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Published in:
IUBMB Life

DOI:
10.1002/iub.2213

Publication date:
2020

Document license:
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Citation for published version (APA):
Unique molecular networks: Formation and role of elastin cross-links

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Abstract
Elastic fibers are essential assemblies of vertebrates and confer elasticity and resilience to various organs including blood vessels, lungs, skin, and ligaments. Mature fibers, which comprise a dense and insoluble elastin core and a microfibrillar mantle, are extremely resistant toward intrinsic and extrinsic influences and maintain elastic function over the human lifespan in healthy conditions. The oxidative deamination of peptidyl lysine to peptidyl allysine in elastin’s precursor tropoelastin is a crucial posttranslational step in their formation. The modification is catalyzed by members of the family of lysyl oxidases and the starting point for subsequent manifold condensation reactions that eventually lead to the highly cross-linked elastomer. This review summarizes the current understanding of the formation of cross-links within and between the monomer molecules, the molecular sites, and cross-link types involved and the pathological consequences of abnormalities in the cross-linking process.

KEYWORDS
aging, desmosine, elastic fibers, elastinopathies, elastogenesis, lysyl oxidase, native cross-links

1 INTRODUCTION

Networks of elastic fibers provide resilience and long-range elasticity to dynamic tissues of all higher vertebrates. Elastic fibers are extremely durable macromolecular assemblies of the extracellular matrix (ECM) and are present in most force-bearing soft tissues. They enable many organs including lungs, skin, or large arteries to stretch and bend with recoil and thus to maintain their physiological functions. The elastic fiber formation, also known as elastogenesis, starts at mid-gestation, reaches its maximum level around birth, and completes during postnatal development.1 Virtually no new fibers are formed in adult tissues2 with the notable exception of the...
uterus, where elastin is rapidly removed after parturition and elastogenesis starts again with every new pregnancy.\textsuperscript{3} The fiber assembly is a complex and only partially understood multistep process, which involves numerous proteins as well as other molecules. The main stages are outlined in Figure 1. The expression of the monomeric elastin precursor protein tropoelastin (TE) takes place in elastogenic cells, including skin fibroblasts, vascular smooth muscle cells, and chondrocytes. TE is transported along the secretory pathways together with a chaperone, the elastin-binding protein (EBP), which protects the TE molecules from intracellular aggregation and degradation.\textsuperscript{4} After secretion, the complex dissociates and TE undergoes self-association through a colloidal phase separation process referred to as coacervation. The underlying noncovalent interactions between hydrophobic regions of TE molecules lead to the formation of distinct globular aggregates and contribute to the subsequent cross-linking process.\textsuperscript{5,6} After these aggregates reach a critical size, they are moved from the plasma membrane through the ECM and are then deposited on a skeleton of fibrillin-rich microfibrils. Further cross-linking leads to the maturation of the elastic fiber.

The cross-links are a fundamental feature of the resultant fiber core of elastin. Its network of interlinked TE molecules distributes the stress and strain forces throughout the biopolymer during deformation and thus helps tissues to resume their shape. The elastin content and the organization of mature elastic fibers are highly tissue-specific and differences in configuration are closely connected to the magnitude and direction of reversible deformation which the connective tissues undergo. In the medial layer of the aorta, for example, elastin forms concentric fenestrated lamellae, whereas the fibers form three-dimensional honeycomb-like structures in elastic cartilage.

The cross-linking is also pivotal for elastin’s insolubility, proteolytic resistance, and remarkable longevity. The latter is reflected by a remarkable half-life that has been determined to be greater than 70 years in humans using aspartic acid racemization analysis and \textsuperscript{14C} turnover.\textsuperscript{7} Although elastin is greatly resistant toward intrinsic and extrinsic influences, it ages and accumulates damage throughout life due to its very low turnover and various enzymatic, chemical, and biophysical influences. Aging of elastin and elastic fibers involves enzymatic degradation, oxidative damage, formation of advanced glycation end products (AGEs), calcification, aspartic acid racemization, lipid accumulation, carbamylation, and mechanical fatigue. These changes can cause a decrease or loss of elastic fiber function and adversely affect morbidity and mortality.

