Posttranscriptional Regulation of LOXL1 Expression Via Alternative Splicing and Nonsense-Mediated mRNA Decay as an Adaptive Stress Response

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Purpose. Alternative mRNA splicing coupled to nonsense-mediated decay (NMD) is a common mRNA surveillance pathway also known to dynamically modulate gene expression in response to cellular stress. Here, we investigated the involvement of this pathway in the regulation of lysyl oxidase-like 1 (LOXL1) expression in response to pseudoexfoliation (PEX)-associated pathophysiologic factors.

Methods. Transcript levels of LOXL1 isoforms were determined in ocular tissues obtained from donor eyes without and with PEX syndrome. Pseudoexfoliation-relevant cell types, including human Tenon's capsule fibroblasts (hTCF) and trabecular meshwork cells (hTMC), were exposed to puromycin, caffeine, TGF-β1, homocysteine, IL-6, retinoic acid, UV-B radiation, oxidative stress, and mechanical stress for up to 48 hours. Western blot analysis was carried out using antibodies against LOXL1, (phosphorylated-) eukaryotic initiation factor 2-α (eIF2-α), and regulator of nonsense transcripts 2 (UPF2). RNA interference was used to knockdown UPF1-3 and Serine/threonine-protein kinase (SMG1).

Results. Constitutive expression of wild-type LOXL1 and alternatively spliced LOXL1-a transcripts was detected in all ocular tissues showing highest levels in trabecular meshwork and differential expression between PEX and control specimens. LOXL1-a transcripts were upregulated in hTCF and hTMC by NMD inhibitors puromycin and caffeine (≥6-fold; P < 0.01) or after knockdown of NMD core factors (≥2-fold; P < 0.05), whereas mRNA and protein levels of LOXL1 were reduced (<0.8 fold; P < 0.05). Exposure of cells to various PEX-associated (stress) factors, including TGF-β1, UV-B light, oxidative stress, mechanical stress, and retinoic acid enhanced LOXL1-a transcript levels (≥1.5-fold; P < 0.05), while partially downregulating LOXL1 levels (≤0.7-fold; P < 0.05). Stress-induced inhibition of NMD was dependent on phosphorylation of eIF2α.

Conclusions. These findings provide evidence for a functional role of alternative splicing coupled to NMD in the posttranscriptional regulation of LOXL1 gene expression and suggest this mechanism to represent a dynamic mode of adapting LOXL1 expression to PEX-associated environmental and nutritional cues.

Keywords: pseudoexfoliation syndrome, glaucoma, LOXL1, regulation, alternative splicing, nonsense-mediated decay, oxidative stress, caffeine, retinoic acid, trabecular meshwork.

One of the most common causes of open-angle glaucoma is the pseudoexfoliation (PEX) syndrome, which is characterized by pathologic deposition of an abnormally crosslinked, extracellular, fibrillar material in many intra- and extracocular tissues including the trabecular meshwork, causing increased aqueous humor outflow resistance and elevated IOP. Pseudoexfoliation syndrome is a late-onset complex disease, involving the combined effects of both genetic and environmental factors. Lysyl oxidase-like 1 (LOXL1) has been identified as a principal genetic risk factor for PEX syndrome/glaucoma through genome-wide association and replication studies in multiple populations worldwide. Although the effect sizes of PEX-associated LOXL1 risk variants were found to be unusually high (odds ratio > 10), they also commonly occurred (>80%) in healthy controls and showed significant reversal in certain populations on a genome-wide level, suggesting that they may have only limited biological significance for PEX pathogenesis. Thus, the existing evidence to date suggests that additional PEX-associated genetic loci.
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IOVS | November 2017 | Vol. 58 | No. 13 | 5931

environmental determinants, and gene-environment interac-
tions remain to be identified.

