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HECT ubiquitin ligase Smurf1 targets the tumor suppressor ING2 for ubiquitination and degradation

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ABSTRACT

Inhibitor of growth 2 (ING2) gene encodes a candidate tumor suppressor and is frequently reduced in many tumors. However, the mechanisms underlying the regulation of ING2, in particular its protein stability, are 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 ING2 for poly-ubiquitination and proteasomal degradation. Intriguingly, the ING2 binding domain in Smurf1 was mapped to the catalytic HECT domain. Furthermore, the C-terminal PHD domain of ING2 was required for Smurf1-mediated degradation. This study provided the first evidence that the stability of ING2 could be regulated by ubiquitin-mediated degradation.

Structured summary:
MINT-7894271: *ING2* (uniprotkb:Q9H160) *binds* (MI:0407) to *Smurf1* (uniprotkb:Q9HCE7) by *pull-down* (MI:0096)
MINT-7894319, MINT-7894339: *ING2* (uniprotkb:Q9H160) *physically interacts* (MI:0915) with *Smurf1* (uniprotkb:Q9HCE7) by *anti tag co-immunoprecipitation* (MI:0007)
MINT-7894301: *Smurf1* (uniprotkb:Q9HCE7) *physically interacts* (MI:0915) with *ING2* (uniprotkb:Q9H160) by *anti bait co-immunoprecipitation* (MI:0006)
MINT-7894358: *ING1b* (uniprotkb:Q9UK53-2) *physically interacts* (MI:0915) with *Smurf1* (uniprotkb:Q9HCE7) by *anti tag co-immunoprecipitation* (MI:0007)
MINT-7894249: *ING2* (uniprotkb:Q9H160) *physically interacts* (MI:0915) with *ubiquitin* (uniprotkb:P62988) by *anti tag co-immunoprecipitation* (MI:0007)

1. Introduction

The human inhibitor of growth (ING) family members, consisting of ING1 to ING5, play a significant role in a broad variety of cellular events, such as growth regulation, senescence, apoptosis, DNA damage repair, transcription regulation and chromatin remodeling [1]. All ING proteins contain a highly conserved C-terminal plant homeodomain (PHD) finger motif which has been shown to recognize histone H3 trimethylated at lysine 4 (H3K4me3). The ING proteins also possess nuclear localization sequences (NLS), which are critical for the ING proteins to be localized in the nucleus [1,2].

Abbreviations: E3, ubiquitin-protein ligase; HECT, homologous to E6AP carboxyl terminus; ING2, inhibitor of growth 2; Nedd4, neural precursor cells-expressed developmentally-downregulated gene 4; NLS, nuclear localization sequences; PHD, plant homeodomain; PtdInsPs, phosphoinositides; Smurf1, Smad ubiquitination regulatory factor 1

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ING proteins exert their biological functions through their associations with specific molecular partners which could be divide into three groups: components of HAT or HDAC complexes, nuclear transcriptional factors (e.g., p53 and NFκB), and the phosphoinositides (PtdInsPs) among the signaling lipids. The tumor suppressor genes are divided into two classes: the caretakers (or type I) and the gatekeepers (or type II). The former are usually DNA repair genes and protect the genome from mutations, whereas the latter prevent cancer through direct control of cell growth. Thus, restoration of missing gatekeeper functions to cancer cells leads to suppression of the neoplastic growth; whereas restoration of caretaker functions will not affect their growth [3]. As type II tumor suppressors, ING genes have been reported to be frequently dysregulated in human tumors, and various mechanisms, including mutations within ING genes, downregulation of gene expression, and protein mislocalization, have been proposed to explain how ING function is altered in the tumors [1,4,5].

ING2 was identified as the second member of ING family. Besides the conserved C-terminal PHD finger domain, ING2 also contains a central NLS sequence and a unique leucine zipper-like (LZL) domain at the N-terminus. The LZL domain is thought to mediate hydrophobic protein–protein interaction [6]. ING2 has been reported to regulate cell growth, apoptosis, DNA repair, chromatin remodeling and gene expression through association with various proteins including mSin3A–HDAC complex, p300, the SWI–SNF–BRG1 complex, RBP1, and H3K4me3 [7–10]. Moreover, ING2 could also interact with PtdInsPs including PtdIns(3)P and PtdIns(5)P via its PHD domain and functions as a specific nuclear PtdInsP receptor. By this means, ING2 is able to activate p53 and cooperates with p53 to induce cellular growth arrest and apoptosis [11].

Previous studies on ING2 status in cancer have indicated that ING2 participation in tumorigenesis is likely to be related to its expression level rather than its mutational status. Using the tissue microarray technology and immunohistochemistry analysis, the nuclear expression of ING2 is reduced in human melanomas compared to dysplastic nevi [12]. Similar results were observed in investigating the status of ING2 in a series of 120 non-small cell lung cancer (NSCLC) samples by using immunohistochemistry. ING2 protein expression is downregulated in more than 50% of NSCLC, with a higher frequency in adenocarcinoma as compared to squamous cell carcinoma. However, neither heterozygosity nor mutation in the ING2 gene could be detected [13]. Furthermore, the protein level of ING2 was also found to be decreased in hepatocellular carcinoma [14]. However, the mechanism responsible for ING2 regulation, in particular its protein stability, is poorly understood.

