second inner cortex (Kielar et al, 2014; Oegema et al, 2019). Patients can also exhibit hydrocephalus (Shaheen et al, 2017). EML1-patients suffer from severe developmental delay, often drug-resistant epilepsy, visual impairment, and intellectual disabilities (Kielar et al, 2014; Shaheen et al, 2017; Oegema et al, 2019). Eml1 mouse models show ectopic proliferating cells in the developing cortical wall, perturbed apical RG cell behavior, and aberrant primary cilia (Kielar et al, 2014; Bizzotto et al, 2017; Uzquiano et al, 2019). Although they exhibit heterotopia, they fall short of recapitulating the complete spectrum of phenotypes observed in humans such as a polymicrogyria-like cortex or megalencephaly (Kielar et al, 2014; Collins et al, 2019), highlighting differences in disease manifestation between humans and non-human model organisms. Here, we explore the function of EML1 during cortical development using human-induced pluripotent stem (iPS) cells and thereof derived cortical cultures.

Results and Discussion

EML1-deficient cerebral organoids exhibit subcortical heterotopia-like phenotypes

To dissect the molecular role of EML1 underlying human cortical development and associated disorders, we reprogrammed fibroblasts from two independent previously characterized patients harboring mutations in the EML1 gene (for more details see Material and Methods and (Kielar et al, 2014)) into iPS cells (two clones

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EML1 patient-specific mutations (patient 1, compound heterozygous, R138X, T243A; patient 2, homozygous, and W225R) were confirmed by sequencing (Fig EV1C and D). To decipher the specific role of EML1 in an isogenic system, we also generated heterozygous EML1 knockout (EML1-heKO) iPS cells from two independent controls by applying CRISPR-Cas9 genome editing. Of note: homozygous EML1-KO clones were prone to die and could not be further cultured. EML1-heKO lines were validated by genotyping (Fig EV1E). Reduced expression of EML1 was confirmed for both EML1-heKO lines (Fig EV1F). We then differentiated iPS cells into cortical progenitors (Shi et al, 2012) and forebrain organoids (Iefremova et al, 2017; Krefft et al, 2018). To investigate whether certain EML1 mutation-induced phenotypic changes are recapitulated within cerebral organoids, we first analyzed patient- and control-derived organoids at day 33 ± 2, the time point at which cerebral organoids have developed ventricular zone (VZ)-, SVZ-, and cortical plate (CP)-like areas as well as cells reminiscent of basal (b)RG (Iefremova et al, 2017). Immunocytochemical analyses revealed that control organoids consist of stratified structures including VZ and CP areas (Fig 1A). In strong contrast, EML1 patient-derived organoids exhibited massive amounts of ectopically localized cells accumulating at the basal side of the VZ areas, in part organized into neural rosettes with accumulation of adherent junction markers such as N-cadherin (NCAD) in their centers (Fig 1B). In addition, β-III-tubulin-positive neurons were organized in two bands, an upper diffuse band located above the ectopic cells and a lower band located in between the VZ area and the ectopic cell population (Fig 1A). To assess the phenotypic changes in all EML1-deficient conditions (EML1-patient 1, EML1-patient 2, EML1-heKO 1, and EML1-heKO 2), we performed immunohistochemistry and quantified the percent of VZ areas with ectopically localized neural rosettes and the percent of heterotopic or disorganized cortical areas in control and EML1-deficient organoids. We confirm a significant increase in neural rosettes and heterotopic clusters of MAPT-positive neurons in all EML1-deficient organoids compared to controls (Fig 1C–E, day 20 ± 2).

Perturbed cell intrinsic properties of EML1-deficient apical radial glial cells

To understand the origin of the perturbed ectopic cell population in the EML1-deficient organoids, we examined RG cell behavior in more detail in EML1-deficient and control organoids at early developmental time points (day 20 ± 2). EML1-deficient in vivo and in vitro models show changes in microtubule-associated processes including mitotic spindle length, cell shape, and primary cilia (Bizzotto et al, 2017; Uzquiano et al, 2019). It was suggested that the changes in spindle length and cell morphology might cause increased mechanical stress in the VZ and by that indirectly encourage RG cell delamination (Bizzotto et al, 2017). To test whether a non-cell autonomous mechanism leads to the accumulation of ectopic progenitor cells in EML1-deficient organoids, we generated hybrid organoids composed of EGFP-EML1-heKO or EGFP-control iPS cells mixed with isogenic control iPS cells before organoid generation. When investigating the hybrid organoids at day 20 ± 2, we found a significant increase in the number of EGFP-positive EML1-heKO cells at the basal side of VZ areas compared to the controls (Fig 2A and B). These data hint toward cell intrinsic mechanisms leading to the ectopic progenitor cells in EML1-deficient organoids. It has been shown that changes in the mitotic spindle and primary cilia can directly impact division mode and RG cell delamination (LaMonica et al, 2013; Mora-Bermúdez et al, 2014; Bizzotto et al, 2017). We first tested whether the division mode is perturbed in EML1-impaired conditions. To that end we visualized mitotic cells (Figs 2C and EV2A) and quantified the plane of cell divisions. While in control organoids the majority of cells exhibit a vertical plane of cell division, we found a significant decrease in vertical division modes in all EML1-impaired conditions. In parallel, EML1-impaired organoids exhibit an increase in oblique and horizontal division angles (Fig 2D, day 20 ± 2). Of note, both oblique and horizontal plane of cell divisions at the VZ surface were described to favor cell delamination (LaMonica et al, 2013). We next investigated the primary cilium and found a significant decrease in the length of the primary cilium in all EML1-deficient-derived cortical progenitor cells upon ARL13B immunostaining (Figs 2E and EV2B) and confirmed perturbed ultrastructure of patient and EML1-heKO-derived primary cilia by electron microscopy (Fig 2F, (Uzquiano et al, 2019)). To investigate whether EML1’s microtubule function causes the observed defects in primary cilia, we stabilized microtubules in control and EML1-deficient cultures using EpothiloneD (Zhang et al, 2012) (EpoD). Immunoblot analyses confirmed a significant increase in stabilized (acetylated) tubulin upon EpoD treatment in all EML1-deficient samples (Fig EV2C and D). When investigating the primary cilia in control- and EML1-deficient conditions, we found a significant increase in stabilized tubulin in control organoids (Fig 2F, EpoD) compared to EML1-deficient samples (Figs 2G and EV2D, EpoD).

Figure 1. Deficiency of EML1 causes ectopic neural rosettes and neuronal heterotopia in cerebral organoids.

A Day 33 ± 2 control- and EML1 patient-derived cerebral organoids stained for β-III-tubulin and DAPI. VZ and CP areas are marked by white brackets, ectopic neural rosettes by red bracket, and heterotrophic neurons by red arrows.

B Day 33 ± 2 control- and EML1 patient-derived cerebral organoid stained for the adherens junction marker N-cadherin (NCAD) expressed at the VZ surface and within the ectopic neural rosettes.

