Supporting Information

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SI Materials and Methods

Animal Experimentation. Protein tyrosine phosphatase (PTP)$\sigma^{-/-}$ mice were generated and backcrossed onto a pure C57BL/6 background as described previously (1). Mice used for experiments were between 8- to 12-wk-old and all control mice were littermates of the PTP$\sigma^{-/-}$ mice. Mice were housed in a specific-pathogen free environment with a standard diet and free access to water. Animal care and experimental protocols were approved by the Animal Care Committee at the Hospital for Sick Children.

Constructs. Full-length human ezrin cDNA was obtained via SIDNET (Hospital for Sick Children) from the Mammalian Gene Collection on a pOTB7 plasmid. This cDNA was cloned into the N-terminal FLAG epitope-tagged mammalian expression plasmid, pcDNA 3.1/nFLAG, using the Gateway system (Invitrogen). Site-directed mutagenesis was performed using the Quikchange II system (Invitrogen) to generate phosphotyrosine (pTyr) ezrin mutants (Y to F or Y to E) at sites Y145, Y191, Y353, and Y477 as outlined in Table S2. Two double mutants, ezrin-Y145F/Y145F and ezrin-Y145E/Y353E, were also generated. All point mutations were confirmed by DNA sequencing.

GST-fusion constructs for the intracellular PTPase domains of rat PTPσ [GST-PTPσ-D1, GST-PTPσ-D1D2, GST-PTPσ-D2, GST-PTPσ-D1(D1478A)] were previously generated in a bacterial expression plasmid, pGEX-2T, as described (2).

Tissue Preparation. Large bowel tissue was collected from PTP$\sigma^{-/-}$, PTP$\sigma^{+/-}$, and PTP$\sigma^{+/+}$ littermates, washed with PBS, and placed in 5 mL of ice cold lysis buffer [150 mM NaCl, 50 mM Hepes, 1% Triton X-100, 10% (vol/vol) glycerol, 1.5 mM $MgCl_2$, 1.0 mM EGTA] supplemented with protease and phosphatase inhibitors [aprotinin (10 μM), leupeptin (10 μM), pepstatin A (10 μM), PMSF (1 mM), sodium orthovanadate (Na_3VO_4; 1 mM)]. The tissue was homogenized using a handheld rotor-stator homogenizer set to medium intensity and left on ice for 30 min. The resulting slurry was centrifuged at 12,500 × *g* at 4 °C for 30 min, and the supernatant was clarified through a 0.45-μm filter.

Tandem Immunoprecipitation of pTyr–Mass Spectrometry. Mouse colon tissue homogenate was incubated overnight at 4 °C with a commercial pTyr immunoprecipitation (IP) kit (Sigma) with anti-pTyr–agarose. The beads were washed twice with IP wash buffer [20 mM Hepes (pH 7.5), 10% glycerol, 0.1% Triton X-100, and 150 mM NaCl] and twice with HPLC-grade water. The bound proteins were eluted using 0.1% trifluoroacetic acid. Eluted proteins were digested with trypsin and desalted, and the resultant peptide mixtures were separated on an automated nanoliter-scale liquid chromatography system (Easy-nLC; Proxeon Biosystems) and then detected using a Thermo-Fisher linear ion trap mass spectrometer system (LTQ), to identify pTyr-containing fragments, as described (3). Over 3,600 unique spectral hits were identified in each of the tissues. To refine this list to isolate potential PTPσ substrates, spectral hit counts were compared between PTP$\sigma^{-/-}$ and PTP$\sigma^{+/+}$ samples (because we demonstrated by immunofluorescence that loss of PTPσ leads to an increase in Tyr phosphorylation in the intestine; Fig. 3). Proteins were ranked according to the difference in unique spectral counts between samples and a minimum threshold of four was assigned to select specifically for phosphoproteins enriched in the PTP$\sigma^{-/-}$ mouse tissue.

