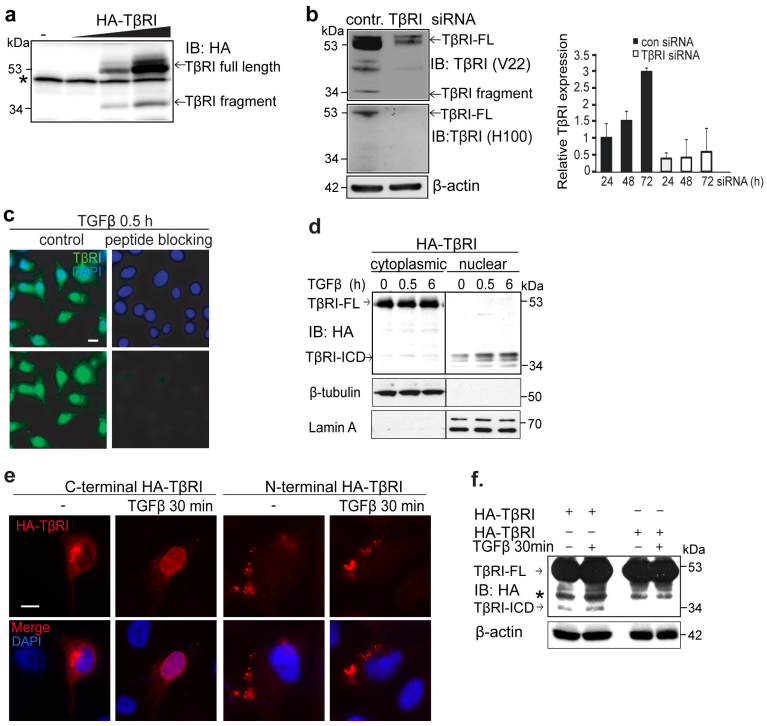
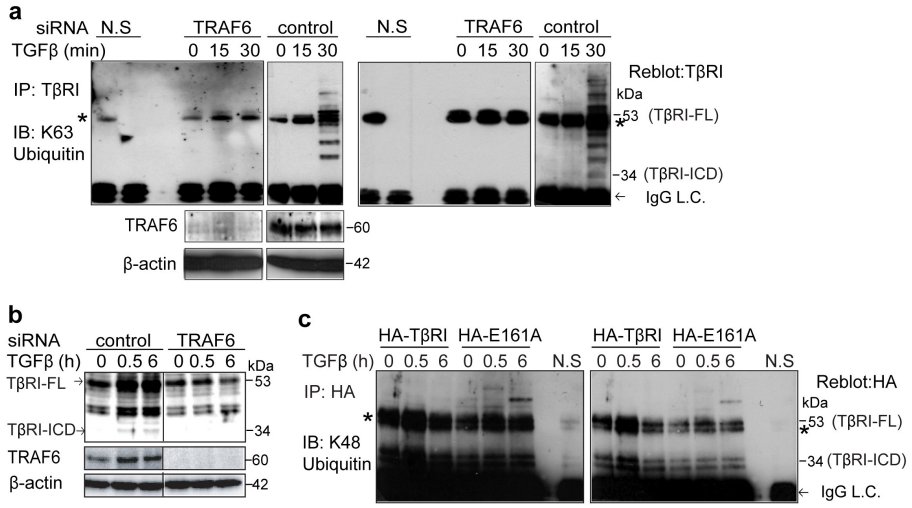
Supplementary Information

TRAF6 ubiquitinates TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer

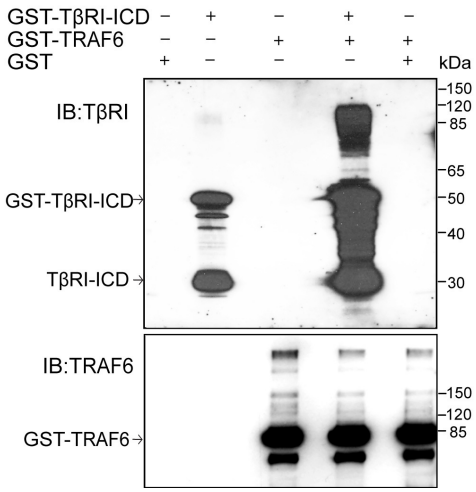
Yabing Mu, Reshma Sundar, Noopur Thakur, Maria Ekman, Shyam Kumar Gudey , Mariya Yakymovych, Helena Dimitrou, Annika Hermansson, Maria Teresa Bengoechea-Alonso, Johan Ericsson, Carl-Henrik Heldin, Marene Landström.



