THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 8, pp. 5137–5147, February 20, 2009

Nucleophosmin Is Cleaved and Inactivated by the Cytotoxic Granule Protease Granzyme M during Natural Killer Cell-mediated Killing*[S]

Received for publication, October 15, 2008 Published, JBC Papers in Press, December 22, 2008, DOI 10.1074/jbc.M807913200

Sean P. Cullen[‡], Inna S. Afonina[‡1], Roberta Donadini[§], Alexander U. Lüthi[‡], Jan Paul Medema[¶], Phillip I. Bird[§], and Seamus J. Martin[‡2]

From the [‡]Molecular Cell Biology Laboratory, Department of Genetics, The Smurfit Institute, Trinity College, Dublin 2, Ireland, the [§]Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria 3800, Australia, and the [¶]Laboratory of Experimental Oncology and Radiobiology, Center of Experimental Molecular Medicine, Academic Medical Center, Meibergdreef 9, 11005 AZ, Amsterdam, The Netherlands

Natural killer (NK) cells kill virus-infected or transformed target cells by delivering cytotoxic proteases called granzymes to the target cell cytosol. One of these proteases, granzyme M, is specifically expressed in NK cells and is thought to instigate a form of cell death distinct from that mediated by granzyme A or granzyme B. However, the mechanism of granzyme M-induced cell death is unclear at present, and few substrates for this granzyme have been reported to date. Here we show that the abundant nucleolar phosphoprotein, nucleophosmin (NPM), is cleaved and inactivated by granzyme M. NPM is essential for cell viability as RNA interference-mediated ablation of NPM expression in human cells resulted in spontaneous apoptosis. Significantly, overexpression of wild-type NPM rescued cells treated with NPM small interference RNA, whereas overexpression of the granzyme M-cleaved form of NPM did not. Because NPM is essential for cell viability, these data suggest that targeting of NPM by granzyme M may contribute to tumor cell eradication by abolishing NPM function.

NK[3] cells are important for the suppression of tumor growth and metastasis (1, 2) and for the clearance of viral infections (3–6). Unlike T cells, NK cells do not require pre-activation by dendritic cells and are thus primed to kill directly upon encounter with an appropriate target (7), promoting death through the granule exocytosis pathway (8, 9).

The granule-dependent pathway to cell death is characterized by perforin-mediated delivery of the contents of cytotoxic granules from an NK cell to the target cell cytosol. Contained within these granules are a number of destructive proteases called granzymes, which promote cell death through the proteolysis of proteins within the target cell (8, 9). Five human granzymes have been described, and each has a different substrate specificity, which suggests that they orchestrate cell death via distinct pathways (10). Granzyme A and granzyme B are the best studied granzymes to date, and, consequently, their mode of action is reasonably well understood (11). Human granzyme B (an Aspase) kills cells by directly activating caspases (12–16), and also, by inducing mitochondrial permeabilization and cytochrome *c* release through proteolysis of the pro-apoptotic BH3-only protein, BID (17–20). The release of cytochrome *c* is a significant event in many apoptotic pathways, because it promotes the formation of a complex between APAF-1 and caspase-9 called the apoptosome, with ensuing caspase-9 activation and a cascade of further caspase activation events (21–23).

Granzyme A (a Tryptase) mediates a caspase-independent form of cell death characterized by single strand DNA nicks and an increase in reactive oxygen species (24). After target cell entry, granzyme A moves to the nucleus where it releases inhibition of the endonuclease NM23-H1 by proteolysis of its inhibitor, SET, thereby freeing NM23-H1 to mediate the single strand DNA nicks synonymous with granzyme A-induced cell death (25).

Granzyme M (a Metase) is highly expressed in NK cells but not in primary T cells (26, 27), which suggests a unique role for this granzyme in innate immune system function. Indeed, studies using mice deficient for granzyme M have suggested a role for this granzyme in the clearance of mouse cytomegalovirus (28). Initial studies suggested that this protease instigated a caspase and BCL-2-independent form of cell death, distinct from that promoted by granzyme A or granzyme B, with dying cells exhibiting a necrosis-like phenotype (29). However, a recent study has indicated that granzyme M may promote target cell death in a manner similar to granzyme B, by directly cleaving both caspases and ICAD (30). Granzyme M has also been proposed to promote the death of target cells through the generation of reactive oxygen species, partly by proteolytic inactivation of heat shock protein 75 (31). Interestingly, the authors of the latter report also found that recombinant granzyme M induced mitochondrial swelling, initiated a loss of mitochondrial transmembrane potential, and promoted the

* This work was supported in part by Science Foundation Ireland Grant PI/B038. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

[1] Supported by a Health Research Board of Ireland scholarship.

[2] A Science Foundation Ireland Principal Investigator. To whom correspondence should be addressed. Tel.: 353-1-896-1289; Fax: 353-1-679-8558; E-mail: martinsj@tcd.ie.

[3] The abbreviations used are: NK, natural killer; NPM, nucleophosmin; siRNA, small interference RNA; z, benzyloxycarbonyl; fmk, fluoromethyl ketone; CFE, cell-free extract; SLO, streptolysin O; PI, propidium iodide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

release of cytochrome *c* (31), which again suggests a mode of action similar to that of granzyme B.

These conflicting data, which highlight inconsistencies concerning our current understanding of the mechanism of granzyme M-mediated killing, prompted us to undertake a detailed analysis of this "orphan" granzyme, paying particular attention to proposed similarities with the mode of action of granzyme B. Here we report that, in contrast to granzyme B, granzyme M induced a predominantly necrosis-like morphology in target cells and was incapable of activating caspases. We also identified the nucleolar phosphoprotein nucleophosmin (NPM) as a direct substrate of granzyme M. We show that NPM is essential for cell survival, because siRNA-mediated ablation of NPM expression led to spontaneous cell death. Importantly, cell death could be rescued by overexpression of full-length but not truncated forms of NPM that mimicked the granzyme M-cleaved forms of this protein. This suggests that proteolysis of this essential protein by granzyme M abolishes its function and thus may limit the oncogenic potential of NPM-associated tumors.

EXPERIMENTAL PROCEDURES

Materials—Antibodies specific to caspase-2, caspase-3, caspase-7, XIAP, signal transducers and activators of transcription-1, RhoGDI, poly(ADP-ribose) polymerase, gelsolin, BCL-x, BCL-2, and BID were purchased from BD Biosciences. Anti-caspase-8 and anti-caspase-9 antibodies were purchased from Oncogene Research Products (UK). Anti-vimentin antibody was purchased from Boehringer Mannheim GmbH (UK). Anti-β-actin and anti-α-tubulin antibodies were purchased from ICN. Anti-fodrin antibody was purchased from Chemicon. Anti-PAK2 antibody was purchased from Cell Signaling Technology. Anti-Co-chaperone p23 antibody was purchased from Affinity Bioreagents (UK). Anti-nucleophosmin antibody was purchased from Zymed Laboratories Inc.. Anti-CD2-associated protein and anti-BAK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-DFF45 antibody was purchased from BIOSOURCE. The peptides, z-VAD-fmk and Ac-DEVD-AFC were purchased from Bachem. SLO was purchased from Aalto Bio Reagents. Recombinant chymostatin was purchased from Calbiochem. Unless otherwise indicated, all other reagents were purchased from Sigma.

Cell-free Reactions—Cell-free cytosolic S-15 extracts (CFEs) were generated from Jurkat cells as previously described (22). Typically, an aliquot of extract of ~20 μg/μl was diluted 2-fold in WCEB (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Where indicated, cytochrome *c*, dATP, and z-VAD-fmk were added to reactions at a final concentration of 50 μg/ml, 1 mM, and 5 μM, respectively. Granzyme M and/or granzyme B was added at the indicated concentrations. Reactions were incubated for 2–3 h at 37 °C to facilitate granzyme-mediated proteolysis. Cell-free reactions were then assessed by immunoblotting.

Fluorometry Assays—Reactions containing Jurkat CFE were typically assembled in a final volume of 10 μl. Following a 1-h incubation at 37 °C, 2.5-μl samples were diluted to a final volume of 200 μl in WCEB containing 50 μM Ac-DEVD. Samples were then measured in an automated fluorometer (Spectrafluor Plus, TECAN, UK) at wavelengths of 430 nm (excitation) and 535 nm (emission).

