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Supplementary Materials for

A Peptide-Based Target Screen Implicates the Protein Kinase CK2 in the Global Regulation of Caspase Signaling

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Other Supplementary Material for this manuscript includes the following:
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Methods

Mass spectrometric analysis of peptide cleavage

Peptides were incubated with caspase-3 in caspase assay buffer for 1 hour at 30°C with gentle agitation. Samples were then spotted onto a MALDI plate (Micromass), and cleavage of the peptides by caspase-3 was assessed by analysis of the peptide products observed in the mass spectra. Mass spectra were acquired on a MALDI-LR mass spectrometer (Micromass) in positive reflectron mode with time-of-flight (TOF) detection. The instrument was calibrated with PEG1000, PEG2000, PEG3000, and sodium iodide, and peptide masses are quoted relative to angiotensin, an internal standard. Spectra were collected between an m/z range of 800 to 2600, and analysis of the spectra was performed with MassLynx 3.5 software.

Determination of phosphorylation sites in caspase-3 by mass spectrometry

One microgram of purified recombinant His-caspase-3 (C163A) was introduced into a 50 μl of buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 mM ATP, 50 ng/μl bovine serum albumin (BSA)]. One hundred nanograms of purified recombinant GST-CK2α were then added to the mixture, which was incubated at 30°C for 2 hours. When the kinase reaction was complete, acetonitrile (ACN) and ammonium bicarbonate were added to final concentrations of 5% and 50 mM, respectively. The solution was then passed through a homemade column consisting of a 200-μl Gel-loader tip loaded with a 1:1 mixture of POROS R1/R2 Applied Biosystems reverse-phase resins, washed extensively with a solution of 50 mM ammonium bicarbonate, 5% ACN, and then eluted with a solution of 50 mM ammonium bicarbonate and 50% ACN. The sample was dried down, and the proteins were then resuspended in 50 mM ammonium bicarbonate, (pH 8.5). The protein sample was reduced with 5 mM DTT for 45 min at 37°C, and then the cysteines were blocked by treatment with 50 mM iodoacetamide. Tryptic peptide fragments were then generated with sequencing-grade trypsin (Promega) in a 12-hour digestion at 37°C. These tryptic peptides were dried down and resuspended in 0.2% formic acid. Peptide separation was performed on a Waters nanoUPLC that was directly coupled to a Micromass QToF Global mass spectrometer equipped with a Zspray Nano-ESI source. The data were acquired in Data Dependent Acquisition (DDA) mode with MassLynx 4.1 software. The data 10 ranges for acquisition were 400 to 1,800 for MS survey, and 50 to 1,800 for MS/MS mode. Cone voltage was 100 V, and the source temperature was set to 80°C. Mascot and PEAKS (Bioinformatics Solutions) software were used to confirm the identity of the phosphorylated peptides. In parallel, a peptide synthesized by EZBiolabs corresponding to the putative phosphorylation sites on caspase-3, GTELDCGIETDSGVDDDMACHK (23 amino acid residues, with a monoisotopic mass of 2309.91), was phosphorylated by following the protocol described earlier, de-salted on POROS R2/R3 resins, loaded into homemade columns, and subsequently analyzed on an Applied Biosystems MALDI TOF/TOF 4700 Proteomics Analyzer in either negative or positive ion modes.

