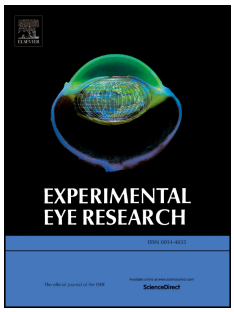

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Ursula Schlötzer-Schrehardt, Matthias Zenkel

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The role of lysyl oxidase-like 1 (LOXL1) in exfoliation syndrome and glaucoma

Review

Ursula Schlötzer-Schrehardt and Matthias Zenkel

Department of Ophthalmology, University of Erlangen-Nürnberg
Schwabachanlage 6, 91054 Erlangen, Germany

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Corresponding author:
Ursula Schlötzer-Schrehardt, PhD
Department of Ophthalmology, University of Erlangen-Nürnberg
Schwabachanlage 6, 91054 Erlangen, Germany
Tel. +49-9131-8534433; Fax. +49-9131-8534631
Email: ursula.schloetzer-schrehardt@uk-erlangen.de

Abstract

Exfoliation syndrome (XFS) is an age-related systemic disease that affects the extracellular matrix. It increases the risk of glaucoma (exfoliation glaucoma, XFG) and susceptibility to diseases of elastin-rich connective tissues. *LOXL1 (lysyl oxidase-like 1)* is still recognized as the major genetic effect locus in XFS and XFG in all populations worldwide, although its genetic architecture is incompletely understood. LOXL1 is a key cross-linking enzyme in elastic fiber formation and remodeling, which is compatible with the pathogenetic concept of XFS as a specific type of elastosis. This review provides an overview on the current knowledge about the role of LOXL1 in the etiology and pathophysiology of XFS and XFG. It covers the known genetic associations at the *LOXL1* locus, potential mechanisms of gene regulation, implications of LOXL1 in XFS-associated fibrosis and connective tissue homeostasis, its role in the development of glaucoma and associated systemic diseases, and the currently available LOXL1-based *in vivo* and *in vitro* models. Finally, it also identifies gaps in knowledge and suggests potential areas for future research.

Keywords

Exfoliation syndrome, pseudoexfoliation syndrome, exfoliation glaucoma, lysyl oxidase-like 1, LOXL1, extracellular matrix, elastic fibers

Introduction

Exfoliation syndrome (XFS) is an age-related systemic disease that manifests primarily in the eyes and represents the most common identifiable cause of open-angle glaucoma (exfoliation glaucoma, XFG) worldwide (Ritch and Schlötzer-Schrehardt, 2001). It is characterized by the excessive production and disordered assembly of microfibrillar components into highly cross-linked and proteolytically resistant fibrillar aggregates, which progressively accumulate throughout the anterior eye segment and connective tissues of various organ systems and blood vessel walls (Schlötzer-Schrehardt and Naumann, 2006). The typical fibrils predominantly contain elastic fiber components, such as elastin, fibrillin-1, microfibril-associated glycoprotein 1 (MAGP-1), fibulins, latent TGF-ß binding proteins (LTBPs) and the cross-linking enzyme lysyl oxidase-like 1 (LOXL1), and appear to be produced by various unrelated epithelial, endothelial and mesenchymal cell types, including lens epithelial cells, ciliary epithelial cells, trabecular meshwork cells, smooth muscle cells and fibroblasts (Zenkel and Schlötzer-Schrehardt, 2014). The pathophysiology involves a complex interplay of pro-fibrotic factors such as TGF-ß1, proteolytic enzymes and inhibitors, pro-inflammatory cytokines, chaperones such as clusterin, and dysregulated stress response pathways including impaired autophagy (Schlötzer-Schrehardt, 2018; Bernstein et al., 2018; Wolosin et al., 2018). Thus, XFS has been characterized as a stress-induced fibrotic

process, an elastic microfibrillopathy, developing from interaction of chronic stress conditions and genetic predisposition in the aging individual, although the exact molecular pathomechanisms still remain unknown.

The genetic architecture of XFS is also incompletely understood, but seems to involve multifactorial complex inheritance including the effects of multiple genes in combination with environmental factors (e.g., geographic latitude, UV exposure, oxidative stress, low antioxidant diet, low folate intake, high coffee consumption) (Pasquale et al., 2018). Large-scale genome-wide association studies (GWAS) have identified robust genetic associations with 7 genetic loci, i.e., *LOXL1* (lysyl oxidase-like 1), *CACNA1A* (calcium voltage-gated channel subunit alpha 1A), *POMP* (proteasome maturation protein), *TMEM136* (transmembrane protein 136), *AGPAT1* (1-acyl-glycerol-3-phosphate acyltransferase alpha), *RBMS3* (RNA binding motif single stranded interacting protein 3) and *SEMA6A* (semaphorin 6A), which provided significant biological insight into disease processes and pointed to an involvement of matrix crosslinking processes, calcium signaling, ubiquitin-proteasome degradation pathway, and blood-aqueous barrier dysfunction in XFS pathophysiology (Thorleifsson et al., 2007; Aung et al., 2015, 2017). Amongst all 7 susceptibility genes, *LOXL1* has been unequivocally confirmed to exert the strongest genetic effect, accounting for up to 30% of heritability of XFS (Aung et al., 2017, 2018) (Fig. 1). LOXL1 is a key cross-linking enzyme in elastic fiber formation and homeostasis, which is compatible with the pathogenetic concept of XFS as a specific type of elastosis (Schlötzer-Schrehardt, 2009, 2018). This review provides an overview on the current knowledge about the role of LOXL1 in the etiology and pathophysiology of XFS and XFG.

LOXL1 **as a genetic risk factor of XFS and XFG**

Protein-coding common variants at *LOXL1*

LOXL1, located on chromosome 15q24.1, was the first major susceptibility gene identified for XFS/XFG through a GWAS in Scandinavian populations (Thorleifsson et al., 2007). This finding has been replicated in multiple geographical populations, which all shared exceptionally high association signals (Aboobakar and Allingham, 2014; Aung et al., 2018). Two common non-synonymous protein-coding variants in exon 1, rs1048661:G>T (p.Arg141Leu) and rs3825942:G>A (p.Gly153Asp), and one intronic variant (rs2165241:T>C), conferring a more than 20-fold increased risk for disease, were initially considered as the causal variants in this locus. However, subsequent studies revealed that the genetic effect conferred by the *LOXL1* alleles was reversed in different ethnic populations, a phenomenon termed allele flipping. For instance, the G allele of rs1048661 was identified as risk allele in most populations studied, while the opposite T allele was reported to increase the risk of XFS in Asian populations. Also, the G allele of rs3825942,

representing the risk allele for the majority of populations, has been shown to be protective in Black South Africans (Aboobakar and Allingham, 2014) (Fig. 2A). Although such allelic reversals may result from interactive effects with other factors that vary between populations, such as genetic background or environmental exposures (Clarke and Cardon, 2010), the observations immediately suggested that the R141L and G153D variants may not be causative factors in XFS development.

This notion was largely confirmed by functional studies. Using a bacterial expression system purified recombinant haplotype variants of R141L and G153D did not show any significant differences in their enzymatic activity against various substrates including elastin and collagen type I (Kim and Kim, 2012). However, the protein variant L141_G153 (T_G), purified from a rat fetal lung fibroblast cell line, had an effect on proteolytic processing of LOXL1 compared to the other haplotype variants (Sharma et al., 2016). This study also presented molecular modelling data suggesting alterations in protein-protein interactions induced by the polymorphisms at positions 141 and 153. Therefore, it cannot be ruled out that the missense variations may affect protein functions such as proteolytic processing or interaction of LOXL1 with any proteins required for elastogenesis, as already predicted by genetic programs (Kuhlenbäumer et al., 2007), thereby disrupting the proper assembly of elastic (micro)fibrils and promoting the disorganized aggregation of XFS fibrils. It is, however, also possible that these sequence variants are rather genetically associated with other functional variants at the *LOXL1* locus.