Expression and Purification of Recombinant NPM in Bacteria—The pET19b expression plasmid encoding full-length human NPM was kindly provided by Dr. B Lambert (Enzymologie et Cinetique, Cachan, France). The expression plasmid was transformed into the bacterial *Escherichia coli* strain BL21(DE3) and expressed and affinity-purified as previously described (65).

Granzyme/Streptolysin O Killing Assays—Typically, streptolysin O (SLO) and granzyme M or granzyme B were added to 2.5×10^5 to 5×10^5 cells/ml. Cells were treated in a final volume of 30 μl in 96-well U-bottomed plates, followed by incubation at 37 °C for 30 min. SLO was then deactivated by diluting cell cultures 1:10 in RPMI containing 10% fetal calf serum, and cells were then transferred to 96-well flat-bottomed plates for further observation. Cell death was subsequently assessed by flow cytometry using annexin V/PI staining (12).

Analysis of Recombinant NPM by MALDI-TOF Mass Spectrometry—Typically, 3 μg of recombinant NPM was incubated for 2 h at 37 °C with varying concentrations of granzyme B or granzyme M in 10-μl reaction volumes. Reaction products were separated by SDS-PAGE and visualized by Coomassie Blue staining. Protein spots were picked from the granzyme M-induced NPM cleavage products and subjected to trypsin digestion and MALDI-TOF mass spectrometry using a Voyager DE Pro mass spectrometer (Applied Biosystems) as previously described (65).

Production of Recombinant Granzymes in Pichia pastoris—Recombinant granzyme B was expressed and purified from *P. pastoris*, and its activity was determined as described previously (66). Recombinant granzyme M was also produced in *P. pastoris*. The activity of recombinant granzyme M used in the study was 165 units/μg, on the substrate *t*-butoxycarbonyl-AAM-thiobenzyl ester (26). A unit is defined as the mass of enzyme required to cause an increase of 0.001 A_{405} nm/min in the hydrolysis of the substrate (50 μM) in a 200-μl reaction at 37 °C.

Plasmids and Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene). All plasmids were verified by DNA sequencing.

Coupled in Vitro Transcription and Translation—[^{35}S]Methionine-labeled proteins were generated using the TnT kit (Promega) as described previously (22).

siRNA-mediated Ablation of Npm Expression—For siRNA-mediated ablation of *Npm* gene expression in human MCF-7 cells, RNA interference duplexes targeting *Npm* mRNA sequence 5′-UGAUGAAAAUGAGCACCAG-3′ (NPM siRNA no. 1) or 5′-UGGUUGCAUUGUCCAUGGC-3′ (NPM siRNA no. 2) were transfected using Oligofectamine (Invitrogen), followed by incubation for 24–96 h. For rescue of NPM knockdown, NPM expression plasmids were transfected into MCF-7 cells 4 h before treatment with siRNA as described above.

One-dimensional Electrophoresis—Jurkat cell-free reactions were prepared with cytochrome *c*/dATP, granzyme B, or granzyme M as described above. Approximately 250 μg of protein extract was then analyzed by large-format one-dimensional electrophoresis and visualized by Coomassie Blue staining.

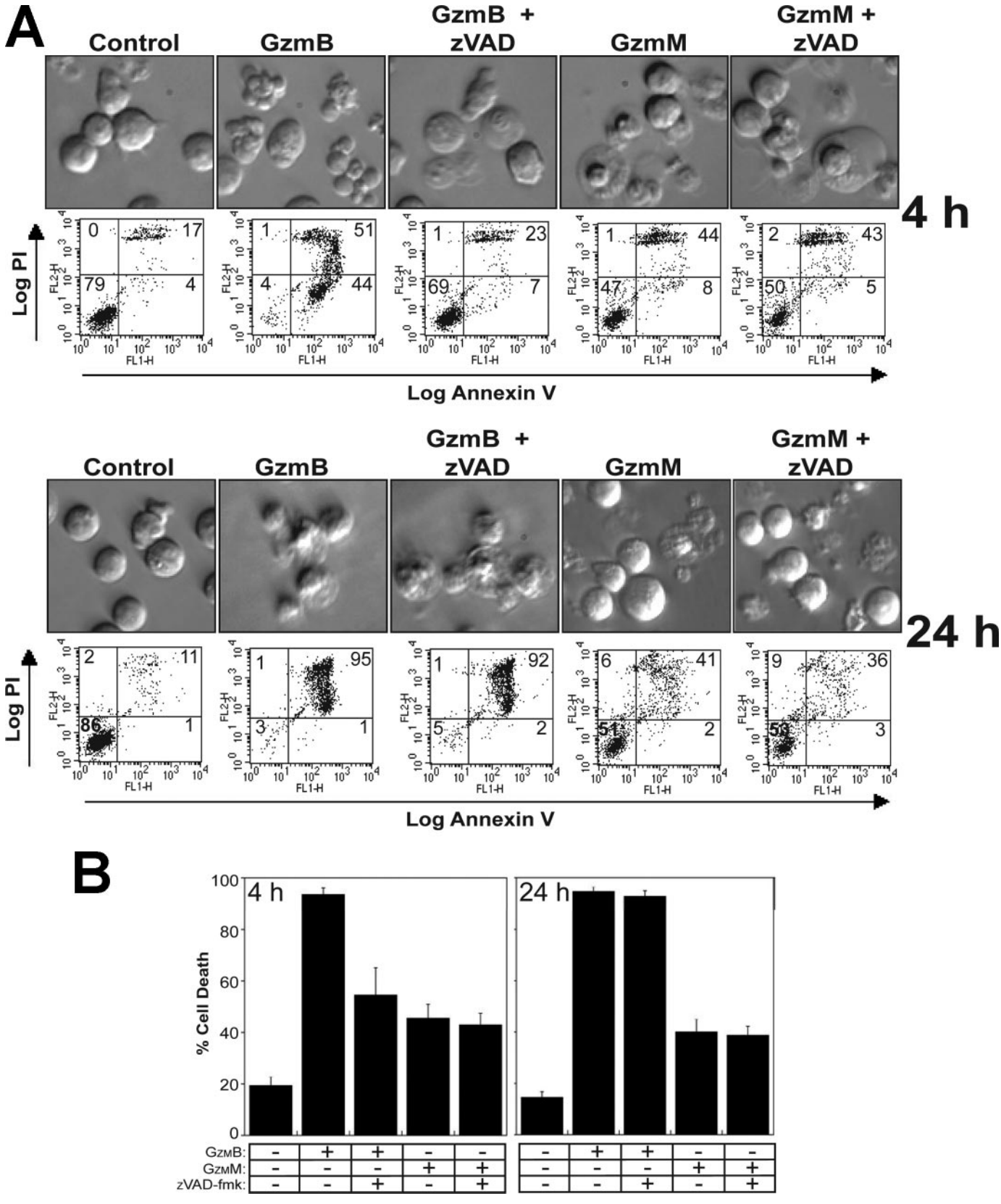


FIGURE 1. **Comparison of cell death induced by human granzyme B and granzyme M.** Jurkat cells were incubated for 30 min at 37 °C, either alone, or with human granzyme B or granzyme M (300 nM), in the presence of streptolysin O (0.75 μg/ml). Cells were then assessed at the indicated time points by phase-contrast microscopy (*A*) and annexin V/PI staining by flow cytometry (*A* and *B*). Where indicated, z-VAD-fmk (100 μM) was included to inhibit endogenous caspases. Results are representative of three independent experiments. *Error bars* represent the mean ± S.E. of triplicate counts on 5000 cells within a representative experiment.

