

Syntaxin 6, a Regulator of the Protein Trafficking Machinery and a Target of the p53 Family, Is Required for Cell Adhesion and Survival^{*[5]}

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The p53 family consists of p53, p63, and p73. It has been well characterized that all of the p53 family proteins are transcription factors and capable of regulating cell cycle and apoptosis. To determine whether the p53 family exerts tumor suppression by other mechanisms, we set to identify novel p53 family target genes. Here, we found that the gene encoding STX6 (syntaxin 6), a vesicle transporter protein, is directly regulated by each of the p53 family proteins. In addition, STX6 can be induced by DNA damage and Mdm2 inhibitor Nutlin-3 in a p53-dependent manner. To examine how STX6 mediates the activity of the p53 family, STX6 is inducibly overexpressed or knocked down in various cell lines. We found that overexpression of STX6 alone has limited effect on cell proliferation. In contrast, we found that knockdown of STX6 inhibits cell proliferation and survival. We also found that knockdown of STX6 leads to cell cycle arrest and apoptosis. Interestingly, we found that p53 is necessary for STX6 knockdown-induced cell cycle arrest and apoptosis. Furthermore, we found that STX6 is necessary for proper expression of focal adhesion kinase and integrin $\alpha 5$ adhesion receptor. Consistent with this observation, STX6 knockdown inhibits cell adhesion. Together, we postulate that STX6 is an effector and a modulator of the p53 family in the regulation of cell adhesion and survival.

As a transcription factor, p53 activates a number of genes, such as *p21*, *MDM2*, *IGFBP3*, *PIDD*, and *Killer/DR5*, to regulate cell cycle arrest and apoptosis (1). p63 and p73, both of which are members of the p53 family, are capable of inducing both common and distinct groups of target genes (1). Interestingly, recent studies showed that p63 can regulate a gene involved in cell adhesion in mammary epithelial cells, and loss of p63 induces cell detachment and death (2, 3). However, it is not clear whether these p63 target genes involved in cell adhesion are also regulated by p53 and p73; nor is certain whether p53 and p73 can regulate a gene involved in cell adhesion.

Soluble *N*-ethylmaleimide-sensitive factor (NSF)³ attachment protein receptor (SNARE) proteins are key mediators of membrane fusion. SNAREs share a 60–70-residue sequence called the SNARE motif, which mediates the interaction between vesicle SNAREs (v-SNAREs) and target membrane SNAREs (t-SNAREs) (4). Syntaxin 6 (STX6), a v-SNARE protein, was identified through its homology to the yeast SNARE pep12p (5) and later found to be the closest ortholog of the yeast Tlg1p (6). STX6 is expressed in *trans*-Golgi and endosomes and found to be associated with a variety of SNARE proteins (7, 8). Recently, STX6 has been reported to regulate the caveolae- but not clathrin-dependent endocytosis (9).

Focal adhesion kinase (FAK), a tyrosine kinase, is tightly linked to cell adhesion through the regulation of the interaction between the extracellular matrix and integrin receptors (10). FAK is found to be associated with integrin receptors and recruits other molecules to the site of their interaction, thus forming a signaling complex that transmits signals from the extracellular matrix to the cell cytoskeleton (11). In addition, the integrin-FAK complex has been implicated in the regulation of anchorage-dependent cell survival (12). Furthermore, studies showed that FAK interacts with p53 and inhibits p53 transcriptional activity and subsequently p53-dependent apoptosis (13, 14). Consistent with this, disruption of FAK signaling results in apoptosis (15). Interestingly, the apoptotic response induced by disruption of FAK and cell adhesion is different from the classic apoptotic response, and thus the cell death is called anoikis (16).

Here, we identified STX6, a novel p53 family target gene. We found that although overexpression of STX6 has no significant effect on cell proliferation, knockdown of STX6 profoundly inhibits cell proliferation and induces cell death. Thus, we postulate that STX6 is an effector of the p53 family in the regulation of cell adhesion and survival.

EXPERIMENTAL PROCEDURES

Plasmids—HA-tagged STX6 was amplified by PCR using an expressed sequence tag clone (GenBankTM number BC009944) as a template. To generate STX6 with an HA tag at its N terminus, the following pair of primers was used: sense primer, 5'-GAAGCTTGCCGCCATGGAGTACCCATAC-

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1.

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³ The abbreviations used are: NSF, *N*-ethylmaleimide-sensitive factor; SNARE, NSF attachment protein receptor; FAK, focal adhesion kinase; HA, hemagglutinin; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; RE, responsive element; nt, nucleotide(s); PARP, poly(ADP-ribose) polymerase; GM1, a ganglioside with one sialic acid.

