Supplementary information

Loss of the cystine/glutamate antiporter in melanoma abrogates tumor metastasis and dramatically increases survival rates of mice

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Supplementary Materials and Methods

Western Blotting

GRP78/BiP (BiP) (Cell Signaling Technology, #3177), LC3 A/B (LC3) (Cell Signaling Technology, #12741) rBAT (GeneTex, # 33506), cystathionine-β-synthase (CBS, GeneTex, # 628777) and cystathionine-γ-lyase (CTH, GeneTex, # 113409)were detected by the same method described in the main text.

Spheroid Formation

Cells cultured under adherent conditions were detached with 0.1% trypsin (#153571, MP Biomedicals SARL, Illkirch, France) and 0.02％EDTA (#345-01865, Dojindo Laboratories, Kumamoto, Japan) solution. Single cells were obtained by passing them through a 20 μm nylon mesh (#NRS-020, Nippon Rikagaku Kikai, Tokyo, Japan), and then seeded onto ultra-low attachment 96-well plates (#3474, Corning) at a density of 3×10^3 cells/well, in medium containing 1.4% methyl-cellulose (#138-05052, Fujifilm Wako Pure Chemical). The number of spheres >40 μm diameter was counted each day, for five days.

Supplementary Table 1| Suppression of subcutaneous tumorigenicity of B16F10 cells by xCT knockout and its recovery through xCT expression.

Cells	Incidence of subcutaneous tumorigenicity	Mean period when the tumor diameter reached 10 mm days)	Mean latency period (days)	Mean survival time (days)	Mean tumor weight at autopsy (gram)
WT	6/6	13.3 ± 1.0	8.0 ± 0**	25.7 ± 5.5*	10.7 ± 2.1*
KO	6/6	36.4 ± 11.9**	26.3 ± 9.2	80.8 ± 45.3	5.7 ± 4.1
R1	6/6	14.3 ± 0.8	10.0 ± 1.3**	22.8 ± 4.7*	5.2 ± 4.0
R2	6/6	14.3 ± 0.8	10.3 ± 0.8**	37.5 ± 17.7	12.3 ± 4.6*
R3	6/6	14.0 ± 0.0	10.0 ± 0**	20.0 ± 1.1	6.9 ± 2.1

2×10^6 tumor cells were injected subcutaneously into mice.

*$P < 0.05$, **$P < 0.01$ vs WT (Mean period when the tumor diameter reached 10 mm (days)).

*$P < 0.05$, **$P < 0.01$ vs KO (for other parameters).

Supplementary Table 2| Suppression of experimental lung metastatic ability of B16F10 cells by xCT-KO and its recovery by xCT expression.

Cells	Incidence of lung metastasis	Mean lung metastatic colony	Mean lung weight (gram)	Mean survival time (days)
WT	6/6**	19.3 ± 15.7*	0.66 ± 0.41*	23.3 ± 3.3**
KO	0/6	0.0 ± 0.0	0.21 ± 0.03	62.5 ± 23.1
R1	6/6**	21.3 ± 8.1**	0.49 ± 0.40	27.8 ± 1.9**
R2	6/6**	24.7 ± 12.6**	1.08 ± 0.34**	25.5 ± 1.4**
R3	4/6	22.0 ± 8.9**	0.88 ± 0.29**	26.2 ± 2.4**

1×10^5 tumor cells were injected intravenously into mice.

*$P < 0.05$, **$P < 0.01$ vs KO.

Supplementary Table 3| Suppression of experimental liver metastatic ability of xCT-KO B16F10 cells after intrasplenic injection into mice and its recovery by xCT induction.

Cells	Incidence of liver metastasis	Mean survival time (Days)	Mean liver weight (gram)	Incidence of peritoneal carcinomatosa	Mean abdominal fluid (mL)
WT	6/6	20.3 ± 3.7	4.87 ± 1.12	5/6**	1.65 ± 0.99**
KO	6/6	32.8 ± 14.8	3.19 ± 1.48	0/6	0.0 ± 0.0
R1	6/6	24.5 ± 10.8	5.11 ± 1.62	4/6*	0.88 ± 0.24**
R2	6/6	17.3 ± 1.2*	5.11 ± 0.52*	5/6**	1.00 ± 0.30**
R3	6/6	18.0 ± 1.3*	4.83 ± 0.16*	2/6	0.86 ± 0.49**

1×10^6 tumor cells were injected into spleen of mice. A few minutes later, the spleen was removed to prevent primary tumor formation in the spleen.

