Atherogenic LOX-1 signaling is controlled by SPPL2-mediated intramembrane proteolysis

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The lectin-like oxidized LDL receptor 1 (LOX-1) is a key player in the development of atherosclerosis. LOX-1 promotes endothelial activation and dysfunction by mediating uptake of oxidized LDL and inducing pro-atherogenic signaling. However, little is known about modulators of LOX-1-mediated responses. Here, we show that the function of LOX-1 is controlled proteolytically. Ectodomain shedding by the metalloprotease ADAM10 and lysosomal degradation generate membrane-bound N-terminal fragments (NTFs), which we identified as novel substrates of the intramembrane proteases signal peptide peptidase-like 2a and b (SPPL2a/b). SPPL2a/b control cellular LOX-1 NTF levels which, following self-association via their transmembrane domain, can activate MAP kinases in a ligand-independent manner. This leads to an up-regulation of several pro-atherogenic and pro-fibrotic targets including ICAM-1 and the connective tissue growth factor CTGF. Consequently, SPPL2a/b-deficient mice, which accumulate LOX-1 NTFs, develop larger and more advanced atherosclerotic plaques than controls. This identifies intramembrane proteolysis by SPPL2a/b as a novel atheroprotective mechanism via negative regulation of LOX-1 signaling.

Introduction

Atherosclerosis and its clinical manifestations represent a leading cause of morbidity and mortality (Herrington et al., 2016). Vascular lesions called atherosclerotic plaques are hallmarks of the disease. Activation and dysfunction of endothelial cells and subendothelial accumulation of oxidized low-density lipoprotein (oxLDL; Steinberg et al., 1989; Di Pietro et al., 2016; Gimbrone and García-Cardeña, 2016) are initiating events for plaque formation (Gimbrone and García-Cardeña, 2016) by triggering immune cell recruitment. oxLDL activates endothelial cells via the lectin-like oxLDL receptor 1 (LOX-1; Sawamura et al., 1997). LOX-1 is a type II transmembrane protein that belongs to the family of C-type lectin receptors (Plato et al., 2013; Xu et al., 2013). The critical role of LOX-1 in atherosclerosis is well documented by in vivo studies in mice. Constitutive deletion or endothelial overexpression of LOX-1 attenuated or exacerbated the development of atherosclerotic plaques (Mehta et al., 2007; White et al., 2011; Akhmedov et al., 2014), establishing a pro-atherogenic function of this protein. This is supported by a significant up-regulation of LOX-1 in human atherosclerotic lesions (Kataoka et al., 1999).

In addition to oxLDL uptake, LOX-1 triggers signaling pathways including the activation of mitogen-activated protein (MAP) kinases (Li and Mehta, 2000) and the NFκB pathway (Cominacini et al., 2000; Matsuenga et al., 2003). By this means, LOX-1 induces expression of adhesion molecules and pro-inflammatory cytokines and promotes atherogenesis (Li et al., 2003; Chen et al., 2005; Mattaliano et al., 2009; Thakkar et al., 2015). Molecular factors regulating LOX-1 stability and signaling functions remain poorly defined. Proteolytic cleavage of LOX-1 liberates a soluble form of this receptor (sLOX-1; Murase et al., 2000). Serum levels of sLOX-1 are modulated in cardiovascular disease (Hayashida et al., 2005). However, the proteolytic enzymes responsible for this have remained controversial (Murase et al., 2000; Mitsuoka et al., 2009; Zhao et al., 2011). Furthermore, identification of the individual cleavage fragments and the impact of proteolysis on LOX-1 signaling are undefined to date.
Proteolysis of transmembrane proteins is a well-established mechanism to control their abundance and function (Lichtenhaller et al., 2011). In a sequential process, referred to as regulated intramembrane proteolysis, a cleavage within the substrate’s ectodomain is followed by the action of an intramembrane-cleaving protease (I-CLIP) processing the residual membrane-embedded stub. The resulting intracellular domain (ICD) is released into the cytosol and can fulfill regulatory functions like in Notch signal transduction (De Strooper et al., 1999).

Signal peptide peptidase–like 2a and b (SPPL2a, SPPL2b) are I-CLIPs functioning in such regulated intramembrane proteolysis sequences (Voss et al., 2013) by cleaving N-terminal fragments (NTFs) derived from type II transmembrane proteins. They are GxGD-type aspartyl I-CLIPs with homology to presenilins (Voss et al., 2013). SPPL2a and SPPL2b exhibit divergent subcellular localizations in lysosomes/late endosomes and at the plasma membrane (Friedmann et al., 2006; Behnke et al., 2011; Schneppenheim et al., 2014b). While most substrates identified to date have been analyzed in cell-based systems, in vivo relevance was shown for SPPL2a-mediated cleavage of the invariant chain (CD74) of the MHCII complex, which is an essential process in development of B cells and dendritic cells documented by a deficiency of these cell types in SPPL2a-deficient mice (Beisner et al., 2013; Bergmann et al., 2013; Schneppenheim et al., 2013). In contrast, the in vivo function of SPPL2b is less clear, and evidence for SPPL2b substrates under endogenous conditions is still lacking.

Here, we show that proteolytic pathways regulate the signaling function of LOX-1. Lysosomal proteolysis and ectodomain shedding contribute to the generation of membrane-bound LOX-1 NTFs, which are capable of inducing ligand-independent proatherogenic and pro-fibrotic signaling. We demonstrate that levels of the LOX-1 NTFs are controlled by SPPL2a/b, accounting for enhanced LOX-1 signaling in the absence of these proteases. Concomitantly, mice with SPPL2a/b deficiency in non-hematopoietic cells are more susceptible to the development of atherosclerotic plaques. Therefore, we identify SPPL2a/b as essential negative regulators of LOX-1 signaling as well as of atherosclerosis.

Results
LOX-1 is processed by ADAM10 and lysosomal proteases
Based on the described soluble form of LOX-1, we investigated proteolytic processing of this protein in more detail. When we expressed N-terminally HA (hemagglutinin) epitope-tagged murine LOX-1 in HeLa (Fig. 1 A) or immortalized murine aortic endothelial cells (iMAECs; Fig. 1 B), we observed the full-length LOX-1 protein (FL) as well as two hitherto unknown fragments of ~25 and 17 kD, which we termed NTF1 and NTF2. Based on the potential glycosylation of NTF1 suggested by its diffuse band, the two N-glycosylation sites N72 and N92 in the stalk domain (Fig. S1 A) were substituted by alanines (Fig. 1 C). Mutation of N72 led to a shift of NTF1, but not of NTF2, indicating that NTF2 does not contain N72. In contrast, blocking glycosylation at N92 did not alter the migration of any of the NTFs, affecting, however, that of the LOX-1 full-length protein. This indicates that NTF1 and NTF2 represent distinct proteolytic fragments. Whereas NTF1 is generated by proteolysis between N72 and N92, the cleavage leading to NTF2 has to occur N-terminally to N72.

With regard to previous reports (Mitsuoka et al., 2009; Zhao et al., 2011), we assessed the role of ADAM proteases in LOX-1 processing using HEK cells deficient for ADAM10 and/or ADAM17 (Fig. 1 D and E). The release of sLOX-1 was completely abolished in cells lacking ADAM10, while absence of ADAM17 had no influence on this process. In line, the production of sLOX-1 in iMAECs was stimulated by ionomycin, an ADAM10 activator, but not by PMA, which stimulates ADAM17 (Fig. 1 F). Even in ADAM10 KO cells, no LOX-1 shedding upon PMA treatment was observed (Fig. S1 B). Unexpectedly, the abolished shedding only led to a minor reduction of both NTFs (Fig. 1, D–F) arguing for additional proteolytic pathways for NTF generation. To define these, we tested a panel of protease inhibitors. Bafilomycin a1, an inhibitor of lysosomal acidification, significantly reduced levels of both NTFs (Fig. 1, G and H; and Fig. S1 C). This pointed to a role of pH-dependent lysosomal proteases in LOX-1 processing. In agreement, we observed a partial colocalization of overexpressed LOX-1 with the lysosomal protein LAMP-2 (Fig. 1 I). Furthermore, the cysteine protease inhibitor E-64d caused a reduction of NTF2 (Fig. S1 C). Interestingly, in this case NTF1 was not depleted, but rather stabilized. Thus, multiple lysosomal proteases are involved in the generation of NTF1 and NTF2. Importantly, NTF-production was not influenced by the LOX-1 ligand oxLDL (Fig. 1, K and L), arguing for a constitutive internalization of the receptor followed by proteolytic degradation in lysosomal compartments. Conversely, inhibiting LOX-1 processing influenced neither oxLDL uptake (Fig. S1 D) nor LOX-1 surface levels (Fig. S1 E).

LOX-1 NTFs undergo intramembrane cleavage by SPPL2a/b
We hypothesized that the LOX-1 NTFs are further processed by intramembrane proteases. Based on their topology and localization, we considered SPPL2a and SPPL2b as candidate I-CLIPs. Co-expression of LOX-1 with either protease, but not their catalytically inactive mutants led to a significant reduction of both NTFs that could be blocked with the SPP/SPPL inhibitor (Z-LL)2-ketone (ZLL; Fig. 2 A). However, NTF depletion induced by SPPL2b was more pronounced (Fig. 2 B). Upon SPPL2a/b coexpression with LOX-1, a smaller band presumably representing the released LOX-1 ICD was enhanced. Immunofluorescence analysis confirmed overlapping distributions between the substrate and both proteases (Fig. 2 C).

