



HOKKAIDO UNIVERSITY

Title	Ceacam1L Modulates STAT3 Signaling to Control the Proliferation of Glioblastoma-Initiating Cells
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Supplementary Information

Supplementary Methods

Neurosphere formation assay

To examine the neurosphere formation activity of the *ceacam1L*-overexpressing hGICs and their parental hGICs, we dissociated the spheres, cultured 1,000 cells per well on the 24-well dish and counted the number of spheres (more than 32 cells) at 7 days. We then re-cultured the cells and counted the sphere number at every 7 days, sequentially.

TMZ sensitivity assay

Two hundreds and fifty cells of the *ceacam1L*-overexpressing hGICs and their parental hGICs were cultured in the NSC medium with a various concentration of TMZ on the 96-well culture dish for 3 days and examined their viability using MTT assay as shown in (7).

Immunohistochemistry

Paraffin-embedded human brain-tumor sections (6 μ m thick) were immunolabeled as described previously (7,8). The following antibodies were used to detect antigens: goat anti-Ceacam1 (1:50; R&D), mouse monoclonal anti-CD68 (1:50, DAKO), rabbit polyclonal anti-EGFR (1:50; Cell Signaling Technology) and rabbit polyclonal anti-phosphorylated STAT3 (1:50, Cell Signaling Technology). Antibodies were detected with Alexa594-conjugated donkey anti-mouse IgG (1:500 Molecular Probe), Alexa488-conjugated donkey anti-goat IgG (1:500; Molecular Probe) and DyLight649-conjugated donkey anti-rabbit IgG (1:500; BioLegend). Cells were counterstained with DAPI (1 μ g/ml) to visualize the nuclei.

Supplementary Figure Legends

Figure S1. Expression of *ceacam1* in mouse and human GIC lines and human

glioma tissues (A) The expression of *ceacam1L* in GBM, anaplastic astrocytoma (AA), anaplastic oligoastrocytoma (AOA), anaplastic oligodendroglioma (AO), hGICs, and control brain tissue (CB) was examined by RT-PCR. (B, C) The expression of *ceacam1L* and *ceacam1S* in mouse GICs (NSCL61 and OPCL61) and human ones (E3 and E6) was examined by RT-PCR. The expression of *gapdh* was used as an internal control.

Figure S2. Knockdown efficiency of *ceacam1* shRNAs

FLAG-tagged mouse and human Ceacam1 expression vectors were transfected with a *contsh* or *ceacam1sh* expression vector into Cos7 cells. Cell extracts were harvested 2 d after transfection, and were analyzed by Western blotting using anti-FLAG and anti-GAPDH (loading control) antibodies.

Figure S3. The Ceacam1L/STAT3 axis regulated the expression of stemness-related genes in NSCL61s (A) Hierarchical clustering of the cells, NSCs, NSC+*ceacam1L*, NSCL61, and NSCL61+*ceacam1sh* using the Agilent Mouse GE 8x60K Microarray. (B) A list of stemness-related genes that were significantly downregulated in NSCL61 by the overexpression of *ceacam1sh* (Fold change: > 3).

Figure S4. Overexpression of *ceacam1L* kept the neurosphere formation activity of hGICs Serial neurosphere formation assay was performed by culturing the dissociated *ceacam1L*-overexpressing hGICs (closed column) and their parental hGICs (open column). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ significantly different from control cells.

Figure S5. Overexpression of *ceacam1L* made hGICs to be TMZ-resistance The *ceacam1L*-overexpressing hGICs (solid lines, closed circles) and their parental hGICs (dashed lines, open circles) were cultured in the presence of various concentration of

TMZ for 3 days and their viability was examined by MTT assay. ** $p < 0.01$ and ***

$p < 0.001$ significantly different from control cells.

Figure S6. Ceacam1+/EGFR+ GBM cells associate with CD68+ microglia (A)

