Smurf2 regulates the degradation of YY1

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1. Introduction

The transcription factor Yin Yang 1 (YY1) plays important roles in cell proliferation and differentiation, and it is highly conserved from insects to mammals [1–6]. YY1 directly or indirectly regulates the expression of target genes, and YY1 can induce or repress target gene expression depending on the cofactors it recruits [6–9]. In addition, YY1 can control gene expression by promoting the post-translational modifications of cofactors. The activity of YY1 itself is also regulated by various post-translational modifications. Acetylation and deacetylation of YY1 by p300 and histone deacetylases modulate the transcriptional activity of YY1 [9–11]. Phosphorylation of YY1 affects its DNA binding ability [12], and sumoylation can alter the specificity of target promoters [13]. YY1 mono-ubiquitination enhances its interaction with C-terminal binding protein (CtBP) and HDAC3, resulting in the formation of a transcription repressor complex that suppresses the expression of Mmp9 [14]. YY1 represses the expression of muscle-specific genes [15,16], and YY1 degradation is a prerequisite for myocyte differentiation [17]. However, little is known about the regulatory mechanism for YY1 degradation and the enzymes that control this process. In ubiquitin-proteasome-mediated degradation, proteins are targeted for degradation by covalent poly-ubiquitination at lysine residues. This requires a coordinated action of three different types of enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase [18]. In this study, we attempted to identify potential E3 ubiquitin ligases for YY1. Among the E3 ligases tested, Smurf2 (Smad ubiquitination regulatory factor 2) affected YY1 protein level significantly. Smurf2 interacted with YY1 and promoted the poly-ubiquitination of YY1. In addition, Smurf2 decreased the protein half-life and transcriptional activity of YY1. Conversely, knockdown of Smurf2 increased the protein level and transcriptional activity of YY1. Smurf2 binding to YY1 and Smurf2-induced degradation of YY1 required the PPxY motif of YY1. PPxY motif is the typical target recognition site of Smurf2. Finally, Smurf2 relieved the suppression of p53 activity by YY1. Taken together, our results suggest a novel regulatory mechanism for YY1 function by Smurf2 in which the protein stability and transcriptional activity of YY1 are regulated by Smurf2 through the ubiquitin-proteasome-mediated degradation of YY1.

2. Materials and methods

2.1. Cell culture

293 human embryonic kidney cells were maintained at 37 °C, 5% CO₂ in DMEM supplemented with 10% FBS, antibiotics and antimycotics. All culture media and supplements were purchased from Life Technologies.
Expression plasmids for N-terminal epitope-tagged human YY1, Smurf2 and p53 were constructed in a CMV promoter-derived mammalian expression vector (pCS4). Deletion mutants of YY1 were generated by PCR-based mutagenesis and confirmed by DNA sequencing. Plasmid for Smurf2 (C716G) mutant was generously provided by Dr. Eek-Hoon Jho (The University of Seoul). YY1-Luc and p53-Luc were generated by inserting 3 tandem repeats of a consensus binding sequence for YY1 (5′-CCG CATTTC-3′) or p53 to pGL3-Basic. For knockdown of Smurf2, oligonucleotides targeting following sense sequences were synthesized: si-Smurf2 #1, 5′-CCT TCT GTG TTT AAT ACA A-3′; si-Smurf2 #2, 5′-GAC CAA CAG CAA CAG CAA G-3′. Sense and antisense oligonucleotides were annealed and ligated into pSuper-retro vector (Oligoengine). Retroviruses were produced according to the manufacturer’s instruction. The following antibodies were used: anti-Flag (M2) from Sigma-Aldrich; anti-GFP (B-2), anti-YY1 (H-414) and anti-Myc (9E10) from Santa Cruz Biotechnology; anti-HA (12CA5) from Roche Applied Science; anti-Smurf2 (2078-1) from Epitomics; and anti-α-tubulin (DM1A) from Cell Signaling Technology.

