Brief Communication

A γ-SECRETASE LIKE INTRAMEMBRANE CLEAVAGE OF TNFα BY THE GxGD ASPARTYL PROTEASE SPPL2b

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Running title: A common principle of intramembrane proteolysis by GxGD type aspartyl proteases

Abbreviations: amu, arbitrary mass units; APH-1, anterior pharynx defective 1; PS, presenilin; SPP, Signal peptide peptidase; SPPL, SPP-like proteases

Key words: presenilin, regulated intramembrane proteolysis, Alzheimer's disease; GxGD-type aspartic protease

γ-Secretase and signal peptide peptidase (SPP) are unusual aspartyl proteases of the GxGD type, which mediate intramembrane proteolysis. Besides SPP, a family of SPP-like proteins (SPPLs) of unknown function was identified. We demonstrate that SPPL2b utilizes multiple intramembrane cleavages to liberate the TNFα intracellular domain into the cytosol and to release the C-terminal counterpart into the lumen. These findings suggest common principles of regulated intramembrane proteolysis by GxGD type aspartyl proteases.

Extensive work on sterol-regulated proteolysis, rhomboids, γ-secretase, and signal peptide peptidase (SPP) [1] led to the novel concept of regulated intramembrane proteolysis. Beside SPP, several SPP homologues (SPP-like proteases; SPPL) of unknown function were identified by database searches [2,3]. We recently mutagenized the aspartate residue within the GxGD motif of SPPL2 and SPPL3 in zebrafish and found that over-expression of such dominant negative constructs phenocopies a loss of function [4]. This strongly indicates that all SPPLs are proteases of the GxGD family. Whereas SPP is known to be involved in clearance of signal peptides as well as in the processing of hepatitis C viral core protein [3], substrates for SPPLs have not been found. In order to identify putative substrates for SPPLs, we focused on SPPL2a/b, since these members of the SPPL family are of specific interest due to their unexpected localization in late endosomes and at the plasma membrane [4] (and accompanying manuscript by Friedmann et al.), which suggests a function independent of signal peptide cleavage. Using our knowledge on the subcellular localization [4] as well as previous work on the substrate requirements for SPP [5] we identified tumor necrosis factor-α (TNFα) as a candidate substrate (see also accompanying manuscript by Friedmann et al.). To verify selective TNFα processing by SPPL2a/b cell lysates from human embryonic kidney 293 cells co-expressing TNFα and SPP or the members of the SPPL family were investigated for the presence of the TNFα intracellular domain (ICD) (for a schematic model of TNFα processing and terminology see Fig. 1F). Indeed, TNFα ICD generation is only observed upon co-expression of SPPL2a or SPPL2b, whereas SPP and SPPL3 did not support TNFα proteolysis (Fig. 1A). Since SPPL2a and b appear to act redundantly (Fig. 1A) and the zebrafish genome encodes only one SPPL2 gene [4], we focused in the following exclusively on SPPL2b. Liberation of the TNFα ICD (sometimes occurring as a closely spaced doublet; Fig. 1B) is dependent on the aspartate residue within the C-terminal active site of SPPL2b, since the TNFα N-terminal fragment (NTF) accumulates upon inactivation of SPPL2b

(Fig. 1B). Moreover, as it is the case for γ-secretase substrates, the TNFα NTF co-immunoprecipitates with SPPL2b upon its inactivation, either by mutagenesis (Fig. 1C lower panel) of the critical aspartate within the GxGD motif or by use of selective inhibitors (Fig. 1C lower panel). Surprisingly, and in contrast to γ-secretase [6], significant amounts of full-length TNFα were also found to specifically co-purify with SPPL2b (Fig. 1C upper panel). Since TNFα FL also co-purifies with SPPL2b it may be cleaved to some extend directly by SPPL2b. This is consistent with the fact that SPPL2b acts independent of a docking protein, such as Nicastrin, which is responsible for the size selection of γ-secretase substrates [7].

TNFα ICD generation predicts that its C-terminal counterpart (TNFα C-domain) is secreted into the media in analogy to processing of γ-secretase substrates [1]. To facilitate isolation of TNFα C-domain, we generated TNFα ΔE, where the ectodomain is deleted. Similar constructs greatly facilitated the investigation of Notch, APP, and CD44 endoproteolysis by γ-secretase [1]. As shown in Fig. 1D (lower panel) cells expressing proteolytically active SPPL2b secreted the TNFα C-domain, whereas no such peptides but large amounts of uncleaved TNFαΔE were observed upon expression of SPPL2b D/A (Fig. 1D upper and lower panel).