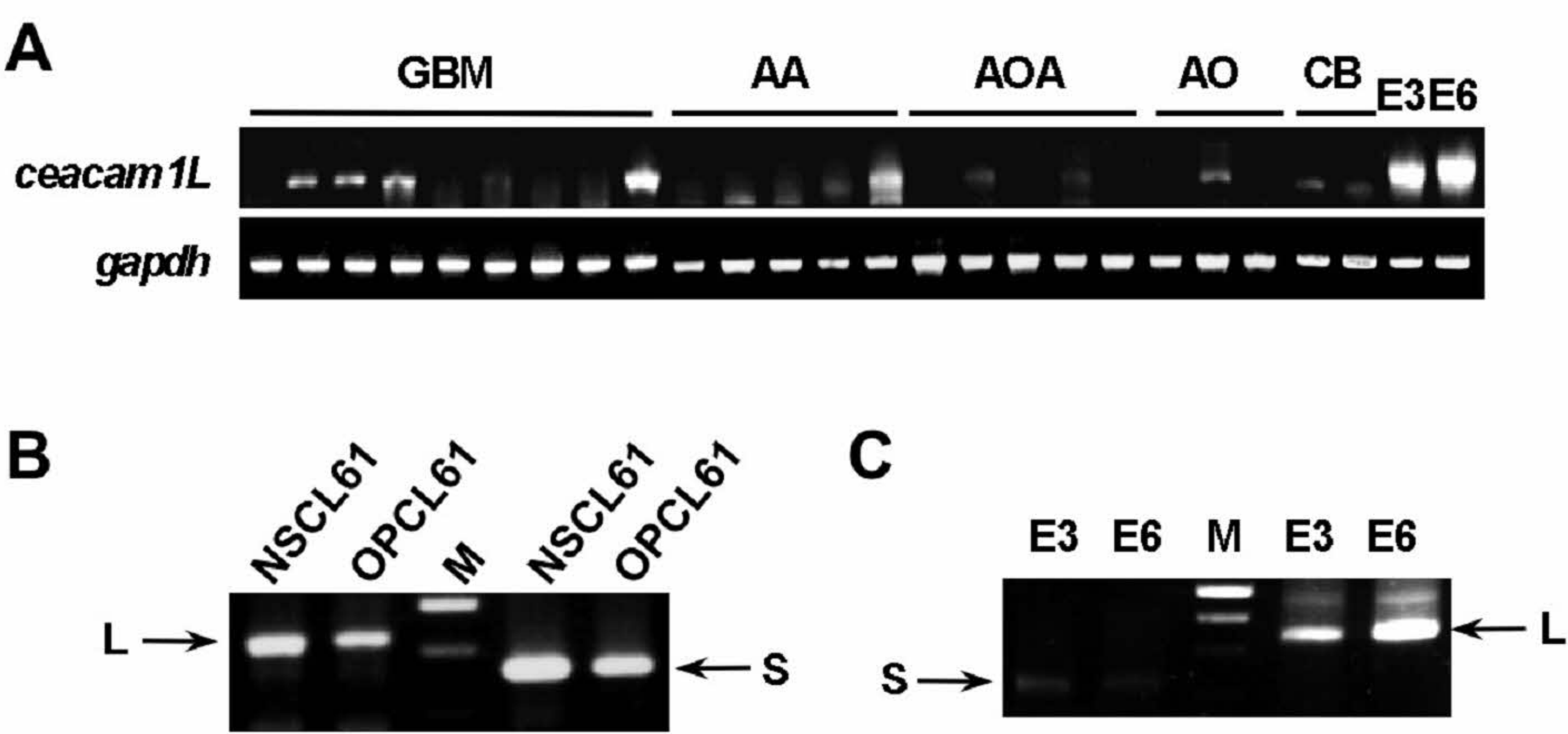
Human GBM tissues were immunolabeled for Ceacam1 (green), CD68 (red) and EGFR

