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# Genetically engineered cerebral organoids model brain tumor formation

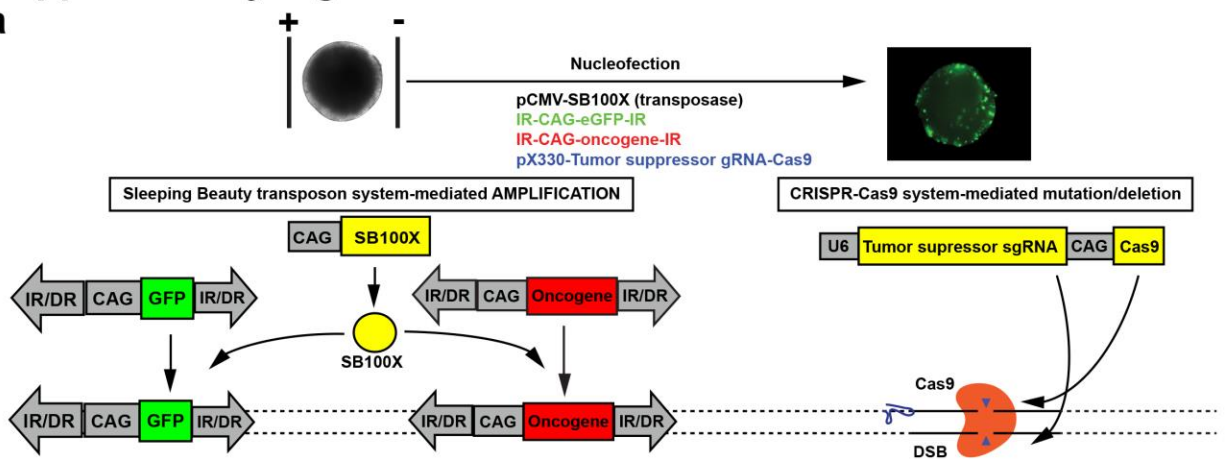
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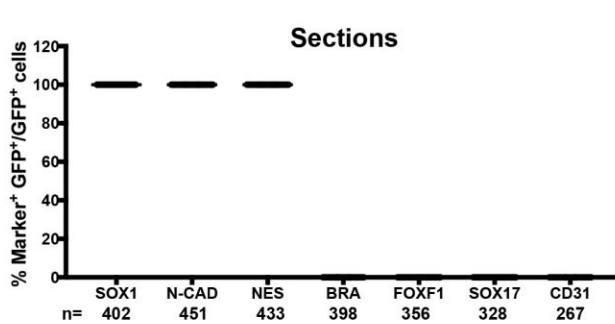
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# Supplementary Fig.1

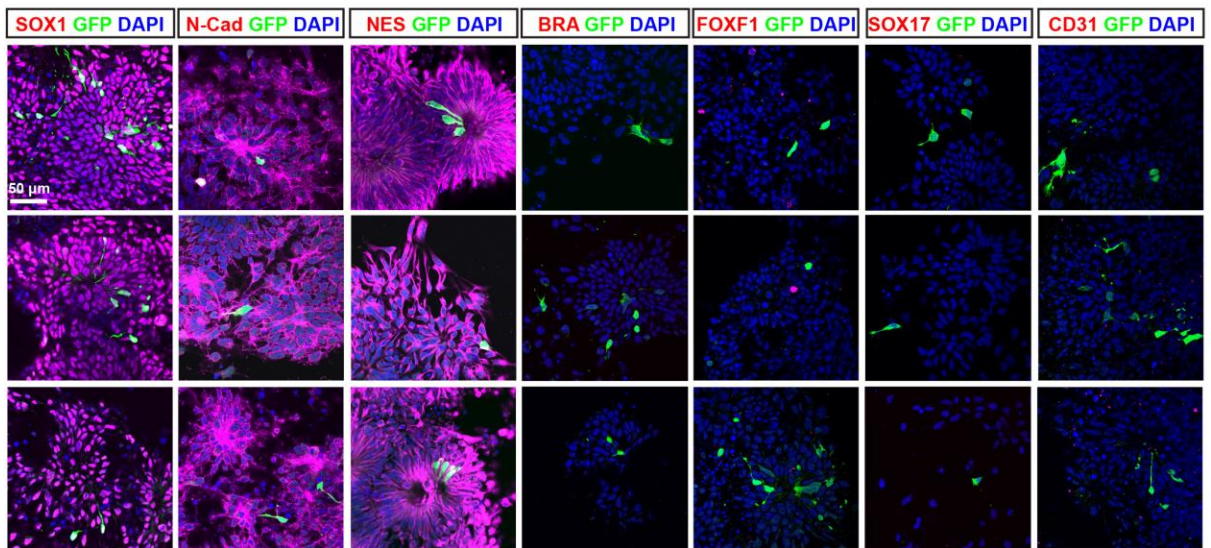
**a**



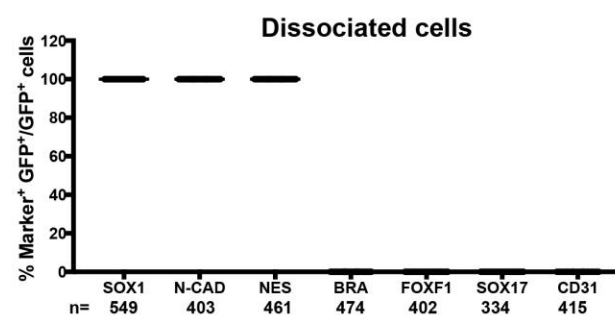
**b**



**c**



**d**



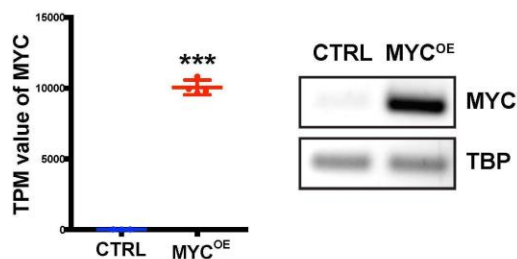
## Supplementary Figure 1

### **The strategy to introduce gene aberrations into neural stem/precursor cells in cerebral organoids.**

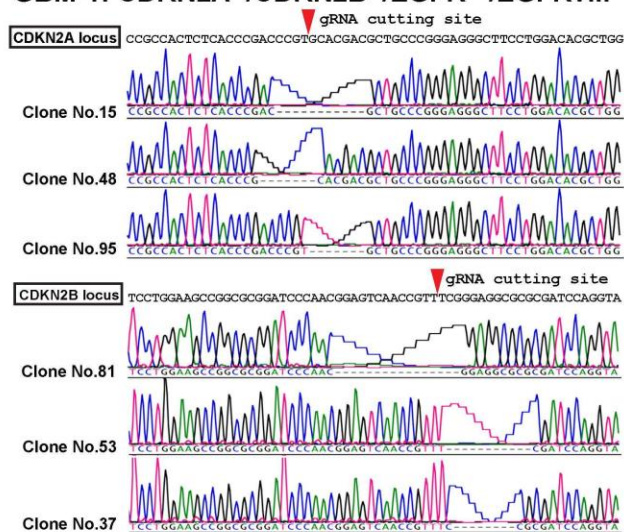
(a) Schematic of the strategy of genome-editing techniques to introduce oncogene amplification and/or tumour suppressor mutation/deletion. Sleeping Beauty transposon system was used to integrate oncogene-expression and GFP-expression elements into genome. CRISPR-Cas9 system was applied to introduce mutation/deletion of tumour suppressors. (b) Quantification of cellular identities of nucleofected cells in EBs one day after nucleofection by immunofluorescence staining on serial cryo-sections. The percentage of marker<sup>+</sup> GFP<sup>+</sup> cells in total GFP<sup>+</sup> cells from different sections were presented. Cell number for different cellular markers analysed in this study was labelled under the graph. This experiment was performed twice independently with same results. (c, d) Immunofluorescence images (c) and quantification (d) of adherent cell culture of dissociated EBs one day after nucleofection stained with different cell markers. The percentage of marker<sup>+</sup> GFP<sup>+</sup> cells in total GFP<sup>+</sup> cells from different EBs were presented. Cell number for different cellular markers analysed in this study was labelled under the graph. This experiment was performed once. All the detailed sample size and mean±s.d. are provided in the source data. Scale bar: c, 50 µm.

