

# Association of genetic variation in *COL11A1* with adolescent idiopathic scoliosis

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## Abstract

Adolescent idiopathic scoliosis (AIS) is a common and progressive spinal deformity in children that exhibits striking sexual dimorphism, with girls at more than five-fold greater risk of severe disease compared to boys. Despite its medical impact, the molecular mechanisms that drive AIS are largely unknown. We previously defined a female-specific AIS genetic risk locus in an enhancer near the *PAX1* gene. Here we sought to define the roles of *PAX1* and newly-identified AIS-associated genes in the developmental mechanism of AIS. In a genetic study of 10,519 individuals with AIS and 93,238 unaffected controls, significant association was identified with a variant in *COL11A1* encoding collagen (α1) XI (rs3753841; NM\_080629.2\_c.4004C>T; p.(Pro1335Leu);  $P=7.07 \times 10^{-11}$ , OR=1.118). Using CRISPR mutagenesis we generated *Pax1* knockout mice (*Pax1*<sup>-/-</sup>). In postnatal spines we found that PAX1 and collagen (α1) XI protein both localize within the intervertebral disc (IVD)-vertebral junction region encompassing the growth plate, with less collagen (α1) XI detected in *Pax1*<sup>-/-</sup> spines compared to wildtype. By genetic targeting we found that wildtype *Col11a1* expression in costal chondrocytes suppresses expression of *Pax1* and of *Mmp3*, encoding the matrix

metalloproteinase 3 enzyme implicated in matrix remodeling. However, this suppression was abrogated in the presence of the AIS-associated *COL11A1*<sup>P1335L</sup> mutant. Further, we found that either knockdown of the estrogen receptor gene *Esr2*, or tamoxifen treatment, significantly altered *Col11a1* and *Mmp3* expression in chondrocytes. We propose a new molecular model of AIS pathogenesis wherein genetic variation and estrogen signaling increase disease susceptibility by altering a *Pax1-Col11a1-Mmp3* signaling axis in spinal chondrocytes.

### eLife assessment

This **valuable** study analyzes a large cohort of Adolescent Idiopathic Scoliosis (AIS) patients, identifying an association with a variant in *COL11A1* (Pro1335Leu). Experimental testing of this potentially pathogenic variant in vitro suggests a connection between *Pax1*, *Col11a1*, *Mmp3*, and estrogen signaling, thus providing **solid** support for the proposed link between hormonal and matrix components in the development of AIS.

## Introduction

The human spinal column is a dynamic, segmented, bony and cartilaginous structure that is essential for integrating the brain and nervous system with the axial skeleton while simultaneously providing flexibility in three dimensions<sup>1</sup>. Idiopathic scoliosis is the most common developmental disorder of the spine, typically appearing during the adolescent growth spurt. Adolescent idiopathic scoliosis (AIS) is reported in all major ancestral groups, with a population prevalence of 1.5-3%<sup>2,3</sup>. Children with AIS usually present with a characteristic right-thoracic major curve pattern and a compensatory lumbar curve. Major thoracolumbar and lumbar curves are less frequent<sup>1</sup>. The three-dimensional nature of the deformity results in torsion in the spine that is most significant at the apex of the major curve, and changes in the structures of the vertebrae and ribs may develop as the curve worsens or progresses<sup>1</sup>. Children with thoracic curves, with larger curves at first presentation, and/or with greater remaining growth potential are at increased risk of progression, but this risk decreases sharply after skeletal maturity<sup>1</sup>. Sex is a recognized risk factor for AIS, with girls having at least a five-fold greater risk of progressive deformity requiring treatment compared to boys<sup>4</sup>. This well-documented sexual dimorphism has prompted speculation that levels of circulating endocrine hormones, particularly estrogen, are important exposures in AIS susceptibility<sup>5</sup>.

The genetic architecture of human AIS is complex, and underlying disease mechanisms remain uncertain. Heritability studies of Northern European<sup>6,7</sup>, North American<sup>8,9</sup>, and South Asian<sup>10</sup> ancestral groups suggest that disease risk is multifactorial, caused by genetic and environmental contributions<sup>2,11</sup>. Accordingly, population-based genome-wide association studies (GWAS) in multiple ancestral groups have identified several AIS-associated susceptibility loci, mostly within non-coding genomic regions<sup>11</sup>. In particular, multiple GWAS have implicated noncoding regions near the *LBX1*<sup>12</sup>, *ADGRG6* (also known as *GRP126*)<sup>13</sup>, and *BNC2*<sup>14</sup> genes. An association with alleles in an enhancer distal to *PAX1*, encoding the transcription factor paired box 1, was primarily driven by females, suggesting that it contributes to the sexual dimorphism observed in AIS<sup>15</sup>. Subsequent meta-analysis of combined AIS GWAS identified additional susceptibility loci. These included variants in an intron of *SOX6*, a transcription factor, that along with *PAX1*, is important in early spinal column formation<sup>16</sup>. Furthermore, gene enrichment analyses found significant correlation of AIS-associated loci with biological pathways involving cartilage and connective tissue development<sup>17</sup>. A more recent GWAS in a Japanese population

identified fourteen additional AIS loci that are candidates for further evaluation<sup>18</sup>. In separate studies, genome sequencing in AIS cases and families identified enrichment of rare variants in the *COL11A2*<sup>19</sup> and *HSPG2*<sup>20</sup> genes, encoding components of the cartilage extracellular matrix (ECM). Hence variation affecting cartilage and connective tissue ECM is an emerging theme in the heterogeneous genetic architecture of AIS.

Pre-clinical animal models are essential tools for accelerating mechanistic understanding of AIS and for therapeutic testing<sup>11</sup>. In zebrafish, several genetic mutants with larval or later-onset spinal deformity have been described, including *ptk7*<sup>21,22</sup>, *c21orf59*<sup>23</sup>, *ccdc40*<sup>24</sup>, *ccdc151*<sup>25</sup>, *dyx1c1*, and *kif6*<sup>26</sup>. In rescue experiments, Rebello et al. recently showed that missense variants in *COL11A2* associated with human congenital scoliosis fail to rescue a vertebral malformation phenotype in a zebrafish *col11a2* knockout line<sup>27</sup>. In mouse, conditional deletion of *Adgrg6* in skeletal cartilage (using *Col2a1-Cre*) produces a progressive scoliosis of the thoracic spine during postnatal development that is marked by herniations within the cartilaginous endplates of involved vertebrae. Progressive scoliosis, albeit to a lesser extent, was also observed when *Adgrg6* was deleted from committed chondrocytes (using ATC-Cre)<sup>28–30</sup>. These studies demonstrate that cartilage and possibly other osteochondroprogenitor cells contribute to the scoliosis phenotype in these models<sup>29</sup>. Taken together, genetic and functional studies in mouse, although limited, support the hypothesis that deficiencies in biogenesis and/or homeostasis of cartilage, intervertebral disc (IVD), and dense connective tissues undermine the maintenance of proper spinal alignment during the adolescent growth spurt<sup>11</sup>.

The combined contribution of reported AIS-associated variants is broadly estimated to account for less than 10% of the overall genetic risk of the disease<sup>18</sup>. To address this knowledge gap, we sought to define novel loci associated with AIS susceptibility in genes encoding proteins of the ECM (i.e. the “matrisome”<sup>31,32</sup>). Here we identify new genetic associations with AIS. Further, our functional assessments support a new disease model wherein AIS-associated genetic variation and estrogen signaling perturb a *Pax1-Col11a1-Mmp3* axis in chondrocytes.

## Results

### Nonsynonymous variants in matrisome genes are associated with increased risk of AIS

The “matrisome” has been defined as “all genes encoding structural ECM components and those encoding proteins that may interact with or remodel the ECM”<sup>33</sup>. Proteins comprising the global ECM as currently defined have been identified by both experimental and bio-informatic methods<sup>31</sup>. We assembled 1027 matrisome genes as previously identified<sup>34</sup>, including 274 core-matrisome and 753 matrisome-associated genes (N=1,027 total). For the genes encoding these 1,027 proteins, we identified all nonsynonymous common variants (MAF > 0.01) queried by the Illumina HumanCoreExome-24v1.0 beadchip and determined their genotypes in a discovery cohort of 1,358 cases and 12,507 controls, each of European ancestry (**Table 1**). After applying multiple quality control measures (see Methods section), we retained 2,008 variants in 597 matrisome genes for association testing (**Supplemental Table 1**). This sample size was estimated to provide at least 80% power to detect significant associations at the matrisome-wide level ( $\alpha \leq 2.5 \times 10^{-5}$ ), for alleles with population frequency  $\geq 0.05$  and OR  $\geq 1.5$  (**Figure 1** – **figure supplement 1a**). Two nonsynonymous variants, in *COL11A1* (rs3753841; NM\_080629.2\_c.4004C>T; p.(Pro1335Leu); odds ratio (OR)=1.236 [95% CI=1.134-1.347],  $P=1.17 \times 10^{-6}$ ) and *MMP14* (rs1042704; NM\_004995.4\_c.817G>A; p.(Asp273Asn); OR=1.239 [95% CI=1.125-1.363],  $P=1.89 \times 10^{-5}$ ) were significantly associated with AIS (**Figure 1a**). Given the sexual dimorphism in AIS and our prior observation of a female-predominant disease locus<sup>15</sup>, we tested the 2,008 variants separately in females (N=1,157 cases

and 7,138 controls). In females, the association with rs3753841 remained statistically significant, whereas rs1042704, near *MMP14*, was not associated with AIS in females (**Figure 1** – **figure supplement 1b**). Our study was not sufficiently powered to test males separately.

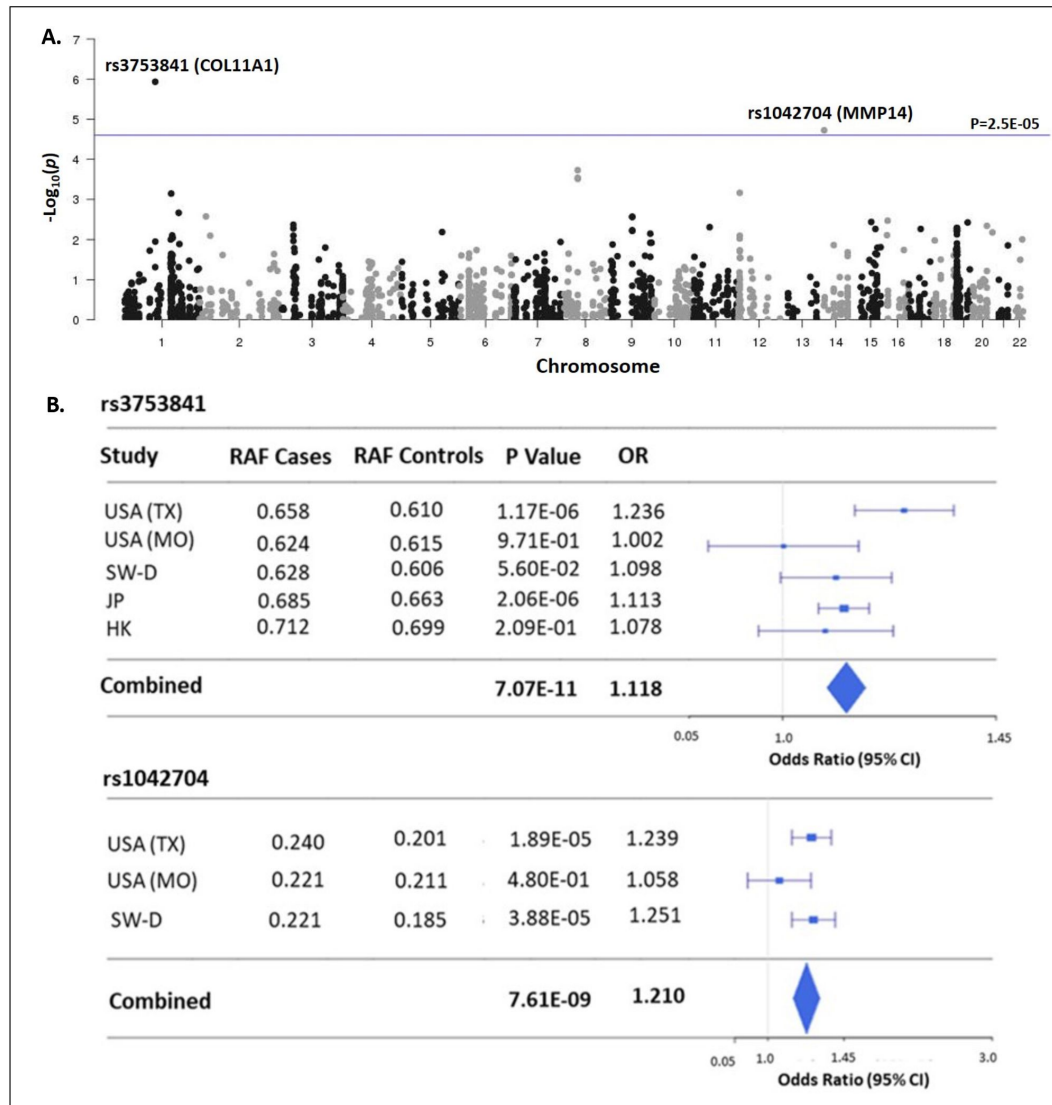
To validate these results, we sought to replicate the associations of rs3753841 and rs1042704 in four independent AIS case-control cohorts, from North America, Europe, and eastern Asia, representing multiple ethnicities (total N = 9,161 AIS cases, 80,731 healthy controls, **Table 1**). Genotypes for both variants were extracted from these datasets and tested for association by meta-analysis together with the discovery cohort (see Methods). Meta-analysis of all cohorts together increased the evidence for association of both variants with AIS risk (**Figure 1b**). While a similar effect size was noted for rs1042704 in Japanese and Han Chinese cohorts, the results were less significant, likely due to lower minor allele frequencies (East Asian MAF = 0.02 compared to total non-Asian cohort MAF = 0.20) in these populations (**Figure 1** – **figure supplement 1c**). Plotting recombination across both regions suggested that these signals were likely confined to blocks of linkage disequilibrium within the *COL11A1* and *MMP14* genes, respectively (**Figure 1** – **figure supplement 1d, e**).

