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

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SUMMARY

Stimulation of death receptors by agonists such as FasL and TNFα activates apoptotic cell death in apoptotic-competent conditions or a type of necrotic cell death dependent on RIP1 kinase, termed necroptosis, in apoptotic-deficient conditions. In a genome-wide siRNA screen for regulators of necroptosis, we identify a set of 432 genes that regulate necroptosis, a subset of 32 genes that act downstream and/or as regulators of RIP1 kinase, 32 genes required for death-receptor-mediated apoptosis, and 7 genes involved in both necroptosis and apoptosis. We show that the expression of subsets of the 432 genes is enriched in the immune and nervous systems, and cellular sensitivity to necroptosis is regulated by an extensive signaling network mediating innate immunity. Interestingly, Bmf, a BH3-only Bcl-2 family member, is required for death-receptor-induced necroptosis. Our study defines a cellular signaling network that regulates necroptosis and the molecular bifurcation that controls apoptosis and necroptosis.

INTRODUCTION

Cell death has been traditionally classified as apoptosis or necrosis. While apoptosis is known as a regulated cellular mechanism, necrosis is known as passive cell death caused by overwhelming stress. Necrosis is characterized by rapid loss of plasma membrane integrity, organelle swelling and mitochondrial dysfunction, and the lack of typical apoptotic features such as internucleosomal DNA cleavage and nuclear condensation. Although necrosis is known to occur under a variety of pathological conditions, little effort has been made to study necrosis due to the belief in its unregulated nature. Support for a regulated necrosis mechanism came from studies of the death receptors. Activation of the Fas and TNFR family of death receptors induces a "prototypic" apoptotic pathway through the recruitment of adaptor proteins, such as FADD, and upstream caspases, such as caspase-8. Interestingly, it was discovered that, in certain cell types, stimulation with FasL or TNFα under apoptosis-deficient conditions could induce cell death with morphological features of necrosis (Kawahara et al., 1998; Vercammen et al., 1997). The fact that the activation of Fas/TNFα receptors may lead to cell death with features of either apoptosis or necrosis argues strongly for the existence of a regulated cellular necrosis mechanism, discrete from apoptosis, which we termed "necroptosis" (Degterev et al., 2005).

RIP1 is a death-domain-containing kinase associated with the death receptors, but its kinase activity is dispensable for the induction of death-receptor-mediated apoptosis (Grimm et al., 1996). In apoptosis-deficient conditions, however, RIP1 kinase activity has been found to be required for the activation of necroptosis by death receptor agonists (Holler et al., 2000). In our previous studies, we have isolated multiple small molecule inhibitors of necroptosis termed necrostatins (Necs) (Degterev et al., 2005, 2008). Importantly, we have shown that Nec-1 is an allosteric inhibitor of RIP1 kinase activity (Degterev et al., 2008). Using Nec-1 as a tool, necroptosis has since been found to contribute to a wide range of pathologic cell death paradigms, including ischemic brain injury, myocardial infarction, excitotoxicity, and chemotherapy-induced cell death (Degterev et al., 2005; Han et al., 2007; Smith et al., 2007; Xu et al., 2007). Here, we have broadly explored the molecular mechanism and functional significance of necroptosis by carrying out a genome-wide siRNA screen for genes required for necroptosis. Our study defines a genetic profile for a cellular necrotic pathway, elucidates the connection between apoptosis and necroptosis, and implicates necroptosis as a critical regulatory pathway for innate immunity and suggests a potential role of necroptosis in human disease.

RESULTS

A Screen for Genes Required for Necroptosis

The treatment of L929 cells with zVAD.fmk has been shown to induce necroptosis, which can be inhibited by Nec-1 (Degterev

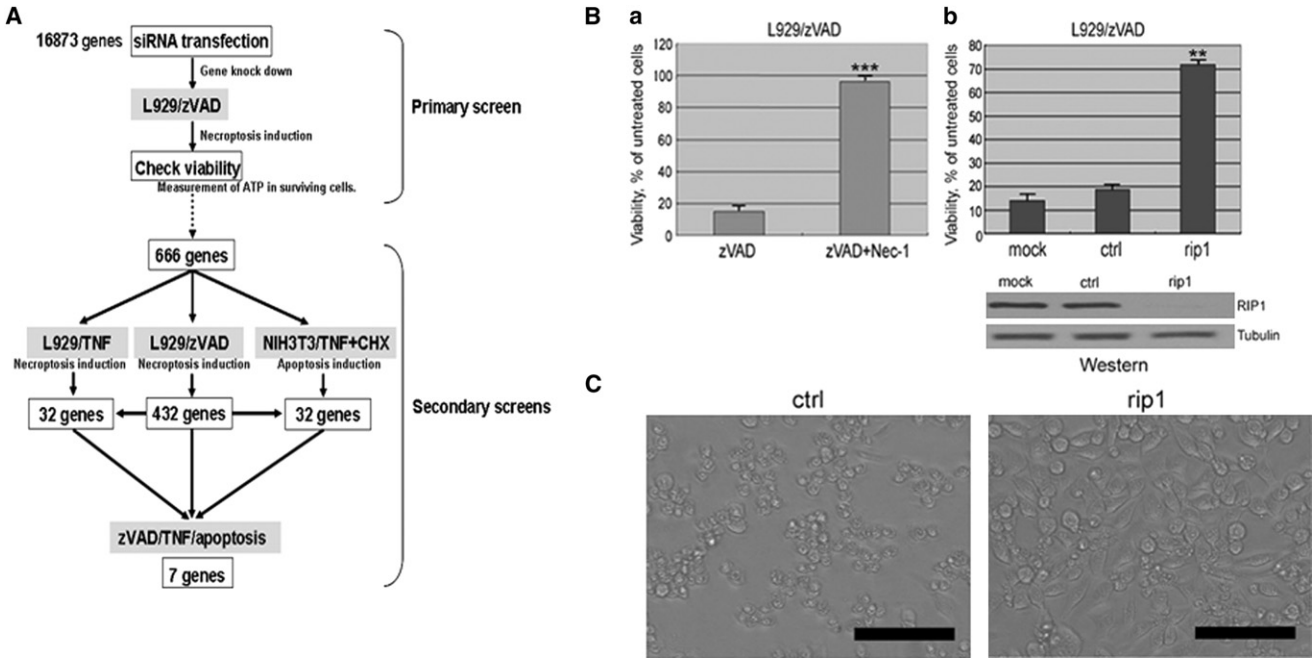

Figure 1. siRNA Screen for Genes Required for Necroptosis

(A) A schematic diagram of first and second screens.

(B) L929 cells not transfected ("a") or transfected with either nontargeting siRNA (ctrl) or *rip1* siRNA at 50 nM for 48 hr ("b") were treated with 20 μM zVAD.fmk with or without 30 μM Nec-1 for 18 hr. (Bottom) Lysates of siRNA-transfected L929 were analyzed by western blotting using anti-RIP1 or anti-Tubulin antibodies.

(C) The morphological change of L929 cells transfected with indicated siRNAs after 18 hr treatment of 20 μM zVAD.fmk were shown by phase-contrast microscopic pictures.

Scale bars, 100 μm. Cellular viability was evaluated by measuring ATP levels in surviving cells using CellTiter-Glo. Error bars, SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$.

et al., 2005). Using this model, we screened the Dharmacon siRNA library covering the mouse genome (16,873 genes) for genes required for necroptosis (Figure 1A). RIP1 siRNA was used as a positive control as knockdown of RIP1 efficiently blocked necroptosis induced by zVAD.fmk (Figure 1B). In the nontargeting siRNA-transfected cells (Dharmacon), the treatment of zVAD.fmk induced ~80% cell death. A siRNA was scored as positive if its ATP level (a surrogate for cell survival) was > 2 standard deviations (SD) above the mean ATP level of the plate. Using this criterion, 666 genes were scored as candidates required for zVAD.fmk-induced necroptosis in L929 cells. As expected, rip1 was scored as a hit in this assay, providing a validation for our approach (Figures 1B and 1C).

