JEM

# Supplemental material

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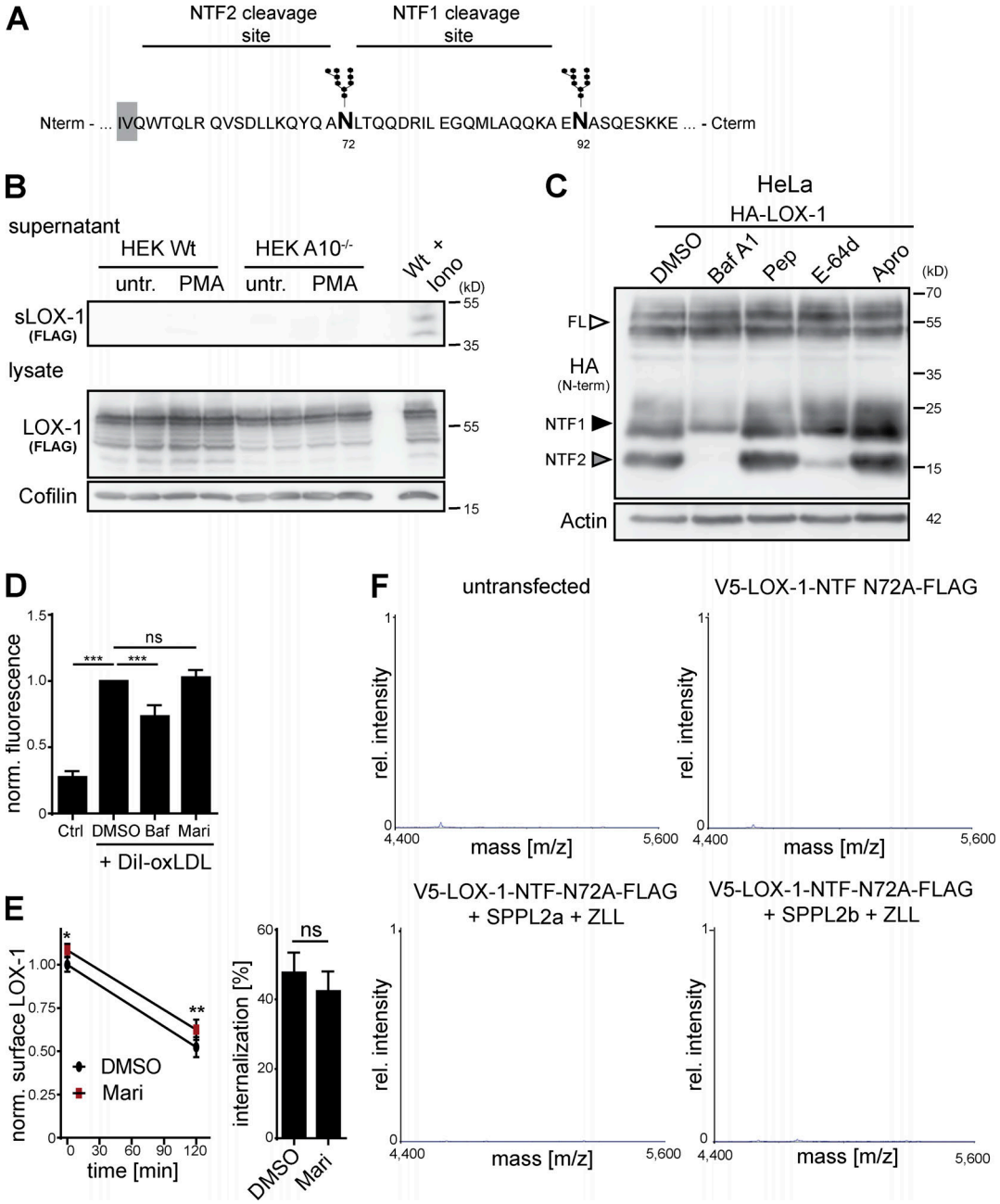



