# Supporting Information

## Takahashi et al. 10.1073/pnas.1106171109

### SI Materials and Methods

**Antibodies, Plasmids, and Reagents.** *Antibodies.* Anti-thrombospondin 1 (anti-TSP1) was from Thermo Scientific. Anti-HA was from Roche. Anti-alkaline phosphatase (anti-AP) was from GenHunter. Anti-phospho EGF receptor (anti-phospho EGFR) (Tyr1173), anti-EGFR, anti-protein tyrosine phosphatase μ (anti-PTPμ), anti–β-actin, and anti-CD148 (clone 143–41) were from Santa Cruz Biotechnology. Affinity-purified anti-CD148 goat polyclonal and phycoerythrin-conjugated anti-CD148 monoclonal (clone 143–41) were from R&D Systems. Anti–leukocyte antigen-related (anti-LAR) was from Millipore. Anti-phospho ERK1/2 (Thr202/Tyr204) was from New England Biolabs. Anti-ERK1/2 was from Upstate Biotechnology. Anti-Flag (clone M2) was from Sigma. Anti-VEGF receptor 2 (anti-VEGFR2) was from Cell Signaling. Anti-phospho VEGFR2 [$pYpY_{1054/1059}$] was from Invitrogen. Anti-tyrosine kinase that contains immunoglobulin-like loops and epidermal-growth-factor-similar domains 2 (anti-Tie2) was from BD Biosciences. Anti-stomach cancer-associated PTP (anti–SAP-1) was kindly provided by T. Matozaki (1). Rabbit anti-vascular endothelial protein tyrosine phosphatase (anti–VE-PTP) antibody was generated in our laboratory. Briefly, the cDNA sequence of the VE-PTP cytoplasmic domain (Asp1723-His1988, Genbank accession no. AY077755) was subcloned to pGEX6×-1 vector (GE Healthcare), and the GST fusion protein was produced and purified according to the manufacturer's instructions (GE Healthcare). Rabbits were immunized with the protein, and the antibody was affinity purified at Alpha Diagnostic Intl., Inc.

***Plasmids.*** Expression plasmids encoding HA-tagged WT or catalytically inactive (cs) CD148 have been described previously (2, 3). HA-tagged CD148 lacking most of cytoplasmic domain (CD148ΔCy) (Asp1002–Ala1337; Genbank accession no. U10886) and HA-tagged CD148 lacking all extracellular fibronectin type-III domains (CD148ΔFN) (Trp139–Tyr789; Genbank accession no. U10886) were generated by PCR and cloned into pSRα (4) and pSecTag2C vectors (Invitrogen), respectively. The cDNA of human LAR was kindly provided by Michel Streuli (Harvard Medical School, Boston, MA) and subcloned into pSRα vector. The expression plasmid of human PTPμ, pMT2-PTPμ, was kindly provided by S. M. Brady-Kalnay (Case Western Reserve University, Cleveland, OH) (5). The SAP-1 and VE-PTP expression vectors were kindly provided by T. Matozaki (Gunma University, Gunma, Japan) (1, 6). The expression vector of Flag-tagged mouse glomerular epithelial protein 1 was kindly provided by T. Shirasawa (Juntendo University, Tokyo, Japan) (7). The cDNA of human TSP1, pGEM2hTSP-1, was purchased from Addgene.

***Reagents.*** HRP-conjugated NeutrAvidin was from Thermo Scientific. Native human TSP1 was from Genway Biotech, Inc. Basic FGF (bFGF) (human, recombinant) was from R&D Systems.

**Cell Culture and Transfection.** Human umbilical vein endothelia cells (HUVEC) were purchased from Lonza and cultured in EGM2 medium (Lonza). Human renal microvascular endothelial cells (HRMEC) have been described previously (4, 8). HEK 293 T and CHO cells were purchased from American Type Culture Collection and cultured as previously described (2). A431D cells were kindly provided by A. Reynolds (Vanderbilt University, Nashville, TN) and cultured as described (9). Transient transfection of HEK 293 or CHO cells was carried out using Fugene HD (Roche) transfection reagent, according to the manufacturer's protocol.

**Recombinant Adenovirus and Its Infection in HUVEC.** Recombinant adenoviruses encoding C-terminally HA-tagged WT CD148 or β-galactosidase (LacZ) were generated using the pAD/CMV/V5 vector and a ViraPower Adenovirus Expression kit (Invitrogen) according to the manufacturer's protocol. Briefly, the HA-tagged CD148 sequence (4) was subcloned to the pENTR 3C vector (Invitrogen) and then was transferred to the pAD/CMV/V5 vector by LR recombination reaction according to the manufacturer's protocol. The resulting adenoviral construct was digested with PacI and transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen). The pAD/CMV/V5/LacZ vector included in the kit was used as a control. The viruses were double-plaque purified, expanded with HEK 293 cells, purified on a cesium chloride gradient, and titered by a plaque assay. For infection HUVEC were cultured in EBM-2 medium and then in serum-free EBM-2 medium, and the adenoviruses were added to subconfluent cells at a multiplicity of infection of 100. The medium was removed 5 h after adenovirus infection, and the cells were washed with PBS, overlaid with fresh growth medium, and cultured for 48 h before study. The efficiency of infection was assessed by anti-HA or X-Gal cell staining. Anti-HA cell staining was carried out as described in Fig. S2. The infection efficiency obtained by this protocol was >80%.