To confirm the screening result, we rescreened the 666 primary siRNA hits using four individual siRNAs for each gene. In order to restrict our analysis to genes that have major impacts on cellular sensitivity to necroptosis, we required that at least two out of the four siRNAs increased cell survival for > 3 SD above that of cells transfected with nontargeting siRNA control and showed at least 60% of the viability of cells expressing the positive control rip1 siRNA. Using these criteria, 432 genes were scored positive. RIP1 was again one of the validated hits, with all four siRNAs showing a protective effect against necroptosis induced by zVAD.fmk (data not shown).

We examined the expression of genes identified in the zVAD.fmk screen across 61 normal mouse tissues using microarray data files from the Novartis GNF mouse expression atlas resource (Su et al., 2004) and found clusters of hits showing increased expression in immune and neuronal cells/tissues (FDR-adjusted $p < 0.05$) (Figure 2A and Tables S1 and S2 available online). To further define the immune cell types, we also analyzed a larger mouse immune microarray panel comprising gene expression profiles from 119 mouse cell/tissue samples, of which 83% represent various types of primary immune cells (Hijikata et al., 2007). We observed a cluster of genes from the zVAD.fmk screen exhibiting increased expression in macrophages, dendritic cells, and NK cells and another cluster showing enhanced expression in B and T lymphocytes (Figure 2B and Tables S3 and S4). The enriched expression in cells of the immune system also suggests a role of necroptosis in regulating immune function.

To further confirm a functional role of necroptosis in the immune system, we tested the sensitivity of primary peritoneal macrophages to Nec-1. As shown in Figure 2C, spontaneous as well as zVAD.fmk-induced cell death of macrophages was inhibited by Nec-1, suggesting that necroptosis plays a role in regulating the survival of activated primary macrophages, one of the key cell types involved in innate immunity. On the other hand, death-receptor-mediated cell death of epithelial cell lines, such as HeLa cells, 293 cells (Degterev et al., 2005), and MCF10A (data not shown), is not sensitive to the inhibition of Nec-1.

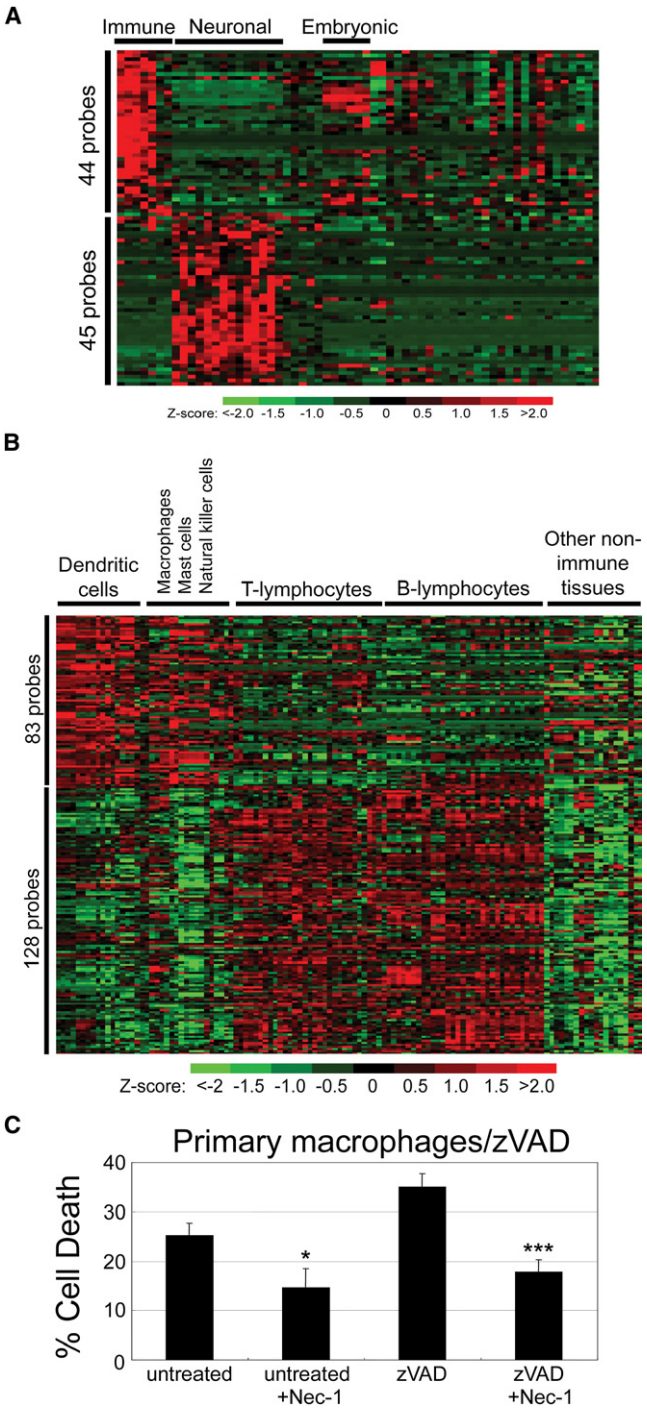

Figure 2. Expression Patterns of the zVAD Hits in Mouse Tissues and Primary Cells

(A) Expression profiles of genes from the zVAD.fmk screen showing significantly higher expression in immune cells or neuronal cells (FDR-adjusted $p < 0.05$) as observed in the Novartis GNF1m microarray data examining expression across 61 mouse tissues.

(B) Expression profiles of genes from the zVAD.fmk screen exhibiting increased expression in mouse macrophages, dendritic cells, and NK cells or in B and Tlymphocytes from a large microarray panel of 119 mouse cell/tissue samples obtained from the RIKEN resource.

CYLD, a tumor suppressor and a deubiquitinating enzyme encoded by a gene that is mutated in familial cylindromatosis (Simonson et al., 2007), was identified in the microarray data sets as having increased gene expression in immune and neuronal cells/tissues (Tables S1 and S2). CYLD is known to be recruited to the TNFα receptor upon its activation, and, furthermore, RIP1 has been identified as a CYLD substrate (Wright et al., 2007), suggesting that CYLD may represent a key regulatory factor in the necroptotic pathway. To confirm the role of CYLD in necroptosis, we compared the efficiency of cyld knockdown with necroptosis inhibition. As shown in Figure 3A, we found an excellent correlation between the efficiency of cyld knockdown by individual siRNAs and protection against zVAD.fmk-induced necroptosis in L929 cells. To further confirm the role of cyld in necroptosis, we used siRNAs against human cyld to inhibit its expression in FADD-deficient Jurkat cells. We found that inhibition of cyld expression in Jurkat cells also attenuated necroptosis (Figure 3B). Execution of necroptosis is associated with activation of autophagy and increased formation of its marker, LC3II, which is efficiently inhibited by Nec-1 (Degterev et al., 2005). Consistently, knockdown of cyld also inhibited LC3II induction in L929 cells treated with zVAD.fmk (Figure 3C).

Since CYLD regulates TNFα signaling (Wright et al., 2007), the requirement of cyld suggests that autocrine regulation of TNFα signaling might be involved in regulating cellular sensitivity to necroptosis induced by zVAD.fmk. Consistent with this possibility, TNFR1 (TNFRSF1a) was identified as one of the genes required for zVAD.fmk-induced necroptosis (Table S6). Since inhibition of caspases by expression of crmA, a caspase inhibitor encoded by cowpox virus, or by multiple peptide caspase inhibitors was found to sensitize L929 cells to TNFα-induced necrosis (Vercammen et al., 1998), our results indicate that an autocrine regulation of TNFα contributes to necroptosis induced by zVAD.fmk.

