Supplemental Data

Article

Regulation of XIAP translation and induction by MDM2 following irradiation

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Supplemental Figures

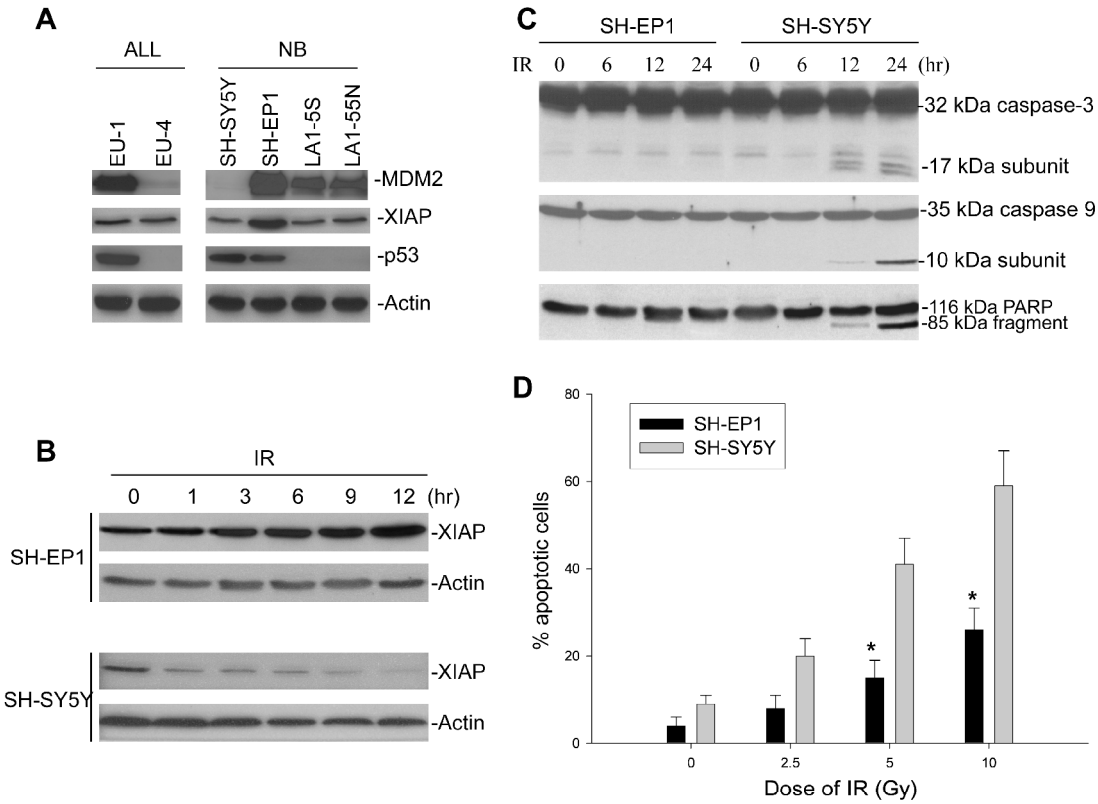

Figure S1. Effect of IR-induced DNA damage on XIAP expression and sensitivity to apoptosis in cells with distinct MDM2 expression. (A) Western blot assay for the protein expression of MDM2, XIAP, and p53 in six cancer cells lines (2 ALL and 4 NB) used as models in this study. (B) Kinetic analyses of XIAP protein expression in SH-EP1 and SH-SY5Y cells following 10 Gy IR treatment, as detected by western blot assay. (C) Results of western blot assay for activation of caspase-3, -9 and cleavage of death substrate PARP in SH-EP1 and SH-SY5Y cells that were treated with 10 Gy IR for the indicated time. (D) Dose-dependent apoptosis induced by IR in SH-EP1 and SH-SY5Y NB cell lines. Cells were exposed to different dose of IR for 24 h, and apoptotic cells were determined by annexin-V staining and analyzed by flow cytometry. Data represents the mean (±SD) percentage of annexin-V positive cells from three independent experiments. * p<0.01.