Here we demonstrate a critical role of Smad ubiquitination regulatory factor 1 (Smurf1) in regulating ING2 protein stability. Smurf1 belongs to the homologous to E6AP carboxyl terminus (HECT) domain-type ubiquitin-protein ligase (E3) and plays a pivotal role in control of cell polarity, maintenance of bone homeostasis and regulation of tumorigenesis through targeting BMP-Smad, Wnt and RhoA signalling pathways [15–18]. In this study, we found that Smurf1 interacts with ING2 both in vivo and in vitro, targets ING2 for poly-ubiquitination and proteasomal degradation. As far as we know this is the first evidence to establish the relevance between the ING2 protein and E3 ligase.

2. Materials and methods

2.1. Plasmid constructs

Full-length, truncated, and point mutations of Smurf1 and ING2 were constructed by inserting PCR amplified fragments into the related vectors. Detailed construct information is available upon request. pCMV/p53 was provided by Dr. Yue Xiong. 6Myc-Smurf1 wild-type, 6Myc-Smurf1-C699A and Flag-Smurf1 were provided by Dr. Kohei Miyazono. Myc-ING1b, Myc-ING2, Flag-ING3, HA-ING4 and Flag-ING5 were gifts from Dr. Karl Riabowol and Dr. Cutis C. Harris.

2.2. Reagents and antibodies

The protein synthesis inhibitor cycloheximide (CHX), anti-Flag and anti-ING2 antibodies were purchased from Sigma. Anti-HA antibody from Roche, anti-Myc antibody from MBL, and anti-Smurf1 antibody was bought from Abcam. Anti-GST and anti-His were from Tiangen. Anti-GAPDH (6C5) and secondary antibodies were purchased from Santa Cruz Biotechnology.

2.3. Cell culture and transfections

Human embryonic kidney HEK293T cells, human breast cancer MCF7 cells were cultured in DMEM medium (Hyclone) containing 10% fetal bovine serum (FBS; Hyclone). Human lung adenocarcinoma H1299 cells were maintained in RPMI 1640 medium (Hyclone) with 10% FBS. Mammalian cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Immunoprecipitation and immunoblotting

Cell lysates were prepared in HEPES lysis buffer (20 mM HEPES, pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF, and 1 mM DTT) supplemented with protease inhibitors. Immunoprecipitations were performed using primary antibody and protein A/G-agarose beads at 4 °C. Lysates and immunoprecipitates were examined using the indicated primary antibodies and then secondary antibody, followed by detection with SuperSignal chemiluminescence kit (Pierce).

2.5. In vivo and In vitro ubiquitination assays

For in vivo ubiquitination assay, cells were treated with MG132 (20 μM) for 8 h before harvested. The cell lysis were prepared in modified RIPA lysis buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 1% sodium deoxycholate, 0.025% SDS, protease inhibitors), and immunoprecipitated with the indicated antibody, and detected by immunoblotting.

For in vitro ubiquitination assay, His-Smurf1, GST-ING2 were expressed in *Escherichia coli* and purified with Ni-nitrilotriacetate-agarose beads (Qiagen) and glutathione-Sepharose 4B beads (Amersham) respectively, as we described [19]. The assays were carried out in 30 μl ubiquitination assay buffer (50 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, and 3 mM ATP), with 0.7 μg of E1, 1 μg of UbcH5c (E2), 15 μg HA-ubiquitin (all from Boston Biochem, MA), 0.7 μg of His-Smurf1 (wild-type or C699A mutant) and 1.5 μg GST-ING2. Samples were incubated at 30 °C for 2 h and terminated with sample buffer.

2.6. Reporter gene assay and apoptosis assay

The luciferase reporter plasmid pG13-Luc (pG13L, containing 13 tandem repeats of p53-binding sites) was a gift from Bert Vogelstein. The luciferase reporter assays were performed as we described previously [20]. Luciferase activity was measured with the Dual Luciferase Assay System (Promega) in accordance with the manufacturer's protocol. Apoptosis assay was performed with Annexin V staining method followed by flow cytometry analysis as described [20].

2.7. RNA interference

The Smurf1-specific siRNA (5′-GCAUCGAAGUGUCCAGAGAAG-3′), ING2-specific siRNA (5′-UCGGGCAAGACAAAUGGAGUU) and non-target control siRNA (5′-UUCUCCGAACGUGUCACGU-3′) were synthesized by Shanghai GenePharm. The siRNAs were transfected with Lipofectamine 2000.

2.8. Real-time RT-PCR

Real-time quantitative PCR was performed as described previously [21]. Reactions were done in triplicate, and relative amounts of cDNA were normalized to GAPDH. Primers used were as follows: Smurf1 F, 5′-CTACCAGCGTTTGGATCTAT-3′ and Smurf1 R, 5′-TGTCTCGGGTCTGTAAACT-3′; ING2 F, 5′-CACAAATGCTCGAATTGGTGG-3′ and ING2 R, 5′-TGCTTTATCTGAGGCTCGTTCA-3′; Puma F, 5′-GACCTCAACGCACAGTA-3′ and Puma R, 5′-CTAATTGGGCTCCATCT-3′; p21 F, 5′-CACCGAGACACCACTGGAGG-3′ and p21 R, 5′-GAGAAGATCAGCCGGCGTTT-3′; GAPDH F, 5′-GGGAAGGTGAAGGTCGGAGT-3′ and GAPDH R, 5′-TTGAGGTCAATGAAGGGGTCA-3′. F and R represent forward and reverse primer, respectively.

