**ABSTRACT**

Collagens are the main structural component of the extracellular matrix (ECM) and provide biomechanical properties to connective tissues. A critical step in collagen fibril formation is the proteolytic removal of N- and C-terminal propeptides from procollagens by metalloproteases of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and BMP1 (bone morphogenetic protein 1)/Tolloid-like families, respectively. BMP1 also cleaves and activates the lysyl oxidase (LOX) precursor, the enzyme catalyzing the initial step in the formation of covalent collagen cross-links, an essential process for fibril stabilization. In this study, using murine skin fibroblasts and HEK293 cells, along with immunoprecipitation, LOX enzymatic activity, solid-phase binding assays and proteomics analyses, we report that the LOX precursor is proteolytically processed by the procollagen N-proteinases ADAMTS2 and ADAMTS14 between Asp-218 and Tyr-219, 50 amino acids downstream of the BMP1 cleavage site. We noted that the LOX sequence between the BMP1 and ADAMTS processing sites contains several conserved tyrosine residues, of which some are post-translationally modified by tyrosine O-sulfation and contribute to binding to collagen. Taken together, these findings unravel an additional level of regulation in the formation of collagen fibrils. They point to a mechanism that controls the binding of LOX to collagen and is based on differential BMP1- and ADAMTS2/14-mediated cleavage of a tyrosine-sulfated domain.

The extracellular matrix (ECM) is a complex scaffold resulting from the association of diverse macromolecules produced and secreted by cells into the surrounding medium. Not only this ECM provides structural support for resident cells, but also influences various cellular processes, including proliferation, adhesion, migration, and differentiation, as well as plays key roles in homeostasis and regeneration of tissues and organs (1). While the composition of the ECM can vary markedly, collagens are the main component conforming the structural matrix core and are fundamental to provide the connective tissues with their biomechanical properties. In vertebrates, 28 types of collagens have been described (I-XXVIII).
which are classified into several families, including the fibrillar collagen family (I-III, V, XI, XXIV, and XXVII) and basement membrane-forming collagen IV (2). Fibrillar collagens form homotrimeric (three identical α-chains) or heterotrimeric (two or three distinct polypeptide chains) molecules. Each α-chain consists of a major uninterrupted triple helical or collagenous domain (characterized by a repetition of Gly-X-Y triplets, where X and Y are commonly proline and hydroxyproline, respectively) flanked by N- and C-terminal non-collagenous domains, the N- and C-propeptides (3). Once synthesized in the endoplasmic reticulum, these collagen chains are brought together in a process governed by the C-propeptides and folded to form the procollagen molecule. Upon transit through the Golgi network and extracellular secretion, the N- and C-propeptides are cleaved off respectively by the procollagen N-proteinases belonging to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family (ADAMTS2, 3 and 14) and by C-proteinases, which are BMP1 (bone morphogenetic protein 1)/Tolloid-like enzymes. These proteolytic events yield triple helical collagen molecules which retain only a short portion of the propeptides, the telopeptides, and are able to assemble into fibrils, the most recognizable signature of collagen-based extracellular structures (3).

The mechanical strength of extracellular collagenous matrix is highly dependent on the formation of covalent cross-links within fibrils, a process initiated by the enzymatic action of lysyl oxidase (LOX) (4). LOX catalyzes the oxidative deamination of lysine and hydroxylysine residues within the telopeptide domains, yielding the corresponding aldehydes, which eventually condense with other oxidized groups or intact lysines to form a variety of, inter- and intrachain cross-linkages. Five different LOX enzymes have been identified in mammals (LOX, and LOX-like 1 to 4), being the canonical LOX the isoform most clearly associated with the remodeling and the stability of the collagen matrix, described to play important roles in fibrosis, tumor microenvironment and tissue repair (5,6).
LOX cleavage regulates collagen binding

By using a polyclonal antibody that specifically recognizes the C-terminal end of LOX protein, we have analyzed the electrophoretic pattern of LOX secreted from human lung fibroblasts and bovine endothelial cells incubated for four days in the presence or absence of the cytokine transforming growth factor-β1 (TGF-β1), a well-known activator of LOX expression (16). As shown in Figure 1A, the supernatant of fibroblasts or endothelial cells showed multiple immunoreactive bands ranging from 25 to 30 kilodalton (kDa), whose levels strongly increased upon incubation with TGF-β1. In order to analyze how this pattern arises, we have generated a human embryonic kidney (HEK) 293 cell clone stably expressing a human LOX construct under tetracyclin-dependent control. Figure 1B shows the LOX immunoreactivity associated to cells (cell extract) or secreted into the extracellular medium (supernatant) in basal conditions or upon stimulation with doxycycline, a tetracycline analog. While only a very faint band of about 50 kDa, corresponding to the intracellular precursor, was observed in the extracts from stimulated cells, significantly higher levels were secreted to the extracellular medium, including the precursor and multiple bands at 25-30 kDa. In contrast to fibroblast and endothelial cultures, where precursor bands were not detected under our experimental conditions, HEK293 cell clone displayed a limited capacity to process LOX as indicated by the accumulation of the unprocessed form, a circumstance providing the possibility to use this cell line to analyze the molecular events associated to the generation of mature forms.

**ADAMTS2 and 14 proteolytically process LOX**

We have previously identified LOX as a potential substrate of ADAMTS14 in the secretome of human fibroblasts (15). Here, we used two methodological approaches to characterize the ability of the procollagen N-proteinases, ADAMTS2, 3 and 14, to cleave LOX. Having generated HEK293 cell clones stably expressing the human forms of these ADAMTS proteases only in the presence of doxycycline (Supplementary Figure 1), we co-cultured them with HEK293 cells able to conditionally secrete LOX. After 48 hours of co-culture with or without doxycycline, the LOX pattern was evaluated by immunoblotting (Figure 2A and B). Expression of ADAMTS2 and ADAMTS14 resulted in a relative enrichment of mature forms of LOX around 25 kDa, at the expense of bands at 30 kDa, strongly suggesting a proteolytic processing. Such cleavage was not observed for ADAMTS3. It has to be mentioned that HEK293 cells display significant endogenous BMP1, which explains the conversion of precursor LOX into forms migrating around 30 kDa. We have also established an in vitro assay for the analysis of LOX cleavage by incubation of concentrated supernatants from LOX-overexpressing clone and purified preparations of ADAMTS2 and ADAMTS14, and compared their effects with that of concentrated supernatants from HEK293 cells overexpressing BMP1 (Supplementary Figure 1 and Figure 2C and D). Similar to data obtained in co-cultures, incubation with ADAMTS2 and ADAMTS14 resulted in a time-dependent reduction of the abundance of the 50 kDa LOX precursor and a concomitant accumulation of the mature form at 25 kDa. In contrast, incubation with BMP1 led to an enrichment of the processed forms around 30 kDa, clearly indicating that ADAMTS enzymes process LOX in a different location, presumably downstream from the established BMP1 site. This aspect was further confirmed by complete proteolysis by BMP1 and addition of ADAMTS14 on top of the BMP1-processed form (Figure 2E).

We then applied a proteomic approach to determine at which site LOX is cleaved by ADAMTS2/14. For that purpose, LOX supernatants exposed to ADAMTS2 were filtered through a 50 kDa-cutoff centrifugal filter in order to enrich the samples in LOX mature forms (Supplementary Figure 2). Flow-through were trypsin-digested and peptides analyzed by mass spectrometry (Figure 3). Several LOX peptides were identified that resulted from trypsin digestion at both their N- and C-terminal ends, except one hemitryptic, *i.e.* cleaved by trypsin at C- but not at N-terminus, presumably obtained by...
ADAMTS2-dependent action. In fact, careful inspection of this potential processing site within the LOX sequence revealed its location 50 residues downstream of the well-established BMP1 site, an observation that fully matches with the molecular weight of the ADAMTS2/14-processed form as observed by immunoblotting. Analysis of LOX protein sequence shows that both BMP1 (MVG/DDP) and ADAMTS (VAD/PYY) sites are well conserved among several organisms (Figure 4). Interestingly, the D/P scissile bond cleaved by ADAMTS2 and 14 is also conserved in LOX forms evolutionary close to the canonical LOX, such as lysyl oxidase-like (LOXL) isoform 1 or the zebrafish LOXL5 (a sort of hybrid LOX:LOXL1 uniquely present in this teleost fish). By contrast, the more evolutionary distant LOXL2, LOXL3 and LOXL4 isoforms display no homology in the corresponding position, in spite of the overall conservation observed in the downstream catalytic domain (5). This observation predicts that LOXL1 and LOXL5 proteins might be also substrates of ADAMTS proteases.