*$P < 0.05$, **$P < 0.01$ vs KO.

Supplementary Table 4| Suppression of tumor forming ability in mice after intraperitoneal injection of xCT-KO B16F10 cells.

Cells	Incidence of tumor formation	Mean survival time (Days)	Mean omentum weight (gram)	Incidence of peritoneal carcinomatosa	Mean abdominal fluid (mL)
WT	6/6	16.5 ± 0.8**	5.27 ± 0.61*	3/6	0.41 ± 0.04*
KO	6/6	23.5 ± 1.4	3.05 ± 2.06	3/6	0.87 ± 0.45
R1	6/6	18.0 ± 1.5**	3.52 ± 1.19	6/6	0.60 ± 0.52
R2	6/6	16.2 ± 1.0**	4.21 ± 1.53	3/6	0.79 ± 0.46
R3	6/6	16.3 ± 2.1**	3.43 ± 1.43	6/6	1.32 ± 2.15

5×10^5 tumor cells were injected intraperitoneally into mice. Primarily forming tumor masses including omentum were weighted.

*$P < 0.05$, **$P < 0.01$ vs KO.

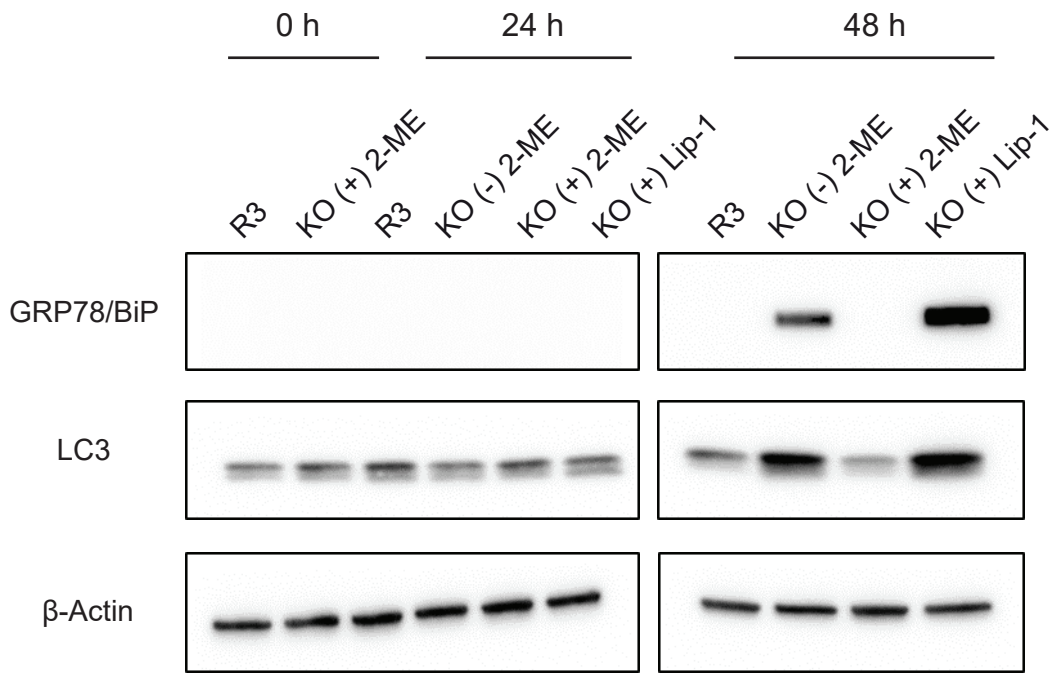
Supplementary Table 5| Suppression of spontaneous lung metastasis of xCT-KO B16F10 cells after intra-footpad injection of mice and its recovery by xCT induction.

Cells	Incidence of primary tumor formation	Mean latency period (Days)	Mean period when the tumor volume reaches 100 mm^3 (Days)	Mean survival period (Days)	Incidence of spontaneous lung metastasis	Mean metastatic nodules
WT	6/6	13.7 ± 0.8*	24.3 ± 1.5**	49.2 ± 14.5**	3/6*	1.2 ± 1.5
KO	6/6	29.0 ± 12.6	38.3 ± 9.6	97.5 ± 8.5	0/6	0.0 ± 0.0
R1	6/6	14.7 ± 1.6*	25.0 ± 1.1**	68.2 ± 20.2**	2/6	1.7 ± 2.6
R2	6/6	14.7 ± 1.6*	25.0 ± 1.7**	74.0 ± 18.1*	2/6	1.0 ± 1.5
R3	6/6	13.3 ± 1.6*	24.0 ± 1.8**	72.3 ± 17.6*	2/6	0.7 ± 1.2

5×10^5 tumor cells were injected into footpad of mice. When the primary tumor volume reaches to over 100 mm^3, the foot including the primary tumor was resected. Mice were sacrificed for autopsy when they become moribund state.