\textbf{FIGURE 1} Schematic representation of the major stages of the elastic fiber assembly: (1) TE is synthesized on the rough endoplasmic reticulum where it binds to the chaperone protein EBP. (2) The EBP-TE complex is transported through the Golgi apparatus and secreted to the cell membrane. (3) TE is released from the chaperone and forms globules at the cell surface, while EBP dissociates as a result of interaction with glycosaminoglycans and is recycled back into the cell. (4) Fibulin-4 is important for the chain alignment of TE mediating the interplay with lysyl oxidase or lysyl oxidase-like enzymes. The oxidation of lysine residues initiates a series of condensation reactions forming covalent intra- and intermolecular cross-links. (5) After clusters of TE molecules reach a critical size, they are moved from the plasma membrane through the ECM and aggregate onto the microfibril scaffold to eventually form the elastic fiber. EBP, elastin-binding protein; ECM, extracellular matrix; TE, tropoelastin
2 | TROPOELASTIN DOMAIN STRUCTURE AND EXPRESSION

In most species, a single gene encodes TE, while teleosts and frogs are known exceptions with two nonallelic genes. The primary transcript of TE undergoes extensive alternative splicing resulting in the production of multiple isoforms. The splicing occurs in phase without affecting the reading frame, and hence, an exon is either included, extended, shortened, or removed. For human TE, at least 18 isoforms have been described, ranging in molecular weights between 49 and 69 kDa. The major exons that have been reported to be alternatively spliced are 3, 10, 11, 13, 22, 23, and 32. The functional consequences of the alternative splicing are still elusive, but some studies suggest that splicing could be tissue-specific or linked to developmental changes of cells. It has been shown that the insertion or deletion of TE domains or the mutation of certain amino acid residues affects diverse mechanisms associated with the assembly of TE monomers into a polymeric network such as coacervation and cross-linking processes as well as the resultant mechanical properties. This suggests that variations in the TE sequence allow tissue-specific alterations in elastin properties or are responsible for abnormal fiber formation under pathological conditions.

TE’s sequence is highly repetitive and about 80% are composed of the four amino acids Gly, Ala, Val, and Pro. The precursor has alternating hydrophobic and more hydrophilic domains, which are encoded by individual exons, so that the domain structure of the protein is reflected in the exon organization of its gene. The hydrophilic domains are involved in cross-linking and contain Lys-Ala (KA) motifs, often embedded in stretches of polyalanine, or Lys-Pro (KP) motifs (see Figure 2). In the KA domains, Lys residues occur mostly as pairs or triplets separated by two or three Ala residues and sometimes another residue, whereas KP domains feature one or two Lys residues and at least one Pro residue in close proximity. Human TE possesses 11 KA and 5 KP cross-linking domains comprising 32 Lys residues. Three further Lys residues are found in the polybasic motif KXXXXKRK of the C-terminal domain, which is encoded by the across-species highly conserved exon 36. These Lys residues are thought to be not involved in cross-links with any of the KA or KP domains. While the exact role of this unique domain still has to be elucidated, it is known to be critical for the assembly and cross-linking during elastogenesis. It has been for instance shown that TE lacking this sequence is less efficiently incorporated into elastin and further exhibits abnormal cross-linking. Further findings suggest that there is virtually no unmodified domain 36 in mature elastin, but it is either cleaved off or otherwise posttranslationally modified.

TE is secreted as soluble, unglycosylated protein by elastogenic cell types such as fibroblasts, smooth muscle cells, endothelial cells, or chondrocytes. TE molecules then interact with each other via their hydrophobic domains in an endothermic, entropically driven process of liquid–liquid phase separation (coacervation). During this key process of microassembly, TE forms distinct globular aggregates on the cell membrane. The regulation of the coacervation as well as the subsequent cross-linking involves several key proteins including fibulin-4 and -5, latent transforming growth factor β binding protein 4 (LTBP4), and microfibril-associated protein 4 (MFAP4).

**FIGURE 2** The domain structure of human TE. The domain numbering shown across the top (hydrophobic domains) and the bottom (hydrophilic domains) is based on exon assignment. Gray squares display hydrophobic domains and light and dark blue squares represent the 16 hydrophilic cross-linking domains. Filled squares indicate exons that are spliced. The sequence motifs of the cross-linking sites within the domains (lysine residues that are mainly separated by alanine or proline residues) are shown above the domain scheme. Human TE lacks exons 34 and 35 that were evolutionarily lost, but are present in nonprimate vertebrates. TE, tropoelastin.
LYSYL OXIDASE-MEDIATED CROSS-LINKING

The cross-link formation in elastin, but also in collagens, is induced by the lysyl oxidase family, which consists of five members: lysyl oxidase (LOX) and the four lysyl oxidase-like enzymes 1-4 (LOXL1-4). Based on homology, the proteins can be divided into two subfamilies: (a) LOX and LOXL1 and (b) LOXL2, −3 and −4. The latter subfamily has a propeptide domain in common, which features four scavenger receptor cysteine-rich domains, whose functions are yet unknown. All LOXs share a conserved C-terminal amine oxidase catalytic domain consisting of a lysyl tyrosyl quinone (LTQ) cofactor and a copper-binding motif (reviewed in Reference 31). The binding of copper is required for the enzyme activity. Besides inducing cross-links in collagens and elastin, LOXs are known to possess a variety of additional biological functions and play for instance pivotal roles in cancer and fibrosis.32

