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Dlg5 interacts with the TGF-β receptor and promotes its degradation

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Abbreviations: ALLN, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal; CD, Crohn's disease; Dlg5, Discs large homolog 5; EMT, epithelial to mesenchymal transition; IBD, inflammatory bowel disease; JNK, c-Jun N-terminal kinases; MUGUK, membrane-associated guanylate kinase; p38, 38 mitogen-activated protein; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; TGF-β, Transforming grows factor-beta

Abstract

Discs large homolog 5 (Dlg5) is a member of the membrane-associated guanylate kinase adaptor family of proteins and is involved in epithelial-to-mesenchymal transition via transforming growth factor-β (TGF-β) signaling. However, the mechanism underlying the regulation of TGF-β signaling is unclear. We show here that Dlg5 interacts and colocalizes with both TGF-β type I (TβRI) and type II (TβRII) receptors at plasma membrane. TβRI activation is not required for this interaction. Furthermore, the overexpression of Dlg5 enhances the degradation of TβRI. Proteasome inhibitors inhibited this enhanced degradation. These results suggest that Dlg5 interacts with TβRs and promotes their degradation.

1. Introduction

Discs large homolog 5 (Dlg5) is a member of the membrane-associated guanylate kinase (MAGUK) adaptor family of proteins. Each MAGUK family member has at least one PDZ (PSD-95, Dlg, ZO-1) domain, an SH3 (Src homology 3) domain, and a guanylate kinase domain. Dlg5 was initially proposed to be one of five human homologs of the *Drosophila* Discs large (Dlg) protein, a tumor suppressor gene which is required for tumor suppression in imaginal discs, based on similarities in their sequences and domain structures [1,2]. However, Dlg5 has its own ortholog in other animals including *Drosophila*, thus it does not belong to the DLG MAGUK subfamily but seems to have its own function [2].We have identified Dlg5 as a binding protein for vinexin [3,4] and shown that Dlg5 associates with β-catenin and that it localizes at cell-cell adhesion sites. Several other Dlg5 binding proteins, including p55 [1], syntaxin 4 [5] and citron kinase [6], have been identified. It has been reported that *Dlg5* polymorphisms are associated with Crohn's disease (CD), which is an inflammatory bowel disease (IBD) [7-11]. *Dlg5* knockout mice showed defects in the kidney and brain [5] as well as abnormal cell polarity. However, these phenotypes cannot explain the pathogenesis of CD.

Recently, we showed that Dlg5 suppresses the epithelial-to -mesenchymal transition (EMT) of renal epithelial cells *via* the inhibition of TGF-β receptor-dependent signaling [12]. TGF-β stimulation induces intracellular signals via two serine-threonine kinase receptors, type I (TβRI) and type II (TβRII) TGF-β receptors. Both TβRI and TβRII form stable homodimers in the absence of TGF-β. Upon TGF-β binding, TβRI homodimers and TβRII homodimers form a heterotetrameric receptor complex, leading to the activation of two downstream signaling pathways [13-15]. The first pathway is Smad signal, in which TGF-β stimulation results in TβRI-mediated phosphorylation of receptor-regulated Smads (Smad2 and Smad3). Phosphorylated Smad2 and Smad3 bind to the common mediator Smad (Smad4). The Smad2/3/4 complexes then accumulate in

the nucleus and regulate target gene transcription. The other pathway is non-Smad signal. TβRI activates c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38 MAPK) through MKK4 and MKK3/6, respectively, independent of Smad signals. Dlg5 depletion in LLc-PK1 renal proximal tubule epithelial cells increases the activation of JNK and p38 MAPK, which are necessary to induce the EMT of these cells [12]. Dlg5 seems to affect TβR because specific inhibition of TβRI or overexpression of Smad7, a physiological inhibitor of TβR-dependent signaling, suppresses Dlg5 depletion-induced EMT. However, the mechanism by which Dlg5 regulates TβR is unknown. Here, we show that Dlg5 interacts with TβRI and enhances its degradation.

2. Materials and methods

2.1 Antibodies and reagents

MG132 and N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN) were purchased from Calbiochem. Bafilomycin and cycloheximide were from WAKO Pure Chemical Industries, Osaka, Japan and Sigma, respectively. The following antibodies were used in this study: anti-Flag (Sigma), anti-GFP (WAKO Pure Chemical Industries), anti-GFP (Santa Cruz Biotechnology), anti-TβRI (Santa Cruz Biotechnology), anti-β-tubulin (Sigma), and anti-vinculin (Sigma) antibodies. The anti-Dlg5 antibody was described previously [3] .