Rare dominant mutations in *COL11A1*, often disrupting a Gly-X-Y sequence, can cause Marshall (MRS) (OMIM# 154780) or Stickler syndromes (STL2) (OMIM# 604841) marked variously by facial anomalies, sensorineural hearing loss, short stature, spondyloepiphyseal dysplasia, eye anomalies, ectodermal features, and scoliosis. Notably, our AIS cohort and particularly individuals carrying the rs3753841 risk allele were negative for co-morbidities or obvious features of Marshall or Stickler syndromes. Thus, variation in *COL11A1* is independently associated with AIS. Notably, we did not detect common variants in linkage disequilibrium ( $R^2 > 0.6$ ) with the top SNP rs3753841 (**Figure 1** – **Supplement 1d**). Further, analysis of 625 exomes from the discovery cohort (46%) identified only three rare variants in five individuals (**Supplemental Table 2**), and rare variant burden testing was not significant as expected (data not shown). These observations suggested that rs3753841 itself could confer disease risk, although our methods would not detect deep intronic variants that could contribute to the overall association signal.

## COL11A1 is expressed in adolescent spinal tissues

We next characterized *COL11A1* in postnatal spine development. *COL11A1* encodes one of three alpha chains of type XI collagen, a member of the fibrillar collagen subgroup and regulator of nucleation and initial fibril assembly, particularly in cartilage<sup>35</sup>. Spinal deformity is well-described in *Col11a1*-deficient (*cho/cho*) embryos<sup>36,37</sup>. In mouse tendon, *Col11a1* mRNA is abundant during development but barely detectable at three months of age<sup>38</sup>. We analyzed RNA-seq datasets derived from adolescent human spinal tissues<sup>39</sup>, finding that *COL11A1* was upregulated in cartilage relative to bone and muscle. In cartilage, *PAX1* and *COL11A2* showed the strongest expression levels relative to other published human AIS-associated genes<sup>13–15,17,19,20,40</sup> (**Figure 2**). In all, most AIS-associated genes showed the strongest expression levels in cartilage relative to other adolescent spinal tissues.

We next sought to characterize *Col11a1* expression in spines of postnatal mice. To detect COL11A1 protein (collagen α1(XI)), we performed immunohistochemistry (IHC) and immunofluorescence (IF) microscopy using a collagen α1(XI) reactive antibody<sup>41</sup> in newborn (P0.5) and adolescent (P28) mice. In spines of P0.5 mice, strong staining was observed in the nucleus pulposus (NP) and in surrounding annulus fibrosus (AF) (**Figure 2b**). In thoracic spines of P28 mice, the compartments of the IVD were more distinct, and strong collagen α1(XI) staining was observed in each (**Figure 2c**). In regions of the cartilage endplate (CEP)-vertebral bone transition, collagen α1(XI) was detected in columnar chondrocytes, particularly in the hypertrophic zone adjacent to condensing bone (**Figure 2c**). We also examined collagen α1(XI) expression in ribs, as these structures are also involved in the scoliotic deformity<sup>1</sup>. In P28 rib growth plates, as in spine, a biphasic pattern was observed in which collagen α1(XI) reactivity was most pronounced around cells of the presumed resting and pre-hypertrophic/hypertrophic zones (**Figure 2** – **figure**



**Figure 1.**

### Matrisome-wide association study.

**a.** Manhattan plot showing  $-\log_{10}$  P-values (y axis) versus chromosomal position (x axis) for the 2,008 common coding variants tested in the discovery study USA (TX). The horizontal line represents the threshold for significance level ( $P$ -value  $< 2.5 \times 10^{-5}$ ) after Bonferroni multiple testing correction. **b.** Tests of association for SNPs rs3753841 and rs1042704 in discovery and independent replication cohorts. RAF-reference allele frequency; OR-odds ratio; CI-confidence interval.

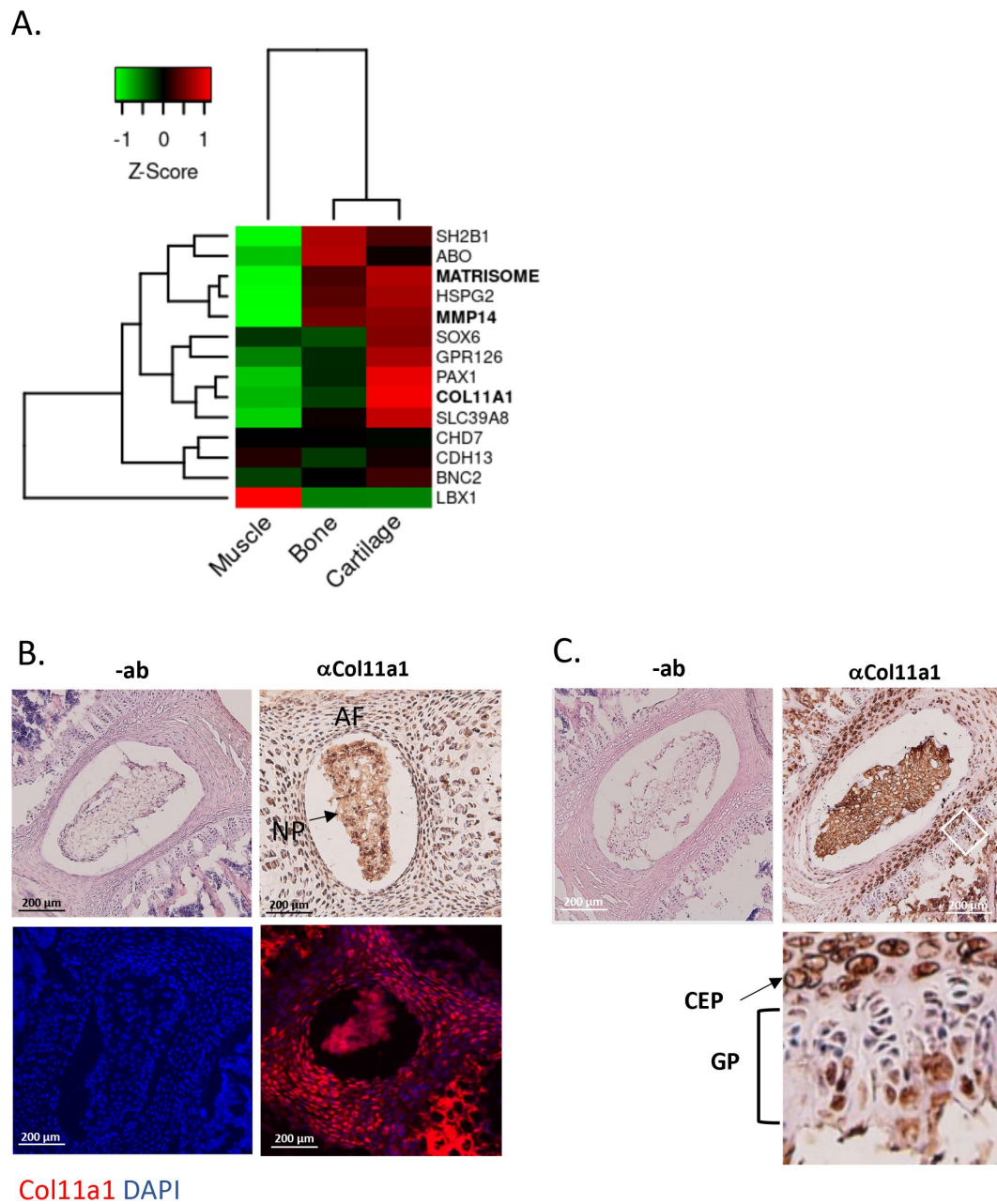
| Cohort   | Ethnicity         | Stage       | Subjects | Cases  |        | Controls |        |
|----------|-------------------|-------------|----------|--------|--------|----------|--------|
|          |                   |             |          | Male   | Female | Male     | Female |
| USA (TX) | NHW               | Discovery   | 13,865   | 201    | 1,157  | 5,369    | 7,138  |
| USA (MO) | NHW               | Replication | 2,951    | 201    | 1,012  | 1,049    | 689    |
| SW-D     | NHW               | Replication | 4,627    | 222    | 1,409  | 505      | 2,491  |
| JP       | EAS (Japanese)    | Replication | 79,211   | 323    | 5,004  | 40,205   | 33,679 |
| HK       | EAS (Han Chinese) | Replication | 3,103    | 178    | 812    | 858      | 1,255  |
| Total    |                   |             | 103,757  | 10,519 |        | 93,238   |        |

**Table 1.**

**Study cohorts. USA (TX): Texas cohort, USA (MO)**

Missouri cohort, SW-D: Swedish-Danish cohort, JP: Japanese cohort, HK: Hong Kong cohort, NHW: Non-Hispanic White, EAS: East Asian.





**Figure 2.**

***Col11a1* and *Mmp14* expression in spine.**

a) A heatmap of transcript per million (TPM) values of *COL11A1*, *MMP14*, and other published genes associated with AIS. The average TPM value of matrisome genes is represented as MATRISOME.

b) Detection of collagen type XI alpha chain 1 in P0.5 mouse spine. Immunohistochemistry (IHC) shown at top, with immunofluorescence (IF) staining below. “-ab” refers to negative controls lacking primary antibody (shown at left). Results are representative of  $N \geq 3$  technical replicates in whole spines.

c) Detection of collagen  $\alpha 1(XI)$  in P28 mouse spine. Negative antibody IHC control shown at left; antibody positive IHC shown at right. Enlarged, rotated view of white boxed area shows a biphasic staining pattern. CEP – cartilage endplate; GP – growth plate. Results are representative of  $N \geq 3$  technical replicates in whole spines.

**supplement a,b**). These data show that in mouse, collagen  $\alpha 1(XI)$  is detectable in all compartments of young postnatal IVD and, at the thoracic level, is particularly abundant in the chondro-osseous junction region of IVD and vertebral growth plate.