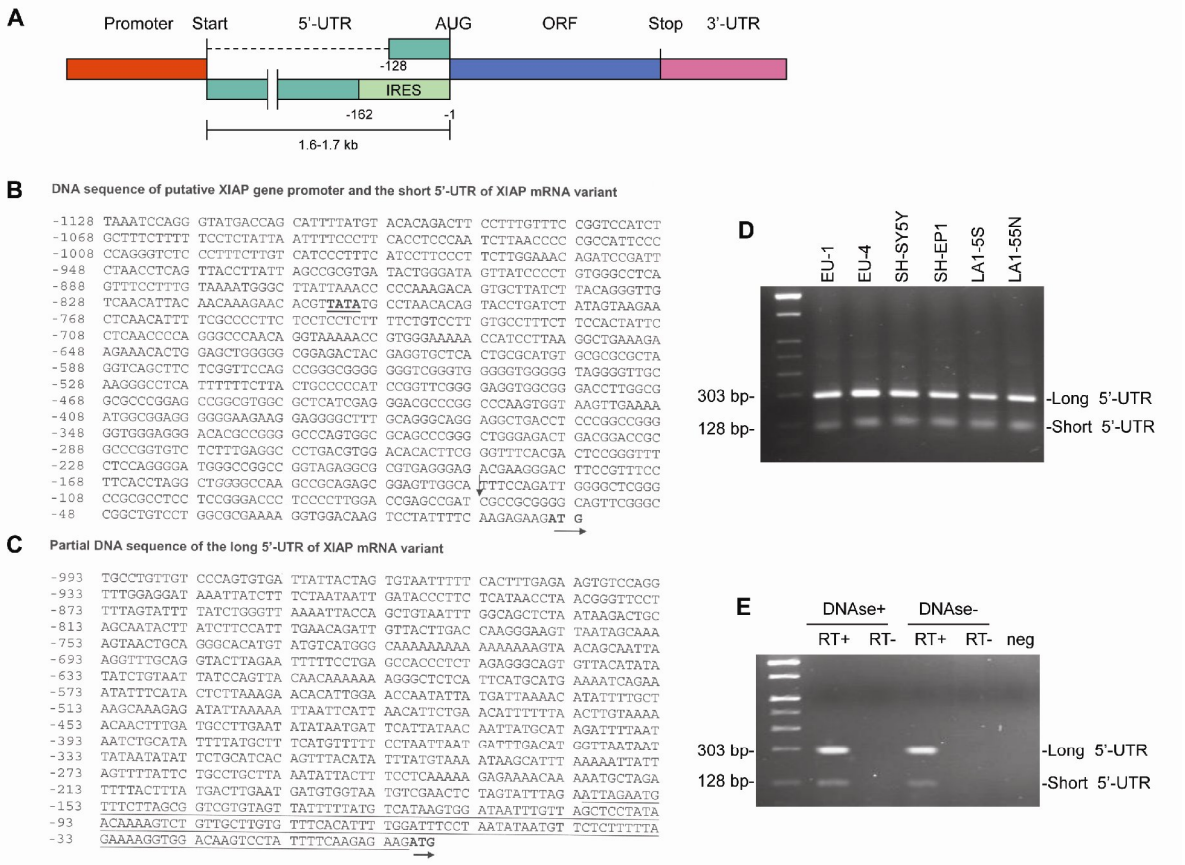

Figure S2. (A) Schematic illustration of XIAP gene containing a putative XIAP gene promoter and two 5'-UTR variants (128 bp and 1.6-1.7 kb) of XIAP mRNA. (B) DNA sequence of XIAP gene promoter and the short 5'-UTR of XIAP mRNA, reported by Liston et al (Liston et al., 1996; http://www.ncbi.nih.gov/entrez, Accession number: NM001167) and submitted by Livingston et al. (http://www.ncbi.nih.gov/entrez, Accession number: AY886519). The potential promoter region (-128 to -1128) contains a TATA box as indicated. The 128 bp short 5'-UTR is located between the cDNA start site (-128, vertical arrow) and the known first codon (-1, horizontal arrow). (C) Partial nucleotide sequence of the long 5'-UTR of XIAP mRNA (1.6 or 1.7 kb) containing a 162-bp IRES (underlined), reported by Holcic et al (Holcic et al., 1999) and submitted by Livingston et al. (AY886519). The horizontal arrow indicates the first codon as same as in (B). The short and long 5'-UTR share same sequence from -1 to -33. (D) Results of RT-PCR using specific primers designed from the short and long 5'-UTR show that all cancer cell lines studied expressed both XIAP mRNAs that contain the short and long 5'-UTR. (E) RT-PCR was performed using the same primers as in (D) and total EU-1 RNA that was digested with or without DNAse, and in the presence or absence of RT. Data confirm that XIAP IRES in the long 5'-UTR is mRNA but not genomic DNA.

