SHORT REPORT

Duplications of *BHLHA9* are associated with ectrodactyly and tibia hemimelia inherited in non-Mendelian fashion

Eva Klopocki,[1,2] Silke Lohan,[1,2] Sandra C Doelken,[1] Sigmar Stricker,[2] Charlotte W Ockeloen,[3] Renata Soares Thiele de Aguiar,[4] Karina Lezirovitz,[4,5] Regina Celia Mingroni Netto,[4] Aleksander Jamsheer,[6,7] Hitesh Shah,[8] Ingo Kurth,[9] Rolf Habenicht,[10] Matthew Warman,[11] Koenraad Devriendt,[12] Ulrike Kordaß,[13] Maja Hempel,[14,15] Anna Rajab,[16] Outi Mäkitie,[17] Mohammed Naveed,[18] Uppala Radhakrishna,[19] Stylianos E Antonarakis,[19,20] Denise Horn,[1] Stefan Mundlos[1,2]

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For numbered affiliations see end of article.

Correspondence to
Dr Eva Klopocki, Institute for Medical Genetics and Human Genetics, Charité Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany; eva.klopocki@charite.de

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ABSTRACT

Background Split-hand/foot malformation (SHFM)—also known as ectrodactyly—is a congenital disorder characterised by severe malformations of the distal limbs affecting the central rays of hands and/or feet. A distinct entity termed SHFLD presents with SHFM and long bone deficiency. Mouse models suggest that a defect of the central apical ectodermal ridge leads to the phenotype. Although six different loci/mutations (SHFM1–6) have been associated with SHFM, the underlying cause in a large number of cases is still unresolved.

Methods High resolution array comparative genomic hybridisation (CGH) was performed in patients with SHFLD to detect copy number changes. Candidate genes were further evaluated for expression and function during limb development by whole mount in situ hybridisation and morpholino knock-down experiments.

Results Array CGH showed microduplications on chromosome 17p13.3, a locus previously associated with SHFLD. Detailed analysis of 17 families revealed that this copy number variation serves as a susceptibility factor for a highly variable phenotype with reduced penetrance, particularly in females. Compared to other known causes for SHFLD 17p duplications appear to be the most frequent cause of SHFLD. A ~11.8 kb minimal critical region was identified encompassing a single gene, *BHLHA9*, a putative basic loop helix transcription factor. Whole mount in situ hybridisation showed expression restricted to the limb bud mesenchyme underlying the apical ectodermal ridge in mouse and zebrafish embryos. Knock down of *bhlha9* in zebrafish resulted in shortening of the pectoral fins.

Conclusions Genomic duplications encompassing *BHLHA9* are associated with SHFLD and non-Mendelian inheritance characterised by a high degree of non-penetrance with sex bias. Knock-down of *bhlha9* in zebrafish causes severe reduction defects of the pectoral fin, indicating a role for this gene in limb development.

Distal limb reduction defect is the generic term for a larger group of congenital malformations which affect the autopod. Among these, split-hand/foot malformations (SHFM) constitute a separate group. SHFM is extremely variable in its phenotypic expression between families, within families, and even between limbs of a single patient, ranging from syndactyly and oligodactyly to the most severe expression—monodactyly with only a single phalanx. Ectrodactyly is not exclusively observed in humans but has also been described in other species such as chicken,[1] frogs[2] and mice.[3] Besides genetic factors studies in rodents show that environmental factors—for example, treatment of pregnant rodents with retinoic acid or cadmium—cause ectrodactyly.[4 5] The teratogenic effect in these cases is due to induction of apoptosis of cells from the apical ectodermal ridge (AER).[4] Currently, six different loci/mutations (SHFM1–6) have been associated with non-syndromic SHFM phenotypes in humans. These include dominant mutations in *TP63* (SHFM4, MIM 605289), recessive mutations in *WNT10B* (SHFM6, MIM 225300), as well as genomic duplications on 10q24 (SHFM3, MIM 246560), and deletions and translocations on 7q (SHFM1, MIM 183600)(reviewed in Duijf *et al*[6]). The genetic cause is still unknown in SHFM2 (MIM 313350) which maps to Xq26, and SHFM5 (MIM 606708) which maps to 2q31. However, a large number of cases remain unsolved. Due to reduced penetrance and variable expressivity, inheritance patterns are often difficult to interpret, and dominant as well as recessive and X-linked inheritance has been postulated. Besides these isolated forms of SHFM, syndromic forms of ectrodactyly have been described such as EEC syndrome (MIM 129900), which is characterised by ectrodactyly, ectodermal dysplasia, and cleft lip/palate. Missense mutations in *TP63* (MIM 603273) cause EEC syndrome.[7 8] In some families SHFM is associated with long bone deficiency involving the tibia and fibula, in which case the condition is referred to as split-hand/foot malformation with long bone deficiency (SHFLD, MIM 119100). Unusual inheritance patterns have made this condition less accessible for mapping approaches with subsequent disease gene discovery.[9]

