Supplementary Information for:

Survival Outcome and EMT Suppression Mediated by a Lectin Domain Interaction of Endo180 and CD147

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Supplementary Methods

Materials

Catalogue numbers for chemicals (Sigma-Aldrich Company Ltd., Gillingham, UK), immunostaining reagents / tissue culture reagents / antibodies (Invitrogen Life Technologies Ltd., Paisley, UK) and other suppliers (where indicated) are stated in the methods.

Transient transfection, constructs and siRNA

3X Flag was cloned into pcDNA3-human Endo180 (C-terminal Xho I site). Truncated Endo180 proteins: ΔCTLD4-C-terminus [from M626 to C-terminus], ΔCTLD5-C-terminus [from A796 to C-terminus] and ΔCTLD6-C-terminus [from M929 to C-terminus] were cloned into p3XFlag-CMV-14 (EcoR I and Xba sites). CTLD-2 [from R508 to K640] and CTLD4 [from A808 to Q958] were cloned into pGex2TK-P (BamH I/Hind III and EcoR I/Hind III sites respectively). Expression vectors were transfected into cells using SuperFect transfection reagent (#301305; Qiagen, Crawley, UK) following manufacturers instructions. siRNA oligonucleotides: Endo180 #1 ($^{5'}$AACCUUUCUCUGUAGUUGAUU$^{3'}$), Endo180 #2 ($^{5'}$CCCAACGUCUUCCUCAUCU$^{3'}$) and reversed Endo180 ($^{5'}$UCUACUCCUUCUGCAACCC$^{3'}$). MT1-MMP ($^{5'}$AACAGGCAAAGCUGAUGCAGA$^{3'}$) and CD147 #1 (Ambion® ID #s2099) and #2 (Ambion® ID #s2098) were also used. siRNA oligonucleotides were transfected into cell monolayers grown to 30% confluency using Oligofectamine^{TM} (#12252-011) following manufacturers instructions. After 24 hours cells were seeded onto neat Cultrex® rBM and overlaid with respective growth medium + 2% v/v Cultrex® rBM as described above.

Immunofluorescent staining procedure

Cells were washed in PBS+ (0.1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and fixed in 4%

v/v paraformaldehyde (PFA) in PBS+ (30 min at room temperature). Reactions were quenched with 75 mM NH_4Cl and 20 mM glycine in PBS+ (5 x 5 min, room temperature). Cells were permeabilized with 0.5% v/v Triton-X in PBS for 30 min and blocked for 2 hours at room temperature with immunofluorescence blocking buffer (IFB) (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% w/v BSA, 0.5% v/v Triton X-100, 0.05% v/v Tween-20) complemented with 20% w/v non-immune goat serum (#ab7481; Abcam). Cells were incubated over night at 4ºC with the primary antibodies diluted in IFB (Supplementary Table S1). After washing with IFB (3 x 10 min) at room temperature cells were incubated with secondary antibodies diluted in IFB + 10% v/v goat serum and incubated for 2 hours at room temperature. To analyze F-actin fibers, phalloidin-Alexa®Fluor-488 was added to the secondary antibody dilution. Samples were washed at room temperature once with IFB + 10% v/v goat serum, 3x with IFB alone and 2x with PBS+. Complexes of antibodies were fixed and the reaction was quenched as described above. After nuclear counterstaining with TOPRO-3 or DAPI, cells were washed with PBS+ (2 x 5 min) and mounted in Vectashield® (#H1000; Vector Laboratories, Peterborough, UK).

GST fusion protein purification

Overnight cultures of E. coli stain BL21 (DE3) pLysS transformed with GST and GST-fusion expression vectors were diluted 1:10 and incubated at 37ºC. Once cultures reached OD^{600} (0.4-0.6 nm) GST and GST-fusion protein expression was induced with 1 mM IPTG (room temperature, 3 h). Cells lysed in 5 ml of GST lysis buffer (1% v/v Triton X-100, 20% v/v glycerol, 10 mM β-mercaptoethanol and Complete™ protease inhibitors [Roche Products Ltd.] in PBS) and sonicated before being centrifuged and incubated under rotation with 50% v/v suspension of glutathione-sepharose beads (4ºC, 1 h). Samples were washed with 5 ml of GST lysis buffer (3x) and GST and GST-fusion

proteins were eluted using 300 ml of glutathione-containing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1% v/v Triton X-100, 20 mM glutathione and proteases inhibitors in PBS) (4ºC, overnight incubation). GST and GST-fusion protein elutes were dialyzed against PBS (4ºC, overnight incubation) and proteins concentration determined using a Pierce BCA protein assay kit (#23225; Thermo Scientific UK) prior to use for cell treatment.