In Vitro Substrate-Trapping Assay. Tissue homogenate from PTP$\sigma^{-/-}$ mouse colon and small bowel was obtained as above. DH5α *Escherichia coli* transformed with the GST-fusion proteins was grown in 1 L of Lennox broth at 37 °C to an OD_{590} of 0.7, followed by addition of 1 mM IPTG to induce expression. After 2 h of induction, the cells were collected by centrifugation, resuspended in PBS supplemented with protease inhibitors (as above) and 1 mM lysozyme, and lysed by sonication. After clarification by centrifugation at 12,500 × *g* for 30 min at 4 °C, the GST-fusion proteins were purified through incubation 200 μL of GST–agarose (Sigma) for 1 h.

To perform the substrate trapping, 5 mg of PTP$\sigma^{-/-}$ mice colonic tissue homogenate in 1 mL of lysis buffer was incubated with 20 μL of the GST–agarose bound to the GST-fusion proteins. Bound proteins were washed twice with lysis buffer and then twice with low-salt HNTG buffer [20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 10% (vol/vol) glycerol]. The beads were compacted by centrifugation, the supernatant was aspirated, and 30 μL of 1× SDS sample buffer was added. Following a brief incubation at 95 °C, the proteins were separated using SDS/PAGE and transferred to a nitrocellulose membrane (GE Healthcare) for immunoblotting.

The antibodies used for immunoblotting were as follows: mouse anti-ezrin (1:1,000; 3C12; Invitrogen), mouse anti-villin (1:1,000; BD Biosciences), mouse anti-pTyr (1:1,000; 3G10; Invitrogen), mouse anti–E-cadherin (1:1,000; BD Biosciences), and mouse anti-p130cas (1:1,000; BD Biosciences). Following a 1 h of incubation with primary antibody, the blots were incubated with horseradish-peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:10,000; Covance) in PBS–Tween 20 and developed with Western Lightning Plus ECL (Perkin-Elmer).

Tissue Culture. Human embryonic kidney (HEK)-293T cells were maintained in Dulbecco's minimum essential minimum (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2; 20 μg of DNA for each of the FLAG-tagged plasmids was transiently expressed using the calcium phosphate-transfection method onto 100-mm dishes. After 2 d, cells were treated for 10 min with 40 μL of sodium pervanadate prepared by adding equal volumes 1 mM sodium orthovanadate and 1% hydrogen peroxide, to enrich for Tyr-phosphorylated proteins. Cells were lysed using 1 mL of lysis buffer supplemented with protease inhibitors (as above).

Localization Experiments in Caco-2 Cells. Caco-2 BBe colorectal adenocarcinoma (C2BBe1; American Type Culture Collection CRL-2102) cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 μg/mL transferrin (Gibco, Invitrogen) at 37 °C and 5% CO_2; 3×10^6 cells were transiently transfected in suspension with FLAG epitope-tagged wild-type (WT) and pTyr ezrin mutants using PolyJet transfection reagent (Signagene Laboratories) and seeded onto 6.5-mm Transwell permeable supports with 3.0-μm pore size (Costar 3145; Corning) in 24-well tissue culture plates. Cells were grown for 4 d in complete media then fixed in 4% paraformaldehyde (PFA) in PBS. Inserts were removed with a scalpel and placed on microscope slides for immunofluorescence microscopy. Cells were immunostained with anti-FLAG antibody (2 μg/mL; M2; Sigma), followed by Alexa Fluor 568 goat anti-mouse IgG (4 μg/mL; Invitrogen) and Alexa Fluor 488 phalloidin (Invitrogen) antibodies. Imaging analysis was performed on

an Olympus IX81 inverted fluorescence microscope equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera and Yokogawa CSU ×1 spinning disk confocal scan head.

***para*-Nitrophenyl Phosphate Phosphatase-Activity Assay.** GST-fusion proteins were purified as above. Thirty microliters of the GST–agarose bound to the GST-fusion proteins were added to 300 μL of PBS with 1 mg/mL *para*-nitrophenyl phosphate (pNPP) (Sigma) in a 96-well plate. The plate was incubated at 37 °C for 30 min, and then the absorbance was read at 405 nm. Absorbance readings were normalized to zero values taken at start of incubation. Readings displayed on Fig. 2*A* are at the 30-min time point.

