Fibromodulin interacts with collagen cross-linking sites and activates lysyl oxidase

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ABSTRACT

The hallmark of fibrotic disorders is a highly cross-linked and dense collagen matrix, a property driven by the oxidative action of lysyl oxidase. Other fibrosis-associated proteins also contribute to the final collagen matrix properties, one of which is fibromodulin - its interactions with collagen affect collagen cross-linking, packing, and fibril diameter. We investigated the possibility that a specific relationship exists between fibromodulin and lysyl oxidase, potentially imparting a specific collagen matrix phenotype. We mapped the fibromodulin-collagen interaction sites using the Collagen II and III Toolkit peptide libraries. Fibromodulin interacted with the peptides containing the known collagen cross-linking sites and the MMP-1 cleavage site in collagens I and II. Interestingly, the interaction sites are closely aligned within the quarter-staggered collagen fibril, suggesting a multivalent interaction between fibromodulin and several collagen helices. Furthermore, we detected an interaction between fibromodulin and lysyl oxidase – a major collagen cross-linking enzyme - and mapped the interaction site to 12 N-terminal amino acids on fibromodulin. This interaction also increases the activity of lysyl oxidase. Altogether, the data suggest a fibromodulin-modulated collagen cross-linking mechanism, where fibromodulin binds to a specific part of the collagen domain and also forms a complex together with lysyl oxidase, targeting the enzyme towards specific cross-linking sites.

Dense collagen matrix in tendons and fibrotic collagen matrix in tumors and atherosclerotic plaques is enriched in a collagen-associated protein fibromodulin, FMOD (1-7). This protein is a member of small leucine-rich proteins whose temporo-spatial expression patterns vary in different tissues and whose absence, in the respective knockout mice, creates tissue-specific collagen matrix phenotypes. Their differential expression and interactions with collagen is attributed to the different ways collagen matrices can be organized, reviewed here (8,9). FMOD itself influences collagen fibrillogenesis and cross-linking: *Fmod-/-* mouse tendons have a disturbed collagen fibril phenotype (1,10) and *Fmod-/-* tendon collagen has a lower acid extractability, excessively oxidized α1(I)-C-telopeptides and aberrantly cross-linked α2(I)-chains (11). Also, the morphological phenotype of *Fmod-/-* tendon collagen fibrils resembles the phenotype of fibrils where lysyl oxidase (LOX) - the major collagen cross-linking enzyme - is inhibited (12). FMOD might therefore be involved in

directing dense collagen cross-linking in tendons and fibrotic matrices, although the exact mechanisms are unknown.

Collagen cross-linking depends on LOX and at least one of its homologues, LOXL2, that catalyze the oxidation of specific collagen lysines (13-18). Through oxidation lysines acquire reactive aldehydes that condense with other lysines, resulting in initial cross-link formation that becomes more complex as the collagens assemble into fibrils (19). LOX is activated through BMP-1 processing of its inactive pro-isoform - a process potentiated by fibronectin (20) and further amplified by BMP-1 itself being incorporated into the fibronectin matrix through interaction with periostin (21). Once activated, LOX oxidizes lysine residues in collagen telopeptides (22,23) while binding to the collagen triple-helical domain (24); this process is more efficient on fibrillar rather than monomeric collagen (25,26) suggesting that proteins that specifically modulate collagen fibrillogenesis could also influence the specificity of LOX-mediated cross-linking. Through excessive cross-linking that imparts stiffness on the collagen matrix, LOX enhances fibrosis-enhanced metastasis (27-29). Despite the dependence of collagen cross-linking on the dynamics of fibril assembly and its restriction to specific collagen domains, research on potential modulators of these processes, especially in relation to LOX, has been scarce. Interestingly, LOX is not a collagen-exclusive enzyme but can oxidize other substrates, e.g. elastin, PDGF-R and TGF-β (30-32), raising the question of whether specific mechanisms exist that target LOX to collagen. Hypothetically, such proteins could modulate LOX activity in the extracellular space during the assembly of collagen fibrils.

In this paper, we hypothesized that FMOD could influence LOX activity near the collagen cross-linking sites, through binding to the specific collagen domains and/or through modulating LOX activity. To investigate this hypothesis, we mapped the collagen-binding sites of FMOD and tested and mapped the potential FMOD-LOX interaction. We also analyzed LOX quantity and distribution in *Fmod-/-* tendons and assayed LOX activity under the influence of FMOD. Our data, together with previously published findings, strongly suggest that FMOD can directly modulate site-specific LOX-mediated collagen cross-linking.

EXPERIMENTAL PROCEDURES

Materials – Collagen Toolkit peptides and their substituted variants were synthesized on TentaGel R Ram resin as C-terminal amides, using either an Applied Biosystems Pioneer or CEM Liberty peptide synthesizer, as previously described (33,34).

The Collagen Toolkits II and III contain 56 or 57 peptides, each with 27 residues of primary collagen sequence with neighboring peptides overlapping by nine amino acids, thus spanning the whole of the corresponding COL domain. The peptides are flanked by $GPP_{(5)}$ repeats and GPC triplets unless stated otherwise - these features impart stable triple-helical conformation on the entire peptide.

Collagen was extracted from mouse tail tendons using 20 mM acetic acid. Recombinant lysyl oxidase protein used in interaction assays was purchased from Origene. For transient expression of lysyl oxidase in 293T cells, for use in LOX activity assays, LOX cDNA was ligated into p3xFLAG-CMV-8 vector (Sigma), replacing the native signal peptide sequence with secretion pre-pro-trypsin leader sequence. Antibodies against lysyl oxidase (EPR4025) and against his-tag (18184) were from Abcam. Antibodies against fibromodulin were from Sigma (SAB1100690).

Reagents – Biotin EZ-link reagent (ThermoFisher) was used to biotinylate recombinant fibromodulins using manufacturer's instructions, then quenched with ethanolamine and dialyzed against TBS. Streptavidin-HRP was from Millipore. TMB substrate kit was from Pierce.

Protein expression and purification – his-tag modified recombinant fibromodulin (human, amino acids 19-376) and its fragments (FMOD90, amino acids 31-376; FMODNT, amino acids 19-95) were expressed in 293T cells using pCEP4 vector (Invitrogen).

Proteins were purified on NiNTA sepharose (Qiagen) and further on Superose 6 (GE Healthcare) in PBS. Bacterial his-tagged FMOD was expressed in BL21 *E.coli* using pET27(b) vector (Novagen) and purified as follows: cells were lysed in 8M urea in 100 mM NaH_2PO_4 and 100 mM Tris pH 8.0 (lysis buffer), the lysate was cleared by sonication and centrifugation, and the supernatant incubated with NiNTA sepharose. The sepharose was then washed in lysis buffer at pH 6.3, and fibromodulin was eluted in lysis buffer at pH 8.0 containing 250 mM imidazole. Fibromodulin was then dialyzed against PBS with 10% glycerol and gradually decreasing urea concentrations (from 8 M to 0 M). In the final step the protein was purified on size exclusion chromatography using PBS. All steps were performed at 4 °C. All protein identities were confirmed by mass spectrometry.