We investigated if LOX-1 NTFs are also substrates of endogenous SPPL2a/b proteases. In LOX-1 expressing iMAECs, ZLL stabilized both LOX-1 NTFs (Fig. 2, D and E). Similarly, when analyzing LOX-1 processing in WT and SPPL2a/b double-deficient (dKO) MEF cells, levels of both NTFs were significantly higher in the KO cells (Fig. 2, F and G). We aimed to delineate the individual contributions of SPPL2a and SPPL2b and included SPPL2a and SPPL2b single-deficient MEF cells. Though a minor increase of the LOX-1 NTF1 was observed in SPPL2b−/− MEFs, only the combined ablation of SPPL2a and SPPL2b led to the described major stabilization of LOX-1 NTF2, arguing for a
Figure 1. **LOX-1 NTFs are generated by ADAM10 and lysosomal proteases.** (A and B) Two LOX-1 NTFs were detected by Western blotting upon overexpression of HA-LOX-1 in either HeLa (A) or iMAEC (B) cells. (C) Usage of the potential glycosylation sites at N72 or N92 was analyzed in HeLa cells using respective mutants. (D) HEK cells deficient for ADAM10, ADAM17, or both and WT cells were transfected with HA-LOX-1-FLAG. sLOX-1 was recovered from conditioned media after 16 h by TCA precipitation. (E) Quantiﬁcation of D. N = 2–3, n = 4–6. One-way ANOVA with Dunnett’s post hoc test. (F) iMAECs stably expressing HA-LOX-1-FLAG were preincubated for 2 h in serum-free DMEM containing 10 µM marimastat (Mari) or DMSO. Cells were treated for 30 min with 1 µM ionomycin (Iono) or 100 nM PMA. Cell lysates and conditioned media were analyzed as in D. (G) Lysosomal processing of HA-LOX-1 was blocked in HeLa cells by incubation with 100 nM bafilomycin a1 (Baf a1) for 24 h. (H) Quantiﬁcation of G. N = 3, n = 3. Student’s t test. (I) Delivery of HA-LOX-1 to LAMP-2–positive compartments was visualized by indirect immunofluorescence in HeLa cells treated with either DMSO or 100 nM bafilomycin a1 for 6 h. Bars, 10 µm. (J) HA-LOX-1–expressing HeLa cells were treated for 4 h with 40 or 80 µg/ml oxLDL, and NTF formation was analyzed by Western blotting. (L) Quantiﬁcation of NTF1+2/FL ratios of cells treated with 40 µg/ml oxLDL. N = 2, n = 4. Student’s t test. ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05; ns, not signiﬁcant. N, the number of independent experiments; n, the number of individual samples for quantiﬁcation. All data are shown as mean ± SD.
synergistic role of endogenous SPPL2a/b in LOX-1 processing (Fig. 2, H and I).

We wanted to confirm that the effect of SPPL2a/b on LOX-1 NTFs represents proteolysis within the transmembrane domain (TMD). Therefore, we expressed a LOX-1 model NTF (Fig. 3 A) comprising amino acids 1–88 together with SPPL2a or SPPL2b in HEK cells and recovered the C-terminal cleavage products for mass-spectrometric analysis from the media. To avoid heterogeneity by N-glycosylation, we mutated the N-glycosylation site at N72. We observed six specific cleavage fragments (Fig. 3 B) that were not present in control samples (Fig. S1 F). The determined peaks were matched with theoretical masses calculated for C-terminal peptides based on the NTF sequence (Fig. 3 C). Both proteases exhibited multiple overlapping cleavage sites within the TMD of LOX-1. Interestingly, SPPL2a showed a strong preference for proteolysis N-terminal to L52, whereas the predominant site used by SPPL2b was shifted to I53.
Figure 3. The LOX-1 TMD is important for SPPL2-dependent intramembrane cleavage. (A–C) Mass-spectrometric determination of SPPL2a/b cleavage sites within the LOX-1 NTF. (A) Amino acid sequence of the employed model substrate. Determined cleavage sites in the TMD (gray) are marked. (B) Secreted C-terminal fragments were purified from conditioned media and analyzed by MS. Arrows indicate peptides increased by protease overexpression with the predominant peaks labeled in red. (C) Peptides assigned to the respective peaks shown in B. Peak 5 corresponds to a peptide with a potential N-terminal

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We aimed to identify determinants within the LOX-1 TMD required for the intramembrane cleavage. Based on the role of helix destabilization for intramembrane proteolysis (Langosch et al., 2015), we tried to increase the stability of the LOX-1 TMD by replacing either selected polar or helix-destabilizing residues (TMDstab) orLocalized acids (32–36I, 37–42I, 43–48I, 49–54I) with isoleucines (Fig. 3 D). All mutants reached the plasma membrane like the LOX-1 WT NTF (Fig. 3 E). Whereas processing of most mutants by coexpressed SPPL2b was not compromised, exchange of residues 43–48 significantly impaired intramembrane cleavage (Fig. 3, F and G). Interestingly, the residues surrounding the cleavage sites between T51, L52, and I53 could be replaced without a significant loss of cleavability.

Endogenous LOX-1 is a substrate of SPPL2a and SPPL2b
To analyze endogenous LOX-1, we generated an antiserum against an N-terminal epitope (aa 2–19) of the protein (Fig. 4, A and B). We confirmed endogenous expression of SPPL2a and SPPL2b in iMAECs and aortic lysates (Fig. 4 C) and observed that inhibition of SPP/SPPL proteases stabilized endogenous LOX-1 NTF2 in iMAECs (Fig. 4, D and E). We also analyzed LOX-1 processing in aortae isolated from WT as well as SPPL2a/b single- and double-deficient mice (Fig. 4, F and G). Similar to iMAECs, we exclusively detected NTF2 in these samples. Only minor amounts of LOX-1 NTF2 were detected in WT aortae, whereas those from dKO mice exhibited a major accumulation. In both the single-deficient aortae, NTF2 abundance was increased. However, levels were much lower than in the dKO aortae, strongly highlighting the importance of both proteases for LOX-1 processing in vivo.

Inhibition of SPPL2a/b enhances ligand-dependent LOX-1–induced MAP kinase signaling
We assessed the impact of SPPL2a/b-mediated LOX-1 proteolysis on the signaling of this receptor. Therefore, we generated an inducible LOX-1–expressing HEK cell line (Fig. 5 A). Treatment with oxLDL triggered phosphorylation of the MAP kinases ERK1/2 (pERK) only when LOX-1 expression was induced (Fig. 5 B). Importantly, inhibition of SPPL2a/b enhanced ERK activation (Fig. 5, B and C). This effect was recapitulated in ZLL-treated...
Figure 5. The LOX-1 NTF regulates MAP kinase activity. (A) T-REx FlipIn cells inducibly overexpressing LOX-1 were generated. LOX-1 expression after induction with 10 µg/ml doxycycline (dox) for 24 h was confirmed by Western blotting. (B) LOX-1 expression was induced in T-REx FlipIn cells (+dox) or cells were left uninduced (−dox). Prior to stimulation with 40 µg/ml oxLDL, cells were cultured in serum-free DMEM for 4 h in the presence of 40 µM ZLL or DMSO. pERK and total ERK1/2 (ERK) levels were determined. (C) Quantification of B. N = 4, n = 4. Student’s t test. (D) iMAECs were incubated with 40 µM ZLL for 24 h. For the last 4 h, cells were transferred to serum-free DMEM before treatment with 40 µg/ml oxLDL and analysis of ERK1/2 activation. (E) Quantification of D. N = 4, n = 4. Student’s t test. (F) LOX-1 surface levels were analyzed by flow cytometry in iMAECs stably overexpressing LOX-1 after 24 h treatment with 40 µM ZLL. N = 2, n = 6. Student’s t test. (G) Stable expression of the LOX-1 NTF in transduced iMAECs was validated. (H) Transduced iMAECs were cultivated for 4 h under serum-free conditions before stimulation with 40 µg/ml oxLDL and assessment of MAP kinase activation. (I) Quantification of H. N = 3, n = 3. Student’s t test. (K) HeLa cells were transfected as indicated with differentially tagged full-length LOX-1 and NTF. Interaction of both proteins was analyzed by coimmunoprecipitation using anti-V5. (L) HeLa cells transfected with 3xFLAG-LOX-1 (3xFLAG-FL) and/or HA-LOX-1 (HA-NTF)
LOX-1 NTF can be considered independent of the full-length oligomers was not considerably modulated by the NTF (Fig. 5, H and I). We analyzed further signaling pathways in unstimulated iMAECs overexpressing the LOX-1 NTF (Fig. 6, D–G). In contrast, mRNA levels of the platelet factor 4 (Pf4) were significantly reduced. In case of CTGF, we considered a direct role of the LOX-1 NTF for homophilic interactions. Differentially tagged WT LOX-1 NTFs could be efficiently coimmunoprecipitated. This interaction was abolished with any of the four TMD mutants (Fig. 6, R and S), indicating that the ability of the NTF to undergo homo- and/or heterophilic protein-protein interactions via the TMD is critical for MAP kinase activation.

Several upstream pathways are involved in LOX-1 NTF induced MAP kinase activation

We aimed to delineate the pathways triggered by the LOX-1 NTF responsible for MAP kinase activation in iMAECs (Fig. 6, H–L). Inhibition of MEK1/2 by U0126 potently reduced activation of ERK, however, had no significant impact on p38. In addition, we also assessed inhibitors against Src family as well as Rho-associated protein kinases (ROCKs). Whereas inhibition of Src kinases impaired activation of both ERK and p38, ROCK inhibition only affected the latter. Thus, not a single, but multiple upstream pathways mediate MAP kinase activation by the LOX-1 NTF. We aimed to identify the molecular determinants within the LOX-1 NTF required for the signaling activation. We mutated all phosphorylatable residues within the cytoplasmic domain of the NTF (Fig. 6 M). However, this mutant was not compromised with regard to MAP kinase activation (Fig. 6, N and O). Furthermore, we evaluated the signaling potential of the isoleucine transmembrane mutants. Surprisingly, their capability to induce signaling was significantly reduced (Fig. 6, P and Q), pointing to a critical role of the TMD, which could act as interaction interface. Based on the described NTF full-length interaction, we tested the ability of the LOX-1 NTF for homophilic interactions. Differentially tagged WT LOX-1 NTFs could be efficiently coimmunoprecipitated. This interaction was abolished with any of the four TMD mutants (Fig. 6, R and S), indicating that the ability of the NTF to undergo homo- and/or heterophilic protein-protein interactions via the TMD is critical for MAP kinase activation.

