# SUPPLEMENTARY MATERIALS AND METHODS

**Spheroid assay**

Cells were seeded, maintained and cultured as described previously ([1](#_ENREF_1)). HCT116 and SW620 cells were cultured and treated with 20 µg/ml control IgG or EpAb2-6. Final data are presented as fold increase in colony number compared to IgG controls. Data are expressed as mean ± standard deviation (SD) from three independent experiments.

**Soft agar assay**

A 0.8% base agar layer was prepared and sterilized, followed by storage at room temperature in conical flasks. The agar was melted and kept in a water bath at 50°C. To make the bottom layer, one part of the melted 0.8% agar (500 μl) was mixed with 500 μl 2× DMEM serum free medium, and the mixture (0.4% agar) was then added to wells at 100 μl/well and allowed to solidify at room temperature. To prepare the top layer (0.3% soft agar), 500 μl of 0.6% agar was mixed with 500 μl 2× DMEM containing cells (500 cells/well). This mixture was then added to the bottom layer (100 μl/well) and allowed to solidify at RT before culturing as described. HCT116 and SW620 cells were cultured and treated with 20 µg/ml control IgG or EpAb2-6. Colonies were photographed after three weeks of growth.

**TACE activity assay**

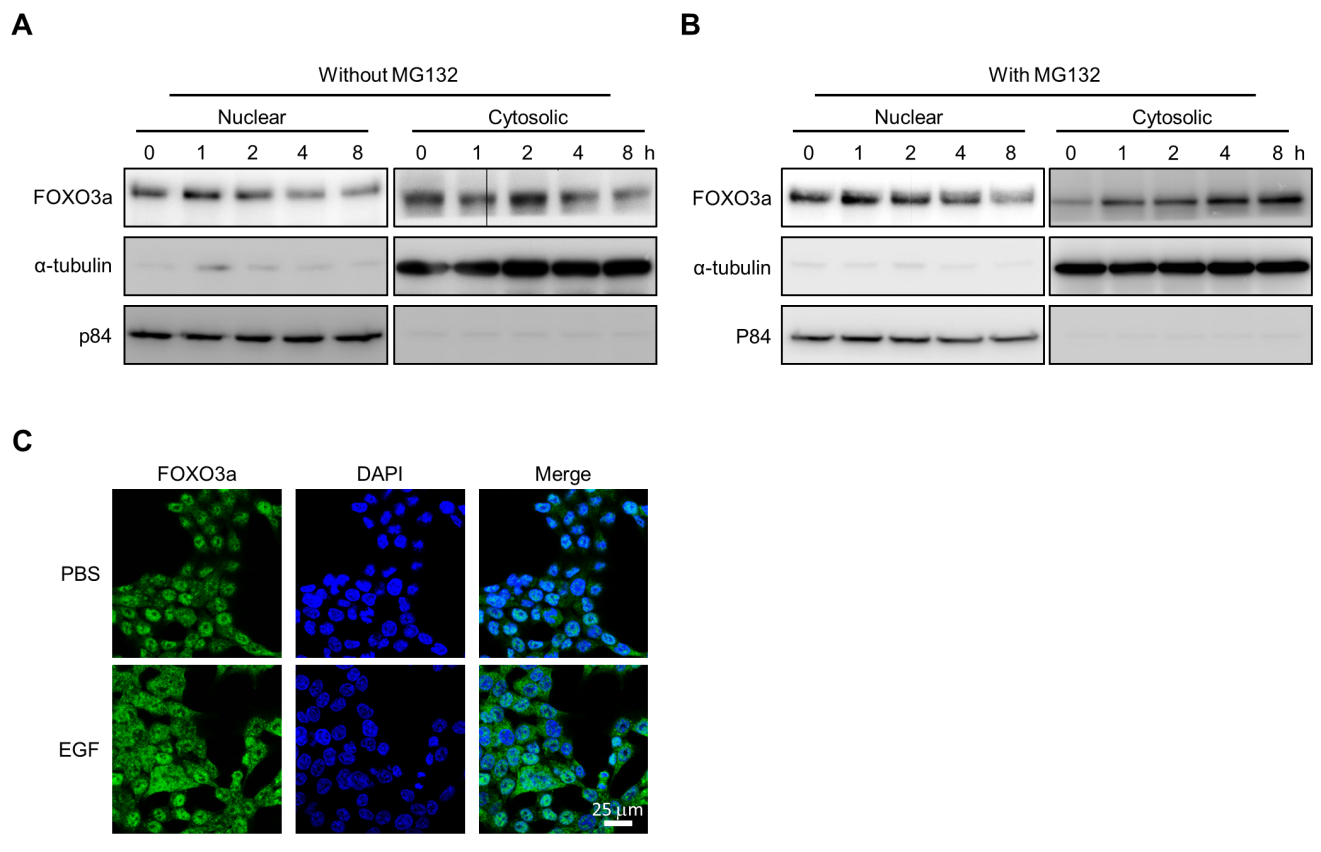
The enzyme activity of ADAM17 was quantified by fluorometric analysis using the InnoZyme™ TACE Activity Kit (Calbiochem #CBA042). To assess activity, 500 μg of a total lysate of HCT116 cells were incubated with anti-ADAM17 antibody-coated well before following the activity kit protocol.