Figure S1. **Characterization of the LOX-1 NTFs. (A)** Murine LOX-1 harbors two potential N-glycosylation sites in its extracellular juxtamembrane stalk region at position N72 and N92, respectively, which were used to determine the part of the protein where LOX-1 NTF1 and NTF2 are generated. Residues being part of the LOX-1 transmembrane segment are highlighted in gray. **(B)** WT or ADAM10-deficient HEK cells transfected with a C-terminally 3xFLAG-tagged LOX-1 variant were cultivated for 30 min in prewarmed DMEM and subsequently treated for 30 min with 100 nM PMA or 1 µM ionomycin (iono) as control. sLOX-1 was recovered from conditioned media by TCA precipitation and detected by Western blotting using the anti-FLAG M2 antibody. Cofilin served as loading control. **(C)** HeLa cells were transiently transfected with HA-LOX-1 and subsequently treated with bafilomycin a1 (Baf a1; 100 nM), pepstatin A-methyl ester (Pep,; 10 µM), E-64d (40 µM), or Aprotinin (Apro; 1 µM) for 24 h. Changes in the levels of different LOX-1 processing products were analyzed by Western blotting using an antibody detecting the HA-epitope fused to the N terminus (Nterm) of LOX-1. **(D)** iMAECs were preincubated with 100 nM bafilomycin a1, 10 µM marimastat, or DMSO as control and subsequently treated for 4 h with 10 µg/ml DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)-oxLDL. oxLDL uptake was finally quantified by fluorescence detection. N = 3, *n* = 9. One-way ANOVA with Dunnett's post hoc test. ***, P ≤ 0.001; ns, not significant. **(E)** iMAECs stably overexpressing LOX-1 were pretreated with DMSO or 10 µM marimastat (Mari) and then labeled for 30 min on ice with an antibody against the C terminus of the receptor in presence of the inhibitor. Cells were subsequently chased for 2 h at 37°C to allow internalization and analyzed for residual LOX-1 at the cell surface by detection with a fluorophore-conjugated secondary antibody followed by flow cytometric analysis. Surface LOX-1 levels were normalized to those of DMSO-treated 4°C controls. The bar diagram shows mean internalization efficacy of LOX-1. N = 2, *n* = 7. Student's *t* test. **, P ≤ 0.01; *, P ≤ 0.05; ns, not significant. **(F)** To verify specificity of the peaks observed upon coexpression of active SPPL2 proteases with the LOX-1 model substrate V5-LOX-1-NTF-N72A-FLAG (Fig. 3, A–C), FLAG-tagged C-peptides were also recovered from media of untransfected or only substrate-expressing cells. Additionally, activity of SPPL2a or SPPL2b was blocked by administration of 20 µM ZLL as indicated. N, the number of independent experiments; *n*, the number of individual samples for quantification. All data are shown as mean ± SD.

