OPEN ACCESS

Original research

Heterozygous pathogenic variants involving *CBFB* cause a new skeletal disorder resembling cleidocranial dysplasia

Tessi Beyltjens,[1] Eveline Boudin ,[1] Nicole Revencu ,[2] Nele Boeckx,[1] Miriam Bertrand,[3] Leon Schütz,[3] Tobias B Haack ,[3] Axel Weber,[4] Eleni Biliouri,[4] Mateja Vinkšel,[5] Anja Zagožen,[5] Borut Peterlin,[5] Shashidhar Pai,[6] Aida Telegrafi,[7] Lindsay B Henderson,[7] Courtney Ells,[8] Lesley Turner,[8,9] Wim Wuyts,[1] Wim Van Hul,[1] Gretl Hendrickx ,[1,10] Geert R Mortier[1,10,11]

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmg-2022-108739).

For numbered affiliations see end of article.

Correspondence to
Dr Gretl Hendrickx, Department of Human Genetics, KU Leuven, Leuven 3000, Belgium; gretl.hendrickx@kuleuven.be

GH and GRM contributed equally.

Received 29 June 2022
Accepted 3 September 2022
Published Online First 14 October 2022

To cite: Beyltjens T, Boudin E, Revencu N, *et al*. *J Med Genet* 2023;**60**:498–504.

ABSTRACT

Background Cleidocranial dysplasia (CCD) is a rare skeletal dysplasia with significant clinical variability. Patients with CCD typically present with delayed closure of fontanels and cranial sutures, dental anomalies, clavicular hypoplasia or aplasia and short stature. Runt-related transcription factor 2 (*RUNX2)* is currently the only known disease-causing gene for CCD, but several studies have suggested locus heterogeneity.

Methods The cohort consists of eight subjects from five unrelated families partially identified through GeneMatcher. Exome or genome sequencing was applied and in two subjects the effect of the variant was investigated at RNA level.

Results In each subject a heterozygous pathogenic variant in *CBFB* was detected, whereas no genomic alteration involving *RUNX2* was found. Three *CBFB* variants (one splice site alteration, one nonsense variant, one 2 bp duplication) were shown to result in a premature stop codon. A large intragenic deletion was found to delete exon 4, without affecting *CBFB* expression. The effect of a second splice site variant could not be determined but most likely results in a shortened or absent protein. Affected individuals showed similarities with *RUNX2*-related CCD, including dental and clavicular abnormalities. Normal stature and neurocognitive problems were however distinguishing features. *CBFB* encodes the core-binding factor β subunit, which can interact with all RUNX proteins (RUNX1, RUNX2, RUNX3) to form heterodimeric transcription factors. This may explain the phenotypic differences between *CBFB*-related and *RUNX2*-related CCD.

Conclusion We confirm the previously suggested locus heterogeneity for CCD by identifying five pathogenic variants in *CBFB* in a cohort of eight individuals with clinical and radiographic features reminiscent of CCD.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Runt-related transcription factor 2 *(RUNX2)* has long been recognised as the only causal gene for cleidocranial dysplasia (CCD), even though the genetic cause of up to 30% of patients with CCD remained elusive, suggesting locus heterogeneity.

WHAT THIS STUDY ADDS

⇒ We were able to identify *CBFB* as a causal gene for a novel disorder resembling CCD in eight subjects from five independent families.

⇒ Features that distinguish *CBFB*-related CCD from *RUNX2*-related CCD are a normal stature and a higher prevalence of developmental delay.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study expands our genotypic, phenotypic and mechanistic knowledge of the so-called CCD spectrum disorder, and will have an impact on the future diagnosis and follow-up of families with CCD.

INTRODUCTION

Cleidocranial dysplasia (CCD, OMIM #119600) is a rare but well-described skeletal disorder with an autosomal dominant inheritance. CCD affects the clavicles and skull, as suggested by its name, and the entire skeleton. Patients with CCD typically present with delayed closure of fontanels and cranial sutures, dental anomalies including supernumerary teeth and eruption failure, clavicular hypoplasia or aplasia and short stature.[1] Craniofacial features are subtle but characterised by prominent parietal and frontal bones, widely spaced eyes, depressed nasal bridge and small maxilla. There is considerable clinical variability, even within families, ranging from mildly affected individuals with solely dental anomalies to individuals presenting with the full spectrum of CCD features. Prevalence has been estimated to be one in 1 million, although one study demonstrated an incidence of up to 1:80 000 births in the Utah population.[1–3] Typical radiographic features include (i) retarded ossification of the skull with partial lack of ossification of the calvaria and skull base and delayed closure of sutures and fontanels with multiple Wormian bones, (ii) partial or total absence of the clavicles, usually bilateral and

more commonly in the lateral and middle thirds, or presence of two fragments of the clavicles with failure to fuse (so-called *clavicula bipartita*), (iii) delayed ossification of the pubic and inferior portion of the ischial bones and (iv) pseudoepiphyses of metacarpal and metatarsal bones and shortening of the distal phalanges.

Based on cytogenetic abnormalities in individuals with a CCD-like phenotype, the disease-causing gene for CCD was initially mapped to chromosome locus 6p21.[4] Subsequently, genomic alterations involving *RUNX2*, encoding the runt-related transcription factor 2 (RUNX2) and residing in this locus, were identified in patients with CCD.[5] Besides chromosomal rearrangements involving *RUNX2*, heterozygous loss-of-function variants in *RUNX2* have been identified in a subset of patients with CCD.[6–8] Currently, *RUNX2* is the only known gene involved in CCD.

RUNX2, alternatively referred to as core-binding factor α subunit 1 (CBFA1), is part of the RUNX protein family (RUNX1, RUNX2, RUNX3) and encodes the α-subunit of the core-binding factor protein. Together with the core-binding factor β subunit (CBFβ), encoded by *CBFB*, this heterodimeric protein complex acts as a master transcriptional regulator for the proliferation and differentiation of mesenchymal stem cells towards chondrocytes and osteoblast-lineage cells.[9–11] This makes the core binding factor indispensable for both endochondral and intramembranous bone formation, which is reflected by the phenotype of patients with CCD.