## Supplementary Fig. 2

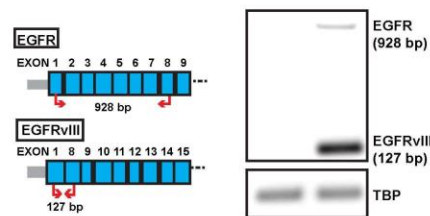
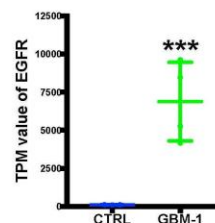
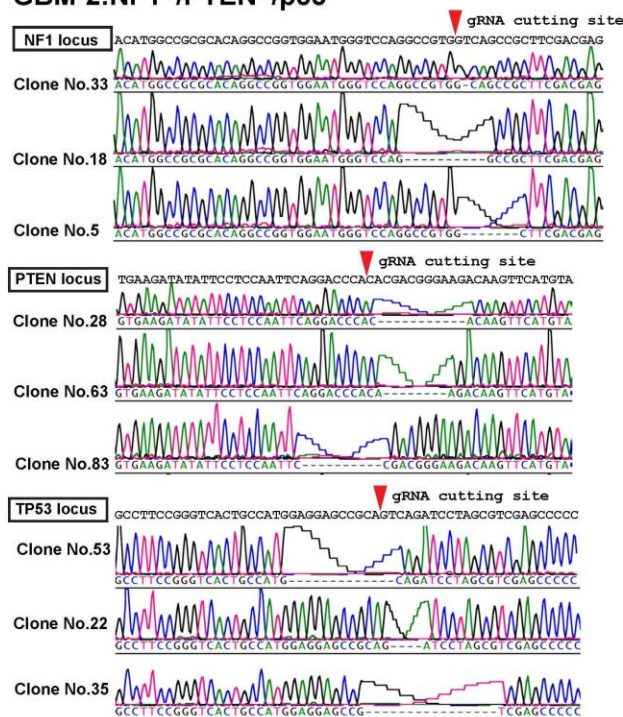
### a MYC<sup>OE</sup>



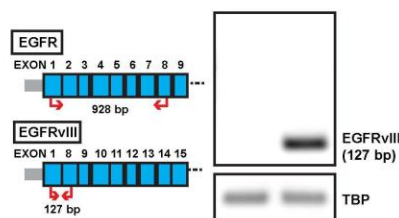
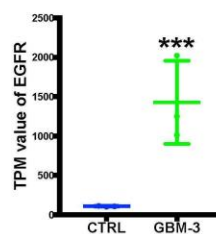
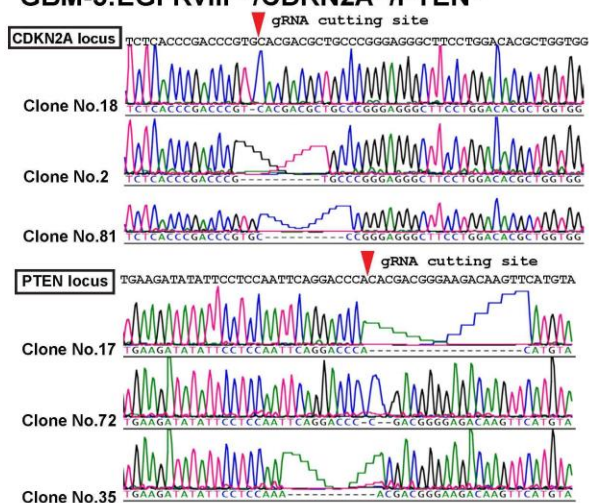
### b GBM-1: CDKN2A<sup>-/-</sup>/CDKN2B<sup>-/-</sup>/EGFR<sup>OE</sup>/EGFRvIII<sup>OE</sup>



### c GBM-2: NF1<sup>-/-</sup>/PTEN<sup>-/-</sup>/p53<sup>-/-</sup>



### d GBM-3: EGFRvIII<sup>OE</sup>/CDKN2A<sup>-/-</sup>/PTEN<sup>-/-</sup>



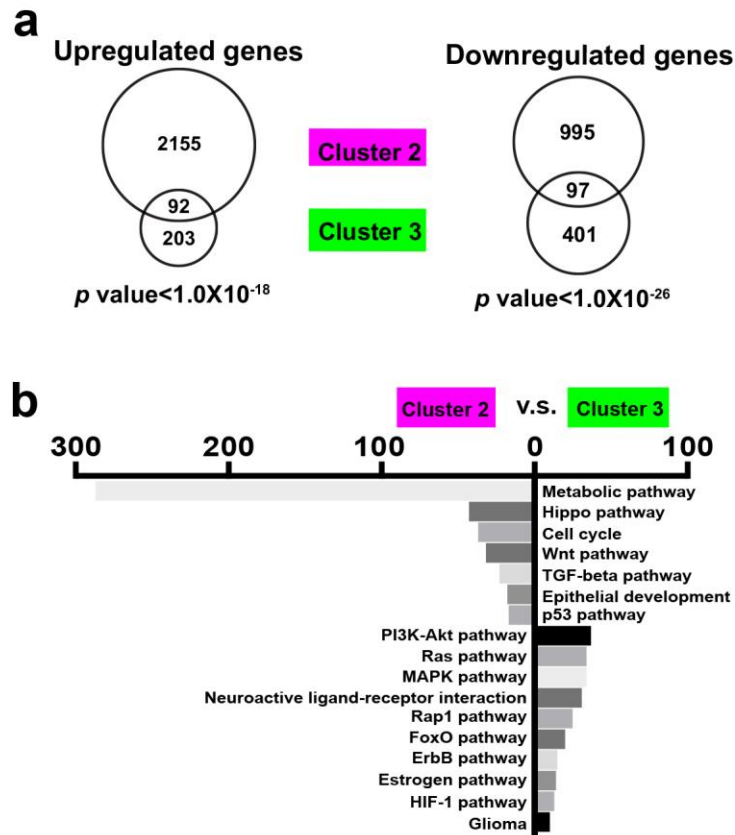
## Supplementary Figure 2

### Verification of gene aberrations introduced by genome-editing techniques.

(a) RNA-seq and RT-PCR analysis showed that tumour cells from MYC<sup>OE</sup> neoCORGs exhibit high MYC expression levels. The TPM value from RNA-seq analysis was analyzed using four organoids from three independent cultures ( $P<0.0001$ ). The TPM values of three organoids from CTRL groups from three independent cultures were presented as control. (b) Three example sequences of CRISPR-Cas9 targeting CDKN2A and CDKN2B locus in tumour cells from GBM-1 neoCORGs. RNA-seq and RT-PCR analysis showed that tumour cells from GBM-1 neoCORGs exhibit high expression levels of both EGFR and EGFRvIII. The TPM value from RNA-seq analysis was analyzed using four organoids from three independent cultures ( $P=0.0068$ ). The TPM values of three organoids from CTRL groups from three independent cultures were presented as control. (c) Three example sequences of CRISPR-Cas9 targeting NF1, PTEN, and p53 locus in tumour cells from GBM-2 neoCORGs. (d) Three example sequences of CRISPR-Cas9 targeting CDKN2A and PTEN locus in tumour cells from GBM-3 neoCORGs. RNA-seq and RT-PCR analysis showed that tumour cells from GBM-3 neoCORGs exhibit high expression level of EGFRvIII, but not EGFR. The TPM value from RNA-seq analysis was analyzed using three organoids from three independent cultures ( $P=0.0124$ ). The TPM values of three organoids from CTRL groups from three independent cultures were presented as control. Statistical analysis of quantification was performed using unpaired two-tailed Student's *t*-test. Data were presented as mean $\pm$ s.d. All the detailed sample size, mean $\pm$ s.d., as well as *P* value were provided in the source data. The percentage of non-homologous end joining (NHEJ) for individual genes by sequencing TA-vector colonies carrying amplified targeting locus are also provided in the source data. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ .



## Supplementary Fig.3

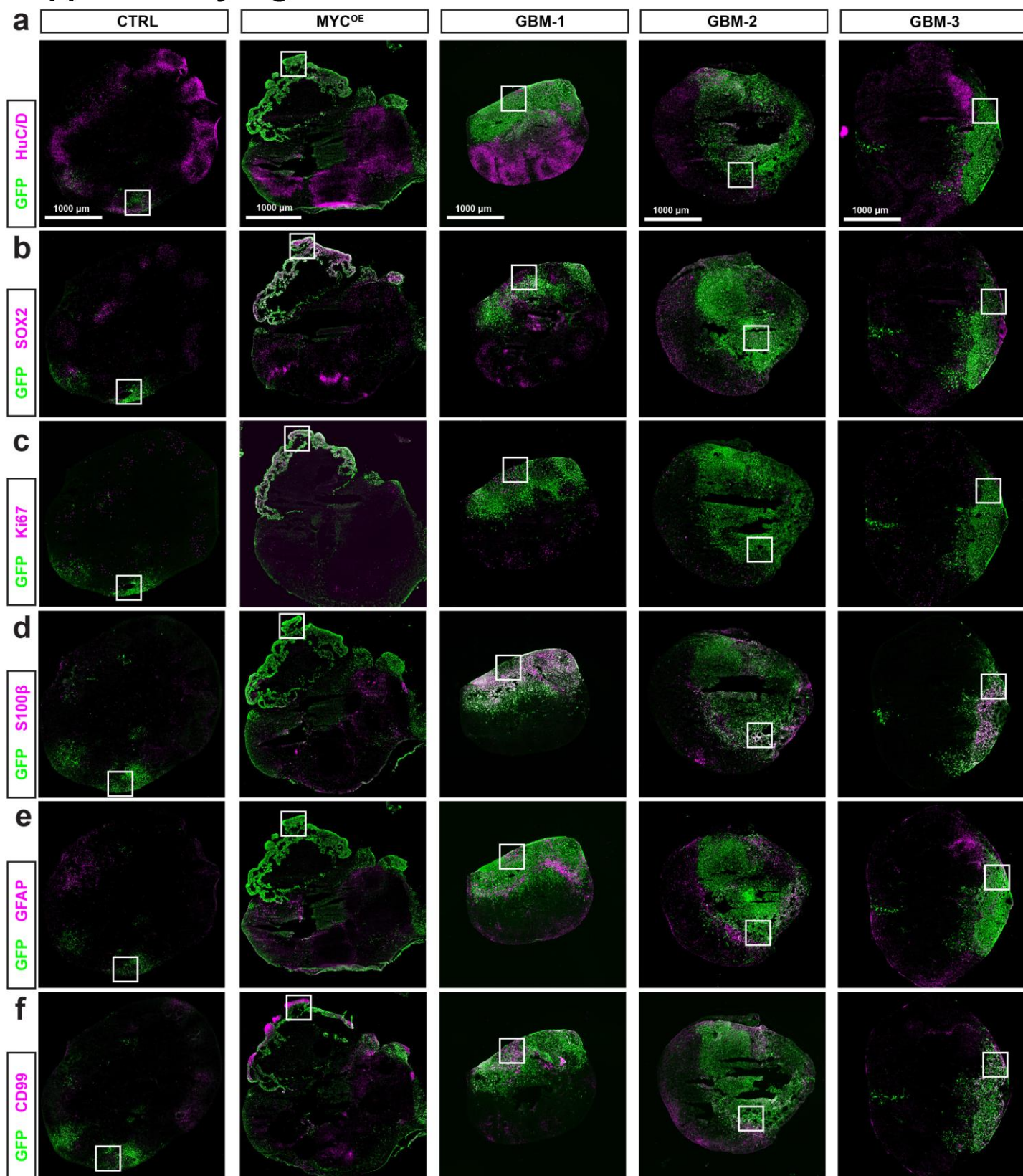


**Supplementary Figure 3**

**Venn diagram hypergeometric and KEGG pathway analysis of tumor cells from the cluster 2 and cluster 3 neoCORS.**

(a) Venn diagram hypergeometric test showed overlap of differentially expressed genes (DESeq, adjusted absolute  $\log_2\text{fc}$  value  $> 0.5$  and adjusted  $P$  value  $< 0.05$ ) in Cluster 2 ( $n=3$  organoids from one experiment) or Cluster 3 ( $n=7$  organoids from one experiment), in each case relative to CTRL organoids ( $n=3$  organoids from one experiment).  $P$  values for overlaps were calculated by hypergeometric test. (b) KEGG pathway enrichment analysis for differentially expressed genes (DESeq, adjusted absolute  $\log_2\text{fc}$  value  $> 0.5$  and adjusted  $P$  value  $< 0.05$ ) in tumour cells from Cluster 2 and Cluster 3 neoCORS.

**Supplementary Fig.4**



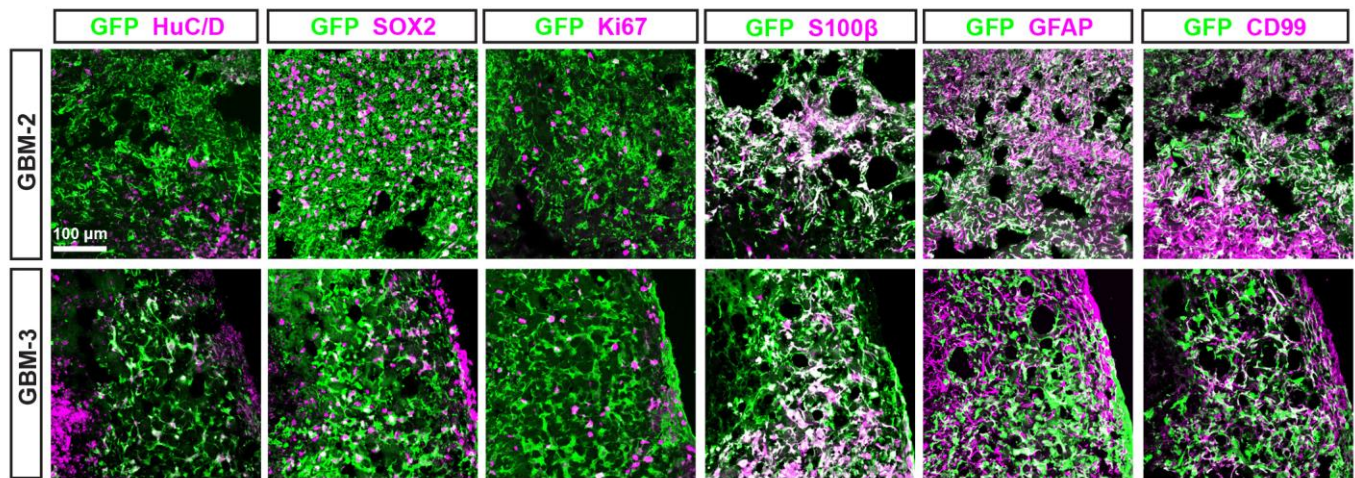
#### Supplementary Figure 4

**Low-magnification images revealed that 4-month-old neoCORGs showed brain-tumor-subtype-specific cellular identities.**

(a-f) Immunofluorescence images and quantification of neuronal marker HuC/D (a, magenta), precursor marker SOX2 (b, magenta), cell cycle marker Ki67 (c, magenta), glial marker S100 $\beta$  (d, magenta) and GFAP (e, magenta), as well as CNS-PNET marker CD99 (f, magenta). The staining was performed from six independent experiments with the similar results. Scale bar: a-f, 1000  $\mu$ m.



## Supplementary Fig.5

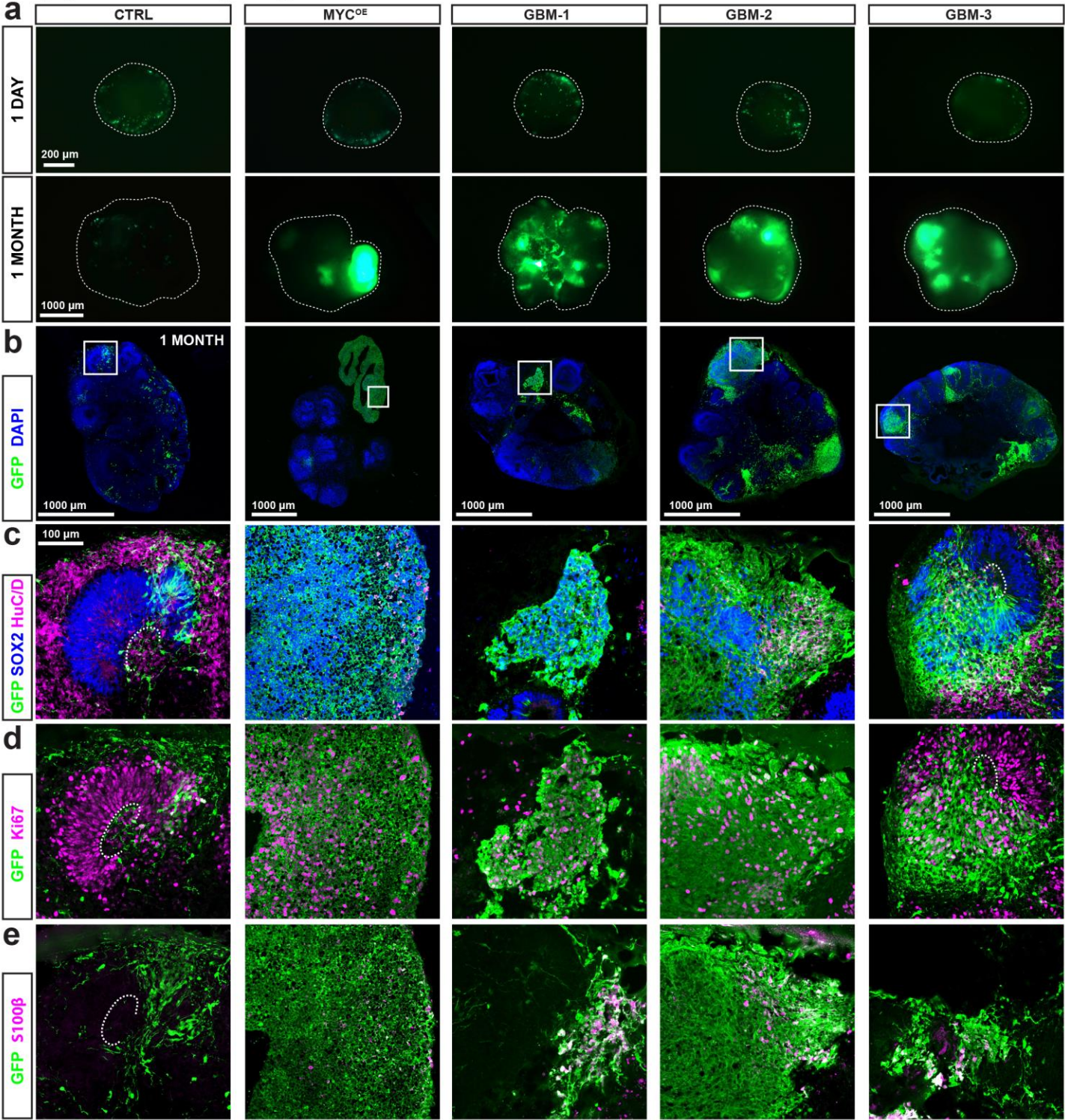


**Supplementary Figure 5**

**High-magnification images revealed that 4-month-old neoCORGs showed brain-tumor-subtype-specific cellular identities.**

Representative immunofluorescence images of four-month-old neoCORGs from GBM-2 and GBM-3 groups. Neuronal marker HuC/D (magenta), precursor marker SOX2 (magenta), cell cycle marker Ki67 (magenta), glial marker S100β (magenta) and GFAP (magenta), as well as CNS-PNET marker CD99 (magenta) were presented. The staining was performed from six independent experiments with the similar results. Scale bar: 100 μm.

