DNA damage stress induces the dissociation of Smurf1/2 from MDM2 in a slow manner

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The tumor suppressor p53 locates at the key point of cell growth or apoptosis balance, and the expression level of p53 is tightly controlled by ubiquitin ligases including MDM2. Upon DNA damage stresses, p53 was accumulated and activated, leading to cell cycle arrest or apoptosis. We previously showed that Smad ubiquitylation regulatory factor 1/2 (Smurf1/2) promotes p53 degradation by interacting with and stabilizing MDM2, and consequently enhancing MDM2-mediated ubiquitylation of p53. However, it is unclear how the Smurf1-MDM2 interaction is regulated in response to DNA damage stress. Here, we show that in response to etoposide treatment Smurf1 dissociates from MDM2, resulting in MDM2 destabilization and p53 accumulation. The negative regulation of Smurf1 on apoptosis is released. Notably, this dissociation is a slow process rather than a rapid response, implicating high expression of Smurf1 might confer the resistance against p53 activation. Consistent with this notion, we observed that Smurf1/2 ligases are highly expressed in colon cancer, esophageal squamous cell carcinoma and pancreatic cancer tissues, suggesting the oncogenic tendency of Smurf1/2.

Smurf1, MDM2, p53, DNA damage stress, apoptosis

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Protein modification post-translation is a critical molecular event in the cellular response to DNA damage, which results in activation, inactivation and degradation of some important components of DNA damage responses. Ubiquitylation is a prominent post-translational protein modification that leads to protein degradation, endocytosis, trafficking of transmembrane proteins. It consists of the covalent attachment of the small protein ubiquitin to target proteins, thereby modifying their biochemical properties and protein partners [1]. E3 ubiquitin ligases are final effectors of the enzyme cascade controlling ubiquitylation [2]. Two main classes of E3s are found, the RING (really interesting

new gene) type and the HECT (homologous to E6AP C-terminus) type. The Nedd4 (Neural precursor cell-expressed developmentally downregulated 4) family of HECT domain E3s typically regulate the stability of both transmembrane receptors and intracellular substrates and endocytosis and trafficking of plasma membrane proteins [3]. Several Nedd4 family members affect key signalling pathways, such as cellular growth and proliferation [1]. For example, Smurf1 and Smurf2 also play key roles in TGF- β /BMP and MEKK2-JNK signalling, controlling bone development, cell polarity and motility [4–9]. Recently, we have demonstrated a critical role of Smurf1/2 in stabilizing the RING type E3 MDM2 (murine double minute 2), which promotes p53 degradation and regulates cell apoptosis [10].

The p53 tumor suppressor gene encodes a transcriptional

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regulator that controls cell cycle progression and apoptosis [11]. In response to DNA damage or cell stress, p53 activates and initiates a cascade of events that block cell division and/or apoptosis. There are many chemotherapeutic agents activate p53 and induce apoptosis in a wide range of human cancer cells [12–14]. We recently identified Smurf1/2 as a novel negative regulator of p53 by enhancing the stability and trans-E3 ligase activity of MDM2. In unstressed cells, Smurf1/2 stabilizes MDM2 by enhancing the heterodimerization of MDM2 with MDMX, which inhibits the autoubiquitylation of MDM2 [10]. However, it is unclear how the Smurf1-MDM2 interaction is regulated in response to DNA damage stress.

In this study, we showed that Smurf1 dissociated from MDM2 and the regulation of Smurf1 on MDM2-p53 was abolished in response to DNA damage. Notably, this dissociation is a slow process rather than a rapid response. Upon a short time treatment with etoposide, Smurf1 could still stabilize MDM2 protein. Moreover, we detected the expression of Smurf1/2 in some types of tumor samples, bringing up the possibility that Smurf1/2 might exhibit some oncogenic activities.

1 Materials and methods

1.1 Plasmids, reagents and antibodies

6Myc-Smurf1 wild-type, 6Myc-Smurf1-C699A and Flag-Smurf1 were described previously [10]. Truncating mutations of Smurf1 were created by inserting PCR-amplified fragments into the related vectors. Anti-Myc antibody was purchased from ClonTech (BD Biosciences). The protein synthesis inhibitor cycloheximide (CHX) was from Sigma. The following antibodies were obtained from Santa Cruz Biotechnology: antibodies against p53 (DO-1), MDM2 (SMP14), GAPDH (6C5).

