Chondrodysplasia-inducing *COL2A1* p.Gly1170Ser causes an ER storage defect without associated unfolded protein response in a human cartilage model

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Abstract

Collagenopathies are a group of clinically diverse disorders caused by defects in collagen folding and secretion. For example, mutations in the gene encoding collagen type-II (*COL2A1*), the primary collagen in cartilage, can lead to chondrodysplasias of various severities. One example is the Gly1170Ser substitution in procollagen-II, which causes precocious osteoarthritis and Legg-Calvé-Perthes disease. Here, we develop and characterize a novel induced pluripotent stem cell-based cartilage model of this disease, including both hetero- and homozygous genotypes. Biochemical characterization reveals that Gly1170Ser procollagen-II is notably slow to fold and secrete. Procollagen-II accumulates intracellularly, consistent with an endoplasmic reticulum (ER) storage disorder. Intriguingly, perhaps due to the pathologic substitution occurring within a triple-helical domain that lacks hydrophobic character, this ER protein accumulation is not recognized by cellular stress responses, such as the unfolded protein response. Interactome studies show that Gly1170Ser procollagen-II interacts to a greater extent with certain ER chaperones and modifying enzymes than wild-type, consistent with its slow folding. These findings provide mechanistic elucidation into the etiology of this disease. Moreover, the cartilage model developed here provides a valuable platform to rapidly screen and develop therapeutic strategies that can restore procollagen folding and secretion in this collagenopathy and others.

Introduction

Collagens are the most abundant proteins in the human body and the molecular scaffold of animal life.[1] Collagenopathies are a collection of genetic diseases caused by defects in the synthesis, folding, quality control, assembly, and/or function of members of the collagen protein family.[2] Autosomal dominant mutations in the 44 human genes that encode the 28 different types of collagen are the most common cause of collagenopathies, with mutations in 29 of these collagen genes already associated with disease.[3, 4] As such, collagenopathies present with clinically diverse phenotypes that manifest most strongly in tissue. Currently, they have no cure. Moreover, effective treatments for the vast majority of collagenopathies are nearly universally lacking.[2, 5]

The fibrillar collagen type-II (collagen-II) is the main proteinaceous component of cartilage, and is thus a central player in both joint function and endochondral bone formation.[6, 7] Mutations in the gene encoding collagen-II (*COL2A1*) often lead to diseases termed type-II collagenopathies, or chondrodysplasias.[8, 9] These disorders encompass a broad range of clinical phenotypes, including skeletal dysplasia, ocular symptoms, and hearing impairment. The spectrum of severity ranges from relatively mild forms of premature arthritis to perinatal lethality. Milder type-II collagenopathies, such as mild spondyloepiphyseal dysplasia with premature arthrosis, often present in adolescence or adulthood. More severe type-II collagenopathies, often diagnosed pre- or neonatally, include disorders like spondyloepiphyseal dysplasia congenita and achondrogenesis type II.[8, 9]

Type-II collagenopathies are overwhelmingly caused by mutations within the procollagen-II triple helix-encoding portion of the *COL2A1* gene.[10] This 1014 amino acid-long domain is composed of Xaa-Yaa-Gly repeats, and is bookended by two smaller globular domains that are cleaved post-secretion.[11] Mutations that encode substitutions of the conserved Gly residues found at every third position in the triple-helical domain are the most common cause of disease.[10]

One exemplary type-II collagenopathy is the chondrodysplasia caused by the autosomal-dominant c.3508 GGT > AGT mutation in exon 50 of *COL2A1,* leading to a p.Gly1170Ser substitution in the C-terminal region of the procollagen-II triple-helical domain (**Fig. 1A**).[12, 13] Patients harboring this mutation present with precocious osteoarthritis and Legg-Calvé-Perthes disease (a juvenile form of avascular necrosis of the femoral head), with symptoms reported as early as the first decade of life, and most often diagnosed in adulthood.[12-16] The disease is typically diagnosed via genetic screening engendered by the familial link. Treatment options are currently extremely limited, with total joint replacement as the standard-of-care.

Conventional and next-generation treatments for the aging-related form of osteoarthritis that aim to reinforce the cartilage extracellular matrix, could be applicable to this disorder. Current research efforts for osteoarthritis focus on cartilage regeneration, ranging from biological and pharmacological interventions to surgical and implantation-based therapies.[17] Methods for stimulating chondrogenesis include biological recombinant agents such as bone morphogenetic protein-7, fibroblast growth factor 18, and autologous platelet rich plasma as inducers of chondrogenesis.[17, 18] Surgical interventions include inducing microfractures by subchondral bone perforation to recruit autologous mesenchymal stem cells to the cartilage defect.[19] Recently, therapies involving implantation of stem cells or stem cell-derived chondrocytes into cartilage defects have garnered traction. Unfortunately, the implanted cells often dedifferentiate and stop depositing a cartilage matrix or form teratomas.[20-23] Thus, much research focuses on engineering chondrogenic substrates[24-26] in which to embed the cells, or optimizing the stages and culture protocols pre-implantation to maximize integration and matrix deposition.[27, 28] For genetic skeletal diseases specifically, research has focused on identifying the disease mechanism and testing pharmacological treatments accordingly,[29] including targeting inflammation and oxidative stress with compounds such as aspirin, ibuprofen, or CoQ10, for example.[30]

A main challenge with studying collagenopathies in general, and type-II collagenopathies in particular, is the paucity of suitable systems to model these disorders. The disease phenotypes are most strongly manifested in tissue, making classical cell culture models insufficient. This challenge is especially acute for collagen-II and cartilage, as primary chondrocytes rapidly de-differentiate in classical culture conditions,[31] and immortalized chondrocytes generally do not deposit an extensive extracellular matrix (ECM).[32] Moreover, mouse models of collagenopathies often fail to recapitulate human phenotypes, with heterozygous mice often showing little to no skeletal phenotypes, and homozygous mice presenting with much more severe pathology than that observed in patients.

Excitingly, recent advances in differentiating induced pluripotent stem cells (iPSC) towards chondrogenic lineages hold considerable promise for addressing this fundamental challenge, allowing us to begin to model type-II collagenopathies *in vitro*. Several protocols now exist for differentiating human pluripotent stem cells (hPSC) to the chondrogenic lineage through various growth factor treatments[33-39] Of note, Craft *et al.* demonstrated the ability to generate chondrocytes from hPSCs by recapitulating key events of cartilage formation during embryonic development. These chondrocytes form a cartilaginous tissue that is stable both *in vitro* and *in vivo*.[36] More recently, members of our team reported a method to guide iPSCs through the process of endochondral ossification, from growth plate cartilage through hypertrophy to ossification.[35] These protocols start from hPSCs, whose inherent ability to proliferate makes these systems highly expandable and thus much more amenable for biochemistry and drug discovery than mouse models. Methods for producing cartilage *in vitro* in the correct developmental context, combined with gene editing technology to create genetically matched cell lines incorporating disease-causing mutations,[40-45] provide an exceedingly promising avenue for

modeling, studying, and discovering potential therapies for genetic skeletal disorders, including the type-II collagenopathies.

Recently, we reported the generation of iPSC lines harboring the heterozygote[42] or homozygote c.3508 GGT > AGT, p.Gly1170Ser mutation in *COL2A1*, created via CRISPR/Cas9 gene editing of a wild-type, isogenic control line (**Fig. 1**, and **Fig.S1**). By differentiating these three iPSC lines to chondrocytes and comparing the properties of the protein, the cells expressing it, and the matrices they deposit, we define the disease phenotype in this *in vitro* cartilage system and use it to understand the pathology in this collagenopathy. We find that slow procollagen-II folding in cells, intracellular retention of the protein without an attendant stress response, and deposition of a deficient extracellular matrix (ECM) underlie Gly1170Ser pathology. Our findings lay the foundation for identifying novel therapeutic targets and strategies for this and other collagenopathies, in a model amenable to drug screening.

Results

Cells expressing Gly1170Ser-substituted collagen-II deposit a cartilaginous ECM

To elucidate the etiology of disease caused by the Gly1170Ser substitution in collagen-II, in prior work we created and characterized genetically matched iPSC lines harboring the Gly1170Ser mutation in *COL2A1* in either one or both alleles using CRISPR/Cas9 gene editing (**Fig. 1A**, and **Figs. S1** and **S2**).[42] These lines, along with the isogenic wild-type control, allow us to unambiguously assess the effect of the Gly1170Ser on collagen-II proteostasis – meaning its synthesis, folding, localization, and function – and how it affects the cells that express it and the matrix they deposit. We began by differentiating all three iPSC lines (wild-type, heterozygous Gly1170Ser, homozygous Gly1170Ser) to chondrocytes using the protocol recently described by Lamandé *et al*.[35] Briefly, we expanded iPSCs in feeder-free monolayer culture, then guided them through differentiation to sclerotome by growth factor treatment over the course of 4 days. To make individual spheroids, we transferred the cells to pellet culture for two weeks and finally to swirler culture, which promotes chondrocyte differentiation and deposition of cartilage ECM components (**Fig. 1B**).[35] We repeated differentiations in three independent differentiations. After 44 days in culture, we harvested the chondronoids from all replicates and processed them for downstream assays.

To evaluate successful differentiation to chondrocytes, we interrogated differentiation-induced changes in the transcriptional profile of chondronoids after 44 days in culture using RNA-sequencing. The gene expression profile for all genotypes was consistent with that of chondrocytes, as evidenced by high expression of cartilage-specific markers such as collagens (*COL2A1*, *COL11A1*, *COL9A1*) and aggrecan (*ACAN*), but not of collagens associated with other tissue types, including *COL1A1*, the major component of bone (**Fig. 2A**). Indeed, cartilage components were the most highly expressed core matrisome genes.[46]

To assess the bulk composition of the ECM deposited by chondrocytes expressing the different procollagen-II variants, we first looked for the most abundant cartilage ECM components, collagen-II and proteoglycans,[6] in histology sections. We observed that the wild-type, clinically relevant heterozygote, and the more severe homozygote mutant abundantly deposited collagen-II in the ECM (**Fig. 2B,** in green). Similarly, toluidine blue staining for proteoglycans revealed that, consistent with the wild-type, the matrices of the heterozygous and homozygous Gly1170Ser variants were also rich in proteoglycans (**Fig. 2B,** in purple). There was no significant difference in the size of the chondronoids between the wild-type and clinically relevant Gly1170Ser heterozygote. However, chondronoids expressing homozygous Gly1170Ser collagen-II were smaller than the wild-type controls (**Fig. 2C**), hinting at a possible defect in secretion of ECM components. Nevertheless, the abundant presence of collagen-II and proteoglycans indicates that, from a bulk composition perspective at least, chondrocytes expressing Gly1170Ser-subsituted procollagen-II deposit a cartilaginous ECM.

