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

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while all referees consider the study as interesting in principle referees 1 and 2 raise major concerns regarding the physiological significance as well as the mechanistic depth of the study that preclude publication of the study here at this stage of analysis. More specifically, both referees feel strongly that cleavage of endogenous sphingomyelinase as well as its co-localisation with caspase-7 and -8 needs to be demonstrated. Furthermore, it becomes clear that the functional and physical interaction between caspase-7 and sphingomyelinase as well as its specificity need to be analysed in more depth. Clearly, the referees and editors recognise your argumentation that specific antibodies against human A-SMase are currently not available. Still, in the view of referees 1 and 2 it is indispensable to present data based on endogenous A-SMase protein and thus to generate a suitable antibody if it is not available. I can see that it will certainly be rather time-consuming to generate such an antibody. Also, the outcome of the required further experimentation cannot be predicted at this point. All of this would thus go far beyond the scope and the time frame of a single revision (3 months); and it is our policy to allow a single round of revision only. I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this point.

Given the high interest the study could potentially spark in principle we would, however, be happy to consider a new version of the study should future studies allow you to strengthen the study considerably along the lines suggested by the reviewers and to provide strong and convincing evidence using (and/or generating) an A-SMase-specific antibody. To be completely clear, however,

I would like to stress that if you wish to send a new manuscript this will need to be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh (involving the original referees if possible), also with respect to the literature and the novelty of your findings at the time of resubmission.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript "Caspase-8 and caspase-7 sequentially mediate proteolytic activation of acid sphingomyelinase in TNF-R1-receptosomes" by B. Edelmann et al. investigates mechanisms of acid sphingomyelinase activation. The authors suggest that at least for TNF-receptor stimulation the internalisation of the receptor complexed with caspase-8 and caspase-7 results in a fusion of the endosomes with trans-Golgi vesicles that contain pro-acid sphingomyelinase and cathepsin D. This brings caspase-8 and caspase-7 in contact with pro-acid sphingomyelinase. Caspase 8 cleaves and activates caspase-7, which in turn cleaves and activates pro-acid sphingomyelinase. The data are interesting, but several issues must be addressed.

1. One of the most important problems of the manuscript is the exclusive use of fusion constructs (EGFP and HA) of the acid sphingomyelinase that are transfected into cells. Thus, none of the studies is performed on endogenous sphingomyelinase and it remains open whether endogenous acid sphingomyelinase is also cleaved and activated by this mechanism. The transfection of such constructs definitely results in over-expression of the acid sphingomyelinase and may also alter the conformation of the enzyme. Such a conformational change may permit cleavage by caspases, which may otherwise not occur. If the authors want to study the activation mechanism of the acid sphingomyelinase they must simply make an antibody against the acid sphingomyelinase. This is a critical experiment. At present the studies are all done in vitro or with over-expression studies and do not prove the suggested mechanism of acid sphingomyelinase activation.

2. The confocal microscopy studies suggest co-localisation of caspase-8 and caspase-7 with acid sphingomyelinase within vesicles. However, these studies do not prove co-localisation, since they can not discriminate whether the caspases are associated with the vesicles at the cytoplasmic side, while the acid sphingomyelinase is presumably localizes to the inner leaflet of the cell membrane. The authors must provide electron microscopy to prove the localisation. Further, how is caspase-7 integrated into these vesicles? Does it associate with TNF-R1?

3. The western blot studies show that only a very minor fraction of the transfected acid sphingomyelinase is activated. This would mean that the specific activity of this fraction of the acid sphingomyelinase is activated much more than 3-fold (approximately 30-fold?). Is that possible? Why is only such a small fraction of the acid sphingomyelinase activated? How much of endogenous acid sphingomelinase does associate with TNF-receptors?

4. How shall caspase-7 associate with acid sphingomyelinase? Just by the enzymatic interaction. This would be very unstable and should not permit a co-immunoprecipitation. Does the acid sphingomyelinase contain a domain that could interact with caspase 7?

5. Fig. 10E shows a stronger signal at 82 kDa for the mutated protein than for the wildtype protein. However, the activity in the samples is the same. How is this possible? Further, the activation of the wildtype form is very minor. Is it possible that this very minor fraction of the acid sphingomyelinase mediates the 3-fold activation of the whole fraction? These data also point out the high importance to study endogenous acid sphingomyelinase.

6. The authors show that an activation of the acid sphingomyelinase occurs within 5 minutes, while the fusion of endosomes with acid sphingomyelinase-containing vesicles was only investigated at 30 and 60 min. These time courses do not fit and the authors should provide (electron microscopy) studies showing a very early fusion of endosomes and trans-Golgi vesicles.

7. The schematic figure 11 is confusing, since the authors presumably investigate multivesicular bodies. However, shown is one large fusion vesicle. The membrane, which contained TNF-R1, disappeared. Is this membrane digested?

8. The late endosomes should have a low pH to permit activity of the acid sphingomyelinase, i.e. a pH of approximately 5. Are caspases active at this pH?

9. Several typographic errors, for instance page 7 "staining instead of staning" or clearify on page 11 should be corrected.

Referee #2 (Remarks to the Author):

In this work, the authors show that caspase-8, caspase-7 and A-SMase, colocalize in internalized TNF receptosomes, and that the sequential activation of caspase-8 followed by caspase-7 is required for A-SMase activation. Furthermore, the authors show that not caspase-8 but caspase-7 directly activates A-SMase through proteolytic cleavage of pro-A-SMase and identified the potential cleavage site of pro-A-SMase. These findings reveal a mechanism of TNF-mediated A-SMase activation.

Specific comments:

1. Fig. 5, to consolidate the specificity of caspase-7, the author should show the results of the incubation with caspase-3.

2. Colocalization of caspase-8, caspase-7 and A-SMase in internalized TNF receptosomes seems to be important. If so, activities of caspase-7 and A-SMase in non-magnetic fractions may be relatively low. In fig. 8a, the authors should show the results of immunoblot analysis in non-magnetic fractions as well as supplementary fig. 4a.

3. In fig. 8a, a high amount of pro-A-SMase is included in non-internalized TNF-receptosome (lane, 0 min). The authors should discuss and confirm this by confocal microscopy.

4. In fig. 10b, cleavage and activation of A-SMase in caspase-7 knockdown cells do not correlat. The authors should comment on this.

5. In fig. 10c, the authors should provide immunoblot analysis for TNF-induced cleavage of A-SMase in capsase-7 deficient MEFs. In addition, the authors should investigate TNF-induced ceramide in capsase-3 and -7 deficient MEFs as well as caspase-8 deficient MEFs shown in fig. 1b.

