Supplementary Materials:

Supplementary methods

Immunofluorescence. Cells attached to coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking with 5% goat serum for 1 h, the slides were incubated with anti-Flag primary antibody (Cell Signaling Technology) at 4 °C overnight, followed by incubation with FITC-conjugated secondary anti-rabbit IgG antibody (Abcam) at room temperature for 1 h. The samples were washed and incubated with DAPI (Sigma-Aldrich) for nuclear counterstaining. Microscopy analysis was performed using Olympus FV1000 confocal microscope (Olympus Co., Tokyo, Japan).

Immunohistochemistry. Paraffin-embedded sections of human or murine breast cancer tissues were deparaffinized and antigen retrieval performed using citrate buffer (pH=6), at a temperature of 97°C for 20 min. After exhaustion of endogenous peroxidase with methanol and hydrogen peroxide, slides were blocked with 0.3% BSA in 0.1 mol/L of tris-buffered saline for 30 min at room temperature, and incubated with primary antibody against CD44ICD (GenScript, Piscataway, NJ, USA), SOX2 (sc-20088, Santa Cruz Biotechnology), OCT4 (ab19857, Abcam) or NANOG (ab80892, Abcam) overnight at 4°C. Antibody binding was detected using respective peroxidase-conjugated secondary antibodies (Sigma-Aldrich) at 37°C for 30 min. A DAB Substrate Kit (Abcam) was used to perform the chromogenic reaction. The images were recorded by an Olympus BX51 Epi-fluorescent microscope (Olympus). The *H*-score for each protein incorporates staining intensity (negative, weak, moderate and strong were graded as 0, 1, 2, 3, respectively) multiplied by the percentage of cells that stained positive based on the images observed under a 10× objective.

CD44s cDNA sequence. The cDNA sequence of human *CD44s* amplified is as follows.

ATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGATTT

GAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGACGG

AGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAAGCTCTGAGC

ATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCACGTGGTGATTCCCCGGATCCACCCCAACTC

CATCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGTATGACACATATTGCT

TCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACCA

ATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGGAGAATACAGAACGAATCCTGA

AGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCTCCTCCAGTGAAAGGAGCAGCACTT

CAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCATCCCAGACGAAGACAGTCCCTGGATCACC

GACAGCACAGACAGAATCCCTGCTACCAGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCAC

TCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTA

TAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCA

GTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGG

AGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGG

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Supplementary Figures:

