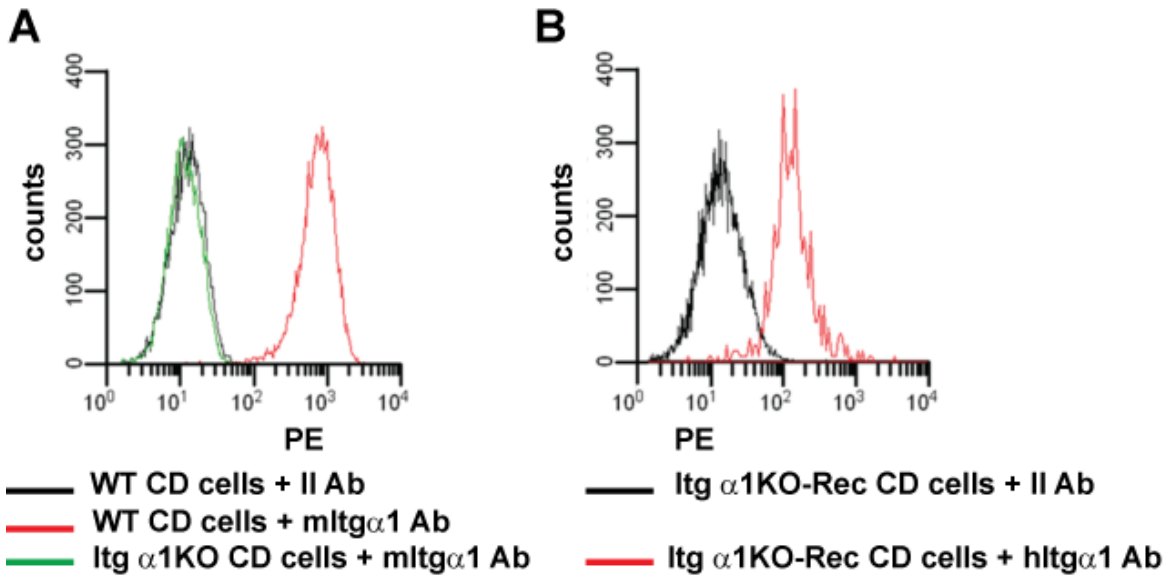
Supplemetal Material

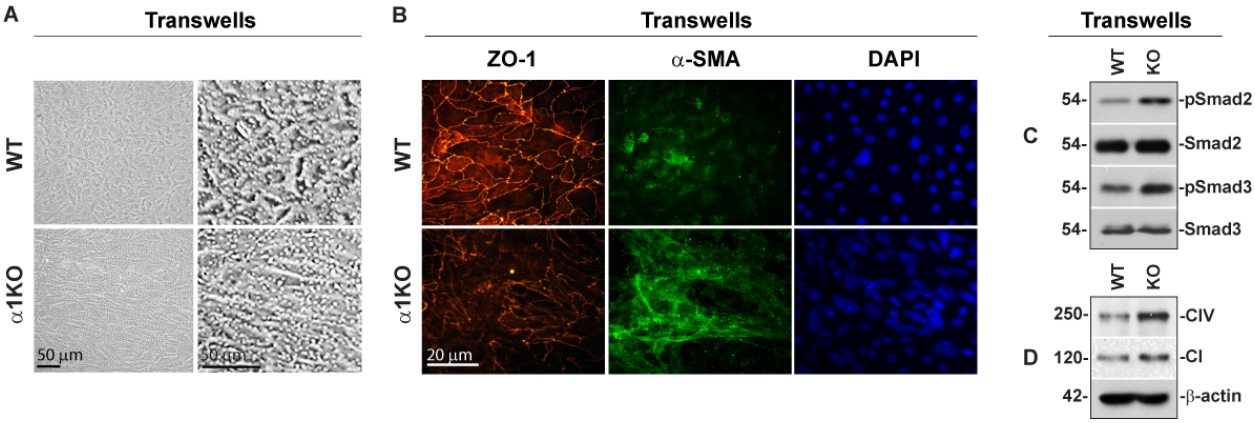
Integrin-mediated type II TGF-β receptor tyrosine dephosphorylation controls SMAD-dependent profibrotic signaling

Chen X et al.



