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Supplemental Information

Differentiation of Hypertrophic Chondrocytes from Human iPSCs for the *In Vitro* Modeling of Chondrodysplasias

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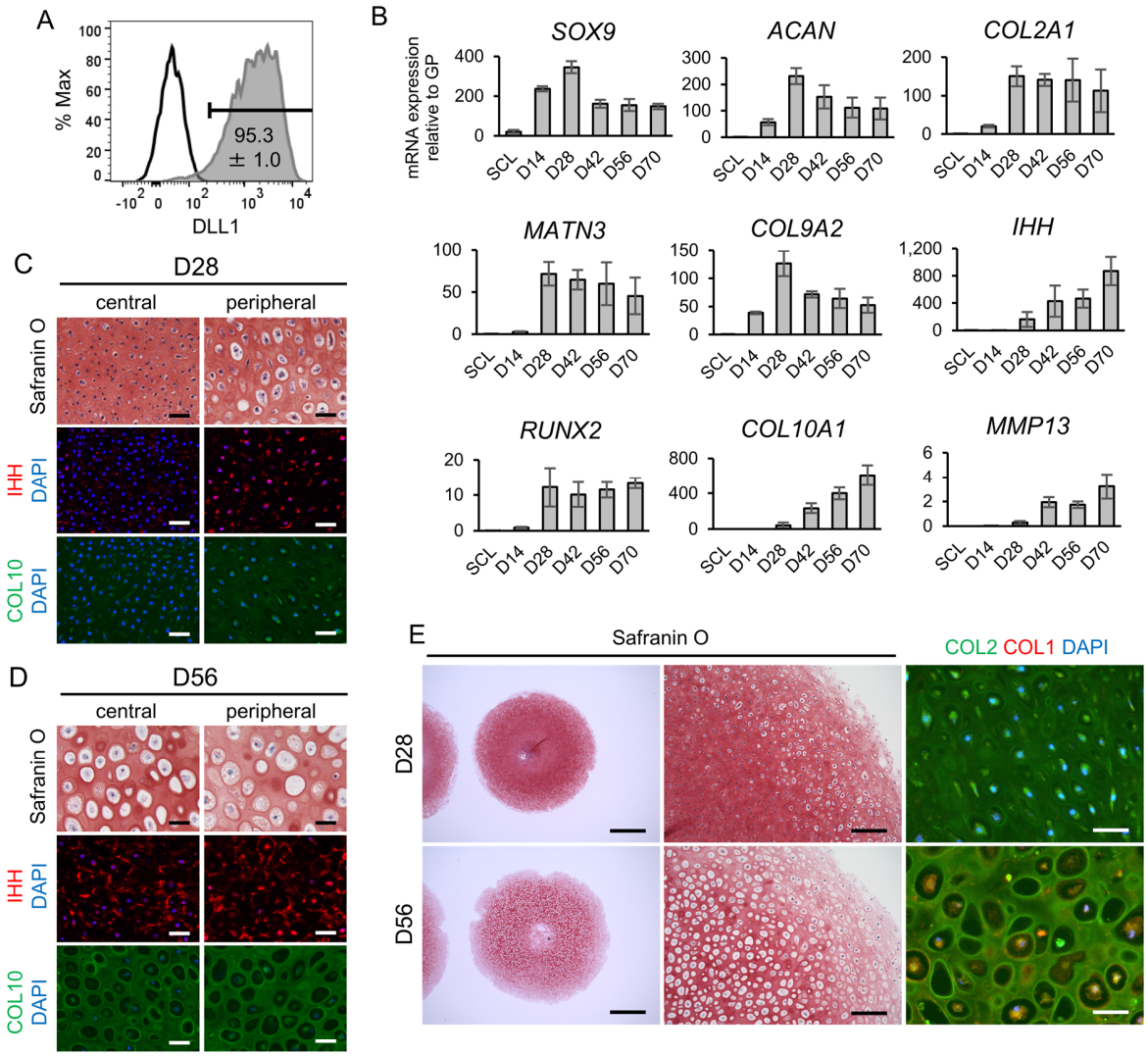
Inventory of Supplemental Information

- Supplemental Figures S1-S6
- Supplemental Tables S1-S6
- Supplemental Experimental Procedures
- Supplemental References



F

		414C2						1231A3						growth
expressed tissue	marker	SCL	D14	D28	D42	D56	D70	SCL	D14	D28	D42	D56	D70	plate
cartilage	*ACAN*	31.8	22.4	19.3	19.0	19.2	20.1	31.5	21.7	19.3	19.4	19.7	20.3	24.4
	COL2A1	23.4	21.6	17.4	16.3	16.6	17.9	23.4	20.3	17.1	16.5	16.6	17.6	22.1
	COL9A2	27.7	22.3	19.5	19.4	19.5	20.7	28.1	21.5	19.6	19.9	20.1	20.6	23.0
	MATN3	30.2	25.2	20.9	19.6	20.7	23.6	30.6	25.4	20.3	19.8	20.2	21.5	23.9
	SOX9	24.0	24.6	22.7	22.5	22.9	23.7	24.3	23.2	22.3	22.7	22.7	23.3	27.9
hypertrophic cartilage	*COL10A1*	37.1	34.9	21.4	16.0	15.2	15.5	42.3	34.9	20.4	16.1	15.2	15.1	21.5
	IHH	37.3	34.2	27.3	23.4	23.2	23.8	37.2	32.5	26.4	24.2	23.5	23.2	30.4
	MMP13	u.d.	40.4	31.9	27.5	27.8	27.2	u.d.	37.7	30.5	26.8	26.8	26.5	24.4
	RUNX2	32.8	29.4	25.8	24.5	24.4	24.9	32.8	29.1	25.1	24.7	24.1	24.4	25.5
bone	*ALPL*	29.6	35.5	23.5	20.3	20.4	22.2	25.9	29.6	22.4	20.1	19.9	20.9	24.0
	COL1A1	23.7	28.9	28.5	28.2	26.7	24.1	23.7	24.7	25.4	23.4	22.1	22.1	19.2
	IBSP	36.0	38.9	27.7	21.6	21.4	21.3	38.9	35.2	28.5	21.4	20.3	20.4	21.1
	SP7	34.6	32.5	25.7	23.1	23.2	23.8	32.9	31.0	25.2	23.6	23.3	23.5	27.1
adipose	*CEBPA*	35.4	40.6	38.4	39.0	35.5	36.3	35.7	38.0	39.2	37.8	38.3	37.5	30.9
	FABP4	41.4	38.5	34.6	35.6	35.6	35.5	38.0	34.8	32.8	31.6	32.3	30.6	21.8
	PPARG	32.7	35.0	30.2	27.6	27.9	27.6	31.8	33.4	28.5	27.0	25.8	26.2	26.0
ligament	*MKX*	32.1	25.1	23.9	24.4	25.7	26.5	30.6	23.7	23.7	24.3	24.9	26.1	30.3
	SCX	27.7	25.9	30.1	32.7	32.4	32.4	26.7	25.2	29.1	29.7	30.3	32.1	29.6
	TNMD	31.9	30.7	32.9	33.9	35.7	35.9	31.7	32.5	33.6	35.5	32.8	32.9	29.7
sclerotome	*PAX9*	24.3	28.7	29.2	30.2	31.5	32.6	24.9	25.3	27.7	28.5	29.2	31.2	33.6
	NKX3-2	25.6	27.8	25.4	25.9	27.7	29.9	25.6	25.4	24.1	24.9	25.9	27.8	29.0
muscle	*MYOD1*	35.9	36.4	34.9	35.2	35.8	35.8	37.0	34.1	34.8	34.6	34.6	34.9	37.8
ectoderm	*SOX1*	32.8	32.7	34.5	33.9	33.9	32.5	28.5	29.7	31.9	33.3	32.4	33.1	38.2
endoderm	*SOX17*	32.9	33.1	29.0	28.4	29.7	31.1	32.3	31.9	27.8	27.6	28.8	29.5	27.9
all	*ACTB*	18.1	21.7	20.5	19.6	20.1	19.8	17.9	20.5	20.2	19.5	19.4	19.9	17.3

Figure S1. Differentiation of hypertrophic chondrocytes from 1231A3 iPSCs. Related to Figure 1

(A) Representative result of DLL1-positive cells (compared to isotype control) on day 2 of sclerotome induction (SI) with the mean and SEM (standard error of the mean) of n=4 independent experiments displayed.

(B) mRNA expression of chondrocyte markers over time from SCL on day -1 to day 70 of HI by qPCR. Values are shown as mean ± SEM (n=3 independent experiments), relative to the mean of 6 pieces of a human distal femoral growth plate (GP).