Flow cytometry and viability assay

Cell culture was harvested and washed 2x with 5 ml of ice cold PBS. Immediately after 10^6 cells were resuspended in 4 ml of cold EtOH 70% v/v added drop wise while vortexing. Fixed cells were stored up to 1 week at 4°C. Cells were spun down, washed with cold PBS and resuspended in 500 µl of chilled propidium iodide solution (40 mg/ml propidium iodide with 40 mg/ml RNAse A). The mix was incubated at 37°C for 45 min to allow total RNA degradation and transferred to a flow cytometry tube to analyze the cell cycle using FACS Calibur (Beckton-Dickinson, Oxford, UK). Linear scale representation of forward and side scattering and DNA content were measured. A total of 10000 events were measured per sample. Percentage of RWPE-1 and PC3 cells in each cell cycle phase and percentage of dead cells was obtained using BD FACStation© software 2007 (Beckton-Dickinson, Oxford, UK). To quantify viable RWPE-1 and PC3 cells 30% confluent cultures were treated with 4 and 10 µg/ml of GST, GST-CTLD2 or GST-CTLD4 for four days. Cells were harvested and the percentage of viable cells calculated using a CASY TT Model cell counter and analyzer system (Roche Innovatis AG).

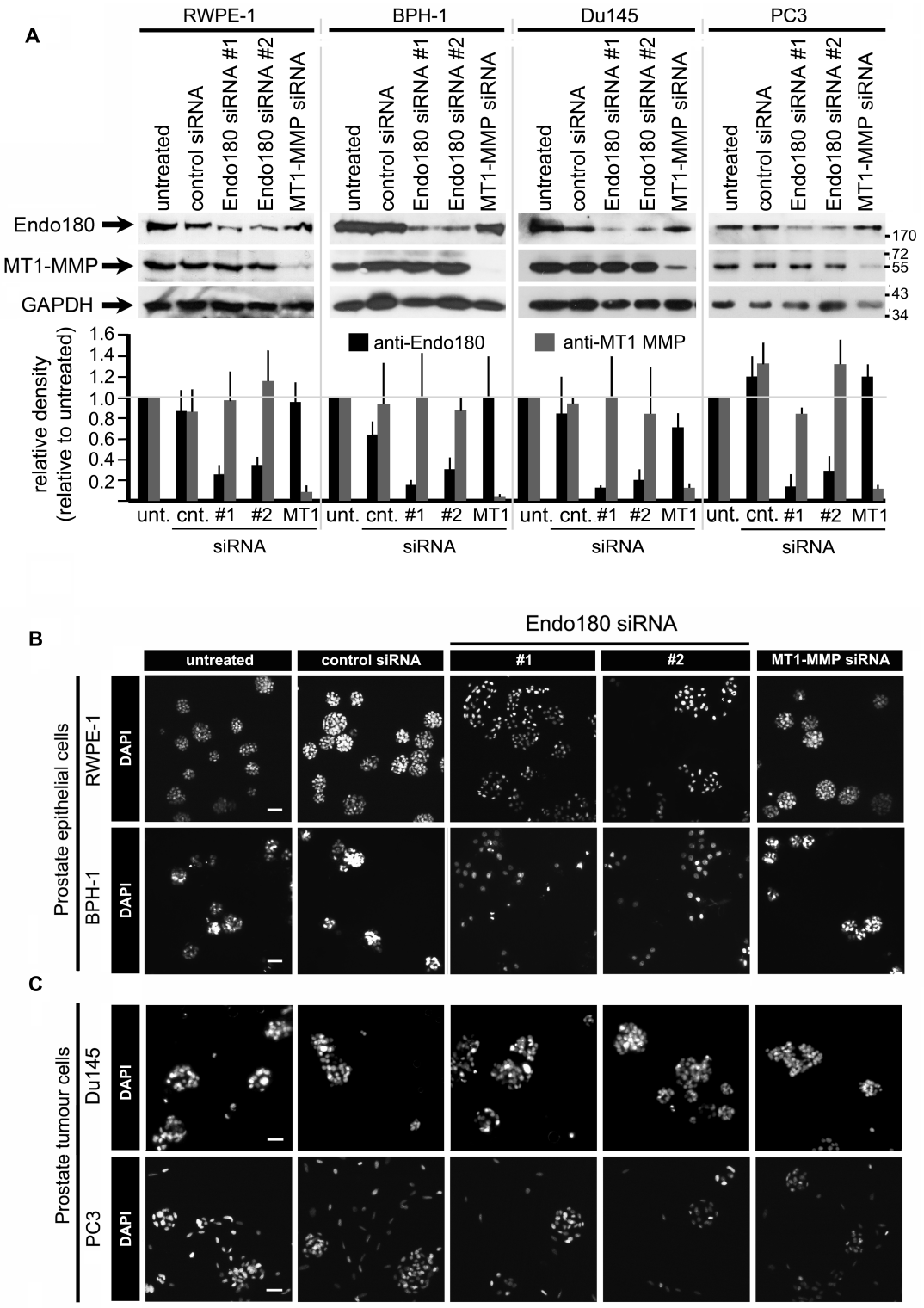

Figure S1. Silencing Endo180 disrupts normal and benign PEC acini, but not PTC spheroids. **A,** Endo180 and MT1-MMP immunoblots show effective silencing of Endo180 (oligonucelotides #1 and #2) and MT1-MMP in RWPE-1, BPH-1, DU145 and PC3 cells using siRNA compared to untreated cells and cells treated with scrambled Endo180 siRNA oligonucleotide #2; MW markers, kDa. (upper panel). Graphs show average of relative levels ± s.d. determined by densitometric analysis of Endo180 (black bars) and MT1-MMP (gray bars), which were adjusted against GAPDH (loading control) and normalized against untreated cells (relative levels = 1) (lower panel). **B,** Normal PECs (RWPE-1) and benign PECs (BPH-1) were untreated or treated with scrambled Endo180 siRNA oligonucleotide #2, Endo180 siRNA oligonucleotides #1 and #2 or MT1-MMP siRNA (three days) and acini were fixed and cell nuclei visualized using DAPI. **C,** Analysis described in panel B. conducted with spheroids of metastatic PTCs (DU145, PC3); magnification, x20; scale bar, 50 µm. Data represents three independent experiments.