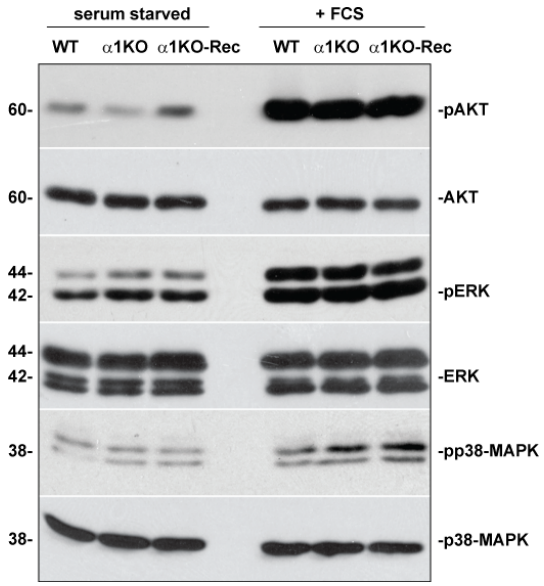
Supplemental Figure 1

FACS analysis of WT, integrin α1KO, and integrin α1KO-Rec CD cells. (**A**) Wild type (WT) and integrin α1KO primary CD cells were incubated with antibodies to mouse integrin α1 subunit (mItga1) or secondary antibody only (II Ab). Integrin expression is displayed by a shift in mean fluorescent intensity relative to no primary antibody incubation using fluorescence-activated cell sorting analysis (PE, Phycoerythrin). (**B**) Integrin α1KO CD cells reconstituted with the human integrin α1 subunit (α1KO-Rec) were incubated with antibodies to human integrin α1 subunit (hItgα1) or secondary antibody only (II Ab) and analyzed as described above.



