Supplementary Information for Manuscript

Caspase-8 and caspase-7 sequentially mediate proteolytic activation of acid sphingomyelinase in TNF-R1-receptosomes

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Supplementary Materials and Methods

Antibodies

Rabbit polyclonal anti-A-SMase antibody was generated by Areta International s.r.l. (Gerenzano, Italy). Rabbit polyclonal anti-caspase-3 antibody, rabbit monoclonal anti-cleaved caspase-7 antibody, and rabbit polyclonal anti-cleaved caspase-3 antibody were obtained from Cell Signaling (Danvers). Rabbit monoclonal anti-caspase-7 antibody E22 and mouse monoclonal anti-caspase-7 antibody 7CSP03 were from abcam (Cambridge, UK). Mouse monoclonal anti-caspase-8 antibody 4H46, mouse monoclonal anti-TNF receptor 1 antibody H5 and rabbit polyclonal anti-Rab5B antibody (A-20) were from Santa Cruz Biotechnology (Santa Cruz). Mouse monoclonal anti-cleaved caspase-8 antibody was from Calbiochem (Bad Soden, Germany). Anti-caspase-8 antibody C15 (Scaffidi et al., 1997) was a gift from Marcus E. Peter (University of Chicago, Illinois, USA). Mouse monolclonal anti-Vti1b antibody was from BD Transduction Laboratories and rat monoclonal anti-mouse Cathepsin D antibody (clon 204712) was from R&D Systems. HRP conjugated anti-GFP antibody was from Miltenyi (Bergisch-Gladbach, Germany). Rat monoclonal anti-HA antibody 3F10 was from Roche (Mannheim, Germany). HRP conjugated rabbit anti-mouse antibody and HRP conjugated goat anti-rabbit antibody were from Dianova (Hamburg, Germany). Rabbit polyclonal anti-GFP antibody, Alexa Fluor 546 labelled goat anti-rat IgG, Alexa Fluor 488 labelled donkey anti-mouse IgG, Alexa Fluor 555 labelled goat anti-mouse IgG, and Alexa Fluor 555 labelled goat anti-rabbit IgG antibodies were from Invitrogen/Molecular Probes (Karlsruhe, Germany).

Cell lines

Mouse embryonic fibroblasts derived from Cathepsin D deficient mice were obtained from Paul Saftig, Universtiy of Kiel, Germany (Saftig et al., 1995).

Cell culture

Jurkat cells carrying a mutated and dysfunctional gene for caspase-8 were kindly provided by John Blenis (Harvard Medical School, Boston). Immortalized MEF cells (wild type and caspase-7 knock-out) were kindly provided by Richard Flavell (Yale School of Medicine, New Haven). HeLa and MEF cells were maintained in DMEM + HEPES culture medium (Invitrogen) and Jurkat cells were maintained in RPMI 1640 medium. Both media were supplemented with 10% fetal calf serum, 10 mM glutamine, and 0.1 mg/ml gentamycin.

Preparation of cell lysates

Adherent cells that had been scraped from the culture flask with a rubber policeman as well as suspension cells were collected by centrifugation at 1.500 x g and washed once with PBS. 1 x 10^7 cells were resuspended in ice cold medium and rested on ice for 60 minutes. The cells were stimulated with 100 ng/ml TNF and incubated at 37°C for diverse time points (0-60 min). The stimulation was stopped by rapidly cooling down the cells in a methanol/dry ice mixture. The samples were centrifuged for 5 min at 1500 x g and the cell pellet was resuspended in 250 mM Na-acetat pH 5.0; 1 mM EDTA, 0.1 % Triton X-100 and homogenized using a syringe and a needle with a 27 gauge. The cell suspension was centrifuged at 4 °C for 10 min at 20,000 x g and protein concentration was measured using a BCA assay kit (Pierce) according to the manufacturers instructions.

