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# COL4A1 and COL4A2 mutations and disease: insights into pathogenic mechanisms and potential therapeutic targets

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**Heterotrimers composed of collagen type IV alpha 1 (COL4A1) and alpha 2 (COL4A2) constitute one of the most abundant components of nearly all basement membranes. Accordingly, mutations in COL4A1 or COL4A2 are pleiotropic and contribute to a broad spectrum of disorders, including myopathy, glaucoma and hemorrhagic stroke. Here, we summarize the contributions of COL4A1 and COL4A2 mutations in human disease, integrate knowledge gained from model organisms and evaluate the implications for pathogenic mechanisms and therapeutic approaches.**

## TYPE IV COLLAGENS

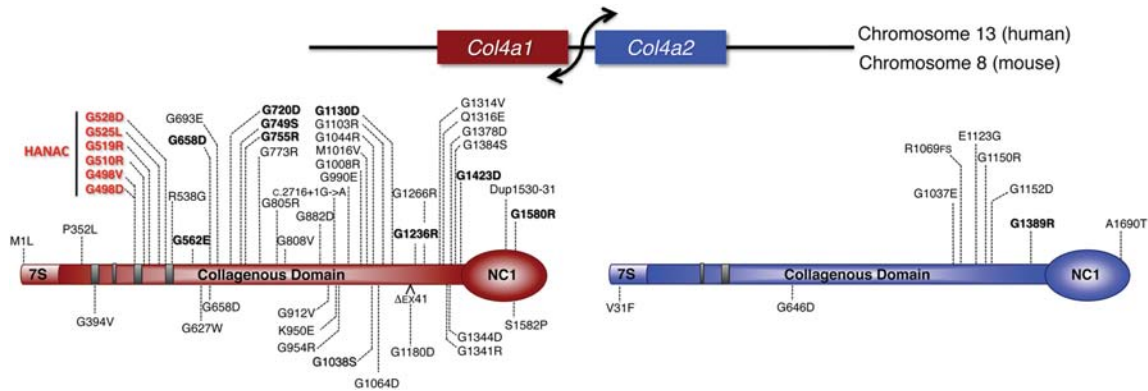
The type IV collagens are encoded by three pairs of paralogous genes [collagen type IV alpha 1 (*COL4A1*) through *COL4A6*]. *COL4A1* and *COL4A2* are highly conserved across species and their protein products are present in almost all basement membranes, whereas *COL4A3* through *COL4A6* are more spatially and temporally restricted (1). The proteins encoded by these six genes associate non-randomly into three distinct heterotrimers *in vivo*:  $\alpha 1\alpha 1\alpha 2$ ,  $\alpha 3\alpha 4\alpha 5$  and  $\alpha 5\alpha 5\alpha 6$  (2–4). Mutations in *COL4A3*, *COL4A4* and *COL4A5* cause Alport Syndrome—a pleiotropic disease affecting the retina, cochlea and kidney that often results in end-stage renal disease (5). Large deletions involving the adjacent *COL4A5* and *COL4A6* genes are reported to cause diffuse leiomyomatosis (6). Here, we review emerging developments regarding the biology and pathogenic mechanisms underlying *COL4A1*- and *COL4A2*-associated diseases.

*COL4A1* (NM\_001845) and *COL4A2* (NM\_001846) comprise 52 and 48 exons, respectively, and are arranged head to head on opposite strands of human Chromosome 13 (13q34). The two genes are separated by 127 nucleotides containing a shared bi-directional promoter that requires additional elements to control tissue specificity and the level and ratio of expression (Fig. 1) (7). Murine *Col4a1* (NM\_009931) and

*Col4a2* (NM\_009932) are located on chromosome 8 (5.0 cM) in a similar genomic organization (8,9). *COL4A1* and *COL4A2* mRNAs are subject to post-transcriptional control, including regulation by a family of microRNAs that down-regulate their expression (10–16) and other microRNAs that indirectly regulate collagen synthesis (17,18). The *Caenorhabditis elegans* *COL4A2* ortholog has a developmentally regulated, alternatively spliced isoform (19). Alternatively spliced *COL4A1* and *COL4A2* isoforms are predicted in humans and mice. One in particular (ENST00000397198) omits amino acids 498–848 which encompass an angiogenesis regulatory domain, putative integrin-binding sites and a region containing an interesting class of mutations in human patients (20) (see below); however, there is currently little empirical evidence to support the existence of alternative splicing *in vivo*.

*COL4A1* and *COL4A2* proteins contain three major domains: an amino-terminal 7S domain, a central triple-helix-forming (collagenous) domain and a carboxy-terminal non-collagenous (NC1) domain (Fig. 1). The 7S domain participates in inter-molecular cross-linking and macromolecular organization. The collagenous domain constitutes the majority of the protein and consists of long stretches of (Gly-X-Y)<sub>n</sub> repeats where X and Y are variable amino

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**Figure 1.** Distribution of COL4A1 and COL4A2 mutations in schematics of human and mouse proteins. The *Col4a1* and *Col4a2* genes are transcribed from a shared, bidirectional promoter. Mature proteins are composed of three distinct domains: 7S, collagenous and non-collagenous (NC1). Mutations identified in humans and in mice are indicated above and below the schematics, respectively, with mutations causing HANAC Syndrome (hereditary angiopathy with nephropathy, aneurysms and muscle cramps) shown in red. Probable pathogenic human mutations, defined as displaying an unambiguous familial inheritance pattern, are in bold while other putative pathogenic human mutations are in plain text.

acids, with proline often occupying the Y position. Unlike fibrillar collagens, the collagenous domains of type IV collagens have frequent interruptions of the Gly-X-Y repeats that are proposed to confer structural flexibility to the collagen IV network (21). Human and mouse COL4A1 have 21 positionally conserved repeat interruptions that divide the collagenous domain into 22 sub-domains. Similarly, human and mouse COL4A2 have 23 conserved repeat interruptions that align with those in COL4A1. All cysteine residues in the collagenous domain of COL4A1 and COL4A2 are present within repeat interruptions, suggesting that interruptions are also important sites for intermolecular cross-linking. The NC1 domains are globular domains responsible for the initiation of heterotrimer assembly (22).

