RESEARCH ARTICLE

Loss of function of Colgalt1 disrupts collagen post-translational modification and causes musculoskeletal defects

Krista A. Geister1,2, Alberto Jose Lopez-Jimenez3,4, Scott Houghtaling1, Tzu-Hua Ho1, Roberto Vanacore3,4 and David R. Beier1,2,*

ABSTRACT

In a screen for organogenesis defects in N-ethyl-N-nitrosourea (ENU)-induced mutant mice, we discovered a line carrying a mutation in Colgalt1 [collagen beta(1-O)galactosyltransferase type 1], which is required for proper galactosylation of hydroxylysine residues in a number of collagens. Colgalt1 mutant embryos have not been previously characterized; here, we show that they exhibit skeletal and muscular defects. Analysis of mutant-derived embryonic fibroblasts reveals that COLGALT1 acts on collagen IV and VI, and, while collagen VI appears stable and its secretion is not affected, collagen IV accumulates inside of cells and within the extracellular matrix, possibly due to instability and increased degradation. We also generated mutant zebrafish that do not express the duplicated orthologs of mammalian Colgalt1. The double-homozygote mutants have muscle defects; they are viable through the larvae stage but do not survive to 10 days post-fertilization. We hypothesize that the Colgalt1 mutant could serve as a model of a human connective tissue disorder and/or congenital muscular dystrophy or myopathy.

KEY WORDS: ENU, Collagen glycosylation, Collagenopathies, Myopathy, GLT25D1

INTRODUCTION

There are nearly 30 different forms of collagen in vertebrates encoded by over 40 genes. This diverse array of proteins is responsible for maintaining the structural integrity of tissues. The vast majority of collagens are secreted by resident fibroblasts. Collagens are ultimately organized into a variety of superstructures, including fibrils, interrupted fibrils, beaded microfilaments, mesh networks and hexagonal lattices, and can even span the cell membrane. Many of them have been shown to interact with a number of additional extracellular matrix (ECM) components (Mienaltowski and Birk, 2014; Ricard-Blum, 2011). The requirement of collagen in bone, cartilage, skin, muscle, ligaments and tendons is evident when one considers the phenotypes including fibrils, interrupted fibrils, beaded microfilaments, mesh networks and hexagonal lattices, and can even span the cell membrane. Many of them have been shown to interact with a number of additional extracellular matrix (ECM) components (Mienaltowski and Birk, 2014; Ricard-Blum, 2011).

The requirement of collagen in bone, cartilage, skin, muscle, ligaments and tendons is evident when one considers the phenotypes including fibrils, interrupted fibrils, beaded microfilaments, mesh networks and hexagonal lattices, and can even span the cell membrane. Many of them have been shown to interact with a number of additional extracellular matrix (ECM) components (Mienaltowski and Birk, 2014; Ricard-Blum, 2011).

Colgalt1

© 2019. Published by The Company of Biologists Ltd | Disease Models & Mechanisms (2019) 12, dmm037176. doi:10.1242/dmm.037176

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.
RESULTS

The fosse mutant phenotype is caused by a loss-of-function allele of Colgalt1

The Colgalt1fosse mutation substitutes an arginine for a highly conserved tryptophan in the nucleotide-diphospho-sugar transferase domain of COLGALT1 (c.T388C:p.W130R), which is in the N-terminal domain of the enzyme (Fig. 1A,B). The Polyphen score for this substitution is 1.0 (highly damaging) (Adzhubei et al., 2010). Western blot analysis of primary mouse embryonic fibroblast (MEF) lysates indicates that this mutation results in a loss of COLGALT1 protein expression (Fig. 1C). In all three independent mutant MEF lines, there is no detectable band corresponding to

---

Fig. 1. Missense mutation in Colgalt1 leads to loss of expression at the protein level. (A) Chromatograms of wild-type and homozygous fosse sequence clearly demonstrates the substitution of a cytosine for a thymine (asterisks). (B) Schematic of COLGALT1 indicates where the missense mutation occurs in the encoded enzyme. (C) Immunodetection of COLGALT1 (top) and COLGALT2 (middle) by western blotting in wild-type and fosse MEF lysates. Ponceau S staining of the proteins transferred to the membrane (bottom) was used as loading control. Underneath these immunoblots is the quantitation by densitometric analyses of Colgalt1 and Colgalt2 chemiluminescent signals. Molecular mass standard protein is shown in kDa for reference. (D,E) Immunoblot detection of PLOD3 (D) and Hsp47 (E) in wild-type and fosse MEF lysates. Signal intensity for bands of interest (n=3 in each group) was quantified with ImageJ and normalized to GAPDH (bottom). Densitometric quantitation for PLOD3 (D) and HSP47 (E) are shown underneath immunoblots. A statistically significant difference (P≤0.05) is indicated by an asterisk (*).
COLGALT1, which is expressed in all wild-type MEF lines (Fig. 1C, top panel). Western blots using primary antibodies against the paralog COLGALT2 (Fig. 1C, middle panel) and the related enzyme procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3) (Fig. 1D) showed that these enzymes are present, but not overexpressed upon COLGALT1 loss of function. Densitometric analysis showed that PLOD3 protein level is even slightly decreased. As shown in Fig. 1F, levels of the collagen-specific chaperone HSP47 was not changed. These results suggest that the loss of Colgalt1 in mutant MEFs did not trigger a major compensatory response by enzymes involved in hydroxylysyl galactosylation or the folding and secretion of collagen molecules.

