Extended Data

Brain mimetic co-culture experiments identify mGluR1 dependence as a vulnerability of lung cancer brain metastasis

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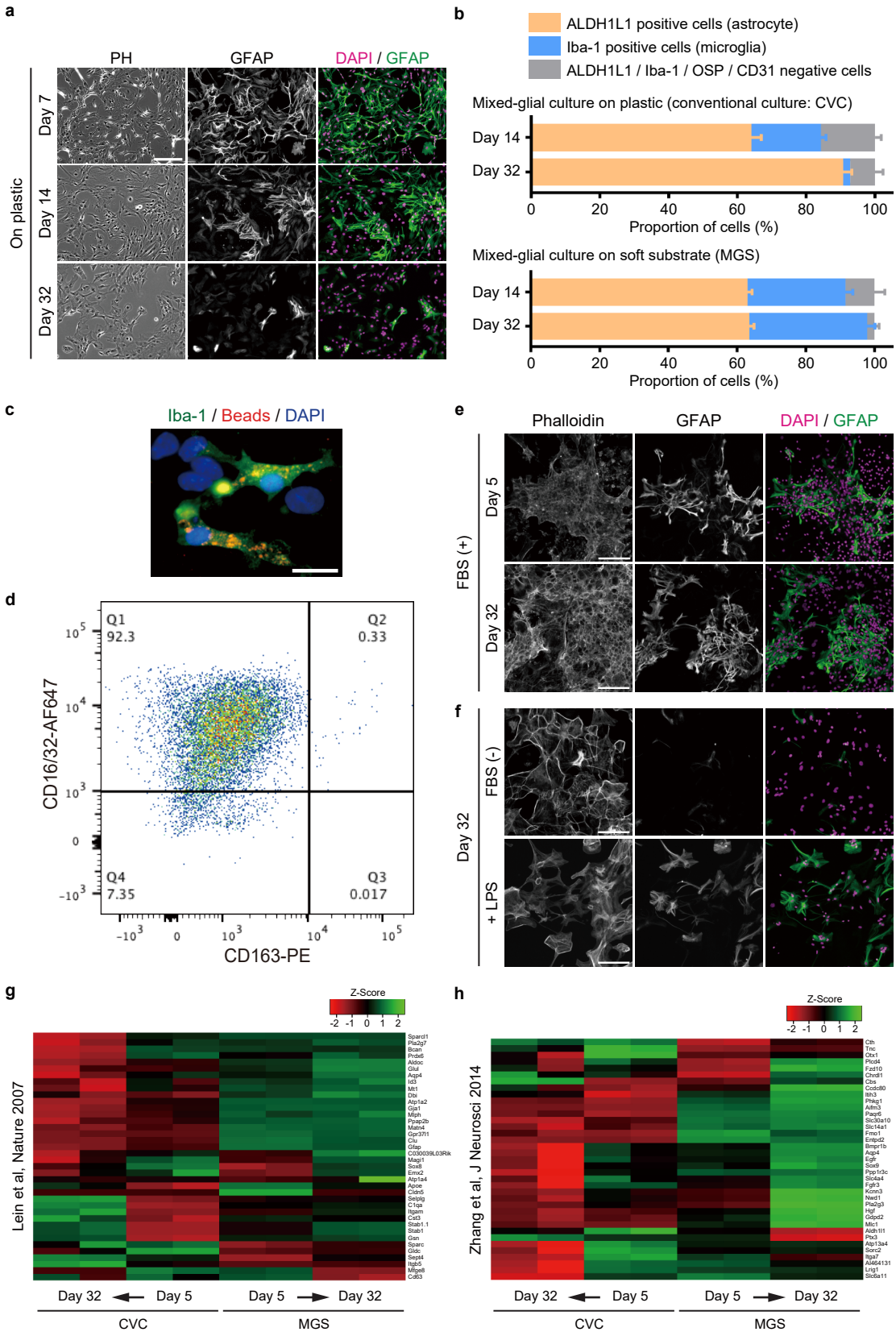
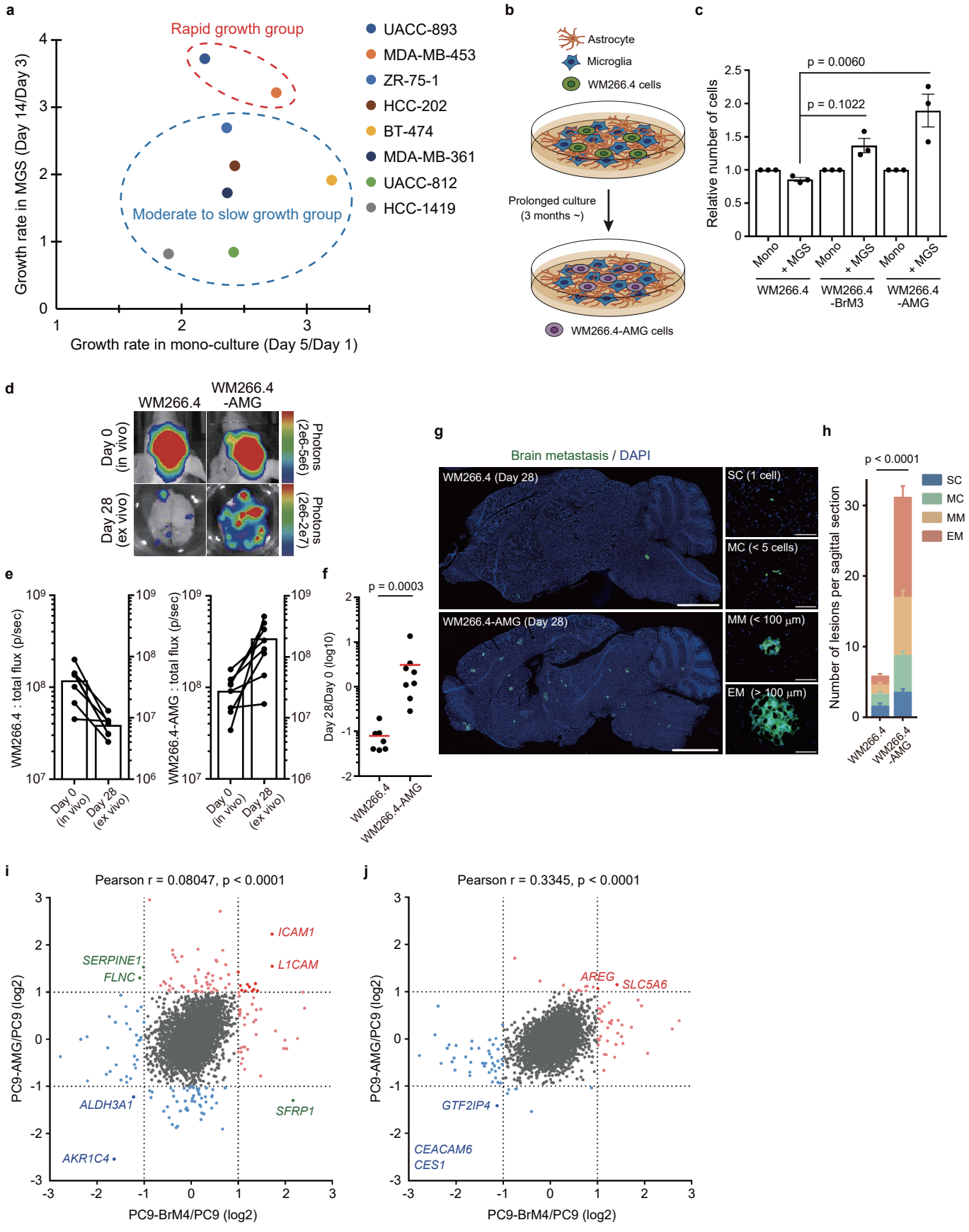
- **Extended Data Figures and Legends 1-9**
- **Supplementary Files S1-S5**