LOX-1 NTF triggered signaling induces pro-atherogenic and -fibrotic targets

A well-established downstream effect of LOX-1 activation is the up-regulation of adhesion molecules like ICAM-1 (Chen et al., 2005; Inoue et al., 2005). Western blotting revealed a significant increase of ICAM-1 in NTF-overexpressing iMAECs (Fig. 7, A and B). This was also reflected in higher ICAM-1 levels at the cell surface (Fig. 7 C) in a similar range as induced by TNF. Importantly, also icam1 mRNA was increased (Fig. 7 D), indicating that the LOX-1 NTF acts by enhancing icam1 expression.

We screened for further targets influenced by NTF overexpression in the same system using qRT-PCR–based arrays (Table S1). 11 targets were selected for further validation (Fig. 7, E and F). We could confirm a significant up-regulation of the chemokine Cxcl2, the CASP8 and FADD-like apoptosis regulator Cflar, the connective tissue growth factor Ctgf, the granulocyte-macrophage colony-stimulating factor Csf2, and the platelet-derived growth factor subunit B Pdgb/b in iMAECs overexpressing the LOX-1 NTF. In contrast, mRNA levels of the platelet factor 4 Pf4 were significantly reduced. In case of CTGF, we confirmed enhanced production of this secreted protein by Western blot analysis of Brefeldin A–treated cells (Fig. 7, G and H).
We assessed the relevance of the described signaling pathways for the identified transcriptional regulation. Therefore, we determined the effect of MEK, Src, and ROCK inhibition on the up-regulation of Icam1 (Fig. 7I), Pdgfb (Fig. 7K), and Ctgf (Fig. 7L). Whereas ROCK inhibition significantly counteracted the increase of Icam1 expression, none of the other compounds had significant effects on this target. In contrast, Src inhibition effectively reduced mRNA levels of Pdgfb and Ctgf. In addition, the NTF-associated Ctgf up-regulation was also responsive to MEK inhibition. Thus, several upstream pathways in individual combinations contribute to the transcriptional effects, highlighting the complexity of the LOX-1 NTF-induced signaling.

Next, we wondered if also an NTF accumulation derived from inhibiting the intramembrane cleavage of endogenous LOX-1 would impact on the identified target genes. Therefore, mRNA levels of the validated targets were determined in ZLL-transduced iMAECs and controls. With the exception of Cflar and F4/80, we observed a significant up-regulation of Icam1, Pdgfb, Csf2, Ctgf, and Cxcl2 upon SPPL2a/b inhibition (Fig. 7M). We also analyzed ICAM-1 surface levels by flow cytometry and found a significant increase in inhibitor-treated cells (Fig. 7N). This further confirms that in iMAECs, SPPL2a/b inhibition phenocopies overexpression of the LOX-1 NTF. These findings strongly suggest that SPPL2a/b deficiency induces a pro-atherogenic and pro-fibrotic state in endothelial cells via the accumulation of LOX-1 NTFS.

Enhanced atherosclerosis in SPPL2a/b double-deficient mice

In healthy aortae from WT mice, low levels of the LOX-1 NTF are present (Fig. 4F), which are increased following induction of atherosclerosis (Fig. S2, A and B). This suggests a need for SPPL2a/b to clear this fragment under such conditions. Therefore, we analyzed if SPPL2a/b deficiency has an impact on the development of atherosclerosis in vivo. We wanted to exclude that the immunological phenotype of SPPL2a/b-deficient (dKO) mice (Schnepenheim et al., 2014b) caused by defective processing of CD74 (Schnepenheim et al., 2013) confounds the analysis. Therefore, dKO and WT mice were reconstituted with WT bone marrow (Fig. S2C), and recovery of normal hematopoiesis was confirmed by flow-cytometric analysis of blood samples (Fig. S2, D and E). At the end of the experiment, analysis of splenocytes demonstrated a full correction of the B cell maturation block of the dKO mice (Fig. 8A and Fig. S2F).

After recovery from transplantation, hypercholesterolemia was induced by adeno-associated viral overexpression of a PCSK9 gain-of-function mutant in combination with a Western-type high cholesterol diet (HCD, Bjørklund et al., 2014). This model has been recently established as an effective approach to eliminate LDL receptor molecules from the cell surface, thereby causing hypercholesterolemia comparable to Ldr−/− mice but circumventing the need for genetic ablation of the corresponding gene (Goettsch et al., 2016; Lu et al., 2016; Rogers et al., 2017; Theodorou et al., 2017). In agreement with this, mice of both genotypes developed significant hypercholesterolemia and hypertriglyceridemia (Fig. 8B). However, plasma cholesterol concentrations in the treated dKO mice were ∼30% lower than in control mice. Strikingly, even despite lower plasma cholesterol levels, atherosclerotic lesion size was significantly increased in the dKO mice (Fig. 8C). While plaque macrophage and smooth muscle cell content were not altered between both genotypes, dKO mice displayed a twofold increase in plaque collagen (Fig. 8C). Furthermore, plaques of these mice had larger necrotic areas than those of controls (Fig. 8D). Histopathological scoring revealed an increased proportion (52% vs. 36%) of advanced atherosclerotic plaques in dKO animals (Fig. 8E), highlighting a pro-atherogenic effect of SPPL2a/b deficiency in nonhematopoietic cells in vivo.

We analyzed MAP kinase activation in atherosclerotic aortic arches (Fig. 8F). In line with the findings in cell-based experiments, phosphorylation of ERK and p38 was significantly enhanced in atherosclerotic aortae from dKO mice (Fig. 8G). Activation of Akt and Nfkb pathways was similar in samples from both genotypes (Fig. S2G). In addition, also ICAM-1 was strongly up-regulated in the aortae from dKO mice after atherosclerosis induction (Fig. 8, F and G). Therefore, the cellular phenotypes linked to SPPL2a/b deficiency and enhanced LOX-1 NTF levels in vitro were recapitulated upon induction of atherosclerosis in vivo.

Despite this obvious correlation, an additional impact of further substrates to the atherosclerotic phenotype of SPPL2a/b-deficient mice is in general conceivable. Therefore, we performed Western blot analysis of TNF-activated iMAECs as well...
Figure 7. Accumulation of the LOX-1 NTF induces a pro-atherogenic state in endothelial cells. (A) Expression of ICAM-1 was analyzed in control (−) or LOX-1 NTF transduced iMAECs by Western blotting. (B) Quantification of A. N = 2, n = 6. Student’s t test. (C) Up-regulation of surface ICAM-1 in LOX-1 NTF transduced iMAECs was validated by flow cytometry. As a control, cells were treated with 5 ng/ml TNF. N = 2, n = 6. One-way ANOVA with Tukey’s post hoc test. (D) Up-regulation of Icam-1 was validated by qPCR. N = 2, n = 6. Student’s t test. (E) Candidate genes from endothelial cell biology and atherosclerosis RT² Profiler arrays with differential regulation between control and iMAEC NTF cells. (F) Differences in mRNA levels of the selected genes were validated by qPCR. N = 2–3, n = 6–9. Student’s t test. (G) Secretion of CTGF was blocked in iMAEC control (−) or NTF cells by incubation with Brefeldin A (1 µg/ml) for 6 h. Intracellular CTGF levels were analyzed by Western blotting. (H) Quantification of G. N = 2, n = 12. Student’s t test. (I–L) iMAEC control or NTF cells were treated for 3 h with 25 µM U0126 (MEK-Inh.), 1 µM Saracatinib (Src-Inh.), or 10 µM Y-27632 (ROCK-Inh.) or left untreated as indicated. Icam-1 (I, N = 3–4, n = 9–12), Ctgf (K, N = 3, n = 8–9), and Pdgfb (L, N = 3, n = 8–9) mRNA levels were quantified by qPCR. One-way ANOVA with Dunnett’s post hoc test. (M) Up-regulation of validated candidate genes was monitored in iMAECs treated for 16 h with either DMSO or 40 µM ZLL. N = 2–3, n = 6–9. Student’s t test. (N) Up-regulation of ICAM-1 upon ZLL administration was validated by flow cytometry. N = 3, n = 11. Student’s t test. ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05; ns, not significant. N, the number of independent experiments; n, the number of individual samples for quantification. All data are shown as mean ± SD.
Figure 8. Enhanced atherosclerosis in SPPL2a/b double-deficient mice. (A–G) Hypercholesterolemia and atherosclerosis were induced in WT and SPPL2a/b-deficient (dKO) mice by adeno-associated viral expression of D377Y-mPCSK9 and an HCD for 9 wk following bone marrow transplantation (BMT) with WT bone marrow. (A) Proportions of transitional stage 1 (1) and 2 (T) as well as mature B cells (% of viable splenocytes) were analyzed by flow cytometry at the end of the atherosclerosis experiment. The dot plots depict viable B220+ splenocytes. (B) Plasma triglyceride (TG) and cholesterol (Chol) levels were determined 3 wk after HCD initiation. N = 2, n = 18–20. (C) Atherosclerotic plaque development was analyzed histologically in H&E-stained aortic root cross-sections. Bars, 400 µm. Plaque macrophage, smooth muscle cell, and collagen contents were quantified based on MAC3, αSMA (alpha smooth muscle actin), and Sirius Red staining, respectively. (D) Plaque necrosis was assessed by evaluating the percentage of early, moderate, and advanced necrotic areas. (E) Western blot analysis of pERK, ERK, p-p38, p38, ICAM-1, and Actin expression in WT and dKO mice. (F) Levels of pERK/ERK ratio were quantified. N = 2, n = 18–20.
as aortae from WT and dKO mice for the best characterized substrates CD74 and TNF. We failed to detect relevant amounts of these proteins in these samples (Fig. S3, A–C). In addition, we performed a quantitative proteomic analysis of aorta from WT and dKO mice (Fig. S3 D and Table S2). We detected 3 known SPPL2a/b substrates and 120 other type II transmembrane proteins as substrate candidates. However, their abundance was not different between both genotypes. Thus, in these approaches we did not obtain evidence for other SPPL2a/b substrates being of pathophysiological importance in endothelial cells and aortae, supporting the critical role of the LOX-1–SPPL2a/b axis.

**Discussion**

Our findings identify proteolysis as a major regulatory mechanism of the oxLDL receptor LOX-1. Two pathways, ectodomain shedding and lysosomal degradation, generate LOX-1 NTFs, which induce pro-atherogenic and -fibrotic signaling and require clearance by SPPL2a/b, representing a novel atherogenic danger signal. The LOX-1 NTF, identifying the accumulation of this fragment as a major regulatory function of ADAM10 for liberation of sLOX-1 in HEK and also murine endothelial cells.

