

# CD109-Mediated Degradation of TGF- $\beta$ Receptors and Inhibition of TGF- $\beta$ Responses Involve Regulation of SMAD7 and Smurf2 Localization and Function

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## ABSTRACT

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a multifunctional cytokine that regulates a wide variety of cellular processes including proliferation, differentiation, and extracellular matrix deposition. Dysregulation of TGF- $\beta$  signaling is associated with several diseases such as cancer and tissue fibrosis. TGF- $\beta$  signals through two transmembrane proteins known as the type I (TGFBR1) and type II (TGFBR2) receptors. The levels of these receptors at the cell surface are tightly regulated by several mechanisms, including degradation following recruitment of the E3 ubiquitin ligase SMAD ubiquitination regulatory factor (Smurf) 2 by SMAD7. In addition, TGF- $\beta$  co-receptors can modulate TGF- $\beta$  signaling receptor activity in a cell-specific manner. We have previously identified a novel TGF- $\beta$  co-receptor, CD109, a glycosyl phosphatidylinositol (GPI)-anchored protein that negatively regulates TGF- $\beta$  signaling. Despite CD109's potential relevance as a regulator of TGF- $\beta$  action *in vivo*, the mechanisms by which CD109 regulates TGF- $\beta$  signaling are still incompletely understood. Previously, we have shown that CD109 downregulates TGF- $\beta$  signaling by promoting TGF- $\beta$  receptor localization into the lipid raft/caveolae compartment and by enhancing TGF- $\beta$  receptor degradation. Here, we demonstrate that CD109 enhances SMAD7/Smurf2-mediated degradation of TGFBR1 in a ligand-dependent manner. Moreover, we show that CD109 regulates the localization and the association of SMAD7/Smurf2 with TGFBR1. Finally, we demonstrate that CD109's inhibitory effect on TGF- $\beta$  signaling and responses require SMAD7 expression and Smurf2 ubiquitin ligase activity. Taken together, these results suggest that CD109 is an important regulator of SMAD7/Smurf2-mediated degradation of TGFBR1. *J. Cell. Biochem.* 113: 238–246, 2012. © 2011 Wiley Periodicals, Inc.

**KEY WORDS:** TGF- $\beta$ ; DEGRADATION; CO-RECEPTOR; SMAD7; SMURF2

The multifunctional growth factor, transforming growth factor- $\beta$  (TGF- $\beta$ ) is composed of three different subtypes: TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 that regulate numerous cellular processes, such as cell growth, differentiation, extracellular matrix deposition, and immune responses. Dysregulation of the TGF- $\beta$  pathway has been implicated in several human diseases, including autoimmune diseases, impaired wound healing, fibrotic disorders, and cancer [Gordon and Blobe, 2008].

TGF- $\beta$  mediates its effect by signaling through a pair of transmembrane serine/threonine kinases, known as type I (TGFBR1)

and type II (TGFBR2) receptors. Upon binding of TGF- $\beta$  to TGFBR2, a constitutively active kinase, TGFBR2 recruits TGFBR1 and induces its phosphorylation on its GS domain [Wieser et al., 1995]. The activated TGFBR1 phosphorylates its intracellular substrates, the R-SMADs, SMAD2, and SMAD3, on their C-terminal SSXS motif. The phosphorylated R-SMADs form complexes with the common SMAD, SMAD4. These complexes accumulate in the nucleus and regulate gene transcription. R-SMADs and SMAD4 possess two conserved domains connected by a linker region: an N-terminal MH1 domain, responsible for DNA binding and a C-terminal MH2

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domain, involved in receptor and SMAD interaction [Feng and Derynck, 2005].

A subclass of SMADs, called the inhibitory SMADs (SMAD6 and SMAD7), also have a C-terminal MH2 domain that does not contain the SSXS phosphorylation site, and lacks the MH1 domain. While SMAD6 inhibits more specifically BMP signaling, SMAD7 antagonizes TGF- $\beta$  signaling by different mechanisms. SMAD7 competes with SMAD2/3 for binding to the activated TGFBR1, thereby preventing the phosphorylation of SMAD2/3 [Imamura et al., 1997; Nakao et al., 1997]. Importantly, via its PY motif located on its linker region, SMAD7 binds to the WW domain of the HECT E3-ubiquitin ligases, SMAD ubiquitination regulatory factor (Smurf) 1 and Smurf 2. Thus, SMAD7 acts as an adaptor protein that recruits Smurf1/2 to the activated receptor complex, resulting in the ubiquitination of TGFBR1, followed by receptor degradation and termination of signaling [Kavsak et al., 2000; Ebisawa et al., 2001].

In most resting cells, SMAD7 localizes in the nucleus. Upon TGF- $\beta$  stimulation or after Smurf1/2 overexpression, SMAD7 is exported out of the nucleus and the SMAD7/Smurf complex interacts with the activated TGFBR1 at the plasma membrane [Suzuki et al., 2002]. Interestingly, because SMAD7 and Smurf2 reside in the lipid-raft/caveolar compartment, internalization of the TGF- $\beta$  receptor via the caveolar route is associated with ubiquitination by Smurf2 followed by TGF- $\beta$  receptor degradation [Di Guglielmo et al., 2003]. Thus, localization of the SMAD7/Smurf2 complex is essential for SMAD7 inhibitory function and molecules that modulate SMAD7/Smurf2 localization could be used as potential target to regulate TGF- $\beta$  signaling.