1.2 Cell culture

Human breast cancer MCF-7 cells were maintained in a DMEM medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine, in a humidified atmosphere of 5% CO₂ at 37°C.

1.3 Immunoprecipitation and immunoblotting

Transfections were performed with Lipofectamine 2000 according to the manufacture's instructions. In order to determine the interaction between Smurf1 and MDM2, immunoprecipitation and immunoblotting were performed as described previously [10].

1.4 Immunohistochemical staining

A tissue array of multiple human colorectal cancers was made from 31 cases of formalin-fixed paraffin-embedded colon cancer tissues and their normal tissues counterparts from patients underwent surgery resection in Cancer Institute and Hospital of Chinese Academy of Medical Sciences (Beijing, China) during the period of June 2001 to November 2003. All samples were collected after obtaining informed consent and approval from the Institutional Review Board. The other kinds of human multiple tissue arrays (MTA) of esophageal squamous cell carcinoma (ESCC) (OD-CT-DgEso 03-002) and pancreatic cancer (OD-CT-DgPan 03-002) were purchased from Shanghai Outdo Biotech (Shanghai, China). The clinical characteristics of all of samples are included in Table 1.

Various cancer tissues and their normal tissues counterparts on multiple tissue arrays (MTAs) were incubated simultaneously with MDM2 (2 µg/mL), Smurf1 (4 µg/mL), Smurf2 (4 µg/mL) antibodies or relative control IgG overnight at 4°C. After washing with PBS, they were reacted with the biotin-labeled second antibody and then visualized using an ultrasensitive streptavidin-peroxidases system (Maxim Biotech, Fuzhou, China). The intensity of MDM2, Smurf1 and Smurf2 for each individual case was quantified as described previously [15] and the percentage of expression were both graded as different scores. If the numerical value was higher or lower than that of the adjacent normal tissue, the expression of MDM2, Smurf1 and Smurf2 in the tumor tissue was considered to be up-/down-regulated, respectively.

1.5 Annexin V-PI staining

Etoposide-induced cell death was determined by an Annexin

Table 1 Smurf1/Smurf2 and MDM2 expression in human cancer tissues

	Smurf1 expression		Smurf2 expression		MDM2 expression	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Colorectal Cancer	65.0% (13/20)	35.0% (7/20)	85.0% (17/20)	15.0% (3/20)	70.8% (17/24)	29.2% (7/24)
ESCC	78.1% (25/32)	21.9% (7/32)	62.5% (20/32)	37.5% (12/32)	68.8% (22/32)	31.2% (10/32)
Pancreatic Cancer	87.5% (21/24)	12.5% (3/24)	71.2% (16/21)	28.8% (5/21)	66.7% (16/24)	33.3% (8/24)
Total	77.6% (59/76)	22.4% (17/76)	72.6% (53/73)	27.4% (20/73)	68.8% (55/80)	31.2% (25/80)

V-PI kit according to the manufacture's instructions. MCF-7 cells incubated with etoposide (40 $\mu\text{mol/L}$) or DMSO for the indicated hours were harvested and twice washed with cold phosphate buffered saline (PBS). Cells were resuspended in binding buffer, treated with annexin V and propidium iodide (PI), and were analyzed on the FACA Calibur.

1.6 RNA interference

The Smurf1 siRNA (5'-UUCUCCGAACGUGUCACGU-3'), Smurf2 siRNA (5'-CCUUCUGUGUUGAACAUAA-3'), and non-targeting siRNAs (5'-UUCUCCGAACGUGUCACGU-3') were synthesised by Shanghai GenePharm. All siRNA transfections were performed with Lipofectamine 2000 (Invitrogen), and the RNA interference efficiency was assessed by western blot analysis.

