ER Stress-Mediated Apoptosis in a New Mouse Model of Osteogenesis imperfecta

Thomas S. Lisse1, Frank Thiele1, Helmut Fuchs1, Wolfgang Hans1, Gerhard K. H. Przemeck1, Koichiro Abe1b, Birgit Rathkolb1,2, Leticia Quintanilla-Martinez2, Gabriele Hoelzlwimmer3, Miep Helfrich4, Eckhard Wolf2, Stuart H. Ralston5, Martin Hrabe de Angelis1*

1 Institute of Experimental Genetics, Helmholtz Zentrum Munchen, German Research Center for Environmental Health, Neuherberg, Germany, 2 Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians University, Munich, Germany, 3 Institute of Pathology, Helmholtz Zentrum Munchen, German Research Center for Environmental Health, Neuherberg, Germany, 4 Department of Medicine and Therapeutics, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, United Kingdom, 5 Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh, United Kingdom

Osteogenesis imperfecta is an inherited disorder characterized by increased bone fragility, fractures, and osteoporosis, and most cases are caused by mutations affecting the type I collagen genes. Here, we describe a new mouse model for Osteogenesis imperfecta termed Aga2 (abnormal gait 2) that was isolated from the Munich N-ethyl-N-nitrosourea mutagenesis program and exhibited phenotypic variability, including reduced bone mass, multiple fractures, and early lethality. The causal gene was mapped to Chromosome 11 by linkage analysis, and a C-terminal frameshift mutation was identified in the Col1a1 (procollagen type I, alpha 1) gene as the cause of the disorder. Aga2 heterozygous animals had markedly increased bone turnover and a disrupted native collagen network. Further studies showed that abnormal proc1(I) chains accumulated intracellularly in Aga2/+ dermal fibroblasts and were poorly secreted extracellularly. This was associated with the induction of an endoplasmic reticulum stress-specific unfolded protein response involving upregulation of BiP, Hsp47, and Gadd153 with caspases-12 and −3 activation and apoptosis of osteoblasts both in vitro and in vivo. These studies resulted in the identification of a new model for Osteogenesis imperfecta, and identified a role for intracellular modulation of the endoplasmic reticulum stress-associated unfolded protein response machinery toward osteoblast apoptosis during the pathogenesis of disease.

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Introduction

Mutations in type I collagen genes (COL1A1/2) typically lead to Osteogenesis imperfecta (OI), the most common heritable cause of skeletal fractures and bone deformation in humans [1]. OI is classified into eight human subtypes, and to date greater than 500 human COL1A1 mutations have been reported representing a clinical heterogeneity dictated by the complex array of mutations. Recently, novel molecules and loci apart from classic type I collagens have been implicated in both murine [2] and human [3–5] alternative recessive forms of OI, thus expanding the genetic heterogeneity.

Type I collagen is the most common ubiquitously expressed fibrous protein in the extracellular matrix (ECM) of connective tissues with both biomechanical and physiological functions [6]. Type I collagen initially exists as a procollagen precursor with NH2- and COOH-terminal propeptide domains with distinct roles. Type I procollagen molecules consist of three polypeptide coiled subunit chains (two proc2(l) and one proc2(l) chain) that self-associate in the endoplasmic reticulum (ER), and require a highly coordinated post-translational regulation. The helical procollagens are deposited into the extracellular space, proteolytically cleaved, and then organized into highly ordered collagen fibrils covalently cross-linked to increase tensile strength and rigidity. Apart from its biomechanical properties, type I collagen stores key factors for remodeling maintenance, and acts as an adhesive substrate with cellular receptors and other matrix-membrane components along its major ligand binding regions [7]. These properties regulate complex intracellular signal transduction pathways for tissue remodeling and repair, immune response, polarization, migration, differentiation, and cell survival within various cellular contexts [8].

Based on detailed radiographic, molecular genetic and morphological analyses, structural collagen mutations are likely associated with lethal (type II) and moderate (types III

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Abbreviations: ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; ECM, extracellular matrix; ENU, N-ethyl-N-nitrosourea; ER, endoplasmic reticulum; OI, Osteogenesis imperfecta; UPR, unfolded protein response

* To whom correspondence should be addressed. E-mail: hrabe@helmholtz-muenchen.de

1a Current address: Bone and Extracellular Matrix Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, United States of America

1b Current address: Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University Medical School, Isehara-shi, Kanagawa, Japan
Author Summary

Osteogenesis imperfecta (OI) is a heterogeneous collection of connective tissue disorders typically caused by mutations in the COL1A1/2 genes that encode the chains of type I collagen, the principle structural protein of bone. Phenotypic expression in OI depends on the nature of the mutation, causing a clinical heterogeneity ranging from a mild risk of fractures to perinatal lethality. Here, we describe a new OI mouse model with a dominant mutation in the terminal C-propeptide domain of Col1a1 generated using the N-ethyl-N-nitrosourea (ENU) mutagenesis strategy. Heterozygous animals developed severe-to-lethal phenotypes that were associated with endoplasmic reticulum stress, and caspases-12 and −3 activation within calvarial osteoblasts. We provide evidence for endoplasmic reticulum stress-associated apoptosis as a key component in the pathogenesis of disease.

