Allelic Heterogeneity Contributes to Variability in Ocular Dysgenesis, Myopathy, and Brain Malformations Caused by *Col4a1* and *Col4a2* Mutations

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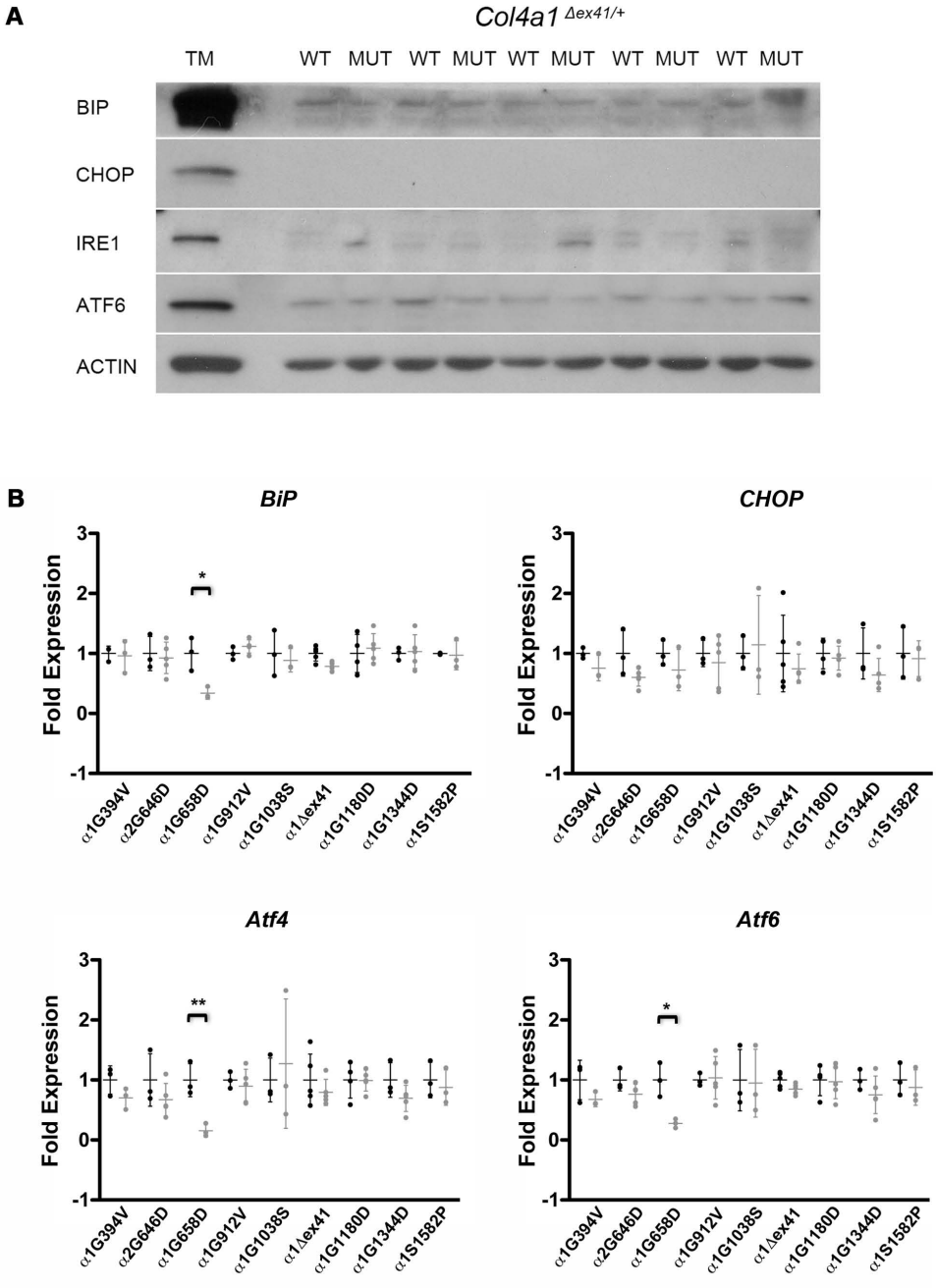
Supplementary Material:

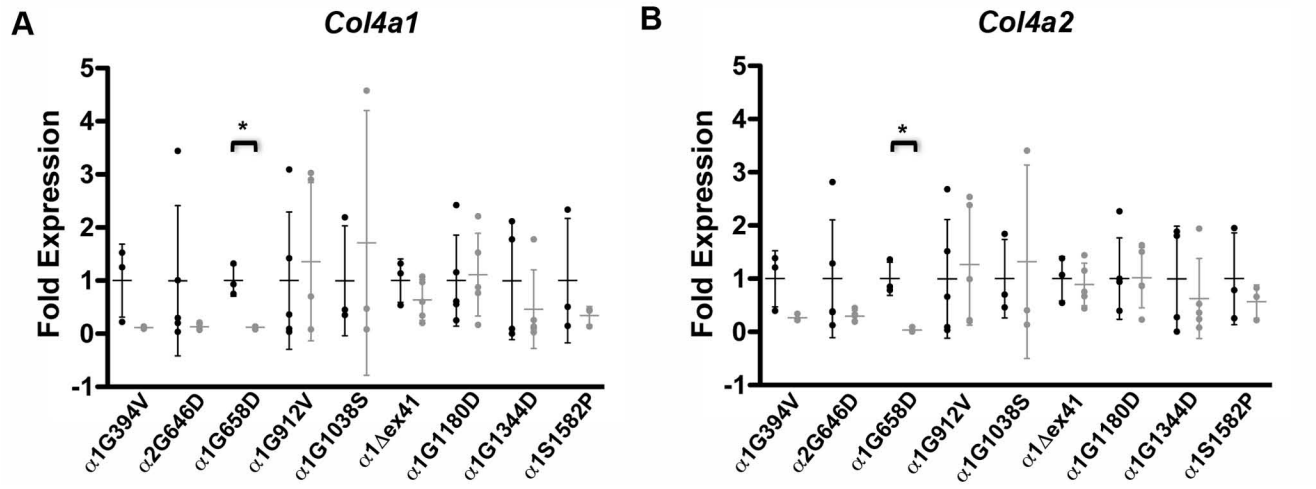
Supplemental Figures 1- 6

Supplemental Table 1. Genotyping Primer Sequences

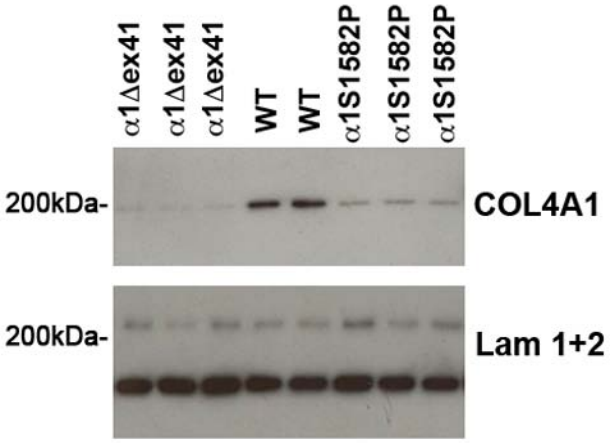
Supplemental Table 2. qRT-PCR Primer Sequences



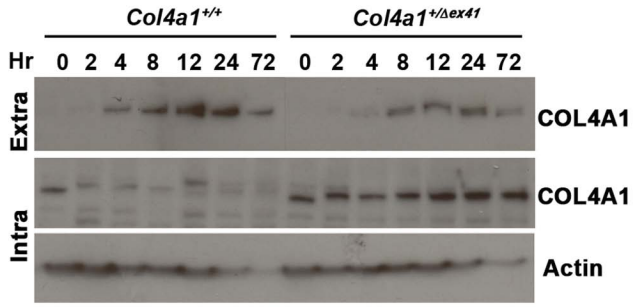
Supplemental Figure 1. *Col4a1* and *Col4a2* mutant MEFs do not show induction of ER stress.
(A) Western blot and (B) quantitative RT-PCR analyses did not detect ER stress response in *Col4a1* and *Col4a2* mutant MEFs. Presence of ER stress was investigated by measuring protein and gene expression levels of the ER chaperone BIP, the ER stress sensor IRE-1, ER stress response pathway transcription factors ATF6 and ATF4, and one of the downstream targets CHOP. (A) Representative Western blot images from *Col4a1*$^{+/\Delta ex41}$ MEFs showing that the expression levels of BiP, CHOP, IRE-1 and ATF6 are extremely low to barely detectable in wild-type (WT) and mutant (MUT) MEFs. Tunicamycin (TM) treated MEFs were used as a positive control to induce expression of ER stress markers. Actin was used as a loading control. Similar results were observed in MEF with all other *Col4a1* and *Col4a2* mutations in the allelic series (data not shown). (B) Quantitative RT-PCR showing that the level of expression of ER stress markers is not significantly different between mutant (grey circles) and WT (black circles) MEFs, with the exception *Col4a1*$^{+/G658D}$ MEFs, which showed reduced expression of BiP, ATF4 and ATF6. Lines show mean +/- standard deviation. Comparisons between wild-type (WT) and mutant from each strain were performed using Student's t-test; *, $p<0.05$; **, $p<0.01$. N = 3 to 5 independent mutant lines per allele.