Based on our previous results indicating that copy number variations (CNVs) may cause congenital malformations,[10–13] we performed high

resolution array comparative genomic hybridisation (CGH) analysis (1M oligo array, Agilent Technologies, Santa Clara, California, USA) in patients with SHFLD. Blood sampling and extraction of DNA was performed by standard methods. All participants gave their consent for molecular testing. This study was approved by the Charité Universitätsmedizin Berlin ethics committee. In a pedigree suggestive of X-linked inheritance (family 10, figure 1A and supplementary figure 2), we identified a duplication of 180 kb on chromosome 17p13.3 (figure 1A). We further analysed a large Brazilian family previously mapped to the same region[14] and identified a 110 kb microduplication (family 16, figure 1A). To identify further families and to test the frequency of 17p13 duplications, we screened a cohort of another 54 families with non-syndromic SHFM including another 11 cases with long bone deficiency (SHFLD). Clinical manifestations and limb anomalies in these patients are summarised in table 1. In this cohort, 17p duplications were identified in 30% (17/56) of the families, *TP63* mutations were detected in 9% (5/56), and 10q24 duplications in 20% (11/56). Of the remaining 23 families of this cohort, nine individuals were screened by array CGH and no deletions at the SHFM1 locus on chromosome 7q were detected. Balanced rearrangements were not excluded. Note that five of 43 patients (12%) presenting with SHFM and no tibial/long bone involvement showed 17p13.3 duplications, which is comparable to the frequency of *TP63* mutations in our non-syndromic SHFM cohort. In summary, 17p13.3 duplications have to be considered the most common aetiology of SHFLD.

