

1 **SUPPLEMENTARY MATERIAL AND METHODS**

2 **Sex as a biological variable**

3 All information regarding the sex origin of the patient material can be found in the table on
4 Supplementary Figure 1A. In regards of GD2 expression and CAR T-therapy response, all patient-
5 derived models, regardless of sex, responded positively and sensitively to the therapy. Sex did
6 also not play a role in the selection of any tissues and models. For the in vivo experiment, all mice
7 were female, due to housing regulations. Additionally, the model engraftment was established in
8 female mice originally.

9

10 **Patient clinical history**

11 Information regarding clinical history and molecular characteristics from the patient tissue of origin
12 at the Máxima can be found in the table in Supplementary Figure 1A.

13

14 **DNA-methylation clustering analysis and data availability**

15 DNA methylation profiles were updated to the Swiss EPDiP Database (<https://epidip.usb.ch>),
16 search numbers can be found atop their respective CNV plots, the lookup numbers are:

17 DKFZ-EP1NS: 208813900021_R08C01

18 PMC-ZFTA-01: 208813900022_R06C01

19 PMC-ZFTA-02: 208813900021_R07C01

20 DKFZ-BT232: 208813900022_R03C0

21 PMC-PFA-01: 208813900022_R01C01

22 PMC-PFA-03: 208813900021_R06C01

23 PMC-PFA-04: 208813900022_R04C01

1 **Tissue processing and cell culturing**

2 Fresh tissue biopsies and CUSA material were disaggregated through repeated mincing and were
3 processed according to a brain tumor dissociation protocol (130-095-942, Miltenyi), and then
4 cultured at 37°C and 5% CO₂ in modified Tumor Stem Medium (TSM), consisting of 48%
5 DMEM/F12, 48% Neurobasal-A medium, 1% HEPES 1M, 1% MEM Non-essential amino acids,
6 1% 100 mmol/L sodium pyruvate, 1% GlutaMAX, supplemented with 2% B27, 1% N2 (all from
7 Thermo Fisher Scientific), 20 ng/mL human EGF, 20 ng/mL human bFGF, 10 ng/mL human
8 PDGF-AA, 10 ng/mL human PDGF-BB (all from Peprotech), and 5 IU/mL heparin (Leo Pharma,
9 Ballerup, Denmark). To achieve long-term culture, adherent monolayers of the models were
10 cultured in TSM with flasks and plates previously coated with poly-L-ornithine (Sigma-Aldrich
11 P4957) and laminin (Sigma-Aldrich L2020), all according to an already established protocol (6).
12 DKFZ-EP1NS and DKFZ-BT232 cells were a kind gift from Prof. Dr. Marcel Kool.

13

14 Of note, PMC-ZFTA-01 and PMC-PFA-04 showed no diagnostic match after methylation profiling.
15 However, deviation from the original classifier diagnosis is a well-known side effect of culture
16 establishment, since the methylation classifier was trained using tissues which are inherently
17 heterogeneous (5). Nonetheless, the PMC-ZFTA-01 CNV methylation profile, as well as the short
18 tandem repeat (STR) profile matched the original patient material, which was confirmed to be a
19 ST-ZFTA EPN.

20

21 **Immunofluorescence**

22 To perform the cytopsin protocol, tumor cells were made single cell suspension using accutase,
23 and then counted with trypan blue (Thermo Fischer T10282,). Cells were then resuspended in
24 cold PBS 2% FCS for a final concentration of 5×10^5 cells/mL. 100 μ L of ice-cold PBS were added
25 to an already mounted cytopsin cuvette and holder, and then centrifuged at 161 RCF for three

1 min to properly pre-wet the carton filter. Then, 200 μ L of cell suspension were added into their
2 respective cytospin cuvette and cells were spun at 161 RCF for seven min. Slides were air dried
3 overnight. The following day, slides were washed three times with TBS 0.1% Tween 20 (Sigma-
4 Aldrich 11332465001), and blocked using western blot (WB) blocking buffer for
5 immunofluorescence (Rockland, MB-070) for 20 min. Subsequently, slides were stained with a
6 pre-conjugated GD2-PE Antibody (BD Biosciences 562100) diluted 1:200 times in WB blocking
7 buffer, and left overnight at 4°C in the dark. Finally, slides were washed three times with TBS 0.1%
8 Tween 20 and three more times with PBS and stained for DAPI using NucBlue Staining Kit
9 (Thermo Fischer R37606), according to the manufacturer's instructions. Slides were then imaged
10 in a DMI Thunder Microscope (Leica Microsystems) at 20x and 63x objectives accordingly.