C Day 20 ± 2 cerebral organoids derived from controls, patients, and EML2-heKOs stained for the neuronal marker MAPT and the adherens junction marker NCAD, counterstained with DAPI. Ectopic neural rosettes and neuronal heterotopia are highlighted with dotted yellow lines.

D Quantification of VZ areas with ectopic neural rosettes (three batches, three organoids analyzed per batch, significance based on Kruskal–Wallis test, P = 0.0001, Dunn’s post hoc test for multiple comparisons performed to define statistical differences between genotypes, and single data points presented are colored by batch).

E Quantification of heterotopic, disorganized, or organized cortical areas (three batches, three organoids per batch, significance based on Kruskal–Wallis test, P = 0.0001 for “organized” and “heterotopic”, no significant difference for “disorganized,” Dunn’s post hoc test performed for multiple comparisons to define statistical differences between genotypes).

Data information: * marks statistical significance in relation to Control 1, # in relation to Control 2. P-values: ***/### < 0.001. VZ, ventricular zone; CP, cortical plate.

Data in graphs are represented as means ± SD. Scale bars: (A) 100 µm; (B) 100 µm, enlarged 25 µm; and (C) 50 µm.

Source data are available online for this figure.
Figure 3.
Figure 2.
cultures upon EpoD treatment, we found a clearly improved primary cilia structure and a significant rescue of the primary cilia length in EML1-deficient EpoD-treated conditions compared to DMSO controls (Figs 2F and G, and EV2B). Our data confirm that EML1-deficiency leads to perturbed spindle orientation and primary cilia, which might directly impact aRG cell division mode and delamination resulting in ectopic progenitor cell localization.

Single-cell RNA sequencing (scRNAseq) and immunohistochemical analyses identify a perturbed progenitor cell population in EML1-deficient organoids

To further investigate the cellular identity of the ectopically located cells in EML1-deficient cerebral organoids, we performed scRNAseq on EML1-heKO 1 and respective isogenic control organoids (pooled dissected areas from nine organoids each, three independent batches, and day 33 ± 2; schematic overview Fig EV3A). We identified different cell populations including RG cells (RG1), RG cells transitioning to bRG cells (RG to bRG), RG cells transitioning to neurons (RG2), intermediate progenitors (IP), and young neurons (YN) based on known marker genes (Pollen et al, 2015; Liu et al, 2017; Nowakowski et al, 2017; Velasco et al, 2019; Fan et al, 2020) (Figs 3A and EV3B). In control organoids we detected expression of EML1 in progenitor cells (Fig EV3C), an expression pattern consistent with that found during early mouse and human brain development (Kielar et al, 2014; Nowakowski et al, 2017; Loo et al, 2019). We also observed the presence of a recently described mesenchymal-like cell cluster (MLC) (Eze et al, 2021) (Figs 3A and EV3B). As the function and role of these cells in cortical development is unclear, we excluded them from further analyses. When comparing the cell type composition between EML1-heKO and isogenic control-derived organoids, we found a clear decrease of 22.5% in RG1 cells and a striking increase of 23.4% in the abundance of RG to brRG cells in the EML1-heKO condition, while only minor variations in the other cell clusters were observed (Fig 3B).

We further investigated molecular characteristics of the EML1-heKO-derived RG to bRG cluster compared to control. Here, we found a set of differentially expressed genes in the EML1-heKO. When further investigating these genes, we identified a clear upregulation of ECM and bRG markers (Pollen et al, 2015; Liu et al, 2017) in EML1-heKO-derived samples (Fig EV3D). The ECM genes COL1A2, COL3A1, and LUM, which are associated with human cerebral cortex expansion and folding (Fietz et al, 2012; Long et al, 2018), were found among the significantly upregulated genes in the EML1-heKO (Figs 3C and EV3D). Notably, a decrease in the expression of ECM genes including COL1A2 was connected to reduced brain convolutions as found in lissencephaly (Kazrhun et al, 2018). It is tempting to speculate that an increase in these ECM genes in the EML1-heKO might play a role in the development of the...
Figure 3.
polymicrogyria-like cortex and megalencephaly observed in patient MRIs (Oegema et al., 2019). We also found that the proneurogenic gene MEIS2 was significantly enriched in this cluster in the EML1-heKO (Fig 3C). GO term analyses reveal significant alterations in positive regulation of neural precursor cell proliferation, ECM assembly, cytoplasmic microtubule organization, establishment of mitotic spindle orientation, and cilia assembly in the EML1-heKO RG to bRG cell cluster compared to control (Fig 3D). When performing immunocytochemical analyses on EML1-heKO and isogenic control organoids (day 33 ± 2) we found in the control a few COL1A2 and MEIS2+ cells in the CP area, some of which co-stain for MAPT, whereas EML1-heKO samples exhibit a broader co-expression of COL1A2 and MEIS2 with hardly any overlap with MAPT within the ectopic cell population (Figs 3E and F, and EV3E and F, day 33 ± 2). To further delineate the nature of the ectopic progenitor cells, we applied our hybrid organoids. This system allows to decipher the morphology of the cells within the ectopic rosettes as well as their marker expression. When investigating EGFP-EML1-heKO hybrid organoids at day 30 ± 2, we found EGFP+ cells organized into ectopic rosettes, which were marked by accumulation of NCAD in their centers (Fig 3G). The EGFP+ cells found around the NCAD centers exhibit different morphologies. Most of the cells do not show a RG-like elongated organization with an apical and/or basal process but rather perturbed morphologies (Fig 3G). Some of the ectopic cells stain positive for the bRG marker FAM107A (Fig 3G). Individual EGFP/FAM107A double-positive ectopic cells contact the centers of the rosettes suggesting an apical domain enriched in NCAD, very unusual for bRG cells during normal brain development (Fig 3G, (Martinez-Martinez et al., 2016)). In control hybrids, the majority of the EGFP+ cells found at a basal location to the VZ areas exhibited a radial morphology with an apical and/or basal process. Taken together, our data suggest that the ectopic progenitors in the EML1-heKO are composed of a perturbed progenitor population, which does not exist as such in control organoids or is very rare and is most likely not reflecting a cell population present during normal human brain development.

Deregulated YAP signaling in EML1-deficient organoids drives ectopic cell expansion

To further dissect progenitor cell behavior that may be responsible for the large numbers of ectopic localized progenitor cells, we examined cell proliferation (day 20 ± 2). We found a significant increase in mitotic cells (labeled with p-VIM) located at the basal side of the VZ areas in all EML1-deficient organoids compared to the controls (Figs 4A and EV4A). No clear change in cell mitosis at the VZ surface was observed between the different conditions (Fig EV4B). We also found that the basally located mitotic cells in the EML1-deficient organoids exhibit an increase in cell cycle re-entry (quantified by BrdU® Ki67® cells) compared to the control (Figs 4B and EV4C). In addition, GO-term analyses confirmed an enhanced positive regulation of cell proliferation in the RG to bRG cell cluster (Fig 3D). The distinct increase in mitotic cell behavior outside the VZ areas raises the intriguing question about the underlying mechanisms. Recent data from mice suggest that premature cortical progenitor delamination, ectopic rosette formation, periventricular neuronal heterotopia, and megalencephaly might result from deregulation of the Hippo signaling pathway (Cappello et al., 2013; Liu et al., 2018; O’Neill et al., 2018; Saito et al., 2018; Najas et al., 2020)—a conserved signaling pathway that controls cell proliferation and tissue development (Camargo et al., 2007; Zhao et al., 2011). To assess whether YAP1, a major downstream effector of the Hippo pathway (Sahu & Mondal, 2021), is altered in EML1-deficient cerebral organoids, we investigated YAP1 expression in our scRNAseq data. We found a significant upregulation of YAP1 in the EML1-KO RG to bRG cell cluster compared to control (Figs 4C and EV4D). We further analyzed expression of the YAP1 interaction partner TEAD2 (Mukhtar et al., 2020) and downstream target genes CCND3 and CYR61 and found that they exhibit significantly increased expression.

