A Protective Role for the Human SMG-1 Kinase against Tumor Necrosis Factor-α-induced Apoptosis

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The human suppressor of morphogenesis in genitalia-1 (hSMG-1) protein kinase plays dual roles in mRNA surveillance and genotoxic stress response pathways in human cells. Here, we report that small interfering RNA-mediated depletion of hSMG-1, but not ATM, TRRAP, hUpf1, or hUpf2, in human U2OS osteosarcoma cells markedly increases the magnitude and accelerates the rate of apoptosis induced by tumor necrosis factor-α (TNFα) stimulation. The increase in TNFα-mediated cell killing observed in hSMG-1-depleted cells is not related to the suppression of nonsense-mediated mRNA decay or to the inhibition of TNFα-induced NF-κB activation. Rather, we observed that loss of hSMG-1 accelerates the degradation of the long form of the FLICE-inhibitory protein (FLIPL), an inhibitor of death-inducing signaling complex-mediated caspase-8 activation, in TNFα-treated cells. These results suggest that hSMG-1 plays an important role in cell survival during TNFα-induced stress.

Members of the phosphoinositide 3-kinase-related kinase (PIKK) family play central roles in cell growth and stress response pathways (1). Mammalian cells express six PIKK family members (mammalian target of rapamycin, ATM, ATR, DNA-dependent protein kinase catalytic subunit, SMG-1, and transformation/transcription domain-associated protein (TRRAP)). The most recently identified member of the PIKK family is human SMG1 (hSMG-1) (2–5). Biochemical studies indicated that immunopurified hSMG-1 preferentially modifies protein substrates containing a Gln residue at the +1 position relative to the Ser or Thr phosphoacceptor residue (termed the “S/T-Q motif”) (2, 4). In this respect, hSMG-1 most closely resembles the genotoxic stress-responsive kinases, ATM, ATR, and DNA-dependent protein kinase. Indeed, hSMG-1 is also activated by DNA damage, and, like ATM and ATR, phosphorylates p53 at Ser-15 during genotoxic stress (4). Nonetheless, the evolutionarily conserved, unique function of hSMG-1 relates to transcriptome rather than genome surveillance.

Nonsense-mediated mRNA decay (NMD) is a conserved mRNA surveillance mechanism that mediates the rapid degradation of mRNA transcripts bearing premature termination codons (PTCs) in their coding sequences (6, 7). NMD protects cells from potential toxicity arising from the accumulation of “damaged,” PTC-bearing mRNAs, which could encode truncated versions of normal cellular proteins. However, NMD is not simply a damage response mechanism, because this process also plays an important role in shaping the transcriptome during normal cell growth and differentiation (8). The molecular components of the NMD pathway were first identified in genetic studies in the worm, Caenorhabditis elegans. Seven genes, designated SMG-1 through SMG-7, are essential for the execution of NMD in C. elegans (9). The NMD machinery relies on the locations of exon-intron boundaries, which are marked by multiprotein exon junction complexes, to determine the location of translation termination codons in fully processed mRNAs (7, 10, 11). The presence of an exon junction complex >50–55 nucleotides downstream of a termination codon renders the mRNA susceptible to NMD. A key step in the initiation of NMD is the binding of a surveillance complex to ribosomes that have encountered translation termination codons (11). The surveillance complex comprises SMG-1, SMG-2 (known as hUpf1 in humans), and the eukaryotic release factors (eRFs)-1 and -3. SMG-1 then phosphorylates SMG-2/hUpf1...
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at multiple S/T-Q sites, leading to dissociation of eRFs and triggering of NMD (6, 12).

Previous studies demonstrated that hSMG-1-depleted human cells display an increased level of spontaneous DNA damage and sensitivity to genotoxic stress (4). Interestingly, hSMG-1 is found in both the cytoplasmic and nuclear compartments (4, 13), unlike the related genotoxic stress-responsive kinases, ATM and ATR, which are confined to the nucleus in most cells (14). The presence of hSMG-1 in the cytoplasm suggested that this PIKK might play additional roles in stress signaling induced by cytokines and other extranuclear stimuli. To address this hypothesis, we examined the sensitivity of hSMG-1-depleted cells to a broadened panel of agents with potential cell-death-inducing activities. These studies unexpectedly revealed that loss of hSMG-1 function dramatically increased the rate and extent of apoptotic cell death induced by tumor necrosis factor-α (TNFα). This cytokine engages the extrinsic pathway of apoptotic death by binding to the type I TNF receptor, which triggers activation of caspases 8 and 10. These initiator caspases, in turn, ignite a proteolytic cascade that activates a set of executioner caspasess (e.g. caspases 3 and 7), which ultimately commit the host cell to apoptotic death (15, 16). Under normal conditions, most cell types are resistant to TNF-α-induced apoptosis due to the rapid activation of the cell survival-promoting NF-κB pathway by a plasma membrane-localized type I TNF receptor signaling complex (termed complex I) (15). Subsequent internalization of this receptor complex leads to the assembly of a distinct, cytoplasmic complex (complex II), which serves as the proximate activator of caspase-8 and -10. In this setting, a key transcriptional target of NF-κB is the gene encoding the FLICE inhibitory protein-long form (FLIP L), which competitively suppresses activation of caspases-8 and -10 by downstream TNF receptor complex II, thereby suppressing the apoptotic cascade.

In the present study, we demonstrate that hSMG-1 protects human cells from TNF-α-induced apoptosis through a mechanism unrelated to the role of hSMG-1 in NMD. In hSMG-1-depleted cells, stimulation with TNFα accelerated the degradation of the anti-apoptotic protein, FLIP L. Hence, loss of hSMG-1 function shifts the balance of anti-apoptotic and pro-apoptotic signals emanating from the TNF receptor toward caspase-8/10 activation and cell death. These findings indicate that the hSMG-1 kinase regulates death receptor signaling in human cells, in addition to its evolutionarily conserved role in the elimination of PTC-containing mRNAs.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—U2OS osteosarcoma cells were cultured in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (fetal bovine serum). The following reagents were used in these studies (sources in parentheses): human recombinant TNFα (R&D Systems); FLAG-tagged TNFα (Apootech); and, wortmannin and cycloheximide (CHX) (Sigma).

Cell Viability, Cleaved Caspase-3, and DNA Fragmentation Assays—Cell viability was monitored by trypan blue dye exclusion. Briefly, U2OS cells were transferred into fresh culture medium immediately prior to exposure to TNFα. Non-adherent cells were collected from the culture medium, and the remaining adherent cells were detached by trypsinization. The total (non-adherent plus adherent) cell population in each sample was washed with phosphate-buffered saline (PBS), and then incubated in 0.1% trypsan blue (Sigma). The percentage of trypan blue-positive cells was determined by microscopic examination with a hemocytometer.

