Ectodomain shedding of TβRIII is required for TβRIII-mediated suppression of TGF-β signaling and breast cancer migration and invasion

Jennifer L. Elderbroom[1], Catherine E. Gatza[2], Tam How[2], Mark Starr[2], Andrew B. Nixon[2], and Gerard C. Blobe[1,2,*]

Author Affiliations

[1]Department of Pharmacology and Cancer Biology, Duke University, Durham, NC 27708

[2]Department of Medicine, Duke University, Durham, NC 27708

[*]Address correspondence to: Gerard C. Blobe, Duke University Medical Center, Box 91004, Durham, North Carolina 27708, USA. Phone: 919-668-1359. Fax: 919-681-6906. E-mail: gerard.blobe@duke.edu.

Abstract

The type III TGF-β receptor (TβRIII), also known as betaglycan, is the most abundantly expressed TGF-β receptor. TβRIII suppresses breast cancer progression through inhibiting migration, invasion, metastasis, and angiogenesis. TβRIII binds TGF-β ligands, with membrane-bound TβRIII presenting ligand to enhance TGF-β signaling. However, TβRIII can also undergo ectodomain shedding, releasing soluble TβRIII, which binds and sequesters ligand to inhibit downstream signaling. To investigate the relative contributions of soluble and membrane-bound TβRIII on TGF-β signaling and breast cancer biology, we defined TβRIII mutants with impaired (ΔShed-TβRIII) or enhanced ectodomain shedding (SS-TβRIII). Relative to wild-type TβRIII, ΔShed-TβRIII increased TGF-β signaling and abrogated TβRIII's ability to inhibit breast cancer cell migration and invasion. Conversely, SS-TβRIII, which increased soluble TβRIII production, decreased TGF-β signaling and increased TβRIII-mediated inhibition of breast cancer cell migration and invasion. Taken together, these studies suggest that the ratio of soluble TβRIII to membrane bound TβRIII is an important determinant for regulation of TβRIII- and TGF-β-mediated signaling and biology.

Introduction

The transforming growth factor β (TGF-β) signaling pathway is a critical regulator of many cellular processes including proliferation, differentiation, migration, invasion and angiogenesis . In normal epithelia and pre-malignant lesions, the TGF-β signaling pathway functions both to maintain tissue homeostasis and suppress malignant initiation and progression. However, once transformation has occurred, cancer cells are able to subvert the actions of TGF-β to promote cancer progression (Siegel and Massague, 2003). During malignant progression, the production of TGF-β ligands in the tumor and stroma increases (Massague, 2008). However, most cancers develop resistance to the homeostatic effects of TGF-β, including TGF-β-induced growth inhibition (Elliott and Blobe, 2005), and respond instead with increased migration, invasion, and metastatic potential (Mooradian et al., 1992).

TGF-β signals through heteromeric cell-surface receptor complexes consisting of a type I and type II receptor that upon ligand binding recruit and phosphorylate the Smad family of transcriptional regulators. In addition, there are a growing number of TGF-β superfamily co-receptors, including the type III TGF-β receptor (TβRIII), which can modulate ligand presentation to the type II receptor (Bernabeu et al., 2009). TβRIII, also known as betaglycan, is the most abundantly expressed TGF-β receptor (Cheifetz et al., 1990). TβRIII has an essential role in regulating TGF-β signaling, mediated through its ability to bind TGF-β ligands with high affinity. TβRIII binds all three isoforms of TGF-β (Wang et al., 1991), as well as bone morphogenetic proteins (BMPs) (Kirkbride et al., 2008), and inhibin (Lewis et al., 2000) through two distinct binding domains in its core protein, as well as bFGF through its heparan sulfate glycosaminoglycan (GAG) chains (Andres et al., 1992). Membrane-bound TβRIII presents ligand

to TβRII to increase signaling (Lopez-Casillas et al., 1993). However, TβRIII can also undergo ectodomain shedding, releasing a soluble form that binds ligand in the extracellular space, thereby reducing ligand availability to the signaling receptors and inhibiting downstream signaling (Lopez-Casillas et al., 1994).

TβRIII has a role as a suppressor of cancer progression in multiple types of cancer, including breast cancer (Dong et al., 2007). Breast cancer is the most common malignancy and the second most common cause of cancer related death in females in the United States (Siegel et al., 2012). In human breast cancers, TGF-β levels are frequently elevated and correlate with a poor patient prognosis (Ghellal et al., 2000). TβRIII expression is decreased in breast cancer cell lines and in human breast cancer patient specimens (Dong et al., 2007). Restoring TβRIII expression suppresses breast cancer progression by inhibiting migration, invasion, metastasis, and angiogenesis (Dong et al., 2007)(Sun and Chen, 1997). Similar to the effects of restoring full-length TβRIII expression, treatment with ectopic soluble TβRIII inhibits tumor growth, angiogenesis and metastasis in breast cancer models (Bandyopadhyay et al., 1999). These data suggest that the tumor-suppressive effects of TβRIII could be mediated, in part, by the production of soluble TβRIII, which antagonizes the tumor promoting effects of TGF-β signaling.

While the role of soluble TβRIII can be investigated by the addition of recombinant soluble TβRIII, the mechanisms regulating ectodomain shedding and generation of soluble TβRIII remain undefined, making it more difficult to delineate the function of cell-surface TβRIII, or the relative contribution of cell-surface TβRIII and soluble TβRIII to signaling and biology. Cleavage of many cell-surface proteins is carried out by a common machinery involving zinc-dependent metalloproteinases of the MMP and ADAM families. These proteases are regulated by several

mechanisms, including protein kinase C activation, intracellular calcium levels, and other activated growth factor signaling pathways (Arribas and Borroto, 2002). However, TβRIII release is largely unaffected by PMA, calcium ionophores and other factors that induce cleavage of canonical transmembrane shedding substrates (Arribas et al., 1997). TβRIII shedding can be reduced, but not blocked, with the pan-metalloproteinase inhibitor TAPI-2, as well as more specific inhibitors against MT1-MMP and MT3-MMP (Velasco-Loyden et al., 2004). However, studies with these inhibitors are complicated by their ability to alter the shedding of many other membrane proteins, making it difficult to use metalloprotease inhibition as a method to specifically determine the role of cell-surface TβRIII. Here we adopted a structure-function approach by creating TβRIII shedding mutants to investigate the significance of TβRIII ectodomain shedding in TGF-β-mediated signaling and TβRIII-mediated biology during breast cancer progression.

Materials and Methods:

Cell culture and reagents, transfections, lentivirus production and infections- COS7, HEK293 and 293FT cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with 10% Fetal Bovine Serum (FBS) (Invitrogen). HMEC cells were maintained in DMEM supplemented with 10% FBS and 0.01 mg/ml recombinant human insulin. MDA-MB-231 cells were grown in Modified Eagle's Medium (MEM) (Gibco) supplemented with 10% FBS, sodium pyruvate (Gibco) and non-essential amino acids (Gibco). All cells were incubated at 37°C with 5% CO_2. For transfections, cells were plated to be ~70% confluent the following day (between 2-3 x 10^5 cells depending on cell line) on 6-well dishes. The following day, cells were transfected with either Fugene 6 (Roche) or X-treme Gene 9 (Roche) transfection reagent and 3 μg plasmid DNA at a ratio of 2.5:1. Experiments were performed on transfected cells 48-72 h post-transfection, as indicated. For lentivirus production, 293FT cells were plated in 10-cm dishes. Cells were transfected with Lipofectamine 2000 (Invitrogen) at a ratio of 3:1 to DNA- 6 μg TβRIII wild-type, mutant or empty vector (pSMPUW-Neo expression vector) (Cell Biolabs) and 3 μg each of 3 third generation lentiviral packaging plasmids (AddGene) in Opti-MEM (Gibco). Media was changed 6 h after transfection. 48 h later, media was collected, spun down to clear, and filtered through a 0.45 μM pore membrane. Viral media was aliquoted and stored at -80°C. For infections, viral media was added to cells in normal growth media at a ratio of either 1:10 or 1:100 in the presence of Polybrene at 6 μg/ml. To create stable lentiviral-expressing cell lines, 48 h post-infection media was changed and complete growth media containing 2 mg/ml G418 (KSE Scientific) was added as a selection agent. Following selection, stable lentiviral cell lines were maintained in complete growth media containing 0.5 mg/ml G418.

Site-directed mutagenesis- Primers for NAAIRS and alanine mutants were designed as in Supplemental Tables 1 and 2, respectively. Mutagenesis PCRs, digests, and transformations were performed using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). NAAIRS and alanine mutants were made in a pDNR-CMV Donor Vector (ClonTech) containing an N-terminally HA-tagged wild-type TβRIII. Sequence analysis of all mutants was performed twice for verification.

Binding and crosslinking- 25,000 cells were plated on 6-well dishes. Cells were transfected 18-20 h later. 24 h later, media was removed and replaced with 1 ml complete growth media. Unless otherwise stated, media was conditioned for 18-20 h before being removed, and both cells and conditioned media incubated with ^{125}I-TGF-β1 (Perkin Elmer), at 100 pM and 25 pM, respectively, in the presence of BSA and protease inhibitors for 3 h at 4°C. After incubation, ligand was chemically crosslinked using 0.5 mg/ml disuccinimidyl suberate and quenched with 1M glycine. Cells were lysed with RIPA buffer supplemented with protease inhibitors, and ligand-receptor complexes were pulled down by immunoprecipitation overnight at 4°C with either an antibody against HA (Roche) or a polyclonal antibody against the extracellular domain of TβRIII (R&D Systems). The resulting complexes were separated via SDS-PAGE and dried gels were exposed to an autoradiograph. Images were acquired with phosphorimaging and were analyzed using ImageJ software (NIH).

Enzyme-linked Immunosorbent Assay (ELISA)- 25,000 cells were plated in 6-well dishes and transfected the following day. After 24 h, media was removed and replaced with 1000 µl full growth media. Culture medium was allowed to condition for 24 h (unless otherwise indicated), then was removed and immediately spun down at 4°C to pellet dead cells and debris. Cleared media was aliquoted and immediately placed at -80°C. For the TβRIII ELISA, capture antibody (R&D Systems) was immobilized onto an E1A/R1A plate (Corning Inc.) overnight. After washing,

samples were loaded onto plate and incubated at room temperature for 2 h. Then detection antibody (R&D systems) was applied and incubated for 2 h, followed by Strepavidin-HRP (R&D systems) incubation for 30 min. Finally, Fast OPD substrate (Sigma) was added, 3M HCl was applied to stop reaction 30 min later, and optical absorbance at 490 nm was recorded immediately.

Western blotting- Cells were plated and transfected as described above. For HEK293 cells, 24 h after transfection, media was removed and replaced with serum-reduced (5% FBS) media. After 18-20 h cells were treated with TGF-β1 or TGF- β2 (R&D Systems), at the concentrations and time-periods indicated. For MDA-MB-231 cells, media was removed and replaced with full serum media overnight before ligand treatment. Following ligand treatment, cells were lysed directly in 2X SB buffer and 20% of the lysate was loaded onto 10% SDS-PAGE gels. After electrophoresis, protein was transferred onto a PVDF membrane, which was blocked in 10% lowfat-milk in phosphate-buffered saline + 0.5% Tween-20 (PBS-T) for 1 h. Blots were probed overnight at 4°C with antibodies in 5% milk/PBS-T against phosphorylated Smad 2 (Cell Signaling), Total Smad2 (Cell Signaling), β-actin (Sigma-Aldrich), or TβRIII-ECD (R & D Systems), and then probed for 1 h with horseradish-peroxide conjugated secondary antibodies (Cell Signaling, Amersham). Bands were visualized using ECL (Western Lightning ECL Pro, Perkin Elmer) and exposure to film, and densitometry was quantified using ImageJ software (NIH).