Collagen-binding assays – each assay included full-length collagen as positive control, and bovine serum albumin (BSA) and a $GPP_{(10)}$ triple-helical peptide, like the Toolkit flanking sequence, as negative controls. Collagen peptides were coated at 10 µg/mL in 20 mM acetic acid overnight at 4 °C. Plates were rinsed three times with TBS, and blocked with 5% BSA in TBS for 1 h at room temperature. After blocking, plates were incubated with biotinylated fibromodulin at 10 µg/ml in TBST with 0.1% BSA for 1 h. After washing with TBST, streptavidin-HRP was added at 1:10,000 dilution in TBST 0.1% BSA and incubated for 1h. After washing with TBST, the binding was detected with TMB substrate, stopped with 2 M sulfuric acid, and absorbance was read at 450 nm.

Assays where fibromodulin binding to coated collagen I was tested in the presence of Toolkit peptides were performed using similar methods, but here acetic acid-extracted tail tendon mouse collagen I was diluted to 10 µg/mL into PBS, distributed into a 96-well plate wells, and incubated at 37 °C for 1 h to induce fibril formation, and next incubated at 4 °C overnight for coating. Fibromodulin (10 µg/mL) was pre-incubated for 2 h with Toolkit peptides of different concentrations before incubating with the coated collagen.

Solid-phase binding assays - either lysyl oxidase or fibromodulin or its variants were coated overnight on a 96-well plate at 5 µg/mL in sodium carbonate buffer pH 9.2. Collagen was coated at 10 µg/mL in PBS. The remaining part of the assay followed the protocol described above (collagen binding assays) but here binding of lysyl oxidase to coated fibromodulin proteins or collagen was detected with rabbit anti-lysyl oxidase and HRP-conjugated anti-rabbit antibody, and binding of fibromodulin proteins to coated lysyl oxidase or collagen was detected with mouse anti-his-tag and HRP-conjugated anti-mouse antibodies.

Immunohistochemistry - sections of paraffin-embedded tail tendons from wild-type and *Fmod-/-* mice were used. Antigen retrieval solution (L.A.B. solution from Polysciences) was used before quenching peroxidase with 0.3% hydrogen peroxide for 15 min and blocking the sections with 10% goat serum in TBS for 1 h. Slides were incubated with anti-fibromodulin (2 µg/mL in TBS and 1% serum) for 2 h, then washed with TBS, and stained with ultra-sensitive ABC rabbit IgG staining kit (Thermo Scientific) and diaminobenzidine peroxidase substrate kit (Vector Labs).

Immunoblots - guanidine-extracted proteins from wild-type and *Fmod-/-* tendons were dissolved in urea/CHAPS solution and quantified using BCA assay (Thermo Scientific). 30 µg total protein was run on 4-12% BisTris SDS-PAGE reducing gels (Thermo Scientific). Proteins were transferred to a nitrocellulose membrane that was immunoblotted using 5% milk in TBS for blocking, TBS with 0.1% Tween-20 for washing, and washing buffer with 0.5% milk for antibody incubations. Anti-lysyl oxidase was used at 1 µg/mL and HRP-conjugated anti-rabbit was used at 0.2 µg/mL. The antibodies were detected with SuperSignal West Dura (Thermo Scientific) and imaged using a charge-coupled device camera.

Lysyl oxidase activity assays – fresh conditioned media (293 serum-free expression medium, Life Technologies) from 293T cells transiently expressing recombinant lysyl

oxidase (from p3xFLAG-CMV-8 vector) or mock control were used. Cells were transfected with either vector using Lipofectamine 3000, and medium was collected after three days. Aliquots of medium were incubated with recombinant fibromodulin or its fragments for 1 h before mixing with lysyl oxidase reaction buffer (Lysyl oxidase activity assay kit, Abcam). Samples were incubated for 15 min at 37 °C and LOX activity was measured by fluorescence detection 530/590 nm excitation/emission.

Proximity ligation assays – Duolink PLA assays were purchased from Sigma and performed according to manufacturer's instructions. Here, we used HFL-1 cells seeded on coverslips and transfected with FMOD-pCEP his-tagged constructs (described above) or, as negative control, his-tagged non-collagen binding domain of FMOD (LRR domains 1-3). His-tagged proteins were detected with mouse anti-his-tag. Endogenous LOX was detected with rabbit anti-LOX. Secondary antibodies coupled to DNA probes were included in the PLA assay kit.

RESULTS

Interaction of fibromodulin with Collagen Toolkit peptides – Because *Fmod-/-* tendons have an altered collagen cross-linking (11), we hypothesized that FMOD could modulate collagen cross-linking by interacting with specific cross-linking sites. To identify the binding sites we used Collagen II and III Toolkits in solid-phase assays. The assays showed FMOD affinity for the helix cross-linking sites (peptides II-5, III-5, II-52, III-53 in both Toolkits, Fig.1 A, B; both Toolkits were used contemporaneously). Other peptides, II-44 and III-44, containing the collagenase cleavage site or the peptide II-8 earlier identified as an MMP interaction site (35,36) also had a high apparent affinity for FMOD (Fig.1 A, B). Finally, FMOD had no affinity for telopeptides (data not shown).

To validate our data, we tested the efficiency of Toolkit peptides in inhibiting FMOD-collagen type I interaction. We selected the peptides with the highest apparent affinity for FMOD, i.e. II/III-44, III-5, III-53, along with a negative control peptide II-26. In a solid-phase binding assay, the cross-linking site, peptides III-5 and III-53, could efficiently inhibit this interaction, while II/III-44 were only weak inhibitors, even at a 30-fold molar excess over FMOD (Fig. 1C). Combining II-44 with III-5 or III-53 did not result in a higher inhibitory effect than when using these peptides alone (Fig. 1C, right). Also, III-53 showed an inconsistent inhibitory effect in different experiments, while III-5 and II/III-44 inhibitions gave similar results.

Sequences of the interacting Toolkit peptides are listed and aligned in Fig. 1D. Among the peptides that contain the known helix cross-linking sites the KGHR motif was the most obvious conserved sequence, making it a likely fibromodulin-binding site. Regarding peptides III-8, II-44 and III-44, a homologous pattern of arginine and hydroxyproline residues could be identified as a second, lower affinity, putative fibromodulin-binding site (Fig. 1D). This charge distribution is the only one occurring in Toolkits II and III. Aligning the II/III-44 peptides with the respective sites on collagen I alpha chains shows a high degree of conservation in the α1(I) chain (only two conservative substitutions) and in α2(I) chain (slightly less conserved) (Fig. 1E). Regarding the helical cross-linking sites, they are all conserved in collagens I, II and III.

To test our hypothesis that FMOD interacts with the helical collagen cross-linking site we tested the binding of FMOD to a peptide containing only this site (GLKGHR) and compared the binding with two mutated variants. As Fig. 1F shows, the interaction with the cross-linking site is high and depends on lysine: FMOD does not bind when lysine is alanine-substituted or acetylated. Also, arginine or histidine alone cannot compensate for the absence of lysine.

Fibromodulin interactions with lysyl oxidase – Because of the collagen cross-linking phenotype in *Fmod-/-* mice, we also hypothesized that FMOD may interact with LOX, an interaction that could modulate the accessibility of LOX to collagen and its cross-linking sites. To identify potential FMOD-LOX interactions we performed proximity

ligation assays on HFL-1 fibroblasts expressing his-tagged FMOD or his-tagged non-collagen-binding fragment of FMOD (negative control) and endogenous LOX. The assays detected an interaction between FMOD and LOX (Fig. 2A).