Non-coding common variants at *LOXL1*

Further sequencing approaches at *LOXL1* led to identification of additional XFS-associated risk variants, rs16958477 and rs12914489, in the *LOXL1* promoter region, suggesting that XFS may be caused by alterations in *LOXL1* gene regulation (Fan et al., 2011). Notably, rs16958477 was found to alter *LOXL1* expression *in vitro* (Ferrell et al., 2009). Hauser and colleagues identified clustering of XFS-associated variants at the exon 1/intron 1 boundary of *LOXL1*, which were shown to modulate the expression of *LOXL1* antisense RNA 1 (*LOXL1-AS1)*, a long non-coding RNA (lncRNA) encoded on the opposite strand of *LOXL1* (Hauser et al., 2015). They also showed that *LOXL1-AS1* expression is regulated in response to oxidative stress and cyclic mechanical stress in human lens epithelial cells and Schlemm's canal endothelial cells, respectively. Although *LOXL1-AS1* is not directly involved in the regulation of *LOXL1*, it may regulate distant target genes involved in the pathogenesis of XFS. Functionality of *LOXL1-AS1* in regulation of NF-κB signaling has been already proven (Wang et al., 2018), and *LOXL1-AS1* expression was found to be upregulated in a variety of tumors to stimulate cell proliferation, cell cycle progression, cell migration and invasion (Chen

他们还发现，在人晶状体上皮细胞和Schlemm管内皮细胞中，LOXL1-AS1的表达分别在氧化应激和循环机械应激反应中受到调节。虽然LOXL1- as1没有直接参与LOXL1的调控，但它可能调控参与XFS发病机制的远端靶基因。LOXL1-AS1在NF-κB信号通路调控中的作用已被证实

et al., 2019). The role of this regulatory lncRNA in XFS pathophysiology needs to be determined, but appears to represent a promising target for further investigations.

A clear regulatory effect on *LOXL1* expression has, however, been demonstrated for another polymorphic locus spanning introns 1 and 2 of *LOXL1*, which has been identified through a GWAS on European and Japanese populations (Pasutto et al., 2017). More specifically, the rs11638944:C>G transversion has been shown to influence expression levels of *LOXL1* through two molecular mechanisms, i.e., differential DNA binding of the transcription factor RXRα (retinoid X receptor alpha) and modulation of alternative splicing of *LOXL1* pre-mRNA. Since the alternatively spliced *LOXL1* transcript is associated with nonsense-mediated decay (NMD), a common post-transcriptional mechanism of gene expression regulation (Smith and Baker, 2015), increased unproductive splicing and higher rates of NMD at the risk sequence resulted in up to 50% reduction of steady-state levels of wild-type *LOXL1* mRNA in cells and tissues of risk allele carriers. In a follow-up study, it was reported that this mechanism of "alternative splicing coupled to NMD" can be modulated by distinct forms of XFS-associated stressors and metabolites, including oxidative stress, caffeine and retinoic acid, and may thus represent a dynamic mode of adapting *LOXL1* expression to varying environmental conditions (Berner et al., 2017).

A recent deep re-sequencing approach at the broad *LOXL1* locus not only confirmed the lack of any "unflipped" common, XFS-associated variant in the intragenic region, but also revealed a single common variant downstream of *LOXL1* without allele effect reversal in populations of different ancestry (Berner et al., 2019). The minor allele G of the intergenic sequence variant rs7173049:A>G was consistently enriched in controls across all populations studied translating to a biologically relevant protective effect (Fig. 2B). Functional analyses showed that this variant influences expression levels of two neighboring genes, *ISLR2* (immunoglobulin superfamily containing leucine-rich repeat protein 2) and *STRA6* (stimulated by retinoic acid receptor 6), and that this effect was mediated by allele-specific binding of the transcription factor THRβ (thyroid hormone receptor beta). The protective allele G was found to correlate with increased tissue expression levels of ISLR2, a membrane protein involved in neural development, and STRA6, a cell surface receptor regulating cellular uptake of vitamin A in ocular tissues. Both genes were significantly downregulated in tissues of XFS patients together with other key components of the STRA6 receptor-driven retinoic acid signaling pathway. Inhibition of retinoic acid signaling in XFS-relevant cell types induced a significant upregulation of LOXL1, TGF-ß1 and many XFS-associated matrix genes *in vitro*. These observations indicated that an impaired retinoid metabolism may be causally involved in the pathophysiology of XFS and that this biological pathway can be potentially targeted to treat fibrotic alterations caused by insufficient tissue retinoid levels in XFS patients.

xfs相关细胞类型维甲酸信号通路的抑制在体外诱导LOXL1、TGF-ß1和许多xfs相关基质基因显著上调。这些观察结果表明，受损的维甲酸代谢可能与XFS的病理生理学有关，该生物学途径可能用于治疗XFS患者组织类维生素a水平不足引起的纤维化改变。

Non-synonymous rare variants at *LOXL1*

A deep re-sequencing effort by the XFS Genetics Consortium revealed a spectrum of 63 rare (minor allele frequency <1%) non-synonymous variants scattered throughout *LOXL1* (Aung et al., 2017). The majority of these variants were significantly enriched in controls compared to cases, suggesting that rare variants within *LOXL1* could confer a protective effect against XFS. In particular, the variant p.Y407F (rs201011613:A>T) showed remarkably strong evidence of protection against XFS on a genome-wide level: the rare T allele was seen in just 2 out of 3803 Japanese patients, but in 68 out of 5338 controls, conferring a 25-fold resistance to disease. Overexpressing the variants in human lens epithelial cells showed that the rare T allele upregulated matrix components such as elastin and fibrillin compared to the wild-type A allele, suggesting that the protective effect could be due to regulation of elastin synthesis and downstream stabilization of the extracellular matrix (Fig. 3). Since, however, the majority of mutations, including p.Y407F, are located in the evolutionary conserved catalytic domain of LOXL1, a direct effect of the amino acid substitutions on enzyme function and catalytic activity could be presumed as well. In view of the fact that rare genetic variants can reveal profound insights into disease biology (Aung et al., 2018) further in-depth investigations into the functional significance of rare variants may improve our understanding on the causal relationship between LOXL1 and XFS development.

LOXL1 as a pathophysiologic risk factor of XFS and XFG

Functional significance of LOXL1

The *LOXL1* gene encodes for a 63-kDa protein with 574 amino acids (http://www.uniprot.org/uniprot/Q08397), which belongs to the lysyl oxidase (LOX) family of enzymes comprising five homologous members: LOX and LOX-like (LOXL) 1-4 (Barker et al., 2012) (Fig. 4A). These are secreted, copper-dependent amine oxidases, which oxidize primary amine substrates to reactive aldehydes. LOX and LOXL1 are structurally related with a high degree of homology and primarily catalyse the covalent cross-linking of collagen and elastin through oxidative deamination of lysine or hydroxylysine side chains, particularly during dynamic processes such as development, tissue injury, fibrosis, and cancer (Csiszar, 2001; Mäki, 2009). The LOXL1 isoform appears to be specifically required for cross-linking of soluble tropoelastin into insoluble elastin during formation and homeostasis of elastic fibers (Liu et al., 2004), although a role in collagen fiber cross-linking has been suggested as well (Kim et al., 1999). Alternative matrix-independent roles, such as suppression or promotion of tumor growth and progression, have also been proposed for LOXL1 (Wu et al. 2007; Zeltz et al. 2019).

Elastic fibers are composed of a central core of cross-linked elastin surrounded by a network of fibrillin-containing microfibrils (Fig. 4C). Elastic fiber formation involves the deposition of tropoelastin globules onto a microfibrillar scaffold for accretion to elastic fibers through cross-linking action of LOX and LOXL1 (Papke and Yanagisawa, 2014) (Fig. 4B). In addition to (tropo)elastin and fibrillin-1, more than 30 ancillary proteins, such as fibulins, emilins, microfibrillar-associated proteins and LTBPs, are involved in mediating important roles in elastic fiber assembly (https://www.wikipathways.org/index.php/Pathway:WP2666; Shin and Yanagisawa, 2019). Preferential binding between fibulin-4 and LOX and between fibulin-5 and LOXL1 has been shown to target both enzymes to elastic fiber assembly sites, to regulate oxidase activity, and to facilitate cross-linking actions (Liu et al., 2004; Papke and Yanagisawa, 2014). The cross-links endow elastic fibers with mechanical stability, high durability, and resistance to proteolytic degradation.

LOXL1 is synthesized as a preproenzyme, and, after cleavage of the N-terminal signal peptide, secreted as proenzyme into the extracellular space (Csiszar, 2001). For its classical function, the proenzyme binds to tropoelastin and fibulin-5 through its N-terminal propeptide domain, followed by proteolytic cleavage of the C-terminal catalytic domain by bone morphogenetic protein-1 (BMP-1) giving rise to the active enzyme (Borel et al., 2001; Thomassin et al., 2005; Northington, 2011). However, a complete picture of the proteases involved in activation, the location of the potential cleavage sites, and the biological functions of proenzyme and active form is still missing. Notably, the putative BMP-1 cleavage site at position 134 is localized in proximity to the XFS-associated coding variants at positions 141 (R141L) and 153 (G153D) (Borel et al., 2001) (Fig. 3A), supporting the notion that alterations in LOXL1 processing may contribute to pathogenesis of XFS (Sharma et al., 2016).