Canonical Pathways in Necroptosis

To gain insights into the function of genes scored in our siRNA screen, we used a gene set approach to identify common pathways associated with these gene clusters. Using the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005), which provides a catalog of some 3000 sets of genes divided into various annotation groupings, we interrogated human orthologs of the 432 genes involved in zVAD.fmk-induced necroptosis against curated gene sets (canonical pathways) and motif-based gene sets (transcription factor targets) from the MSigDB. We identified canonical pathways that were found to be significantly enriched ($p < 0.05$) among zVAD.fmk hits compared to the full set of genes screened in the siRNA library to which human orthologs could be mapped. Involvement of the TNFR1 pathway is very clear, as multiple hits in the zVAD.fmk screen, such as *tnfr1* (*tnfrsf1a*), *ripk1*, *cyld*, *jun*, and *grb2*, have established roles in

(C) Involvement of necroptosis in primary macrophage cell death induced by zVAD.fmk. Isolated peritoneal macrophages from mice stimulated by thioglycollate were untreated or treated with 100 μM zVAD.fmk with or without 30 μM Nec-1 for 24 hr, and cell death was measured by Sytox assay (Invitrogen). Error bars, SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$.

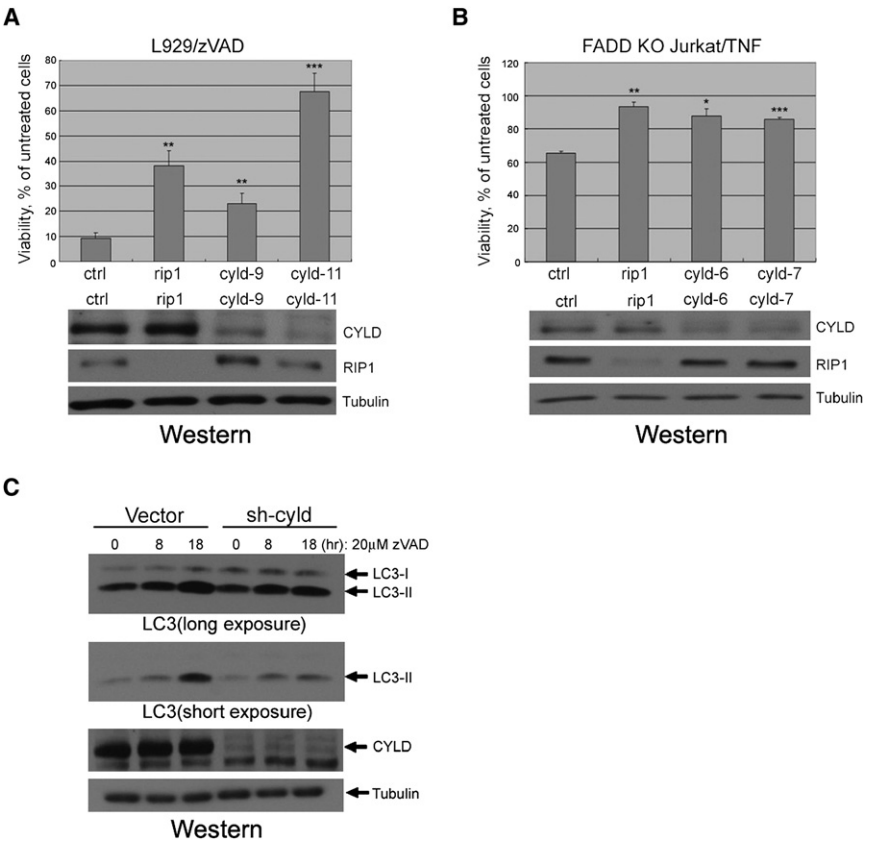

Figure 3. CYLD Knockdown Inhibits Necroptosis

(A) L929 cells were transfected with three different siRNAs against cyld (cyld9 and cyld11) and rip1 (50 nM) for 48 hr, treated with 20 μM zVAD.fmk for an additional 18 hr, and the viability was measured as in Figure 1. Knockdown efficiency of cyld was confirmed by western blot using anti-CYLD, anti-RIP1, or anti-Tubulin antibodies. Error bars, mean SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$.

(B) Human FADD-deficient Jurkat cells were electroporated with siRNAs against human cyld (cyld6 and cyld7). At 48 hr later, the cells were treated with 10 ng/ml TNFα for an additional 16 hr, and the viability was measured as in Figure 1. The cell lysates were analyzed by western blotting as in (A).

(C) Inhibition of LC3-II induction in cyld knockdown cells. L929 cells stably expressing shRNA for cyld (sh-cyld) or vector alone (vector) were treated with 20 uM zVAD.fmk for indicated periods, and the cell lysates were analyzed by western blotting using anti-LC3-II, anti-CYLD, or anti-Tubulin antibodies.

TNF signaling. In addition, the toll-like receptor (TLR) pathway, which was identified to be significantly enriched in the canonical pathway analysis, is also likely to be involved in necroptosis, as several zVAD.fmk hits, such as *ripk1*, *cyld* (Meylan et al., 2004; Yoshida et al., 2005), multiple members of the interferon family (*ifna1*, *ifna7*, and *ifna13*), and an IFN-induced gene, LRG47/Irgm1, are also known to have roles in the TLR pathway. Interestingly, our analysis identified that at least 70 out of 432 genes identified in the zVAD.fmk screen are connected through an extensive network surrounding innate immune pathways to TNFR1 and TLR signaling using pathway components, *ripk1*, *jun*, *cd40*, and *spp1*, which are also zVAD.fmk hits, as anchor points (Figure 4A). An important role of necroptosis in innate immunity is consistent with the enriched expression of zVAD.fmk hits in the immune system. This network provides a framework for future explorations into the mechanisms by which cellular sensitivity to necroptosis is regulated in response to multiple TLR and death receptor family members.

Although it was not known that the treatment with zVAD.fmk would lead to the activation of some of the signaling processes involved in the TNFR1 and TLR pathways, the agonists of TNFR1 and TLR have been previously shown to induce caspase-independent necrosis that we termed necroptosis (Degterev et al., 2005; Kalai et al., 2002; Temkin et al., 2006; Vercammen et al., 1998; Xu et al., 2006). Consistent with a role of TNFR1 in zVAD-induced necroptosis, neutralization of TNFα by antibody protected L929 cells from necroptosis induced by zVAD.fmk (Figure 4B). Furthermore, we found that L929 cells can be induced to die by cotreatment with IFNγ and polyI:C (polyinosinic:polycytidylic acid), a synthetic double-stranded RNA agonist of TLR3 commonly used to mimic viral infection, and the cell death was inhibited by Nec-1 (Figure 4C). Thus, consistent with the connection of necroptosis and innate immunity, the cell death induced by the activation of TLR3 in L929 cells in the presence of IFNγ requires RIP1 kinase activity to induce necroptosis.

The canonical pathway enrichment analysis also uncovered the involvement of the glutathione metabolic pathway, the glycosylphosphatidylinositol pathway, proteins involved in translation (including translation initiation factors), and ribosomal proteins (Table S5). The protection offered by reducing the levels of glutathione peroxidase (*gpx4*) and glutathione S-transferases (*gsta3* and *gsto2*), which is expected to increase the availability of free glutathione, is consistent with the fact that RIP1 activation leads to increases in the cellular levels of free radical species, which plays an important role in mediating necroptosis (Xu et al., 2006; Yu et al., 2006). Similarly, inhibition of glycosylphosphatidylinositol (GPI) anchor biosynthesis by knockdown of *pgap1*, *pigl*, and *pigm* is consistent with the requirement of GPI in TNFα signaling (Fukushima et al., 2004). Identification of these canonical pathways provides new insights into the molecular mechanism of necroptosis induced by zVAD.fmk as well as a validation for our screen.

