ARTICLE

Ellis–van Creveld Syndrome and Weyers Acrodental Dysostosis Are Caused by Cilia-Mediated Diminished Response to Hedgehog Ligands

VICTOR L. RUIZ-PEREZ* AND JUDITH A. GOODSHIP**

Ellis–van Creveld syndrome (EvC; OMIM 225500) is a recessive disorder comprising chondrodysplasia, polydactyly, nail dysplasia, orofacial abnormalities and, in a proportion of patients, cardiovascular malformations. Weyers acrodental dysostosis (Weyers; OMIM 193530) is an allelic dominant disorder comprising polydactyly, nail dysplasia, and orofacial abnormalities. EvC results from loss-of-function mutations in *EVC* or *EVC2*, the phenotype associated with the mutations in these two genes being indistinguishable. Three convincing causative mutations have been identified in patients with Weyers acrodental dysostosis, which are clustered in the last coding exon of *EVC2* and lead to production of a truncated protein lacking the final 43 amino acids. Localization and function of EVC and EVC2 are inferred from studying the murine orthologs. Both Evc and Evc2 proteins localize to the basal bodies of primary cilia and analysis of an Ellis–van Creveld mouse model, which includes the limb shortening and tooth abnormalities of EvC patients, has demonstrated Hedgehog signaling defects in the absence of Evc. The loss of Evc2 has not been studied directly, but Hedgehog signaling is impaired when a mutant murine Evc2 Weyer variant is expressed in vitro. We conclude that the phenotypic abnormalities in EvC and Weyers syndrome result from tissue specific disruption of the response to Hh ligands.

KEY WORDS: Ellis–van Creveld syndrome; Weyers acrodental dysostosis; EVC; EVC2; hedgehog

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INTRODUCTION

Ellis–van Creveld Syndrome

Ellis–van Creveld syndrome (chondroectodermal dysplasia) was recognized as a distinct syndrome more than 70 years ago when Ellis and van Creveld reported three children with chondrodysplasia, nail dysplasia, oral abnormalities, polydactyly and, in two of the three, a heart murmur [Ellis and van Creveld, 1940]. These authors recorded that intelligence in these three children was normal and noted that the parents of two of the three cases were consanguineous indicating recessive inheritance.

The bone abnormalities in EvC consist of disproportionate short stature with shortening of the limbs and narrow chest. Generally birth length is below the third centile and height continues to be below the third centile to adulthood [McKusick et al., 1964]. However, this is not always the case, for example, 7 out of 12 affected individuals in one of the large pedigrees used to map the disorder were said to be of normal height in their population [da Silva et al., 1980]. The limb shortening is acromesomelic. Affected individuals are unable to make a tight fist because shortening of the phalanges is also disproportionate with the distal phalanges being more affected than the proximal phalanges. Genu valgum is a frequent though not constant finding. In addition to the chondrodysplasia there are skeletal patterning abnormalities; bilateral type A postaxial polydactyly of the hands is present in almost all affected individuals, and polydactyly of the feet is present in approximately 10% of cases. Polydactyly is typically postaxial and it is unusual to

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have more than six digits to a limb. There are occasional reports of interdigital polydactyly of hands or feet and syndactyly between fingers or toes can occur. Nail dysplasia is a distinctive, almost invariant finding, though again it was not reported in the Brazilian pedigree that contributed to mapping the disorder [da Silva et al., 1980].

The radiographic features of EvC include postaxial hexadactyly, cone-shaped epiphyses of the phalanges and fusion of the carpals, especially the capitate and hamate, in later childhood and adolescence (Fig. 1). In the majority of cases there is shortening of the ribs and progressive distal shortening of the limbs (Fig. 1). In the pelvis there are hook-like protrusions or spikes from the medial and lateral borders of the acetabulum giving rise to the terms "trident acetabulum" (Fig. 1). The appearances of the ribs and the pelvis become more normal in later childhood and adolescence. The proximal tibial metaphyses is slanted with hypoplasia of the lateral part of the proximal tibial epiphysis (Fig. 1) giving rise to a genu valgum deformity which becomes more obvious as the child grows. There is little information on the radiographic appearance in the fetus.

All individuals with EvC have oral features (Fig. 2) [Hattab et al., 1998; Cahuana et al., 2004; Mostafa et al., 2005]. There is often a notch in the center of the upper lip due to tethering of the upper lip to the gingival margin. There are multiple labial-gingival frenulae and in some cases partial ankyloglossia can lead to a bifid appearance when the tongue is protruded. Natal teeth are not uncommon. Teeth are small and abnormal in shape, conical or with abnormal cusp pattern, and position. There are abnormalities in eruption of primary and permanent teeth, usually delayed though premature eruption of permanent teeth has also been reported. Hypodontia is quite frequently observed, typically absence of primary and/or permanent maxillary and mandibular incisors, though supernumary teeth and fused teeth also occur. Clefts of the mandibular and/or maxillary alveolar ridge may be seen at the lateral incisor region. Tooth enamel may be hypoplastic. Although teeth and nails are abnormal, EvC is not a generalized ectodermal dysplasia as skin and hair are normal.