Results

Identification and Lethality of Aga2/+

The original Aga2/+ mouse was identified in the Munich ENU dominant mutagenesis screen [14], and displayed an abnormal gait due to deformity of the hind limbs at five weeks of age among the F1 mutagenized progeny. Aga2/+ males have general reproductive success and the mutation segregated in a dominant manner with complete penetrance (Table 1). Aga2/+ females produced smaller litter sizes due to a reduction in body size. A large subset of Aga2/+ animals succumbed to postnatal lethality (Table 1), and featured severe bone deformities and fractures. Aga2/+ inter se mating yielded non-Mendelian ratios for dominant inheritance suggesting embryonic lethality. Various embryonic stages were investigated to determine gestational arrest in homozygotes, which was estimated to occur around embryonic day 9.5 post coitum (data not shown).

Positional Cloning and Characterization of the Aga2 Mutation

Genetic mapping of the Aga2 locus was performed following the standard outcross-backcross breeding strategy. The mutation was mapped to a 700 kb domain on Chromosome 11 (Figure S1). Within the Aga2 candidate region, two skeletal genes were identified, Col1a1 (procollagen type I, alpha1) and Chad (chondroadherin). Both candidate genes were sequenced and a novel T to A transversion mutation within intron 50 of Col1a1 was identified (Figure 1A). The Aga2 substitution generated a novel 3′ splice acceptor site and predicted a terminal frameshift beyond the endogenous stop (Figure 1C). On the transcript level, cDNA from homozygous embryos revealed a 16 bp expanded transcript, whereas in heterozygous embryos equal levels of two transcripts are present (Figure 1B). We further confirmed the mutation at the genomic level. The T to A exchange disrupted the endogenous MspA1I restriction site, generating a cleavage-resistant allele in Aga2/+ samples (Figure 1B).

The C-terminal portion of human, and murine as well as Aga2 mutant Col1a1 was aligned highlighting specific residues of importance (Figure 1C). Of interest, the most terminal conserved cysteine C244 (aa 1451) was ablated, and 48 endogenous amino acids including the stop were frameshifted, concomitantly predicting 90 new amino acids beyond the termination position. The Aga2 frameshift begins 56

Table 1. Mode of Inheritance and Postnatal Lethality

| Mating | Breeding Pairs | Litter Size | Postnatal Progeny |
|--------|----------------|-------------|-------------------|
|        |                |             | AGA2+/+           |
|        |                | +/+         | +/+               |
|        |                | Female      | Male              |
|        |                | Male        | Female            |
|        |                |              |                   |
| Aga2/+ × C3H | 14             | 7.1 ± 0.42  | 19 26 26 29       |
|          | Lethal to weaning |      | 2 (11%) 11 (42%) 3 (12%) 17 (58%) |
| C3H × Aga2/+  | 18             | 5.4 ± 0.51  | 23 25 22 29       |
|          | Lethal to weaning |      | 3 (13%) 15 (60%) 1 (5%) 20 (69%) |
| Aga2/+ × Aga2/+ | 4              | 4.3 ± 0.54  | 3 8 2 5           |
|          |                |              | No homozygous     |

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amino acids away from the chain selectivity sequence [15]. Additionally, five new cysteines and a potential N-linked oligosaccharide (CHO) attachment site were introduced. The secondary structure of the Aga2 product was predicted to remove the hydrophobicity of the short loop formed by the last intra-chain disulphide bond (data not shown).

Aga2 Skeletal and Growth Phenotypes

Among all Aga2+ animals, the gross skeletal phenotype was discernable starting between days 6 - 11 after birth. Heterozygous animals that survived to adulthood were classified as moderately-to-severely affected and displayed the hallmark dystrophic limb(s), long bone and pelvis fractures, reduced body size, and generalized decreases in DXA-based bone parameters (Figure 2 and Table S1; data were collected from the German Mouse Clinic dysmorphology primary screen [16]). Lethal animals developed thin calvaria, hemorrhaging at joint cavities and intracranial sites, scoliosis, provisional rib and long bone calluses and deformities, body size deficit, pectus excavatum, gasping, cyanosis, platyspondyly, edema of the eyes, greasy skin, and eczema (Figure 2 and data not shown). Comminuted fractures incurred signifying the severe brittleness of Aga2+ bones accompanied by many repair blastemas characteristic of a type II OI phenotype.