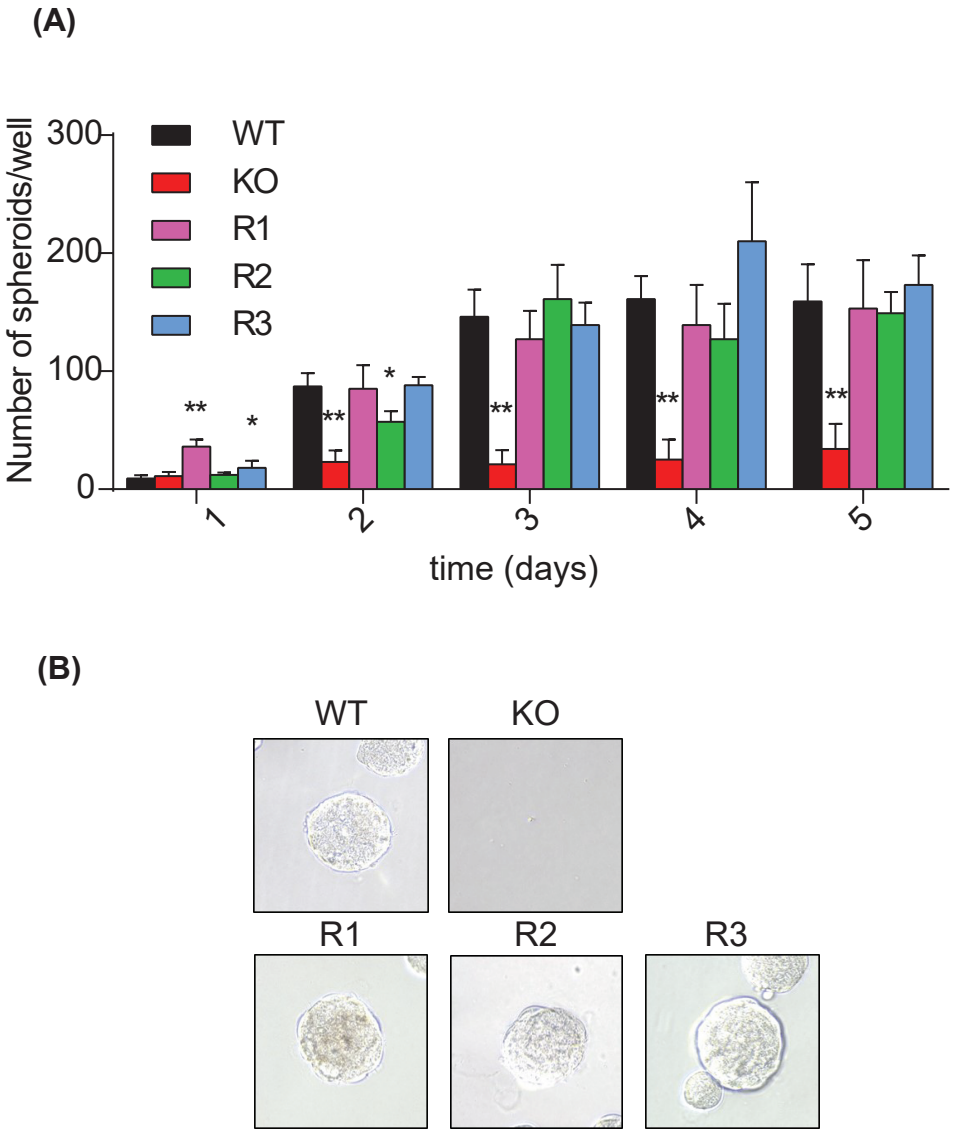
*$P < 0.05$, **$P < 0.01$ vs KO.