The Cleaved Caspase-3 Assay (Meso Scale Systems) was performed according to the manufacturer’s protocol. This assay utilizes an MSD plate coated with α-cleaved-caspase-3 and SULFOTAG-labeled α-caspase-3 antibodies. For detection of cleaved caspase-3, 20 μg of cellular extract was used per well and subjected to electric current. The light emission of the SULFOTAG-labeled antibody was captured by a charge-coupled device camera and quantified using a SECTOR SI6000 instrument.

The Cell Death Detection ELISA plus kit (Roche Applied Science) was used to detect DNA fragmentation, an indicator of apoptosis. This assay captures and detects nucleosomes with α-histone and peroxidase-labeled α-DNA antibodies, respectively. The cell extracts (15,000 cells per sample) were mixed with the 2,2’-azinobis-(3-benzthiazolone-6-sulfonic acid) substrate, and the plate was incubated for 10 min with gentle shaking at room temperature. The absorbance was then read on a ThermoMAX plate reader (Molecular Devices), and the data were plotted as the absorbance difference between 405 nm and the reference wavelength, 490 nm.

Antibodies—hSMG-1-specific antibodies were raised by immunizing rabbits (Cocalico Biologicals, Inc.) with bacterially expressed and purified glutathione S-transferase fusion proteins containing hSMG-1 amino acids 2281–2339 (Ab-1) or amino acids 1691–1790 (Ab-2). The α-hSMG-1 (Ab-2) anti-serum was affinity-purified over Affi-Gel 15 (Bio-Rad) coupled to the glutathione S-transferase-hSMG-1 1691–1790 fusion protein. The PLC-γ1-specific antibodies were as described in a previous study (17). Additional antibodies were obtained from Covance Research Products (α-HA clone 12CA5), Sigma (α-FLAG-M2, α-β-Actin, and α-Bax clone 6A7), Santa Cruz Biotechnology (α-Bax clone N20, α-Bcl-2 clone N-19, α-IκBα clone C-21, α-caspase-8 clone C-20, α-TRAF-2 clone C20, and α-type I TNF receptor clone H5), BD Transduction Laboratories (α-TRADD and α-FADD), Cell Signaling Technology (α-phospho-p44/42 MAPK and α-p44/42 MAPK), BD Pharmingen (α-Topo IIß), Apootech (α-FLIP clone Dave II), and Alexis Biochemicals (α-FLIP clone NF6 and α-caspase-8 clone 12F5).

Cell Transfections—For NMD assays, U2OS cells were seeded into 100-mm dishes (1 × 10⁶ cells per dish). After 20 h, cells were transfected with 1.5 μg of human β-globin-luciferase test plasmid, either Luc-BGG-WT or Luc-BGG-39PTC, and 1.5 μg of TK- Renilla reference plasmid DNA using the FuGENE 6 transfection reagent (Roche Applied Science). Two days later, the cells were replated and selected in puromycin (1 μg/ml). After 14–20 days, single colonies were isolated and propagated to generate clonal cell lines.

For siRNA transfection experiments, U2OS cells were plated in 60-mm dishes (1 × 10⁵ per dish) in complete medium. After 24 h, cells were transfected with the indicated siRNA duplexes (Dharmacon) at a final concentration of 67 nM using Oligo-
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fectamine (Invitrogen). The following siRNA sequences were used (target mRNAs in parentheses): 5'-CGUACGCAGGAU-ACUCGCA-3' (Luciferase), 5'-GGCUACGUCCAGGAGCG- GCA-3' (green fluorescent protein), 5'-CCAGACACGAGG- AAAUG-3' (hSMG-1), 5'-AAUUCGUGGGAGCUUUGGA-3' (hSMG-1), 5'-GCACACGUGGAUUGGC-3' (ATM), 5'- CGAGACUUGCGGAAUGC-3' (ATR), 5'-GAUGCGAU- UCCGUCAUU-3' (hUpf-1), and 5'-GGCUUUUGUGCCCA- GCCAUCA-3' (hUpf-2). Cells were used in experiments after 48–72 h of siRNA treatment.

Immunoprecipitation and Immunoblotting—For DISC immunoprecipitations, U2OS cells were exposed to 1 μg of FLAG-TNFα, and cell extracts were prepared by resuspending washed cell pellets in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Tween 20, 1 mM dithiothreitol) plus protease and phosphatase inhibitors. Samples were incubated for 30 min on ice, and lysates were precleared for 2 h with 20 μl of Sepha- rose-6B (Sigma). The cell extracts were incubated with 20 μl of protein G-Sepharose CL-4B (Amersham Biosciences), and sig- naling complexes were immunoprecipitated with 2 μg of α-FLAG-M2 or 2 μg of α-caspase-8 (C-20) antibody.

For detection of active BAX, cellular extracts were prepared in CHAPS lysis buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1% CHAPS) plus protease inhibitors. The active form of BAX was immunoprecipitated from cell extracts with 2 μg of α-BAX (6A7) antibody.

After separation by SDS-PAGE, proteins were detected by immunoblotting. Antibody-bound proteins were immuno- blotted with protein A-horseradish peroxidase (Amersham Biosciences) for rabbit antibodies or sheep anti-mouse IgG- horseradish peroxidase (Amersham Biosciences) to detect mouse monoclonal antibodies. Immunoreactive proteins were illuminated with the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

Immunofluorescence Microscopy—For immunofluorescence microscopy of endogenous hSMG-1, U2OS cells were plated in 4-well covered chamber slides (Nalge Nunc). After the indicated treatments, the cells were fixed for 15 min at room temperature in 2% paraformaldehyde, then washed with PBS and quenched with 0.1 M glycine. The fixed cells were permeabilized for 5 min at room temperature with 0.2% Triton X-100 containing 1% normal goat serum. The permeabilized cells were blocked with PBS plus 1% normal goat serum and then stained with 3 μg/ml affinity-purified α-hSMG-1 (Ab-2). After 2–3 h at room temperature, the sample wells were washed with PBS plus 1% normal goat serum. The samples were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG) in the presence of 4 μg/ml 4',6'-diamidino-2-phenyl- indole. The slides were mounted with Mowiol (Hoechst).

Immunofluorescence microscopy was performed with a Leica DMIRE2 inverted automated digital imaging microscope. Image deconvolution was accomplished with the nearest-neighbor algorithm of the Simple PCI software (Compix, Inc.).

NMD Assays—A β-globin NMD reporter (Luc-BBG-39PTC) was created by inserting the luciferase open reading frame into the human β-globin gene (BGG), which was mutated to contain a premature translation termination codon at codon 39 (39PTC). The control reporter plasmid (Luc-BBG-WT) con- tained the wild-type BGG genomic sequence. Expression of β-globin from both plasmids was controlled by the β-globin promoter (pβ-globin). U2OS cells were transfected with the control and NMD reporter constructs, and stably transfected clones were isolated and expanded. For assays, the cells were transiently transfected with a TK-Reporter reference plasmid, and luciferase activities were measured with the Dual Luciferase Assay system (Promega, Madison, WI) according to the manufacturer’s instructions.

