Supplemental Materials

Molecular Biology of the Cell

Meyer et al.

Supplementary Materials and Methods

Reagents and antibodies

All antibodies were purchased from Cell Signaling Technology, with the exception of PALS, which was purchased from Santa Cruz Biotechnology (sc-33831). Catalog numbers are: pSmad2 (3101), total Smad2 (3103), pSmad3 (9520), total Smad3 (9523), p-p38 (4511), total p38 (9212), pErk1/2 (9101), total Erk1/2 (4695), pPI3K p85 (4228), total PI3K p110 (4249), pAkt (4051), total Akt (4691), pIκBα (9246), and total IκBα (9242). The small molecule inhibitors CI1040 (MEK), U0126 (MEK), SB202190 (p38), and SB203580 (p38) were purchased from Cell Signaling Technology. PP2 (Src) was purchased from Sigma, and PD98059 (MEK), IKK Inhibitor VII (NFκB), LY294002 (PI3K), and SP600125 (JNK) were purchased from Calbiochem.

Induction of WT or P826A TβRIII expression

Cells were grown in 6-well dishes and were treated with 1.5 μg/mL doxycycline for 0, 3, 6, and 12 hrs. The level of TβRIII cell surface expression was visualized via binding and crosslinking.

Cell signaling analysis

NMuMG cells were plated in 6-well format. At ~50% confluency, cells were incubated for 6 hrs with serum free media and were left untreated or were stimulated with 100 pM TGF-β for 30 min. For pSmad2 analysis, cells were left untreated or were treated for 30 min with varying doses of TGF-β (0, 10, 25, 50, 75, and 100 pM). To verify decreased pSmad2 levels for the TGF-β RI kinase inhibitor and the TGF-β neutralizing antibody, serum starvation was accompanied by a 6 hr pre-treatment with 10 μg/mL concentrations of either inhibitor prior to stimulation with 0, 10, 20, or 50 pM TGF-β for 30 min.

Small molecule inhibitor screens: invasion and migration

The following concentrations of inhibitors were incubated with the cells in the upper chamber for both assays:

U0126: 10 μM
PD98059: 20 μM
CI1040: 1 μM
SB202190: 10 μM
SB203580: 1 μM
LY294002: 20 μM
PP2: 10 μM
IKK Inhibitor VII: 2 μM
SP600125: 15 μM

Appropriate volumes of DMSO were used as controls, and the assays were performed as described in the main text.

TGF-β1 ELISA

Cells were incubated with fresh media, and TGF-β1 was allowed to accumulate for 24 hrs. Media samples were collected, spun down to remove dead cells and debris, and frozen at -80^{o}C.

Multiplex assays were done in a 96-well format according to the SearchLight protocol. Samples were thawed on ice, centrifuged at 20,000*g* for 5 min to remove any residual precipitate, and were appropriately diluted before placement onto SearchLight plates. Samples and standards were incubated at room temperature for 1 hr while shaking using a Labline Titer Plate Shaker (Model No. 4625). Plates were washed 3 times using an automated plate washer (Biotek Instruments, Inc., Model ELx405), the biotinylated secondary antibody was added, and the plates were then incubated for an additional 30 min. After three more washes, streptavidin-HRP was added to the plates, the plates were incubated for 30 min, washed again, and SuperSignal substrate was added. Images of the plates were taken within 10 min, followed by image analysis using SearchLight array analyst software (Version 2.1). Analyte concentrations were calculated based on a standard curve derived by performing four serial dilutions of the corresponding protein standard on each plate. Samples were tested in duplicate, and the mean value was used for analysis.

TβRIII immunohistochemistry and TUNEL staining

For TβRIII IHC, slides were baked at 60^{o}C for 30 min and deparaffinized in two changes of xylene (5 min each) followed by 100%, 90%, 80%, and 70% ethanol solutions (5 min each). Slides were washed in running water for 5 min and blocked for 30 min in 1% H_2O_2 in methanol. Slides were washed in running water for 2 min, blocked in 10% normal goat serum (Vector Laboratories, S-1000) in PBS/0.06% Brij, and blocked with the Avidin/Biotin Blocking Kit (Vector Laboratories, SP-2001) according to the manufacturer's instructions. Tissues were incubated with a 1:200 dilution of primary antibody (anti-TβRIII cytosolic domain, described in Dong et al. 2007) overnight in 10% normal goat serum in PBS. The next day, slides were washed 3 times in PBS/0.06% Brij for 5 min each and incubated for 30 min with a 1:100 dilution of biotinylated anti-rabbit secondary (Vector Laboratories, BA-1000) in 10% normal goat serum in PBS. Slides were washed 5 times in PBS for 5 min and incubated with Vectastain rabbit ABC reagent (Vector Laboratories, PK-4001) for 30 min. Slides were further washed 5 times in PBS for 5 min. Staining was visualized by DAB reagent (Vector Laboratories, SK-4100). Tissues were further stained with hematoxylin, washed in running water for 2 min, and rehydrated in 70%, 80%, 90%, and 100% ethanol for 1 min each, followed by a 3 min incubation in xylene. Slides were mounted using VectaMount (Vector Laboratories, H-5000).