2.2 Cell culture, plasmids, and transfection

The 293T and Caco-2 cells (RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan) were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. PC3 cells (obtained from Dr. Yoshiyuki Kakehi of Kagawa University) were maintained in RPMI-1640 medium containing 10% fetal bovine serum. The longest variant of Dlg5 (Dlg5α, see Fig. 1E), which includes 1,919

amino acids containing CARD (caspase recruitment domain), coiled-coil, SH3, and GUK domains, as well as four PDZ domains, was purchased from Promega (Madison, USA). A shorter variant (Dlg5β), which lacks the CARD domain, was described previously [3]. Dlg5ΔCC, which lacks the CARD and coiled-coil domains and corresponds to an open reading frame of KIAA0583 (AB011155), was obtained from the Kazusa DNA Research Institute. Plasmids encoding the TGF-β receptors (TβRI-FLAG and TβRII-HA, both of which are epitope-tagged at C-terminus) were gifts from Dr. S. Aota (RIKEN, Japan). Transfection of plasmid DNA was performed using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions.

2.3 RNA interference (RNAi)

The stealth siRNAs for Dlg5/lp-dlg (GGAUUCCAUGGAGUGGGAAACGGAA) were designed based on the sequence of the human Dlg5 cDNA (NP_004738) and synthesized by Invitrogen. The transfection of siRNAs (10 nM) was performed using Lipofectamine RNAiMAX (Invitrogen) as previously described [16].

2.4 Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described [12,17]. Briefly, cells cultured under various conditions were washed with PBS. The cells were then lysed in RIPA buffer without SDS (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 0.1 mg/mL *p*-amidinophenyl methanesulfonyl fluoride, 5 μg/mL leupeptin, and 10 μg/mL aprotinin) on ice for 30 minutes. Lysates were centrifuged at 18,700 × g for ten minutes. For immunoprecipitation, equal amounts of cell lysate were incubated with the indicated antibodies for 1 hour and then with Protein G sepharose beads for 1 hour. For immunoblotting, cell lysates or immunoprecipitates were electrophoresed and transferred onto PVDF membranes (Millipore). After blocking with BLOCKING ONE (Nacalai Tesque, Kyoto, Japan), the membranes were incubated with primary antibodies

and then with the appropriate secondary antibodies. Antigen detection was performed with an LAS 3000 image analyzer (FUJIFILM, Tokyo, Japan) using the ImmunoStar LD kit (Wako, Osaka, Japan). Quantification was performed by Multi Gauge (FUJIFILM, Tokyo, Japan).

2.5 Immunostaining

Caco-2 cells were transfected with plasmids encoding GFP-Dlg5ΔCC, TβRI-Flag and TβRII-HA. The cells were cultured on coverslips coated with type IV collagen for 24 hours. Cells were fixed with methanol for 20 minutes at -20^{o}C and blocked with 10% goat serum in PBS+ for 30 minutes. They were then incubated with anti-Flag antibody and anti-HA antibody for 1 hour at room temperature, followed by incubation with Alexa 633-labeled goat anti-rabbit IgG (Invitrogen) and Alexa 546-labeled goat anti-rat IgG for 1 hour. Fluorescence imaging was performed with a LSM700 confocal microscopy system (Carl Zeiss Co., Ltd.).

2.6 Statistical analysis

The statistical analysis was performed using Student's paired *t*-test.

3. Results

3.1 Dlg5 interacts with TβRI and TβRII

Previously, we showed that Dlg5 suppresses EMT through the inhibition of TGF-β receptor-mediated signaling [12]. Several molecules, including Smad7 and Smurf1/2, attenuate TGF-β signaling via binding to the TβRs [18]. To examine whether Dlg5 can interact with TβRs, TβRI-Flag and TβRII-HA were cotransfected with GFP-tagged Dlg5 variant (GFP-Dlg5ΔCC) into 293T cells, followed by coimmunoprecipitation using an anti-GFP antibody. As shown in Fig. 1A, both TβRI and TβRII were coprecipitated with Dlg5ΔCC. To check whether Dlg5 could bind TβRI independent of

TβRII and vice versa, we performed immunoprecipitation assays. GFP-Dlg5ΔCC was transfected into 293T cells with TβRI-Flag and then immunoprecipitated with anti-GFP antibody. TβRI (Fig. 1B) was coprecipitated with Dlg5ΔCC. The reciprocal coimmunoprecipitation experiment confirmed this interaction (data not shown). We also examined whether the interaction of Dlg5 with TβRII depends on TβRI. GFP-Dlg5ΔCC was transfected into 293T cells with TβRII-HA and then immunoprecipitated with anti-GFP antibody. TβRII (Fig. 1C) was coprecipitated with Dlg5ΔCC. These results indicate that Dlg5 can interact with both TβRI and TβRII independently. Dlg5 has splice variants, such as Dlg5α and β (Fig. 1E, also see Materials and Methods). To determine which Dlg5 splice variants can interact with the TβRs, coimmunoprecipitation experiments with Dlg5α, -β, and -ΔCC were performed. TβRI coprecipitated with all these Dlg5 variants (Fig. 1D), suggesting that these variants all contain the TβRI interaction region. Because TβRI coprecipitated the most effectively with Dlg5ΔCC, possibly because Dlg5α and Dlg5β were less soluble in the lysis buffer than Dlg5ΔCC (Fig. 1D), primarily Dlg5ΔCC was used in subsequent experiments.