Metabolic Labeling—U2OS cells were washed and starved in methionine- and cysteine-free medium (Cellgro) for 1 h at 37 °C. Baseline control samples were pretreated with 100 μg/ml CHX for 3 h, and both control and test cell populations were labeled with 100 μCi/ml [35S]methionine/ cysteine (EXPRESS-35S Protein labeling mix, PerkinElmer Life Sciences). At the indicated times, cells were washed extensively with cold PBS and were harvested in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol) plus protease inhibitors. Incorporation of [35S]methionine/cysteine was determined by trichloroacetic acid precipitation and analyzed in an LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA).

NF-κB Reporter Assays—U2OS cells were seeded in 6-well dishes (1 × 105 per dish) and were transfected 24 h later with the indicated siRNA duplexes. After 24 h, the cells were cotransfected with 15 ng of Renilla luciferase, 300 ng of firefly luciferase, and 500–2000 ng of HA-TRAF-2 plasmid DNA with the FuGENE 6 transfection reagent (Roche Applied Science). The total amount of DNA per well was equalized by co-transfection with f pcDNA3.1 (Invitrogen) empty vector plasmid as a control and NMD reporter constructs, and stably transfected clones were isolated and expanded. For assays, the cells were transfected with a TK-Reporter reference plasmid, and luciferase activities were measured with the Dual Luciferase Assay system (Promega) according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Assays—NF-κB DNA binding activity was measured with a probe containing the κB enhancer sequence (5'-TAGTTGAGGGGACTTTCCCAATGC-3'). NF-κB DNA binding activity was measured as a control and was measured using a probe derived from the adenovirus-2 origin of replication (5'-CTTATTTTGGATTGACCAATAT-3'). The 5' overhangs of these probes were filled in with Klenow (exo-) in the presence of [α-32P]ATP for 30 min at 37 °C. Unincor- porated nucleotides were removed with a gel filtration column (Roche Applied Science). The radiolabeled probe (20–40 kcpm per sample) was incubated with 5 μg of protein extract and 1 μg of pol(dIdC) for 20 min at room temperature. Protein-DNA complexes were resolved on a 5% non-denaturing polyacryl- amide gel and detected by autoradiography.

Cellular Fractionation—Prior to cell fractionation, U2OS cells were treated for 48 h with the indicated siRNA oligonu- cleotides, and were exposed to 20 ng/ml TNFα. After 90 min, the cells were washed once in cold PBS, and once in cold hypo- tonic buffer (10 mM HEPES (pH 6.9), 10 mM KCl, 2 mM sodium orthovanadate, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 50 mM microsystin). Cells were then harvested in 500 μl of hypotonic buffer, incubated on ice for 30 min, and lysed by Dounce homogenization (40–50 strokes). Lysates were centrifuged under refrigeration for 3 min at 1,000 × g. The resulting supernatant was centrifuged at 14,000 g
for 30 min at 4 °C, and the supernatant was collected and stored (cytoplasmic fraction). The insoluble pellet (nuclear plus membrane fraction) was resuspended in NETNT lysis buffer (10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 plus protease, and phosphatase inhibitors as listed above), sonicated on ice for ~2–4 cycles, 10 s/cycle, and stored. Proteins were separated by SDS-PAGE and analyzed by Western blot analysis.

**RESULTS**

**hSMG-1 Protects U2OS Cells against TNFα-induced Apoptosis**—Preliminary studies aimed at defining the subcellular localization of hSMG-1 revealed that this PIKK is predominantly expressed in the cytoplasm, as reported previously (13). Interestingly, more detailed immunofluorescence microscopic analyses revealed that a subpopulation of hSMG-1 molecules localizes to mitochondria in human U2OS osteosarcoma cells (supplemental Fig. S1). Based on the central role of mitochondria in apoptotic cell death, we examined the effects of several apoptosis-inducing stimuli in U2OS cells depleted of hSMG-1 by transfection with multiple specific siRNAs. In preliminary experiments, we observed that hSMG-1 depletion dramatically accelerated and magnified the apoptotic response induced by the combination of TNFα plus the protein synthesis inhibitor, CHX (results not shown). In most cell types, inhibition of protein synthesis is required to block the expression of NF-κB-dependent, anti-apoptotic proteins that normally counteract the caspase-8/10-dependent cell death pathway engaged during TNF receptor ligation (16).

To further characterize the role of hSMG-1 in the cellular response to TNFα, we determined whether hSMG-1 knockdown sensitized U2OS cells to TNFα in the absence of CHX. U2OS cells were treated with luciferase or hSMG-1 siRNAs, and then exposed to 20 ng/ml TNFα, and the percentage of cell death was determined with a Trypan blue vital dye exclusion assay. Relative to control (luciferase) siRNA-transfected cells, hSMG-1-depleted U2OS cells displayed a dramatic increase in cell death after 2–4 h of exposure to TNFα (Fig. 1A). The loss of cell viability was accompanied by the appearance of a hypodiploid cell sub-population, consistent with apoptotic death (results not shown). Morphologic evaluation of these samples indicated that a significant proportion of the hSMG-1-depleted cells contained nuclei with condensed chromatin after only 2 h of TNFα treatment (Fig. 1B). The increase in TNFα-induced apoptosis in hSMG-1-depleted cells was confirmed in both caspase 3 activation (Fig. 1C) and DNA fragmentation (Fig. 1D) assays. Cleavage of the receptor-proximal initiator caspase, caspase-8, was evident after 4 h in TNFα plus CHX-treated U2OS cells (Fig. 1E). In contrast, significant caspase-8 cleavage was observed after only 2 h in hSMG-1-deficient U2OS cells that were exposed to TNFα alone (Fig. 1F). These studies were reproduced in two additional human cancer cell lines, A549 and HeLa, indicating that sensitization to TNFα-induced cell death was not unique to the U2OS cell line (results not shown). In a subsequent experiment, we tested whether the pro-apoptotic Bcl-2 family member BAX could be activated by TNFα in hSMG-1-deficient cells (Fig. 1G). The active form of BAX was immunoprecipitated from hSMG-1-depleted cells treated with TNFα for 2 h, but not from similarly treated control cells. This result suggests that loss of hSMG-1 significantly augments the coupling of TNF receptor engagement to the “intrinsic” pathway of apoptosis provoked by BAX-mediated mitochondrial damage.

In subsequent experiments, we examined the effects of siRNAs targeted against other PIKKs or components of the NMD pathway on cellular sensitivity to TNFα. In contrast to the dramatic increase in TNFα-induced cell death observed in hSMG-1-depleted cells, knockdown of the related PIKKs, ATM and ATR, or the NMD pathway components, hUpf1 or hUpf2, failed to sensitize the cells to death signaling by TNFα (Fig. 2A and supplemental Fig. S2). Similar results were obtained at time points later than the 2-h treatment interval shown in this experiment (data not shown). Thus, these findings indicate that the hSMG-1-related PIKKs, ATM and ATR, do not modulate pro-apoptotic signaling through the TNF receptor. Furthermore, the failure of hUpf1 or hUpf2 depletion to alter TNFα sensitivity suggests that the pro-apoptotic effects of hSMG-1 depletion is unrelated to the inhibition of NMD. This conclusion is further substantiated by the results described in Fig. 3 below.