Implication of LOXL1 in connective tissue disorders

Functional elastic fibers confer elasticity and resilience to load-bearing connective tissues, such as blood vessel walls, lungs, skin, and female reproductive tissues, to dynamically accommodate the fluctuating and repetitive forces and structural distortions. Whereas elastic fiber formation (elastogenesis) is usually completed during postnatal development, elastic fiber remodeling (homeostasis), occurs in adult tissues in response to mechanical stress, and depends on a balance between enzymatic degradation and re-synthesis (Papke and Yanagisawa, 2014). Thus, elastic fiber remodeling requires appropriate synthesis of tropoelastin and ancillary proteins, proper cross-linking by LOXL1 and LOX, and regulation of elastolytic activities by endogenous inhibitors of elastases and matrix metalloproteinases (Liu et al., 2006). Mutations and polymorphisms in genes involved in elastogenesis as well as a range of environmental factors, such as UV light, can disturb this balance towards a gradual loss of functional elastic fibers.

Abundant evidence suggests a correlation between reduced levels of LOXL1 and defects in elastic fiber remodeling and repair, eventually leading to alterations in biomechanical properties and stability of connective tissues (Rodriguez et al., 2008; Mäki, 2009). Mice deficient in LOXL1 do not deposit normal elastic fibers and present many defects in elastic tissues resulting in lung emphysema, loose skin, vascular abnormalities including aortic aneurysms, skeletal malformations as well as pelvic floor dysfunction and pelvic organ prolapse in female animals (Liu et al., 2004, 2006; Lee et al., 2008; Remus et al. 2012; Alsofi et al., 2016). In line with this, decreased expression and/or activity of LOXL1 have been linked to both systemic and ocular connective tissue disorders in patients suffering from aortic aneurysms, venous insufficiency, pulmonary emphysema, cutis laxa, pelvic floor disorders, inguinal hernia, choroidal neovascularization and age-related macular degeneration (Yu et al., 2008; Pascual et al., 2008, 2009; Jung et al., 2009; Debret et al., 2010; Busnadiego et al., 2015; Besiktepe et al., 2017). Downregulation of LOXL1 during physiological aging (Pascual et al., 2008; Langton et al., 2012) may also contribute to an age-related decline in tissue elasticity.

大量证据表明，LOXL1水平的降低与弹性纤维重塑和修复缺陷之间存在相关性，最终导致结缔组织生物力学性能和稳定性的改变

在主动脉瘤、静脉供血不足、肺气肿、皮肤松弛、盆底疾病、腹股沟疝、脉络膜新生血管和年龄相关性黄斑变性患者中，LOXL1表达和/或活性的降低与系统性和眼部结缔组织疾病有关

In contrast, increased expression levels of LOXL1 have been linked to fibrotic disorders of liver, lungs, heart and other organs (Rodriguez et al., 2008; Tjin et al., 2017). In these conditions, LOXL1 becomes transiently activated at early stages of fibrogenesis or during remodeling of fibrotic tissue in association with newly synthesized extracellular matrix components (Decitre et al., 1998). In animal models of organ fibrosis, selective inhibition of LOXL1 prevented fibrosis or arrested disease progression by reducing TGF-ß activation, myofibroblast transformation, and extracellular matrix expression and cross-linking (Zhao et al., 2018; Bellaye et al., 2018).

在器官纤维化动物模型中，选择性抑制LOXL1可以通过减少TGF-ß激活、肌成纤维细胞转化、细胞外基质表达和交联来阻止纤维化或阻止疾病进展

Increased amine oxidase activity has been also suggested to contribute to increased matrix accumulation and cross-linking in the trabecular meshwork of glaucoma patients (Sethi et al., 2011). All LOX isoforms including LOXL1 were expressed in cultured human trabecular meshwork cells and were upregulated by TGF-ß via canonical Smad and non-Smad signaling pathways. TGF-ß-mediated elevation of intraocular pressure (IOP) and increased outflow resistance in glaucoma patients has, therefore, been attributed to excessive cross-linking processes of matrix molecules, which is consistent with increased trabecular meshwork stiffness in glaucomatous eyes compared with non-glaucomatous control eyes (Last et al., 2011).

Implication of LOXL1 in XFS pathophysiology

LOXL1 is ubiquitously expressed in all ocular tissues of the anterior and posterior segment (Hewitt et al., 2008; Schlötzer-Schrehardt et al., 2008). Based on its functional implications in both elastotic-degenerative and fibrotic diseases, LOXL1 dysregulation has been also

proposed to play a dual role in XFS pathogenesis, both in the fibrotic process of XFM accumulation and in structural weakening of elastic connective tissues (Schlötzer-Schrehardt, 2009; Whigham & Allingham 2011). Whereas other lysyl oxidase isoforms were not differentially expressed in tissues of XFS/XFG patients, dysregulation of LOXL1 expression has been shown to be a hallmark of the disease and to depend on tissue type and disease stage.

In tissues of the anterior eye segment, LOXL1 expression is significantly increased in early stages of the disease, paralleled by an upregulation of elastic fiber components, obviously to participate in the early cell-associated processes of abnormal matrix formation and cross-linking (Schlötzer-Schrehardt et al., 2008). Pro-fibrotic factors including growth factors (TGF-ß1), pro-inflammatory cytokines (IL-6), and UV light have been suggested to drive a fibrotic stress response in the anterior segment (Zenkel et al., 2011). Using immunohistochemistry, direct mass spectrometry and atomic force microscopy, LOXL1 has been identified as a major and integral component of aggregated XFM deposits (Schlötzer-Schrehardt et al., 2008; Sharma et al., 2009; Creasey et al., 2011; Ronci et al., 2013), where it co-localized with (tropo)elastin, fibrillin-1, LTBP-1/2 and fibulin-4 on the typical exfoliation fibrils (Fig. 5). These observations suggest a distinct role of LOXL1 in the formation and cross-linking of XFM fibrils, their aggregation and stable accumulation, and resistance to proteolytic degradation.

Aggregate formation of LOXL1 within XFM deposits may also contribute to this fibrotic process, since LOXL1 has been characterized as an aggregation-prone protein owing to its intrinsically disordered domain structure of the entire N-terminus with a peak around amino acid 153 (Bernstein et al., 2018). Slight disturbances in LOXL1 protein folding and solubility, caused by failure of protein quality control machinery, accelerated senescence, compromised cellular stress responses, and/or expression of mutant protein variants, may increase the risk for formation of protein aggregates in the extracellular space. In support of this novel concept of XFS as a LOXL1 aggregopathy, Tenon capsule fibroblasts derived from XFG patients displayed many signs observed in other protein aggregation diseases, including abnormalities in lysosomal and autophagosomal arrangement, microtubule organization, mitochondrial function and autophagic clearance (Want et al., 2016; Wolosin et al., 2018). Inhibition of the lysosome/autophagy pathway led to an increase in LOXL1 protein expression in XFS cells indicating that LOXL1 is in fact directed to the autophagy pathway. It has therefore been speculated that accumulation of misfolded LOXL1 protein caused by LOXL1 risk variants in combination with age-related defects in protein degradation by the lysosomal and autophagy pathway are centrally involved in XFM formation (Bernstein et al., 2018). Moreover, there is also evidence for impaired proteasome function in tissues of XFS patients, as reflected by reduced expression levels of the proteasome maturation protein

POMP and the ubiquitin-conjugating enzymes UBE2A and UBE2B (Zenkel et al., 2007; Aung et al., 2017) as well as reduced proteasome activity (Hayat et al., 2019), which may contribute to accumulation of misfolded protein aggregates. Finally, defective proteins derived from improper synthesis can trigger an unfolded protein response in the endoplasmic reticulum, a cellular stress response intended to degrade misfolded proteins and to promote correct protein folding. Increased expression of endoplasmic reticulum-related stress markers, indicating unfolded protein response activation, has been also shown in lens epithelial cells of XFS patients (Hayat et al., 2019).

Altogether, existing data support the notion that a proteostasis imbalance involving LOXL1 plays a critical role in abnormal XFM formation and aggregation. The gradual build-up of XFM in anterior segment tissues predisposes to a broad spectrum of ocular manifestations with IOP elevation and glaucoma development representing the most serious complications (Ritch and Schlötzer-Schrehardt, 2001).