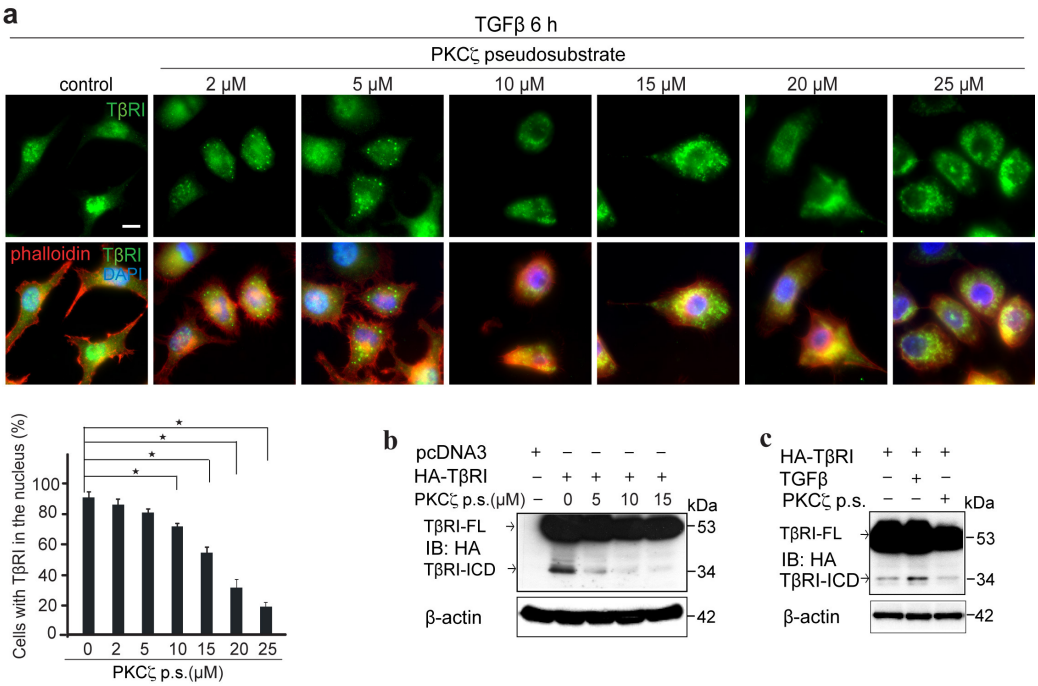
Supplementary Figure S1. Cleavage of the TGFβ type I receptor (TβRI)
(a) PC-3U cells transiently transfected with increasing amounts of C-terminally tagged HA-TβRI. The full-length HA-tagged TβRI migrated as a 53 kDa protein in SDS-gel electrophoresis and a shorter fragment was noticed with a size of approximately 40 kDa (the asterisk indicates a background band). Molecular weight markers are indicated. The PC-3U cells produce TGFβ in an autocrine fashion[23] which promotes generatation of an intracellular fragment of the HA-tagged TβRI. **(b)** Knockdown of TβRI by siRNA was performed to demonstrate the specificity of the V22 and H100 antibodies, which recognize the intracellular and extracellular domain of TβRI, respectively, used in immunoblotting and immunofluorescence (as shown in Fig. 1 a). To verify the efficiency of knock-down of TβRI, RNA was isolated from untreated cells or siRNA treated cells after 24, 48 and 72 h, and the TβRI mRNA was measured by qRT-PCR (mean ± s.d., $n = 3$ independent experiments). **(c)** Specificity of the V22 TβRI antibody used to detect the nuclear localization of TβRI. PC-3U cells were treated with TGFβ for 30 minutes and subjected to immunofluorescence staining for endogenous TβRI (control). The V22 antibody was incubated with the peptide used for immunization for 1 h in room temperature to show its specificity (peptide blocking). Staining with DAPI was used to visualize cell nuclei. Scale bar 20 µm. **(d)** Cell lysates from PC-3U cells transiently transfected with C-terminally HA-tagged TβRI were treated as indicated, then fractionated into cytoplasmic and nuclear proteins. The samples were then subjected to SDS-gel electrophoresis, followed by immunoblotting using HA-antibody, which recognize the intracellular domain of HA-tagged TβRI. β-tubulin and lamin A served as controls for the cytoplasmic and nuclear fractions, respectively. **(e)** PC-3U cells ectopically expressing C-terminally HA-tagged wt TβRI or the N-terminal HA-tagged TβRI were stimulated with TGFβ for 30 min and thereafter stained with HA antibody. Staining with DAPI was used to visualize cell nuclei. Scale bar 20 µm. **(f)** Cell lysates from PC-3U cells transiently transfected with C- or N- terminally HA-tagged TβRI and treated as indicated, were subjected to SDS-gel electrophoresis, followed by immunoblotting using HA-antibody. * indicates a background band.



