A Genome-Wide RNA Interference Screen Identifies Caspase 4 as a Factor Required for Tumor Necrosis Factor Alpha Signaling

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Tumor necrosis factor alpha (TNF- α) is a potent inflammatory cytokine secreted upon cellular stress as well as immunological stimuli and is implicated in the pathology of inflammatory diseases and cancer. The therapeutic potential of modifying TNF- α pathway activity has been realized in several diseases, and antagonists of TNF- α have reached clinical applications. While much progress in the understanding of signaling downstream of the TNF- α receptor complex has been made, the compendium of factors required for signal transduction is still not complete. In order to find novel regulators of proinflammatory signaling induced by TNF- α , we conducted a genome-wide small interfering RNA screen in human cells. We identified several new candidate modulators of TNF- α signaling, which were confirmed in independent experiments. Specifically, we show that caspase 4 is required for the induction of NF- κ B activity, while it appears to be dispensable for the activation of the Jun N-terminal protein kinase signaling branch. Taken together, our experiments identify caspase 4 as a novel regulator of TNF- α -induced NF- κ B signaling that is required for the activation of I κ B kinase. We further provide the genome-wide RNA interference data set as a compendium in a format compliant with minimum information about an interfering RNA experiment (MAIRE).

Inflammation is essential for an efficient innate immune response, helping to alert the body to potential intruders and enabling immune cells to access the site of an infection. However, when inflammatory processes become chronic or systemic, tissue damage and diseases can arise (e.g., Crohn's disease or psoriasis) (12, 30).

The cytokine tumor necrosis factor alpha (TNF- α) is the major mediator of inflammation (4). TNF- α can bind to both TNF- α receptor 1 (TNFR1) and TNFR2. Upon binding of TNF- α to TNFR1, it induces an intracellular signaling cascade that can induce either inflammation or apoptosis, depending on the cell type. Molecularly, the ligand-receptor complex first recruits TRADD and TRAF2/5, followed by cellular inhibitors of apoptosis protein (cIAPs). cIAPs are responsible for forming K63- and K11-linked ubiquitin chains on RIP1 (23, 29, 55, 58). These lead to the recruitment of the linear ubiquitin chain assembly complex (LUBAC) and the linear ubiquitination of RIP1, NEMO, and possibly other components (59). The ubiquitin chains on RIP1 allow binding of further signaling factors, leading to the activation of NF- κ B (through I κ B kinase [IKK]) and AP-1 (through mitogen-activated protein kinase/Jun N-terminal protein kinase [JNK]) transcription factors (59).

Recently, mass spectrometric analysis revealed that LUBAC is an essential regulator of TNF- α receptor complex ubiquitination (19, 24). In addition, RNA interference (RNAi) screens identified several novel TNF- α signaling components, including the cylindromatosis tumor suppressor (CYLD) (13) in human cells and IAP2 and akirins as conserved modulators of TNF- α -like signaling pathways in *Drosophila* (20, 22). Yet, to date, no RNAi screen for TNF- α -induced activation of NF- κ B covering the complete human genome has been reported (13, 16, 17, 36, 41, 65).

Here, we present the results of a functional genomic screen with the aim to identify novel regulators of TNF- α signaling. We established a quantitative assay to measure NF- κ B signaling activity after TNF- α stimulation and screened a genome-wide small interfering RNA (siRNA) library in human cells. This approach

identified several novel candidates that were confirmed with independent siRNAs and in independent cell lines. Specifically, we focused on caspase 4 (CASP4), which is required for robust activation of NF- κ B. Transcriptional profiling showed that CASP4 is required for the expression of endogenous NF- κ B target genes. We used epistasis analysis to map the role of CASP4 upstream of or at the level of IKK activation. Taken together, our experiments identified CASP4 as a novel positive regulator of TNF- α -induced NF- κ B signaling. Furthermore, we provide the full RNAi screening data set as a resource for further exploration.

MATERIALS AND METHODS

Cell lines and reagents. Human embryonic kidney 293T (HEK293T), HeLa, and HepG2 cells were kindly provided by C. Niehrs (DKFZ) and T. Dick (DKFZ). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco). TNF- α was obtained from Biosource. The sequences of the siRNAs used are listed in Table S1 supplemental material.

Plasmids. In order to monitor NF- κ B transcriptional activity, a cell-based dual-luciferase assay in HEK293T cells was established. As a pathway-specific reporter, an NF- κ B-dependent firefly luciferase (FL) expression plasmid (4-4-FL) was cloned. Eight NF- κ B binding sites (8 $\times$ 5'-GG ACTTTCC-3', in concordance with the degenerate NF- κ B binding site

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5'-GGGRNWWYCC-3', where G stands for a purine base, N denotes any base, W is an adenine or thymine, Y denotes a pyrimidine base [15, 35, 51]) were fused upstream of the firefly luciferase gene (pGL3 promoter, Promega). A constitutively expressed *Renilla* luciferase (RL) driven from the β -actin promoter (act-RL) served as a coreporter for transfection efficiency and cell viability. The β -actin promoter, an approximately 1,100-bp sequence 5' of the transcription start site of the β -actin gene, was amplified by PCR and then ligated into pRL null (Promega). Results of proof-of-principle experiments are shown in Fig. S1A to C in the supplemental material. The JUN reporter plasmid was kindly provided by P. Krammer (DKFZ) [38].

Transfections and luciferase assays. siRNAs (final concentration, 50 nM) were transfected by reverse transfection using DharmaFECT1 transfection reagent (Dharmacon/Thermo Fisher Scientific) following the manufacturer's instructions. After 24 h, plasmids were transfected using TransIT transfection reagent (Mirrus), and then plasmid expression was allowed for 48 h. Sixteen hours (HEK293T) or 24 h (HepG2) prior to the luciferase readout, cells were stimulated with 20 ng (HEK293T) or 50 ng (HepG2) per ml TNF- α (final concentrations). For overexpression studies, HEK293T or HepG2 cells were reverse transfected with siRNAs using the DharmaFECT1 reagent (Dharmacon/Thermo Fisher Scientific) following the manufacturer's instructions. Twenty-four hours later, cells were transfected with plasmids pEF-DEST51-Caspase4 (based on the pEF-DEST51 gateway vector; Invitrogen) or empty control using TransIT transfection reagent (Mirrus), allowing plasmid expression for 48 h. Cells were either left untreated or treated with TNF- α (20 ng/ml) for 2 h prior to the luciferase readout. Calcein readout was used for normalization (21).