In addition to ectodomain shedding, we have identified a so far unknown lysosomal pathway of LOX-1 processing. Based on NTF production, this lysosomal pathway exerts a significantly higher contribution to LOX-1 turnover than ADAM10. Constitutive internalization of LOX-1 has been observed before (Murphy et al., 2008b), but not linked to lysosomal proteolysis. Cellular levels of several receptors are controlled by lysosomal degradation, including the EGF receptor (Eden et al., 2009) and many G protein–coupled receptors (Hanyaloglu and von Zastrow, 2008). Usually this involves sorting into intraluminal vesicles by the Endosomal Sorting Complexes Required for Transport pathway (Babst and Odorizzi, 2013). In contrast, LOX-1 processing by lysosomal proteases and SPPL2a/b rather takes place in the limiting membrane of such compartments, thereby releasing the LOX-1 ICD into the cytosol (Fig. 2 A). Whether this cleavage fragment exerts regulatory functions remains to be investigated.

The enhanced pro-atherogenic signaling upon SPPL2a/b inhibition or deficiency was phenocopied by overexpression of the LOX-1 NTF, identifying the accumulation of this fragment as an underlying mechanism. The NTF-induced signaling was ligand-independent and also observed in a cell type with no response to oxLDL without overexpression of the full-length receptor. This argues for an autonomous signaling by the LOX-1 NTF. This was driven by MAP kinases, including ERK and p38, in contrast to the ligand induced by the full-length receptor, which also activates NFκB (Cominacci et al., 2000; Matsuoka et al., 2003). This supports a model of two different LOX-1 signaling modes. With detectable levels of the NTF in aortae from WT mice (Fig. 4 F) and its increase under atherogenic conditions (Fig. S2 A), this novel mode of LOX-1 signaling is likely of pathophysiological importance.

We showed that the TMD of the LOX-1 NTF is critical for its ability to activate MAP kinases, but also for homophilic interactions. Multimerization could be a prerequisite for recruitment of cytosolic proteins or transmembrane signaling adaptors. However, even unbiased immunoprecipitation in combination with mass spectrometry (IP-MS) approaches have so far failed to identify potential candidates. Thus, the direct molecular link between the LOX-1 NTF and the activation of ERK and p38 remains to be identified. Our inhibitor experiments indicated that multiple pathways including MEK1/2, Src family kinases, and ROCK1/2 are involved in this process, suggesting that different protein interactions of the NTF are involved. Whereas an impact of MEK1/2 inhibition on LOX-1 signaling has been described (Hu et al., 2008b; Yang et al., 2017), a role of Src family kinases is a
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SPPL2 proteases control LOX-1 signaling

A

HeLa

B

C

HeLa

D

E

MGKPIIPNPLLGDSTTFDDLKDQTKQDQDEKINSKAKGKLQLFYSPFVWCLAAATLGVCLGIVTVMTYGLMQLSQVSDDLTTQLPAALTHQKKKGQISARDYKDDDDKAP

SPPL2a

F

V5 tag

hLOX-1 (2-88)

V5-hLOX-1-NTF-73A-FLAG + SPPL2a

V5-hLOX-1-NTF-73A-FLAG + SPPL2b

G

Peak

No.

fragment

sequence

obs. mass [Da]

SPPL2a

obs. mass [Da]

SPPL2b

calc. mass [Da]

1

T52

TIMVLGMLQSVSDDLTTQEAALTHQKKKGQISARDYKDDDDKAP

5160

5163

5155

2

V55

VLGMQLQSVSDDLTTQEAALTHQKKKGQISARDYKDDDDKAP

4917

4918

4914

3

L56

LGMLQSVSDDLTTQEAALTHQKKKGQISARDYKDDDDKAP

4814

4814

4814

4

Q59

QLSQVSDLTTQEAALTHQKKKGQISARDYKDDDDKAP

4497*

4500*

4513

5

L60

SQVSDDLTTQEAALTHQKKKGQISARDYKDDDDKAP

4385

4389

4385

H

K

SPPL2a

SPPL2b

GAPDH

SPPL2a

SPPL2b

Luminal ECs + foam cells

Microvessel ECs

I

norm. mRNA

2a

2b

SPPL2a

SPPL2b

Luminal ECs + foam cells

Microvessel ECs
The role of SPPL2a/b for processing of LOX-1 NTFs is conserved in humans. (A) HeLa cells were transfected with HA-tagged hLOX-1 alone or in combination with human SPPL2a (hSPPL2a) or SPPL2b (hSPPL2b-myc). Where indicated, cells were treated with 20 µM ZLL for 6 h before lysis and Western blot analysis. (B) Quantification of A. N = 3–4, n = 3–4. One-way ANOVA with Dunnett’s post hoc test. (C) Accumulation of hLOX-1 NTFs in HeLa cells upon inhibition of endogenous SPPL2 proteases with 10, 20, and 40 µM ZLL. (D) Quantification of C for 40 µM ZLL treatment. N = 4, n = 8. Student’s t test. (E–G) The cleavage sites of human SPPL2a and SPPL2b in the TMD of hLOX-1 were analyzed by MS using V5-hLOX-1 NTF N73A-FLAG as model substrate. (E) Bar graphs reveal the percentage of each cleavage site relative to total cleavage (left). Arrowheads indicate the start and stop of cleavage sites (right). (F and G) The cleavage sites of human SPPL2a and SPPL2b in the TMD of hLOX-1 were analyzed by MS using V5-hLOX-1 NTF N73A-FLAG as model substrate. G) The cleavage sites of human SPPL2a and SPPL2b in the TMD of hLOX-1 were analyzed by MS using V5-hLOX-1 NTF N73A-FLAG as model substrate.

Several of the identified LOX-1 NTF downstream targets (Fig. 7 E) have been linked with LOX-1 before, including Cxcl2 (Mattaliano et al., 2009), Csf2 (Yang et al., 2017), Ctgf (Hu et al., 2009), and Icam1 (Zhu et al., 2005). These genes cover a wide range of biological functions including endothelial adhesion molecules (Icam1), cytokines/chemokines (Cxcl2, Csf2), and growth factors (Ctgf, Pdgfb). With regard to atherosclerosis, the up-regulation of these targets triggers multiple axes promoting leukocyte recruitment (Wolpe et al., 1989), immune cell and smooth muscle cell proliferation (Biwa et al., 2000; Raines, 2004; Zhu et al., 2009), cell migration (Raines, 2004; Al-Alwan et al., 2013), and extracellular matrix deposition (Fan et al., 2000). In particular, the adhesion molecule ICAM-1 plays a crucial role in atherosclerotic plaque development (Kitagawa et al., 2002). Ctgf is overexpressed in plaques and enhances plaque fibrosis (Cicha et al., 2005). In sum, the effects of increased LOX-NTF levels are not only pro-atherogenic but also pro-fibrotic. This agrees well with a reduced collagen deposition in atherosclerotic lesions of LOX-1−/− mice (Hu et al., 2008a). Importantly, the pro-fibrotic activity of LOX-1 is not limited to atherosclerosis, but has also been observed in other disease models (Hu et al., 2009; Lu et al., 2012; Wang et al., 2012; Dai et al., 2014; Deng et al., 2016). It will be of interest if also in these contexts LOX-1 signaling is controlled by SPPL2a/b proteases.

Our findings further establish SPPL2a/b and 1-CLIPs as regulators of membrane-associated signaling. This was previously suggested by identifying the CD74 NTF, a substrate of SPPL2a (Schnepenheim et al., 2013), as a negative regulator of B cell receptor signaling (Hüttl et al., 2015). Further examples are the receptor proteins TREM-2 (Wunderlich et al., 2013; Glebov et al.,...
Materials and methods

Experimental animals

Generation of SPPL2a/b dKO mice has been described previously (Schneppeheim et al., 2014b). All mice were backcrossed for 10 generations in a C57BL/6N Crl background. For all experiments, WT mice with the same genetic background bred and housed in the same animal facility were used as controls. Breeding of mice has been approved by the Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume of Schleswig-Holstein (V 242.7224.121–3), and animal care and handling were performed in accordance with local and national guidelines. Mice were housed in individually ventilated cages in the animal facility of the Christian-Albrechts-University Kiel, Kiel, Germany. The atherosclerosis experiment was conducted with ethical approval by the Animal Ethics Committee of Maastricht University, Maastricht, Netherlands (permit number 2014–097).

Plasmids

Expression constructs encoding WT murine SPPL2a and SPPL2b fused to a C-terminal myc epitope or their catalytically inactive mutants D416A or D414A as well as corresponding constructs for human SPPL2a were described before (Behnke et al., 2011; Schneppeheim et al., 2013, 2014a, b). C-termially myc-tagged constructs encoding either WT or catalytically inactive (D421A) human SPPL2b were derived from previously described cDNAs (Flührer et al., 2006) and inserted into pcDNA3.1 Hydro+ vectors (Thermo Fisher Scientific) using HindIII and XhoI restriction sites. The open reading frame coding for murine LOX-1 was amplified from a LOX-1 expression plasmid that was a kind gift of Tatsuya Sawamura (National Cerebral and Cardiovascular Center, Osaka, Japan). The PCR product comprising an N-terminally appended HA epitope was ligated into pcDNA3.1 Hydro+ via BamHI and XbaI restriction sites. Based on this plasmid, glycosylation mutants of the murine oxLDL receptor were generated by mutating N72 and N92 to alanines. With the exception of Fig. 9, where human LOX-1 (hLOX-1) and SPPL2a/b (hSPPL2a, hSPPL2b) constructs were employed; in all other experiments the murine cDNAs were used. A GFP-encoding pMSCV puro plasmid was a kind gift of Michael Engelke (University of Göttingen, Göttingen, Germany). Untagged murine full-length LOX-1 or the putative murine LOX-1 NTF consisting of amino acids 1–88 of the receptor was cloned into the pMSCV puro vector (Clontech) using BglII and XhoI restriction sites. Correspondingly, a HA-LOX-1-FLAG construct in this vector was generated. A murine LOX-1 cDNA carrying an N-terminal or C-terminal 3xFLAG-tag was ligated into pcDNA4.0 TO. The coding sequence of hLOX-1 was obtained from Sinobiological, fused to an N-terminal HA tag, and cloned into pcDNA3.1 Hydro (+) via BamHI and XbaI restriction sites. For mass-spectrometric determination of the SPPL2a/b cleavage sites in human and murine LOX-1 NTFs, coding sequences of NTFs comprising amino acids 1–88, with appended N-terminal V5- and C-terminal FLAG epitopes, were inserted into pcDNA3.1 Hydro+. In these constructs, the glycosylated asparagine residues N72 (murine) and N73 (human) were mutated to alanine to reduce complexity of mass spectrometric analysis. HA- or untagged TMD mutants of murine LOX-1 (TMDstab, B1I, B2I, B3I, B3I) as well as the
unphosphorylatable LOX-1 NTF mutant (T2A, S18A, SS30/31AA) were cloned into the pMSCV puro vector using its BglII and XhoI restriction sites.

