

SHORT REPORT

Microduplications upstream of *MSX2* are associated with a phenocopy of cleidocranial dysplasia

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ABSTRACT

Background Cleidocranial dysplasia (CCD) is an autosomal dominant skeletal disorder characterised by hypoplastic or absent clavicles, increased head circumference, large fontanelles, dental anomalies and short stature. Although CCD is usually caused by mutations leading to haploinsufficiency of *RUNX2*, the underlying genetic cause remains unresolved in about 25% of cases.

Methods Array comparative genomic hybridisation was performed to detect copy number variations (CNVs). Identified CNVs were characterised by quantitative PCR and sequencing analyses. The effect of candidate genes on mineralisation was evaluated using viral overexpression in chicken cells.

Results In 2 out of 16 cases, the authors identified microduplications upstream of *MSX2* on chromosome 5q35.2. One of the unrelated affected individuals presented with a phenocopy of CCD. In addition to a classical CCD phenotype, the other subject had a complex synpolydactyly of the hands and postaxial polydactyly of the feet which have so far never been reported in association with CCD or CNVs on 5q35.2. The duplications overlap in an ~219 kb region that contains several highly conserved non-coding elements which are likely to be involved in *MSX2* gene regulation. Functional analyses demonstrated that the inhibitory effect of *Msx2* overexpression on mineralisation cannot be ameliorated by forced *Runx2* expression.

Conclusions These results indicate that CNVs in non-coding regions can cause developmental defects, and that the resulting phenotype can be distinct from those caused by point mutations within the corresponding gene. Taken together, these findings reveal an additional mechanism for the pathogenesis of CCD, particularly with regard to the regulation of *MSX2*.

Cleidocranial dysplasia (CCD, MIM 119600) is an autosomal dominant skeletal disorder that affects bones derived from endochondral as well as intramembranous ossification such as the cranium, the clavicles and, to a lesser degree, long bones. Characteristic features of the skull are enlarged fontanelles with delayed closure, Wormian bones, frontal bossing and mid-face hypoplasia. The most prominent clinical feature is narrow, sloping shoulders that can be apposed at the midline due to aplastic or hypoplastic clavicles. Further features include various dental anomalies, including supernumerary teeth and delayed eruption of permanent teeth,

short stature and hand abnormalities, as well as other skeletal changes.¹

CCD is mainly caused by loss-of-function mutations in runt-related transcription factor 2 (*RUNX2*, MIM 600211), also known as core-binding factor A1.^{1–2} The osteoblast-specific transcription factor *RUNX2* is a member of the runt-domain gene family and promotes the differentiation of mesenchymal cells into osteoblasts.^{3–4} Point mutations in *RUNX2* can be detected in about 60%–70% of all affected individuals with CCD, and heterozygous deletions can be identified in about 10%.^{2,5}

Some other hereditary skeletal disorders resemble CCD to a certain degree, and at least some of these disorders result from mutations in genes that affect the action of *RUNX2* on its target genes. Abnormalities in core-binding factor β (*CBFB*, MIM 121360), which interacts with *RUNX2*, cause skeletal anomalies such as large fontanelles, hypoplasia of the distal phalanges and delayed ossification.^{6–7} Parietal foramina with cleidocranial dysplasia (PFMCCD, MIM 168550) is caused by loss-of-function mutations of *Drosophila* muscle segment homeobox gene homologue 2 (*MSX2*, MIM 123101) and is therefore another entity aetiologically and phenotypically distinct from *RUNX2*-related CCD. Characteristic clinical features of PFMCCD are mild clavicular hypoplasia, craniofacial abnormalities such as a broad forehead and central bossing, and symmetrical oval defects of the parietal bones which are termed parietal foramina.⁸ Parietal foramina can occur in combination with clavicular hypoplasia in case of *MSX2* mutations or isolated due to mutations in *MSX2* or aristaless-like 4 (*ALX4*, MIM 605420).^{9–10}

Genome-wide screening methods such as microarray-based comparative genomic hybridisation (array CGH) are a valuable tool to detect submicroscopic copy number variations (CNVs). To further investigate the underlying genetic cause of CCD, we performed array CGH (244 K or 1M oligonucleotide array, Agilent Technologies, Santa Clara, California, USA) in 16 individuals in whom mutations and CNVs in *RUNX2*, *CBFB* and *MSX2* had been excluded. Data analysis was carried out as described previously.¹¹ Using this approach, we detected microduplications of different sizes on chromosome 5q35.2 in two unrelated affected individuals (Supplementary figure S1, Supplementary figure S2).