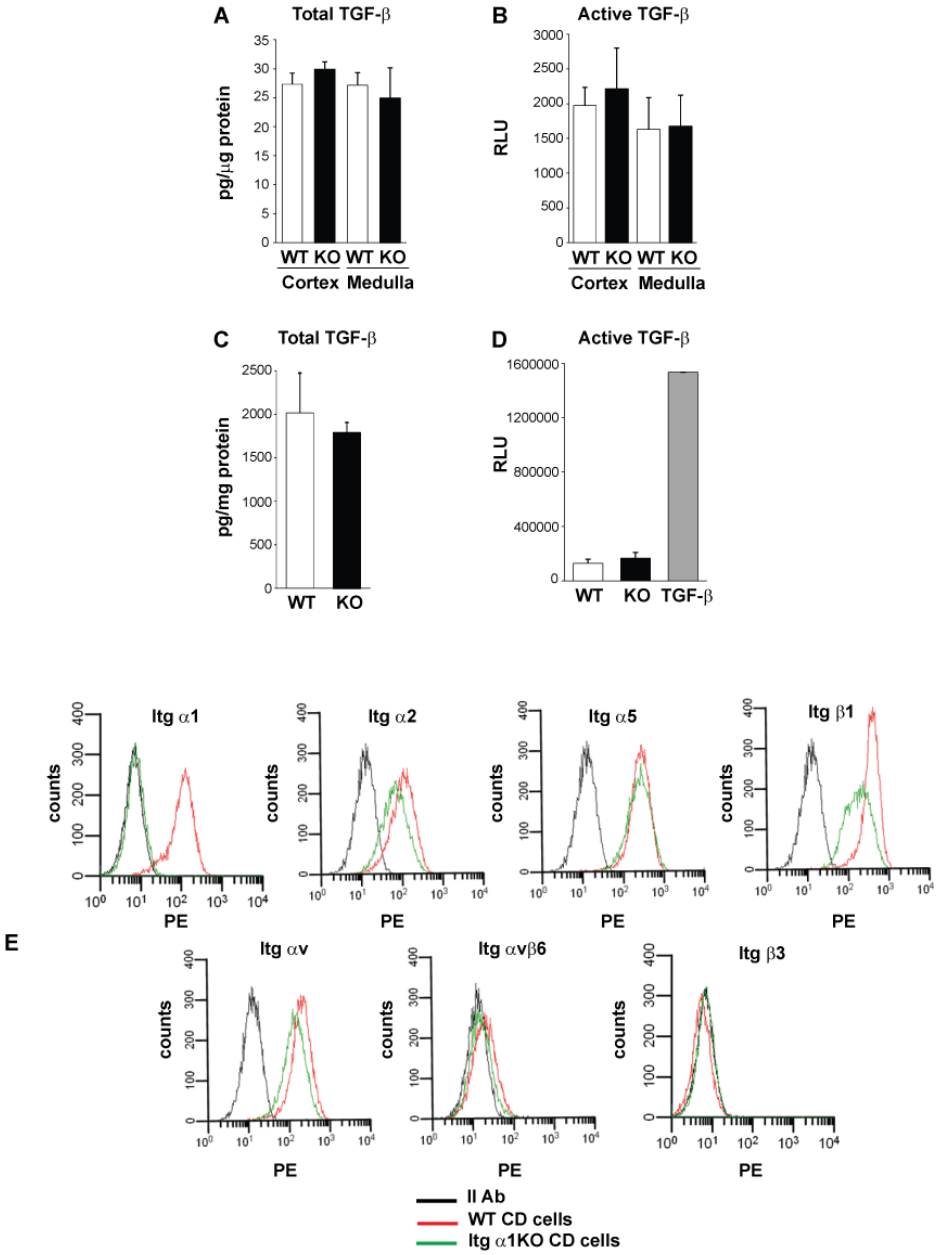
Supplemental Figure 2

Characterization of CD cells grown on transwells. (**A**) Morphology of wild type (WT) and integrin α1KO CD cells cultured in complete medium on transwells for 3 days. (**B**) The CD cells indicated were grown on transwells in complete medium for 3 days and then stained with anti-ZO-1 and anti-αSMA antibodies to visualize levels and localization of epithelial and myofibroblast markers. (**C, D**) The cells indicated were grown in complete medium on transwells. Three days later the cells were serum starved for 24 hours and then analyzed by Western blot for levels of activated and total Smad2 and Smad3, as well as collagen I and collagen IV levels (20 μg/lane total cell lysates were used for analysis).



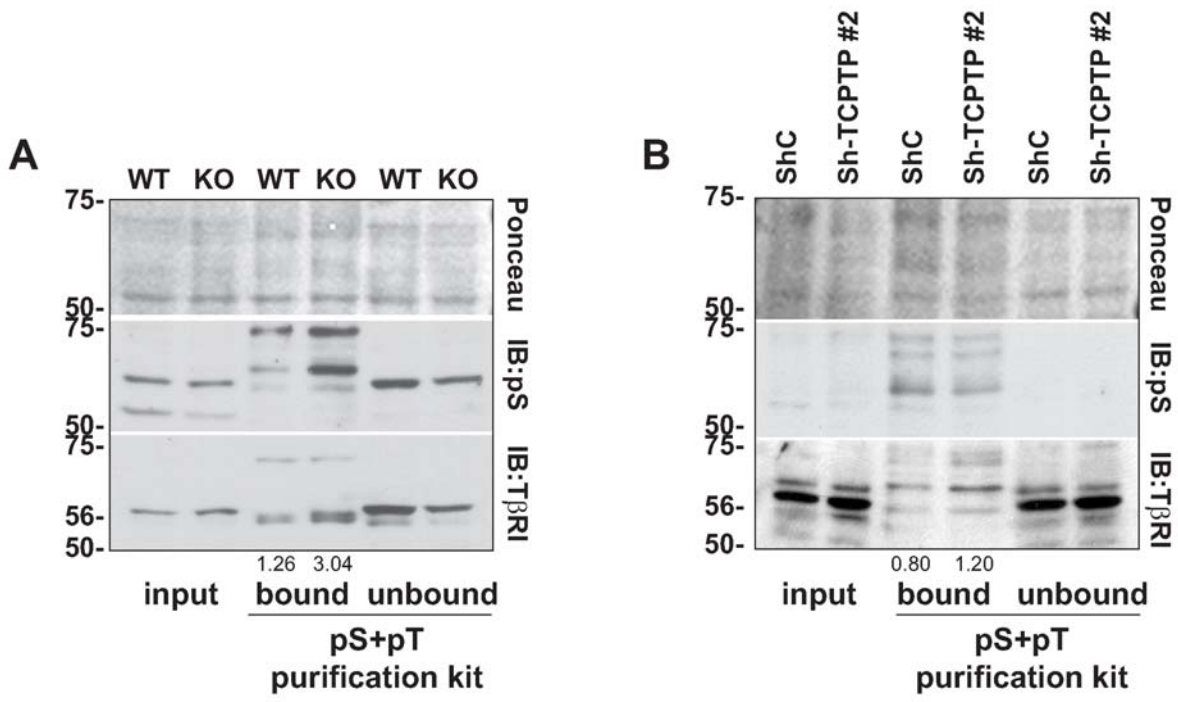
Supplemental Figure 3

Analysis of TGF-β non-canonical signaling in CD cells. The CD cells indicated were grown on plastic in complete medium. After 3 days they were serum starved for 24 hours and either left untreated or treated with 10% FCS (used as positive control) for 30 minutes. Cell lysates (20 μg/lane) were analyzed by western blot for levels of phosphorylated and total AKT, ERK and p38-MAPK.



Supplemental Figure 4

Analysis of TGF-β1 levels and integrin α1KO CD cells and mice. (**A-D**) Total levels of TGF-β1 and bioactive levels of TGF-β (all isoforms) were analyzed in kidneys of wild type (WT) and integrin α1KO mice 7 days after UUO as well as from 3-day-conditioned medium from WT and integrin α1KO CD cells by ELISA (**A, C**) and PAI/L assays (**B, D**). The values in A and B represent the mean ± s.d. of 3 mice per genotype. The values in C and D represent the mean ± s.d. of one representative experiment performed in quadruplicate. Three independent experiments were performed with similar results. (**E**) Integrin expression by WT and integrin α1KO CD cells. Cells were incubated with antibodies to α1, α2, α5, αv, β1, β3, or αvβ6 integrin subunits. Integrin expression is displayed by a shift in mean fluorescent intensity compared with no primary antibody incubation using fluorescence-activated cell sorting analysis (PE, Phycoerythrin).