Extended Data Fig. 1 Ishibashi et al.

Extended Data Fig. 1: MGS stably maintains astrocytes and microglia. a, Astrocytes derived from C57BL/6 neonatal mouse brains were cultured on a plastic dish in DMEM supplemented with 10% FBS. Cells were fixed and stained with GFAP and DAPI at days 7, 14 and 32. Scale = 100 μm. **b,** The proportion of astrocyte and microglia cultured on a plastic dish (upper graphs) or in MGS (lower graphs). Mixed-glial cells were fixed stained with the indicated antibodies at days 14 and 32 (n = 3, independent experiments). The bar indicates mean ± SEM. **c,** A representative image of Iba-1-positive microglia that phagocytize carboxylate-modified polystyrene fluorescent latex beads. Green; Iba-1, Red, carboxylate-modified polystyrene fluorescent latex beads, Blue; DAPI. **d,** CD11b-positive microglia in MGS were fractionated with CD163 (x-axis) and CD16/32 (y-axis) expression. **e,** Representative images of MGS stained with phalloidin, GFAP, and DAPI at days 5 (upper panels) and 32 (lower panels). Scale = 100 μm. **f,** Representative images of MGS stained with an anti-GFAP antibody, phalloidin and DAPI after serum removal (upper panels) and re-stimulation with LPS (lower panels) at day 32. Scale = 100 μm. **g** and **h,** Comparative gene expression analysis of astrocytes cultured with the conventional method (CVC) or in MGS. RNAs were extracted at days 5 and 32. The astrocyte gene sets are from Lein et al., Nature 2007 (**g**), and Zhang et al., J Neurosci 2014 (**h**). Please also see Supplementary File S1.