In order to evaluate LOX cleavage by ADAMTS2 and ADAMTS14 in a relevant cell model, we used skin fibroblasts isolated from wild type (WT) and Adamts2-Adamts14-deficient mice (17). These fibroblasts were left untreated or were incubated with TGF-β1 (5 ng/ml during 48 hours). Conditioned media were then analyzed by immunoblotting. As shown in Figure 5, for wild type fibroblasts incubated with TGF-β1, the vast majority of the LOX signal was detected around 25 kDa, which corresponds to the ADAMTS2/14 processed forms. By contrast, upon induction with TGF-β1, fibroblasts from the double knockout showed a diminishing of the 25 kDa product with respect to wild type, without significantly affecting the BMP1 product at 30 kDa, which demonstrates the participation of ADAMTS2/14 in the maturation of LOX.

Identification of tyrosine sulfation in the region of LOX protein flanked by BMP1 and ADAMTS2/14 processing sites

Examination of the LOX immunoreactive bands throughout the present study showed that they are actually formed by multiple molecular species slightly differing in their electrophoretic mobility, a behavior typically arising from extensive post-translational modifications (PTM). We have interrogated this possibility by two-dimensional (2D) electrophoresis followed by immunoblotting. As shown in Figure 6, precursor and mature forms of LOX are visualized as several spots. Interestingly, LOX precursor at 50 kDa, with a theoretical isoelectric point (pI) of 7.99, gave rise to a train of spots at much more acidic pI, an observation indicating the presence of PTM adding extra negative charges (Figure 6A). Upon cleavage with BMP1 enzyme, the most acidic part of this series of spots are kept, whereas the digestion with ADAMTS2 collapsed these species into a single spot at more basic pI (Figure 6B and C). These observations are compatible by the presence of the PTM causing the acidic shift within the protein region flanked by BMP1 and ADAMTS sites. This region, depicted in Figure 7A, represents a unique tract within LOX sequence as it contains 12 tyrosines in a 50 amino acids long segment, most of them well conserved among different LOX orthologs (see Figure 4). Tyrosine O-sulfation is one of the PTM known to add extra negative charges and has been increasingly recognized as an important regulator of protein-protein interactions in the extracellular space (18,19). In fact, tyrosine sulfation of LOX in this particular location has been previously suggested, but, to our knowledge, not thoroughly investigated (20). Analysis of LOX sequence using Sulfinator, a software used to predict tyrosine sulfation sites, revealed candidate tyrosines for this modification in a cluster within this region (Figure 7A) (21). As a first approach to investigate whether these tyrosine residues become sulfated, we have altered LOX sequence by changing tyrosines into phenylalanines, which blocked sulfation, and analyzed the mutant protein by immunoblotting under control conditions and after processing with BMP1 and ADAMTS enzymes. As shown in Figure 7B (left), the precursor of this LOX
mutant in the tyrosine cluster showed a slightly increased electrophoretic mobility compared to the wild type precursor, an effect much more evident in the forms processed by BMP1. In contrast, the products resulting from ADAMTS2 cleavage migrated similarly for both wild type and mutant proteins. Altogether, these observations suggest that these tyrosine residues are the target of a particular chemical modification, and its mutation abolishes this effect. This interpretation is further supported by 2D-electrophoresis showing that LOX spots corresponding to the precursor band and the BMP1-processed form were shifted to the right in the mutant, whereas both wild type and mutant forms accumulated into the same spot after proteolysis with ADAMTS2 (Supplementary Figure 3). Evidences for a sulfotyrosine modification were additionally obtained by using an antibody recognizing this particular PTM. As shown in Figure 7B (right), this antibody stained positive the form processed by BMP1 in the wild type but not in the mutant protein, leaving unstained the ADAMTS2-processed forms. Since our antibodies are not efficient for immunoprecipitation, we then generated LOX fused to green fluorescent protein (GFP). First, as shown in Figure 7C, we confirmed that LOX-GFP chimeras behaved essentially as the corresponding forms without the GFP-tag, i.e. both forms are substrates for BMP1 and ADAMTS2 proteases, and tyrosine residues are the subject of a PTM modifying the electrophoretic mobility of precursor and BMP1-processed forms. Then, these cell clones were metabolically labeled with inorganic \[{}^{35}\text{S}\]-sulfate and the labeling of LOX proteins was determined after immunoprecipitation by electrophoresis and autoradiography. As shown in Figure 7D (left), sulfated polypeptides corresponding to BMP1-processed forms were identified for the wild type LOX-GFP, while no labeling of the ADAMTS2 cleaved product was observed. In spite of the proven ability of the anti-GFP beads to pull down the LOX proteolytic fragments in the LOX-GFP mutant protein (Fig. 7D, right), no radioactive-labeled bands were detected for this chimera. Finally, we have also performed a proteomic characterization of immunoprecipitated LOX-GFP bands resulting from proteolysis with BMP1 by Parallel-Reaction Monitoring (PRM). Due to restrictions imposed by sequence, instead of performing a conventional digestion with trypsin, we processed protein bands with AspN, and therefore, we could only analyze the peptide DDNPYYNY (Supplementary Figure 4 and 5). By comparison with synthetic peptides harboring sulfotyrosines in different positions, we were able to unambiguously detect the sulfuryl moiety in the last tyrosine residue of this peptide (Tyr187), and also in one of the previous doublet, with no distinction of the exact position (Tyr183 or Tyr184). Disulfotyrosine peptides were not detected, most likely because negatively charge residues cannot stay in close proximity.

Taken together, these results demonstrate that tyrosine residues in the LOX sequence flanked by cleavage sites for BMP1 and ADAMTS2/14 undergo sulfation, a modification that potentially can influence the biological properties of LOX enzyme.

**Biological effect of the proteolytic processing of LOX. Contribution of the tyrosine sulfated domain for binding to collagen**

Having demonstrated that LOX is processed by ADAMTS2/14, we investigated the contribution of this cleavage to the enzymatic activity while comparing with the effect of BMP1. LOX enzymatic activity was determined in an *in vitro* assay using a soluble substrate through detection of peroxide in a horseradish peroxidase (HRP)-coupled real time reaction (22). Using this method, we observed that supernatants from doxycycline-induced HEK293 cells displayed strong increases in LOX activity compared with supernatants from cells left under basal conditions, clearly attributed to the induction of the expression of LOX protein (Fig. 8A). The presence of \(\beta\)-aminopropionitrile (BAPN, LOX inhibitor) resulted in a complete inhibition of this induction, which further demonstrated the specificity and relevance of the assay (Fig. 8A and B). As also shown in this Figure, incubation of LOX with ADAMTS2 did not
significantly modify its activity. To our surprise, treatment with BMP1, the well-established activator of LOX, led the activity essentially unchanged. Therefore, in contrast to previous assumptions, these observations suggest that the precursor is catalytically active and that the processing by BMP1 or ADAMTS enzymes does not substantially enhance this intrinsic enzymatic activity. In addition to elastin and collagen substrates, LOX enzyme can also oxidize a variety of mono- or diamines of various carbon chain lengths (23,24). Given the apparent flexibility of substrate requirements, which has permitted the development of a convenient (and widely used) HRP-coupled assay, our observations suggest that, rather than the enzymatic capacity of the LOX protein, proteolysis may determine the interaction with substrates, and thereby restrict its activity in vivo on collagen and elastin (22). We have therefore investigated the capacity of the precursor and processed forms of wild type and tyrosine-mutated LOX to bind collagen in a modified solid phase binding assay. HEK293-derived secretomes used in our studies do not contain pure LOX species, but rather a mixture resulting from the action of endogenous proteases, a confounding variable that can potentially give erroneous values in a standard colorimetric assay. For this reason, LOX-containing supernatants were incubated with immobilized collagens, either bearing telopeptides (telocollagen) or not (atelocollagen). Bound material was then collected and analyzed by SDS-PAGE coupled to immunoblotting. Using this approach, we could precisely determine the percentage of binding of the various LOX species (Fig 8). LOX precursor showed a limited capacity to bind telocollagen and this was not modified by mutation of the tyrosine sulfated domain. In contrast, BMP1-processed wild type LOX displayed a higher binding capacity, a property that was markedly reduced for the tyrosine-mutated LOX, indicating that a substantial part of the interaction goes through these modified residues. Interestingly, the form processed by ADAMTS2 showed lower binding and this was not altered in the mutant. Binding of LOX forms to atelocollagen was low and remained essentially unchanged upon proteolysis or mutation of tyrosine residues. Taken together, these results suggest that the differential cleavage of a tyrosine sulfated domain by BMP1 and ADAMTS2/14 determines the capacity of an active LOX catalytic domain to interact with collagen, and thereby to direct it towards specific lysine residues in the telopeptide domain.