Thymidine Incorporation Assay- 1500 cells were plated in 96-well plates, either in the absence or presence of 50 pM TGF-β1 (each condition in triplicate), in pre-conditioned media (conditioned for 24 h from cells expressing EV or corresponding TβRIII mutants). 20 h after plating, 1 μCi of ^{3}H-Thymidine (Perkin Elmer) was added to each well, and allowed to incubate for 4 h. After incorporation, cells were washed 2x with cold PBS, 1x with cold 10% trichloroacetic acid (TCA),

then rocked at 4°C for 1 h in 10% TCA. Then, cells were washed 1x with 10% TCA, and 0.2N NaOH was added to cells to lyse overnight. The following day, lysates were added to 2 ml scintillation fluid (Ultima-Gold, Perkin Elmer) and the amount of incorporation was determined by scintillation counting.

Transwell Migration Asssay- 25,000 cells in serum-free conditioned media (24 h from corresponding cell lines) were plated in the upper chamber of a 50 μg/ml fibronectin-coated transwell with 8 μM pores (Corning Inc.). Cells were either left untreated, or treated with 50 pM TGF-β1 (each condition in duplicate). Media containing serum was used as a chemoattractant in the bottom well, and cells were allowed to migrate for 24 h. After migration, the cells remaining on the top of the filter were removed by gently washing with a cotton swab, and migrated cells on the bottom of the filter were fixed in methanol and stained with H&E. Filters were cut out, mounted onto slides and examined microscopically. 3 random fields of cells were chosen and counted.

Matrigel Invasion Assay- 75,000 cells in serum-free conditioned media (24 h from corresponding cell lines) were plated in the upper chamber of a Matrigel-coated transwell with 8 μM pores (BD Biosciences). Cells were either left untreated, or treated with 50 pM TGF-β1 (each condition in duplicate). Media containing serum was used as a chemoattractant in the bottom well, and cells were allowed to invade for 24 h. After invasion, the cells remaining on the top of the filter were removed by gently washing with a cotton swab, and invaded cells on the bottom of the filter were fixed in methanol and stained with H&E. Filters were cut out, mounted onto slides and examined microscopically. 3 random fields of cells were chosen and counted.

Dual-Luciferase Reporter Assay- 100,000 cells were plated in 6-well plates. 24 h later, cells were transfected with 2.3 μg of pE2.1 luciferase reporter plasmid and 0.2 μg of pRL-SV40 renilla

plasmid. The next day, cells were treated with 24 h pre-conditioned serum-free media, and 50 pM of TGF-β1 or TGF-β2. 20 h later cells were lysed and the dual-luciferase reporter assay (Promega) was performed per kit instructions.

Statistical Analysis- Data are presented as mean ± SEM. One-way or two-way ANOVAs were performed followed by either a one-sample Student's *t*-test for values compared to a normalized control, or either a Tukey's Test or a Two-tailed Student's *t*-test for comparing two experimental values. P values < 0.05 are considered significant.

Results

Mutations in the juxtamembrane region of TβRIII alter ectodomain shedding

To investigate the significance of TβRIII ectodomain shedding on TGF-β-mediated signaling and biology, we set out to identify TβRIII mutants with altered ectodomain shedding. Human TβRIII is an 851 amino acid transmembrane proteoglycan with a large 766 amino acid extracellular domain, a single hydrophobic transmembrane region, and a short 42 amino acid cytoplasmic domain (Lopez-Casillas et al., 1991). Endogenous soluble TβRIII has nearly the same electrophoretic mobility as full-length, membrane bound TβRIII (Figure 1A), suggesting that cleavage occurs just proximal to the plasma membrane, consistent with what has been demonstrated with other shed receptors (Perez-Torres et al., 2008). A substitution mutagenesis approach replacing 6 amino acids of the endogenous TβRIII protein sequence with the NAAIRS amino acid sequence (Asparagine-Alanine-Alanine-Isoleucine- Arginine-Serine) was chosen, as this sequence can adopt multiple secondary stuctures, potentially minimizing structural changes (Hamad et al., 2002). To identify regions important for TβRIII shedding, a series of TβRIII NAAIRS mutants beginning 95 amino acids upstream of the transmembrane region and spanning the entire juxtamembrane region was created (Figure 1B, Supp. Table 1).

We initially assessed and confirmed expression of all 17 mutants (M0-M16) in COS7 cells, which lack endogenous TβRIII (Figure 1C). To evaluate processing to the cell surface, ligand binding ability and ectodomain shedding, we performed iodinated TGF-β1 binding and crosslinking assays on both the conditioned media from COS7 cells and on COS7 cells expressing either controls (pDNR-EV or pDNR-WT-TβRIII) or one of the membrane-proximal TβRIII NAAIRS mutants (M0-M16), followed by immunoprecipitation of TβRIII and soluble TβRIII. While all TβRIII NAAIRS mutants trafficked to the cell surface and bound ligand, there were significant

differences in the electrophoretic mobility of the mutants (Figure 1D), suggesting differential post-translational processing of the TβRIII mutants. However, by directly comparing levels of ligand binding to soluble TβRIII in the conditioned media to levels of ligand binding to membrane-bound TβRIII on the cell surface, ectodomain shedding of each mutant could be assessed independently of these factors. Four NAAIRS mutants (M1, M2, M9 and M11) consistently exhibited decreased ectodomain shedding compared to wild-type TβRIII controls (Figure 1D, E). Interestingly, three NAAIRS mutants (M13, M14, M15) exhibited increased ectodomain shedding compared to wild-type TβRIII controls (Figure 1D, E). These differences in shedding were independently confirmed by performing ELISA analysis for soluble TβRIII (Figure 1F).

A single amino acid substitution at m742 significantly inhibits TβRIII ectodomain shedding

To further define the distinct amino acid residues regulating ectodomain shedding of TβRIII, we created individual alanine point mutations in TβRIII of each amino acid contained within the NAAIRS mutants M1, M2, M9 and M11 (Supp. Table 2). Expression of each mutant was confirmed via western blotting (Figure 2A, Supp. Figure 1A). Ectodomain shedding of each mutant was evaluated via both ^{125}I-TGF-β1 binding and crosslinking assays and ELISA. Perturbing any amino acid residue within the M9 mutant disrupted ectodomain shedding to some degree, and substituting methionine 742 to alanine (M742A) fully recapitulated the decreased ectodomain shedding phenotype of the M9 NAAIRS mutant (Figure 2B-D). Mutation of isoleucine 738 to alanine (I738A) also significantly decreased ectodomain shedding (Figure 2B-D). In contrast, no single point mutations within M1, M2 or M11 NAAIRS mutants significantly altered ectodomain shedding (Supp. Figure 1B, C).

As the M9 NAAIRS, M11 NAAIRS, M742A and I738A mutants all significantly diminished but did not abrogate ectodomain shedding, we created mutants that combined the M742A and I738A mutations within M9 and the L752A and V754A mutations within M11. However, neither of these mutants exhibited any further decrease in shedding compared to the M9 or M742A mutations alone (data not shown). Therefore, we utilized the M9 NAAIRS and the M742A mutants as our models for shedding-deficient TβRIII (Δshed-TβRIII), and M13 as a 'super-shedder' (SS-TβRIII) to investigate the effects of altering the ratio of soluble to cell-surface TβRIII. To investigate whether the shedding properties of these mutants were retained in different cell contexts, we transiently expressed the mutants in human embryonic kidney epithelial cells (HEK-293) and in mink lung epithelial cells (Mv1Lu). In all cases, the M9 NAAIRS and M742A mutants exhibited reduced ectodomain shedding, and the M13 NAAIRS mutant exhibited increased ectodomain shedding (Supp. Figure 2A).

To investigate the effects of increased or decreased TβRIII ectodomain shedding in breast cancer, we utilized the well-characterized breast cancer cell line MDA-MB-231, in which we and others have already demonstrated the ability of TβRIII and soluble TβRIII to regulate its cancer biology, including migration and invasion *in vitro* and *in vivo* (Bandyopadhyay et al., 1999; Dong et al., 2007). MDA-MB-231 cells exhibit low endogenous TβRIII levels, consistent with loss of TβRIII expression during breast cancer progression. WT-TβRIII, Δshed-TβRIII, SS-TβRIII or an empty-vector DNA control were stably incorporated into MDA-MB-231 cells via lentiviral infection and single clones were selected, expanded and examined for TβRIII expression and ectodomain shedding via ^{125}I-TGF-β1 binding and crosslinking assays. Monoclonal MDA-MB-231 cell lines with equivalent levels of cell-surface TβRIII were chosen for further study (Figure 2E), and the

expected levels of soluble TβRIII production were confirmed via binding and crosslinking (Figure 2E) and ELISA (Figure 2F).

Effects of altered TβRIII ectodomain shedding on TGF-β signaling

In many cell lines, including MDA-MB-231 cells, increasing TβRIII expression results in decreased TGF-β signaling, as measured by Smad2 phosphorylation and transcription of TGF-β-responsive genes (Dong et al., 2007). As increasing TβRIII expression can result in increased soluble TβRIII (Lopez-Casillas et al., 1991), and soluble TβRIII is sufficient to inhibit downstream TGF-β signaling (Arribas and Borroto, 2002), this TβRIII-mediated decrease in TGF-β responsiveness has been hypothesized to be the result of increased production of soluble TβRIII. To directly address this hypothesis, and to examine the effects of high levels of cell-surface TβRIII in the absence of soluble TβRIII on TGF-β signaling, we utilized the stable MDA-MB-231 TβRIII ectodomain shedding mutant cell lines. Consistent with prior studies, compared to empty vector control MDA-MB-231 cells, WT-TβRIII expressing cells exhibited slightly decreased TGF-β1-mediated Smad2 phosphorylation (Figure 3A). In contrast, in Δshed-TβRIII cells, TGF-β1-mediated Smad2 phosphorylation was increased, suggesting that in the absence of significant ectodomain shedding, cell-surface TβRIII is able to present ligand and enhance signaling (Figure 3A). Consistent with this hypothesis, relative to WT-TβRIII cells, TGF-β1-mediated Smad2 phosphorylation was further reduced in SS-TβRIII cells (Figure 3A). A dose response curve from 0-100 pM TGF-β1 demonstrated a similar pattern of responsiveness at all concentrations tested (Figure 3B). To determine whether alterations in Smad2 phosphorylation were leading to changes in gene transcription, dual-luciferase reporter assays were performed using the TGF-β-responsive pE2.1 promoter. While there was no significant difference between WT-TβRIII and EV expressing cells,

SS-TβRIII cells treated with TGF-β1 had decreased TGF-β-induced transcription (Figure 3C). Conversely, cells stably expressing the Δshed-TβRIII had significantly increased TGF-β1-mediated pE2.1 transcription (Figure 3C). To establish whether the observed changes in signaling were due to changes in soluble TβRIII levels, we treated cells with ectopic recombinant soluble TβRIII. Recombinant soluble TβRIII reduced TGF-β signaling in a dose-dependent manner in each cell line, with the exception of the SS-TβRIII expressing cells (Supp. Figure 3), perhaps because signaling is already maximally inhibited by the increased levels of soluble TβRIII produced by this cell line. Together, these data demonstrate that the decrease in TGF-β responsiveness frequently observed when TβRIII is expressed requires TβRIII ectodomain shedding and generation of soluble TβRIII.

We next examined the effects of the shedding mutants on mediating TGF-β2 signaling. As TGF-β2 cannot bind to TβRII on its own (De Crescenzo et al., 2006), TGF-β2 requires TβRIII for presentation to TβRII and functional signaling. Consistent with this role, both WT-TβRIII and the Δshed mutant increased TGF-β2 responsiveness in MDA-MB-231 cells (Supp. Figure 4). However, the high ratio of soluble TβRIII relative to cell-surface TβRIII in the SS-TβRIII expressing cells shifted the balance from TβRIII-mediated promotion of signaling to inhibition of signaling (Supp. Figure 4).