**Affinity Purification and Mass Spectrometry.** HA-tagged CD148 or LacZ (mock) was introduced to HUVEC using the recombinant adenoviruses as described above. Cells in ten 100-mm dishes were surface-labeled with 2 mM biotin (EZ-Link Sulfo-NHS-LC-Biotin; Thermo Scientific) in PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ ($PBS^+$) for 1 h at 4 °C. Excess biotin was quenched by washing twice in 100 mM glycine/$PBS^+$ on ice. After three washes in ice-cold $PBS^+$, the cells were lysed in lysis buffer [1% Nonidet P-40, 150 mM NaCl, 20 mM Hepes (pH 7.5), 1 mM EDTA, 5 mM NaF, 5 mM iodoacetic acid, 1 mM $Na_3VO_4$, complete protease inhibitor mixture from Roche], and clarified cell lysates were incubated with anti-HA affinity matrix (Roche) for 4 h at 4 °C. The bound proteins were eluted using 1 mg/mL HA peptide (Roche) in buffer [20 mM Tris (pH 7.5), 100 mM NaCl, 0.1 mM EDTA]. Then the eluate was incubated with streptavidin-immobilized agarose (Sigma) to collect cell-surface proteins in CD148 complex. The resulting proteins were eluted with Laemmli sample buffer containing 62.5 mM DTT, separated with Ready-Gel 4–20% (Bio-Rad), and stained with colloidal blue (Invitrogen). Protein bands of interest were excised from the gel, cut into 1-mm cubes, and equilibrated in 50 mM $NH_4HCO_3$. Proteins were reduced within the gel pieces with DTT (3 mM in 100 mM $NH_4HCO_3$, 37 °C for 15 min) followed by alkylation with iodoacetamide (6 mM in 50 mM $NH_4HCO_3$ for 15 min). The gel pieces then were dehydrated with acetonitrile and rehydrated with 15 μL of 12.5 mM $NH_4HCO_3$ containing 0.01 μg/μL trypsin (Trypsin Gold; Promega), and trypsin digestion was carried out for >2 h at 37 °C. Peptides were extracted with 60% acetonitrile, 0.1% formic acid, dried by vacuum centrifugation, and reconstituted in 15 μL 0.1% formic acid. Five microliters of peptide hydrosylate were analyzed by C18 reverse-phase liquid chromatography-tandem MS (LC-MS/MS) using a Thermo linear trap quadrupole ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, nanospray source and Xcalibur 2.0 instrument control using standard triple-play methods. Tandem MS data were analyzed with the Sequest algorithm to search a human subset of the UniRef100 database (January 23, 2007; 223,514 entries) using Xcorr cutoffs of γ1.8 for $[M+2H]^{2+}$/2

ions and γ2.5 for $[M+3H]^{3+}/3$ ions. In addition, the database contained a concatenated reverse-decoy database to estimate false-discovery rates, which were at 5% or below. The peptide sequences obtained from the LacZ (mock)-transduced cells through the same procedure were considered as background.

**Fusion Proteins.** The construct for AP-TSP1 fusion protein was generated by inserting a full-length TSP1 sequence (nucleotides 234–3689; Genbank accession no. NM003246) in the C-terminal region of AP in APtag-5 vector (GenHunter) using PCR. The construct for the CD148-Fc fusion protein was generated by linking the CD148 ectodomain sequence (nucleotides 340–3276; Genbank accession no. U10886) to the human Fc (IgGk) sequence (10) using PCR. The resulting CD148-Fc sequence or human Fc (IgGk) sequence was inserted into pTT vector (11). The plasmids were transiently transfected into HEK 293 6E cells using polyethylenimine, and the fusion proteins were produced by suspension cell culture as described (11). The amount of AP-TSP1 or AP protein in culture supernatant was quantified by AP activity assay (GenHunter), according to the manufacturer's instructions. The CD148-Fc fusion protein or human Fc was purified using a HiTrap protein A HP column (GE Healthcare) according to the manufacturer's instruction. Purity and quality of the proteins were assessed by immunoblot analysis as well as by colloidal blue staining (Invitrogen).

**Binding Assays.** ***Cell-free binding assay.*** Purified CD148-Fc (5 μg, 22 pmol) or equal molar Fc alone (control Fc) was incubated with the medium containing 5 nM of AP or AP-TSP1 at 4 °C for 4 h. The Fc complex was pulled down using protein-G Sepharose (GE Healthcare Biosciences), washed with HBSS containing 0.5 mg/mL BSA and 20 mM Hepes (pH 7.0) (HBAH), and assayed for bound AP activity using AP assay reagents (GenHunter) as described (12). Saturation binding and Scatchard analysis were performed using Reacti-Bind Protein A-coated plates (Thermo Scientific) as described (13). Wells were blocked with HBAH for 15 min. CD148/Fc (5 μg) or equal molar control Fc (0.6 μg) was allowed to bind to protein A for 1.5 h at room temperature. Wells then were washed with HBAH, incubated with a range of AP-TSP1 concentrations for 1.5 h, washed, and assayed for bound AP activity using AP assay reagents (GenHunter) as described (14).