In spite of these unresolved issues, pathogenic analyses
have confirmed a direct and fundamental role for LOXL1 in the
pathophysiology of PEX syndrome and its associated glauco-
a. LOXL1 encodes a member of the lysyl oxidase family of
enzymes (LOX, LOXL1–4), which catalyze the generation of
lysine-derived cross-links in extracellular matrix molecules,
such as collagen and elastin. 12 A major function of LOXL1 is
crosslinking of tropoelastin monomers into elastin polymers
during the formation and maintenance of elastic fibers, with
the crosslinks providing tensile strength and elasticity for
connective tissues required to sustain mechanical load. 13 Thus,
dysregulated expression of this key enzyme has been linked to
both fibrotic and elastotic-degenerative connective tissue
disorders, including myocardial infarction, aortic aneurysms,
and pelvic organ prolapse. 14–17 Dysregulated expression of
LOXL1 has also been shown to be a hallmark of PEX syndrome,
contributing to disease development and predisposing to PEX-
associated ocular and systemic complications. 16–22 Increased
expression levels of LOXL1 in early disease stages have been
related to aggregation and crosslinking of PEX fibrils in anterior
segment tissues, such as the trabecular meshwork, 18 whereas
reduced expression levels of LOXL1 in advanced disease stages
have been related to pronounced structural elastotic and
biomechanical alterations of elastin-rich, load-bearing tissues,
such as the lamina cribrosa. 19, 22 LOXL1 has, therefore, been
considered a major susceptibility factor for the development of
PEX glaucoma by increasing resistance to aqueous humor
outflow through cross-linked PEX fiber aggregates and by
increasing the risk for pressure-induced optic nerve damage
through elastin fiber destabilization. 23 Several PEX-associated
pathogenetic factors, such as TGF-β1, oxidative stress, and
ultraviolet (UV)-B radiation, have been shown to influence
LOXL1 gene expression and may act as comodulating factors
in etiopathogenesis of PEX and its associated glaucoma. 24
Accordingly, increased lysyl oxidase activity in the trabecular
meshwork has been suggested to account for TGF-β-mediated
IOP elevation, because TGF-β induces both expression and
activity of LOXL1 and all other LOX isoforms in human
trabecular meshwork cells. 25

In a genome-wide association study on European PEX
patients, we recently identified a four component polymorphic
locus positioned in intron 1 and 2 of LOXL1 within a genomic
region with enhancer-like chromatin features. One of these
SNPs, rs11638944:C>G, was found to act as an expression
quantitative trait locus affecting transcriptional output of
LOXL1, mediated by differential transcription factor binding
and alternative pre-mRNA splicing. 26 We showed that
increased transcriptional activity at this locus is associated with
reduced binding of retinoid X receptor alpha and with
enhanced alternative splicing coupled with nonsense-mediated
decay (NMD), which altogether reduces the levels of
LOXL1 mRNA in cells and tissues with the alternative allele.
The alternatively spliced transcript, LOXL1-a, includes an addition-
al exon (Ex1A) in the first intron of LOXL1, introducing a
premature termination codon (PTC) in exon 2 via frameshift,
subjecting this unproductive isoform to NMD.

The NMD pathway is a common mRNA control mechanism
that degrades unproductive PTC-containing mRNA variants
during translation. 27–29 Recognition and targeting of these
transcripts to NMD requires a core machinery consisting of
three UPF-shareshirt proteins (UPF1–UPF3) along with other
factors (e.g., serine/threonine-protein kinase [SMG1], eukary-
otic release factor), which are part of exon–exon junction
complexes. Alternative mRNA splicing coupled to NMD is
also known to dynamically modulate gene expression,
particularly during development, in response to various forms
of cellular stress, and in nutrient homeostasis pathways, and
has emerged as a common posttranscriptional mechanism to
regulate fine adjustment of RNA levels. 30–32 Thus, by coupling
alternative splicing to NMD, cells can functionally downreg-
ulate gene expression levels under certain conditions,
particularly stresses like heat, hypoxia, oxidative stress, or
amino acid deprivation. 33 An essential intermediary step, by
which stress factors inhibit the NMD pathway to downreg-
ulate gene expression, is the phosphorylation of the
cukaryotic translation initiation factor 2α (eIF2α), known
to be a critical factor for the initiation of many stress
pathways. 34 Based on our previous finding that constitutive
NMD-coupled alternative splicing is influenced by PEX-
associated genetic variants in LOXL1, 26 we hypothesized that
this pathway represents a means to regulate steady-state
levels of LOXL1 mRNA and protein as an adaptive response to
PEX-associated cues and stress factors.

Here, we show that constitutive expression levels of the
alternatively spliced transcript LOXL1-a parallel those of wild-
type LOXL1 with highest expression levels in trabecular
meshwork tissue, and with differential expression patterns in
PEX and control specimens. We further confirm that the
LOXL1-a splice variant is a target of the NMD pathway in
multiple PEX-relevant cell types including trabecular mesh-
work cells, and that stabilization of LOXL1-a by NMD
inhibition is associated with reduced mRNA and protein levels
of wild-type LOXL1. We provide additional evidence that
various forms of PEX-associated stressors and factors including
TGF-β1 enhance LOXL1-a transcript levels, and that the stress-
induced inhibition of NMD is dependent on phosphorylation
of eIF2α. Some of these NMD-suppressing agents (i.e.,
oxidative stress, retinoic acid, and caffeine) simultaneously
downregulate mRNA and protein levels of wild-type LOXL1,
suggesting that alternative splicing coupled to NMD represents
a novel mechanism of adapting LOXL1 expression to environ-
mental and metabolic cues involved in the pathophysiology of
PEX syndrome/glaucoma.