Although in most CCD cases pathogenic *RUNX2* variants or chromosomal aberrations at the 6p21 locus can be identified, the genetic cause in 10%–30% of patients with CCD remains elusive,[12 13] suggesting locus heterogeneity. In this paper, we report on eight individuals, originating from five unrelated families and presenting with features reminiscent of CCD, in whom we identified heterozygous pathogenic variants in *CBFB*.

METHODS

Study subjects

The case cohort consists of the proband (subject 1) with whom the study started and seven additional individuals who were identified using GeneMatcher.[14 15] The study was conducted according to the World Medical Association Declaration of Helsinki on ethical principles for medical research involving human subjects.[16]

Genetic analyses

CBFB variants of interest (NC_000016.10; NM_022845.3; GRCh38) were identified through trio-exome (family I, IV, V), trio-genome (family II) or single-exome sequencing (family III) and confirmed with Sanger sequencing, if necessary. In general, the genome analyses all followed a similar workflow of DNA sequencing, bioinformatic analysis, variant filtering and variant interpretation/prioritisation. MutationTaster, Combined Annotation Dependent Depletion (CADD) scores and PolyPhen-2 were used to assess the pathogenic potential of the variants.[17–19] In each case, no genomic alteration in *RUNX2* could be identified.

For family I, the effect of the splice site variant was evaluated by isolating RNA from blood of the affected individual and his healthy parents by using the PAXgene Blood RNA System (Qiagen). RNA was reverse transcribed to cDNA by using the SuperScript III First-Strand Synthesis System (Life Technologies) according to manufacturer's instructions. Sanger sequencing was performed using primers binding exon 1 (forward 5'-CCCGACCAGAGAAGCAAGTT-3') and exon 3 (reverse 5'-GTTTGTCGCTGTTCTCCCTG-3') to amplify the cDNA region surrounding the splice site variant in *CBFB*. For family II, RNA was extracted from PAXgene Blood RNA Tubes (Qiagen) on a QIAsymphony platform (Qiagen) using a QIAsymphony PAXgene Blood RNA Kit (Qiagen) for automated purification of intracellular RNA (including miRNAs) from stabilised blood following the manufacturer's protocol. Subsequent library preparation and RNA-sequencing (RNA-seq) were performed as previously described.[20] In brief, RNA quality was assessed with an Agilent 2100 Fragment Analyzer total RNA kit (Agilent Technologies, Santa Clara, California, USA) and 100 ng of total RNA (RNA integrity number 9) was used for library preparation with a NEBNext Ultra II Directional RNA Library Prep kit for poly(A)-selected sequencing libraries. Generated libraries were sequenced on the Illumina NovaSeq 6000 platform as 2×100 bp paired-end reads with approximately 50 million clusters. The quality of raw RNA-seq data in FASTQ files was assessed using ReadQC (V.2022_04) to identify potential sequencing cycles with low average quality and base distribution bias. Reads were preprocessed using SeqPurge (V.2022_04) and aligned using STAR (V.2.7.10a), which allowed alignment of spliced reads to the human reference genome (build GRCh38). The quality of the alignment was analysed using MappingQC (ngs-bits V.2022_04) and visually verified using Broad Integrative Genome Viewer (V.2.11.9). Based on Ensembl genome annotation (GRCh38, Ensembl Release 104), read counts for all genes were determined using subread (V.2.0.3). For comparative gene expression analysis, in-house transcriptome datasets from 145 blood RNA-seq datasets from individuals with unrelated phenotypes were used as controls. For comparative gene expression analysis, the transcripts per million values were used as normalisation for raw gene counts. Z-scores were calculated based on log2-transformed normalised expression values and to calculate p values a cumulative normal distribution of the z-score was used.

RESULTS

Subject 1

The first subject is a boy aged 4 years referred to the diagnostic lab because of a clinical suspicion of CCD. An overview of all clinical features is summarised in table 1. He presented with unilateral 'swelling' of the left clavicular region (figure 1). A chest radiograph revealed bilateral *clavicula bipartita* (figure 2). The diagnosis of CCD was suspected and for that reason he was referred to the clinical genetics' outpatient clinic. On clinical evaluation, mild facial dysmorphism was noted with maxillary hypoplasia and a pointy chin. A bony prominence was seen on the lateral edge of both clavicles, which was more pronounced on the left side (figure 1). Dental exam only showed mild enamel abnormalities. Growth was within normal range with a height of 97.5 cm (10th percentile), weight of 14.8 kg (10th–25th percentile) and head circumference of 51 cm (50th–75th percentile). Psychomotor development was normal and there was no significant medical history. More detailed radiographic evaluation revealed additionally shortening of the distal phalanges and presence of pseudoepiphyses at the second and fifth metacarpals (figure 1). Since no genomic alterations were found in *RUNX2*, trio-exome sequencing was performed which revealed a heterozygous splice site variant (c.78+1G>T) in intron 1 of *CBFB*, which was not present in both healthy parents (table 1). Based on RNA extracted from blood of the proband and his mother, cDNA analysis showed that the c.78+1G>T variant creates an alternative splice donor site (online supplemental figure 1A), causing a deletion of the last five nucleotides (r.74_78del)