TGF- $\beta$  signaling can be modulated at the receptor level by TGF- $\beta$  co-receptors such as betaglycan, endoglin, or CD109, in a cell-specific manner [Bernabeu et al., 2009]. CD109 is a glycosyl phosphatidylinositol (GPI)-anchored protein of 180 kDa that belongs to the  $\alpha$ 2-macroglobulin/complement family [Lin et al., 2002]. It binds the TGF- $\beta$ 1 subtype with high affinity, but displays lower affinity for the other subtypes [Tam et al., 1998]. Moreover, CD109 forms a heteromeric complex with the TGF- $\beta$  signaling receptors and negatively regulates TGF- $\beta$  signaling in numerous cell types [Finsson et al., 2006]. The importance of CD109 in homeostasis is underscored by the observation that CD109 is mutated in colorectal cancer [Sjoblom et al., 2006] and that CD109 expression is deregulated in many cancers [Hashimoto et al., 2004; Zhang et al., 2005; Finsson et al., 2006; Hasegawa et al., 2007; Sato et al., 2007; Hagiwara et al., 2008; Hasegawa et al., 2008] and in psoriasis [Litvinov et al., 2011]. Given the potential significance of CD109 in regulating TGF- $\beta$  signaling in various diseases, delineating its mechanism of action is important, as it may lead to the development of novel therapeutic strategies. Recently, we have demonstrated that CD109 promotes the localization of TGF- $\beta$  receptors into the caveolae, a compartment of signaling down-regulation. Moreover, we have shown that CD109 promotes TGF- $\beta$  receptor degradation [Bizet et al., 2011]. Here, we investigate whether SMAD7 and Smurf2 are involved in this process. We demonstrate that CD109 enhances SMAD7/Smurf2-dependent degradation of TGFBR1 in a ligand-dependent manner, likely by increasing the association of SMAD7/Smurf2 with the activated TGF- $\beta$  receptors, thereby inhibiting TGF- $\beta$  signaling and responses.

## MATERIALS AND METHODS

### CELL LINES

The human keratinocyte cell line HaCaT, kindly provided by P. Boukamp (Heidelberg, Germany), human embryonic kidney (HEK) 293 cells and COS-1 cells, both purchased from the American Type Culture Collection, were cultured as described previously [Finsson et al., 2006]. HaCaT clones stably expressing CD109 (or its empty vector, EV) were selected and cultured in the presence of 0.5 mg/ml Geneticin (Invitrogen, Carlsbad, CA).

### TRANSIENT TRANSFECTIONS AND siRNA TREATMENT

HaCaT, 293, and COS-1 cells were transfected with different combinations of the following plasmids: CD109 or its empty vector (EV, pCMVSPORT6), 6myc-SMAD7-WT (gift from T. Imamura, The JFCR Cancer Institute, Tokyo), TGFBR2 and TGFBR1-WT or T204D-HA, HA-SMAD7-WT or  $\Delta$ PY, Flag-Smurf2WT or C716A (gifts from J. Wrana, University of Toronto) using Superfect (Qiagen, Mississauga, ON, Canada). Alternatively, HaCaT cells were transfected with CD109 siRNA (ID#129083), SMAD7 siRNA # 1 (ID#17186), SMAD7 siRNA #2 (ID#17280) or a negative control siRNA (ID#4611) (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen).

### CO-IMMUNOPRECIPITATION AND WESTERN BLOT

Lysates from 293 cells were immunoprecipitated with a mouse monoclonal anti-HA (hemagglutinin) antibody (12CA5, Abgent, San Diego, CA). Western blot analyses were conducted with the following antibodies: Mouse monoclonal anti-CD109 (TEA 2/16, BD Biosciences), anti-myc (9E10, ebiosciences, San Diego, CA) or anti-Flag-M2 (Sigma Aldrich), anti-PAI-1 (Plasminogen Activator Inhibitor -1) and anti-fibronectin (both from BD Biosciences), anti- $\beta$ -actin antibodies (Santa Cruz Biotechnology) and rabbit polyclonal anti-TGFBR1 (Cell Signaling Technologies, Danvers, MA).

### IMMUNOFLUORESCENCE MICROSCOPY

293 cells were incubated with 250 pM biotinylated-TGF- $\beta$ 1 (R&D, Minneapolis, MN) for 2 h at 4°C, then with 10  $\mu$ g/ml streptavidin-AF647 for 1 h at 4°C and placed at 37°C in D-MEM, 10% FBS, for 30 min as described in Bizet et al. [2011]. Cells were fixed with 4% paraformaldehyde and permeabilized with methanol. Non-specific sites were blocked with 10% normal goat serum in PBS. Cells were stained using an anti-CD109 antibody followed by an Alexa-Fluor488 (AF488) conjugated anti-mouse IgG (Invitrogen) or by a Cy5-conjugated Fab fragment goat anti-mouse IgG (Jackson ImmunoResearch Lab, West Grove, PA) if another mouse antibody was used in the following steps. Cells were then stained with a rabbit anti-Flag antibody followed by a Cy3 conjugated anti-rabbit IgG (all from Sigma Aldrich) or with an AF488 conjugated mouse anti-HA antibody (Invitrogen). Cells were mounted in Mowiol as described previously [Blanc et al., 2005]. Images were acquired with a LSM 510 META Axioplan 2 confocal laser scanning microscope (Carl Zeiss, Toronto, ON, Canada). After background removal by median filtering [Landmann and Marbet, 2004], Pearson correlation coefficient was calculated using the JaCoP plug-in for ImageJ [Bolte and Cordelières, 2006].

## LUCIFERASE ASSAY

Cell lysates from 293 cells transfected with (CAGA)<sub>12</sub>-lux [Denner et al., 1999] and pCMV- $\beta$ -galactosidase, CD109, TGFBR1-T204D, Smurf2-WT, or Smurf2C716A (or their empty vectors) were analyzed for luciferase activity and the values were normalized to  $\beta$ -galactosidase activity.

## STATISTICAL METHODS

Numerical results are represented as means of *n* independent experiments  $\pm$  SEM. A two-tailed Student *t*-test was used to determine statistical significance between two groups. Comparisons within more than two groups were made by one-way analysis of variance and multiple comparisons were made by Holm-Sidak test (post-hoc) using the SigmaPlot software. A value of  $P < 0.05$  was considered significant.

## RESULTS

### CD109 FACILITATES SMAD7/Smurf2-MEDIATED DEGRADATION OF TGFBR1 IN A TGF- $\beta$ -DEPENDENT MANNER

We have previously demonstrated that overexpression of CD109 in HaCaT and 293 cells accelerates TGF- $\beta$  receptor degradation [Bizet et al., 2011]. Here, we show that knockdown of CD109 expression using siRNA in HaCaT cells significantly ( $P < 0.05$ ) slows down TGFBR1 degradation following TGF- $\beta$  treatment, as compared to control siRNA transfected cells (Fig. 1A). This demonstrates that endogenous CD109 is able to promote TGFBR1 degradation in the presence of ligand. Because we have previously demonstrated that CD109 promotes localization of TGF- $\beta$  receptors to the caveolar compartment in a ligand-dependent manner [Bizet et al., 2011], we decided to investigate whether CD109's effect on TGFBR1 degradation is also ligand-dependent. TGFBR1 levels decrease after TGF- $\beta$  addition (Fig. 1B, lane 1 vs. 5), but this decrease is not observed when CD109 expression is knocked down using CD109-specific siRNA (Fig. 1B, lane 5 vs. 6). In contrast, no significant difference in TGFBR1 level is detected in the absence of ligand between control and CD109 siRNA transfected cells (Fig. 1B, lane 1 vs. 2). This result suggests that endogenous CD109 promotes TGFBR1 degradation, in the presence but not in the absence of ligand.

