Supporting Information

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SI Materials and Methods

Cell Culture. HeLa, Jurkat, K562, 721.221, and NK-92(MI) cells were obtained from ATCC. HeLa cells were maintained in DMEM plus 10% (vol/vol) heat-inactivated FCS plus supplement (100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, 6 mM Hepes-free acid, 1.6 mM L-glutamine, 50 μM β-mercaptoethanol) (D10). Jurkat, 721.221, and K562 cells were maintained in RPMI-1640 plus 10% (vol/vol) heat-inactivated FCS with supplement (K10). NK-92(MI) cells were maintained in RPMI-1640 plus 10% (vol/vol) heat-inactivated human AB serum plus supplement.

Reagents/Antibodies. The following antibodies were used for immunoblotting and/or immunofluorescence: mouse antibodies to β-actin (JLA20; EMD Bioscience; 1/1,000), caspase 3 (3G2; Cell Signaling Technology; 1/1,000), heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (4B10; Sigma; 1/5,000 for Western blotting; 1/2,000 for immunofluorescence), hnRNP A2/B1 (DP3B3; Sigma; 1/2,000), hnRNP C1/C2 (4F4; Sigma 1/5,000), hnRNP U (3G6; Santa Cruz Biotechnology; 1/1,000), lamin A/C (BD Biosciences; 1/1,000), tubulin-α (B5-1–2; Sigma; 1/5,000), and c-Fos (2G9C3; Abcam; 1/2,000); rat antisera to HA (3F10; Roche; 1/2,000) and BrdU (BU1/75; Abcam; 1/500); rabbit antisera to PARP-1/2 (H-250; Santa Cruz Biotechnology; 1/1,000), SET [Lieberman antibody (1); 1/1000], and c-Jun (Santa Cruz Biotechnology; 1/1,000); goat antisera to GST (GE; 1/2,000) and Ku80 (Santa Cruz Biotechnology; 1/1,000). Secondary antibodies were sheep anti–mouse-HRP (GE; 1/2,000), donkey anti-rabbit-HRP (GE, 1/2000), donkey anti-goat-HRP (Santa Cruz Biotechnology, 1/1,000), donkey anti–rat-HRP (Zymed; 1/2,000), donkey anti–mouse-488 (Invitrogen; 1/5,000), donkey anti-mouse-647 (Invitrogen; 1/2,000), donkey anti–rat-488 (Invitrogen; 1/5,000). Rabbit anti-mouse IgG (Jackson Immunoresearch), mouse IgG (Jackson Immunoresearch), and protein A-agarose beads (Roche) were used for immunoprecipitation. Mouse anti-Fas (CH11; Immunotech) was used at 1 μg/mL final concentration. Doxorubicin hydrochloride was from Fluka. zVAD-fmk was from Calbiochem. Recombinant granzyme A (GzmA) and catalytically inactive granzyme A (S-AGzmA) were purified from *Escherichia coli* as previously described (1, 2). Native granzyme B (GzmB) was purified from YT-Indy natural killer (NK) cells as previously described (3). Native rat PFN was purified from ascites fluid as previously described (1). Recombinant granzymes (Gzms) generated in a recently introduced mammalian expression system for high-yield protein (4) were used in experiments in Fig. 5 and Figs. S2*C* and S5. Briefly, GzmA or GzmB cDNA was cloned into pHLseq (kind gift of Yvonne Jones, University of Oxford, Oxford, UK) at the AgeI and KpnI sites using the forward primers 5′GAAACCGGTGACGACGACGACAAGATTATTGGAGGAAATGAA for GzmA and 5′GAAACCGGTGACGACGACGACAAGATCATCGGGGGACATGAG for GzmB (introducing enterokinase sites before the N terminus of the active proteases) and the reverse primers 5′GTGCTTGGTACCAACTGCTCCCTTGATAGTCATAAT for GzmA and 5′GTGCTTGGTACCGTAGCGTTTCATGGTTTTCTT for GzmB. The plasmid was transfected into 293T cells by calcium phosphate precipitation. The transfected cells were grown in serum-free medium (ExCell 293; Sigma) for 4 d. Recombinant protein in culture supernatants was purified by immobilized metal affinity chromatography using Nickel-NTA (Qiagen) following the manufacturer's instructions. Eluted granzymes were treated with enterokinase (0.05 IU/mL supernatant) (Sigma) for 16 h at room temperature. Active Gzms were purified on an S column, concentrated, and quality tested as previously described (5).