(C, D) Safranin O and IHH or COL10 immunostaining on day 28 (C) and day 56 (D) of HI in the central or peripheral area of each pellet. Scale bars, 50 µm.

(E) Safranin O staining (left, middle columns) and COL2 (green) with COL1 (red) immunostaining (right column) of pellets from day 28 and 56 of HI. Scale bars, (left column) 1 mm, (middle column) 200 µm, (right column) 50 µm.

(F) Ct values of markers of different tissue types from qPCR. Values are the mean from n=4 (414C2) or n=3 (1231A3) independent experiments or 6 pieces of the human distal femoral growth plate. For values indicated in red, the signal was undetected in at least one replicate. u.d., undetected in all replicates. For (C), (D), (E), similar results were obtained in n=4 independent experiments.

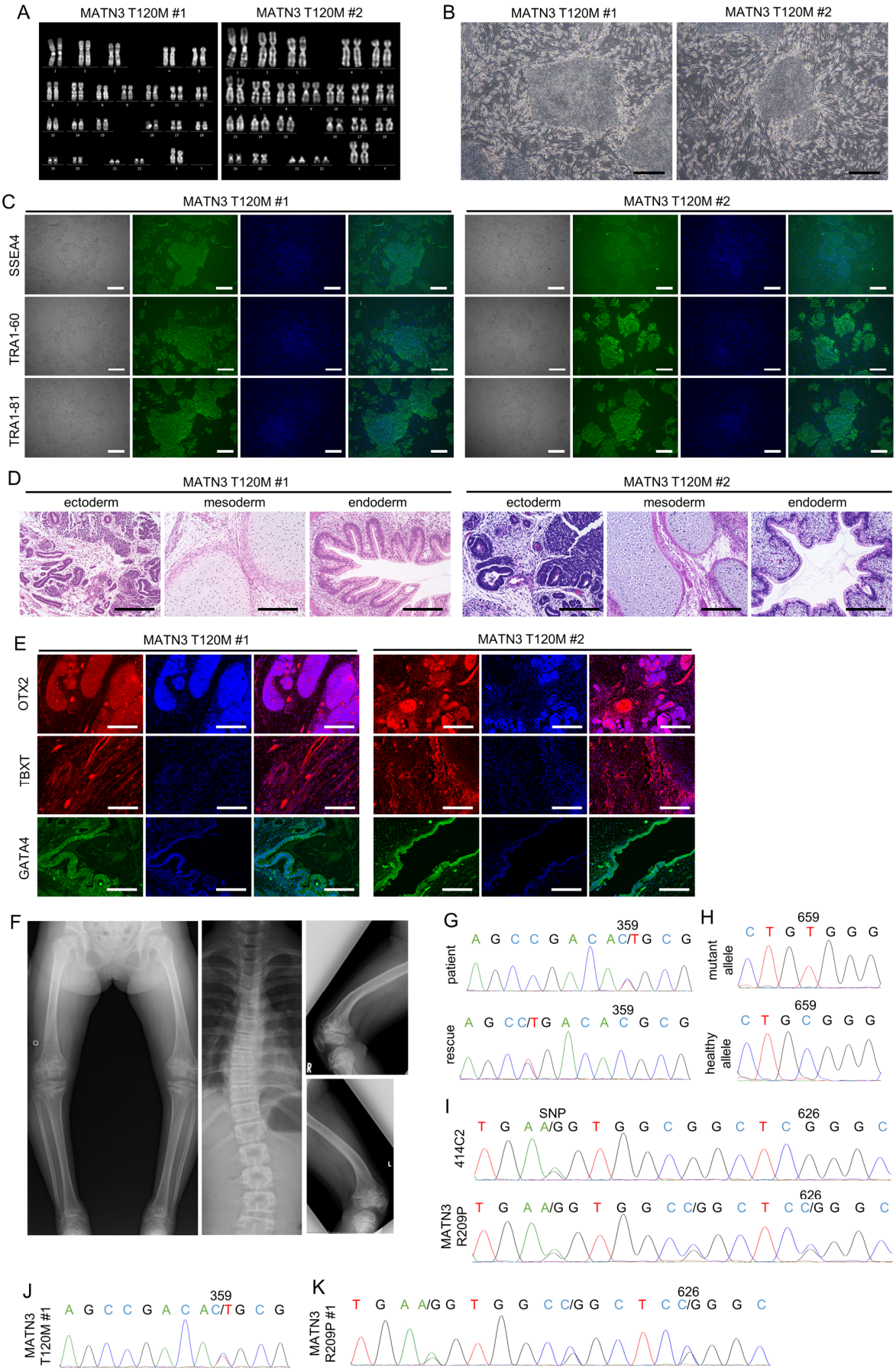

A
MATN3 T120M #1
MATN3 T120M #2
B
MATN3 T120M #1
MATN3 T120M #2
C
MATN3 T120M #1
MATN3 T120M #2
SSEA4
TRA1-60
TRA1-81
D
MATN3 T120M #1
MATN3 T120M #2
ectoderm
mesoderm
endoderm
ectoderm
mesoderm
endoderm
E
MATN3 T120M #1
MATN3 T120M #2
OTX2
TBXT
GATA4
F
G
patient
359
A G C C G A C AC/T G C G
rescue
359
A G C C/T G A C A C G C G
H
mutant allele
659
C T G T G G G
healthy allele
659
C T G C G G G
I
414C2
SNP
626
T G A A/G G T G G C G G C T C G G G C
MATN3 R209P
626
T G A A/G G T G G C C/G G C T C C/G G G C
J
MATN3 T120M #1
359
A G C C G A C AC/T G C G
K
MATN3 R209P #1
626
T G A A/G G T G G C C/G G C T C C/G G G C

Figure S2. Multiple epiphyseal dysplasia (MED) patient phenotype, validation of iPSC clones and sequence of an additional *MATN3* mutant clone. Related to Figure 2

(A) Karyotype analysis of MED patient-derived clones #1 and #2, showing a normal karyotype of 46,XX.

(B) Phase-contrast image of iPSC clones (on-feeder culture at the time of validation). Scale bars, 100 µm.

(C) Brightfield image (left) and immunostaining (right) of each clone. Pluripotent markers are shown in green with the nuclear counterstain DAPI in blue. Scale bars, 100 µm.

(D) HE staining of tissue formed from teratomas of each clone, showing differentiation into ectoderm, mesoderm and endoderm. Scale bars, 200 µm.

(E) Immunostaining of ectoderm marker OTX2 (red), mesoderm marker TBXT (red), and endoderm marker GATA4 (green) with DAPI (blue) in teratoma tissue. Scale bars, 200 µm.

(F) Patient x-ray image of the lower limbs (left), spine (middle), right femur (upper right) and left femur (lower right).

(G) Sequence of the patient showing the heterozygous *MATN3* c.359C>T (p.T120M) mutation (top) and the gene-corrected rescue (bottom).

(H) Single allele sequence of the mutant allele (top) and healthy allele (bottom), showing the healthy allele containing the SNP rs187943382, *MATN3* c.659T>C (p.V220A).

(I) Sequence of the wild type iPSC line 414C2 (top), showing the SNP in *MATN3* used for allele-specific targeting, and the mutant 414C2 with the heterozygous *MATN3* c.626G>C (p.R209P) mutation (bottom).

(J, K) Sequence of cDNA reverse-transcribed from RNA on day 56 of HI of the mutant clones showing that the mutant RNA is expressed.

