Supervillin-mediated Suppression of p53 Protein Enhances Cell Survival*S

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Background: Supervillin is a membrane-associated actin- and myosin II-regulatory protein that promotes cell growth, adhesion, and invasion. Supervillin inversely regulates p53 levels, binds the p53-stabilizing protein USP7, and regulates the USP7-p53 interaction. Supervillin regulation of p53 through USP7 contributes to cell survival. This study describes a new locus for cross-talk between cytoskeletal proteins and p53-regulated survival signaling.

Integrin-based adhesions promote cell survival as well as cell motility and invasion. We show here that the adhesion regulatory protein supervillin increases cell survival by decreasing levels of the tumor suppressor protein p53 and downstream target genes. RNAi-mediated knockdown of a new splice form of supervillin (isoform 4) or both isoforms 1 and 4 increases the amount of p53 and cell death, whereas p53 levels decrease after overexpression of either supervillin isoform. Cellular responses to DNA damage induced by etoposide or doxorubicin include down-regulation of endogenous supervillin coincident with increases in p53. In DNA-damaged supervillin knockdown cells, p53 knockdown or inhibition partially rescues the loss of cell metabolic activity, a measure of cell proliferation. Knockdown of the p53 deubiquitinating enzyme USP7/HAUSP also reverses the supervillin phenotype, blocking the increase in p53 levels seen after supervillin knockdown and accentuating the decrease in p53 levels triggered by supervillin overexpression. Conversely, supervillin overexpression decreases the association of USP7 and p53 and attenuates USP7-mediated p53 deubiquitination. USP7 binds directly to the supervillin N terminus and can deubiquitinate and stabilize supervillin. Supervillin also is stabilized by derivatization with the ubiquitin-like protein SUMO1. These results show that supervillin regulates cell survival through control of p53 levels and suggest that supervillin and its interaction partners at sites of cell-substrate adhesion constitute a locus for cross-talk between survival signaling and cell motility pathways.

Cross-talk between signaling pathways involved in cell motility, invasion, and cell survival is of great interest in the search for novel cancer therapies (1). Cell migration and proliferation are controlled by integrin-based substrate interaction sites called focal adhesions (2, 3). Focal adhesions also facilitate cell survival during stressful conditions, e.g. DNA damage, by decreasing levels of the p53 tumor suppressor protein (4–6). Adhesion is proposed to mediate a feedback loop involving direct binding of p53 protein to the focal adhesion kinase (FAK)* protein and to the FAK promoter (7). In addition, the FAK-related protein Pyk2, which can be expressed at increased levels after FAK knockdown (8), increases cell proliferation by decreasing p53 levels (9).

Integrin signaling also is required for adhesion and matrix invasion by F-actin-enriched structures known as podosomes and invadopodia, or collectively, as invadosomes (10, 11). Downstream signaling involving FAK and Src family tyrosine kinases, which include Lyn, promotes cell proliferation as well as invasion and correlates with poor prognosis in cancer patients (12). Depending on the cellular context (13), Lyn can promote cell survival by down-regulating p53 levels (14). Interestingly, wild-type p53 negatively regulates cell migration and invasion in vascular smooth muscle cells (15), and mutant p53 drives invasion of lung cancer cells by promoting integrin recycling (16). Taken together, these reports suggest cross-regulation of p53 and adhesion-based signaling pathways (17).

In previous studies, we found that the focal adhesion-regulatory, Lyn-associated protein supervillin inversely regulates tight cell-substrate adhesion and is required for normal cell division, cell motility, and matrix degradation (18–24). Supervillin is tightly associated with cholesterol-rich lipid raft membranes and co-immunoprecipitates with Lyn and other signaling proteins (21). As is observed after FAK knockdown (25, 26), supervillin knockdown increases the numbers of large, mature focal adhesions (23). Supervillin also increases podosome turn-

* The abbreviations used are: FAK, focal adhesion kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qRT-PCR, quantitative real-time PCR; SUMO, small ubiquitin-related modifier; SV1, supervillin isoform 1 (accession NM_003174.3); SV4, supervillin isoform 4 (accession JX467682).
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Reagents and Antibodies—Glutathione-Sepharose was from Amersham Biosciences. Etoposide, doxorubicin, mouse anti-FLAG M2 affinity gel, rabbit polyclonal anti-FLAG, rabbit polyclonal anti-archvillin (A1355), anti-supervillin (S8695), rabbit polyclonal antibody, and mouse monoclonal anti-\beta-tubulin (TUB2.1) antibodies were from Sigma-Aldrich. Rabbit anti-USP7 was from Abcam, and rabbit anti-FLAG, anti-GFP, and mouse anti-HA tag antibody were from Cell Signaling Technology. Mouse anti-p53 antibody was from Invitrogen, and mouse anti-actin and rabbit anti-MAP kinase 1/2 were from Millipore. Mouse anti-FAK antibody was from Upstate. Mouse Alexa Fluor 568-labeled secondary antibody was purchased from Invitrogen; horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch. Rabbit polyclonal anti-supervillin (H340) has been described (21, 33).