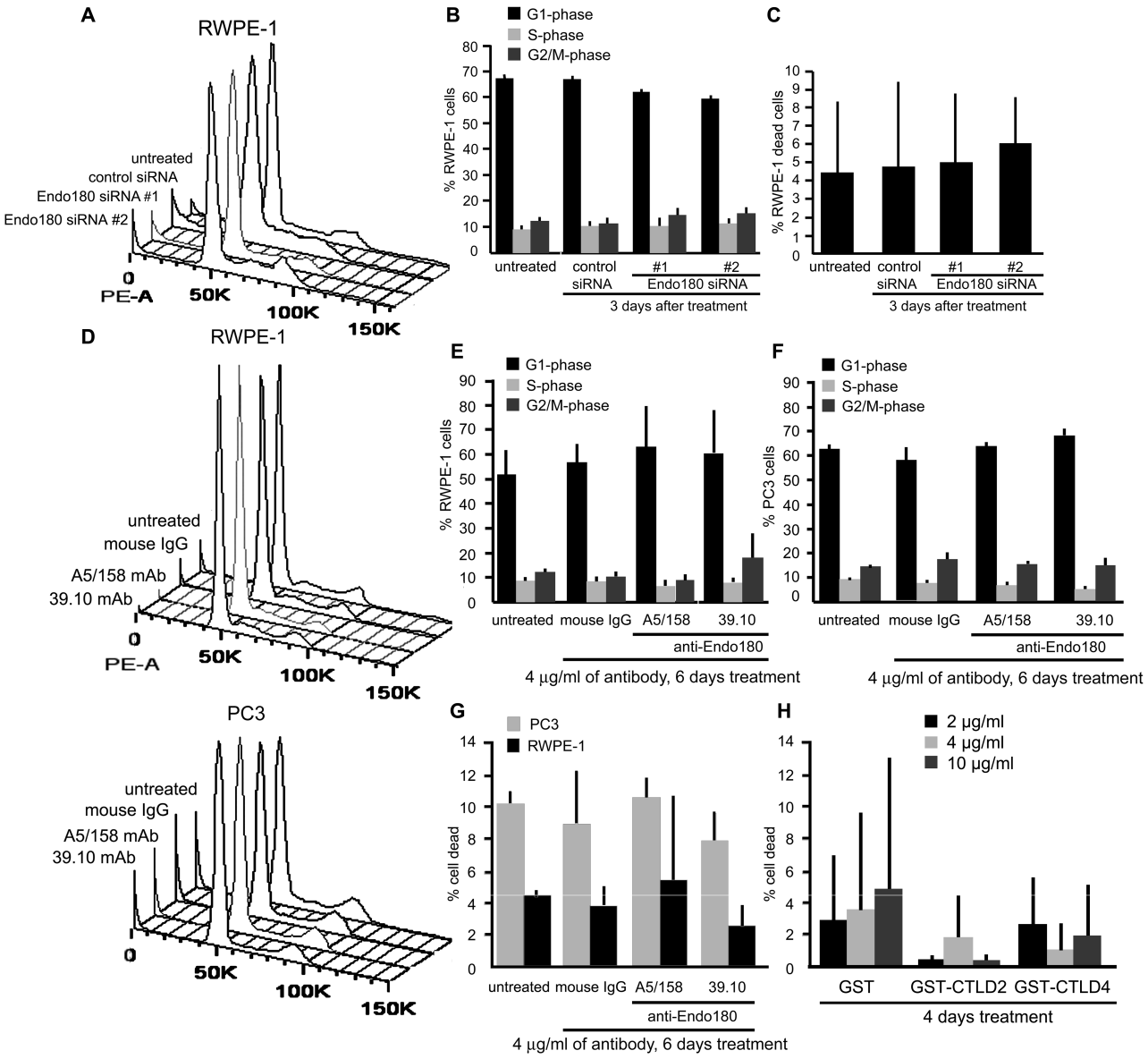

Figure S2. None of the treatment used in culture affected cell cycle and/or cell viability. **A,** Cell cycle profiles of RWPE-1 cells untreated or treated with scrambled Endo180 siRNA oligonucleotide #2 or Endo180 siRNA oligonucleotides #1 and #2 (three days) and analyzed by FACS (1 x 10^5 events counted; FlowJo 8.8.6 software). **B,** Graph shows % RWPE-1 cells in G1-phase (black bars), S-phase (light gray bars) and G2/M-phase (dark gray bars). **C,** Graph shows % dead cells following treatment; data shown is average ± s.d. of three independent experiments. **D,** Targeted blockade of Endo180 does not affect the cell cycle or viability of normal PECs or PTCs. PECs (RWPE-1) and PTCs (PC3) untreated or treated with mouse IgG, A5/158 mAb and 39.10 mAb (4 µg/ml per day for six days) and analyzed by FACS (1 x 10^5 events counted; FlowJo 8.8.6 software). **E,** Graphs show % RWPE-1 cells in G1-phase (black bars), S-phase (light gray bars) and G2/M-phase (dark gray bars); representative cell cycle profiles are shown. **F,** Same as panel E with PC3 cells (right panel). **G,** Graph shows % dead cells after treatment; RWPE-1 (gray bars); PC3 (black bars); data shown is average ± s.d. of three independent experiments. **H,** Targeted blockade of CTLD2 and CTLD4 does not affect normal PEC viability. Graph shows % dead RWPE-1 cells untreated or treated with purified GST, GST-CTLD2 or GST-CTLD4 (2, 4 or 10 µg/ml a day for four days) calculated using a Casy cell counter with TT system (Innovatis, Roche Diagnostics GmbH); 2 ug/ml treated cells black bars; 4 µg/ml treated cells light gray bars; 10 µg/ml treated cells dark gray bars; data shown is average ± s.d. of three independent experiments.