In Vitro Dephosphorylation Assay. Activity of GST-fusion proteins was confirmed using the pNPP assay above. FLAG-tagged proteins were precipitated from cell lysate using 20 μL of Anti-M2 FLAG-agarose (Sigma) for 1 h at 4 °C. Bound proteins were washed twice with lysis buffer and twice with low-salt HNTG. The GST- and FLAG-fusion proteins were eluted from the beads using 50 mM Tris·HCl (pH 8.0) with 10 mM reduced glutathione and 0.1 M glycine (pH 3.5) quenched with 10% vol/vol 100 mM Tris·HCl (pH 8.0), respectively. Fifty microliters of each of the eluted GST-fusion proteins and the eluted FLAG-fusion proteins were combined in 50 μL of PBS and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 30 μL of 5× SDS sample buffer. To inhibit the activity of the phosphatase domain, pervandate (PV) was included with the incubation (Fig. S3). Proteins were separated on SDS/PAGE gel and immunoblotted as described above. To conduct mass spectrometry (MS) analysis to determine pTyr targets of PTPσ on ezrin, the dephosphorylation experiment was repeated without eluting the FLAG-tagged proteins from the beads. Following the incubation, these beads were washed and then processed for tandem MS as described above. To enrich for pTyr-containing tryptic peptide fragments, a pTyr IP kit (PScan no. 7902-S; Cell Signaling Technology) was used before tandem MS analysis.

Immunohistochemistry. Fixed intestinal tissue sections were dissected to isolate regions of interest then embedded in paraffin. Five-micron sections were obtained using a microtome and placed on Superfrost Plus (Fisher Scientific) microscope slides. Following incubation at 60 °C for 30 min, the tissues sections were deparaffinized and rehydrated using sequential washes (100% xylenes, 5 min × 2; 50% xylenes:50% ethanol, 5 min; 100% ethanol, 5 min × 2; 95% ethanol, 3 min; 75% ethanol, 3 min; 50% ethanol, 3 min; cold H_2O, 5 min). Antigen retrieval was performed using sodium citrate buffer (pH 6.0) heated to 95 °C in a pressure cooker for 35 min. Membrane solubilization and blocking was performed using SS-PBS (PBS plus 0.1% saponin, 10% goat serum). Primary antibodies were incubated overnight at 4 °C in a humidified chamber. Primary antibodies used were as follows: mouse anti-ezrin (1:300; 3C12; Invitrogen), mouse anti–E-cadherin (1:400; BD Biosciences), rabbit anti-CD71 (1:50; Cell Signaling Technologies), rabbit anti-CD163 (1:50; M-96; Santa Cruz Biotechnology), and mouse anti-pTyr (1:250; 4G10 Invitrogen). Following washes in PBS, the slides were incubated with secondary antibodies in SS-PBS for 1 h at room temperature. Secondary antibodies used were goat anti-rabbit Alexa488 (1:1,000; Invitrogen) and goat anti-mouse Alexa555 (1:1,000; Invitrogen). Slides were further stained with DAPI (1 mg/mL), and coverslips were mounted using Dako Fluorescent Mounting reagent (Dako).

Ussing Chamber Analyses. Mice were killed by cervical dislocation, and the colon and small bowel were excised and placed into oxygenated Kreb's buffer (115 mM NaCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.0 mM KH_2PO_4 and 25 mM $NaHCO_3$ at pH 7.35) at 37 °C. Small bowel sections were obtained proximal to the cecum, whereas large bowel sections were obtained proximal to the anus. Excised tissue were opened by dissection along the mesentery axis and mounted on 2.8 mm × 11.2 mm oblong sliders (P2304; Physiological Instruments) with the luminal side down. The sliders were loaded into a four-chamber Ussing chamber system (EM-CSYS-4; Physiological Instruments) that was precalibrated. Kreb's buffer was added to both chambers, whereas 10 mM glucose was added to the serosal side as an energy source and 10 mM mannitol was added to the luminal side to maintain osmotic balance. Agar-salt bridges were used to both monitor potential difference across the membrane and to apply the appropriate short-circuit current (I_{sc}) to maintain the potential difference at zero as controlled through an automated voltage clamp. The system is controlled through the Acquire and Analyze software (Physiological Systems), which modulates voltage and current controls remotely. The tissue was allowed to equilibrate for 15 min before I_{sc} readings were taken. Whereas we were able to measure transepithelial electric resistance (TEER) in colonic epithelial tissues obtained from WT and PTPσ$^{-/-}$ mice (described above), we were not able to measure TEER in colonic Caco-2 cells following knockdown of PTPσ, because such knockdown disrupted the integrity of the epithelial monolayer.