## Col11a1 is downregulated in the absence of Pax1 in mouse spine and tail

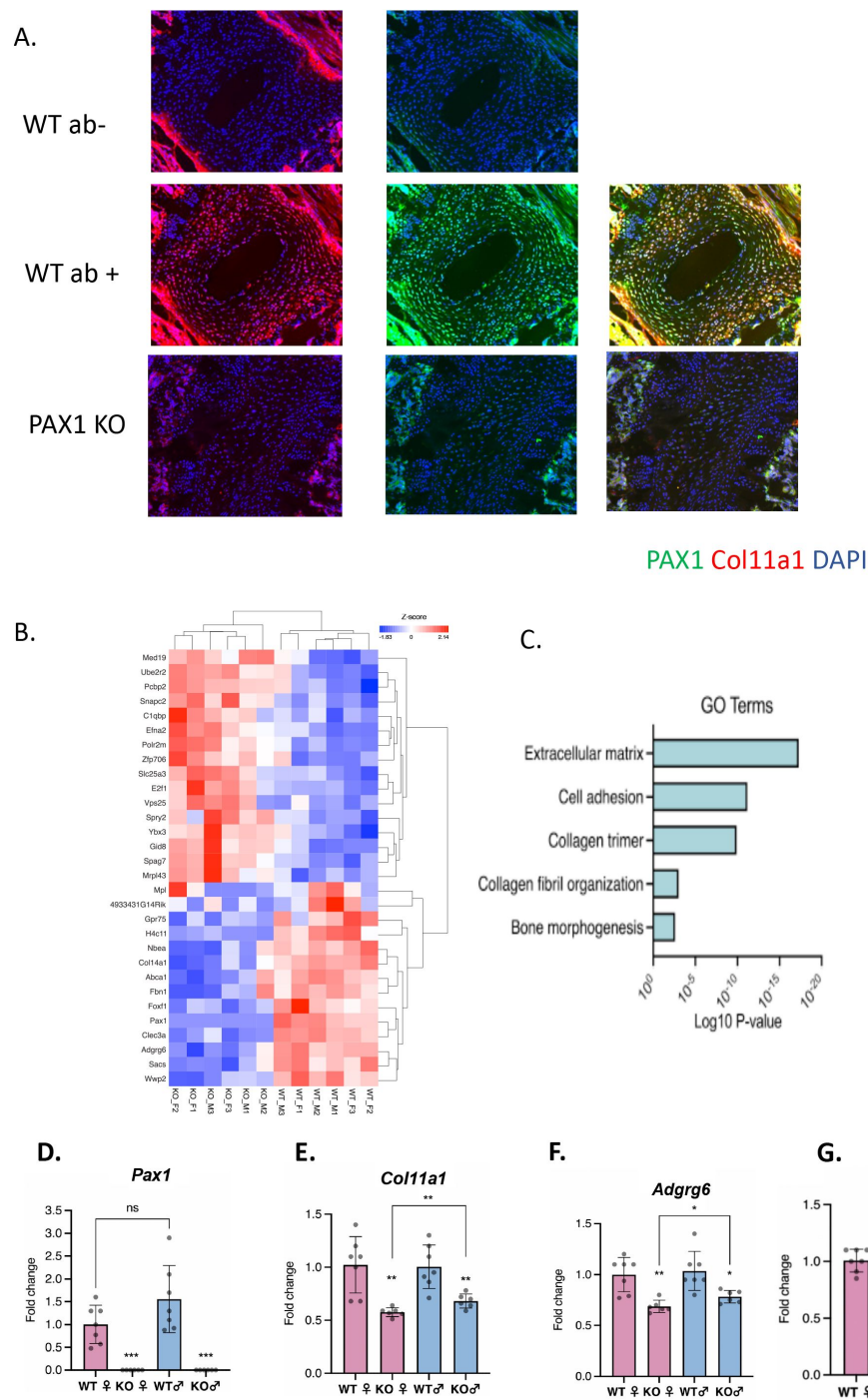
We previously identified AIS-associated variants within a putative enhancer of *PAX1* encoding the transcription factor Paired Box 1<sup>15,17</sup>. *Pax1* is a well-described marker of condensing sclerotomal cells as they form segments that will eventually become the IVD and vertebrae of the spine<sup>42–44</sup>. We generated *Pax1* knock-out mice (*Pax1*<sup>−/−</sup>) using CRISPR-Cas9 mutagenesis and validated them using sequencing and Southern blot (**Figure 3** – **figure supplement 3a-d**). Homozygous *Pax1*<sup>−/−</sup> mice were viable and developed kinks in the tail, as observed in other *Pax1*-deficient mice<sup>45</sup>. We next compared the expression of collagen  $\alpha 1(XI)$  protein in IVD and condensing bone of wildtype and *Pax1*<sup>−/−</sup> mice by performing IF staining in P28 spines (**Figure 3a**). In wildtype IVD, strong overlapping expression of collagen  $\alpha 1(XI)$  and PAX1 cells was observed, mostly within the CEP and chondro-osseous interface (**Figure 3a**). PAX1 staining was negative in *Pax1*<sup>−/−</sup> mice as expected, and collagen  $\alpha 1(XI)$  staining was dramatically diminished in CEP and the chondro-osseous vertebral borders. Moreover, the IVD in *Pax1*<sup>−/−</sup> mice was highly disorganized, without discernable NP, AF, and CEP structures as has been reported (**Figure 3** – **figure supplement e**)<sup>46</sup>. To test the effect of *Pax1* on expression of *Col11a1* and other AIS-associated genes during embryonic development, RNA was isolated from vertebral tissue dissected from the tails of E12.5 wild-type and *Pax1*<sup>−/−</sup> mice and subjected to bulk RNA-seq and quantitative real-time PCR (qRT-PCR) (**Figure 3b**). Gene-set enrichment analysis of RNA-seq was most significant for the gene ontology term “extracellular matrix” (**Figure 3c**). By qRT-PCR analysis, expression of *Col11a1*, *Adgrg6*, and *Sox6* were significantly reduced in female and male *Pax1*<sup>−/−</sup> mice compared to wild-type mice (**Figure 3d-g**). These data show that loss of *Pax1* leads to reduced expression of *Col11a1* and the AIS-associated genes *Adgrg6* and *Sox6* in affected tissue of the developing tail.

## Col11a1 regulates Mmp3 expression in chondrocytes

*COL11A1* has been linked with ECM remodeling and invasiveness in some cancers<sup>47</sup>. In solid tumors, *COL11A1* has been shown to alter ECM remodeling by enhancing *MMP3* expression in response to *TGF $\beta$* <sup>47</sup>. *MMP3* encodes matrix metalloproteinase 3, also known as stromelysin, an enzyme implicated in matrix degradation and remodeling in connective tissues<sup>48</sup>. We confirmed strong *MMP3* mRNA expression, relative to *COL11A1*, in human spinal cartilage and bone, but minimal expression in spinal muscle (**Figure 4** – **figure supplement 4a**). We next cultured costal chondrocytes from P0.5 *Col11a1*<sup>fl/fl</sup> mice<sup>41</sup> and subsequently removed *Col11a1* by treating with Cre-expressing adenoviruses. After confirming *Col11a1* excision (**Figure 4a**), we compared *Mmp3* expression in these cells to cells treated with GFP-expressing adenoviruses lacking Cre activity. We found that *Mmp3* expression was significantly increased in cells where *Col11a1* mRNA expression was downregulated by about 70% compared to untreated cells (**Figure 4b**). Furthermore, Western blotting in these cells demonstrated a ~2-5-fold increase in pro-, secreted, and active forms of *Mmp3* protein when collagen  $\alpha 1(XI)$  was reduced. The proteolytic processing *per se* of precursor *MMP3* into active forms<sup>49</sup> did not appear to be affected by *Col11a1* expression (**Figure 4c**). These results suggest that *Mmp3* expression is negatively regulated by *Col11a1* in mouse costal chondrocytes.

To test whether *Col11a1* affects *Mmp3* expression *in vivo*, we bred *Col11a1*<sup>fl/fl</sup> female mice with *Col11a1*<sup>fl/fl</sup> ATC males carrying the Acan enhancer-driven, doxycycline-inducible Cre (ATC) transgene<sup>50</sup>. ATC has been shown to harbor Cre-mediated recombination activity in most differentiated chondrocytes and in nucleus pulposus within two days of treating pregnant mothers with doxycycline starting at E15.5<sup>50</sup>. ATC activity was confirmed by crossing this line to





**Figure 3.**

#### Assessing *Pax1* regulation of *Col11a1*.

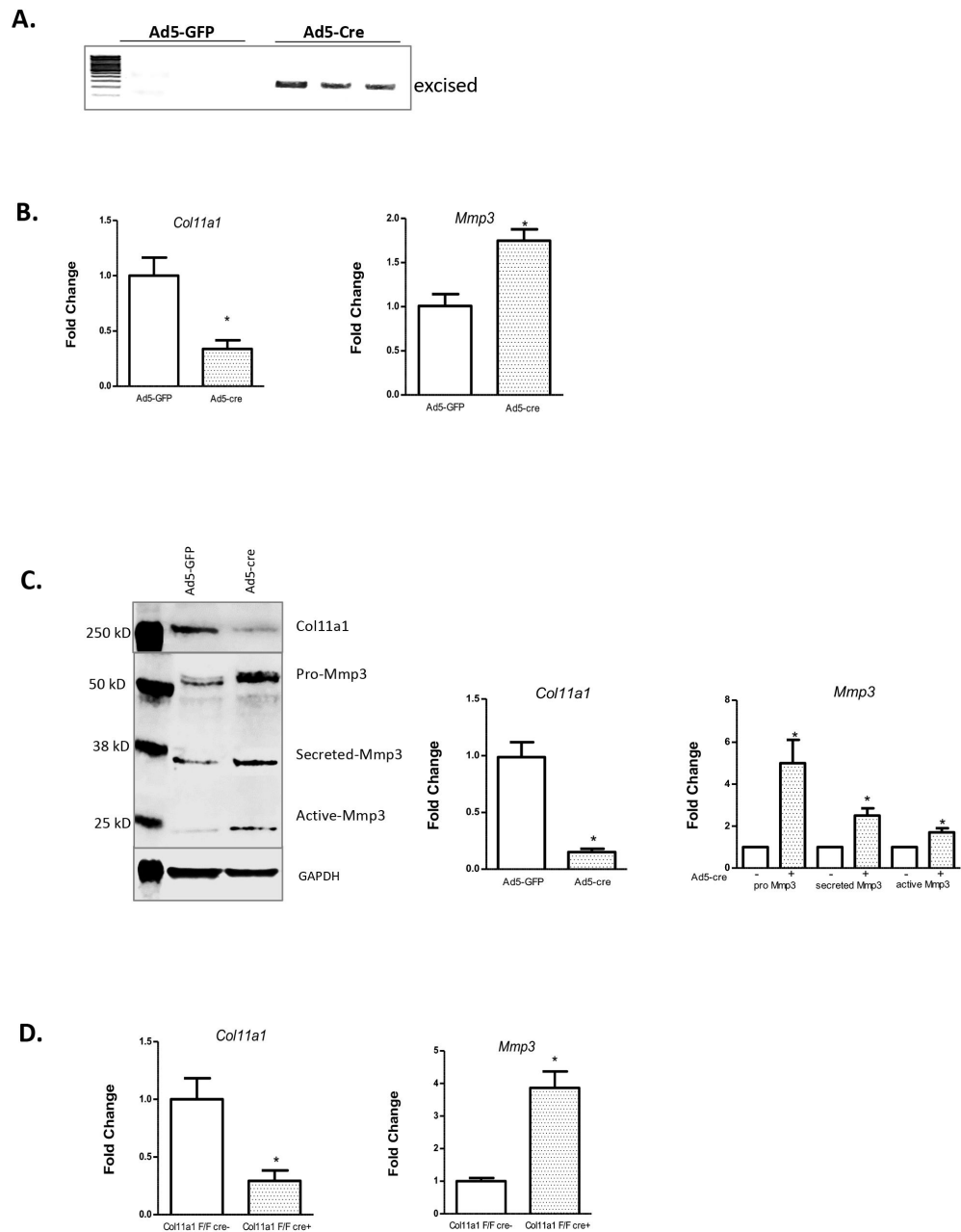
a) IF staining of P28 IVD from thoracic regions of *Pax1*<sup>-/-</sup> (bottom) and wildtype littermate (middle, top) mice using PAX1- (green) and collagen α1(XI)-specific (red) antibodies and DAPI nuclear counterstain. Antibody-negative controls are shown at top as (-ab). Results are representative of N≥3 technical replicates in whole spines.

b) Heatmap of differentially expressed genes (*P*-value<0.0001) in E12.5 tails of WT and *Pax1*<sup>-/-</sup> mice

c) Gene ontology (GO) analysis of differentially expressed genes in E12.5 tail WT and *Pax1*<sup>-/-</sup> null mice

d-g) Gene expression levels dissected from E12.5 mouse tail from wild type (WT) and *Pax1*<sup>-/-</sup> (KO) mice as determined by qRT-PCR. Each value represents the ratio of each gene expression to that of β-actin, and values are mean ± standard deviation.

The expression value of WT female group was arbitrarily set at 1.0. Each dot represents one embryo and statistical differences were determined using a two-sided unpaired t-test (*P*\*<0.05, *P*\*\*<0.01, *P*\*\*\*<0.001).



**Figure 4.**

***Col11a1* regulation of *Mmp3* expression in cartilage.**

a) PCR assay of *Col11a1* excision in *Col11a1<sup>fl/fl</sup>* cultured costal chondrocytes.

b) Gene expression levels from *Col11a1<sup>fl/fl</sup>* cultured costal chondrocytes transduced with GFP (Ad5-GFP, left) or Cre-expressing adenovirus (Ad5-cre, right) as determined by qRT-PCR. Values represent the ratio of each gene expression to that of *GAPDH*, and values are mean  $\pm$  standard deviation. The expression value of control Ad5-GFP results was arbitrarily set at 1.0. Statistical differences were determined using a two-sided paired t test ( $P^* < 0.05$ ). Results shown for  $N \geq 3$  biologic replicates, each including 3 technical replicates.

c) Western blot detection of collagen  $\alpha 1$ (XI), MMP3, and GAPDH loading control in cultured costal chondrocytes after Ad5-GFP or Ad5-cre transduction. Results are representative of  $N = 4$  biologic replicates. Protein size ladder is shown in lane 1. Quantification of bands detected by Western blotting, where scramble results were set to 1.0, is shown at right. Statistical differences were determined using a two-sided paired t test ( $P^* < 0.05$ ).

d) Gene expression levels from dissected *Col11a1<sup>fl/fl</sup>* ATC costal cartilage, analyzed as described in a). Results shown for  $N = 3$  biologic replicates, each including 3 technical replicates.

the R26td[Tomato] reporter that ubiquitously expresses the fluorescent gene Tomato after Cre recombination. Strong Cre activity was seen in P0 pups of mothers treated with doxycycline at E15.5 in the NP, CEP, and annulus fibrosus (AF) of the IVD and in chondrocytes of the growth plates (**Figure 4** – **figure supplement 4b**). Pregnant *Col11a1*<sup>fl/fl</sup> females were treated with doxycycline water from E15.5 to induce Cre expression in differentiated chondrocytes. Excision of *Col11a1* was confirmed in DNA from costal cartilage of *Col11a1*<sup>fl/fl</sup>ATC cre-positive offspring (**Figure 4** – **figure supplement 4c**). Consistent with results obtained by *in vitro* excision of *Col11a1*, cartilage from mice deficient in *Col11a1* showed ~4-fold upregulation of *Mmp3* mRNA expression relative to *Col11a1*<sup>fl/fl</sup> mice (**Figure 4d**).