The matrix of disease variant chondronoids is deficient

We hypothesized that, despite the deposition of an ECM rich in collagen-II and proteoglycans, and given the osteoarthritic and avascular necrotic phenotype of patients,[12-14] expression of the disease-causing Gly1170Ser variant could lead to defects in the deposited ECM that were not apparent in the immunohistochemistry.

To assess the quality of the deposited matrix, we used transmission electron microscopy (TEM) to examine the chondronoid matrix at higher magnification and resolution. In the wild-type chondronoids, we observed an extensive network of connecting fibrils (**Fig. 3**), consistent with *in vivo* cartilage samples.[6] TEM imaging of the heterozygous Gly1170Ser chondronoids, in contrast, revealed a comparatively defective matrix (**Fig. 3**). There were fewer collagen-II fibrils in the ECM, leading overall to a deficient network. This reduced matrix could be caused by insufficient or improperly folded collagen-II being secreted from cells and deposited in the ECM, by defective crosslinking of the collagen that is secreted into the ECM, or by a combination of both. This phenotype observed in the clinically relevant heterozygous Gly1170Ser variant could help to explain the joint symptoms of patients.[12-14] Such a deficient matrix would likely lead to a defective coating of articular cartilage at joints, leaving the joint as a whole more susceptible to injury or the stress of repeated loading.[47]

When compared to the wild-type chondronoids, and even the heterozygotes, homozygous Gly1170Ser matrices showed striking deficiencies via TEM (**Fig. 3**). The fibrils in chondronoids from this genotype were generally shorter, yielding a very sparse network. A healthy cartilage matrix is required not only for joint health, but also for proper skeletal development, as it is the tissue that precedes bone during the process of endochondral ossification.

Gly1170Ser-substituted collagen-II is slow to fold, a defect that is exacerbated in homozygotes

Since expression of Gly1170Ser-substituted collagen-II leads to a deficient cartilage matrix, we next sought to elucidate the underlying cause(s) of this deficiency. We hypothesized that the Gly→Ser substitution causes a delay or defect in the proper folding and assembly of the procollagen-II triple helix,[2, 5] which in turn could propagate to slow secretion of the protein into the ECM.

As procollagen-II folds in the ER, it interacts with modifying enzymes that install post-translational modifications on the individual polypeptide chains, such as hydroxyl groups on proline and lysine residues, as well as *O*-glycans on hydroxylysine residues.[1, 48] Slower folding of the polypeptide chains leads to hyper accumulation of these modifications. Thus, a key signature of slow-folding collagen variants is reduced electrophoretic mobility

on SDS-PAGE[49] with *O*-glycosylation of the excessively hydroxylated lysine residues contributing to the shift in molecular weight.

To test the hypothesis that the Gly1170Ser pathologic substitution causes a delay in collagen-II folding, we extracted collagen from the tissues by flash-freezing chondronoids and homogenizing the tissue, followed by a pepsin digestion to extract the pepsin-resistant collagen fraction. After separation via SDS-PAGE, we observed that the heterozygous, pathological Gly1170Ser substitution did indeed lead to reduced electrophoretic mobility, indicating that the substituted protein was excessively post-translationally modified and, therefore, slow to fold within cells when compared to the wild-type protein (**Fig. 4**).

In the homozygote, where all three procollagen-α1(II) chains contain the Gly1170Ser substitution, we observed that the delay in folding was further exacerbated (**Fig. 4**). That is, the homozygous Gly1170Ser collagen-II displayed even further reduced electrophoretic mobility on SDS-PAGE compared to the heterozygous variant.

Gly1170Ser-substituted procollagen-II is intracellularly retained and causes ER dilation

The deficient ECM combined with the slow folding kinetics strongly suggests that Gly1170Ser-substituted procollagen-II presents a challenge for cells to fold and secrete. We next sought to determine the fate of this variant in cells. We hypothesized that the slow folding of Gly1170Ser procollagen-II would lead to accumulation of the protein within cells and, specifically, within the ER, where this secreted protein folds. Intracellular retention is commonly observed in other diseases that impact protein folding and assembly.[50]

To test this hypothesis, we visualized procollagen-II inside the chondrocytes. We observed that a significantly larger proportion of Gly1170Ser-expressing chondrocytes retained procollagen-II intracellularly as compared to the wild-type, as evidenced by collagen-II immunohistochemistry (IHC) (**Fig. 5A**). We performed three independent, blinded scorings of multiple IHC images to quantify the fraction of cells with intracellularly retained procollagen-II in each genotype. In the clinically relevant heterozygous variant, 64% of cells showed signs of intracellular procollagen-II retention, as compared to just 34% of cells expressing wild-type procollagen-II (**Fig. 5B**). In the more severe homozygous variant, >80% of cells exhibited intracellular procollagen-II retention (**Fig. 5B**).

To better localize the intracellular protein, we co-stained with antibodies against collagen-II and an ER marker, calreticulin. We found that much of the intracellularly retained procollagen-II co-localized with calreticulin, suggesting that a portion of the protein is retained within the ER. These observations, along with the slow folding kinetics (**Fig. 4**), indicate that this pathologic variant is challenging for cells to properly fold and secrete, presumably because the sterically bulky residue in place of the much smaller Gly residue hinders triple helix

assembly and disrupts a key stabilizing interchain hydrogen bond within the helix.[1] Instead, procollagen-II begins to strongly accumulate and aggregate within the ER.

We next sought to ascertain whether this intracellular retention dysregulates ER homeostasis, thereby exacerbating pathology. In other protein folding disorders, including other collagenopathies, intracellular retention of misfolded or aggregated protein is often accompanied by dilation of the ER.[50] Indeed, high magnification TEM revealed that expression of the pathologic Gly1170Ser variant did indeed lead to dilated ER in the chondrocytes (**Fig. 6**), a phenotype that was exacerbated in the homozygote. Consistent with ER storage diseases, this pathologic ER distention confirms the Gly1170Ser substitution leads to defects in procollagen-II proteostasis, which in patients would eventually propagate to tissue-level defects in cartilage and pathologic joints.

Cells do not robustly respond at the transcript level to the Gly1170Ser-substituted collagen-II ER storage defect

Our data indicate that Gly1170Ser-substituted procollagen-II is slow to fold, accumulates in the ER, and exhibits a secretion defect. That secretion defect produces pathologic ER phenotypes, including extensive dilation. We next asked whether this protein folding challenge was accompanied by a transcriptional stress response. The unfolded protein response (UPR) is generally responsible for maintaining ER proteostasis by increasing levels of ER chaperones, quality control factors, and secretion machineries through signaling mediated by the transcription factors XBP1s and ATF6(f), and reducing non-essential translation via the PERK pathway.[51] Upon sustained and unresolved UPR signaling, cellular apoptosis can also be induced by the PERK signaling pathway.[52]

We tested for upregulation of UPR target genes[53] in Gly1170Ser-expressing chondronoids versus wild-type using RNA-sequencing of the different genotypes at two different timepoints during chondronoid growth: day 34 and day 44 (**Fig. 7**). These timepoints were selected to reflect an early and a late stage of cartilage growth, but with both timepoints harvested post-differentiation as UPR activation is known to be initiated during that process.[54] Notably, we observed that the UPR was not detectably induced in the clinically relevant heterozygote line at either timepoint. For the more severe homozygote line, there was no sign of UPR activation at the later timepoint. At the early timepoint, there was a very modest upregulation of UPR genes, which we discuss further later in this section.

Bypassing the UPR despite extensive ER accumulation of a misfolding protein can occur if the accumulating protein evades detection by UPR signaling pathways. The primary mechanism to activate the UPR relies on the ER chaperone BiP. Downstream UPR signaling is triggered when BiP is sequestered off the three UPR

sensors, IRE1, ATF6 and PERK, and instead binds un- or mis-folded proteins through hydrophobic interactions with exposed hydrophobic cores. Procollagen, however, is a long hydrophilic protein lacking such a core. Moreover, its triple helix – where the Gly1170Ser substitution is located – is adorned with many hydrophilic post-translational modifications.[48, 55] Indeed, there is no evidence that the collagen triple-helical domain itself binds BiP (present evidence indicates BiP largely or perhaps exclusively binds procollagen's C-propeptide domain during folding, especially for misfolding-prone C-propeptide domain variants[5, 56-59]), and thus aggregating or unfolded triple-helical domains that accumulate in the ER need not be expected to activate the UPR and indeed often do not.[60]

We next sought to determine if chondrocytes were instead raising any other organized transcriptional stress response when expressing Gly1170Ser procollagen. We looked for the non-canonical stress response to procollagen-I misfolding first described by Mirigian *et al.*,[61, 62] but we did not detect any significant upregulation of genes associated with this response (**Fig. 7**). We also scrutinized activation of the integrated stress response (ISR)[61, 63] but observed no robust activation of this pathway for the clinically relevant heterozygote. There was, however, modest upregulation of this response in the more severe but clinically irrelevant homozygote variant specifically at the later timepoint. Similarly, we investigated genes related to oxidative stress and inflammation, but again did not observe any significant differences in expression levels in the Gly1170Ser-expressing cells compared to wild-type, at either the early or late timepoints (**Fig. 7**).

We then turned to gene set enrichment analysis (GSEA) as a more global approach to identify differentially expressed pathways between Gly1170Ser-expressing lines and the wild-type (**Figs. S3** and **S4**). GSEA similarly did not identify any differentially expressed stress pathways. It did, however, highlight subtle differences in a few cellular processes. For the clinically relevant heterozygote, the main differences were upregulation of gene sets related to cellular respiration, as well as a downregulation of gene sets associated with translation and mitosis at the late timepoint when compared to the wild-type. These differences could suggest a UPR-independent reshaping of energetic allocations for cellular functions as an adaptive response to the accumulation of slow-folding procollagen-II. Moreover, the downregulation of genes involved in translation could be related to an attempt to alleviate the protein folding challenge in the ER, though the lack of apparent UPR/ISR activation makes it unclear how this response is initiated. Leiken and co-workers have described a non-canonical response,[61, 62] but as noted above that response is not observed in our transcription data.