Figure 4. YAP signaling drives ectopic progenitor cell expansion in EML1-deficient organoids.

A Quantification of mitotic cells located at the basal side of VZ areas in control and EML1-deficient conditions (three batches and three organoids analyzed per batch).
B Quantification of BrdU® Ki67® cells located at the basal side of VZ areas in control and EML1-deficient conditions (three batches and three organoids analyzed per batch).
C Normalized expression of YAP1, TEAD2, CCND3, and CYR61 in RG to bRG cells. Separate violins show expression in control and EML1-heKO. Asterisks indicate Bonferroni corrected P-values. Percentages indicate amount of cells in each group expressing the respective gene (three pooled batches and three organoids each).
D Immunofluorescence staining for (D) p-VIM and YAP1 or (E) Pax6 and YAP1 in control- and EML1-heKO-derived organoids (day 20 ± 2; squares indicate areas enlarged in adjacent part of the panel, respectively).
F Quantification of basally located p-Vim+ cells with nuclear YAP1 signal per VZ area in control- and EML1-heKO-derived organoids (three batches and three organoids analyzed per batch).
G Quantification of PAX6+ cells with nuclear YAP1 signal per VZ area in control- and EML1-heKO-derived organoids (three batches and three organoids analyzed per batch).
H Immunofluorescence staining for p-Vim in EML1-heKO- and control-derived organoids in DMSO-, Verteporfin-, or Fluvastatin-treated conditions, counterstained with DAPI. Dotted line indicates VZ areas.
I Quantification of p-VIM+ cells located at the basal side of VZ areas in control- or EML1-heKO-derived organoids under DMSO-, Verteporfin-, or Fluvastatin-treated condition (three batches and three organoids analyzed per batch).
J Quantification of ectopic neural rosettes per VZ area in EML1-heKO-derived organoids in DMSO-, Verteporfin-, or Fluvastatin-treated conditions (three batches and three organoids analyzed per batch).

Data information: Data in graphs are represented as means ± SD. Significance based on Kruskal–Wallis test (A–B, F, G, J), or two-way ANOVA on log10 normalized data (I) or Wilcoxon rank sum test (C). Dunn’s post hoc (A, F, G, J) or Tukey post hoc (I) test for multiple comparisons was performed to define statistical differences between genotypes. P-values ***< 0.001, **< 0.01, < 0.05. Scale bars: (D and E) 50 μm, enlarged 10 μm, (H) 50 μm.
Figure 4.
levels in the RG to bRG cluster of the EML1-KO (Figs 4C and EV4D). To further test whether YAP1 signaling is indeed associated with the massive expansion of the ectopic progenitor cells, we stained control and EML1-heKO sections with an antibody against YAP1 and p-VIM (day 20 ± 2). Here, we found significantly more basally located p-VIM-positive cells exhibiting nuclear YAP1, suggesting active YAP1, in EML1-heKO-derived organoids compared to the isogenic control (Fig 4D and F). We also observed a significant increase in nuclear YAP1 in PAX6+ cells within the VZ areas in the EML1-heKO compared to controls (Fig 4E and G). Of note, most of the nuclear YAP1 PAX6 co-expressing cells were located in distal positions to the VZ surface (Fig 4E). To further determine the functional role of YAP1 in driving defective RG cell behavior and cortical malformation, we pharmacologically inhibited YAP1 function using Verteporfin (Kostic et al, 2019) or its nuclear translocation using Fluvastatin (Oku et al, 2015). Decrease in nuclear YAP1 upon Fluvastatin treatment was confirmed by immunohistochemistry (Fig EV4E). When assessing progenitor cell proliferation, we found a significant decrease in mitotic cells at the basal side of the VZ areas in Verteporfin- and Fluvastatin-treated EML1-heKO organoids. No difference in ab-ventricular cell mitosis could be observed in control organoids upon Verteporfin or Fluvastatin treatment (Fig 4H and I). In addition, pharmacological inhibition of YAP1 significantly reduced the numbers of ectopic neural rosettes in EML1-heKO organoids (Figs 4J and EV4F). YAP1 was reported to contribute to the evolutionary expansion of the neocortex by promoting bRG cell proliferation (Kostic et al, 2019). It is tempting to speculate that YAP1 is activated in the ectopic progenitor cells due to their basal location in the tissue, by that representing an indirect effect of EML1 deficiency. Of note, additional signaling pathways might be impacted by EML1 deficiency and it would be interesting to further investigate them in the future.

Concluding remarks

Using a human in vitro model, we identified for the first time that deficiency in EML1 leads to the formation of ectopic neural rosettes and occurrence of perturbed progenitor cells, which show increased ECM production and YAP1-mediated expansion. In addition, we confirmed a role of EML1 in primary cilia formation and progenitor cell proliferation. Although this study shows the formation of ectopic cells (heterotopia) and allowed us to decipher new underlying pathomechanisms in EML1-deficient cerebral organoids, which were not yet identified in Eml1-mouse models, the developmental stage of the model used does not fully enable us to investigate the entire scope of MCD in humans caused by EML1 impairment. For instance, the direct correlation between perturbed EML1-derived progenitor cells and the observed polymicrogyria-like cortex and megalencephaly in EML1 patients needs to be further investigated. It is also of note that the organoid system is prone to heterogeneity and even though we could reproduce major findings in two EML1 patient-derived and two EML1-heKO lines, key experiments should be repeated in additional iPS lines to ensure full reproducibility. Nevertheless, our human in vitro approach allowed insight into so far not described pathological features and pathomechanisms of early stages of heterotopia formation.