To confirm that the increase in TNFα-induced apoptosis observed in hSMG-1-depleted cells was directly related to an on-target effect of the siRNA, we determined whether this response was reversed by forced overexpression of the full-length hSMG-1 protein (Fig. 2B). The results showed that transfection with increasing amounts of hSMG-1-encoding plasmid DNA caused a progressive decrease in TNFα-dependent cell death in the siRNA-transfected cells. These results confirm that the sensitizing effects of hSMG-1-targeted siRNA are directly related to the knockdown of hSMG-1 expression.

**Effect of NMD Suppression on TNFα-induced Apoptosis**—The results presented in Fig. 2A demonstrated that hUpf1 depletion failed to increase cellular sensitivity to TNFα-induced apoptosis. Because both hSMG-1 and hUpf1 are involved in NMD, the Fig. 2A results suggest that hSMG-1 depletion enhances the apoptotic response to TNFα through a mechanism independent of its role in the NMD pathway. To further address this issue, we generated a U2OS subclone that stably expressed a NMD activity reporter (Luc-BGG-39PTC) in which the luciferase cDNA is fused to the mutated BGG gene sequence bearing a PTC (Fig. 3A). We also prepared a U2OS subclone expressing a NMD-insensitive construct containing...
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A. TNFα-induced cell death. Cells were transfected with luciferase or hSMG-1 siRNA, and, after 48 h, were exposed to 20 ng/ml TNFα for the indicated times. Cell viability was determined by trypan blue dye exclusion. Results (mean ± S.E.) were obtained from three independent trials. B. hSMG-1 immunostaining. U2OS cells were treated with the indicated siRNAs for 48 h, exposed to TNFα for the indicated times, and stained with a polyclonal α-SMG-1 antibody (green). Cell nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (blue), and images were obtained by immunofluorescence microscopy. C. Cleaved caspase-3 levels. U2OS cells were treated with luciferase- or hSMG-1-siRNAs for 48 h, or left untreated, and were exposed to 20 ng/ml TNFα and/or 10 μg/ml cycloheximide (CHX). At the indicated times, the relative amount of cleaved caspase-3 was determined with a cleaved caspase-3 assay (meso scale discovery). Data are presented as mean ± S.D. from triplicate samples. D. DNA fragmentation. U2OS cells were transfected with the indicated siRNAs, and, after 48 h, the cells were stimulated for 2 h with 20 ng/ml TNFα and/or 10 μg/ml CHX. The cells were then harvested, and DNA fragmentation was evaluated with the Cell Death Detection ELISA plus kit (Roche Applied Science). Data are presented as mean ± S.D. from triplicate samples. E. caspase-8 cleavage. U2OS cells were treated with 10 μg/ml CHX and/or 20 ng/ml TNFα and were harvested at the indicated times. Detergent-soluble proteins were resolved by SDS-PAGE and immunoblotted with α-hSMG-1, α-PLC-γ1, and α-caspase-8 antibodies. In the α-caspase-8 immunoblot, the p43/41 bands denote the cleaved forms of caspase-8. F, the experiment was performed as described in C, except that the cells were transfected with the indicated siRNA prior to TNFα stimulation. G. BAX activation. Whole cell extracts were prepared from siRNA-transfected U2OS cells, and detergent extracts were immunoprecipitated with an antibody that selectively recognizes the active conformation of BAX (α-Active BAX).

The wild-type BGG sequence (Luc-BGG-WT) as a control. Treatment of the Luc-BGG-39PTC-expressing cells with either hSMG-1- or hUpf1-targeted siRNA increased luciferase activity in these cells, consistent with suppression of NMD-mediated degradation of the reporter gene-derived mRNA (Fig. 3B). Quantitatively greater increases in luciferase activity were reproducibly found in the hUpf1-depleted cells relative to their hSMG-1-depleted counterparts. This outcome may reflect variation in the efficiency of gene knockdown by the two respective siRNAs, or may indicate a more critical role for hUpf1 in NMD. Support for the latter possibility stems from the observation that treatment of the Luc-BGG-39PTC-expressing cells with wortmannin, at a concentration (10 μM) known to fully and irreversibly inhibit hSMG-1 kinase activity (2, 4), elicited an increase in luciferase activity that was virtually identical to that induced by hSMG-1 depletion (Fig. 3B). Neither the siRNA nor the drug treatments had any significant effect on the expression of luciferase in Luc-BGG-WT-transfected cells, indicating that the changes observed in the Luc-BGG-39PTC-expressing cells were related to the suppression of NMD activity. We conclude that the increase in TNFα-induced cell killing seen in hSMG-1-depleted cells cannot be attributed to interference with the turnover of PTC-bearing mRNAs via NMD.

Loss of hSMG-1 Does Not Reduce General Protein Synthesis—As previously reported, inhibition of RNA or protein synthesis by agents such as actinomycin D and CHX, respectively, sensitizes cells to TNFα-induced apoptosis by blocking the expression of pro-survival proteins that suppress initiator caspase activation. In the NMD assays shown in Fig. 3B, luciferase protein expression from the Luc-BGG-39PTC reporter gene was actually increased in the hSMG-1-depleted cells, indicating that the translational machinery was not generally impaired in these cells. To substantiate this conclusion, we measured global protein synthesis activity in siRNA-treated U2OS cells. Relative to luciferase siRNA-treated control cells, hSMG-1-depleted cells displayed a modest reduction in the rate of protein synthesis, as measured by incorporation of [35S]methionine into trichloroacetic acid-precipitable protein (Fig. 4). In contrast, exposure of non-transfected U2OS cells to CHX, at the same drug concentration used to sensitize these cells to TNFα, completely sup-