On the other hand, for the homozygote line, GSEA revealed upregulation of genes associated with collagen folding and extracellular matrix deposition (**Fig. S3**). The implications of the observed differences in ECM-related processes will be interesting to follow-up on in future studies, albeit bearing in mind the reduced clinical relevance of this genotype. We also note that, possibly because cartilage-related pathways are generally

poorly annotated, with few, if any, comprehensive gene sets, no cartilage-specific gene sets were identified as being differentially expressed in the Gly1170Ser-expressing cells. The most substantial transcriptional difference we observed, again present exclusively in the more severe and clinically irrelevant homozygous variant, was downregulation of genes associated with chondrocyte hypertrophy,[35] indicating that the homozygous chondrocytes were slower to mature when compared to wild-type (**Fig. 7**). Chondrocyte hypertrophy, characterized by enlarged chondrocytes expressing collagen-X, is a key step in long bone formation, when the cartilaginous, collagen-II rich matrix shifts to a mineralized, collagen-I bone matrix in a process known as endochondral ossification.[7] Such slow maturation could also explain the very modest upregulation of UPR targets observed exclusively at the early timepoint in the homozygous variant, as transient UPR signaling has been implicated in chondrocyte proliferation, differentiation, and hypertrophy.[54, 64, 65] Interestingly, the clinically relevant heterozygote showed the converse trend in hypertrophic gene expression, with certain genes for hypertrophy being upregulated compared to the wild-type — a sign of accelerated maturation — although this effect was more muted (**Fig. 7**). These differences in maturation speed likely have important implications for the overall functionality of the skeletal ECM deposited.[2] Further studies on the effect of maturation rate (specifically in the case of accelerated hypertrophy) are needed to better understand its role in pathogenesis of disease.

Quantitative comparative interactome analysis of Gly1170Ser procollagen-II variant reveals increased interactions with certain ER enzymes and folding clients

Given the absence of robust, well-organized transcriptional responses, we next asked whether the cellular proteostasis machinery can recognize Gly1170Ser-induced procollagen-II misfolding. We used quantitative mass spectrometry (MS) to elucidate and compare the interactomes of wild-type versus Gly1170Ser-substituted procollagen-II (**Fig. S5**).[66] For this experiment, we transiently overexpressed epitope-tagged procollagen-II in HT-1080 cells to enable MS-grade immunoprecipitations. This cell line was selected because it expresses the cellular machinery necessary for folding procollagen, but does not express endogenous procollagen-II which could confound our findings.[67] **Figure S5** outlines the experimental design and workflow.

Using tandem mass tag (TMT)-based MS, we quantitatively compared the interactomes of wild-type and Gly1170Ser procollagen-II to identify proteins that differentially interact with the bait.[56, 66, 68, 69] We identified multiple high confidence procollagen-II interactors (**Table 1**). After normalizing to bait, the fold interaction enrichment was calculated as $Fold\ Enrichment = \frac{\Sigma\ abundance\ in\ Gly1170Ser\ samples}{\Sigma\ abundance\ in\ wild\text{-}type\ samples}$.

The data reveal that the cellular proteostasis machinery does indeed differentially engage wild-type versus Gly1170Ser procollagen-II. Most notably, the pathogenic variant interacted to a greater extent with modifying enzymes known to engage with collagen in the ER.[66] These interactors included procollagen-lysine,2-

oxoglutarate 5-dioxygenase 2 (*PLOD2*), protein disulfide-isomerase (*P4HB*), prolyl 3-hydroxylase 1 (*P3H1*), peptidyl-prolyl cis-trans isomerase (*FKBP10*), and peptidyl-prolyl cis-trans isomerase B (*PPIB*) (**Table 1**). Heightened interactions with procollagen-modifying enzymes are consistent with the substituted variant being hypermodified owing to slow folding.

Other differentially interacting proteins included known collagen chaperones calreticulin (*CALR*) and the collagen-specific molecular chaperone serpin H1 (*SERPINH1*) (**Table 1**). Increased interaction with these chaperones is consistent with Gly1170Ser being more challenging for cells to fold. Another notable interactor was the ER chaperone BiP (*HSPA5*) which interacted to the same extent with both wild-type and pathologic procollagen-II (presumably at their respective globular domains[56-58]). This lack of differential interaction is consistent with the failure of Gly1170Ser procollagen-II to trigger the UPR, as seen in the RNA-sequencing data (**Fig. 7**).

Beyond modifying enzymes and chaperones, other interactors that differentially engaged Gly1170Ser procollagen-II were also proteins destined for secretion, such as cartilage-associated protein (*CRTAP*) and fibronectin (*FN1*) (**Table 1**). These secreted proteins are, like procollagen-II, folded in the ER, where they apparently develop more extensive interactions with the slow-folding Gly1170Ser procollagen-II variant than with wild-type procollagen-II. Intriguingly, other disease-causing variants of procollagen-II were previously shown to have increased interactions with fibronectin, a finding suggested to underly abnormal ECM formation.[70]

Thus, the cellular proteostasis machinery can recognize slow-folding Gly1170Ser through increased interactions with certain ER proteostasis network components. These interactions do not alleviate the challenges associated with folding this variant, but they may present an opportunity for pharmacologic tuning of the ER proteostasis network to address the ER storage defect.[5, 60, 71]

Discussion

Since it was first described in 2005,[12-16, 72, 73] little progress has been made towards understanding the pathogenic mechanisms in the chondrodysplasia caused by the Gly1170Ser substitution in procollagen-II.[13, 74-76] Here, we define the effects of the Gly1170Ser substitution in a robust human iPSC-based tissue model of cartilage and elucidate the proteostasis defects that arise from expression of this pathologic variant, in both the clinically relevant heterozygous model and the more severe but less clinically relevant homozygote. We find that, despite their ability to deposit a cartilage matrix rich in collagen-II and proteoglycans, cells expressing Gly1170Ser-substituted collagen-II show signs of pathology, in both the hetero- and homozygous models. Their matrix is deficient and lacks much of the structural collagen network. At the cellular level, Gly1170Ser procollagen-II is slow to fold and accumulates excessive post-translational modifications. Delayed triple-helix folding also results in a large amount of procollagen being retained within a dilated ER. This ER accumulation, however, is not accompanied by any substantive UPR.

In a complementary approach, Liang *et al*. previously generated a knock-in mouse model of the Gly1170Ser substitution, and characterized wild-type, heterozygous, and homozygous mice at birth or earlier.[74] Heterozygous pups showed no skeletal phenotype, while homozygous pups presented with severe skeletal malformations and died shortly after birth from respiratory distress. Consistent with the findings from our human cartilage model, they observed a sparse ECM deposited by transgenic mice, as well as ER dilation in both transgenic genotypes, and intracellular retention of collagen-II, albeit only in chondrocytes homozygote for the substitution. Conversely, they also observed activation of the UPR in homozygous chondrocytes by quantitative RT-PCR of six UPR-related genes (and upregulation of two of those genes in heterozygous chondrocytes). These differences highlight the intricacies of various model systems and underline the potential benefits of studying collagenopathies in models that most closely resemble the human context.

From a biophysical perspective, the pathologic Gly→Ser substitution disrupts the triple helix because it replaces the conserved, small glycine residue at the interior of the helix with a much larger serine, causing steric hindrance. Moreover, it disrupts a key interchain hydrogen bond that stabilizes the helix because the amide hydrogen of serine is not in the proper orientation to serve as a donor site.[1] Such disruption slows triple-helix folding, causing the protein to accumulate posttranslational modifications, which we detected by reduced electrophoretic mobility. The hypermodification may itself be problematic, as we will discuss later, but it is the slow folding leading to procollagen-II accumulation within the ER that is the hallmark of an ER storage disease.[77]

Notably, even as Gly1170Ser procollagen-II accumulates in the ER, it does not trigger a chronic UPR. Repeated, but transient, activation of this signaling response would likely be beneficial to cells, as it would

promote folding and clearance of accumulated procollagen-II by upregulating chaperones, quality control and clearance factors, and by lightening the incoming protein folding load.[5] However, there is no evidence that the hydrophilic triple-helical domain of procollagen binds BiP,[60] the main detector of accumulated misfolded proteins in the ER, and therefore helix mutations may not activate the UPR signaling pathway. Our data suggest that an underlying cause of pathology is the slow folding and accumulation of Gly1170Ser procollagen-II intracellularly occurring in the absence of any chronic UPR or other stress response. Indeed, for many of the common Gly→Ser substitutions that induce collagenopathies, UPR activation is, at most, inconsistently observed.[60] Our results add weight to the view that chronic, pathologic UPR is by no means a universal mechanism underpinning the collagenopathies.[5, 60] Instead, procollagen-II accumulates within the ER, causing extensive dilation as is commonly observed in ER storage diseases, and the chondrocytes fail to productively resolve the issue.

From a therapeutic perspective, this finding that cells expressing the pathologic variant do not activate beneficial aspects of the UPR makes those pathways a compelling target for this, and likely many other, collagenopathies.[5, 71, 78] Exogenous, branch-specific activation of the pro-folding IRE1 and ATF6 arms of the UPR could promote procollagen folding by upregulating chaperones and quality control factors, as well as clearing accumulated protein by upregulation of clearance machinery.[79, 80] Even just one of these outcomes could greatly benefit cells expressing Gly1170Ser procollagen-II. This strategy has already been applied in other protein folding diseases,[79] and many small molecules are already known to specifically target different nodes within the UPR.[81]

Further evidence that promoting proteostasis, whether via the UPR or more generally, may be a viable therapeutic strategy for this disease, derives from the location of the substitution within the collagen-II triple helix. Position 1170 does not lie in any known binding site for other proteins,[82-84] and therefore likely does not impede important signaling or structural functions mediated by its interactors. Rather, it seems likely that pathology is due directly to proteostasis defects.

