Supporting Information

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SI Materials and Methods

Reagents. Specialized chemicals were obtained from the following sources: G418, isopropyl-β-D-thiogalactopyranoside and cell culture media were from Wisent; GelCode blue stain and *N*-hydroxysuccinimidyl (NHS)-fluorescein were from Thermo Scientific; glutathione and chelating Sepharose 4B fast flow resins were from GE Healthcare; Trail was from Enzo Life Sciences; Tropix I-BLOCK was from Life Technologies; and thrombin, streptavidin-HRP, dATP, digitonin, horse cytochrome *c*, and general protease inhibitors were from Sigma-Aldrich. Caspase inhibitors and caspase substrates were obtained from BioMol Intl. Other chemicals and molecular biology reagents and enzymes were obtained from general sources.

Plasmids. Human caspase-7 (GenBank accession no. NM_001227) and human caspase-3 (GenBank accession no. NM_032991.2) cDNAs were used for all constructs. All recombinant caspase proteins were expressed using the pET-23b(+) (Novagen) plasmid, as previously described (1). For mammalian cell expression, all caspase-7 and caspase-3 proteins were expressed using a modified pcDNA3 plasmid that allowed fusion of the cDNA to a sequence encoding a Flag epitope following an XhoI restriction site. Consequently, all expressed proteins contain a C-terminal ELDYKDDDDK sequence and have the native or engineered N-terminus.

Truncation mutants for mammalian expression were generated using standard PCR and an oligonucleotide encoding a Kozak sequence as well as a 3′ reverse primer. All other mutants were generated by overlapping PCR with a pair of oligonucleotides containing the appropriate substitutions and outside primers. Caspase-3/caspase-7 chimeras were obtained by overlapping PCR with oligonucleotides encompassing the chimeric DNA sequence and outside primers. Oligonucleotide sequences are available upon request.

The p23 cDNA was cloned from a human fetal brain cDNA library using a pair of oligonucleotides that inserted a 5′ BamHI restriction site and a 3′ XhoI site. The amplified cDNA was subcloned into the same sites of the pGEX-KG plasmid for bacterial expression.

A short-hairpin RNA construct targeting caspase-7 was created using the following sequences: 5′-gatccccAGACCGGTCCTCGTTTGTAttcaagagaTACAAACGAGGACCGGTCTttttta-3′ and 5′-AGCTtaaaaaAGACCGGTCCTCGTTTGTAtctcttgaaTACAAACGAGGACCGGTCTGGG-3′ (capital letters denote the interfering sequence). Both primers (20 μM each) were annealed in buffer [30 mM Hepes-KOH (pH 7.4), 100 mM potassium acetate, 2 mM magnesium acetate] and ligated into the BglII-HindIII sites of pSuper (2). Caspase-7 resistance to RNA interference was provided by the silent mutations of three nucleotides within the recognition motif of the mature RNAi (underlined in the above sequences).

Cell Culture and Transfection. AD-293 (human embryonic kidney cells; Invitrogen) cells were grown in DMEM supplemented with 10% vol/vol FBS, 2 mM L-glutamine and penicillin/streptomycin. Every 3 d, the cells were either split or provided with fresh medium. MCF-7 cells (human mammary gland adenocarcinoma pleural effusion; ATCC), which do not express caspase-3, were propagated in EMEM medium supplemented as described for AD-293 cells. MCF-7^{sh7} stable cells were established by selecting transfected cells for 14 d using 0.5 mg·mL^{-1} G418. Clonal cell lines were obtained from the stable population using cloning rings or 96-well plate seeding (9–11 cells per mL^{-1}, 0.1 mL per well). Cells were expanded in the appropriate medium and caspase-7 levels were characterized by immunoblotting.

Cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. High-level expression was achieved using 2 (six-well plates) or 3–4 μg (6-cm dishes) of plasmid DNA. Lower levels of expression were obtained using 0.25–0.5 μg of the caspase plasmid DNA and empty plasmid (3–4 μg final quantities). Cells and debris were harvested at 24 h or the indicated time in PBS with 2 mM EDTA/EGTA, pelleted by centrifugation, washed in PBS, and kept frozen at −80 °C in 10 μL of PBS to facilitate the lysis step. We routinely obtained >80% transfection efficiency based on a β-galactosidase assay.

Caspases were labeled in intact cells with 20 μM biotinyl-VAD-fmk in culture media for 1 h at 37 °C. Then, cells were harvested and kept as described above.

