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Extracellular processing of lysyl oxidase-like 2 and its effect on amine oxidase activity

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Abstract

Over-expression of lysyl oxidase-like 2 (LOXL2) is associated with several hepatic and vascular fibrotic diseases and tumor progression in some aggressive cancers. Secreted LOXL2 promotes extracellular matrix crosslinking by catalyzing the oxidative deamination of peptidyl lysine. A great deal remains to be learned about the post-translational modifications of LOXL2, including whether such modifications modulate enzymatic and disease-promoting activities; such knowledge would inform the development of potential therapies. We discovered that upon secretion in cell culture, LOXL2 undergoes proteolytic processing of the first two of four scavenger receptor cysteine-rich domains at the N-terminus. A similar pattern of processing was also evident in tissue extracts from an invasive ductal carcinoma patient. Processing occurred at ^{314}Arg-^{315}Phe-^{316}Arg-^{317}Lys↓–^{318}Ala-, implicating proprotein convertases. siRNA-mediated knockdown of proprotein convertases (furin, PACE4, and PC5/6), as well as incubation with their recombinant forms, showed that PACE4 is the major protease that acts on extracellular LOXL2. Unlike LOX, which requires cleavage of its propeptide for catalytic activation, cleavage of LOXL2 was not essential for tropoelastin oxidation nor for crosslinking of collagen type IV *in vitro*. However, in the latter case, processing enhanced the extent of collagen crosslinking ~2-fold at [LOXL2] ≤ 10 nM. These results demonstrate an important difference in the regulatory mechanisms for LOX and LOXL2 catalytic activity. Moreover, they pave the way for further studies of potential differential functions of LOXL2 isoforms in fibrosis and tumor progression.

Lysyl oxidase-like 2 (LOXL2) belongs to the lysyl oxidase (LOX) family of proteins, which consists of Cu^{2+}-and lysine tyrosylquinone (LTQ)-dependent amine oxidases. LOX catalyzes the oxidative deamination of the ε-amino group of lysine and hydroxylysine

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Author contributions

M.M. conceived the project and drafted, edited and completed the manuscript. K.O. performed most of the experiments and took part in drafting the manuscript. H-J.M. conducted the kinetic assay. H-J.M. and J.F. performed preliminary studies on inhibitor screening and generated mutants and selected HEK293 cells stably expressing mutant LOXL2s. J.F. and A.M. helped edit the manuscript. All authors read and approved the manuscript.

ASSOCIATED CONTENT

Supporting Information

Notes

The authors declare no competing financial interest.

residues in collagen and elastin to promote crosslinking of these molecules, which is essential for stabilization of the extracellular matrix (ECM).[1, 2] LOXL2 has been considered as a therapeutic target for cardiac, pulmonary and hepatic fibrosis[3–5] as well as for aggressive tumors (e.g. breast, colon, gastric, liver, lung, etc.).[6–8] In these diseases, LOXL2 is highly upregulated and mostly detected in fibrotic foci.[8–10] In addition to LOXL2, LOX is often upregulated in these diseases; however, LOX was also proposed to be a tumor suppressor in gastric cancer.[11] LOXL2-knockdown and a specific antibody against LOXL2 have been shown to effectively reduce disease progression in mice models, whereas suppression of LOX by a specific antibody was reported to be ineffective.[3, 10,12,13] LOX and LOXL2 share a highly conserved C-terminal amine oxidase catalytic domain; therefore, LOXL2 is generally presumed to have the LOX amine oxidase activity. However, LOX differs from LOXL2 at its N-terminus, where the latter has four scavenger receptor cysteine-rich (SRCR) domains (Fig. 1).[14] The SRCR domains are mostly found in cell-surface or secreted proteins, where they are proposed to function in ligand binding.[15] However, the molecular function of the SRCR domains of LOXL2 remains to be determined.

LOX is catalytically activated in the ECM when bone morphogenetic protein-1 (BMP-1) cleaves the LOX pro-domain between Gly168 and Asp169 (Fig. 1).[16–19] The BMP-1 cleavage site is not conserved in LOXL2 nor other SRCR domain-containing LOXLs (i.e. LOXL3 and LOXL4). While proteolytic cleavage of SRCR domains has been reported for other extracellular proteins such as macrophage receptor CD163 and Type I and II bovine macrophage receptors, the protease responsible for the processing has not been identified.[20, 21] Akiri *et al*. first reported that a recombinant LOXL2 (rLOXL2) was secreted as a mixture of ~100-kDa and ~60-kDa proteins from MCF-7 breast cancer cells stably transfected with a rLOXL2 expression construct; they raised the possibility of proteolysis.[22] Hollosi *et al*. reported that the processing of rLOXL2 in the culture media occurred in a time-dependent manner, and a protease-inhibitor cocktail inhibited the processing.[23] We have worked to define the posttranslational modifications of LOXL2 and their impact on LOXL2 subcellular localization, enzyme activity and biological functions. We reported that *N*-glycosylation, but not LTQ cofactor formation, is essential for LOXL2 secretion.[24–26] Recently, we used liquid chromatography -tandem mass spectrometry (LC-MS/MS) to characterize the *N*-linked glycosylation profile of WT-LOXL2 isolated from a suspension culture of HEK293F cells.[27] All three predicted *N*-glycosylation sites (e.g. Asn288, Asn455, and Asn644, Fig. 1) were occupied with complex and acidic glycans, where a variety of unique glycopeptide compositions (33 kinds at Asn288, 8 kinds at Asn455, and 34 kinds at Asn644) were identified. Here, we focused on rLOXL2 secreted from HEK293 cells to define the mode of proteolytic processing of LOXL2 and assess the importance of the processing on the LOX amine oxidase activity of LOXL2 *in vitro.*

MATERIALS AND METHODS

Materials.

MDA-MB-231 and PFHR-9 cells were purchased from the American Type Culture Collection (Manassas, VA). HEK and HEK 293T cells were purchased from Thermo Fisher Scientific (Lenexa, KS). Antipain, phenylmethylsulfonyl fluoride (PMSF), leupeptin,

pepstatin A, EDTA, EGTA, and tropoelastin were purchased from Sigma-Aldrich (St. Louis, MO). A proprotein convertase inhibitor {#537076, 4-(Guanidinomethyl)phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide} was purchased from EMD Millipore (Burlington, MA). Decanoyl-RVKR-CMK and Hexa-D-Arg were purchased from R&D Systems (Minneapolis, MN). The source of antibodies used in this study are as follows: anti-LOXL2, -LOXL3, -LOXL4, -furin, -PACE4, -PC5/6, and -Strep-tag II were purchased from Abcam (Cambridge, MA); anti-β-actin, HRP-conjugated anti-rabbit IgG, and HRP-conjugated anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA); anti-collagen type IV was purchased from Assay Biotechnology (Fremont, CA). HALT protease inhibitor, Hygromycin B, L-glutamine, penicillin/streptomycin, fetal bovine serum (FBS), MEM, DMEM and Lipofectamine 3000 were purchased from Thermo Fisher Scientific. Each set of three unique 27-mer siRNA duplexes targeting furin, PACE4, and PC5/6 (si-furin: 5'-rGrGrArCrArUrGrArGrArUrArArUrGrUrUrArGrArGrGrUTT-3'; siPACE4 (si-PCSK6): 5'-rGrGrUrCrCrArArCrArUrGrUrGrGrUrArCrCrUrGrCrArUTG-3'; si-PC5/6 (si-PCSK5): 5'-rGrUrCrArUrArUrCrArGrGrArUrArCrCrArArGrArArArArAAT-3') were purchased from Origene (Rockville, MD).