RT-PCR Cloning of Supervillin Isoform 4—Messages expressed from the SVIL gene in human U2OS cells were obtained by reverse-transcription (RT-PCR) of first-strand cDNA. Human supervillin isoforms 1 (hSV1) and 4 (hSV4) were cloned by PCR from first-strand cDNA (Superscript III First-strand Synthesis SuperMix; Invitrogen) in two steps, using Platinum TaqDNA polymerase (Invitrogen). hSV1 was cloned as described (34). For hSV4, a forward primer in the 5’-UTR (primer 1: 5’-CACGAAAGGAAATCGATGTCAAGC-3’) was balanced with a reverse primer in coding exon 4 (primer 2: 5’-GCTTGGAACTGCCAGTCAAGC-3’), and a forward primer in coding exon 4 (primer 3: 5’-GGAGATGTGTG-CTTCACGAGAAGGCTTCAGC-3’) was paired with a reverse primer in the 3’-UTR (primer 4: 5’-CTGGGCCTGCTC-CCACGCTCAAGAC-3’). The 1.5-kb and 5.3-kb products were cloned into pCR2.1-TOPO vectors and sequenced in both directions. Theoretical pl and molecular weight were determined using the Compute pl/MW program at the Expasy Web site.

Mammalian Expression Vectors—EGFP-tagged hSV1 (34) and hSV4 were ligated into pEGFP-C2 (Clontech) between EcoRI and XbaI in two steps. First, the common 3’-coding sequence digested with EcoRV-XbaI was ligated into pEGFP-C2 vector digested with Smal-XbaI. This vector was then digested with EcoRI and ligated with EcoRI-digested hSV1 and hSV4 5’-coding sequences. 3’-FLAG-tagged hSV1 and hSV4 were generated similarly. The EcoRV/Xhol-digested supervillin 3’ sequence was ligated into p3’-FLAG-CMV-14 vector (Sigma-Aldrich) digested with EcoRV and Sall. The vector was then cut with EcoRI, and the EcoRI-digested hSV1 and SV4 5’ sequences were inserted. We generated EGFP-hSV1 (amino acids 1–675) by cloning the EcoRI-cut N-terminal hSV1 sequence into pEGFP-N2. EGFP-hSV1 (amino acids 675–1270) was ligated into pEGFP-C2 between the EcoRI and Smal sites. EGFP-hSV1 (amino acids 1034–1788) was ligated into pEGFP-C2 between Sall and XbaI. Predicted SUMoylation sites were mutagenized using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Plasmids encoding FLAG-HA-tagged human USP7 (plasmid 22591) (35), GFP-ubiquitin (plasmid 11928) (36), and HA-ubiquitin (plasmid 18712) (37) were purchased from Addgene. Full-length USP7 and USP7(210–1102) were generated by PCR and ligated into p3’-FLAG-Myc-CMV25 between HindIII and BamHI sites. USP7(1–350) was ligated into HindIII-EcoRV sites. The GFP-SUMO1 plasmid was a generous gift from Dr. Yao Xuebiao (38).

Cell Culture and Transfection—Human U2OS, HeLa, and HEK293 (ATCC) cell lines were grown in DMEM, 2 mM l-glutamine, 10% FBS, and penicillin/streptomycin. DNA plasmids were transfected using Lipofectamine 2000, and Stealth dsRNA was transfected with Lipofectamine RNAi MAX, all from Invitrogen. Stealth double-stranded (ds)RNAs were made against sequences found in both supervillin isoforms 1 and 4: (5’-CCCCUGGAAGAUACUGAAGCCGAC-3’ (exon 16) and 5’-GCGAUACUACCUCUACCUUAAUA-3’ (3’-UTR)) and against sequences unique to hSV4: 5’-GAUGGUUCUCAUCAUAAAGUGAG-3’ (exon 4) and 5’-UCCUGCGAGUUAAGGUUGGGU-3’ (exon 5). The Stealth duplex sequence, 5’-GAAACUAUGAGGACCCAGAGAA-3’, was used as a control. Human USP7 was targeted with the dsRNAs 5’-GAAAGCUAAUUGGUGAAAUA-3’ (USP7-1) and 5’-GAUCCUGAGUUGGGUGAUAUU-3’ (USP7-2). Stably expressing EGFP-hSV1 and EGFP-hSV4 U2OS cell lines were selected by G418, and multiple single clones were sorted by FACS and cloned in 96-well plates.

