

Cellular hierarchies of embryonal tumors with multilayered rosettes are shaped by oncogenic microRNAs and receptor–ligand interactions

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Embryonal tumor with multilayered rosettes (ETMR) is a pediatric brain tumor with dismal prognosis. Characteristic alterations of the chromosome 19 microRNA cluster (C19MC) are observed in most ETMR; however, the ramifications of C19MC activation and the complex cellular architecture of ETMR remain understudied. Here we analyze 11 ETMR samples from patients using single-cell transcriptomics and multiplexed spatial imaging. We reveal a spatially distinct cellular hierarchy that spans highly proliferative neural stem-like cells and more differentiated neuron-like cells. C19MC is predominantly expressed in stem-like cells and controls a transcriptional network governing stemness and lineage commitment, as resolved by genome-wide analysis of microRNA–mRNA binding. Systematic analysis of receptor–ligand interactions between malignant cell types reveals fibroblast growth factor receptor and Notch signaling as oncogenic pathways that can be successfully targeted in preclinical models and in one patient with ETMR. Our study provides fundamental insights into ETMR pathobiology and a powerful rationale for more effective targeted therapies.

Embryonal tumor with multilayered rosettes (ETMR) is a malignant childhood tumor that occurs throughout the central nervous system (CNS). ETMR carries a dismal prognosis, with a median survival of 14 months and a 5-year overall survival of approximately 20% despite intensive multimodal treatment^{1–3}. Most patients are diagnosed before the age of 3 years.

ETMR often exhibits a distinctive histological pattern, with primitive tumor regions forming multilayered rosettes, surrounded by more differentiated areas rich in neoplastic neuropil⁴. The tumor's variable CNS locations, characteristic architecture, marker proteins and transcriptional profiles suggest that ETMR originates from pluripotent neuronal cells at the early stages of brain development^{5–7}. The coexistence of primitive and differentiated regions in the same tumor reflects a retained potential for cellular differentiation—a feature observed in other pediatric and adult brain tumors^{7–10}. In fact, neuronal differentiation, whether spontaneous or treatment-induced, is one of the few factors associated with disease

stabilization or long-term survival in patients with ETMR^{11,12}. Harnessing this differentiation potential is thus a critical therapeutic avenue. In addition, identifying and disrupting cellular cooperation between malignant cell types could offer a specific therapeutic strategy.

A common feature of ETMR is the ectopic activation of the chromosome 19 miRNA cluster (C19MC) by structural aberrations involving focal amplifications or fusions with the nearby *TTYH1* gene^{13–15}. In nonmalignant tissues, C19MC is highly expressed in trophoblast cells of the placenta^{16,17}. During fetal CNS development, distinct miRNAs have important roles in regulating early neural stem and progenitor cell differentiation¹⁸. However, the role of individual C19MC miRNAs and their collective impact on ETMR formation and progression remain poorly understood. Deciphering the downstream effects of specific C19MC members and identifying their target genes could provide valuable insights into this unique oncogenic event and unveil tumor-specific therapeutic opportunities.

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In this study, we performed single-cell transcriptomic profiling and multiplexed spatial imaging in a comprehensive cohort of 11 patient samples to decipher the malignant cell types of ETMR, investigate their spatial distribution in tumor tissues and define cellular correlates in the healthy developing brain. We further conducted miRNA immunoprecipitation (IP) and antisense targeting experiments in patient-derived ETMR models to study the role of C19MC in shaping the malignant cellular hierarchy. Finally, we analyzed receptor–ligand interactions between malignant cell types to nominate ETMR-specific vulnerabilities and exploit these vulnerabilities using targeted therapies in preclinical models and in a patient.

Results

ETMR shows pronounced cellular heterogeneity

To resolve the intertumoral and intratumoral heterogeneity of ETMR, we conducted single-cell RNA sequencing (scRNA-seq) and single-nucleus RNA sequencing (snRNA-seq) of six fresh and five frozen patient tumors, respectively (Fig. 1a, Extended Data Fig. 1a and Supplementary Table 1a). Tumors were selected based on histopathological diagnosis and DNA methylation-based classification^{19,20}; they included samples with different C19MC activation states ($n = 9$ amplified, $n = 2$ nonamplified). Biallelic mutations affecting *DICER1* were detected in both nonamplified cases (Extended Data Fig. 1b). Samples were derived from primary ($n = 7$) and recurrent ($n = 4$) tumors of the supratentorial ($n = 7$) or infratentorial ($n = 3$) region of the brain and one extracranial metastasis from a total of nine patients. Four patients were female and five patients were male, with an average age of 25 months. The age and sex distribution, tumor locations and high rate of recurrence in our cohort were consistent with previously described ETMR epidemiology^{1,21}.

In total, we profiled 2,571 cells from the six fresh patient tumors that passed stringent quality controls (Extended Data Fig. 1c and Methods). To identify malignant cells in this dataset, we inferred genome-wide copy-number variations (CNVs) (Extended Data Fig. 1d). Only 51 cells lacked CNVs and were considered nonmalignant, indicating high tumor purity in all samples (>95%). CNVs in malignant cells most frequently included gains of chromosome 2 (four of six tumors), in two cases accompanied by gains of chromosomes 7 and 8, and partial or total gains of chromosome 19 (three of six tumors), consistent with previous studies^{1,4,6}.

Unsupervised clustering of malignant single-cell transcriptomes ($n = 2,520$) revealed two major malignant cell populations, indicating pronounced cellular heterogeneity in ETMR (Fig. 1b,c). Notably, all six samples contained cells mapping to both cell populations (malignant cluster 1: 35–86%; malignant cluster 2: 14–65%). We next determined the transcriptional gene signatures specific to each cluster. Malignant cluster 1 expressed genes involved in undifferentiated cell growth and proliferation (for example, *VIM*, *CCND1*, *PAX6*, *PLTP*, *PROM1*), whereas

malignant cluster 2 was associated with genes involved in neurogenesis and cell differentiation (for example, *STMN2*, *TUBB3*, *ENO2*; Fig. 1d and Supplementary Table 2a). Therefore, we labeled the gene signatures as stem-like and differentiated signatures, respectively. Similar cellular heterogeneity was observed in five ETMR tumors from our extended frozen cohort ($n = 886$ cells), which included two tumors without a C19MC amplification (Extended Data Fig. 2a–c).

To extend our findings to a larger cohort of ETMR samples, we quantified our gene signatures in 26 tumor samples analyzed using bulk transcriptome profiling (Fig. 1e)²². Signature scores were anticorrelated (Pearson's $r = -0.66$). Notably, recurrent ETMRs had higher stem-like scores and lower differentiated scores compared to tumors at diagnosis ($P < 0.001$, Student's t -test). This is exemplified in one patient for which the primary (ET1) and two recurrent tumors (ET1R1, ET1R2) were profiled. We did not identify statistically significant associations with C19MC status, histological subtypes, tumor locations or age at diagnosis in this dataset.

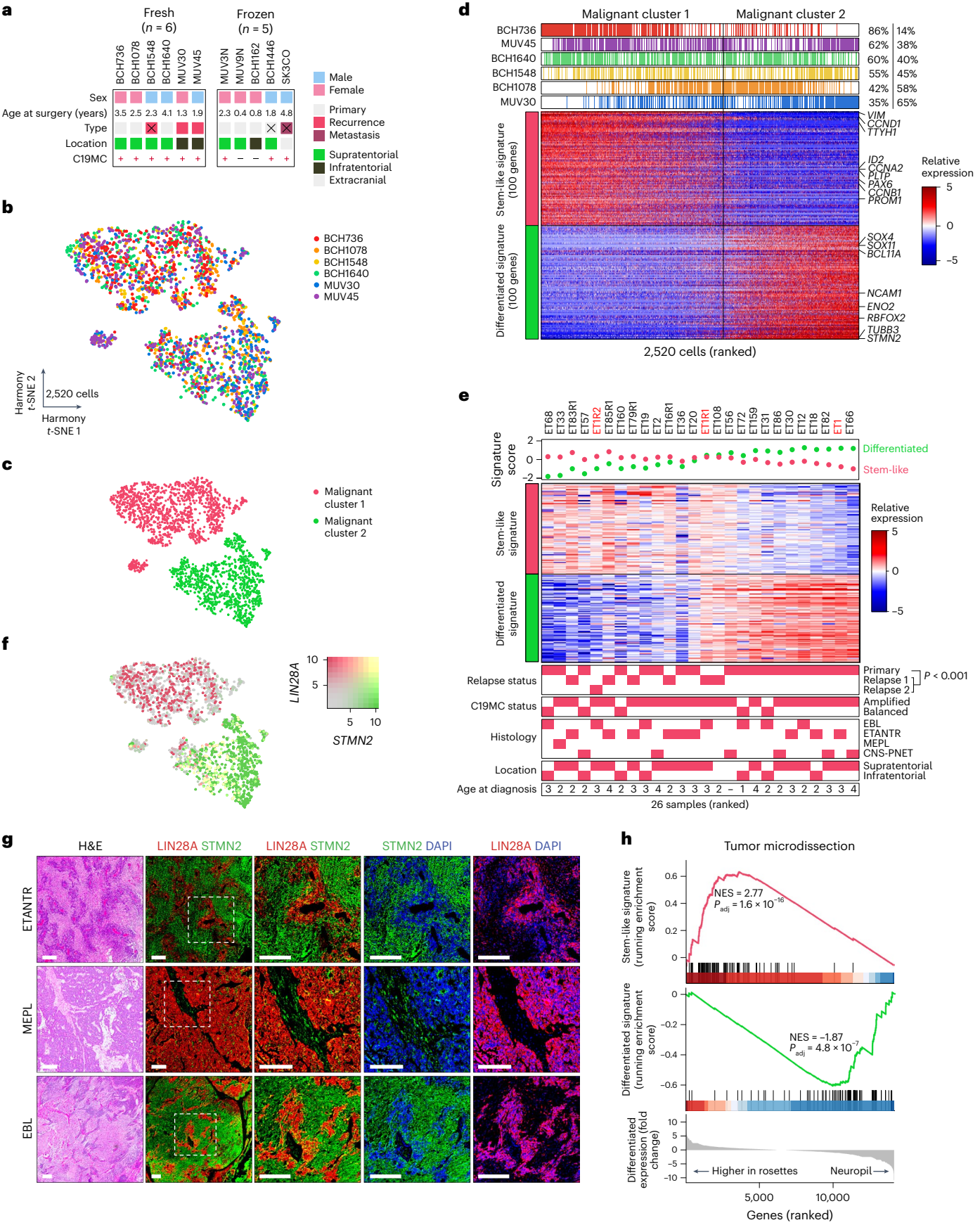
To validate the transcriptional signatures and spatially resolve malignant cell populations, we used immunofluorescence (IF) microscopy with markers specific to each malignant population (Fig. 1f and Extended Data Fig. 2d). Staining on patient tumor sections across three histological subtypes revealed a mutually exclusive pattern: LIN28A⁺, PLTP⁺ and PAX3⁺ stem-like cells were confined to hypercellular rosette-like structures, while STMN2⁺, NSE⁺ and TUBB3⁺ differentiated cells were located in neuropil-abundant, less cell-dense areas (Fig. 1g and Extended Data Fig. 2e). These spatial patterns were further validated by mapping malignant signatures onto transcriptomes from microdissected rosette-like and neuropil-abundant structures²². Gene set enrichment analysis (GSEA) confirmed substantial enrichment of the stem-like signature in rosette-like structures and the differentiated signature in neuropil-rich areas (Fig. 1h). Together, these findings highlight pronounced cellular heterogeneity and a clear alignment between transcriptional and histological phenotypes in ETMR.

Malignant hierarchies resemble fetal cortical neurogenesis

To investigate parallels between ETMR and normal brain development, we compared transcriptional states using our malignant signatures on a human fetal neocortex dataset (12–13 weeks after conception; Extended Data Fig. 3a)²³. We found that the ETMR stem-like signature was enriched in immature neural stem cells (NSCs) that resembled apical progenitor cells located in the ventricular zone. In contrast, the differentiated ETMR signature was enriched in mature neurons located in the outer subventricular zone and the cortical plate. Using a random forest classifier trained on cell types from the fetal neocortex dataset²³, we identified a third ETMR cell population resembling basal progenitor cells, a derivative of apical progenitors of the inner subventricular zone (Fig. 2a and Extended Data Fig. 3b)²⁴. This population

Fig. 1 | scRNA-seq and multiplexed imaging of ETMR tumors. a, Overview of clinical and molecular characteristics of the ETMR scRNA-seq cohort ($n = 11$ tumor samples). Sex, age at sample collection, tumor type, location and C19MC activation status are indicated. Samples indicated with X originated from the same patient at different disease stages. **b**, t -SNE visualization of high-quality malignant cells ($n = 2,520$ cells) from six samples of the fresh cohort, integrated using Harmony. Cells are colored according to the sample of origin. **c**, t -distributed stochastic neighbor embedding (t -SNE) visualization of $n = 2,520$ cells colored according to two major malignant cell clusters, identified using hierarchical clustering. **d**, Heatmap showing the relative expression of 100 genes specific to malignant clusters 1 (stem-like signature) and 2 (differentiated signature). Single cells ($n = 2,520$ cells) are ranked according to the difference in signature scores. The percentages of cells belonging to malignant clusters 1 and 2 are indicated above. Selected genes of interest are indicated on the right. **e**, Heatmap showing the relative expression of signature genes in $n = 26$ ETMR samples profiled using bulk expression arrays. Samples are ranked according to the difference in signature scores, shown above the heatmap. Red indicates

the primary lesion (ET1) and two recurrences (ET1R1, ET1R2) collected from the same patient. Correlations to clinical and molecular annotations are indicated below the heatmap. Age at diagnosis is given in years. A two-sided Student's t -test was used to determine the statistical significance between scores for malignant cluster 1 in primary and recurrent ETMR. PNET, primitive neuroectodermal tumor. **f**, t -SNE visualization of $n = 2,520$ cells colored according to the expression of the marker genes LIN28A (red) and STMN2 (green). **g**, Representative hematoxylin and eosin (H&E) and IF staining for LIN28A (red), STMN2 (green), and 4',6'-diamidino-2-phenylindole (DAPI) (blue) across the histological subtypes ETANTR, MEPL and EBL. Staining was performed on at least $n = 3$ different tumor samples for each subtype. EBL, ependymoblastoma; ETANTR, embryonal tumor with abundant neuropil and true rosettes; MEPL, medulloepithelioma. **h**, GSEA of malignant gene signatures in transcriptional profiles generated from microdissected rosette-like and neuropil-abundant tumor regions. NES, normalized enrichment score; P_{adj} , adjusted P value. GSEA P values were calculated using a permutation test and adjusted for multiple comparisons using the Benjamini–Hochberg procedure. **g**, Scale bars, 300 μ m.



represents an intermediate between NSCs and differentiated neurons and is defined by expression of transcriptional regulators, including *ASCL1*, *INSM1*, *NHLH1* and *NEUROD1* (Fig. 2b and Extended Data Fig. 3c). These results suggest striking parallels between cellular states in ETMR and the developing brain.

These findings prompted us to systematically investigate transcriptional regulation in ETMR using single-cell regulatory network inference and clustering (SCENIC) analysis²⁵. Analyzing gene coexpression module (regulon) activity and transcription factor (TF) expression across malignant ETMR cells, we identified 55 regulons of which 37 were shared among all six ETMR samples, including many regulons defined by key neurodevelopmental TFs (Fig. 2c,d and Extended Data Fig. 3d–f). Based on their activity in single cells, we clustered shared regulons into four groups. A first group of regulons was defined by members of the E2F and TFDP TF groups, which are major regulators of the cell cycle in mammalian cells. Cells positive for these regulons largely overlapped cells positive for a second group of regulons defined by the *SOX2*, *SOX3* and *NUAK2* TFs. As these TFs have important roles in NSCs, we labeled this population NSC-like cells. A third group of regulons was defined by the *TCF4*, *TCF12*, *NEUROD1*, *NEUROG1* and *NHLH1* TFs, which were positive in the intermediate population of cells. These TFs are key regulators of neuronal lineage commitment and differentiation and are specific to migrating basal progenitors in normal brain development (Extended Data Fig. 3c). This intermediate population connected NSC-like cells to cells that were positive for a fourth group of regulons defined by TFs specific to differentiated neurons (for example, *SOX4*, *SOX11*, *KLF7*, *ZEB1*)^{23,26,27}.

SCENIC analysis allowed us to refine our previous malignant cell populations and suggested a cellular hierarchy ranging from NSC-like cells (overlapping malignant cluster 1) to neuron-like cells (malignant cluster 2) via an intermediate cell population (cells from both clusters 1 and 2) that was detected in every patient sample (Fig. 2e and Extended Data Fig. 4a). Consequently, we generated three transcriptional gene signatures reflecting these refined cell populations, in addition to a cycling signature (Extended Data Fig. 4b,c and Supplementary Table 2b–d). Interestingly, cells positive for the cycling signature were highly enriched in the NSC-like population and absent from the neuron-like population (Fig. 2f and Extended Data Fig. 4d). To evaluate the putative cellular hierarchy, we inferred cellular trajectories based on the relative abundance of spliced and unspliced RNA transcripts using *velocyto*²⁸. This analysis revealed unambiguous differentiation trajectories of the intermediate populations toward neuron-like cells (Fig. 2g and Extended Data Fig. 4e), akin to neuronal differentiation in normal brain development.

To investigate the spatial distribution of refined cell populations, we performed multiplexed spatial phenotyping on more than 45,000 cells from three primary tumor tissues using representative TFs (Extended Data Fig. 4f,g). PAX6⁺ NSC-like cells localized to rosette-like tumor structures, while PEG3⁺ neuron-like cells were found in neuropil-rich areas (Fig. 2h). *NEUROD1*⁺ cells were more evenly distributed throughout the tumor. Nearest neighbor distance (NND)

analysis showed the largest distances between NSC-like and neuron-like cells (mean NND = 30.8 μ m), with intermediate cells positioned closer to both (15.3 μ m and 22.6 μ m, respectively; Fig. 2i). This spatial pattern corroborates our inferred developmental hierarchy in ETMRs, in which undifferentiated NSC-like cells form rosettes and retain some differentiation potential as they migrate to form neuropil-rich areas, similar to normal neurogenesis²³.

In summary, these results suggest that ETMR cells closely recapitulated sequential developmental programs during normal human brain development. NSC-like and intermediate ETMR cells correlated to apical and basal progenitors, respectively, while neuron-like ETMR cells correlated to more mature basal neurons. Malignant cell types are controlled by master transcriptional regulators and form spatially distinct niches within ETMRs, reminiscent of those found in normal brain development.

C19MC expression is restricted to ETMR stem-like cells

The defining molecular feature of more than 90% of ETMRs is the activation of C19MC via amplifications or fusions involving the nearby gene *TTYH1* (ref. 14). C19MC is the largest cluster of miRNAs in the human genome that covers a region of approximately 100 kb containing 46 miRNA genes grouped into 16 miRNA families (miR-498, miR-512, miR-515 to 527, miR-1283 and miR-1323, expressed as a single primary transcript; Fig. 3a)²⁹. Prior studies implicated C19MC members in regulating cell cycle, tumor invasion and progression^{1,15,30,31}. However, because of its size, it is challenging to comprehensively define the targets of C19MC.

Building on our findings on ETMR intratumoral heterogeneity, we examined whether C19MC is differentially expressed in specific malignant cell types. Quantifying C19MC primary transcript levels across single cells in our patient cohort with ETMR revealed high expression in malignant NSC-like cells, with much lower levels in intermediate and neuron-like cells (Fig. 3b and Extended Data Fig. 5a,b). Expression of the C19MC fusion partner *TTYH1* was highly correlated (Spearman's $r = 0.41$; Fig. 3c). To validate and spatially map C19MC expression, we used RNA in situ hybridization (ISH). We selected miR-512, a highly abundant mature miRNA in ETMR primary tissues as identified by small RNA-seq (Extended Data Fig. 5c)²². In a C19MC-amplified tumor (MUV3N) from our cohort, miR-512 expression was confined to rosette-like regions enriched with undifferentiated NSC-like cells (Fig. 3d and Extended Data Fig. 5d). In contrast, miR-124, a neuron-specific miRNA^{32,33} not part of C19MC, was highly expressed in neuropil-rich tumor regions but absent in rosettes.

