Expanded View Figures

Figure EV1. Generation and validation of *SPPL2c*$^{-/-}$ mice and SPPL2c antisera.

A Scheme of the murine SPPL2c genomic locus and the employed targeting strategy of the allele designed by Velocigene. Upon deletion of the entire SPPL2c coding region, a β-galactosidase reporter gene (*β-Gal*) and a floxed neomycin resistance cassette (*Neo*) were inserted. The latter was excised by breeding with Cre-Deleter mice. Prior to analysis of *SPPL2c*$^{-/-}$ mice, the Cre transgene was removed again by breeding. Positions of primers used for genotyping or RT–PCR are indicated. For genotyping, two PCRs amplifying specific fragments from the wild type (WT) or the post-Cre Knockout allele were performed as depicted from a representative set of mice.

B HEK293 cells were transiently transfected with SPPL2c isoform A or B fused to a C-terminal Myc epitope or empty vector. Western blot detection was performed with the newly generated, affinity-purified antisera against an N- or C-terminal epitope of SPPL2c. In parallel, anti-Myc was employed as a control and cofilin was detected to confirm equal protein loading. *non-specific band.

C SPPL2c protein was not detected in the major murine tissues. Total tissue lysates from wild-type (+/+) or *SPPL2c*$^{-/-}$ mice as indicated were analysed by Western blotting using the SPPL2c antiserum generated against the C-terminus of the protein. Testis lysates were included as positive control, and Actin or EEF2 was visualised to confirm equal protein loading. *non-specific band.

D Additional deglycosylation controls for Fig 1E. To control for proper deglycosylation of lysates of SPPL2c-transfected HEK cells, shifts in the bands for Tetraspanin-3 (Tspan3) and Transferrin receptor 1 (TfR1) were detected by Western blotting using specific antibodies.

E N-glycosylation of SPPL2c isoform B is similar to that of isoform A. Lysates of HEK293 cells transiently expressing murine SPPL2c isoform B were treated with endoglycosidase H (Endo H) or peptidyl N-glycosidase F (PNGase F) prior to Western blot analysis with the SPPL2c antiserum against an N-terminal epitope, which also detects isoform B. As control for successful deglycosylation, we also visualised the lysosomal integral membrane protein LIMP-2. *, non-specific band.

F Murine SPPL2c is N-glycosylated at N106. HEK293 cells were transiently transfected with wild-type SPPL2c isoform A (Iso A) or a N106A mutant inactivating the putative N-glycosylation consensus site. To reveal N-glycosylation of the expressed proteins, lysates were treated with Endo H or PNGase F prior to Western blotting. SPPL2c was detected with the antiserum against the C-terminal epitope validate in (B) and LIMP-2 served as control for the deglycosylation. *, non-specific band.

G Additional deglycosylation controls for Fig 1F. To control for proper deglycosylation of murine testis lysates, shifts in the bands for Tspan3, TfR1 and SPPL2b were detected by Western blotting using specific antibodies. *, non-specific band.

Source data are available online for this figure.