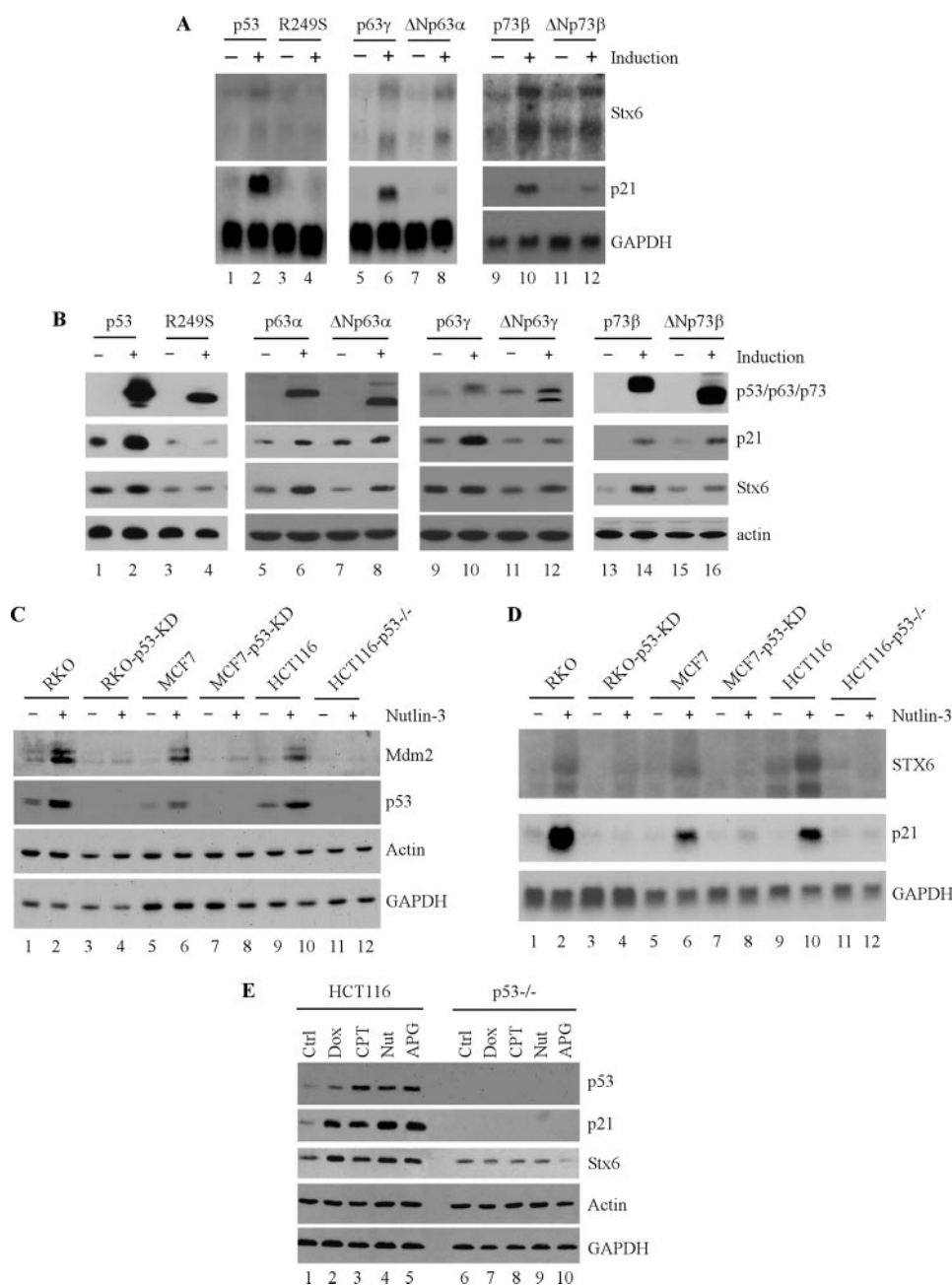


FIGURE 1. STX6 is induced by p53. *A*, Northern blots were prepared with RNAs purified from MCF7 cells that were uninduced (–) or induced (+) to express p53, p53(R249S), p63γ, ΔNp63α, p73β, or ΔNp73β. The blots were probed with cDNAs derived from the *STX6*, *p21*, and *GAPDH* genes, respectively. *B*, Western blots were prepared using extracts from MCF7 cells that were uninduced (–) or induced (+) to express wild-type p53, mutant p53(R249S), Myc-tagged p63α, Myc-tagged ΔNp63α, Myc-tagged p63γ, Myc-tagged ΔNp63γ, HA-tagged p73β, or HA-tagged ΔNp73β. p53 was detected with anti-p53 antibody. p63 was detected by anti-Myc antibody. p73 was detected with anti-HA antibody. p21, STX6, and actin were detected by their respective antibodies. *C*, Western blots were prepared using extracts from RKO, RKO-p53-KD, MCF7, MCF7-p53-KD, HCT116, and HCT116 (p53^{–/–}) cells that were mock-treated (–) or treated (+) with 5 nM Nutlin-3 for 20 h. Mdm2, p53, actin, and GAPDH were detected with their respective antibodies. *D*, Northern blots were prepared with RNAs purified from RKO, RKO-p53-KD, MCF7, MCF7-p53-KD, HCT116, and HCT116 (p53^{–/–}) cells that were mock-treated (–) or treated (+) with 5 nM Nutlin-3 for 20 h. The blots were analyzed as in *A*. *E*, Western blots were prepared using extracts from HCT116 and HCT116 p53^{–/–} cells that were untreated (*ctrl*) or treated with doxorubicin (*Dox*), camptothecin (*CPT*), Nutlin-3 (*Nut*), or apigenin (*APG*). p53, STX6, and actin were detected by their respective antibodies.

GACGTACCAGATTACGCTATGTCCATGGAGGACCCCTTC-3'; antisense primer, 5'-ggaattcTCACAGCACTAAGAAGAGGATG-3'. To generate STX6 with an HA tag at its C terminus, the following pair of primers was used: sense primer,

5'-gaagctt GCC GCC ATGTCCA-TGGAGGACCCCTTC-3'; antisense primer, 5'-ggaattcctcaAGCGTAATCTGGTACGTCGTATGGGTACTCCAGCACTAAGAAGAGGATGAG-3'. To generate a construct that expresses siRNA against *STX6* under the control of the tetracycline-regulated H1 promoter, two 65-base oligonucleotides targeting *STX6* from nucleotide +356 to +375 (GenBankTM number NM_005819) were designed as previously described (17, 18). The pair of oligonucleotides was then annealed and cloned into pBabe-H1 at HindIII and BglII sites, and the resulting construct was designated pBabe-H1-siSTX6. pBabe-H1 is a PolIII promoter-driven vector with a tetracycline operon sequence inserted before the transcription start site (19). To generate a luciferase reporter under the control of a potential p53-responsive element in the *STX6* gene, the *STX6* promoter fragment from nucleotide –762 to –343 (the first nucleotide upstream of the putative transcription start site is designated as –1) was amplified and cloned into O-Fluc reporter vector upstream of a *c-fos* basic promoter and a luciferase reporter (20). A mutant p53-responsive element (p53-RE) with three point mutations (C-369A, C-360T, and G-357A) was also amplified and cloned into O-Fluc. The fragments were confirmed by sequencing.

Cell Culture—RKO, MCF7, and HCT116 cells were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum. HCT116 (p53^{–/–}) is a derivative of HCT116 in which the *p53* gene was somatically knocked out (21, 22). RKO-p53-KD is a derivative of RKO in which p53 was stably knocked down by RNA interference (18). The H1299 and MCF7 cell lines, which inducibly express p53, p53 (R249S), p63α, ΔNp63α, p63γ, ΔNp63γ, p73β, or ΔNp73β, were as previously described (23–25). RKO

and MCF7 cell lines, which inducibly express HA-tagged STX6, were generated as previously described (23). To generate inducible STX6 knockdown cell lines, pBabe-H1-siSTX6 was transfected into MCF7 and RKO cells in which a tetracycline repres-

sor is expressed by pcDNA6 (26). The STX6 knockdown cell lines were selected with puromycin and confirmed by Western blot analysis. To generate inducible STX6 knockdown cell lines with p53 stable knockdown, pBabe-H1-STX6 siRNA was cotransfected with pBabe-U6-p53 siRNA into MCF7 cells, in which a tetracycline repressor is expressed by pcDNA6. The resulting STX6 and p53 dual knockdown cell lines were selected with puromycin. Both STX6 knockdown and p53 knockdown were confirmed by Western blot analysis.

Luciferase Assay—A dual luciferase assay was performed in triplicate according to the manufacturer's instructions (Promega). Briefly, p53-null H1299 cells were plated at 5×10^4 cells/well in a 24-well plate and allowed to recover overnight. Cells were then cotransfected with 200 ng of O-Fluc reporter vector along with pcDNA3 or a pcDNA3 vector expressing wild-type p53, p53 (R249S), p63 γ , Δ Np63 γ , p73 β , or Δ Np73 β . As an internal control, 5 ng of pRL-CMV, a *Renilla* luciferase vector (Promega), was also cotransfected per well. Thirty-six hours post-transfection, luciferase activity was measured with the dual luciferase kit and Turner Designs luminometer. The -fold increase in relative luciferase activity is a product of the luciferase activity induced by a p53 family protein divided by that induced by empty pcDNA3 vector.

ChIP Assay—The ChIP assay was performed as previously described (27, 28). RKO cells were mock-treated or treated with doxorubicin for 24 h, whereas MCF7 cells were uninduced or induced to express Myc-tagged p63 γ or HA-tagged p73 β for 24 h. Chromatins in these cells were then cross-linked with 1% formaldehyde for 10 min at room temperature, sonicated to generate 200–1000-bp DNA fragments, and immunoprecipitated with anti-p53 antibody to capture p53-DNA complexes, with anti-Myc antibody to capture Myc-tagged p63 γ -DNA complexes, or with anti-HA antibody to capture HA-tagged p73 β -DNA complexes. After reverse cross-linking and phenol-chloroform extraction, the bound DNA fragments were purified by a Qiagen column. PCR was performed to visualize enriched DNA fragments. The primers designed to amplify the p53-RE within the STX6 promoter were as follows: sense, 5'-CCTGAAAAGCCGACTGCCTAGT-3'; antisense, 5'-GCGGGGCTTACGCCTGCGTCCC-3'. The primers designed to amplify the upstream p53-RE1 within the p21 promoter and GAPDH were as previously described (29, 30). The following two pairs of primers were used to amplify the STX6-upstream control region and the STX6-downstream control region: upstream sense (5'-TATAACAGGCTAGTGAAGGAGCAGG-3') and upstream antisense (5'-CAGAAATAAGGAGATGGCAACACAG-3'); downstream sense (5'-TTCTGTTGCCAGACTGGAGTGC-3') and downstream antisense (5'-CCGAGACAGGTGGATCACAA-GGTC-3').