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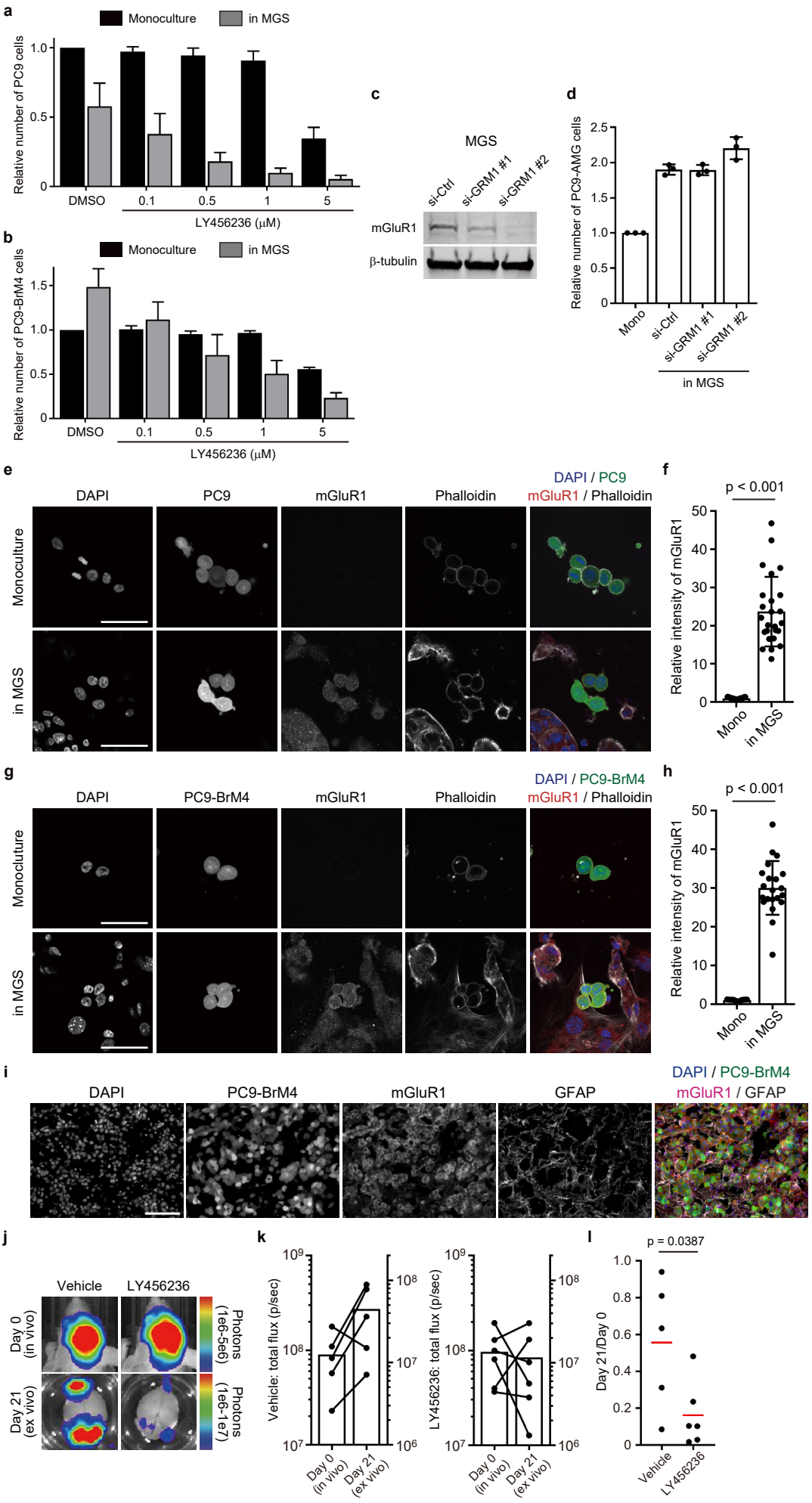
Extended Data Fig. 2: Cancer cells cultured in MGS acquire brain metastatic capabilities. a, A panel of HER2-positive breast cancer cell lines were subjected to in vitro proliferation assay. The x-axis indicates the growth rate in monoculture on soft collagen gels (Day 5/Day 1) and the y-axis in MGS (Day 14/Day 3). **b,** An illustration of prolonged culture of WM266.4 cells in MGS to establish WM266.4-AMG cells. **c,** Relative growth of WM266.4, WM266.4-BrM3 and WM266.4-AMG cells cultured on soft collagen gels (mono) or in MGS for 72 hours (n = 3). The bars indicate mean ± SD. **d-f,** Representative images of bioluminescence detection from mouse brains with WM266.4 or WM266.4-AMG brain metastases (day 0, in vivo and day 28, ex vivo). (**d**) The total photon flux at day 0 (left y-axis) and day 28 (right y-axis) (**e**) and the ratio of the total photon flux (Day 28/Day 0) (**f**) are shown (n = 7 for WM266.4 and n =8 for WM266.4-AMG). The bars indicate the mean. **g,** Representative images of sagittal brain sections with WM266.4 or WM266.4-AMG brain metastases. The four stages of brain metastasis progression are shown in the small panels. SC: single-cell, MC: micro-cluster, MM: micro-metastasis, EM: established metastasis. Scale = 2.5 mm (large panels), 100 μm (small panels). **h,** The number of metastatic lesions per a sagittal brain section at day 28 is shown. 28 slices from 7 mice (WM266.4) and 32 slices from 8 mice (WM266.4-AMG) were used for the quantification. The bars indicate mean ± SEM. **i, j,** A scatter plot showing the fold change of gene expression in PC9-BrM4 (x-axis) and PC9-AMG (y-axis) compared with parental PC9 cells. RNAs were extracted from the cells cultured in a conventional manner (**i**) or co-cultured in MGS (**j**). Genes upregulated > 2-fold in PC9-BrM4 or PC9-AMG are represented with red spots and downregulated < 0.5-fold are represented with blue spots. Bright-red and bright-blue spots indicate genes upregulated > 2-fold or downregulated < 0.5-fold on one side, respectively. Green spots indicate genes upregulated > 2-fold on one side and downregulated < 0.5-fold on the other side. Genes with the highest expression levels, in the top 20% (average of PC9, PC9-BrM4, and PC9-AMG), were used for analysis. Please also see Supplementary Files S2 and S3.