The enzymes proven to be essential for the proper cross-linking of TE are LOX and LOXL1. However, we have recently shown that also LOXL2 interacts with TE in vitro. The co-distribution of both proteins in the vascular wall further suggests that LOXL2 may contribute to elastogenesis.33 LOX and LOXL1 are secreted as proforms and require proteolytic processing by procollagen C-proteinases.34 After activation, they extracellularly catalyze the oxidative deamination of lysine residues.35 More specifically, the ε-amino group of Lys residues located in KA or KP domains condenses with one of the LTQ carbonyl groups to form a covalent intermediate. Subsequent hydrolysis releases the amino-modified LTQ and α-aminoadipic acid-δ-semialdehyde (see Figure 3), generally referred to as allysine (Lya). The subsequent LTQ regeneration is mediated by Cu²⁺ and associated with the release of one molecule of hydrogen peroxide and ammonia.36,37

Lya is highly reactive and participates in the subsequent formation of nonenzymatic cross-links. On average, ~90% of all Lys residues per TE molecule are modified, that is, they are either derivatized to Lya or incorporated into cross-links.38 This indicates that the modification is partial, which is fundamental for the subsequent condensation reactions, of which most require the availability of free Lys residues.

3.1 Cross-link structures

Different types of covalent cross-links are formed by the spontaneous condensation of Lys and Lya that are referred to as bi-, tri-, tetra-, and pentafunctional with respect to the number of involved Lys moieties. Some studies investigated the mechanisms of cross-link formation with Lys and Lya analogs and demonstrated that low-functional cross-linking structures are intermediate stages in the formation of higher functional linkages.39,40 Figure 4 gives an overview on the proposed pathways of sequential cross-link formation that are further discussed.

The simplest cross-linking structures found in mature elastin but also in collagen are bifunctional cross-linking amino acids, of which two chemically distinct types exist in elastin. They can be formed by either the condensation of Lys and Lya or two Lya moieties. The condensation of Lya with a nonmodified Lys residue forms a Schiff base-type (imine) linkage termed 6,7-dehydrolysinonorleucine (ΔLNL). ΔLNL can be reduced to the secondary amine cross-linker lysinonorleucine (LNL), whose chemical structure is symmetrical with respect to the central nitrogen atom. Aldol condensation of two Lya residues yields allysine aldol (AA), which is an α,β-unsaturated aldehyde (enal) and can therefore easily react with another unmodified Lys residue to form the trifunctional cross-link dehydromerodesmosine (ΔMDES). The Schiff base linkage in ΔMDES can, as in ΔNL, undergo further reduction to form merodesmosine (MDES). It has also been shown in model reactions that ΔMDES can emerge from the condensation of the enamine tautomer of ΔLNL and Lya.40 The reactions of ΔLNL and AA or ΔMDES and Lya yield the tetrafunctional cross-links desmosine

![Figure 3](image-url) Cross-linking is initiated by oxidative deamination of the side chains of lysine residues by members of the family of lysyl oxidases. The reaction produces a reactive aldehyde termed allysine under consumption of oxygen and the release of ammonia and hydrogen peroxide.
(DES) and isodesmosine (IDES), which are structural isomers and unique to elastin among mammals. Hence, ΔLNL, AA, and ΔMDES can be considered as intermediates, which further condense to higher-functional cross-links. Upon imine reduction, LNL and MDES remain as stable cross-links in mature elastin. To date, the mechanism of imine reduction in the ECM is elusive in terms of the identity of the reducing agent. Raju and Anwar suggested that dihydropyridines (the initial condensation products of DES and IDES) in near proximity to imine-type cross-links may serve as reducing agent and initiate the transformation of imines to secondary amines in ΔLNL and ΔMDES.41 Another trifunctional cross-linking amino acid identified from elastin hydrolysates is cyclopentenosine that comprises a 2-cyclopenten-1-one structure. It is formed through cyclization of an α,β,γ,δ-unsaturated aldehyde resulting from the aldol condensation of three Lya residues.42,43