Site-directed Mutagenesis.

pcDNA3.1-WT-LOXL2 was used as the template for Quikchange site-directed mutagenesis to generate R314P-, R314A-, R316A-, K317A-, K317Q-, and K317R-point mutations as described.[26] The following primer pairs from Eurofins Genomics (Louisville, KY) were used to introduce point mutations. K317A-forward: 5'-CCCTCAAGATTCCGGGCCGCGCTAAAGCCAG AG-3'; K317A reverse: 5'CTCTGGCTTGTACGCGGCCCGGAATCTTGAGGG-3'; K317Q-forward: GGACCCTCAA GATTCCGGCAGGCGTACAAGCCAGAGCAAC; K317Q-reverse: 5'-GTTGCTCTGGCTTGTACGC CTGCCGGAATCTTGAGGGTCC-3'; K317R-forward:5'-CCTCAAGATTCCGGAGAGCGTACAAGC CAG-3'; K317R-reverse: 5'-CTGGCTTGTACGCTCTCCGGAATCTTGAGG-3'; R314P-foward: 5'-AGC CCTGACGGACCCTCACCCTTCCGGAAAGCGTACAAG-3'; R314P-reverse: 5'-CTTGTACGCTTTC CGGAAGGGTGAGGGTCCGTCAGGGCT-3'. R314A/R316A/K317A (AAA)-LOXL2 and R314A/ R316A/K317Q (AAQ)-LOXL2, were generated using the K317Q-LOXL2 construct using the following primer pairs. R314A/R316A/K317A-foward: 5'-CCTGACGGACCCTCAGCCTTCGCCGCCGCGTACA AGCCA-3'; R314A/R316A/K317A-reverse: 5'-TGGCTTGTACGCGGCGGCGAAGGCTGAGGGTC CGTCAGG-3'; R314A/R316A/K317Q-forward: 5'-CCTGACGGACCCTCAGCCTTCGCCCAGGCGTA CAAGCCA-3'; R314A/R316A/K317Q-reverse: 5'-TGGCTTGTACGCCTGGGCGAAGGCTGAGGGTC CGTCAGG-3'. Sequences were validated by DNA sequencing (Genewiz, South Plainfield, NJ).

Cell lines and cell culture.

HEK293 cells stably expressing WT-LOXL2 (HEK-WT-LOXL2) were selected in our lab.[26] HEK cells stably expressing K317A-, K317Q-, K317R-, R314P-, AAA-, and AAQ-LOXL2s were selected using the same procedure. HEK stable cells were maintained in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 150 μg/mL of Hygromycin B at 37°C in a 5% CO_2 humidified

atmosphere. MDA-MB-231 and PFHR-9 cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Detection of recombinant LOXL2 (rLOXL2) in conditioned media of HEK stable cells.

HEK-LOXL2 cells were seeded in MEM at 1×10^7 cells per 100 mm Petri dish or 3×10^6 cells per 50 mm Petri dish. After 24 hours, the growth medium was replaced with serum-free medium (SFM). The conditioned media were harvested at the indicated time and concentrated using 30-kDa cut-off Amicon Ultra-4 centrifugal filter units (EMD Millipore, Burlington, MA). The concentrated media were mixed with 2× Laemmli sample buffer (Bio-Rad, Hercules, CA), loaded onto an 8% polyacrylamide gel, and proteins were separated by SDS-PAGE. The separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and subjected to Western blot analysis using anti-LOXL2 antibody (1:5,000) and HRP-conjugated anti-rabbit IgG (1:5,000). Proteins were visualized with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).

Detection of native LOXL2 and LOXL4 in conditioned media of MDA-MB-231 cells.

MDA-MB-231 cells were seeded in a 6-well plate or a 50 mm Petri dish, respectively, for detection of LOXL2 and LOXL4. At 80% confluency, growth media were replaced with SFM. Conditioned medium was harvested 1–3 days later, concentrated, and subjected to SDS-PAGE and Western blot analysis as described above. For detection of LOXL4, anti-LOXL4 antibody (1:5,000) was used.

Detection of recombinant LOXL3 (rLOXL3) in conditioned media of HEK cells transiently expressing rLOXL3.

The ORF of WT-LOXL3 in pCMV6-XL6 (Origene, Rockville, MD) was subcloned into pEXPR-IBA42 (IBA, Göttingen, Germany) at the BsaI sites and then the coding sequence for LOXL3-Strep II was subcloned into pcDNA3.1-Hygro(−) (Thermo Fisher Scientific), using KpnI and XhoI restriction enzymes (pcDNA-WT-LOXL3-StrepII). The DNA sequence was validated by Sanger sequencing. HEK-293T cells were seeded in MEM at 0.5×10^6 cells/well in a 6-well plate. After 24 hours, cells were transfected with 5 μg of pcDNA-WT-LOXL3-StrepII by Lipofectamine 3000. Two days later, culture media were replaced with SFM and cells were incubated three more days. The culture media were harvested, concentrated, and subjected to SDS-PAGE and Western blot analysis as described above, except anti-Strep-tag II antibody (1:50,000) was used as the primary antibody.

Extraction of proteins from breast cancer tissue.

Flash-frozen biospecimen of human breast cancer tissues were obtained from the Biospecimen Repository Core Facility at the University of Kansas Medical Center. Frozen breast tissue (0.1 – 0.2 g) was suspended in urea buffer (8 M urea, 50 mM DTT, 1% SDS, Halt protease inhibitors) and 30% (V/V) glass beads were added. Each sample was lysed by ultrasonication on ice and the soluble fraction was isolated as supernatant after centrifugation at 20,000 × g for 20 min at 4°C. The protein concentration of the supernatant was determined by BCA assay. A 6.4 μg of total protein was subjected to SDS-PAGE and Western blot analysis as described above. For detection of LOXL2, anti-LOXL2 antibody

(1:1,000) was used as primary antibody. The Western blot X-ray film was scanned on a Brother MFC-J870DW and the image was saved as a jpg file (600 dpi). The intensity values of protein bands of interest and background of the digitized image were calculated by ImageJ (ver. 1.48, NIH). The background intensity was calculated from three separate areas on the same film and normalized.

Chemical inhibition of LOXL2 processing.

HEK-LOXL2 cells were seeded in MEM in 6-well plates at 1×10^6 cells/well. At 24 hours post-seeding, the growth media were replaced with SFM containing antipain (100 μM), PMSF (1 mM), leupeptin (100 μM), pepstatin A (100 μM), EDTA (1 mM), EGTA (1 mM), #537076 (50 μM), Decanoyl-RVKR-CMK (50 μM), or Hexa-D-Arg (50 μM). Two days later, conditioned media were collected and concentrated. A 10 μL of the concentrated media was subjected to SDS-PAGE and Western blot analysis as described above.

siRNA transfection.

HEK-WT-LOXL2 and HEK-K317R-LOXL2 cells were seeded in MEM at $0.3 – 0.5 \times 10^6$ cells/well in 6-well plates, and allowed to grow until the cell density reached 20–30% confluency. Cells were transfected with 50 nM siRNA duplexes using Lipofectamine 3000 following the manufacturer's protocol. Three days after transfection, the growth medium was replaced with SFM containing the corresponding siRNA and incubated for one day (HEK-K317R-LOXL2) or 3 days (HEK-WT-LOXL2). The conditioned medium was prepared and subjected to SDS-PAGE and Western blot analysis as described above.

Preparation of whole cell lysate.

After siRNA transfection, cells were trypsinized and harvested by centrifugation at 1,000 × g at 4°C for 10 min. Cells were washed with ice-cold PBS and then lysed in RIPA buffer containing HALT protease inhibitors with brief ultrasonication on ice. After incubating lysates on ice for 30 min, insoluble materials were removed by centrifugation at 20,000 × g for 10 min at 4°C. The protein concentration of the supernatant fluids was determined by BCA assay and adjusted to 1 mg/mL with RIPA buffer. Proteins (typically 5–10 μg) were separated on on a 8% acrylamide gel by SDS-PAGE and subjected to Western blot analysis as described above. The following antibodies were used: furin (1:5,000); PACE4 (1:2,000); PC5/6 (1:2,000); β-actin (1:5,000); HRP-conjugated anti-rabbit IgG (1:5,000); HRP-conjugated anti-mouse IgG (1:5,000).

Preparation of rLOXL2.

WT-, AAQ-and K317R-LOXL2s were produced in Freestyle™ HEK suspension culture (Thermo Fisher Scientific) and purified following the published method,[25] with some optimization. Full-length (~100-kDa) and Δ1–2SRCR-LOXL2s were isolated by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column on an AKTA FPLC (GE Healthcare Life Science, Pittsburgh, PA). Proteins were eluted at 1 mL/min in 0.1 M Tris buffer (pH 8.0) containing 1% Pluoronic F68 and 0.15 M NaCl.

Tropoelastin oxidation.

The amine oxidase activity of rLOXL2 was performed using the standard HRP coupled Amplex™ Red H_2O_2 detection assay in 50 mM sodium borate buffer (pH 8) at 37°C as described.[25]

Processing of rLOXL2 by proprotein convertases.

Recombinant human PCs (furin, PACE4 and PC5/6) were produced and purified as described.[28] Purified WT-, K317R-, and AAQ-LOXL2 (100 nM) were incubated with 1 U of PCs in 20 mM HEPES buffer (pH 7.5) containing 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 0.5 % Triton X-100 for 3 hours at 37°C. The reaction was quenched by addition of 2× Laemmli sample buffer, then subjected to SDS-PAGE and Western blot analysis as described above.

Collagen type IV crosslinking.

