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CHAPTER 2

Molecular Insights Into Prolyl And Lysyl Hydroxylation Of Fibrillar Collagens In Health And Disease

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ABSTRACT

Collagen is a macromolecule that has versatile roles in physiology, ranging from structural support to mediating cell signaling. Formation of mature collagen fibrils out of procollagen α-chains requires a variety of enzymes and chaperones in a complex process spanning both intracellular and extracellular post-translational modifications. These processes include modifications of amino acids, folding of procollagen α-chains into a triple helical configuration and subsequent stabilization, facilitation of transportation out of the cell, cleavage of propeptides, aggregation, cross-link formation, and finally the formation of mature fibrils. Disruption of any of the proteins involved in these biosynthesis steps potentially result in a variety of connective tissue diseases because of a destabilized extracellular matrix. In this review we give a revised overview of the enzymes and chaperones currently known to be relevant to the conversion of lysine and proline into hydroxyproline and hydroxylysine, respectively, and the O-glycosylation of hydroxylysine, and give insights on the consequences when these steps are disrupted.
INTRODUCTION

The extracellular matrix (ECM) is composed of several macromolecules, such as collagens, fibronectin, elastin, laminins, hyaluronan and proteoglycans. ECM molecules are synthesized and secreted locally by specialized cells predominantly belonging to the mesenchyme lineage (e.g. (myo)fibroblasts, chondrocytes, osteoblasts, tendocytes). The classical view of EMC function pertains its role in supporting tissues, either by maintaining its form, or by withstanding biomechanical forces. Beyond this, ECM molecules have roles in a wide variety of physiological functions, such as cell-cell signaling, adhesion, migration, cell differentiation and many more. Disruptions in ECM biosynthesis can therefore negatively influence all these processes.

Collagen molecules are composed of three α-chains that are, at least in part, folded into a triple helical conformation (triple helix). A collagen molecule can be composed of three α-chains expressed from a single gene (homotrimers), or from two or three different genes (heterotrimers) (Figure 1). The 28 known collagen types can be subdivided into nine families based on their structural features (1). One of these families are the fibril-forming collagens (type I, II, III, V, XI, XXIV and XXVII). The fibrillar collagen family can be further subdivided into clade A, B or C collagens, based on their evolutionary origin (2). Despite their structural differences, all collagen molecules (irrespective of its type) show stretches of helical formations of the three α-chains, and these stretches show repeated Gly-X-Y motifs (where X and Y can be any amino acid). Due to this resemblance, most of the collagen types rely on similar biosynthesis pathways. Biosynthesis of fibril-forming collagens is a multistep process, spanning both intracellular and extracellular post-translational modifications facilitated by various enzymes and chaperones (Figure 2). Mutations in several of these enzymes are
responsible for a number of heritable diseases that display extensive collagen defects. In addition, mutations in collagen genes and aberrant collagen deposition (as a result of aberrant synthesis, degradation, and/or modification) have also been reported in connective tissue diseases, as well as in pathologies ranging from fibrosis to cancer and arthritis.

Collagen molecules show a variety of post-translational modifications (PTMs), such as hydroxylation of many Pro and Lys residues into hydroxyproline (Hyp) and hydroxylysine (Hyl), and the subsequent glycosylation of Hyl. Even though these collagen PTMs have been studied in considerable detail in the last two decades, quite some question marks remain with regard to e.g. substrate specificities and structure/function relationships. Emerging evidence indicate that highly interconnected processes are fundamental to collagen PTMs. In this review we present a revised up-to-date view on which proteins are needed to work together in an assembly-line fashion for generating Hyp, Hyl and glycosylated Hyl, and the consequences these PTMs have on connective tissues.

**Figure 2. Biosynthesis of fibrillar collagens at a glance.**
After procollagen α-chains are imported into the ER, three individual α-chains associate to form a triple helix structure while simultaneously being subjected to post-translational modifications of Pro and Lys residues. Trimer formation and folding into a triple helix conformation starts with the association of the carboxyl-terminal propeptides and subsequent formation of disulfide bonds between the propeptides (I). During these processes, collagen chaperones prevent aggregation within the cell or premature association of procollagen chains. Following transport from the late-Golgi, the N-terminal pro-peptide is cleaved from procollagen (II). When deposited into the extracellular space (III), the triple helical procollagen is converted to collagen by cleavage of its remaining C-terminal propeptide (IV). Next, the resulting triple helical collagen molecule aggregate into fibrils that are subsequently covalently cross-linked to increase fibril stability (V).
HYDROXYLATION OF PROLYL RESIDUES

Prolyl 4-hydroxylation and Prolyl 3-hydroxylation
The triple helical part of collagen molecules consist of repetitive Gly-X-Y motifs, where the X and Y positions can contain any amino acid, but mostly are occupied by a proline (Pro) and a 4-hydroxyproline (4Hyp), respectively. One of the first steps in post-translational modification of procollagen is the conversion of triple helical Pro into Hyp. The Pro in the telopeptides are not converted in Hyp because of the lack of the Gly-X-Y motif. Two enzyme families, that reside in the lumen of the ER, catalyze the hydroxylation of helical proline residues (Figure 3). It are collagen prolyl-4-hydroxylases (C-P4Hs), which catalyze proline 4-hydroxylation (4Hyp) at the Y position in the Gly-X-Y motif of the alpha chains, and prolyl-3-hydroxylases (C-P3Hs), which catalyze proline 3-hydroxylation (3Hyp) at the X position in the Gly-X-4Hyp sequence of the alpha chains. Both families are 2-oxoglutarate dioxygenases: they need 2-oxoglutarate, O2, ascorbate and Fe²⁺ for its activity.

Collagen prolyl-4-hydroxylases: tetrameric proteins composed of P4H and PDI
The human collagen prolyl-4-hydroxylase (C-P4H) is a tetrameric enzyme composed of two α-subunits and two β-subunits. The β-subunit is encoded by P4HB; it is known as protein disulphide isomerase (PDI). Three isoforms are known from the α-subunit (designated as α(I), α(II), and α(III)), that are encoded by P4HA1, P4HA2 and P4HA3.

Figure 3. Collagen prolyl hydroxylation.
Prolyl (P) 4-hydroxylation at numerous positions is performed by the tetrameric complex P4HA/PDI, while PDI alone catalyzes disulfide bonds in the propeptides (both reactions indicated by the green arrow). Prolyl 3-hydroxylation is performed by the three different P3H isoforms at a few predetermined locations only. The complex of P3H1/CypB/CRTAP is responsible for P986 hydroxylation of the α-chains, whereas P3H2 hydroxylates P707 and P944 of the α-chains. P3H3 also hydroxylates Pro707, and additionally the C-terminal (GPP)n repeat. During these processes CypB performs cis-trans isomerization of collagen prolyl peptide bonds, and together with FKBP65 prevents premature association of procollagen α-chains.
respectively. The type I, II and III C-P4Hs are tetramers composed of \([\alpha(I)]2\beta2\), \([\alpha(II)]2\beta2\) and \([\alpha(III)]2\beta2\), respectively. Tetramers containing a mixture of two types of \(\alpha\)-subunits are unlikely to occur (3-6). The \(\alpha\)-subunit facilitates substrate recognition and enzymatic activity. The substrate recognition of C-P4Hs is mostly performed by the peptide-substrate-binding domain located at the N-terminus of the \(\alpha\)-subunits, whereas the catalytic domain is located at the C-terminus (7). After a hydroxylation reaction the affinity to the peptide-substrate-binding domain weakens and results in dissociation of the C-P4H (7). PDI monomers perform disulfide isomerase activity, however this activity is not required for C-P4H tetramer formation or its prolyl 4-hydroxylase activity (8, 9). Instead, as the \(\beta\)-subunit of C-P4Hs, PDI is responsible for retaining the tetramer in the rER through its C-terminus via its KDEL retention signal (8, 10). In addition, PDI performs a chaperone function by preventing aggregation of nascent procollagen chains (11, 12). Also, without the \(\beta\)-subunit the \(\alpha\)-subunit forms aggregates and is restrained from catalytic activity (13, 14).