Material and Methods

Generation of human iPS cells

Skin fibroblasts were obtained from the Coriell Biorepository (Control 1, 2-year-old female, catalog ID GM00969), from a healthy donor (Control 2, 44-year-old healthy female derived with given informed consent within the collaborative research center project SFB636 B7 (ID number B7.0284)) and from two patients harboring mutations in the EML1 gene (Patient 1, P135, 14 years male; Patient 2, 3489, newborn (8 days) male), both with given informed consent and obtained according to the guidelines of the local institutional review boards (IRBs APHP-Délegation Interrégionale à la Recherche Clinique, Paris and Erasmus Medical Center, Rotterdam). Research on human cells was approved by the French Ministry of Health (L.1243.3, DC-2015-2559). Patient 1 carries a compound heterozygous EML1 mutation. An A c.481C>T nucleotide mutation in exon 5, changing an arginine residue (Arg138) to a stop codon, and a c.796A>G mutation in exon 8, changing a threonine into an alanine residue, leading to an impaired association of EML1 with microtubules. Patient 2 carries a c.673T>C mutation—W225R, in which a hydrophobic nonpolar residue changed to highly basic hydrophilic residue—this residue can be found deep in the beta propeller structure where the HELP domain is present (Kielar et al, 2014; Richards et al, 2014). Fibroblasts were reprogrammed by non-integrative delivery of OCT4, SOX2, KLF4, and c-MYC using Sendai virus (SeV) vectors (CytoTune-iPS 2.1 Sendai Reprogramming Kit, Thermo Fisher, Ban et al, 2011); Ethics Committee II of Heidelberg University approval no. 2009-350N-MA for hiPS generation). Pluripotent stem cells were validated as described in Irefanova et al (2017). iPSCs were cultured as colonies in Essential 8 (E8) medium on Geltrans-coated (GT, Thermo Fisher Scientific) cell culture plates with daily medium change. Cells were passaged using EDTA (Thermo Fisher Scientific) and seeded in a 1:3 to 1:10 ratio. Following passaging, medium was supplemented with 5 µM Y-27632 (Cell Guidance Systems) to promote cell survival. All human iPS cell lines were regularly checked and confirmed negative for mycoplasma. Reagents and resources used in this study can be seen in Table 1.

Generation and validation of EML1-heKO lines

EML1-heKO lines were generated using CRISPR/Cas9 and homology-directed repair (HDR) (Santa Cruz). Successful site-specific double-strand break followed by integration of the HDR sequence leads to the disruption of the EML1 gene and the integration of a puromycin selection cassette. In brief, 1 million iPS cells derived from either control 1 or control 2 were transfected with three different gRNAs (1 µg in total) directed against early exons of the EML1 gene (Exons 2 and 5) alongside the respective HDR plasmids using the NucleofectorTM2b (Lonza) and the Cell Line Nucleofector® Kit V (Lonza) according to the manufacturer’s protocol. Following nucleofection, cells were plated on GT-coated cell culture plates in E8 medium supplemented with 5 µM Y-27632, Puromycin (1 µg/ml, Merck Millipore) selection was initiated 48 h following transfection. Clones were manually picked 7–12 days following nucleofection into GT-coated 48-well cell culture plates. Integration of the HDR cassette was validated on genomic DNA by PCR. PCR primers were designed to recognize the EML1 wild-type allele.
Table 1. Reagents and resources used in this study.

| Reagent or Resource | Vendor                  | Identifier         |
|---------------------|-------------------------|---------------------|
| A83-01              | Biomol                  | Cay9001799          |
| B27-Supplement      | Thermo Fisher Scientific| 17504044            |
| BCA Protein Assay Kit | Thermo Fisher Scientific| 23225               |
| Blasticidin S Hydrochlorid | Carl Roth                | CP14.2              |
| Boric acid          | Thermo Fisher Scientific| B0994               |
| BrdU                | BD-Bioscience           | S50891              |
| BSA                 | Sigma Aldrich           | A3294               |
| cAMP                | Sigma Aldrich           | D0627               |
| Chromium Single Cell 3’ Library & Gel Bead Kit v2 | 10xGenomics | PN-120267          |
| DAPI                | Biolegend               | 422801              |
| DMEM/F12            | Thermo Fisher Scientific| 11320074            |
| DMEM/F-12 with Glutamin and HEPES | Thermo Fisher Scientific | 11330-057        |
| DNase               | Sigma Aldrich           | AMPD1-1KT           |
| PBS                 | Sigma Aldrich           | D8537               |
| EDTA                | Thermo Fisher Scientific| 15575020            |
| EML1 CRISPR/Cas9 KO Plasmid (h) | Santa Cruz | sc-406445          |
| EML1 HDR Plasmid (h) | Santa Cruz          | sc-406445-HDR       |
| EpithioneD          | Abcam                   | ab143616            |
| Epoxy resin         | Polyscience             | EPON218             |
| Extractme Genomic DNA Kit | 7Bioscience     | EM13                |
| Fetal Bovine Serum (FBS) | Thermo Fisher Scientific | 10270106        |
| FGF-2 (154)         | Cell Guidance Systems   | GFH146              |
| Fluvastatin         | Sigma Aldrich           | SML0038             |
| Geltrex, hESC-Qualified | Thermo Fisher Scientific| A1413302           |
| Glucose             | Carl Roth               | HN06.2              |
| GlutaMAX            | Thermo Fisher Scientific| 35050038            |
| Glutaraldehyde      | Sigma Aldrich           | CS882               |
| HCL                 | Thermo Fisher Scientific| 15538334            |
| Heparin             | Sigma Aldrich           | H3149               |
| Insulin             | Sigma Aldrich           | 91077C              |
| iScript cDNA synthesis kit | Bio-Rad            | 1708890             |
| KnockOut Serum Replacement (KOSR) | Thermo Fisher Scientific | 10828028        |
| LAAP                | Sigma Aldrich           | A8960               |
| L-cysteine          | Sigma Aldrich           | 168149              |
| LDN-193189          | Cell Guidance Systems   | SM23                |
| Mowiol 4-88         | Carl Roth               | 0713.1              |

(continued)

| Reagent or Resource | Vendor                  | Identifier         |
|---------------------|-------------------------|---------------------|
| N2-Supplement       | Thermo Fisher Scientific| 17502048            |
| Natriumselinit      | Sigma Aldrich           | S5261               |
| NEAA                | Thermo Fisher Scientific| 11140035            |
| Papain              | Sigma Aldrich           | P3125               |
| Paraformaldehyd (PFA) | Sigma Aldrich         | P6148               |
| Penicillin/Streptomycin | Thermo Fisher Scientific| 15140122           |
| pegGOLD TriFast     | VWR                     | 30-2010             |
| PierceTM Protease inhibitor | Thermo Fisher Scientific | A32955          |
| Pluronic F-127      | Sigma Aldrich           | P2443               |
| Puromycin           | Merck Millipore         | 540222              |
| SOD                 | Carl Roth               | CN301               |
| Sendai Reprogramming Kit (CytoTune-IPS 2.1) | Thermo Fisher Scientific | A16517         |
| Taq Polymerase      | Biozym                  | 331610              |
| TGFβ1               | Cell Guidance Systems   | GFH39               |
| Transferin          | Sigma Aldrich           | T3705               |
| Triton X-100        | Merck Millipore         | 108603              |
| TrypLE Express      | Thermo Fisher Scientific| 12605-028           |
| Verteporfin         | Sigma Aldrich           | SML0534             |
| XAV999              | Cell Guidance Systems   | SM38                |
| Y-27632             | Cell Guidance Systems   | SM02                |

or the integration of the puromycin cassette. Of note: only heterozygous EML1-(he)KO iPSC clones could be expanded, stored, and further differentiated into cerebral organoids.