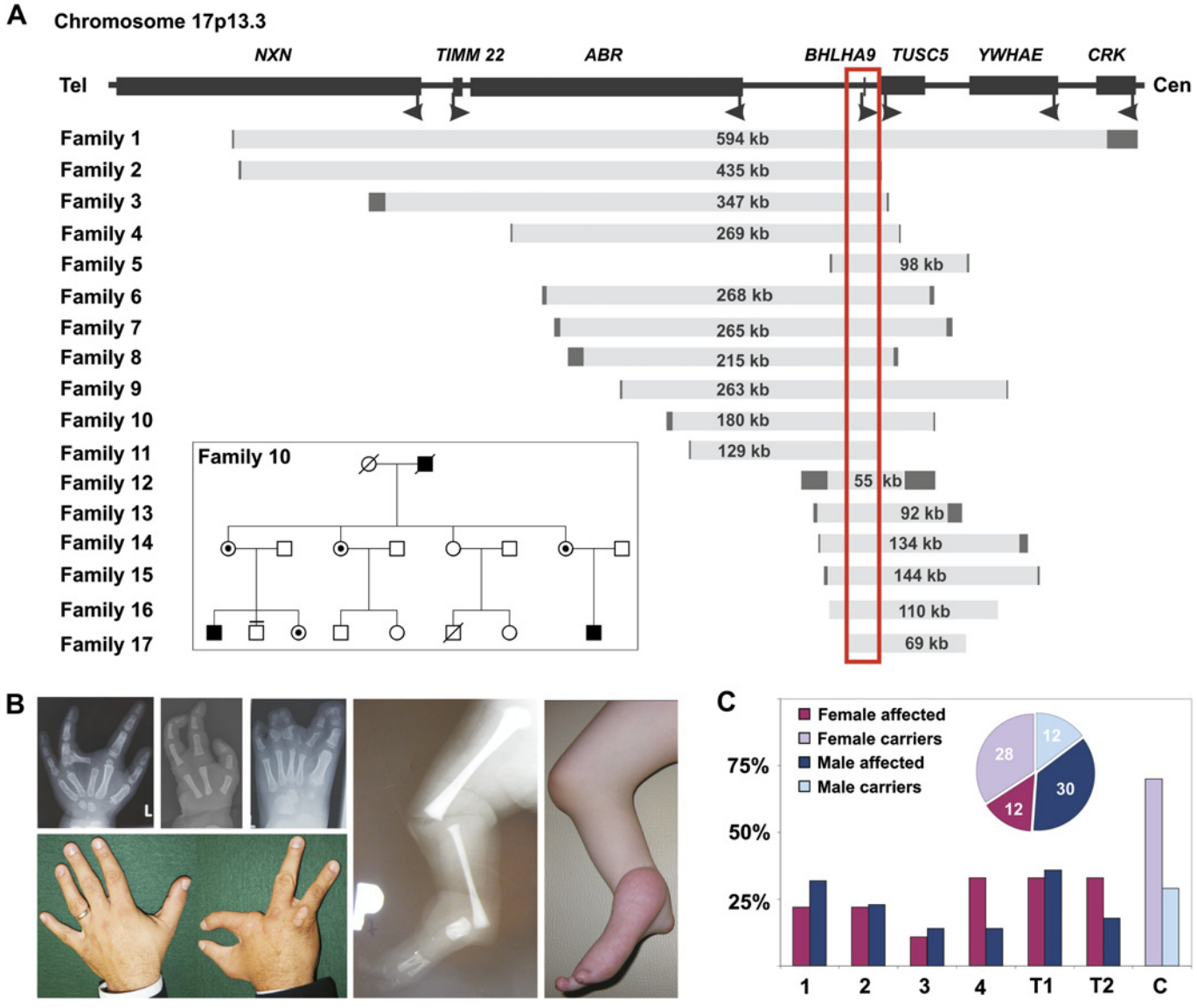

Figure 1 Schematic overview of microduplications in 17p13.3 associated with split-hand/foot malformation with long bone deficiency (SHFLD) (families 1–17), clinical phenotypes, and distribution. (A) The smallest region of overlap (SRO) is indicated by the red box and encompasses *BHLHA9*. Pedigree family 10 is example of a pedigree showing dominant inheritance with reduced penetrance and sex bias. Duplicated regions—light grey; breakpoint regions—dark grey. Genomic positions according to hg18. (B) Examples of clinical phenotypes. Note strong intraindividual variability, for example, split right hand and only mildly affected left hand (lower left). Typical presentation of tibial aplasia (right). (C) Pie chart showing distribution of 17p duplication among healthy and affected carriers (82 individuals from 17 families). Bar chart illustrates range in phenotypic variability in 31 affected from 16 families. Each bar depicts the percentage of affected for the specific phenotype with respect to patients' gender. As underlying scoring criteria we chose the number of affected limbs (that is, 1–4) and the presence of tibia hemimelia (T1, unilateral; T2, bilateral). In addition, sex distribution in healthy carriers (C) is shown.

Table 1 Clinical manifestations in our split-hand/foot malformations (SHFM) cohort

