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Binding of RhoA by the C2 domain of E3 ligase Smurf1 is essential for Smurf1-regulated RhoA ubiquitination and cell protrusive activity

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ABSTRACT

Smurf1-mediated RhoA ubiquitination and degradation plays key roles in regulation of cell polarity and protrusive activity. However, how Smurf1 recognizes RhoA is still not clear. Here we report that the C2 domain of Smurf1 is necessary and sufficient for binding RhoA, and therefore is crucial for targeting RhoA for ubiquitination. In contrast, the C2 domain is dispensable for Smurf1-mediated ubiquitination of Smad1. Consistent with its biochemical specificity, the C2 domain is essential for Smurf1-regulated protrusion formation but not BMP signaling. Therefore, our study reveals the mechanism of the C2 domain of Smurf1 in substrate selection.

Structured summary of protein interactions:
SMURF1 physically interacts with **Smad1** by pull down (View interaction)
SMURF1 physically interacts with **RhoA** by pull down (View interaction)
SMURF1 physically interacts with **Smad1** by anti tag coimmunoprecipitation (View interaction)
SMURF1 physically interacts with **RhoA** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

The Nedd4 (neural precursor cell-expressed developmentally down-regulated 4)-like family of E3 ubiquitin ligases, which are a special subgroup of the HECT E3 ubiquitin ligases that all contain a C2 domain on the N-terminus, 2–4 WW domains in the middle, and a C-terminal HECT domain, and are thus also referred to as C2-WW-HECT E3 ubiquitin ligases [1]. The HECT (homologous to E6-AP C-terminus) domain possesses intrinsic catalytic activity for transferring ubiquitin to substrates through a conserved cysteine [2]. The WW domains are known to mediate the C2-WW-HECT E3s to interact with their substrates that contain PPXY (PY) or phospho-Ser/Thr–Pro (pS/TP) motifs [3]. The C2 domain was first recognized in classical PKCs as a Ca^{2+}-dependent phospholipid-binding domain in controlling protein subcellular localization. It is one of the most common lipid binding domains in mammals. However, there also exist a large number of C2 domains that show low or no affinity for Ca^{2+}, acting in a Ca^{2+}-independent manner [4]. In addition to membrane binding, some C2 domains, Ca^{2+}-dependent or Ca^{2+}-independent, are also involved in protein–protein interactions [5]. The Nedd4-like family of E3 ubiquitin ligases in fact contain the Ca^{2+}-independent C2 domains [6].

Smurf1 (Smad ubiquitination regulatory factor 1) and Smurf2 are two closely related members of the Nedd4-like family of E3 ligases, which were originally identified as negative regulators of TGFβ/BMP signaling through targeting the receptors or downstream Smads for ubiquitination and degradation [7]. Smurfs also regulate many other cellular functions such as cell polarity, epithelial-to-mesenchymal transition (EMT), and bone development by regulating the stability of RhoA, Rap1B, Prickle1, MEKK2, RUNX, and TRAF family proteins [8–15]. Smurfs utilize their WW domains to interact with the PY motifs present in diverse substrates including Smads, MEKK2, RUNX, and TRAFs. However, it remains unclear as to how Smurfs recognize the substrates that do not contain any PY motif, such as RhoA and Rap1B. A recent study showed that Smurf1 could target hPEM-2, a GEF for Cdc42, through its C2 domain in a Ca^{2+}-independent manner, suggesting a role of C2 domain in substrate selection for Smurf1 [16].

In this study, we report that the C2 domain is necessary and sufficient for Smurf1 binding RhoA but not Smad1, which is the founding substrate for the Smurfs. In agreement with such a discriminatory pattern of interaction, deletion of the C2 domain only affects Smurf1-mediated RhoA degradation and cytoskeleton remodeling, but not BMP signaling. These results reveal the recognition mechanism of RhoA by Smurf1 and further demonstrate that Smurf1 utilizes distinct regions for targeting diverse substrates.

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2. Materials and methods

2.1. Constructs and reagents

Full length human Smurf1 (wild type and C699A) and Smurf1ΔC2 were described previously [6,17]. Additional truncated mutants were constructed by inserting PCR fragments into appropriate vectors. Human RhoA, $RhoA^{N19}$ and Smad1 were described previously [12,17]. RhoA and Smad1 were separately inserted into pGEX-4-TEV vector [18] for bacterial expression of GST-RhoA and GST-Smad1 fusion protein, with a TEV protease cleavage site between GST and target protein. LLnL was from Boston Biochem.