2 Results

2.1 Combination of either the N-lobe or the C-lobe of Smurf1 HECT with WW2 was sufficient to stabilize MDM2

We previously showed that ubiquitin ligase Smurf1 interacts with MDM2 through its WW2 domain and this interaction is required for MDM2 stabilization by Smurf1 [10]. The C-terminal HECT domain of Smurf1 is required for this process, although the ubiquitin ligase activity of Smurf1 is not necessary [10]. Consistent with this notion, overexpression of WW plus HECT domain with MDM2 resulted in a dramatic stabilization of MDM2 (Figure 1(a), lane 5), whereas overexpression of either HECT or WW domain alone, or a ΔHECT deletion mutant only slightly increased the protein level of MDM2 (Figure 1(a), lanes 2, 3 and 6). The ubiquitin ligase-inactive mutant C699A of Smurf1 had similar stabilizing effect to wild-type Smurf1 on MDM2 (Figure 1(a), lanes 7 and 8). A typical HECT domain consists of

an N-terminal lobe and a C-terminal lobe connected by a short hinge (605–607 aa of Smurf1), including the Smurf1/2 HECT. To further investigate whether the N-lobe or the C-lobe of Smurf1 is involved in MDM2 regulation, a series of Smurf1 mutants were generated and coexpressed with MDM2. Notably, the combination of either the N-lobe or the C-lobe of HECT with WW2 was sufficient to stabilize MDM2 (Figure 1(a), lanes 10–12 vs lane 9). Therefore, on the one hand, both the WW2 and the HECT domains are required for Smurf1 to stabilize MDM; on the other hand, either the N-lobe or the C-lobe of HECT was sufficient to coordinate with WW2 of Smurf1 to stabilize MDM2 (Figure 1(b)).

2.2 Smurf1 dissociates from MDM2 gradually in response to DNA damage

MDM2 is a short-lived protein due to its autoubiquitylation and proteasomal degradation. To ensure the p53 accumulation and activation, MDM2 itself is degraded rapidly following DNA damage. We next examined whether the stabilizing activity of Smurf1 on MDM2 was inactivated by DNA damage. After etoposide treatment, p53 was significantly accumulated whereas MDM2 was degraded (Figure 2(a), lane 3 vs lane 1). In the presence of ectopic Smurf1, under unstressed conditions Smurf1 stabilized MDM2 (Figure 2(a), lane 2 vs lane 1, MDM2 panel); upon etoposide treatment, the activity of Smurf1 was abrogated completely after 16 h (Figure 2(a), lane 4 vs lane 3, MDM2 panel). Notably, the abundance of Smurf1 was constant (lane 4 vs lane 2, Smurf1 panel), indicating that Smurf1 stability was not regulated by DNA damage. In addition, depletion of Smurf1/2 by RNA interference (RNAi) resulted in an increase of cell apoptosis (Figure 2(b), top panels), possibly due to the increased p53 activity as we previously showed [10]. Upon etoposide treatment for 24 h, the effect of Smurf1 depletion on cell apoptosis was abolished (Figure 2(b), bottom

Figure 1 Mapping the regions of Smurf1 required for MDM2 stabilization. The indicated Myc-tagged deletion mutants of Smurf1 were coexpressed with CMV-MDM2 in MCF7 cells, and the lysates were analyzed by immunoblotting with anti-MDM2 and anti-Myc antibodies (a). The effect of Smurf1 mutants on MDM2 stability was summarised in (b). GFP plasmid was co-transfected into the cells in all of the plasmid transfections. The expression of GFP was analyzed to indicate the transfection efficiency and GAPDH was used as the loading control.

Figure 2 The stabilizing effect of Smurf1 on MDM2 is abolished in response to DNA damage. (a) DNA damage stress abolished the stabilizing effect of Smurf1 on MDM2 protein. MCF7 cells, transfected with MDM2, control vector or Smurf1, were treated with DMSO or etoposide (40 $\mu\text{mol/L}$) for 16 h, harvested and analysed by IB with MDM2 antibody. ETO, etoposide. (b) Apoptosis analysis of untreated and etoposide-treated MCF7 cells transfected with Smurf1 and/or Smurf2-specific siRNAs. Annexin-V staining and flow cytometry analysis was performed to determine the cell apoptosis ratio. (c) MCF7 cells, transfected with MDM2, control vector or Smurf1, were treated with etoposide for the indicated times, harvested and analysed by immunoblotting with anti-MDM2 and anti-Myc antibodies. (d) Myc-Smurf1 and CMV-MDM2 were co-transfected into MCF7 cells, treated with MG132 for 8 h before harvesting, together with etoposide (40 $\mu\text{mol/L}$) for the indicated times. Cell lysates were immunoprecipitated with anti-Myc antibody, and samples were detected by immunoblotting with anti-MDM2.

panels). Collectively, Smurf1/2 activity on MDM2 stability control was negatively regulated by DNA damage stress.