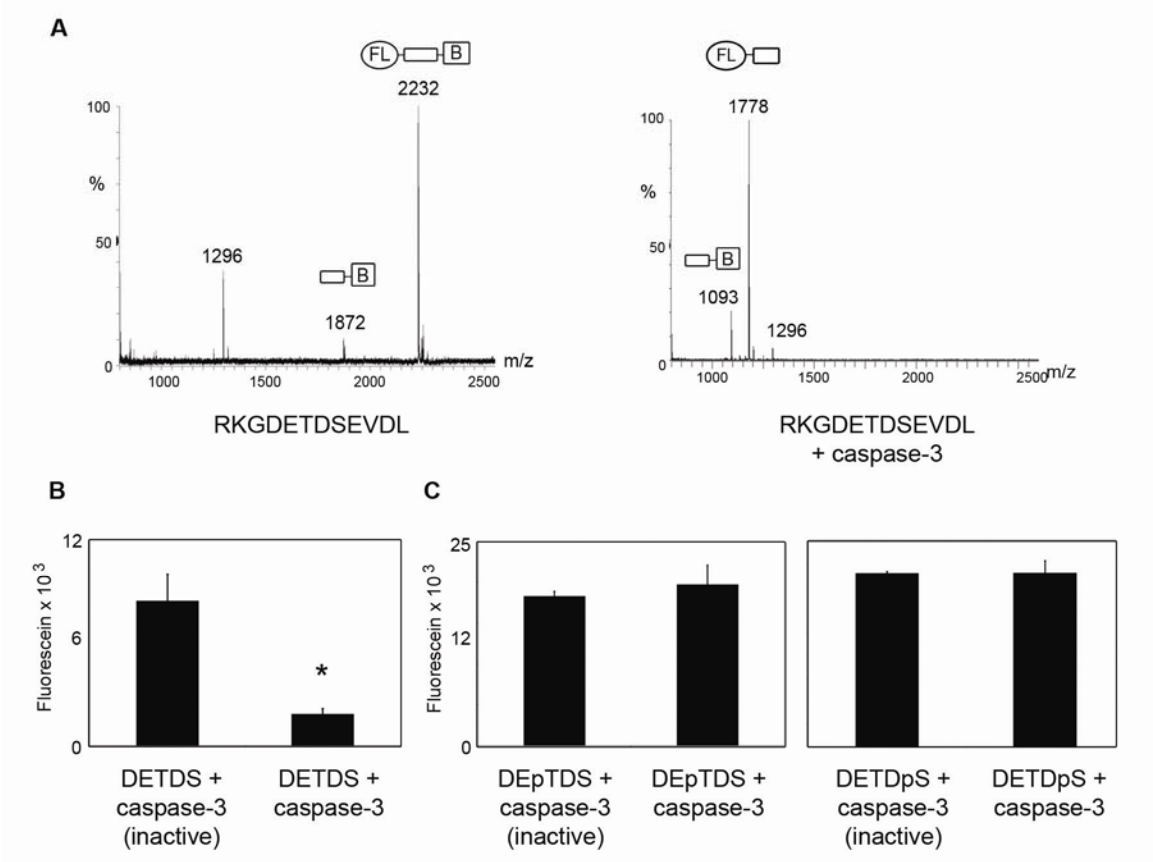


Fig. S1. Phosphorylation of the P2 or P1′ residue prevents cleavage by caspases. (**A**) N-terminal fluorescein-labeled and C-terminal biotinylated peptides corresponding to an engineered sequence (DETDS) of the caspase-3 target PARP1 were treated with active or inactive caspase-3, and cleavage of the peptides was detected by MALDI TOF mass spectrometric analysis. After treatment with caspase-3, cleaved peptides were identified corresponding to the appropriate m/z value for the N-terminal fluorescein-labeled and the C-terminal biotinylated proteolytic cleavage products. (**B**) By way of comparison, the same PARP1 (DETDS) peptides were analyzed by CSI. All of the treatments were performed in triplicate; error bars represent the SD. (**C**) Protection of the phosphorylated PARP1 peptides phosphorylated at the P2 and P1′ residues (DEpTDS and DETDpS) was determined by CSI. A statistical difference in fluorescein counts/0.1s bound to streptavidin-coated plates was determined by comparing treatments with active or inactive caspase-3 by ANOVA ($P < 0.01$). All of the treatments were performed in triplicate; error bars represent the SD.