3. Results and discussion

3.1. Smurf1 negatively regulates the protein level of ING2 in a proteasome-dependent manner

The ubiquitin-proteasome pathway is one of the most important pathways that regulate protein degradation. To explore the mechanism responsible for ING2 stability regulation, we first detected whether ING2 protein turnover is regulated through the proteasome pathway. The level of endogenous ING2 protein increased significantly after treatment with MG132, a potent proteasome inhibitor (Fig. 1A), suggesting that ING2 protein could be degraded in a proteasome system.

The process of ubiquitination involves the sequential transfer of ubiquitin between a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a E3. E3s are final effectors of the enzyme cascade controlling ubiquitination and determine the specificity of substrate recognition. We next screened possible E3 regulator of ING2 from its interacting proteins. A high-throughput screening of TGF-β signalling network system identified ING1L (i.e. ING2) could interact strongly with both the wild-type (WT) Smurf1 ligase and the C699A ligase-inactive mutant [22]. Notably, this interaction is specific since ING2 neither interacts with Smurf2, the close member of Smurf1 among the neural precursor cells-expressed developmentally-downregulated gene 4 (Nedd4) family, nor with Smads and the TGF-β/BMP receptors among the TGF-β/BMP network. On the other hand, Smurf1 specifically interacts with ING2 but not with ING3 or ING4 [22]. Smurf1 has been demonstrated to target Smad1, Smad5, RhoA, MEKK2, Prickle1, PEM-2 and TRAFs for ubiquitination and degradation [15–18,23–25]. Then we hypothesized that Smurf1 might function as an E3 ligase in the ING2 degradation.

To test our hypothesis, ING2 protein level was analysed in the presence of ectopic Smurf1 or its CA mutant. Smurf1-WT significantly reduced the endogenous ING2 protein levels; in contrast, Smurf1-CA mutant slightly increased it which might be caused by a dominant negative effect (Fig. 1B, lanes 1–3). MG132 treatment blocked the downregulation of ING2 by Smurf1 (lane 4),

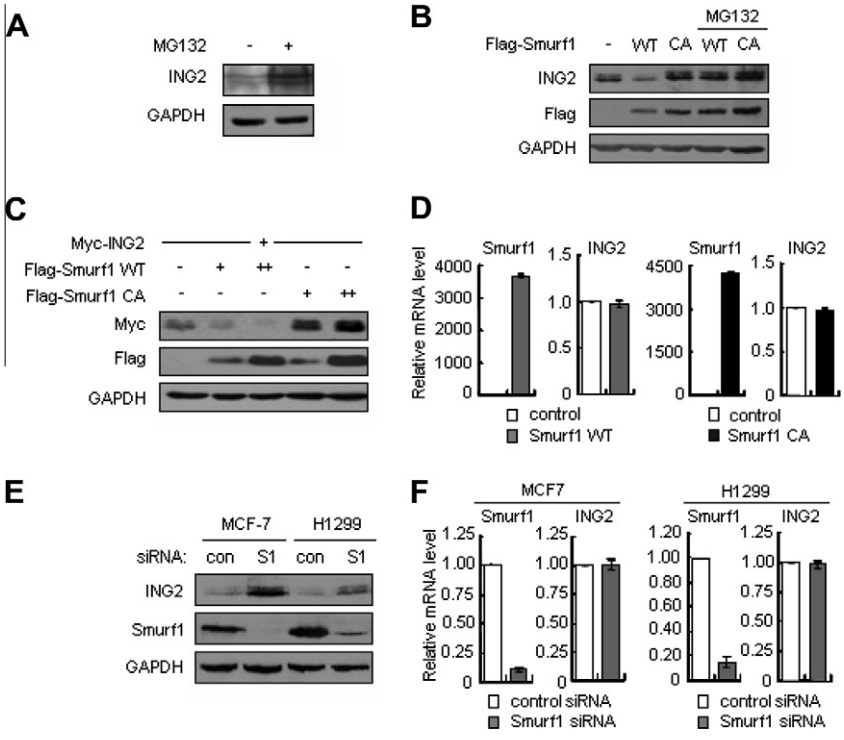

Fig. 1. Smurf1 negatively regulates the protein level of ING2. (A) MG132 treatment upregulates ING2 protein level. MCF7 breast cancer cells were treated with MG132 (20 μM) for 8 h before harvest. Endogenous ING2 level was analyzed by immunoblotting (IB). GAPDH was analyzed as an internal control. (B) Smurf1 decreases the endogenous ING2 protein level. MCF7 cells were transfected with Flag-tagged wild-type (WT) Smurf1 or its ligase-inactive mutant (C699A). The cells were then treated with or without the proteasome inhibitor MG132 (20 μM) for 8 h before harvest. The endogenous ING2 level was analyzed. (C) Smurf1 decreases the exogenous ING2 protein level in a dose-dependent manner. MCF7 cells were transfected with the increasing amount of Smurf1 together with ING2, and the ING2 level was detected. (D) Smurf1 has no effect on the mRNA level of ING2. The ING2 mRNA prepared from the transfected MCF7 cells was analyzed by real-time PCR assay. Data are presented as mean ± standard deviation (S.D., $n = 3$). (E and F) Smurf1 depletion increased the endogenous ING2 protein level but had no effect on ING2 mRNA level. MCF7 or H1299 cells were transfected with non-targeted control (NC) or Smurf1–specific (S1) siRNA, and the endogenous ING2 and Smurf1 level were analyzed by IB (E) or real-time PCR (F). Data are presented as mean ± S.D. ($n = 3$).