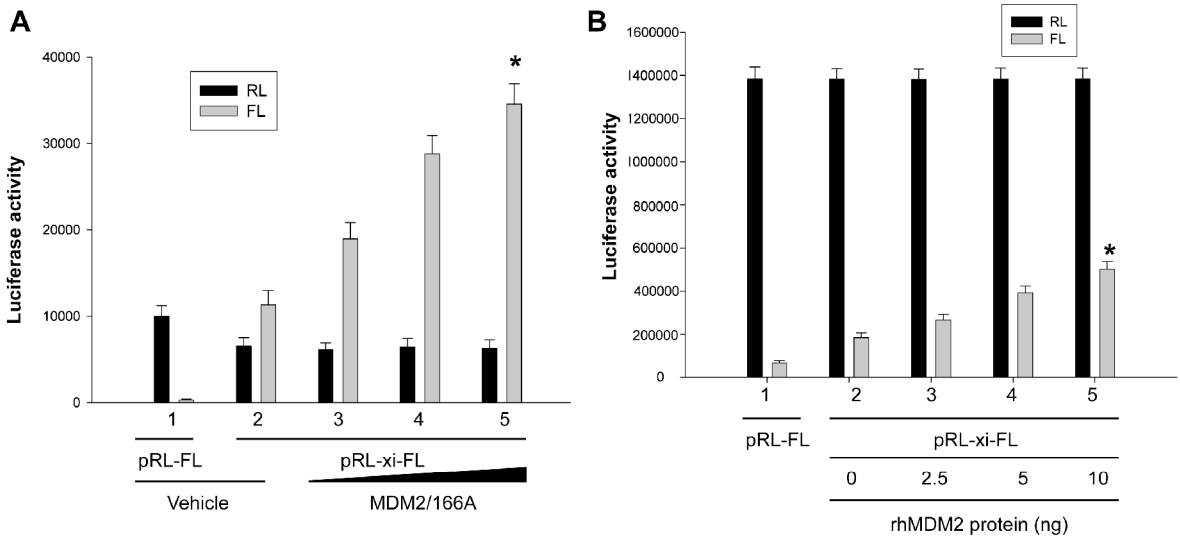

Figure S3. (A) Induction of XIAP IRES (xi)-mediated translation by MDM2 in transfection of EU-4 cells, using the dicistronic plasmid pRL-xi-FL. Cells were co-transfected with 5 μg pRL-xi-FL and increasing amounts of MDM2/166A (2.5, 5, and 10 μg), and a control prepared without MDM2 (vehicle, pcDNA empty vector). Controls included transfection of the pRL-FL empty vector alone. Quantitative RL and FL activities were detected using the Dual-Luciferase Reporter System. The bars represent the average ±SD of three independent experiments. * $p< 0.01$ for the comparison between lanes 5 and 2. (B) Induction of XIAP IRES activity by MDM2 in an *in vitro* translation assay. The *in vitro* translation assay was performed using either the pRL-xi-FL plasmid or the pRL-FL vector as a control, and the TnT® T7 Quick Coupled Transcription/Translation System (Promega) in the presence or absence of recombinant human MDM2 (rhMDM2) protein (Calbiochem). The bars represent the mean ±SD of three independent experiments. *p<0.01 for the comparison with *bar* 2.