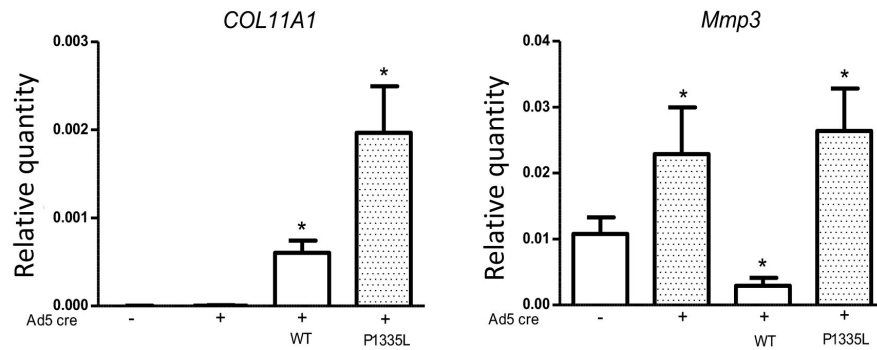
## AIS-associated variant in COL11A1 perturbs its regulation of MMP3

Although low-resolution structures currently available for collagen triple helices are not useful for modeling the effects of individual variants on protein stability, we noted that the AIS-associated variant P1335L occurs at the third position of a Gly-X-Y repeat and consequently could be structurally important in promoting stability of the triple helix, particularly if it is hydroxylated. We also noted that this variant is predicted to be deleterious by Combined Annotation Dependent Depletion (CADD)<sup>51</sup> and Genomic Evolutionary Rate Profiling (GERP)<sup>52</sup> analysis (CADD CADD=25.7; GERP=5.75). Further, *COL11A1* missense variants have been shown to evoke transcriptional changes in ECM genes in cancer cells.<sup>53</sup> We therefore tested whether the *COL11A1*<sup>P1335L</sup> sequence variant alters its regulation of *Mmp3* in chondrocytes. For this, SV40-immortalized cell lines were established from *Col11a1*<sup>fl/fl</sup> mouse costal chondrocytes and transduced with lentiviral vectors expressing green fluorescent protein (GFP) and *COL11A1*<sup>wt</sup>, *COL11A1*<sup>P1335L</sup>, or vector alone. After transduction, GFP-positive cells were grown to 50% confluence and treated with Cre-expressing adenovirus (ad5-Cre) to remove endogenous mouse *Col11a1* (**Figure 5a**). Using a human-specific *COL11A1* qRT-PCR assay, we detected overexpression of *COL11A1*<sup>wt</sup> and *COL11A1*<sup>P1335L</sup> compared to untransduced cells regardless of Cre expression (**Figure 5a**). Western blotting with an antibody directed against the HA epitope tag confirmed overexpression of human collagen α1(XI) protein (**Figure 5b**). Endogenous *Mmp3* mRNA and protein upregulation was evident by qRT-PCR and Western blotting, respectively, in untransduced cells treated with Ad5-Cre, as expected. Overexpressing human wildtype *COL11A1* suppressed *Mmp3* expression, consistent with the negative regulation we previously observed (**Figure 5 a,b**). However, the *COL11A1*<sup>P1335L</sup> mutant failed to downregulate *Mmp3* expression despite being overexpressed (**Figure 5a,b**). Thus, regulation of *Mmp3* appeared to be perturbed in the presence of the *COL11A1*<sup>P1335L</sup> variant in these cells.

## *Col11a1* and *Mmp3* are responsive to estrogen receptor signaling in chondrocytes

The expression of *Col11a1*, and of other ECM genes, is known to be estrogen-responsive in certain tissues, such as ovarian follicular cells.<sup>54</sup> Because of the suspected role of endocrine hormones in AIS, we investigated whether *Col11a1* expression was responsive to estrogen receptor siRNA-mediated knockdown in cultured chondrocytes. We first validated that *Mmp3* mRNA and protein levels were significantly increased after *Col11a1* knockdown in wildtype chondrocytes, as observed by Cre-mediated deletion in *Col11a1*<sup>fl/fl</sup> chondrocytes (**Figure 6a**). Estrogen receptor 2 (*Esr2*), but not estrogen receptor alpha (*Esr1*), was detected in mouse chondrocytes by qRT-PCR (data not shown). We therefore tested the consequences of *Esr2* siRNA-mediated knockdown on gene expression in chondrocytes. After *Esr2* knockdown, *Col11a1* as well as *Pax1* were significantly upregulated compared to scramble control, while *Mmp3* expression was significantly downregulated (**Figure 6b**). We also performed *Col11a1* knockdowns in these cells and noted upregulation of *Pax1* expression, suggesting a negative feedback loop between *Pax1* and *Col11a1* in these cells (**Figure 6b**). Simultaneous knockdown of *Col11a1* and *Esr2* expression reduced *Mmp3* expression to normal levels, supporting a possible interaction between *Col11a1* and *Esr2* in regulating *Mmp3*. Treating chondrocytes with tamoxifen, an estrogen receptor modulator, also

A.



B.

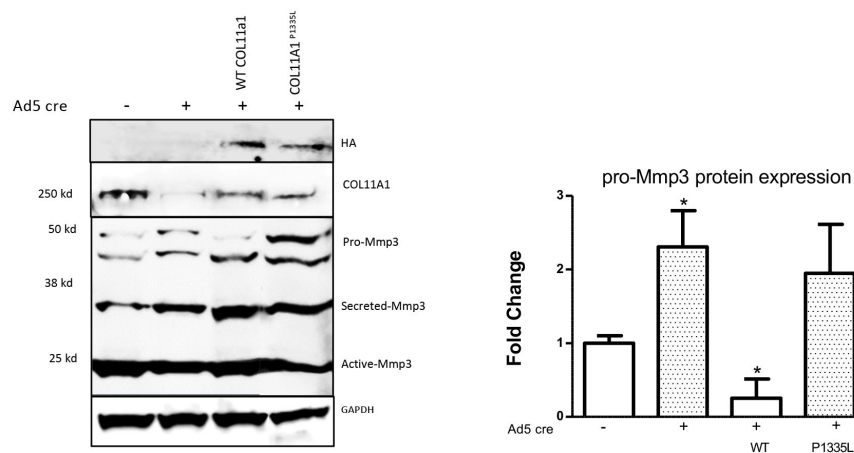


Figure 5.

### *Col11a1*<sup>P1335L</sup> regulation of *Mmp3* expression in lentiviral transduced mouse GPCs.

a) qRT-PCR of human *COL11A1* and endogenous mouse *Mmp3* in SV40 immortalized mouse costal chondrocytes transduced with the lentiviral vector only (lanes 1,2), human WT *COL11A1* (lane 3), or *COL11A1*<sup>P1335L</sup>. Values represent the ratio of each gene expression to that of *GAPDH*, and values are mean  $\pm$  standard deviation. Significant quantitative changes ( $P \leq 0.05$ ) relative to vector-only transfected cells as measured by unpaired t-tests are shown by \*. Results shown for N=4 biologic replicates, each including 3 technical replicates.

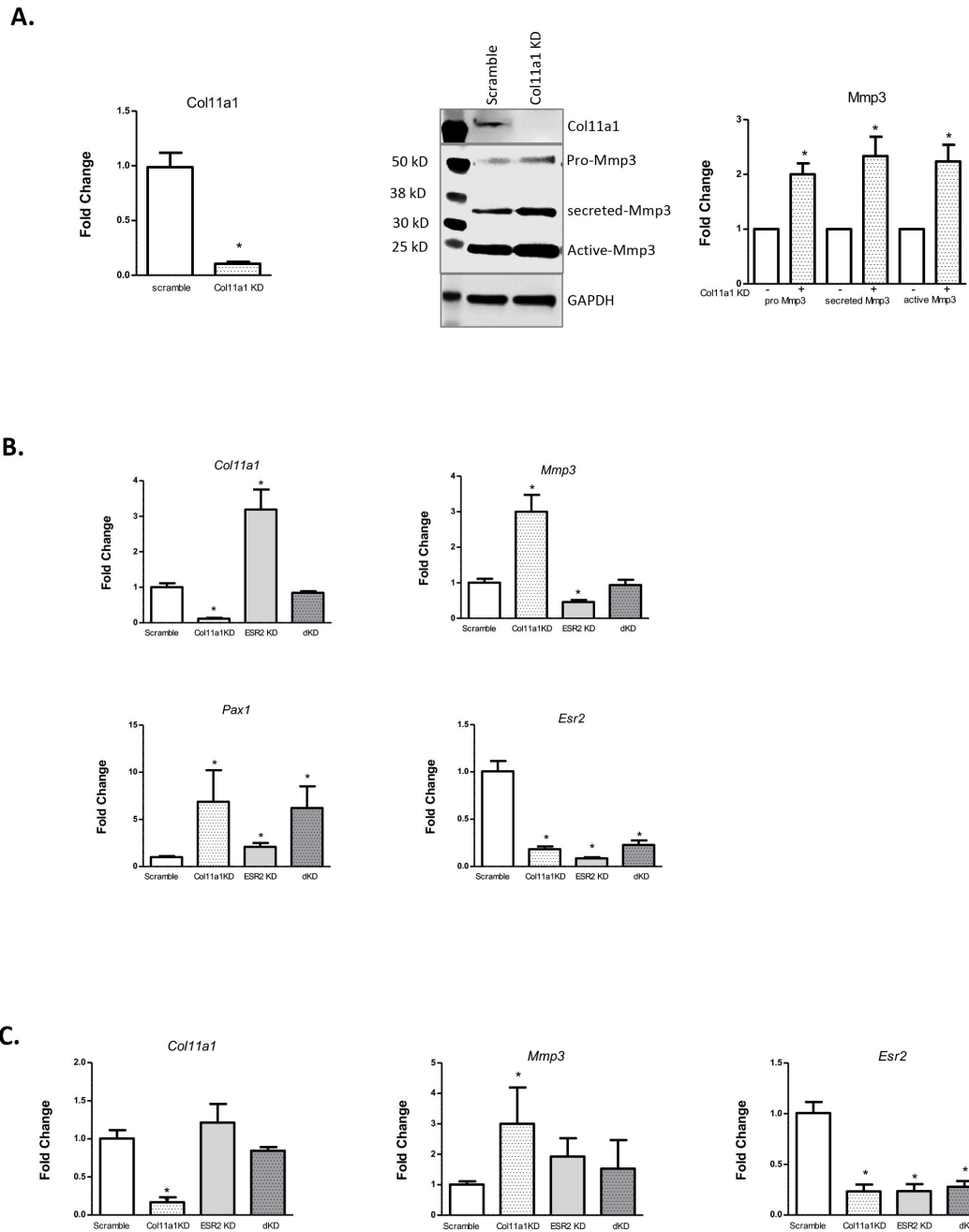
b) Western blot corresponding to experiments shown in (a) using HA antibody to detect epitope-tagged human collagen  $\alpha 1(XI)$ , *COL11A1* antibody to detect mouse and human collagen  $\alpha 1(XI)$ , MMP3 antibody to detect endogenous mouse MMP3, and *GAPDH*. Values are mean after normalization to *GAPDH*,  $\pm$  standard deviation. Significant differences ( $P \leq 0.05$ ) relative to vector-only, Ad5-negative transfected cells as measured by unpaired t-tests are shown by \*.

upregulated *Col11a1* expression to similar levels as observed after *Esr2* knockdown, compared to cells treated with DMSO carrier (**Figure 6** [↗](#)-**figure supplement 6a**). These results suggest that estrogen signaling suppresses *Col11a1* expression. In cultured rat CEP cells, *Esr2* mRNA was downregulated, and *Mmp3* mRNA was upregulated after *Col11a1* knockdown, as observed in mouse chondrocytes (**Figure 6c** [↗](#), **Figure 6** [↗](#) – **figure supplement 6b**). However, *Esr2* knockdown did not significantly impact *Col11a1* or *Mmp3* expression in these cells (**Figure 6c** [↗](#)). Hence, we conclude that in cultured mouse chondrocytes, ESR2 signaling disrupts the suppression of *Mmp3* by *Col11a1*.

## Discussion

Adolescent idiopathic scoliosis has been described in the medical literature for centuries, yet its underlying etiology has remained enigmatic<sup>55</sup> [↗](#). Given that AIS originates in children who appear to be otherwise healthy, even its tissue of origin has been difficult to discern, and long debated<sup>11</sup> [↗](#). The advent of powerful genotyping and sequencing methods in the last two decades has led to breakthrough discoveries of genetic loci associated with AIS, most in non-coding regions of the genome that are difficult to interpret biologically<sup>11</sup> [↗](#). Aggregating these results, however, provided supportive evidence that pathways of cartilage and connective tissue ECM development are relevant in AIS etiology<sup>11</sup> [↗](#),<sup>17</sup> [↗](#). Here, in the largest multi-ethnic human cohort studied to date, we elected to test the hypothesis that alterations in ECM proteins themselves contribute to AIS susceptibility. This approach yielded most significant evidence for a common protein-altering variant in the *COL11A1* gene encoding collagen α1(XI), a minor yet critical component of cartilaginous ECM. Moreover, our studies define a *COL11A1*-mediated disease pathway (**Figure 7** [↗](#)) and point to the chondro-osseous junction of IVD and vertebrae spine as a relevant cellular compartment in AIS etiology.

