Smurf1-mediated Axin Ubiquitination Requires Smurf1 C2 Domain and Is Cell Cycle-dependent*

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Background: Smurf1 ubiquititates Axin via K29 poly-ubiquitin chains, and interferes with Wnt/β-catenin signaling.

Results: The C2 domain of Smurf1 is responsible for interacting with Axin, and Smurf1-mediated Axin ubiquitination attenuates in the G2/M phase of cell cycle.

Conclusion: Smurf1-Axin interaction follows a non-canonical pattern and is under the control of cell cycle.

Significance: Further clarification of the characteristic of Smurf1-mediated K29-linked poly-ubiquitination of Axin.

Previously, Smad ubiquitination regulatory factor 1 (Smurf1)-mediated Lys29 (K29)-linked poly-ubiquitination of Axin has been identified as a novel regulatory process in Wnt/β-catenin signaling. In this work, we discovered that the C2 domain of Smurf1 is critical for targeting Axin for ubiquitination. We found that the C2 domain-mediated plasma membrane localization of Smurf1 is required for Axin ubiquitination, and interfering with that disturbs the co-localization of Smurf1 and Axin around the plasma membrane. Moreover, the C2 domain of Smurf1, rather than its WW domains, is involved in Smurf1’s interaction with Axin; and the putative PPXY motifs (PY motif) of Axin are not essential for such an interaction, indicating that Smurf1 binds to Axin in a non-canonical way independent of WW-PY interaction. Further, we found that Smurf1-Axin interaction and Axin ubiquitination are attenuated in the G2/M phase of cell cycle, contributing to an increased cell response to Wnt stimulation at that stage. Collectively, we uncovered a dual role of Smurf1 C2 domain, recruiting Smurf1 to membrane for accessing Axin and mediating its interaction with Axin, and that Smurf1-mediated Axin ubiquitination is subjected to the regulation of cell cycle.

Smurf1 belongs to the C2-WW-HECT subfamily of HECT-type E3 ubiquitin ligases and plays essential roles in the bone morphogenetic protein (BMP), Wnt-PCP, and MEKK-JNK pathways by promoting ubiquitin-dependent degradation of their key regulators (1–4). It contains a C2 domain at the N terminus, two WW domains in the middle for mediating protein-protein interactions and a HECT domain at the C terminus (5). Most of the substrates of Smurf1, such as Smad7 and phospho-MEK2, bind to the WW domains of Smurf1 through their PY motifs (1, 3), and this WW-PY-mediated interaction could be considered as the canonical Smurf1-substrate interaction. However, recently, the involvement of Smurf1’s C2 domain in substrate recognition was emphasized by the discovery that Smurf1 interacts with RhoA via its C2 domain. This finding indicates that other non-canonical interactions may exist between Smurf1 and its substrates, especially those that do not have PY motifs (6). Meanwhile, Smurf1’s C2 domain is also important for its plasma membrane localization, destruction of which prevents Smurf1 from locating to the plasma membrane (3). For example, the aforementioned RhoA ubiquitination was considered to occur around the plasma membrane, during which the membrane localization of Smurf1 by its C2 domain was indispensable (7).

The Wnt/β-catenin signaling pathway is involved in embryogenesis and is also linked to tumorigenesis and many other human diseases (8, 9). Recently, the regulations of Wnt/β-catenin signaling, including ubiquitin-mediated ones, have been intensively studied. The first demonstrated ubiquitination event in Wnt/β-catenin signaling is about its core component β-catenin, which is ubiquitinated by the E3 ligase β-TrCP for degradation (10). However, several non-proteolytic ubiquitination have also been discovered in regulating this pathway. For instance, HectD1-mediated K63-linked polyubiquitination of APC facilitates Axin-APC interaction and therefore inhibits Wnt signaling transduction (12), and monoubiquitination of Groucho by XIAP promotes Wnt/β-catenin signaling via disturbing Groucho-TCF/LEF1 interaction (13).

