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Smurf1 ubiquitin ligase targets Kruppel-like factor KLF2 for ubiquitination and degradation in human lung cancer H1299 cells

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ABSTRACT

Krüppel-like factor 2 (KLF2) has been demonstrated to be essential for normal lung development, erythroid differentiation, T-cell differentiation, migration and homing. However, the mechanisms underlying the regulation of KLF2, in particular its responsible E3 ligase is still unclear. Here we show that the homologous to E6AP carboxyl terminus (HECT)-type ubiquitin ligase Smad ubiquitination regulatory factor 1 (Smurf1) interacts with and targets KLF2 for poly-ubiquitination and proteasomal degradation specifically in lung cancer H1299 cells. The catalytic ligase activity of Smurf1 is required for it to regulate KLF2. Consequently, Smurf1 represses the transcriptional factor activity of KLF2 and regulates the expression its downstream genes such as CD62L and Wee1. This study provided the first evidence that Smurf1 functions as an E3 ligase to promote the ubiquitination and proteasomal degradation of KLF2.

1. Introduction

Krüppel-like factors are a subclass of the zinc-finger family of transcription factors implicated in the regulation of cellular growth and differentiation. Since the initial discovery of EKLF in 1993, a total of 17 mammalian Krüppels have been identified and designated based on the chronological order of discovery (i.e., KLF1–17) [1,2]. Human KLF2, owing to its high expression in lung tissues, was initially termed lung KLF (LKLF), and KLF2 has been demonstrated to be essential for normal lung development. The *KLF2* knock-out mice exhibit impaired blood vessel formation attributable to the lack of smooth muscle cell recruitment [3,4]. KLF2 can function as either a transactivator or repressor [5]. For example, KLF2 has been found to upregulate the expression of G1/S checkpoint gene p21 to inhibit leukemia cell growth [6]. On the contrary, KLF2 inhibits the expression of G2/M tyrosine kinase Wee1 to cause DNA damage-induced apoptosis [7]. Additionally, KLF2 is known to regulate the signaling pathways involved in thymocyte and T-cell trafficking. In this regard, KLF2 directly activates the promoters of CD62L and sphingosine-1-phosphate receptor 1, whose expression is critical for T cell egress from the thymus and homing to the lymph nodes [8].

Although the functions and the downstream targets have been widely studied, less is known about the stability control of KLF2 protein. Previous studies have indicated that KLF2 protein can be targeted to proteasome system for degradation. Although the HECT-type ubiquitin ligase WWP1 (WW domain-containing protein 1) was shown to promote the ubiquitination and degradation of KLF2, the catalytic activity of WWP1 enzyme seems to be not required for this degradation [9]. Therefore, one could speculate that WWP1 may not function as a direct E3 ligase for KLF2. At present, the identity of the ligase for KLF2 still remains unclear.

Here we provide evidence to show that Smad ubiquitination regulatory factor 1 (Smurf1) interacts with KLF2 both in vivo and in vitro, and directly targets the ubiquitination and proteasomal degradation of KLF2 specifically in lung cancer H1299 cells. Both Smurf1 and WWP1 belong to the Nedd4 family of HECT-type ligases and contain similar C2-WW-HECT structure. Smurf1 has been demonstrated to play a pivotal role in control of cell polarity, maintenance of bone homeostasis and regulation of tumorigenesis through targeting BMP-Smad, RhoA signaling pathways [10,11]. The current study adds KLF2 to the substrate list of Smurf1 and establishes the functional relationship between Smurf1 and KLF2-mediated transcriptional control.

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2. Materials and methods

2.1. Plasmid constructs, antibodies and reagents

Full-length and truncated forms of KLF2 and Smurf1 were constructed by PCR, followed by subcloning into various vectors. Anti-Myc antibody was from Clontech. Anti-Flag M2 monoclonal antibody, the protein synthesis inhibitor cycloheximide (CHX) and the proteasome inhibitor MG132 were from Sigma. Anti-KLF2 polyclonal antibody (ab-28526) was from Abcam. Anti-GFP antibody was from Cell Signaling Technology and anti-HA antibody was from Roche. Anti-GST and his antibodies were from Tiangen. GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology.