The effect of the mutation on volumetric bone mineral density (vBMD) and content (vBMC) was evaluated in adult mice using in vivo peripheral quantitative computed tomography (pQCT; Table S2). In Aga2+ distal femora, trabecular and cortical vBMD as well as cortical vBMC were substantially decreased compared to controls. In contrast, the trabecular vBMC was unchanged due to enlarged medullar areas (suggesting elevated resorption in Aga2+). Collectively, these results suggest potential defects in mineralization and/or bone formation in Aga2+ mice.

Figure 1. The C-Propeptide Col1a1Agag2 Mutation

(A) Mutation scheme. In Aga2, splicing occurs at the new splice acceptor site (ag, highlighted red). The taat domain (underlined) harbors a putative splicesosome branch site (*).

(B) Genotyping and presence of the extended transcript.

(C) Alignment of human and murine Col1a1 terminal C-propeptides and prediction of the Aga2 frameshifted sequence. The start of mouse exon 51 (S2 in human) is marked by a blue line. The frameshifted cysteine (C244) is boxed in red, newly introduced cysteines are underlined in blue; the potential N-linked oligosaccharide (CHO) attachment site is boxed in blue. Start of the human frameshift mutation described by Willing et al. [30] is boxed in black.

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Clinical Chemical and Hormone Analysis

Several serum biochemical and hormonal markers were evaluated to detect possible bone metabolic disturbances (Table S3). Total alkaline phosphatase (ALP) and osteocalcin levels were significantly increased in both sexes in Aga2+/ compared to littermate controls. In addition, circulating TRACP 5b (an osteoclast marker) was significantly elevated in Aga2+. No changes in inorganic phosphate levels were observed (data not shown).

Aga2+/ animals depicted a significant increase in PTH levels. Furthermore, Aga2+/ mice yielded a distinct increase in calcitonin, which was more pronounced in females. Taken together, the significantly increased bone formation and resorption markers indicate that in Aga2+/ mice metabolic bone turnover is elevated. This was further supported by histomorphometric analysis of bone formation and resorption showing increased numbers of endogenous osteoblasts and osteoclasts, an increased bone formation rate (BFR) and a reduced mineral apposition rate (MAR) in Aga2/+ bone samples (Figures S2, S3 and Table S4).

Altered Cellular and Collagen Structures in Aga2+/+

Fibroblast and type I collagen features in Aga2+/+ connective tissues were evaluated by transmission and scanning electron microscopy (TEM and SEM). Aga2+/+ dermis contained more heterogeneous populations of fibroblasts with smaller nuclei, aberrant dilated electron-dense ERs, lysosomes and empty autophagic-like vacuoles interspersed throughout the cells (Figure 3A and 3B). Aga2+/+ ERs appeared contiguous with secretory vesicles signifying the formation of ER associated compartments. SEM studies on cortical nanostructure of Aga2+/+ samples depicted a less-parallel, less-densely packed network of collagen bundles when compared to controls (Figure 3C–3F).

Procollagen trafficking was assessed in immunofluorescence studies using dermal fibroblasts (Figure 4). To uncover mature type I collagen integrity we used an antibody that was directed against the triple helical domain and was also capable of detecting procollagens (Figure 4A). Wild-type cells depicted both intracellular and intact extracellular surface staining (Figure 4B). In Aga2+/+ cells, the intact type I collagen surface stain was largely reduced while intracellularly retaining procollagen molecules were stained (Figure 4C). Double immunofluorescence staining of proα1(I) chains was performed using antiserum LF-67 (Figure 4A), protein disulfide isomerase (PDI), and Golgi phosphoprotein 4 (GOLPH4) for detection of the ER and cis-Golgi apparatus, respectively. Merged stainings showed proα1(I) chain retention within the ER (Figure 4E) but not in the cis-Golgi (Figure 4G), when compared to controls (Figure 4D and 4F). It is speculated that aberrant procollagen molecules aggregate into vesicular structures (Figure 4E, merged green dots), which may be part of the proteasome. To further clarify the intracellular abnormalities double immunofluorescence staining of proα1(I) chains was performed using antiserum LF-41 and β-actin antibody for the cytoskeleton. The LF-41 epitope resides within the wild-type terminal C-propeptide...
overlapping segment of the Aga2 frameshifted region and captures only molecules that contain chains with the normal C-propeptide (Figure 4A). Using antiserum LF-41, control fibroblasts depict dynamic intracellular procollagen and isoform trafficking to the infoldings of the plasma membrane (Figure 4H). Contrarily, Aga2+/+ fibroblasts retained intracellular (wild-type) procollagen molecules at near perinuclear regions (Figure 4I, arrow). Surface staining in both wild type and Aga2+/+ cells was limited given that the LF-41 antiserum was unable to discriminate mature, proteolysed type I collagen in the ECM. Based on our results, the mutation affected proα1(I) chain processing by blocking its ER-to-Golgi anterograde transport, thus inhibiting vesicular exocytosis to the matrix.