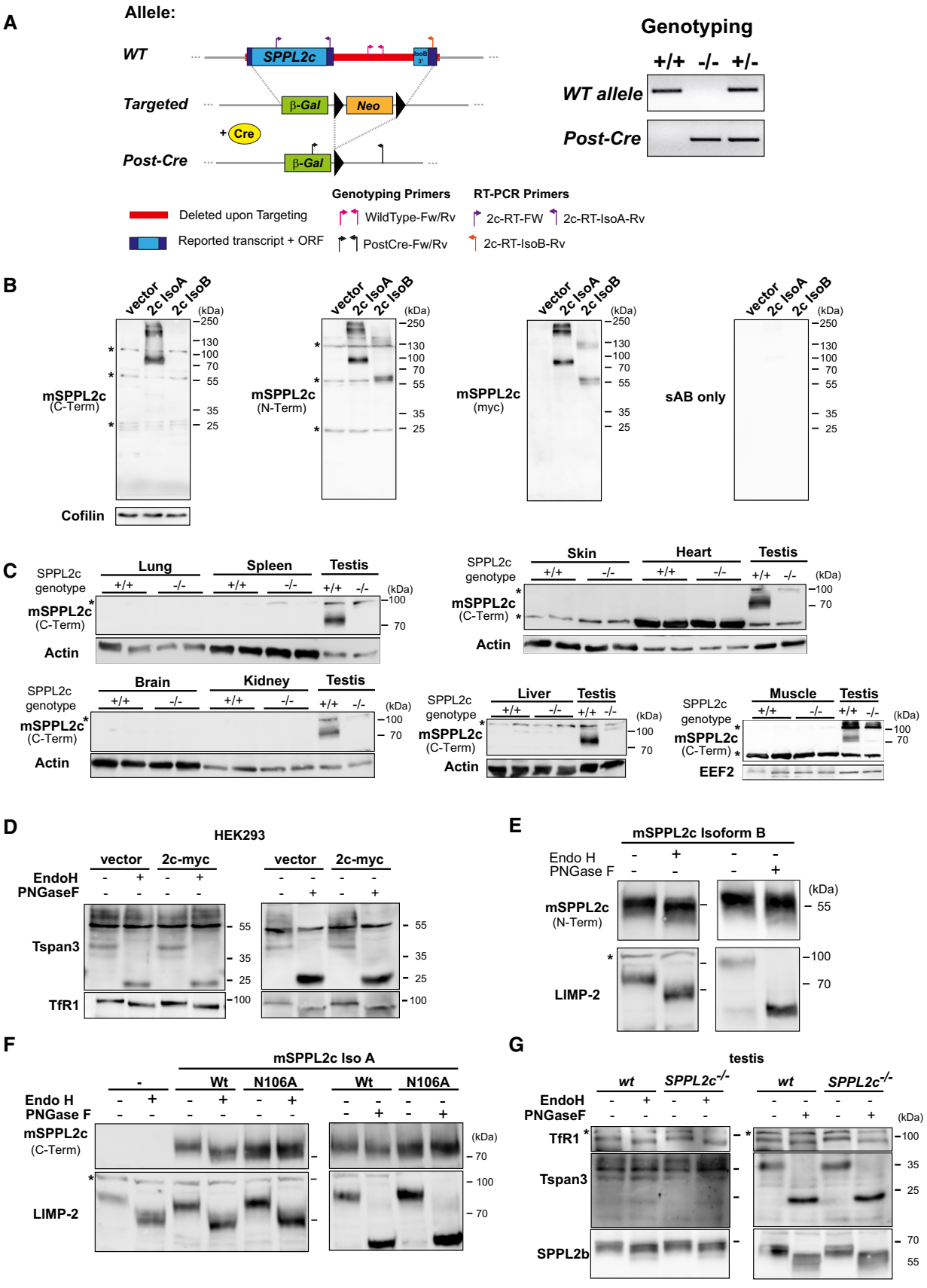


Figure EV1.