Hydroxylation of proline residues occurs exclusively at individual unfolded procollagen \(\alpha\)-chains. Once the \(\alpha\)-chains are folded in a triple helical configuration, C-P4Hs are not able anymore to convert Pro into 4Hyp (15, 16). The three isoforms of P4HA are expressed in a tissue-dependent manner: \(P4HA1\) is expressed in most tissues, \(P4HA2\) is the main form in chondrocytes, osteoblasts as well as endothelial and epidermal cells (17-19), whereas \(P4HA3\) mRNA has been detected in many tissues, albeit in very low amounts (20). The overall amino acid sequence identity between the \(\alpha\)-subunits is rather low and ranges between 35 and 65%. The highest degree of similarity is observed at the C-terminal end, where the catalytic site is located. Nothing is known about substrate specificities of the \(\alpha\)-subunits in terms of affinities towards the different collagen types. Furthermore, two forms of both the \(\alpha(I)\) and \(\alpha(II)\) subunits are known, due to alternative splicing of \(P4HA1\) and \(P4HA2\), respectively. It remains to be seen whether these splice variant products display specific biological functions.

**Consequences of prolyl 4-hydroxylation**

4-Hydroxylation of collagen proline residues is essential for proper assembly and (thermal) stabilization of collagen triple helices through intramolecular hydrogen bonding (21). When collagen prolyl residues are underhydroxylated, the processed collagen molecules undergo abnormal assembly, being unable to form higher-ordered structures. In contrast to heterozygotes, \(P4ha1\) null mice show developmental delay and die between E10.5 and E11.5, presumably caused by ruptured capillaries resulting from a disturbed deposition of the basement membrane-specific collagen type IV (22). Isolated fibroblasts from these null mice were still able to secrete fibril-forming collagens (type I and III), but the resulting fibrils show a slightly increased diameter. Interestingly, \(P4ha2\) null mice are viable (discussed in (1)). In humans no mutations are found in the genes coding for any of the P4HA \(\alpha\)-subunits, perhaps indicating that in humans a loss of C-PH4 enzyme function leads to a premature death of the embryo. A heterozygous missense mutation in the C-terminal disulfide isomerase domain of P4HB, affecting disulfide isomerase activity but not prolyl-4-hydroxylation, leads to the osteogenesis imperfecta-like Cole-Carpenter syndrome (23).
Prolyl-3-hydroxylases
Prolyl-3-hydroxylases (also known as leprecans) are represented in three isoforms in humans, namely P3H1, P3H2 and P3H3, that are encoded by LEPRE1, LEPREL1 and LEPREL2, respectively. All contain a similar N-terminal domain, and a C-terminal 2-oxoglutarate-dependent dioxygenase domain with hydroxylase activity that shares homology with prolyl-4-hydroxylases and lysyl hydroxylases (24). Prolyl 3-hydroxylases have both unique and overlapping distribution in adult and embryonic tissues (25-27). In general, P3H1 is present at tissues rich in fibrillar collagens, whereas P3H2 is predominantly seen in basement membrane-rich tissues (26). P3H1 is identified as a member of a trimeric complex formed together with cartilage-associated protein (CRTAP) and cyclophilin B (CypB) (24), a complex that hydroxylates the α1(I) Pro986 residue of fibrillar collagen type α1(I) and collagen α1(II) (28-30). Another complex that resides in the ER consist of four different proteins, namely P3H4/P3H3/CypB/LH1, a complex that facilitates the activity of LH1 (31).

Prolyl-3-hydroxylation varies among fibrillar collagen types and tissue types
3-Hydroxyproline sites within GPP (specifically: Gly-Pro-4Hyp) sequences have been determined in the human α1(I), α2(I), α1(II), α1(III) and α2(V) chains. The following 3Hyp residues were found in these clade A collagen chains: Pro986 (also denoted as A1 site; 99% occupancy in α1(I), α1(II), and α1(V); 0% occupancy in α1(III), no GPP available in α2(II)), Pro944 (also denoted as A2 site; 60% occupancy in α2(V), 10-87% occupancy in α1(II), 0% occupancy in α1(I), α2(I) and α1(III)), Pro707 (also denoted as A3 site; 80% occupancy in α2(I) and α2(V), 0% occupancy in α1(I), α1(II) and α1(III)), and Pro470 (also denoted as A4 site; 13% occupancy in α2(V), 0% occupancy in α1(I), α2(I) and α1(III), no GPP available in α1(III)) (32, 33). Especially remarkable is the complete absence of 3Hyp in collagen type III, and the full occupation of 3Hyp at position Pro986 in α1(I), α1(II) and α2(V). 3Hyp has also been found in the (GPP)n repeat located C-terminally of Pro986 of α1(I), α2(I) and α1(II) (34). Clearly, there are major differences in the percentage of 3Hyp site abundancy between the different collagen chains, even though the sites are homologous. In addition, variations of 3Hyp levels are seen at the same residue of identical collagen chains from different tissues. Most strikingly are the differences in the degree of occupation of Pro944 by 3Hyp in α1(II) (highest in the cornea, lowest in articular cartilage, intermediate in nucleus pulposus) (32) and in the extreme C-terminal (GPP)n repeat in α1(I) and α2(I) (high in tendon, absent in skin and bone) (34) or α1(II) (high in cornea, low in cartilage) (35). It should be stressed that the above refers to mammalian species only, as the 3Hyp patterns can be different in lower invertebrates. For example, the A1 site of collagen type III is fully hydroxylated in Xenopus and chicken skin, whereas no such hydroxylation was seen in mammals. Further dissimilarities were already previously discussed (36).

Specificity of prolyl-3-hydroxylases
P3H depletion experiments deciphered the substrate specificity of P3H1, P3H2 and P3H3 towards collagen type II and the α2 chain of collagen type V (33). The authors
demonstrated that P3H1 is the primary enzyme for Pro986, that P3H2 is responsible (at least in part) for 3-hydroxylation of Pro944, Pro707 and the C-terminal (GPP)n repeat, and that P3H3 is partially involved in the 3-hydroxylation of Pro707 and the C-terminal (GPP)n repeat. The substrate specificity of P3H1 and P3H2 towards Pro986 and Pro707 in collagen type I, respectively, was confirmed in P3h1 null mice (37), in mice carrying a single amino acid substitution in the catalytic site of P3h1 (38), and in P3h2 null mice (35).

3Hyp residues have also been found in clade B collagen chains, namely α1(V), α1(XI) and α2(XI). Major hydroxylation sites (25-75% occupancy) are Pro434, Pro665 and Pro692 (32). However, nothing is known about the substrate specificity of P3H isoenzymes towards these clade B collagen chains. Furthermore, a high number of 3Hyp residues have been reported for collagen type IV: over 10 residues per α-chain (39). P3H1 does not seem to hydroxylate collagen type IV, but P3H2 does (27, 35).

Consequences of prolyl 3-hydroxylation
Although all three isoenzymes can be found together, the differences in expression at various developmental stages and some of the unique tissue specificities suggest major functional roles for each of the modifications. Indeed, mutations in LEPRE1, resulting in an absence of hydroxylation of Pro986, results in severe or lethal autosomal recessive osteogenesis imperfecta (29, 40, 41), whereas mutations of LEPRE2 results in high myopia in the absence of musculoskeletal abnormalities (35, 42, 43). An absence of Hyp986 might lead to decreased collagen-protein interactions, as has been shown for decorin (44), whose binding is near the collagen residues 961/962, which is in close proximity to 986 (45).

Based on the position of 3Hyp in the sequence of fibrillar α-chains, it was postulated that this amino acid is involved in the lateral association of collagen molecules as well as the D-staggered alignment of molecules in the initial assembly of collagen fibrils (36). The outward pointing direction of 3Hyp in the triple helix (46) may promote short-range hydrogen bonding between individual triple helices, thereby facilitating supramolecular assembly (32). Tendons from P3h1 null mice show that the basic molecular packing of collagen molecules along the fibril axis is the same and that there are no differences in the D-spacing, but that there is a more disordered packing (D-stagger distortions). In new-born P3h1 null mice the fibril diameter in tendons is increased, whereas in adult mice the fibrils are disorganized due to branching and fusing, most probably due to a failure of the postnatal lateral fusion of smaller fibrils (47). Interestingly, an increase in 5-hydroxylysine (Hyl) in its glycosylated form is seen in skin and tendon of P3h1 null mice (most likely due to an increased occupancy of normally modified Hyl, not due to additional modification sites), and especially in bone. The excess of glycosylation in bone might have a severe impact on the tight packing and mineralization of collagen, and it has been put forward that this excess glycosylation results in more severe disturbances than the lack of 3Hyp itself (47).