To confirm the interaction between endogenous Dlg5 and the TβRs, we performed coimmunoprecipitation experiments using PC3 prostate cancer cells in which Dlg5 is expressed at high levels. The PC3 cell lysates were subject to immunoprecipitation with anti-TβRI antibody and examined for coimmunoprecipitation of endogenous Dlg5. As shown in Fig. 2, Dlg5 was detected when anti-TβRI antibody, but not control IgG, was used for the immunoprecipitation. Transfection of Dlg5 siRNA reduced the expression of Dlg5 in lysates as well as in the coprecipitation of Dlg5 with TβRI. We could not detect coimmunoprecipitation of endogenous TβRII with Dlg5 because it could not be immunoprecipitated efficiently (data not shown). These results suggest that Dlg5 binds TβRI under physiological conditions. Thus, we focused on TβRI in subsequent experiments.

3.2 TβRI activation and kinase activity are not necessary for Dlg5 binding

The interactions of TβRI with Smad7, SARA (Smad anchor for receptor activation), and TAK1 (TGF-β activated kinase 1) are regulated by TβRI activation [18-21]. To investigate whether TβRI activation affects its interaction with Dlg5, coimmunoprecipitation experiments using constitutively active (CA-TβRI T204D) and kinase-dead (KD-TβRI D351A) TβRI mutants were performed. As shown in Fig. 3A, CA-TβRI and KD-TβRI were coimmunoprecipitated with Dlg5 similarly to WT-TβRI. Next, we examined the effect of TGF-β stimulation on the Dlg5-TβRI interaction. After cotransfection of TβRI with GFP-Dlg5ΔCC, the cells were incubated with TGF-β for 30 or 180 minutes before immunoprecipitation was performed (Fig. 3B). TβRI was coimmunoprecipitated with GFP-Dlg5ΔCC before TGF-β stimulation. Thirty minutes after TGF-β stimulation, phosphorylated Smad2 was detected, thus indicating the activation of TβRI. However, the amount of TβRI coimmunoprecipitated was comparable to that before TGF-β stimulation, even after TGF-β stimulation for 30 or 180 minutes (Fig. 3B). We also examined the effect of TGF-β stimulation on the interaction of endogenous proteins. PC3 cells were treated with or without TGF-β, then cell lysates were immunoprecipitated with anti-TβRI antibody. Coimmunoprecipitated Dlg5 with TβRI in TGF-β treated cells was comparable to that in untreated cells (Fig. 3C). These results suggest that TβRI activation and kinase activity are not necessary for binding to Dlg5.

3.3 Dlg5 colocalizes with TβRs at the plasma membrane

To test for colocalization of Dlg5 with the TβRs in cells, Caco-2 colon epithelial cells were used. Caco-2 cells have well-organized cell-cell adhesion structures where Dlg5 localizes (data not shown). GFP-Dlg5ΔCC, TβRI-Flag, and TβRII-HA were cotransfected into Caco-2 cells. Cells were fixed and immunostained with anti-Flag and anti-HA antibodies. GFP-Dlg5ΔCC localized to the plasma membrane as well as

diffusely (Fig. 4), which is consistent with a previous report [3]. Both TβRI-Flag and TβRII-HA were also detected at the plasma membrane as well as in the cytosol (Fig. 4). The merged image indicates the colocalization of TβRI, TβRII, and GFP-Dlg5ΔCC at the plasma membrane. These results suggest that GFP-Dlg5ΔCC, TβRI-Flag, and TβRII-HA colocalize at the plasma membrane in Caco-2 cells.

3.4 Dlg5 overexpression promotes TβRI degradation

The degradation of TβRI and TβRII is one of main regulatory mechanisms of TGF-β signaling [18,22-25]. To investigate whether Dlg5 affects TβRI degradation, GFP-Dlg5ΔCC and TβRI-Flag were cotransfected into 293T cells. Twenty-four hours after transfection, cycloheximide was added to the culture medium to inhibit protein synthesis. After further incubation for 0-8 hours, TβRI expression was examined by immunoblotting with anti-Flag antibody. As shown in Fig. 5A, TβRI levels decreased only slightly over time in control cells. In contrast, TβRI levels decreased significantly faster in Dlg5ΔCC-expressing cells. A quantitative analysis showed that the amount of TβRI was reduced to 80 % and 50 % in control and Dlg5ΔCC-expressing cells, respectively, 4 hours after the addition of cycloheximide (Fig. 5B). Consistent with this overexpression experiments, Dlg5 knockdown in PC3 cells significantly increased the TβRI expression both under TGF-β-stimulated and -unstimulated conditions (Fig. 5C, D). TβRII degradation after cycloheximide treatment was also examined in 293T cells, but it was very slow and no decrease in TβRII protein levels was detected, even eight hours after cycloheximide treatment (data not shown). These results suggest that Dlg5 promotes the degradation of TβRI.