DNA Histogram Analysis—Cells induced or uninduced to express or knock down STX6 for various times were collected and fixed in 75% ethanol for at least 1 h at 4 °C. Cells were then washed with PBS and resuspended in a staining buffer containing 100 μ g/ml RNase A and 50 μ g/ml propidium iodine (Invitrogen). The percentage of cells in each phase of the cell cycle (sub-G₁, G₁, S, and G₂-M) was analyzed by a FACS-Caliber cell sorter (BD Biosciences) along with CellQuest software.

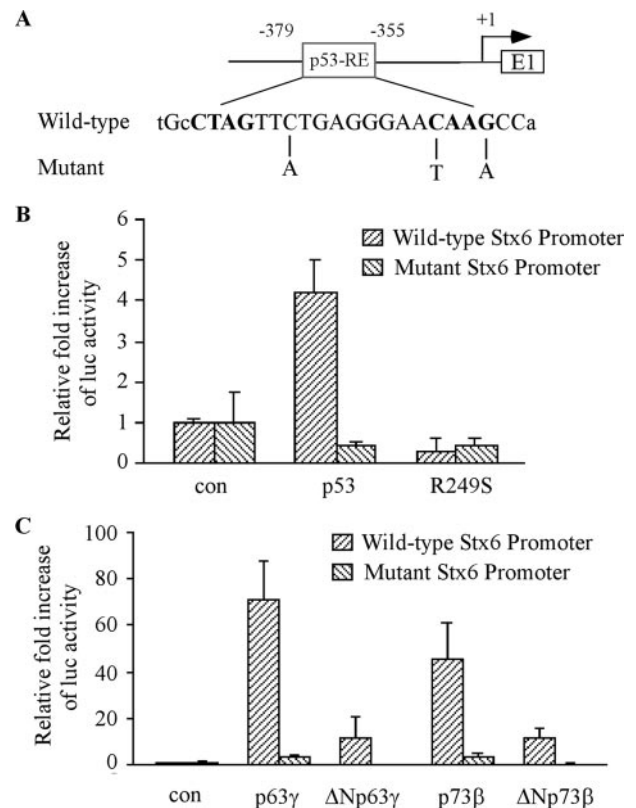


FIGURE 2. Identification of a potential p53RE in the STX6 gene. A, schematic presentation of the STX6 locus containing a potential p53-RE located in the STX6 promoter (nt -379 to -355) (top). The potential p53-RE sequence and mutated p53-RE sequence are shown in the bottom two panels. B and C, the wild-type, but not the mutant, p53-RE in the STX6 gene is responsive to p53, p63 γ , Δ Np63 γ , p73 β , and Δ Np73 β but not mutant p53(R249S). The luciferase assay was performed as described under "Experimental Procedures." con, control.

Cell Attachment Assay—Six-well plates were coated with 5 μ g/ml fibronectin at 37 °C for 1 h, washed twice with PBS, and then incubated with 1% bovine serum albumin at 37 °C for 1 h, followed by PBS wash twice. The coated plates were kept at 4 °C until use. MCF7 cells, which were induced or uninduced to knock down STX6 for 36 h, were seeded at 20,000 cells on the fibronectin-coated plates with 2 ml of culture medium/well and incubated in the 37 °C, 5% CO₂ incubator for 30 min. The unattached cells in the medium were collected, gently washed twice with 1 ml of PBS, and then counted by a Beckman Coulter counter. The attached cells were trypsinized and also counted. The ratio of the attached cells to the total number of cells at the control condition was set at 100%.

Northern Blot Analysis—Total RNAs were isolated from H1299, RKO, MCF7, and HCT116 cells using TRIzol reagent (Invitrogen). Northern blot analysis and preparation of p21 and GAPDH probes were as described previously (31). The STX6 probe, a 1.5-kb XhoI-EcoRI fragment, was made from an expressed sequence tag clone (GenBank™ number BC009944).

Antibodies and Immunoblotting—Cells were collected from culture plates in PBS, resuspended in 2 \times sample buffer, and boiled for 7 min at 95 °C. Anti-Stx6 antibody was purchased from synaptic system (Germany). Other antibodies used were anti-p53 monoclonal antibodies (DO-1, PAb1801, PAb240, and

STX6 Mediates p53 Family-dependent Cell Adhesion

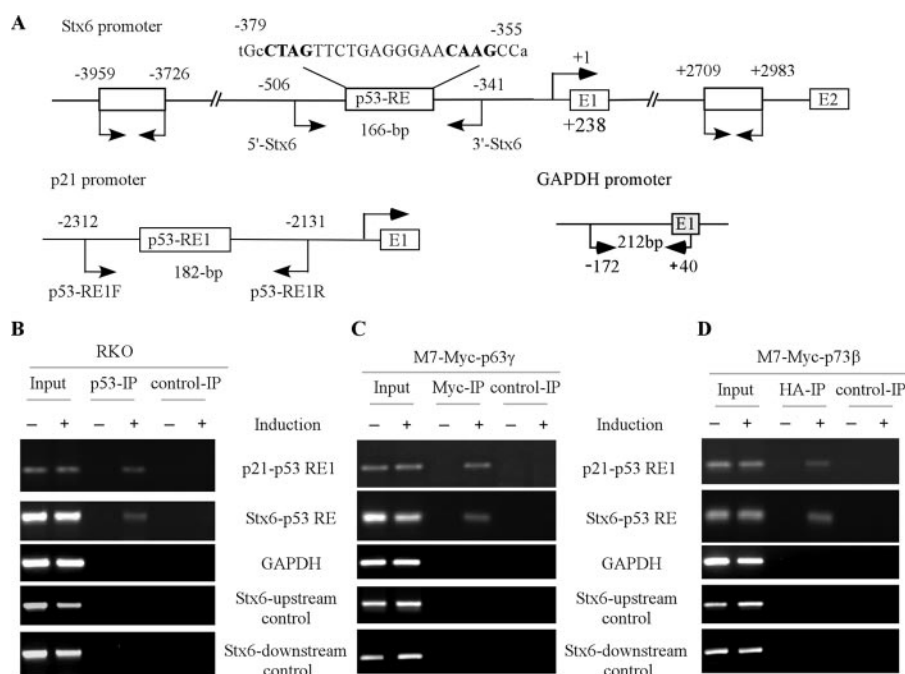


FIGURE 3. The p53 family proteins bind to the potential p53-RE in the *STX6* gene. *A*, schematic presentation of the *STX6*, *p21*, and *GAPDH* promoters along with the location of the p53-RE and various PCR primers used for ChIP assay. *B–D*, the p53 family proteins bind to the p53-RE in the *STX6* and *p21* genes *in vivo*. The ChIP assay was performed as described under “Experimental Procedures.” Upon induction or no induction of various p53 family proteins, RKO and MCF7 cells were cross-linked with formaldehyde followed by sonication. Chromatin was immunoprecipitated (IP) with anti-p53 to detect p53-DNA complexes, with anti-Myc to detect Myc-tagged p63-DNA complexes, or with anti-HA to detect p73-DNA complexes along with a control IgG for nonspecific binding. p53-REs in the *STX6* and *p21* genes were amplified by PCR. Potential binding of the p53 family proteins to the upstream and downstream regions in the *STX6* gene and the *GAPDH* promoter was also examined.

PAb421), anti-actin (Sigma), anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p21 (C-19) (Santa Cruz Biotechnology), anti-PCNA (Santa Cruz Biotechnology), anti-Mdm2 (Santa Cruz Biotechnology), anti-FAK (Santa Cruz Biotechnology), anti-integrin $\alpha 5$ (Chemicon), anti-PYK kinase (Santa Cruz Biotechnology), anti-clathrin heavy chain (Santa Cruz Biotechnology), anti-HA monoclonal antibodies (12CA5 and HA11) (Covance), and anti-Myc monoclonal antibody (9E10) (Cell Signaling).

