REPORT

Mutations in the SPARC-Related Modular Calcium-Binding Protein 1 Gene, SMOC1, Cause Waardenburg Anophthalmia Syndrome

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Waardenburg anophthalmia syndrome, also known as microphthalmia with limb anomalies, ophthalmoacromelic syndrome, and anophthalmia-syndactyly, is a rare autosomal-recessive developmental disorder that has been mapped to 10p11.23. Here we show that this disease is heterogeneous by reporting on a consanguineous family, not linked to the 10p11.23 locus, whose two affected children have a homozygous mutation in SMOC1. Knockdown experiments of the zebrafish smoc1 revealed that smoc1 is important in eye development and that it is expressed in many organs, including brain and somites.

Anophthalmia describes a rare developmental anomaly in which the eye is absent as the result of a deficiency in the development of the primary optic vesicles. Severe microphthalmia occurs at a prevalence of 3 in 10,000. Microphthalmia can be isolated or syndromic. Genetic studies of isolated cases have identified mutations in, among others, RAX,[1] VSX2[2] (MIM 142993) PAX6[3] (MIM 607106), MITF[4] (MIM 156845), MAF[5] (MIM 177075), OTX2[6] (MIM 600037), SOX2[7] (MIM 184429), and SIX6[8] (MIM 606326). All these genes play a role in early ocular development. Mutations in members of the Wnt signaling and TGF-β1 (MIM 190180) superfamily, including members of the bone morphogenetic proteins and growth-differentiation factors, have also been implicated.[9,10] For recent reviews, see [11–13]. More recently, TMX3, a member of the thioredoxin family of proteins, was shown to be mutated in unilateral microphthalmia.[14]

At least ten forms of syndromic microphthalmia have been reported so far: MCOPS1 (MIM 309800), Lenz microphthalmia;[15] MCOPS2 (MIM 300166), oculo-facio-cardio-dental syndrome;[16] MCOPS3 (MIM 206900), anophthalmia-esophageal-genital syndrome;[7] MCOPS4 (MIM 301590), microphthalmia-ankyloblepharon-mental retardation;[17] MCOPS5 (MIM 610125), microphthalmia-optic nerve agenesis, corpus callosum agenesis, joint laxity;[6] MCOPS6 (MIM 607932), microphthalmia-pituitary anomalies;[18] MCOPS7 (MIM 309801), microphthalmia-dermal aplasia-sclerocornea;[19] MCOPS8 (MIM 601349), microcephaly-microphthalmia-ectrodactyly of lower limbs-prognathism;[20] and MCOPS9 (MIM 601186), pulmonary agenesis, microphthalmia-diaphragmatic defect.[21] Several of these entities have overlapping phenotypes, and whether they all represent different syndromes remains to be shown.