Primary genome-wide screening. Genome-wide RNAi experiments were performed with a synthetic siRNA library (Qiagen) in HEK293T cells. siRNA sequences were annotated using the RefSeq database, release 27. siRNA concentrations of 20 nM were used. In each well of a 384-well plate, 0.05 μ l DharmaFECT1 was gently mixed with 4.95 μ l RPMI and the plate was incubated for 10 min at room temperature before another 10 μ l RPMI was added. This mix was then pipetted to 5 μ l 200 nM (Qiagen) siRNA (in siRNA buffer), followed by another incubation for 30 min at room temperature. Cells (4×10^3) in 30 μ l (antibiotic-free) culture medium (DMEM plus 10% FCS) were seeded on top of the siRNAs, and the plates were briefly spun (Centrifuge 5804; Eppendorf) to collect all liquid at the bottom of the wells and then kept in the incubator (Binder) for 3 days to allow protein depletion. siRNAs against the receptor TNFRSF1A (siTNFR1) and RelA (siRelA), i.e., siRNAs that diminish activation of NF- κ B transcriptional activity, served as positive controls. An example of the performance of screening of replicates is shown in Fig. S2 in the supplemental material. The complete data set is available in Tables S2 and S3 in the supplemental material.

Secondary screening. Screening results were validated by making use of deconvoluted siRNA pools (50 nM; siGENOME set of four upgrade; Dharmacon/Thermo Fisher Scientific) and siRNA pools picked from the genome-scale siRNA library from Qiagen (20 nM). For further experiments, siRNAs were purchased from Dharmacon/Thermo Fisher Scientific and used at a concentration of 50 nM.

ELISA. For enzyme-linked immunosorbent assay (ELISA), cells were reverse transfected with siRNAs in a 384-well format. The interleukin-8 (IL-8) concentration in cell culture supernatants was assessed using a human CXCL8/IL-8 ELISA kit (R&D) and by following the manufacturer's instructions exactly. Measurements were carried out in technical duplicates in a 96-well format, pooling eight equally treated wells. HEK293T cells were treated with 20 ng/ml TNF- α for 16 h, while HepG2 cells were stimulated with 25 ng/ml TNF- α for 24 h. The absorbance was measured with a Mithras LB940 plate reader (Berthold Technologies), with the filter set at 450 nm (for proof of principle, see Fig. S1D in the supplemental material).

Expression profiling and qRT-PCR. Total RNA was extracted using an RNeasy kit (Qiagen).

(i) Expression profiling. RNA quality was assessed by measuring mRNA depletion mediated by respective siRNAs and TNF- α -mediated induction of target genes by quantitative real time (qRT)-PCR before RNA was handed over to the DKFZ Genomics and Proteomics Core Facilities. There, RNA was hybridized onto Illumina Human Sentrix-8 chips.

(ii) qRT-PCR. One microgram of RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). cDNA corresponding to 110 ng of starting RNA was used for two technical replicates. qRT-PCR was performed in a 384-well format using a Universal Probe Library system (Roche) and a LightCycler 480 system (Roche). HRPT1 was used as the housekeeping gene. The sequences of the qRT-PCR primers are given in Table S4 in the supplemental material.

Antibodies. **(i) Antibodies for immunoblotting.** The anti- α -tubulin antibody was purchased from Sigma (antibody DM1A); the anti-phospho-I κ B- α antibody (antibody 9246), the total I κ B- α antibody (antibody 9242), the anti-phospho-JNK antibody (antibody 4668), and the total JNK antibody (antibody 9252) were purchased from Cell Signaling; the caspase 4 antibody was purchased from Santa Cruz (antibody sc-1229); the β -actin antibody (antibody AC-15) was from Abcam; and secondary antibodies were purchased from Southern Biotech (anti-mouse IgG1) and GE Healthcare (anti-mouse antibody).

(ii) Antibodies for fluorescence-activated cell sorting (FACS). The anti-TNFRSF1A antibody (antibody MABTNFR1-B1) was purchased from BD Pharmingen, ubiquitinated anti-mouse antibody [F(ab')₂ IgG (H+L)] was from Southern Biotech, and streptavidin-phycoerythrin (PE) was from BD. The isotype control antibody was kindly provided by H. Walczak (Imperial College, London, United Kingdom).

Immunoblotting. For immunoblotting experiments, HEK293T, HeLa, or HepG2 cells were reverse transfected with siRNAs in a 24-well or 12-well format. After 2 days of siRNA treatment, cells were starved for 3 to 4 h by exchanging the culture medium with serum-free DMEM. Then, medium was exchanged for culture medium or 50 ng/ml TNF- α -containing medium to stimulate cells for either 0, 5, 10, or 20 min. After stimulation, cells were trypsinized and lysed for 30 min in 70 μ l lysis buffer (1 $\times$ phosphate-buffered saline [PBS], 10% Triton X-100, protease and phosphatase inhibitors). Protein concentration was determined using a bicinchoninic acid assay (Pierce), following the manufacturer's instructions. Proteins were separated on 10% NuPage bis-Tris nongradient gels (Novex; Invitrogen) in 1 $\times$ MOPS (morpholinepropanesulfonic acid) buffer (50 mM Tris base, 50 mM MOPS, 1.025 mM EDTA, 69.3 mM SDS) and transferred onto nitrocellulose membranes by wet transfer (XCell II blot module; Invitrogen) in 2 $\times$ transfer buffer (0.5 M bis Tris, 0.5 M bicine, 0.02 M EDTA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)-Tween (TBS-T; 0.137 M NaCl, 0.02 M Tris-HCl, pH 7.8) before being probed with antibodies.

