

there is simply not yet enough information to make an informed decision about this issue.

Most of the mutations in *MLL2* are predicted to result in haploinsufficiency. However, it is unclear by what mechanism(s) haploinsufficiency of *MLL2* could cause Kabuki syndrome. *MLL2* encodes a histone 3 lysine 4 (H3K4) methyltransferase, one of at least 10 proteins (genes for which have not to our knowledge yet been screened in Kabuki cases in which *MLL2* mutations were not found) that have been identified to specifically modify the lysine residue at the fourth amino acid position of the histone H3 protein [Kouzarides, 2007]. *MLL2* has a SET domain near its C-terminus that is shared by yeast Set1, *Drosophila* Trithorax (TRX) and human MLL1 [FitzGerald and Diaz, 1999]. *MLL2* appears to regulate gene transcription and chromatin structure in early development [Prasad et al., 1997]. In mice, loss of *MLL2* results in embryonic lethality before E10.5, and while *MLL2*<sup>+/-</sup> mice are viable, they are smaller than wild-type [Ng et al., 2010].

Kabuki syndrome is the most common of a small, but growing group of multiple malformation syndromes accompanied by developmental delay that are caused by mutations in genes that encode proteins involved in histone methylation [De Sario, 2009]. The most notable of these is CHARGE syndrome, which is one of the syndromes often considered in the differential diagnosis of children ultimately diagnosed with Kabuki syndrome. CHARGE syndrome is caused by mutations in *CHD7*, which encodes a chromodomain protein that recognizes the trimethylated H3K4 side chain [Vissers et al., 2004]. Other disorders caused by defects of histone methylation status include several intellectual disability syndromes, some of which are also characterized by malformations (e.g., cleft lip/palate) that overlap with those found in individuals with Kabuki syndrome.

Kabuki syndrome is one of the most common causes of heritable developmental delay. Discovery that mutations in *MLL2* are the most common cause of Kabuki syndrome highlights the role that disrupted regulation of histone methylation plays as a cause of human birth defects. Characterizing the spectrum of mutations in *MLL2* is a small but important first step toward understanding the mechanism(s) that underlies Kabuki syndrome.

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## *SMOC1* Is Essential for Ocular and Limb Development in Humans and Mice

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Microphthalmia with limb anomalies (MLA) is a rare autosomal-recessive disorder, presenting with anophthalmia or microphthalmia and hand and/or foot malformation. We mapped the MLA locus to 14q24 and successfully identified three homozygous (one nonsense and two splice site) mutations in the SPARC (secreted protein acidic and rich in cysteine)-related modular calcium binding 1 (*SMOC1*) in three families. *Smoc1* is expressed in the developing optic stalk, ventral optic cup, and limbs of mouse embryos. *Smoc1* null mice recapitulated MLA phenotypes, including aplasia or hypoplasia of optic nerves, hypoplastic fibula and bowed tibia, and syndactyly in limbs. A thinned and irregular ganglion cell layer and atrophy of the anteroventral part of the retina were also observed. Soft tissue syndactyly, resulting from inhibited apoptosis, was related to disturbed expression of genes involved in BMP signaling in the interdigital mesenchyme. Our findings indicate that *SMOC1/Smoc1* is essential for ocular and limb development in both humans and mice.

### Introduction

Microphthalmia with limb anomalies (MLA [MIM 206920]), also known as Waardenburg anophthalmia syndrome or ophthalmoacromelic syndrome, is a rare autosomal-recessive disorder first described by Waardenburg.<sup>1</sup> It is characterized by ocular anomalies ranging from mild microphthalmia to true anophthalmia and by limb anomalies such as oligodactyly, syndactyly, and synostosis of the 4<sup>th</sup> and 5<sup>th</sup> metacarpals.<sup>2–4</sup> The genetic cause for MLA has remained unknown.