After demonstrating selective TNFα ICD and TNFα C-domain generation, we investigated whether these TNFα ICDs can be generated endogenously. Since the TNFα ICD is very unstable (data not shown), which makes the *in vivo* detection difficult, we turned to in vitro assays similar to those we introduced to isolate the highly unstable APP ICD (AICD) [8]. After demonstrating specific *in vitro* generation of TNFα ICD upon overepression of SPPL2b (Fig. 1E, left panel), we repeated the same type of experiments with membrane extracts obtained from cells not transfected with SPPL2b. This revealed that under endogenous conditions, TNFα ICD is generated and its production is inhibited by the SPP/SPPL specific inhibitor $(Z\text{-}LL)_2$-ketone (this inhibitor does not block γ-secretase) [9] (Fig. 1E, right panel). Together with the data in Fig. 1A, which demonstrate selective TNFα ICD generation by SPPL2a/b, this strongly suggests that endogenous SPPL2a/b generate TNFα ICD.

Next, the cleavage sites of the ICD and the TNFα C-domain were determined by mass spectrometry and radiosequencing. Mass spectrometry of purified ICDs revealed major intramembrane cleavage sites after amino acid 34 and 39 (Fig. 2A,C). Additional peptides ranging from 25 to 29 amino acids (Fig. 2A,C) suggest degradation by a cytoplasmic carboxypeptidase (blue arrows in Fig. 2C). This is supported by the observation that TNFα ICD

can be detected as a doublet in some cases (Fig. 1B). Furthermore, over time the higher molecular weight peptide is trimmed to the smaller peptide (data not shown). Radiosequencing of the TNFα C-domain demonstrates a major cleavage after Cys 49 (evident from large peaks of ^{3}H-Phe at cycle 4, ^{3}H-Val at cycle 6, ^{3}H-Leu at cycle 1 and 2 and ^{3}H-Ile at cycle 7) as well as a lesser abundant cleavage after Leu 51 (evident from ^{3}H-Phe peak at cycle 2, ^{3}H-Val peak at cycle 4 and ^{3}H-Ile at cycle 5) (Fig. 2B,C). Both cleavage sites were also confirmed by mass spectrometry (data not shown). Similar data were obtained upon sequencing of ^{3}H-Phe-labeled TNFα C-domain generated by cells expressing endogenous SPPL2 (Suppl. Fig. S2 B). ICDs with C-termini around amino acids 49 or 51 were not obtained. Likewise, we obtained no evidence for longer TNFα C-domains.

Our data thus demonstrate a surprising similarity in the cleavage pattern of γ-secretase and SPPL2b, although both proteases show fundamental differences with respect to complex formation (γ-secretase requires PS, Aph-1, Nct, and Pen-2 [10], while SPPL2 activity is massively increased upon overexpression of a single cDNA), substrate orientation and limited sequence homology. SPPL2b, like γ-secretase, performs multiple intramembrane cleavages separated by a number of amino acids (Fig. 2C). It is even tempting to speculate that this may include an ε- (after amino acid 34) [11] and ζ-like [12] cut (after amino acid 39) as well as a γ-like cleavage (after amino acids 49 and 51) at the luminal side of the membrane. Interestingly, the latter occurs as two cuts very similar to the γ-secretase cleavage, which liberates the 40 and 42 amino acid amyloid β-peptide. Moreover, similar to γ-secretase, RIP of TNFα by SPPL2 generates a cytoplasmic cleavage product, which is required for cellular signaling (see accompanying manuscript by Friedmann et al.). Strikingly, for SPPL2b, the active sites are reversed within the membrane to accommodate type-2-oriented substrates. As for γ-secretase, the question arises of how one protease activity can cut its substrate at several sites. Like presenilin [13], the catalytically active component of the γ-secretase complex [1], SPP and all SPPLs appear to occur as homodimers [4,14]. Moreover, at least for SPP, it has been shown that dimerization facilitates the binding of an active-site directed photoaffinity labeled γ-secretase inhibitor, suggesting that dimerization is required to form the fully active catalytic site of SPP [14]. Our findings thus demonstrate a common multiple intramembrane cleavage by GxGD-type aspartic proteases, which is required for the liberation of a signaling factor into the cytoplasm and the secretion of Aß-like domains in the extracellular space.

