

Multiple Pathways are Impacted by Variations in RAS

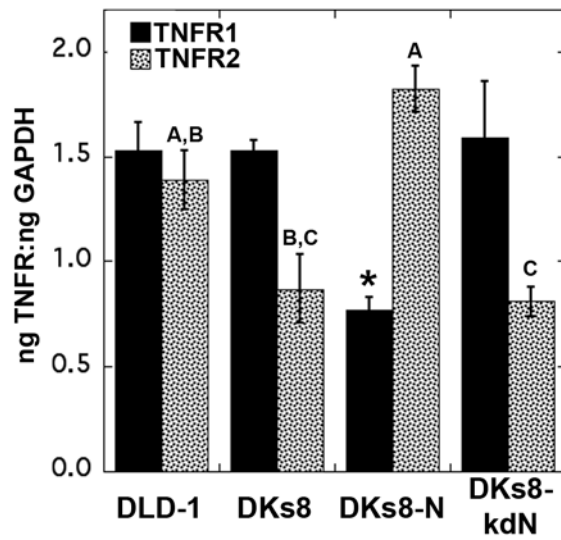


Figure S1. TNFR1 and TNFR2 expression relative to GAPDH. mRNA was isolated from IFN- γ -sensitized cells using RNeasy Mini kits (Qiagen, Valencia, CA) and quantified on a NanoDrop (Thermo Scientific, Waltham, MA). 1 μ g of RNA was transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). 25ng of cDNA was analyzed by RT-PCR using QuantiTect SYBR Green and Primers (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). For TNFR1, DKs8-N cells have significantly decreased levels compared to the other three cell lines (indicated by *, Tukey-HSD, $p < 0.05$). TNFR2 levels also vary between the cell lines (cell lines without a common letter are significantly different, Tukey-HSD, $p < 0.05$).

Although the cell lines show different expression patterns of TNFR1 (which contains a death domain) and TNFR2 (a soluble form that may antagonize TNFR1 action), these patterns do not correlate precisely to the extent of apoptosis (1). For example, DKs8 (a cell line with low apoptosis) and DKs8-kdN (a cell line with high apoptosis) have similar expression profiles. These observations motivate the examination of the impact of RAS mutants on downstream kinases that interpret the input from the TNF receptors.

Multiple Pathways are Impacted by Variations in RAS

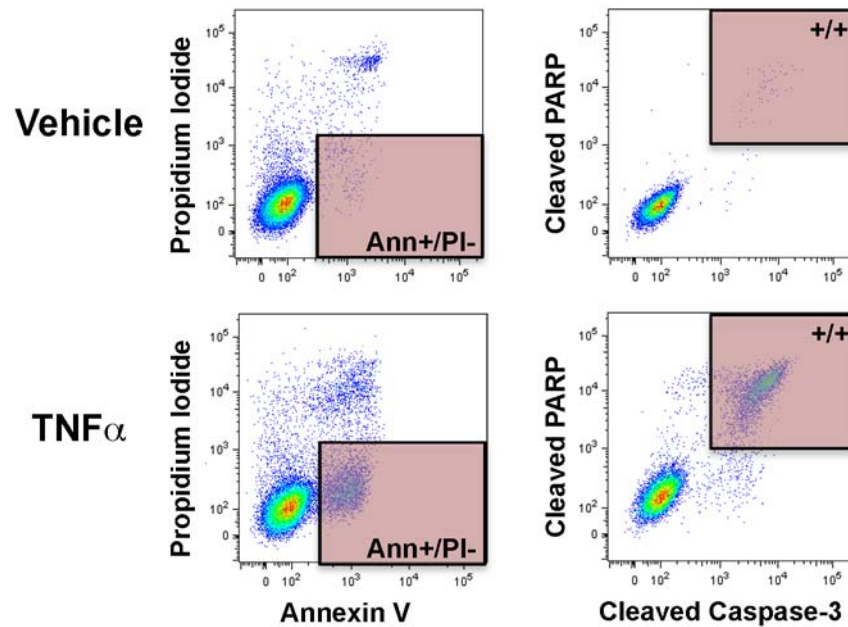


Figure S2. Example gatings for the two apoptotic assays for DLD-1 cells at 12 hours. The Annexin+/propidium iodide- population is an early event in apoptosis, while cleaved caspase-3+/cleaved PARP+ cells are a later event (2). Alexa-350 Annexin V (1:20) and propidium iodide (1 μ g/mL, Invitrogen) were used to stain half of the collected cells. The remaining cells were fixed with 4% paraformaldehyde, permeabilized and stained using anti-cleaved caspase-3 (1:500) and anti-cleaved PARP (1:250, BD Pharmingen, Franklin Lakes, NJ), followed by Alexa 488-donkey-anti-rabbit IgG and Alexa 647-donkey-anti-mouse IgG (both at 1:250, Invitrogen).