***Cell-surface binding assay.*** Transiently transfected CHO cells or A431D or A431D/CD148 WT cells were grown on 12-well plates. The cells were incubated with 20 nM of AP-TSP1 or AP followed by the AP assay reagents (GenHunter), and the bound AP was assessed by cell staining as well as by the AP activity measure as described (14). The cells were photographed using the Nikon Diaphot microscope package.

***ELISA-based binding assay.*** Anti-human IgG antibody (Fc-specific; Sigma) was coated on 96-well flat-bottomed Nunc ELISA plates at 1:1,500 dilution in PBS for 1 h at room temperature and blocked with 1% BSA, 0.1% Tween 20 in PBS for 20 min. Then CD148-Fc (0.5 μg per well) was added and incubated for 1 h at room temperature. After washing with 0.1% Tween 20 in PBS, the wells were incubated with varying doses of AP-TSP1 or TSP1 overnight at 4 °C. Then the wells were incubated with a monoclonal TSP1 antibody (Thermo Scientific) and subsequently were incubated with HRP-conjugated anti-mouse IgG antibody (Fc-specific; Sigma). After washing, 50 μL of ABTS substrate (Thermo Scientific) was added to each well and incubated for 20 min. Color reaction was measured at 405 nm in the plate reader.

***Competition assay.*** A 96-well plate was coated with CD148-Fc as described above. AP-TSP1 (6.25 nM) was added to the wells in the presence of competing concentrations of TSP1 overnight at 4 °C. Bound AP-TSP1 was detected using anti-His6 antibody (Roche) and HRP-conjugated anti-mouse IgG antibody (Fc-specific, Sigma). Color reaction was carried out as described above. Note: AP-TSP1 is C-terminally tagged with His6 sequence.

**Stable Cell Preparation.** A431D cells stably expressing HA-tagged CD148 (WT and cs) were prepared using the LZRS retroviral vector as described (15). In brief, HA-tagged CD148 WT and cs sequences were subcloned into the pLZRS-IRES-zeo vector (provided by A. Reynolds, Vanderbilt University, Nashville, TN); retrovirus was produced using Phoenix packaging cells, and cells were infected as described (15). Stably transfected cells were selected with 400 μg/mL Zeocin (Invitrogen), stained with phycoerythrin-conjugated anti-CD148 antibody (clone 143–41; R&D Systems), and cells whose CD148 level was comparable to that in HRMEC were sorted using a BD FACSAria II flow cytometer (BD Biosciences). $CD148^-$ cells also were sorted and used as a control.

**shRNA-Mediated CD148 Knockdown.** HRMEC were plated in six-well plates at a density of 50%, cultured overnight, and the CD148-specific or scramble (control) shRNA lentivirus particles ($1 \times 10^6$ infectious units (IFU); Sigma) were added to the cells in growth medium containing 5 μg/mL Polybrene (Santa Cruz Biotechnology) and incubated for 18 h. After the medium was changed to fresh growth medium, the cells were used for proliferation assay, immunoblot analysis, and flow cytometry. Immunoblot analysis was carried out as previously described (4). CD148 knockdown also was assessed by flow cytometry; cells were trypsinized and washed with 0.5% BSA/PBS, and $1 \times 10^5$ cells were blocked with 1 μg of human IgG (Thermo Scientific) and stained with 10 μL of phycoerythrin-conjugated CD148 antibody (R&D Systems) for 45 min at 4 °C. After washing, cells were analyzed using the three-laser BD LSRII flow cytometer (Becton Dickinson).

**Cell Proliferation Assay.** A431D or A431D/CD148 WT or cs cells were plated in 98-well plates ($2 \times 10^3$cells per well). When the cells were attached, serum was reduced (0.1% FBS) overnight (day 0). Then cells were cultured in growth medium [2.5% (vol/vol) FBS] in which native human TSP1 (Genway Biotech, Inc.) or vehicle was added with or without CD148-Fc or control Fc. The medium was replaced with fresh agents every 2 d. Cell number was assessed at the indicated time points using the CyQUANT NF cell proliferation assay kit (Invitrogen) according to the manufacturer's instructions. HRMEC were plated in 98-well plates ($2 \times 10^3$cells per well), starved in 0.1%FBS medium for 8–10 h, and then cultured in DMEM containing 1% FBS and 20 ng/mL bFGF in which TSP1 or vehicle was added with or without CD148-Fc or control Fc. The medium was replaced with fresh agents every 2 d. Cell number was assessed using the CyQUANT NF cell proliferation assay kit (Invitrogen).