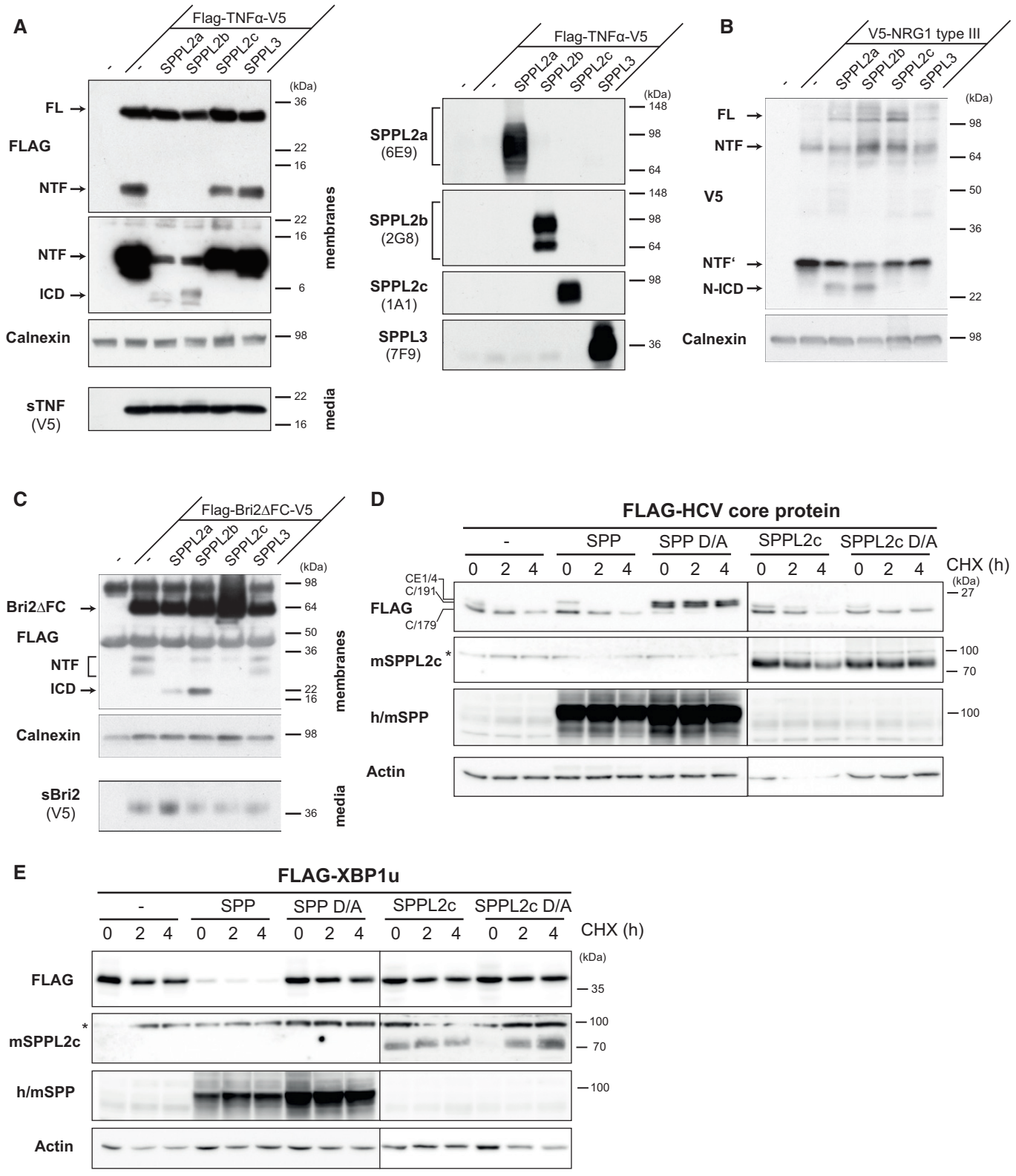


Figure EV2.

Figure EV2. SPPL2c does not cleave known substrates of SPPL2a, SPPL2b and SPP.

A–C The SPPL2a/b substrates TNF-α (A), neuregulin 1 (NRG1) type III (B) and Bri2 (C) are not processed by co-expressed SPPL2c. T-Rex™-293 cells stably expressing catalytically active human SPPL2a, SPPL2b, SPPL2c or SPPL3 under a doxycycline-inducible promoter were transiently transfected with the indicated epitope-tagged substrates. Expression of the proteases upon doxycycline induction was confirmed by Western blotting using the indicated protein-specific antibodies. Full-length (FL) and processed forms (NTF, ICD) of substrates were detected by Western blotting of total membrane preparations. To detect Bri2ΔFC and N-terminal fragments (NTFs), an immunoprecipitation with anti-FLAG was performed prior to Western blot analysis. In addition, cell culture media of TNF-α- and Bri2ΔFC-expressing cells (A, C) were analysed to confirm proteolytic release of the ectodomain (sTNF-α, sBri2), which is a prerequisite for the cleavage by SPPL2a/b. Western blot detection with anti-FLAG (TNF-α, Bri2) or anti-V5 (NRG1 type III; sTNF-α and sBri2) directed against the respective tag-epitopes was conducted. By this means, full-length forms of the substrates as well as the N-terminal fragments (NTF) derived from processing of the ectodomain (sTNF-α, sBri2) or the hairpin structure of NRG1 type III were revealed. Upon expression of SPPL2a or SPPL2b, an intracellular domain (ICD) was released from all three substrates, which was not observed upon SPPL2c expression.