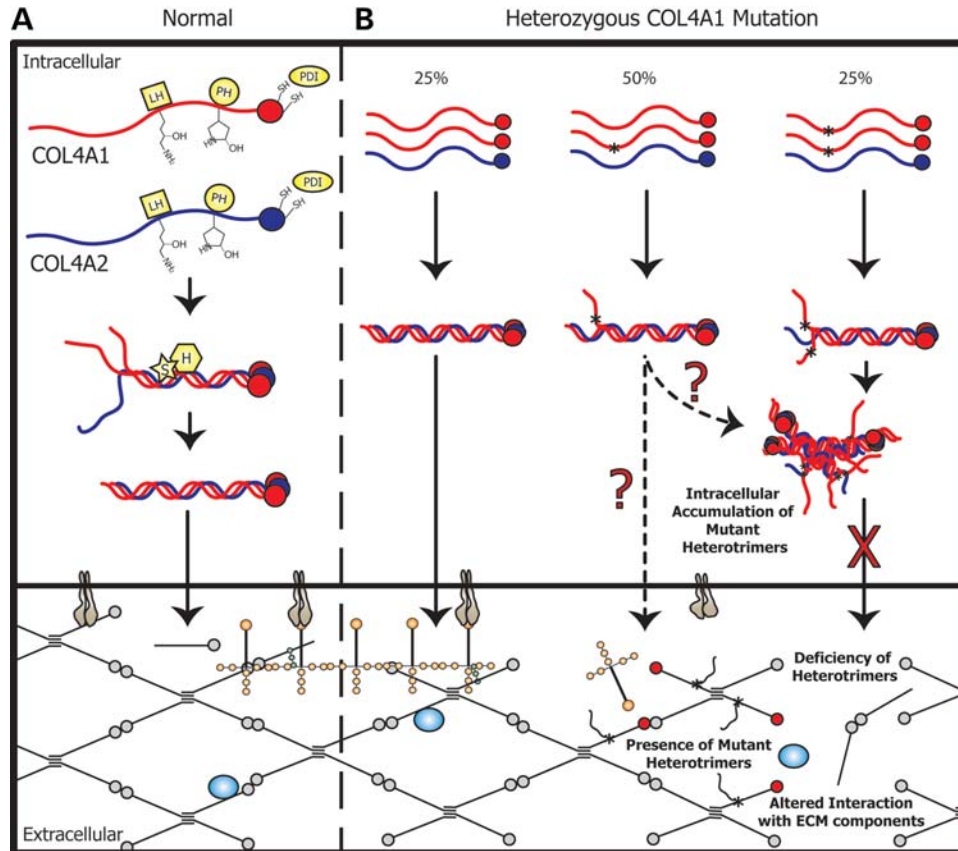
## BIOSYNTHESIS OF $\alpha 1\alpha 1\alpha 2$ HETEROTRIMERS

COL4A1 and COL4A2 are translated at the rough endoplasmic reticulum (ER) where nascent peptides interact with ER resident proteins to ensure proper folding, post-translational modification and heterotrimer assembly (Fig. 2A). NC1 domains are folded and stabilized by intra-molecular cross-links formed by protein disulfide isomerase (PDI) before determining the register of the triple helix and initiating heterotrimer formation with one COL4A2 and two COL4A1 peptides ( $\alpha 1\alpha 1\alpha 2$ ) (3,23). Prior to triple helix formation, the individual peptides of the trimeric complex undergo several post-translational modifications, including hydroxylation of prolyl and lysyl residues and N-linked and O-linked glycosylation.

Hydroxylation of proline to hydroxyproline (Hyp) is critical for triple helix stabilization. Without Hyp, melting temperatures of triple helices are near physiological temperatures. Hydroxylation of Y-position prolines to 4-Hyp is catalyzed by prolyl 4-hydroxylase (P4H). In mammals, P4H is an  $\alpha 2\beta 2$  tetramer in which PDI is the  $\beta$ -subunit and the  $\alpha$ -subunit possesses the substrate recognition domain and the enzymatic active site. Vertebrates have three  $\alpha$ -subunit isoforms, *P4HA1-3*, suggesting that there may be redundancy or

substrate specificity. *Caenorhabditis elegans* lacking P4H activity or mice that are deficient for *P4ha1* have basement membranes that lack type IV collagen and rupture easily, leading to lethality (24–26) (Table 1). Mice deficient for *P4ha2* have no obvious phenotype (27) and mice deficient for *P4ha3* have not been reported. Vertebrates also have three prolyl 3-hydroxylase (P3H) isoforms, *P3H1-3*, that catalyze the less common modification of X-position proline to 3-Hyp. Mutations in *P3H1* (officially *LEPRECAN 1*: leucine proline-enriched proteoglycan) cause severe autosomal recessive osteogenesis imperfecta type VIII (28). The *P3H2* gene (*LEPREL1*: leprecan-like 1) appears to encode the P3H responsible for modifying type IV collagens (29) and is expressed in the developing lens capsule (30). Mutation in *P3H2* causes high myopia and variable early onset cataract and peripheral vitreoretinal degeneration (31), and mutation of *P3H3* has not been described. P4H and P3H require ascorbate as a co-factor and insufficient dietary ascorbic acid renders both classes of enzymes inactive, leading to collagen instability and scurvy (32,33).

Hydroxylation of lysine to hydroxylysine is also vital to triple helix stabilization and provides O-linked glycosylation sites. Nearly 90% of the Y-position lysine residues of type IV collagens are hydroxylated and glycosylated by lysyl hydroxylases. Vertebrates have three lysyl hydroxylase isoforms, *PLOD1-3*. Mutations in *PLOD1* (procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1) cause Ehlers–Danlos Syndrome, type VI (34,35), mutations in *PLOD2* cause Bruck Syndrome, type 2 (36) and mutations in *PLOD3* cause multi-system connective tissue disorder (37) (Table 1). *PLOD3* is notable in that it can be secreted (38,39) and possesses hydroxylysyl galactosyltransferase (GT) and galactosylhydroxylysyl glucosyltransferase (GGT) activity (40). Mice completely deficient for *Plod3* have disrupted basement membranes (41) and type IV collagen accumulates within dilated ER [similar to *C. elegans* with mutation of the lone lysyl hydroxylase ortholog (42)]. Elegant biochemical dissection of the functional domains revealed that pathology results from deficiency of GGT activity and not lysyl hydroxylase activity, demonstrating that glycosylation is necessary for intracellular trafficking (43,44).



