Supporting Information

10.1073/pnas.0908486107

SI Materials and Methods

Yeast Interaction Trap System, Interaction Site Mapping. The HeLa cDNA expression library was purchased from Takara. The LexA DNA binding domain fusions (bait) were constructed in the vector plex202 (1), the activation domain constructs (prey) in the vector pB42AD (Takara). The coding region of murine nSMase2 (amino acids 1–655) as well as fragments coding for amino acids 1 to 317 (containing the N terminus with the two membrane-anchoring regions plus the collagenous domain, nSMase2ND) or 318 to 655 (containing the C-terminal catalytic domain, nSMase2CD; see refs. 2 and 3) were amplified by PCR and subcloned into both plex202 (EcoRI/SalI) and pB42AD (EcoRI/XhoI) vectors. By the same strategy, deletion constructs for human EED were generated that lacked the individual WD repeats I (EEDΔI, encompassing amino acids 75–441), I and II, (EEDΔI-II, 184–441), I to III, (EEDΔI-III, 234–441), IV to VII (EEDΔIV-VII, 2–233), V to VII, (EEDΔV-VII, 2–293), VI and VII (EEDΔVI-VII, 2–353), and VII (EEDΔVII, 2–404). Clones containing the coding region of human EED (2-441) in pB42AD were isolated from the HeLa cDNA expression library and subcloned into plex202 (EcoRI/SalI). Bait and prey constructs for full-length RACK1 ($RACK1_{1–317}$), $RACK1_{144–317}$, $RACK1_{198–317}$ and $RACK1_{204–317}$ have been described previously (4). $RACK1_{1–278}$ lacking the C-terminal WD repeat VII was generated by PCR as outlined above and cloned into plex202. All constructs were verified by sequencing. Transformation of the yeast strain EG48/JK103 (5) with bait constructs and, subsequently, with the library DNA or selected activation domain fusion constructs was performed as described (6). Transformants were grown on Ura^- His^- Trp^- glucose plates, before selection for leucine prototrophy and β-galactosidase expression on Ura^- His^- Trp^- Leu^- galactose X-gal plates was used to test for positive interaction. In addition, controls for nonspecific transactivation of bait constructs, nonspecific interaction of prey constructs with unrelated proteins (the protein kinase SYK) (1) or with empty bait vector plex202, as well as for nonspecific β-galactosidase expression in bait/prey-transfectants on Ura^- His^- Trp^- X-gal glucose plates were all negative.

Cell Culture. Jurkat (human leukemic T), 293 (human embryonic kidney), HeLa (human cervix carcinoma), COS-7 (African green monkey kidney), and NIH 3T3 (murine fibroblast) cells were originally obtained from the American Type Culture Collection. The murine pre-B cell lines 70Z/3TR55Δ212–308/346 and 70Z/3TR55Δ308–340 have been described previously (7). COS-7, 293, and NIH 3T3 cells were grown in high glucose DMEM, Jurkat and 70Z/3 cells in a mixture of Click's/RPMI 1640 (50/50% vol/vol), and HeLa cells in RPMI 1640, all supplemented with 10% (vol/vol) FCS, 2 mM glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin (Invitrogen) in a humidified incubator at 5% (wt/vol) CO_2. Activated primary human T cells were isolated from buffy coats of healthy donors as previously described (8) The scientific use of buffy coat cells was approved by the Ethics Commission of the Medical Faculty of the Christian-Albrechts-University, Kiel.