For TUNEL staining, the TumorTACS *in situ* Apoptosis Detection Kit (Trevigen, 4815-30-K) was used according the manufacturers' instructions.

Supplementary Figure 1. P826A TβRIII cells have disrupted PALS polarity protein staining. Cells were grown on coverslips to confluency, allowed to polarize, and were fixed and stained with an anti-PALS primary antibody followed by an Alexa-488 labeled secondary antibody (green). Images were obtained at 400x magnification.

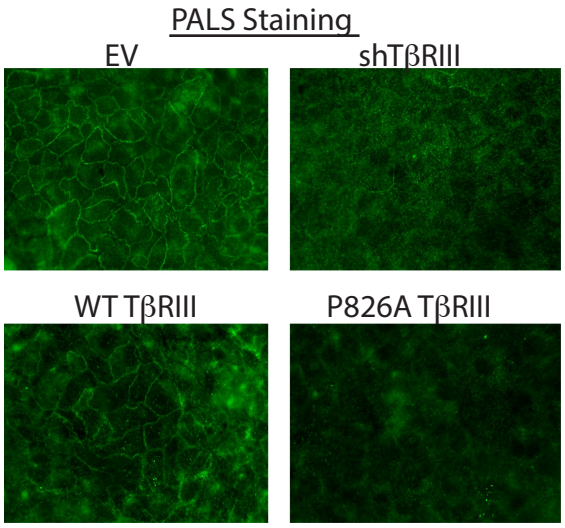
Supplementary Figure 2. Smad2 activation is enhanced by P826A TβRIII expression. (A) Cells were grown to ~75% confluency in 6-well dishes and were serum starved for 6 hours in the presence (+Ab) or absence (-Ab) of 10 μg/mL TGF-β neutralizing antibody. Cells were then treated with the indicated concentrations of TGF-β1 ligand for 30 minutes, lysed, and analyzed by western blotting for phospho Smad2 (pSmad2) and total Smad2 (T Smad2). (B) Cells were grown in 6-well dishes to 75% confluency. Fresh media was added and was allowed to condition for 24 hours. The media was then harvested and analyzed by ELISA for TGF-β1 ligand. The graph shows the concentration of TGF-β1 detected in the media from each cell line and represents an average of 3 independent experiments. N.S.=not significant (Student's t-test). (C) Cells were grown to 75% confluency and were serum starved for 6 hours. Following a 30 min treatment with 100 pM TGF-β1, the levels of phospho (pSmad3) and total Smad3 (T Smad3) were analyzed by western blotting. (D) Cells were grown as in (C), with the exception that varying concentrations of TGF-β1 ligand were added for 30 minutes. Lysates were analyzed for the levels of phospho and total Smad2. (E) Cells were plated as in (A) but in the presence(+SB) or absence (-SB) of 10 μg/mL of the TβRI kinase inhibitor SB431542.

Supplementary Figure 3. Inhibition of non-canonical TGF-β signaling does not affect P826A TβRIII cell migration or invasion. (A) Cells were grown in 6-well format until they reached ~75% confluency, serum starved for 6 hours, and treated with 100 pM TGF-β1 for the indicated times. The lysates were analyzed for the indicated proteins. The black line denotes intervening lanes that were removed for clarity. (B) For migration assays (left panel), 15x10^3 P826A TβRIII cells were plated in 200 μl of serum free media containing DMSO (control) or the indicated chemical inhibitors. Cells were allowed to migrate for 24 hours toward 500 μl of media containing 10% FBS. The number of migrated cells in 3 independent fields of view (100x) was counted for each cell line. Data are graphed as the number of migrating cells/the total plated cell number (% migration). U0=U0126 (10 μM); CI=CI040 (1 μM); PD= PD98059 (20 μM); SB202=SB202190 (10 μM); SB203=SB203580 (1 μM); SP600=SP600125 (15 μM); LY = LY294002 (20 μM); IKK=IKK Inhibitor VII (2 μM); PP2 (10 μM). The signaling pathways inhibited by each chemical are noted below the graph. For invasion assays, 50x10^3 P826A TβRIII cells were plated in 500 μl of serum free media containing DMSO (control) or the indicated chemical inhibitors at the same concentrations listed above. Cells were allowed to invade for 24 hours toward 600 μl of media containing 10% FBS. The number of invasive cells in 3 independent fields of view (100x) was counted for each cell line. Data are graphed as the number of invasive cells/the total plated cell number (% invasion). N.S.-not significant; ***p<0.001; **p<0.01; *p<0.05 (C) 75x10^3 WT TβRIII cells were plated