Molecular cloning and transfection of cell lines

Stable retransfection of caspase-8 into caspase-8-deficient Jurkat C8- cells was performed by retroviral transduction of full length caspase-8 or empty vector as control construct into Jurkat C8- cells as described previously (Ungefroren et al., 2003). For the transfection of HeLa cells with EGFP-tagged A-SMase an expression vector based on pEGFP-N3 (Clontech) was obtained from H. Kashkar (University of Cologne, Germany), in which the coding sequence for human acid sphingomyelinase (nucleotides 175-2067 from Genbank accession no: NM_000543) was inserted between the EcoR I and Sal I site of the vector. The A-SMase coding sequence in this construct contained an in frame deletion of 18 nucleotides (298-315) leading to a deletion of a ALALAL (amino acids 41-47) hexapeptide from the repetitive region at the C-terminal end of the signal peptide. Two further mutations in the A-SMase coding sequence, namely a C/A exchange at position 1054 leading to a Q294K mutation and a C/T exchange at position 1145 (as in Genbank accession number X52678) leading to a T324I mutation, were removed using Quikchange site-directed mutagenesis kit (Stratagene) and appropriate primers (see Supplementary Table 1). In the same way mutations of the aspartic acid residues at positions 220, 222, 225, and 253 were introduced. A-SMase coding sequences of resulting plasmids were confirmed by dideoxynucleotide sequencing using BigDye 3.1 (Applied Biosystems) sequencing kits and an ABI 310 capillary sequencer (Applied Biosystems).

A double hemaglutinin-tag (RPYPYDVPDYAGYPYDVPDYA) was added to the C-terminus of the A-SMase coding region by insertion of a stretch of double stranded DNA composed of four pre-annealed 5'-phosphorylated overlapping oligonucleotides (see Supplementary Table 1) between the Sal I and Sac II site of the A-SMase-EGFP expression vector. $2x10^6$ HeLa cells in 200µl RPMI 1640 medium were transfected with 20 µg of vector DNA (Qiagen Maxiprep kit) of these plasmids using electroporation with a BioRad Genepulser II with RF-

module and the following parameters: 5 pulses of 240 V and 5 ms duration at a frequency of 40 Hz with 50% modulation and an interval time of 1 s.

Caspase-7 knock-down

For caspase-7 knock-down, a set of three siRNA's (invitrogen, see Supplemental Table 2) was used to transfect HeLa wild-type cells and HeLa cells stably expressing GFP-tagged A-SMase. After cultivation for 96 hours stimulation with TNF as previously described was performed.

Confocal microscopy

For colocalization of TNF-R1 with activated caspase-8, active caspase-7, caspase-3, or Vti1b, respectively, cells were grown on coverslips coated with fibronectin (Beckton Dickinson, Heidelberg) and first incubated with biotinylated-TNF (120 ng/ml) in combination with 240 ng/ml Avidin–FITC (R&D Systems, Wiesbaden, Germany) for 60 min at 4°C. The temperature was shifted to 37°C for the times indicated in the figure legends to allow for receptor internalization. Cells were fixed in paraformaldehyde (4% in PBS) for 20 min, permeabilized in PBS supplemented with 0.1% Saponin, 0.2% BSA, and stained with mouse monoclonal antibodies against the active fragment of caspase-8 (Calbiochem) or Vti1b (BD Transduction Laboratories), rabbit monoclonal antibody against the pro-caspase-7 (abcam), the active fragment of caspase-7 (cell signalling), or the active fragment of caspase-3 (cell signalling), respectively. Primary antibodies were diluted 1:50 and incubated for 1 h at RT followed by secondary antibody Alexa Fluor 555 coupled anti-mouse IgG (Molecular Probes; Invitrogen) or Alexa Fluor 555 coupled anti-rabbit IgG (Molecular Probes; Invitrogen), respectively.

Colocalization of caspase-7 and A-SMase was investigated using HeLa cells transiently transfected with EGFP-tagged A-SMase. The cells were incubated with 100 ng/ml TNF for times indicated and after fixation and permeabilization as above an antibody directed against

pro-caspase-7 or cleaved caspase-7, respectively, diluted 1:50 in PBS was incubated for 1 h at RT followed by Alexa FLuor 555 coupled anti-rabbit IgG secondary antibody.

For colocalization studies of tagged A-SMase and active caspase-8, cells were incubated with 100 ng/ml TNF for times indicated. After fixation and permeabilization, staining of HA-tagged A-SMase in transfected cells was performed using the 3F10 anti-HA rat monoclonal antibody (Roche) at a dilution of 1:50 in PBS for 1 h at RT followed by Alexa Fluor 546 labeled goat anti-rat IgG secondary antibody (invitrogen) for 1 h at RT. Co-staining of the active fragment of caspase-8 was performed using the cleaved caspase-8 specific mouse monoclonal antibody 11G10 (Calbiochem) as primary antibody and an AlexaFluor-488 labeled donkey anti-mouse IgG antibody (Molecular Probes) as described above. Cells were visualized using a Zeiss LSM 510 confocal laser scanning microscope equipped with an Axiovert 100M (Carl Zeiss, Jena, Germany).

