Supplemental Information

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1. Supplemental Materials and methods

Antibodies. Mouse anti-trimeric Tn (tTn) antibody (MLS128) was generated by Numata et al. (1). Goat anti-ppGalNAc-T13 antibody (T-18), rabbit anti-Syndecan-1 (Sdc1) antibody (H-174), rabbit anti-integrin α5 antibody (H-104) and anti-integrin β1 antibody (M-106), hamster anti-integrin β1 antibody (Hmβ1-1), rabbit anti-FAK antibody (C-20), rabbit anti-phospho-FAK antibodies (Tyr-576, Tyr-577, Tyr-861, and Tyr-925), anti-phospho-paxillin antibodies (Tyr-31 and Tyr-181) and normal Syrian hamster IgG were purchased from Santa cruz Biotechnology (Santa cruz, CA). Rat anti-Syndencan-1 antibody (281-2) and rabbit anti-phospho-FAK antibody (Tyr-397) were from BD Transduction Laboratories (San Jose, CA). Mouse anti-phospho-paxillin (Tyr-118) and anti-rabbit IgG antibody conjugated with HRP were from Cell Signaling Technology (Beverly, MA). Anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) was purchased from Amersham Bioscience. Anti-rabbit IgG antibody conjugated with HRP was from Cell Signaling Technology. FITC-labeled anti-mouse IgG antibody was purchased from ICN/Cappel (Durham, NC). FITC-labeled streptavidin was from EY Laboratories, Inc. (San Mateo, CA). Anti-mouse IgG conjugated with HRP (Mouse TrueBlot^{TM} ULTRA) was from Bay bioscience (Kobe, Japan). Anti-rat IgG antibody conjugated with Alexa405, anti-rabbit IgG antibody conjugated with Alexa488 and anti-mouse IgG antibody conjugated with Alexa564 were purchased from Invitrogen (Carlsbad, CA).

Cell lines and culture. Establishment of high metastatic sublines (C4-ly; lymph node, C4-sc; lung) from Lewis lung cancer cell line (sublines) H7 and C4 was as described (2). These sublines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. Establishment of stable transfectant cells of *ppGalNAc-T13* cDNA (*T13*-TF) and control cells was as described (3). These clones were cultured in DMEM

supplemented with 7.5% FBS and G418 (350 μg/ml). Establishment of stable knockdown cells of *ppGalNAc-T13* gene (*T13*-KD) and control cells was as described (3). These clones were cultured in DMEM supplemented with 7.5% FBS and blasticidin (2 μg/ml). GM1 synthase RNAi transfectant (GM1-Si) cells and control cells were cultured in DMEM supplemented with 7.5% FBS and puromycin (6 μg/ml) (Calbiochem).

Cell adhesion assays using RT-CES system. Cells (2.5×10^4) were added on ACEA e-plates coated with ECM proteins. The adhesion of cells was monitored continuously using the RT-CES system (Wako Pure Chemical, Osaka, Japan) for 24 h. In order to block the cell adhesion in which tTn antigen is involved, cells were pretreated with anti-tTn mAb (10 μg/ml) or control IgG in serum-free medium for 30 min.

Immunofluorescence. Cells were plated on pre-coated plates with FN after being treated and rotated under serum-free conditions. After incubation for 0, 5, 15 and 30 min, cells were fixed in paraformaldehyde (4% in PBS for 10 min) and treated with 0.1% Triton X-100 in PBS for 10 min at room temperature. Nonspecific binding was blocked with 2.5% BSA in PBS for 60 min at room temperature. Cells were stained with the first antibodies (rat anti-Sdc1 pAb, rabbit anti-integrin β1 pAb or mouse anti-tTn mAb) in PBS containing 0.5% BSA for 60 min at room temperature, then with anti-rat IgG conjugated with Alexa405, anti-rabbit IgG conjugated with Alexa488 and anti-mouse IgG conjugated with Alexa564 in PBS containing 0.5% BSA for 45 min at room temperature. The resulting staining patterns were imaged using a confocal microscope (Fluoview FV10i, Olympus, Tokyo, Japan). It was confirmed that there is no cross-reaction between the authentic antigens and non-relevant secondary reagents. DIC indicates images of differential interference contrast microscope.