Congenital heart defects were recognized as being a feature of the condition from the earliest reports and occur in approximately 60% of EvC patients [Giknis, 1963; Digilio et al., 1999]. The commonest cardiac malformations are atrioventricular canal defects and common atrium, which each account for approximately a third of the cardiac defects. Additional

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malformations reported in combination with atrioventricular canal defects are hypoplastic left ventricle and left superior vena cava. Additional malformations reported in combination with common atrium are hypoplastic left ventricle, left superior vena cava, VSD, transposition of the great arteries and tricuspid atresia. The abnormalities in the remaining third of patients with a cardiac malformation are atrial or ventricular septal defects either as isolated anomalies or in combination with additional malformations including pulmonary stenosis, aortic coarctation,

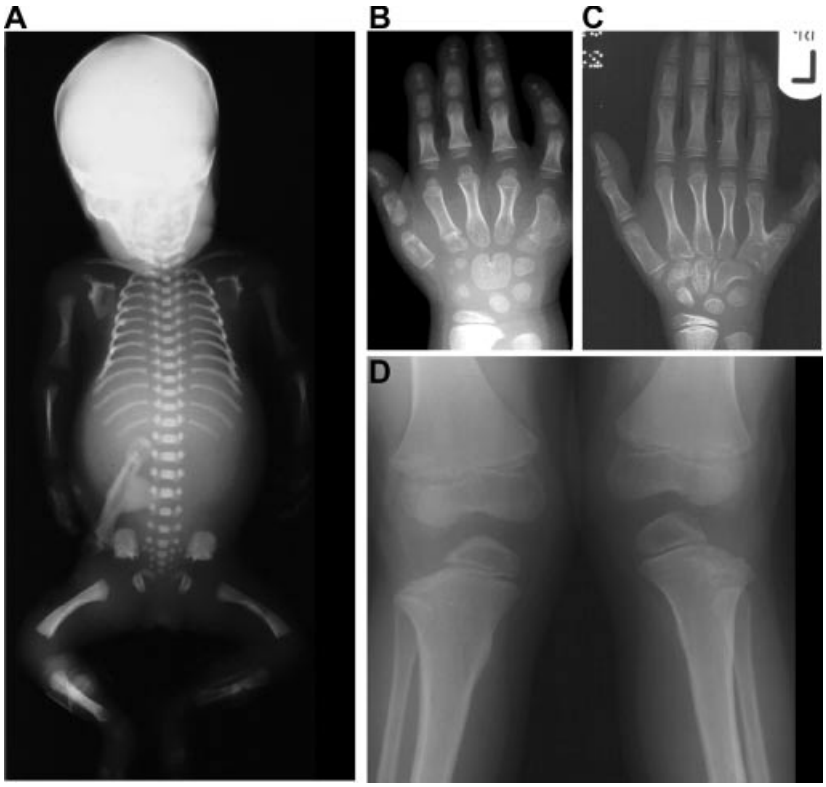

Figure 1. Radiological abnormalities in Ellis–van Creveld syndrome. **A**: Short ribs, short long bones, and acetabular spikes with narrow sacrosciatic notches. **B**: Postaxial polydactyly, short middle, and distal phalanges with cone shaped epiphyses, and accessory carpals with carpal fusion. **C**: Postaxial polydactyly, short distal phalanges, and broad hamate. **D**: Absence of lateral component of proximal tibial epiphyses and sloping of lateral tibial metaphyses giving rise to genu valgum deformity.

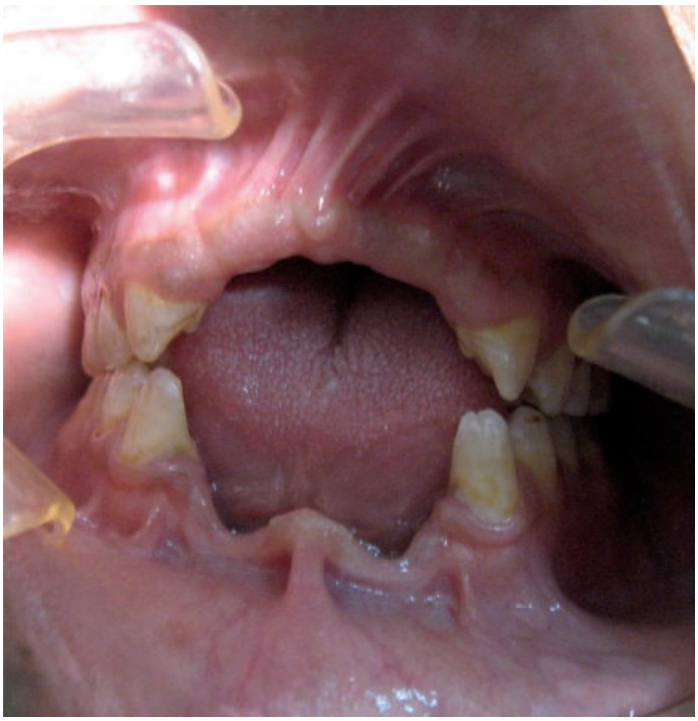

Figure 2. Oral abnormalities in a patient with Ellis–van Creveld syndrome. Congenital absence of the incisors, conical shaped teeth and multiple frenulae.