MATERIALS AND METHODS

Study Approval

Ethics approval for this study was obtained from the
institutional review board of the Medical Faculty of the
University of Erlangen-Nürnberg (No. 4218-CH). Informed
consent to tissue donation was obtained from the donors or
their relatives, and investigations were performed in accord-
dance with the principles of the Declaration of Helsinki for
experiments involving human tissues and samples.

Human Tissues

Human donor eyes used for corneal transplantation with
appropriate research consent were obtained from Caucasian
donors and processed within 20 hours after death. For RNA
and DNA extractions, 20 donor eyes with manifest PEX
syndrome (79 ± 7 years), 19 eyes with early stages of PEX
syndrome (82 ± 8 years), and 59 healthy appearing age-
matched control eyes without any known ocular disease (75 ±
10 years) were used. The presence of characteristic PEX
material deposits in manifest disease (= late stages of PEX
syndrome) was assessed by macroscopic inspection of anterior
segment structures and confirmed by electron microscopic
analysis of small tissue sectors. Early stages of PEX syndrome
were defined by a frosted appearance of the zonules as
described previously. 35 In these cases, subtle PEX material
deposits were visible exclusively on the zonules by macro-

scopic inspection of donor eyes (Supplementary Fig. S1). Ocular tissues (cornea, trabecular meshwork, lens, iris, ciliary body, retina, choroid, lamina cribrosa, and optic nerve) were prepared under a dissecting microscope and shock frozen in liquid nitrogen.

**Cell Culture**

Tenon’s capsule biopsies from four patients without (71 ± 7 years) and with PEX syndrome (78 ± 10 years), respectively, were obtained during cataract surgery. Additionally, eight biopsy samples were obtained from younger patients (6 ± 2 years) during strabismus surgery. Primary human Tenon’s capsule fibroblast (hTCF) cultures were established and maintained as previously described.24 All hTCF were homogenous for the risk alleles of the two coding SNPs, rs1048661T>G and rs3825942A>G, as well as the four intronic SNPs, rs12905253G>A, rs11638944C>G, rs12441130T>C, rs11631579A>G, which had been previously shown to influence alternative splicing of LOXL1.26 Primary human optic nerve head astrocyte (hONHA) cultures were generated from lamina cribrosa tissue of five healthy donor eyes (74 ± 9 years) with appropriate research content as previously described.19 Primary human trabecular meshwork cells (hTMC) were obtained from Provitro (Berlin, Germany) and grown in Dulbecco’s modified Eagle’s medium (DMEM; Pan Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; Pan Biotech) and 1% antibiotic (penicillin/streptomycin/amphotericin B; Pan Biotech). The immortalized human lens epithelial cell (hLEC) line B-3 was obtained from ATCC (Manassas, VA, USA) and grown in Eagle’s Minimum Essential Medium (ATCC) supplemented with 20% FBS and 0.5% antibiotic (gentamycin; Life Technologies, Carlsbad, CA, USA). The immortalized human nonpigmented ciliary epithelial cell (hNPEC) line ODM-2 was kindly supplied by Miguel Coca-Prados (Fundación de Investigación Oftalmológica, Oviedo, Spain), grown in DMEM (with 4.5 g/L glucose; Pan Biotech), supplemented with 10% FBS and 50-μg/mL gentamicin.

To analyze relative expression levels of LOXL1 and LOXL1-a, cells were grown to subconfluence (90%), kept in serum-free medium for 24 hours, and then exposed to either TGF-β1 (5 ng/mL; R&D Systems, Wiesbaden, Germany), IL-6 (10 ng/mL; Peprotech, Hamburg, Germany), homocysteine (500 μM; Sigma-Aldrich, Munich, Germany), or retinoic acid (2 μM; Sigma-Aldrich) for 48 hours under serum-free conditions and t-butylhydroperoxide (tBHP, 100 μM; Sigma-Aldrich) for 5 hours in serum-containing medium. For inhibition of NMD, cells were treated with puromycin (100 μg/mL; Life Technologies) or caffeine (12 mM; Sigma-Aldrich) for 14 hours in serum-containing medium. Exposure to UV-B radiation (40 mJ/cm²) was carried out in Grey’s balanced salt solution (Sigma-Aldrich) using an Alphalanger 3400 (Alpha Innotech Corporation, San Leandro, CA, USA) as UV-B source (wavelength 302 nm), followed by incubation in serum-containing medium for 24 hours. Cells were exposed to mechanical stress (55 mm Hg) for 48 hours using a computer-controlled, open pressurized chamber system with dynamic humidified air flow as previously described (Schlotzer-Schrehardt U, et al. IOVS 2014;55:ARVO E-Abstract 4233). Parallel cultures maintained under normal conditions without exposure to stress factors at atmospheric pressure served as controls.