Proteasomal and lysosomal degradation pathways mediate TβRI degradation, which is enhanced by its interaction with Smad7 [18,24]. To test which degradation pathway is engaged by Dlg5, cells were cotransfected with GFP-Dlg5ΔCC and TβRI-Flag and then treated with the proteasome inhibitor MG132 or the lysosome inhibitor bafilomycin for

8 hours. Consistent with Fig. 5, Dlg5ΔCC overexpression significantly decreased TβRI protein levels (Fig. 6A, D). Treatment with MG132 significantly increased TβRI levels in Dlg5ΔCC-overexpressing cells but not in control cells (Fig. 6B, E). Quantitative analysis showed that MG132 treatment increased TβRI levels 1.3-fold. Another proteasome inhibitor, ALLN, also increased TβRI levels in GFP-Dlg5ΔCC expressing cells (data not shown). In contrast, bafilomycin increased TβRI levels in both control and Dlg5ΔCC-overexpressing cells (Fig. 6C, F). These results suggest that Dlg5 enhances the proteasome-dependent degradation of TβRI.

4. Discussion

Dlg5, a member of the MAGUK family of adaptor proteins, localizes at cell-cell adhesion sites. We previously showed that Dlg5 suppresses TβR-mediated signaling and EMT in renal epithelial cells [12]. However, the mechanism of this suppression is unknown. In this research, we found that both TβRI-Flag and TβRII-HA were clearly coprecipitated with Dlg5ΔCC. Furthermore, endogenous Dlg5 was also coimmunoprecipitated with endogenous TβRI in PC3 cells. GFP-Dlg5 was colocalized with TβRI-Flag and TβRII-HA at cell-cell adhesion sites in Caco-2 cells. These results indicate that Dlg5 forms a signaling complex with TβRs at the plasma membrane.

We showed here that treatment with proteasome inhibitors suppressed Dlg5ΔCC-enhanced TβRI degradation. Lysosome inhibitor increased the TβRI expression in Dlg5ΔCC-expressing cells but it also increased the expression in control cells, implying that Dlg5 enhanced TβRI degradation via proteasome-dependent mechanisms. In some cases, negative regulators of TGF-β signaling associate with TβRs and promote TβR degradation *via* proteasome- and lysosome-dependent pathways simultaneously [18,24,25]. In other cases, only proteasomal degradation contributes to the TβR degradation [26]. Cellular context may explain the different pathway for the TβRI degradation.

Expression of the TGF-β-signaling inhibitor Smad7 is enhanced by TGF-β stimulation itself. The interaction of TβRI with Smad7 is stimulated by TβRI activation [18-20]. Thus, Smad7 seems to function in a negative feedback loop regulating TGF-β signaling. In contrast, the interaction of Dlg5 with TβRI was not stimulated by TβRI activation. Both the constitutively active mutant of TβRI (CA-TβRI) and the kinase-dead mutant KD-TβRI bind Dlg5 in a manner comparable to the wild type receptor. TGF-β stimulation for 30 or 180 minutes did not affect these interactions. Furthermore, Dlg5 expression decreases after TGF-β stimulation [12]. Thus, Dlg5 seems to participate in positive feedback (specifically double-negative type feedback [27,28]) loop regulating TGF-β signaling but not in a normal negative feedback loop. Alternatively, Dlg5 may suppress leaky (non-specific) activation of TGF-β signaling by binding TβRs, in a mechanism similar to FKBP12 [29].

The dysregulation of TGF-β signaling increases the risk of CD. TGF-β1 knockout mice develop systemic inflammation, including in the gut [30]. High levels of Smad7 are observed in CD patients [31], and its knockdown attenuates experimental colitis in mice [32]. We found that Dlg5 interacts with TβRs and promotes their degradation. These results may explain how *Dlg5* polymorphisms affect the development of IBD, including CD. Future experiments will be directed towards understanding the relationship between Dlg5 polymorphisms and the regulation of TβR signaling.

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Figure legends

Figure 1. GFP-Dlg5 is coimmunoprecipitated with TβRI-Flag and TβRII-HA. A-C: GFP or GFP-Dlg5ΔCC were cotransfected into 293T cells together with Flag-tagged TβRI and HA-tagged TβRII (A), Flag-tagged TβRI (B) or HA-tagged TβRII (C). Cell lysates were subject to immunoprecipitation (IP) with an anti-GFP antibody. Immune complexes were examined using the antibodies indicated. Arrows mean differentially glycosylated forms of TβRII**. D:** TβRI-Flag, as well as either GFP, GFP-Dlg5α, GFP-Dlg5β or GFP-Dlg5ΔCC, was transfected into 293T cells. Cell lysates were immunoprecipitated with anti-GFP antibody, and immunoblotting was performed with the antibodies indicated. **E:** Schematic diagram showing the protein domains of Dlg5α, -β, and -ΔCC. Dlg5α contains CARD, coiled-coil, SH3, and GUK domains, as well as four PDZ domains. Dlg5α and β are splice variants. Dlg5ΔCC is a deletion mutant that lacks the CARD and coiled-coil domains.