left superior vena cava, and anomalous pulmonary return. There has been a case report of single ventricle with ASD. Right isomerism sequence and situs inversus have also been reported. This incidence and spectrum of malformations in the literature is very similar to that in a group of 41 patients in whom we identified EVC or EVC2 mutations [Tompson et al., 2007]. Cardiovascular malformations (CVM) were present in 12/20 patients with *EVC* mutations, comprising 7 AVSD, 1 partial AVSD, 1 common atrium, 2 primum ASDs, and 1 complex cardiac defect. Cardiovascular malformations were present in 16/21 patients with *EVC2* mutations, comprising 4 AVSDs, 2 partial AVSDs, 1 common atrium, 4 primum ASD, 1 secundum, 1 unspecified ASD, 1 complex cardiac malformation and 2 coarctations of the aorta. Thus there is no apparent difference in the pattern or incidence of CVM between patients with *EVC* and *EVC2* mutations. Prognosis in EvC is related to the severity of cardiovascular malformations and in the absence of cardiovascular malformations lifespan is normal with reports of families including affected individuals in their seventh and eighth decade.

There have been a number of reports of affected males having children but no reports of affected females having children [McKusick et al., 1964; da Silva et al., 1980; Mostafa et al., 2005]. Parents of EvC-affected individuals do not have features of the condition.

Weyers Acrodental Dysostosis

Weyers [1952] described the radiological findings in three infants with postaxial polydactyly and oral abnormalities similar to, but milder than, those reported in EvC. In contrast to EvC in which a large number of case reports followed the first description, relatively few Weyers families have been described

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[Curry and Hall, 1979; Roubicek and Spranger, 1984; Ye et al., 2006; Zannolli et al., 2008]. Weyers is transmitted as a dominant disorder, and it was mapped to the same chromosomal region as EvC in a family with eight affected individuals from four generations who had postaxial polydactyly, nail dystrophy, mild short stature, and tooth abnormalities. The proband of this family, in addition to polydactyly, nail dystrophy, and tooth abnormalities, had disproportionate short stature and a cardiovascular malformation [Howard et al., 1997]. This proband is the only case described in a dominant pedigree with a cardiovascular malformation. It is not clear whether the proband in this family extends the phenotypic spectrum of Weyers syndrome or whether he has EvC. *EVC* and *EVC2* mutation analysis in the proband and mildly affected individuals in this family would clarify this. In the families reported, height has generally been in the lower half of the normal range and there have been no reports of genu valgum. Whilst postaxial polydactyly type A has been reported in hands and feet, it has not been always been present in the hands and seems more common in the feet, which is the converse of the situation in EvC. Polydactyly type B and 2–3 toe syndactyly have also been reported [Ye et al., 2006; Zannolli et al., 2006; Zannolli et al., 2008; Valencia et al., in press]. The onychodystrophy and oral manifestations are very similar to those reported in EvC.

IDENTIFICATION OF THE EvC GENES

The gene for EvC was mapped to chromosome 4p16 using nine Amish

families along with single families from Ecuador, Mexico, and Brazil [Polymeropoulos et al., 1996]. The positional cloning exercise that followed led to identification of mutations in a novel gene (*EVC*) in seven EvC families including an intronic change (IVS13 + 5 G>T) in the Amish pedigree that was subsequently demonstrated to affect splicing [Ruiz-Perez et al., 2000; Tompson et al., 2007]. However, no mutations were identified in this gene in the other families used to map the disorder or in two additional consanguineous families that were homozygous across the region. Furthermore, on systematic screening of *EVC* in a panel of samples we identified causative mutations in only a third of affected individuals. Thus we continued to clone and investigate additional genes in the region. Meanwhile Takeda and coworkers mapped autosomal recessive bovine chondrodysplastic dwarfism in Japanese brown cattle to the region orthologous to human chromosome 4p16 [Yoneda et al., 1999] and after excluding bovine *Evc* went on to identify two mutations in a contiguous novel gene that they named Limbin, *LBN*, which accounted for the bovine phenotype [Takeda et al., 2002]. Mutations in the human ortholog, *EVC2*, were identified in EvC patients shortly thereafter [Ruiz-Perez et al., 2003; Galdzicka et al., 2002].

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EVC and *EVC2* are in divergent orientation in close proximity to each other; for example, in humans the transcription start sites are separated by 2624 bp, and this genomic organization has been conserved through evolution, being present not only in vertebrates but also in amphioxus, sea urchin and snail (Chris Ponting, personal communication). Initially we thought that the two genes were not homologous as no resemblance was detected in classical BLAST searches but further PSI-BLAST analysis has revealed homology indicating that they arose from an ancient duplication event (Chris Ponting, personal communication).

EVC encodes a novel 992 amino acid protein and *EVC2* encodes a novel 1308 amino acid protein. Analysis of the predicted peptide sequence shows that both proteins have multiple coiled-coil regions, that EVC has a predicted transmembrane domain at its N-terminus and that EVC2 has a predicted transmembrane domain at the N-terminus with a second transmembrane domain (amino acids 299–321) but no other recognized motifs.