RESULTS

STX6 Is a Novel Target Gene of the p53 Family—As a transcription factor, the p53 family exerts its function by regulating various genes involved in cell proliferation and survival. To determine whether a gene involved in cell adhesion can be regulated by the entire p53 family proteins, we performed a microarray array using RNAs purified from MCF7 cells uninduced or induced to express p63 γ . Many genes were found to be up-regulated. Among these is the *STX6* gene. To confirm the microarray analysis, Northern blot analysis was performed and showed that the expression level of *STX6* was markedly increased by p63 γ in MCF7 cells (Fig. 1A, compare lanes 5 and 6). In addition, Δ Np63 α was also capable of inducing *STX6* (Fig. 1A, compare lanes 7 and 8). To examine whether *STX6* is a common p53 family target, the level of *STX6* was examined in MCF7 cells uninduced or induced to express p53, p53 (R249S), p73 β , or Δ Np73 β . We found that *STX6* was induced by wild-type p53, p73 β , and Δ Np73 β but not mutant p53 (R249S) (Fig.

1A, compare lanes 2, 4, 10, and 12 with lanes 1, 3, 9, and 11, respectively). In addition, the expression level of *p21* was measured as a positive control. As expected, *p21* was induced markedly by wild-type p53, p63 γ , and p73 β ; induced mildly by Δ Np63 α and Δ Np73 β ; and not induced by mutant p53 (R249S) (Fig. 1A). The expression level of *GAPDH* was measured as a loading control (Fig. 1A). We note that *STX6* is located on chromosome 1Q25.3. Based on the NCBI data base, the *STX6* gene encodes two transcripts through alternative splicing at the 3'-untranslated region. One transcript has 4,500 nucleotides (NM_005819), whereas the other has 1,500 nucleotides (BC009944). As shown in Fig. 1A, both the long and the short transcripts were expressed in MCF7 cells, and the expression levels for both transcripts were increased by the p53 family.

Next, we wanted to know whether the increased levels of *STX6* transcripts correlate with an increase in the levels of *STX6* protein.

Western blot analysis was performed and showed that upon induction, the level of *STX6* protein was increased by wild-type p53, p63 α , Δ Np63 α , p63 γ , Δ Np63 γ , p73 β , and Δ Np73 β but not mutant p53(R249S) (Fig. 1B). In addition, the level of p21 was measured and found to be increased by wild-type p53, p63 α , Δ Np63 α , p63 γ , Δ Np63 γ , p73 β , and Δ Np73 β but not mutant p53(R249S) (Fig. 1B). The level of actin was measured as a loading control (Fig. 1B).

To further make certain that *STX6* is a true target of p53, we examined whether activated endogenous p53 is able to induce *Stx6*. To test this, tumor cells with endogenous wild-type p53 or in which endogenous p53 was stably knocked down by siRNA or somatically knocked out were treated with Nutlin-3, an inhibitor of Mdm2. Since Mdm2 is a negative inhibitor of p53, inhibition of Mdm2 by Nutlin-3 leads to p53 activation (32). Indeed, we showed that upon treatment with Nutlin-3, p53 was stabilized along with induction of Mdm2 in RKO, MCF7, and HCT116 cells, all of which carry an endogenous wild-type p53 (Fig. 1C, compare lanes 1, 5, and 9 with lanes 2, 6, and 10, respectively). In contrast, when p53 was stably knocked down by siRNA (RKO-p53-KD and MCF-p53-KD) or somatically knocked out (HCT116-p53 $^{-/-}$), Nutlin-3 treatment had no effect on p53 accumulation and Mdm2 induction (Fig. 1C, compare lanes 3, 7, and 11 with lanes 4, 8, and 12, respectively). Next, Northern blot analysis was performed to measure the level of *STX6* transcripts upon p53 activation. We showed that the levels of *STX6* were markedly increased in p53-proficient cells (RKO, MCF7, and HCT116 cells) (Fig. 1D, compare lanes

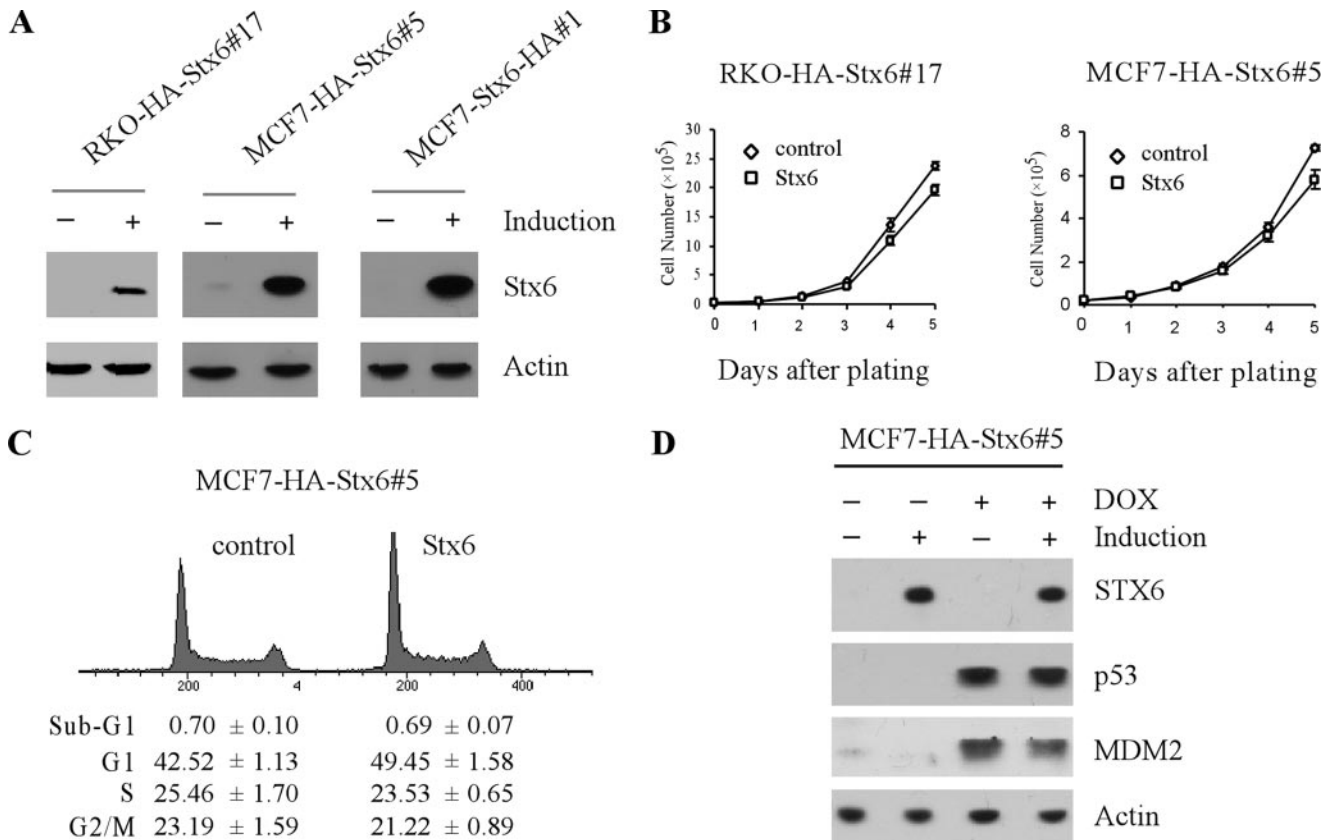


FIGURE 4. Overexpression of STX6 has a limited effect on cell proliferation. *A*, generation of RKO and MCF7 cell lines that inducibly express HA-tagged STX6. The levels of HA-tagged STX6 were quantified with anti-HA. *B*, the growth rate of RKO and MCF7 cells that were uninduced or induced to express HA-tagged STX6 was measured over a 5-day period. *C*, MCF7 cells were uninduced (–) or induced (+) to express HA-STX6 for 3 days and then stained with propidium iodide for DNA histogram analysis as described under “Experimental Procedures.” *D*, Western blots were prepared using cell extracts from MCF7 cells uninduced (–) or induced (+) to express HA-STX6 for 24 h, followed by treatment with or without doxorubicin (DOX; 0.4 μ g/ml) for 20 h. The levels of p53, MDM2, STX6, and actin were quantified with their corresponding antibodies.

