Daxx is a multifunctional protein that regulates a variety of cellular processes, including transcription, cell cycle, and apoptosis. SPOP is a BTB (Bric-a-brac/Tramtrack/Broad complex) protein that constitutes Cul3-based ubiquitin ligases. Here we show that SPOP serves as an adaptor of Daxx for the ubiquitination by Cul3-based ubiquitin ligase and subsequent degradation by the proteasome. Expression of SPOP with Cul3 markedly reduced Daxx level, and this degradation was blocked by SPOP-specific short hairpin RNAs. Inhibition of the proteasome by MG132 caused the prevention of Daxx degradation in parallel with the accumulation of ubiquitinated Daxx. Expression of SPOP with Cul3 reversed Daxx-mediated repression of ETS1- and p53-dependent transcription, and expression of SPOP with Cul3 markedly reduced Daxx level, potentially binding to Cul3 to form a novel Cul3-interacting domain. Most BTB proteins, but not all, have additional domains for protein-protein interaction, such as zinc fingers, Kelch repeats, and MATH motifs. Furthermore, a subset of proteins containing BTB domain has been identified to function as substrate-specific adaptors that bind to Cul3. Specifically, MEL-26, a homolog of human SPOP (speckle-type POZ protein) in Caenorhabditis elegans, was first identified as a BTB protein that serves as a specific adaptor of MEI-1 for the ubiquitination by Cul3-based Ub ligase and subsequent degradation by the proteasome (13). MEI-1 is a subunit of the katin-like microtubule-associating heterodimer MEI-1/MEI-2 that localizes to the spindles and the chromosomes during meiosis (14). SPOP BTB protein has also been shown to mediate the ubiquitination of the Polycomb group BMI and the variant histone MacroH2A (15). In addition, Keap1 BTB protein was shown to recruit Nrf2 to Cul3 (16–19). Nrf2 is a transcription factor that regulates the expression of anti-oxidant genes upon oxidative stress. In Schizosaccharomyces pombe, Btb1p, Btb2p, and Btb3p interact with Cul3, but their functions remain unknown (20). Therefore, so far only a few protein substrates have been shown to interact with BTB proteins for their ubiquitination by Cul3-based Ub ligases.

Daxx was originally identified as a protein that binds to the death domain of Fas receptor by yeast two-hybrid screening (21). Daxx interacts with the apoptosis signal-regulating kinase 1 (ASK1) and promotes Fas-mediated apoptosis through the activation of Jun N-terminal kinase (22). Subsequent studies have also shown that Daxx behaves as a pro-apoptotic protein under various stress conditions (21, 23–25). On the contrary, homoyzogous deletion of the Daxx gene has been shown to cause extensive apoptosis and embryonic lethality, suggesting that Daxx is an anti-apoptotic protein (26). Recent studies also support that Daxx is anti-apoptotic rather than pro-apoptotic (27–29).

Daxx also plays a role as a transcription regulator by interacting with various nuclear proteins involved in transcription. Daxx interacts with HDAC-II, core histones, and a chromatin-associate protein, Dek, suggesting that Daxx represses basal transcription through chromatin remodeling (30). Daxx has also been shown to form a ternary complex with homeodomain proteins Pax-3 and Pax-7 to inhibit transcription,
whereas Pax-3/4 forkhead in human rhabdomyosarcoma (FKHR) fusion protein in alveolar rhabdomyosarcoma cells is resistant to Daxx inhibition (31, 32). Similarly, Daxx interacts with ETS1 to repress transcriptional activation of the MMP1 gene (33). It has also been demonstrated that Daxx suppresses cell death induced by p53 overexpression and p53-dependent stress response, thus acting as a negative regulator of p53 (34). Interestingly, the level of Daxx protein has been shown to decrease in HeLa cells upon induction of apoptosis by actinomycin D or exposure to UV (28). Moreover, Daxx has recently been shown to interact with SPOP (35). These reports suggest a possibility that the functions of Daxx can be regulated by proteolysis.