Our previous study has demonstrated that Smurf1 ubiquitiates Axin through K29-linked poly-ubiquitination, which interrupts Axin-LRP5/6 association and then inhibits Wnt/β-catenin signaling (14). In the present work, we showed that the C2 domain of Smurf1 was crucial for its interaction with Axin, suggesting a non-canonical WW-PY-independent interaction for the two proteins. Moreover, the function of Smurf1 C2 domain in localization was also essential for Smurf1’s activity on Axin. Finally, we found that Smurf1-mediated Axin ubiquitination, as well as its inhibitory effect on Wnt/β-catenin sig-
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EXPERIMENTAL PROCEDURES

Plasmids, siRNA, Antibodies, and Purified Wnt3a Protein—Truncation mutants of human Smurf1 and Axin were constructed by PCR, and subcloned into mammalian expression vectors (pCMV-Flag and pCMV-HA) or bacterial expression vectors (pET-28C and pGEX-4T2). Point mutations of Axin and Smurf1 were generated using a Stratagene QuikChange site-directed mutagenesis kit. Other plasmids have been applied previously (14). Specific siRNA oligos against human Smurf1 and Axin were synthesized in GenePharma. Their sequences were as follows: Smurf1 siRNA: CCGACACUGUG-AAAAACACTT; Axin siRNA: CGAGAGCCAUCUAACCGA-AATT. Detailed construct information is available upon request. The commercial antibodies used for Western blotting (co-IP) have been described previously (14). Wnt3a protein was expressed and secreted by Drosophila S2 cells, and then purified using Blue Sepharose and gel filtration chromatography.

Cell Culture, Transfection, and Luciferase Assay—Drosophila S2 cells that stably express Wnt3a protein were cultured in Express Five Serum Free medium (SFM) plus 10% FBS, 125 μg/ml hygromycin, penicillin-streptomycin, and glutamine. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS). Axin-stable cells (HEK293T cells stably transfected with Flag-Axin) were cultured in HEK293T medium plus 50 μg/ml hygromycin. Plasmid or siRNA transfection and luciferase assay were carried out as previously described (14, 15).

Co-IP Assay and in Vivo Ubiquitination Assay—Co-IP assay, in vivo ubiquitination assay and Western blotting were performed as described before (11, 14). The results of Western blotting were viewed by FujiFilm Las 4000 (FujiFilm).

In Vitro Binding—Recombinant proteins were expressed and purified from Escherichia coli, and GST pull-down assay was applied as previously described (14).

In Vitro Ubiquitination Assay—In vitro ubiquitination was applied in 30 μl of reaction mixture containing human E1 (125 ng), UbH5C (500 ng), ubiquitin (10 μg) (Boston Biochem), GST-Axin (500 ng), and His6-Smurf1 (300 ng) or its truncations. 3 μl of 10× reaction buffer and 3 μl of ATP-Mg2+ provided by the Ubiquitin Conjugation Rxn Buffer Kit (Boston Biochem) were added as well. The reaction was taken place at 30°C for 2 h, and then terminated by adding the stop buffer (Boston Biochem).

Immunofluorescence Staining—As previously described (16), HEK293T cells grown on coverslips were transfected with GFP-tagged or HA-tagged plasmids, and then fixed and stained with anti-HA antibody (1:1000) where necessary. Nuclear staining by DAPI was applied.

Cell Cycle Assay—To arrest cells at the G2/M stage, Axin-stable cells were treated with nocodazole (100 ng/ml) for 16 h. For double thymidine block, cells were incubated in 2 mM thymidine for 2 × 17 h exposure time, separated by a 9-h recovery interval, and then released for the indicated times for subsequent assays. To evaluate the corresponding cell cycle stages, FACS analysis was performed as previously mentioned (17).

RESULTS

Plasma Membrane Localization of Smurf1 Facilitates Its Modification on Axin—Our previous study identified Smurf1 as a negative regulator in the Wnt/β-catenin signaling pathway by ubiquitinating Axin via K29-linked poly-ubiquitination and subsequently disrupting its association with the Wnt co-receptor LRP6 (14). To assess whether Smurf1-mediated Axin ubiquitination occurred around the plasma membrane, we generated a truncation mutant of Smurf1 (residues 38–731, noted as Smurf1-ΔN) (Fig. 1A, upper panel), deleting the sequence important for Smurf1’s plasma membrane localization from the C2 domain (3). Consistent with the previous report, full-length Smurf1 was predominantly distributed around the plasma membrane, while Smurf1-ΔN was mainly located in the cytosol (Fig. 1A, bottom panel) (3). However, Smurf1-ΔN retains the ability to interact with and ubiquitinate Axin, as evidenced by a co-IP assay in HEK293T cells and GST pull-down assays using purified proteins, as well as in vitro ubiquitination experiments (Fig. 1, B–D).