Generally, the nature and location of the Gly1170Ser substitution would suggest a much more severe pathology than that observed in both patients and in our cartilage model because (1) it involves a Gly substitution, which are generally more detrimental to procollagen folding than substitutions of Xaa- or Yaa-position residues,[2] and (2) it is C-terminal, and thus, since procollagen folds from the C- to N-terminus, would disrupt the folding of a much larger portion of the protein than if it were located near the N-terminus.[85] The symptoms of this disease, however, fall on the milder side of the spectrum, which is likely attributed to the proline-rich region located directly N-terminal to the substitution. The propensity of peptides to assemble into triple helices increases with the number of prolines,[1] and thus, the proline-rich region likely helps procollagen-II to more rapidly resume folding despite the presence of a large, interchain hydrogen bond-breaking amino acid.

Despite its “favorable” position, Gly1170Ser still causes pathology. The ER storage phenotype associated with expression of Gly1170Ser procollagen-II propagates to the tissue, as these cells deposit a deficient ECM, lacking many of the network-forming fibrils observed in the wild-type. In patients, such failure likely leads to cartilage degradation ultimately requiring total joint replacement. From a molecular perspective, multiple factors could be causing the deficient matrix that ultimately contributes to symptoms in patients. One possibility is that cells expressing the pathologic variant secrete less collagen into the extracellular space. Another is that the secreted collagen is structurally abnormal and unable to correctly assemble into fibrils. Alternatively, or in conjunction, the deficient matrix could be a product of a defect in crosslinking. Hypermodification of lysines in collagen is known to affect crosslinking, as the process requires both a lysine and a hydroxylysine devoid of *O*-glycans in order to form a covalent crosslink.[55] Our data indicate that Gly1170Ser-substituted procollagen-II is hypermodified due to slow folding, which likely leads to a higher proportion of *O*-glycosylated hydroxylysines, and therefore could disrupt crosslinking and overall matrix health.

Interestingly, while the slow folding of Gly1170Ser procollagen-II, intracellular accumulation of the protein, and deficient ECM phenotypes all display a stepwise difference in severity between the heterozygous and homozygous variants, our RNA-sequencing data reveal that the homozygous cells are not, at least from a transcriptome standpoint, simply a more severe version of the heterozygotes. Both heatmaps and GSEA analyses revealed that different genes and pathways were differentially expressed depending on the genotype. This finding has implications for mouse models where homozygotes are often used to model autosomal dominant disorders in humans, owing to a lack of disease phenotype in mice heterozygous for the mutation. Nonetheless, our finding that homozygous chondronoids displayed a delay in hypertrophy is consistent with the severe skeletal dysplasia and disorganized growth plate observed in homozygous Gly1170Ser mice.[74]

In this work, we focused on modeling growth plate cartilage to understand how the mutation impacts development of cartilage and the skeleton, and how those relate to pathology, in both the clinically relevant heterozygous variant and the more severe homozygote. In future work, it will be equally interesting to investigate the phenotype in articular cartilage, which can also be grown from iPSCs,[36] to further understand how the mutation relates to the osteoarthritic phenotype in particular.

The comparison in a human model with isogenic background enabled by the chondronoid system allowed us to define the pathologic phenotype and elucidate the defects in procollagen-II proteostasis. Moreover, by virtue of being highly expandable and modeling tissue, chondronoids constitute an ideal platform for screening small molecules and genetic interventions to enhance procollagen-II proteostasis. The model is equally conducive to in depth biochemical analyses, and, with recent advances in iPSC-based modeling of the endochondral ossification process,[35] could enable similar studies in collagenopathies involving bone disorders.

Methods

General Reagents

All media and cell culture reagents were obtained from Corning/Cellgro, unless otherwise noted. Restriction enzymes, ligases, and polymerases were obtained from New England BioLabs. HA-Agarose beads (A2095) were obtained from Millipore.

Plasmids

The plasmid encoding wild-type human *COL2A1* (variant IIB, consensus sequence) was obtained from Origene (#RG221644, NM_033150). The C-terminal GFP tag was removed and replaced by a stop codon. To the N-terminus, a pre-protrypsin signal sequence, HA epitope tag, and NotI site were inserted. The Gly1170Ser *COL2A1* plasmid was made using site directed mutagenesis of the wild-type plasmid.

Antibodies

Antibodies were obtained from the following suppliers, and used at the specified dilutions: anti-Collagen-II (Millipore MAB8887, 1:150), anti-Calreticulin (Invitrogen AB325990, 1:200), Goat Anti-Rabbit Alexa Fluor 488 (Abcam ab150077, 1:500), Goat Anti-Mouse Alexa Fluor 568 (Abcam ab175473, 1:500).

Induced pluripotent stem cell lines growth and maintenance

Generating the gene edited lines from ATCC fibroblast line 1502 is covered by the Human Research Ethics Committee of the Royal Children's Hospital, Victoria, Australia (HREC #33118).[42] iPSC lines MCRIi019-A (wild-type control) and MCRIi019-A-2 (*COL2A1* p.Gly1170Ser heterozygote) and the homozygote variant iPSCs were grown in a feeder-free manner on Matrigel (Corning)-coated 6-well plates in Essential 8 (E8) medium (ThermoFisher Scientific). Media was changed daily. Cells were routinely passaged (1:6 split) every 3 – 4 d and gently detached from plates using 0.5 mM EDTA in PBS.

Chondrocyte differentiation

Undifferentiated iPSCs at 70–90% confluency were dissociated using 0.5 mM EDTA in PBS and passaged into 6-well plates (~2×10^5 cells per well) pre-coated with Matrigel, and cultured for 48 h in E8 expansion medium. Differentiation to sclerotome was preformed as described,[35] with APEL2 (StemCell Technologies) containing 5% Protein Free Hybridoma Medium (PFHM II; ThermoFisher Scientific). On day 0, medium was changed to anterior primitive streak inducing medium containing 30 ng/ml Activin A (R&D Systems), 4 μM CHIR99021 (Tocris), 20 ng/mL FGF2 (PeproTech) and 100 nM PIK90 (Merck Millipore). After 24 h this medium was replaced with medium containing 3 μM CHIR99021, 20 ng/mL FGF2, 1 μM A8301 (Tocris) and 0.25 μM LDN193189 (Cayman Chemical) to induce paraxial mesoderm for 24 h. Early somite development was then induced with medium containing 1 μM A8301 and 0.25 μM LDN193189, 3 μM C59 (Tocris) and 0.5 μM

PD0325901 (Selleck Chemicals). After 24 h, sclerotome induction was initiated with 1 μM C59 and 2 μM purmorphamine (Sigma-Aldrich). After the first day of sclerotome induction (day 4) cells were dissociated from monolayer culture using Tryple Select (ThermoFisher Scientific), resuspended in sclerotome differentiation media, 300 μL aliquots containing 2×10^5 cells were dispensed into 96-well low-attachment, round-bottom plates (Corning), and pelleted by centrifugation at 400 × *g* for 3 min using a swing-out rotor. Pellets were incubated in sclerotome differentiation media for a further 48 h to complete sclerotome differentiation in the 96-well static culture format. Differentiations were performed in biological triplicate, where individual replicates originated from cells of separate plates differentiated with individually prepared differentiation media from different media bottles.

Chondrocyte maturation and extracellular matrix deposition

To promote chondrocyte differentiation, maturation, and ECM deposition, sclerotome cell pellets were cultured in 96-well round bottom plates in APEL2 medium supplemented with 5% PFHM II, 20 ng/mL FGF2 (PeproTech), 1× Penicillin-Streptomycin (ThermoFisher Scientific), and 200 μM freshly added sodium ascorbate (Amresco). On day 20, pellets were transferred to 6 cm non-adherent culture dishes (Greiner) with 15 – 20 pellets per dish in 5 mL of APEL2/PFHM II medium lacking FGF2 with orbital rotation at 60 rpm. Media was changed every 2 – 3 d for an additional 24 d of maturation. For harvesting, chondronoids were rinsed once in PBS and snap-frozen or processed for downstream assays. For each assay, chondronoids were harvested from all three biological replicates. Chondronoids originating from the same differentiation were considered technical replicates. A micrometer was used for precise diameter measurement of each of 10 chondronoids per genotype.

Histology and toluidine blue staining

Cultured chondronoids were fixed overnight at 4 °C in 10% neutral-buffered formalin (Millipore), washed in 70% ethanol, processed on a tissue processor (Sakura Tissue Tek VIP5) with a biopsy processing schedule as follows: 70% Ethanol – 10 mins, 95% Ethanol × 2 – 10 mins each, 100% Ethanol × 3 – 10 mins each, Xylene × 2 – 10 mins each, Paraffin wax × 4 – 10 mins each, and then embedded in “Paraplast X-tra”rrections paraffin wax using a Leica embedding station. Serial 4 μm sections were cut from the chondronoids using a Leica rotary microtome. Sections were floated onto a 45 °C water bath and mounted on Leica “X-tra” adhesive slides (Leica biosytems), drained, and dried at room temperature overnight. Before staining, sections were heated at 60 °C for 30 min. Sections were treated with xylene to remove the paraffin wax and then with an ethanol series comprising 100%, 90%, 70% ethanol then water to rehydrate the sections. Sections were stained with toluidine blue solution (1% toluidine blue, 0.2 M sodium phosphate pH 9, filtered) for 10 min to detect a cartilage proteoglycan matrix, then rinsed in distilled water. Microscope slides were cover slipped using ProLong Gold Antifade Reagent (Invitrogen) and sealed with nail polish. Images were captured using a Leica DM 2000 LED

microscope with Leica Application Suite (LAS) software version 4.9.0. Image processing was performed using ImageJ.