**PTP Activity Assay.** A431D or A431D/CD148 WT or cs cells were plated in 100-mm dishes at 30% confluence, serum reduced [2.5% (vol/vol) FBS] for 12 h, and then treated with 2.5 μg/mL of native human TSP1 (Genway Biotech, Inc.) or vehicle for 15 min, with or without CD148-Fc (5.0 μg/mL) or control Fc (0.6 μg/mL). The cells in two 100-mm dishes were lysed in buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 5 mM NaF, 5 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF), and CD148 was immunoprecipitated from clarified lysate (800 μg) using anti-HA affinity matrix (Roche) for 4 h at 4 °C. The washed immunocomplexes were incubated in reaction buffer (50 mM sodium acetate, 0.5 mg/mL BSA, 0.5 mM DTT, 5 mM paranitrophenyl phosphate for 30 min at 30 °C, with or without 0.1 mM $Na_3VO_4$, and the reaction was stopped with 0.2 N NaOH. The amount of cleaved substrate was assessed by measuring OD values at 410 nm as described (16). The beads then were washed and pelleted, and the proteins were eluted with Laemmli sample buffer. The amount of CD148 in the beads was assessed by immunoblot using anti-HA antibody. HRMEC were plated in 100-mm dishes at a density of 30%, serum reduced [2.5% (vol/vol) FBS] for 4 h, and exposed to TSP1 (10 μg/mL) or vehicle for 15

min with or without CD148-Fc (10 μg/mL) or control Fc (1.2 μg/mL). CD148 was immunoprecipitated using anti-CD148 antibody (goat polyclonal; R&D System), and the PTP activity assay was performed in the same manner.

**Immunoprecipitation and Immunoblot Analysis.** Immunoprecipitation and immunoblotting were performed essentially as previously described (2, 8). Coimmunoprecipitation studies were carried out as follows. For HRMEC or HUVEC, subconfluent cells were lysed in Nonidet P-40 lysis buffer [1% Nonidet P-40, 150 mM NaCl, 20 mM Hepes (pH 7.5), 1 mM EDTA, 5 mM NaF, 5 mM iodoacetic acid, 1 mM $Na_3VO_4$, complete protease inhibitor mixture from Roche], and immunoprecipitation was carried out using anti-CD148 antibody (goat polyclonal; R&D Systems). Species-matched IgG (Santa Cruz Biotechnology) was used as a control. The immunocomplexes were separated by SDS-6% acrylamide gel electrophoresis and immunoblotted using anti-TSP1. The membrane was reprobed with anti-CD148. HEK 293 cells were transfected with the expression vectors, cultured in medium for 48 h, then incubated with 2 μg/mL of native human TSP1 for 15 min at 37 °C. After washing, cells were incubated with 2 mM 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) cross-linker (Thermo Scientific) for 60 min, washed, and lysed in Nonidet P-40 lysis buffer. Immunoprecipitation was carried out using the antibodies indicated in the figure (Fig. 1). DTSSP was cleaved with 50 mM DTT before SDS/PAGE. The immunocomplexes were separated by SDS-6% acrylamide gel electrophoresis and immunoblotted using anti-TSP1. The membrane was reprobed with the indicated antibodies. Phosphorylation of EGFR was assessed as follows: A431D or A431D/CD148 WT or cs cells were plated in 100-mm dishes at 30% confluence, serum reduced [2.5% (vol/vol) FBS] for 12 h, and then incubated with 2.5 μg/mL of TSP1 protein or vehicle for 15 min at 37 °C. The cells were lysed in Nonidet P-40 lysis buffer, and EGFR was immunoprecipitated using anti-EGFR antibody and immunoblotted using the phospho-specific EGFR antibody. The membrane was reprobed with anti-EGFR antibody. Phosphorylation of ERK1/2 was assessed using crude protein cell lysates as previously described (8).

**VEGF Treatment and Immunoblot Analysis of VEGFR2 Phosphorylation.** HRMEC ($2 \times 10^6$ cells) were plated in 100-mm dishes. After achieving 60% confluence, cells were starved overnight (0.1% FBS) and then were incubated with 80 ng/mL of human recombinant VEGF (R&D Systems) or vehicle for 10 min together with 10 μg/mL of TSP1 or vehicle in the presence of CD148-Fc (10 μg/mL) or control Fc (1.2 μg/mL). Cells were lysed in a lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 2 mM $Na_3VO_4$, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, complete protease inhibitor from Roche], and clarified lysates (80 μg) were separated by SDS-6% acrylamide gel electrophoresis and immunoblotted using the phospho-specific anti-VEGFR2 [$pYpY_{1054/1059}$] antibody (Invitrogen). The membrane was reprobed with anti-VEGFR2 antibody (Cell Signaling). Phosphorylation of ERK1/2 was assessed using the same protein cell lysates. Immunoblotting was carried out as previously described (8).

**Statistical Analysis.** Data are expressed as means ± SEM. Statistical analysis was performed with Prism4 (GraphPad Software). For two-group comparisons, the unpaired Student's *t* test was used to calculate the *P* value.