To investigate whether Smurf1-ΔN was able to ubiquitinate Axin in vivo, we performed in vivo ubiquitination assay in HEK293T cells transfected with Axin, ubiquitin, and Smurf1 or Smurf1-ΔN. As shown in Fig. 1E, Smurf1-ΔN failed to elicit the ubiquitination of exogenous Axin. Consistently, in Axin-stable cells stably expressing a low level of Axin-Flag, Smurf1-ΔN also lost its ability to link ubiquitin chains to Axin (Fig. 1F). As a control, Smad7, a well known substrate of Smurf1 (3), was able to be modified by Smurf1-ΔN (Fig. 1G). These results implied that the subcellular localization of Smurf1 was important for Axin ubiquitination. Actually, the co-localization of Smurf1 and Axin was readily detected and mainly observed around the plasma membrane, whereas that of Smurf1-ΔN and Axin was largely disrupted (Fig. 1H). Altogether, the above evidence supported our hypothesis that Smurf1-Axin interaction occurred around the plasma membrane and the C2 domain-mediated plasma membrane localization of Smurf1 was critical for Smurf1-mediated Axin ubiquitination.

Plasma Membrane Localization of Smurf1 Is Important for Its Function in Regulating Wnt/β-catenin Signaling—Next, we examined the potential role of Smurf1’s subcellular localization in its antagonistic effect in Wnt/β-catenin signaling. Firstly, we carried out co-IP assays to examine the effect of Smurf1 and Smurf1-ΔN on Axin-LRP6 interaction. As displayed in Fig. 2, A and B, Smurf1-ΔN no longer disrupted the interaction, neither between overexpressed LRP6 and Axin nor between endogenous LRP6 and Axin-Flag in Axin-stable cells. Smurf1-ΔN also failed to reduce Wnt3a-induced LRP6 phosphorylation (Fig. 2C), but it still retained the activity in catalyzing the degradation of ectopically-expressed Smad7 and endogenous Smad1 (Fig. 2, D and E). We then used TOPFlash/FOPFlash reporter system and found that the inhibitory effect of Smurf1-ΔN on Wnt3a-stimulated TOPFlash activity was significantly impaired compared with that of full-length Smurf1, while both of them had no apparent effect on the control FOPFlash (Fig. 2F). Finally, we assessed the functions of Smurf1 and Smurf1-ΔN on the
expression levels of two Wnt target genes: Axin2 and N KD1. As shown in Fig. 2G, Smurf1-ΔN lost the ability to down-regulate the expression of Axin2 and N KD1 levels induced by Wnt. Moreover, when endogenous Smurf1 was knocked down and replaced by RNAi-resistant(r) Smurf1 or Smurf1-ΔN, Smurf1 knockdown-induced promotion of LR6 phosphorylation could only be reversed by the introduction of rSmurf1, but not by rSmurf1-ΔN (Fig. 2H). Therefore, we concluded that the plasma membrane localization of Smurf1 was important for its inhibitory function in Wnt/β-catenin signaling.

Smurf1 Interacts with Axin in a Non-canonical Way in Which Its C2 Domain Is Required—Normally, the WW domains of Smurf1 are responsible for recognizing and interacting with the PY motif of its targeted proteins (5, 18, 19). Therefore, we asked whether Smurf1-Axin interaction is also mediated by WW-PY interaction. To answer this question, several fragments of Smurf1 were used: Sf1-N (residues 1–233, containing the C2 domain), Sf1-WW (residues 147–351, containing the WW domains), Sf1-C (residues 314–731, containing the HECT domain) and Sf1-ΔWW, a truncated mutant lacking the WW domains (Fig. 3A). To our surprise, it is the Sf1-N, but not Sf1-WW, that was bound to Axin (Fig. 3, A and B), and Sf1-C could also interact with Axin but with a much lower binding affinity. As a control, Sf1-WW could interact with Smad7 (Fig. 3C), while depletion of Smurf1 WW domains dramatically attenuated its association with Smad7, but not that with Axin (Fig. 3D). These results indicated that Smurf1-Axin interaction is probably mediated by Sf1-N encompassing the C2 domain rather than the WW domains. To further confirm this, we performed in vitro binding assays. As shown in Fig. 3, E and F, GST pull-down assays confirmed the direct interactions of Axin with Sf1-N and Sf1-ΔWW. Additionally, Sf1-N and Sf1-N-ΔN (residues 38–233) had similar binding affinities when interacted with Axin (Fig. 3E), which was consistent with our previous data (Fig. 1C). Moreover, in vitro ubiquitination assay showed that Sf1-ΔWW could promote Axin ubiquitination as effectively as did the full-length Smurf1 (Fig. 3G). To further delineate the interacting regions of Smurf1 with Axin, we split the N domain of Smurf1 into two segments: Sf1-C2 (residues 1–159) and Sf1-N-ΔC2 (residues 119–233) (Fig. 3F). Their interactions with Axin were tested by co-IP assays. As shown in Fig. 3H, Sf1-C2 was able to co-immunoprecipitate Axin, while Sf1-N-ΔC2 could not. In keeping with the above finding, a purified truncation of Smurf1 lacking its C2 domain (residues 160–731,
noted as Sf1-ΔC2) lost most of its binding affinity with GST-Axin (Fig. 3F), as well as its ability to ubiquitinate Axin (Fig. 3I). Taken together, these findings demonstrated that the C2 domain of Smurf1, but not the WW domains, was crucial for its binding and ubiquitinating Axin. Smurf2, the homologous protein of Smurf1, is reportedly also an E3 ligase of Axin, but to promote the ubiquitin-dependent degradation of ectopically-expressed Smad7 and endogenous Smad1. HEK293T were transfected with the indicated plasmids and protein levels were measured by Western blotting, indicative of a WW-PY-independent—in interacting with Axin.