Multiple Pathways are Impacted by Variations in RAS

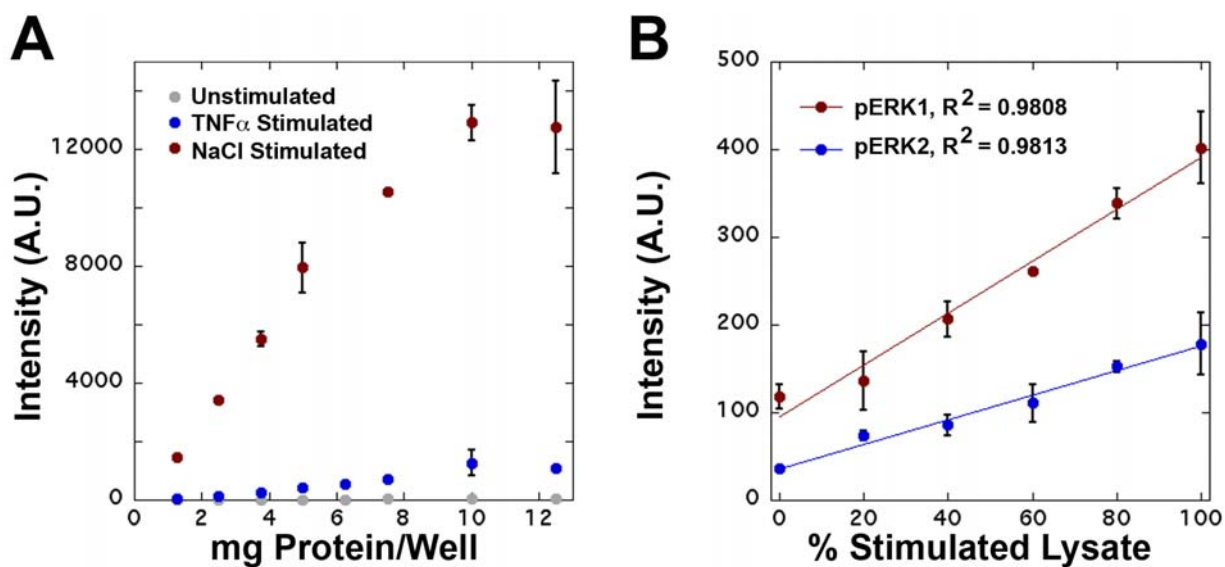


Figure S3. Luminex assays for phosphorylated proteins are linear, allowing quantitative comparisons. *A*, Increasing amounts of lysate show a linear response for pHSP27 levels across three different stimuli. *B*, When lysates from strong and weak stimuli are mixed at a set concentration, the level of phosphorylated protein detected is linear (assayed at 5 μg per well). For the collected data set, 1.8 μg (pAKT) or 5 μg (all others) of clarified lysate was assayed for each sample in duplicate.