D, E HEK293 cells were transiently transfected with N-terminally FLAG-tagged HCV core-E1/4 protein (D) or XBP1u (E) either alone or in combination with active or inactive (D/A) murine SPP or SPPL2c. To compare the turnover and stability of the respective substrates in the absence or presence of the protease, cycloheximide (CHX) chase experiments were performed. Therefore, transfected cells were treated for 0, 2 or 4 h with 100 μg/ml CHX to block protein synthesis prior to cell harvest. The overexpressed murine SPPL2c was detected with anti-SPPL2c (C-terminal epitope). For detection of overexpressed murine (m) SPP, a polyclonal antiserum cross-reacting with endogenous human SPP was employed. In (D), the different processed forms (C/191, C/179)) of the HCV core-E1/4 protein (CE1/4) were labelled as described previously [13]. In these experiments, we predominantly detected a dimeric form of SPP. *, non-specific band.

Source data are available online for this figure.

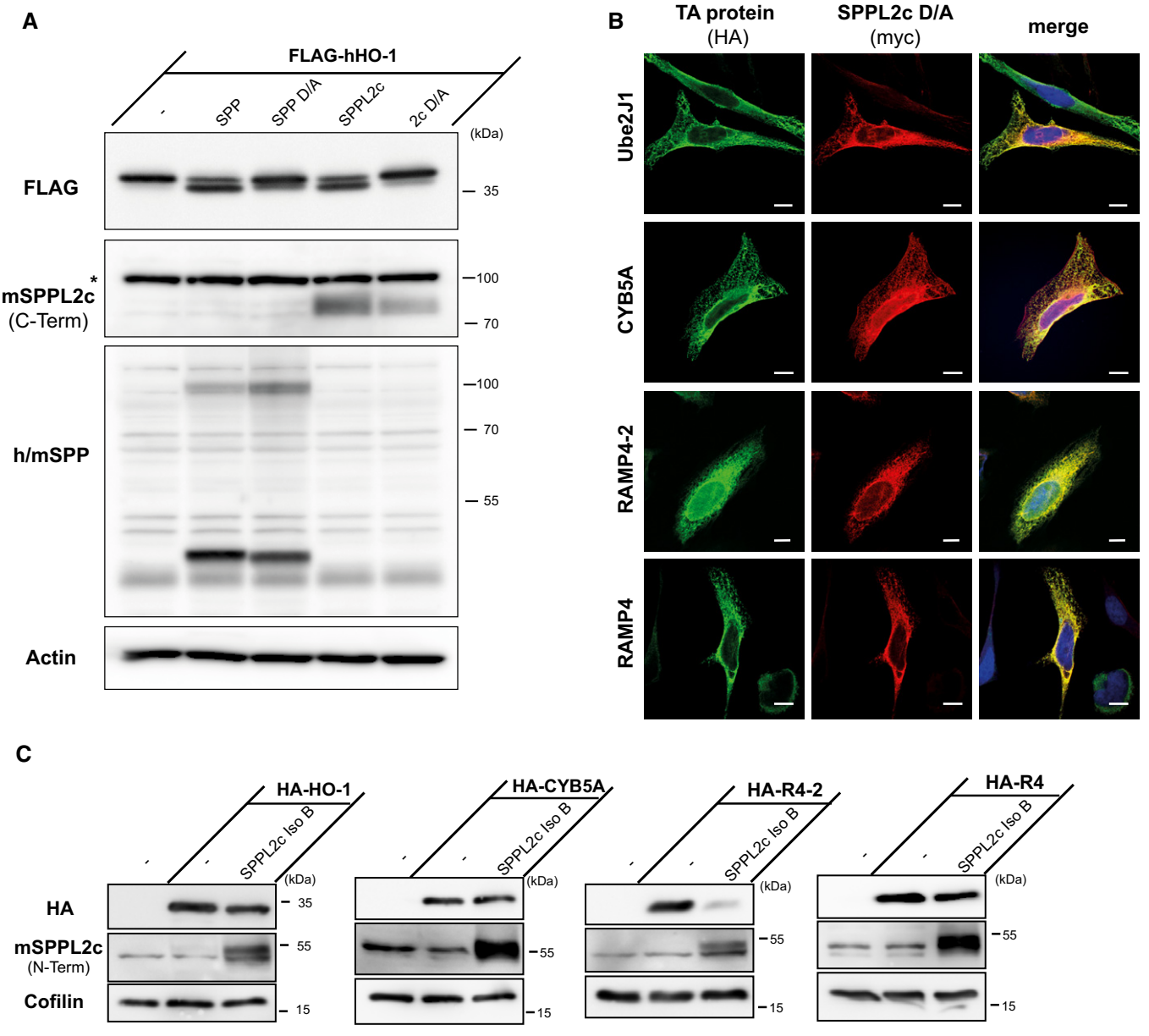


Figure EV3. SPPL2c cleaves selected tail-anchored (TA) proteins.