FACS. HeLa cells were transfected with siRNAs in a 6-well format. After 2 days of incubation to allow protein depletion, cells were scraped off the wells. Cell pellets were resuspended in 50 μ l FACS buffer (4% FCS in 1 $\times$ PBS) and stained with primary and secondary antibodies for 30 min each on ice. Then, cells were stained for 20 min with streptavidin-PE before being analyzed using a FACSArray apparatus (BD). An unspecific antibody of the same isotype served as a control.

Computational analyses. **(i) Screening analysis.** Screening data were analyzed using the R/Bioconductor software package cellHTS2 (11). Data were log₂ transformed and shorth normalized. Then, the ratio of the experimental reporter (firefly luciferase) to the invariant coreporter (*Renilla* luciferase) value was determined in order to exclude possible artifacts such as cell death affecting both experimental and invariant reporter genes. Replicate measurements were averaged before a *z* score for each siRNA pool was calculated. The *z* score is a statistical means to express how many standard deviations that an observation is above or below the mean of all measurements. Here, a negative *z* score reflects a reduction in signaling pathway activity; a positive *z* score reflects an increase.

(ii) **Microarray analysis.** Microarray data were read into R using the package *beadarray* (18). After quality control of the chips using the R package *arrayQualityMetrics* (32), data were normalized using the *vsr* method (27, 28). Then, two different TNF- α -induced target gene expression signatures were defined: (i) genes altered after 2 h of TNF- α treatment and (ii) genes altered after 8 h of TNF- α treatment compared to gene expression by unstimulated cells. In order to define a TNF- α -induced gene expression signature, first, genes differentially expressed in TNF- α -stimulated cells that were treated with the negative-control siRNA were determined. Moderated *t* statistics for these differentially expressed genes were calculated using the empirical Bayes method (54). Only those genes that passed a false discovery rate (FDR; calculated by the Benjamini and Hochberg method) with a maximum of 0.1 were considered in the gene expression signature and were reported to be TNF- α target genes (<http://bioinfo.lifl.fr/NF-KB/>). Differential gene expression was assessed using the package *limma* (54). Figure S3 in the supplemental material shows the result of unsupervised hierarchical clustering of all samples by TNF- α target genes, demonstrating that expression of these genes discriminates untreated from TNF- α -treated samples.

Microarray data accession number. The microarray data from the present study have been submitted to ArrayExpress and can be found under accession no. E-MTAB-900.

RESULTS

A genome-wide RNAi screen identifies novel components of TNF- α signaling. The application of TNF- α to many vertebrate cell types leads to the rapid induction of NF- κ B target genes, including proinflammatory cytokines and signaling regulators (26). In order to quantitatively measure NF- κ B activity in a high-throughput format, we constructed a sensitive reporter gene construct by fusing eight NF- κ B binding sites to a firefly luciferase gene (Fig. 1A). In addition, we generated a constitutively expressed *Renilla* luciferase coreporter based on the β -actin promoter to normalize reporter gene activity for variation in transfection efficiency and changes in cell viability. We tested these reporters in HEK293T cells and showed a robust 16-fold induction after TNF- α stimulation sufficient for high-throughput experiments (see Fig. S1B in the supplemental material).

Next, we tested known components of the TNF- α signaling pathway for their ability to reduce relative reporter gene activity. As shown in Fig. 1B, siRNAs targeting TNFR1, but not TNFR2, led to a >70% reduction in relative reporter activity, indicating that TNF- α signaling in HEK293T cells is primarily transmitted through TNFR1. Similarly, knockdown of RelA led to a strong reduction in relative reporter activity (Fig. 1C; see Fig. S1C in the supplemental material). These experiments validated the NF- κ B reporter for use in high-throughput screening experiments.

Next, we screened a genome-wide siRNA library (Qiagen) in HEK293T cells for novel regulators of TNF- α signaling pathway activity. In brief, HEK293T cells were reverse transfected with 20 nM siRNAs prealiquoted in 384-well plates. At 24 h after siRNA transfection, cells were transfected with reporter plasmids, followed by another incubation for 48 h before cell lysis. At 56 h, cells were treated with 20 ng/ml TNF- α for 16 h. After addition of the lysis buffer, cell lysates were frozen at -80°C to ensure constantly timed stimulation conditions across the whole screen. Cell lysates were thawed, and luciferase levels were assessed. Each 384-well plate contained positive controls (siTNFR1, siRelA) and negative controls (siRNA against LRP5 [siLRP5]) to monitor transfection efficiency and cell viability across the screen (see the work flow in Fig. 1D and Materials and Methods). The performance of the screen was evaluated by assessing the separation of measurements

for negative and positive controls. As shown in Fig. 1E, the data were of good quality (9), with measurements for the negative control (siLRP5) clearly separated from those for the strongest positive control (siTNFR1). A quantile-quantile (QQ) plot (Fig. 1F) indicated that the screen recovered several candidate modifiers of TNF- α signaling.

Computational analysis of the genome-wide RNAi screen. Overall, more than 40,000 experimental data points were collected. In order to normalize for experimental variation and exclude cell viability modifiers, we performed a stepwise analysis and filtering process (9, 11). First, raw screening data were analyzed using the R/Bioconductor software package *cellHTS2* (11). Data were $\log_2$ transformed and shortly normalized before the ratio of the experimental reporter (FL) to the invariant coreporter (RL) value was determined. Phenotypes of screened siRNAs were expressed as *z* scores, with a negative *z* score reflecting a reduction in signaling pathway activity and a positive *z* score reflecting an increase. The full data set, including raw and processed data, is available as supplemental material in a form compliant with minimum information about an RNAi experiment (MIARE) (see Table S3 in the supplemental material).

To identify the strongest and most robust modifiers, we applied stringent thresholds: (i) reduction in FL levels by at least 50% compared to the mean of the experiment and (ii) a concomitant reduction of RL expression of not more than 30%. We further required a *z*-score cutoff of ≤ -2.5 . In this way, we recovered 49 potential positive regulators of TNF- α signaling, among them the known pathway components TNFRSF1A (TNFR1) and RelA. Nineteen candidates were selected for secondary validation experiments, on the basis of research in the literature (e.g., prior reports on a role in inflammation or immune signaling), the presence of signaling-related protein domains, and protein-protein interactions (Fig. 2A).