Supplementary Fig.6



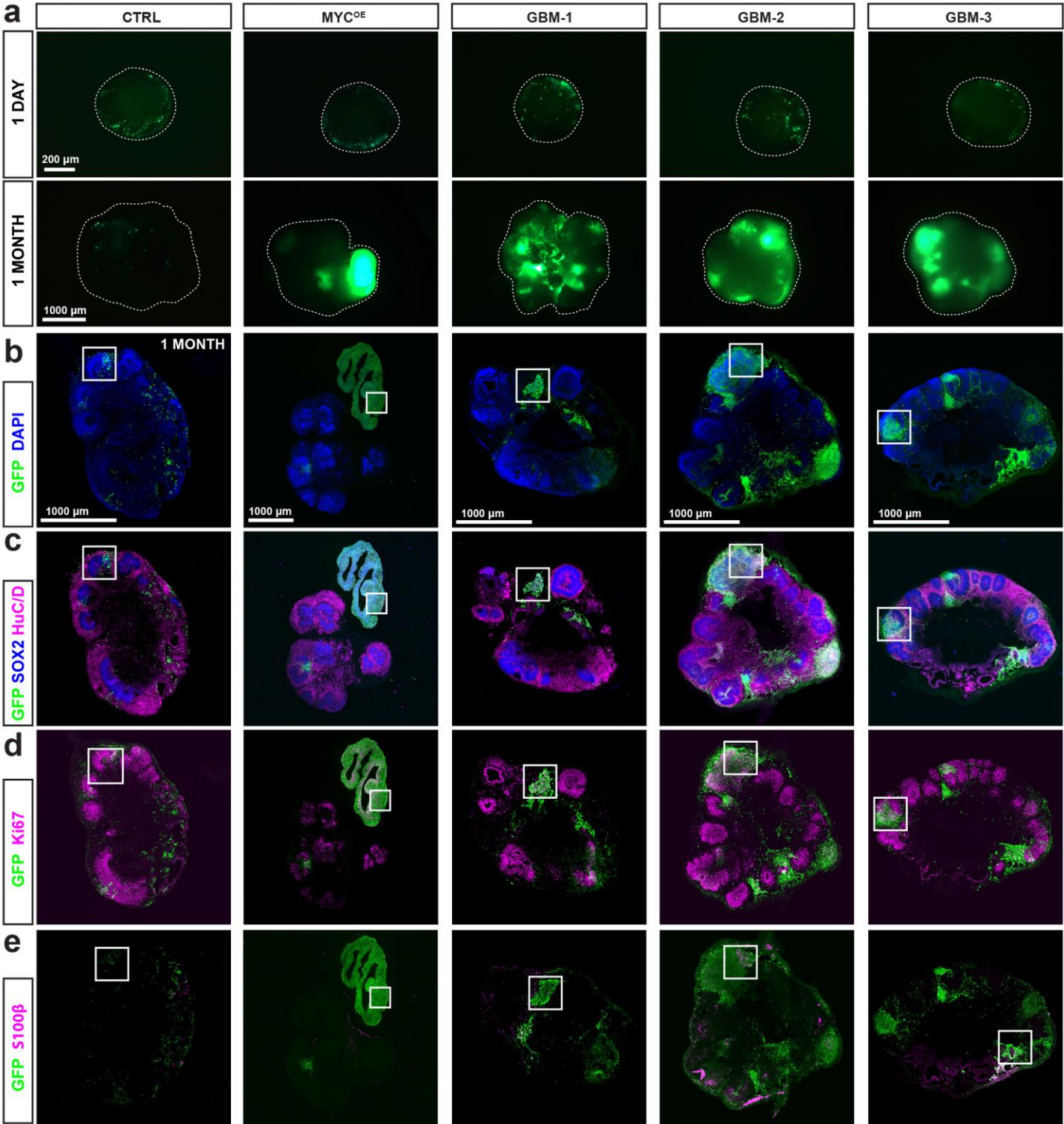
Supplementary Figure 6

High-magnification images revealed that 1-month-old neoCORs showed brain-tumor-subtype-specific cellular identities.

**(a)** Immunofluorescence images of control and neoplastic groups one day and one month after nucleofection confirmed the tumour-initiation capability of genetic disruptions. **(b)** Immunofluorescence images of DAPI (blue) and GFP (green) of control and tumour groups one month after nucleofection. **(c-e)** Immunofluorescence images and quantification of neuronal marker HuC/D (c, magenta), precursor marker SOX2 (c, blue), cell cycle marker Ki67 (d, magenta), as well as glial marker S100 $\beta$  (e, magenta). The staining was performed from three independent experiments with the similar results. Scale bar: a, upper panel: 200  $\mu\text{m}$ , lower panel: 1000  $\mu\text{m}$ ; b, 1000  $\mu\text{m}$ ; c-e, 100  $\mu\text{m}$ .



Supplementary Fig.7



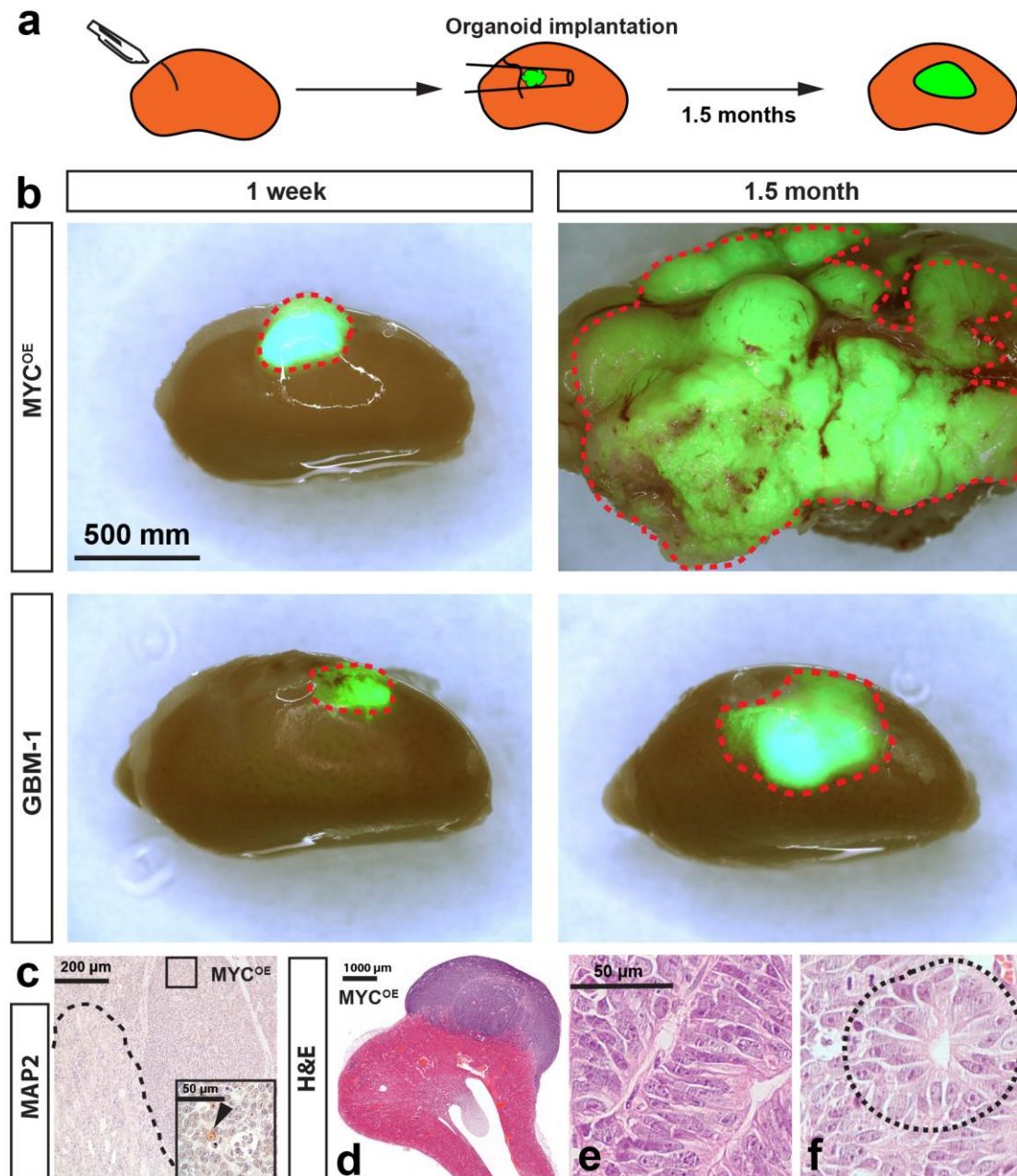
Supplementary Figure 7

Low-magnification images revealed that 1-month-old neoCORGs showed brain-tumor-subtype-specific cellular identities.

**(a)** Immunofluorescence images of control and neoplastic groups one day and one month after nucleofection confirmed the tumour-initiation capability of genetic disruptions. **(b)** Immunofluorescence images of DAPI (blue) and GFP (green) staining of control and neoplastic groups one month after nucleofection. **(c-e)** Immunofluorescence images and quantification of neuronal marker HuC/D (c, magenta), precursor marker SOX2 (c, blue), cell cycle marker Ki67 (d, magenta), as well as glial marker S100 $\beta$  (e, magenta). The staining was performed from three independent experiments with the similar results. Scale bar: a, upper panel: 200  $\mu\text{m}$ , lower panel: 1000  $\mu\text{m}$ ; b-e, 1000  $\mu\text{m}$ .



## Supplementary Fig.8



Supplementary Figure 8

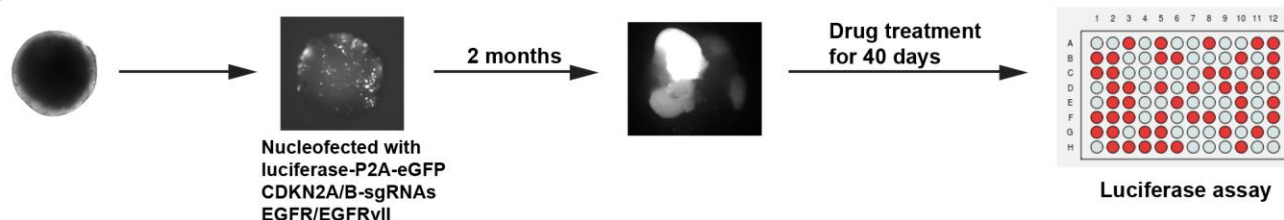
### In vivo expansion of neoCORs after renal subcapsular implantation.