FIGURE 1. hSMG-1 depletion sensitizes U2OS cells to TNFα-induced killing. A, TNFα-induced cell death. Cells were transfected with luciferase or hSMG-1 siRNA, and, after 48 h, were exposed to 20 ng/ml TNFα for the indicated times. Cell viability was determined by trypan blue dye exclusion. Results (mean ± S.E.) were obtained from three independent trials. B, hSMG-1 immunostaining. U2OS cells were treated with the indicated siRNAs for 48 h, exposed to TNFα for the indicated times, and stained with a polyclonal α-SMG-1 antibody (green). Cell nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (blue), and images were obtained by immunofluorescence microscopy. C. Cleaved caspase-3 levels. U2OS cells were treated with luciferase- or hSMG-1-siRNAs for 48 h, or left untreated, and were exposed to 20 ng/ml TNFα and/or 10 μg/ml cycloheximide (CHX). At the indicated times, the relative amount of cleaved caspase-3 was determined with a cleaved caspase-3 assay (meso scale discovery). Data are presented as mean ± S.D. from triplicate samples. D. DNA fragmentation. U2OS cells were transfected with the indicated siRNAs, and, after 48 h, the cells were stimulated for 2 h with 20 ng/ml TNFα and/or 10 μg/ml CHX. The cells were then harvested, and DNA fragmentation was evaluated with the Cell Death Detection ELISA plus kit (Roche Applied Science). Data are presented as mean ± S.D. from triplicate samples. E. caspase-8 cleavage. U2OS cells were treated with 10 μg/ml CHX and/or 20 ng/ml TNFα and were harvested at the indicated times. Detergent-soluble proteins were resolved by SDS-PAGE and immunoblotted with α-hSMG-1, α-PLC-γ1, and α-caspase-8 antibodies. In the α-caspase-8 immunoblot, the p43/41 bands denote the cleaved forms of caspase-8. F, the experiment was performed as described in C, except that the cells were transfected with the indicated siRNA prior to TNFα stimulation. G. BAX activation. Whole cell extracts were prepared from siRNA-transfected U2OS cells, and detergent extracts were immunoprecipitated with an antibody that selectively recognizes the active conformation of BAX (α-Active BAX).
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Reduced Expression of DISC-associated FLIP<sub>L</sub> in hSMG-1 siRNA-treated Cells—Stimulation of type I TNF receptor triggers the formation of two multiprotein DISCs (15). The plasma membrane-bound complex (DISC I) contains TRADD, TRAF2, and the protein kinase RIP1. After a temporal delay, DISC II is assembled in the cytoplasm through association of TRADD and RIP1 with FADD, caspase-8, and caspase-10. DISC II in turn activates downstream caspases, leading to apoptosis. The pro-apoptotic function of DISC II is inhibited by FLIP<sub>L</sub>, which structurally resembles caspase-8, except that it lacks protease activity. We hypothesized that hSMG-1 protects cells against the apoptotic activity of TNFα by regulating DISC assembly and/or function in human cells. Interestingly, caspase-8 contains two potential hSMG-1 phosphorylation sites at Ser-219 and Ser-338, which suggested that caspase-8 might be a novel hSMG-1 substrate. However, immune complex kinase assays with His-tagged, caspase-8<sub>216–478</sub> as the substrate failed to show phosphorylation of either of the Ser-Gln motifs by hSMG-1, suggesting that caspase-8 was not a physiological substrate for the hSMG-1 kinase (supplemental Fig. S3).

We next examined TNFα-induced DISC assembly in U2OS cells stimulated with a FLAG-tagged version of human TNFα. Cell extracts were immunoprecipitated with either anti-FLAG antibody, to purify DISC I, or with caspase-8 antibody, to pull down DISC II. Loss of hSMG-1 function had no detectable effect on the assembly of DISC I in TNFα-stimulated cells (Fig. 6A), consistent with the impaired NFκB activation response observed in these cells (Fig. 5). Interestingly, a significant amount of co-precipitating FLIP<sub>L</sub> was observed in the anti-caspase-8 immunoprecipitates from the luciferase siRNA-treated control cells prior to TNFα stimulation (Fig. 6B, 0-min time point). Neither FADD nor TRAF2 were found in this sam-

pressed protein synthesis. These results strongly suggest that the increase in TNFα sensitivity observed in hSMG-1-deficient cells is not attributable to a global reduction in mRNA translation.

NF-κB Function Is Not Impaired in hSMG-1-depleted Cells—The activation of NF-κB during TNF receptor engagement triggers the expression of various anti-apoptotic genes such as FLIP<sub>L</sub>, Bcl-X<sub>L</sub>, A1, and X-chromosome-linked inhibitor of apoptosis (16, 18, 19). In addition, NF-κB limits the duration of Jun N-terminal kinase (JNK) activation through the induction of antioxidants that maintain the activities of JNK-inactivating phosphatases (20). To determine whether hSMG-1 was required for TNFα-stimulated NF-κB activation, we examined IκBα degradation and NF-κB-dependent transcriptional activity in hSMG-1 siRNA-treated U2OS cells. Loss of hSMG-1 had no effect on either the early degradation of IκBα, or the subsequent re-expression of this protein (Fig. 5A). The latter event is itself dependent on the transcriptional activity of NFκB (21). Moreover, the increase in NFκB-dependent reporter gene transcription triggered by transient overexpression of the TNF receptor-associated protein, TRAF2, was unaffected by siRNA-mediated depletion of hSMG-1 (Fig. 5B). Finally, we examined the formation of DNA-bound NFκB complexes by electrophoretic mobility shift assay. Treatment of U2OS cells with hSMG-1-targeted siRNA had no effect on the nuclear translocation and DNA-binding activity of NFκB provoked by TNFα stimulation (Fig. 5C). Cellular exposure to TNFα also stimulates the activation of MAPKs, including the ERKs, which could influence apoptotic sensitivity (22). However, immunoblot analysis of the cellular extracts used for the electrophoretic mobility shift assay revealed no impairment of TNFα-induced ERK activation in the hSMG-1-depleted cells (Fig. 5C). We conclude from these studies that the increase in TNFα-induced apoptosis observed in hSMG-1-depleted cells is not related to impaired coupling of the TNF receptor to either the NFκB or ERK signaling pathways.

FIGURE 2. Loss of hSMG-1 selectively sensitizes U2OS cells to TNFα-induced apoptosis. A, selective induction of apoptosis by hSMG-1 gene silencing. U2OS cells were transfected with the indicated siRNAs, and, after 48 h, the cells were stimulated for 2 h with 20 ng/ml TNFα. Percentage of cell death was determined by trypan blue dye exclusion. Results (mean ± S.E.) were obtained from four independent trials. Whole cell extracts from parallel samples were resolved by SDS-PAGE and immunoblotted with α-caspase-8 antibody. B, reversal of TNFα-induced cell killing in hSMG-1-depleted cells by forced expression of hSMG-1. U2OS cells were transfected with luciferase (Luc)- or hSMG-1-targeted siRNA, followed by secondary transfection with the indicated amounts of expression plasmids encoding HA-tagged, wild-type hSMG-1 (HA-hSMG-1<sup>WT</sup>) or kinase inactive hSMG-1 (HA-hSMG-1<sup>KI</sup>), HA-hSMG-1<sup>WT</sup> contains an Asp → Ala substitution at a conserved residue (Asp-2195) in the hSMG-1 catalytic domain. The cells were stimulated with TNFα at 48 h after the initial transfection, and cell death was scored as described in A. Results are depicted as mean ± S.E. from three independent trials. The bottom panel shows immunoblots of the endogenous and ectopically expressed (HA-tagged) hSMG-1 proteins.