To exclude phenotypes caused by primary screening artifacts (Fig. 2A), we repeated the original screening assay to reproduce screening results (Fig. 2B, secondary screening). In addition, we also employed an ELISA detecting protein levels of the endogenous TNF- α target gene IL-8 for independent phenotypic validation. To exclude siRNA off-target effects, follow-up experiments were performed with several individual siRNAs targeting the same gene. Five candidates (MEN1, ROBO4, LRRC59/PRO1855, PTK9, and CASP4) that showed a robust reduction both in independent assays and with independent siRNAs were tested in a second cell line (Fig. 2A). Among the top five candidates, CASP4 displayed one of the most robust phenotypes in all assays (see Tables S5 and S6 in the supplemental material).

CASP4 is required for TNF- α signaling. CASP4 belongs to the family of inflammatory caspases, fulfilling functions in both apoptosis (endoplasmic reticulum [ER] stress induced) and inflammation (Toll-like receptor 4 [TLR4] signaling and inflammasome) (62). In the secondary assays, silencing of CASP4 resulted in an $\sim 50\%$ reduction in TNF- α signaling activity in both the luciferase reporter assay and the IL-8 ELISA in HEK293T cells (Fig. 3A and B) and HepG2 cells (Fig. 3C and D). These phenotypes were consistent with efficient siRNA-mediated depletion of CASP4 (Fig. 3E and F).

For both the luciferase assays and ELISAs, cells had been treated with TNF- α for 16 h to monitor the NF- κ B transcriptional activity induced by extended TNF- α signaling. To investigate a possible role for CASP4 in the early, feedback loop-independent

FIG 1 Genome-wide RNAi screens for TNF- α -induced NF- κ B signaling using a cell-based assay in HEK293T cells. (A) The screening procedure is based on a dual-luciferase reporter system. TNF- α signaling is monitored using an NF- κ B binding-dependent firefly luciferase construct. A constitutively expressed coreporter (*Renilla* luciferase) monitors transfection efficiency and cell viability. (B) TNF- α signaling in HEK293T cells was mediated by TNFRSF1A (TNFR1). Knockdown of TNFRSF1B (TNFR2) did not decrease relative signaling pathway activity (compared to that for the mock-infected control). Cells were transfected with four individual siRNAs (1 to 4) or with pools of four siRNAs (pool, siLRP5). R1 + R2, double knockdown of both receptors (siRNA pools); siLRP5, negative control. One representative result of two independent experiments is shown. (C) Phenotypes of the siRNA controls that were used in the RNAi screens. Induction of TNF- α signaling and, thus, NF- κ B transcriptional activity leads to an approximately 16-fold induction of firefly luciferase expression. siTNFR1 and siRelA strongly reduced NF- κ B reporter activity and served as positive controls. siLRP5 did not target a known component of the TNF- α pathway and was used as a negative control. siRNA against Con (siCon) slightly induced the pathway and was therefore not further used. Pools of four siRNAs were used. One representative result of at least 3 independent experiments is shown. (D) Summary of the screening approach. HEK293T cells were reverse transfected with siRNAs prealiquoted in 384-well plates. Twenty-four hours after siRNA transfection, cells were transfected with reporter plasmids. After 32 h, cells were treated with 20 ng/ml TNF- α for 16 h before luciferase expression was assessed. (E) Distribution of normalized luciferase intensities of controls. The Z' factor is a statistical means for expressing the separation of the distribution of luciferase expression values measured for positive controls (yellow, siTNFR1; red, siRelA) or negative controls (blue, siLRP5). Z' factors from 0 to 0.5 depict acceptable assays; those from 0.5 to 1 depict very good assays (9, 64). (F) Quantile-quantile plot of normally distributed quantiles against actual pathway screening result quantiles. A fit to a normal distribution is represented by the solid line. siRNAs in the tails represent RNAi reagents with significant phenotypes. The dashed line depicts where the z-score cutoff in the individual screening analysis was set to define hits (z score ≤ -2.5).

FIG 2 Secondary screening procedure to reduce number of high-confidence candidates. (A) Forty-nine high-confidence hits were determined by applying stringent filters to the primary screening hit list. Subsequently, 19 candidates were selected by data mining and entered into secondary screening. (B) Phenotypes of these 19 candidates in the dual-luciferase assay in HEK293T cells. Relative TNF- α signaling activity compared to that in mock-transfected cells is shown, using pools of 4 siRNAs for each candidate. Mean values of two independent experiments are shown. Error bars depict standard deviations of the two biological replicates. The four candidates (marked in bold) were subjected to secondary screening in HepG2 cells.

cascade from TNFR1 to NF- κ B (5, 52), we next measured the expression of early response genes, which are induced within 15 to 30 min upon TNF- α stimulation (26).

We first assessed the global effect of CASP4 depletion on TNF- α target gene expression by expression profiling using Illumina Human Sentrix-8 arrays (Fig. 4A). Expression of early response genes was assayed after 2 h of TNF- α treatment; the late response was evaluated after 8 h of stimulation. The microarray experiment revealed that 2 h of stimulation with TNF- α induced the expression of known TNF- α target genes in the negative control (small interfering RL [siRL]) but not in the positive controls (siRelA, siTNFR1) or in cells transfected with siRNA against CASP4 (siCASP4). After 8 h of stimulation, expression of genes that were differentially regulated in cells treated with the negative control was nearly unchanged in positive controls. Interestingly, CASP4 silencing led to a reversed phenotype, as genes whose expression was normally induced after exposure to TNF- α were strongly repressed instead (Fig. 4A). These results strongly suggest that CASP4 is required for the regulation of TNF- α target gene expression.