Collagen type IV was prepared based on the published procedure[3] with some modification. For ECM deposition, $2 – 3 \times 10^6$ or $0.5 – 1 \times 10^7$ of PFHR-9 cells (ATCC, CRL-2423) were seeded on a fibronectin-coated 50-mm or 100-mm Petri dish and grown 8–10 days past confluence in 5 mL or 15 mL of DMEM containing 50 μg/mL ascorbic acid and 1 mM BAPN at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was replaced with fresh medium every 24 hours. PFHR-9 cells were detached from the dish by incubating with 1% (w/v) deoxycholate in water for 10 min at room temperature. The insoluble ECM was washed three times with PBS lacking divalent cations overlaid with 1×10^7 cells of HEK-LOXL2 or MDA-MB-231 cells (harvested at 50 – 80% confluency in MEM or DMEM, respectively) in 15 mL of SFM in 100-mm dishes, and incubated at 37°C for 3 days. To examine the effect of rLOXL2, the insoluble ECM was prepared as described above, and incubated with rLOXL2 in 5 mL of 20 mM sodium phosphate buffer (pH 8) in 50-mm dishes and incubated at 37°C for 5 days. The ECM was throughly scraped off the dish in 1 mL of 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1% (w/v) deoxycholate, 5 mM benzamidine, 25 mM 6-aminocaproic acid, and 0.5 mM PMSF. The ECM was homogenized by sonication and the insoluble fraction was isolated by centrifugation at 15,000 × g for 20 min at 4°C. This process was repeated one more time to remove soluble fractions. Finally, the pellets were vortexed for 10 min at 4°C and the insoluble fraction was isolated by centrifugation at 15,000 × g for 20 min at 4°C. The pellets were then suspended in 200 μL of 50 mM Tris-HCl (pH 7.4) containing 0.1 mg/mL collagenase, 5 mM $CaCl_2$, 5 mM benzamidine, 25 mM 6-aminocaproic acid, and 0.5 mM PMSF, and incubated for 24 hours at 37°C. The reaction mixture was centrifuged at 15,000 × g for 30 min at 4°C to remove collagenase-resistant materials. The supernatant was mixed with 4× Laemmli sample buffer, boiled for 5 min and subjected to SDS-PAGE analysis on an 8% acrylamide gel. The 7S monomer and oligomers (dimer, trimer, tetramer, pentamer and hexamer) of collagen IV were detected by Western blot analysis using anti-collagen IV antibody (1:2,000). The Western blot X-ray film was scanned on a Brother MFC-J870DW and the image was saved as a jpg file (600 dpi). The intensity value of protein bands of interests of the digitized image was calculated by ImageJ (ver. 1.48, NIH) and statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Data are presented as mean ± SEM

of three independent experiments. Statistical significance was analyzed with two-way ANOVA followed by multiple comparisons with Bonferroni correction. * represents $P < 0.05$. ** represents $P < 0.005$.

RESULTS AND DISCUSSION

LOXL2 undergoes proteolytic processing upon secretion.

WT-LOXL2 secreted from HEK-WT-LOXL2 cells was initially detected as a single ~100-kDa protein; however, a ~60-kDa form appeared in the culture medium upon extended culture (Fig. 2A, Fig. S1), as reported.[23] This time-dependent processing was also detected for endogenous LOXL2 secreted from MDA-MB-231 breast cancer cells (Fig. 2B). Importantly, these two forms of LOXL2 also were readily detected in tissue extracts prepared from a biopsy sample of an invasive ductal carcinoma (IDC) patient (Fig. 2C). Both LOXL2 isoforms were also detected in a biopsy sample from one ductal carcinoma *in situ* (DCIS) patient, albeit in significantly reduced amounts (Fig. 2C). The endogenous LOXL2s detected in the culture media of breast cancer cells (Fig. 2B) and in tissue extracts (Fig. 2C) had slightly smaller apparent molecular masses than those of the rLOXL2s secreted from HEK-WT-LOXL2 cells (Fig. 2A). This is most likely due to alterations in the *N*-glycan profile, as has been commonly observed for proteins secreted from cancer cells and tissues.[29, 30] In the IDC tissue extract, an additional strong band at ~50-kDa was detected. Future study is necessary to determine whether this ~50-kDa protein is a LOXL2 isoform, and if so, how it is generated. The N-terminal sequence of the ~60-kDa form (isolated from HEK-WT-LOXL2 cell culture) was determined by Edman degradation analysis (PAN Facility, Stanford University), revealing that ^{317}Lys↓^{318}Ala-(in between the second and the third SRCR domains) is the cleavage site (Fig. 2D).

Inhibitor screening and site-directed mutagenesis studies suggest a proprotein convertase.

Cleavage immediately C-terminal to a Lys residue implicates a trypsin-type serine protease. Concordantly, common serine protease inhibitors such as antipain and leupeptin inhibited the processing in cell culture, whereas the aspartyl protease inhibitor pepstatin A had no effect (Fig. 3A). Curiously, the serine protease inhibitor PMSF was also ineffective, although this might be attributed to the extremely short half-life of PMSF in aqueous solution.[31] EDTA also inhibited the processing, but EGTA was less effective (Fig. 3A). We incubated WT-LOXL2 with a few known secreted serine proteases such as trypsin and HtrA1, but none of them produced the 60-kDa form. After incubating WT-LOXL2 with a few known secreted serine proteases, such as trypsin and HtrA1, we observed that trypsin gradually degraded WT-LOXL2 into small peptides, whereas HtrA1 produced 35-, 50-, and 55-kDa forms of LOXL2 (Fig. S2). While we never observed increased abundance of the 60-kDa form after incubation with trypsin or HtrA1, we cannot fully exclude the possibility that these proteases produced the 60-kDa form as an intermediate product. However, our collective results indicated that the responsible protease was a serine protease, but probably not of the trypsin type.

We then selected HEK293 cells stably expressing cleavage-site variants such as K317A-, K317Q-, and K317R-LOXL2s. Like WT-LOXL2, K317A-and K317Q-LOXL2s were secreted as ~100-kDa protein on Day 1 (Fig. 3B). Unexpectedly, they both underwent time-dependent processing, suggesting that one or more proteases most likely also process LOXL2 at two other Arg residues near Lys317 (i.e. Arg314 and Arg316). To support this idea, the processing was completely inhibited for HEK293 cells stably expressing triple mutants (R314A/R316A/R317A-and R314A/R316A/R317Q-LOXL2s, abbreviated as AAA-, and AAQ-LOXL2, respectively) (Fig. 3C). Moreover, whereas the efficiency of WT-LOXL2 processing increased when HEK-WT-LOXL2 cells were at passage numbers ≥10, AAA-and AAQ-LOXL2s resisted proteolytic cleavage under any tested culture condition (up to cell passage number 6 or 21, respectively; Fig. 3C and 3D). On the other hand, K317R-LOXL2 underwent complete processing on Day 1 (Fig. 3B), regardless of cell passage number. On the other hand, K317R-LOXL2 underwent complete processing on Day 1. These data indicated that the protease(s) strongly prefer Arg over Lys at P1. Based on these results, Arg-X-Arg-Lys/Arg seemed to be the recognition sequence for the endogenous protease(s), implicating one or more subtilisin-like proprotein convertases (PCs).[32, 33]

PC1/3, PC2, furin, PC4, PC5/6, paired basic amino acid cleaving enzyme 4 (PACE4), and PC7 are known to cleave precursor proteins at specific single or paired basic amino acid residues within the conserved polybasic site, $(R/K)X_{2n}(R/K)\downarrow$.[33, 34] We examined the effect of three commercially available cell-permeable PC-family inhibitors (Inhibitor #537076, Decanoyl-RVKR-CMK, and Hexa-D-Arg) on LOXL2 cleavage. Inhibitor #537076 inhibits furin ($K\hat{i}$ = 16 pM), PC4, PC5/6, and PACE4 with similar potency but does not inhibit PC2, PC7, or trypsin-like serine proteases.[35] Decanoyl-RVKR-CMK irreversibly inhibits all seven PCs (PC1, PC2, furin, PC4, PACE4, PC5/6, PC7).[36] Hexa-D-Arg competitively inhibits furin (K_i = 0.106 mM), PACE4 ($K\hat{i}$ = 0.58 mM) and PC1 ($K\hat{i}$ = 13.2 mM).[37] All three inhibitors effectively suppressed LOXL2 cleavage (Fig. 3D), implicating PC1/3, furin, PC4, PC5/6, and/or PACE4 as the responsible protease(s). Among these PCs, secreted isoforms have been reported for furin, PC5/6 and PACE4.[38–41]

siRNA knockdown and treatment with recombinant PCs indicate PACE4 is the primary protease in LOXL2 processing.