A Ectopically expressed murine SPPL2c also cleaves human heme oxygenase 1 (HO-1). HEK293 cells were transiently transfected with human N-terminally FLAG-tagged HO-1 (FLAG-hHO-1) alone or in combination with active or inactive (D/A) murine SPP or SPPL2c. Western blot analysis was performed with anti-FLAG. SPP was detected with a polyclonal serum detecting the overexpressed murine and the endogenous human (h/m) SPP. SPPL2c was visualised with the antiserum against the C-terminus of the protein. To control for equal protein loading, actin was visualised. *, non-specific band.

B All TA proteins analysed in Fig 2 co-localise with SPPL2c. HeLa cells were transiently transfected with N-terminally HA-tagged Ube2J1, CYB5A, RAMP4-2 or RAMP4 together with inactive (D/A) murine SPPL2c fused to a Myc epitope at its C-terminus. Substrates and the inactive proteases were visualised with anti-HA and anti-Myc, respectively, in conjunction with fluorophore-conjugated secondary antibodies. Scale bars, 10 μm.

C Substrate specificity of SPPL2c Isoform B is similar to isoform A. The indicated proteins were transiently expressed in HEK293 cells either alone or together with murine SPPL2c isoform B (IsoB). The expressed substrate candidate proteins were detected with anti-HA. Expression of SPPL2c was confirmed with anti-SPPL2c directed against an N-terminal epitope. Cofilin was used to confirm equal protein loading.

Source data are available online for this figure.

Figure EV4. Analysis of *SPPL2c*$^{-/-}$ testis.

A No change in SPP, SPPL2a and SPPL2b expression in testis of SPPL2c-deficient mice. Total RNA was isolated from testis of wild-type and *SPPL2c*$^{-/-}$ mice (n = 3). In the resulting cDNA, *SPP*, *SPPL2a* and *SPPL2b* transcript abundance was quantified by qRT–PCR and normalised to that of *Tuba1a*. Bars depict mean values normalised to those of wild-type samples ± SD.

B Western Blot analysis of SPP protein in total lysates from wild-type and *SPPL2c*$^{-/-}$ testis. To facilitate identification of murine endogenous SPP, HEK293 cells transiently expressing murine SPP carrying a C-terminal Myc epitope or just transfected with empty vector (–) were analysed. Due to the strong expression in these cells, only 1/8 of the protein amount was loaded from these samples as compared to the testis lysates. In addition to the SPP monomer (open arrowhead), we predominantly detected dimeric SPP (closed arrowhead), which exhibits a high stability under these experimental conditions. Actin was detected as control for protein loading.

C β-galactosidase reporter expression in *SPPL2c*$^{-/-}$ testis. Total lysates from wild-type and *SPPL2c*$^{-/-}$ testis were analysed by Western blotting for *E. coli* β-galactosidase, SPPL2c and Actin.

D Gating strategy for sorting of individual germ cell populations based on their DNA content as determined by Hoechst 33342 staining. Cells were first roughly gated based on their forward (FSC) and sideward scatter (SSC) prior to exclusion of PI-positive dead cells. Finally, 1C (haploid cells including spermatids), 2C (spermatogonia, secondary spermatocytes, Sertoli cells, other somatic cells) and 4C cells (primary spermatocytes, G2 spermatogonia) were gated based on their individual Hoechst staining as depicted.

E Immunohistochemical visualisation of SPP in paraffin sections from Bouin-fixed wild-type and *SPPL2c*$^{-/-}$ testis. Prior to immunostaining, sections were subjected to epitope retrieval in citrate buffer. A polyclonal SPP antiserum generated against an internal epitope of murine SPP was used as primary antibody. As a control for antibody specificity, stainings with normal rabbit polyclonal IgG were performed in parallel. Scale bars, 100 μm.