Implication of LOXL1 in XFG development

In manifest stages of XFS, LOXL1 expression is significantly decreased below normal homeostatic levels in all anterior segment tissues, i.e. cornea, trabecular meshwork, iris and ciliary body, in spite of ongoing fibrosis (Schlötzer-Schrehardt et al., 2008; Khan et al., 2010; Pasutto et al., 2017). The reason for these differential expression patterns in early and late stages of the disease is not known, but may involve a counterregulation of LOXL1 in response to the steady build-up of extracellular aggregates and/or a gradual prevalence of (epi)genetic mechanisms of LOXL1 suppression. The resulting deficiency of LOXL1 protein, which appears to be all trapped within XFM deposits, may adversely affect the structural stability of elastin-rich tissues of the anterior segment, particularly the trabecular meshwork and the zonules. In the trabecular meshwork, a well-developed elastic fiber system is present in the juxtacanalicular tissue of inner and outer walls of Schlemm's canal, which plays a significant role in maintaining structural integrity and flexibility of Schlemm's canal in outflow regulation (Hann and Fautsch, 2011). The zonular fibers represent extensible bundles of fibrillin-rich microfibrils, not associated with elastin, which are obviously cross-linked by remarkable amounts of LOXL1 (De Maria et al., 2017). Thus it is conceivable that a shortage of LOXL1 and subsequent degenerative changes of elastic (micro)fibrils may contribute to known ocular complications in XFS patients, i.e., disorganization of the juxtacanalicular tissue and collapse of Schlemm's canal predisposing to XFG as well as progressive disintegration of the zonular fibers predisposing to lens subluxation (Ritch & Schlötzer-Schrehardt, 2001; Schlötzer-Schrehardt and Naumann, 2006).

In contrast to anterior segment tissues, most tissues of the posterior segment, including choroid, retina and sclera, displayed no differential expression patterns of LOXL1 in XFS,

with exception of the lamina cribrosa, which revealed a consistent downregulation of LOXL1 and elastic proteins throughout early and late stages of the disease (Schlötzer-Schrehardt et al., 2012). The cause of this tissue-specific downregulation is currently not known, but may be related to the constant mechanical load acting on this structure. The XFS-specific LOXL1 deficiency in the lamina cribrosa has been associated with a fragmentation of elastic fiber networks observed in the laminar beams and peripapillary sclera (Fig. 6) as well as with a significantly reduced tissue stiffness (Young's modulus) in XFS eyes compared to age-matched control eyes (Braunsmann et al., 2012). Mechanistically, it may be speculated that the primary LOXL1 deficiency can provoke increased proteolytic degradation and impaired remodelling of elastic fibers upon increased mechanical stress caused by elevated levels and fluctuations of IOP. Consistently, downregulation of LOXL1 has been shown to interfere with elastic fiber assembly by optic nerve head astrocytes in vitro (Schlötzer-Schrehardt et al., 2012).

Based on these observations, it has been suggested that these biomechanical alterations in XFS eyes render the lamina cribrosa more vulnerable to IOP-induced optic nerve damage and that LOXL1 deficiency is a major susceptibility factor for a XFS-specific risk of glaucoma development. Progressive thinning of the lamina cribrosa, occurring early in XFS patients, has been also suggested to put XFS patients at a higher risk of glaucomatous damage and faster progression compared to pimary open-angle glaucoma patients (Kim et al., 2013; Moghimi et al., 2018).

Implication of LOXL1 in XFS-associated systemic disease

XFS-associated systemic diseases may be also caused by dysfunctional elastin metabolism and biomechanical impairment of elastin-rich connective tissues. Using a robust medical dataset, the Utah Population Database, XFS has been shown to be associated with pelvic organ prolapse (Wirostko et al., 2016), inguinal hernias (Besch et al., 2018), and chronic obstructive pulmonary disease (Wirostko et al., 2018). XFS has been also associated with cardiovascular diseases, including arterial hypertension, arrhythmia, cardiomyopathy, coronary artery disease, and particularly with aneurysms of the abdominal aorta, a potentially life-threatening complication (Schumacher et al., 2001; French et al., 2012; Katsi et al., 2013). Aortic wall specimens derived from XFS patients showed significantly reduced expression levels of LOXL1 compared with control specimens (Pasutto et al., 2017). Accordingly, elastic lamellae in walls of elastic arteries, such as aorta, and larger veins, such as saphenous vein, appeared discontinuous, fragmented and rarefied (Fig. 6). Thus, a common mechanism underlying these systemic associations may be a combination of increased mechanical stress and weakened elastic tissues due to LOXL1 deficiency and inadequate elastin repair processes.

Potential mechanisms of LOXL1 dysregulation in XFS and XFG

Precisely regulated expression and activity of LOX family oxidases are prerequisites to their critical functions in connective tissue homeostasis. Gene expression is generally regulated at three levels: 1. transcriptional level (e.g. epigenetic modifications, transcription factor binding), 2. post-transcriptional level (e.g. alternative pre-mRNA splicing, RNA processing), and 3. post-translational level (e.g. post-translational modifications, proteolytic processing). All of these mechanisms have been shown to be involved in *LOXL1* regulation and to be modulated in response to external or environmental factors.

On the transcriptional level, hypermethylation of the *LOXL1* promoter by DNA methyltransferase (DNMT) has been shown to downregulate or even silence *LOXL1* expression in human skin fibroblasts in cutis laxa and during ageing as well as in bladder cancer cells (Wu et al., 2007; Debret et al., 2010; Moulin et al., 2017). This mechanism may be responsible for the marked downregulation of LOXL1 in elastic tissues during physiological aging (Pascual et al., 2008; Langton et al., 2012), and appears to contribute to reduced LOXL1 expression levels in ocular tissues of manifest XFS patients (Ye et al., 2015). Notably, DNA hypermethylation can be reversed through the use of DNMT inhibitors such as 5-aza-dC, leading to normalization of LOXL1 expression levels (Debret et al., 2010). Allele-specific effects on *LOXL1* gene regulation have been shown to be mediated by differential binding of the transcription factor RXRα and by alternative pre-mRNA splicing coupled to NMD, thereby contributing to reduced expression levels of LOXL1 in tissues of XFS patients (Pasutto et al., 2017). LOXL1 is predicted to undergo post-translational modifications such as disulfide bond formation (http://www.uniprot.org/uniprot/Q08397). Regulation of LOXL1 on the post-translational level also requires proteolytic processing by BMP-1 and other proteases for enzymatic activation (Borel et al., 2001), which may be also influenced by XFS-associated coding variants at *LOXL1* (Sharma et al., 2016). Altogether, available data suggests that epigenetic modification and sequence variation at *LOXL1,* increasing the risk of XFS, are associated with reduced LOXL1 expression at baseline in tissues of risk allele carriers, which can, however, be stimulated in the presence of pro-fibrotic triggering factors.

One of the best established pro-fibrotic factors stimulating LOXL1 expression and activity is TGF-ß1, which is known to have a key role in the pathophysiology of XFS/XFG (Schlötzer-Schrehardt, 2018). This growth factor induces LOXL1 expression, secretion and activity in ocular and non-ocular cell types through Smad and non-Smad signalling pathways (Zenkel et al., 2011; Sethi et al., 2011; Berner et a., 2017; Ma et al., 2018). Conversely, silencing of LOXL1 suppressed TGFß-induced fibrogenesis by inhibiting Smad2/3 phosphorylation (Ma et al., 2018; Bellaye et al., 2018). These observations suggest a regulatory feed-back loop

between TGF-ß signalling and LOXL1 activity, which may play an important role in trabecular meshwork tissue homeostasis (Sethi et al., 2011) and in the pathophysiology of XFS and XFG (Liravi et al., 2016, ARVO Abstract 4685). In addition to TGF-ß1, external stress factors including oxidative stress, UV light, hypoxia, and mechanical stress, have been shown to upregulate expression of LOXL1 in Tenon's capsule fibroblasts and trabecular meshwork cells *in vitro* (Zenkel et al., 2011; Berner et al., 2017). Induction of LOXL1 was even found to correlate with the *LOXL1* haplotype formed by the R141L and G153D variants (Zenkel et al., 2011). LOXL1 expression can be also regulated by dietary factors, with folate and vitamin D3 upregulating and caffeine and retinoic acid downregulating its expression levels in Tenon's capsule fibroblasts and trabecular meshwork cells (Pasutto et al., 2017; Berner et al., 2017; Zenkel et al., 2018, ARVO Abstract 3513). Finally, LOXL1 expression and activity appears to be influenced by hormones such as estradiol (Zong et al., 2015). These findings provide a potential link between epidemiologic risk factors for XFS incidence and prevalence and its underlying pathophysiology involving LOXL1 (Aboobakar et al., 2017; Pasquale et al., 2018).

