INTRODUCTION

The TGFβ (transforming growth factor β) signalling pathway is necessary for the normal functioning of a variety of cells, its cell-type-specific responses often mediate growth inhibition, extracellular-matrix synthesis and cell migration. Whereas this signalling pathway is crucial for normal development, it also plays a much more sinister role in a number of pathologies, including cancer. There are three prototypical TGFβ receptors: TβRI (TβR type I), TGFβR2 (TβR type II) and TGFβRIII (TβR type III), also known as betaglycan). Whereas the role of TβRII and TβRI in TGFβ signal propagation has been established, the contribution of TGFβRIII to TGFβ signalling is less well understood. At the cell surface, TβRI and TβRII receptors can be internalized by clathrin-mediated endocytosis and clathrin-independent membrane-raft-dependent endocytosis. Interestingly, the endocytic route of the receptors plays a direct role in TGFβ-dependent Smad signal transduction; receptors endocytosed via clathrin-mediated endocytosis activate Smad signalling, whereas receptors endocytosed via membrane rafts are targeted for degradation. The objective of the present study was to evaluate the contribution of TGFβRIII to TGFβRI membrane partitioning, receptor half-life and signalling.

Using sucrose-density ultracentrifugation to isolate membrane-raft fractions, we show that TβRIII recruits both TβRII and TβRI to non-raft membrane fractions. Immunofluorescence microscopy analysis demonstrated that overexpression of TβRIII affects intracellular trafficking of TGFβRII by recruiting TβRII to EEA1 (early endosome antigen 1)- and Rab5-positive early endosomes. Using 125I-labelled TGFβ1 to follow cell-surface receptor degradation we show that overexpression of TβRIII also extends the receptor half-life of the TβRII–TβRII complex. Interestingly, we also show, using a luciferase reporter assay, that TβRIII increases basal TGFβ signalling. As numerous pathologies show aberrant activation of TGFβ signalling, the present study illustrates that TβRIII may represent a novel therapeutic target.

Key words: clathrin-mediated endocytosis, immunofluorescence microscopy, intracellular trafficking, membrane raft, subcellular fractionation, transforming growth factor β receptor (TGFβR).
Recently we have shown that the extracellular domain of TβRII is necessary for entrance into membrane-raft domains [13]. Furthermore, we have shown that the glycosylation state of the cell, though not of TβRII itself, mediates TβRII endocytosis [13]. This suggests that TβRII may interact with glycosylated protein(s) at the cell surface to direct receptor partitioning.

Although many studies have evaluated the contribution of the TβRII and TβRII to Smad signalling, few have investigated the role of TβRIII. TβRIII is a highly glycosylated proteoglycan with a large extracellular domain [14,15]. TβRIII was thought previously to simply function in presentation of ligand to TβRII [16]; however, several recent studies illustrate that TβRIII may play a crucial role in TGFβ-dependent cancer metastasis. Expression levels of TβRIII have been correlated with a number of cancers, including prostate cancer [17], ovarian cancer [18], granulosa tumours [19] and non-small cell lung adenocarcinomas [20]. In some instances, TβRIII overexpression appears to contribute to cancer cell motility and invasion [21], whereas in others knockdown of TβRIII increases tumour cell metastasis [22]. Furthermore, TβRIII endocytosis has been implicated in the activation of TGFβ signalling [23]. These studies highlight the fact that TβRIII may play a crucial role in TGFβ signalling, particularly in cancer.

The objective of the present study is to evaluate the role of TβRIII in TGFβ endocytosis and degradation. Using immunofluorescence microscopy and sucrose-density ultracentrifugation, we show that TβRIII directs TβRII and TβRI to undergo clathrin-mediated endocytosis. TβRIII also increases trafficking of TβRII into early endosomal compartments. Furthermore, this re-directed trafficking increases the half-life of the TβRII–TβRI complex and basal TGFβ signalling levels.