Caspase Expression, Purification, and Characterization. Recombinant caspases were expressed as C-terminal His_6-tagged proteins in the BL21(DE3)*pLysS Escherichia coli* strain (Novagen) in 2xYT medium containing 100 μg·mL^{-1} ampicillin at 30 °C for 5–20 h to obtain a fully processed active protein. Proteins were purified using immobilized metal affinity chromatography as described elsewhere (3). With the exception of the caspase-7 5A4 alanine mutant and the casp3:casp7 chimera, for which enzyme concentrations were estimated based on 80% of the absorbance at 280 nm, all enzymes were active site-titrated using the irreversible inhibitor Z-VAD-fmk, as described elsewhere (3). The kinetic parameters (k_{cat}, K_M and k_{cat}/K_M) were determined by nonlinear regression using the Michaelis-Menten equation on the velocity data obtained in caspase buffer [10 mM Pipes (pH 7.4), 100 mM NaCl, 10% sucrose (wt/vol), 0.1% CHAPS, 1 mM EDTA and 10 mM DTT] using a substrate concentration range of 0–300 μM AcDEVD-Afc.

Poly(ADP Ribose) Polymerase 1 Cleavage Assays. Cellular extracts containing poly(ADP ribose) polymerase (PARP) were prepared from clonal MCF-7 cells stably expressing an anticaspase-7 short-hairpin RNA (see above) to reduce potential interference by endogenous executioner caspases. Cells were grown to confluence, washed with PBS (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), harvested in PBS containing 2 mM EDTA and EGTA and recovered by low-speed centrifugation. The following protocol was adapted from previously published work (4), and is described for a 150-mm plate. We routinely prepared extracts from eight 150-mm plates. The cell pellet was resuspended in 0.2 mL of ice-cold solution 1 [50 mM Hepes (pH 7.4), 150 mM NaCl and 25 μg·mL^{-1} digitonin] and incubated on ice for 10 min. After centrifugation at 2,000 × *g*, the pellet was resuspended by vigorous mixing in 0.2 mL of ice-cold solution 2 [50 mM Hepes (pH 7.4), 150 mM NaCl, 1% Nonidet P-40] and incubated on ice for 30 min. The soluble material (detergent extract) was recovered by centrifugation at 7,000 × *g* for 10 min and kept at −80 °C in small aliquots. The general noncaspase protease inhibitors (1 mM 1,10-*ortho*-phenanthroline, 10 μM E-64, 10 μM leupeptin, 10 μM 3,4-dichloroisocoumarin, 1 μM MG-132) were added to all buffers. The PARP cleavage assay was then performed as follows. Unless otherwise mentioned, a 20- to 30-μL reaction mixture containing detergent extract (1.5–3.5 mg·mL^{-1}) was used with 0.5–1.0 nM active-site-titrated protease in caspase buffer at 37 °C for 30–60 min.

Assays that used recombinant caspase-9 or p35-C2A were performed similarly by substituting the extracts with purified proteins. Proteins were TCA-precipitated and analyzed by SDS/PAGE. Flag-tagged PARP protein was produced in 293^{sh7} cells by transfection. PARP was recovered by immunoprecipitation using the M2 anti-flag antibody, protein A/G beads and eluted using 100 $\mu g \cdot mL^{-1}$ flag peptide.

PARP hydrolysis rate were estimated using the relation $P = 1 - e^{kEt}$, which correlates the proportion of substrate cleaved to enzyme concentration (E), time (t), and cleavage rate (k). Importantly, the same extracts preparation containing PARP was used for all determinations, which allows relative comparison.