2.2. Yeast two-hybrid screening

Yeast two-hybrid screening was performed with the Pro-Quest-TM two-hybrid system (Invitrogen, CA) in the human brain cDNA library (Invitrogen) as we described [12]. The WW domains region (aa 236–340) of human Smurf1 was used as the bait.

2.3. Cell culture and transfection

Human embryonic kidney HEK293T cells were cultured in DMEM medium (Hyclone). Human lung cancer cell H1299 cell lines were maintained in 1640 (Hyclone). All cells were supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Hyclone). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions.

2.4. Fluorescence microscopy

At 24 h post-transfection, the cells were fixed with 2% paraformaldehyde for 10 min at room temperature, rinsed with PBS, and permeabilized with 3% Triton X-100/PBS for 10 min. The cells were then rinsed with PBS and incubated with monoclonal antibody for 1 h at room temperature, followed by incubation with goat anti-mouse IgG secondary antibody for 1 h at room temperature. The nuclei of the cells were stained with 0.1 g/ml DAPI, the cells were observed using a fluorescence microscope.

2.5. In vivo ubiquitination assays

Cells were lysed in RIPA lysis buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 1% sodium deoxycholate, 0.025% SDS, protease inhibitors], and then incubated with indicated antibody for 3 h at 4 °C and protein A/G-agarose beads (Santa Cruz) for a further 8 h at 4 °C. After three washes, proteins were detected by immunoblotting.

2.6. Immunoprecipitation and immunoblotting

Cells were harvested and lysed in HEPES lysis buffer (20 mM HEPES pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF, 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (Roche). The lysate was incubated with indicated antibody 3 h at 4 °C, then added protein A/G-plus agarose and rotated gently more than 8 h at 4 °C. The immunoprecipitates were washed at least three times in lysis buffer, and analyzed by western blotting followed by detection with a Super Signal chemiluminescence kit (Pierce).

2.7. In vitro GST pull-down assay

Bacteria-expressed GST or GST–KLF2 proteins were immobilized on glutathione–Sepharose 4B beads (Amersham) and washed, then beads were incubated with His-Smurf1. Beads were washed with GST binding buffer (100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% Nonidet P40 and protease inhibitor cocktail) and proteins were eluted, followed by western blotting [13].

2.8. Luciferase reporter assay

The 3.7-kb *Mlu*I-*Stu*I fragment of L-selectin promoter which contains the KLF2 binding site was inserted into pGL3-basic (Promega). KLF2 luciferase reporter assay was carried out as described before [14]. Cells were lysed using passive lysis buffer (Promega), and luciferase activities in cell extract were determined using a Dual-Luciferase assay system (Promega).

2.9. RNA interference

The Smurf1-specific siRNA (5′-GCAUCGAAGUGUCCAGAGAAG-3′), and non-target control siRNA (5′-UUCUCCGAACGUGUCACGU-3′) were synthesized by Shanghai GenePharm.

2.10. Real-time RT-PCR

Real-time quantitative PCR was performed as described previously [15]. Reactions were done in triplicate and relative amounts of cDNA were normalized to GAPDH. Primers used as follows: CD62L F, 5′-GAAGGACCAAGCAAAGCC-3′ and CD62L R, 5′-CCAT GATGTGCCAGGAAA-3′; Wee1 F, 5′-ATTTCTCTGCGTGGGCAGAAG-3′ and Wee1 R, 5′-CAAAAGGAGATCCTTCAACTCTGC-3′; KLF2 F, 5′-GCGGCAAGACCTACACCAAG-3′ and KLF2 R, 5′-GCACAGATGG CACTGGAATG-3′; GAPDH F, 5′-GGGAAGGTGAAGGTCGGAGT-3′ and GAPDH R, 5′-TTGAGGTCAATGAAGGGGTCA-3′.

