

Supplementary Information

Figure Legends

Figure S1. Endogenous SPP co-purified with ectopically expressed Derlin1 and TRC8.

(A) Immunoprecipitation (IP) of HA-tagged Derlin1 (Derlin1-HA) and western blot (WB) analysis with indicated antibodies. Filled triangle, SPP monomer; open triangle, SDS-stable dimer; asterisks, cross-reacting immunoglobulin chains.

(B) IP/WB analysis of HA-tagged TRC8 (TRC8-HA) as in (A).

Figure S2. XBP1u is inserted to the ER membrane within a type II-oriented TMD.

(A) For FPP assay, XBP1u-N-GFP was coexpressed with the luminal ER marker RFP-KDEL in Hek293T cells and 18h post-transfection cells were permeabilized with digitonin and subsequently treated with the protease trypsin. GFP and RFP signals before and after trypsin treatment for 30 sec, 60 sec, 90 sec and 120 s are shown as indicated. For quantification of three independent experiments see (C). Scale bar: 5 μ m

(B) FPP assay of XBP1u-C-GFP and CD3 δ -C-GFP as in (A).

(C) Quantification of FPP assays as in (A) and (B) shows that fluorescence intensity of XBP1u-C-GFP was protected in the range of the luminal ER marker RFP-KDEL, whereas XBP1-N-GFP and CD3 δ -mCherry were accessible to exogenously added trypsin (means \pm SEM, n=3).

(D) Hek293T cells expressing XBP1u harboring an N-terminal FLAG-tag and a C-terminal HA-tag were either treated with digitonin, which permeabilizes

the plasma membrane that is rich in cholesterol but leaves the ER membrane unaffected, or Triton X-100 to fully solubilize all membranes. Subsequently, accessibility of the FLAG and HA epitope was probed by immunofluorescence microscopy. For control, cells were permeabilized with either digitonin or Triton X-100 and probed with antibodies specific for the cytosolic C-terminal tail of Derlin1 and the ER luminal chaperone BiP.

(E) *In vitro* translation of XBP1u^{R232N} in presence of ER-derived rough microsomes (RM) and proteinase K (PK) protection. Note that the panel on the right was analyzed on a Tris-bicine gel, which did not resolve the unmodified XBP1u (filled triangle) from its glycosylated form (open triangle). Filled and open circles indicate the protease protected C-terminal stub without and with N-linked glycan, respectively. Asterisk, unrelated second initiation product.

(F) Glycosylation of potential acceptor sites in XBP1u as indicated. Of note, a naturally occurring N-linked glycosylation site at position 185 is also not recognized.

(G) Treatment of Hek293T cells with epoxomicin (5 μ M) for 16 h induces the UPR, whereas (Z-LL)₂-ketone (50 μ M) and L-685,458 (5 μ M) showed no effect. Distribution of spliced and unspliced *XBP1* mRNA was assessed by RT-PCR. As positive control for chemically induced UPR, cells were treated with 5 μ g/ml tunicamycin for 90 min.

(H) Western blot analysis of endogenous XBP1u in isolated microsomal membrane fractions from HeLa and U2OS cells in presence of 5 μ M epoxomicin, 50 μ M (Z-LL)₂-ketone and 5 μ M L-685,458 for 16 h, respectively. CLIMP63 was used as loading control.

(I) Immunofluorescence analysis of untransfected Hek293T cells showed that endogenous XBP1u co-localized with the ER protein BAP31. Treatment of cells with epoxomicin (1 μ M) and L-685,458 (5 μ M) both increased steady-state level of XBP1u. Whereas inhibition of SPP with L-685,458 stabilized only the ER-resident pool, epoxomicin-treatment also led to detection of a fuzzy XBP1u signal adjacent to the ER staining (BAP31).

Figure S3. FPP assay of XBP1u^{mt1} **(A)** and XBP1u^{mt2} **(B)** as in (Fig S2A).

Figure S4. p97-associated deubiquitinases are dispensable for XBP1u turnover.

(A) Cells transfected with FLAG-tagged XBP1u or CD3 δ were co-transfected either with empty vector (mock), the dominant negative mutants Ataxin3^{C14A} or YOD1^{C160S} as indicated and subjected to cycloheximide (CHX) chase. Western blot quantification is shown (means \pm SEM, n=3).

(B) Hek293T cells expressing FLAG-tagged XBP1u were treated with 5 μ M epoxomicin and ubiquitination of XBP1u-N-FLAG was assessed by immunoprecipitation (IP) and western blotting (WB). Mono-, and poly-ubiquitinated forms are indicated with open triangles. Cross-reacting immunoglobulin chains are indicated by asterisks.