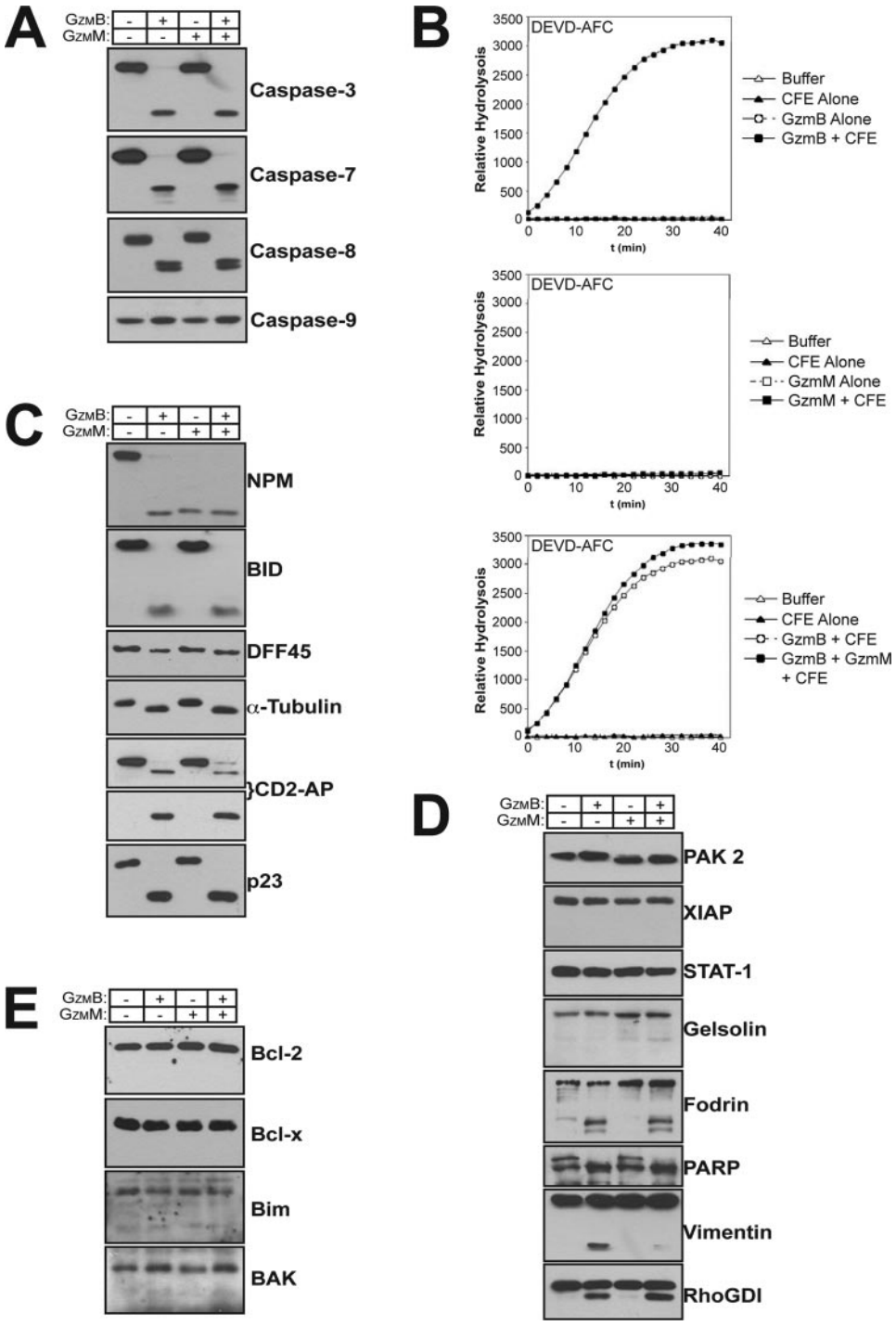


FIGURE 2. **Granzyme M targets NPM for proteolytic degradation.** Jurkat CFEs were treated for 2 h at 37 °C with granzyme B (300 nM) and/or granzyme M (300 nM) as indicated, followed by analysis of caspase-3, caspase-7, caspase-8, and caspase-9 processing by Western blotting (*A*), proteolytic processing of granzyme B substrates (*C*), proteolytic processing of caspase substrates (*D*), or proteolysis of BCL-2 family proteins (*E*). z-VAD-fmk (5 μM) was included in all reactions to suppress endogenous caspase activation. *B*, Ac-DEVD-AFC hydrolysis in Jurkat CFE treated with granzyme B (300 nM) and/or granzyme M (300 nM) for 1 h at 37 °C. Note that incubation of either granzyme with Ac-DEVD-AFC in the absence of CFE did not result in peptide hydrolysis. Results shown are representative of at least three independent experiments.

Image Acquisition and Analysis—The cell images presented in Figs. 1, 3, and 6 were taken on an inverted microscope (Olympus IX71) using a 40× objective, and images were captured using a Colorview II camera (Soft Imaging System) equipped with Analysis image acquisition software. All images were acquired at room temperature. All Western blot images were scanned from x-ray film using a scanner (Epson Perfection) and Adobe Photoshop software. All figures were prepared using Adobe PageMaker 7.0 software.

RESULTS

Granzyme M Promotes Lysis of Target Cells That Is Distinct from Granzyme B-mediated Killing—To investigate the mechanism of granzyme M-mediated killing, we loaded *P. pastoris*-derived human granzyme M into human Jurkat cells using the pore-forming protein, SLO. For comparison, we also delivered *P. pastoris*-derived human granzyme B into Jurkat cells in a similar manner. As shown in Fig. 1*A*, loading of Jurkat cells with granzyme B led to rapid cell death (within 4 h), as assessed by annexin V staining and propidium iodide (PI) uptake. Granzyme B-treated cells exhibited all of the typical features of apoptosis, with distinctive membrane blebbing, and became annexin V-positive in the absence of PI uptake (Fig. 1*A*). Inhibition of caspases, by inclusion of the polycaspase inhibitor z-VAD-fmk in the assay, transiently delayed apoptosis induced by granzyme B, as expected (Fig. 1, *A* and *B*). After 24 h however, all granzyme B-treated cells had died (Fig. 1, *A* and *B*), regardless of the inclusion of caspase inhibitor, most likely due to BID-mediated targeting of mitochondria by granzyme B (17–20).

In marked contrast, loading of cells with granzyme M initiated rapid cell death, characterized by rapid cell swelling and extensive cell lysis typical of necrosis, and cells became simultaneously double-positive for annexin V and PI (Fig. 1*A*). Furthermore, preincubation of target cells with z-VAD-fmk did not afford protection from granzyme M-mediated killing (Fig. 1, *A* and *B*). These data strongly suggest that granzyme M promotes a lytic form of cell death, which does not involve caspase activation or display features of apoptosis (Fig. 1, *A* and *B*).

Granzyme M Fails to Cleave Caspases or BID—To further explore whether granzyme M is capable of activating caspases,

we added this granzyme to Jurkat CFE and monitored caspase activation. Granzyme B was used as a control, because this granzyme readily processes caspases. Western blot analysis demonstrated that, although 300 nM granzyme B efficiently cleaved caspase-3, -7, and -8, an identical concentration of granzyme M did not (Fig. 2*A*). As a more sensitive readout of caspase activation, we measured hydrolysis of the caspase-3/-7 fluorogenic peptide substrate, DEVD-AFC. Fig. 2*B* illustrates that, although granzyme B promoted very efficient DEVD hydrolysis in cell-free extracts, no caspase activity was detectable in response to granzyme M. Granzyme M has recently been shown to promote the release of cytochrome *c* from mitochondria (31) and, in light of this, we asked whether this granzyme could mediate cytochrome *c* release by cleaving BID, in a manner similar to granzyme B. Fig. 2*C* demonstrates that, although 300 nM granzyme B efficiently cleaved BID, an equal concentration of granzyme M did not. Together, these data strongly suggest that granzyme M does not activate caspases or BID and thus, promotes cell death in a fashion distinct from that mediated by granzyme B.

Nucleophosmin Is a Substrate for Granzyme M—As recent studies have demonstrated a similarity between the killing mechanism of granzyme B and granzyme M, we explored whether these granzymes might share similar substrates. Therefore, we screened a panel of known granzyme B substrates for proteolysis by granzyme M, with Jurkat cell-free extract used as a source of human substrate proteins. Fig. 2*C* demonstrates that the abundant nucleolar phosphoprotein, NPM, is a direct granzyme M substrate, with proteolysis going to completion at 300 nM. A small difference in the molecular weight of the granzyme B and granzyme M-mediated cleavage products suggested that granzyme M cleaved NPM at a site close to that recognized by granzyme B (Fig. 2*C*). Although granzyme B efficiently cleaved a range of other known substrates, including α-Tubulin, granzyme M did not. Of note, granzyme M failed to process both ICAD and poly(ADP-ribose) polymerase, which is at odds with a previous report (30).