2.2. Cell culture and transfection

HEK293T cells were grown in high-glucose DMEM (HyClone) containing 10% FBS (HyClone) at 37 °C in a humidified 5% CO_2 incubator. Mv1Lu lung epithelial cells were grown in MEM (HyClone) containing 10% FBS and 1% NEAA (non-essential amino acids) (HyClone). HEK293T cells were transiently transfected using the calcium–phosphate method as previously described [18]. Mv1Lu cells were transiently transfected using TurboFect (Fermentas) following the manufacturer's protocol.

2.3. Immunoprecipitation, immunoblotting, GST pull-down, and ubiquitination assays

Immunoprecipitation, immunoblotting, GST pull-down, in vivo and in vitro ubiquitination assays were all carried out as described previously [17,18]. The antibodies used in this work were anti-Flag M2 monoclonal antibody (Sigma), rat anti-HA monoclonal antibody or anti-HA monoclonal antibody conjugated with peroxidase (Roche 3F10), anti-Myc monoclonal antibody (Santa Cruz), anti-ubiquitin P4D1 monoclonal antibody (Santa Cruz), anti-Smad1 monoclonal antibody (Santa Cruz), and anti-β-actin monoclonal antibody (Santa Cruz).

2.4. Immunofluorescence assay

Mv1Lu cells grown on glass coverslips were transfected with Flag-tagged Smurf1 or its mutants. Cells were fixed with 4% paraformaldehyde, and stained using anti-Flag M2 monoclonal antibody and Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody. Texas Red conjugated Phalloidin was used to visualize F-actin. Alexa Fluor-conjugated secondary antibodies and Texas Red-conjugated Phalloidin were from Invitrogen. Images were obtained using a Leica TCS SPII confocal microscope and Leica confocal software.

2.5. Luciferase assay

BMP-responsive IBRE-Lux reporter plasmid [19] was transiently transfected into HEK293T cells together with pCMV-Gal. Luciferase activity was measured using the luciferase assay system (Promega) in a Varioskan Flash microplate reader (Thermo Scientific). Galactosidase activity was measured to ascertain the transfection efficiency as previously described [19].

3. Results

3.1. The C2 domain is critical for Smurf1-mediated RhoA ubiquitination and degradation

To investigate the function of C2 domain in Smurf1 towards its substrates, we examined the activities of wild-type Smurf1 and Smurf1ΔC2 on Smad1 and RhoA, two known substrates with or without PY motif, respectively. Interestingly, both wild-type Smurf1 and Smurf1ΔC2 could effectively down-regulate Smad1 (Fig. 1A). However, only wild-type Smurf1, but not Smurf1ΔC2, decreased the steady-state levels of RhoA (Fig. 1B), suggesting that the C2 domain of Smurf1 is required specifically for the Smurf1-regulated RhoA degradation. We next determined the ubiquitination levels of RhoA and Smad1 after co-expression with Smurf1 or its ΔC2 mutant. HEK293T cells were co-transfected with Smurf1 or its mutants and the substrates as indicated (Fig. 1C and D). Both wild type Smurf1 and Smurf1ΔC2 could dramatically enhance the ubiquitination of Smad1 (Fig. 1C). In contrast, only wild-type Smurf1 but not Smurf1ΔC2 markedly increased the ubiquitination levels of RhoA (Fig. 1D). We also performed in vitro ubiquitination assays using bacterially expressed proteins to examine the role of C2 domain in Smurf1 regulated substrate ubiquitination. Consistent with the in vivo ubiquitination assays, both wild type Smurf1 and Smurf1ΔC2 could induce Smad1 ubiquitination, whereas only wild type Smurf1 could ubiquitinate RhoA (Fig. 1E and F). Thus, the C2 domain is necessary for Smurf1-mediated RhoA ubiquitination and degradation.