**Generation of cerebral organoids**

Cerebral organoids were generated as described with slight adaptations (Iefremova et al., 2017; Kreft et al., 2018). In brief, U-bottom 96-well plates were coated with 5% Pluronic F-127 (Sigma Aldrich) in phosphate buffered saline (PBS) for 15 min to create low attachment wells. iPS cell colonies were dissociated using TrypLE Express (Thermo Fisher Scientific) and 6,000 cells were plated per low attachment well in 150 µl E8 medium supplemented with 50 µM Y-27632. Medium was changed every other day. At day 5, medium was changed to neural induction medium (Table 2). Medium was changed every other day. On days 9–11, when translucent neural ectoderm was visible, organoids were embedded in a 3:2 ratio of GT to neural induction medium and further cultured in Pluronic F-127-coated 6 cm dishes in neural differentiation medium (Table 3) under continuous agitation at 70 rpm on an orbital shaker (Infors Celltriton HD) with a medium change every 3 to 4 days. When indicated, organoids were exposed to 100 nM Verteporfin (Sigma
organoids were incubated for 2 h in medium containing 10 ng/ml EML1-EGFP-labeled iPS cells (16 onwards with daily medium changes. A83-01, 0.5 mM; XAV939, 2 µM; GlutaMAX, 1% (v/v); NEAA, 1% (v/v); D-Glucose, 4.44 mM; Heparin, 10 µg/ml; KOSR, 2% (v/v); Penicillin/Streptomycin, 1% (v/v)).

### Generation of hybrid organoids

EGFP-labeled iPS cells (EML1-heKO and isogenic control) were generated using a lentiviral construct expressing EGFP under the PGK promoter as well as a blasticidin resistance cassette (pLentiPGK-EGFP-SV40-blasticidin (Koch et al., 2006)). Forty-eight hours post-transfection, iPS cells were cultured for at least 2 weeks using E8 medium supplemented with 10 µg/ml blasticidin (Carl Roth). Homogeneous EGFP expression was validated by visual monitoring using epifluorescence microscopy. Hybrid organoids were generated by mixing either control-EGFP- or EM1.1-heKO-EGFP-derived iPS cells with control iPS cells in a 1:1,000 ratio before organoid generation.

### BrdU labeling of cerebral organoids

For 5-bromo-2′-deoxyuridine (BrdU) labeling, day 30 ± 2 cerebral organoids were incubated for 2 h in medium containing 10 µM BrdU (BD Bioscience). After 2 h of incubation, cerebral organoids were washed three times with fresh medium and transferred into a new 6 cm culture dish. Following 24 h, cultivation time cerebral organoids were fixed and further processed for immunohistochemistry.

### Generation of iPS cell-derived cortical progenitors

Differentiation of iPS cells into cortical progenitors was performed as described with slight adaptations (Shi et al., 2012; Iefremova et al., 2017; Uzquiano et al., 2019). In brief, iPS cell colonies cultured in E8 medium were dissociated using TrypLE (Thermo Fisher Scientific) and seeded as single cells onto GT-coated cell culture plates in E8 medium supplemented with 5 µM Y-27632. Once the cell culture reached 98% confluence, neural induction was initiated by changing the culture medium to neural induction medium containing DMEM/F12 (Thermo Fisher Scientific), 0.5% N2 supplement, 1% B27 supplement, (Thermo Fisher Scientific), cAMP (300 ng/ml, Sigma Aldrich), LDN-193189 (0.2 mM, Cell Guidance Systems), A83-01 (0.5 mM, Biomol), XAV939 (2 µM, Cell Guidance Systems), 1% GlutaMAX (Thermo Fisher Scientific), 1% NEAA (Thermo Fisher Scientific), and 4.44 mM Glucose (Carl Roth). Cells were maintained in this medium for 8–11 days, collected by dissociation with TrypLE and replated in neural differentiation media containing DMEM/F12, 0.5% N2 supplement, 1% B27 supplement, and cAMP (300 ng/ml) on GT-coated cell culture plates. Cells were split in a 1:2 ratio when cultures reached 100% confluence using TrypLE. Electron microscopy analyses were performed on cortical progenitors passaged twice. When indicated, 0.5 nM EpothiloneD (EpoD, abcam) was added to the medium for 72 h with one medium change after 48 h before cells were fixed and analyzed or harvested for Western blot analysis.

### Electron microscopy of neural progenitor cells

Samples were processed and imaged as previously described in Uzquiano et al. (2019). Briefly, cells were fixed for 1 h in phosphate buffer (PB), 0.1 M buffer containing 4% paraformaldehyde (PFA, Sigma Aldrich), and 2.5% glutaraldehyde (Sigma Aldrich) at 4°C. Following fixation, cells were postfixed in 2% osmium tetroxide diluted in 0.2 M Palade buffer. After osmication, cells were dehydrated in a series of ethanol baths and flat embedded in epoxy resin (EPON 812, Polysciences). After resin polymerization, small pieces were dissected from flat-embedded cultures, mounted in plastic stubs and sectioned. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM100 electron microscope. Digital images were obtained with a CCD camera (Gatan Orius).

### Immunofluorescence and specific antibody information

Cells were washed twice with PBS, fixed for 10 min with 4% PFA, and washed again twice with PBS and used either directly for immunostaining or stored at 4°C. Organoids were fixed, embedded, and cryosectioned into 20 µm sections as described previously (Iefremova et al., 2017; Krefet et al., 2018). For detection of the pluripotency-associated markers, TRA-1-60, TRA-1-81, and SSEA-3 samples were incubated with primary antibodies at room temperature for 4 h, washed three times, incubated with secondary antibody for 45 min, counterstained with DAPI (Biolegend), and mounted with Mowiol 4-88 mounting solution (Carl Roth). For detection of BrdU and Ki67,
slices were permeabilized using 0.5% Triton X-100 in PBS for 30 min, washed with PBS, treated with 2 N HCl (Thermo Fisher Scientific) for 10 min, and washed twice with PBS. Then, slices were treated with 0.1 M boric acid (Thermo Fisher Scientific) for 10 min and washed three times with PBS. Slices were then blocked with 10% fetal bovine serum and 0.1% Triton X-100 in PBS for 1 h at room temperature and subsequently stained overnight in blocking solution at 4°C with antibodies against BrdU and Ki67. On the next day, slices were washed three times with PBS and secondary antibodies were applied in blocking solution for 1 h at room temperature, counterstained with DAPI, and mounted with Mowiol. For all other antibodies, cells or organoid sections were blocked and permeabilized in 10% fetal bovine serum (Thermo Fisher Scientific) in PBS with 0.3% Triton X-100 for 1 h at room temperature (RT), incubated with primary antibodies for 16 h at 4°C, washed three times with PBS, incubated with secondary antibodies for 1 h, counterstained with DAPI, and mounted with Mowiol. Images were acquired with either the confocal microscope Leica TCS SP5II or the fluorescence microscope Leica DM6 B microscope and processed using the software Leica Application Suite AF, Leica Application Suite X, as well as ImageJ.

Primary and secondary antibodies and dilutions used in this study can be seen in Tables 4 and 5. Software and algorithms used for analysis can be seen in Table 6.

