

LETTER TO THE EDITOR

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# Deficiency for the ER-stress transducer OASIS causes severe recessive osteogenesis imperfecta in humans

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## Abstract

Osteogenesis imperfecta (OI) is a clinically and genetically heterogeneous brittle bone disorder. Whereas dominant OI is mostly due to heterozygous mutations in either *COL1A1* or *COL1A2*, encoding type I procollagen, recessive OI is caused by biallelic mutations in genes encoding proteins involved in type I procollagen processing or chaperoning. Hitherto, some OI cases remain molecularly unexplained. We detected a homozygous genomic deletion of *CREB3L1* in a family with severe OI. *CREB3L1* encodes OASIS, an endoplasmic reticulum-stress transducer that regulates type I procollagen expression during murine bone formation. This is the first report linking *CREB3L1* to human recessive OI, thereby expanding the OI gene spectrum.

**Keywords:** Osteogenesis imperfecta, Type I collagen, OASIS, CREB3L1, Endoplasmic reticulum stress

## Background

Osteogenesis imperfecta (OI) is a genetically heterogeneous brittle bone disorder with varying degrees of clinical severity, ranging from perinatal lethality to generalized osteopenia [1]. The predominant autosomal dominant forms display mutations in either *COL1A1* or *COL1A2*, encoding the  $\alpha 1$ - and  $\alpha 2$ -chains of type I procollagen, while rarer autosomal recessive forms mostly result from defective endoplasmic reticulum (ER)-resident proteins involved in post-translational processing or chaperoning of these  $\alpha(I)$ -chains [1,2]. Processing defects prevent normal collagen fibrillogenesis and on biochemical analysis often show perturbed modification of the collagen  $\alpha$ -chain. Known defects include biallelic mutations in *LEPRE1* [3-5], *CRTAP* [5,6], *PPIB* [7,8], *BMP1* [9,10], and *PLOD2* [11]. Mutations in chaperones (including Hsp47 (*SERPINH1*) and FKBP10) impair intracellular collagen trafficking with intracellular retention or aggregation of collagen molecules and show dilation of the ER on electron microscopy, resulting in OI or related phenotypes [12-14]. Finally, rare other defects linked

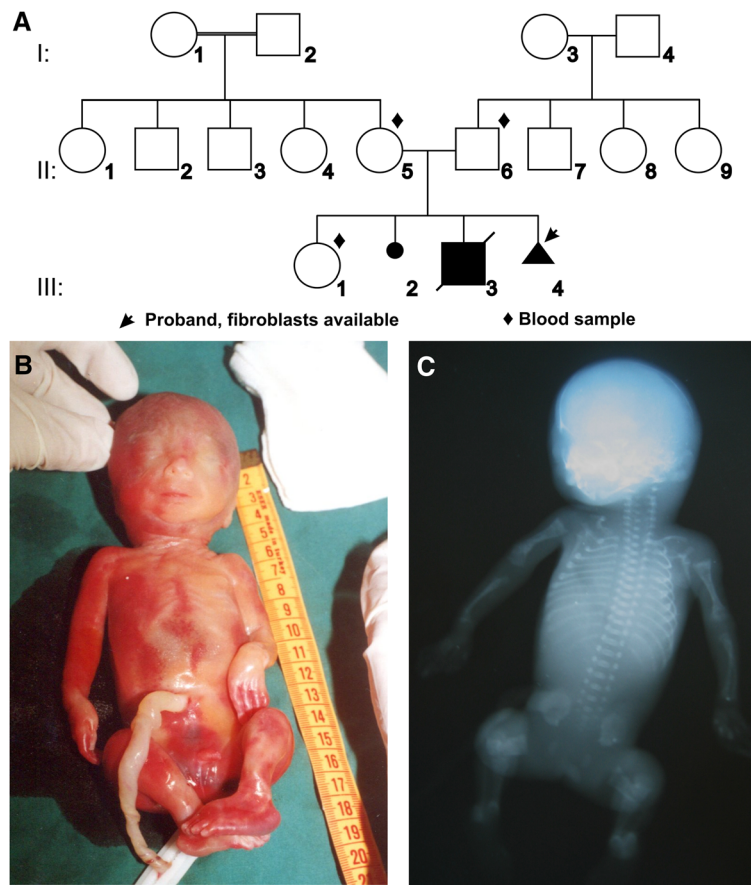
to distinct mechanisms involve the transcription factor osterix (*SP7*) [15], pigment epithelium derived factor (*SERPINF1*) [16] and transmembrane protein 38B (*TMEM38B*) [17,18]. A recurrent mutation in a gene encoding the Interferon-inducible transmembrane protein 5 (*IFITM5*), which is involved in bone growth during prenatal murine development, was recently shown to cause autosomal (AD) dominant OI [19-21]. Recently, heterozygous and homozygous mutations in *WNT1* (*WNT1*), which is a key signalling molecule in osteoblast function and bone development, were shown to underlie certain forms of AD early-onset osteoporosis and AR OI, which was in some patients associated with severe intellectual disability [22-26]. However, a small proportion of OI patients remain molecularly unexplained.