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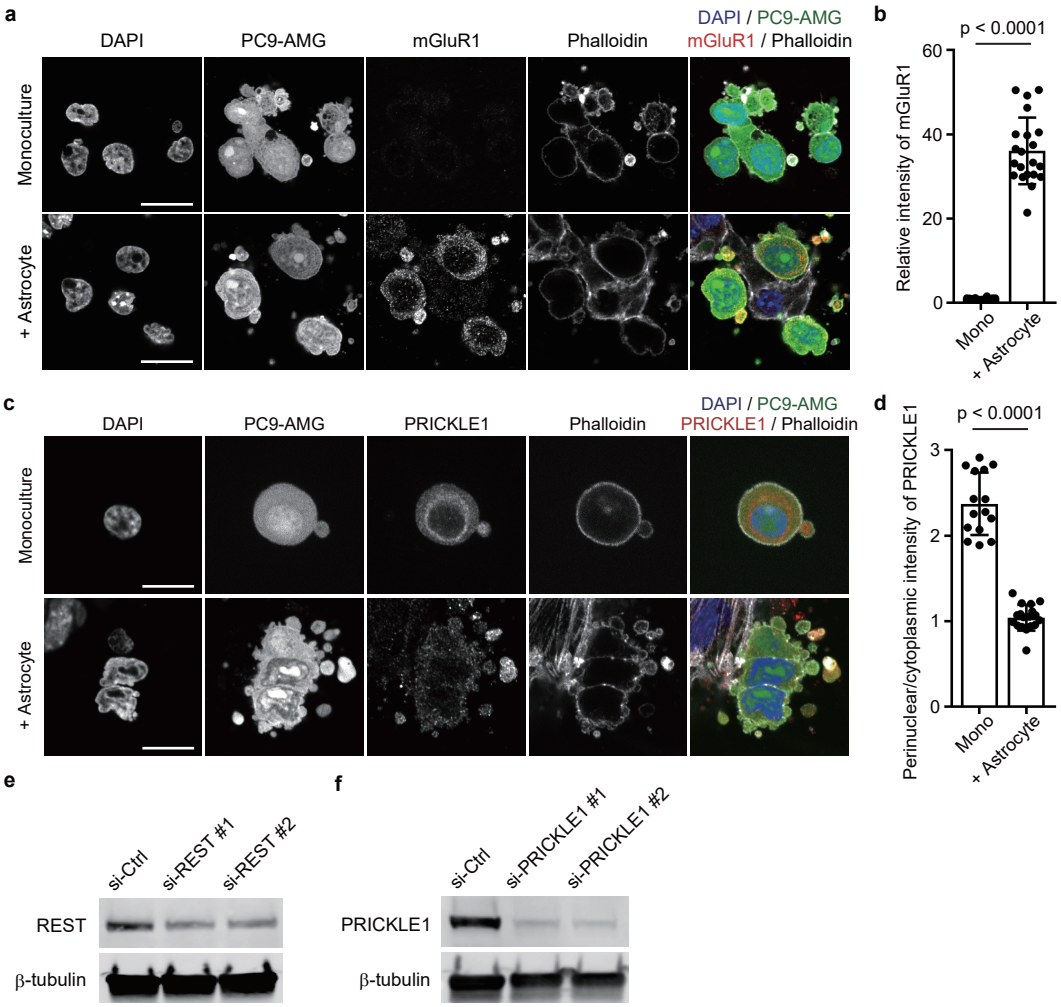
Extended Data Fig. 3: LY456236 suppresses the growth of PC9 and PC9-BrM4 cells cultured in MGS. a, b, PC9 (**a**) or PC9-BrM4 (**b**) cells cultured in 3D collagen gels (monoculture) or in MGS were treated with DMSO (0.01%) or LY456236 (0.1-5 μM) for 48 hours. The relative number of viable PC9 or PC9-BrM4 cells was quantified (n = 3). The bars indicate mean ± SD. **c, d,** Mixed-glial cells were treated with control siRNA or siRNA targeting *GRM1* (**c**), and PC9-AMG cells were monocultured or co-cultured with these mixed-glial cells in 3D collagen gels. The relative number of viable PC9-AMG cells was quantified (n = 3) (**d**). The bars indicate mean ± SD. **e, f,** Representative images of PC9 cells cultured in 3D collagen gels (monoculture) or co-cultured in MGS, stained with an anti-mGluR1 antibody, DAPI and phalloidin (**e**). The relative intensity of mGluR1 was quantified (n = 19 for monoculture condition and n = 25 for MGS co-culture condition) (**f**). Scale = 50 μm. **g, h,** Representative images of PC9-BrM4 cells cultured in 3D collagen gels (monoculture) or co-cultured in MGS, stained with an anti-mGluR1 antibody, DAPI and phalloidin (**g**). The relative intensity of mGluR1 was quantified (n = 13 for monoculture condition and n = 22 for MGS co-culture condition) (**h**). Scale = 50 μm. **i,** Representative images of PC9-BrM4 cells in the mouse brain (day 28), stained with an anti-mGluR1 antibody, anti-GFAP antibody and DAPI. Scale = 100 μm. **j-l,** PC9-BrM4 cells metastasized to the mouse brain were treated with vehicle or LY456236 (50 mg/kg/day) for 14 days. Representative images of bioluminescence detection (day 0, in vivo and day 21, ex vivo) (**j**), the total photon flux from the brain at day 0 (left y-axis) and day 21 (right y-axis) (**k**), and the ratio of the total photon flux (Day 21/Day 0) (**l**) are shown (n = 5 for vehicle and n = 6 for LY456236). The bars indicate the mean.