***LOXL1*-based models for XFS/XFG**

In vivo models

Ocular abnormalities of *Loxl1* knockout mice include structural defects of Bruch's membrane leading to increased choroidal neovascularization, cataract development, blood-aqueous barrier disruption, and markedly decreased elastic fibers in the iris, ciliary body and chamber angle (Yu et al., 2008; Wiggs et al., 2014). A transgenic mouse model overexpressing the mouse ortholog of *Loxl1* in the lens displayed considerable accumulations of insoluble LOXL1 protein aggregates within the rough endoplasmic reticulum of lens fiber cells, which were not seen in wildtype lenses (Zadravec et al., 2019). This study confirmed a strong tendency of LOXL1 to form intracellular aggregates, which may be caused by misfolded protein conformation, high protein concentrations exceeding solubility limit, or lack of molecular chaperones required for LOXL1 solubility and proper secretion. Both *Loxl1* knockdown or overexpression models could, however, not replicate the formation of abnormal extracellular matrix deposits nor develop a lasting increase in IOP and glaucoma.

In contrast, mice transiently overexpressing Wnt5a in ocular tissues accumulated microfibrillar material positive for fibrillin and LOXL1 on surfaces of anterior segment structures. They also displayed certain features characteristic of XFS in patients, i.e. saw-tooth appearance and disrupted basement membrane of the posterior iris pigment epithelium, iris stromal atrophy and disorganized ciliary zonules (Yuan et al., 2019).

Overall, none of the currently available *LOXL1*-based mouse models recapitulates the key features of human XFS, i.e. XFM deposition, elevated IOP and glaucoma (Anderson et al., 2018). These observations suggest that mechanisms other than excess or lack of LOXL1

are necessary to mimic the XFS/XFG phenotype. Further studies are needed to examine a potential relationship between Wnt signaling, LOXL1 metabolism and XFS pathogenesis.

In vitro models

Fibroblasts are an easy-to-handle and useful model system to analyse the effects of LOXL1 overexpression or deficiency in a more controlled *in vitro* setting, because these cells secrete and assemble an abundant extracellular matrix including an extensive elastic fiber network stabilized by LOXL1 (Zenkel et al., 2011; Sommer et al., 2013). A rat fetal lung fibroblast cell line (RFL-6) has been previously used to overexpress LOXL1 haplotype variants, formed by the two common coding variants, which were secreted and accumulated in the extracellular space (Sharma et al., 2016). We have demonstrated the suitability and efficiency of stably transfected primary human Tenon's capsule fibroblasts as a model system to analyze synthesis, secretion, and targeting of LOXL1 to the extracellular matrix (Krysta et al., ARVO 2012, Abstract #3852) (Fig. 7). Upon stimulation with TGF-ß1, fibroblasts secreted biologically active LOXL1 and assembled an elastic microfibrillar network with deposition of cross-linked insoluble elastin after 3 weeks in culture. This cell-based assay may represent a useful strategy to unravel critical functional differences of LOXL1 variants in extracellular matrix formation and aggregation in response to environmental factors, and may reveal potential downstream effects of LOXL1 overexpression and silencing on gene expression patterns. In fact, mouse lung fibroblasts showed apparent changes in gene expression patterns after siRNA-mediated knockdown of Loxl1 (Mizikova et al., 2017). This study provided evidence that lysyl oxidases including Loxl1 can impact on expression levels of matrix-related genes, particularly elastin, collagen, and matrix metalloproteinases.

Conclusion / future directions

To date, *LOXL1* is still recognized as the major genetic effect locus in XFS and XFG in all populations worldwide. However, the functional mechanisms by which the associated variants confer risk for disease still remain unknown. There is only one single functional variant downstream of *LOXL1* that is consistently shared among all populations, which evidently impacts on the retinoic acid signalling pathway warranting further exploration. Functional analysis of rare variants in *LOXL1* that are also conserved across ethnic groups would be an important next step to reveal insights into disease biology. Also, transcriptome analyses on tissue and single cell level are needed to identify additional biologically relevant genes and pathways and their potential relationship to LOXL1.

然而，相关变异带来疾病风险的功能机制仍然未知。在所有种群中，只有一个LOXL1下游的单一功能变异是一致的，这显然影响维甲酸信号通路，值得进一步研究。对少数民族群体中也具有保守性的LOXL1的罕见变异进行功能分析，将是揭示疾病生物学的重要下一步。此外，还需要在组织和单细胞水平上进行转录组分析，以确定其他生物学上相关的基因和途径，以及它们与LOXL1的潜在关系。

In spite of the unsolved complexity of its genetic architecture, LOXL1 remains a promising candidate molecule to elucidate the pathophysiology of the abnormal matrix process on the one hand and elastic fiber remodeling failure on the other hand. There are

still many open questions concerning its regulation in a time- and context-specific manner, maintenance of proteostasis, intra- and extracellular processing, interaction with other matrix components as well as with other genetic and environmental factors, which may be addressed in appropriate *in vitro* models.

Normalizing dysregulated LOXL1 expression in a tissue-specific manner appears as a potential target for intervention, but it remains to be determined whether stimulation or inhibition of LOXL1 should be prioritized. Activation of *LOXL1* promoter activity, e.g. by reversal of methylation, could serve as a strategy to stimulate LOXL1 expression in load-bearing elastic tissues. Plant compounds, such as emodin, dill extract, *Origanum* extract or *Hamamelis* extract, have been used to enhance LOXL1 expression and activity and thereby stabilize extracellular matrix (Cenizo et al., 2006; Jian et al., 2014; Moulin et al., 2017; Pain et al., 2018). The irreversible lysyl oxidase inhibitor, β-aminopropionitrile (BAPN), is currently used as an anti-fibrotic strategy in animal models, but unwanted side effects including arterial aneurysm and dissection may occur (Mäki, 2009). To the best of our knowledge, there is no specific inhibitor of LOXL1 commercially available, which could be applied in tissues affected by XFM accumulation. However, apart from directly tackling LOXL1 expression, regulatory pathways may be also targeted to treat XFS-specific fibrotic alterations, e.g. caused by aberrant TGF-ß1 and retinoic acid signaling.

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Figure legends

Fig. 1. Manhattan plot of the results from the GWAS comprising 13,620 XFS cases and 109,837 controls. SNP markers at seven independent loci surpass genome-wide significance (defined as $p \leq 5x10^{-8}$) (Figure from Aung et al. (2017)).

Fig. 2. A. Forest plot showing genome-wide significant allele effect reversal observed for the classical *LOXL1* common polymorphisms rs3825942:G>A and rs1048661:G>T. **B.** Forest plot showing significant consistent association for the variant rs7173049:A>G. Squares represent the estimated odds ratios per-copy of the risk allele for each country collection and in the meta-analysis.The area of the square is scaled in proportion to the variance of the estimate, and the horizontal lines represent the 95% confidence intervals (Figure adapted from Aung et al. (2017)).

Fig. 3. A. Schematic illustration of the LOXL1 protein with the signal peptide (red), the pro-peptide (grey) and the lysyl oxidase domain (blue) indicating positions of BMP-1 cleavage sites, common variants R141L and G153D, and the rare variant Y407F. **B.** Immunofluorescent staining of HA-tagged LOXL1 haplotype variants formed by the two common and the rare variant, overexpressed in human lens epithelial cells, and labelled with anti-HA for detection of LOXL1 (red), elastin (green) and DAPI (blue) (Figure adapted from Aung et al. (2017)).

Fig. 4. A. Structural homology of the family of lysyl oxidase enzymes (LOX, LOXL1-4). All LOX proteins are highly conserved in their C-terminal copper-binding catalytic domain, which is responsible for the extracellular conversion of primary amines of lysine side chains into reactive aldehydes, and are variable in their N-terminal regions, which are important for substrate binding and enzyme maturation. Both LOX and LOXL1 contain pro-sequences, which are necessary for deposition onto elastic microfibrils. These sequences are cleaved to generate catalytically active enzymes. The black square indicates the cytokine receptor-like (CRL) domain. LTQ: lysyl-tyrosyl-quinone co-factor (Figure from Barker et al. (2012)). **B.** Simplistic model illustrating the function of LOXL1 during elastogenesis. The N-terminal region of LOXL1 binds to the C-terminal domain of fibulin-5 to activate tropoelastin by converting it to its lysyl-deaminated form. Subsequently, covalent cross-linking of lysyl-deaminated tropoelastin occurs spontaneously to form mature, cross-linked elastin polymers (Figure from Northington (2011)). **C.** Immunogold localization of LOXL1 (20 nm gold particles) and elastin (10 nm gold particles) on an elastic fiber (ec: elastin core; mf: microfibrils).