FUNCTION OF EVC AND EVC2

An EvC mouse model was generated by replacing the first exon of *Evc* with a *Lac*Z cassette fused to the first ATG of the gene [Ruiz-Perez et al., 2007]. This strategy created a null allele that mimics the effect of the mutations seen in EvC patients, most of which introduce nonsense codons with the transcripts predicted to undergo nonsense-mediated decay and hence absence of protein. As the *Lac*Z reporter was driven by the *Evc* promoter the *Evc* targeted allele was also used to obtain information about the *Evc* expression pattern. X-gal staining of $Evc^{+/-}$ and $Evc^{-/-}$ mouse embryos showed *Lac*Z expression in the orofacial region at E11.5 and in the orofacial mesenchyme and all the forming cartilage elements from E12.5. *Lac*Z expression is also present in developing nails. Thus X-gal staining correlated with the phenoyptic abnormalities of EvC. In addition to the tissues stained by X-gal, which should correspond with those having the highest levels of *Evc* transcription, RT-PCR analysis and immunostaining have demonstrated that Evc was expressed at low levels in most cells. While the animals heterozygous for the targeted allele were normal, mice lacking Evc phenocopied the human condition. Like EvC patients, the mice had short limbs, short ribs, and dental abnormalities but did not have polydactyly or obvious cardiovascular malformations. Radiographic measurements clearly demonstrated that the skeletal shortening was more pronounced in the distal part of the limbs. On histological analysis of the long bones, there was epiphyseal shortening and delayed periosteal induction with the reduction in length of the growth plate being mainly due to a shortening of the columns of proliferative chondrocyte though the size of the hypertrophic

region is also reduced. This appearance was consistent with defective Ihh signaling and analysis of the expression of *Indian Hedgehog* (*Ihh*) and its downstream targets by in situ hybridization demonstrated normal *Ihh* expression but diminished mRNA levels of the Ihh downstream targets, *Ptch1*, *Gli1*, and *Pthrp*. In vitro studies treating murine embryonic fibroblasts (MEFs) and chondrocytes with the Hh agonist purmophamine confirmed that hedgehog signal transduction was defective in cells lacking Evc [Ruiz-Perez et al., 2007]. However not all aspects regulated by Ihh in the growth plate are equally affected. Chondrocyte proliferation which is reduced to approximately 50% in Ihh mutants was shown by BrdU labeling to be normal in the Evc knockout mice. The reduced proliferation rate in Ihh mutants is due to increased levels of Gli3R as proliferation is restored in the Ihh; Gli3 double knockouts [Hilton et al., 2005; Koziel et al., 2005]. Gli3R was shown not to be increased in Evc knockout mice explaining why chondrocyte proliferation was unaffected in these mice. Comparable studies have not been reported for mice lacking *Evc2* but we deduce that EVC2 also plays a role in hedgehog signaling as the phenotype in patients lacking EVC2 is indistinguishable from the phenotype of patients lacking EVC. Furthermore, hedgehog signaling was disrupted when a Weyers mutant version of Evc2 was expressed in vitro providing evidence for its involvement in hedgehog signaling [Valencia et al., in press].

Consistent with their role in Hh signaling, on immunofluorescence microscopy both proteins localized to the base of primary cilia [Ruiz-Perez et al., 2007; Sund et al., 2009]. This is of particular interest as hedgehog signaling is mediated through primary cilia [Huangfu et al., 2003; Huangfu and Anderson, 2005; Haycraft et al., 2007]. Evc is not required for ciliogenesis as cilia appear normal in the mouse mutant [Ruiz-Perez et al., 2007]. However, the precise role of these two proteins in hedgehog signal transduction remains to be elucidated.

GENOTYPE–PHENOTYPE: EvC

EVC and *EVC2* mutations each account for approximately half of patients with EvC, the phenotype associated with mutations in each gene being indistinguishable. Initially it had been thought that there may be further locus heterogeneity, but mutations were identified in all cases in a panel of 36 EvC patients containing a high proportion of consanguineous cases screened recently [Tompson et al., 2007; Valencia et al., in press]. Thus any additional genes account for only a small proportion of cases.

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In both genes the majority of mutations have been nonsense mutations or frameshift mutations that introduce a nonsense codon, and for these it is likely that transcripts undergo nonsense-mediated decay (Fig. 3A). In total 41 independent *EVC* mutations have been reported, eight of which have been seen in more than one family (Table I). Whilst the majority have introduced directly or indirectly nonsense codons, approximately a quarter have occurred within consensus splice sites. Investigation of a homozygous mutation present in only one parent revealed segmental uniparental disomy of a region on the short arm of chromosome 4 in one patient [Tompson et al., 2001]. There has been one deletion removing the last 10 exons and another one removing the last 12. Six missense changes or in-frame deletions have been reported in EVC: p.K302del, p.S307P, p.T372_G374del, p.L620_L626del, p.L623P, and p.Q896H. The protein effect of the c.2T>A mutation which changes the first ATG to AAG is unknown. In total 46 independent *EVC2* mutations have been reported associated with the EvC phenotype, 6 of which have been seen in more than one family (Table II). Three changes have been at consensus splice sites and four have been deletions of more than one exon. cDNA analysis of the Del-520E13-c.2448 showed that it led to skipping of exons13 and 14 resulting in an in frame deletion p.A630_M834del that removes 205 amino acids [Tompson et al., 2001]. cDNA was not available to study the effects of the other three deletions. Three missense changes have been reported in EVC2: p.D207Y, p.I283R, and p.A1045V. Two mutations, p.S1220RfsX3 and p.S1245VfsX20, introduce nonsense codons in the final coding exon of *EVC2* and are thus predicted to produce truncated proteins. The mechanism by which the missense and in-frame deletions in the proteins lead to the phenotype has not been studied.