We considered two potential explanations for the cell-type-specific expression of C19MC. First, genetic heterogeneity might exist in ETMR cells, with C19MC-driving alterations restricted to NSC-like cells but absent in intermediate and neuron-like cells. Alternatively, genetic alterations involving C19MC might be present in all malignant cell types, with its expression regulated in a cell-type-specific manner. To investigate this, we performed DNA fluorescence in situ hybridization (FISH) targeting the C19MC region in patient tumors with C19MC

Fig. 2 | Transcriptional regulation of malignant cell-type transitions. **a**, *t*-SNE visualization of $n = 2,520$ malignant ETMR cells colored for cells predicted as basal progenitor cells (green) using a random forest classifier trained on cells from the fetal neocortex. **b**, *t*-SNE visualization of $n = 2,520$ malignant ETMR cells, highlighting normalized expression of the basal progenitor cell marker genes *ASCL1*, *INSM1*, *NHLH1* and *NEUROD1*. **c**, Heatmap showing normalized area under the curve (AUC) values (indicating regulon activity) from the SCENIC analysis. Four groups of regulons were formed by hierarchical clustering across patients. The annotation above the heatmap indicates the classification of $n = 2,520$ cells into three malignant cell types. **d**, Network showing similarity scores (thickness of connecting lines) determined using pairwise comparison of regulons shown in **c**. **e**, *t*-SNE visualization of $n = 2,520$ malignant ETMR cells colored according to refined cell types. **f**, *t*-SNE visualization of $n = 2,520$ malignant ETMR cells colored

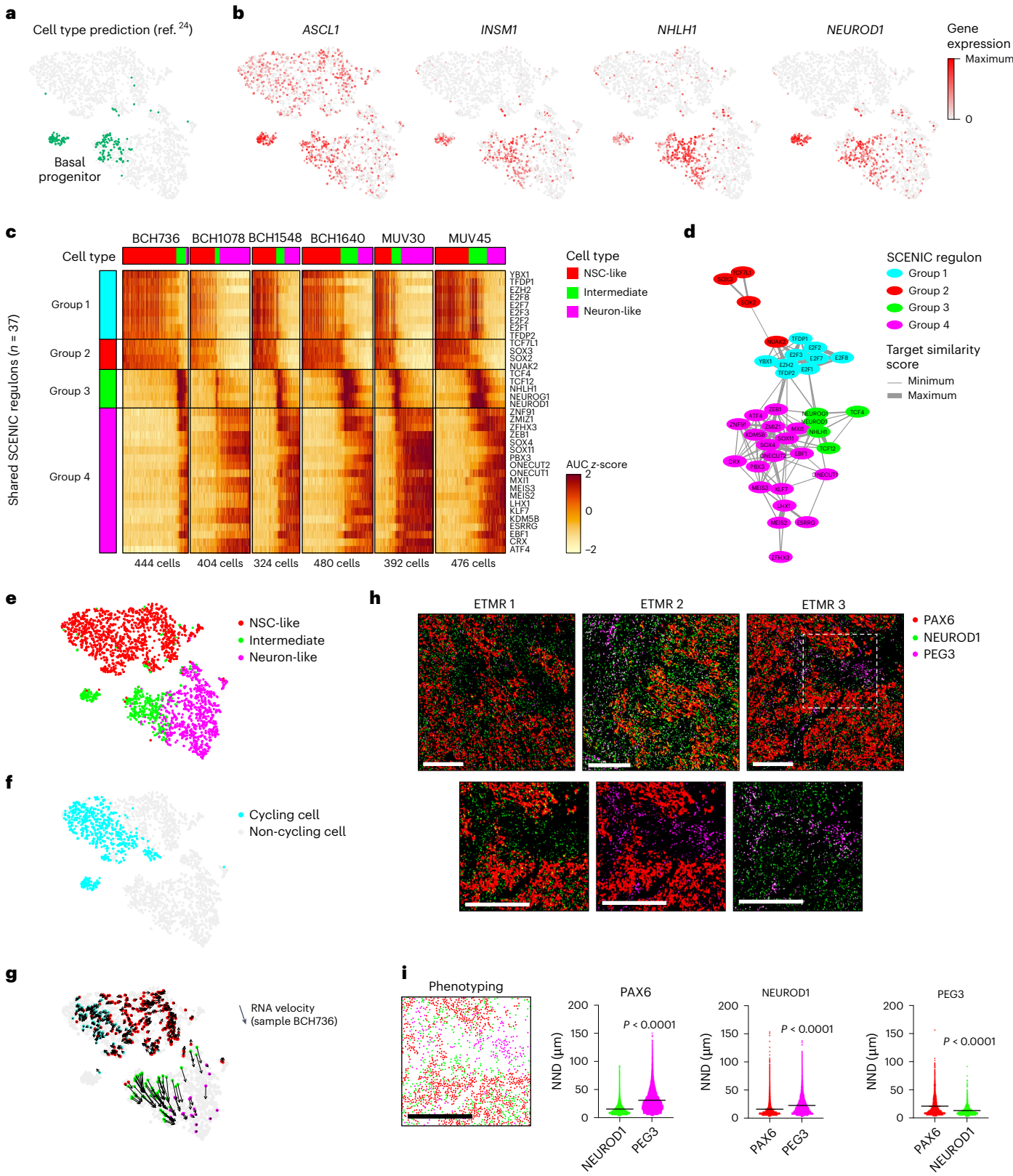
according to cell cycle state. **g**, *t*-SNE visualization of $n = 2,520$ malignant ETMR cells overlaid with differentiation trajectories generated by RNA velocity analysis of a representative ETMR sample (BCH736). **h**, Representative multiplexed IF images of $n = 3$ different primary ETMR samples for the marker TFs PAX6 (NSC-like cells, red), *NEUROD1* (intermediate cells, green) and PEG3 (neuron-like cells, magenta; top). Magnification of the indicated region (dashed outline) in ETMR3 is shown as pairwise contrasts (bottom). **i**, Pseudo image (left) showing a reconstruction of cells analyzed using in silico phenotyping for the same region as in **h**, colored according to marker gene expression. The violin plots show the nearest neighbor distance analysis between different ETMR cell types identified using multiplexed phenotyping. A total of $n = 46,621$ cells were phenotyped. *P* values were calculated using a two-sided, unpaired Student's *t*-test. The lines represent the mean values. **h**, Scale bars, 300 μ m.

amplification (Fig. 3e and Extended Data Fig. 5d). Analyzing 35,585 nuclei from patient MUV45, we detected three or more C19MC foci in nuclei from both low-density (neuropil-abundant) and high-density (rosette-like) regions, indicating that C19MC amplifications are widespread across malignant cell types (Fig. 3f and Extended Data Fig. 5e). Similar results were observed in a second patient tumor (MUV30) (Extended Data Fig. 5f). These findings suggest that C19MC genetic

alterations occur in all malignant cells, while its cell-type-specific expression is regulated by its fusion partner TTYH1, which itself is cell-type-specifically controlled^{7,26,34}.

Widespread target gene regulation by C19MC

Next, we set out to study C19MC target gene regulation in functional experiments. For this purpose, we evaluated C19MC expression levels



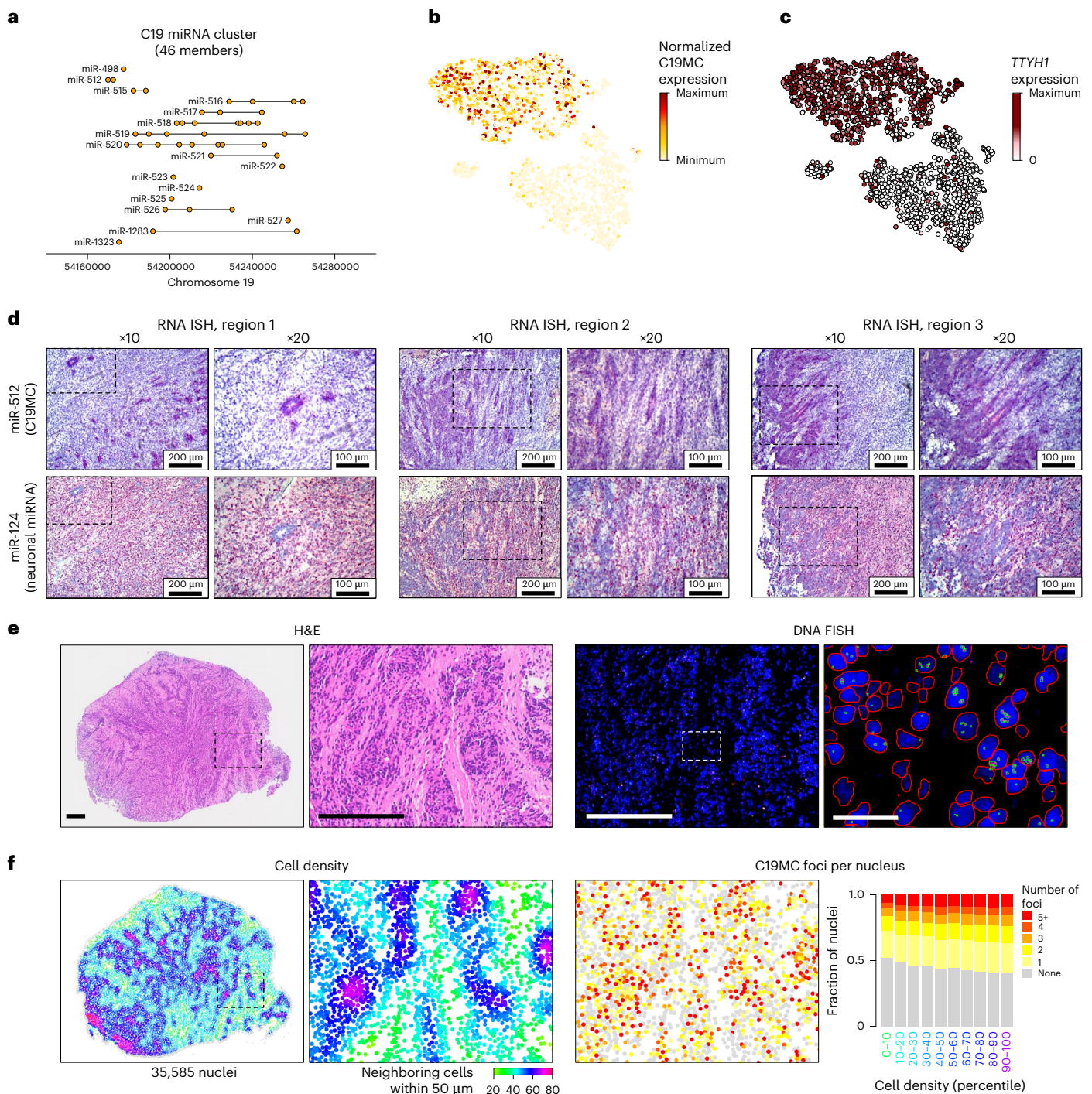


Fig. 3 | Spatial analysis of C19MC expression across malignant cell types. **a**, Schematic illustrating the genomic location of 46 miRNA members of C19MC, grouped into 17 miRNA families. **b**, *t*-SNE representation of $n = 2,520$ malignant ETMR cells highlighting the normalized expression of the C19MC pri-miRNA, as determined using scRNA-seq. **c**, *t*-SNE representation of $n = 2,520$ malignant ETMR cells colored for the relative expression of the C19MC fusion partner *TTYH1*. **d**, Microscopy images of RNA ISH analysis of the ETMR sample MUV3N stained for miR-512 (C19MC marker) and miR-124 (marker for neuron-like cells) on two consecutive tumor slices. Three tumor regions of the same slides are shown. The dashed outline indicates regions that are shown at higher magnification. Images show representative regions of one sample with a total of $n = 3$ patient tumors. **e**, Microscopy images of H&E (left) and DNA FISH (right)

analysis of the C19MC-amplified sample MUV45 on consecutive tumor slices. The dashed outline indicates regions that are shown at higher magnification. The red and green boundaries in the rightmost image show automated segmentation of nuclei and subcellular C19MC foci, respectively. DNA FISH was performed and quantified on three individual ETMR samples. Images shown are representative of the three samples. **f**, Pseudo images showing systematic analysis of cell density and the number of distinct C19MC foci per nucleus derived from the DNA FISH images shown in **e**. A total of $n = 35,585$ nuclei were analyzed. The bar plot summarizes the numbers of C19MC foci per nucleus grouped according to cell density. **e**, Scale bars, 250 μ m (H&E and DNA FISH, left) and 25 μ m (DNA FISH, right).

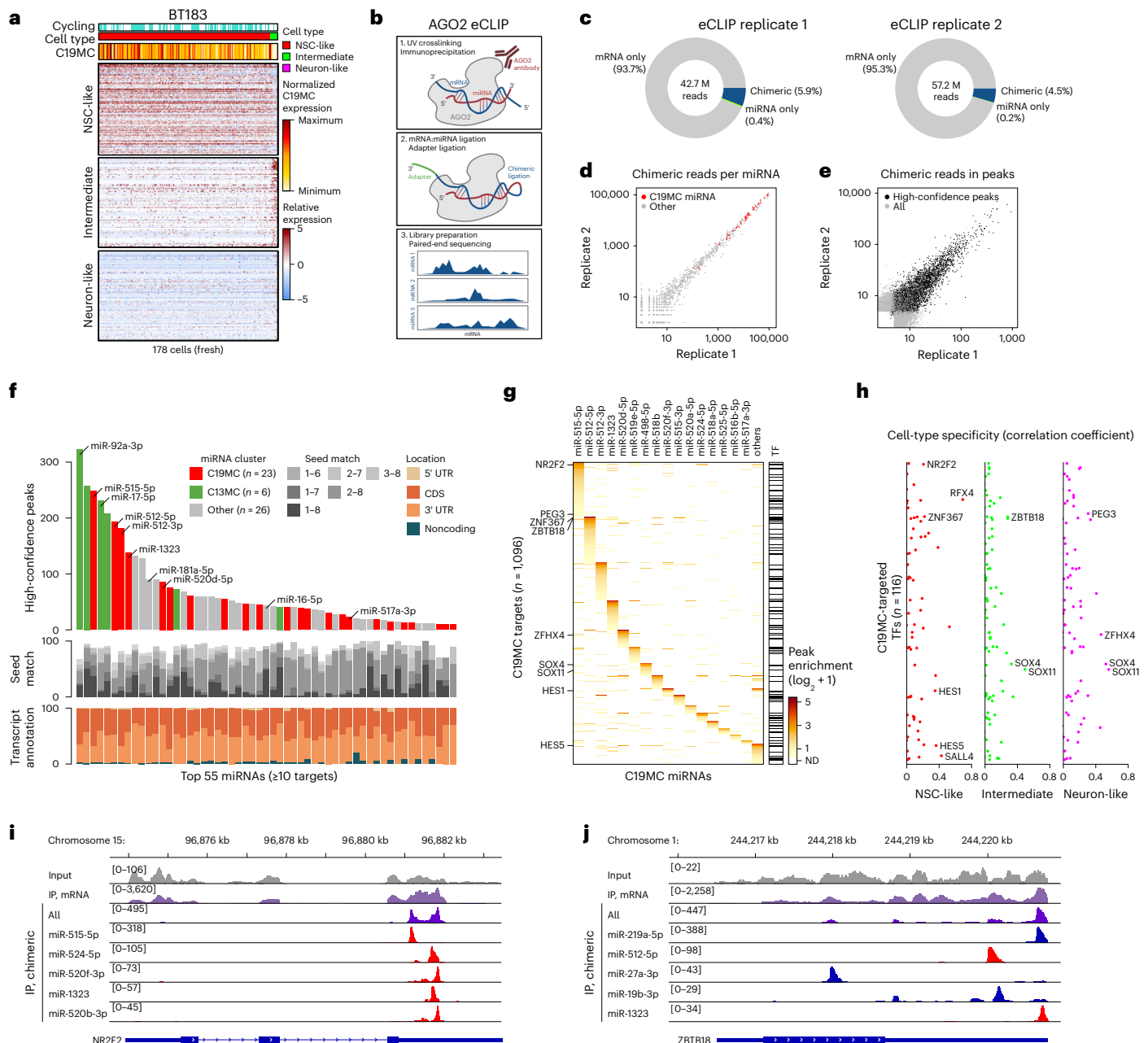


Fig. 4 | Genome-wide identification of miRNA targets using chimeric eCLIP. **a**, Heatmap showing the relative expression of refined signature genes ($n = 100$ per signature) in $n = 179$ cells from the BT183 patient-derived in vitro model. Normalized expression levels of the pri-miRNA of C19MC and cell-type assignments are indicated above the heatmap. **b**, Schematic illustration of the AGO2 eCLIP experimental workflow. **c**, Pie charts showing the fractions of mRNA, miRNA and chimeric mRNA-miRNA in both eCLIP replicates ($n = 42.7$ M and 57.2 M reads). **d**, Scatter plot showing the number of chimeric reads per miRNA in both technical replicates. C19MC members are highlighted in red. **e**, Scatter plot showing the number of chimeric reads per peak in both technical replicates. High-confidence peaks are highlighted ($n = 4,009$). **f**, Top, Bar plot showing the number of high-confidence peaks for C19MC (red, $n = 23$ miRNAs), C13MC (green, $n = 6$ miRNAs) and other (gray, $n = 26$ miRNAs) miRNAs with at least ten target genes. Middle, Bar plot indicating the percentage of peaks containing the respective seed motifs. Bottom, Bar plot indicating the percentage of peaks in UTRs (5' and

3' UTRs), CDS or noncoding transcripts. **g**, Heatmap showing peak enrichment for high-confidence target genes ($n = 1,096$) of C19MC members. Target genes for miRNAs with fewer than 20 target genes are summarized in the rightmost column. TFs are highlighted next to the heatmap. Selected TFs are labeled on the left. **h**, Dot plots showing the correlation of C19MC-targeted TFs ($n = 116$) to cell-type-specific signature scores in single cells of the patient samples, in the same order as shown in **g**. Selected TFs are labeled. **i**, Genome plot of the region on chromosome 15, including the C19MC-targeted TF NR2F2. The track shows coverage in input control; the second track shows coverage of nonchimeric mRNA-only reads; the third track shows coverage of all chimeric reads, combined for all miRNAs; the remaining tracks show the chimeric reads associated with five separate C19MC members (shown in red). **j**, Genome plot of region on chromosome 1 encompassing the TF ZBTB18. Coverage tracks of chimeric reads associated with C19MC members miR-512-5p and miR-1323, and three non-C19MC miRNAs, are indicated in red and blue, respectively. Schematic in **b** created with BioRender.com.

and malignant cell-type composition in the ETMR cell model BT183 (ref. 6). Small RNA-seq showed high expression of C19MC members, with 29 of the 100 most highly expressed miRNAs belonging to the cluster (19.5% of all reads; Extended Data Fig. 6a). scRNA-seq revealed

that in vitro cultured cells consisted almost entirely of NSC-like cells ($>95\%$), a small fraction of intermediate cells and no neuron-like cells (Fig. 4a and Supplementary Table 3a). As with primary tumors, the C19MC primary transcript was highly detected in NSC-like cells. Based

on these results, we concluded that BT183 is a suitable cell model for functional experiments of C19MC target gene regulation.

Determining the target genes of individual miRNAs is challenging and often based on computational algorithms that rely on complementarity to the miRNA seed sequence (6–8 bp) and evolutionary conservation of the target site. To experimentally identify the bona fide mRNA targets of all miRNAs expressed in BT183 cells, we performed enhanced crosslinking and IP (eCLIP) using Argonaute-2 (AGO2) pulldown and next-generation sequencing of chimeric reads to map genome-wide miRNA-mRNA interactions (Fig. 4b)^{35,36}. Out of 42.7 M and 57.2 M sequenced reads, we detected 5.9% and 4.5% of chimeric reads in replicates 1 and 2, respectively (Fig. 4c and Extended Data Fig. 6b). The number of chimeric reads associated with a specific miRNA in both replicates was highly correlated (Pearson's $r = 0.99$; Fig. 4d). Of all chimeric reads, 37.8% and 41.5% were associated with C19MC members in replicate 1 and 2, respectively.

Defining miRNA-specific binding sites along coding and noncoding mRNA transcripts, we detected a total of 12,787 peaks with at least five chimeric reads in either replicate (Pearson's $r = 0.88$; Fig. 4e and Extended Data Fig. 6c). Of these, 4,009 were deemed high-confidence peaks (Supplementary Table 3b). The number of peaks per miRNA varied greatly, with some highly expressed miRNAs associated with hundreds of peaks (Fig. 4f top). Of the 55 miRNAs associated with ten or more peaks, 23 were members of C19MC (37.0% of peaks in total). Interestingly, all six members of the chromosome 13 miRNA cluster (C13MC, also known as the *miR-17–92* cluster) were also among the miRNAs with the most detected peaks (28.3%). Most peaks (84.2%) contained a match to the miRNA seed sequence (20.8% 8 bp match; 43.8% 7 bp match; 19.6% 6 bp match; Fig. 4f middle). Peaks were highly enriched in 3' untranslated regions (UTRs) (3' UTR, 49.4%) or in the coding sequence (CDS) (46.8%) of the mRNA targets, with fewer miRNAs binding to the 5' UTR or noncoding transcripts, in line with expectations (Fig. 4f bottom).

Considering their importance for ETMR biology, we specifically investigated the target genes of C19MC. Of 1,096 identified target genes, most (79.7%) were associated with a unique C19MC member (Fig. 4g and Supplementary Table 3b). Only few genes were associated with multiple members of the same C19MC family (for example, *miR-518b/c*, *miR-520b/f*) with overlapping peaks (Extended Data Fig. 6d). Most remaining genes were associated with multiple members of different families that had distinct binding sites along the transcript, underscoring the complexity of C19MC target gene regulation. As C19MC expression is specific to NSC-like cells, we hypothesized that target genes may include key regulators that govern cell proliferation, stemness and lineage commitment. As such, we focused on 116 TFs that were identified as C19MC targets (10.5% of all target genes) and integrated their cell-type-specific expression in the tumors of ETMR patients (Fig. 4h).

A prime target gene resulting from our systematic analysis that is specifically expressed in NSC-like cells is *NR2F2* (also known as *COUP-TFII*). We detected highly enriched peaks for *miR-515-5p*, *miR-524-5p*, *miR-520b/f-3p* and *miR-1323* in the 3' UTR, suggesting pronounced posttranscriptional repression (Fig. 4i and Supplementary Table 3b). *NR2F2* encodes an evolutionary conserved TF that is transiently expressed in the ventricular zone during embryonic development and has important roles in regulating neuronal differentiation³⁷. Interestingly, we found several members of the C2–H2 zinc finger protein (ZNF) TF family (for example, *ZNF367*, *ZBTB18*, *EGR1*, *ZFXH4* and *PEG3*) to be targeted by C19MC members (Fig. 4j and Extended Data Fig. 6e,f). C2–H2 ZNF family members are key regulators of brain development and neuronal lineage commitment³⁸. In addition to C2–H2 ZNF family members, we identified *SOX4* and *SOX11* among the target mRNAs of several C19MC members (including *miR-498-5p*, *miR-524-5p*, *miR-520d-5p* and *miR-1323*) with expression specific to intermediate and neuron-like cells (Extended Data Fig. 6g,h). Prior research established a critical role of *SOX4* and *SOX11* in promoting neuronal differentiation and migration^{39,40}. Lastly, we also detected peaks for multiple C19MC members in *HES1* (targeted by *miR-512-3p*, *miR-515-5p*, *miR-520b-3p*, *miR-520d-3p*, *miR-520f-3p*, *miR-525-3p* and *miR-1323*) and *HES5* (targeted by *miR-520d-5p* and *miR-523-3p*) TFs (Extended Data Fig. 6i,j). HES TFs are critical regulators of telencephalic patterning⁴¹ and neuronal differentiation⁴², and are important downstream effectors of the Notch signaling pathway⁴³. Reanalysis of a public dataset confirmed that many of the identified TFs (*NR2F2*, *ZNF367*, *SOX4*, *SOX11*, *HES1*) are downregulated upon CRISPR-guided transcriptional activation of C19MC in HEK 293 cells (Extended Data Fig. 7a,b)¹⁷.

Taken together, our genome-wide analysis of miRNA-mRNA interactions uncovers a widespread regulatory role of individual C19MC members in ETMR cells. We showed that TFs are frequently targeted, underscoring the importance of C19MC in dysregulating crucial developmental processes that include repression of positive regulators of lineage commitment and differentiation, as well as repression of negative regulators of stemness and proliferation.