Intracellular Colocalization Studies. The coding region of EED was amplified by PCR and ligated into the HindIII/EcoRI sites of the eukaryotic expression vector pFLAG-CMV2 (Sigma-Aldrich) to generate the plasmid $pFLAG.EED_{2–441}$. By the same approach, the plasmid $pMYC.nSMase2_{1–655}$ was constructed, containing full-length nSMase2 N-terminally fused to the myc tag of pcDNA3.1 (−)/Myc-His A (via EcoRI/HindIII; Invitrogen). Both constructs were sequenced. Next, 1.5×10^6 293 cells were transiently transfected with 5 μg of either construct by the calcium phosphate precipitation method and grown to subconfluency on coverslips for 48 h before fixation in 2.5% (wt/vol) paraformaldehyde in PBS for 30 min at room temperature. The coverslips were washed three times in PBS for 10 min to remove the fixative followed by permeabilization and blocking of nonspecific sites with 0.05% (wt/vol) Saponin and 0.1% (wt/vol) BSA in PBS containing 0.1% (wt/vol) glycine for 1 h at room temperature and three washes in PBS for 5 min each. Cells were incubated with primary rabbit anti-myc polyclonal antibody (1:100; sc-789, Santa Cruz) or mouse anti-FLAG M2 monoclonal IgG1 antibody (1:100; Sigma-Aldrich) in PBS for 1 h at 37°C. After washing three times in PBS (5, 10, and 15 min), cells were incubated with rhodamine-conjugated donkey anti-rabbit or goat anti-mouse secondary antibody (1:100; Dianova) for 1 h in PBS at 37°C. For staining of nuclei, DAPI (Sigma-Aldrich, 1 μg/mL) was added and the cells were further incubated for 30 min at 37°C. Cells were again washed three times in PBS (5, 10, and 15 min) before stained proteins were visualized using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss). Jurkat cells were left untransfected or transfected with $pFLAG.EED_{2–441}$ alone or in combination with $pMYC.nSMase2_{1–655}$ by Amaxa nucleofection (Lonza), using solution V and program C-16, and incubated for 48 h at 37°C. Cells were stimulated with hTNF (100 ng/mL, BASF Bioresearch) as indicated, washed with ice-cold PBS containing 0.1% (wt/vol) sodium azide, and fixed in 1 mL of 3% (wt/vol) paraformaldehyde in PBS for 15 min on ice. Subsequently, the cells were washed two more times with ice-cold PBS, resuspended in PBS containing 1% (wt/vol) BSA, and cytofuged onto glass slides. After drying for 1 h, the cells were permeabilized by addition of ethanol/acetone (1:1) for 5 min at −20°C. After three washes for 10 min each in PBS, the glass slides were preincubated in incubation solution [0.2% (wt/vol) BSA in PBS] for 1 h and incubated with primary mouse anti-FLAG monoclonal IgG1 antibody, rabbit anti-myc polyclonal antibody, rabbit anti-EED polyclonal antibody (ab4469, Abcam), goat anti-nSMase2 polyclonal antibody (sc-67692, Santa Cruz), or mouse anti-RACK1 IgG2a monoclonal antibody (sc-17754, Santa Cruz; all 1:100 in incubation solution, overnight, 4°C). The next day, the glass slides were washed three times with PBS for 10 min and incubated with Alexa Fluor 488- or 555-conjugated goat anti-mouse, donkey anti-goat or donkey anti-rabbit antibodies (all from Invitrogen, 1:500 in incubation solution, 1 h, 37°C). Parallel control reactions were performed using secondary antibody alone to exclude nonspecific reactivity. After addition of DAPI (1 μg/mL) and further incubation for 5 min at 37°C to stain the nuclei, the glass slides were washed again three times with PBS for 10 min each and analyzed by confocal laser scanning microscopy. Staining for endogenous proteins in 293, COS-7, NIH 3T3, HeLa, activated primary human T cells, and 70Z/3 pre-B cells was carried out as above, using antibodies specific for endogenous EED (ab4469, Abcam, or sc-28701, Santa Cruz), nSMase2 (sc-67305, Santa Cruz) or RACK1 (610177, BD Biosciences). For generation of $H\text{-}2K^K$-EED, the extracellular and transmembrane region of the mouse MHC class I molecule $H\text{-}2K^k$ was amplified by PCR from pMACS K^k.II (Miltenyi) and inserted in frame between the FLAG-tag and the EED coding region of $pFLAG.EED_{2–441}$. By the same strategy, EEDΔI–III and EEDΔIV–VII were inserted into pFLAG-CMV2. All constructs were verified by sequencing. Transfection of the constructs into Jurkat cells and confocal immunofluorescence analysis was carried out as above, using a FITC-conjugated mouse IgG2a anti-$H\text{-}2K^k$ monoclonal antibody (Miltenyi), or anti-FLAG monoclonal antibody to detect the constructs. Final digital images were processed using Adobe Photoshop CS (Adobe Systems), with identical settings applied to all images from one experiment. For each experiment, representative cells are shown out of at least 200 inspected cells per sample.