6.The immunoblot analysis in fig 10e is not clear. What is the 82kDa fragment under the non-cleaved form of A-SMase? The authors should comment on this.

7. Does overexpressed cleaved, but not non-cleaved A-SMase generate ceramide and activate cathepsin D? The authors should show convincing evidence that the cleaved form of A-SMase is active form.

8. General remark: cleavage of A-SMase by caspase-7 has to be shown at the endogenous levels.

According to the literature, antibodies are available that detect endogenous A-SMase (Santa-Cruz). If the authors believe that these antibodies are not good enough,new A-SMase antibodies have to be generated as this is the most crucial tool for this study.

Referee #3 (Remarks to the Author):

In recent years a variety of details concerning the molecular mechanisms of TNF-R1/p60 induced cell death have been elucidated. However, this did not result in a uniform picture but rather revealed previously complexity and plasticity in TNF-R1 death signalling. So, in context of TNF-alpha induced killing of cells, RIPK1-dependent and -independent caspase-8 activation as well as RIPK1-mediated necrosis and a role of JNK and acid sphingomyelinase (ASM) have been described. Unfortunately, the relation of these mechanisms and their potential integration are still ill-defined.

In this respect, the study of Edelmann and coworkers makes a significant step ahead by identifying a proteolytic cascade linking caspase-8 activation to activation of ASM. They give convincing evidence that sequential activation of caspase-8 and caspase-7 results in activation of ASM by non-canonical cleavage by caspase-7 and thus put together two principle mechanisms (caspase signalling versus ASM) operating in TNF-alpha induced cell death. In the current form the work of Edelmann et al only shows data related to the caspase-8 caspase-7 ASM cascade, but does not reveal its relevance for TNF-alpha induced cell death. I think it is mandatory to include experiments analyzing cell death in TNF-alpha stimulated cells in which the caspase-8 caspase-7 ASM cascade is selectively blocked (caspase-7 MEFs, caspase-7 siRNA, both already used in the current version of the manuscript).

Minor points:

- There is only a transient appearance of the pro-A-SMase cleavage product while processed casapse-7 accumulates (e.g. figure 8A). Can the authors comment on that?
- TNF-alpha is a rather weak activator of caspase-8 in HeLa cells. Is there a stronger activation of the caspase-8 caspase-7 ASM cascade in response to TRAIL or CD95L?
- The authors should indicate which of the several caspase-7 antibodies listed in the MM section are used in the various experiments/figures.
- Figure 9 shows 12 different IP/WB bands each individually cut from an unknown number of gels and experiments. This is substandard and does not meet the good quality of the remaining data. The specific IPs plus a control Ab IP should be analyzed and shown as a whole on one blot for each of the antibodies used in the WB detection.

1st Revision - authors' response 31 August 2010

Reply to reviewer #1

We thank the reviewer for his comments and suggestions.

Comment 1:
One of the most important problems of the manuscript is the exclusive use of fusion constructs (EGFP and HA) of the acid sphingomyelinase that are transfected into cells. Thus, none of the studies is performed on endogenous sphingomyelinase and it remains open whether endogenous acid sphingomyelinase is also cleaved and activated by this mechanism. The transfection of such constructs definitely results in over-expression of the acid sphingomyelinase and may also alter the conformation of the enzyme. Such a conformational change may permit cleavage by caspases, which may otherwise not occur. If the authors want to study the activation mechanism of the acid sphingomyelinase they must simply make an antibody against the acid sphingomyelinase. This is a critical experiment. At present the studies are all done in vitro or with over-expression studies and do not prove the suggested mechanism of acid sphingomyelinase activation.

Reply 1:

We completely agree and were lucky to find a suitable and established anti-A-SMase antibody generated by Cristiana Perrotta and the company Areta international (Gerenanzo, Italy) (see Perrotta et al., *Cancer Res.* 67: 7559-7564, 2007; Bianco et al., *EMBO-J.* 28: 1043-1054, 2009). We used this antibody to detect endogenous A-SMase and to reproduce all our results obtained by overexpressed A-SMase-EGFP or A-SMase-HA constructs, either by confocal laser scan microscopy (**Fig. 2B, C and 5C**), by Western blotting (**Fig. 3A, B; 4G; 6A; 7B; 8C; 9A and Supplementary Fig. 6**) and by immunoprecipitation experiments (**Fig. 7B**). By confocal microscopy we now show the colocalization of endogenous A-SMase with TNF-R1 already after 5 min of TNF-receptor internalization (**Fig. 2B**), colocalization of endogenous A-SMase with active caspase-8 (**Fig. 2C**) and with caspase-7 (**Fig. 5C**). Most importantly, we reproduced the cleavage of endogenous 72 kDa pro-A-SMase, resulting in the appearance of a 57 kDa A-SMase molecule and enhanced A-SMase enzymatic activity (**Fig. 3A**). In an independent approach, using cell fractionation by OptiPrep-density gradient centrifugation we demonstrated that the cellular 57 kDa protein corresponds to the mature lysosomal A-SMase, exhibiting a higher activity than the 75 / 72 kDa pre-pro- and pro-A-SMase enzymes, respectively (**Fig. 3B**). Furthermore, we could show that exogenous caspase-7 is able to directly cleave pro-A-SMase in anti-A-SMase immunoprecipitates (**Fig. 4G**), we detected recruitment, processing, and activation of endogenous A-SMase in isolated TNF-receptosomes (**Fig. 6A, B, C**). In addition we found that caspase-7 co-immunoprecipitates with endogenous pro-A-SMase and vice versa (**Fig. 7B**), demonstrating direct interaction of caspase-7 with endogenous pro-A-SMase. The functional link between caspase-7 and endogenous A-SMase is now shown by experiments, where the TNF-induced processing and activation of A-SMase is blocked after downmodulation of caspase-7 by siRNA (**Fig. 8C, D**), and by analyzing murine embryonic fibroblasts derived from caspase-7 knock-out mice, which also did not respond to TNF by cleavage of pro-A-SMase and enzymatic activation of A-SMase (**Fig. 9A, B**).

Comment 2:
The confocal microscopy studies suggest co-localisation of caspase-8 and caspase-7 with acid sphingomyelinase within vesicles. However, these studies do not prove co-localisation, since they can not discriminate whether the caspases are associated with the vesicles at the cytoplasmic side, while the acid sphingomyelinase is presumably localizes to the inner leaflet of the cell membrane. The authors must provide electron microscopy to prove the localisation.
Further, how is caspase-7 integrated into these vesicles? Does it associate with TNF-R1?