The formation of the tetrafunctional cross-linking amino acids DES and IDES is facilitated by the pairwise arrangement of Lys residues within the primary structure of TE (see Figure 2). DES and IDES were first isolated from elastin hydrolysates by Partridge, Elsden, and Thomas in the early 1960s and initially denoted as “compound A” and “compound M.”44 Further analyses by the same group revealed those compounds to be isomeric pyridinium derivatives, that is, they comprise a nitrogen-containing aromatic six-atom heterocycle that is substituted by the remains of the former Lys side chains at positions 1, 3, 4, and 5 in DES as well as 1, 2, 3, and 5 in IDES.45 In total, the formation of DES and IDES requires one nonmodified Lys residue and three Lya moieties. The ring is then formed by the nonmodified nitrogen atom of the ε-amino group of Lys, the ε-carbon of a Lya residue, and the δ- and ε-carbons of the other two Lya residues (see Figure 4). The valence of the nitrogen heteroatom causes an intrinsic positive charge, which is advantageous for molecular cross-link analysis as described in the next section. Apart from DES/IDES, other pyridinium-derived cross-linking structures were identified in elastin including neodesmosine (trifunctional),46 pentasine,47 and allodesmosine (pentafuctional).48

In addition to the functionality determined by the number of involved amino acid residues, cross-links can also be categorized by the number of involved TE monomers (intramolecular vs. intermolecular) or cross-linking domains (intradomain vs. interdomain). While intradomain linkages are always intramolecular, interdomain linkages can be formed both intra- and intermolecularly. The unique tetrafunctional cross-links in

**FIGURE 4** The formation of the major bi-, tri-, and tetrafunctional cross-links in elastin. Cross-linking is initiated by oxidative deamination of the side chains of lysine residues. The reaction produces a reactive aldehyde termed allysine. The aldehyde groups subsequently condense spontaneously with another allysine residue by an aldol condensation forming allysine aldol, or with another lysine residue to form dehydrolysinonorleucine. These bifunctional cross-links can further condense with each other to form the tetrafunctional cross-links desmosine and its isomer isodesmosine, which are unique to elastin. Alternatively, other intermediates such as the trifunctional merodesmosine can give rise to desmosine and isodesmosine formation by condensation with an allysine residue.
elastin are at least bi-domain linkages, but can theoretically link up to four domains. The formation of DES and IDES is generally considered occurring by the condensation of an AA with a ΔLNL-type intradomain cross-link to eventually connect two peptide strands.49

3.2 | Analysis of cross-links

The analysis of elastin cross-links is challenging because of their diversity, the vast number of possible combinations, and the repetitive nature of TE’s primary structure. Further complicating are elastin’s insolubility, its resistance to specific proteases, and the presence of different splice variants and posttranslational modifications such as prolyl hydroxylation.50 Analytical difficulties have hampered the comprehensive analysis of the cross-links in the past, but recent advantages have allowed new insights into the molecular structure of elastin. All analytical approaches described in the literature require the initial purification of elastin. The different techniques take advantage of elastin’s insolubility as well as its resistance to high temperatures, chemicals, and many proteases.51,52 Purified elastin can be enzymatically or chemically hydrolyzed into peptides or amino acids, which make the protein indirectly accessible for analytical investigations.

The pioneering qualitative and quantitative studies of cross-linking amino acids as well as the identification of cross-linking structures were accomplished through total hydrolysis of elastin followed by amino acid analysis. As mentioned above, the investigation of the TE domains involved in cross-linking as well as the exact determination of the cross-linking sites within the domains requires proteolytic hydrolysis. Elastases including some serine, cysteine, and metalloproteases serve this purpose,53 and cross-linking sites can be identified by analyzing the released cross-linked peptides. While peptide sequences and cross-link locations have been determined in the past with classical methods such as Edman degradation,54,55 more recent studies utilized sensitive high-resolution mass spectrometric (MS) analyses in combination with customized software.38,56,57 Bifunctional intradomain cross-links release internally cross-linked peptides with a cyclic structure upon hydrolysis, whereas bi- and higher functional interdomain cross-links are released as interconnected peptide species, which are challenging with respect to their analyses. The MS-based identification of cross-linked elastin-derived peptides can partly be performed with the help of specialized software. Intradomain linkages can be identified by any sequencing software that allows the incorporation of customized posttranslational modifications and databases as described.56 Additionally, customized software for the analysis of cross-linked peptides is available. StavroX/MeroX, for example, is a suitable freeware that allows for the analysis of MS data for both bifunctional intrapeptidal and interpeptidal cross-links, but requires manual validation.58 Even more complicated is the elucidation and assignment of higher functional cross-links. The analysis of such species, for instance peptides containing DES/IDES, benefits from a prior chromatographic enrichment. This method takes advantage of the peptide hydrophilicity caused by the intrinsic positive charge of the pyridinium ring. The detection and identification of tetrafunctional cross-links can be further assisted by a customized MS method, in which each peptide molecular ion is subjected to two consecutive tandem MS measurements. The first one is carried out at elevated collision energy, leading to the release of specific reporter ions upon dissociation of the pyridinium core. This facilitates the targeted screening for DES/IDES-containing peptides. The second tandem MS experiment uses lower collision energy and the respective spectra contain sequence-relevant information.57 To date, the locations of a high number of bi- and tetrafunctional cross-links have been identified. The involvement of distinct domains in tri- and pentafucational cross-linking as well as their quantitative contribution is still not known.