F The reduction of SPPL2c-deficient spermatids is not caused by apoptosis. TUNEL staining was performed on paraffin sections from wild-type and *SPPL2c*$^{-/-}$ testis (n = 3 per genotype), as shown here from a representative example. Labelled apoptotic cells were observed among the spermatogonia (arrow) and primary spermatocytes (arrowhead), however at a similar frequency in both genotypes. No relevant labelling was seen in spermatids neither in wild-type nor in *SPPL2c*$^{-/-}$ sections. Scale bars, 100 μm.

Source data are available online for this figure.

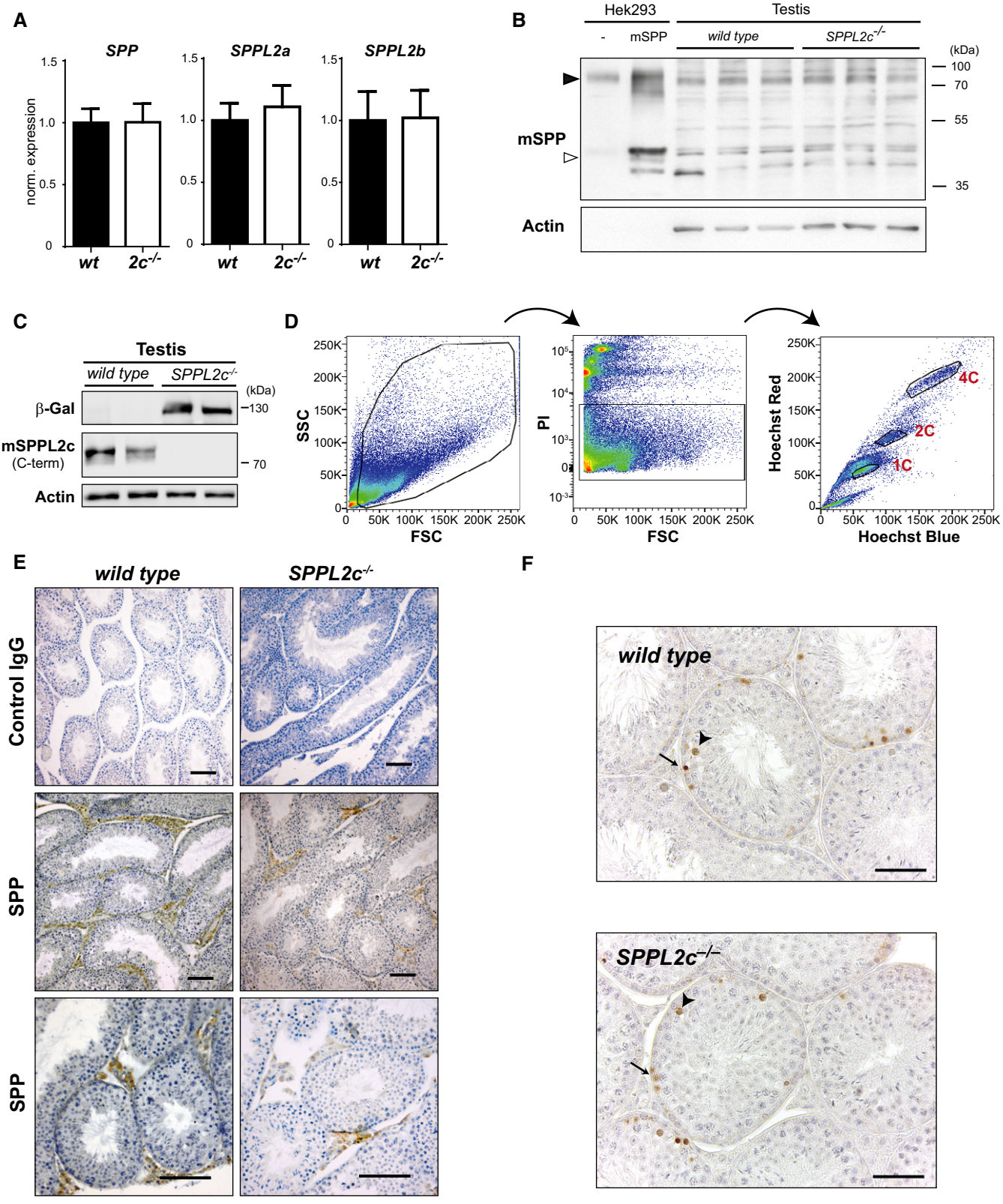
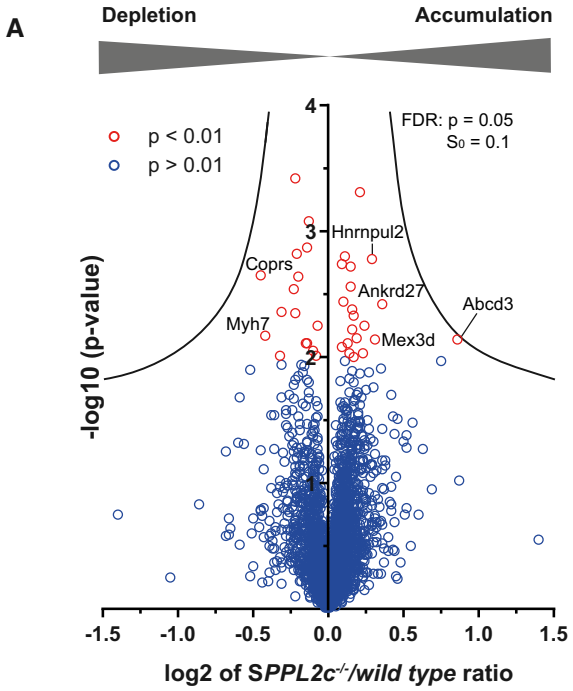
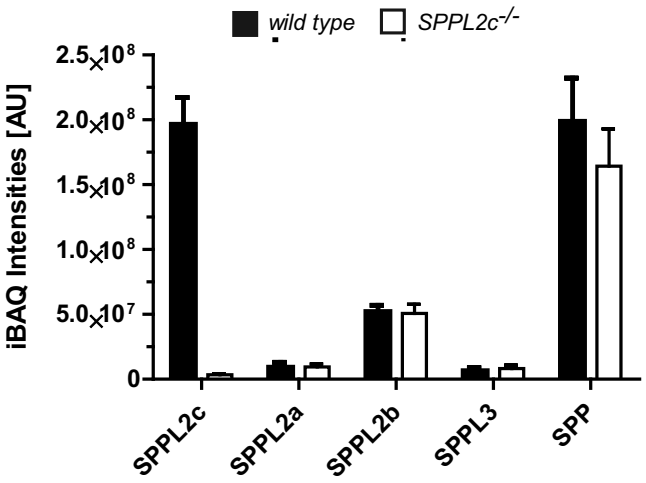