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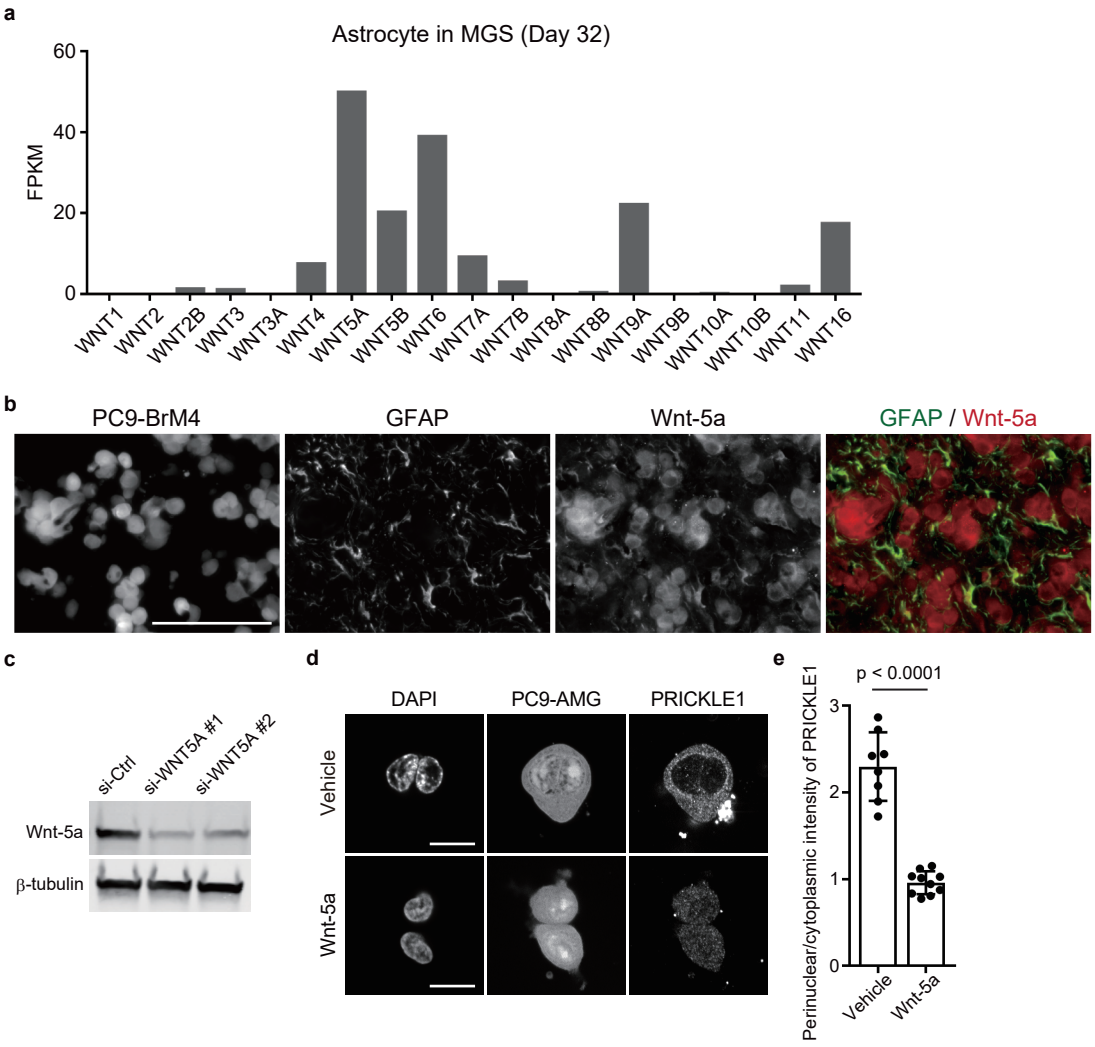
Extended Data Fig. 4: Astrocytes induce mGluR1 expression in PC9-AMG cells through PRICKLE1 translocation. a, b, Representative images of PC9-AMG cells cultured with or without astrocytes in 3D collagen gels, stained with an anti-mGluR1 antibody, DAPI and phalloidin (**a**). The relative intensity of mGluR1 was quantified (n = 14 for monoculture condition and n = 21 for astrocyte co-culture condition) (**b**). Scale = 20 μm. **c, d,** Representative images of PC9-AMG cells cultured with or without astrocytes in 3D collagen gels, stained with an anti-PRICKLE1 antibody, DAPI and phalloidin (**c**). The ratio of perinuclear and cytoplasmic intensity of PRICKLE1 was quantified (n = 15 for monoculture condition and n = 18 for astrocyte co-culture condition) (**d**). Scale = 20 μm. **e, f,** PC9-AMG cells treated with control siRNA or siRNAs targeting *REST* (**e**) or *PRICKLE1* (**f**) were immunoblotted with the indicated antibodies.