2.2. Plasmids, antibodies and reagents

Aliquots of cell lysates corresponding to approximately 10^7 cells were incubated with glutathione-Sepharose beads carrying 10 μg of GST-fusion protein (GST-YY1 or GST-Smurf2) for 5 h at 4 °C. Bound proteins were analyzed by immunoblotting.

3. Results

3.1. Smurf2 can down-regulate YY1

In order to identify potential E3 ubiquitin ligases for YY1, we examined the effects of various E3 ligases on YY1 protein level. Among the E3 ligases tested, two types of E3 ligases affected YY1 reproducibly (data not shown). One was the HECT domain type E3 ligase family that includes Smurf1/2 and Itch, and the other was the RING finger type E3 ligase family that includes Cbl-b and c-Cbl. The HECT domain type and the RING finger type E3 ligases differ in their ubiquitin ligation chemistry [19]. We investigated the ability of these two types of E3 ligases for modulating YY1 protein level, and found that YY1 protein level is reduced significantly by the HECT domain type E3 ligases (Fig. 1A). We decided to investigate the function of Smurf proteins further, since YY1 contains the PPxY motif which is the typical target recognition binding site of Smurf1/2.

2.3. DNA transfection and reporter assays

Transient transfection was performed using the calcium phosphate-mediated method or the polyethyleneimine (Polysciences, Inc.)-mediated method. Unless otherwise specified, cells were analyzed 48 h after transfection. For luciferase assays, cells were transfected with indicated plasmids along with pCMV-β-Gal. 36 h later, luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized with corresponding β-galactosidase activities.

2.6. GST pull down assay

Cells were lysed in an ice-cold lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_2VO_4, 250 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. Lysates were cleared by centrifugation. For immunoblotting, α-tubulin was used as a loading control. For immunoprecipitation, the supernatants were incubated with appropriate antibodies and protein A or G-sepharose beads. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and visualized using appropriate antibodies and chemiluminescence Western blotting reagent (GE Healthcare).

2.4. Immunoblotting and immunoprecipitation

2.5. RNA preparation and semi-quantitative RT-PCR

Fig. 1. Smurf2 down-regulates YY1. (A) 293 cells were transfected with Myc-tagged YY1 and indicated E3 ubiquitin ligases. Levels of overexpressed YY1 protein are compared by anti-Myc immunoblotting [Myc (YY1)]. Levels of overexpressed E3 ligases are also compared by anti-Flag [Flag (Smurf1/2, Itch)] and anti-HA [HA (Cbl)] immunoblotting. Tubulin was used as a loading control. (B) 293 cells were transfected with a YY1-responsive luciferase reporter (YY1-Luc) and indicated combinations of YY1, Smurf2, or Smurf2 siRNA #1 (si-Smurf2). 36 h later, cells were assayed for luciferase activities. Results were analyzed using Student’s t-test, with p < 0.05. Experiments were performed in triplicate and repeated three times. The averages and standard deviations of a representative experiment are shown.
Next, we examined whether Smurf2 also affects the transcriptional activity of YY1. Smurf2 decreased YY1-induced expression of an YY1-responsive reporter (YY1-Luc) whereas Smurf2 knockdown increased it (Fig. 1B). However, Smurf1 knockdown did not affect the protein levels of endogenous and overexpressed YY1 (data not shown). Taken together, these results indicate that Smurf2 can negatively regulate the protein level and transcriptional activity of YY1.