**EXPERIMENTAL**

**Antibodies**

Primary monoclonal anti-Myc (Santa Cruz Biotechnology; catalogue no. sc-40), anti-FLAG (Sigma–Aldrich; catalogue no. F3165) and anti-EA1 (early endosome antigen-1) (BD Transduction Laboratories; catalogue no. 610457) antibodies, and primary polyclonal anti-HA (haemagglutinin) (Santa Cruz Biotechnology; catalogue no. sc-805) and anti-caveolin-1 (BD Transduction Laboratories; catalogue no. 610060) antibodies were used for protein detection. Secondary HRP (horseradish peroxidase)-conjugated goat anti-(mouse Ig) (eBioscience; catalogue no. 18-8817) and goat anti-(rabbit Ig) (eBioscience; catalogue no. 18-8816) were used for immunoblotting experiments. Fluorescently conjugated anti-EA1–FITC (BD Transduction Laboratories; catalogue no. 612007), goat anti-mouse Ig)-Cy5 (indodicarbocyanine) (Jackson ImmunoResearch; catalogue no. 115-175-205) and goat anti-(rabbit Ig)–Cy3 (indocarbocyanine) (Jackson ImmunoResearch, catalogue no. 711-165-152) antibodies were used for immunofluorescence studies. All antibodies were used according to the manufacturers’ instructions.

**Cell culture**

HEK-293T cells [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] were maintained in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10% (v/v) FBS (fetal bovine serum). Mv1Lu (Mink lung) cells stably transfected with HA-tagged TβRII (Mv1Lu HAT cells) were cultured in MEM (minimal essential medium) supplemented with non-essential amino acids, 10% (v/v) FBS and 0.3% hygromycin. All cells were maintained at 37°C in a 5% CO₂ atmosphere.

**Constitutively active receptor constructs**

Constructs encoding the C-terminally HA-tagged TβRII (TβRII–HA), the intracellularly truncated receptor (TβRII–Δcyt) and an extracellularly truncated receptor (TβRII–ΔEX) were previously described [13,24]. The GFP (green fluorescent protein)-tagged wild-type Rab5, Rab5–Q79L and Rab5–S34N constructs were used as described previously [25].

**Transfection**

HEK-293T cells were transiently transfected by calcium phosphate precipitation. Mv1Lu HAT cells were transiently transfected using PEI (polyethyleneimine).

**Isolation of caveolae- and membrane-raft-enriched membrane fractions**

Membrane rafts were isolated as described previously [12,13]. Briefly, transfected HEK-293T cells were grown to confluence in 100-mm-diameter dishes. Cells were washed twice with cold 1× PBS and lysed in 0.5 M NaCl, pH 11.0, containing protease inhibitors. After the cells were scraped, the cell lysate was homogenized with three 10 s bursts using a Polytron tissue homogenizer (Brinkmann Instruments). Cells were then sonicated three times for 20 s each time with a Vibra Cell sonicator (Sonic and Materials). The homogenates were then adjusted to 40% (w/v) sucrose, and overlaid with 30% (w/v) sucrose and 5% (w/v) sucrose solutions. The samples were centrifuged for 16 h at 40000 rev./min at 4°C using a Beckman SW41 rotor. Following centrifugation, 12 aliquots (1 ml samples) were collected and an aliquot of each sample was denatured with Laemmli sample buffer, boiled and subjected to SDS/PAGE (10% gels) followed by immunoblotting.

**Immunoblotting**

Proteins were separated by SDS/PAGE (10% gels) and transferred on to nitrocellulose membranes by electrophoretic transfer. Blots were incubated for 1 h in 5% (w/v) low-fat milk powder/TBST (Tris-buffered saline with 0.1% Tween 20). After incubation with primary and secondary antibodies, bound antibodies were detected using SuperSignal chemiluminescence reagent (Pierce) and a VersaDoc imager (Bio-Rad Laboratories).

**Immunoprecipitation**

HEK-293T cells transiently transfected with cDNA were lysed in TNTE buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF and protease inhibitors) and centrifuged at 14000 g for 10 min at 4°C. A protein assay was conducted on total cell lysates for analysis of protein concentration. The remaining cell lysates were then incubated with 1 μg of anti-HA primary antibody for 16 h at 4°C followed by incubation with Protein-G–Sepharose beads for 2 h at 4°C. The precipitates were washed three times with the lysis buffer containing 0.1% Triton X-100, eluted with Laemmli sample buffer and subjected to SDS/PAGE (10% gels) and immunoblotting.