Figure S1

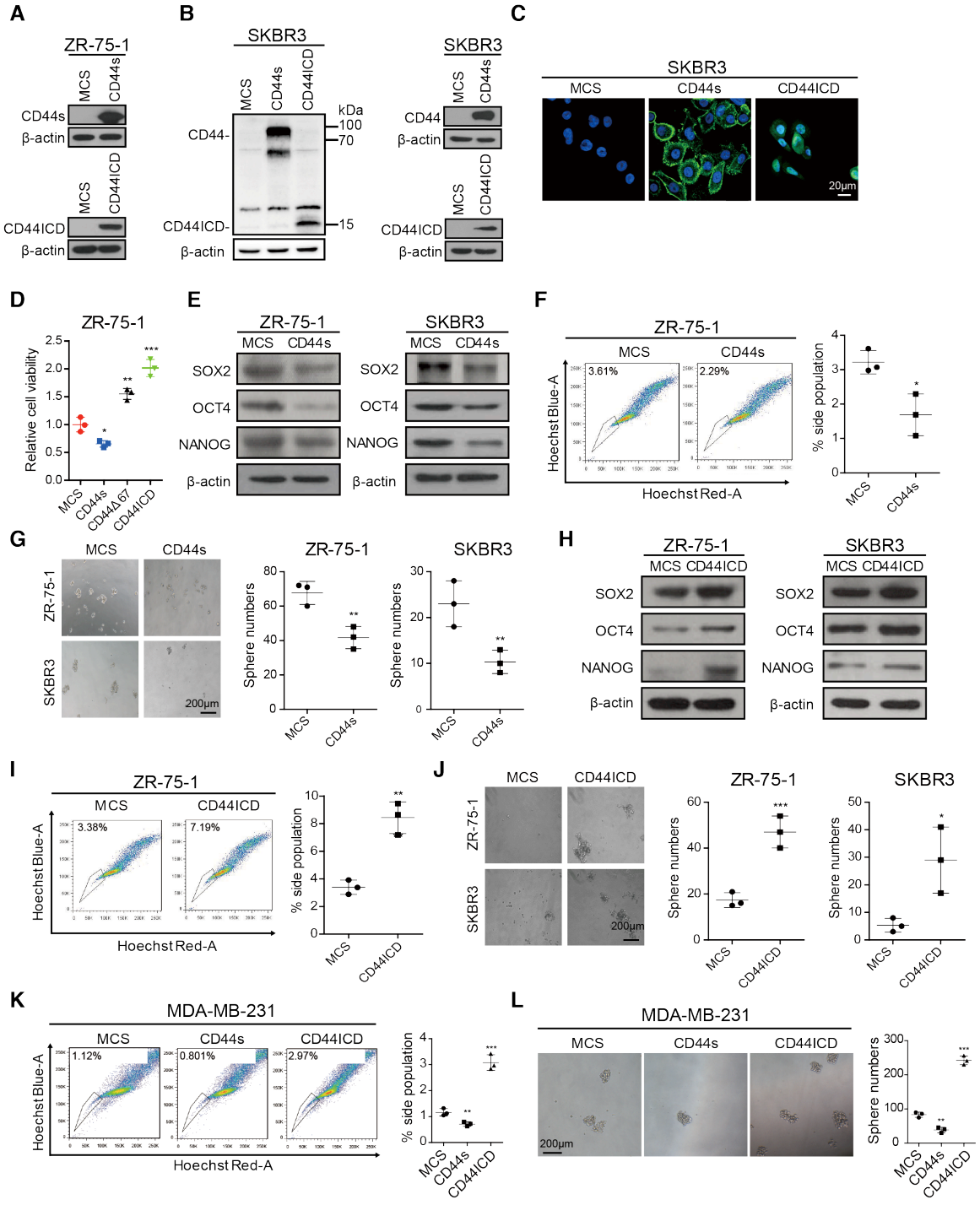

Figure S1 CD44ICD promotes stem cell-like characteristics of breast cancer cells

(A) Western blot analysis of CD44s and CD44ICD expression in ZR-75-1 cells transduced with vector control (MCS), CD44s or CD44ICD. **(B)** Western blot analysis of CD44s and CD44ICD expression in SKBR3 cells transduced with vector control (MCS), CD44s or CD44ICD. **(C)** Immunofluorescence staining of CD44s and CD44ICD in SKBR3 cells transduced with vector control (MCS), CD44s or

CD44ICD. **(D)** Trypan blue exclusion analysis of cell viability in ZR-75-1 cells transduced with vector control (MCS), CD44s, CD44Δ67 or CD44ICD. **(E)** Western blot analysis of SOX2, OCT4 and NANOG expression in ZR-75-1 and SKBR3 cells transduced with vector control (MCS) or CD44s. β-actin serves as a loading control. **(F)** Flow cytometric analysis of SP in ZR-75-1 cells transduced with vector control (MCS) or CD44s. The graph on the right shows the statistical results. **(G)** Sphere formation ability of ZR-75-1 and SKBR3 cells transduced with vector control (MCS) or CD44s. The graph on the right shows the statistical results. **(H)** Western blot analysis of SOX2, OCT4 and NANOG expression in ZR-75-1 and SKBR3 cells transduced with vector control (MCS) or CD44ICD. β-actin serves as a loading control. **(I)** Flow cytometric analysis of SP in ZR-75-1 cells transduced with vector control (MCS) or CD44ICD. The graph on the right shows the statistical results. **(J)** Sphere formation ability of ZR-75-1 and SKBR3 cells transduced with vector control (MCS) or CD44ICD. The graph on the right shows the statistical results. **(K)** Flow cytometric analysis of SP in MDA-MB-231 cells transduced with vector control (MCS), CD44s or CD44ICD. The graph on the right shows the statistical results. **(L)** Sphere formation ability of MDA-MB-231 cells transduced with vector control (MCS), CD44s or CD44ICD. The graph on the right shows the statistical results. Student's *t*-test was used for statistical analysis, data are shown as mean ± SD. Data are representatives of at least three independent experiments.