3.2. The C2 domain of Smurf1 directly interacts with RhoA both in vivo and in vitro

We next examined the role of C2 domain in recognition of RhoA. For this purpose, we performed immunoprecipitation assays using a series of Smurf1 truncated mutants to map the RhoA binding domain in Smurf1 (Fig. 2A). In our previous study, we found that Smurf1 interacts with wild type RhoA directly in vitro, but that the interaction in vivo could only be detected with the inactive form of RhoA, $RhoA^{N19}$ [12]. Therefore, we detected the interaction between $RhoA^{N19}$ and Smurf1 or its mutants by co-immunoprecipitation assays. Indeed, similar to the full length Smurf1, both the C2 domain alone and a ΔHECT mutant that contains the C2 domain can associate with RhoA (Fig. 2B). In contrast, the Smurf1 C2 domain does not interact with Smad1; instead, the WW domains of Smurf1 are important for its interaction with Smad1 (Fig. 2C). We further carried out GST pull-down assays using proteins purified from bacteria to test whether the C2 domain could directly interact with RhoA or Smad1. Consistent with the co-immunoprecipitation result, the C2 domain alone can bind to RhoA (Fig. 2D), but not to Smad1 (Fig. 2E). Interestingly, although the ΔC2 mutant and WW domains each bind Smad1 with comparable affinity to the full length Smurf1 in the GST pull-down assays (Fig. 2E), they showed less association with Smad1 than the full length Smurf1 in the co-immunoprecipitation assays (Fig. 2C and E), suggesting that the C2 domain of Smurf1 in the cell might be needed to stabilize Smurf1/Smad1 complex, or to exclude other competing proteins for the Smad1 binding. In addition, the HECT domain could also co-immunoprecipitate with RhoA and Smad1 (Fig. 2B and C), but failed to associate with RhoA or Smad1 in the GST pull-down assays (Fig. 2D and E), indicating that the interaction between the HECT domain and RhoA or Smad1 might be indirect, and/or that post-translational modifications are involved. Taken together, these results identified the C2 domain of Smurf1 as a direct RhoA binding domain that is necessary for specific targeting of RhoA for ubiquitination and degradation.

3.3. The C2 domain is important for Smurf1-regulated protrusive activity but not for attenuating BMP signaling

Smurf1 has been shown to be a negative regulator for BMP signaling through targeting Smad1 for degradation, as well as a key regulator for cell polarity and protrusive activity by downregulating local RhoA at the leading edge [12,17]. To test for functional specificity of the Smurf1 C2 domain in the different biological