		Phenotype of index patient					
		Ectrodactyly		Monodactyly		Tibia aplasia/	
Family	Genotype	Hands	Feet	Hands	Feet	hypoplasia	Other features
1	17p13.3 duplication	+/+	−/−	−/−	v	+/−	−
2		NA	NA	NA	NA	+	NA
3		+/+	+/+	−/−	−/−	+/+	−
4		+/+	−/+	−/−	−/−	−/−	−
5		+/+	+/−	v	−/−	+/−	−
6		NA	NA	NA	NA	+	NA
7		+/+	+/+	−/−	−/−	+/+	−
8		+/+	−/+	−/−	−/−	+/+	−
9		+/+	−/−	−/−	−/−	−/−	−
10		+/+	+/+	−/−	−/−	−/−	−
11		+/−	−/−	−/−	−/−	+/−	−
12		+/+	−/+	−/−	−/−	+/+	−
13		+/−	−/−	−/−	−/−	−/+	−
14		+/−	+/+	−/−	−/−	+/+	−
15		+/+	−/+	−/−	−/−	−/−	−
16		+/+	+/+	−/−	−/−	+/+	−
17		+/+	+/+	−/−	−/−	−/−	−
18	10q24 duplication	+/+	+/+	−/−	−/−	−/−	Oligodontia, learning disability
19		+/+	+/+	−/−	−/−	−/−	−
20		+	+	−/−	−/−	−/−	−
21		−/+	+/+	−/−	−/−	−/−	−
22		−/−	−/−	+/+	+/+	−/−	−
23		+/+	+/+	−/−	−/−	−/−	Preaxial polydactyly of hand unilateral
24		+/+	+/+	−/−	−/−	−/−	−
25		+/+	+/+	−/−	−/−	−/−	Preaxial polydactyly of hand unilateral
26		−/+	−/−	+/−	+/+	−/−	−
27		+/+	+/+	−/−	−/−	−/−	Intellectual disability
28	*TP63* mutation	−/−	+/+	−/−	−/−	−/−	−
29		+/+	+/+	−/−	−/−	−/−	−
30		+/+	+/+	−/−	−/−	−/−	−
31		+/−	−/−	−/−	−/−	−/−	−
32		+/+	+/+	−/−	−/−	−/−	−
33	dup10q24, dup17p13.3	−/−	+/+	−/−	−/−	−/−	−
34	and *TP63* mutation excluded	−/−	+/+	−/−	−/−	−/−	−
35		−/−	+/+	−/−	−/−	−/−	−
36		+/+	+/+	−/−	−/−	−/−	Aplasia/hypoplasia of tibiae and fibulae, aplasia/hypoplasia of radius and ulna unilateral
37		−/−	+/+	−/−	−/−	−/−	Aplasia/hypoplasia of fibulae
38		−/−	+/+	−/−	−/−	−/−	−
39		−/−	+/+	−/−	−/−	−/−	−
40		−/+	+/+	−/−	−/−	−/−	−
41		−/−	+/+	−/−	−/−	−/−	−
42		−/−	+/+	−/−	−/−	−/+	Hypoplasia of the third toes, hypoplasia of the tibia/fibula unilateral
43		+/+	+/+	−/−	−/−	−/−	Reduction defects of lower legs and forearms
44		+/+	+/+	−/−	−/−	−/−	−
45		+/+	+/+	−/−	−/−	−/−	−
46		+/+	+/+	−/−	−/−	−/−	Renal duplication, bifid uvula
47		+/+	+/+	−/−	−/−	−/−	Hypoplasia of fibulae
48		+/+	+/+	−/−	−/−	−/−	Aplasia of fibulae
49		−/+	−/−	+/−	−/−	−/−	Hypoplasia of ulna unilateral
50		+	+	−/−	−/−	−/−	Short stature, ectodermal dysplasia
51		+/+	−/−	−/−	−/−	−/−	Postaxial polydactyly of hands, sparse blond hair, small teeth, cleft palate, dysplastic kidneys, imperforate anus, micropenis, microcephaly, intellectual disability
52		−/−	+/−	−/−	−/−	−/−	−
53		+	+	−/−	−/−	−/−	Familial cleft lip/palate
54		+/+	+/+	−/−	−/−	−/−	Short stature, mild intellectual disability
55		+	+	−/−	−/−	−/−	Light hair and skin
56		+/+	−/−	−/−	−/−	−/−	Pre-axial polydactyly of feet

+, present; −, absent; ±/±, right/left.

The duplication breakpoints in the 17 families were non-recurrent and duplication sizes varied from 69 kb to 594 kb (figure 1A; data submitted to DECIPHER database). The overlapping region was identified between positions 1 117 153 and 1 128 992 corresponding to ~11.8 kb of sequence (red box, figure 1A). The duplications were arranged in direct tandem orientation as demonstrated by breakpoint sequencing (supplementary figure 1). In the 17 families all of the 42 affected individuals showed the duplication and 40 individuals were identified as non-affected carriers by quantitative real-time PCR (pedigrees in supplementary figure 2).