Isolation of raft fraction. GEM/rafts were isolated using a detergent extraction method essentially as described by Mitsuda *et al.* (4). Cells (1.0×10^7) were plated in 15-cm culture dishes and cultured up to 90% confluency, and three dishes of cells were used for each preparation. After washing twice with ice-cold PBS, the cells were lyzed in 1 ml of MNE/Triton X-100 buffer (1% Triton X-100, 25 mM MES-NaOH, pH 6.5, 150 mM NaCl, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin), and then Dounce-homogenized 15 times. Samples were placed on the bottom of

Ultra-Clear™ centrifuge tubes (Beckman Instruments) and mixed with an equal volume of 80% (w/v) sucrose in MNE buffer without Triton X-100. Then, 2 ml of 30% sucrose (w/v) in MNE buffer without Triton X-100 was overlaid, and 1 ml of 5% (w/v) sucrose in MNE buffer without Triton X-100 was layered on the top. The samples were centrifuged at 100,000 × g in an SW50.1 rotor for 16 h at 4 °C. The entire procedure was performed at 4 °C. From the top of the gradient, 0.5 ml of each fraction was collected to yield 10 fractions.

References

1. Ref. 8 in text.
2. Ref. 6 in text.
3. Ref. 7 in text.
4. Ref. 9 in text.

2. Supplemental Table S1.

Sequences of siRNAs used in this study

Gene symbol	accession No.	(*bp)	Sequences	(*bp)
Sdc1	NM_011519	73	5’-TGGCTGTAAATGTTCCTCC-3’	92

* bp indicates relative to start codon.

3. Figure legends for Supplemental Figures 1-8.

Supplemental Fig. S1. tTn antigen on Sdc1 modulates invasion activity of Lewis lung cancer cells

Transfection of *ppGalNAc-T13* cDNA in pCMV3B expression vector into C4 resulted in the establishment of stable transfectant lines (TF-1 and TF-2). Vector control lines (Vc-1 and Vc-2) were also generated. Transfection of anti-*ppGalNAc-T13* shRNA vector into C4-sc resulted in the establishment of stable transfectant lines (KD-1 and KD-2). Vector control lines (C-1 and C-2) were also generated. *Aa and Ab*, Immunoblotting was performed with anti-ppGalNAc-T13 pAb in *T13*-TF (a) and *T13*-KD (b) clones. *Ac and Ad*, Expression of tTn was analyzed by flow cytometry, and the results were presented by mean fluorescence intensity (MFI) of *T13*-TF (c) and *T13*-KD (d) clones. *Ae and Af,* Invasion assay, in which 5×10^5 cells were seeded in the upper chamber in the absence of serum. After 24 h, invaded cell numbers were counted. Columns represent means ± S.D. (n=3). *B,* Cells were solubilized in the lysis buffer containing Triton X-100, and the lysates were immunoprecipitated with 2 μg of anti-tTn mAb or normal mouse IgG at 4 °C. After SDS-PAGE of the immunoprecipitates with an anti-tTn mAb, immunoblotting was performed with each Ab (a). IgG, normal mouse IgG; IP, immunoprecipitation with anti-tTn mAb. This is essentially same as reported previously (Ref. 7 in text). Band intensities of Sdc1 precipitated by anti-tTn antibody were scanned by a densitometry and presented as relative Sdc1/tTn (b).

Supplemental Fig. S2. Trimeric Tn antigen-expressing cells showed increased cell adhesion to FN only, not other ECM proteins.

Cells were seeded in 96-well e-plates at 1.0×10^4 cells/well, and cell attachment and spreading were monitored by the RT-CES system. The e-plates were pre-coated with fibronectin (FN), laminin (LN), collagen type I (CLI) or poly-L-lysine (PLL) as described in "Experimental Procedures."

Supplemental Fig. S3. Expression of tTn antigen on Sdc1 increased cell adhesion to FN in an integrin-dependent manner

Cells were seeded in the wells of 96-well e-plates at 2.5×10^4 cells/well, and cell attachment and spreading were monitored by the RT-CES system. *A,* Morphology of

T13-KD lines (a, C-1; b, KD-1). *B,* The e-plates were pre-coated with poly-L-lysine (PLL) or fibronectin (FN) as described under "Experimental Procedures". *C,* Knockdown with siRNA against Sdc1 (Sdc1) in *T13*-KD lines was also analyzed by using FN-coated plates. A representative of three independent experiments was shown.