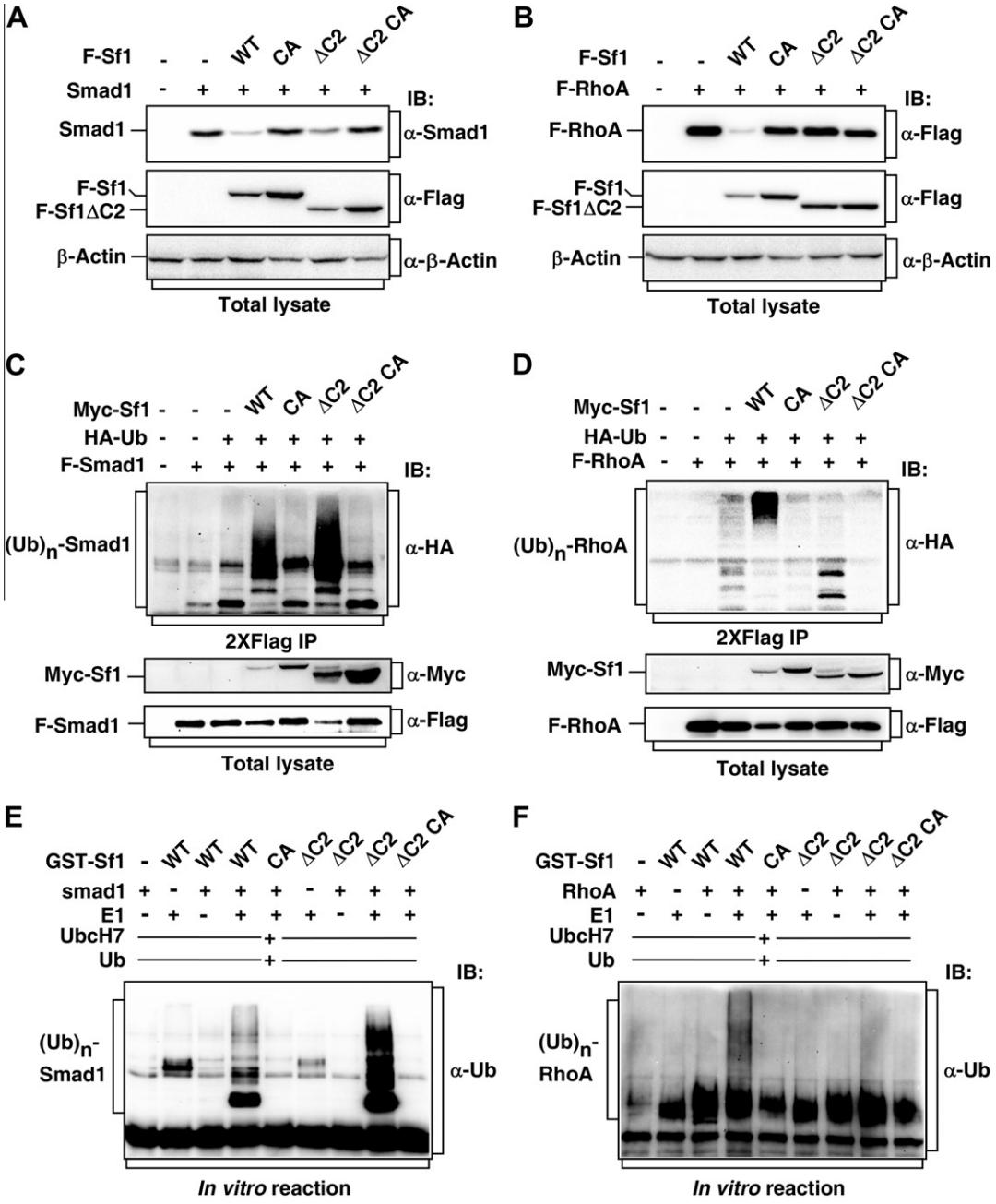

Fig. 1. The C2 domain is necessary for Smurf1 to target RhoA but not Smad1 for ubiquitination and degradation. (A) The C2 domain is dispensable for Smurf1-mediated Smad1 degradation. HEK293T cells were transiently transfected with Smad1 and Flag-tagged wild-type or its catalytically inactive form Smurf1 (F-Sf1 WT or CA) or their C2 domain deletion mutants (F-Sf1 ΔC2 or ΔC2 CA) in different combinations. The steady-state protein levels were determined by immunoblotting (IB) total cell lysates with indicated antibodies. (B) The C2 domain is necessary for Smurf1-mediated RhoA degradation. The same assay was performed as in (A), except that Flag-tagged RhoA was used instead of Smad1. (C) Smurf1 mediated Smad1 ubiquitination in vivo. HEK293T cells transfected with indicated combinations of Flag tagged Smad1 (F-Smad1), HA-tagged ubiquitin (HA-Ub) and Myc-tagged Smurf1 (Myc-Sf1) or its mutants. After overnight treatment with 20 μM LLnL, cell lysates were subjected to ubiquitination assay. Ubiquitin-conjugated Smad1 [$(Ub)_n$-Smad1] were detected with anti-HA antibody. (D) Smurf1 mediated RhoA ubiquitination in vivo. Same as (C) except that Flag-tagged RhoA (F-RhoA) was used. (E) Smurf1-mediated Smad1 ubiquitination in vitro. Smad1 and GST-tagged Smurf1 (GST-Sf1) or its mutants were purified from bacteria and subjected to in vitro ubiquitination assays. Ubiquitinated Smad1 proteins [$(Ub)_n$-Smad1] were detected with anti-ubiquitin antibody. (F) Smurf1-mediated RhoA ubiquitination in vitro. Same as in (E) except that RhoA was used.

processes, we transiently transfected Mv1Lu mink lung epithelial cells with catalytically active or inactive form of Smurf1 and Smurf1ΔC2. In agreement with the previous study [12], more than 70% Mv1Lu cells transfected with wild-type Smurf1 but not the catalytically inactive Smurf1-CA showed large numbers of long protrusions (Fig. 3A). However, neither the catalytically active form nor the inactive form of Smurf1ΔC2 induced long protrusions, indicating that the C2 domain is important for Smurf1 to modulate endogenous RhoA (Fig. 3A). By contrast, Smurf1ΔC2 still retains the capability to suppress BMP signaling. As shown in Fig. 3B, overexpression of Smurf1ΔC2 effectively inhibited BMP signaling in a dose-dependent manner, suggesting that the C2 domain is dispensable for Smurf1 to regulate BMP signaling. Therefore, the Smurf1 C2 domain exerts a specific role in modulating cell motility as opposed to its function in BMP signaling.