3. Results

3.1. Smurf1 interacts with KLF2 both in vitro and in vivo

The WW domains of Nedd4 family of ubiquitin ligases are usually responsible for substrate recognition. In a yeast two-hybrid screen with the WW domains of human Smurf1 as bait in a human brain cDNA library, we identified the full length of the KLF2 as an interactor candidate. This prey attracted our interest for further investigation because KLF2 is a nuclear transcriptional factor whereas most known Smurf1 substrates including Smad1/5, MEKK2, Prickle 1, TRAF4, and RhoA are localized in the cytoplasm or at the plasma membrane. To confirm the interaction between Smurf1 and KLF2, in vitro GST pull-down assays with recombination His-Smurf1 and GST-KLF2 were first performed. A specific interaction of Smurf1 with GST-KLF2, but not with GST alone was observed (Fig. 1A). To assess whether KLF2 interacts with Smurf1 in vivo, a co-immunoprecipitation (Co-IP) assay was performed in lung cancer H1299 cells and the result revealed an association between Flag-Smurf1 and Myc-KLF2 in the presence of the proteasome inhibitor MG132 (Fig. 1B). As a positive control, the auxiliary factor of Smurf1, CKIP-1 was also co-immunoprecipitated with Smurf1 (Fig. 1B). Also, ectopic KLF2 proteins were co-immunoprecipitated with endogenous Smurf1 protein (Fig. 1C).

The finding that KLF2 interacts with Smurf1 in cultured cells leads us to speculate these two types of proteins may localize in the same subcellular compartment. To assess the subcellular localization of KLF2 and Smurf1, H1299 cells were co-transfected with GFP-KLF2 and Flag-Smurf1 in the presence of MG132. When coex-