We wanted to identify the binding site of fibromodulin to LOX. Because we earlier identified the collagen-binding site to be in the LRR domain (37), we hypothesized that LOX binding could be in the N-terminal domain of FMOD. We therefore made a recombinant N-terminal fragment of FMOD (FMODNT, amino acids 19-95), and truncated FMOD (FMOD90, amino acids 31-376) where we deleted the 12 N-terminal amino acids. We expressed the proteins in 293T cells, purified them (Fig. 2B) and used them in solid-phase assays. The assays show that LOX binds to full-length FMOD and to the N-terminal fragment (FMODNT), but not to the truncated FMOD90 (Fig. 2C,D). The binding to LOX is therefore sequestered to the N-terminal flank of FMOD, and the apparent K_D of FMOD-LOX interaction is ≅150 nM (Fig. 2E). We also analyzed if tyrosine sulfation on FMOD could affect this interaction: here we used bacterially expressed FMOD (lacking tyrosine sulfation) to assess its affinity for LOX. As Fig. 2F shows, the apparent affinity of LOX for FMOD with or without tyrosine sulfation is similar. Lastly, we also compared the relative affinities of FMOD and LOX for collagen. In our assay LOX affinity for collagen was low even at high molarity, whereas FMOD affinity for collagen had an apparent K_D ≅ 250 nM (Fig. 2G). We also tested if FMOD could increase or inhibit LOX-collagen affinity but the data were inconclusive (not shown).

Lysyl oxidase in fibromodulin-null mice – Because we detected an interaction between FMOD and LOX we then investigated if LOX processing, distribution, or quantity is altered in *Fmod-/-* mice. We used guanidine extracts from tail tendon tissue from wild-type and *Fmod-/-* mice to immunoblot for LOX. We could not detect any major deviation in quantity or processing of LOX in *Fmod-/-* mice; the latter was evident from observing only a 32 kDa band (processed LOX) but no 55 kDa pro-LOX band in either mice (Fig. 3A). Staining for LOX in tail tendon sections of wild-type and *Fmod-/-* mice did not reveal any obvious changes in distribution of LOX (Fig. 3B).

Lysyl oxidase activity influenced by the N-terminal flank of fibromodulin – Next, we wanted to investigate if the presence of FMOD could stimulate or inhibit LOX activity. Unfortunately, we could not measure LOX activity directly in protein extracts from mouse tendons due to a very low signal. We therefore used conditioned cell culture medium from 293T cells overexpressing LOX and mixed it with different amounts of FMOD, FMOD90 or FMODNT. Here, we used fresh total cell culture medium from cells expressing LOX, as we observed that purification of this enzyme dramatically decreased its activity, as reported previously (38). Fig. 4 shows that addition of 1 μg full-length FMOD or the N-terminal part of FMOD to LOX-containing medium can increase LOX activity: FMOD gives 62% ($p<0.05$) and FMODNT gives 47% ($p<0.05$) increase, while the truncated FMOD90 protein can achieve this effect only marginally (18%, N.S.).

DISCUSSION

We reasoned that FMOD could function as a LOX modulator during collagen fibrillogenesis because *Fmod-/-* mice tendons have an altered cross-linking pattern (11) and FMOD modulates collagen fibrillogenesis, i.e. when LOX is also recruited to collagen (1,6,25). Here, we present data that shows an FMOD interaction with collagen cross-linking sites, as well as an interaction of FMOD with LOX that results in increased LOX activity. This, together with the mentioned reports, supports a previously unknown role of FMOD as a LOX-modulating protein that can influence site-specific cross-linking of collagen.

FMOD, along with other homologous Small Leucine-Rich Proteins (SLRPs), has tissue-specific modulatory functions during collagen fibrillogenesis, these functions being evident in the different SLRP-deficient mice (8,39). Modulating fibril assembly could in

itself secondarily influence the LOX-mediated collagen cross-linking, but our data show that FMOD can exert this effect through direct binding to some of the collagen helix cross-linking sites, of which there are two sites on each of the fibrillar collagens, represented here by peptides II-5, II-52 and III-5/6 and III-52/53 (shown in Fig. 1A, B, D). While, all carry the putative KGHR binding sequence, the Toolkit peptides are not equivalent in the binding assays. III-6 and III-52 have low thermal stability(33,34), 30 and 39 °C, compared with 47 °C for the Toolkits as a whole (DA Slatter, unpublished), perhaps accounting for their low FMOD binding. We have observed that the N-terminal site (peptide III-5) appears to have a higher affinity for FMOD, judged from the higher inhibitory effect of peptide 5 on the FMOD-collagen interaction (Fig. 1C). This might be explained by the influence of the neighboring amino acids and suggests a fine-tuned mechanism of cross-link modulation. We also observed that the lysine in the KGHR sequence is essential for binding of FMOD to the cross-linking site (Fig. 1F), suggesting also that FMOD may not bind to an already cross-linked site, i.e. when lysine has reacted with a telopeptide allysine. This would suggest FMOD involvement in collagen fibril assembly before cross-linking takes place.

We also observe that FMOD binds peptides previously identified as MMP-binding (peptides II-44 and III-44, Fig. 1A,B) (35,36) a site in both collagens that is adjacent to the cross-linking site on a neighboring tropocollagen molecule in a quarter-stagger assembled microfibril (Fig. 5A). Similarly, both these sites align with C-telopeptides (Fig. 5) that are excessively oxidized by LOX in *Fmod-/-* mice (11). The span across the three sites (three tropocollagens) is approximately 5 nm, which is roughly the size of the concave face of the LRR pocket in SLRPs (40,41), and it is tempting to suggest a multivalent binding of FMOD to two interaction sites (III-5, III-44) across the three collagen helices (proposed in Fig. 5B). Therefore, FMOD could modulate collagen cross-linking by binding or possibly cross-bridging three collagen monomers around their cross-linking sites. Against this conclusion is the lack of potentiation of the inhibitory effect of both III-5 and III-44 on FMOD-collagen interaction; on the other hand, inhibition by III-5 alone is very efficient and any additive effect of III-44 may be marginal and hard to detect. Alternatively, bound FMOD could switch to an adjacent collagen monomer at some critical point during fibrillogenesis, thus switching from regulation of, perhaps, lateral assembly of collagens to regulation of cross-linking. The discrepancy between solid-phase binding and ability of peptides to inhibit FMOD binding to collagen is intriguing. Peptide III-5 supports strong FMOD binding and, in solution, blocks FMOD binding to collagen I. In contrast, although II-44 and III-44 are also strong binders of FMOD, both only slightly inhibit its binding to collagen I. This suggests two binding modes, perhaps separate collagen-binding sites on FMOD, a first, high-affinity site that recognises the KGHR sequence, and a second remote site that recognises the MMP-interaction motifs in II-44 and III-44. Whether allosteric or co-operative effects allow these sites to regulate one another remains to be established.

