

NAK is recruited to the TNFR1 complex in a TNF α -dependent manner and mediates the production of RANTES:

Identification of endogenous TNFR-interacting proteins by a proteomic approach

**Jun Kuai¹, Joe Wooters², J. Perry Hall¹, Vikram R. Rao¹, Elliott Nickbarg^{2#}, Bilian Li³,
Moitreyee Chatterjee-Kishore³, Yongchang Qiu², and Lih-Ling Lin^{1§}**

¹Inflammation, ²Protein Chemistry and Proteomics, and ³Genomics
Wyeth Research, 200 Cambridge Park Drive, Cambridge, MA 02140.

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Abbreviations: TNF, tumor necrosis factor; TNFR1, TNF receptor type I; TNFR2, TNF receptor type II; TRADD, TNFR1-associated death domain; FADD, Fas-associated death domain; RIP, receptor interacting protein; TRAF2, TNF receptor associated factor 2; TRAP2, Tumor necrosis factor receptor-associated protein 2; NAK, NF- κ B-activating kinase; T2K, TRAF2-associated kinase; TBK1, TANK-binding kinase 1; TRCP1, TNF receptor complex protein 1; TRCP2, TNF receptor complex protein 2; IL-1, IL-6 and IL-8: interleukin-1, interleukin-6 and interleukin-8; RANTES, regulated upon activation normal T-cell expressed and secreted; siRNA: small interference RNA.

[§] Address correspondence to: Lih-Ling Lin, Ph.D., Inflammation Signaling, Wyeth Research, 200 Cambridge Park Drive, Cambridge, MA 02140. Phone: 617-665-5476, Fax: 617-665-5499, e-mail: llin@wyeth.com

[#] Current address: Neogenesis Pharmaceuticals, Inc., 840 Memorial Dr., Cambridge, MA 02139

SUMMARY

Tumor necrosis factor α (TNF α) is a proinflammatory cytokine with pleiotropic immunological and biological activities. TNF α signaling is triggered by the engagement of soluble TNF α to two types of cell surface receptors, TNFR1 and TNFR2. This recruits cytosolic proteins to the intracellular domains of the receptors and initiates signaling to downstream effectors. In this study, we use a proteomic approach to identify these cytosolic proteins from affinity-purified, endogenous TNF α -TNFR complexes in human myelomonocytic U937 cells. Seven proteins are identified, including TRADD, TRAP2, and TRAF2, which are three proteins known to be recruited to TNF α receptors. NAK, RasGAP3, TRCP1, and TRCP2 are also identified. We further show that NAK is recruited to TNFR1 in a temporally regulated and TNF α -dependent manner, and that mediates the TNF α -induced production of the chemokine RANTES. These data demonstrate that NAK is a component of the TNF α -TNFR1 signaling complex and confirm the physiological role of NAK in the TNF α -mediated response.

INTRODUCTION

Tumor necrosis factor α (TNF α) is a key proinflammatory cytokine that controls immune and inflammatory responses and regulates cell proliferation and apoptosis (1-4). It is produced by many cell types including macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts in response to inflammation or infection. Human diseases such as rheumatoid arthritis and inflammatory bowel disease are associated with inappropriate production of TNF α or sustained activation of TNF α signaling. Therefore, understanding the regulation of TNF α production and signals transmitted by TNF α is necessary for the development of effective treatments for these diseases.

The pleiotropic biological activities of TNF α are mediated by its binding to two different cell surface receptors, the TNF receptor type I (TNFR1) and the TNF receptor type II (TNFR2) (4). Neither of these receptors possess enzymatic activities. Signals transduced from these receptors are mediated by cytosolic proteins recruited to the receptors, and their intracellular domains share no significant homology suggesting that they may interact with different cellular proteins. Gene knockout experiments confirm that TNFR1 and TNFR2 control different signaling cascades, and that the majority of TNF α signaling events are mediated by TNFR1 (4).

Binding of TNF α to TNFR1 induces homo-trimerization of the receptors and recruits cytosolic proteins (4). These proximal events activate multiple downstream pathways, including activation of two transcription factors NF- κ B and AP-1 and activation of those pathways that lead to apoptosis. Over the last decade, many of the molecular mechanisms of TNF α signaling have begun to be deciphered through the identification of proteins that are recruited to the TNF receptors. Upon TNF α binding, TRADD is first recruited to TNFR1 and provides a platform

for the recruitment of other death domain-containing proteins, such as RIP and FADD (5-8). TRAF2, a member of the TRAF family of proteins that does not contain the death domain, is recruited to TNFR1 via TRADD (9). TRAF2 is also an adaptor protein of TNFR2 (10). Given the very low abundance of the endogenous receptors and proteins recruited to the receptors, together with the limits of the protein identification, most of these proteins were identified under the conditions of overexpression utilized in yeast two-hybrid systems or by affinity purification using recombinant proteins. Therefore, little is known about the composition of the endogenous receptor complex.