Because SMAD7 is known to be a critical adaptor for Smurf2-mediated degradation of TGFBR1, we then examined whether CD109's effect on TGFBR1 degradation involves SMAD7 expression. When SMAD7 expression is knocked down, (Supplementary Fig. 1 shows siRNA efficiency) TGFBR1 levels increase in the presence of TGF- $\beta$  (Fig. 1B, lane 7 vs. 5), which is consistent with SMAD7's ability to mediate TGF- $\beta$ -induced TGFBR1 degradation. Importantly, CD109 has no effect on TGFBR1 levels when SMAD7 expression is knocked down (Fig. 1B, lane 7 vs. 8), demonstrating that CD109's ability to promote TGFBR1 degradation requires SMAD7 expression. Taken together, these results suggest that CD109 promotes ligand-dependent degradation of TGFBR1 by a mechanism that requires SMAD7.

Next, we sought to determine whether CD109's effect on TGFBR1 degradation involves both SMAD7 and the E3 ubiquitin ligase Smurf2. Overexpression of Smurf2 and SMAD7 in COS-1 cells leads

to a decrease in TGFBR1 levels, consistent with their known ability to induce TGFBR1 degradation (Fig. 1C, lane 3 and 4 vs. lane 1 and 2 and lane 7 and 8 vs. 5 and 6). Importantly, in the presence of TGF- $\beta$ , CD109 overexpression further decreases the level of TGFBR1 when SMAD7 and Smurf2 are co-transfected (Fig. 1C, lane 8 vs. 7), suggesting that CD109 acts synergistically with SMAD7 and Smurf2 to mediate TGFBR1 degradation. However, in the absence of TGF- $\beta$ , CD109 has no effect on TGFBR1 degradation (Fig. 1C, lane 4 vs. 3). The lack of significant effect of CD109 overexpression on TGFBR1 levels observed in the absence of SMAD7/Smurf2 transfection, but in the presence of ligand (Fig. 1C, lane 5 vs. 6) might be due to the short duration of treatment (90 min), which might be insufficient to induce CD109-mediated degradation in COS-1 cells, and/or to the low level of endogenous SMAD7 in these cells. This raises the possibility that CD109 may require transcription of SMAD7 to facilitate TGFBR1 degradation. Interestingly, in the presence of TGF- $\beta$ , CD109 overexpression also decreases SMAD7 and Smurf2 levels (Fig. 1C, lane 7 vs. 8). This observation is consistent with previous reports demonstrating that both SMAD7 and TGFBR1 are ubiquitinated by Smurf2 [Kavsak et al., 2000] and that Smurf2 mediates its own auto-ubiquitination [Ogunjimi et al., 2005]. Importantly, this result suggests that CD109 promotes the degradation of SMAD7/Smurf2/TGFBR1 complex, in the presence of TGF- $\beta$ .

We further examined the ability of CD109 to promote the degradation of a constitutively active TGFBR1. CD109 overexpression significantly decreases the level of activated TGFBR1 (TGFBR1-T204D) in both COS-1 and 293 cells transfected with SMAD7 and Smurf2 (Fig. 1D), indicating that CD109 enhances degradation of activated TGFBR1.

### CD109 PROMOTES THE ASSOCIATION OF SMAD7/ Smurf2 WITH TGFBR1

Next, we explored the possibility that CD109 promotes TGFBR1 degradation by altering SMAD7/Smurf2 association with the TGF- $\beta$  receptors. Co-immunoprecipitation experiments reveal that the association between TGFBR1 and SMAD7 is enhanced when CD109 is overexpressed, as compared to EV transfected cells, in 293 cells (Fig. 2A). This result indicates that CD109 may promote or stabilize the interaction between SMAD7 and TGFBR1. Moreover, both SMAD7 and CD109 are co-immunoprecipitated with TGFBR1, suggesting that CD109 may form a complex with TGFBR1 and SMAD7. Consistent with the above result, CD109 is able to associate with SMAD7 as shown by co-immunoprecipitation of CD109 with SMAD7 (Fig. 2B). In addition, immunofluorescence and confocal microscopy analysis reveals a strong colocalization signal between CD109 (visualized in blue), SMAD7 (in green), and Smurf2 (in red) (Fig. 2C), consistent with the notion that a complex between CD109, SMAD7 and Smurf2 is formed. Next, we analyzed whether CD109 modulates the association of SMAD7 with Smurf2-WT or with Smurf2-C716A [Kavsak et al., 2000], a catalytically inactive mutant of Smurf2. Interestingly, CD109 overexpression increases the association between SMAD7 and Smurf2-WT (Fig. 2D, lane 2 vs. 3, visualized on a high exposure film) or Smurf2-C716A (Fig. 2D, lane 4 vs. 5, visualized on a lower exposed film), as compared to EV transfection. When SMAD7 is co-transfected, the