A
SPP
SPPL2a
SPPL2b
norm. expression
1.5
1.0
0.5
0
wt
2c-/-
B
Hek293
Testis
-
mSPP
wild type
SPPL2c-/-
(kDa)
100
70
55
35
mSPP
Actin
C
Testis
wild type
SPPL2c-/-
(kDa)
β-Gal
130
mSPPL2c
(C-term)
70
Actin
D
SSC
FSC
PI
Hoechst Red
Hoechst Blue
4C
2C
1C
E
wild type
SPPL2c-/-
Control IgG
SPP
SPP
F
wild type
SPPL2c-/-

Figure EV4.





C

SPPL2c Isoform A

```
        10         20         30         40         50
MACLGSLHPL GSLLLLFLLL LLSPEARGEY GLVRVVSKNW SKDYCVLYSS
        60         70         80         90        100
DYVNLPRDLH HAPLLSLHDG TKTPWCPDED SFHQAQDSSP RQRPLHQTTT
       110        120        130        140        150
MVTRGNCSFY AKGWLAQDQG AQGLLIVSRA RNQQCSDTIS KPQDPSKPWP
       160        170        180        190        200
ALTIPVAVLR YTDMLDIVSH TYGDTDVRVA MFAPLEPVTD YNMAIIFILA
       210        220        230        240        250
VGTVAAGGYW AGLMEANKLQ RRQAQRGGGL GGHNQQQTVA AERSQRAWED
       260        270        280        290        300
DDFEDAPMDF TPAMTGAVVT MSCSIMILLY FFYDCFVYVM IGIFSLGAST
       310        320        330        340        350
GLYSCLAPIL CHLPLWRYQW VLPGQRVSVT WPLLLLAGLC AMVTVLWVIH
       360        370        380        390        400
RNEDHWAWLL QDTLGVAYCL FVLRRVRLPT FKNCTLFLLA LLAFDVFFVF
       410        420        430        440        450
ITPLFTKTGE SIMVEVASGP ADSSSHERLP MVLKVPRLSF SALTLCNQPF
       460        470        480        490        500
SILGFGDIVV PGFLVAYCHR FDMQVQSRQV YYMACTVAYA VGLLVTFVAM
       510        520        530        540        550
ILMQMGQPAL LYLVSSTLLT SLAVATCRQE FTLFWTGQGR AKIPAEPVAQ
       560        570        580        590        600
PCIASAVGSK MKLEDAKDSR TTNRFEQAVD GESGDLESST GDDMAEMVTL
       610        620        630        640        650
SEDEATSPEG HSESSEGWSD TNLDPNELPS GSPMALEAML IPLIQPIPHP
       660        670        680        690
SELGHIRTQS RVHDSSLPWM GLHKRKGLKV KKSMSAQAPL
```