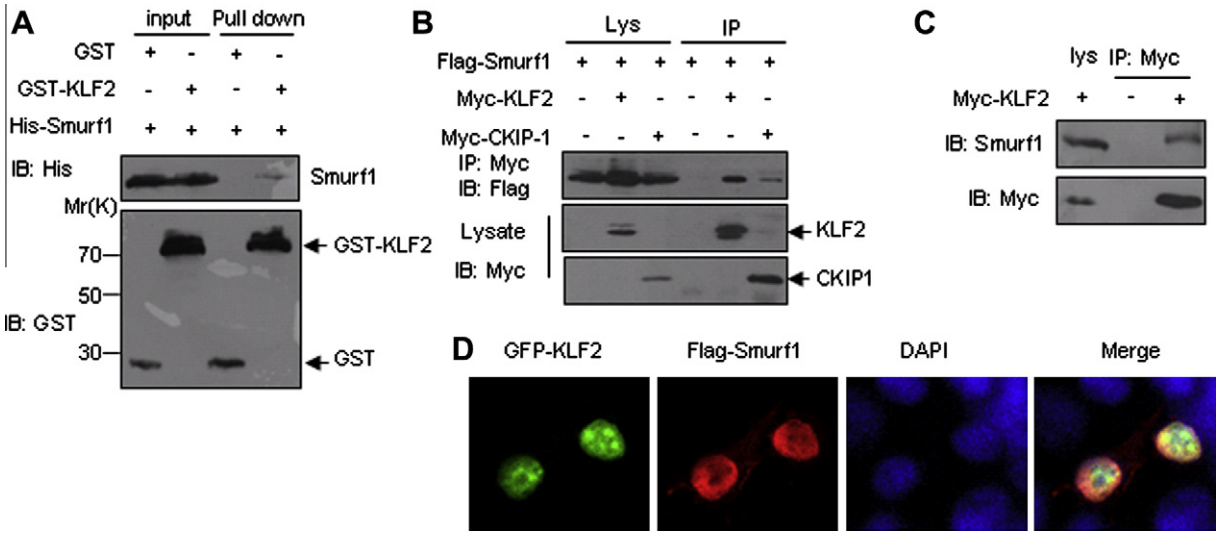

Fig. 1. Smurf1 interacts with KLF2 in vitro and in vivo. (A) Direct interaction between Smurf1 and KLF2 is revealed by GST pull-down assays. Input and pull-down samples were both subjected to immunoblotting with anti-GST and anti-His antibodies. Input represents 10% of that used for pull-down. IB, immunoblotting. (B and C) Co-immunoprecipitation of Smurf1 and KLF2 in H1299 cells. To avoid the degradation of KLF2, MG132 (20 μM) were added for 8 h before harvested. Cell lysates were immunoprecipitated with anti-Myc antibody and analyzed by immunoblotting. (D) KLF2 and Smurf1 colocalize in the nucleus of H1299 cells. Green represents the Smurf1 proteins and nuclei were stained with DAPI.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pressed, KLF2 and Smurf1 were co-localized in the nucleus in H1299 cells (Fig. 1D). Thus, Smurf1 interacts with KLF2 both in vitro and in vivo.

3.2. Mapping the binding regions between Smurf1 and KLF2

To clearly characterize the association between Smurf1 and KLF2, we generated several Smurf1 deletion mutants to map the KLF2-interacting region. Co-IP assays indicated that the WW domain but not the C2 or HECT domains of Smurf1 mediated the interaction with KLF2 (Fig. 2A). This notion is consistent with the yeast two-hybrid screening since the WW domain of Smurf1 is the bait. KLF2 protein contains an N-terminal transcriptional activation domain (AD, aa 1–110) and a central inhibitory domain (ID, aa 111–267), and the C-terminal zinc finger domain (ZF, aa 268–354). The inhibitory domain has been shown to interact with WWP1, resulting in ubiquitination and proteasomal degradation of KLF2 [9]. We next mapped the binding region of KLF2 with Smurf1. We observed that both the ID and the ZF domains were sufficient for binding to Smurf1, whereas the AD domain alone cannot mediate the interaction (Fig. 2B).

3.3. Smurf1 promotes the proteasomal degradation of KLF2 in H1299 cells

Smurf1 has been demonstrated to target Smad1/5, RhoA, MEKK2, Prickle1, RunX2, and ING2 for ubiquitination and degradation [10,11,16–19]. WW domains are usually responsible for sub-