**γ-secretase activity assay**

The enzyme activity of γ-secretase was quantified by fluorometric analysis using the fluorogenic γ-secretase substrate (Calbiochem #565764). The cell lysate was extracted with 0.5% CHAPSO to avoid breaking the structure of γ-secretase subunits. Each well contained 1 mg total lysate from HCT116 cells and 32.5 mM substrate; the reaction was carried out at 37°C in Corning® 96 well plates (black flat bottom). Fluorescence was determined with a microplate reader (Molecular Devices, SpectraMax M5) at an excitation wavelength of 355 nm and an emission wavelength of 440 nm under kinetic mode for 3 h, with a 3 min interval time.

**Reference**

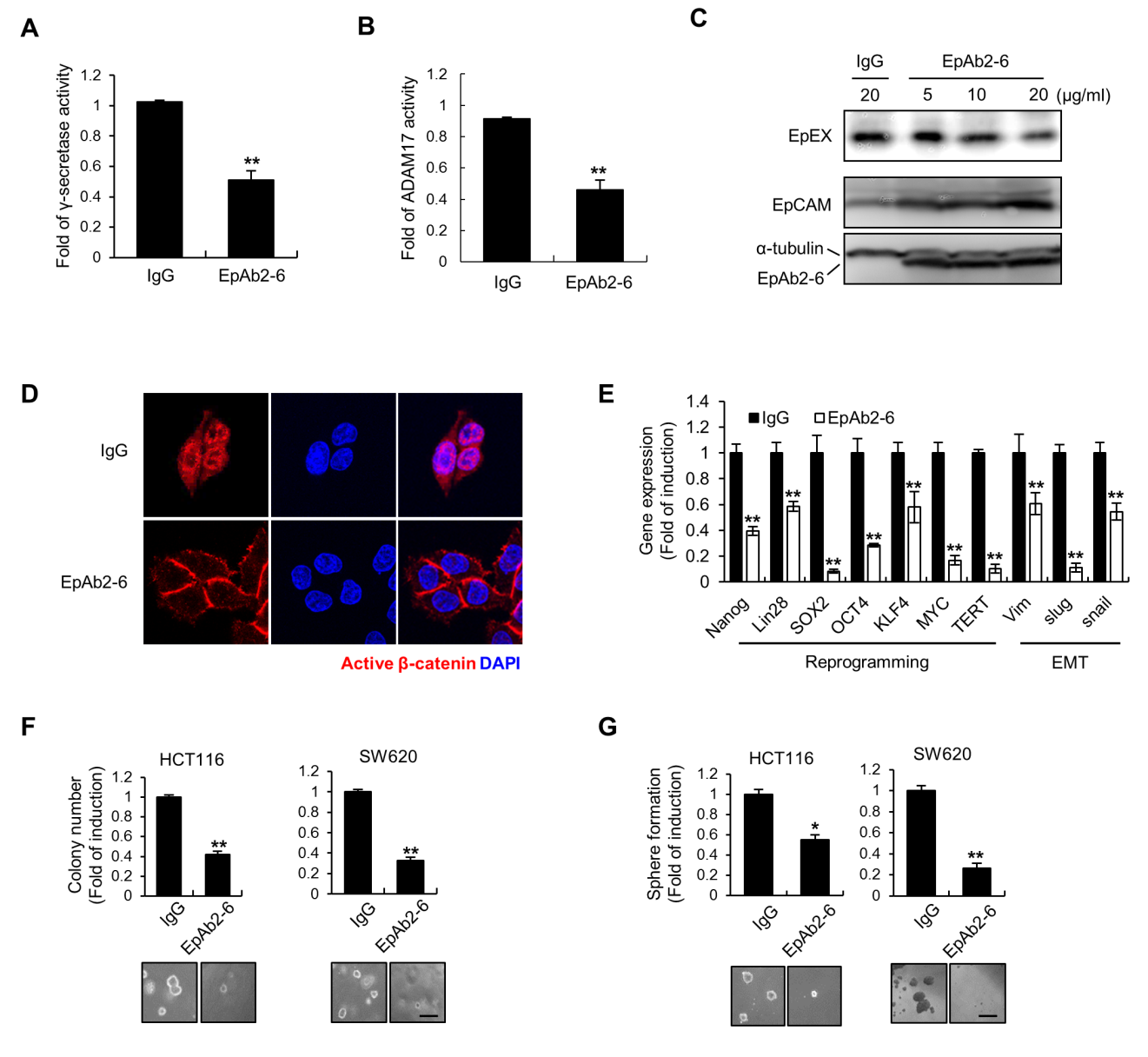
1. Lin CW, Liao MY, Lin WW, Wang YP, Lu TY, Wu HC. Epithelial cell adhesion molecule regulates tumor initiation and tumorigenesis via activating reprogramming factors and epithelial-mesenchymal transition gene expression in colon cancer. The Journal of biological chemistry **2012**;287:39449-59