Figure 2. Endogenous Dlg5 is coimmunoprecipitated with TβRI in PC3 cells. PC3 cells were transfected with Dlg5 siRNA and incubated for three days. The cells were then lysed and immunoprecipitated with anti-TβRI antibody or control IgG. The immune complexes were examined with the antibodies indicated.

Figure 3. TβRI activation and kinase activity are not necessary for binding to Dlg5. A: GFP-Dlg5ΔCC, together with Flag-tagged wild type (WT), constitutively active (CA) or kinase-dead (KD) TβRI, was transfected into 293T cells. Cell lysates were immunoprecipitated (IP) with anti-GFP antibody and immunoblotted with the antibodies indicated. The expression levels of KD-TβRI were low for unknown reasons. **B:** 293T cells were transfected with Flag-tagged TβRI and GFP-tagged Dlg5ΔCC. The cells were then treated with TGF-β for 30 or 180 minutes, followed by immunoprecipitation and immunoblotting with the antibodies indicated. **C:** PC3 cells were treated with or without TGF-β for 30 minutes, followed by immunoprecipitation and immunoblotting with the antibodies indicated.

Figure 4. Dlg5 colocalizes with TβRI and TβRII. Caco-2 cells were cotransfected with TβRI-Flag, TβRII-HA and GFP-Dlg5ΔCC. The cells were fixed and immunostained with anti-Flag and anti-HA antibodies. Dlg5 was visualized by GFP fluorescence.

Figure 5. Dlg5 promotes TβRI degradation. A: GFP-Dlg5ΔCC and TβRI-Flag were cotransfected into 293T cells. Twenty-four hours after transfection, 100 μg/ml cycloheximide was added to the culture medium to inhibit protein synthesis. After further incubation for 0-8 hours, TβRI expression was determined by immunoblotting with an anti-Flag antibody. **B:** The relative TβRI expression in A was quantified. The values represent the mean ± S.E. from three independent experiments. **C:** PC3 cells were transfected with Dlg5 siRNA and incubated for three days. The cells were then treated with TGF-β for 30 minutes, followed by immunoblotting with the indicated antibodies. Vinculin was used as a loading control. **D:** TβRI expression in C was quantified. The values represent the mean ± S.E. from three independent experiments.

Figure 6. Dlg5-enhanced TβRI degradation involves proteasome. A-C: Cells were cotransfected with GFP-Dlg5ΔCC and TβRI-Flag, then treated without (A) or with MG132 (B) or bafilomycin (C). After incubation for eight hours, TβRI levels were determined by immunoblotting with the antibodies indicated. Vinculin was used as a loading control. **D-F:** TβRI expression in A-C was quantified. The values represent the mean ± S.E. from three independent experiments.