Activation of NF- κ B is one of the major pathways by which TNF α induces the expression of genes involved in immune and inflammatory responses (4). NF- κ B is composed of homo- or hetero-dimers of Rel family members (11), but the most common form consists of a p50/p65 heterodimer. NF- κ B is retained in the cytoplasm by a family of inhibitory proteins known as I- κ B proteins. In response to TNF α , the I- κ B proteins are phosphorylated by the I- κ B kinase (IKK) complex, which contains IKK α , IKK β , and IKK γ subunits (12). Phosphorylated I- κ B is ubiquitinated and subsequently degraded by the 26S proteasome. NF- κ B then translocates to the nucleus where it binds to specific promoter sequences termed κ B elements and thereby activates the transcription of NF- κ B-driven genes. The IKK complex phosphorylates I- κ B α on serine 32 and serine 36. NAK (also known as T2K or TBK1) shares a high degree of homology with IKK α and IKK β and phosphorylates I- κ B α on serine 36 but not serine 32 *in vitro* (13-15). Thus, it has been proposed to be a member of the IKK family kinases (13). *Nak*^{-/-} mice have similar phenotypes to *Ikkb*^{-/-}, *Ikk γ* ^{-/-}, and *RelA*^{-/-} mice, in that they all die at embryonic day 14.5 from massive liver degeneration and apoptosis, which is consistent with this proposal (15-20). Moreover, in *Nak*^{-/-} mouse embryonic fibroblast cells the transcription of two NF- κ B-driven

genes, TLR2 and ICAM, are deficient in response to TNF α (15). Surprisingly, however, in these cells I- κ B α degradation and the binding of NF- κ B to κ B DNA elements are normal. Based on these observations, it has been suggested that NAK may phosphorylate the p65 subunit of NF- κ B or act through an unidentified factor to regulate the activation of the NF- κ B (13). Although NAK clearly plays an essential role in the transcription of a subset of TNF α -induced NF- κ B driven genes, the precise involvement of NAK in TNF α signaling has remained elusive.

In this study, we explored the composition of the endogenous TNF α -TNFR1 complex using a proteomic approach. Compared to conventional protein identification technologies, the mass spectrometry-based proteomic approach used here has a high sensitivity (down to femtomole detection levels) and can handle a high complexity of samples, which allowed us to identify low abundance proteins from an endogenous complex without purifying each individual protein. We affinity-purified the endogenous TNF α -TNFR receptor complex from U937 cells using Flag-tagged TNF α . Proteins co-purified in the complex were first eluted with Flag-peptides and then with 8M Urea. The eluted proteins were resolved by SDS-PAGE and identified by tandem mass spectrometry. Seven proteins, TRADD, TRAF2, TRAP2, NAK, RasGAP3, and two novel proteins, were identified in the complex. We also demonstrate that recruitment of NAK to TNFR1 is temporally regulated and TNF α -dependent, and that endogenous NAK is required for the TNF α -induced production of the chemokine RANTES.

MATERIALS & METHODS

Cell Culture, antibodies and reagents- U937 and HEK293 cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Gibco BRL) and DME (Gibco BRL), respectively, supplemented with 10 % FBS. Anti-TNFR1 antibody (AF225) was

purchased from R&D Systems. Anti-TRADD, -TRAF2 and -NAK (M-375) antibodies were purchased from Santa Cruz Biotechnology. Anti-Flag (M2) antibody and affinity beads were obtained from Sigma. Anti-HA antibodies were purchased from Roche Molecular Biochemicals. All chemical reagents otherwise specified were purchased from Sigma. Human Flag-NAK was cloned by PCR from a human ovary cDNA library (BD Biosciences Clontech, Palo Alto, CA) into pENTR/SD/D-TOPO vector using directional TOPO cloning kits from Invitrogen, and subsequently cloned into expression vector pAdori-DV (a Wyeth proprietary vector). Mutation of K38M was introduced by site-directed mutagenesis using PCR and cloned into pAdori-DV vector as above.

Purification of endogenous TNF α -receptor complex- 1×10^{10} U937 cells were harvested and washed twice with warm PBS (37 °C), then resuspended to 1×10^7 cells/ml. Cells were either left untreated or treated with 100 ng/ml of Flag-TNF α (Alexis) for 10 min at 37 °C. Cells were then lysed in 50 ml of lysis buffer (20mM Tris.HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 30 mM NaF, 1mM NaVO₄ and protease inhibitor cocktails [Roche Molecular Biochemicals]), and gently rocked at 4 °C for 30 min. Cell debris was removed by centrifugation twice at 10,000 \times g for 30 min. The lysate was preclarified by incubation with Gamma Binding beads (Amersham Biosciences) for 1 hour. The resulting lysate was immunoprecipitated using M2-affinity beads (Sigma). The immuno-complex was first eluted with Flag peptide (Sigma) at a concentration of 2 mg/ml. The residual binding proteins were then further eluted with 8M Urea. One half of the peptide-eluted proteins or 1/10 of the 8M Urea-eluted proteins was subjected to immunoblot analysis using anti-TRADD or -TRAF2 antibody. The remainders of the samples were separated by SDS-PAGE and visualized by silver staining.