**Affinity labelling**

Transiently transfected HEK-293T cells were labelled for 2 h with 250 pM [125I]TGFβ1 ligand in 0.5% BSA/KRH (Krebs-Ringer Hepes) solution at 4°C. Cells were cross-linked to ligand

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Luciferase reporter assay

HepG2 cells were transiently transfected using the calcium phosphate precipitation method with ARE (activin-response element)–Luc (luciferase), β-galactosidase and FoxH1 (forkhead box H1) reporter plasmids or with TβRI, TβRII and/or increasing concentrations of TβRIII. Cells were serum-starved in 0.2% FBS/DMEM for 4 h prior to treatment. Cells were incubated in the presence or absence of 100 pmol of TGFβ for 16 h. Luciferase activity was normalized to β-galactosidase activity prior to analysis.

RESULTS

TβRIII is concentrated in non-raft membrane fractions

Our previous work has shown that the extracellular domain of TβRI is necessary for membrane-raft partitioning [13]. Deletion of the extracellular domain decreases the endocytosis of TβRII via membrane-raft-dependent mechanisms [13]. As both TβRI and TβRII interact with TβRIII, we first sought to identify the membrane localization of the three TβR subtypes.

To evaluate the membrane localization of the TβRs, membrane-raft fractions were isolated using sucrose-density ultracentrifugation as described previously [12, 13]. Briefly, HEK-293T cells were transiently transfected with Myc–TβRII, TβRII–HA or TβRII–FLAG cDNA. Cells were lysed in 1 M Na2CO3 with protease inhibitors, homogenized and sonicated, then overlaid with a sucrose gradient. Following overnight high-speed ultracentrifugation, fractions were collected and subjected to SDS/PAGE. Immunoblotting for endogenous caveolin-1, a marker of membrane rafts, was performed to ensure membrane-raft isolation. As shown in Figure 1(A), membrane rafts were concentrated in fractions 4–6. Membrane-raft and non-raft fractions were pooled, adjusted to the same volume and subjected to SDS/PAGE as shown in Figure 1B. Interestingly, we observed that TβRII and TβRII largely partition in membrane-raft fractions, with approx. 70% of TβRII and 75% of TβRII found in membrane-raft fractions (Figure 1B). However, TβRIII was more heavily concentrated in non-raft fractions with only approx. 30% of receptors found in membrane-raft fractions (Figure 1B).

We also evaluated the partitioning of endogenous TβRII and TβRII in HepG2 cells by sucrose-density ultracentrifugation (Supplementary Figure S1A at http://www.BiochemJ.org/bj/429/bj4290137add.htm). We first assessed the membrane-raft content of HepG2 cells by immunoblotting collected fractions for caveolin-1 (Supplementary Figure S1A). To visualize endogenous receptors, we used [125I]TGFβ1 ligand to label cell-surface receptors, subjected lysates to sucrose-density ultracentrifugation and performed autoradiography. Similar to previous results in MvILU cells [12], endogenous TβRII was found primarily concentrated in membrane-raft fractions (Supplementary Figure S1B), as in our overexpression studies. TβRI is found in both membrane-raft and non-raft fractions, with slightly more receptors found in non-raft fractions (Supplementary Figure 1B). Although we found more TβRI in membrane-raft fractions in our overexpression studies (Figure 1B), this difference may be due to the relative levels of TβRI and TβRII in HepG2 cells.

TβRIII forms a stable interaction with TβRII in the presence and absence of ligand

Having found that TβRIII was more concentrated in non-raft fractions, we sought to evaluate whether TβRIII could alter the partitioning of TβRII. We initially wanted to assess the ability of TβRIII to form a stable interaction with TβRII, as previous studies had simply suggested that the role of TβRIII was to present TGFβ ligand to the receptor. To address this question, we used a co-immunoprecipitation approach in HEK-293T cells transiently transfected with TβRII–HA, Myc–TβRIII and TβRII–FLAG cDNA (Figure 2A). Following transfection, cells were serum-starved and then either treated with 500 pmol of TGFβ for 1 h or left untreated. Immunoprecipitation of cell lysates with an anti-HA antibody and subsequent immunoblotting with an anti-FLAG antibody (left-hand panel), receptors levels were quantified using QuantityOne software (right-hand panel). Results are means ± S.D. (n = 3). R, raft fractions; NR, non-raft fractions.