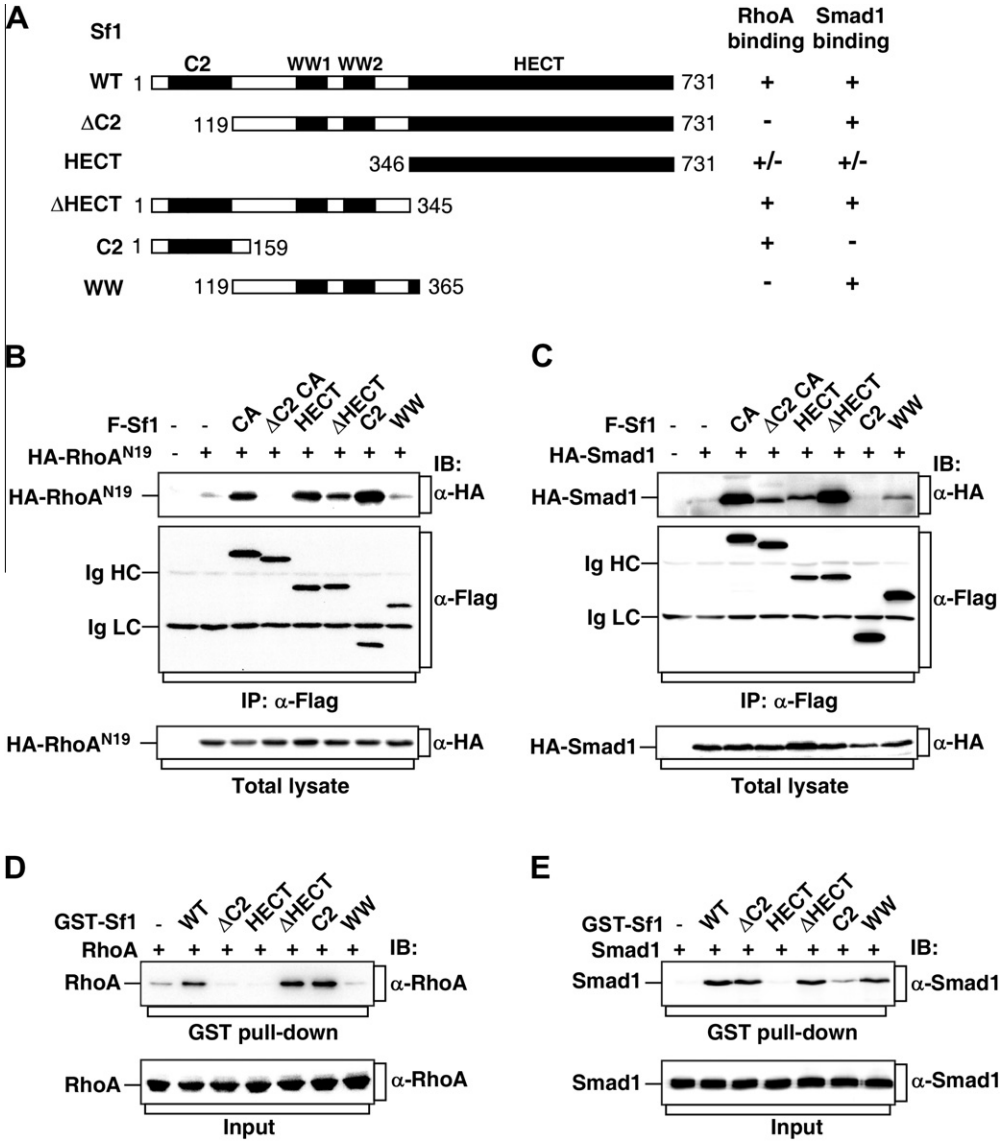

Fig. 2. The C2 domain of Smurf1 directly interacts with RhoA. (A) Schematics of Smurf1 domains and truncated mutants. (B) Domain mapping for Smurf1 and RhoA interaction by immunoprecipitation (IP) assay. HEK293T cells were transiently transfected with combinations of HA-tagged RhoA^{N19} (HA-RhoA^{N19}) and Flag-tagged Smurf1 (F-Sf1) or its mutants as indicated. Cell lysates were subjected to IP using anti-Flag antibody, followed by immunoblotting (IB) with rat anti-HA antibody to detect associated RhoA. (C) Domain mapping for Smurf1 and Smad1 interaction by IP. HA-Smad1 and various Flag-tagged Smurf1 were cotransfected into HEK293T cells, followed by IP and Western blotting analysis. (D) GST pull-down assay for mapping RhoA binding domain of Smurf1. RhoA, and GST-tagged Smurf1 (GST-Sf1) or its truncated mutants purified from bacteria were subjected to GST pull-down assay. Associated RhoA was detected with anti-RhoA antibody. (E) GST pull-down assay for mapping Smad1 binding domain of Smurf1. Same as (D) except that bacterially expressed Smad1 was used instead of RhoA.

4. Discussion

To date, multiple Smurf1 substrates in diverse signaling pathways have been identified. Smurf1 can ubiquitinate Smad1/5 and TGFβ receptors to inhibit TGFβ/BMP signaling [7], TRAFs to mediate NFκB signaling [15], RUNX2/3 and MEKK2 to control bone homeostasis [9,13,14], and RhoA to regulate cell migration and TGFβ-induced EMT [8,12]. Unlike the substrates with PY