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Mutant p53 Disrupts the Stress MAPK Activation Circuit Induced by ASK1-Dependent Stabilization of Daxx

Tetsuya Kitamura,[1,2] Yayoi Fukuyo,[1] Masahiro Inoue,[3] Nobuko T. Horikoshi,[1] Masanobu Shindoh,[2] Buck E. Rogers,[1] Anny Usheva,[4] and Nobuo Horikoshi[1]

[1]Department of Radiation Oncology, Washington University School of Medicine, St. Louis, Missouri; [2]Department of Oral Pathology and Biology, Hokkaido University Graduate School of Dental Medicine, Sapporo, Japan; [3]Department of Parasitology, Kurume University Medical School, Kurume, Japan; and [4]Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston Massachusetts

Abstract

Daxx is a regulatory protein for apoptosis signal–regulating kinase 1 (ASK1) which activates c-*Jun* NH_2-terminal kinase (JNK) and p38 pathways in response to stressors such as tumor necrosis factor-α (TNFα). Here, we show that TNFα treatment induces the accumulation of Daxx protein through ASK1 activation by preventing its proteasome-dependent degradation. ASK1 directly phosphorylates Daxx at Ser^{176} and Ser^{184} and Daxx is required for the sustained activation of JNK. Tumorigenic mutant p53, which binds to Daxx and inhibits Daxx-dependent activation of ASK1, prevents Daxx phosphorylation and stabilization. When mutant p53 was depleted in cancer cells, Daxx was accumulated and the cell-killing effect of TNFα was restored. Our results indicate that Daxx not only activates ASK1 but also is a downstream target of ASK1 and that accumulated Daxx further activates ASK1. Thus, the Daxx-ASK1 positive feedback loop amplifying JNK/p38 signaling plays an important role in the cell-killing effects of stressors, such as TNFα. Tumorigenic mutant p53 disrupts this circuit and makes cells more tolerable to stresses, as its gain-of-function mechanism. [Cancer Res 2009;69(19):7681–8]

Introduction

Daxx was identified as a Fas death domain–interacting protein and shown to enhance Fas-induced apoptosis through the activation of c-*Jun* NH_2-terminal kinase (JNK; ref. 1). Daxx binds to and activates apoptosis signal–regulating kinase 1 (ASK1), a member of the stress-responsive mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K) family (2, 3). ASK1 activates the MKK4/JNK and MKK6/p38 MAPK pathways through their phosphorylation. ASK1 displays proapoptotic activity in responding to diverse stimuli such as tumor necrosis factor-α (TNFα), anti-Fas antibody, and oxidative stress (3–5).

Daxx is a ubiquitously expressed gene (6) and has an essential developmental function (7). Based on a recent study, *Daxx*-silencing in cells compels a resistance to cell death induced by UV irradiation and oxidative stress, and an impaired JNK activation (8), suggesting that Daxx is a regulatory upstream factor of JNK to induce apoptosis. In addition, $ASK1^{-/-}$ and $JNK^{-/-}$ cells are resistant to TNFα-induced and H_2O_2-induced apoptosis (9, 10). Nevertheless, controversial results have been reported that Daxx has an antiapoptotic effect (11) or sensitizes cells to Fas-induced apoptosis (12). Blocking of JNK activation and apoptotic induction has been observed in cells overexpressing a dominant-negative kinase-inactive mutant of ASK1 (K709M; refs. 3, 4). Furthermore, ASK1 is required for sustained activation of JNK/p38 and apoptosis (10). These findings suggest a physiologic significance in the network of Daxx-ASK1-JNK signaling for apoptotic cell death.

Translocation of Daxx shuttling between the nucleus and cytoplasm has been implicated in its apoptotic function (13). Daxx is mainly located in the nucleus, whereas it is a proapoptotic signal mediator in the cytoplasm when ASK1 is overexpressed (14). This seems to be consistent with the report describing the cytoplasmic localization of Daxx upon exposure to stresses such as Fas cross-linking (15). Homeodomain-interacting protein kinases (HIPK) have been found to phosphorylate Daxx. HIPK1 phosphorylates Daxx at Ser^{667} and consequently relocalizes Daxx from promyelocytic leukemia protein-nuclear bodies. The relocalization of Daxx by HIPK1 is dependent on the HIPK1 active kinase domain, however, independent of Daxx phosphorylation (16). The translocated Daxx binds to ASK1 and leads to ASK1 oligomerization (17). HIPK2 also binds to Daxx and induces its phosphorylation, which in turn leads to JNK activation.

Here, we report that TNFα treatment induces the phosphorylation and stabilization of Daxx protein through ASK1 activation, which is essential for sustained JNK activation and resultant apoptosis. This positive feedback loop mechanism is inhibited by tumorigenic mutant p53.

Materials and Methods

Plasmid construction and short interfering RNA synthesis. Various Daxx substitution mutants were generated by PCR with the primers T174A/F (5′-CAAATGCTGAAAACGCTGCCTCTCAG-3′), T174A/R (5′-CTGAGAGGCAGCGTTTTCAGCATTTG-3′), S176A/F (5′-GAAAACACTGCCGCTCAGTCTCCAAG-3′), S176A/R (5′-CTTGGAGACTGAGCGGCAGTGTTTTC-3′), T181A/F (5′-GTCTCCAAGGGCCCGTGGTTCCCGGCGGCAG-3′), T181A/R (5′-CTGCCGCCGGGAACCACGGGCCCTTGGAGAC-3′), S184A/F (5′-GTCTCCAAGGACCCGTGGTGCCCGGCGGCAG-3′), S184A/R (5′-CTGCCGCCGGGCACCACGGGTCCTTGGAGAC-3′), T174AS176AS178AT181AS184A/F (5′-GAAAACGCTGCCGCTCAGGCGCCAAGGGCCCGTGGTGCC-3′), and T174AS176AS178AT181AS184A/R (5′-GGCACCACGGGCCCTTGGCGCCTGAGCGGCAGCGTTTTC-3′). For generating GST-Daxx 70 to 216 wild-type and its substitution mutants, the NH_2-terminal region of Daxx was amplified using primers (5′-GGAGGAGGATCCCCTTCCTTGAACTTTGTAAGATGCAG-3′) and (5′-GGAGGAGAATTCCTAATCCAATTCTGAGAGATCCAACTC-3′). The short interfering RNAs (siRNA) for Daxx and negative control were obtained from Invitrogen.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

T. Kitamura and Y. Fukuyo contributed equally to this work.

Current address for N. Horikoshi and Y. Fukuyo: Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215.