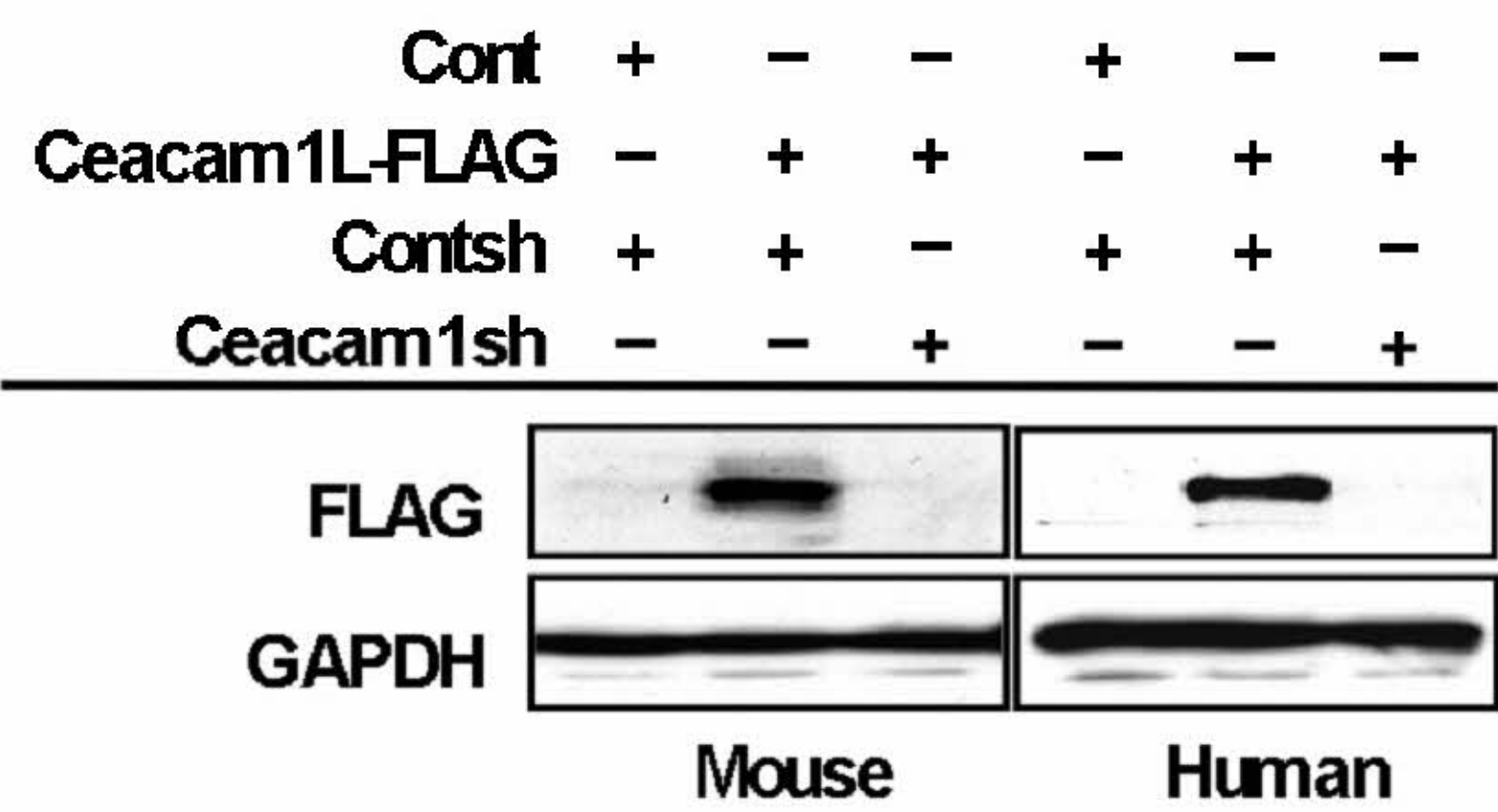
(purple). Arrows point CD68+ cells that associate with a Ceacam1+ cell. (B) Human