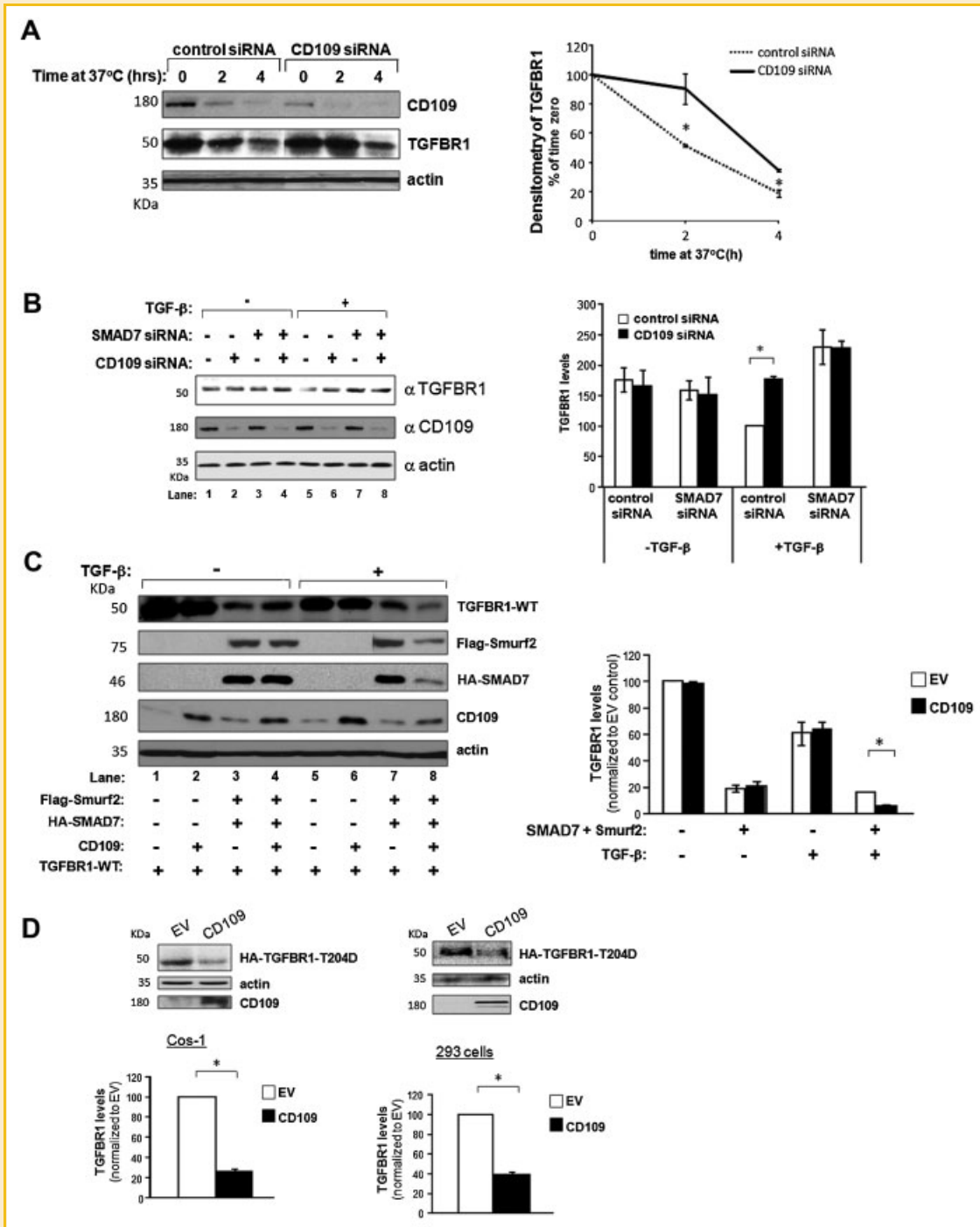


Fig. 1. CD109 increases SMAD7/Smurf2-mediated TGFBR1 degradation. A: Left panel: HaCaT cells transfected with control or CD109 siRNA were pre-incubated with 100 pM TGF-β at 4°C, and incubated at 37°C for 0–4 h. Cell lysates were analyzed by western blot using the indicated antibodies. Right panel: Densitometry of TGFBR1 (expressed as a percentage of its level at time 0 h; mean of n = 3 independent experiments, ±SEM, \*P < 0.05). B: Left panel: HaCaT cells transfected with CD109 siRNA, SMAD7 siRNA#1, or negative control siRNA were treated with 100 pM TGF-β for 16 h to observe the degradation of TGFBR1 without any pre-incubation. Cell lysates were analyzed by western blot using the indicated antibodies. Right panel: Densitometry of TGFBR1 (mean of n = 3 independent experiments, ±SEM, \*P < 0.05). C: Left panel: Cell lysates from COS-1 cells co-transfected with the indicated cDNA and treated with or without 100 pM TGF-β for 90 min were analyzed by western blot using anti-TGFBR1, anti-HA, anti-Flag, and anti-CD109 antibodies. Right panel: Densitometry of TGFBR1 (mean of n = 3 independent experiments, ±SEM, \*P < 0.05). D: Lysates from COS-1 cells (top left panel) and 293 cells (top right panel), co-transfected with HA-TGFBR1-T204D, HA-SMAD7, Flag-Smurf2 and CD109 or EV were analyzed by western blot using the same antibodies as in (C), except that HA-TGFBR1-T204D was detected using an anti-HA antibody. Bottom panel: Densitometry of TGFBR1 (mean of n = 3 independent experiments, ±SEM, \*P < 0.05).