In addition to screening known granzyme B substrates, we also assessed proteolysis of a panel of caspase substrates by granzyme M. Fig. 2*D* demonstrates that PAK2 was also cleaved by granzyme M. None of the other caspase substrates tested were cleaved by granzyme M, but interestingly, fodrin, vimentin, and RhoGDI were cleaved by granzyme B, albeit at a high granzyme concentration (300 nM) (Fig. 2*D*). We also undertook Western blot analysis of members of the BCL-2 family; however, this failed to yield novel substrates for either granzyme (Fig. 2*E*). In addition, adding both granzyme B and M to cell-free extracts did not have an additive effect on caspase activation or substrate proteolysis (Fig. 2). Of note, we found granzyme M to be non-promiscuous at the concentrations used in this study as incubation of this protease with Jurkat. CFE failed to reveal any detectable changes to the Jurkat cell proteome as assessed by one-dimensional gel analysis, whereas caspase activation induced many detectable alterations to the proteome when analyzed by the same method (supplemental Fig. S1).

Nucleophosmin Is an in Vivo Granzyme M Substrate—To assess the efficiency of proteolysis of NPM and PAK2 by granzyme M, we titrated granzyme M and granzyme B into Jurkat

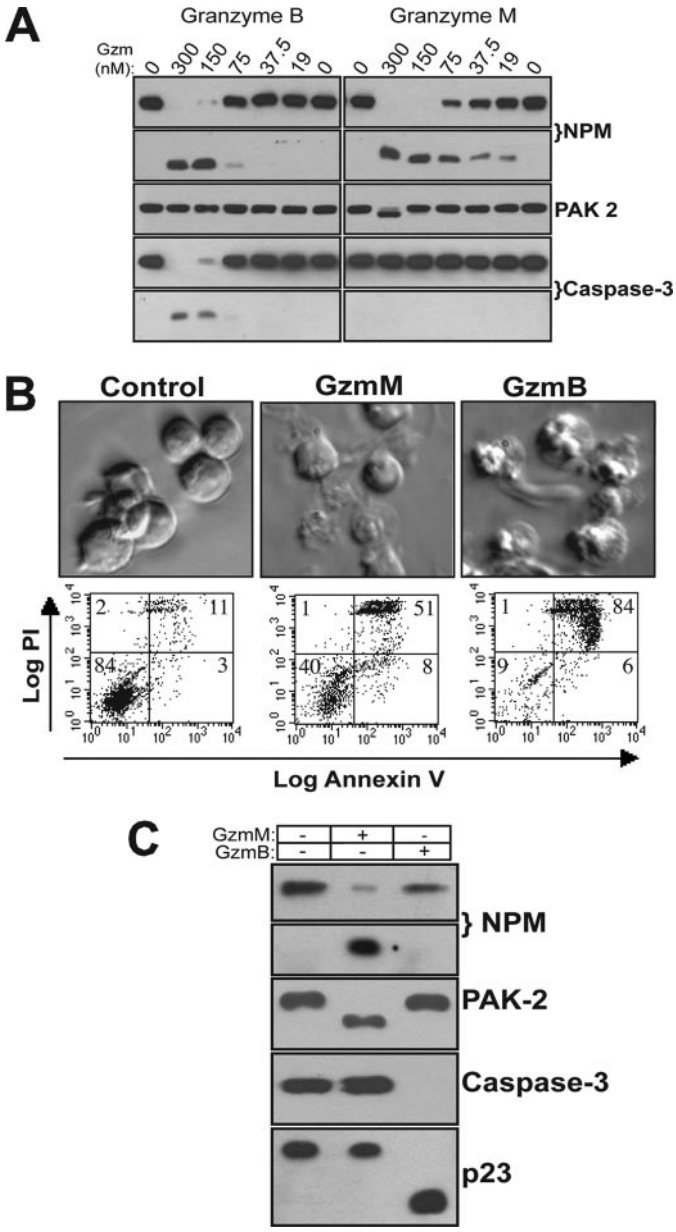
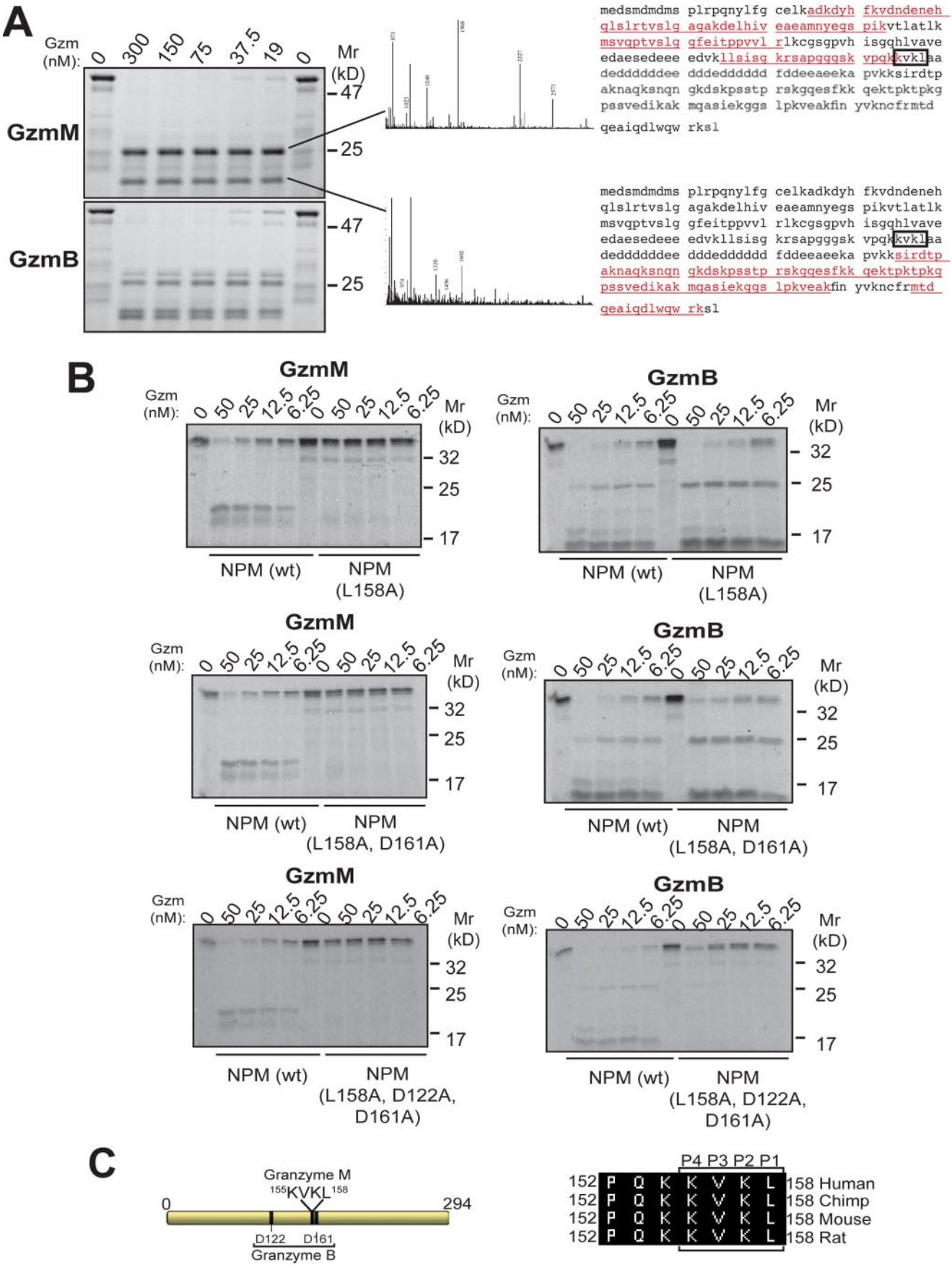


FIGURE 3. **NPM is an *in vivo* granzyme M substrate.** *A*, titration of granzyme B, *versus* granzyme M, in Jurkat CFEs. Extracts were treated with the indicated concentrations of granzyme B or granzyme M and incubated for 2 h at 37 °C, followed by Western blotting for the indicated proteins. *B*, Jurkat cells were incubated for 30 min at 37 °C with granzyme B (300 nM), or granzyme M (300 nM), in the presence of streptolysin O (0.75 μg/ml). z-VAD-fmk (100 μM) was included in all treatments to inhibit endogenous caspases. After 6 h, cell death was assessed by phase-contrast microscopy and annexin V/PI staining by flow cytometry. *C*, Jurkat cells were treated as described in *B*, and samples were then analyzed by Western blotting for proteolytic processing of the indicated proteins.

cell-free extract. Granzyme M induced efficient proteolysis of NPM, down to 19 nM, whereas in contrast, 150 nM granzyme B was required for NPM proteolysis (Fig. 3*A*). Thus, NPM is a better substrate for granzyme M than for granzyme B. Granzyme M also cleaved PAK-2; however, proteolysis was much less efficient than for NPM, with cleavage observed only at 300 nM (Fig. 3*A*).