11

12 In order to perform the GD2 staining on primary patient material, resection-derived FFPE
13 materials from EPN patients were processed and stained for GD2 by the Prinses Maxima Center's
14 diagnostic department, according to an already established protocol by Kailangiri et al. (PMID:
15 22374462, PMID: 39046881). The primary GD2 antibody was obtained from BD Pharmingen
16 (Cat# 554272) and the secondary antibody was an anti-mouse Opal 520 reagent
17 (Cat#FP1487001KT). Slides were then imaged in the research department in a DMI Thunder
18 Microscope (Leica Microsystems) at 20x objective.

19

20 **Flow cytometry**

21 GD2 membrane stainings were obtained by making a single cell suspension of the tumor cells
22 using accutase. Cells were then allotted to 96-well plates ($\sim 1 \times 10^5$ cells/well). Zombie Violet
23 (BioLegend 423113) was used to exclude dead cells, and staining for GD2 was done using a pre-
24 conjugated GD2 PE Antibody (BD Biosciences 562100), diluted 1:200 times in cold PBS. Cells

1 were then washed two times with ice-cold PBS and Analyzed on a Cytoflex LS (Supplementary
2 Figure 3).

3

4 To perform the GD2-CAR T-cell co-culture, tumor cells were made single cell suspension using
5 accutase at RT, counted with trypan blue and stained with 5 μ M CellTrace Violet Cell Proliferation
6 Kit™ (Thermo Fischer C34557). Cells were then plated in an already-coated Poly-L-
7 Ornithine/laminin 96 well u-bottom Sarstedt plate, according to an already published protocol (5),
8 at a density of 2×10^4 cells/well. One day later, GD2-CAR T-cells and the matched donor
9 untransduced T-cell controls were counted and added according to their respective effector to
10 target (E:T) ratios. Finally, after 48 h, cells were centrifuged and supernatants were stored at -
11 80°C for further analysis (ELISA), and tumor killing was measured using a 7AAD-Staining solution
12 (Miltenyi 130-111-568) according to the manufacturer's instructions.

13

14 **ELISA**

15 ELISA was performed using an IFN- γ Human uncoated ELISA kit (Invitrogen 15531107) according
16 to the manufacturer's instructions.

17

18 **Incucyte live imaging**

19 Tumor cells were made single cell using accutase at RT and counted with trypan blue. Cells were
20 then plated in an already-coated Poly-L-Ornithine/laminin 96 well u-bottom Sarstedt plate,
21 according to an already published protocol (5), at a density of 2×10^4 cells/well. One day later,
22 GD2-CAR T-cells and matched donor untransduced T-cells were counted and added at an E:T of
23 1:2. Killing was then monitored for 5 days using the IncuCyte® S3 Live-Cell Analysis System cell
24 imaging system by Sartorius. GUI version 2023Arev2.

1

2 **Statistics**

3 All statistical analyses were performed using GraphPad Prism version 10.4.1 for Windows,
4 (GraphPad Software, Boston, MA, USA, www.graphpad.com). Two-tailed unpaired Student's t-
5 test was used to compare data from the untransduced controls vs the GD2-CAR T-cell at the
6 highest ratio. Two-way-ANOVA was used to perform analysis in tumor-killing and IFN- γ secretion
7 of the tumor models against a GD2-negative control (VUMC-ATRT-03). A p value less than 0.05
8 was considered significant. Non-linear regression fit was used to assess the anti-tumor effect of
9 the therapeutic agents. Multilevel regression analysis (MLR) was performed to assess the survival
10 benefit and control tumor growth in the *in vivo* experiments.

11

12 **Study approval**

13 Patient materials were collected at the Princess Máxima Center for Pediatric Oncology, according
14 to national and institutional guidelines and in accordance with the declaration of Helsinki. All
15 included patients provided written informed consent for participation in the biobank (International
16 Clinical Trials Registry Platform, NL7744). Prinses Maxima Centrum voor kinderoncologie,
17 Heidelberglaan 25, 3584 CS, Utrecht. All animal experiments were carried out according to
18 governmental and institutional guidelines and approved by the Regierung-spräsidium Karlsruhe
19 (permit number: 35-918.81/G-4/20). Department 35, Schlossplatz 4-6, 7631 Karlsruhe.