Mass spectrometry and protein identification- Protein bands of interest were manually excised from the gel, reduced, and alkylated with iodoacetamide, and then digested *in situ* with trypsin using an automated digestion robot (ABIMED, Germany) as previously described (21). The peptide digests were then sequenced using a high-throughput tandem mass spectrometer (ThermoQuest LCQ-DECA, San Jose CA) equipped with a micro-electrospray reversed phase liquid chromatography interface. Data were acquired in automated MS/MS mode using the data acquisition software provided with the LCQ to detect and sequence each peptide as it eluted from the column. The dynamic exclusion and isotope exclusion functions were employed to increase the number of peptide ions that were analyzed. During the LS-MS/MS run, typically >1000 fragmentation spectra were collected from each sample and matched against the nonredundant databases (NCBI) using the Sequest software package (ThermoQuest).

Immunoprecipitation and Western analysis- For immunoprecipitation, 1×10^8 U937 cells were treated with Flag-TNF α at 100 ng/ml for different times, or left untreated. Cell lysates were prepared as described above and subjected to immunoprecipitation using either M2-beads or TNFR1 antibody. Immunoprecipitates were analyzed by immunoblots probed with anti-NAK, -TRADD, or -TRAF2 antibodies. Signals were detected with HRP-conjugated secondary antibody and ECL detection kits (Amersham Pharmacia Biotech).

Analysis of RANTES production and kinase assays- 293T cells were seeded on 6-well plates and transfected with either vector alone, NAK or NAK(K38M) expression vectors using calcium phosphate (ProFection Mammalian Transfection System, Promega). After 48 hours, cells were

stimulated with 100 ng/ml of TNF α for 24 hours, or left untreated. Cell supernatants were collected and analyzed for RANTES by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (BioSource International). Cells were harvested and lysed as described above. Flag-NAK proteins were immunoprecipitated with M2-beads and analyzed for kinase activity using biotin-AKELDQGSLCTSFVGTLLQKKK peptides derived from IKK β as substrates (22). Samples were incubated at 30°C for 30 min in a 30 μ l reaction mixture containing 50 mM HEPES (pH 8.0), 10 mM MgCl₂, 2.5 mM EGTA, 1mM DTT, 20 mM β -glycerophosphate, 1mM NaF, 1 mM vanadate, 33 μ M ATP, 330 μ Ci ³³P-ATP and 267 μ M peptide. Peptides were then separated from each reaction mixture by passing the reaction mixture through a P81 phosphocellulose membrane (Whatman) under a vacuum manifold. The extent of peptide phosphorylation on each membrane was determined by standard liquid scintillation counting. The immunoprecipitates were also subjected to immunoblot analysis to assess the relative amounts of immunoprecipitated proteins.

Small interfering RNA -A pool of small interfering RNAs (siRNAs) targeting NAK was purchased from Dharmacon Inc. (M-003788-02, Lafayette, Chicago). Human HEK293 cell line was transfected with the siRNA smart pool targeting NAK or scrambled RNAs (D-001200-01-80, Dharmacon) using lipofectamine 2000 (Invitrogen). After 24 hours, cells were treated with TNF α for 40 hours. RANTES production was measured in the medium by ELISA. The levels of NAK, IKK α and IKK β transcripts were determined by Taqman using standard manufacturer's protocols. The fold changes of the mRNAs were all normalized to the housekeeping gene beta-2-microglobulin. The primers and probes for NAK (Hs00179410_m1), IKK β (Hs00233287_m1), and beta-2-microglobulin (Hs99999907_m1) were purchased from Applied

Biosystems (Foster City, California). Primers for IKK α (Forward: CAGGTTCTCTTCCTGGCATCA, Reverse: CCTTCGATGAGAAGGGGATACTG) were ordered from Integrated DNA Technologies (Coralville, Iowa) and probe (TGATTACACCAACTCCTT) was ordered from Applied Biosystems (Foster City, California).

RESULTS and DISCUSSION

Purification of the endogenous TNF α -TNFR complex from U937 cells.

Both TNFR1 and TNFR2 are expressed in U937 cells (23). The binding of TNF α to these receptors triggers their homo-trimerization and the recruitment of cytosolic proteins to the receptor tails. The activated receptors, together with the recruited cytosolic proteins, constitute the TNF α -TNFR complex. In order to understand the composition of the endogenous TNF α -TNFR complex, we treated U937 cells with Flag-tagged TNF α and affinity purified the TNF α -TNFR complex using anti-Flag antibody-conjugated Sepharose beads. TNF α interacts with both receptors, so it was assumed that the TNF α -TNFR complex contains both TNF α -TNFR1 and TNF α -TNFR2, and that proteins associated with either of these receptors would be co-purified.

After purification, the bound complex was eluted with Flag peptides. Proteins eluted from the beads were resolved by SDS-PAGE and visualized by silver staining (Figure 1A). Approximately twelve protein bands were seen in the eluted complex but none of these bands were present in the sample from cells without TNF α stimulation. Thus, these proteins were specifically associated with the TNF α -TNFR complex. As a control, TRADD and TRAF2 were detected by immunoblotting in the eluates from TNF α -treated cells (Figure 1B). Therefore, we conclude that the active TNF α -TNFR complex was formed and purified.