Macromolecular Permeability. After the tissues had equilibrated in the Ussing chamber and baseline I_{sc} readings were acquired, 10^{-5} M horseradish peroxidase (HRP) (Type VI; Sigma) was added to the luminal chambers to act as a probe for macromolecular permeability; 500-μL samples were taken from the serosal chambers at 30-min intervals for 2 h and replaced by 500 μL of fresh Kreb's buffer to maintain constant volume. A modified Worthington method was used to evaluate the enzymatic activity of the HRP in the serosal samples as described (4).

Fluorescein Isothiocyanate–Dextran Assay. PTPσ$^{-/-}$, PTPσ$^{+/-}$, and PTPσ$^{+/+}$ littermates were fasted for 4 h and then administered 15 mg of fluorescein isothiocyanate (FITC)-dextran (molecular mass, 3,000–5,000 or 40,000 Da; Sigma) by orogastric gavage. Needle size and gavage volume used were determined according to weight of the mouse based on standard practices. After 4 h, the mice were killed by cervical dislocation and bled by cardiac puncture. Serum was isolated using centrifugation, and serum FITC levels were evaluated using fluorometry in a 96-well plate.

Dextran Sodium Sulfate Model. PTPσ$^{-/-}$, PTPσ$^{+/-}$, and PTPσ$^{+/+}$ littermates were treated with 3% dextran sodium sulfate (Sigma) in their drinking water ad libitum for 4 d. The mice were monitored daily for weight loss, stool consistency, rectal bleeding, and general health. On the fourth day, the mice were killed, and the small bowel and colon were excised and fixed using 4% wt/vol PFA in PBS.

1. Wallace MJ, et al. (1999) Neuronal defects and posterior pituitary hypoplasia in mice lacking the receptor tyrosine phosphatase PTPsigma. *Nat Genet* 21(3): 334–338.
2. Siu R, Fladd C, Rotin D (2007) N-cadherin is an in vivo substrate for protein tyrosine phosphatase sigma (PTPsigma) and participates in PTPsigma-mediated inhibition of axon growth. *Mol Cell Biol* 27(1):208–219.
3. Tong J, et al. (2008) Tandem immunoprecipitation of phosphotyrosine-mass spectrometry (TIPY-MS) indicates C19ORF19 becomes tyrosine-phosphorylated and associated with activated epidermal growth factor receptor. *J Proteome Res* 7(3):1067–1077.
4. Gareau MG, Jury J, Perdue MH (2007) Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. *Am J Physiol Gastrointest Liver Physiol* 293(1):G198–G203.