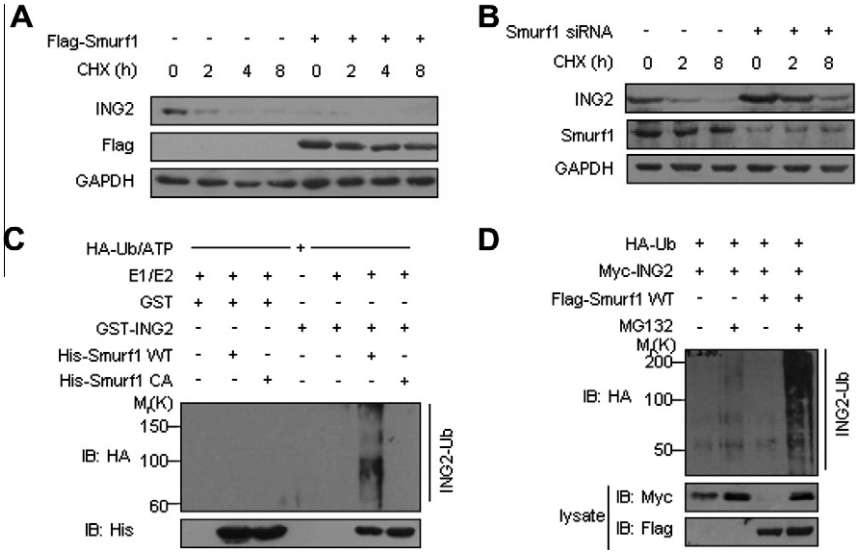

Fig. 2. Smurf1 promotes the ubiquitination and degradation of ING2. (A) Smurf1 shortens the half life of endogenous ING2 protein. MCF7 cells were transfected with control plasmid or Flag-Smurf1, and cells were treated with the protein synthesis inhibitor cycloheximide (CHX, 10 μg/ml) for the indicated times before harvest. ING2 protein expression was analyzed. (B) Depletion of Smurf1 prolonged the half life of endogenous ING2 protein. MCF7 cells were transfected with control siRNA or Smurf1 siRNA, treated with CHX and ING2 protein expression was analyzed. (C) Smurf1 catalyzes the ubiquitination of ING2 in vitro. Purified HA-ubiquitin, E1, E2 (UbcH5c), bacterial-expressed and purified His-Smurf1 WT or CA, GST-ING2 or GST were mixed for in vitro ubiquitination assays and immunoblotted with anti-HA. (D) Smurf1 enhances the ubiquitination of ING2 in vivo. MCF7 cells were transfected with HA-Ub, Myc-ING2, control vector or Flag-Smurf1, and treated with MG132 as indicated. Ubiquitinated ING2 was immunoprecipitated (IP) by anti-Myc antibody and analyzed by IB.

indicating that Smurf1 promoted the proteasome-mediated ING2 degradation. Smurf1 also negatively regulated the levels of exogenous ING2 protein in a dose-dependent manner (Fig. 1C). This regulation was not due to the change of ING2 mRNA abundance (Fig. 1D). These results suggested that Smurf1 promoted ING2 degradation dependent of its E3 ligase activity. To confirm the role of Smuf1 in regulating the ING2 protein stability in vivo, we decreased Smurf1 expression using small interfering RNA (siRNA). Downregulation of endogenous Smurf1 significantly increased ING2 levels in both MCF7 and H1299 cells (Fig. 1E). Again, Smurf1 depletion had no significant effect on the mRNA level of ING2 (Fig. 1F).

3.2. Smurf1 promotes the poly-ubiquitination of ING2

To examine whether the effect of Smurf1 on the ING2 protein levels is through stabilization of the protein, we measured the half life of ING2 protein. When cells were treated with cycloheximide (CHX), the protein synthesis inhibitor, the turnover of endogenous ING2 was dramatically accelerated by co-expression of Smurf1 (Fig. 2A), while depletion of Smurf1 prolonged the half life of ING2 (Fig. 2B). To determine whether Smurf1 could directly catalyze the ubiquitination of ING2, we reconstituted an in vitro ubiquitination system using purified E1 and E2 (UbcH5c), bacteria-expressed His-Smurf1 and GST-ING2 or GST alone as a control. In this system, Smurf1-WT but not Smurf1-CA efficiently catalyzed the poly-ubiquitination of ING2 (Fig. 2C). In vivo ubiquitination assay showed that overexpression of Smurf1 significantly increased the poly-ubiquitination of ING2 in the presence of MG132 (Fig. 2D). These results suggested that Smurf1 functions as a candidate E3 ligase of ING2 for ubiquitination and degradation.

3.3. Smurf1 associates with ING2 both in vitro and in vivo

E3 ligase can interact with its substrates and facilitate the transfer of ubiquitin to one or more lysine residues in the substrates. To confirm the interaction between Smurf1 and ING2, in vitro GST pull-down assays with recombination His-Smurf1 and GST-ING2 were first performed, and the specific interaction of Smurf1 with GST-ING2, but not with GST alone was observed (Fig. 3A). To assess whether ING2 interacts with Smurf1 in vivo, a co-immunoprecipitation (Co-IP) assay was performed and the result revealed an association between endogenous Smurf1 and ING2 in the presence of MG132 (Fig. 3B). Thus, Smurf1 interacts with ING2 both in vitro and in vivo.