Forty years ago, Waardenburg reported an autosomal-recessive anophthalmia with hand and foot malformations.[22] This syndrome (Waardenburg anophthalmia syndrome, also known as microphthalmia with limb anomalies, ophthalmoacromelic syndrome, and anophthalmia-syndactyly [MIM 206920]) is characterized by unilateral or bilateral microphthalmia, clinical anophthalmia, syndactyly, polydactyly, synostosis, and/or oligodactyly. In addition, other organs may be affected (long-bone hypoplasia; renal, venous, and vertebral anomalies). Most patients are mentally retarded and are born from consanguineous parents. Since the first description by Waardenburg, more than 35 cases have been identified.[22–30] Recently, Hamanoue et al. used homozygosity mapping to identify a 433 kb homozygous region on chromosome 10p11.23 between STS9 and STS12, but no mutated gene was identified.[31]
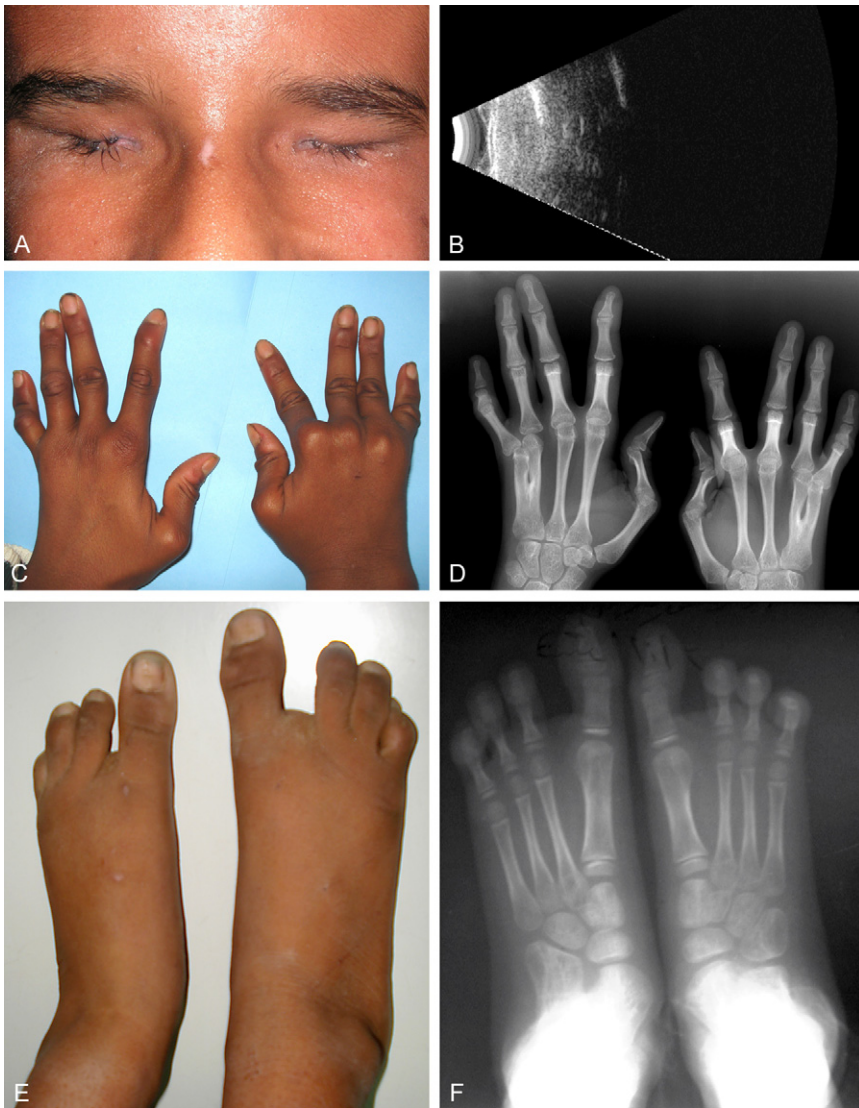
We report on a consanguineous family of Egyptian origin with four children, two of whom are affected with Waardenburg anophthalmia syndrome (Figure 1). The first child (V.2 in Figure 1), born to healthy parents, was a 12-yr-old girl when last seen at our clinics. At birth, her father (IV.7) was 33 yr old and her mother (IV.8) 30 yr old. The second affected child (V.4) was 8 yr old. Both were born at full term after an uneventful pregnancy and an uncomplicated vaginal delivery. Birth weights were within normal limits, but lengths are unknown. Both had global delay in developmental milestones: responsive smile was first seen at 10 and 8 mo in patients V.2 and V.4, respectively, and they walked at 3 yr. A Binet test performed on V.4 at 7 yr of age gave a score of 80, and her older sister was similarly mentally retarded. On clinical examination, both had broad lateral eyebrows, sparse eyelashes, short palpebral fissures, and bilateral

anophthalmia (Figure 1A). Transpalpebral ultrasonography (12 MHz) (Figure 1B) and ultrasound biomicroscopy (50 MHz) confirmed the absence of an eye globe in both affected individuals, and no cysts were observed. Clinically observed skeletal abnormalities included malar flattening, a high palate, bilateral proximal placement of the thumb, bilateral F45 osseous syndactyly, bilateral F5 radial clinodactyly, bilateral camptodactyly of F15 (Figures 1C and 1D), an absent ray in both feet with sandal gap and pes planum (Figure 1e), and mild scoliosis in both affected children. Hand and wrist X-rays (Figure 1D) revealed fusion of the carpal bones (capitate and hamate). Elbow X-rays were normal. X-rays of the feet (Figure 1F) and legs showed bilateral partial fusion of both middle and medial cuneiform bones, an absent ray, and normal tibia and fibula. These features were common to both patients. Orbital and brain MRI scans were performed in both girls as well. No eye globe or cysts were reported on orbital MRI, but extraocular muscles normal in signal and size were present. On brain MRI, complete absence of the optic nerves, chiasma, and optic tracts was observed, and no other malformations were noted, especially of the corpus callosum and pituitary gland. The karyotype was normal (46,XX) in both patients, as was the renal ultrasound examination. The pedigree is described in Figure 2. This study was approved by the ethics committees of the Faculty of Medicine, University of Alexandria and the Faculty of Biology and Medicine, University of Lausanne.