**Altered Ex Vivo Osteogenic Activity in Aga2+/+ Calvarial Culture System**

OB metabolism and function were characterized utilizing the primary calvarial culture system. We determined the growth curve of Aga2+/+ primary OBs and observed a limited saturation density within the stimulated samples (Figure 5A) that was accompanied by a relative increase in ALP levels until day 26 (Figure S4A). Also, Aga2+/+ total protein and mitochondrial reductase activity levels were significantly increased (Figure S4B). Consistent with our immunofluorescence studies there was a clear reduction in the total amount of secreted acid-soluble collagens tested in Aga2+/+ media (Figure S4B). Of note, unstimulated Aga2+/+ OBs depicted no significant change in cellular protein and metabolic activity levels (data not shown).

To address the functional defects in Aga2+/+ OBs, nodular formation, growth and binding capacities were studied over time (Figure S4C). The formation of bone-like nodules was persistently reduced between days 9 and 16 in Aga2+/+. In addition, the average area of individual Aga2+/+ nodules was reduced compared to controls. Lastly, a 3.2 fold comparative increase in nodular dye-binding capacity was observed in Aga2+/+ (Figure S4C). Taken together, these results demon-

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**Figure 3.** Electron Microscopical Findings in Dermal Fibroblasts and Cortical Bone

(A,B) TEM micrographs of wild-type and Aga2+/+ fibroblasts. The rough endoplasmatic reticulum (rER) is engorged in Aga2+/+ cells (bars, 2 μm for main, 1 μm for inset). Increased intracellular empty vacuoles (arrow heads) and lysomes (L) were present in Aga2+/+. (C–F) SEMs of femoral endosteal surfaces reveal differences in organization of the collagen matrix (C,D) Outer cortical surface. (E,F) Inner cortical surface.

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strate clear functional and metabolic disturbances in Aga2+/OBs.

Initiation of ER Stress-Induced Apoptosis in Aga2+/OBs

To show involvement of key regulators of ER stress response pathways, we performed qPCR studies using primary calvarial OBs (Figure 5B). At days 16 and 26 in culture (11 and 21 days after induction, respectively), expression of the molecular chaperones genes Hspa5 (also known as BiP/GRP78) and Serpinh1 (also known as Hsp47) was upregulated; 1.5 – 2.2 folds above control. The expression of Ddit3 (also known as Gadd153/chop), a transcription factor involved in the induction of apoptosis, was ~3.7 folds higher in Aga2+/cells at day 26 compared to control levels. To confirm the induction of cytoprotective unfolded protein response (UPR) in Aga2+/ cells, Western analysis of BiP protein expression was performed using primary calvarial OBs. As shown in Figure 5C BiP protein levels were increased compared to controls.

Within the context of ER-induced stress we investigated cell apoptosis. The Aga2 mutation led to a combined relative increase in the number of early-late stage TUNEL-positive picnotic OBs compared to control (Figure 6E). At 16 days in culture, Aga2+/ preparations depicted a 10 % relative increase in the number of OBs, which contained aberrant caspase-12-immunoreactivity (Figure 6A and 6B; 3.3 ± 0.9 % +/- and 12.9 ± 1.9 % Aga2+/; p = 0.0007 Student t-test). For specificity, proteolytic processing of procaspase-12 was evident only in Aga2+/ samples via Western analysis (Figure 6G). Caspase-3/7 activation was independently confirmed depicting a significant 9 % comparative increase in activity in Aga2+/ OBs (Figure 6F). Lastly, activated caspase-3 immunoreactivity was observed within femoral periosteum (Figure 6C and 6D). Aga2+/ tissue contained elevated relative numbers of activated caspase-3-positive OBs (28.9 ± 2.7 % +/- and 42.6 ± 4.1 %
increased bone fractures, fragility, deformity, osteoporosis,
and disorganized trabecular and collagen structures. Com-
pared to the lethal transgenic lines [17,18], the Aga2+/+
phenotype is stronger and associated with increased lethality,
but comparable to the BritIV knock-in (Col1a1G310C) line [19].
The biochemical serum and histomorphometric results of
Aga2+/+ mice indicated an elevated bone turnover that is
described similarly for OI patients [20–23]. Our ex vivo
studies showed that Aga2+/+ OBs deposited less collagen
matrix, and were overly active, affecting nodule growth and
function.