Cell Fractionation. K562 cells, maintained in exponential phase, were harvested by centrifugation at 400 × *g* for 10 min. The cell pellet was washed 3× in PBS, resuspended in 5× pellet volume of Buffer A [10 mM Tris (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA], and incubated on ice for 30 min. Cells were disrupted (as monitored by trypan blue staining) by three strokes of a Dounce homogenizer on ice, and intact cells were pelleted by centrifugation (100 × *g*, 30 s, 4 °C). The supernatant then was centrifuged at 16,000 × *g* for 1 min at 4 °C. The nuclear pellet was washed 3× with 5 mL cold Buffer A, centrifuged at 16,000 × *g* for 30 s, and resuspended in 0.5–1 mL PBS plus 1 mM $MgCl_2$ (PBS+Mg). Cytosolic supernatant was clarified further by centrifugation at 16,000 × *g* for 10 min to remove organelles and insoluble material. Protein content was determined using the Pierce BCA Assay. HeLa cells, transfected 18 h earlier in six-well tissue-culture plates using 2 μg DNA and 5 μL Lipofectamine 2000 (Invitrogen), were washed three times in PBS before fractionation according to ref. 6. Two micrograms of total protein were resolved per lane by SDS/PAGE. After Gzm and PFN treatment, HeLa cells were harvested by centrifugation at 400 × *g* for 3 min at 4 °C. The cell pellet was resuspended gently in 150 μL RNase-free fractionation buffer (PARIS Kit; Ambion) and kept on ice for 5 min. Nuclei were pelleted by spinning at 500 × *g* for 3 min at 4 °C. The nuclear pellet was resuspended in 125 μL fractionation buffer and centrifuged immediately at 500 × *g* for 1 min at 4 °C. After 25 μL was saved for SDS/PAGE analysis to confirm the fractionation, the pooled supernatant was mixed with 750 μL TRIzol-LS (Invitrogen). The nuclear pellet was resuspended in 250 μL disruption buffer (PARIS Kit) and mixed with 750 μL TRIzol-LS after samples were saved for protein analysis.

Two-Dimensional Electrophoresis Sample Preparation. Whole K562 nuclei (1–3 mg protein equivalents), suspended in PBS+Mg to a final concentration of 0.5 mg/mL, were left untreated or treated with 1 μM GzmA for 30 min at 37 °C. Reactions were stopped by adding 1 mM PMSF. Nuclei then were treated with 300 U/mL Benzonase (Sigma) for 30 min at room temperature. Nuclease activity was quenched by adding 10 mM EDTA for 5 min at room temperature. Nuclei then were harvested by centrifugation at 16,000 × *g* for 30 s at 4 °C, solubilized in 200–400 μL of 2D solubilization buffer [4% (wt/vol) CHAPS, 7 M urea, 2 M thiourea], and incubated on ice for 30 min before pulse sonication for 3 s using a Misonix S-4000 sonicator set to 30% maximum. Insoluble material was pelleted by Eppendorf microfuge Model 5415D at top speed for 30 min at room temperature. The supernatant was harvested, and protein was precipitated using the 2D Clean-up Kit (GE). Pellets were air dried before resuspension in 100 μL 2D solubilization buffer followed by incubation at 37 °C overnight. Protein concentration was determined using the RcDc Assay (Bio-Rad).

Two-Dimensional Gel Electrophoresis. Two hundred fifty micrograms of protein were diluted in 300 μL solubilization buffer containing 40 mM DTT and 0.2% carrier ampholytes (pH 3–10) (Bio-Rad). Samples were actively loaded under 50-μA current onto pH 3–10 nonlinear 17-cm ReadyStrip immobilized pH gradient strips (Bio-Rad) at 20 °C for 24 h. After 1 h hydration, strips were overlaid with mineral oil. After hydration was complete, strips were blotted with distilled water and placed in

a clean focusing tray with water-wetted electrode wicks in place. After mineral oil was layered on top of the strip, the focusing program was initiated (step 1: 250 V, linear slope, 1 h; step 2: 500 V, rapid slope, 1 h; step 3: 8,000 V, rapid slope, 60,000 Vh; 50 μA limit/gel) using a Bio-Rad Protean isoelectric focusing cell. After isoelectric focusing, strips were reduced in 2D equilibration Buffer 1 [6 M urea, 375 mM Tris (pH 7.4), 2% (wt/vol) SDS, 2% (vol/vol) glycerol, 10 mg/mL DTT] for 10 min at room temperature. Buffer 1 was exchanged for Buffer 2 [6 M urea, 375 mM Tris (pH 7.4), 2% (wt/vol) SDS, 2% (vol/vol) glycerol, 25 mg/mL iodoacetamide] for 10 min at room temperature. Strips were cleaned in 1× TGS [25 mM Tris (pH 8.6), 192 mM glycine, 0.1% SDS] before casting on top of a 17-cm 10% (wt/vol) PAGE gel with a 5% (wt/vol) polyacrylamide stacking gel. Focusing strips and molecular-weight markers (Bio-Rad) were cast in low-melt agarose with bromophenol blue (Bio-Rad) and placed at 4 °C until set, about 5 min. Second-dimension electrophoresis was performed at 150 V until the dye front was less than 0.5 cm from gel bottom. Gels were uncast and were silver stained using the SilverQuest staining kit (Invitrogen) using 200 mL solution per gel per staining step.