Figure 1 Membrane partitioning of TβRs

(A) HEK-293T cells transiently expressing Myc–TβRIII, TβRII–HA or TβRII–FLAG were subjected to sucrose-density ultracentrifugation as described in the Experimental section. Fractions 1–12 were then collected and immunoblotted using anti-Myc, anti-HA and anti-FLAG antibodies as indicated. Fractions were also immunoblotted for endogenous caveolin-1 (α-cav1), a marker for membrane rafts. (B) Quantification of TβR membrane partitioning. Fractions 4–6 (membrane raft) and 8–12 (non-raft) from each condition were pooled, adjusted to the same volume and subjected to SDS/PAGE. Following immunoblotting with anti-Myc, anti-HA or anti-FLAG antibodies (left-hand panel), receptors levels were quantified using QuantityOne software (right-hand panel). Results are means ± S.D. (n = 3). R, raft fractions; NR, non-raft fractions.
illustrate that the interaction of TβRII with TβRIII increases the proportion of TβRII found in non-membrane-raft fractions. Quantification of membrane-raft partitioning was performed by pooling raft and non-raft fractions and performing SDS/PAGE (Figure 2B) followed by analysis of this differential partitioning using Quantity One software. As shown in the histogram in Figure 2(D), upon co-expression with TβRIII, 72% of TβRII was found in non-membrane-raft fractions, compared with 33% in the absence of TβRIII. This suggests that the association of TβRII with TβRIII increases the endocytosis of TβRII by clathrin-mediated mechanisms. As clathrin-dependent endocytosis promotes TGFβ signalling, TβRIII expression may increase downstream signalling events.

TβRIII alters the endocytosis of a cytosolic-truncation mutant TβRI

We have shown previously that the cytosolic-truncation mutant of TβRII, TβRII-D cyt, is found nearly exclusively in membrane-raft fractions [13]. To assess whether TβRIII can also re-direct the partitioning of TβRII-D cyt, we first performed a co-immunoprecipitation experiment to assess the interaction of TβRII with HA-tagged TβRII-D cyt and TβRII-D EX, which lacks the extracellular domain. As TβRIII has a large glycocalyceal extracellular domain, we predicted that it would primarily interact with the extracellular domain of TβRII. We used anti-HA primary antibodies to immunoprecipitate full-length TβRII and the truncation mutants, and to evaluate their association with Myc–TβRIII. We observed that although TβRIII can interact with both truncation mutants, it forms a more robust interaction with TβRII-D cyt (Figure 3A).

To address whether TβRIII can re-direct the partitioning of TβRII-D cyt, we used sucrose-density ultracentrifugation to concentrate membrane rafts from HEK-293T cells overexpressing TβRII-D cyt and TβRII-D cyt in the presence of TβRIII (Figure 3B). Interestingly, we found that, similar to full-length TβRII, TβRIII also shifts TβRII-D cyt from membrane rafts into non-membrane-raft fractions (Figure 3B). As an internal control, we also confirmed that TβRIII was able to alter the membrane partitioning of full-length TβRII (Figure 3B). As TβRIII can re-direct the partitioning of TβRII-D cyt, this illustrates that the ability of TβRIII to re-direct the partitioning of TβRII is not dependent on the intracellular domain of TβRII, which has binding sites for clathrin [27].

TβRIII associates with TβRI in the absence of ligand and affects its partitioning

As TβRI plays a crucial role in the propagation of TGFβ signalling by phosphorylating downstream R-Smads (regulatory Smads), we also wanted to assess whether TβRIII affected the partitioning of TβRI.

We first assessed whether TβRIII could interact with TβRI using a co-immunoprecipitation approach. We used anti-FLAG antibodies to immunoprecipitate TβRIII and, similar to our results with TβRII, we found that TβRI can form a robust interaction with TβRIII in the absence of ligand (Figure 4A). Importantly, this interaction occurs in the absence of TβRII, as HEK-293T cells express very few endogenous TβRIIs.