Figure S2

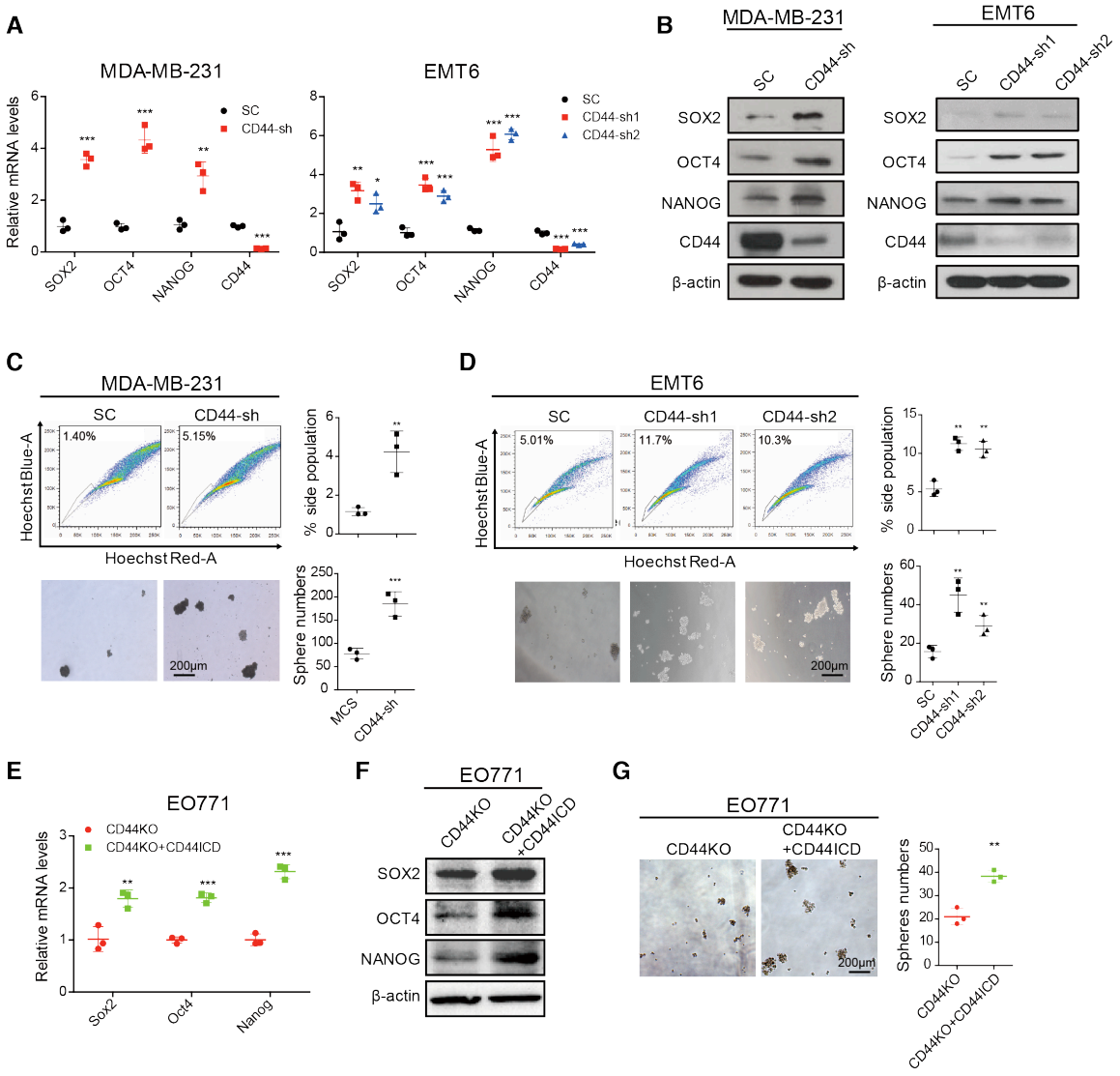

Figure S2 CD44 knockdown enhances stem cell-like characteristics of breast cancer cells
(A) qPCR analysis of relative mRNA expression of SOX2, OCT4 and NANOG in MDA-MB-231 and EMT6 cells transduced with scramble control (SC) or shRNA for CD44. **(B)** Western blot analysis of SOX2, OCT4, and NANOG expression in MDA-MB-231 and EMT6 cells transduced with scramble control (SC) or shRNA for CD44. β-actin serves as a loading control. **(C)** Flow cytometric analysis of SP and sphere formation ability of MDA-MB-231 cells transduced with scramble control (SC) or shRNA for CD44. The graph on the right shows the statistical results. **(D)** Flow cytometric analysis of SP and sphere formation ability of EMT6 cells transduced with scramble control (SC) or shRNA for CD44. The graph on the right shows the statistical results. **(E)** qPCR analysis of relative mRNA expression of SOX2, OCT4 and NANOG in CD44KO EO771 cells and CD44KO EO771 cells transduced with CD44ICD. **(F)** Western blot analysis of SOX2, OCT4, and NANOG expression in CD44KO EO771 cells and CD44KO EO771 cells transduced with CD44ICD. **(G)** Sphere formation ability of CD44KO EO771 cells and CD44KO EO771 cells transduced with CD44ICD. The graph on the right shows the statistical results. For panel A and D, one-way ANOVA was used for statistical analysis, for panel C, E and G, Student's *t*-test was used for statistical analysis, data are shown as mean ± SD. Data are representatives of at least three independent experiments.