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FIGURE LEGENDS

Figure 1: Endoproteolytic processing of TNFα

(A) The TNFα ICD is selectively generated by SPPL2a/b. **(B)** TNFα ICD production is abolished upon co-expression of an inactive SPPL2b mutant. **(C)** TNFα NTF (lower panel) and TNFαFL (upper panel) co-isolate with inactivated SPPL2b. Asterisk: IgG light chain. Full length blot including input control is presented as Suppl. Fig. S1. **(D)** Generation of the TNFα C-domain by cells expressing wt SPPL2b but not by cells expressing SPPL2b D421A. **(E)** In vitro generation of TNFα ICD. Note endogenous TNFα ICD generation (longer exposure in the right bottom panel). **(F)** Model illustrating the processing of TNFα.

Figure 2: Intramembrane cleavage of TNFα

(A) MALDI-TOF-MS analysis of TNFα ICDs. For calculation of the respective masses see Suppl. Fig. S2 A. **(B)** Cells cotransfected with TNFα and SPPL2b wt were labelled with either ^{3}H-Phenylalanine (◆), ^{3}H-Valine (▲),^{3}H-Leucine (■) or ^{3}H-Isoleucine (●). Radiosequencing of the isolated TNFα C-domain reveals a major cleavage after Cys 49 and a lesser cleavage after Leu 51. **(C)** Model showing the multiple cleavages of TNFα by SPPL2b.

MATERIALS AND METHODS

Cell culture, cDNAs and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax (DMEM; Gibco Life Sciences) supplemented with 10% fetal calf serum (Gibco Life Sciences). The cell lines stably overexpressing SPP, SPPL2b and SPPL3 have been described previously [4]. The inactive SPPL2b D421A mutant was generated by polymerase chain reaction mutagenesis and subcloned into the EcoRI/XhoI sites of pcDNA4/ myc-His (Invitrogen Life Sciences).

The TNFα cDNA was purchased from ATCC (clone AAA61198) and after addition of a N-terminal Flag tag (DYKDDDDK) after the starting methionine and a C-terminal HA tag (YPYDVPDYA) subcloned into the Hind III/ Not I sites of Peak 12. The C-terminally truncated TNFα ΔE construct was generated by inserting a stop codon after amino acid 80. This construct also contains a N-terminal Flag tag and a C-terminal HA tag. TNFα ΔE was subcloned into the Hind III/ Xho I sites of pcDNA 3.1. Hygro + (Invitrogen Life Sciences). TNFα cDNA was cotransfected with the respective SPP/ SPPLs using Lipofectamine 2000 (Invitrogen Life Sciences) according to the supplier's instructions. All cDNA constructs were sequenced for verification.

Antibodies, immunoprecipitation and immunoblotting

The monoclonal anti-myc antibody 9E10 was obtained from the hybridoma bank and the anti-HA-peroxidase coupled 3F10 antibody from Roche. The monoclonal anti-Flag M2 and the polyclonal HA 6908 antibody were obtained from Sigma (St. Louis, MO). For co-immunoprecipitation cells were lysed in assay buffer (25 mM HEPES-KOH, pH 7,6, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM DTT) containing 1% CHAPSO on ice for 30 min. Lysates were clarified by centrifugation for 20 min at 16000 x g and immunoprecipitated for 4 hrs at 4 °C.