Reply 2:
The exact localization of caspase-7 and A-SMase at the TNF-receptosomes is at present unknown. We extended our confocal microscopy colocalization studies to earlier time points and detect recruitment of A-SMase to TNF-receptosomes already after 5 min (**Fig. 2B**) and within the same time frame also colocalization of A-SMase with caspase-8 (**Fig. 2C**) and caspase-7 (**Fig. 5C**). In addition, also within the same time frame, we observed colocalization of TNF-receptosomes and subcellular compartments positive for Vti1b, a SNARE-protein associated with trans-Golgi vesicles by confocal microscopy (**Supplementary Fig. 3B**) and in isolated TNF-receptosomes by Western blotting (**Supplementary Fig. 3A**). We also performed immunoprecipitation experiments to investigate a possible interaction of caspase-7 with the TNF-R1 and found that caspase-7 does not bind to TNF-R1 (data not shown). From this, we can conclude, that caspase-7 rather is located at the plasma membrane in close vicinity to the TNF-receptor and is co-internalized with the TNF-receptosomes associated with the cytosolic face of the vesicle membrane (as depicted in our scheme in **Fig. 11**).

A microsomal localization of caspase-7 has been described in the studies of Zhivotovski et al. (*1999; Cell Death Differ* 6: 644-651) and Chandler et al. (1998; *J. Biol. Chem* 273: 10815-10818). Aouad et al. (2004, *J. Immunol*. 172: 2316-2323) detected caspase-3 in lipid rafts at the plasma membrane in untreated Jurkat cells and a Fas-induced recruitment of caspase-3 together with caspase-8 and FADD to the Fas receptor, suggesting that caspase-3 was in close proximity to caspase-8 and became part of the death-inducing signalling complex after Fas-triggering. A similar mechanism might be possible in the case of caspase-7 recruitment to TNF-R1 in our scenario. Here, this topology allows for contact between TNF-R1 associated caspase-8 and caspase-7, resulting in caspase-8 mediated caspase-7 activation as well as providing contact to pro-A-SMase after fusion of TNF-receptosomes and trans-Golgi vesicles which contain the A-SMase zymogen. Following the suggestions of this reviewer, we also performed electron microscopy and post-fixation immuno-

staining for caspase-7 and A-SMase to answer this intriguing question, but unfortunately our first experiments did not give clear results. These analyses appear to require substantial additional experimentation and, given the time limitations of this revision, we kindly ask the reviewer to allow for answering this important question in a separate follow-up report in the near future.

Comment 3:
The western blot studies show that only a very minor fraction of the transfected acid sphingomyelinase is activated. This would mean that the specific activity of this fraction of the acid sphingomyelinase is activated much more than 3-fold (approximately 30-fold?). Is that possible?Why is only such a small fraction of the acid sphingomyelinase activated? How much of endogenous acid sphingomelinase does associate with TNF-receptors?

Reply 3:
We agree with the reviewer´s notion that the specific activity of the cleaved A-SMase molecule, indeed must be much higher than that of the pro-A-SMase, which is believed to exhibit only low enzymatic activity compared to the mature enzyme (Ferlinz et al., 1994; *Biochem J* 301: 855-862). Based on our new data obtained by cell fractionation experiment shown in **Fig. 3B**, the 57 kDa protein represents the mature lysosomal enzyme and we think that it is well possible, that a mature enzyme in lysosomes exhibits a higher activity than the pro-form. From densitometric analysis of our Western blot data we calculated that the amount of the cleaved form of pro-A-SMase ranges between 25– 46 % and by comparing the amount of cleaved A-SMase detected and quantitated by Western blotting with the increases in enzymatic A-SMase activity, we calculated an approximately 5 to 14 -fold activation for endogenous A-SMase in response to TNF.

As discussed on **page 17** of the revised manuscript the restricted amounts of total A-SMase cleaved and activated in response to TNF or caspase-7 may be based on the following reasons:

(**1.**) The accessibility of the pro-A-SMase to cleavage by caspase-7: in whole cells, TNF-receptosomes fuse only to a certain portion of pro-A-SMase containing compartments, as can be seen in the confocal micrographs in Fig. 2B and also when comparing the amounts of pro-A-SMase recruited to TNF-receptosomes and the portion that remains in the non-magnetic fractions in the flow-through after magnetic separation of the TNF-receptosomes, shown in **Fig. 6A**. Based on our Western blot and confocal microscopical data, we calculated that approximately 1-2% of the total cellular (endogenous) A-SMase and 0.4-0.6 % of the total overexpressed A-SMase is recruited by receptosomes.

(**2.**) The cleavage and activation of pro-A-SMase should be a regulated process, i.e. regulated by posttranslational modifications such as phosphorylation or glycosylation of the enzyme (reviewed by Jenkins, R.W., Canals, D., Hannun,Y.A., 2009, *Cell. Signal*. 21:836-846).
As an example for the regulatory function of substrate-phosphorylation, the requirement of phosphorylation of the reticulon protein Nogo-B at Ser 16 within the noncanonical caspase-7 cleavage site was described recently (Schweigreiter et al., 2007, *Proteomics* **7**: 4457-67).
(**3.**) The cleaved and thereby activated mature A-SMase must also be regulated with regard to its activity in order to terminate the reactions. One possible mechanism to terminate enzymatic activity is the proteolytic degradation of the enzyme. Thus the stability and half-life of the cleaved, active 57 kDa A-SMase protein would be reduced compared with the pro-A-SMase form. This scenario is indicated by the transient appearance of the cleaved A-SMase forms either of the native (**Fig. 4G; Fig. 6A**) or the recombinant protein (**Fig. 3C, Fig. 4A; Fig. 4F, Fig. 6A; Fig. 8A**).

Comment 4:
How shall caspase-7 associate with acid sphingomyelinase? Just by the enzymatic interaction. This would be very unstable and should not permit a co-immunoprecipitation. Does the acid sphingomyelinase contain a domain that could interact with caspase 7?

Reply 4:
We agree that association by enzymatic interaction is unlikely. We could not find consensus interaction motifs deposited in public data bases within the A-SMase sequence. Notably, we detected interaction of the pro-form of caspase-7 with A-SMase in our co-immunoprecipitation experiments, i.e. pro-caspase-7 should bind A-SMase prior to activation by cleavage (see **Fig. 7**).

This fact may point to a possible interaction of A-SMase with the pro-peptide of pro- caspase-7 or at least a contribution of this pro-peptide in the binding. We will perform binding studies using deletion mutants of pro-A-SMase constructs in the future to identify the caspase-7 binding domain in pro-A-SMase.