20

21 ***In vivo* experiments**

22 Overall, two different pilot experiments were performed. For the first trial, 5×10^5 luciferase-
23 expressing DKFZ-BT232 cells (kindly gifted by Prof. Dr. Marcel Kool) were orthotopically
24 transplanted into the cerebellum of 12 NOD.Cg-*Prkdc*^{scid} Il2rg^{tm1Wjl}/SzJ mice purchased from

1 Charles River Laboratories (Sulzfed, Germany). Two weeks after injection, BLI was monitored
2 weekly using the IVIS Lumina (Perkin Elmer) imaging system. Once the average BLI signal
3 exceeded 1×10^7 (large tumor burden), treatment was started. For the second trial, another 12
4 NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were transplanted identically. However, once the
5 average BLI signal in this study reached 1×10^6 (small tumor burden), treatment was started. All
6 mice were then intravenously primed with 1×10^6 GD2-CAR T-cells per mice, followed by an
7 intraventricular injection of 7×10^6 GD2-CAR T-cells per mice one week later. BLI was then
8 monitored weekly. Both experiments were discontinued 150 days after tumor injection due to local
9 legislation. During both intervention trials, no treatment related toxicities were observed.

10

11 **Schematics**

12 For the GD2-CAR T-cell co-culture, the symbols used were: cancerous-cell-5 icon by Servier
13 <https://smart.servier.com/> is licensed under CC-BY 3.0 Unported
14 <https://creativecommons.org/licenses/by/3.0/>, T Cell. NIAID NIH BIOART Source.
15 bioart.niaid.nih.gov/bioart/508, simple_receptor_2 icon by Helicase 11 undefined is licensed
16 under CC-BY 4.0 Unported <https://creativecommons.org/licenses/by/4.0/>, NIAID Visual & Medical
17 Arts. (07/10/2024). Flow Cytometer. NIAID NIH BIOART Source. bioart.niaid.nih.gov/bioart/160.

18 For the in vivo experiment set up, the symbols used were:
19 T_Maze_Test_Mouse_Placed_in_the_Start_Arm icon by DBCLS
20 <https://togotv.dbcls.jp/en/pics.html> is licensed under CC-BY 4.0 Unported
21 <https://creativecommons.org/licenses/by/4.0/>, NIAID Visual & Medical Arts. (07/10/2024). TCell
22 Receptor. NIAID NIH BIOART Source. bioart.niaid.nih.gov/bioart/510, NIAID Visual & Medical
23 Arts. (07/10/2024). Lab Mouse. NIAID NIH BIOART Source. bioart.niaid.nih.gov/bioart/279, NIAID
24 Visual & Medical Arts. (07/10/2024). T Cell. NIAID NIH BIOART Source.
25 bioart.niaid.nih.gov/bioart/508

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5 foundation (<https://stophersentumoren.nl/>), as well as the KiTZ Máxima Collaborative Grant, call
6 22.

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8 **Conflict of interest**

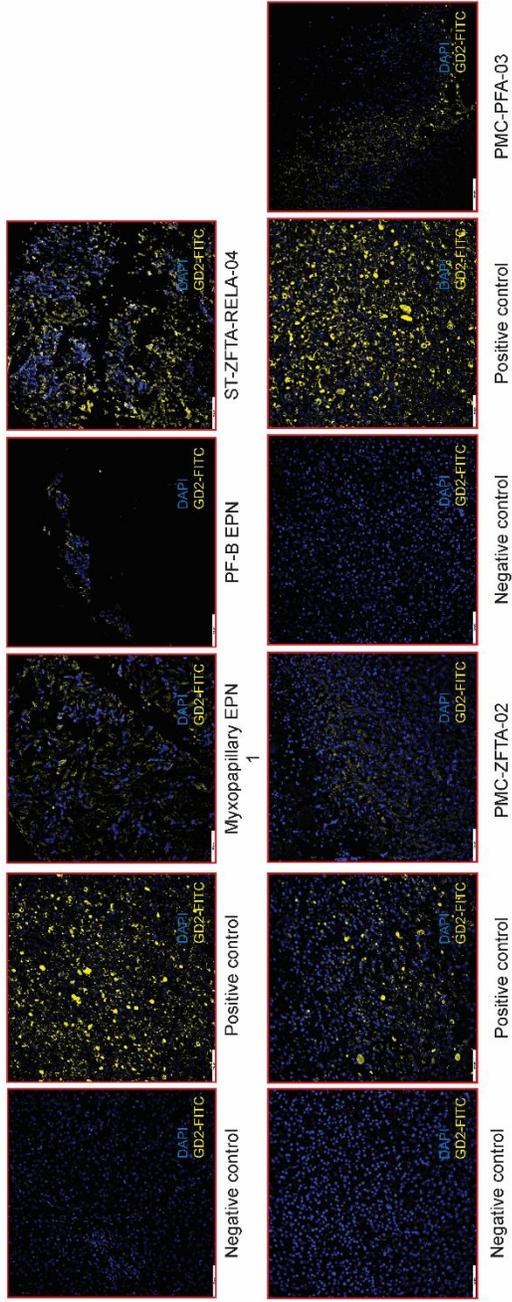
9 The authors declare no conflict of interest.