In the present study we demonstrate that SPOP recruits Daxx to Cul3 and promotes the ubiquitination of Daxx and subsequent degradation by the proteasome. Moreover, SPOP/Cul3-mediated degradation of Daxx reversed Daxx-mediated repression of ETS1- and p53-dependent transcription. We also demonstrate that Daxx degradation triggers apoptosis. Taken together, we suggest that SPOP/Cul3-Ub E3 ligase plays an essential role in the control of Daxx level and, thus, in the regulation of Daxx-mediated cellular processes, including transcriptional regulation and apoptosis.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Cells**—pGL3-MMP1-Luc and various vectors expressing human SPOP were prepared as described (35). SPOP cDNA was cloned into pEYFP-C1 (BD Biosciences Clontech) and pcDNA3.1-Myc-His (Invitrogen). The cDNAs for SPOP deletion fragments were cloned into p3XFLAG-CMV10 (Sigma). The cDNAs for human Roc1 and Cul3 were cloned into pcDNA3-FLAG, and Ub cDNA was cloned into pcDNA4-HisMax (Invitrogen). To prevent SPOP deletion fragments were cloned into p3XFLAG-CMV10 (Sigma). For assaying the transcription of p53-dependent luciferase reporter gene, 293T cells were cultured on 24-well plates and transiently transfected with pGL3-MMP1-Luc and various combinations of expression vectors for HA-Daxx, FLAG-ETS1, FLAG-SPOP, FLAG-Cul3, and FLAG-Roc1. For assaying the transcription of p53-dependent luciferase reporter gene, PG13-Luc was used as a reporter vector. The luciferase (Luc) activity was then determined using a luciferase assay kit (Promega). Total amounts of transfected vector DNAs were kept constant by supplementing the appropriate amount of empty pcDNA vector. The luciferase activity was then normalized relative to the co-expressed β-galactosidase activity.

**Short Hairpin RNAs (shRNA) Construction and Reverse Transcription-PCR**—SPOP-specific shRNAs were synthesized and cloned into pSilencer 3.0 vector driven by the human H1 promoter (Ambion) with the following target sequences: 1, sense, 5′-GAT CGG TTC CCT GAC GCT CAC AAG GCT ACT TTC CTT AAG AAA CAT GG-3′ (forward) and 5′-CC TTT ATG CAA CAC ATG TGG ACC TGG ATC TCT CCT TCT GCT CAT CTA GAG-3′ (reverse); K711S, 5′-GCC ATA GTG CGG ATA ATG CCA TCT AAG AAG ATG CAG C-3′ (forward) and 5′-G CTG CAT CCT CTT TCT TGG ACA TGG CAT TAT CGG CAC TAT AGC-3′ (reverse). 293T, HeLa, and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C under a humidified atmosphere. Transient transfections were performed using Lipofectamine Plus (Invitrogen) according to the manufacturer’s instruction.

**Immunoprecipitation and Immunoblot**—For immunoprecipitation, cells were lysed in 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM Na3PO4, 10 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 1X protease inhibitor mixture (Roche Applied Science). Cell lysates were incubated with the appropriate antibodies for 2 h at 4°C and then with 50 μl of 50% slurry of protein A/G-Sepharose for the next hour. Bound proteins were eluted by boiling and subjected to SDS-PAGE followed by immunoblot with mouse anti-HA antibody (Roche Applied Science), rabbit anti-HA-antibody (Upstate Biotechnology, Inc.), mouse anti-FLAG M2 monoclonal antibody (Sigma), anti-Xpress antibody (Invitrogen), rabbit anti-Daxx-antibody (Calbiochem or Upstate), rabbit anti-Cul3-antibody (Zymed Laboratories Inc.), or anti-SPOP antisemur. Rabbit polyclonal anti-SPOP antiserum was raised against a recombinant C-terminal fragment of SPOP (amino acids 162–375). For in vitro interaction assay, HA-Daxx, MycHis-SPOP, and FLAG-Cul3 were in vitro translated using [35S]Met and TNT quick-coupled reticulocyte lysate system (Promega). 35S-Labeled proteins (20 μl) were incubated for 2 h with anti-HA antibody and then pulled down by treatment with protein A/G-Sepharose beads. Precipitates were subjected to SDS-PAGE followed by autoradiography.