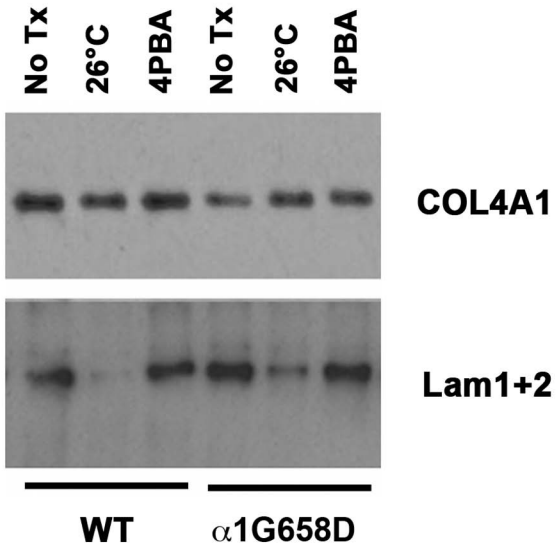
Supplemental Figure 2. *Col4a1* and *Col4a2* gene expression in mutant MEFs.
Quantitative RT-PCR for *Col4a1* and *Col4a2* were performed on MEFs derived from the different *Col4a1* and *Col4a2* mutant mice. With the exception of *Col4a1*$^{+/G658D}$, which showed reduced levels of both *Col4a1* and *Col4a2* RNA, *Col4a1* and *Col4a2* mutations did not significantly alter the levels of *Col4a1* and *Col4a2* gene expression. Lines show mean +/- standard deviation. Student's t-test was used to compared the relative expression between *Col4a1*$^{+/+}$ (black circles) and *Col4a1*$^{+/mut}$ or *Col4a2*$^{+/mut}$ (grey circles) MEFs; *, $p<0.05$. N = 3 to 5 independent mutant lines per allele.



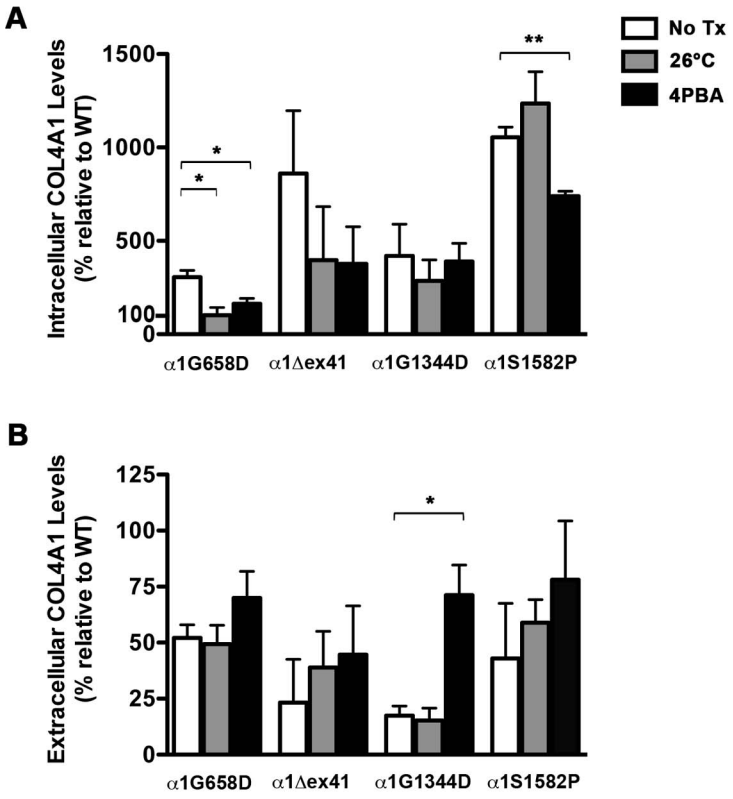
Supplemental Figure 3. Validation of loading volume of conditioned medium using laminin. Representative Western blot showing extracellular levels of COL4A1 and Laminin 1+2 in *Col4a1*$^{+/+}$ and *Col4a1*$^{+/mut}$ MEFs. Conditioned media from *Col4a1*$^{+/+}$ and *Col4a1*$^{+/mut}$ MEFs was collected after induction of collagen secretion and loading volumes were normalized to the total protein concentration of the cellular fraction and ran under reducing conditions to evaluate levels of laminin, another major basement membrane component, in *Col4a1*$^{+/+}$ and *Col4a1*$^{+/mut}$ MEFs. Similar results were observed for *Col4a1*$^{+/G658D}$ and *Col4a1*$^{+/G1344D}$ MEFs (data not shown). N= 3 MEF lines per mutation and 8 MEF lines for *Col4a1*$^{+/+}$.