It is widely known that secreted signaling molecules such as Sonic hedgehog (Shh), wntless-type MMTV integration site family (Wnt), transforming growth factor  $\beta$  (Tgf- $\beta$ ), bone morphogenetic proteins (Bmps), and fibroblast growth factor (Fgf) are involved in the development of many organs and tissues, including the eyes and limbs.<sup>5,6</sup> In particular, mutations in *BMP4* (MIM 112262) have resulted in anophthalmia with systemic manifestations, including polydactyly and/or syndactyly (also known as microphthalmia, syndromic 6, MCOPS6 [MIM

607932]),<sup>7</sup> highlighting importance of BMP signaling in both the developing eye and limb.

*SMOC1* (MIM 608488), which encodes SPARC (secreted protein acidic and rich in cysteine)-related modular calcium binding 1, is a member of the SPARC (also known as BM-40) matricellular protein family that modulates cell-matrix interaction by binding to many cell-surface receptors, the extracellular matrix, growth factors, and cytokines.<sup>8,9</sup> SMOCs are extracellular glycoproteins with five domains: an N-terminal follistatin-like (FS) domain, two thyroglobulin-like (TY) domains, a domain unique to SMOC, and an extracellular calcium-binding (EC) domain.<sup>9</sup> *SMOC1* is widely expressed in various tissues with localization to basement membranes.<sup>9,10</sup> Although the biological function of *SMOC1* remains largely unknown, it has been recently reported that *Xenopus smoc* protein, the ortholog of human *SMOC1*, acts as a BMP antagonist,<sup>11</sup> suggesting that human *SMOC1* can also modulate BMP signaling.

Here, we demonstrate that *SMOC1* mutations cause MLA. We also show that *Smoc1* null mice recapitulated

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MLA phenotypes, indicating that *SMOC1* plays essential roles in both eye and limb development in humans and mice.

## Subjects and Methods

### Subjects

A total of four families with one or two cases of MLA were analyzed in this study, including three previously reported families (A, B, and C).<sup>12,13</sup> Family X from Turkey, which has been previously described,<sup>14</sup> was newly recruited to this study. Detailed clinical information of all the patients is available in the literature,<sup>12,14</sup> and phenotypes of patients with confirmed mutations are summarized in Table S1 (available online). A total of five affected and 16 unaffected members from the four families were analyzed in the linkage study. Genomic DNA was obtained from peripheral-blood leukocytes with the use of QuickGene 610-L (Fujifilm, Tokyo, Japan) after informed consent had been given. Experimental protocols were approved by the institutional review board of Yokohama City University School of Medicine.

### SNP Genotyping, and Fine Mapping with Short Tandem Repeat Markers

Whole-genome SNP genotyping, with the use of GeneChip Human Mapping 50K Array XbaI (Affymetrix, Santa Clara, CA), and fine mapping of possible candidate regions, with the use of additional microsatellite markers, were performed as previously described.<sup>12,15</sup> The list of primers used for fine mapping is presented in Table S2.

### Linkage Analysis

Multipoint linkage analyses using aligned SNPs were performed with ALLEGRO software.<sup>16</sup> Two-point linkage analyses of candidate regions were performed with the LINKAGE package MLINK (FASTLINK software, version 5.1). In each program, an autosomal-recessive model of inheritance with complete penetrance and a disease-allele frequency of 0.001 were applied.

### Mutation Analysis of Candidate Genes

All coding exons and exon-intron boundaries of *RAD51L1* (MIM 602948), *ACTN1* (MIM 102575), *ERH* (MIM 601191), *SRSF5* (MIM 600914), *DCAF5* (MIM 603812), *COX16*, *EXD2*, *GALNTL1*, *SLC39A9*, *KIAA0247*, *MED6* (MIM 602984), *TTC9* (MIM 610488), *MAP3K9* (MIM 600136), and *SMOC1* (transcript variant 1, GenBank accession number NM\_001034852.1) were analyzed in the probands of families A, C, and X. The transcript variant 2 of *SMOC1* (GenBank accession number NM\_022137.4) is 3 bp shorter than the variant 1, leading to an in-frame amino acid deletion at position 431. PCR was cycled 35 times at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 30–90 s in a total volume of 20 µl containing 30 ng genomic DNA as a template, 0.5 µM forward and reverse primers, 200 µM each deoxyribonucleotide triphosphate (dNTP), 1 × ExTaq buffer, and 0.25 U ExTaq (Takara). All primers were designed with Primer3 software. Detailed information of primers is available upon request. PCR products were purified with ExoSAP (USB) and sequenced with BigDye Terminator 3.1 (Applied Biosystems) on a 3100 Genetic Analyzer. Sequences of patients were compared to reference genome sequences in the UCSC Genome Browser (February 2009

assembly) with Seqscape software, version 2.1 (Applied Biosystems).