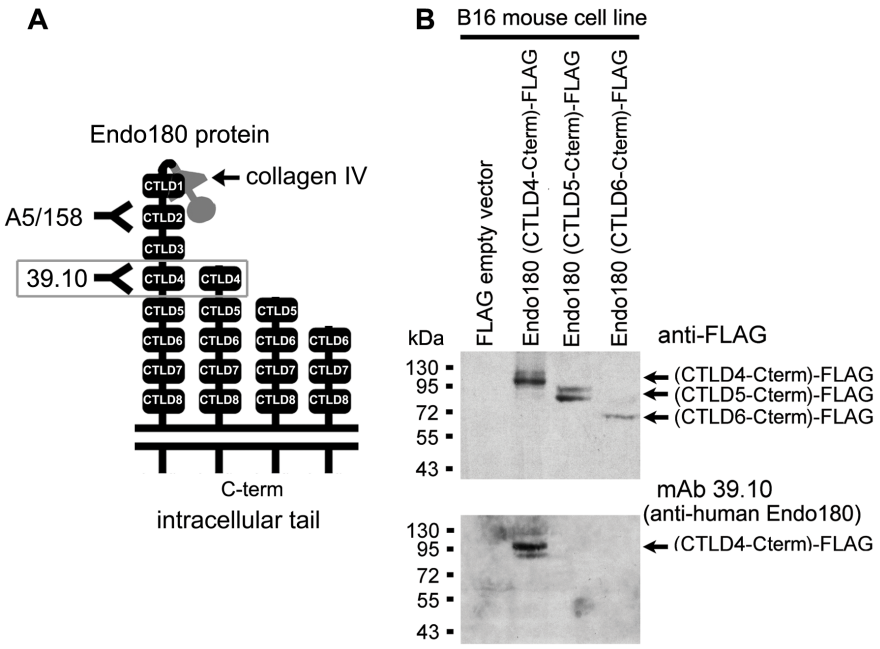

Figure S3. Epitope mapping of the Mouse monoclonal anti-Endo180 39.10. **A,** Epitopes in human Endo180 ectodomain recognized by A5/158 mAb and 39.10 mAb. **B,** Transfection of truncated human Endo180-Flag proteins (Endo180-ΔCTLD4-C-terminus, Endo180-ΔCTLD5-C-terminus and Endo180-ΔCTLD6-C-terminus) in B16F10 mouse melanoma cells and immunodetection with anti-Flag (Sigma mouse anti-Flag mAb clone M2) (upper panel) and 39.10 mAb (lower panel). MW markers are indicated in kDa.

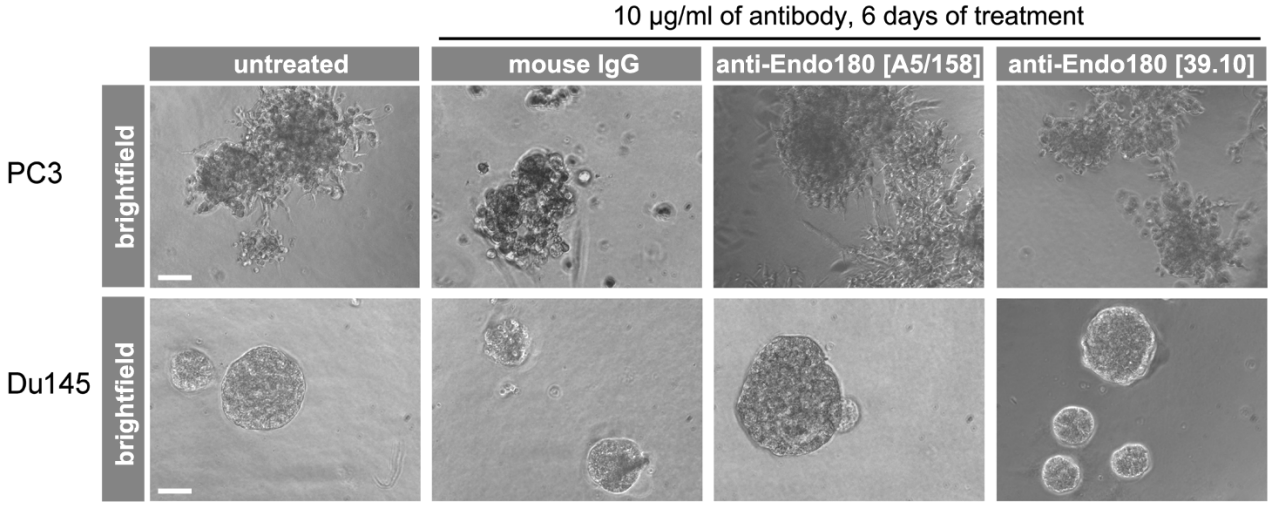

Figure S4. Targeted blockade of Endo180 does not alter spheroid formation by PTCs. Brightfield images of PTCs (PC3, DU145) that were untreated or treated with IgG control, A5/158 mAb or 39.10 mAb (10 µg/ml per day for six days); magnification x20; scale bar, 100 µm.