**Cell culture and transfection**

HeLa cells (DSMZ), Flip-In T-Rex 293 (Invitrogen), HEK293 ADAM10/17 KO cells, MEFs, and Platinum-E retroviral packaging cells (Cell Biolabs, Inc.) were cultivated in DMEM (GIBCO) supplemented with 10% FCS (Biochrom) as well as 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich). ADAM10 and ADAM17 single- and double-deficient HEK293 cells were generated by CRISPR/Cas9 genome editing and initially described in Riethmüller et al. (2016). iMAECs were a kind gift of Prof. Hanjoong Jo (Emory University, Atlanta, GA) and cultured as described in Bond et al. (2010).

MEFs were isolated from 13.5-d-old embryos from WT, SPPL2a+/−, SPPL2b−/−, and SPPL2a/b double-deficient mice. After removing the head and internal organs, embryos were cut into small pieces and incubated in Trypsin-EDTA for 15 min at 37°C. The resulting tissue pieces were dissociated by pipetting and the cell suspension transferred to 12 ml prewarmed DMEM supplemented with 10% FCS and antibiotics. Cells were sedimented, washed once in fresh medium, and plated out for culture. For immortalisation, MEFs were transfected at an early passage with an expression plasmid of the SV40 large T antigen (pMSSVLT; Schuermann, 1990). After an average of 10 passages, immortalized cells were used for experiments.

Bone marrow–derived dendritic cells (BMDCs) were generated as described in Schneppenheim et al. (2014b) based on the protocol published by Lutz et al. (1999). For detection of N-terminal fragments of TNF, BMDCs were activated for 6 h with 500 ng/ml lipopolysaccharides from Escherichia coli (Sigma-Aldrich).

For transient transfection of HeLa, iMAEC, MEF, and Platinum-E cells, Turbofect transfection reagent (Thermo Fisher Scientific) was applied. Flip-In T-Rex 293 cells stably expressing murine LOX-1 were generated by transfection with the respective construct and the pOG44 vector encoding the Flp recombinase followed by selection with 100 µg/ml hygromycin and 10 µg/ml blasticidin (both Invivogen). After selection, surviving clones were combined and further analyzed as polyclonal cell lines. To induce transgene expression, 10 µg/ml doxycycline (Sigma-Aldrich) was added to the medium for 24 h. To ensure homogenous expression of the LOX-1 NTF in transiently transfected HEK293 cells, when analysis of signaling pathways was intended, cells were transfected in 6-cm cell culture dishes with 2 µg of either a LOX-1 NTF coding construct or a corresponding empty vector harboring a puromycin resistance cassette (pMSCV). 1 d after transfection, fresh medium containing 10 µg/ml puromycin was added, and cells were incubated for 4 d before further processing for Western blot analysis.

In LOX-1–expressing cell lines, LOX-1–dependent signal transduction was stimulated with 40 µg/ml oxLDL obtained from Hycultec. To influence proteolytic processing of LOX-1, several compounds were added, if not indicated differently, 24 h before analysis to the medium of cultured cells. The lysosomal acidification inhibitor bafilomycin a1 (Sigma-Aldrich) was used in a final concentration of 100 nM. To inhibit cysteine and serine proteases, E-64d (40 µM; Enzo), Leupeptin (2.5 µM; Roth), and AEBSF (500 µM; Sigma-Aldrich) were applied. The cell-permeable Pepstatin A–methyl ester (10 µM; Merck) was used to interfere with the activity of aspartyl proteases. Activity of the metalloproteinases ADAM10 and ADAM17 was blocked by marimastat (10 µM; Sigma-Aldrich) or stimulated by treatment with ionomycin (1 µM; Sigma-Aldrich) or PMA (100 nM; Sigma-Aldrich), respectively. SPPL2 proteases were inhibited by application of the SPP family inhibitor ZLL (Peptanova) in concentrations ranging from 10 to 40 µM. For kinase inhibition, the MEK1/2–inhibitor U0126 (25 µM; Sigma-Aldrich), the Src-family inhibitor Saracatinib (1 µM; Selleckchem), or the ROCK inhibitor Y-27632 (10 µM; Cayman Chemicals) was applied. To allow for detection of CTGF in lysates of empty vector or LOX-1 NTF–expressing iMAECs, Brefeldin A (Sigma-Aldrich) was applied in a concentration of 1 µg/ml to the culture medium for 6 h before lysis of the cells. Except for Leupeptin, E64-d, and Y-27632, which were applied as aqueous solutions, all small molecule inhibitors were dissolved in DMSO, which was used as solvent control in respective experiments.

**Retroviral transduction**

For generation of ecotropic retroviruses for transduction of iMAEC and MEF cells, Platinum-E cells were transfected using Turbofect transfection reagent. After 48 h, virus-containing supernatants were removed from Platinum-E cultures and passed through a 20-µm filter (BD Biosciences). Subsequently, supernatants supplemented with 8 µg/mL polybrene (Sigma-Aldrich) were added to the respective cells seeded a day before in 10-cm culture dishes. 1 d after transduction, supernatants were replaced by fresh culture medium containing 10 µg/ml puromycin (Invivogen). Stably transduced cells were maintained as polyclonal cell lines without subcloning.

**Protein extraction and Western blot analysis**

Cell and tissue lysis was performed as described in Schneppenheim et al. (2013). In brief, cells were harvested by centrifugation and subsequently extracted in lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 0.1% SDS) supplemented with Complete protease inhibitor mix (Roche), 0.5 µg/mL Pepstatin A (Sigma-Aldrich), 4 mM EDTA, and 4 mM Pefabloc SC Protease Inhibitor (Roth). Aortic vessels were flushed with PBS and cleaned from adherent adipose and connective tissue. For lysis, 150 µL of lysis buffer and ceramic beads was added to each aorta, which was homogenized by vigorous shaking of the mixture in a Precellys homogenizer (Peqlab). Afterward, cell and tissue suspensions were sonicated using a Branson Sonifier 450 (Emerson Industrial Automation) and incubated at 4°C for 1 h. The remaining debris was pelleted by centrifugation at 13,000 g for 10 min at 4°C, and protein concentrations of supernatants were determined using the Fierce BCA Protein Assay Kit (Thermo Fisher Scientific). After lysis, protein lysates were complemented with the required amount of reducing SDS-PAGE sample buffer according to Laemmli (1970) and denatured for 10 min at 56°C.

For recovery of sLOX-1 from conditioned media of HA-LOX-1–FLAG or mLOX-1-3xFLAG expressing iMAEC or HEK293 cells,
these were cultured in serum free medium for 30 min before stimulation with ionomycin or PMA for 30 min. After stimulation, 800 µl supernatant was supplemented with 200 µl 100% (wt/vol) TCA for 1 h at 4°C. Precipitated proteins were sedimented by centrifugation at 13,000 g for 10 min at 4°C. The resulting pellet was washed once with ice-cold acetone before resuspension in reducing SDS-PAGE sample buffer and denaturation at 95°C for 10 min.

Electrophoretic separation by SDS-PAGE with a standard Tris-glycine buffer system (Laemmli, 1970), semidry transfer to nitrocellulose membranes, and immunodetection were conducted as described previously (Schroeder et al., 2010). Monoclonal antibodies against HA (3F10), myc (9B11), V5, and FLAG (M2) epitope tags were obtained from Roche, Cell Signaling, or Sigma-Aldrich, respectively. sLOX-1 was detected applying a polyclonal goat antibody directed against the C terminus of this protein (AF1564; R&D Systems). To allow detection of LOX-1 NTPs, rabbits were immunized with a synthetic peptide corresponding to the amino acids 2–19 of the protein (Pineda Antikörper Service). Similarly, a polyclonal antiserum against residues 575–592 of human SPPL2b was raised. In both cases, antisera were affinity-purified against the immobilized immunogens before use. Polyclonal antibodies for detection of murine SPPL2a (Behnke et al., 2011), murine SPPL2b (Schnepenheim et al., 2014b), and human SPPL2a (Schnepenheim et al., 2014a) were described before. The polyclonal anti-TNF antibody has been described earlier (Mentrup et al., 2015). Murine CD74 was detected using the monoclonal antibody YN1/1.7.4 from BioLegend. CTGF was detected from the membrane. Murine ICAM-1 was detected with a polyclonal antiserum against murine ICAM-1 (YN1/1.7.4) conjugated to the fluorophore Alexa 488 (Molecular Probes). After detachment with Accutase (E Bioscience), Alexa 594-conjugated goat anti-mouse antibody was added and the membrane was washed five times with IP buffer, and finally eluted by boiling in reducing SDS-PAGE sample buffer.

Indirect immunofluorescence
Immunocytochemical stainings were performed as described previously (Schroeder et al., 2010). Cells grown on coverslips were fixed with 4% (wt/vol) paraformaldehyde. Lysosomal compartments were visualized with the 2D5 monoclonal LAMP-2 antibody (Radons et al., 1992). Alexa 488- and 594-conjugated secondary antibodies were purchased from Molecular Probes. Nuclei were stained with DAPI (Sigma-Aldrich), which was included in the embedding medium at a concentration of 1 µg/ml. Images were acquired with an Olympus FV1000 confocal laser scanning microscope and further processed with Olympus Fluoview software and Adobe Photoshop software.