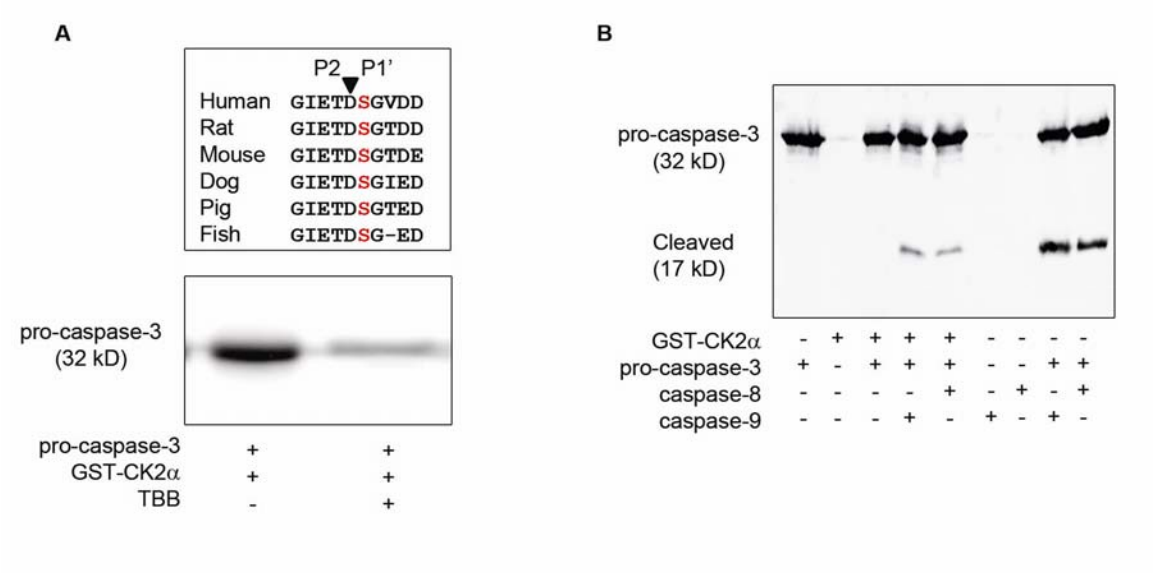
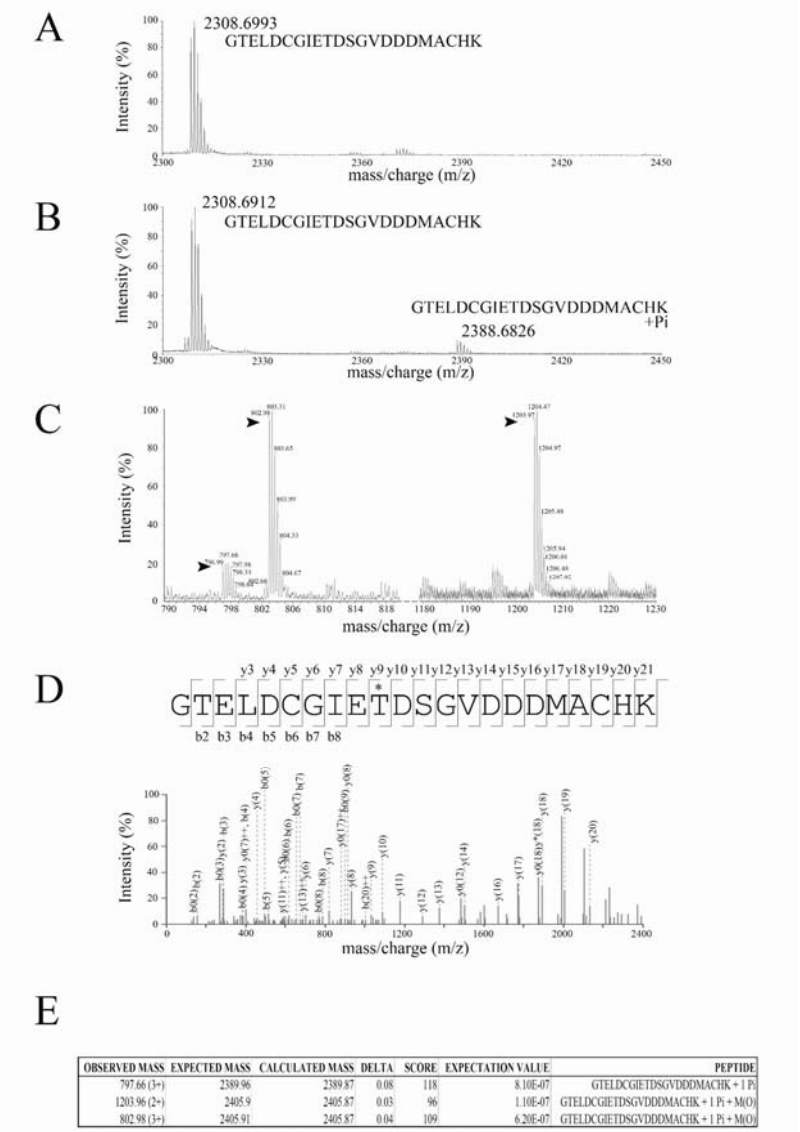