Colgalt1fosse/fosse embryos exhibit musculoskeletal phenotypes

Colgalt1fosse/fosse embryos have a rounded body, appear slightly swollen and are smaller than unaffected littermates (Fig. 2A). The forepaws of mutant embryos are distinctly bent downward at the wrist (Fig. 2A). Staining of skeletal elements using Alcian Blue to label cartilage and Alizarin Red to stain mineralized tissue revealed that the carpals in the wrist and the rib cage are smaller than in wild-type mice (Fig. 2B). Hematoxylin and Eosin (H&E) staining of histological sections through isolated tibiae revealed normal epiphyseal growth plate architecture (Fig. 2C). Staining of sections through the limbs revealed a readily observable muscle defect (Fig. 2D,E). The muscle fibers

Fig. 2. Loss of function of Colgalt1 leads to defects in musculoskeletal structure. (A) Colgalt1fosse/fosse embryos are smaller, rounded and swollen compared to unaffected littermates at E18.5. (B) Skeletal preparations of Colgalt1fosse/fosse embryos at E18.5 clearly demonstrate a reduction in the size of the bones in the wrist and the rib cage. (C) Hematoxylin and Eosin (H&E)-stained sections through the proximal tibiae indicate that there is no gross disorganization in the growth plates of this skeletal element at E18.5. (D) H&E-stained sagittal sections through the forelimbs show disorganized, ragged and shorter muscle fibers in the bicep and brachioradialis compared to wild type. Right hand panels show magnified views of sections in left hand panels. Scale bars: 200 μm (C, 10×; D, left) and 100 μm (C 20×; D, right).
in major muscle groups of the arms and legs appear shorter, more disorganized and with increased interfiber space (Fig. 2D). Unsurprisingly, muscle groups also appear smaller in size overall (Fig. 2E).

**Collagen I accumulates in MEFs and is reduced in MEF ECM**

Collagen I is one of the most abundantly expressed and widely distributed collagen types (Mienaltowski and Birk, 2014; Ricard-Blum, 2011; Yamauchi and Sricholpech, 2012). To test whether type I collagen expression is altered in *Colgalt1f0sse/f0sse*, we conducted western blot analyses using a primary antibody against type I collagen (Fig. 3A,B).

Analysis of cell lysates would provide information about general expression and processing of type I collagen. The analysis of isolated ECM would determine whether there was impaired secretion or integrity, or a predisposition to degradation. Our western blot analyses clearly demonstrated that type I collagen accumulates intracellularly (Fig. 3A). Consistent with that observation, while its precursor (procollagen) and intermediate (pN- or pC-procollagen) forms are present, processed type I collagen is dramatically reduced in the culture supernatants of *f0sse* compared to wild-type cells (Fig. 3B).

Fig. 3. The *Colgalt1f0sse* mutation impairs secretion of collagen type I. (A) Immunoblot detection of the α1 chain of type I collagen in wild-type and *f0sse* MEF lysates. The electrophoretic migration of procollagen (i.e. full length, unprocessed form of the α1 chain of type I collagen) is indicated. Quantitation of the procollagen signal by densitometric analysis is shown adjacent to the immunoblot. (B) Immunoblotting analysis of type I collagen in culture medium of wild-type and *f0sse* MEF lines. The migration of procollagen, amino (pN-) or carboxyl (pC-) procollagen proteolytic intermediates and processed collagen are indicated. Bands of interest were normalized to Ponceau S staining and quantified using ImageJ software (Schindelin et al., 2012). Quantitation of the processed collagen signal by densitometric analysis is shown adjacent to the immunoblot. The graphs show results from three independent experiments as mean (s.d.). Statistically significant difference was determined by unpaired *t*-test and it is indicated by an asterisk (*) above the data points (n=3 in each group; *P*≤0.05).
collagen is significantly decreased in the culture medium of mutant MEFs (Fig. 3B).