Supplemental Figure 5

Analysis of serine and threonine phosphorylated TβRI in CD cells. Cell lysates (0.5 mg) from serum-starved WT and integrin α1KO CD cells (**A**) or WT CD cells transfected with shRNAi control (ShC) or TCPTP shRNAi (Sh-TCPTP) (1 clone each shown) (**B**) were immunoprecipitated using the phosphoserine/phosphothreonine protein purification columns (Qiagen, #37101). Bound and unbound proteins were then analyzed by Western blot for levels of phosphoserine products and TβRI. A band corresponding to TβRI (~56kDa) was more prominent in integrin α1KO and sh-TCPTP transfected CD cell lysates bound to the purification columns (lower panel). Serine phosphorylated products with molecular weights 50-75 kDa were detected with anti-phosphoserine antibodies (middle panel). The values represent the intensity of the TβRI band normalized to the intensity of the 50 kDa band detected in the Ponceau staining.

A

MGRGLLRGLWPLHIVLWTRIASTIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFC
DVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHDPKLPY
HDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPDLLLVI
FQVTGISLLPPLGVAISVIIIFYCYRVNRQQKLSSTWETGKTRKLMEFSEHCAIILE
DDRSDISSTCANNINHNTELLPIELDTLVGKGRFAEVYKAKLKQNTSEQFETVAV
KIFPYEEYASWKTEKDIFSDINLKHENILQFLTAEERKTELGKQYWLITAFHAKGN
LQEYLTRHVISWEDLRKLGSSLARGIAHLHSDHTPCGRPKMPIVHRDLKSSNILV
KNDLTCCLCDFGLSLRLDPTLSVDDLANSGQVGTARYMAPEVLESRMNLENVE
SFKQTDVYSMALVLWEMTSRCNAVGEVKDYEPPFGSKVREHPCVESMKDNVL
RDRGRPEIPSFWLNHQGIQMVCETLTECWDHDPEARLTAQCVAERFSELEHLD
RLSGR SCSEEKIPEDGSLNTTK

B

	Position	sequence	Y phosphorylation sites	Features of Y phosphorylation sites
1	151 - 153	EEY	[E/D]XpY	SHP1 substrate motif
2	151 - 154	EEYN	X[E/D]pYX	EGFR substrate motif
3	152 - 153	EY	[E/D/Y]pY	TC-PTP substrate motif
4	257 - 259	EVY	[E/D]XpY	SHP1 substrate motif
5	281 - 282	YE	pY[A/G/S/T/E/D]	Src substrate motif
6	282 - 284	EEY	[E/D]XpY	SHP1 substrate motif
7	282 - 285	EEYA	X[E/D]pYX	EGFR substrate motif
8	283 - 284	EY	[E/D/Y]pY	TC-PTP substrate motif
9	284 - 285	YA	pY[A/G/S/T/E/D]	Src substrate motif
10	321 - 324	YWLI	pYXX[L/I/V]	JAK2 substrate motif
11	334 - 337	QEYL	X[E/D]pYX	EGFR substrate motif
12	334 - 337	QEYL	X[E/D]pY[I/L/V]	EGFR substrate motif
13	335 - 336	EY	[E/D/Y]pY	TC-PTP substrate motif
14	446 - 448	DVY	[E/D]XpY	SHP1 substrate motif
15	448 - 449	YS	pY[A/G/S/T/E/D]	Src substrate motif
16	468 - 471	KDYE	X[E/D]pYX	EGFR substrate motif
17	469 - 470	DY	[E/D/Y]pY	TC-PTP substrate motif
18	470 - 471	YE	pY[A/G/S/T/E/D]	Src substrate motif