**Figure 2.** Schematic representation of type IV collagen biosynthesis and potential sites for pathogenic insults. (A) Collagen proteins undergo extensive post-translational modifications and assemble into heterotrimers for secretion into the ECM where they polymerize into a network and interact with other extracellular and membrane bound molecules [LH, lysyl hydroxylase; PH, prolyl hydroxylase; PDI, protein disulphide isomerase; S, secreted protein, acidic, cysteine-rich (SPARC); H, heat shock protein 47 (HSP47)]. (B) Assuming random assembly within the ER of cells heterozygous for *COL4A1* mutations, 25% of heterotrimers will be normal, 50% of heterotrimers will incorporate one mutant *COL4A1* protein and 25% of heterotrimers will incorporate two mutant *COL4A1* proteins. Normal heterotrimers (left) are presumed to be secreted, while heterotrimers containing two mutant proteins (right) are not. It is unknown if heterotrimers with one normal and one mutant *COL4A1* are secreted. (Heterozygous mutations in *COL4A2* would produce only the first two classes of heterotrimers at 50% each.) The primary pathogenic insult may be intracellular (cytotoxic accumulation of mutant heterotrimers) or extracellular (either the presence of mutant heterotrimers or the deficiency of heterotrimers in basement membranes). Either putative extracellular insult could directly or indirectly alter interactions with signaling molecules such as BMPs (represented as blue circles) or cell-surface receptors such as integrins (represented as grey structures), which can in turn lead to autocrine or paracrine intracellular signaling defects.

Two additional proteins, HSP47 and SPARC, have important roles in collagen IV biosynthesis. HSP47 is an ER resident glycoprotein of the serpin family (official name is *SERPINH1*) (45) that acts as a collagen-specific molecular chaperone induced by heat shock but not ER stress. Whether HSP47 binds nascent peptides, monomers, unhydroxylated trimers or hydroxylated triple helices is debated (46–49); however, it appears to transiently associate with Y-position arginine motifs (i.e. Gly-X-Arg) (47,49,50) and dissociate in a pH-dependent manner after trafficking to the more acidic cis-Golgi (48). Although the precise role of HSP47 is unresolved, in its absence, collagen heterotrimers are misfolded and secretion is delayed (51–53) and *HSP47* mutations cause severe, recessive Osteogenesis Imperfecta type X (54). SPARC (secreted protein, acidic, cysteine-rich) is a calcium-binding matricellular glycoprotein that recognizes non-denatured triple helical collagen (55). SPARC-deficient mice have early onset cataracts owing to disrupted collagen and laminin networks in the lens capsule (56–59) as well as decreased bone remodeling and severe osteopenia (60)

(Table 1). SPARC has four predicted  $\alpha 1\alpha 1\alpha 2$ -binding sites and interacts with collagen in the ER and possibly in the extracellular matrix (ECM) (61). In *Drosophila*, cell-intrinsic SPARC is required and deficiency causes intracellular  $\alpha 1\alpha 1\alpha 2$  accumulation (62). Collectively, these data suggest that SPARC influences collagen IV folding, assembly and/or modification in the ER.

Following post-translational modification, heterotrimers are transported from the ER to the Golgi where they are packaged into vesicles for secretion into the ECM (63). In the extracellular space, heterotrimers assemble into higher order macromolecular networks. NC1 domains of two heterotrimers are cross-linked via special S-hydroxylysyl-methionine bonds (64). At the N-terminus, 7S domains of four heterotrimers form lateral, anti-parallel interactions directed by hydroxylysine-linked disaccharides and stabilized by lysyl-derived cross-links (44,65).

Outside the cell, the  $\alpha 1\alpha 1\alpha 2$  network interacts with other ECM components (laminins, perlecan, nidogens and proteoglycans), provides structural integrity and participates in

**Table 1.** Consequences of mutations in proteins involved in type IV collagen biosynthesis

Protein	Phenotypes in model organisms	Phenotypes in humans
P4HA1	Lethality Basement membranes lack type IV collagen	Not reported
P4HA2	No obvious phenotype	Not reported
P4HA3	Not reported	Not reported
P3H1	Disruption in fibrillar collagen rich tissues	Osteogenesis imperfecta, type VIII
P3H2	Not reported	High myopia Early onset cataract Peripheral vitro-retinal degeneration Retinal detachment
P3H3	Not reported	Not reported
PLOD1	Abnormal collagen fibrils Hypotonia Aortic rupture	Ehlers–Danlos syndrome, type VI
PLOD2	Not reported	Bruck syndrome, type 2
PLOD3	Lethality Disrupted basement membranes Accumulation of type IV collagen in ER	Flat facial profile Deafness Myopia  Cataract Arterial rupture Osteopenia Joint contractures and fractures Skin blistering Nail abnormalities
HSP47	Collagen misfolding and delayed secretion	Osteogenesis imperfecta, type X
SPARC	Disruption of collagen and laminin networks Early cataract formation Osteopenia	Not reported

cell–matrix and cell–cell communication. Collagens can signal directly to cells via integrins, discoidin domain receptors, mannose receptors and cell surface heparan sulfate proteoglycans (20,66). Type IV collagens can also influence intercellular communication and orchestrate developmental and homeostatic processes by modulating the distribution and bioavailability of diffusible morphogens (67–71). Additionally, cleaved fragments of type IV collagens such as arresten (the 229 amino acid NC1 domain of COL4A1) and canstatin (the 227 amino acid NC1 domain of COL4A2) can be biologically active and suppress angiogenesis and tumor growth (72–78). Thus, through their interactions with ECM components, cell surface receptors, and morphogens, or via proteolytic cleavage, type IV collagens can dynamically influence a broad range of biological processes. The extents to which each of these processes is perturbed in *COL4A1*- and *COL4A2*-related diseases constitute a growing area of research.