Fig. 5. Immunofluorescence labeling of elastic proteins and LOXL1 in anterior segment tissues of an XFS eye showing co-localization within exfoliation material (XFM) deposits. Positive signals are indicated by red (LOXL1) or green (LTBP-1, tropoelastin) fluorescence in

contrast to blue fluorescence of nuclei stained with 4,′6-diamidino-2-phenylindole (ABL, anterior border layer; BV, blood vessel; CE, ciliary epithelium).

Fig. 6. Light microscopic appearance of representative areas of the lamina cribrosa, aortic wall, and wall of saphenous vein analyzed for purple-stained elastin fibers using Weigert's resorcin-fuchsin in normal and XFS tissue specimens. Inserts show details of elastic lamellae in the tunica media; arrows point to ruptures in the internal elastic lamina.

Fig. 7. Cell culture model using primary human Tenon's capsule fibroblasts stably transfected with pCMV6 vectors containing full-length LOXL1. **A.** Immunofluorescence labeling of LOXL1 (green fluorescence) and LTBP-2 (red fluorescence) showing assembly of an elastic microfibrillar network after 14 days in culture. **B.** Immunofluorescence labeling of LOXL1 (green fluorescence) and elastin (red fluorescence) showing co-localization along elastic fibers. **C.** Transmission electron microscopic immunogold labeling of LOXL1 (10 nm gold particles) and elastin (20 nm gold particles) showing elastogenesis in the cell periphery.

Fig. 1.

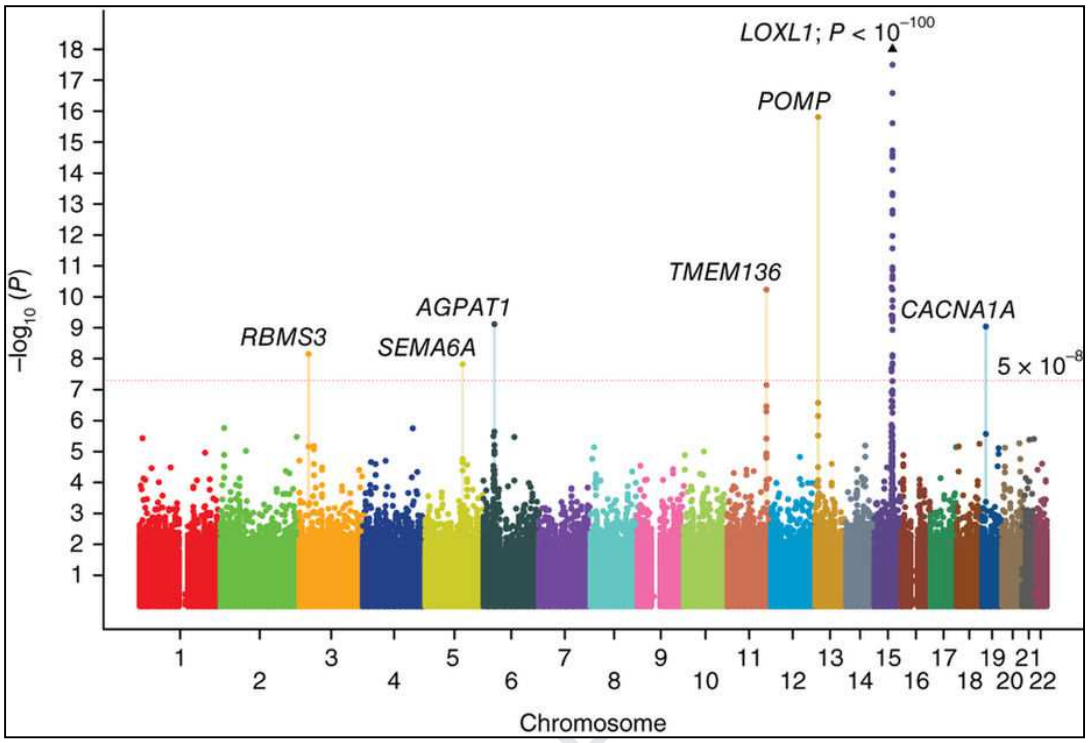
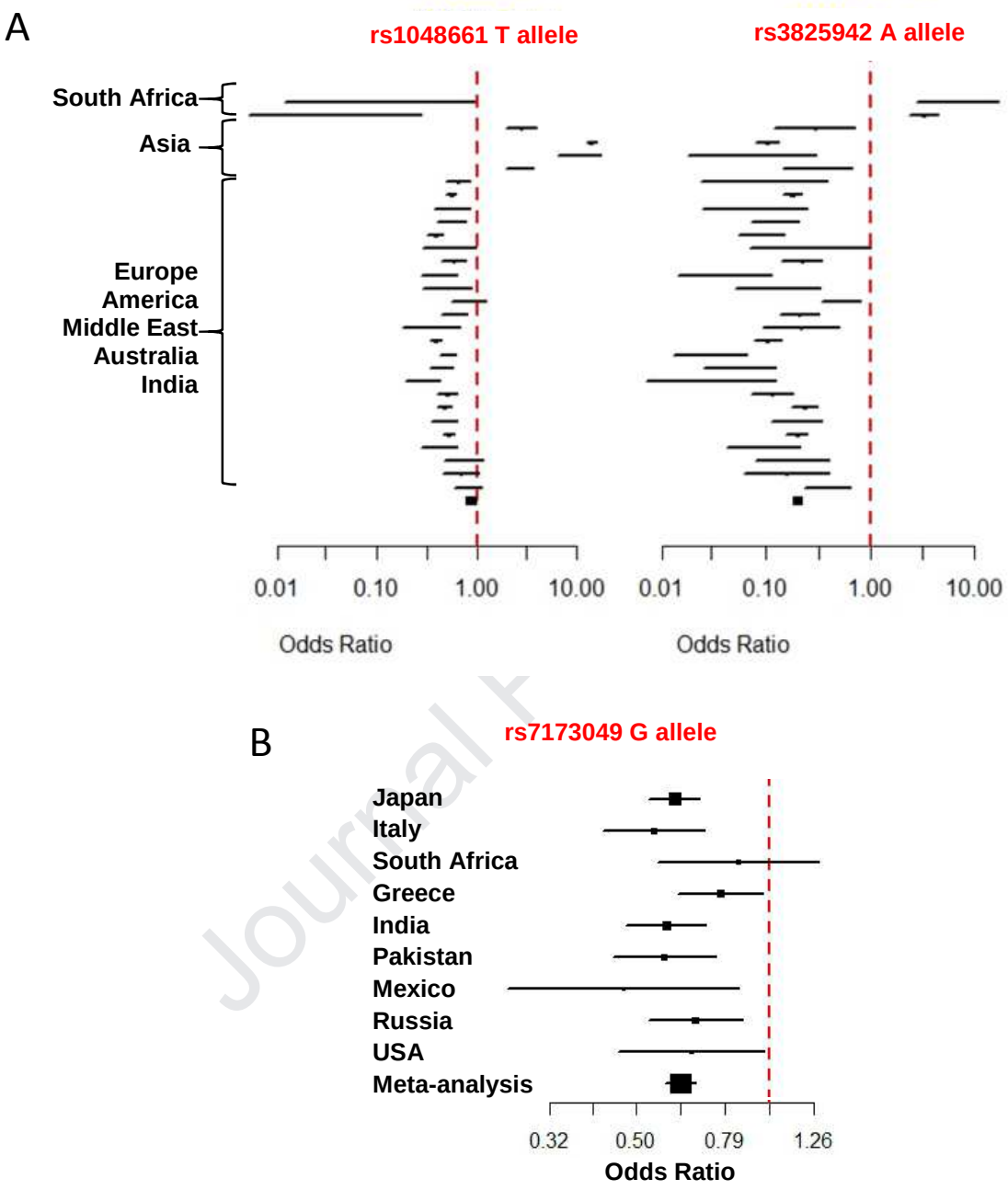


Fig. 2.