Next, we explored the interface regions in Axin for Smurf1 binding. As illustrated in Fig. 4, A and B, Axin-N2 (residues 1–757) and Axin-M (residues 437–757) bound to Smurf1 with a similar affinity as did the full-length Axin, while Axin-N1 (residues 1–437) showed significantly decreased binding with Smurf1. By contrast, Axin-N1 and -N2, but not Axin-M, were capable of interacting with Smurf2, albeit with a much weaker binding capability when compared with the full-length Axin (Fig. 4C). Sequence analysis suggested three putative PY motifs in human Axin—PASY (residues 39–42), PRTY (residues 372–375), and PIPY (residues 757–760). However, Axin mutations targeting, either one single PY motif or all the three, showed no obvious impact on Axin’s interaction with Smurf1 (Fig. 4, D and E), while the association between Smurf2 and Axin was drastically impaired when the three PY motifs were mutated (Fig. 4F).

Taken together, these results further supported a WW-PY-independent interaction between Smurf1 and Axin, which was distinct from that of Smurf2-Axin.

**The Inhibitory Function of Smurf1 in Wnt/β-catenin Signaling Is Cell Cycle-dependent**—In our previous work, we found that Wnt3a stimulation led to a decrease of K29-linked polyubiquitination of Axin (14), suggesting that Smurf1-mediated Axin ubiquitination might be a constitutive event in resting cells to prevent the recruitment of Axin to the LRP5/6 co-receptors. Considering that the level of phospho-LRP6 fluctuates during cell cycle process and peaks in the G2/M phase (21), we wondered whether Smurf1 regulated Wnt/β-catenin signaling in a cell cycle-dependent manner. To resolve this question, firstly, we monitored Axin ubiquitination in Axin-stable cells by performing double thymidine block at G1/S stage and then releasing for various times. FACs analysis was applied to mark
the corresponding cell cycle stage. As depicted in Fig. 5, A and B, LRP6 phosphorylation peaked during G2/M, concomitant with a low Smurf1–Axin interaction and Axin ubiquitination. Consistently, both Axin ubiquitination (Fig. 5C, lane 2 and lane 4) and Smurf1–Axin interaction (Fig. 5D) were down-regulated in G2/M cells synchronized by nocodazole. Besides, we noticed...
that depletion of Smurf1 resulted in efficient attenuation of Axin ubiquitination (Fig. 5C, lane 2 and lane 3), whereas further impact of nocodazole was not apparent (Fig. 5C, lane 3 and lane 5). Together, these data showed that Smurf1-mediated Axin ubiquitination is specifically inhibited in the G2/M phase.

Then we asked whether the observed attenuation of Axin ubiquitination at G2/M stage contributed to the G2/M peak of LRP6 phosphorylation. Actually, in Axin-stable cells released from double thymidine block, Axin-LRP6 interaction was elevated in the G2/M phase (Fig. 5E). Moreover, as shown in Fig. 5F, although phospho-LRP6 level was increased in Smurf1-deficient cells across the cell cycle process, the expected G2/M peak was diminished, indicating a G2/M relief of the inhibitory function of Smurf1. Thus, these results altogether suggested that the aforementioned G2/M inhibition of Smurf1-mediated Axin ubiquitination facilitates Axin-LRP6 interaction and the G2/M peak of phospho-LRP6, contributing to an increase in the response to Wnt stimulation at that stage.