Next we asked whether this abrogation was a rapid or a slow response. Detailed time-course analysis showed that the attenuation effect of Smurf1 activity in response to etoposide treatment was a slow process (Figure 2(c)). As indicated, at 2, 4 and 6 h post-treatment with etoposide, Smurf1 still retained the ability to stabilize the MDM2 protein. It has been well-characterized that p53 was rapidly accumulated within 0.5 h in response to DNA damage and the MDM2 destabilization precedes p53 accumulation [16,17]. Therefore, the ectopic expression of Smurf1 should prevent the rapid accumulation and activation of p53 due to the slow destabilization of MDM2.

To investigate how the MDM2 was released from Smurf1 control in response to DNA damage stresses, we examined the interaction between Smurf1 and MDM2 in the indicated times. Four hours after etoposide treatment, the interaction between Smurf1 and MDM2 was significantly reduced (Figure 2(d)), indicating a gradual dissociation of

Smurf1 from MDM2.

2.3 The N-lobe of Smurf1 HECT domain is involved in the dissociation of Smurf1 from MDM2 in response to DNA damage

Next we utilized the deletion mutants of Smurf1 to explore the possible mechanism by which the stabilizing effect of Smurf1 on MDM2 was attenuated by etoposide. Like the case of full-length Smurf1, the stabilizing effect of WW2+HECT (283–731) form of Smurf1 on MDM2 was also abrogated by etoposide treatment (Figure 3(a), lane 7 vs lane 6). Strikingly, deletion of the HECT N-lobe generated a mutant of Smurf1 which constitutively stabilized MDM2 despite the etoposide treatment (Figure 3(a), lane 8). Half-life analysis of MDM2 showed that the 283–731- ΔN mutant retained the strong ability to stabilize MDM2 and prolong the half-life of MDM2 even in the presence of etoposide (Figure 3(b)), implicating that the HECT N-lobe might include the information responsive to DNA damage.

Figure 3 The N-lobe of Smurf1 HECT domain is involved in the attenuation of Smurf1 activity in response to DNA damage. (a) MCF7 cells were co-transfected with MDM2, Smurf1 WT or mutants, treated with etoposide for 2 h. The protein levels of MDM2 and Smurf1 WT or mutants were analyzed by immunoblotting. Both long and short time exposure data were shown. (b) MCF7 cells were cotransfected with MDM2, Smurf1 WT or mutants, treated with etoposide for 2 h, and then chased by cycloheximide (CHX) for the indicated times. The cell lysates were analyzed by immunoblotting. Both long and short time exposure data were shown.

2.4 The correlation of Smurf1, Smurf2 and MDM2 expressed in human cancer samples

P53 functions as a potent tumor suppressor whereas MDM2 as an oncoprotein. P53 gene has been found widely mutated in various tumors and MDM2 amplified in certain tumors. Given that Smurf1/2 stabilizes MDM2 and destabilizes p53, we hypothesized that Smurf1/2 might exhibit some oncogenic activities. To gain further insights into the physiological relevance of MDM2 regulation by Smurfs, we examined the levels of Smurf1, Smurf2 and MDM2 proteins in human cancer samples. Immunohistochemical staining of paired colorectal cancer (CC), esophageal squamous cell carcinoma (ESCC) and pancreatic cancer (PC) samples showed that Smurf1, Smurf2 and MDM2 were mainly localized in the cytoplasm of tumor cells (Figure 4). By analysis of the effective stained tissue samples, the expression of Smurf1 was found up-regulated in colorectal cancer

(65.0%, 13/20), ESCC (78.1%, 25/32) and pancreatic cancer (87.5%, 21/24) tissues; Smurf2 was up-regulated in colorectal cancer (85.0%, 17/20), ESCC (62.5%, 20/32) and pancreatic cancer (71.2%, 16/21); and MDM2 was also over-expressed in colorectal cancer (70.8%, 17/24), ESCC (68.8%, 22/32) and pancreatic cancer (66.7%, 16/24). Overall, Smurf1, Smurf2 and MDM2 were validated in 76, 73 and 80 tumor patients with 3 different types of cancers, respectively. The results showed that they were over-expressed in 77.6% (59/76), 72.6% (53/73) and 68.8% (55/80) tumor tissues, respectively (Table 1), indicating that Smurfs expression was correlated with MDM2.