**Immunocytochemistry**—HeLa cells plated on gelatin-coated coverslips were fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature followed by permeabilization with 0.5% Triton X-100 in PBS. All subsequent dilutions and washes were carried out with PBS containing 0.1% Triton X-100. Nonspecific binding sites were saturated by incubation with a blocking solution (10% goat serum, 1% bovine serum albumin, and 1% gelatin in PBS) for 30 min. Cells were incubated with primary antibody for 1 h and washed with PBS containing 0.1% Triton X-100 four times with 10-min intervals. They were then incubated with fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-conjugated secondary antibody for 1 h and washed 4 times with 10-min intervals. 4,6-Diamidino-2-phenylindole was used for counterstaining the nuclei. The cover-glasses were mounted in Vectashield (Vector Laboratories, Inc.), and cells were observed under a Zeiss Axiosplan II microscope or Image Restoration Microscope (Applied Precision).

**Transcription Assay**—For assaying the transcription of ETS1-dependent luciferase reporter gene, 293T cells were cultured on 24-well plates and transiently transfected with pGL3-MMP1-Luc and various combinations of expression vectors for HA-Daxx, FLAG-ETS1, FLAG-SPOP, FLAG-Cul3, and FLAG-Roc1. For assaying the transcription of p53-dependent luciferase reporter gene, PG13-Luc was used as a reporter vector. The luciferase (Luc) activity was then determined using a luciferase assay kit (Promega). Total amounts of transfected vector DNAs were kept constant by supplementing the appropriate amount of empty pcDNA vector. The luciferase activity was then normalized relative to the co-expressed β-galactosidase activity.
Surprisingly, neither MATH domain (N-terminal MATH domain indicated by m3) or the C-terminal region of SPOP deletion mutants. The SPOP mutants that lack either the N- or C-terminal sequence adjacent to BTB domain may also contribute to the interaction of SPOP with Cul3. Fig. 1C confirms that SPOP specifically interacts with Cul3 but not with the other Cul family members. Notably, SPOP ran as a doublet on the gel when expressed with Cul3 but not with the other Culs, and only one of the two bands interacted with Cul3. Therefore, we examined which of the SPOP doublet interacts with Cul3. We also examined whether SPOP interacts with any or both of Cul3 and its Nedd8-conjugated form and whether Daxx interacts with any or both of the SPOP doublet. Immunoprecipitation analysis shows that Cul3 interacted only with the slow-migrating form of SPOP (Fig. 1D, left panels). SPOP interacts with both Cul3 and its Nedd8-conjugated form (middle panel), and Daxx interacts with both of the SPOP doublet (right panel). However, the nature of the fast-migrating form of SPOP remains unknown, although it is unlikely that the fast-migrating form is a cleavage product of SPOP whose C-terminal fragment was removed, since the SPOP mutant (m2) that lacks the C-terminal 297–375 sequence is still capable of interacting with Cul3.

SPOP-mediated Interaction of Daxx with Cul3—Daxx was shown to interact with the N-terminal MATH domain of SPOP (35). To determine whether SPOP can act as an adaptor for the interaction between Cul3 and Daxx, FLAG-Cul3 was expressed in 293T cells with HA-Daxx or both FLAG-SPOP and HA-Daxx. Immunoprecipitation with anti-HA antibody shows that Daxx interacts with Cul3 in the presence of SPOP but much more weakly in its absence (Fig. 2A). To confirm the requirement of SPOP for the interaction between Daxx and Cul3, HA-Daxx was expressed with FLAG-Cul3 or both HA-SPOP and FLAG-Cul3. Immunoprecipitation with anti-FLAG antibody again shows that Cul3 interacts with Daxx in the presence of SPOP but much more weakly in its absence (Fig. 2B). We then examined whether endogenous SPOP is also capable of interacting with Cul3 and Daxx. Lysates were obtained from

The nuclei were counterstained with 4,6-diamidino-2-phenylindole for counting total cell numbers.

RESULTS

Interaction of Human SPOP with Cul3—SPOP is a BTB protein that was initially identified as an auto-antigen of scleroderma patients (36). To define the sites within the human SPOP and Cul3 proteins for their interaction, we first generated Cul3 mutants based on the previous reports. The replacement of Tyr-50 by Gly of Pcu3p, a Cul3 homolog in S. pombe, prevents its ability to interact with BTB proteins (20). The replacement of Lys-711 by Ser in human Cul3 blocks its modification by Ned8 (37, 38). Therefore, mutagenesis was performed to generate Y62G (Tyr-62 in Cul3 corresponds to Tyr-50 in Pcu3p) and K711S (Fig. 1A, upper panel). HA-Cul3 was expressed in 293T cells with FLAG-Cul3 or its mutant forms. Cell lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblot with anti-HA antibody. Wild-type Cul3, but not Y62G, was co-precipitated with SPOP (Fig. 1A, lower panel), indicating that Tyr-62 in human Cul3 is also essential for the interaction between Cul3 and SPOP. On the other hand, K711S could be co-precipitated with SPOP, indicating that the interaction between Cul3 and SPOP is independent of Nedd8 modification.