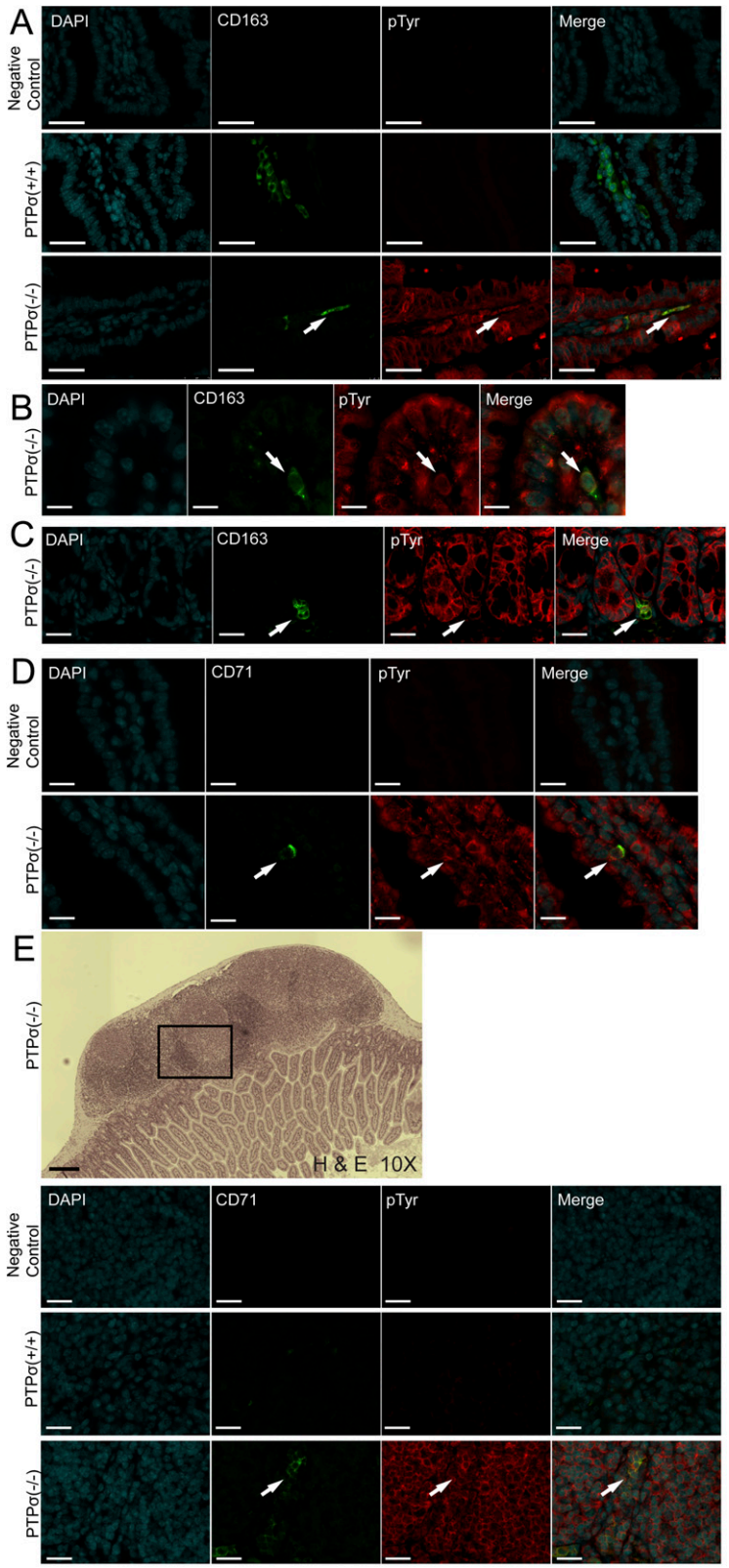

Fig. S1. Macrophages/monocytes and lymphocytes present in the lamina propria regions of the PTPσ$^{-/-}$ mice show positive staining for pTyr. Small bowel tissue sections from PTPσ$^{-/-}$ mice were coimmunostained for pTyr (red) and CD163 or CD71 (green), two markers of immune cell populations. (*A*) CD163^{+} (marker for macrophages and monocytes) immune cell present in the lamina propria demonstrated positive staining for pTyr (white arrow). (Scale bars: 30 μm.) (*B*) Zoomed view of lamina propria depicting positive pTyr staining in CD163^{+} cell. (Scale bars: 15 μm.) (*C*) Macrophage present at the base of the intestinal crypts immunostained positive for both pTyr and CD163 (white arrow). (Scale bars: 20 μm.) (*D*) CD71^{+} (marker for activated T and B lymphocytes) cell in the lamina propria adjacent to the epithelia demonstrated positive staining for pTyr (white arrow). (Scale bars: 15 μm.) (*E*) Peyer's patch (identified by morphology) present in the small bowel of the PTPσ$^{-/-}$ mouse showed positive staining for pTyr in addition to containing regions with activated lymphocytes, as evidenced by positive staining for CD71. [Scale bars: 150 μm for H&E; 25 μm for immunofluorescence.] All negative control images represent incubation with secondary antibody only.