TNFα in *vitro* assay

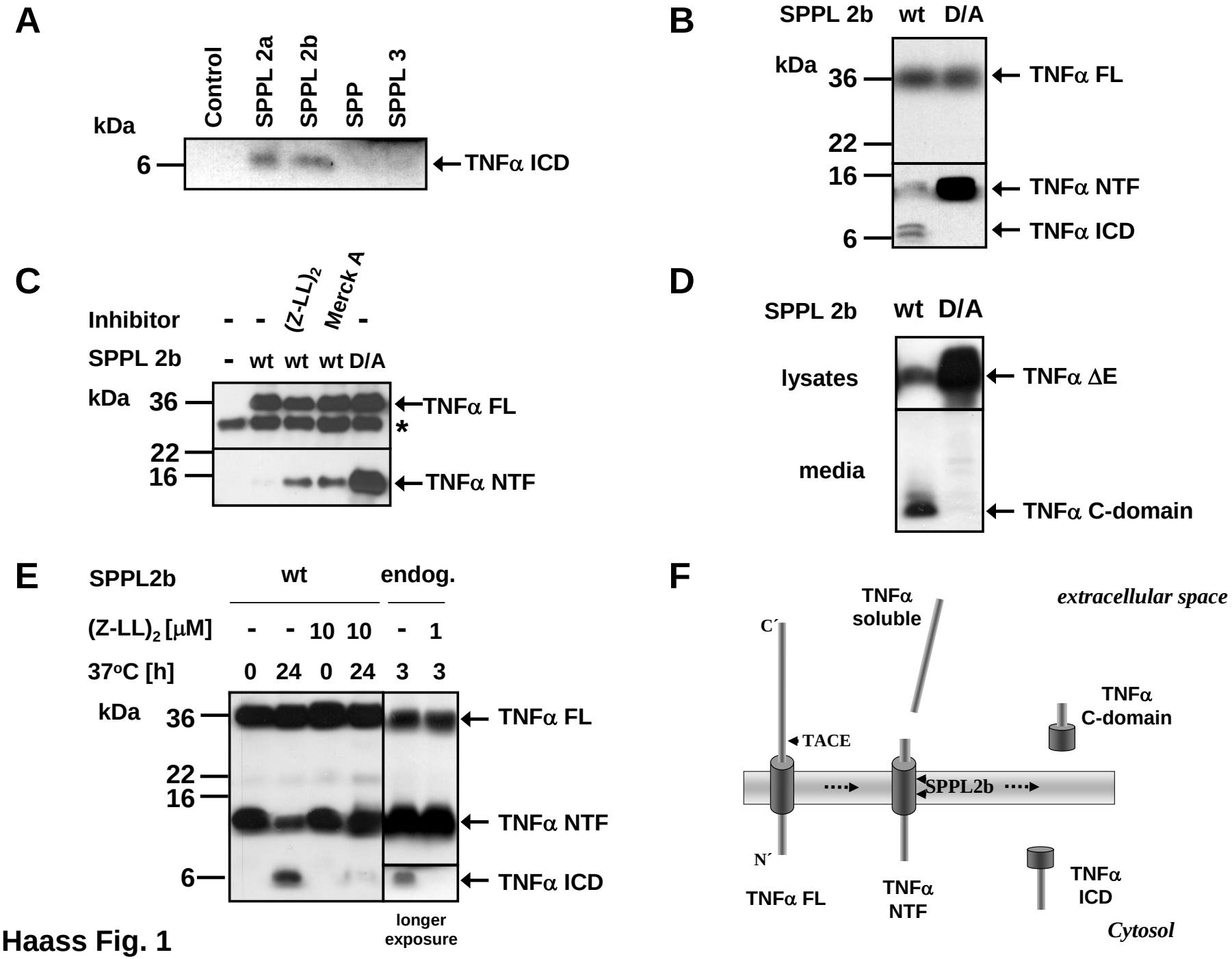
Membranes of HEK 293 cells stably expressing the indicated SPPL2b derivative and transiently expressing TNFα were prepared as described before [10]. Membranes were subsequently re-suspended in assay buffer (see above) and incubated for the respective time points at 37 ^{o}C. After incubation lipids were extracted using Chloroform/ Methanol (1:2) and proteins were subjected to SDS-polyacrylamid electrophoresis and immunoblotting. Were indicated $(Z\text{-}LL)_2$-Ketone (Calbiochem) a selective inhibitor of the SPP-family [3] was added prior to incubation at 37 ^{o}C degrees.

Mass spectrometry

TNFα in vitro assays were performed as described above. After incubation at 37 ^{o}C membranes were solubilized in STEN-Lysis buffer (50 mM Tris, pH 7,6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (NP-40), 1% Triton X-100) and TNFα ICD peptides were immunoprecipitated using anti-Flag M2 agarose, affinity gel (Sigma). Immunoprecipitations were carefully washed 6 times with 1x PBS and 2 times with dH_2O. Protein was subsequently eluted by using formic acid. Samples were subjected to analysis by mass spectrometry using as matrix α-cyano-4-hydroxycinnamic acid (Sigma) as described [10]. Mass spectra were recorded on a Voyager-DE STR mass spectrometer (Applied Biosystems) in the linear mode with external calibration.