Requests for reprints: Nobuo Horikoshi, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 3 Blackfan Circle, CLS-728, Boston, MA 02215. Phone: 617-735-3308; Fax: 617-735-3327; E-mail: nhorikos@bidmc.harvard.edu.

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The siRNA sequence for Daxx-1 was described previously (11): 5′-GGAAAAG-GAGUUGGAUCUCUCAGAA-3′. The siRNA sequence for Daxx-2 is 5′-CCC-UCCCACACACCUCUCC-3′.

Cell culture and transfection. HeLa, ME180, A431, A2058, and 293 cells were cultured in DMEM (HyClone) supplemented with 10% FCS. HT29 cells were maintained in McCoy's5A (HyClone) supplemented with 10% FCS. For transfection with plasmid DNA, 293 cells were transfected by using FuGene 6 (Roche) and transfection into HeLa and ME180 cells was performed by electroporation with Nucleofector (Amaxa Biosystems). Duplex siRNAs were transfected by using OligofectAMINE (Invitrogen). HT29 cells ($\sim 2 \times 10^5$) were infected with control or p53 shRNA (Imgenex) adenoviruses (multiplicity of infection = 200) for 5 d.

Production of anti-phosphorylated Daxx–specific antibody. Anti-rabbit phosphorylated Daxx–specific antibody was generated by Open Biosystems with a synthetic phosphorylated peptide (CNTApSQSPRTRGpSRR), which corresponded to $pSer^{176}$ and $pSer^{184}$-containing Daxx.

Immunoprecipitation and Western blotting. Cell lysate preparations, Western blotting, and immunoprecipitation were described previously (18). For immunoprecipitation of phosphorylated Daxx at Ser^{176} and Ser^{184}, 1 mg of cell lysates were incubated with 5 μg of anti-phosphorylated specific Daxx antibody or control rabbit immunoglobulin for 2 h at 4°C. Immunoprecipitated materials were separated by SDS-PAGE. The primary antibodies were anti-FLAG (M5, Sigma), anti-HA (3F10, Roche), anti–enhanced green fluorescent protein (EGFP; Santa Cruz Biotechnology), anti-phosphorylated JNK (Cell Signaling), anti-JNK (Cell Signaling), anti-p53 (FL393, DO-1, Santa Cruz Biotechnology), anti-Daxx (Sigma), anti-actin (Santa Cruz Biotechnology), anti-PARP (Affinity Bioreagents, Inc.), and anti-GST (Santa Cruz Biotechnology).

Immunoprecipitation kinase assay. Immunoprecipitation kinase assay was performed as described (18). Briefly, cells were lysed and subjected to immunoprecipitation with the 3F10 anti-HA antibody (Roche) for 2 h at 4°C. The beads were washed and then incubated with 0.2 μg of substrate for 10 min at 30°C in the kinase reaction buffer supplemented with 100 μmol/L of ATP and 0.3 μCi of [γ-^{32}P]ATP. The reactions were terminated by the addition of SDS sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and measured by PhosphorImager (Molecular Dynamics).

Results

Accumulation of cellular Daxx protein in response to TNFα treatment coincides with the activation of ASK1. TNFα exposure induces two types of JNK activation, i.e., early and sustained induction, mediated by a mammalian MAP3K, ASK1 (3, 4, 19). Daxx enhances apoptosis in cells by interacting with and activating ASK1, which activates the JNK/p38 pathway. Interestingly, we observed that the cellular protein level of Daxx was significantly increased in TNFα-treated cells (Fig. 1*A*; Supplemental Fig. S1). Therefore, we investigated the effect of TNFα on cellular Daxx at the protein level. Treatment with TNFα increased endogenous Daxx expression levels in a dose-dependent manner. These results indicate that there is a TNFα-dependent mechanism to regulate Daxx levels in cells.

Because ASK1 is activated by TNFα (4), ASK1 activity is likely to be critical in cellular Daxx accumulation. 293 cells were transfected with either control vector or expression plasmids for ASK1-HA or a dominant-negative kinase-dead mutant, ASK1 K709M (KM)-HA (4), together with EGFP-Daxx and control EGFP to test the effect of