We found that the processing of WT-LOXL2 was suppressed by siRNA targeting *PCSK6* (PACE4), whereas siRNAs targeting *FURIN* or *PCSK5* (PC5/6) were not effective (Fig. 4A). On the other hand, the processing of K317R-LOXL2 was ~50% suppressed by siRNAs targeting all three PCs (Fig. 4B). To further evaluate these results, we first used size-exclusion chromatography to isolate 100-kDa forms of WT-, K317R-, and AAQ-LOXL2 from suspension-adapted HEK293-F cell cultures (Fig 4C and Fig. S3). In these suspension cultures, the extent of cleavage in crude rLOXL2 was substantially decreased, probably because PC expression in the suspension-adapted cell line was much lower than in our adherent HEK293 cell line (unpublished results). We then incubated the rLOXL2s with purified recombinant forms of furin, PACE4, and PC5/6 in HEPES buffer at pH 7.5. 100-kDa WT-LOXL2 was processed only by PACE4 (Fig. 4D, left), whereas 100-kDa K317R-LOXL2 was processed by all three recombinant PCs (Fig. 4D, middle). None of these recombinant PCs were able to process the triple mutant AAQ-LOXL2 (Fig. 4D, right).

We reasoned that examination of the other SRCR domain-containing LOX family members (i.e., LOXL3 and LOXL4) might provide some insight into the mechanism by which PCs recognize and cleave LOXL2. While the sequence of amino acids surrounding the processing site is conserved for all mammalian LOXL2 orthologs, the recognition sequence is altered in LOXL3 and LOXL4 (Fig. 5A). Although both LOXL3 and LOXL4 contain paired basic amino acid residues at P2-P1, neither of them have a basic residue at P4. Concordantly, rLOXL3 and LOXL4 secreted from HEK293 cells and MDA-MB-231 cells, respectively, were detected as full-length ~85-kDa and ~90-kDa proteins (Fig. 5B, left and middle). This suggests that PACE4 requires a basic residue at P4 for LOXL2 cleavage. Since LOXL4 contains the –Arg(P2)-Lys(P1)-sequence of LOXL2 but is secreted as a single, full-length protein, we hypothesized that Pro at the P4 position may block proteolytic cleavage. To test this, we generated R314P-LOXL2. This mutation completely inhibited the processing (Fig. 5B, right). Thus, PACE4 recognizes and cleaves a highly specific sequence within LOXL2 [e.g. Arg(P4)-X-Arg(P2)-Lys(P1)↓-site]. This tightly regulated cleavage is unique to LOXL2 among the LOX-family of proteins.

Taken together, our results indicate that PACE4 is the primary protease responsible for processing WT-LOXL2 at the -Arg(P4)-X-Arg(P2)-Lys(P1)↓-site in between the 2nd and 3rd SRCR domains. Two other secreted PCs, furin and PC5/6, are excluded from participating in WT-LOXL2 cleavage, due to their strong preference for Arg over Lys at P1. Moreover, our findings are consistent with published data regarding the sensitivity of PCs to various protease inhibitors.[39] In that study, 2 mM PMSF inhibited furin and PACE4 only ~30%; we determined that 1 mM PMSF did not appreciably inhibit the processing of LOXL2 (Fig. 3A). We did not test higher concentrations of PMSF due to its cytotoxicity. Additionally, it was reported that PACE4 was inhibited 30% by 1 mM EDTA but not at all by 1 mM EGTA, whereas furin was completely inhibited by EDTA or EGTA at 1 mM. The authors proposed either that PACE4 does not require a stoichiometric amount of Ca^{2+} for its activity, or that Ca^{2+} is bound much more tightly to PACE4 than furin, therefore even EGTA is not able to fully chelate out Ca^{2+}. These results are consistent with ours, where 1 mM EDTA completely inhibited the processing of LOXL2 but EGTA at the same concentration was ineffective (Fig. 3A).

Proteolytic processing is not essential for the amine oxidase activity of LOXL2.

LOXL2 is generally presumed to have the same biological functions as LOX, due to their conserved LOX catalytic domain. However, whereas it is known that secreted pro-LOX undergoes ~3-fold proteolytic activation via BMP-1-mediated cleavage of the pro-peptide in cell culture studies,[17, 42, 43] the impact of PC-mediated cleavage on LOXL2 activity has not been determined. If LOXL2 is regulated in a similar manner to LOX, then the ~60-kDa form of LOXL2 would be expected to have significantly increased amine oxidase activity compared to the ~100-kDa form. To test this, WT-and AAQ-LOXL2s were isolated as the 100-kDa form, and 60-kDa Δ1–2SRCR-LOXL2 was purified from K317R-LOXL2 (a mixture of ~100-kDa and ~60-kDa) by size-exclusion chromatography (Fig. 4C, Fig. S3). Importantly, each rLOXL2 contained nearly stoichiometric amounts of LTQ cofactor and Cu^{2+}, as determined by a UV-vis spectroscopic titration assay that we recently developed (manuscript in preparation). The basic kinetic parameters for the oxidation of tropoelastin by

these three kinds of purified rLOXL2 are summarized in Table 1. Both k_{cat} and K_m values differed by < 2-fold among the three rLOXL2s. Thus, removal of the first two SRCR domains had an insignificant impact on amine oxidase activity as measured by tropoelastin oxidation by LOXL2 in solution.

We also sought to determine the effect of LOXL2 cleavage on LOXL2-mediated crosslinking of collagen type IV. To this end, we applied to our system a recently described method[44] with some modification: we used the ECM deposited from PFHR-9 cells without isolation of collagen type IV 7S dodecamer, because lower order 7S domains (i.e. monomer, dimer, trimer, tetramer, pentamer, and hexamer) were detectable by immunoblot analysis using a collagen type IV antibody. The tetramer, pentamer, and hexamer of 7S domains were detected in PFHR-9-derived ECM overlaid with MDA-MB-231, HEK-WT-LOXL2, and HEK-K317R-LOXL2 cells (Fig. 6A). However, no crosslinking was detected with negative controls, including HEK293 cells stably transfected with an empty-vector, and HEK-WT-LOXL2 cells in the presence of BAPN, a LOX inhibitor. Likewise, we observed crosslinking of collagen type IV when PFHR-9-derived ECM was treated with purified WT-LOXL2 (Fig. 6B). The amine oxidase-activity of LOXL2 was required for this crosslinking, since we did not detect any hexamer or pentamer in the presence of BAPN or with a phenylhydrazine (PH)-inhibited form of rLOXL2.

To assess whether proteolytic cleavage of LOXL2 plays a critical role for collagen type IV crosslinking, we added varying amounts of purified rLOXL2s (WT-, AAQ-, and Δ1–2SRCR-LOXL2, Fig. 4C) in 20 mM phosphate buffer (pH 8.0) to the ECM deposited from PFHR-9 cells (Fig. 7A). Due to the potential for LOXL2 to undergo processing during the 5-day incubation with ECM, we took aliquots of LOXL2-containing supernatant on day 0 and on day 5 (immediately prior to isolation of the 7S domains), and subjected these to Western blot analysis. Indeed, WT-LOXL2 underwent substantial processing during the 5-day incubation, whereas the cleavage-invulnerable AAQ-LOXL2 and the already-cleaved Δ1–2SRCR-LOXL2 remained essentially unchanged (Fig. 7B, compare top and bottom). For each rLOXL2, the amount of crosslinked collagen type IV (pentamer and hexamer of 7S domains) increased in a LOXL2-dose-dependent manner. At lower concentrations (5 and 10 nM) of rLOXL2, Δ1–2SRCR-LOXL2 was ~2-fold more active than full-length LOXL2, although these differences were not statistically significant ($P = 0.1916$, $P = 0.0539$, respectively). At higher concentrations (20 and 40 nM), the difference in catalytic activity was diminished (Fig. 7C), most likely due to saturation of LOXL2 activity. Further study is necessary to evaluate whether the modest activating effect imparted by cleavage of the first two SRCR domains is biologically significant.