Although collagen type IV has the highest number of 3Hyp residues reported so far in collagens, the function of 3Hyp in collagen type IV is less understood. Half of these 3Hyp residues are located in the 7S domain (48). Both collagen type IV is and 3Hyp are ancient molecules, as both are found in sponges (49), which are the most
primitive multicellular organisms of the animal kingdom.

**P3H1 activity requires CRTAP and CypB**

Complex formation with CRTAP and CypB is necessary for P3H1 to perform proline-3-hydroxylation *in vivo* of unfolded collagen chains, which is decreased when either CRTAP or CypB is knocked-out or mutated (28, 50, 51). P3H1, CRTAP and CypB form a 1:1:1 complex (28) which is responsible for the 3-hydroxylation of Pro986 (due to P3H1). Mutations in P3H1 results in the loss of 3Hyp at position 986. Interestingly, mutations in CRTAP, a protein that show a high homology with the N-terminal end of P3H1 (but lacks the C-terminal catalytic domain), also results in the loss of 3-hydroxylation of Pro986. Finally, mutations in CypB result in decreased levels of 3Hyp at position Pro986, indicating that all components of the complex contribute to the functioning of P3H1.

The formation of complexes with CRTAP and CypB has not been confirmed for P3HA2 and P3HA3. Since there are no differences in 3-hydroxylation other than the residue Pro986 in osteogenesis imperfecta cases or null mice (41, 52), and since co-expression of CRTAP does not influence 3-prolyl hydroxylase activity of recombinant P3H2 *in vitro* (53), it is highly plausible that complex formation with CTRAP and CypB is not needed for P3H2 or P3H3 activity.

**HYDROXYLATION OF LYSYL RESIDUES**

**Lysyl 5-hydroxylation occurs in the telopeptides and in the triple helical part**

Lysyl hydroxylases (LHs) are present in the rER where they catalyze the conversion of lysine (Lys) into 5-hydroxylysine (Hyl) in the α-chains of procollagen. Hyl can be found in the triple helix and in the telopeptides (Figure 4A). Some of these Hyl residues are essential in the forming of certain covalent cross-links; furthermore, Hyl can serve as an attachment site for carbohydrates, resulting in galactosylhydroxylysine (GHyl) or glucosylgalactosylhydroxylysine (GGHyl). Additionally to collagens, LHs are also able to target other proteins with collagenous sequences, such as adiponectin (54). Just as the C-P4Hs and P3Hs, the lysyl hydroxylases belong to the 2-oxoglutarate dependent dioxygenases. In humans, as in rats and mice, three isoenzymes exist: LH1, LH2 and LH3, encoded by *PLOD1*, *PLOD2* and *PLOD3*, respectively. The expression of the LH genes is tissue-type dependent (55-57). All three isoforms are expressed during embryonic development of mice, but the levels of expression varies immense between the isoforms in different developmental stages. Whilst LH1 is expressed highly throughout development, LH2 is only highly expressed until day 7 and LH3 until day 7 and re-emerges at day 15, showing that the LH genes are specifically regulated during organogenesis (58).

Hydroxylation of Lys in the triple helix of collagen occurs exclusively on Lys present in the sequence Gly-X-Lys; a Lys in the X position is not hydroxylated. The hydroxylated Lys in the telopeptides is embedded in an entirely different amino acid sequence. In view thereof, the existence of two classes of enzymes has been postulated: a class of enzymes that converts the Lys in the triple helical sequence into Hyl (helical lysyl hydroxylase) and a class of enzymes that is responsible for the conversion of Lys
in the telopeptides into Hyl (telopeptide lysyl hydroxylase).

The Lys (and consequently Hyl) in the telopeptides can, after it is converted into allysine or hydroxyallysine by lysyl oxidases, interact with Lys, Hyl or His in the triple helix, leading to various divalent and trivalent cross-links (Figure 4B). Here, we restrict ourselves to certain cross-links derived from Lys and/or Hyl. The cross-link hydroxylysylpyridinoline (HP) is formed out of two hydroxyallysine residues from the telopeptides and a specific Hyl of the triple helix, whereas lysylpyridinoline is made of two hydroxyallysine from the telopeptides and a specific Lys of the triple helix. An increase of the total amount of HP and LP crosslinks reflects an increase in lysyl hydroxylation of the telopeptides, an increase of HP at the expense of LP reflects an increase in hydroxylation of the specific Lys residues of the triple helix that are involved in cross-linking. Likewise, an increase of LP at the expense of HP reflects a diminished hydroxylation of the same Lys residues in the triple helix. The triple helical Lys of α1(I) involved in cross-linking is K87 or K930, for α2(I) this is K87 or K933, for α1(II) this is K87 or K930 and for α1(III) this is K96 or K939. The N-telopeptide of α1(I), α2(I), α1(II) or α1(III) cross-links with the mentioned C-terminal Lys/Hyl in the triple helix, whereas the C-telopeptide of α1(I), α1(II) or α1(III) cross-links with the mentioned N-terminal Lys/Hyl in the triple helix (there is no Lys in the α2(I) C-telopeptide).

Figure 4. Collagen lysyl hydroxylation and cross-linking.
(A) Specific 5-hydroxylysine residues are reactive groups towards oxidation reactions that generate pyridinoline cross-links between two K9 residues and one K930 residue, and one K87 and two K1208 residues. (B) Lysyl hydroxylases (LH) need to form dimers for activity. For LH2, FKBP65 assists in the dimerization, whereas for LH1 the P3H4/P3H1/CypB complex could potentially drive this dimerization. LH2 specifically performs 5Hyl at both telopeptides regions at K9 α1(I), K5 α2(I) and K1208 α1(I). In collagen type I, LH1 seems to be the predominant isoform responsible for 5-hydroxylation of lysine in the helical domain of the α-chains. Here it performs 5-hydroxylation of at least K87 α2(I) and K930 α1(I) needed for hydroxylysylpyridinoline cross-linking.
The most direct evidence that a telopeptide lysyl hydroxylase must exists has been derived from cross-link studies in Ehlers-Danlos type VIA syndrome and Bruck syndrome. EDS-VIA patients show a decreased level of Hyl in the triple helix in certain collagen types in certain tissues, but show a normal level of pyridinoline or normal excretion levels of pyridinoline in urine (59, 60). Pyridinoline is a cross-link derived from the hydroxylysine route. Thus, in EDS-VIA, despite the deficiency of Hyl in the triple helix, a normal amount of Hyl is present in the telopeptides. From this it follows, that the mutated gene in EDS-VIA is a helical lysyl hydroxylase. In Bruck syndrome the opposite is seen: in bone, normal Hyl levels of the triple helix of collagen is seen, whereas hydroxylysine-derived cross-links (relying on telopeptide Hyl) are virtually absent (61). Thus, in Bruck syndrome, despite normal levels of triple helical Hyl, a deficiency of Hyl in the telopeptides is observed, showing the existence a telopeptide lysyl hydroxylase. The gene mutated in EDS-VIA is \textit{PLOD1} (62, 63), indicating LH1 is a helical lysyl hydroxylase, whereas the lysyl hydroxylase mutated in Bruck syndrome is \textit{PLOD2} (64-67), indicating LH2 is a telopeptide lysyl hydroxylase.

\textbf{Substrate specificity of LH1 and LH3}

Despite normal pyridinoline levels in EDS-VIA patients, the ratio of HP to LP has changed dramatically as LP is formed at the expense of HP. This indicates that the triple helical Lys residues involved in cross-linking collagen (\(\alpha1(I): K87\) or \(K930\); \(\alpha2(I): K87\) or \(K933\); \(\alpha1(II)\) \(K87\) or \(K930\); \(\alpha1(III): K96\) or \(K939\)) are not hydroxylated, meaning that LH1 specifically hydroxylate these residues and that LH 3 (or LH2) is not able to compensate for this. In normal tissues, most of the Lys residues in collagen IV and V are hydroxylated, whereas the lysyl hydroxylation level of collagen type I and III is much less. Mutations in LH3 gives rise to 30\% lower Hyl levels in a preparation of collagens IV and V, whereas no such decrease is seen in a preparation containing collagen I and III (68). Thus, LH3 does not seem to play a major role in the lysyl hydroxylation of collagen I and III. Indeed, no differences were found in the ratio HP/LP of bone (68), indicating that the Lys residues in collagen type I that participate in crosslinking are not hydroxylated by LH3. This is in agreement with the observation that a loss of LH1 leads to a replacement of HP into LP, showing that LH1 is primarily involved in hydroxylating the triple helical Lys residues involved in cross-linking (59).