3.2. Smurf2 enhances the proteasome-mediated degradation of YY1

We investigated the effect of Smurf2 on YY1 protein level in more detail. In 293 cells, overexpressed Smurf2, but not c-Cbl, decreased the level of endogenous YY1 protein (Fig. 2A). Down-regulation of YY1 by Smurf2 was abolished in the presence of a proteasome inhibitor MG132, suggesting that Smurf2 reduces YY1 protein level through the proteasome-mediated degradation. Smurf2 also decreased the level of overexpressed YY1 in dose-dependent and MG132-sensitive manners (Fig. 2B). Mutation that impedes the E3 ubiquitin ligase activity of Smurf2, substitution of cysteine716 to glycine (C716G), abolished the ability of Smurf2 to decrease YY1 protein level (Fig. 2C). Knockdown of Smurf2 resulted in a significant increase of endogenous YY1 protein level (Fig. 2D). However, overexpression or knockdown of Smurf2 did not affect the level of endogenous YY1 mRNA significantly (Fig. 2E). Next, we examined the effect of Smurf2 on the half-life of YY1 protein in the presence of a translation inhibitor cycloheximide. In the absence of Smurf2, the half-life of YY1 exceeded 6 h (Fig. 2F). Overexpression of Smurf2 reduced the half-life of YY1 to approximately 3 h, indicating that Smurf2 modulates the stability of YY1 protein. Taken together, these results suggest that Smurf2 negatively regulates YY1 through the proteasome-mediated degradation of YY1, but not through the reduction of YY1 transcription.

3.3. Smurf2 enhances the poly-ubiquitination of YY1 and interacts with YY1

We also examined the effect of Smurf2 on YY1 poly-ubiquitination. Wild type Smurf2, but not the E3 ubiquitin ligase-defective mutant, increased the poly-ubiquitination of YY1 (Fig. 3A).

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**Fig. 2.** Smurf2 reduces YY1 protein level through the proteasome-mediated degradation. (A–B) 293 cells were transfected with HA-Smurf2 or HA-c-Cbl (A), or Myc-YY1 and increasing amounts of HA-Smurf2 (B). Cells were then treated with MG132 (2 μM) or vehicle alone (DMSO) for 8 h. (C) 293 cells were transfected with indicated combinations of Myc-YY1 and HA-tagged wild type (WT) or E3 ubiquitin ligase-defective C716G mutant (CG) Smurf2. (D) 293 cells were transfected with retrovirus expressing Smurf2 siRNA (si-Smurf2) #1 or #2. For panels A–D, levels of endogenous YY1, overexpressed Myc-YY1 and endogenous Smurf2 are compared by immunoblotting (IB). Arrow heads indicate overexpressed HA-Smurf2. For panel A, the level of overexpressed HA-Smurf2 is also examined by anti-Smurf2 IB. (E) 293 cells were transfected with Myc-YY1 alone or with Myc-Smurf2. 48 h later, cells were treated with cycloheximide (CHX, 40 μg/ml) for indicated amounts of time. Left panel: The levels of overexpressed YY1 are compared by IB. Right panel: The relative intensities of YY1 bands in the left panel are measured by densitometry.
Next, we examined the possibility of interaction between Smurf2 and YY1. When transfected in 293 cells, YY1 co-immunoprecipitated Smurf2 and vice versa (Fig. 3 B). In addition, overexpressed Smurf2 co-immunoprecipitated endogenous YY1 (Fig. 3 C). YY1 also interacted with Smurf2 (C716G mutant) (Fig. 3 D), indicating that the E3 ubiquitin ligase activity of Smurf2 is not necessary for their interaction. We then investigated whether YY1 can bind to purified Smurf2 protein by GST pull-down assay. Bacterially expressed, purified GST-YY1 interacted with Smurf2, suggesting a potential direct interaction between YY1 and Smurf2 (Fig. 3 E). However, c-Cbl did not interact with YY1 (Fig. 3 F). Taken together, these results suggest that Smurf2 interacts with YY1 and enhances poly-ubiquitination of YY1.

3.4. PPxY motif containing region of YY1 is necessary for the interaction with Smurf2

Next, we attempted to identify the specific domains of YY1 that are important for the interaction with Smurf2 using a series of YY1 deletion mutants. Results of co-immunoprecipitation (Co-IP) assays revealed that deletion of a region (amino acids 196–295) containing the PPxY motif of YY1 (PYPD in the case of YY1) abolished the interaction with Smurf2 (Fig. 4 B). Next, we examined the binding between YY1 deletion mutants and Smurf2 by GST pull-down assay. Similar to the results of Co-IP assays, GST-Smurf2 bound to all but YY1 deletion mutants (1–154 and 81–195) lacking the PPxY motif containing region (Fig. 4 C and D). PPxY motif is the typical recognition and interaction site for Smurf2 E3 ubiquitin ligase domain. These results suggest that the PPxY motif containing region of YY1 is necessary for the interaction with Smurf2.