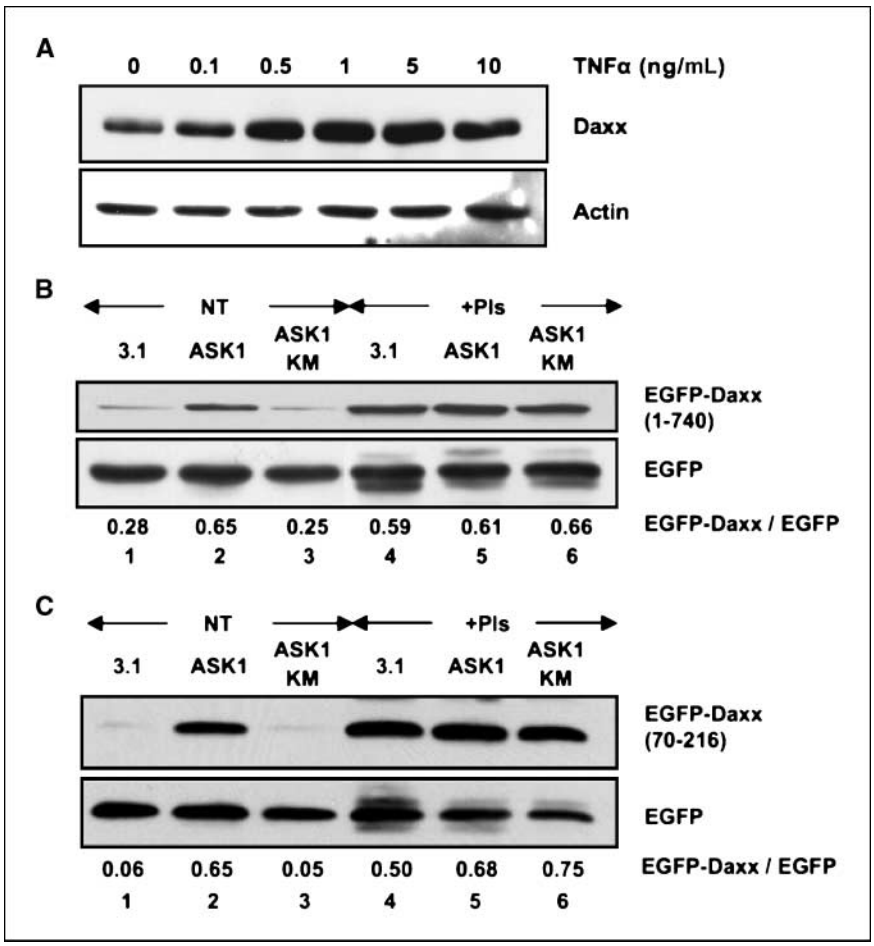

Figure 1. ASK1 increases Daxx expression by preventing proteasome-dependent degradation. *A,* TNFα treatment increases the expression level of Daxx. HeLa cells were treated with TNFα at the indicated concentrations for 18 h. The cell extracts were subjected to Western blotting for Daxx and actin. *B* and *C,* ASK1 stabilizes Daxx. 293 cells were transfected with expression plasmids for EGFP-Daxx, either full length (*B*) or a deletion mutant (70–216; *C*), and EGFP together with control vector (3.1), ASK1-HA or kinase inactive mutant K709M (KM)-HA, and then cultivated for 2 d. Twelve hours after the treatment with or without proteasome inhibitors (*Pls*), cell extracts were prepared and subjected to Western blotting with anti-EGFP antibody. Relative EGFP-Daxx protein levels are shown as a ratio over the EGFP protein levels (*bottom*). *NT,* no treatment. Note that the expression level of EGFP serves as an internal reference of ectopic EGFP fusion protein expression.

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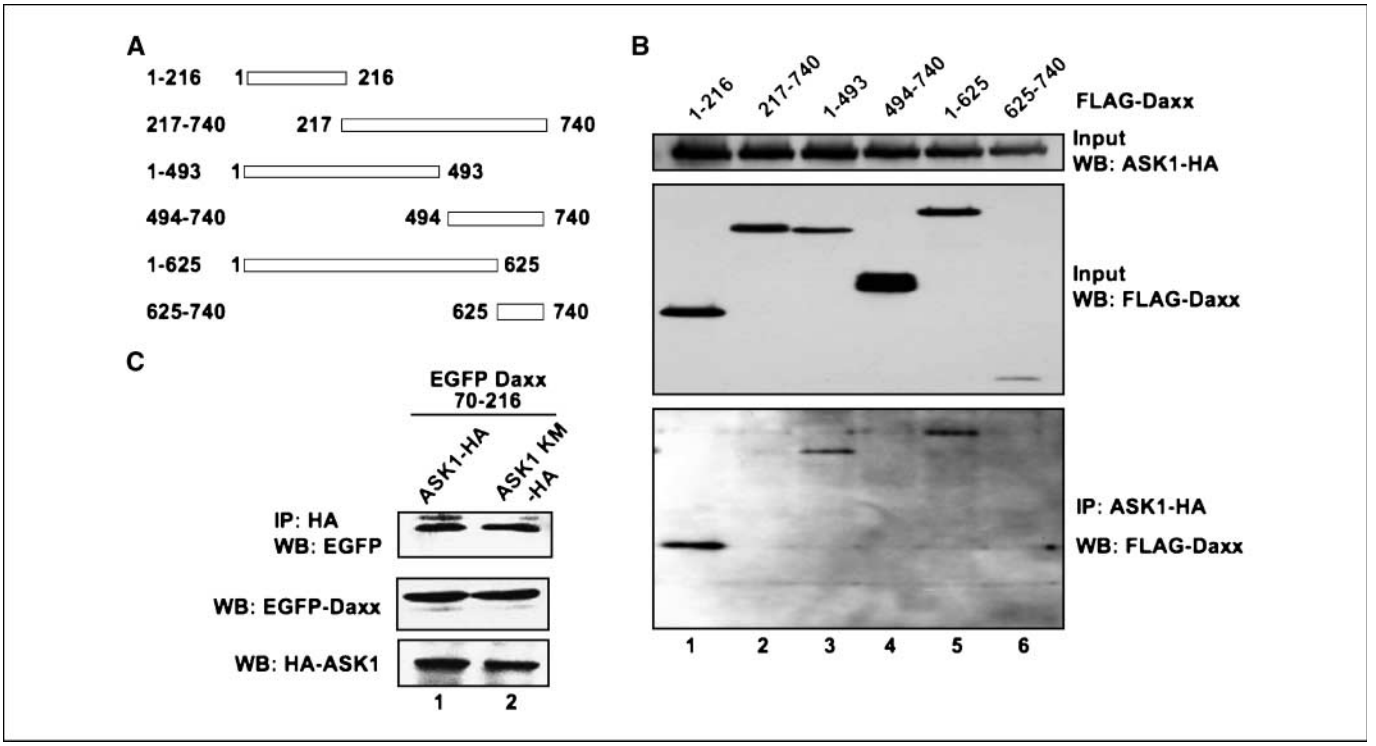

Figure 2. ASK1 binds to the NH_2-terminal region of Daxx. *A,* schematic diagram of deletion mutants of Daxx. Numbers indicate amino acid residues. *B,* mapping of the ASK1-interacting region of Daxx. 293 cells were transfected with the plasmids expressing ASK1-HA and various deletion mutants of FLAG-Daxx, as indicated in *A*. Two days posttransfection, cell extracts were subjected to immunoprecipitation with anti-HA antibody, and the precipitated materials were analyzed by Western blotting with anti-FLAG antibody. The 20% inputs for immunoprecipitation were analyzed for FLAG-Daxx and ASK1-HA proteins by Western blotting (*Input WB*). *C,* ASK1 kinase activity is dispensable for the interaction of ASK1 with Daxx. Cell extracts from 293 cells expressing EGFP-Daxx 70 to 216 and either ASK1-HA or ASK1 KM-HA were prepared and the expression levels of EGFP-Daxx 70 to 216 and ASK1-HA were determined by anti-EGFP and anti-HA antibodies, respectively. Because the expression level of EGFP-Daxx 70 to 216 was higher in cells when expressed with ASK1-HA than with ASK1 KM-HA, the amounts of lysates for immunoprecipitation were adjusted based on the Western blotting data for EGFP-Daxx 70 to 216 and ASK1. Cell lysates were immunoprecipitated with anti-HA antibody followed by Western blotting for EGFP. *IP,* immunoprecipitation; *WB,* Western blotting.