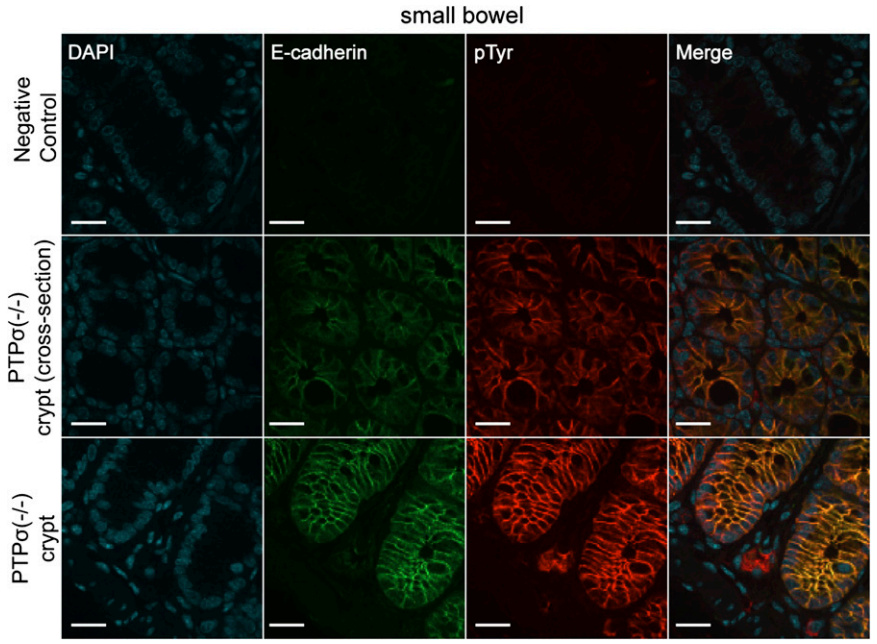

Fig. S2. Tyr-phosphorylated proteins present at the plasma membrane in the PTPσ$^{-/-}$ mouse intestine colocalize with the apical junction marker E-cadherin. Paraffin-embedded small bowel tissue sections from PTPσ$^{-/-}$ mice were coimmunostained for pTyr (red) and the apical junction marker E-cadherin (green). Colocalization was observed in the intestinal crypts. Negative control represents incubation with secondary antibody only. Two different views of the crypts are depicted. (Scale bars: 45 μm.)

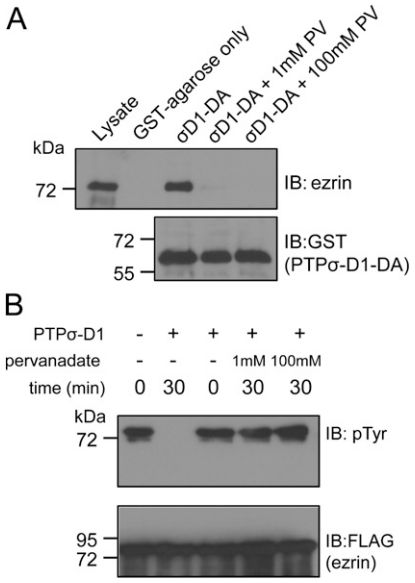

Fig. S3. Pervanadate (PV) treatment abrogates binding and dephosphorylation of ezrin by the PTPσ-D1 domain in vitro. (*A*) Transiently transfected FLAG tagged ezrin was precipitated from HEK293T cell lysate in a GST pull-down assay using the GST–PTPσ-D1(D1472A) substrate-trapping mutant in the presence or absence of PV. The untreated pull down (σD1-DA) demonstrated ezrin coprecipitation with the PTPσ-D1 domain, as shown by positive ezrin immunostaining by Western blot. Following PV treatment at 1 or 100 mM concentrations, this interaction is lost, because ezrin does not appear in the pull-down lysate. Pull down using GST–agarose alone was used as a negative control to ensure binding specificity. (*B*) Transiently transfected FLAG-tagged ezrin was immunoprecipitated from HEK293T cells and then incubated with the D1 domain of PTPσ in vitro for 30 min with or without the addition of PV. Thirty minutes of incubation with 1 or 100 mM PV showed no change in pTyr-staining intensity compared with initial levels, whereas the incubation without PV showed almost complete loss of pTyr.