Aga2+/+ proα1(I) Chain Defects and Human Case
Correlations

The procollagen triple helix proceeds from the carboxyl to
amino end, and this association is modulated by correct
folding via intra-chain disulfide bonds in the C-propeptide
region before chain association and inter-chain disulfide
linkage [24,25]. In certain C-propeptide OI-II lethal con-
ditions, the mutated proα1/2(I) chains can associate with
normal chains to form secreted triple helical procollagen
molecules despite alterations in endogenous disulfide bonds
[12,26], while in other lethal cases chain formation with the
mutated propeptide is entirely or largely precluded [27,28].
Based on these results, the lethal OI phenotypes are
heterogeneously derived from structural and/or cellular
metabolic defects.

At the biochemical level, we report significant increased
retention of aberrant procollagen molecules within Aga2+/+
cells, compromising cellular metabolism and the ECM.
Moreover, we show that the majority of accumulated chains
were wild type in nature. Gel analysis of cDNA showed
significant splicing of the new Aga2 splice site. Although
cryptic mRNA transcripts were readily available for trans-
lation, unstable mRNA decay and/or anomalies in transport
from the nucleus to the cytoplasm due to secondary structure
may have mediated low-level translation of mutant chains.
The presumptive elongated chain with newly introduced
cysteins and disruption of a crucial beta sheet due to the
mutation likely destabilized protein conformation, thus
affecting preferential chain assembly and proteolysis. Also,
a N-linked oligosaccharide unit was introduced capable of
altering intracellular processing and secretion of procol-
lagen as well [29].

Our findings were consistent with observations made by
Willing et al. [30] who investigated a human OI family that
harbored a mutation in the 3′ end of COL1A1, which ablated
the intra-chain disulfide cysteine as in the Aga2 condition.
Fitzgerald et al. [13] reported that the fate of mutated
unassembled C-propeptide chains from the proband de-
scribed by Willing et al. were intracellularly degraded in
the proteasome. Similar to Aga2, it was unclear if the mutated
chains ever incorporated into procollagen molecules causing
protein suicide and the expected downstream effects [31], or
involved an alternative mechanism to influence the clinical
outcome.

ER Stress and Degradation within Aga2+/+ Cells

Many disorders result from the cell’s inability to export
mutated proteins and enzymes from the ER, including OI
[32,33]. Depending on the nature of the mutation, incorrectly
folded proα1/2(I) molecules are managed via multipartite

Discussion

ENU mutagenesis is a powerful approach to identify mouse
models for human skeletal disorders by generating novel
phenotypes. Human mutations affecting the α1/2(I) C-
propeptide coding and non-coding regions have been
identified less often than other types of mutations and reflect
strong variability in clinical outcomes. Here, we provide the
first report on the cloning and phenotypic description of the
novel mouse line Aga2, which bear a chemically induced
generically stable mutation affecting the terminal C-propep-
tide domain of Col1a1. We provide evidence that abnormal
proα1(I) chains accumulated intracellularly and were poorly
secreted extracellularly. Furthermore, we show for the first
time that this was associated with the induction of an ER
stress-specific unfolded protein response (UPR) with caspases
12 and 3 activation and apoptosis of osteoblasts both in vitro
and in vivo. The Aga2 line provides a unique tool for
understanding the underlying mechanisms of the incurable
debilitating human disease Osteogenesis imperfecta (OI) and the
efficacy of novel therapeutic strategies.

Aga2+/+ Skeletal Phenotypes

Aga2+/+ mice feature phenotypic variability ranging from
postnatal lethality to moderate-to-severe changes including

Figure 5. Anomalies in Aga2+/+ Osteoblast Growth and Induction of
Cytoprotective Unfolded Protein Response

(A) Growth curve analysis of +/- and Aga2+/+ primary calvarial culture
system. (B) UPR, qPCR analysis of stimulated calvarial OBs. Values represent
means ± SEM, where n = 4–5 experiments (*p < 0.01 Student t-test). (C)
Western analysis of BiP using primary OBs.
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Aga2+/+, p = 0.02 Student t-test), suggesting a 14 % comparative
basal increase in apoptosis. Also, TUNEL experiments
revealed increased numbers of DNA–fragmented OBs within
Aga2+/+ periosteum (data not shown).
highly regulated sorting pathways in order to retain ER homeostasis and to prevent the secretion of abnormal proteins. The cytoprotective unfolded protein response (UPR) involves the activation of complex regulatory ER stress signaling mechanisms that can either repress protein synthesis or upregulate ER-resident chaperons and other translation regulators [34]. BiP/Grp78, a central regulator of ER function, was shown to bind to proα(I) chains from cell strains, which harbored unique C-propeptide mutations that inhibited chain association [11] and mediated intracellular ER-degradation [12]. Contrarily, mutations that inhibit folding in the triple helical domain of type I procollagen chains do not result in abnormal BiP binding or induction even though the aberrant molecules are retained within the ER [35].