Fig. S2. Phosphorylation of procaspase-3 by CK2 results in its protection from caspase-mediated cleavage. (**A**) An alignment of the protein sequences of procaspase-3 from different species revealed a conserved CK2 phosphorylation motif (Ser^{176}) within the caspase activation sequence of procaspase-3 at the P1′ position adjacent to Asp^{175}. In vitro [γ-^{32}P] kinase assays with GST-CK2α and purified procaspase-3 (C163A) as a substrate were performed to determine whether procaspase-3 was an in vitro target of CK2. Kinase reactions that had been treated with TBB (60 μM) were used as a control. (**B**) In parallel, an in vitro "cold" kinase assay containing GST-CK2α and procaspase-3 followed by treatment with active caspase-8 or caspase-9 was also performed. The generation of a 17-kD band that corresponded to the cleaved caspase-3 fragment after treatment of procaspase-3 with active caspase-8 and caspase-9 was detected by Western blotting analysis with antibodies against caspase-3. This verified that caspase-8 and caspase-9 were active in the in vitro caspase cleavage assays. Procedures were performed in triplicate as described for Fig. 5.



OBSERVED MASS	EXPECTED MASS	CALCULATED MASS	DELTA	SCORE	EXPECTATION VALUE	PEPTIDE
797.66 (3+)	2389.96	2389.87	0.08	118	8.10E-07	GTELDCGIETDSGVDDDMACHK + 1 Pi
1203.96 (2+)	2405.9	2405.87	0.03	96	1.10E-07	GTELDCGIETDSGVDDDMACHK + 1 Pi + M(O)
802.98 (3+)	2405.91	2405.87	0.04	109	6.20E-07	GTELDCGIETDSGVDDDMACHK + 1 Pi + M(O)

Fig. S3. Mass spectrometric determination of the phosphorylation site of caspase-3. A caspase-3 peptide with the sequence GTELDCGIETDSSGVDDDMACHK was synthesized to determine the site of phosphorylation by CK2. After incubation with CK2 for 2 hours, the peptide was then desalted and analyzed on a MALDI-TOF/TOF in negative ion mode, as described earlier. (**A**) Spectrum of the non-treated control peptide. (**B**) Spectrum of the caspase-3 peptide after phosphorylation by CK2. (A and B) The nonphosphorylated and phosphorylated peptide sequences, as well as their corresponding mono-isotopic masses are shown. Recombinant, phosphorylated caspase-3 was also subject to mass spectrometric analysis. Briefly, intact caspase-3 protein was incubated with purified CK2 for 2 hours. The sample was subsequently digested with trypsin and subjected to electrospray mass spectrometry. The data collected were then analyzed to determine the site of phosphorylation on caspase-3, as described earlier. (**C**) Mass spectrum illustrating three parent ions that were selected for MS/MS ion sequencing (denoted by the arrows). The three ions selected were 797.66 (2+), 802.98 (3+), and 1203.96 (2+). (**D**) The MS/MS peptide fragmentation pattern of the triply-charged ion of mass 797.66. This fragmented ion corresponded to a caspase-3 peptide that was phosphorylated at Thr^{174} (denoted by the asterisk). The peptide sequence is shown above, with the corresponding y- and b-ions that were used to make the positive identification. (**E**) A table listing the three parent ions that were selected for fragmentation and were phosphorylated at Thr^{174}. Mascot values obtained from the analysis are categorized for each selected ion. Pi, phosphate; M(O), methionine oxidation. The experiments were performed a minimum of three times.