Radiosequencing

HEK 293 cells stably expressing SPPL2b wt and transiently expressing TNFαΔE were metabolically labeled with [^{3}H]valine, [^{3}H]phenylalanine, [^{3}H]leucine or [^{3}H]isoleucine (Hartmann Analytic, Germany) for 16 hours, supernatants were collected and TNFα C-domain peptides were isolated by immunoprecipitation with antibody HA6908 (Sigma) separated by SDS-PAGE and transferred to PVDF membrane. Radiolabeled proteins were detected by autoradiography, excised, and subjected to automated Edman chemistry in an Applied Biosystems Model 475A sequenator. Amino-acid anilinothiazolinones were extracted with n-butyl chloride, transferred into 7 ml scintillation vials, and assayed for radioactivity after addition of 5 ml scintillation cocktail (Econofluor, NEN).


A
Control
SPPL 2a
SPPL 2b
SPP
SPPL 3
kDa
6
TNFα ICD
B
SPPL 2b wt D/A
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36
22
16
6
TNFα FL
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SPPL 2b - wt wt wt D/A
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TNFα FL
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SPPL 2b wt D/A
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(Z-LL)2 [μM] - - 10 10 - 1
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kDa
36
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TNFα FL
TNFα NTF
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longer exposure
F
TNFα soluble
extracellular space
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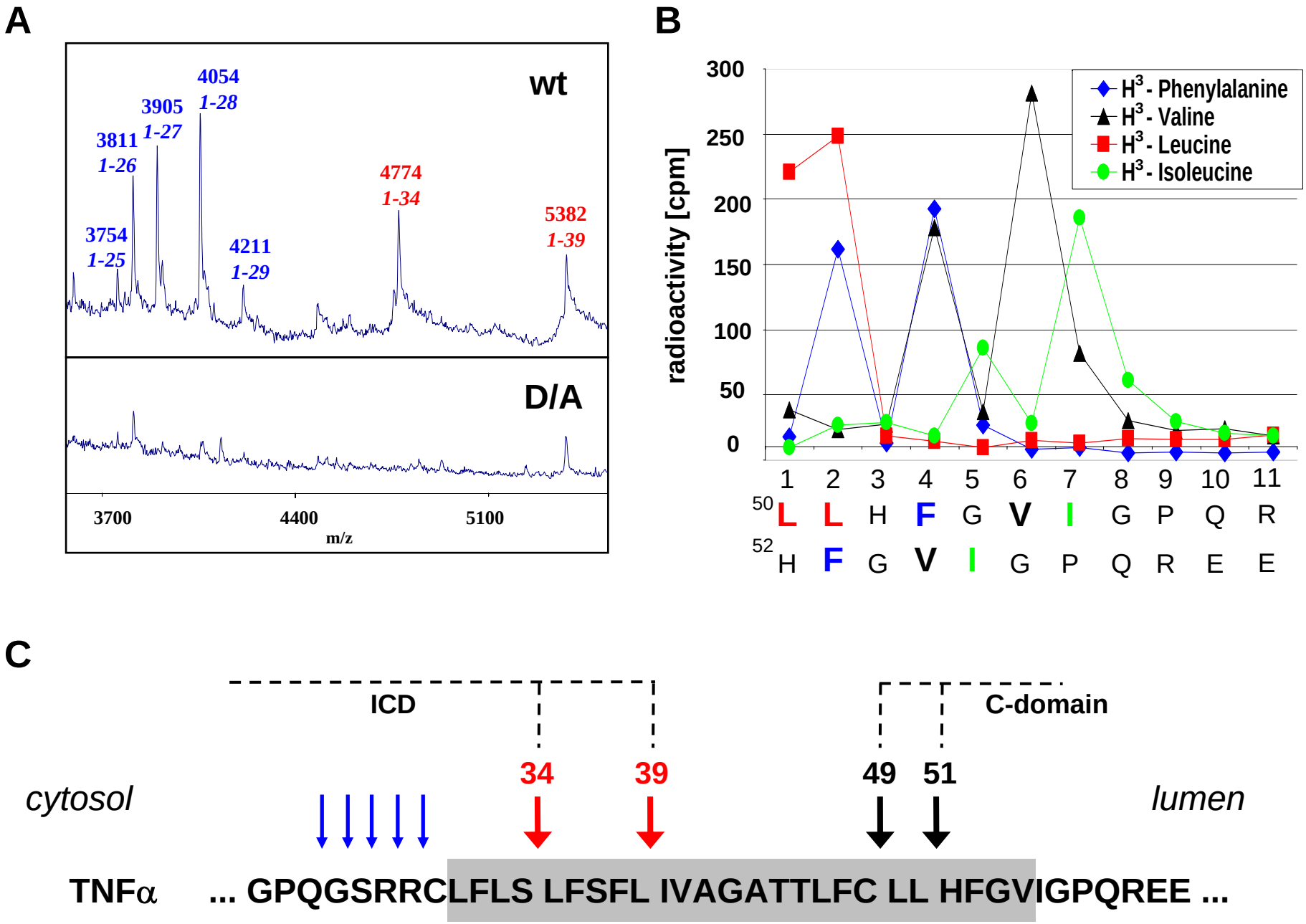
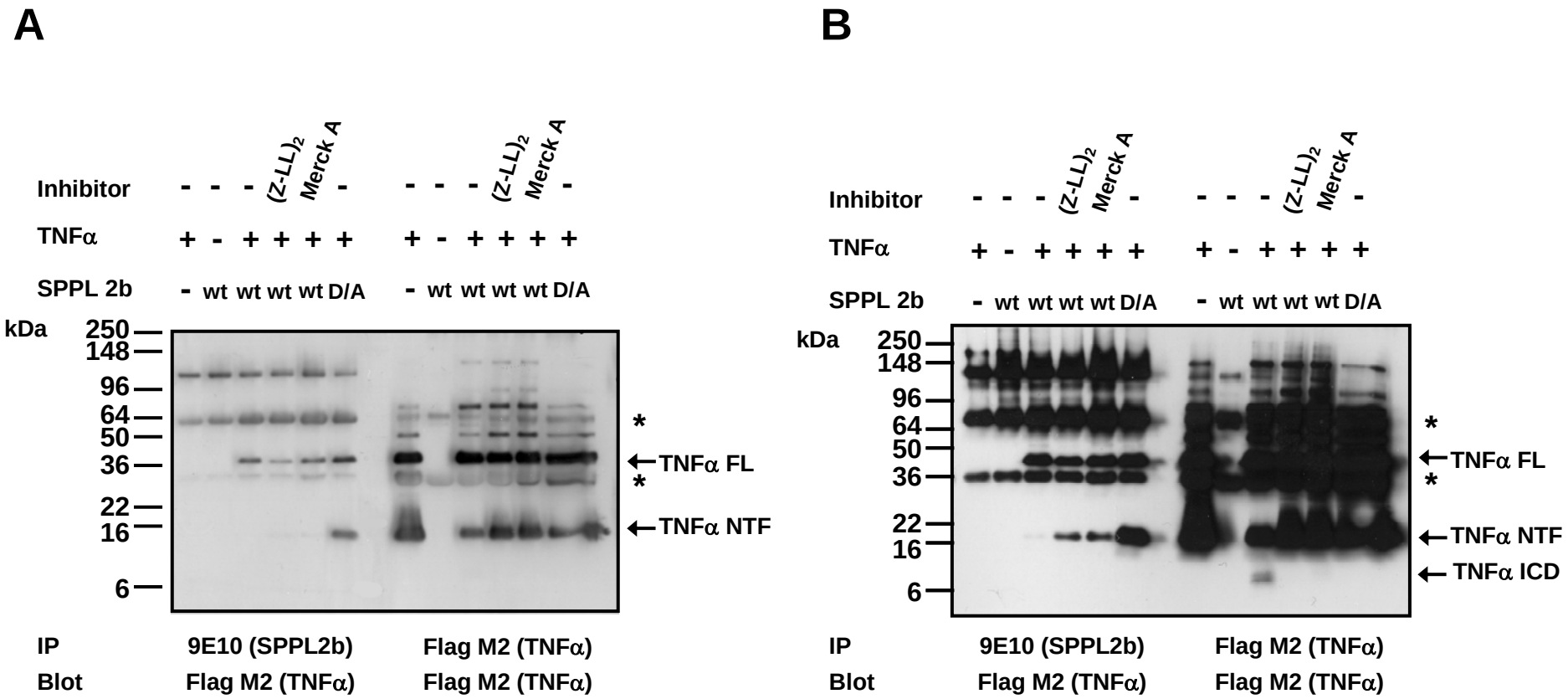
TNFα C-domain
SPPL2b
N´
TNFα FL
TNFα NTF
TNFα ICD
Cytosol