Supplemental Materials and methods

Cells

SH-EP1 is a substrate-adherent (S) type clone, while the SH-SY5Y cell line is a neuroblastic (N) type clone of SK-N-SH (Ciccarone et al., 1989). Both LA1-55N (N-type) and LA1-5S (S-type) were cloned from the LA-N-1 cell line (Ross et al., 2004). All four NB lines were obtained from the American Type Culture Collection (ATCC). The EU-1 and EU-4 leukemia cell lines were established in our laboratory from pediatric ALL patients at Emory University. Cultured EU-1 and EU-4 cells resembled the primary leukemic cells: EU-1 cells have wt-p53 and overexpress MDM2, whereas EU-4 cells lack p53 expression and express very low levels of MDM2 (Zhou et al., 1995). All cell lines were grown in standard culture medium (RPMI 1640 containing 10% FBS, 2 mmol/L of L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin) at 37^0C, in 5% CO_2 - in-air.

Plasmids

Human wt MDM2 cDNA was cloned into a pDsRed1-C1 vector between EcoR1 and BamH1, to generate the plasmid pDsRed1-C1/MDM2. The Quick Change site directed mutagenesis kit (Stratagene) was used to mutate the Akt phosphorylation site from serine 166

to either alanine or glutamic acid (MDM2/166A or MDM2/166E) in pDsRed1-C1/MDM2. The wt and various C-terminal truncated and mutated GST-tagged MDM2 constructs were generated by polymerase chain reaction and cloned into the bacterial pGEX expression vector. The mutated constructs included 415-491 Δ1 with a Ser at position 428 changed to Gly, 415-491 Δ2 with a mutation of Gly at 448 to Ser, and 415-491 Δ3 having a substitution of Leu at 487 by Ser. The Tet-On gene expression systems including pTet-On and its response (pTRE2hyg) plasmids were purchased from Clontech (Palo Alto, CA). The human MDM2 cDNA was cloned into the pTRE2hyg at the Bam H1 restriction site. The pSUPER/MDM2 siRNA plasmids were constructed by inserting several specific 19 nt MDM2 sequence (GAAGTTATTAAAGTCTGTT, ACACTTATACTATGAAAGA, CAACATATTGTATATTGTT) into an expression plasmid, pSUPER-neo, purchased from OligoEngine (Seattle, WA). The monocistronic plasmid pGL3-xp-xi was constructed by first inserting the XIAP promoter (xp) into the pGL3-basic vector at the Xho1 and Hand III sites, then inserting a 670 bp cDNA fragment from the 5'-UTR of XIAP containing the 162-bp XIAP IRES (xi) immediately downstream of xp, at the Hind III and Nco1 sites. Various monocistronic plasmids containing deleted xi or xi in a reverse orientation served as controls. All the generated plasmids were DNA sequenced in order to confirm the presence of the expected deletions and mutations.

Gene transfection and reporter assay

To establish an MDM2-inducible model, both pTet-On and pTRE2hyg-MDM2 plasmids were stably transfected into EU-4 cells to generate a cell line EU-4/Tet-On/MDM2, where MDM2 expression was induced by addition of doxycycline in the cell culture. EU-4 cells in exponential growth were transfected with pTet-On plasmid by electroporation at 300 V, 950 μF, using a Gene Pulser II System (Bio-Rad, Hercules, CA). The cells were seeded 48 hours post-transfection into culture dishes for the selection of G418-resistant colonies. Colonies were grown in methylcellulose medium containing G-418 (500 μg/ml) for 2-3 weeks, and the clones were picked and grown in RPMI medium with or without G-418 for the subsequent transfection of pTRE2hyg-MDM2 plasmid. Transfection was performed as described above, and stably transfected colonies were selected by hygromycin B. For stable MDM2 siRNA transfection, LA1-55N cells in exponential growth were transfected with pSUPER/MDM2 or pSUPER containing a scrambled 19-nt (control) plasmid by electroporation as described above.