We next sought to confirm these findings by assaying the expression of selected TNF- α target genes by qRT-PCR. Figures 4B to E show that the expression of TNF- α target genes in positive controls and the expression in siCASP4-treated cells resembled each other, with a decreased or lost induction of genes that were differentially regulated in the negative control. This phenotype could be confirmed in HepG2 cells (see Fig. S4 in the supplemental material). In addition, nuclear translocation of NF- κ B in response to TNF- α was diminished upon knockdown of CASP4 (see Fig. S5 in the supplemental material). Taken together, these data support the requirement of CASP4 for TNF- α -induced target gene expression.

CASP4 knockdown interrupts signaling from TNFR1 to NF- κ B at the level of the IKK complex. To establish where in the TNF- α pathway CASP4 function was required, we performed epistasis experiments. Decreased signaling activity could potentially be caused by reduced sensitivity to TNF- α as a consequence of decreased TNFR1 levels at the cell surface. We therefore examined the levels of TNFR1 at the cell surface by FACS of HeLa cells (which exhibit high TNFR1 cell surface expression; Novartis Gene Expression Atlas, <http://biogps.org/>) following 48 h of siRNA knockdown. As shown in Fig. 5A and B, siCASP4-treated cells and negative-control cells displayed similar amounts of TNFR1 on their surfaces. In contrast, cells treated with siTNFR1 (positive control) exhibited reduced TNFR1 cell surface expression. Thus, we conclude that knockdown of CASP4 had no influence on TNFR1 cell surface levels.

We then assessed whether CASP4 expression is sufficient to induce NF- κ B transcriptional activity by overexpressing CASP4 in HEK293T and HepG2 cells (see Fig. S6 in the supplemental material). In both cell types, CASP4 overexpression led to a strong induction of NF- κ B reporter expression. This gain-of-function phenotype was independent of prior stimulation with TNF- α and could also be observed while concomitantly silencing TNFR1. These results indicate that CASP4 acts downstream of TNFR1.

Early phosphorylation events are central to the regulation of TNF- α signaling pathway activity. To determine whether silencing of CASP4 affected these signaling steps, we evaluated I κ B- α phosphorylation by immunoblotting of HeLa cell extracts. Cells were transfected with siRNAs for 48 h and then stimulated for 0, 5, 10, or 20 min with 50 ng/ml TNF- α . I κ B- α phosphorylation reached its maximum level after 5 min of TNF- α stimulation in siRL-treated cells (Fig. 5C). I κ B- α degradation was detected 20 min after TNF- α stimulation. In siTNFR1-transfected cells, I κ B- α phosphorylation was strongly decreased. Likewise, knockdown of CASP4 abolished phosphorylation of I κ B- α in HEK293T (Fig. 5C) and HeLa cells (see Fig. S7A in the supplemental material). A similar phenotype was also observed in HepG2 cells (see Fig. A7B in the supplemental material). We therefore conclude that CASP4 acts at the level or upstream of the activation of IKK, which is required for I κ B- α phosphorylation (53, 63).

Next, we assessed whether silencing of CASP4 also abolished signaling via JUN transcription factors. Using a JUN luciferase reporter, we found that signaling via JNK was not affected by CASP4 knockdown (Fig. 5D). This observation was confirmed by immunoblot analysis of the JNK phosphorylation status (Fig. 5E). In conclusion, the phenotype observed upon CASP4 silencing was not caused by interrupted shuttling of the TNFR1 to the cell surface. Instead, CASP4 depletion interrupted the intracellular

FIG 3 CASP4 is required for TNF- α signaling. (A to D) HEK293T (A and B) or HepG2 (C and D) cells were reverse transfected with siRNAs, and the dual-luciferase assay (A and C) and IL-8 ELISA (B and D) were performed. Depicted is relative signaling activity, i.e., FL/RL (luciferase assay) or relative IL-8 secretion (ELISA) compared to mock-transfected cells (medium; HEK293T) or negative-control transfected cells (HepG2). RIP1, TNFR1, and RelA, siRNA pools against RIP1, TNFRSF1A, and RelA, respectively (positive controls). Mean values of two independent experiments are shown. Error bars depict standard deviations of the two biological replicates. ctrl, mean of negative siRNA controls. (E) siRNA-mediated mRNA depletion assessed by qRT-PCR in HEK293T cells. Relative CASP4 mRNA levels compared to those in cells transfected with the negative control are shown. Error bars depict standard deviations of the two technical replicates. Data representative of 3 independent experiments are shown. (F) siRNA-mediated protein depletion assessed by immunoblotting in HeLa cells. Staining for α -tubulin served as a loading control. Representative data of 4 independent experiments are shown.

TNF- α signaling cascade, specifically, the signaling branch activating NF- κ B transcription factors.

DISCUSSION

Because of its role in numerous pathologies, different strategies have been pursued to characterize the TNF- α signaling cascade and find potential new intervention points (49). Here we report the first genome-wide mammalian screen for TNF- α -mediated activation of NF- κ B, which expands the candidate lists of previ-

ously targeted RNAi screens (13, 16, 36, 41, 65) to a genome-wide compendium of putative modulators of TNF- α signaling modulators, including known pathway components such as TNFR1 and RelA. Our genetic screening strategy also encompassed an extensive follow-up analysis using a range of secondary screening experiments and identified five high-confidence candidates. We particularly focused on the role of CASP4 in TNF- α signaling. Our experiments confirmed that silencing of CASP4 led to reduced TNF- α signaling pathway activity in multiple cell lines, as evi-

FIG 4 Silencing of CASP4 interferes with the induction of TNF- α target genes. Expression of TNF- α target genes in HEK293T cells after 2 and 8 h of TNF- α treatment assessed by expression profiling (A) and qRT-PCR (B to E). (A) Expression profiles of selected TNF- α target genes. Microarray data were transformed logarithmically before induction of target genes, and similarity of expression was assessed from the Euclidean distance between data points. Rows of the heat map are known TNF- α target genes; columns of the heat map are different samples. Blue, lower expression than that by unstimulated cells; yellow, higher expression. Data summarized from two biological replicates are shown. siRL, siRNA against *Renilla* luciferase (negative control). (B to E) Effect of CASP4 knockdown on TNF- α -induced BIRC3 (B), TNF- α (C), CCL20 (D), and NFKBIA (E) expression. HEK293T cells were reverse transfected with siRNAs, and 3 days later, target gene mRNA levels were measured by qRT-PCR after 0, 2, and 8 h of induction using 20 ng/ml TNF- α . Expression levels were normalized to expression in the negative-control sample (siRNA against *Renilla* luciferase): for each time point, mRNA expression levels were set to 100% in the control sample, and mRNA levels in candidate samples were calculated in relation to the control. Data derived from five independent experiments are shown, with error bars indicating the standard deviations of the biological replicates. *P* values were calculated using Student's *t* test. **, *P* < 0.01; ***, *P* < 0.001. (A to E) siTNFR1 and siRelA, siRNA against TNFRSF1A and RelA, respectively (positive controls); siCASP4, siRNA pool against CASP4.