3.3 | Molecular sites

About four decades ago, Gerber and Anwar54,59 as well as Baig et al.60 used Edman degradation to analyze tetrafunctionally cross-linked peptides and were able to determine the amino acid sequence of peptide strands C-terminal to DES/IDES in bovine, porcine, and human elastin. They concluded that DES and IDES are formed by two Lys pairs after modification by the action of LOX. They found that three of these Lys residues are C- and N-terminally flanked by Ala or other small residues (e.g., Ser), while the fourth Lys residue is followed by a hydrophobic residue, such as Phe or Leu, which prevents it from LOX modification. This in turn means that DES/IDES are formed by the alignment of two KA domains after LOX-induced Lys modification. KP domains appear to be much less involved in DES/IDES formation, probably because of their relatively unordered secondary structure. The only proof for DES/IDES comprising a KP domain is a peptide from bovine elastin identified by Baig et al.60

In the 1990s, Brown-Augsburger et al. published the results of a study on cross-linked peptides from incompletely cross-linked elastin isolated from copper-deficient
pigs. They identified a complex cross-linked peptide comprising three peptide strands joined by one DES/IDES and two LNL cross-links. The strands originated from the DES/IDES-linked domains 19 and 25 of a TE monomer, where the third Lys residue of each of these two domains was additionally linked by LNL to one of the two Lys residues of domain 10 of another TE monomer. Based on these findings and the low-resolution three-dimensional structure of recombinant human TE, Baldock and coauthors proposed a head-to-tail model for TE polymerization suggesting a uniform molecular interconnection of primary TE fibers that presumably maturate through lateral cross-linking and hydrophobic interactions to form larger assemblies.

In two recent studies, we utilized sophisticated tandem mass spectrometric methods on proteolytically derived mixtures of noncross-linked and cross-linked peptides of mature bovine and human elastin. We identified a broad variety and high number of cross-links and gained additional information on the manifold nature of the cross-linking process. While bifunctional cross-links were expectedly found to connect two different cross-linking domains, we surprisingly also identified a high number of bifunctional intradomain cross-links, whose formation was shown before only in in vitro studies. As mentioned, those structures are thought to be intermediates in the formation of tetrafunctional cross-links, but had not been described before to exist in mature elastin. A stable intradomain cross-linking is rather unexpected for two reasons. On the one hand, those types of linkages can only stabilize local secondary structure elements within a single TE monomer and do not contribute to the spreading of impacting forces throughout the whole polymer as interdomain and especially intermolecular cross-links do. On the other hand, this stabilization of secondary structural elements is related to a steric compression of the hydrophilic cross-linking regions, and one could speculate that this might be beneficial for hydrophobic hydration, which is a basic requirement for elastin’s elasticity. Studies have shown that bifunctional LNL and AA intradomain cross-links can be found in every TE cross-linking domain. So far, intradomain linkages and their influence on elastin’s structure and properties have not been investigated, and they are considered as remains of the multistep pathway of higher functional cross-linking.

In our recent studies of bovine and human elastin, we sequenced several tetrafunctionally cross-linked peptides, in which all assignable peptide strands were derived from KA domains. In human elastin, the single-Lys cross-linking domain 14 was found to be involved in DES/IDES formation together with two other KA domains, which is in contrast to the previously described assumptions that postulated the involvement of only two KA domains in DES/IDES formation.

In conclusion, recent studies have shown that distinct Lys residues can be involved in different types of cross-links or even remain unaltered. One of the many examples for this diversity is Lys-104 in domain 6 of human elastin (see Figure 5, left half). The residue was found in its unaltered state (I), but also intramolecularly cross-linked to the adjacent Lys residue via AA (II) and LNL (III). It was furthermore involved in two different interdomain cross-links with domains 13 and 14 via LNL (IV) and AA (V), respectively, and took part in DES/IDES formation (VI). In contrast to some earlier postulated pathways and models referring to cross-link formation and TE polymerization, these findings reveal that the cross-linking pattern of elastin is heterogeneous with, if any, only a few restrictions. KP domains form intra-and interdomain bifunctional cross-links and would, thus, generally be capable of participating in tetrafunctional cross-linking. However, apart from one exception, KP domains have not been found in DES/IDES-containing peptides, suggesting they play a minor role. Despite the fact that most of the identified peptide species propose the formation of tetrafunctional cross-links from only two KA domains, the existence of tri-domain cross-linking via DES/IDES was shown. Uncondensed intermediate linkages remain as stable bifunctional cross-links in mature elastin and are another indicator of a stochastic cross-link pattern. The concept of an overall heterogeneous cross-linking of elastin is in high agreement with recently published findings of studies on TE’s self-assembly and the structural influence of Lys-Lya conversion. Modeling and in silico analysis of the coacervation process based on a coarse-grained model of TE revealed head-to-tail, head-to-head, tail-to-tail but also lateral alignment prior to cross-linking (illustrated in Figure 5, right part). Additionally, Lys-Lya conversion gives rise to the structural dynamics of TE that was found to be associated with a decrease in the population of a distinct set of three-dimensional structures, when the number of incorporated Lya residues per molecule was increased.