## Findings

We describe a Turkish family (Figure 1A) with three sibs, two of whom were affected by severe OI (written informed consent of the family was obtained and the study was approved by the Ethics Committee of the Ghent University Hospital (Ghent, Belgium)). Consanguinity was not reported, but the parents originated from neighbouring villages. The first affected child (III:3) developed several fractures *in utero* and was small for

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**Figure 1 Pedigree and clinical findings.** **A.** Pedigree of the Turkish family. **B.** Post-mortem examination of foetus III:4 at 19 weeks of gestation showed bowed extremities and pes equinovarus. **C.** X-rays of foetus III:4 revealed beaded ribs and multiple fractures of tubular bones.

gestational age. His birth length was 40 cm (<P3). At the first day of life he was hospitalized for hyperbilirubinemia and O-bain-like deformities, soft calvarial bones and widely open fontanelles were noticed. He developed several fractures after birth and multiple fractures healed with extremity deformities. He also had a right inguinal hernia. X-rays showed beaded ribs, callus formation and multiple fractured tubular bones with an accordion-like broadened appearance. He was hospitalized several times due to recurrent constipation and pulmonary infections (bronchopneumonia). During this period, he developed abdominal distention and hepatomegaly, the latter due to cardiac insufficiency. No signs of T-cell dysfunction or other immune deficiencies have been noted. He died at 9 months of age. The second affected sib (III:4, Figure 1A) was a male foetus from a pregnancy that was medically terminated at 19 weeks of gestation. Post-mortem examination showed thin ribs and fractures at bowed humerus and femora (Figure 1B-C).