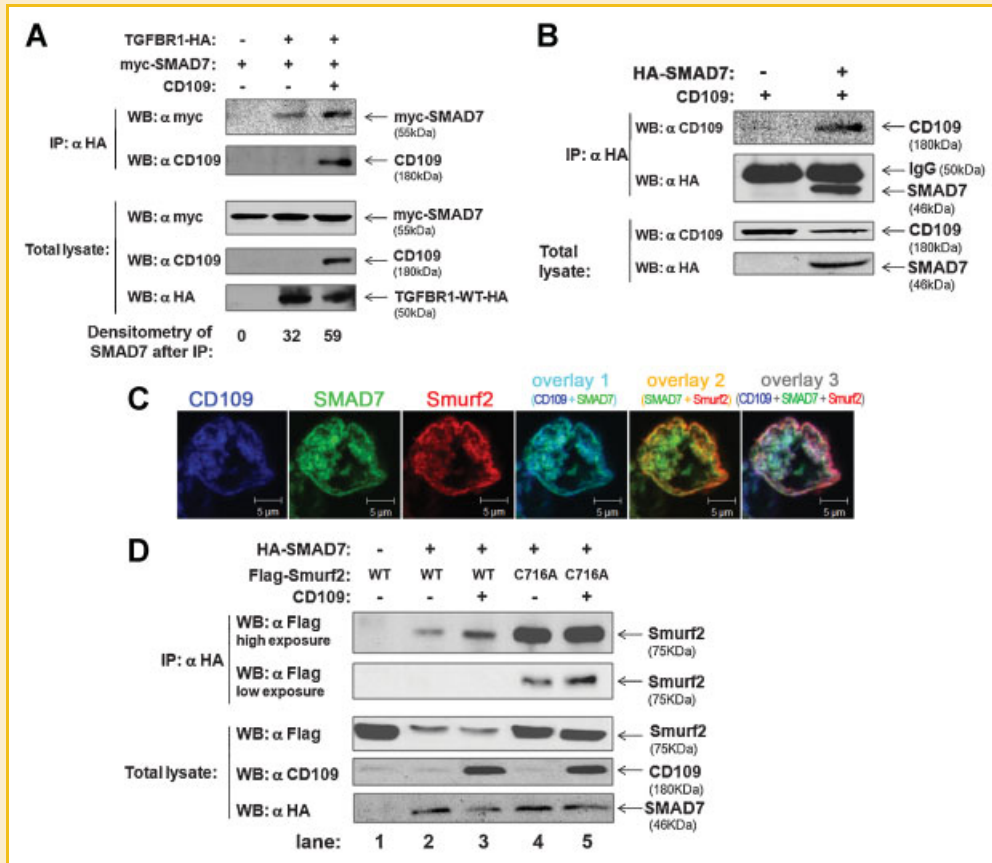


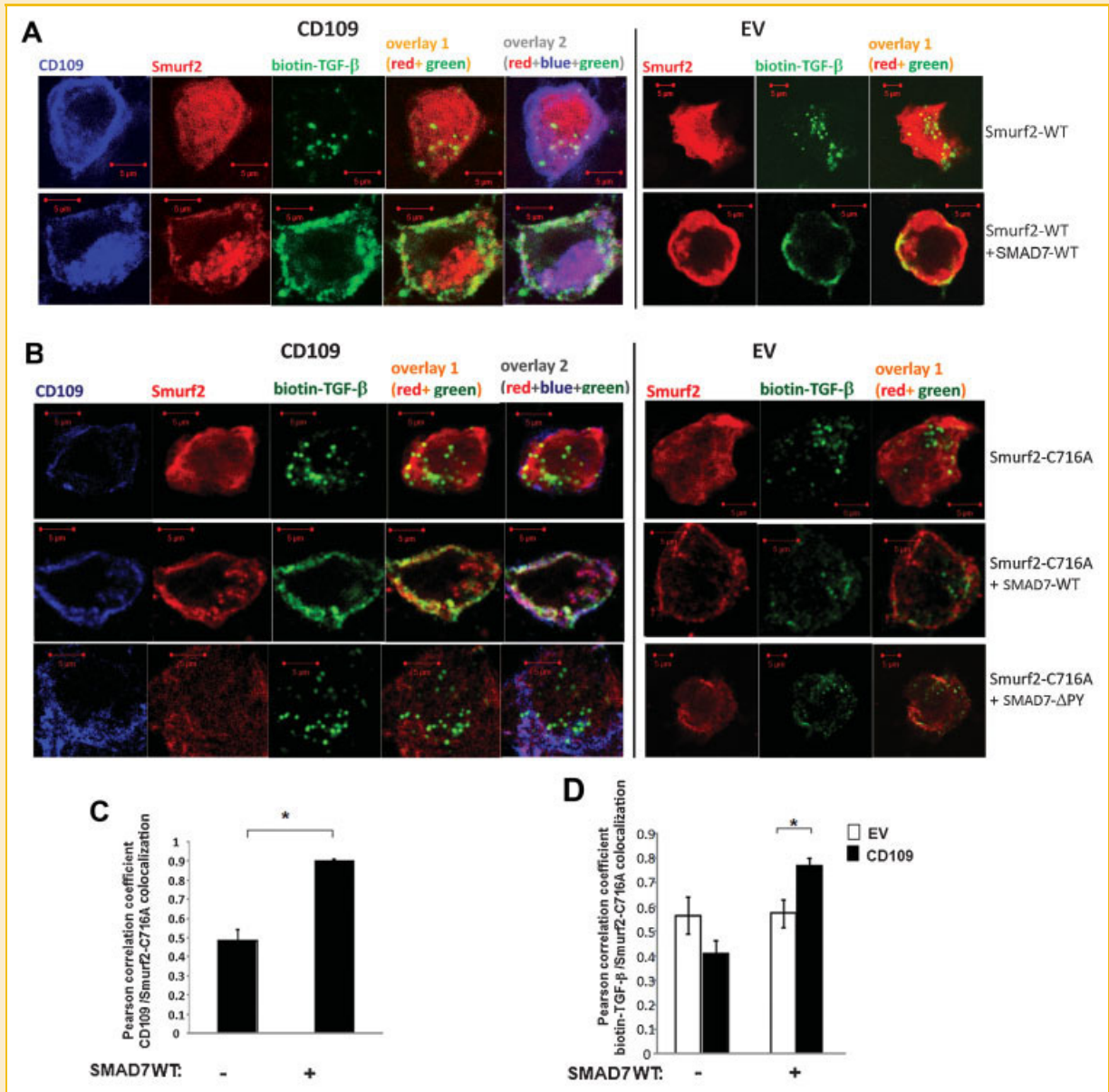
Fig. 2. CD109 promotes the association of SMAD7/Smurf2 with TGFBR1. A, B, D: Co-Immunoprecipitation: Lysates from 293 cells transfected with TGFBR1-HA (A) or TGFBR1 (B and D) and TGFBR2 and the indicated cDNAs and treated with TGF- $\beta$  for 90 min were subjected to immunoprecipitation with an anti-HA antibody followed by western blot using the indicated antibodies. 6myc-SMAD7 run at the expected size of 55 kDa (6myc tag: 10 kDa + SMAD7: 46 kDa). Densitometry of myc-SMAD7 levels in the immunoprecipitates (A) is indicated at the bottom. (C) Immunofluorescence: 293 cells transfected with CD109, Flag-Smurf2-C716A, and HA-SMAD7 were stained for CD109 (blue), Flag (red), and HA (green) and analyzed by confocal microscopy. Colocalization of CD109 and SMAD7 appears in turquoise (overlay 1), while colocalization of SMAD7 and Smurf2 appears in yellow (overlay 2). Triple colocalization appears in white (overlay 3). A representative cell from  $n = 3$  independent experiments is shown.

level of Smurf2-WT (Fig. 2D, lane 1 vs. 2 and 3, total lysate) decreases, likely due to SMAD7's ability to relieve autoinhibition of Smurf2-WT [Wiesner et al., 2007]. Moreover, CD109 overexpression leads to a greater decrease in the level of Smurf2-WT (but not Smurf2-C716A) (Fig. 2D, lane 2 vs. 3, total lysate) and a decrease in SMAD7 (Fig. 2D, lane 2 vs. 3, total lysate), as seen in Figure 1C, indicating that CD109 promotes the degradation of the SMAD7/Smurf2 complex. Collectively, our results suggest that CD109 enhances TGFBR1 degradation by promoting and/or stabilizing the association of SMAD7/Smurf2 with the TGF- $\beta$  receptors.