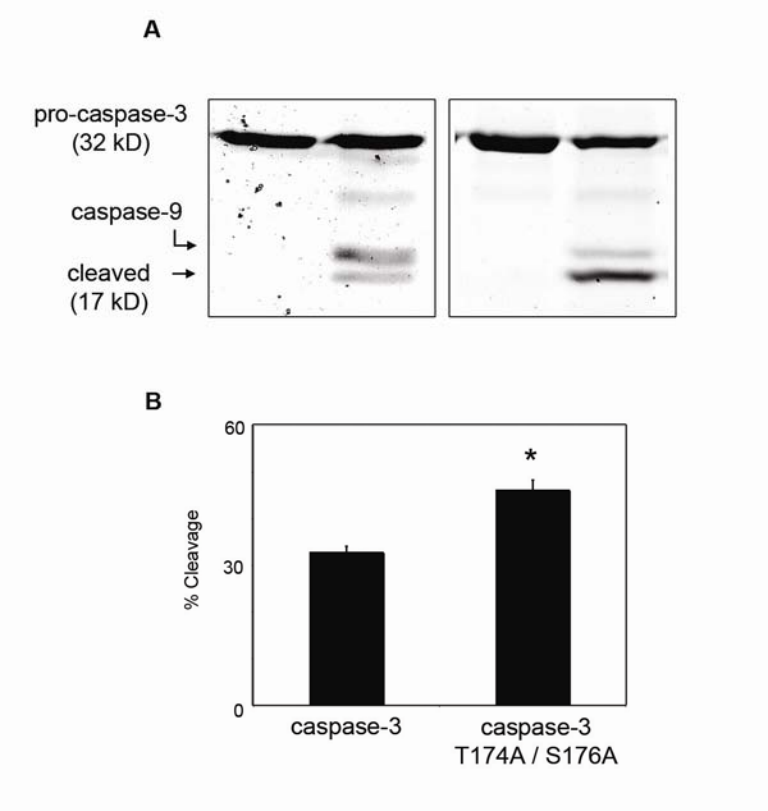


Fig. S4. Phosphorylation of procaspase-3 at Thr^{174} and Ser^{176} protects it from caspase-mediated cleavage. (**A**) Cleavage of procaspase-3 (left panel) and the procaspase-3 T174A/S176A mutant (right panel) were visualized on Gel Code Blue–stained gels after treatment with CK2, caspase-9, or both. (**B**) The abundance of the cleaved 17-kD band was calculated as a percentage of that of the full-length, 32-kD procaspase-3 band, and ANOVA analysis ($P \leq 0.05$) was performed to compare these percentages between samples containing either procaspase-3 or the procaspase-3 (T174A/S176A) mutant. Experiments were performed in triplicate. Error bars represent the SD. Procedures were performed as described for Fig. 5.

Table S3. Proteins identified in the peptide match search that have overlapping CK2 and caspase recognition motifs that are cleaved by caspases in cells.

Name	**Protein ID**	Sequence	Caspase
Apoptosis regulator BAX, membrane isoform alpha*	BAXA_HUMAN	klseclkrigDELDSNMElqrmiaav	unknown
Eukaryotic translation initiation factor 4H	IF4H_HUMAN	kfkgfcyvefDEVDSLKEaltydgal	3
Caspase-3 precursor*	CASP3_HUMAN	acrgteldcgIETDSGVDddmachk	8 / 9
DNA-binding protein SATB1	SATB1_HUMAN	khfkktkdmmVEMDSLSElsqqg	6
Protein Max	MAX_HUMAN	msdnddIEVESDEEqprfqsaadk	5
Caspase-8 precursor*	CASP8_HUMAN	qgdnyqkgipVETDSEeqpylemd	8
PH and SEC7 domain-containing protein 4	PSD4_HUMAN	wtldasqsslLETDGEqpsslkkkea	unknown

*Protein essential in the progression of apoptosis.