Cell viability was assessed using a fluorescent kit (Live/Dead Viability/Cytotoxicity kit; Molecular Probes, Eugene, OR, USA) and a plate reader (Fluoroscan Ascent 2.4; Thermo Scientific, Bonn, Germany) as previously described.26

**siRNA Silencing**

Human Tenon’s capsule fibroblast (0.65 × 10⁶ cells) were transiently transfected with specific siRNA (ON-TARGETplus SMARTpool; GE Healthcare Dharmacon, Freiburg, Germany) for UPF1 (600 pmol), UPF2 (150 pmol), UPF3A and UPF3B (150 pmol), SMG1 (150 pmol), or LOXL1 (120 pmol) by electroporation using the Nucleofector II transfection device (Lonza, Köln, Germany) and the Amaxa Basic Fibroblast Nucleofector Kit (Lonza). Transfections with scrambled siRNA (ON-TARGETplus Non-targeting pool; GE Healthcare Dharmacon) served as controls. Transfected cells were seeded into 6-well plates in duplicate and harvested at 48 hours posttransfection for real-time PCR analysis.

**Real-Time PCR**

Ocular tissues and cultured cells were extracted using the Precellys 24 homogenizer and lysing kit together with the AllPrep DNA/RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions including an on column DNasel digestion step using the RNase-free DNase Set (Qiagen). First-strand cDNA synthesis from 0.5 μg of total RNA was carried out using 200U 1st strand Perfect script reverse transcriptase (Thermo Scientific) and 200 ng of random primers (Roche Life Science, Mannheim, Germany) in a 20-μL reaction. Quantitative real-time PCR was performed using the CFX Connect thermal cycler and software (Bio-Rad Laboratories, München, Germany). Polymerase chain reactions (25 μL) were run in duplicate and contained 2 μL of first-strand cDNA, 0.48 μM each of upstream- and downstream-primer, and SsoFast EvaGreen Supermix (Bio-Rad). Exon-spanning primers (Eurofins Genomics, Ebersberg, Germany), designed by means of Primer 3 software (in the public domain, http://bioinfo.ut.ee/ primer3/), are summarized in Supplementary Table S1. For quantification, probes were run in parallel and analyzed with the ΔΔCt method. Averaged data represents at least three biological replicates. Amplification specificity was checked using melt curve, agarose gel, and sequence analyses with the Prism 3100 DNA-sequencer (Applied Biosystems, Foster City, CA, USA).

For normalization of gene expression levels, mRNA ratios relative to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxanthine phosphoribosyltransferase-1 (HPRT) were calculated.

**Western Blot Analysis**

Western blot analysis using antibodies against LOXL1 (kindly provided by Takako Sasaki, Erlangen), hUPF2 (RENT2, ab153830; Abcam, Cambridge, UK), eIF2α (9722; Cell Signalling, Danvers, MA, USA), phospho-eIF2α (P-eIF2α, 9721; Cell Signalling), and β-actin (AC-15; Sigma) was performed as described previously.24 In negative control experiments, the primary antibody was replaced by PBS. Immunodetection was performed with a horseradish peroxidase-conjugated secondary antibody and the Super Signal West Femto (LOXL1, UPF2, eIF2α, and Phospho-eIF2α) or Pico (β-actin) ECL kit (Thermo Scientific). Specific protein bands were quantitatively analyzed with the LAS-3000 (Fujifilm, Düsseldorf, Germany) chemiluminescence detection system and software (Multi Gauge V1.1; Fujifilm).

Equal loading of samples was verified by immunodetection of β-actin. For normalization of LOXL1 protein expression levels, protein ratios relative to the housekeeping gene β-actin were calculated. Data represents at least three biological replicates.
Statistical Analysis
Group comparisons were performed using an unpaired two-tailed t-test or a Mann-Whitney U test using SPSS v.19 software (IBM, Ehningen, Germany). P less than 0.05 was considered statistically significant.

RESULTS
Expression Patterns of LOXL1 Transcripts in Ocular Tissues
First, we analyzed expression patterns of wild-type LOXL1 and alternative LOXL1-a transcripts (Fig. 1A) in various ocular tissues, including cornea, lens, trabecular meshwork, iris, ciliary body, choroid, retina, lamina cribrosa, and optic nerve, of healthy donor eyes (n = 4). The relative abundance of transcript levels of LOXL1-a was determined by qPCR using exon Ex1A-specific forward and exon 2-specific reverse primers (Supplementary Table S1) and identity of qPCR fragments was confirmed by sequence analysis. LOXL1-a transcripts, although low in abundance, were detected in all ocular tissues examined showing lowest expression levels in retina and cornea, and highest expression levels in trabecular meshwork, lens, choroid, and lamina cribrosa (Fig. 1B). Relative expression levels of wild-type LOXL1 largely paralleled this expression pattern.