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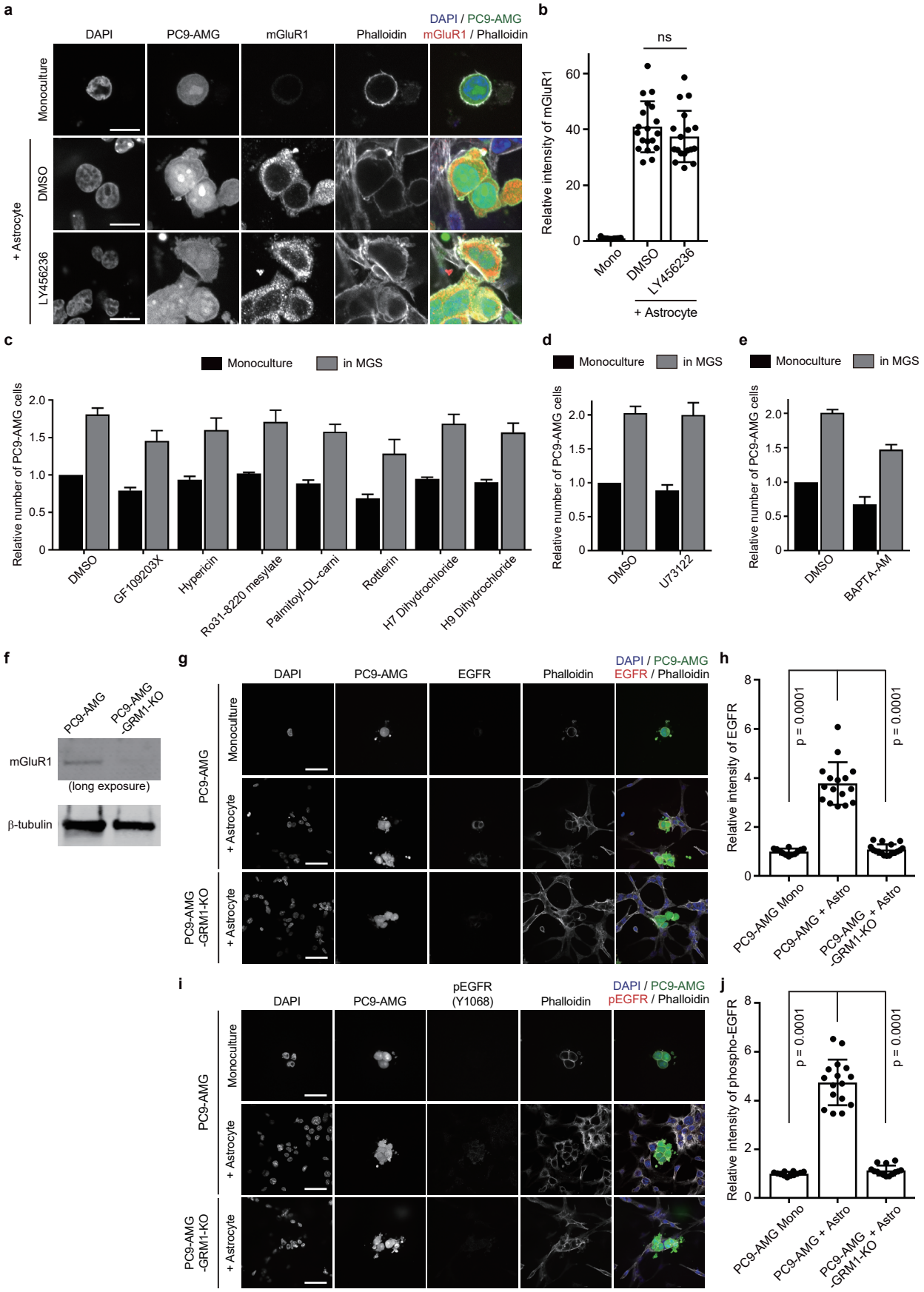
Extended Data Fig. 5: Wnt-5a translocate PRICKLE1 and REST in cancer cells. a, mRNA expression (FPKM) of *WNT* family genes in astrocytes in MGS at day 32. Please also see Supplementary File S1. **b,** Representative images of PC9-BrM4 cells in the mouse brain (day 28), stained with an anti-GFAP antibody, anti-Wnt-5a antibody and DAPI. Scale = 100 μm. **c,** Immunoblotting of astrocytes treated with control siRNA or siRNA targeting *WNT5A*. **d, e,** Representative images of PC9-AMG cells cultured in 3D collagen gels and treated with vehicle (PBS with 0.01% BSA) or Wnt-5a (500 ng/ml) for 24 hours. Cells were fixed and stained with an anti-PRICKLE1 antibody, counterstained with DAPI (**d**). The ratio of perinuclear and cytoplasmic intensity of PRICKLE1 was quantified (n = 8 for monoculture condition and n = 10 for astrocyte co-culture condition) (**e**). Scale = 20 μm.



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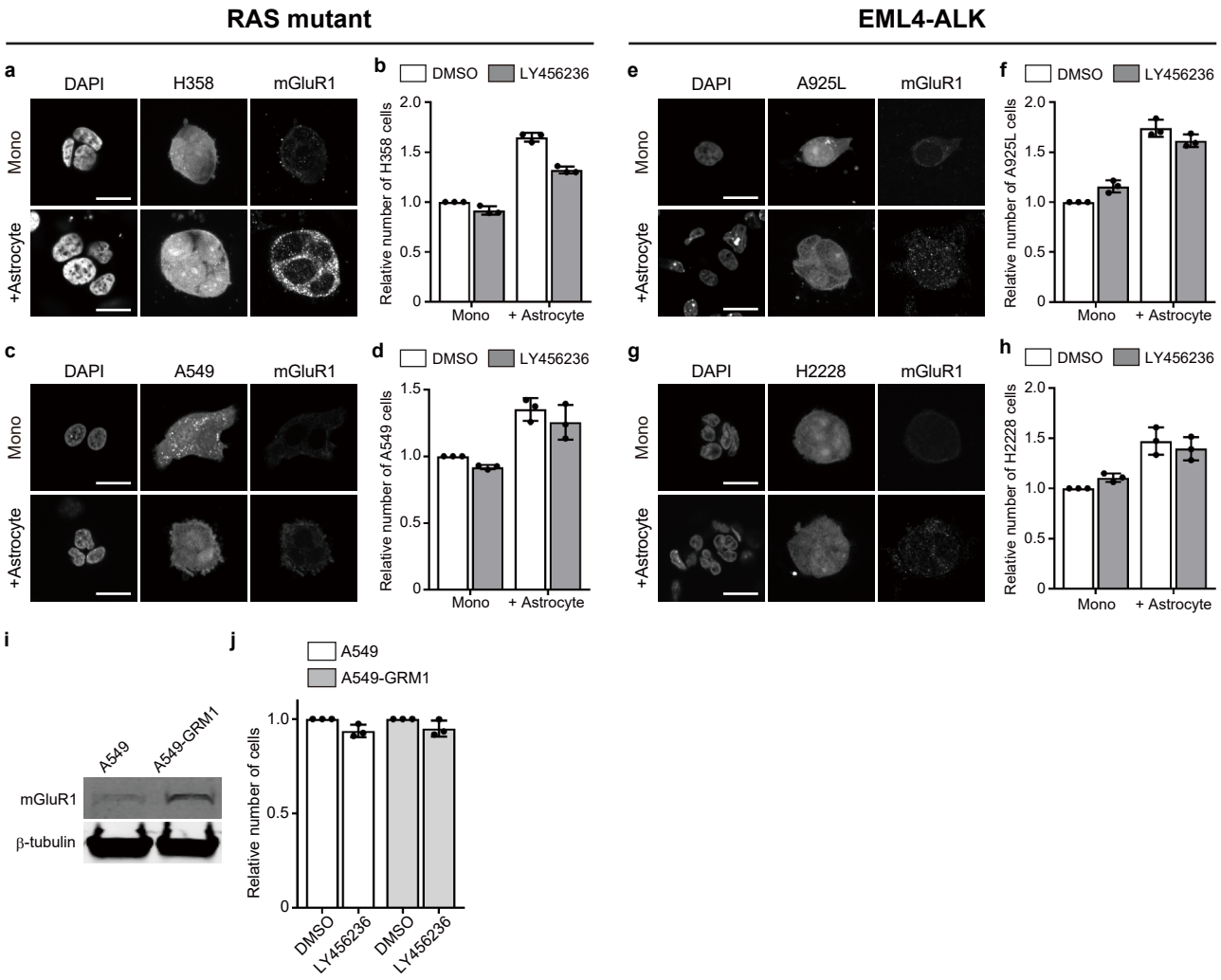
Extended Data Fig. 6: Loss of mGluR1 signalling attenuates EGFR expression and phosphorylation in PC9 cells. a, b, PC9-AMG cells monocultured or co-cultured with astrocytes in 3D collagen gels were treated with DMSO (0.01%) or LY456236 (1 μM). The cells were stained with an anti-mGluR1 antibody, DAPI and phalloidin (**a**). The relative intensity of mGluR1 was quantified (from left to right, n = 13, 19, 18). Scale = 20 μm. **c,** PC9-AMG cells cultured in 3D collagen gels (monoculture) or co-cultured in MGS were treated with DMSO (0.01%) or the indicated drug (1 μM) for 48 hours and the relative number of viable PC9-AMG cells was quantified (n = 3). The bars indicate mean ± SD. **d,** PC9-AMG cells cultured in 3D collagen gels (monoculture) or co-cultured in MGS were treated with DMSO (0.01%) or U73122 (5 μM) for 48 hours. The relative number of viable PC9-AMG cells was quantified (n = 2). The bars indicate mean ± SD. **e,** PC9-AMG cells cultured in 3D collagen gels (monoculture) or co-cultured in MGS were treated with DMSO (0.01%) or BAPTA-AM (10 μM) for 48 hours. The relative number of viable PC9-AMG cells was quantified (n = 2). The bars indicate mean ± SD. **f-j,** PC9-AMG and PC9-AMG-GRM1-KO cells (**f**) were monocultured or co-cultured with astrocytes in 3D collagen gels. The cells were fixed and stained with an anti-EGFR antibody (**g**) or anti-phospho-EGFR antibody (**i**), counterstained with DAPI and phalloidin. The relative intensities of EGFR (from left to right, n = 12, 16, 17) (**h**) and phospho-EGFR (from left to right, n = 12, 16, 14) (**j**) were quantified. Scale = 50 μm.