Fig. S1



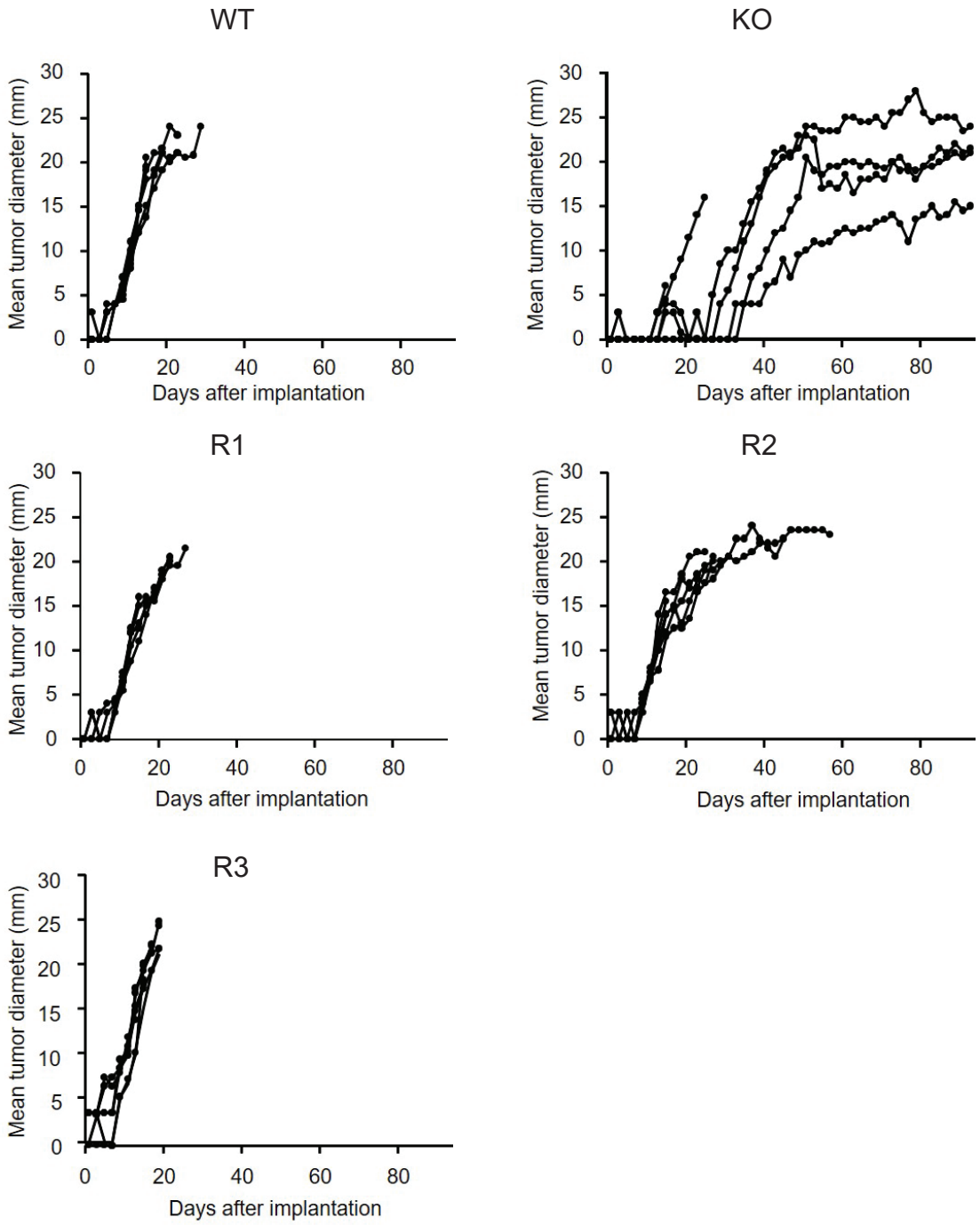
Supplementary Fig. 1| ER stress state under the absence of 2-ME in B16F10 xCT KO cells. ER stress relative protein (BiP) and one of an autophagy marker (LC3) were determined by immunoblot analysis. 10×10^4 of B16F10 KO and one of xCT reconstituted clones (R3) were plated and incubated for two days in the presence of 2ME (50 μM), then washed with pre-warmed PBS to remove 2-ME (0 h). Subsequently, cells were kept in fresh medium containing either 2-ME (50 μM), Lip-1 (500 nM) or DMSO as control for another 24-48 hours.