3 Discussion

The tumor suppressor p53 protein is tightly regulated by the ubiquitin-proteasomal system. Several E3s, including MDM2

Figure 4 The correlation of Smurfs and MDM2 expression in human cancer tissues. Representative immunohistochemical staining of MDM2, Smurf1 and Smurf2 in human cancer tissues. The representative fields from expression of Smurf1 (left), Smurf2 (middle) and MDM2 (right) in human colorectal cancer (CC), esophageal squamous cell carcinoma (ESCC) and pancreatic cancer (PC) tissues. Various cancer tissues (C1 & C2) and their normal tissues counterparts (N) were analyzed simultaneously and all photos were taken at 200× (N, C1) or 400× (C2).

[18], COP1 [19], JFK/FBXO42 [20], have been reported to mediated p53 ubiquitylation and degradation. The p53-MDM2 negative feedback loop constitutes the core module of a network of regulatory interactions activated under cellular stress. In unstressed cells, the level of p53 protein is kept low mainly by MDM2, and MDM2 negatively regulates the activity of p53. In response to DNA damage, ATM kinase is activated and phosphorylates both MDM2 and p53 proteins, promoting the dissociation of MDM2-p53 and leading to p53 accumulation. The p53-mediated pathways are further activated by various post-translational modifications including phosphorylation and acetylation, leading to cell cycle arrest and repair of the DNA damage. If repair is not possible due to excessive damage, the p53-mediated apoptotic pathway is activated bringing about cell death [21]. In our previous study, we have proved that Smurf1/2 does not bind with p53 directly but promotes p53 degradation by enhancing the stability of MDM2, and the regulation on MDM2 is not dependent on the E3 activity of Smurf1/2. Additionally, we found that Smurf1/2 inhibits cell apoptosis through p53 [10]. Here we show that in cells exposed to DNA damaging agents, overexpressed Smurf1 retained the ability to stabilize MDM2 protein in early stage. When the cells were exposed to DNA damage for a relative long time, the regulatory effects of Smurf1 on MDM2 stability and cell apoptosis were completely abolished. We further provided evidence that the N-terminal lobe of Smurf1 HECT domain contains the responsive element regulated by DNA damage. At the present stage, we do not exactly know the molecular mechanism by which Smurf1 was negatively regulated by DNA damage. Notably, the stability of Smurf1 protein was not affected significantly by DNA damage stress, both for the exogenous Smurf1 (Figure 2) and for the endogenous Smurf1 [22]. By contrast to DNA damage, the endoplasmic reticulum (ER) stress induced the proteasomal degradation of Smurf1 [22]. Although our data showed that DNA damage treatment destroyed the interaction between Smurf1 and MDM2 in a slow manner, it was still unclear how DNA damage stress altered their interaction. There may be some post-translational modifications, or protein translocation which needs further investigations.

MDM2 has been found amplified in human sarcomas. Amplification of the MDM2 gene is now reported in more than 10% of 8000 human cancers from various tissues, including lung and stomach [23]. Both Smurf1 and Smurf2 are key regulators in TGF- β signaling pathway. Insensitivity to TGF- β results in uncontrolled cell proliferation and contributes to tumorigenesis. As Smurf1/2 are essential modulators of the TGF- β cascade, it is not surprising that dysregulation or dysfunction of Smurfs hampers TGF- β signaling. Genetic amplification of Smurf1 and overexpression of Smurf2 are associated with pancreatic and esophageal squamous cell carcinomas, respectively [24]. Strikingly, we observed that Smurf1/2 overexpression occurs in colorectal

cancer, esophageal squamous cell carcinoma and pancreatic cancer samples, consistent with the abnormal high expression of MDM2 in these cancers. We speculate Smurf1/2 and MDM2 may have overlapped function in regulating tumor development. And the stabilizing effects of Smurf1/2 on MDM2 might contribute to the high level of MDM2 in cancer tissues, which might alter the sensitivity of cancer cells to DNA damaging-drugs in cancer therapy. Whether Smurf1/2 belongs to the novel oncogenes and the pathophysiological relevance between Smurf1/2 and MDM2 in cancers need deeper investigations in the future.

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