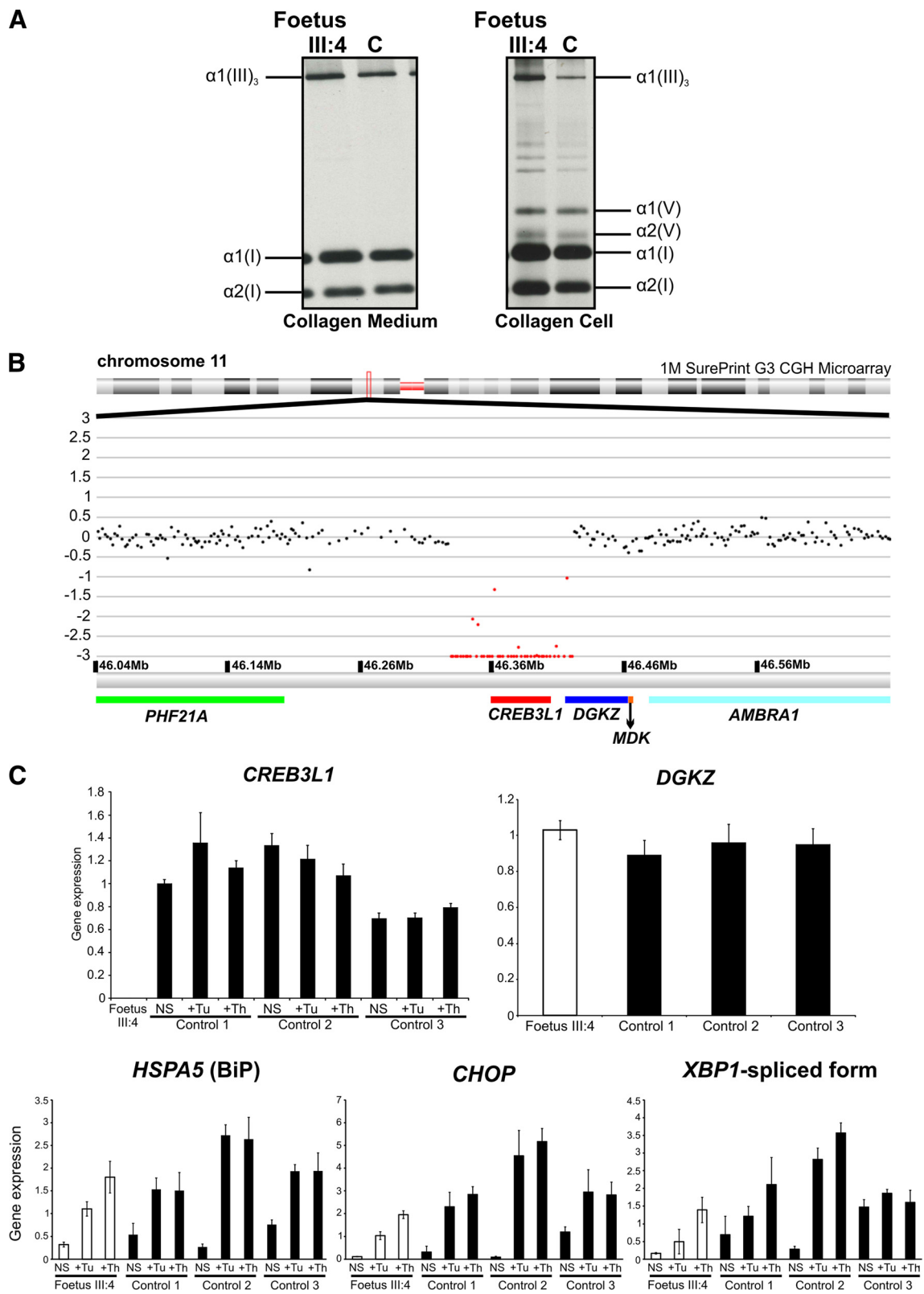
The parents have a healthy daughter (III:1) and have had one miscarriage (III:2, cause unknown). The adolescent daughter has blue sclerae but had not experienced

any fractures. The mother (II:5) at 38 years of age and the father (II:6) at 47 years have blue sclerae, a soft and velvety skin and normal teeth. While the mother has small joint hypermobility, the father has conductive hearing loss.

Biochemical (pro)collagen SDS-PAGE analysis was performed on the medium and cellular fractions of cultured skin fibroblasts of foetus III:4. No obvious quantitative or qualitative abnormalities of <sup>14</sup>C-labelled type I procollagen (data not shown) and mature secreted and intracellular type I collagen (Figure 2A) were detected.

Subsequently, all known OI genes (*COL1A1*, *COL1A2*, *BMP1*, *LEPRE1*, *CRTAP*, *PIIB*, *PLOD2*, *SERPINH1*, *FKBP10*, *SP7*, *SERPINF1*, *TMEM38B*, *IFITM5* and *WNT1*) were sequenced by direct Sanger sequencing (ABI3730XL automated sequencer, Applied Biosystems), but no causal mutation(s) were detected.

We selected the *CREB3L1* gene [GenBank:NM\_052854.2], encoding the ER-stress transducer OASIS (Old Astrocyte Specifically Induced Substance), as an excellent candidate gene based on the observation that *OASIS*<sup>-/-</sup> mice were born with severe osteopenia and spontaneous fractures



**Figure 2** (See legend on next page.)

(See figure on previous page.)