Table S4. Proteins identified from the peptide match screen that have overlapping CK2 and caspase recognition motifs and were previously identified to be phosphorylated in cells. Phosphorylation was determined with the Phospho-Elm and Phosida databases.

Swiss-Prot ID	Sequence	Kinase
MPP10_HUMAN	sdeditnvhdDELDSNKEddeiaeeeae	unknown
UB2R1_HUMAN	eeeadscfgdDEDDSGTEe	**CK2**
CHD8_HUMAN	DEDDSDSEldls	unknown
NFIA_HUMAN	sstkrlksveDEMDSPGEepfytgqgrs	unknown
CV019_HUMAN	lfkppedsqdDESDSDAEeeqttkrrrp	unknown
NOLC1_HUMAN	qpvessedssDESDSSSEeekkpptkav	unknown
VPS72_HUMAN	eyqgdqsdteDEVDSDFDidegdepssd	**CK2**
NOL8_HUMAN	sgklfdssddDESDSEDDsnrfkikpqf	unknown
BAZ1B_HUMAN	rkkfpdrlaeDEGDSEPEavgqsrgrrq	unknown
VAMP4_HUMAN	gsvkserrnlLEDDSDEEedfflrgpsg	unknown
NUCL_HUMAN	pkkmapppkeVEEDSEDEemsedeedds	unknown
RBL2_HUMAN	pasttrrrlfVENDSPSDggtpgrmppq	**CDK2**
CO039_HUMAN	gpvmygklprLETDSGLEhslphsvgnq	unknown
NOL8_HUMAN	ddrfrmdsrfLETDSEEEqeevnekkta	unknown
MY18A_HUMAN	vtkyqkrknkLEGDSDVDseledrvdgv	unknown
AVEN_HUMAN	ggwgagasapVEDDSDAEtygeendeqg	unknown
SDA1_HUMAN	apgkcqkrkyIEIDSDEEprgellslrd	unknown
MBD2_HUMAN	sraadteemdIEMDSGDE	unknown
RRP15_HUMAN	kdhfysdddaIEADSEGDaepcdkenen	unknown
DDEF2_HUMAN	yewrllhedlDESDDDmdeklqpspn	unknown
MRE11_HUMAN	tknysevievDESDVEedifpttskt	**CK2**
CV019_HUMAN	lfkppedsqdDESDSDaeeeqttkrr	unknown
NIPBL_HUMAN	knntaaetedDESDGEdrgggtsgsl	unknown
NOL8_HUMAN	sgklfdssddDESDSEddsnrfkikp	unknown
CPSF2_HUMAN	qskeadidssDESDIEedidqpsahk	unknown
MAN1_HUMAN	gskvllgfssDESDVEasprdqaggg	unknown
MPP10_HUMAN	nlkykdffdpVESDEDitnvhddeld	unknown
L1CAM_HUMAN	kdetfgeyrsLESDNEekafgssqps	**CK2**
MAX_HUMAN	msdnddieVESDEEqprfqsaadk	**CK2**
CHD4_HUMAN	rssseddd1dVESDFDdasinsysvs	unknown
NOL8_HUMAN	ddrfrmdsrfLETDSEeeqeevnekk	unknown
CA052_HUMAN	arllpegeetLESDDEkdehtskkrk	unknown
SETD2_HUMAN	rgplkkrrqeIESDSEsdgelqdrkk	unknown
WDR70_HUMAN	ktqpktmfaqVESDDEeaknepewkk	unknown
CBPD_MOUSE	kksllshefqDETDTEeetlyssk	unknown
CTNA2_MOUSE	avlmirtpeeLEDDSDFEqedydvrsrt	unknown
ENSP00000307525	tipVESDDDegap	unknown
SF3A1_HUMAN	fgeseevemeVESDEEddkqekaeep	unknown
MST4_HUMAN	rfkrwkaeghSDDESDsegsdsests	unknown

Table S6. Identification of candidate CK2 and caspase peptide sequences that are sensitive to cleavage by caspases. Cleavage by a caspase was determined with the CSI assay. Statistical differences in fluorescein counts bound to streptavidin-coated plates between untreated samples and those treated with caspases 3, 8, or 9 were determined by ANOVA ($P \leq 0.05$). Protein ID was obtained from Swiss-Prot or NCBI.