Two-Dimensional Gel Image Analysis and Mass Spectrometry. Silver-stained gels were scanned, and images were analyzed using RedFin Image analysis software (Ludesi). Spots that decreased at least 10-fold after GzmA treatment were chosen as hits if they matched unambiguously, based on nearby spots, and had a minimal volume of 100 in the untreated sample. Additional negatively stained spots (negative volume by RedFin) were added manually based on visual analysis of decreased volume after GzmA treatment. Chosen spots were excised, trypsin-digested in gel, and then analyzed on a nanoscale reverse-phase HPLC by electrospray ionization and LTQ linear ion-trap mass spectrometry at the Taplin Biological Mass Spectrometry Facility, Harvard Medical School.

GzmA Treatment of Intact Nuclei. Small-scale treatments were performed as above using 0.5 mg/mL protein equivalents of nuclei in PBS+Mg with the indicated dose of GzmA for the indicated time at 37 °C. Reactions were stopped by adding 10 mM PMSF and 5× SDS loading buffer and boiling for 5 min.

Gzm and Perforin Treatment. K562 cells (5×10^4) or HeLa cells (2×10^5) in cell buffer (10 mM Hepes, 4 mM $CaCl_2$, 0.4% BSA in HBSS) were treated for the indicated time at 37 °C with a sublytic concentration of rat perforin (PFN) (defined as the concentration that causes 5–15% cell death by propidium iodide staining in the absence of Gzms) and the indicated GzmA or GzmB concentration [prediluted in PFN buffer (10 mM Hepes in HBSS)]. Reactions were stopped by boiling in 5× SDS loading buffer or by transfer to 4 °C before nuclear/cytoplasmic fractionation. Samples were resolved by SDS/PAGE and immunoblot. For caspase inhibition, cells were preincubated with 75 μM of zVAD-fmk in loading buffer before Gzms and PFN were added. To assess cell death, 2×10^4 cells were labeled with ^{51}Cr, and ^{51}Cr release was assessed after 4 h or were stained with annexin V and propidium iodide and analyzed 1 h after treatment by flow cytometry as previously described (5). Dead cells were defined as annexin V-positive and/or propidium iodide-positive.

Cytotoxicity Assay. 721.221 cells (5×10^4) were coincubated with NK-92 cells at an effector:target ratio of 5:1 for 4 h at 37 °C. At indicated times, cells were harvested by centrifugation (250 × g for 3 min) before lysis on ice in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA]. After 5× SDS loading buffer was added, samples were boiled for 1 min and analyzed by SDS/PAGE and immunoblot.

Immunopurification of hnRNP Complexes. hnRNP complexes were purified as described (7). Immunoprecipitates were resuspended in 40 μL of 2× SDS sample buffer and analyzed by immunoblot or silver staining.

Caspase Activation. Jurkat cells (0.5×10^6/mL in K10) were treated with 1 μg/mL anti-Fas (CH11 mAb) or 1 μM doxorubicin in the presence or absence of 75 μM zVAD-fmk for the indicated time. HeLa cells were treated with 1 mM staurosporine for the indicated time. Cell death and caspase inhibition were verified by annexin V/propidium iodide staining. Cells were harvested and resuspended in RIPA buffer plus 5× sample loading buffer containing β-mercaptoethanol.

Recombinant hnRNP. hnRNP genes were amplified from HeLa cDNA using primers that added 5′-EcoRI and 3′-NotI restriction sites for insertion into pGEX-4T1 (GE). Insertions were verified by sequencing. GST-hnRNP fusion proteins were purified from BL21(DE3) *E. coli* according to the supplier's instructions. Protein purity was assessed by Coomassie blue staining of SDS/PAGE gels.

Cleavage Site Determination. GST-hnRNP A1 was treated with increasing doses of GzmA and resolved on SDS/PAGE gels stained with Coomassie blue. Bands corresponding to cleavage fragments were excised, in-gel digested with chymotrypsin, and analyzed by mass spectrometry to identify GzmA cleavage sites.

Construction of hnRNP A1 mutants. Site-directed mutagenesis introduced point mutations of R196, R232, and R284 to alanine using the following primers:

R196A forward: 5′CATCCAGCCAAAGAGGTGCAAGTGGTTCTGGAAAC
R196A reverse: 5′GTTTCCAGAACCACTTGCACCTCTTTGGCTGGATG
R232A forward: 5′GGCTTTGGTGGCAGCGCTGGTGGTGGTGGATATG
R232A reverse: 5′CATATCCACCACCACCAGCGCTGCCACCAAAGCC
R284A forward: 5′GGAAATTTTGGAGGCGCAAGCTCTGGCCCCTATGG
R284A reverse: 5′CCATAGGGGCCAGAGCTTGCGCCTCCAAAATTTCC