Figure S3

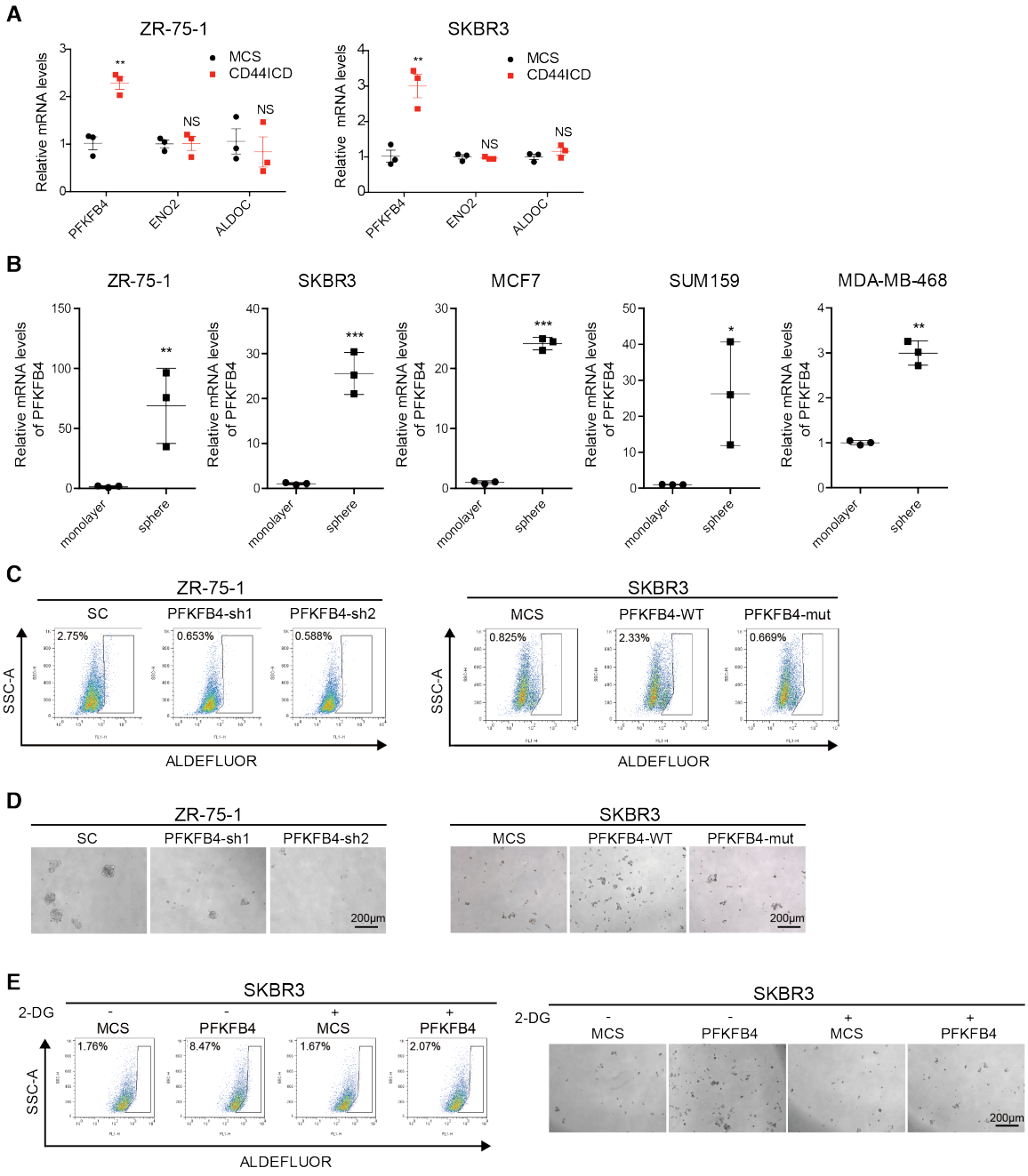

Figure S3 PFKFB4 enhances stem cell-like characteristics of breast cancer cells

(A) qPCR analysis of PFKFB4, ENO2 and ALDOC expression in ZR-75-1 and SKBR3 cells transduced with vector control (MCS) or CD44ICD. **(B)** qPCR analysis of PFKFB4 expression in breast cancer cell spheres and monolayers. **(C)** Representative graphs of flow cytometric analysis of ALDH activity in ZR-75-1 cells transduced with scramble control (SC) or shRNA for PFKFB4, and in SKBR3 cells transduced with vector control (MCS), PFKFB4-WT or PFKFB4-mut. **(D)** Representative graphs of sphere formation ability of ZR-75-1 cells transduced with scramble control (SC) or shRNA for PFKFB4, and SKBR3 cells transduced with vector control (MCS), PFKFB4-WT or PFKFB4-mut. **(E)** Representative graphs of flow cytometric analysis of ALDH activity and sphere formation ability of PFKFB4 ectopic expression and

vector control (MCS) SKBR3 cells treated with 5mM 2-DG. Student's *t*-test was used for statistical analysis, data are shown as mean ± SD. Data are representatives of at least three independent experiments.