The results of this study together with the previous observation of *COL11A2* rare variant enrichment in AIS supports a role for the collagen α1(XI) heterotrimer itself in its pathogenesis<sup>19</sup> [↗](#). Collagen type XI, composed of three chains encoded by the *COL11A1*, *COL11A2*, and *COL2A1* genes (OMIM #s 120280, 120290, 120140, respectively), is a minor component of collagen type II fibrils that are abundant in cartilage. Collagen type XI is also broadly expressed in testis, trachea, tendons, trabecular bone, skeletal muscle, placenta, lung, brain neuroepithelium, the vitreous of the eye, and intervertebral discs<sup>56</sup> [↗](#). In the pericellular space, collagen α1(XI) initiates fibrillogenesis with collagen type II fibrils, maintaining regular spacing and diameter of the collagen fibrils, while organizing the pericellular surface by interaction with cartilage proteoglycans<sup>57</sup> [↗](#),<sup>58</sup> [↗](#). Purified human collagen type XI, when added back to chondrocytes in *in vitro* culture, stimulates chondrogenesis while inhibiting hypertrophy, as measured by histologic staining, proliferation assays, and relative expression of chondrogenic early marker genes<sup>59</sup> [↗](#). In newborn and one-month old mice, we found that collagen α1(XI) was abundant in IVD and at the chondro-osseous junction of IVD and vertebrae, particularly concentrated in pre-hypertrophic/hypertrophic chondrocytic cells. In long bone growth plates, Long et al.<sup>60</sup> [↗](#) recently identified eight distinct cell clusters after unsupervised analysis of single cell (scRNAseq) of flow-sorted hypertrophic chondrocytes from Col10a1Cre;Rosa26fs-tdTomato mice. At embryonic stage 16.5 (E16.5), *Col11a1* expression was highest in cells with signatures of pre-hypertrophic to hypertrophic transition, and lowest in cells with osteogenic signatures (M. Hilton, personal communication)<sup>60</sup> [↗](#). Taken together, these results suggest that collagen α1(XI) normally participates in maintaining growth plate cells in a hypertrophic, pre-osteogenic state, although little is known about its precise molecular function in that compartment, or in the IVD, during spinal development. Spines of *Col11a1*-deficient mice (*cho/cho*) show incompletely formed vertebral bodies, spinal curvatures, and decreased separation between vertebrae, which are themselves less mineralized than in wildtype mice<sup>36</sup> [↗](#). Notably, common *COL11A1* variants also have been associated with adult lumbar disc herniation (LDH) and degeneration (LDD), as well as DXA-measured bone size, spinal stenosis and spondylolisthesis<sup>61</sup> [↗](#)-<sup>63</sup> [↗](#). Although gain of function



**Figure 6.**

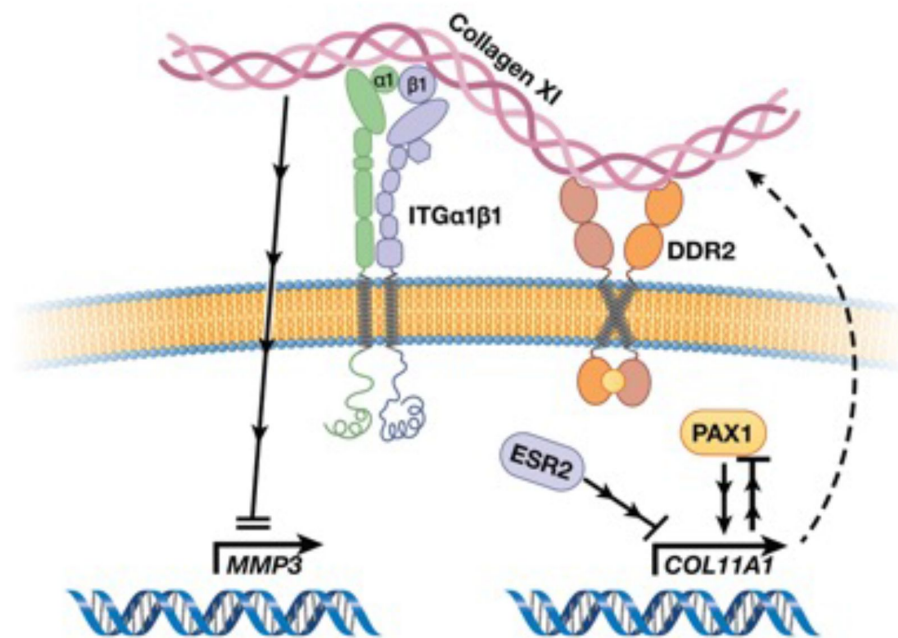
**Effects of estrogen receptor beta on *Col11a1*-*Mmp3* signaling axis.**

a) Representative Western blot (of N=4 biologic replicates) of cultured costal chondrocytes after scramble or *Col11a1*-specific siRNA knockdown. Protein size ladder is shown in lane 1. Quantification of bands detected by Western blotting are shown at right, where scramble results were set to 1.0. Values are mean after normalization to GAPDH,  $\pm$  standard deviation.

b) Gene expression levels of *Col11a1* (left), *Mmp3* (middle), and *Esr2* (right) mRNA in cultured costal chondrocytes showing fold change relative to the scramble control. dKD = double *Col11a1*-*Esr2*-specific siRNA knockdowns. Each value represents the ratio of each gene expression to that of GAPDH, and values are mean  $\pm$  standard deviation. Results are representative of N $\geq$ 3 biologic replicates, each including 3 technical replicates.

c) Gene expression levels from rat CEP cells, as described in (b).





**Figure 7.**

Cartoon depiction of a collagen XI-mediated signaling axis in chondrocytes. Collagen XI is held in the pericellular space by integrins and DDR2. COL11A1, under the regulation of ESR2 and PAX1, signals through unknown mechanisms and inhibits MMP3 transcription.

or dominant negative effects of the rs3753841 variant would not have been revealed in our assays, the spinal deformity noted in the *cho/cho* loss of function model, and failure of missense variants in *Col11a2* to rescue congenital scoliosis, lead us to surmise that reduction in the components of collagen type XI disrupt spinal development.

*Pax1* is a well-described marker of early spine development, where it activates a gene expression cascade starting at E12.5-13.5 in mouse development<sup>45,64,65</sup>. Our data showed that loss of *Pax1* leads to decreased expression of *Col11a1*, *Sox6*, and *Adgrg6* in E12.5 tails of both male and female mice. The downregulation of *Col11a1* is consistent with a prior study of gene expression in flow-sorted GFP-labeled *Pax1*<sup>-/-</sup> embryonic IVD cells<sup>65</sup>. However, from these experiments we cannot discern if *Pax1* directly regulates *Col11a1* in *cis*, or by an indirect effect. It is likely, however, that *Col11a1* expression in developing tail is directly activated by binding SOX transcription factors, as a prior genomic study using chromatin immunoprecipitation and sequencing (ChIP-seq) in rat chondrosarcoma cells identified super enhancers near the *Col11a1* gene that were bound multiple times by SOX9 and SOX6<sup>66</sup>. The SOX5/6/9 trio is known to regulate many of the same genes as PAX1<sup>65</sup>, but whether this includes *Col11a1* is unknown.

In mouse postnatal spines, we observed co-localization of collagen α1(XI) and PAX1 proteins specifically within the cartilaginous endplate-vertebral junction region that includes the vertebral growth plate. The endplate, which is important as the site allowing diffusion of nutrients from the circulation into the avascular inner IVD, harbors subpopulations of cells expressing type II collagen presumably organized by collagen type XI<sup>42,44</sup>. While the endplate is continuous with the vertebral growth plate in mice, it is important to note that in humans the endplate and vertebrae become distinctly separate structures with closure of the growth plates at puberty<sup>42</sup>. This is also the site of the ring apophyses that form the insertion of the IVD into vertebrae<sup>67</sup>. Lagging maturity of the ring apophysis, combined with mechanical forces across the IVD in the horizontal plane, has been proposed as an initiating factor leading to rotatory decompensation in the adolescent spine in AIS<sup>67,68</sup>. Recently, Sun et al. reported the discovery of a vertebral skeletal stem cell (vSSC) residing in the endplate and marked by expression of the genes *Zic1* and *Pax1*, along with other cell surface markers<sup>69</sup>. These vSSCs also express high levels of *Col11a1* (M. Greenblatt, personal communication). It is interesting to consider that AIS-associated variation in collagen α1(XI), perhaps together with mechanical forces, could alter the differentiation trajectory of this cell niche. Altogether, extant data and our results strongly suggest that cell populations at the IVD-vertebral junction region are relevant in AIS pathogenesis. Further investigation is warranted to understand the developmental programmes of cells in this region of the spine.

Matrix metalloproteinase 3, also known as stromelysin, is a secreted enzyme expressed in connective tissues and in regions of endochondral ossification<sup>70</sup>. MMP3 has degradative activity toward a variety of ECM components, including proteoglycans, fibronectin, laminin, but notably not type I collagen<sup>71</sup>. Additionally, in chondrocytes MMP3 also has been shown to translocate to the nucleus, where it activates transcription of connective tissue growth factor (*CTGF/CCN2*) by binding to an element known as transcription enhancer dominant in chondrocytes (TRENDIC)<sup>72,73</sup>. Our observations of a *Col11a1-Mmp3* signaling axis in chondrocytes and CEP cells raise the possibility that *Col11a1* variation may have consequences for both MMP3 enzymatic activity levels and for MMP3-mediated transcriptional programming in these cells. *COL11A1* missense variants, usually altering glycine or proline in Gly-X-Y repeats in the collagen α1(XI) helical domain as with *COL11A1*<sup>P1335L</sup>, are reported to be frequent in cutaneous squamous cell carcinomas and have been linked to transcriptional changes and tumor invasiveness<sup>53</sup>. The mechanisms by which chondrocytes or other cells sense such single amino acid changes in collagen α1(XI) and induce subsequent transcriptional responses are unknown but may involve direct interaction with integrins in the pericellular space<sup>53</sup>.

We found that *Col11a1* expression is sensitive to estrogen receptor blockade or knockdown in chondrocytes. Type XI collagen is also a key player in organizing the pericellular space, which is critical for transmitting mechanical forces from the ECM to the cell<sup>74</sup>. Thus, it is interesting to consider that type XI collagen may effectively act as a receptor for environmental cues, i.e., mechanical forces and estrogen signaling, in the adolescent spine. Our study provides new insights into the regulation and signaling role of *Col11a1* in chondrocytes, and it suggests potential mechanisms by which its genetic variation contributes to AIS susceptibility.

## Methods

### Discovery study

The cases in the discovery stage (USA TX: n=1,358) were recruited at Scottish Rite for Children as approved by the Institutional Review Board of the University Texas Southwestern Medical Center as previously described<sup>75</sup>. Subjects were genotyped on the Illumina HumanCoreExome BeadChip (Illumina, San Diego, CA, USA). For controls, we utilized 12,507 non-AMD GRU (non-age related macular degeneration general research use) subjects of the European ancestry downloaded from dbGaP web site (<https://www.ncbi.nlm.nih.gov/gap/>) from the International Age-Related Macular Degeneration Genomics Consortium study (IAMDGC: phs001039.v1.p1.c1; [https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study\\_id=phs001039.v1.p1](https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001039.v1.p1)). The subjects from the IAMDGC study were also genotyped on the Illumina HumanCoreExome Beadchip-24v1.0 platform<sup>76</sup>. We merged cases and controls and applied quality controls to the genotypes for 468,801 overlapping SNPs using PLINK.1.9<sup>77</sup> as described in<sup>17</sup>. In summary, samples with sex inconsistencies or from duplicated or related individuals or ancestral outliers as identified by principal component analysis (PCA) were removed, leaving 13,865 samples in the analysis. Genotypes were corrected for strand direction, and SNPs with call-rate per marker <95%, deviating from Hardy-Weinberg equilibrium (HWE) (cutoff P-value =  $10^{-4}$ ), or with significant missingness rate between cases and controls (cutoff P-value =  $10^{-4}$ ) were removed, leaving 341,759 SNPs in the analysis. Genotypes for SNPs across autosomal chromosomes were imputed using Minimac3 with the 1000G-Phase3.V5 reference panel as described in the instructions available from the software website<sup>78</sup>. Protein-coding changes were annotated with ANNOVAR using RefSeq-based transcripts<sup>79</sup>. External databases included allele frequencies from gnomAD<sup>80</sup>, variant pathogenicity in Clinvar<sup>81</sup>, Combined Annotation Dependent Depletion (CADD) scores<sup>82</sup>, Genomic Evolutionary Rate Profiling (GERP) scores<sup>83</sup> and protein domains in IntroPro<sup>84</sup>. Only bi-allelic common (MAF>0.01) protein-altering SNPs with imputation quality Rsq >=0.3 within matrisome genes<sup>34</sup> were included for further analysis. Matrisome genes used can be found in the Molecular Signature Database (MsigDB)<sup>85,86</sup> ([https://www.gsea-msigdb.org/gsea/msigdb/cards/NABA\\_MATRISOME](https://www.gsea-msigdb.org/gsea/msigdb/cards/NABA_MATRISOME)). Genetic association for the imputed allele dosages in the discovery cohort (USA TX) was performed in Mach2dat<sup>87</sup> using logistic regression with gender and ten principal components as covariates. The genomic regions of the associated loci were visualized with LocusZoom software<sup>88</sup> utilizing linkage disequilibrium (LD) information from 1000 Genomes EUR populations.