To investigate the effects of TβRIII ectodomain shedding on the kinetics and duration of TGF-β signaling, we performed time-course experiments. In the absence of significant soluble TβRIII (EV and Δshed-TβRIII), Smad2 phosphorylation peaked at ~ one hour and signaling persisted out to six hours (Figure 4A). In contrast, in cells with higher levels of soluble TβRIII (WT-TβRIII and SS-TβRIII), Smad2 phosphorylation peaked earlier (~ 30 minutes), and signaling persisted only out to two to three hours (Figure 4A,B). Similar results were obtained by transient transfection of these TβRIII shedding mutants in the normal human epithelial cell line HEK293

(Supp. Figure 5). Interestingly, we also consistently observed evidence of a biphasic signaling pattern in the Δshed-TβRIII cell line, with signal diminishing between three to four hours, but then returning to nearly peak levels at five hours post-treatment (Figure 4A,B). Integrating the signaling that occurred over the six hour period demonstrated a 50% reduction in total signaling in WT-TβRIII expressing cells compared to Δshed-TβRIII (Figure 4C). These data establish an important role for the ratio of soluble and cell-surface TβRIII in regulating the kinetics and magnitude of TGF-β signaling.

Effects of altered TβRIII ectodomain shedding on TGF-β-mediated migration and invasion

TβRIII inhibits epithelial and cancer cell motility and invasion in multiple human cancers, including breast cancer (Finger et al., 2008; Lambert et al., 2011; Mythreye and Blobe, 2009; Turley et al., 2007). Increasing wild-type TβRIII expression in MDA-MB-231 cells inhibited TGF-β-induced transwell migration and invasion (Dong et al., 2007). Similarly, plating MDA-MB-231 cells in conditioned media from COS7 cells over-expressing TβRIII also reduced TGF-β-induced invasion (Dong et al., 2007), suggesting that the production of soluble TβRIII is one mechanism for TβRIII-mediated inhibition of cell motility and invasion. To directly determine the contribution of TβRIII ectodomain shedding and soluble TβRIII production on TβRIII-mediated inhibition of migration and invasion, we utilized the monoclonal stable MDA-MB-231 cell lines expressing the TβRIII shedding mutants. As these experiments are usually performed in serum-free media, we first examined the effect of serum on TβRIII ectodomain shedding. In the absence of serum, ectodomain shedding was potently inhibited (Supp. Figure 6). Therefore, for these experiments, cells were plated in media that had been pre-conditioned for 24 hours, to more accurately reflect the differential levels of ectodomain shedding. We next investigated whether expression of the

TβRIII shedding mutants altered the proliferation rates of the stable cell lines using ^{3}H-thymidine incorporation assays. Over 24 hours, the time frame for both the invasion and migration assays, there was a decrease in the basal proliferation rates of WT-TβRIII, Δshed-TβRIII and SS-TβRIII compared to EV control cells of ~40%, 30% and 50%, respectively (Supp. Figure 7). TGF-β treatment also slightly reduced proliferation rates in all four cell lines (Supp. Figure 7). To reflect these changes, migration and invasion results were normalized to the proliferative index of each corresponding condition.

While TGF-β1 stimulated migration of the MDA-MB-231 cells expressing EV, cells expressing WT-TβRIII were unresponsive to ligand (Figure 5A,B). Conversely, cells expressing Δshed-TβRIII demonstated a 3-fold increase in basal migration and an increase in ligand-mediated migration relative to EV control cells (Figure 5A,B). Expressing SS-TβRIII had an even more potent effect on motility than WT-TβRIII, producing a significant decrease in ligand-induced transwell migration (Figure 5A,B). Similar results were obtained by transient transfection of the TβRIII shedding mutants in normal human mammary epithelial cells (HMECs) (Supp. Figure 8).

We also evaluated the effects of altered TβRIII ectodomain shedding on MDA-MB-231 cell invasion via Matrigel-coated transwell invasion assays. TGFβ-mediated invasion was significantly inhibited in both the WT-TβRIII and SS-TβRIII expressing cells (Figure 5C,D). In contrast, compared to EV, there was an increase in both basal and TGF-β-mediated invasion in Δshed-TβRIII expressing cells (Figure 5C,D). Together, these data demonstrate that ectodomain shedding is required for TβRIII-mediated inhibition of TGF-β induced breast cancer cell migration and invasion.

Discussion

Here we have demonstrated that mutating the juxtamembrane region of TβRIII can alter its ectodomain shedding, and that inhibiting production of soluble TβRIII results in increased TGF-β responsiveness, increased duration of TGF-β signaling, and abrogation of TβRIII's ability to inhibit TGF-β-mediated migration and invasion.

Ectodomain shedding of transmembrane proteins is a common phenomenon that often contributes to regulation of signal transduction. Ligands and growth factors, including TGF-α, can be activated for autocrine signaling by release from the membrane (Teixido et al., 1990), and cell-surface signaling receptor levels can be altered to either increase or decrease cellular responsiveness. Recent studies demonstrated that the type I TGF-β receptor is released from the membrane by TACE, resulting in decreased TGF-β signaling (Liu et al., 2009). Following this shedding event, a secondary γ-secretase cleavage releases the intracellular domain (ICD) of TβRI, which can then accumulate in the nucleus and interact with transcriptional regulators to alter TGF-β-induced gene transcription and subsequent tumor cell invasion (Mu et al., 2011). In addition, it was recently reported that TβRIII is also a substrate for γ-secretase cleavage and that its ICD is stable after this event (Blair et al., 2011); however, it is still unknown whether the ICD is involved in downstream signal transduction. If the TβRIII-ICD functions similarly to the TβRI-ICD, it will be interesting to determine whether any of the alterations in signaling and biology reported here are regulated by increased or decreased activity of the ICD resulting from the Δshed- or super-shed-TβRIII mutation.

While we have established discrete mutations in the TβRIII juxtamembrane region that regulate TβRIII shedding, we have not elucidated how these mutations result in increased or decreased ectodomain shedding. The M9 NAAIRS mutation and M742A substitution could

decrease shedding either by disrupting a potential metalloprotease consensus sequence, or by altering the structure of TβRIII, so that it is unable to physically interact with or be recognized as a substrate by the appropriate proteases. Knowledge regarding the precise site of endogenous TβRIII cleavage and structural information regarding the extracellular domain of TβRIII would both be useful in determining how these alterations regulate TβRIII shedding. We used the protease specificity prediction server (PROSPER) to perform *in silico* analysis of the TβRIII juxtamembrane domain sequence (Song et al., 2012). The region containing the M9 NAAIRS mutant revealed several potential cleavage sites at A740, M741 and M742 by MMP3, MMP9 and cathepsin, suggesting that the M9 and M742A mutants could be disrupting these recognition sequences. The region containing the NAAIRS M13-M15 mutants, which increased TβRIII ectodomain shedding, is highly proline-rich. Due to the unique biochemical and structural properties of proline, mutating this region may alter the structure of the extracellular domain of TβRIII to make it more accessible to an endogenous, constitutive sheddase. Indeed, while there were no protease consensus sites within the M13-M15 region, when the M13 super-shedder sequence containing the NAAIRS substitution was queried in the PROSPER software, two novel consensus sites for MMP9 and elastase at were introduced at that site. Whether these proteases are responsible for shedding TβRIII is currently being explored.

As we and others have demonstrated an important role for TβRIII and soluble TβRIII in regulating breast cancer progression, both through effects on breast cancer cells (Sun and Chen, 1997; Dong et al., 2007), as well as effects on the tumor microenvironment, including the local immune response (Hanks et al., 2013) and angiogenesis (Bandyopadhyay et al., 1999; Dong et al., 2007), understanding the regulation and mechanism of TβRIII shedding and how this process might be altered during cancer progression could yield insight into targeting TGF-β signaling in

cancer patients. While TβRIII does not appear to be a substrate of the canonical ectodomain shedding machinery (Arribas et al., 1997), determining what factors do regulate TβRIII shedding could be quite informative. Ectodomain shedding of other cell surface receptors can be activated by numerous triggers, including UV irradiation, inflammation, and growth factor stimulation (Killock and Ivetic, 2010; Seo et al., 2007). In addition to decreased TβRIII expression in human cancers, the balance of cell-surface and soluble TβRIII could be altered by aberrant processes or signaling within the tumor that potentially could be targeted therapeutically. The current studies suggest that increasing TβRIII ectodomain shedding and soluble TβRIII levels could be beneficial in reducing the pro-tumorigenic effects of TGF-β in established cancers. Indeed, receptor trap molecules based partially on soluble TβRIII have been described (Verona et al., 2008) and could be developed for this indication.

In addition to TGF-β ligands, TβRIII binds other TGF-β superfamily members, including multiple BMPs, and can enhance binding of these ligands to signaling receptors (Kirkbride et al., 2008). Studies using the shedding mutants defined here demonstrate that soluble TβRIII is able to sequester BMP and reduce downstream signaling and BMP-mediated biology, while Δshed-TβRIII enhances BMP-mediated signaling and biology (Gatza and Blobe, unpublished data). These studies suggest that TβRIII ectodomain shedding plays a critical role in regulating BMP signaling as well as TGF-β. Future studies will determine whether soluble and cell-surface TβRIII have differential effects on other TβRIII binding proteins, including inhibin and bFGF.

TGF-β levels are frequently elevated in human breast cancers (Ghellal et al., 2000), and most human breast cancers become resistant to the antiproliferative effects of TGF-β, despite an intact core signaling pathway (Riggins et al., 1997). These data support an important role for the TGF-β signaling pathway in mammary carcinogenesis. Accordingly, there are currently several

strategies being explored for targeting TGF-β signaling in breast cancer patients (Connolly et al., 2012). However, the highly contextual nature and dichotomous functions of TGF-β signaling during breast cancer progression suggest that further definition of this pathway is required to safely and effectively target the TGF-β signaling pathway. As we have demonstrated that there is increased TGF-β responsiveness when ectodomain shedding is inhibited, our work suggests that anti-TGF-β therapies may be more effective on tumors with a low ratio of soluble/cell-surface TβRIII. Understanding the distinct roles of soluble and membrane-bound TβRIII and how they interact to regulate TGF-β-mediated signaling and biology at different ratios will be useful when considering using TGF-β-targeted therapies for cancer and other diseases. The work presented here contributes to this understanding by defining differential effects of a low vs. high ratio of soluble TβRIII/cell-surface TβRIII on TGF-β signaling and TGF-β-mediated migration and invasion in breast cancer cells.

Acknowledgements

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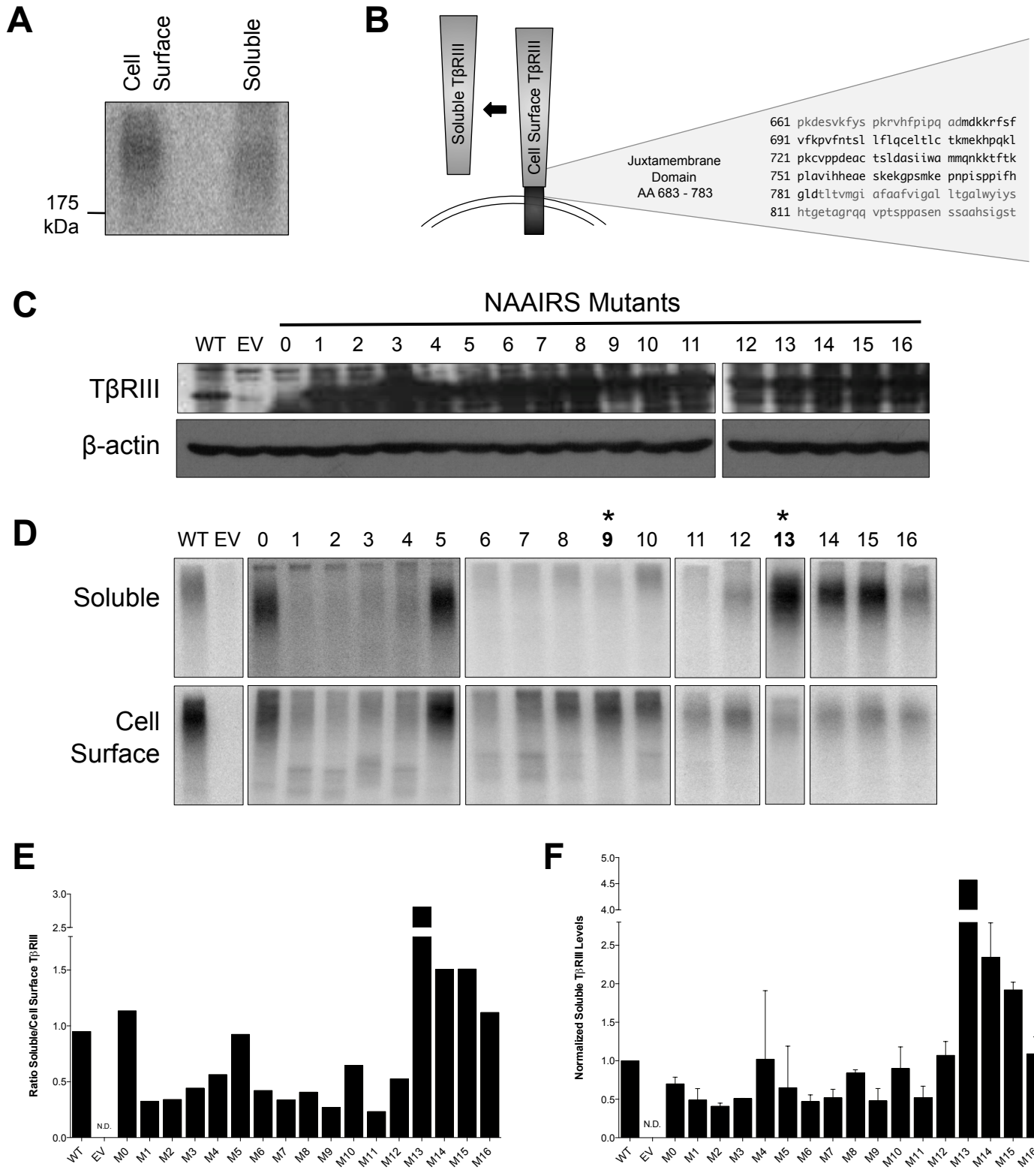