### **COL4A1 AND COL4A2 MUTATIONS CAUSE CLINICALLY DIVERSE DISEASES IN HUMANS**

*COL4A1* and *COL4A2* mutations cause highly penetrant multi-system disorders (79–84) (Tables 2 and 3). A rapidly increasing number of *COL4A1* mutations are being identified and the

first *COL4A2* mutations were recently reported (85–87) (Fig. 1). *COL4A1* mutations were initially found to cause type I porencephaly, which is characterized by cystic cerebral cavities that communicate with the ventricles and are thought to arise from germinal matrix hemorrhages. Porencephaly commonly associates with infantile hemiparesis, hydrocephalus, seizures, poor or absent speech development, mental retardation and cerebral palsy. In addition to pre- and peri-natal hemorrhages, *COL4A1* and *COL4A2* mutations also cause sporadic and recurrent intracerebral hemorrhages (ICH) in young and old patients. Some hemorrhages occur spontaneously while others are induced by triggering events such as prenatal and birth trauma, head trauma, participation in sports, and anticoagulation use. This increased susceptibility to develop ICH is thought to result from systemic small-vessel disease, which has been confirmed by tissue biopsy. Skin and kidney biopsy samples from patients with *COL4A1* and *COL4A2* mutations were often normal on light microscopy evaluation but demonstrated significant ultrastructural abnormalities with focal interruptions or expanded and thickened, fragmented basement membrane capillaries on electromicroscopy. Even in the absence of overt porencephaly or hemorrhagic stroke, some individuals with *COL4A1* mutations have clinically silent defects, such as diffuse or periventricular leukoencephalopathy and calcification, intracranial aneurysms and cerebral microbleeds, suggesting that *COL4A1* and *COL4A2* play an insidious and underappreciated role in a broad spectrum of cerebrovascular disease (CVD) (79–82,84–96). Still other patients with *COL4A1* mutations have been identified without any evidence of CVD, which underscores the range in the severity of pathology from porencephaly and ICH that cause long-term disability or death to asymptomatic mutation carriers.

In addition to CVD, *COL4A1* or *COL4A2* mutations are confirmed to cause ocular, cerebral, renal and muscular defects (Table 3). Ophthalmologic examination is recommended in evaluating for *COL4A1*- and *COL4A2*-associated diseases as retinal vascular tortuosity is highly penetrant in patients with *COL4A1* mutations, and may have predictive value for more serious CVD. Multiple patients have also been described with cataracts, ocular anterior segment dysgenesis (ASD), including Axenfeld Rieger Syndrome, and juvenile-onset glaucoma. Nephropathy manifests diversely, ranging from gross and microscopic hematuria to renal cysts to agenesis of the kidney. Functional renal defects have been reported in patients with small-vessel disease, leukoencephalopathy and congenital cataracts (95), and a multi-system syndrome referred to as HANAC: hereditary angiopathy with nephropathy, aneurysms and muscle cramps (90,94). Nearly, all families with HANAC syndrome have muscle cramps or elevated serum creatine kinase. Consistent with mutations causing myopathy, we identified two putative *COL4A1* mutations in patients with congenital muscular dystrophy and cerebral cortical malformations, including lissencephaly, that are consistent with diagnoses of Walker–Warburg Syndrome or muscle–eye–brain disease (97).

To date, the clinical manifestations of *COL4A1* mutations in patients represent only a subset of the phenotypes observed in mice with *Col4a1* mutations. The high prevalence of CVD reported in patients with *COL4A1* or *COL4A2* mutations

**Table 2.** Cerebrovascular features associated with human COL4A1 and COL4A2 mutations

	Reference	Porencephaly	ICH/stroke	Leukoencephalopathy	Microbleeds	ICA
<b>COL4A1 mutations</b>						
p.M1L	Breedveld <i>et al.</i> (81)	4/5	1/1	3/3		1/1
p.P352L	Weng <i>et al.</i> (84)		1/1		1/1	
p.G498D	Plaisier <i>et al.</i> (94)	0/3	1/3	1/2		
p.G498V	Plaisier <i>et al.</i> (90), Alamowitch <i>et al.</i> (91)	0/8	3/6	5/6	2/6	3/6
p.G510R	Plaisier <i>et al.</i> (94)	0/3		0/2		0/2
p.G519R	Plaisier <i>et al.</i> (90), Alamowitch <i>et al.</i> (91)	0/4	1/2	1/2		1/2
p.G525L	Plaisier <i>et al.</i> (94)	0/5	1/5	4/4		1/4
p.G528D	Plaisier <i>et al.</i> (90), Alamowitch <i>et al.</i> (91)	0/2	0/1	1/1	1/1	1/1
p.R538G	Weng <i>et al.</i> (84)		1/1			
p.G562E	Gould <i>et al.</i> (80), Vahedi <i>et al.</i> (89)	2/6	2/6	6/6	3/4	0/1
p.G658D	Livingston <i>et al.</i> (95)			2/2		
p.G693E	Livingston <i>et al.</i> (95)	1/1		0/1		
p.G720D	Sibon <i>et al.</i> (88)	1/5	2/5	5/5	1/5	1/1
p.G749S	Gould <i>et al.</i> (79), Aguglia <i>et al.</i> (2004) (123), Vermeulen <i>et al.</i> (2011) (124)	4/11	1/2			0/3
p.G755R	Shah <i>et al.</i> (92) Rouaud <i>et al.</i> (93) Shah <i>et al.</i> (96)	0/3	4/5	5/5	2/4	1/3
p.G773R	Shah <i>et al.</i> (96)	1/2	1/2	2/2		
p.G805R	Vahedi <i>et al.</i> (82)	1/1	1/1	1/1	1/1	0/1
p.G808V	Meuwissen <i>et al.</i> (2011) (125)	1/1	1/1			
p.G882D	Shah <i>et al.</i> (96)			1/2		
c.2716+1 G>A	Meuwissen <i>et al.</i> (2011) (125)	1/1	1/1			
p.G990E	Livingston <i>et al.</i> (95)	1/1		1/1		
p.G1008R	Meuwissen <i>et al.</i> (2011) (125)	1/1	1/1			
p.M1016V	Labelle-Dumais <i>et al.</i> (97)					
p.G1044R	Meuwissen <i>et al.</i> (2011) (125)	1/1	1/1			
p.G1103R	Lichtenbelt <i>et al.</i> (83)	1/1	1/1			
p.G1130D	Breedveld <i>et al.</i> (81)	3/3		2/2		
p.G1236R	Gould <i>et al.</i> (79) van der Knaap <i>et al.</i> (2006) (126)	3/3	1/3	3/3	1/3	0/3
p.G1266R	Shah <i>et al.</i> (96)	2/2		2/2	1/1	
p.G1314V	Livingston <i>et al.</i> (95)	1/1		1/1		
p.Q1316E	Labelle-Dumais <i>et al.</i> (97)					
p.G1378D	Livingston <i>et al.</i> (95)	1/1		1/1		
p.G1384S	Vermeulen <i>et al.</i> (2011) (124)	1/1	1/1			
p.G1423R	Breedveld <i>et al.</i> (81)	2/2		2/2		
p.P1530_M1531dup	Bilguvar <i>et al.</i> (2009) (127)		2/4			
p.G1580R	de Vries <i>et al.</i> (2009) (128)	4/4	3/4	4/4	0/1	0/3
<b>COL4A2 mutations</b>						
p.G1037E	Yoneda <i>et al.</i> (87)	1/1	1/1			0/1
p.R1069fs	Verbeek <i>et al.</i> (86)	2/3		0/2		0/2
p. E1123G	Jeanne <i>et al.</i> (85)		2/2			
p.Q1150K	Jeanne <i>et al.</i> (85)		1/1			
p.G1152D	Yoneda <i>et al.</i> (87)	3/4				0/1
p.G1389R	Verbeek <i>et al.</i> (86)	1/3		2/3		1/3
p.A1690T	Jeanne <i>et al.</i> (85)		1/1			