Multiple Pathways are Impacted by Variations in RAS

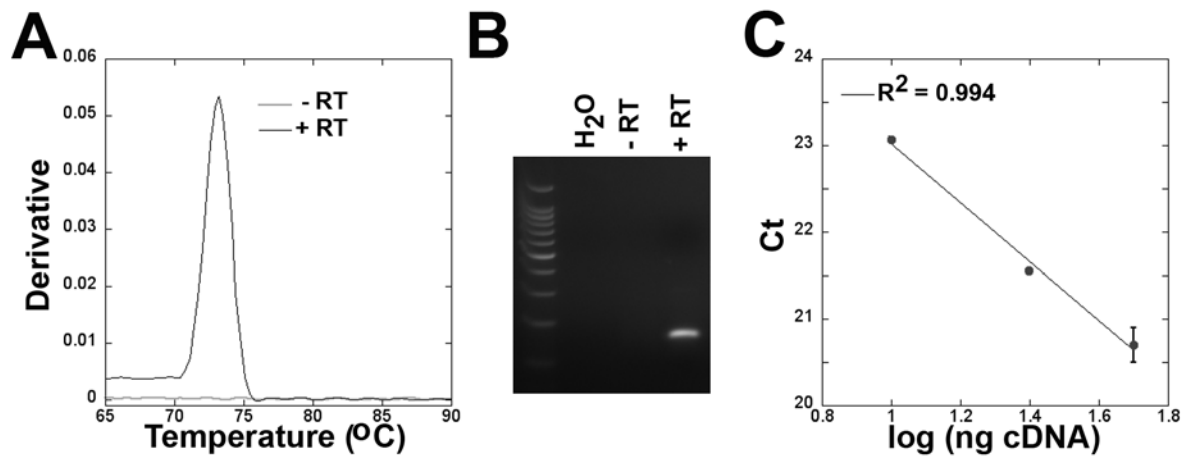


Figure S4. mRNA was isolated using RNeasy Mini kits (Qiagen) and quantified on a NanoDrop (Thermo Scientific). 1 μ g of RNA was transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). 25ng of cDNA was analyzed by RT-PCR using QuantiTect SYBR Green and Primers (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems). Each DUSP primer set was validated *A*, for clean melting curves, *B*, production of a band of the correct length by gel electrophoresis, and *C*, linearity for increasing levels of cDNA. Data shown is for DUSP6.

Multiple Pathways are Impacted by Variations in RAS

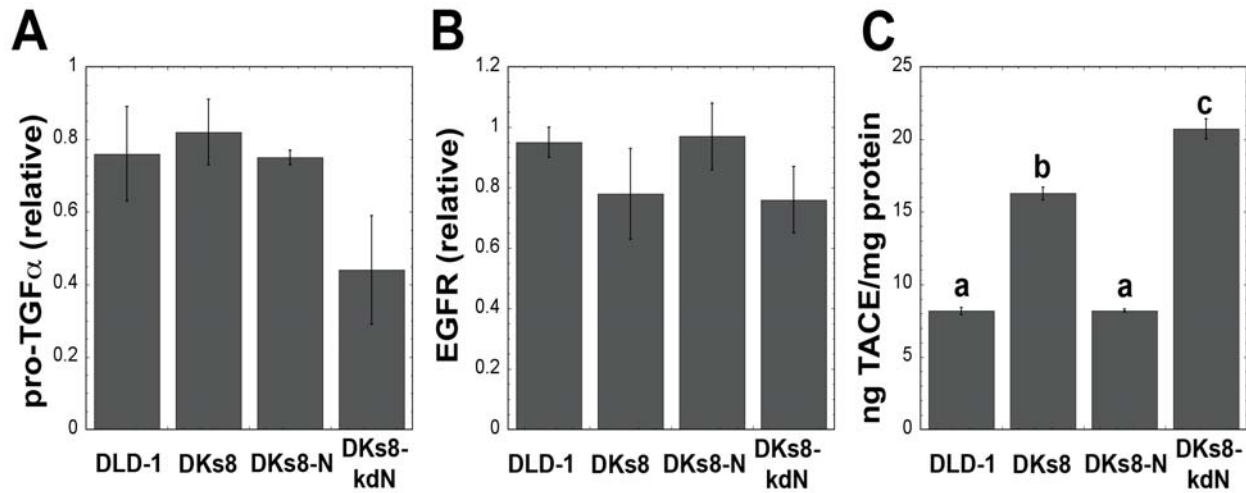


Figure S5. RAS-variant cell lines had differences in levels of TACE, but not pro-TGF α or EGFR. *A,B*, Whole cell lysates were examined by quantitative Western Blot for pro-TGF α and EGFR (Cell Signaling Technology, Danvers, MA, #3715, #2232). 15 μ g of total protein was loaded per lane. Background-subtracted signal was normalized to concurrently measured GAPDH (Cell Signaling Technology #2118). Blots were probed with IR-dye labeled secondary antibodies and detected using the LI-COR Odyssey (LI-COR, Lincoln, NE). Normalized levels were not significantly different, $p > 0.05$. *C*, Cell lysates were analyzed for levels of TACE by ELISA (R&D Systems). Levels were normalized to total cellular protein measured by BCA assay (Bio-Rad). Different letters represent significantly different levels by TUKEY-HSD, $p < 0.05$.