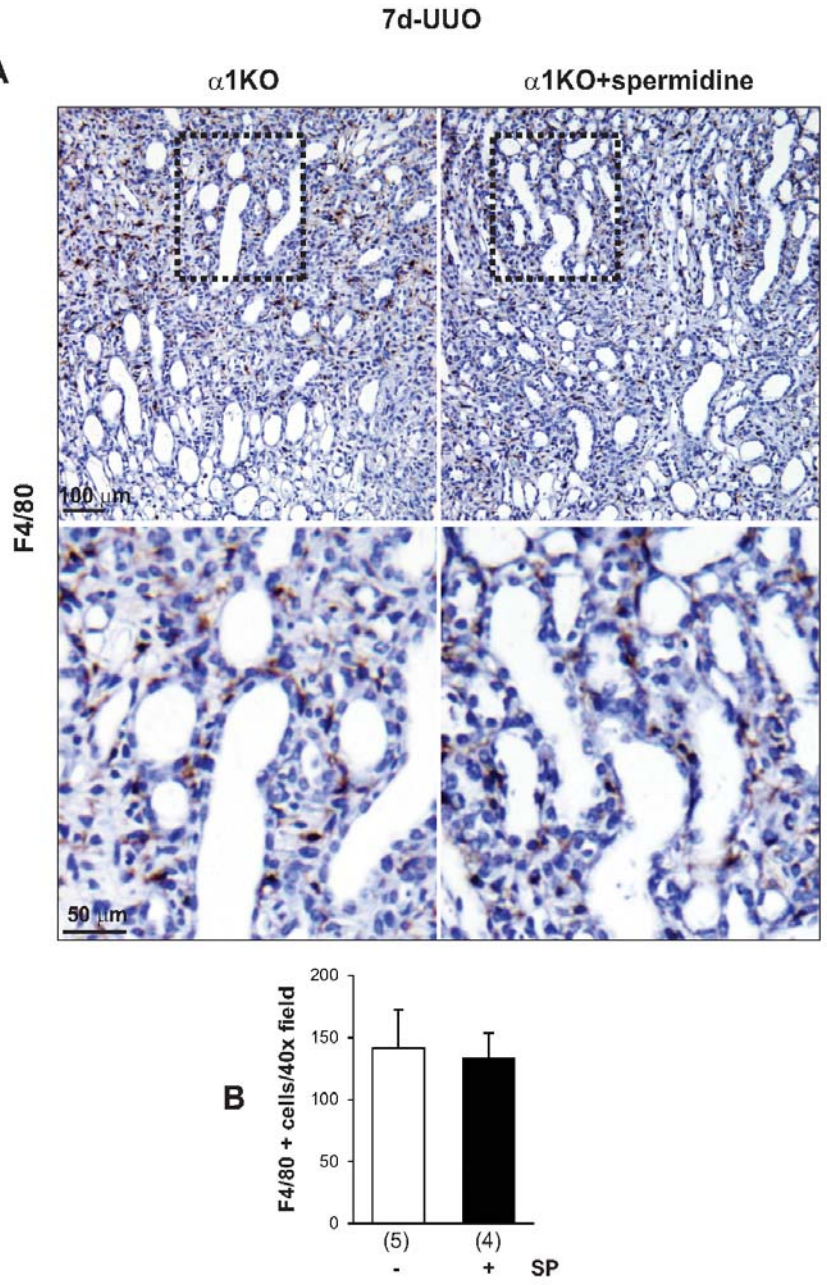
cytoplasmic domain

C

Mouse 215-RVHRQQKLSPSWESSKPRKLMDFSDNCAIILEDDRSDISSTCANNINHNTELLPIELDTLVGKGRFAEVYKAKLKQNTSE
Human 190-RVNRQQKLSSTWETGKTRKLMEFSEHCAIILEDDRSDISSTCANNINHNTELLPIELDTLVGKGRFAEVYKAKLKQNTSE
259
QFETVAVKIFPYEEYSSWKTEKDIFSDINLKHENILQFLTAEERKTELGKQYWLITAFHAKGNLQEYLTRHVISWEDLRKLG
QFETVAVKIFPYEEYASWKTEKDIFSDINLKHENILQFLTAEERKTELGKQYWLITAFHAKGNLQEYLTRHVISWEDLRKLG
284 336
SSLARGIAHLHSDHTPCGRPKMPIVHRDLKSSNILVKNDLTCCLCDFGLSLRLDPTLSVDDLANSGQVGTARYMAPEVLE
SSLARGIAHLHSDHTPCGRPKMPIVHRDLKSSNILVKNDLTCCLCDFGLSLRLDPTLSVDDLANSGQVGTARYMAPEVLE
424
SRMNLENVESFKQTDVYSMALVLW EMTSRCNAVGEVKDYEPPFGSKVREHPCVESMKDSVLRDRGR-507
SRMNLENVESFKQTDVYSMALVLW EMTSRCNAVGEVKDYEPPFGSKVREHPCVESMKDNVLRDRGR-497
470