To assess whether TβRIII can re-direct membrane partitioning of TβRI, we again used sucrose-density ultracentrifugation to isolate membrane rafts. Similar to the results shown in Figure 1(A), TβRI was found to be heavily concentrated in membrane-raft fractions (Figure 4B). Interestingly, and complementary to our findings with TβRII, TβRIII also shifted TβRI into non-raft fractions (Figure 4B). Our results evaluating

As TβRIII was found robustly in non-membrane raft fractions and could stably associate with TβRII, we speculated that the interaction of TβRIII with TβRII might increase the partitioning of TβRII into non-membrane-raft fractions.

To evaluate the ability of TβRIII to differentially partition TβRII, HEK-293T cells transiently transfected with TβRII–HA and Myc–TβRIII cDNA were subjected to sucrose-density ultracentrifugation as described above. Figures 2(B) and 2(C)
the partitioning of both TβRI and TβRII in the presence of TβRIII suggest that TβRIII is able to direct the partitioning of the TβRII–TβRI complex, but importantly can also interact with either receptor independently.

**TβRIII decreases entry of TβRII into caveolin-1-positive vesicles**

The intracellular trafficking of TβRI is also directly influenced by their endocytosis. When TβRs are endocytosed via membrane rafts/caveolae, the receptors enter into caveolar vesicles and are targeted for ubiquitination and degradation [12]. Furthermore, no signal transduction occurs in caveosomes, as TβRI is blocked from interacting with Smad2/3 by the inhibitory Smad, Smad7 [12]. Thus we predicted that as TβRIII shifted TβRII out of membrane-raft fractions less TβRII would also be found in caveolin-1-positive vesicles.

To address this question, we used an immunofluorescence approach to visualize co-localization of TβRI and TβRIII with GFP-tagged caveolin-1. Mv1Lu HA T cells were transiently transfected with Myc–TβRII and incubated with anti-HA antibodies at 4°C to label HA-tagged TβRII at the cell surface. Following labelling with fluorescent secondary antibodies, receptors were permitted to internalize by warming the cells to 37°C. We then performed standard immunofluorescence microscopy to visualize TβRIII and caveolin-1.

Figure 5(A) (upper panels) shows that in the absence of TβRIII, a large fraction of TβRII co-localized with caveolin-1. However, upon addition of TβRIII, much less TβRII was found co-localized with caveolin-1 (Figure 5A). Furthermore, very little co-localization between TβRIII and caveolin-1 was found. Not only do these results confirm our ultracentrifugation results, they also illustrate that TβRIII can direct TβRII out of the caveolin-1-positive vesicles, and therefore may also have a direct effect on TβRII half-life.

**TβRIII increases early endosomal trafficking of TβRII**

Having shown that TβRIII directs TβRII out of caveolar vesicles, we sought to evaluate whether TβRII also increases TβRII entrance into the early endosome. It has been shown previously that receptors internalized via clathrin-coated-pit-mediated endocytosis traffic into early endosomes, where they can interact with Smad transcription factors to propagate TGFβ signal transduction [12]. Therefore if TβRIII can re-direct the intracellular trafficking of TβRII, then it may also have a direct effect on TGFβ signal transduction.

To evaluate the ability of TβRIII to affect TβRII trafficking we used an immunofluorescence-based approach to evaluate the co-localization of the receptors. As in the experiments above, Mv1Lu HA T cells were transiently transfected with Myc–TβRIII cDNA. At approx. 36 h post-transfection, receptors were labelled at the cell surface by cooling cells to 4°C and incubating with an anti-HA antibody. After labelling with fluorescent secondary antibodies, receptors were permitted to internalize by warming to 37°C. Cells were also labelled with an anti-Myc primary antibody, to visualize TβRII. To evaluate early endosomal trafficking of receptors two markers for the early endosome, EEA1–FITC (Figure 5B) and Rab5–GFP (Figure 5C), a GTPase involved...
in early endosomal sorting, were evaluated in terms of co-localization with receptors.