Coimmunoprecipitations. The plasmid pMYC.nSMase2$_{318–655}$ containing myc-tagged nSMase2CD was constructed by PCR as outlined above for full-length nSMase2 and verified by sequencing. 293 cells were transiently transfected with pMYC.nSMase2$_{1–655}$, pMYC.nSMase2$_{318–655}$ or pcDNA3.1(−)/Myc-His A in combination with pFLAG.EED$_{2–441}$ or pFLAG.CMV2. After 24 h, cells were treated with 100 ng/mL hTNF as indicated and harvested in lysis buffer [50 mM hepes pH 7.2, 250 mM NaCl, 10% (vol/vol) glycerol, 2 mM EDTA, 0.1% (vol/vol) Nonidet P-40, Complete protease inhibitor mixture (Roche)]. Cellular lysates were precleared with GammaBind G-Sepharose (GE Healthcare) and immunoprecipitation was performed over night on ice using 1 μg of anti-myc IgG1 monoclonal antibody (Invitrogen) or 1 μg of anti-FLAG IgG1 monoclonal antibody. After collection of the immunecomplexes with GammaBind G-Sepharose and four washing steps in lysis buffer (the second step containing 1M NaCl), the immunoprecipitated proteins were separated on 10% (wt/vol) gels by SDS/PAGE. For detection of expressed tagged nSMase2, nSMase2CD and EED protein in total cell lysates, 20 μg of cell protein per lane were resolved by SDS/PAGE. After electrophoretic transfer to nitrocellulose (Whatman-Biometra), reactive proteins were visualized using monoclonal anti-myc IgG1 or anti-FLAG antibody and the ECL detection kit (GE Healthcare). For detection of EED coimmunoprecipitating with full-length nSMase2, mouse TrueBlot horseradish peroxidase anti-mouse IgG (eBioscience) was used as secondary antibody to reduce interfering signals of comigrating Ig heavy chains. Alternatively (for EED and nSMase2CD), the immunoprecipitation was performed with rabbit anti-myc polyclonal antibody to reduce Ig heavy chain interference. For coimmunoprecipitation of endogenous proteins, lysates were prepared as above from 1×10^8 Jurkat or HeLa cells stimulated with 100 ng/mL hTNF as indicated. EED or nSMase2 were immunoprecipitated from 1 mg of protein with 2 μg mouse monoclonal anti-EED antibody 05–1320 (Millipore) or 4 μg rabbit anti-nSMase2 polyclonal antibody sc-67305 using μMACS protein G MicroBeads according to the instructions of the manufacturer (Miltenyi). Coimmunoprecipitating EED, nSMase2 or RACK1 were subsequently detected by Western blot with anti-EED antibody 05–1320, rabbit anti-nSMase2 polyclonal antibody, or anti-RACK1 antibody 610177. Immunoprecipitated nSMase2 or EED were visualized with rabbit anti-nSMase2 polyclonal antibody or anti-EED antibody 05–1320. Equal loading as well as efficiency of transfer was routinely verified for all Western blots by Ponceau S-staining.

Preparation of Membrane, Cytoplasmic, and Nuclear Fractions. Membrane, cytoplasmic and nuclear fractions were generated from Jurkat cells stimulated with 100 ng/mL hTNF for 0 or 3 min using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit or the ProteoJET Membrane Protein Extraction Kit (Fermentas) according to the manufacturer's instructions. Endogenous EED, actin, tubulin, pan-cadherin, and histone were detected with mouse monoclonal antibody 05–1320 (Millipore), goat polyclonal antibody sc-1615 (Santa Cruz), mouse monoclonal antibody ab7291, rabbit polyclonal antibody ab16505, and rabbit polyclonal antibody ab1791 (all from Abcam).