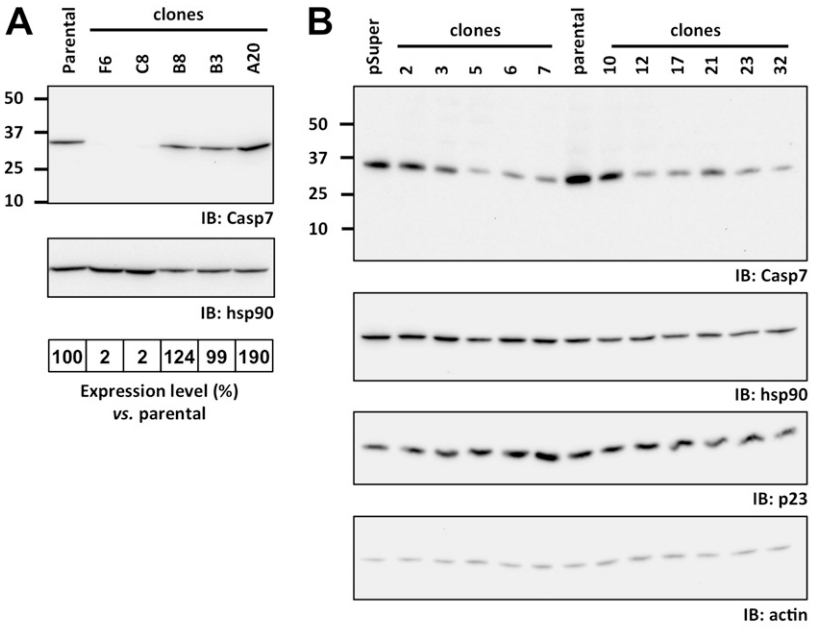
GST Pull-Down Assay. Residues 24–62 from WT caspase-7 were expressed in BL21(DE3) *Escherichia coli* as a GST-fusion protein using the pGEX-KG system. Proteins were purified using glutathione resin in PBS containing 1% Triton X-100. Pull-down assays were performed in caspase buffer containing 2 mM DTT (supplemented with the general protease inhibitors listed above and 10 μM AcDEVD-CHO) using ~10 μg (~3 nmols) of bound protein, 15 μL of resin, and 200 μg of MCF-7^{sh7} detergent extract (1 $\mu g \cdot mL^{-1}$) overnight at 4 °C. The resin was washed three times with caspase buffer, and the proteins were analyzed by immunoblotting. The postpull-down supernatant was also analyzed to estimate the pull-down efficacy.

Hypotonic Extracts. Extracts from transfected 293^{sh7} cells were prepared as described previously (5) in a Pipes buffer [20 mM Pipes (pH 7.4), 20 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$ and 2 mM DTT]. Caspases activation was programmed by the addition of 1 μM horse cytochrome *c* and 1 mM dATP to 40 μL of extracts and 20 μL of dialyzed (8,000 molecular weight cutoff membrane; Spectrum Laboratories) detergent extracts as a source of PARP. Ectopically expressed caspase-7 levels were adjusted using extracts from empty plasmid-transfected cells. Caspase-3 and -7 activity was measured using 200 μM of the chromogenic substrate Ac-DEVD-pNA.

Recombinant p23 and Fluorescein Labeling. Full-length p23 was expressed in BL21(DE3) *E. coli* as a GST fusion protein using the pGEX-4T-1 system (GE Healthcare). Proteins were purified in PBS containing 1% Triton X-100 using glutathione resin, washed with PBS and cleaved with thrombin (10 U/mL of resin) at 4 °C for 16 h. The free amines in purified p23 were trace labeled using NHS-fluorescein in a 2:1 fluorescein:p23 molar ratio. Unreacted labeling reagent and buffer were removed using a 3,000 molecular weight cutoff Amicon Ultra spin filter (Millipore).

SDS/PAGE and Immunoblotting. Cell extracts were prepared by boiling PBS-washed cells for 10 min in 50 mM Tris (pH 7.4), 4 M urea and 1% SDS. Clarified lysates were resolved on continuous SDS polyacrylamide gels using the ammediol buffer system and transferred to polyvinylidene difluoride (PVDF) membrane in 10 mM CAPS (pH 11) and 10% methanol (vol/vol) at constant current (0.4 A for 40–60 min). The ensuing blots were processed for immunoblotting with the indicated antibodies and the corresponding HRP-conjugated secondary antibodies (1:7,500; GE Healthcare) and Luminata Crescendo Western HRP substrate (Millipore). The chemiluminescence was monitored using a VersaDoc 4000MP imaging system (BioRad), and the quantification analyses were performed on the nonsaturated raw images using the QuantiOne software (BioRad). The following primary antibodies were used: caspase-7 (1:1,500; 9492; Cell Signaling Technologies), M2 anti-Flag (0.5 $\mu g \cdot mL^{-1}$; F-3165; Sigma-Aldrich); Hsp90 (0.025 $\mu g \cdot mL^{-1}$; 610419; BD Transduction Laboratories), inhibitor of caspase-activated Dnase (ICAD; 0.17 $\mu g \cdot mL^{-1}$; PX023A; Cell Sciences); p23 (1:2,000; MA3-414; Thermo Scientific); and PARP [(C-terminus; 1:7,500; 556362; BD Pharmingen) or (N-terminus; 1:7,500; ALX-804–211; Enzo Life Sciences)]. To detect biotinylated proteins, samples were prepared, resolved on SDS/PAGE and transferred onto PVDF membrane as described above, membranes were blocked using 0.2% Tropix I-BLOCK in PBS and revealed using streptavidin-HRP (0.2 $\mu g \cdot mL^{-1}$) and HRP substrate as above. Broad Range or dual color Precision Plus protein standards were used.