Supplemental Fig. S4. Co-localization of Sdc1, integrin β1 and tTn antigen during adhesion to FN.

A, C4-sc-derived C-1 and KD-1 cells were incubated for 0, 5, 15 and 30 min, and cells were fixed in paraformaldehyde (4% in PBS for 10 min) and treated with 0.1% Triton X-100 in PBS for 10 min at room temperature. Then, cells were stained for Sdc1 (blue), ITGβ1 (green) and tTn (red), and their images were observed using a con-focal microscope. DIC, differential interference contrast microscope. *B,* Images of the y-z axis in Y (purple line). The right side in the image is the adhesion site. Images of the x-z axis in X (yellow line). The bottom side in the image is the adhesion site. Red boxes indicate co-localizing sites.

Supplemental Fig. S5. Knockdown of ppGalNAc-T13 resulted in the suppression of lung metastasis.

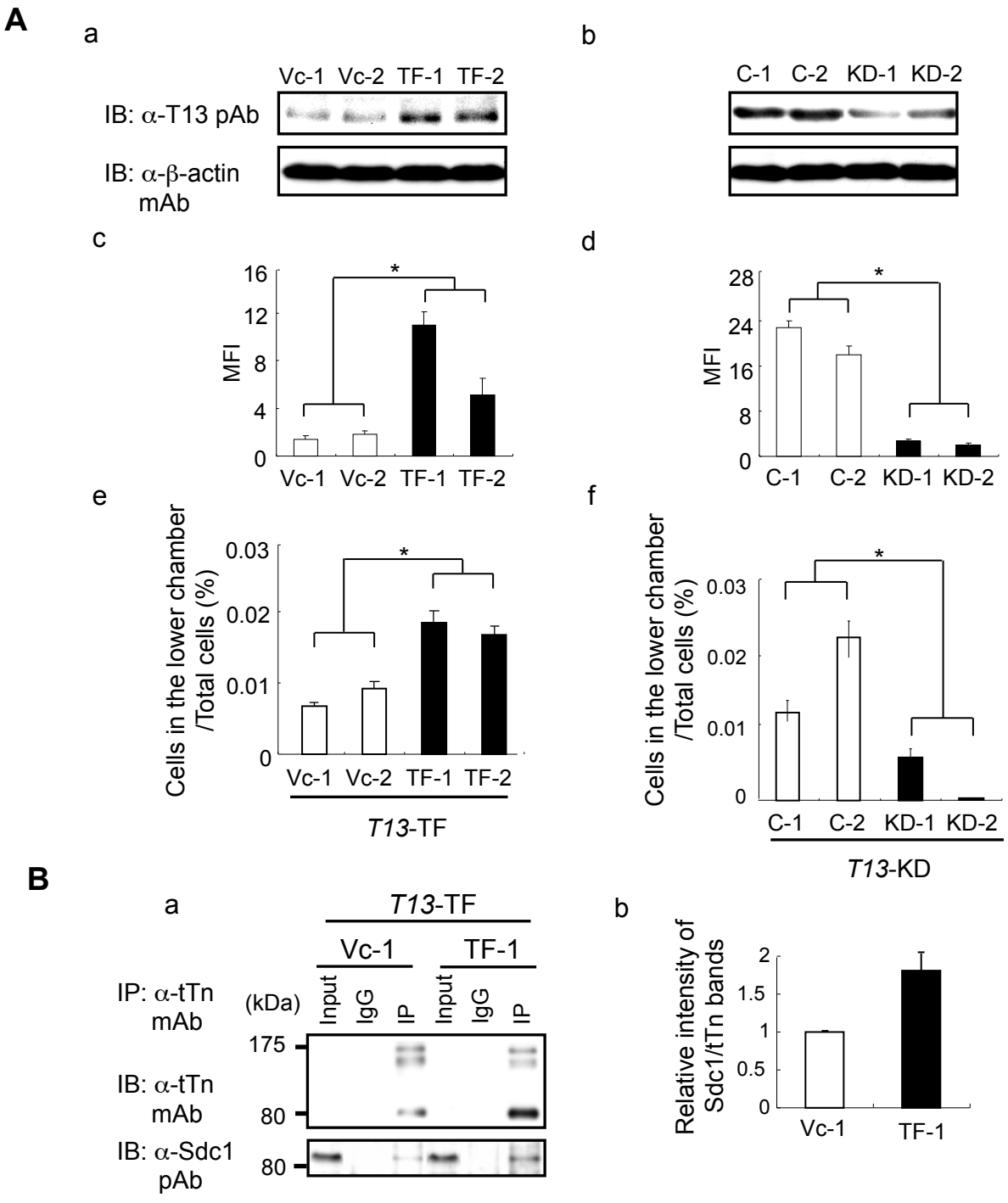
Metastasis experiments were performed using C4-sc-derived *T13*-KD lines. *A,* A histogram to depict the percentage of mice that developed lung metastasis after injection of each cell line. Bars represent ± S. D. (C, KD; n=15). *B,* Primary tumors coalescent to fascia and peritoneum (a) and lung metastasis (b) in *T13*-KD. Arrows indicate metastasis foci. *C,* H&E staining and immunohistochemical staining by tTn mAb was performed using primary tumors (a) and lung sections (b) in *T13*-KD lines. Scale bar, 30 μm.