**Collagen IV modification and stability are disrupted in Colgalt1fosse/fosse MEFs, but expression in mutant tissue is normal**

Collagen IV is one of the most highly glycosylated forms of collagen (Yamauchi and Sricholpech, 2012; Basak et al., 2016) and is crucial for maintenance of the basal lamina of epithelia (Poschl et al., 2004; Khoshnoodi et al., 2008; Brown et al., 2017). Mutations in collagen IV α-chain-encoding genes are known to cause vascular, eye and kidney defects (Pedchenko et al., 2010; Gould et al., 2006; Hudson et al., 1993). Additionally, it is one of the major forms of collagen disrupted upon loss of Plod3 function in mutant mice (Rautavuoma et al., 2004; Sipila et al., 2007), which have a phenotype we found to be strikingly similar to that of Colgalt1fosse/fosse mutants (Geister et al., 2017). Taken together, these observations led to the hypothesis that collagen IV could be perturbed in Colgalt1fosse/fosse embryos.

To test this hypothesis, we conducted western blots on MEF cultures to identify any anomalies in collagen IV processing, secretion or stability (Fig. 4A,B). Collagen IV accumulates in mutant MEFs (Fig. 4A), and the protein itself runs at a lower molecular mass (Fig. 4A,B). This shift is likely due to the loss of COLGALT1-mediated galactosylation of hydroxylysines. In the medium, the band corresponding to full-length collagen IV is more robust in mutant cells (Fig. 4B, arrows). Consistent with the increased...
secretion of collagen IV, an independent assay showed its increased deposition as evidenced by increased levels of non-collagenous (NC1) domain solubilized from the ECM produced by fosse MEFs (Fig. 4C,D). However, the collagen IV that is present in the mutant cell lines’ media exhibits a banding pattern that is characteristic of protein degradation (Fig. 4B, bracket). These results suggest that fosse MEF cultures increase collagen IV production and deposition likely in response to its decreased stability due to defective lysine glycosylation.

Given that collagen IV is expressed in muscle and we have a muscle defect in our mutant mouse embryos, we decided to test expression of collagen IV by immunohistochemistry (IHC) in sections through Colgalt1fosse/fosse limbs. We used the same primary antibody used for western blots, which recognizes the NC1 domain of COL4A2 (Fig. 4E). Collagen IV should be expressed around individual muscle fibers and the group itself. Our IHC images clearly demonstrate that, in spite of the tissue organization aberrations in collagen VI processing or secretion.

Using a primary antibody against COL6A1, we conducted western blot analyses on MEF lysates and cell culture medium. In both cases, we observed a reduction in molecular mass in mutant samples (Fig. 5A,B). As with collagen IV, this likely indicates the loss of galactosylation of hydroxylysines in COL6A1 upon loss of function of Colgalt1. Secretion of COL6A1 appears to be normal, as the bands are of the same intensity as in wild-type cell lysates and medium (Fig. 5A,B). The presence of a single band with minimal variation in molecular mass and lack of degradation fragments suggest that, unlike collagen IV, defective glycosylation does not significantly impact the stability of collagen VI.

Given that we have an observable defect in the musculature of our mutant embryos, and the fact that collagen VI does appear to be of a lower molecular mass in these mutants, we decided to test whether expression of COL6A1 was altered in mutant tissues by conducting IHC. We applied a primary antibody against COL6A1 to frozen sections through Colgalt1fosse/fosse limbs and observed normal expression in skin and muscle (Fig. 5C).

**Mutation of colgalt1 in zebrafish causes early lethality**

To complement our work in Colgalt1fosse/fosse mouse embryos, we generated colgalt1 mutant lines of zebrafish using CRISPR-Cas9 genome-editing techniques (Varshney et al., 2015; Varshney et al., 2016). Like many genes in the zebrafish, colgalt1 is duplicated, with loci corresponding to the colgalt1 sequence on chromosomes 1 and 3. Hereafter, these loci are referred to as colgalt1b and colgalt1a, respectively. We selected single-guide RNAs (sgRNAs) that target the fifth exons of both genes (Varshney et al., 2016) and injected them into zebrafish embryos at the one-cell stage. This led to the generation of a 1 bp deletion in colgalt1b on chromosome 1 (colgalt1b11bpdel19bpins5) and an 11 bp deletion/19 bp insertion in colgalt1a on chromosome 3 (colgalt1a11bpdel19bpins5) (Fig. S1). Predicted open reading frames for the encoded proteins indicate that both mutations cause frameshifts followed by premature stop codons, which should lead to loss of colgalt1a and colgalt1b activity in double-homozygous fish (Fig. S1).