### Animals

*Smoc1* mutant mice, created with the use of the *Sleeping Beauty* transposon system, have been previously described.<sup>17</sup> Line PV384 was provided by the RIKEN BioResource Center through the National BioResource Project of MEXT, Japan. Three independent mouse lines (no. 1 to no. 3), each with a single insertion in intron 1 of *Smoc1*, were bred as heterozygotes. Lines 1 and 3 were backcrossed for at least four generations to a C57BL/6J background. Line 2 was maintained with a mixed background of C57BL/6J and ICR. We mainly analyzed line 1, but we confirmed similar phenotypes in lines 2 and 3. Animals were housed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Yokohama City University, School of Medicine. PCR genotyping of mice was performed with the use of genomic DNA from yolk-sac, ear, or tail biopsies. The following primers were used: PV384-WF, 5'-AAAGGCTGGGAATTGTTG A-3'; PV384-WR, 5'-TGCAGCTGAACTGTCTCTCC-3'; PV384-MF, 5'-TGTCCCTAACTGACTTGCCAAA-3'. The PV384-WF/PV384-WR primers amplified a 441 bp wild-type (WT) product, and the PV384-MF/PV384-WR primers amplified a 218 bp mutant product.

### Southern Hybridization

Genomic DNA was extracted from livers or tail biopsies of PV384 heterozygous (*Smoc1*<sup>Tp/+</sup>) mice via standard protocols. The gene-trap insertions were analyzed by Southern hybridization with the use of 10 µg of *SacI*-, *NdeI*-, *BglIII*-, and *EcoRI*-digested DNA. The probe (451 bp), which hybridized to the internal ribosome entry site (IRES) in the gene-trap vector, was synthesized with the DIG PCR Probe Synthesis Kit (Roche) with the use of the following primers: 5'-CTAACGTTACTGGCCGAAGC-3' and 5'-CCCAGATCAGATCCCATACAA-3'. Hybridization, washing, and detection of probes were performed according to the manufacturer's protocol. Images were captured with the FluorChem system (Alpha Innotech).

### Cloning of Gene-Trap Insertion Sites

After identification of aberrant DNA fragments by Southern hybridization, *NdeI*-, *SacI*-, and *EcoRI*-digested DNA from PV384 mice was fractionated by electrophoresis, and appropriately sized fragments containing *O11* (*other locus 1*), *O12*, and *O13* were isolated with a QIAEXII Gel Extraction Kit (QIAGEN). The isolated DNA was self-ligated by Ligation High ver.2 (Toyobo), precipitated with ethanol, and dissolved in 20 µl EB buffer (QIAGEN). Inverse PCR was performed in 25 µl reactions, containing 2 µl ligated DNA, 1 × PCR buffer for KOD FX, 0.4 mM each dNTP, 0.5 µM each primer, and 0.5 U KOD FX DNA polymerase (Toyobo). Primers common to *O11*, *O12*, and *O13* were as follows: Inv-F, 5'-ATCGCCAGTTCTGTATGAACGGTCTGGTCTT-3'; Inv-R, 5'-CCCTCTTACGTGCCAGCCATCTTAGAGATAC-3'. Confirmatory PCR of gene-trap insertion sites for *O11*, *O12*, and *O13* loci was performed with the use of the following primers: *O11*-F, 5'-GAGTGGTATTCA TTGGATTCTGCTGAT-3'; *O12*-F, 5'-AAATCCAGCTGGCCAACAGACTAAG-3'; *O13*-F, 5'-TTGCCGGGTAGACTCTATCAAGAACCA-3'; *TBAL*-R, 5'-CTTGTGTCATGCACAAAGTAGATGTCC-3'. Primer sets of *O11*-F/*TBAL*-R, *O12*-F/*TBAL*-R, and *O13*-F/*TBAL*-R could amplify 175 bp, 607 bp, and 767 bp products, respectively. These PCR primer pairs were also used for genotyping of mice harboring a single insertion at the *Smoc1* locus.