Figure 1: *Mutations in the juxtamembrane domain of TβRIII alter ectodomain shedding.* (A) Binding and crosslinking of transiently transfected HA tagged WT-TβRIII in COS7 cells. Following ^{125}I-TGF-β1 binding and crosslinking, cell lysate and conditioned media were immunoprecipitated with an antibody against HA. (B) Schematic of TβRIII with the amino acid sequence of the juxtamembrane domain. (C) Western blot of WT-TβRIII and indicated NAAIRS mutants transiently transfected in COS7 cells. EV, empty vector. β-actin was used as a loading control. (D) Binding and crosslinking of COS7 cells transiently transfected with the indicated constructs. Cells were grown in full growth media for 20 h. Following ^{125}I-TGF-β1 binding and crosslinking, cell lysates and conditioned media were immunoprecipitated with an antibody against HA. * denotes the mutants that were further utilized in these studies. Representative images from 2 independent experiments. (E) Quantification of *(D)*. Densitometric analysis was performed in ImageJ software, and the ratio of soluble/cell-surface TβRIII was determined. (F) ELISA analysis of soluble TβRIII from COS7 cells transiently transfected with the indicated contructs. Media were conditioned for 24 h. Concentration of soluble TβRIII was determined from a standard curve. Soluble TβRIII levels were then normalized to TβRIII expression determined via western blotting from control lysates. Data are from 2 independent experiments and shown as mean ± SEM normalized to WT-TβRIII.

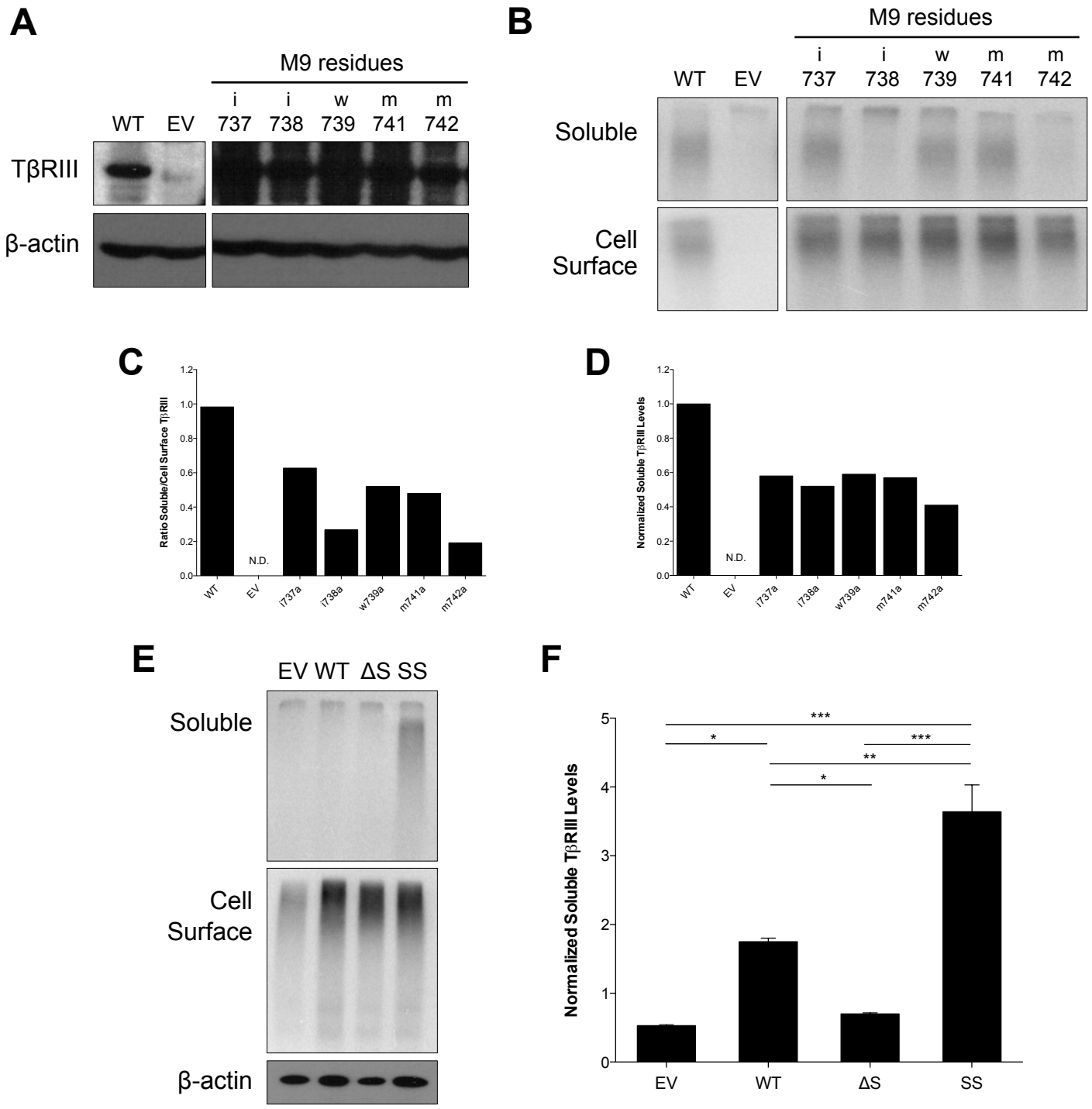

Figure 2: *A single amino acid substitution at M742 significantly inhibits ectodomain shedding.* (A) Western blot showing expressing of WT-TβRIII and M9 alanine point mutants transiently transfected in COS7 cells. β-actin was used as a loading control. (B) Binding and crosslinking of COS7 cells transiently transfected with the indicated contructs. Cells were grown in full growth media for 20 h. Following ^{125}I-TGF-β1 binding and crosslinking, cell lysates and conditioned media were immunoprecipitated with an antibody against HA. Representative images from 2 independent experiments. (C) Quantification of *(B)*. Densitometric analysis was performed in ImageJ software, and the ratio of soluble/cell-surface TβRIII was determined. (D) ELISA analysis of soluble TβRIII from COS7 cells transiently transfected with the indicated contructs. Media were conditioned for 24 h. Concentration of soluble TβRIII was determined from a standard curve. Soluble TβRIII levels were then normalized to TβRIII expression determined via western blotting from control lysates. Representative data from 2 independent experiments. (E) Binding and crosslinking of monoclonal stable lentiviral MDA-MB-231 cell lines made with EV, WT-TβRIII, Δshed-TβRIII (M9 mutant) or super-shed TβRIII (M13 mutant). Following ^{125}I-TGF-β1 binding and crosslinking, cell lysates and conditioned media were immunoprecipitated with an antibody against the extracellular domain of TβRIII. β-actin was used as a loading control. Representative data from 3 independent experiments. (F) ELISA analysis of soluble TβRIII from stable MDA-MB-231 cell lines. Media were conditioned for 24 h. Concentration of soluble TβRIII was determined from a standard curve of known amounts. Soluble TβRIII levels were then normalized to β-actin expression determined via western blotting from control lysates. Data are from 3 independent experiments and shown as mean ± SEM. One-way ANOVA $p < 0.0001$. Tukey's multiple comparisons tests: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