Crosses of double-heterozygous colgalt1a and colgalt1b mutant fish revealed that no double homozygotes were obtained at 3 months of age (Table 1). Single colgalt1a or colgalt1b homozygous fish were obtained at expected numbers, with no evident developmental defects. To more specifically determine the day of death, intercrosses were done between fish that were homozygous for one colgalt1 allele and heterozygous for the other; one-quarter of the progeny should be double homozygotes. These were found to be present at days 7-9 post-fertilization and absent by day 10 (Table 2). Assessment of double-homozygous larvae at 8 and 9 days post-fertilization by phalloidin staining revealed gross morphological defects in muscle fiber organization (Fig. 5D), and larvae demonstrated reduced mobility at these ages.

**The Colgalt1fosse/fosse skin barrier is intact**

Reduction of type I collagen in the skin could disrupt the organ’s ability to reduce fluid loss, leading to severe dehydration and death. To test the barrier function of Colgalt1fosse/fosse skin, we conducted a skin barrier assay (Indra and Leid, 2011). This test makes use of the intrinsic β-galactosidase activity of the skin at its basal layers. Fresh skin tissue is incubated with X-gal solution. Normal skin will keep the X-gal out of the basal layers of the skin, while a disrupted skin barrier will allow the X-gal to reach these basal layers, where β-galactosidase will catalyze the substrate to a blue by-product, turning the skin blue. Examination of homozygous mutant mice reveals that the skin barrier is intact (Fig. S2).

**DISCUSSION**

We have previously reported the discovery of an N-ethyl-N-nitrosourea (ENU)-induced mutation of Colgalt1 in the mouse (Geister et al., 2017) (Fig. 1A). Our characterization of its phenotype here provides valuable insight into the functions and tissue requirements for this enzyme in collagen processing. Clearly, COLGALT1 activity is required in muscle for proper architecture of muscle fibers within individual muscle groups (Fig. 2D,E). Previous work has also shown that COLGALT1 is capable of galactosylating hydroxylysine residues in collagens IV in vitro (Schegg et al., 2009). Here, we have confirmed that COLGALT1 galactosylates hydroxylysine residues in collagen IV (Fig. 4A,B), and provide the first evidence indicating that COLGALT1 acts on collagen VI (Fig. 5A,B).

The Colgalt1 fosse mutation is a missense mutation that substitutes an arginine for a highly conserved tryptophan residue (p.W130R, Fig. 1) (Geister et al., 2017). Previous works on the functional domains of human COLGALT1 have shown that the C-terminal domain is required for galactosyltransferase activity (Perrin-Tricaud et al., 2011) and that the N-terminal domain shares homology with other galactosyltransferases and PLOD3 (Liefhebber et al., 2010). Our analysis of COLGALT1 expression indicates that this mutation leads to a loss of COLGALT1 in mutant MEFs (Fig. 1C). As it is a missense mutation, this change in protein expression is not likely due to nonsense-mediated decay. It is possible that the substitution of a basic amino acid for a hydrophobic amino acid in this highly conserved region results in protein instability and degradation.

Colgalt1 fosse mutant embryos exhibit many structural phenotypes (Fig. 2), including bent wrists. While smaller carpals (Fig. 2B)
could contribute to the wrist phenotype, it could also be due to a defect in muscle or connective tissue. Since we have observed a muscle defect, this phenotype could be indicative of contractures (Fig. 2D,E). It is worth noting that removing the skin of the lower, but not upper, limbs frequently results in complete disarticulation of the tibia from the femur, suggesting a weakening of the connective tissues in the knee joint. Upon dissection, the integument also feels weakened and is frequently marked with areas of hemorrhaging. In addition, at least one, if not both, of the eyelids are open in most mutant embryos (Fig. 2A). Analysis of skin barrier function revealed this to be normal (Fig. S2).

Mutant embryos also variably express cleft palate and exencephaly (Fig. S2); these phenotypes are never observed in wild-type littermates. Chi-square analysis indicates that this association is highly significant, demonstrating that cleft palate and exencephaly are contingent on the presence of the fosse mutation (Geister et al., 2017). Genomic analyses followed by Sanger sequencing of candidate ENU variants have suggested that it
is unlikely that additional genetic loci are acting in a recessive or dominant manner to cause cleft palate, or in a dominant manner to cause exencephaly (Geister et al., 2017). We have concluded that these phenotypes are likely due to stochastic events during embryonic development.

We also investigated the role of Colgal1 in the development of zebrafish. The Danio rerio genome is partially duplicated, and zebrafish carry two orthologs of Colgal1. These appear to be functionally redundant, as zebrafish homozygous for null mutations in either of these alleles survive to adulthood, appear normal and are fertile. This is also true for zebrafish that are homozygous for one mutant allele and heterozygous for the other. However, zebrafish homozygous for both null mutant alleles demonstrate defects in muscle development and do not survive to 10 days post-fertilization, potentially due to reduced mobility and inability to feed.