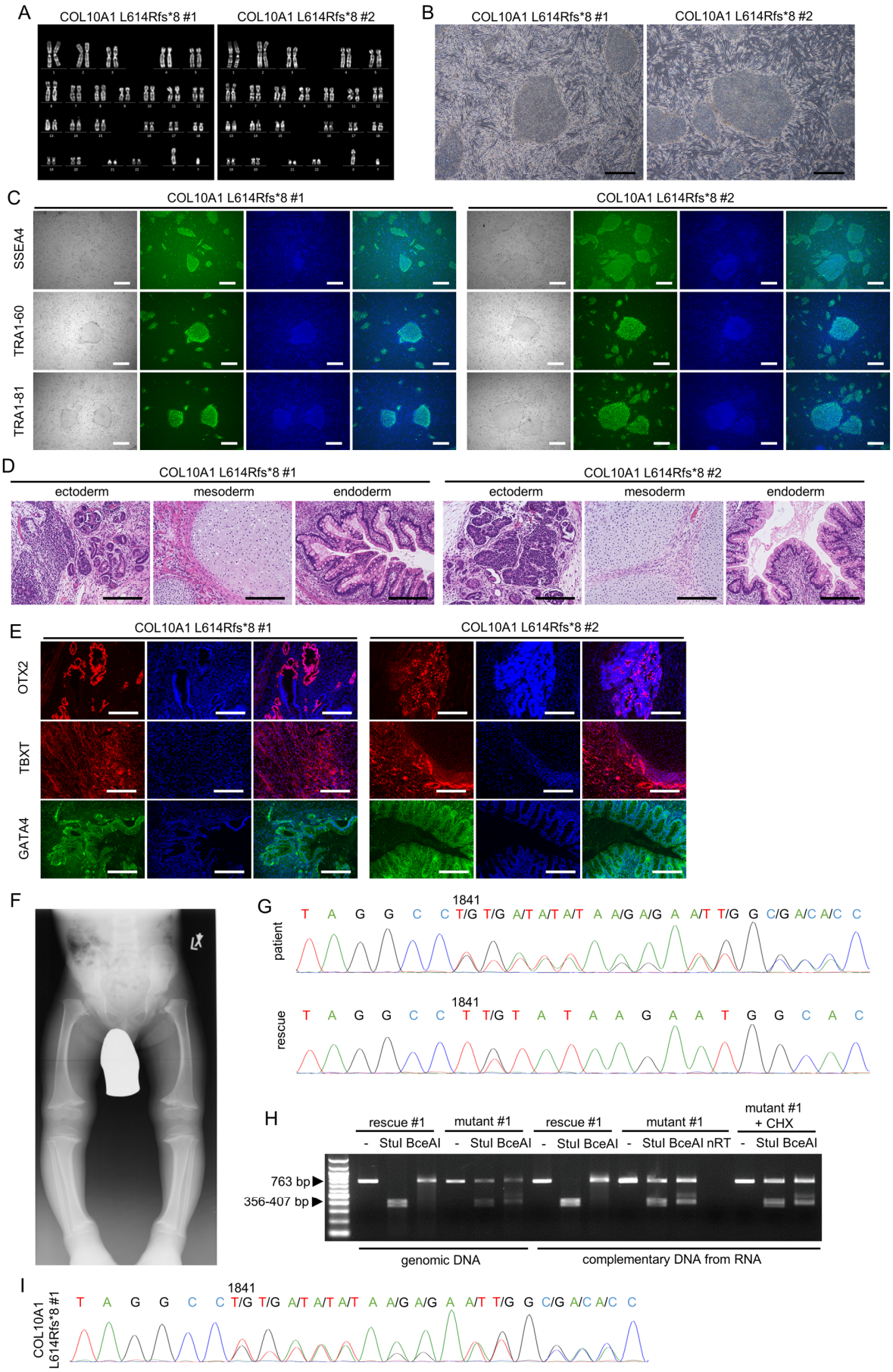

A
COL10A1 L614Rfs*8 #1
COL10A1 L614Rfs*8 #2
B
COL10A1 L614Rfs*8 #1
COL10A1 L614Rfs*8 #2
C
COL10A1 L614Rfs*8 #1
COL10A1 L614Rfs*8 #2
SSEA4
TRA1-60
TRA1-81
D
COL10A1 L614Rfs*8 #1
ectoderm
mesoderm
endoderm
COL10A1 L614Rfs*8 #2
ectoderm
mesoderm
endoderm
E
COL10A1 L614Rfs*8 #1
COL10A1 L614Rfs*8 #2
OTX2
TBXT
GATA4
F
G
1841
T A G G C C T/G T/G A/T A/T A/T A A/G A/G A A/T T/G G C/G A/C A/C C
patient
1841
T A G G C C T T/G T A T A A G A A T G G C A C
rescue
H
rescue #1
mutant #1
rescue #1
mutant #1
mutant #1
+ CHX
- StuI BceAI - StuI BceAI - StuI BceAI - StuI BceAI nRT - StuI BceAI
763 bp
356-407 bp
genomic DNA
complementary DNA from RNA
I
1841
COL10A1
L614Rfs*8 #1
T A G G C C T/G T/G A/T A/T A/T A A/G A/G A A/T T/G G C/G A/C A/C C

Figure S3. Metaphyseal chondrodysplasia type Schmid (MCDS) patient #1 phenotype and validation of iPSC clones. Related to Figure 2

(A) Karyotype analysis of clones #1 and #2, showing a normal karyotype of 46,XY.

(B) Phase-contrast image of iPSC clones (on-feeder culture at the time of validation). Scale bars, 100 µm.

(C) Brightfield image (left) and immunostaining (right) of each clone. Pluripotent markers are shown in green with the nuclear counterstain DAPI in blue. Scale bars, 100 µm.

(D) HE staining of tissue formed from teratomas of each clone, showing differentiation into ectoderm, mesoderm and endoderm. Scale bars, 200 µm.

(E) Immunostaining of ectoderm marker OTX2 (red), mesoderm marker TBXT (red), and endoderm marker GATA4 (green) with DAPI (blue) in teratoma tissue. Scale bars, 200 µm.

(F) Patient x-ray image of the lower limbs.

(G) Sequence of the patient showing the heterozygous *COL10A1* c.1841_1841delT (p.L614Rfs*8) mutation (top) and the gene-corrected rescue (bottom).

(H) Electrophoresis after reverse transcribing RNA, amplifying the *COL10A1* region containing the mutation by PCR, and processing the amplicons by restriction enzyme StuI (recognizing only the wild type allele) or BceAI (recognizing only the mutant allele). The mutant allele is clearly visible and not changed by NMD (nonsense-mediated decay) inhibitor CHX (cycloheximide), showing that the early stop codon in L614Rfs*8 does not lead to NMD. The nRT (no reverse transcriptase) negative control shows no band, indicating that no DNA contamination is present. RNA was taken from samples on day 56 of HI. Similar results were obtained in three biologically independent experiments.

(I) Sequence of cDNA reverse-transcribed from RNA on day 56 of HI of the mutant clone showing that the mutant RNA is expressed.