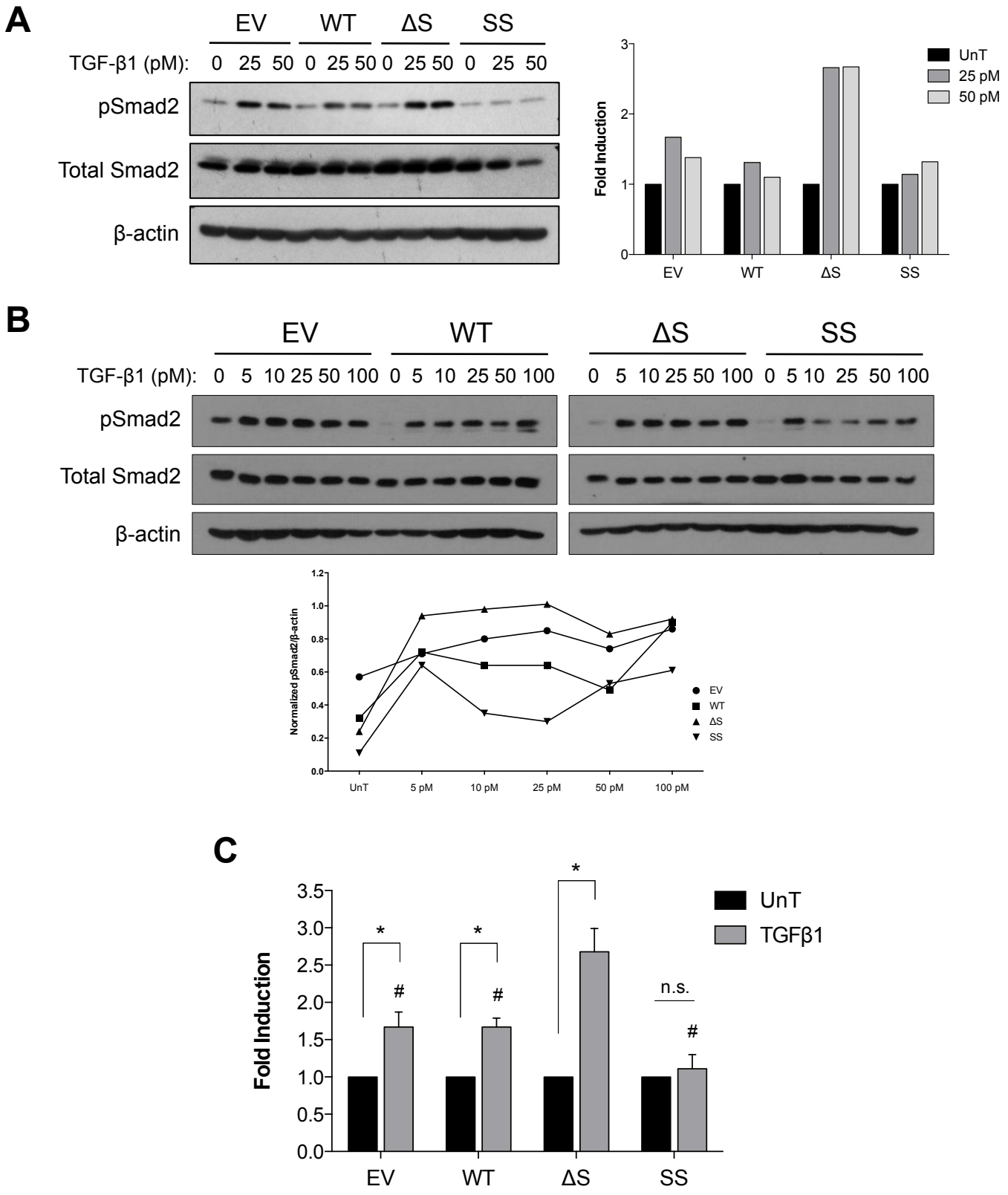

Figure 3: *Effects of altered TβRIII ectodomain shedding on TGF-β signaling.* (A,B) Lentiviral stable MDA-MB-231 cell lines expressing either EV, WT-TβRIII, Δshed-TβRIII or super-shed TβRIII were plated in full serum media and allowed to condition for 20 h before treatment with the indicated concentrations of TGF-β1 for 30 minutes. Western blot analysis was performed with the indicated antibodies. Total Smad2 and β-actin were used as loading controls. Quantification of densitometric analysis shown as levels of phosphorylated Smad2/β-actin. Data are representative of ≥ 3 independent experiments. (C) Stable MDA-MB-231 cell lines were transfected with a pE2.1 responsive luciferase construct and a renilla construct. The following day cells were treated with serum-free media that had been pre-conditioned from the corresponding cell line for 24 h and 50 pM TGF-β1. Cells were treated for 24 h. Results from 4 independent experiments are shown as pE2.1/renilla activity, and normalized to ligand untreated condition of each cell line. Two-way ANOVA $p < 0.001$. * One-sample *t*-test $p < 0.05$; # Two-tailed *t*-test $p < 0.05$ relative to ΔS + TGF-β1.

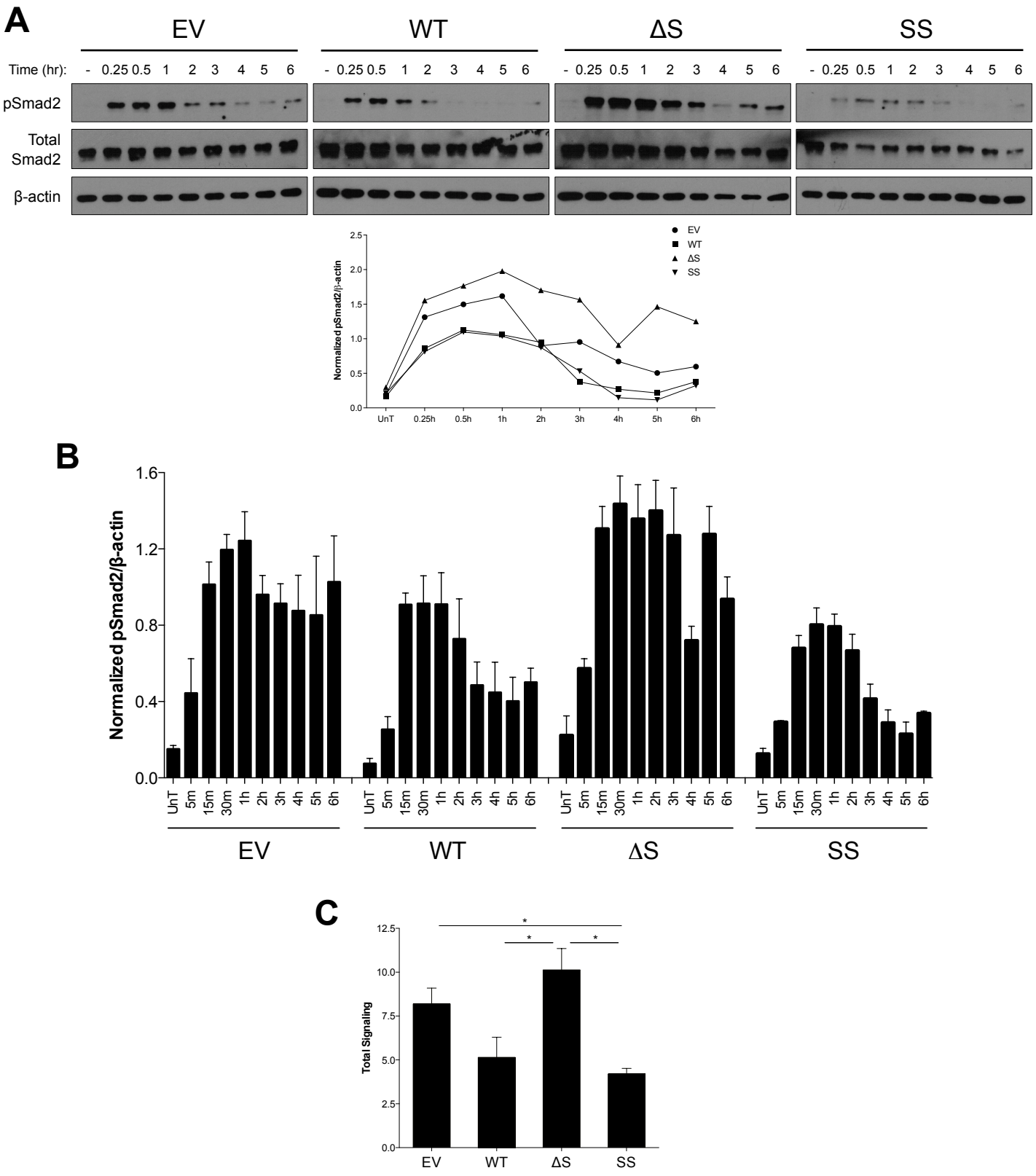

Figure 4: *TβRIII ectodomain shedding regulates the kinetics and magnitude of TGF-β signaling in MDA-MB-231 cells.* (A) Lentiviral stable MDA-MB-231 cell lines expressing either EV, WT-TβRIII, Δshed-TβRIII or super-shed TβRIII were plated in full serum media and allowed to condition for 20 h before treatment with 50 pM of TGF-β1 for the indicated times. Western blot analysis was performed with indicated antibodies. Total Smad2 and β-actin were used as loading controls. Quantification of densitometric analysis shown as levels of phosphorylated Smad2/β-actin is shown below. Representative data from 4 independent experiments. (B) Summary of time-course experiment data. Densitometric analysis of phosphorylated Smad2/β-actin. Data for ≥ 3 independent experiments for each time point shown as mean ± SEM. One-way ANOVA $p < 0.05$ for 15m, 30m, 1h, 2h, 3h, 4h, 5h, and 6h timepoints. (C) Integrated signaling over 6h time-course. Densitometric analysis of phosphorylated Smad2/β-actin of each experiment plotted as line graphs, and the area under the curve was calculated for each cell line. Data from 4 independent experiments shown as mean ± SEM. One-way ANOVA $p < 0.05$. * Two-tailed *t*-test $p < 0.05$.

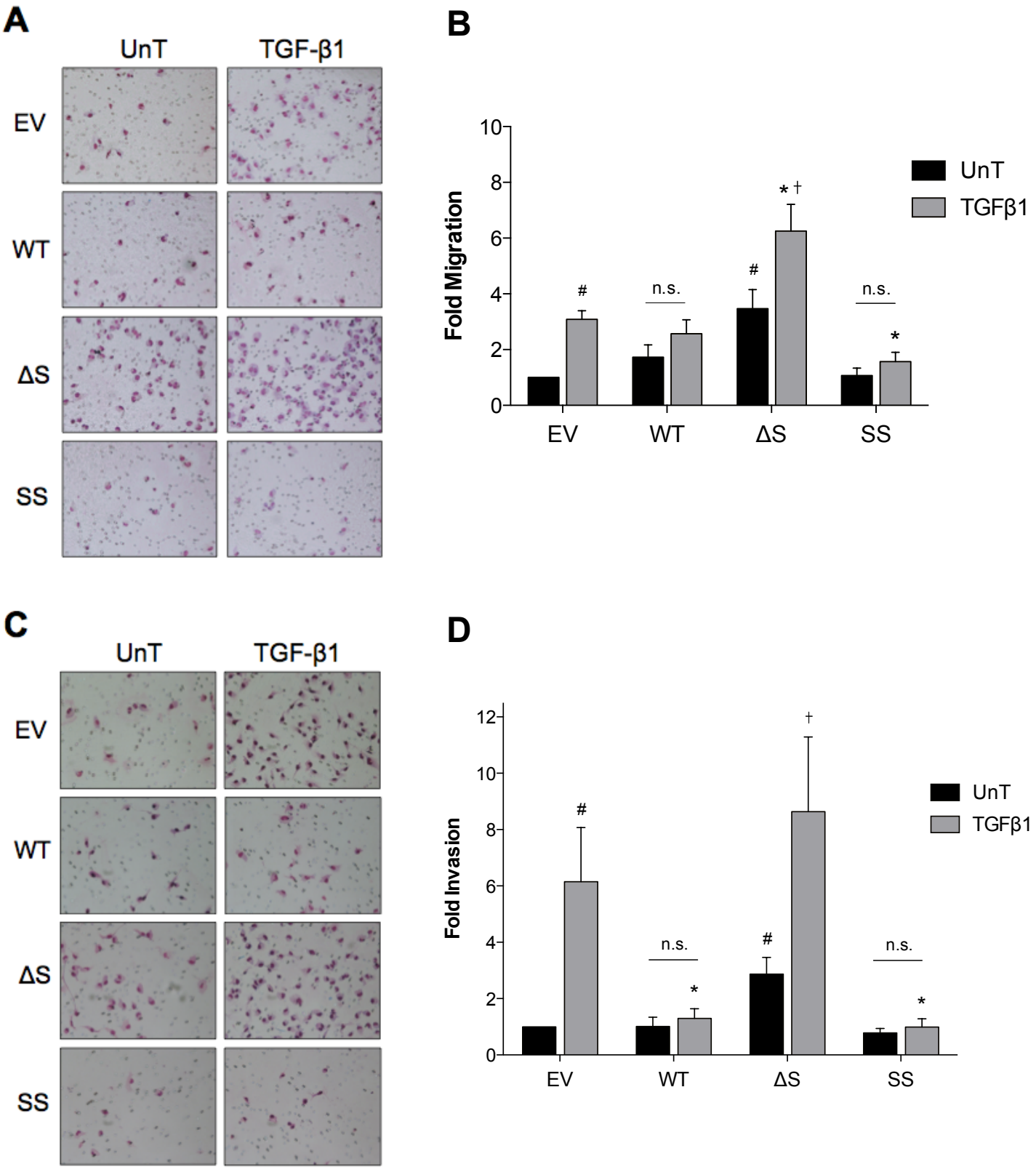

Figure 5: *Effects of altered TβRIII ectodomain shedding on TGF-β-mediated migration and invasion.* Stable MDA-MB-231 cell lines were plated in media that had been pre-conditioned from the corresponding cell line for 24 h in (A,B) fibronectin coated transwell chambers or (C,D) Matrigel-coated transwell chambers in either the absence (UnT) or presence of 50 pM TGF-β1. Cells were allowed to migrate or invade for 24 h. (A) Representative images of migrated cells. (B) Summary of n=4 experiments. Data normalized to EV UnT and shown as mean ± SEM. Two-way ANOVA for cell line and treatment $p < 0.05$. Tukey's multiple comparisons tests: # $p < 0.05$ relative to EV UnT; * $p < 0.05$ relative to EV + TGF-β1; † $p < 0.05$ relative to ΔS UnT. (C) Representative images of invaded cells. (D) Summary of n=5 experiments. Data normalized to EV UnT and shown as mean ± SEM. Two-way ANOVA for interaction, cell line and treatment $p < 0.05$. Tukey's multiple comparisons tests: # $p < 0.05$ relative to EV UnT; * $p < 0.05$ relative to EV + TGF-β1; † $p < 0.05$ relative to ΔS UnT.