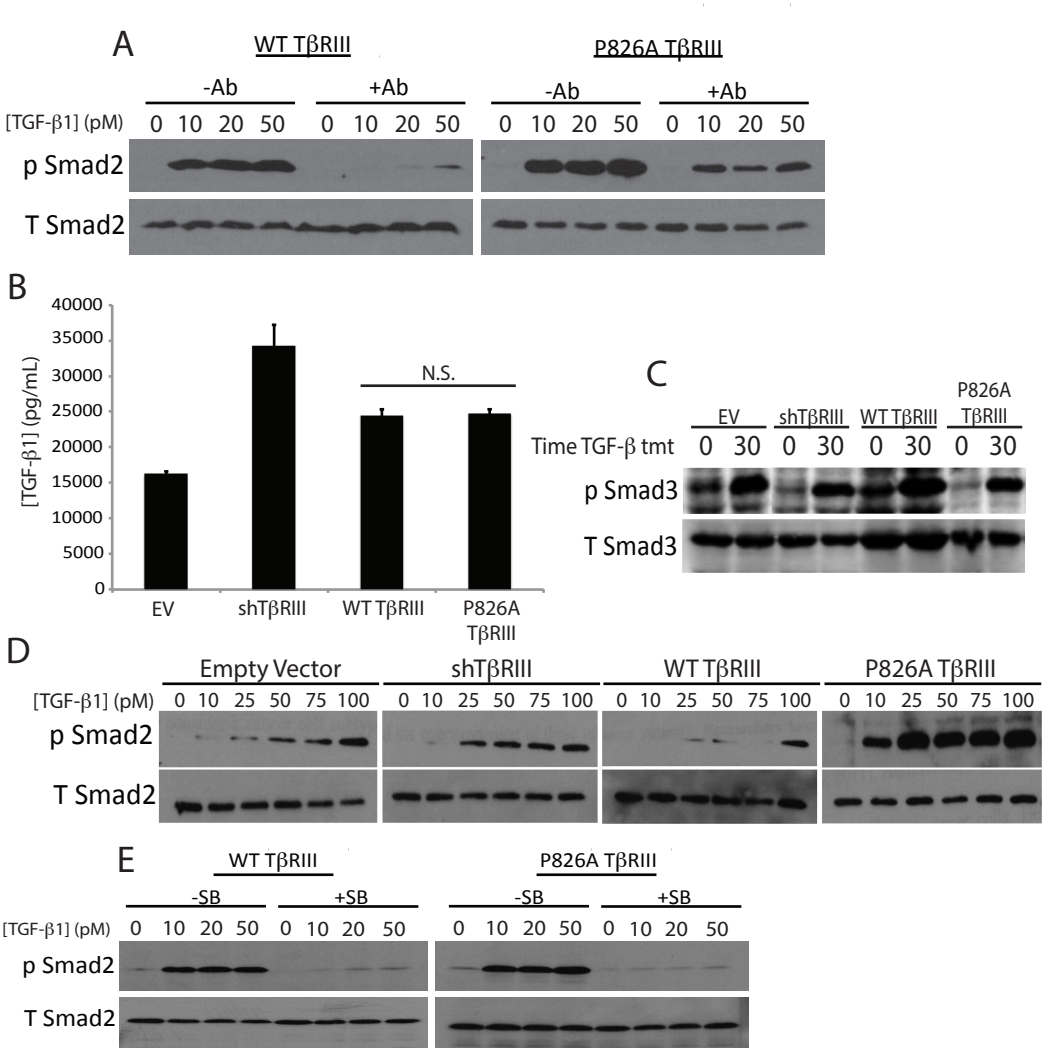
and counted as described in (B). N.S.=not significant; ***p<0.001; **p<0.01 (Student's t-test).

Supplementary Figure 4. WT and P826A TβRIII tumors maintain TβRIII expression. (A) IHC was performed to detect TβRIII expression levels (brown) in tumor samples. shTβRIII tumors were used as a control for staining. Images were taken at 400x magnification. Representative images are shown. (B) TUNEL staining was performed to analyze the levels of apoptosis in each tumor sample. No apoptosis was detected in the single EV sample that was large enough for analysis. N=9 for shTβRIII; N=4 for WT TβRIII; N=10 for P826A TβRIII. N.S.=not significant (Mann-Whitney test).