This is the first characterization of the embryonic phenotype of a Colgal1 null allele in the mouse. A recent characterization of heterozygous phenotypes in a Colgal1 ‘knock out’ also reports that the homozygous mutants are lethal prior to embryonic day (E)13.5 (Ye et al., 2018). This difference is likely due to the ENU-induced missense mutant retaining a low amount of activity (despite our observation that the protein is not detectable by western blot analysis).

Of note, Colgal1 has the potential to functionally and phenotypically overlap with the lysyl hydroxylases Plod1, Plod2 and Plod3 (Yamauchi and Sricholpech, 2012). As mentioned previously, COLGALT1 and Plod3 colocalize in the ER and are thought to work together to hydroxylate and glycosylate lysine residues in collagens (Schege et al., 2009; Liefhebber et al., 2010). Furthermore, while categorized as a lysyl hydroxylase, PLOD3 also exhibits collagen galactosyl- and glucosylgalactosyltransferase activity (Heikkinen et al., 2000; Rautavuoma et al., 2002; Wang et al., 2002a,b).

Intriguingly, in the screen in which we discovered Colgal1fosse/fosse, we also discovered mutant mice with an ENU-induced missense mutation of a conserved valine residue in Plod3 (Plod3H9Gly/Plod3H9Gly, c.T695A:p.V232E) that phenocopies Colgal1fosse/fosse (Geister et al., 2017). The Plod3H9Gly mutation occurs in exon 7, closer to the N-terminus and is only five residues downstream of a mutation reported in a PLOD3 compound heterozygous human patient with multiple abnormalities (p.N223S; Salo et al., 2008). Below, we compare our two mutant phenotypes with those that have been previously described to illustrate how these new mutant alleles fit within the current model of post-translational modification of collagens.

Colgal1fosse/fosse and Plod3H9Gly/Plod3H9Gly mutant embryos survive until late gestation (E18.5), and presumably die at or around the time of birth, as we have never observed a homozygous live pup from either line. This is in contrast to all of the Plod3 mutant alleles that have been described, which die between E8.5 and E14.5 (Rautavuoma et al., 2004; Ruotsalainen et al., 2006). Therefore, we have discovered two mutant alleles that allow enough glucosylgalactosyltransferase activity of PLOD3 and galactosyltransferase activity of COLGALT1 to ensure survival past midgestation, but ultimately insufficient levels to allow survival past birth. Given the nearly identical phenotypes of Colgal1fosse/fosse and Plod3H9Gly/Plod3H9Gly, it is likely that both are acting in the same cell types and catalyzing hydroxylation and glycosylation of the same collagen α-chains.

Specifically, we present evidence that COLGALT1 acts on collagen IV and V1 (Figs 4 and 5). Both of these types exhibit a reduction in molecular mass that is likely due to the loss of glycosylation of hydroxylsines (Figs 4A,B; 5A,B). Galactose is the first sugar to be added in the collagen glycosylation cascade (Sipio et al., 2007; Bushby et al., 2014; Kalluri, 2003). We also suggest that it is likely that mutations of COLGALT1 would cause connective tissue disorders in humans, similar to the patient with a defective PLOD3 expression whose phenotype included bone fragility and contractures (Salò et al., 2008). Of note, a recent report described compound heterozygous mutations of COLGALT1 in two pediatric patients with cerebral small vessel disease (Miyatake et al., 2018). COLGALT1 was investigated because of the known role of COL4A1/COL4A2 in similar diseases (Weng et al., 2012; Jeanne et al., 2012). Neither patient was reported to have primary muscle disease. Given the role of COLGALT1 in the metabolism of many collagens, including those such as COL6A1, which is associated with primary myopathies (Kirschner, 2013), we speculate that these patients retained sufficient low-level enzyme activity to spare them from the embryonic defects seen in our mutant and the related Plod3 mutants.

Addional studies of Colgal1 and related enzymes could add clarification to the complex process of collagen post-translational modification. Although we know the cellular locale, key players and tissue requirements for collagen glycosylation, the benefit that these modifications provide remains elusive. Future studies of

**Table 1. Double-mutant colgal1a−/−;colgal1b−/− zebrafish do not survive to adulthood**

| colgal1a | colgal1b | Observed no. at 3 months | Expected no. at 3 months |
|---------|---------|--------------------------|-------------------------|
| +/+     | +/+     | 10                       | 11.5                    |
| +/+     | −/+     | 30                       | 23                      |
| +/+     | −/−     | 13                       | 11.5                    |
| +/+     | −/+     | 22                       | 23                      |
| +/+     | +−/−    | 52                       | 46                      |
| −/−     | −/+     | 23                       | 23                      |
| −/−     | −/+     | 14                       | 11.5                    |
| −/−     | −/+     | 20                       | 23                      |
| −/−     | −/+     | 0                        | 11.5                    |
| Total   |         | 184                      | 184                     |
| P-value |         | 0.0456                   |                         |