The observation that the triple helical region of collagens II, IV and V is hydroxylated normally in EDS-VI patients, whereas collagen type I and III is not, already suggested the presence of two lysyl hydroxylases with different substrate specificities (69), as was the finding that the residual lysyl hydroxylase activity of EDS-VIA cells preferentially hydroxylated type IV collagen (70). From the available data it seems that LH1 hydroxylates most lysine in the triple helical region of collagen I and III (especially the residues involved in cross-linking), and that LH3 hydroxylates most lysine in the triple helical region of collagen types II, IV and V. However, this does not explain why EDS-VIA patients show differences in the triple helical lysyl hydroxylation of collagen type I derived from various tissues. Here, collagen type I from skin and bone in EDS-VIA is Hyl deficient, whereas collagen type I from tendon, kidney and lung is not (69). This observation still waits an explanation.

The notions above show that LH3 cannot compensate for the loss of LH1 in
the case of collagen I and III, and that the other lysyl hydroxylases cannot compensate for the loss of LH3 in the case of lysyl hydroxylation of collagen type IV and V. The loss of LH1 and LH3 activity does not lead to a decreased level of pyridinoline cross-linking, showing that neither enzyme is involved in the lysyl hydroxylation of the N- and C-telopeptides (only Hyl in the telopeptides can generate pyridinoline cross-links). This job seems to be restricted to LH2.

Substrate specificity of LH2
LH2 has two splice forms, LH2a (short) and LH2b (long), that result from alternative splicing of PLOD2, with LH2b containing an additional exon 13A of 63 bps encoding 21 amino acids. LH2b is ubiquitously expressed in almost all tissues examined, whereas LH2a is only expressed in lower amounts and always together with LH2b in kidney, liver, spleen, placenta and cartilage (55, 71-73). Inclusion of exon 13A is tissue-specific, and depends on the expression of RBFOX2, TIA1 and TIAL1 proteins that mediate splicing towards LH2b by binding to intronic cis-elements flanking exon 13A (55, 74-76). Suppression of these trans-acting factors by siRNAs decreased the levels of LH2b(long) and increased the levels of LH2a(short). Recently, also exonic cis-enhancer elements were found inside exon 13A that regulate splicing of LH2 via a currently unknown trans-acting protein factor (77). Whole tissue splicing patterns of LH2 changes during embryogenesis of mice. Until E11.5 LH2a is the main isoform present, where the expression of LH2b starts at E11.5 being the main isoform from then on (71).

LH2b is mostly seen in tissues rich in fibrillar collagens, whereas LH2a is mostly absent in such tissues. Expression studies in (precursors of) osteoblasts revealed that the elevation of Lys hydroxylation in the telopeptides of type I collagen coincide with a higher expression of LH2 mRNA (78), suggesting that LH2b is a telopeptide lysyl hydroxylase. This was indeed confirmed in later studies (64, 73, 79, 80). An absence of LH2b has a major impact on musculoskeletal tissues, especially bone, as illustrated by mutations in PLOD2. So far, nine mutations have been found in PLOD2, resulting in Bruck syndrome type 2 as well as an autosomal recessive form of osteogenesis imperfecta (64-67). All mutations are located in the C-terminal part of PLOD2, and are located between exon 12 and 19 (67). An absence of LH2 activity results in decreased levels of pyridinoline in bone. An elevated expression of LH2b has been recognized as a general phenomenon in fibrotic conditions of various tissues, where specifically LH2b is correlated to elevated levels of pyridinoline cross-links per collagen since LH1 and LH3 do not show a similar increased expression (73). These two examples describe an important role for LH2b in the formation of collagen telopeptide-derived pyridinoline cross-links.

Whether LH2b is also able to hydroxylate triple helical Lys is an as yet unanswered question. Unfortunately, the hydroxylation status of the triple helix has never been investigated in Bruck syndrome type 2 patients, but the Lys residues in the triple helix are hardly affected in Bruck syndrome type 1 patients (characterized by FKB10 mutations). Upregulation of LH2b by a variety of cytokines resulted in a substantial increase in hydroxylation of the telopeptides, but not in the triple helix (81). It thus seems likely, that LH2b only hydroxylates the telopeptides. On the other hand, co-expression of LH2b with proα1(I) in insect cells resulted in a substantial
amount of Hyl residues in the triple helix, as was the case for LH1 and LH3. In this expression system only LH2b was able to hydroxylate the N-telopeptide; hydroxylation of the C-telopeptide was not investigated. Addition of LH1 and LH2b in vitro to non-hydroxylated α1(I) as produced in Pichia pastoris resulted in comparable amounts of triple helical Hyl (82). In addition, LH2b is able to hydroxylate synthetic peptides exhibiting sequences derived from the triple helix (82, 83). Interestingly, LH2b was not able to hydroxylate the α1(I) N-telopeptide in a peptide sequence format (as was the case for the C-telopeptide), but it was able to do so when the N-telopeptide sequence was present in the proα1(I) procollagen chain (82). It thus seems that LH2b somehow requires the collagenous domain in order to hydroxylate the telopeptides.

Even less is known about LH2a. Since it is mainly absent in tissues rich in fibrillar collagens, it does not seem to play a role in lysyl hydroxylation of fibrillar collagens. Of the nine reported mutations of LH2, eight affect both transcripts, i.e. the mutations can have an effect on both LH2a and LH2b. One mutation specifically affects the splicing, and the patient only expressed LH2a but not LH2b (66). As the presence of LH2a (it replaces LH2b) leads to Bruck syndrome, it indicates that LH2a is not able to hydroxylate the telopeptides. However, the evidence for this statement is only indirect, as cross-link studies have not been performed, and expression analysis of LH2a in bone was not conducted.

**Lysyl hydroxylases are only active as dimers**

All three lysyl hydroxylases need to form homodimers for lysyl hydroxylase activity whereas this is not obligatory for the glucosyltransferase activity of LH3 (84, 85). Interestingly, dimerization between different isoforms was observed in vitro where LH3 was able to form a heterodimer with LH1 (85). However, there was a much higher tendency to form homodimers than heterodimers. We have shown that LH2a and LH2b also can form a heterodimer with LH1 and LH3, but that LH2a cannot form a heterodimer with LH2b (86). The ability to form heterodimers could broaden the specificity of the performed lysyl hydroxylase reactions for different collagens, and might partly explain the striking differences in lysyl hydroxylation levels between the different collagen types or the same collagen type between different tissues.

**P3H1 and CypB influence lysyl hydroxylase activity**

Strangely, mutations in proteins other than LHs can affect lysyl hydroxylation. Transgenic mice carrying a single amino substitution in the catalytic site of P3h1 did not show evidence of a generalized over-modification of collagen type I. The total amount of HP+LP was the same, but there was an increase in the HP/LP ratio. This ratio reflects the hydroxylation status of K87 and K930 in α1(I) and/or K87 and K933 in α2(I) that participates in cross-link formation. Since the hydroxylation status of K87 and K930 in α1(I) was unaltered, the difference in HP/LP ratio may be due to an increased hydroxylation at the homologous sites in the α2(I) chain (38). If so, the observation is still difficult to explain, as the triple helical cross-link sites of α1(I) and α2(I) are both hydroxylated by LH1. Is there a role of P3H1 in this phenomenon by affecting specific LH1 activities?