**Figure S1. EGFR-AKT signaling pathway decreases nuclear localization of FOXO3a**

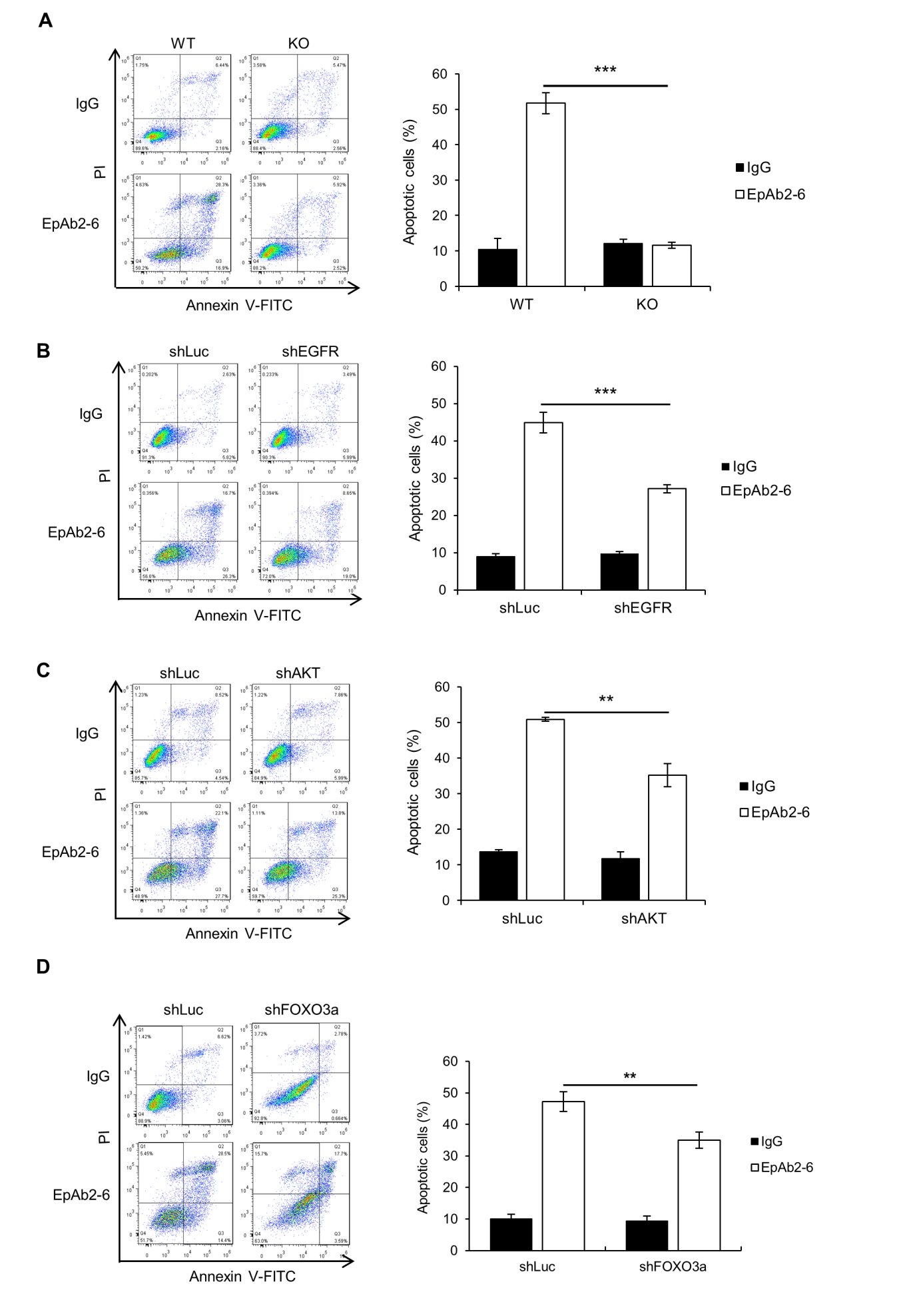
(A, B) HCT116 cells were serum starved then pre-treated with or without 10 μM MG132 for 1 h before being treated with EGF (100 ng/ml) and extracted at the indicated times. Nuclear and cytoplasmic fractions were analyzed by immunoblotting with the indicated antibodies. (C) HCT116 cells were treated with EGF for 2 h, and the subcellular localization of FOXO3a was assessed by immunofluorescence staining.