Comment 5:
Fig. 10E shows a stronger signal at 82 kDa for the mutated protein than for the wildtype protein. However, the activity in the samples is the same. How is this possible? Further, the activation of the wildtype form is very minor. Is it possible that this very minor fraction of the acid sphingomyelinase mediates the 3-fold activation of the whole fraction? These data also point out the high importance to study endogenous acid sphingomyelinase.

Reply 5:
The basal specific activities of wild-type (29.0 nmol/mg/h) and mutated pro-A-SMase-GFP (33.7 nmol/mg/h) (**Fig. 10**) in cell lysates are indeed similar.

In different preparations of cell lysates from untreated cells that expressed A-SMase-EGFP fusion proteins we found varying proportions of the 82 kDa band to the full-length protein, which did not correlate with the respective basal A-SMase activities. These discrepancies may be explained by varying amounts of unglycosylated pro-A-SMase molecules: the constitutive bands at 82 kDa (A-SMase-EGFP) and 57 kDa (endogenous A-SMase) most likely represent the unglycosylated forms of the respective pro-A-SMase molecules. As we demonstrated in **Fig. 3D**, the deglycosylation of pro-A-SMase-EGFP by PNGase leads to a protein band of almost the same molecular weight as the cleaved glycosylated A-SMase-EGFP protein, and it is unfortunately not always possible to distinguish between these two molecuar species in the Western blots.

In the Western blot of the wild-type A-SMase-EGFP expressing samples in the new **Figure 10**, the 82 kDa band is actually a double band, which represents a mixture of a cleavage product of glycosylated A-SMase produced by caspase-7 and the unglycosylated form of uncleaved pro-A-SMase. In the Western blot of the D253A-mutant samples in **Figure 10** there is only a single band at 82 kDa, which represent in this case only the unglycosylated form of uncleaved pro-A-SMase, which is constitutively present and largely unchanged by TNF stimulation. In contrast the upper band of the doublet at 82 kDa from wild-type A-SMase-EGFP expressing samples shows a clear peak in intensity 30 min after TNF stimulation, which we think is due to an increased generation of the cleavage-fragment from glycosylated pro-A-SMase by caspase-7.
Only the changes of intensity of this protein in response to TNF are considered to be caused exclusively by proteolytic cleavage. Because caspase-7 is a protease without known glycohydrolase activity, it is rather unlikely that caspase-7 might also be able to deglycosylate pro-A-SMase. The functional link between caspase-7 proteolytic activity and generation of the 82 kDa A-SMase-EGFP as well as the 57 kDa endogenous A-SMase enzymes, respectively, was demonstrated by down-modulation of caspase-7 by siRNA (**Fig. 8A-D**) and by using caspase-7 knock-out MEFs (**Fig. 9A, B**). In both cases, lack of caspase-7 correlated with lack of pro-A-SMase processing and enzymatic activation.

Comment 6:
The authors show that an activation of the acid sphingomyelinase occurs within 5 minutes, while the fusion of endosomes with acid sphingomyelinase-containing vesicles was only investigated at 30 and 60 min. These time courses do not fit and the authors should provide (electron microscopy) studies showing a very early fusion of endosomes and trans-Golgi vesicles.

Reply 6:
See reply 2: We extended our confocal microscopy colocalization studies to earlier time points and detected recruitment of A-SMase to TNF-receptosomes already after 5 min (**Fig. 2B**) and within the same time frame also colocalization of A-SMase with caspase-8 (**Fig. 2C**) and caspase-7 (**Fig. 5C**). In addition, also within the same time frame, we observed colocalization of TNF-receptosomes and subcellular compartments positive for Vti1b, a SNARE-protein associated with trans-Golgi vesicles by confocal microscopy (**Supplementary Fig. 3B**) and in isolated TNF-receptosomes by Western blotting (**Supplementary Fig. 3A**). We are currently trying to characterize the temporal and spatial

compartmentalization of the fusion events by electron microscopy, but this will take more time and extended experimentation and we kindly ask the reviewer to allow us to present the results in a separate report in the near future.

Comment 7:
The schematic figure 11 is confusing, since the authors presumably investigate multivesicular bodies. However, shown is one large fusion vesicle. The membrane, which contained TNF-R1, disappeared. Is this membrane digested?

Reply 7:
In our first submission we omitted the membranes of the ingested receptosome vesicles to show the presumed situation at late time points, where digestion of multivesicular structures most likely occurs. We now changed the figure to show the multivesicular compartment shortly after fusion of early receptosomes with trans-Golgi vesicles, which, according to our new data occurs as early as 5 min after TNF-receptor internalization (**Fig. 2B, C; Fig. 5C, 6A, Supplementary Fig. 3A, B**).

Comment 8:
The late endosomes should have a low pH to permit activity of the acid sphingomyelinase, i.e. a pH of approximately 5. Are caspases active at this pH?

Reply 8:
Again based on our new data (see reply 7 above), we believe that TNF-receptosome fusion is an early event and the initial activation of caspase-8, caspase-7 and pro-A-SMase cleavage occur within 3 min (**Fig. 6A**, endogenous A-SMase in TNF-receptosome preparations) to 5 min (**Fig. 2B, C; 5C**, endogenous A-SMase, active caspase-8 and caspase-7 colocalization by confocal microscopy). At these early time points, the pH within the multivesicular compartments should not be very low. We measured the activity of caspase-7 *in vitro* at various pH-values and found approximately 50% activity at pH 6.5 compared with 100% at pH 7.5 (**Supplementary Fig. 5**).

Comment 9:
Several typographic errors, for instance page 7 "staining instead of staning" or clearify on page 11 should be corrected.

Reply 9:
We corrected the typographic errors.

Referee #2 (Remarks to the Author):

We also thank this reviewer for his comments and suggestions.

Comment 1:
Fig. 5, to consolidate the specificity of caspase-7, the author should show the results of the incubation with caspase-3.

Reply 1:
Following the reviewer´s suggestion, we now show the data obtained after treating whole cell lysates or immunoprecipitated A-SMase-EGFP preparations with caspase-3, demonstrating pro-A-SMase cleavage and activation occurring in lysates, but not in purified pro-A-SMase preparations (**Fig. 4C, D**). Thus pro-A-SMase seems not to be a direct substrate for caspase-3.