(a) Schematic of renal subcapsular xenograft procedure. (b) NeoCORs from MYC<sup>OE</sup> group and GBM-1 group were implanted into kidney capsule. Engrafted kidneys were analysed at one week and one and half months after xenograft to evaluate the *in vivo* expansion of neoCORs from MYC<sup>OE</sup> and GBM-1 groups. (c) Immunohistochemical staining of neuronal marker MAP2 in the MYC<sup>OE</sup> implant. Arrowhead shows a neuron. (d-f) H&E staining of implanted MYC<sup>OE</sup>

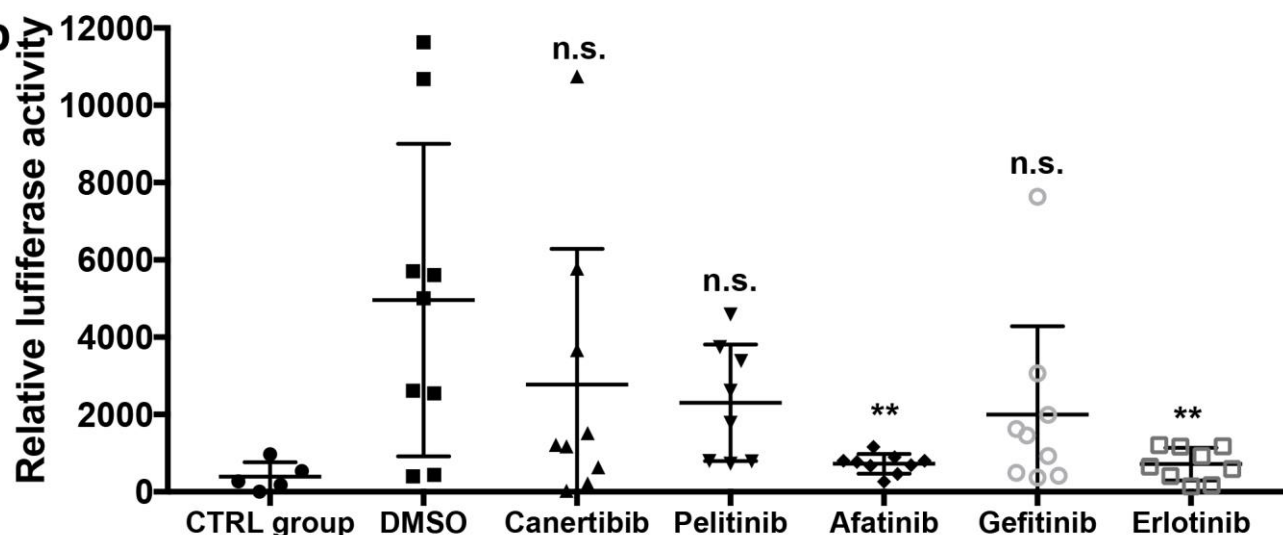
organoids showing cell sheet (e) and rosette (f) structures. The implantation experiments were performed three times independently with similar results. Scale bar: b, 500  $\mu\text{m}$ ; d, 1000  $\mu\text{m}$ ; e,f, 50  $\mu\text{m}$ .

## Supplementary Fig.9

**a**



**b**

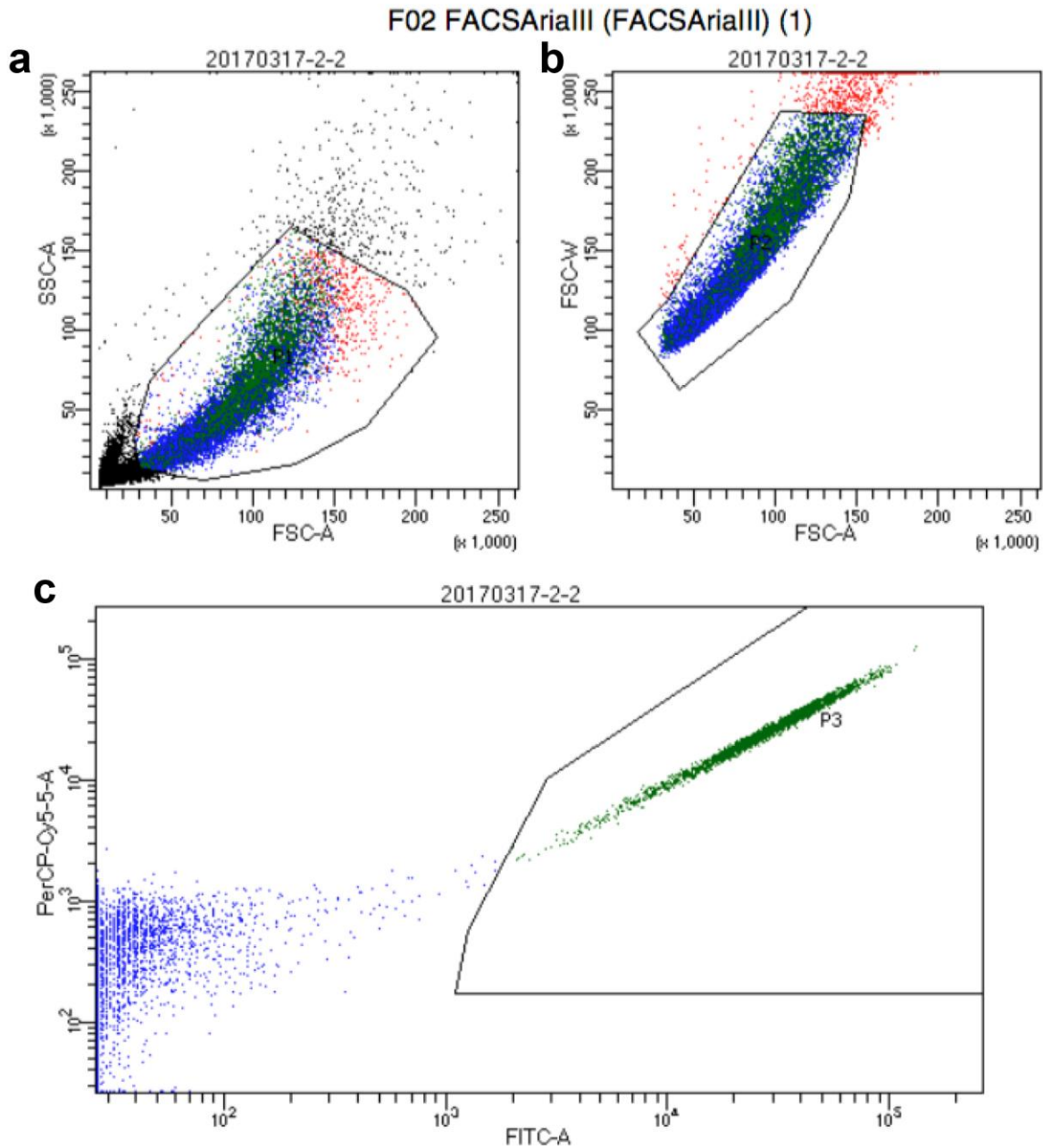


Supplementary Figure 9

**Drug testing assay showed the drug-screening potential of neoCORGs.**

(a) Schematic of luciferase assay-based drug testing strategy on neoCORGs from GBM-1 group. (b) Quantification of relative luciferase activity revealed that EGFR inhibitors Afatinib ( $725.57 \pm 253.71$ ;  $P=0.0076$ ) and Erlotinib ( $716.10 \pm 424.94$ ;  $P=0.0074$ ) significantly reduced luciferase activity in GBM-1 (CDKN2A<sup>-</sup>/CDKN2B<sup>-</sup>/EGFR<sup>OE</sup>/EGFRvIII<sup>OE</sup>) neoCORGs (DMSO: n=9; Canertinib: n=9; Pelitinib: n=8; Afatinib: n=9; Gefitinib: n=9; Erlotinib: n=9). Normalized luciferase activity was presented. This experiment was performed once. Statistical analysis of quantifications was performed using one-way ANOVA with Dunnett's test. Data were presented as mean $\pm$ s.d. All the detailed sample size, mean $\pm$ s.d., as well as  $P$  value are provided in the source data. \*\*,  $P<0.01$ .

## Supplementary Fig.10



Supplementary Figure 10

An example of the gating strategy for FACS analysis.

(a) Gating for live cells. (b) Gating to exclude doublets and cell aggregates. (c) Gating for GFP<sup>+</sup> cells.