Immunofluorescence—U2OS cells stably expressing EGFP-tagged hSV1 or hSV4 were fixed with paraformaldehyde, permeabilized, and stained, as described previously (23, 28), using Alexa Fluor 568-phallolidin (Invitrogen) or with mouse anti-FAK antibody (diluted 1:500) and Alexa Fluor 568-labeled anti-mouse secondary antibody. Confocal images were acquired with a 100x Plan Apo objective lens (NA 1.4) on a Nikon TE-2000E2-inverted microscope (Nikon Instruments) with a Yokogawa CSU10 Spinning Disk Confocal Scan Head (Solamere Technology Group), a Rolera-MGI Plus camera (QImaging),
and MetaMorph 7.6 software. Images were adjusted uniformly for contrast and brightness and merged using Adobe Photoshop software.

**Live Cell Imaging**—U2OS cells on 25-mm² coverslips were transfected with dsRNA, incubated at 37 °C with 5% CO₂ for 48 h, and sealed into live-cell imaging chambers. Time-lapse images were acquired every 3 min for 36 h using a 10× objective lens (NA 0.30) on a DMIRE 2 inverted microscope with a mechanical stage (Leica Microsystems), a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics) and Simple PCI 6 software (Compix Inc., Sewickley, PA). Images were exported as AVI videos at 15 frames/s; selected stills were sized and contrast-enhanced in Adobe Photoshop.

**Flow Cytometry**—U2OS cells were trypsinized and resuspended with phosphate-buffered saline before overnight fixation at 4 °C in 95% ethanol and staining with propidium iodide. DNA content was determined using FACS Calibur (BD Biosciences) and FlowJo software (Tree Star).

**MTT Cell Proliferation and Survival Assay**—U2OS cells transfected with dsRNA were incubated at 37 °C with 5% CO₂ in 96-well flat-bottom microplates in a final volume of 100 µl of DMEM/well. Cells were treated with etoposide or doxorubicin and assayed for tetrazolium reductase activity, which is proportional to cell proliferation and viability (39), according to the manufacturer’s instructions (ATCC, kit 30-1010K). Cellular metabolic activity was determined as a percentage relative to untreated control cells.

**Quantitative Real-time PCR (qRT-PCR)**—Total RNA was extracted from U2OS or HeLa cells using the RNeasy Mini Kit reagent (Qiagen). For qRT-PCR, we used the Power SYBR Green RNA-to-Ct-1-Step Kit (Applied Biosystems) in an Applied Biosystems StepOnePlus Real-time PCR System (Applied Biosystems). Primers were: p21_Foreward, 5′-TCAC-TGTCTTTGACTCCCTGTGGCCT-3′ and p21_Reverse, 5′-AGGCTCTTCTTGGAGAAGATCAGTA-3′; BAX_Foreward, 5′-TTTCTTGAGGCAACTTCAACTGGG-3′ and BAX_Reverse, 5′-TGTCAGCCACCCATTAGGTTCTGAT-3′; NAPDH_Foreward, 5′-TGCTGCTCCTCCTCATGTTCATCT-3′ and NAPDH_Reverse, 5′-TCCAGAAAGTTGGGCCCATGAAAGA-3′; p53_Foreward, 5′-AGATGAAGCTCCCGCAGATGC-3′ and p53_Reverse, 5′-TCACAGACTTGCTGGCTCCGAGA-3′; MD2_Foreward, 5′-AGATTGTTGGGCCCTT CGTGAGAAT-3′ and MD2_Reverse, 5′-AGCCCTCCT CACGCTTGTGTTGAT-3′. Relative mRNA levels were normalized first to the levels of control NAPDH mRNA and then to the level of the mRNA in the corresponding control sample.

**Recombinant Proteins and Pulldown Assays**—Expression vectors encoding GST-USP7 (1–350) and GST-hSV1 (1–400) were generated by ligation into EcoRI/Smal sites of pGEX-5P-1 vector (Amersham Biosciences). GST fusion proteins were produced in *Escherichia coli* BL21 bacteria, affinity purified on glutathione-Sepharose, dialyzed into 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% β-mercaptoethanol, and 0.1 mM EGTA with protease inhibitors, and stored frozen at −80 °C.

In pulldown experiments, GFP- or FLAG-tagged mammalian expression plasmids expressed for 24 h in HEK293 cells were extracted with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 5 mM NaF, 1 mM PMSF and a protease inhibitor mixture (Sigma-Aldrich). Cell lysates were clarified for 20 min at 13,800 × g at 4 °C, and supernatants were incubated at 4 °C for 2 h with Sepharose-bound GST-USP7 (1–350), GST-hSV1 (1–400), or GST alone. Beads were washed five times with extraction buffer, and bound proteins were released at 95 °C for 10 min with Laemmli sample buffer (40). Beads were frozen and stored at −80 °C or directly loaded onto SDS-PAGE for immunoblotting.