Flow cytometry
FACS analysis of stably transfected, inhibitor- or TNF (Immunotools)-treated iMAECs was facilitated by an antibody against murine ICAM-1 (YNI/1.7.4) conjugated to the fluorophore APC. For determination of surface LOX-1 levels in receptor overexpressing iMAECs, LOX-1 was first marked with the AF-1564 antibody (R&D Systems) before labeling of the primary antibody with an anti-goat–Alexa 647 conjugate (Molecular Probes). After detachment with Accutase (E Bioscience) for
5 min, cells were washed once in FACS buffer (PBS + 2% FCS + 2 mM EDTA) and subsequently stained with the indicated antibodies against propidium iodide (BD Biosciences). Cells were resuspended in 200 µl FACS buffer and analyzed using a FACS Canto flow cytometer (BD Biosciences) and the FACS Diva software (BD Biosciences). Further data processing was performed with the FlowJo Software (Tree Star).

To assess the reconstitution of B cell development after bone marrow transplantation in the atherosclerosis experiment, cell suspensions were prepared from harvested spleens by passing the tissue through a 70-µm cell strainer (Greiner). Splenocytes were incubated in erythrocyte lysis buffer (150 mM NH₄Cl and 10 mM NaHCO₃, pH 7.4) for 2 min on ice, washed, and filtered through a 70-µm cell strainer. Subsequently, splenic cells were blocked with anti-CD16/32 (clone 93; eBioscience), washed, and stained with antibodies against B220 (RA3-6B2; PE-Cy7; eBioscience), CD21 (8D9; PE; eBioscience), and CD24 (30–F1; APC; eBioscience). Cells were analyzed using a FACS Canto II flow cytometer (BD Biosciences) and FACS Diva software v6 (BD Biosciences) for data interpretation.

For quantification of LOX-1 internalization, iMAECs stably overexpressing LOX-1 were incubated for 30 min on ice with an antibody against the C terminus of the protein in the presence of 10 µM marimastat or DMSO as control. Cells were washed twice with ice-cold PBS and subsequently chased for 2 h at 37°C. After detachment of the cells from the culture dishes, remaining surface LOX-1 was labeled with an anti-goat–Alexa 647 antibody and detected using a FACS Canto cytometer as described above.

Cleavage site determination
Epitope-tagged LOX-1 NTFs (V5-LOX-1-NTF-N72A-FLAG, V5-hLOX-1-NTF-N73A-FLAG) were transiently expressed in T-REx 293 cells with or without stable overexpression of SPPL2a or SPPL2b. The C-peptides released by the SPPL2-mediated intramembrane cleavage were recovered from conditioned media using anti-FLAG M2-conjugated agarose beads (Sigma-Aldrich). After repeated washing with IP-MS buffer (0.1% n-octyl glucoside, 10 mM Tris/HCl, pH 8.0, 5 mM EDTA, and 140 mM NaCl) and sterile water, peptides were eluted using a trifluoroacetic acid/acetonitrile/water mixture (1:20:20, vol/vol/vol) saturated with α-cyano-4-hydroxycinnamic acid. The dissolved samples were dried on a stainless plate and subjected to Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)–MS analysis using a VoyagerDE STR instrument (Applied Biosystems).

Proteomic analysis of murine aorta
The total proteome of three biologically independent aortic samples from WT and SPPL2a/b-deficient mice was analyzed and quantitatively compared. Each sample was prepared from pooled aortae of two age- and sex-matched mice of the respective genotype, which were extracted in 150 µl lysis buffer. Lysate corresponding to 150 µg protein was mixed with 100 µl 30% acrylamide, 1.25 µl TEMED (N,N,N’,N’-tetramethylethylenediamine) and 5 µl of 10% ammonium persulfate to trap proteins in a gel matrix, fixed (50% methanol and 10% acetic acid), washed with water, and cut into pieces. After dehydration, proteins were reduced with 10 mM dithiothreitol at 60°C for 30 min and alkylated using 55 mM iodoacetamide. Subsequently, proteins were digested by adding 200 µl of trypsin solution (10 ng/µl) in 50 mM ammonium bicarbonate (Shevchenko et al., 2006). After extraction using 10% formic acid and 50% and 100% acetonitrile, peptides were purified using the C18 SepPak column system (Waters) and lyophilized.

Peptides were then labeled using TMT 6plex (Thermo Fisher Scientific) according to the manufacturer. Samples were named WT pool #1 (TMT126), WT pool #2 (TMT128), WT pool #3 (TMT130), dKO pool #1 (TMT127), dKO pool #2 (TMT129), and dKO pool #3 (TMT131) and purified using the C18 SepPak column system. Then, peptides were combined and fractionated by high pH reversed-phase chromatography on a Phenomenex Gemini 3u C18 110A 250 × 3 mm column using a Dionex Ultimate 3000 HPLC as described (Treitz et al., 2015), reconstituted in 20 µl 5% formic acid and submitted to liquid chromatography–tandem MS analysis, which was performed using a Dionex U3000 nanoUHPLC coupled to a Q Exactive Plus mass spectrometer (both from Thermo Scientific). 8 µl of each fraction was loaded on a trap column (10 µm × 300 µm; 3 µm; 100 Å; Acclaim Pepmap 100 C18; Dionex), and separation was performed using an Acclaim PepMap 100 C18 analytical column (50 cm × 75 µm) with a flow-rate of 300 nl/min using a 180-min gradient. MS data were acquired from 5 to 145 min with MS full scans between 300 and 1,800 (m/z) at a resolution of 70,000 at m/z 200. The 15 most intense precursors were subjected to high-energy collisional fragmentation. MS data were searched using the SequestHT algorithm in Proteome Discoverer 2.2 (Thermo Scientific) against the entire reviewed Ensembl protein database of Mus musculus (v. 6.7.2017) with full enzyme specificity. Static modifications were carbamidomethylation on cysteine residues, TMT on lysines and peptide N termini, while oxidation of methionine residues was set as dynamic modifications. The C-terminal pyroglutamyl residue was set as static modification and missed cleavages were allowed. Search tolerances were set to 0.01 Da for MS and 0.02 Da for MS/MS. The protein group identifications were further filtered based on a false discovery rate (FDR) confidence ≤ 0.01. TMT reporter ion ratio quantification (Proteome Discoverer 2.2) was reported for three independent biological replicates (WT/dKO). Reporter ion abundances for the six channels were used to perform a two-tailed t-test, and analysis of the local FDR showed that a t test P value of 0.04 corresponded to a FDR of 5%, hereby determining our significance cut-off for reproducibility of differential protein abundance. Boundaries for differential abundances between WT and dKO were set to statistically significant fold changes of more than 1.4 (Table S2).

RT-PCR and quantitative gene expression analysis
Total cellular RNA was isolated with the Nucleospin RNA Kit (Macherey Nagel) according to the manufacturer’s recommendations. Subsequently, RNA was transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). RT-PCR analysis of human SPPL2 protease expression was performed with the following primers: hSPPL2a-RT-fw: 5’-AGTTGCCCAATCTCAAGTCATG-3’, hSPPL2a-RT-rv: 5’-CCA AATGCTCCATCAGTAG-3’, hSPPL2b-RT-fw: 5’-ATCTTC AATGCTCCATCAGTAG-3’.
ACGCGTTTGGCCGCA-3', hSPPL2b-RT-: 5'-CCAGGCCCACACTG
GTCTTGGTGG-3', hAPDH-RT-fw: 5'-AGGGGAGTCAAGCGAT
TTG-3', hAPDH-RT-rv: 5'-TGATTGCAATGACTGGTTTGTG-3'. To
analyze potential differences in the expression of atherosclerosis-
related genes due to overexpression of the putative LOX-1 NTF,
mouse endothelial cell biology and mouse atherosclerosis RT²
Profiler PCR arrays (Qiagen) were employed using 500 ng of
total RNA isolates obtained from two independently generated
IMAEc lines stably transduced either with an untagged LOX-1-188
(NTF) coding construct or a corresponding empty vector control.
Arrays were performed in a 384-well format according to the
manufacturer’s recommendations. ΔCT-values were compared
between the four lines, and candidate genes were selected based
on an increased or decreased mean NTF-empty vector ratio and
low variability between the individual lines per construct. Genes
with highly different expression in equally transduced lines
were excluded for further follow-up.