SPOP has been shown to interact with Cul3 via BTB domain (12). To determine whether BTB domain itself is sufficient for interaction with Cul3, various deletion mutants of SPOP were constructed (Fig. 1B, upper panel). HA-Cul3 was expressed in 293T cells with each of the FLAG-SPOP deletion mutants. The SPOP mutants that lack either the N-terminal MATH domain (indicated by m3) or the C-terminal region (m2) were co-immunoprecipitated with Cul3 (Fig. 1B, lower panel). Surprisingly, neither MATH domain (m1) nor BTB domain alone (m4) was capable of interacting with Cul3. Nor could the C-terminal region alone (m5) interact with Cul3. These results suggest that the N-or C-terminal sequence adjacent to BTB domain may also contribute to the interaction of SPOP with Cul3. Fig. 1C confirms that SPOP specifically interacts with Cul3 but not with the other Cul family members. Notably, SPOP ran as a doublet on the gel when expressed with Cul3 but not with the other Culs, and only one of the two bands interacted with Cul3. Therefore, we examined which of the SPOP doublet interacts with Cul3. We also examined whether SPOP interacts with any or both of Cul3 and its Nedd8-conjugated form and whether Daxx interacts with any or both of the SPOP doublet. Immunoprecipitation analysis shows that Cul3 interacted only with the slow-migrating form of SPOP (Fig. 1D, left panels). SPOP interacts with both Cul3 and its Nedd8-conjugated form (middle panel), and Daxx interacts with both of the SPOP doublet (right panel). However, the nature of the fast-migrating form of SPOP remains unknown, although it is unlikely that the fast-migrating form is a cleavage product of SPOP whose C-terminal fragment was removed, since the SPOP mutant (m2) that lacks the C-terminal 297–375 sequence is still capable of interacting with Cul3.

SPOP/Cul3-mediated Degradation of Daxx
Results further demonstrate that SPOP serves as an adaptor molecule throughout the nucleoplasm as if each was expressed separately. These however, Cul3 remained in the cytoplasm, whereas Daxx was found of all three proteins within the nucleus but as clump-like structures that expression of Daxx with both Cul3 and SPOP resulted in co-localization was recruited to nuclear speckles upon co-expression with SPOP. Co-ligase for degradation by the proteasome, Cul3 or its mutant forms were shown to decrease when cells are induced for apoptosis (28). In an attempt to determine whether Daxx is ubiquitinated by Cul3-based Ub protein that bridges Daxx to Cul3. Collectively, these results demonstrate that SPOP functions as an adaptor protein that bridges Daxx to Cul3.

To determine whether Daxx co-localizes in cells with Cul3 through their interaction with SPOP, HeLa cells were transfected with each or varied combinations of expression vectors for the proteins. Immunocytochemical analysis reveals that Cul3, which alone localizes predominantly in the cytoplasm (Fig. 3A), was recruited to the nucleus and co-localized as speckles with SPOP upon co-expression with SPOP (Fig. 3B). Likewise, Daxx, which alone resides throughout the nucleoplasm, was recruited to nuclear speckles upon co-expression with SPOP. Co-expression of Daxx with both Cul3 and SPOP resulted in co-localization of all three proteins within the nucleus but as clump-like structures that appear distinct from nuclear speckles. Without expression of SPOP, however, Cul3 remained in the cytoplasm, whereas Daxx was found throughout the nucleoplasm as if each was expressed separately. These results further demonstrate that SPOP serves as an adaptor molecule that recruits Cul3 to the nucleus for interaction with Daxx.