**Figure 2 Biochemical and molecular results.** **A.** Biochemical collagen analysis was performed on collagens produced by the patients dermal fibroblasts, which were grown for 16 hrs in the presence of  $^{14}\text{C}$ -Proline. Radioactively labelled intracellular and secreted fibrillar collagen proteins were isolated and mature collagens were obtained by pepsin digestion. Foetal secreted (left panel) as well as intracellular (right panel) mature type I collagen revealed a normal electrophoretic pattern when compared to a control (C) sample. Also for the unprocessed, secreted type I procollagen a normal electrophoretic migration pattern was observed (data not shown). **B.** ArrayCGH analysis on a 1M SurePrint G3 Human CGH Microarray revealed a homozygous deletion of the entire *CREB3L1* gene in the affected foetus III:4. **C.** Expression level analysis by RT-qPCR was performed in duplicate on total RNA extracted from three biological replicates of the fibroblast cell lines from foetus III:4 and three controls (C1, C2 and C3) (LightCycler480 and RealTime ready DNA Probe Master Mix, Roche). The expression level of each investigated gene was quantified using *qbase*<sup>PLUS</sup> (Biogazelle)[27]. *HPRT1*, *RLP13a* and *YWHAZ* were applied as reference targets. RT-qPCR for foetus III:4 confirmed the total absence of *CREB3L1* expression when compared to control samples (C1, C2 and C3). *DGKZ* has two alternative (tissue-specific) isoforms [28].

[29], reminiscent of severe human OI. In those mice, OASIS was shown to be crucial for bone formation through activating *coll1a1* transcription and facilitating the secretion of matrix proteins. Treatment of murine osteoblasts with BMP-2 (bone morphogenic protein 2) causes mild ER-stress and is associated with accelerated RIP (regulated intramembrane proteolysis) of OASIS. The N-terminal part of OASIS is subsequently translocated to the nucleus, where it binds to the osteoblast-specific UPRE (unfolded protein response element) regulatory region in the murine *Col1a1* promoter thereby causing high levels of type I procollagen expression [29]. While the amount of type I procollagen is normal in the murine OASIS<sup>-/-</sup> skin, reduced amounts of type I procollagen were detected in OASIS<sup>-/-</sup> calvaria and tibia, which suggested tissue-specific decrease of type I procollagen in the bone matrix but also failure of the OASIS<sup>-/-</sup> osteoblasts to produce high levels of type I procollagen [29]. OASIS further functions as a tissue-specific ER-stress transducer that alters transcription of target genes involved in developmental processes, differentiation, or maturation upon mild ER-stress. PCR amplification of all exons and flanking introns of *CREB3L1* failed in foetus III:4, suggesting a homozygous whole gene deletion. ArrayCGH analysis (1M SurePrint G3 Human CGH Microarray, Agilent Technologies) and copy number profiling (arrayCGHbase) confirmed this genomic deletion, which encompasses *CREB3L1* and the first exon of *DGKZ* (arr11p11.2(46268141-46359490)×0, Figure 2B) [30,31]. Whereas the arr11p11.2(46268141-46359490)×0 homozygous deletion was not reported before, heterozygous deletions or gains of this genomic region are described in the Decipher database [32] and the Database of Genomic Variants [33] but encompassing large genomic regions comprising multiple genes (6 to 86 genes and/or multiple chromosomal abnormalities) which, in some cases, are associated with intellectual disability. Both parents and the healthy sister were heterozygous for the deletion (data not shown). *DGKZ* encodes diacylglycerol kinase zeta, an ubiquitously expressed enzyme that is most abundantly present in the brain, thymus and skeletal muscle [34] and which has a regulatory role in T-cell receptor signalling and T-cell