In Aga2+/+, the presence of dilated ERs and accumulation of aberrant procollagens in the ER strongly support intracellular trafficking defects and degradation within the proximal region of the secretory pathway. We speculate that aberrant procollagen molecules aggregated into vesicular structures (Figure 4E), which are part of the proteasome. Degradation did occur within more distal secretory compartments by the presence of lysosome-like structures [36]. In Aga2+/+ OB cultures treated with ascorbate, BiP levels were slightly upregulated implicating its constitutive involvement during ER stress. Previous studies from Chessler et al. suggested that in skin fibroblasts from OI patients with mutations in the C-terminal propeptide BiP peaked about 48 hours after exposure to ascorbate and that longer exposure resulted in decreasing levels [11]. As cells studied here were
exposed to ascorbate continuously during growth as part of their medium enrichment, we performed Western analysis and confirmed increased BiP protein levels. Hsp47, another molecular chaperone, which is part of the ‘quality control’ system for procollagens, is enhanced in certain OI cases [37]. In our studies, the degree of Hsp47 induction was also elevated. Thus, in Aga2+/+ both BiP and Hsp47 seemed to choreograph the intracellular regulation of mutant type I procollagens with concomitant induction of apoptosis.

**Ex Vivo and In Vivo Evidence for Increased ER Stress-Induced Apoptosis**

Many diseases are associated with either inhibition or increase of apoptosis [34,38], but to date the active involvement of apoptosis during OI pathogenesis is inconclusive. Caspase-12 is a specific mediator of the ER stress-induced UPR within skeletal tissue [39]. In primary Aga2+/+ OBs, removal of the procaspase-12 adaptor protein-binding domain was evident, and the processing of procaspase-12 demonstrated the ER stress origin of mutated pro-Ca(1(I) initiator signals. In addition, the presence of increased activated caspase-3 and TUNEL-positive OBs in Aga2+/+ primary culture and periosteous also implicated a tendency toward apoptosis commitment and cell death, respectively.

Although several models have been proposed for the direct activation of caspase-12 the entire process has yet to be described. The Bcl2 family-proteins are well-established components of the apoptotic machinery, some of which are associated with ER stress pathways. For example, Bim (Bcl2-interacting mediator of cell death) activates caspase-12 via translocating from the dynein-rich ER compartment upon stress [40]. BiP is known to inhibit caspase-mediated cell death by forming complexes with procaspases 7 and 12, and BiP disassociation facilitates procaspase activation [41]. Gadd153/CHOP is a key transcription factor in the regulation of cell growth, differentiation and ER stress-induced apoptosis. Gadd153 induces death by promoting ER client protein load and oxidation via transcription of target genes, which mediate apoptosis, presumably leading to caspase cascades [34]. In our studies, Gadd153 was highly induced over time, and was presumably triggered by mutated procollagens and the modulation of ER stress signaling. Of interest, the anti-apoptotic component Bcl2 is down regulated during ER stress conditions attributed to Gadd153 upregulation, leading to enhanced oxidation and apoptosis [42]. Thus, given the upregulation of Gadd153 within Aga2+/+ OBs, Bcl2 was likely down regulated, exacerbating and contributing to cell death. Further studies are necessary to elucidate the pro- and anti-apoptotic components that modulated the severe clinical outcome in Aga2+/+ animals. In addition to severe long bone deformities we observed immunological, blood, heart, lung and energy metabolic defects pointing to a systemic effect of the Col1a1 mutation. Further experiments will be necessary to elucidate the influence of the systemic defect on bone and early lethality (unpublished data).

The molecular diversity of apoptosis in OI is unknown, and it is unclear if the apoptotic program is a phenomenon of type I procollagen mutations that severely affect chain assembly and retention caused by triple helical versus C-propeptide structural defects, or by both. Recently, Forlino et al. [43] evaluated phenotype heterogeneity within the BtllW knock-in OI mouse line and identified increased Gadd153 transcript and protein levels within calvaria of lethal animals. In this study we isolated and characterized a unique mouse model for OI, and provided evidence that the Aga2 C-propeptide Ca(1(I) mutation induced ER-mediated osteoblast apoptosis that affected cellular function and metabolism, which we now suggest to be a key component, among others, that influenced disease severity in Osteogenesis imperfecta.

**Materials and Methods**

**Antibodies and kits.** Activated caspase-3 (R&D Systems, Germany), caspase-12 (BD Pharmingen, Germany), anti-α-tubulin (Sigma, Germany), β-actin, type I collagen, and BiP/GRP78 (all Abcam, UK), and fluorescently labeled secondary antibodies (Molecular Probes, Germany) were commercially purchased. Antisera LF-41 and -67 [44] were generous gifts provided by Dr. L. Fisher (NIH, USA). The serum biochemical analysis has been previously described [45]. The mouse TRACP 5b (IDS Ltd), osteocalcin ELISA (BTI), and mouse/ rat intact PTH as well as calcitonin immunoradiometric assay (Immundiagnostik, Germany) kits were commercially purchased. All measurements were performed based on the manufacturers’ recommendations.