Immunohistochemistry and confocal microscopy

Rehydrated histological sections underwent antigen retrieval by incubation with 1 mg/mL pepsin in 0.5 M acetic acid (Millipore) at 37 °C for 30 min. Samples were washed 3× with PBS, then incubated with 0.1% Triton X-100 for 30 min to permeabilize the cells. Samples were then incubated in a solution of 5% bovine serum albumin (BSA, Millipore) in PBS at RT for 1 h to block non-specific antibody binding. Samples were labeled simultaneously with anti-Col-II antibody (Millipore MAB8887, 1:150) and anti-Calr antibody (Invitrogen AB325990, 1:200) at in 1% BSA for 1 h at RT. Samples were washed 3 × with PBS. Both secondary antibodies, Alexa Fluor 568-conjugated anti-mouse and Alexa Fluor 488-conjugated anti-rabbit (Invitrogen; 1:500), were applied simultaneously in 1% BSA solution for 30 min – 1 h at RT. Samples were washed 3 × with PBS. Cover slips were mounted on the microscope slides using ProLong Gold Antifade Reagent with DAPI (Invitrogen) and sealed with nail polish. Images were acquired at the W. M. Keck Microscopy Facility at the Whitehead Institute on a Zeiss AxioVert200M microscope with a 10 ×, 63 ×, and 100 × oil immersion objective with a Yokogawa CSU-22 spinning disk confocal head with a Borealis modification (Spectral Applied Research/Andor) and a Hamamatsu ORCA-ER CCD camera. The MetaMorph software package (Molecular Devices) was used for image acquisition. The excitation lasers used to capture the images were 405 nm, 488 nm, and 561 nm. Image processing was performed using ImageJ.

Transmission electron microscopy

Samples were washed with PBS and fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 100 mM cacodylate buffer pH 7.2 for 2 h at 4 °C, then post-fixed with 1% osmium tetroxide in 1.25% potassium ferrocyanide. They were then en-bloc stained with 2% uranyl acetate in 0.05 M maleate buffer pH 5.2 overnight at RT, followed by serial dehydrations with ethanol, then embedded in resin at 60 °C for 48 h. 60 nm sections were obtained using a diamond knife on a Leica UC67 Ultramicrotome and observed at 120 kV on a T12 Spirit Transmission Electron Microscope (ThermoFisher Instruments). Micrographs were captured using a digital camera from Advanced Microscopy Techniques (AMT).

RNA-sequencing

For RNA-sequencing, individual chondronoids were snap frozen in liquid nitrogen and pulverized using a liquid nitrogen-cooled tissue grinder. RNA was extracted with TRIzol (Invitrogen), followed by purification using Direct-zol RNA Microprep kit spin columns (Zymo Research) according to manufacturer's instructions. RNA samples were run on a fraction analyzer at MIT's BioMicro Center for quality control. All RNA samples were processed at the same time using the same batch of reagents. RNA samples were quantified using an Advanced Analytical Fragment Analyzer. 10 ng of total RNA was used for library preparation on a Tecan ECO150. Custom primers were used to append a 6 bp barcode specific to each sample, as well as a unique

molecular identifier (Integrated DNA technologies). Maxima H Minus Reverse Transcriptase (Thermo) was added per manufacturer's recommendations with a template-switching oligo, incubated at 42 °C for 90 min and inactivated by incubation at 80 °C for 10 min. Following the template switching reaction, cDNA from 24 wells containing unique well identifiers were pooled together and cleaned using RNA Ampure beads at 1.0×. cDNA was eluted with 17 μL of water, digested with Exonuclease-I (New England Biolabs) at 37 °C for 30 min, and inactivated by incubation at 80 °C for 20 min. Second strand synthesis and PCR amplification was done by adding Advantage 2 Polymerase Mix (Clontech) and the SINGV6 primer (Integrated DNA Technologies, 10 pmol, 5'-/5Biosg/ACACTCTTTCCCTACACGACGC-3') directly to half of the exonuclease reaction volume. Eight cycles of PCR were performed followed by clean up using regular SPRI beads at 0.6×, and elution with 20 μL of RSB. Successful amplification of cDNA was confirmed using the Fragment Analyzer. Illumina libraries were then produced using Nextera FLEX tagmentation substituting P5NEXTPT5-bmc primer (25 μM, Integrated DNA Technologies, (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG*A*T*C*T*-3' where * = phosphorothioate bonds.) in place of the normal N500 primer. Final libraries were cleaned using SPRI beads at 0.7 × and quantified using the Fragment Analyzer and qPCR before being loaded for paired-end sequencing using the Illumina NextSeq500 in high-output paired-end mode (20/57 nt reads). Analyses were performed using previously described tools and methods. Reads were aligned against hg19 (February 2009) using BWA mem v.0.7.12-r1039 (RRID:SCR_010910) with flags –t 16 –f, and mapping rates, fraction of multiply-mapping reads, number of unique 20-mers at the 5' end of the reads, insert size distributions and fraction of ribosomal RNAs were calculated using BEDTools v. 2.25.0 (RRID:SCR_006646).[86] In addition, each resulting bam file was randomly down-sampled to a million reads, which were aligned against hg19, and read density across genomic features were estimated for RNA-Seq-specific quality control metrics. For mapping and quantitation, reads were scored against GRCh38/ENSEMBL 101 annotation using Salmon v.1.3 with flags quant -p 8 -l ISR –validateMappings.[87] The resulting quant.sf files were imported into the R statistical environment using the tximport library (tximport function, option "salmon"), and gene-level counts and transcript per milllion (TPM) estimates were calculated for all genes.[88] Samples were clustered based on $\log_2$-transformed TPMs of protein-coding genes and obvious outliers were excluded from downstream analyses.

Differential expression was also analyzed in the R statistical environment (R v.3.5.1) using Bioconductor's DESeq2 package (RRID:SCR_000154).[89] Dataset parameters were estimated using the estimateSizeFactors(), and estimateDispersions() functions; read counts across conditions were modeled based on a negative binomial distribution, and a Wald test was used to test for differential expression (nbinomWaldtest(), all packaged into the DESeq() function), using the treatment type as a contrast. Shrunken $\log_2$ fold-changes were calculated using the lfcShrink function, based on a normal shrinkage estimator.[89] Fold-changes, *p*-values and false-discovery rates were reported for each gene. In the heterozygous line, X-chromosome deactivation was eroded, as is a common occurrence in iPSC lines,[90-92] so hits caused by

differential expression of X-linked genes were disregarded. All the gene sets considered were either previously published,[35, 53, 61-63] or curated from previously described gene ontologies.

Gene Set Enrichment Analysis (GSEA)

Differential expression results from DESeq2 were retrieved, and the "stat" column was used to pre-rank genes for GSEA analysis. These "stat" values reflect the Wald's test performed on read counts as modeled by DESeq2 using the negative binomial distribution. Genes that were not expressed were excluded from the analysis. GSEA (linux desktop version, v4.1)[93] was run in the pre-ranked mode against MSigDB 7.0 C5 (Gene Ontology) set, and ENSEMBL IDs were collapsed to gene symbols using the Human_ENSEMBL_Gene_ID_MSigDB.v7.4.chip (resulting in 25162 and 26199 genes for WT vs. mutant sample in early and late time-point comparisons, respectively). In addition, a weighted scoring scheme, meandiv normalization, cutoffs on MSigDB signatures sizes (between 5 and 2000 genes, resulting in 9,476 and 9,588 gene sets retained for early and late time-point wild-type vs. mutant comparisons, respectively) were applied and 5,000 permutations were run for *p*-value estimation.

Collagen extraction and SDS-PAGE analysis

Chondronoids of the same genotype were rinsed with PBS, blotted dry with a Kim wipe, pooled, snap frozen in liquid nitrogen, and pulverized using a liquid nitrogen-cooled tissue grinder. The powder obtained was added to 100 μL chondroitinase ABC solution (50 mM Tris HCl pH 8, 50 mM sodium acetate, 0.1 mU/μL chondroitinase ABC (Millipore)) and incubated in a thermomixer set to 37 °C and 1,500 rpm for 6 h. 900 μL of denaturing solution (50 mM sodium acetate, 10 mM EDTA, 4 M guanidine hydrochloride, 65 mM dithiothreitol) was added, and samples were rotated end-over-end at 4 °C overnight. The next day, samples were centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was removed and the pellet resuspended in 200 μL of cold pepsin solution (0.1 mg/mL pepsin (Millipore) in 0.5 M acetic acid) and rotated end-over-end at 4 °C overnight. The next day, samples were centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was saved, neutralized with 10 M NaOH, and lyophilized. Lyophilized samples were reconstituted in loading buffer for SDS-PAGE (50 mM Tris HCl pH 6.8, 2 M urea, 0.1% SDS, 0.1% sucrose, 0.01% bromophenol blue (Millipore)) and boiled for 15 min. Samples were separated on homemade 4/8% tris SDS-PAGE gels. Gels were stained in Coomassie Blue solution (0.1% Coomassie Blue (Millipore), 10% acetic acid, 50% methanol, 40% water) and destained in the same solution without Coomassie Blue. Gels were imaged on an Epson scanner.

Intracellular collagen-II quantification

A survey of confocal images of immunohistochemistry samples stained for collagen-II was used to quantify the percent of cells with intracellularly retained collagen-II. 75 images of the different genotypes at different magnifications were arranged in a random order. Three independent and blinded participants were asked to count the total number of cells and the number of cells with intracellular staining in each image. The proportion

of positive cells was averaged between all participants for each genotype, and the error bars represent the standard error of the mean. Statistical significance between genotypes was calculated using a heteroscedastic student's *t*-test for each participant, and the largest of the three *p*-values obtained is displayed on the chart.

Proteomics

Cell Culture, transfections, and immunoprecipitation. HT-1080 cells (ATCC) were cultured in complete DMEM supplemented with 10% FBS. Transfections of wild-type and Gly1170Ser *COL2A1*-encoding plasmids were performed using Transit-2020 transfection reagent (Mirus) according to manufacturer's instructions. The next day, media was replaced with fresh complete DMEM supplemented with 50 μM sodium ascorbate (Amresco). Two days post-transfection, cells were harvested and washed three times with 1× phosphate buffered saline (PBS) and crosslinked in plate with 0.2 mM dithiobis(succinimidyl propionate) (DSP; Lomant's Reagent, Thermo) in PBS at rt for 30 min. Crosslinking was quenched by addition of 0.1 M Tris at pH 8.0 (final concentration) for 15 min at rt. Cells were scraped from plates in PBS using a cell scraper, then pelleted and resuspended in RIPA: 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), protease inhibitor tablets and 1.5 mM phenylmethylsulfonyl fluoride (PMSF; Amresco). Cells were lysed for 30 min on ice, then centrifuged at 21,100 × g for 15 min at 4 °C. Supernatants were collected, quantified for protein content (BCA assay, Pierce), normalized, and incubated for 16 h with the HA antibody conjugated beads, rotating end-over-end at 4 °C. Samples were washed with RIPA 3 × and eluted by boiling for 10 min in 300 mM Tris at pH 6.8 with 6% SDS and 600 mM dithiothreitol (DTT, Millipore) to release crosslinks. Eluates were prepared for mass spectrometry analysis.