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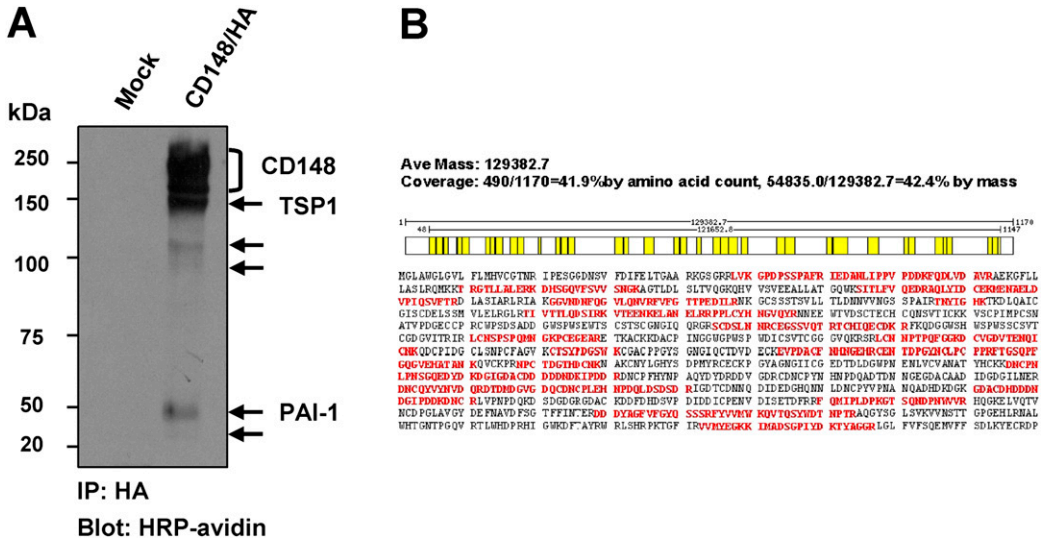


**Fig. S1.** Identification of TSP1 as a CD148-interacting extracellular protein in HUVEC. (*A*) HA-tagged WT CD148 (CD148/HA) or LacZ (mock) was introduced into HUVEC using recombinant adenovirus, and the CD148-interacting extracellular protein(s) were isolated by biotin surface labeling and subsequent affinity purifications as described in *SI Materials and Methods*. Arrows indicate the cell-surface protein(s) coprecipitated with CD148/HA. These proteins were stained, excised, and subjected to LC-MS/MS analysis. Other than TSP1, an ~50-kDa protein was identified as plasminogen activator inhibitor-1 (PAI-1). IP, immunoprecipitation. (*B*) TSP1 peptide sequences (red) identified by LC-MS/MS.

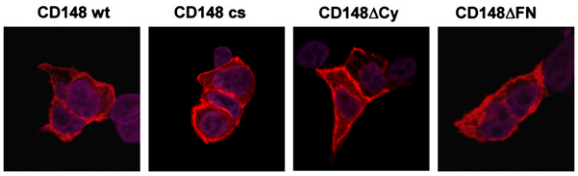


**Fig. S2.** Localization of HA-tagged CD148 forms in HEK 293 cells. HA-tagged CD148 forms (WT, cs, ΔCy, ΔFN) were transiently transfected to HEK 293 cells using the Fugene HD transfection reagent (Roche). The cells were fixed with 2% paraformaldehyde, permeabilized with 0.02% saponin, and stained with anti-HA mouse monoclonal antibody (Covance) followed by incubation with a secondary antibody (Alexa Fluor 568 goat anti-mouse IgG antibody; Invitrogen) as previously described (1). The nucleus (purple) was stained with TO-PRO-3 reagent (Invitrogen). The cells were observed by confocal microscopy (Zeiss LSM410). All CD148 forms (red) are present on plasma membrane. Note: The ΔFN mutant also is present in cytoplasm.

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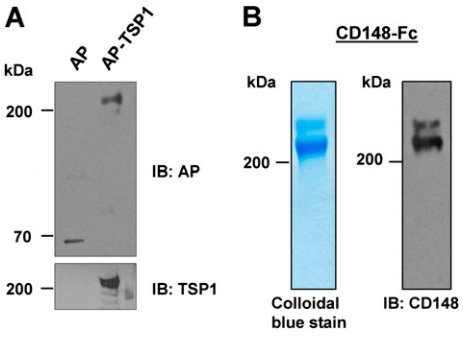


**Fig. S3.** Preparation of fusion proteins. AP-TSP1 and CD148-Fc proteins were produced by suspension culture of HEK 293 6E cells as described in *SI Materials and Methods*. (*A*) Immunoblot analysis of the culture supernatant showing bands with expected size for AP (67 kDa) or AP-TSP1 (210 kDa). (*B*) Colloidal blue stain and immunoblot analysis of the purified CD148-Fc (5 μg). IB, immunoblot.