of antioxidants that maintain the activities of JNK-inactivating phosphatases (20). To determine whether hSMG-1 was required for TNFα-stimulated NF-κB activation, we examined IκBα degradation and NF-κB-dependent transcriptional activity in hSMG-1 siRNA-treated U2OS cells. Loss of hSMG-1 had no effect on either the early degradation of IκBα, or the subsequent re-expression of this protein (Fig. 5A). The latter event is itself dependent on the transcriptional activity of NFκB (21). Moreover, the increase in NFκB-dependent reporter gene transcription triggered by transient overexpression of the TNF receptor-associated protein, TRAF2, was unaffected by siRNA-mediated depletion of hSMG-1 (Fig. 5B). Finally, we examined the formation of DNA-bound NFκB complexes by electrophoretic mobility shift assay. Treatment of U2OS cells with hSMG-1-targeted siRNA had no effect on the nuclear translocation and DNA-binding activity of NFκB provoked by TNFα stimulation (Fig. 5C). Cellular exposure to TNFα also stimulates the activation of MAPKs, including the ERKs, which could influence apoptotic sensitivity (22). However, immunoblot analysis of the cellular extracts used for the electrophoretic mobility shift assay revealed no impairment of TNFα-induced ERK activation in the hSMG-1-depleted cells (Fig. 5C). We conclude from these studies that the increase in TNFα-induced apoptosis observed in hSMG-1-depleted cells is not related to impaired coupling of the TNF receptor to either the NFκB or ERK signaling pathways.
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A pβ-globin Luc-BGG WT/39PTC

B U2OS WT

U2OS 39PTC

FIGURE 3. Effect of hSMG-1 versus hUpf1 depletion on the PTC-dependent degradation of β-globin mRNA. A, β-globin NMD reporter. A schematic diagram of the structure of human β-globin-luciferase reporter constructs, Luc-BGG-WT and Luc-BGG-39PTC, is shown. B, NMD reporter assays. U2OS cells were stably transfected with wild-type or PTC-containing β-globin reporter plasmid and a TK-Renilla reference plasmid. Stable clones were then treated with the indicated siRNAs, or 10 μM Wortmannin (Wm) for 12 h. DMSO was added to the indicated sample as a vehicle control (VC). At 72 h post-transfection, cells were harvested, and luciferase activities were measured with a luciferase assay system (Promega), and each sample was normalized to the Renilla luciferase control activity.

FIGURE 4. Rate of protein translation in hSMG-1-depleted cells. U2OS cells were treated with luciferase- or hSMG-1-siRNAs for 72 h, starved in fetal bovine serum-, methionine-, and cysteine-free medium for 3 h, labeled with [35S]methionine/cysteine, and harvested at the indicated times. Cycloheximide (CHX)-treated cells were subjected to a similar experimental protocol, and trichloroacetic acid-precipitable radioactivity was measured by scintillation counting. Results (mean ± S.E.) were obtained from three independent trials.

ple, suggesting that U2OS cells constitutively express considerable amounts of the FLIP_L-caspase-8 heterodimer, which functions independently as a signaling complex in pro-survival pathways (18). In these luciferase siRNA-treated cells, TNFα stimulation provoked a rapid decrease in the level of caspase-8-associated FLIP_L, the assembly of the FADD-containing DISC II, and the appearance of cleaved FLIP_L, indicating that caspase-8 was partially activated (18). In contrast, hSMG-1-depleted cells contained dramatically reduced amounts of the FLIP_L-caspase-8 heterodimer; however, in response to TNFα, these cells readily assembled DISC II, as indicated by the appearance of FADD in the anti-caspase-8 immunoprecipitates. Direct immunoblotting of the cellular extracts used in these assays revealed no alterations in the overall levels of any of these proteins (Fig. 6B), indicating that alterations observed in Fig. 6B were due to specific changes in the levels of these proteins in the α-caspase-8 immunoprecipitates. In contrast to the luciferase siRNA-treated control cells, the hSMG-1-deficient cells constitutively expressed the cleaved form of FLIP_L (denoted with an arrow in Fig. 6B), indicating that these cells displayed a basal level of caspase-8 activation. In these cells, TNFα stimulation elicited the appearance of the fully cleaved caspase-8 fragment after 90 min, indicating robust apoptotic signaling associated with the loss of hSMG-1. We were unable to detect co-precipitating hSMG-1 in either the FLAG or caspase-8 immunoprecipitates, suggesting that hSMG-1 is not stably associated with DISC I or DISC II (Fig. 6, A and B).

In subsequent experiments, we examined the effect of hSMG-1 depletion on the expression and localization of FLIP_L in U2OS cells. Interestingly, FLIP_L was localized exclusively to the membrane-enriched subcellular fraction, which contains nuclei, mitochondria, and other organelles, in both the luciferase- and hSMG-1-siRNA-transfected cells (Fig. 7A). Stimulation of the hSMG-1-depleted cells with TNFα caused a striking decrease in the level of FLIP_L in membrane-enriched fractions prepared from the hSMG-1-treated cells. The decrease in membrane-bound FLIP_L was not accompanied by the appearance of FLIP_L in the soluble fraction, which suggested that the protein underwent cytokine-induced degradation, rather than re-localization, in the hSMG-1-deficient cells. Consistent with a specific role of hSMG-1 in the cellular response to TNFα, no decrease in FLIP_L protein expression was observed in U2OS cells depleted of ATM, ATR, hUpf1, or hUpf2 after exposure to TNFα (supplemental Fig. S2).

In subsequent studies, we examined the role of the proteasome in the decrease in FLIP_L expression observed in TNFα-stimulated, hSMG-1-depleted cells. The siRNA-transfected cells were pretreated with either MG132 or PS-341 (Velcade™) to block proteasomal activity, and then were stimulated for 90 min with TNFα. Prior exposure of the cells to either proteasome inhibitor prevented the loss of FLIP_L provoked by TNFα in the hSMG-1-deficient cells (Fig. 7B). These results suggest that hSMG-1 protects cells from TNFα-induced apoptosis, at least in part, by maintaining expression of the anti-apoptotic protein, FLIP_L, during TNF receptor signaling.

To determine the effect of hSMG-1 depletion on basal FLIP_L turnover, we treated cells with CHX to block protein synthesis and monitored the total level of FLIP_L protein over time. Depletion of hSMG-1 had no effect on the basal turnover of FLIP_L in
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U2OS cells (Fig. 7C, upper panels). In contrast, stimulation of the hSMG1-depleted cells with TNFα in the presence of CHX (to block new FLIPL protein synthesis) resulted in an abrupt and dramatic decrease in FLIPL levels (Fig. 7C, lower panels). Moreover, a similar stimulation had no effect on FLIPL protein expression in the luciferase siRNA-treated control cells. Real-time quantitative reverse transcription-PCR analyses of mRNA extracted from luciferase or hSMG1 siRNA-transfected cells revealed comparable levels of FLIPL mRNA transcripts in both knockdown cells after 90 min of exposure to TNFα (Fig. 7D). These results suggest that the reduction in FLIPL protein observed in hSMG1-depleted cells during TNF-α stimulation cannot be attributed to a decrease in the steady-state FLIPL mRNA level.