**SUMOylation Assay**—HEK293T cells were co-transfected with FLAG-tagged supervillin and GFP-tagged SUMO1 plasmids. Twenty-four hours later, cells were lysed in denaturing lysis buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 0.2% SDS, 1% Triton X-100, 1 mM EGTA, 1 mM NaF, 1 mM Na₃VO₄, 10% glycerol) with 20 mM N-ethylmaleimide, 1 mM PMSF, and protease inhibitors. Lysates were clarified (16,000 × g, 10 min, 4 °C) and incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 2 h at 4 °C. Immunoprecipitates were washed three times with lysis buffer and eluted in Laemmli sample buffer.

**Ubiquitination Assay**—HEK293T cells were co-transfected with FLAG-tagged supervillin and HA-tagged ubiquitin plasmids for 24 h and lysed in denaturing lysis buffer containing 10 mM MG132, 1 mM PMSF, and protease inhibitors. Competition assays were performed similarly with FLAG-p53 and GFP-tagged USP7 and SV4.FLAG-tagged proteins were recovered, as described above.

**Immunoblotting**—Total cell proteins were extracted with 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 5 mM sodium fluoride, 1 mM PMSF, and protease inhibitor mixture (Sigma-Aldrich), separated by SDS-PAGE and electrotransferred to nitrocellulose blots. Blots were probed for 2 h at 20–22 °C or overnight at 4 °C with primary antibodies and dilutions as follows: anti-p53, 1:500; anti-FLAG, 1:2000; anti-GFP, 1:2000; anti-HA, 1:1000; anti-supervillin (H340), 1:1000; anti-supervillin (A1355), 1:500; anti-supervillin (S8695), 1:1000, anti-myosin heavy chain IIβ, 1:10,000; anti-ERK1/2, 1:2000; anti-actin, 1:2000; and anti-USP7, 1:500. HRP-conjugated secondary antibodies and dilutions were: anti-rabbit IgG, 1:10,000 and anti-mouse IgG, 1:10,000. Band densities were quantified using ImageJ (67).

**RESULTS**

**A New Supervillin Isoform Regulates p53 Levels and Cell Survival**—Many noncellular line contain a previously undocumented supervillin splice form, isoform 4 (SV4, Fig. 1). In addition to the nonmuscle supervillin isoform 1 (SV1) with a molecular mass of ~201 kDa (Fig. 1A) (accession NM_003174.3) (34), a larger ~245-kDa polypeptide is detected by the well characterized affinity-purified anti-H340 antibody directed against the first 340 residues of human supervillin (H340) (Fig. 1B) (21, 33) and by two commercial anti-supervillin antibodies (Fig. 1B and supplemental Fig. S1, A and B). Each of six supervillin-specific dsRNAs reduces levels of the ~245-kDa polypeptide (supplemental Fig. S1, A and B), which is present in HeLa cells (Fig. 1B, lane 1) and prominent in A549 lung carcinoma, U2OS osteosarcoma, and retinal pigment epithelial RPE-1 cells (Fig. 1B, lanes 4–6). The low levels of this polypeptide in other cell lines (Fig. 1B, lanes 3, 7, and 8) suggest cell type
Many cell lines contain two supervillin isoforms. FIGURE 1. Supervillin isoform 1 (SV1) is predicted to be 2183 residues with a molecular weight of 244,639 and a pI of 6.5 (accession JX467682). This combination of exons distinguishes SV4 from striated muscle archvillin (isoform 2), which contains all four differentially spliced exons (accession NM_021738.2) (33), and from smooth muscle archvillin (SmAV, isoform 3), which contains coding exon 4 but not coding exons 3, 5, or 9 (accession AY380816.1) (30). As described previously for bovine SV1 (19, 23, 24, 41), stably expressed human SV1 localizes with actin filaments and dot-like podosomes at the basal plasma membrane (Fig. 1C, a–a’). Stably expressed full-length SV4 localizes similarly (Fig. 1C, b–b’ and d–d’).