Magnetic Labeling of TNF Receptors, Isolation of TNF Receptor Magnetic Fractions, and Immunoprecipitation of TNF Receptor-Associated Proteins. Cells were incubated in a total volume of 250 μL cold PBS with 100 μL (400 ng) of biotinylated TNF (FluoroKine-Kit, R&D) for 1 h on ice. Thereafter, 200 μL of 50 nm MACS Streptavidin Microbeads solution (Miltenyi) was added and cells were incubated for 1 h on ice. Formation of magnetically labeled TNF receptor complexes was achieved by incubation of cells at 37°C for the indicated times. Cells were subsequently homogenized mechanically using glass beads in a 0.25 M sucrose buffer, supplemented with 15 mM hepes, 0.5 mM $MgCl_2$, pH 7.4 and the Protease Inhibitors Set (Roche) at 4°C. A postnuclear supernatant was submitted to separation of TNF receptor magnetic fractions in a high-gradient magnetic field generated in a custom-built free-flow magnetic chamber (S. Schütze, German patent No. DE 101 44 291). Magnetic fractions were analyzed by SDS/PAGE and Western blotting using antibodies against TNF-R1 (mouse monoclonal IgG2b antibody sc-8436, Santa Cruz), RACK1 (610177), EED (ab4469), and nSMase2 (sc-67305). For immunoprecipitation of TNF receptor-associated proteins, control and TNF receptor magnetic fractions were pelleted by centrifugation for 1 h at 20,000 × *g*. Next, 20 μl of each pellet were lysed in 200 μl of lysis buffer [50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 0.25% (wt/vol) Na-deoxycholate, 1% (vol/vol) Triton X-100, 1 mM EDTA and protease inhibitors] for 1 h on ice. The microbeads-coupled immunoprecipitates were pelleted and washed twice with 1 mL of homogenization buffer as above, then resuspended in 100 μl of homogenization buffer and analyzed by Western blotting for the presence of TNF-R1, RACK1, EED, and nSMase2.

RNA Interference, Measurement of nSMase2 Activity. The predesigned siRNAs EED_1, -_2, and -_3 specific for human EED (ID # 115460, 115461, and 115462) as well as the negative control siRNA were obtained from Applied Biosystems. The efficiency of the siRNAs was evaluated by transfection of 293 cells with pFLAG.EED$_{2–441}$ for 24 h followed by retransfection with 150 pmol of siRNA with X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's instructions. Forty hours later, down-regulation of EED was assessed by Western blot using anti-FLAG monoclonal antibody and densitometric quantification. To determine the impact of EED down-regulation on the activation of nSMase2 by TNF, Jurkat cells were nucleofected with 150 pmol siRNA, as outlined above, and subsequently cultured for 40 h. Next, cells were analyzed for expression of endogenous EED by immunofluorescence (ab4469), as outlined above. In parallel, cells were stimulated in triplicates with 100 ng/mL hTNF for the indicated times and homogenized in 20 mM Hepes, 10 mM $MgCl_2$, 5 mM DTT, and 0.2% (vol/vol) Nonidet P-40 pH 7.4, including 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, and 750 μM ATP. The activity of nSMase2 in the lysates was measured by incubating 20 μg of protein for 2 h at 37°C in the presence of *N*-methyl-[^{14}C]-sphingomyelin (GE Healthcare, 0.2 μCi/mL in 20 mM Hepes, 10 mM $MgCl_2$, pH 7.4). Radioactive phosphocholine was extracted using chloroform/methanol (2:1) and routinely determined in the aqueous phase by scintillation counting.