Role of Transcription and Translation in Necroptosis

Of the 432 genes involved in zVAD.fmk-induced necroptosis, 291 (67%) and 281 (65%) could be classified into broad molecular function (Figure 5A and Table S6) and biological process (Figure 5B and Table S7) categories for mouse genes, respectively. There appears to be an enrichment trend for nucleic

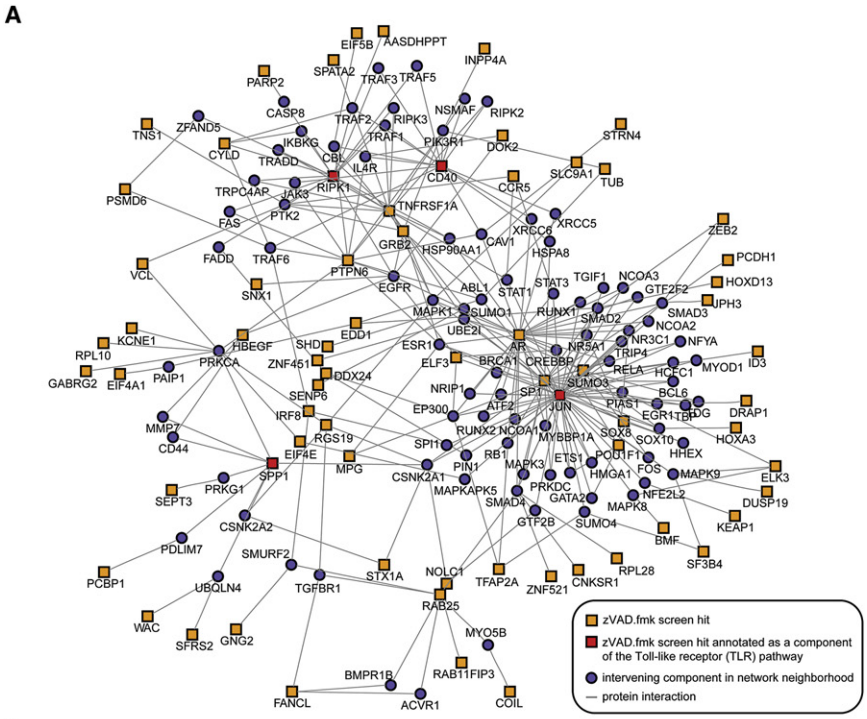

Figure 4. Network Extension of the Innate Immune Toll-Like Receptor Signaling Pathway and Its Participation in Necroptosis

(A) Network was constructed by anchoring on TLR pathway components (CD40, RIPK1, JUN, and SPP1) that are also zVAD.fmk hits using protein-protein interaction data curated from literature and high-throughput yeast two-hybrid screens.

(B) Autocrine production of TNFα is involved in zVAD-induced necroptosis. L929 cells were pre-treated with indicated concentrations of anti-mouse TNFα antibody (μg/ml) for 1 hr followed by treatment with or without 20 μM zVAD.fmk or 30 μM Nec-1 for 16 hr.

(C) Involvement of TLR pathway in necroptosis. L929 cells were treated with indicated chemicals for 19 hr. Cellular viability was measured as described in Figure 1. polyI:C; 25 μg/ml, interferon gamma (IFNγ); 1000U/ml, Nec-1; 30 μM.

Error bars, SD. *p < 0.05; **p < 0.01; ***p < 0.001; n = 4.

acid binding (unadjusted hypergeometric p = 0.01) and nucleic acid metabolism (hypergeometric p = 0.003) categories in the set of genes involved in zVAD.fmk-induced necroptosis relative to their representation in the global set of genes examined in the siRNA screen. None of the functional enrichments were significant at the 0.05 level after being adjusted using Benjamini and Hochberg's (BH) method, but this may be overly conservative since the functional categories are not independent. Interestingly, 43 genes in the zVAD.fmk hit list encode transcription factors (Table S6).

To begin to characterize the regulation of necroptosis, we explored the promoter regions of genes identified in our siRNA screen to see whether we could find shared transcription factor binding sites as defined in the TRANSFAC database (www.gene-regulation.com) and the MSigDB collection of transcription factor (TF) targets (i.e., motif-based gene sets) (Xie et al., 2005). We found an enrichment of binding sites (unadjusted hypergeometric p < 0.05) for TFs such as *myog/nf1*, *meisb1/hoxa9*, *nrf2*, *hnf4*, *lef1*, *ar*, *pax4*, and *pparg* in the promoters of genes involved in zVAD.fmk-induced necroptosis (Figure 5C), suggesting the involvement of these *cis* elements in the underlying transcriptional regulatory control of genes involved in zVAD.fmk-induced necroptosis. Consistent with this proposal, a binding site for the androgen receptor (AR) was found to be enriched in the promoters of genes involved in necroptosis, and AR was also a hit in the screen for genes involved in zVAD.fmk-induced necroptosis (Table S6). This provides further evidence for the importance of transcriptional control of necroptosis.

In contrast to death-receptor-mediated apoptosis, which is sensitized by the inhibition of protein synthesis via inhibition of the NF-κB transcriptional response, inhibition of protein synthesis by CHX has been shown to inhibit necroptosis induced by zVAD.fmk (Yu et al., 2004), but not by TNFα (Figure S1). Thus, the protection provided by the inhibition of some of the essential

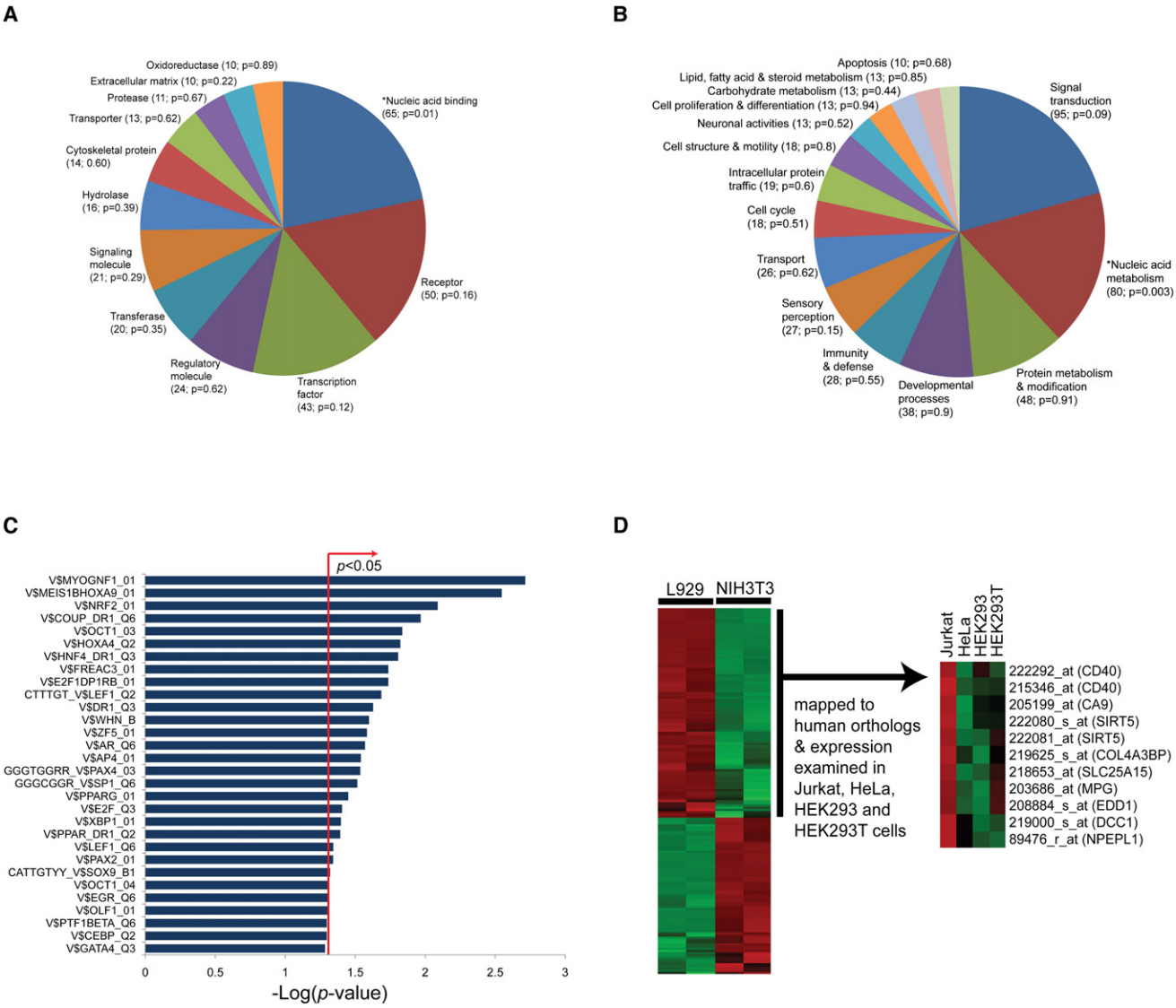

Figure 5. Enrichment of Transcription Factor Binding Sites and Nucleic Acid Binding Function of Genes Involved in Necroptosis