**Supplementary Table 1. Genetic aberrations tested in this study**

Groups with gene aberrations	Tumor subtypes
CDKN2A	GBM
CDKN2B	GBM
NF1	GBM
PTEN	GBM
p53	GBM, Pediatric GBM
ATRX	Pediatric GBM
RB1	GBM
CDK4	GBM, Pediatric GBM
MDM2-B	GBM, Pediatric GBM
EGFR	GBM
EGFRvIII	GBM
PDGFRA	GBM, Paediatric GBM
H3F3A-K27M	Paediatric GBM
H3F3A-G34R	Paediatric GBM
MYC	GBM, CNS-PNET, MB
SMARCB1	AT/RT
PTCH1	MB
CTNNB1	MB
CDKN2A/CDKN2B	GBM
CDKN2A/CDKN2B/EGFR	GBM
CDKN2A/CDKN2B/EGFRvIII	GBM
CDKN2A/CDKN2B/EGFR/EGFRvIII	GBM
CDKN2A/CDKN2B/PTEN	GBM
CDKN2A/CDKN2B/p53	GBM
CDKN2A/CDKN2B/PDGFRA	GBM
EGFR/CDK4	GBM
EGFRvIII/CDK4	GBM
EGFR/EGFRvIII/CDK4	GBM
MDM2-B/CDK4	GBM
NF1/PTEN/p53	GBM
EGFRvIII/CDKN2A/PTEN	GBM
H3F3A-K27M/ARTX/p53	Paediatric GBM
H3F3A-G34R/ARTX/p53	Paediatric GBM

**Abbreviation**

GBM: glioblastoma;

CNS-PNET: center nervous system primitive neuroectodermal tumor;

MB: medulloblastoma

AT/RT: atypical teratoid/rhabdoid tumor



**Supplementary Table 2. Primers for cloning oncogenes into sleeping beauty construct**

Gene symbols	Primers	
MYC	upstream	GACGGCGCGCCCGCCACCATGCTGGATTTTTTCGGGTAG
	downstream	GACACCGGTTTACGCACAAGAGTTCCGTAG
EGFR/EGFRvIII	upstream	GACGGCGCGCCCGCCACCATGCGACCTCCGGGACG
	downstream	GACACCGGTTTCATGCTCCAATAAATTCACTG
PDGFRA	upstream	GACGGCGCGCCCGCCACCATGGGGACTTCCCATCCGGCGTTC
	downstream	GACACCGGTTTACAGGAAGCTGTCTTCCACCAG
CDK4	upstream	GACGGCGCGCCCGCCACCATGGCTACCTCTCGATATGAGC
	downstream	GACACCGGTTCACTCCGGATTACCTTCATCC
MDM2-B	upstream	GACGGCGCGCCCGCCACCATGTGCAATACCAACATGTCTG
	downstream	GACACCGGTCTAGGGGAAATAAGTTAGCAC
H3F3A-K27M/ H3F3A-G34R	upstream	CATTTTGGCAAAGAATTCCCTCGATACCGGGGCGCGCCCGCCACCATGGCTCGTACAAAGCAGACTGC
	downstream	CGGGAATGCTAGCAATCATTGGTTGATCAGCTTTGTTACCGGTTTAAGCACGTTCTCCACGTATG

**Supplementary Table 3. Primers for cloning tumor suppressor guide RNAs into CRISPR-Cas9 construct**

Gene symbols	Primers	
CDKN2A	Top	CACCGTCCCGGGCAGCGTCGTGCAC
	Bottom	AAACGTGCACGACGCTGCCCCGGGAC
CDKN2B	Top	CACCGACGGAGTCAACCGTTTCGGG
	Bottom	AAACCCCGAAACGGTTGACTCCGTC
NF1	Top	CACCGCTCGTCGAAGCGGCTGACCA
	Bottom	AAACTGGTCAGCCGCTTCGACGAGC
PTEN	Top	CACCGAACTTGTCTTCCCGTCGTGT
	Bottom	AAACACACGACGGGAAGACAAGTTC
p53	Top	CACCGTCGACGCTAGGATCTGACTG
	Bottom	AAACCAGTCAGATCCTAGCGTCGAC
RB1	Top	CACCGCGGTGGCGGCCGTTTTTCGG
	Bottom	AAACCCGAAAAACGGCCGCCACCGC
ATRX	Top	CACCGAAATTCGAGTTTCGAGCGA
	Bottom	AAACTCGCTCGAAACTCGGAATTC
SMARCB1	Top	CACCGAGAACCTCGGAACATACGG
	Bottom	AAACCCGTATGTTCCGAGGTTCTC
PTCH1	Top	CACCGCAGATAGTCCCGGTCCGGCG
	Bottom	AAACCGCCGGACCGGGACTATCTGC
CTNNB1	Top	CACCGAAACAGCTCGTTGTACCGCT
	Bottom	AAACAGCGGTACAACGAGCTGTTTC

**Supplementary Table 4. Primers for RT-PCR**

Gene symbols	Primers	
MYC	Top	TCGGATTCTCTGCTCTCCTC
	Bottom	CCTGCCTCTTTTCCACAGAA
EGFR/EGFRvIII	Top	CGGGCTCTGGAGGAAAAG
	Bottom	GCCCTTCGCACTTCTTACAC
TBP	Top	GGGCACCACTCCACTGTATC
	Bottom	CGAAGTGCAATGGTCTTTAGG

**Supplementary Table 5. Primers for amplification of CRISPR-Cas9 targeting gene locus**

Gene symbols	Primers	
CDKN2A	Top	CAGTGCTCTCTGCCTGTGAC
	Bottom	TGTGCTGGAAAATGAATGCT
CDKN2B	Top	CGTTAAGTTTACGGCCAACG
	Bottom	GGAATCCCGTCTCATTCTCA
NF1	Top	CGTGGAAGGATCCCACTT
	Bottom	CCCCTAAACGTTACCCACCT
PTEN	Top	TGCTTGAGATCAAGATTGCAG
	Bottom	GCCATAAGGCCTTTTCCTTC
TP53	Top	TTTAGTGGTGGGAAGGTTGG
	Bottom	GGGGACTGTAGATGGGTGAA

**Supplementary Table 6. Primary Antibodies**

Antigen	Species	Company	Catalog No.	Dilution	Application	Datasheet links
BRACHYURY	Goat	R&D Systems	AF2085	1:200	IF	<a href="https://resources.rndsystems.com/pdfs/datasheets/af2085.pdf">https://resources.rndsystems.com/pdfs/datasheets/af2085.pdf</a>
CD31	Mouse	Dako	M0832	1:200	IF	<a href="http://www.finelis.com/product/up_files/M0823.pdf">http://www.finelis.com/product/up_files/M0823.pdf</a>
CD99	Rabbit	Abcam	ab108297	1:500	IF	<a href="http://www.abcam.com/CD99-antibody-EPR3096-ab108297.pdf">http://www.abcam.com/CD99-antibody-EPR3096-ab108297.pdf</a>
FOXF1	Goat	R&D Systems	AF4798	1:200	IF	<a href="https://resources.rndsystems.com/pdfs/datasheets/af4798.pdf">https://resources.rndsystems.com/pdfs/datasheets/af4798.pdf</a>
GFAP	Rabbit	DAKO	Z0334	1:500	IF&IHC	<a href="https://www.agilent.com/en/products/immunohistochemistry/antibodies-controls/multipurpose-antibodies/glia-fibrillary-acidic-protein">https://www.agilent.com/en/products/immunohistochemistry/antibodies-controls/multipurpose-antibodies/glia-fibrillary-acidic-protein</a>
GFP	Chicken	Abcam	ab13970	1:500	IF&IHC	<a href="http://www.abcam.com/GFP-antibody-ab13970.pdf">http://www.abcam.com/GFP-antibody-ab13970.pdf</a>
HuCD	Mouse	Thermo Fisher Scientific	A-21271	1:100	IF	<a href="https://www.thermofisher.com/order/genome-database/generatePdf?productName=HuCD/HuCD&amp;assayType=PRANT&amp;detailed=true&amp;productId=A-21271">https://www.thermofisher.com/order/genome-database/generatePdf?productName=HuCD/HuCD&amp;assayType=PRANT&amp;detailed=true&amp;productId=A-21271</a>
Ki67	Mouse	BD Biosciences	550609	1:100	IF&IHC	<a href="http://www.bdbiosciences.com/ds/pm/tds/550609.pdf">http://www.bdbiosciences.com/ds/pm/tds/550609.pdf</a>
MAP2	Rabbit	Merck Millipore	MAB3418	1:500	IHC	<a href="http://www.merckmillipore.com/AT/de/product/Anti-MAP2-Antibody-clone-AP20_MM_NF-MAB3418">http://www.merckmillipore.com/AT/de/product/Anti-MAP2-Antibody-clone-AP20_MM_NF-MAB3418</a>
MMP2	Rabbit	Abcam	ab92536	1:200	IF	<a href="http://www.abcam.com/MMP2-antibody-EPR1184-ab92536.pdf">http://www.abcam.com/MMP2-antibody-EPR1184-ab92536.pdf</a>
N-CADHERIN	Mouse	BD Biosciences	610920	1:500	IF	<a href="http://www.bdbiosciences.com/ds/pm/tds/610920.pdf">http://www.bdbiosciences.com/ds/pm/tds/610920.pdf</a>
NESTIN	Mouse	BD Biosciences	611658	1:200	IF	<a href="http://www.bdbiosciences.com/ds/pm/tds/611658.pdf">http://www.bdbiosciences.com/ds/pm/tds/611658.pdf</a>
PLAU	Rabbit	Abcam	ab24121	1:200	IF	<a href="http://www.abcam.com/Urokinase-antibody-ab24121.pdf">http://www.abcam.com/Urokinase-antibody-ab24121.pdf</a>
S100 $\beta$	Rabbit	Abcam	ab52642	1:200	IF	<a href="http://www.abcam.com/S100-beta-antibody-EP1576Y-ab52642.pdf">http://www.abcam.com/S100-beta-antibody-EP1576Y-ab52642.pdf</a>
SOX1	Goat	R&D Systems	AF3389	1:200	IF&IHC	<a href="https://resources.rndsystems.com/pdfs/datasheets/af3369.pdf">https://resources.rndsystems.com/pdfs/datasheets/af3369.pdf</a>
SOX2	Rabbit	Abcam	ab97959	1:1000	IF	<a href="http://www.abcam.com/SOX2-antibody-ab97959.pdf">http://www.abcam.com/SOX2-antibody-ab97959.pdf</a>
SOX17	Goat	R&D Systems	AF1924	1:100	IF	<a href="https://resources.rndsystems.com/pdfs/datasheets/af1924.pdf">https://resources.rndsystems.com/pdfs/datasheets/af1924.pdf</a>
VIM	Mouse	Santa Cruz Biotechnology	sc-6260	1:100	IF	<a href="https://datasheets.scbt.com/sc-6260.pdf">https://datasheets.scbt.com/sc-6260.pdf</a>