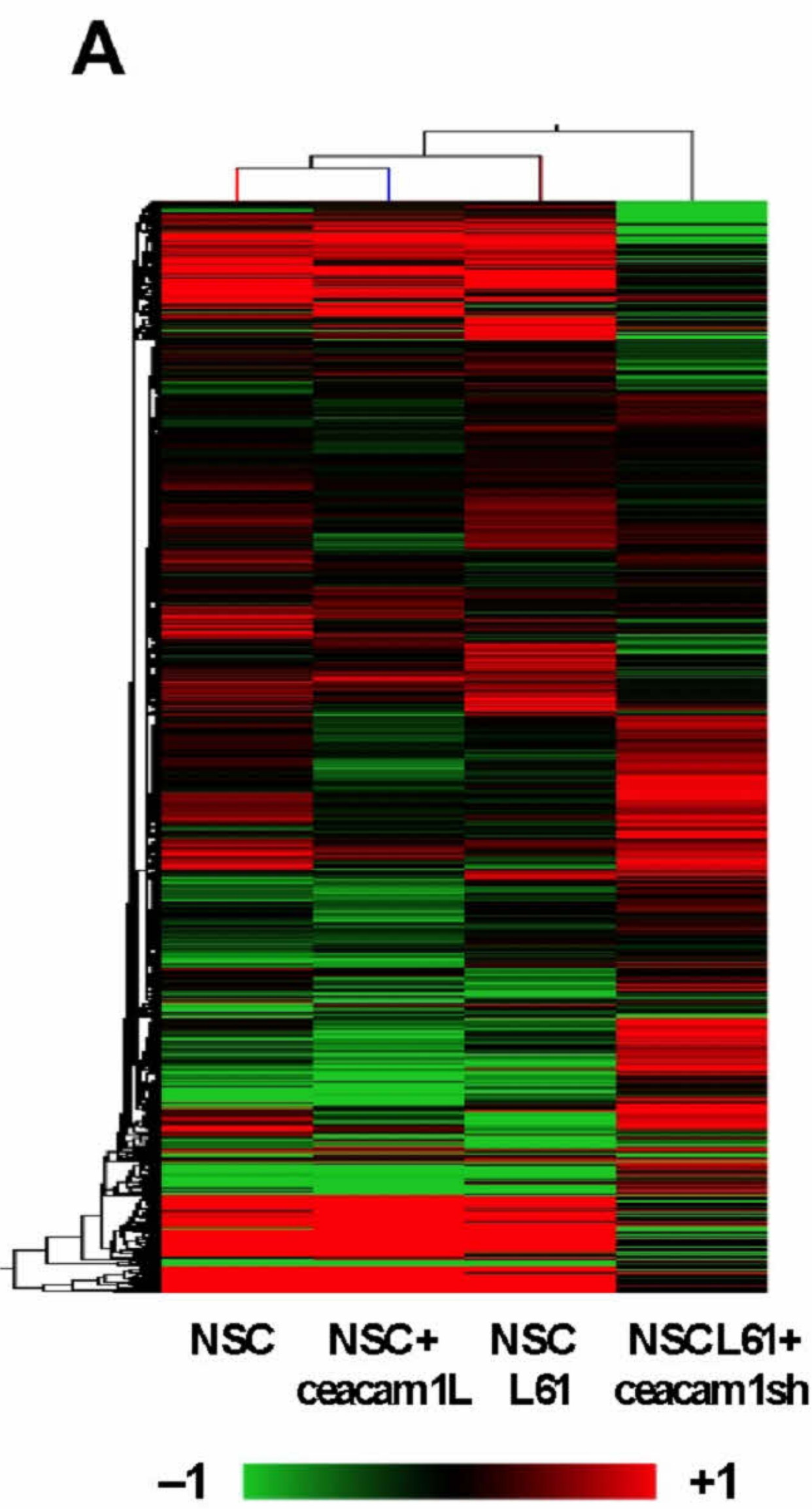
GBM tissues were immunolabeled for Ceacam1 (green), CD68 (red) and p-STAT3

(purple). Arrow points a CD68+ cell that associate with a Ceacam1+ cell. Nuclei were

counterstained with DAPI (blue). Scale bar: 50 μm , 20 μm (insets).







B

No	Gene symbol	Gene name	Fold change (NSCL61/ Ceacam1sh- NSCL61)	STAT3-binding sites
1	Dll3	Delta-like 3	140.77	O
2	Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	121.40	—
3	Notch3	Notch gene homolog 3	100.18	O
4	Dll1	Delta-like 1	80.07	O
5	Aldh1l1	Aldehyde dehydrogenase 1 family, member L1	77.33	ND
6	Sox1	SRY-box containing gene 1	67.39	O
7	Sox3	SRY-box containing gene 3	42.97	—
8	Hey1	Hey/Enhancer of split related with YRPW motif 1	41.66	O
9	Lgr5	Leucine rich repeat containing G protein coupled receptor 5	32.43	—
10	Abcg4	ATP-binding cassette, sub-family G (WHITE), member 4	29.61	—
11	Aldh1l2	Aldehyde dehydrogenase 1 family, member L2	14.71	—
12	Angpt1	Angiopoietin 1	13.12	O
13	Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	12.47	—
14	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	11.57	O
15	Aldh1a3	Aldehyde dehydrogenase family 1, subfamily A3	8.28	—
16	Egfr	Epidermal growth factor receptor	7.88	O
17	Notch4	Notch gene homolog 4	7.66	O
18	Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7	7.66	ND
19	Cxcr4	Chemokine (C-X-C motif) receptor 4	6.34	O
20	Abca3	ATP-binding cassette, sub-family A (ABC1), member 3	6.20	—
21	Abcc5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	5.98	O
22	Sox4	SRY-box containing gene 4	5.73	—
23	Ptch1	Patched homolog 1	4.37	O
24	Abcc10	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	4.34	—
25	Sox9	SRY-box containing gene 9	4.08	O
26	Sox2	SRY-box containing gene 2	3.30	O
27	Stat3	Signal transducer and activator of transcription 3	3.25	O
28	Abcd1	ATP-binding cassette, sub-family D (ALD), member 1	3.17	—
29	Abcb8	ATP-binding cassette, sub-family B (MDR/TAP), member 8	3.11	O
30	Aldh3b1	Aldehyde dehydrogenase 3 family, member B1	3.02	—

O: one or more STAT3 binding sites; —: no site; ND: not detected