Mutant sequences then were PCR amplified to substitute an N-terminal HA epitope tag for GST. HA-hnRNP genes were cloned into the EcoRI and XhoI sites of pcDNA4/V5-His (Invitrogen) and pBABEpuro to obtain pBABEpuro-HA-hnRNP A1 WT or mutant. Retrovirus-containing supernatant was obtained by harvesting 293T cells 48 h after transfection with pBABEpuro-HA-hnRNP A1 (WT or mutant), pgagpol, and pMG2D. Insertions and mutations were confirmed by sequencing. To construct hnRNP A1 truncations from the pGEX4T1:hnRNP A1 WT template, an N-terminal HA tag, 5′ EcoRI restriction site, and 3′ stop codon and XhoI restriction site were added using PCR and the following primers:

HA-A1 forward:
5′ATGGAATTCATGTACCCCTACGACGTGCCCGACTACGCCTCTAAGTCAGAGTCTCC
A1 FL reverse: 5′ATGCTCGAGTCAAAATCTTCTGCCACTGCC
A1(1-196) reverse: 5′ATGCTCGAGTCATCGACCTCTTTGGCTGG
A1(1-232) reverse: 5′ATGCTCGAGTCAACGGCTGCCACCAAAGCC
A1(1-284) reverse: 5′ATGCTCGAGTCATCTGCCTCCAAAATTTCC

HA-hnRNP genes were cloned into the EcoRI and XhoI sites of pcDNA4/V5-His (Invitrogen). Truncations were confirmed by sequencing.

Fluorescence Microscopy. HeLa cells were transfected on collagen-coated coverslips with 1 μg DNA using 3 μL FuGene (Roche). Cells were fixed 18 h posttransfection with 2% paraformaldehyde for 20 min at room temperature. Fixative was removed and replaced with 50 mM ammonium chloride in PBS for 20 min at room temperature. Cells were washed 3× with wash buffer (0.05% Triton X-100 in PBS) and then were permeabilized for 15 min at room temperature with Perm buffer (0.2% Triton X-100 in PBS). After blocking with 10% FCS in wash buffer for 30 min, slides were stained with primary antibodies for 1 h at room temperature in wash buffer. Cells were washed 3× before incubation with fluorophore-conjugated secondary antibodies for 1 h at room temperature in wash buffer. Cells were washed 3× before being mounted on glass slides using VectaShield mounting medium (Vector Laboratories) with DAPI. Image acquisition by epifluorescence microscopy was performed using an Axiovert 200M microscope, equipped with a 63× lens (Pan Apochromat, 1.4 NA; Carl Zeiss). Images were analyzed with SlideBook 4.2 (Intelligent Imaging Innovations Inc.) to generate 3D stacks of optical sections acquired 0.2 μm apart before nearest-neighbor deconvolution.

BrU Incorporation. HeLa cells grown overnight in D10 on collagen-coated coverslips were washed 3× with HBSS and placed in 100 μL cell buffer containing 2 mM bromouridine (Sigma) with 100 μL PFN buffer containing PFN and GzmA (final concentration, 1 μM). Cells were incubated for 30 min at room temperature before 300 μL cell buffer was added and were incubated at room temperature for an additional 30 min. Medium was aspirated, and cells were fixed with 2% paraformaldehyde and stained as above.

Quantitative RT-PCR. Total RNA was reverse transcribed using random hexamers and superscript III reverse transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed in duplicate samples using SYBR Green FastMix (Quanta) on a BioRad CFX96. mRNA levels were normalized to *GAPDH*. Primers to amplify spliced mRNAs were chosen at neighboring exons separated by a large intron that would not be amplified unless the intervening intron was removed by splicing. Primers to amplify unspliced transcripts were chosen in an adjacent exon and intron. Primers to amplify total RNA were chosen within a single exon. The following primers were used:

MYC (spliced and unspliced) forward 5′GGAGGCTATTCTGCCCATTT
MYC (spliced) reverse 5′CACCGAGTCGTAGTCGAGGT
MYC (unspliced) reverse 5′GCATTCGACTCATCTCAGCA
MYC (total) forward 5′TTGCCGCATCCACGAAACTT
MYC (total) reverse 5′TGCAAGGAGAGCCTTTCAGA
FOS (spliced and unspliced) forward 5′TTGTGAAGACCATGACAGGAGG
FOS (spliced) reverse 5′TCCTTTCCCTTCGGATTCTCCT
FOS (unspliced) reverse 5′TTCCCAGGAAGAGTACGCTAGA
FOS (total) forward 5′TCCAGTGCCAACTTCATTCCCA
FOS (total) reverse 5′TGTCATGGTCTTCACAACGCCA
DUSP5 (spliced and unspliced) forward 5′AATGTCAGCTACAGGCCAGCTT
DUSP5 (spliced) reverse 5′AACTCGCACTTGGATGCATGGT
DUSP5 (unspliced) reverse 5′AGCCCAGAATACCACTCAGGAT
DUSP5 (total) forward 5′ATCCTGAGTGTTGCGTGGATGT
DUSP5 (total) reverse 5′AGCTGGCCTGTAGCTGACATTT
E2F1 (spliced and unspliced) forward 5′AGCTGGACCACCTGATGAAT
E2F1 (spliced) reverse 5′TGCAATGCTACGAAGGTCCTGA
E2F1 (unspliced) reverse 5′AATCCAAGCCTCTCTAGTCCCA
E2F1 (total) forward 5′TTGACCCAGGACCTCCGACAG
E2F1 (total) reverse 5′TGTCAGTGTCCTCGGAGAGCAG
JUN forward 5′TGCGTGCGCTCTTAGAGAAACT
JUN reverse 5′TCACGTGAGGTTAGTTTGGGCT
IFNA1 forward 5′ACCTTGATGCTCCTGGCACAAA
IFNA1 reverse 5′TGGTTGCCATCAAACTCCTCCT