An EvC family with a homozygous contiguous gene deletion removing both *EVC* and *EVC2*, *c4orf6* and *STK32B* has recently been reported [Temtamy et al., 2008]. All three affected individuals in the family had postaxial polydactyly of hands and feet, nail dysplasia, the characteristic pattern of bone shortening, genu valgum, and dental anomalies. None of the three affected individuals had a cardiovascular malformation. All three had borderline intelligence, which could be due to deletion of both *EVC* and *EVC2* or due to loss of one of the other two genes removed by the deletion or possibly, though less likely, due to an unrelated recessive disorder in this consanguine-

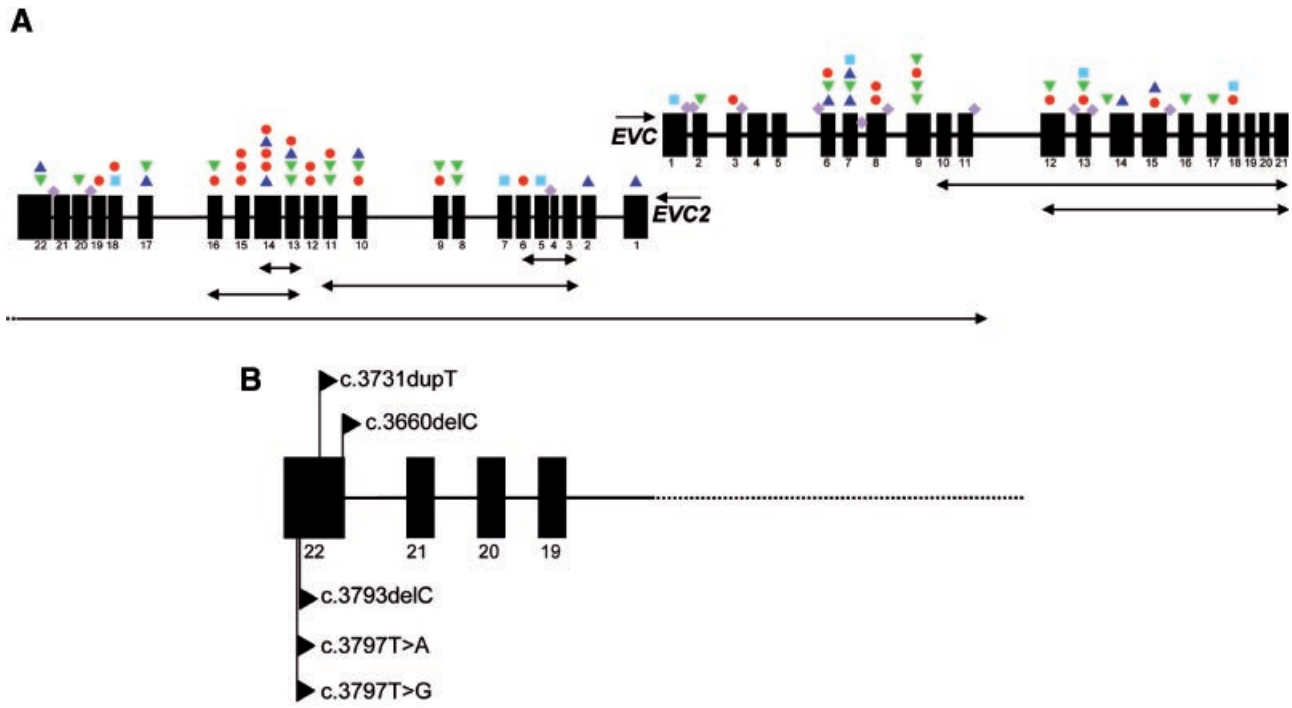

Figure 3. Distribution of the mutations described in *EVC* and *EVC2*. **Panel A** illustrates the exon composition of *EVC* and *EVC2* with the mutations found in each exon in Ellis–van Creveld patients shown above. Code: Nonsense mutations are represented by red circles, microinsertions by blue triangles, microdeletions by green inverted triangles, missense mutations by light blue squares and splice site by violet rhombi. Large deletions spanning more than one exon are represented by arrows which cover the deleted exons. The *STK32B-EVC* deletion spanning both genes is also represented. The picture is drawn so that 4p telomere is on the left. **Panel B** indicates the position of the EvC recessive mutations (above) and Weyers dominant mutations (below) exon22 of *EVC2*.

ous family. The fact that the skeletal, orofacial, and cardiac phenotypes were typical in this family suggests that neither gene compensates for the other, that is, there is no functional redundancy. The same chromosomal deletion was found in conjunction with a splice site mutation in a patient of different ethnic origin who had classical EvC and normal intelligence [Temtamy et al., 2008]. The observation that none of the carriers of this deletion had phenotypic features of EvC, formally excludes *EVC/EVC2* digenic inheritance as a mechanism for EvC.