activation [35]. Two different isoforms (*DGKZ1* in immune cells and *DGKZ2* in other cells) are known, in which exon 1 is either present or absent and which have a tissue- and developmental stage-specific expression [28]. Hitherto, no known function in bone formation has been ascribed to *DGKZ* and thus a possible contributing role to (the severity of) the bone phenotype of patient III:3 and foetus III:4 cannot completely be excluded. Expression analysis by real time-quantitative PCR (RT-qPCR) on total RNA isolated from dermal fibroblasts of foetus III:4 confirmed complete absence of the *CREB3L1* transcript. In order to investigate the expression of the two *DGKZ* isoforms (*DGKZ1* and *DGKZ2*), two different primer pairs were designed, of which one was specific for exon 1 that is only present in the *DGKZ1* isoform. RT-qPCR experiments revealed no amplification for the primer pair specific for exon 1 in cultured dermal fibroblasts, suggesting that the *DGKZ1* isoform is not expressed in these cells. For the second primer pair normal *DGKZ* expression was observed, which implies normal expression of the *DGKZ2* isoform in cultured human dermal fibroblasts (Figure 2C). RT-qPCR analysis of the ER-stress markers BiP, CHOP and the spliced form of XBP1 showed levels comparable to controls, even after stimulation of confluent fibroblasts for 4 hours with the ER-stress inducers Tunicamycin (Tu, 10 µg/ml, Sigma-Aldrich) and Thapsigargin (Th, 1 µM, Sigma-Aldrich) (Figure 2C). This is in accordance to the observations in OASIS<sup>-/-</sup> mice. The expression level of *CREB3L1* was unchanged in control fibroblasts after treatment with Tu and Th (Figure 2C), suggesting that OASIS does not play a major role in the ER-stress pathways previously linked to disease pathogenesis [1]. Additionally, our finding that type I (pro)collagen production is normal in human dermal fibroblasts (Figure 2A) confirms that OASIS has a tissue-specific effect on type I (pro)collagen production [29].

In conclusion, the identification of *CREB3L1* (encoding the ER-stress transducer OASIS) as a novel gene for autosomal recessive OI expands the spectrum of genes linked to OI and reinforces the role of ER-stress in the pathophysiology of OI.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

Conceived and designed the experiments: SS, PC. Identified and recruited human subjects, obtained ethical approvals, coordinated collection of samples, and provided clinical information: FM, BC, HK, ADP. Performed the experiments: SS, SD, AD, WS. Analyzed the data: SS, PC. Wrote the paper: SS, FM, BC, ADP, PC. All authors have read and approved the final manuscript.