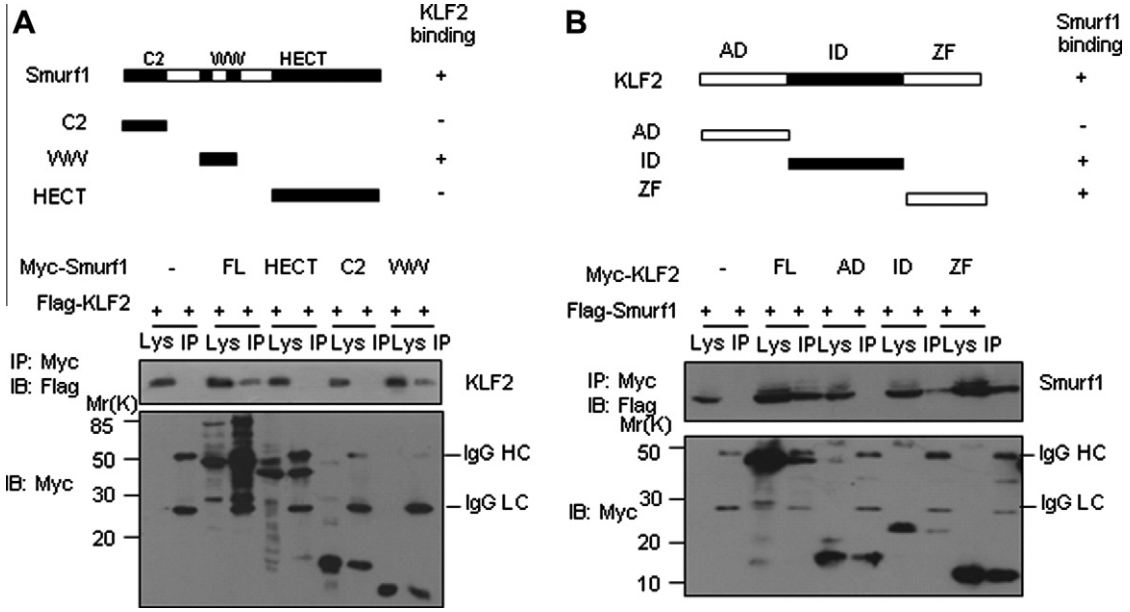

Fig. 2. Mapping the binding region between Smurf1 and KLF2. (A) Smurf1 WW domain mediates the interaction with KLF2 protein. KLF2 and Smurf1 deletion mutants were transfected into H1299 cells. Cell lysates were immunoprecipitated with anti-Myc antibody and detected. (B) The inhibitory domain and zinc finger domain of KLF2 binds to Smurf1. Smurf1 and the indicated deletion mutants of KLF2 were transfected into H1299 cells. Cell lysates were immunoprecipitated and analyzed by immunoblotting.

strate recognition. Interestingly, the WW domains of Smurf1 mediate the interaction with KLF2 (Fig. 2A). Then we hypothesized that Smurf1 might function as an E3 ligase in the KLF2 degradation. To test this, KLF2 was analyzed in the present of ectopic wild-type (WT) Smurf1 or its ligase-inactive C699A mutant. Smurf-WT significantly reduced KLF2 protein levels in human lung cancer H1299 cells; in contrast, Smurf1 CA mutant hardly affected the levels of KLF2 (Fig. 3A, lanes 1–3). The reduction effect of Smurf1 on KLF2 was blocked by treatment with the proteasome inhibitor MG132 (Fig. 3A, lanes 4–6), indicating this is mediated by the proteasome system. Smurf1 expression had no significant effects on the mRNA levels of KLF2 (Fig. 3B). To examine whether the effect of Smurf1 on the KLF2 protein levels is through stabilization of the protein, we measured the half life of KLF2. When cells are treated with the protein synthesis inhibitor cycloheximide (CHX), the turnover of KLF2 was dramatically accelerated by co-expression of Smurf1 (Fig. 3C).

However, the degradation-promoting effect of KLF2 by Smurf1 was not observed in human embryonic kidney HEK293T cells (Fig. 3D). Careful analysis of the subcellular localization of Smurf1 in these two cell lines showed that ectopic Smurf1 was predominantly localized in the nucleus of H1299 cells whereas mainly at the plasma membrane in HEK293T cells (Fig. 3E). KLF2 has been well-defined as a nuclear transcription factor; thus, it seems like that only when Smurf1 entered into the nucleus it promoted the degradation of KLF2 significantly.

Next, we asked whether the endogenous Smurf1 protein affects the KLF2 protein stability. To this end, Smurf1 was depleted by small interfering RNA (siRNA) duplex in H1299 cells. By western blotting, there was a two-fold increase in the KLF2 protein level in cells depleted of Smurf1 relative to control cells (Fig. 3F). Again, we did not observe any significant alteration on the mRNA level of KLF2 when Smurf1 was knockdown (Fig. 3G).

We previously showed that CKIP-1 functions as an auxiliary factor of Smurf1 ligase to promote the degradation of the substrates of Smurf1. CKIP-1 deficient mice exhibited decreased Smurf1 activity and increased levels of Smurf1 substrate such as the phosphorylated MEKK2 [13]. If KLF2 is really the physiological substrate of Smurf1, its protein level should be upregulated in CKIP-1 deficient mice. We then analyzed the protein expression of endogenous KLF2 in liver and lung tissue extracts derived from CKIP-1 WT and knockout (KO) mice. As shown in Fig. 3H, KLF2 is easily detected in mice lung but undetectable in the liver. As expected, the KLF2 expression was increased significantly in the lung from CKIP-1 KO mice compared with the WT mice. Collectively, these data indicate that KLF2 is a bona fide substrate of Smurf1 ligase.

3.4. Smurf1 promotes the poly-ubiquitination of KLF2 and represses its transcriptional activation

Because the ubiquitin ligase activity of Smurf1 was required for KLF2 degradation (Fig. 3A), we next sought to determine whether Smurf1-mediated KLF2 degradation is a consequence of ubiquitination. The in vivo ubiquitination assay showed that overexpression of Smurf1 significantly increased the poly-ubiquitination of KLF2 (Fig. 4A). In contrast, Smurf1 C699A mutant did not trigger poly-ubiquitination of KLF2. This data suggested that Smurf1 functions as an E3 ligase of KLF2 for ubiquitination.