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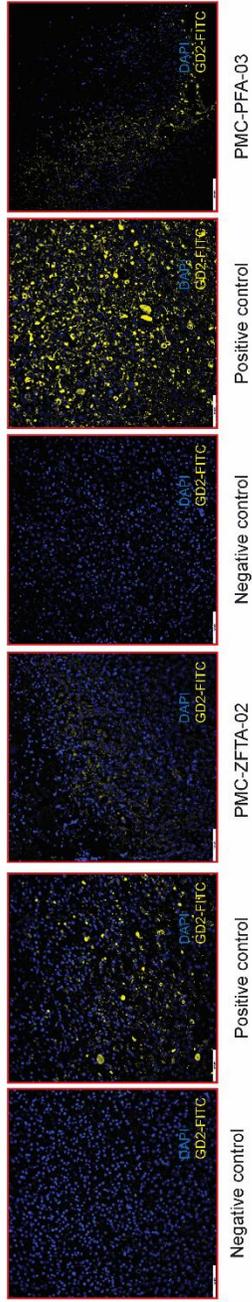
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Celline	Patient characteristics										Histology	Molecular characteristics							IHC							
	Patients age	Sex	Location of tumor	Primary tumor or relapse	Location of relapse	Therapy previous to relapse	Relapse in RT field	Total resection	Outcome	Localized/ metastasized disease at diagnosis		Grade I, II or III	Methylation	Score	1q gain	6q loss	Mutations	Fusions	Losses	Amplifications	EZH4p	H3K27me3	H3K27M1	Ki67	pS6	L1CAM
PMC-PFA-01	5	F	posterior fossa	primary tumor	n.a.	decanmethason	n.a.	yes	in follow up	localised	Grade III	PFAgroup IA, subclass II	0.89	no	no	HCC3(NM_025254.2):c.85A>T (p.S28W) and A3XL1.	none	none	none	n.a.	loss	positive 100%	n.a.	n.a.	n.a.	n.a.
PMC-PFA-03	4	F	frontal lobe	relapse	distant	RT	No	No	in follow up	metastasized	Grade III	PFA	0.89	yes	yes	none	none	none	Stochy positive	loss	negative 80%	n.a.	n.a.	n.a.	n.a.	
PMC-PFA-04	3	M	posterior fossa	primary tumor	n.a.	decanmethason	n.a.	yes	in follow up	localised	Grade III	PFA	0.89	yes	no	none	none	none	Strongly positive	loss	negative 100%	n.a.	n.a.	n.a.	n.a.	
PMC-ZFTA-01	15	M	high/l frontal lobe	relapse	local	radiotherapy	yes	yes	in follow up	localised	Grade III	ZFTA-RELA fusion-positive subtype A	0.98/0.88	no	no	none	C11orf95(NM_001144836.1) exon 3 and RELA(NM_021975.4) exon 2	Homozypes loss of CDKN2A/B	none	n.a.	n.a.	n.a.	50% positive	positive		
PMC-ZFTA-02	6	M	left parietal lobe	primary tumor	n.a.	decanmethason	n.a.	yes	in follow up	localised	Anaplastic ependymoma grade III	ZFTA-RELA fusion-positive subtype A	0.89	no	no	none	C11orf95(NM_001144836.1) exon 3 and RELA(NM_021975.4) exon 2	Homozypes loss of CDKN2A/B	none	n.a.	n.a.	n.a.	30% positive	strongly positive		