Discussion

The biosynthesis of collagen is a highly complex process involving numerous events occurring intra- and extracellularly (3). The final step, fundamental to provide the collagen network with its biomechanical properties, is the introduction of covalent cross-links, a process initiated by the enzymatic action of lysyl oxidase (LOX) (4). Current knowledge of the biological properties and functions of LOX is based upon in vitro investigations, mostly done during early works on matrix biology over the past century, and more recently generated in vivo knockout models (reviewed in (25)). Based on this experimentation, it has been shown that LOX protein is secreted as an inactive precursor that is cleaved by BMP1 to yield the mature form responsible for the cross-linking of fibrillar collagens and elastin in the extracellular matrix of different tissues, mainly the vasculature, the skin and lung (7-10,26-28). The present study adds new players in this picture and provides novel structural information about LOX that is crucial for its biological activity. Here we demonstrate that ADAMTS2 and ADAMTS14, two enzymes initially known for their aminoprocollagen peptidase activity, process LOX protein in a position downstream of the well-characterized BMP1 cleavage site, and that the sequence between these sites contains a cluster of sulfated tyrosines essential for efficient collagen binding. Taken together, these data are consistent with the observation of multiple bands persistently reported in numerous studies (11-13). They show also that the differential cleavage of LOX by BMP1 and ADAMTS2/14 modifies its affinity towards collagen and most probably its capacity to oxidize lysine residues in the telopeptide (Figure 9). Analysis of the enzymatic activity of the
various LOX species revealed that BMP1- and ADAMTS-mediated proteolysis did not significantly modify the capacity of an otherwise active precursor to oxidize a soluble substrate. The intriguing observation that the LOX precursor exhibits full activity in this assay opposes the widely accepted assumption of an inactive form becoming active upon cleavage by BMP1 (7-10). However, to our knowledge, no single study in the literature has shown in line the cleavage of LOX by BMP1 and the effect on its enzymatic activity in vitro, but rather indirect effects such as a reduced LOX activity in supernatants from protease-deficient cells, or enhanced LOX activity and processing, together with augmented insoluble collagen deposition in cells expressing higher levels of BMP1 mRNA (8,29). In this respect, considering the flexibility of substrate requirements, with LOX capable of oxidizing not only collagen and elastin, but also a variety of nonpeptidyl amines, we hypothesize that LOX activity is regulated by the extent of interaction with its different substrates, rather than by modification of its intrinsic enzymatic capacity (23,24). Consistent with this hypothesis, we observed increased binding to immobilized collagen upon BMP1-mediated cleavage as compared to the unprocessed precursor. Previous studies have shown that LOX protein has a higher affinity, and thereby increased oxidation rate, on collagen present as fibrils, rather than in soluble or denatured form (30,31). Therefore, processing by BMP1 potentiates the capacity of LOX to interact with collagen fibrils, positioning the catalytic active site close to the target lysines in the telopeptides. In fact, our experiments showed that LOX is capable of binding to both telo- and atelocollagen, with BMP1 action influencing specifically the capacity to interact with telopeptide-containing collagen. This is somewhat in contrast with previous studies showing that LOX exclusively interacts with the triple helical portion since the apparent binding affinity for native collagen was found to be quite similar to that for fibrils prepared from pepsin-digested collagen (30). It should be however taken into account that these experiments were done with bovine aorta LOX preparations reported to be a mixture of multiple LOX species for which precise information about their individual binding features was not available (11).

An interesting observation of our results is the fact that cleavage by ADAMTS2 and 14 substantially diminished the capacity of LOX to bind telocollagen, indicating that an important part of the binding occurs through sequence determinants located between the BMP1 and ADAMTS cleavage sites. Using different methodological approaches, we demonstrate here that some tyrosine residues within this protein segment are modified by sulfation. While our study could not make a complete characterization of the tyrosine residues present in this cluster, we succeeded in demonstrating sulfation of at least two different tyrosines. However, based on our 2D electrophoresis analyses, it can be anticipated that additional tyrosines outside this protein segment can also be modified. The importance of this PTM in the biological function of LOX is highlighted by the significant reduction in the collagen binding capacity observed for the mutant construct. In fact, tyrosine sulfation has evolved as an important PTM mediating extracellular protein-protein interactions (18,19). In the context of the extracellular matrix, several molecules, including fibromodulin, lumican, osteoadherin and opticin, have been reported to contain clusters of sulfated tyrosine residues (32,33). Particular attention deserves fibromodulin as its tyrosine-sulfated domain has been reported to bind collagen and accelerate fibril formation (34). Molecular characterization of the binding properties of fibromodulin has revealed several areas of binding on the helical portion of type I collagen, with sulfotyrosines contributing to this interaction. Further mapping using triple-helical collagen toolkit peptides showed these sites partially overlap with the helical cross-linking sites (35). In fact, fibromodulin has turned to be an important regulator of collagen cross-linking since its deficiency results in a dysregulated enhancement of collagen cross-links which is associated with altered mechanical properties of tendinous tissues (36,37). Interestingly, fibromodulin was also reported to bind LOX, this interaction
resulting in an apparent increase in LOX activity (35). Based on these pieces of information, a model of fibromodulin-regulated collagen cross-linking has been proposed where this small leucine-rich protein, by forming a ternary complex at the helical cross-linking sites, directs LOX activity toward specific lysine residues on the telopeptide of an adjacent microfibril. Our results are in line with this hypothesis by endowing LOX protein with the capacity to interact directly with the telopeptides. Nevertheless, further experimentation is necessary to understand how the same PTM drives fibromodulin to the helical portion or makes LOX able to interact with the telopeptide ends, as well as whether tyrosine sulfated domains are involved or not in the interaction between LOX and fibromodulin.