Using immunofluorescence microscopy, we found that in the absence of TβRII, TβRII co-localized with EEA1; however, a substantial proportion of receptors did not localize with EEA1 (Figure 5B). One possibility is that receptors are internalized by both clathrin- and non-clathrin-mediated mechanisms. However, upon co-expression with TβRII, more TβRII was found co-localized with EEA1, suggesting an increase in clathrin-mediated endocytosis or endosomal retention (Figure 5B). Co-localization experiments with Rab5 also showed that, in the presence of TβRII, more TβRII was found in early endosomes (Figure 5C). As Rab5 has been shown to cause endosomal enlargement [27a], we also performed co-localization studies with GFP-labelled Rab5–S34N or Rab5–Q79L mutants (Supplementary Figure S2 at http://www.BiochemJ.org/bj/429/bj4290137add.htm). Our results indicate that though Rab5–Q79L can cause endosomal enlargement, this does not appear to increase the localization of TβRII with the early endosomal compartment (Supplementary Figure S2).

These immunofluorescence experiments further illustrate that TβRIII and TβRII may form a stable interaction, as shown by their co-localization (Figure 5). Overall, the present study strongly suggests that TβRIII is able to direct the trafficking of TβRII.

TβRIII extends the half-life of TβRII

Efficient turnover of TβRs is essential for optimal TGFβ signal transduction, as TβRs and ligand may be ubiquitously expressed. It has been shown previously that the intracellular compartmentalization of TβRs directs receptor degradation and recycling. Receptors in early endosomal compartments are recycled to the cell surface, whereas receptors localized in caveolin-1-positive compartments are targeted for ubiquitination and degradation [12]. Therefore having found that TβRIII redirects trafficking of TβRII into early endosomal compartments, we predicted that TβRIII expression would also increase the half-life of TβRII.

To assess this further, we used 125I-labelled TGFβ1 ligand to track cell-surface TβR half-life in both the presence and absence of TβRIII. Briefly, HEK-293T cells were transiently transfected with TβRI, TβRII, TβRIII, Smurf2 (Smad ubiquitination regulatory factor 2) and Smad7, as outlined in Figure 6. Smurf2 and Smad7 were transiently transfected to promote receptor ubiquitination and degradation, as in the absence of Smurf2 and Smad7 receptors have a prolonged half-life (Figure 6, top panel). Post-transfection, cells were labelled with 125I[TGFβ1 at 4°C. The ligand was then cross-linked to receptors and the cells were warmed to 37°C to promote receptor internalization. Cells were lysed after 0, 2, 4 and 8 h of internalization, and lysates were subjected to SDS/PAGE and visualized using autoradiography.

The top panel of Figure 6 illustrates that, in the absence of Smurf2 and Smad7, TβRII had a prolonged half-life both in the presence and absence of TβRIII. However, upon addition of Smurf2 and Smad7, which promote receptor ubiquitination and degradation, TβRIII greatly extends the half-life of both TβRI and TβRII (Figure 6, middle and bottom panels). These results confirm our receptor trafficking studies and imply that TβRIII can have direct effects on TGFβ signal transduction by altering the TβRII–TβRI complex half-life.

TβRIII enhances basal TGFβ signalling

As it has been shown previously that clathrin-mediated endocytosis enhances TGFβ signal transduction [12], we sought to identify whether TβRIII expression could affect TGFβ signalling. To address this question, we used a TGFβ-responsive ARE promoter upstream of a luciferase construct to quantitatively assess the role of TβRIII expression on signalling. HepG2 cells were transiently transfected with ARE–Lux, FoxH1 and/or TβRI, TβRII or TβRIII, Smurf2 (Smad ubiquitination regulatory factor 2) and Smad7, as outlined in Figure 6. TβRIII expression could affect TGFβ-dependent signalling in the absence of TβRII (Figure 7). Interestingly, we found that TβRIII expression could increase TGFβ-dependent signalling in the absence of TβRII (Figure 7). This result was surprising, as TβRIII is best known for its role in ligand presentation. We hypothesize that the ability of TβRIII to enhance basal TGFβ signalling is due to enhancing clathrin-mediated endocytosis of the TβRII–TβRI complex. Indeed, it has been shown that TβRs can signal at a basal level in the absence of ligand [28]. In contrast with this result, we observed that increasing levels of TβRII cDNA transfection decreases TGFβ signalling in the presence of ligand (Figure 7).