Supplemental Table 1: Mutagenesis primers for TβRIII NAAIRS mutants

Mutation:	*Location:*	*Forward Primer Sequence:*	*Reverse Primer Sequence:*
M0	AA 683-688	cccgcaagctgac aatgctgctatacgatcg ctttgtcttcaagc	gcttgaagacaaag cgatcgtatagcagcatt gtcagcttgcggg
M1	AA 689-694	gataagaagcgattc aatgctgctatacgatcg gtcttcaacacc	ggtgttgaagac cgatcgtatagcagcatt gaatcgcttcttatc
M2	AA 695-700	gtcttcaagcct aatgctgctatacgatcg ctctttctacag	ctgtagaaagag cgatcgtatagcagcatt aggcttgaagac
M3	AA 701-706	caacacctcactg aatgctgctatacgatcg ctgacgctgtgtacg	cgtacacagcgtcag cgatcgtatagcagcatt cagtgaggtgttg
M4	AA 707-712	ctacagtgtgag aatgctgctatacgatcg atggagaagcacc	ggtgcttctccat cgatcgtatagcagcatt ctcacactgtag
M5	AA 713-718	gctgtgtacgaag aatgctgctatacgatcg aagttgcctaag	cttaggcaactt cgatcgtatagcagcatt cttcgtacacagc
M6	AA 719-724	gaagcacccccag aatgctgctatacgatcg cctcctgacgaagc	gcttcgtcaggagg cgatcgtatagcagcatt ctgggggtgcttc
M7	AA 725-730	cctaagtgtgtg aatgctgctatacgatcg acctcgctggac	gtccagcgaggt cgatcgtatagcagcatt cacacacttagg
M8	AA 731-736	gacgaagcctgc aatgctgctatacgatcg ataatctgggcc	ggcccagattat cgatcgtatagcagcatt gcaggcttcgtc
M9	AA 737-742	ctggacgcctcg aatgctgctatacgatcg cagaataagaag	cttcttattctg cgatcgtatagcagcatt cgaggcgtccag
M10	AA 743-748	ctgggccatgatg aatgctgctatacgatcg accaagccccttg	caaggggcttggt cgatcgtatagcagcatt catcatggcccag
M11	AA 749-754	gaagacgttc aatgctgctatacgatcg atccaccatgaagc	gcttcatggtggat cgatcgtatagcagcatt gaacgtcttc
M12	AA 755-760	ccccttgctgtg aatgctgctatacgatcg tctaaagaaaaagg	ccttttttctttaga cgatcgtatagcagcatt cacagcaagggg
M13*	AA 761-766	* Made by Mutagenex	
M14	AA 767-772	gaaaaaggtcca aatgctgctatacgatcg ccaatttctccacc	ggtggagaaattgg cgatcgtatagcagcatt tggacctttttc
M15	AA 773-778	gaaggaaccaaat aatgctgctatacgatcg ttccatggtctgg	ccagaccatggaa cgatcgtatagcagcatt atttggttccttc
M16	AA 778-783	ccaatttctccacca aatgctgctatacgatcg accctaaccgtgatg	catcacggttagggt cgatcgtatagcagcatt tggtggagaaattgg

Supplemental Table 2: Mutagenesis primers for TβRIII alanine mutants

Mutation:	*Forward Primer Sequence:*	*Reverse Primer Sequence:*
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s699a	gtcttcaacacc gcc ctgctctttctac	gtagaaagagcag ggc ggtgttgaagac
l700a	cttcaacacctca gcc ctctttctacag	ctgtagaaagag ggc tgaggtgttgaag
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w739a	gcctcgataatc gcc gccatgatgcag	ctgcatcatggc ggc gattatcgaggc
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m742a	ctgggccatg gcc cagaataagaag	cttcttattctg ggc catggcccag
t749a	gaagacgttc gcc aagccccttgc	gcaaggggctt ggc gaacgtcttc
k750a	gaagacgttcacc gcc ccccttgctgtg	cacagcaagggg ggc ggtgaacgtcttc
p751a	cgttcaccaag gcc cttgctgtgatc	gatcacagcaag ggc cttggtgaacg
l752a	caccaagccc gcc gctgtgatccac	gtggatcacagc ggc gggcttggtg
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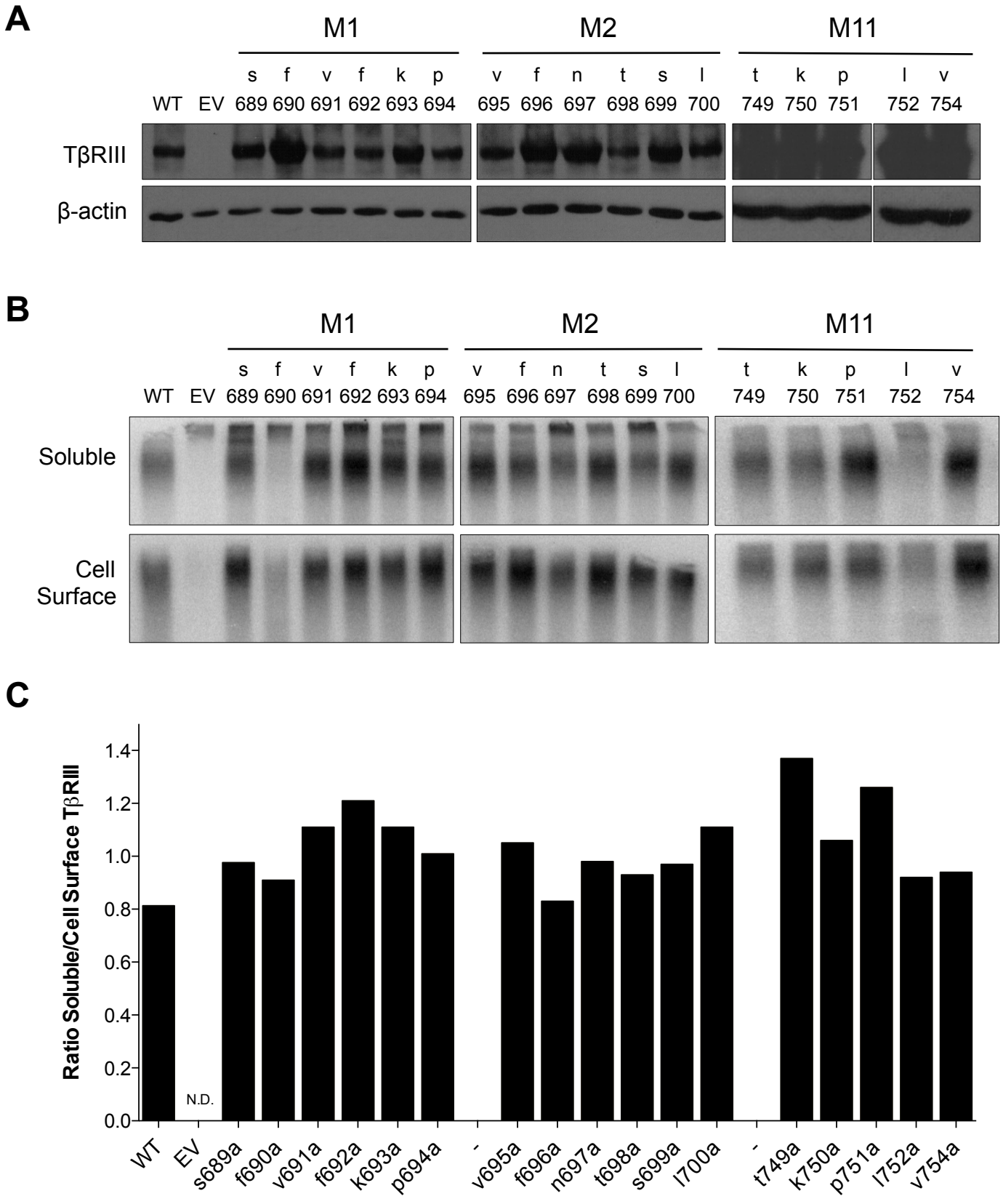
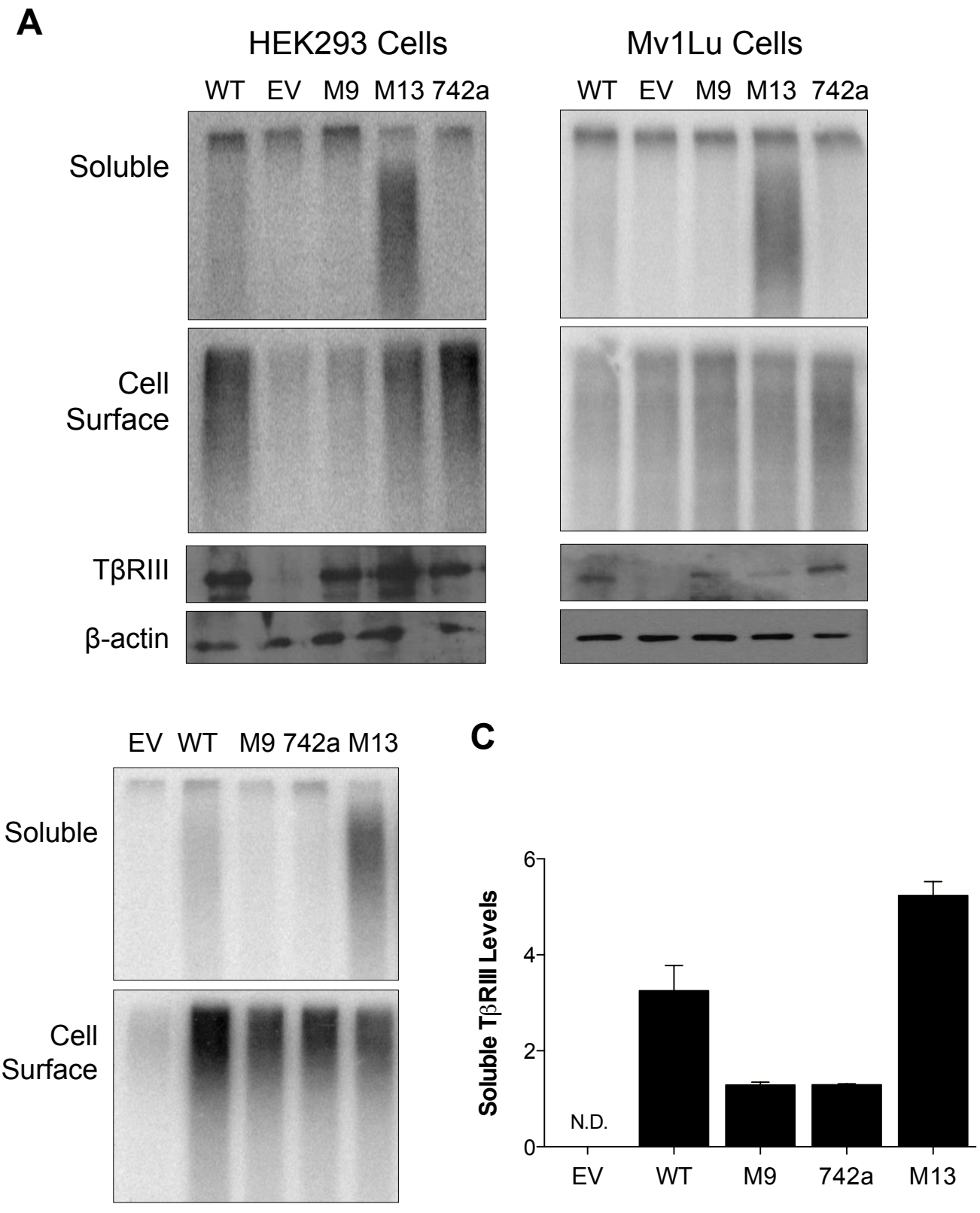
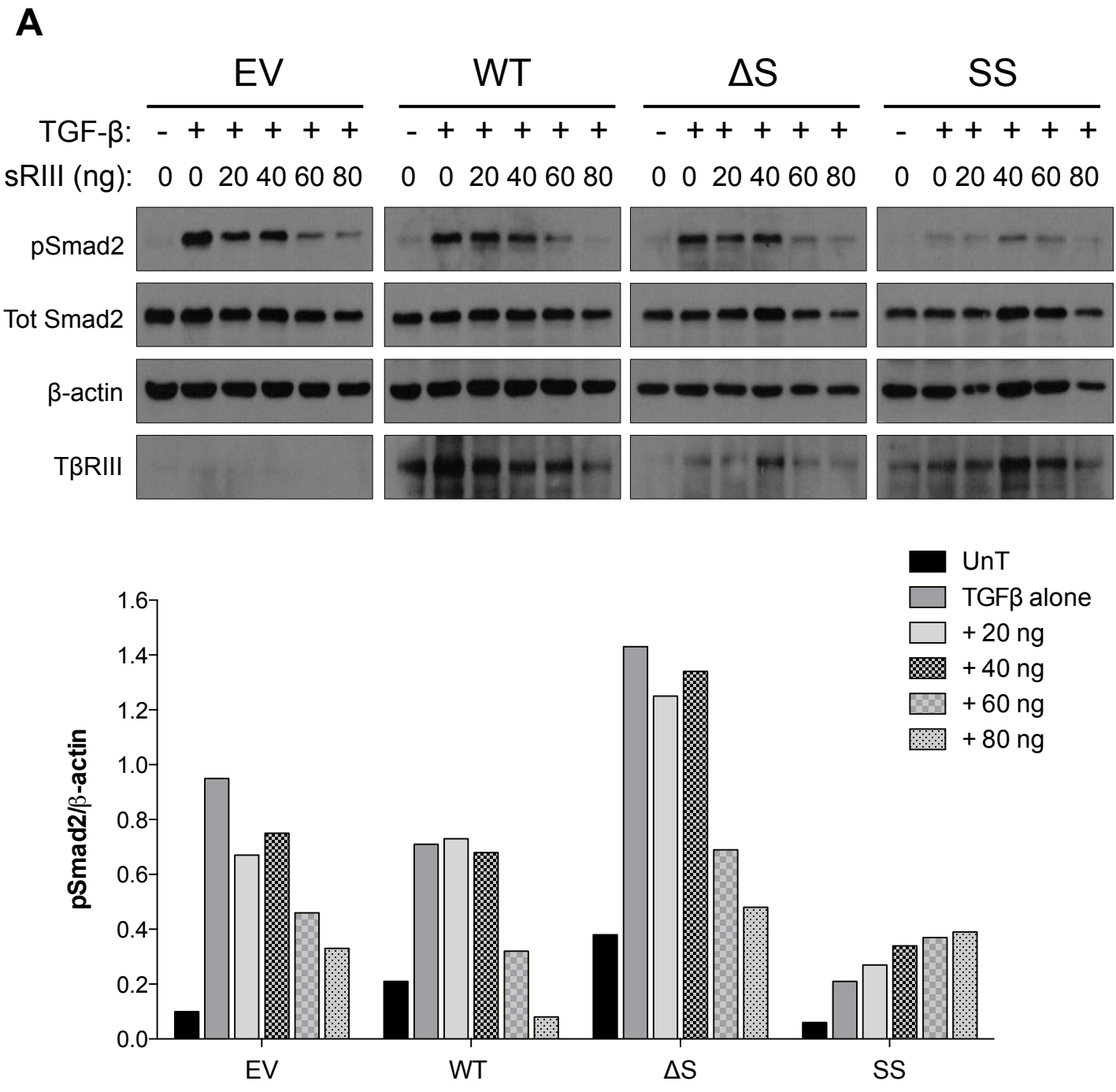