Silencing of C19MC attenuates tumor cell proliferation

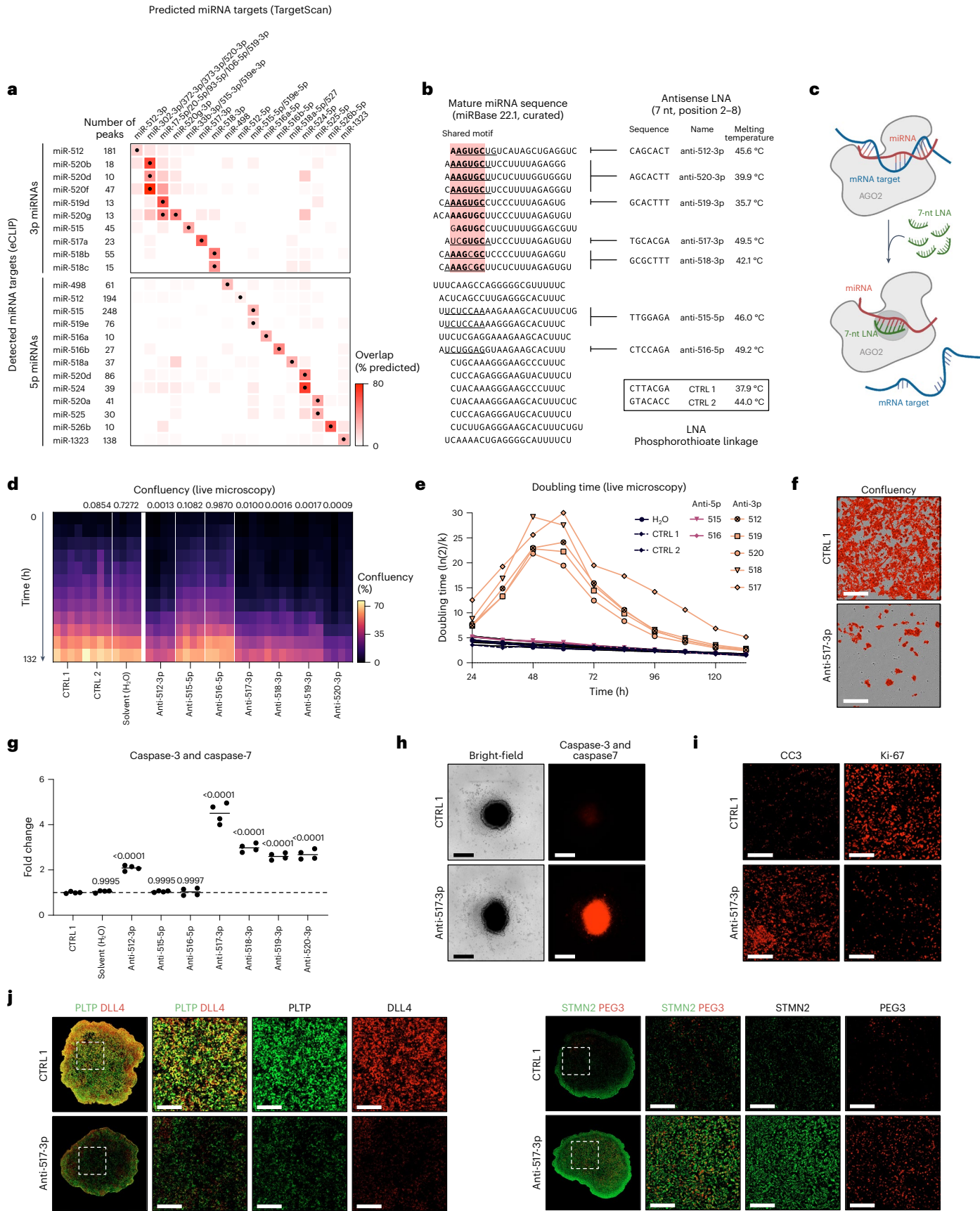
Because of the complexity of C19MC target regulation and its restricted expression to few healthy and malignant tissues, understanding the functional consequences of C19MC expression is challenging¹. Therefore, we asked if C19MC targets may overlap with those of other miRNAs with well-described biology. We compared detected peaks from our eCLIP experiment with predicted binding sites for all human miRNAs using TargetScan (Fig. 5a)^{44,45}. The highest overlap for C19MC members was generally observed with their respective predicted targets. However, the C19MC members *miR-520b/d/f-3p* shared high

Fig. 5 | Targeting C19MC families with short antisense oligonucleotides. a, Heatmap showing the overlap of C19MC targets detected in eCLIP with predicted targets from TargetScan. C19MC members are grouped into 3p (top, $n = 10$) and 5p (bottom, $n = 13$) miRNAs. The black dots indicate the largest overlap with predicted targets. The number of high-confidence peaks for each member are indicated. **b,** Mature C19MC miRNAs as in **a**. The AAGUGC seed motif in 3p miRNAs is highlighted in red. LNAs complemented the seed sequences of one or more C19MC members (7-nt-long, complementary to position 2–8). Nontargeting controls were designed (CTRL 1, CTRL 2). Predicted melting temperatures are indicated. **c,** Schematic illustration of the miRNA targeting mechanism of LNAs. **d,** Heatmap showing the confluency of BT183 ETMR cells treated with LNAs over time, tracked using live-cell microscopy in two-dimensional (2D) culture with four biological replicates. P values were calculated with a one-way analysis of variance (ANOVA) and adjusted P values are provided. The results of $n = 4$ biological replicates are shown. **e,** Longitudinal doubling time estimation for BT183 growth treated with the indicated LNAs. Doubling times were calculated using the log of exponential growth equation in Prism 8. The results of $n = 4$ independent experiments are shown. **f,** Bright-field

images showing BT183 cell confluency after treatment with anti-517-3p or CTRL 1 LNAs for 72 h. Identified cell areas are highlighted in red. This experiment achieved reproducible results in four biological replicates. **g,** Dot plot showing the caspase-3 and caspase-7 signal after 72 h of LNA treatment in three-dimensional BT183 cells. The means are indicated. Statistical analysis was performed on $n = 4$ biological replicates using a one-way ANOVA, comparing each condition to CTRL 1. P_{adj} values are given. **h,** Bright-field and fluorescence images showing the caspase-3 and caspase-7 signal after 72 h of treatment with anti-517-3p or CTRL 1 LNAs in BT183 cells. Images shown are representative of $n = 4$ biological replicates. **i,** IF image analysis of BT183 cells for Ki-67 and cleaved caspase-3 (CC3). Cells were treated with anti-517-3p or CTRL 1 LNAs for 72 h. This experiment achieved reproducible results in three biological replicates. **j,** Representative IF staining for the indicated markers. Cells were treated with anti-517-3p or CTRL 1 LNAs for 72 h. This experiment achieved reproducible results in three biological replicates. Left, The dashed boxes indicate the tumor area shown in the magnified panels on the right. **f,** Scale bars, 400 μm . **h,** Scale bars, 500 μm . **i,j,** Scale bars, 300 μm . Schematic in **c** created with BioRender.com.

overlap with the predictions for the broadly conserved miR-302 family. In addition, the C19MC members miR-519d and miR-520g shared high overlap with the broadly conserved miR-17 family. Both miRNA families have been described as key regulators of embryonic and neural stem

cell maintenance and proliferation^{46–49}. We noted that many expressed C19MC 3p miRNAs share a common 6-nt-long AAGUGC or related motif overlapping the seed sequence (Fig. 5b left). Previous studies found that miRNAs containing the AAGUGC motif, which include members of



the miR-302 and miR-17 families, drive proliferation in different cancer entities and consequently described it as an oncomotif^{50,51}. Expressed C19MC 5p miRNAs did not share a motif sequence.

To assess the functional roles of different C19MC families, we designed seven 7-nt-long locked nucleic acid (LNA) antisense oligonucleotides directed against the seed sequence (position 2–8) of mature miRNAs (Fig. 5b right and Supplementary Table 4a). These short oligonucleotides form stable heteroduplexes with their target miRNAs, thereby inhibiting their function (Fig. 5c)⁵². We designed five LNAs to target a total of eight C19MC 3p miRNAs containing the AAGUGC oncomotif (anti-512-3p, anti-519-3p, anti-520-3p) or a related sequence (anti-517-3p, anti-518-3p). Two additional LNAs were designed to target three C19MC 5p miRNAs that did not contain the oncomotif (anti-515-5p, anti-516-5p). We exposed C19MC-amplified ETMR cells (BT183) to these LNAs in live cultures and found that all 3p LNAs had a pronounced effect on cell proliferation, resulting in significantly reduced growth rates over a 132-h time course (Fig. 5d). Interestingly, the growth inhibitory effect of LNAs wore off after 60–72 h, with doubling times peaking around that time point and then normalizing again (Fig. 5e). LNAs directed against the 5p C19MC members had no discernible effect compared to two nontarget controls. Among the effective LNAs, anti-517-3p had the most durable growth-inhibiting effect on ETMR cells (Fig. 5f and Extended Data Fig. 7c).

In addition, we investigated the pro-apoptotic effect of LNAs on ETMR cells by assessing caspase-3-mediated and caspase-7-mediated apoptosis with a fluorescence live-cell assay. Exposure to anti-3p LNAs resulted in a pronounced induction of apoptosis in ETMR cells after 72 h, with the strongest effect observed for the miR-517-targeting LNA (Fig. 5g,h and Extended Data Fig. 7d). Anti-5p LNAs (anti-515-5p and anti-516-5p) did not show an effect. Assessing protein expression in anti-3p LNA-treated ETMR cells using multiplexed immunohistochemistry (IHC) analysis indicated marked decrease of the proliferation marker Ki-67, while the apoptosis marker cleaved caspase-3 showed increased levels of expression (Fig. 5i and Extended Data Fig. 7e). Furthermore, we observed decreased protein levels of NSC-like and intermediate markers (PLTP, DLL4) and increased expression of neuron-like markers (PEG3, STMN2) on anti-3p LNA treatment (Fig. 5j). The strongest effects were observed for the miR-517-targeting LNA.

In summary, we showed that specific C19MC members are reminiscent of other broadly conserved miRNA families with important regulatory roles in human development. Targeting miRNAs that contain the AAGUGC oncomotif or a related sequence with short antisense oligonucleotides led to a pronounced antiproliferative and pro-apoptotic effect, and promoted differentiation of ETMR cells in vitro.

Cellular cooperation between malignant cell types

While antisense targeting of C19MC miRNAs holds promise as a future therapeutic strategy, we aimed to identify alternative targets

in ETMR with immediate clinical potential. Cell surface receptors are established therapeutic targets in cancer, with several inhibitors already approved for pediatric use^{8,33,54}. To pinpoint such targets in ETMR, we systematically analyzed receptor and ligand expression and inferred their interactions across ETMR cell types using CellPhoneDB (Fig. 6a and Extended Data Fig. 8a)⁵⁵. This genome-wide analysis highlighted interactions in the fibroblast growth factor receptor (FGFR) and Notch signaling pathways across all six samples of our fresh tumor cohort (Fig. 6b,c). We found that NSC-like cells exhibited high expression of three FGF receptors (*FGFR1*, *FGFR2* and *FGFR3*), while their ligands (*FGF5*, *FGF7*, *FGF9*) were predominantly expressed in more differentiated neuron-like ETMR cells (Fig. 6d and Extended Data Fig. 8b–d). Similarly, NSC-like cells showed elevated expression of Notch receptors (*NOTCH1*, *NOTCH2*, *NOTCH3*), while canonical Notch ligands (*DLL1*, *DLL3*, *DLL4*) were expressed in intermediate ETMR cells (Fig. 6e and Extended Data Fig. 8e,f). As the FGFR and Notch pathways have central roles in neural development and are implicated in brain cancers, our findings suggest that both pathways are active in tumor-driving NSC-like cells. Furthermore, the data imply a specific role for more differentiated intermediate and neuron-like cells in providing essential growth factors, maintaining a cooperative network across malignant cell types^{56,57}.

This hypothesis raised the question why in vitro models of ETMR were predominantly found in an NSC-like state (Fig. 4a). We considered that cells may have adapted to the artificial supply of FGF2 and epidermal growth factor (EGF) in growth-factor-supplemented (full) medium that does not necessitate more differentiated tumor cells to supply these factors. Compared to BT183 cells cultured in full medium, growth factor deprivation (base medium) resulted in stalled cell proliferation over a time course of 138 h (Fig. 6f left). Next, we systematically interrogated the effect of 18 different FGF ligands (Supplementary Table 4b). We observed similar degrees of cell proliferation in FGF subfamilies (Fig. 6f right). The most pronounced effects were observed for the FGF1 subfamily ligands (FGF1 and FGF2, specific to *FGFR1* and *FGFR3*), FGF4 subfamily ligands (FGF4 and FGF6, specific to *FGFR1* and *FGFR2*) and FGF9 subfamily ligands (FGF9, FGF16 and FGF20, specific to *FGFR3*)⁵⁸. In contrast, FGF7 and FGF19 subfamily ligands (specific to *FGFR4*) did not rescue cell proliferation. Supplementing the soluble Notch ligand sDLL1 or sDLL4 to the growth medium also resulted in a clear acceleration of cell proliferation (Fig. 6g).

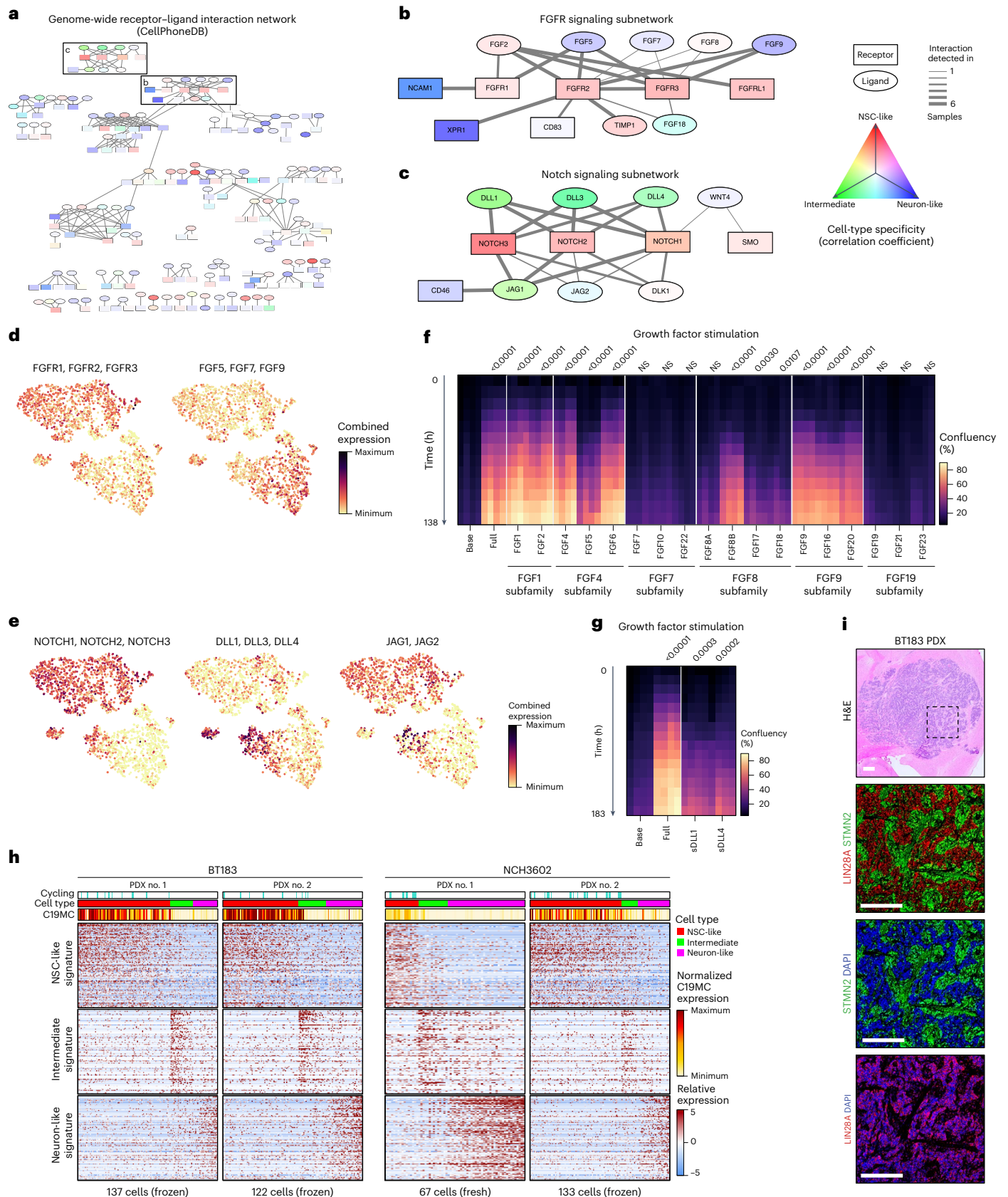
Next, we tested the hypothesis that ETMR cell models, in the absence of artificial growth factors, may develop more differentiated cell types to endogenously produce their own growth factors. Therefore, we analyzed two orthopic patient-derived xenograft (PDX) models using scRNA-seq (two tumors per model) and scored cells using our cell-type-specific signatures derived from patient primary tumors. Compared to in vitro cultured cells, PDX models showed

Fig. 6 | Systematic analysis of tumor-promoting receptor–ligand interactions. **a**, Network graph showing the interactions between receptors (rectangles) and ligands (ellipses) across our single-cell ETMR cohort ($n = 6$ samples), as inferred using genome-wide CellPhoneDB analysis performed separately on each sample. The different fill colors indicate the cell-type specificity of receptors and ligands. Line width indicates the number of samples in which the interaction was detected ($P < 0.05$). A larger version of the network graph is shown in Extended Data Fig. 8a,b. **b**, The network graph shows the FGFR signaling subnetwork. Receptors are specifically expressed in NSC-like tumor cells, while several ligands are specific to neuron-like cells. Line width indicates the number of samples in which the interaction was detected ($P < 0.05$). **c**, Network graph showing the Notch signaling network. Receptors are specifically expressed in NSC-like tumor cells, while Delta ligands (*DLL1*, *DLL3*, *DLL4*) are specific to intermediate cells. **d**, t -SNE representation of $n = 2,520$ malignant ETMR cells colored according to the mean relative expression of FGF receptors (left) and ligands (right). **e**, t -SNE representation of $n = 2,520$ malignant ETMR cells colored according to the mean relative expression of Notch receptors (left), Delta ligands (center) and Jagged

ligands (right). **f**, Heatmap showing cell confluency after FGF growth factor stimulation in BT183 ETMR cells over a time course of 138 h. Cells were seeded as 2D cultures and cell growth was followed by live-cell microscopy. Four biological replicates were analyzed and different FGF ligands were grouped according to subfamilies. Statistical analysis was performed using a one-way ANOVA of the AUCs. P_{adj} values are given. The results of $n = 4$ biological replicates are shown. **g**, Heatmap showing cell confluency after soluble Delta ligand stimulation over a time course of 183 h. Four biological replicates were analyzed. Statistical analysis was performed using a one-way ANOVA of the AUCs. P_{adj} values are given. The results of $n = 4$ biological replicates are shown. **h**, Heatmaps showing the relative expression of refined signature genes ($n = 100$) per ETMR cell type in $n = 3$ PDX models. Top, Cell-type assignment and cells in a cycling state are indicated. **i**, Representative H&E and IF staining of BT183 PDX for the indicated markers. Scale bars, 300 μ m. This experiment achieved reproducible results in three biological replicates. The dashed box in the H&E image indicates the tumor area shown in the IF staining below.

notably higher proportions of intermediate and neuron-like cells (Fig. 6h and Supplementary Table 3a). In line with our findings in the patient tumor samples, IF staining of PDXs demonstrated biphasic expression of the markers LIN28A (rosette-like areas) and STMN2 (neuropil-abundant areas; Fig. 6i). These data show that the cellular

and spatial heterogeneity found in patient tumors is reproduced in orthotopic PDX models. The finding that PDX models reconstitute a full cellular hierarchy suggests that BT183 cells maintained the potential to differentiate toward more differentiated cell types in the absence of artificially supplied growth factors.