During the course of our study, Lopez-Jimenez *et al.* published complementary findings, identifying the cleavage site of LOXL2 as ^{317}Lys↓–^{318}Ala and proposing that a serine protease was responsible for LOXL2 cleavage.[45] Here, we demonstrated that the proprotein convertase PACE4 is the principal catalyst of LOXL2 cleavage. PACE4 specifically cleaves LOXL2, but not LOXL3 nor LOXL4, by recognizing the polybasic sequence comprising ^{314}Arg-^{315}Phe-^{316}Arg-^{317}Lys↓–^{318}Ala-), which is absent in the latter LOXLs. In our experiments, the proteolytic processing of LOXL2 did not substantially alter its LOX amine oxidase activity in solution, when assayed by tropoelastin oxidation using isolated rLOXL2s

(Table 1). Furthermore, we observed that processing only modestly increased LOXL2 activity in an *in vitro* assay of type IV collagen crosslinking (Fig. 7A, 7C). In contrast, Lopez-Jimenez *et al.* reported that a cleavage-invulnerable mutant LOXL2 (R257G/R316G/K317E-LOXL2: GGE-LOXL2) was unable to crosslink collagen type IV, and concluded that proteolytic cleavage is necessary for physiological function.[45] However, crude media from HEK cells stably expressing GGE-LOXL2 exhibited amine oxidase activity towards cadaverine (*i.e.,* a small molecule). Therefore, the authors proposed that LOXL2 processing exerts a regulatory effect other than catalytic activation, perhaps permitting LOXL2-collagen interaction by alleviating steric hindrance from the first two SRCR domains. Currently, no crystal structure of the full-length WT-LOXL2 is available, and further study is necessary to understand how GGE-LOXL2 might differ structurally from AAQ-LOXL2, our processing-invulnerable mutant that is competent for type IV collagen crosslinking.

Conclusion

LOXL2 undergoes time-dependent proteolytic processing of the first two SRCR domains at the N-terminus upon secretion in cell culture. LOXL2 processing was also detected in tissue extracts from an invasive ductal carcinoma patient and from a ductal carcinoma in situ patient. Site-directed mutagenesis experiments identified the protease recognition site as ^{314}Arg-Phe^{315}-Arg^{316}-Lys^{317}↓-Ala^{318}-, which is unique to LOXL2 in the LOX-family of proteins. siRNA-mediated knockdown of proprotein convertases (furin, PACE4, and PC5/6), as well as incubation with their recombinant forms, showed that PACE4 is the major protease that processes extracellular LOXL2. The proteolytic processing of LOXL2 is not essential for its activity in tropoelastin oxidation nor in crosslinking of collagen type IV *in vitro*. However, processing enhanced the extent of collagen crosslinking ~2-fold at [LOXL2] ≤ 10 nM. In summary, these results demonstrate an important difference in the mechanism for regulation of LOX and LOXL2 catalytic activity. We have begun to study the biological and physiological significance of the processing of the first two SRCR domains. PACE4 has been shown to play a critical role in cell proliferation and tumor progression in prostate, ovarian, and both estrogen receptor-positive and -negative breast cancers.[26, 46–49] LOXL2 is highly upregulated in triple negative breast cancer cells and has been demonstrated to promote cell proliferation, tumor progression/invasion *in vitro* and *in vivo*.[10, 22, 26, 50] It will be important to determine whether LOXL2 collaborates with PACE4 in these roles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BAPN	β-aminopropionitrile
LOX	lysyl oxidase
LOXL2	lysyl oxidase-like 2
LOXL3	lysyl oxidase-like 3
LOXL4	lysyl oxidase-like 4
PCSK	proprotein convertase subtilisin/kexin family gene
PC	proprotein convertase
PACE4	proprotein convertase subtilisin/kexin type 6
SRCR	scavenger receptor cysteine-rich

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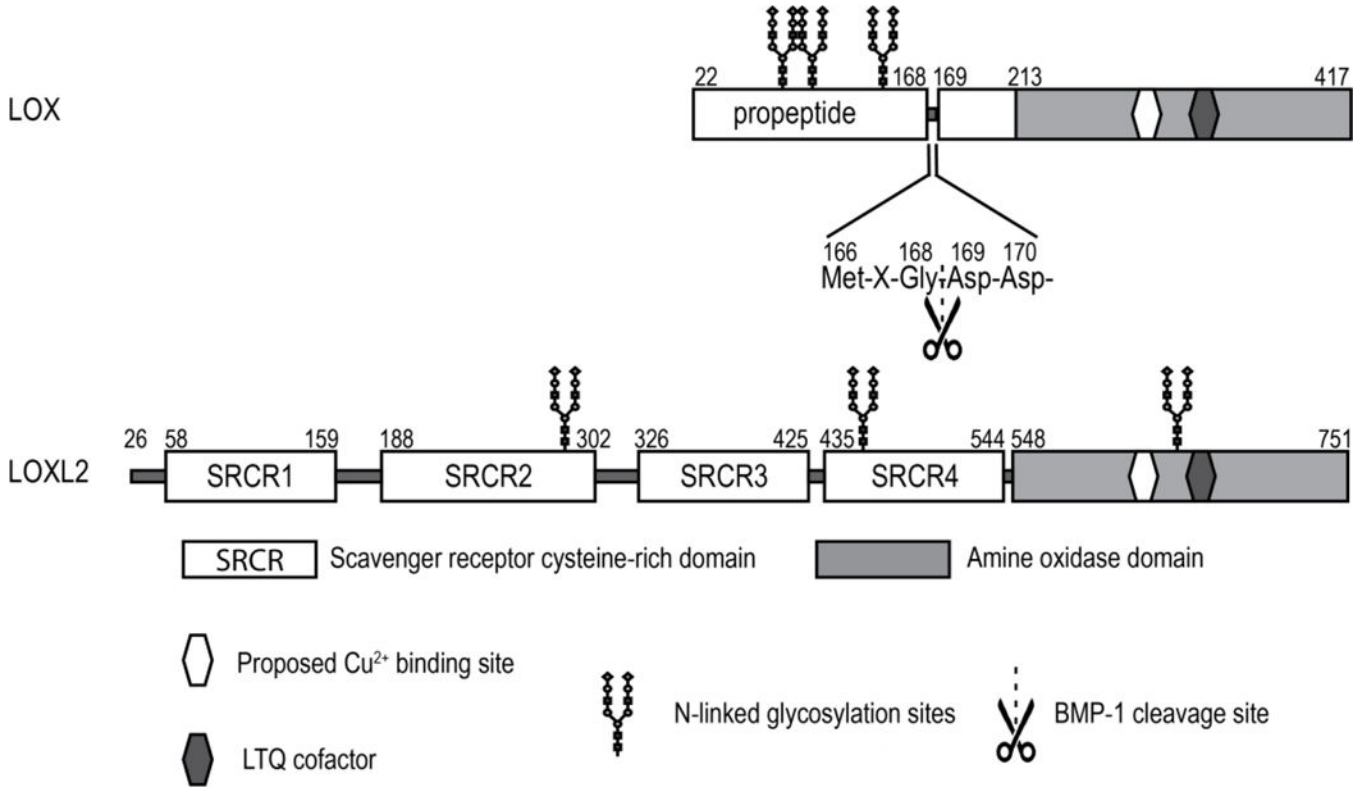

Figure 1. A schematic representation of human LOX and LOXL2.

The *N*-linked glycosylation sites identified in LOXL2 are Asn288, Asn455 and Asn644.[27] The proposed Cu^{2+} binding site for LOXL2 is His626-X-His628-X-His630.