**Table 2. Double-mutant colgal1a−/−;colgal1b−/− zebrafish do not survive past 9 days**

|          | 7 days | 8 days | 9 days | 10 days | 11 days | 12 days |
|----------|--------|--------|--------|---------|---------|---------|
| Total    | 96     | 133    | 208    | 50      | 40      | 26      |
| Expected | 24     | 33.25  | 54     | 12.5    | 10      | 6.5     |
| Observed | 26     | 32     | 52     | 0       | 0       | 0       |
| P-value  | 0.83735| 0.80235| 0.7877 | 0.00004 | 0.00026 | 0.00324 |
COLGALT1 and related enzymes will expand our understanding of collagen biosynthesis and its involvement in disorders of collagen glycosylation.

**MATERIALS AND METHODS**

**Generation, care and use of animals**

All animal work was reviewed and approved by the Seattle Children’s Research Institute’s Institutional Care and Use Committee (IACUC). The discovery and positional cloning of the Colgalt1 mutant line was previously described (Geister et al., 2017). The line was generated and maintained on the C57BL/6J background. Genotype analysis was done by PCR with primers (F: 5′-GCCACCTCTGGAGAATCTCCTC-3′, R: 5′-CATCTCTCCTCCTGGTGAA-3′) to generate a 265 bp product followed by digestion with XspI. The ENU mutation introduces an XspI site.

**Generation and culture of mouse embryonic fibroblasts**

MEFs were derived from E14.5 embryos using standard procedures (Joyer, 2000; Nagy, 2003). MEFs were cultured at 37°C and 5% CO2 in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 50 µg/ml ascorbic acid. After reaching confluency, cells were cultured in FBS-free medium for an additional 40 h before collecting culture media and preparing whole-cell extracts. For analyses of collagen biosynthesis, MEFs were cultured in FBS-free medium for an additional 40 h before collecting culture media and preparing whole-cell extracts. For analyses of collagen biosynthesis and its involvement in disorders of collagen glycosylation, MEFs were cultured for 7 days past confluency with culture media and preparing whole-cell extracts. For analyses of collagen biosynthesis, MEFs were cultured in FBS-free medium for an additional 40 h before collecting culture media and preparing whole-cell extracts. For analyses of collagen biosynthesis, MEFs were cultured in FBS-free medium for an additional 40 h before collecting culture media and preparing whole-cell extracts.

**Western blotting protocols**

For lysate preparation, MEFs were washed twice with PBS and lysed with RIPA buffer supplemented with HALT Protease Inhibitor Cocktail (Thermo Fisher Scientific). Culture media was harvested and concentrated about 200-fold. Frozen tissue samples were homogenized using a Dounce homogenizer. Protein content was quantified by BCA assay (Pierce). Western blotting protocols were performed as previously described (Indra and Leid, 2011).

**Histological analyses**

Tissues were collected from embryos at E18.5 and fixed overnight in 4% paraformaldehyde. They were then either processed for paraffin or frozen sectioning. For paraffin-embedded tissue processing, they were incubated overnight in PBS, dehydrated in ethanol to 70% and embedded in paraffin. For frozen sections, fixed tissues were incubated in 30% sucrose until the tissue sank, and embedded in OCT freezing medium on dry ice. Sections were then cut and mounted on slides. Paraffin-embedded sections were stained with H&E, whereas frozen sections were used for immunohistochemistry, the protocols for which are detailed below.

For immunofluorescence studies, frozen tissue sections were allowed to equilibrate to room temperature, washed in 1× PBS for 5 min to dissolve the OCT, and incubated with blocking solution for 1 h (blocking solution: 1× PBS, 3% bovine serum albumin, 0.3% Triton X-100, 0.02% sodium azide, 3% goat serum). Slides were then incubated with primary antibodies against COL4A2 (1:1000, rat monoclonal H22 antibody, Chondex, cat. # 7071), or COL6A1 (1:100, Abcam, #6588). The next day, slides were washed in PBS and incubated with secondary antibodies. For COL4A2, the slides were incubated with goat anti-rabbit Alexa-Fluor-647 from Life Technologies (cat. # A21247) diluted 1:1000 for 1 h at room temperature. For COL6A1, we used goat anti-rabbit Alexa-Fluor-488 from Life Technologies (#a11008), diluted 1:500 and incubated for 1 h at room temperature. After washing the slides, the tissues that reacted with anti-COL4A2 antibodies were incubated with Vectashield® antifade mounting medium with DAPI (Vector Laboratories, cat. # H-1200), whereas those that reacted with anti-COL6A1 antibodies were incubated with Hoechst 33258 solution (Life Technologies Corp., cat. # H3569) diluted 1:5000 in PBS for 5 min, rinsed in PBS and mounted with aqueous mounting medium.