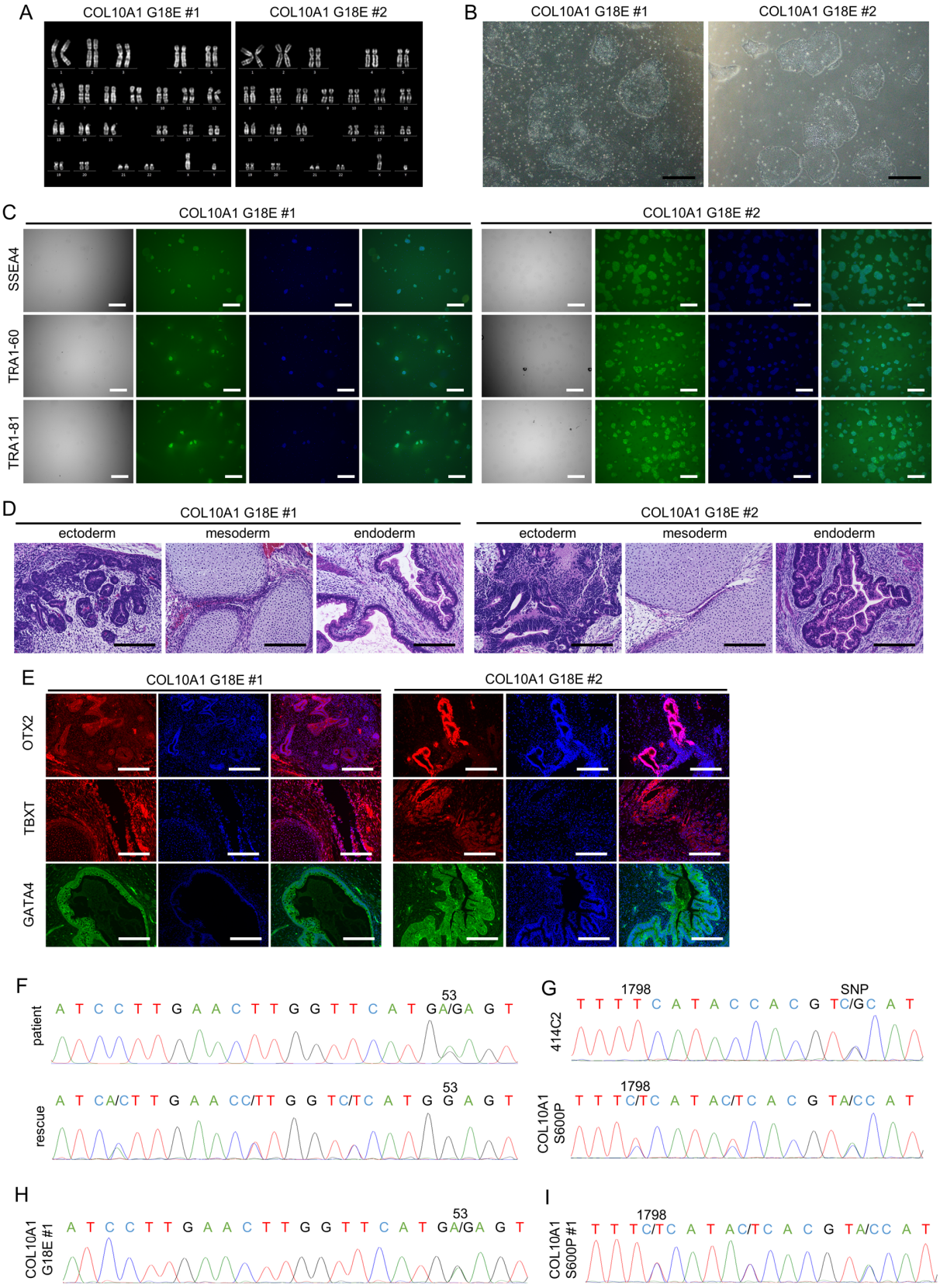

A
COL10A1 G18E #1
COL10A1 G18E #2
B
COL10A1 G18E #1
COL10A1 G18E #2
C
COL10A1 G18E #1
COL10A1 G18E #2
SSEA4
TRA1-60
TRA1-81
D
COL10A1 G18E #1
ectoderm
mesoderm
endoderm
COL10A1 G18E #2
ectoderm
mesoderm
endoderm
E
COL10A1 G18E #1
COL10A1 G18E #2
OTX2
TBXT
GATA4
F
patient
A T C C T T G A A C T T G G T T C A T GA/GA G T
53
rescue
A T CA/CT T G A A CC/TT G G TC/TC A T G G A G T
53
G
1798
SNP
414C2
T T T T C A T A C C A C G TC/GC A T
COL10A1 S600P
1798
T T TC/TC A T AC/TC A C G TA/CC A T
H
COL10A1 G18E #1
A T C C T T G A A C T T G G T T C A T GA/GA G T
53
I
COL10A1 S600P #1
1798
T T TC/TC A T AC/TC A C G TA/CC A T

Figure S4. Validation of iPSC clones from MCDS patient #2 and sequence of an additional *COL10A1* mutant clone. Related to Figure 2

(A) Karyotype analysis of clones #1 and #2, showing a normal karyotype of 46,XY.

(B) Phase-contrast image of iPSC clones (feeder-free culture at the time of validation). Scale bars, 100 µm.

(C) Brightfield image (left) and immunostaining (right) of each clone. Pluripotent markers are shown in green with the nuclear counterstain DAPI in blue. Scale bars, 100 µm.

(D) HE staining of tissue formed from teratomas of each clone, showing differentiation into ectoderm, mesoderm and endoderm. Scale bars, 200 µm.

(E) Immunostaining of ectoderm marker OTX2 (red), mesoderm marker TBXT (red), and endoderm marker GATA4 (green) with DAPI (blue) in teratoma tissue. Scale bars, 200 µm.

(F) Sequence of the patient showing the heterozygous *COL10A1* c.53G>A (p.G18E) mutation (top) and the gene-corrected rescue (bottom).

(G) Sequence of the wild type iPSC line 414C2 (top), showing the SNP in *COL10A1* used for allele-specific targeting, and the mutant 414C2 with the heterozygous *COL10A1* c.1798T>C (p.S600P) mutation (bottom).

(H, I) Sequence of cDNA reverse-transcribed from RNA on day 56 of HI of the mutant clones showing that the mutant RNA is expressed.

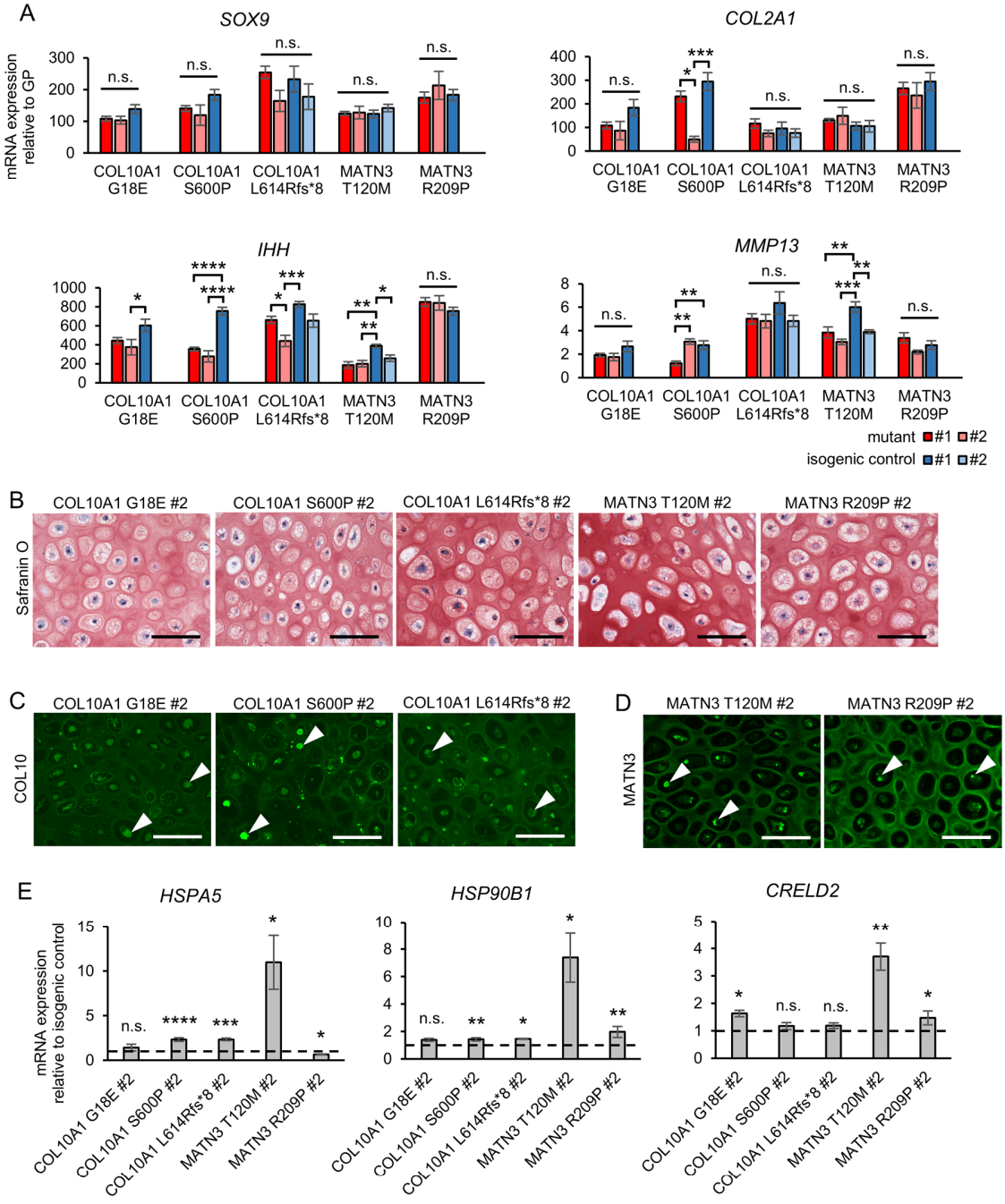

A
SOX9
COL2A1
IHH
MMP13
mRNA expression relative to GP
COL10A1 G18E
COL10A1 S600P
COL10A1 L614Rfs*8
MATN3 T120M
MATN3 R209P
n.s.
mutant #1 #2
isogenic control #1 #2
B
COL10A1 G18E #2
COL10A1 S600P #2
COL10A1 L614Rfs*8 #2
MATN3 T120M #2
MATN3 R209P #2
Safranin O
C
COL10
D
MATN3
E
HSPA5
HSP90B1
CRELD2
mRNA expression relative to isogenic control

Figure S5. Additional mutant clones also show intracellular retention and ER stress without disruption of chondrogenic differentiation. Related to Figures 2, 3, and 4

(A) mRNA expression of chondrocyte markers of various stages by qPCR. Values are relative to the mean of 6 pieces of the human distal femoral growth plate (number of independent experiments shown in Table S2). Statistical analysis by ANOVA with post-hoc Tukey HSD.

(B) Safranin O staining. Similar results were obtained in n=3 independent experiments. Scale bars, 100 µm.