### Meta-analysis study

For the meta-analysis stage we utilized four cohorts [USA MO: n=2,951(1,213 cases and 1,738 controls), Swedish-Danish populations [SW-D: n= 4,627 (1,631 cases and 2,996 controls), as described in<sup>17,89</sup>], Japan [JP: n=79,211(5,327 cases and 73,884 controls)] and Hong Kong [HK: n=3,103 (990 cases and 2,113 controls)] to check significant candidates from the discovery study. Summary statistics across the discovery study and the 4 replication cohorts [total N=103,757 (10,519 cases and 93,238 controls)], were combined as previously described<sup>17</sup> using METAL<sup>90</sup>.

## Usa mo

cohort: Whole exome sequencing (WES) data from 1,213 unrelated idiopathic scoliosis cases of European ancestry with spinal curvature greater than 10° Cobb angle were derived from the Adolescent Idiopathic Scoliosis 1000 Exomes Study (dbGAP accession number: phs001677), and recruited from St. Louis Children's Hospital, and St. Louis Shriners Hospital for Children. Patients and/or parents provided study consent and IRB approval was obtained from each contributing institution. For controls, exome data from 1,738 unrelated samples of European ancestry were provided by investigators at Washington University School of Medicine in St. Louis, MO (dbGAP accession numbers: phs000572.v8.p4 and phs000101.v5.p1), and Oregon Health & Science University in Portland, OR (<https://gemini.conradlab.org/>). Exome data were aligned to the human genome reference (GRCh37) using BWA-MEM (v0.7.15). Variant calling of single nucleotide variants (SNVs) and insertion and deletion variants (INDELs) were generated first for each single sample in cases and controls and then combining all samples with joint genotyping method, described in GATK Best-Practices (Genome Analysis Toolkit (GATK v3.5) <https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-Workflows>). All cases and controls samples were classified as unrelated and of European ancestry using relationship inference<sup>91</sup> and principal component analysis<sup>77</sup>. Association analysis of variants rs3753841 and rs1042704 were performed using logistic regression adjusting for sex and PCs in PLINK<sup>77</sup>.

## JP cohort

Informed consents were obtained from all the subjects or their parents and the ethics committees of RIKEN and participating institutions approved this study. 5,327 case subjects were recruited from collaborating hospitals (Japanese Scoliosis Clinical Research Group) as previously described<sup>18</sup>. For controls, 73,884 subjects were randomly selected from the BioBank Japan Project, and subjects were genotyped on Illumina Human BeadChips as previously described<sup>14,18</sup>. Imputation and association analyses in JP were performed as previously described<sup>92</sup>.

## HK cohort

3,103 subjects were recruited at The Duchess of Kent Children's Hospital as approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB approval number: UW 08-158). All 990 cases were characterized by Cobb angles greater than 40 degrees with onset age between 10 and 18 years old. Congenital, neuromuscular and syndromic scoliosis samples were excluded. We used 2,113 controls from the Chinese population with no spinal deformities on MRI scans<sup>93</sup>. Cases and controls were genotyped using the Illumina Infinium OmniZhongHua-8 BeadChip and analyzed with GenomeStudio 2.0 software. The quality control approach adopted the GWA tutorial developed by Andries et al.<sup>94</sup>. The filtered genotyping data of cases and controls was phased and imputed using SHAPEIT<sup>95</sup> and IMPUTE2<sup>96</sup>, respectively. Logistic model association analysis was performed using PLINK 1.9<sup>77</sup>.

## Stratification-by-sex test

To investigate sex-specificity in the *COL11A1* and *MMP14* loci, we performed stratification-by-sex analysis in the discovery study (USA\_TX). Association for the imputed allele dosages in rs3753841 and rs37538 were computed separately for females (1,157 cases and 7138 controls) using logistic regression with ten principal components as covariates in Mach2dat<sup>87</sup>.

## RNAseq of human tissues

RNAseq was performed as previously described<sup>97</sup>. Read counting and transcript quantification were performed using HTSeq<sup>98</sup>. Finally, reads were normalized using DESeq2 tools<sup>99</sup> and TPM values were generated using the Kalisto pipeline<sup>100</sup>.

## Animal studies

All mouse and rat work was conducted per IACUC approved protocols and in accordance with AALAC and NIH guidelines.

### Generation of Pax1 knockout mice

Mouse work was approved by the UCSF IACUC, protocol number AN181381. Two gRNAs were designed to target the 5' and 3' ends of *Pax1* gene (gRNA sequence shown in **Figure 5** [figure supplement 5a](#)) using the gRNA design tool on the Integrated DNA Technologies (IDT, Newark, NJ, USA) website and selected based on low off-target and high on-target scores. The knockout allele was generated using *i*-GONAD<sup>101</sup> [as previously described](#) <sup>102</sup> [.](#)

To validate proper generation of knockout, mice were analyzed by genotyping with primers shown in Appendix A Key Resources Table, Sanger sequencing of PCR-amplified DNA, and Southern blot (**Figure 5** [figure supplement 5a](#)). For Southern blot analyses, genomic DNA were treated with NcoI (Cat #R0193, New England Biolabs, MA, USA) and fractionated by agarose gel electrophoreses. Following capillary transfer onto nylon membranes, blots were hybridized with Digoxigenin (DIG)-labeled DNA probes (corresponding to chr2:147,202,083-147,202,444; mm9) amplified by the PCR DIG Probe Synthesis Kit (Cat #11636090910, Sigma-Aldrich, MO, USA). The hybridized probe was immunodetected with antidigoxigenin Fab fragments conjugated to alkaline phosphatase (Cat # 11093274910, Sigma-Aldrich, MO, USA) and visualized with a CDP star (Cat #11685627001, Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. Chemiluminescence was detected using the FluorChem E (Cat #92-14860-00, ProteinSimple, CA, USA).

### Col11a1<sup>fl/fl</sup> mice

The *Col11a1*<sup>fl/fl</sup> mouse line was kindly provided by Dr. Lou Soslowsky with permission from Dr. David Birk. All *Col11a1*<sup>fl/fl</sup> and wildtype mice were handled per UTSW IACUC, protocol number 2016-101455.

### Other mice

Cartilage was harvested from C57B46 wild type mice for siRNA-mediated knockdowns experiments.

## Histologic methods

For thin cryostat sections, P0.5 mouse whole body was fixed in 4% paraformaldehyde for 6 hours followed by 10% sucrose for 12 hours, then transferred to 18% sucrose for 24 hours. Tissues were then embedded in optimal cutting temperature compound (OCT) and sectioned using low-profile blades on a Thermo Shandon Cryostar NX70 cryostat and all sections were lifted on APES clean microscope slides. For whole mount images, samples were treated similarly with the addition of 2% polyvinylpyrrolidone (PVP) during the cryoprotection step and frozen in 8% gelatin (porcine) in the presence of 20% sucrose and 2% PVP. Samples were sectioned at a thickness of 10 μm. Slides were stored at -80° C until ready for use. For P28 and older mice, spines were removed then fixed, decalcified, and embedded in OCT. Spines were processed by making 7 μm thick lateral cuts the length of the spine.

Collagen α1(XI) was detected by immunohistochemistry staining using affinity-purified antisera against peptide (C) YGTMEPYQTETPRR-amide (Genescript, NJ, USA) as described<sup>41</sup> [.](#) and secondary horseradish peroxidase (HRP) conjugated affinity purified secondary antibody (Cat # AP187P, Millipore-Sigma Aldrich, MO, USA). Briefly, frozen sections were equilibrated to room temperature for 1 hour, then fixed with 4% paraformaldehyde in PBS at 4 degrees Celsius for 20 minutes. Slides were washed, treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 minutes to block

endogenous peroxidase, washed, and transferred to PBS with 0.05% TWEEN 20 (Cat #P3563-10PAK, Sigma-Aldrich, MO, USA) pH 7.4. Slides were blocked with 0.5 % goat serum in PBS mix with 0.2% Triton 100 (Cat #T8787, Sigma-Aldrich, MO, USA) at room temperature for 1.5 hours. The primary collagen  $\alpha 1(XI)$  affinity purified antibody was applied at 0.40 mg/ml and slides were incubated overnight at 4°C. Afterward slides were washed in PBS Tween 20 for three times and treated with goat anti-rabbit-HRP for 1.5 hours, then washed three times in PBS Tween 20. After applying 3,3'-diaminobenzidine (DAB) solution, slides were washed and counterstained with Mayer's hematoxylin (Cat #MHS80, Sigma-Aldrich, MO, USA), washed, dehydrated, and mounted.

For collagen  $\alpha 1(XI)$  and PAX1 IF studies, P0.5 mice, P28 spine and ribs sections were fixed in 4% paraformaldehyde (PFA) for 20Lminutes then washed with PBS + 0.1% Triton three times, before incubation with 10% normal goat serum in PBS + 0.1% Triton for 30Lmin to block the background. Slides were incubated with goat anti-mouse collagen  $\alpha 1(XI)$  antibody at 1:500 dilution and mouse anti-rat PAX1(Cat #MABE1115M, Sigma-Aldrich, MO, USA), in PBS + 0.1% Triton + 1% normal goat serum at 4L°C overnight. Secondary antibodies used were 1:5000 anti-rat Alexa488 and anti-mouse Alexa594 conjugated antibodies (Cat #A32740 Invitrogen, CA, USA). The sections were mounted using ProLong Gold with DAPI (Cat #S36964 Invitrogen, CA, USA) for imaging as described<sup>103</sup>. All images were taken with Carl Zeiss Axio Imager.M2 fluorescence microscope (Zeiss, Oberkochen, DE).

## Rib cartilage and IVD cell culture

Mouse costal chondrocytes were isolated from the rib cage and sternum of P0.5 mice. Rat IVD was removed intact from 1-month female rats and immediately separated into NP, AF, and CEP isolates. Subsequently, tissues were incubated and shaken with 2 mg/ml Pronase solution (Cat #10165921001 Sigma-Aldrich, Inc., St. Louis, MO, USA) for 1 hour, followed by 1.5 hours digestion with 3 mg/ml Collagenase D solution. (Cat #11088882001 Sigma-Aldrich, Inc., St. Louis, MO, USA), then 5 hours digestion with 0.5 mg/ml Collagenase D solution before 3 times PBS wash. Filtered, dissociated cells were seeded in Dulbecco's modified Eagle's medium (DMEM; Cat #MT15017CV Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS), 100 $\mu$ g/ml streptomycin and 100IU/ml penicillin. Remaining cartilage tissues underwent further digestion in 86LU/ml type 1 collagenase (Cat # SCR103 Sigma-Aldrich, Inc., St. Louis, MO, USA) overnight. Cells were collected and cultured in DMEM with 10% FBS plus 100 $\mu$ g/ml streptomycin and 100IU/ml penicillin.

## SV40 immortalization and transfection of primary chondrocytes

*Col11a1<sup>fl/fl</sup>* mouse costal chondrocytes were isolated from the rib cage and sternum of P0.5 mice. The cells were transduced with pRRLsin-sv40 T antigen-IRES-mCherry lenti-virus<sup>104</sup> for 48 hours, then sorted for mCherry-positive cells by flow cytometry. mCherry-positive cells were then infected with plv-eGFP, plv-eGFP-COL11A1-HA, plveGFP-COL11A1<sup>P1335L</sup>-HA constructs. After expansion, GFP-positive cells were sorted by flow cytometry and seeded in 24-well plates.

## Adenovirus treatment

SV40 induced *Col11a1<sup>fl/fl</sup>* mouse costal chondrocytes were grown to 50% confluency. Afterward, cells were treated with 2ul Ad5-CMV-cre adenovirus (titer  $1.8 \times 10^{11}$  pfu/ml) and Ad5-CMV-eGFP (titer  $1.65 \times 10^{10}$  pfu/ml) as control. Both virus strains were from the Gene Vector Core facility, Baylor College of Medicine. After 48-hours the cells were harvested for mRNA and protein lysate.

## RNA-seq and qRT-PCR

For *Pax1* knockout studies, total RNA was collected from E12.5 tails using TRIzol (Cat #15596026, Thermo Fisher Scientific, MA, USA) and converted to cDNA using ReverTra Ace qPCR-RT master mix with genomic DNA (gDNA) remover (Cat #FSQ-301, Toyobo, Osaka, Japan). Sequencing was done using an Illumina Novaseq platform and the data were analyzed using Partek Flow (Version



10.0) and Gene ontology<sup>105</sup>, qPCR was performed using SsoFast EvaGreen supermix (Cat #1725205, Bio-Rad, CA, USA). Primer sequences used for qPCR are shown in Appendix A, Key Resources table.