Figure S4

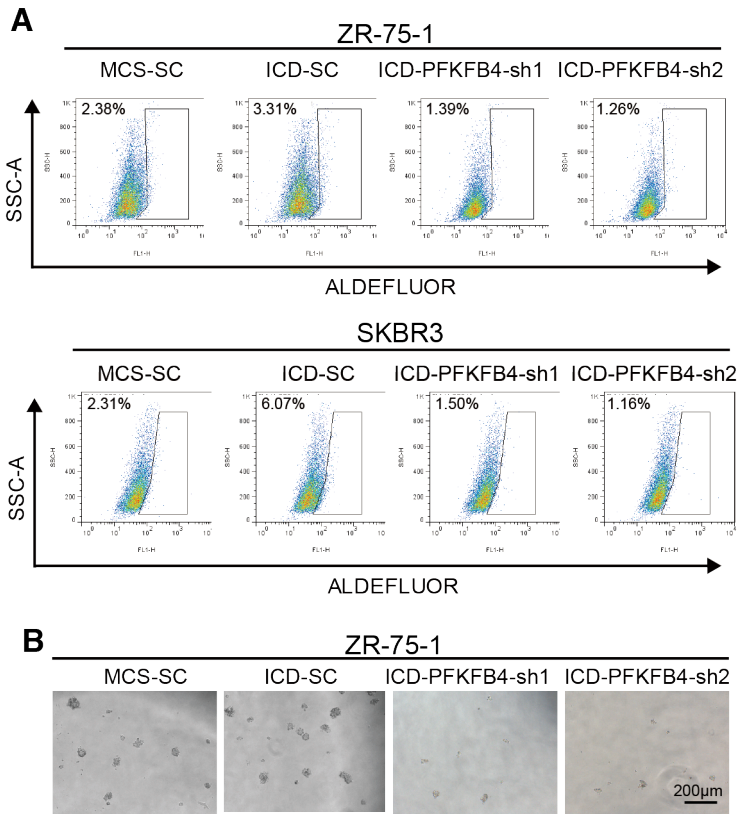

Figure S4 PFKFB4 is required for CD44ICD-mediated up-regulation of stemness
(A) Representative graphs of flow cytometric analysis of ALDH activity in ZR-75-1 and SKBR3 cells transduced with vector control (MCS) or CD44ICD and scramble control (SC) or shRNA for PFKFB4. **(B)** Sphere formation ability of ZR-75-1 cells transduced with vector control (MCS) or CD44ICD and scramble control (SC) or shRNA for PFKFB4.

Supplementary table 1. Primers for gene overexpression and shRNAs for gene knockdown

Gene	Primer Sequences (5'→3')
CD44s (Forward)	CGTCTAGAGCCACCATGGACAAGTTTTGGTGGC
(Reverse)	CGGCTAGCTTACACCCCAATCTTCATGTC
CD44s-Flag (Forward)	CGTCTAGAGCCACCATGGACAAGTTTTGGTGGC
(Reverse)	CGACGCGTTTACTTATCGTCGTCATCCTTGTAATC CCCCAATCTTCATGTC
CD44Δ67 (Forward)	CGTCTAGAGCCACCATGGACAAGTTTTGGTGGC
(Reverse)	CGACGCGTTCACCTTCTTCGACTGTTGAC
CD44ICD (Forward)	CGTCTAGAGCCACCATGGCAGTCAACAGTCGAA AGGTGTG
(Reverse)	CGACGCGTTTACACCCCAATCTTCATGTCC
CD44ICD-Flag (Forward)	CGTCTAGAGCCACCATGGCAGTCAACAGTCGAA GAAGGTGTG
(Reverse)	CGACGCGTTTACTTATCGTCGTCATCCTTGTAATC CACCCCAATCTTCATGTCC
PFKFB4 (Forward)	CGTCTAGAGCCACCATGGCGTCCCCACGGGAATT G
(Reverse)	CGACGCGTTCACTGGTGAGCAGGCACCGTG
PFKFB4-mut (Forward)	GCCAACATCGTGCAAGTGGCACTGGGCAGCCCT GACTATG
(Reverse)	CATAGTCAGGGCTGCCCAGTGCCACTTGCACGAT GTTGGC
CD44-sh	AAAAGAACGAATCCTGAAGACATCTTTGGATCC