Transient transfection was performed to examine the effect of MDM2 on XIAP IRES activity: EU-4 cells were co-transfected with various MDM2 expression plasmids and either monocistronic or bicistronic XIAP IRES reporter plasmids. The subcellular distribution of transfected MDM2 in pDsRed1-C1 vector was detected by confocal microscopy. The pRL (*Renilla* luciferase)-CMV vector was co-transfected in the transfection of monocistronic plasmid to provide an internal control. Transfected cells were resuspended in 10 ml of RPMI containing 10% FBS and incubated 24-36 hours. Cell extracts were prepared with 1 x lysis buffer, then 20 μl aliquots of the supernatant were mixed first with 100 μl of Luciferase Assay Reagent II (Promega) to measure the FL activity and next the RL activity was determined by adding Stop & Glo® Reagent to the same sample. These luciferase activities were analyzed on a Microplate Luminometer (Turner Designs). In the transfection with bicistronic plasmid, cells were washed in 1 ml phosphate-buffered saline (PBS) and harvested in 300 μl of CAT ELISA kit lysis buffer. β-Galactosidase (βgal) enzymatic activity was determined by the chemiliminescent reporter gene assay system (Applied Biosystems, Bedford, Massachusetts). CAT levels were determined using the CAT ELISA kit, according to the protocol provided by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). In the transfection with dicistronic plasmid pRL-xi-FL, the RL and FL activities were determined as described above.

Western blot assay

Whole cell protein were prepared by lysing cells for 30 min at 4^0 C in a buffer composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5mM EDTA, 1% (v/v) Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 25 μg/ml leupeptin. To detect the cellular localization of MDM2, both nuclear and cytoplasmic fractions were isolated using the NE-PER kit (Pierce) according to the instructions of the manufacturer. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose filter. After blocking with buffer containing 5% non-fat milk in 20 mM Tris-HCl (pH7.5) with 500 mM NaClfor 1 h at room temperature, the filter was incubated with specific antibodies for 1 h at room temperature, followed by HRP-labeled secondary antibody. The blots were developed using a chemiluminescent detection system (ECL, Amersham Life Science, Buckinghamshire, England).

Northern blot assay

Total RNA was electrophoresed using a 1% agarose/6% formaldehyde gel and transferred to a nylon filter. Probes were prepared by a randomized-labeling approach using α-^{32}P-dCTP. Hybridization was performed in 50% (v/v) formamide/5x SSC/1% SDS/5x Denhardt's/20% dextran sulfate/100μg/ml sheared salmon sperm DNA solution at 42^0 C for 16 h. A final wash was carried out in 1x SSC/0.1% SDS at 65^0 C for 30 min. After washing, the filter was autoradiographed for 24 hours.

Reverse transcription (RT)-PCR

The total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and was digested with DNAse for examining the expression of both short and long 5'-UTR of XIAP mRNA. RT-PCR was performed with an Access RT-PCR Kit (Promega Corporation) according to the manufacturer's protocols. Reactions without addition of reverse transcriptase (RT-) served as controls. To specifically amplify and detect short and long 5'-UTR of XIAP mRNA, two forward primers 5'-TTTCCAGATTGGGGCTCG-3' and 5'-TTTATGTAAAATAAGCAT-3' for short and long 5'-UTR of XIAP respectively, and one common reverse primer 5'-CTTCTCTTGAAAATAGGA-3' were used. Expected sizes of PCR products for short and long 5'-UTR were 128 bp and 303 bp respectively. The PCR products were mixed and visualized with ethidium bromide staining under UV light after electrophoresis on a 1.5% agarose gel. For testing XIAP mRNA expression, First-strand cDNA synthesis was performed with mixture of random nonamers and oligo-dT as primers (Qiagen). Amplification of XIAP was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using QuantiFast SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions. The XIAP primers and the house-keeper gene GAPDH were purchased from Qiagen (sequences of the primers not provided).