We next investigated that whether Smurf1 influences the transcriptional activity of KLF2. Reporter gene assay showed that the KLF2 transcriptional activity was significantly repressed by exogenous Smurf1 in H1299 cells (Fig. 4B). Importantly, depletion of Smurf1 in H1299 cells resulted in a dramatic enhancement of KLF2 transcriptional activity (Fig. 4C), indicating that endogenous Smurf1 negatively regulates KLF2 activity.

As a transcription factor, KLF2 activates or represses the expression of its target genes. For example, KLF2 promotes the expression of the selectin CD62L which is involved in T cell trafficking [8], while it represses the expression of Wee1 tyrosine kinase which regulates the G2/M cell cycle transition [7]. To gain further insight into the Smurf1-mediated regulation of KLF2, the ability of Smurf1

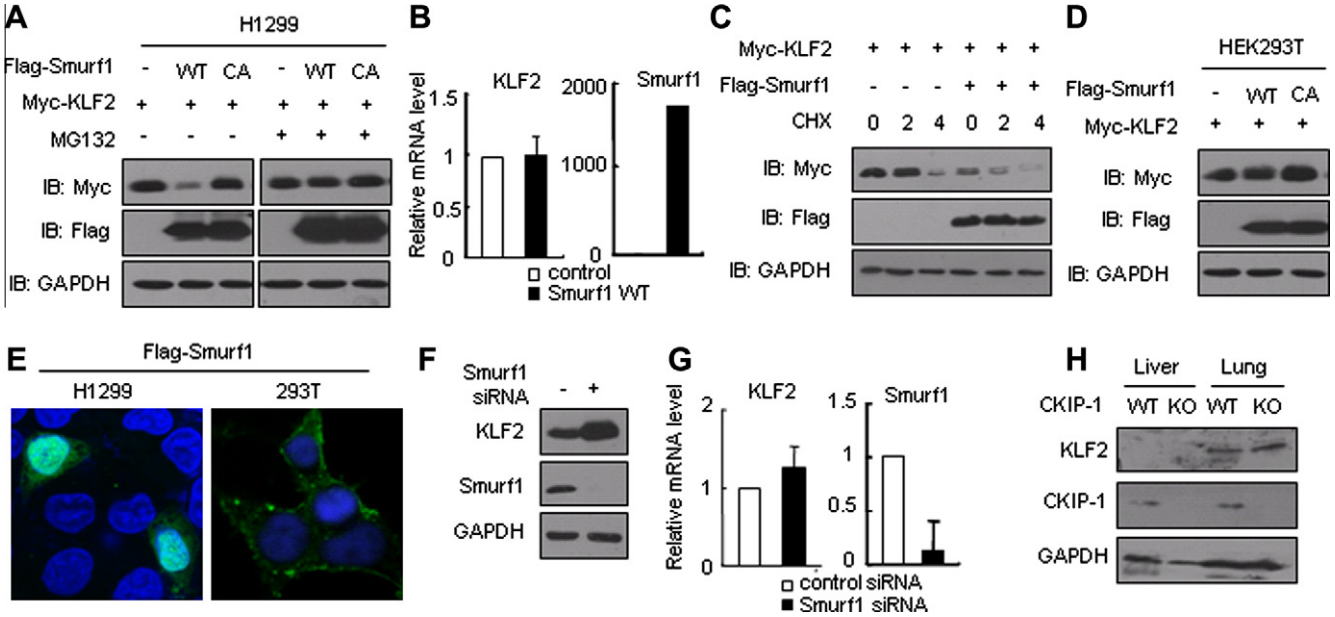

Fig. 3. Smurf1 destabilizes KLF2 in H1299 cells. (A) H1299 cells were transfected with Myc-KLF2, Flag-tagged wild type (WT) Smurf1 or its ligase-inactive mutant (C699A). The cells were then treated with or without MG132 before harvest. (B and G) H1299 cells were transfected with control plasmid or Flag-Smurf1 (B), or with non-targeted control or Smurf1-specific siRNA (G), and KLF2 mRNA level was analyzed by real-time PCR assay. (C) H1299 cells were transfected with control plasmid or Flag-Smurf1, and cells were treated with the protein synthesis inhibitor cycloheximide (CHX, 10 μg/ml) for the indicated