ASK1 activity on the amount of cellular Daxx. The cellular level of EGFP-Daxx was evaluated by Western blotting with EGFP as a reference (EGFP-Daxx/EGFP ratio). Daxx accumulated in cells only when transfected with ASK1-HA wild-type, but not with control vector or ASK1 KM-HA (Fig. 1*B, lanes 1–3*), and this was due to Daxx stabilization because proteasome inhibitors eliminated this effect (Fig. 1*B, lanes 4–6*). Importantly, ASK1-dependent stabilization on Daxx level was also observed in three of its deletion mutants (amino acids 1–625, 1–418, and 70–216), but not in other mutants (amino acids 1–69, 625–740, 419–740, 279–740, and 217–740; Fig. 1*C*; Supplemental Fig. S2). These results suggest that the NH_2-terminal region, amino acids 70 to 216, of Daxx is the only domain required for Daxx stabilization. The ASK1-dependent phosphorylation in this region might lead to Daxx accumulation by preventing proteasomal degradation.

Therefore, it is reasonable to assume that the same region of Daxx directly interacts with ASK1. ASK1-Daxx interaction was studied by immunoprecipitation of ASK1-HA from 293 cell lysates expressing both ASK1-HA and the various truncated forms of FLAG-Daxx (Fig. 2*A*). ASK1-HA efficiently coimmunoprecipitated with FLAG-Daxx 1 to 216, 1 to 493, and 1 to 625 (Fig. 2*B*, *lanes 1, 3*, and *5*), but not with 217 to 740, 494 to 740, and 625 to 740 (*lanes 2, 4*, and *6*) of Daxx lacking the NH_2-terminal region. Thus, the NH_2-terminal 216 amino acids of Daxx are sufficient for the interaction with ASK1. We were also able to detect an association between deleted region 70 to 216 of Daxx and ASK1, regardless of the ASK1 activity (Fig. 2*C*). These results indicate that amino acids 70 to 216 of Daxx are responsible for the interaction with ASK1. The kinase activity of ASK1 is not required for the interaction.

ASK1 phosphorylates Daxx on Ser^{176} and Ser^{184}. We next asked whether Daxx was a direct substrate for ASK1 kinase. An *in vitro* ASK1 kinase assay was performed with bacterially produced GST-Daxx 70 to 216 as a substrate. Wild-type ASK1 immunopurified from 293 cell extracts clearly phosphorylated GST-Daxx 70 to 216 (Supplemental Fig. S3).

There are 21 serine/threonine residues within amino acid position 70 to 216 of Daxx (Fig. 3*A*). We generated various substitution mutants of 21 serine (S)/threonine (T) to alanine (A) within amino acids 70 to 216 of Daxx to determine the phosphorylation sites (Fig. 3*B*; Supplemental Figs. S4 and S5). The *in vitro* phosphorylation of GST-Daxx 70 to 216 wild-type was evident by ASK1-ΔN, a constitutively active mutant of ASK1 (Fig. 3*C, lanes 2* and *5*; ref. 5). Among mutants, GST-Daxx ST0, which lacks five serine/threonine residues within amino acids 176 to 184 (Fig. 3*B*), nearly diminished its phosphorylation (Fig. 3*C*, *lanes 4* and *6*). Likewise, GST-Daxx S176/184A, which is a double-substituted mutant at Ser^{176} and Ser^{184} also drastically reduced its phosphorylation level (Fig. 3*C, lane 3*; Supplemental Fig. S5). Because these phosphorylation-deficient mutants still interact with ASK1-ΔN, reduced phosphorylation is not due to the lack of interaction with ASK1 (Supplemental Fig. S6). Conversely, GST-Daxx ST0-176/184S, which restored both Ala^{176} and Ala^{184} residues to serines in ST0, regained its phosphorylation level. Again, there was no difference between ST0-176/184S and wild-type in the

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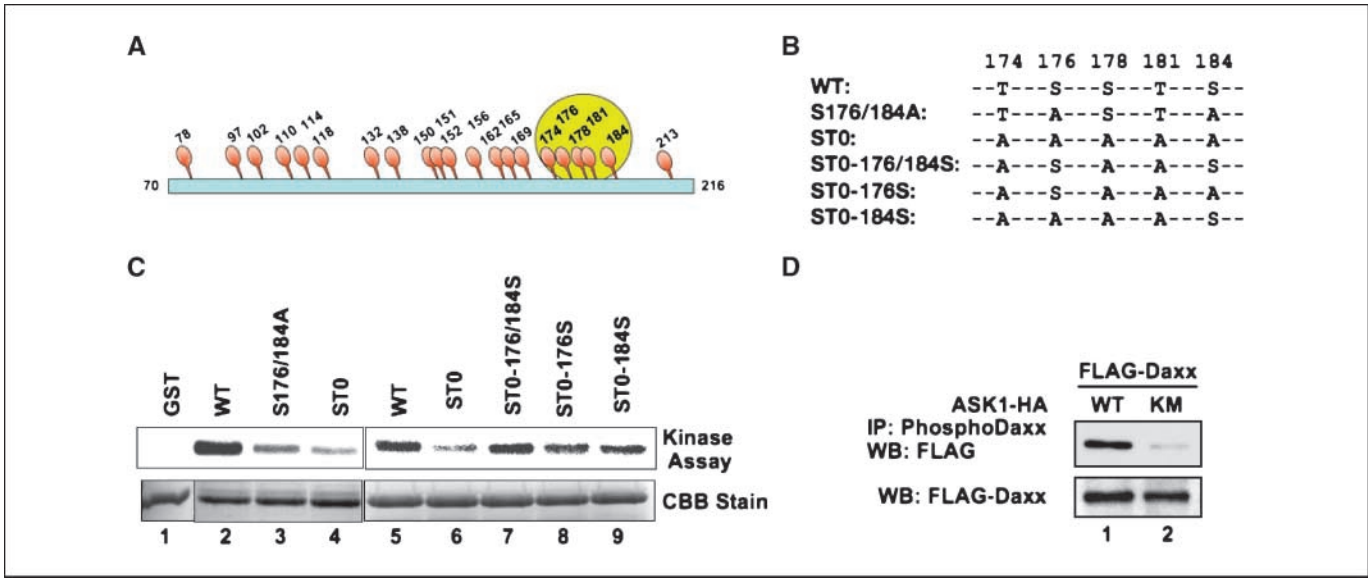