### 4 CROSS-LINKING ABNORMALITIES AND NONENZYMATIC CROSS-LINKING

Different inherited or acquired pathological conditions influence the structure, distribution, and abundance of elastic fibers in organs and tissues that are rich in elastin including the skin, lungs, or cardiovascular system. In the following, elastic-fiber pathologies affecting the cross-linking of elastin are summarized, which are mainly associated with mutations in the genes of LOX or participants of elastogenesis that interact with LOX and/or TE
during cross-linking (see Table 1). The proper function of LOX and consequently the cross-linking in mature elastin are vital for the survival of organisms. In fact, homozygous LOX−/− mice suffer perinatal death from aortic aneurysm and spontaneous dissection, and show highly abnormal aortic histology characterized by fragmented elastic fibers and aberrant smooth muscle cell layers due to deficient cross-linking.65,66

4.1 | Heritable diseases

Heritable diseases can be divided into fibrillinopathies and elastinopathies. While fibrillinopathies are caused by mutations in fibrillin genes and affect the formation of elastic fibers, elastinopathies are directly associated with changes to the elastin gene. Two fibrillinopathies that are connected to a deficient function of LOX are Menkes disease (MD; OMIM 309400) and occipital horn syndrome (OHS; OMIM 304150). MD and OHS are caused by mutations in the copper-transporting ATPase gene ATP7A, influencing different copper-dependent enzymes such as LOX, which leads to the formation of smaller, sparse, and fragmented elastic fibers.67,68 While MD is a lethal multisystemic disorder inherited as an X-linked recessive trait, thus affecting mainly male individuals, who die in their early childhood,69 OHS shows milder symptoms, has a later age of onset and shows less severe neurodegeneration.68

Another set of fibrillinopathies associated with alterations in cross-linking is autosomal recessive cutis laxa (ARCL) 1, of which subtype A (OMIM 219100) is caused by mutations in the genes encoding fibrillin-5, subtype B (OMIM 614437) is related to mutations in the fibrillin-4 gene, and subtype C (Urban-Rifkin-Davis syndrome, OMIM 613177) occurs as a result of mutation in the LTBP4 gene. ARCL1A, which goes along with loss of function mutations in FBLN5, is associated with severe cardiovascular manifestations such as supravalvular aortic stenosis, pulmonary artery stenosis, tortuosity of the arteries, and emphysematous changes in the lung. Interaction of elastin with the microfibril scaffold during elastogenesis is compromised in ARCL1A, because folding and secretion of fibrillin-5 are impaired. As a consequence, disorganized elastic fibers with granular appearance form Reference 70. It is not clear yet, which exact role fibrillin-5 has during elastogenesis, however, it is known that it binds LOX, fibrillin-1, TE, LTBP2, and

**FIGURE 5** Molecular and macromolecular structure of elastin. The analysis of cross-linked peptides from enzymatic digests of human elastin revealed that single lysine residues were involved in various types of cross-links or even remained unaltered. This is demonstrated by the example of the N-terminal lysine residue 104 (red) of the cross-linking motif of domain 6 in the left part of the figure. This residue was found in its unaltered state (I), but also cross-linked via allysine aldol (II), and lyasinonorleucine (III) to the adjacent lysin residue of domain 6. Moreover, it was found cross-linked to domain 13 (IV) and 14 (V) via lysinonorleucine and allysine aldol, respectively, and was also involved in DES/IDES formation (V). Amino acid residues depicted in gray were not part of the identified peptides and are just shown for clarity. The right part of the figure shows the structure of an elastic fiber consisting of an amorphous elastin core (yellow) and fibrillin-containing microfibrils (blue). The magnification symbolically shows an unordered network of tropoelastin monomers in the elastin polymer. The random orientation of individual tropoelastin monomers causes head-to-tail (N–C), tail-to-tail (C–C), head-to-head (N–N), and lateral interactions that give rise to a randomized cross-linking structure. DES, desmosine; IDES, isodesmosine
LTBP4 and facilitates elastic fiber formation through enhancing coacervation and cross-linking of TE. ARCL1B shows various systemic symptoms including vascular tortuosity, aortic aneurysm, developmental emphysema, skin and joint laxity, and arachnodactyly. Since fibulin-4 binds LOX, TE, fibrillin-1, and LTBP1, cross-linking is affected and elastic fibers are underdeveloped in the skin of ARCL1B patients. In fact, histological analysis of fibulin-4−/− mice revealed the presence of irregular elastin aggregates instead of intact elastic fibers, and DES content was strongly diminished. Homozygous fibulin-4−/− mice were further found to die perinatally. Symptoms of ARCL1C include developmental emphysema, tortuosity, and gastrointestinal malformations such as diverticulosis. Moreover, elastin was found to be located in large globular aggregates instead