#### CD109 INCREASES COLOCALIZATION OF Smurf2 WITH THE TGF- $\beta$ RECEPTORS, IN A SMAD7-DEPENDENT MANNER

Because localization of SMAD7 and Smurf2 to TGF- $\beta$  receptors is a critical step in the process by which Smad7/Smurf2 mediates TGF- $\beta$  receptor degradation, we next examined whether CD109 promotes colocalization of Smurf2 with the TGF- $\beta$  receptors in a SMAD7-dependent manner. We have previously validated the use of biotin-TGF- $\beta$  followed by streptavidin treatment to specifically detect TGF- $\beta$ -bound to TGFBR1/TGFBR2 in 293 cells [Bizet et al., 2011].

Thus, we used this system to analyze the colocalization of CD109 (blue), biotin-TGF- $\beta$  (green), and Smurf2 (red), in the presence or absence of SMAD7, by confocal microscopy (Fig. 3). Compared to previous experiments (Figs. 1 and 2), cells were treated with TGF- $\beta$  for a shorter period (30 min) to observe the early event of the complex formation (Fig. 3A), before too much degradation occurs. Moreover, because Smurf2-WT transfection induces TGF- $\beta$  receptor degradation (even at time 0 of TGF- $\beta$  treatment, see Fig. 1C), quantitative colocalization analyses were conducted with Smurf2-C716A, a mutant unable to ubiquitinate the receptors [Kavsak et al., 2000]. No major differences between Smurf2-WT and Smurf2-C716A transfected cells are observed in the cellular localization of Smurf2, CD109 or biotin-TGF- $\beta$  (Fig. 3A vs. B). As reported previously [Kavsak et al., 2000; Suzuki et al., 2002], transfection of SMAD7-WT induces a relocalization of Smurf2-C716A at the plasma membrane where it colocalizes with biotin-TGF- $\beta$  (i.e., activated receptors) (Fig. 3B, middle panel). Interestingly, overexpression of SMAD7-WT increases the colocalization of Smurf2-C716A with CD109 (Fig. 3B,C), suggesting that CD109 forms a complex with Smurf2, in the presence of SMAD7. Importantly, in the presence of SMAD7-WT, CD109 overexpression



**Fig. 3.** CD109 promotes colocalization of Smurf2 with TGF- $\beta$  receptors, in a SMAD7-dependent manner. **A:** 293 cells transfected with CD109, TGFBR1/TGFBR2, Flag-Smurf2-WT, HA-SMAD7-WT, or their corresponding empty vectors were treated with biotin-TGF- $\beta$ /streptavidin (green) and incubated for 30 min at 37°C. After fixation, cells were stained for CD109 (blue) and Flag (red) and analyzed by confocal microscopy. The colocalization of biotin-TGF- $\beta$  and Flag-Smurf2-WT appears in yellow (overlay 1). The colocalization of CD109 and Flag-Smurf2-WT appears in purple and triple colocalization of TGF- $\beta$ , Smurf2 and CD109 appears in white (overlay 2). **B:** 293 cells transfected with CD109, TGFBR1/TGFBR2, Flag-Smurf2-C716A, HA-SMAD7-WT, or HA-SMAD7- $\Delta$ PY or their corresponding empty vectors were treated with biotin-TGF- $\beta$ /streptavidin and stained as in (A). The colocalization of biotin-TGF- $\beta$  and Flag-Smurf2-C716A appears in yellow (overlay 1). The colocalization of CD109 and Flag-Smurf2-C716A appears in purple and triple colocalization of TGF- $\beta$ , Smurf2 and CD109 appears in white (overlay 2). Representative cells from  $n = 3$  independent experiments are shown. **C** and **D** Quantitative analysis (using Pearson correlation coefficient) of the colocalization between CD109 and Flag-Smurf2-C716A (**C**) and between biotin-TGF- $\beta$  and Flag-Smurf2-C716A (**D**), mean  $\pm$  SEM, \* $P < 0.05$ .

enhances the colocalization of Smurf2-C716A with the biotin-TGF- $\beta$ -bound TGF- $\beta$  receptors (Fig. 3B, middle panel and D), as compared to EV transfected cells. In contrast, in the absence of SMAD7, TGF- $\beta$  receptors are found mainly in cytoplasmic vesicles (Fig. 3B, top panel). These results suggest that CD109 can increase the association between the E3 ubiquitin ligase Smurf2 and the activated TGF- $\beta$  receptors in a SMAD7-dependent manner. The mechanism(s) involved in the localization of biotin-TGF- $\beta$

(activated receptors) mainly at the plasma membrane and not in cytoplasmic vesicles in CD109/SMAD7-WT overexpressing cells remains to be delineated (Fig. 3B, middle panel). Interestingly, in the presence of SMAD7- $\Delta$ PY, a mutant unable to bind Smurf2, biotin-TGF- $\beta$  is located in cytoplasmic vesicles in both EV and CD109 transfected cells (Fig. 3B, bottom panel), indicating that CD109's ability to enhance Smurf2/TGF- $\beta$  receptor colocalization requires the interaction of SMAD7 with Smurf2.

## INHIBITION OF TGF- $\beta$ SIGNALING BY CD109 INVOLVES SMAD7 AND Smurf2 UBIQUITIN LIGASE ACTIVITY

The results presented so far show that CD109 promotes SMAD7/Smurf2-dependent degradation of TGF- $\beta$  receptors. We next investigated if this mechanism is involved in mediating CD109's inhibitory effect on TGF- $\beta$  signaling and responses. First, we examined whether CD109's inhibitory effect on transcriptional activity induced by a constitutively active TGFBR1 (TGFBR1-T204D) requires Smurf2 ubiquitin ligase activity using the SMAD3-responsive (CAGA)<sub>12</sub>-lux reporter construct, in 293 cells. CD109 inhibits TGFBR1-T204D-induced signaling in control (pcDNA3 transfected) cells and in the presence of Smurf2-WT, but not in the presence of Smurf2-C716A (Fig. 4A). The decreased transcriptional activity in Smurf2-WT (compared to pcDNA3 transfected cells) is likely due to an increase in TGFBR1 degradation, while the increased transcriptional activity in Smurf2-C716A (compared to pcDNA3 transfected cells) may be due to a decrease in TGFBR1 (endogenous TGFBR1 and transfected TGFBR1-T204D) degradation (Fig. 4A).