Mass spectrometry and interactome characterization. Samples were reduced, alkylated, and digested with sequencing-grade pepsin (Promega) using an S-Trap Micro (Protifi) following standard protocol. After digestion, the samples were labeled with TMT 10plex™ reagents (Thermo) according to manufacturer's instructions. Briefly, the dried tryptic peptides were resuspended in 100 mM triethylammmonium bicarbonate (TEAB), vortexed. Acetonitrile was added to each of the 10-plex TMT label reagents, vortexed, and briefly centrifuged. Peptides and labeling reagents were then combined in a 1:2 ratio and incubated for 1 h at rt, after which the reaction was quenched with 5% hydroxylamine for 15 min. Equal amounts of each channel were combined, dried by speed-vacuum, and resuspended in TEAB. Samples were then fractionated with Pierce High pH Reversed-Phase peptide Fractionation Kits (cat#84868) using standard protocols, then dried and resuspended in 0.2% formic acid for injection on LC-MS/MS. Labeled tryptic peptides were separated by reverse phase HPLC (Thermo Ultimate 3000) using a Thermo PepMap RSLC C_{18} column (2 μm tip, 75 μm × 50 cm PN# ES903) over a gradient before nano-electrospray injection onto an Orbitrap Exploris 480 mass spectrometer (Thermo).

The mass spectrometer was operated in a data-dependent mode. The parameters for the full scan MS were: resolution of 120,000 across 375-1600 *m/z* and maximum IT of 25 ms. The full MS scan was followed by MS/MS for as many precursor ions in a three second cycle with a NCE of 32, dynamic exclusion of 30 s and resolution of 45,000. Raw mass spectral data files (.raw) were searched using Sequest HT in Proteome Discoverer (Thermo). Sequest search parameters were: 10 ppm mass tolerance for precursor ions; 0.05 Da for fragment ion mass tolerance; 2 missed cleavages of trypsin; fixed modification were carbamidomethylation of cysteine and TMT modification on the lysines and peptide N-termini; variable modifications were methionine oxidation, methionine loss at the N-terminus of the protein, acetylation of the N-terminus of the protein, methylation of histidine, arginine, and glutamine, hydroxylation of proline, and methionine-loss plus acetylation of the protein N-terminus; total PTM per peptide was set to 8. The false discovery rate was set at 0.01. Data was searched against a human database and a common MS contaminant database made in house. Human Database: https://www.uniprot.org/proteomes/UP000005640

Raw abundance values were used for quantification. First, abundance of each protein in the negative control was determined as the average abundance of the three no transfection control conditions. High confidence interactors were identified as any protein detected with an abundance of at least 2 × that of the negative control samples across all three replicates. Next, for each experimental sample, the abundance of each protein was normalized to the abundance of bait (collagen-II) to obtain a normalized abundance value. The fold enrichment was calculated as the ratio of the average of normalized abundance values in the Gly1170Ser conditions to the average of normalized abundance values in the wild-type conditions.[69] Significance was evaluated using a homoscedastic student's *t*-test across the three biological replicates, as well as the two-stage step-up method of Benjamini, Krieger, and Yekutieli with a desired FDR set to 10%.

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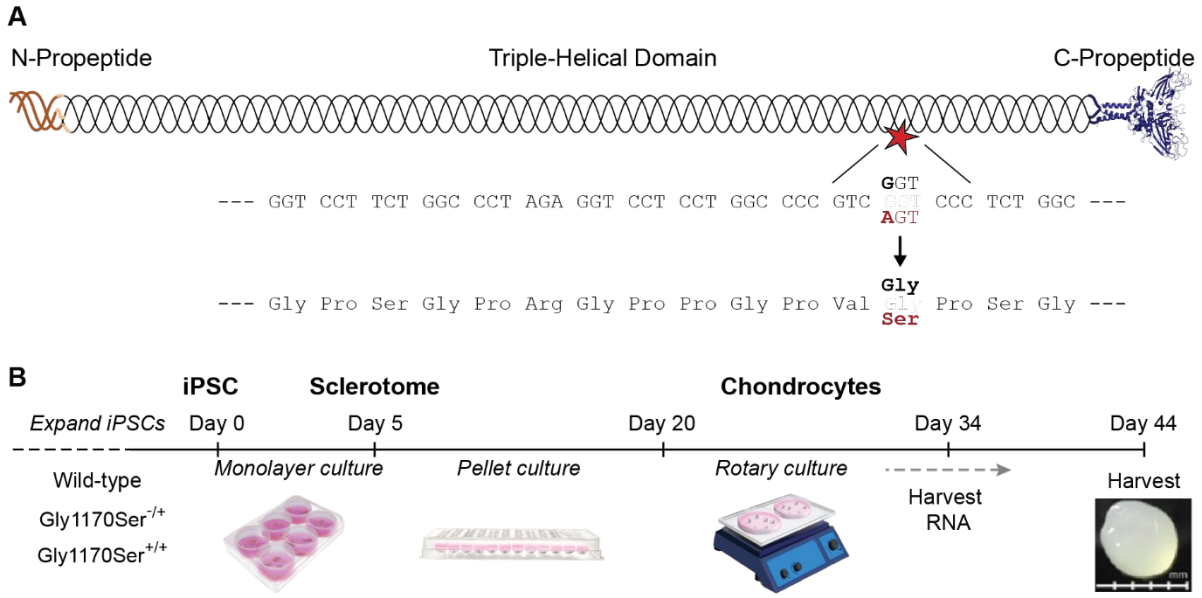

Fig. 1. A. Procollagen-II is composed of a lengthy (1,014 amino acid) triple-helical domain sandwiched by two smaller propeptide domains. The c.3508 GGT > AGT mutation in exon 50 results in a Gly→Ser substitution at position 1170 in the procollagen-II amino acid sequence (shown in red). **B**. Differentiation scheme from iPSCs to chondronoids.

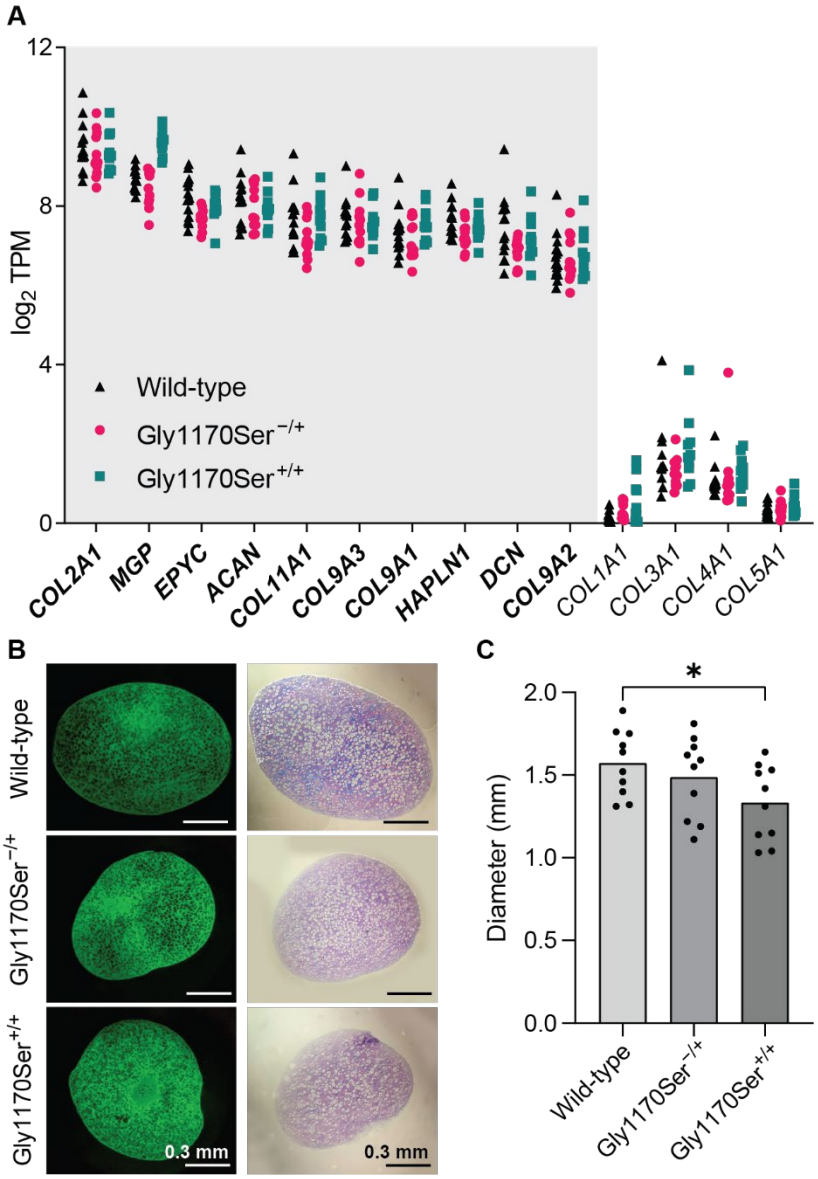

Fig. 2. A. Ten most highly expressed core matrisome genes (bolded) demonstrating high expression of cartilage-specific markers. Low to no expression of collagens of other tissue types highlights the faithful differentiation to chondrocytes. RNA samples were harvested 44 days after initiation of the chondrocyte differentiation. TPM: transcripts per million. **B.** Immunohistochemistry of collagen-II on histological sections of chondronoids shows that all genotypes deposit a matrix rich in collagen-II (green). Toluidine blue staining (purple) reveals an extensive proteoglycan matrix in all genotypes. **C**. Size distribution of chondronoids by genotype. Homozygous Gly1170Ser chondronoids are significantly smaller than wild-type ($p < 0.05$).