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<td>Menkes disease (OMIM 309400)</td>
<td>Copper-transporting ATPase gene ATP7A</td>
<td>Collagen- and elastic-fiber abnormalities such as smaller collagen fibers and smaller, sparse and fragmented elastic fibers.</td>
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<tr>
<td>Occipital horn syndrome (OMIM 304150)</td>
<td>Copper-transporting ATPase gene ATP7A</td>
<td>Formation of smaller, sparse and fragmented elastic fibers, laxity of skin and joints, connective tissue disorders such as occipital exostosis (occipital horns)</td>
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<td>Autosomal recessive cutis laxa 1A (OMIM 219100)</td>
<td>Mutations in the genes encoding fibulin-5</td>
<td>Disorganized elastic fibers with granular appearance, supravalvular aortic stenosis, pulmonary artery stenosis, tortuosity of the arteries, emphysematous changes in the lung</td>
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<tr>
<td>Autosomal recessive cutis laxa 1B (OMIM 614437)</td>
<td>Mutations in the fibulin-4 gene</td>
<td>Underdeveloped elastic fibers and collagen bundles reduced in size, vascular tortuosity, aortic aneurysm, hypertension, developmental emphysema, skin and joint laxity, arachnodactyly</td>
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<tr>
<td>Autosomal recessive cutis laxa 1C (OMIM 613177)</td>
<td>Mutation in the LTBP4 gene</td>
<td>Dermal elastic fibers fragmented, elastin less present in microfibril bundles, but located external to the bundles in large globular aggregates, developmental emphysema, tortuosity, gastrointestinal malformations such as diverticulosis</td>
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<td>Acquired pseudoxanthoma elasticum</td>
<td>Unclear cause</td>
<td>Papular eruption, cutaneous mineralization, and fragmentation of elastic fibers leading to a lax redundant skin, in contrast to inherited pseudoxanthoma elasticum often limited to the skin and is not associated with systemic involvement</td>
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<tr>
<td>Age-related macula degeneration 3 (OMIM 608895)</td>
<td>Missense mutations that affect fibulin-5</td>
<td>Alterations of elastogenesis in the Bruch’s membrane of the macula</td>
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of microfibrillar bundles, and elastic fibers were fragmented. LTBP4 binds fibrillin-5, fibrillin-4, and fibrillin-1, which in turn binds to TE, mainly influencing elastin deposition onto the microfibrillar scaffold during elastogenesis with only an indirect impact on cross-linking.

Further heritable diseases include thoracic aortic aneurysms and dissections (TAAD), a large group of heterogeneous conditions, which in some cases are caused by missense mutations in the LOX gene that consequently go along with insufficient cross-linking of elastin and collagen in the aortic wall. Introduced into mouse genome, the mutation was found to cause disorganized ultrastructural properties of the aortic wall with fragmented elastic lamellae in heterozygous mice, whereas homozygous mice died after parturition from ascending aortic aneurysm and spontaneous hemorrhage.