To quantify the expression level of *Col11a1*, *Mmp3*, and marker genes in IVD compartments and rib cartilage, cultured cells were collected in RNeasy (Qiagen, Inc.) for RNA purification. Taqman Reverse Transcription Kit (Cat #4387406 Thermo Fisher Scientific, MA, USA) was used to reverse transcribe mRNA into cDNA. Following this, RTLqPCR was performed using a Power SYBR Green PCR Master Mix Kit (Cat #1725271, Bio-Rad, CA, USA). The primer sequences for the genes used in this study are listed in the Appendix A Key Resources Table. Gene expression was calculated using the  $\Delta\Delta CT$  method after normalizing to GAPDH.

## siRNA knockdown

Mouse rib cartilage cells seeded in 6 well-plates were 60-80% confluent at transfection. Lipofectamine RNAiMAX reagent (Cat #13778030 Thermo Fisher, Waltham, MA, USA) was diluted (9ul in 500ul) in Opti-MEM Medium (Cat # 31985070 Thermo Fisher MA, USA). 30 pmol siRNA was diluted in 500ul Opti-MEM medium, then added to diluted Lipofectamine RNAiMAX Reagent. siRNA-lipid complex was added to cells after 5-minute incubation at room temperature. Cells were harvested after 72 hours.

## Western blotting

For MMP3 western blotting, a total of 30  $\mu$ g protein mixed with SDS-PAGE buffer was loaded on 12% SDS-polyacrylamide gel for electrophoresis. For collagen  $\alpha 1(XI)$  western blotting, 50  $\mu$ g protein mixed with SDS-PAGE buffer was loaded on 4-20% SDS-polyacrylamide gel. The separated proteins were then transferred to nitrocellulose membranes (Cat #77010 Thermo Fisher Waltham, MA, USA) at 100 volts for 2-3 hours. The membrane was first incubated with blocking buffer containing 5% defatted milk powder, and then exposed to 0.1 mg/mL mouse anti-rabbit Mmp3 (Cat #ab214794 Abcam, Cambridge, MA, USA) or anti-rabbit Col11a1 (Cat #PA5-38888 Thermo Fisher Waltham, MA, USA) overnight. The samples were then washed thoroughly with TBS buffer, followed by incubation with HRP-labeled antirabbit IgG secondary antibodies 1:5000, (Cat #32460 Thermo Fisher Waltham, MA, USA) overnight. The membranes were then washed with TBS buffer. GAPDH was detected by a rabbit anti-mouse antibody (Cat #14C10 Cell Signaling, MA, USA) and used as the internal loading control.

## Data availability

Summary data for GWAS3 is deposited in the NHGRI-EBI GWAS catalog (accession #GCST90179478). Otherwise, data generated or analyzed in this study are included in the manuscript.

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## Author contributions

H.Y., A.M.K., A.U., X-Y. L, N.O, Y.K., E.E., Y.F., L.A., Y.H.K., R.C., R.S, Y.Z., J.P. performed genetic, bioinformatic, or proteomic analyses or molecular biology experiments. N.V.G., B.M.E., J.P.Y.C., and J.A.H. provided technical or clinical advice. C.T., Y.-Q.S., C.A.G., P.G., and S.I. supervised genetic experiments and provided summary data. J.J.R., N.A., and C.A.W. supervised the work. C.A.W. conceived the study and wrote the manuscript. All co-authors reviewed and approved the final manuscript.

## Competing interests

The authors declare no competing interests.

## Appendix A. Key Resources Table

### siRNA and Primer Sequences

|                                 |                          |
|---------------------------------|--------------------------|
| Mouse Esr2 siRNA                | CAAGUGUUACGAAGUAGGAdT    |
| Mouse Col11a1 siRNA             | GAAAGAAGGUGCAAAGGGUdT    |
| Mouse Mmp3 F                    | CTCTGGAACCTGAGACATCACC   |
| Mouse Mmp3 R                    | AGGAGTCCTGAGAGATTTGCGC   |
| Mouse Col11a1 F                 | AGGAGAGTTGAGAATTGGGAATC  |
| Mouse Col11a1 R                 | TGGTGATCAGAATCAGAAATT    |
| Mouse Col11a2 F                 | CTCATCTTCCTGCATCAGAC     |
| Mouse Col11a2 R                 | ACTTGGAAGCGAGGTCCT       |
| Mouse Adgrg6 F                  | AGAGGATGGACTGAGGCTGTGT   |
| Mouse Adgrg6 R                  | CCAGGCTTGTTTGGACATGGTTG  |
| Mouse Sox6 F                    | GCATAAGTGACCGTTTTGGCAGG  |
| Mouse Sox6 R                    | GGCATCTTTGCTCCAGGTGACA   |
| Mouse Mmp14 F                   | GCCTTCTGTTCTCCTGATAA     |
| Mouse Mmp14 R                   | CCATCCTTCCTCTCGTAG       |
| Mouse Pax1 F                    | AACCAGCACGGAGTATACAGC    |
| Mouse Pax1 R                    | TGTAAGCTACCGAGTGCATCC    |
| Mouse Esr2 F                    | GGTCCTGTGAAGGATGTAAGGC   |
| Mouse Esr2 R                    | TAACACTTGCGAAGTCGGCAGG   |
| Mouse Gapdh F                   | CATCACTGCCACCCAGAAGACTG  |
| Mouse Gapdh R                   | ATGCCAGTGAGCTTCCCGTTTCAG |
| Rat Sfrp2 F                     | CGTGAAACGGTGGCAGAAG      |
| Rat Sfrp2 R                     | CGGATGCTGCGGGAGAT        |
| Rat Krt19 F                     | AAGACACACTGGCAGAAACG     |
| Rat Krt19 R                     | GATTCTGCCGCTCACTATCA     |
| Rat Mmp12 F                     | TTGGCCATTCTTGGGGCTGC     |
| Rat Mmp12 R                     | TGTTGGTGGCTGGACTCCCAGG   |
| Mouse Pax1 F (Fig. 5)           | CCGCACATTCAGTCAGCAAC     |
| Mouse Pax1 R (Fig. 5)           | CATCTTGGGGGAGTAGGCAG     |
| Mouse Col11a1 F (Fig. 5)        | CACAAAACCCCTCGATAGAAGTG  |
| Mouse Col11a1 R (Fig. 5)        | CCTGTGATCAGGAAGTCTGAA    |
| Mouse Adgrg6 F (Fig. 5)         | TCCTGTCCATCTCTGGCTCA     |
| Mouse Adgrg6 R (Fig. 5)         | CACAAGACAGAGCTGCTCCA     |
| Mouse Sox6 F (Fig. 5)           | TGCGACAGTTCTTCACTGTGG    |
| Mouse Sox6 R (Fig. 5)           | CGTCCATCTTCATACCATACG    |
| Mouse $\beta$ -Actin F (Fig. 5) | GGCACCACACCTTCTACAATG    |
| Mouse $\beta$ -Actin R (Fig. 5) | GGGGTGTTGAAGGTCTCAAAC    |
| Pax1-genotyping F               | CAGAACCTGGAATGCTGTGCTC   |
| Pax1-genotyping R               | AAAGGGTTGCAGTGCCTTCAC    |

## Appendix B. Clinical groups

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**Editors**

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**Sofia Araújo**

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**Reviewer #1 (Public Review):**

Summary:

This revised study follows up on previous work showing a female-specific enhancer region of PAX1 is associated with adolescent idiopathic scoliosis (AIS). This new analysis combines human GWAS analysis from multiple countries to identify a new AIS-associated coding variant in the COL11A1 gene (COL11A1P1335L). Using a Pax1 knockout mouse they go on to find that PAX1 and Collagen XI protein are expressed in the intervertebral discs (IVDs) and robustly in the growth plate, showing that COL11A1 expression is reduced in Pax1 mutant growth plate. Moreover, other AIS-associated genes, Gpr126 and Sox6, were also reduced in Pax1 mutant mice, suggesting a common pathway is involved in AIS.

Using SV40 immortalized costal cartilage cells, derived from floxed Col11a1 mice primary rib cage cartilage, they go to show that removal of Col11a1 leads to reduction of Mmp3 expression. In this context, the expression of wild-type Col11a1 restored regular levels of Mmp3 expression, while expression of the AIS-associated Col11a1P1335L allele failed to restore normal Mmp3 expression. This supports a model that the AIS-associated Col11a1P1335L allele leads to the dysregulation of ECM in vivo.

Using this culture system, they go on to test the role of the estrogen receptor ESR2, showing that loss of this receptor leads to reduced Mmp3 and Pax1 expression, and increased Col11a1

expression. They support this by showing similar gene expression changes and estrogen receptor function in Rat cartilage endplate cell culture.

Altogether, this study nicely brings together an impressive number of human genetic data from multi-ethnic AIS cohorts and controls from across the globe and functionally tests these findings in cell culture and animal models. This study wonderfully integrates other findings from other human and mouse work in AIS and supports a new molecular mechanism by which estrogen can interact and synergize with COL11A1/PAX1/MMP3 signaling to change ECM development and dynamics, thus providing a tangible model for mutations and dysregulation of this pathway can increase the susceptibility of scoliosis.

**Strengths:**

This work integrates a large cohort of human genetic data from AIS patient and control from diverse ethnic backgrounds, across the globe. This work attempts to functionally test their findings in vivo and by use of cell culture.

**Weaknesses:**

Many of the main functional work was done in cell culture and not in vivo.

- <https://doi.org/10.7554/eLife.89762.3.sa2>

**Reviewer #2 (Public Review):**

**Summary:**

In this manuscript, Yu and colleagues sought to identify new susceptibility genes for adolescent idiopathic scoliosis (AIS). The significance of this work is high, especially given the still large knowledge gap of the mechanistic underpinnings for AIS. In this multidisciplinary body of work, the authors first performed a genetic association study of AIS case-control cohorts (combined 9,161 cases and 80,731 controls) which leveraged common SNPs in 1027 previously defined matrisome genes. Two nonsynonymous variants were found to be significantly associated with AIS: MMP14 p.Asp273Asn and COL11A1 p.Pro1153Leu, the latter of which had the more robust association and remained significant when females were tested independent of males. Next, the authors followed a series of functional validation experiments to support biological involvement of COL11A1 p.Pro1153Leu in AIS through expression, biochemical, and histological studies in physiologically relevant cell and mouse models. Together, the authors propose a hitherto unreported model for AIS that involves the interplay of the COL11A1 susceptibility locus with estrogen signaling to alter a Pax1-Col11a1-Mmp3 signaling axis at the growth plate.

**Strengths:**

The manuscript is clearly written and follows a series of logical steps toward connecting multiple matrisome genes and putative AIS effectors in a new framework of pathomechanism. The multidisciplinary nature of the work makes it a strong body of work wherein multiple models offer multiple lines of supportive data. Thus, this manuscript remains an important multidisciplinary study of the genetic and functional basis of adolescent idiopathic scoliosis (AIS). To the benefit of the overall manuscript quality, the reviewers have addressed most concerns to satisfaction. Please include the list of three rare missense variants mentioned in the response to reviewers as a supplementary table. Please also include methods for the SKATO rare variant burden analysis.

- <https://doi.org/10.7554/eLife.89762.3.sa1>

**Reviewer #3 (Public Review):****Summary:**

This article demonstrates a Pax1-Col11a1-Mmp3 signaling axis associated with adolescent idiopathic scoliosis and finds that estrogen affects this signaling axis. In addition, the authors have identified a new COL11A1 mutation and verified its effect on the Pax1-Col11a1-Mmp3 axis.

**Strengths:**

1. Col11a1P1335L is verified in multicenter cohorts with high confidence.
2. The article identified a potential pathogenesis of AIS.

**Weaknesses:**

The SV40-immortalized cell line established from Col11a1fl/fl mouse rib cartilage was applied in the study, and overexpression system was used to confirm that P1335L variant in COL11A1 perturbs its regulation of MMP3. However, due to the absence of P1335L point mutant mice, it cannot be confirmed whether P1335L can actually cause AIS, and the pathogenicity of this mutation cannot be directly verified.

- <https://doi.org/10.7554/eLife.89762.3.sa0>

**Author Response**

The following is the authors' response to the previous reviews.