3.5. The PPxY motif of YY1 is important for Smurf2-induced degradation of YY1

Next, we examined whether the PPxY motif is also important for Smurf2-enhanced degradation of YY1. Smurf2 did not reduce the protein level of a YY1 mutant lacking the PPxY motif (∆PPxY) (Fig. 5 A). In addition, knockdown of Smurf2 did not affect the protein level of wild type YY1 (∆PPxY) mutant, whereas it increased the protein level of wild type YY1 (Fig. 5 B). It is also noteworthy that the basal level of YY1 (∆PPxY) protein was higher than that of wild type YY1, even when equal amounts of DNA were used for transfection. We also tested the interaction between Smurf2 and YY1 (∆PPxY) mutant by GST pull-down assay. Purified GST-Smurf2 interacted with wild type YY1 but it failed to interact with YY1 (∆PPxY) mutant, suggesting that the PPxY motif of YY1 is important for the interaction with Smurf2 (Fig. 5 C). Consistently, deletion of the PPxY motif significantly prolonged the half-life of YY1 protein (Fig. 5 D). These results suggest that the PPxY motif of YY1 is important for Smurf2-induced degradation of YY1.

3.6. Smurf2 relieves the suppression of p53 activity by YY1

Previous studies have reported that YY1 can inhibit p53 activity [20,21]. Therefore, we analyzed the effect of Smurf2 on YY1-mediated suppression of p53 activity using a p53-responsive luciferase reporter
The PPxY motif of YY1 is important for the interaction between YY1 and Smurf2. (A) Schematic representation of regions removed in YY1 deletion mutants. Relative location of the PPxY motif (amino acids 248–251) is indicated by a red asterisk. (B) 293 cells were transfected with HA-Smurf2 and indicated Myc-YY1 deletion mutants. The interaction between YY1 and Smurf2 is determined by anti-Myc IP followed by anti-HA IB. (C–D) Lysates from 293 cells transfected with indicated Myc-YY1 mutants were incubated with GST-Smurf2 fusion protein bound to glutathione-sepharose beads. YY1 proteins brought down with the beads are analyzed by anti-Myc IB (GST-Smurf2 pull down).

PPxY motif has been shown to serve as the recognition site for Smurf2 WW domains [22,23]. Smurf2 contains a HECT domain, a C2 domain, and three WW domains (WW1, WW2, and WW3). Studies have shown that the WW domains of Smurf2 are responsible for the substrate recognition and interaction. These results and ours suggest that the poly-ubiquitination-mediated degradation of YY1 can be a novel regulatory mechanism for YY1 function. YY1 consists of several functional domains: an N-terminal transcriptional activator domain (amino acids 1–154), a glycine-lysine-rich transcriptional repressor domain (amino acids 154–226), and a C-terminal transcriptional repressor domain (amino acids 226–414) containing four C2H2 type zinc-finger domains [24]. The PPxY motif is located at amino acids 248–251 of YY1. Although YY1 has been reported to interact with various transcription factors and transcription regulators including Smad1, HDAC and p300 through its functional domains, protein interaction with YY1 PPxY motif has not been reported so far. Our results suggest a novel regulatory mechanism of YY1 function involving its PPxY motif.

Controlling the degradation of YY1 is a critical step for myocyte differentiation. Smurf2 may play a key role in this process. Smurf2 was first recognized as a negative regulator of the BMP/TGF-β signaling pathway. Smurf2, through its E3 ubiquitin ligase activity, down-regulates BMP/TGF-β signaling by targeting receptors for BMP/TGF-β signaling pathway and receptor-regulated Smads for destruction [25–27]. In addition to the signaling components, Smurf2 has been shown to regulate the downstream effectors of TGF-β/BMP signaling.