Supplementary References

Saftig P, Hetman M, Schmahl W, Weber K, Heine L, Mossmann H, Köster A, Hess B, Evers M, von Figura K, et al. (1995) Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. *EMBO J* **14**: 3599-608

Scaffidi C, Medema JP, Krammer PH, Peter ME (1997) FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. *J Biol Chem* **272**: 26953-26958

Ungefroren H, LenschowW, Chen wb, Faendrich F, Kalthoff H. (2003) Regulation of biglycan gene expression by transforming growth factor-beta requires MKK6-p38 mitogen-activated protein kinase signaling downstream of Smad signaling, *J Biol Chem* **278**: 11041-11049.

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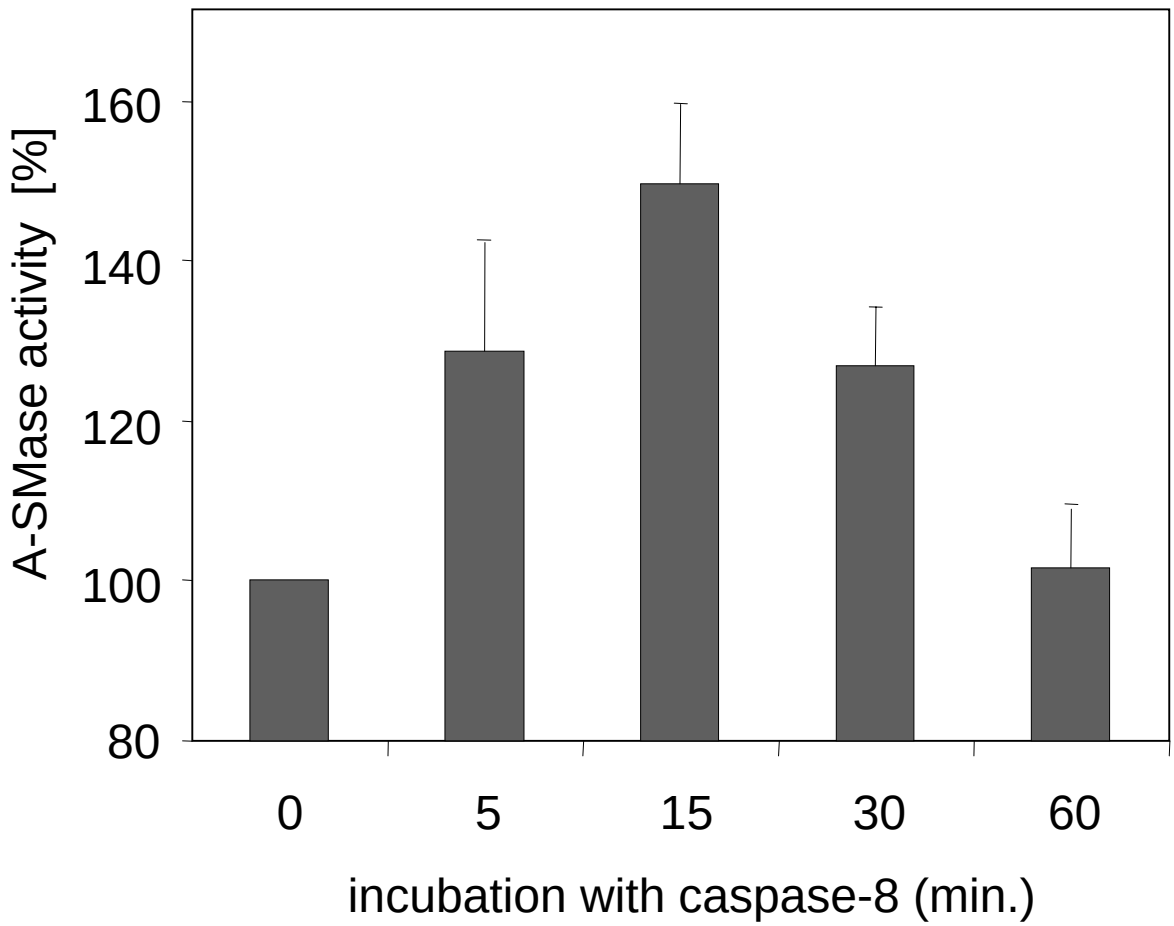
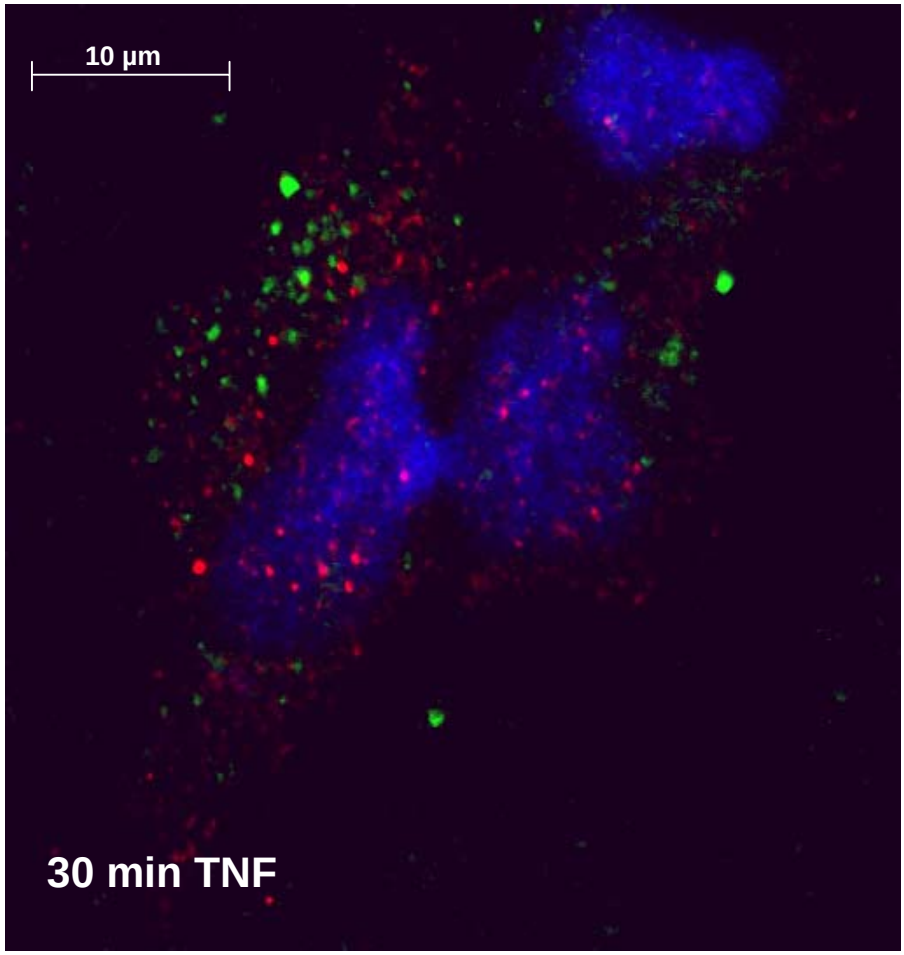
Supplementary Figures and Tables