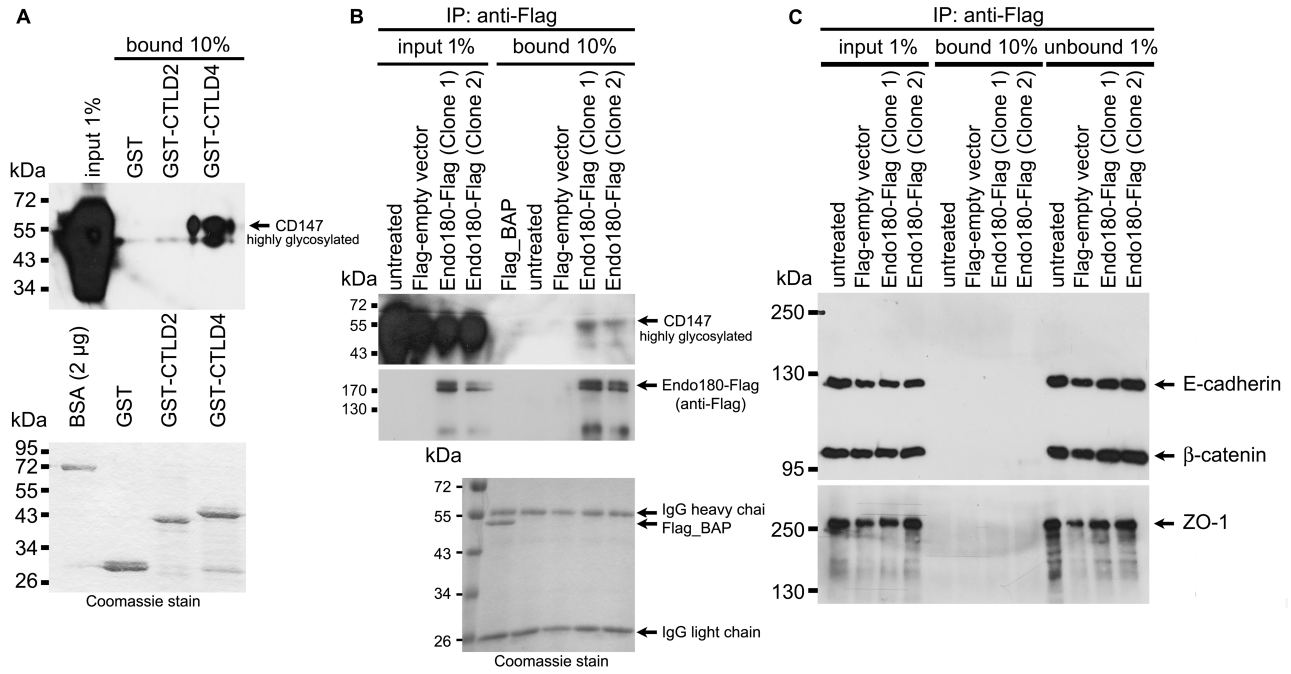

Figure S5. Highly glycosylated CD147 can be precipitated from normal PEC lysates using CTLD4 as bait. **A,** Upper panel: Immunoblot shows levels of CD147 in GST-CTLD2 and GST-CTLD4 pull-downs from PEC (RWPE-1) whole cell lysates compared to GST alone (negative control). Lower panel: coomassie blue stain shows levels of protein bound to glutathione-agarose beads in precipitates. **B,** Highly glycosylated CD147 immunoprecipitation of from normal PEC lysates using Endo180-Flag. Immunoblot show CD147 (upper panel) and Flag (middle panel) immunoprecipitated using anti-Flag from untransfected PECs (RWPE-1) or PECs transfected with Flag-empty vector or Endo180-Flag vectors (clones 1 and 2); internal controls include binding buffer, anti-Flag M2-agarose affinity gel and 50 ng/µl of bacteria alkaline phosphatase protein tagged with Flag (Flag_BAP); 1% of input and 10% of bound fraction were loaded; coomassie stain shows quantities of anti-Flag M2-Agarose affinity gel in each reaction; IgG heavy and light chain of anti-Flag indicate that equal amount of anti-Flag M2 was used in each immunoprecipitation (lower panel). **C,** Immunoblots show lack of E-cadherin, β-catenin (upper blot) and ZO-1 (lower blot) following immunoprecipitation; 1% of input, 10% of bound and 1% of unbound fraction were loaded; MW markers, kDa.