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2. Beresford PJ, et al. (2001) Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem* 276: 43285–43293.
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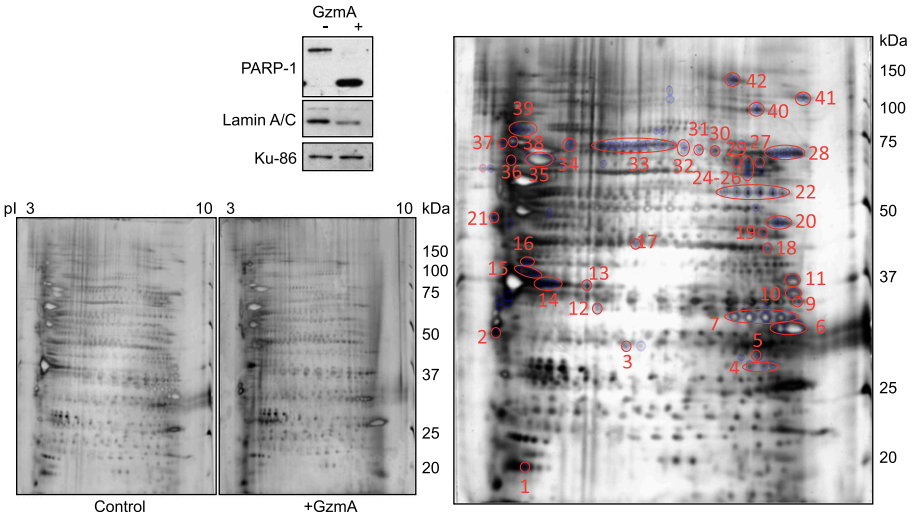

Fig. S1. Gels used to identify candidate nuclear GzmA substrates. K562 nuclei were treated with 1 μM GzmA or buffer for 30 min at 37 °C, and nuclear proteins were resolved using 2D gel electrophoresis and visualized by silver staining. GzmA nuclear activity was verified by assessing cleavage of known GzmA nuclear substrates, PARP-1, and lamin A/C, by immunoblot. Ku80, which is unaffected by GzmA, was used as a loading control. Most spots are unchanged by GzmA treatment. Spots that changed in intensity at least 10-fold after GzmA treatment were grouped into 42 spots that migrated with similar apparent molecular weight that were analyzed by mass spectrometry.