**Immunoblot and specific antibody information**

Cells were washed twice with ice-cold PBS, scraped off into PBS, and collected via centrifugation. Cell pellets were lysed in RIPA buffer (150 mM NaCl, 0.2% SDS (Carl Roth), 0.2% Triton X-100 (Merck Millipore), 25 mM EDTA, 50 mM Tris–HCl, pH 7.4) containing PierceTM protease inhibitor (Thermo Fisher Scientific) and PierceTM phosphatase inhibitor (Thermo Fisher Scientific) for 1 h on ice. Genomic DNA was sheared by sonication. Subsequently, cell debris was precipitated by centrifugation at 16,000 rcf for 15 min at 4°C. Protein concentration of cleared cell lysates was determined using the BCA protein assay kit (Thermo Fisher Scientific). For immunoblotting, 25 µg of protein was boiled in 6× SDS sample buffer for 5 min at 95°C. Lysates were resolved on 10% gels and transferred onto 0.2 µm nitrocellulose membranes by semi-dry blotting. Nitrocellulose membranes were blocked in 5% BSA in TBST for 1 h at RT and subsequently incubated overnight with primary antibody in blocking solution at 4°C. The next day, membranes were washed three times with TBST, incubated with IR-dye-conjugated secondary antibodies (DyLight™, Cell Signaling Technology) diluted 1:15,000 in TBST for 1 h at room temperature. Subsequently, membranes were washed three times before visualization of target proteins using an Odyssey IR imaging system (LI-COR Biosciences). Primary antibodies and concentrations were as follows: Acetylated α-tubulin (AC-TUB) (Cell Signaling Technology, 1:1,000) and β-Actin (Cell Signaling Technology, 1:15,000). Of note: due to the lack of a reliable EML1 antibody, we were not able to include data on EML1 protein levels.

**Single-cell RNA sequencing experiments**

Organoids at day 33 ± 2 were microdissected to enrich for cortical areas. The dissected tissue was dissociated by incubating in papain (Sigma Aldrich) solution containing papain buffer (1 mM L-cysteine and 0.5 mM EDTA in Earle’s balanced salt solution), 20 units

| Antibodies or Resource | Vendor | Identifier |
|------------------------|--------|------------|
| **Antibodies IF**       |        |            |
| AFP                    | Holzel | Cat# 12177-MM27 |
| ARL13B                 | Antibodies Incorporated | Cat# 75-287, RRID: AB_2341543 |
| Anti-BrdU              | BD-Biosciences | Cat# 347580, RRID: AB_10015219 |
| COL1A2                 | Abcam  | Cat# ab96723, RRID: AB_10679394 |
| FAM107A                | Sigma Aldrich | HPAD5888 |
| Anti-GFP               | Aves Labs | Cat# GFP-1020, RRID: AB_10000240 |
| Ki67                   | Cell Signaling | Cat# 9129, RRID: AB_2687446 |
| MAPT                   | Synaptic Systems | Cat# 314 004, RRID: AB_1547385 |
| MEIS2                  | Sigma Aldrich | Cat# WH0004212/M1, RRID: AB_1842419 |
| N-cadherin             | BD-Bioscience | Cat# 610921, RRID: AB_398236 |
| PAX6                   | Biolegend | Cat# 901303, RRID: AB_2565003 |
| p-Vimentin             | MBL    | Cat# D076-3, RRID: AB_529263 |
| SMA                    | abcam  | Cat# ab5694, RRID: AB_223021 |
| TPX2                   | Novus Biologicals | Cat# NB500-179, RRID: AB_10002747 |
| TRA-1-60               | Merck Millipore | Cat# MAB48380, RRID: AB_2119183 |
| TRA-1-81               | Merck Millipore | Cat# MAB48381, RRID: AB_177638 |
| TUBB3                  | Biolegend | Cat# 802001, RRID: AB_2564645 |
| SSEA3                  | abcam  | Cat# ab16286, RRID: AB_882700 |
| YAP1                   | Cell Signaling | Cat# 14074, RRID: AB_2650491 |
| Alexa Fluor 568 - Goat anti-mouse IgG (H + L) | Thermo Fisher Scientific | Cat# A-11004, RRID: AB_2534072 |
| Alexa Fluor 488 - Goat anti-Rabbit IgG (H + L) | Thermo Fisher Scientific | Cat# A-11008, RRID: AB_143165 |
| Alexa Fluor 555 - Goat anti-Rabbit IgG (H + L) | Thermo Fisher Scientific | Cat# A-21428, RRID: AB_2538489 |
| Alexa Fluor 647 - Goat anti-Guinea Pig IgG (H + L) | Thermo Fisher Scientific | Cat# A-21450, RRID: AB_2795091 |
| Alexa Fluor 488 - Goat anti-Chicken IgY (H + L) | Thermo Fisher Scientific | Cat# A-11039, RRID: AB_2534096 |

| Antibodies immunoblot: |        |            |
|------------------------|        |            |
| Acetylated α-tubulin (AC-TUB) | Cell Signaling Technology | 5335, RRID: AB_1054494 |
| β-Actin                | Cell Signaling Technology | 3700, RRID: AB_2242334 |
papain, and 10 µg/ml of DNase (Sigma Aldrich) for 20 min at 37°C. Following incubation, excessive papain solution was removed, 3 ml organoid differentiation medium was added, and samples were mechanically dissociated using wide-bore 1,000 µl pipette tips coated with 1% bovine serum albumin (BSA) in PBS. The cell suspension was centrifuged at 400 g for 4 min at 4°C. The supernatant was removed, the cell pellet was resuspended in 1 ml ice-cold PBS + 0.04% BSA, and filtered through a 30 µm cell strainer. Counting and viability were assessed using Trypan blue staining (Countess automatic cell counter, Thermo Fisher Scientific). Single cell library preparation was performed using the 10x Genomics Chromium platform according to the 10x Genomics Chromium Single Cell 3’ Library & Gel Bead Kit v2 chemistry user guide (10x Genomics).

The prepared cDNA libraries were processed by the High Throughput Sequencing Unit of the Genomics & Proteomics Core Facility of the German Cancer Research Center (DKFZ). The libraries were sequenced on two lanes on the Illumina HiSeq 4K platform with a protocol specific for 10x scRNA libraries (paired-end 26 + 74).