Figure 3. ASK1 phosphorylates on Ser^{176} and Ser^{184} in Daxx. *A,* schematic diagram of 21 serine/threonine residues within amino acids 70 to 216 of Daxx. Numbers indicate the position of amino acid residues. *B,* five serine (*S*) and threonine (*T*) residues between 174 and 184 (*circled in A*) were characterized in detail. Substitution mutants between amino acids 174 and 184 are shown. *C,* ASK1 phosphorylates Daxx on Ser^{176} and Ser^{184} *in vitro*. Recombinant mutant Daxx GST-fusion proteins were incubated with a constitutively active mutant ASK1 ΔN-HA in the presence of [γ-^{32}P] ATP as described in Materials and Methods. The reaction mixtures were separated in SDS-PAGE, and phosphorylated GST-fusion proteins were determined by PhosphorImager. The protein levels of GST-Daxx wild-type and its substitution mutants in the kinase reaction were visualized by Coomassie brilliant blue staining. *D,* phosphorylation of Daxx at Ser^{176} and Ser^{184} *in vivo*. FLAG-Daxx was expressed with full-length wild-type ASK1-HA or ASK1 KM-HA and the phosphorylation status of FLAG-Daxx was determined. The cell extracts were immunoprecipitated with anti-phosphorylated Daxx–specific antibody followed by Western blotting with anti-Daxx or FLAG antibodies. Because the expression level of FLAG-Daxx was increased by the presence of ASK1, the amounts of cell lysate in the immunoprecipitation reactions were adjusted to contain equal amounts of Daxx protein (WB:FLAG-Daxx).

interaction with ASK1 (Supplemental Fig. S6). Single revertant mutants ST0-176S and ST0-184S, carrying restored residues Ala^{176} and Ala^{184} to serine of ST0, respectively, displayed partial restoration of phosphorylation (Fig. 3*C, lanes 8* and *9*).

ASK1-dependent Daxx phosphorylation was monitored *in vivo* with an anti-phosphorylated Daxx–specific antibody raised against a synthetic phosphopeptide encompassing pSer^{176} and pSer^{184} of Daxx. Western blotting of precipitated materials with anti-phosphorylated Daxx antibody showed that FLAG-Daxx was phosphorylated at Ser^{176} and Ser^{184} by ASK1 wild-type but not by kinase-dead ASK1 KM (Fig. 3*D*). Similar results were obtained for endogenous Daxx when constitutively active ASK1ΔN or ASK1 KM was expressed (Supplemental Fig. S7*A*). Daxx was phosphorylated at Ser^{176} and Ser^{184} by endogenous ASK1 when cells were treated with TNFα (Supplemental Fig. S7*B*). Similar experiments were performed with a phosphorylation-deficient Daxx mutant FLAG-Daxx S176/184A. The amount of immunoprecipitated Daxx was not increased either by ASK1 wild-type or TNFα-treatment, indicating that the anti-phosphorylated Daxx antibody recognizes phosphorylated Daxx on Ser^{176} and Ser^{184} (Supplemental Fig. S7*C* and *D*). These *in vitro* and *in vivo* results show that Daxx is a direct substrate of ASK1 and the Ser^{176} and Ser^{184} of Daxx are phosphorylated. Furthermore, ASK1-dependent phosphorylation of Daxx is induced by TNFα treatment, indicating that Daxx is a physiologic substrate of ASK1 in cells.

ASK1-dependent phosphorylation of Daxx is critical for its stabilization. We investigated the effect of Daxx phosphorylation by ASK1 on its accumulation. The null-phosphorylation mutant FLAG-Daxx ST0 failed to be accumulated by ASK1-ΔN (Fig. 4, *lane 8*), whereas the revertant mutant FLAG-Daxx ST0-176/184S, phosphorylatable by ASK1-ΔN, restored its ASK1-dependent accumulation to a similar extent as that of wild-type FLAG-Daxx

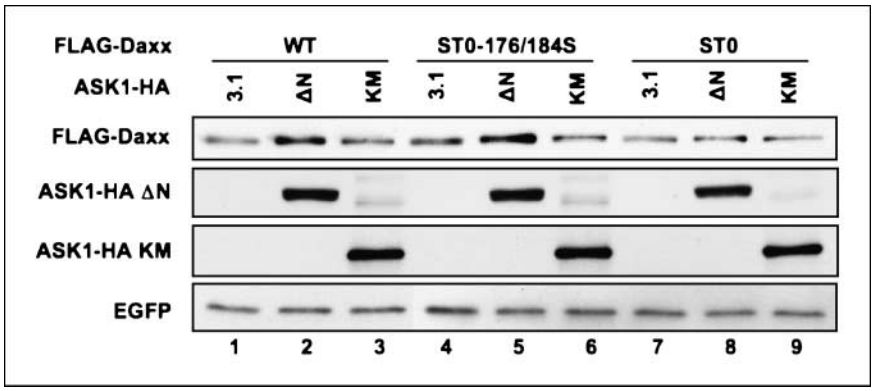

Figure 4. Phosphorylation on Ser^{176} and Ser^{184} in Daxx is critical for Daxx stabilization. 293 cells were transfected with expression plasmids for FLAG-Daxx (wild-type, ST0-176/184S, or ST0) and EGFP together with either empty vector (3.1), or expression plasmid for ASK1 ΔN-HA or ASK1 KM-HA. Two days after transfection, cell extracts were prepared and FLAG-Daxx levels were compared by Western blotting with anti-FLAG antibodies using the expression level of EGFP as a reference. The expression levels of ASK-HA ΔN and ASK1-HA KM are also shown by Western blotting with anti-HA antibody.