1. Adam-Klages S, et al. (1996) FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. *Cell* 86:937–947.
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8. Paulsen M, et al. (2008) Interaction with XIAP prevents full caspase-3/-7 activation in proliferating human T lymphocytes. *Eur J Immunol* 38:1979–1987.

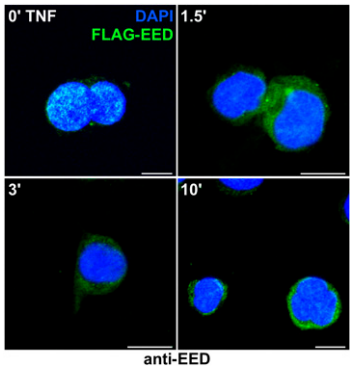

Fig. S1. Translocation of FLAG-EED in response to TNF. Jurkat cells were analyzed as outlined for Fig. 2*B*, except that antibody ab4469 specific for EED was used to detect transfected pFLAG.EED$_{2–441}$ (*green*, anti-EED). Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.)

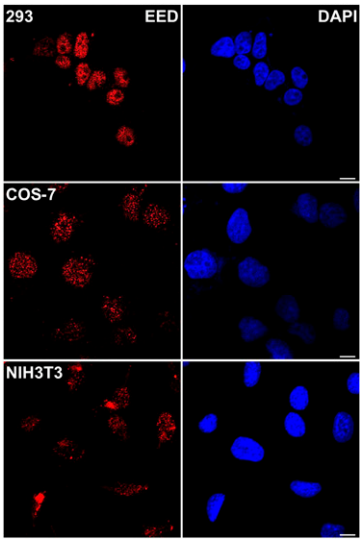

Fig. S2. Nuclear localization of endogenous EED. The intracellular localization of EED was determined in 293, COS-7, and NIH 3T3 cells by staining for endogenous EED (ab4469, *red*). In the same experiment, nuclei of cells were stained with DAPI (*blue*). (Scale bars, 10 μm.)

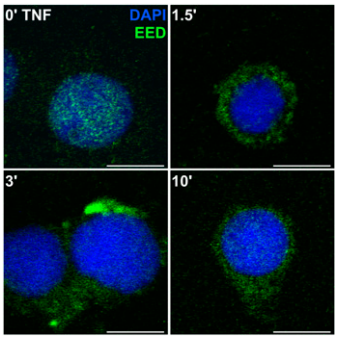

Fig. S3. TNF-induced translocation of endogenous EED. Analysis of Jurkat cells was performed as outlined for Fig. 3*A*, except that a different rabbit polyclonal antibody specific for EED (sc-28701) was used to detect the endogenous EED protein (*green*). Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.)

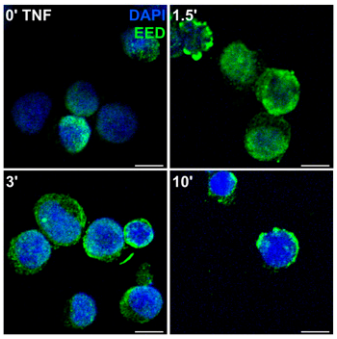

Fig. S4. TNF induces translocation of endogenous EED in vivo in activated primary human T cells. The localization of endogenous EED (*green*) was detected in activated primary human T cells after treatment with TNF for the indicated times with anti-EED antibody ab4469. Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.)

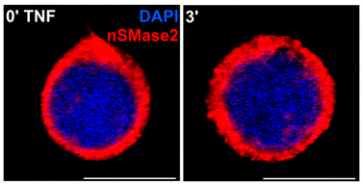

Fig. S5. Localization of endogenous nSMase2 in the cytoplasm and at the plasma membrane. Endogenous nSMase2 (*red*) was detected in Jurkat cells treated with TNF for 0 and 3 min as in Fig. 3*B*, but using the distinct rabbit polyclonal antibody sc-67305. Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.)

