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Supplemental Data

Identification of a Molecular Signaling Network that Regulates a Cellular Necrotic Cell Death Pathway

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Supplemental Experimental Procedures

Cell Lines and Culture Media

Murine fibrosarcoma L929 and murine fibroblast NIH-3T3 cells (ATCC) were cultured in DMEM supplemented with 10%FBS and antibiotics. FADD-deficient Jurkat cells (kind gift from J. Blenis, Harvard Medical School) were grown in RPMI1640 with 10%FBS and antibiotics.

shRNAs, siRNAs, and Plasmids

shRNA against mouse cyld was cloned into retroviral pSRP vector (Degterev, A. et al., 2005) digested with Bgl2 and Xho1. The following sequence of cyld was used for the knockdown: sh-cyld: AGAGAAGAATGATATAGAG. All of the following sequences of mouse siRNAs were purchased from Dharmacon.

Non-targeting siRNA#2(D-001210-02-20)

rip1: GAAUGAGGCUUACAACAGAUU

cyld9: UGAAAUGACUGAGCGAUAAUU

cyld11: CUGCAUUGAUGAUACGAUAUU

parp2-1: GGGAAAGGCUCAUGUGUAU

parp2-3: GCAAGAAGAUGCGCACGUG

bmf1: CCAGAAAGCUUCAGUGUAU

bmf4: CGCAACAACACCAGCAGAA

tipe1-3: UACCAGCAGUGAGGUCUUG

tipe1-4: GGAGCCAUCUGUGCCGCAU

tnfr10:GGAAGGAGUUCAUGCGUUU

tnfr11: CAAUGCAGACCUUGCGAUU

Human cyld6: AGAGAUAUCUACAGACUUU

Human cyld7: GGAGAGUACUUGAAGAUGU

Antibodies

Antibody against CYLD is a generous gift from Dr. S. C. Sun (University of Texas MD Anderson Cancer Center). Anti-LC3, anti-RIP1, anti-Tubulin, and anti-TNFα antibodies were purchased from Novus Biologicals, BD Biosciences, Sigma, and R&D Systems, respectively.

RT-PCR

Total RNA of cells transfected with siRNAs were extracted by using a RNeasy column (Qiagen) and the cDNAs were synthesized from 500ng of the RNA template using SuperScript First-Strand Synthesis System for RT-PCR (Invitogen) following protocols from the manufacturers.

Analysis of Gene Expression across 61 Tissues

Microarray data files were obtained from the Novartis GNF mouse expression atlas resource (Su et al., 2004) , and expression values of 36,182 probe sets from the GNF1M custom chip were analyzed. The dataset was normalized at the probeset level using the GC Robust Multi-array Average (GCRMA) algorithm (Wu et al., 2004), and we filtered the data by excluding from the analysis those probe sets with 100% 'absent' calls (MAS5.0 algorithm) across all 61 tissues. The dataset was further filtered by setting a minimum threshold value >20 in at least one sample for each probe set and a maximum-minimum expression value >100. Data analysis and linear modeling was performed in the R programming language, utilizing functions from Linear Models for Microarray Data (limma) (Smyth, 2004). The moderated t-statistic for each probe (i.e. with the standard errors moderated across genes) was computed using a Bayesian model. The associated *p*-value was adjusted to control the false discovery rate (FDR) in

multiple testing using the Benjamini and Hochberg's method (Benjamini and Hochberg, 1995). Genes that are differentially expressed in immune tissues and neuronal tissues compared to other tissues were identified as having an FDR-adjusted *p*-value <0.05. Hierarchical clustering (centroid linkage method) was performed with Cluster 3.0 using Pearson's correlation coefficient as the similarity metric (Eisen et al., 1998). Z-score transformation was applied to each probeset across all arrays prior to generating 'heatmaps' for visualization using Java TreeView (Saldanha, 2004).

Microarray Analysis of a Large Panel of Mouse Immune Samples

Microarray data was obtained from the RIKEN (Hijikata et al., 2007) containing gene expression data from 119 mouse samples of which immune cells constitute 83%. The dataset was GCRMA-normalized (Wu et al., 2004) and linear models applied using functions from limma (Smyth, 2004) implemented in the R programming language (as above).

Microarray Analysis of Gene Expression in Mouse L929 and NIH3T3 Cells

Total RNA of L929 cells or NIH3T3 cells were extracted by Trizol (Invitrogen) followed by RNeasy (Qiagen). RNA analyses were performed at the microarray core facility at the Harvard Medical School and Partners Healthcare Center for Genetics and Genomics. The quantity, purity and integrity of RNA were evaluated by UV spectrophotometry and

RNA-nano Bioanalyzer (Agilent). Sample processing and hybridization on Mouse Genome 430 2.0 GeneChip microarrays (Affymetrix) were performed according to manufacturer's instructions. Probe-level normalization, filtering and data analysis performed as described in the previous section. Genes that are differentially expressed in L929 cells relative to NIH3T3 cells were identified as having an FDR-adjusted *p*-value <0.05 and a fold-change >1.5. The GEO accession number of this microarray analysis: GSE13335.

Microarray Analysis of Gene Expression in Jurkat, HeLa, HEK293, and HEK293T Cell Lines

The MAS5.0-processed microarray (Affymetrix U133A-based) dataset was obtained from the Genomics Institute of the Novartis Foundation (GNF) website (http://wombat.gnf.org). This dataset contains expression data from the NCI60 cell lines collection as well as other human cell lines including necroptosis-sensitive Jurkat, and necroptosis-resistant HeLa, HEK293 and HEK293T. We examined in these four cell lines, the expression profiles of probesets corresponding to 9 genes which represent human orthologs of mouse genes showing higher expression in L929 cells versus NIH3T3 cells, and whose probes on the GNF microarray indicate elevated expression in Jurkat (at least 1.5-fold) relative to HeLa and HEK293/HEK293T. Expression data for

these 4 cell lines were z-score transformed, clustered and a heatmap generated as described in sections above. Two additional human orthologs, SUMO3 and POLD3 were not included because those probes did not show a consistent change in Jurkat versus HeLa and HEK293/HEK293T.

Figure S1

Supplementary Figure 1

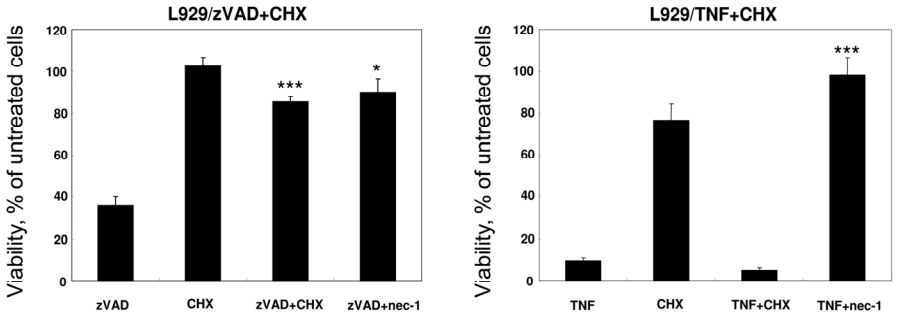

Figure S1. Inhibition of zVAD.fmk-Induced Necroptosis by Inhibitor of Protein Synthesis

L929 cells were treated with 20μM zVAD.fmk (left panel) for 20hr, or with 10ng/ml TNFα for 48rh (right panel) with or without co-treatment of 1.0μg/ml cycloheximide (CHX) and 30μM nec-1. The cellular viability was measured as described in Figure 1. Error bars, SD. *p < 0.05; **p < 0.01; ***p < 0.001; n = 4.

Supplemental References

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