ICH, intracerebral hemorrhage; ICA, intracranial aneurysm.

might reflect ascertainment bias since the original reports of mouse *Col4a1* mutations described CVD. We predict that *COL4A1* and *COL4A2* mutations will be identified in diverse diseases and contribute to multiple, clinically distinct, developmental or acquired disorders as demonstrated in mouse models. The first *COL4A2* mutations were only recently identified in patients with ICH and porencephaly (85–87). Evidence from model organisms suggests that *COL4A2* mutations will phenocopy *COL4A1* mutations and contribute to equally diverse disorders. However, for stoichiometric reasons, it is possible that *COL4A2* mutations may be less severe or even sub-clinical and thus, although equally

abundant, may contribute less frequently to human diseases than *COL4A1* mutations.

### **COL4A1 AND COL4A2 MUTATIONS CAUSE PLEIOTROPIC PHENOTYPES IN MICE**

Mice completely deficient for both *Col4a1* and *Col4a2* (*Col4a1*<sup>-/-</sup>; *Col4a2*<sup>-/-</sup>) die at mid-gestation and exhibit various defects, including neuronal ectopias, disorganization of the capillary network during angiogenesis and impaired placental development (98) (Tables 4 and 5). Interestingly, mice

**Table 3.** Developmental, ocular, cardiac, renal, and musculoskeletal features associated with human COL4A1 and COL4A2 mutations

	Reference	MCD	RAT	Cataract	ASD	ONH	Cardiac abnormalities	Nephropathy	Urinary retention	Muscle cramps/ elevated CK
<b>COL4A1 mutations</b>										
p.M1L	Breedveld <i>et al.</i> (81)									
p.P352L	Weng <i>et al.</i> (84)						1/1 (aortic valve replacement)			
p.G498D	Plaisier <i>et al.</i> (94)		3/3		0/3		1/3 (SVA)	1/3 (H)		0/3
p.G498V	Plaisier <i>et al.</i> (90), Alamowitch <i>et al.</i> (91)		8/8				3/8 (SVA)	8/8 (H, C)		8/8
p.G510R	Plaisier <i>et al.</i> (94)		3/3		0/3		1/2 (SVA)	1/3 (mild RI)		3/3
p.G519R	Plaisier <i>et al.</i> (90), Alamowitch <i>et al.</i> (91)		4/4					1/2 (mild RI, C)		2/2
p.G525L	Plaisier <i>et al.</i> (94)		5/5		0/5		2/4 (SVA)	2/4 (mild RI, C)		5/5
p.G528D	Plaisier <i>et al.</i> (90), Alamowitch <i>et al.</i> (91)		2/2				1/1 (paroxysmal SVA)	1/1 (mild RI, C)		1/1
p.R538G	Weng <i>et al.</i> (84)									
p.G562E	Gould <i>et al.</i> (80), Vahedi <i>et al.</i> (89)		6/6					2/6 (H, mild RI)		
p.G658D	Livingston <i>et al.</i> (95)		0/1	1/2	0/1	0/1		1/1 (H)		1/1
p.G693E	Livingston <i>et al.</i> (95)	1/1		1/1						
p.G720D	Sibon <i>et al.</i> (88)		0/5	5/5	5/5 (GLC)		0/2	0/1		
p.G749S	Gould <i>et al.</i> (79), Aguglia <i>et al.</i> (2004) (123), Vermeulen <i>et al.</i> (2011) (124)						4/6 (mitral valve prolapse)			
p.G755R	Shah <i>et al.</i> (92), Rouaud <i>et al.</i> (93), Shah <i>et al.</i> (96)		0/5	5/5			0/2	0/2	1/1	0/1
p.G773R	Shah <i>et al.</i> (96)	1/1	0/2	2/2						
p.G805R	Vahedi <i>et al.</i> (82)		1/1	1/1			0/1	0/1		
p.G808V	Meuwissen <i>et al.</i> (2011) (125)							1/1 (agenesis, C)		
p.G882D	Shah <i>et al.</i> (96)	1/1	0/2	2/2	1/1			0/1		1/1
c.2716 + 1 G>A	Meuwissen <i>et al.</i> (2011) (125)									
p.G990E	Livingston <i>et al.</i> (95)	1/1		1/1	1/1		0/1			1/1
p.G1008R	Meuwissen <i>et al.</i> (2011) (125)			1/1						
p.M1016V	Labelle-Dumais <i>et al.</i> (97)	1/1				1/1		0/1		1/1
p.G1044R	Meuwissen <i>et al.</i> (2011) (125)			1/1						
p.G1103R	Lichtenbelt <i>et al.</i> (83)		1/1							
p.G1130D	Breedveld <i>et al.</i> (81)									
p.G1236R	Gould <i>et al.</i> (79), van der Knaap <i>et al.</i> (2006) (126)		1/3	3/3			0/3			
p.G1266R	Shah <i>et al.</i> (96)	1/1	0/1	0/1						
p.G1314V	Livingston <i>et al.</i> (95)	1/1	0/1	0/1						1/1
p.Q1316E	Labelle-Dumais <i>et al.</i> (97)	1/1				1/1				1/1
p.G1378D	Livingston <i>et al.</i> (95)					1/1				1/1
p.G1384S	Vermeulen <i>et al.</i> (2011) (124)									
p.G1423R	Breedveld <i>et al.</i> (81)									
p.P1530_M1531dup	Bilguvar <i>et al.</i> (2009) (127)									
p.G1580R	de Vries <i>et al.</i> (2009) (128)		0/3	0/3				0/3		
<b>COL4A2 mutations</b>										
p.G1037E	Yoneda <i>et al.</i> (87)			0/1				0/1		1/1
p.R1069fs	Verbeek <i>et al.</i> (86)	1/2				1/3	0/1			
p. E1123G	Jeanne <i>et al.</i> (85)									
p.Q1150K	Jeanne <i>et al.</i> (85)									
p.G1152D	Yoneda <i>et al.</i> (87)		0/1	0/1	0/1	0/1		0/1		0/1
p.G1389R	Verbeek <i>et al.</i> (86)					0/2				
p.A1690T	Jeanne <i>et al.</i> (85)									

MCD, malformations of cortical development; RAT, retinal artery tortuosity or hemorrhage; ASD, anterior segment dysgenesis; GLC, congenital glaucoma; ONH, optic nerve hypoplasia; SVA, supraventricular arrhythmia; H, hematuria; C, renal cysts; RI, renal insufficiency.