TGF- $\beta$  is a potent inducer of extracellular matrix protein, such as fibronectin and PAI-1 (plasminogen activator inhibitor-1), and we have previously shown that CD109 inhibits TGF- $\beta$ -induced extracellular matrix synthesis [Finsson et al., 2006]. Thus, we

examined whether Smurf2 ubiquitin ligase activity is required for CD109's inhibitory effect on TGF- $\beta$ -induced fibronectin synthesis in HaCaT cells. CD109 knockdown increases TGF- $\beta$ -induced fibronectin expression in control transfected cells (Fig. 4B, lane 3 vs. 4) and in Smurf2WT (Fig. 4B, lane 5 vs. 6) but not Smurf2-C716A transfected cells (Fig. 4B, lane 7 vs. 8), indicating that endogenous CD109 dampens TGF- $\beta$  responses by a mechanism that involves Smurf2 ubiquitin ligase activity. In addition, transfection of CD109 siRNA leads to an increase in TGF- $\beta$ -induced PAI-1 levels (Fig. 4C, lane 3 vs. 4) in control siRNA transfected cells, but not in SMAD7 siRNA transfected cells (Fig. 4C, lane 7 vs. 8 and lane 11 vs. 12). These results suggest that CD109's inhibition of TGF- $\beta$  responses require SMAD7 expression. Altogether, our results suggest that CD109 negatively regulates TGF- $\beta$  action by enhancing SMAD7/Smurf2-mediated TGFBR1 degradation.

## DISCUSSION

The novel TGF- $\beta$  co-receptor, CD109, is a GPI-anchored protein with high affinity for the TGF- $\beta$ 1 subtype and forms a complex with the TGF- $\beta$  signaling receptors, inhibiting TGF- $\beta$  signaling in vitro

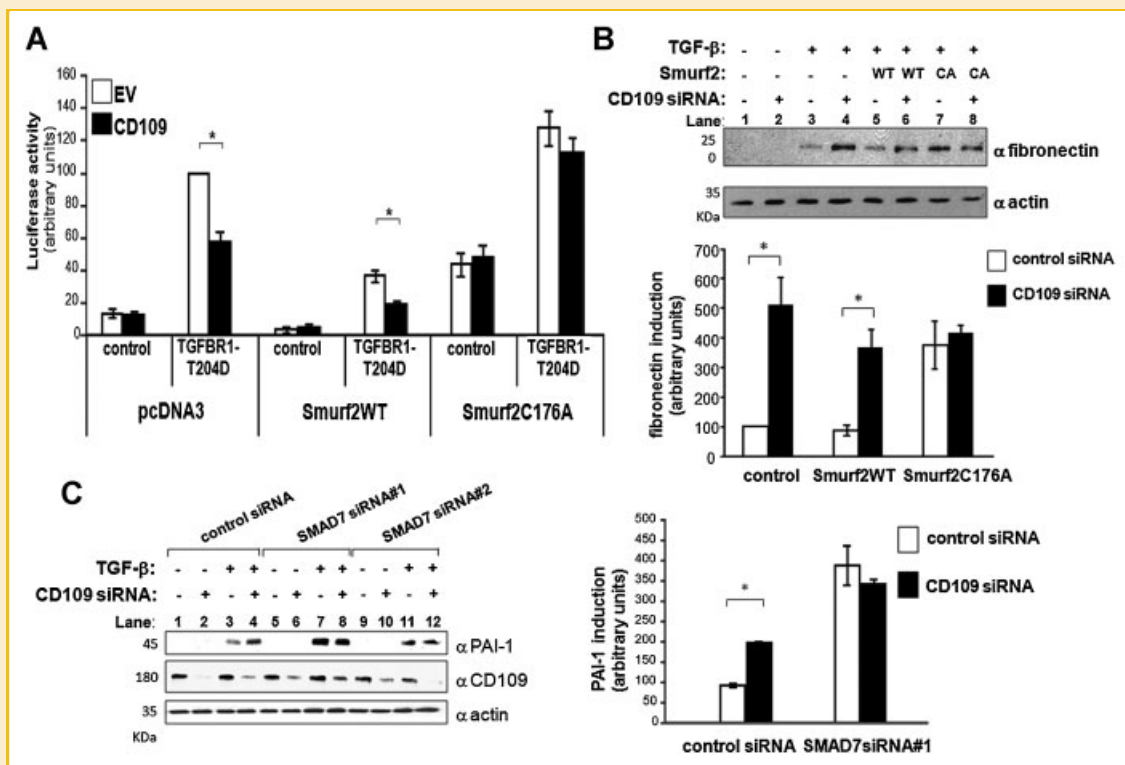


Fig. 4. CD109's inhibition of TGF- $\beta$  responses involves Smurf2 ubiquitin ligase activity and SMAD7. A: Cell lysates from 293 cells transfected with CD109, TGFBR1-T204D, Smurf2-WT, Smurf2-C716A or their empty vectors, (CAGA)<sub>12</sub>-lux and  $\beta$ -galactosidase were analyzed for luciferase and  $\beta$ -galactosidase activities. Luciferase activity was normalized to  $\beta$ -galactosidase and results were expressed as percentage of EV + TGFBR1-T204D control (mean of  $n = 4$  independent experiments  $\pm$  SEM,  $^*P < 0.05$ ). B: HaCaT cells transfected with CD109 siRNA or negative control siRNA, Smurf2-WT or Smurf2-C716A were treated for 16 h with or without 100 pM TGF- $\beta$ 1 and were analyzed by western blot for fibronectin and actin. Bottom panel: Densitometry for TGF- $\beta$  induced fibronectin of  $n = 3$  independent experiments,  $^*P < 0.05$ . C: Left panel: HaCaT cells transfected with CD109 siRNA, SMAD7 siRNAs or negative control siRNA were treated with 100 pM TGF- $\beta$ 1 for 16 h, and were analyzed by western blot for PAI-1, CD109, and actin. Bottom panel: Densitometry for TGF- $\beta$ -induced PAI-1 of  $n = 3$  independent experiments,  $^*P < 0.05$ .