times before harvest. (D) HEK293T cells were transfected Myc-KLF2, with Flag-tagged Smurf1 WT or C699A. KLF2 level was analyzed by immunoblotting. (E) Flag-Smurf1 was transfected into H1299 and HEK293T cells. Green represents the Smurf1 proteins and nuclei were stained with DAPI. (F) H1299 cells were transfected with non-targeted control or Smurf1-specific siRNA, and the endogenous KLF2 and Smurf1 level were detected. (H) Endogenous KLF2 protein level in lung tissue of *CKIP-1*−/− mice was markedly upregulated. After the organs were lysised, KLF2, CKIP-1 and GAPDH protein level were analyzed by immunoblotting.

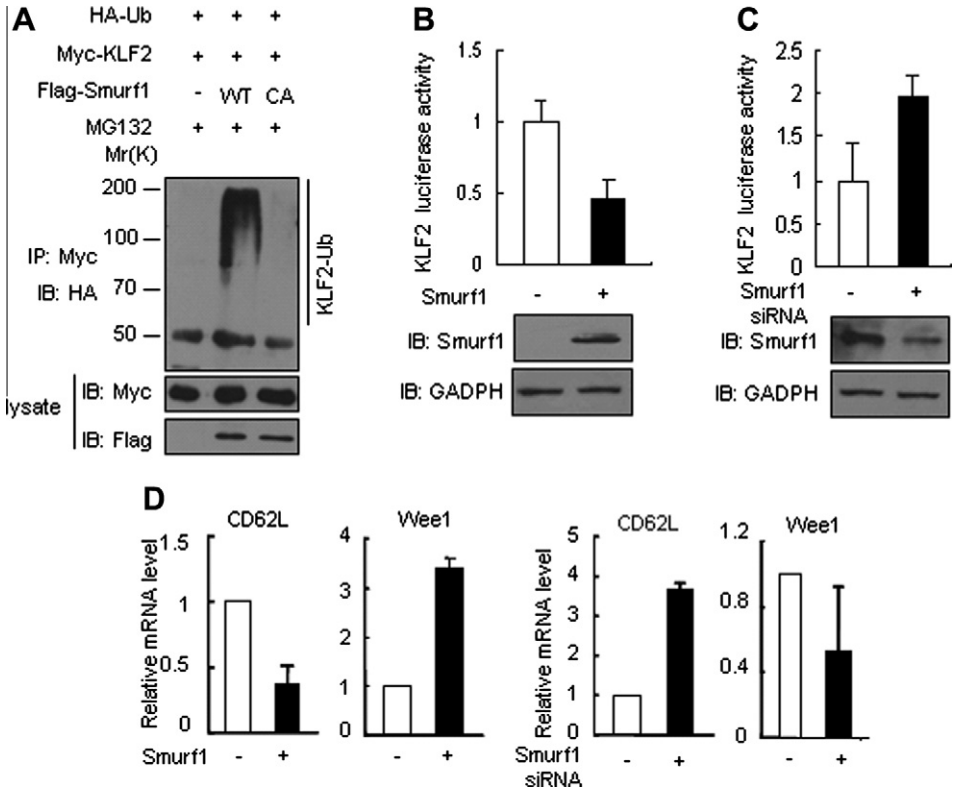

Fig. 4. Smurf1 promotes the ubiquitination of KLF2 and represses the transcriptional activity of KLF2. (A) H1299 cells were transfected with HA-Ub, Myc-KLF2, control vector or Flag-Smurf1 WT or CA, and treated with MG132 as indicated. Ubiquitinated KLF2 was immunoprecipitated (IP) with anti-Myc antibody and detected by immunoblotting with anti-HA antibody. (B and C) H1299 cells transfected with Smurf1 or mock vector, with non-targeted control or Smurf1-specific siRNA. KLF2 reporter activity was assayed as described in Materials and Methods. (D) Smurf1 regulates the expression of KLF2 downstream genes. H1299 cells were transfected with Smurf1 or mock vector, and non-targeted control or Smurf1-specific siRNA. Total RNA was subjected to real-time RT-PCR analysis. Mean values and SD (error bar) are depicted.