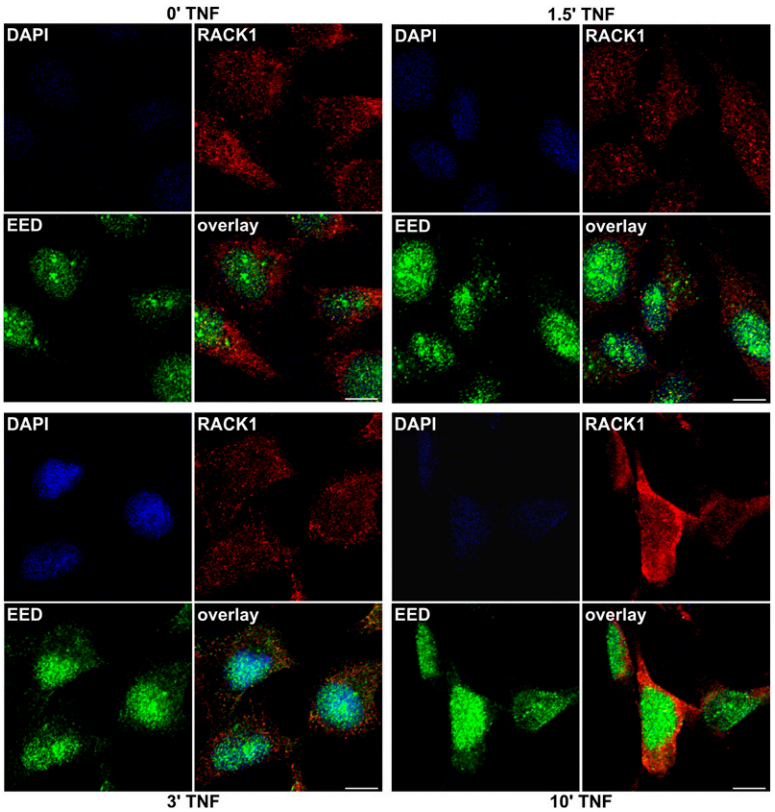

Fig. S6. TNF-induced colocalization of EED and RACK1 in HeLa cells. Untransfected HeLa cells were analyzed for colocalization of EED (*green*) and RACK1 (*red*) by antibodies against the endogenous proteins (ab4469, 610177) after treatment with TNF for the indicated times. Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.) Despite a higher amount of EED retained in the nucleus than in Jurkat cells [Witte V, et al. (2004) HIV-1 Nef mimics an integrin receptor signal that recruits the polycomb group protein Eed to the plasma membrane. *Mol Cell* 13:179–190], the cytoplasmic translocation and colocalization of EED with RACK1 is clearly visible.

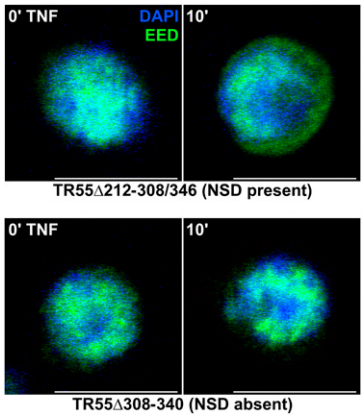

Fig. S7. TNF-R1 mediates translocation of endogenous EED through the NSD but not through the death domain. The localization of endogenous EED (*green*) was detected after treatment with TNF for 0 and 10 min with anti-EED antibody ab4469 in 70Z/3 pre-B cells that express a deletion mutant of TNF-R1 still containing the NSD but unable to signal through the death domain (TR55Δ212–308/346, *Upper*), as well as in 70Z/3 cells which express a TNF-R1 deletion mutant that lacks the NSD (TR55Δ308–340, *Lower*). Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.) Similar to HeLa cells (Fig. S6), 70Z/3 cells retain a higher amount of EED in their nucleus than Jurkat cells. Nevertheless, the cytoplasmic translocation of EED is clearly visible in the upper right panel but not in the lower right panel.