1. Denault JB, Salvesen GS (2003) Human caspase-7 activity and regulation by its N-terminal peptide. *J Biol Chem* 278:34042–34050.
2. Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296:550–553.
3. Denault JB, Salvesen GS (2002) Unit 21.13: Expression, purification, and characterization of caspases. *in Curr. Prot. Prot. Sci.*, eds Coligan JE, Dunn BM, Speicher DW, Wingfield PT (John Wiley & Sons, Inc., Hoboken).
4. Holden P, Horton WA (2009) Crude subcellular fractionation of cultured mammalian cell lines. *BMC Res Notes* 2:243.
5. Stennicke HR, et al. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem* 273:27084–27090.



	casp7/hsp90	casp7/actin1	casp7/actin2	casp7/p23
pSuper empty	33%	82%	76%	82%
clone 2	31%	46%	53%	57%
clone 3	17%	24%	27%	33%
clone 5	10%	6%	6%	8%
clone 6	11%	10%	15%	13%
clone 7	14%	11%	14%	10%
Parental cells	100%	100%	100%	100%
clone 10	65%	53%	53%	51%
clone 12	14%	14%	11%	12%
clone 17	22%	16%	13%	14%
clone 21	34%	24%	52%	37%
clone 23	23%	14%	11%	15%
clone 32	**11%**	**11%**	**7%**	**16%**

Fig. S1. Short-hairpin RNA-mediated repression of caspase-7 expression in clonal MCF-7 and AD-293 cells. (*A*) Clonal MCF-7 cell lines were generated using empty pSuper (clones B8 or B3) or pSuper containing a short-hairpin RNA against caspase-7 (other clones) as described in *Materials and Methods*. Clones were analyzed by immunoblotting (IB) with anti-caspase-7 and anti-Hsp90 antibodies. Caspase-7 expression was normalized with the control proteins and compared with the parental cells. Clone C8 was chosen to prepare cellular extracts. (*B*) Clonal AD-293 cell lines were generated using empty pSuper (first lane) or pSuper containing the same short hairpin RNA as in *A*. Clones were analyzed as in *A* using a panel of antibodies (two independent actin quantifications). Clone 32 was chosen.