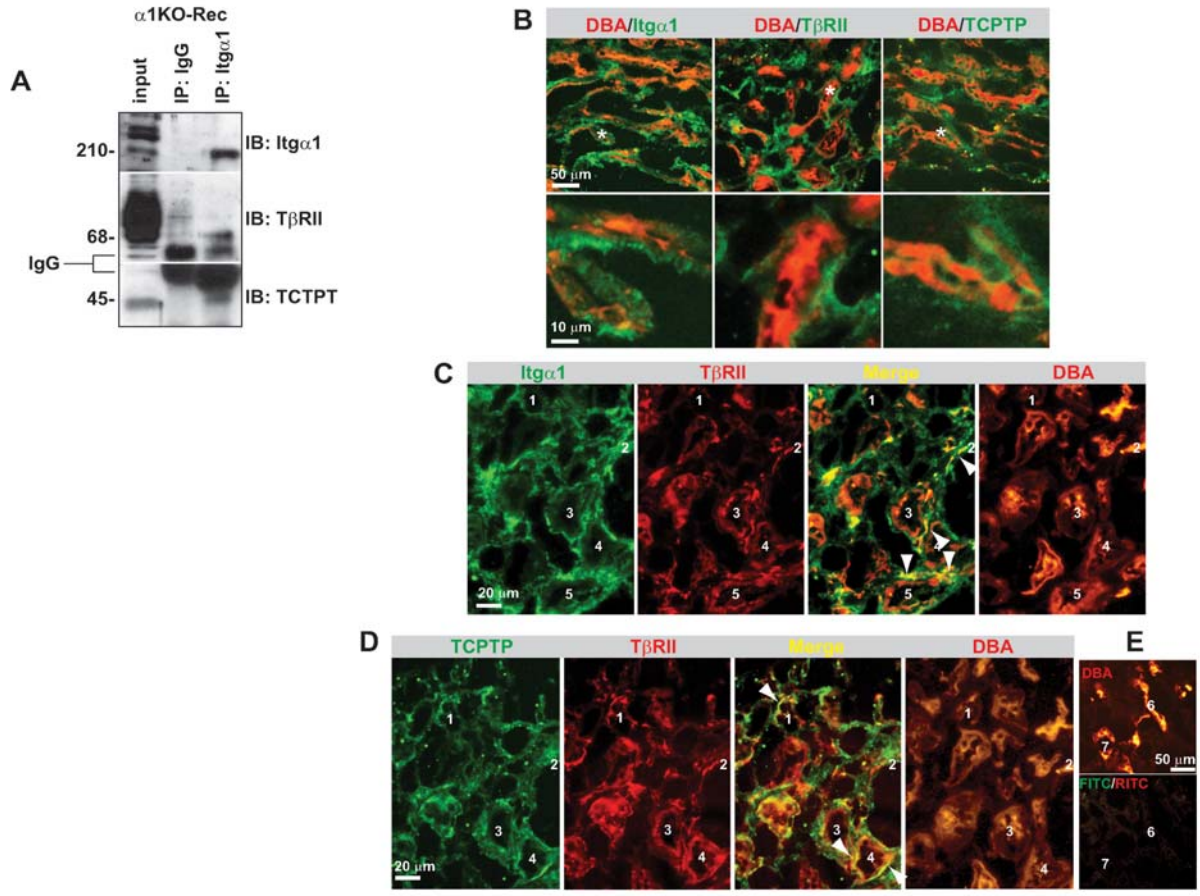
Supplemental Figure 6

The human TβRII cytoplasmic domain contains TCPTP substrate motifs. The PhosphoMotif Finder (http://www.hprd.org/PhosphoMotif_finder) was used to identify potential tyrosine kinase and tyrosine phosphatase motifs in the human TβRII cDNA (full-length sequence indicated in **A**). Analysis revealed 18 potential tyrosine phosphorylatable sites of which 3, located in the cytoplasmic domain and highlighted in yellow (Y284), green (Y336) and blue (Y470), are TCPTP dephosphorylation substrates (**B**). Note that although Y424 (in the black box in **A**) has been described as a potential phosphorylatable tyrosine (6), it does not appear in the list as it does not contain typical tyrosine phosphorylation and/or dephosphorylation motifs. (**C**) Portion of the mouse (red) and human (black) TβRII cytoplasmic domain showing 5 highly conserved phosphorylatable tyrosines.