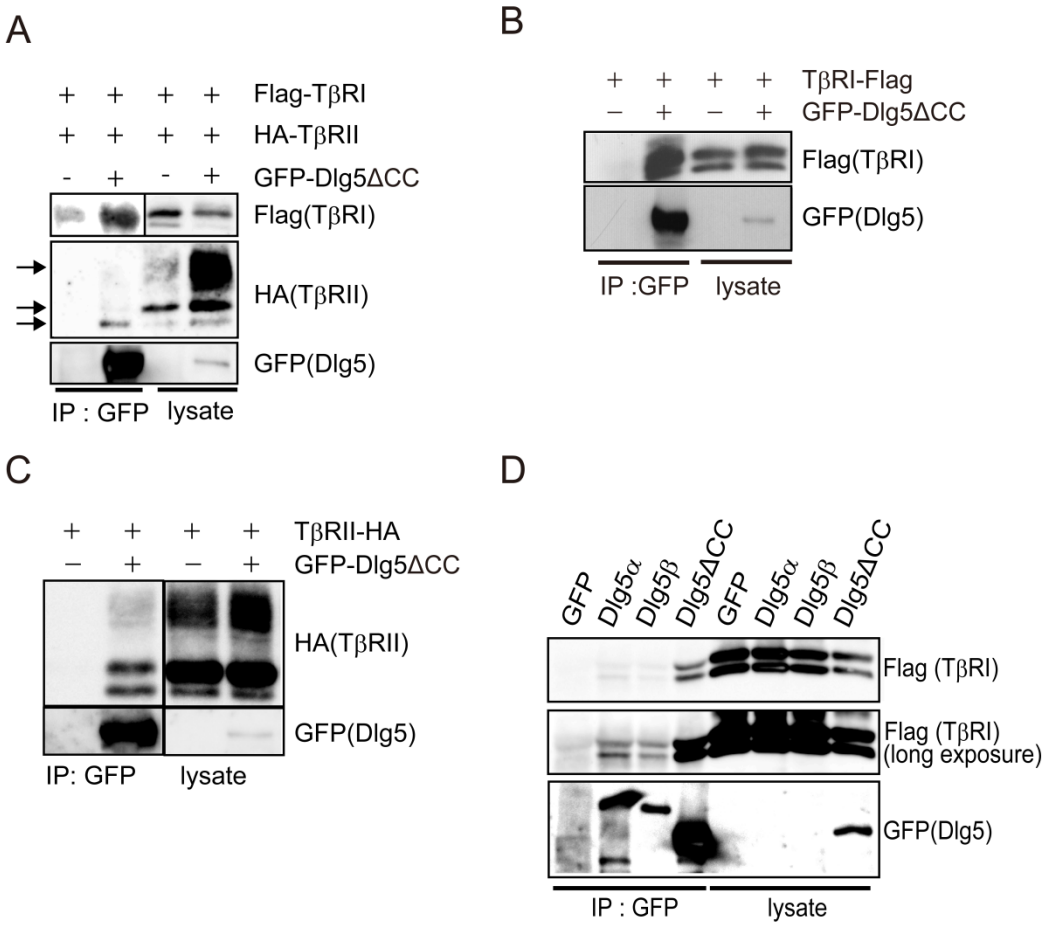

Fig. 1 Sezaki et al.

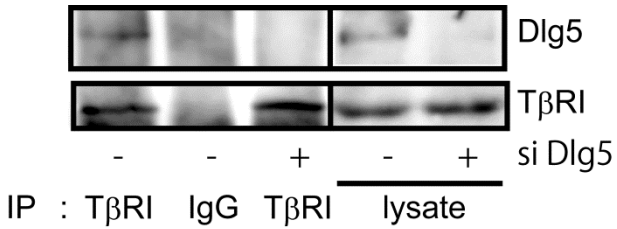

Fig. 2 Sezaki et al.

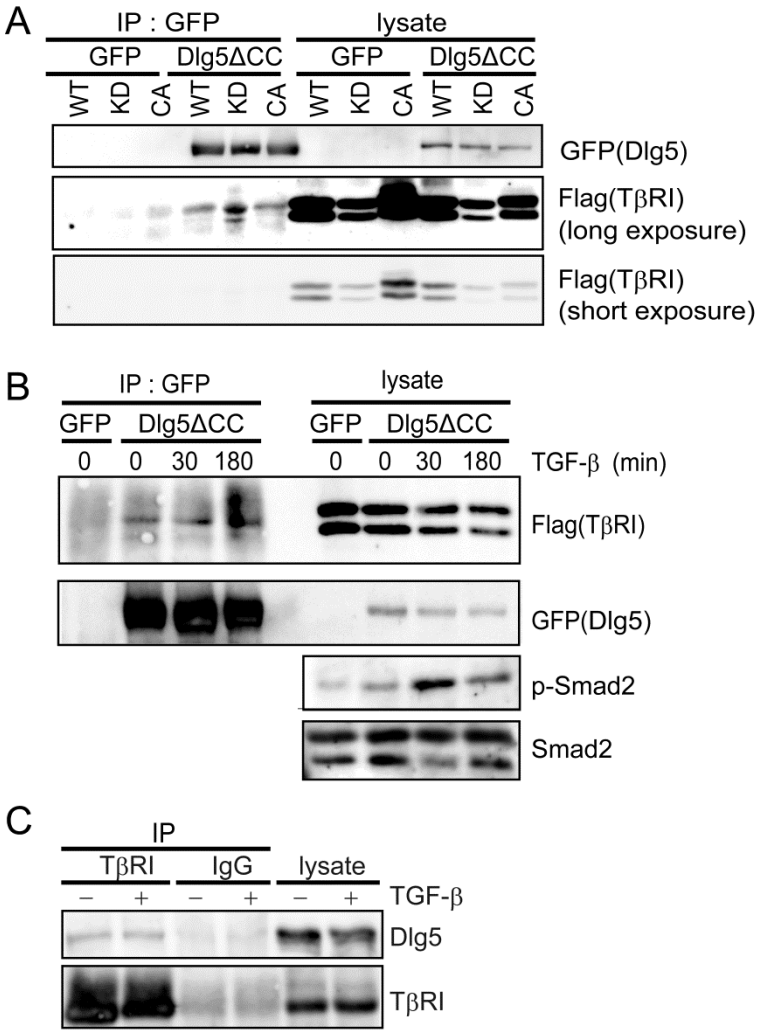

Fig 3. Sezaki et al.