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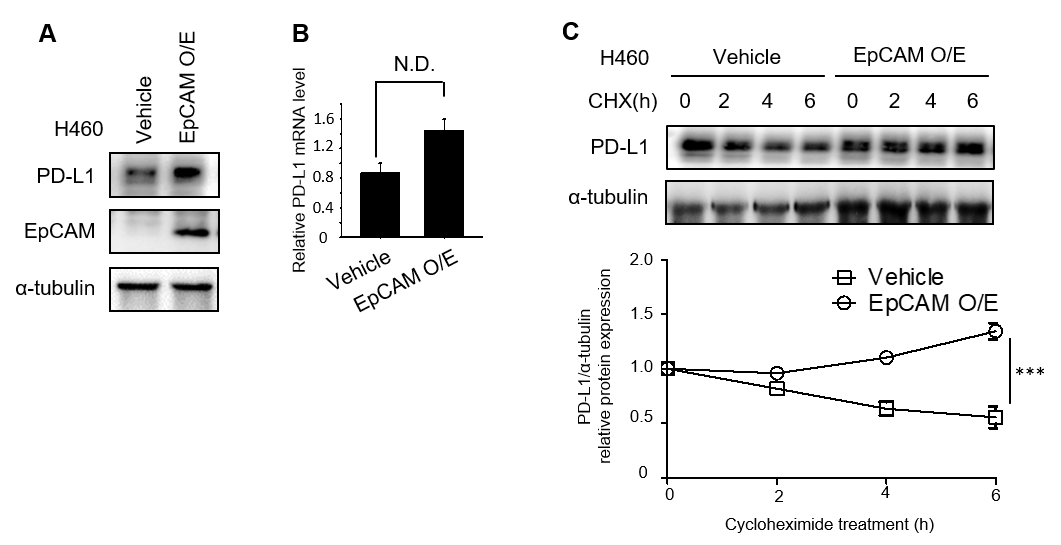
**Figure S2. EpAb2-6 decreases EpICD shedding and β-catenin nuclear translocation**

HCT116 cells were treated with either control IgG (20 µg/ml) or EpAb2-6 (20 µg/ml) for 24 h. Cell proteins were subsequently examined using (A) γ-secretase and (B) TACE activity assay kits. (C) HCT116 cells were treated with EpAb2-6 (0-20 µg/ml) for 24 h, and the culture supernatants were immunoblotted with anti-EpEX antibody. (D) HCT116 cells were treated with EpAb2-6 (20 µg/ml) for 6 h, and the subcellular localization of active β-catenin was assessed by immunofluorescence staining. (E) Real-time qPCR analysis mRNA levels of reprogramming and EMT genes in EpAb2-6 treated HCT116 cells. (F) The effect of EpAb2-6 on colon cancer cells was examined by the anchorage-independence assay. Results from one representative field in each group are shown. Quantification of results of colony formation data is presented. (G) The effect of EpAb2-6 on colon cancer cells was examined by spheroid formation. Spheroid formation from colon cancer cells was counted in triplicate. Representative images of tumorspheres after treatment with IgG or EpAb2-6 are shown. Bar, 5 mm. N = 3 independent experiments. Error bars indicate ± SD. \**p* < 0.05, \*\**p* < 0.01.

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**Figure S3. EGFR, AKT or FOXO3a knockdown and EpCAM knockout decrease EpAb2-6-induced apoptosis**

(A) EpCAM-knockout (KO), (B) EGFR-, (C) AKT-, (D) FOXO3a-knockdown HCT116 cells were treated with EpAb2-6, and the apoptotic cells were quantified by fluorescein-conjugated annexin V-FITC/PI double labeling. N = 3 independent experiments. Bar graphs show mean ± SEM. \**p* < 0.05, \*\**p* < 0.01.