Table 1 Genetic and clinical data on individuals with pathogenic *CBFB* variants

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8
Family	I	II	III	III	IV	IV	V	V
Gender	Male	Male	Female	Female	Female	Female	Female	Female
Age (years)	4	7	37	62	5	32	4	41
CBFB variants (NM_022845.3)	c.78+1G>T r.74_78del	c.283-1039_400-7568del r.283_399del	c.295_296dup	c.295_296dup	c.247C>T	c.247C>T	c.283-2A>G	c.283-2A>G
	p.(Cys25Tyrfs*2)	p.(Val95_Gln133del)	p.(Pro100Leufs*3)	p.(Pro100Leufs*3)	p.(Arg83*)	p.(Arg83*)	NA	NA
Variant effect	Frameshift	Deletion exon 4	Frameshift	Frameshift	Nonsense	Nonsense	NA	NA
Inheritance	De novo	De novo	Maternal	Unknown	Maternal	Unknown	Maternal	Unknown
Suspicion of CCD	+	+	+	+	+	+	+	–
Clinical features								
Height (percentile)	10th	50th–75th	75th–90th	50th–75th	75th–90th	25th–50th	50th–75th	NA
History of large anterior fontanel	–	–	NA	NA	+	–	+	NA
Maxillary hypoplasia	+	–	NA	NA	+	–	–	NA
Other craniofacial dysmorphism	Pointed chin	–	NA	NA	–	–	Prominent forehead, plagiocephaly	NA
Dental anomalies	–	Delayed eruption of deciduous teeth	4 supernumerary teeth	NA	Eruption failure of 5 deciduous teeth	13 super numerary teeth	–	–
Sloping shoulders	–	–	NA	+	+	–	–	–
Hearing loss	–	–	NA	NA	+	+	–	–
Developmental delay	–	Mild	NA	NA	Mild	–	Moderate	–
Other				Meningioma, broad thumbs	Pes planus, genua valga	Pes planus	Broad thumbs, clinodactyly, FTT, hypercalcaemia, CKD	
Radiographic features								
Abnormal clavicles	Bilateral *clavicula bipartita*	Unilateral *clavicula bipartita*, contralateral hypoplasia	Unilateral *clavicula bipartita*	Bilateral aplasia	Bilateral *clavicula bipartita*	-	Unilateral *clavicula bipartita*	NA
Delayed ossification of pubic and ischial bones	–	–	NA	NA	–	+	–	NA
Pseudoepiphyses of metacarpals/metatarsals	+ (second and fifth MC)	+ (second MC)	NA	NA	-	+	+ (second to fifth MC and MT)	NA
Short distal phalanges	+	+	NA	NA	NA	NA	+	NA
Retarded carpal ossification	+	+	NA	NA	NA	NA	+	NA
Other		Coxa valga					Generalised osteopenia, DDH	

CKD, chronic kidney disease; DDH, developmental dysplasia of the hip; FTT, failure to thrive; MC, metacarpal; MT, metatarsal; NA, not available.

of exon 1 with premature stop codon (p.(Cys25Tyrfs*2)) as a result (online supplemental figure 1B-C). *In silico* predictions by MutationTaster and PolyPhen-2 scored the c.78+1G>T variant as respectively disease-causing (score 1.0) and possibly damaging (score 0.913).

Subject 2

Subject 2 is a boy aged 7 years referred because of the suspicion of CCD. He had delayed eruption of deciduous teeth and showed hypermobility of the shoulders (table 1). Height was 126 cm (50th–75 percentile), weight 21.5 kg (10th percentile) and head circumference 52.5 cm (75th–90th percentile). His motor development was delayed. He started to crawl at 14 months and began to walk at 24 months of age. Speech development was also delayed. He started to vocalise simple words such as 'mama' and 'papa' well after 3 years and 6 months of age. At age 6 years, his development was assessed according to the Wechsler Preschool and Primary Scale of Intelligence, showing an average intelligence with partial performance deficits and a mild expressive and receptive language developmental disorder. Skeletal survey showed left-sided *clavicula bipartita* and right-sided clavicular hypoplasia, shortening of the distal phalanges, pseudoepiphyses at the second metacarpals and delayed carpal ossification (figure 2). Genetic analysis revealed a large intragenic and presumably in-frame deletion of 9003 nucleotides encompassing exon 4 of *CBFB* (g.67065643_67074645del; c.283-1039_400-7568del). The deletion was absent in both healthy parents and predicted to be disease-causing (score 1) by MutationTaster. Based on RNA extracted from blood of subject 2, *CBFB* mRNA was analysed using RNA-seq. RNA-seq data showed the presence of the heterozygous in-frame deletion r.283_399del (online supplemental figure 2). No significant changes (z=0.656; p=0.512) were observed in overall *CBFB* expression levels compared with controls, suggesting that the transcripts carrying the *CBFB* r.283_399del deletion are stable and not subject to nonsense-mediated decay.

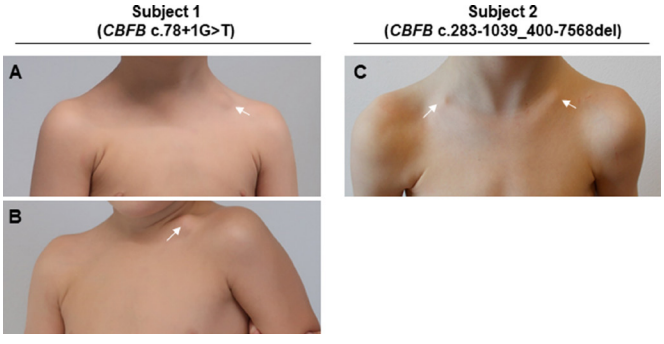

Figure 1 Clinical features of subject 1 (A, B) and subject 2 (C) at the age of 4 years and 7 years, respectively. Note the bony prominences at the shoulders (shown by white arrows) in both cases, clearly visible on the left side in subject 1 (A, B) and less pronounced on both sides in subject 2 (C).