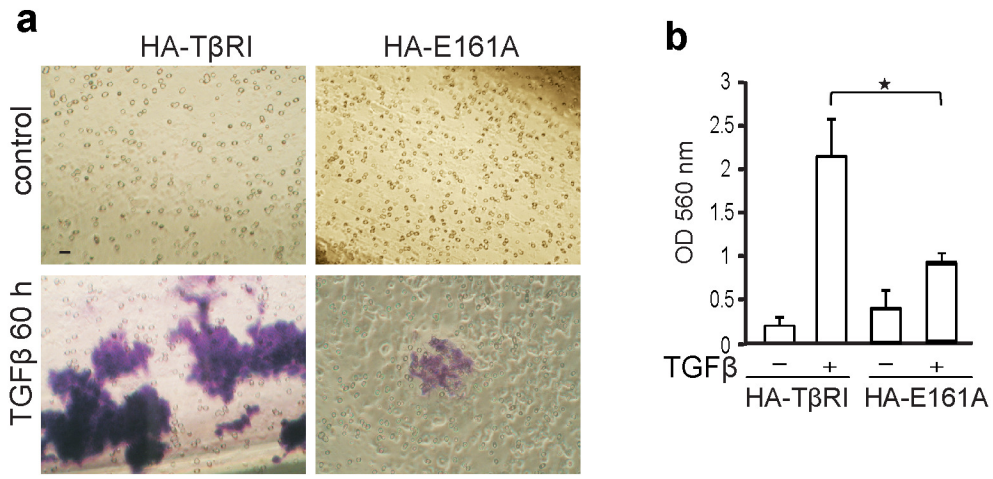
Supplementary Figure S2. TRAF6 promotes TGFβ-induced Lys63-linked polyubiquitination of TβRI and its cleavage. (a) PC-3U cells, in which endogenous TRAF6 was silenced by its siRNA or not, were treated or not with TGFβ, whereafter ubiquitination of TβRI was examined by an *in vivo* ubiquitination assay in Sorrentino et al.[5]. A fraction of cell lysates were subjected to immunoprecipitation (IP) with the V22 TβRI antibody followed by immunoblotting (IB) for Lys63 (K63)-linked polyubiquitin-specific antibodies. A light-chain specific antibody was used to avoid cross-reaction with IgG heavy chain. The IP-filter was reblotted with the V22 antibody to verify its specificity in IP. * indicates a background band. Note that the Lys63-linked polyubiquitination occurs only in the presence of TRAF6. Note also that the TβRI-FL migrates just above the background band from IgG heavy chain. **(b)** Cell lysates from PC-3U cells transiently transfected with control or TRAF6 siRNA, were treated with TGFβ, as indicated and subjected to immunoblotting with the V22 antibody to detect endogenous TβRI. Note the shorter fragment of TβRI in control cells stimulated with TGFβ, but not in cells where endogenous TRAF6 was knocked-down by siRNA. **(c)** PC-3U cells transiently transfected with C-terminally HA-tagged wt TβRI, or E161A mutant TβRI deficient in binding of TRAF6, were treated or not with TGFβ, whereafter ubiquitination of TβRI was examined by an *in vivo* ubiquitination assay. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA followed by immunoblotting (IB) for a Lys48 (K48)-linked polyubiquitin-specific antibody. A light-chain specific secondary antiserum was used to avoid cross-reaction with IgG heavy chain. The IP-filter was reblotted with HA to verify equal expression levels of wt and E161A mutant TβRI. * indicates a background band.