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### References

- Forlino A, Cabral WA, Barnes AM, Marini JC: **New perspectives on osteogenesis imperfecta.** *Nat Rev Endocrinol* 2011, **7**:540–557.
- Marini JC, Forlino A, Cabral WA, Barnes AM, San Antonio JD, Milgrom S, Hyland JC, Korkko J, Prockop DJ, De Paepe A, et al: **Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans.** *Hum Mutat* 2007, **28**:209–221.
- Cabral WA, Chang W, Barnes AM, Weis M, Scott MA, Leikin S, Makareeva E, Kuznetsova NV, Rosenbaum KN, Tiffit CJ, et al: **Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta.** *Nat Genet* 2007, **39**:359–365.
- Van Dijk F, Nikkels PG, den Hollander NS, Nesbitt IM, van Rijn RR, Cobben JM, Pals G: **Lethal/severe osteogenesis imperfecta in a large family: a novel homozygous LEPRE1 mutation and bone histological findings.** *Pediatr Dev Pathol* 2010, **14**(3):228–234.
- Baldrige D, Schwarze U, Morello R, Lennington J, Bertin TK, Pace JM, Pepin MG, Weis M, Eyre DR, Walsh J, et al: **CRTAP and LEPRE1 mutations in recessive osteogenesis imperfecta.** *Hum Mutat* 2008, **29**:1435–1442.
- Barnes AM, Chang W, Morello R, Cabral WA, Weis M, Eyre DR, Leikin S, Makareeva E, Kuznetsova N, Uveges TE, et al: **Deficiency of cartilage-associated protein in recessive lethal osteogenesis imperfecta.** *N Engl J Med* 2006, **355**:2757–2764.
- Barnes AM, Carter EM, Cabral WA, Weis M, Chang W, Makareeva E, Leikin S, Rotimi CN, Eyre DR, Raggio CL, Marini JC: **Lack of cyclophilin B in osteogenesis imperfecta with normal collagen folding.** *N Engl J Med* 2010, **362**:521–528.
- van Dijk FS, Nesbitt IM, Zwikstra EH, Nikkels PG, Piersma SR, Fratantoni SA, Jimenez CR, Huizer M, Morsman AC, Cobben JM, et al: **PPIB mutations cause severe osteogenesis imperfecta.** *Am J Hum Genet* 2009, **85**:521–527.
- Martinez-Glez V, Valencia M, Caparros-Martin JA, Aglan M, Temtamy S, Tenorio J, Pulido V, Lindert U, Rohrbach M, Eyre D, et al: **Identification of a mutation causing deficient BMP1/mTLD proteolytic activity in autosomal recessive osteogenesis imperfecta.** *Hum Mutat* 2012, **33**:343–350.
- Asharani PV, Keupp K, Semler O, Wang W, Li Y, Thiele H, Yigit G, Pohl E, Becker J, Frommolt P, et al: **Attenuated BMP1 function compromises osteogenesis, leading to bone fragility in humans and zebrafish.** *Am J Hum Genet* 2012, **90**:661–674.
- Bank RA, Robins SP, Wijmenga C, Breslau-Siderius LJ, Bardeol AF, van der Sluijs HA, Pruijs HE, Tekoppele JM: **Defective collagen crosslinking in bone, but not in ligament or cartilage, in Bruck syndrome: indications for a bone-specific telopeptide lysyl hydroxylase on chromosome 17.** *Proc Natl Acad Sci U S A* 1999, **96**:1054–1058.
- Alanay Y, Avaygan H, Camacho N, Utine GE, Boduroglu K, Aktas D, Alikasifoglu M, Tuncbilek E, Orhan D, Bakar FT, et al: **Mutations in the gene encoding the RER protein FKBP65 cause autosomal-recessive osteogenesis imperfecta.** *Am J Hum Genet* 2010, **87**:572–573.
- Kelley BP, Malfait F, Bonafe L, Baldrige D, Homan E, Symoens S, Willaert A, Elcioglu N, Van Maldergem L, Verellen-Dumoulin C, et al: **Mutations in FKBP10 cause recessive osteogenesis imperfecta and Bruck syndrome.** *J Bone Miner Res* 2011, **26**:666–672.
- Christiansen HE, Schwarze U, Pyott SM, AlSwaid A, Al Balwi M, Alrasheed S, Pepin MG, Weis MA, Eyre DR, Byers PH: **Homozygosity for a missense mutation in SERPINH1, which encodes the collagen chaperone protein HSP47, results in severe recessive osteogenesis imperfecta.** *Am J Hum Genet* 2010, **86**:389–398.
- Lapuzina P, Aglan M, Temtamy S, Caparros-Martin JA, Valencia M, Leton R, Martinez-Glez V, Elhossini R, Amr K, Vilaboà N, Ruiz-Perez VL: **Identification of a frameshift mutation in Osterix in a patient with recessive osteogenesis imperfecta.** *Am J Hum Genet* 2010, **87**:110–114.
- Becker J, Semler O, Gilissen C, Li Y, Bolz HJ, Giunta C, Bergmann C, Rohrbach M, Koerber F, Zimmermann K, et al: **Exome sequencing identifies truncating mutations in human SERPINF1 in autosomal-recessive osteogenesis imperfecta.** *Am J Hum Genet* 2011, **88**:362–371.
- Shaheen R, Alazami AM, Alshammari MJ, Faqeih E, Alhashmi N, Mousa N, Alsinani A, Ansari S, Alzahrani F, Al-Owain M, et al: **Study of autosomal recessive osteogenesis imperfecta in Arabia reveals a novel locus defined by TMEM38B mutation.** *J Med Genet* 2012, **49**:630–635.
- Volodarsky M, Markus B, Cohen I, Staretz-Chacham O, Flusser H, Landau D, Shelef I, Langer Y, Birk OS: **A deletion mutation in TMEM38B associated with autosomal recessive osteogenesis imperfecta.** *Hum Mutat* 2013, **34**:582–586.
- Cho TJ, Lee KE, Lee SK, Song SJ, Kim KJ, Jeon D, Lee G, Kim HN, Lee HR, Eom HH, et al: **A single recurrent mutation in the 5'-UTR of IFITM5 causes osteogenesis imperfecta type V.** *Am J Hum Genet* 2012, **91**:343–348.
- Semler O, Garbes L, Keupp K, Swan D, Zimmermann K, Becker J, Iden S, Wirth B, Eysel P, Koerber F, et al: **A mutation in the 5'-UTR of IFITM5 creates an in-frame start codon and causes autosomal-dominant osteogenesis imperfecta type V with hyperplastic callus.** *Am J Hum Genet* 2012, **91**:349–357.
- Hanagata N, Li X, Morita H, Takemura T, Li J, Minowa T: **Characterization of the osteoblast-specific transmembrane protein IFITM5 and analysis of IFITM5-deficient mice.** *J Bone Miner Metab* 2011, **29**:279–290.
- Fahiminiya S, Majewski J, Mort J, Moffatt P, Glorieux FH, Rauch F: **Mutations in WNT1 are a cause of osteogenesis imperfecta.** *J Med Genet* 2013, **50**:345–348.
- Keupp K, Beleggia F, Kayserili H, Barnes AM, Steiner M, Semler O, Fischer B, Yigit G, Janda CY, Becker J, et al: **Mutations in WNT1 cause different forms of bone fragility.** *Am J Hum Genet* 2013, **92**:565–574.
- Pyott SM, Tran TT, Leistritz DF, Pepin MG, Mendelsohn NJ, Temme RT, Fernandez BA, Elsayed SM, Elsobky E, Verma I, et al: **WNT1 mutations in families affected by moderately severe and progressive recessive osteogenesis imperfecta.** *Am J Hum Genet* 2013, **92**:590–597.
- Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, Lu JT, Pekkinen M, Wessman M, Heino TJ, et al: **WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta.** *N Engl J Med* 2013, **368**:1809–1816.
- FaQEih E, Shaheen R, Alkuraya FS: **WNT1 mutation with recessive osteogenesis imperfecta and profound neurological phenotype.** *J Med Genet* 2013, **50**:491–492.
- Hellems J, Mortier G, De Paepe A, Speleman F, Vandesompele J: **qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.** *Genome Biol* 2007, **8**:R19.
- Ding L, Bunting M, Topham MK, McIntyre TM, Zimmerman GA, Prescott SM: **Alternative splicing of the human diacylglycerol kinase zeta gene in muscle.** *Proc Natl Acad Sci U S A* 1997, **94**:5519–5524.
- Murakami T, Saito A, Hino S, Kondo S, Kanemoto S, Chihara K, Sekiya H, Tsumagari K, Ochiai K, Yoshinaga K, et al: **Signalling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone formation.** *Nat Cell Biol* 2009, **11**:1205–1211.
- Buysse K, Delle Chiaie B, Van Coster R, Loeys B, De Paepe A, Mortier G, Speleman F, Menten B: **Challenges for CNV interpretation in clinical**