Comment 2:
Colocalization of caspase-8, caspase-7 and A-SMase in internalized TNF receptosomes seems to be important. If so, activities of caspase-7 and A-SMase in non-magnetic fractions may be relatively low. In fig. 8a, the authors should show the results of immunoblot analysis in non-magnetic fractions as well as supplementary fig. 4a.

Reply 2:
We performed the experiments suggested and now show the distribution of TNF-R1, caspase-8, caspase-7, caspase-3, endogenous A-SMase, and recombinant A-SMase-EGFP in magnetically isolated TNF-receptosomes as well as the corresponding non-magnetic fractions obtained after the magnetic separation of the lysates, depicted in the new **Fig. 6A**. In the non-magnetic fractions, we indeed could detect no caspase-7 activation and also no cleavage / activation of A-SMase in contrast to the events observed in the corresponding magnetic receptosome preparations. A delayed activation of caspase-8 and caspase-3 was observed in the non-magnetic fractions.

Comment 3:
In fig. 8a, a high amount of pro-A-SMase is included in non-internalized TNF-receptosome (lane, 0 min). The authors should discuss and confirm this by confocal microscopy.

Reply 3:
A-SMase as a lysosomal protein is distributed in vesicles throughout the entire cytosolic compartment (**Fig. 2B, C, and D**). At 0 min internalization TNF-receptosomes are formed from resealed plasma membrane patches which contain TNF-receptors attached to paramagnetic beads as well as all the proteins located at the plasma membrane. In A-SMase-EGFP or A-SMase-HA overexpressing cells it is possible that the respective recombinant protein becomes mislocated at the plasma membrane as soon as mannose-6-phosphate receptors become saturated with overexpressed lysosomal proteins. Although overexpressed A-SMase-EGFP or -HA should get secreted, if its retrieval to the lysosome by mannose-6-phosphate receptors becomes saturated, we can not exclude that a substantial portion of the secreted A-SMase-EGFP remains attached to the exterior surface of the plasma membrane and gets co-internalized or at least gets incorporated into resealed membrane patches together with the TNF-receptor at 0 min internalization. This may explain the occurrence of a quite substantial amount of A-SMase-EGFP in the 0 min TNF-receptosome sample in **Fig. 6A**.
Also a substantial amount of endogenous A-SMase is observed in the 0 min sample of TNF-receptosomes. By confocal microscopy, we also observed that in some unstimulated HeLa-cells a substantial percentage of endogenous A-SMase is detected at or in close proximity to the plasma membrane by the A-SMase-specific antibody (see **Supplementary Fig. 4**). Such membrane associated A-SMase can be incorporated similarly into resealed plasma membrane patches in the 0 min TNF-receptosome samples after homogenization of the cells (see manuscript **page 12**, second paragraph).

Comment 4:
In fig. 10b, cleavage and activation of A-SMase in caspase-7 knockdown cells do not correlate. The authors should comment on this.

Reply 4:
The very slight increase of the 82 kDa cleavage product of A-SMase-EGFP in caspase-7 knock-down cells visible at 30 min and 45 min in **Fig. 8B** of the revised manuscript may not have lead to a detectable increase in A-SMase activity in these samples, because the basal activity of the pro-ASMase-EGFP, which is much more abundant in these samples, was already too high to allow a substantial elevation of total activity by the very small amount of the cleavage product. So the effect of residual caspase-7 cleavage becomes obscured by experimental error in this case. In the samples from control siRNA transfected cells, where A-SMase activation becomes detectable, the ratio of cleavage product to pro-form is at least 2-6 times higher than in any of the caspase-7 knock-down samples.

Comment 5:
In fig. 10c, the authors should provide immunoblot analysis for TNF-induced cleavage of A-SMase in capsase-7 deficient MEFs. In addition, the authors should investigate TNF-induced ceramide in caspase-3 and -7 deficient MEFs as well as caspase-8 deficient MEFs shown in fig. 1b.

Reply 5:
(1) We performed Western blot analysis with TNF stimulated MEF wild type cells and caspase-7 deficient MEFs and analysed the cleavage of endogenous A-SMase using the anti-A-SMase antibody. **Fig. 9A** and **B** show that after TNF stimulation endogenous A-SMase is cleaved and activated in MEF wild type cells whereas no cleavage and activation was detectable in caspase-7 deficient MEFs. The analysis of the proapoptotic lipid ceramide showed that there is no enhanced generation of C16/18 ceramide after TNF stimulation in caspase-7 deficient MEFs whereas in wild type cells high amounts of ceramide were produced (**Fig. 9C**). As ceramide is known to activate cathepsin D (CTSD), we further investigated the cleavage of CTSD. As can be seen in **Fig. 9D**, CTSD is cleaved in wild type MEFs whereas caspase-7 deficient MEFs do not generate the active 32kDa fragment of CTSD. With respect to apoptosis, we investigated the apoptotic behaviour of these cells by FACS analysis and as shown in **Fig. 9E** found that the fraction of caspase-7 deficient MEFs responding with apoptosis was reduced by 31.7 % in comparison to wild type cells (19% vs. 29%). The residual capacity to undergo apoptosis of the caspase-7 deficient cells is most likely mediated by the direct caspase-8 / caspase-3 cascade, which is independent of A-SMase activity and ceramide generation and which is still working in these cells.

(2) Since we did not find cleavage and activation of immunoprecipitated A-SMase-EGFP by caspase-3 *in vitro* (**Fig. 4D**), nor an association of caspase-3 with TNF-receptosomes (Fig. **6A),** or a direct association of A-SMase-EGFP with caspase-3 (**Fig. 7A**), nor a colocalization of caspase-3 with internalized TNF in confocal microscopy (**Supplementary Fig. 2**) we have no indication for a specific function of caspase-3 in TNF-induced A-SMase activation. We therefore would like to convince the reviewer that it makes little sense to perform further experiments to explore the role of caspase-3 in TNF-induced ceramide generation or cathepsin D-activation.
(3) Caspase-8 deficient Jurkat cells and wild type Jurkat cells were used to investigate A-SMase activation and ceramide production (**Fig. 1A and B**). After TNF stimulation, wild type Jurkat cells showed an activation of A-SMase as well as production of C16/18 ceramide. The A-SMase in caspase-8 deficient Jurkat cells was not activated and also additional ceramide was not generated. As caspase-8 is an initiator caspase for apoptosis we investigated the response of both cell types after prolonged stimulation with TNF/CHX. **Fig. 1C** shows that caspase-8 deficient Jurkat cells do not undergo apoptosis whereas 57% of the wild type Jurkat cells became apoptotic after this treatment.

Comment 6:
The immunoblot analysis in fig 10e is not clear. What is the 82kDa fragment under the non-cleaved form of A-SMase? The authors should comment on this.