Copy-number variation

Affected individual 1 (P1) presented with typical signs of CCD. As shown in figure 1A, he was able to appose his shoulders at the midline due to a complete absence of both clavicles (figure 1B). At the age of 4 years, he showed a persistent anterior fontanelle (figure 1C) but no signs of parietal foramina or other abnormalities of the skull (figure 1D). X-rays of the hands (figure 1E) and the pelvis (figure 1F) revealed signs of delayed bone age. The anlagen of some deciduous teeth were missing (figure 1G). Cardiac arrhythmia had been repeatedly found during cardiological examination including Holter ECG monitoring, but no structural heart malformation was detectable by ultrasound.

Array CGH identified an ~ 1.7 Mb duplication upstream of *MSX2* in this individual (Supplementary figure S1; Decipher BER264221). Quantitative PCR (qPCR) was used to refine the breakpoint intervals and to confirm de novo occurrence of the duplication (Supplementary figure S3A). The centromeric breakpoint was localised within an ~ 0.7 kb interval

(chr5:172,411,905-172,412,552) flanked by qPCR amplicons A3 and A4, and the telomeric breakpoint within an ~ 2.1 kb interval (chr5:174,066,198-174,068,279) flanked by amplicons A9 and A10 (figure 2A; genomic positions are given according to hg19). Analysis by PCR with primers A9-forward and A4-reverse (Supplementary table S1) on genomic DNA level allowed amplification and sequencing analysis of a junction fragment which contained a homology of 37 bp beginning at the telomeric site on position 174,067,202 and at the centromeric site on position 172,412,091 (Supplementary figure S4A). Both breakpoints were localised within *AluY* elements. These data are consistent with unequal homologous recombination as a conceivable mechanism leading to the ~ 1655 kb microduplication oriented in direct tandem and localised ~ 69 kb upstream of *MSX2* (figure 2B).

Affected individual 2 (P2) presented with typical features of CCD. Both clavicles were absent (figure 1H), and at the age of