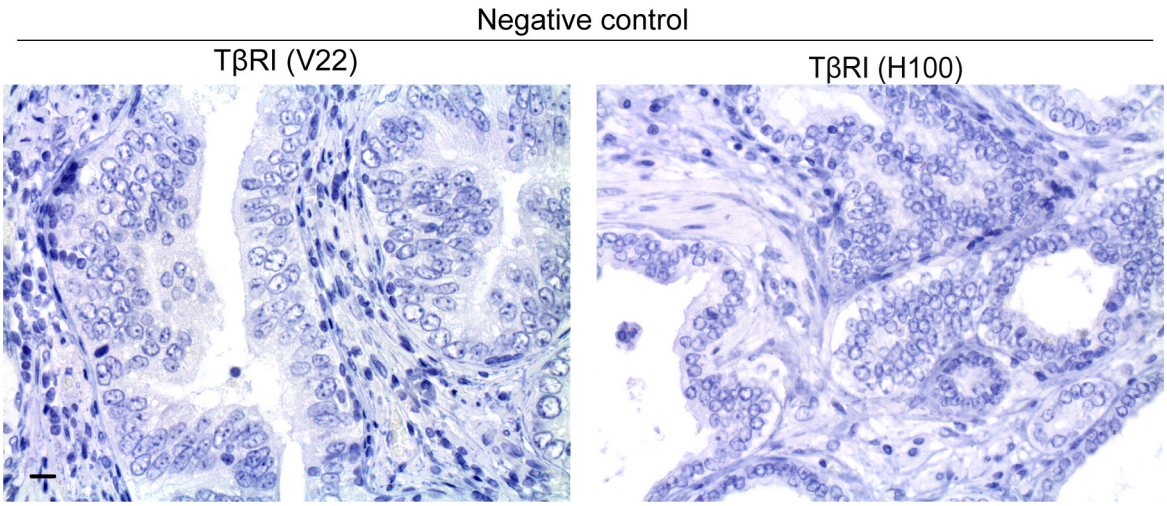
Supplementary Figure S3. TRAF6 and Ubc13-Uev1A promotes polyubiquitination of TβRI *in vitro.* GST-TβRI intracellular domain proteins were incubated in the presence or absence of recombinant GST-TRAF6 proteins (approximally 0.1 µg at maximum concentration), in a reaction mixture containing 20 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl2, 10 mM dithiothreitol, 10 mM ATP, 0.5 µg/µl ubiquitin (Sigma), 2 µM ubiquitin aldehyde (BIOMOL), 100 µM MG132 (Sigma), 0.1 µg E1 (human recombinant from Biomol), 0.2 µg E2 Ubc13–Uev1A (Biomol) at 30 °C for 1 h, then subjected to SDS–PAGE, followed by immunoblotting with antibodies against TβRI (V22 from Santa Cruz (1:500) and TRAF6 from ZYMED Laboratories (1:500). Molecular weight markers are indicated. For further details of the *in vitro* ubiquitination assay see also Sorrentino et al.[5]. Note the polyubiquitination of TβRI ICD proteins in the presence of GST-TRAF6.



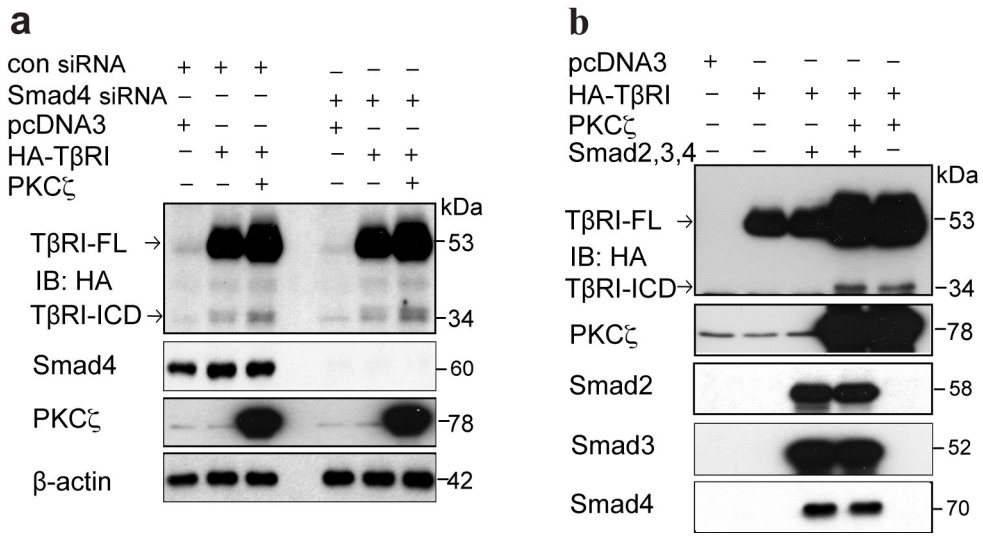
Supplementary Figure S4. PKCζ promotes nuclear accumulation of TβRI. (a) PC-3U cells were treated with TGFβ for 6 h and subjected to immunofluorescence staining for endogenous TβRI in the presence or absence of increasing amounts of the PKCζ pseudosubstrate (p.s), to analyze the subcellular localization of the endogenous TβRI visualized by the V22 antibody. Quantification of the number of cells showing endogenous TβRI in the nucleus (mean ± s.d., *n*=3 independent experiments where N= 200 - 300 cells were counted, *p<0.0003, ANOVA). Staining with DAPI was used to visualize cell nuclei. Scale bar 20 μm. **(b,c)** Cell lysates from PC-3U cells transiently transfected and treated as indicated, were subjected to immunoblotting to visualize TβRI-FL and TβRI-ICD. The filters were reprobed with actin to confirm equal loading of proteins in all lanes.