molecular karyotyping: lessons learned from a 1001 sample experience. *Eur J Med Genet* 2009, **52**:398–403.

31. Menten B, Pattyn F, De Preter K, Robbrecht P, Michels E, Buysse K, Mortier G, De Paepe A, van Vooren S, Vermeesch J, *et al*: **arrayCGHbase: an analysis platform for comparative genomic hybridization microarrays.** *BMC Bioinforma* 2005, **6**:124.
32. Firth HV, Richards SM, Bevan AP, Clayton S, Corpas M, Rajan D, Van Vooren S, Moreau Y, Pettett RM, Carter NP: **DECIPHER: database of chromosomal imbalance and phenotype in humans using ensembl resources.** *Am J Hum Genet* 2009, **84**:524–533.
33. Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, Lee C: **Detection of large-scale variation in the human genome.** *Nat Genet* 2004, **36**:949–951.
34. Rincon E, Gharbi SI, Santos-Mendoza T, Merida I: **Diacylglycerol kinase zeta: at the crossroads of lipid signaling and protein complex organization.** *Prog Lipid Res* 2012, **51**:1–10.
35. Kanoh H, Yamada K, Sakane F: **Diacylglycerol kinase: a key modulator of signal transduction?** *Trends Biochem Sci* 1990, **15**:47–50.

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