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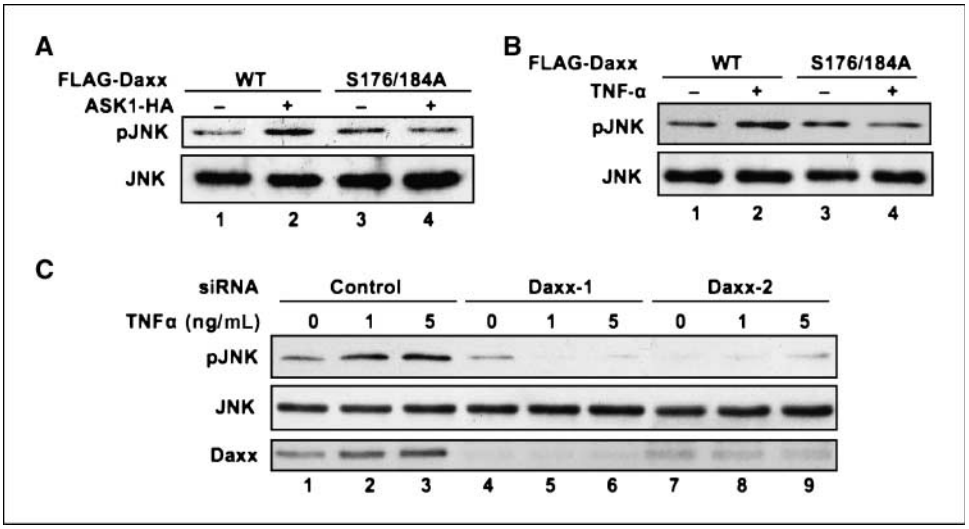

Figure 5. The activation of JNK by TNFα treatment requires Daxx, and the phosphorylation on Ser^{176} and Ser^{184} in Daxx is critical. *A,* phosphorylation on Ser^{176} and Ser^{184} in Daxx is essential for ASK1-dependent sustained JNK activation. HeLa cells were transfected with the indicated expression plasmids. Cell extracts were prepared and analyzed for expression levels of phosphorylated JNK and total JNK by Western blotting. *B,* phosphorylation on Ser^{176} and Ser^{184} in Daxx is essential for TNFα-induced sustained JNK activation. FLAG-Daxx wild-type or FLAG-Daxx S176/184A mutant expression plasmid was transfected into HeLa cells. Twelve hours posttransfection, cells were incubated in the presence or absence of TNFα (1 ng/mL) for 18 h. The cellular levels of phosphorylated JNK and total JNK were determined by Western blotting. *C,* Daxx is essential for sustained activation of JNK. siRNA for control, Daxx-1, or Daxx-2 were transfected into HeLa cells. Three days after transfection, cells were incubated with TNFα (0, 1, or 5 ng/mL) for 18 h. The activation of JNK was determined by Western blotting with anti-phosphorylated JNK antibody. Total JNK and Daxx were determined by Western blotting using anti-JNK and Daxx antibodies, respectively.

(Fig. 4, *lanes 5* and *2*, respectively). These results indicate that ASK1-dependent phosphorylation of Daxx at Ser^{176} and Ser^{184} is required for its accumulation in cells.

We next determined the functional significance of ASK1-dependent Daxx phosphorylation in JNK activation. Ectopic expression of ASK1, together with phosphorylation-deficient FLAG-Daxx S176/184A, failed to activate JNK (Fig. 5*A*, *lane 4*). Moreover, JNK was not activated with mutant FLAG-Daxx S176/184A expression by TNFα treatment (Fig. 5*B, lane 4*). Because sustained late phase JNK activation mediated through ASK1 enhances apoptosis in mouse embryonic fibroblasts (10), it is likely that Daxx also plays a critical role in ASK1-dependent sustained JNK activation and apoptosis in human cells. We determined the role of Daxx on JNK activation induced by TNFα in HeLa cells. Cells were transfected with Daxx-1, Daxx-2, or control siRNA and the activation of JNK was determined (Fig. 5*C*). JNK was effectively activated in response to an increasing concentration of TNFα. When Daxx protein was reduced by >90% with siRNA, the activation of JNK by TNFα was completely abolished, and the JNK activation levels became even lower than the basal levels. Because the total JNK protein level was not changed during TNFα treatment, the reduction of the phosphorylated form of JNK was due to the inhibition of the ASK1-dependent JNK activation. These results indicate that Daxx is required for the TNFα-dependent sustained activation of JNK. We also determined the requirement of Daxx in the apoptosis induced by TNFα. When Daxx expression was depleted by siRNA, both the induction levels of PUMAα (20) and the cleavage of PARP (11) were clearly reduced (Supplemental Fig. S8). These results indicate that Daxx plays a critical role in TNFα-dependent apoptotic cell death. The biological significance of the phosphorylation of Daxx in TNFα-dependent apoptosis was also addressed. When S176/184A mutant Daxx was expressed, the cleavage of PARP was not enhanced by TNFα treatment (Supplemental Fig. S9). These results indicate that the phosphorylation of Daxx on Ser^{176} and Ser^{184} plays a critical role in cellular response induced by TNFα treatment.

Tumorigenic mutant p53 inhibits ASK1-dependent phosphorylation of Daxx and prevents TNFα-dependent accumulation of Daxx. We have previously reported that the inhibition of Daxx-dependent ASK1 activation is a mechanism for mutant p53 gain-of-function (18). We therefore tested the effect of mutant p53 on the ASK1-dependent phosphorylation of Daxx. We observed that mutant p53 inhibits the ASK1-dependent phosphorylation of Daxx in a dose-dependent manner (Fig. 6*A, lanes 2–5*). The wild-type p53, however, failed to inhibit Daxx phosphorylation (Fig. 6*A, lanes 6–8*). These results indicated that the inhibitory effect is mutant p53–specific. Furthermore, the Daxx-ASK1 association was not dependent on ASK1 kinase activity and tumorigenic mutant p53 inhibits the complex formation between Daxx and ASK1 (Supplemental Fig. S10).