Comparing control samples (n = 25) and samples obtained from eyes with early (n = 10) and manifest PEX syndrome (n = 10), expression levels of both LOXL1 and LOXL1-a were found to be significantly increased in early stages and significantly decreased in late stages of disease in anterior segment tissues, such as trabecular meshwork and ciliary body (P < 0.05; Fig. 1C). In contrast, posterior segment tissues, such as the choroid, did not show any differences in expression levels of both transcripts between PEX and control specimens.

LOXL1-a is a Target of the NMD Pathway in Multiple Cell Types
To confirm that LOXL1-a is a direct target of NMD, different PEX-relevant cell types, (i.e., hTCF, hTMC, hONHA, hNPEC, and hLEC; n = 5 each), were treated with the protein synthesis inhibitor puromycin and the SMG1 kinase inhibitor caffeine, which are known to selectively stabilize NMD transcripts. 37,38 To exclude cytotoxic effects of the drugs, cell viability assays were performed in parallel, revealing no significant differences between treated and untreated control cells (data not shown). Both puromycin and caffeine induced a significant increase of LOXL1-a (up to 7-fold) and a significant decrease of LOXL1 (up to 0.5-fold) in all cell types compared with untreated cells (P < 0.05; Fig. 2A). Maximum effects were observed in hTCF and hTMC.

Western blot analyses performed with cell lysates from hTCF and hTMC treated with puromycin, caffeine, or TGF-β1 (n = 3 each) confirmed that LOXL1-a is neither translated into a truncated (by PTC termination) or elongated (by PTC read-through) protein product (Fig. 2B). Using an antibody directed against the N-terminal region of LOXL1, we observed a specific band at 63 kDa corresponding to the full-length form of wild-type LOXL1, which was significantly downregulated by both puromycin and caffeine and significantly upregulated by TGF-β1 (P < 0.01). Apart from a presumably unspecific band at 52 kDa, which persisted after siRNA-mediated knockdown of LOXL1 (Supplementary Fig. S2), no other bands corresponding to either truncated LOXL1 protein (expected band at 46 kDa) or elongated protein (expected band at 76 kDa) were observed (Fig. 2B). Altogether, these observations confirm that LOXL1-a transcript is sensitive to NMD and suggest that NMD functions in a regulatory mode to influence LOXL1 expression levels.

NMD-Coupled Alternative Splicing Regulates LOXL1 Expression Levels
To confirm a regulatory role of NMD on LOXL1 expression, we analyzed the relative expression levels of LOXL1-a and LOXL1 following siRNA-mediated knockdown of various key NMD factors (i.e., UPF1, UPF2, UPF3A/B, and SMG1) in hTCF (n = 3 each). Efficient knockdown of all factors was confirmed by qPCR (Supplementary Fig. S3). Knockdown of all NMD factors resulted in significantly increased expression levels of LOXL1-a (up to 2-fold) compared with scramble siRNA-transfected controls (P < 0.05), whereas expression levels of LOXL1 were significantly decreased (up to 0.5-fold; P < 0.05; Fig. 3A). Western blot analyses of hTCF transfected with UPF2 siRNA (n = 3) confirmed a significant downregulation of wild-type LOXL1 on the protein level compared with scramble siRNA-transfected control cells (P < 0.05; Fig. 3B). These findings indicate that suppression of NMD represents a mechanism to downregulate LOXL1 gene and protein expression.

NMD-Coupled Alternative Splicing is Modulated by External Stresses and Cues
To investigate the effect of PEX-associated stress factors and cues on the expression of LOXL1-a and LOXL1, hTCF were treated with TGF-β1, IL-6, homocysteine, or retinoic acid, 39 and exposed to UV-B radiation, 31 oxidative stress (tBHP), 12 or mechanical stress 33 for 5 to 48 hours. Compared with untreated control cells, LOXL1-a was found to be consistently upregulated in response to most exposures except IL-6 and homocysteine (P < 0.05; Fig. 4A). Exposure to TGF-β1, UV-B light, and mechanical stress resulted in significant increases of both LOXL1-a (up to 8-fold) and LOXL1 (up to 4-fold) transcript levels compared with untreated control cells, whereas exposure to retinoic acid and oxidative stress had opposite effects on transcript levels upregulating LOXL1-a (up to 2-fold and downregulating LOXL1 (up to 0.4-fold). These observations could be confirmed in hTMC (Fig. 4B). Because GAPDH has been reported to be induced by stress factors, such as oxidative and mechanical stress, 44 we validated the effect of oxidative stress, UV-B radiation, and mechanical stress on the expression of LOXL1-a and LOXL1 in hTCF and hTMC using HPRT as a stable reference gene. 45 The results were, however, not different from those using GAPDH as reference gene (Supplementary Fig. S4).