B

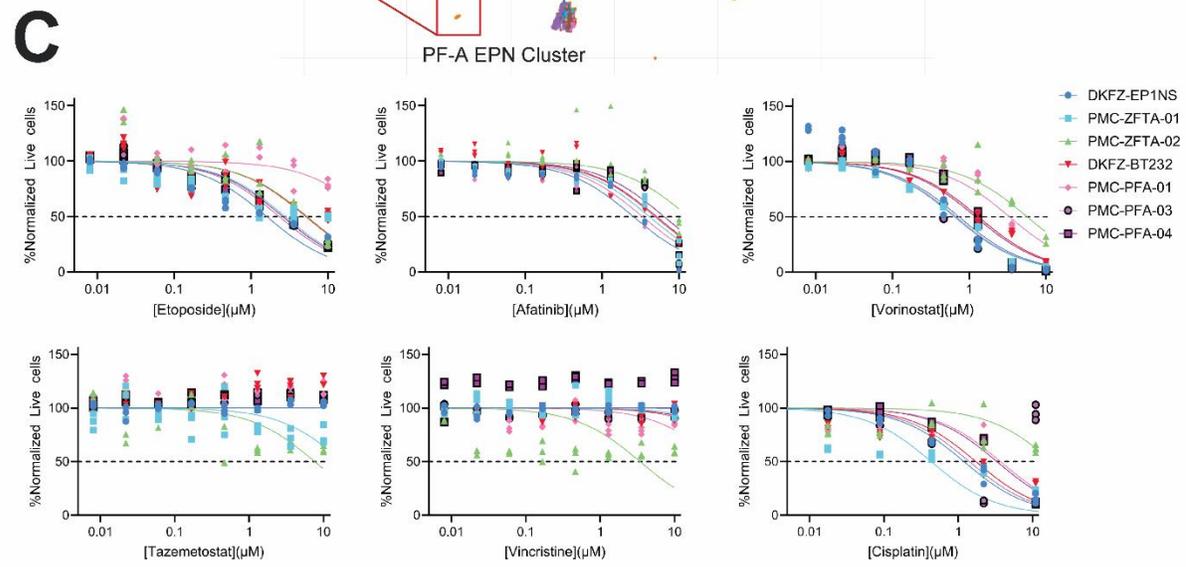