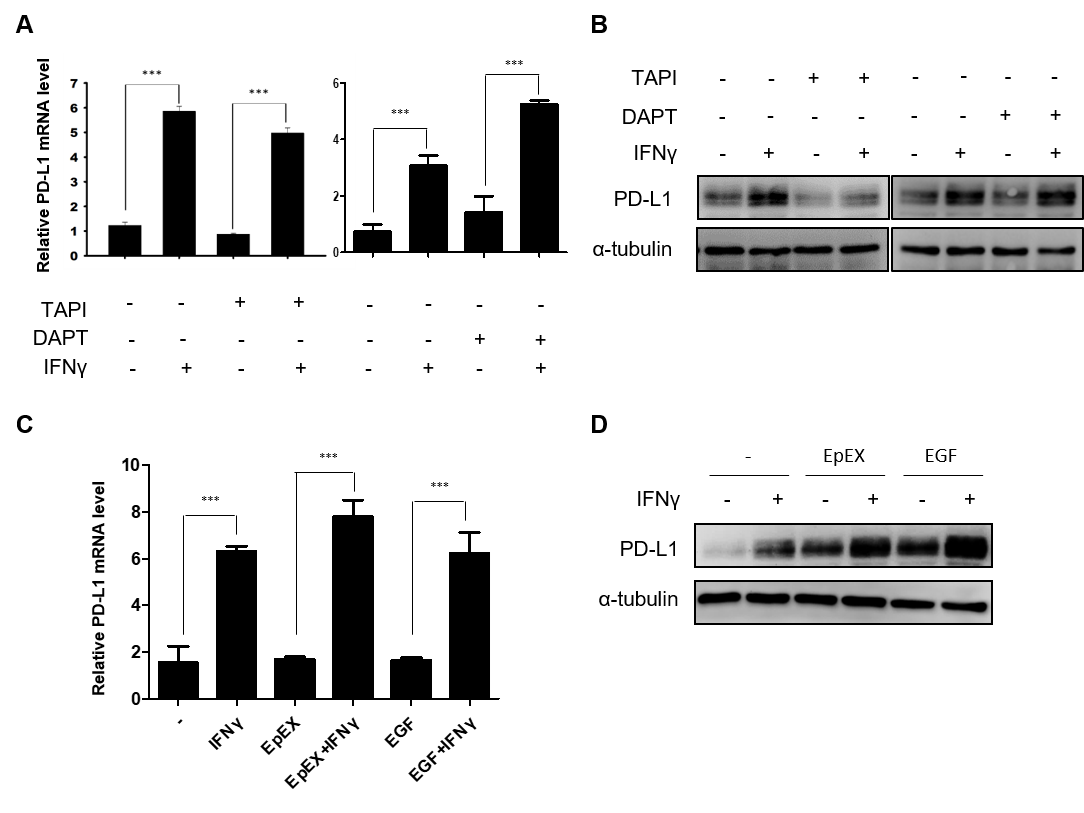
**Figure S4. EpCAM expression stabilizes PD-L1 protein in H460 cells.**

After transfection with vehicle or EpCAM expression vector, the PD-L1 protein and mRNA expression levels were analyzed by (A) Western blotting or (B) qRT-PCR. (C) H460 cells