1, 5, and 9 with lanes 2, 6, and 10, respectively). In contrast, when p53 was stably knocked down by siRNA or somatically knocked out, the levels of *STX6* transcripts were not significantly altered (Fig. 1C, compare lanes 3, 7, and 11 with lanes 4, 8, and 12, respectively). Again, the transcript levels of *p21* and *GAPDH* were measured as positive and loading controls, respectively (Fig. 1D). Finally, we examined the levels of STX6 protein in HCT116 and p53-null HCT116 cells upon treatment with various chemotherapy agents (doxorubicin, camptothecin, and apigenin) or the Mdm2 inhibitor Nutlin-3. We found that the level of STX6 protein was increased in HCT116, but not p53-null HCT116, cells (Fig. 1E). We note that upon comparing the basal levels of STX6 in p53-proficient and -deficient cells, the levels of STX6 were lower in p53-knockdown and p53-null cells than that in p53-proficient cells (Fig. 1D, compare lanes 1, 5, and 9 with lanes 3, 7, and 11, respectively; Fig. 1E, compare lane 1 with lane 6), suggesting that the basal expression level of STX6 is regulated by p53.

As a transcription factor, the p53 family proteins bind to a specific DNA sequence in a target gene and activate the gene expression. Thus, we searched for a potential p53 family RE in the *STX6* gene. One potential binding site (designated as *STX6* p53-RE) was found to be located at nt –379 to –355 in the *STX6* promoter (the first nt upstream of the transcription start site is designated as –1) with the following sequence: tGc

CTAG TTC TGA GG GAA CAAG CCa. Upon alignment with the consensus p53 binding site, the p53-RE in the *STX6* gene has three mismatches in the noncritical region (the lowercase letters represent mismatches) (Fig. 2A).

To investigate whether the p53 family can activate the *STX6* transcription through this potential p53-RE, the promoter fragment from nt –913 to –344, which carries one copy of the potential p53-RE, was cloned into O-Fluc reporter vector under the control of the *c-fos* basic promoter to drive the expression of luciferase. A mutant *STX6* promoter fragment (G–358A, C–361T, and C–369A) was also cloned into O-Fluc (Fig. 2A). We found that the wild-type, but not mutated, *STX6* promoter was responsive to wild-type p53 (Fig. 2B). Mutant p53 (R249S) was inert (Fig. 2B). Similarly, the wild-type, but not mutated, *STX6* promoter was responsive to p63 γ , Δ Np63 γ , p73 β , and Δ Np73 β (Fig. 2C). These results are consistent with the expression patterns of STX6 upon induction of various p53 family proteins (Fig. 1).

Next, we performed a ChIP assay to identify and confirm the direct binding of various p53 family proteins to the potential p53-RE in the *STX6* gene. To examine p53 binding to the *STX6* gene, RKO cells were untreated or treated with doxorubicin to activate p53. After cross-linking with formaldehyde and sonication, the p53-DNA complexes were immunoprecipitated by anti-p53 antibody. Anti-HA antibody was used as a negative

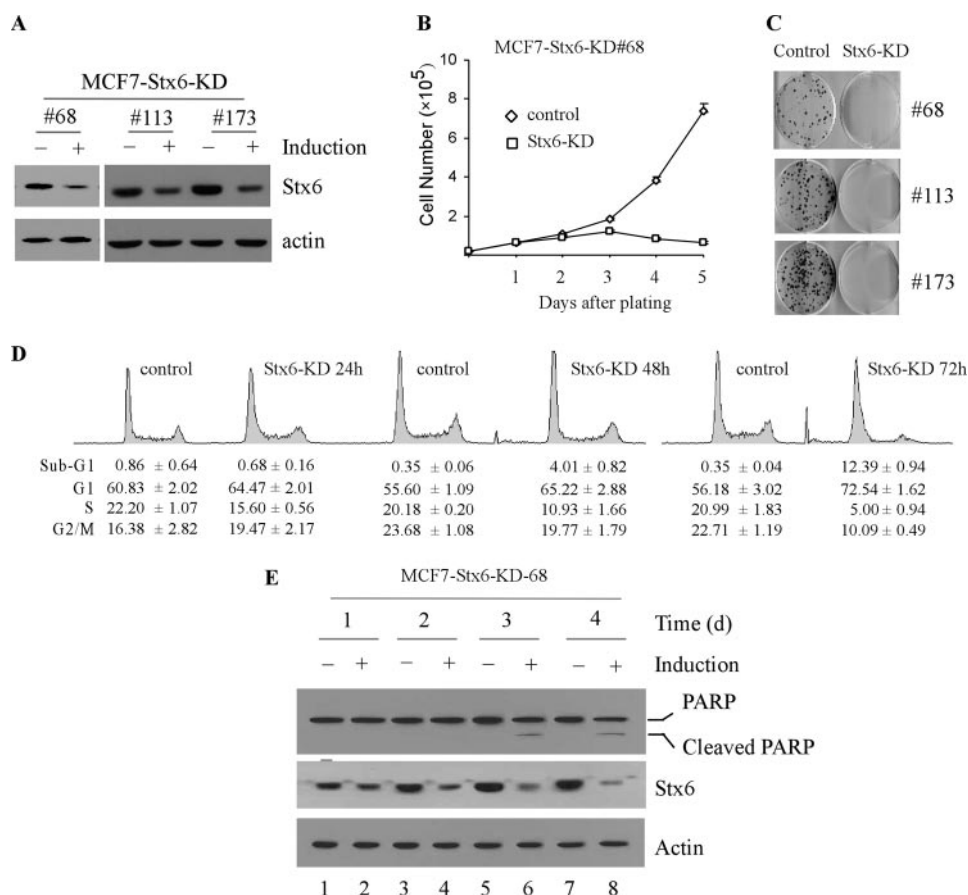


FIGURE 5. STX6 is required for cell proliferation and survival. A, generation of MCF7 cell lines in which endogenous STX6 is inducibly knocked down using a tetracycline-inducible H1 promoter. B, the growth rate of MCF7 cells that were uninduced or induced to express siRNA against STX6 was measured over a 5-day period. C, the colony-forming ability of MCF7 cells uninduced (–) or induced (+) to knock down STX6 over a 14-day period. D, knockdown of STX6 leads to cell cycle arrest and apoptosis. MCF7 cells, which were uninduced (control) or induced (Stx6-KD) to express siRNA against STX6 for 1, 2, or 3 days, were stained with propidium iodide for DNA histogram analysis as described under “Experimental Procedures.” E, Western blots were prepared using extracts from MCF7 cells that were uninduced (–) or induced (+) to knock down STX6 for 1, 2, 3, or 4 days. The blots were probed with antibodies against PARP, STX6, and actin, respectively.

control. PCR was used to amplify the potential STX6 DNA fragment associated with p53 protein (Fig. 3A). The p53 binding to p53-RE1 in the p21 promoter was used as a positive control. Additionally, a region within the promoter of the GAPDH gene was amplified as a control for nonspecific binding. We also designed two pairs of primers to amplify the regions upstream and downstream of the potential p53-RE, respectively (Fig. 3A). As shown in Fig. 3B, we found that the STX6 fragment containing the potential p53-RE was markedly enriched (Fig. 3B). In addition, the p21 fragment containing p53-RE1 was also enriched (Fig. 3B). No enrichment was observed by control anti-HA (Fig. 3B). In addition, no p53 binding to GAPDH or to the upstream and downstream regions in the STX6 gene was detected (Fig. 3B).