denced by both reporter constructs and endogenous target genes. This effect was not mediated by reduced TNFR1 cell surface levels after CASP4 knockdown. We mapped CASP4 function through epistasis experiments upstream or at the level of the activation of the IKK complex. CASP4 RNAi led to a striking repression of TNF- α target genes, similar to the phenotype described for IKK- α / β -double-knockout cells (43). These results support the notion that CASP4 and IKK might act at the same level in TNF- α signaling. Although knockdown of CASP4 interfered with the IKK-mediated phosphorylation of I κ B- α upon treatment with TNF- α , TNF- α -induced signaling via JNK to JUN transcription factors was not affected.

Caspases are mainly known for their role in apoptosis induction. In previous studies, CASP4 was shown to be activated upon ER stress; however, its role in ER stress-induced apoptosis remained controversial (3, 6–8, 25, 37, 40, 42, 44, 45, 56, 57). Recently, other functions beyond induction of apoptosis have begun to emerge for caspases (1). CASP4 belongs to the family of inflammatory caspases, which also comprises human CASP1, CASP5, and CASP12 (39), and is involved in the response to inflammatory and danger signals, such as lipopolysaccharide (LPS) and ATP (62). CASP1, CASP4, and CASP5 were shown to be components

of the inflammasome, a large protein complex sensing intracellular danger signals (33, 50). CASP12 is normally expressed as a truncated protein, and a mutation leading to full-length transcription of the *casp12* gene has recently been linked to a diminished response to endotoxins and sepsis (48).

Several lines of evidence from previous studies support our findings that CASP4 is required for TNF- α signaling. First, it has been shown that CASP4 expression is induced by interferons, supporting an immune-regulatory function of CASP4 (2, 61). Second, Bian et al. have recently described that CASP4 expression and activation are induced by several proinflammatory stimuli, including TNF- α and LPS, and that CASP4 has a dual role in apoptosis and proinflammatory signaling (6). Third, CASP4 contains a caspase recruitment domain (CARD), which is also found in other proteins required in the regulation of NF- κ B signaling (10). In addition to mediating homotypic protein interactions (60), CARD domains promote interactions with RING, kinase, and DD domains (47). Since all these domains are found in components of the TNF- α signaling cascade, CASP4 possesses several putative interaction partners in the signaling cascade from TNFR1 to NF- κ B. Fourth, CASP4 has been linked to TLR4 signaling (34), an immune pathway that utilizes signaling molecules also found in

FIG 5 Silencing of CASP4 interferes with direct signaling from TNFR1 to the IKK but not with TNFR1 cell surface expression or TNF- α -induced activation of JNK. (A) Knockdown of CASP4 does not affect TNFR1 cell surface expression. HeLa cells were transfected with control and candidate siRNAs and were stained for cell surface TNFR1. TNFR1 was detected using a specific antibody labeled with PE. PE staining was assessed by FACS. The histograms represent the intensity of the TNFR1-PE stain (x axis) dependent on cell number (y axis). An overlay of all samples is shown. Blue curve, siCASP4-treated cells; black curve, siTNFR1-treated cells; red curve, siRL-treated cells. Data shown are representative of two independent experiments. (B) Quantification of the results from panel A. PE-positive and -negative cells were quantified as a percentage of all analyzed cells. For quantification, two populations, PE positive and PE negative, were defined on the basis of the fact that siRL-treated cells were stained with the isotype-matched control antibody (isotype control). For each sample, the percentage of cells in either of the two populations was calculated. Control antibody-stained cells showed background staining. Anti-TNFR1 staining in siTNFR1-transfected cells was decreased to background, while cells treated with siRNAs against CASP4 or the negative control exhibited similar staining. (C) Knockdown of CASP4 abolishes I κ B- α phosphorylation upon TNF- α stimulation in HEK293T cells. HEK293T cells were transfected with siRNA pools, before I κ B- α phosphorylation upon TNF- α treatment (50 ng/ml) was assessed by immunoblotting. β -Actin served as a loading control. p-I κ B- α , phospho-I κ B- α . (D) Knockdown of CASP4 does not affect TNF- α -induced activation of JNK signaling. HEK293T cells were reverse transfected with siRNAs, and a dual-luciferase assay was performed, using a JUN-dependent firefly luciferase as a pathway-specific reporter and the β -actin promoter-driven *Renilla* luciferase as a coreporter. Depicted is relative signaling activity, i.e., FL/RL compared to negative-control transfected cells. ctrls, mean of negative siRNA controls; RelA, siRNA pools against RelA (positive control). Mean values of two independent experiments are shown. Error bars depict standard deviations of the two biological replicates. (E) Phosphorylation of JNK is unaffected by siCASP4 treatment. HeLa cells were transfected with siRNA pools, before JNK phosphorylation upon TNF- α treatment (25 ng/ml) was assessed by immunoblotting. β -Actin served as a loading control. Data shown are representative of two independent experiments. p-JNK, phospho-JNK. (A and E) siRL, siRNA pool against *Renilla* luciferase (negative control); siTNFR1, siRNA pool against TNFRSF1A (positive control); siCASP4, siRNA pools against caspase 4. (F) Model of CASP4's role in TNF- α signaling. Epistasis experiments showed that CASP4 is needed for the activation of IKK; hence, CASP4 is probably located at TNFR complex I (TNFR-C). At TNFR-C, two different branches of signaling are initiated: (i) both TRAF2 and TRAF5 together with RIP1 are required for the activation of IKK and, finally, NF- κ B; (ii) TRAF2 alone is sufficient to mediate the activation of JNK signaling activating transcription factors such as JUN. Knockdown of CASP4 does not affect this second signaling branch but affects signaling only to NF- κ B.

the TNF- α pathway, such as DD proteins and members of the TRAF family. This suggests that CASP4 might be another protein shared between TNF- α and TLR signaling. Finally, a caspase 4 (caspase 11)-knockout mouse which is resistant to LPS-induced sepsis has recently been reported (33). While there is currently no mechanistic explanation for the *in vivo* phenotype, it is tempting to speculate that LPS-induced inflammatory feedback loops via TNF- α are constrained in CASP4-knockout animals.