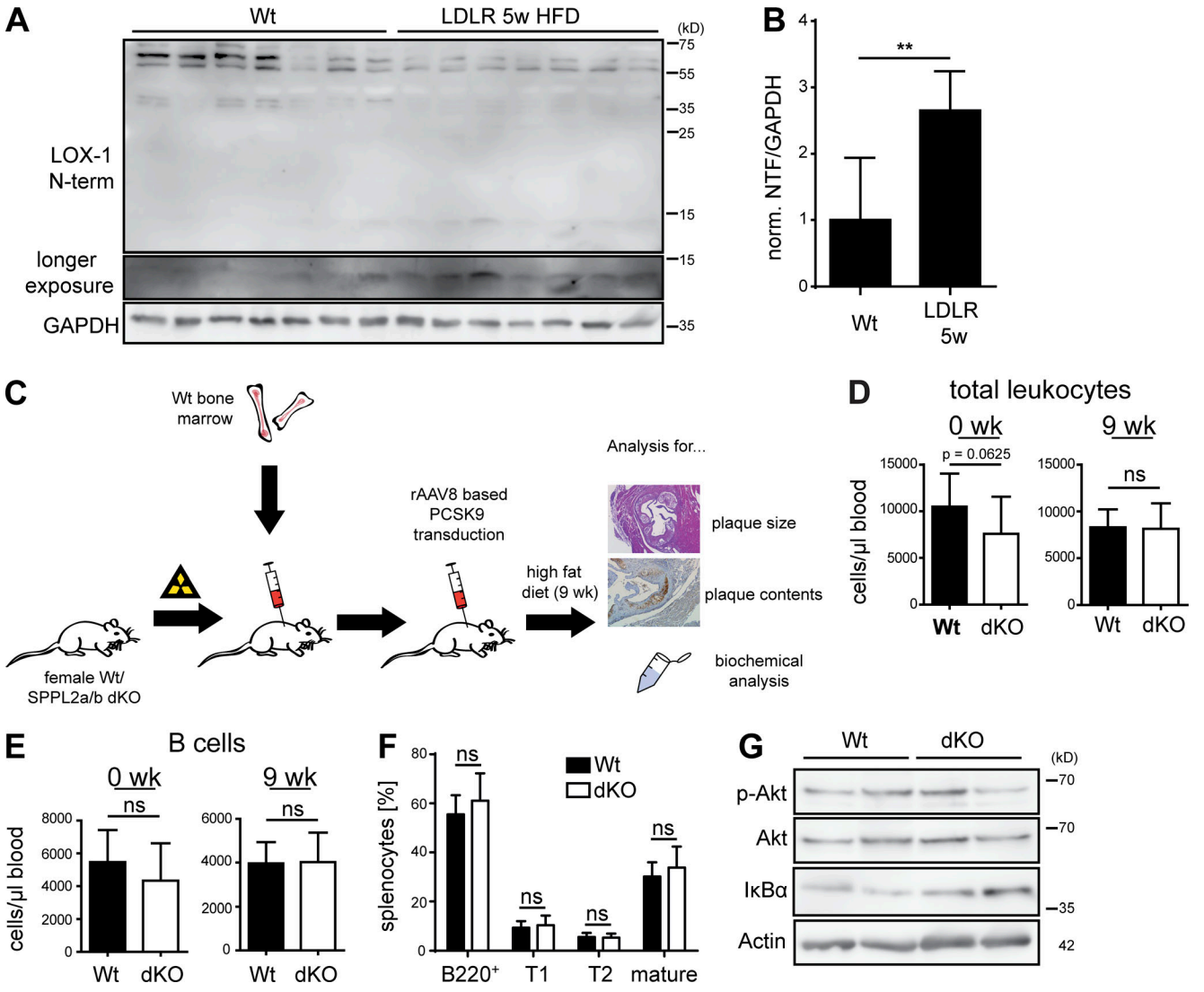
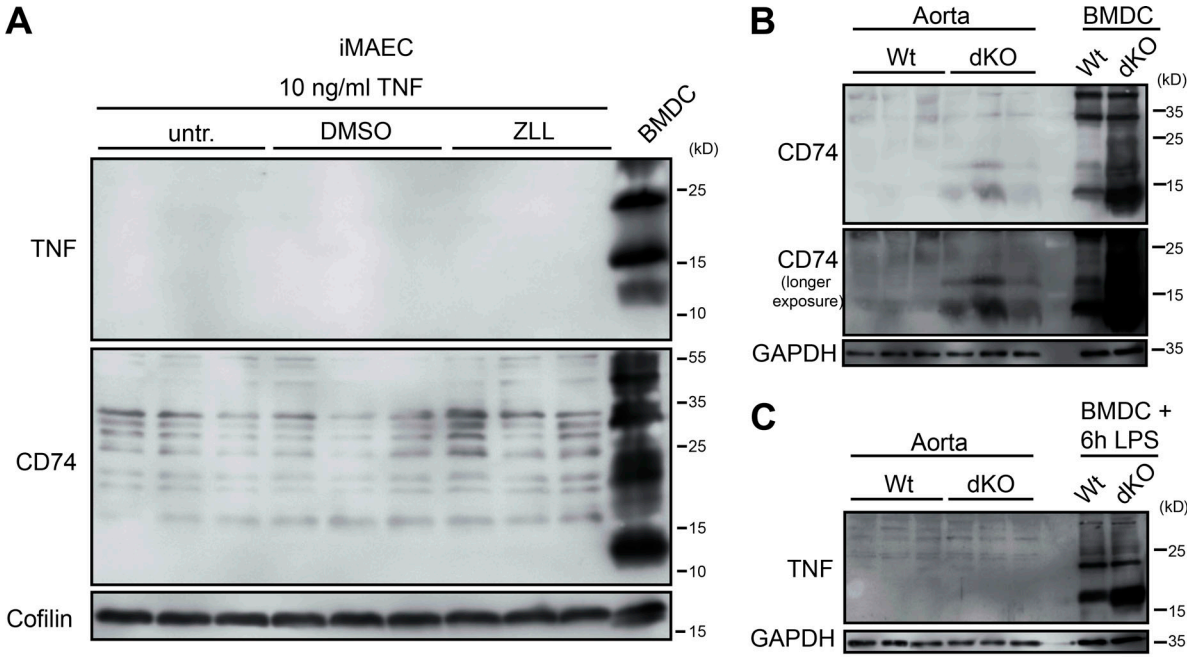