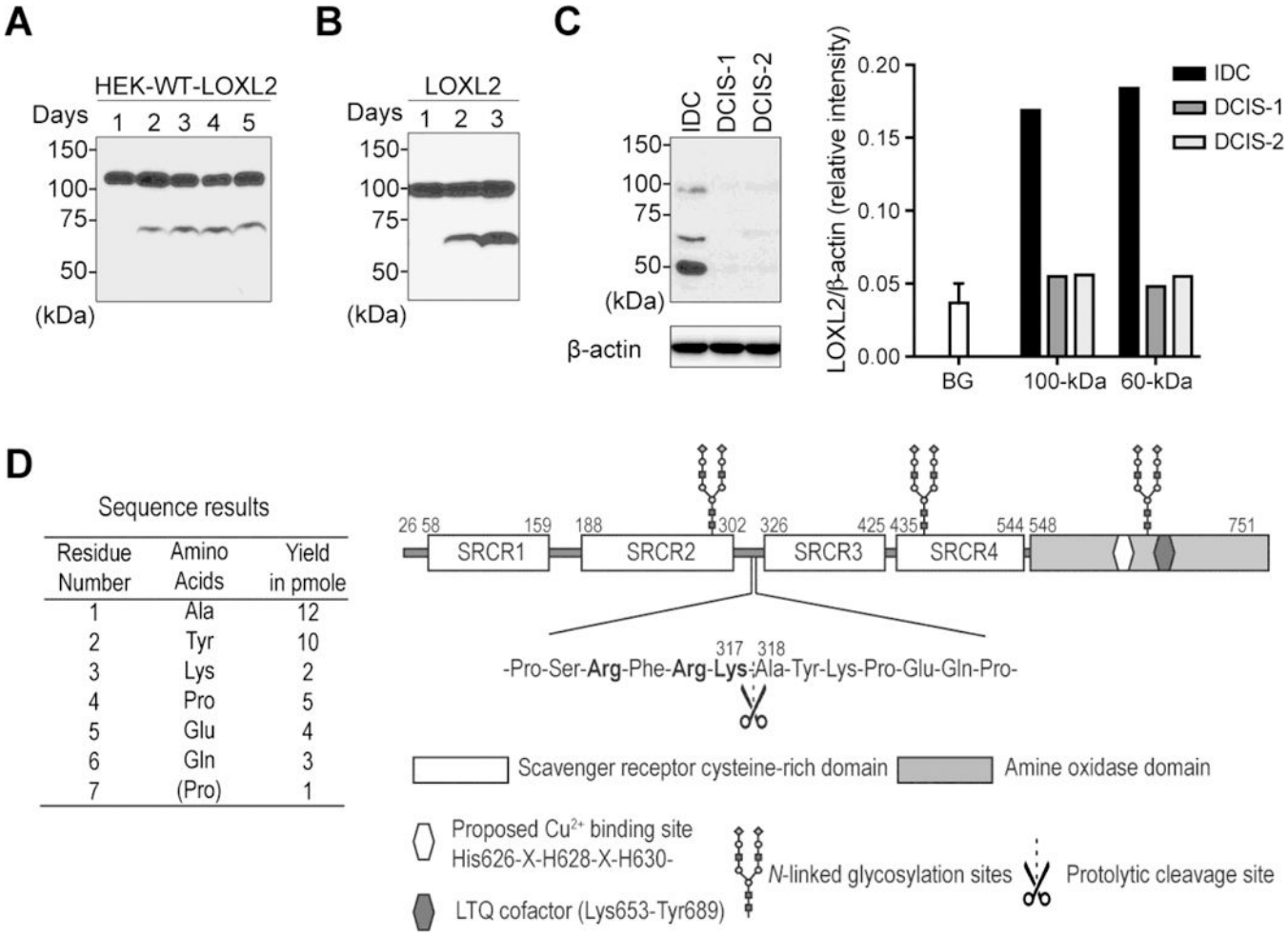

Figure 2. Secreted LOXL2 undergoes proteolytic processing.

A, Time-dependent processing of WT-LOXL2 detected in the culture media of HEK-WT-LOXL2. ***B***, Time-dependent processing of endogenous LOXL2 secreted from MDA-MB-231 cells ***C,*** Detection of the two forms (~100-kDa and ~60-kDa) of LOXL2 in tissue extracts prepared from biopsy samples of breast cancer patients. Western blot analysis of total extracts prepared from a sample of IDC (invasive ductual carcinoma) and two samples of DCIS (ductural carcinoma in situ) tissues dissected from breast cancer patients. β-Actin staining was included as a loading control (left panel). Signal relative intensity was normalized to β-actin (right panel). BG: background intensity (mean ± S.D.) from three independent areas from the same X-ray film. ***D,*** *N*-terminal sequence of 60-kDa LOXL2 revealed that the processing occurs in between the second and the third SRCR domains at ^{317}Lys-^{318}Ala. *Left*, N-terminal sequence of the 60-kDa LOXL2 by Edman degradation. *Right*, Schematic representation of the proteolytic cleavage site of LOXL2 identified in this study.

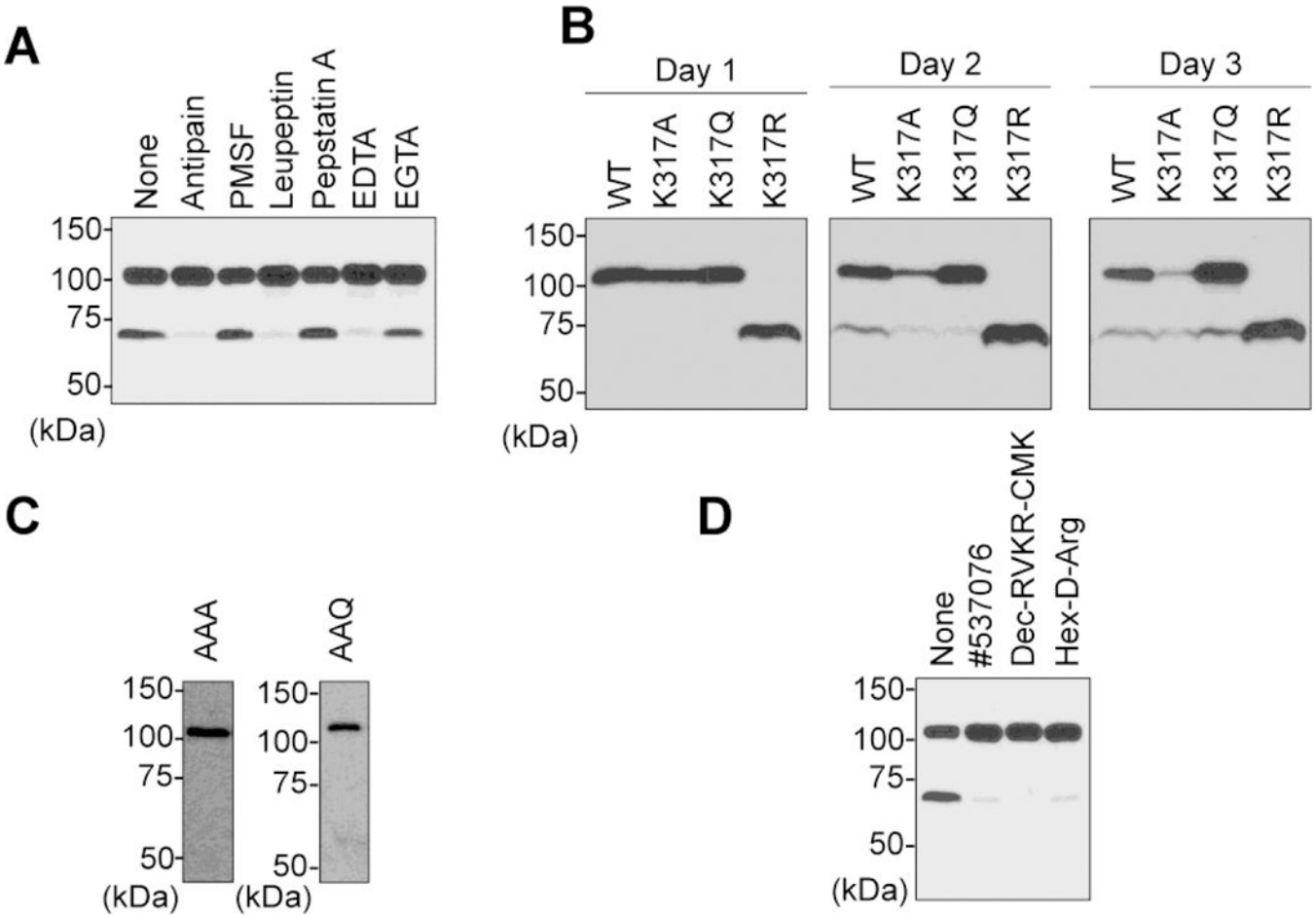

Figure 3. Effect of protease inhibitors and site-directed mutagenesis on the processing of LOXL2.

A, Effect of protease inhibitors on the proteolytic processing of LOXL2. ***B,*** Time-dependent processing of LOXL2 point mutants in the conditioned media of the corresponding HEK293 stable cells. ***C***, No processing was observed for triple mutants,AAA-LOXL2 and AAQ-LOXL2 in cell culture at day 3. ***D,*** Effect of proprotein convertase inhibitors on WT-LOXL2 processing in conditioned media of HEK-WT-LOXL2 (day 2).