Proteolysis plays an important role in the homeostasis of connective tissues, not only by allowing the degradation of structural components but also by regulating the capacity of a large array of macromolecules to assemble into a well-organized physiological ECM. BMP1 and ADAMTS enzymes, responsible for the processing of procollagen and, as studied here, of the precursor of LOX protein, have been described to cleave a wide variety of substrates, most of them belonging to the ECM category. BMP1 and its related tolloid-like enzymes were reported to cleave small leucine-rich proteoglycans (biglycan, decorin), basement membrane components (such as laminin) and mineralization factors, including dentin sialoprotein and phosphophoryn (38). On the other hand, a recent work from our group has significantly expanded the substrate repertoire of ADAMTS procollagenases with the identification of fibronectin, some fibulins, thrombospondin-1, decorin and perlecan, among many others (15). These studies, altogether, permitted to establish preferential cleavage sites for these proteases. Processing by BMP1 seems to be quite restricted, with most substrates displaying an aspartate in P1′ position (39). For ADAMTS procollagenases, however, while identified cleavage sites were found to be enriched in proline and glycine residues, a clear consensus sequence has not yet been well-defined (15). LOX precursor fulfills the strict aspartate requirement for BMP1 proteolysis, whereas proline is well represented within the well-conserved ADAMTS cleavage site in orthologs from different species (see Figure 4). Given the critical role of these proteases in collagen biosynthesis, gene defects that alter their expression and/or activity are expected to cause disease. Recessive mutations in BMP1 are linked with recessive forms of osteogenesis imperfecta (OI), a heterogeneous group of bone disorders characterized by fragile bones that break easily (40). ADAMTS2 gene is associated with the dermatosparactic type of Ehlers-Danlos syndrome, a rare condition characterized by extreme skin fragility (41-43). At the molecular level, both clinical conditions display defective procollagen processing, claimed to be responsible for the production of collagen molecules with altered structure leading to aberrant matrix in affected tissues. Collagen cross-linking is likely to be dysregulated in these connective tissue disorders. However, considering the overall matrix involvement and the fact that these proteases process other matrix components, this aspect has been poorly investigated. Rare variants in the LOX gene have been identified that disrupt enzyme function and promote thoracic aortic aneurysms because of insufficient elastin and collagen cross-linking in the aortic wall (44,45). Sequence variations have been also found that fall within and around BMP1 and ADAMTS cleavage sites, or even alter the tyrosine residues modified by sulfation (46). Their functional consequences have not been investigated yet. However, it can be speculated that they can alter the proteolytic activation of LOX or the binding characteristics of the sulfated domain. Further studies in patient or animal models will be required to gain insight into how BMP1, ADAMTS2 and ADAMTS14 coordinately regulate collagen processing and cross-linking, as well as the contribution of these genetic variants to the process.

In conclusion, our data provides novel knowledge about the proteolytic regulation of LOX functions, linking the differential action of BMP1 and ADAMTS procollagenases on a sulfated tyrosine
domain to the capacity of LOX to bind and oxidize collagen in the context of extracellular matrix synthesis and deposition.

**Experimental Procedures**

**Cell culture**

Tetracycline (Tet)-inducible human embryonic kidney 293 (HEK293) cells stably expressing LOX, ADAMTS and BMP1 constructs were generated and maintained in culture according to protocols previously published (15,47). Human lung fibroblasts from the cell line CCD-19Lu were obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture medium as already described (48). Primary bovine aortic endothelial cells were isolated from thoracic aortas and maintained in culture using previously described methods (49).

Skin fibroblast cultures from mice doubly deficient for Adamts2 and Adamts14 were established as followed (17). After sacrifice, mice were shaved and a skin fragment from the belly of approximately two cm$^2$ was collected, washed in 70% ethanol, transferred in culture hood, rinsed in sterile PBS and cut into one mm$^2$ pieces. Skin fragments were then incubated at 37°C in 10 ml of complete Dulbecco's Modified Eagle Medium (DMEM) growth medium supplemented with collagenase (Sigma, St. Louis, MO) at 1000 U/ml. After 2 hours, 10 ml of complete medium supplemented with 10% fetal bovine serum (FBS) was added and centrifugation was performed (5 min at 500g). The pellet was homogenized by pipetting in 10 ml of warm complete medium containing 15% FBS, transferred into a 10 cm tissue culture dishes and placed into an incubator under hypoxic conditions (at 37°C, 5% CO$_2$, 5% O$_2$). Fibroblasts expand from tissue fragments within 2-5 days and can be amplified (1 to 3 ratio) for up to 10 passages when kept in hypoxia.

**Construction of LOX, ADAMTS and BMP1 constructs and generation of stable clones for overexpression**

A full-length human LOX construct in pYX-Asc vector was obtained from Imagenes GmbH (Berlin, Germany). A full-length human BMP1 construct in pBabe vector was kindly provided by Víctor L. Ruiz-Pérez (Instituto de Investigaciones Biomédicas “Alberto Sols”, Madrid, Spain) (50). Both constructs were cloned into the vector pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA) to obtain the corresponding pcDNA5/FRT/TO-LOX and –BMP1 plasmids. These constructs were then cotransfected with the Flp recombinase expression plasmid pOG44 into the Flp-In T-REx 293 cell line using Lipofectamine 2000 (Invitrogen). These cells stably express the Tet repressor and contain a single integrated FRT (Flp recombination target) site. Flp recombinase expression from the pOG44 vector mediates insertion of the cDNA cassettes into the genome at the integrated FRT site through site-specific DNA recombination. After 48 hours, cells were selected for hygromycin B resistance (Roche Diagnostics, Barcelona, Spain), and clones appeared after 10–15 days. Isogenic pooled clones were expanded and checked for transgene expression after 48 hours of incubation in the absence or presence of doxycycline (tetracycline analog) at 1 µg/ml. A construct for the overexpression of a fusion protein of human LOX and green fluorescent protein (LOX-GFP) was generated by cloning of the LOX insert into pEGFP-N1 (Life Technologies) and further transfer of the LOX-GFP chimera to pcDNA5/FRT/TO for expression in the Flp-In T-REx 293 system. Specific mutations in the tyrosine cluster of LOX or LOX-GFP proteins were introduced by site-directed mutagenesis using the primer CCTACAAGTACTCTGACGACAACCCTTTCTTCACTTCTCGAAGCCAGGACCTGGGGG (changed nucleotides shown in bold).

ADAMTS-overexpressing HEK293 cell clones were generated as described previously (15). Briefly, cells were transfected (Novagen GeneJuice® Transfection Reagent, EDM Millipore, Billerica, MA) with the pcDNA™ 6/Tr expression vector (Invitrogen) and selected using blasticidin. Clones expressing high level of the tetracycline repressor (TR) protein were identified by immunoblotting (Rabbit polyclonal #TET01, MoBiTec) and further transfected by the pcDNA4/TO expression vector (TetOn system, Invitrogen) containing the complete coding
sequence of ADAMTS2, ADAMTS3 or ADAMTS14 inserted in the “multiple cloning site” (NotI-XbaI sites for ADAMTS2 and ADAMTS14, and PmeI-PmeI sites for ADAMTS3). After selection (Zeocin, 300 µg/ml), subcloning was performed to identify clones producing ADAMTS2, ADAMTS3 or ADAMTS14 only in the presence of doxycyclin. Semipurified preparations of ADAMTS enzymes were obtained as previously reported (51).

**Protein analysis**

To induce transgene expression in HEK293, cells were seeded on 100 mm culture dishes and incubated at 90% confluence with 5 ml of serum- and phenol red-free medium containing 1 µg/ml doxycycline (Dox) for 48 hours. Cell supernatants were collected and concentrated down to about 200 µl using Amicon Ultracentrifugal filters (Ultracel-10K, EDM Millipore). Separately, cell monolayers were washed with PBS and lysed with 750 µl Tris-SDS buffer (60 mM Tris-HCl, pH 6.8, 2% SDS) to obtain total cell lysate. Protein concentration was determined by the BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). For analysis of LOX expression by standard one-dimensional (1D) electrophoresis coupled to immunoblotting, proteins were fractioned on SDS-polyacrylamide gels. Proteins were also analysed by two-dimensional (2D) electrophoresis. For this type of studies, samples were precipitated with the methanol/chloroform method and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS and 1% carrier ampholites pH 3-10 L) and solubilized for 1 hour at room temperature with agitation (52). Protein concentration was determined using Pierce™ 660 nm Protein Assay (Thermo Scientific). Protein extracts were diluted in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTT, 1,2% DeStreak and 1% carrier ampholites pH 3-11 NL) up to final volume of 200 µl, and applied by active rehydration to 11 cm IPG strips pH 3-11 NL (GE Healthcare). The first dimension was run at 75 µA/IPG strip in the IPGphor IEF System (GE Healthcare) following a voltage increase in eight steps. After the first dimension, strips were equilibrated with SDS equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of bromophenol blue) containing 1% (w/v) DTT for 12 min and thereafter in the same buffer containing 4% (w/v) iodoacetamide for 10 additional min. Then the proteins were separated on home-casted 12.5% Tris-glycine gels using an Mini Protean electrophoresis cell (BIO-RAD) at 25°C until the tracking dye had migrated off the bottom of the gel. 1D and 2D gels were then transferred onto nitrocellulose membranes at 12 V for 20 min in a semi-dry Trans-Blot Turbo system (Bio-Rad). Membranes were blocked by incubation for 30 min with 1% BSA in Tris-buffered saline (TBS) containing 0.5% Tween-20, and antigens were detected using specific primary antibodies (LOX: ab31238, Abcam, Cambridge, United Kingdom; BMP1: AF1927, R&D Systems; anti-Sulfotyrosine antibody: Sulfo-1C-A2, ab136481, Abcam; anti-GFP: Cat.No. 11814460001, mouse monoclonal, Roche; ADAMTS2: homemade mouse monoclonal antibody recognizing the fourth TSP1 repeat; ADAMTS3: 11-867-423-001, Roche, specific for human influenza hemagglutinin (HA)-tag inserted in the spacer domain; ADAMTS14: sc-67436, Santa Cruz Biotechnology, Dallas, TX). Blots were then developed and quantified using corresponding IRDye 680 or Li-Cor IRDye 800 labeled secondary antibodies with the Odyssey infrared imaging system (Li-Cor).