For quantitative PCR (qPCR), 0.5 µl cDNA was analyzed using
the Universal Probe Library System (Roche) and a Light Cycler
480II (Roche) as described in Mentrup et al. (2015). The fol-
lowing primers were used: mCtgf (probe 71) fw: 5'-TGACCTGGA
GAAAAACATTAAGA-3'; mCtgf (probe 71) rev: 5'-AGGCCCTGTA
TGCTTCAACTGT-3'; mlcm1 fw (probe 64): 5'-GCTACCATC
ACCGTGTACTTCG-3', mlcm1 rev (probe 64): 5'-TGAGGCTCT
TGCTTCAACTGT-3'; mPdgfrb fw (probe 74): 5'-CGAGGGAGGAG
AGCTTA-3', mPdgfrb rev (probe 74): 5'-GTCTTGACCTGGCG
ATT-3'; mCxxcl2 fw (probe 26): 5'-AAAATCATCAGAAGAT
CATGAAAC-3', mCxxcl2 rev (probe 26): 5'-CTGGTGTCTTCCGT
TGAGG-3'; mCcl5 fw (probe 110): 5'-TGACAGAGAGCTGAC
AGC-3', mCcl5 rev (probe 110): 5'-GAGTGTGTGGCCAGCCATA-
3'; mKdr fw (probe 18): 5'-CCCCCAAATTTCCATATTGACA-3',
mKdr rev (probe 18): 5'-CGGCTCTTTGCCTACTGTG-3';
mPdgfrb fw (probe 67): 5'-TCAAGCTGCAAGTCTAATGTC-3',
mPdgfrb rev (probe 67): 5'-CCATTTGCAAGGTGGTACT-3';
mP4f1 fw (probe 64): 5'-TCTGGGACTATTTAATGAC-3',
mP4f1 rev (probe 64): 5'-CCATTTTCTAGGTTCTAT-3';
mCsf2 fw (probe 18): 5'-TGAATGAGCTCCTACCCAG-3',
mCsf2 rev (probe 18): 5'-GAGTGGTGTCCGAGCCATA-
3'; mTuba1a afw (probe 88): 5'-GTG-3',
m T u b a 1 a ar rev (probe 51): 5'-GTG-3',
mCOL3A1 fw (probe 49): 5'-GCCGTTTCGGCCGCA-3',
mCOL3A1 rev (probe 49): 5'-GGAAAACATTAAGA-3',
mPDGFB fw (probe 18): 5'-CTGGGACTATTTAATGAC-3',
mPDGFB rev (probe 18): 5'-GAGTGGTGTCCGAGCCATA-
3'; mFGF1 fw (probe 41): 5'-AGC-3',
mFGF1 rev (probe 41): 5'-TTGGGATCCATCTTAAGCAC-3',
mFGFR1 fw (probe 88): 5'-TCAAGCTGCAGGTCAATGTC-3',
mFGFR1 rev (probe 88): 5'-GAGTGGTGTCCGAGCCATA-
3'; mICAM1 fw (probe 64): 5'-CCATTGGCAGGGTGACTC-3',
mICAM1 rev (probe 64): 5'-GTGATGGCATGGACTGTGGT-3';
mFgf1 fw (probe 41): 5'-AGC-3',
mFgf1 rev (probe 41): 5'-TTGGGATCCATCTTAAGCAC-3',
mFGF1R1 fw (probe 88): 5'-GAGTGGTGTCCGAGCCATA-
3'; mKDR fw (probe 18): 5'-GCCGTTTCGGCCGCA-3',
mKDR rev (probe 18): 5'-GGAAAACATTAAGA-3',
mCOL3A1 fw (probe 49): 5'-GCCGTTTCGGCCGCA-3',
mCOL3A1 rev (probe 49): 5'-GGAAAACATTAAGA-3',
mICAM1 fw (probe 64): 5'-CCATTGGCAGGGTGACTC-3',
mICAM1 rev (probe 64): 5'-GTGATGGCATGGACTGTGGT-3';
mFGF1 fw (probe 41): 5'-AGC-3',
mFGF1 rev (probe 41): 5'-TTGGGATCCATCTTAAGCAC-3',
mFGFR1 fw (probe 88): 5'-GAGTGGTGTCCGAGCCATA-
3'; mFGF1R1 rev (probe 88): 5'-TCAAGCTGCAGGTCAATGTC-3',
mTuba1a afw (probe 88): 5'-GTG-3',
mTuba1a ar rev (probe 51): 5'-GTG-3'; mCOL3A1 fw (probe 49):
5'-GCCGTTTCGGCCGCA-3', mCOL3A1 rev (probe 49):
5'-GGAAAACATTAAGA-3', mICAM1 fw (probe 64):
5'-CCATTGGCAGGGTGACTC-3', mICAM1 rev (probe 64):
5'-GTGATGGCATGGACTGTGGT-3'; mFGF1 fw (probe 41):
5'-AGC-3', mFGF1 rev (probe 41): 5'-TTGGGATCCATCTTAAGCAC-
3', mFGFR1 fw (probe 88): 5'-GAGTGGTGTCCGAGCCATA-
3'; mFGFR1 rev (probe 88): 5'-TCAAGCTGCAGGTCAATGTC-3'.

Bone marrow transplantation and atherosclerosis model
1 wk before bone marrow transplantation, female WT and
SPPL2a/b DKO mice received water supplemented with anti-
biotics (neomycin, 100 mg/liter, and Polymyxin B sulfate,
60,000 U/liter, both from Gibco), which was continued for
5 wk after the procedure. Mice were exposed to 6 Gy total
body irradiation 1 d before and on the day of transplantation
with 5 × 10⁶ bone marrow cells isolated from WT mice. After
a recovery period of 5 wk, hypercholesterolemia was induced by
adeno-associated viral expression of a gain-of-function mutant
of PCSK9 (rAAV8-D377Y-mPCSK9) according to Bjørklund et al.
(2014). Viral particles produced by the University of North
Carolina Vector Core were injected into the tail vein of the mice
(10¹ genome copies). Concurrently, mice were fed an HCD
containing 0.25% cholesterol in addition to 15% cacao butter,
1% corn oil, 40.5% sucrose, 10% corn starch, and 5.95% cellu-
lose (824171; Special Diets Services) for 9 wk. Plasma con-
centrations of triglycerides and cholesterol were determined
at baseline just before HCD initiation as well as after 9 wk
using standard enzymatic kits (Cholesterol FS’10; Trigly-
cerides FS 5’ Ecoline; Diagnostic Systems GmbH) according to
the manufacturer’s instructions. Prior to blood sampling from
the tail vein, mice were fasted for 4 h.

Female LDL receptor-deficient (Ldlr⁻/⁻) mice (n = 4, C57Bl6
background) were obtained from in-house breeding, irradiated
2+ with 6 Gy, and reconstituted with WT bone marrow, followed
by HCD (824171; Special Diets Services) feeding for 3 wk (permit
number 2014-019; Maastricht University, Netherlands).

Histological analysis
After 9 wk of HCD feeding, mice were anesthetized, euthanized,
and perfused with PBS containing nitroprusside (0.1 mg/ml;
Sigma-Aldrich). Hearts were excised and fixed in 1% parafor-
maldehyde overnight. Serial paraffin sections of the aortic root
were cut (4 µm) and stained with H&E (Sigma-Aldrich). Hearts were excized and
speciﬁcations. Plaques were classiﬁed as early (foam cell-rich but lacking a necrotic
core), moderately advanced (containing a ﬁbrotic cap and often a necrotic core, but no medial macrophage inﬁltration), and advanced
lesions (typiﬁed by medial macrophage inﬁltrates, elastic lamina degradation, and more pronounced necrosis and ﬁbrosis). Atherosclerotic lesions were further analyzed for abundance of
macrophages (1:200; MAC3; clone M3/84; BD Biosciences),
smooth muscle cells (1:3,000; aSMA; clone 1A4; Sigma-Aldrich)
and collagen content (Sirius Red; Polysciences). Sections (MAC3,
aSMA) were subjected to heat-induced antigen retrieval using
citrate buffer (pH 6; DAKO), and speciﬁc antigen-antibody
binding was visualized using appropriate biotinylated secondary
antibodies, ABC horseradish peroxidase (Vector Laboratories),
and DAB substrate kit (DAKO). Cell nuclei were counterstained
with hematoxylin. Pictures were taken using a Leica DM3000
light microscope, and sections were analyzed in a blinded
manner using Adobe Photoshop CS6 software.

Immunohistochemistry of human plaque sections
Collection, storage, and use of human carotid artery tissue in the
Maastricht Pathology Tissue Collection and patient data confi-
dentiality were performed after informed consent and in
agreement with the “Code for Proper Secondary Use of Tissue
in the Netherlands,” which is in accordance with the

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guidelines of and approved by the medical and ethical committee of Maastricht University Medical Centre, Maastricht, Netherlands. Human carotid endarterectomy segments were fixed in paraformaldehyde and paraffin-embedded. Sections were incubated with polyclonal antisera against human SPPL2a or SPPL2b (described above), followed by detection with Brightvision secondary antibodies (ImmunoLogic) and Vector Red (Vector Laboratories). Cell nuclei were counterstained with hematoxylin. Pictures were taken using a Leica DM3000 light microscope.

Statistical analysis
For statistical analysis, an unpaired Student’s t test or a one-way ANOVA with either Tukey’s or Dunnett’s post hoc testing was performed as indicated in the individual figure legends using GraphPad Prism. All data are shown as mean ± SD. Where stably transduced iMAECs were used, data quantification is based on at least three independently generated cell lines. n describes the number of independent experiments, and n depicts the number of individual samples for quantification.

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R. Fluhrer, A. Fukumori, and H. Steiner conducted the cleavage site determination. A.O. Helbig and A. Tholey contributed the proteomic analysis of aortic samples. B. Rabe provided reagents and protocols including technical advice. K. Theodorou and M. Donners performed the atherosclerosis experiments and contributed to the overall design of the study. T. Mentrup analyzed and interpreted data. B. Schröder designed, conceptualized, and supervised the research. B. Schröder and T. Mentrup wrote the manuscript. All authors contributed to the manuscript editing.