Identification of proteins co-purified with the receptor complex by mass spectrometry.

To identify the proteins co-purified with the TNF α -TNFR complex, we excised the twelve bands from the silver stained gel in Figure 1A and analyzed them by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) after in-gel digestion (see Methods). As expected, TNF α and TRADD were detected in band 12 and band 9, respectively (Table 1). Surprisingly, we were unable to detect any peptides of TRAF2 at the expected position of band 7 where we had detected it by immunoblotting (Figure 1B). Proteins in other bands either could not be determined due to the poor quality of the spectrum or later turned out to be non-specific binding proteins, such as Hsp90 and Hsp71 in band 4 and tropomyosin in band 10.

In a previous study, we found that Flag peptides only elute one tenth of the proteins that bound to the beads (24). We observed the same phenomenon in the present study as indicated by the low recovery (~10%) of total TRADD on the beads judged by immunoblot analysis (data not shown). This low efficiency of elution was probably due to the multimeric and high affinity interactions between M2-antibodies and trimerized Flag-TNF α -TNFR complex. To increase the amount of proteins eluted from the beads, we treated the beads further with 8M Urea and resolved the eluted proteins by SDS-PAGE. However, 8M Urea also eluted non-specific binding proteins resulting in a pattern that was indistinguishable between the untreated and TNF α -treated samples on the silver stained gel (data not shown). Therefore, we excised the areas that corresponded to the peptide-eluted bands in Figure 1A and then analyzed these bands by mass spectrometry. Proteins identified from the 8M Urea eluates of the untreated sample were proteins that bound non-specifically to the M2-beads. These proteins were subtracted from the

total proteins identified in the 8M Urea eluates of the TNF α -treated sample. This resulted in a list of proteins that specifically associated with the TNF α -TNFR complex (Table1).

In the 8M Urea eluates of the TNF α -treated sample, seven peptides of TRAF2 were detected at the band 7 position, and these peptides were totally absent in the control. Peptides of TNF α and TRADD were also detected in the 8M Urea eluates, as were two peptides of TNFR1 and one peptide of TNFR2. The presence of TNFR1, TNFR2, TRADD, and TRAF2 in the 8M Urea eluates demonstrates that the 8M Urea eluates contained components of both TNF α -TNFR1 and TNF α -TNFR2 complexes.

Five additional proteins were detected in the 8M Urea eluates (Table 1). Among them, TRAP2, also known as subunit 2 of 26S proteasome, was previously identified in a yeast two-hybrid screen for proteins that interact with the intracellular domain of TNFR1 (25,26). The detection of TRAP2 in our endogenous TNF α -TNFR1 complex demonstrates that TRAP2 associates with TNFR1 under physiological conditions. Together, these observations suggest a direct interaction of TNF α signaling with the 26S proteasome-mediated protein degradation. Given the proteasome-mediated degradation of phosphorylated I- κ B during the activation of NF- κ B, it is conceivable that the regulation of proteasome activity is coupled to the activation of NF- κ B during TNF α signaling.

NAK, an IKK family kinase which has been reported to play a critical role in the TNF α -induced activation of NF- κ B (13,15), was also detected. Thus, there exists a physiological association of NAK with the TNF α -TNFR complex, which confirms and extends the previous observations and unequivocally demonstrates a role for NAK in TNF α signaling under physiological conditions.

We also detected RasGAP3, and two novel proteins KIAA0143 (gi2495710) and FLJ20758 (gi17433930), which we named TRCP1 and TRCP2 (TNF Receptor Complex Protein 1 and 2), respectively. RasGAP3 is an inositol-1,3,4,5-tetrakisphosphate (IP₄) binding protein that belongs to the GTPase-activating protein-1 (GAP1) family (27). RasGAP3 contains a highly conserved RasGAP domain and a pleckstrin-homology domain that localizes it to the plasma membrane (28,29). RasGAP3 has not been previously implicated in TNF α signaling; however, MADD, another death domain containing protein that interacts with TNFR1, contains a guanine nucleotide exchange domain of the small GTPase Rab (30,31). The association of the TNFR complex with proteins that regulate the activities of small GTPases links TNF α signaling to small GTPases. TRCP1 encodes 885 amino acids and six peptides were identified in the 8M Urea eluates (Table1). TRCP2 has 689 amino acids and eight peptides were identified (Table 1). We did not find any functional domains in TRCP1 and TRCP2. Identification of the specific TNFR receptors that TRCP1, TRCP2, and RasGAP3 interact with, and their functions in TNF α signaling, require further investigation.

NAK is recruited to the TNFR1 receptor in a TNF α -dependent manner and mediates the TNF α -induced production of the chemokine RANTES.

We next tested whether NAK associates with TNFR1 using immunoprecipitation assays with an anti-TNFR1 antibody. As seen in Figure 2, similar patterns of NAK recruitment were observed in immunoprecipitates using either anti-TNFR1 antibody or anti-Flag (M2) antibody, confirming that NAK was indeed recruited to TNFR1. Moreover, this recruitment was temporally regulated. It peaked between 5 and 10 minutes, later than TRADD and TRAF2, which peaked as early as 2 minutes. NAK has been reported to associate with TRAF2 (13,14);

therefore, it is likely that TRAF2 mediates the recruitment of NAK to the complex. Importantly, similar to the TNF α -dependent recruitment of TRADD and TRAF2, the recruitment of NAK was also dependent upon TNF α treatment (Figure 2B).