RNAi-mediated reduction in the level of SV4 leads to >3-fold increases in the level of the cell guardian protein p53 and to increased cell death (Fig. 2, supplemental Fig. S2, and supplemental Movies S1–S3). Stealth dsRNA targeting coding exon 4, 5, or 16 reduced the amount of SV4 to ~10% of endogenous levels within 48 h (Fig. 2A, lanes 2 and 3 versus lane 1) and to ~5% of control levels after 72 h (see below). Only the dsRNA targeting coding exon 16 effectively reduces the level of SV1 within 48 h (Fig. 2A, lane 4) although all three dsRNAs reduce both isoforms to ~5% of endogenous levels by 72 h (see below). All three supervillin-specific dsRNAs cause a ~3-fold increase in the level of p53 protein relative to actin and ERK1/2 loading controls within 48 h (Fig. 2, A and B). Consistent with supervillin knockdown experiments in HeLa cells (22), dsRNA targeting either coding exon 5 or coding exon 16 decreases the percentage of U2OS cells that undergo apparently normal cell division 24–60 h later (Fig. 2, C and D, and supplemental Movies S1–S3). However, rather than failing primarily during early cytokinesis, as is seen in HeLa cells (22), supervillin knockdown in U2OS cells predominantly increases cell death (Fig. 2, C and D, and supplemental Movies S2 and S3). The supervillin knockdown U2OS cells mostly die after rounding without attempting cleavage or after a sudden, apparently apoptotic, fragmentation of cytoplasm and nuclei. The differences between cells treated with control and supervillin-specific dsRNAs are even more pronounced after 36 h of filming in supplemental Movies S1–S3, suggesting an increased effect as residual supervillin dwindles. As predicted by the numbers of phase-bright dead cells at the end of filming (Fig. 2C), cytometric analyses show increased amounts of fragmented, sub-G1 DNA.
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FIGURE 2. Knockdown of SV4 alone or with SV1 promotes increased levels of p53 and cell death. A, immunoblots of U2OS cells showing down-regulation of SV4 after 48 h of treatment with RNAi against coding exon 4 or 5 and knockdown of both SV1 and SV4 after similar targeting of exon 16. Actin and ERK1/2 served as loading controls. B, quantification of p53 protein levels after RNAi-mediated reduction in levels of SV4 alone (exons 4 and 5) or both isoforms (exon 16). n = 3; **, p < 0.001. C, stills from time-lapse videos showing transient rounding leading to normal cytokinesis for most U2OS cells treated with control dsRNA (supplemental Movie S1) and division failure and/or death of most rounding cells treated with dsRNA against either coding exon 5 (supplemental Movie S2) or coding exon 16 (supplemental Movie S3). Scale bar, 200 μm. D, quantification of the fates of rounding cells from videos, such as those shown in C. n = 428 (control); n = 245 (exon 5); n = 269 (exon 16). Cells treated with supervillin-specific dsRNAs were more likely to die after rounding than were cells treated with control dsRNAs. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars, S.D. E, DNA content histograms showing increased amounts of fragmented, sub-G1 DNA (arrows) in U2OS cells treated with dsRNA targeting supervillin coding exon 5 or 16 versus cells treated with control dsRNA.
DNA in cells deficient in SV4 only (Fig. 2E, Exon 5) or in both SV1 and SV4 (Fig. 2E, Exon 16). Taken together, these experiments show that p53 protein levels and the amount of cell death both increase in the absence of supervillin and suggest enhancement of p53-dependent cell cycle checkpoints (42).

Conversely, supervillin overexpression leads to reductions in the level of p53 protein (Fig. 3) and message (supplemental Fig. S2), which is up-regulated by p53 protein (43). Transient overexpression of FLAG-tagged SV4 or SV1 in the readily transfectable HeLa CLL-2 cells (Fig. 3A) decreases p53 protein levels ∼3-fold and ∼2-fold, respectively (Fig. 3B). Message levels of the downstream p53 target proteins MDM2, p21, and BAX also inversely with p53 protein levels and p53 function.

Supervillin-p53 Cross-talk during DNA Damage—Supervillin levels decrease as p53 protein levels increase following DNA damage by the topoisomerase II inhibitors etoposide (Fig. 4A) and doxorubicin (Fig. 4B). These reagents cause DNA damage that triggers p53-mediated cell cycle arrest (44). Down-regulation of SV4 alone by a dsRNA that targets coding exon 5 or the down-regulation of both SV4 and SV1 with dsRNA against coding exon 16 or 18 exacerbates cell death following DNA damage (Fig. 4, C and D), without itself causing increased nuclear staining for the DNA damage marker γH2AX (data not shown). This increase in cell death is partially reversed by simultaneous RNAi-mediated depletion of p53 (Fig. 4, E and F) or by inhibition of p53 mitochondrial or transcriptional functions with pifithrin-μ (PFTμ) or pifithrin-α (PFTα), respectively (Fig. 4, G and H) (45, 46). These results suggest that part of the effect of supervillin on cell survival is through control of p53 protein levels. However, cell metabolic activity after double knockdown of p53 and supervillin is lower than after knockdown of p53 alone (Fig. 4, E and F), consistent with p53-independent effects of supervillin.