(C) Comparison of N-terminal cleavage product of XBP1u-N-FLAG generated by SPP wt with reference peptides (Rf.) corresponding to the indicated N-terminal fragment reveals main cleavage site in the center of TM domain. Position of alternative cleavage and trimming events, and whether SPP cleaves at multiple sites as it has been observed for other GxGD proteases

(Lichtenthaler et al, 2011), however, remains to be investigated. Asterisks, higher molecular weight form possibly representing ubiquitinated fragment.

(D) In order to detect the C-terminal fragment, Hek293T cells expressing XBP1u with a C-terminal HA-tag were incubated for 8 h with 5 μ M epoxomicin. Additional treatment of cells with 50 μ M (Z-LL)₂-ketone or 5 μ M L-685,458 blocked generation of this fragment. Gray triangles, putative ubiquitinated forms.

Figure S5. Membrane-tethered XBP1u triggers degradation of XBP1s.

(A) FLAG-tagged XBP1u wt and the XBP1u^{mt2} co-immunoprecipitated with HA-tagged XBP1s.

(B) Stable cells expressing XBP1s were transfected either with empty vector (mock), XBP1u wt or XBP1u^{mt2} as indicated and subjected to cycloheximide (CHX) chase. Western blot quantification of XBP1s is shown (means \pm SEM, n=3).

Figure S6. SPP^{D265A} traps XBP1u in a membrane-spanning orientation.

(A) Hek293T cells co-expressing Myc-tagged SPP^{D265A} and XBP1u harboring an N-terminal FLAG tag and a C-terminal HA tag (XBP1u-N-FLAG-C-HA) were permeabilized with either digitonin or Triton X-100 and probed with the indicated antibodies.

(B) Western blot quantification of XBP1u co-purified with SPP wt and SPP^{D265A} was normalized to the respective total fraction (means \pm SEM, n=5).

Figure S7. TRC8 and Derlin1 are required for XBP1u degradation.

(A) SPP-HA was co-expressed either with Derlin1-GFP or Derlin1^{G180V}-GFP, immunoprecipitated and analyzed by BN-PAGE and western blot (WB) analysis as shown in (Fig 1D).

(B) HA-tagged TRC8 (TRC8-HA) and TRC8 Δ R (TRC8 Δ R-HA) co-immunoprecipitate with XBP1u-N-FLAG but not with RAMP4.

(C) Hek293T cells were transfected with XBP1u-FLAG, TRC8-HA and TRC8 Δ R-HA as indicated and ubiquitination of XBP1u-N-FLAG was assessed by immunoprecipitation (IP) and western blotting (WB). Mono-, and poly-ubiquitinated forms are indicated with open triangles. Cross-reacting immunoglobulin chains are indicated by asterisks.

(D) The cellular localization of XBP1u truncated version XBP1u/194 (amino acid 1-194) and XBP1u Δ (amino acid 1-206) were analyzed by cellular fractionation.

(E) XBP1u Δ -N-FLAG is cleaved by SPP as shown by western blot analysis of Hek293T cells co-expressing SPP wt (wt) or SPP^{D265A} (DA).

(F) SPP-HA was co-expressed either with XBP1u or XBP1u Δ , immunoprecipitated and analyzed by BN-PAGE/WB analysis as shown in (Fig 1D).

Figure S8. XBP1u tail is recognized by Derlin1.

(A) CD74 wt was co-transfected either with empty vector (mock), SPP^{D265A}, TRC8 Δ R or Derlin1^{G180V} as indicated and subjected to ³⁵S-pulse-label chase analysis (means \pm SEM, n=3). Gray triangles, CD74 missing one or two N-linked glycans.

(B) GFP-tagged Derlin1^{G180V} co-immunoprecipitates with FLAG-tagged CD74-XBP1u. See Fig 8A for outline of the fusion construct.

(C) Knockdown of Derlin1 by siRNA showed no effect on XBP1u Δ turnover (means \pm SEM, n=3). CHX, cycloheximide chase; nt, non-targeting control siRNA.