In conclusion, analysis of both membrane fractionation and receptor trafficking illustrate that TβRIII promotes clathrin-mediated endocytosis of both TβRII and TβRI, and directs TβRII into the early endosome. TβRII expression therefore has functional consequences on TGFβ signal transduction, as it extends receptor half-life by re-directing the TβRII–TβRI complex out of the degradative membrane-raft pathway and enhances basal TGFβ signalling.
TGFβRIII alters TGFβRII and TGFβRI trafficking

DISCUSSION

The mechanism of endocytosis at the cell membrane can have immediate downstream effects in signal transduction. In the canonical TGFβ signalling pathway, clathrin-mediated endocytosis increases TGFβ signalling through enabling the association of the receptors with SARA (Smad anchor for receptor activation) in the early endosome. SARA is able to mediate the interaction of the TβRs with Smad proteins, which are the downstream effectors of TGFβ signal transduction [12]. Membrane-raft/caveolar endocytosis, however, decreases TGFβ signalling through promoting the degradation of the receptors [12]. In membrane rafts, Smurf2 associates with the receptors, promotes the ubiquitination and degradation of the TβRs, and prevents the association of Smad proteins (reviewed in [7]).

Therefore, given the endocytic mechanism of TβRs can have such profound effects on TGFβ signalling and a number of pathologies, including metastatic cancers and fibrotic diseases, show aberrant TGFβ signalling, an in-depth study evaluating the mechanism through which TβRs are directed to endocytose was warranted.

To address the issue of endocytic partitioning in TGFβ signalling, we first attempted to evaluate the contribution of TβRs to the raft and non-raft partitioning of TβRII.
Interestingly, using co-immunoprecipitation studies, we showed that TβRIII, the least well-characterized TβR, was able to associate with both TβRII and TβRI even in the absence of ligand. We confirmed these results with sucrose-density ultracentrifugation, which quantitatively illustrated that the association of TβRIII with TβRII and TβRI can significantly shift the partitioning of TβRII and TβRI into non-membrane-raft/clathrin fractions. In support of our subcellular fractionation studies, we also showed that TβRIII directs TβRII into the early endosome and out of the degradative pathway using immunofluorescence microscopy. Using 125I-labelled TGFβ1 to track TβRII and TβRI half-life, we showed that TβRIII can have a direct effect on the signalling capacity of the TβRII–TβRI complex, as its association can extend the half-life of TβRII–TβRI. Finally, we showed that TβRIII increases basal TGFβ signalling, but decreases signalling in the presence of ligand.

Although our present study evaluates the contribution of TβRIII to TβR trafficking, other studies have also attempted to evaluate factors that affect TGFβ receptor half-life. Koli and Arteaga [29] illustrated that binding of TGFβ to TβRII can shorten its half-life. Interestingly, it has also been shown recently that inhibiting clathrin-mediated endocytosis of TβRII extends receptor half-life and promotes TGFβ signalling [30]. In that study, the authors illustrated that inhibitors of clathrin-mediated endocytosis prevent internalization of TβRIs, but allow the association of SARA and TβRI at the cell surface; this then promotes and extends TGFβ signalling [30].

Another study has recently attempted to evaluate the contribution of TβRIII to TβRII membrane partitioning [23]. Those authors concluded that TβRIII was endocytosed via membrane rafts in Cos7 and HepG2 cells [23]. Furthermore, they reported that membrane-raft-associated TβRIII regulates phosphorylation of Smad2 and p38 [23]. Although we present differing results in the present paper, in that TβRIII is primarily found in non-membrane-raft fractions and is able to differentially partition the TβRII–TβRI complex, this result may be due to the cell types used in both studies. Differences in membrane-raft content between HepG2 and HEK-293T cells may account for some of the discrepancies observed. The present study nonetheless illustrates that although TβRIII endocytosis can affect TGFβ signalling, there may be other interacting protein partners that influence the effect of TβRIII. Indeed, a recent review highlights several small GTPases that can have a modulating effect on TGFβ endocytosis and signal transduction [31].