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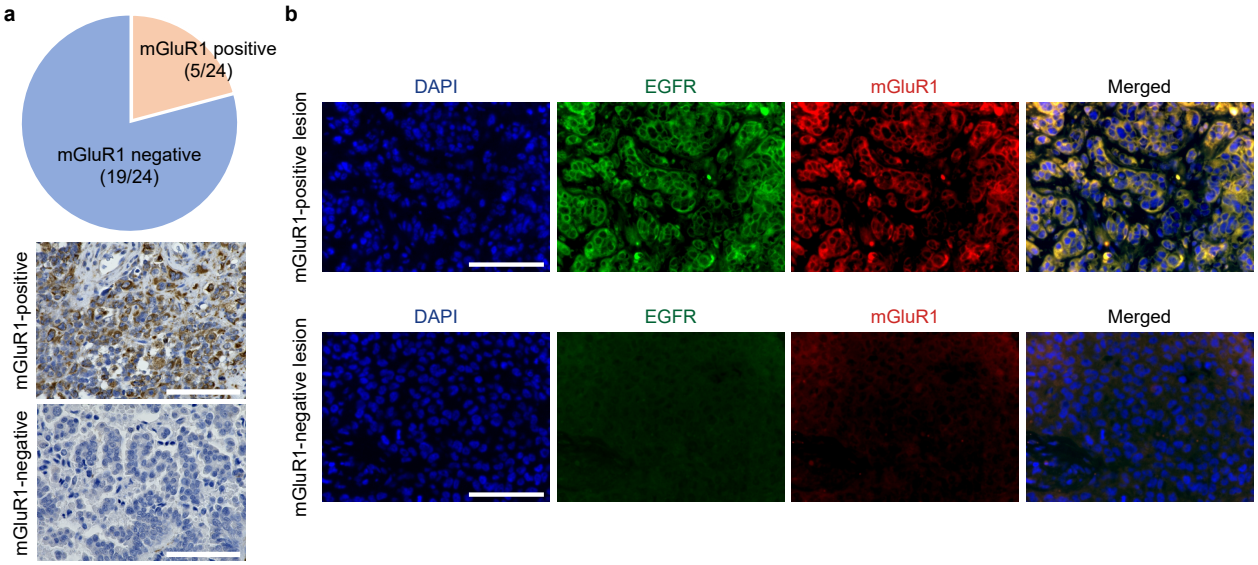
Extended Data Fig. 7: EGFR-wt lung cancer cells do not become mGluR1 signalling dependent. a, c, e, g, Ras-mutant human lung cancer cells, H358 (**a**) and A549 (**c**), and EML4-ALK fusion human lung cancer cells, A925L (**e**) and H2228 (**g**) were monocultured or co-cultured with astrocytes in 3D collagen gels. The cells were fixed and stained with an anti-mGluR1 antibody and DAPI. Scale = 20 μm. **b, d, f, h,** H358 (**b**), A549 (**d**), A925L (**f**) and H2228 (**h**) cells were monocultured or co-cultured with astrocytes in 3D collagen gels and treated with DMSO (0.01%) or LY456236 (1 μM) for 48 hours. The relative number of viable cancer cells was quantified (n = 3 in each group). The bars indicate mean ± SD. **i, j,** Parental or mGluR1-overexpressing A549 cells (**i**) were cultured in 3D collagen gels with DMSO (0.01%) or LY456236 (1 μM) for 48 hours. The relative number of viable cells was quantified (**j**). The bars indicate mean ± SD.