Supplementary Materials and Methods

Plasmids and RNA Interference

Human SPP (ORF Gateway clone 164956320; NCBI reference sequence EL734970) was cloned into pcDNA3.1 (Invitrogen) with a triple HA-tag inserted between residue 373 and the C-terminal KKXX ER-retention signal (SPP-HA). The active site mutant D265A of SPP and the following point mutations were introduced by Quick Change site-directed mutagenesis (Stratagene, La Jolla, CA, USA). The construct encoding Myc-tagged SPP^{D265A} had been described previously (Schrul et al, 2010). Human Derlin1 (ORF Gateway clone 142772246; NCBI reference sequence BC002457), and Derlin1^{G180V} (Greenblatt et al, 2011) were cloned with an N-terminal triple HA-tag into pcDNA3.1. The catalytic mutant D271A of human SPPL3 (IMAGE 4177751) was cloned with a C-terminal HA-tag followed by a KKXX ER retention signal. For ectopic expression of human XBP1u (ORF Gateway clone 149791320; NCBI reference sequence BC000938) pcDNA3.1 expression constructs harboring an N-terminal triple HA-tag or a triple FLAG-tag were generated, respectively. In order to block IRE1-mediated splicing without affecting the amino acid sequence of XBP1u, the 3'-splice site of these constructs were blocked by mutating guanine at -1 position to cytosine

(Yoshida et al, 2001). Splice deficiency of these XBP1u constructs was confirmed in Hek293T cells. Human XBP1s (IMAGE 3856898) was cloned into pcDNA3.1 with an N-terminal triple HA-tag. To increase sensitivity of pulse-label chase experiments (see below), an (AUG)₈-sequence was inserted in frame after the triple FLAG-tag in order to encode eight additional methionines (M8). Glycosylation sites were introduced into XBP1u by mutating T212N, C215N, L220S, P221N and R232N, respectively. XBP1u^{mt1} (S187L, P188L, A192L) and XBP1u^{mt2} (Q197L, Q199L, S200L, S203L) were generated by overlap assembly PCR. To generate construct coding for N-terminally triple FLAG-tagged truncated versions of XBP1u, the respective coding region was amplified by PCR and cloned into pcDNA3.1. Human invariant chain/CD74 (High et al, 1993) was cloned with an N-terminal FLAG3-M8-tag into pcDNA3.1. The constructs coding CD74-XBP1u was cloned by overlap assembly PCR, fusing residues 1 to 56 of CD74 to residues 203 to 261 of XBP1u harboring an N-terminal FLAG3-M8-tag. XBP1u-CD74 was generated accordingly by fusing residues 1 to 202 of XBP1u to residues 57 to 216 of CD74. Plasmids encoding triple FLAG-tagged CD3 δ and RI332 (Fleig et al, 2012), ssRFP-KDEL (Lorenz et al., 2006), RAMP4 with an opsin-tag (Schrul et al, 2010), OP91* (Crawshaw et al, 2004), Ataxin3^{C14A} (Wang et al, 2006), TRC8 Δ R (Stagg et al, 2009) had been described previously. For detection by fluorescence microscopy, CD3 δ was cloned into pmCherry-N1 (Clontech). YOD1^{C160S} described previously (Ernst et al, 2009) was cloned with a C-terminal HA-tag into pcDNA3.1. To generate a UPR reporter plasmid, the 311-bp fragment of the *BiP* promoter (-304 to +7) region (Yoshida et al, 2009) was amplified by PCR from Hek293T genomic DNA and cloned into

pGL3-Basic vector (Promega) followed by firefly luciferase sequence to generate pGL3-*BiP*-FL. As a control, pCIneo-RL constitutively expressing *Renilla* luciferase was used. For stable expression, SPP-HA, FLAG3-XBP1s, FLAG3-XBP1u and FLAG3-XBP1u^{mt2} were subcloned into pcDNA5/FRT/TO (Invitrogen). The miRNA-based construct harboring a p97-specific small hairpin RNA (shRNA) was generated using the BLOCK-iT system (Invitrogen) by cloning the targeting sequence 5'-AATGAGATCCATCTTCTTGCG into pcDNA6.2-GW/EmGFP-miR as described in the manufacturer's protocol. Subsequently, the miRNA cassette was amplified by PCR and subcloned into pcDNA5/FRT/TO for the generation of inducible stable cells. The sequences of all generated plasmids were verified by DNA sequencing. Small interfering RNA (siRNA)-oligonucleotides targeting Derlin1 (ID s35605) and a non-targeting control siRNA (ID 4390843) were purchased from Ambion.

Antibodies

The following antibodies were used: mouse monoclonal anti-FLAG (M2; Sigma), rabbit polyclonal anti-FLAG (F7425; Sigma-Aldrich), monoclonal anti-FLAG M2-Peroxidase (A8592; Sigma), mouse monoclonal anti-HA (HA.11; Covance), rat monoclonal Anti-HA High Affinity (11867423001; Roche), mouse monoclonal anti GFP (11814460001; Roche), mouse monoclonal anti-BAP31 (A1/182; Alexis), mouse monoclonal anti-CLIMP63 (ALX-804-604; Enzo Life Sciences), mouse monoclonal anti-AMFR/gp78 (ab54787; Abcam), mouse monoclonal anti-Derlin1 (SAB4200148; Sigma), rabbit polyclonal anti-BiP (ab21685; abcam), mouse monoclonal anti-TRC8 (H00011236-M01; Abnova), rabbit polyclonal anti- α -actin (ab1801; Abcam), rabbit polyclonal

anti-calnexin (ab22595; Abcam), goat polyclonal anti-XBP1 (sc-32136; Santa Cruz). Rabbit polyclonal anti-SPP was a gift from Chica Schaller; monoclonal mouse anti-opsin, and polyclonal rabbit anti-p97 antibodies were obtained from Bernhard Dobberstein. The polyclonal rabbit anti-RHBDL4 has been described previously (Fleig et al, 2012).