Duplications overlapping with the smallest region of overlap (SRO) are not listed in the database of genomic variants. In the literature, however, 17p13.3 duplications have been described in patients with mental retardation and autistic manifestations.[15 16] The duplications in some of the described individuals overlap the critical region described here, but skeletal defects were not observed. The absence of limb anomalies in these individuals is probably due to non-penetrance. Our cases with duplications on 17p have been assessed for developmental delay and autistic features by pediatricians and/or clinical geneticists. Since none of our affected individuals presented with autistic features or developmental delay, it is likely that different risk alleles are located within the distal region of chromosome 17p.

Recently, Armour *et al*[17] described three SHFLD families with overlapping duplications on chromosome 17p13.3. Taken together with our results, the SRO is located between 1 117 153–1 128 916 and encompasses only a single putative gene, *BHLHA9*, with a one-exon open reading frame which translates to a protein containing the characteristic structural motif of basic helix loop helix (bHLH) transcription factors. Transcription factors of the bHLH type are involved in regulation of cell proliferation, differentiation, and various developmental processes (reviewed by Jones[18]). To investigate the natural expression pattern of *BHLHA9*, we identified the mouse and zebrafish orthologues and performed whole mount in situ hybridisation at different embryonic stages according to standard procedures (mouse transcript BCO48728; zebrafish transcript wu:fb99e06). These experiments revealed that *Bhlha9* expression in both species is restricted to the distal limb bud mesenchyme underlying the AER, supporting the idea of a role in limb development (figure 2A–I). We did not observe any other major expression domains. To investigate the role of *BHLHA9* in limb development we performed morpholino (MO) knockdown experiments in zebrafish embryos (AB stock maintained at 28.5°C). Antisense custom-morpholino oligonucleotides (MO_bhlha9) were targeted around the start codon of bhlha9 (5′-CAGACGCTCGCTGGAGTCATGGATA-3′) and ordered from Gene Tools (Gene Tools, LLC, Philomath, Oregon, USA). For negative control experiments the standard control oligonucleotide (MO_scrambled) from Gene Tools was used. Embryos were injected with 5 μM per embryo of 1 mM solution at the one cell stage (MO_bhlha9: n=83; MO_scrambled: n=42). Uninjected embryos (n=92) from the same pool were used as wild-type controls (WT). Embryos were fixed at different time points with 4% paraformaldehyde overnight at 4°C, subsequently washed twice with PBST, and stored in 100% methanol. All 83 MO_bhlha9 injected embryos showed severely truncated pectoral fins compared to controls at 72 h post-fertilisation (hpf) (figure 2J–O), indicating that Bhlha9 is important for limb development in a dosage dependent manner.

Outgrowth of the limb is regulated via diverse mechanisms including signals from the AER, an ectodermal cell lining, and the underlying progress zone (PZ). Without the AER the limb does not grow because cells of the PZ do not receive the necessary signal for proliferation as shown by experimental removal of the AER in chicken embryos.[19] The importance of the AER in limb outgrowth and its relation to SHFM has been demonstrated by investigations of the mouse mutant *Dactylaplasia* (*Dac*), which shows an ectrodactyly phenotype due to degeneration of the central AER starting after developmental stage E11.5.[20] As a consequence, a reduction in proliferation of the underlying mesenchyme was observed which ultimately results in missing central rays and a phenotype resembling SHFM. Thus, SHFM is proposed to result from a failure to maintain AER activity in the median region of the autopod. Based on the phenotypic similarities between cases with 17p13.3 duplication, *Dac* and other SHFM mouse models (reviewed in Duijf *et al*[6]), we hypothesise that the transcription factor BHLHA9 regulates target genes important for survival and/or proliferation of cells in the PZ. This regulation might either be under control of the AER or BHLHA9 may influence the AER directly. Since we observed pectoral fin outgrowth in the morpholino, we conclude that the initial induction of the AER is not influenced by the knockdown of bhlha9. However, the massive truncation of the pectoral fins suggests that further growth of the fin is severely disturbed, possibly due to interference with AER maintenance. In this scenario a change in dosage of *BHLHA9* induced by the morpholino in zebrafish or by the duplication in patients may disturb the signalling network and the interactions between AER and PZ in the limb bud.