Multiple Pathways are Impacted by Variations in RAS

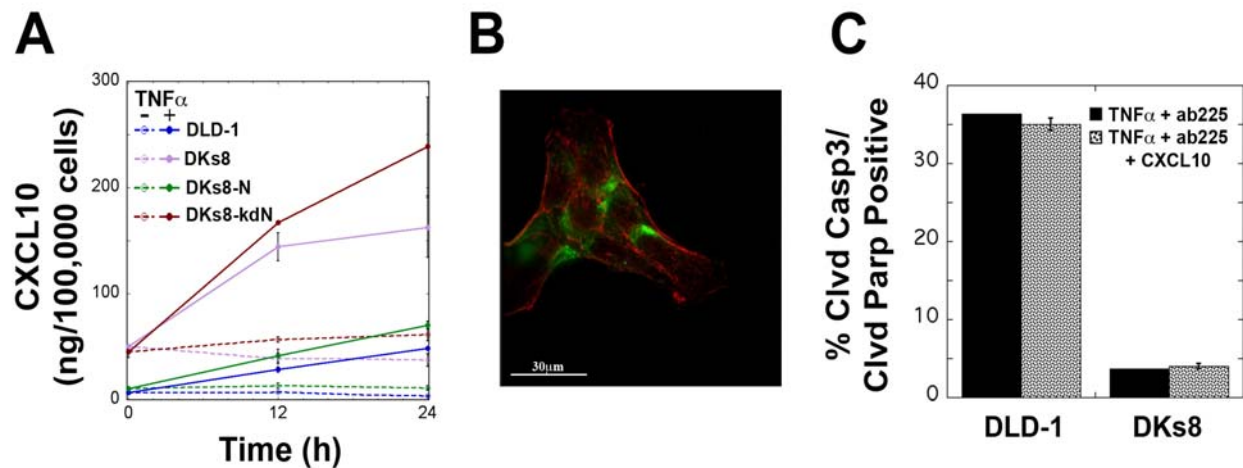


Figure S6. RAS variant cells express CXCR3 and produce CXCL10 in response to TNF α , but the potential autocrine loop does not appear to impact apoptosis. **A**, CXCL10 was quantified by Luminex assay and normalized to concurrent cell counts. **B**, CXCR3 was observed in DLD-1 cells by immunofluorescence (described in materials and methods, using 1:5 anti-CXCR3, R&D Systems). Green = CXCR3; Red = phalloidin for actin filaments. **C**, Co-treatment with ab225 decreases CXCL10 levels (data not shown). However, exogenous treatment with CXCL10 to supplement TNF α + ab225 treated cells had no effect on apoptosis at 24 hours. Cells were stained for cleaved caspase-3 and cleaved PARP and analyzed by flow cytometry for double positive (apoptotic) cells.

Multiple Pathways are Impacted by Variations in RAS

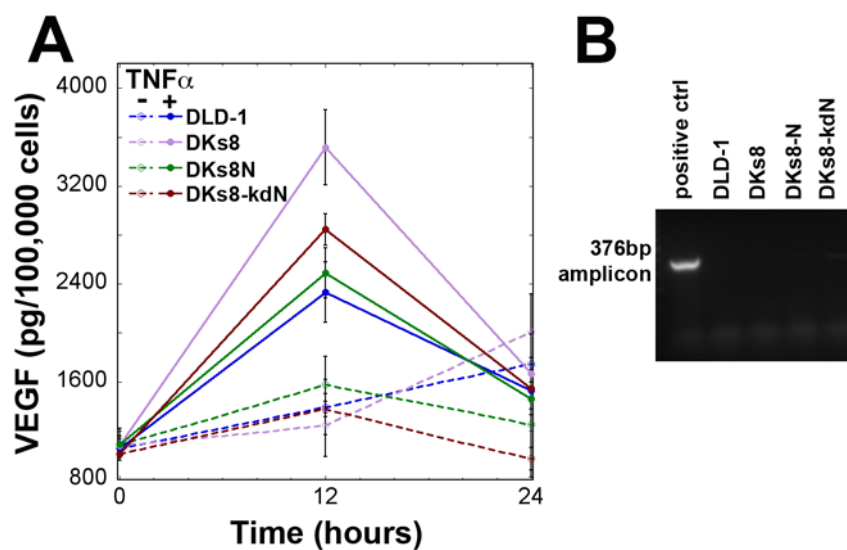


Figure S7. RAS variant cells produce VEGF in response to $TNF\alpha$, but do not express the VEGF-R2. *A*, VEGF was quantified by Luminex assay and normalized to concurrent cell counts. *B*, VEGF-R2 cDNA was not detected following 35 cycles of PCR.