**Table 4.** Developmental, neurologic and vascular features associated with mouse Col4a1 and Col4a2 mutations

COL4A1 mutations	Reference	Embryonic lethality (homozygous)	Growth retardation	Porencephaly/porencephalic lesions	Intracerebral hemorrhage	Malformations of cortical development	Vascular defects/systemic hemorrhages
COL4A1/COL4A2 double null	Pöschl <i>et al.</i> (2004) (98)	+	+ (embryonic)			+	+ (capillary organization abnormalities)
$\Delta$ ex41	Gould <i>et al.</i> (79, 80, 101), Labelle-Dumais <i>et al.</i> (97)	+	+ (reduced body size)	+	+	+	+ (cerebral and systemic vasculature abnormalities)
p.G394V	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G627W	Van Agtmael <i>et al.</i> (99)	+	+ (reduced body size)				+ (bruising at birth)
p.G658D	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G912V	Favor <i>et al.</i> (100)	+		+	+	+	+
p.K950E	Van Agtmael <i>et al.</i> (99,102)	+			+	+	
p.G954A	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G1038S	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G1064D	Van Agtmael <i>et al.</i> (99)	+	+ (reduced body size)				+ (bruising at birth)
p.G1180D	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G1341A	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G1344D	Favor <i>et al.</i> (100)	+		+	+	+	+
p.S1582P	Favor <i>et al.</i> (100)	+		+	+	+	+
COL4A2 Mutations	Reference	RAT	ASD	ONH	Other ocular defects	Nephropathy	Muscle defects/elevated CK
p.V31F	Favor <i>et al.</i> (100)	+		+	+	+	+
p.G646D	Favor <i>et al.</i> (100), Jeanne <i>et al.</i> (85)	+		+	+	+	+



**Table 5.** Ocular, renal, muscular, pulmonary, and reproductive features associated with mouse Col4a1 and Col4a2 mutations

Reference	RAT	ASD	ONH	Other ocular defects	Nephropathy	Muscle defects/ elevated CK	Pulmonary defects	Reduced reproductive functions
<b>COL4A1 mutations</b>								
COL4A1/ COL4A2 double null								
$\Delta$ ex41	Pöschl <i>et al.</i> (2004) (98) Gould <i>et al.</i> (79, 80, 101), Labelle-Dumais <i>et al.</i> (97), unpublished data <sup>a</sup>	+	+	+	+ (hyphema)	+ (glomerular BM abnormalities, MA, H)	+ (perinatal)	+ (males)
p.G394V	Favor <i>et al.</i> (100), unpublished data	+	+	+	+(M, B, NV, LO, C)	+ (MA, H)		+ (females)
p.G627W	Van Agtmael <i>et al.</i> (99)				+ (RD, RGC degeneration, B, optic nerve cupping, C)	+ (Bowman's capsule abnormalities)		
p.G658D	Favor <i>et al.</i> (100), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (MA, H)		+ (females)
p.G912V	Favor <i>et al.</i> (100), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (MA, H)		+ (females)
p.K950E	Van Agtmael <i>et al.</i> (99)				+ (retinal arteriolar silvering)	-		
p.G954A	Favor <i>et al.</i> (100)				+ (M, B, NV, LO, C)			+ (females)
p.G1038S	Favor <i>et al.</i> (100), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (MA, H)		+ (females)
p.G1064D	Van Agtmael <i>et al.</i> (99)				+ (RD, RGC degeneration, C)			
p.G1180D	Favor <i>et al.</i> (100), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (MA, H)		+ (females)
p.G1341A	Favor <i>et al.</i> (100)				+ (M, B, NV, LO, C)			+ (females)
p.G1344D	Favor <i>et al.</i> (100), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (MA, H)		+ (females)
p.S1582P	Favor <i>et al.</i> (100), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (H)		+ (females)
<b>COL4A2 mutations</b>								
p.V31F	Favor <i>et al.</i> (100)				+ (M, B, NV, LO, C)	-		+ (females)
p.G646D	Favor <i>et al.</i> (100), Jeanne <i>et al.</i> (85), unpublished data <sup>a</sup>	+	+	+	+ (M, B, NV, LO, C)	+ (MA, H)		+ (females)

RAT, retinal arteriolar tortuosity; ASD, anterior segment dysgenesis; ONH, optic nerve hypoplasia; M, microphthalmia; B, buphthalmos; NV, preretinal neovascularization; LO, lens opacity; C, cataracts; RD, retinal detachment; RGC, retinal ganglion cell layer; MA, microalbuminuria; H, hematuria.

<sup>a</sup>Unpublished data provided by Dr Mao Mao and Dr Marion Jeanne.

double heterozygous for the *Col4a1* and *Col4a2* null alleles (*Col4a1*<sup>+/-</sup>; *Col4a2*<sup>+/-</sup>) are viable and without any overt phenotype. In contrast, mice heterozygous for missense or splicing mutations in *Col4a1* or *Col4a2* develop genetically complex, pleiotropic phenotypes. As such, mutagenesis screens in mice have been invaluable for understanding the roles of *COL4A1* and *COL4A2* in development and disease. To date, 13 *Col4a1* mutations and 2 *Col4a2* mutations have been identified in mice (79,99,100). The majority of these mutations are missense mutations of glycine residues occurring in the collagenous domain (Fig. 1). We identified a splice acceptor site mutation that leads to skipping of exon 41 during mRNA processing, which results in a 17-amino-acid deletion (p.G1169\_K1185del) that contains a Gly-X-Y repeat interruption (79). (Note: The affected exon had been annotated as exon 40 and therefore this mutation was named  $\Delta$ ex40 in previous publications. Subsequent builds of the mouse genome identify the exon as exon 41 and hereafter we refer to the mutation as *Col4a1* <sup>$\Delta$ ex41</sup>.)