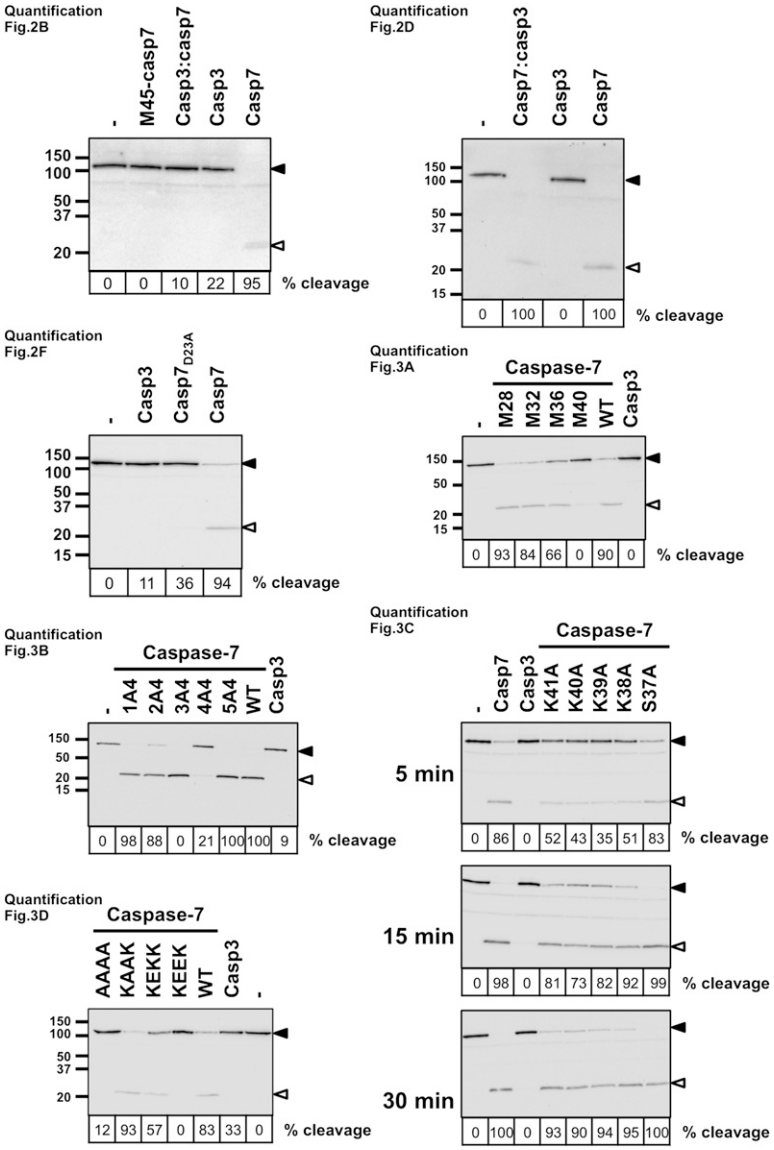


Fig. S2. Quantification of PARP proteolysis from key panels of Figs. 2 and 3. Results from Fig. 2 *B*, *D*, and *F* and Fig. 3 were further analyzed to determine the percentage of PARP proteolysis. The intensity of the 116-kDa band was quantified using Quantity One software (BioRad) from nonsaturated exposure acquired on a VersaDoc 4000MP imager. Values are percentages of cleavage compared with the untreated sample figuring on each blots. Blots are labeled as in the main text. Closed arrowhead, full-length protein; open arrowhead, cleaved fragment.

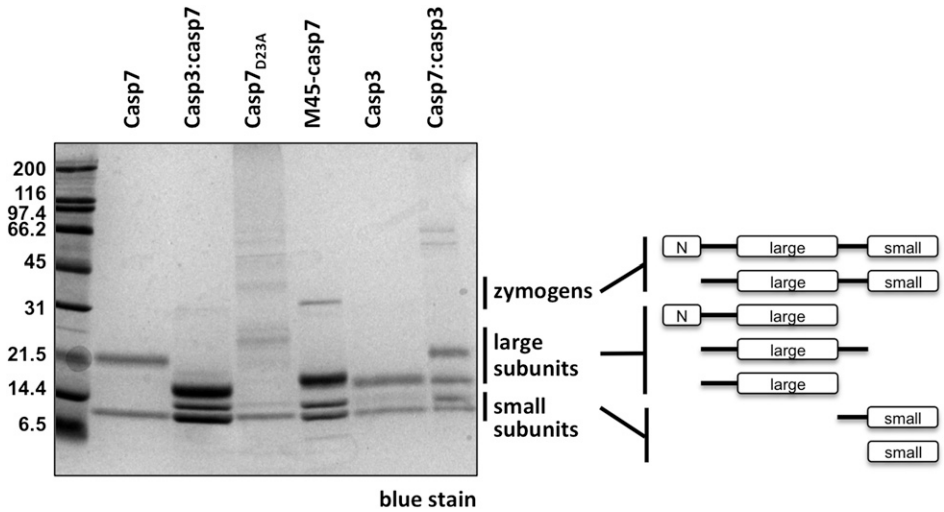


Fig. S3. Bacterial expression and autoactivation of wild-type caspase-3 and -7 and chimeric caspases. The indicated caspase were analyzed on poly-acrylamide gel and visualized using GelCode blue staining reagent. Schematics of proteins obtained after N-peptide removal and cleavage at activation sites are presented on the right.

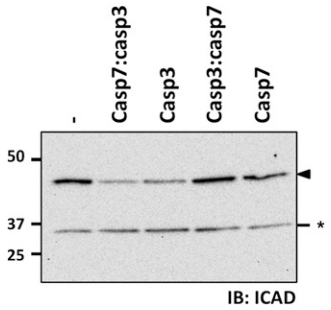


Fig. S4. Caspase-3 or -7's N-terminal domain (NTD) does not confer preference for ICAD cleavage. Detergent extracts were incubated for 15 min in the presence of 10 nM of the indicated caspase in caspase buffer, and then analyzed by immunoblotting using an anti-ICAD antibody. The asterisk indicates a nonspecific band recognized by the antibody and serves as a loading control. Closed arrowhead, full-length protein.