		AAAGATGTCTTCAGGATTCGTTC
	PFKFB4-sh1	AAAAGCCCAACTCTCATTGTCATGGTTGGATCCA ACCATGACAATGAGAGTTGGGC
	PFKFB4-sh2	AAAAGCTGGAGAGGCAAGAGAATGTTTGGATCC AAACATTCTCTTGCCTCTCCAGC
	CREB-sh1	AAAAGCAGCTCATGCAACATCATCTTT GGATCCAAAGATGATGTTGCATGAGCTGC
	CREB-sh2	AAAAGGCCCAGCCATCAGTTATTCATTGGATCCA ATGAATAACTGATGGCTGGGCC
	Cd44-sh1	AAAAGCAATGTGCTACTGACTATTTGGATCCAAA TAGTCAGTAGCACATTGC
	Cd44-sh2	AAAAGGTAATTCCGAGGATTCATTTG GATCCAAATGAATCCTCGGAATTACC

Supplementary table 2. Primers for qRT-PCR

	Primer	**Sequences (5'→3')**
Human	SOX2 (Forward)	GCCTGGGCGCCGAGTGGA
	(Reverse)	GGGCGAGCCGTTCATGTAGGTCTG
	OCT4 (Forward)	GCTCGAGAAGGATGTGGTCC
	(Reverse)	CGTTGTGCATAGTCGCTGCT
	NANOG (Forward)	TCTGGACACTGGCTGAATCCT
	(Reverse)	CGCTGATTAGGCTCCAACCAT
	CD44 (Forward)	CGGACACCATGGACAAGTTT
	(Reverse)	GAAAGCCTTGCAGAGGTCAG
	PFKFB4 (Forward)	GGGTGCCTCTTGGCCTTAAA

	(Reverse)	GCCCACACGGCATACTTTTC
	ALDOC (Forward)	TCACGTAGCTCTGCGACATC
	(Reverse)	CAGAAAGGGCTGGGTACGAG
	ENO2 (Forward)	TTGGGGGAACGATGTGTCTG
	(Reverse)	CGCAGGCTTCAGTGAGTACA
	GAPDH (Forward)	CTCTGATTTGGTCGTATTGGG
	(Reverse)	TGGAAGATGGTGATGGGATT
	CREB (Forward)	AACCAGCAGAGTGGAGATGC
	(Reverse)	TCTGTGTTCCGGAGAAAAGTCT
Mouse	Sox2 (Forward)	TCCAAAAACTAATCACAACAATCG
	(Reverse)	GAAGTGCAATTGGGATGAAAA
	Oct4 (Forward)	GTTGGAGAAGGTGGAACCAA
	(Reverse)	CTCCTTCTGCAGGGCTTTC
	Nanog (Forward)	CCTCAGCCTCCAGCAGATGC
	(Reverse)	CCGCTTGCACTTCACCCTTTG
	Cd44 (Forward)	CTTGGCCACCAGAGATCGAG
	(Reverse)	GTGGTCACTCCACTGTCCTG
	Gapdh (Forward)	AGAGGGAAATCGTGCGTGAC
	(Reverse)	AAGAAGGAAGGCTGGAAAA

Supplementary table 3. Primers for ChIP assay

Primer	Sequences (5'→3')
CR1 (Forward)	CAGGTTGAAGCCGTGTTGGT
(Reverse)	GGGATGTGCACATTGTCTATCTTAC
CR2 (Forward)	TAATACTGTAAGATAGACAGTGC

	(Reverse)	TCTCGAACTCCTGACCTCAGGTGA
CR3 (Forward)		TGTAATCCCAGCACTTTGGAAG
	(Reverse)	CTCTTTCTTTGCTTTGGCTG
CR4 (Forward)		AACAGGAGCGAAACTCCGTCTC
	(Reverse)	TGGGACGAGGCCTACGTGA
CR5 (Forward)		TGCAGGCCTACTGGCCCCT
	(Reverse)	CATGCTCAACTCCGGATTGTACT
CR6 (Forward)		GGGCGGGACCAGTACAATC
	(Reverse)	AGTCGGACTGCGCCGCTTC
CR7 (Forward)		GGAAGATAGACATGGGCTGTCG
	(Reverse)	GTGCACCTCCCACCTCCTC