DISCUSSION

The C2-WW-HECT E3 ubiquitin ligase Smurf1 has been defined as an important component in multiple signaling pathways and cellular processes by regulating the protein stability of its substrates. To date, among these substrates of Smurf1, Axin is a special one because of its atypical ubiquitination type and non-degradation fate (14). In this study, we showed that the C2 domain of Smurf1 was functionally important for Smurf1-mediated Axin ubiquitination, as well as for the inhibitory effect of Smurf1 in Wnt signaling transduction (Figs. 1 and 2). On one hand, Smurf1’s C2 domain recruits Smurf1 to the plasma membrane and promotes its in vivo interaction with Axin (Fig. 1). The function of the C2 domain in Smurf1 localization has been well clarified by the study of T/H9252R-I (3). In our work, we showed that Smurf1-mediated Axin ubiquitination took place around the plasma membrane. Although partial destruction of Smurf1 C2 domain did not affect its physical binding with Axin, their co-localization in cells was largely impaired, and Axin ubiquitination by Smurf1 was also accordingly declined. On the other hand, the C2 domain of Smurf1 is involved in binding Axin (Fig. 3). Although WW-PY-mediated interaction is prevalently for Smurf1 and its substrates, such as Traf4 (22) and phospho-MEK2 (1), alternative ways of interaction may happen especially when the substrates contain no PY motifs. For example, TRIB2 was discovered to interact with Smurf1 through its TDD domain, which was critical for the subsequent ubiquitination (23). Here, our work provided a new example of WW-PY-independent Smurf1-substrate interaction which is mediated by Smurf1’s C2 domain. Different from the case of RhoA that does not have any PY motifs, Axin has three potential PY motifs.
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However, all of these PY motifs were not involved in Axin’s interaction with Smurf1 (Fig. 4, D and E). Meanwhile, the destinations of ubiquitinated RhoA and Axin were also distinct: degradation for RhoA but non-degradation for Axin. The regulatory mechanisms leading to distinct destinies of these two proteins warrant further investigation.

Another interesting question raised by this work is that whether the non-canonical way of interaction contributes, at least partially, to the assembly of K29 poly-Ub chains in whether the non-canonical way of interaction contributes, at least partially, to the assembly of K29 poly-Ub chains in at least partially, to the assembly of K29 poly-Ub chains in whether the non-canonical way of interaction contributes, at least partially, to the assembly of K29 poly-Ub chains in the degradation of Axin (20), and in this work, we showed that Smurf2-Axin interaction occurs most likely in the canonical WW-PY-dependent way (Figs. 3/4 and 4F). Therefore, it is possible that the non-canonical interaction we observed between Smurf1 and Axin might confer them unique conformations which prefers a K29 Ub-linkage on Axin and subsequently affects Axin’s function in Wnt/β-catenin signaling. Structural insights into the Smurf1/2-Axin complexes will undoubtedly provide invaluable information for addressing this question. Moreover, considering the involvement of attenuated Wnt activities in many diseases, such as osteoporosis, the inhibitory role of Smurf1 through Axin in Wnt/β-catenin signaling may be exploited for designing new drugs, especially ones targeting the C2 domain of Smurf1 without affecting its WW domains. These candidates may specifically interfere with Smurf1-Axin interaction and release the inhibitory effect of Smurf1 on Wnt/β-catenin signaling.

Finally, we demonstrated that Smurf1-mediated Axin ubiquitination, as well as its negative effect on LRPE6 phosphorylation and Wnt/β-catenin signaling was under the control of cell cycle. The Wnt/β-catenin signaling has been reported to be cell cycle-regulated; a G2/M cyclin/CDK complex is responsible for the G2/M peak of LRPE6 phosphorylation and therefore permits an increased cell response to Wnt signaling at that stage (21). Our work here provided another possible mechanism contributing to the cell cycle-regulated Wnt signaling. On one hand, Axin-LRP6 interaction was enhanced in the G2/M phase (Fig. 5E), and in Axin-deficient cells, phoso-LRP6 level, and its G2/M peak were attenuated (Fig. 5G). According to these data, we speculate that besides the cyclin/CDK-mediated LRPE6 phosphorylation, an additional amplification step requiring Axin-LRP6 interaction was involved in the G2/M elevation of phoso-LRP6. On the other hand, Smurf1-mediated Axin ubiquitination, as well as its effect toward LRPE6 phosphorylation, is cell cycle-dependent and minimized in the G2/M phase (Fig. 5, A, C, and F). The attenuated activity of Smurf1 in the G2/M phase may facilitate the G2/M peak of phoso-LRP6 and contribute to an increase in the response of cells to Wnt stimulation. Currently, it remains unclear about the regulatory mechanisms how Smurf1-mediated Axin ubiquitination is regulated by cell cycle. Considering the fact that Smurf1-Axin interaction is accordingly decreased at G2/M stage (Fig. 5, B and D), it is possible that some other interacting partners may participate in at G2/M to disrupt their interaction. Another possibility is that post-translational modifications on Smurf1 may occur at G2/M, regulating its function and impairing its binding to Axin. Further studies are required to address these problems.

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