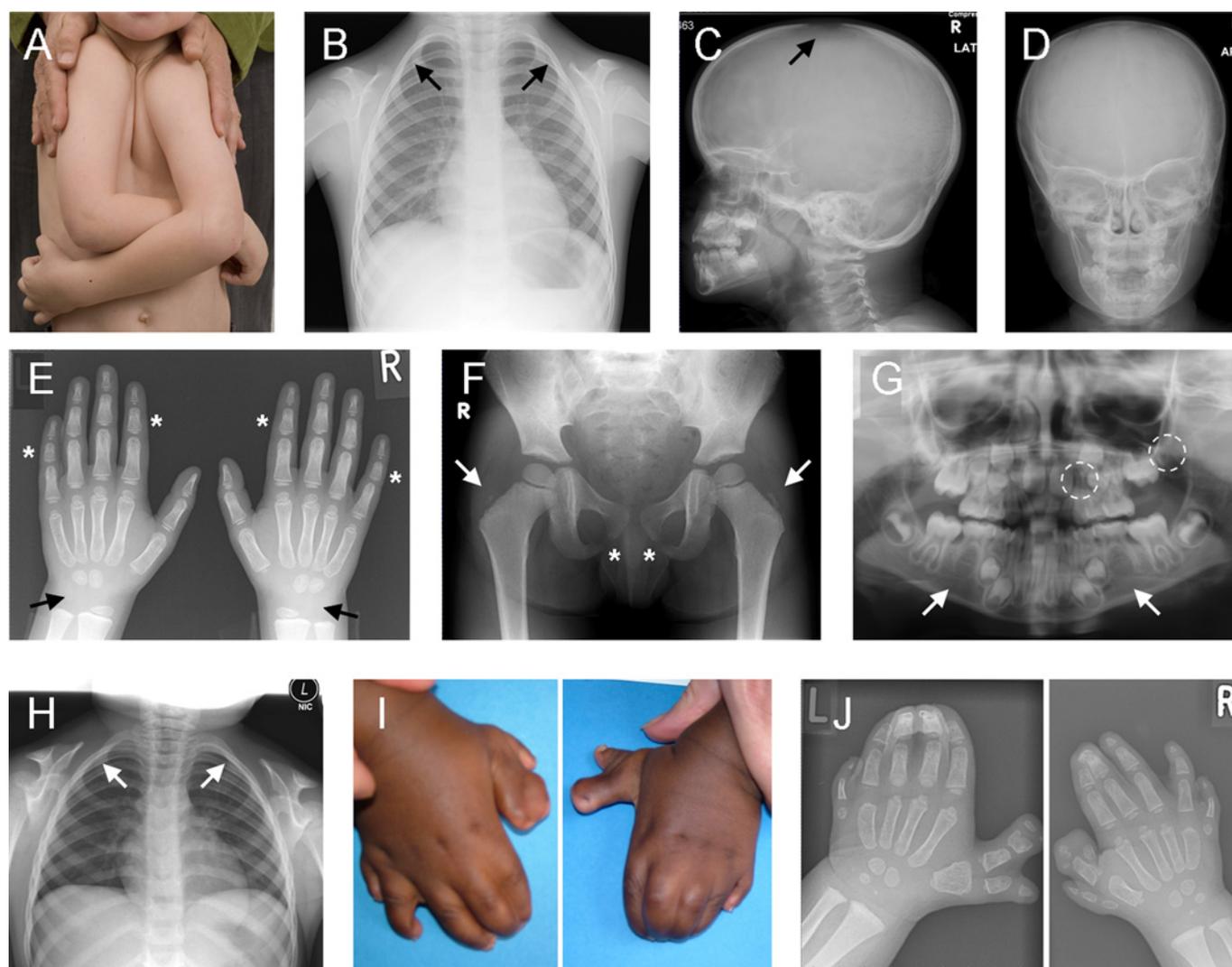
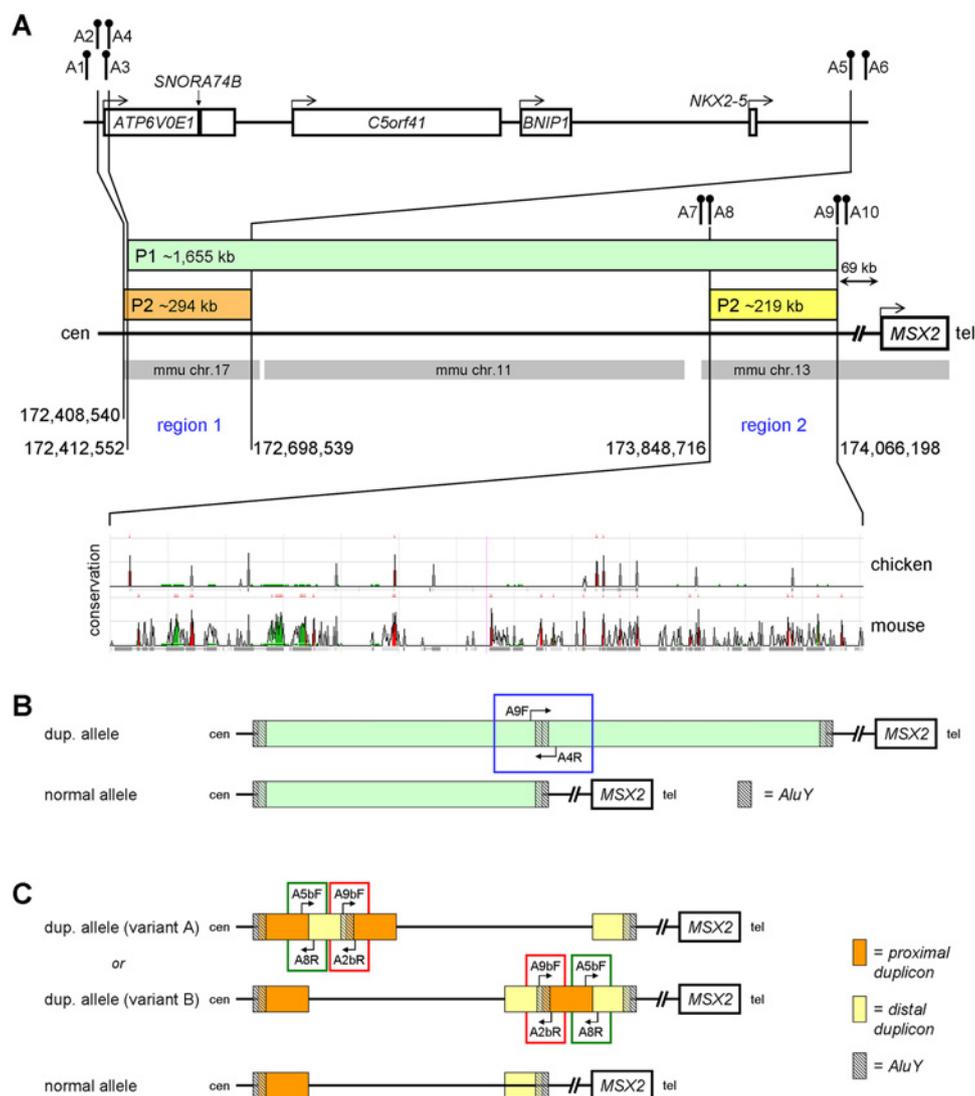