SPOP/Cul3-mediated Degradation of Daxx—Although it was unknown whether Daxx is degraded in cells, the level of Daxx protein has been shown to decrease when cells are induced for apoptosis (28). In an attempt to determine whether Daxx is ubiquitinated by Cul3-based Ub ligase for degradation by the proteasome, Cul3 or its mutant forms were expressed in 293T cells with SPOP and Roc1. Fig. 4A shows that Daxx level is markedly reduced by expression of wild-type Cul3 but much less significantly by that of Y62G or K711S. These results suggest that the degradation of Daxx requires the interaction of Cul3 with SPOP in addition to Nedd8 modification of Cul3. However, Daxx level in cells expressing Y62G or K711S with SPOP was also lower than that in cells expressing Daxx only. Thus, the partial degradation of Daxx that occurred in cells expressing Y62G or K711S with SPOP might be mediated by the interaction of endogenous Cul3 with SPOP.

Among the SPOP deletions, the mutants lacking the N- and C-terminal regions (m3 and m2, respectively) could interact with Cul3 (see Fig. 1B). To determine whether the deletion mutants are capable of mediating the degradation of Daxx, they were expressed in 293T cells with Cul3 and Roc1. Unlike wild-type SPOP, neither of the deletion mutants could mediate the degradation of Daxx (Fig. 4B). These results indicate that the full-length SPOP is required for its ability to mediate Daxx degradation.

We then examined whether Daxx is degraded by the proteasome. An expression vector for HA-Daxx was transfected to 293T cells with or without vectors encoding FLAG-tagged SPOP, Cul3, and Roc1. After incubation for 24 h, cells were treated with MG132, a specific inhibitor of the proteasome. At each time point, cell lysates were prepared and subjected to immunoblot with anti-HA antibody. The level of Daxx decreased when cells were induced for apoptosis (28). In an attempt to determine whether Daxx is ubiquitinated by Cul3-based Ub ligase for degradation by the proteasome, Cul3 or its mutant forms were expressed in 293T cells with SPOP and Roc1. Fig. 4A shows that Daxx level is markedly reduced by expression of wild-type Cul3 but much less significantly by that of Y62G or K711S. These results suggest that the degradation of Daxx requires the interaction of Cul3 with SPOP in addition to Nedd8 modification of Cul3. However, Daxx level in cells expressing Y62G or K711S with SPOP was also lower than that in cells expressing Daxx only. Thus, the partial degradation of Daxx that occurred in cells expressing Y62G or K711S with SPOP might be mediated by the interaction of endogenous Cul3 with SPOP.
treatment with MG132, Daxx was completely stabilized even in cells expressing SPOP with Cul3 and Roc1. These results strongly suggest that SPOP/Cul3-Ub ligase mediates the proteasome-dependent degradation of Daxx.

**Prevention of Daxx Degradation by SPOP-specific shRNAs**—To determine whether SPOP is indeed required for the proteasome-dependent degradation of Daxx, we constructed SPOP-specific shRNAs. Analysis by reverse transcription-PCR revealed that two shRNA vectors (referred to as shRNA-1 and -2), but not a negative control vector (shControl), effectively reduced the level of SPOP mRNA (Fig. 5A). On the other hand, the level of β-actin mRNA remained the same whether shControl or shRNA vectors were transfected. To confirm whether shRNAs could knock down the SPOP protein, each shRNA vector was transfected to cells with or without a vector encoding FLAG-SPOP. The level of ectopically expressed SPOP was markedly reduced by transfection of shRNA vectors, whereas the level of β-actin remained unchanged (Fig. 5B, left panel). The level of endogenous SPOP was also gradually decreased upon increasing the amount of transfected shRNA-1 (right panel). These results indicate that shRNAs specifically knock down the SPOP protein.

We then examined the effect of shRNA-mediated knock down of SPOP on the stability of endogenous and ectopically expressed Daxx. shControl or shRNA-1, which reduced the SPOP level more effectively than shRNA-2, was transfected to HeLa cells with or without a vector encoding HA-Daxx. The level of endogenous Daxx (Fig. 5C, right panel) as well as of ectopically expressed Daxx (left panel) was markedly decreased upon overexpression of SPOP with Cul3 and Roc1. However, co-expression of shRNA-1 with the Ub E3 components resulted in stabilization of both endogenous and ectopically expressed Daxx. Expression of shRNA-1 without the E3 components also led to stabilization of endogenous Daxx. These results indicate that SPOP plays an essential role in the degradation of Daxx.