Homozygous mapping via the Human660W-Quad DNA Analysis BeadChip (Illumina) was performed on individuals IV.7, IV.8, V.2, V.3, and V.4. We first concentrated on the region around the membrane protein palmitoylated 7 gene (*MPP7* [MIM 610973]) that was reported to be a potential candidate on chromosome 10.[31] However, the largest homozygous stretch of DNA in that region was only 60 kb. Therefore, the molecular anomaly involved in the family that we describe must be located elsewhere. Analysis of the rest of the data identified several homozygous regions in the genome of the patients, but many were also present in the nonaffected sibling and were therefore discarded. Three regions of homozygosity larger than 1 Mb and specific to the two patients were identified (Table S1, available online). The largest region was located between rs11158442 (position 62,198,792) and rs4899594 (position 76,031,821), defining a 14 Mb region on chromosome 14q23. Two genes in which mutations had already been implicated in microphthalmia and anophthalmia, *BMP4* (MIM 112262) and *OTX2* (MIM 600037), were located close to the homozygous region but were clearly outside of it.[6,32] According to Ensembl, this interval

Figure 2. Pedigree and Mutation Analysis
(A) Pedigree showing consanguinity.
(B) Electropherogram of part of *SMOC1* exon 3 showing normal (1), heterozygous (2), and homozygous (3) c.378+1G>T mutation.

contains more than 100 protein-coding sequences. As its name implies, the ophthalmo-acromelic syndrome shows aberration in the development of the eyes and bones. We therefore restricted the list of candidate genes to those expressed in the eyes, bones, and connective tissue. Expression of each candidate was checked in the EST profile of the UniGene database. Thirty-three genes passed this filter. Among these genes, *MPP5* (MIM 606958), *RDH11* (MIM 607849), *ZFP36L1* (MIM 601064), *SRSF5* (MIM 600914), *SMOC1* (MIM 608488), and *ZFYVE1* (MIM 605471) were further evaluated. *MPP5* (ENSG00000072415) is a member of the peripheral membrane-associated guanylate kinase (MAGUK) family and may play a role in tight junction formation and cell-polarity establishment. Interestingly, *MPP7*, another member of the MAGUK family, was the only gene identified in the locus reported by Hamanoue et al.[31] *RDH11* (ENSG00000072042) is implicated in the reduction of all-trans-retinal, a molecule with high relevancy to the eye. The zinc finger protein 36, C3H type-like 1 gene (*ZFP36L1* [ENSG00000185650]) is a member of the *TIS11* family of early-response genes and is induced by, among others, the polypeptide mitogen EGF. It could thus regulate the response to growth factors. SRSF5 (ENSG00000100650) is a member of the serine- and arginine-rich family of pre-mRNA splicing factors. Mutations in splicing factors have been implicated in retinal pigmentosa. Zinc finger FYVE domain-containing protein 1 (ENSG00000165861) is a member of the FYVE domain proteins. They mediate the recruitment of proteins involved in membrane trafficking and cell signaling to phophatidylinositol 3-phosphate-containing membranes. Mutations in PIKFYVE, another member of the FYVE domain proteins, are responsible for the François-Neetens fleck corneal dystrophy (MIM 121850). Sequencing of the exons and intron-exon boundaries of these genes did not show any causative variants.

However, sequencing of *SMOC1* (ENSG00000198732) revealed a c.378+1G>T (IVS3+1G>T) mutation in the canonical splice donor site of intron 3 (Figure 2). Both parents were heterozygous for this mutation, and the only available non-affected child was homozygous for the normal sequence. This mutation was looked for in 556 control chromosomes from individuals of Egyptian, North African, and European descent by denaturing high-performance liquid chromatography and was never observed. For primers used in *SMOC1* sequencing, see the Supplemental Data.