We also examined whether p63 and p73 bind to the potential p53-RE in the STX6 gene. To this end, MCF7 cells were uninduced or induced to express Myc-tagged p63 γ or HA-tagged p73 β . The p63-DNA complexes were immunoprecipitated with anti-Myc antibody along with anti-HA as a control. The p73-DNA complexes were immunoprecipitated with anti-HA antibody along with anti-Myc as a control. We found that both p63 γ and p73 β bound to the potential p53-RE in the STX6 gene

(Fig. 3, C and D). In addition, the p21 promoter fragment containing p53-RE1 was also enriched (Fig. 3, C and D). Furthermore, no enrichment was observed by anti-HA control for p63 and anti-Myc control for p73 (Fig. 3, C and D). Furthermore, no p63 or p73 binding to GAPDH or to the upstream and downstream regions in the STX6 gene was detected (Fig. 3, C and D).

Overexpression of STX6 Has No Major Effect on Cell Proliferation—As a p53 family target, overexpression of STX6 may be able to inhibit cell proliferation by inducing cell cycle arrest and apoptosis. To test this, we generated multiple cell lines, including MCF7 and RKO, that inducibly express STX6 under the control of tetracycline-inducible promoter. One representative RKO cell line (RKO-HA-STX6#17) and two representative MCF7 cell lines (MCF7-HA-STX6#5 and MCF7-STX6-HA#1) are shown in Fig. 4A. Growth rate analysis was performed and showed that over a 5-day testing period, overexpression of STX6 had only a mild effect on cell proliferation in RKO and MCF7 cells (Fig. 4B). Consistent with this, DNA histogram analysis was performed and showed that upon induction of STX6 for 3 days, the number of cells in G₁ was slightly increased (Fig.

4C). Furthermore, overexpression of STX6 had no significant effect on the activation and transcriptional activity of p53 in MCF7 cells upon treatment with doxorubicin (Fig. 4D).

Physiologically Relevant Levels of STX6 Are Necessary for Cell Proliferation and Survival—To further determine the biological function of STX6 in the p53 family pathway, we generated MCF7 cell lines in which endogenous STX6 can be inducibly knocked down. Three representative cell lines (MCF7-STX6-KD#68, MCF7-STX6-KD#113, and MCF7-STX6-KD#173) were chosen for further analysis (Fig. 5A). Western blot analysis showed that upon induction of siRNA against STX6, the level of STX6 protein was reduced by 60–80%. To determine whether STX6 is necessary for cell survival, growth rate and colony formation assays were performed. We showed that upon knockdown of STX6, MCF7 cells failed to proliferate over a 5-day testing period (Fig. 5B). In addition, MCF7 cells failed to form colonies (Fig. 5C). Furthermore, we observed that 48 h after STX6 knockdown, some MCF7 cells started to detach from the plate, and on the third day, many cells lost their ability to attach to the plates. Thus, we performed DNA histogram analysis to examine whether STX6 knockdown leads to cell cycle arrest and apoptosis. As shown in Fig. 5D, upon STX6 knockdown for

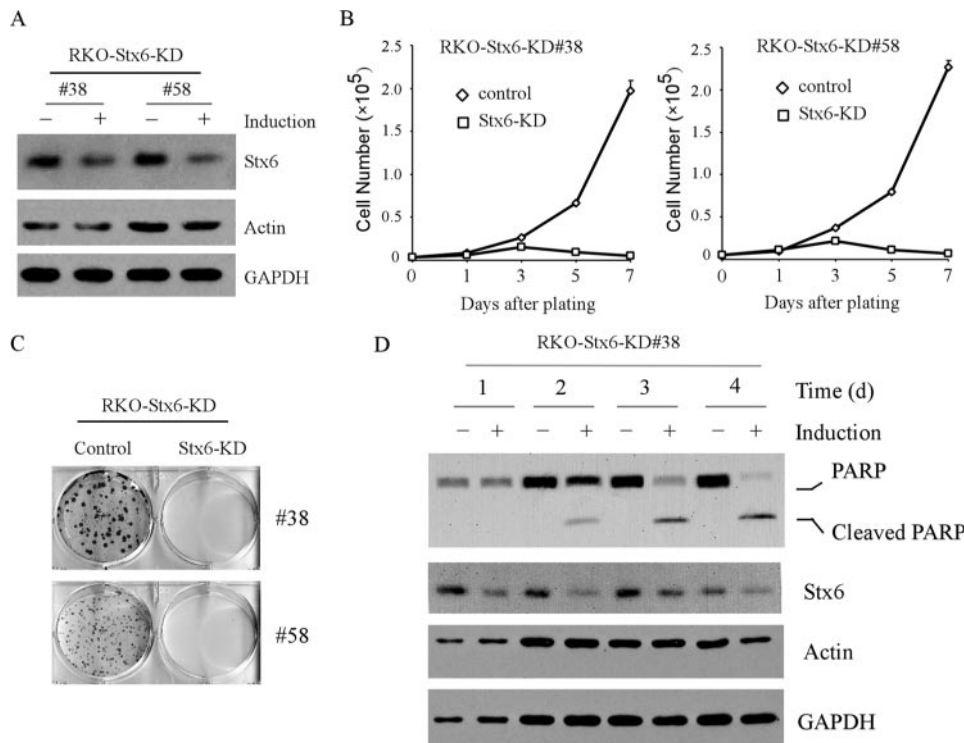


FIGURE 6. The requirement of STX6 for cell proliferation and survival is not cell type-specific. A, generation of RKO cell lines in which endogenous STX6 is inducibly knocked down using a tetracycline-inducible H1 promoter. Western blots were prepared using extracts from RKO cells that were uninduced (–) or induced (+) to knock down STX6 for 2 days and then probed with antibodies against Stx6, actin, and GAPDH, respectively. B, the growth rate of RKO cells that were uninduced or induced to express siRNA against STX6 was measured over a 7-day period. C, the colony-forming ability of RKO cells uninduced (–) or induced (+) to knock down STX6 over a 14-day period. D, PARP cleavage is induced by STX6-KD. Western blots were prepared using extracts from RKO cells that were uninduced (–) or induced (+) to knock down STX6 for 1, 2, 3, or 4 days. The blots were probed with antibodies against PARP, STX6, actin, and GAPDH, respectively.

24 h, MCF7 cells started to undergo cell cycle arrest as the number of cells in the G₁ phase was increased by ~4% along with a concomitant decrease of cells in the S phase. Interestingly, upon prolonged knockdown of STX6 for 48 and 72 h, both numbers of cells in sub-G₁ and G₁ phases were markedly increased (Fig. 5D). Cells with a sub-G₁ DNA content are a conservative measurement of cells undergoing apoptosis. To further demonstrate that knockdown of STX6 leads to apoptosis, we measured the level of cleaved PARP, one of the caspase substrates during apoptosis. We showed that PARP was cleaved upon knockdown of STX6 (Fig. 5E, compare lanes 6 and 8 with lanes 5 and 7).