Reply 6:
As already explained in reply 4 the 82 kDa band is actually a double band (as can be observed in the Western blot of the wild-type A-SMase-EGFP expressing samples in **Fig. 10** of the revised manuscript), which represents a mixture of a cleavage product of glycosylated A-SMase produced

by caspase-7 and the unglycosylated form of uncleaved pro-A-SMase (see **Fig. 3D**). In the Western blot of the D253A-mutant samples in **Fig. 10** there is only a single band at 82 kDa which represents in this case only the unglycosylated form of uncleaved pro-A-SMase, which is constitutively present and largely unchanged by TNF stimulation. In contrast the double band at 82 kDa from wild-type A-SMase-EGFP expressing samples shows a clear peak in intensity 30 min after TNF stimulation, which we think is due to an increased generation of the cleavage-fragment from glycosylated pro-A-SMase by caspase-7.

Comment 7:
Does overexpressed cleaved, but not non-cleaved A-SMase generate ceramide and activate cathepsin D? The authors should show convincing evidence that the cleaved form of A-SMase is active form.

Reply 7:
Actually we do not propose that cleaved A-SMase is the only enzymatically active A-SMase species, but we rather suggest that the cleaved A-SMase has a higher enzymatic activity over the glycosylated, uncleaved precursor, which possesses a low basal activity. Unfortunately we have no means to separate cleaved from uncleaved A-SMase-EGFP, while preserving their enzymatic activities. We performed cell fractionation experiments to further characterize the 57 kDa cleavage form and found a co-sedimentation of the 57 A-SMase together with the lysosomal fraction (characterized by expression of the marker proteins Lamp-1 and mature 32 kDa cathepsin D) depicted in new **Fig. 3B.** This lysosomal fraction also contained the highest enzymatic A-SMase activity. Thus we suggest that the 57 kDa cleavage product represents the mature lysosomal A-SMase.

Additionally, in all other experiments performed, increases in the cleaved A-SMase form correlated with enhanced enzymatic A-SMase activity, strongly supporting the fact, that this enhanced A-SMase activity is causally related to the cleaved A-SMase molecule.
Along this line and according to your suggestions we analyzed the ceramide levels of cells unable to cleave and activate A-SMase (i.e. caspase-7 deficient MEFs). Based on the data presented in **Fig. 9** (see reply 5), where we compare caspase-7 deficient MEFs with wild type MEFs, we found that only cleaved A-SMase generates enough ceramide to activate CTSD whereas the non-cleaved A-SMase is unable to do so.

Comment 8:
General remark: cleavage of A-SMase by caspase-7 has to be shown at the endogenous levels. According to the literature, antibodies are available that detect endogenous A-SMase (Santa-Cruz). If the authors believe that these antibodies are not good enough,new A-SMase antibodies have to be generated as this is the most crucial tool for this study.

Reply 8:
We completely agree with this view. Unfortunately, the currently available lots of the anti-A-SMase antibody from Santa-Cruz in our hands turned out to be completely unspecific and could not be used for the detection of endogenous A-SMase. As already detailed in our reply to comment 1 of the first referee, we were lucky to find a suitable anti-A-SMase antibody generated by Cristiana Perrotta and the company Areta international s.r.l. (Gerenzano, Italy), (see Perrotta et al., *Cancer Res.* 67, 7559-7564, 2007; Bianco et al., *EMBO-J.* 28, 1043-1054, 2009). We used this antibody to detect endogenous A-SMase and to reproduce all our results obtained by overexpressed A-SMase-EGFP or A-SMase-HA constructs, and to recapitulate most of the experiments that had been performed with the recombinant A-SMase-EGFP: in **Fig. 2B, C and 5C** by confocal laser scan microscopy, in **Fig. 3A, B; 4 G; 6A; 7B; 8C; 9A and Supplementary Fig. 6** by Western blotting and in **Fig. 7B** by immunoprecipitation experiments. By confocal microscopy we now show the colocalization of endogenous A-SMase with TNF-R1 already after 5 min of TNF-receptor internalization (**Fig. 2B**), colocalization of endogenous A-SMase with active caspase-8 (**Fig. 2C**) and with caspase-7 (**Fig. 5C**). As critical results, we reproduced the cleavage of endogenous 72 kDa pro-A-SMase, resulting in the appearance of a 57 kDa A-SMase molecule and enhanced A-SMase enzymatic activity (**Fig. 3A**). Using cell fractionation by OptiPrep-density gradient centrifugation we demonstrated that the cellular 57 kDa protein corresponds to the mature lysosomal A-SMase, exhibiting a higher activity

than the 75/72 kDa pre-pro- and pro-A-SMase enzymes, respectively (**Fig. 3B**). Furthermore, we could show that exogenous caspase-7 is able to directly cleave pro-A-SMase in anti-A-SMase immunoprecipitates (**Fig. 4G**). We detected recruitment, processing, and activation of endogenous A-SMase in isolated TNF-receptosomes (**Fig. 6A, B, C**). Additionally, caspase-7 co-immunoprecipitated with endogenous pro-A-SMase and vice versa (**Fig. 7B**) demonstrating direct interaction of caspase-7 with endogenous pro-A-SMase. The functional link between caspase-7 and endogenous A-SMase is now shown by experiments, where the TNF-induced processing and activation of A-SMase is blocked after down-modulation of caspase-7 by siRNA (**Fig. 8C, D**), and by analyzing murine embryonic fibroblasts derived from caspase-7 knock-out mice, which also did not respond to TNF by cleavage of pro-A-SMase and enzymatic activation of A-SMase (**Fig. 9A, B**).

Referee #3 (Remarks to the Author):

We thank the reviewer for his comments and suggestions.

Comment 1:
In the current form the work of Edelmann et al only shows data related to the caspase-8 caspase-7 ASM cascade, but does not reveal its relevance for TNF-alpha induced cell death. I think it is mandatory to include experiments analyzing cell death in TNF-alpha stimulated cells in which the caspase-8 caspase-7 ASM cascade is selectively blocked (caspase-7 MEFs, caspase-7 siRNA, both already used in the current version of the manuscript).