To comparatively analyze the NMD response in PEX and control cells, we treated hTCF derived from control and PEX patients (n = 4 each) with NMD inhibitors (i.e., puromycin and caffeine), as well as typical PEX-associated pathogenetic factors (i.e., UV-B light and TGF-β1). Basal expression levels of both transcripts were not different between hTCF derived from PEX and control patients (Fig. 4C). Expression levels of LOXL1-a were equally upregulated in both groups in response to puromycin, caffeine, and UV-B, but showed significant differences in response to TGF-β1, which induced a 5-fold increase in control cells and a 20-fold increase in PEX cells (P < 0.05; Fig. 4C). Similarly, expression levels of LOXL1 were only differentially regulated in response to TGF-β1, which stimulated a 2.5-fold increase in control cells and a 4.5-fold increase in PEX cells (P < 0.05; Fig. 4C).

Phosphorylation of eIF2α has been shown to be an essential intermediary step for stress-induced inhibition of NMD. 46 Therefore, we examined the phosphorylation state of eIF2α in normal hTCF and hTMC following treatment with puromycin.
FIGURE 1. Expression levels of wild-type LOXL1 and alternatively spliced LOXL1-a transcripts in ocular tissues of PEX and control subjects. (A) Schematic representation of two LOXL1 transcripts (LOXL1, LOXL1-a); exons are indicated by numbers (Ex1 to Ex7); the alternatively spliced transcript LOXL1-a includes an additional exon (Ex1A). (B) Relative expression levels of LOXL1 and LOXL1-a mRNA in ocular tissues of normal donors (n = 4) using real-time PCR technology; data are presented as mean values ± SD relative to expression levels in the retina (set to 1). (C) Relative expression levels of LOXL1 and LOXL1-a mRNA in ocular tissues derived from healthy human donors (control, n = 25) and donors with early (n = 10) and manifest (n = 10) stages of PEX syndrome using real-time PCR technology; data are presented as mean values ± SD. The relative expression levels were normalized relative to GAPDH (*P < 0.05, **P < 0.001, ***P < 0.0001; unpaired 2-tailed Student’s t-test).
cin, caffeine, and TGF-β1. As expected, increased protein levels of phosphorylated eIF2α (p-eIF2α) were observed in both cell types after exposure to all factors compared with unstimulated control cells (P < 0.05), whereas total eIF2α protein levels remained unchanged (Fig. 4D).

Altogether, these observations suggest that certain stress conditions (e.g., exposure to oxidative stress) and dietary factors (e.g., caffeine and retinoic acid) suppress the NMD pathway, thereby providing a means to dynamically adapt LOXL1 gene expression by hTMC and other cell types to certain external stress factors and internal cues.

**DISCUSSION**

Current evidence supports a fundamental role for LOXL1 in connective tissue homeostasis and stability, and dysregulated expression of this key enzyme has been linked to both fibrotic and elastotic-degenerative connective tissue disorders including myocardial infarction, myelofibrosis, lung emphysema, aortic aneurysms, and pelvic organ prolapse as well as ageing. Studies have also provided evidence for a dysregulated expression of LOXL1 in tissues of PEX patients, which appears to contribute to both formation of fibrillar PEX material aggregates and elastotic-degenerative connective tissue alterations, in dependence of tissue type and disease stage. On the one hand, LOXL1 expression was found to be transiently upregulated during the initial stages of the fibrotic matrix process in tissues of the anterior segment including the trabecular meshwork, obviously to become involved in PEX fiber aggregation and crosslinking. This fibrotic process may be stimulated by profibrotic triggering factors, such as TGF-β1, present in the aqueous humor of PEX patients. On the other hand, LOXL1 expression was found to be significantly downregulated below homeostatic levels in elastin-rich intra- and extraocular connective tissues, such as the lamina cribrosa and aortic wall. Reduced LOXL1 expression levels were found to be associated with pronounced elastotic and biomechanical alterations, which have been suggested to predispose to the development of glaucoma and cardiovascular diseases.
problems in PEX patients, possibly augmented by increased mechanical load acting on these compromised connective tissues (Schlötzer-Schrehardt U, et al. IOVS 2014;55:ARVO E-Abstract 4233). In addition to its supposed role in PEX glaucoma, increased LOXL1 expression and activity in trabecular meshwork cells has been also suggested to be involved in TGFβ-mediated matrix accumulation in the outflow tissues, and thereby contribute to increased aqueous humor outflow resistance and IOP elevation in the development of POAG. Thus, understanding how LOXL1 is regulated in health and disease, particularly in interaction with external and internal factors, seems to be the key to providing clues for susceptibility to PEX syndrome, glaucoma, and other connective tissue disorders.