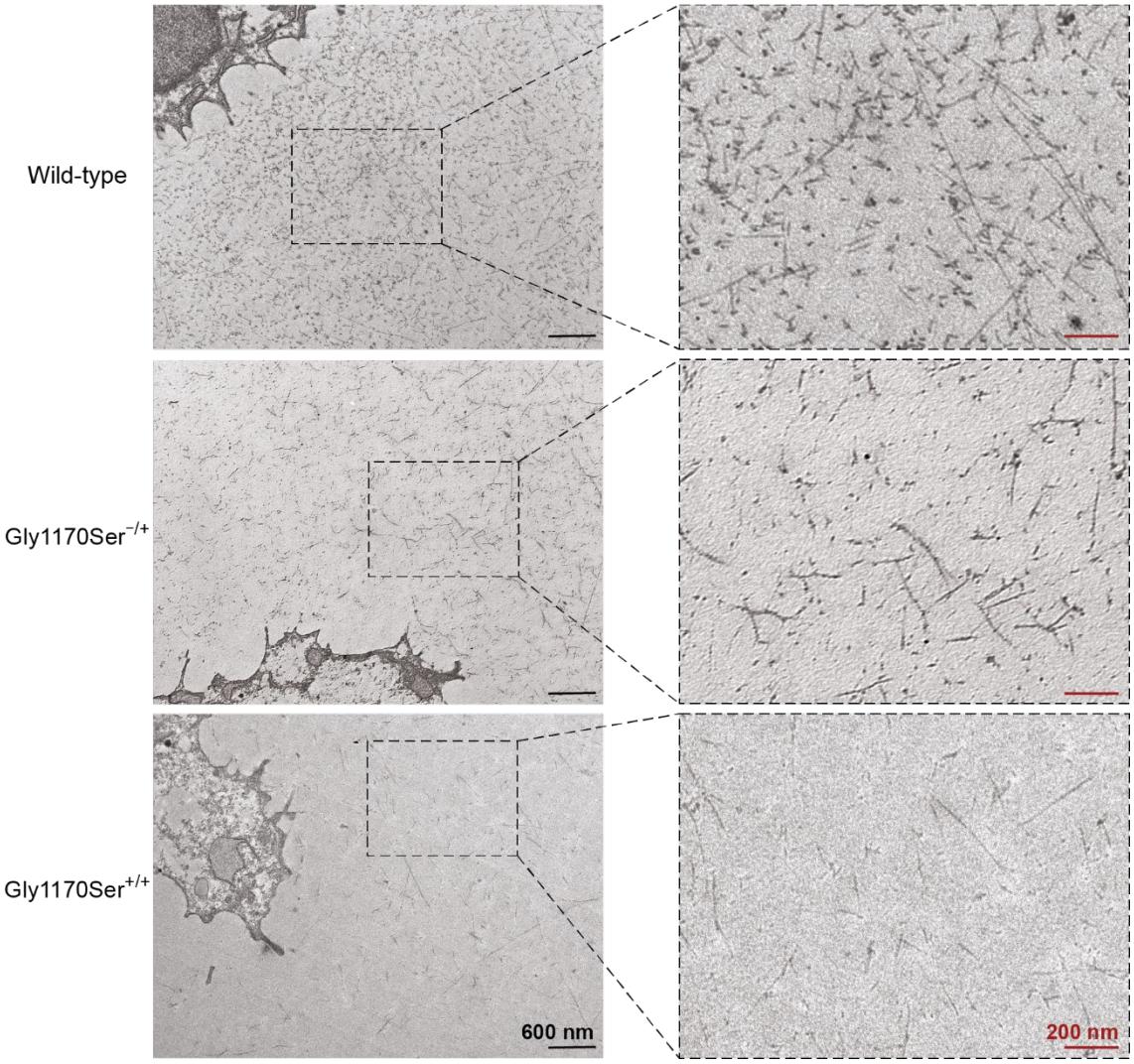

Fig. 3. TEM imaging of chondronoid ECMs. While the wild-type matrix is rich in network-forming collagen fibers, the clinically relevant heterozygous matrix is comparatively deficient, a phenotype that is further exacerbated in the homozygous chondronoids.

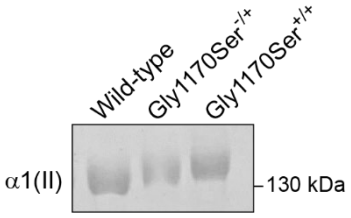

Fig. 4. SDS-PAGE separation of chondronoid-extracted collagen showing reduced electrophoretic mobility for the heterozygous and homozygous variants compared to wild-type collagen-II.

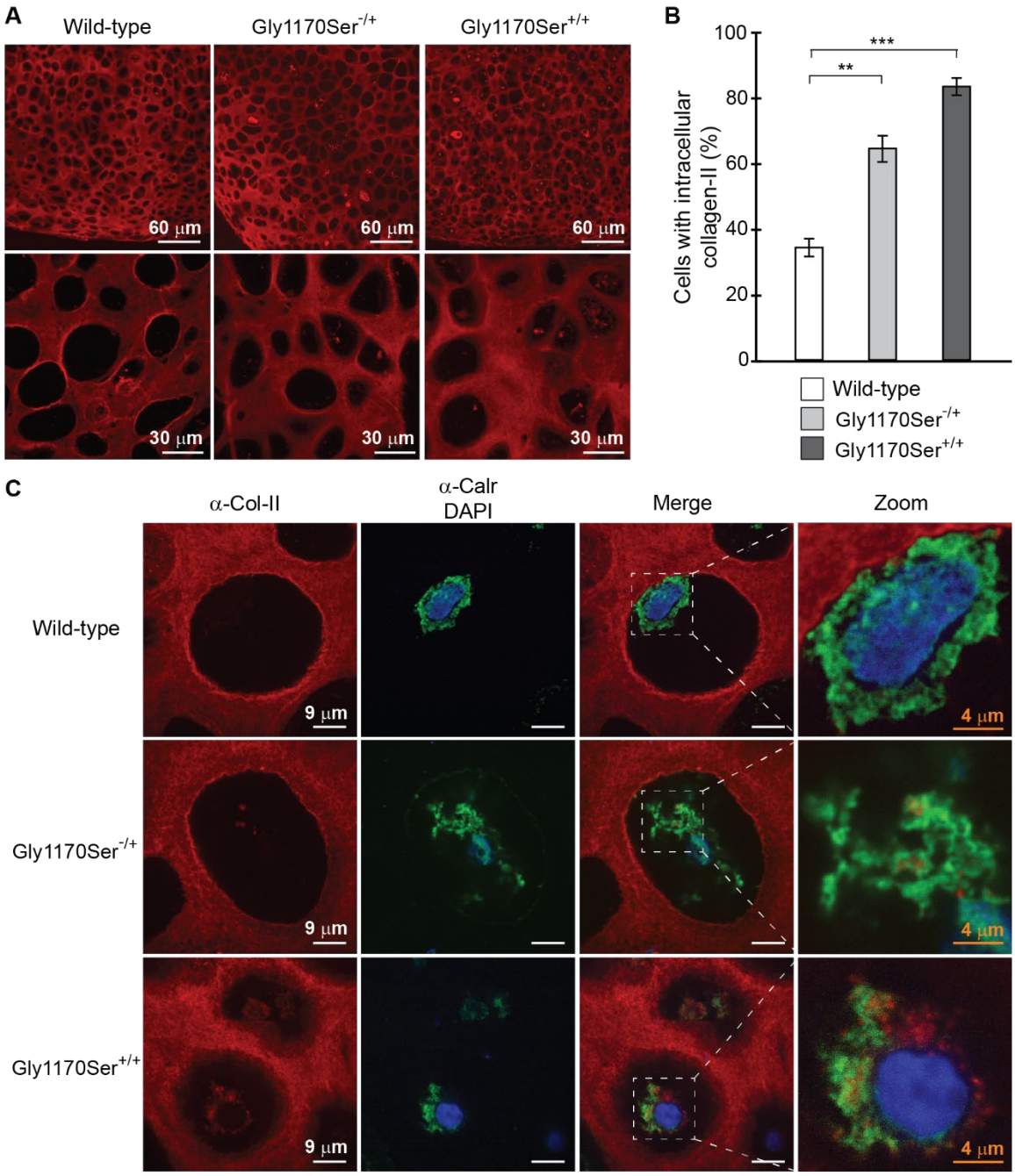

Fig. 5. A. Confocal images of collagen-II IHC at 10× and 63× magnification show an increase in intracellular procollagen-II retention across genotypes. **B.** Blinded quantification of the extent of procollagen-II intracellular retention. 34% of wild-type cells have intracellular procollagen-II, 64% of heterozygous cells, and 83% of homozygous cells. **C.** Confocal images of chondronoids stained for collagen-II, the ER marker calreticulin, and the nuclear stain DAPI reveal that much of the intracellularly retained procollagen-II in disease variants is localized in the ER.