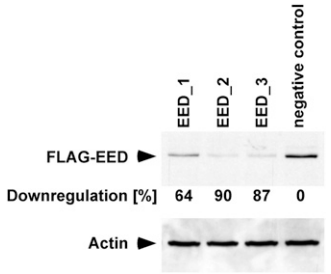

Fig. S8. EED is required for activation of nSMase2 by TNF. 293 cells transiently overexpressing pFLAG.EED$_{2–441}$ were transfected with siRNAs specific for EED (EED_1, EED_2 or EED_3) or with an siRNA that does not elicit an RNA interference response (negative control). The down-regulation of EED (*arrow*) was determined 40 h after transfection by Western blot analysis in combination with densitometric analysis and is indicated relative to the negative control. Equal loading was verified by detection of actin.

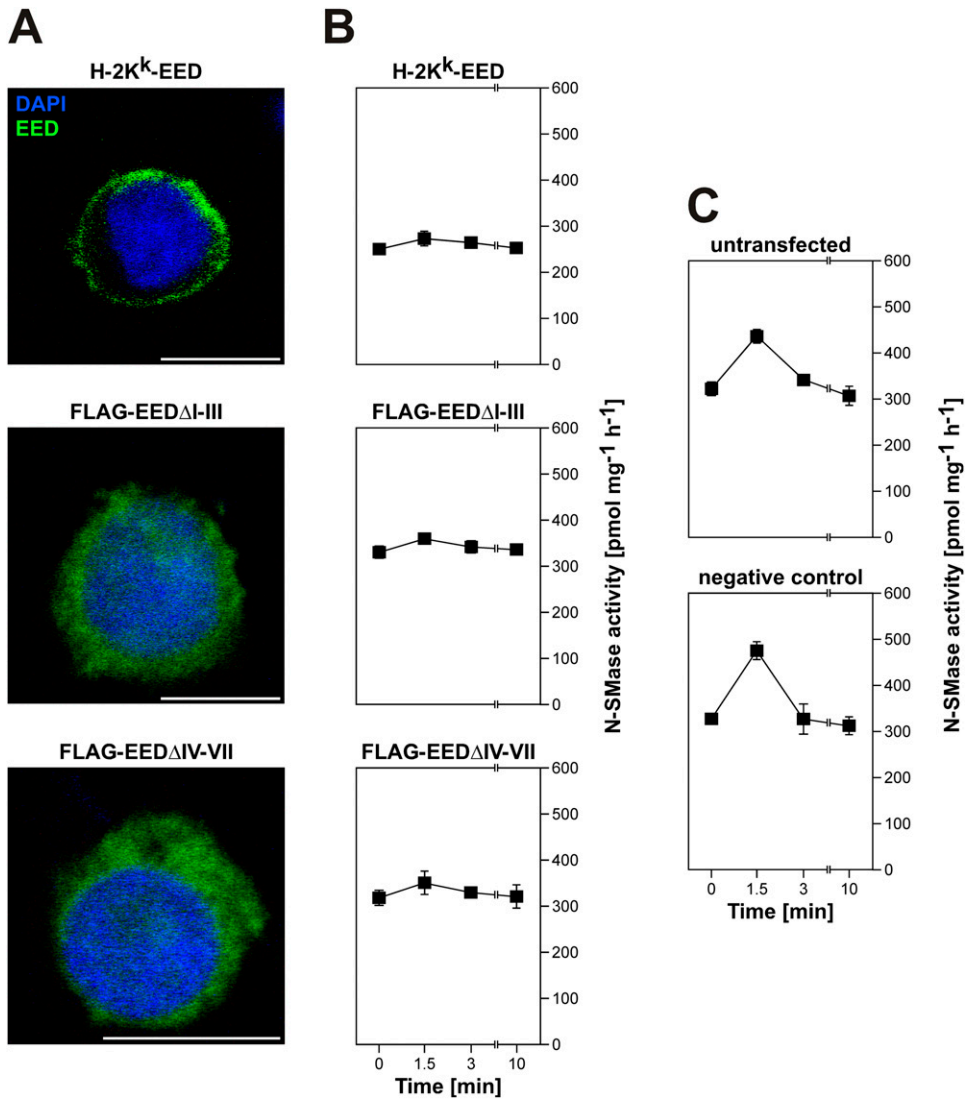

Fig. S9. TNF-dependent activation of nSMase2 requires recruitment of EED to the plasma membrane in the context of the TNF-R1•FAN•RACK1-complex and all domains of EED. (*A*) Jurkat cells were nucleofected with an EED construct constitutively targeted to the plasma membrane (H-2K^k-EED, *Top*), with the EED C-terminal region (FLAG-EEDΔI-III, *Middle*), or with the nSMase2-interaction domain of EED (FLAG-EEDΔIV–VII, *Bottom*). The transfected cells were analyzed for expression of the respective construct with a H-2K^k-specific antibody (H-2K^k-EED) or with a monoclonal anti-FLAG antibody (FLAG-EEDΔI–III, FLAG-EEDΔIV–VII) by confocal laser scanning microscopy (*green*). Cell nuclei were stained with DAPI (*blue*). (Scale bars, 10 μm.) (*B*) Alternatively, cells were treated with TNF for the indicated times before N-SMase activity was measured. (*C*) For control, N-SMase activity was additionally measured in untransfected and in Jurkat cells transfected with empty vector only (pFLAG-CMV2; negative control). The values shown represent the means from triplicate determinations performed in parallel; error bars indicate the respective standard deviations. One out of two independent experiments with similar results is shown.