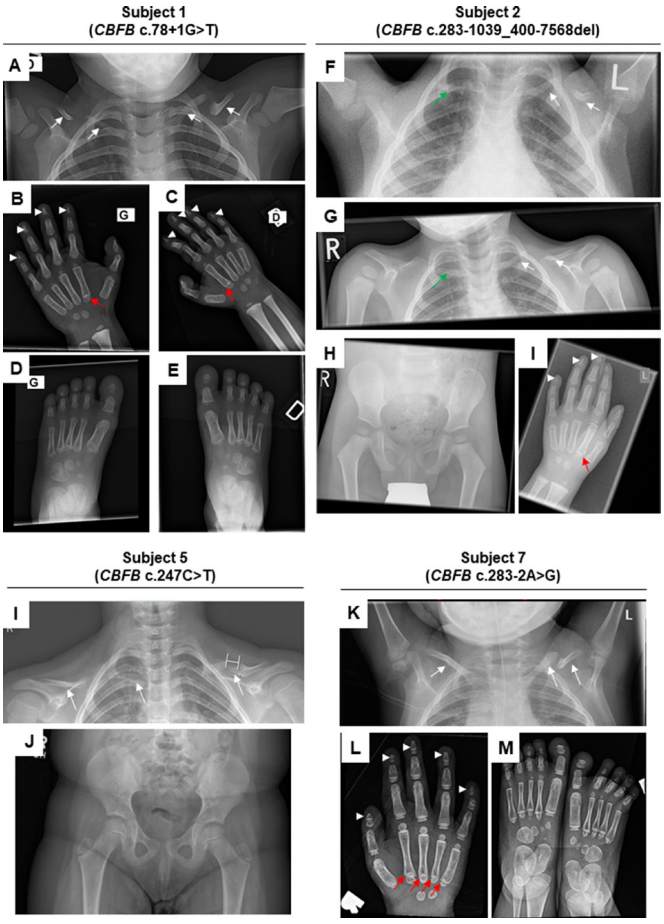

Figure 2 Radiographic features of subject 1 (A–E, age: 3 years), subject 2 (F–I, age: 7 years), subject 5 (I, J, age: 5–7 years) and subject 7 (K–M, age: 3 years 7 months). Clavicular abnormalities (*clavicula bipartita*) are clearly visible (white arrows in A, F, G, I and K). The right clavicle of subject 2 is hypoplastic and misses its acromial or distal part (green arrows in F and G). The right clavicle of subject 7 (K) shows mild middiaphyseal bowing, probably a remnant of the traumatic birth. Hand radiographs show hypoplasia of the distal phalanges (arrowheads in B, C, I and L) and pseudoepiphyses at several metacarpals (red arrows in B, C, I and L). Note the rather round and ballooned epiphyses of the distal phalanges in subject 7 (L). Feet radiographs (D, E) also show hypoplasia of the distal phalanges and pseudoepiphyses at the metatarsals. Radiographs of the pelvis in subject 1 (H) and subject 5 (J) show a normal ossification of the pubic and ischial bones.

Subjects 3 and 4

Subject 3 is an adult woman presenting with clinical signs suspicious for CCD, including right-sided *clavicula bipartita* and four supernumerary teeth (table 1). Her height was 172 cm. She had a clinical diagnosis of fibromyalgia without other significant medical history. Exome sequencing revealed a heterozygous duplication of two nucleotides (c.295_296dup) resulting in a frameshift (p.(Pro100Leufs*3)) in exon 4 of *CBFB* (MutationTaster: disease-causing, score 1; PolyPhen-2: probably damaging, score 1.000). This frameshift variant was also identified in subject 4, her mother, who showed more prominent features of CCD with absent clavicles. The mother also had broad thumbs and a history of meningioma. Her height was 168 cm.

Subjects 5 and 6

Subject 5 is a girl aged 5 years who was referred to a clinical geneticist because of developmental and neurobehavioural concerns after a head injury around the age of 1 year. She had a history of chronic otitis media. Clinical examination revealed features reminiscent of CCD (table 1). She had bilateral sloping and hypermobile shoulders that could be adducted across the midline and she missed five deciduous teeth. Height was 112.4 cm (75th–90th percentile), weight 29.5 kg (97th percentile) and head circumference 53.3 cm (>97th percentile). She also suffered from bilateral sensorineural hearing loss. Skeletal survey revealed bilateral *clavicula bipartita* (figure 2). Brain MRI showed no post-traumatic lesions or other explanation for the developmental and neurobehavioural problems. Exome sequencing revealed a heterozygous nonsense c.247C>T (p.(Arg83*)) variant in exon 3 of *CBFB* (CADD score: 36; MutationTaster: disease-causing, score 1). The nonsense variant was also present in the mother (subject 6) who also showed features of CCD, including the presence of 13 supernumerary teeth and bilateral hearing loss at a young age. Her height was 165.1 cm. Her radiographs showed normal clavicles.

Subjects 7 and 8

Subject 7 was referred to the genetics clinic at the age of 11 months because of bilateral congenital cataracts, global developmental delay and possible dysmorphic features. She also had a history of traumatic birth with right shoulder dystocia, suspicion of bilateral clavicular fractures and multifocal intraparenchymal and subarachnoid haemorrhages. There had been an acute event on day 1 of life with profound hypoglycaemia, seizures and apnoeas with need for neonatal intensive care unit admission and invasive ventilation. Brain MRI at day 12 did not show signs of hypoxic ischaemic injury. Furthermore, there was a history of bilateral developmental dysplasia of the hip. At clinical examination, she showed a large anterior fontanel (8×3 cm) and mild dysmorphic features including a prominent forehead and mid-posterior plagiocephaly. There was bilateral mild fifth finger clinodactyly and hypoplasia of the fifth toenails. She showed normal growth with a height of 73.7 cm (50th percentile), weight 9.6 kg (50th–75th percentile) and head circumference of 46 cm (75th percentile). There was developmental delay, as she only began rolling at 10 months and is not yet able to sit independently at 11 months of age. At 3 years of age, she presented with feeding problems, failure to thrive and recurrent symptomatic hypercalcaemia with hypercalciuria and bilateral nephrocalcinosis. Parathyroid hormone level was decreased. A skeletal survey was performed that showed generalised osteopenia. It also revealed left-sided *clavicula bipartita* (figure 2). The right clavicle showed a sharp curve at the mid-diaphysis, possibly as a consequence of clavicular fracture due to the traumatic birth. There was hypoplasia of the distal phalanges in both hands and feet with ballooning of the epiphyses most pronounced in digit I, II and V. Pseudoepiphyses were present in the second through fifth metacarpals and metatarsals bilaterally. Ossification of the carpal bones and distal radius was delayed (figure 2). At 4 years and 10 months of age, progression towards stage 4 chronic kidney disease was noted. The girl still showed moderate developmental delay with gross motor as well as fine motor and language delay. There is some question as to whether she fit the criteria for autism spectrum disorder. There had been no more seizures since day 1 of life. Trio-exome sequencing revealed a heterozygous splice site variant (c.283-2A>G) in intron 3 of *CBFB* (CADD score: 34; MutationTaster: disease-causing, score 1; Splice ADA score:

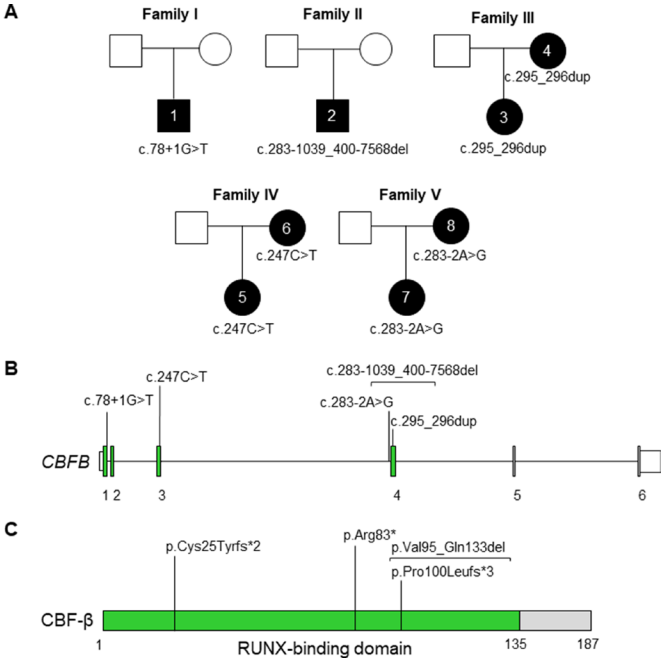

Figure 3 Pedigree and genetic findings in the five families with a pathogenic heterozygous *CBFB* variant. (A) The pedigrees (families I–V) with the affected individuals (numbered 1–8) are shown together with the pathogenic *CBFB* variant. (B) Schematic representation of the *CBFB* gene with localisation of the *CBFB* variants. Exons are shown as vertical bars. Exons shown in green code for the RUNX-binding domain. (C) Schematic representation of the CBFβ protein with indication of the two frameshift and one nonsense variants. The RUNX-binding domain is shown in green.

0.999; Splice RF score: 0.926). MutationTaster predicts a full loss of the splice acceptor sequence motif and a gain of an alternative acceptor splice site after the first 6 nucleotides of exon 4 (score 0.37). This c.283-2A>G variant appeared to be inherited from the mother (subject 8). The mother does not recall any abnormal skeletal concerns, although she did report dental abnormalities at the age of 8 years, resulting in the removal of all teeth. Her adult teeth have been normal.

Table 2 Phenotypic comparison between cases with pathogenic *CBFB* variants and 16q22.1 deletion cases

	Our cohort (n=8)	16q22.1 deletions including *CBFB* (n=8)
Age at last evaluation (average (range))	24 years (4 years–62 years)	17 months (2 months–5 years)
Clinical features		
Growth <3rd percentile	0/8	6/8
History of large anterior fontanel	2/5	7/8
Maxillary hypoplasia	2/5	2/8
Other craniofacial dysmorphism	2/5	8/8
Dental anomalies	4/7	0/8
Sloping shoulders	2/7	1/8
Hearing loss	2/6	1/8
Developmental delay	3/6	8/8
Radiographic features		
Delayed ossification of the skull	0/2	2/4
Wormian bones	0/2	2/4
Abnormal clavicles	6/7	0/4
Delayed ossification of pubic and ischial bones	1/5	0/2
Pseudoepiphyses of metacarpals/metatarsals	4/5	0/3
Short distal phalanges	3/3	2/3
Retarded carpal ossification	3/3	0/3

DISCUSSION

We report on eight individuals, originating from five different families, who all demonstrated clinical and/or radiographic features reminiscent of CCD. Dental and clavicular abnormalities were the most consistent clinical features (table 1). Considerable phenotypic variability, even within families, was present. Clavicular abnormalities ranged from unilateral *clavicula bipartita* to bilateral clavicular aplasia. Two individuals (subjects 5 and 7) had a history of delayed closure of fontanels and cranial sutures. Skeletal survey in two subjects revealed additional signs of CCD such as retarded carpal ossification, presence of pseudoepiphyses in the metacarpals and shortening of the distal phalanges. Two other cases had pseudoepiphyses of metacarpal or metatarsal bones and one had delayed ossification of the pubic and ischial bones. Other musculoskeletal features, such as pes planus, genua valga, coxa vara and generalised osteopenia, were present in some subjects (table 1). Interestingly, developmental delay was noted in three patients, a feature that is usually not observed in *RUNX2*-related CCD.[21 22] Another distinguishing feature from *RUNX2*-related CCD is the normal stature with a height above average.

All subjects in this study cohort tested negative for genomic alterations or variations affecting *RUNX2*. In all subjects, we identified heterozygous pathogenic variants in *CBFB*, encoding CBFβ, an essential interactor of RUNX2. None of these variants were listed in the ExAC,[23] gnomAD[24] or dbSNP databases. These variants included two splice site variants (c.78+1G>T; c.283-2A>G), one duplication of two nucleotides (c.295_296dup; p.(Pro100Leufs*3)), one nonsense variant (c.247C>T; p.(Arg83*)) and a large intragenic deletion (c.283-1039_400-7568del; p.(Val95_Gln133del)) (figure 3). A premature stop codon, resulting in either nonsense-mediated decay or a truncated protein, was the consequence for at least three of these variants. The large intragenic deletion (c.283-1039_400-7568del) was found to result in skipping of exon 4 on mRNA level (r.283_399del), without affecting overall *CBFB* expression levels. RNA samples were not available to investigate the outcome of the splice site variant in intron 3 (c.283-2A>G). All variants were located in that portion of the gene that codes for the RUNX-binding domain of CBFβ (the first four (out of six) exons and corresponding introns) (figure 3).