Protein Name	Protein ID	Sequence	Caspase	Cleavage
PARP1*	PARP1_HUMAN	rkgDEVDGvdev	3	Yes
Presenilin-2*	PSN2_HUMAN	meeDSYDSFGEps	3	Yes
PTEN*	PTEN_HUMAN	svtpDVSDNEpdh	3	Yes
PTEN*	PTEN_HUMAN	ysDTTDSDPEnep	3	Yes
Fodrin*	SPTA2_HUMAN	mprDETDSktasp	3	Yes
Bid^{+}	BID_HUMAN	egydeLQTDGnrs	8	Yes
Bid^{+}	BID_HUMAN	egydeLQTpDGnrs	8	No
Procaspase-3^{+}	CASP3_HUMAN	cgIETDSGVDddm	3 / 8 / 9	Yes
Procaspase-3^{+}	CASP3_HUMAN	cgIETpDSGVDddm	3 / 8 / 9	No
Procaspase-3^{+}	CASP3_HUMAN	cgIETDSpGVDddm	3 / 8 / 9	No
Serine/threonine-protein kinase MST4	MST4_HUMAN	hsdDESDSEgsds	3	No
Carboxypeptidase D precursor	CBPD_HUMAN	fqDETDTEEEtlyss	3	No
M-phase phosphoprotein 10	MPP10_HUMAN	dDELDSNKEddei	3	Yes
transcription factor-like 1	VPS72_HUMAN	eDEVDSDFDideg	3	Yes
nucleolar protein 8	NOL8_HUMAN	dDESDSEDDsnrf	3	No
THO complex 5	THOC5_HUMAN	dDESDSDAEeeqt	3	No
zinc finger and BTB domain containing 45	ZBT45_HUMAN	esdDETDGEdgeg	3	Yes
F-box only protein 3 isoform 1	FBX3_HUMAN	admDESDEDdeee	3	No
chromodomain helicase DNA binding protein 8	CHD8_HUMAN	DEDDSDSEldls	3	Yes
development- and differentiation-enhancing factor 1	DDEF1_HUMAN	edlDESDDDmdek	3	Yes
Serine/threonine-protein kinase LMTK1	LMTK1_HUMAN	edsDESDEElrcy	3	No
NKF3 kinase family member	148368962	eswDESDEEllam	3	No
meiotic recombination 11 homolog A isoform 2	24234690	ievDESDVEedif	3	No
interferon alpha/beta receptor 2 isoform a	46488937	nydDESDSDteaa	3	No
NMD3 homolog	113419592	tipVESDDDegap	3	No
nucleolar protein 8	NOL8_HUMAN	srfLETDSEeeqe	3	No
chromodomain helicase DNA binding protein 4	CHD4_HUMAN	dldVESDFDdasi	3	No
vesicle-associated membrane protein 4	VAMP4_HUMAN	eLEDDSDFEqedy	3	No
cell death regulator aven	AVEN_HUMAN	pVEDDSDAEtyge	3	
echinoderm microtubule associated protein	EMAL1_HUMAN	shsDESDSDlsdv	3	Yes
huntingtin interacting protein B	SETD2_HUMAN	eIESDSESDgelq	3	No
histone deacetylase 4	HDAC4_HUMAN	qepIESDEEeaep	3	No
Procaspase-8	CASP8_HUMAN	grpVETDSEsefp	3	No
nucleolar protein with MIF4G domain 1	NOM1_HUMAN	dLESDSQDEseee	3	No
Random control sequence, no caspase-3 site	CONTROL	rkgAEVAGVdev	3	No

*Cleaved by caspase 3 in cells. $^{+}$Cleaved by caspase 8, caspase 9, or both in cells.