(A and B) The 432 hit genes from the secondary screen for zVAD.fmk-induced necroptosis were classified into (A) molecular function and (B) biological process categories for mouse genes according to the PANTHER classification system. Genes for which no annotations could be assigned were excluded from the analysis for both the hits and the global set (i.e., genes examined in the siRNA screen). Categories with at least ten genes are displayed in the pie charts. The number of genes assigned to each category and enrichment p values are shown in brackets. An asterisk denotes categories found enriched (unadjusted hypergeometric $p < 0.05$) relative to the global set of genes examined in the screen. Lists of assigned genes grouped by molecular function or biological process categories are provided in Tables S6 and S7, respectively.

(C) Enrichment analysis of *cis* regulatory elements, in particular transcription factor (TF) binding sites in the promoters of genes involved in zVAD.fmk-induced necroptosis (vertical axis of graph), using motif-based gene sets from the MSigDB and TF binding sites defined in the TRANSFAC database. TF binding motifs were examined for enrichment among human orthologs of zVAD.fmk hits compared to the global set of genes screened in the siRNA library to which human orthologs could be mapped. The bar chart displays the negative log of the enrichment p values for each pathway using the hypergeometric distribution.

(D) Expression profiles of probes against zVAD.fmk screen hits showing elevated expression in necroptosis-sensitive L929 cells compared to necroptosis-resistant NIH 3T3 cells were mapped to human orthologs and expression trends examined in four human cell lines: necroptosis-sensitive Jurkat cells, necroptosis-resistant HeLa, HEK293 cells, and HEK293T cells (from the GNF microarray collection). Expression values were z-score-transformed for each probe across arrays.

cellular machinery involved in protein translation, such as *eif3s10*, *eif4a1*, *eif4e*, *eif4ebp2*, *eif5b*, and *pcbp1*, as well as multiple ribosomal proteins and proteins involved in mRNA splicing (Table S6), is likely due to the inhibition of protein translation. Consistent with necroptosis being a cellular program of necrotic cell death, transcription and translation may be essential for the progression of necroptosis, at least under certain conditions. Furthermore, these data suggest an intriguing possibility that

availability of the translational machinery might represent another factor determining cellular choice in executing cell death.

Gene Expression Profile of Cells Sensitive to Necroptosis

To examine the transcriptional profile associated with cellular sensitivity to necroptosis, we performed microarray analysis on L929 cells, which are sensitive to necroptosis, and NIH 3T3 cells, which we have shown previously to be unable to undergo necroptosis (Degterev et al., 2005). By comparing the expression profiles of the 432 hit genes from the zVAD.fmk screen in these two cell types, we identified 60 zVAD.fmk hits that were expressed at significantly higher levels in L929 cells relative to NIH 3T3 cells (FDR-adjusted $p < 0.05$; fold-change > 1.5) (Table S8).

To determine whether this transcriptional profile is preserved in other cell lines known to be sensitive or resistant to necroptosis, we next examined gene expression data for four human cell lines (Jurkat, HeLa, HEK293, and HEK293T) from the Genomics Institute of the Novartis Foundation (GNF) cell line collection. The trend appears to be consistent for the nine genes that we examined (*edd1*, *mpg*, *ca9*, *slc25a15*, *sirt5*, *npepl1*, *dcc1*, *cd40*, and *col4a3bp*) (Figure 5D). These genes exhibited elevated expression in necroptosis-sensitive Jurkat cells relative to HeLa, HEK293, and HEK293T cells, which are resistant. These results suggest that increased expression of a subset of zVAD hit genes might convey cellular sensitivity to necroptosis.

Core Components of the Necroptotic Pathway

Treatment of L929 cells with TNFα strongly induces necroptosis (Fiers et al., 1995). TNFα, however, also induces the activation of NF-κB, which requires the intermediate domain of RIP1 (Ting et al., 1996). Since activation of NF-κB is prosurvival, necroptosis of L929 cells induced by TNFα cannot be inhibited by RIP1 siRNA (Figure 6A). On the other hand, since Nec-1 targets RIP1 kinase activity specifically and does not affect the activation of NF-κB, Nec-1 inhibits TNFα-induced necroptosis of L929 cells (Degterev et al., 2005, 2008) (Figure 6A). Since RIP1 is specifically recruited to the TNFα receptor in the activation of necroptosis (Zheng et al., 2006), the genes required for TNFα-induced necroptosis are predicted to be specifically downstream and/or regulators of RIP1 kinase activity.

We screened the 666 primary siRNA hits from the zVAD.fmk screen for genes required for necroptosis induced by TNFα (Figure 1A). In nontargeting siRNA-transfected cells, TNFα caused ~80% cell death. tnfr1 siRNA, which provided a strong protection against necroptosis induced by TNFα, was used as a positive control. A gene was scored as positive if at least two out of four of its siRNAs increased cell survival to at least 50% of the level provided by tnfr1 siRNA. This screen identified 32 genes required for necroptosis induced by TNFα (Table 1). Since these 32 genes are also required for zVAD.fmk-induced necroptosis, we hypothesize that these 32 genes represent potential core components of the necroptotic pathway. Consistent with this proposal, CYLD, which has been shown previously to interact with RIP1 and regulate RIP1 ubiquitination (Wright et al., 2007), is required for necroptosis induced by either zVAD.fmk or TNFα. Importantly, PARP-2, a member of poly-ADP ribose polymerase family (Menissier de Murcia et al., 2003), and Bmf, a member of the Bcl-2 family, is also required for necroptosis induced by either zVAD.fmk or TNFα (Figures 6B and 6C). In addition, TIPE1 is also required for both zVAD.fmk and TNFα-induced necroptosis (Figure 6D). TIPE1 is a close homolog of TIPE2 that has been shown to play an important role in mediating death-receptor-mediated apoptosis and innate immunity (Sun et al., 2008).

Common Regulators of Apoptosis and Necroptosis

Because multiple hits in our screen, such as *cyld* and *bmf*, have been previously shown to contribute to apoptosis, we hypothesized that apoptosis and necroptosis may share certain common regulators. To ask whether any of the hits from the zVAD.fmk screen may also be required for apoptosis, we screened 666 primary hits from the zVAD.fmk screen against apoptosis induced by TNFα and CHX in NIH 3T3 cells (Figure 1A). We chose this system because we have previously found that cell death induced by TNFα and CHX in NIH 3T3 cells cannot be inhibited by Nec-1 but is efficiently prevented by zVAD.fmk, as is characteristic for apoptosis (Degterev et al., 2005). In control nontargeting siRNA-transfected cells, the treatment with TNFα induced at least 60%–70% cell death, which is blocked by knockdown of cyld and tnfr1, but not by that of rip1 (Figure 6E). tnfr1 siRNA was again used as a positive control. A gene was scored as positive if at least two out of four of its siRNAs increased cell survival to at least 80% of that of positive control cells expressing tnfr1 siRNA on the same plate. This screen identified 32 genes required for both apoptosis of NIH 3T3 cells induced by TNFα and CHX and necroptosis of L929 cells induced by zVAD.fmk (Table 1). The expression of these 32 genes may have a general impact on cellular sensitivity to multiple cell death stimuli.