Reverse Transcription and *XBP1* Splicing Assay

Hek293T cells were lysed and total RNA was collected by extraction with NP40 and SDS as had been described (Gough, 1988). PolyA mRNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and used as template for PCR amplification across the fragment of the *XBP1* cDNA bearing the intron target of IRE1 α ribonuclease activity (Yoshida et al, 2001). Primers used were 5'-CCTGGTTGCTGAAGAGGAG and 5'-CCATGGGGAGATGTTCTGG, leading to a 145 bp amplicon from unspliced *XBP1* and a 119 bp amplicon from spliced *XBP1* cDNA. PCR conditions were: 95°C for 5 min; 95°C for 1 min; 50°C for 1 min; 72°C for 45 sec; 72°C for 5 min with 40 cycles of amplification. PCR products were resolved on a 2.5% agarose/1x TAE gel and stained with ethidium bromide.

Peptide synthesis and Circular Dichroism (CD) Spectroscopy

Peptides were synthesized by Boc chemistry (PSL, Heidelberg, Germany) and were >90 % pure as judged by mass spectrometry. For better solubility, the hydrophobic residues of the predicted TM domains were tagged with KKK as follows. *XBP1u* wt, KKKISPWILAVLTLQIQSLISCWAFKKK; *XBP1u*^{mt1}, KKKILLWILLVLTQIQSLISCWAFKKK; *XBP1u*^{mt2},

KKKISPWILAVLTLILLILLCWAFKKK. Peptide concentrations were determined via UV spectroscopy using extinction coefficients of 11,000 $M^{-1}cm^{-1}$.

For CD spectroscopy in the TFE/buffer mixture, peptides originally dissolved in 100 % 2,2,2-trifluoroethanol (TFE) were brought to 40 % (v/v) TFE by adding 10 mM Tris/HCl pH 7.0 while maintaining a peptide concentration of 50 μM . CD spectra were obtained using a Jasco J-710 CD spectrometer from 190 nm to 260 nm in a $d = 1.0$ mm cuvette at 20°C using a response of 1 sec, a scan speed of 100 nm/min and a sensitivity of 100 mdeg/cm. Spectra were the signal-averaged accumulation of 10 scans after subtraction of the spectra recorded for pure solvent. Mean molar ellipticities were calculated using peptide concentrations determined experimentally from Trp-fluorescence of the peptides as described (Hofmann et al, 2004). Secondary structure contents were estimated by deconvoluting the spectra using the program CDNN-PEPFIT (Bohm et al, 1992). The experiments were carried out in triplicate.

Luciferase Reporter Assay

To study activity of ectopically expressed XBP1s, approx. 5×10^5 Hek293 T-Rex-FLAG-XBP1s cells were transiently transfected with pGL3-BiP-FL (0.1 μg), pCIneo-RL (0.1 μg) and XBP1u constructs or empty vector as indicated. For induction of XBP1s, 4 h after transfection cells were grown in presence of 1 $\mu g/ml$ doxycyclin. After 48 h, cells were lysed in 500 μL of passive lysis buffer (dual-luciferase reporter assay system; Promega) and cellular debris

were removed by centrifugation for 2 min at 13,000 rpm. For the analysis of the endogenous arm of the UPR, Hek293 T-REx cells, Hek293 T-REx-FLAG-XBP1u and Hek293 T-REx-FLAG-XBP1u TMD^{mt2} cells under doxycycline-induction were treated with 1 µg/mL tunicamycin for 18 h, and cells were lysed as described above. A total of 20 µl of the supernatant was mixed with 50 µl of substrates from the dual-luciferase reporter assay system. Firefly and *Renilla* luciferase activities were measured on an Appliskan Multimode Microplate Reader (Thermo). After subtraction of background noise, the activity of firefly luciferase was normalized by *Renilla* luciferase. The induction fold was analyzed by comparing -/+ doxycycline treatment for ectopically expressed XBP1s or -/+ tunicamycin treatment for endogenous UPR activation.

Supplemental References

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