We observed high phenotypic variability in 31 cases with 17p13.3 duplication in which clinical data were available. A scoring system that quantifies the degree of pathology in each limb was applied (figure 1B,C). The results show that nine of 31 affected (29%) have only one affected limb (score=1), in six (19%) all limbs are affected (score=4), and in 11 (35%) unilateral (T1) or seven (23%) bilateral tibial hemimelia (T2) was present in addition to SHFM (figure 1C). Interestingly more than half of the women with tibial involvement have both lower limbs affected (T2; figure 1C). This observation suggests that females, when affected, are generally more severely affected. The common clinical feature in all affected individuals was ectrodactyly of one or more extremities. While tibial involvement was present in approximately 60% of affected 17p duplication carriers, it has so far not been described in any patient with 10q24 duplication or *TP63* mutations. Thus, tibia hemimelia is a strong indicator of 17p duplication as the underlying genetic cause.

We studied affected and non-affected SHFLD family members by quantitative real-time PCR and identified a high degree of non-penetrance. In total, duplications were observed in 82 individuals, of whom 42 were affected, but also in 40 unaffected carriers. Furthermore, a clear sex bias was observed resulting in more males (30/42) being affected than females (12/42) (pie chart figure 1C). Together, these effects result in a non-Mendelian inheritance pattern with reduced recurrence risk and many mothers who are healthy carriers. Based on our calculations, the recurrence risk of unaffected carriers to have clinically affected children is 36% for male and 15% for female offspring.

The reason for this distortion is unknown. An imprinting effect is unlikely since affected individuals were observed in both paternal and maternal inheritance of the duplication. The presence of intraindividual variability suggests that stochastic factors play an important role in the pathogenesis of this condition. However, as yet unidentified modifier(s) either at the same locus or elsewhere in the genome are likely to be important as well. The sex ratio distortion could be due to a common