CypB also seems to interact with LH1, thereby having a positive effect on
LH1 activity (87, 88). A mutation of PPIB (CypB) in horses suffering from hyperelastosis cutis resulted in a decrease in Hyl and Glc-Gal-Hyl in skin and tendon, despite a delay in folding of procollagens in the rER (88). A delay in folding normally results in an overmodification. A dramatic decrease in Hyl was confirmed in skin of CypB null mice (89), whereas the amount of GHyl and GGHyl remained the same. A less striking decrease in Hyl levels, but still significant, was found in tendons (90), and the percentage of GHyl and GGHyl was higher, indicating an overmodification of the existing Hyl residues. Interestingly, in bone there was no decrease in Hyl, GHyl was increased, and GGHyl was, as Hyl, unchanged. A more detailed analysis of bone collagen type I revealed a systematic underhydroxylation of triple helical Lys involved in cross-linking (α1(I)K87, α2(I)K87, α2(I)K933), whereas most of the triple helical Lys residues that are not involved in cross-linking are normally hydroxylated or overhydroxylated. As a consequence, the ratio LP/HP in bone increased (89). An increased ratio of urinary LP to HP has also been reported in hyperelastosis cutis horses (88), again indicating an underhydroxylation of the bone triple helical Lys involved in cross-linking. A preferential underhydroxylation of α1(I)K87 and α2(I)K87 was also found in tendons and, interestingly, an increase in the hydroxylation of the N- and C-telopeptides was observed in the CypB null mice (90). It was shown with immunoprecipitation that CypB interacts with LH1, LH2 and LH3; the modification patterns in CypB mice show that CypB somehow regulates the function of LHs.

**SC65 influence LH1 but not LH2 activity**

Interestingly, also the protein synaptonemal complex 65 (SC65 = LEPREL4 = P3H4) plays a role in lysyl hydroxylation. It forms a complex with P3H3, CypB and LH1. An absence of P3H4 does not affect the lysyl hydroxylation level in the telopeptides, but results in severe under-hydroxylation of the helical-domain lysines that control whether HP of LP cross-links are formed, resulting in increased levels of LP at the expense of HP. Thus, P3H4 is required for the activity of LH1, but not LH2 (31). Since LH1 is only active as a dimer, it means that either the P3H4/P3H3/LH1/CypB complex is of a pentameric nature, or the P3H4 complex assists in LH1 dimerization as has recently been observed for FKBP65 in LH2 dimerization (86).

**FKBP65 influences lysyl hydroxylase activity**

Another protein that indirectly affects lysyl hydroxylation, namely in the telopeptides, is FKBP65, encoded by FKBP10. The N- and C-telopeptide of α1(I) were specifically underhydroxylation in Fkbp10 null mice, while there were no differences in the helical domain (91). Interestingly, postnatal FKBP10 expression is limited to bone and ligament, reflecting the bone and contracture phenotype of Bruck syndrome (91). Bruck syndrome type 1 and some autosomal recessive cases of OI are caused by mutations in FKBP10 (61, 67, 92-100). Intriguingly, its phenotype is similar to that of Bruck syndrome type 2 that is caused by mutations in PLOD2. Both syndromes show the same biochemical defect, namely a strong underhydroxylation of the telopeptides of collagen type I (61, 97, 98, 101), resulting in a pyridinoline content that is 5-10% of that in control bone. In contrast, in all of the human subjects a normal or a slight increase in the hydroxylation of the triple helical Lys residues is seen (61, 97, 98, 101).
Remarkably, in a patient with a FKBP65 null mutation a HP:LP ratio of 1.0.27 was observed, whereas in control bone this ratio was 2.6:1 (101), despite the fact that peptide mapping showed a normal lysyl hydroxylation level of α1(I)87 and α1(I)930 of the non-crosslinked peptides. Schwarze et al. (2013) explained this by postulating that the few hydroxyallysine in the telopeptides show preferential bonding to Lys over Hyl of the partially hydroxylated triple helical sites. However, what makes this explanation less likely is the normal HP:LP ratio seen in bone of a case described by Bank et al. (1999), the case that showed the c.344G>A (p.Arg114Gln) point mutation in FKBP10 (101). Another remarkable observation is, that collagen type I in ligaments showed a normal pyridinoline crosslink level (61), as was the case for collagen type II in cartilage (61, 101). It thus seems that FKBP65 is required for LH2b activity in bone tissue, but not in other tissues, although such a mechanism is difficult to explain.

A deletion of Tyr293 in the third PPIase domain of FKBP10 has been found in the autosomal recessive Kuskokwim syndrome (98), a disorder found in Yup’ik Eskimos living around the Kuskokwim River (Alaska, USA). This syndrome is characterized by congenital contractures of large joints with spinal, pelvic and foot deformities but with only minor skeletal manifestations. It thus extends the phenotypic spectrum of FKBP10 from OI alone, and OI with contractures (Bruck syndrome), to a predominantly congenital contracture syndrome. A residual (2-10%) lysyl hydroxylation of the telopeptide Lys1208 residue was observed, and patients with a 2%-3% residual telopeptide hydroxylation had more severe skeletal symptoms than that of patients with 8%-10% hydroxylation. This residual lysyl hydroxylation might explain the minor skeletal features of the Kuskokwim syndrome compared to Bruck syndrome (98), where an essentially undetectable hydroxylation of Lys1208 is seen. It has been postulated that the impaired telopeptide hydroxylation perhaps disturbs the specific linear secretion and deposition through fibripositors of collagen in tendons (a distinctive pathway for tendons), explaining the congenital contractures.

We have recently shown that FKBP65 forms a complex with LH2 but not with LH1 and LH3, and that inhibiting the PPIase activity of FKBP65 prevents the formation of LH2 dimers. As only dimers show lysyl hydroxylase activity, this explains why mutations in FKBP65 has such a dramatic impact on LH2 (86). Surprisingly, also CypB can bind to LH2, as well as to FKBP65. However, in contrast to FKBP65, CypB seems to inhibit LH2 activity, as an absence of CypB results in higher hydroxylation levels of the telopeptides (90).

**SLC39A13 is involved in collagen modification**

The gene encoding the membrane-bound zinc transporter ZIP13 (SLC39A13) is mutated in the spondylocheiro-dysplastic type of EDS (102). These patients have an increased lysyl pyridinoline / hydroxylysyl pyridinoline (LP/HP) ratio in the urine, indicating that the triple helical lysyl residues involved in cross-linking are underhydroxylated. Collagen produced by fibroblasts showed an overall underhydroxylation of Lys residues of the triple helix (i.e. it was not confined to the triple helical Hyl involved in cross-linking), and also lower levels of 4Hyp and 3Hyp. Thus, hydroxylation of collagen as a whole was generally lower. It is not known how ZIP13 is involved in this process, but it has been hypothesized that a zinc deficiency in the ER leads to a more general
ER dysfunction rather than reducing the hydroxylase activity of the prolyl- and lysyl hydroxylases (103).

GLYCOSYLATION OF HYL RESIDUES

Two glycosylated forms of Hyl
The heterogeneity of collagen is also markedly enhanced by O-glycosylation of Hyl via the 5-hydroxyl group with a monosaccharide (β-D-galactopyranose, Gal) or a disaccharide (α-D-glucopyranosyl-(1->2)-β-D-galactopyranose, GlcGal). Thus, two glycosylated forms of Hyl exist: galactosylhydroxylysine (GHyl) and glucosylgalactosylhydroxylysine (GGHyl). The first step, the addition of galactose to Hyl, is catalysed by glycosyl transferase 25 domain 1 (GLT25D1) and glycosyl transferase 25 domain 2 (GLT25D2), whereas the second step, the addition of glucose on GHyl, is catalysed by LH3, finally resulting in GGHyl (Figure 5).

GLT25D1 and GLT25D2 catalyzes the formation of GHyl
Glycosyl transferase 25 domain 1 (GLT25D1) and glycosyl transferase 25 domain 2 (GLT25D2) are ER localized enzymes that show high hydroxylysine galactosyltransferase activity towards collagen and mannose binding lectin (104, 105). The distribution of these two collagen β(1-O)galactosyltransferase enzymes is tissue-dependent; GLT25D1 is expressed in almost all tested human adult and fetal tissues except for tissues belonging to the nervous system, whereas GLT25D2 is expressed specifically in the nervous system (104). Whether both enzymes target similar substrates is currently unknown. Both enzymes contain three DXD (Asp-any residue-Asp) sugar-binding motifs, but only the first two motifs (amino acids 166-168 and 461-463) are needed for galactosyltransferase activity in GLT25D1 (106). Based on their overall homology it is likely that the same motifs are responsible for the activity of GLT25D2. Reducing GLT25D1 expression (31-67%) via RNA interference resulted in a concomitant (35-65%) decrease in total collagen galactosyltransferase activity. Interestingly, GLT25D1 and GLT25D2 failed to show any significant glucosyltransferase activities. As recombinant GLT25D1 and GLT25D2 had high galactosyltransferase activities towards denatured (deglycosylated) collagen α-chains (104), GLT25D1 and GLT25D2 are considered the major contributors to galactosylate hydroxylysine residues of collagens prior to LH3 glucosylation of these galactosylhydroxylysine residues. This was confirmed by the observation that in bone, collagen type I galactosylation of Hyl is carried out by GLT25D1 whereas the subsequent glucosylation is performed by LH3 (107, 108).