SMOC1 (secreted modular calcium-binding 1) is a 48 kDa secreted modular protein containing an EF-hand calcium-binding domain and is a member of the SPARC family of proteins characterized by the presence of follistatin and EF-hand calcium-binding domains.[33] Analysis of the EST profile in UniGene indicates that it is widely expressed, including in the eyes and bones. *SMOC1* has 12 exons and several large introns. Intron 3 has a size of 22.2 kb, and a mutation in the canonical G nucleotide of the splice donor might either greatly perturb mRNA synthesis or activate nonsense-mediated decay. Unfortunately, we were unable to amplify *SMOC1* by using RT-PCR with RNA extracted from normal blood and could not therefore investigate whether nonsense-mediated decay was happening or whether a truncated protein with an aberrant C terminus was generated.

SMOC1 is well conserved among various species, including zebrafish, in which we could identify a retired contig containing the 3′ region of a putative *smoc1* (LOC795519). Protein-homology analysis between human SMOC1 and the putative zebrafish smoc1 gave an overall score of 63%, with some regions having a score over 80%. Except for the missing (or undiscovered) signal peptide, the putative *smoc1* has a structure very similar to that of the human (see Figure S1). It also contains an FS domain, two TY domains, and a C-terminal SPARC calcium-binding domain containing two EF hands. Compared to *SMOC1*, *smoc1* contains an additional region coding for a 27-amino-acid-long stretch between the FS domain and the first TY domains, as well as a second, 17-amino acid-long region situated just after the first TY domain and before the first *SMOC1*-specific domain (Figure S1). Interestingly, the *SMOC1*-specific domains are
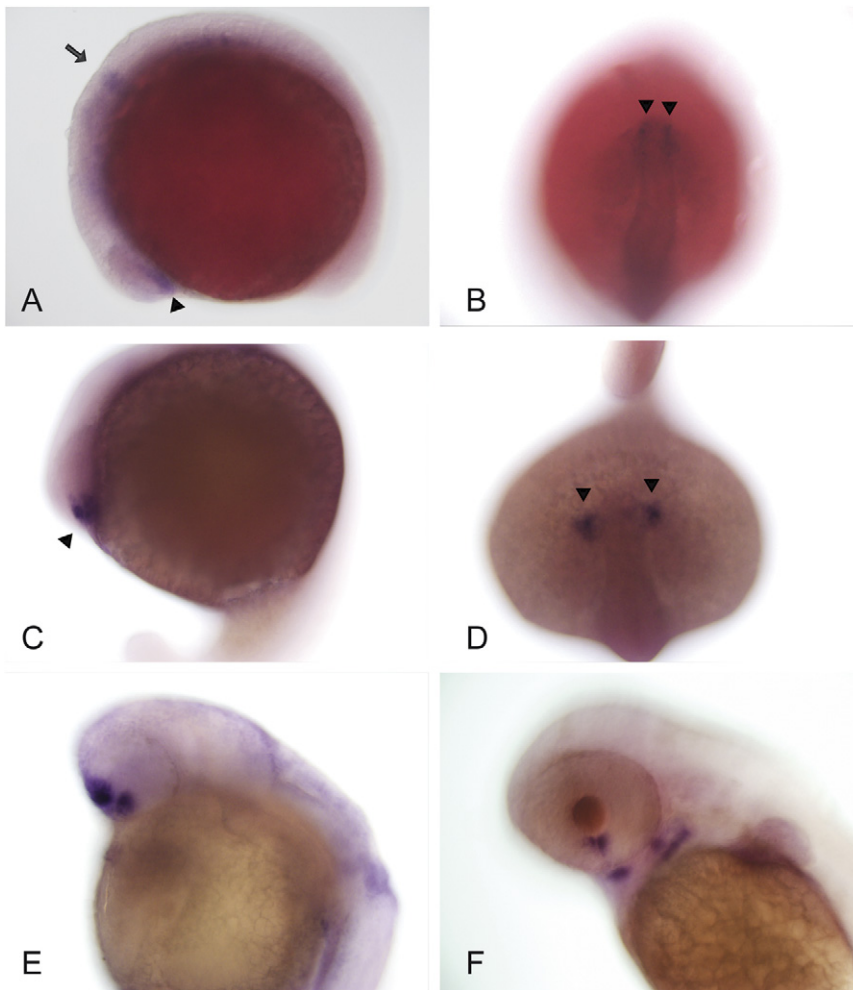
(A–D) Whole-mount in situ hybridization experiments showing early expression of *smoc1* in the brain (arrow) and in the anterior retina (arrowhead) at 10 ss and 18 ss, respectively.