Mice heterozygous for semi-dominant *Col4a1* or *Col4a2* mutations have multi-system disorders including ocular, renal, pulmonary, muscular, vascular, reproductive and central nervous pathology (Tables 4 and 5). *Col4a1*<sup>+/ $\Delta$ ex41</sup> mutant mice have pre- and peri-natal ICH, including porencephalic lesions. Independent of porencephaly, mutant mice also have highly penetrant tortuosity of the retinal vasculature, and fully penetrant multi-focal and recurrent ICH as adults (79,80).

*Col4a1* and *Col4a2* mutations were originally identified because of severe, highly penetrant cataracts and ocular ASD. Subsequent ocular analysis revealed optic nerve hypoplasia, retinal vascular tortuosity and progressive defects including retinal degeneration and elevated intraocular pressure that may advance to glaucoma (79,80,99–102). The relatively high penetrance of ocular dysgenesis in mice compared with humans may reflect selection bias due to the ease with which this pathology is detected in phenotype-driven mutagenesis screens. *Col4a1* mutant mice also have thinning and splitting of the glomerular basement membrane and functional renal pathology, including microalbuminuria and hematuria (80). In addition to glomerular defects, abnormalities in Bowman's capsule are also reported (99).

The NC1 domains of  $\alpha$ 1 $\alpha$ 1 $\alpha$ 2 have a role in synaptogenesis of the neuromuscular junction (103) and, more recently, we detected myopathy including muscle weakness, elevated creatine kinase, split muscle fibers and increased non-peripheral nuclei in *Col4a1*<sup>+/ $\Delta$ ex41</sup> mice (97). This observation along with ocular dysgenesis and focal lamination defects in the cerebral cortex, resembling lissencephaly observed in human patients (97), established *Col4a1* mutant mice as a novel model for a spectrum of congenital muscular dystrophy disorders that includes Walker–Warburg Syndrome and muscle–eye–brain disease. Pathology has been detected in every organ examined to date and the collection of pleiotropic phenotypes in *Col4a1* and *Col4a2* mutant mice reflects the nearly ubiquitous distribution of *COL4A1* and *COL4A2* in basement membranes. Additional disease-related defects are likely to be revealed as further detailed characterizations are conducted.

Genetic modifiers almost certainly contribute to variable expressivity of disease in patients. In mice, the penetrance and severity of pathology is genetic-context dependent. Although

not all phenotypes have been evaluated, pathology resulting from the *Col4a1* <sup>$\Delta$ ex41</sup> mutation is more severe in C57BL/6J mice compared with mice that have been crossed to either 129SvEvTac or CAST/EiJ (97,101). In a pilot experiment, a locus on CAST/EiJ Chromosome 1 was identified that rescued ocular dysgenesis (101). Identifying modifier genes and determining how they prevent pathology will reveal cellular pathways that may be amenable to therapeutic interventions that prevent or delay progressive diseases, such as glaucoma, myopathy and hemorrhagic stroke.

## GENERAL PATHOGENIC MECHANISMS

There are several plausible, non-mutually exclusive, mechanisms by which *COL4A1* and *COL4A2* mutations might be pathogenic (Fig. 2B). Moreover, the mechanisms might be tissue-specific or even heterogeneous whereby different mechanisms play greater or lesser roles in different diseases. Thus, dissecting the primary cellular mechanisms underlying various *COL4A1*- and *COL4A2*-related diseases are active and important areas of research.

Mice heterozygous for null alleles of *Col4a1* and *Col4a2* had no detectable pathology, and supplemental transgenic expression of normal *Col4a1* was insufficient to fully complement *Col4a1* mutations in *Drosophila* (104). Together, these data argue against amorphic or hypo-morphic insults and support that pathogenicity occurs, at least in part, via anti-morphic or neo-morphic effects where there is a requirement for the presence of a mutant protein. Broadly, the pathogenic insult(s) could be intracellular and/or extracellular. Many mutations result in accumulation of  $\alpha$ 1 $\alpha$ 1 $\alpha$ 2 heterotrimers within cells and, in some cases, lead to activation of an ER stress response (79,85,101,105) which may produce acute or chronic cytotoxic effects. Alternatively, intracellular sequestration of mutant heterotrimers could lead to extracellular deficiency of  $\alpha$ 1 $\alpha$ 1 $\alpha$ 2 heterotrimers. In cells heterozygous for *COL4A1* mutations, 75% of heterotrimers are expected to be abnormal, which could reduce the levels of extracellular heterotrimers below a critical threshold. However, for *COL4A2* mutations, 50% of heterotrimers should be normal and secreted, and yet, *COL4A2* mutations phenocopy *COL4A1* mutations. This observation does not reconcile easily with the absence of a phenotype in heterozygous null mice (although a reduction in extracellular  $\alpha$ 1 $\alpha$ 1 $\alpha$ 2 has never been demonstrated in these mice). A simple model of extracellular deficiency also cannot explain the inability for supplemental *COL4A1* expression to fully rescue pathology in mutant *Drosophila* (104). A third possibility is a dominant negative effect of extracellular mutant proteins. Although intracellular retention suggests that mutant heterotrimers might not reach the ECM, these observations are based upon relatively insensitive techniques and mutant  $\alpha$ 1 $\alpha$ 1 $\alpha$ 2 may indeed be secreted. Therefore, understanding the fate of mutant  $\alpha$ 1 $\alpha$ 1 $\alpha$ 2 heterotrimers is an unresolved issue. More complex factors might also influence outcomes, including feedback mechanisms and transcriptional control that might alter the overall biology of mutant cells. Perhaps, cells heterozygous for null alleles simply produce more protein from the normal allele to compensate, whereas cells expressing pathogenic mutations are

instructed to stop this compensation. To date, the quantity or quality of heterotrimers reaching basement membranes have not been determined.

There are multiple pathways by which extracellular insults could cause disease. Either deficiency of normal heterotrimers or presence of mutant heterotrimers could lead to compromised basement membranes that perturb cell–matrix signaling via cell surface receptors or growth factor-mediated cell–cell signaling. Importantly, these deleterious effects could also be indirect via other matrix molecules if mutant basement membranes are altered in structure or composition. Observations from a subset of patients with *COL4A1* mutations diagnosed with HANAC syndrome raise the intriguing possibility that some mutations might involve integrin signaling (see below). Alternatively, in *Drosophila*, type IV collagens directly bind to bone morphogenetic proteins (BMPs), a class of TGF $\beta$  signaling molecules, and participate in morphogen gradients and modulate their signaling (67,68). These data are interesting given that TGF $\beta$  signaling is necessary for angiogenesis and mutations in genes encoding proteins involved in this pathway cause hereditary hemorrhagic telangiectasia in which defects in angiogenesis predispose to ICH. Such a mechanism may be analogous to in Marfan Syndrome whereby mutations in the gene for another structural ECM protein, fibrillin-1, result in perturbed TGF $\beta$  signaling which causes some of the major clinical features of the disease (106–108). Finally, the reduction in proteolytically processed, biologically active NC1 domains of type IV collagens themselves may also be involved.