To determine whether the reduced expression of FLIPL is causally related to the increase in TNFα-induced apoptosis in hSMG1-deficient cells, we determined the effect of forced FLIPL overexpression on this response. U2OS cells were sequentially transfected with hSMG1-targeted siRNA and a FLIPL expression plasmid. The results showed that transfection of the hSMG1-depleted cells with increasing amounts of the FLAG-tagged FLIPL expression plasmid resulted in a progressive increase in cellular resistance to TNFα-induced death (Fig. 7E). Thus, the protective function of hSMG1 in TNFα-stimulated cells is due, at least in part, to the maintenance of FLIPL expression, and reduced activation of the pro-apoptotic DISC II in these cells.

DISCUSSION

The results of this study describe an unanticipated role for the PIKK family member, hSMG1, in the regulation of cellular sensitivity to apoptotic death induced by TNF receptor engagement. In particular, our findings point toward the anti-apoptotic protein, FLIPL, as a key effector of the cytoprotective function of hSMG1 in TNFα-stimulated cells. Depletion of hSMG1-sensitized human cancer cell lines to killing by TRAIL as well as TNFα (see supplemental Fig. S4A). However, these cells did not display increased sensitivity to killing by Fas receptor ligation (supplemental Fig. S4A), or by the cytotoxic agent, doxorubicin (results not shown). As expected, increased sensitivity to TRAIL-induced apoptosis correlated with the accelerated degradation of FLIPL in hSMG1-depleted cells (see supplemental Fig. S4B). While the present report was under review, Llobet et al. (23) demonstrated that the inhibition of casein kinase (CK2) sensitizes endometrial carcinoma cells to TRAIL- and Fas-induced death via a proteasome-mediated degradation of FLIPL. Hence, at least two protein kinases, CK2 and hSMG1, modulate cellular sensitivity to the pro-apoptotic activities of TNF family ligands by regulating the stability of the cytoprotective FLIPL protein during engagement of the receptors for these cytokines.

Previous studies identified hSMG1 as a central player in NMD, which functions in both normal transcriptome pattern-
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![Graph](Image)

A siRNA: Luciferase hSMG-1
Flag-TNFα (min): α-hSMG-1 α-TNFR1 α-TRAF-2 α-TRADD α-FADD α-Caspase-8 α-FLIP-L 0 5 15 60 90 0 5 15 60 90

B siRNA: Luciferase hSMG-1
Flag-TNFα (min): α-hSMG-1 α-TRAF-2 α-FLIP-L α-TNFR1 α-FADD α-Caspase-8 0 5 15 60 90 0 5 15 60 90

FIGURE 6. DISC composition in hSMG-1-depleted cells. A, DISC I. U2OS cells were treated with luciferase or hSMG-1 siRNAs for 48 h, stimulated with recombinant FLAG-tagged human TNFα (FLAG-TNFα), and immunoprecipitated with a FLAG-specific monoclonal antibody. Samples were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. B, DISC II. After FLAG-TNFα stimulation, extracts from luciferase and hSMG-1-depleted cells were immunoprecipitated with α-caspase-8 antibody and immunoblotted with the indicated antibodies. The arrows point to proteolytically cleaved proteins.

C siRNA: Luciferase hSMG-1
Flag-TNFα (min): α-hSMG-1 α-TNFR1 α-Caspase-8 α-FLIP-L α-PLC-γ1 0 5 15 60 90 0 5 15 60 90

so such as CHX, is typically required to observe TNFα-stimulated apoptosis. CHX unmasks the caspase-8-activating potential of the TNF receptor, in part, by blocking the synthesis of the anti-apoptotic protein, FLIPL, which competitively inhibits caspase-8/10 activation by the TNFα-induced DISC II (28). In the absence of FLIPL, TNF receptor engagement triggers caspase-8/10 activation, setting in motion a caspase-mediated proteolytic cascade that eventually commits the host cell to die by apoptosis. Our results indicate that hSMG-1 protects TNFα-exposed cells from death, at least in part, by stabilizing the FLIPL protein, and dampening activation of the caspase 8/10-dependent apoptotic cascade during type I TNF receptor signaling. We speculate that this hSMG-1-dependent response represents an early, fail-safe mechanism that protects cells from apoptosis until the expression of NFκB-dependent pro-survival genes (including FLIPL itself) reach fully cytoprotective levels in TNFα-stimulated cells. Although the increased FLIPL stability observed in hSMG-1-proficient cells might be sufficient to protect cells from the pro-apoptotic actions of TNFα, we cannot rule out the possibility that hSMG-1 influences other TNFα-dependent signaling mechanisms that may dampen the apoptotic signaling through the type I TNF receptor.

The rapid activation of NF-κB by the TNF receptor-associated DISC I underlies the suppression of TNFα-induced apoptosis in mammalian cells (15). In these cells, NFκB controls the expression of a diverse set of genes that increase cellular resistance to apoptosis and mediate many of the actions of TNFα, including its pro-inflammatory properties. As part of its anti-apoptotic function, NFκB stimulates the expression of FLIPL (18, 28), which suggested that hSMG-1 might regulate FLIPL protein levels by promoting either the activation or transcriptional activity of NFκB. However, deletion of hSMG-1 by RNA interference had no effect on TNFα-induced NFκB activation, either at the level of κB degradation or NFκB transcriptional activity. Furthermore, the increase in TNFα sensitivity observed in hSMG-1-deficient cells was not the result of a CHX-like global inhibition of protein translation. Finally, cell death induced by TNFα in hSMG-1-deficient cells was not a general consequence of NMD pathway suppression, because siRNA-mediated silencing of the hUpf1 gene strongly inhibited NMD but failed to sensitize cells to TNFα-induced apoptosis.
Our findings point toward alterations in FLIPL expression and function as underlying events leading to increased TNFα sensitivity in hSMG-1-depleted cells. Knockdown of hSMG-1 expression virtually abolished the basal expression of FLIPL-caspase-8 heterodimers in U2OS cells, without impeding the assembly of the pro-apoptotic DISC II. The absence of caspase-8-associated FLIPL was not explained by an increase in basal FLIPL protein turnover in these cells. The cellular functions of FLIPL are pleiotropic and only partially understood. In immune cells, the caspase-8-FLIPL heterodimer plays a positive role in the activation of NFκB through certain immune receptors (18). Association of FLIP1 with caspase-8 triggers the autocleavage of caspase-8 and the generation of a moderately active form of this protease (18). This form of caspase-8 appears to act as a signaling protein, rather than an inducer of apoptosis. The function of the caspase-8-FLIP1 heterodimer in U2OS osteosarcoma cells is unknown, but one possibility is that it mediates NFκB activation in response to specific stress-inducing stimuli. Our studies show that TNFα-induced NFκB activation is not perturbed in hSMG-1-depleted cells; hence, TNF receptor coupling to NFκB is not dependent on the presence of an intact caspase-8-FLIP1 heterodimer, at least in the U2OS cells used in the present study. Clearly, additional research is needed to define the impact of caspase-8-FLIP1 heterodimer disruption on TNFα sensitivity and other aspects of cell fitness in hSMG-1-deficient cells.