Based on the specific colocalization of GLT25D1 with other ER specific proteins (105), it is highly likely that galactosylation of collagen hydroxylysine residues by these two enzymes occurs in the early protein secretory pathway, even before the transport of procollagen to the Golgi apparatus. Indeed, GLT25D1 showed a comparable subcellular localization as LH3, and of one of its substrates mannose binding lectin (105). A protein that is similar to GLT25D1 and GLT25D2 in terms of sequence identity and domain organization is the cerebral endothelial cell adhesion molecule (CEECAM1). However, no collagen glycosyltransferase activity could be detected (105);(106), most
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LH3 catalyzes the formation of GGHyl
The glycosyltransferase activity of LH3 is located in the N-terminus of the molecule, whereas the LH activity resides in the C-terminal part (84, 109-111). A loss of glycosyltransferase activity of LH3 does not affect its lysyl hydroxylase activity. From a phylogenetic point of view, LH3 is by far the oldest of the three LH iso-enzymes (112, 113), while during evolution LH1 and LH2 lost the glycosyltransferase activity seen in LH3.

Although galactosyltransferase activity for LH3 has been detected in vitro (111), the galactosyltransferase activity of LH3 in vivo is much lower than its glucosyltransferase activity (84). It thus seems that LH3 does not play a significant role in the formation of GalHyl in collagen. Indeed, LH3 null mice tissues show unaltered enzymatic galactosyltransferase activities (114), indicating the presence of other enzymes with such activities. These other enzymes are most likely the above mentioned GLT25D enzymes, as LH1 and LH2 do not show any glycosyltransferase activity (84, 109, 110). In this context it is of interest to note, that a deficiency of LH3 activity leads to a decrease in urinary GGHyl levels, but not GHyl levels (115).

The combination of lysyl hydroxylase and glycosyltransferase activity in a single enzyme is not unique for LH3. A bifunctional lysyl hydroxylase and glycosyltransferase enzyme has been discovered in mimivirus, a giant virus recovered from Acanthamoeba polyphaga, namely the protein encoded by LR230 open reading frame (LR230 ORF) (116). Interestingly, LR230 ORF transfers glucose to Hyl, whereas in the animal kingdom, ranging from sponges to humans, galactose is transferred to Hyl. So far, glucosylhydroxylysine has only been found in viral collagenous sequences.

Figure 5. Glycosylation of hydroxylysines and collagen assembly.
Hydroxylysines can be subjected to successive glycosylation. First, GLT25D1 or GLT25D2 converts 5Hyl into galactosylhydroxylysine (GHyl). Afterwards, LH3 converts GHyl into glucosylgalactosylhydroxylysine (GGHyl). During and after addition of the various PTMs, collagen folding occurs from the C-terminus to N-terminus during which FKBP65 and HSP47 control the aggregation.
Biological role of glycosylated Hyl

The biological significance of collagen glycosylation is still under investigation. The presence of $\text{GHy}l$ in the $\alpha_1(\text{IV})1263-1277$ region results in a dramatic reduction of melanoma cells adhesion, as it inhibits CD44 binding (117). Glycosylation of Hyl in the sequence $\alpha_1(\text{IV})531-543$ also results in significant inhibition of melanoma cell $\alpha_3\beta_1$ integrin binding (118). As melanoma cell adhesion to basement membranes (which are rich in collagen type IV) is a critical step in the onset of metastasis, one may conclude that glycosylation of collagen type IV diminishes tumor cell invasion.

The collagen receptor Endo180 (uPARAP) is involved in the endocytic uptake of collagen. It was found that the CTLD-2 lectin domain of Endo180 binds to glycosylated Hyl, leading to an increased endocytic efficiency of Endo180 toward highly glycosylated collagen (such as collagen type IV). Remarkably, binding of collagen to the mannose receptor (MRC1 = CD206), being another collagen receptor involved in the endocytic uptake of collagen and a member of the same protein family as Endo180, is not influenced by glycosylated Hyl (119). The other two family members of Endo180, namely the phospholipase A2 receptor and DEC-205, do not directly bind to collagen (120). As Endo180 is involved in collagen turnover during malignant progression (121), it seems that glycosylation of Hyl enhances tumor cell invasion, which is opposite to the finding presented above with regard to melanoma cell invasion.

Glycosylated Hyl is also important in the induction of collagen-induced arthritis. Immunization with underglycosylated collagen type II results in a lower severity of collagen-induced arthritis (122, 123), and a subset of T cells specifically recognized glycosylated Hyl (124, 125).

The presence of Hyl is not required for collagen fibril formation, as collagen devoid of Hyl is able to form native-type fibrils (126, 127). However, glycosylated Hyl has an effect on fibril diameter: increased glycosylation levels results in thinner fibrils (128, 129). Hyl or glycosylated Hyl does not seem to have an effect on the thermal stability of collagen (130).

There are major differences in the glycosylation status of Hyl in position $\alpha_1(\text{I})87$. In bovine bone and skin, and in mouse bone more than 90% of the Hyl is glycosylated. However, the ratio $\text{GGHyl}$ to $\text{GHy}l$ in bovine bone is 1:5, in mouse bone 3:1, and in bovine skin 1:1. In rat tail tendon less than 10% of the Hyl in $\alpha_1(\text{I})87$ is glycosylated, in a $\text{GGHyl}$ to $\text{GHy}l$ ratio of 1:1 (131). Thus, there are tissue-specific differences in the glycosylation status of $\alpha_1(\text{I})87$, but there are also species-specific differences within the same tissue. In addition, within the same tissue, the glycosylation pattern of homologous sites on the $\alpha_1(\text{I})$ and $\alpha_2(\text{I})$ chains differ markedly (132): $\alpha_1(\text{I})87$ is a major glycosylation site whereas $\alpha_2(\text{I})$ is hardly glycosylated. For $\alpha_1(\text{I})174$ and $\alpha_2(\text{I})174$ the situation is reversed. Impairment of the glycosylation reaction (carried out by LH3) on $\text{GHy}l$ results in a faster self-aggregation of collagen molecules and in thicker fibrils. In addition, alterations are seen in cross-link formation and maturation, as well as a delayed mineralization of the collagen fibrils (132, 133).

It has been postulated that glycosylated Hyl is positively involved in the binding of DDR2 towards collagen (134, 135), as DDR2 was unable to bind to collagen that was treated with periodate to deglycosylate collagen. However, periodate also
oxidases unsubstituted Hyl, and it is likely that this oxidation is the reason for not binding of DDR to collagen, rather than the absence of glycosylated Hyl (136).

Mutations in the glycosyltransferase domain of LH3 has major implications for the formation of collagen type VI and VI structures, both collagens normally being highly glycosylated. Lack of glycosyltransferase activity of LH3 in mice results in early lethality because of a defective basal membrane (68, 137) due to the diminished secretion of collagen type IV and defects in the assembly mechanism of collagen IV tetramers in the extracellular space. An absence of the lysyl hydroxylase in Caenorhabditis elegans and Sophophora (possessing glycosyltransferase activity) is also lethal because of a lack of basement membranes (138, 139). Furthermore, in mice, defects in tetramer formation of collagen VI as a consequence of the lack of glycosylated Hyl was obvious (137), and in addition defects are seen in the extracellular aggregation and distribution of collagen VI. Tetramer formation of collagen VI takes place in the cell itself and is a unique property of collagen VI. Outside the cell collagen IV segregates into higher-order structures, namely beaded microfibrillar networks. In man, mutations of LH3 have been found in two families (115, 140), resulting in a syndrome of congenital malformations severely affecting many connective tissues with skin blisters that resemble epidermolysis bullosa. In zebrafish, it has been shown that the presence of LH3 is critical for motor axon migration and neural crest cell migration (141, 142).

Knock-out studies in mice revealed that the lack of glucosyltransferase activity of LH3 causes embryonic lethality, whereas the lack of LH activity of LH3 resulted in a normal embryonic development (68). Since galactosyltransferase activity of LH3 is likely to be minimal, it shows that the glucosylation of galactosylhydroxylysine by LH3 has a major functional role. Interestingly, a reduction of LH3 leads to changes into the cytoskeletal structure, most likely because of the impaired extracellular matrix organization (140).