The importance of the effect of TβRIII on TGFβ signal transduction is only beginning to be explored. This TβR has recently drawn attention due to its aberrant expression in several cancers. Indeed, TβRIII overexpression is found in seminomas [32], and knockdown of TβRIII decreases invasiveness and motility of breast cancer cells [21]. However, it has also been shown that loss of TβRIII can promote metastasis and invasiveness in a number of cancers, including non-small cell lung cancer [20], pancreatic cancer [22] and prostate cancer [17] [33]. Our finding that TβRIII promotes basal TGFβ signalling, but decreases ligand-dependent signalling, may help explain some of the duality of its function in cancer. Perhaps the levels of TβRIII may play a role: a total loss of TβRIII may increase TGFβ signalling by preventing ligand sequestration from the TβRII–TβRI complex and thus may promote the metastatic effects of TGFβ signalling. Although the opposing effects of TβRIII warrant further investigation of this pathway, our studies suggest that TβRIII expression may play a critical role in control of TGFβ signal transduction.

Overall, in the present study we have shown that TβRIII directs TβRII and TβRI to undergo clathrin-mediated endocytosis. This
altered endocytosis directs TβRII into early endosomal pathways, extends TβRII and TβRI half-life and enhances basal TGF-β signalling. As TβRIII is aberrantly expressed in a number of pathologies, the present study suggests that TβRIII may mediate TGF-β signal transduction by altering TβR endocytosis.

**AUTHOR CONTRIBUTION**
Sarah McLean performed the experimental work except for the experiments presented in Figure 7, which were performed by Gianni Di Guglielmo. Both authors planned the experiments and wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

TGFβ (transforming growth factor β) receptor type III directs clathrin-mediated endocytosis of TGFβ receptor types I and II

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Figure S1 Partitioning of endogenous TβRI and TβRII

(A) HepG2 cells were subjected to sucrose-density subcellular fractionation. Fractions were immunoblotted for a marker for the early endosome (EEA1) or a marker for membrane rafts (caveolin-1; cav-1) to ensure isolation of membrane-raft fractions. A non-specific band is seen underneath the caveolin-1 protein band and is indicated (*). (B) HepG2 cells were affinity-labelled with [125I]TGFβ1 prior to sucrose-density subcellular fractionation. Following subcellular fractionation, lysates were subjected to SDS/PAGE. Receptor partitioning was visualized and quantified by phosphoimaging. TβRI and TβRII partitioning into raft and non-raft fractions was quantified using QuantityOne software (lower panel). Results are means ± S.D. (n = 3). R, raft fractions; NR, non-raft fractions.

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Figure S2  TβRII trafficking in the presence of Rab5-S34N or Rab5-Q79L

(A) Upper panel: Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII and transiently expressing Rab5-S34N–GFP (Rab5-GDP) were incubated with anti-HA antibodies at 4°C. Following incubation with Cy3-labelled secondary antibodies (red), the cells were incubated at 37°C for 1 h to allow receptor endocytosis. TβRII co-localizing with Rab5-GDP results in a yellow colour and non-Rab5-GDP localized TβRII is also found in the cytoplasm (red). Lower panel: Mv1Lu HAT cells transiently expressing Myc–TβRIII and Rab5-S34N–GFP (Rab5-GDP) were incubated with anti-HA and anti-Myc antibodies at 4°C and processed for immunofluorescence microscopy as described for the upper panel. (B) Upper panel: Mv1Lu HAT cells were also assessed for their co-localization with transiently expressed Rab5-Q79L–GFP (Rab5-GTP), shown in yellow. Lower panel: Mv1Lu HAT cells expressing HA-tagged TβRII and Myc–TβRIII were also assessed for their co-localization with Rab5-Q79L–GFP (Rab5-GTP). As before, receptors were labelled at the cell surface, internalized, then assessed for their co-localization. TβRII co-localizing with Rab5–GTP results in a yellow colour, TβRII co-localizing with TβRII results in a magenta colour, and all three co-localizing results in white colour. Scale bar = 10 μm.