Supplemental Fig. S6. GM1-Si clones also exhibited increased invasion activity by formation of trimeric Tn antigen on Sdc1 and modulation of lipid rafts.

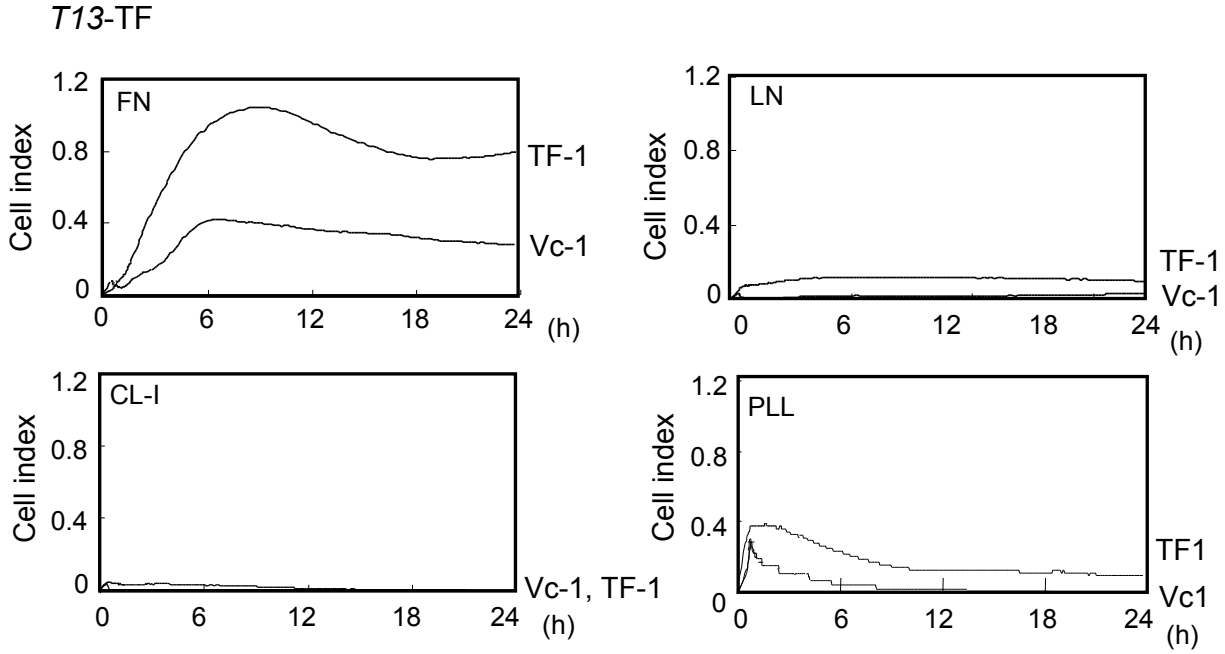
A, Immnoblotting was performed with anti-T13 pAb in GM1-Si lines (a) and invasion assay (b) was performed as described in "Experimental Procedures." Columns represent means ± S. D. (n=3). *, $P < 0.01$. *B,* Expression levels of tTn were analyzed by flow cytometry with anti-tTn mAb. *C,* The lysates were immunoprecipitated with 2 μg of anti-tTn mAb or normal IgG at 4 °C. Antibodies used for immunoblotting were as