In addition to the ER, LH3 is found in the extracellular space as well, in serum, and at the cell surface where it seems to associate with collagenous proteins (143). The secretion is dependent on LH3 glucosyltransferase activity; secretion into the cell medium was nearly eliminated when this active site was mutated. Two ways for actively secreting LH3 have been identified, either via a Golgi-dependent or via a Golgi-independent pathway (144). The exact function of extracellular LH3 glycosyltransferase activity is not completely understood, although differences are reported in cell growth and viability when there is an extracellular deficiency of LH3 (145). Interestingly, LH3 was able to modify (hydroxylate and glycosylate) extracellular proteins in their native conformation in vitro (143). Wang et al. speculate for an extracellular role of LH3 in regulating cell behavior via cell-matrix interactions or receptor activation, but the LH3 binding partners are yet to be identified.

REGULATION OF COLLAGEN FOLDING

**Procollagen folding rate affects the level of lysyl and prolyl hydroxylation**

Already during post-translational hydroxylation of proline and lysine residues and glycosylation of hydroxylsines, three selected alpha chains associate at the C-terminus
and are connected by disulfide bonds induced by PDI. Triple helix formation continues from the carboxy-terminus in a zipper-like fashion towards the amino-terminus (Figure 5). Hydroxylation of Pro into 3Hyp and 4Hyp, hydroxylation of Lys into Hyl, and subsequent glycosylation of Hyl into GHyl or GGHyl occurs on the nascent α-chains. Once the chains are folded into a triple helix the enzymes responsible for these modifications cannot catalyze their reactions anymore. As a correct functioning of collagen molecules in the extracellular space is to a large extent determined by the quality as well as quantity of the mentioned post-translational modifications, it is clear that a correct rate of folding of procollagen is of considerable importance. Slowing down the collagen folding rate, e.g. due to mutations in the collagen α-chains, results in overmodification, as is seen in osteogenesis imperfecta.

During the process of synthesis and triple helix formation cis-trans isomerization of peptide bonds are catalyzed by peptidyl-prolyl cis-trans isomerases (PPIase). In fact, the rate-limiting step of triple-helix formation of collagen (a process that proceeds from the carboxyl-terminal end toward the amino-terminal end in a zipper-like fashion) is the cis-trans isomerization of peptide bonds. So far, three PPIases have been reported to act on procollagen. It is FK506 binding protein 22 (FKBP22, encoded by FKBP14), FK506 binding protein 65 (FKBP65, encoded by FKBP10) and cyclophilin B (CypB, encoded by PP1B) (146, 147). FKBP22 and FKBP65 are members of the immunophilins, a group of proteins that exhibit PPIase activity and that can bind to immunosuppressive drugs such as FK506 (Tacrolimus) and rapamycin (Sirolimus).

**Immunophilins Hardly Affect Procollagen Folding Rate**
FKBP22 binds to collagen type III, VI and X, but not with collagen type I, II or V (148). It seems to prefer 4Hyp (148,149), so this chaperone likely acts mainly after prolyl-4-hydroxylation has taken place. Substrates other than collagen have so far not been reported for FKBP22. Mutations in, or a complete absence of, FKBP22 results in Ehlers-Danlos syndrome that resembles the kyphoscoliotic type (150-152). The substrate specificity of FKBP22 fits with the observed phenotypes; bone and articular cartilage are not affected in these patients, which is in agreement that FKBP22 does not bind to type I, II, or V collagen.

Another PPIase that associates with collagen is FKBP65. A role of FKBP65 as a molecular chaperone of collagen by assisting in protein folding via the conversion of cis- to trans-isomers of peptidyl-prolyl bonds seems attractive. Such a chaperone function for FKBP65 has been described for tropoelastin (153). However, the PPIase activity of FKBP65 does not seem to be as important for the formation of the procollagen triple helix, as inhibitors of the PPIase activity of FKBP65 had only a marginal effect on the rate of folding of the triple helix (154,155). Also, mutant FKBP65 does not result in collagen overmodification (i.e. higher levels of (glycosylated) Hyl). In addition, it does not affect the normal 3-hydroxylation of Pro986 of the α1(I) chain (91,92,97,101), suggesting that it acts after the prolyl-3-hydroxylation complex (156).

FKBP65 interacts both with unfolded α-chains (gelatin) and triple helical collagen, and it is able to delay fibril formation in vitro (i.e. it inhibits collagen aggregation) (157). Therefore, it is assumed that FKBP65 prevents premature association between procollagen chains during synthesis, as well as premature aggregation.
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between triple helical procollagen molecules. This chaperone function of FKBP65 is not inhibited by FK506 or cyclosporin A, whereas both drugs inhibits its PPIase activity, indicating that PPIase activity is not involved in the chaperone function (157). Interestingly, FKBP65 initiates coacervation of tropoelastin, a chaperone function that is also independent of its PPIase activity (158,159). Homozygous knock-down of the FKBP10 gene in mice is lethal. Secretion of collagen was not delayed, but intracellular trafficking was abnormal as cells obtained a dilated ER with embedded matrix as well as intracytoplasmic collagen fibers (91), hence indicating the chaperoning function of FKBP65. In the human situation, type I procollagen aggregates are either seen (92) or not seen (101). As in null mice, secretion of collagen was normal or only slightly delayed (92,97,98,101); trimerization and the folding rate were normal as well (97).

Cyclophilin B Affects Procollagen Folding Rate
CypB has PPIase activity towards collagen both in its free form as well as in the P3H1/CRTAP/CypB complex form (160). PPIase activity of CypB is markedly higher as that of FKBP22 or FKBP65. Since the rate-limiting step of triple-helix formation of collagen (a process that proceeds from the carboxyl-terminal end toward the amino-terminal end in a zipper-like fashion) is the cis-trans isomerization of peptide bonds, CypB is involved in the speed of triple helix formation. Almost all CRTAP and P3H1 mutations result in overmodification of the collagen helical region (i.e. a delayed folding of the procollagen chains is seen due to a decreased peptidyl-prolyl cis-trans isomerase activity), indicating that these components of the complex on its turn contribute to the functioning of CypB. Indeed, the PPIase activity of CypB is highest when it forms a complex with P3H1 and CRTAP (160), revealing an interdependence of the complex members. About 16% of the Pro and 8% of the Hyp in nascent collagen type I show the cis-configuration (161). Inhibition of the activity of CypB by means of cyclosporin A disrupted the stability, folding and assembly of collagen, showing that the cis-bonds must be converted into the trans-bonds (155,162). Although CypB has long been attributed as the only collagen isomerase, the observation that an absence of CypB can result in a normal helical modification (indicating normal folding), favours the hypothesis that there is more than one collagen isomerase (163). It seems the P3H1/CRTAP/CypB complex acts as a molecular chaperone during collagen synthesis (164), and behaves as a disulfide isomerase in the rER (147). Alternative roles for CypB are suggested as a retro-translocator of abnormal folded collagen to facilitate proteasomal degradation in the cytosol, a role that was discovered in CypB null mice, which show abnormally localized collagen in the ER consistent with a delay in processing or translocation for proteasomal degradation (165). Finally, CypB seems to be involved in the initiation of the triple helix formation via the folding of the C-propeptides of type I collagen (166).

Pathological Consequences Of Aberrations In Prolyl And Lysyl Hydroxylation
Several heritable connective tissue diseases result in aberrations of prolyl and lysyl hydroxylation of fibrillar collagens, due to mutations in collagen (and subsequent problems in folding rate of the α-chains), to mutations in lysyl hydroxylases or prolyl hydroxylases, or due to mutations in proteins that somehow affect the activity of one of
the hydroxylases (Table 1). Also acquired diseases, such as osteoporosis, osteoarthritis, tendinitis, cancer and fibrosis, show changes in the quality of the collagen molecules, mainly because of altered lysyl hydroxylation levels.

The kyphoscoliotic type of Ehlers-Danlos syndrome (EDS type VIA) is caused by mutations in \textit{PLOD1}, but mutations in \textit{SLC39A13} (102), \textit{PLOD3} (115) and FKBP14 (150-152) have been correlated to EDS-like phenotypes. The first is now classified as the spondylocheiro-dysplastic type of EDS, whereas the latter has not yet been officially classified as an EDS subtype.