Fastq files were parsed to cellranger (10x Genomics) count in order to generate a count matrix. FastQC was used for general sequencing quality control (Andrews, 2015). If not stated otherwise, data analysis was performed using the Seurat (v3.2.2) package in R (Stuart et al, 2019). Count matrix was filtered with following parameters: Any feature that was expressed in less than three cells was removed from the analysis. For the control sample, any cell with < 2,000 expressed features, more than 10% mitochondrial genes expressed, or less than 5,000 total UMI counts was removed from further analysis. For the EML1/heKO sample, any cell with < 1,500 expressed features, more than 10% mitochondrial genes expressed, or < 3,000 total UMI counts was removed from further analysis. The data were normalized using sctransform. Dimensional reduction was performed using UMAP with dims = 1:30. Shared nearest-neighbor graph was constructed with dims = 1:30. Clusters were generated with resolution = 0.4. Cells were defined by interpreting expression of known marker genes. One group of cells that was defined as

| Table 4 (continued) |
|---------------------|
| **Reagent or Resource** | **Vendor** | **Identifier** |
| Anti-mouse IgG (H + L) (DynLight 680 Conjugate) | Cell Signaling Technology | S470, RRID: AB_10696895 |
| Anti-rabbit IgG (H + L) (DynLight 800 4X PEG Conjugate) | Cell Signaling Technology | S151, RRID: AB_10697505 |

**Table 5. Primary antibodies and dilutions.**

| Antibodies | Vendor | Cat. No. | Raised against | Dilution |
|------------|--------|----------|----------------|----------|
| **Primary antibodies** | | | | |
| AFP | Holzel | Cat# 12177-MM27 | mouse | 1:300 |
| ARCL38 | Antibodies Incorporated | Cat# 75-287 | mouse | 1:100 |
| Anti-BrdU | BD-Biosciences | Cat# 347580 | mouse | 1:50 |
| COL1A2 | Abcam | Cat# ab96723 | rabbit | 1:300 |
| FAM107A | Sigma Aldrich | Cat# HP058888 | rabbit | 1:400 |
| Anti-CFP | Aves Labs | Cat# GFP-1020 | chicken | 1:500 |
| KI67 | Cell Signaling | Cat# 9129 | rabbit | 1:500 |
| MAPT | Synaptic Systems | Cat# 314 004 | guinea pig | 1:1,000 |
| MEIS2 | Sigma | Cat# WH0004212M1 | mouse | 1:200 |
| N-cadherin | BD-Bioscience | Cat# 610921 | mouse | 1:500 |
| PAX6 | Biolegend | Cat# 901301 | rabbit | 1:500 |
| p-Vimentin | MBL | Cat# D076-3 | mouse | 1:1,000 |
| SMA | Abcam | Cat# ab5934 | rabbit | 1:300 |
| TPX2 | Novus Biologicals | Cat# N8500-179 | rabbit | 1:500 |
| TRA-1-60 | Merck Millipore | Cat# MAB4360 | mouse | 1:300 |
| TRA-1-81 | Merck Millipore | Cat# MAB4381 | mouse | 1:300 |
| TUBB3 | Biolegend | Cat# 802001 | rabbit | 1:1,000 |
| SSEA3 | Abcam | Cat# ab16286 | rat | 1:500 |
| YAP1 | Cell Signaling | Cat# 14074 | rabbit | 1:300 |

| **Secondary antibodies** | | | | |
| Alex Fluor 568-Goat anti-mouse IgG (H + L) | Thermo Fisher Scientific | Cat# A-11004 | goat anti-mouse | 1:1,000 |
| Alex Fluor 488-Goat anti-Rabbit IgG (H + L) | Thermo Fisher Scientific | Cat# A-11008 | goat anti-rabbit | 1:1,000 |
| Alex Fluor 555-Goat anti-Rabbit IgG (H + L) | Thermo Fisher Scientific | Cat# A-21428 | goat anti-rabbit | 1:1,000 |
| Alex Fluor 647-Goat anti-Guinea Pig IgG (H + L) | Thermo Fisher Scientific | Cat# A-21450 | goat anti-guinea | 1:1,000 |
| Alex Fluor 488-Goat anti-Chicken IgY (H + L) | Thermo Fisher Scientific | Cat# A-11039 | goat anti-chicken | 1:1,000 |
samples were isolated using peqGOLD TriFast (VWR) following the manufacturers protocol. Triplicate total mRNA was isolated using the Extractme genomic DNA kit (7Bioscience) of Genomic DNA (for patient mutations and EML1 PCR analysis).

Software and algorithms used for analysis can be seen in Table 6. https://github.com/ahoffrichter/Jabali_et_al_2021_scRNAseq_analysis. For detailed information about the data analysis pipeline in R, refer to the expression per cell was calculated as proportion of feature set. Human. R package version 3.12.0.). For each GO term of interest, from org.Hs.eg.db (org.Hs.eg.db: Genome wide annotation for Human. R package version 3.12.0.) gen plots, the genes that are collected in a GO term were retrieved (Hadley, 2016) and show normalized expression values. For GO violin plots were generated with Seurat (Stuart et al 2019) and show normalized expression values. For GO violin plots were generated with Seurat (Stuart et al, 2019) and ggplot2 (Hadley, 2016) and show normalized expression values. For GO violin plots, the genes that are collected in a GO term were retrieved from org.Hs.eg.db (org.Hs.eg.db: Genome wide annotation for Human. R package version 3.12.0.). For each GO term of interest, the expression per cell was calculated as proportion of feature set. For detailed information about the data analysis pipeline in R, refer to https://github.com/ahoffrichter/Jabali_et_al_2021_scRNAseq_analysis. Software and algorithms used for analysis can be seen in Table 6.

PCR analysis

Genomic DNA (for patient mutations and EML1-heKO validation) was isolated using the Extractme genomic DNA kit (7Bioscience) according to the manufacturers protocol. Triplicate total mRNA samples were isolated using peqGOLD TriFast (VWR) following the supplier’s instructions. One microgram total mRNA was used for reverse transcription with the iScript cDNA synthesis kit (BioRad) following the manufacturer’s protocol. Semi-quantitative PCR reactions were run in at least triplicates using Taq Polymerase (Biozym). PCR conditions and cycle numbers were adjusted to each primer pair for specific DNA amplification on cDNA obtained from commercially available human fetal (single donor, female, 19 weeks of gestation). For quantitative RT–PCR (qRT–PCR), PCR products were assessed by dissociation curve and gel electrophoresis. Data were normalized to 18S rRNA levels. Primers used to validate EML1–patient-specific mutations and the EML1-heKO as well as EML1 expression can be seen in Table 7.

Quantitative assessment of 2D and 3D cell cultures

Ectopic neural rosettes were quantified based on NCAD staining and localization in the organoid structure. More precisely, accumulation of NCAD at the basal side of VZ areas was defined as ectopic neural rosettes and quantified per VZ area using the cell counter tool in ImageJ. The percent of VZ areas with ectopic neural rosettes was then calculated for the different conditions. Organization of neurons was investigated per cortical area (composed of a VZ and CP area) based on MAPT staining and localization. If the majority of MAPT-positive neurons were found within the CP area, we called them organized. If in case MAPT-positive neurons distributed in the CP as well as in the VZ area, we defined that as disorganized. Criteria for heterotopia were met in case clusters or bands of MAPT-positive cells found between the VZ area and the CP region. The percent of heterotopic, disorganized, or organized cortical areas was then calculated for the different conditions. The number and localization of EGFP-positive cells within hybrid organoids were investigated by defining VZ and CP areas and quantifying the EGFP-positive cells in the respective areas using the cell counter tool in ImageJ. Mitotic planes were quantified at the VZ surface by analyzing 20-µm-thick tenner serial sections stained for p-vimentin (to identify dividing cells) and TPX2 (a spindle assembly factor which plays a role in inducing microtubule assembly and growth during M phase used to visualize the plane of dividing