motifs for Smurf1 binding via WW–PY interaction, there is no PY motif in RhoA, which leaves the molecular mechanism of Smurf1 and RhoA recognition elusive. The C2 domains of Smurf1 and Smurf2 can bind phospholipids in a Ca^{2+}-independent manner [6,20]. When C2 domain is deleted, Smurf1 could still bind and translocate Smad7 from nucleus to cytosol, but failed to recruit the complex to the plasma membrane [20], indicating that the C2 domain is important for membrane recruitment of Smurf1. In this study, we found that the C2 domain of Smurf1 is essential for its selective targeting of RhoA for degradation, but has little effect on Smad1 degradation as shown by that Smurf1ΔC2 mutant could effectively ubiquitinate Smad1 but not RhoA.

Our study is in good agreement with a recent report by Lu et al. that the Smurf1 C2 domain has different roles in mediating Smurf1 activity toward RhoA, Smad5, and Runx2 [21]. Although C2 domain has been reported involved in protein–protein interaction [5], studies of the C2 domains of C2-WW-HECT family members have been mainly focused on their roles in regulation of subcellular localization and catalytic activities [6,21–26], and current functional understanding of C2 domains in substrate selection is very limited. Accordingly, Lu et al. suggested that the reason of C2 domain in exerting opposing effects on ubiquitination of RhoA, Smad5, and Runx2 is due to its role in regulation of Smurf1 subcellular localization. However, in our study, we revealed that the