### 4.2 Acquired diseases

Acquired elastic fiber disorders lead to an impairment of the structure and function of elastic fibers. Most of them such as acquired cutis laxa (CL), solar/actinic elastosis, acquired pseudoxanthoma elasticum (PXE), and age-related macular degeneration (ARMD) are related to the destruction of elastic fibers, but it is not clear if or to what extent cross-linking is changed. It is, however, likely that some peptides formed during breakdown of elastic fibers contain cross-links and eventually get excreted, which may change the amount of cross-links present in elastin. Acquired CL is associated with an inflammatory phase and systemic elastolysis and leads to skin laxity and is characterized by the appearance of erythematous papules or plaques. In the cardiovascular system, aortic rupture, emphysema, intestinal diverticula, and hernias can be observed. UV-induced extrinsic skin aging (photoaging) induces solar elastosis, which is characterized by coarse wrinkling, furrowing, and loss of elasticity along with an apparent thickening of the skin due to the accumulation of elastotic material in the upper and middle dermis. Elastotic material contains elastin, fibrillin, versican, as well as hyaluronic acid, that is, principal components of the elastic fibers. However, the supramolecular organization of elastotic material and hence its functionality are severely impaired. In fact, elastic fibers of elastotic materials seem to be cross-linked differently compared with normal elastic fibers as DES contents were found to be different. Acquired PXE only appears in the skin, where cutaneous mineralization and fragmentation of elastic fibers lead to a lax redundant skin. ARMD is the most common pathology that leads to irreversible visual loss in the Western world. It shows a complex pathology, and there is great genetic heterogeneity among the 15 types of ARMD that are caused by multiple mutations and polymorphisms of genes. ARMD3 (OMIM 608895) has been reported to be connected to missense mutations in the FBLN5 gene, resulting in decreased fibrillin-5 secretion and reduced elastogenesis followed by severe alterations in the structure of the Bruch’s membrane of the macula.

### 4.3 Nonenzymatic changes in cross-linking during aging

During aging of an individual, nonenzymatic glycation occurs in mature elastin as a consequence of its low turnover in human organs and tissues. After binding of sugar carbonyl groups to free amino groups in elastin, Schiff bases are formed during glycation and rearrange to ketoamines (Amadori’s product). Irreversible AGEs are eventually formed through chemical rearrangements and further oxidative processes (glycoxidation) from Amadori’s products and their degradation products glyoxal and 3-deoxy-glucosone. AGEs such as Nϵ-carboxy-methyl-lysine, Nϵ-carboxy-ethyl-lysine, and pentosidine are able to produce reactive oxygen species or interact with various cell surface structures. Overall, mature elastin exhibits only few glycation sites, that is, free ϵ-amino groups, as only few Arg residues are present and Lys residues are mainly involved in cross-linking. It has, however, been shown that elastin binding of glucose and ribose has a strong impact on the physical properties of elastin. Moreover, the amount of pentosidine in intervertebral disc elastin has been shown to increase with age. In aortic tissue, glycation enhances the stiffness of the elastic fibers of vessel walls, which has been described to occur in early stages of atherosclerosis and diabetes. On the molecular level, this is linked to conformational changes in elastin, the loss of basic groups, and alterations in interionic interactions. It has further been described that elastin shows differences in susceptibility toward enzymatic degradation and increased calcium deposition after reaction with glucose and ribose. AGEs may further lead to mechanical malfunction because of the formation of cross bridges between matrix molecules, and the progression of atherosclerosis may be accelerated through enhanced adhesion of circulating blood cells to the vessel walls. Typical elastin cross-links such as DES and IDES can also be modified by oxidation to oxodesmosin (OXO) and isooxodesmosine (ISOXO). The formation of OXO and ISOXO is not only age-related but also connected to pathophysiological conditions.
5 SUMMARY AND FUTURE DIRECTIONS

The unique cross-links in elastin are crucial for vertebrate life as they are responsible for the recoil of elastic fibers and thus contribute to structural integrity and biomechanics of dynamic tissues. Elastin's exceptional properties have made investigating its molecular assembly and structure one of the most interesting tasks in matrix biology. Technical and computational advances in the biological sciences in recent years enabled studies that have greatly enhanced the knowledge of TE's gene expression and synthesis as well as its assembly to mature elastin. The latest work on the coacervation and cross-linking stages highlighted in this review sheds new light on the molecular complexity of these processes and suggests heterogeneous interactions and cross-linking of the monomers.

Yet, many questions remain to be answered before a complete picture of the formation of elastic fiber networks is gained. Although numerous molecules directly or indirectly involved in elastogenesis have been discovered, little is known about their exact roles and underlying molecular mechanisms. Future research needs to focus on these and still unknown interactions, their order, timing, and tissue-specific differences. Variations in the monomer due to alternative splicing or prolyl hydroxylation and their impact on the formation and properties of polymeric elastin are further questions to be addressed.

A better understanding of elastogenesis and knowledge of the interactions with elastic fiber proteins and their degradation products is required to advance the recognition and treatment of genetic and acquired elastic fiber diseases. Detailed mechanistic information is further needed to develop strategies for reinitiating the formation of functional elastic fibers in regenerating tissues and to design advanced biomaterials and tissue-engineered constructs that resemble the ECM.

ACKNOWLEDGMENTS

The work was supported by the Fraunhofer Internal Programs under Grant No. Attract 069-608203 (C.E.H.S.) and by the LEO Fondet grant LF17063 (A.H.).

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REFERENCES