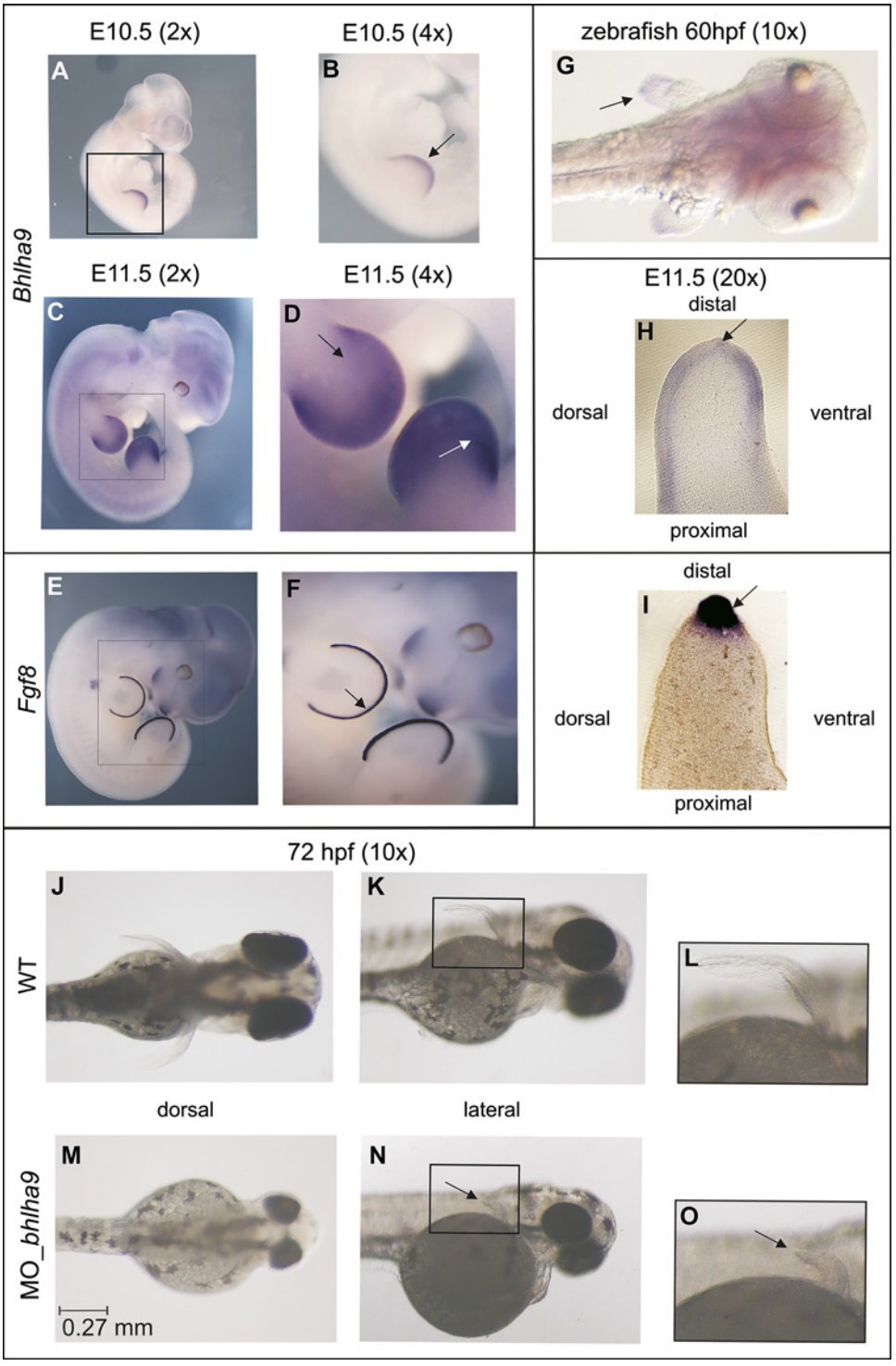

Figure 2 *Bhlha9* is required for limb development. (A–D) Limb specific *Bhlha9* expression (mouse transcript BC048728) in the distal mesenchyme below the apical ectodermal ridge (AER) is detected in forelimb and hindlimb. As a marker of the AER we used *Fgf8* (E, F, I). Longitudinal sections of forelimb at stage E11.5 show *Bhlha9* expression restricted to the subridge mesenchymal layer as well as in the dorsal and ventral regions (H). The AER indicated by strong *Fgf8* expression (I, arrow) shows no detectable *Bhlha9* expression. WT zebrafish embryos at 60 h post-fertilisation (hpf) show a similar *bhlha9* expression pattern in the mesenchyme of the fin field (arrow in G; zebrafish transcript wu:fb99e06). Antisense morpholino (MO) oligonucleotides (MO_bhlha9) were designed to analyse *bhlha9* function in zebrafish. At 72 hpf overall growth was retarded in *bhlha9* MO. Pectoral fins are clearly visible in wild-type (WT) larvae (J–L, uninjected controls) whereas the pectoral fins in *bhlha9* MO injected larvae were severely truncated (M–O, arrows).

polymorphism in a hormone responsive promoter/enhancer that is 'dosage sensitive' and leads to either gene repression or expression in a sex dependent manner. Alternatively, the presence of a modifier on the X chromosome could lead to a 'dilution' effect in females via random X inactivation. In this case, females will only be affected if both X chromosomes carry the modifier, or if the X chromosome that does not carry the modifier is over proportionally inactivated in the affected limb. Thus, the duplication on 17p can be seen as a susceptibility factor for SHFLD which is necessary but not sufficient for development of this malformation. A similar situation has been described for thrombocytopenia-absent-radius (TAR) syndrome (MIM 274000).[21] In TAR syndrome a microdeletion of variable size on chromosome 1q encompassing at a minimum 200 kb and 11 genes serves as a susceptibility factor and, similar to SHFLD, only ~50% of individuals who carry the deletion show malformations. However, TAR syndrome does not show the sex bias observed in association with the 17p duplication. Thus, SHFLD and TAR syndrome are examples of congenital malformations with complex, non-Mendelian inheritance. The penetrance in these conditions appears to be dependent on yet to be identified modifiers. Congenital malformations are relatively common, affecting 3–5% of newborns. In the great majority of cases the origin is unknown. Since most of these cases are

Web resources

- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
- Database of Genomic Variants (DGV), http://projects.tcag.ca/variation/
- Ensembl Genome Browser, http://www.ensembl.org/index.html
- Decipher, https://decipher.sanger.ac.uk/ (Patient numbers: family 1, BER258552; family 2, BER258553; family 3, BER258555; family 4, BER258557; family 5, BER258558; family 6, BER258559; family 7, BER258560; family 8, BER258561; family 9, BER258562; family 10, BER258563; family 11, BER258564; family 12, BER258565; family 13, BER258566; family 14, BER258567; family 15, BER258569; family 16, BER258570; family 17, BER258571).

thought to be sporadic, environmental factors and/or polygenic inheritance have been considered as the underlying pathology. Our finding shows that rare CNVs can serve as a susceptibility factor for congenital disease, a mechanism which may explain increased recurrence risk in conditions otherwise considered to be sporadic.