(p53-Luc). p53 induced the expression of p53-Luc (Fig. 6 A), YY1 reduced p53-induced expression of p53-Luc, and Smurf2 relieved this suppression in a dose-dependent manner. We then examined the effect of Smurf2 on YY1-mediated suppression of p53 target gene expression. p53-induced expression of p21, BAX and NOXA was reduced by YY1, and concurrent knockdown of Smurf2 further reduced the expression of p53 target genes (Fig. 6 B). These results suggest that the suppressive ability of YY1 on p53 activity can be negatively regulated by Smurf2.

### 4. Discussion

In this report, we provided evidences that Smurf2 can function as an E3 ubiquitin ligase for YY1, and regulates the protein stability and transcriptional activity of YY1. The PPxY motif of YY1 is important for the recognition and degradation by Smurf2.

We showed that wild type YY1 overexpressed in 293 cells, but not the YY1 (ΔPPxY) mutant, interacts with purified Smurf2 protein. We attempted to examine the direct interaction between purified YY1 and Smurf2 proteins. However, we were unable to detect such interaction. Although we cannot rule out the possibility of direct interaction between YY1 and Smurf2, as E3 ubiquitin ligases need to recognize and bind to the target protein for ubiquitin conjugation, the inability to detect the direct interaction between them may reflect the requirement of other adaptor molecules/proteins.
Fig. 5. The PPxY motif of YY1 is important for Smurf2-induced degradation of YY1. (A) 293 cells were transfected with Myc-YY1 [wild type or PPxY motif deleted (ΔPPxY) mutant] and increasing amounts of Flag-Smurf2. (B) 293 cells were transduced with retrovirus expressing Smurf2 siRNA (si-Smurf2) #1 or #2. Cells were then transfected with Myc-tagged wild type or ΔPPxY mutant YY1. For panels A and B, the levels of overexpressed Myc-YY1 protein and endogenous Smurf2 are compared by IB. (C) Lysates from 293 cells transfected with Myc-tagged wild type or ΔPPxY mutant YY1 were incubated with GST-Smurf2 fusion protein bound to Glutathione-sepharose beads. YY1 proteins brought down with the beads are analyzed by anti-Myc IB (GST-Smurf2 pull down). (D) 293 cells were transfected with Myc-tagged wild type or ΔPPxY mutant YY1. 48 h later, cells were treated with cycloheximide (CHX, 40 μg/ml) for indicated amounts of time. Left panel: The levels of overexpressed Myc-YY1 are compared by anti-Myc IB. Right panel: The relative intensities of YY1 bands in the left panel are measured by densitometry.

Fig. 6. Smurf2 relieves the suppression of p53 activity by YY1. (A) 293 cells were transfected with a p53-responsive luciferase reporter (p53-Luc) and indicated combinations of p53, YY1 and Smurf2 (numbers indicate amounts of DNA used in μg). 36 h later, cells were assayed for luciferase activities. Results were analyzed using Student’s t-test, with p < 0.05. Experiments were performed in triplicate and repeated three times. The averages and standard deviations of a representative experiment are shown. (B) 293 cells were transduced with retrovirus expressing Smurf2 siRNA #1 (si-Smurf2). Cells were then transfected with indicated combinations of p53 and YY1. The levels of p21, BAX and NOXA mRNA are compared by RT-PCR. Numbers indicate the relative intensities of corresponding bands measured by densitometry.
For example, Smurf2 regulates the function of Runx2, a key transcription factor for BMP-stimulated osteoblast differentiation [28,29]. YY1 has been shown to mediate the regulation of cell growth and differentiation by BMP and TGF-β signaling pathways [30]. These results indicate that BMP and TGF-β signaling may be involved in the interaction/regulation between Smurf2 and YY1. Although we identified Smurf2 as a potential regulator of YY1, it is still unknown whether BMP/TGF-β signaling pathways regulate the interaction between Smurf2 and YY1. Therefore, further investigation is needed to understand the functional significance of Smurf2-induced poly-ubiquitination/degradation of YY1 in the regulation of cell growth and differentiation by BMP and TGF-β signaling.

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