Activation of endogenous A-SMase by exogenous caspase-8 in lysates from caspase-8- knock-out Jurkat cells.

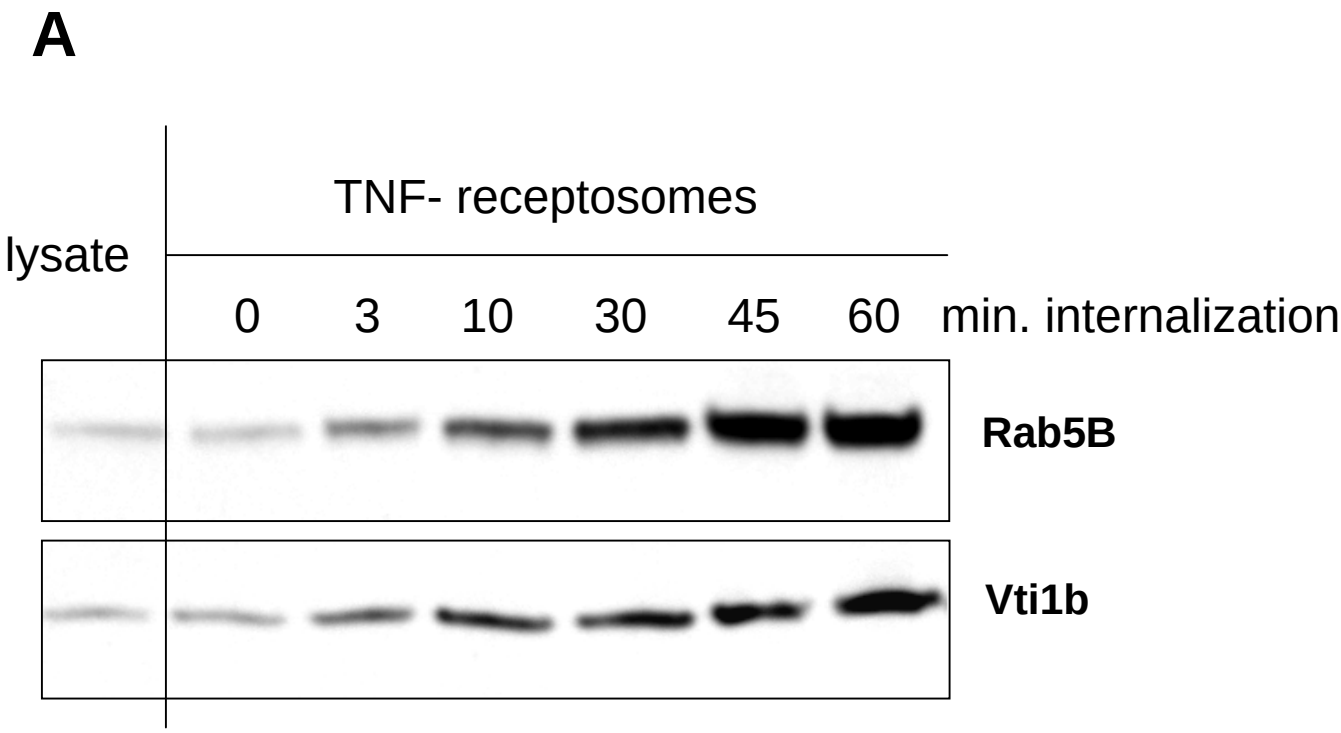
Cell lysates were treated with purified caspase-8 for indicated times and the enzymatic activity of A-SMase was estimated by the mixed micellar A-SMase assay using N-methyl-[^{14}C] sphingomyelin as substrate. Data shown are representative of three experiments (±SEM)

Supplementary Figure 1



Localization of cleaved caspase-3 in TNF-stimulated HeLa cells. Merged confocal laser-scanning microscopic images of HeLa cells fluorescence labeled with biotin-TNF/FITC-avidin complexes (shown in green) and anti-cleaved caspase-3 antibody (shown in red) at 30 min after synchronized temperature shift to 37°C for TNF-receptor internalization. Colocalization of TNF and cleaved caspase-3 could not be observed.