**Skin barrier assay**

Embryos were collected at E18.5 and the skin barrier assay was performed as previously described (Indra and Leid, 2011).

**CRISPR-Cas9 genome editing in zebrafish**

Mutations in the sgRNA target site 5′-GGGCGTTTTGCCGTTGCGGA-3′ in exon 5 of the colgalt1 isoform on chromosome 1 and target site 5′-GGGATGTTTGGCCGTTCAA-3′ in exon 5 of the colgalt1 ortholog on chromosome 3 were generated using CRISPR-Cas9. Cas9 mRNA was prepared from plasmid MLM3613 using the mMESSAGE mMACHINE™ T7 Transcription Kit (Thermo Fisher Scientific/Invitrogen, #AM1344). The sgRNA was prepared using a cloning-free synthesis method (Varshney et al., 2015). The DNA template was generated by annealing oligos and extending using High Fidelity Phusion Polymerase (New England Biolabs, #M0530S). In vitro transcription was performed using HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, #E2050S).

Plasmid MLM3613 was a gift from Keith Joung (Addgene plasmid # 42251) (Hwang et al., 2013). A total of 2 µl of solution containing Cas9 mRNA at 336 ng/µl and sgRNA at 24 ng/µl was injected into the yolk sac of single-celled zebrafish embryos. Screening of F1 progeny from injected embryos and genotyping of F2 progeny from double-heterozygous crosses was performed by PCR and sequencing with F: 5′-ATTGTGGCACACACCTGCTAT-3′, R: 5′-TGAACTTTATCTTTGCGTTTGACT-3′ mutation in the sgRNA target site.

**Phallolidin staining**

Phallolidin assay was performed as previously described (Goody et al., 2012).

**Acknowledgements**

We thank Mohamed Rafi for his skillful technical assistance and Gist H. Farr, III for help with zebrafish analysis.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: K.A.G., R.V., D.R.B.; Methodology: K.A.G., A.J.L.-J., S.H., R.V., D.R.B.; Validation: D.R.B.; Formal analysis: A.J.L.-J., D.R.B.; Investigation: K.A.G., A.J.L.-J., R.V., D.R.B.; Resources: R.V., D.R.B.; Writing - original draft: K.A.G., D.R.B.; Writing - review & editing: K.A.G., A.J.L.-J., R.V., D.R.B.; Supervision: R.V., D.R.B.; Project administration: D.R.B.; Funding acquisition: R.V., D.R.B.

**Funding**

This work was supported in part by the National Institutes of Health (HD036404 to D.R.B. and DK099467 to R.V.).
Mienaltowski, M. J. and Birk, D. E. (2014). Structure, physiology, and biochemistry of collagens. Adv. Exp. Med. Biol. 802, 5-29. doi:10.1007/978-1-4939-07793-1_2

Miyatake, S., Schneebeger, S., Koyama, N., Yokochi, K., Ohmura, K., Shinya, M., Mori, H., Koshimizu, E., Imegawa, E., Uchiyama, Y. et al. (2016). Biallelic COL1A1 variants are associated with cerebral small vessel disease. Ann. NeuroL. 84, 843-853. doi:10.1016/j.nia.2015.08.002

Nagy, A. (2003). Manipulating the Mouse Embryo: a Laboratory Manual. Vol. 10, 3rd ed, pp. 74-105. Cold Spring Harbor Laboratory Press.

Ovchinnikov, D. (2008). Alcian blue/azur stained sheets of cartilage and bone in mouse collagen IV. Cold Spring Harb. Protoc. 2008, pdb.prot5170. doi:10.1101/pdb.prot5170

Pedchenko, V., Bondar, O., Fogo, A. B., Vanacore, R., Voziyan, P., Kitching, A. R., Wieslander, J., Kashtan, C., Borza, D.-B., Neilson, E. G. et al. (2010). Molecular architecture of the Goodpasture autoantigen in anti-GBM nephritis. J. Biol. Chem. 285, 343-351.