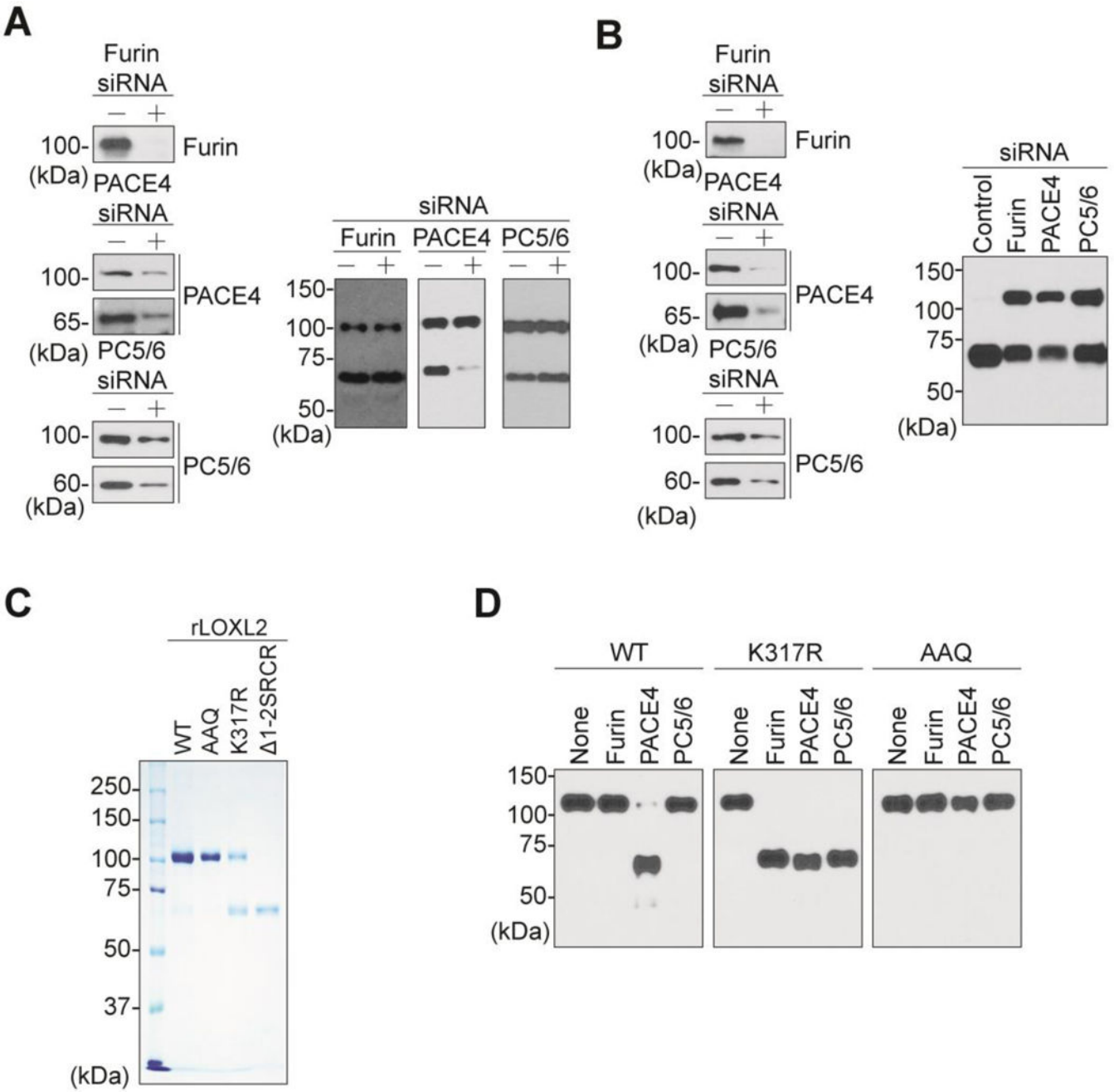

Figure 4. PACE4 catalyzes the proteolytic processing of WT-LOXL2.

A, Furin, PACE4 or PC5/6 were knocked down by specific siRNAs, and the knockdown efficacy was determined by probing whole cell lysates with anti-furin, anti-PACE4 or anti-PC5/6 antibodies, respectively (left panel). The effect of PC-specific knockdown on WT-LOXL2 processing was determined by probing the conditioned media of siRNA-treated HEK-WT-LOXL2 cells with anti-LOXL2 antibody (right panel). ***B,*** Furin, PACE4, or PC5/6 were knocked down by specific siRNAs, then the knockdown efficacy was determined (left panel) as described in Fig. 4A. The impact of PC-specific knockdown on K317R-LOXL2 processing was determined in the conditioned media of HEK-K317R-LOXL2 cells (right

panel). ***C***, SDS-PAGE analysis of purified rLOXL2s (2 μg) used for *in vitro* assays in this study. The 100-kDa form of K317R used in this study was further isolated from the mixture of 100-kDa and 60-kDa (***C***) by size-exclusion chromatography (Fig. S3). ***D,*** Cleavage of 100-kDa rLOXL2s by recombinant PCs.

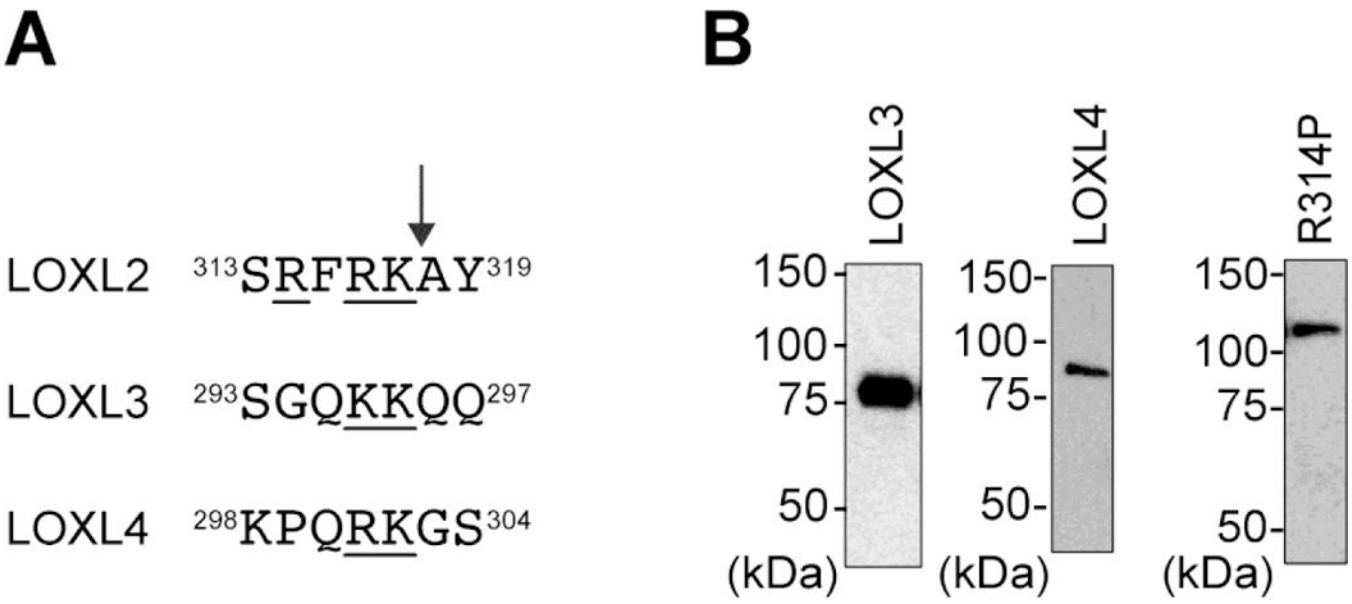

Figure 5. The -Arg(P4)-X-Arg(P2)-Lys(P1)↓-site is unique to LOXL2.

A, Sequence alignment of the processing site of LOXL2 (underline + arrow) with the corresponding sequences of LOXL3 and LOXL4 (paired basic residues are underlined). ***B,*** Detection of recombinant LOXL3, endogenous LOXL4 and R314P-LOXL2 in the day 3 culture media of rLOXL3-transfected HEK293 cells, MDA-MB-231 cells and HEK-R314P-LOXL2, respectively.