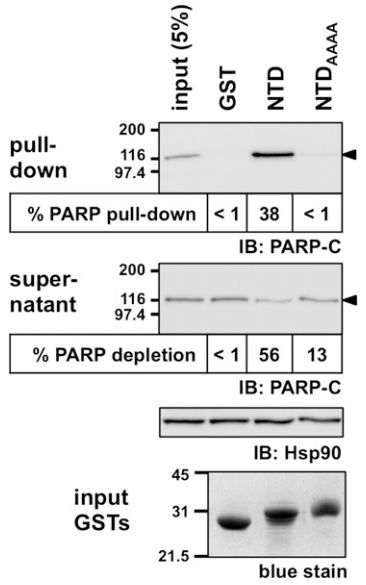


Fig. S5. Caspase 7's NTD binds PARP. Ten micrograms of GST, GST-NTD, or GST-NTD$_{AAAA}$ on beads were incubated with 200 μg (1 mg·mL^{-1}) of MCF-7^{sh7} extract for 16 h at 4 °C. Pull-down proteins (*Top*) and equal proportion of post pull-down supernatant (*Middle*) were analyzed by immunoblotting with the indicated antibodies. Blots were quantified to determine pull-down and depletion efficacy and are presented as percentage of total PARP protein used in each samples. SDS/PAGE analysis of GST proteins is presented (*Bottom*).

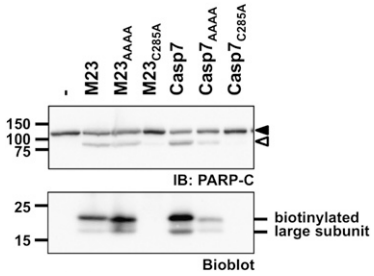


Fig. S6. Labeling of caspase-7 and caspase-7$_{AAAA}$ in intact cells. Twenty-four hours posttransfection, cells as in Fig. 4*A* were incubated for 1 h with 20 μM of the cell permeable biotinyl-VAD-fmk irreversible caspase inhibitor. Extracts were prepared and analyzed by immunoblot using an anti-PARP antibody or using streptavidin-HRP to revealed biotinylated (active) caspase-7 (bioblot). Closed arrowhead, full-length protein; open arrowhead, cleaved fragment.

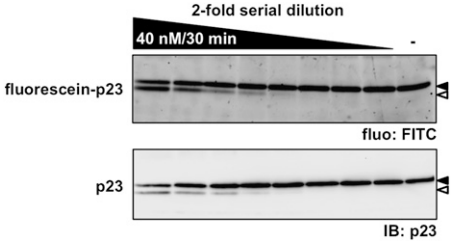


Fig. S7. FITC-labeled and unlabeled p23 are cleaved as efficiently by caspase-7. Unlabeled or fluorescein labeled purified recombinant p23 (50 nM) was incubated for 30 min with twofold serial dilution of recombinant caspase-7 in caspase buffer starting at a caspase concentration of 40 nM. Samples were analyzed by fluorescence imaging (*Upper*) or immunoblotting (*Lower*) using an anti-p23 antibody. It is noted that no more than a twofold difference in cleavage efficiency was observed. Closed arrowhead, full-length protein; open arrowheads, cleaved fragments.