The TNFα-dependent accumulation of Daxx was determined in two cell lines (ME180 and HeLa) with wild-type p53 and three cell lines (HT29, A431, and A2058) carrying endogenous mutant p53. Daxx accumulation was observed in ME180 and HeLa cells (Fig. 6*B, lanes 1–6*) but not in HT29, A431, or A2058 (Fig. 6*B, lanes 7–15*) suggesting that the expression of mutant p53 interferes with Daxx from TNFα-dependent accumulation.

If this is the case, depletion of mutant p53 should restore the TNFα-dependent accumulation of Daxx and induces apoptosis in cells. HT29 cells which express R273H (Arg to His substitution mutant at amino acid 273) were infected with adenoviruses expressing shRNA for p53 knockdown followed by treatment with TNFα. The amount of Daxx was not increased by TNFα treatment when control adenoviruses were infected (Fig. 6*C, lanes 1* and *2*). However, treatment with TNFα induced the accumulation of Daxx when mutant p53 was depleted (Fig. 6*C, lanes 3–6*). Furthermore, when the accumulation of Daxx was restored, TNFα-dependent apoptosis became evident as judged by the cleaved PARP. These results suggest that HT29 cells fail to undergo apoptosis by TNFα

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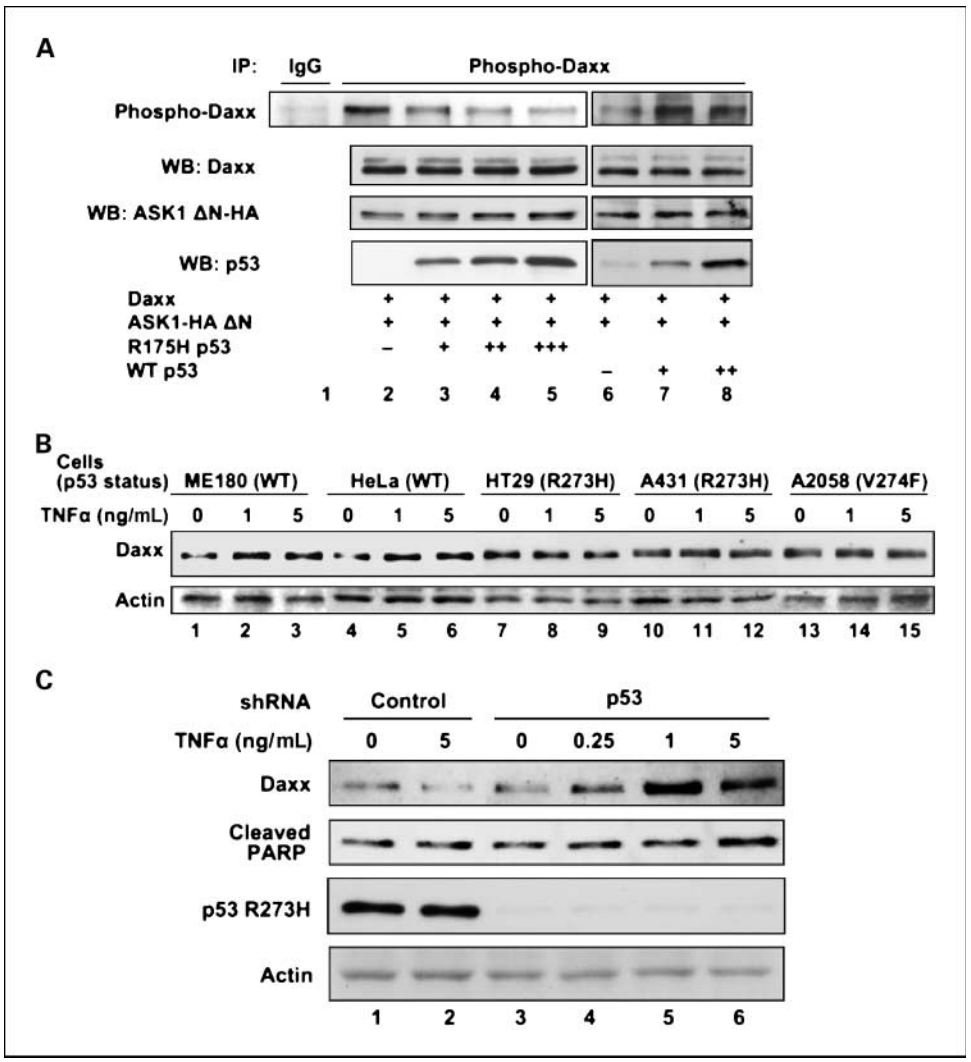

Figure 6. Mutant p53 inhibits ASK1-dependent phosphorylation of Daxx and prevents Daxx accumulation induced by TNFα. *A,* mutant p53 inhibits ASK1-dependent phosphorylation of Daxx. HeLa cells were transfected with expression plasmids, including wild-type and mutant p53 plasmids, in the indicated combinations. Thirty hours after transfection, cell extracts were subjected to immunoprecipitation with anti-phosphorylated Daxx–specific antibody followed by Western blotting with anti-Daxx antibody. Because the expression level of Daxx was reduced by the presence of mutant p53 (18), the amounts of cell lysate in the immunoprecipitation reactions were adjusted to contain equal amounts of Daxx protein (WB:Daxx). *B,* TNFα does not induce the accumulation of Daxx in cells expressing natural tumorigenic mutant p53. Two cell lines with wild-type p53 (ME180 and HeLa) and three cell lines with expressing mutant p53 (HT29, A431, and A2058) were examined for the accumulation of Daxx in response to TNFα treatment. Cells were incubated with TNFα (0, 1, and 5 ng/mL) for 18 h and the cell extracts were subjected to Western blotting for Daxx and actin. *C,* depletion of mutant p53 restores TNFα-induced accumulation of Daxx and apoptosis. HT29 cells were infected (multiplicity of infection = 200) with adenoviruses encoding control or p53 shRNA for 5 d followed by treatment with TNFα at the indicated concentration for 18 h. The cell extracts were analyzed by Western blotting with the indicated antibodies.

treatment because mutant p53 blocks JNK activation by preventing Daxx-ASK1 interaction and the accumulation of Daxx in cells.