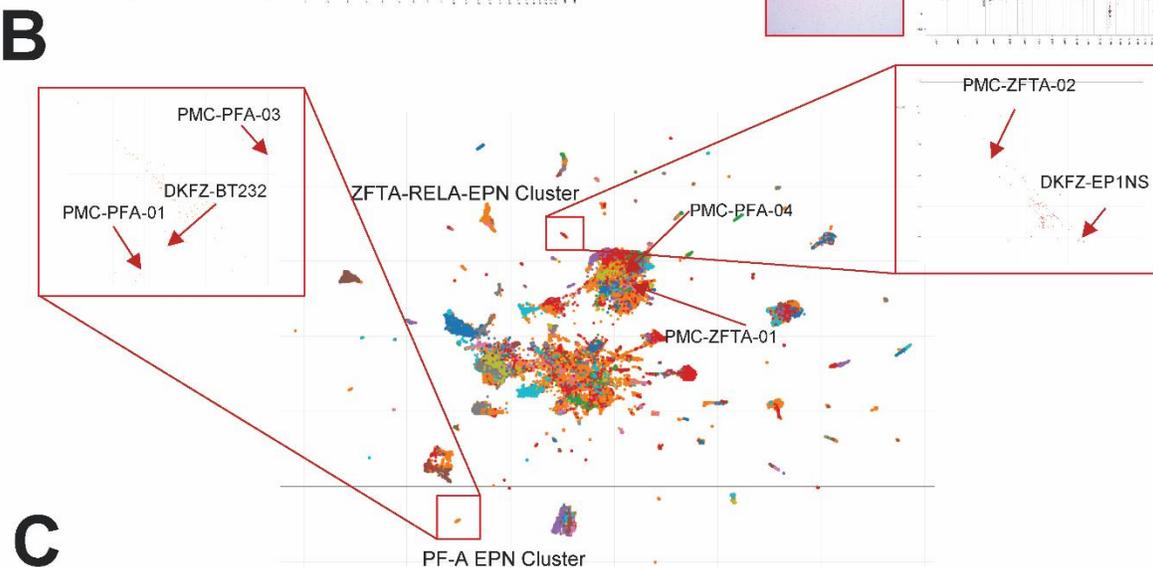
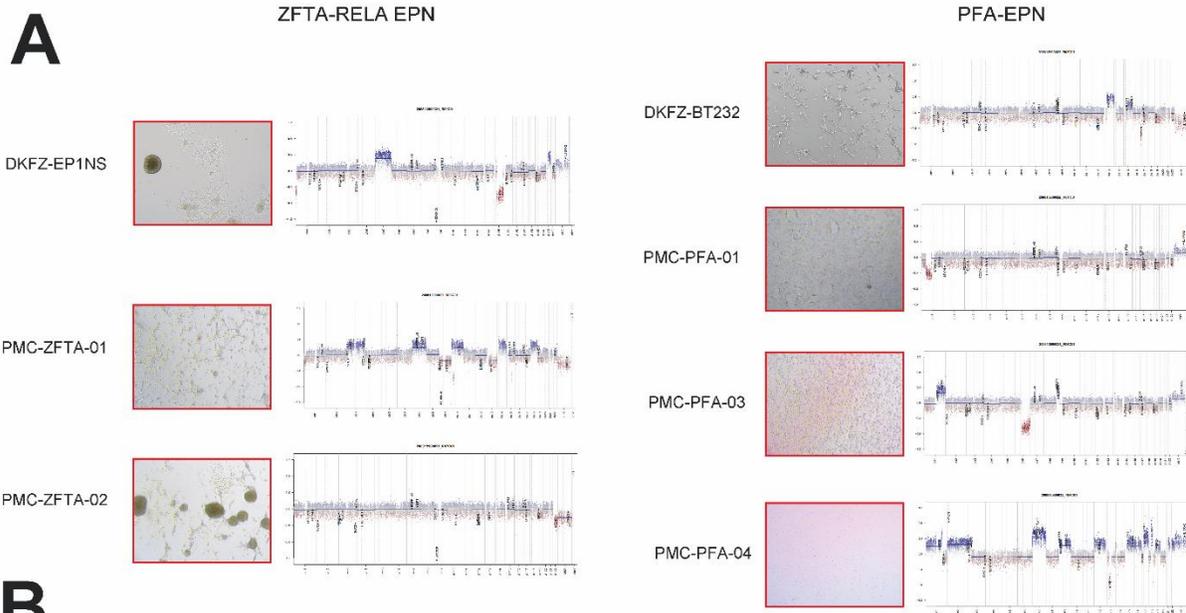