Haass Fig. 1


A
wt
D/A
4054
1-28
3905
1-27
3811
1-26
3754
1-25
4211
1-29
4774
1-34
5382
1-39
3700
4400
5100
m/z
B
radioactivity [cpm]
300
250
200
150
100
50
0
H3 - Phenylalanine
H3 - Valine
H3 - Leucine
H3 - Isoleucine
1 2 3 4 5 6 7 8 9 10 11
50 L L H F G V I G P Q R
52 H F G V I G P Q R E E
C
ICD
C-domain
34
39
49
51
cytosol
lumen
TNFα ... GPQGSRRCLFLS LFSFL IVAGATTLFC LL HFGVIGPQREE ...

Haass Fig. 2



Supplementary Information Figure S1: TNFα NTF co-isolates with inactivated SPPL2b

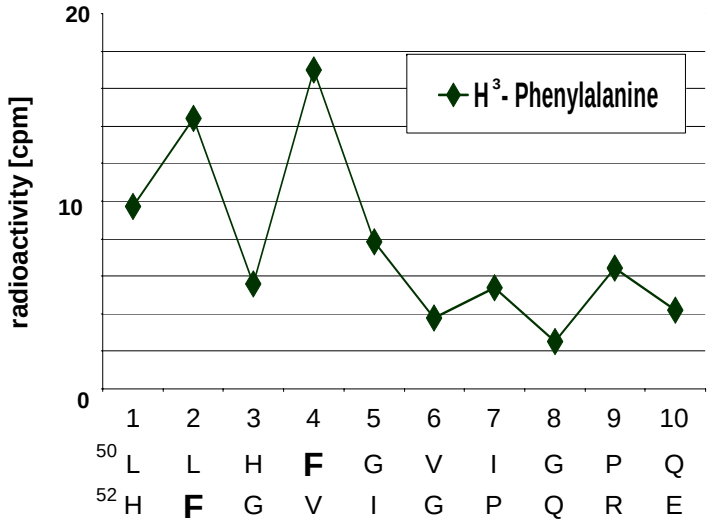
Co-immunoprecipitations of TNFα with SPPL2b (left side) reveal significant amounts of TNFa NTF co-isolating with inactivated SPPL2b. TNFαFL also co-immunoprecipitates with SPPL2b wt as well as with inactivated SPPL2b. Parallel samples were immoprecipitated with anti flag M2 agarose (Sigma) and bloted with the monoclonal M2 antibody against the N-terminal flag Tag of TNFa, to show the respective input (right side).**Asterisk**: IgG

A) short exposure, **B)** long exposure

A

TNFa species	*Sequence*	*mass calc. [Da]*	*mass calc. incl. acetyl group [Da]*
1-39	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFL	5340	5382
1-34	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSRRCLFLS	4732	4774
1-29	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSRR	4169	4211
1-28	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSR	4013	4055
1-27	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGS	3857	3899
1-26	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQG	3770	3812
1-25	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQ	3713	3755

B



Supplementary Information Figure S2:
A) MALDI-TOF-MS analysis of TNFα ICDs: Calculation of the peptide masses refers to the sequences of TNFα including a N-terminal Flag-tag (**bold**). The mass spectra show TNFα peptides, likely carrying an N-terminal acetyl group, which results in an increase in mass by 42 amu. The respective peptides were not detected upon expression of SPPL2b D421A. The smaller fragments (1-25 to 1-29) are likely due to degradation by a carboxypeptidase activity. *mass calc.: Calculated molecular mass.* **B) Radiosequencing of TNFα C-domain generated by endogenous SPPL2:** Cells only transfected with TNFα were labelled with 3H-Phenylalanine (◆). Radiosequencing of the isolated TNFα C-domain reveals identical cleavage sites as found for overexpressed SPPL2b.