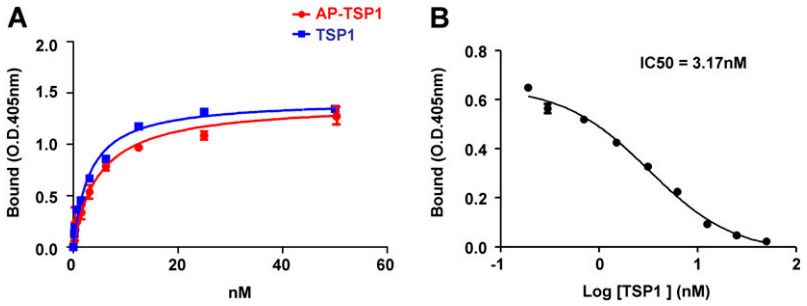


**Fig. S4.** Comparison of AP-TSP1 and TSP1 in binding to CD148-Fc. (*A*) CD148-Fc (0.5 μg per well) were coated on microtiter plates and blocked with 1% BSA, 0.1% Tween 20 in PBS. A range of concentrations of AP-TSP1 or TSP1 was applied to the wells, and binding was detected by ELISA using anti–TSP-1 antibody as described in *SI Materials and Methods*. (*B*) Competition assay of AP-TSP1 and TSP1. Binding of AP-TSP1 (6.25 nM) to CD148-Fc was determined in the presence of competing concentrations of TSP1 protein. Bound AP-TSP1 was detected by ELISA using anti-His6 antibody. AP-TSP1 binding to CD148-Fc is inhibited dose-dependently by TSP1. Data are means ± SEM of quadruplicate determinations.

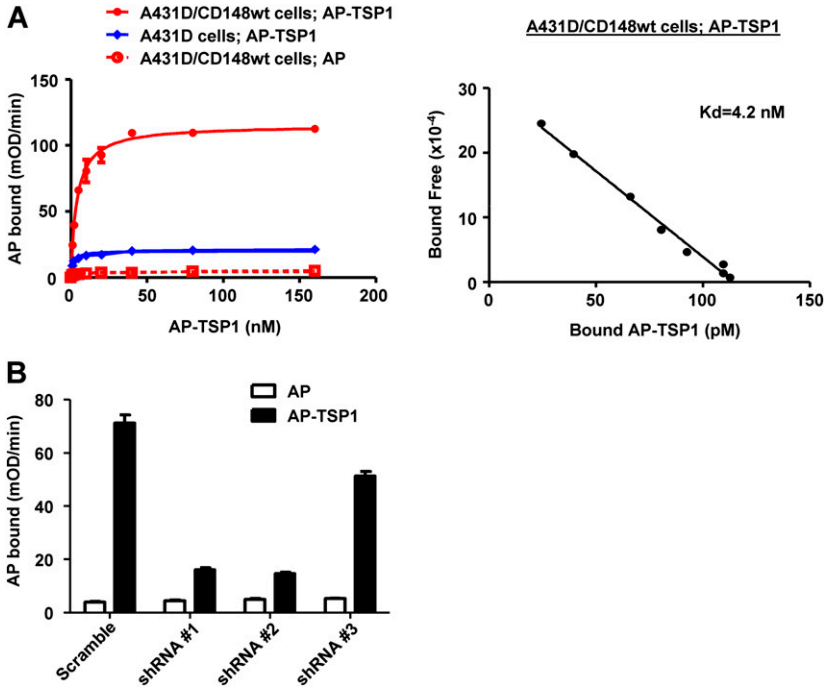


**Fig. S5.** AP-TSP1 binds to A431D/CD148 WT cells at high affinity. (*A*) (*Left*) A431D/CD148 WT and A431D cells were incubated with varying concentrations of AP-TSP1 or AP, and the bound AP proteins were quantified by AP activity assay as described in *SI Materials and Methods*. (*Right*) Scatchard analysis of AP-TSP1 binding to A431D/CD148 WT cells. (*B*) A431D/CD148 WT cells were plated in six-well plates at a density of 50% and then were treated with lentivirus ($1 \times 10^6$ IFU) encoding CD148-targeting (#1, #2, #3) or scramble shRNA with 5 μg/mL polybrene. At 72 h after infection, cells were incubated with 20 nM of AP-TSP1 or AP, and the bound AP proteins were assessed by AP activity assay. AP-TSP1 binding to A431D/CD148 WT cells is reduced remarkably by CD148 knockdown. Data are means ± SEM of quadruplicate determinations.

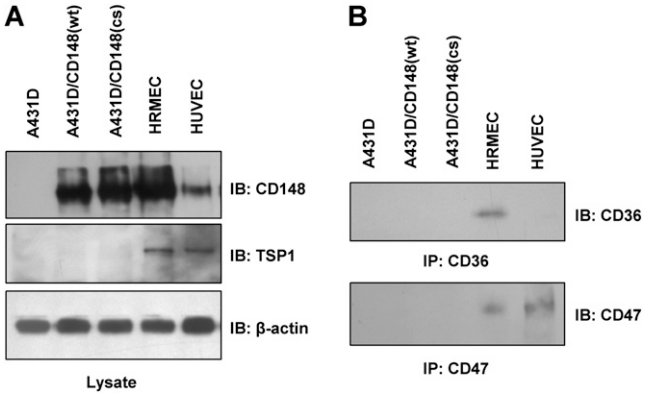


**Fig. S6.** Expression of CD148, CD36, and CD47 in A431D stable cells and primary human endothelial cells. (*A*) A431D stable cells, HRMEC, and HUVEC were lysed in SDS buffer [0.1 M Tris (pH 6.8), 2% SDS, 4% glycerol], and 40 μg of protein cell lysate was subjected to immunoblot analysis using anti-CD148 or anti-TSP1 antibodies. Equal loading was confirmed by reprobing for β-actin. (*B*) A431D stable cells, HRMEC, and HUVEC were lysed in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, protease inhibitor mixture (Roche)], and CD36 or CD47 was immunoprecipitated from clarified lysates (500 μg) using anti-CD36 or anti-CD47 antibodies (Santa Cruz Biotechnology). The immunoprecipitates were separated by SDS-7% acrylamide gel electrophoresis and immunoblotted with anti-CD36 or anti-CD47 antibodies.