Figure S2. **Atherosclerosis study in WT and SPPL2a/b-deficient mice. (A)** Aortic arches of WT animals on a regular chow diet or *Ldlr*$^{-/-}$ mice, which had received an HCD for 5 wk, were lysed and analyzed for LOX-1 full-length and NTF levels by Western blotting. GAPDH was detected to control protein loading. **(B)** Quantification of NTF/GAPDH levels from *n* = 7 animals per genotype. Student's *t* test. **(C)** Experimental design of the performed study. Female WT or SPPL2a/b dKO mice were subjected to irradiation and bone marrow transplantation with male WT bone marrow to correct the immunological phenotype of SPPL2a/b-deficient mice. Finally, hypercholesterolemia was induced by adeno-associated virus–mediated overexpression of the PCSK9 D377Y gain-of-function mutant to deplete LDL receptors and administration of an HCD. After 9 wk, mice were sacrificed and analyzed for development of atherosclerotic lesions. **(D–F)** Bone marrow transplantation restored normal hematopoesis in irradiated WT and SPPL2a/b-deficient mice. **(D)** Total leukocyte counts were analyzed in whole blood obtained from tail vain bleedings after 0 or 9 wk of HCD. N = 2, *n* = 9. Student's *t* test. **(E)** B220$^{+}$ B cells were quantified by flow cytometry in the same blood samples as in B from WT or SPPL2a/b-deficient mice. N = 2, *n* = 9. Student's *t* test. **(F)** Total B cells (B220$^{+}$) as well as T1, T2, and mature B cell subsets were analyzed by flow cytometry applying CD21-PE, CD24-APC, and B220-PE-Cy7 antibodies in splenocytes of mice at the end of the experiment. The proportion (%) of the respective populations among all viable splenocytes was quantified and depicted as mean ± SD. N = 2, *n* = 18–20. Student's *t* test. **(G)** A potential activation of the Akt and NFκB pathway was evaluated in aortic lysates from WT and dKO mice from the atherosclerosis experiment by Western blotting. Phosphorylated and total Akt as well as IκBα were detected. Actin was used to control equal protein loading. As described in Materials and methods, phosphorylated and total forms of Akt were detected from the same membrane. After detection of pAkt, membranes were stripped and reprobed to detect total Akt. **, P ≤ 0.01; ns, not significant. N, the number of independent experiments; *n*, the number of individual samples for quantification.




**D** substrates

| protein | identifier | Wt1/dKO1 | Wt2/dKO2 | Wt3/dKO3 | mean Wt/dKO |
|---|---|---|---|---|---|
| Itm2b | O89051 | 1.216 | 1.053 | 0.988 | 1.09 |
| TfR1 | Q62351 | 0.853 | 0.961 | 0.929 | 0.91 |
| Tmem106b | Q80X71 | 0.972 | 0.889 | 1.194 | 1.01 |

Figure S3. **Cleavage of established SPPL2 substrates in nonhematopoietic cells. (A)** iMAECs were treated for 16 h with 10 ng/ml TNF to induce expression of SPPL2 substrates. Simultaneously, cells were coincubated with DMSO or ZLL to block SPPL2 protease activity. Finally, intramembrane proteolysis of TNF or CD74 was monitored by Western blotting using antibodies directed against the N termini of both proteins. A lysate from WT BMDCs was used as positive control. **(B and C)** Expression and potential proteolysis of CD74 (B) and TNF (C) was analyzed by Western blotting in aortae of WT or SPPL2a/b dKO mice using antibodies directed against the N termini of the individual proteins. For comparison, lysates of either LPS-activated (500 ng/ml, 6 h; C) or unstimulated (B) WT or SPPL2a/b double-deficient BMDCs were loaded on the same gels. **(D)** Abundance ratios of established SPPL2 substrates in aortae comparing WT and SPPL2-deficient mice as determined by quantitative proteomics.

**Tables S1 and S2 are provided online as Excel files. Table S1 shows endothelial cell biology and atherosclerosis RT$^2$ Profiler array results of iMAEC (–) vs. NTF cells. Table S2 shows quantitative comparative proteomic analysis of aortae from WT and SPPL2a/b-deficient mice.**