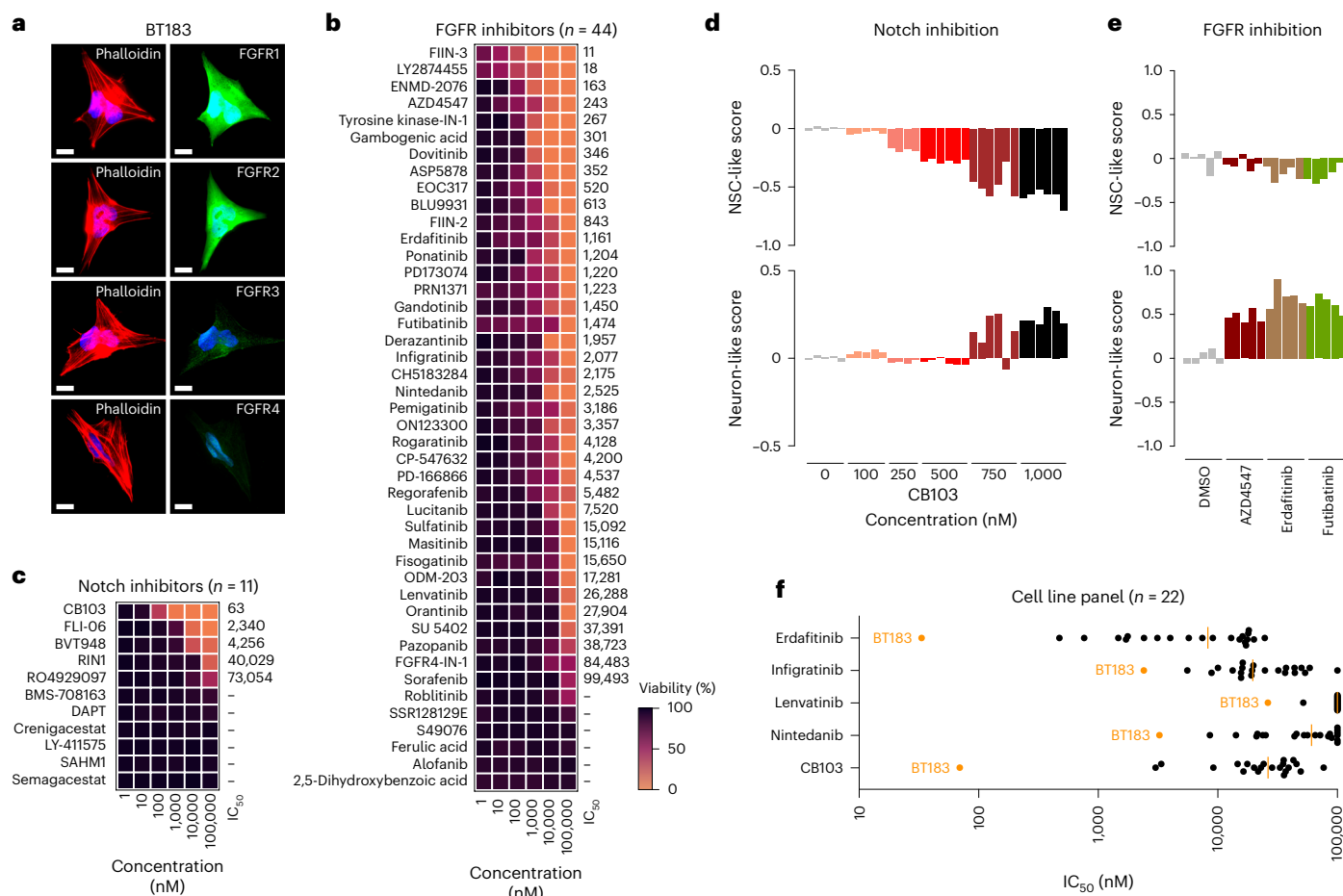


Fig. 7 | Small-molecule inhibitor screening uncovers promising drug candidates. **a**, Images showing IF staining of BT183 cells for actin cytoskeleton (red) and four FGFRs (green). The images shown are representative of $n = 3$ biological replicates. Scale bars, 10 μm . **b**, Heatmap indicating the viability of BT183 cells screened for 44 FGFR inhibitors at different concentrations. Right, Half-maximal inhibitory concentration (IC_{50}) values given in nanomolar concentrations. Inhibitors are sorted from top to bottom according to their respective IC_{50} . The results of $n = 4$ biological replicates are shown. **c**, Heatmap indicating the viability of BT183 cells screened for 11 Notch inhibitors, sorted according to their respective IC_{50} . The results of $n = 4$ biological replicates are shown. **d**, Bar plots showing NSC-like (top) or neuron-like (bottom) signature

scores of BT183 cells treated with CB103 at the indicated concentrations for 7 days. The individual bars indicate technical replicates. This experiment was performed in $n = 1$ biological replicate. **e**, Bar plots showing the NSC-like (top) or neuron-like (bottom) signature scores of BT183 cells treated with 1,000 nM of the FGFR inhibitors AZD4547, erdafitinib and futibatinib for 3 days. The individual bars indicate technical replicates. This experiment was performed in $n = 1$ biological replicate. **f**, Dot plot showing the screening of five therapeutic drug candidates in a panel of 22 cancer cell lines. The ETMR cell line BT183 is highlighted in orange; the mean IC_{50} across the cell lines is indicated by the vertical bars. The results of $n = 4$ biological replicates for each cell line are shown.

Taken together, our results indicate the presence of an oncogenic cellular cooperation network that mediates ETMR growth. More differentiated ETMR cells express high levels of growth factors (that is, *DLL1*, *DLL3* and *DLL4*, and *FGF5*, *FGF7* and *FGF9*, in intermediate and neuron-like cells, respectively), which may bind to their respective receptors (*NOTCH1*, *NOTCH2* and *NOTCH3*, and *FGFR1*, *FGFR2* and *FGFR3*) that are specifically expressed in tumor-driving NSC-like cells.

Therapeutic targeting of oncogenic signaling pathways

Having confirmed the growth-promoting roles of FGFR and Notch signaling in ETMR, we evaluated whether these pathways could be therapeutically targeted. IF microscopy of the BT183 cell line revealed high protein levels of FGFR1 and FGFR2, moderate FGFR3 expression and weak FGFR4 expression (Fig. 7a and Extended Data Fig. 9a), consistent with mRNA expression patterns in NSC-like ETMR cells observed through scRNA-seq in primary tumors (Fig. 6d and Extended Data Fig. 8c). To assess FGFR inhibition, we tested 44 FGFR inhibitors in BT183 cells (Supplementary Table 4c) and found many to be effective

at nanomolar-to-low-micromolar concentrations (Fig. 7b). Sensitivity was highest for FGFR1-specific, FGFR2-specific and FGFR3-specific inhibitors, while FGFR4-specific inhibitors had minimal effects on cell viability. In addition, we tested 11 Notch inhibitors (Fig. 7c and Supplementary Table 4d). Most gamma secretase inhibitors showed no significant effect on BT183 cell viability, except for RO4929097, which reduced viability only at high concentrations. In contrast, CB103, a Notch transcription complex inhibitor, potentially reduced cell viability at nanomolar concentrations³⁹.

RNA expression profiling after CB103 treatment revealed a dose-dependent increase in the neuron-like signature score and decreases in NSC-like, intermediate and cycling signature scores, suggesting a shift toward a more differentiated phenotype in the remaining tumor cells (Fig. 7d and Extended Data Fig. 9b–d). Similarly, profiling after treatment with FGFR inhibitors (AZD4547, erdafitinib and futibatinib) showed reduced cycling signature scores and increased neuron-like signature scores (Fig. 7e). Notably, treated cells also exhibited high intermediate signature scores and upregulation of Notch signaling pathway genes (Extended Data Fig. 9e–j).

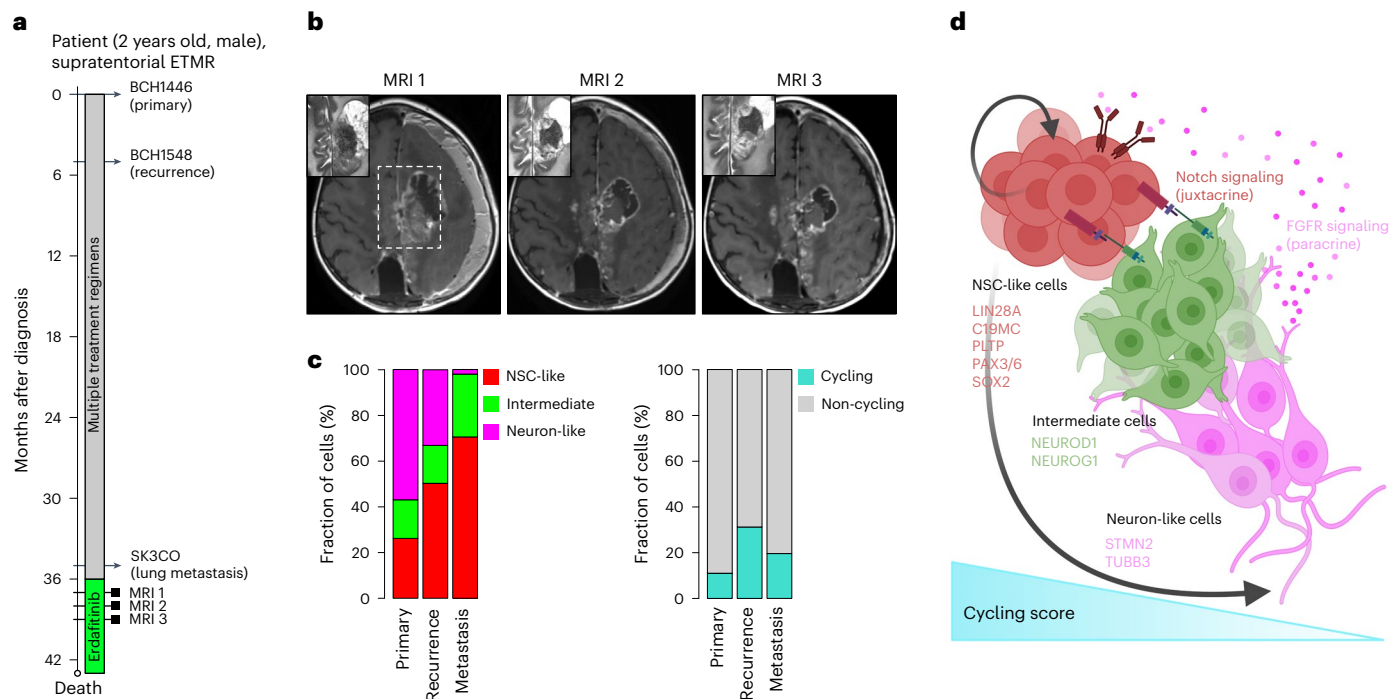


Fig. 8 | Partial response in one patient with ETMR upon erdafitinib treatment.

a, Schematic of the clinical history of $n = 1$ patient with ETMR. Therapeutic strategies, including surgeries (indicated by the arrows), chemotherapy and erdafitinib (the colored bars), and magnetic resonance tomographies (MRI) (indicated with squares) are depicted. Sample IDs are linked to the respective tumor lesions. **b**, Postcontrast axial T1 images of the brain with inset axial T2 images from the left paracentral lobule tumor site (dashed box) taken at monthly intervals: after 1.5 months of erdafitinib (left); after 2.5 months of erdafitinib (center); and after 3.5 months of erdafitinib and 1 month of talzoparib treatment (right).

Note the resolution of measurable tumoral enhancement and marked reduction of T2 hypointense tumor by 2.5 months of erdafitinib (center). **c**, Bar plots indicating the proportions of malignant cell types (left) and cell cycle state (right) in $n = 3$ samples collected over the course of disease, analyzed using scRNA-seq. **d**, Graphical summary of cellular hierarchies identified in ETMR, and the proposed signaling interactions between different malignant cell types and potential targets for pharmacological intervention. Schematic in **d** created with BioRender.com.

To assess the potency and selectivity of these inhibitors across other tumor types, we tested four U.S. Food and Drug Administration-approved FGFR inhibitors (lenvatinib, erdafitinib, infigratinib, nintedanib) and CB103 on 22 preclinical models spanning brain and non-brain cancers (Supplementary Table 5). Remarkably, BT183 cells demonstrated the highest sensitivity to all inhibitors, being approximately 300 times more sensitive to erdafitinib and more than 400 times more sensitive to CB103 compared to the average sensitivity across all tested models (Fig. 7f and Supplementary Table 5).

Finally, we sought to translate our findings into clinical application. A 5-year-old male with recurrent right parietal ETMR, distant disease in the left parafalcine brain region and extracranial lung metastases was treated with erdafitinib under compassionate use after multiple chemotherapeutic regimens over 3 years (Fig. 8a and Extended Data Fig. 10a). Two months after starting erdafitinib, combined with PD-1/CTLA4 and PARP inhibitors, magnetic resonance imaging (MRI) showed regression of the left parafalcine brain lesion, constituting a partial response (Fig. 8b). This lesion and other previously affected brain sites remained stable for 5 months, marking the longest interval without progression since the patient's first recurrence. However, as multiple targeted therapies were used concurrently, the response could not be attributed to erdafitinib alone. The patient eventually succumbed to treatment-resistant lung metastases.

To investigate disease progression despite multimodal therapy, we analyzed longitudinal tumor samples from this patient, including the primary tumor (BCH1446), recurrent brain tumor (BCH1548) and metastatic lung lesion (SK3CO) using scRNA-seq and snRNA-seq (Fig. 8a). All samples were collected before erdafitinib treatment. Over the disease course, we observed an increasing proportion of

NSC-like tumor cells and a decreasing proportion of neuron-like cells (Fig. 8c). NSC-like cells in progressive disease showed higher expression of C19MC and FGFR1, while neuron-like cells expressed higher levels of *FGF5* and *FGF9* (Extended Data Fig. 10b–d). A comparison of NSC-like cells from primary and metastatic samples revealed overexpression of genes related to protein synthesis (ribosomal proteins) and RNA processing (*CNOT3*, *PABPC1*, *SRSF1*) in metastatic samples (Extended Data Fig. 10e,f). These findings suggest that chemotherapeutic regimens failed to target tumor-driving NSC-like cells effectively. The high proportion of FGFR⁺ cells at the start of erdafitinib therapy may have contributed to its ability to stall tumor growth.

In summary, we show preclinical and preliminary clinical evidence for the efficacy of FGFR inhibition in ETMR that targets the cellular cooperation between malignant cell types toward therapeutic benefit.

Discussion

Curative options for patients with ETMR remain limited because of incomplete understanding of its molecular mechanisms, resulting in poor outcomes. To address this, we analyzed 11 patient samples along with in vitro and in vivo models. Single-cell transcriptome profiling and spatial imaging revealed cellular hierarchies in ETMR resembling the developing brain, with undifferentiated malignant cells mirroring fetal ventricular zone apical progenitors. This explains ETMR's exclusive manifestation in young children and lack of differentiation into glia-like cells, unlike other pediatric brain tumors⁶⁰. Our findings on the cellular heterogeneity of ETMR build on prior reports describing the histological phenotype of both undifferentiated and neuron-like structures within tumors^{4,22}. Jessa and colleagues previously studied the cellular identity of ETMR cell populations using scRNA-seq for

selected cases⁷. Our analyses on a larger number of samples expand on these findings to link single-cell-resolved transcriptional states with histological phenotypes and to decipher their underlying regulatory networks.

A hallmark of ETMR is the overexpression of the miRNA cluster C19MC, whose individual targets we identified using genome-wide eCLIP profiling. Surprisingly, prominent targets like ZBTB18, SOX4 and SOX11 were mostly expressed in C19MC-low expressing, differentiated cell populations, suggesting that C19MC might delay differentiation in NSC-like cells. We hypothesize that high C19MC expression in NSC-like cells delays the onset of expression of these transcriptional regulators, thereby imposing a differentiation block. In cells that overcome this differentiation block, target genes are no longer repressed by C19MC and therefore are highly detected. Based on our eCLIP results, we designed specific antisense LNAs, which exerted potent antitumor effects on ETMR cells *in vitro*. Because of its exclusive expression in ETMR, targeting C19MC promises little off-target effects. Therefore, inhibition of C19MC members presents a promising therapeutic option for patients with ETMR. Currently, LNA-based inhibitors such as LNA-i-miR221 are being evaluated in clinical trials for other types of cancer (NCT04811898).

Cell–cell interactions between tumors and their microenvironment, and their role in driving tumor progression and metastasis, are well documented in many cancers⁶¹. However, the principles guiding oncogenic interactions among malignant cells in a tumor remain largely unexplored. We discovered that FGFR and Notch receptors are expressed by proliferative NSC-like cells, while more differentiated malignant cells in the same tumor supply the corresponding ligands, indicating a coordinated role in sustaining tumor growth (Fig. 8d). Targeting these interactions may represent a critical vulnerability in ETMR. Testing over 50 FGFR and Notch inhibitors revealed several drugs with clinically meaningful inhibitory concentrations in the low nanomolar range, showing the highest efficacy in ETMR cells compared to other brain tumors or non-brain cancers. While CB103, the most potent Notch inhibitor identified, is still in clinical development, multiple U.S. Food and Drug Administration-approved FGFR inhibitors for other cancers could be repurposed for off-label use as second-line therapy in pediatric patients with brain cancer^{62–64}.

In conclusion, we showed that ETMR has a distinct cellular architecture, reminiscent of developmental processes occurring during early fetal neurogenesis. Our study further demonstrates how specific C19MC members, and the developmental signaling pathways such as Notch and FGFR, are the drivers of cellular self-renewal and impaired differentiation. We anticipate that future studies of these phenomena will expose other vulnerabilities and hold promise for the development of key therapeutic strategies for this highly malignant type of childhood brain tumor.

Methods

Human participants and ethical considerations

Our research complies with all relevant ethical regulations. ETMR samples were collected from patients treated at the Boston Children's Hospital (DFCI IRB 10-417), the Medical University of Vienna (MUV IRB 1244/2016) or via waiver of consent as appropriate. Tumors were diagnosed as ETMR based on histology, positivity for LIN28A expression using IHC, C19MC amplification or DNA methylation-based tumor classification using the app.epignostix.com website²⁰. All samples used in the study were de-identified. Clinical and molecular features, including age, sex, tumor location and C19MC amplification status are presented in Fig. 1a and Supplementary Table 1a. Patients or their legal representatives, who did not receive compensation, provided written informed consent to the use of tumor tissue for research purposes and publication of de-identified data preoperatively according to the respective institutional review board guidelines. All procedures on human participants were in accordance with the 2024 version of

the Declaration of Helsinki. All animal experiments were performed according to German Laws for Animal Protection and approved by the regional authorities (approval no. G91/20).

Sample collection, cell dissociation and sorting

Tumor tissues were collected at the time of surgery and processed directly for scRNA-seq ($n = 6$) or snap-frozen and processed later for snRNA-seq ($n = 5$). Fresh tissues were mechanically dissociated and additional enzymatic digestion was performed using the Brain Tumor Dissociation Kit (Miltenyi Biotec). Single-cell suspensions were filtered through a 70- μ m strainer and subsequently centrifuged (500g) for 5 min. For nucleus extraction from snap-frozen tissues or optimal cutting temperature-embedded tumor samples, the tissue was disaggregated using mild chopping in 1 ml 0.49% CHAPS extraction buffer on ice⁶⁵. Single-nucleus suspensions were filtered through a 40- μ m strainer and subsequently centrifuged (500g) for 5 min at 4 °C. Pellets were resuspended in PBS-bovine serum albumin (PBS-BSA 1%) sorted into 96-well plates as stated in the Reporting Summary and as described in ref. 10. A figure exemplifying the gating strategy is shown in Extended Data Fig. 1a.

Single-cell data generation

The whole transcriptomes of single cells were amplified, libraries were generated and sequencing was performed according to a modified Smart-seq2 protocol^{9,66}. RNA purification was performed using Agencourt RNAClean XP beads (Beckman Coulter). Reverse transcription was carried out using oligo(dT) primers and Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific) in the presence of a template-switching oligonucleotide (QIAGEN). Complementary DNA was amplified with the KAPA HiFi HotStart ReadyMix (Roche) and purified using Agencourt AMPure XP Beads (Beckman Coulter). The Nextera XT Library Prep Kit (Illumina) was used for library generation. Multiplexed libraries (up to eight 96-well plates) were paired-end sequenced (2 × 38 bp) on a NextSeq 500/550 instrument using the High Output Kit v2.5 (Illumina).

Single-cell processing

Raw sequencing reads were aligned to the RefSeq human transcriptome database using Bowtie v.0.12.7 (ref. 67). Gene expression was quantified using RSEM v.1.2.19 as transcripts per million (TPM)⁶⁸. Downstream processing was performed using R v.4.2.0 as described previously unless otherwise stated⁹. Briefly, expression levels were calculated using log-transformation of the TPM ($TPM_{i,j}$) for each gene (i) and cell (j): $E_{i,j} = \log_2(TPM_{i,j}/10 + 1)$. To filter out low-quality cells in fresh samples, cells with fewer than 2,000 detected genes or more than 15% mitochondrial reads were removed. For the frozen samples, a filtering threshold of fewer than 1,500 genes was used. In total, 3,406 high-quality cells (2,520 derived from fresh and 886 from frozen tissue samples) were obtained (Extended Data Fig. 1c). For these cells, we computed the average expression of each gene as $Ea_i = \log_2(\text{average}(TPM_{i,1...n}) + 1)$. For the downstream analysis, we only considered expressed genes with an average expression greater than four, obtaining 7,795 and 10,809 genes for fresh and frozen tissues, respectively. We defined the relative expression by centering the expression levels using $Er_{i,j} = E_{i,j} - \text{average}[E_{i,1...n}]$ for cells and genes.

CNVs were inferred from expressed genes as described previously⁶⁹ and used for separating malignant cells (cells containing CNVs) from nonmalignant cells (cells without detectable CNVs) (Extended Data Fig. 1d). For normalization, we used a set of 284 nonmalignant cells (oligodendrocytes, microglia, T cells)^{8,70}.

To quantify the primary C19MC transcript in single cells (Fig. 3b and Extended Data Fig. 5a,b), we aligned sequencing reads to the hg38 reference genome using STAR v.2.7.3a, supplying RefSeq transcriptome annotations⁷¹. Uniquely aligned reads in the genomic region encompassing C19MC were counted and normalized to the total number of

reads aligning to all of chromosome 19. STAR alignments were also used to generate loom files for velocity analysis.

Data harmonization and unsupervised clustering

The $\log_2$ -transformed expression matrices containing 7,795 genes and 2,520 cells for the fresh-sorted dataset, or 10,809 genes and 886 cells for the frozen-sorted dataset, were used as input for the unsupervised *t*-SNE analysis. Genes associated with cell cycle activation⁷² were excluded from the analysis, resulting in 7,414 and 10,717 genes for the fresh-sorted and frozen-sorted datasets, respectively. Data were harmonized by applying the HarmonyMatrix function of the Harmony package v.0.1.0 (ref. 73). The resulting matrix was fed into the Rtsne function using standard parameters, except for *pca* = FALSE, and resulted in two malignant clusters. Marker gene signatures (top 100 enriched genes) were defined by calculating the mean expression difference of cells assigned to malignant cluster 1 and malignant cluster 2 (that is, stem-like and differentiated signatures). Gene signatures were used to score cells in a similar way as described previously⁹. Using the gene signatures (G_j), we calculated a score for each cell (i), $SC_j(i)$, quantifying the relative expression (Er) of the genes in G_j , compared to the average relative expression of a control gene set G_j^{cont} : $SC_j(i) = \text{average}[Er(G, i)] - \text{average}[Er(G, i)]_{jjj}^{cont}$. The control gene set contained 100 genes with the most similar aggregate expression.

Integration of bulk ETMR datasets

mRNA expression data from $n = 26$ ETMR samples were assessed using the Affymetrix GeneChip Human Genome U133 Plus2.0 arrays²². Samples were scored by applying the gene signatures ($n = 100$) for malignant cluster 1 or 2. The RNA-seq data on microdissected neuropil and rosette-like regions from ETMR tissue were analyzed²². Specifically, the differential gene expression of rosette-like minus neuropil areas was calculated. Enrichment of malignant cluster 1 (stem-like) or 2 (differentiated) signature genes (top 100 genes per cluster; Supplementary Table 2a) was assessed using GSEA, using the GSEA function of clusterProfiler v.4.4.4 (ref. 74). The GSEA results were visualized using the gseaplot2 function of the enrichplot package v.1.16.1. The GSEA of FGFR-inhibitor-treated versus dimethyl sulfoxide (DMSO)-treated control BT183 cells were performed using Kyoto Encyclopedia of Genes and Genomes v.7.5.1 Pathways annotations.