D

SPPL2c Isoform B

```
        10         20         30         40         50
MACLGSLHPL GSLLLLFLLL LLSPEARGEY GLVRVVSKNW SKDYCVLYSS
        60         70         80         90        100
DYVNLPRDLH HAPLLSLHDG TKTPWCPDED SFHQAQDSSP RQRPLHQTTT
       110        120        130        140        150
MVTRGNCSFY AKGWLAQDQG AQGLLIVSRA RNQQCSDTIS KPQDPSKPWP
       160        170        180        190        200
ALTIPVAVLR YTDMLDIVSH TYGDTDVRVA MFAPLEPVTD YNMAIIFILA
       210        220        230        240        250
VGTVAAGGYW AGLMEANKLQ RRQAQRGGGL GGHNQQQTVA AERSQRAWED
       260        270        280        290        300
DDFEDAPMDF TPAMTGAVVT MSCSIMILLY FFYDCFVYVM IGIFSLGAST
       310        320        330        340        350
GLYSCLAPIL CHLPLWRYQW VLPGQRVSVT WPLLLLAGLC AMVTVLWVIH
       360        370        380        390        400
RNEDHWAWLL QDTLGVAYCL FVLRRVRLPT FKNCTLFLLA LLAFDVFFVF
       410        420        430        440        450
ITPLFTKTGE SIMVEVASGP ADSSSHERLP MVLKVPRLSF SALTLCNQPF
       460        470        480        490        500
SILGFGDIVV PGFLVAYCHR FDMQVQSRQV YYMACTVAYA VGLLVTFVAM
       510        520        530        540        550
ILMQMGQPAL LYLVSSTLLT SLAVATCRQE FTLFWTGQGR AKIPAEPVAQ
       560        570        580        590        600
PCIASAVGSK MKLEDAKDSR TTNRMPEEDF V
```

Figure EV5. Identification of endogenous SPPL2c substrates by proteomics.

A A label-free quantitative proteome analysis of cytosolic fractions from wild-type and *SPPL2c*$^{-/-}$ testis (n = 5) was performed. For each quantified protein, a log2 intensity ratio between wild-type and SPPL2c-deficient samples was calculated. In the depicted volcano plot for each protein, the negative log10 of the *P*-value (*y*-axis) is plotted versus the log2 ratio of intensity values of *SPPL2c*$^{-/-}$ versus *wild-type* samples (*x*-axis). All proteins above the significance level of $P < 0.01$ (Student's *t*-test) are coloured in red. The hyperbolic curve indicates the threshold for a permutation-based FDR correction for multiple hypotheses with $P = 0.05$ and s0 = 0.1.

B Comparison of the abundance of different SPPL proteases and SPP in membrane preparations from wild-type and *SPPL2c*$^{-/-}$ testis. Protein levels were estimated on the basis of the MS data using intensity-based absolute quantification (iBAQ). Mean ± SD, n = 5 per genotype.

C, D Mapping of peptides identified by mass spectrometry in testis membrane preparations from wild-type mice to isoform 1 (C, UniProt A2A6C4-1) and isoform 2 (D, UniProt A2A6C4-2) of murine SPPL2c. Identified peptide sequences are indicated in yellow. Isoform-specific peptides for the respective isoforms of SPPL2c are marked in turquoise. Transmembrane domains as annotated in UniProt are indicated in purple.