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S1. DUSP Screen

Δ Ct relative to t=0									
	DLD-1			Dks8			Dks8N		
	30	90	240	30	90	240	30	90	240
DUSP1	-0.72	-0.62	-0.36	-1.26	-1.38	-0.36	-0.44	-0.29	-0.75
DUSP2	0.36	0.75	0.85	-0.69	-1.04	-0.16	-0.47	-0.08	0.65
DUSP3	0.23	0.18	0.30	-0.86	-1.38	-1.21	0.07	0.77	0.62
DUSP4	0.44	-0.16	0.34	-0.39	-1.38	-1.33	-0.46	-0.66	-0.81
DUSP5	-1.23	-1.08	-0.75	-1.39	-3.26	-2.78	-0.46	-1.98	-2.24
DUSP6	-0.01	-0.21	0.34	-0.11	-0.66	-0.27	-0.25	-0.04	0.16
DUSP7	0.04	0.05	0.76	-0.13	-0.89	-0.52	-0.33	-0.20	-0.10
DUSP9	Levels too low to reliably detect								
DUSP14	0.27	0.49	0.50	0.37	0.15	0.43	0.10	0.45	0.23

Expression levels of DUSP genes were screened by RT-PCR for nine phosphatases that recognize ERK as a substrate (3). Each RAS-variant line was plated and treated with IFN γ and TNF α as described in the materials and methods. At time 0, 30, 90, and 240 minutes, mRNA was isolated using RNeasy Mini kits (Qiagen) and quantified on a NanoDrop (Thermo Scientific). 1 μ g of RNA was transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). 25ng of cDNA was analyzed by RT-PCR using QuantiTect SYBR Green and Primers (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Only DUSP5 showed a consistent up-regulation across the three RAS-variants examined.

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S2. Top 20 most informative signals in the PLSR models.

Apoptosis			CXCL1 and CXCL8		
VIP ¹	Signal	Time (min)	VIP	Signal	Time (min)
1.49	Clvd Casp-8 (MW = 41)	720	1.39	pJNK	15
1.44	plkB α	60	1.36	pJNK	30
1.44	Clvd Casp-8 (MW = 41)	480	1.35	plkB α	120
1.39	plkB α	240	1.35	pHSP27	15
1.39	pHSP27	0	1.35	plkB α	90
1.35	pERK2	720	1.35	pHSP27	30
1.32	pJNK	0	1.33	pHSP27	60
1.31	pERK1	480	1.33	pERK2	0
1.30	pAkt	240	1.32	plkB α	15
1.28	plkB α	480	1.32	pJNK	60
1.27	plkB α	5	1.23	plkB α	720
1.24	pERK1	30	1.23	pERK1	0
1.23	plkB α	720	1.23	pAkt	15
1.23	plkB α	90	1.21	pERK1	15
1.22	pERK1	120	1.21	pERK2	720
1.19	pERK1	720	1.20	plkB α	5
1.19	pERK1	60	1.19	plkB α	60
1.18	Clvd Casp-8 (MW = 43)	720	1.18	plkB α	480
1.16	plkB α	120	1.16	plkB α	240
1.12	pERK1	240	1.12	pERK1	30

¹Variable importance of projection (4) .

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S3. Top 20 loadings in the first principal component of the apoptosis model

w₁c₁	Signal	Time (min)
0.224	plkB α	60
0.212	pERK2	720
0.208	plkB α	240
0.207	pERK1	480
0.190	plkB α	480
0.190	plkB α	90
0.186	plkB α	5
0.186	pERK1	120
0.182	plkB α	720
0.179	pERK1	720
-0.176	pHSP27	0
0.175	plkB α	120
0.169	pERK1	240
0.164	plkB α	15
0.162	pERK2	240
0.161	pERK2	480
0.159	pERK1	60
0.156	pERK1	30
0.147	pAkt	240
0.140	pERK1	90