Figure 1 Clinical phenotypes associated with microduplications at the *MSX2* locus on 5q35.2. (A–G) Clinical phenotype of P1. (A) Hypermobility of the shoulders. (B) X-rays of the thorax showing absent clavicles on both sides (arrows). (C) The skull defect at the age of 4 years and 3 months is marked by an arrow (lateral view). (D) AP picture. There are no signs of parietal foramina or craniosynostosis. (E) X-rays of the hands at the age of 4 years and 6 months. The middle phalanges of digit II and V appear slightly shortened (asterisks). Os triquetrum and os lunatum are not visible in both wrists (arrows) which correspond to a carpal age of 2 years. (F) X-rays of the pelvis. On both sides, the synchondrosis ischiopubica is still open (asterisks) and the ossification centres of trochanter major are relatively small (arrows). (G) Orthopantomogram at the age of 7 years and 11 months. The anlagen of the deciduous teeth 22 and 27 (circles) as well as 35 and 45 (arrows) are missing. H–J Clinical phenotype of P2. (H) X-rays of the thorax showing absent clavicles on both sides (arrows). (I) Limb phenotype with synpolydactyly of both hands and (J) corresponding x-rays.

Figure 2 Schematic overview of microduplications at the *MSX2* locus on 5q35.2. The duplicated sequences observed in the affected individuals with CCD are shown as coloured rectangles (green, duplication P1; orange, proximal duplication P2; yellow, distal duplication P2). Coloured boxes in B and C correspond to the junction fragments shown in Supplementary figure S4. (A) The approximate positions of quantitative PCR amplicons used to refine the breakpoint intervals are indicated (A1–A10). On top, the proximal overlapping duplicated sequence (region 1) is enlarged showing the genes within this interval. The conservation of the distal overlapping duplicated sequence (region 2) is depicted in the ECR browser plot. Note that there are no RefSeq genes in this interval. The sequence upstream of the mouse homologue *Msx2* is located on different chromosomes as indicated by the grey bars. For clarity, the four genes between region 1 and region 2 duplicated only in P1 (cf, Supplementary figure S1) are not illustrated. (B) Direct tandem orientation of the microduplication in P1. (C) As heterozygous SNPs could be identified both at the centromeric end of the proximal duplication and at the telomeric end of the distal duplication, there remain two variants (variant A or variant B) for the arrangement of the duplicons in P2. The genuine position of the duplicons on the normal allele is shown at the bottom. (B and C) A9F, A4R, A5bF, A8R, A9bF and A2bR refer to the PCR primers used to amplify the different junction fragments.