To demonstrate that the effect of STX6 knockdown on cell cycle arrest and apoptosis is not cell type-specific, we generated RKO cell lines in which STX6 can be inducibly knocked down. Two representative cell lines, RKO-STX6-KD#38 and RKO-STX6-KD#58 are shown in Fig. 6A. Similar to the observations in MCF7 cells, knockdown of STX6 led to growth suppression (Fig. 6, B and C) and apoptosis (Fig. 6D).

It is well established that the p53 family proteins and some of their targets positively or negatively regulate each other, forming a feedback loop (1). Thus, we wanted to examine whether p53 plays a role in cell cycle arrest and apoptosis induced by STX6 knockdown. To test this, we generated multiple cell lines in which STX6 can be inducibly knocked down and endogenous wild-type p53 was stably knocked down by siRNA. West-

ern blot analysis was performed to measure the levels of STX6 and p53. Two representative cell lines (M7-(p53-KD)-STX6-KD#68 and STX6-KD#72) were identified in which levels of STX6 protein were inducibly knocked down (Fig. 7A, compare lanes 5, 7, 9, and 11 with lanes 6, 8, 10, and 12, respectively), and the levels of p53 protein were markedly decreased in cells mock-treated or treated with doxorubicin (Fig. 7A, lanes 5–12). For p53-competent MCF7 cells in which STX6 can be inducibly knocked down (M7-STX6-KD#68), the level of STX6 was reduced regardless of treatment with doxorubicin (Fig. 7A, compare lanes 1 and 3 with lanes 2 and 4, respectively). However, p53 was stabilized upon DNA damage (Fig. 7A, compare lanes 3 and 4). We note that upon STX6 knockdown, the basal level of p53 protein was slightly increased (Fig. 7A, compare lanes 1 and 2). In addition, the extent of p53 stabilization induced by DNA damage was further increased upon STX6 knockdown (Fig. 7A, compare lanes 3 and 4). Next, a short term cell growth rate assay was performed and showed

that the cell growth inhibition was reversed by stable p53 knockdown (Fig. 7B), compared with p53-competent cells in which STX6 was inducibly knocked down (Figs. 5B and 6B). Consistent with this observation, a colony formation assay showed that upon constitutive knockdown of p53, STX6-KD-induced growth suppression was markedly decreased compared with that in p53-competent cells (Fig. 7C, compare left panel with middle and right panels). Moreover, the extent of PARP cleavage induced by STX6 knockdown was also markedly reduced by p53 knockdown (Fig. 7D, compare lane 2 with lanes 6 and 10 for 3-day STX6-KD; compare lane 4 with lanes 8 and 12 for 4-day STX6-KD).

Due to detachment of cells from culture plates and the role of STX6 in the membrane remodeling and protein trafficking, we examined several cell adhesion receptors and focal adhesion kinase. We found that upon knockdown of STX6, the expression level of FAK protein was dramatically decreased and kept at a very low level over a 4-day testing period (Fig. 8, A and B, FAK panel, compare lanes 1, 3, 5, and 7 with lanes 2, 4, 6, and 8, respectively). In addition, upon knockdown of STX6, the expression levels of integrin $\alpha 5$ were also decreased (Fig. 8, A and B). However, PYK kinase, another member of the FAK kinase family, was not affected (data not shown). Furthermore, knockdown of Stx6 had no effect on the expression levels of EEA1, one of the STX6-associated proteins in the endosome, and clathrin heavy chain (data not shown). Since p53 is found to

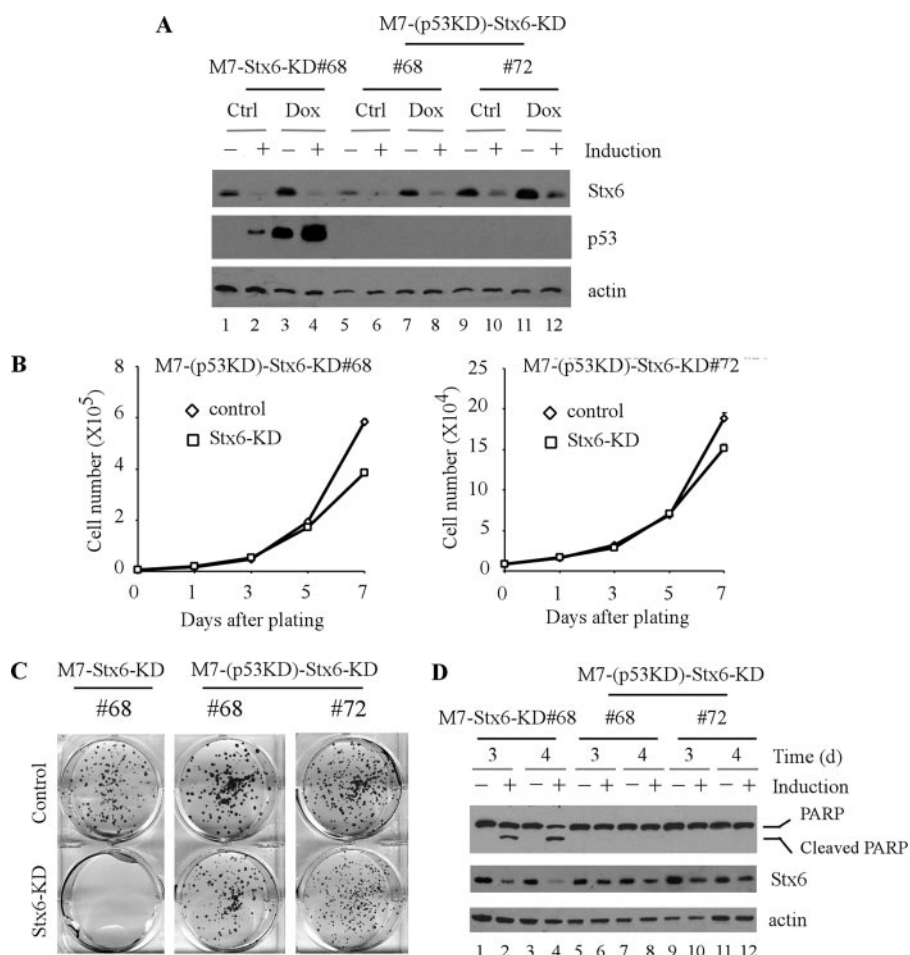


FIGURE 7. STX6-KD-induced growth suppression is p53-dependent. A, generation of MCF7 cell lines in which endogenous p53 was stably knocked down and STX6 can be inducibly knocked down. Western blots were prepared using extracts from p53-competent and p53-KD MCF cells that were uninduced (–) or induced (+) to knock down STX6 for 2 days along with mock treatment (ctrl) or treatment with doxorubicin (Dox). The blots were probed with antibodies against STX6, p53, and actin, respectively. B, the growth rate of p53-KD MCF cells that were uninduced or induced to express siRNA against STX6 was measured over a 7-day period. C, the colony-forming ability of p53-competent MCF7 (left) and p53-KD MCF7 cells (middle and right) uninduced (–) or induced (+) to knock down STX6 over a 14-day period. D, PARP cleavage is induced by STX6-KD. Western blots were prepared using extracts from p53-competent and p53-KD MCF cells that were uninduced (–) or induced (+) to knock down STX6 for 3 or 4 days. The blots were probed with antibodies against PARP, STX6, and actin, respectively.

FAK and integrin $\alpha 5$ expression was abrogated (Fig. 8C, compare lanes 1, 3, 5, and 7 with lanes 2, 4, 6, and 8, respectively).