To confirm that NPM was an authentic substrate for granzyme M, we assessed the extent of proteolysis during granzyme



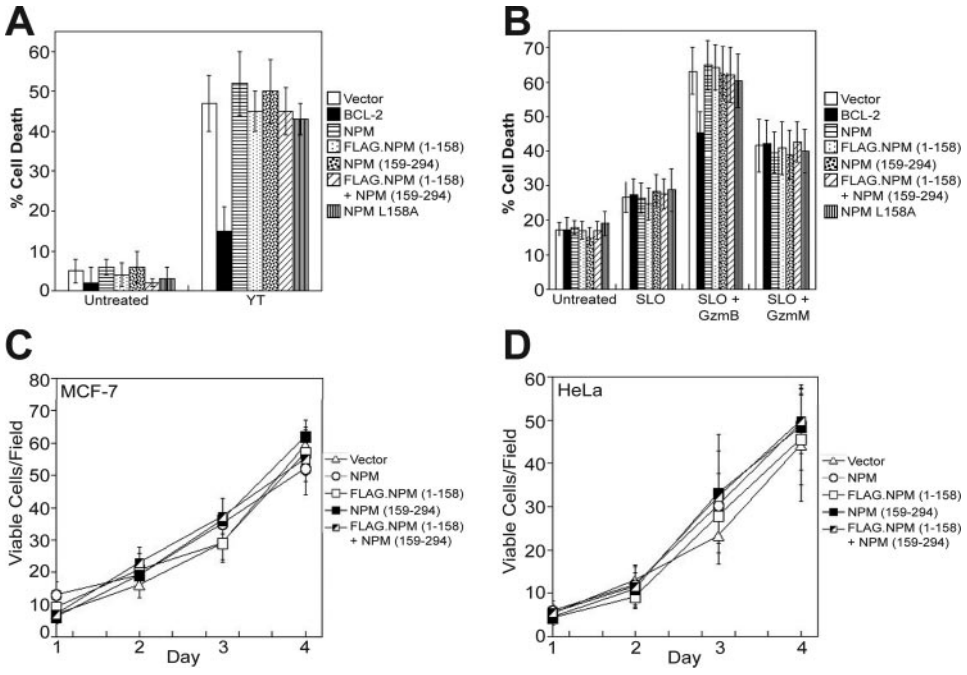


FIGURE 5. **NPM overexpression does not modulate granzyme M-mediated target cell death.** *A*, overexpression of NPM fails to modulate NK cell-mediated apoptosis. HeLa cells were transfected with the indicated pcDNA3-based plasmids together with pAAV-GFP reporter plasmid. 24 h later, cells were exposed to an effector:target ratio of 2.5:1 for 10 h, followed by assessment of apoptosis in the green fluorescent protein-positive cell population. *B*, overexpression of NPM fails to modulate direct granzyme M-induced apoptosis. HeLa cells, transfected as indicated in *A*, were incubated for 30 min with granzyme B or granzyme M (300 nM), in the presence of SLO (0.75 μg/ml) to permit granzyme entry to cells. After 4 h, cell viability was assessed by annexin V/PI staining by flow cytometry. *C* and *D*, overexpression of NPM fails to modulate the proliferation of MCF-7 and HeLa cells. Human MCF-7 (*C*) or HeLa cells (*D*) were transfected with the indicated NPM constructs, and proliferative rates were then assessed by hemocytometer counting at the indicated time points. Results are representative of three independent experiments. *Error bars* represent the mean of triplicate counts of at minimum of 100 cells per field ±S.E.

M-mediated killing of Jurkat cells. 300 nM granzyme M was sufficient to induce 60% cell death (Fig. 3*B*), and efficient proteolysis of NPM was detected by Western blot under these conditions (Fig. 3*C*). In contrast, 300 nM granzyme B killed 90% of the cells but only weakly cleaved NPM, demonstrating that NPM is a substantially better *in vivo* substrate for granzyme M than for granzyme B (Fig. 3, *B* and *C*). PAK-2 was also processed by granzyme M under these conditions (Fig. 3*C*). In an effort to determine the contribution of granzyme M to NPM proteolysis in a NK cell context, we also explored a number of commercially available calpain and lysosomal protease inhibitors in an effort to perturb granzyme M activity. However, we were unable to inhibit granzyme M in Jurkat CFEs, while z-VAD-fmk efficiently inhibited caspases under the same conditions (supplemental Fig. S2).

Granzyme M Cleaves NPM at Leu-158—To identify the granzyme M cleavage site within NPM, we cleaved recombinant NPM with granzyme M and analyzed the cleavage products by mass spectrometry (Fig. 4*A*). This revealed that granzyme M cleaved NPM somewhere between amino acids Lys-155 and Ser-195 (Fig. 4*A*). Using a peptide-based screening approach, two groups have independently determined the substrate specificity of granzyme M (32, 33). Both groups identified a strong preference of this granzyme for either leucine or methionine in the P1 position of the cleavage site (32, 33). Additionally, lysine may be preferred in the P4 position (33), Ala, Ser, or Asp may be comfortably accommodated at P3 (32), whereas Pro may be preferred at P2 (32, 33). This knowledge, in combination with our mass spectrometry data, led us to investigate a potential granzyme M cleavage site within NPM at Leu-158, KVKL (Fig. 4*A*). In addition, it was likely that granzyme B cleaved NPM at a site in close proximity to the granzyme M site as the SDS-PAGE mobility of the granzyme M and granzyme B-derived NPM cleavage products were similar (Figs. 2*C* and 3*A*). As granzyme B cleaves NPM at Asp-161 (34), this lent further credence to Leu-158 as a potential site for proteolysis by granzyme M. Indeed, subsequent mutation of the candidate Leucine residue (Leu-158) using site-directed mutagenesis confirmed that granzyme M cleaves NPM at Leu-158 (Fig. 4*B*). Notably, this motif is highly conserved in other mammals, which suggests that NPM is a conserved target of granzyme M in other species (Fig. 4*C*). In addition, the NPM (L158A) mutant was still cleaved by granzyme B, indicating that this protease cleaves the protein at a different site to granzyme M (Fig. 4*B*). However, cleavage of NPM (L158A) by granzyme B was slightly abrogated, most likely because the granzyme B cleavage site contains the mutated leucine residue at the P4 position. We also mutated both granzyme B sites along with the granzyme M site and, as Fig. *4B* illustrates, this mutant was not cleaved by either granzyme.

Previous reports have indicated that overexpression of NPM may lead to inhibition of cell death (35–37). To examine the possibility that NPM may regulate the susceptibility of target cells to cytotoxic T lymphocyte/NK-mediated apoptosis, we transfected HeLa cells with expression plasmids encoding wild-

FIGURE 4. **NPM is cleaved by granzyme M at Leu-158.** *A*, recombinant NPM, incubated alone or with the indicated concentrations of granzyme M or granzyme B, for 2 h at 37 °C, was resolved by SDS-PAGE then visualized by Coomassie Blue staining. The indicated bands, representing the N- and C-terminal cleaved forms of NPM, were analyzed by MALDI-TOF mass spectrometry. Peptides identified from the cleaved fragments are underlined on the amino acid sequence of the protein. The putative granzyme M cleavage site is *boxed*. *B*, ^{35}S-labeled *in vitro* transcribed/translated wild-type NPM, NPM L158A, NPM L158A/D161A, and NPM L158A/D122A/D161A point mutants were incubated with the indicated concentrations of granzyme M or granzyme B for 2 h at 37 °C. Samples were then resolved by SDS-PAGE and visualized by fluorography. *C*, schematic diagram of NPM indicating the granzyme M and granzyme B cleavage sites. The granzyme M cleavage site within NPM is completely conserved in other mammals. The P4, P3, P2, and P1 residues of the granzyme M cleavage site are indicated on the alignment.