(C, D) Immunostaining of COL10 (C) or MATN3 (D). Similar results were obtained in n=3 independent experiments. Arrowheads indicate intracellular aggregates. Scale bars, 100 µm.

(E) mRNA expression of ER stress and UPR markers by qPCR. Values are relative to the respective isogenic control (number of independent experiments shown in Table S2). Dotted lines indicate the value=1 of the isogenic controls. Statistical analysis by unpaired two-sided t-test.

All results are from day 56 of HI and expressed as the mean ± SEM. (n.s. no significant difference, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

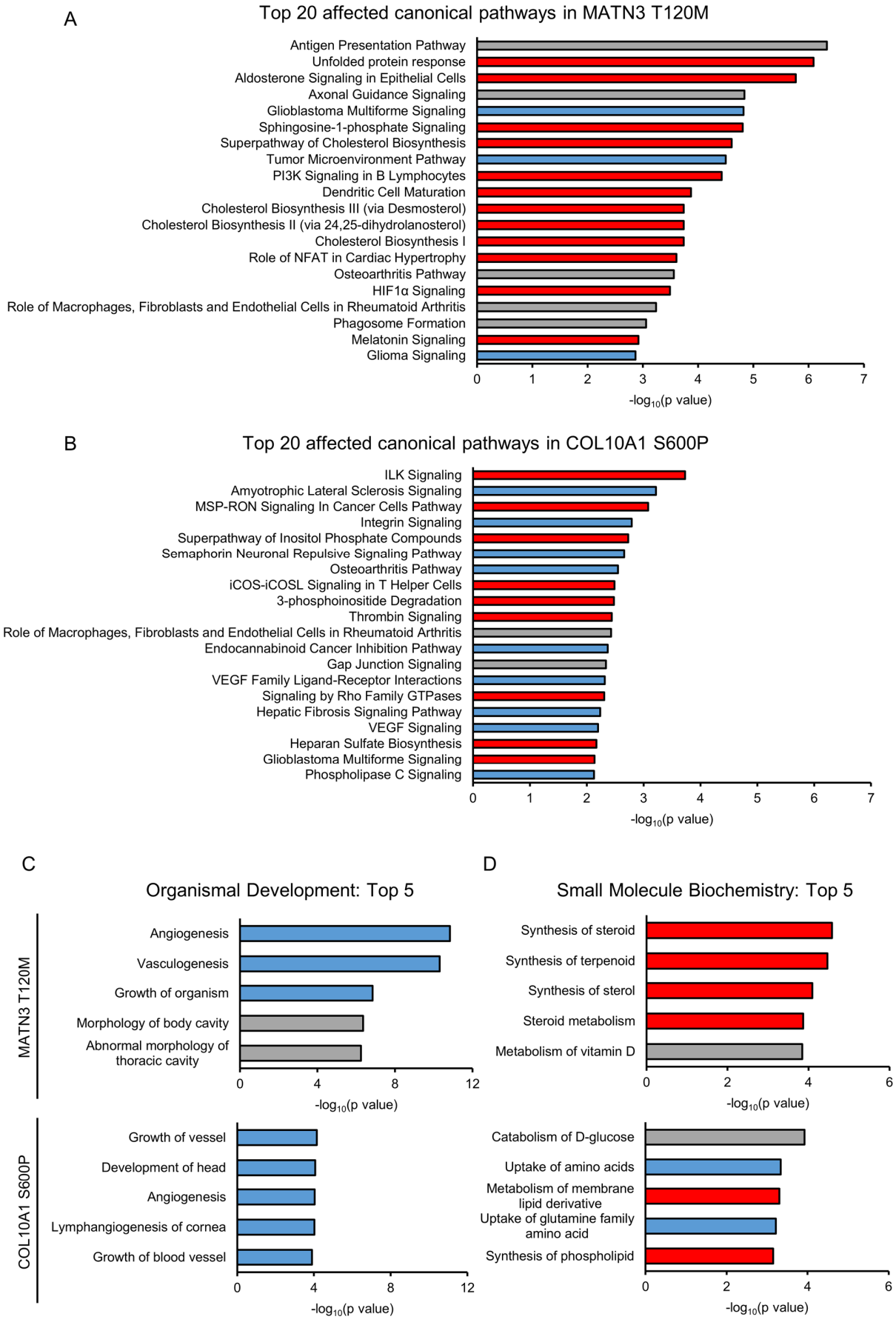

A
Top 20 affected canonical pathways in MATN3 T120M
Antigen Presentation Pathway
Unfolded protein response
Aldosterone Signaling in Epithelial Cells
Axonal Guidance Signaling
Glioblastoma Multiforme Signaling
Sphingosine-1-phosphate Signaling
Superpathway of Cholesterol Biosynthesis
Tumor Microenvironment Pathway
PI3K Signaling in B Lymphocytes
Dendritic Cell Maturation
Cholesterol Biosynthesis III (via Desmosterol)
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)
Cholesterol Biosynthesis I
Role of NFAT in Cardiac Hypertrophy
Osteoarthritis Pathway
HIF1α Signaling
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
Phagosome Formation
Melatonin Signaling
Glioma Signaling
0 1 2 3 4 5 6 7
-log10(p value)
B
Top 20 affected canonical pathways in COL10A1 S600P
ILK Signaling
Amyotrophic Lateral Sclerosis Signaling
MSP-RON Signaling In Cancer Cells Pathway
Integrin Signaling
Superpathway of Inositol Phosphate Compounds
Semaphorin Neuronal Repulsive Signaling Pathway
Osteoarthritis Pathway
iCOS-iCOSL Signaling in T Helper Cells
3-phosphoinositide Degradation
Thrombin Signaling
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
Endocannabinoid Cancer Inhibition Pathway
Gap Junction Signaling
VEGF Family Ligand-Receptor Interactions
Signaling by Rho Family GTPases
Hepatic Fibrosis Signaling Pathway
VEGF Signaling
Heparan Sulfate Biosynthesis
Glioblastoma Multiforme Signaling
Phospholipase C Signaling
0 1 2 3 4 5 6 7
-log10(p value)
C
Organismal Development: Top 5
MATN3 T120M
Angiogenesis
Vasculogenesis
Growth of organism
Morphology of body cavity
Abnormal morphology of thoracic cavity
0 4 8 12
-log10(p value)
COL10A1 S600P
Growth of vessel
Development of head
Angiogenesis
Lymphangiogenesis of cornea
Growth of blood vessel
0 4 8 12
-log10(p value)
D
Small Molecule Biochemistry: Top 5
Synthesis of steroid
Synthesis of terpenoid
Synthesis of sterol
Steroid metabolism
Metabolism of vitamin D
0 2 4 6
-log10(p value)
Catabolism of D-glucose
Uptake of amino acids
Metabolism of membrane lipid derivative
Uptake of glutamine family amino acid
Synthesis of phospholipid
0 2 4 6
-log10(p value)

Figure S6. Different pathways and functions are affected in the MATN3 T120M and COL10A1 S600P mutants. Related to Figure 6

(A, B) Top 20 IPA Canonical Pathways in the MATN3 T120M and COL10A1 S600P mutants vs their isogenic controls by microarray analysis (n=3 independent experiments).

(C, D) Top 5 Organismal Development (C) and Small Molecule Biochemistry (D) terms in IPA Diseases and Functions for the MATN3 T120M (top) and COL10A1 S600P (bottom) mutants vs their isogenic controls by microarray analysis (n=3 independent experiments).

All results are from day 56 of HI. Genes with adjusted p value < 0.05 by moderated t-test were used for IPA. Red bars, positive activation z-score; blue bars, negative activation z-score; gray bars, zero or unavailable activation z-score.

Supplemental Tables

Table S1. Transcriptome analysis details. Related to Figures 1 and 6

(see Table S1.xlsx)

Table S2. Number of independent experiments for mRNA expression in Figures 2B, 4F, 4G, and Supplemental Figures S5A, S5E

Sample name	Mutant or isogenic control	Number of independent experiments
COL10A1 G18E	mutant (#1)	12
	mutant #2	4
	isogenic control (#1)	8
COL10A1 S600P	mutant (#1)	12
	mutant #2	4
	isogenic control (#1)	12
COL10A1 L614Rfs*8	mutant (#1)	8
	mutant #2	4
	isogenic control (#1)	4
	isogenic control #2	4
MATN3 T120M	mutant (#1)	4
	mutant #2	4
	isogenic control (#1)	4
	isogenic control #2	4
MATN3 R209P	mutant (#1)	8
	mutant #2	4
	isogenic control (#1)	12

[a]Unless stated otherwise, all mutants and isogenic controls in the main and supplemental figures refer to mutants #1 and isogenic controls #1.