Polysome preparation and analysis

Polysomes profiling was carried out essentially as described previously (Feng et al., 1997) with slight modifications. Briefly, EU-1 cells, with or without exposure to IR, and SH-SY5Y cells, transfected with MDM2-166A or with empty vector as described in the corresponding experiments, were incubated with 100μg/ml CHX for 15 min to arrest polyribosome migration. Cells were then lysed to isolate cytoplasmic RNA in a buffer containing 20mM Tris-HCl at pH 8.0, 100mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 500U/ml RNAsin, and a cocktail of protease inhibitors, followed by fractionation on 15-45% (w/v) sucrose gradient. The gradient was centrifuged in a SW41Ti rotor at 39,000rpm for 1 hr. Fractions were collected from each gradient tube by up-ward replacement with monitored absorption at OD_{254} by using a fractionator (Isco, Lincoln, NE). The RNA in each fraction was extracted and subjected to Real-Time PCR as described above, using primers: XIAP (forward, 5′-

ATGACTTTTAACAGTTTTGAAGG; reverse, 5′-GCTCGTGCCAGTGTTGATGCTG) and Actin (forward, 5'-GGACTTCGAGCAAGAGATGG; reverse, 5'-AGCACTGTGTTGGCGTACAG) served as control.

mRNA degradation rate

RNA synthesis was terminated in IR treated cell cultures by addition of 5 mg/ml actinomycin D. At different times after actinomycin D addition, the cells were harvested, and total RNA was isolated. The amount of XIAP mRNA remaining was determined by Northern blotting. Comparison between experiments was carried out by normalizing the starting XIAP mRNA levels (defined as 1 unit).

In vitro translation assay

In vitro translation was performed using TnT® T7 Quick Coupled Transcription/Translation System (Promega), according to the manufacturer's protocol. The reaction mixture (50 µl) containing 1 µg of pRL-xi-FL plasmid was incubated for 60 min at 30^0C in the presence or absence of different amounts of recombinant human MDM2 (rhMDM2) protein (Calbiochem). The translation level of RL and FL was determined by Dual-Luciferase Reporter Assay System using luminometer as described above.

Expression of GST-tagged proteins

The expression and purification of GST-fused MDM2 proteins were performed as described previously (Lai et al., 1998). Briefly, after transfection of various GST-fused MDM2 plasmids into BL21 *Escherichia coli*, the cells were incubated in LB medium. The cells transfected with plasmids containing the last 11 mostly hydrophobic amino acids were incubated at 30^0C for soluble protein expression, whereas cells transfected with a plasmid with these 11 amino acids deleted were incubated at 37^0C. The cells were harvested after incubation with 0.1 mM IPTG for 2 h. Purification of the GST-fused MDM2 proteins was performed by lysing the induced cells with sonication, followed by isolation with glutathione-agarose beads (Pharmacia). The purity and correct expression of each of the GST-fused MDM2 proteins were analyzed by gel electrophoresis and Coomassie G250 staining as well as western blot assay using anti-GST antibody.

UV cross-linking and RNA binding assays

UV cross-linking and immunoprecipitation assays were performed as described previously (Holcik et al., 2000a). Briefly, the DNA templates for synthesis of the XIAP IRES RNA probe (probe 1) and a control RNA probe (probe 2) from non-IRES upstream 5'-UTR of XIAP mRNA were generated by PCR using XIAP IRES-specific primer pairs (probe 1, forward: 5′-TAATACGACTCACTATAGGGCGAAATTAGAATGTTTCTTAGCGGTC-3', reverse: 5′-CTTCTCTTGAAAATAGGAC-3'; probe 2, forward: 5′-TAATACGACTCACTATAGGGCGATTATTCTGCCTGCTTAAATATTAC-3', reverse: 5′-CTAAATACTAGAGTTCGACATTAC-3'. The forward primers incorporated the T7 promoter sequence (underlined). A previously identified MDM2-binding RNA, clone A RNA (Elenbaas et al., 1996), (probe 3) was chemically synthesized with the T7 promoter included: this was used as a positive control. Internally labeled RNA probes were synthesized by *in vitro* transcription with T7 polymerase (MAXIScript T7 RNA polymerase kit, Ambion) in the presence of [α-^{32}P] UTP (Amersham). The extracts from SH-EP1 and SH-SY5Y cells, rhMDM2 and various GST-fused MDM2 proteins were mixed with ^{32}P-labeled RNA probes. UV cross-linking of the RNA-protein complexes was performed using a 254-nm UV light source set at 400,000µJ/cm^2. The UV-irradiated RNA-protein complexes were then treated with RNase T1 and resolved by 10% SDS-PAGE gel and visualized by autoradiography.