Comparison to human fetal brain development

Single-cell mRNA expression was assessed using the Smart-seq2 technology from $n = 220$ cells from the developing fetal neocortex²³. Brain regions were separated using laser microdissection, separating the tissue into ventricular zone, outer subventricular zone, inner subventricular zone or cortical plate. Cell annotations into apical and basal progenitors or neurons, as well as signature genes for the respective cell populations, were retrieved from the original publication. Raw expression values were processed similarly to the ETMR scRNA-seq data, using the pipeline described above. We filtered for genes that were detected in both datasets (5,482 genes with an average $\log_2$ -expression value > 0.5). To classify ETMR cells according to the described cell populations, we trained a random forest classifier using the randomForest package v.4.7-1.1. We first trained an outer classifier for feature selection using the following non-default parameters: *samplesize* = *rep*(19,3) and *ntree* = 1,000. For the model with seven cell-types, we used *samplesize* = *rep*(8,7). We then selected the 100 probes with the highest MeanDecreaseAccuracy value to train a final random forest classifier using the same parameters. The ETMR cell classifications are shown in Fig. 2a and Extended Data Fig. 3b.

SCENIC analysis and identification of malignant cell types

SCENIC analysis was performed on high-quality malignant ETMR cells using the SCENIC v.1.3.1 (ref. 25) across all fresh-sorted ETMR samples. The expression matrix was filtered for genes not expressed in any cell.

The motif database searching for motifs in the hg19 genome 10 kb around the transcription start site (seven species) was used. The gene coexpression network was built using GENIE3 v.1.18.0. TF motifs were recognized using RcisTarget v.1.16.0 and annotated to the matching TF. AUC values (indicating regulon activity) were assessed using AUCCell v.1.18.1. The relative AUC (AUCr) scores of every regulon (i) per sample (j) were centered and normalized using $AUCr_{ij} = \text{AUC}_{ij} - \text{average}(\text{AUC}_{i,1..n}) / \text{s.d.}(\text{AUC}_{i,1..n})$. Active regulons detected between 10% and 90% of cells were selected ($n = 164$). Hierarchical clustering of the AUC scores was performed to identify common groups of regulons across cell populations. Regulon groups were selected by cutting the dendrogram using 0.3 as the cutoff, resulting in four regulon groups (containing 38 regulons) that were shared and five groups (containing 18 regulons) that were not shared among samples. For the network graph in Fig. 2c, the Jaccard index of target genes per regulon was assessed and plotted using Cytoscape v.3.9.1. Refined gene signatures were defined by correlating (Pearson correlation) normalized gene expression values to the average regulon scores across single cells. Genes were ranked according to the highest difference to other cell programs, excluding the cycling program. The top 100 genes according to regulon group were defined as refined gene signatures (Supplementary Table 2b). Malignant cells were classified into refined cell types (Fig. 2e) by applying the scoring strategy described above. Cellular hierarchies were assessed using the velocity v.0.6 package²⁸ comparing the abundances of spliced and unspliced mRNAs. Combined loom files were generated for every plate from genome-wide STAR alignments.

Receptor–ligand interactions

Receptor–ligand interactions of high-quality malignant ETMR cells were inferred using CellPhoneDB v.4.0.0 in Python v.3.7 (ref. 55) in every individual fresh-sorted ETMR sample and merged afterwards. Interactions were filtered using $P < 0.05$ to select for statistically significant interactions. Genes were color-coded according to their cell-type-specific expression. Interaction networks were exported and plotted in Cytoscape v.3.9.1. The number of interactions (number of tumors exerting interactions) is indicated by line width in the network graphs.

Multiplexed IF imaging

Formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed using a pressure cooker and 1× citrate buffer, pH 6.0. Subsequently, sections were stained using the Opal Polaris 7 Color Manual IHC Detection Kit (Akoya Biosciences). All antibodies used are stated in the Reporting Summary.

Fluorescence microscopy slide scans were taken with PhenolImager Fusion (Akoya Biosciences). Image analysis was performed using inForm (Akoya Biosciences) and QuPath for image and spatial analysis. Briefly, regions of interest polygons were drawn onto the scans of several tumors. Tissue folds and stain artifacts were manually excluded. Cells were segmented on the DAPI signal using the cell detection tool and phenotyped by creating single measurement classifiers, which were manually set and optimized for each staining. Spatial analysis was performed using the detect centroid distance 2D tool. Data were exported to Prism 8 (Graph Pad Software) for plotting.

Conventional IF imaging

For the analysis of the FGF receptors shown in Fig. 7a, BT183 cells were subcultured onto chambered coverslips (cat. no. 80826, ibidi) and incubated for 48 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 (cat. no. 3051.3, Carl ROTH) and blocked with 5% BSA (cat. no. 8076.2, Carl ROTH) for 1 h. All antibodies used are listed in the Reporting Summary. Cells were then counterstained with a 1:1,000 dilution of Phalloidin CruzFluor 647 Conjugate (cat. no. sc-363797, Santa Cruz Biotechnology) and mounted with ProLong Diamond Antifade Mountant with DAPI (cat. no. P36966,

Thermo Fisher Scientific). Imaging was performed using a BZ-X800E fluorescence microscope (Keyence Corporation). After image acquisition, digital images were analyzed using the Keyence software. Fluorescence signals from FGFR1, FGFR2, FGFR3 and FGFR4 were normalized to the cell area detected by phalloidin staining. The numbers of analyzed cells were determined using DAPI nuclear staining. Over 12,000 cells from three independent experiments were analyzed.

miRNAscope HD (RED) assay (RNA ISH)

ETMR FFPE tissue sections (4 μm) from tumors were obtained from the Medical University of Vienna (Departments of Pediatrics and Adolescent Medicine, and Neurosurgery), and from Boston Children's Hospital (Department of Pathology). SuperFrost Plus glass slides (cat. no. 12-550-15, Thermo Fisher Scientific) were used and stored at -80°C . The miRNAscope HD (RED) kit was used for sample staining (cat. no. 324510, Advanced Cell Diagnostics) according to the manufacturer's recommendations. Slides were baked for 1 h at 60°C followed by deparaffinization using xylene and ethanol. Post-fixation of dehydrated tissue was done for 2 h using 12% formaldehyde. Subsequently, RNAscope hydrogen peroxide was applied for tissue pretreatment at room temperature for 10 min. RNAscope 1X Target Retrieval Reagent was used for target retrieval in a steamer (FS20, Braun) at 99°C for 15 min, followed by RNAscope Protease III treatment for 30 min at 40°C . Probes targeting miR-512 (SR-hsa-miR-512-3p-S1; 897511-S1) and miR-124 (SR-hsa-miR-124-3p-S1; 728471-S1) were included for C19MC and neuronal cell populations, respectively, and were hybridized at 40°C for 2 h. Amplification rounds were performed according to the miRNAscope HD (RED) Assay protocol. Gill's Hematoxylin I (cat. no. HXGHEILT, StatLab) was used for tissue counterstaining at room temperature for 2 min followed by bluing solution (0.02% ammonia water). Slides were air-dried at 60°C for 30 min. Thereafter, slides were covered with coverslips using VectaMount Mounting Medium (H-5000, Vector Laboratories). Stained tissue sections were imaged using a DMi8 bright-field microscope and LAS-X software (Leica Microsystems) and processed using the ImageJ software (research resource ID: SCR_003070, 2.0.0-rc-69/1.52p).

DNA FISH

Fresh ETMR FFPE tissue sections (4 μm) from tumors were used for staining with the C19MC/TPM4 FISH Probe Kit (cat. no. CT-PAC033-10-OG, CytoTest) according to the manufacturer's guidelines. The signal for the C19MC probe, located on chromosome 19q, was visualized in red. The signal for the TPM4 probe, located on chromosome 19p and serving as an internal control, was shown in green. Slides were scanned using PhenolImager Fusion (Akoya Biosciences) with a $\times 40$ objective. Image analysis was performed by using QuPath for image analysis. Briefly, regions of interest were drawn onto the scans of two tumor sections. Cells were segmented on the DAPI signal using the cell detection tool. FISH signals were detected using the subcellular detection tool, resulting in a subcellular detection count (that is, the number of FISH foci per nucleus). Cells with three or more subcellular detections of C19MC foci were considered as high-confidence tumor cells carrying the C19MC amplification. We expect that most cells with two or less foci also represent malignant cells in which nuclei are only partially captured in the tissue sections or in which C19MC foci overlap. The cell density for each segmented nucleus was calculated as the number of neighboring nuclei within a radius of 50 μm .

Animal experiments

BT183 (100,000)⁶ or NCH3602 (1,000,000)⁷⁵ ETMR cells were injected into the cerebral cortex of 6–8-week-old female NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ). Tumor tissues were collected fresh and processed directly or frozen and processed at a later time. Animal experiments were performed according to German Laws for Animal Protection and approved by the regional authorities

(approval no. G91/20). Mice were checked daily and weighed three times per week. Specific neurological endpoint criteria were defined to ensure animal welfare. The study design included daily monitoring of the animals by trained personnel, with particular attention to neurological symptoms that could indicate tumor progression. Mice were euthanized when signs of physical (for example, 20% body weight loss) or neurological sickness appeared. No animals or data points were excluded from the analyses for any reason. In our study, all animal experiments were conducted in accordance with institutional and governmental ethical guidelines and were approved by the responsible ethics committee. The maximum tumor burden permitted by our institutional animal protocols (1,500 mm^3) was strictly adhered to and was not exceeded.

Cell culture

Cell sources are stated in the Reporting Summary and Supplementary Table 5a. BT183 cells were cultured as neurospheres in ultralow attachment flasks (Thermo Fisher Scientific) using serum-free NeuroCult NS-A Basal Medium (STEMCELL Technologies) supplemented with NeuroCult Cell Proliferation Supplement, $2\text{ }\mu\text{g ml}^{-1}$ heparin, 20 ng ml^{-1} EGF, 20 ng ml^{-1} basic FGF (all from PeproTech) and $1\times$ antibiotic-antimycotic solution (Thermo Fisher Scientific). Cells were maintained at 37°C with 5% CO_2 and fed every 3–4 days. When 90% of neurospheres exceeded 200 μm in diameter, cells were split using ACCUTASE (STEMCELL Technologies).

A431, PC9, U87, DAOY, SF8628, SUPB15, JURKAT, IMR32, MBT032, MBT026, MBT037 and REH cells were cultured adherently in cell-culture-treated flasks (Sarstedt) using Roswell Park Memorial Institute 1640 medium with glutamine (Thermo Fisher Scientific) supplemented with 10% FCS, $1\times$ nonessential amino acids, $1\times$ sodium pyruvate, 100 U ml^{-1} penicillin and $100\text{ }\mu\text{g ml}^{-1}$ streptomycin (all from Thermo Fisher Scientific). These cells were maintained at 37°C with 5% CO_2 and fed every 3–4 days. Cells were split with Trypsin-EDTA (Thermo Fisher Scientific) once 80–90% confluency was reached.

SUIV, MBT021, BT690, DIPGXIIIIP*, MBT011, DIPG007, MBT028, MBT014 and MBT030 cells were cultured as neurospheres in ultralow attachment flasks (Thermo Fisher Scientific) using a serum-free medium comprising Neurobasal-A medium and DMEM/F-12 (Thermo Fisher Scientific) supplemented with 1 M HEPES solution, $1\times$ nonessential amino acids, $1\times$ sodium pyruvate, $1\times$ GlutaMAX-1 supplement, $1\times$ antibiotic-antimycotic solution, $1\times$ B-27 supplement minus vitamin A (all from Thermo Fisher Scientific), $2\text{ }\mu\text{g ml}^{-1}$ heparin, 20 ng ml^{-1} EGF, 20 ng ml^{-1} basic FGF, 20 ng ml^{-1} platelet-derived growth factor-AA and 20 ng ml^{-1} platelet-derived growth factor-BB (all from PeproTech). Cells were fed every 3–4 days and split using ACCUTASE when 90% of neurospheres exceeded 200 μm in diameter.

Small RNA-seq

Genome-wide quantification of miRNAs from the BT183 cell line was performed according to the vendor's instructions (Arraystar). The sequencing library was sequenced on an Illumina NextSeq 500 instrument (50 bp, single-end, approximately 7.5 M reads) and analyzed using miRBase v.22 annotations.

miRNA crosslinking and IP

Preparation for the AGO2 eCLIP analysis was performed according to the vendor's instructions (Eclipsebio). Briefly, BT183 ETMR cells were expanded in ultralow attachment flasks. Cell viability was measured using Trypan blue exclusion (cat. no. 15250-061, Thermo Fisher Scientific). Only cells with at least 95% viability were pooled and centrifuged at 200g for 5 min. The medium was removed and cells were resuspended in 25 ml of Dulbecco's PBS medium. For ultraviolet (UV) crosslinking, 60 million cells were aliquoted into a 10-cm tissue-culture-grade plate that had been chilled in a cooling box to 4°C and placed into the UV crosslinker (254 nm UV with $400\text{ mJoules cm}^{-2}$).

After crosslinking, cells were transferred to a 50-ml conical tube and counted using an automated cell counter. Cells were centrifuged, the supernatant was removed and cells were flash-frozen in liquid nitrogen at a concentration of 20×10^6 cells ml^{-1} in a 1.5-ml Eppendorf Safe-Lock Tube. After flash-freezing, pellets were stored at -80°C before further processing using the no-gel chimeric eCLIP protocol³⁶. Through optimized ligation conditions, eCLIP produces chimeric reads that contain the mature miRNA (5'-end) and targeted mRNA (3'-end), which allows for mapping of genome-wide miRNA-mRNA interactions. Two replicates were produced (independent cell expansion and crosslinking).

The resulting libraries (input and IP) were sequenced on an Illumina instrument (150 bp, paired-end). Sequencing reads were first processed using fastp v.0.22.0⁷⁶ to cut adapter sequences and merge overlapping paired-end reads. Only merged reads were used for the downstream analysis. We next annotated mature miRNAs (miRBase v.22.1, trimmed to 20 bases) to the 5'-end of sequencing reads (starting at position 11–15, allowing for up to one mismatch). Reads that contained an annotated miRNA (chimeric reads, miRNA-only reads) and reads that did not contain an annotated miRNA (mRNA-only reads; Extended Data Fig. 6b) were aligned to the human genome (hg19) with RefSeq gene annotations using STAR v.2.7.10a⁷¹ using the `--clipSpNbases 36` parameter (14 bases for mRNA-only reads). Only reads associated with a mapping quality of 255 and aligning to an annotated coding or noncoding mRNA transcript were retained. Furthermore, reads that were overlapping an annotated miRNA gene were removed. Potential PCR duplicates were removed by only considering one random read per unique molecular identifier sequence (first ten base pairs of read 1) and gene.

Alignments of chimeric reads were split by annotated miRNAs; genomic coverage tracks were generated using genomecov from bedtools v.2.29.0 (ref. 77) and bedGraphToBigWig v.2.8 from UCSC. Coverage tracks were visualized using the Integrative Genomics Viewer browser v.2.12.2 (ref. 78). Peaks were called using a sliding window approach that considers the average chimeric read coverage in 75-bp windows along transcripts for every annotated miRNA. Windows that had a higher average coverage than the neighboring windows on either side (shifted by ± 1 bp) and a minimum average coverage of five were defined as peaks ($n = 12,787$). Some genes contained multiple distinct binding sites for the same miRNA. Peaks detected in both replicates that were enriched at least twofold compared to the average coverage in the input libraries (normalized by the total number of aligned reads) were merged and defined as high-confidence peaks for downstream analysis ($n = 4,009$). A list of all high-confidence peaks and their annotations is provided in Supplementary Table 3b. The genome coordinates of predicted miRNA binding sites were downloaded from the TargetScan website (release 8.0, <https://www.targetscan.org/>).

For the reanalysis of the CRISPR-based C19MC activation experiment in HEK 293 cells¹⁷, we downloaded the raw sequencing data of all 12 samples from the Sequence Read Archive. We then quantified gene expression values similar to our single-cell datasets and filtered for genes expressed in the control experiment (11,983 genes with a $\log_2$ -expression value > 0.5). Genes with an average $\log_2$ fold change smaller than -0.25 across both guide RNAs were deemed downregulated ($n = 509$; Extended Data Fig. 7a,b).

Antisense miRNA inhibition

LNA antisense oligonucleotides were designed with a fully phosphorothioate-modified backbone to prevent enzymatic degradation. All LNAs were synthesized and purchased from Integrated DNA Technologies. The exact LNA sequences are listed in Supplementary Table 4a. LNAs were reconstituted in sterile H_2O to a stock concentration of 100 μM . BT183 cells were plated onto cell-culture-treated 24-well plates (Corning) and immediately treated with either a targeting LNA, a nontargeting control or solvent only. The final LNA concentration

on the cells was 4 μM . Cell growth was assessed using an IncuCyte S3 live-cell imaging system (Sartorius) and confluence was determined using the IncuCyte software from ten bright-field images taken every 12 h. Induction of apoptosis was achieved by adding caspase-3/7 Red Apoptosis Reagent (Sartorius) at a final concentration of 2.5 μM to LNA-treated ETMR cells. Bright-field and fluorescent images were taken by the IncuCyte S3 live-cell imaging system at 72 h after LNA treatment. Fluorescence image data were analyzed using the IncuCyte software. To assess protein changes upon LNA treatment, BT183 cells were embedded in Matrigel droplets at 1,000 cells per μl . Cell-containing droplets were cultured for 48 h on an orbital shaker before treatment was initiated. Cells were treated with LNAs for 72 h with complete medium change and retreatment every 24 h. All droplets were fixed with 4% paraformaldehyde, dehydrated and embedded in paraffin. Protein levels were assessed via multiplexed IF imaging as described above.

FGF and Notch ligand assays

FGFs and soluble Notch ligands were purchased from several vendors (Supplementary Table 4b). BT183 cells were plated onto cell-culture-treated 24-well plates in basal medium containing NeuroCult NS-A Basal Medium supplemented with NeuroCult Cell Proliferation Supplement, 2 $\mu\text{g ml}^{-1}$ heparin (STEMCELL Technologies) and 1 $\times$ antibiotic-antimycotic solution. Growth factors were added immediately after cell seeding at a final concentration of 20 ng ml^{-1} . No growth factors were added to the control. Cell growth was assessed using an IncuCyte S3 live-cell imaging system and confluence was determined using the IncuCyte software from ten bright-field images taken every 12 h.

In vitro drug screening

Cells from 22 lines were dissociated using ACCUTASE or Trypsin (Thermo Fisher Scientific) and subsequently plated on 384-well plates (Thermo Fisher Scientific) at 1,000 cells per well. Drugs were pre-diluted on several 96-well V-bottom plates (Sarstedt) and added to the cell-containing wells 24 h after seeding. All inhibitors were commercially purchased and are listed in Supplementary Table 4c,d. Cells were incubated with the drugs or solvent controls for 72 h. Cell viability was determined by adding CellTiter-Glo 2.0 (Promega Corporation) to each well and measuring the luminescence signals with a Varioskan LUX Plate Reader (Thermo Fisher Scientific). Luminescence signals were normalized to the solvent controls. Dose-response curves and IC_{50} values were calculated using Prism 8. Each condition was tested in two biological replicates. To quantify transcriptional signatures in CB103-treated cells, we performed regular Smart-seq2 profiling on 100 viable cells per replicate.

Erdafitinib therapy as compassionate use

Erdafitinib was commercially prescribed after medical insurance coverage approval was obtained. After an appropriate washout period from the last tumor-directed treatment, the patient began oral erdafitinib at 4 mg by mouth daily, in combination with concurrent radiation therapy to distant non-cranial sites of disease recurrence (pulmonary). Erdafitinib was continued through further progression 2 months later and then sequentially combined with commercially available agents, including a PARP inhibitor and checkpoint inhibitors. Combinations were well tolerated with no notable adverse events. With ongoing evidence of progressive growth, erdafitinib was continued until 7 months from the start of the agent, at which time substantial distant progressive disease prompted discontinuation of all tumor-directed therapy.

Statistics and reproducibility

Several available ETMR tissue samples from our research environment were analyzed in this study. No statistical method was used to pre-determine sample size. No data were excluded from the analyses. Each experiment was performed in at least three biological replicates unless

otherwise stated in the text. Statistical significance was calculated as specified in the figure legends. $P < 0.05$ was considered statistically significant. The RNA ISH and FISH experiments were performed in the same ETMR tissue samples that were part of our scRNA-seq cohort as indicated. The Shapiro–Wilk test confirmed the normal distribution of data for Fig. 1e, justifying the use of the Student's *t*-test. If not stated otherwise, data distribution was assumed to be normal, but this was not formally tested. The experiments were not randomized. The investigators were not blinded to allocation during the experiments and outcome assessment.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The expression matrices of high-quality cells and raw RSEM files (including malignant and nonmalignant cells) of all samples generated in this study have been deposited in the Gene Expression Omnibus under accession no. [GSE231614](#). Raw fastq files have been deposited in the European Genome-Phenome Archive under accession no. [EGAS50000000937](#). All datasets used in this study are summarized in Supplementary Table 1b. Source data are provided with this paper.

Code availability

No new algorithms were generated in this study. The R v.4.2.0 statistical environment was used and supplemented with packages as indicated.

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Author contributions

A. Beck, L.G.-P., M. Kool, V.H. and M.G.F. conceived the study, designed and performed the experiments, interpreted the results and wrote the manuscript with the input of all authors. L.G.-P. and V.H. performed the scRNA-seq, snRNA-seq and eCLIP data analysis, the bulk mRNA expression array and the DNA methylation array data analysis. G.A.V.C. and S.L. designed the experiments and interpreted the results. M.L.S., O.A.H., I.L., R.D.H., A. Baumgartner and A.D.N.S. performed the tumor tissue processing, generated the scRNA-seq and snRNA-seq data, and performed the eCLIP experiments. L.G.-P., V.H. and C.A.O.d.B. performed the trajectory analyses. B.E. performed the RNA ISH experiments. N.A. and K.B. performed the FISH experiments. A. Beck performed the multiplex IHC, spatial phenotyping and analysis, and the in vitro LNA, ligand and drug screening experiments. M. Klenner and P.S.F. performed the cell culture and drug screening of non-ETMR cells. L.v.B., J.C.T. and N.T. collected and processed the tumor tissue and established the cell models. S.M., L.M., D.S., D.L., R.G., C.D., A.P., C.H., I.S., L.C.B. and S.N.C. collected and processed the tumor tissue. B.S. and N.M. performed the PDX experiments. E.Y. reviewed the MRI images and assessed the radiographic response. J.G., S.A., J.H.,

S.M.P. and A.K. provided the primary tissue resources and pathology consultation. All authors read, reviewed and approved the final manuscript.