LOX-GFP proteins were immunoprecipitated using GFP-Trap agarose beads (ChromoTek, Planegg-Martinsried, Germany) following manufacturer’s protocol. Immunoprecipitated proteins were analysed as described above. For analysis of tyrosine sulfation, corresponding LOX-GFP-overexpressing HEK293 cell clones were plated on 100 mm culture dishes and grown until 90% confluence. Then, cells were rinsed 3 times with sulfate-free medium in which 0.8 mM MgSO₄ had been replaced with 0.8 mM MgCl₂. The dishes were then incubated for 48 hours at 37°C with sulfate-free medium containing 0.2 mCi of [³²S]-sulfate (Perkin-Elmer, Boston, MA) in the presence of 1 µg/ml Dox. After the
incubation, the supernatants were collected and processed as described above for immunoprecipitation and electrophoresis analysis. Gels containing [35S]-sulfate-labeled proteins were soaked for 20 minutes in fixing solution (20 % acetic acid, 10 % methanol) and then dried and exposed to autoradiographic films (Agfa Healthcare, Mortsel, Belgium) or using a Fujifilm BAS-1500 phosphorimagor (Fuji Photo Film Co. Ltd., Kanagawa, Japan).

Proteomic analysis

For the analysis of the proteolytic processing site of LOX by ADAMTS2 enzyme, LOX supernatants were exposed to the protease for 4 hours at 37°C and then filtered through a 50 kDa-cutoff Amicon Ultracentrifugual filter (Ultracel-50K, Millipore) in order to enrich the sample in low molecular weight polypeptides. Eluates were then applied onto 1.2-cm wide wells of a conventional SDS-PAGE gel (0.75 mm-thick, 4% stacking, and 10% resolving). Electrophoresis was continued until proteins became concentrated at the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (2 x 2 mm), and placed in 0.5 ml microcentrifuge tubes. The gel pieces were destained in acetonitrile:water (ACN:H2O, 1:1), incubated with 10 mM DTT for 1 h at 56 °C for disulfide bond reduction and with 50 mM iodoacetamide for 1 h at room temperature in darkness for thiol group alkylation, and then digested in situ with sequencing grade trypsin (Promega, Madison, WI) as previously described (53). Digestion products were desalted onto OMIX Pipette tips C18 (Agilent Technologies) and analyzed by reverse phase-liquid chromatography-tandem mass spectrometry (RP-LC-MS/MS) in an Easy-nLC II system coupled to an ion trap linear trap quadrupole (LTQ)-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). Peptide identification from raw data was carried out using PEAKS Studio 6 software (Bioinformatics Solutions Inc.) (54). Database search was performed against UniProt-Homo Sapiens.fasta (decoy-fusion database). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, allows non-specific cleavage at one end of the peptide, and tolerances of 20 ppm for precursor ions and 0.6 Da for MS/MS fragment ions. The variable modifications allowed were methionine oxidation and cysteine carbamidomethylation. False discovery rate was set as < 1% (FDR < 0.01). Detected LOX sequences were screened for non-tryptic internal peptides (no K or R in P1) as potential sites for ADAMTS processing.

To characterize the potential modification by tyrosine sulfation, LOX-GFP supernatants were exposed to BMP1 protease and immunoprecipitated with GFP-Trap agarose beads as described above. Afterwards, immunoprecipitates were then fractioned by SDS-PAGE and analysed by immunoblotting with anti-GFP antibody and stained for total protein with Coomassie Blue. Gel bands matching LOX-GFP precursor and processed forms were excised from the gel and manually digested with sequencing-grade AspN (Promega). For this digestion, gel plugs were alternatively washed several times with 25 mM ammonium bicarbonate and acetonitrile; thereafter, samples were reduced with 10 mM DTT for 1 h at 37°C, followed by alkylation with 55 mM of iodoacetamide for 30 min at room temperature before incubating with 0.1 µg AspN for 18 h at 37°C. Following digestion, peptides were extracted, pooled, dried by speed-vacuum centrifugation and resuspended in 0.2% TFA in water for Parallel Reaction Monitoring (PRM) analysis using an Eksigent 1D-nanoHPLC coupled to a 5600TripleTOF QTOF mass spectrometer (Sciex, Framingham, MA, USA), equipped with a nano-ESI source and controlled by Analyst v.1.7 software (ABSciex). HPLC was equipped with a trap column Acclaim PepMap 100, 5 µm particle diameter, 100 Å pore size (ThermoFisher Scientific), switched on-line with a silica-based reversed phase column nanoACQUITY UPLC 75 µm × 15 cm, 1.7 µm particle size (Waters, Milford, Massachusetts). The loading pump delivered a solution of 0.1% formic acid in 98% water / 2% acetonitrile (Scharlab, Barcelona, Spain) at 3 µL/min. The nanopump provided a flow-rate of 250 nL/min and was operated under gradient
elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in 100% acetonitrile as mobile phase B. Gradient elution was performed according the following scheme: isocratic conditions of 96% A: 4% B for five minutes, a linear increase to 40% B in 25 min, a linear increase to 95% B in two minutes, isocratic conditions of 95% B for five minutes and return to initial conditions in 10 min. Injection volume was 5 µL.

The LC system was coupled via a nanospray source to the mass spectrometer. PRM analysis was conducted in negative ion mode with the ion spray voltage set at 2800 V. Initial PRM tests were also performed in positive ion mode. Drying gas temperature was set to 150°C at a flow rate of 20 L/min. Full scan MS spectra (m/z 230-1500) were acquired during 100 ms. Product ion scans of precursors with m/z values 611.7, 651.8 and 691.8, corresponding to the unmodified, monosulfonated and disulfonated variants of DDNPYYNYY (M-2H+), respectively, were acquired during 100 ms/cycle in the 100-1500 m/z range. Acquisition mode was high-sensitivity and both MS and MSMS spectra were analyzed using PeakView v1.2 software (Sciex).

Sulfated synthetic DDNPYYNYY peptides were used to validate PRM analysis and to confirm the sequence identity. These peptides were synthesized in-house using standard Fmoc chemistry in an Intavis Multiple peptide synthesizer (INTAVIS, Cologne, Germany).

Analysis of LOX enzymatic activity

LOX supernatants were assayed for enzymatic activity using the Lysyl Oxidase Activity Assay Kit (Fluorometric) from Abcam. This assay is based on the widely used method described by Palamakumbura et al and uses a proprietary soluble LOX substrate in an horseradish peroxidase-coupled reaction with concomitant detection of hydrogen peroxide with Amplex red reagent (22). Briefly, fifty µl of concentrated supernatant were mixed with an equivalent volume of reaction mixture in the presence or absence of 0.3 mM β-aminopropionitrile (BAPN), a pan-inhibitor of lysyl oxidases. H2O2 release was measured every 3 min for a total period of 1 hour at excitation and emission wavelengths of 525 and 580-640 nm, respectively, on a Glomax Multi Detection system (Promega). Enzyme activities were expressed as fluorescence arbitrary units and corrected for background levels determined in the presence of BAPN.