(E and F) *smoc1* is localized in the ventral retina, on both sides of the choroid fissure at 24 hr (E), and in the pharyngeal arches at 48 hpf (F).

(A, C, E, and F) Top and left represent dorsal and frontal parts of the animal. (B) and (D) are top views. Magnification: 200×.

also conserved in zebrafish. Database searching and PCR amplifications using various primers located in the coding regions of the gene allowed us to identify only one copy of *smoc1* in the *D. rerio* genome.

In order to evaluate the role of smoc1 in the development of the eye, we first evaluated its expression in zebrafish by in situ hybridization and then observed the effect of a transient knockdown of its translation by using a morpholino targeting the donor splice site of intron 3, the location that is mutated in the patients.

On the basis of the sequence of LOC795519, we cloned, after RT-PCR of zebrafish embryo mRNA using primers F (5′-ATGCC TCAT CGCTT CAT-3) and R (5′-CTAAC CCTCC TTATT GACTCC-3′), a 1359 bp cDNA coding for 453 amino acids. From this clone, we generated two dioxygenin-labeled probes for in situ hybridization that covered the 5′ and 3′ regions of *smoc1* with primers F100 (5′-GGTTC AGACG GACGC AGTTATG-3′) and R628 (5′-TCTGC CTCTC CTGGT CACAA-3′ and F716 (5′-GCCAC CAATC TACAG GTTAC-3′) and R1063 (5′-CGTGA CTGCC GTTAT TATCC-3′), respectively. and examined *smoc1* expression in zebrafish embryos as described previously.[34] Expression of *smoc1* by whole-
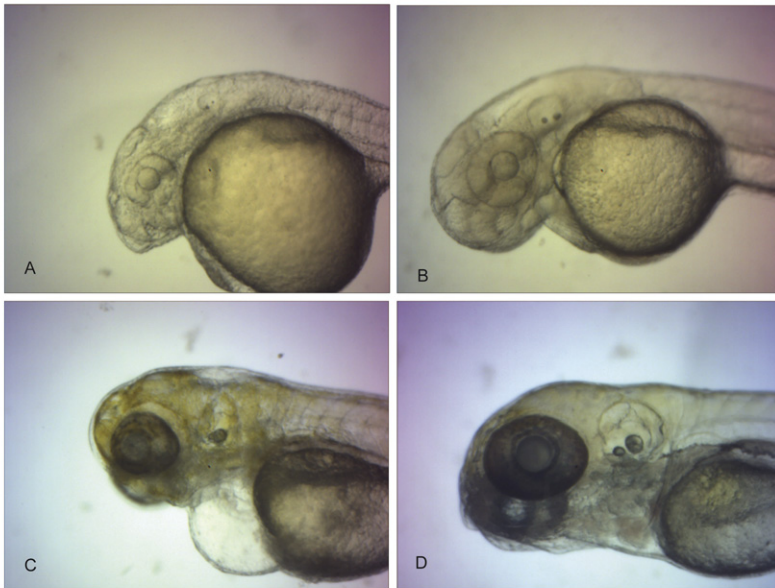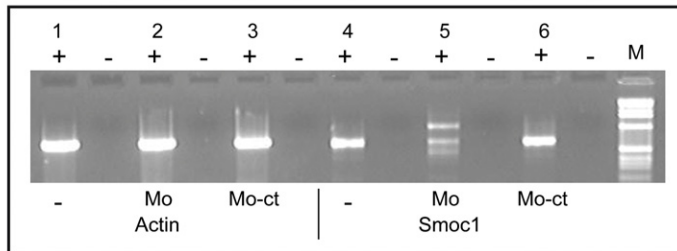
mount in situ hybridization was first evident at the 10-somite stage (10 ss). In the optic vesicles, transcripts were first detected in the ventrolateral diencephalon (Figures 3A and 3B), then in the most anterior part of the retina at 18 ss (Figures 3C and 3D). At 24 and 48 hr postfertilization (hpf), smoc1 was expressed in the ventral retina, on both sides of the choroid fissure (Figure 3E and 3F). Signals were seen in the brain at 10 ss and 18 ss as well as in pharyngeal arches at 48 hpf (Figure 3F). Functional analysis of *smoc1* was assessed with the use of morpholino injections. Microinjections were performed with a Femtojet (Eppendorf). The smoc1 morpholino covering the exon 3-intron 3 splice site (5′-CCGGA ACTCT GACAG ACCTG AGCAA-3′) was synthesized by Gene Tools. For control injections, the standard control morpholino provided by Gene Tools was used. The stock solutions were diluted to 750 μM in H_2O with 0.1% phenol red (Acros Organics, Brunschwig AG). Two nanoliters were injected in the yolk at 1- to 2-cell stage. First, efficiency of the morpholino targeting the exon 3-intron 3 splice site of *smoc1* was controlled by RT-PCR with the use of primers F100 and R628 (Figure 4B). Additional amplicons larger and smaller than the normal 660 bp product were observed. They may correspond to fragments containing part of intron 3 or abnormal splice variants. Knockdown of smoc1 revealed microphthalmia associated with defects, especially in the ventral retina at 2 days postfertilization (dpf): the choroid fissure remained widely open compared to the untreated or control morpholino-injected larvae (Figures 4A and 4B). The coloboma was no longer detectable at 5 dpf, but microphthalmia was still severe, and the retina was especially underdeveloped at the ventral and nasal parts of the eye (Figure 4C). Smoc1 may also be important for brain development, because defects in the forebrain were observed at 5 dpf (Figure 4C). Pharyngeal