Although LOXL1 has long been recognized as the major effect locus in PEX syndrome and glaucoma, the mechanisms by which the associated variants confer risk for disease still remain unknown. Given that the two missense variants do not appear to contribute to PEX pathophysiology,10 it has been suspected that functional regulatory variants in noncoding regions may have a role in disease susceptibility by influencing transcriptional output of LOXL1 expression. In fact, a functional variant within the LOXL1 promoter region, rs16958477, which was associated with increased risk for PEX in a US Caucasian population, has been previously shown to affect LOXL1 gene transcription.26,54 Hauser and colleagues5 identified PEX-associated variants at the exon 1/intron 1 boundary of LOXL1 in black South African, US Caucasian, German, and Japanese patients, which were shown to modulate the expression of LOXL1 antisense RNA 1 (LOXL1-AS1), a long noncoding RNA of LOXL1, in response to oxidative and mechanical stress. However, these variants had no effect on LOXL1 expression.

Recently, we identified four PEX-associated functional variants located in a genomic region of intron 1 of LOXL1 with regulatory potential, which modulated transcriptional output of LOXL1 on the transcriptional and posttranscriptional level. In particular, we found that rs11638944, located in close proximity to a splice site, exerts a cis-acting effect on the expression levels of LOXL1 by enhancing splicing of an alternative LOXL1-a transcript, which differs from the wild-type transcript by inclusion of an additional exon in intron 1 and is degraded by NMD. This splicing event is, therefore, unproductive and may serve the purpose to downregulate the normal LOXL1 mRNA and protein levels.26,54 Genotype-phenotype correlations showed that hTCF cell lines homozygous for the risk allele G of rs11638944 express significantly higher constitutive levels of LOXL1-a and significantly lower levels of wild-type LOXL1 mRNA than cell lines homozygous for the protective allele C.26 These initial observations suggested that alternative splicing of LOXL1 pre-mRNA is influenced by sequence variation and that higher rates of mRNA degradation by NMD may lead to reduced steady-state levels of LOXL1. Because coupling of alternative splicing and
FIGURE 4. Effects of PEX-associated pathophysiologic factors on expression levels of LOXL1 and LOXL1-a. (A) Relative expression levels of LOXL1 and LOXL1-a in hTCF after exposure to TGF-β1 (5 ng/mL), IL-6 (10 ng/mL), homocysteine (500 μM), and retinoic acid (2 μM) for 48 hours, tBHP (100 μM) for 5 hours, UV-B light (40 mJ/cm²), and mechanical stress (55 mm Hg) for 48 hours. Expression levels were determined by quantitative real-time PCR, normalized against GAPDH, and expressed relative to controls (dotted line). Data represent mean values ± SD of three independent experiments; *P < 0.05, **P < 0.001, ***P < 0.0001; unpaired 2-tailed Student’s t-test. (B) Relative expression levels of LOXL1 and LOXL1-a in hTMC after exposure to TGF-β1, retinoic acid, tBHP, UV-B light, and mechanical stress. (C) Relative expression levels of LOXL1 and LOXL1-a in hTCF derived from healthy human donors (control, n = 4) and donors with manifest PEX syndrome (PEX, n = 4) at basal condition (left panel) and after exposure to puromycin (100 μg/mL), caffeine (12 mM), UV-B light (40 mJ/cm²), or TGF-β1 (5 ng/mL) (right panel). Expression levels were determined by quantitative real-time PCR, normalized against GAPDH, and expressed relative to controls (dotted line). (D) Western blot analysis of eIF2α and phospho-eIF2α (p-eIF2α) in cell lysates from hTCF and hTMC without (Co) or after stimulation with caffeine (Caf), TGF-β1, and puromycin (Pu). Specific bands indicating eIF2α and p-eIF2α appear at 38 kDa. Equal loading of samples was verified by immunodetection of β-actin, and expression levels were normalized to β-actin expression. Densitometry analysis of band intensities of p-eIF2α shows results of three independent experiments (data represent mean values ± SD; *P < 0.05, **P < 0.001, ***P < 0.0001; unpaired 2-tailed Student’s t-test).
LOXL1 Regulation by NMD-Coupled Alternative Splicing

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NMD has been reported as a general dynamic mode of controlling and adapting gene expression in response to a variety of cellular stresses.\textsuperscript{41,53} We expanded our previous findings to analyze the NMD pathway in the regulation of LOXL1 in response to known PEX-associated cues using PEX-relevant cellular models.