Competing interests

M.G.F. is a consultant for Twentyeight-Seven Therapeutics and Blueprint Medicines. J.C.T. serves as a consultant for Advanced Accelerator Applications-Novartis. The other authors declare no competing interests.

Additional information

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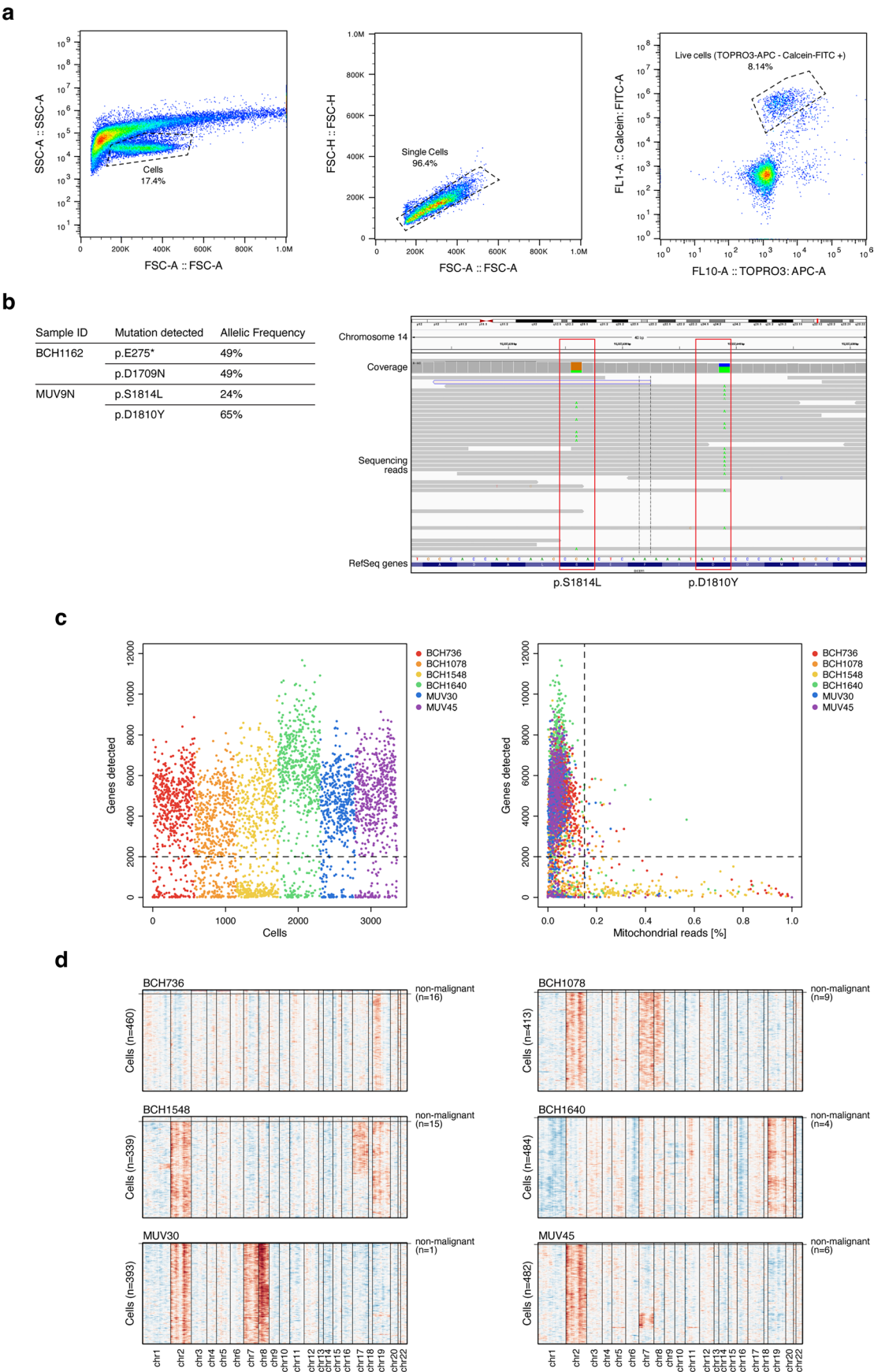
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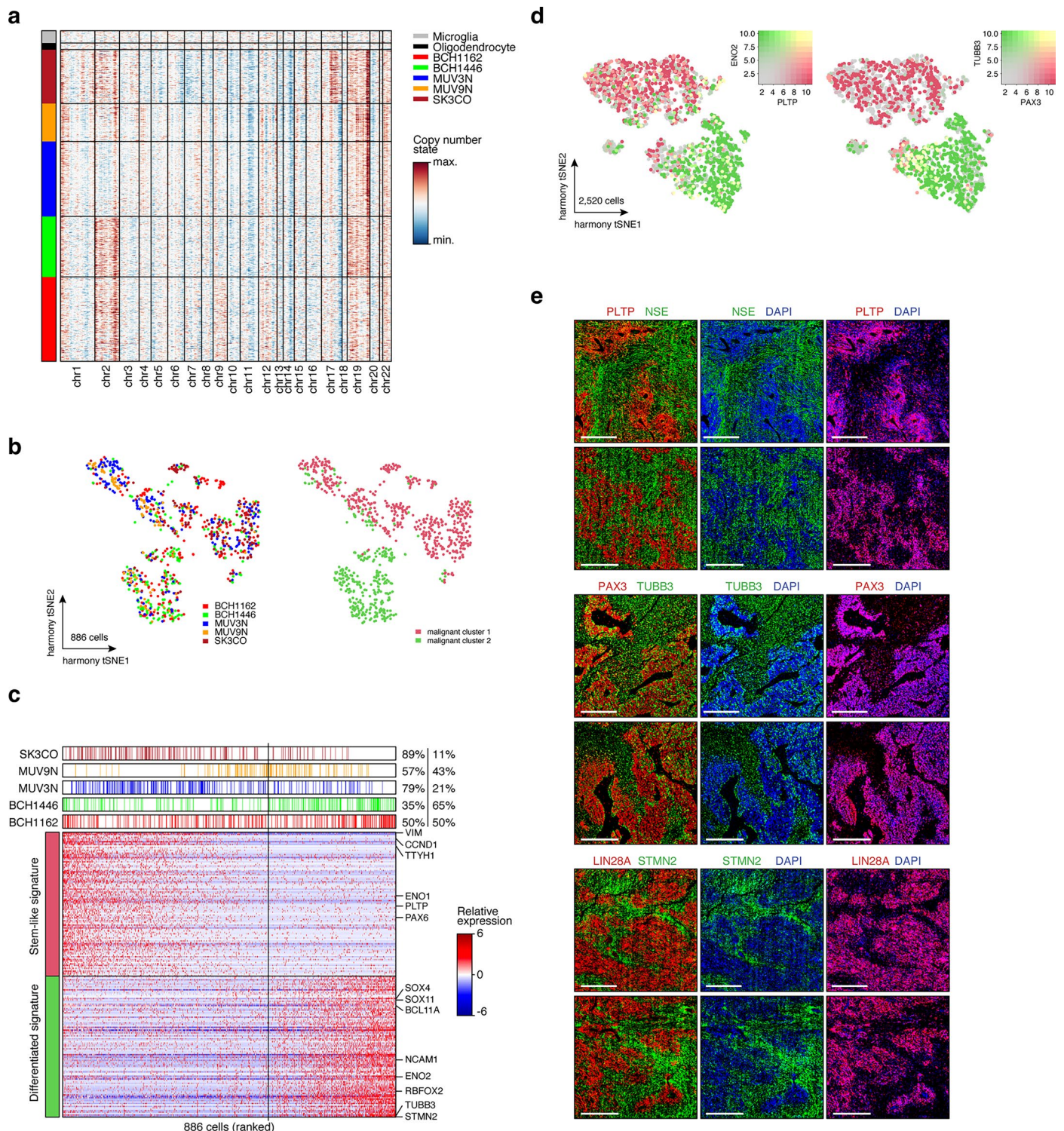
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Extended Data Fig. 1 | Sample genetics and single-cell quality control.

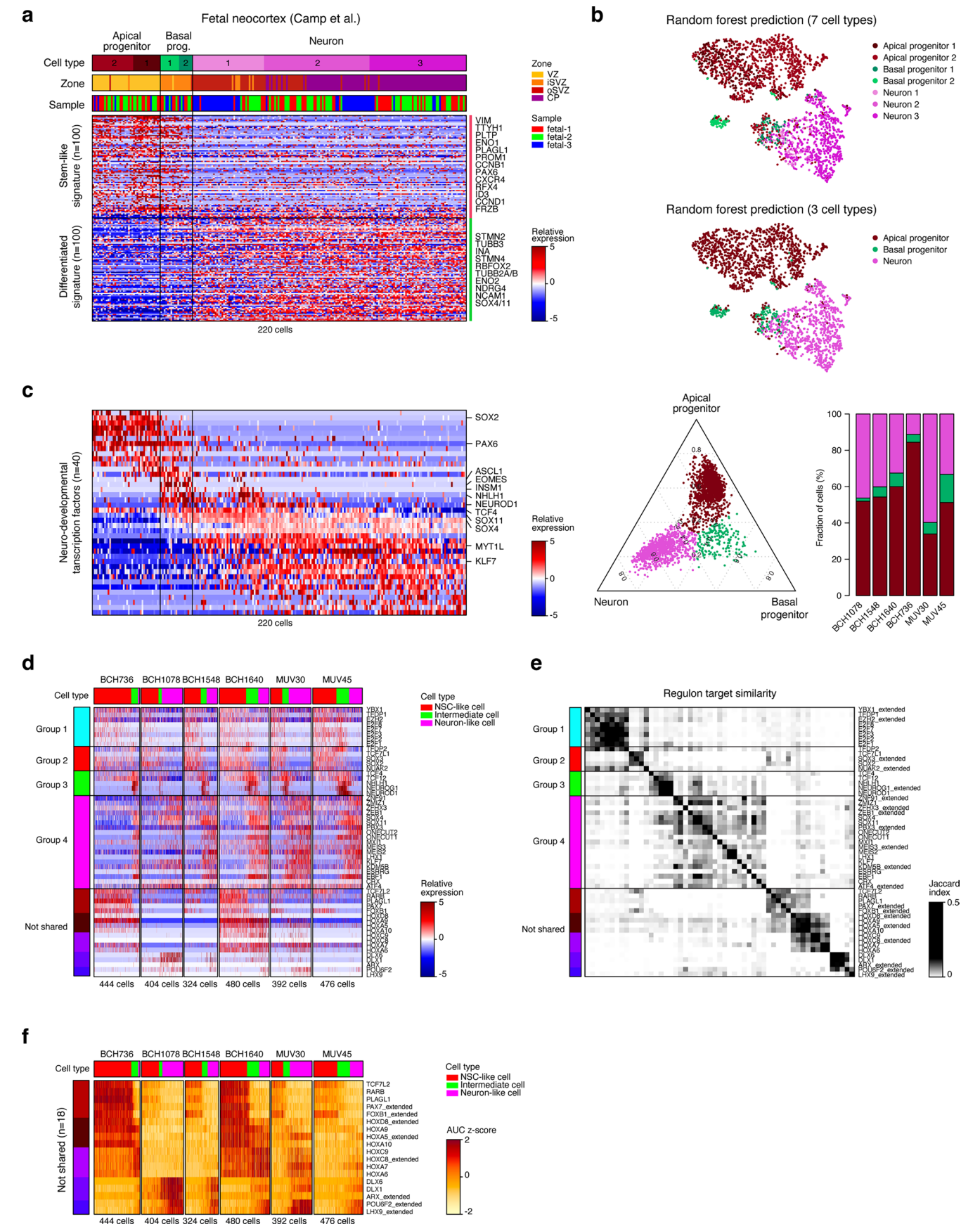
a) Flow cytometry plots exemplify the gating strategy for single cell sorting of fresh patient samples into 96-well plates. **b)** Table shows overview of mutations detected in DICER1 in the C19MC non-amplified samples BCH1162 and MUV9N by genome sequencing. IGV screenshot on the right shows that nearby mutations S1810Y and S1814L in sample MUV9N are not detected within the same sequencing read, indicating that two different alleles are affected. **c)** Dot plots show gene number per cell (left) and per percent mitochondrial reads (right).

Samples are indicated by color. Cells that expressed less than 2,000 genes or more than 15% mitochondrial RNA, as indicated by dashed lines, were excluded from further analysis. A total of $n = 2,571$ high-quality cells were retained. **d)** Heatmaps show scRNA-seq derived copy-number variations in every cell (y axis) along the genome (x axis). One heatmap per sample is shown. $n = 51$ cells without discernible copy-number variations were labeled as non-malignant cells and excluded from further analysis. Related to Fig. 1.



Extended Data Fig. 2 | Cellular heterogeneity in the extended snRNA-sequenced ETMR sample cohort. a) Heatmap shows copy number profiles derived from snRNAseq of cells from the frozen-sorted tumor samples ($n = 886$ cells) and non-malignant reference cells. **b)** tSNE representations of high-quality ETMR cells ($n = 886$ cells) from frozen-sorted tumor samples highlighting sample distribution (top) and malignant clusters (bottom). Gene signatures derived from fresh-sorted tumor samples were used to score and classify cells into malignant clusters 1 or 2. **c)** Heatmap shows the relative expression of 100 genes specific to malignant clusters 1 (stem-like signature) and 2 (differentiated signature) in frozen-sorted ETMR cells ($n = 886$ cells). Percentages of cells

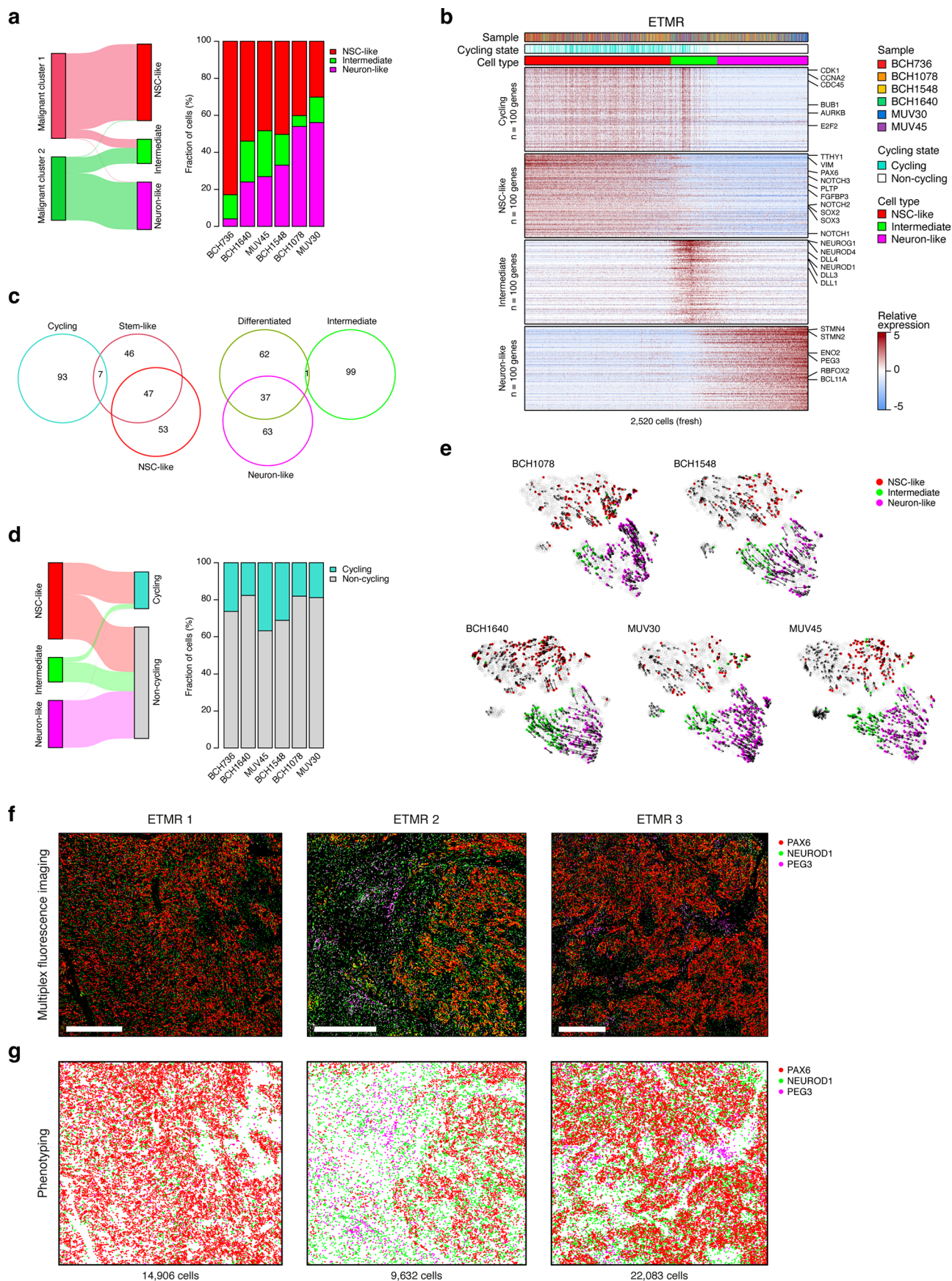
belonging to malignant clusters 1 and 2 are indicated above. Genes of interest are indicated on the right. **d)** tSNE representations of fresh-sorted ETMR cells ($n = 2,520$ cells), colored according to marker genes specific to malignant clusters 1 (stem-like signature: *PLTP*, *PAX3*) and 2 (differentiated signature: *ENO2*, *TUBB3*). **e)** Representative multiplex immunofluorescence images of ETMR tissue stained for markers for malignant clusters 1 (stem-like signature: *PLTP*, *PAX3*, *LIN28A* shown in red) and 2 (differentiated signature: *NSE*, *TUBB3*, *STMN2* shown in green). DAPI staining was used to highlight the nuclei. Scale bars = 300 μm . This experiment achieved reproducible results in three biological replicates. Related to Fig. 1.



Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Developmental correlates and transcriptional regulation of malignant cell types. **a)** Heatmap shows relative expression levels of marker genes for malignant clusters 1 (stem-like signature) or 2 (differentiated signature) in fetal neocortex cells (n = 220 cells) 24. Samples were laser-microdissected and different germinal zones are annotated (top). Cell annotations for apical progenitor cells (API/2), basal progenitor cells (BP1/2), or mature neurons (NI/3) are indicated above the heatmap. **b)** tSNE visualization, colored by predictions from the random forest-based classifier for 7 (top) or 3 (middle) fetal neocortex cell types. Bottom row shows a ternary plot of random forest prediction scores of ETMR cells (n = 2,520 cells). Cells are colored according to their maximum prediction score for apical progenitors (brown), basal progenitors (green) or neurons (pink). Bar plot shows the fraction of cells classified as apical progenitors (brown), basal progenitors (green) or

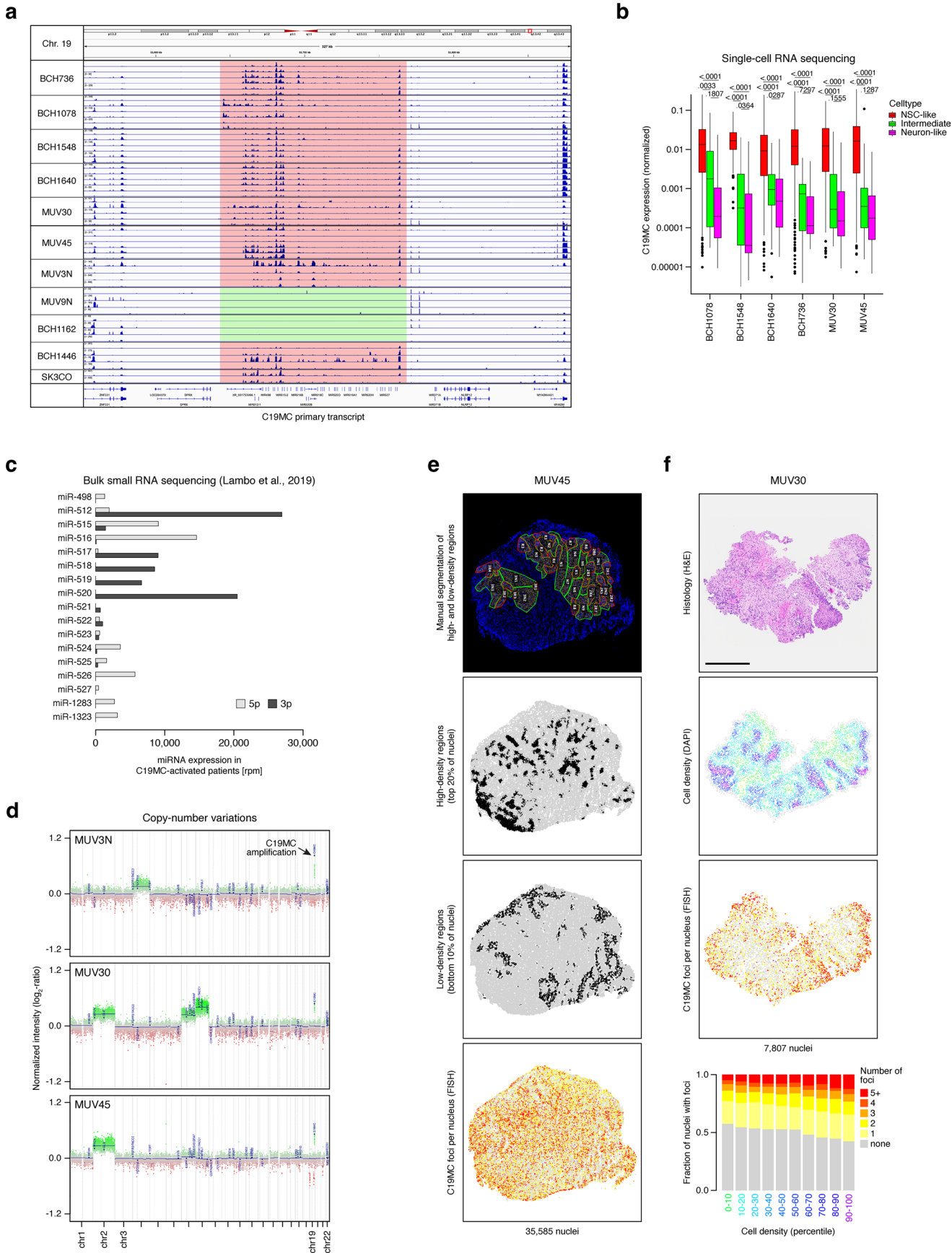
neurons (pink) per ETMR sample. **c)** Heatmap shows relative expression of neurodevelopmental TFs in fetal neocortex cells (n = 220 cells). Cell ordering is identical with panel **(a)**. **d)** Heatmap shows relative expression levels of TFs associated with regulons derived from SCENIC analyses of the fresh-sorted ETMR scRNA-seq dataset (n = 2,520 cells). Four groups were shared across samples and five groups were not shared across samples. Annotations above the heatmap indicate classification of cells into malignant cell types. **e)** Heatmap shows pairwise similarity (Jaccard index) between target genes of regulons detected by SCENIC analysis (n = 55 regulons). **f)** Heatmap shows normalized Area Under the Curve (AUC) values of n = 2,520 cells (indicating regulon activation) derived from SCENIC analyses for five regulon groups that were not shared across samples. Related to Fig. 2.



Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Gene signatures of malignant cell types. **a)** Sankey plot (left) shows the distribution and relationship of $n = 2,520$ cells assigned to malignant clusters 1 and 2 versus NSC-like, intermediate, and neuron-like clusters. NSC-like and neuron-like cells almost entirely derive from malignant clusters 1 and 2, respectively. The intermediate population derives from both malignant clusters. Bar plot (right) shows the fractions of malignant cell types per fresh-sorted ETMR sample. **b)** Heatmap shows relative expression of signature genes specific to cycling, NSC-like, intermediate, and neuron-like cells in the fresh-sorted ETMR scRNAseq dataset ($n = 2,520$ cells). Sample annotation, cycling cells, and cell type assignment are indicated on top of the heatmap. Genes of interest are highlighted on the right. **c)** Proportional venn diagrams highlight the overlap of genes associated with different single cell-derived signatures. **d)** Sankey plot (left) shows the distribution and relationship between $n = 2,520$ cells assigned to the NSC-like, intermediate, and neuron-like clusters versus the

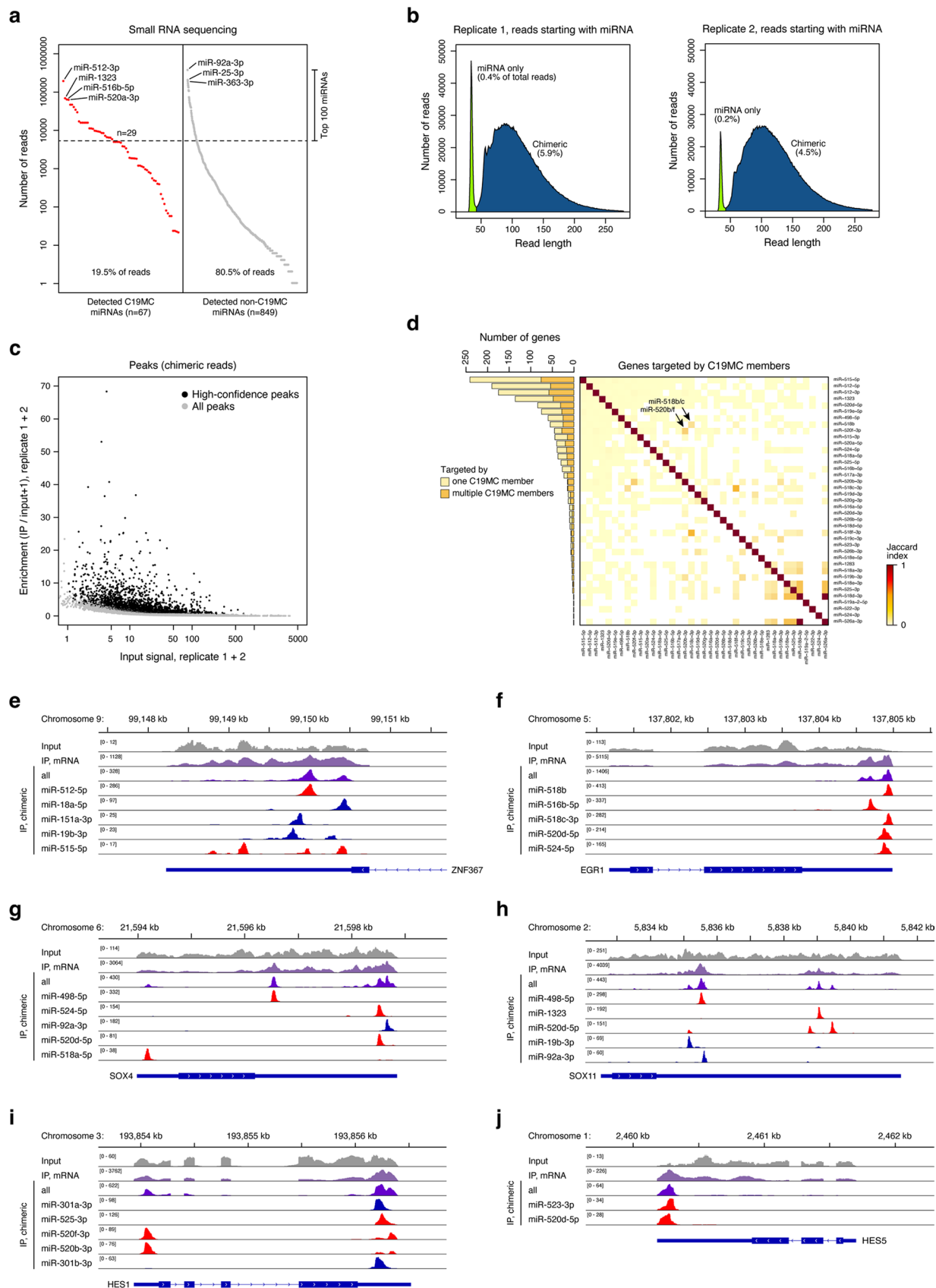
cell cycle state. Bar plot (right) shows the fractions of cycling and non-cycling cells per fresh-sorted ETMR sample. **e)** tSNE visualization of the fresh-sorted ETMR scRNAseq dataset ($n = 2,520$ cells), overlaid with differentiation trajectories defined by RNA velocity analysis. **f)** Images show representative multiplexed immunofluorescence stainings of three primary ETMR samples for PAX6 (NSC-like cells, red), NEUROD1 (Intermediate cells, green) and PEG3 (neuron-like cells, magenta) TFs. Scale bars = $500\ \mu\text{m}$. This experiment was performed at least three times for each tumor sample. **g)** Pseudo images of *in silico* phenotyped cells of the images shown in panel (f). Coordinates and phenotypes of cells were determined as described in the methods. Pseudo images are resolved by marker genes PAX6 (NSC-like cells, red), NEUROD1 (Intermediate cells, green) and PEG3 (neuron-like cells, magenta). This experiment was performed at least three times for each tumor sample. Related to Fig. 2.



Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Expression of C19MC in patient samples. a) Genome plot showing combined scRNA-seq coverage of a 330 kb region encompassing the C19MC locus for each ETMR sample, grouped by plate. Approximate boundaries of the C19MC primary transcript are highlighted in red (C19MC activated samples, $n = 9$) and green (non-C19MC activated samples, $n = 2$). **b)** Boxplot showing normalized expression levels of the C19MC primary transcript (pri-miRNA) per cell ($n = 2,520$ cells) in a cell type- and sample-resolved manner, as quantified by scRNA-seq. Cell numbers per cell type and sample are given in Supplementary Table 2c. Boxes indicate the median and the lower and upper hinges of the boxes correspond to the first and third quartiles. Outliers are indicated by individual dots outside of the whiskers. P values upon two-sided student's t-tests are given. n.s. Not significant. **c)** Bar plot shows average expression levels of C19MC members in C19MC-amplified ETMR, assessed by small RNA sequencing of bulk tumor samples ($n = 7$). Expression levels of the mature 5p and 3p miRNAs arms are shown. **d)** Copy-number variation profile

of three ETMR tumor samples that were analyzed by RNA in-situ hybridization (RNA-ISH) or DNA fluorescence in-situ hybridization (DNA-FISH). Genomic location of the C19MC amplification is indicated. **e)** Additional images of DNA-FISH analysis of sample MUV45. Top panel shows manual segmentation of rosette-like structures (red boundaries) and neuropil-abundant areas (green boundaries). Second and third panels indicate nuclei in high- and low-density regions, respectively (black). Bottom panel indicates the number of distinct C19MC foci per nucleus. **f)** Microscopy images of H&E (top panel) and DNA fluorescence in situ hybridization (DNA-FISH, other panels) analysis of C19MC-amplified sample MUV30 on consecutive tumor slices. Pseudo images show systematic analysis of cell density and number of distinct C19MC per nucleus. Bar plot summarizes the numbers of distinct C19MC foci per nucleus grouped according to cell density. DNA-FISH was performed and quantified on three individual ETMR samples. Scale bars = 500 μm . Related to Fig. 3.

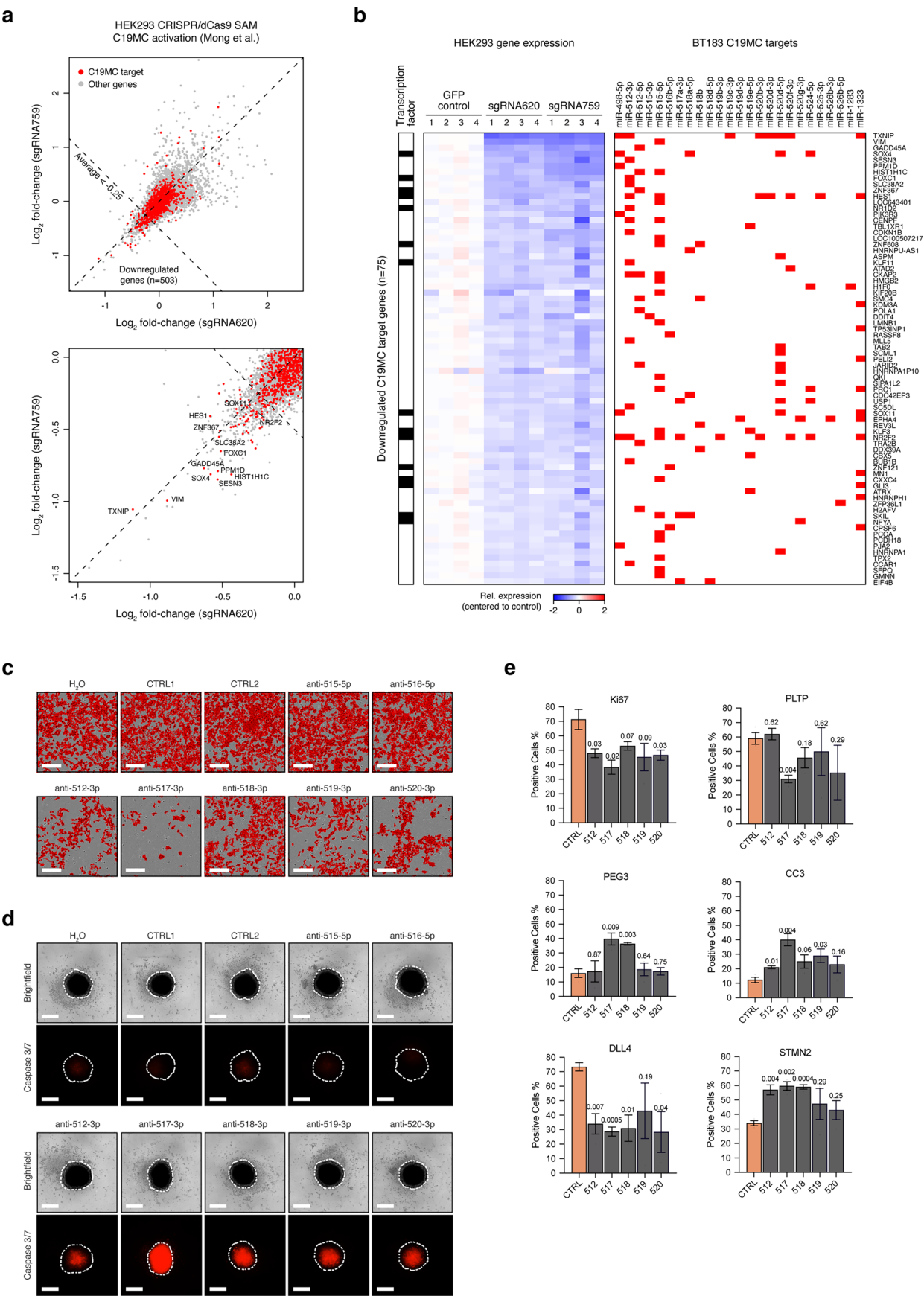


Extended Data Fig. 6 | See next page for caption.

Extended Data Fig. 6 | Identification of miRNA-mRNA interactions using eCLIP.

a) Dot plot shows expression of mature miRNAs in BT183 cell line assessed by small-RNA sequencing. Detected C19MC members are colored in red ($n = 67$). Most highly expressed miRNAs of interest are highlighted. **b)** Histograms show the number of detected reads as a function of paired-end read length for replicate 1 and 2 of the eClip experiment in BT183 cells. Only reads that contain a mature miRNA sequence at the 5' end (miRNA-only and chimeric reads) are shown. Percentages of reads for each population relative to all reads (chimeric and non-chimeric) are indicated. Over 5 million chimeric reads were detected across replicates. **c)** Dot plot shows the enrichment of chimeric reads in detected peaks over normalized reads in the input control, combining both replicates. $n = 4,009$ high-confidence peaks are indicated in black. **d)** Heatmap shows overlap (Jaccard index) in genes targeted by individual C19MC members. The total number of target genes per C19MC member is indicated in the bar plot on

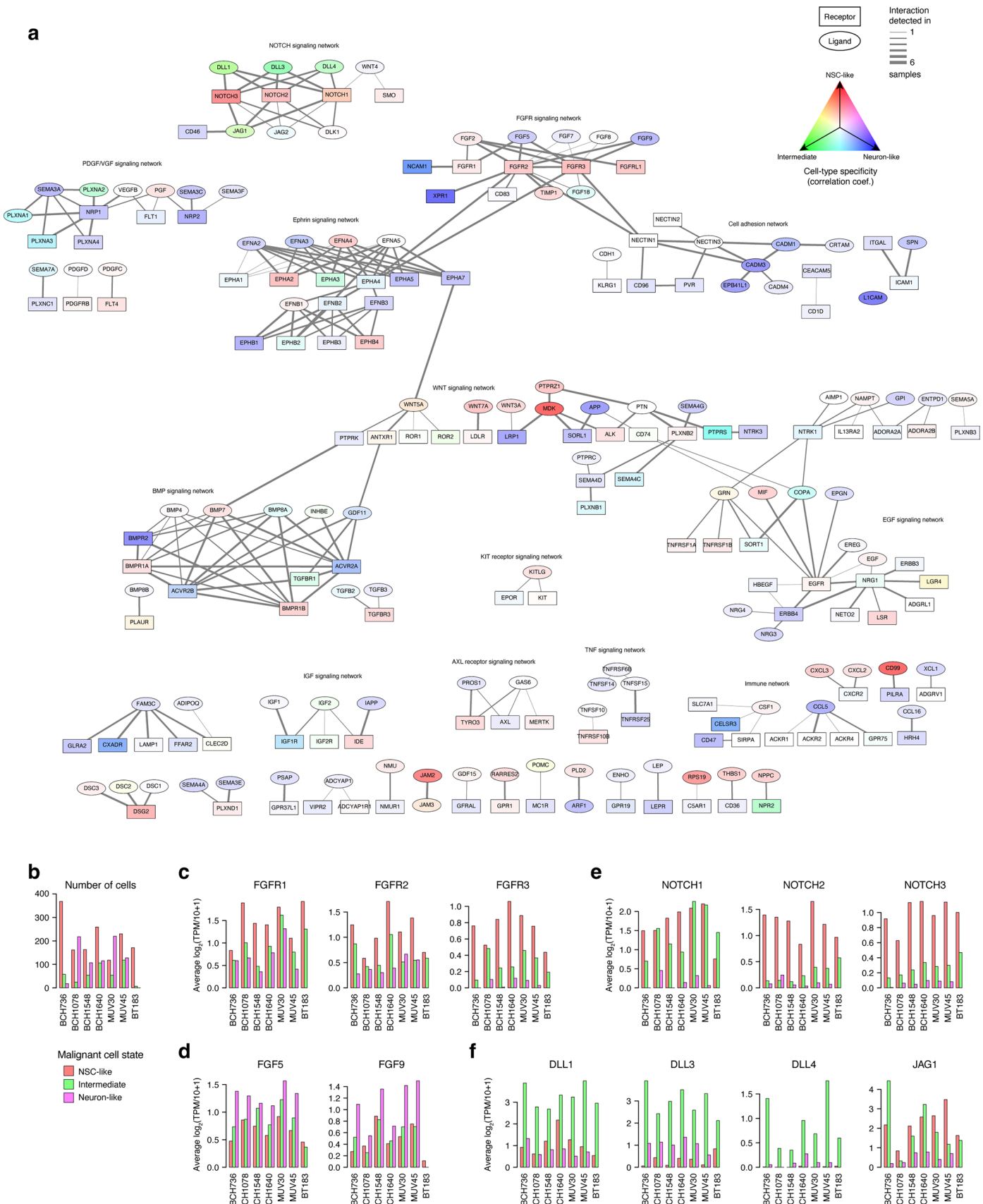
the left, split by genes that are targeted by a single or multiple members. miRNAs of interest are highlighted by arrows. **e)** Genome plot of region on chromosome 9 encompassing the 3' UTR of TF transcription factor *ZNF367*. Top track shows coverage in input control, second track shows coverage of non-chimeric mRNA-only reads. Third track shows coverage of all chimeric reads, combined for all miRNAs. Remaining tracks show coverage of chimeric reads associated with C19MC members miR-512-5p and miR-515-5p and three non-C19MC miRNAs, indicated in red and blue, respectively. **f)** Genome plot of region on chromosome 5 encompassing *EGR1*, similar to panel (e). **g)** Genome plot of region on chromosome 6 encompassing *SOX4*, similar to (e). **h)** Genome plot of region on chromosome 2 encompassing *SOX11*, similar to (e). **i)** Genome plot of region on chromosome 3 encompassing *HES1*, similar to (e). **j)** Genome plot of region on chromosome 1 encompassing *HES5*, similar to (e). Related to Fig. 4.



Extended Data Fig. 7 | Functional analyses of C19MC in cell line models.

a) Scatter plot shows a re-analysis of the dataset published by Mong et al., 2020. C19MC is expressed in HEK293 cells using a CRISPR activation system and two different gRNAs specific for its promoter region. Downregulated genes ($n = 509$, putative C19MC targets) are located at the bottom left. C19MC targets identified by eCLIP in BT183 cells are indicated in red. Scatter plot at the bottom shows a magnified view of downregulated genes, with key C19MC targets labeled. **b)** Heatmap on the left shows relative gene expression of 75 downregulated genes that were also identified as C19MC targets by eCLIP. Heatmap on the right shows a detailed view for which C19MC member eCLIP peaks were detected. **c)** Brightfield images showing BT183 ETMR cell confluency after treatment

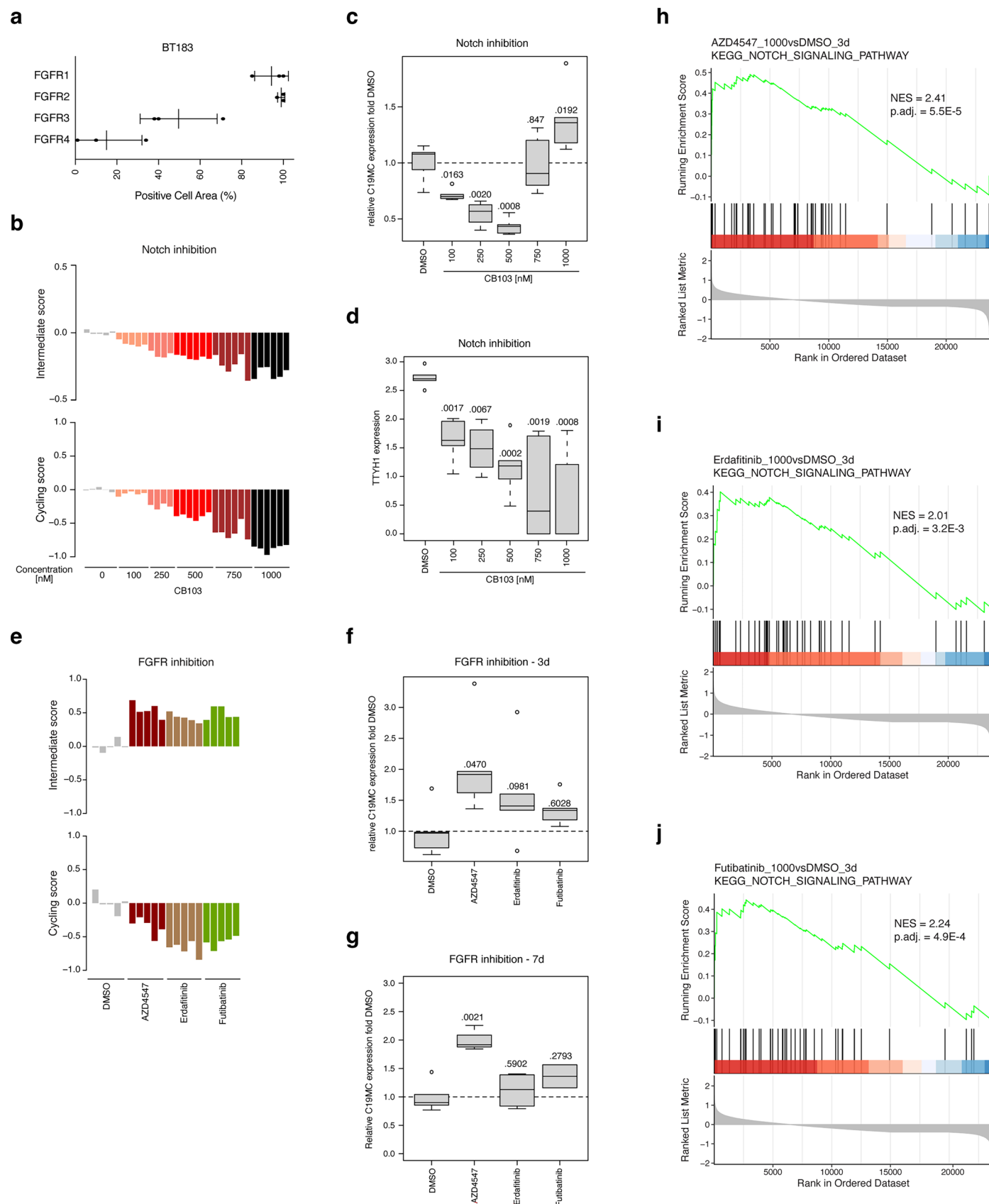
for 72 h with LNAs targeting seven C19MC families, non-targeting CTRL-LNAs (CTRL1, CTRL2), or solvent control (H₂O). Identified cell areas are highlighted in red. Scale bars = 400 μm . Images shown are representative of the $n = 4$ biological replicates. **d)** Brightfield and fluorescence images showing BT183 cell caspase 3/7 signal after treatment for 72 h with LNAs targeting seven C19MC families, non-targeting CTRL-LNAs (CTRL1, CTRL2), or Solvent control (H₂O). Scale bars = 500 μm . Images shown are representative of the $n = 4$ biological replicates. **e)** Bar plots show quantification of protein expressing cells for the indicated markers assessed by immunofluorescence analysis. Percentages of positive cells from three replicates are shown as mean values with SEM. P-values were calculated using the two-sided, unpaired Student's t-test. Related to Figs. 4 and 5.



Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | CellPhoneDB analysis reveals receptor-ligand interactions. **a)** Network graph of CellPhoneDB analysis shows inferred ligand (ellipses) and receptor (rectangles) interactions in our single-cell dataset ($P < 0.05$, $n = 6$ samples). Colors indicate correlation of expression levels to cell type-specific signature scores. CellPhoneDB analysis was performed on each sample separately. The width of edges indicates the number of samples the interaction was detected in. **b)** Bar plot shows the number of cells detected per

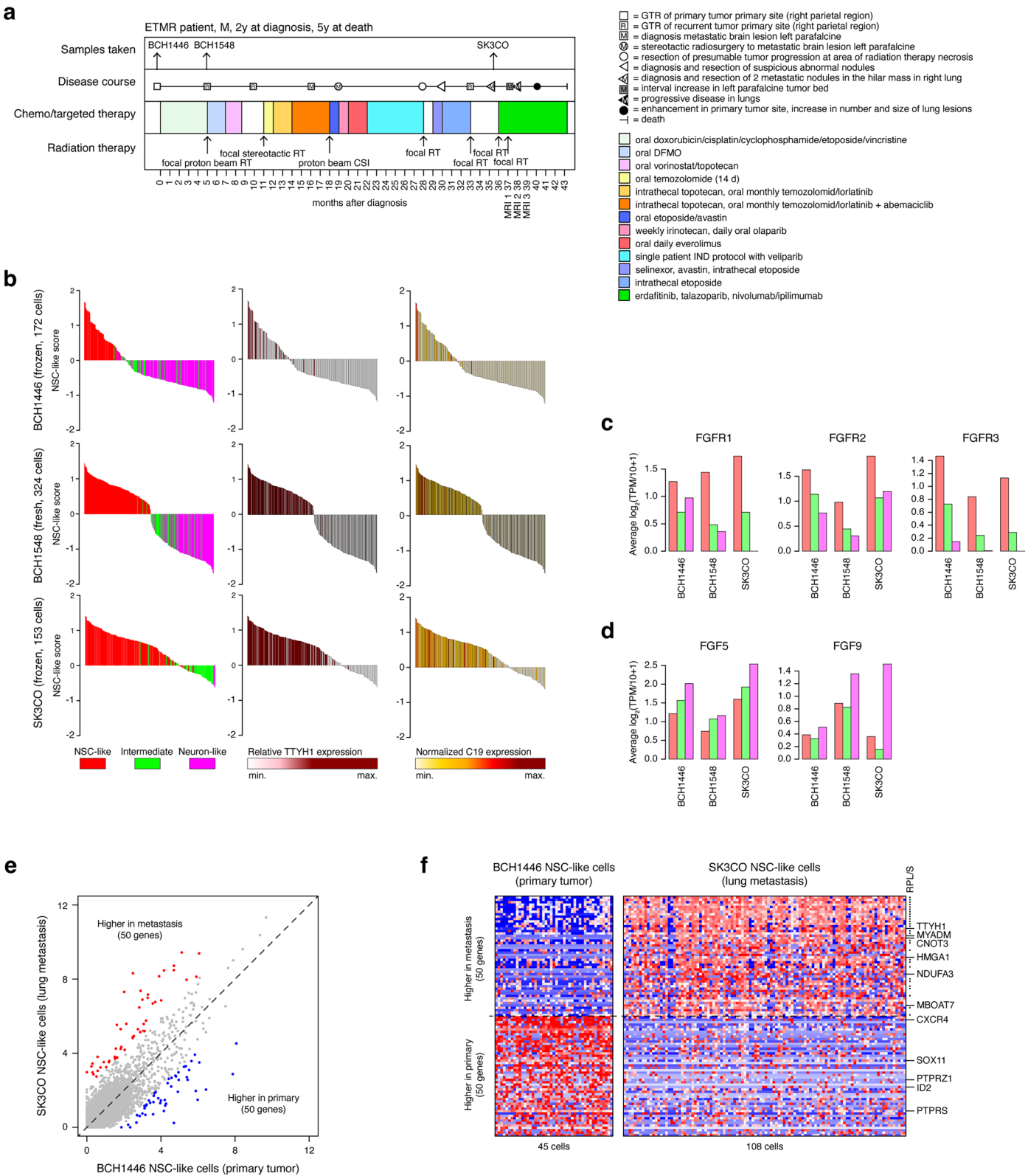
malignant cell type across fresh-sorted ETMR samples. **c)** Bar plot shows average expression of FGFR genes in single cell data of fresh-sorted ETMR samples, separated by malignant cell type. Average expression in the BT183 cell line is indicated on the right. **d)** Bar plot shows expression of FGF ligands. **e)** Bar plot shows expression of NOTCH receptors. **f)** Bar plot shows expression of Delta ligands. For barplots shown in panels **(b-f)** $n = 2,520$ cells from tissue samples and $n = 179$ cells from BT183 were plotted. Related to Fig. 6.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | FGFR and Notch inhibition in ETMR cells. **a)** Bar plots show quantification of fluorescent signals for four FGF receptors in BT183 cells. Signals from over 12,000 cells were analyzed and normalized to the cell area determined by Phalloidin. Percentages of positive areas for FGFR1, 2, 3 and 4 are shown as mean values with SEM. **b)** Bar plots showing intermediate (top) and cycling (bottom) signature scores in BT183 cells that were treated with CB103 at the indicated concentrations for 7 days. Individual bars for each indicate technical replicates. This experiment was performed in one biological replicate. **c)** Boxplot shows C19MC expression levels in BT183 cells upon treatment with different concentrations of the NOTCH inhibitor CB103 or DMSO control. Two-sided student's t-tests were performed. *P* values are indicated. Each box represents the median $\pm$ interquartile range of $n = 4-6$ wells as in **b)** treated with the inhibitor at indicated concentrations. **d)** Boxplot shows expression of TTYH1, the presumed fusion partner of C19MC upon treatment as in **c)**. Two-sided student's t-tests were performed. *P* values are indicated. Each box represents the median $\pm$ interquartile range of $n = 4-6$ wells as in **b)** treated with

the inhibitor at indicated concentrations. **e)** Bar plots show intermediate (top) and cycling (bottom) signature scores in BT183 cells treated with 1000 nM of the FGFR inhibitors AZD4547, erdafitinib, or futibatinib for 3 days. This experiment was performed in one biological replicate. **f)** Boxplot shows C19MC expression levels after 3 days of treatment with three different FGFR inhibitors (1,000 nM) or DMSO control. Two-sided student's t-tests were performed. *P* values are indicated. Each box represents the median $\pm$ interquartile range of $n = 5$ wells treated with the inhibitor at indicated concentrations. **g)** Boxplot as in **f)** after 7 days of treatment. Two-sided student's t-tests were performed. *P* values are indicated. Each box represents the median $\pm$ interquartile range of $n = 5$ wells treated with the inhibitor at indicated concentrations. **h)** Gene set enrichment analysis plot of the KEGG Notch signaling pathway gene set in transcriptional profiles generated from AZD4547-treated BT183 ETMR cells versus DMSO controls. NES Normalized enrichment score, *p*.adj adjusted *P*-value. **i)** Gene set enrichment analysis plot for cells treated with erdafitinib. **j)** Gene set enrichment analysis plot for cells treated with futibatinib. Related to Fig. 7.



Extended Data Fig. 10 | See next page for caption.

Extended Data Fig. 10 | Clinical translation of a previously unreported drug candidate and longitudinal tumor profiling. **a)** Illustration of clinical history of an ETMR patient treated with erdafitinib on a compassionate use basis. Therapeutic interventions (radiation, chemo-/targeted therapy, surgical resections) as well as radiological examinations are indicated by symbols and colors. Sample IDs are linked to the respective tumor lesions. CSI = Craniospinal Irradiation; DFMO = Difluoromethylornithine; GTR = Gross Total Resection; IND = Investigational New Drug; MRI = Magnetic Resonance Imaging; RT = Radiotherapy; **b)** Bar plots show the NSC-like signature score in cells from the primary tumor (BCH1446, n = 172 cells, top row), the recurrent lesion (BCH1548, n = 324 cells, middle row), as well as the metastatic tumor (SK3CO, n = 153 cells, bottom row). Bars are colored by cell type (left column), TTYH1 expression (middle column), and C19MC pri-miRNA expression (right column). **c)** Bar plot

shows the expression of FGFR genes in longitudinal samples from the same patient, separated by malignant cell types. The primary tumor (BCH1446, n = 172 cells, top row), the recurrent lesion (BCH1548, n = 324 cells, middle row), as well as the metastatic tumor (SK3CO, n = 153 cells, bottom row) were analyzed. **d)** Bar plot shows the expression FGF ligands. The primary tumor (BCH1446, n = 172 cells, top row), the recurrent lesion (BCH1548, n = 324 cells, middle row), as well as the metastatic tumor (SK3CO, n = 153 cells, bottom row) were analyzed. **e)** Scatter plot shows comparison of average gene expression profiles from different samples of the same patient (primary tumor and lung metastasis). Only NSC-like cells were used (n = 153 cells). **f)** Heatmap shows the top 50 up- and downregulated genes between both samples. Columns represent individual NSC-like cells (n = 45 and n = 108 cells). Genes of interest are indicated on the right. Genes encoding for ribosomal proteins are indicated by dots. Related to Fig. 8.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	Single cell RNA sequencing data was generated using the Illumina NextSeq 500/550 (38bp paired-end sequencing) instrument according to manufacturer's guidelines. Small RNA sequencing data using a library of genome-wide miRNAs was generated using the Illumina NextSeq 500 system (50bp single-end sequencing). eCLIP data were generated on an Illumina instrument using 150bp paired-end sequencing according to manufacturer's instructions.
Data analysis	To analyze the data, we used publicly available open-source software such as RSEM, Bowtie, Samtools, and GATK. Downstream analysis was performed in R and python using several publicly available packages and tools such as CellPhoneDB, SCENIC, and velocity. For image analysis the following software was used: QuPath version 0.4.2, inForm version 2.4.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Expression matrices of high-quality cells (including malignant and non-malignant cells) of all samples generated within this study are deposited in the Gene Expression Omnibus (GEO) repository under accession code GSE231614. Raw scRNA-seq data will be made available upon reasonable request to the corresponding authors. All datasets that were used within this study are summarized in Supplementary Table 1b. Provided source data files include further raw data such as annotation, embedding coordinates, and cell scores.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	Tumor samples from 6 female and 5 male participants were collected and subjected to single-cell RNA sequencing. We did not observe sex-related differences concerning biological tumor properties.
Reporting on race, ethnicity, or other socially relevant groupings	We did not collect data on race, ethnicity, or other socially relevant groupings on the subjects that were included in this study.
Population characteristics	A description of the population characteristics is included in the manuscript. Data on patient ages, tumor location, tumor specificities (primary or metastatic lesion) were collected. Patient ages ranged from 0.4-4.8 years.
Recruitment	Participants were recruited based on availability of fresh and fresh-frozen biopsy material (tumor) taken at the time of neurosurgery. Patients were recruited in the course of their presentation in the clinics. All available ETMR samples were included. There was no selection bias. Tumor tissue was isolated in the course of the patient's routine therapeutic and diagnostic surgeries. Samples from three international clinical institutions were included.
Ethics oversight	The local Institutional Review Boards of the respective institutions (Boston Children's Hospital DFCI IRB 10-417, Medical University of Vienna MUV IRB 1244/2016) approved the collection of tumor material. Patients and/or their legal representatives, who did not receive compensation, provided written consent to the use of tumor tissue for research purposes and publication of de-identified data preoperatively according to respective Institutional Review Board guidelines. All procedures on human participants were in accordance with the Helsinki declaration.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was defined by sample availability. There was no sample-size calculation performed.
Data exclusions	Initial tumor diagnosis as ETMR was confirmed by board certified pathologists and DNA methylation-based classification (www.molecularneuropathology.org). No data were excluded from the analyses
Replication	Each experiment was performed in at least three biological replicates unless otherwise stated in the text. RNA-ISH and FISH experiments were performed in the same ETMR tissue samples that were part of our scRNAseq cohort as indicated.
Randomization	No human or animal trials requiring randomization are included in this study.
Blinding	No human or animal trials requiring blinding are included in this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Anti-PAX6 antibody [AD2.38] (Abcam, Ab78545), STMN2 Polyclonal Antibody (Thermo Fisher, 720178), Purified Mouse Anti-Ki-67 Clone B56 (BD, 550609), LIN28A Monoclonal Antibody [14E6-4E6] (Thermo Fisher, MA1-016), NEUROD1 Monoclonal Antibody [3H8] (Abnova, H00004760-M01), PEG3 Polyclonal Antibody (Thermo Fisher, PA5-55282), NSE Monoclonal Antibody [5G10] (Thermo Fisher, MA1-22730), Purified anti-Tubulin β 3 (Biolegend, 801201), PLTP Polyclonal Antibody (Thermo Fisher, PA5102820), PAX3 Monoclonal Antibody [16H22L10] (Thermo Fisher, 701147), FGFR1 Polyclonal Antibody (Thermo Fisher, PA5-97748), FGFR2 Polyclonal Antibody (Thermo Fisher, PA5-14651), FGFR3 Recombinant Rabbit Monoclonal Antibody [JM110-33] (Thermo Fisher, MA5-32620), FGFR4 Recombinant Polyclonal Antibody [14HCLC] (Thermo Fisher, 710210), Cleaved Caspase-3 (Asp175) (D3E9) Rabbit mAb (Cell Signaling, 9579), DLL4 (D7N3H) Rabbit mAb (Cell Signaling, 96406), Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488 (Thermo Fisher, A32731). All primary antibodies were used at a 1:100 dilution. For validation appropriate positive controls were used and specific staining patterns were evaluated.

Validation

All the antibodies used in this study are commercially available and extensively validated by the respective company. Validation data and links to publications using these antibodies are available in each of these company's website. All antibodies used in this study exhibited expected staining patterns in accordance with the existing literature.

Anti-PAX6 antibody [AD2.38] (Abcam, Ab78545): <https://www.abcam.com/en-us/products/primary-antibodies/pax6-antibody-ad238-ab78545>

STMN2 Polyclonal Antibody (Thermo Fisher, 720178): <https://www.thermofisher.com/antibody/product/STMN2-Antibody-Polyclonal/720178>

Purified Mouse Anti-Ki-67 Clone B56 (BD, 550609): <https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/purified-mouse-anti-ki-67.550609>

LIN28A Monoclonal Antibody [14E6-4E6] (Thermo Fisher, MA1-016): <https://www.thermofisher.com/antibody/product/LIN28A-Antibody-clone-14E6-4E6-Monoclonal/MA1-016>

NEUROD1 Monoclonal Antibody [3H8] (Abnova, H00004760-M01): https://www.abnova.com/en-global/product/detail/H00004760-M01?gad_source=1&gclid=Cj0KCQiAouG5BhDBARIsAOc08RT0hGzwHGemJP0wxMP4NYtUrQLmFS0t7sbCC2yHu7BIUWfqTHHIPQkaAm3DEALw_wcB

PEG3 Polyclonal Antibody (Thermo Fisher, PA5-55282): <https://www.thermofisher.com/antibody/product/PEG3-Antibody-Polyclonal/PA5-55282>

NSE Monoclonal Antibody [5G10] (Thermo Fisher, MA1-22730): <https://www.thermofisher.com/antibody/product/NSE-Antibody-clone-5G10-Monoclonal/MA1-22730>

Purified anti-Tubulin β 3 (Biolegend, 801201): <https://www.biolegend.com/de-de/products/purified-anti-tubulin-beta-3-tubb3-antibody-11580>

PLTP Polyclonal Antibody (Thermo Fisher, PA5102820): <https://www.thermofisher.com/antibody/product/PLTP-Antibody-Polyclonal/PA5-102820>

PAX3 Monoclonal Antibody [16H22L10] (Thermo Fisher, 701147): <https://www.thermofisher.com/antibody/product/PAX3-Antibody-clone-16H22L10-Recombinant-Monoclonal/701147>

FGFR1 Polyclonal Antibody (Thermo Fisher, PA5-97748): <https://www.thermofisher.com/antibody/product/FGFR1-Antibody-Polyclonal/PA5-97748>

FGFR2 Polyclonal Antibody (Thermo Fisher, PA5-14651): <https://www.thermofisher.com/antibody/product/FGFR2-Antibody-Polyclonal/PA5-14651>

FGFR3 Recombinant Rabbit Monoclonal Antibody [JM110-33] (Thermo Fisher, MA5-32620): <https://www.thermofisher.com/antibody/product/FGFR3-Antibody-clone-JM110-33-Recombinant-Monoclonal/MA5-32620>

FGFR4 Recombinant Polyclonal Antibody [14HCLC] (Thermo Fisher, 710210): <https://www.thermofisher.com/antibody/product/FGFR4-Antibody-clone-14HCLC-Recombinant-Superclonal/710210>

Cleaved Caspase-3 (Asp175) (D3E9) Rabbit mAb (Cell Signaling, 9579): <https://www.cellsignal.com/products/9579/datasheet?images=1&protocol=0&size=A4>

DLL4 (D7N3H) Rabbit mAb (Cell Signaling, 96406): <https://www.cellsignal.com/products/96406/datasheet?images=1&protocol=0&size=A4>

Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488 (Thermo Fisher, A32731): <https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32731>

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	BT183 were provided by Marcel Kool (German Cancer Research Center (DKFZ), Heidelberg, Germany); SUIV, DIPG007 and DIPGXIII* were provided by Michelle Monje (Stanford University School of Medicine, Stanford, CA); BT690 were provided by Keith Ligon (Dana-Farber Cancer Institute, Boston, MA); MBT011, 014, 021, 026, 028, 030, 032 and 037 were generated from primary patient tumor tissue in Alexander Beck's lab, U87, DAOY, SUPB15, JURKAR, REH and IMR32 were purchased from ATCC; A431, PC9 and SF8628 were purchased from Sigma-Aldrich. Sex of the primary cell lines generated from human participants are as follows: Male: BT183, MBT011, 026, 028, 030 Female: MBT014, 021, 032 and 037
Authentication	All cells purchased from ATCC or Sigma-Aldrich came with STR-authentication. Cells from Monje, Kool and Ligon lab were not further authenticated. All MBT cell lines from the Beck lab were authenticated by DNA methylation-based profiling and classification.
Mycoplasma contamination	No cultures tested positive for mycoplasma.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Female NOD-scid IL2rgnull (NSG) mice were used for patient-derived xenograft experiments. Animals were 6-8 weeks-old at the time of tumor cell injection.
Wild animals	No wild animals were used.
Reporting on sex	We did not consider sex-related differences in tumor engraftment.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	Animal experiments were performed according to German Laws for Animal Protection and approved by the regional authorities (Approval number G91/20).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Single-cell suspensions obtained from fresh tumors in PBS with 1% BSA were stained with 0.5-1 μ M calcein AM (Life Technologies, C3100MP) and 0.33 μ M TO-PRO3 iodide (Life Technologies, T3605) for 15 min at RT and kept on ice. Single-cell sorting was performed on a SH800 (Sony) sorter using 488 nm (calcein AM, 530/30 emission filter) and 633 nm (TO-PRO-3, 665/30 emission filter) lasers. Viable cells were identified by positive staining for calcein AM and negative staining for TO-PRO-3. Doublets were discriminated based on back scatter area (BSC-A) versus back scatter width (BSC-W). Singlet viable cells were sorted into 96-well plates containing cold TCL buffer (Qiagen, 1031576), briefly spun down, snap frozen on dry ice, and stored at -80°C. Single-nuclei suspensions extracted from frozen tumors were stained with 0.5 μ M Vybrant DyeCycle™ Ruby Stain (Invitrogen, V10309) immediately before FACS. Intact nuclei were selected by positive staining for Ruby Stain on the SH800 sorter (633 nm laser, 665/30 nm emission filter). Doublets were excluded in the Ruby Stain area versus Ruby Stain height setting. Singlet nuclei were sorted into 96-well plates containing TCL buffer and 1% beta-mercaptoethanol, briefly spun down, snap frozen on dry ice and stored at -80°C.
Instrument	SH800 (SONY) fluorescence-activated cell sorter with a 100 μ m nozzle

Software	SONY SH800 software
Cell population abundance	FACS was performed to select for viable tumor cells as described previously (Patel 2014, Tirosh 2016, Venteicher 2017, Filbin 2018, Hovestadt 2019). We did not include information on the tumor purity in this study.
Gating strategy	Live, single cells were identified by size (forward scatter), granularity (back scatter), singlet gating (back scatter area vs. back scatter width), positive staining for calcein and negative staining for TO-PRO-3. Single nuclei were identified by positive staining for Vybrant Ruby Stain and singlet gating (Ruby Stain laser light area vs. Ruby Stain laser light height).

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.