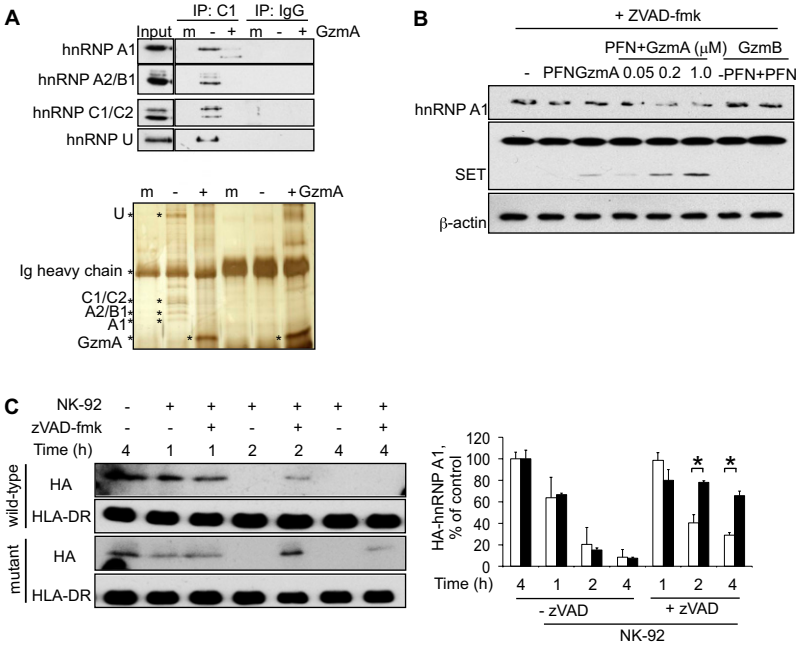

Fig. S2. GzmA cleaves multiple hnRNP proteins. (*A*) hnRNP complexes immunopurified from HeLa nuclei using anti-hnRNP C1/C2 monoclonal antibody or IgG control were mock treated or treated with 0.5 μM GzmA for 15 min at 37 °C, and protein degradation was monitored by immunoblot (*Upper*) or silver stain (*Lower*). The presumed identity of hnRNP species, Ig heavy chain, and GzmA are denoted with asterisks. m, immunoprecipitations performed without adding ("minus") nuclear lysates. Most hnRNP protein is degraded under these conditions. (*B*) K562 cells were treated with PFN and/or GzmA or GzmB at the indicated dose and time in the presence of zVAD-fmk to inhibit caspase activation. hnRNP A1 cleavage was assessed by immunoblot. Control samples were treated with buffer, PFN only, or GzmA only for 1 h. Caspase inhibition does not affect GzmA cleavage of hnRNP A1 but largely abrogates the effect of GzmB shown in Fig. 2*C*. SET is a known GzmA substrate (1); β-actin served as loading control. (*C*) (*Left*) 721.221B cells, transfected to express HA-tagged WT (*Upper*) or triple-mutant (*Lower*) hnRNP A1, were left untreated or treated with zVAD-fmk and coincubated with NK-92 cells at an effector cell:target cell (E:T) ratio of 5:1 for 1–4 h. hnRNP A1 cleavage was monitored by HA-immunoblot. HLA-DR served as a loading control. A representative blot is shown. (*Right*) Quantification by densitometry (mean± SEM, normalized to HLA-DR and then relative to untreated cells) of three independent experiments. Statistical differences between WT and mutant hnRNP A1-expressing cells were calculated using Student's *t* test (*$P < 0.05$). In this experiment, which used an E:T ratio of 5:1, hnRNP A1 cleavage in the target cell was assessed by HA-tag immunoblot of exogenously expressed HA-tagged hnRNP A1. HA-tagged hnRNP A1 became undetectable within 2 h of natural killer cell incubation unless zVAD-fmk was added to inhibit caspase activation. HA-hnRNP A1 levels declined significantly even in the presence of caspase inhibition, suggesting that both caspase-dependent and -independent cleavage of hnRNP A1 occurred.

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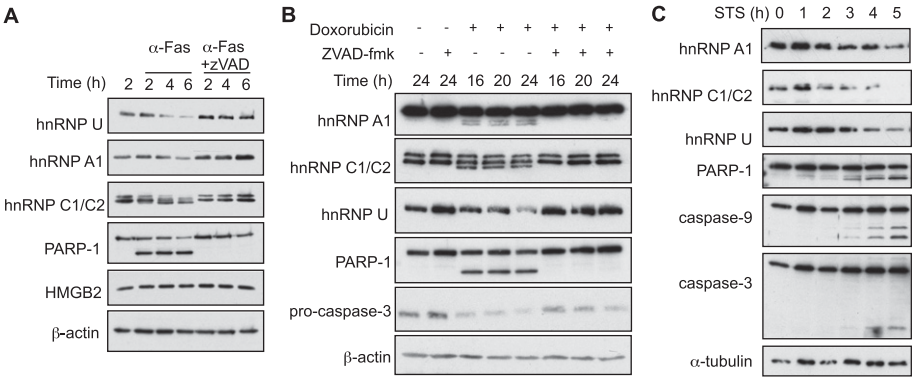

Fig. S3. Multiple hnRNPs are degraded during caspase-mediated apoptosis. Jurkat cells were treated with Fas antibody (*A*) or doxorubicin (*B*) for the indicated time in the absence or presence of zVAD-fmk. (*C*) HeLa cells were treated with staurosporine (STS) for the time indicated. hnRNP degradation was monitored by immunoblot. PARP-1 (a known caspase substrate) and procaspase-3/9 processing were monitored for caspase activation. β-Actin and tubulin served as loading controls.