Thus, current data support that the pathogenic insult(s) of *COL4A1* and *COL4A2* mutations are anti-morphic or neo-morphic, may be intracellular or extracellular and, if extracellular, could be caused by deficiency or presence of mutant proteins. None of these potential mechanisms is mutually exclusive and may be differentially involved depending on the specific mutation. Understanding the extents to which mutant collagens induce intracellular stresses, affect signaling via cell surface receptors or perturb intercellular communication via morphogens are unresolved and important questions.

### ALLELIC HETEROGENEITY TO EXPLAIN VARIABLE EXPRESSIVITY AND UNDERSTAND PATHOGENIC MECHANISM(S)

Emerging evidence from human patients and mouse models supports that allelic heterogeneity contributes to phenotypic variability. Understanding the functional differences between mutations can also be exploited to determine cellular mechanisms of disease. Glycine residues are required at every third position of the collagenous domain because the absence of a side chain allows glycine to fit into the core of the triple helix (109). However, both the position of the mutation and the residue replacing glycine can influence the biosynthetic consequence of the mutation (110). Non-glycine missense mutations are potentially less disruptive to triple helix formation and may lead to milder phenotypes (99) or act via different mechanisms such as disruption of post-translational modification or impacting functional sub-domains that interact with other matrix molecules, cell surface receptors or growth

factors (20). For instance, the *COL4A1* <sup>$\Delta$ ex41</sup> mutant protein, which is missing 17 amino acids from the collagenous domain, including a repeat interruption, has a very strong effect on intracellular retention of *COL4A1* and *COL4A2* and mice with this mutation tend to have more severe phenotypes (79,101,105). However, it is somewhat premature to compare the effects of different murine mutations because, to date, the mutations have not been systematically evaluated on a uniform genetic context. It is even more perilous to compare the clinical consequences of mutation in patients because they are usually identified in one or few people and the genetic context is even less defined. Despite this caution, emerging evidence supports allelic heterogeneity in human patients. Six families with *COL4A1* mutations have a diagnosis of HANAC syndrome, which is suggested to be clinically distinguishable. Interestingly, all six mutations cluster within a 31-amino-acid region of the *COL4A1* protein (located in exons 24 and 25) that contains multiple putative integrin-binding sites as well as other potential protein–protein interaction domains (20,94) (Fig. 1), suggesting that pathology might involve interactions with integrins or other extracellular proteins. Further studies to understand how allelic heterogeneity contributes to phenotypic heterogeneity and the extent to which it might reflect mechanistic heterogeneity are needed. Such insights could hold significant value as prognostic tools and for developing targeted treatments for different disease processes.

### THERAPEUTIC STRATEGIES FOR *COL4A1*- AND *COL4A2*-ASSOCIATED DISEASES

There is evidence for external factors playing a role in the expressivity of *COL4A1*- and *COL4A2*-associated diseases. Environmental factors that contribute to disease often represent modifiable risk factors. For example, surgical delivery of *Col4a1* mutant pups greatly reduced the incidence of perinatal ICH, suggesting that Cesarean delivery could improve clinical outcomes in patients with *COL4A1* mutations (80). We have also observed an exercise-dependent difference in serum creatine kinase activity in *Col4a1* <sup>$\Delta$ ex41/+</sup> mice (97). Moreover, head trauma, sports activity and use of anticoagulants have all been reported as factors contributing to ICH in patients with *COL4A1* mutations. These reports suggest that management of modifiable risk factors may improve quality of life in patients with *COL4A1* or *COL4A2* mutations.

The next frontier for therapeutic strategies will capitalize on specific disease mechanism(s). Thus, the impetus for understanding the molecular mechanisms of *COL4A1* and *COL4A2* pathogenesis is to ultimately develop targeted therapeutic approaches to prevent, delay or diminish disease. For example, if heterotrimer deficiency in basement membranes is pathogenic, then efforts to up-regulate expression might be beneficial. However, if misfolded proteins are cytotoxic, then increasing production of *COL4A1* and *COL4A2* might exacerbate the insult and efforts to promote degradation of intracellular accumulations might be beneficial. Misfolded proteins within the ER are generally eliminated via the proteasomal (ER-associated degradation) or the lysosomal pathway (autophagy) (111,112). Misfolded *COL1A1* heterotrimers are

degraded via autophagy, and inducing autophagy with rapamycin decreased detergent-insoluble protein aggregates (113). There are several FDA-approved autophagy-inducing drugs (114,115) whose efficacy in preventing or treating COL4A1- and COL4A2-related diseases can be tested.

Other therapeutic approaches include promoting protein folding to shift the fate of mutant heterotrimers and alleviate intracellular and extracellular insults. Proof of principle for this approach comes from key experiments in *C. elegans* where conditions that promote protein folding decreased intracellular accumulation, restored secretion and rescued the viability of mutant animals (116). Similar results could be achieved using chemical chaperones (117) which are currently being explored as treatment options in a variety of diseases associated with protein misfolding and are worthy of exploration for their efficacy in treating COL4A1- and COL4A2-related disorders (118–122).

## FUTURE DIRECTIONS

In a relatively brief period of time, COL4A1 and COL4A2 mutations have been discovered to cause a broad spectrum of debilitating or fatal diseases. Pathology varies in penetrance and severity among tissues and can be modulated by genetic and environmental factors. Model organisms will be key for understanding the molecular mechanisms associated with specific mutations. Mouse models can be used to understand genotype–phenotype correlations, provide insight into the contribution of allelic heterogeneity and identify genetic modifiers that could lead to the discovery of novel molecular pathways involved in the etiology of COL4A1- and COL4A2-associated disorders. The use of conditional mutants will be valuable to genetically dissect the tissue-specific effects of different mutations. Understanding the biological consequences of COL4A1 and COL4A2 mutations is critical to the development of personalized therapeutic strategies. As the molecular mechanisms of disease are discovered, we can better predict and address the effects of particular mutations, and potentially provide targeted therapeutic options to patients with COL4A1 and COL4A2 mutations.

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