Finally, interpretation of a previously reported *EVC* mutation warrants comment. A father and daughter, not with classical EvC but with postaxial polydactyly of the hands, a partial atrioventricular canal with common atrium and agenesis of the upper lateral incisors with enamel hypoplasia, were reported as having a heterozygous missense change, p.R443Q [Ruiz- Perez et al., 2007]. This change has subsequently been reported as a rare polymorphism, *rs35953626*, that is more common in African populations and thus does not account for their phenotype.

GENOTYPE–PHENOTYPE: WEYERS ACRODENTAL DYSOSTOSIS

Weyers commented on the similarity between the oral and hand and feet abnormalities in the patients that he described and those observed in EvC [Weyers, 1952]. A heterozygous frameshift c.3793delC in the last coding exon of *EVC2* reported in a Chinese pedigree with six living affected individuals in different generations with Weyers acrodental dysostosis provided definitive evidence that the conditions are allelic [Ye et al., 2006]. This mutation changes leucine 1265 to tyrosine and introduces a stop in the next codon. Mutations have recently been identified in three further Weyers families (Table III) [Valencia et al., in press]. In one Caucasian family, the mutation segregating in the family is c.3793delC, identical to that reported in the Chinese family. In the remaining two families the changes were found at nucleotide 3797, c.3797T>A and c.3797T>A, which also truncate the protein at L1266, a striking clustering of mutations. Of these two mutations, one segregated in the family and the other occurred as a de novo event providing further evidence for its pathogenicity.

The initial description of the *EVC* gene included investigation of a child who had classical EvC whose father's height was on the second centile and who had dysplastic nails and widely spaced conical-shaped teeth but did not have polydactyly [Spranger and Tariverdian, 1995]. This father was found to carry EVC p.S307P, and it was suggested that this accounted for his clinical features which were consistent with Weyers syndrome [Ruiz-Perez et al., 2000]. However, this is one of the few recurrent EvC mutations and no other

TABLE I. Mutations Identified in *EVC*

Exon/intron	Nucleotide change	Protein effect	Number of cases	Refs.
Exon1	c.2T>A	p.M1?	1	9
Intron1	c.174 + 1G>A		1	5
Intron1	c.175 − 2A>G		1	9
Exon2	c.203delA	p.N68IfsX48	1	9
Exon3	c.363C>A	p.Y121X	1	9
Intron3	c.384 + 5_6GA>AC		1	5
Intron5	c.703 − 1G>C		1	5
Exon6	c.708dupT	p.I237YfsX5	1	9
Exon6	c.735delT	p.D246TfsX27	1	1
Exon6[a]	c.770T>A	p.L257X	1	8
Exon7	c.873dupT	p.E292X	3	5, 9
Exon7	c.904_906del	p.K302del	2	1, 9
Exon7	c.910dupA	p.R304KfsX5	1	1
Exon7	c.919T>C	p.S307P	3	1, 5
Intron7	c.940 − 150T>G		1	9
Exon8	c.1018C>T	p.R340X	4	1, 5, 9
Exon8	c.1060G>T	p.E354X	1	9
Intron8	c.1098 + 1G>A		2	6,9
Exon9	c.1114_1122del	p.T372_G374del	1	9
Exon9	c.1217delT	p.L406RfsX94	1	9
Exon9	c.1255G>T	p.E419X	1	9
Exon9	c.1269_1278del	p.Q424RfsX73	1	9
Exons10−21	Ex10_21del		1	5
Intron11	c.1563 + 1G>C		1	9
Exons12−21	Ex12_21del		1	1
Exon12	c.1678G>T	p.E560X	1	9
Exon12	c.1694delC	p.A565VfsX23	3	5
Intron12	c.1777 − 2A>G		1	5
Exon13	c.1813C>T	p.Q605X	1	5
Exon13	c.1858_1878del	p.L620_L626del	2	9
Exon13	c.1868T>C	p.L623P	2	7, 9
Intron13	c.1886+5G>T		1	1
Intron13−Exon14	Del_IVS13 (−9 to +14)		1	8
Exon14	c.2088_2089dupCA	p.R697TfsX15	1	5
Exon15	c.2200C>T	p.Q734X	1	5
Exon15	c.2277_2280dupCCGG	p.A761PfsX7	1	5
Intron15	c.2304 + 2T>G		1	5
Exon16	[c.2344_2345del; c.2357_2370del]	p.T782QfsX26	1	9
Exon17	c.2457delG	p.M820WfsX108	1	1
Exon18	c.2635C>T	p.Q879X	1	1
Exon18	c.2688G>C	p.Q896H	1	5

Some changes have been adapted to the current Human Genome Variation Society specifications for describing sequence changes (http://www.hgvs.org/mutnomen/). Mutations are named using NM_153717.2 as the reference sequence taking the A of the ATG translation initiation codon as nucleotide 1.

References code: 1, Ruiz-Perez et al. [2000]; 2, Ruiz-Perez et al. [2003]; 3, Galdzicka et al. [2002]; 4, Ye et al. [2006]; 5, Tompson et al. [2007]; 6, Temtamy et al. [2008]; 7, Ulucan et al. [2008]; 8, Sund et al. [2009]; 9, Valencia et al. [in press].

[a]This mutation introduces a stop codon when the *rs6446393* C>T polymorphism at c.769 is a T. This is the case for the reference sequence used in the original report (AF216184) of this mutation.