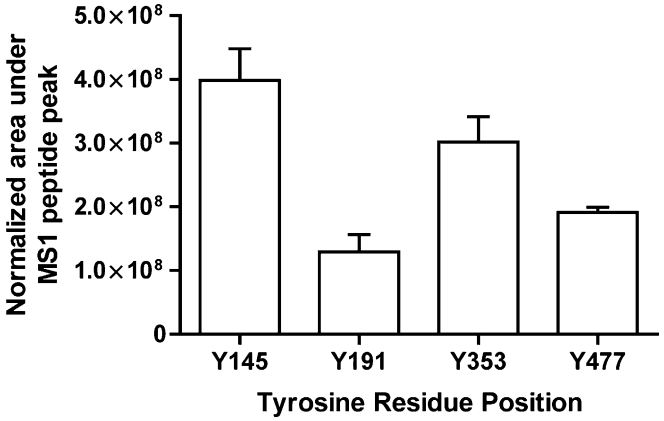

Fig. S4. Relative levels of Tyr phosphorylation at various sites in ezrin isolated from the colon of PTP$\sigma^{-/-}$ mice. For each site, the area was calculated under the MS1 peak for each pTyr-containing peptide analyzed by tandem MS. The abundance of each peptide was normalized to the total abundance of ezrin in the sample to allow comparison between experiments. Data are means ± SD. ANOVA between each Tyr residue revealed a statistically significant difference in relative phosphorylation levels [$F(3,8) = 46.85$; $P < 0.0001$].

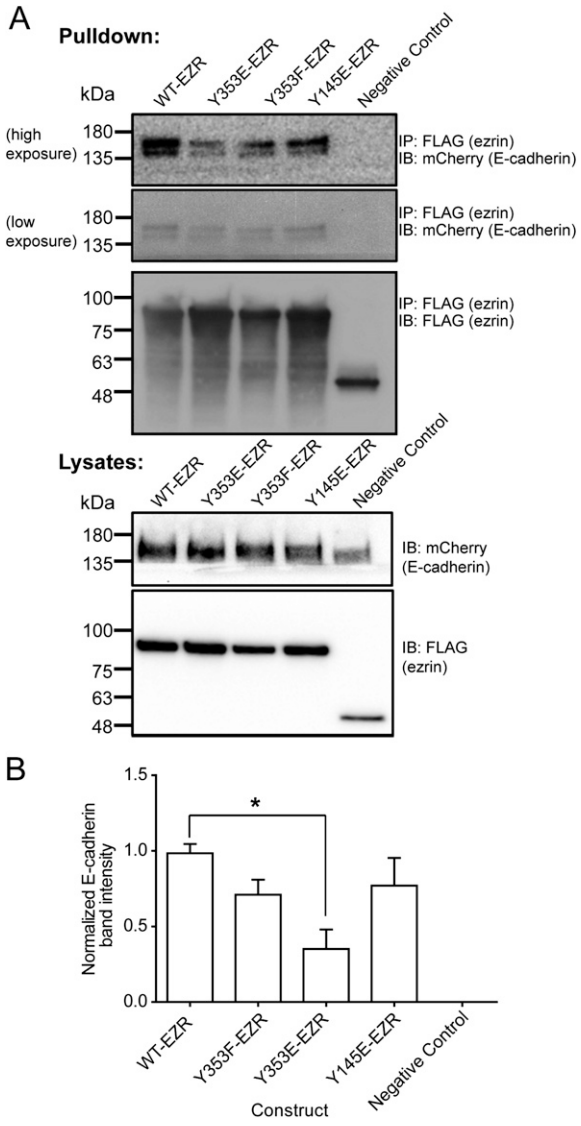

Fig. S5. Ezrin (EZR) weakly binds to E-cadherin, and this interaction is reduced in the Y353E mutant. WT or pTyr mutant FLAG-tagged EZR constructs were transiently cotransfected with mCherry-tagged E-cadherin into HEK293T cells. EZR was immunoprecipitated (IP) from cell lysates using anti-FLAG agarose. Bound proteins were eluted using 1× SDS sample buffer, separated by SDS/PAGE, and immunoblotted (IB) using anti-FLAG and anti-mCherry antibodies. (*A*) Immunoblot demonstrating co-IP of mCherry–E-cadherin with the FLAG-EZR. The negative control lane corresponds to a FLAG-tagged control protein (N-terminal fragment of villin) that does not interact with E-cadherin. (*B*) Quantitation of the mCherry–E-cadherin signal intensity for the immunoblot in *A*, demonstrating reduced binding for the Y353E-EZR mutant. Values were normalized to the amount of FLAG-EZR IP. Each phosphomutant is illustrated as a ratio relative to WT-EZR. All quantitation was conducted using ImageJ. Data are means ± SD ($n = 4$ experiments; $^*P < 0.0001$; Student *t* test).