Based on the effect of STX6 knockdown on the expression of FAK and integrins and detachment of cells from culture plates, we wanted to examine whether cell adhesion is affected by knockdown of STX6. Since fibronectin is known to participate in the integrin-mediated cell adhesion (33), we performed a cell adhesion assay with fibronectin-coated plates. To test this, MCF7 cells, which were uninduced or induced to knock down STX6 for 36 h, were seeded on fibronectin-coated plates for 30 min, and the cells were then gently washed twice with PBS. The attached cells were then trypsinized. Both the attached and unattached cells were collected and counted, respectively. The ratio of the attached cells to total seeded cells was calculated. For the control uninduced MCF7 cells, the ratio was set at 100%. We found that cell adhesion was markedly inhibited by knockdown of STX6 (Fig. 9, compare the *third* and *fourth* columns), whereas treatment with tetracycline alone had a mild but insignificant effect (Fig. 9, compare the *first two* columns). These data suggest that knockdown of STX6 inhibits cell proliferation and induces apoptosis by blocking cell adhesion in MCF7 cells.

DISCUSSION

In this study, we have identified STX6 as a common p53 family target. Although many p53 family target genes have been identified, STX6 is unique in many ways. STX6, a vesicle transporter protein, is critical for a number of cellular protein trafficking machineries, especially ones for membrane remodeling (8). Indeed, we found that knockdown of STX6 leads to decreased expression of FAK kinase and cell adhesion receptors, integrin $\alpha 5$. As a result,

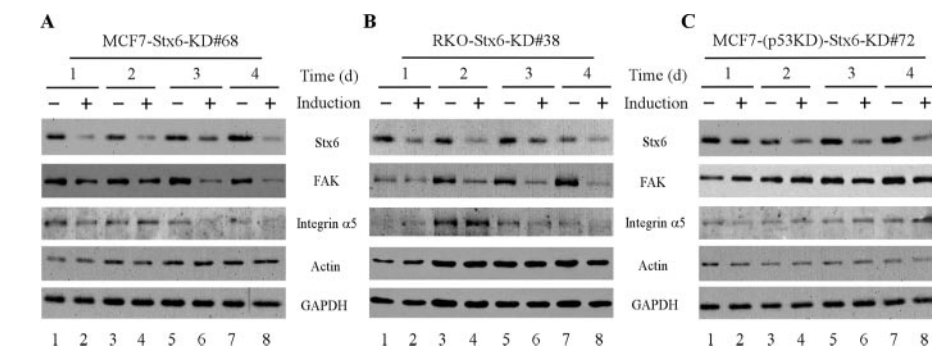


FIGURE 8. STX6 is required for proper expression of FAK and integrin $\alpha 5$ in a p53-dependent manner. Western blots were prepared using extracts from MCF7 cells (A and B) or p53-KD MCF7 cells (C) that were uninduced (–) or induced (+) to knock down STX6 for 1, 2, 3, or 4 days. The blots were probed with antibodies against STX6, FAK, integrin $\alpha 5$, actin, and GAPDH, respectively.

be required for growth suppression induced by STX6-KD, we examined whether p53 plays a role. Interestingly, we found that upon knockdown of p53, STX6-KD-mediated suppression of

STX6 knockdown inhibits cell adhesion and subsequently apoptosis. Interestingly, we found that STX6-KD-mediated growth suppression is p53-dependent, since constitutive knockdown of

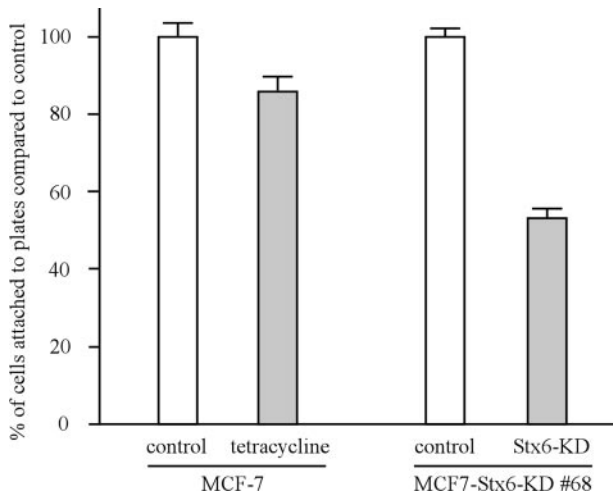


FIGURE 9. **STX6 is required for cell adhesion.** Parental MCF7 cells and MCF7-STX6-KD#68 cells, which were untreated or treated with tetracycline for 36 h, were seeded on fibronectin-coated plates. The attached and unattached cells were counted, and the ratio of attached cells to total seeded cells under the control condition was set at 100% as described under "Experimental Procedures."

p53 rescues the proliferative defect induced by lack of STX6. In addition, through searching the Oncomine data base, STX6 expression was found to be markedly increased in primary tumor tissues of lung, prostate, and bladder but decreased in glioma and leukemia (supplemental Table 1). Taken together, these data suggest that STX6 is an effector and a modulator of the p53 family in the regulation of cell adhesion and survival.

It is well established that as a transcription factor, the p53 family proteins induce several groups of genes involved in both extrinsic death receptor-mediated and mitochondria-mediated apoptotic pathways. These include genes that encode Fas, FasL, and Killer/DR5 and genes that encode Bax, Puma, and Noxa (1). Here, we showed that STX6 modulates the p53 family-dependent apoptosis via another default apoptotic pathway, called anoikis. Like lack of survival signals for anoikis (34), STX6 knockdown inhibits cell adhesion via decreased expression of FAK and integrin $\alpha 5$. Thus, upon exposure to stress signals, p53 is activated and induces STX6, which would then counter the anoikis apoptotic pathway. In this regard, STX6 mediates p53 prosurvival functions, especially to counter the effect of stress signals that activate anoikis, such as lack of nutrients and damages to microenvironments necessary for cell adhesion.

STX6 is involved in multiple secretory and endocytic pathways, such as endosomal maturation (35), GM1 ganglioside membrane delivery, and Glu4 (glucose transporter-4) reinternalization and subendosomal Glu4 sorting (36, 37). STX6 has also been found to interact with several SNARE proteins, including Syn7, Vti1b, and VAMP7 and -8 (vesicle-associated membrane protein 7 and 8, respectively) (38). In addition, STX6 is found to specifically regulate the delivery of microdomain-associated lipids and proteins to the cell surface via caveolae (9). Depletion of STX6 caused a significant drop in the internalization of lactosylceramide, a known indicator of caveolar endocytosis (39). A recent study showed that syntaxin 6 is positively involved in nerve growth factor-dependent neurite outgrowth in PC12 cells potential via its first coiled-coil domain (40). Here,

we found that STX6 is required for maintaining proper expression levels of FAK and integrin $\alpha 5$. This is not surprising, given the fact that STX6 has been found to be expressed in the trans-Golgi network, endosomal structures, and post-Golgi immature secretory granules (7, 8). As a key regulator of these intracellular protein trafficking complexes, it is likely that knockdown of STX6 causes inappropriate targeting of FAK and integrin $\alpha 5$, which leads to their premature degradation. Alternatively, since STX6-KD-induced suppression of FAK and integrin $\alpha 5$ can be blocked by constitutive p53 knockdown, it is possible that STX6 knockdown activates the p53 family pathway, which in turn inhibits the expression of key cell adhesion molecules, such as FAK and integrin $\alpha 5$. Indeed, both the basal and DNA damage-induced levels of p53 were increased upon STX6 knockdown. There are at least two possibilities of how p53 is stabilized by STX6 knockdown. First, the effect of STX6 on p53 might be mediated via inhibition of FAK expression, since FAK facilitates p53 turnover via enhanced Mdm2-dependent p53 ubiquitination (41). Second, the effect of STX6 on p53 might be mediated via inhibition of integrin expression, since various integrins, including integrin $\alpha 5$, are found to inhibit p53 through a pathway involving MEK1 signaling (42, 43). Nevertheless, how lack of STX6 leads to decreased expression of these key cell adhesion molecules and increased expression of p53 warrants further investigation, which is beyond the scope of this report.

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