Several other proteins besides FMOD, including fibronectin, fibrin, MMPs or collagen C-telopeptide bind to II/III-44 or its homologous site on collagen I (42-45). A cell might use such an overlapping interaction hotspot to differentially regulate collagen fibrillogenesis, cell adhesion or contraction. Within the sequence of II-44 (Fig 1E) binding of MMP13 to the canonical ¾-¼ cleavage site required residues spanning the first leucine and second arginine (35,36), and whilst the only residues critical for MMP1 binding were the two leucines, contacts were observed in the MMP1-peptide co-crystal including the next hydroxyproline (35,36), whilst fibronectin binding embraces the first and second arginine residues (44). In summary, the sites in collagens I and II that bind MMPs, FN and FMOD are not identical, but all overlap, and it would be anticipated that their binding would be mutually exclusive.

To speculate on one example, C-telopeptide binding to the site that corresponds to II-44/III-44 stimulates collagen

fibrillogenesis (42,46). FMOD could, through binding to this site, delay fibrillogenesis that is stimulated by the C-telopeptide. In this manner, the C-telopeptide oxidation by LOX would also be delayed, because LOX activity increases on assembled collagen fibrils (25). Such a mechanism could explain the increased C-telopeptide oxidation in *Fmod-/-* mice (11), i.e. absence of FMOD would allow for a faster, uncontrolled, C-telopeptide-driven fibrillogenesis, thus triggering LOX to excessively oxidize C-telopeptides.

Binding of FMOD to II-44/III-44 also supports the previous observation that FMOD can inhibit MMP cleavage of collagen (47). Such an interaction could protect collagen from being digested before it starts to assemble into fibrils. FMOD could of course also protect the already assembled fibers by shielding the II-44 site from MMP.

Our interaction data are largely in line with the previously reported interactions of FMOD with collagen I CNBr fragments (48). In this report, FMOD bound collagen fragment CB6 (that includes sequence from Toolkit peptide III-53), CB7 (includes III-44), and CB8 (includes III-8), but did not bind CB5, which contains the N-terminal helical cross-linking site. The explanation for this discrepancy could be a conformational unfolding of the KGHR sequence: KGHR is preceded by a methionine where CNBr cleaves, and the cleavage would leave the KGHR site without flanking, helix-inducing GXY residues, likely to cause fraying of the new N-terminal end of the peptide, disrupting the binding site.

In the same paper (48) the authors also report on FMOD binding to collagen II CNBr fragments. CB9,7 fragment, containing the II-52 sequence, was not used in the binding assay. One apparent discrepancy with our data is the absence of binding to CB10 (which contains the II-44 sequence) and binding to CB11 (where we have not found any binding). Here, one should consider that collagen II is more glycosylated, while Toolkit peptides are not – this could affect FMOD-collagen II interaction. Our data, as discussed above, point to conserved interaction sites across collagens I, II and III (Fig. 1E).

The binding of FMOD to collagen cross-linking sites suggested that FMOD might interact with the cross-linking enzyme, LOX. Indeed, we were able to identify their interaction in a fibroblast cell culture using PLA assays (Fig. 2A). We have mapped this interaction to the N-terminal 12 amino acids of FMOD (Fig. 2B-E) and observed no apparent effect of FMOD tyrosine sulfation on the interaction (Fig. 2F). We also observed that the binding of LOX to FMODNT was less pronounced than binding to FMOD (Fig. 2E); we think this could be due to a minor misfolding of FMODNT.

Our data suggest that FMOD forms a complex with LOX and collagens, where LOX binds at one end of FMOD (as shown here) and collagen(s) bind in the LRR domain (37). We did, however, observe that FMOD increased LOX activity even in the absence of collagen and this activity depended on the N-terminal tail of FMOD (Fig. 4). This activity increase may be considered marginal, and possibly we could have had obtained a more potentiating effect had we used purified lysyl oxidase rather than cell medium. In our hands though, purification or storage of LOX is detrimental to its activity (although its interaction with FMOD remains unaffected), and therefore we used fresh cell medium for the activity assay. There may, of course, be other components in the cell medium that affect the extent of FMOD-stimulated LOX activity.

We suggest a model of FMOD-regulated collagen cross-linking where FMOD restricts premature fibril assembly and dysregulated cross-linking of collagens. This is achieved by binding to the surface of a collagen microfibril at its cross-linking site, while simultaneously binding LOX and directing its activity towards a specific lysine residue on an adjacent microfibril (Fig. 5B). Such an interaction may explain tissue-specific cross-linking found in collagen matrices populated by FMOD, including tendons, ligaments, or fibrotic tissues. One should keep in mind that LOX-FMOD interaction does not ensure LOX-mediated cross-linking, but appears to direct it towards specific collagen lysine residues.

Previously we reported excessive oxidation of collagen C-telopeptides in *Fmod-/-* mice (11). We have not detected any changes in LOX processing or quantity in *Fmod-/-* tendons, but we show here that FMOD interacts with LOX, changes its activity, and binds to collagen cross-linking sites. We reason that FMOD, by binding across the C-telopeptides, could shield LOX from excessively oxidizing telopeptides within one microfibril, a reaction that would prompt uncontrolled cross-linking between C-telopeptide allysines, forming aldols interlinking other microfibrils. In FMOD-controlled packing and cross-linking of microfibrils, LOX could be directed towards the telopeptides of another microfibril by binding to one end of FMOD, triggering the formation of a specific inter-fibril cross-link. It is also possible that LOX becomes positioned towards N-telopeptides in the same microfibril (Fig. 5B). This would ensure concerted, tissue-specific packing of microfibrils into fibrils, and regulated intrafibrillar cross-linking.

In summary, our current and previous data show that FMOD can modulate LOX activity on specific sites on collagen, and support a novel theory regarding control of specific collagen cross-linking through collagen-associated proteins, likely crucial in the proper assembly of tendons but also of fibrotic collagen matrices. The exact cross-links being formed in healthy and fibrotic tissues remain to be shown, as does the identity of the collagen-associated proteins that modulate their formation. This effort could open new perspectives and reveal new targets for treating fibrotic conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

SK conceived the study, designed, performed and analyzed the experiments, and wrote the paper. RWF designed the Toolkit peptides, synthesized by DB and AB, and revised the manuscript with SK. KR analyzed the experiments and revised the manuscript with SK. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Identification of fibromodulin binding sites on collagen. Collagen Toolkit II (*A*) and Toolkit III (*B*) peptides were coated on a 96-well plate. The plate was blocked with BSA, and incubated with biotinylated FMOD at 10 μg/ml (170 nM) for 2 h. Binding was detected with streptavidin-HRP followed by TMB substrate, quenching with sulfuric acid, and measuring A_{450}. BSA was used as a negative control. *C.* Inhibition of FMOD binding to coated collagen I. The assay was performed as above, but collagen I was coated on a 96-well plate, and FMOD was pre-

incubated with increasing amounts of the interacting Toolkit peptides III-5, III-44, III-53, or with a negative control peptide II-26. *D.* Sequences of the interacting Toolkit peptides are listed, with homologue amino acids underlined (except the repetitive glycines). The non-binding peptides are in italics. *E.* Homology alignment of FMOD-binding Toolkit peptides and the corresponding sequences on collagen I α1- and α2-chains. *F.* Solid-phase binding assay to assess interaction of FMOD with cross-linking site-containing collagen peptides and their mutated variants. The assay was performed as in *A*. Peptide sequences are listed on the x-axis (K(Ac) = acetylated lysine), except III-5 sequence that is listed in *E.* These shorter peptides were synthesised with GCP terminal triplets. In *A, B, C, F* error bars represent standard deviation (n=3, technical replicates). All experiments were performed three times.