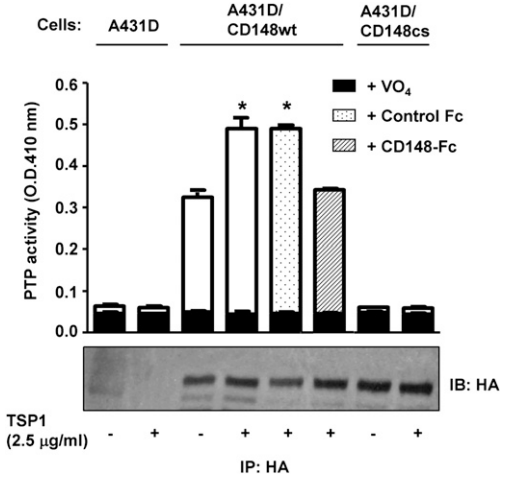


**Fig. S7.** TSP1 increases CD148 catalytic activity in A431D/CD148 WT cells. A431D and A431D/CD148 WT or cs cells were plated in 100-mm dishes, serum reduced (2.5% FBS), and exposed to TSP1 (2.5 μg/mL) or vehicle for 15 min with or without CD148-Fc (5 μg/mL) or control Fc (0.6 μg/mL). CD148 was immunoprecipitated using anti-HA, and the washed immunocomplexes were assayed for PTP activity with or without 1 mM sodium orthovanadate ($VO_4$). The amount of CD148/HA in the immunocomplexes also was assessed by anti-HA immunoblot. The data show means ± SEM of quadruplicate determinations. *$P < 0.01$ vs. vehicle-treated A431D/CD148 WT cells.

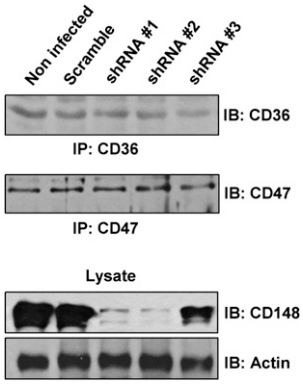


**Fig. S8.** Expression of CD36 and CD47 in CD148 knocked-down HRMEC. (*Upper*) HRMEC were treated with CD148-targeting shRNA (#1, #2, #3) or scramble shRNA lentivirus as described in *SI Materials and Methods*. At 72 h after infection, CD36 or CD47 was immunoprecipitated from clarified lysates (500 μg) and immunoblotted with anti-CD36 or anti-CD47 antibodies as described in Fig. S6. (*Lower*) Expression levels of CD148 in each sample. Fifty micrograms of protein cell lysates were subjected to immunoblot analysis using anti-CD148 antibody. Equal loading was confirmed by reprobing for β-actin. Representative results of three independent experiments are shown.