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S4. Top 20 loadings in the second principal component of the apoptosis model

w₂C₂	Signal	Time (min)
-0.330	pJNK	0
-0.293	pHSP27	0
-0.292	pJNK	5
0.262	pAkt	240
-0.255	pAkt	15
-0.236	pJNK	720
-0.235	pHSP27	5
-0.203	pHSP27	720
-0.201	pJNK	480
-0.200	pJNK	90
-0.177	pERK2	5
-0.168	pHSP27	15
-0.167	pHSP27	480
-0.164	pJNK	15
-0.159	pJNK	60
-0.158	pERK1	5
-0.154	pERK2	0
-0.153	pERK1	0
0.153	pERK1	30
0.147	plkB α	0

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S5. Top 20 loadings in the third principal component of the apoptosis model

w₃C₃	Signal	Time (min)
-0.250	pAkt	90
-0.249	pAkt	720
0.208	pHSP27	240
0.207	pERK1	30
-0.204	pAkt	120
-0.199	pAkt	60
0.199	pHSP27	120
0.186	pERK1	60
0.185	pHSP27	90
-0.178	pAkt	480
0.173	pJNK	240
-0.167	pAkt	240
-0.167	pAkt	5
-0.165	pAkt	0
-0.162	pERK2	0
0.160	pJNK	120
-0.154	pERK1	0
0.153	pERK2	60
-0.153	pI κ B α	5
-0.151	pAkt	30

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S6. Top 20 loadings in the first principal component of the CXCL1/CXCL8 model

w₁c₁	Signal	Time (min)
0.204	pJNK	15
0.201	plkB α	120
0.195	pJNK	30
0.194	pHSP27	15
0.194	plkB α	90
0.193	pHSP27	30
0.192	plkB α	15
0.192	pHSP27	60
0.190	pJNK	60
0.185	pERK1	15
0.182	pERK2	0
0.181	plkB α	5
0.179	plkB α	720
0.178	pERK2	720
0.172	plkB α	480
0.171	plkB α	60
0.163	plkB α	240
0.162	pERK2	15
0.160	pERK1	0
0.153	pERK1	720

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S7. Top 20 loadings in the second principal component of the CXCL1/CXCL8 model

w₂C₂	Signal	Time (min)
0.244	pERK1	30
-0.236	pHSP27	0
-0.223	pAkt	15
-0.208	pJNK	5
-0.207	pAkt	480
-0.202	pAkt	5
-0.201	pAkt	0
-0.196	pJNK	0
-0.184	pAkt	30
0.184	Clvd Casp 8 (MW = 41)	480
0.183	pERK1	60
0.172	pHSP27	240
-0.169	pERK1	0
-0.168	pHSP27	5
-0.162	pAkt	60
0.159	pHSP27	120
-0.158	pHSP27	720
-0.157	pERK2	0
-0.155	pERK2	5
0.148	pERK1	120

Multiple Pathways are Impacted by Variations in RAS

Supplementary Table S8. Top 20 loadings in the third principal component of the CXCL1/CXCL8 model

w₃C₃	Signal	Time (min)
-0.247	pERK2	90
-0.233	Clvd Casp 8 (MW = 43)	480
-0.224	pERK1	5
0.223	pHSP27	60
0.219	pJNK	30
0.218	plkB α	30
0.212	pHSP27	30
0.201	plkB α	15
-0.195	pAkt	120
0.191	pJNK	60
-0.187	pERK2	120
0.181	plkB α	90
-0.181	pHSP27	5
-0.178	pERK2	5
0.174	plkB α	720
-0.173	pERK1	90
-0.163	pJNK	480
0.156	plkB α	240
-0.154	pHSP27	480
0.152	plkB α	120

Supplemental References

1. Balkwill F. Tumour necrosis factor and cancer. *Nat Rev Cancer* 2009; 9: 361-71.
2. Janes KA, Albeck JG, Gaudet S, Sorger PK, Lauffenburger DA, Yaffe MB. A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis. *Science* 2005; 310: 1646-53.
3. Owens DM, Keyse SM. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* 2007; 26: 3203-13.
4. Gaudet S, Janes KA, Albeck JG, Pace EA, Lauffenburger DA, Sorger PK. A compendium of signals and responses triggered by prodeath and prosurvival cytokines. *Mol Cell Proteomics* 2005; 4: 1569-90.