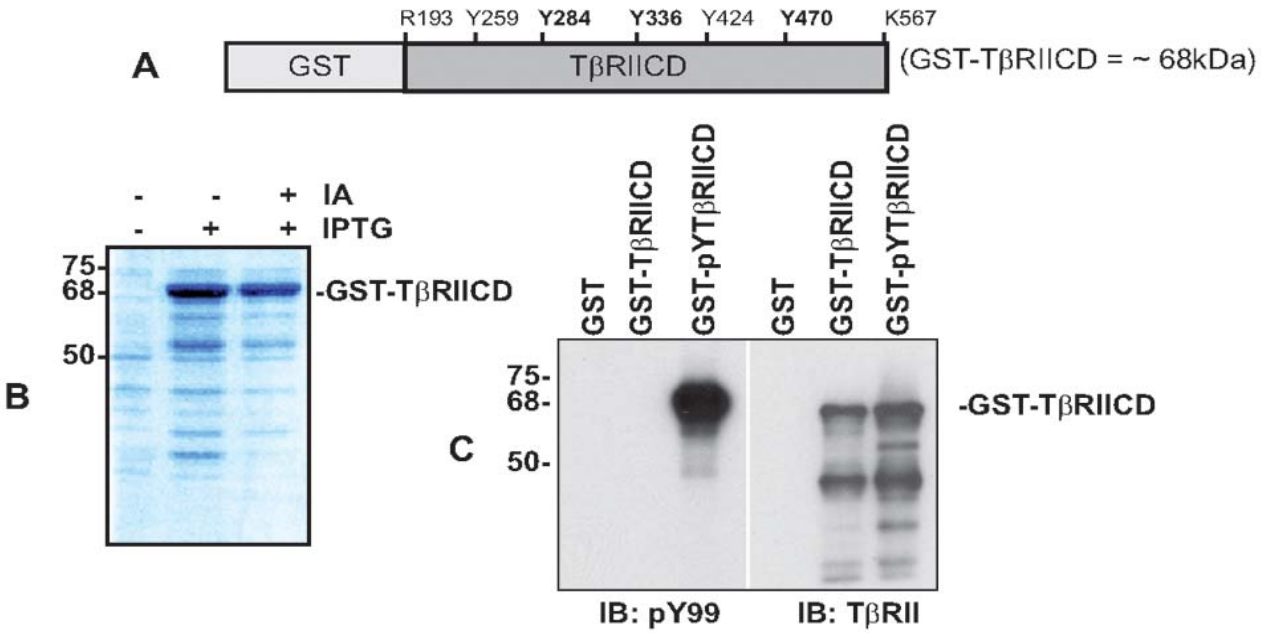
Supplemental Figure 7

Evaluation of macrophage infiltration in the kidneys of UUO-injured integrin α1KO mice untreated or treated with spermidine. (**A**) Paraffin sections of kidneys from integrin α1KO mice untreated or treated with spermidine (30 mM via gavage) at 7 days post-UUO were stained with anti-F4/80 antibodies. (**B**) The number of macrophages in spermidine treated and untreated mice was evaluated and expressed as F4/80 positive cells/microscopic field. Values are the mean ± sem of the mice indicated in parenthesis (5 field/kidney were counted).