Supplemental Figure 4. Kinetics of COL4A1 secretion.
Representative Western blot images depicting extracellular and intracellular levels of COL4A1 in *Col4a1*$^{+/+}$ and *Col4a1*$^{+/\Delta ex41}$ MEFs at different time points (expressed in hours) after the induction of COL4A1 secretion (serum deprivation and ascorbic acid supplementation). All mutations examined displayed a similar kinetic pattern of secretion and accumulation although the relative intracellular and extracellular levels were allele–dependent. Actin was used as a loading control. Intra: Intracellular, Extra: Extracellular.



Supplemental Figure 5. Effects of reduced temperature and 4PBA on COL4A1 secretion in *Col4a1*$^{+/+}$ and *Col4a1*$^{+/mut}$ MEFs.
Representative Western blot images showing extracellular levels of COL4A1 and Laminin 1+2 in conditioned medium of *Col4a1*$^{+/+}$ and *Col4a1*$^{+/G658D}$ MEFs run under non-reducing conditions. Similar results were observed for other mutant MEF lines examined: *Col4a1*$^{+/\Delta ex41}$, *Col4a1*$^{+/G1344D}$ and *Col4a1*$^{+/S1582P}$. N= 3 *Col4a1*$^{+/+}$ and 3 *Col4a1*$^{+/mut}$ MEF lines per strain.



Supplemental Figure 6. Effects of reduced temperature and 4PBA on COL4A1 biosynthesis in *Col4a1* mutant MEFs relative to wild-type MEFs.
Quantitative analysis of intracellular and extracellular COL4A1 levels in *Col4a1*$^{+/mut}$ MEFs cultured under normal growth conditions (No Tx), at reduced temperature (26°C) or in the presence of 4PBA compared to their *Col4a1*$^{+/+}$ counterparts. COL4A1 intracellular and extracellular levels were normalized on tubulin and laminin 1+2 levels respectively. Values are presented as mean +/- standard error of the mean. For statistical analyses (Student's t-tests; *, $p<0.05$, N= 3 MEF lines per mutation) comparisons are made between each treatment and non–treated cells with the same mutation.

Supplemental Table 1. Genotyping Primer Sequences

Amino Acid	Line	SNP Forward Primer	SNP Reverse Primer
α1G394V	*Col4a1*^{ENU4004}	ATTACACAGCGCTGCTTCTAGC	CACCTTGTGTGACTCCTTAGCA
α1G658D	*Col4a1*^{Acso}	CTGTGGTAGCCTTCTTGTGGT	TGAATGCGAAACACTTAAGGA
α1G912V	*Col4a1*^{ENU911}	GATTGCTGGCAGATAATGA	CGTAAGCAATTGCTGTAG
α1G1038S	*Col4a1*^{ENU6024}	CTTCTTCCTCCCTGTGATATG	TAAGCAGAGCGATTTGTGTTG
α1Δex41	*Col4a1*$^{del.G1169\text{-}K1185}$	TTTCCCAGTCACGACGTTGAAACTGAGCTTGGGTCCTCTG	GTGTCTTGGATCAGAACCAGTGGGACA
α1G1180D	*Col4a1*^{ENU6005}	TTGGCAGGTACCACATATCACA	CTGTCCTGCCTCTGTAGGAGAA
α1G1344D	*Col4a1*^{ENU6019}	TGTTGAGTTGTAAGCCTCTGGTC	CATTTGATGAGGCTCTGTGATG
α1S1582P	*Col4a1*^{ENU6009}	AAGGGATGATCTGGGAAGTTTG	CAGGAGGGACTTTGTCCTCAG
α2G646D	*Col4a2*^{ENU4003}	CACTGTGCTGAAAGTCAGTGCT	CTGGTCACATGTGCATGAATCT

Supplemental Table 2. qRT-PCR Primer Sequences

Gene	Forward Primer	Reverse Primer
Gapdh	GGGAAGCCCATCACCATCTT	GCCTTCTCCATGGTGGTGAA
Hprt1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
Col4a1	GCAGGAGAGAAGGGTGAAC	GGTCCTCGGTCTCCTTTGG
Col4a2	CATCCGTCGGAGATGAAGAT	TAGCCCACTCTCCCTTCTGA
BiP	TCATCGGACGCACTTGGAA	CAACCACCTTGAATGGCAAGA
ATF6	TTTGATGCCTTGGGAGTCAG	GATGGAGCAACTGGAGGAAG
ATF4	GCGTATTAGAGGCAGCAGTG	GAAGAGCGCCATGGCTTAG
CHOP	GTCCCTAGCTTGGCTGACAGA	TGGAGAGCGAGGGCTTTG