3 years the anterior fontanelle was still open. In addition to classical features of CCD, she presented with a complex synpolydactyly of the hands (figure 1I,J) and postaxial polydactyly of the feet which have so far never been described in patients with CCD or in association with CNVs on 5q35.2. By sequencing analysis, no mutation was identified in genes known to be associated with limb malformations such as *GLI3* (MIM 165240), *ROR2* (MIM 602337), *HOXD13* (MIM 142989), *TWIST1* (MIM 601622), *ALX4* (MIM 605420) and *EFNB1* (MIM 300035). Array CGH identified an ~ 0.3 Mb duplication ~ 1.5 Mb upstream of *MSX2* and, further telomeric, a second ~ 0.2 Mb duplicated region ~ 0.1 Mb upstream of *MSX2* (Supplementary figure S2; Decipher BER264214). qPCRs with amplicons indicated in figure 2A confirmed both duplications. Analysis of the parents revealed normal results, thus, confirming de novo occurrence of both duplicated sequences (Supplementary figure S3B).

The four breakpoint intervals were narrowed down by qPCR. Further analysis by PCR on genomic DNA level using different primer combinations both for inverted or tandem orientation of the duplicated sequences in region 1 and region 2 allowed no amplification of a junction fragment, whereas PCR with primers A9b-forward and A2b-reverse and with primers A5b-forward

and A8-reverse (Supplementary table S1) allowed the identification and sequencing analysis of two different junction fragments. Junction 1 connects the telomeric end of the distal duplication (A9→A10) with the centromeric end of the proximal duplication (A1←A2) and contains a homology of 21 bp beginning at the telomeric site on position 174,067,232 and at the centromeric site on position 172,406,206 (Supplementary figure S4B). The telomeric breakpoint in junction 1 is almost identical with the telomeric breakpoint identified in P1. Similar to the situation in P1, unequal homologous recombination between *Alu* repeats would be a conceivable mechanism.

In junction 2 (Supplementary figure S4C), the proximal duplicated sequence ends at position 172,700,622 (A5→A6) and the distal duplicated sequence begins at position 173,847,989 (A7←A8). In between there is a short insertion of eight nucleotides. These findings can best be explained by break-induced non-homologous DNA end joining as underlying mechanism. Two possibilities for the complex genomic rearrangement in P2 remain to be considered with either one copy of the ~ 219 kb distal duplicon localised in direct orientation in between two copies of the ~ 294 kb proximal duplicon or vice versa (figure 2C). Unfortunately, it was not possible to finally clarify the arrangement of the duplicons by FISH analysis. Additional

Copy-number variation

information on the complex rearrangement observed in P2 can be found at the end of the Supplementary information.

The microduplications identified in the affected individuals are most likely to be causative since they occurred *de novo* and similar microduplications are not described as CNVs in the Database of Genomic Variants. As shown in figure 2A, the microduplications overlap in an ~287 kb proximal region (region 1) and share an ~219 kb distal interval (region 2). Region 1 contains exons 2–4 of *ATP6V0E1* (MIM 603931), the entire coding sequence of the genes *SNORA74B* (MIM 611331, localised within intron 3 of the *ATP6V0E1* gene), *BNIP1* (MIM 603291) and *NKX2-5* (MIM 600584), as well as the open reading frame *C5orf41*. None of these genes has a known role in skeletal development, and we found no expression of these genes in the developing limb (data not shown). Mutations in *NKX2-5* are associated with congenital defects of the heart such as atrial septal defect, tetralogy of Fallot and atrioventricular conduction defects. It is possible that the duplication of *NKX2-5* accounts for the cardiac arrhythmia observed in P1.