Table S1. Proteins with enriched Tyr phosphorylation in PTPσ$^{-/-}$ mouse colon

Gene	Protein	Difference in unique spectral counts, PTPσ$^{-/-}$ vs. PTPσ$^{+/+}$
Myh11	Myosin, heavy chain 11, smooth muscle	244
Myh9	Myosin, heavy polypeptide 9, nonmuscle	67
Vil1	Villin 1	36
Gsn	Gelsolin	24
Myh14	Myosin, heavy chain 14, nonmuscle	24
Acta1	Actin, α1, skeletal muscle	23
Actb	Actin, β	16
Bcar1	Breast cancer anti-estrogen resistance 1, CRK-associated substrate	16
Myl6	Myosin, light polypeptide 6, alkali, smooth muscle and nonmuscle	15
Myh10	Myosin, heavy chain 10, nonmuscle	13
Hist1h1e	Histone cluster 1, H1e	10
Tubb5	Tubulin, β5 class I	10
Cttn	Cortactin c	7
Mylc2b	Myosin light chain, regulatory B	7
Dbn1	Drebrin 1	6
Lpp	LIM domain-containing preferred translocation partner in lipoma	5
Tuba1b	Tubulin, α1B	5
Lasp1	LIM and SH3 protein 1	4
Ezr	Ezrin	4
Cdh1	E-cadherin	4
Ctnnb1	β-catenin	4
Inpp5d	Inositol polyphosphate-5-phosphatase	4
Ptpn18	Protein tyrosine phosphatase, nonreceptor type 18	4
Egfr	Epidermal growth factor receptor	4
Flt3	FMS-like tyrosine kinase 3	4
Tpm1	Tropomyosin 1, α	4
Hcls1	Hematopoietic cell specific Lyn substrate 1	4
Nedd9	Neural precursor cell expressed, developmentally down-regulated gene 9	4
Actr3	ARP3 actin-related protein 3 homolog	3
Pecam1	Platelet/endothelial cell adhesion molecule 1	3
Krt8	Keratin 8	3
Hnrpab	Heterogeneous nuclear ribonucleoprotein A/B	3
Fau	Finkel–Biskis–Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	3
Tjp2	Tight junction protein 2	3
Tpm3	Tropomyosin 3, γ	3
Eps8	Epidermal growth factor receptor pathway substrate 8	3

Table S2. Primers for site-directed mutagenesis

Point mutation	Primers
Y145F	
Forward	GTGCACAAGTCTGGGTTTCTCAGCTCTGAGC
Reverse	GCTCAGAGCTGAGAAACCCAGACTTGTGCAC
Y145E	
Forward	GTGCACAAGTCTGGGGTGCTCAGCTCTGAGC
Reverse	GCTCAGAGCTGAGCACCCCAGACTTGTGCAC
Y191F	
Forward	GGCTTGATGGTAGTTTTGACAGAAGGATTCC
Reverse	GGAATCCTTCTGTCAAAACTACCATCAAGCC
Y191E	
Forward	GGCTTGATGGTAGTGAGGACAGAAGGATTCC
Reverse	GGAATCCTTCTGTCCTCACTACCATCAAGCC
Y353F	
Forward	CTGCGGCTGCAGGACTTTGAGGAGAAGACAAAG
Reverse	CTTTGTCTTCTCCTCAAAGTCCTGCAGCCGCAG
Y353E	
Forward	CTGCGGCTGCAGGACGAGGAGGAGAAGACAAAG
Reverse	CTTTGTCTTCTCCTCCTCGTCCTGCAGCCGCAG
Y477F	
Forward	CACCCCCCGTGTTCGAGCCGGTGAGC
Reverse	GCTCACCGGCTCGAACACGGGGGGTG
Y477E	
Forward	CACCCCCCGTGGAGGAGCCGGTGAGC
Reverse	GCTCACCGGCTCCTCCACGGGGGGTG