TABLE II. Mutations Identified in *EVC2* in EvC patients

Exon/intron	Nucleotide change	Protein effect	Number of cases	Refs.
Exon1	c.194_198dupGGCGG	p.S67GfsX17	1	2
Exon2	c.273dupT	p.K92X	1	5
Exons3–6	Ex3_6del		1	9
Introns3–11	Del_IVS3 + 1086_IVS11-431		1	5
Intron4	c.519 + 2T>C		1	5
Exon5	c.619G>T	p.D207Y	1	8
Exon6	c.745C>T	p.Q249X	2	5, 9
Exon7	c.848T>G	p.I283R	2	2, 9
Exon8	c.893delA	p.H298PfsX15	1	5
Exon8	c.983delG	p.G328EfsX27	1	5
Exon9	c.1024A>T	p.K342X	1	8
Exon9	c.1028_1034del	p.L343PfsX10	1	5
Exon10	c.1195C>T	p.R399X	1	2
Exon10	c.1386_1387del	p.R463KfsX26	1	5
Exon10	c.1467_1468dupGA	p.S491GfsX4	1	5
Exon11	c.1541_1542del	p.L514RfsX22	1	5
Exon11	c.1655_1658del	p.G552DfsX2	1	5
Exon11	c.1708C>T	p.Q570X	2	5
Exon12	c.1828C>T	p.Q610X	1	9
Exon12	c.1855C>T	p.Q619X	1	2
Intron12–Exon14	Del-520Ex13_c.2448	p.A630_M834del	1	5
Exons13–16	Ex13_16del		1	9
Exon13	c.1918delA	p.M640CfsX21	1	9
Exon13	c.2010delA	p.K670NfsX2	2	5
Exon12	c.2019dupT	p.K674X	1	5
Exon13	c.2029C>T	p.R677X	1	9
Exon14	c.2056dupC	p.Q686PfsX3	1	2
Exon14	c.2263C>T	p.Q755X	1	5
Exon14	c.2365G>T	p.E789X	1	9
Exon14	c.2447_2451dupAGGCC	p.V818RfsX3	1	5
Exon14	c.2476C>T	p.R826X	1	9
Exon15	c.2620C>T	p.R874X	1	8
Exon15	c.2652G>A	p.W884X	1	9
Exon15[a]	c.2698G>T	p.E900X	1	8
Exon16	c.2710C>T	p.Q904X	1	9
Exon16	c.2746delA	p.S916AfsX6	1	5
Exon17	c.2854dupA	p.R952KfsX52	1	5
Exon17	c.2885delG	p.G962AfsX17	1	9
Exon18	c.3134C>T	p.A1045V	1	5
Exon18	c.3265C>T	p.Q1089X	1	3
Exon19	c.3283G>T	p.E1095X	1	9
Intron19	c.3360 + 1G>A		1	9
Exon20	c.3405_3411del	p.G1136RfsX6	1	9
Intron21	c.3659 + 2T>C		2	5, 8
Exon22	c.3660delC	p.S1220RfsX3	8	2, 5, 9
Exon22	c.3731dupT	p.S1245VfsX20	1	9

Some changes have been adapted to the current specifications of the Human Genome Variation Society for describing sequence changes (http://www.hgvs.org/mutnomen/). The mutations are named using NM_147127.3 as the reference sequence taking the A of the ATG translation initiation codon as nucleotide 1.

Reference code: 1, Ruiz-Perez et al. [2000]; 2, Ruiz-Perez et al. [2003]; 3, Galdzicka et al. [2002]; 4, Ye et al. [2006]; 5, Tompson et al. [2007]; 6, Temtamy et al. [2008]; 7, Ulucan et al. [2008]; 8, Sund et al. [2009]; 9, Valencia et al. [in press].

[a]This mutation differs form the original report as the amino acid at position 900 is E instead of Q.

TABLE III. Mutations Identified in *EVC2* in Weyers Patients

Exon	Nucleotide change	Protein effect	Number of cases	Refs.
Exon22	c.3793delC	p.L1265YfsX2	2	4, 9
Exon22	c.3797T>A	p.L1266X	1	9
Exon22	c.3797T>G	p.L1266X	1	9

The mutations are named using NM_147127.3 as the reference sequence taking the A of the ATG translation initiation codon as nucleotide 1.
Reference code: 1, Ruiz-Perez et al. [2000]; 2, Ruiz-Perez et al. [2003]; 3, Galdzicka et al. [2002]; 4, Ye et al. [2006]; 5, Tompson et al. [2007]; 6, Temtamy et al. [2008]; 7, Ulucan et al. [2008]; 8, Sund et al. [2009]; 9, Valencia et al. [in press].

carriers of this mutation have been reported as having a phenotype. In view of the findings in subsequent Weyers families, it would be interesting to study *EVC2* in this man.

FUNCTIONAL STUDIES OF *EVC2* EXON22 MUTATIONS

It is intriguing that three of the five mutations that have been identified in the last exon of *EVC2* have been associated with the Weyers phenotype and are dominant mutations whilst the other two mutations have been identified in EvC pedigrees with no phenotypic manifestations in the heterozygous parents or siblings (Fig. 3B).