A

B

arches seemed to be underdeveloped as well, but additional studies will be needed to confirm this.

The exact role of SMOC1 is still poorly understood. It was identified through its homology with secreted protein acidic and rich in cysteine (SPARC [MIM 182120]), also known as osteonectin, a protein important for bone calcification.[35] Sparc-deficient mice show cataract and rupture of the lens capsule around the age of 6 mo.[36] SMOC1 is localized in many tissues, where it associates with basement membranes, and interaction between the FS domain and the EC domain influences calcium binding.[37] Calcium is critical for many developmental programs and is required for continuous function of the visual cycle, in which, among other roles, it regulates the guanylyl cyclase activating protein (*GUCA1A* [MIM 600364]), a calcium-binding protein with four EF-hand domains. Interestingly, Jalili syndrome (MIM 217080), another malformation syndrome affecting ocular development and the formation of the tooth, a tissue closely related to bone, is due to mutations in *CNNM4*, a gene implicated in Ca^{2+} control through Mg^{2+} exchange at GUCA1A.[34] Novinec et al. also showed that SMOC1 binds many proteins, including C-reactive protein (CRP [MIM 123260]), fibulin-1 (MIM 135820), and vitronectin (MIM 193190).[37] Fibulins are proteins involved in elastic-fiber assembly, cell proliferation, migration, adhesion, and angiogenesis, in which fibulin-1 stabilizes newly formed blood vessels.[38,39] SMOC1 has also been involved in osteoblast differentiation and *SMOC1* knockdown by shRNA-inhibited mineralization and expression of osteoblast-differentiation markers in human bone-marrow-derived mesenchymal stem cells.[40] Interestingly, the same authors also identified EFEMP1 (MIM 601548), a member of the fibulin family, as implicated in osteoblast differentiation. Mutation in *EFEMP1* causes malattia leventinese or Doyne honeycomb retinal dystrophy (MIM 126600), a disease characterized by the early development of drusen, similar to age-related macular degeneration.[41] It is not known how SMOC1 participates in the development of bones, but on the basis of the phenotype associated with Waardenburg anophthalmia, it may be required for proper individualization of hand and foot rays.

In the zebrafish, *smoc1* is highly expressed in the brain, choroidal fissure, pharyngeal arches, and somites. Knockdown experiments showed a coloboma at 2 dpf. At 5 dpf, the eye was microphthalmic and the brain showed gross malformations. Additional studies need to be done to see whether it is also implicated in the development of fin rays. Our analysis showed that Waardenburg anophthalmia syndrome is genetically heterogeneous. In addition to the previously described locus on chromosome 10, we identified a second locus on chromosome 14 and showed that mutations in *SMOC1* also cause this syndrome.

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cell.com/AJHG/.

Web Resources

The URLs for data presented herein are as follows:

Ensembl, http://www.ensembl.org
Entrez, http://www.ncbi.nlm.nih.gov/sites/entrez
NCBI, http://www.ncbi.nlm.nih.gov
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim
UniGene, http://www.ncbi.nlm.nih.gov/unigene

Accession Numbers

LOC795519 information was obtained from NCBI. Zebrafish *smoc1* accession number: HQ665031.

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