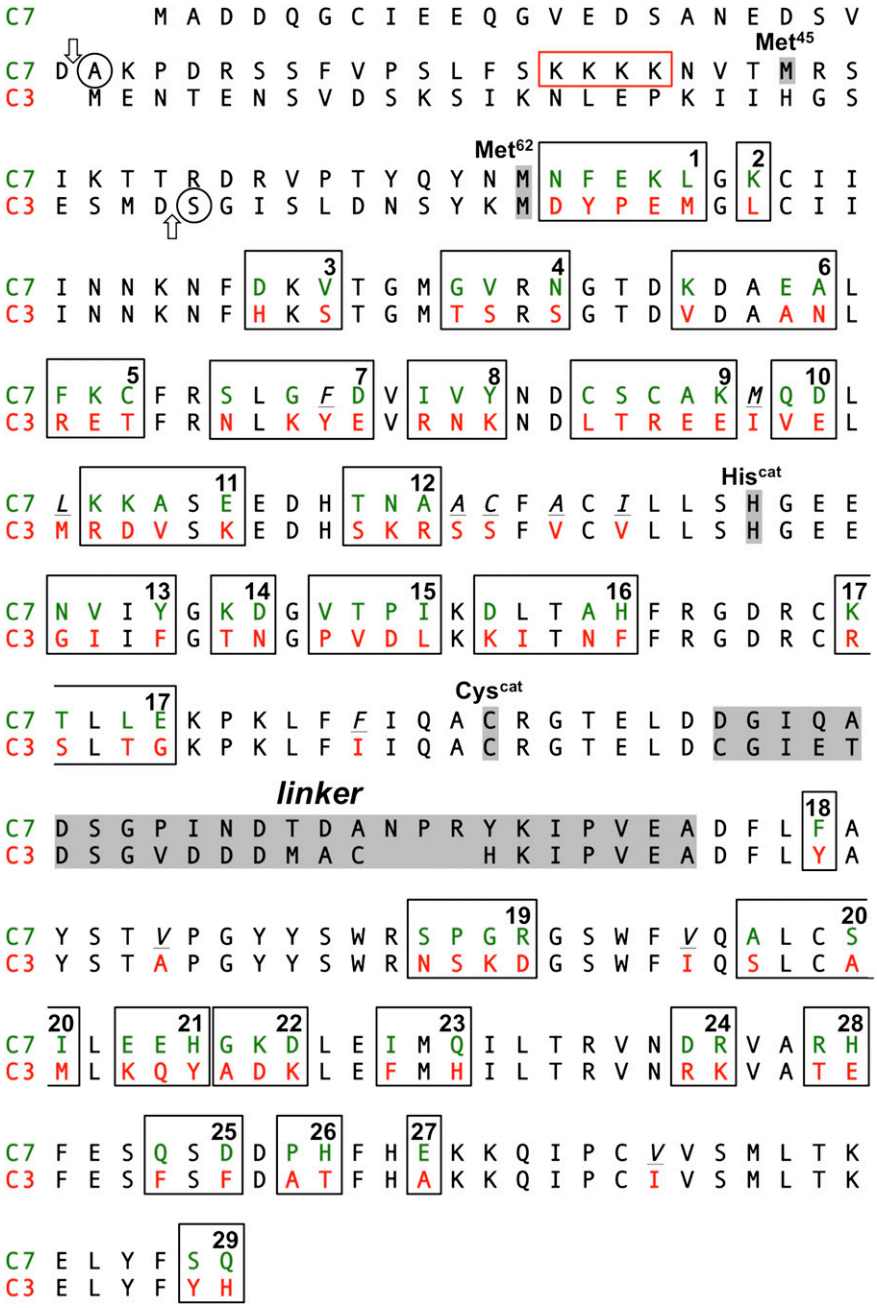


Fig. S8. Rational design of the caspase-7:caspase-3 mutant library. Alignment of caspase-7 and caspase-3 primary sequence. Boxed residues correspond to mutants in which amino acids from caspase-3 were substituted in caspase-7. Italicized and underlined residues were not mutated because they are buried into the structure. The catalytic cysteine and histidine along with the methionine 62, which was used to create chimeric caspases, and methionine 45 are indicated. The N-terminal residue of the mature form of each caspases resulting from the removal of the N-peptide is circled. A red box indicates the tetrabasic patch. The linker that separates the large and small subunit is shadowed and was left unchanged.

Table S1. Kinetic parameters on AcDEVD-Afc substrate of wild-type and mutant caspases used in this study