As a key proinflammatory cytokine, TNF α can stimulate the production of various cytokines and chemokines, such as TNF α itself, IL-1, IL-6, IL-8 and RANTES, which mediate and elevate the inflammatory response (32). RANTES is a chemokine that is able to recruit leukocytes to the sites of inflammation (33). In response to TNF α stimulation, a high level of RANTES (3000 to 4000 pg/ml) can be produced from synovial fibroblasts and monocytic cells (e.g., THP-1) (data not shown). However, a low level of RANTES (less than 200 pg/ml) can be detected in HEK293 cells in response to extensive TNF α stimulation (up to 40 hours) (Figure 4). Importantly, overexpression of NAK dramatically promoted both basal (about 20 fold increase) and TNF α -induced (about 40 fold increase) RANTES production in HEK293 cells (Figure 3A). This event was dependent upon the catalytic activity of NAK because the kinase inactive mutant NAK(K38M) (13) caused no induction of RANTES despite a level of protein expression equal to the wild type NAK (Figure 3). The induction of RANTES increased with increasing TNF α (Figure 4A), and the reduction of endogenous NAK by a siRNA smart pool targeting NAK mRNA decreased this TNF α -induced RANTES production up to 70% (Figure 4A). This siRNA smart pool reduced 40% of the mRNA of NAK (Figure 4B) but caused no decrease of the mRNA of IKK α and IKK β (data not shown). Therefore, these results demonstrate that NAK is required for TNF α -induced RANTES production.

Expression of RANTES is regulated by multiple transcription factors including NF- κ B and IRF3 (33). TNF α -induced activation of NF- κ B has been well established. Although deficiency of NAK impairs TNF α -induced transcription of NF- κ B driven genes, it does not seem

to block the phosphorylation and degradation of I κ B and the DNA binding activity of NF- κ B. Recently, NAK has been shown to play an essential role in the virus-induced expression of RANTES via the activation of IRF3 (34). However, TNF α -induced activation of IRF3 has not been reported. It could be that the basal TNF α -induced activation IRF3 is very low due to the low endogenous level of NAK. It is also possible that recruitment of NAK activates an yet unknown factor that leads to the activation of NF- κ B or IRF3 for the expression of RANTES. Further investigations are needed to clarify the role of NAK in control of the TNF α -induced expression of RANTES.

In summary, we have successfully isolated endogenous TNF α -TNFR complexes and identified proteins in these complexes using a mass spectrometry-based proteomic approach. The TNF α receptors TNFR1 and TNFR2 and seven potential signaling proteins were identified. Based on our data and previous findings, we conclude that TRADD, TRAF2, TRAP2 and NAK can associate with TNFR1 under physiological conditions, and that TRAF2 and NAK, via association with TRAF2, may form a complex with TNFR2. Our data also indicate that RasGAP3 and the two novel proteins TRCP1 and TRCP2 interact with a TNF α -TNFR complex, although the specific TNFR that these proteins associate with remains unknown. Identification of these molecules in TNF α -TNFR complex furthers our understanding of the molecular events in various TNF α -mediated cellular responses, such as the production of the chemokine RANTES (via NAK), the regulation of proteasome function (via TRAP2), and signaling from small GTPases (via Ras GAP3).

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Figure 1. Multiple proteins associated with TNF α -TNFR complex. U937 cells were treated with Flag-TNF α (+), or untreated (-). Immunoprecipitations were performed with anti-Flag antibody M2-conjugated Sepharose beads. After extensive washing, the immuno-complexes were eluted with Flag peptides. The eluted proteins were resolved by SDS-PAGE and visualized by silver staining (A). The presence of TRADD and

TRAF2 in the complex was detected by Western Blots with anti-TRADD or anti-TRAF2 antibodies (B).

Figure 2. Time- and TNF α -dependent recruitment of NAK to TNFR1 complex.

U937 cells were treated with Flag-TNF α for different times, or left untreated.

Immunoprecipitations were performed with either M2 beads or TNFR1 antibody (see details in Methods), followed by immunoblotting with anti-NAK (A, B), anti-TRAF2 (C), anti-TRADD (D) antibodies, respectively. As a control, the detection of a non-specific binding protein CBP80 on M2 beads using anti-CBP80 antibody indicated that similar amounts of cell lysate were present in each immunoprecipitation (E).

Figure 3. Overexpression of NAK promotes TNF α -induced RANTES production.

293T cells were transfected with NAK or NAK(K38M) expression plasmids, or vector alone. After 48 hours, cells were treated with 100 ng/ml of TNF α for 16 hours or left untreated. Cell supernatants were collected and measured for the production of RANTES by ELISA (A). Cells were harvested and lysed for kinase assays using a peptide substrate derived from IKK β (B). Expression of proteins was detected in the cell lysates by immunoblotting with anti-Flag antibody (D).