indicated (a: anti-tTn mAb, b: anti-Sdc1 pAb). IgG, normal mouse IgG; IP, immunoprecipitation with anti-tTn mAb. *D,* GM1-Si clones were lysed using Triton X-100, and the extracts were fractionated with discontinuous sucrose density gradient centrifugation. Fractions were subjected to immunoblotting using the antibodies against the proteins indicated.

Supplemental Fig. S1



Supplemental Fig. S2



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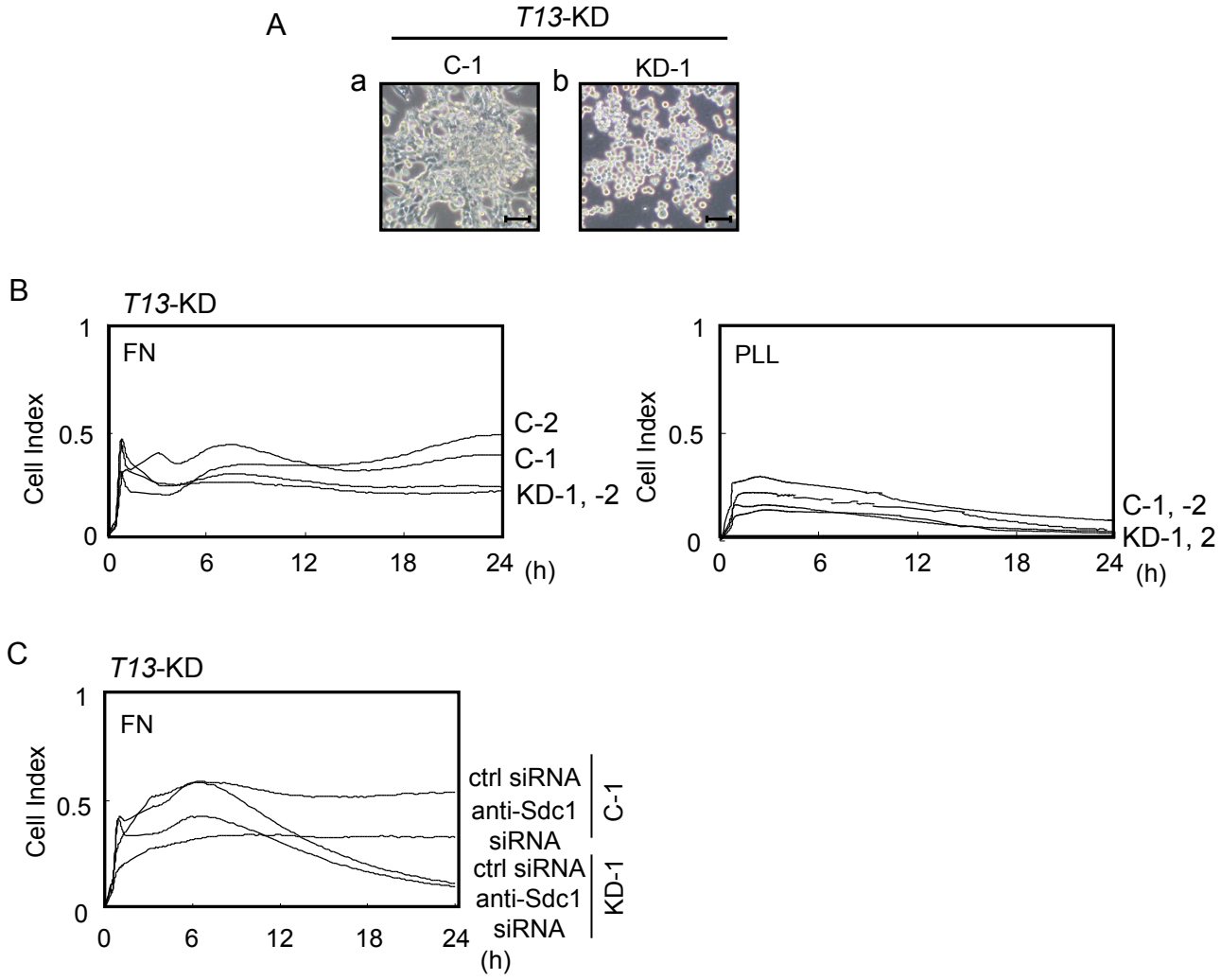