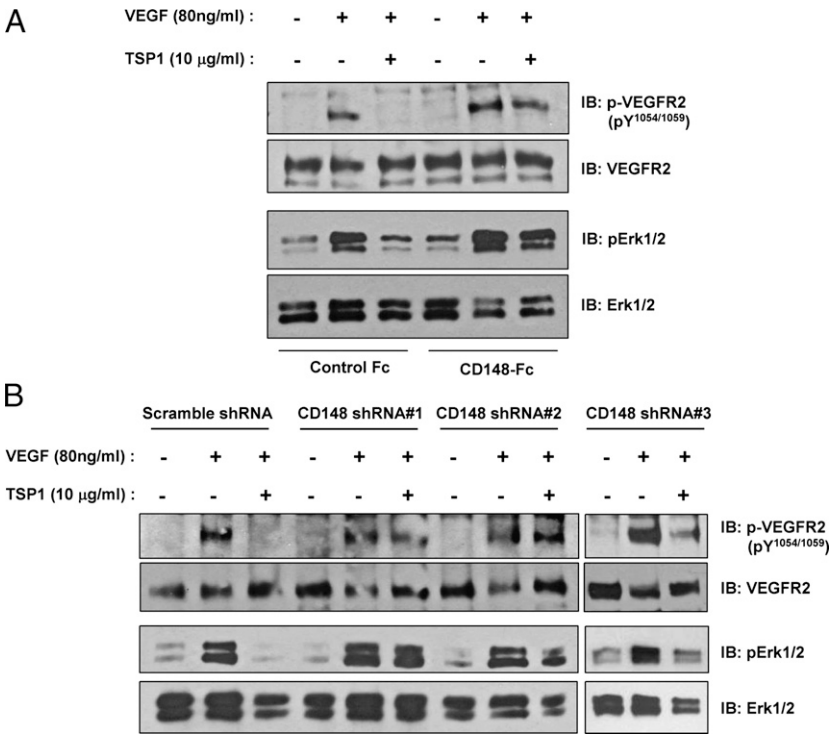


**Fig. S9.** TSP1 inhibition of VEGFR2 is reduced by CD148-Fc or CD148 knockdown in HRMEC. (*A*) HRMEC were treated with VEGF (80 ng/mL) with or without TSP1 (10 μg/mL) in the presence of CD148-Fc (10 μg/mL) or control Fc (1.2 μg/mL) as described in *SI Materials and Methods*. Phosphorylation of VEGFR2 and Erk1/2 was assessed by immunoblot analysis using phospho-specific VEGFR2 ($pYpY_{1054/1059}$) or ERK1/2 (T202/Y204) antibodies. The membranes were reprobed with antibodies to total VEGFR2 or Erk1/2. (*B*) HRMEC were treated with lentivirus encoding CD148-targeting shRNA (#1, #2, #3) or scramble shRNA. At 24 h after infection, cells were plated in 100-mm dishes at a density of 50%, starved in 0.1% FBS medium, and then treated with VEGF (80 ng/mL) or vehicle for 10 min. Phosphorylation of VEGFR2 and Erk1/2 was assessed by immunoblot analysis as in *A*. Results shown are representative of three independent experiments.

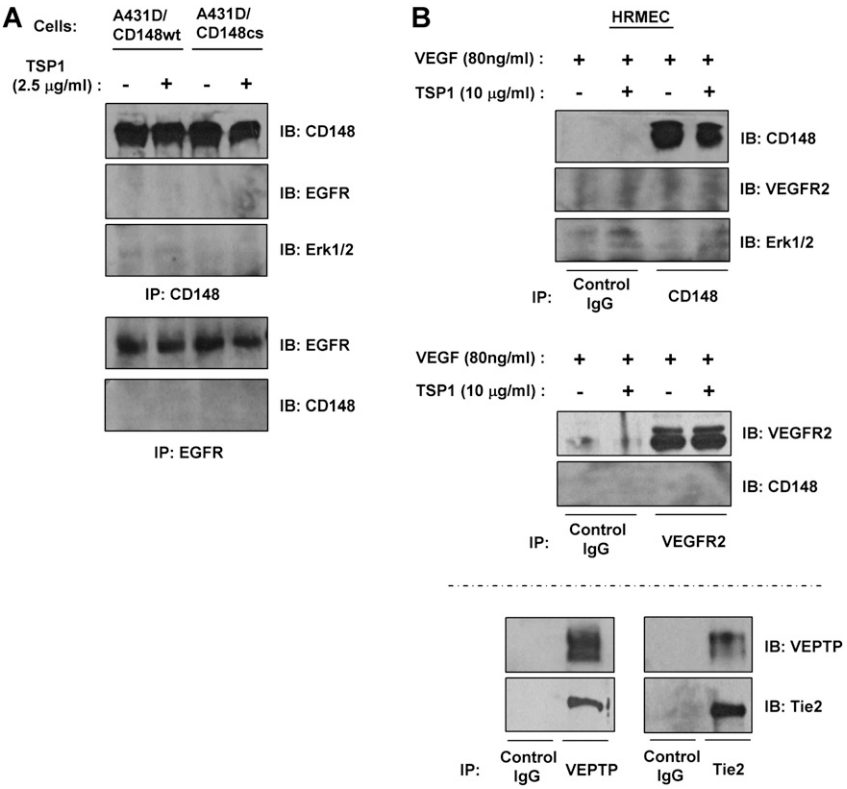


**Fig. S10.** Association of CD148 with EGFR or VEGFR2 is not observed in TSP1-treated cells. (*A*) A431D/CD148 WT or cs stable cells were plated in 100-mm dishes, serum reduced (2.5% FBS), and exposed to TSP1 (2.5 μg/mL) or vehicle for 15 min. Cells were lysed in Nonidet P-40 lysis buffer, and CD148 or EGFR was immunoprecipitated using anti-CD148 or anti-EGFR as described in *SI Materials and Methods*. The immunocomplexes were immunoblotted for EGFR, Erk1/2, and CD148. (*B*) HRMEC were plated in 100-mm dishes at a density of 50%, starved in 0.1% FBS medium, and then treated with VEGF (80 ng/mL) and TSP1 (10 μg/mL) or vehicle for 10 min. Cells were lysed in Nonidet P-40 lysis buffer, and CD148 or VEGFR2 was immunoprecipitated. The immunocomplexes were immunoblotted for VEGFR2, Erk1/2, and CD148. VE-PTP interaction with Tie2 receptor (1) also was examined in HRMEC as a positive control. Results shown are representative of three independent experiments. Association between CD148 and Erk1/2 also was not observed in TSP1- and vehicle-treated cells, suggesting a transient interaction (enzyme-substrate reaction) of these proteins.

1. Winderlich M, et al. (2009) VE-PTP controls blood vessel development by balancing Tie-2 activity. *J Cell Biol* 185:657–671.