Supplementary Figure 2

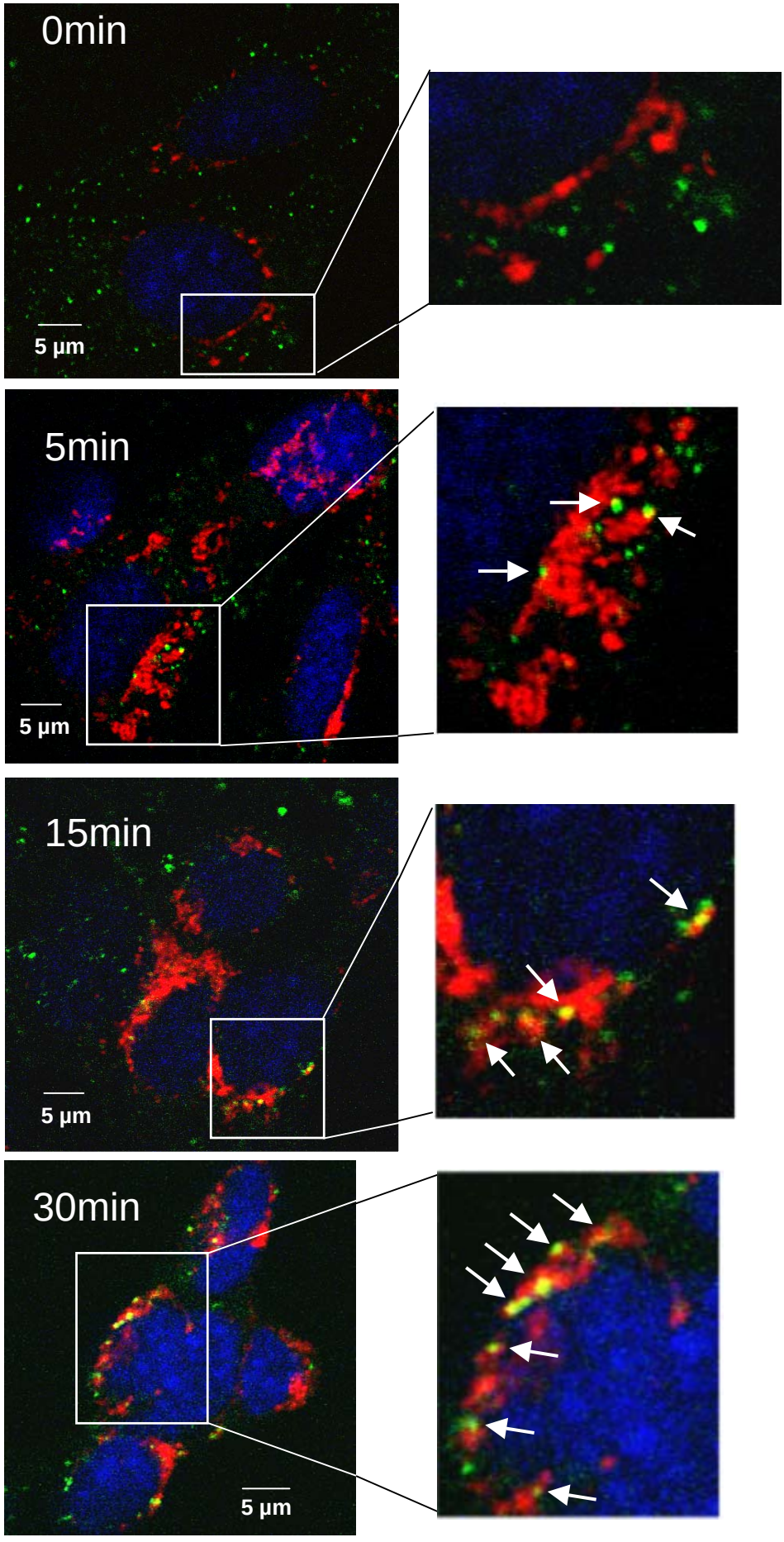


Early kinetics of TNF receptor internalization and fusion of TNF-receptosomes with trans-Golgi vesicles.

(A) TNF-receptosomes were isolated at various time points as described in Materials and Methods and analyzed by Western blotting for recruitment of endosomal marker protein Rab5B and trans-Golgi marker protein Vti1b.