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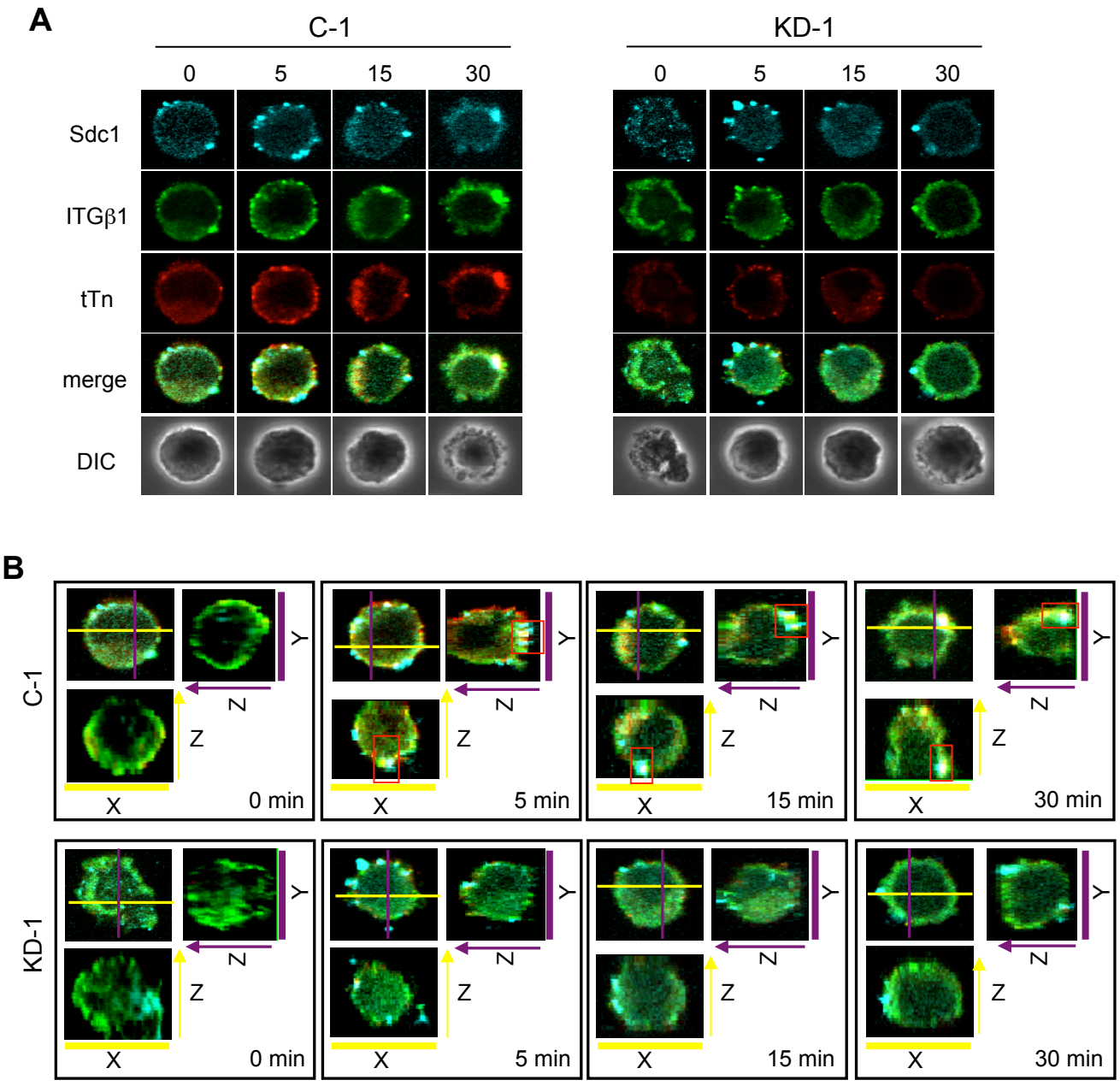