**SPOP/Cul3-mediated Ubiquitination of Daxx**—To determine whether Daxx can be ubiquitinated by SPOP/Cul3-Ub ligase, HA-Daxx and His-Max-Ub were expressed in COS-7 cells with different combinations of SPOP, Cul3, and Roc1. Cell lysates were prepared and subjected to immunoprecipitation with anti-HA antibody followed by immunoblot with anti-FLAG antibody. Daxx protein was ubiquitinated by expression of SPOP with Cul3 and Roc1 (Fig. 6A). On the other hand, little or no ubiquitination of Daxx was observed when any of the Ub E3 components was missing. These results indicate that SPOP/Cul3-Ub E3 ligase is responsible for the ubiquitination of Daxx.

To clarify further the requirement of SPOP for Daxx ubiquitination, an expression vector for wild-type Cul3 or Y62G that cannot interact with Daxx was transfected to COS-7 cells with vectors encoding SPOP and Roc1. To determine the requirement of Nedd8 modification of Cul3 for Daxx ubiquitination, cells were transfected with a vector encoding K711S that could not be modified by Nedd8. After incubation for 24 h, cells were treated with MG132 for the next 12 h. The level of ubiquitinated Daxx in cells expressing Y62G or K711S was much lower than that seen in cells expressing wild-type Cul3 (Fig. 6B). These results clearly show that the ubiquitination of Daxx requires both the interaction of Cul3 with SPOP and the Nedd8 modification of Cul3. Notably, accu-
SPOP/Cul3-mediated Degradation of Daxx

Reversal of Daxx-mediated Transcriptional Repression by SPOP—MMP1 is a collagenase-1 that plays a crucial role on ECM degradation, and the MMP1 gene is known to be a target of ETS1 (39–41). In addition, Daxx has been shown to repress the promoter activity of MMP1 gene in cultured cells (33, 35). Therefore, we examined whether SPOP/Cul3-mediated degradation of Daxx prevents its transcriptional repression function. pGL3-MMP1-Luc was transfected to 293T cells with pSV-β-Gal. ETS1, SPOP, and Roc1 were also expressed in cells with wild-type Cul3, Y62G, or K711S. Cell lysates were prepared and subjected to assay for the transcription of luciferase reporter gene. Co-expression of SPOP and Roc1 with wild-type Cul3 significantly enhanced the transcription of ETS1-dependent reporter gene (Fig. 7A, left panel), suggesting that SPOP/Cul3-mediated degradation of Daxx reverses the endogenous Daxx-mediated transcriptional repression of luciferase reporter gene driven by MMP1 promoter. Furthermore, transcription of ETS1-dependent reporter gene that was strongly repressed by overexpression of Daxx could effectively be recovered by co-expression of SPOP and Roc1 with wild-type Cul3 but not with Y62G or K711S. Because Daxx acts as a repressor for p53 (34), we examined whether SPOP/Cul3-mediated degradation of Daxx also leads to a promotion of p53-dependent transcription. Experiments were performed as above except that PG13-Luc was used as a reporter vector. Nearly identical patterns of the results were obtained for the effect of SPOP overexpression on Daxx-mediated transcriptional repression of luciferase reporter gene driven by PG13 (Fig. 7A, right panel).

To clarify further the effect of Daxx degradation on the transcriptional repression, shRNA-1 was transfected to cells with (Fig. 7B) or without a vector expressing Daxx (Fig. 7C). shRNA-mediated knock down of SPOP, which stabilizes both ectopically expressed and endogenous Daxx, led to repressing of ETS1- and p53-dependent transcription of reporter genes, whereas the control vector showed little or no effect. Collectively, these results suggest that SPOP/Cul3-mediated degradation of Daxx could change the transcriptional repression function of Daxx by mediating the proteosome-dependent degradation of Daxx.

Induction of Apoptosis by Daxx Degradation—p53 is known to induce apoptosis (42–44). ETS1 also induces the expression of apoptotic genes, including MMP1 (41). Therefore, we examined whether SPOP/Cul3-mediated degradation of Daxx induces apoptosis by reversing Daxx-mediated repression of ETS1- and p53-dependent transcription. HeLa cells were transfected with vectors encoding Cul3, SPOP, and Roc1. After incubation for various periods, the apoptotic signal was assessed by immunoblot analysis for the cleavage of poly(ADP-ribose) polymerase (PARP) using anti-PARP antibody. The cleavage of PARP occurred in a time-dependent manner in cells expressing Cul3, SPOP, and Roc1 (Fig. 8A, left panel). Immunoblot analysis of the same lysates using anti-Daxx antibody reveals that the level of endogenous Daxx is decreased in a time-dependent fashion. In contrast, the Daxx protein level remained unchanged in cells transfected with empty vectors. These results suggest that SPOP/Cul3-mediated degradation of Daxx induces apoptosis at least in part by reversing Daxx-mediated repression of ETS1- and p53-dependent transcription.