**Animal housing and handling.** Mouse husbandry was conducted under a continuously controlled specific-pathogen-free (SPF) hygiene standard according to the Federation of European Laboratory Animal Science Associations (FELASA) protocols. Standard rodent diet and water were provided ad libitum. All animal experiments were conducted under the approval of the responsible animal welfare authority.

**Linkage analysis and Aga2 genotyping.** The F1 dominant ENU mutagenesis screen was conducted on the inbred strain C3HeB/FeJ and has been previously described [14]. Aga2 was backcrossed to wild-type C57BL/6 mice for five generations, and then outcrossed to C57BL/6 (F1), and backcrossed to the outcrossed strain (N1). Phenotype-positive N2 progeny were tail-genotyped with microsatellite markers. Recombination fractions and map distances were calculated using Mapmanager QT2. All primers for sequencing are available upon request.

**Skeletal and radiological analysis.** Skeletal preparations were generated as previously described [46]. Simple and compound fractures were grossly examined. Compression fractures were evaluated using a dissecting microscope. For μCT scans (Tomoscope 10010m; VAMP, Germany), image reconstruction and visualization were performed using the ImpactCB and ImpactView software (VAMP, respectively). pQCT analysis was performed as previously described [47].

**Electron microscopy, histology, and histomorphometry.** SEM was performed as previously described [48]. For TEM, lower dorsal skin was prepared as previously described [49]. Sample preparation, embedding and double labeling experiments were performed as previously described [50]. For the demonstration of endogenous ALP and TRAP, the procedure of Xiao and Scott [51] was performed with modifications. All bone histomorphometric parameters were derived from the standardized nomenclature [52]. Non-sequential sections were collected every 70 - 100 μm. All images and measurements were captured and determined using an Axioplan2 workstation (AxioVision v3.1; Zeiss, Germany) and the ImageJ program (v1.36; NIH, USA). Automatic color thresholding was applied to stacked image sets. Labels were traced with minimal operator bias. 6 - 20 repeated measurements were made per section using four non-sequential sections, where n = 4 - 6 equals the number of animals examined.

**Western analysis.** Dermal fibroblasts were cultured as previously described [53]. For caspase-12 and BiP detection, cells were lysed in RIPA buffer with protease inhibitors. Supernatants were analyzed using the NuPAGE Novex gel system (Invitrogen, UK).

**Skeletal and cellular assays.** OBs were prepared from 3-day-old pups with similar appearance as previously described [54]. Cells were plated at 2 × 10^3 cells/cm² for all experiments. OB differentiation was initiated via media exchange (i.e. 2-MEM w/10 % FCS, 2 mM glutamine, 1 % pen/strep, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate). A minimum of four calvaria/genotype was analyzed using the ImpactCB and ImpactView software (VAMP, respectively). pQCT analysis was performed as previously described [47].

**Primary OB culture and cellular assays.** OBs were prepared from 3-day-old pups with similar appearance as previously described [54]. Cells were plated at 2 × 10^3 cells/cm² for all experiments. OB differentiation was initiated via media exchange (i.e. 2-MEM w/10 % FCS, 2 mM glutamine, 1 % pen/strep, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate). A minimum of four calvaria/genotype was prepared presenting an independent experiment (n) with four repeated measurements. DNA, total protein, ALP and MTT (kit) measurements were performed in 24-well plates as previously described [55]. Caspase-3/7 activity was monitored using a fluorogenic
substrate (Ac-DEVD-AMC, ANXXORA, Germany) at excitation 380 nm and emission 460 nm. Mineral content was quantified by establishing an alizarin red standard curve and measuring dye release using 10% hexadecylpyridinium in 6-well plates at 570 nm. For nodule characterization, samples were analyzed using the ImageJ software.

**Indirect immunofluorescence and TUNEL analysis.** Cells were cultured on glass coverslips coated with poly-L-lysine. For immunohistochemical analysis, sodium citrate (10 mM, pH 6.0 in microwave) and proteinase K (10 μg/ml, 37°C) antigen retrieval methods were performed, and the Vectastain ABC kit (Vector Labs, USA) utilized. The TUNEL assay was performed with an ALP-conjugated kit (Roche, Germany). Independent preparations (n = 4) were analyzed with repeated measurements. For cell culture, 300-400 cells were counted per image field. For in vivo caspase-3 analysis, a region 0.4 mm from the growth plate was analyzed, and 400-480 total cells were evaluated within a set region (i.e., magnification area and field).

**qPCR.** RNA and cDNA were prepared as previously described [56]. qPCR was performed using the TaqMan and QuantiTect ready-to-use primers (Qiagen, Germany). Duplicate crossing points per marker were averaged per independent experiment (n = 4). Significance was depicted between time points. Values were normalized to levels of β-actin from the same pool for fold differences.