Supervillin Interacts with USP7—A potential regulatory mechanism is suggested by the physical and functional interaction of the supervillin N terminus with the p53 deubiquitinating enzyme USP7/HAUSP (32) (Fig. 5 and supplemental Figs. S3 and S4). Thirteen potential USP7 binding sites are predicted for SV1, and 20 USP7 binding sites are predicted for SV4 by the ELM resource, using a cut-off value of 0.013 (47) (Fig. 5A, top). GFP-tagged SV1 residues 1–675 (Fig. 5A, bottom, lane 1) and SV4 residues 1–1250 (supplemental Fig. S3, lane 3) each bind strongly to the GST-tagged USP7 N terminus (amino acids 1–350). By contrast, internal and C-terminal sequences common to both isoforms (GFP-SV1 residues 675–1270, GFP-SV1 residues 1034–1788) exhibit no or weak binding, respectively (Fig. 5A, bottom, lanes 2 and 3). GST-tagged SV1 residues 1–400, but not GST alone, pull down FLAG-tagged full-length USP7 (Fig. 5B, lane 3). Furthermore, GFP-tagged full-length SV1 co-precipitates with FLAG-tagged full-length USP7 and with USP7 amino acids 1–350 (Fig. 5C, lanes 1 and 3), but not with the FLAG-tagged USP7 C terminus or FLAG alone (Fig. 5C, lanes 2 and 4). Knockdown of USP7 reduces p53 protein levels by increasing the extent of ubiquitin-mediated p53 degradation (48, 49). Overexpression of SV4 may accentuate the already strong decrease in p53 levels caused by USP7 reduction (supplemental Fig. S4, lanes 4 and 5 versus lanes 7 and 8). Conversely, USP7 knockdown blocks the increase in p53 levels caused by reduction of SV1 and SV4 (Fig. 5, D and E). Taken together, these experiments suggest that interactions between the N termini of USP7 and supervillin may coordinately regulate p53 levels, e.g. through supervillin-mediated sequestration of USP7 and subsequent increases in the ubiquitination and degradation of p53.

Supervillin Inhibits the USP7-p53 Interaction—Overexpression of SV4 decreases co-sedimentation of USP7 with p53 and increases p53 ubiquitination (Fig. 6). GFP-USP7 specifically coprecipitates with FLAG-p53 and anti-FLAG antibody from nonadenating cell lysates (Fig. 6A, lane 2 versus lane 1), an interaction that is reduced ∼3-fold (36% ± 4%, n = 2) by the presence of co-transfected GFP-SV4 (Fig. 6A, lane 3). The reduced USP7-p53 binding is reflected by an increase in p53 degradation, with GFP-SV4 counteracting the ability of GFP-USP7 to protect p53 from covalent modification by HA-tagged ubiquitin (Fig. 6B). GFP-USP7 levels are relatively unaffected by GFP-SV4 levels (Fig. 6, A and B, WCE). These results suggest a direct competition between supervillin and p53 for binding to USP7 although indirect mechanisms cannot be excluded.

Supervillin Itself Can Be Regulated by Ubiquitination—The stability of the supervillin protein itself can be regulated by USP7 and by covalent modification with ubiquitin and SUMO1 (Fig. 7), a small ubiquitin-related modifier peptide that functionally cross-talks with ubiquitin (50). The UbPred program (51) predicts 19 high confidence ubiquitination sites in SV1 and 34 high confidence sites in SV4, nearly all in the N terminus. In agreement with this prediction, supervillin levels increase in cells that overexpress GFP-USP7 (Fig. 7A). Immunoprecipitated FLAG-tagged SV1 (Fig. 7B) and SV4 (Fig. 7C) are both modified by HA-tagged ubiquitin (Fig. 7, B and C, lanes 3). The amount of HA-ubiquitin covalently bound to immunoprecipitated SV1 (Fig. 7B) and, especially, to SV4 (Fig. 7C) is reduced in the presence of GFP-USP7 (Fig. 7, B and C, lanes 4), consistent with USP7-mediated deubiquitination. As predicted by the SUMOplot™ Analysis Program, FLAG-tagged SV1 also is covalently modified by GFP-tagged SUMO1 (Fig. 7D, lane 2, SUMO-SV1). Most SUMO1 binding is eliminated by mutagen-
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DISCUSSION

We show here that the lipid raft-associated protein supervillin promotes cell survival by suppressing levels of p53. The knockdown of the newly described supervillin isoform 4 by itself or with supervillin isoform 1 increases cell death and p53 protein (Fig. 7G). Taken together, these results suggest that supervillin levels are regulated by ubiquitination, USP7-mediated deubiquitination, and SUMOylation.