Discussion

We showed that the expression level of Daxx in cells was up-regulated by ASK1 and that the ASK1 kinase activity is required for this effect. Indeed, Daxx serves as a direct substrate for ASK1 and is phosphorylated at Ser^{176} and Ser^{184} by ASK1. The serine residues at 176 and 184 in human Daxx are well conserved in mammals, including chimpanzee, monkey, dog, cow, mouse, and rat, suggesting the importance of these residues, and thus, the regulation of Daxx function through the phosphorylation of these serines might also be conserved. The molecular mechanisms for TNFα-dependent sustained JNK activation have been proposed involving caspase-dependent mammalian sterile 20-like kinase 1 (MST1) cleavage or reactive oxygen species production (21, 22). We found that Daxx is essential for the sustained activation of JNK and apoptosis induced by TNFα in cells and Daxx phosphorylations are necessary for TNFα-responsive cellular Daxx accumulation as well as JNK activation. These results are consistent with what was shown in *ASK1*$^{-/-}$ mouse embryonic fibroblasts, in which ASK1 was essential for TNFα-induced sustained JNK activation (10). In our Daxx knockdown experiment, TNFα treatment further reduced the level of phosphorylated JNK when the protein level of cellular Daxx was largely undetectable. This could be due to the activation of MAPK phosphatases by TNFα as reported previously (23).

Previously, it was reported that ASK1 interacts with the COOH-terminal region of Daxx during glucose deprivation (17). We found, however, that ASK1 interacted with the NH_2-terminal region of Daxx and that the interaction was independent from the ASK1 kinase activity because both the kinase-inactive mutant of ASK1 (ASK1 KM) and wild-type ASK1 bind Daxx equally well. Most likely the mode of ASK1-Daxx interaction is different from the ASK1-MKKs complexation because Daxx is missing the DVD docking domain present at the COOH terminus of MKK3/MKK6 and MKK4/MKK7 (24). The sequence surrounding the phosphorylation sites, Ser^{176} and Ser^{184}, in Daxx differs from the previously identified sequences in MKKs or the autophosphorylation sites on ASK1 (25). Mutations on both serines did not affect Daxx-ASK1 interaction, indicating that these residues are dispensable for the interaction. Furthermore, these results also confirm that the interaction between ASK1 and Daxx is not affected by the Daxx phosphorylation status of both serines.

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The involvement of ubiquitination in regulating MAPKs has been recently illustrated (26). Because proteasome inhibitors abolish ASK1-supported accumulation of Daxx, it suggests that Daxx accumulation or degradation is proteasome-dependent. We and others found that Daxx degradation is ubiquitin-dependent (27, 28). However, a ubiquitin-independent proteasome-dependent Daxx degradation initiated by human cytomegalovirus pp71 tegument protein was also reported (29). It is reasonable to speculate that the phosphorylation of Daxx by ASK1 affects the ubiquitin status of Daxx (27).

Phosphorylation of proteins often plays a critical role in ubiquitinating target proteins and the consequent protein degradation (30, 31). Recently, the phosphorylation of Daxx has been shown to induce its cellular degradation. Peptidyl-prolyl isomerase (Pin1) binds to the phosphorylated Ser^{178}-Pro^{179} motif, although this is only present in human, in the Daxx protein, and induces the rapid degradation of Daxx via the ubiquitin-proteasome pathway (28). Pin1 catalyzes the cis-trans isomerization of phosphorylated Ser/Thr-Pro motifs within its specific target substrates. ASK1 phosphorylates two serines close to each other in Daxx, thus, if Pin1 twists the Daxx molecule at Pro^{179} flipping Ser^{176} and Ser^{184} in trans position, it might prevent ASK1 from phosphorylating Daxx. The phosphorylation on Ser^{178} and Ser^{176}/Ser^{184} could be a tug-of-war situation to regulate Daxx metabolism.

It has been recently reported that in response to DNA damage, Daxx undergoes ubiquitination and subsequent proteasomal degradation. Daxx degradation promotes the translocation of RASSF1C from the nucleus to the cytoplasm (32). Cellular inhibitor of apoptosis protein 1 (c-IAP1), a regulator of TNF signaling, is a ubiquitin ligase for ASK1, and p38 and JNK phosphorylations are prolonged in *c-IAP1*$^{-/-}$ B cells (33). Because p38/JNK activation by Daxx depends on ASK1, c-IAP1 is also a key factor in modulating Daxx activity and stability in stress signaling.

Mutations in p53 have been found in >50% of all human cancer cells. Mutant p53s escape from MDM2/HDM2-dependent degradation with the help of $p16^{INK4a}$ inactivation (34) and accumulate to induce the malignant transformation (35, 36). Tumorigenic mutant p53 inhibits the interaction between Daxx and ASK1, and thus, inhibits the ASK1-dependent phosphorylation of Daxx. As a result, mutant p53 inhibits Daxx-dependent JNK activation (18). We propose a mechanism in which mutant p53 inhibits Daxx accumulation by preventing the phosphorylation of Daxx in response to stress signals. Heat shock protein 27 has been reported to block apoptosis by inhibiting the interaction between Daxx and ASK1 (15), providing another example for the biological significance of the cooperative activation between Daxx and ASK1 in JNK signaling cascade. Therefore, blocking the Daxx-ASK1 circuit could be one molecular mechanism for the gain-of-function of mutant p53 which contributes to tumorigenesis.

Based on our results, we propose a positive feedback loop mechanism between Daxx and ASK1 (Supplemental Fig. S11). When ASK1 is activated by stresses, such as oxidative stress, UV and cytotoxic cytokines like TNFα, ASK1 phosphorylates Daxx and stabilizes it. This accumulated Daxx, in turn, further activates ASK1 which reinforces the amplification of downstream kinase activations including JNK and p38. Tumorigenic mutant p53 and possibly heat shock protein 27 may break the circuit between Daxx and ASK1 and thus cells will become more tolerant to stresses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tetsuya Kitamura, Yayoi Fukuyo, Masahiro Inoue, et al.

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