Here, we show that constitutive expression levels of LOXL1-a parallel those of wild-type LOXL1 with differential expression in early and late stages of PEX as well as highest expression levels in trabecular meshwork tissue, which is of major importance for aqueous humor outflow and IOP regulation,\textsuperscript{51,55} supposed to be influenced by LOXL1-mediated extracellular matrix crosslinking and tissue stiffness.\textsuperscript{25} We further confirm that the LOXL1-a splice variant is a target of NMD in PEX-relevant cell types, as evidenced by increased transcript levels after NMD suppression by treatment with translational inhibitors or siRNA-mediated knockdown of NMD core factors. Stabilization of LOXL1-a transcript was associated with reduced expression levels of wild-type LOXL1 on the mRNA and protein level indicating an association between NMD-coupled alternative splicing and LOXL1 expression regulation. Notably, inhibition of UPF1 phosphorylation by caffeine\textsuperscript{58} resulted in significantly increased LOXL1-a levels and significantly decreased LOXL1 levels compared with untreated cells, particularly in hTMC. Because coffee consumption has been shown to represent a significant risk factor for PEX incidence and prevalence in a prospective study of over 120,000 US health professionals,\textsuperscript{59} this effect of caffeine on LOXL1 expression regulation may be part of a complex network of gene-environment interactions in the pathogenesis of PEX syndrome and glaucoma.

We provide additional evidence, that LOXL1-a transcript levels in hTMC and hTCF are enhanced by various forms of external stress factors, including UV-B light, oxidative stress, and mechanical stress, as well as internal (patho)physiological factors, including TGF-β1 and retinoic acid, which were previously shown to modulate LOXL1 expression levels.\textsuperscript{24-26} In agreement with a previous study, IL-6, and homocysteine had no significant effect on expression levels of LOXL1 and LOXL1-a. This observation together with inconsistent clinical reports on plasma homocysteine levels in patients with PEX, which are elevated in many but not in all study populations,\textsuperscript{57} suggests that hyperhomocysteinemia may not be causally involved in PEX pathophysiology, but may only serve as a biomarker or innocent bystander of disease.\textsuperscript{38}

Stress-induced inhibition of NMD by UV-B light, oxidative and mechanical stress was shown to depend on eIF2α phosphorylation, which represents a critical step in NMD-coupled stress response.\textsuperscript{54,48} Some of these NMD-suppressing agents (i.e., oxidative stress, retinoic acid, and caffeine) simultaneously downregulated cellular levels of wild-type LOXL1, suggesting that this pathway provides a posttranscriptional mechanism of adapting LOXL1 expression levels to certain environmental and dietary cues. Functional mechanisms of how LOXL1-a may influence LOXL1 expression could involve an autoregulatory negative feedback loop or action as a noncoding regulatory RNA, as has been described for regulation of CDA1 gene expression.\textsuperscript{54} Because LOXL1 may have multiple functions besides matrix crosslinking,\textsuperscript{2,25} and because alterations in LOXL1 expression have been shown to broadly influence cellular transcriptomes,\textsuperscript{60} fine-tuning of LOXL1 expression in response to certain stimuli may be of biological significance for cellular function.

Even if the precise mechanism of action still has to be clarified, the described pathway may be part of a complex network of LOXL1-environment interactions in the pathogenesis of PEX syndrome and glaucoma, which has been clearly linked to oxidative stress and caffeine.\textsuperscript{4,56,61} The suggested mechanism is a common tool to dynamically and rapidly modulate gene expression, particularly in response to physiological or microenvironmental changes.\textsuperscript{53} Apart from this newly described regulatory pathway, LOXL1 gene regulation may certainly involve a wide range of other mechanisms on the transcriptional (e.g., epigenetic modifications, transcription factor binding) and posttranscriptional (e.g., mRNA processing and stability, small and long noncoding regulatory RNAs) level, which can be influenced by LOXL1 genetic variants and environmental factors.\textsuperscript{62} As an instance, DNA hypermethylation of the LOXL1 promoter has been reported to occur in PEX patients and to downregulate LOXL1 expression in skin fibroblasts in a case with cutis laxa.\textsuperscript{21,63} The precise delineation of the roles played by these mechanisms in modulating steady-state LOXL1 mRNA levels awaits further discovery.

Acknowledgments

The authors thank Miguel Coca-Prados, Fundación de Investigación Oftalmológica, Oviedo, Spain, for providing the human nonpigmented ciliary epithelial cell line ODM-2; Olga Zwenger, Ekaterina Gedova, Petra Koch, Angelika Mößner, Myriam Eitl, and Elke Meyer for excellent technical assistance, and Takako Sasaki for the supply of LOXL1 antibody. Supported by grants from the Interdisciplinary Center for Clinical Research (IZKF) of the University Hospital, Erlangen, Germany, and the German Research Foundation (SCHL 366/8-1). Disclosure: D. Berner, None; M. Zenkel, None; F. Pasutto, None; U. Hoja, None; P. Liravi, None; G.C. Gusek-Schneider, None; F.E. Kruse, None; J. Schödel, None; A. Reis, None; U. Schlötzer-Schrehardt, None

References