FIGURE 4. DNA damage decreases the amount of endogenous supervillin, with supervillin reduction accentuating p53-dependent loss of cell viability. A and B, immunoblots showing the amounts of SV4, SV1, p53, and actin loading control in U2OS cell lysates after induction of DNA damage with increasing amounts of etoposide (A) or doxorubicin (B). Supervillin levels were visualized with H340 antibody (A) or A1355 antibody (B) (supplemental Fig. S1B), which better visualizes SV4. Amounts of SV4, SV1, and p53 were normalized relative to actin and shown as percentages of their 0-h control values. C and D, absorbance at 590 nm in the MTT assay for cell viability after a 48-h treatment with control dsRNA or dsRNA specific for SV4 (exon 5) or for both supervillin isoforms (exons 16 and 18) followed by 24 h of treatment with 50 μM etoposide (C) or 2 μM doxorubicin (D). E and F, cell viability after a 48-h treatment with control dsRNA or dsRNA specific for SV4 (exon 5) or for both isoforms (exon 16), with (+) or without (−) simultaneous knockdown of p53 followed by 24 h of treatment with 50 μM etoposide (E) or 2 μM doxorubicin (F). G and H, cell viability after a 48-h knockdown with control dsRNA or dsRNA specific for both supervillin isoforms (exon 16) followed by a 24-h treatment with either vector alone, 10 μM pifithrin-α (PFT-α), or 2 μM pifithrin-α (PFT-α) and either 50 μM etoposide (G) or 2 μM doxorubicin (H). Statistical significance was assessed by ANOVA (C, D, G, and H) or Student’s two-tailed t test (E and F). n = 3; *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars, S.E.
protein levels. Conversely, overexpression of SV1 or SV4 decreases the level of p53. In DNA-damaged cells, endogenous supervillin proteins decrease as p53 levels rise; further reduction of supervillin by RNAi accentuates p53-dependent effects on metabolic activity. RNAi-mediated knockdown of p53 or addition of a p53 inhibitor significantly reverses the supervillin-knockdown effect, strongly suggesting that increased cellular p53 is an important intermediate in the control of cell proliferation by supervillin.

One mechanism of action is the association of supervillin with the p53 deubiquitinating enzyme USP7 (Fig. 8). USP7 binds to the supervillin N terminus and is required for increased levels of p53 protein after supervillin knockdown. A, upper, predicted USP7 binding sites (bars) within SV4-specific exons 3–5 and in EGFP-tagged full-length SV1 (GFP-SV1(1–1788)) and SV1 deletion mutants. A, lower, anti-GFP immunoblots of GFP-SV1 constructs bound (Bound) to the GST-tagged USP7 N terminus (amino acids 1–350; lanes 1–3) or to GST alone (lane 4) and input proteins in whole cell lysates (WCL). Protein Stain, Coomassie Blue staining of GST-tagged proteins from the lanes containing bound and eluted fraction. MW, molecular mass markers (lane 5); sizes on the right in kDa. B, immunoblots stained for FLAG-tagged full-length USP7 in whole cell lysates (lane 1) and in fractions bound to GST only (lane 2) or to the GST-tagged SV1 N terminus, residues 1–400 (lane 3). Protein Stain, Coomassie Blue. C, upper, diagram of full-length USP7(1–1102) and USP7 deletion mutants. C, lower, immunoblots showing co-immunoprecipitation of GFP-full-length SV1 with anti-FLAG antibody and FLAG-tagged full-length USP7 (lane 1), USP7 residues 210–1102 (lane 2), USP7 residues 1–350 (lane 3), or FLAG without USP7 sequences (lane 4). D, immunoblots of whole cell lysates stained for endogenous SV1 and SV4, USP7, p53, and actin after 48 h of treatment with the indicated dsRNAs. E, quantification of relative p53 levels in lysates treated with the indicated dsRNAs. *, p = 0.0349; n = 3.
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ently due to apoptosis, is the predominant phenotype after 48 h of supervillin knockdown in U2OS cells (Fig. 2). The difference between these cell lines likely arises from the presence in HeLa cells, but not in U2OS cells, of human papillomavirus oncoproteins, which target p53 for degradation by a pathway that is unaffected by USP7 (48, 52). The supervillin contributions to other survival signaling pathways (28, 31) may contribute to the incomplete rescue by p53 knockdown of the supervillin knockdown effect in U2OS cells.

The intracellular location(s) for the supervillin/USP7/p53 functional interactions remain an open question. At physiological levels, supervillin associates primarily with membrane-bound cortical myosin II and F-actin and overlaps with focal adhesion proteins, including the p53-binding protein FAK (18, 19, 21, 24, 27, 41), whereas most USP7 and p53 are concentrated within the nucleus (53, 54). On the other hand, USP7 and p53 can be cytoplasmic (55, 56), and the central region of supervillin contains a strong nuclear localization signal that can target supervillin fragments to the nucleus (24). This central supervillin region also promotes androgen receptor signaling at an undetermined intracellular location (57). We have been unable to document localization of USP7 at focal adhesions or physiological levels of supervillin in the nucleus. However, we cannot exclude the possibility that a minor fraction of supervillin or a proteolytic fragment translocates to the nucleus, where it could interact with nuclear populations of USP7, FAK, and/or Lyn (13, 53, 58). Alternatively, supervillin may affect the deubiquitination and/or nuclear translocation of cytoplasmic p53, either alone or in complexes with other proteins, such as FAK or Lyn (13, 14, 58).