It has been well-defined that the WW domains of HECT E3s recognize the PPxY or PxxY (PY) motif of substrate [26]. However, no PY motif exists within ING2 protein. To clearly characterize the association between Smurf1 and ING2, we generated several Smurf1 deletion mutants to map the ING2-interacting region. Co-IP assays indicated that the HECT domain but not the C2 or WW domains of Smurf1-mediated the interaction with ING2 (Fig. 3C). Importantly, the HECT domain was both required and sufficient for Smurf1 to promote the degradation of ING2 (Fig. 3D) and to catalyze the poly-ubiquitination of ING2 in vitro (Fig. 3E) and in MCF7 cells (Fig. 3F).

We next mapped the binding region of ING2 with Smurf1. As shown in Fig. 3G, deletion of PHD domain together with the extreme C-terminus had no significant effect on the interaction between ING2 and Smurf1 (lane 5). Similarly, deletion of LZL region also had no significant effect on the interaction (lane 3). The central region (ΔLZLΔPHD, aa 64–220) was sufficient for Smurf1 binding although the affinity seems to decrease slightly (lane 4). We also tested the truncate with either the LZL or the PHD alone but failed due to the unsuccessful expression of these truncate constructs with undetermined reasons. These data suggested that the LZL or PHD domain was not required for ING2 binding to Smurf1 (although we cannot rule out this possibility) and the central region was sufficient for this binding.

3.4. The PHD domain of ING2 is critical for Smurf1-mediated degradation

To further explore the regulation mechanism of ING2 by Smurf1, we detected the effect of Smurf1 on the ING2 mutants to narrow down the region where ING2 is targeted by Smurf1.