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References
Akmedov, A., I. Rozenberg, F. Paneni, G.G. Camici, Y. Shi, C. Doerrries, A. Sledzinska, F. Mocharia, A. Breitenstein, C. Lohmann, et al. 2014. Endothelial overexpression of LOX-1 increases plaque formation and promotes atherosclerosis in vivo. Eur. Heart J. 35:2839–2848. https://doi.org/10.1093/euheartj/het352
Al-Alwan, L.A., Y. Chang, A. Mogas, A.J. Halyko, C.J. Bagole, J.G. Martin, S. Rousseau, D.H. Eidelberg, and Q. Hamid. 2013. Differential roles of CXCL2 and CXCL3 and their receptors in regulating normal and asthmatic airway smooth muscle cell migration. J. Immunol. 191:2731–2741. https://doi.org/10.4049/jimmunol.1203421
Aoyama, T., K. Takeda, B. Schröder, K. Yamamoto, X.W. Cheng, J.K. Liao, and T. Murohara. 2009. Gamma-Secretase inhibitor reduces diet-induced atherosclerosis in apolipoprotein E-deficient mice. Biochem. Biophys. Res. Commun. 383:216–221. https://doi.org/10.1016/j.bbrc.2009.03.154
Babst, M., and G. Odorizzi. 2013. The balance of protein expression and degradation: an ESCRTs point of view. Curr. Opin. Cell Biol. 25:489–494. https://doi.org/10.1016/jceb.2013.05.003
Behnke, J., J. Schneppenheim, F. Koch-Nolte, F. Haag, P. Saftig, and B. Schröder. 2011. Signal-peptide-peptidase-like 2a (SPPL2a) is targeted to lysosomes/late endosomes by a tyrosine motif in its C-terminal tail. FEMS Lett. 586:2951–2957. https://doi.org/10.1016/j.femsle.2011.08.043
Beisner, D.B., P. Langeraker, A.E. Parker, C. Dahlberg, F.J. Otero, S.E. Sutton, L. Poirot, W. Barnes, M.A. Young, S. Niessen, et al. 2013. The intramembrane protease SPPL2a is required for B cell and DC development and survival via cleavage of the invariant chain. J. Exp. Med. 210:23–30. https://doi.org/10.1084/jem.20121072
Bergmann, H., M. Labas, A. Short, L. Mioso, N. Barthel, C.E. Teh, C.M. Roots, K.R. Bull, Y. Jeslali, K. Horikawa, et al. 2013. B cell survival, surface BCR and BAFFR expression, CD74 metabolism, and CD8- dendritic cells require the intramembrane endopeptidase SPPL2a. J. Exp. Med. 210:31–40. https://doi.org/10.1084/jem.20121076
Biwa, T., M. Sakai, M. Shichiri, and S. Horiiuchi. 2000. Granulocyte/macrophage colony-stimulating factor plays an essential role in oxidized low density lipoprotein-induced macrophage proliferation. J. Atheroscler. Thromb. 7:14–20. https://doi.org/10.5551/jat1994.7.14
Bjerklund, M.M., A.K. Hollensen, M.K. Hagensen, F. Dagnaes-Hansen, C. Christoffersen, J.G. Mikkelson, and J.F. Bentzon. 2014. Induction of atherosclerosis in mice and hamsters without germline genetic engineering. Circ. Res. 114:1684–1689. https://doi.org/10.1161/CIRCRESAHA.114.302937
Bond, A.R., C.W. Ni, H. Jo, and P.D. Weinberg. 2010. Intimal cushions and endothelial nuclear elongation around mouse aortic branches and their spatial correspondence with patterns of lipid deposition. Am. J. Physiol. Heart Circ. Physiol. 298:H536–H544. https://doi.org/10.1152/ajpheart .00917.2009
Chen, K.J., Y. Chen, Y. Liu, J. Xie, D. Li, T. Sawamura, P.L. Hermonat, and J.L. Mehta. 2005. Adhesion molecule expression in fibroblasts: alteration in fibroblast biology after transfection with LOX-1 plasmids. Hypertension. 46:622–627. https://doi.org/10.1161/01.HYP.000017945.99515.00
Cichla, I., A. Yilmaz, M. Klein, D. Raithel, D.R. Brigtostock, W.G. Daniel, M. Gottfert-Streubel, and C.D. Garlicks. 2005. Connective tissue growth factor is overexpressed in complicated atherosclerotic plaques and

Mentrup et al.
SPPL2 proteases control LOX-1 signaling

https://doi.org/10.1084/jem.20171438
Sawamura, T., N. Kume, T. Aoyama, H. Moriwaki, H. Hoshikawa, Y. Aiba, T. Tanaka, S. Miwa, Y. Katsuma, T. Kita, and T. Masaki. 1997. An endothelial receptor for oxidized low-density lipoprotein. Nature. 386:73–77. https://doi.org/10.1038/386073a0

Schnepfheim, J., R. Dressel, S. Hüttil, R. Lüllmann-Rauch, M. Engelke, K. Dittmann, J. Wienands, E.L. Eskelinen, I. Hermans-Borgmeyer, R. Fluhrer, et al. 2013. The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. J. Exp. Med. 210:41–58. https://doi.org/10.1084/jem.201210069

Schnepfheim, J., S. Hüttil, A. Kruchen, R. Fluhrer, I. Müller, P. Safig, R. Schnepfheim, C.L. Martin, and B. Schröder. 2014a. Signal-peptide-peptide-like 2a is required for CD74 intramembrane proteolysis in human B cells. Biochem. Biophys. Res. Commun. 451:48–53. https://doi.org/10.1016/j.bbrc.2014.07.051

Schnepfheim, J., S. Hüttil, T. Mentrup, R. Lüllmann-Rauch, M. Rothaug, M. Engelke, K. Dittmann, R. Dressel, M. Araki, K. Araki, et al. 2014b. The intramembrane proteases signal Peptide peptidase-like 2a and 2b have distinct functions in vivo. Mol. Cell. Biol. 34:1398–1411. https://doi.org/10.1128/MCB.00038-14

Schröder, B., W. Rinkelgabe, A. Hasilik, and P. Safig. 2010. Molecular characterisation of ‘transmembrane protein system 192′ (TMEM192), a novel protein of the lysosomal membrane. Biol. Chem. 391:695–704. https://doi.org/10.1515/bc.2010.062

Schermer, M. 1990. An expression vector system for stable expression of B cell receptors. J. Immunol. 144:48–54. https://doi.org/10.1155/1003.0198

Schwartz, M. 2004. Rhe signalling at a glance. J. Cell Sci. 117:5457–5458. https://doi.org/10.1242/jcs.0198

Shevchenko, A., H. Tomas, J.V. Olsen, and M. Mann. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1:2856–2860. https://doi.org/10.1038/nprot.2006.468

Singer, C.A., B. Lontay, H. Uruh, A.J. Halayko, and W.T. Gerthoffer. 2011. Src mediates cytokine-stimulated gene expression in airway myocytes through ERK MAPK. Cell Commun. Signal. 9:14. https://doi.org/10.1186/1478-811X-9-14

Steinberg, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. 320:915–924.

Thakkar, S., X. Wang, M. Khaidakov, Y. Dai, K. Gokulan, J.L. Mehta, and K.I. Vanniasinkam. 2008. Oxidised LDL receptor-1 associates with lectin-like oxidized LDL receptor-1 and mediates oxidized lipoprotein receptor-1 as a biochemical marker for atherosclerosis-associated diseases. Dis. Markers. 25:131–141. https://doi.org/10.1155/2008/2005315

Thakkar, S., X. Wang, M. Khaidakov, Y. Dai, K. Gokulan, J.L. Mehta, and K.I. Vanniasinkam. 2008. Oxidised LDL receptor-1 associates with lectin-like oxidized LDL receptor-1 and mediates oxidized lipoprotein receptor-1 as a biochemical marker for atherosclerosis-associated diseases. Dis. Markers. 25:131–141. https://doi.org/10.1155/2008/2005315

van der Vorst, E.P., M. Jeurissen, I.M. Wolfs, A. Keijbeck, K. Theodorou, E. Wunderlich, P., K. Glebov, N. Kemmerling, N.T. Tien, H. Neumann, and W. Rinkelgabe. 2010. The molecular physiology of signal peptide peptidase (SPP) and SPP-like proteases. Mol. Cell. Biol. 30:3087–3096. https://doi.org/10.1128/MCB.00038-14

Wang, X., M. Khaidakov, Z. Ding, Y. Dai, K. Gokulan, J.L. Mehta, and T. Harada. 2007. Lectin-like oxidized low-density lipoprotein receptor-1 as a biochemical marker for atherosclerosis-associated diseases. Dis. Markers. 25:131–141. https://doi.org/10.1155/2008/2005315

White, S.J., G.B. Sala-Newby, and A.C. Newby. 2011. Overexpression of ADAM17 degrades the α7 nicotinic acetylcholine receptor and modulates atherosclerotic plaque composition by shifting the balance of inflammatory and nematicidal Bacillus thuringiensis. J. Proteomics. 113:337–350. https://doi.org/10.1016/j.jprot.2014.09.027

van der Vorst, E.P., M. Jeurissen, M.L. Wolfs, M. Jeurissen, T. L. Theelen, J.C. Blumer, E. Wijnands, J.P. Cleutjens, Y. Li, et al. 2017. Whole body and hematopoietic ADAMS deficiency does not influence advanced atherosclerotic lesion development, despite its association with human platelet aggregation. Sci. Rep. 7:11670. https://doi.org/10.1038/s41598-017-10549-x

Treich, C., L. Cassidy, A. Höckendorf, M. Leippe, and A. Tholey. 2015. Quantitative proteome analysis of Caenohabditis elegans upon exposure to nematocidal Bacillus thuringiensis. J. Proteomics. 113:337–350. https://doi.org/10.1016/j.jprot.2014.09.027

van der Vorst, E.P., M. Jeurissen, M.L. Wolfs, M. Jeurissen, T. L. Theelen, J.C. Blumer, E. Wijnands, J.P. Cleutjens, Y. Li, et al. 2017. Whole body and hematopoietic ADAMS deficiency does not influence advanced atherosclerotic lesion development, despite its association with human platelet aggregation. Sci. Rep. 7:11670. https://doi.org/10.1038/s41598-017-10549-x

Voss, M., B. Schröder, and R. Fluhrer. 2013. Mechanism, specificity, and physiology of signal peptide peptidase (SPP) and SPP-like proteases. Biochim. Biophys. Acta. 1828:2828–2839. https://doi.org/10.1016/j.bbamem.2013.03.033

Wang, X., M. Khaidakov, Z. Ding, S. Mitra, L. Yu, Y. Dai, and J.L. Mehta. 2012. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and cardiac fibroblast growth. Hypertension. 60:1437–1442. https://doi.org/10.1161/HYPERTENSIONAHA.112.200659

White, S.J., G.B. Sala-Newby, and A.C. Newby. 2011. Overexpression of scavenger receptor LOX-1 in endothelial cells promotes atherogenesis in the ApoE(−/−) mouse model. Cardiovasc. Pathol. 20:369–373. https://doi.org/10.1016/j.carpath.2010.08.007

Wolfe, S.D., B. Sherry, D. Juers, G. Davatelis, R.W. Yurt, and A. Cerami. 1989. Identification and characterization of macrophage inflammatory protein-2. Proc. Natl. Acad. Sci. USA. 86:612–616. https://doi.org/10.1073/pnas.86.2.612

Wunderlich, F., K. Glebov, N. Kenmeringer, N.T. Tien, H. Neumann, and J. Walter. 2013. Sequential proteolytic processing of the triggering receptor expressed on myeloid cells-2 (TREM2) protein by ectodomain shedding and γ-secretase-dependent intramembranous cleavage. J. Biol. Chem. 288:33027–33036. https://doi.org/10.1074/jbc.M113.517540

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