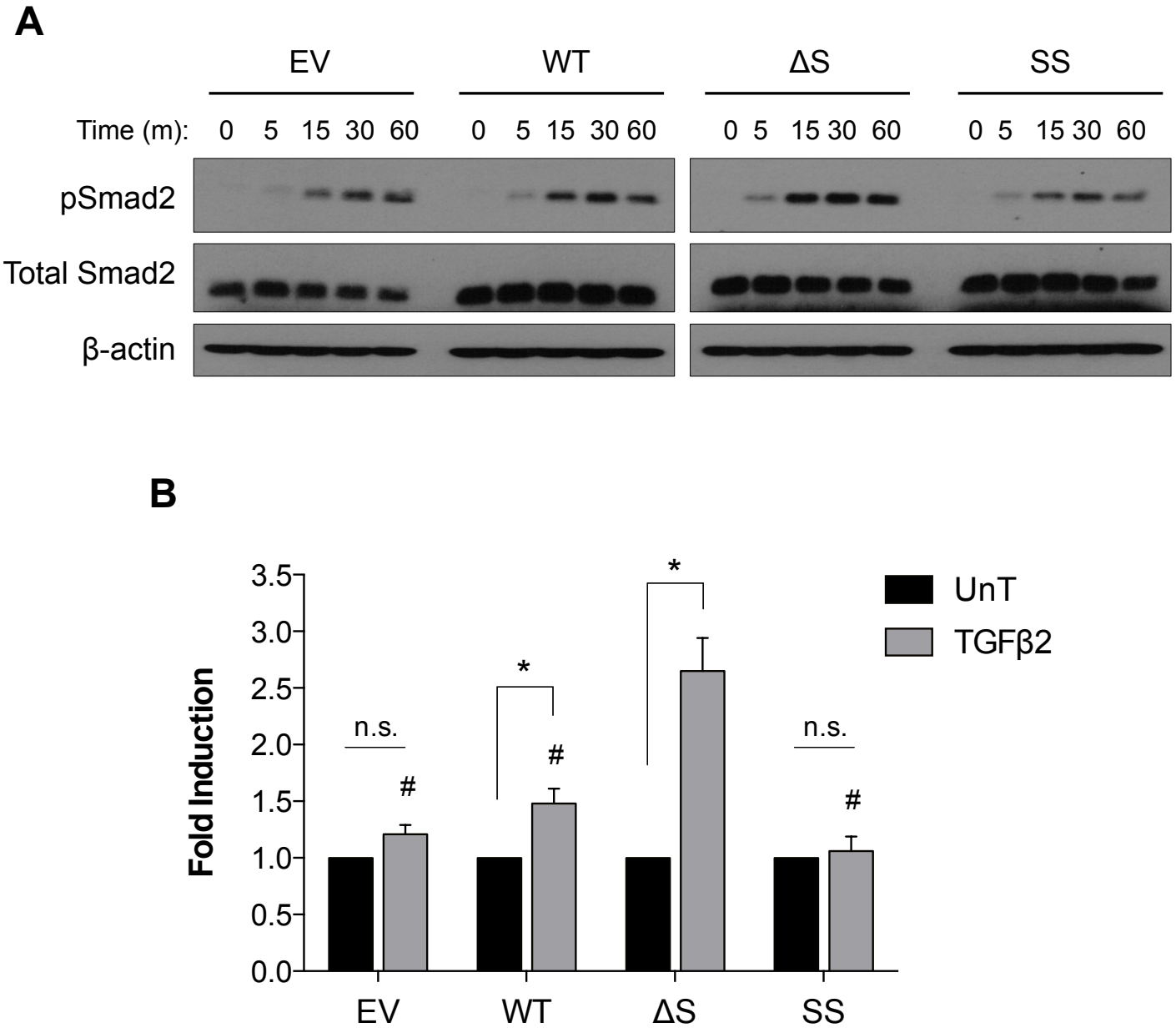
Supplemental Figure 1: *No single point mutations within M1, M2 or M11 significantly altered TβRIII ectodomain shedding.* (A) Western blot of transiently transfected WT-TβRIII or alanine mutants in COS7 cells. β-actin was used as a loading control. (B) Binding and crosslinking of transiently transfected WT-TβRIII or alanine mutants in COS7 cells. 24 h after transfection media was changed to full serum media and allowed to condition overnight. Following ^{125}I-TGF-β1 binding and crosslinking, lysates and conditioned media were immunoprecipitated with an antibody against HA. (C) Quantification of *(B)*. Data shown as densitometry of soluble TβRIII/cell surface TβRIII. Representative image from 2 independent experiments.



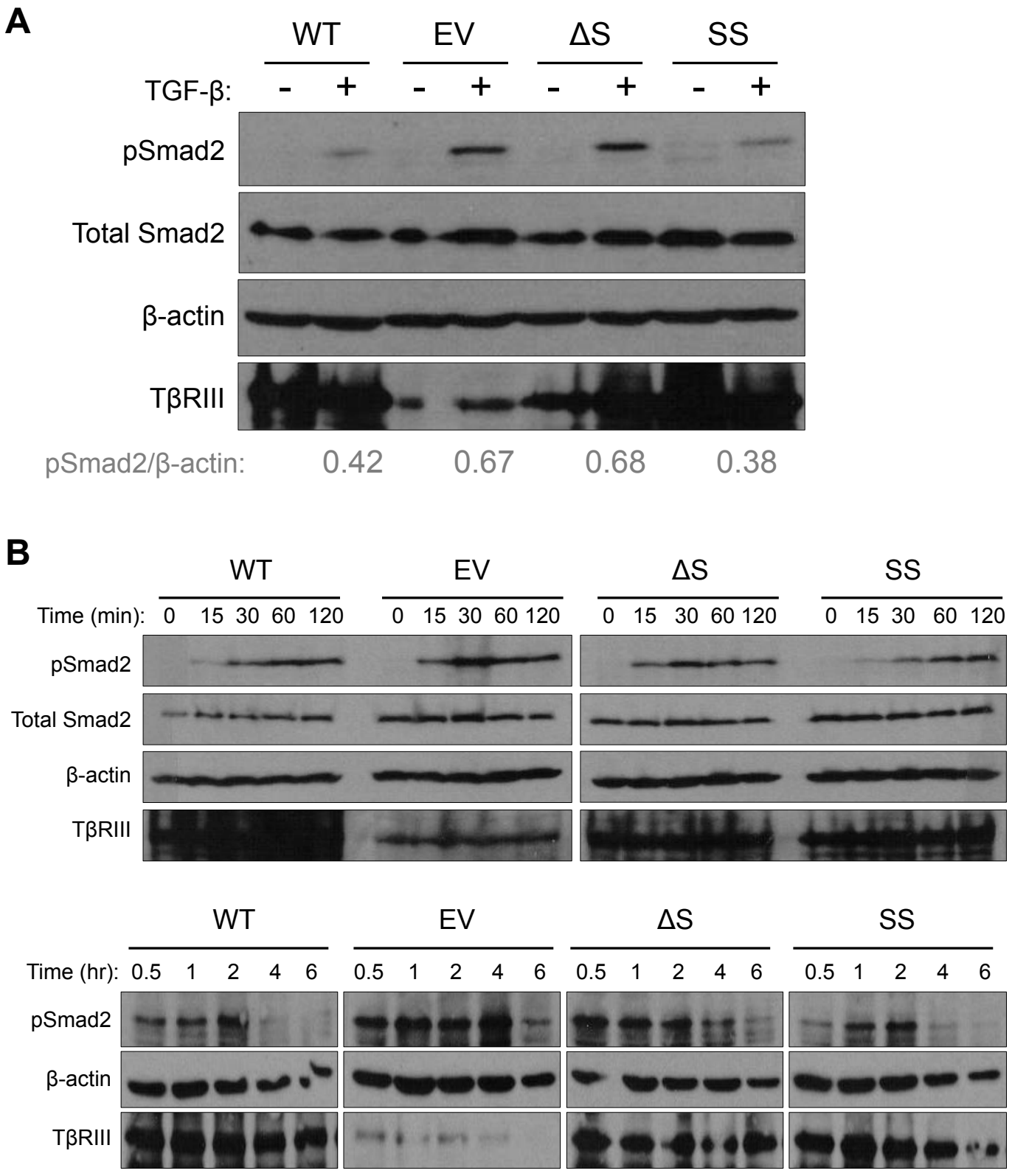
Supplemental Figure 2: *Alterations in TβRIII ectodomain shedding in shedding mutants is maintained in multiple cell lines.* (A) Binding and crosslinking of HEK293 and Mv1Lu cells. Cells were transiently transfected with EV, WT-TβRIII or shedding mutants and 24 h later media was replaced and allowed to condition for 20 h. Following ^{125}I-TGF-β1 binding and crosslinking, lysates and conditioned media were immunoprecipitated with an antibody against TβRIII. TβRIII and β-actin western blots shown as loading controls. Representative images from 2 independent experiments. (B) Binding and crosslinking of final shedding mutants in COS7 cells, performed as in *(A)*. Representative images from 2 independent experiments. (C) ELISA data from media conditioned from transiently transfected COS7 cells for 20 h. Data from 3 independent experiments are shown as levels of soluble TβRIII in the conditioned media normalized to β-actin loading controls from the corresponding cell lysates.