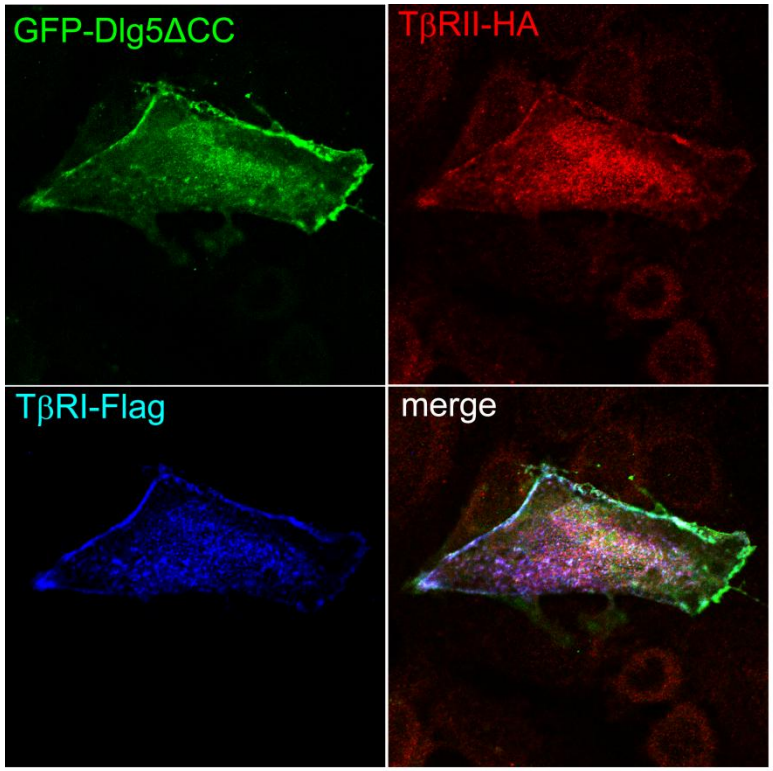

Fig. 4 Sezaki et al.