Figure 2. Identification and characterization of FMOD-LOX interaction. *A.* Proximity ligation assay on HFL-1 cells expressing FMOD (*left*) or non-collagen-binding His-tagged FMOD fragment as negative control (*right*). Anti-His-tag and anti-LOX antibodies were used to detect the interaction (red fluorescence); nuclear staining with DAPI is blue. *B.* Gel image of FMOD variants used in the solid-phase assays in *C-F*. The proteins were expressed in 293T cells and purified on Ni-NTA sepharose. The amino acid range of each construct is listed. *C.* Solid-phase assays showing interactions between FMOD variants and LOX. LOX was coated on a 96-well plate, which was blocked with BSA, and incubated with 170 nM FMOD variants, whose binding was detected using anti-His-tag antibody. *D.* As in *C,* but here FMOD variants were coated on the plate followed by incubation with 150 nM LOX and detection with anti-LOX antibody. The experiments in *C* and *D* were repeated twice giving similar results. *E.* As in *D.* but here serial dilutions of LOX were used to determine apparent K_D. Similar results were obtained in three experiments. *F.* Solid-phase assay to assess binding of LOX to coated FMOD (expressed in 293 cells), bactFMOD (expressed in *E. coli*) and FMOD90. The assay was performed as in *D. G.* Solid-phase assay to compare binding of serially diluted FMOD and LOX to coated collagen in a 96-well plate. Binding was detected using anti-His-tag or anti-LOX antibodies. Error bars in *C-G* represent standard deviation (n=3, technical replicates). Experiments in *F* and *G* gave similar results at three occasions.

Figure 3. Lysyl oxidase quantity and distribution in *Fmod-/-* mice. *A.* Guanidine-extracted proteins from wild-type and *Fmod-/-* tail tendons were precipitated with ethanol, run on SDS-PAGE, transferred to a nitrocellulose membrane, and stained with Ponceau, or detected with anti-FMOD or –LOX antibodies (n=3, biological replicates). *B*. Immunodetection of LOX was performed on formalin-fixed tail tendons of wild-type and *Fmod-/-* mice. The sections were deparaffinized, hydrated, and processed for antigen retrieval and peroxidase quenching, before blocking with 10% goat serum and incubating with rabbit anti-LOX (or isotype control antibody) followed by HRP-conjugated anti-rabbit. The staining was developed using diaminobenzamidine peroxidase.

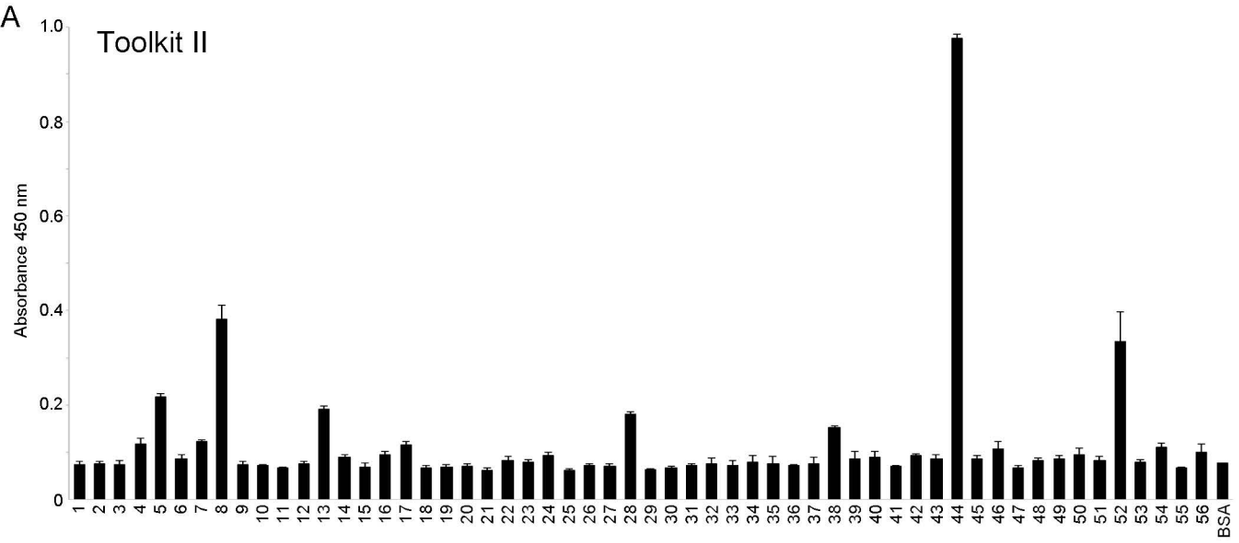
Figure 4. Lysyl oxidase activity in the presence of fibromodulin. Lysyl oxidase activity was measured with a commercial kit. As a sample, conditioned medium from 293T cells expressing LOX (or from LOX-negative control cells) was used. The medium was pre-incubated for 1 h with FMOD or its variants and then mixed with the substrate and incubated for 15 min at 37 °C. Enzyme activity was measured by fluorescence emission at 590 nm after excitation at 530 nm. The plotted values are biological duplicates. Similar results were obtained in three independent experiments.

Figure 5. Proposed model for collagen cross-linking modulation by fibromodulin. *A.* Alignment of the collagen III monomers is derived from 234-residue D-periods. The identified high-affinity FMOD-binding sites in collagen are marked in *dark green* (III-5), *yellow* (III-8),

blue (III-44), *light green* (III-53). D-periods are delimited with black lines. Collagen monomers are arranged in quarter-stagger, which reveals vertical alignment of III-44 and III-5 sites on neighboring collagen monomers. The two telopeptides (*red*) also align with FMOD-binding sites. *B.* Proposed binding of the FMOD-LOX complex to a microfibril. FMOD binds across three monomers, interacting with the vertically aligned collagen-binding sites. LOX binds to the N-terminal end of FMOD and is positioned against the N-telopeptide in the microfibril.

Figure 1

A



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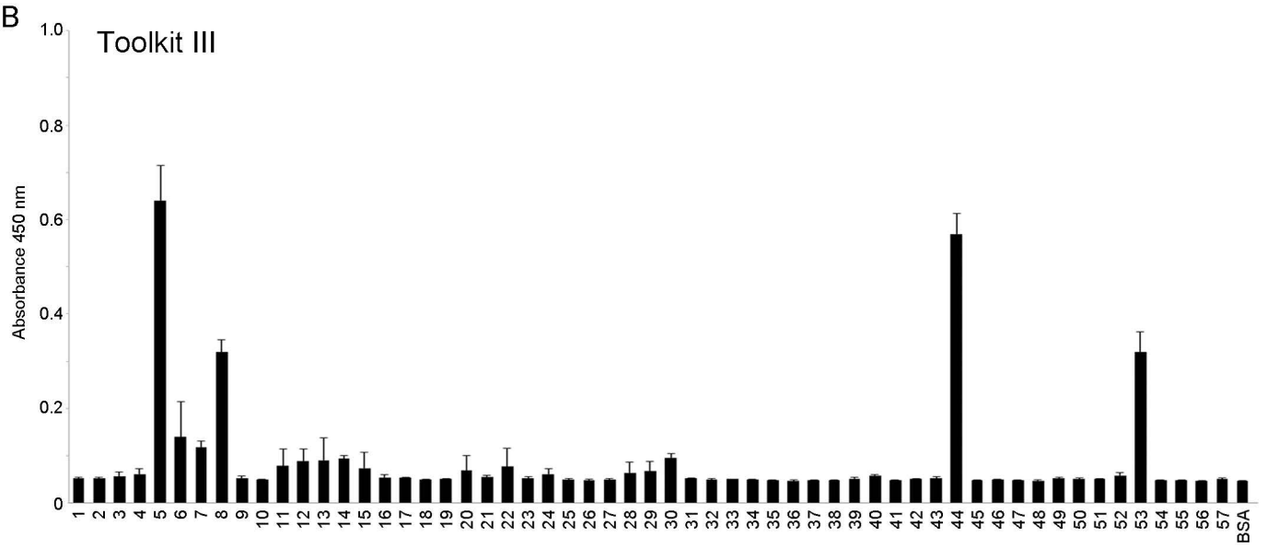