Table S3. Guide RNAs and repair templates used for gene editing. Related to Experimental Procedures

Gene editing	Guide RNA (protospacer)	Single-stranded oligonucleotide repair template (100 nt)	Restriction enzyme
Creation of COL10A1 S600P	CGTGCATGTGAA AGGGACTC	CCCAAGGACTGGAATCTTTACTTGTCAG ATACCAGGAATATACTATTTTCCATATCAC GTACATGTGAAAGGGACTCATGTTTGGG TAGGCCTGTATAAG	PciI (ACATGT)
Creation of MATN3 R209P	AGGTGAATGAAG TGGCGGCT	ATCATTGTTACAGATGGGAGGCCCCAGG ACCAGGTGAATGAAGTGGCCGCTCCGG CCCAAGCATCTGGTATTGAGCTCTATGC TGTGGGCGTGGACCGGG	BsrBI (CCGCTC)
Rescue of COL10A1 G18E	TGAACTTGGTTCA TGAAGTG	AGAATATGCTGCCACAAATACCCTTTTTG CTGCTAGTATCATTGAACCTGGTCCATG GAGTGTTTTACGCTGAACGATACCAAAT GCCCACAGGCATAAA	NcoI (CCATGG)
Rescue of COL10A1 L614Rfs*8	TTGGGTAGGCCG TATAAGAA	TTCATACCACGTGCATGTGAAAGGGACT CATGTTTGGGTAGGCCTTTATAAGAATG GCACCCCTGTAATGTACACCTATGATGA ATACACCAAAGGCTAC	PsiI (TTATAA)
Rescue of MATN3 T120M	ACATGCGGGTGG CAGTGGTG	AACTTTTGTCTCCCGGATAATCGACACT CTGGACATTGGGCCAGCTGACACGCGG GTGGCAGTGGTGAACTATGCTAGCACTG TGAAGATCGAGTTCCAA	PvuII (CAGCTG)

[a]Underlined nucleotides in the repair template indicate the recognition sites of the restriction enzymes.

Table S4. Primers for sequencing. Related to Experimental Procedures

Amplified domain	Direction	Sequence
COL10A1 NC1 domain	forward	CAGCAGGAGCAAAGGGAATG
	forward	GCTGGCATAGCAACTAAGG
	forward	CCACCAGGTCAAGCAGTCATG
	reverse	TTGAATGGGAGGCACAAGG
	reverse	GGGAAGGTTTGTTGGTCTG
	reverse	TGTACTCACATTGGAGCCACTAG
COL10A1 NC2 domain	forward	TCAGTCTGTGGATGATAGTC
	forward	CACTTGATTACCTGAGTATAGC
	forward	CAAGGCACCATCTCCAGGAAC
	forward	CATCTCCAGGAACTCCCAGC
	reverse	AGTTTCATTTGCCTGCTTG
	reverse	CAGAAGTTGGAAAGTAACACC
	reverse	TATGGTCCTCTCTCTCCTGG
	reverse	CCTTGCTCTCCTCTTACTGC
MATN3 vWFa domain	forward	GCAGAGGGATGAGGTTCTAAG
	forward	GCAGGCAGTGGTGATGTTGG
	forward	AGATAAGCAGTCCCTGAAGC
	forward	GACTCCTCCTGCTGCTCTGG
	reverse	GTTGGAAGCAAAGACTGACC
	reverse	GGCTTCGTCCATTGCTGTCTG
	reverse	TCACAGTGGTGCTTGCCTTC

Table S5. Primers for qPCR. Related to Experimental Procedures

Amplified gene	Direction	Sequence
ACTB	forward	CACCATTGGCAATGAGCGGTTC
	reverse	AGGTCTTTGCGGATGTCCACGT
COL10A1	forward	CCCAGCACGCAGAATCCATC
	reverse	AGTGGGCCTTTTATGCCTGT
COL2A1	forward	CGAGGCAACGATGGTCAGCC
	reverse	TGGGGCCTTGTTCACCTTTGA
MATN3	forward	GTTCAGTCCGTGACAAGTGTG
	reverse	AGTCTTCGTGCTTCCTCAGTG
SOX9	forward	GACTTCCGCGACGTGGAC
	reverse	GTTGGGCGGCAGGTACTG
MMP13	forward	CATGAGTTCGGCCACTCCTT
	reverse	CCTGGACCATAGAGAGACTGGA
IHH	forward	CGGTGGACATCACCACATCA
	reverse	CGTGGGCCTTTGACTCGTAA
ALPL	forward	GGAAGACACTCTGACCGTGG
	reverse	GGGGGCCAGACCAAAGATAG
SP7	forward	ATCCAGCCCCCTTTACAAGC
	reverse	TAGCATAGCCTGAGGTGGGT
ACAN	forward	TCGAGGACAGCGAGGCC
	reverse	TCGAGGGTGTAGCGTGTAGAGA
RUNX2	forward	TTACTTACACCCCGCCAGTC
	reverse	TATGGAGTGCTGCTGGTCTG
COL9A2	forward	GCGCTATCGGTGCCACTGGG
	reverse	GGGGGCCCGTTGCTCCTTTC
HSPA5	forward	GTTTGCTGAGGAAGACAAAAAGCTC
	reverse	CACTTCCATAGAGTTTGCTGATAATTG
CRELD2	forward	CCAAGTACGAGTCCAGCGAG
	reverse	TGCTCCTCCTGCGCCTCTAG
HSP90B1	forward	ATGGAGCAGCAAGACTGAAAC
	reverse	TCTTCTTCTTCTTCCTCTACTGC
XBP1 spliced	forward	TGCTGAGTCCGCAGCAGGTG
	reverse	GCTGGCAGGCTCTGGGGAAG
EDEM3	forward	GATGGAGAAGGATGAGCTATGAC
	reverse	GAACGTGGTTGTTCATCACC
DERL3	forward	CCTCGTGGACCTGCTGG
	reverse	GGTCTGCAGGAGCCTCTTG

HERPUD1	forward	CGTTGTTATGTACCTGCATC
	reverse	TCAGGAGGAGGACCATCATTT
COL1A1	forward	GGACACAGAGGTTTCAGTGGT
	reverse	GCACCATCATTTCCACGAGC
IBSP	forward	AAGGACAAGGCTACGATGGC
	reverse	CGGATGCAAAGCCAGAATGG
CEBPA	forward	CGGTGGACAAGAACAGCAAC
	reverse	CGGAATCTCCTAGTCCTGGC
FABP4	forward	ACCAGGAAAGTGGCTGGCAT
	reverse	CAGGTCAACGTCCCTTGGCT
PPARG	forward	GCTGTTATGGGTGAAAACTCTG
	reverse	ATAAGGTGGAGATGCAGGCTC
MKX	forward	CGCACAGACACTCTGGAAAA
	reverse	AGCGGCACTTTGACAGTCTT
SCX	forward	CCCAAACAGATCTGCACCTTC
	reverse	GCGAATCGCTGTCTTTCTGTC
TNMD	forward	CCATGCTGGATGAGAGAGGT
	reverse	CTCGTCCTCCTTGGTAGCAG
PAX9	forward	ACAGTGCTGCTCCTTCTGGT
	reverse	ATGTGAGACCTGGGAATTGG
NKX3-2	forward	CAGAAATTCTCCCAAAGATGC
	reverse	TCTCCCTACAGTTTCGCCG
MYOD1	forward	GTGTCGAGCCTAGACTGCCT
	reverse	CTCAGAAGGCACGTCCGC
SOX1	forward	TACAGCCCCATCTCCAACTC
	reverse	GCTCCGACTTCACCAGAGAG
SOX17	forward	CGCTTTCATGGTGTGGGCTAAGGACG
	reverse	TAGTTGGGGTGGTCCTGCATGTGCTG

Table S6. Key reagents used in this study. Related to Experimental Procedures

Induction reagents and drugs	Source	Identifier
AK02N	Ajinomoto	AJ100
iMatrix-511 silk	Nippi	892021
Matrigel	BD	354230
PenStrep	Gibco	15140-163
bFGF	Wako	068-04544
CHIR99021	Axon	Axon1386
Activin A	R&D	338-AC01-M
SB431542	Selleck Chemicals	S1067
LDN193189	Stemgent	04-0074
PD173074	Tocris	3044
XAV939	Tocris	3748/10
SAG	Calbio	566660
Y-27632	Wako	034-24024
Ham's F12	Gibco	11765-054
BSA	Sigma	A8806-5G
IMDM	Sigma	I3390
CD Lipid	Life Tech	11905-031
Apo-transferrin	Sigma	T1147-500MG
Monothioglycerol	Sigma	M6145-25ML
Insulin	Wako	097-06474
Dexamethasone	Wako	047-18863
PDGF-BB	R&D	520-BB
TGFβ3	R&D	243-B3
BMP4	R&D	314-BP
T3	Sigma	T-074-1ML
β-glycerophosphate	Sigma	G6501-100G
ITS premix	Corning	354352
L-ascorbic acid 2-phosphate	Sigma	A8960
Proline	Sigma	P-5607
Glucose	Sigma	G8769
Sodium pyruvate	Sigma	S8636