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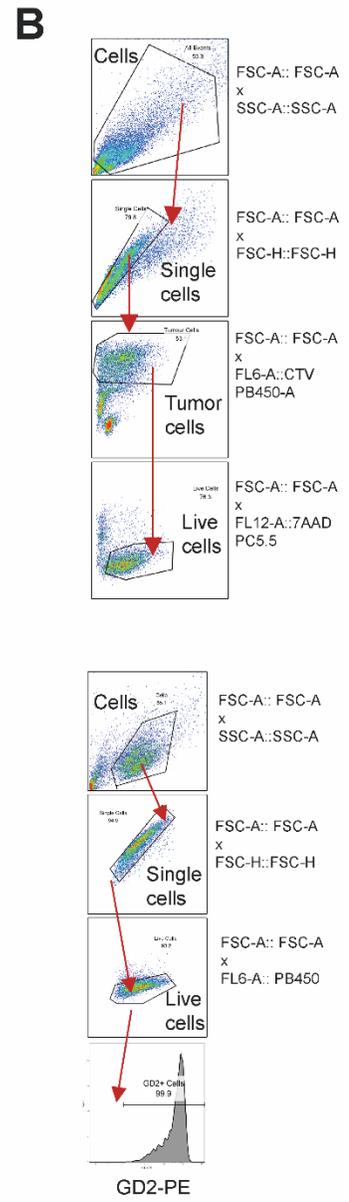
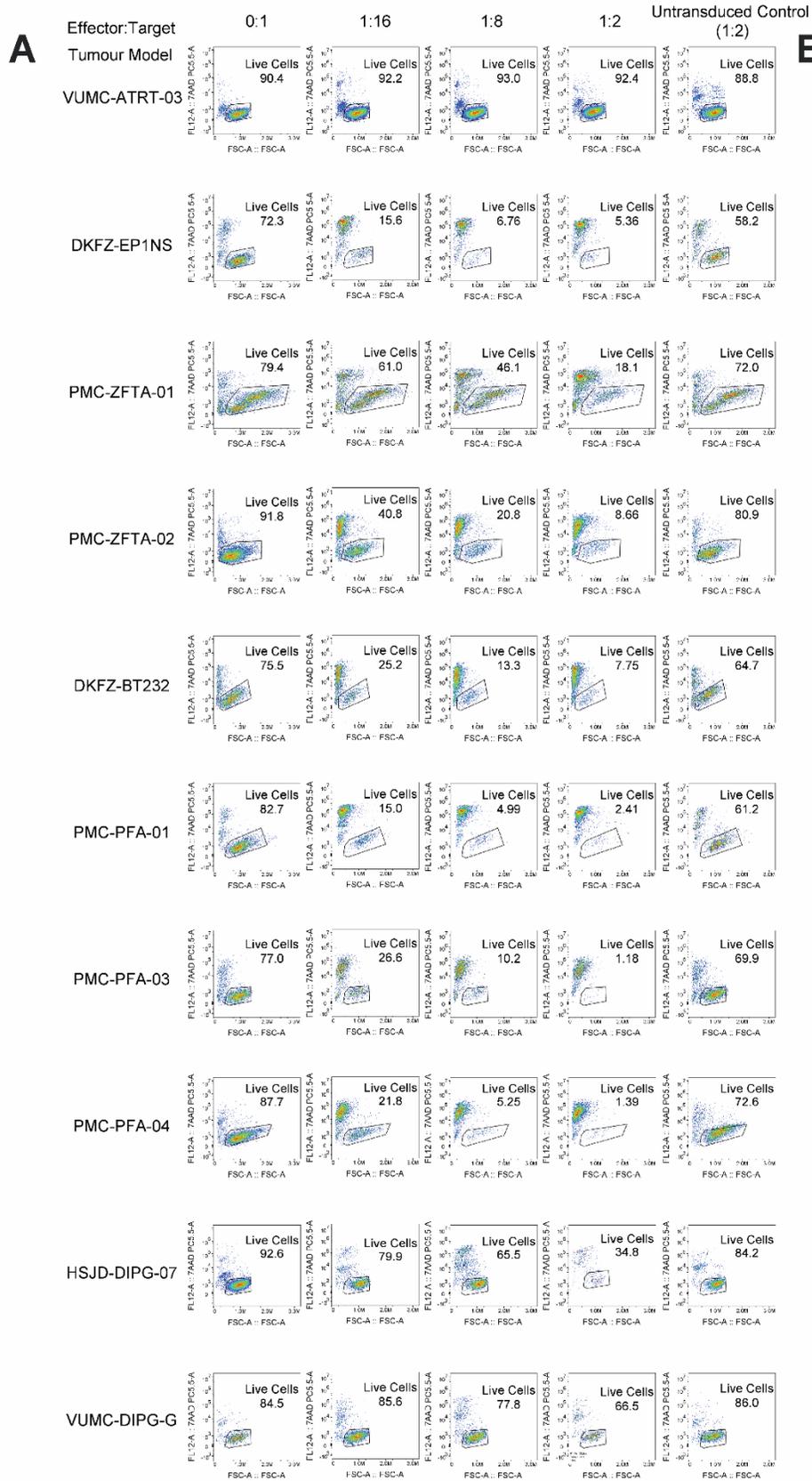
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Supplementary Figure 1. Patient characteristics and GD2 stainings across multiple EPN tissues. **A** Table containing each patient characteristics. **B** GD2 Patient stainings of an ependymoma tissue microarray. **C** GD2 Staining of original biopsy material from PMC-ZFTA-02 and PMC-PFA-03. Bar represents 100 μm . Each slide had its own in-slide positive and negative control. Positive controls are neuroblastoma tumor tissue. Negative controls are healthy liver tissue. An objective of 20x was used.