**Statistics.** Level of significance was set at p < 0.5 by ANOVA test on the influence of genotype and sex, and subsequent pairwise mean comparisons performed by Student t- or post hoc (Bonferroni) tests. All statistics were calculated using the Prism 4 (GraphPad, USA) and SigmaStat v. 3.1 (Systat Software, Germany) software.

**Supporting Information**

**Figure S1.** Genome-Wide Linkage and High-Resolution Haplotype Analysis of the Aga2 Locus

(A) Genome-wide linkage analysis revealed Aga2 linkage on mouse distal Chromosome 11.

(B) Haplotype analysis identified the most probable Aga2 candidate region between D11Mit288 and D11Lis2. Log of odds ratio (LOD), centimorgan (cM), megabase (Mb), and (black) heterozygous (C3HeB/Fej & C57BL6J alleles), (white) homozygous (C57BL6J allele).

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**Figure S2.** In Vivo Bone Formation and Resorption in 16-Wk-Old Male Mice

(A,B) Bone formation and mineral accretion were monitored by dual calcein (red arrows) and tetracycline (white arrows) in vivo labeling (dl). Tibial secondary cancellous (c, inset) and endocortical (not shown) regions were characterized. Bar, 100 μm for main in (A) and (B) combined. Bracketed bars within the corner brackets represent mean ± SEM of sample dl lengths (17.9 ± 0.6 mm ± dl, ±; and 11.7 ± 1.0 mm 7 dl, Aga2+).

(C,D) Undecalcified tibial sections in control and Aga2+ mice, respectively, depict increased resorptive bays with multinucleated OCs (black arrows) at the osteogenic zone, and extended hypertrophic zone of the growth plate (Gp; horizontal arrow) in Aga2+/+. Bar, 100 μm combined; stained with von Kossa and counterstained with Paragon. Bone marrow (Bm), trabecula (Tb).

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**Figure S3.** Quantitative Analysis of Physiological Bone Growth and Remodeling in 16-Wk-Old Male Tibiae

(A) Percentage of secondary trabecular surface encompassed with OBs. (B) MAR calculated within both secondary cancellous (c) and endocortical (EC) bones, while BFR was calculated for mid-cancellous trabeculae.

(C) Relative number of TRAP-positive OCs within secondary trabeculae. (D) Cell density within femora and tibiae. Bars represent mean ± SEM, where n = 4 in (A–C), and n = 6 experiments in (D). p-Values in (A–C) derived from the Student t-test, while in (D), *p < 0.0001 one-way ANOVA and **p < 0.001 post hoc test. Diaphysis (d), metaphysis (m), cortex (Cx), trabeculae (Tb).

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**Figure S4.** Aga2+/+ Osteoblastic Ex Vivo Response and Function

(A) ALP activity measurement in primary OB lysates. (B) OB total protein, reduced MTT, and secreted acid-soluble collagen levels at 21 days in culture.

(C) Time-course analysis of the density and individual area of bone-like nodules, and the amount of bounded alizarin red dye per total nodule area at 16 days. Asterisks show significant differences at *p < 0.05, ***p < 0.001, and ****p < 0.0001 (Student t-test), while stimulated-unstimulated p-values were derived from ANOVA with post hoc test. Values represent mean ± SEM, where n = 4–5 independent experiments. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), unstimulated (unstim.).

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**Table S1.** Dysmorphology

Found at doi:10.1371/journal.pgen.0040007.s001 (106 KB DOC).

**Table S2.** In Vivo pQCT Analysis in Paternal-Derived 16-Wk-Old Male Femur

Found at doi:10.1371/journal.pgen.0040007.s002 (61 KB DOC).

**Table S3.** Clinical Chemical Analysis at 12–16 Wk of Age

Found at doi:10.1371/journal.pgen.0040007.s003 (54 KB DOC).

**Table S4.** Histomorphometry

Found at doi:10.1371/journal.pgen.0040007.s004 (55 KB DOC).

**Accession Numbers**

Colla1, MGI:198467 (murine) and HGNC:2187 (human); Chod, MGI:1096860; Hip5, MGI:59835; Serpin1 (Hip47), MGI:88283; Dild1 (Gadd153), MGI:109247. Data depicted from Mouse Genome Informatics (MGI; www.informatics.jax.org) and HUGO Gene nomenclature Committee (HGNC; http://www.genenames.org).

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**Author contributions.** TSL and MHdA designed the experiments and wrote the paper. TSL, FT, WH, BR, GH, and MH performed the experiments. TSL, FT, IQ, HF, WH, GKH, KA, BR, SHR, and MHdA analyzed the data. EW provided reagents/materials/analysis tools.

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**Competing interests.** The authors have declared that no competing interests exist.

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