Supplementary Information

Materials & Methods

Plasmids and shRNA
The expression vectors pcDNA3-HA-PIASy, pcDNA-HA-PIASy-C/A (Shigeki Miyamoto, University of Wisconsin), pEGFP-SUMO-2 (Hans Will, Heinrich-Pette-Institut Hamburg), pcDNA3-His-SUMO-2 (Ron T. Hay, University of Dundee), pcDNA3-TANK, pCMV-Myc-TANK, pcDNA3.1-Flag-TANK and pcDNA3-Myc-IKKε (Alain Chariot, Université de Liège) were kindly provided by the indicated persons. Plasmids for bacterial expression of GST-Ubiquitin, GST-SUMO-1 and GST-4×Ubiquitin were gifts from Ivan Dikic (Goethe University Frankfurt), while the vector encoding Flag-tagged NEMO was provided by Daniel Krappmann (GSF, Munich). The plasmids pSUPER-puro-Scramble, pSG5-His-SUMO-1, pSG5-His-SUMO-1-G97A, pcDNA3-Flag-HA-SENP1, pEGFP-SUMO-1, pcDNA3-HA-Ubc9, pcDNA3-Flag-IKKε and constructs derived thereof (Renner et al, 2010), pcDNA3-Myc-PIASy, pcDNA3-Myc-Pc2 (Roscic et al, 2006), pcDNA3-Flag-TBK1, pcDNA3-Flag-TBK1-K38A (Buss et al, 2004) and pcDNA3-TBK1-Myc (Gatot et al, 2007) were previously described. The plasmids pEGFP-IKKε and pEGFP-IKKε-K38A were generated by ligating the IKKε cDNA excised from pcDNA3-Flag-IKKε via BamHI and XbaI into pEGFP-C3 digested with BglII and XbaI. An expression vector for untagged IKKε was generated by excising the Flag-Tag from pcDNA3-Flag-IKKε using HindIII and BamHI and religating the vector after Klenow fill-in. TANK point mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

Antibodies and reagents
The mouse monoclonal anti-TANK (D-2) and anti-SUMO-1 (D-11) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal antibodies against the Myc (9E10), HA (3F10) and GFP (7.1 and 13.1) epitopes were from Roche Diagnostics. The mouse monoclonal anti-IKKε antibody and the rabbit polyclonal anti-SUMO-2/3 and anti-TANK antibodies were from Abcam, the anti-Cox2 antibody was a donation from Bernd Fiebich (Freiburg) and the rabbit monoclonal anti-phospho-IRF-3 (Ser396)
antibody was purchased from Cell Signaling Technology. IgGs against Flag and tubulin (tub 2.1) were purchased from Sigma. Secondary horseradish peroxidase coupled anti-rabbit, anti-mouse or anti-rat antibodies were purchased from Dianova. R-848 was purchased from InVivoGen, while the crosslinker dimethyl-3-3’-dithiobispropionimidate 2-HCl was from Pierce.

**Cell culture, cell lines and transient transfections**

Human embryonic kidney 293T cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. HEK293 cells stably transfected to express the human TLR7 receptor (kindly gifted by Stefan Bauer, University of Marburg) were grown in complete DMEM in the presence of 0.3 mg/ml G418. Transient transfections were done with Rotifect according to the instructions given by the manufacturer (Roth). Ramos Burkitt lymphoma cells were grown in RPMI-1640 medium and transfected by electroporation.

**Cell extracts and Western blotting**

NP-40 lysates were prepared by resuspending cell pellets in NP-40 buffer (20 mM Tris-HCl/pH 7.5, 1% (v/v) NP-40, 150 mM NaCl, 1 mM MgCl₂, 10% Glycerol supplemented with protease and phosphatase inhibitors), followed by 20 min incubation on ice and clearance of the lysates by centrifugation. Western blotting was done by SDS-PAGE and semi-dry transfer to polyvinylidene difluoride membranes. After incubation with primary and secondary peroxidase-coupled antibodies, protein detection was done using the enhanced chemoluminiscence (ECL) system (GE Healthcare) according to the instructions given by the manufacturer.

**Immunoprecipitation**

For immunoprecipitation, cleared cell extracts were supplemented with 1 µg precipitating antibody and 20 µl (bed volume) protein A/G agarose. After 4 h rotating at 4°C, immunoprecipitates were washed 5 times in NP-40 buffer containing 50 mM Tris-HCl/pH 7.5 and precipitated proteins were eluted by boiling the protein A/G beads for 5
Precipitates were then analyzed by SDS-PAGE and Western blotting.