Table S1. Mapping of the interaction sites of nSMase2 (bait) and EED (prey)

	Prey							
Bait	EED	EEDΔI	EEDΔI-II	EEDΔI-III	EEDΔIV-VII	EEDΔV-VII	EEDΔVI-VII	EEDΔVII
nSMase2CD	+++	++	–	–	++	+++	+++	+++
nSMase2ND	–	–	–	–	–	–	–	–
nSMase2	–	–	–	–	–	–	–	–

Strength of interaction: very strong (+++), strong (++), weak (+), and not detectable (–).

Table S2. Mapping of the interaction sites of EED (bait) and nSMase2 (prey)

	Prey		
Bait	nSMase2CD	nSMase2ND	nSMase2
EED	+	–	+
EEDΔI	–	–	–
EEDΔI-II	–	–	–
EEDΔI-III	–	–	–
EEDΔIV-VII	+++	–	+++
EEDΔV-VII	+++	–	+++
EEDΔVI-VII	+++	–	+++
EEDΔVII	+++	–	+++

Strength of interaction: very strong (+++), strong (++), weak (+), and not detectable (–).

Table S3. Mapping of the interaction sites of RACK1 (bait) and EED (prey)

	Prey							
Bait	EED	EEDΔI	EEDΔI-II	EEDΔI-III	EEDΔIV-VII	EEDΔV-VII	EEDΔVI-VII	EEDΔVII
$RACK1_{1–317}$	+++	–	–	–	+	+	+++	+++
$RACK1_{144–317}$	+++	–	–	–	+++	+++	+++	+++
$RACK1_{198–317}$	–	–	–	–	+	++	+++	+++
$RACK1_{204–317}$	–	–	–	–	+	++	++	++
$RACK1_{1–278}$	–	–	–	–	–	–	–	–

Strength of interaction: very strong (+++), strong (++), weak (+), and not detectable (–).

Table S4. Mapping of the interaction sites of EED (bait) and RACK1 (prey)

	Prey			
Bait	$RACK1_{1–317}$	$RACK1_{144–317}$	$RACK1_{198–317}$	$RACK1_{204–317}$
EED	–	–	–	–
EEDΔI	–	–	–	–
EEDΔI-II	–	–	–	–
EEDΔI-III	–	–	–	–
EEDΔIV-VII	++	+++	+++	++
EEDΔV-VII	++	+++	+++	++
EEDΔVI-VII	++	+++	+++	++
EEDΔVII	++	+++	+++	++

Strength of interaction: very strong (+++), strong (++), weak (+), and not detectable (–).