Glutamax	Life Tech		35050-061	
DMEM/F12	Gibco		11320-033	
Carbamazepine	Sigma		C4024-1G	
Trimethylamine N-oxide	Sigma		317594-5G	
Valproic acid	Sigma		P4543-10G	
Rapamycin	MedChemExpress		HY10219	
Antibodies	Host	Clonality	Source	Identifier
COL1	Rabbit	Polyclonal	Novus Biologicals	NB600-408-0.1mg
COL2	Mouse	Monoclonal	Thermo	MS235P0
COL10	Mouse	Monoclonal	Thermo	14-9771-82
MATN3	Rabbit	Polyclonal	abcam	ab106388
PDI	Mouse	Monoclonal	Thermo	MA3-019-A647
IHH	Rabbit	Polyclonal	abcam	ab39634
COL1	Goat	Polyclonal	SBA	1310-01
COL2	Goat	Polyclonal	SBA	1320-01
Ki67	Rabbit	Monoclonal	Nichirei	718071
DLL1	Mouse	Monoclonal	R&D	FAB1818A
APC-conjugated mouse IgG2B	Mouse	Monoclonal	R&D	IC0041A
SSEA4	Mouse	Monoclonal	Chemicon	MAB4304
TRA1-60	Mouse	Monoclonal	Chemicon	MAB4360
TRA1-81	Mouse	Monoclonal	Chemicon	MAB4381
OTX2, TBXT, GATA4	Goat	Polyclonal	R&D	SC022
ATF6	Mouse	Monoclonal	abcam	ab122897
ACTB, peroxidase conjugate	Mouse	Monoclonal	Sigma	A3854-200UL
Phospho-EIF2A	Rabbit	Monoclonal	CST	3398T
EIF2A	Rabbit	Monoclonal	CST	5324T
ACTB	Rabbit	Monoclonal	CST	4970S
HSPA5	Rabbit	Monoclonal	CST	3177T
PDI	Rabbit	Monoclonal	CST	3501T
ERO1A	Rabbit	Polyclonal	CST	3264T

Supplemental Experimental Procedures

Establishment of isogenic iPSC lines using CRISPR/Cas9

In order to create gene-corrected rescues, the 20 nt protospacer sequence for the guide RNA targeting the mutated site was inserted into the vector pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene). Cloning was performed as previously described[1] using DH5α cells (Toyobo). The vector was isolated using the Midi Prep Kit (Macherey-Nagel) and electroporated into iPSCs (1×10^6 cells) using the NEPA 21 (Nepa Gene), together with a repair template consisting of 100 nt including the gene correction, a recognition site for restriction enzymes to confirm the insertion, and silent mutations for PAM or guide RNA blocking. Cells were cultured in six well plates with 10 μM Y-27632 overnight, after which they were treated with 0.5-0.7 μg/ml puromycin for 24 hours to select for cells incorporating the vector. One week after sparsely re-seeding the cells, 48-96 single colonies were randomly picked and expanded. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN) with proteinase K treatment, upon which the relevant regions were amplified with PCR using KOD Plus Neo (Toyobo). Amplicons were digested by the restriction enzymes to confirm insertion of the repair template. Samples for which insertion was confirmed were then purified using the FastGene Gel/PCR Extraction kit (Nippon Genetics) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the 3500xL Genetic Analyzer (Applied Biosystems).

Mutants were established from the wild type iPSC line 414C2 similarly to the gene-corrected rescues. Here, the guide RNA was designed to target a SNP near the intended site of mutagenesis. The 100 nt repair template included the new mutation, a recognition site for restriction enzymes, and silent mutations for PAM or guide RNA blocking. All primers, guide RNAs, repair templates, and restriction enzymes used in this gene editing are listed in Tables S3 and S4.

iPSC culture and validation

All patient-derived iPSC lines were established on SNL feeder cells in primate embryonic stem cell medium (ReproCELL) supplemented with 4 ng/ml bFGF (Wako), 50 U penicillin and 50 μg/ml streptomycin (Gibco). After several passages as whole colonies on feeder cells, iPSCs were moved to feeder-free culture on dishes coated with laminin (Nippi) in StemFit AK02N (Ajinomoto) and passaged as single cells once a week at a density of 1.1×10^3 to 3.2×10^3 cells/cm^2.

Newly established iPSC clones were validated for pluripotency by immunostaining either before switching to feeder-free culture (MATN3 T120M and COL10A1 L614Rfs*8) or after the switch (COL10A1 G18E). After fixation in 4% paraformaldehyde, cells were PBS washed and incubated for

1 hour at 4°C in a blocking solution of PBS with 0.2% TritonX-100 (Sigma) and 5% Blocking One Histo (Nacalai Tesque). Pluripotency markers SSEA4, TRA1-60, and TRA1-81 (all Chemicon) were diluted in the blocking solution at 1:400, 1:1000, and 1:1000, respectively, and used to stain the cells overnight at 4°C. On the next day, cells were further incubated for 1 hour at 4°C in blocking solution containing Alexa 488-conjugated goat anti-mouse IgG antibody (Thermo) at 1:500. The nucleus was counterstained using DAPI.

All iPSC clones were further validated for their ability to differentiate into all three germ layers through teratoma formation. 1×10^6 cells were injected into each testis of at least three NOD-SCID mice, which were sacrificed after 8 to 12 weeks to excise the tissue. After fixation in 4% paraformaldehyde, the tissue was embedded in paraffin, sectioned, and stained with H&E. Markers for the three germ layers were stained using the Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D) according to the manufacturer's instructions.

Sclerotome induction (SI) and hypertrophic chondrocyte induction (HI)

For SI, CDMi (chemically defined medium with insulin) was used as base medium.[2] This medium consists of a 1:1 mixture of Iscove's modified Dulbecco's medium (IMDM) (Sigma) and Ham's F12 (Gibco), 5 mg/ml BSA (Sigma), 1x CD Lipid (Life Tech), 15 µg/ml apo-transferrin (Sigma), 450 µM monothioglycerol (Sigma), 50 U penicillin, 50 µg/ml streptomycin, and 7 µg/ml insulin (Wako). SI was performed as previously described.[3] In brief, on day 0 of induction, primitive streak cells were induced with 20 ng/ml bFGF, 10 µM CHIR99021 (Axon), and 50 ng/ml Activin A (R&D) for 24 hours. Then, presomitic mesoderm cells were induced with 10 µM SB431542 (Selleck Chemicals), 3 µM CHIR99021, 250 nM LDN193189 (Stemgent), and 20 ng/ml bFGF for 24 hours. Following this, somitic mesoderm cells were induced with 100 nM PD173074 (Tocris) and 1 µM XAV939 (Tocris) for 24 hours. Finally, sclerotome cells were induced with 100 nM SAG (Calbio) and 600 nM LDN193189 for 72 hours.

For HI, we used the same basal chondrogenic medium as previously described,[4] except for the lack of dexamethasone. This medium consists of 1% (v/v) ITS premix (Corning), 0.17 mM L-ascorbic acid 2-phosphate (Sigma), 0.35 mM proline (Sigma), 0.15% (v/v) glucose (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM Glutamax (Life Tech), 100 U penicillin, and 100 µg/ml streptomycin in DMEM/F12 (Gibco). To this medium, the factors described in the Experimental Procedures were sequentially added, and the medium was changed every 2-3 days.

mRNA expression analysis

For RNA extraction, cartilage pellets were first powderized using the Multi-beads shocker (Yasui

Kikai). Total RNA was extracted using the RNeasy Micro or Mini Kit (QIAGEN) with DNase treatment and reverse transcribed using ReverTra Ace (Toyobo) in a total volume of 20 µL with up to 300 ng of RNA. cDNA was diluted 1:10 and quantitative PCR (qPCR) was performed with 1 µl cDNA in duplicate reactions using the Thunderbird SYBR qPCR Mix (Toyobo) and QuantStudio 12K Flex Real-Time PCR System (Thermo). Primers are listed in Table S5. All results were normalized by the β-actin expression in each sample. For statistical analysis, unpaired two-sided t-tests were performed for comparisons between two groups. For comparisons between more than two groups, ANOVA with post-hoc Tukey HSD was used. Normal human growth plate tissue was obtained from the distal femoral physis of an 11-year-old female patient with a malignant bone tumor after total femoral replacement and used as control for differentiation markers.