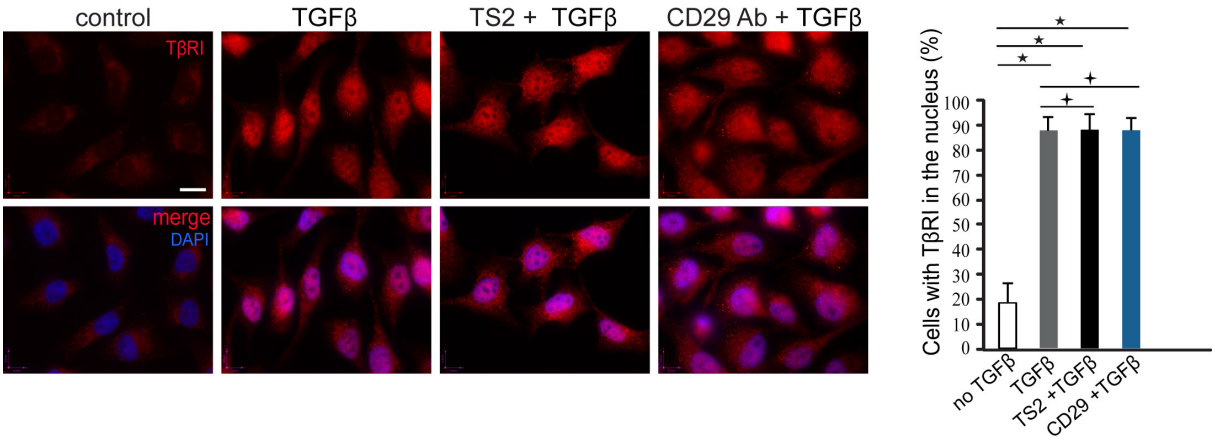
Supplementary Figure S5. TβRI promotes invasion of human prostate cancer cells. **(a)** Invasion assay for LNCaP cells, transiently transfected with wt or E161A mutant TβRI, treated as indicated. Cells were visualized by staining with crystal violet cell stain solution. Scale bar 20 μm. **(b)** Quantification of invasive LNCaP cells transiently transfected with wt or E161A mutant TβRI and treated with TGFβ, measured by optical density (O.D) at 560 nm in invasion assays (mean ± s.d., *n*=3 independent experiments, *p<0.01 Student's *t* test).



Supplementary Figure S6. Sections from prostate cancer tissues were subjected to identical immunohistochemical staining procedure as shown in Figure 8, while the primary antibodies (V22 and H100) were omitted, in order to serve as negative controls. Scale bar 20 μm.



Supplementary Figure S7. Low or high levels of Smads do not influence PKCζ-dependent generation of TβRI-ICD. (a) Cell lysates from PC-3U cells transiently transfected with control or Smad4 siRNA, pcDNA3, C-terminally HA-tagged TβRI alone or together with PKCζ, as indicated and subjected to immunoblotting with HA antibodies to detect TβRI-ICD. Total cell lysates were also subjected to immunoblotting with Smad4 and PKCζ antisera. One of the filters were stripped and reprobed with β-actin antibodies to serve as a control for equal loading of proteins. **(b)** Cell lysates from PC-3U cells transiently transfected with C-terminally HA-tagged TβRI alone or together with Smad2,3,4 and PKCζ as indicated and subjected to immunoblotting with HA antibodies to detect TβRI-ICD. Total cell lysates were also subjected immunoblotting with Smad2, Smad3, Smad4 and PKCζ antibodies.



Supplementary Figure S8. TGFβ-induced nuclear accumulation of TβRI ICD is not dependent on integrin signalling. PC-3U cells were treated with TGFβ for 30 min, in the presence or absence of TS2 or CD29 antibodies, respectively. Endogenous TβRI is shown by immunofluorescence using the V22 antibody. Quantification of the number of cells showing endogenous TβRI in the nucleus is shown the panel below (mean ± s.d., *n* = 3 independent experiment, where N = 200-300 cells where counted in each group, *p<0.0002, +p>0.96, ANOVA). Staining with DAPI was used to visualize cell nuclei. Scale bar 20 μm.