Solid phase binding assay

Binding of the various LOX species to collagen was performed by a modification of the standard solid phase binding assay (55). Telocollagen (RatCol, rat tail type I collagen solution) and atelocollagen (Nutragen, bovine type I collagen solution) (Advanced Biomatrix, San Diego, CA) were coated overnight on Maxisorb 96-well immunoplates (Nunc, Thermo Scientific) at 50 µg/ml in 16 mM acetic acid buffer and washed three times with HBS buffer (10 mM HEPES, 0.135 M NaCl, pH 7.4). Afterwards, wells were blocked with 3% BSA in HBS buffer for 1 hour and washed three times before the incubation with LOX-containing concentrated supernatants for 1 hour at room temperature in HBS buffer containing 2 mM CaCl2 and 15 µM ZnSO4. Unbound proteins were extensively washed out with HBS buffer and remaining bound material was removed by addition of 30 ml of SDS-loading buffer and 5 min-heating at 60°C. Unbound and bound fractions were then analyzed for LOX immunoreactivity by immunoblotting as described above. LOX proteins were quantified by densitometry and the extent of binding expressed as percentage of total.

Statistical analysis

Data are presented as the mean ± SD of n independent measurements as indicated in the corresponding figure legends. Statistical comparisons between groups were calculated by one-way ANOVA analysis followed by Bonferroni’s post-test for multiple-group comparisons using GraphPad Prism 6. The P values obtained are indicated in the figure legends when statistically significant (P < 0.05).

Protein alignments were performed with Clustal Omega at the European Bioinformatics Institute (EMBL-EBI) using sequences available in the NCBI (National Research Council of Canada).
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Center for Biotechnology Information
Protein database.
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Author Contribution: F. R-P. and T.R-G. designed and performed the experiments, analyzed the data and contributed to manuscript text and reviewed its final version. A.C. also contributed to design the experiments and to write and review the manuscript. L.D. and M.B. generated the ADAMTS-expressing vectors and performed protein preparations. A.P. and G.B. performed the proteomic analyses and contributed to manuscript text.
LOX cleavage regulates collagen binding

References
1. Engel, J., and Chiquet, M. (2011) An Overview of Extracellular Matrix Structure and Function, in The Extracellular Matrix: an Overview (RP, M. ed.), Springer-Verlag, Berlin Heidelberg, pp 1-39
2. Kadler, K. E., Baldock, C., Bella, J., and Boot-Handford, R. P. (2007) Collagens at a glance. Journal of Cell Science 120, 1955-1958
3. Canty, E. G., and Kadler, K. E. (2005) Procollagen trafficking, processing and fibrillogenesis. Journal of Cell Science 118, 1341-1353
4. Eyre, D. R., and Wu, J. J. (2005) Collagen cross-links. in Topics in Current Chemistry
5. Grau-Bové, X., Ruiz-Trillo, I., and Rodriguez-Pascual, F. (2015) Origin and evolution of lysyl oxidases. Scientific Reports 5, 10568
6. Rodriguez-Pascual, F., and Slatter, D. A. (2016) Collagen cross-linking: insights on the evolution of metazoan extracellular matrix. Sci Rep 6, 37374
7. Trackman, P. C., Bedell-Hogan, D., Tang, J., and Kagan, H. M. (1992) Post-translational glycosylation and proteolytic processing of a lysyl oxidase precursor. The Journal of biological chemistry 267, 8666-8671
8. Uzel, M. I., Scott, I. C., Babakhanlou-Chase, H., Palamakumbura, A. H., Pappano, W. N., Hong, H. H., Greenspan, D. S., and Trackman, P. C. (2001) Multiple bone morphogenetic protein 1-related mammalian metallocollagenases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryonic fibroblast cultures. The Journal of biological chemistry 276, 22537-22543
9. Cronshaw, A. D., Fothergill-Gilmore, L. A., and Hulmes, D. J. S. (1995) The proteolytic processing site of the precursor of lysyl oxidase. Biochemical Journal 306, 279-284
10. Panchenko, M. V., Stetler-Stevenson, W. G., Trubetskoy, O. V., Gacheru, S. N., and Kagan, H. M. (1996) Metallocollagenase Activity Secreted by Fibroblastic Cells in the Processing of Prolysyl Oxidase: POTENTIAL ROLE OF PROCOLLAGEN C-PROTEINASE. Journal of Biological Chemistry 271, 7113-7119
11. Kagan, H. M., Sullivan, K. A., Olsson, T. A., and Cronlund, A. L. (1979) Purification and properties of four species of lysyl oxidase from bovine aorta. Biochemical Journal 177, 203-214
12. Vidal, G. P., Shieh, J. J., and Yasunobu, K. T. (1975) Immunological studies of bovine aorta lysyl oxidase: Evidence for two forms of the enzyme. Biochemical and Biophysical Research Communications 64, 989-995
13. Kuivaniemi, H. (1985) Partial characterization of lysyl oxidase from several human tissues. Biochemical Journal 230, 639-643
14. Rosini, S., Pugh, N., Bonna, A. M., Hulmes, D. J. S., Farndale, R. W., and Adams, J. C. (2018) Thrombospondin-1 promotes matrix homeostasis by interacting with collagen and lysyl oxidase precursors and collagen cross-linking sites. Science Signaling 11
15. Bekhouche, M., Leduc, C., Dupont, L., Janssen, L., Delolme, F., Vadon-Le Goff, S., Smargiasso, N., Baiwir, D., Mazzucchelli, G., Zanella-Cleon, I., Dubail, J., De Pauw, E., Nusgens, B., Hulmes, D. J. S., Moali, C., and Colige, A. (2016) Determination of the substrate repertoire of ADAMTS2, 3, and 14 significantly broadens their functions and identifies extracellular matrix organization and TGF-β signaling as primary targets. The FASEB Journal 30, 1741-1756
16. Boak, A. M., Roy, R., Berk, J., Taylor, L., Polgar, P., Goldstein, R. H., and Kagan, H. M. (1994) Regulation of lysyl oxidase expression in lung fibroblasts by transforming growth factor-beta 1 and prostaglandin E2. American Journal of Respiratory Cell and Molecular Biology 11, 751-755
17. Dupont, L., Exh, G., Chantry, M., Monseur, C., Leduc, C., Janssen, L., Cataldo, D., Thiry, M., Jerome, C., Thomassin, J. M., Nusgens, B., Dubail, J., Baron, F., and Colige, A. (2018) Spontaneous atopic dermatitis due to immune dysregulation in mice lacking Adamts2 and 14. Matrix Biology 70, 140-157
18. Kehoe, J. W., and Bertozzi, C. R. (2000) Tyrosine sulfation: a modulator of extracellular protein–protein interactions. Chemistry & Biology 7, R57-R61
LOX cleavage regulates collagen binding

19. Yang, Y. S., Wang, C. C., Chen, B. H., Hou, Y. H., Hung, K. S., and Mao, Y. C. (2015) Tyrosine sulfation as a protein post-translational modification. *Molecules (Basel, Switzerland)* 20, 2138-2164

20. Atsawasuwan, P., Mochida, Y., Katafuchi, M., Tokutomi, K., Mocanu, V., Parker, C. E., and Yamauchi, M. (2011) A Novel Proteolytic Processing of Prolysyl Oxidase. *Connective Tissue Research* 52, 479-486

21. Monigatti, F., Gasteiger, E., Baircho, A., and Jung, E. (2002) The Sulfinator: predicting tyrosine sulfation sites in protein sequences. *Bioinformatics (Oxford, England)* 18, 769-770