were treated with 50 μM CHX for the indicated intervals, and PD-L1 expression was analyzed by Western blotting. Protein expression over time is shown in the graph.



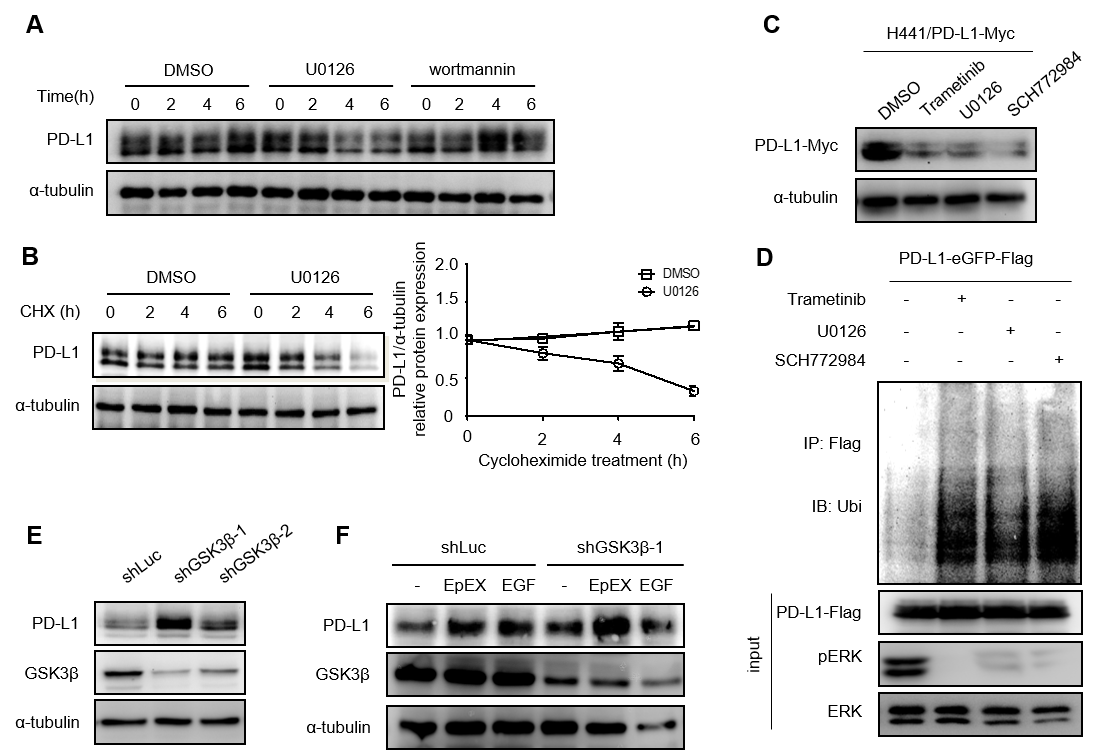
**Figure S5. Expression of EpCAM and PD-L1 in a colon tumor tissue array**