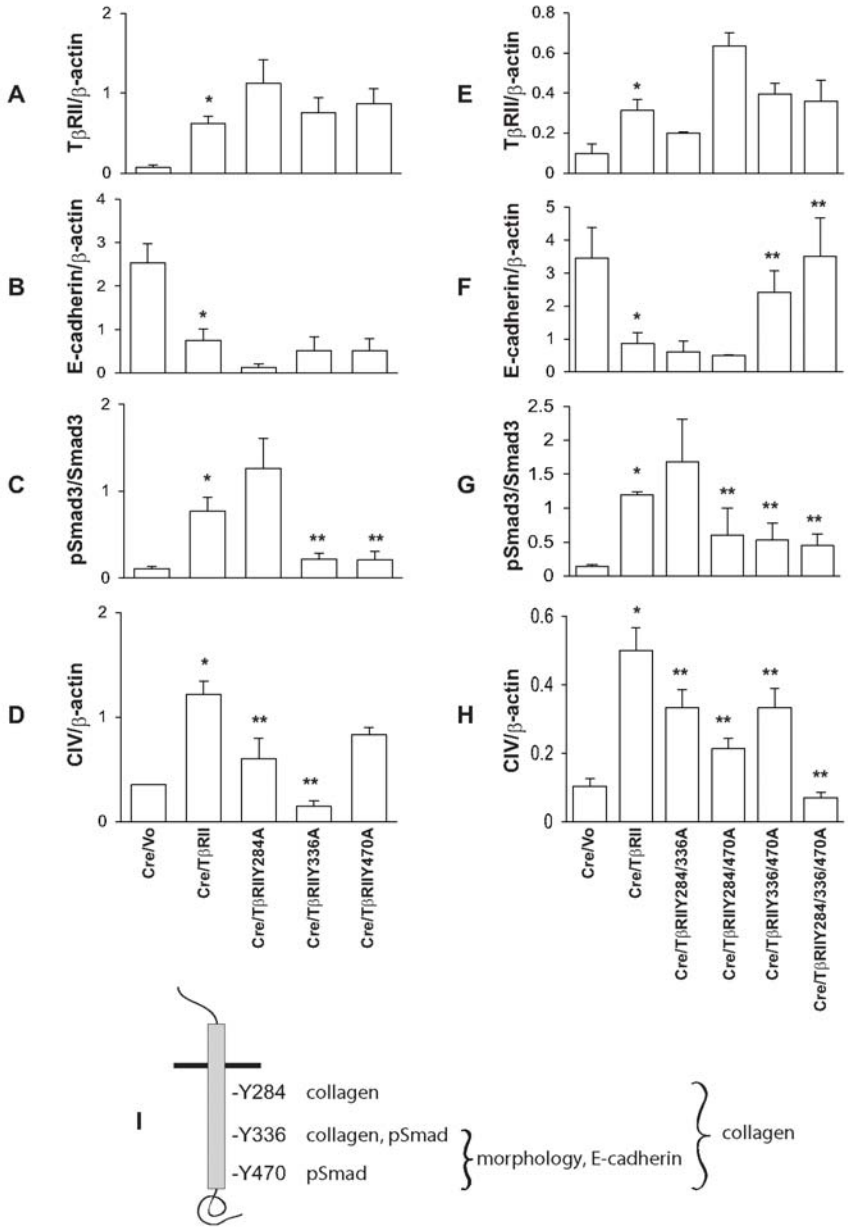
Supplemental Figure 8

TβRII forms a complex with integrin α1β1 and TCPTP. (**A**) Cell lysates (0.5 mg) derived from integrin α1KO CD cells expressing the human integrin α1 subunit (α1KO-Rec) were incubated with either anti-human integrin α1 antibodies (20 μg) or mouse IgG isotype controls (20 μg), followed by incubation with Protein G beads. The immunoprecipitation complexes were analyzed by Western blot, and products were detected with antibodies to the human integrin α1 subunit (Itgα1), TβRII, and TCPTP. (**B-D**) Human kidney frozen sections (5 μm) were stained with Dolichos Biflorus Agglutinin (DBA, red) and mouse anti-human integrin α1 (green), rabbit anti-human TβRII (green) and mouse anti-human TCPTP antibodies (green) to evaluate expression of these proteins in collecting ducts. The asterisk indicates ducts magnified and shown in the lower panel. (**C, D**) Human kidney serial frozen sections were stained with mouse anti-human integrin α1 (green) together with rabbit anti-human TβRII (red) (**C**); mouse anti-human TCPTP (green) together with rabbit anti-human TβRII (red) (**D**); or DBA alone (red) (**C, D**) to evaluate integrin α1/TβRII and TβRII/TCPTP co-localization (arrowhead) within collecting ducts (indicated by the numbers 1-5). (**E**) Human kidney serial frozen sections (5 μm) were stained with DBA alone (red) or with FITC-conjugated anti-mouse together with RITC-conjugated anti-rabbit antibodies to evaluate background staining in collecting ducts (indicated by the numbers 6 and 7).



Supplemental Figure 9

Generation of TβRII cytoplasmic domain constructs. (**A**) Schematic representation of the 68 kDa GST-TβRII construct used to generate non-phosphorylated and tyrosine phosphorylated human GST-TβRII cytoplasmic domain (CD, R193-K567). This domain contains 5 potential phosphorylatable tyrosines, of which 3 (in bold) are potential TCPTP dephosphorylation sites. (**B**) Coomassie staining showing expression of bacterial non-phosphorylated (-IA, indoleacrylic acid) and tyrosine phosphorylated (+IA) GST-TβRIICD upon induction with isopropyl β-d-thiogalactoside (IPTG). (**C**) Purified GST, unphosphorylated GST-TβRIICD or tyrosine phosphorylated GST-pYTβRIICD constructs were analyzed by Western blot for levels of tyrosine phosphorylation (IB:pY99) or expression levels (IB:TβRII). The GST-pYTβRIICD construct (68 kDa) is highly phosphorylated on tyrosine residues.



Supplemental Figure 10

Quantification of cell signaling regulated by TβRII carrying single or multiple Y-to-A mutations in the TCPTP dephosphorylation sites Y284, Y336 or Y470. (**A-H**) AdenoCre-treated α1KO/TβRII$^{fl/fl}$ cells were transfected with empty vector (Cre/Vo), full-length TβRII (Cre/TβRII), or the Y-to-A TβRII mutations indicated. Cell lysates (20 μg/lane) from serum-starved cells were analyzed by Western blot for levels of TβRII, E-cadherin, pSmad3 and collagen IV (CIV) as well as β-actin and total Smad3 (**see Figures 9C, 9D)**. TβRII, E-cadherin, CIV, and β-actin (**A, B, D, E, F, H**) as well as pSmad3 and Smad3 (**C, G**) bands were quantified by densitometry analysis and values are expressed as described in the Methods. Values are presented as mean ± sem of three experiments. Differences between Cre/Vo and Cre/TβRII cells (*) or cells expressing wild type vs. Y-to-A mutated TβRII (**) were significant ($p \leq 0.05$). (**I**) Schematic representation of the downstream targets and/or signaling regulated by Y284, Y336, and/or Y470 in the TβRII cytoplasmic tail.