[Finsson et al., 2006]. Deregulation of CD109 expression or mutation of CD109 gene occurs in many cancers [Hashimoto et al., 2004; Zhang et al., 2005; Sjoblom et al., 2006; Hasegawa et al., 2007; Sato et al., 2007; Hagiwara et al., 2008; Hasegawa et al., 2008], underscoring its potential relevance in vivo. We have previously shown that CD109 downregulates TGF- $\beta$  signaling by promoting TGF- $\beta$  receptor localization into the lipid raft/caveolae compartment and by enhancing TGF- $\beta$  receptor degradation [Bizet et al., 2011]. However, the mechanisms by which CD109 promotes TGF- $\beta$  receptor degradation are not known. Our findings in the current study show that CD109 acts synergistically with SMAD7 and Smurf2 to decrease TGFBR1 levels in the presence of ligand and that SMAD7 expression is essential for CD109's effect in promoting TGFBR1 degradation, indicating that CD109 enhances SMAD7/Smurf2-mediated degradation of the activated TGF- $\beta$  receptors. Moreover, we demonstrate that CD109 increases the association of SMAD7/Smurf2 with the activated TGF- $\beta$  receptors. In addition, we show that CD109's inhibitory effect on TGF- $\beta$  signaling and responses involve SMAD7 expression and Smurf2 ubiquitin ligase activity. Taken together, these results suggest that CD109 inhibits TGF- $\beta$  signaling by promoting and/or stabilizing that association of SMAD7/Smurf2 to the TGF- $\beta$  receptors, thereby enhancing their degradation in the presence of ligand.

Previously, we have shown that CD109 promotes the localization of TGFBR1 into caveolae [Bizet et al., 2011]. Our results in the present study demonstrating that CD109 enhances TGF- $\beta$  receptor degradation mediated by SMAD7/Smurf2 is consistent with the notion that localization to caveolae promotes TGF- $\beta$ -receptor degradation due to the presence of SMAD7 and Smurf2 in the caveolar compartment [Di Guglielmo et al., 2003]. Moreover, our finding that CD109 colocalizes with SMAD7 and Smurf2 is in agreement with the previous reports that CD109 [Bizet et al., 2011] and SMAD7 [Di Guglielmo et al., 2003] localize to caveolae. The association between CD109 and SMAD7 is likely indirect since CD109 is a cell-surface GPI-anchored protein and SMAD7 is a nuclear/cytosolic protein. It is likely that TGFBR1 links CD109 with SMAD7, since TGFBR1 has been shown to interact directly with CD109 [Finsson et al., 2006] and SMAD7 [Hayashi et al., 1997]. By promoting TGFBR1 localization to the caveolae in the presence of TGF- $\beta$  [Bizet et al., 2011], CD109 may facilitate the recruitment of SMAD7/Smurf2 to TGF- $\beta$ -bound receptors leading to their degradation.

The subcellular localization of SMAD7 and Smurf2 is a critical determinant of their function. CD109, by regulating the localization of SMAD7/Smurf2 to the plasma membrane (and to the TGF- $\beta$ -bound receptors), may act as a key modulator of SMAD7/Smurf2 function. CD109 may thus control TGF- $\beta$  receptor levels both by promoting the localization of TGFBR1 to the caveolar compartment and by linking it to SMAD7/Smurf2-mediated degradation of TGFBR1. Therefore, it is likely that an aberrant expression or localization of CD109 may lead to abnormal TGFBR1 degradation, resulting in uncontrolled TGF- $\beta$  signaling.

We have previously demonstrated that the CD109-mediated caveolar localization of TGFBR1 occurs in a TGF- $\beta$ -dependent manner [Bizet et al., 2011]. Here, we provide evidence that CD109 promotes TGFBR1 degradation also in a ligand-dependent manner.

Our results showing that (overexpressed and endogenous) CD109 decreases the level of TGFBR1 in the presence, but not in the absence, of ligand and that CD109 reduces the level of a constitutively active TGFBR1 suggests that CD109 targets preferentially the activated TGFBR1 to the caveolae and thus stabilizes the complex formation of activated TGFBR1 with SMAD7/Smurf2. Therefore, CD109 accelerates TGFBR1 degradation in the presence of ligand. However, it remains to be determined whether and why CD109 is unable to facilitate SMAD7/Smurf2 complex formation with TGFBR1 in the absence of ligand. CD109 does not sequester TGF- $\beta$  receptor to caveolae where SMAD7/Smurf2 localize, in unstimulated cells. Moreover, it is possible that when TGF- $\beta$  levels are below threshold, SMAD7 expression might be too low to observe an effect of CD109 on TGFBR1 degradation. TGF- $\beta$  has been shown to induce SMAD7 expression [Nakao et al., 1997], which acts in a negative feedback loop by recruiting the E3 ubiquitin ligase Smurf2 to activated TGFBR1, leading to TGF- $\beta$  receptor degradation and termination of signaling [Kavak et al., 2000]. Our findings that CD109 enhances SMAD7/Smurf2 association with the TGF- $\beta$  receptors and that CD109 enhances SMAD7/Smurf2-mediated degradation of the activated TGFBR1 suggest that CD109 may participate in the SMAD7 negative feedback loop to decrease the intensity and duration of TGF- $\beta$  signaling. Furthermore, our observation that CD109's inhibition of fibronectin and PAI-1 expression involves Smurf2 activity and SMAD7 expression indicate that CD109 downregulates TGF- $\beta$  responses by promoting SMAD7/Smurf2-dependent TGF- $\beta$  receptor degradation. Together, our results suggest that CD109 plays a critical role in TGF- $\beta$  signal termination.

Escape from CD109's negative regulation of TGF- $\beta$  signaling and thus its dampening effect on TGF- $\beta$  responses may result in aberrant TGF- $\beta$  action that often leads to many human diseases such as cancer metastasis and tissue fibrosis. Thus, because alterations in CD109 expression and function can result in abnormal TGF- $\beta$  responses, CD109 may represent a novel therapeutic target for treatment of diseases in which TGF- $\beta$  is known to play a pathophysiological role.

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