**Supplementary Table 7. Secondary Antibodies**

<b>Host</b>	<b>Recognizes</b>	<b>Fluophore</b>	<b>Company</b>	<b>Catalog No.</b>	<b>Dilution</b>	<b>Application</b>
Donkey	Chicken	Alexa Fluor 488	Jackson Immuno	703-605-155	1:500	IF
Donkey	Rabbit	Alexa Fluor 568	Invitrogen	A10042	1:500	IF
Donkey	Rabbit	Alexa Fluor 647	Invitrogen	A31573	1:500	IF
Donkey	Mouse	Alexa Fluor 647	Invitrogen	A31571	1:500	IF
Donkey	Mouse	Alexa Fluor 568	Invitrogen	A10036	1:500	IF
Donkey	Goat	Alexa Fluor 568	Invitrogen	A11057	1:500	IF
Goat	Mouse IgG2b	Alexa Fluor 568	Invitrogen	A21144	1:500	IF
Goat	Rabbit	n/a	Dako	E0432	1:500	IHC
Goat	Chicken	n/a	Abcam	Ab97135	1:500	IHC
Rabbit	Goat	n/a	Dako	F0250	1:500	IHC

**Title:**

Genetic engineering to initiate tumorigenesis in cerebral organoids

**Abstract:**

Brain tumours are among the most lethal and devastating cancers. Their treatment is currently limited by the genetic heterogeneity and the incompleteness of available laboratory models. Cancer sequencing projects have identified large numbers of DNA aberrations in brain tumours. But their individual relevance for cancer initiation and progression cannot easily be addressed using existing models. Recently developed three-dimensional culture system that recapitulate early development of human brains, named cerebral organoids, allow us to study various human brain disorders. Here we describe a method combining genome-editing techniques and cerebral organoid culture system to initiate tumorigenesis. This protocol not only provides a platform to test the driver gene aberrations of brain tumours in a rapid, systematic manner, but also give us a great tool to study the initiation mechanism of various brain tumours, including region-specific brain tumour if the starting organoids were pre-patterned.

**Introduction:**

Malignant brain tumours are among the most devastating cancers with almost negligible survival rates. Although the fundamental biology and therapeutic investigations of brain tumours have been explored in a variety of experimental model systems, their survival rate has not improved in decades. In addition, brain tumours are characterized by a wide variety of DNA aberrations that either cause oncogene overexpression or loss of tumour suppressor gene function<sup>1-3</sup>, which is difficult to recapitulate using existing models. Thus, complementary models using state-of-the-art experimental model systems are required for brain tumour investigation.

Three-dimensional organoid culture technology allows the development of complex, organ-like tissues reminiscent of in vivo development<sup>4,5</sup>. Based on this cerebral organoid culture system, we introduced gene aberrations via two different genome-editing techniques, Sleeping Beauty transposon system<sup>6,7</sup> for gain-of-function, and CRISPR-Cas9 system for loss-of-function<sup>8,9</sup>. By combining those two genome-editing techniques, we could recapitulate a wide variety of tumorigenic events. This protocol introducing various combinations of clinical-relevant gene aberrations in cerebral organoids to mimic the brain tumour initiation provides us a powerful tool to study human brain tumour biology and to investigate therapeutic strategies.

**Reagents:****CELLS**

- Feeder-free (FF) and Feeder-dependent (FD) H9 human embryonic stem cells (hESCs, WiCell Research Institute, Wisconsin, USA) were both used to generate cerebral organoids in this study.
- CF-1-gamma-irradiated mouse embryonic stem cells (MEFs) (GSC-6001G, Global Stem) was used for FD H9 hESC culture.

**PLASMIDS**

- Overexpression constructs for eGFP/oncogene integration were based on Sleeping Beauty Transposase system. pCAG-SB100X were cloned from pCMV(CAT)T7-SB100 (Addgene cat. No.: 34879)<sup>7</sup> and pCAGEN (Addgene cat. No.: 11160)<sup>10</sup>. pCAG-GS/IR was cloned from pT2/LTR7-GFP (Addgene cat. No.: 62541)<sup>11</sup> and pCAGEN.
- For introduction of gene mutations, short guide RNAs of tumour suppressors were cloned into CRISPR/Cas9 vector pX330-U6-Chimeric\_BB-CBh-hSpCas9 (Addgene cat. No.: 42230)<sup>8</sup>.

**REAGENTS**

- mTeSR1 medium (Stem Cell Technologies, cat. no. 05850)

- DMEM/F12 (Invitrogen, cat. no. 31330-038)
- Neurobasal medium (Invitrogen, cat. no. 21103049)
- N2 supplement (Invitrogen, cat. no. 17502048)
- B27 without vitamin A supplement (- Vit. A) (Invitrogen, cat. no. 12587010)
- B27 with vitamin A supplement (+Vit. A) (Invitrogen, cat. no. 17504044)
- Knockout serum replacement (KOSR) (Invitrogen, cat. no. 10828-028)
- hESC-quality FBS (Gibco, cat. no. 10270-106)
- GlutaMAX (Invitrogen, cat. no. 35050-038)
- Heparin (Sigma, cat. no. H3149)
- Rock inhibitor Y27632 (RI) (Millipore, cat. no. SCM075)
- Insulin solution (Sigma, cat. no. I9278-5ML)
- Matrigel, hESC-Qualified (Corning, cat. no. 354277)
- Matrigel (Corning, cat. no. 354234)
- CELLBANKER<sup>®</sup> 2 (Amsbio, cat. no. 11891)
- Sterile PBS (DPBS without Ca<sup>2+</sup>/Mg<sup>2+</sup>; Thermo Fisher Scientific, cat. no. 14190-169)
- Water For Injection (WFI) for Cell Culture; Thermo Fisher Scientific, cat. no. A1287301)
- Penicillin/Streptomycin (P/S) (Sigma, cat. no. P0781)
- Minimal essential medium non-essential amino acids (MEM-NEAA): (Sigma cat. no. M7145)
- 2-Mercaptoethanol (2-ME) (Merck, cat. no. 8057400005)
- bFGF (FGF2; Peprotech, cat. no. 100-18B)
- Collagenase IV (Gibco, cat. no. 17104-019)
- Dispase (Sigma, cat. no. 17105-041)
- Trypsin-EDTA (Gibco, cat. no. 25300-054)
- Trypsin inhibitor (Sigma, cat. no. T6414-100ML)
- Accutase solution (Sigma, cat. no. A6964-100ML)
- EDTA (Sigma-Aldrich, cat. no. E6758)
- Human Stem Cell Nucleofector<sup>®</sup> Kit 1 (Lonza, cat. no. LONVVPH-5012)

### **Equipments:**

- CO<sub>2</sub> incubators (New Brunswick, model Galaxy 170s)
- Biological safety cabinet (Faster Safefast Premium 212)
- Sterile microcentrifuge tubes (1.5-ml size; Fisher Scientific, cat. no. 05-408-129)
- Stericup 0.2-µm filter unit (500 and 250 ml; Millipore, cat. nos. SCGVU02RE SCGVU05RE, respectively)
- Steriflip 50 ml filter unit (Millipore, SCGP00525)
- U-bottom ultra-low attachment plates, 96 well (Corning, cat. no. 7007)
- Conical tubes, 15 ml (Greiner Cell Star, cat. no. 188271)
- Parafilm (Sigma-Aldrich, cat. no. P7793)
- Six-well tissue culture dishes (Eppendorf, cat. no. 0030720113)
- Tissue culture dish, 60 mm (Eppendorf, cat. no. 00307701119)
- Tissue culture dish, 100 mm (Eppendorf, cat. no. 0030702115)
- Gilson Pipetman (P1000, P200 and P10)
- Sterile filter pipette tips (P1250, P300, P20, P10 µl; Biozym, cat. nos. VT0270, VT0250, VT0220, respectively)
- Orbital shaker (Infors Celltron orbital shaker, cat. no. INF-69222)
- Pipet boy (Integra Biosciences, cat. no. 155 000)
- Serological pipettes, 5, 10, 25 ml (BD Falcon, cat. nos. 357543, 357551, 357525, respectively)
- Sterilized scissors
- Water bath, 37 °C (Fisher Scientific, Isotemp water bath, model 2333, cat. no. 15-462-21Q)
- Inverted tissue culture microscope (Zeiss, model Axio Vert.A1)
- Automated cell counter (Invitrogen, Countess II)

- Cell counter slides (Countess Cell Counting Chamber Slides, Thermo Fisher Scientific, cat. no. C10228)
- Trypan blue (included with cell counting slides)
- Benchtop centrifuge (Eppendorf, cat. no. 5810)
- Vacuum pump (Integra, Vacusafe)
- 2 ml Aspiration pipettes (Falcon, cat. no. 35755)
- Nucleofector™ 2b (Lonza, cat. no. AAB-1001)
- Tissue embedding mold (Thermo Fisher Scientific, cat. no. 1220)

## **Procedure:**

### **REAGENT SETUP**

- hESC medium

To prepare 500 ml of hESC medium, 400 ml of DMEM/F12, 100 ml of KOSR, 15 ml of FBS, 5 ml of GlutaMAX, 5 ml of MEM-NEAA, and 3.5 µl of 2-ME were mixed together and sterile-filtered with a 22 µm filter bottle. FGF2/bFGF and/or RI were added freshly just before usage. Medium can be stored at 4 °C for up to 2 weeks after preparation.