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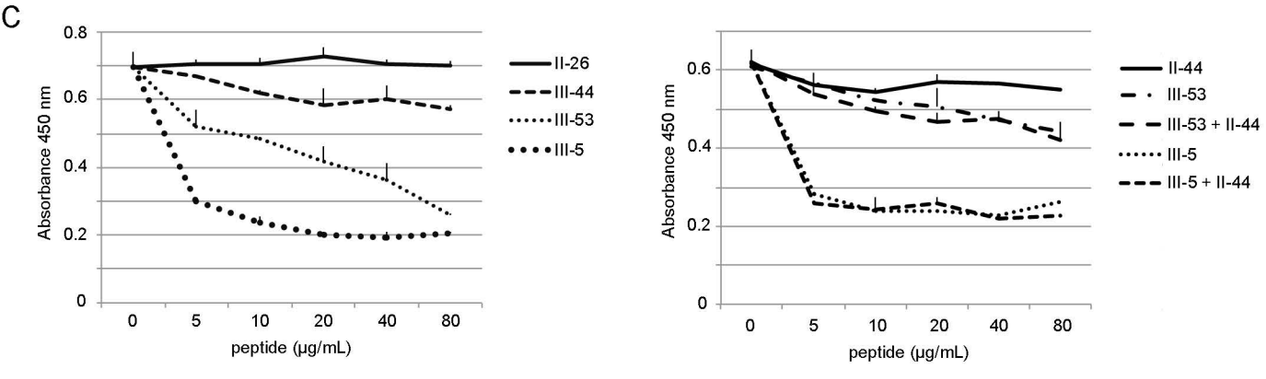

D

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III-8          GAOGPMGPRGAOGERGROGLOGAAGAR
III-44         GAOGPLGIAGITGARGLAGPOGMOGPR
II-8                    GLOGERGRTGPAGAAGARGNDGQOGPA
II-44                   GLAGQRGIVGLOGQRGERGFOGLOGPS

III-5 GERGLOGPOGIKGPAGIOGFOGMKGHR
III-53                       GIKGHRGFOGNOGAOGSOGPAGQQGAI
II-5             GARGFOGTOGLOGVKGHRGYOGLDGAK
II-52               GEAGEOGERGLKGHRGFTGLQGLOGPO
III-6                     GFOGMKGHRGFDGRNGEKGETGAOGLK
III-52   GPQGPRGDKGETGERGAAGIKGHRGFO
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E

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α1(I)                GIAGQRGVVGLOGQRGERGFOGLOGPS
α2(I)                GLLGAPGILGLOGSRGERGLOGVAGAV
II-44                GLAGQRGIVGLOGQRGERGFOGLOGPS
III-44      GAPGPLGIAGITGARGLAGPOGMPGPR

α1(I)                GARGLOGTAGLOGMKGHRGFSGLDGAK
α2(I)                GARGFOGTPGLOGFKGIRGHNGLDGLK
II-5                 GARGFOGTPGLOGVKGHRGYPGLDGAK
III-5      GERGLPGPPGIKGPAGIPGFOGMKGHR

α1(I)                GLOGERGRPGAQGPAGARGNDGATGAA
α2(I)                GLOGERGRVGAQGPAGARGSDGSVGPV
II-8                 GLOGERGRTGPAGAAGARGNDGQPGPA
III-8      GAPGPMGPRGAOGERGRPGLQGAAGAR

α1(I)      GETGEQGDRGIKGHRGFSGLQGPOGPO
α2(I)      GEOGEKGPRGLPGLKGHNGLQGLOGIA
II-52      GEAGEPGERGLKGHRGFTGLQGLOGPO
III-53               GIKGHRGFPGNPGAOGSOGPAGQQGAI
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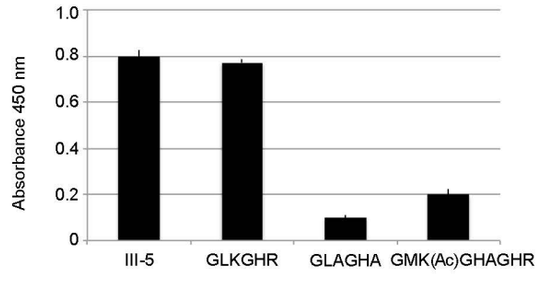
F