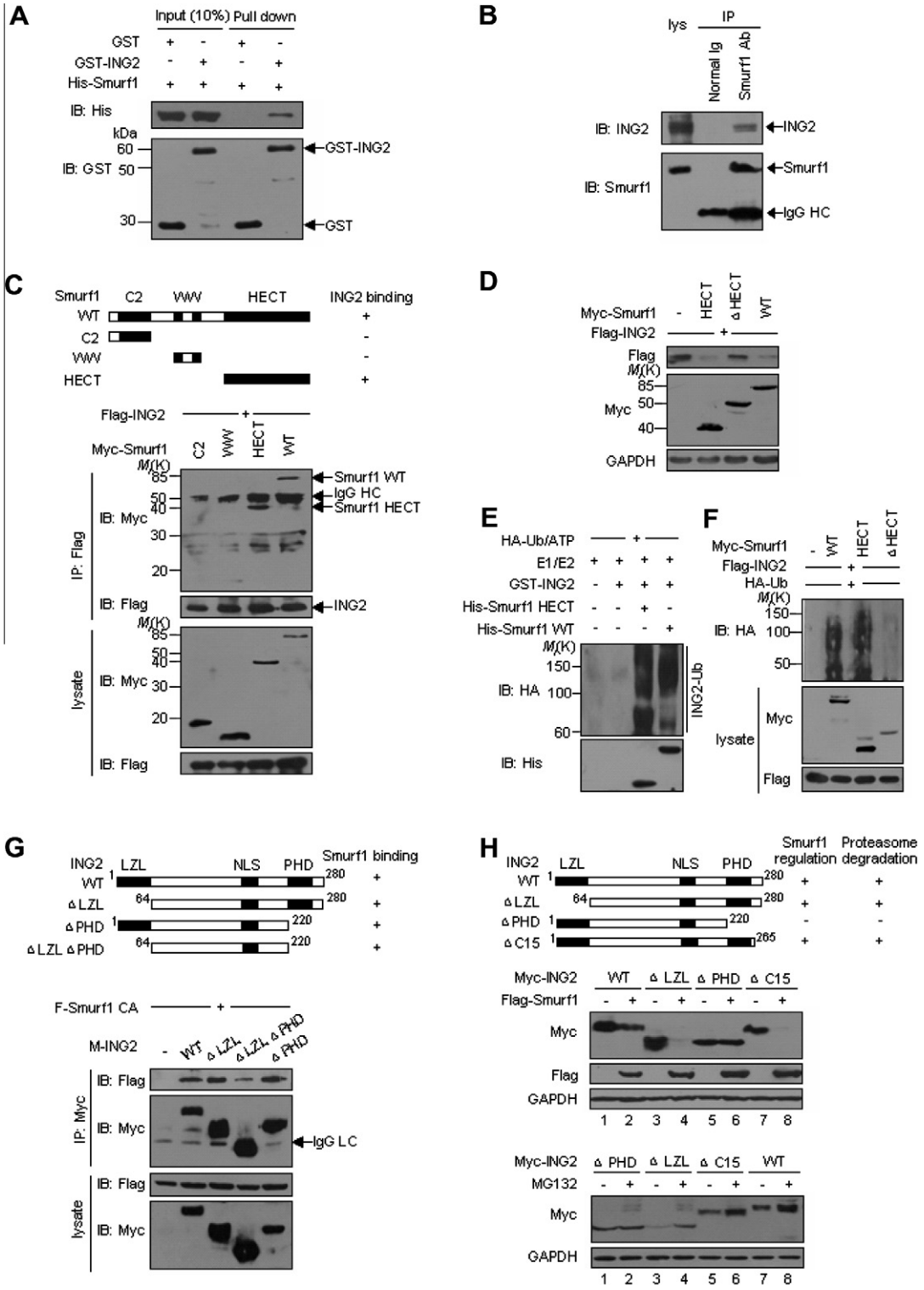

Fig. 3. Smurf1 interacts with ING2 in vitro and in vivo. (A) Direct interaction between Smurf1 and ING2 is revealed by GST pull-down assays. Input and pull-down samples were both subjected to immunoblotting with anti-GST and anti-His antibodies. Input represents 10% of that used for pull-down. (B) Co-immunoprecipitation of endogenous Smurf1 and ING2. To avoid the degradation of ING2, MG132 were added in MCF7 cells for 8 h before harvested. Cell lysates were immunoprecipitated with anti-Smurf1 antibody or a normal control IgG and all samples were analyzed by IB. IgG HC, heavy chain of IgG. (C) Smurf1 HECT domain mediates the interaction with ING2 protein. ING2 and Smurf1 deletion mutants were transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody. Both the lysate (bottom) and immunoprecipitates (upper) were analyzed by IB. (D) Smurf1 HECT domain determines the regulation of ING2. Flag-ING2 and Smurf1 WT or mutants were cotransfected into MCF7 cells. Cell lysates were analyzed by IB. (E) Smurf1 HECT domain catalyzed the poly-ubiquitination of ING2 in vitro. Purified HA-ubiquitin, E1, E2 (UbcH5c), bacterial-expressed and purified Smurf1 WT or mutant, GST-ING2 were mixed for in vitro ING2 ubiquitination assays and immunoblotted with anti-HA. (F) Smurf1 HECT domain promoted the ubiquitination of ING2 in vivo. HA-Ub, Flag-ING2 and Smurf1 constructs were cotransfected into MCF7 cells, and treated with MG132. Ubiquitinated ING2 was immunoprecipitated with anti-HA antibody and analyzed by IB. (G) The central region (aa 64–220) of ING2 binds to Smurf1. Smurf1 and the indicated deletion mutants of ING2 were transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-Myc antibody. Both the lysate (bottom) and immunoprecipitates (upper) were analyzed by IB. (H) The PHD domain of ING2 is crucial for the degradation of ING2 by Smurf1. Upper: MCF7 cells were transfected with Flag-Smurf1 and Myc-ING2 WT or mutants, and the cell lysates were subjected to immunoblot analysis. Lower: MCF7 cells were transfected with Myc-ING2 WT or mutants, and treated with or without MG132 (20 μM) for 8 h before harvest. Cell lysates were analyzed by IB.

Western blot analysis indicated that the PHD region of ING2 was crucial for Smurf1-mediated degradation (Fig. 3H). A deletion mutant lack of the extreme C-terminal 15 residues (named as ΔC15) was further generated in order to examine whether the PHD domain itself or the extreme C-terminus was critical in the degradation. Co-expression of Smurf1 significantly decreased the level of ING2-ΔC15 (Fig. 3H upper, lane 8), indicating that the extreme C-terminus was not required for this degradation. Thus, the PHD domain itself should contain the degradation signal recognized by Smurf1. We also observed that the protein levels of ING2 ΔLZL, ΔC15 and WT were significantly increased in the presence of proteasome inhibitor MG132, while the level of ΔPHD was unchanged (Fig. 3H lower panels), further indicating that the PHD domain was important for proteasome-mediated ING2 degradation.

3.5. Smurf1 specifically regulates ING2 and ING1b among ING family members and inhibits ING2-mediated activation of p53

ING family consists of five members. We next examined whether Smurf1 played a universal role in targeting ING family proteins for degradation. There are three splicing isoforms of ING1 gene, p33^{ING1b}, p47^{ING1a}, p27^{ING1d}, and another variant