Haploinsufficiency of *CBFB* has previously been observed in patients with a 16q22.1 deletion encompassing *CBFB*. This interstitial microdeletion syndrome (OMIM #614541) is a multiple congenital anomaly disorder, characterised by poor growth, delayed psychomotor development and distinct craniofacial dysmorphism. Skeletal features overlap with CCD and include hypoplastic distal phalanges, a narrow thorax and delayed closure of the fontanels and cranial sutures.[25] On review of the literature, we identified eight cases with a heterozygous 16q22.1 deletion that includes *CBFB*.[25–28] Phenotypic comparison to our cases with a heterozygous pathogenic variant in *CBFB* shows similarities as well as some striking differences (table 2). Interestingly, no dental or clavicular anomalies were reported in any of these published 16q22.1 deletion cases. Although one patient showed a narrow thorax, clavicles were radiographically normal.[28] However, radiographic evaluation was performed in only six out of the eight 16q22.1 deletion cases, usually at a relatively young age (median age 17 months), which means that some skeletal features could have been missed.[29 30] Neurodevelopmental

delay was reported in all cases, which could be explained by haploinsufficiency of one or more contiguous genes in the deleted interval. In at least five cases *CTCF* was deleted and it is known that heterozygous loss-of-function variants in this gene are responsible for cognitive impairment (OMIM #615502). Given the phenotypic differences between the *CBFB* cases and the 16q22 deletion cases, one could speculate that the variants in *CBFB* do not simply result in haploinsufficiency due to nonsense-mediated RNA decay (NMD). This is further supported by our analyses of RNA from subject 1, where the allele with predicted premature stop codon (r.74_78del; p.(Cys25Tyrfs*2)) could be amplified, without the use of a NMD inhibitor, and from subject 2, where skipping of exon 4 (r.283_399del) did not affect overall *CBFB* expression levels (online supplemental figure 1, online supplemental figure 2).

It is possible that the heterozygous *CBFB* variants in our cohort result in a truncated protein that fails to interact properly with the RUNX proteins or, alternatively, exerts a new function (neomorphic effect). CBFβ and RUNX2 are subunits of the core-binding factor (CBF) family of heterodimeric transcription factors. These transcription factors consistently contain a DNA-binding RUNX protein (RUNX1, RUNX2, RUNX3) and the non-DNA-binding CBFβ protein that ensures high-affinity DNA binding of the RUNX proteins and stability of the CBF complex. The most conserved part of the RUNX proteins is the Runt domain (RHD) that enables DNA binding and binding to CBFβ.[31–34] Interestingly, most *RUNX2* variants causing CCD are located within this Runt domain.[35] Also the *CBFB* variants identified in our case cohort all affect the RUNX-binding domain. Thus, hampering or destabilising the binding of CBFβ to the RUNX proteins might impair the function of the CBF transcription factor complex.

CBF complexes regulate gene expression through binding to promoters or enhancer elements. The effects of RUNX-CBFβ regulation can be lineage-specific and stage-specific, and affect crucial processes such as cellular proliferation and differentiation.[31] RUNX2 is a master transcriptional regulator during osteoblast and chondrocyte differentiation, as was also demonstrated by the general lack of ossification in *Runx2*-deficient (*Runx2*$^{-/-}$) mice.[10] Interestingly, multiple studies demonstrated that CBFβ is a necessary cofactor of RUNX2 during bone formation.[36–38] Moreover, conditional knockout mouse models of *Cbfb* in chondrocytes, osteoblasts or mesenchymal stem cells, also show an impairment of both intramembranous and endochondral bone development.[36 38–42] Based on these findings, Kundu *et al*. already hypothesised that pathogenic variants in *CBFB* may be responsible for some cases of CCD that are not linked to pathogenic variants in *RUNX2*.[36] It is however clear that the phenotype we observe in our patient cohort is different from classical *RUNX2*-related CCD, especially in terms of their normal stature and developmental delay. This could be attributed to the disturbed interaction of CBFβ with the other RUNX proteins (RUNX1 and RUNX3). So far, it has been demonstrated that they have a primary function in haematopoiesis (RUNX1), in nociceptive (RUNX1) and proprioceptive neurons (RUNX3) of the dorsal root ganglia, in CD8^{+} T-cell development (RUNX1, RUNX3) and a supportive role during bone formation (RUNX1) and chondrocyte maturation (RUNX3).[43] Future functional studies are needed to examine how *CBFB* variants affect the function of the different CBFβ-RUNX transcription factor complexes, and how this may contribute to the development of the phenotype we observe in our cohort.

In conclusion, we confirm the previously suggested locus heterogeneity for CCD by identifying five novel pathogenic variants in *CBFB* in eight individuals with a new skeletal disorder with phenotypic features overlapping with CCD. We also demonstrate that the *CBFB*-related phenotype in our patient cohort is more extensive than classical *RUNX2*-related CCD. This study therefore expands our genotypic, phenotypic and mechanistic knowledge of the so-called cleidocranial dysplasia spectrum disorder.

Author affiliations
[1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium
[2]Center for Human Genetics, Cliniques universitaires Saint-Luc and University of Louvain, Brussels, Belgium
[3]Institute of Medical Genetics and Applied Genomics, University of Tübingen, Tübingen, Germany
[4]Institute of Human Genetics, Justus Liebig University, Giessen, Germany
[5]Clinical Institute of Genomic Medicine, University Medical Centre Ljubljana Division of Internal Medicine, Ljubljana, Slovenia
[6]Children's Health, Division of Genetics, Medical University of South Carolina, Charleston, South Carolina, USA
[7]GeneDx Inc, Gaithersburg, Massachusetts, USA
[8]Provincial Medical Genetics Program, Eastern Health, St. John's, Newfoundland, Canada
[9]Memorial University of Newfoundland, St. John's, Newfoundland, Canada
[10]Department of Human Genetics, KU Leuven, Leuven, Belgium
[11]Center for Human Genetics, University Hospital Leuven, Leuven, Belgium

Acknowledgements We would like to thank all subjects and their families for their participation in the study.