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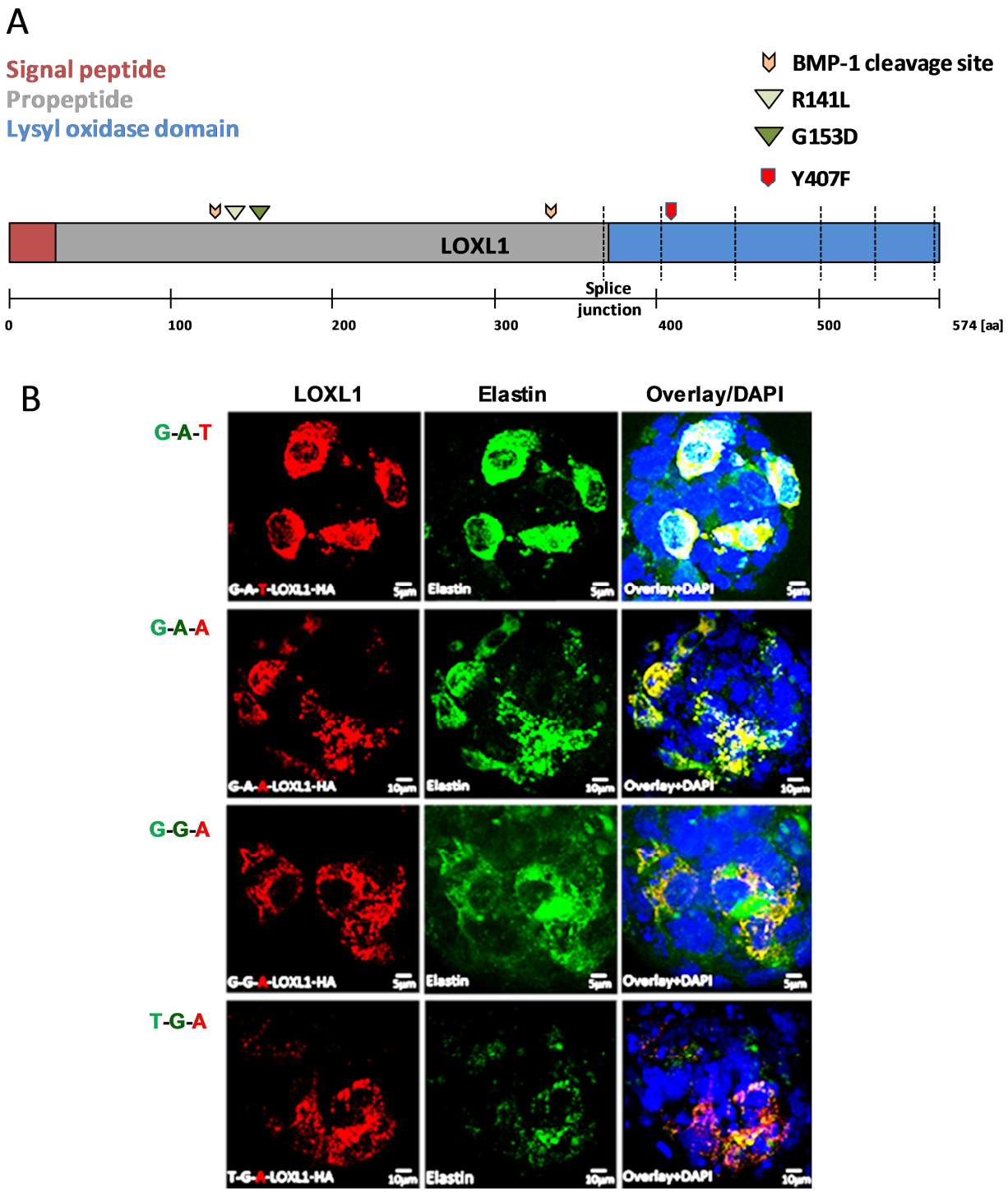

A
Signal peptide
Propeptide
Lysyl oxidase domain
BMP-1 cleavage site
R141L
G153D
Y407F
LOXL1
Splice junction
0
100
200
300
400
500
574 [aa]
B
LOXL1
Elastin
Overlay/DAPI
G-A-T
G-A-A
G-G-A
T-G-A
G-A-T-LOXL1-HA
G-A-A-LOXL1-HA
G-G-A-LOXL1-HA
T-G-A-LOXL1-HA
Elastin
Overlay+DAPI
5μm
10μm

Fig. 4.

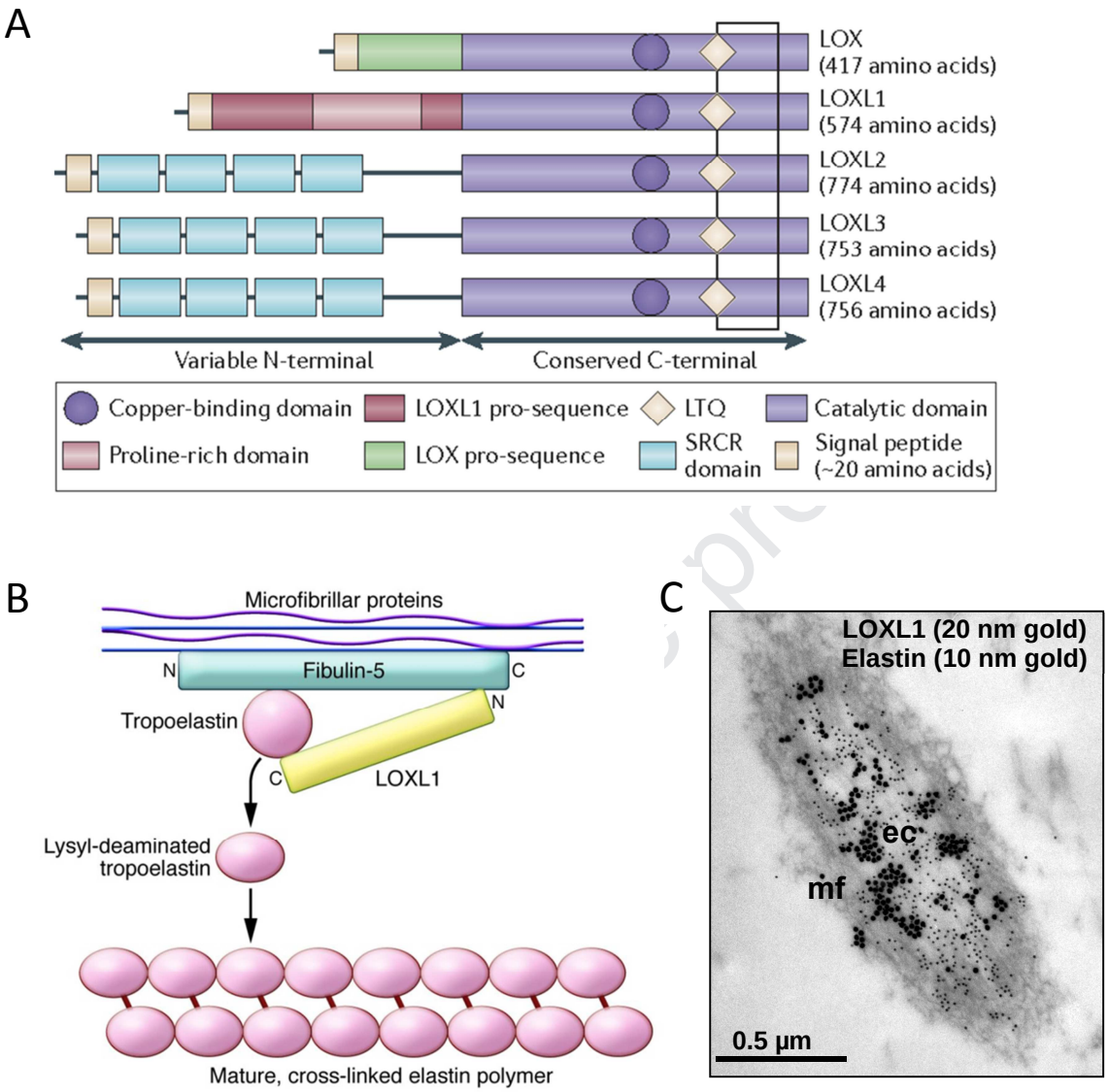
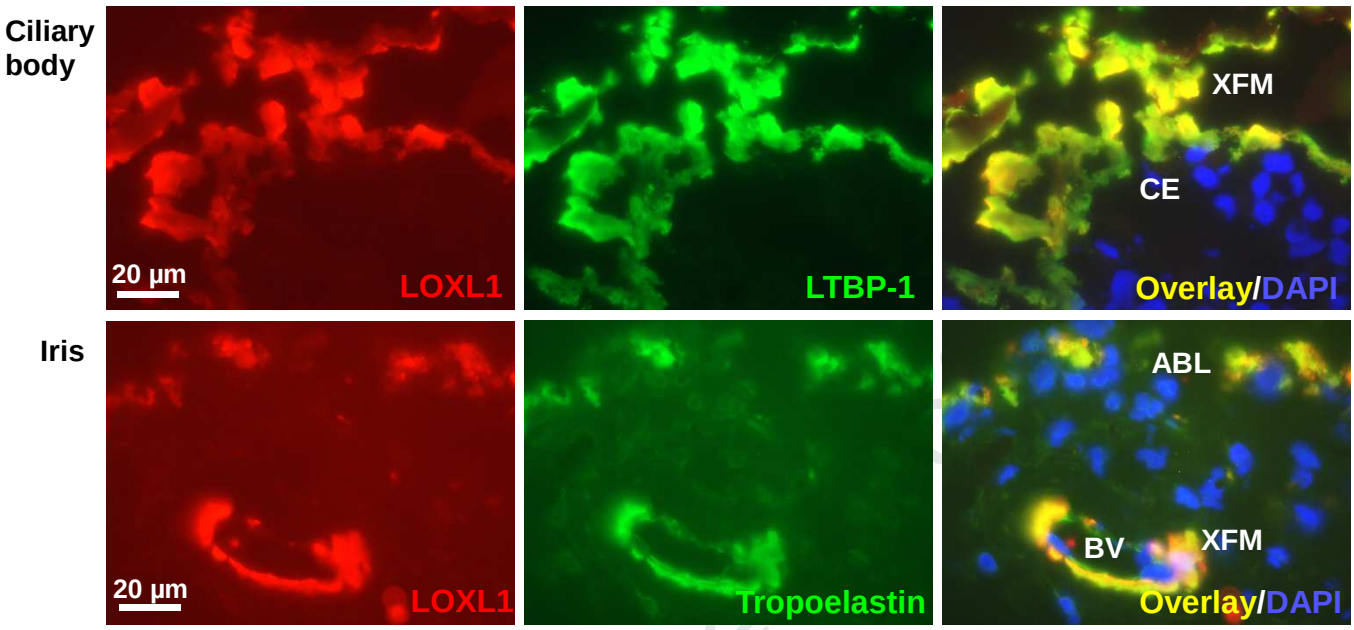