Perrin-Tricaud, C., Rutschmann, C. and Hennet, T. (2011). Identification of domains and amino acids essential to the collagen galactosyltransferase activity of GLT25D1. J. Mol. Biol. 415, 61-72. doi:10.1016/j.jmb.2011.06.043

Poschl, E., Schlote-Schrardt, U., Bruchvogl, B., Saito, K., Ninomiya, Y. and Mayer, U. (2004). Collagen I is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development 131, 1619-1628. doi:10.1242/dev.01037

Rautavauxa, K., Takaloum, K., Passoja, K., Pirskanen, A., Kivist, A.-P., Kivirikko, K. I. and Myllyharju, J. (2002). Characterization of three fragments that constitute the monomers of the human lysyl hydroxylase isoenzymes 1-3. The 30-kDa N-terminal fragment is not required for lysyl hydroxylase activity. J. Biol. Chem. 277, 23084-23091. doi:10.1074/jbc.M110277200

Rautavauxa, K., Takaloum, K., Sormunen, R., Myllyharju, J., Kivirikko, K. I. and Soininen, R. (2004). Premature aggregation of type IV collagen and early lethality in lysyl hydroxylase null mice. Proc. Natl. Acad. Sci. USA 101, 14120-14125. doi:10.1073/pnas.0409961101

Ricard-Blum, S. (2011). The collagen family. Cold Spring Harb. Perspect. Biol. 3, a004978. doi:10.1101/cshperspect.a004978

Ruotsalainen, H., Siipila, L., Vapola, M., Sormunen, R., Salo, A. M. and Uitto, L. (2004). Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes. J. Cell. Sci. 119, 625-635. doi:10.1242/jcb.200307280

Salo, A. M., Cox, H., Farndon, P., Moss, C., Grindulis, H., Risteli, M., Robins, S. P. and Myllyharju, R. (2008). A connective tissue disorder caused by mutations of the lysyl hydroxylase 3 gene. Am. J. Hum. Genet. 83, 495-503. doi:10.1016/j.ajhg.2008.09.004

Schepp, B., Hulsmeier, A. J., Rutschmann, C., Maag, C. and Hennet, T. (2009). Core glycosylation of collagen is initiated by two beta(1-O)-galactosyltransferases. Mol. Cell. Biol. 29, 943-952. doi:10.1128/MCB.02085-07

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676-682. doi:10.1038/nmeth.2019

Siipila, L., Ruotsalainen, H., Sormunen, R., Baker, N. L., Lamadé, S. R., Vapola, M., Wang, C., Sado, Y., Aszodi, A. and Myllyharju, R. (2007). Secretion and assembly of type IV and VI collagens depend on glycosylation of hydroxylysines. J. Biol. Chem. 282, 33338-33348. doi:10.1074/jbc.M704198200

Spiro, R. G. (1967). The structure of the disaccharide unit of the renal glomerular basement membrane. J. Biol. Chem. 242, 4813-4823.

Varshney, G. K., Pei, W., Laffel, J. M., Xu, L., Gallardo, V., Carrington, B., Bishopp, K., Jensen, M. P., Li, M. M. et al. (2005). High-throughput gene targeting and phenotyping in zebras using a CRISPR-Cas system. Genome Res. 15, 1030-1042. doi:10.1101/gr.26931.105

Varshney, G. K., Zhang, S., Pei, W., Adomako-Ankoham, A., Fohtung, J., Schaffer, K., Carrington, B., Maskeri, A., Slevin, C., Wolfsberg, T. et al. (2016). CRISPRz: a database of zebrafish validated sgRNAs. Nucleic Acids Res. 44, D282-D282. doi:10.1093/nar/gkw998

Wang, C., Luosuajärvi, H., Heikinlampi, J., Risteli, M., Uitto, L. and Myllyharju, R. (2002a). The α3(1) chain of the collagen type IV α1(1) chain is an epitope for autoantibodies in sera of patients with idiopathic collapsing glomerulonephritis. J. Biol. Chem. 277, 5599-5608. doi:10.1074/jbc.M201392200

Weng, Y.-C., Sonni, A., Labelle-Dumas, C., De Laeu, M., Kauffmann, W. B., Jeanne, M., Biffi, A., Greenberg, S. M., Rosand, J. and Gould, D. B. (2012). CollA4A1 mutations in patients with sporadic late-onset intracerebral hemorrhage. J. NeuroL. 285, 18573-18579. doi:10.1016/j.jnrl.2011.09.007

Yamashita, Y. and Ichihara, K. (2004). Role of Collagen type VI in the formation of the basement membrane. Pediatr. Surg. 29, 1226-1232. doi:10.1016/j.purol.2004.06.004

Ye, X., He, L., Ma, J., Li, Y., Zhang, M., Yang, J., Zhang, J., Xiao, F. and Wei, H. et al. (2018). Downregulation of Glit2d1 aggravates carbon tetrachloride-induced acute hepatic injury through activation of the TGFβ1/Smad signaling pathway. Mol. Med. Rep. 18, 3611-3618. doi:10.3892/mmr2018.9392