Immunohistochemical staining for (A) EpCAM and (B) PD-L1 was performed on a colon tumor tissue array.



**Figure S6. EpCAM is crucial for PD-L1 protein expression**

(A, B) After starvation and treatment with IFNγ for 16 h, H441 cells were treated with EpEX or EGF for 1 h and the PD-L1 expression was analyzed by (A) qRT-PCR and (B) Western blotting. (C, D) H441 were treated with or without TAPI, DAPT and IFNγ for 24 h, and PD-L1 expression was analyzed by (C) qRT-PCR and (D) Western blotting.



**Figure S7. MAPK pathway increases PD-L1 protein stability**

(A) H441 cells were treated with DMSO, U1026 or wortmannin for the indicated intervals, and PD-L1 expression was analyzed by Western blotting. (B) H441 cells were treated with U0126 for 6 h, and CHX for the indicated intervals, and PD-L1 expression was analyzed by Western blotting. (C) H441 cells expressing PD-L1-Myc were treated with trametinib, U0126 or SCH772984 for 6 h, and total cell lysate was subjected to Western blotting. (D) H441 cells expressing PD-L1-eGFP-Flag were treated with MG132 for 5 h. Total cell lysate was immunoprecipitated with anti-Flag antibody and the polyubiquitin of PD-L1-eGFP-Flag analyzed by Western blotting. (E) PD-L1 expression was analyzed in shLuc- or shGSK3β-expressing cells by Western blotting. (F) shLuc or shGSK3β cells were starved for 16 h and then treated with EpEX or EGF for 1 h. The total cell lysate was subjected to Western blotting.

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**Figure S8. EpAb2-6 treatment shows no significant body weight change in the orthotopic model with HCT116-Luc tumors**

Average body weight of each group is shown on the indicated days. Error bars show mean ± SD.