Contributors TB, GH and GRM were responsible for conceptualisation, visualisation, writing (original draft) and writing (review and editing). All other authors participated in writing (review and editing). All authors evaluated and examined families and/or performed genetic analyses. EBo, GH and GRM performed additional experiments and/or evaluated data collected for subject 1. MB, LS and TBH performed the additional experiments and/or evaluated data collected for subject 2. GH and GRM act as guarantor of the study and hereby accept full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

Funding This study was supported by a Methusalem-OEC grant—'GENOMED' (grant number: FFB190208) to TB, NB, WW, WVH, GH and GRM.

Competing interests AT and LBH are employees of GeneDx, Inc.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval The study was conducted according to the World Medical Association Declaration of Helsinki on ethical principles for medical research involving human subjects. Written informed consent was obtained from all affected subjects, parents or legal representatives participating in this study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iDs
Eveline Boudin http://orcid.org/0000-0003-4818-6804
Nicole Revencu http://orcid.org/0000-0002-7120-4903
Tobias B Haack http://orcid.org/0000-0001-6033-4836
Gretl Hendrickx http://orcid.org/0000-0001-6715-9241

REFERENCES

1 Farrow E, Nicot R, Wiss A, Laborde A, Ferri J. Cleidocranial dysplasia: a review of clinical, radiological, genetic implications and a guidelines proposal. *J Craniofac Surg* 2018;29:382–9.
2 Stevenson DA, Carey JC, Byrne JLB, Srisukhumbowornchai S, Feldkamp ML. Analysis of skeletal dysplasias in the Utah population. *Am J Med Genet A* 2012;158A:1046–54.
3 Machol K, Mendoza-Londono R, Lee B. Cleidocranial Dysplasia Spectrum Disorder. In: Adam MP, Ardinger HH, Pagon RA, eds. Seattle (WA): GeneReviews((R)), 1993.
4 Mundlos S, Mulliken JB, Abramson DL, Warman ML, Knoll JH, Olsen BR. Genetic mapping of cleidocranial dysplasia and evidence of a microdeletion in one family. *Hum Mol Genet* 1995;4:71–5.
5 Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell* 1997;89:773–9.
6 Jaruga A, Hordyjewska E, Kandzierski G, Tylzanowski P. Cleidocranial dysplasia and RUNX2-clinical phenotype-genotype correlation. *Clin Genet* 2016;90:393–402.
7 Narahara K, Tsuji K, Yokoyama Y, Seino Y. Cleidocranial dysplasia associated with a t(6;18)(p12;q24) translocation. *Am J Med Genet* 1995;56:119–20.
8 Nienhaus H, Mau U, Zang KD, Henn W. Pericentric inversion of chromosome 6 in a patient with cleidocranial dysplasia. *Am J Med Genet* 1993;46:630–1.
9 Komori T. Molecular mechanism of Runx2-dependent bone development. *Mol Cells* 2020;43:168–75.
10 Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of CBFA1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997;89:755–64.
11 Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. CBFA1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 1997;89:765–71.
12 Baumert U, Golan I, Redlich M, Aknin J-J, Muessig D. Cleidocranial dysplasia: molecular genetic analysis and phenotypic-based description of a middle European patient group. *Am J Med Genet A* 2005;139A:78–85.
13 Dinçsoy Bir F, Dinçkan N, Güven Y, Baş F, Altunoğlu U, Kuvvetli SS, Poyrazoğlu Şükran, Toksoy G, Kayserili H, Uyguner ZO. Cleidocranial dysplasia: clinical, endocrinologic and molecular findings in 15 patients from 11 families. *Eur J Med Genet* 2017;60:163–8.
14 Sobreira N, Schiettecatte F, Boehm C, Valle D, Hamosh A. New tools for mendelian disease gene identification: PhenoDB variant analysis module; and GeneMatcher, a web-based tool for linking Investigators with an interest in the same gene. *Hum Mutat* 2015;36:425–31.
15 Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. *Hum Mutat* 2015;36:928–30.
16 World Medical Association. World medical association declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA* 2013;310:2191–4.
17 Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* 2013;76.
18 Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 2014;46:310–5.
19 Steinhaus R, Proft S, Schuelke M, Cooper DN, Schwarz JM, Seelow D. MutationTaster2021. *Nucleic Acids Res* 2021;49:W446–51.
20 Deschauer M, Hengel H, Rupprich K, Kreiß M, Schlotter-Weigel B, Grimmel M, Admard J, Schneider I, Alhaddad B, Gazou A, Sturm M, Vorgerd M, Balousha G, Balousha O, Falna M, Kirschke JS, Kornblum C, Jordan B, Kraya T, Strom TM, Weis J, Schöls L, Schara U, Zierz S, Riess O, Meitinger T, Haack TB. Bi-allelic truncating mutations in *VWA1* cause neuromyopathy. *Brain* 2021;144:574–83.
21 Cooper SC, Flaitz CM, Johnston DA, Lee B, Hecht JT. A natural history of cleidocranial dysplasia. *Am J Med Genet* 2001;104:1–6.
22 McBrien H, Turk J, Letch N. The management of ADHD and associated problems in a young person with cleidocranial dysostosis (CCD) and mild intellectual disability. *Clin Child Psychol Psychiatry* 2006;11:445–56.
23 Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won H-H, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG, Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 2016;536:285–91.