A
LOX
(417 amino acids)
LOXL1
(574 amino acids)
LOXL2
(774 amino acids)
LOXL3
(753 amino acids)
LOXL4
(756 amino acids)
Variable N-terminal
Conserved C-terminal
Copper-binding domain
LOXL1 pro-sequence
LTQ
Catalytic domain
Proline-rich domain
LOX pro-sequence
SRCR domain
Signal peptide (~20 amino acids)
B
Microfibrillar proteins
N
Fibulin-5
C
Tropoelastin
N
C
LOXL1
Lysyl-deaminated tropoelastin
Mature, cross-linked elastin polymer
C
LOXL1 (20 nm gold)
Elastin (10 nm gold)
ec
mf
0.5 µm

Fig. 5.



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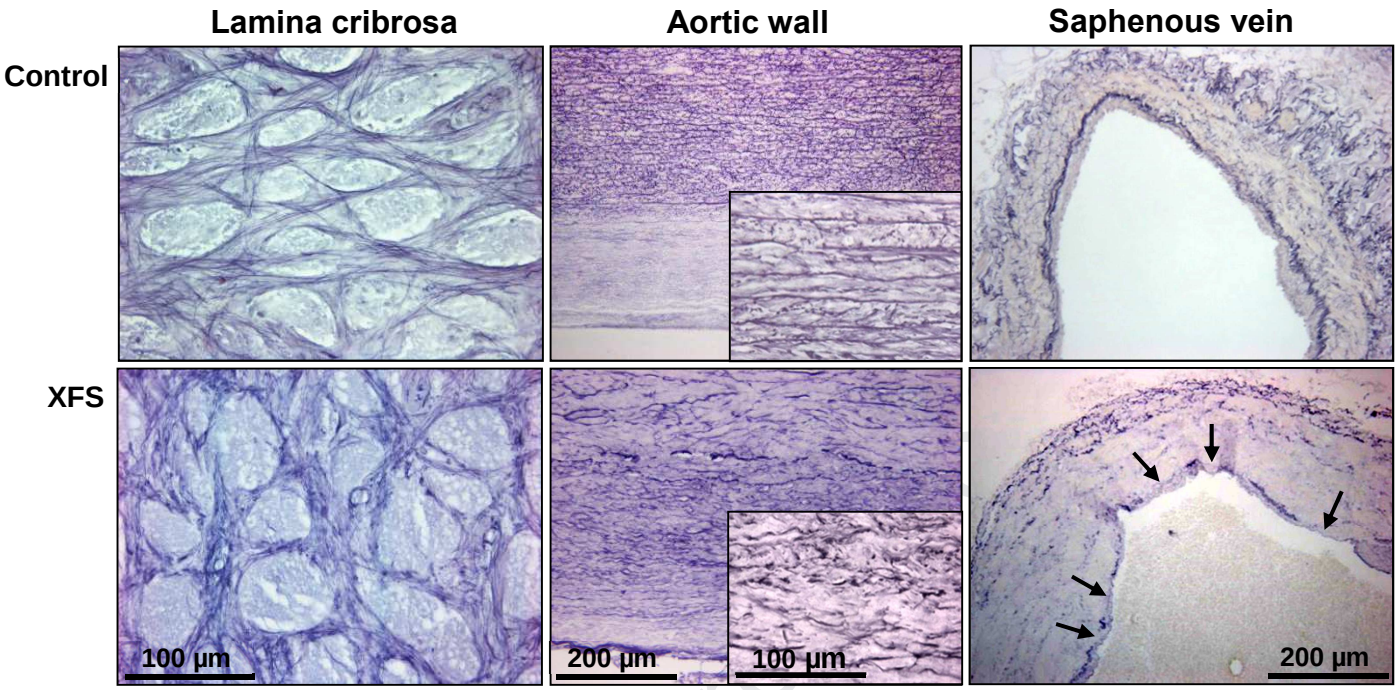

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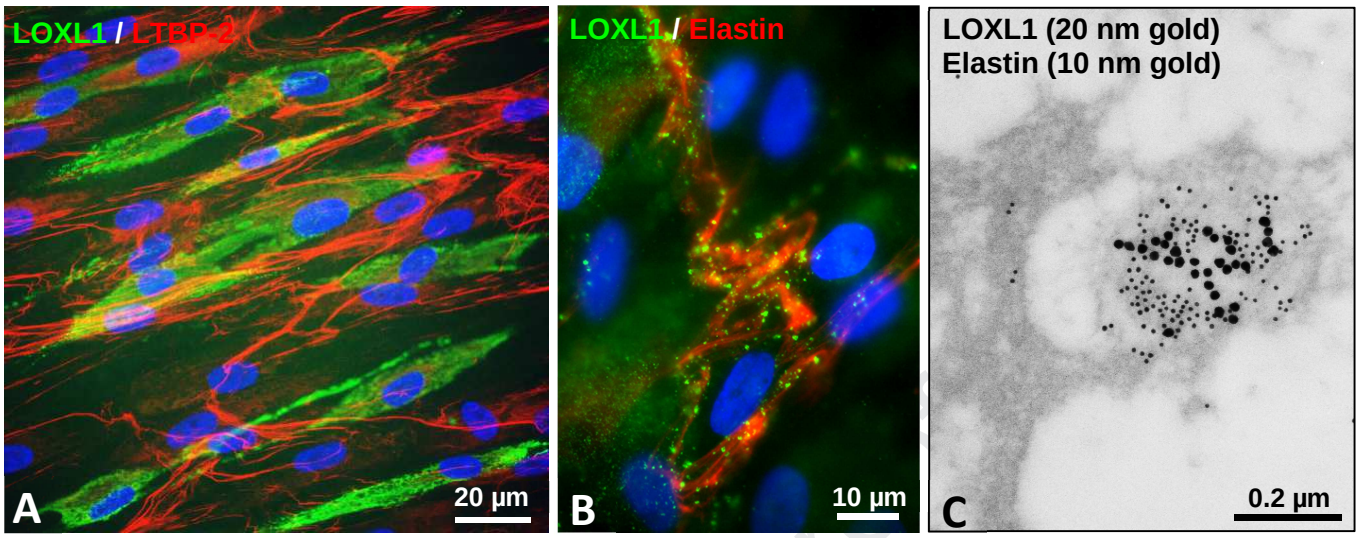

Highlights

This review provides an overview on the current knowledge about the role of LOXL1 in the etiology and pathophysiology of XFS and XFG. It covers

- the known genetic associations at the *LOXL1* locus
- potential mechanisms of gene regulation
- implications of LOXL1 in XFS-associated fibrosis and connective tissue homeostasis
- its role in the development of glaucoma and associated systemic diseases
- currently available LOXL1-based *in vivo* and *in vitro* models
- gaps in knowledge and suggestions of potential areas for future research.