XIAP mRNA was co-immunoprecipitated from whole-cell extracts by using the modified method of Seto *et al,* as described previously (Seto et al., 1999). Briefly, cultured SH-EP1 cells were harvested by low-speed centrifugation at 4°C. The cell pellets were resuspended in 100 µl of RNA binding buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40, 50 µM $ZnCl_2$, 2% glycerol, 1mM DTT) that was supplemented with 10 U of RNase inhibitor (5 Prime-3 Prime), then cell extracts were prepared by the freeze-thaw method. To whole-cell extracts, 5 µl of monoclonal anti-MDM2 antibody, anti-La antibody, or the anti-actin antibody (Amersham), 20 µl of protein A plus G agarose beads (Calbiochem), and 5 U of RNase inhibitor were added, after which the samples were incubated for 60 min at room temperature. Next, the beads were washed extensively with RNA binding buffer supplemented with RNase inhibitor. The RNA associated with the antibody-antigen complexes was isolated by repeat phenol-chloroform extractions, and precipitation with 2 M ammonium acetate and 3 volumes of cold ethanol. The RNA was then analyzed by RT-PCR, using XIAP-specific primers (forward, 5′-ATGACTTTTAACAGTTTTGAAGG; reverse, 5′-GCTCGTGCCAGTGTTGATGCTG).

Metabolic labeling, pulse-chase assay and cycloheximide treatment

For testing protein synthesis by metabolic labeling, EU-1 cells were pre-incubated in methionine-free DMEM containing 10% dialyzed fetal calf serum for 1 h. When indicated, the pre-incubated cells were treated with 50 µM of the proteosome inhibitor MG132 for 1 h, and irradiated with 5 Gy; then 30 min after irradiation, labeled with 100 µCi of [^{35}S]methionine (Trans ^{35}S-Label; ICN) for 5 min. The XIAP protein was immunoprecipitated onto protein A-Sepharose beads with an XIAP-specific monoclonal antibody. Finally, radiolabeled XIAP protein was assessed by SDS-PAGE analysis.

The irradiation-induced XIAP translation was also tested by protein-synthesis inhibitor cycloheximide (CHX) assay. EU-1 cells were irradiated and incubated for different times before lysis in the presence or absence of 50 µg/ml CHX (Sigma), and the expression of XIAP was detected by Western blot analysis as described above.

The half-life of XIAP in EU-4 cells transfected with MDM2 and with control plasmid was detected by pulse-chase experiments, metabolic labeling, and immunoprecipitation. The metabolic labeling was performed as described above, but the incubation time was extended to 60 min without pretreatment with MG132 and irradiation. After [^{35}S]methionine labeling, the cells were washed once in phosphate-buffered saline, and then 4 ml of complete medium (cold DMEM-10% fetal calf serum) was added. Cells were harvested after 0, 15, 30, and 60 min using NP-40 lysis buffer. Radiolabeled XIAP protein was assessed as described above and quantified by densitometric analysis. Western blot analysis was performed to determine immunoprecipitated XIAP levels to assess load, and levels were quantified by densitometric analysis. All values were corrected relative to the load control and represented graphically.

Cytotoxicity and apoptosis assays

Cells were cultured in 96-well microtitre plates with different concentrations of antisense for 44 hr. WST (25 µg/well) was then added and cells were incubated for an additional 4 hr. The optical density (OD) of the wells was then read with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 620 nm. Appropriate controls lacking cells were included to determine background absorbance.

An annexin-V assay (Oncogene, San Diego, CA) was used to quantitate apoptotic cells. Briefly, cells with or without treatment were washed once with PBS and stained with FITC-annexin-V and PI for 30 min according to the manufacturer's instructions. Stained cells were detected using the FACScan (Becton Dickinson) and analyzed using WinList software (Verity Software House Inc).

Supplemental References

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