Interestingly, the genomic architecture upstream of *MSX2* is not fully conserved between mouse and human. As indicated by the grey bars in figure 2A, the first block including region 1 corresponds to mouse chromosome 17, the second block between region 1 and region 2 to mouse chromosome 11, and the third block including region 2 as well as *MSX2* is located on mouse chromosome 13. This makes it unlikely that region 1 contains *cis*-regulatory elements relevant for the regulation of *MSX2*. In contrast, region 2 contains no RefSeq genes, but several evolutionary conserved non-coding elements (CNEs) which may constitute tissue specific enhancers for *MSX2*.

During skeletal development complex regulatory mechanisms are needed that dynamically control spatial and temporal expression of developmentally important genes such as secreted signalling molecules and key transcription factors. In principle, gene regulation can be mediated by regulatory promoters located immediately upstream of their target genes to direct correct gene expression. However, more complex regulation can be achieved by the so-called *cis*-regulatory elements that can be localised as far as 1.5 Mb upstream or downstream of the target gene.¹² *Cis*-regulatory elements are important mediators of gene regulation as they have the potential to activate or inhibit gene expression in distinct cell types or tissues at certain time points. In line with the particular importance of these elements, an emerging number of genetic alterations affecting regulatory elements have been identified.^{13–14} Such long-range regulatory effects appear to be of particular importance for developmental genes which often show complex temporal and spatial expression patterns. A novel mutational mechanism involving the duplication of CNEs has recently been described for a number of genes including *SHH* (MIM 600725), *IHH* (MIM 600726), *BMP2* (MIM 112261) and *SOX9* (MIM 608160).¹⁴ These duplications are likely to exert their effect by altering gene expression of the target gene. Based on these observations, we hypothesise that the CNVs described here cause a CCD-like phenotype by interfering with *MSX2* gene regulation. Studies addressing the regulation of *MSX2* gene expression focused on phylogenetically conserved *cis*-regulatory elements within the *Msx2* promoter region.^{15–18} The large gene desert containing multiple CNEs 5' of *MSX2* is highly suggestive of extensive long range regulation. However, how these elements contribute to the spatial and temporal expression of *Msx2* is unknown, and we cannot exclude that other sequences within the duplicated region 2 might have an effect on skeletal development.

MSX2 is one of three members of the *Msx* homeobox gene family, and *Msx1* (MIM 142983) and *Msx2* are believed to be functionally redundant. During embryogenesis and development, both genes are expressed at multiple sites and in a variety of tissues including the craniofacial processes and branchial arches, the tooth and limb buds, as well as the heart.¹⁹ *Msx* genes frequently show overlapping expression and are believed to promote lineage commitment and proliferation, but inhibit cell-type specific differentiation unless developmental patterns are established. *Msx* genes are highly expressed at the margins of bones in osteoblasts. Multiple studies have shown that they are, together with Runx2, Dlx5 (MIM 600028), Dlx6 (MIM 600030) and other transcription factors, involved in the regulation of osteoblast differentiation.²⁰

It has been shown *in vitro* that overexpression of *Msx2* inhibits osteoblastic differentiation and mineralisation of the extracellular matrix. In contrast, expression of antisense *Msx2* RNA leads to decreased protein levels of *Msx2* and thereby to reduced proliferation, accelerated differentiation and enhanced mineralisation.²¹ In an *in vivo* model, it has been demonstrated that overexpression of an *Msx2* transgene resulted in an increased number of alkaline phosphatase-positive cells in the osteogenic front of the sagittal suture and enhanced parietal bone growth.²² Using a luciferase reporter containing the osteocalcin promoter, it has been shown that *Msx2* represses the activity of *Runx2* and that *Dlx5* counteracts this repressor activity. The authors suggest the possibility that the effects of *Msx2* overexpression are mediated through the repression of *Runx2* binding to its target genes.²³ Further investigation of the interactions among *Dlx5*, *Msx2* and *Runx2* revealed that the inductive action of *Dlx5* on the *Runx2* promoter can also be antagonised by *Msx2* suggesting an inhibitory effect of *Msx2* on *Runx2* transcription.²⁴