Overall, comparing the lists of genes required for necroptosis of L929 cells induced by zVAD.fmk and TNFα and apoptosis of NIH 3T3 cells induced by TNFα and CHX, we identified seven genes that are involved in all three cell death paradigms (Table 1). Consistent with the role of TNFα in all three cell death paradigms, three out of seven factors have been previously shown to regulate TNFα signaling: TNFR1, CYLD, which directly interacts with the TNFR1 complex, and TIPE1, which is also very likely to be a component of the apical death receptor signaling complexes.

Potential Roles of Necroptosis in Human Disease

To further explore the significance of necroptosis in human disease, we analyzed the 432 genes from zVAD.fmk screen against a database of known human disease genes. Interestingly, we found 33 genes from the zVAD.fmk screen that are also implicated in human diseases (Table S9). For example, junctophilin-3 (*jph3*) has been implicated in Huntington's disease-like 2 (HDL2). HDL2 is an autosomal-dominant, progressive, adult-onset neurodegenerative disorder similar to Huntington's disease pathologically. Although the mechanism of neurodegeneration of HDL2 is still not yet clear, a CAG/CTG expansion mutation was found in a variably spliced exon of *jph3* that is responsible for the disease (Holmes et al., 2001). Such connections of genes involved in necroptosis and human diseases suggest the possible involvement of necroptosis in these human diseases that needs to be examined in future.

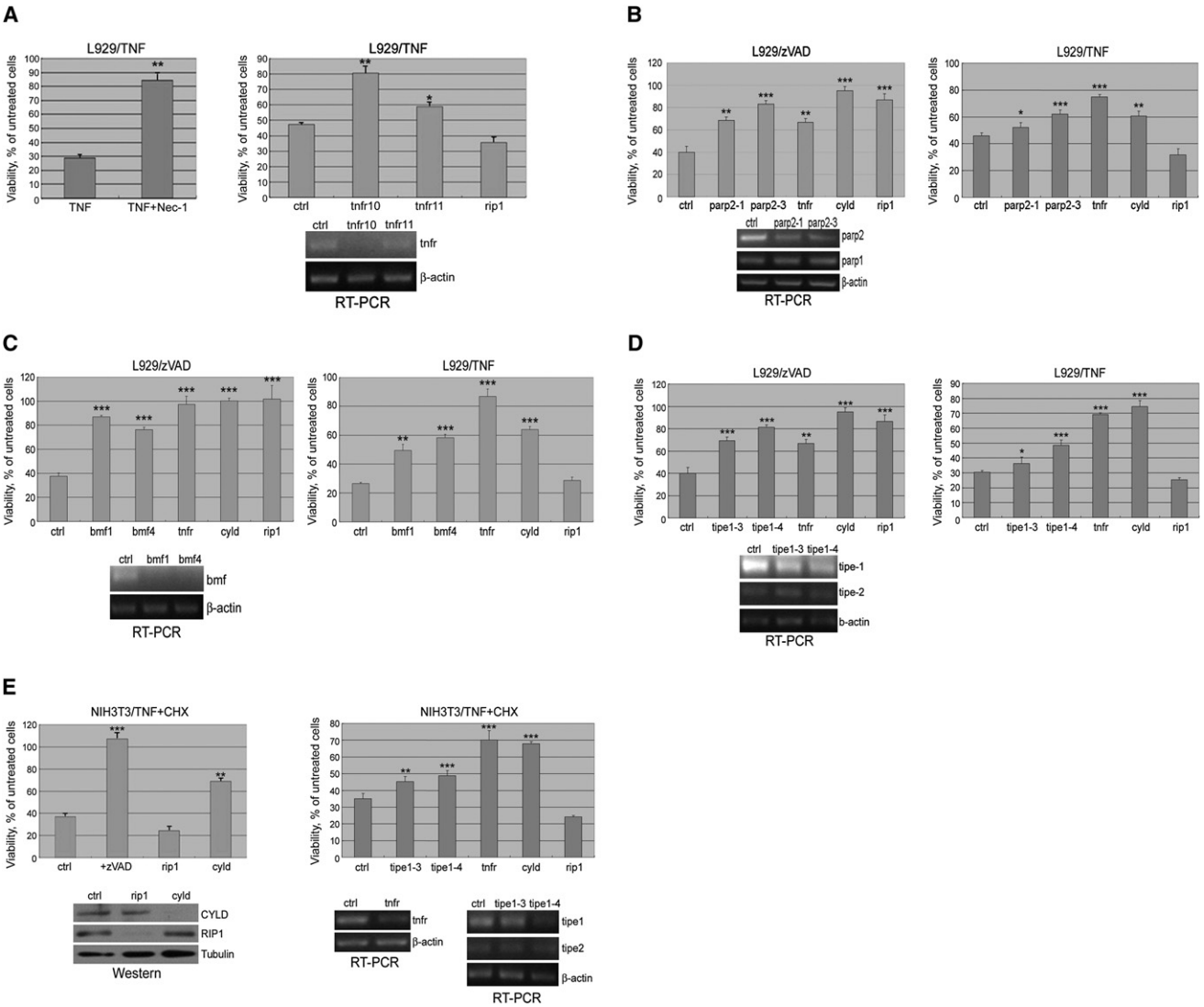

Figure 6. The Genes Regulate Necroptosis and Apoptosis

(A) L929 cells were treated with 40 ng/ml TNFα for 20 hr with or without treatment of 30 μM Nec-1. L929 cells, transfected with indicated siRNAs (tnfr, tnf receptor; ctrl, nontargeting siRNA), were treated with 10 ng/ml TNFα for 18 hr. Knockdown efficiency of tnfr in L929 cells was determined by RT-PCR (right bottom panel).

(B) Knockdown of *parp-2* inhibits necroptosis. L929 cells transfected with indicated siRNAs treated with 20 μM zVAD.fmk (left) for 18 hr or with 10 ng/ml TNFα for 20 hr (right). The knockdown efficiency was determined by RT-PCR using Parp-1 as a control (left bottom).

(C) Knockdown of *bmf* inhibits necroptosis. L929 cells transfected with indicated siRNAs (bmf, ctrl, cyld, and rip1) were treated as in (B). Knockdown efficiency of *bmf* was determined by RT-PCR (left bottom panel).

(D) Knockdown of *tipe1* inhibits necroptosis and apoptosis. L929 cells transfected with indicated siRNAs (tipe1, tnfr, cyld, and rip1) were treated as in (B).

(E) NIH 3T3 cells transfected with indicated siRNAs (tipe1, tnfr, rip1, and cyld) (40 nM) were treated with 10 ng/ml TNFα + 1.0 μg/ml CHX for 12 hr with or without treatment of 100 μM zVAD.fmk (upper panels). Knockdown efficiency was determined by RT-PCR for *tipe1* using *tipe2* as a control (right bottom panels) or western blotting for RIP1 and CYLD (left bottom panel). Cellular viability was measured as described in Figure 1.

Error bars, SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$.