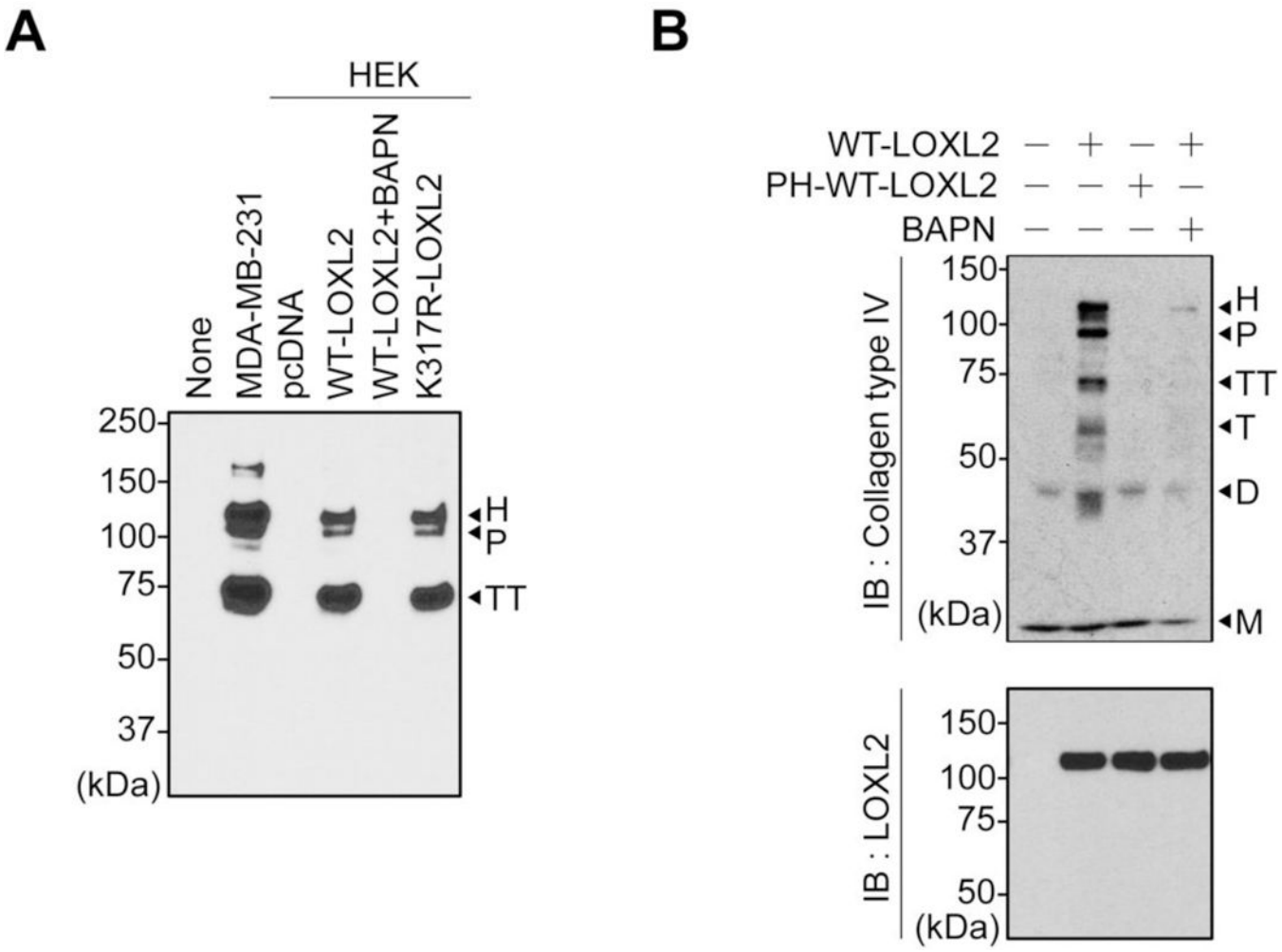

Figure 6. LOXL2 catalyzes the crosslinking of collagen type IV *in vitro*.

A, Detection of collagen type IV 7S domains in the collagenase-resistant fraction of ECM overlaid with MDA-MB-231, HEK-WT-LOXL2, and HEK-K317R-LOXL2 cells. None: no cells were overlaid (negative control). The ECM overlaid with empty vector-transfected cells (HEK-pcDNA) and HEK-WT-LOXL2 incubated with BAPN (1 mM) also served as negative controls. M: monomer, D: dimer, T: trimer, TT: tetramer, P: pentamer, H: hexamer of collagen type IV 7S domains. ***B***, Crosslinking of collagen type IV by purified WT-LOXL2 (20 nM) was dependent on LOX amine oxidase activity. Neither BAPN (1 mM)-inhibited rLOXL2 nor phenylhydrazine-derivatized rLOXL2 (PH-WT-LOXL2) could catalyze crosslinking of collagen type IV.

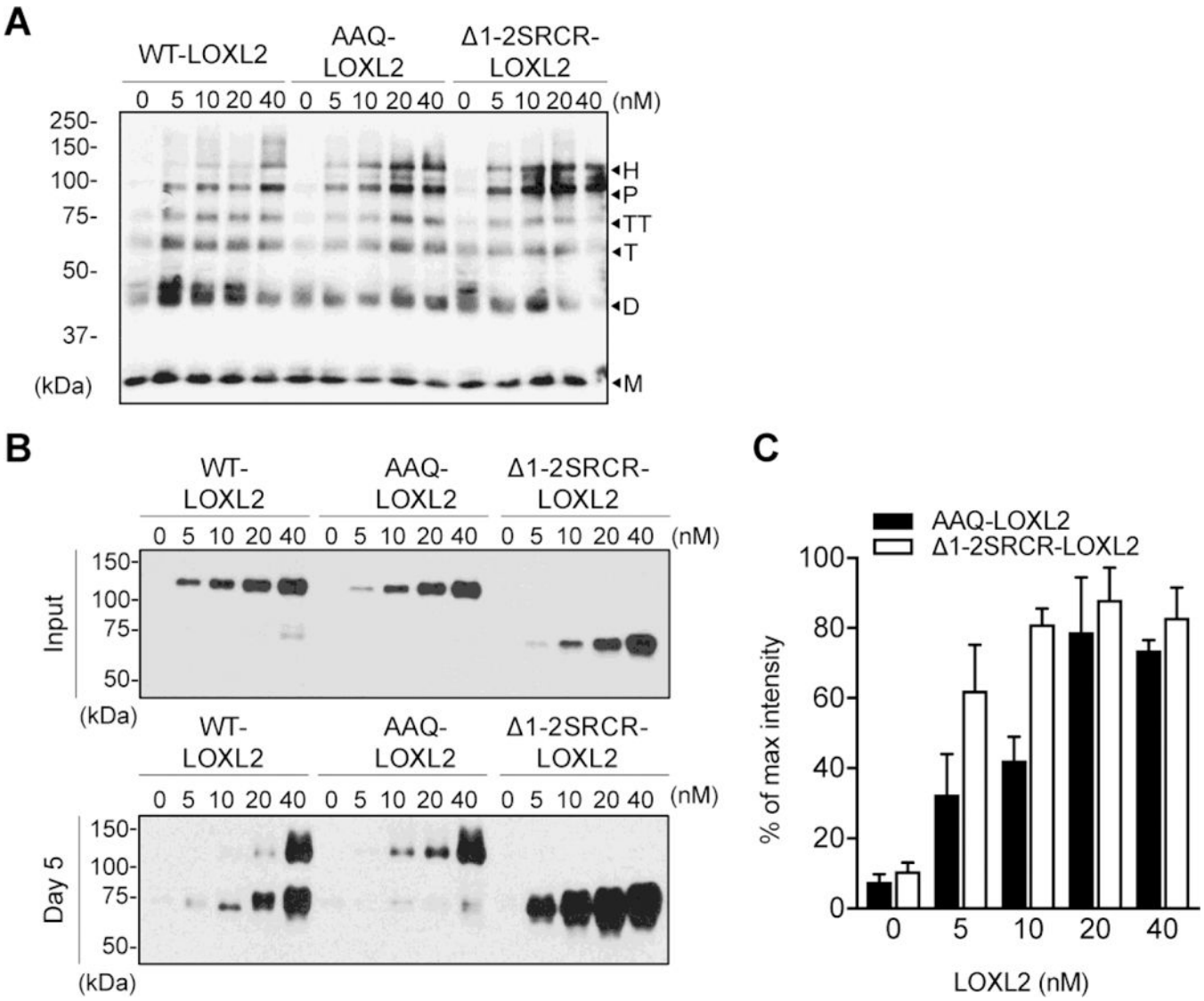

Figure 7. Proteolytic processing of LOXL2 does not significantly impact collagen type IV-crosslinking activity *in vitro*.

A, Accumulation of crosslinked 7S domains (H, P) was observed in a concentration-dependent manner when ECM was incubated in 20 mM sodium phosphate (pH 8.0) containing purified WT-, AAQ-, or Δ1–2SRCR-LOXL2. ***B***, The purity of rLOXL2 being added to PFHR-9-ECM as determined by Western blot analysis. Top panel: Immediately after addition to ECM. Bottom panel: After 5 days incubation with ECM. ***C,*** Quantitation of crosslinked 7S domains (H, P) formed by incubating ECM with AAQ-or Δ1–2SRCR-LOXL2. Error bars indicate ± SEM.

Table. 1

Kinetic parameters of the oxidation of tropoelastin by rLOXL2.

LOXL2	V_{max} (mAbs min^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}min^{-1}$)
WT-	311.7 ± 13.0	11.2 ± 0.5	$(2.8 \pm 0.4) \times 10^{-4}$	$(4.0 \pm 0.6) \times 10^{4}$
R314A/R316A/K317Q-	234.5 ± 18.0	9.2 ± 0.7	$(4.8 \pm 1.0) \times 10^{-4}$	$(1.9 \pm 0.4) \times 10^{4}$
Δ1–2SRCR-	176.8 ± 16.1	7.0 ± 0.6	$(4.4 \pm 1.1) \times 10^{-4}$	$(1.6 \pm 0.4) \times 10^{4}$