Nonsense-mediated decay (NMD) detection

Genomic DNA was extracted from iPSCs and RNA was extracted on day 56 of HI of the COL10A1 L614Rfs*8 mutant and isogenic control. For the cycloheximide (CHX)-treated group, cartilage pellets were treated with 100 µg/ml CHX (Sigma) for 6 hours before RNA extraction. RNA was reverse transcribed with a negative control (no reverse transcriptase), and the cDNA was amplified for the NC1 domain with PCR. The amplicons were digested by the restriction enzyme StuI (NEB), which only recognizes the wild type sequence, and BceAI (NEB), which only recognizes the mutant sequence.

Protein expression analysis

On day 56 of HI, cartilage pellets were collected, PBS washed, and stored at -80°C. After adding 100 µl SDS sample buffer with 1% (v/v) protease inhibitor cocktail (Nacalai Tesque), the pellets were homogenized using homogenizer pestles (Kenis) and sonicated until complete dissolution. The samples were centrifuged and the supernatant was collected. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo). HSPA5, PDI, and ERO1A protein expression was detected with Simple Western (Wes) using the 12-230 kDa Separation Module (ProteinSimple) according to the manufacturer's instructions. For each lane, 5 µg of protein was used at a concentration of 1 µg/µl and antibodies were used at 1:50 for HSPA5, PDI, or ERO1A and at 1:100 for β-actin (all CST). All results were normalized by the β-actin values of each sample. ATF6, EIF2A, and phospho-EIF2A protein expression was detected using Western blotting. 12 µg of protein was used per lane of 10% polyacrylamide gel at a concentration of 2 µg/µl. After SDS-PAGE and transfer to PVDF membranes, blocking was performed for 1 hour using 5% skim milk (Nacalai) or 3% polyvinylpyrrolidone (Sigma). Primary antibodies were used at 1:1000 in 5% skim milk or Can Get

Signal 1 (Toyobo) for overnight staining with shaking at 4°C. The secondary antibodies, HRP-conjugated goat anti-rabbit or horse anti-mouse IgG (both CST), were used at 1:5000 in 5% skim milk or Can Get Signal 2 (Toyobo) for 1 hour at room temperature while shaking. The ECL Prime Western Blotting Detection Reagent (GE Healthcare) and the BIO-RAD Chemidoc XRS+ were used to detect the signal. After detection, membranes were stripped of the antibodies using WB Stripping Solution (Nacalai), stained with peroxidase-conjugated β-actin antibody (Sigma), and visualized as above. The signal intensities were quantified using ImageJ. For statistical analysis, unpaired two-sided t-tests were performed.

Microarray analysis

After RNA extraction, RNA quality was confirmed using the RNA 6000 Nano Kit (Agilent Technologies). The RNA was reverse transcribed into cDNA and amplified with the Ambion WT Expression Kit, fragmented and labeled with the GeneChip WT Terminal Labeling and Controls kit, and hybridized to Human Gene 1.0ST Arrays using the GeneChip Hybridization Wash and Stain Kit according to the manufacturer's protocol (Affymetrix). The arrays were scanned and the raw data were imported into GeneSpring GX 14.9 for analysis. The data were normalized using the RMA-16 algorithm and the baseline was adjusted to the median of all samples. A moderated t-test was performed on all samples (n=3 independent experiments) to stabilize the variance estimate in the low number of replicates.[5] The Benjamini-Hochberg correction was used to account for multiple testing. For Figure 1, all genes with adjusted p value < 0.05 and fold change > 1.5 in day 56 vs day 28 pellets were used for Ingenuity Pathway Analysis (IPA). In IPA, Canonical Pathways and Diseases & Functions were analyzed. Heatmaps were generated in Genespring using the averaged normalized intensity values for each sample. For Figure 6, all genes with adjusted p value < 0.05 were used for IPA. Heatmaps were generated in Microsoft Excel using each mutant's log2 fold change of UPR genes.

Flow cytometry analysis

To determine the induction efficiency of presomitic mesoderm, cells were analyzed by flow cytometry on day 2 of SI. Cells were detached and washed with FACS buffer containing 0.1% (w/v) BSA in PBS. Approximately 5×10^5 cells were stained with either APC-conjugated DLL1 antibody (R&D) or APC-conjugated mouse IgG2B (R&D) as isotype at a 1:200 dilution in FACS buffer for 30 minutes at 4°C. Following another wash with FACS buffer, the cells were stained with 1 μg/ml DAPI (R&D) to label dead cells. Analysis was performed using the FACS Aria II and the FACSDiva software (BD). Graphs were created using the FlowJo software. For statistical analysis, unpaired two-sided t-

tests were performed.

Histological analysis

Paraffin-embedded tissue sections were deparaffinized and stained with H&E, von Kossa, and Safranin O. TUNEL staining was performed using the ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Millipore) with proteinase K treatment according to the manufacturer's instructions. For immunofluorescence staining, antigen retrieval was performed by 40 minutes incubation at 37°C in 10 mg/ml hyaluronidase (Sigma) and 15 minutes incubation at 80°C in 1 mM EDTA (Wako). After blocking in 10% FBS (Nichirei) for 1 hour, the tissue was stained at 4°C overnight using antibodies against COL1 (Novus Biologicals) at 1:600, COL2 (Thermo) at 1:1000, COL10 (Thermo) at 1:300, IHH (abcam) at 1:800, and MATN3 (abcam) at 1:500 in Can Get Signal Immunostain Solution B (Toyobo). On the next day, the samples were incubated at room temperature for 1 hour in the same solution with Alexa 488-conjugated goat anti-mouse IgG antibody (Thermo) at 1:1000, Alexa 555-conjugated goat anti-rabbit IgG antibody (Thermo) at 1:1000, Alexa 647-conjugated donkey anti-mouse IgG antibody (Thermo) at 1:1000, and Alexa 647-conjugated PDI antibody (Thermo) at 1:500. DAPI was used as a nuclear counterstain at 10 μg/ml and the samples were observed with the BZ-X810 (Keyence). For immunohistochemistry, antigen retrieval was performed by 40 minutes incubation in EDTA at 98°C or 5 minutes incubation in proteinase K at room temperature, and blocking was performed using 3% BSA. Samples were incubated for 30 minutes at room temperature with antibodies against Ki67 (Nichirei), or overnight at 4°C with antibodies against COL1 (SBA) at 1:500, COL2 (SBA) at 1:600, or HSPA5 (CST) at 1:1600. Simple Stain MAX PO (R) or (G) (Nichirei) was used to detect the signal.

Image analysis and quantification

All samples were quantified using the BZ-X800 Analyzer software and statistical analysis was performed using unpaired two-sided t-tests. Pellet size was quantified using images taken of live pellets from 3 technical replicates each of 4 independent experiments on day 56 of HI with the BZ-X810 (Keyence). The size was calculated by taking the average of the major axis of each pellet.

For quantification of the COL2 and TUNEL stainings, 4 images each were taken of 4 independent experiments at x20 magnification, with the images being taken at the top, bottom, left, and right sides of the pellet, at a depth of approximately 500-1000 μm from the surface to avoid both the necrotic center and the disintegrating periphery. Dead cells of each sample were quantified by counting the number of TUNEL-positive cells per field of view (FOV). Cell size was quantified by selecting the inverse of COL2 staining and measuring the average area of the selection.

For the quantification of COL10, MATN3, and PDI stainings, 4 images each were taken of 4 independent experiments at x40 magnification randomly throughout the pellet at a depth of approximately 500-1000 μm from the surface to avoid both the necrotic center and the disintegrating periphery. Intracellular retention of COL10 and MATN3 was calculated by dividing the total green fluorescence intensity of the area co-staining with PDI (red) by the total green fluorescence intensity of the whole image. The ER size was quantified from the average area of PDI fluorescence.

Transmission electron microscopy (TEM)

On day 56 of HI, pellets were PBS washed and fixed in 2.5% glutaraldehyde for 8 hours at 4°C. After PBS washing and post-fixation in 2% osmium tetroxide for 2 hours, the pellets were dehydrated and embedded in Quetol 812 resin. 1 μm sections were taken and stained with toluidine blue. Ultra-thin sections (120 nm) were taken and stained on grids with uranyl acetate and lead citrate. Images were taken using the H-7500 transmission electron microscope (Hitachi) with the NanoSprint500 (AMT).

Supplemental References

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