to regulate transcription of the KLF2 target genes was determined. Quantitative real-time RT-PCR analysis showed that overexpression of Smurf1 significantly decreased the mRNA level of CD62L and increased that of Wee1, while depletion of Smurf1 resulted in a dramatic increase of CD62L mRNA level and a decrease of Wee1 (Fig. 4D). These data indicated that Smurf1 inhibited the transcriptional activity of KLF2 to control its target genes.

4. Discussion

In this study, we identified the HECT-type ubiquitin ligase Smurf1 could target the Krüppel-like factor KLF2 for ubiquitination and degradation in a proteasome-dependent manner. Notably, this regulation seems to be cell type-dependent since it was observed in human lung cancer H1299 cells but not human embryonic kidney HEK293T cells. The possible cause might be the different distribution pattern of Smurf1 in these cells (Fig. 3). Depletion of Smurf1 in H1299 resulted in a significant upregulation of KLF2 protein level. In vivo ubiquitination assay showed that KLF2 was a new nuclear substrate of E3 ligase Smurf1. As far as we know, only Runx2 and JunB have been shown as the nuclear substrates of Smurf1 [17,19]. Therefore, our current findings provide new clues of Smurf1 nuclear functions.

Although KLF2 has been demonstrated to be degraded by the ubiquitin–proteasome system, the responsible ubiquitin ligase for KLF2 remains unclear. A previous suggested candidate is WWP1, another member of Nedd4 family; however, mutation of WWP1's catalytic cysteine (C886) did not affect its ability to promote ubiquitin-modification and proteasomal degradation of KLF2 [9]. By contrast to WWP1, the catalytic activity of Smurf1 was required for KLF2 degradation (Fig. 3). Whether WWP1 cooperates with Smurf1 to promote KLF2 degradation under physiological conditions need to be further investigations. Interestingly, both WWP1 and Smurf1/2 have been shown to target Smads of growth-inhibitory transforming growth factor β (TGF-β) signaling pathway for degradation [20]. Although Smurf1 may have a direct catalytic function in ubiquitination of KLF2, we can not obviate the possible role of WWP1 in this proteasomal degradation progress.

Nedd4 family members contain an N-terminal C2 domain, two to four WW domains and a C-terminal HECT domain [21]. It has been well-characterized that the central WW domains mediate the recognition of most of the defined substrates, and our data also indicate that the WW domains of Smurf1 function as the substrate recognition module. Most WW domains of HECT E3s recognize the PPxY or PxxY (PY) motif of substrate. Although one PPPY (aa 81–84) motif exist in the N-terminal transactivation domain of KLF2, our data suggest that the transactivation domain is not required for the interaction with Smurf1 (Fig. 2B). Instead, the inhibitory domain and zinc finger domain mediate the interaction. Additionally it is also the inhibitory domain that is recognized by WWP1 [9]. Therefore, we speculate that the canonical WW domain-PY motif rule might be not utilized by Smurf1-KLF2 pair.

KLF2 functions as either a transactivator or repressor. Smurf1 represses the transcriptional factor activity of KLF2 and regulates its down-stream genes such as CD62L and Wee1. CD62L is critical for KLF2 regulating T cell egress from the thymus and homing to the lymph nodes [8,22]. The involvement of Nedd4 family members in the regulation of the immune response has been widely studied in the case of Itch [23]. Our results implicated that Smurf1 might also play a role in immunity control by regulating KLF2, which is worthy to be deeply investigated in the future.

Acknowledgments

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