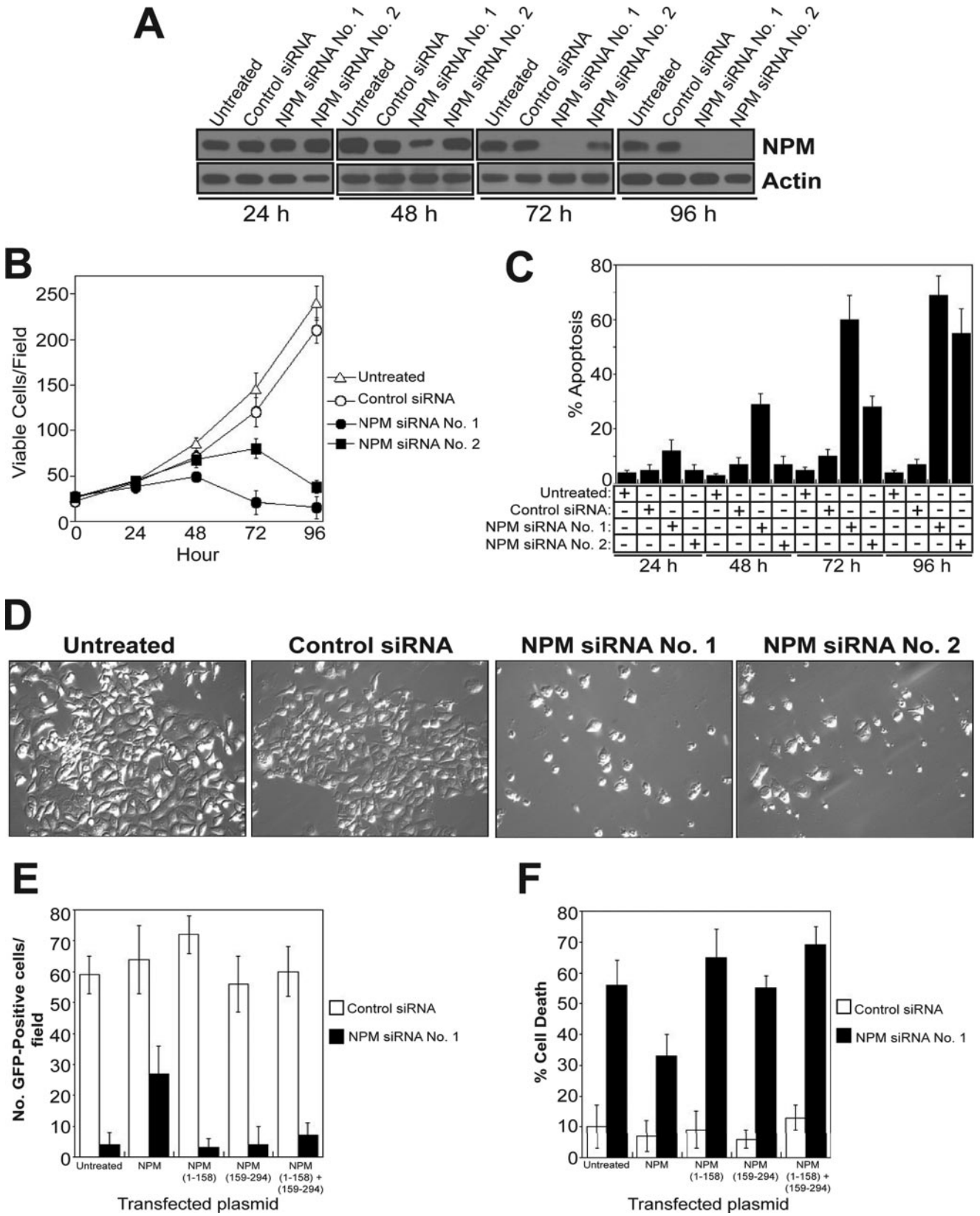


FIGURE 6. **Proteolysis of NPM by granzyme M disables its function.** *A*, MCF-7 cells were treated for 24, 48, 72, or 96 h with two different siRNA oligonucleotides targeted against NPM mRNA. Lysates were prepared at each time point, and samples were subjected to SDS-PAGE and Western blotting with anti-NPM and anti-actin antibodies. Cell viability (*B*) and % cell death (*C*) were determined at the indicated time points, and cell cultures were assessed by phase-contrast microscopy at 96 h (*D*). *E* and *F*, MCF-7 cells were transfected with the indicated constructs, followed by treatment 4 h later with siRNA oligonucleotides directed against NPM. Cell viability (*E*) and cell death (*F*) were assessed after 96 h. All cell counts represent triplicate determinations of at least 100 cells per field (±S.E.) from representative experiments.

type NPM, as well as N- and C-terminal NPM truncation proteins, which mimic the fragments generated by granzyme M-mediated degradation. We also transfected the non-cleavable NPM mutant (L158A), which is not cleaved by granzyme M. Cells transfected with these constructs were then subjected to attack by the human NK cell line, YT. However, as illustrated in Fig. 5*A*, NPM failed to protect HeLa cells from YT-mediated death. Because NK cells harbor five granzymes, including granzyme B and granzyme M, it was possible that granzyme B-mediated proteolysis of NPM (L158A) in this assay may disable the protein, thus masking a possible effect of this substrate in response to granzyme M. To address this issue, we overexpressed the panel of NPM constructs in HeLa cells and used SLO to deliver individual granzymes to ask whether NPM could modulate cell death in a purely granzyme B- or granzyme M-dependent system. However, NPM expression again failed to modulate cell death in response to either granzyme (Fig. 5*B*). To further determine whether NPM conferred protection from NK-mediated cell death, we transfected HeLa cells with an NPM construct in which both the granzyme M and granzyme B cleavage sites were mutated and subjected these cells to attack by YT cells. However, this construct offered no protection against YT cells (supplemental Fig. S3), which is probably not surprising given the multiplicity of cellular substrates for granzymes. Thus, the failure to cleave a particular substrate is most likely compensated for by the availability of other substrates that are also required for cell viability. Of note, overexpression of NPM also failed to protect HeLa cells from apoptosis induced by a panel of pro-apoptotic drugs and pro-apoptotic BCL-2 family members (data not shown). These data suggest, contrary to recent studies (35–37), that NPM is unlikely to act as a direct suppressor of cell death.

Some reports have suggested that high levels of NPM may drive cell proliferation (38, 39). Indeed, the *Npm* gene is a target of the proto-oncogene *myc* (40, 41). These data prompted us to ask whether overexpression of NPM could enhance cell proliferation. Accordingly, we overexpressed the panel of NPM constructs in MCF-7 and HeLa cells and monitored proliferation rates over 4 days. However, we failed to detect any discernable effect of NPM overexpression on the proliferation rate of either human cell line (Fig. 5, *C* and *D*).

NPM Function Is Abolished by Granzyme M-mediated Proteolysis—NPM is essential for development, because mice lacking the *NPM* gene suffer embryonic lethality at mid-gestation (42, 43). In addition, cells generated from these embryos are unstable and undergo p53-dependent senescence followed by widespread apoptosis in culture (42, 43). These observations prompted us to explore the importance of NPM for cell viability. To this end, we used two different siRNA constructs directed against the *Npm* coding sequence to ablate NPM expression in MCF-7 cells, with NPM knockdown confirmed by Western blot analysis (Fig. 6*A*). Ablation of NPM expression led to a profound decrease in cell numbers over 96 h, coupled with a dramatic increase in spontaneous cell death (Fig. 6, *B–D*). Significantly, cell death was rescued by restoring NPM levels through overexpression of wild-type NPM but not by proteins that mimic the granzyme M cleavage products of NPM, suggesting that proteolysis by granzyme M abolishes NPM function and may contribute to cell death (Fig. 6*E*). It should be noted that rescue was incomplete as overexpression of NPM was simultaneously counteracted by siRNA treatment (supplemental Fig. S4). Collectively, these data suggest that disruption of NPM function by granzyme M may contribute to the NK cell-mediated eradication of tumor and/or virus-infected cells.