Author affiliations
[1]Institute for Medical Genetics and Human Genetics, Charité—Universitätsmedizin Berlin, Berlin, Germany
[2]Max-Planck-Institute for Molecular Genetics, Berlin, Germany
[3]Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[4]Centro de Estudos do Genoma Humano, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil
[5]Laboratório de Otorrinolaringologia/LIM32, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil
[6]Department of Medical Genetics, University of Medical Sciences, Poznan, Poland
[7]NZOZ Center for Medical Genetics GENESIS, Poznan, Poland
[8]Department of Orthopaedics, Paediatric Orthopaedic Service, Kasturba Medical College, Manipal, Karnataka, India
[9]Institut für Humangenetik, Universitätsklinikum Jena, Jena, Germany
[10]Kath. Kinderkrankenhaus Wilhemstift, Hamburg, Germany
[11]Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, The Howard Hughes Medical Institute, Children's Hospital, Boston, Massachusetts, USA
[12]Department of Medical Genetics, Leuven University Hospital, Leuven, Belgium
[13]Institute for Human Genetics, Universitätsmedizin Greifswald, Greifswald, Germany
[14]Institute of Human Genetics, Technische Universität München, Munich, Germany
[15]Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[16]Department of Genetics, Directorate General of Health Affairs, Ministry of Health, Muscat, Sultanate of Oman
[17]Children's Hospital, Helsinki University Central Hospital, University of Helsinki, and Folkhälsan Institute of Genetics, Helsinki, Finland
[18]Center for Arab Genomic Studies (CAGS), Dubai, United Arab Emirates
[19]Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland
[20]Division of Medical Genetics, Geneva University Hospitals, Geneva, Switzerland

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics committee of Charité Universitätsmedizin Berlin.

Contributors EK conducted the study and analysed the data, drafted the manuscript, approved the final version of the manuscript; SL performed the in situ hybridisation as well as zebrafish experiments, approved the final version of the manuscript; SCD carried out the clinical analysis of the cases and participated in drafting the manuscript, approved the final version of the manuscript; SS participated in in situ hybridisation experiments, approved the final version of the manuscript; CWO performed qPCR experiments, approved the final version of the manuscript; RSTA participated in analysis of family, approved the final version of the manuscript; KL provided patient samples and clinical data, approved the final version of the manuscript; RCMN provided patient samples and clinical data, approved the final version of the manuscript; AJ provided patient samples and clinical data, approved the final version of the manuscript; HS provided patient samples and clinical data, approved the final version of the manuscript; IK provided patient samples and clinical data, approved the final version of the manuscript; RH provided patient samples and clinical data, approved the final version of the manuscript; MW provided patient samples and clinical data, approved the final version of the manuscript; KD provided patient samples and clinical data, approved the final version of the manuscript; UK provided patient samples and clinical data, approved the final version of the manuscript; MH provided patient samples and clinical data, approved the final version of the manuscript; AR provided patient samples and clinical data, approved the final version of the manuscript; OM provided patient samples and clinical data, approved the final version of the manuscript; MN provided patient samples and clinical data, approved the final version of the manuscript; UR provided patient samples and clinical data, approved the final version of the manuscript; SEA contributed to design of the study and critically revised the manuscript, approved the final version of the manuscript; DH carried out the clinical analysis of the cases and critically revised the manuscript, approved the final version of the manuscript; SM participated in the design of the study and drafted the manuscript, approved the final version of the manuscript.

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Eva Klopocki, Silke Lohan, Sandra C Doelken, Sigmar Stricker, Charlotte W Ockeloen, Renata Soares Thiele de Aguiar, Karina Lezirovitz, Regina Celia Mingroni Netto, Aleksander Jamsheer, Hitesh Shah, Ingo Kurth, Rolf Habenicht, Matthew Warman, Koenraad Devriendt, Ulrike Kordaß, Maja Hempel, Anna Rajab, Outi Mäkitie, Mohammed Naveed, Uppala Radhakrishna, Stylianos E Antonarakis, Denise Horn and Stefan Mundlos

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