**Table S1. Primers for quantitative real-time PCR**

|  |  |
| --- | --- |
| Genes | Sequence |
| Nanog | Forward: 5'-ATGCCTCACACGGAGACTGT-3'  Reverse: 5'-AGGGCTGTCCTGAATAAGCA-3' |
| Sox2 | Forward: 5'-TATTTGAATCAGTCTGCCGAG-3'  Reverse: 5'ATGTACCTGTTATAAGGATGATATTAGT-3' |
| Oct4 | Forward: 5'-AGCAAAACCCGGAGGAGT-3'  Reverse: 5'-CCACATCGGCCTGTGTATATC-3' |
| Lin28 | Forward: 5'-CCAGTGGATGTCTTTGTGCACC-3'  Reverse: 5'-GTGACACGGATGGATTCCAGAC-3' |
| Klf4 | Forward: 5'- GCGAACTCACACAGGCGAGAAACC-3'  Reverse: 5'- TCGCTTCCTCTTCCTCCGACACA-3' |
| Myc | Forward: 5'-AAACACAAACTTGAACAGCTAC-3'  Reverse: 5'-ATTTGAGGCAGTTTACATTATGG-3' |
| Slug | Forward: 5'-TGGTTGCTTCAAGGACACAT-3'  Reverse: 5'-GTTGCAGTGAGGGCAAGAA-3' |
| Snail | Forward: 5'-CTTCGGCTCCAGGAGAGTC-3'  Reverse: 5'-TTCCCACTGTCCTCATCTGAC-3' |
| Vimentin | Forward: 5'-GTTTCCCCTAAACCGCTAGG-3'  Reverse: 5'-AGCGAGAGTGGCAGAGGA-3' |
| TERT | Forward: 5'-CCTGCTCAAGCTGACTCGACACCGTG-3'  Reverse: 5'-GGAAAAGCTGGCCCTGGGGTGGAGC-3' |
| BIM | Forward: 5'-GAGCCACAAGACAGGAGC-3'  Reverse: 5'-AAGGGCAATTCTGAGGGA-3' |
| p21 | Forward: 5'-CACAGCTCAGTGGACTGGAA-3'  Reverse: 5'-ACCCTAGACCCACAATGCAG-3' |
| FasL | Forward: 5'-AGCAAATAGGCCACCCCAGTCC-3'  Reverse: 5'-TGGCTCAGGGGCAGGTTGTTG-3' |
| HtrA2 | Forward: 5'-GTGGCAGACATCGCAACGCT-3'  Reverse: 5'-ACCCACTGATTCCGGAGGAG-3' |
| GAPDH | Forward: 5'-CTTCACCACCATGGAGGAGGC-3'  Reverse: 5'-GGCATGGACTGTGGTCATGAG-3' |

**Table S2. Primers for ChIP assay**

|  |  |
| --- | --- |
| Name | Sequence |
| FOXO3a | Forward: 5'-TGCAAAGAAACAATCC-3'  Reverse: 5'-TATATCAAGGTTCTACC-3' |

**Table S3. Primers for HtrA2 promoter cloning**

|  |  |
| --- | --- |
| Name | Sequence |
| pGL4.18-HtrA2-1 | Forward: 5'-AAAGAGCTCTGCTCTGCTTTGTCAGCTGG-3'  Reverse: 5'-AAACTCGAGACTGCTTCCTACTGCTCGTGC-3' |
| pGL4.18-HtrA2-2 | Forward: 5'-AAAGAGCTCCTTGACCACCTACCCAGCTGG-3'  Reverse: 5'-AAACTCGAGACTGCTTCCTACTGCTCGTGC-3' |

**Table S4. The effect of gene knockdown on EpAb2-6-induced apoptosis**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | A | B | A-B | C (A-B/A) |
| shEGFR | 35.9 | 17.4 | 18.5 | 51.5 |
| shc-Met | 37.1 | 28.2 | 8.9 | 24.0 |
| shAKT | 37.1 | 23.4 | 13.7 | 36.9 |
| shFOXO3a | 37.2 | 25.7 | 11.5 | 30.9 |
| shHtrA2 | 47.7 | 29.3 | 18.4 | 38.6 |

A: EpAb2-6-induced apoptosis in control knockdown cells (%)

B: EpAb2-6-induced apoptosis in indicated knockdown cells (%)

A-B: The decrease of EpAb2-6-induced apoptosis in indicated knockdown cells (%)

C: The decrease of EpAb2-6-induced apoptosis in indicated knockdown cells compared to apoptosis in control knockdown cells (%), (A-B/A)