To test whether the inhibitory effect of *Msx2* on mineralisation can be antagonised by *Runx2* overexpression, we created two RCAS virus constructs containing *Msx2* and *Runx2*. Chicken cells were transfected with each construct or co-transfected with both in parallel, and mineralisation was assayed using alizarin red staining. As shown in Supplementary figure S5, the inhibitory effect of *Msx2* on mineralisation cannot be ameliorated by *Runx2* overexpression and is thus dominant over *Runx2*-mediated osteoblastic induction and bone formation. These data suggest that prolonged or increased expression of *MSX2* during craniofacial development may affect the spatial and temporal control of proliferation and differentiation of osteoblast precursor cells.

Haploinsufficiency of *MSX2* due to loss-of-function mutations or heterozygous deletions encompassing the entire gene causes parietal foramina (MIM 168500).¹⁰ In mice inactivation of *Msx2* results in multiple abnormalities including persistent calvarial defects.²⁵ In contrast, transgenic mice overexpressing *Msx2* exhibit enhanced calvarial bone growth and suture overgrowth suggesting that *Msx2* dosage influences osteogenic growth in the skull, possibly by influencing the rate of cell differentiation in the sutures.^{21–22} This hypothesis is supported by the observation that additional copies of the *MSX2* gene are associated with craniosynostosis in humans.^{26–27}

In some individuals, parietal foramina can be associated with hypoplasia of the clavicles.⁸ However, as noted by the authors, the clavicular involvement in these cases was mild and difficult to assess on physical examination whereas the clavicles were absent in the cases presented here. Furthermore, the affected individuals described here do neither show parietal foramina nor craniosynostosis, the phenotypes generally associated with *MSX2* loss-of-function or gain-of-function, respectively.

Web resources

The URLs for data presented herein are as follows: Decipher, <https://decipher.sanger.ac.uk/>; UCSC Genome Browser, <http://genome.ucsc.edu/>; Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim>; ECR browser, <http://ecrbrowser.dcode.org/>; and Database of Genomic Variants (DGV), <http://projects.tcag.ca/variation/>.

Although the dental phenotype observed in P1 is not typical for CCD, hypodontia has been linked to mutations in several genes including *MSX1*. The complex synpolydactyly of the hands in P2 is reminiscent of hand malformations caused by spatiotemporal disruption of Hedgehog signalling. Recently, it has been shown that *Msx1* and *Msx2* expression in the limb mesenchyme modulates digit number and identity via the *Shh* and *Bmp* signalling pathways.²⁸ The forelimb phenotype in these mice shows striking similarities to the complex synpolydactyly of the hands which we observed in P2. In addition, the hindlimbs of the *Msx* double conditional mutant mice showed a skeletal phenotype similar to the one observed in P2, that is, postaxial polydactyly. Although the limb phenotype in P2 primarily points to a disturbance of the Hedgehog pathway, it is notable that Hedgehog signalling also plays a role in the regulation of bone growth in the sutures.¹¹

Based on previously described CNVs involving the *MSX2* coding sequence, the effect observed here appears to be more complex than either increased or decreased *MSX2* gene dosage. A similar situation has been reported for duplications involving regulatory sequences at the *IHH* and *SOX9* loci.^{11 29} We conclude that the pathogenic effect of the microduplications can best be explained by a misregulation of spatiotemporal *MSX2* expression patterns. Such overexpression/misexpression is likely to result in a loss of *RUNX2* activity due to the antagonistic properties of *MSX2*, thus, mimicking *RUNX2* haploinsufficiency.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by the Charité-Universitätsmedizin Berlin ethical board.

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Data sharing statement Primer sequences are available in supplementary data and on request. Detailed protocol information is available upon request.

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