Figure 4. Endogenous NAK is required for TNF α -induced RANTES production.

The HEK 293 cell line was transfected with a pool of four siRNAs targeting NAK (siRNA smart pool from Dharmacon). After 24 hours of transfection, cells were treated with 100 ng/ml of TNF α for 40 hours. RANTES production in the culture medium was

then measured (A). The endogenous NAK mRNA was measured by Taqman using standard protocols as summarized in the Material and Methods section (B).

Table 1. List of proteins identified in TNF α -TNFR1 complex. Proteins listed in the table were detected in the TNF α -treated sample but not in controls. * F stands for bands in FLAG-eluted sample; U stands for bands in 8M Urea-eluted sample. Numbers correspond to what is indicated in Figure 1.

Proteins	GenBank identification number (gi)	Position *	Number of peptides detected
TNF α	13645336	F12/U12	5
TNFR1	4507575	U7	2
TNFR2	4507577	U5	1
TRADD	808915	F9/U9	9
TRAF2	10863939	U7	7
TRAP2	4506227	U3	8
NAK	7019547	U4	15
RASGAP3	7110713	U3	6
TRCP1	2495710	U4	6
TRCP2	17433930	U4	8

Figure 1

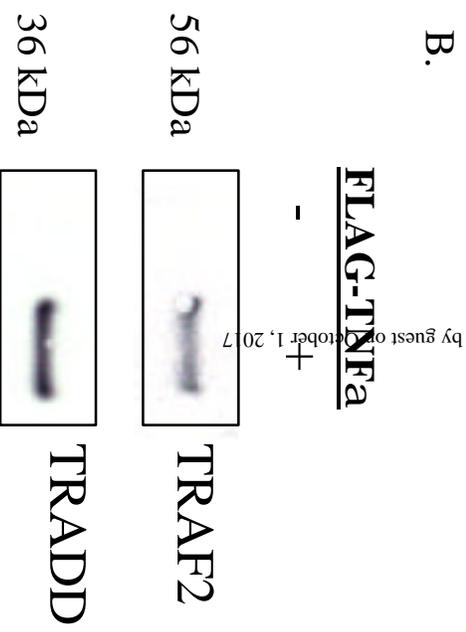
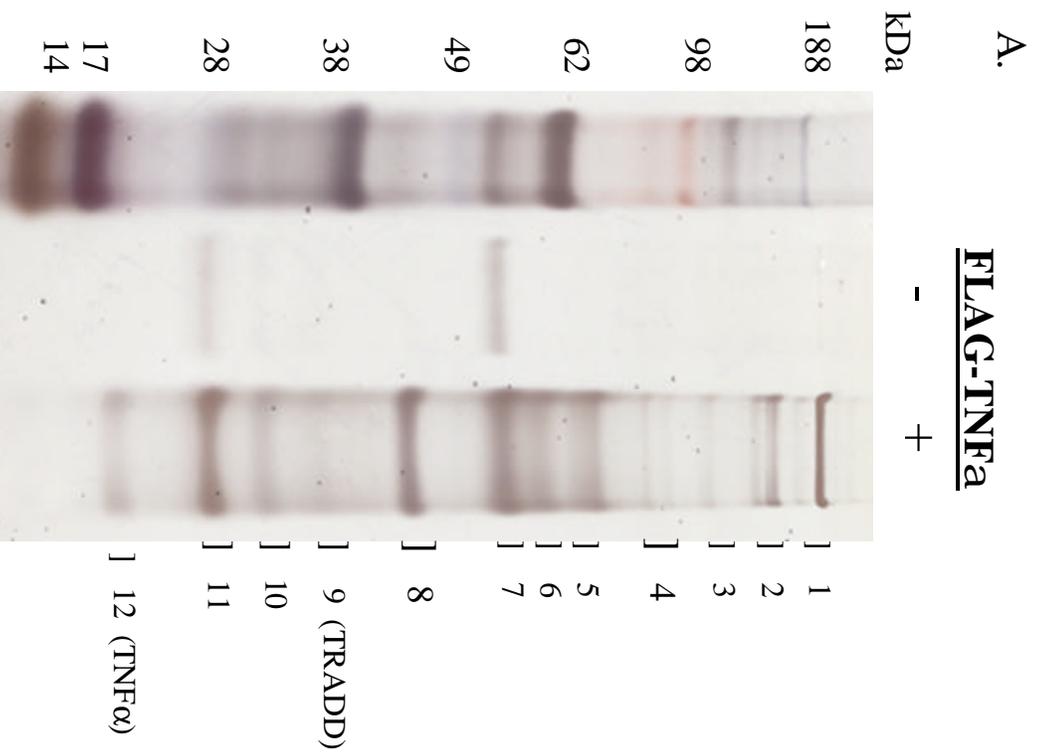
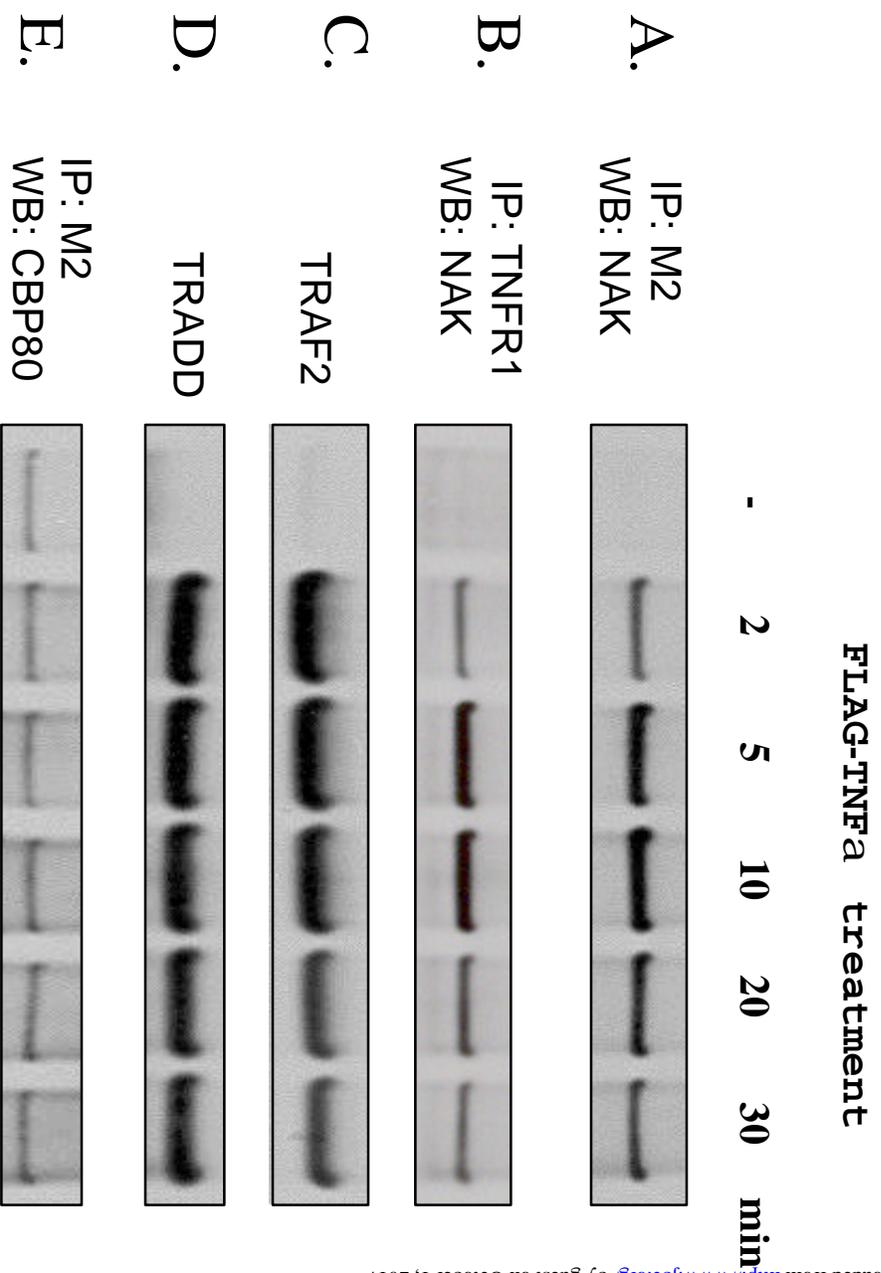
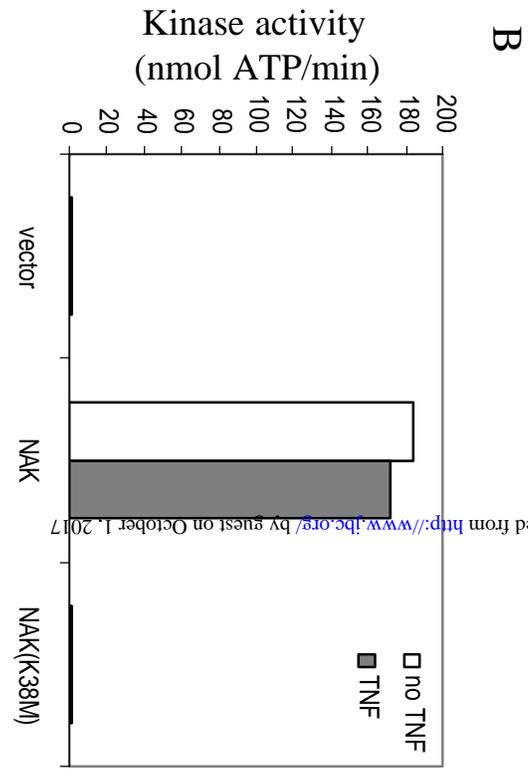
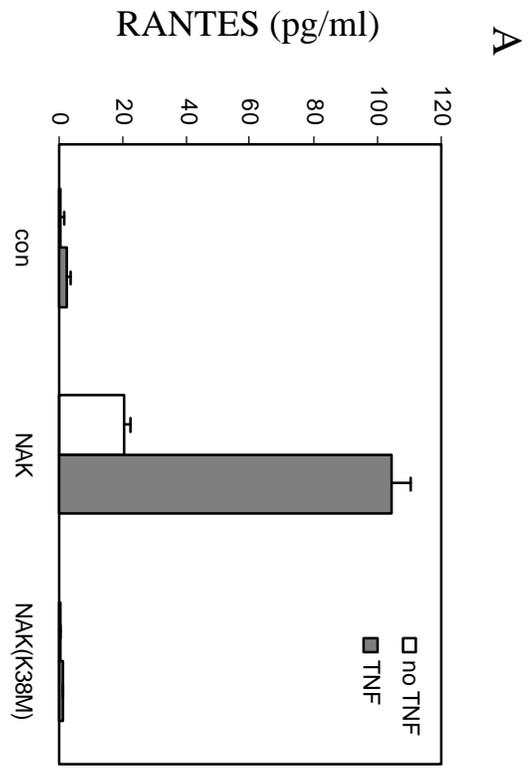