That said, the results reported here are consistent with the associations and functions of supervillin at cell-substrate adhesion sites. Like FAK and Src family kinases (25, 59, 60), super-

![FIGURE 6. Supervillin isoform 4 decreases co-sedimentation of USP7 with p53 and increases p53 ubiquitination in the presence of USP7. A, immunoblots of anti-FLAG immunoprecipitates (IP:FLAG) and whole cell extracts (WCE) from HEK293 cells expressing GFP-USP7 and the FLAG vector (lane 1) or FLAG-tagged p53 (lanes 2 and 3), without (lane 2) or with (lanes 1 and 3) EGFP-SV4. The anti-FLAG IgG heavy chain (IgG HC) served as a loading control. B, immunoblots of anti-FLAG immunoprecipitates and WCE from HEK293 cells expressing FLAG-p53 with HA-ubiquitin (Ub), GFP-USP7, and GFP-SV4, as indicated.](image)

![FIGURE 7. Supervillin is ubiquitinated under regulation by USP7 and SUMOylation. A, immunoblots of whole cell extracts (WCE) from HeLa cells expressing GFP alone (lane 1) or GFP-USP7 (lane 2). B and C, immunoblots of anti-FLAG immunoprecipitates (IP:FLAG) and WCE from cells expressing FLAG-tagged SV1 (B) or SV4 (C) and GFP alone (lanes 1 and 3) or GFP-USP7 (lanes 2 and 4), without (lanes 1 and 2) and with (lanes 3 and 4) HA-ubiquitin (HA-Ub). The positions of presumably monoubiquitinated SV1 and SV4 are indicated (Ub-SV1 and Ub-SV4). Loading controls were antibody against β-actin (A) or anti-FLAG staining (B and C). D, immunoblots of anti-FLAG immunoprecipitates and WCE from HeLa cells that co-express FLAG-tagged SV1 or FLAG-SV1 with the indicated point mutations in one or both of two predicted SUMOylation sites (K968R, K1479R) and either GFP alone (lane 1) or GFP-SUMO1 (lanes 2–5). Blots were stained for Flag or GFP, as shown. E, immunoblots of WCE from cells that co-express FLAG-SV1 (lanes 1 and 2) or FLAG-SV4 (lanes 3 and 4) and either GFP (lanes 1 and 3) or GFP-SUMO1 (lanes 2 and 4) after staining with antibodies against FLAG, USP7, actin, and GFP. F, immunoblots of IP or WCE from HeLa cells co-expressing either empty vector (lane 1) or HA-tagged ubiquitin (HA-Ub, lanes 2–5) and FLAG-SV4 without (lanes 1 and 2) or with mutations in SUMOylation sites that correspond to the SV1 mutations in D (lanes 3–5). G, immunoblots of WCE from HeLa cells expressing either empty vector (lane 1) or wild-type (lane 2) or SUMOylation-deficient (lanes 3–5) FLAG-SV4 mutants stained for supervillin (H340 antibody), p53 and actin. Molecular mass markers, in kDa, are on the left.](image)
Supervillin suppresses p53

We propose that the inverse relationship between levels of supervillin and p53 is caused by direct or indirect inhibition by supervillin of the ability of USP7 to deubiquitinate ubiquitinated p53 (Ub-p53). Decreases in USP7 enzymatic activity or accessibility to Ub-p53 will increase p53 degradation and cell survival, especially after stressors like DNA damage. Supervillin SUMOylation and deubiquitination by bound USP7 could accentuate this effect by stabilizing supervillin itself against ubiquitin-mediated degradation.

Supervillin increases cell motility and promotes the turnover of cell-substrate adhesions (18, 23, 28). Although other processes also occur (19, 60–62), adhesion turnover mediated by FAK, Src, and supervillin involves activation of myosin II contractility by myosin light chain kinase (18, 23, 26). We now show that supervillin also regulates p53 levels, as do FAK, PYK2, and Src family kinases. Taken together, these observations raise questions about commonality in signaling mechanisms that link decreases in p53 levels with increased rates of adhesion turnover and cell motility.

Signaling cross-talk between cell proliferation pathways and sites of cell-substrate adhesion goes in both directions. Overexpression of wild-type p53 or activation of p53 with doxorubicin can down-regulate expression of the α5 integrin subunit and inhibit Src-induced podosome formation, cell motility, and cell invasion (15, 63). Conversely, mutated p53 proteins can promote cell invasion and prolong survival signaling by increased recycling of integrins and growth factor receptors (16). In addition, the stability of many focal adhesion proteins, including FAK and Src, is regulated by ubiquitination (64), and many deubiquitinas, although not USP7, are required for epithelial cell scattering (65). A role for supervillin in the regulation of cell survival, as well as adhesion and matrix degradation (18–20, 23), sheds new light on the observation that missense somatic mutations in supervillin occur in ~22% of sequenced tumors (26/120 unique samples) (66). We thus suggest that the machinery involved in adhesion protein turnover may be integrally involved in the poorly understood survival signaling pathways promoted by integrin-based cell-substrate adhesion.

In summary, we report the cloning of a new supervillin isoform and show for the first time a role for supervillin in regulation of cell survival through control of p53 levels. This study provides new insights into cross-talk between the cytoskeletal motile machinery and cell survival mechanisms.

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