24 Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, Collins RL, Laricchia KM, Ganna A, Birnbaum DP, Gauthier LD, Brand H, Solomonson M, Watts NA, Rhodes D, Singer-Berk M, England EM, Seaby EG, Kosmicki JA, Walters RK, Tashman K, Farjoun Y, Banks E, Poterba T, Wang A, Seed C, Whiffin N, Chong JX, Samocha KE, Pierce-Hoffman E, Zappala Z, O'Donnell-Luria AH, Minikel EV, Weisburd B, Lek M, Ware JS, Vittal C, Armean IM, Bergelson L, Cibulskis K, Connolly KM, Covarrubias M, Donnelly S, Ferriera S, Gabriel S, Gentry J, Gupta N, Jeandet T, Kaplan D, Llanwarne C, Munshi R, Novod S, Petrillo N, Roazen D, Ruano-Rubio V, Saltzman A, Schleicher M, Soto J, Tibbetts K, Tolonen C, Wade G, Talkowski ME, Neale BM, Daly MJ, MacArthur DG, Genome Aggregation Database Consortium. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* 2020;581:434–43.
25 Fujiwara M, Yoshimoto T, Morita Y, Kamada M. Interstitial deletion of chromosome 16q: 16q22 is critical for 16q- syndrome. *Am J Med Genet* 1992;43:561–4.
26 Goto T, Aramaki M, Yoshihashi H, Nishimura G, Hasegawa Y, Takahashi T, Ishii T, Fukushima Y, Kosaki K. Large fontanelles are a shared feature of haploinsufficiency of RUNX2 and its co-activator CBFB. *Congenit Anom* 2004;44:225–9.
27 Khan A, Hyde RK, Dutra A, Mohide P, Liu P. Core binding factor beta (CBFB) haploinsufficiency due to an interstitial deletion at 16q21q22 resulting in delayed cranial ossification, cleft palate, congenital heart anomalies, and feeding difficulties but favorable outcome. *Am J Med Genet A* 2006;140:2349–54.
28 Callen DF, Eyre H, Lane S, Shen Y, Hansmann I, Spinner N, Zackai E, McDonald-McGinn D, Schuffenhauer S, Wauters J. High resolution mapping of interstitial long arm deletions of chromosome 16: relationship to phenotype. *J Med Genet* 1993;30:828–32.
29 Golan I, Baumert U, Hrala BP, Müssig D. Dentomaxillofacial variability of cleidocranial dysplasia: clinicoradiological presentation and systematic review. *Dentomaxillofac Radiol* 2003;32:347–54.
30 Ishii K, Nielsen IL, Vargervik K. Characteristics of jaw growth in cleidocranial dysplasia. *Cleft Palate Craniofac J* 1998;35:161–6.
31 Blyth K, Cameron ER, Neil JC. The Runx genes: gain or loss of function in cancer. *Nat Rev Cancer* 2005;5:376–87.
32 Kagoshima H, Shigesada K, Satake M, Ito Y, Miyoshi H, Ohki M, Pepling M, Gergen P. The runt domain identifies a new family of heteromeric transcriptional regulators. *Trends Genet* 1993;9:338–41.
33 Park J, Gebhardt M, Golovchenko S, Perez-Branguli F, Hattori T, Hartmann C, Zhou X, deCrombrugghe B, Stock M, Schneider H, von der Mark K. Dual pathways to endochondral osteoblasts: a novel chondrocyte-derived osteoprogenitor cell identified in hypertrophic cartilage. *Biol Open* 2015;4:608–21.
34 Tahirov TH, Inoue-Bungo T, Morii H, Fujikawa A, Sasaki M, Kimura K, Shiina M, Sato K, Kumasaka T, Yamamoto M, Ishii S, Ogata K. Structural analyses of DNA recognition by the AML1/Runx-1 runt domain and its allosteric control by CBFbeta. *Cell* 2001;104:755–67.
35 Zhou G, Chen Y, Zhou L, Thirunavukkarasu K, Hecht J, Chitayat D, Gelb BD, Pirinen S, Berry SA, Greenberg CR, Karsenty G, Lee B. CBFA1 mutation analysis and functional correlation with phenotypic variability in cleidocranial dysplasia. *Hum Mol Genet* 1999;8:2311–6.
36 Kundu M, Javed A, Jeon J-P, Horner A, Shum L, Eckhaus M, Muenke M, Lian JB, Yang Y, Nuckolls GH, Stein GS, Liu PP. Cbfbeta interacts with Runx2 and has a critical role in bone development. *Nat Genet* 2002;32:639–44.
37 Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G. Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with CBFbeta. *Mol Cell Biol* 1998;18:4197–208.
38 Yoshida CA, Furuichi T, Fujita T, Fukuyama R, Kanatani N, Kobayashi S, Satake M, Takada K, Komori T. Core-binding factor beta interacts with Runx2 and is required for skeletal development. *Nat Genet* 2002;32:633–8.
39 Chen W, Ma J, Zhu G, Jules J, Wu M, McConnell M, Tian F, Paulson C, Zhou X, Wang L, Li Y-P. Cbfβ deletion in mice recapitulates cleidocranial dysplasia and reveals multiple functions of Cbfβ required for skeletal development. *Proc Natl Acad Sci U S A* 2014;111:8482–7.
40 Jiang Q, Qin X, Kawane T, Komori H, Matsuo Y, Taniuchi I, Ito K, Izumi S-I, Komori T. Cbfb2 isoform dominates more potent Cbfb1 and is required for skeletal development. *J Bone Miner Res* 2016;31:1391–404.
41 Wu M, Li C, Zhu G, Wang Y, Jules J, Lu Y, McConnell M, Wang Y-J, Shao J-Z, Li Y-P, Chen W. Deletion of core-binding factor β (Cbfβ) in mesenchymal progenitor cells provides new insights into Cbfβ/Runxs complex function in cartilage and bone development. *Bone* 2014;65:49–59.
42 Wu M, Li Y-P, Zhu G, Lu Y, Wang Y, Jules J, McConnell M, Serra R, Shao J-Z, Chen W. Chondrocyte-specific knockout of Cbfβ reveals the indispensable function of Cbfβ in chondrocyte maturation, growth plate development and trabecular bone formation in mice. *Int J Biol Sci* 2014;10:861–72.
43 Cohen MM. Perspectives on Runx genes: an update. *Am J Med Genet A* 2009;149A:2629–46.