Osteogenesis imperfecta (OI) is heterogeneous heritable connective tissue disorder manifested by low bone mass and strength that result in bone fragility, deformity and growth defects. The underlying cause of OI is directly related to a deficiency of normal collagen type I deposition in the ECM. The mildest and most prevalent form of the autosomal dominant OI types, result from diminished synthesis of structurally normal procollagen type I caused by heterozygous mutations in either the \textit{COL1A1} or the \textit{COL1A2} gene. The more severe to lethal forms of the autosomal dominant OI types result from structural defects of the procollagen type I chains (167). Genetic disruptions in several non-collagen genes have currently been identified that result in an autosomal recessive OI phenotype (168) (reviewed by (169-171)); six of these loci (\textit{LEPRE1}, \textit{CRTAP}, \textit{PPIB}, \textit{SERPINH1}, \textit{FKBP10} and \textit{BMP1/mTLD}) seems to have a central function in collagen type I biosynthesis.

Bruck syndrome is a rare autosomal recessive disorder characterized as osteogenesis imperfecta with congenital joint contractures. Bruck syndrome is subdivided into two types that are phenotypically indistinguishable, and classification is rather based on underlying genetic mutations. Bruck syndrome type 1 is caused by mutations of \textit{FKBP65} encoded by \textit{KFBP10} (67,92,94,96,101,172,173), whereas Bruck syndrome type 2 is due to mutations in \textit{LH2} encoded by \textit{PLOD2} (64-67). In contrast to OI, Bruck syndrome patients show normal secretion of collagen type I, and no mutations in \textit{COL1A1} or \textit{COL1A2} are detected (174). The molecular background of Bruck syndrome development is a reduction in trifunctional cross-linking of collagen type I caused by an under-hydroxylation of lysyl residues in the telopeptides but with normal helical lysyl hydroxylation (61). It is LH2 that hydroxylates the telopeptides. FKBP65 is required to form dimers of LH2, being the active form of LH2 (86). A disease related to Bruck syndrome is Kuskokwim syndrome, in which \textit{FKBP65} is mutated as well (98). In this syndrome the congenital contractures of large joints is prominent, but in contrast to Bruck syndrome, there are only minor skeletal manifestations.

Apart from heritable connective diseases, changes in prolyl hydroxylation and lysyl hydroxylation/glycosylation are seen in various common pathologies. For example, an increased lysyl hydroxylation/glycosylation of the triple helix is seen in osteoarthritic cartilage (175-177), in osteopenia (129) and in tendinitis (178), resulting in an impairment of the functional integrity of the extracellular matrix. An increase in pyridinoline cross-linking (due to the increased lysyl hydroxylation of the telopeptides) is seen in fibrosis, and has been associated with a decreased degradability of collagen, thus contributing to the accumulation of collagen and the irreversibility of scarring (73,179,180). Furthermore, an increase in pyridinoline facilitates metastasis of
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Collagen related activities</th>
<th>Associated genetic disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolyl 4-hydroxylase 1 (P4HA1)</td>
<td>P4HA1</td>
<td>4-hydroxylation of proline residues at the Y position of the Gly-X-Y-Gly motif</td>
<td>Unknown</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase 2 (P4HA2)</td>
<td>P4HA2</td>
<td>Beta subunit of prolyl-4-hydroxylase proteins ensures activity of P4HA1-3; in addition: disulfide isomerase</td>
<td>Cole-Carpenter syndrome type 1</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase 3 (P4HA3)</td>
<td>P4HA3</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase beta polypeptide (PDI)</td>
<td>P4HB</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Prolyl 3-hydroxylase 1 (P3H1)</td>
<td>LEPRE1</td>
<td>3-hydroxylation of proline residues at the X position of the Gly-X-4Hyprolyl Gly motif; P3H1 complexes with CRTAP and CypB</td>
<td>Osteogenesis imperfecta type VIII</td>
</tr>
<tr>
<td>Prolyl 3-hydroxylase 2 (P3H2)</td>
<td>LEPREL1</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Prolyl 3-hydroxylase 3 (P3H3)</td>
<td>LEPREL2</td>
<td>Affects activity of LH1 (but not LH2)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Synaptonemal complex 65 (P3H4)</td>
<td>SC65</td>
<td>Complexes with LH1 and CypB; Cofactor for 3Hyp modification and collagen folding</td>
<td>Severe recessive osteogenesis imperfecta type VII</td>
</tr>
<tr>
<td>Cartilage associated protein (CRTAP)</td>
<td>CRTAP</td>
<td>Complexes with CRTAP and P3H1; Cofactor for 3Hyp modification and collagen folding; CypB binds to LH1-3 and stimulates LH1 activity</td>
<td>Severe recessive osteogenesis imperfecta type IX</td>
</tr>
<tr>
<td>Peptidylprolyl isomerase B (CypB)</td>
<td>PPIB</td>
<td>Conforms collagen 5Hyl to galactosylhydroxylysine</td>
<td>Unknown</td>
</tr>
<tr>
<td>Glycosyltransferase 25 domain containing 1 (GLT25D1)</td>
<td>GLT25D1</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Glycosyltransferase 25 domain containing 2 (GLT25D2)</td>
<td>GLT25D2</td>
<td>No official classification: Ehlers-Danlos syndrome with progressive kyphoscoliosis, myopathy, and hearing loss</td>
<td>Osteogenesis imperfecta type XI, Kuskokwim syndrome, Bruck syndrome type 1</td>
</tr>
<tr>
<td>FKS06 binding protein 22 (FKBP22)</td>
<td>FKB14</td>
<td>Presumed procollagen chaperone</td>
<td>Unknown</td>
</tr>
<tr>
<td>FKS06 binding protein 65 (FKBP65)</td>
<td>FKB10</td>
<td>Procollagen chaperone, LH2 dimerization</td>
<td>Unknown</td>
</tr>
<tr>
<td>Lysyl hydroxylase 1 (LH1)</td>
<td>PLOD1</td>
<td>5-hydroxylation of triple helical lysyl residues</td>
<td>Ehlers-Danlos syndrome type VIA</td>
</tr>
<tr>
<td>Lysyl hydroxylase 2 (LH2)</td>
<td>PLOD2</td>
<td>5-hydroxylation of telopeptide lysyl residues by LH2b; function LH2a unknown</td>
<td>Bruck syndrome type 2</td>
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<tr>
<td>Lysyl hydroxylase 3 (LH3)</td>
<td>PLOD3</td>
<td>5-hydroxylation of triple helical lysyl residues and O-glycosylation of galactosylhydroxylysine</td>
<td>No official classification: EDS-like phenotypes</td>
</tr>
<tr>
<td>Membrane-bound zinc transporter, member 13 (ZIP13)</td>
<td>SLC39A13</td>
<td>Affects lysyl + prolyl hydroxylation by affecting general ER function</td>
<td>No official classification: Spondylocheirodysplasia, Ehlers-Danlos syndrome-like</td>
</tr>
</tbody>
</table>
tumor cells (181-183). These are only some examples of collagen modifications in non-heritable pathologies, illustrating the major impact of collagen modifications in health and disease.

CONCLUSIONS

Spanning many intracellular and extracellular post-translational modifications, the path from a single collagen α-chain to a supramolecular fibril structure is both complex and fragile. With so many proteins required to work together in order to form a single end product, failure to comply can result in a variety of inherited and acquired diseases. Collagen prolyl and lysyl hydroxylation can be traced back to one of the first steps on the evolutionary ladder of complex multicellular organisms, what underlines its significance. A few of these genes have no known human pathology since no inheritable mutations are known, suggesting that they are essential for embryonic development. Null mice of several collagen biosynthesis-related genes confirm their importance in the early steps of development. Science is just starting to understand the individual implications on biochemical and molecular level, and a deepened knowledge of collagen biosynthesis in general and hydroxylation in particular can make important contributions to treatments in major fields such as cancer, fibrosis and regenerative medicine.
REFERENCES

hydroxylation of -X-Pro-Gly- sequences. Biochimica et biophysica acta, 1079, 103-111.


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recessive forms of osteogenesis imperfecta, inhibit the hydroxylation of telopeptide lysines in bone collagen. Hum Mol Genet, 22, 1-17.


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