Supplementary Figure 3 A

B

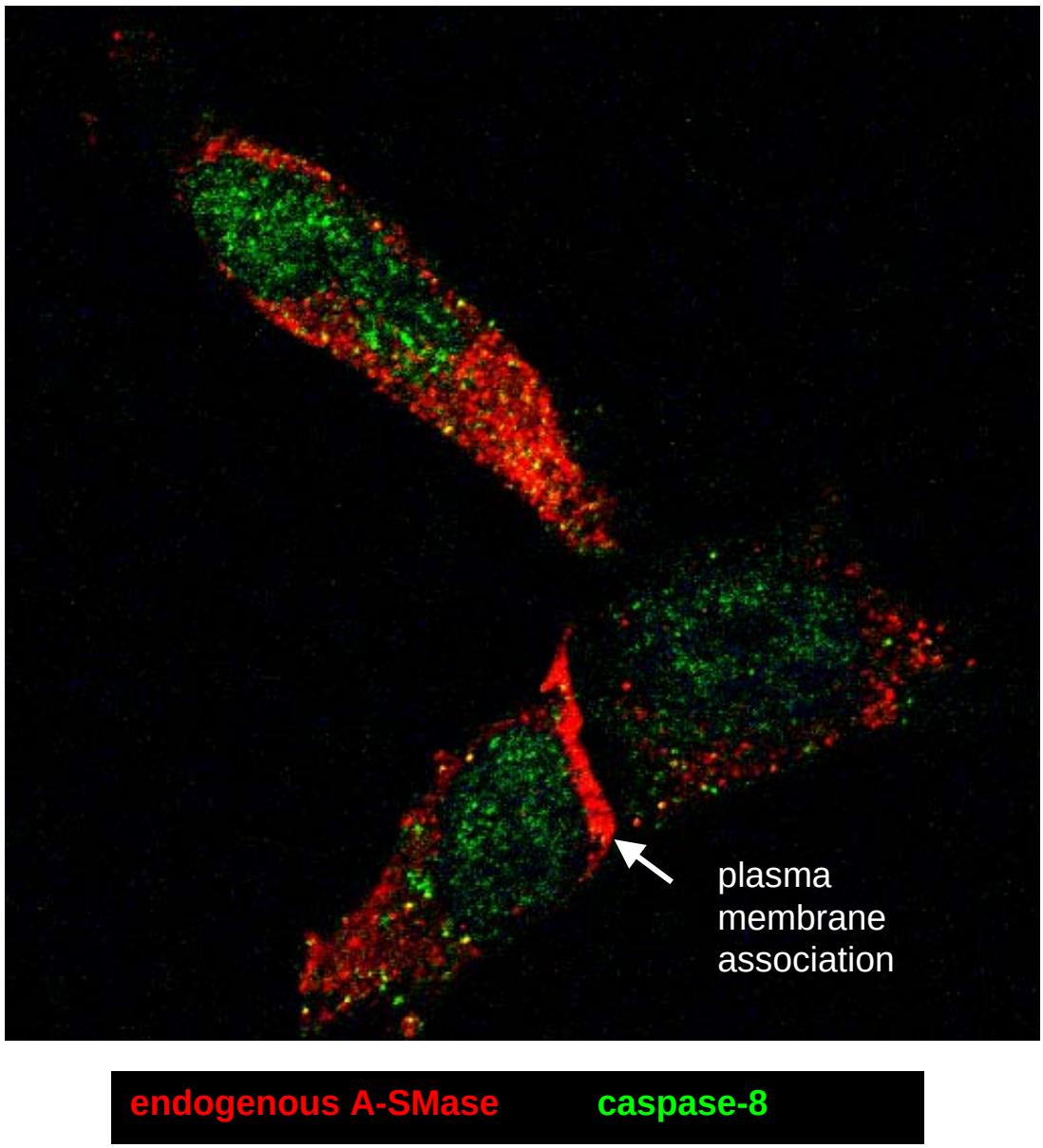


Supplementary Figure 3 B

Supplementary Figure 3 B

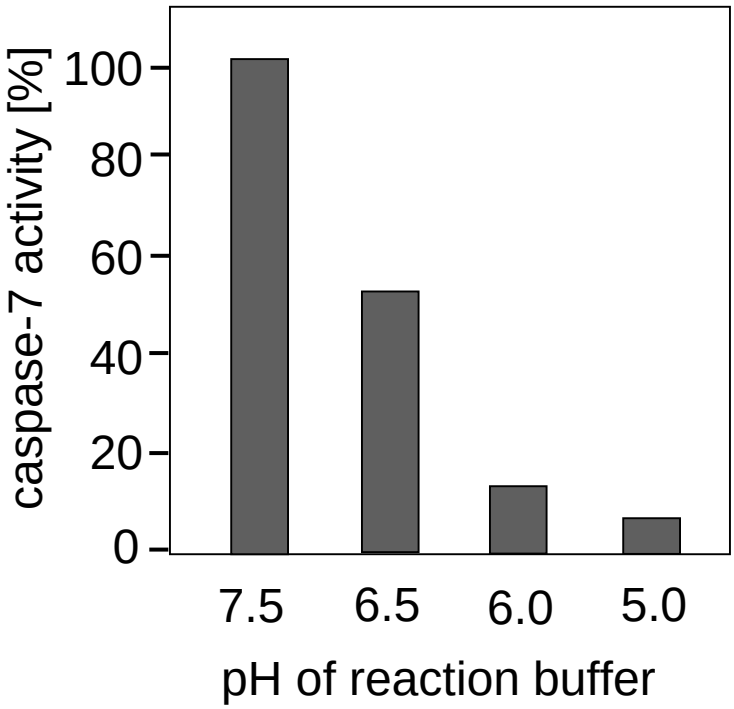
Early kinetics of TNF receptor internalization and fusion of TNF-receptosomes with trans-Golgi vesicles.

(**B**) Murine cathepsin D-knock-out fibroblasts were stimulated with biotin-TNF / streptavidin-FITC for indicated times and analyzed for distribution of internalized TNF / TNF-receptor complexes (green) and trans-Golgi compartments, stained with anti-Vti1b antibodies (red). Colocalization of TNF and Vti1b is indicated by yellow color of superimposed green and red fluorescence (indicated by arrows). Early fusion of TNF receptosomes with trans-Golgi vesicles becomes apparent already at 5 min. after TNF receptor internalization, in line with the immunoblot results shown in (**A**).



Plasma membrane association of endogenous A-SMase in HeLa cells. Wilde-type HeLa cells incubated on ice with TNF (100 ng/ml) for 1 h and immediately fixed in formaldehyde are stained with anti-A-SMase antibody (red) and with anti-caspase-8 antibody (green) as described in Materials and Methods. At the contact area of two cells an association of the endogenous A-SMase with the plasma membrane even without TNF-internalization (0 min) is visible.