DISCUSSION

There has been some confusion regarding the killing mechanism of granzyme M, with some reports suggesting that this granzyme kills in a manner similar to granzyme B, while others propose a killing mechanism akin to necrosis (29–31). Here we have shown that granzyme M promotes cell death with a predominantly necrosis-like phenotype, as distinct from granzyme B, which contradicts recent reports in the literature (30, 31). Whereas granzyme B-mediated killing involved both direct activation of target cell caspases and BID cleavage, granzyme M killed cells in a caspase and BID-independent manner, further distinguishing its mode of action from that of granzyme B. Using a candidate screening approach, we identified NPM as a direct substrate for granzyme M. NPM was efficiently cleaved in cells treated with SLO/granzyme M at a site distinct from that recognized by granzyme B.

NPM was originally identified as an abundant phosphoprotein with nucleolar localization, although it constantly shuttles between the nucleus and the cytoplasm (44, 45). NPM is essential for development, as transgenic mice lacking NPM suffer embryonic lethality at mid-gestation (42, 43). NPM is a multifunctional protein and has been implicated in a diverse array of cellular functions; however, a large body of recent work has implicated this protein in the progression of human cancer. NPM overexpression in tumors of diverse origin suggests that high levels of this protein may drive tumorigenesis (46–49). In light of this, a proposed role in ribosome biogenesis (50, 51) may link NPM with tumor proliferation, because it is formally possible that high levels of this protein in tumor cells may support tumor growth by increasing rates of ribosome biogenesis. Significantly, the *Npm* gene is frequently translocated in hematopoietic tumors, resulting in the formation of oncogenic fusion proteins (52–56), with NPM thought to act as a dimerization partner for various oncogenic kinases. In addition, mutation of NPM is the most common alteration associated with acute myelogenous lymphoma (53).

These data suggest that mutation of NPM can promote tumor growth, thus implicating *Npm* as a proto-oncogene. As such, disabling NPM function may impair tumor growth. Indeed, NPM-deficient cells are profoundly unstable, undergoing p53-dependent senescence and apoptosis, as a result of spontaneous aneuploidy and DNA damage (42, 43). Loss of NPM is likely to weaken genomic stability, because this protein has also been implicated in the control of centrosome duplication (57) and in DNA repair (35, 36). We have shown here that ablation of NPM expression results in spontaneous cell death. Importantly, proteolysis of this protein by granzyme M is likely to disable NPM function, because cell death due to NPM loss could be rescued by overexpression of wild-type, but not truncated forms of NPM, which mimic the fragments generated by

granzyme M-mediated degradation. In light of the role of NK cells in the eradication of experimental tumors and in tumor immune surveillance (1, 2), these data suggest that proteolysis of NPM, by granzyme M, may contribute to the anti-tumor activity of NK cells. It should be noted that, because siRNA-mediated ablation of NPM resulted in apoptosis while granzyme M induced a mode of cell death more reminiscent of necrosis, this strongly suggests that granzyme M also targets additional proteins to kill target cells.

In addition to their anti-tumor action, NK cells are recognized as pivotal players in the innate immune response to viral infection (4–6) and are required for the control of herpesviruses (3, 58–60). NPM has been implicated in viral progression, with a number of viral proteins reported to use the shuttling capabilities of NPM to gain access to the host cell nucleus (61–64). Indeed, granzyme M-deficient mice are partially defective for murine cytomegalovirus clearance, which suggests that NPM may play an important role in murine cytomegalovirus progression (28). Collectively, these data suggest that proteolysis of NPM by granzyme M may contribute to the control of tumors and viral infections.

Acknowledgement—We thank Dr. B Lambert for the nucleophosmin bacterial expression plasmid.

REFERENCES

1. Kim, S., Iizuka, K., Aguila, H. L., Weissman, I. L., and Yokoyama, W. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2731–2736
2. Street, S. E., Hayakawa, Y., Zhan, Y., Lew, A. M., MacGregor, D., Jamieson, A. M., Diefenbach, A., Yagita, H., Godfrey, D. I., and Smyth, M. J. (2004) *J. Exp. Med.* **199,** 879–884
3. Daniels, K. A., Devora, G., Lai, W. C., O'Donnell, C. L., Bennett, M., and Welsh, R. M. (2001) *J. Exp. Med.* **194,** 29–44
4. Brown, M. G., Dokun, A. O., Heusel, J. W., Smith, H. R., Beckman, D. L., Blattenberger, E. A., Dubbelde, C. E., Stone, L. R., Scalzo, A. A., and Yokoyama, W. M. (2001) *Science* **292,** 934–937
5. Martin, M. P., Gao, X., Lee, J. H., Nelson, G. W., Detels, R., Goedert, J. J., Buchbinder, S., Hoots, K., Vlahov, D., Trowsdale, J., Wilson, M., O'Brien, S. J., and Carrington, M. (2002) *Nat. Genet.* **4,** 429–434
6. Khakoo, S. I., Thio, C. L., Martin, M. P., Brooks, C. R., Gao, X., Astemborski, J., Cheng, J., Goedert, J. J., Vlahov, D., Hilgartner, M., Cox, S., Little, A. M., Alexander, G. J., Cramp, M. E., O'Brien, S. J., Rosenberg, W. M., Thomas, D. L., and Carrington, M. (2004) *Science* **305,** 872–874
7. Moretta, A., Marcenaro, E., Parolini, S., Ferlazzo, G., and Moretta, L. (2008) *Cell Death Differ.* **15,** 226–233
8. Russell, J. H., and Ley. T. J. (2002) *Annu. Rev. Immunol.* **20,** 323–370
9. Cullen, S. P., and Martin, S. J. (2007) *Cell Death Differ.* **15,** 251–262
10. Grossman, W. J., Revell, P. A., Lu, Z. H., Johnson, H., Bredemeyer, A. J., and Ley, T. J. (2003) *Curr. Opin. Immunol.* **5,** 544–552
11. Lieberman, J., and Fan, Z. (2003) *Curr. Opin. Immunol.* **15,** 553–559
12. Martin, S. J., Aramante-Mendes, G. P., Shi, L., Chuang, T. H., Casiano, C. A., O'Brien, G. A., Fitzgerald, P., Tan, E. M., Bokoch, G. M., Greenberg, A. H., and Green, D. R. (1996) *EMBO J.* **15,** 2407–2416
13. Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) *Nature* **377,** 446–448
14. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., and Ni, J. (1996) *Cell* **85,** 817–827
15. Medema, J. P., Toes, R. E., Scaffidi, C., Zheng, T. S., Flavell, R. A., Melief, C. J., Peter, M. E., Offringa, R., and Krammer, P. H. (1997) *Eur. J. Immunol.* **27,** 3492–3498
16. Adrain, C., Murphy, B. M., and Martin, S. J. (2005) *J. Biol. Chem.* **280,** 4663–4673
17. Heibein, J. A., Goping, I. S., Barry, M., Pinkoski, M. J., Shore, G. C., Green, D. R., and Bleackley, R. C. (2000) *J. Exp. Med.* **192,** 1391–1402
18. Sutton, V. R., Davis, J. E., Cancilla, M., Johnstone, R. W., Ruefli, A. A., Sedelies, K., Browne, K. A., and Trapani, J. A. (2000) *J. Exp. Med.* **192,** 1403–1414
19. Alimonti, J. B., Shi, L., Baijal, P. K., and Greenberg, A. H. (2001) *J. Biol. Chem.* **276,** 6974–6982
20. Pinkoski, M. J., Waterhouse, N. J., Heibein, J. A., Wolf, B. B., Kuwana, T., Goldstein, J. C., Newmeyer, D. D., Bleackley, R. C., and Green, D. R. (2001) *J. Biol. Chem.* **276,** 12060–12067
21. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
22. Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin S. J. (1999) *J. Cell Biol.* **144,** 281–292
23. Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008) *Nat. Rev. Mol. Cell. Biol.* **9,** 231–241
24. Beresford, P. J., Xia, Z., Greenberg, A. H., and Lieberman, J. (1999) *Immunity* **10,** 585–594
25. Fan, Z., Beresford, P. J., Zhang, D., Xu, Z., Novina, C. D., Yoshida, A., Pommier, Y., and Lieberman, J. (2003) *Nat. Immunol.* **4,** 145–153
26. Smyth, M. J., Sayers, T. J., Wiltrout, T., Powers, J. C., and Trapani, J. A. (1993) *J. Immunol.* **151,** 6195–6205
27. Smyth, M. J., Wiltrout, T., Trapani, J. A., Ottaway, K. S., Sowder, R., Henderson, L. E., Kam, C. M., Powers, J. C., Young, H. A., and Sayers, T. J. (1992) *J. Biol. Chem.* **267,** 24418–24425
28. Pao, L. I., Sumaria, N., Kelly, J. M., van Dommelen, S., Cretney, E., Wallace, M. E., Anthony, D. A., Uldrich, A. P., Godfrey, D. I., Papadimitriou, J. M., Mullbacher, A., Degli-Esposti, M. A., and Smyth, M. J. (2005) *J. Immunol.* **175,** 3235–3243
29. Kelly, J. M., Waterhouse, N. J., Cretney, E., Browne, K. A., Ellis, S., Trapani, J. A., and Smyth, M. J. (2004) *J. Biol. Chem.* **279,** 22236–22242
30. Lu, H., Hou, Q., Zhao, T., Zhang, H., Zhang, Q., Wu, L., and Fan, Z. (2006) *J. Immunol.* **177,** 1171–1178
31. Hua, G., Zhang, Q., and Fan, Z. (2007) *J. Biol. Chem.* **282,** 20553–20560
32. Rukamp, B. J., Kam, C. M., Natarajan, S., Bolton, B. W., Smyth, M. J., Kelly, J. M., and Powers, J. C. (2004) *Arch. Biochem. Biophys.* **422,** 29–32
33. Mahrus, S., Kisiel, W., and Craik, C. S. (2004) *J. Biol. Chem.* **279,** 54275–54282
34. Ulanet, D. B., Torbenson, M., Dang, C. V., Casciola-Rosen, L., and Rosen, A. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 12361–12366
35. Wu, M. H., Chang, J. H., and Yung, B. Y. (2002) *Carcinogenesis* **23,** 93–100
36. Wu, M. H., Chang, J. H., Chou, C. C., and Yung, B. Y. (2002) *Int. J. Cancer* **97,** 297–305
37. Li, J., Zhang, X., Sejas, D. P., Bagby, G. C., and Pang, Q. (2004) *J. Biol. Chem.* **279,** 41275–41279
38. Gubin, A. N., Njoroge, J. M., Bouffard, G. G., and Miller, J. L. (1999) *Genomics* **59,** 168–177
39. Dergunova, N. N., Bulycheva, T. I., Artemenko, E. G., Shpakova, A. P., Pegova, A. N., Gemjian, E. G., Dudnik, O. A., Zatsepina, O. V., and Malashenko, O. S. (2002) *Immunol. Lett.* **83,** 67–72
40. Zeller, K. I., Haggerty, T. J., Barrett, J. F., Guo, Q., Wonsey, D. R., and Dang, C. V. (2001) *J. Biol. Chem.* **276,** 48285–48291
41. Boon, K., Caron, H. N., van Asperen, R., Valentijn, L., Hermus, M. C., van Sluis, P., Roobeek, I., Weis, I., Voûte, P. A., Schwab, M., and Versteeg, R. (2001) *EMBO J.* **20,** 1383–1393
42. Grisendi, S., Bernardi, R., Rossi, M., Cheng, K., Khandker, L., Manova, K., and Pandolfi, P. P. (2005) *Nature* **437,** 147–153
43. Colombo, E., Bonetti, P., Lazzerini Denchi, E., Martinelli, P., Zamponi, R., Marine, J. C., Helin, K., Falini, B., and Pelicci, P. G. (2005) *Mol. Cell. Biol.* **25,** 8874–8886
44. Borer, R. A., Lehner, C. F., Eppenberger, H. M., and Nigg, E. A. (1989) *Cell* **56,** 379–390
45. Yun, J. P., Chew, E. C., Liew, C. T., Chan, J. Y., Jin, M. L., Ding, M. X., Fai, Y. H., Li, H. K., Liang, X. M., and Wu, Q. L. (2003) *J. Cell. Biochem.* **90,** 1140–1148
46. Tanaka, M., Sasaki, H., Kino, I., Sugimura, T., and Terada, M. (1992) *Cancer Res.* **52,** 3372–3377
47. Nozawa, Y., Van Belzen, N., Van der Made, A. C., Dinjens, W. N., and