Figure 2

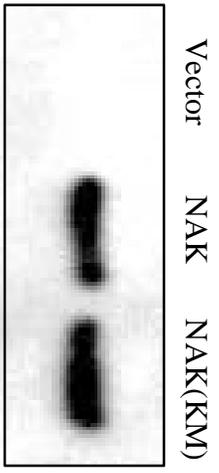


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Figure 3

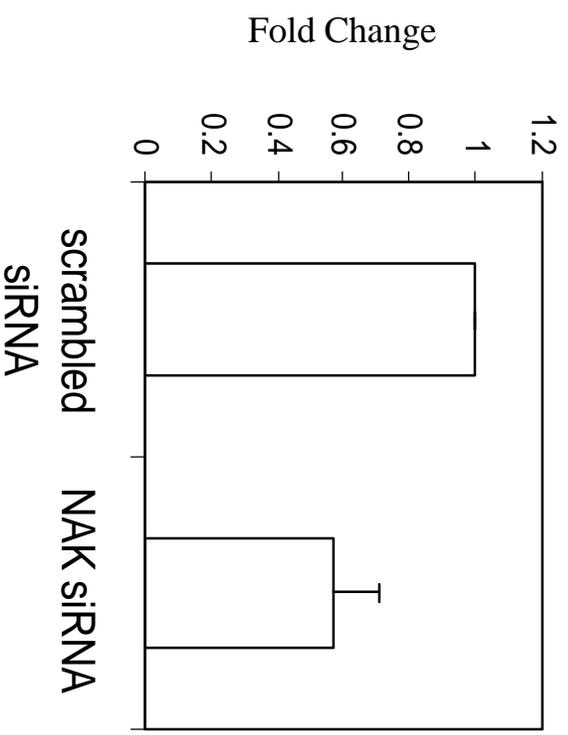
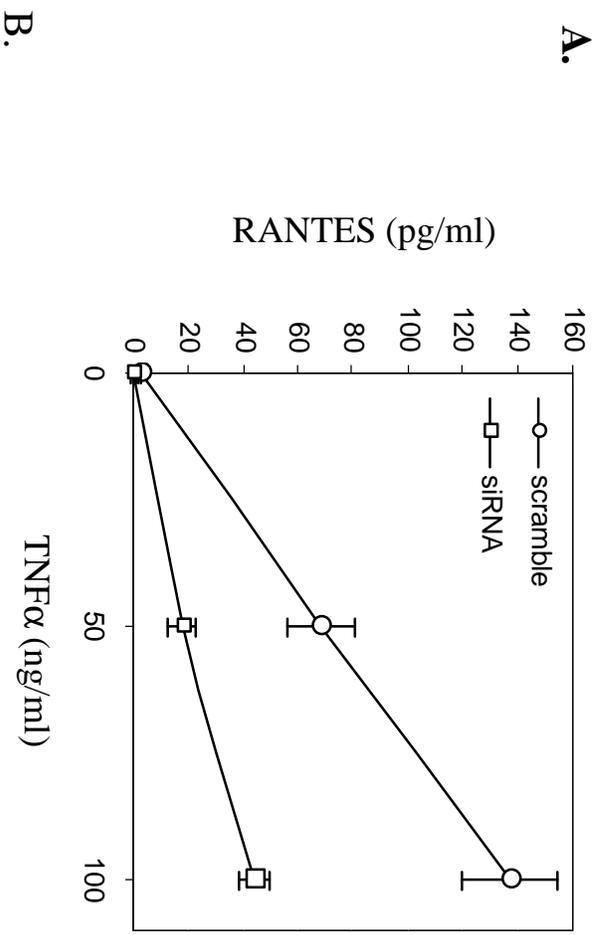


C. WB: Flag



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Figure 4



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NAK is recruited to the TNFR1 complex in a TNF α -dependent manner and promotes the production of RANTES: Identification of endogenous TNFR-interacting proteins by a proteomic approach

Jun Kuai, Joe Wooters, James Perry Hall, Vikram R. Rao, Elliott Nickbarg, Bilian Li, Moitreyee Chatterjee Kishore, Yongchang Qiu and Lih-Ling Lin

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