Although hSMG-1 knockdown had no effect on basal FLIP1 protein expression, stimulation of the hSMG-1-depleted cells with TNFα triggered a rapid decline in the level of FLIP1, due to proteasome-dependent degradation. In hSMG-1-deficient
cells, FLIP\(_L\) failed to associate normally with the TNF\(\alpha\) induced, pro-apoptotic DISC II. Moreover, TNF\(\alpha\) stimulation caused a dramatic decrease in FLIP\(_L\) protein expression in the absence of hSMG-1; as expected, this response was accompanied by the caspase-8 autocleavage and the onset of apoptosis.

The mechanism whereby loss of hSMG-1 destabilizes FLIP\(_L\) during TNF receptor stimulation remains to be defined. The decrease in FLIP\(_L\) expression was reversed by proteasome inhibition, suggesting that hSMG-1 protects FLIP\(_L\) from degradation via the ubiquitin-proteasome pathway. A recent report identified the Itch E3 ligase as an effector of FLIP\(_L\) ubiquitination in TNF\(\alpha\)-stimulated cells (29). Interestingly, these studies also demonstrated that the JNK kinase, which is activated during TNF receptor stimulation, phosphorylates Itch, thereby increasing its ubiquitin ligase activity toward FLIP\(_L\). Integration of these earlier results with the present findings suggests a model in which hSMG-1 delivers a signal that suppresses the degradation of FLIP\(_L\), possibly by inhibition of JNK-dependent Itch activation. Again, more research is needed to define the mechanism(s) through which hSMG-1 regulates the expression and function of FLIP\(_L\) during TNF receptor engagement.

In summary, the present studies define an NMD-independent function of hSMG-1 that dampens pro-apoptotic signal output from TNF receptors. We have found that a subpopulation of hSMG-1 molecules is localized to mitochondria, and, in light of the pivotal function of these organelles in apoptotic signaling, the contribution of the mitochondria-associated pool of hSMG-1 to life and death decisions merits particular attention. In addition, the potential regulatory interaction between hSMG-1 and the JNK-Itch pathway should be examined in detail. Interestingly, a visual inspection of the primary amino acid sequence of Itch reveals 9 S/T-Q sites that could be targeted for phosphorylation by hSMG-1. Future studies will undoubtedly clarify the mechanism through which hSMG-1 protects cells against TNF\(\alpha\)-induced apoptosis, and will likely reveal additional roles for this PIKK in stress responses in human cells.

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REFERENCES
1. Abraham, R. T. (2004) DNA Repair (Amst.) 3, 883–887
2. Yamashita, A., Ohnishi, T., Kashima, I., Taya, Y., and Ohno, S. (2001) Genes Dev. 15, 2215–2228
3. Denning, G., Jamieson, L., Maquat, L. E., Thompson, E. A., and Fields, A. P. (2001) J. Bio. Chem. 276, 22709–22714
4. Brumbaugh, K. M., Otterness, D. M., Geisen, C., Oliveira, V., Brognard, J., Li, X., Lejeune, F., Tibbetts, R. S., Maquat, L. E., and Abraham, R. T. (2004) Mol. Cell 14, 585–598
5. Abraham, R. T. (2004) DNA Repair (Amst.) 3, 919–925
6. Isken, O., and Maquat, L. E. (2007) Genes Dev. 21, 1833–8356
7. Conti, E., and Izaurralde, E. (2005) Curr. Opin. Cell Biol. 17, 316–325
8. Mendell, J. T., Shariﬁ, N. A., Meyers, J. L., Martinez-Murillo, F., and Dietz, H. C. (2004) Nat. Genet. 36, 1073–1078
9. Pulak, R., and Anderson, P. (1993) Genes Dev. 7, 1885–1897
10. Maquat, L. E. (2005) J. Cell Sci. 118, 1773–1776
11. Garneau, N. L., Wilusz, J., and Wilusz, C. J. (2007) Nat. Rev. Mol. Cell. Biol. 8, 113–126
12. Yamashita, A., Kashima, I., and Ohno, S. (2005) Biochem. Biophys. Acta 1754, 305–315
13. Ohnishi, T., Yamashita, A., Kashima, I., Schell, T., Anders, K. R., Grimson, A., Hachiya, T., Hentze, M. W., Anderson, P., and Ohno, S. (2003) Mol. Cell 12, 1187–1200
14. Abraham, R. T. (2001) Genes Dev. 15, 2177–2196
15. Miechau, O., and Tschopp, J. (2003) Cell 114, 181–190
16. Aggarwal, B. B. (2003) Nat. Rev. Immunol. 3, 745–756
17. Secrist, J. P., Karnitz, L., and Abraham, R. T. (1991) J. Biol. Chem. 266, 12135–12139
18. Budd, R. C., Yeh, W. C., and Tschopp, J. (2006) Nat. Rev. Immunol. 6, 196–204
19. Park, S. M., Schickel, R., and Peter, M. E. (2005) Curr. Opin. Cell Biol. 17, 610–616
20. Shen, H. M., and Pervaiz, S. (2006) FASEB J. 20, 1589–1598
21. Hayden, M. S., and Ghosh, S. (2004) Genes Dev. 18, 2195–2224
22. Saklatvala, J., Davis, W., and Guesdon, F. (1996) Philos. Trans. R. Soc. Lond. B Biol. Sci. 351, 151–157
23. Llobet, D., Eritja, N., Encinas, M., Llecha, N., Yeramian, A., Pallares, J., Sorolla, A., Gonzalez-Tallada, F. J., Matias-Guiu, X., and Dolcet, X. (2007) Oncogene, Epub ahead of print
24. Azzalin, C. M., and Lingner, J. (2006) Curr. Biol. 16, 433–439
25. Azzalin, C. M., and Lingner, J. (2006) Cell Cycle 5, 1496–1498
26. Kaygun, H., and Marzluff, W. F. (2005) Nat. Struct. Mol. Biol. 12, 794–800
27. Rodriguez-Gabriel, M. A., Watt, S., Bahl, J., and Russell, P. (2006) Mol. Cell. Biol. 26, 6347–6356
28. Kreuz, S., Stiegmand, D., Scheurich, P., and Wajant, H. (2001) Mol. Cell. Biol. 21, 3964–3973
29. Chang, L., Kamata, H., Solinas, G., Luo, J. L., Maeda, S., Venuprasad, K., Liu, Y. C., and Karin, M. (2006) Cell 124, 601–613