- Neural Induction (NI) medium

To prepare 500 ml of NI medium, 500 ml of DMEM/F12, 5 ml of N2 supplement, 5 ml of GlutaMAX, 5 ml of MEM-NEAA, and 500 µl of Heparin solution were mixed and sterile-filtered using a 22 µm filter bottle. Medium can be stored at 4 °C for up to 2 weeks after preparation.

- Differentiation Medium

To prepare 500 ml of differentiation medium, 250 ml of DMEM/F12, 250 ml of Neurobasal, 2.5 ml of N2 supplement, 5 ml of B27 (with or without vitamin A supplement), 125 µl of Insulin, 175 µl of a 1:100 solution of 2-ME (in DMEM/F12), 5 ml of GlutaMAX, 2.5 ml of MEM-NEAA, and 5 ml of P/S solution were mixed and sterile-filtered using a 22 µm filter bottle. Medium can be stored at 4 °C for up to 2 weeks after preparation.

- FGF2/bFGF stock solution

To prepare FGF2/bFGF stock solution (10 µg/ml), 50 µg of FGF2/bFGF was reconstituted in 5 ml PBS +0.1% BSA, and aliquoted into 50 or 100 µl aliquots. Aliquots can be stored at -20 °C for up to 1 year.

- Heparin stock solution:

Heparin stock solution (1 mg/ml) were prepared in PBS, and stored at -20°C for up to 1 year.

- Rock Inhibitor (RI) stock solution

To prepare RI stock solution, 5 mg of RI was reconstituted in 2.96 ml of H<sub>2</sub>O, and aliquoted into 0.5-1 ml aliquots. Aliquots can be stored at -20 °C.

- Gelatin solution for coating

Gelatin solution was prepared as 0.1% wt/vol in H<sub>2</sub>O. For 500 ml solution, 0.5 g of Gelatin were reconstituted in H<sub>2</sub>O at 50 °C, and sterile-filtered with a 22 µm filter bottle. Gelatin solution can be stored at 4 °C for up to 1 year.

- Collagenase IV solution

Collagenase IV solution was prepared as 1 mg/ml in DMEM/F-12 medium, and sterile-filtered with a 22 µm filter. Aliquots can be stored at -20 °C for up to 6 months.

- Dispase solution

Dispase solution was prepared as 0.5 mg/ml in DMEM/F-12 medium, and sterile-filtered with a 22 µm filter. Aliquots can be stored at -20 °C for up to 6 months.

## PROCEDURE

### A) Cell maintenance

- FF hESCs

FF hESCs were cultured in a feeder-free manner on Matrigel-coated plate with mTeSR medium in a 5% CO<sub>2</sub> incubator at 37 °C. For coating, low-growth-factor Matrigel (0.5 mg per 6-well plate) was dissolved in ice-cold DMEM/F12. FF hESCs were routinely splitted using 0.5 mM EDTA in PBS.

- FD hESCs

FD hESCs were maintained with hESC medium containing 20 ng/ml FGF2/bFGF in a 5% CO<sub>2</sub> incubator at 37 °C on the gelatin- and MEF (1.87×10<sup>5</sup> cells/well)-coated 6-well cell culture plates. FD hESCs were routinely passaged using collagenase IV solution (0.1% wt/vol in H<sub>2</sub>O).

### B) Generation of Cerebral organoids

#### Day 0, Embryoid body (EB) formation

1. Single cell suspension was prepared as described previously using hESCs cultured in either feeder-independent or feeder-dependent manner <sup>4</sup>.
2. Cell density was counted using automated cell counter. Nine thousand live cells/well in 150 µl hESC medium containing RI (1:100) and low FGF2/bFGF (1:2500, 4 ng/ml) were seeded in a 96-well low-attachment U-bottom cell culture plate.

#### Day 3, Exchanging medium

3. The medium was exchanged with fresh hESC medium without RI and FGF2/bFGF.

#### Day 5 or 6, Neural induction (NI)

4. hESC medium was replaced by NI medium to induce neural lineage differentiation when the sizes of EBs are more than 500 µm.

#### Day 5 or 6 to Day 11 or 12, Neuroectoderm expansion

5. NI medium was exchanged every second days with fresh NI medium for 6 days.

! Attention: EBs with expanded radialized neuroepithelial structure were selected for the further procedure.

#### Day 11 or 12, Nucleofection of plasmid cocktails to introduce gene mutations/amplifications

6. Nucleofector solution is prepared according to manufacturer's protocol by mixing 82 µl of solution 1 and 18 µl of supplement 1 for one reaction. Maximum 5 µg of plasmid cocktail, including 500 ng of transposase expression vector pCAG-SB100X, 1 µg of transposon vector for pCAG-eGFP, and 1 µg of each transposon vectors to express oncogene and/or CRISPR-Cas9 vectors were added into nucleofector solution.
7. About 15 EBs were collected, washed with PBS, resuspended with nucleofection solution with plasmid cocktails, and transferred into nucleofection cuvettes.

! Attention: a) Using widened pipette tips to transfer EBs.

b) Gentle operation is required for entire procedure to avoid the damage of EBs.

c) More than 15 EBs performed with nucleofection using Nucleofector™ 2b (Lonza) will significantly reduce the nucleofection efficiency.

8. EBs in the nucleofection cuvettes were nucleofected under program A-023 using Nucleofector™ 2b.
9. EBs in the nucleofection cuvettes were gently added 1 ml of NI medium, and poured out into 6-cm dishes containing NI medium, and incubated in a 5% CO<sub>2</sub> incubator at 37 °C for overnight.



#### Day 12 or 13, EB embedding

10. EBs were embedded into matrigel based on previous described procedure. Briefly, EBs were transferred onto parafilm in 6- or 10-cm petri dish. The excess medium was carefully removed.
11. EBs were embedded into a droplet of matrigel, and adjusted into the center of the droplet before the matrigel droplet solidifies. Embedded EBs then were incubated into 37 °C incubator for 20 min to solidify the matrigel.
12. Differentiation medium (-A) was added onto EBs to wash embedded EBs off the parafilm. The parafilm was discarded from the petri dish.
13. Embedded EBs were cultured in a 5% CO<sub>2</sub> incubator at 37 °C without shaking.

#### Day 16 or 17, Differentiation of cerebral organoids

14. Differentiation medium (-A) were replaced by differentiation medium (+A).
15. The dish was transferred onto an orbital shaker in a 5% CO<sub>2</sub> incubator at 37 °C.

#### Day 16 or 17 to Day 40, initiation of neoplastic cerebral organoids

16. Medium was changed twice a week with differentiation medium (+A).

#### Day 40 and later, Neoplastic cerebral organoids selection for further analysis

17. Neoplastic cerebral organoids with overgrowth of GFP-positive mutated cells will be collected for further analysis.
18. Differentiation medium (+A) was exchanged twice per week for neoplastic cerebral organoid culture to the desired age of collection for subsequent analyses such as immunofluorescence staining, RNA-seq, renal implantation, drug testing, and viral infection.

#### **Timing:**

- Steps 1-2, making EBs: 1-2 hours
- Step 3, feeding EBs with hESC medium: 2-3 days
- Steps 4-5, feeding EBs with NI medium: 6 days
- Steps 6-8, nucleofection of EBs: 2-3 hours
- Step 9, EB recovery from nucleofection: overnight
- Steps 10-13, EB embedding into matrigel droplet: 1-3 hours
- Steps 14-15, Static culture of embedded EBs: 4 days
- Step 16, culture of neoplastic cerebral organoids on orbital shakers: 24 days
- Step 17, selection of neoplastic cerebral organoids for further analysis: 2 hours
- Steps 18-19, culture of neoplastic cerebral organoids for desired analyses: for different analyses, neoplastic cerebral organoids were cultured for different times. To analyse more “matured” neoplastic organoids, the RNA-seq and immunofluorescence staining were performed on neoplastic organoids older than 120 days. To investigate the ZIKV tropism toward tumour cells, 130-160-days old neoplastic cerebral organoids were used.

#### **Troubleshooting:**

Problem: Nucleofection efficiency is too low.

Solution 1: Reduce the numbers of EBs numbers per nucleofection cuvettes

Solution 2: Using different nucleofector such as NEPA21 super electroporator (NEPAGENE)

Problem: Nucleofected EBs were destroyed during embedding

Solution 1: Operate gently when transfer EBs, especially when pour EBs out after nucleofection

Solution 2: Recover nucleofected EBs in the 5% CO<sub>2</sub> incubator at 37 °C for longer period

Problem: Tumour sizes are variable among different neoplastic cerebral organoids

Solution: As current experimental setup, the mutation cells undergoes a self-selection process for tumour growth. Although similar amount cells were nucleofected among EBs, how many cells indeed carrying mutations after nucleofection is not controllable. To reduce the variability for certain analysis such as drug testing, the organoids containing similar tumour regions will be selected.

### **Anticipated Results:**

The overgrowth of GFP-labelled neoplastic cells could be observed two weeks after introduction of gene aberrations in MYC<sup>OE</sup> groups, while the overgrowth of neoplastic cells in other groups could be observed 1 month after nucleofection. Along the culture, tumour regions in neoplastic cerebral organoids become bigger and bigger. In four-months-old neoplastic organoids, tumour regions in most neoplastic organoids are dominant.

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### **Associated Publications:**

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##### Competing financial interests

The authors declare no competing financial interests.

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