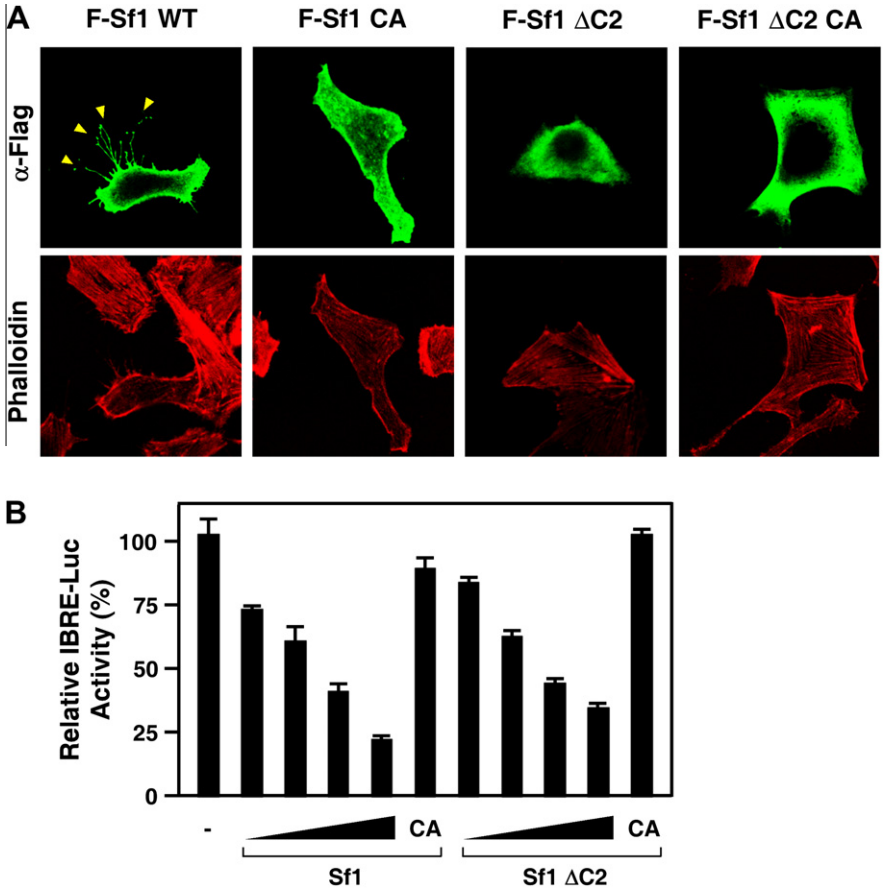

Fig. 3. The C2 domain is important for Smurf1-induced protrusive activity but not for Smurf1-regulated BMP signaling. (A) Deletion of the C2 domain disables Smurf1-induced protrusive activity. Twenty-four hours after transfection with Flag-tagged wild-type or its catalytically inactive form Smurf1 (F-Sf1 WT or CA) or their C2 domain deletion mutants (F-Sf1 ΔC2 or ΔC2 CA), Mv1Lu cells were visualized by immunostaining with anti-Flag antibody followed by Alexa Fluor 488-conjugated secondary antibody for Smurf1 (green), and Texas red-phalloidin for actin cytoskeleton (red). Smurf1-induced protrusions are marked with yellow arrowheads. (B) Smurf1ΔC2 retains the ability to downregulate BMP signaling. HEK293T cells were transiently transfected with the IBRE-lux reporter together with different amounts of Smurf1 (Sf1) or Smurf1ΔC2 (Sf1 ΔC2) in triplicate and the resulting relative luciferase activities were determined 40 h after transfection. The mean +/− S.D. of a representative result of three independent experiments is shown.

Smurf1 C2 domain alone is sufficient for RhoA binding in vivo and in vitro, whereas no direct interaction was detected between C2 domain and Smad1. Furthermore, the C2 domain is critical for Smurf1-dependent regulation of cytoskeleton restructuring, but not for the regulation of BMP signaling. Therefore, the interaction between the C2 domain and RhoA may play a key role in Smurf1-mediated RhoA degradation. As Smurf1 is recruited to the leading edge of cell to ubiquitinate RhoA locally for degradation to promote protrusive activity [12], we speculate that the C2 domain of Smurf1 has a dual function in regulation of cell protrusive activity by recruiting Smurf1 to the plasma membrane and subsequently recognizing RhoA for its ubiquitination and degradation. Hence, our study, together with the previous study that showed C2 domain is important for Smurf1 binding hPEM-2 [16], strongly suggested that the C2 domains are not only crucial for membrane recruitment through selectively binding phospholipids, but also important for substrate selection through direct interaction with certain substrates of Smurf1.

In summary, our study revealed the molecular mechanism of RhoA recognition by Smurf1 through the binding of C2 domain and RhoA. As the C2 domains are fairly conserved in the family of Nedd4-like E3 ubiquitin ligases [6], it is possible that other family members may also use the C2 domain to bind substrates. For instance, Smurf2 might target Rap1B through the interaction between C2 domain and Rap1B, which is also a Ras family small GTPase. Moreover, our study also suggests that C2 and WW domains may cooperatively determine substrate specificity, which renders multiple biological functions to the C2-WW-HECT family E3 ubiquitin ligases, particularly in different biological contexts. This modular recognition of substrates could be exploited for selective manipulation of different signaling pathways by using small molecules to target the distinct C2 or WW domains of the E3 ubiquitin ligases.

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