Reply 1:
We completely agree with your suggestions that we also have to show the relevance of caspase-8 and caspase-7 for TNF-alpha induced cell death. Following your suggestions, we used caspase-8 deficient Jurkat cells and wild type Jurkat cells to investigate A-SMase activation, ceramide production and apoptosis (**Fig. 1A, B, and C**). **Fig. 1C** shows that caspase-8 deficient Jurkat cells do not undergo apoptosis whereas 57% of the wild type Jurkat cells become apoptotic. This result clearly indicates that caspase-8 is essential for the activation of apoptosis.
As suggested we also compared apoptosis induction by TNF in wild type MEFs and caspase-7 deficient MEFs. As can be seen in **Fig. 9E**, caspase-7 deficient MEFs show a rate of apoptosis that is reduced by 31.7% when compared to wild type cells (19% vs. 29%). However, the apoptotic programme in these cells is not completely blocked. This result indicates, that caspase-7, after all necessary for A-SMase activation after TNF stimulation, is indeed involved and contributes to apoptosis induction after prolong TNF-incubation. But in the absence of caspase-7, however, other pathways of apoptosis signalling independent of A-SMase activation (e.g. direct activation of caspase-3 by caspase-8) can partially compensate for this deficiency.

Comment 2:
Minor points:
There is only a transient appearance of the pro-A-SMase cleavage product while processed caspase-7 accumulates (e.g. figure 8A). Can the authors comment on that?

Reply 2:
The transient appearance of the A-SMase cleavage product can be taken as an indication of two characteristics of the proposed caspase-7 / A-SMase signalling cascade (discussed on **page 17** of the revised manuscript):

1.) Obviously caspase-7 can only cleave a limited fraction of the total pro-A-SMase present. In the context of intact cells this may in part reflect a restricted accessibility of pro-A-SMase for caspase-7. Particularly in the case of overexpressed A-SMase-EGFP (see e.g. **Fig. 3C**) a substantial proportion of the precursor seems to be located in compartments (e.g. trans-Golgi vesicles or lysosomes) that are inaccessible for caspase-7. In the case of the endogenous A-SMase the proportion of cleaved fragment versus precursor is higher (see e.g. **Fig. 3A**), indicating that under physiological conditions a higher proportion of pro-A-SMase becomes accessible for caspase-7. But even under *in vitro* conditions in cell lysates or in immunoprecipitated material incubation with exogenous caspase-7 can only cleave a limited proportion of the total pro-A-SMase (-EGFP). The reason for the partial

cleavage in these cases can only be found in a restricted cleavage competence of the pro-A-SMase substrate. Such a lack of cleavage competence of the substrate could be due to partial alterations by posttranslational modifications (e.g. glycosylation or phosphorlyation) of pro-A-SMase that may either be blocking the cleavage site for caspase-7, or that may by contrast be missing and be necessary for formation of an optimal cleavage site.

2.) The cleavage fragment of A-SMase- (EGFP) has a decreased stability, which leads to its transient appearance. As soon as the maximal amount of cleavable pro-A-SMase (-EGFP) has been processed by caspase-7 the instability of the fragment (or a proteolytic degradation event) leads to its disappearance at later time points. Physiologically the decreased stability of the active fragment may serve to limit the extent and duration of A-SMase activation after TNF-stimulation.

Comment 3:
TNF-alpha is a rather weak activator of caspase-8 in HeLa cells. Is there a stronger activation of the caspase-8 caspase-7 ASM cascade in response to TRAIL or CD95L?

Reply 3:
For other death-receptor ligands, like TRAIL and CD95L, an involvement of caspase-7 in the activation of A-SMase after ligand stimulation has not yet been described.

Previously it has been shown, that binding of Fas ligand to the CD95-receptor recruits small amounts of caspase-8 leading to A-SMase activation and translocation to the cell surface (Grassme et al., 2003; *Oncogene* 22: 5457). However, it is not known, if caspase-8 activates A-SMase directly or if there are other factors (like caspase-7) in between. In the case of TRAIL even less is known about A-SMase activation. Dimitru et al. (2006, *Oncogene* 25: 5612-5625) reported that after binding of TRAIL to its receptor, A-SMase translocates to the outer leaflet of the plasma membrane and generates ceramide, which leads to subsequent receptor clustering and induction of apoptosis.
For the receptors of both ligands it is known that they can be internalized after ligand binding. However, it has not yet been investigated, whether there are any fusion processes with trans-Golgi vesicles containing A-SMase and whether A-SMase is activated in the respective receptosomes. The characterization of the molecular mechanisms of A-SMase activation by CD95L and TRAIL is the topic of a current project in our lab.

Comment 4:
The authors should indicate which of the several caspase-7 antibodies listed in the MM section are used in the various experiments/figures.

Reply 4:
We have indicated the respective antibodies used in all figure legends.

Comment 5:
Figure 9 shows 12 different IP/WB bands each individually cut from an unknown number of gels and experiments. This is substandard and does not meet the good quality of the remaining data. The specific IPs plus a control Ab IP should be analyzed and shown as a whole on one blot for each of the antibodies used in the WB detection.

Reply 5:
We have displayed the IP data in a matrix arrangement neglecting the original Western blot context and the relevant controls, because we found it easier for the reader to grasp the relevant messages from this type of arrangement. However, we agree that for the sake of quality assessment of the displayed data it is indespensible to show the entire original Western blots including all controls. We added an assembly of all original blots as **Supplementary Figure 6** and marked the regions displayed in **Fig. 7**.

2nd Editorial Decision 25 September 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Referee 1 feels that there are a few issues that still need to be addressed (see below), and I would therefore like to ask you to deal with these issues in an amended version of the manuscript.

Still, there is one remaining editorial issue that needs further attention.

Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there are a number of panels that do not fully meet these requirements: Figure 3D, Figure 6A, Figure 8A; Figure 7 A+B/Supplementary Figure 6 (it should be stated in the legend that Supplementary Figure 6 refers to Figure 7A+B; and cutting/pasting should be indicated in Supplementary Figure 6)

I therefore like to kindly ask you to send us a new version of the manuscript that contains suitably amended versions of these figures. It would also be important to explain in the figure legends that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final print version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors performed extensive revisions. There are still a few open issues:
The authors state that 25-46% of the A-SMase are cleaved. However, only 1-2% of the endogenous A-SMase are recruited to receptosomes. This seems to be a discrepancy? Further, how is it possible that such a low percentage of recruited protein has such a high activity? Is it possible that the authors measure one, but not the only mechanism of activation? I think the data very much suggest a scenario of several activation mechanisms of the A-SMase. This should be stated in the abstract and the discussion.

The 2nd open issue is the topology. I agree that electron microscopy data are very difficult to obtain and the exact analysis might be an issue for another manuscript. However, the topology is an open issue and this should be clearly stated in the manuscript.