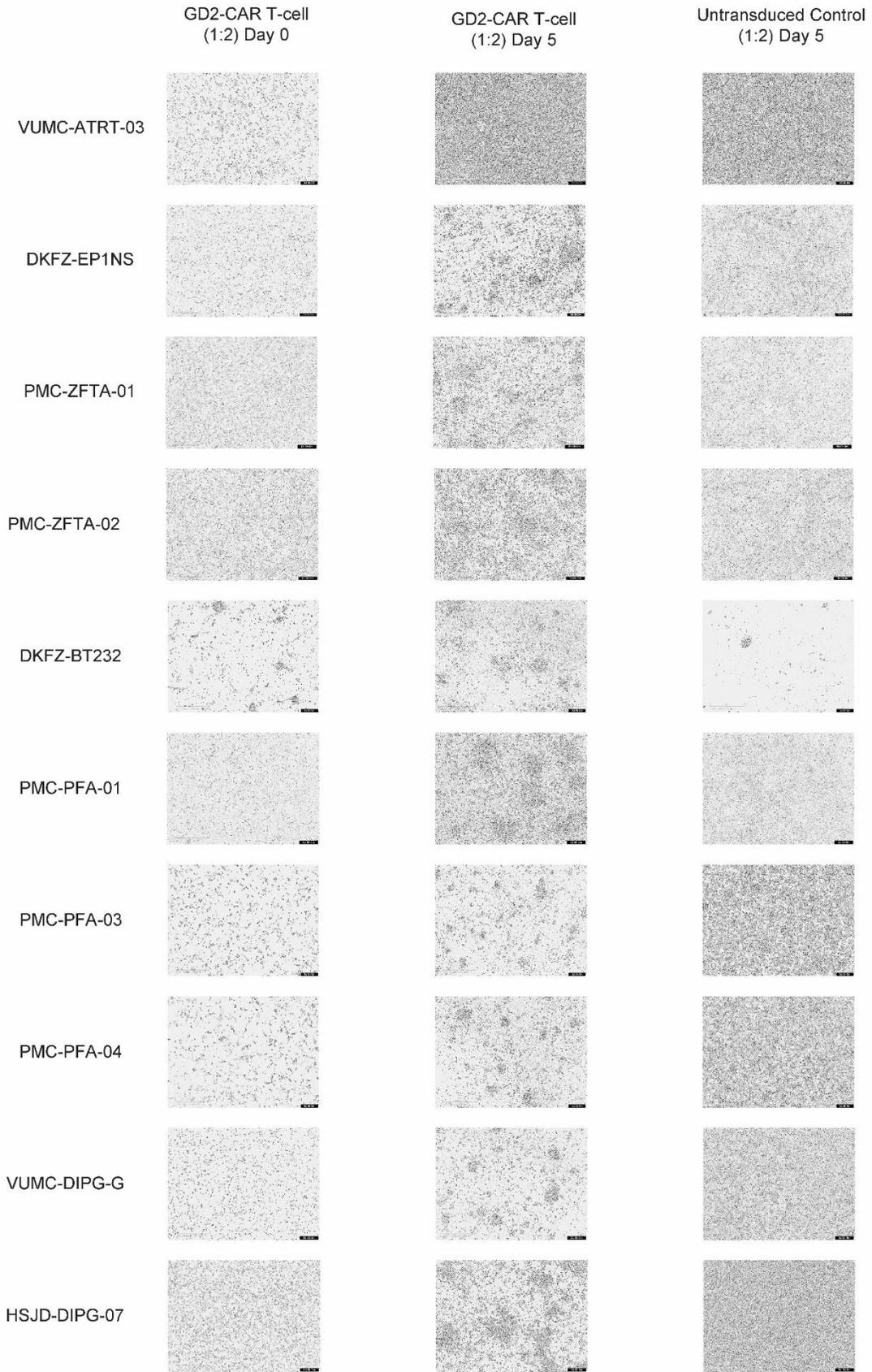


1 **Supplementary Figure 2.** Validation of the EPN models through DNA methylation and drug screening. **A** Pictures of
2 EPN models along with their respective CNV plots. **B** DNA-methylation unbiased clustering analysis in the EPDiP Swiss
3 database for tumor classification. PMC-ZFTA-01 shows up as PXA (0.55 score) due to location of the recurrence, short
4 tandem repeats as well as CNV plot match those of the original patient material. **C** Measurement of the cell viability
5 CellTiterGlo assay after treatment with increasing concentrations of chemotherapeutics etoposide, afatinib, vorinostat,
6 tazemetostat, vincristine and cisplatin. Each dots represents a technical replicate.

7



- 1 **Supplementary Figure 3.** Representative raw dot plots of GD2-CAR T- cell co-culture and gating strategy. **A:**
- 2 Representative raw dot plots for the quantification of killing. **B:** Gating strategy for GD2-CAR T-cell co-culture (upper)
- 3 and of GD2 assessment (lower).



- 1 **Supplementary Figure 4.** *Real-time tumor clearance of GD2-CAR T-cells. Image of all models in co-culture with either*
- 2 *GD2-CAR T-cells (left and center) or matched donor untransduced T-cells (right). At day 0 and day 5 after co-culture.*
- 3 *The videos showing the real-time tumor clearance can be found at Mendeley Repository URL:*
- 4 *<https://data.mendeley.com/datasets/z8rrkwckxm/1>.*