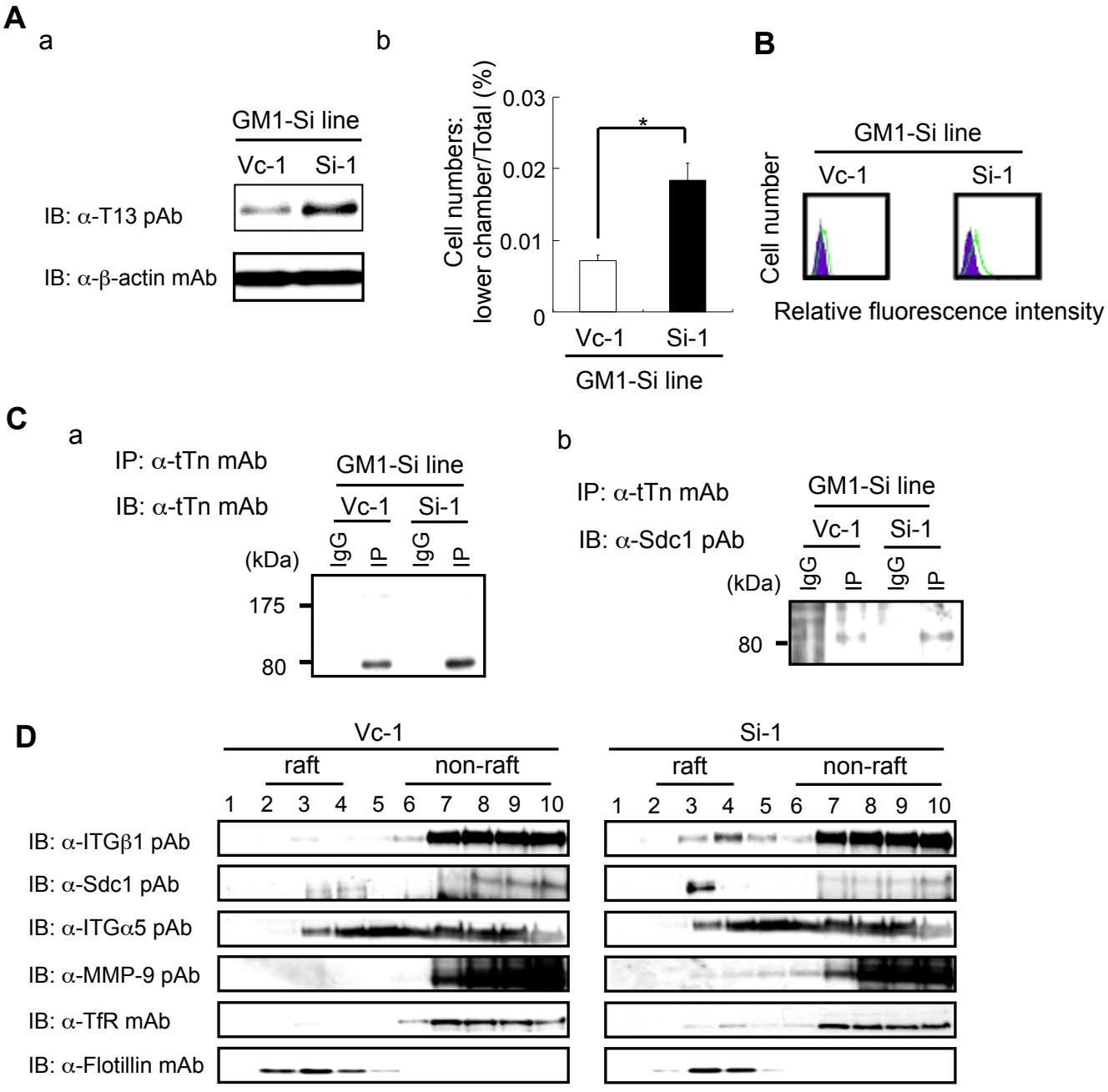


Supplemental Fig. S5



Supplemental Fig. S6