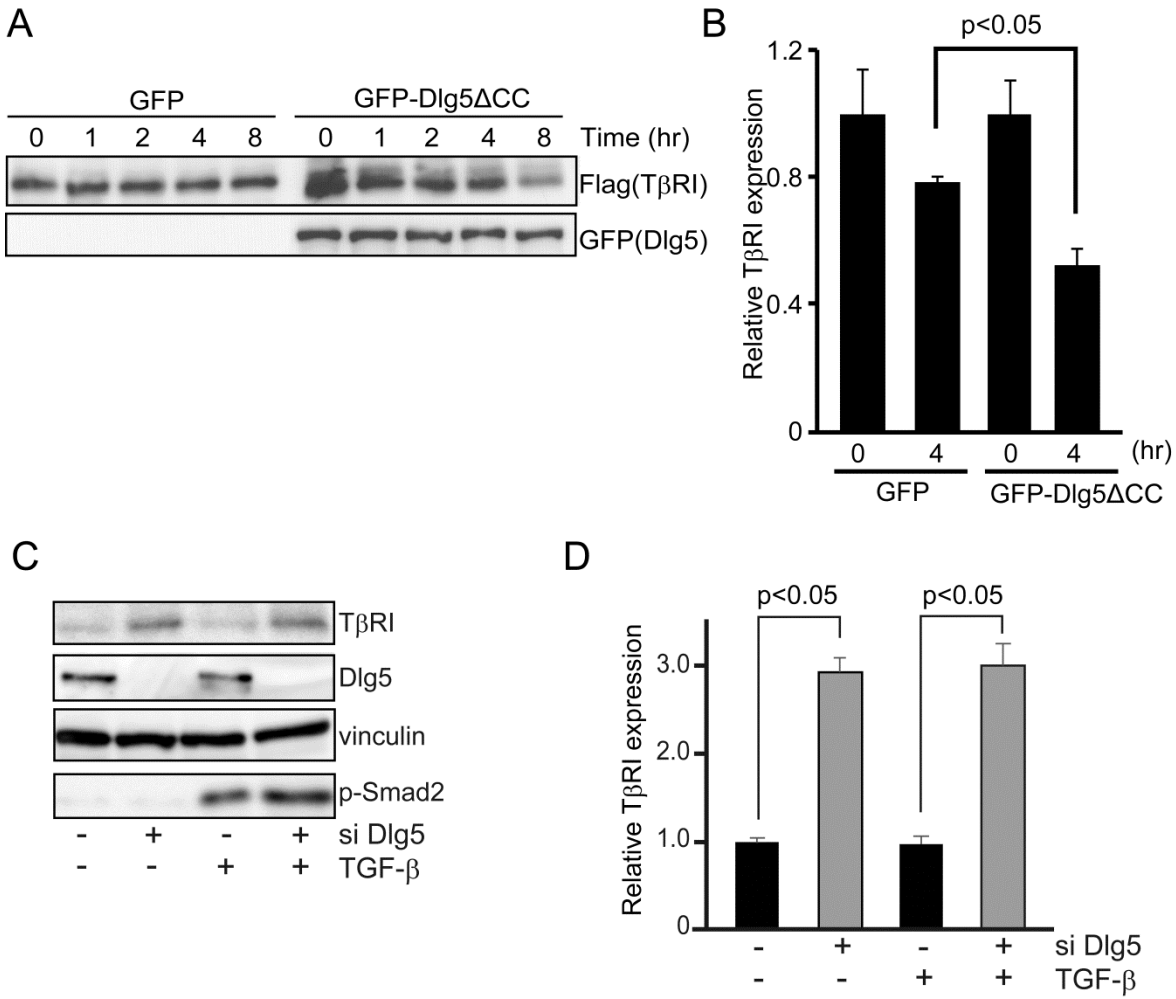

Fig. 5 Sezaki et al.

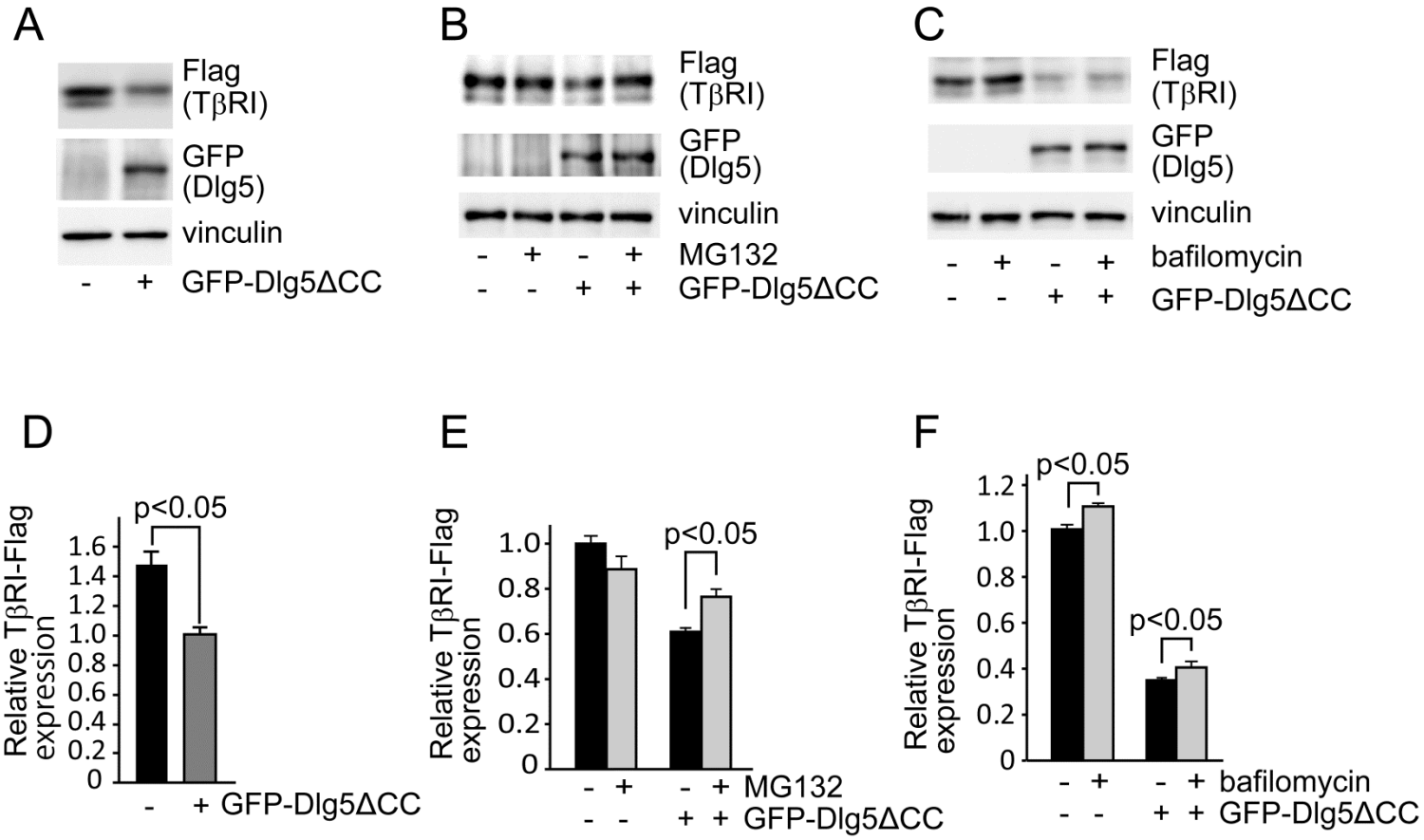

Fig. 6 Sezaki et al.