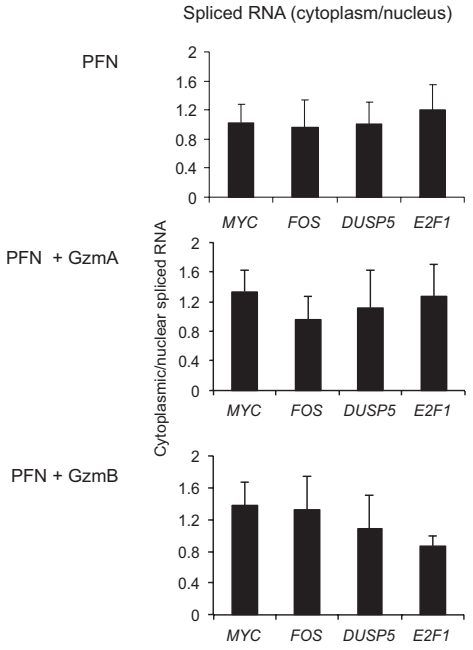

Fig. S4. Newly synthesized RNA that is spliced is exported from the nucleus efficiently during Gzm-mediated cell death. HeLa cells were left untreated or treated with PFN and/or 0.5 μM GzmA or GzmB for 1 h before RNA isolation from fractionated nuclei and cytoplasm. PCR primers were chosen to amplify unspliced, spliced, or total RNA. qRT-PCR results first were normalized to *GAPDH,* and then the ratios were normalized to their value in untreated cells. The ratio of cytoplasmic:nuclear spliced RNA is shown. The data shown are mean ± SD from four independent experiments. There was no significant difference in nuclear export of spliced RNA after Gzm treatment.

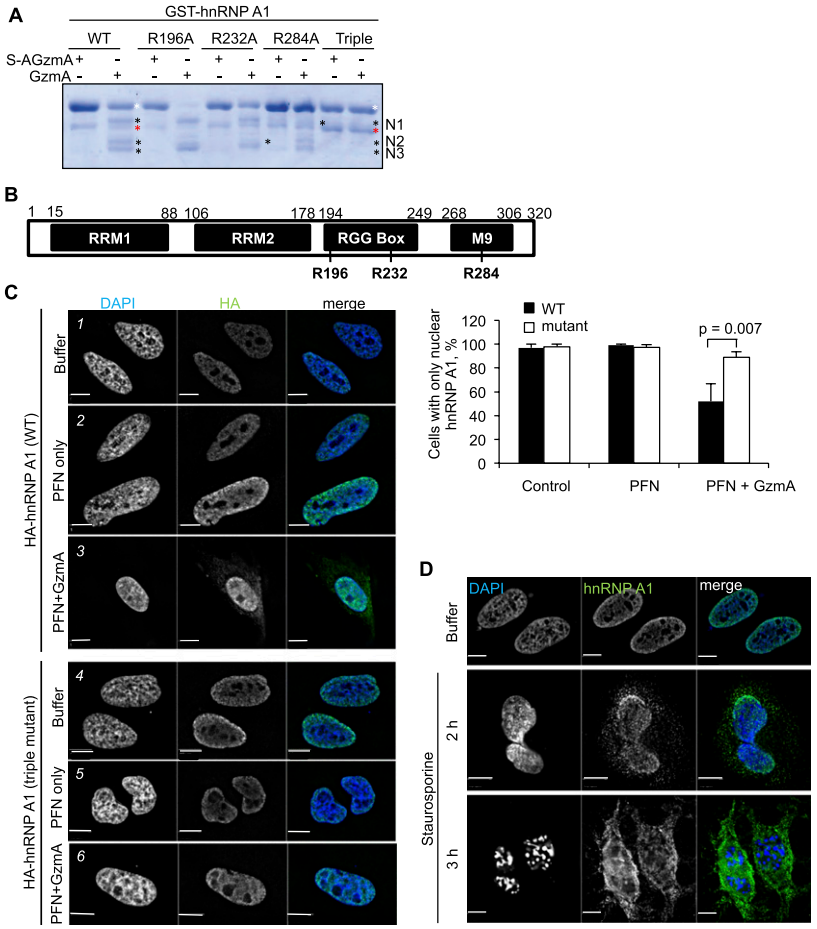

Fig. S5. GzmA cleaves hnRNP A1 after R196, R232, and R284, resulting in mislocalization to the cytoplasm. (*A*) Recombinant purified GST-hnRNP A1 (GST was added to the N terminus) was treated with 0.4 μM GzmA or S-AGzmA for 30 min at 37 °C and examined by Coomassie blue staining. Three N-terminal cleavage products (black asterisks) appear within 10 min. Yellow asterisk indicates full-length GST-hnRNP A1; red asterisk indicates a contaminating band. Peptide sequencing of these N-terminal fragments after in-gel chymotrypsin digestion identified cleavage after R196, R232, and R284. Single-point mutants (R196A, R232A, or R284A) and a triple-point mutant (R196/232/284A) of GST-hnRNP A1 also were treated with GzmA or S-AGzmA. The triple mutant was resistant to GzmA. (*B*) Domain structure of hnRNP A1 indicating GzmA cleavage sites. M9, nuclear localization signal; RGG, Arg-Gly-Gly–rich region; RRM, RNA recognition motif. (*C*) (*Left*) HeLa cells transfected with WT (panels 1–3) or uncleavable mutant (panels 4–6) of HA-hnRNP A1 were treated with buffer, PFN, and/or 1 μM GzmA for 1 h and stained for HA (green) and DAPI (blue). (Scale bars, 10 μm.) (*Right*) Quantitative analysis of proportion of cells without detectable cytoplasmic HA-hnRNP A1. Data shown are mean ± SD for three independent experiments. (*D*) Immunofluorescence localization of endogenous hnRNP A1 after staurosporine treatment. Untransfected HeLa cells were treated with 1 μM staurosporine for the indicated time and stained for hnRNP A1 (green) and DAPI (blue).