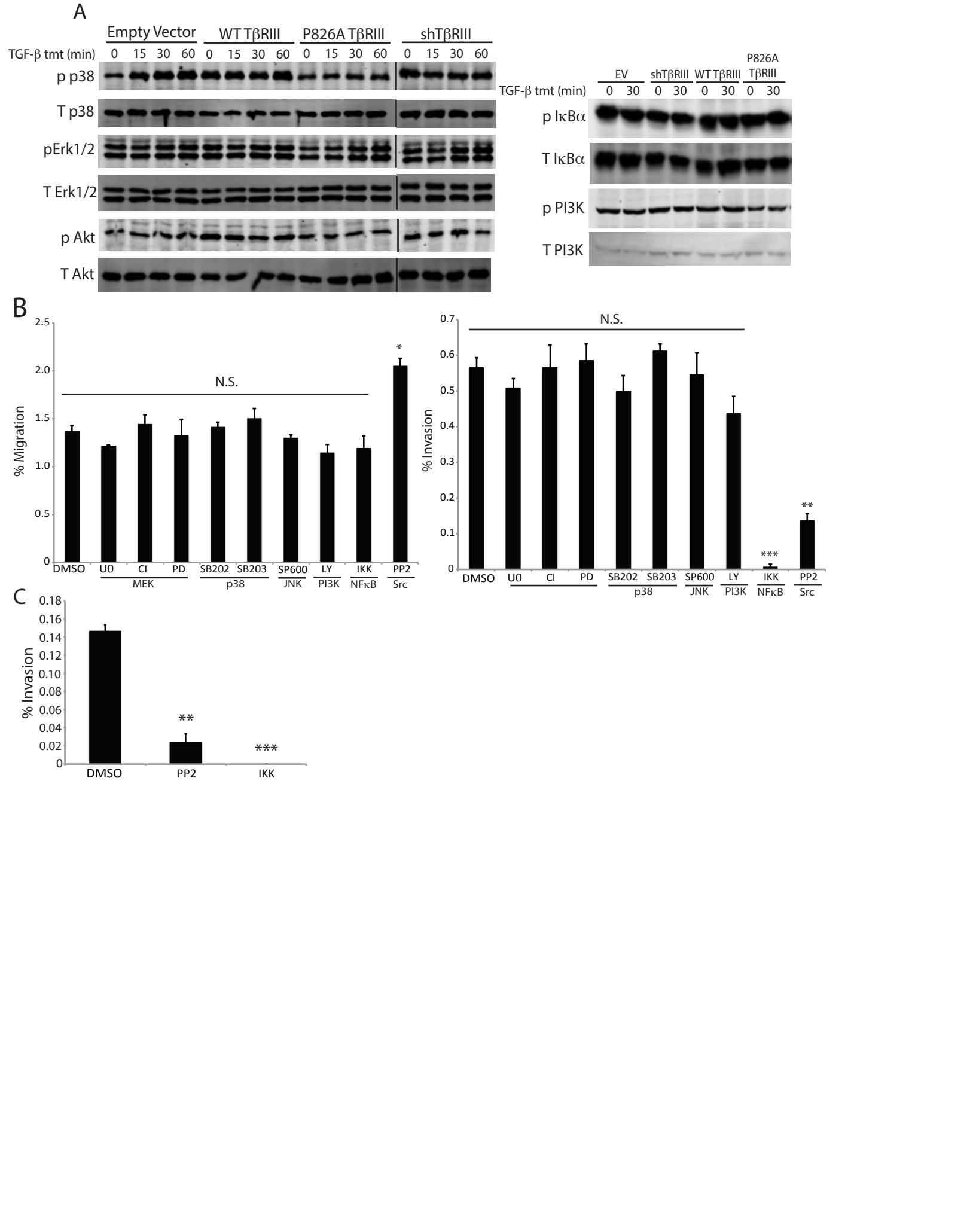
Supplementary Figure 1



Supplementary Figure 2

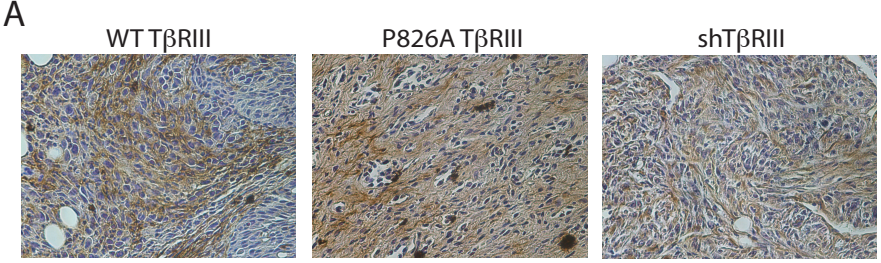


Supplementary Fig. 3



Supplementary Figure 4

A



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