DISCUSSION

In this study, we show an extensive signaling network that regulates necroptosis, a cellular caspase-independent necrotic cell death pathway. The enriched expression of the genes required for necroptosis in the immune and nervous systems suggests that necroptosis may function as a physiological cell death mechanism in these two compartments. Interestingly, cell death with necrotic features, termed "type III cell death," has been described to occur during normal development of the nervous system (Clarke, 1990). We propose that necroptosis may represent a type of type III cell death. On the other hand, the ability of Nec-1 to extend the life span of activated primary macrophages suggests that necroptosis could function as a cellular mechanism

Table 1. The Genes that Regulate Necroptosis and Apoptosis

SYMBOL	ID	NAME	
Tmem57	66146	transmembrane protein 57	
Grb2	14784	growth factor receptor bound protein 2	
Pvr	52118	poliovirus receptor	
Foxi1	14233	forkhead box I1	
Jph3	57340	junctophilin 3	
Rab25	53868	RAB25, member RAS oncogene family	
S100a7a	381493	S100 calcium binding protein A7A	
4933439F11Rik	66784	RIKEN cDNA 4933439F11 gene	
4732429D16Rik	217305	RIKEN cDNA 4732429D16 gene	
9430015G10Rik	230996	RIKEN cDNA 9430015G10 gene	
Bmf	171543	Bcl2 modifying factor	
Defb1	13214	defensin beta 1	
Eif5b	226982	eukaryotic translation initiation factor 5B	
Dpysl4	26757	dihydropyrimidinase-like 4	
Commd4	66199	COMM domain containing 4	zVAD/TNF
Txnl4b	234723	thioredoxin-like 4B	
F730015K02Rik	319526	RIKEN cDNA F730015K02 gene	
Hspbap1	66667	Hspb associated protein 1	
Mag	17136	myelin-associated glycoprotein	
Mrcl	353287	mannose receptor-like precursor	
Hoxa3	15400	homeo box A3	
Kcnip1	70357	Kv channel-interacting protein 1	
Galnt5	241391	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N–acetylgalactosaminyltransferase 5	
Olfr1404	258881	olfactory receptor 1404	
Parp2	11546	poly (ADP-ribose) polymerase family, member 2	
Tnfrsf1a	21937	tumor necrosis factor receptor superfamily, member 1a	zVAD/TNF/apoptosis
Sycp2	320558	synaptonemal complex protein 2	
Atp6v1g2	66237	ATPase, H+ transporting, lysosomal V1 subunit G2	
Nudt13	67725	nudix (nucleoside diphosphate linked moiety X)-type motif 13	
Spata2	263876	spermatogenesis associated 2	
cyld	74256	cylindromatosis 1	
Tnfaip8l1	66443	tumor necrosis factor, alpha-induced protein 8-like 1	
Grid2	14804	glutamate receptor, ionotropic, delta 2	
Ephx1	13849	epoxide hydrolase 1, microsomal	
Insm2	56856	insulinoma-associated 2	
Stx1a	20907	syntaxin 1A (brain)	
Rasa4	54153	RAS p21 protein activator 4	
Plrg1	53317	pleiotropic regulator 1, PRL1 homolog (Arabidopsis)	
Sfrs2	20382	splicing factor, arginine/serine-rich 2 (SC-35)	
Rpl37	67281	ribosomal protein L37	
Trmt11	73681	tRNA methyltransferase 11 homolog (S. cerevisiae)	
0610009D07Rik	66055	RIKEN cDNA 0610009D07 gene	
Tmem107	66910	transmembrane protein 107	
Sft2d2	108735	SFT2 domain containing 2	
Srcap	546001	Snf2-related CREBBP activator protein	zVAD/apoptosis
Psmd6	66413	proteasome (prosome, macropain) 26S subunit, non–ATPase, 6	
Cwc15	66070	CWC15 homolog (S. cerevisiae)	
Adam26a	13525	a disintegrin and metallopeptidase domain 26A (testase 3)	
A130070M06	230050	hypothetical protein A130070M06	
Atp6ap2	70495	ATPase, H+ transporting, lysosomal accessory protein 2	
Abce1	24015	ATP-binding cassette, sub-family E (OABP), member 1	
A530021J07Rik	330578	Riken cDNA A530021J07 gene	
Olfr380	259027	olfactory receptor 380	
Rai14	75646	retinoic acid induced 14	
Rpl10	110954	ribosomal protein 10	
Crebl2	232430	cAMP responsive element binding protein-like 2	
Gpr152	269053	G protein-coupled receptor 152	

A siRNA library against 16,873 genes in the mouse genome was screened for ability to protect against zVAD.fmk-induced necroptosis in L929 cells. The 666 primary hits were selected for having achieved viability more than 2 SD above the mean plate viability. A total of 432 genes were scored positive in secondary confirmatory screen with zVAD.fmk-induced necroptosis. The siRNAs against 666 genes were screened against TNFα-induced necroptosis in L929 cells and TNFα-CHX-induced apoptosis in NIH 3T3 cells, respectively. A set of 32 genes was scored positive (zVAD/TNF box) in TNFα-induced necroptosis. Another set of 32 genes was scored positive (zVAD/apoptosis box) in TNFα + CHX-induced apoptosis. A set of seven genes (in both boxes) was scored positive in all three screens (zVAD.fmk- or TNFα-induced necroptosis and TNFα/CHX-induced apoptosis).

to limit and regulate the numbers of active macrophages during infection. The sensitivity of cells involved in innate immunity to treatment with zVAD.fmk suggests that caspase inhibition might represent a cellular signal of being invaded by virus, and, therefore, such cells must be destroyed by activating a cellular suicide mechanism such as necroptosis. Thus, necroptosis might serve as a cellular defense mechanism in protecting mammals against the invasion of foreign organisms.

Core Regulators of Necroptosis

Our analysis identified a group of 25 genes, including *parp2*, *bmf*, *pvr*, *rab25*, *jph3*, *mag*, and *foxi1* (Table 1), that are required for necroptosis induced by both zVAD.fmk and TNFα, but not for apoptosis. These results provide a first insight into the composition of the "core" necroptotic machinery downstream and/or regulators of RIP1. Regulation of necrotic cell death is a new function for these genes, emphasizing the notion that necroptosis is a separate mechanism of regulated cell death distinct from apoptosis.

Interestingly, this screen identified Grb2, an SH2 and SH3 domain-containing adaptor molecule that has been known to bind to TNFR1-mediating TNFα-dependent activation of c-Raf-1 kinase (Hildt and Oess, 1999), as required for both zVAD.fmk and TNFα-induced necroptosis. The involvement of Grb2 suggests a role of MAP kinases in the signaling of necroptosis. The identification of multiple known targets of MAP kinase, such as *edd*, *sp1*, *slc9a1*, and *rgs19*, is consistent with this possibility.

Our screen uncovered a role of PARP-2 as required for zVAD.fmk and TNFα-induced necroptosis. PARP-2 is the closest homolog of PARP-1 in the PARP family, sharing 60% identity in the catalytic domain. Previous studies have implicated PARP-1 in necrosis induced by alkylating agent MNNG (N-methyl-N′-nitrosoguanidine), which requires RIP1 (Xu et al., 2006). PARP-2 and PARP-1 can heterodimerize and have partially redundant functions, as indicated by the embryonic lethality of $parp1^{-/-}$; $parp2^{-/-}$ double, but not singly, mutant mice (Menissier de Murcia et al., 2003). Oligomerization of PARP1 and PARP2 has been shown to stimulate PARP catalytic activity in assisting the base excision repair after DNA nicks. PARP-2 is also cleaved in apoptosis, albeit with a delayed time course compared to that of PARP-1, suggesting that PARP-2 is not an ideal substrate for caspases as is PARP-1. Interestingly, expression of a caspase-cleavage-resistant form of PARP-1 (D214A) has been shown to lead to necrosis (Kim et al., 2000). The activation of PARP1 that catalyzes the hydrolysis of NAD+ into nicotinamide and poly-ADP ribose has been proposed to cause depletion of NAD+, which, in turn, may contribute to the energy failure in necrosis (Zong et al., 2004). Thus, it is possible that persistent elevated levels of PARP-2 activity resistant to caspase cleavage may contribute to the execution of necroptosis.

Transcriptional factor FOXI1 is also required for both zVAD.fmk- and TNFα-induced necroptosis. Mutant mice with a targeted disruption of FOXI1 display tubular acidosis resulting from a defect in cell differentiation characterized by the absence of vacuolar H(+)-ATPase expression (Kurth et al., 2006). Interestingly, an H(+)-ATPase, *atp6v1g2*, was also a hit required for necroptosis induced by both zVAD.fmk and TNFα. The vacuolar H(+)-ATPase (V-ATPase) is a ubiquitous multisubunit pump responsible for the acidification of intracellular organelles. Inhibition of V-ATPase is expected to increase the pH in acidic intracellular organelles such as lysosomes, which, in turn, obligates the activities of lysosomal hydrolytic enzymes.