22. Palamakumbura, A. H., and Trackman, P. C. (2002) A Fluorometric Assay for Detection of Lysyl Oxidase Enzyme Activity in Biological Samples. *Analytical Biochemistry* 300, 245-251

23. Trackman, P. C., and Kagan, H. M. (1979) Nonpeptidyl amine inhibitors are substrates of lysyl oxidase. *Journal of Biological Chemistry* 254, 7831-7836

24. Kagan, H. M., Williams, M. A., Williamson, P. R., and Anderson, J. M. (1984) Influence of sequence and charge on the specificity of lysyl oxidase toward protein and synthetic peptide substrates. *Journal of Biological Chemistry* 259, 11203-11207

25. Rodriguez-Pascual, F., and Rosell-Garcia, T. (2018) Lysyl Oxidases: Functions and Disorders. *Journal of Glaucoma* 27, S15-S19

26. Cronlund, A. L., Smith, B. D., and Kagan, H. M. (1985) Binding of lysyl oxidase to fibrils of type I collagen. *Connect Tissue Res* 14, 109-119

27. Siegel, R. C. (1974) Biosynthesis of Collagen Crosslinks: Increased Activity of Purified Lysyl Oxidase with Reconstituted Collagen Fibrils. *Proceedings of the National Academy of Sciences of the United States of America* 71, 4826-4830

28. Tillgren, V., Mörgelin, M., Önnerfjord, P., Kalamajski, S., and Aspberg, A. (2016) The Tyrosine Sulfate Domain of Fibromodulin Binds Collagen and Enhances Fibril Formation. *Journal of Biological Chemistry* 291, 23744-23755

29. Svensson, L., Aszödi, A., Reinhold, F. P., Fässler, R., Heinegård, D., and Oldberg, Å. (1999) Fibromodulin-null Mice Have Abnormal Collagen Fibrils, Tissue Organization, and Altered Lumican Deposition in Tendon. *Journal of Biological Chemistry* 274, 9636-9647
LOX cleavage regulates collagen binding

37. Kalamajski, S., Liu, C., Tillgren, V., Rubin, K., Oldberg, A., Rai, J., Weis, M., and Eyre, D. R. (2014) Increased C-telopeptide cross-linking of tendon type I collagen in fibromodulin-deficient mice. The Journal of biological chemistry 289, 18873-18879

38. Moali, C., and Hulmes, D. J. (2012) Roles and regulation of BMP1/Tolloid-like proteases: collagen/matrix assembly, growth factor activation, and beyond. in Extracellular Matrix: Pathobiology and Signaling. (Karamanos, N. ed.), Walter de Gruyter, Berlin/Boston. pp 539-561

39. Rawlings, N. D., Waller, M., Barrett, A. J., and Bateman, A. (2014) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Research 42, D503-D509

40. Marini, J. C., Forlino, A., Bächinger, H. P., Bishop, N. J., Byers, P. H., Paepe, A. D., Fassier, F., Fratzl-Zelman, N., Kozloff, K. M., Krakow, D., Montpetit, K., and Semler, O. (2017) Osteogenesis imperfecta. Nature Reviews Disease Primers 3, 17052

41. Colige, A., Sieron, A. L., Li, S. W., Schwarze, U., Petty, E., Wertelecki, W., Wilcox, W., Krakow, D., Cohn, D. H., Reardon, W., Byers, P. H., Lapiere, C. M., Prockop, D. J., and Nusgens, B. V. (1999) Human Ehlers-Danlos syndrome type VII C and bovine dermatosparaxis are caused by mutations in the procollagen I N-proteinase gene. American journal of human genetics 65, 308-317

42. Bekhouche, M., and Colige, A. (2015) The procollagen N-proteinases ADAMTS2, 3 and 14 in pathophysiology. Matrix biology : journal of the International Society for Matrix Biology 44-46, 46-53

43. Le Goff, C., Somerville, R. P., Kesteloot, F., Powell, K., Birk, D. E., Colige, A. C., and Apte, S. S. (2006) Regulation of procollagen amino-propeptide processing during mouse embryogenesis by specialization of homologous ADAMTS proteases: insights on collagen biosynthesis and dermatosparaxis. Development (Cambridge, England) 133, 1587-1596

44. Guo, D.-c., Regalado, E. S., Gong, L., Duan, X., Santos-Cortez, R. L. P., Arnaud, P., Ren, Z., Cai, B., Hostetter, E. M., Moran, R., Liang, D., Estrera, A., Safi, H. J., Leal, S. M., Bamshad, M. J., Shendure, J., Nickerson, D. A., Jondeau, G., Boyleau, C., and Milewicz, D. M. (2016) LOX Mutations Predispose to Thoracic Aortic Aneurysms and Dissections. Circulation research 118, 928-934

45. Lee, V. S., Halabi, C. M., Hoffman, E. P., Carmichael, N., Leshchiner, I., Lian, C. G., Birhals, A. J., Vuzman, D., Mechem, R. P., Frank, N. Y., and Stitziel, N. O. (2016) Loss of function mutation in LOX causes thoracic aortic aneurysm and dissection in humans. Proceedings of the National Academy of Sciences 113, 8759-8764

46. Hunt, S. E., McLaren, W., Gil, L., Thormann, A., Schuijlenburg, H., Sheppard, D., Parton, A., Armean, I. M., Trevanion, S. J., Flick, P., and Cunningham, F. (2018) Ensembl variation resources. Database 2018

47. Busnadiego, O., González-Santamaría, J., Lagares, D., Guinea-Viniegra, J., Pichol-Thieven, C., Muller, L., and Rodriguez-Pascual, F. (2013) LOXL4 Is Induced by Transforming Growth Factor β1 through Smad and JunB/Fra2 and Contributes to Vascular Matrix Remodeling. Molecular and Cellular Biology 33, 2388-2401

48. Puig, M., Lugo, R., Gabasa, M., Gimenez, A., Velasquez, A., Galgoczy, R., Ramirez, J., Gomez-Caro, A., Busnadiego, O., Rodriguez-Pascual, F., Gascon, P., Reguart, N., and Alcaraz, J. (2015) Matrix stiffening and betal integrin drive subtype-specific fibroblast accumulation in lung cancer. Molecular cancer research : MCR 13, 161-173

49. Rodriguez-Pascual, F., Redondo-Horcajo, M., and Lamas, S. (2003) Functional cooperation between Smad proteins and activator protein-1 regulates transforming growth factor-beta-mediated induction of endothelin-1 expression. Circulation research 92, 1288-1295

50. Martinez-Glez, V., Valencia, M., Caparros-Martin, J. A., Aglan, M., Temtamy, S., Tenorio, J., Pulido, V., Lindert, U., Rohrbach, M., Eyre, D., Giunta, C., Lapunzina, P., and Ruiz-Perez, V. L. (2012) Identification of a mutation causing deficient BMP1/mTLD proteolytic activity in autosomal recessive osteogenesis imperfecta. Human mutation 33, 343-350
LOX cleavage regulates collagen binding

51. Colige, A., Ruggiero, F., Vandenberghhe, I., Dubail, J., Kesteloot, F., Van Beeumen, J., Beschin, A., Brys, L., Lapiere, C. M., and Nusgens, B. (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. *The Journal of biological chemistry* **280**, 34397-34408

52. Wessel, D., and Flugge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* **138**, 141-143

53. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical chemistry* **68**, 850-858

54. Zhang, J., Xin, L., Shan, B., Chen, W., Xie, M., Yuen, D., Zhang, W., Zhang, Z., Lajoie, G. A., and Ma, B. (2012) PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. *Molecular & cellular proteomics : MCP* **11**, M111 010587