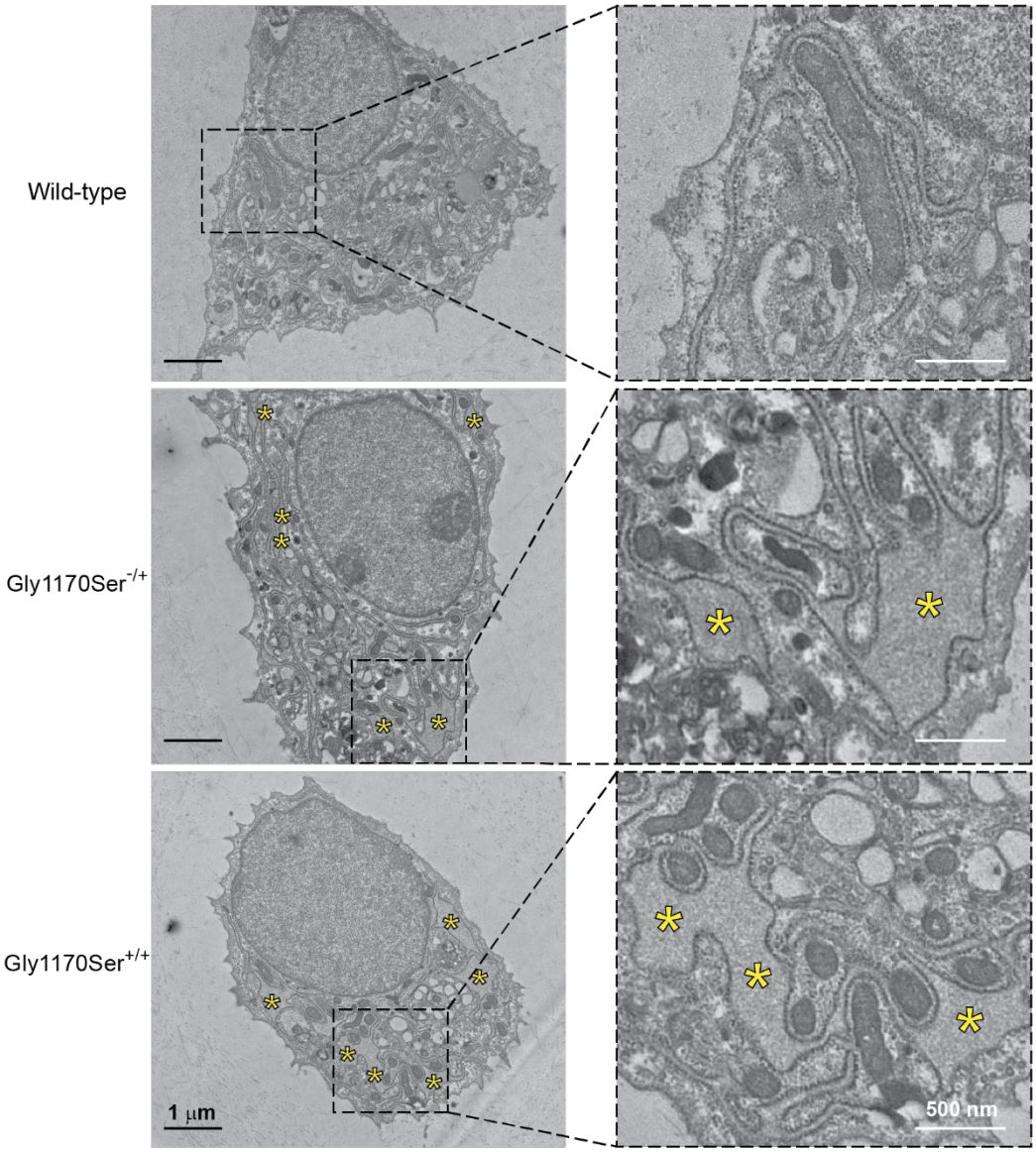

Fig. 6. Representative TEM images reveal the presence of distended ERs (marked with yellow stars) in cells expressing Gly1170Ser-substituted procollagen-II. The frequency and extent of ER dilation is further increased in homozygous cells compared to heterozygotes.

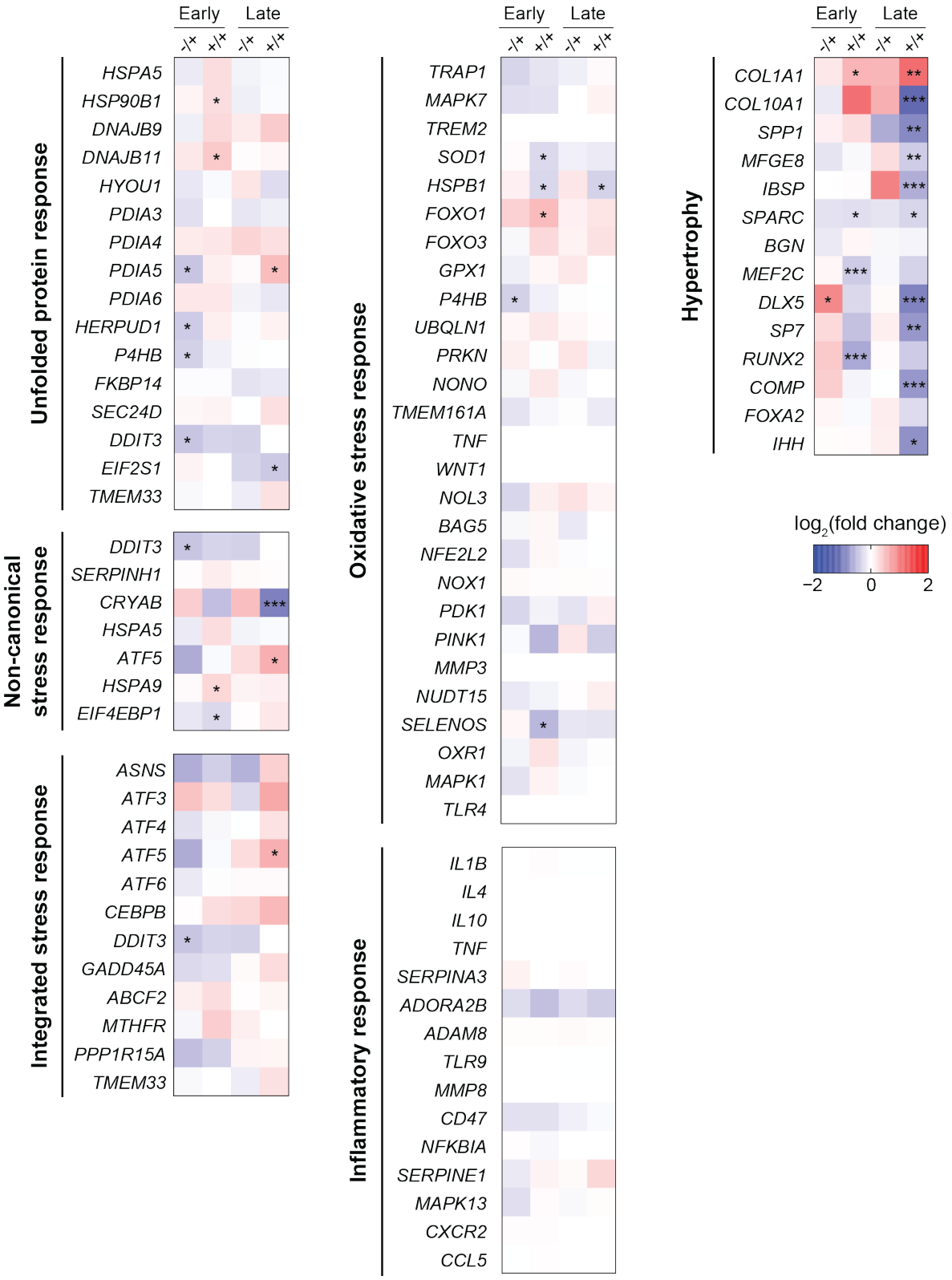

Fig. 7. Heatmap plotting the $\log_2$(fold change) of selected genes expressed in the heterozygous (−/+) and homozygous (+/+) Gly1170Ser chondronoids compared to wild-type chondronoids, at the early and late time points. * *p adjusted* < 0.05; ** *p adjusted* < 0.01; *** *p adjusted* < 0.001.

Table 1. High-confidence interactors differentially engage with wild-type and Gly1170Ser procollagen-II across three biological replicates.[a]

Protein (*Gene*)	**Fold-enrichment**	***p*-value[c]**	***q*-value[d]**
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 (*PLOD2*)	1.95	0.0013	0.0186
Protein disulfide-isomerase (*P4HB*)	1.67	0.0004	0.0128
Prolyl 3-hydroxylase 1 (*P3H1*)	1.55	0.0329	0.1504
Cartilage-associated protein (*CRTAP*)	1.52	0.0360	0.1504
Fibronectin (*FN1*)	1.49	0.0375	0.1504
Peptidyl-prolyl cis-trans isomerase FKBP10 (*FKBP10*)	1.45	0.0421	0.1504
Serpin H1 (*SERPINH1*)	1.45	0.1239	0.2952
Peptidyl-prolyl cis-trans isomerase B (*PPIB*)	1.41	0.0300	0.1504
Prolyl 4-hydroxylase subunit alpha-1 (*P4HA1*)	1.41	0.0892	0.2836
Protein canopy homolog 2 (*CNPY2*)	1.36	0.3542	0.5065
Peptidyl-prolyl cis-trans isomerase FKBP9 (*FKBP9*)	1.36	0.1109	0.2952
Glucosidase 2 subunit beta (*PRKCSH*)	1.33	0.1198	0.2952
Prolyl 4-hydroxylase subunit alpha-2 (*P4HA2*)	1.32	0.1685	0.3442
Calumenin (*CALU*)	1.31	0.1823	0.3475
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (*PLOD1*)	1.29	0.2444	0.4111
Calreticulin (*CALR*)	1.28	0.0124	0.1183
Multifunctional procollagen lysine hydroxylase and glycosyltransferase LH3 (*PLOD3*)	1.25	0.3520	0.5065
Procollagen galactosyltransferase 1 (*COLGALT1*)	1.24	0.3770	0.5134
Cytoskeleton-associated protein 4 (*CKAP4*)	1.17	0.4391	0.5708
Thioredoxin domain-containing protein 5 (*TXNDC5*)	1.17	0.5423	0.6743
Protein-glutamine gamma-glutamyltransferase 2 (*TGM2*)	1.16	0.5872	0.6845
Endoplasmic reticulum chaperone BiP (*HSPA5*)	1.16	0.1554	0.3418
Protein disulfide-isomerase A3 (*PDIA3*)	1.14	0.2728	0.4335
Endoplasmin (*HSP90B1*)	1.14	0.2332	0.4111
Protein ERGIC-53 (*LMAN1*)	1.12	0.6222	0.6845
Protein disulfide-isomerase A4 (*PDIA4*)	1.11	0.6076	0.6845
Protein disulfide-isomerase A6 (*PDIA6*)	1.11	0.9680	0.9887
Collagen alpha-1(I) chain (*COL1A1*)	0.94	0.8438	0.8938

[a]Proteins displaying a >2-fold increase in signal compared to negative control conditions in at least four out of the six experimental samples. See S3 data for complete, unfiltered data set.

[b]Fold-enrichment was calculated as the ratio of bait-normalized abundance in Gly1170Ser to the bait-normalized abundance in wild-type.

[c]*p*-value was calculated using a homoscedastic student's *t*-test across the three biological replicates.

[d]*q*-value was calculated using a two-stage step-up method of Benjamini, Krieger, and Yekutieli with a desired false discovery rate set to 10%.