Fig. S2



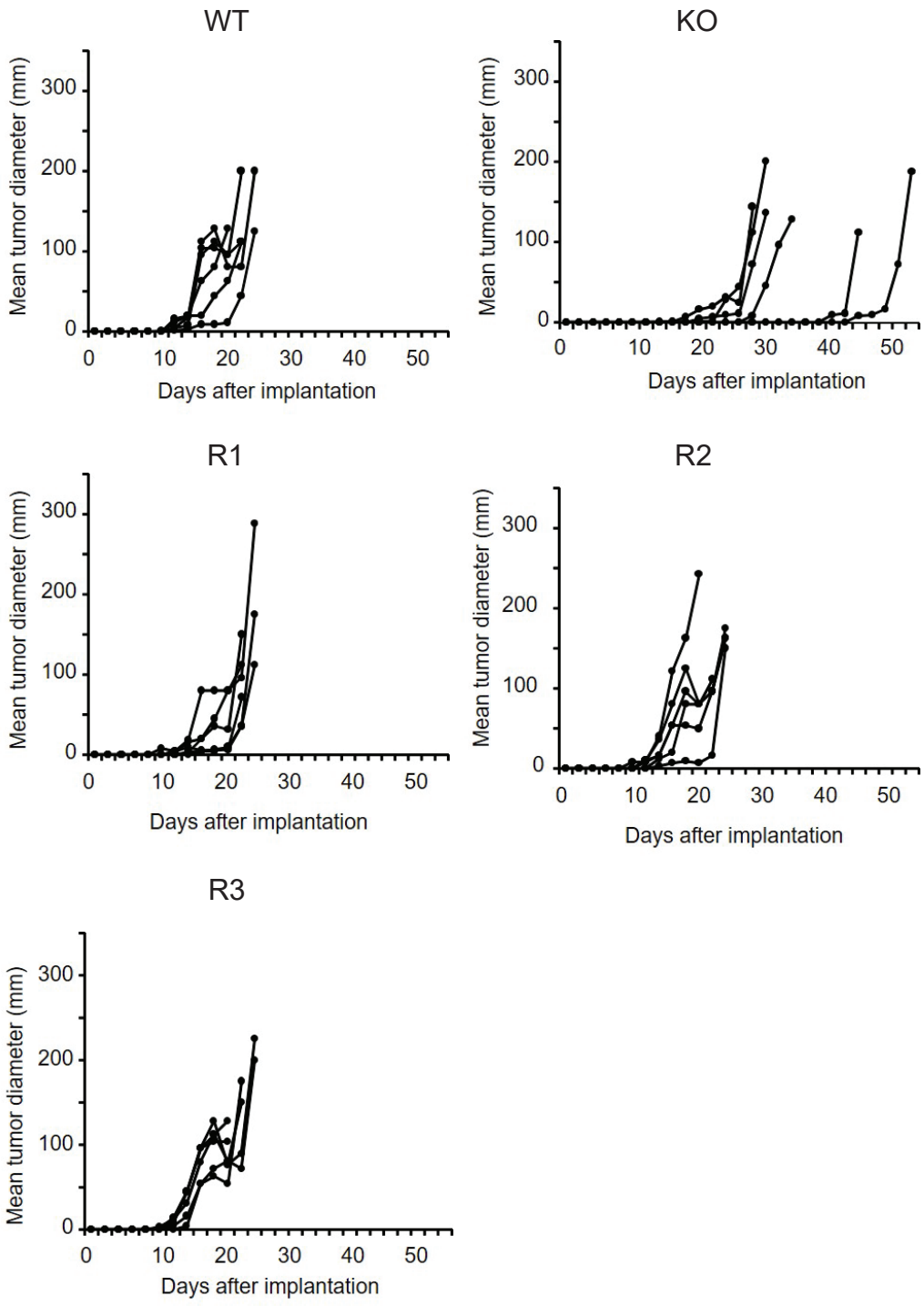
Supplementary Fig. 2| Alternation in spheroid formation in B16F10 xCT KO cells. (A) Spheroid formation was assessed by counting the number of spheroids with a diameter of over 40 μm at each time point (days). (B) Representative pictures of one single spheroid derived from each cell line are shown.

Fig. S3



Supplementary Fig. 3| Tumor growth in subcutaneous (corresponding to Fig. 3 e and f). Tumor growth (diameter) was measured using calipers after the implantation of 2 × 10^6 cells subcutaneously into the right flank of mice until it reached the humane endpoint of the experiment.

Fig. S4



Supplementary Fig. 4| Tumor growth in footpad (corresponding to Fig. 5-g). The size of tumor (diameter) after 5×10^5 of B16F10 cells injected into the right footpad of mice is shown.