### Table 6. Software and algorithms used in this study.

| Software and Algorithms | Source | Identifier |
|-------------------------|--------|------------|
| Cellranger v3.0.1       | 10x Genomics | https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest |
| Fiji software (Image J 1.52i) | Wayne Rasband, NIH, USA | Fiji, RRID:SCR_002285 |
| ggplot2                 | Hadley (2016) | RRID:SCR_014601 |
| Seurat v3.0.0           | Stuart et al (2019) | https://github.com/satijalab/seurat/releases/tag/v3.0.0 |
| SPSS                    | https://www.ibm.com/IBM SPSS statistics 25 |
| R                       | R Core | https://www.r-project.org/ |

### Table 7. Primers used in this study.

| Primer | Forward | Reverse | Source |
|--------|---------|---------|--------|
| P135 Mut. Exon 5 (T<sub>a</sub> 45°C) | GACCTCTATGATATATATTT | TGTGTTATAGTCTTATAAA | IDT |
| P135 Mut. Exon 8 (T<sub>a</sub> 60°C) | CGTCCATGCTTTTGGCG | TGGACGTTCTATGTATATTT | IDT |
| 3489 Mut. (T<sub>a</sub> 60°C) | GCTGGGCACTGAGTATCTT | ACCACACGTATTGTCGAGGA | IDT |
| Validation of EML1-heKO: | | | |
| T<sub>a</sub> 60°C for all primer combinations: | | | |
| Before Cas. gRNA 1 | AGGGAACATGCTGAATACGACA | AAAAGAGCCGGAGCCATACA | IDT |
| In Cas. gRNA 1 | CCTGTACGATTGTCGCCACG | IDT |
| Before Cas. gRNA 2/3 | CGGAGCGATCCTGATATTTG | IDT |
| In Cas. gRNA 2/3 | TCACTCAAGCCGACACCTT | IDT |
| EML1 WT behind Cas. gRNA 1 | TCACTCAAGCCGACACCTT | IDT |
| EML1 WT behind Cas. gRNA 2/3 | TCACTCAAGCCGACACCTT | IDT |
| EML1 expression: | | | |
| T<sub>a</sub> 60°C for both pairs: | | | |
| EML1-qPCR | GCGTCTATGGCTACAGGC | ACTGCTAGGCACTTACG | IDT |
| 18s-qPCR | TCTGGGAGCGGCGGAGG | GGGCGCATGCGGAGTGG | IDT |
cells). The angle of the spindle of apical radial glia cells in relation to the prospective ventricular surface was investigated using the ImageJ angle tool. Cell proliferation at the VZ surface or basal to the VZ areas was quantified based on the distribution of p-Vim-positive cells in the respective localization using the cell counter tool in ImageJ. BrdU+ KI67+ cells at the basilar side of the VZ areas were quantified using ImageJ plugin Cell Counter. PAX6-positive cells displaying nuclear YAP1 were quantified per VZ area following immunohistochemistry. More specifically, the total amount of PAX6-positive cells within a VZ area was determined and divided by the amount of PAX6-positive cells displaying a nuclear YAP1 signal within this area. All quantitative assessments were performed on at least three different organoids derived from at least three independent batches. The length of the primary cilia was investigated in cortical progenitors following ARL13B immunostaining using the length measurement tool in ImageJ on three biological replicates. Images for all quantitative assessments were acquired using the confocal microscope Leica TCS SP5II or the fluorescence microscope Leica DM6 B microscope. In order to make the different batches analyzed distinguishable from one another, different shapes and color codes were utilized in the graphs displayed in this study. VZ areas or organoids analyzed from one batch for example are represented by black dots, grey dots, white dots with black border, or grey dots with black border.

Statistical analysis

All quantitative data were generated based on biological triplicates and tested for normal distribution using the D’Agostino & Pearson omnibus normality test. If criteria for normal distribution were met, statistical significance was tested using parametric tests in the form of one-way ANOVA or two-way ANOVA tests followed by post hoc testing (Sidak’s multiple-comparison test or Tukey’s multiple-comparison test) based on the experimental design (*P < 0.05, **P < 0.01, and ***P < 0.001). If criteria for normal distribution were not met, non-parametric testing in the form of a two-tailed Mann–Whitney- or Kruskal–Wallis test followed by post hoc testing (Dunn’s multiple-comparison test) was conducted. All deviations from means are depicted as mean with SD or SEM. All analyses were performed with the help of R statistical software package, IBM SPSS statistics 25, and GraphPad Prism 6. Gene expression was compared between control and EML1-heKO cells within cell type using the Wilcoxon rank sum test. Values show Bonferroni-corrected P-values (*P < 0.05, **P < 0.01, and ***P < 0.001). Comparison of proportion of GO feature sets was performed using the Wilcoxon rank sum test (*P < 0.05, **P < 0.01, and ***P < 0.001). Software and algorithms used for analysis can be seen in Table 6.

Generation of schemes

Synopsis image as well as schemes in Figs 2C and EV3A were created using biorender.com.

Data availability

The sc-RNA-seq data will be deposited in NCBI’s Gene Expression Omnibus and will be accessible through a GEO Series accession number. For detailed information about the data analysis pipeline in R and the scRNAseq datasets generated and/or analyzed during the current study, refer to the GitHub repository https://github.com/ahoffrichter/Jabali_et_al_2021_scRNAseq_analysis. Any additional information required to reanalyze the data reported in this study is available from the lead contact upon request.

Expanded View for this article is available online.

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Author contributions

Ammar Jabali: Data curation; Validation; Investigation; Visualization; Methodology. Writing—original draft; Writing—review & editing. Anne Hoffrichter: Data curation; Software; Formal analysis; Investigation; Visualization. Ana Uzquiano: Investigation; Methodology. Fabio Marsoner: Investigation. Ruven Wilkens: Investigation; Visualization; Methodology. Marco Siekmann: Validation; Methodology. Bettina Bohl: Investigation; Visualization; Methodology. Andrea, Carlo Rossetti: Validation. Sandra Horschitz: Methodology. Philipp Koch: Resources; Funding acquisition; Validation; Writing—original draft; Writing—review & editing. Fiona Francis: Resources; Writing—original draft; Writing—review & editing. Julia Ladewig: Conceptualization; Resources; Supervision; Funding acquisition; Validation; Investigation; Visualization; Writing—original draft; Writing—review & editing.

In addition to the CRediT author contributions listed above, the contributions in detail are: JL conceived and designed the research project; AJ, AU, PK, FF, and JL designed experiments; AJ, AU, FM RW, and MS performed experiments and collected data; AJ, AH, AU, BB, ACR, FF, PK, and JL analyzed data; and AJ and SH programmed EML1 patient samples. FF communicated with clinicians for patient sample collection. FF and PK were involved in ongoing critical discussion. JL and AJ wrote the manuscript with critical help from FF, AU, and PK. All authors provided ongoing critical review of experiments, results, and commented on the manuscript.

Disclosure and competing interest statement

The authors declare that they have no conflict of interest.

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