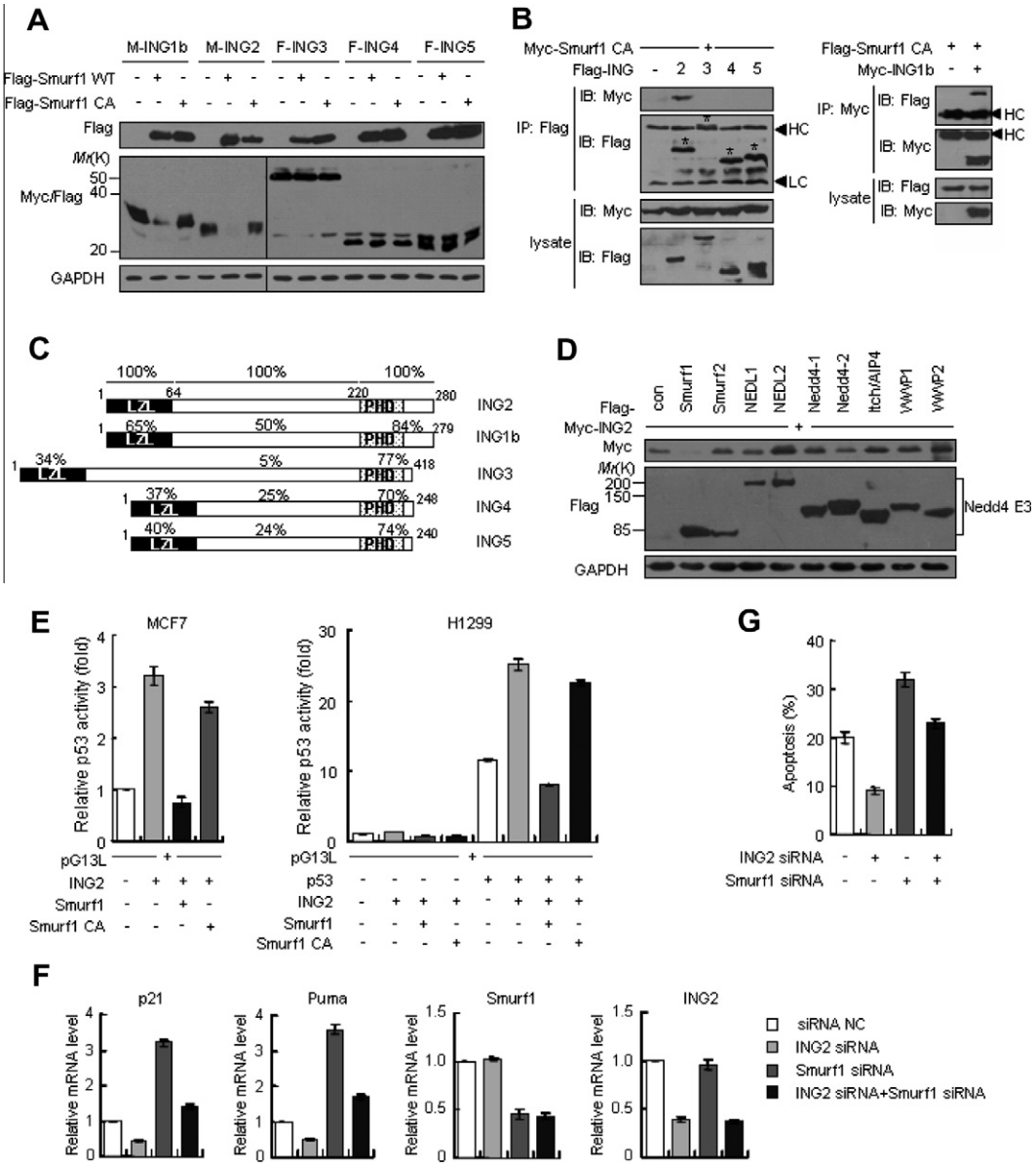

Fig. 4. Smurf1 regulates the stability of ING2, ING1b and inhibits ING2-mediated p53 transactivation. (A) The possible regulation of Smurf1 on ING family members. The indicated INGs plasmids were transfected into MCF7 cells together with or without Smurf1. The protein levels of INGs were analyzed. (B) Smurf1 interacts with ING2 and ING1b, but not with ING3, ING4 or ING5. Smurf1-CA and INGs constructs were transfected into HEK293T cells as indicated. Cell lysates were immunoprecipitated with anti-Flag (or Myc) antibody. Both the lysate (bottom) and immunoprecipitates (upper) were analyzed by IB. HC and LC represent heavy and light chain of IgG, respectively. The asterisks indicate ING proteins (left). (C) Protein sequence alignment of ING2 with the other human ING proteins. Take the LZL, central region, and PHD plus the extreme C-terminus sequence of ING2 as 100%, and blast the corresponding regions of other ING proteins with ING2, respectively. (D) ING2 specifically interacts with Smurf1 among Nedd4 family E3 ligases. Flag-tagged nine members of Nedd4 family of E3 ligases were each coexpressed with ING2 in MCF7 cells. The expression levels of ING2 and Nedd4 E3s were analyzed by immunoblotting with the indicated antibodies. (E) Effects of Smurf1 on p53 activity. p53-wild-type MCF7 (left) or p53-deficient H1299 cells (right) were transfected with the indicated constructs. p53 activity was measured by reporter gene assay. Representative results of three independent experiments are shown. Data are mean ± S.D. (n = 3). (F) The effect of Smurf1 on the expression of p53 targeted genes. Total RNA from ING2 RNAi or/and Smurf1 RNAi-treated MCF7 cells was subjected to quantitative real-time PCR analysis. Data are mean ± S.D. (n = 3). (G) The effect of Smurf1 on cell apoptosis. ING2 RNAi or/and Smurf1 RNAi-treated MCF7 cells were incubated with etoposide (100 μM) for 20 h. Apoptosis was determined by staining with Annexin V followed by flow cytometry analysis. Data are mean ± S.D. (n = 3).

$p24^{ING1c}$, among which $p33^{ING1b}$ is the major and most intensively characterized isoform [27]. ING2 exhibits 70% sequence homology to $p33^{ING1b}$ [28]. Phylogeny studies have shown that ING1 and ING2 on the one hand, whereas ING4 and ING5 on the other hand, have an overall high homology and, therefore, could have closely related functions [29]. Indeed, Smurf1 was able to promote the degradation of ING1 as well. By contrast, Smurf1 had no significant effect on the protein levels of ING3, ING4 and ING5 (Fig. 4A). Co-immunoprecipitation assays showed that Smurf1 interacted with ING2 and ING1b, but not with ING3, ING4 or ING5 (Fig. 4B), consistent with previous study [22]. Sequence alignment clearly indicates that the homology of the central region (aa 64–220) between ING2 and ING1b is 50%, much higher than that of ING3, ING4 and ING5 (5%, 25%, 24%, respectively), while the PHD region is conserved in all ING proteins (Fig. 4C). Given that the central region of ING2 is responsible for Smurf1 binding (Fig. 3G), the differential homology of this region among ING family explains why ING2 and ING1b interact with Smurf1, whereas ING3, ING4 and ING5 do not. In addition, among the Nedd4 family is Smurf1 which specifically downregulated the stability of ING2 protein (Fig. 4D).