Supplementary Figure 4



pH-dependent activity of caspase-7. One unit of exogenous caspase-7 was diluted in buffer containing 50 mM Hepes, 100 mM NaCl, 0.1% CHAPS with pH varying from pH 7.5 to pH 5. Caspase-7 activity was measured using the Apo-ONE® Homogenous caspase assay (Promega) as described in Materials and Methods.

Supplementary Figure 5

Putative caspase-7 cleavage sites within the pro-A-SMase amino acid sequence

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  1 mprygaslrq scprsgreqg qdgtagapgl lwmglvlala lalalalals
 51 dsrvlwapae ahplspqghp arlhrivprl rdvfgwgnlt cpickglfta
101 inlglkkepn varvgsvaik lcnllkiapp avcqsivhlf eddmvevwrr
151 svlspseacg lllgstcghw difsswnisl ptvpkpppkp psppapgapv
201 srilfltdlh wdhdylegtd pdcadplccr rgsglppasr pgagywgeys
251 kcdlplrtle sllsglgpag pfdmvywtgd ipahdvwhqt rqdqlraltt
301 vtalvrkflg pvpvypavgn hestpvnsfp ppfiegnhss rwlyeamaka
351 wepwlpaeal rtlriggfya lspypglrli slnmnfcsre nfwllinstd
401 pagqlqwlvg elqaaedrgd kvhiighipp ghclkswswn yyrivaryen
451 tlaaqffght hvdefevfyd eetlsrplav aflapsatty iglnpgyrvy
501 qidgnysgss hvvldhetyi lnltqanipg aiphwqllyr aretyglpnt
551 lptawhnlvy rmrgdmqlfq tfwflyhkgh ppsepcgtpc rlatlcaqls
601 aradspalcr hlmpdgslpe aqslwprplf c
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Aspartate to alanine exchanges within the suspected caspase-7 cleavage sites

D225 → A225 (wdhdylegtd pdcaddplccr → wdhdylegtd pdcaaplccr)
3D220 → 3A220 (wdhdylegtd pdcaddplccr → wdhdylegta pacaaplccr)
D253 → A253 (pgagywgeys kcdlplrtle → pgagywgeys kcalplrtle)

Supplementary Figure 6

Supplementary Fig. 6

Identification of putative caspase-7 cleavage site in pro-A-SMase. Pro-A-SMase amino acid sequence was screened for putative cleavage sites of caspase-7. The aspartates 220, 222, 225 and 253 were identified as possible cleavage sites that could be responsible for the generation of a fragment with the observed molecular weight. Accordingly to this, three mutants were generated, where one (D225 or D253) or three (D220, D222 and D225) of the suspected aspartates were exchanged to alanine.

Supplementary Table 1

Primer sequences used for site-directed mutagenesis of pro-A-SMase-EGFP constructs

primer name	primer sequence
HA-tag1_for	tcgacaggccttacccatacgacgttccagacta
HA-tag1_rev	ccagcgtagtctggaacgtcgtatgggtaaggcctg
HA-tag2_for	cgctggttacccatacgacgttccagactacgcttgaccgc
HA-tag2_rev	ggtcaagcgtagtctggaacgtcgtatgggtaa
D225A_for	gggcacggaccctgcatgcgcagacccactgtgc
D225A_rev	gcacagtgggtctgcgcatgcagggtccgtgccc
3D220A_for	cctggagggcacggcgcctgcatgcgcagcaccactgtgctgcc
3D220A_rev	ggcagcacagtggtgctgcgcatgcaggcgccgtgccctccagg
D253A_for	acagcaagtgtgcactgcccctgagga
D253A_rev	tcctcaggggcagtgcacacttgctgt
K294Q_for	gactcgtcaggaccaattgcgggccctgac
K294Q_rev	gtcagggcccgcaattggtcctgacgagtc
I324T_for	gtaaccatgaaagtactcctgtcaatagcttc
I324T_rev	gaagctattgacaggagtactttcatggttac

Supplementary Table 2

RNA-Primer sequences used for RNAi of caspase-7

primer name	primer sequence
CASP7HSS101381	GGCCCAUCAAUGACACAGAUGCUAA
CASP7HSS101381	UUAGCAUCUGUGUCAUUGAUGGGCC
CASP7HSS101382	UCCACGGUUCCAGGCUAUUACUCGU
CASP7HSS101382	ACG AGU AAU AGC CUG GAA CCG UGG A
CASP7HSS1188916	CAC CCG GGA CCG AGU GCC UAC AUA U
CASP7HSS1188916	AUA UGU AGG CAC UCG GUC CCG GGU G