CASP4 could fulfill its function in TNF- α signaling both as a protease (31) and as a scaffold protein. Both mechanisms have been described to play a role for TNF- α -induced activation of NF- κ B for other caspases and organisms. Recently, the *Drosophila* orthologue of CASP8, DREDD, was shown to cleave *Drosophila* IMD (46). This cleavage enabled the interaction of components of the TNFR complex and thereby the activation of IKK and NF- κ B (46). However, a scaffold function of CASP8 has also been described to be required for TNF- α signaling to NF- κ B (14).

According to our results, depletion of CASP4 does not interfere with JNK activation. Thus, we propose that CASP4's mode of action in TNF- α signaling is probably located in the signaling branch required for the activation of IKK and, finally, NF- κ B (Fig. 5F). CASP4 could act as either a protease or a scaffold molecule at the TNFR complex. However, further studies will be necessary to characterize its molecular function.

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REFERENCES

- Abraham MC, Shaham S. 2004. Death without caspases, caspases without death. *Trends Cell Biol.* 14:184–193.
- Ahn EY, Pan G, Vickers SM, McDonald JM. 2002. IFN- γ upregulates apoptosis-related molecules and enhances Fas-mediated apoptosis in human cholangiocarcinoma. *Int. J. Cancer* 100:445–451.
- Ariazi EA, et al. 2011. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc. Natl. Acad. Sci. U. S. A.* 108:18879–18886.
- Baud V, Karin M. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* 11:372–377.
- Beutler B, Milsark IW, Cerami AC. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* 229:869–871.
- Bian ZM, Elnor SG, Elnor VM. 2009. Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 50:6006–6014.
- Binet F, Chiasson S, Girard D. 2010. Arsenic trioxide induces endoplasmic reticulum stress-related events in neutrophils. *Int. Immunopharmacol.* 10:508–512.
- Binet F, Chiasson S, Girard D. 2010. Evidence that endoplasmic reticulum (ER) stress and caspase-4 activation occur in human neutrophils. *Biochem. Biophys. Res. Commun.* 391:18–23.
- Birmingham A, et al. 2009. Statistical methods for analysis of high-throughput RNA interference screens. *Nat. Methods* 6:569–575.
- Bouchier-Hayes L, Martin SJ. 2002. CARD games in apoptosis and immunity. *EMBO Rep.* 3:616–621.
- Boutros M, Bras LP, Huber W. 2006. Analysis of cell-based RNAi screens. *Genome Biol.* 7:R66. doi:10.1186/gb-2006-7-7-r66.
- Bradley JR. 2008. TNF-mediated inflammatory disease. *J. Pathol.* 214:149–160.
- Brummelkamp TR, Nijman SM, Dirac AM, Bernards R. 2003. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF- κ B. *Nature* 424:797–801.
- Chaudhary PM, et al. 2000. Activation of the NF- κ B pathway by caspase 8 and its homologs. *Oncogene* 19:4451–4460.
- Chen FE, Ghosh G. 1999. Regulation of DNA binding by Rel/NF- κ B transcription factors: structural views. *Oncogene* 18:6845–6852.
- Chew J, et al. 2009. WIP1 phosphatase is a negative regulator of NF- κ B signalling. *Nat. Cell Biol.* 11:659–666.
- Choudhary S, et al. 2011. High throughput short interfering RNA (siRNA) screening of the human kinome identifies novel kinases controlling the canonical nuclear factor-kappaB (NF- κ B) activation pathway. *J. Biol. Chem.* 286:37187–37195.
- Dunning MJ, Smith ML, Ritchie ME, Tavare S. 2007. beadarray: R classes and methods for Illumina bead-based data. *Bioinformatics* 23:2183–2184.
- Gerlach B, et al. 2011. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* 471:591–596.
- Gesellchen V, Kutenkeuler D, Steckel M, Pelte N, Boutros M. 2005. An RNA interference screen identifies inhibitor of apoptosis protein 2 as a regulator of innate immune signalling in *Drosophila*. *EMBO Rep.* 6:979–984.
- Gilbert DF, et al. 2011. A novel multiplex cell viability assay for high-throughput RNAi screening. *PLoS One* 6:e28338. doi:10.1371/journal.pone.0028338.
- Goto A, et al. 2008. Akirins are highly conserved nuclear proteins required for NF- κ B-dependent gene expression in *Drosophila* and mice. *Nat. Immunol.* 9:97–104.
- Grell M, Wajant H, Zimmermann G, Scheurich P. 1998. The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proc. Natl. Acad. Sci. U. S. A.* 95:570–575.
- Haas TL, et al. 2009. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. *Mol. Cell* 36:831–844.
- Hitomi J, et al. 2004. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J. Cell Biol.* 165:347–356.
- Hoffmann A, Levchenko A, Scott ML, Baltimore D. 2002. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298:1241–1245.
- Huber W, von Heydebreck A, Suettmann H, Poustka A, Vingron M. 2003. Parameter estimation for the calibration and variance stabilization of microarray data. *Stat. Appl. Genet. Mol. Biol.* 2:Article 3.
- Huber W, von Heydebreck A, Suettmann H, Poustka A, Vingron M. 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 18(Suppl 1):S96–S104.
- Jiang Y, Woronicz JD, Liu W, Goeddel DV. 1999. Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science* 283:543–546.
- Karin M, Greten FR. 2005. NF- κ B: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* 5:749–759.
- Karki P, Dahal GR, Park IS. 2007. Both dimerization and interdomain processing are essential for caspase-4 activation. *Biochem. Biophys. Res. Commun.* 356:1056–1061.
- Kauffmann A, Gentleman R, Huber W. 2009. arrayQualityMetrics—a bioconductor package for quality assessment of microarray data. *Bioinformatics* 25:415–416.
- Kayagaki N, et al. 2011. Non-canonical inflammasome activation targets caspase-11. *Nature* 479:117–121.
- Lakshmanan U, Porter AG. 2007. Caspase-4 interacts with TNF receptor-associated factor 6 and mediates lipopolysaccharide-induced NF- κ B-dependent production of IL-8 and CC chemokine ligand 4 (macrophage-inflammatory protein-1). *J. Immunol.* 179:8480–8490.
- Le Bail O, Schmidt-Ullrich R, Israel A. 1993. Promoter analysis of the gene encoding the I kappa B-alpha/MAD3 inhibitor of NF-kappa B: pos-