We thank the reviewers for truly valuable advice and comments. We have made multiple corrections and revisions to the original pre-print accordingly per the following comments:

1. *Pro1153Leu is extremely common in the general population (allele frequency in gnomAD is 0.5). Further discussion is warranted to justify the possibility that this variant contributes to a phenotype documented in 1.5-3% of the population. Is it possible that this variant is tagging other rare SNPs in the COL11A1 locus, and could any of the existing exome sequencing data be mined for rare nonsynonymous variants?*

*One possible avenue for future work is to return to any existing exome sequencing data to query for rare variants at the COL11A1 locus. This should be possible for the USA MO case-control cohort. Any rare nonsynonymous variants identified should then be subjected to mutational burden testing, ideally after functional testing to diminish any noise introduced by rare benign variants in both cases and controls. If there is a significant association of rare variation in AIS cases, then they should consider returning to the other cohorts for targeted COL11A1 gene sequencing or whole exome sequencing (whichever approach is easier/less expensive) to demonstrate replication of the association.*

Response: Regarding the genetic association of the common COL11A1 variant rs3753841 (p. (P1335Leu)), we do not propose that it is the sole risk variant contributing to the association signal we detected and have clarified this in the manuscript. We concluded that it was worthy of functional testing for reasons described here. Although there were several common variants in the discovery GWAS within and around COL11A1, none were significantly associated with AIS and none were in linkage disequilibrium ( $R^2 > 0.6$ ) with the top SNP rs3753841. We next reviewed rare ( $MAF \leq 0.01$ ) coding variants within the COL11A1 LD region of the associated SNP (rs3753841) in 625 available exomes representing 46% of the

1,358 cases from the discovery cohort. The LD block was defined using Haploview based on the 1KG\_CEU population. Within the ~41 KB LD region (chr1:103365089- 103406616, GRCh37) we found three rare missense mutations in 6 unrelated individuals, Table below. Two of them (NM\_080629.2: c.G4093A:p.A1365T; NM\_080629.2:c.G3394A:p.G1132S), from two individuals, are predicted to be deleterious based on CADD and GERP scores and are plausible AIS risk candidates. At this rate we could expect to find only 4-5 individuals with linked rare coding variants in the total cohort of 1,358 which collectively are unlikely to explain the overall association signal we detected. Of course, there also could be deep intronic variants contributing to the association that we would not detect by our methods. However, given this scenario, the relatively high predicted deleteriousness of rs3753841 (CADD= 25.7; GERP=5.75), and its occurrence in a GlyX-Y triplet repeat, we hypothesized that this variant itself could be a risk allele worthy of further investigation.

#### Author response table 1.



We also appreciate the reviewer's suggestion to perform a rare variant burden analysis of COL11A1. We did conduct pilot gene-based analysis in 4534 European ancestry exomes including 797 of our own AIS cases and 3737 controls and tested the burden of rare variants in COL11A1. SKATO P value was not significant (COL11A1\_P=0.18), but this could be due to lack of power and/or background from rare benign variants that could be screened out using the functional testing we have developed.

1. COL11A1 p.Pro1335Leu is pursued as a direct candidate susceptibility locus, but the functional validation involves both: (a) a complementation assay in mouse GPCs, Figure 5; and (b) cultured rib cartilage cells from Col11a1-Ad5 Cre mice (Figure 4). Please address the following:

2A. Is Pro1335Leu a loss of function, gain of function, or dominant negative variant? Further rationale for modeling this change in a Col11a1 loss of function cell line would be helpful.

Response: Regarding functional testing, by knockdown/knockout cell culture experiments, we showed for the first time that Col11a1 negatively regulates Mmp3 expression in cartilage chondrocytes, an AIS-relevant tissue. We then tested the effect of overexpressing the human wt or variant COL11A1 by lentiviral transduction in SV40-transformed chondrocyte cultures. We deleted endogenous mouse Col11a1 by Cre recombination to remove the background of its strong suppressive effects on Mmp3 expression. We acknowledge that Col11a1 missense mutations could confer gain of function or dominant negative effects that would not be revealed in this assay. However as indicated in our original manuscript we have noted that spinal deformity is described in the cho/cho mouse, a Col11a1 loss of function mutant. We also note the recent publication by Rebello et al. showing that missense mutations in Col11a2 associated with congenital scoliosis fail to rescue a vertebral malformation phenotype in a zebrafish col11a2 KO line. Although the connection between AIS and vertebral malformations is not altogether clear, we surmise that loss of the components of collagen type XI disrupt spinal development. in vivo experiments in vertebrate model systems are needed to fully establish the consequences and genetic mechanisms by which COL11A1 variants contribute to an AIS phenotype.

2B. Expression appears to be augmented compared WT in Fig 5B, but there is no direct comparison of WT with variant.



Response: Expression of the mutant (from the lentiviral expression vector) is increased compared to mutant. We observed this effect in repeated experiments. Sequencing confirmed that the mutant and wildtype constructs differed only at the position of the rs3753841 SNP. At this time, we cannot explain the difference in expression levels. Nonetheless, even when the variant COL11A1 is relatively overexpressed it fails to suppress MMP3 expression as observed for the wildtype form.

*2C. How do the authors know that their complementation data in Figure 5 are specific? Repetition of this experiment with an alternative common nonsynonymous variant in COL11A1 (such as rs1676486) would be helpful as a comparison with the expectation that it would be similar to WT.*

Response: We agree that testing an allelic series throughout COL11A1 could be informative, but we have shifted our resources toward in vivo experiments that we believe will ultimately be more informative for deciphering the mechanistic role of COL11A1 in MMP3 regulation and spine deformity.

*2D. The y-axes of histograms in panel A need attention and clarification. What is meant by power? Do you mean fold change?*

Response: Power is directly comparable to fold change but allows comparison of absolute expression levels between different genes.

*2E. Figure 5: how many technical and biological replicates? Confirm that these are stated throughout the figures.*

Response: Thank you for pointing out this oversight. This information has been added throughout.

*1. Figure 2: What does the gross anatomy of the IVD look like? Could the authors address this by showing an H&E of an adjacent section of the Fig. 2 A panels?*

Response: Panel 2 shows H&E staining. Perhaps the reviewer is referring to the WT and Pax1 KO images in Figure 3? We have now added H&E staining of WT and Pax1 KO IVD as supplemental Figure 3E to clarify the IVD anatomy.

*1. Page 9: "Cells within the IVD were negative for Pax1 staining ..." There seems to be specific PAX1 expression in many cells within the IVD, which is concerning if this is indeed a supposed null allele of Pax1. This data seems to support that the allele is not null.*

Response: We have now added updated images for the COL11A1 and PAX1 staining to include negative controls in which we omitted primary antibodies. As can be seen, there is faint autofluorescence in the PAX1 negative control that appears to explain the "specific staining" referred to by the reviewer. These images confirm that the allele is truly a null.

*1. There is currently a lack of evidence supporting the claim that "Col11a1 is positively regulated by Pax1 in mouse spine and tail". Therefore, it is necessary to conduct further research to determine the direct regulatory role of Pax1 on Col11a1.*

Response: We agree with the reviewer and have clarified that Pax1 may have either a direct or indirect role in Col11a1 regulation.

1. There is no data linking loss of COL11A1 function and spine defects in the mouse model. Furthermore, due to the absence of P1335L point mutant mice, it cannot be confirmed whether P1335L can actually cause AIS, and the pathogenicity of this mutation cannot be directly verified. These limitations need to be clearly stated and discussed. A Col11a1 mouse mutant called chondroplasia (cho), was shown to be perinatal lethal with severe endochondral defects (<https://pubmed.ncbi.nlm.nih.gov/4100752/>). This information may help contextualize this study.

Response: We partially agree with the reviewer. Spine defects are reported in the cho mouse (for example, please see reference 36 Hafez et al). We appreciate the suggestion to cite the original Seegmiller et al 1971 reference and have added it to the manuscript.

1. A recent article (PMID37462524) reported mutations in COL11A2 associated with AIS and functionally tested in zebrafish. That study should be cited and discussed as it is directly relevant for this manuscript.

Response: We agree with the reviewer that this study provides important information supporting loss of function I type XI collagen in spinal deformity. Language to this effect has been added to the manuscript and this study is now cited in the paper.

1. Please reconcile the following result on page 10 of the results: "Interestingly, the AIS-associated gene Adgrg6 was amongst the most significantly dysregulated genes in the RNA-seq analysis (Figure 3c). By qRT-PCR analysis, expression of Col11a1, Adgrg6, and Sox6 were significantly reduced in female and male Pax1<sup>-/-</sup> mice compared to wild-type mice (Figure 3d-g)." In Figure 3f, the downregulation of Adgrg6 appears to be modest so how can it possibly be highlighted as one of the most significantly downregulated transcripts in the RNAseq data?

Response: By "significant" we were referring to the P-value significance in RNAseq analysis, not in absolute change in expression. This language was clearly confusing, and we have removed it from the manuscript.

1. It is incorrect to refer to the primary cell culture work as growth plate chondrocytes (GPCs), instead, these are primary costal chondrocyte cultures. These primary cultures have a mixture of chondrocytes at differing levels of differentiation, which may change differentiation status during the culturing on plastic. In sum, these cells are at best chondrocytes, and not specifically growth plate chondrocytes. This needs to be corrected in the abstract and throughout the manuscript. Moreover, on page 11 these cells are referred to as costal cartilage, which is confusing to the reader.

Response: Thank you for pointing out these inconsistencies. We have changed the manuscript to say "costal chondrocytes" throughout.

#### Minor points

- On 10 of the Results: "These data support a mechanistic link between Pax1 and Col11a1, and the AIS-associated genes Gpr126 and Sox6, in affected tissue of the developing tail." qRT-PCR validation of Sox6, although significant, appears to be very modestly downregulated in KO. Please soften this statement in the text.

Response: We have softened this statement.

- *Have you got any information about how the immortalized (SV40) costal cartilage affected chondrogenic differentiation? The expression of SV40 seemed to stimulate Mmp13 expression. Do these cells still make cartilage nodules? Some feedback on this process and how it affects the nature of the culture what be appreciated.*

Response: The “+ or –” in Figure 5 refers to Ad5-cre. Each experiment was performed in SV40-immortalized costal chondrocytes. We have removed SV40 from the figure and have clarified the legend to say “qRT-PCR of human COL11A1 and endogenous mouse Mmp3 in SV40 immortalized mouse costal chondrocytes transduced with the lentiviral vector only (lanes 1,2), human WT COL11A1 (lane 3), or COL11A1P1335L. Otherwise we absolutely agree that understanding Mmp13 regulation during chondrocyte differentiation is important. We plan to study this using in vivo systems.

- *Figure 1: is the average Odds ratio, can this be stated in the figure legend?*

Response: We are not sure what is being asked here. The “combined odds ratio” is calculated as a weighted average of the log of the odds.

- *A more consistent use of established nomenclature for mouse versus human genes and proteins is needed.*

*Human: GENE/PROTEIN Mouse: Gene/PROTEIN*

Response: Thank you for pointing this out. The nomenclature has been corrected throughout the manuscript.

- *There is no Figure 5c, but a reference to results in the main text. Please reconcile.*  
*-There is no Figure 5-figure supplement 5a, but there is a reference to it in the main text. Please reconcile.*

Response: Figure references have been corrected.

- *Please indicate dilutions of all antibodies used when listed in the methods.*

Response: Antibody dilutions have been added where missing.

- *On page 25, there is a partial sentence missing information in the Histologic methods; “#S36964 Invitrogen, CA, USA)). All images were taken...”*

Response: We apologize for the error. It has been removed.

- *Table 1: please define all acronyms, including cohort names.*

Response: We apologize for the oversight. The legend to the Table has been updated with definitions of all acronyms.

- *Figure 2: Indicate that blue staining is DAPI in panel B. Clarify that “-ab” as an abbreviation is primary antibody negative.*

Response: A color code for DAPI and COL11A1 staining has been added and “-ab” is now defined.

- *Page 4: ADGRG6 (also known as GPR126)...the authors set this up for ADGRG6 but then use GPR126 in the manuscript, which is confusing. For clarity, please use the gene name Adgrg6 consistently, rather than alternating with Gpr126.*

Response: Thank you for pointing this out. GPR126 has now been changed to ADGRG6 throughout the manuscript.

- *REF 4: Richards, B.S., Sucato, D.J., Johnston C.E. Scoliosis, (Elsevier, 2020). Is this a book, can you provide more clarity in the Reference listing?*

Response: Thank you for pointing this out. This reference has been corrected.

- *While isolation was addressed, the methods for culturing Rat cartilage endplate and costal chondrocytes are poorly described and should be given more text.*

Response: Details about the cartilage endplate and costal chondrocyte isolation and culture have been added to the Methods.

- *Page 11: 1st paragraph, last sentence "These results suggest that Mmp3 expression"... this sentence needs attention. As written, I am not clear what the authors are trying to say.*

Response: This sentence has been clarified and now reads “These results suggest that Mmp3 expression is negatively regulated by Col11a1 in mouse costal chondrocytes.”

- *Page 13: line 4 from the bottom, "ECM-clearing"? This is confusing do you mean ECM degrading?*

Response: Yes and thank you. We have changed to “ECM-degrading”.

- *Please use version numbers for RefSeq IDs: e.g. NM\_080629.3 instead of NM\_080629*

Response: This change has been made in the revised manuscript.

- *It would be helpful for readers if the ethnicity of the discovery case cohort was clearly stated as European ancestry in the Results main text.*

Response: “European ancestry” has been added at first description of the discovery cohort in the manuscript.

- *Avoid using the term "mutation" and use "variant" instead.*

Response: Thank you for pointing this out. “Variant” is now used throughout the manuscript.

- *Define error bars for all bar charts throughout and include individual data points overlaid onto bars.*

Response: Thank you. Error bars are now clarified in the Figure legends.