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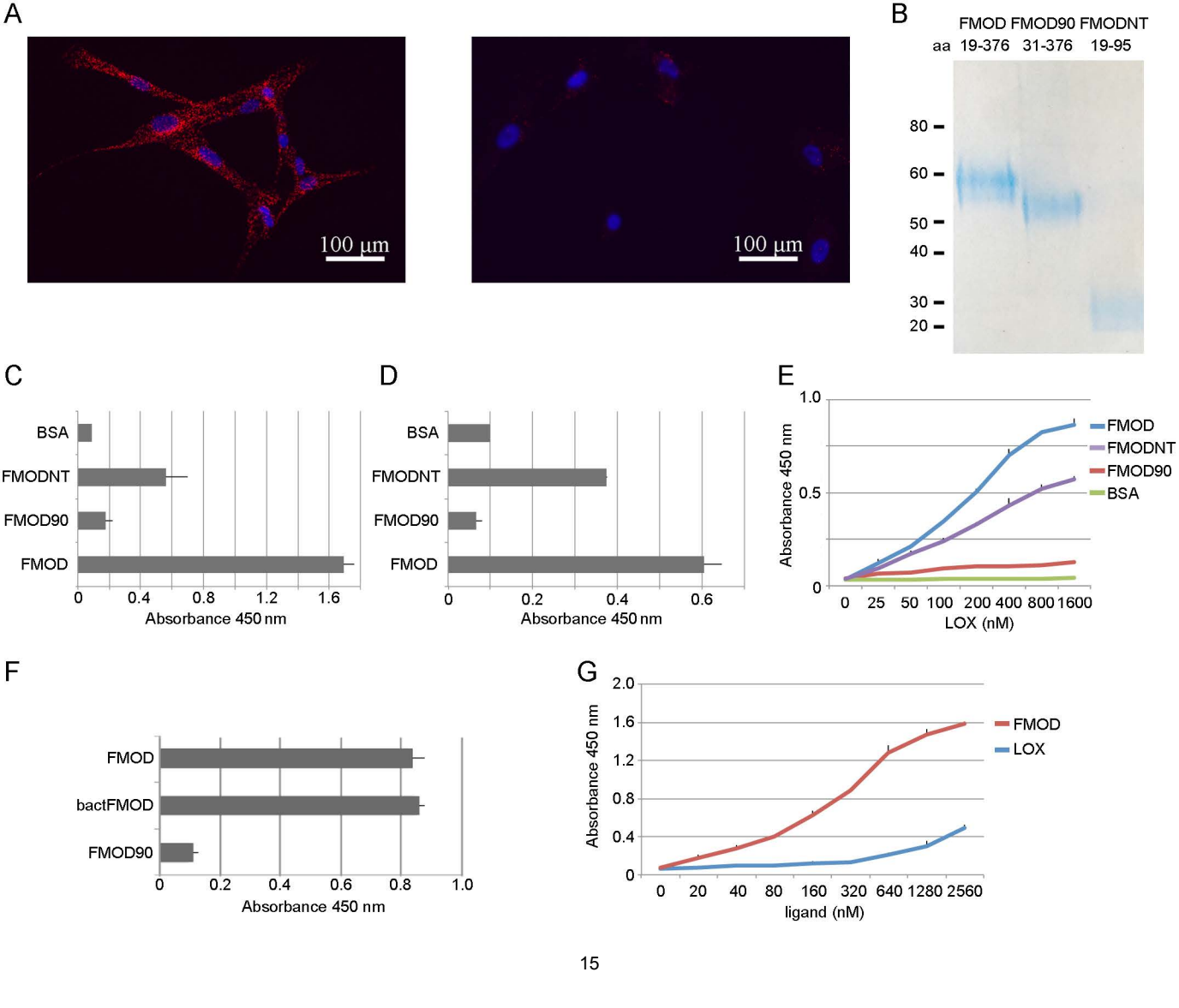
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100 µm
B
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40
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FMODNT
FMOD90
FMOD
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Absorbance 450 nm
D
BSA
FMODNT
FMOD90
FMOD
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Absorbance 450 nm
E
Absorbance 450 nm
1.0
0.5
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LOX (nM)
FMOD
FMODNT
FMOD90
BSA
F
FMOD
bactFMOD
FMOD90
0 0.2 0.4 0.6 0.8 1.0
Absorbance 450 nm
G
Absorbance 450 nm
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ligand (nM)
FMOD
LOX

Figure 3

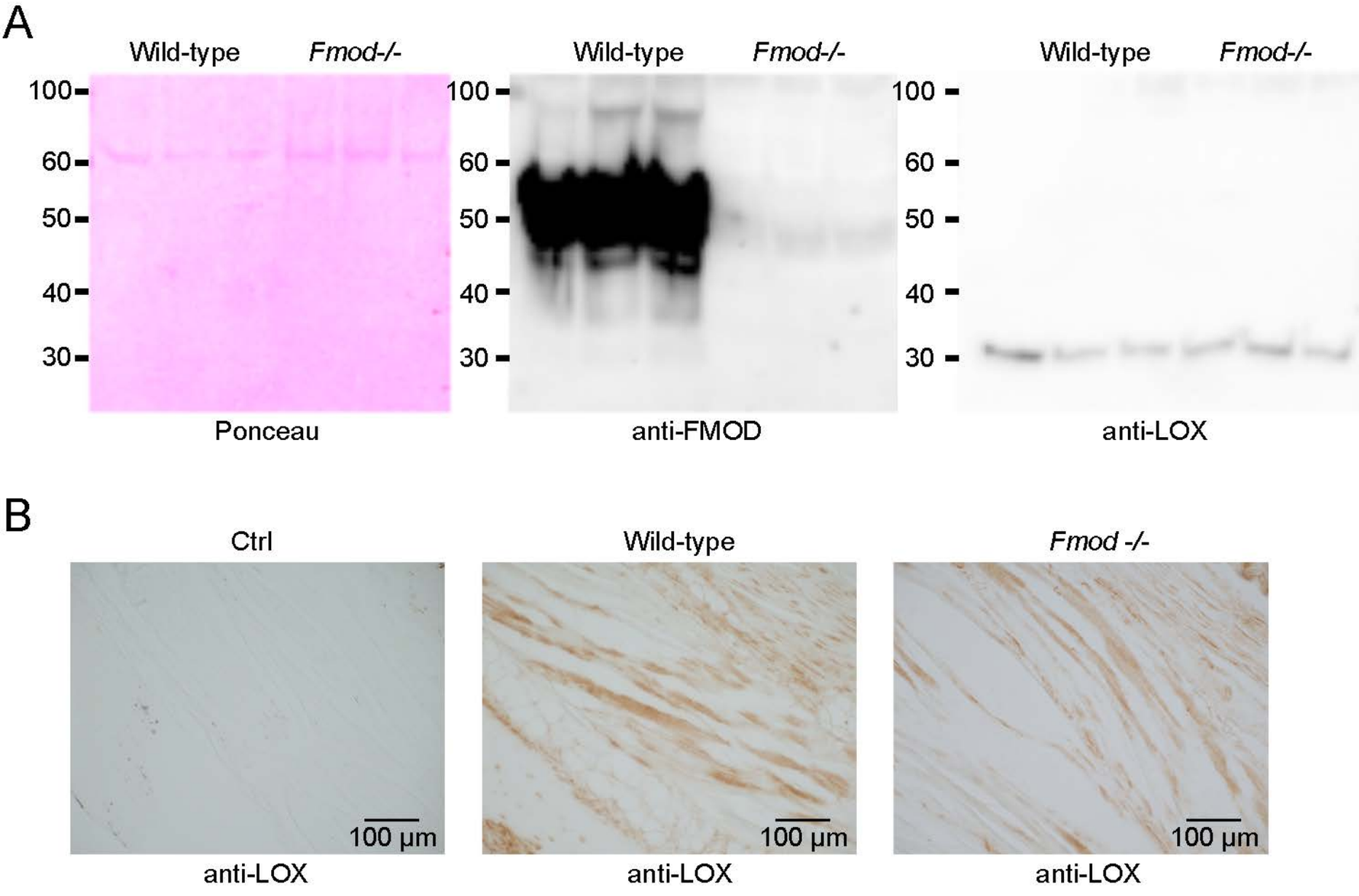
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Figure 4