Because the level of Daxx protein has been shown to decrease in HeLa cells upon the induction of apoptosis by actinomycin D (28), we performed the same experiment as above but in the presence of actinomycin D. Without overproduction of SPOP, Cul3, and Roc1, the cleavage of PARP evidently occurred in parallel with a decrease in Daxx levels by about 24 h after the drug treatment (Fig. 8A, right panel). Moreover, expression of the Ub E3 ligase components led to a marked acceleration of Daxx degradation as well as of PARP cleavage. These results demon-
strate that actinomycin D induces the loss of Daxx, and SPOP/Cul3-based Ub ligase is responsible for the drug-induced loss of Daxx.

To determine whether Daxx degradation indeed triggers apoptosis, Cul3 and Roc1 were expressed in cells with or without SPOP. After incubation for 24 h, cells were subjected to a TUNEL assay for staining the apoptotic cells. The number of apoptotic cells was markedly increased upon expression of SPOP with Cul3 and Roc1 (Fig. 8B). Expression of Cul3 and Roc1 without SPOP also increased the number of apoptotic cells but to a much lesser extent than that seen with it. On the other hand, overexpression of Daxx reversed the apoptotic effect seen by ectopic expression of SPOP, Cul3, and Roc1. These results again show that SPOP/Cul3-mediated degradation of Daxx triggers apoptosis. These results also suggest that Daxx is an anti-apoptotic protein.

DISCUSSION

The present studies have demonstrated that SPOP BTB protein serves as an adaptor of Daxx for the ubiquitination by Cul3-based Ub ligase and subsequent degradation by the proteasome. In addition to BTB domain that mediates the interaction with Cul3, SPOP has a MATH domain that functions in protein-protein interaction (12, 17). Deletion of the MATH domain in SPOP, which blocks the interaction between SPOP and Daxx (35), abrogated SPOP/Cul3-mediated degradation of Daxx, indicating that the MATH domain of SPOP is responsible for recruiting Daxx to Cul3-based Ub ligase. Furthermore, shRNA-mediated knock down of SPOP was found to block the degradation of Daxx. We also demonstrated that the inhibition of the proteasome by
SPOP/Cul3-mediated Degradation of Daxx

Enigmatically, Daxx has been reported to function as a pro-apoptotic as well as anti-apoptotic protein (46). Although initial studies reportedly suggested that Daxx is pro-apoptotic (21, 22), later studies have demonstrated that Daxx is anti-apoptotic. In addition to the critical demonstration that the knock-out of Daxx gene causes an extensive apoptosis with early embryonic lethality (26), Daxx has been shown to inhibit CD43-mediated apoptosis in acute myeloid leukemia cells (27). Furthermore, small interfering RNA-mediated Daxx silencing has been shown to sensitize cells to Fas- and stress-induced apoptosis (28). We also demonstrate here that SPOP/Cul3-mediated degradation of Daxx induces apoptosis. Expression of SPOP with Cul3 and Roc1 led to the degradation of endogenous Daxx with concomitant cleavage of PARP and to a marked increase in the number of TUNEL-stained apoptotic cells. Activation of caspase-3 also occurred under the same conditions (data not shown). Moreover, SPOP/Cul3-mediated degradation of Daxx reversed Daxx-mediated repression of transactivation function of ETS1 and p53, both of which are known to induce the expression of pro-apoptotic genes, such as MPM1 and Bax, respectively (39, 42, 47). Consistently, Daxx has been shown to suppress p53-induced cell death, thus acting as a negative regulator of p53 (34). Therefore, the present study provides additional evidence that Daxx acts as an anti-apoptotic protein at least under the conditions tested.

Beside apoptosis, Daxx has been implicated in numerous biological processes, such as acute promyelocytic leukemia, chrmosomal segregation, tumor suppression, heat shock response, viral infection, and embryonic development. Therefore, we suggest that SPOP/Cul3-mediated degradation of Daxx could be an important mechanism for the regulation of Daxx-mediated biological processes.

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