Referee #3 (Remarks to the Author):

The authors have substantially improved their manuscript by including additional data related to the analysis of endogenous aSMase. They have also carefully considered my comments made on the

first version. I feel that the authors have also responded well to the two other reviewers.

2nd Revision - authors' response 10 November 2010

Reply to reviewer #1

We thank this reviewer for his comments and suggestions.

Comment 1:

The authors state that 25-46% of the A-SMase are cleaved. However, only 1-2% of the endogenous A-SMase are recruited to receptosomes. This seems to be a discrepancy? Further, how is it possible that such a low percentage of recruited protein has such a high activity? Is it possible that the authors measure one, but not the only mechanism of activation? I think the data very much suggest a scenario of several activation mechanisms of the A-SMase. This should be stated in the abstract and the discussion.

Reply 1:

We agree with the reviewer's comment, that apparently our data point to a discrepancy between the amount of pro-A-SMase cleavage / activation induced by TNF in whole cells (Figure 3A) and in isolated TNF-receptosomes (Figure 6A, B). Aside from the possibility of several activation mechanisms for A-SMase inside the cell (an argument which we of course accept and will give reference to), the reason for this discrepancy may also be explained by the following arguments:

(1.) The results obtained with isolated TNF-receptosomes represent a snap-shot of the *in vivo*-situation, since we isolate a distinct population of TNF-receptor complexes that were coupled to magnetically labelled TNF at a given time after synchronized receptor-internalization.
TNF-treatment of whole cells without subsequent isolation of a distinct TNF / TNF-receptor population will result in a much higher biological effect reflecting the sum of all possible TNF-receptor internalization events taking place in the cell.

(2.) Thus, does the calculated amount of A-SMase recruited to TNF-receptosomes really reflect the *in vivo*-situation in whole cells?

- We evaluated the amount of fusion events between internalized TNF-receptosomes and A-SMase positive compartments analyzed by confocal microscopy in whole cells (Figure 2B) and estimated a colocalization rate of 5-20% for TNF-receptosomes and A-SMase positive vesicles. This indicates, that the 1-2% recovery of A-SMase in isolated TNF-receptosomes is significantly lower than the expected amount of receptosome-associated A-SMase, either due to (i) the fact, that we did not isolate all of the TNF-receptosomes present in the cell lysates, either because not all were magnetically labeled, or because some of the labeled receptosomes were lost during the isolation procedure, or due to (ii) degradation of A-SMase during the isolation procedure, which takes up to 6 hours at 4°C and may also diminish the amount and activity of the enzyme. The latter is unavoidable because protease inhibitors in the homogenization buffer will not be able to block intraluminal proteases of endosomes and lysosomes since the homogenization procedure and the magnetic separation are performed without the use of detergents, thus leaving vesicle membranes intact.

(3) Therefore, it is questionable, whether the results obtained from analyzing cell lysates and isolated receptosomes can be compared with a 1:1 stochiometry. So, we have to ask whether the assay conditions for estimation of A-SMase cleavage and enzymatic activity in cell lysates and isolated TNF-receptosomes are identical to allow a direct comparison in quantitative terms.
- With cell lysate samples Western blots and enzyme assays are performed with fresh material, directly after TNF-treatment of the cells followed by homogenization in buffers containing protease inhibitors and detergents. In contrast, preparation of TNF-receptosomes takes up to 6 hours, which may cause A-SMase degradation by proteolysis through largely uninhibited endosomal or lysomal

proteases and/or secondary modifications of the activation status of A-SMase in the different receptosome preparations.

Since at present we cannot answer these open questions, following the reviewers suggestion we agree to incorporate this scenario in the Discussion section on page 19 of the amended revision by stating that:

"……However, the high amount of A-SMase cleaved and activated by TNF in whole cells compared to the ratio of TNF-receptosome / A-SMase fusion events and A-SMase-activation observed in isolated TNF-receptosomes may also reflect the fact that additional mechanisms of activation exist for A-SMase."

Referring to the second part of comment 1: we are well aware that other mechanisms of A-SMase activation exist, which have been described in the past (Schütze *et al.,* 1992; Zeidan and Hannun, 2007; Lang *et al*., 2007; Charruyer *et al*., 2005; Reinehr *et al*. 2006; Won and Singh, 2006, *Free Radical Biol Med. 40, 1875-1888 for review on sphingolipid signaling and redox regulation*) and of course novel mechanisms may also be detected in the future. Related to this discussion, and to follow the reviewer's suggestion, we now state in the abstract on page 2, that our data are

"…confirming proteolytic cleavage as one further mode of A-SMase activation"

Comment 2:

The 2nd open issue is the topology. I agree that electron microscopy data are very difficult to obtain and the exact analysis might be an issue for another manuscript. However, the topology is an open issue and this should be clearly stated in the manuscript.

Reply 2:

We thank the reviewer for accepting our explanation of the technical difficulties related to electron microscopic evaluations of the exact topology of caspase-7 / A-SMase interaction in whole cells. Following the reviewers suggestion we now incorporate the following sentences in the discussion on page 19:

"It is presently unknown how A-SMase and pro-caspase-7 can come into direct contact inside the cell, since A-SMase in the lumen of the endo-lysosomal compartment and caspase-7 at the cytosolic surface of the TNF-receptosomes are supposed to be separated by a lipid membrane. Whether an intraluminal membrane break-down after vesicular fusion or a translocation of proteins through the lipid bilayer within the multivesicular compartment will lead to contact with each other is an open question. The same topology problem is unresolved for the proposed regulation of A-SMase secretion and activiation by PKC-d (Zeidan and Hannun, 2007; Jenkins et al., 2010) as well as for the observed association of caspase-3 and A-SMase upon NO exposure (Castillo et al., 2007)." ……. "We are currently trying to solve this important question by immuno-electron microscopic analysis."

Reply to Editor:

...we repeated the experiments shown in Figure 7A+B and Supplementary Figure 6 (different IPs run on the same gel with input and bead controls and the respective Western blots) as well as siRNA experiments run on the same gel together with the control (Figure 8A) ad show ehses data in new Figure 7A+B and new Figure 8A. The primary scans of these experiments are shown in the Supplemental Information "Primary Scans". Since the original scans of the IP experiments in Figure 7 are now depicted in the Primary Scan supplements, we deleted former Supplementary Figure 6.

...we included appropriate indications in the figure legends in response to your requests concerning figures containing lanes of gels that were assembled from cropped lanes (Figure 3D, Figure 6A,

Figure 8A, Figure 7A+B). In addition, we included the primary scans of Figure 3D, 6A, 7A+B, and 8A for supplementary information as requested.