Previous studies revealed that ING2 could interact with p53 and enhance its transcriptional activity. We next examined whether Smurf1-dependent degradation of ING2 interfered p53 activity. As expected, the transcriptional activity of endogenous p53 in MCF7 cells was increased when ING2 was overexpressed. This elevation was reduced by Smurf1-WT but not the Smurf1-CA mutant (Fig. 4E, left), indicating that Smurf1 affects ING2 function dependently of its ligase activity. To confirm the effect of Smurf1 on ING2-mediated p53 transactivation, the p53-deficient H1299 cells was used. P53 activity could not be detected in these cells; when exogenous p53 was reintroduced, the activity of p53-binding promoter-driven luciferase was easily detected. ING2 enhanced the p53 activity and Smurf1 further inhibited the ING2 function dependently of the ligase activity (Fig. 4E, right).

Knockdown of Smurf1 by siRNA transfection significantly upregulated the mRNA levels of p53 downstream target genes including p21 and Puma (Fig. 4F), and enhanced the cell apoptosis induced by etoposide (Fig. 4G). ING2 knockdown had the opposite effect, consistent with previous reports [11]. Importantly, abrogation of ING2 together with depletion of Smurf1 partially (but still significantly) inhibited Smurf1 knockdown-induced p53 target gene upregulation and apoptosis augmentation (Fig. 4F and G), indicating that Smurf1 functions partially through ING2 to regulate p53 activity and apoptosis.

Taken together, we identified the HECT-type ubiquitin ligase Smurf1 could target the tumor suppressor ING2 for ubiquitination and proteasome-dependent degradation and further influence the effect of ING2 on p53 activity. Depletion of Smurf1 resulted in a significant upregulation of ING2 protein level. In vivo and in vitro ubiquitination assay showed that ING2 was a new substrate of E3 ligase Smurf1. Recently, it has been demonstrated that ING3 is degraded by the ubiquitin-proteasome pathway through the RING finger-type E3 ligase SCF^{Skp2} complex and lysine 96 residue is essential for ING3 ubiquitination [30]. Moreover, $p33^{ING1b}$ is degraded in the 20S proteasome and NAD(P)H quinone oxidoreductase 1 (NQO1) inhibits the degradation of $p33^{ING1b}$ [31]. ING4 is also degraded by the ubiquitin-proteasome pathway and nucleolar accumulation of ING4 prolongs its half life although the responsible E3 ligase remains unclear [32]. These findings illustrate that most of ING family proteins undergo ubiquitin-proteasome-mediated degradation. Our study for the first time shows that ING2 can be targeted for ubiquitin-proteasomal degradation through the HECT domain-type E3 ligase Smurf1, and thus provides a functional crosstalk between ING family of tumor suppressors and Nedd4 family of ubiquitin ligases.

Notably, Smurf1 interacts with ING2 and targets ING2 for degradation completely dependent on the HECT domain. It has been well-characterized that the central WW domains mediate the recognition of most of the defined substrates including Smad1/5, RhoA, MEKK2, Prickle1 and TRAFs [15,17,18,23,24], although a most recent study indicated that the N-terminal C2 domain might also mediate the interaction with the substrate PEM-2, a guanine nucleotide exchange factor for Cdc42 [25]. Our data indicate that the HECT domain could also function as the substrate recognition module and thus, contribute to deepen the understandings of the functions of HECT domain. How the HECT domain itself coordinates both the substrate binding and the ubiquitin transfer is worthy of precise investigations.

Our study also gained further insight into the novel role of PHD finger domain in the stability control of ING2. Although a subclass of putative PHD fingers were shown to have E3 ubiquitin ligase activity, the PHD fingers in ING family have not been suggested to possess such a function. Instead, they were found to mediate the interaction with lipid including PtdIns(5)P in vivo and also PtdIns(3)P in vitro, and with histone H3 trimethylated at lysine 4. We revealed that the PHD finger domain was required for Smurf1-mediated degradation. Although studies with the other ING proteins (such as ING3 and ING4) reported that the N-terminal region was involved in ING ubiquitination [30,32], the C-terminal PHD domain of ING2 was targeted for ubiquitination (Fig. 3H). However, unlike ING1b and ING2, the members ING3, 4, and 5 could not be degraded by Smurf1 (Fig. 4A). Notably, although the PHD domain is highly conserved among the ING family, the central region is not so conserved, and the central region of ING1b exhibits 50% homology to ING2, which is much higher than that of other ING proteins with ING2 (Fig. 4C). We showed that ING2 interacts with Smurf1 through this central region but not the highly conserved PHD domain (Fig. 3G). So we suggested that the failure of Smurf1 to mediate the degradation of ING3, 4 and 5 might be caused by the low similarity of the central region, which leads to the inability of ING3, 4, and 5 to interact with Smurf1. Indeed, we revealed that Smurf1 interacted with ING2 and ING1b, but not with ING3, 4 or 5, as revealed by co-immunoprecipitation assays (Fig. 4B). Consistent with our results, a previous report showed that Smurf1 interacted with ING2 but not with ING3 and ING4 [22]. Based on these data, we proposed that Smurf1 interacts with the central region of ING2 and targeted the PHD domain of ING2 for degradation. Therefore, both the E3 ligase and the degradation targeting site of distinct ING family members are likely to be different. In addition, mutations within PHD fingers are found in tumor tissue [5]. Whether these mutations affect the stability of ING should be further explored in the future.

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