55. Mould, P. A. (2009) Solid phase assays for studying ECM protein-protein interactions. *Methods in molecular biology (Clifton, N.J.)* **522**, 195-200
Figure 1. LOX isoform is expressed in multiple forms. A) Human lung fibroblasts and bovine vascular endothelial cells were incubated for four days under basal conditions or in the presence of 5 ng/ml of TGF-β1. Then, cell supernatants were taken and concentrated using a 10 kDa-cutoff centrifugal filter. An aliquot was fractioned by SDS-PAGE and assayed by immunoblotting with a specific LOX antibody recognizing the C-terminal catalytic domain. Multiple immunoreactive bands were observed with molecular weights ranging from 25 to 30 kDa. B) Generation of HEK293 cells overexpressing human LOX in a tetracycline inducible manner. LOX protein in the total cell extract and in the supernatant was assessed by immunoblotting under basal conditions and upon induction with the tetracycline analogue, doxycycline (Dox). The precursor form at 50 kDa and multiple shorter forms (at 25-30 kDa) are identified in the culture medium of Dox-treated cells. The blots shown correspond to representative experiments performed twice with two independent preparations.
Figure 2. Cleavage of LOX by ADAMTS2 and 14. LOX cleavage was assayed by immunoblotting in LOX/ADAMTS-overexpressing co-cultures after incubation with doxycycline for 48 hours (A and B) and after in vitro incubation assays (C and D) for the indicated times. For comparison, LOX fragments generated upon incubation with BMP1 are also shown in panel C. ADAMTS2 and ADAMTS14, either in co-culture or using purified proteins, promoted the accumulation of a mature form of about 25 kDa (red arrow), whereas BMP1 cleavage gave rise to bands in the 30 kDa range (blue arrow), indicating distinct processing sites. ADAMTS3 co-culture did not modify the relative levels of both fragments, suggesting it was not able to process LOX under our experimental conditions. E) Sequential incubation with BMP1 and ADAMTS14. LOX supernatant was first incubated with BMP1 for one hour. Then, ADAMTS14 was added into the reaction mixture for the indicated times.
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Figure 3. Proteomic characterization of the ADAMTS-mediated cleavage site in LOX protein. LOX supernatants exposed to ADAMTS2 were enriched in LOX mature forms by passing through a 50 kDa-cutoff centrifugal filter. Then, the flow-through were trypsin digested and the resulting peptides fractionated by liquid chromatography and analyzed by mass spectrometry. A) Peptide coverage along the human LOX sequence showing identified tryptic fragments (shown in bold) as well as a hemitryptic peptide that resulted from the action of ADAMTS2 (yellow, fragment ion spectra in B). C) Schematic representation of the cleavage site by BMP1 (blue arrow and arrowhead in panel A) and ADAMTS2/14 (red arrow and arrowhead in panel A) within LOX. The cleaved sequences and the theoretical molecular weights of the LOX forms generated by these cleavages are also provided.
Figure 4. Multiple sequence alignments of ADAMTS cleavage site and flanking sequences. 
A) Sequence alignment of LOX orthologs from the indicated species showing high homology at the ADAMTS and BMP1 cleavage sites, and also in between. B) Sequence alignment of ADAMTS cleavage site and downstream sequences from LOX orthologs and paralogs from the indicated species. Of note is the high degree of homology observed between the cleavage site characterized in human LOX and corresponding sequences of human LOXL1 and the zebrafish LOX, LOXL1 and LOXL5 isoforms. On the contrary, no significant homology was found among LOXL2, LOXL3 and LOXL4 paralogs, in spite of the conservation observed in the downstream histidine-rich catalytic core. Protein alignments were done with the ClustalW2 program (http://www.ebi.ac.uk).
Figure 5. LOX cleavage in skin fibroblasts from wild type and *Adams2-Adams14*-deficient mice. Accumulation of LOX mature forms in the extracellular medium was assayed by immunoblotting in cultured skin fibroblasts from wild type (WT) and doubly deficient *Adamts2-Adamts14* knockout mice (TS2−/TS14−) upon induction with TGF-β1 or basal. Note that ADAMTS2/TS14-deficient cells showed a diminishing of the 25 kDa product (red arrow) with respect to wild type, without significantly affecting the 30 kDa BMP1 product (blue band) (ratio of band intensities ADAMTS/BMP1: 3.71 in the wild type; 1.45 in *Adamts2-Adamts14*-deficient fibroblasts).
Figure 6. Analysis of LOX proteolysis by two-dimensional electrophoresis coupled to immunoblotting. LOX supernatants kept under basal conditions (A) or incubated with BMP1 (B) or ADAMTS2 (C) were fractioned by 1D- (left) or 2D- (right) electrophoresis and analyzed by immunoblotting using a specific C-terminal LOX antibody. LOX precursor was visualized as a train of spots with isoelectric point (pI) more acidic than predicted from amino acid sequence (7.99). BMP1-cleaved mature form gave a similar pattern (indicated by a red dashed oval), whereas the cleavage by ADAMTS2 resulted in a single, prominent spot at more basic pI (red arrowhead). The blots shown correspond to representative experiments performed twice with two independent preparations.
Figure 7. Identification of tyrosine sulfation in the LOX protein sequence flanked by BMP1 and ADAMTS2 cleavage sites. A) LOX protein sequence flanked by BMP1 and ADAMTS cleavage sites. Tyrosine residues conserved among LOX/LOXL1 orthologs are marked in red. Asterisks indicate tyrosines that are predicted to undergo sulfation by the algorithm Sulfinator. The sequences of the wild type and mutant construct with the cluster of tyrosines changed to phenylalanine are also shown. B) Cleavage of wild type and mutant LOX
by BMP1 (BM) and ADAMTS2 (TS) as assessed by immunoblotting with anti-LOX (left) and anti-sulfotyrosine (right) antibodies. The asterisk indicates the position of an unspecific band recognized by the antibody in both wild type and mutant samples treated with BMP1. C) BMP1- and ADAMTS2-mediated cleavage of wild type and mutant LOX chimera C-terminally fused to green fluorescent protein (GFP) as assessed by immunoblotting with an anti-GFP antibody. D) Sulfation of tyrosines in LOX variants. HEK293 cells overexpressing wild type (left panels) and mutant (right panels) LOX/GFP constructs were metabolically labelled with $[^{35}\text{S}]$-sulfate. The supernatants were then kept untreated (−) or incubated with BMP1 or ADAMTS2 before immunoprecipitation using anti-GFP beads. Inputs and immunoprecipitates were analyzed by immunoblotting with an anti-GFP antibody (upper panels) and by autoradiography (lower panels). Note that only slow-migrating bands resulting from the action of BMP1 were shown to incorporate radioactivity, whereas the fragment with higher electrophoretic mobility specifically generated by ADAMTS2 was not. Radioactively labeled bands observed in the supernatant treated with ADAMTS2 likely represent fragments generated as a result of endogenous BMP1 secreted by HEK293 cells. No specific radioactive labeling was detected in the immunoprecipitates from mutant LOX/GFP.
LOX cleavage regulates collagen binding capacity. Supernatants from LOX-overexpressing cells under control conditions (LOX) or exposed to BMP1 or ADAMTS2 were assessed for LOX enzymatic activity in a time-lapse fluorescence assay performed in the absence (left panel) or presence (right panel) of the LOX inhibitor β-aminopropionitrile (BAPN, 0.3 mM). Basal tracing represents the activity from a supernatant of a culture in the absence of induction with doxycycline. Representative data (A) and quantification from three independent experiments (B). Values are shown as fluorescent arbitrary units (mean ± SD, n=6, *P<0.05 vs. basal). C) Solid phase binding assay to telocollagen (with intact telopeptides) or atelocollagen (without telopeptides) of the various LOX species shown in (D). Binding capacity is shown as percentage of total (mean ± SD, n=6, *P<0.05 vs. the corresponding control without protease treatment, *P<0.05 vs. the corresponding wild type). Statistical comparisons between groups were calculated by one-way ANOVA analysis followed by Bonferroni’s post-test.
Figure 9. Schematic model describing LOX regulation by BMP1- and ADAMTS-mediated proteolysis in the context of collagen processing. The diagram summarizes the ability of these proteases to cleave LOX into two different locations yielding mature LOX species with different capabilities to bind collagen telopeptides based on the presence of a sulfotyrosine domain.
Differential cleavage of lysyl oxidase by the metalloproteinases BMP1 and ADAMTS2/14 regulates collagen binding through a tyrosine sulfate domain
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