Table S1. GzmA candidate nuclear substrates identified by 2D differential proteomics

Name	Other names	MW	pI	Spot	Spot MW/pI	Peptides	% coverage
AURKB	Aurora/IPL1-related kinase 2	39.3	9.4	11	37.8/9	9	24.1
DCJ11	DNAJ homolog subfamily c member 11	63.3	8.5	25	69.2/7.5	3	6.4
DDX17	p72	72.4	8.8	23	68.7/8	10	13.2
DDX21	GU	87.3	9.3	29	72.5/7	3	3.8
DDX41		69.8	6.8	33	81/6	5	9.5
DDX5	p68	69.1	9.1	27	73.1/8	23	37
DNL3	DNA ligase 3, LIG3	102.7	9.0	40	97/8	4	4.4
ELAV1	HuR	36.1	9.2	6	28.5/9	11	31.3
FIP1	Pre-mRNA 3′-end-processing factor FIP1	66.5	5.4	37	80.7/3.5	3	6.7
FUS		53.4	9.4	23	68.7/8	5	8.6
HNRCL	hnRNP C-like 1	32.1	4.9	15	38.9/4.2	11	25.9
HNRPC	hnRNP C1/C2	33.7	5.0	16	41.1/4.2	8	27.1
HNRPD	hnRNP D0/hnRNP D	38.4	7.6	17	45.4/6	3	9
HNRPG	hnRNP G	42.3	10.1	18	44/8.5	8	19.9
HNRPL	hnRNP L	60.2	6.7	29	72.5/7	8	17.9
HNRPM	hnRNP M	77.4	8.9	31	77.7/6.2	39	36.9
HNRPQ	hnRNP Q	69.6	8.6	33	81/6	3	4.5
HNRPU	hnRNP U	90.5	6.0	41	104/9.5	7	12.6
HS71L	HSP70 like protein 1	70.4	6.0	35	74/4.8	3	5.1
IMB1	Importin beta-1 subunit (Importin 90)	97.2	4.7	38	82/3.5	4	7.5
LMNA	Lamin A/C	74.1	6.6	33	81/6	47	57.8
LMNB1	Lamin B1	66.4	5.2	35	74/4.8	67	66
LMNB2	Lamin B2	67.7	5.4	35	74/4.8	7	12.7
NKRF	NFκB-repressing factor	77.7	8.9	40	97/8	5	7
NOLC1	Nucleolar phosphoprotein p130, NOPP140	73.7	9.5	42	115/7	7	10
NONO	p54nrb	54.2	9.0	22	60/8	10	23.8
NPM	Nucleophosmin	32.6	4.6	13	33.2/5.5	6	24.5
NUCL	Nucleolin	76.5	4.6	31	77.7/6.2	3	4.7
NUP37	Nucleoporin NUP37	36.7	5.6	13	33.2/5.5	3	11
NUPL2	Nucleoporin-like 2, NLP-1, HCG1	44.9	9.3	18	44/8.5	4	11.1
PHB2	Prohibitin-2	33.3	9.8	7	30/8	10	33.1
PININ		81.5	6.8	42	115/7	5	6.1
ROA1	hnRNP A1	38.7	9.3	6	28.5/9	13	36.9
ROA2	hnRNP A2/B1	37.4	9.0	7	30/8	22	54.5
ROA3	hnRNP A3	40	9.1	11	37.8/9	9	21.4
ROA0	hnRNP A0	30.8	9.3	8	31.7/8	3	16.7
SF3B4	Splicing factor 3b subunit 4	44.4	8.6	19	48.2/8.2	3	12.7
SFPQ	H-splicing factor, PSF	76.1	9.5	40	97/8	17	17.3
SFRS1	SF2, ASF-1	27.6	10.4	5	23.7/8	5	20.2
THOC1	THO complex subunit 1, THO1	75.7	4.9	39	88.5/4	5	9.1
U5S1	116 kDa U5 small nuclear ribonucleoprotein component	109.5	4.8	39	88.5/4	3	3.4
UTP18	U3 small nucleolar RNA-associated protein 18	62	8.9	29	72.5/7	6	13.8
WDR18	WD repeat protein 18	47.4	6.2	17	45.4/6	8	21.8
ZN326	Zinc finger protein 326	65.7	5.1	34	80.7/5.2	6	11.2

The calculated molecular weight (MW) and isoelectric point (pI) are compared with their values from the GzmA-untreated 2D gel (Fig. S1). The number of peptides identified by mass spectrometry and their coverage of the protein are given. Hits were required to have at least three peptides, to be localized to the nucleus, and to be predicted to migrate roughly like the spot from which they were retrieved. MW, molecular weight.

Dataset S1. Summary of mass spectrometry results

Dataset S1

All proteins recovered by at least three peptides are shown.