Role of Bmf in Necroptosis

We identified a member of the *bcl-2* family, *bmf*, to be required for necroptosis induced by either zVAD.fmk or TNFα. Bmf is a member of the prodeath BH3-only subgroup of Bcl-2 family proteins (Puthalakath et al., 2001). BH3-only proteins serve as integrators of upstream signaling in both apoptosis and autophagy. Our result suggests that Bmf might play a similar role in necroptotic signaling. Overexpression of Bmf has been shown to reduce the colony formation of L929 cells (Puthalakath et al., 2001); however, the mechanism by which Bmf induces the death of L929 cells has not been directly examined. $Bmf^{-/-}$ mice develop a B-cell-restricted lymphadenopathy caused by the abnormal resistance of these cells to a range of apoptotic stimuli and have accelerated development of γ-irradiation-induced thymic lymphomas (Labi et al., 2008). The phenotype of $Bmf^{-/-}$ mice is consistent with a role of necroptosis in mediating homeostasis of the immune system. We propose that Bmf may function as a tumor suppressor by mediating the execution of both apoptosis and necroptosis.

Interestingly, knockdown of Bmf blocked necroptosis induced by zVAD.fmk and TNFα, but not apoptosis of TNFα/CHX-treated NIH 3T3 cells. Although Bmf has been implicated as a proapoptotic molecule, this result suggests that, at least in the death receptor signaling pathway, Bmf is primarily involved in mediating necroptosis, but not apoptosis. It is possible that the activation of Bmf may induce either apoptosis or necroptosis in a stimulus and cellular context-dependent manner.

Necroptosis in Tumorigenesis?

The role of two tumor suppressor genes including cyld and edd1 and four Ras-related proteins—*rab25*, *rasa4*, *rassf7*, and *rassf8*—in regulating necroptosis suggests a possible function of necroptosis in tumorigenesis. The Rab proteins are involved in regulating intracellular membrane trafficking (Pfeffer, 2007). Dysregulation of Rab25 gene expression has been noted in multiple cancers, including ovarian and breast cancers (Cheng et al., 2004). On the other hand, RASA4/CAPRI (RAS p21 protein activator 4), a suppressor of epithelial cell transformation (Westbrook et al., 2005), functions as a Ca(2+)-dependent Ras GTPase-activating protein (GAP) to inactivate the Ras-MAPK pathway following a stimulus that elevates intracellular calcium (Lockyer et al., 2001). A common role of RASA4 in regulating both necroptosis and apoptosis may provide a potential mechanism for its proposed role as a tumor suppressor. DAB2IP/AIP1, another Ras-GAP and an ASK1-interacting protein, has been previously shown to bind to RIP1 to mediate the activation of ASK1-JNK/p38 signaling in endothelial cells (EC) (Zhang et al., 2007). We found that knockdown of DAB2IP partially inhibited apoptosis but had no effect on necroptosis (data not shown). Since two GAPs, DAB2IP and RASA4, have been found to mediate the signaling of RIP1, it is possible that multiple Ras-GAP proteins may be involved in mediating downstream signaling of RIP1 in different cell types to regulate cell death. Finally, Rassf7

and Rassf8 are members of the Ras association domain family (RASSF) that are Ras effectors characterized by a conserved motif (the RalGDS/AF6 Ras association [RA] domain) and are known as putative tumor suppressors that modulate some of the growth inhibitory responses mediated by Ras. Overexpression of some members of RASSF family, such as Rassf6, has been shown to trigger both caspase-dependent and caspase-independent cell death. Rassf family has also been implicated in regulating death-receptor-mediated cell death (Ikeda et al., 2007).

We propose that necroptosis may be selectively activated when there is a failure of caspase activation, which may occur under pathological conditions such as viral infection or oncogenic mutations. Since deficiency in apoptosis has been noted as one of the hallmarks of cancers (Hanahan and Weinberg, 2000), necroptosis may play a very important role in tumorigenesis as a backup cell death mechanism in apoptosis-deficient cancer cells.

EXPERIMENTAL PROCEDURES

siRNA Screen

siRNA screen was performed using 16,873 siRNA pools covering most of the mouse genome (Dharmacon mouse kinase and phosphatase, G-protein-coupled receptor, druggable genome, and remaining genome libraries [Thermo Fisher Scientific]). Mouse fibrosacroma cell line L929 cells cultured in 384-well white plates (Corning) were robotically transfected with 50 nM siRNA by reverse transfection method using HiPerFect transfection reagent according to a protocol from the manufacturer (QIAGEN). At 48 hr after the transfection, the cells were treated with 20 μM pan-caspase inhibitor zVAD.fmk and cultured for an additional 18 hr to induce necroptosis. Viability was measured using luminescence-based ATP levels as a surrogate marker in surviving cells using CellTiter-Glo ATP assay (Promega). Positive controls (ripk1, Dharmacon) and negative controls (nontargeting control siRNA: siCONTROL nontargeting no. 2, Dharmacon) were present in every plate. The screen was performed in duplicate. siRNAs for each gene were classified as hits if their viability showed > 2 SD above the mean ATP value of the plate on duplicate plates.

In the confirmation screen, four siRNAs of each pool were individually transfected into cells and screened using the same procedure as in the primary screen. In the TNFα tertiary screen, L929 cells were transfected with individual siRNAs and, 48 hr later, treated with 40 ng/ml TNFα for an additional 72 hr. In the apoptosis tertiary screen, NIH 3T3 cells were transfected with individual siRNA and, 48 hr later, treated with 10 ng/ml TNFα + 1.0 μg/ml cycloheximide (CHX) for an additional 12 hr to induce apoptosis.

Enrichment Analyses

Sets of hit genes were classified into functional categories such as biological process, molecular function (PANTHER classification system) (Mi et al., 2005), canonical pathways (MSigDB) (Subramanian et al., 2005), and transcription factor (TF)-binding sites (MSigDB and TRANSFAC v7.4 [www.gene-regulation.com]. Enrichment analyses of canonical pathways and TF-binding motifs were performed on gene sets that were mapped to human orthologs (Homologene) (Wheeler et al., 2008). To assess the statistical enrichment or overrepresentation of these categories for the hit genes relative to their representation in the global set of genes examined in the siRNA screen, p values were computed using the hypergeometric test (Rivals et al., 2007) as described in the GOHyperGAll module (Horan et al., 2008) implemented in the R programming language. Comprehensive background information about the hypergeometric distribution can be found here: http://mathworld.wolfram.com/hypergeometricdistribution.html.

Briefly, the hypergeometric distribution describes the probability of finding at least S genes associated with a particular category in a set of *g* genes involved in zVAD.fmk-induced necroptosis, given that there are *S* genes associated with that same category in the global set of *G* genes examined in the genome-wide siRNA screen. For each category, *c*, and the list of genes, *l*, the p value was calculated as:

$$P(c,l) = \left(1 - \sum_{k=0}^{s-1} \frac{\binom{g}{k}\binom{G-g}{S-k}}{\binom{G}{S}}\right)$$

Here,

$$\binom{n}{r}$$

represents the binomial coefficient. An unadjusted p value < 0.05 was considered significant. Categories assigned with at least ten genes are displayed in Figures 5A and 5B.

Human Protein Interaction Network

The network was constructed by iteratively connecting interacting proteins, with data extracted from a collection of genome-wide interactome screens and curated literature entries in HPRD (Mishra et al., 2006). For the network analysis, mouse components were mapped to human orthologs (Homologene) (Wheeler et al., 2008). The network uses graph theoretic representations, which abstract components (gene products) as nodes and relationships (e.g., interactions) between components as edges, implemented in the Perl programming language.

SUPPLEMENTAL DATA

The Supplemental Data article include Supplemental Experimental Procedures, one figure, and nine tables and can be found with this article at http://www.cell.com/supplemental/S0092-8674(08)01382-2.

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