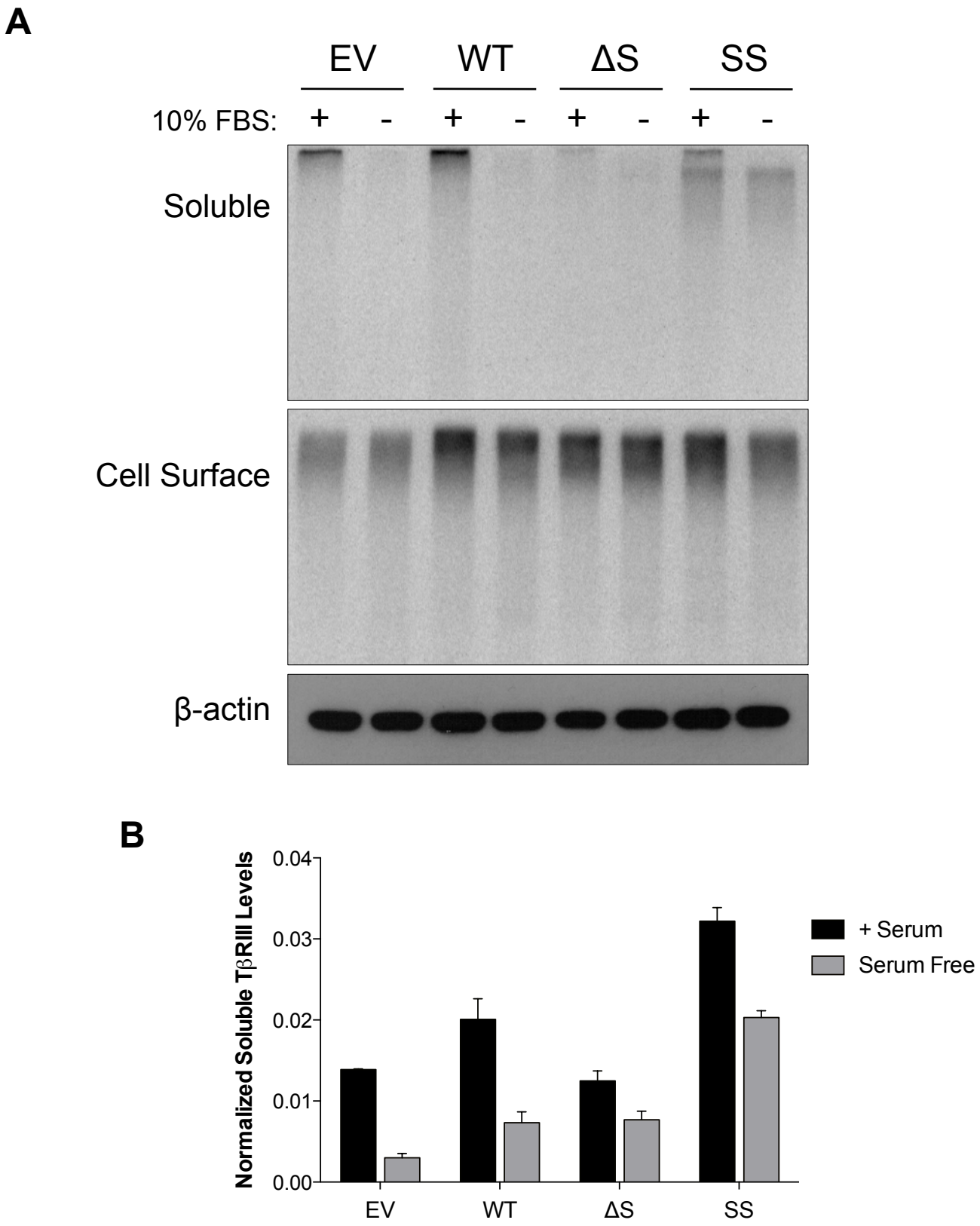
Supplemental Figure 3: *TGF-β signaling is reduced by exogenous soluble TβRIII.* (A) Lentiviral stable MDA-MB-231 cell lines were plated in full serum media and allowed to condition for 20 h before treatment with 50 pM TGF-β1 and indicated amounts of exogenous soluble TβRIII for 30 minutes. Western blot analysis performed with indicated antibodies. TβRIII and β-actin western blots shown as loading controls. Representative image from 2 independent experiments. Densitometric analysis of pSmad2/β-actin control shown below.



Supplemental Figure 4: *WT-TβRIII and ΔS-TβRIII sensitize MDA-MB-231 cells to TGF-β2.* (A) Lentiviral stable MDA-MB-231 cell lines were plated in full serum media and allowed to condition for 20 h before treatment with 50 pM TGF-β2 for the indicated time periods. Western blot analysis performed with indicated antibodies. Representative images from 2 independent experiments. (B) Stable MDA-MB-231 cell lines were transfected with a pE2.1 responsive luciferase construct and a renilla construct. The following day cells were treated with media that had been pre-conditioned from the corresponding cell line for 24 h and 50 pM TGF-β2. Cells were treated for 24 h. Results from 4 independent experiments are shown as pE2.1/renilla activity, and normalized to ligand untreated condition of each cell line. * One-sample t-test $p < 0.05$ relative to UnT. # Two-tailed *t*-test $p < 0.05$ relative to ΔS + TGF-β2.



Supplemental Figure 5: *TβRIII ectodomain shedding regulates the kinetics and magnitude of TGF-β signaling in HEK293 cells.* HEK293 cells were transiently transfected with EV, WT-TβRIII or shedding mutants. Following overnight serum starvation, cells were treated with (A) 50 pM TGF-β1 for 30 minutes or (B) 50 pM TGF-β1 for indicated time period. Western blot analysis performed with indicated antibodies. TβRIII and β-actin western blots shown as loading controls. Representative images from 3 independent experiments shown.



Supplemental Figure 6: *TβRIII ectodomain shedding is affected by the presence of serum in MDA-MB-231 cells.* (A) Binding and crosslinking of lentiviral stable MDA-MB-231 cell lines expressing either EV, WT-TβRIII or the shedding mutants. Cells were plated and the following day media was changed to either full serum media (10% FBS) or serum-free media and allowed to condition for 24 h. Following ^{125}I-TGF-β1 binding and crosslinking lysates and conditioned media were immunoprecipitated with an antibody against TβRIII. β-actin western blot shown as a loading control. Representative images from 2 independent experiments shown. (B) ELISA data from media conditioned from lentiviral stable cell lines for 24 h in the absence or presence of 10% FBS. Data from 3 independent experiments are shown as levels of soluble TβRIII in the conditioned media normalized to β-actin loading controls from the corresponding cell lysates. Two-way ANOVA for cell line and treatment $p < 0.001$.

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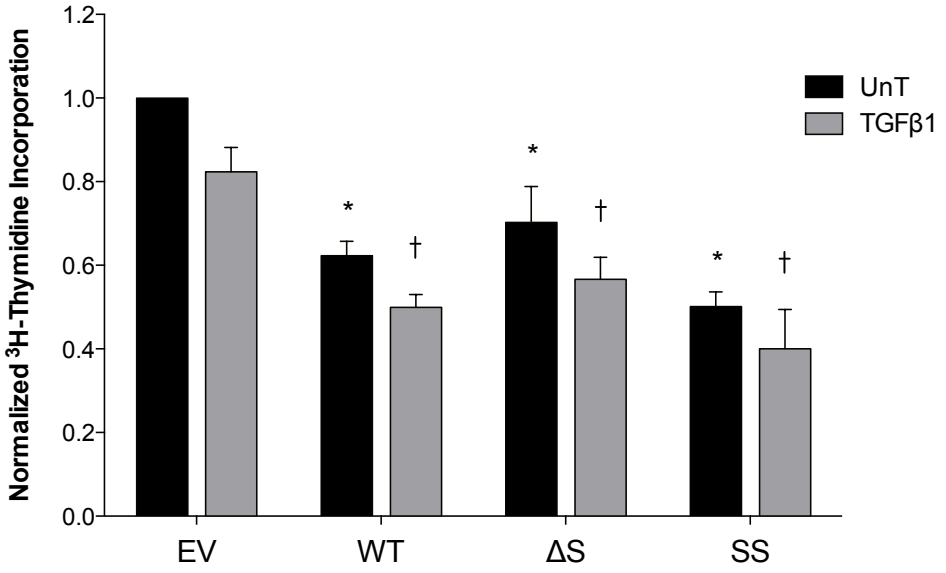

Supplemental Figure 7: *Effects of TβRIII and shedding mutants on proliferation at 24 hours.* (A) MDA-MB-231 lentiviral stable cell lines expressing either EV, WT-TβRIII or the shedding mutants were plated in media pre-conditioned for 24 h from corresponding cells in 96 well plates in triplicate in the absence or presence of 50 pM TGF-β1. 24 hours later proliferation was determined via ^{3}H-Thymidine incorporation. Data from 4 independent experiments are shown as CPM normalized to EV UnT. Two-way ANOVA for cell line $p < 0.002$. * One-sample *t*-test $p < 0.05$ relative to EV UnT. † Two-tailed *t*-test $p < 0.05$ relative to EV + TGF-β1.

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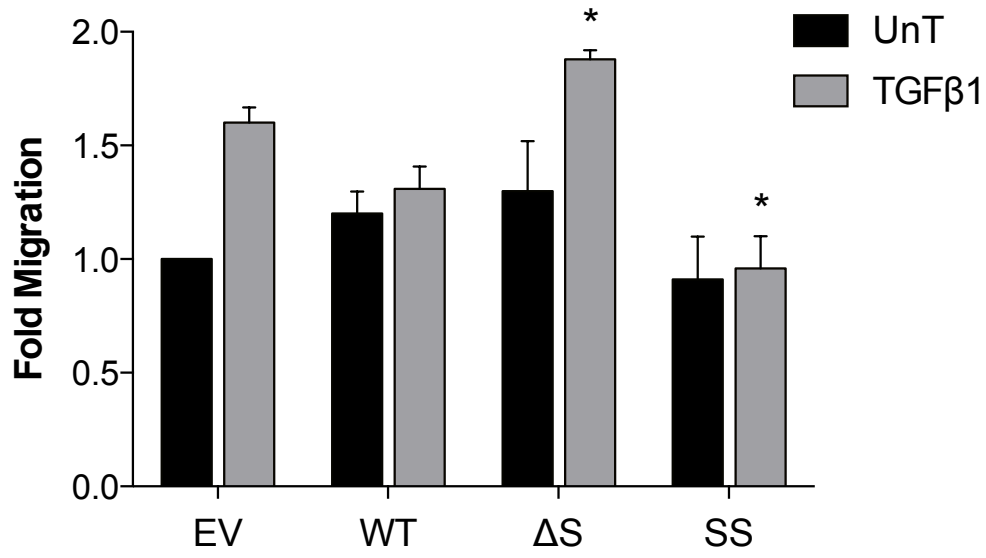

Supplemental Figure 8: *TβRIII ectodomain shedding regulates TGF-β-mediated migration in HMECs.* (A) Human Mammary Epithelial Cells (HMECs) transiently transfected with EV, WT-TβRIII, or the shedding mutants were plated onto fibronectin-coated transwells and allowed to migrate for 24 h in the absence or presence of 100 pM TGF-β1. Data from 3 independent experiments are shown as number of migrated cells normalized to EV UnT. * Two-tailed *t*-test $p < 0.05$ relative to EV + TGF-β1.