**Enrichment of His-tagged proteins on Ni-NTA columns**

His-SUMO-modified proteins were enriched after lysis under denaturing conditions using Ni-NTA agarose beads (Qiagen). The cells were collected by centrifugation and the cell pellets were lysed in 800 µl Gu-HCl lysis buffer (6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl, 10 mM imidazole, 1 mM β-Mercaptoethanol, pH 8.0). The DNA was sheared by sonification and lysates were cleared by centrifugation (10 min, 16000 × g). The supernatant was mixed with 40 µl of equilibrated Ni-NTA agarose, followed by incubation for at least 4 h at room temperature. Subsequently, the beads were washed once in Gu-HCl lysis buffer and then in consecutive steps once in Urea wash buffer 1 (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl, 10 mM imidazole, 1 mM β-Mercaptoethanol, pH 8.0) and twice in Urea wash buffer 2 (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl, 10 mM imidazole, 1 mM β-Mercaptoethanol, 0.1 % Triton-X100, pH 6.3). The bound proteins were eluted by boiling the beads for 5 min in 50 µl 2× SDS sample buffer containing 200 mM imidazole. The purified His-SUMO-modified proteins were further analyzed by immunoblotting.

**GST-pull down experiments**

Recombinant GST fusion proteins for *in vitro* interaction studies were produced in the *E. coli* strain BL21(DE) and purified on GSH-coupled beads by standard methods. Proteins potentially interacting with any of the recombinant GST fusion proteins were expressed in HEK293T cells. After NP-40 lysis of the transfected HEK293T cells, each sample was split into aliquots which were used for incubation with 5 µg of GST or the GST fusion proteins, respectively. After addition of 50 µl GSH sepharose and incubation on a rotating device for at least 4 h at 4°C, beads were washed four times in NP-40 lysis buffer. Bound proteins were eluted by the addition of 50 µl 2× SDS sample buffer and subsequent boiling for 5 min. Proteins associated with GST fusion proteins were analyzed by Western blotting, while the recombinant GST proteins were controlled by SDS-PAGE and Coomassie staining.
Real-time quantitative PCR

Cells were pelleted and immediately lysed in RNA lysis buffer RLT (Qiagen). Cell lysis and total RNA extraction was performed using the RNeasy mini kit as recommended by the manufacturer (Qiagen). After confirming the integrity of RNA by agarose gel electrophoresis, 1 µg of RNA was used for cDNA synthesis using Oligo (dT) 20 primers and the Superscript II first strand synthesis system (Invitrogen). Real-time PCR was performed using specific primers (PIASy-f TCTCGACCTTCAGATGCTC, PIASy-r TCAGGGCTACAGTCAAACG) using the absolute SYBR Green ROX Mix (Thermo Scientific) and analyzed using an Applied Biosystems 7300 real time PCR system. Experiments were done in triplicate, data were normalized to the housekeeping gene β-actin and the relative abundance of transcripts was calculated by the comparative ΔΔ CT method.

Supplementary Literature


**Supplementary Figures**

**Supplementary figure 1.** Myc-TANK was expressed together with GFP-SUMO-1 and wildtype or inactive mutants of Flag-IKKε as shown. SUMOylation of Myc-TANK was examined by immunoblot analysis of 1× SDS lysates, while TANK phosphorylation was revealed after lysis in NP-40 buffer.

**Supplementary figure 2.** TBK1 and His-SUMO-1 were expressed either with TANK or a phosphorylation-deficient TANK mutant. Phosphorylation and SUMOylation of the TANK proteins was revealed with specific antibodies as shown.

**Supplementary figure 3.** The TANK sequence harboring the SUMO attachment site was compared between the indicated species. Identical amino acids are marked by stars, homologous and related amino acids are indicated. The lower part shows the SUMO consensus sequence where Ψ stands for a large, uncharged amino acid and x for any amino acid.
**Supplementary figure 4.** Cells were transfected as shown and lysed, followed by analysis of TANK SUMOylation by Western blotting with specific antibodies.

**Supplementary figure 5.** Cells were transfected to express a TANK-specific shRNA (shTANK) or an adequate control (shScramble) along with vectors encoding the indicated TANK proteins or mutated versions thereof (Flag-TANK-mut or Flag-TANK-K282R-mut) that were codon-exchanged to become shRNA resistant. Cell lysates were analyzed for TANK expression and comparable loading as shown.

**Supplementary figure 6.** Cells transfected to express His-SUMO-2 and Flag-TANK were grown in an incubator at 37°C or exposed for 30 min to 43°C. The levels of SUMO-modified TANK were analyzed after enrichment of SUMOylated proteins on Ni-NTA beads, while 1× SDS lysates were used to control protein expression levels.
Supplementary figure 7. Cells transfected with a PIASy-specific siRNA were re-transfected to express Myc-PIASy for one day. Equal amounts of proteins contained in cell lysates were tested for expression of PIASy and tubulin as shown.