Bosman, F. T. (1996) *J. Pathol.* **178,** 48–52
48. Shields, L. B., Gerçel-Taylor, C., Yashar, C. M., Wan, T. C., Katsanis, W. A., Spinnato, J. A., and Taylor, D. D. (1997) *J. Soc. Gynecol. Investig.* **4,** 298–304
49. Subong, E. N., Shue, M. J., Epstein, J. I., Briggman, J. V., Chan, P. K., and Partin, A. W. (1999) *Prostate* **39,** 298–304
50. Olson, M. O., Wallace, M. O., Herrera, A. H., Marshall-Carlson, L., and Hunt, R. C. (1986) *Biochemistry* **25,** 484–491
51. Itahana, K., Bhat, K. P., Jin, A., Itahana, Y., Hawke, D., Kobayashi, R., and Zhang, Y. (2003) *Mol. Cell* **12,** 1151–1164
52. Yoneda-Kato, N., Look, A. T., Kirstein, M. N., Valentine, M. B., Raimondi, S. C., Cohen, K. J., Carroll, A. J., and Morris, S. W. (1996) *Oncogene* **12,** 265–275
53. Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettirossi, V., Saglio, G., Mandelli, F., Lo-Coco, F., Pelicci, P. G., and Martelli, M. F. (2005) *N. Engl. J. Med.* **352,** 254–266
54. Grimwade, D., Biondi, A., Mozziconacci, M. J., Hagemeijer, A., Berger, R., Neat, M., Howe, K., Dastugue, N., Jansen, J., Radford-Weiss, I., Lo Coco, F., Lessard, M., Hernandez, J. M., Delabesse, E., Head, D., Liso, V., Sainty, D., Flandrin, G., Solomon, E., Birg, F., and Lafage-Pochitaloff, M. (2000) *Blood* **96,** 1297–1308
55. Redner, R. L. (2002) *Leukemia* **16,** 1927–1932
56. Cazzaniga, G., Dell'Oro, M. G., Mecucci, C., Giarin, E., Masetti, R., Rossi, V., Locatelli, F., Martelli, M. F., Basso, G., Pession, A., Biondi, A., and Falini, B. (2005) *Blood* **106,** 1419–1422
57. Okuda, M. (2002) *Oncogene* **21,** 6170–6174
58. Biron, C. A., Byron, K. S., and Sullivan, J. L. (1989) *N. Engl. J. Med.* **320,** 1731–1735
59. Lee, S. H., Girard, S., Macina, D., Busà, M., Zafer, A., Belouchi, A., Gros, P., and Vidal, S. M. (2001) *Nat. Genet.* **28,** 42–45
60. Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., and Lanier, L. L. (2002) *Science* **296,** 1323–1326
61. Li, Y. P. (1997) *J. Virol.* **71,** 4098–4102
62. Huang, W. H., Yung, B. W., Syu, W. J., and Lee, Y. H. (2001) *J. Biol. Chem.* **276,** 25166–25175
63. Okuwaki, M., Iwamatsu, A., Tsujimoto, M., and Nagata, K. (2001) *J. Mol. Biol.* **311,** 41–55
64. Mai, R. T., Yeh, T. S., Kao, C. F., Sun, S. K., Huang, H. H., and Wu Lee, Y. H. (2006) *Oncogene* **25,** 448–462
65. Hill, M. M., Adrain, C., Duriez, P. J., Creagh, E. M., and Martin, S. J. (2004) *EMBO J.* **23,** 2134–2145
66. Cullen, S. P., Adrain, C., Luthi, A. U., Duriez, P. J., and Martin, S. J. (2007) *J. Cell Biol.* **176,** 435–444

Molecular Basis of Cell and Developmental Biology:

Nucleophosmin Is Cleaved and Inactivated by the Cytotoxic Granule Protease Granzyme M during Natural Killer Cell-mediated Killing

Sean P. Cullen, Inna S. Afonina, Roberta Donadini, Alexander U. Lüthi, Jan Paul Medema, Phillip I. Bird and Seamus J. Martin

J. Biol. Chem. 2009, 284:5137-5147.
doi: 10.1074/jbc.M807913200 originally published online December 22, 2008