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The two EvC-associated mutations are 5′ of the Weyers mutations. Given that these five mutations occur in the final exon, the corresponding transcripts are predicted to escape nonsense-mediated decay. This has been confirmed by cDNA analysis for the c.3660delC (p.S1220RfsX3) EvC-associated mutation and for the c.3793delC Weyers-associated mutation for which primary fibroblasts were available. The effect on Hh signaling of these two changes have been investigated in vitro [Valencia et al., in press]. The equivalent murine versions of these two truncated proteins and wild-type Evc2 were expressed in NIH 3T3 mouse fibroblasts along with a luciferase reporter of hedgehog signaling, and the response to the hedgehog agonist SAG assayed. The hedgehog pathway was fully active in cells expressing wild-type Evc2 and cells expressing the protein mimicking the EvC mutation, however, there was almost no activation of the Hh responsive luciferase reporter in the cells expressing the protein mimicking the Weyers mutation indicating that expression of this protein disrupts hedgehog signaling. This is in keeping with manifestation of a phenotype in individuals heterozygous for the Weyers mutation but not in individuals heterozygous for the EvC mutation. The mechanism by which removal of the last 43 amino acids disturbs the function of the normal protein produced from the other chromosome, whilst deletion of the last 87 amino acids does not is not yet known. In the absence of antibodies that detect EVC2 on Western blot analysis of proteins from human fibroblasts, production of protein corresponding to the transcripts containing the c.3660delC and c.3731dupT recessive changes has not been demonstrated and it remains possible that the exon22 EvC mutations lead to less stable truncated proteins than the Weyers changes and thus to a reduction in EVC2 protein as for the majority of EvC associated mutations. Nevertheless the cluster of mutations observed in association with the dominant Weyers phenotype indicates that the final 43 amino acids of EVC2 are necessary for normal protein function.

CONCLUSION

The data from the analysis of the Evc knockout and the in vitro studies using Evc2 dominant mutations indicate that EVC and EVC2 are required together for a normal level of response to hedgehog ligands in certain tissues. The two proteins do not have redundant functions as the phenotypes resulting from the loss of EVC, the loss EVC2 or the loss of EVC and EVC2 are equivalent.

It has been demonstrated that the bone abnormalities in EvC result from diminished response to Indian hedgehog. The two phenotypes that have been shown to result from mutations within Indian hedgehog itself differ from the EvC skeletal phenotype. Brachydactyly A1 results from heterozygous mutations restricted to a specific region of the N-terminal active fragment of Indian hedgehog that has been shown to be a calcium-binding site essential for interaction with its receptor and cell-surface partners [McLellan et al., 2008; Byrnes et al., 2009]. Modeling a brachydactyly A1 mutation in the mouse has confirmed that impaired interaction with the receptor decreases signaling efficiency and also increases the range of Ihh signaling in the developing digit [Gao et al., 2009]. In brachydactyly A1 the middle phalanges are most affected in contrast to the progressively distally shortening that occurs in EvC. The absence of long bone and rib abnormalities in patients with brachydactyly A1 implies that the EvC phenotype cannot be completely explained by diminished signaling efficiency and increased range of signaling. Acrocapitofemoral dysplasia is a recessive disorder featuring short stature, brachydactyly and narrow thorax but in which the most striking

radiological abnormalities are in the head of the femur [Hellemans et al., 2003; Mortier et al., 2003]. In contrast, the head of the femur is relatively normal in EvC patients. Furthermore whilst the typical knee deformity in EvC is genu valgum, in acrocapitofemoral dysplasia the knee deformity is genu vara. Elucidating the precise role of EVC and EVC2 in modulating response to hedgehog ligands remains a challenge.

The orofacial and nail abnormalities observed in EvC are expected to be the consequence of diminished response to Sonic hedgehog signal (Shh) in tissues in which EVC expression is high. The oral frenulae seen in EvC patients are very similar to those seen in oro-facio-digital syndrome type 1 (OFD1). As hedgehog signaling is mediated through primary cilia and as the protein mutated in OFD1 plays a role in ciliogenesis [Ferrante et al., 2006] it is likely that some aspects of the OFD1 oral phenotype are due to aberrant response to sonic hedgehog. The cardiovascular defects found in EvC patients are also likely to arise from disruption of Shh signaling as atrioventricular septal defects are seen following conditional abrogation of Shh in the mouse [Goddeeris et al., 2008].

EvC is grouped together with Jeune syndrome and the short rib-polydactyly syndromes (SRP) I–IV on the basis of their overlapping radiological phenotypes. Mutations in *IFT80*, which encodes an intraflagellar transport protein, account for a small proportion of patients with Jeune syndrome [Beales et al., 2007] and mutations of the gene encoding a second cilia-related protein, DYNC2H1, which is a component of the cytoplasmic dynein complex, have recently been identified in some patients with the Jeune phenotype and patients with short rib-polydactyly type 3 [Dagoneau et al., 2009]. The mechanism by which mutations in these cilia-related proteins lead to chondrodysplasia is likely to be aberrant response to Hedgehog signaling. Putting together these findings and considering the central role of Ihh signaling in the growth plate, the chondrocyte primary cilia is a crucial organelle for skeletal development.

The mechanism by which mutations in these cilia-related proteins lead to chondrodysplasia is likely to be aberrant response to Hedgehog signaling. Putting together these findings and considering the central role of Ihh signaling in the growth plate, the chondrocyte primary cilia is a crucial organelle for skeletal development.

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