Supplementary Table S1 - Antibodies and reagents used for Immunoblotting, Immunofluorescence and Immunohistochemistry

Antibody	Concentration		
	Immunoblotting	Immunofluorescence	Immunohistochemistry
Mouse anti-human Endo180 monoclonal antibody clone A5/158	1:5 dilution of hybridoma supernatants	0.5 mg/ml	
Mouse anti-human Endo180 monoclonal antibody clone 39.10	1:5 dilution of hybridoma supernatants	0.5 mg/ml	10 µg/ml
mouse anti-human MT1-MMP clone LEM-2/15.8 mAb (#MAB3328; Merck Millipore UK)	1/2000		
rabbit anti-human E-cadherin pAb (#3195; Cell Signaling Technology, Denver, USA)	1/1000	1/250	
rabbit anti-human GAPDH pAb (#ab9485; Abcam)	1/10000		
mouse anti-human β-catenin clone E5 mAb (#sc-7963; Santa Cruz Biotechnology)	1/500		
rabbit anti-human ZO-1 pAb (#40-2200, Invitrogen Life Technologies Ltd., Paisley, UK)	1/1000		
mouse anti-human ERK2 clone C-14 mAb (#sc-154; Santa Cruz Biotechnology)	1/2000		
mouse anti-human CD147 clone 1S9-2A mAb (#MAB2623; Merck Millipore UK)	1/10000	1/1000	
anti-Flag mouse mAb clone M2 (#F1804; Sigma Aldrich Ltd.)	1/5000	1/100	
HRP-conjugated AffiniPure goat anti-mouse IgG (H+L) (#115-035-174; Jackson Immuno Research Laboratories Inc., Pennsylvania, USA)	1/5000		
HRP-conjugated goat anti-rabbit IgG #sc-2004 (Santa Cruz Biotechnology, Heidelberg, Germany)	1/5000		
Alexa®Fluor-conjugated IgGs [H+L]		1/500	
phalloidin Alexa®Fluor-488 (#A12379, Invitrogen Life Technologies Ltd., Paisley, UK)		1/100	
TO-PRO®-3 iodide nuclear counterstain (#T3605, Invitrogen Life Technologies Ltd., Paisley, UK)		1/1000	
4',6-diamidino-2-phenylindole (DAPI) (#D3571, Invitrogen Life Technologies Ltd., Paisley, UK)		1/5000	

Supplementary Table S2 - NCLPC1/4 prostate cancer tissue microarray patient characteristics, N = 157

Patient characteristics	NCLPC1	NCLPC4
No. patients	76	81
Median age, range	-	72, 55-89
Years of study	1989-1995	1988-1997
No. tumor cores per patient	2	2
Tumor-adjacent non-malignant cores per patient	0	0-2
Gleason range	6-10	6-10
Metastatic disease	Yes	Yes
Survival	Yes	Yes
Cause of death	Yes	Yes

-, not known

Supplementary Table S3. NCLPC1/4 prostate cancer tissue microarray - patient deaths [censored] after three, five, seven and ten years.

NCLPC1/4 arrays combined, N (%)					
Groups	**N total**	**N of death after 3, 5, 7 y 10 years**			
		3 years	**5 years**	**7 years**	**10 years**
Endo180 negative	33	8 (24%)	13 (39%)	18 (55%)	23 (70%)
Endo180 positive	93	44 (47%)	60 (65%)	69 (74%)	74 (80%)
Gleason grade 5-7	41	11 (27%)	21 (51%)	27 (66%)	32 (78%)
Gleason grade 8-10	93	44 (47%)	58 (62%)	66 (70%)	70 (75%)
Gleason grade 5-7					
Endo180 negative	14	1 (7%)	3 (21%)	6 (43%)	9 (64%)
Endo180 positive	24	9 (38%)	16 (67%)	19 (79%)	21 (88%)