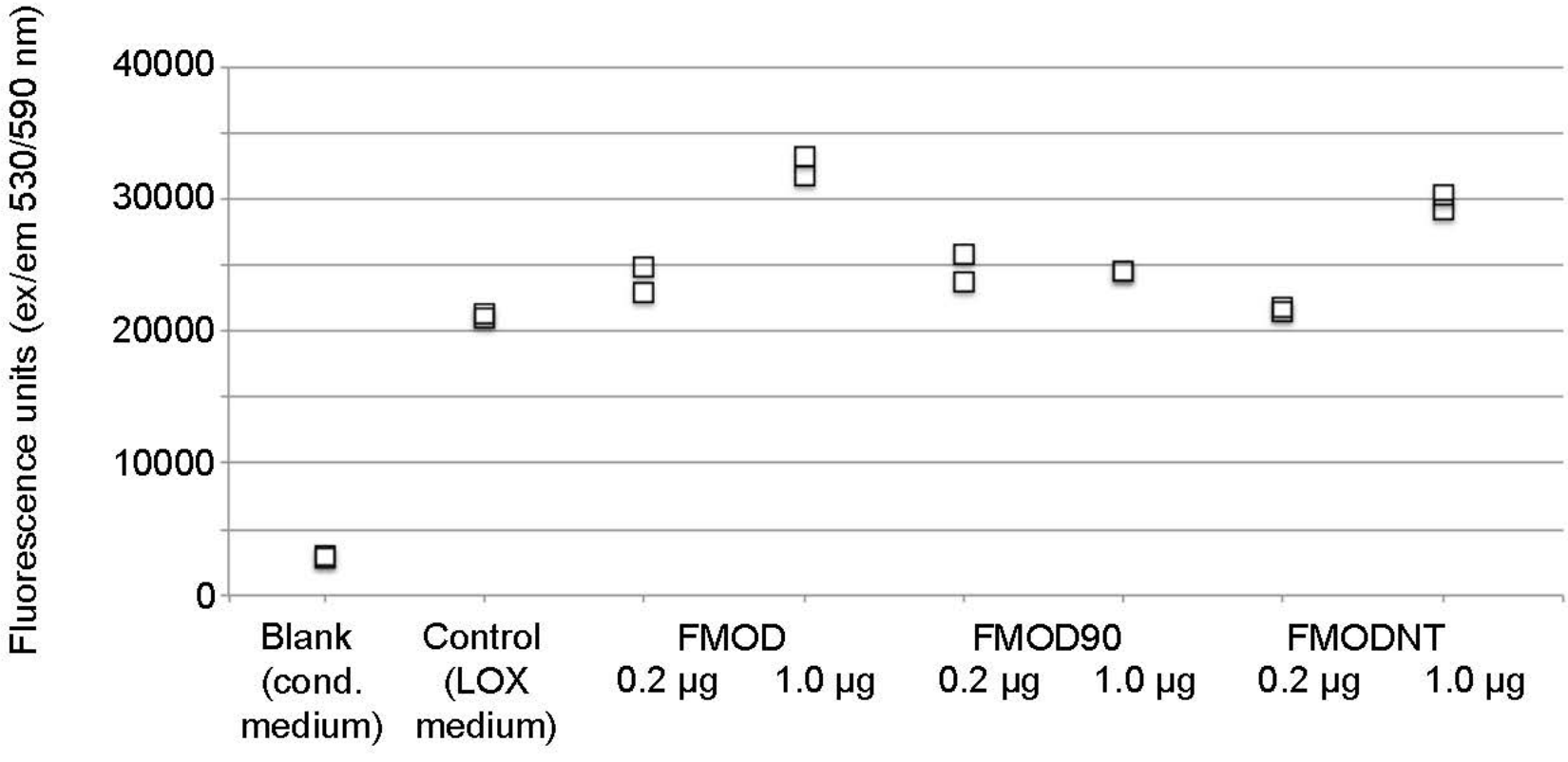
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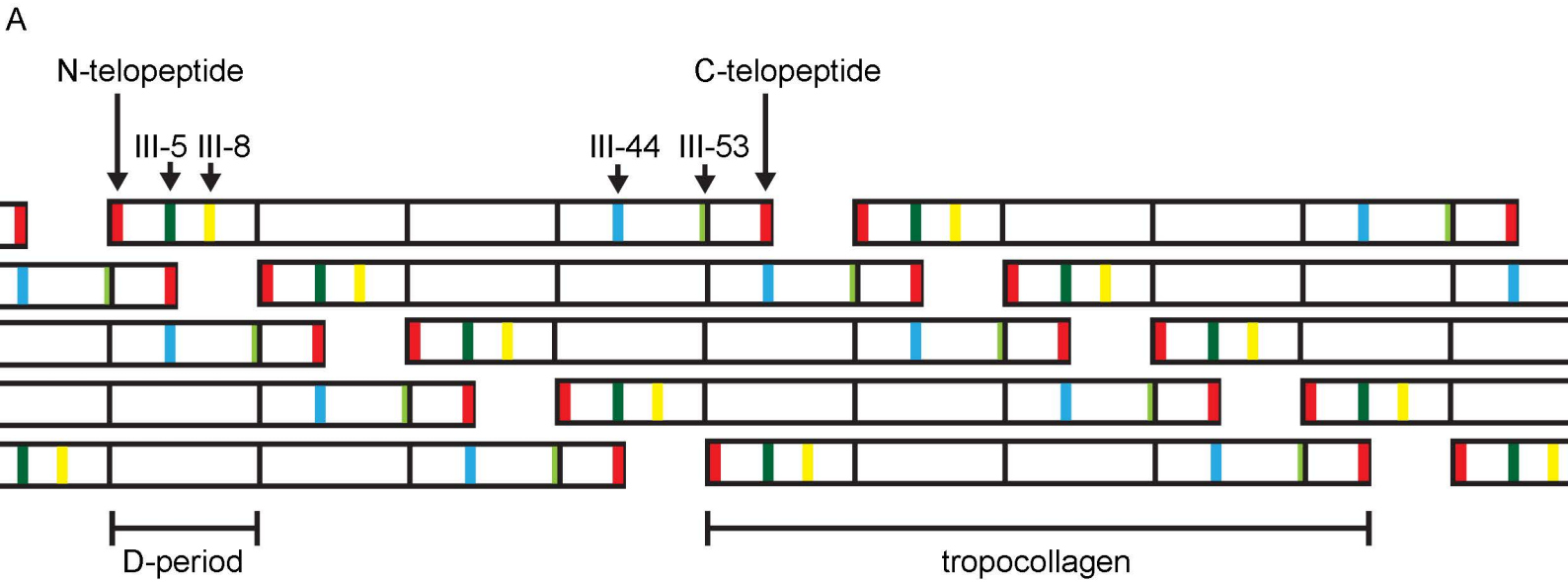
Figure 5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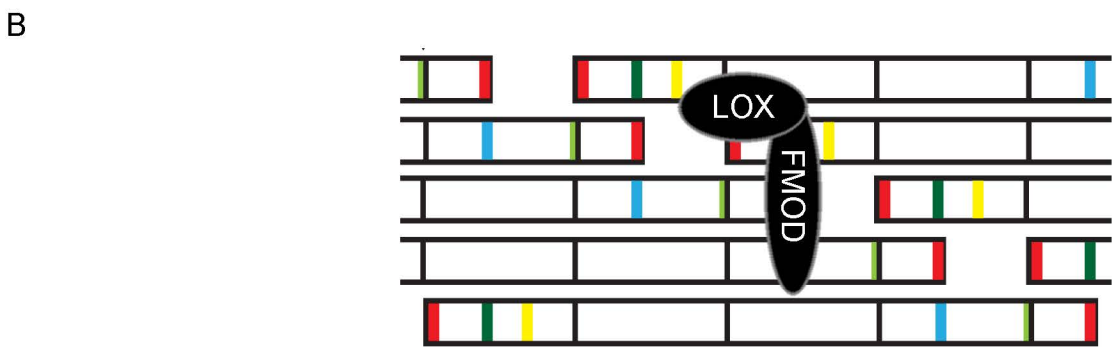
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