- itive regulation by members of the rel/NF-kappa B family. *EMBO J.* 12: 5043–5049.
36. Li S, Wang L, Berman MA, Zhang Y, Dorf ME. 2006. RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. *Mol. Cell* 24:497–509.
  37. Lin R, Sun Y, Li C, Xie C, Wang S. 2007. Identification of differentially expressed genes in human lymphoblastoid cells exposed to irradiation and suppression of radiation-induced apoptosis with antisense oligonucleotides against caspase-4. *Oligonucleotides* 17:314–326.
  38. Li-Weber M, Giaisi M, Baumann S, Treiber MK, Krammer PH. 2002. The anti-inflammatory sesquiterpene lactone parthenolide suppresses CD95-mediated activation-induced-cell-death in T-cells. *Cell Death Differ.* 9:1256–1265.
  39. Martinon F, Tschopp J. 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 117: 561–574.
  40. Matsuzaki S, Hiratsuka T, Kuwahara R, Katayama T, Tohyama M. 2010. Caspase-4 is partially cleaved by calpain via the impairment of Ca²⁺ + homeostasis under the ER stress. *Neurochem. Int.* 56:352–356.
  41. Metzger M, et al. 2011. An RNAi screen identifies USP2 as a factor required for TNF-alpha-induced NF-kappaB signaling. *Int. J. Cancer* 129:607–618.
  42. Miller SD, et al. 2007. Tauroursodeoxycholic acid inhibits apoptosis induced by Z alpha-1 antitrypsin via inhibition of Bad. *Hepatology* 46: 496–503.
  43. Molestina RE, Sinai AP. 2005. Host and parasite-derived IKK activities direct distinct temporal phases of NF-kappaB activation and target gene expression following *Toxoplasma gondii* infection. *J. Cell Sci.* 118:5785–5796.
  44. Mulugeta S, et al. 2007. Misfolded BRICHOS SP-C mutant proteins induce apoptosis via caspase-4- and cytochrome c-related mechanisms. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293:L720–L729.
  45. Oda T, Kosuge Y, Arakawa M, Ishige K, Ito Y. 2008. Distinct mechanism of cell death is responsible for tunicamycin-induced ER stress in SK-N-SH and SH-SY5Y cells. *Neurosci. Res.* 60:29–39.
  46. Paquette N, et al. 2010. Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for *Drosophila* NF-kappaB signaling. *Mol. Cell* 37:172–182.
  47. Reed JC, Doctor KS, Godzik A. 2004. The domains of apoptosis: a genomics perspective. *Sci. STKE* 2004:re9. doi:10.1126/stke.2392004re9.
  48. Saleh M, et al. 2004. Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms. *Nature* 429:75–79.
  49. Scheinfeld N. 2004. A comprehensive review and evaluation of the side effects of the tumor necrosis factor alpha blockers etanercept, infliximab and adalimumab. *J. Dermatolog. Treat.* 15:280–294.
  50. Schroder K, Tschopp J. 2010. The inflammasomes. *Cell* 140:821–832.
  51. Sen R, Baltimore D. 1986. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47:921–928.
  52. Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV. 1990. Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J. Exp. Med.* 171:35–47.
  53. Sizemore N, Lerner N, Dombrowski N, Sakurai H, Stark GR. 2002. Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. *J. Biol. Chem.* 277:3863–3869.
  54. Smyth GK. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3:Article 3.
  55. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 81:513–523.
  56. Suzuki M, Endo M, Shinohara F, Echigo S, Rikiishi H. 2009. Enhancement of cisplatin cytotoxicity by SAHA involves endoplasmic reticulum stress-mediated apoptosis in oral squamous cell carcinoma cells. *Cancer Chemother. Pharmacol.* 64:1115–1122.
  57. Szaraz P, Banhegyi G, Benedetti A. 2010. Altered redox state of luminal pyridine nucleotides facilitates the sensitivity towards oxidative injury and leads to endoplasmic reticulum stress dependent autophagy in HepG2 cells. *Int. J. Biochem. Cell Biol.* 42:157–166.
  58. Ting AT, Pimentel-Muinos FX, Seed B. 1996. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J.* 15:6189–6196.
  59. Walczak H. 2011. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. *Immunol. Rev.* 244:9–28.
  60. Weber CH, Vincenz C. 2001. The death domain superfamily: a tale of two interfaces? *Trends Biochem. Sci.* 26:475–481.
  61. Yamamoto-Yamaguchi Y, Okabe-Kado J, Kasukabe T, Honma Y. 2003. Induction of apoptosis by combined treatment with differentiation-inducing agents and interferon-alpha in human lung cancer cells. *Anticancer Res.* 23:2537–2547.
  62. Yazdi AS, Guarda G, D'Ombra MC, Drexler SK. 2010. Inflammatory caspases in innate immunity and inflammation. *J. Innate Immun.* 2:228–237.
  63. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* 91:243–252.
  64. Zhang JH, Chung TD, Oldenburg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4:67–73.
  65. Zheng L, et al. 2004. An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 101:135–140.