Caspases	K_M (μM)	±	k_{cat} (s^{-1})	±	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	±
WT and chimeric caspases						
Caspase-7*	31.9	4.6	3.4	0.2	1.1×10^5	0.2×10^5
Caspase-3*	8.8	2.3	5.2	0.4	5.9×10^5	2.0×10^5
Casp3:casp7*	21.8	4.3	7.8	0.5	3.6×10^5	0.9×10^5
Casp7:casp3*	12.5	2.3	9.3	0.5	7.4×10^5	1.7×10^5
Caspase-7† (AcPEVD-Afc)					3.9	
Caspase-3 (AcPEVD-Afc)	95.1	7.7	0.2	0.0	1.9×10^3	1.6×10^2
Deletion mutants						
M23‡	68.3	3.8	9.5	0.2	1.4×10^5	N/A
M28	40.8	8.4	14.7	1.2	3.5×10^5	1.1×10^5
M32	32.2	2.2	5.1	0.1	1.6×10^5	1.4×10^4
M36	51.1	3.7	5.6	0.2	1.1×10^5	1.2×10^4
M40	26.2	2.0	6.0	0.2	2.3×10^5	2.5×10^4
M45*	20.6	4.4	2.8	0.2	1.3×10^5	0.4×10^5
Tetra-alanine mutants						
1A4	39.8	3.1	3.5	0.1	8.8×10^4	1.0×10^4
2A4	29.7	3.3	3.5	0.1	1.2×10^5	1.6×10^4
3A4	24.2	2.9	5.6	0.2	2.3×10^5	3.6×10^4
4A4	22.3	1.0	4.7	0.1	2.1×10^5	1.4×10^4
5A4	36.8	1.6	6.65	0.1	1.8×10^5	1.0×10^4
Single alanine mutants						
D23A‡	68.7	4.5	9.5	0.2	1.4×10^5	N/A
S37A	37.5	2.4	4.4	0.1	1.2×10^5	1.0×10^4
K38A	35.7	1.4	4.3	0.1	1.2×10^5	0.7×10^4
K39A	36.6	1.5	3.9	0.1	1.1×10^5	6.7×10^3
K40A	29.8	1.6	2.9	0.1	9.7×10^4	6.8×10^3
K41A	47.8	2.9	4.1	0.1	8.6×10^4	7.0×10^3
KKKK mutants						
AAAA	31.3	2.0	6.1	0.1	2.0×10^5	5.2×10^4
KAAK	41.1	2.5	8.9	0.2	2.2×10^5	7.7×10^3
KEEK	13.5	0.9	6.2	0.1	4.6×10^5	3.8×10^4
KEKK	25.1	1.1	4.6	0.1	1.8×10^5	1.1×10^4
Library						
Mutant 1	36.6	3.6	1.2	0.1	3.4×10^4	4.7×10^3
Mutant 2	41.8	1.7	1.1	0.0	2.6×10^4	1.4×10^3
Mutant 3	31.6	1.6	2.3	0.0	7.2×10^4	5.0×10^3
Mutant 4	81.8	7.0	4.6	0.2	5.6×10^4	6.8×10^3
Mutant 5	35.9	1.9	4.1	0.1	1.2×10^5	8.4×10^3
Mutant 6	81.2	8.1	13.4	0.1	1.7×10^5	3.0×10^4
Mutant 7	32.0	4.4	1.3	0.1	4.0×10^4	7.4×10^3
Mutant 8			No detectable activity			
Mutant 9	29.5	1.4	2.4	0.1	8.3×10^4	5.3×10^3
Mutant 10	39.8	1.6	3.6	0.1	9.1×10^4	5.1×10^3
Mutant 11	30.2	2.3	3.3	0.1	1.1×10^5	1.1×10^4
Mutant 12	31.7	2.3	3.1	0.1	9.9×10^4	9.5×10^3
Mutant 13	22.4	1.7	2.9	0.1	1.3×10^5	1.0×10^4
Mutant 14	31.1	1.8	2.8	0.1	8.8×10^4	6.8×10^3
Mutant 15	29.3	1.1	3.5	0.1	1.2×10^5	6.0×10^3
Mutant 16	31.7	4.4	2.6	0.1	8.2×10^4	1.5×10^4
Mutant 17	37.7	2.6	0.7	0.0	2.0×10^4	1.8×10^3
Mutant 18	76.4	5.4	1.7	0.1	2.2×10^4	2.2×10^3
Mutant 19	22.8	1.3	6.9	0.1	3.0×10^5	2.3×10^4
Mutant 20	26.8	3.2	1.9	0.1	7.2×10^4	1.1×10^4
Mutant 21	39.6	2.6	2.8	0.1	7.1×10^4	6.2×10^3
Mutant 22	45.8	2.9	3.4	0.1	7.5×10^4	6.4×10^3
Mutant 23	12.2	1.0	2.1	0.0	1.7×10^5	1.7×10^4
Mutant 24	54.7	6.2	1.7	0.1	3.1×10^4	5.0×10^3
Mutant 25	51.6	4.1	3.6	0.1	6.9×10^4	8.2×10^3
Mutant 26	90.9	6.5	1.4	0.1	1.5×10^4	1.6×10^3
Mutant 27	18.2	0.8	8.5	0.1	4.6×10^5	2.6×10^4
Mutant 28	47.5	3.4	2.3	0.1	4.8×10^4	4.8×10^3
Mutant 29	10.6	1.1	3.7	0.1	3.5×10^5	4.6×10^4

*Kinetic parameters were compiled from at least three independent determinations and SEs are reported.
†Kinetic parameters were obtained in pseudofirst order conditions because the K_M is > 100 μM.
‡Taken from Denault and Salvesen (1). N/A, not available.