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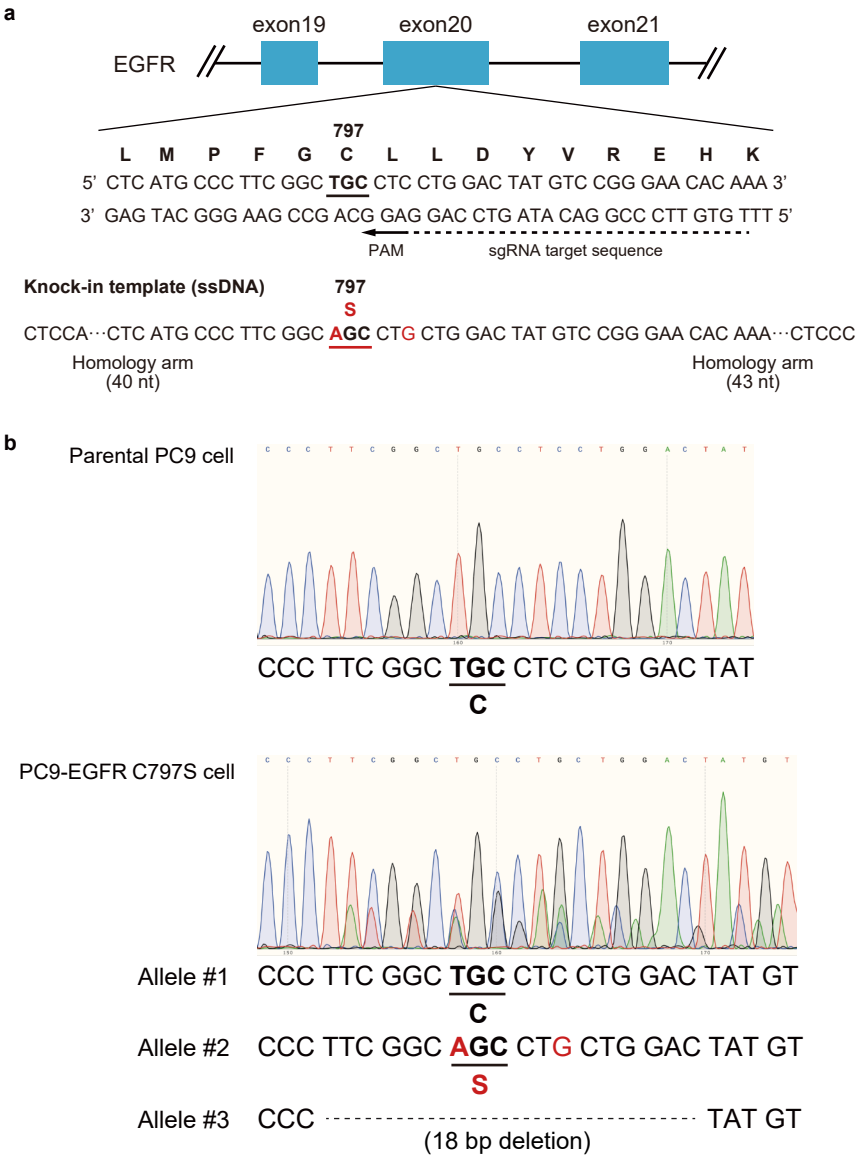
Extended Data Fig. 8: Expression of mGluR1 and EGFR in human lung cancer brain metastasis. a, A pie chart showing the mGluR1 positivity rates of brain metastatic lung cancer surgical specimens and representative images of mGluR1 positive and negative immunostaining. **b**, Representative images of brain metastatic lung cancer co-stained with an anti-EGFR and anti-mGluR1 antibodies, counterstained with DAPI. The upper panels show the mGluR1-positive lesion from case #3 and the lower panels show the mGluR1-negative lesion from case #5. Scale = 200 μm. Please also see Supplementary File S4.



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Extended Data Fig. 9: Generation of PC9 cells with EGFR C797S mutation. a, A blueprint for CRISPR/Cas9-mediated gene editing of EGFR. The sequences of sgRNA and knock-in template are shown. The base mutation to place the C797S amino acid mutation (AGC) and a silent mutation to ensure template knock-in (CTG) are highlighted in red. **b**, Results of EGFR sequencing of parental PC9 cells (top panel) and established PC9-EGFR-C797S cells (bottom panel). At least three types of EGFR alleles were detected in the PC9-EGFR-C797S cells.

Supplementary File S1. Gene expression data of astrocytes cultured with a conventional method or in MGS (at days 5 and 32).

Supplementary File S2. Gene expression data of PC9-Luc-mEGFP, PC9-Luc-mEGFP-BrM4 and PC9-Luc-mEGFP-AMG cells cultured in a conventional manner.

Supplementary File S3. Gene expression data of PC9-Luc-mEGFP, PC9-Luc-mEGFP-BrM4 and PC9-Luc-mEGFP-AMG cells cultured in MGS.

Supplementary File S4. Twenty-four clinical cases of lung cancer brain metastasis assessed for mGluR1 and EGFR expression.

Supplementary File S5. Primers, siRNAs, CRISPR/Cas9-related DNA/RNA templates, antibodies, and reagents.