## Confirmation of Promoter- and Poly(A)-Trapped Transcripts

Whole embryos at embryonic day 10.5 (E10.5) and E11.5 were stored in RNAlater solution (QIAGEN). Total RNA was extracted from WT, *Smoc1<sup>TP/+</sup>*, and *Smoc1<sup>TP/TP</sup>* embryos with the use of RNeasy Plus Mini (QIAGEN). One microgram total RNA was subjected to reverse transcription with the use of a PrimeScript 1<sup>st</sup> Strand Synthesis Kit with random hexamers (Takara). A control reaction with no reverse transcriptase was included in each experiment. PCR was performed in 20  $\mu$ l reactions, containing 1  $\mu$ l cDNA, 1  $\times$  PCR Buffer for KOD FX, 0.4 mM each dNTP, 0.3  $\mu$ M each primer, and 0.4 U KOD FX (Toyobo). Primers used are listed below: *Smoc1*-E, 5'-GTCCCCACCTCCCCAAGTGCTTTGA-3'; *LacZ*-R, 5'-TGCCAAAAGACGGCAATATGGTGGAAA-3'; *GFP*-E, 5'-T CACATGGTCTGCTGGAGTTCGTGAC-3'; *Smoc1*-R, 5'-ACACT TGCTCTGGCCAGCATCTTTGCAT-3'. Primer sets of *Smoc1*-E/*Smoc1*-R, *Smoc1*-E/*LacZ*-R, and *GFP*-E/*Smoc1*-R could amplify native *Smoc1* (366 bp), promoter-trapped transcripts (Tp-LacZ, 500 bp) and poly(A)-trapped transcripts (Tp-GFP, 308 bp), respectively. The PCR conditions were 98°C for 10 s, 68°C for 1 min, for 30 cycles. Primers for *ACTB*<sup>18</sup> were used as an internal control. PCR for *ACTB* was cycled 20 times at 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s in a total volume of 10  $\mu$ l containing 0.5  $\mu$ l cDNA, 0.4  $\mu$ M each primer, 0.2 mM each dNTP, 1  $\times$  ExTaq buffer, and 0.5 U ExTaq HS (Takara). All PCR products were electrophoresed on 2% agarose gels.

## In Situ Hybridization

Embryos were collected between E9.5 and E13.5. Whole-mount in situ hybridization was carried out as previously described.<sup>19,20</sup> Two fragments of *Smoc1* cDNA were obtained as probes by RT-PCR, with the use of total RNA extracted from livers of E16.5 mouse embryos, and subcloned into pCR4-TOPO (Invitrogen). Primer sequences were as follows: probe 1-F, 5'-GTCTGCTCAGCCCC ACT-3'; probe 1-R, 5'-CCTGAACCATGTCTGTGGTG-3'; probe P-F, 5'-CAGGAACAGGAAAGGGGAAGA-3'; probe P-R, 5'-AAGGGAAA ACCACACAGCAC-3'. PCR products were 1023 bp and 1578 bp, corresponding to nucleotide positions 275–1297 and 1849–3426 of the mouse *Smoc1* cDNA (GenBank accession number NM\_001146217.1), respectively. The cDNA fragment amplified with probe P-F and probe P-R primers was identical to the probe used in a previous report.<sup>21</sup> Digoxigenin-labeled sense and antisense riboprobes were synthesized with the use of a digoxigenin RNA labeling kit (Roche). These two different antisense probes demonstrated identical staining patterns, and the control sense probes showed no staining. The expression pattern was confirmed with more than three embryos. In addition, the following probes were used: *Bmp2* (gift from Y. Takahashi),<sup>22</sup> *Sox9* (gift from A. Yamada),<sup>22</sup> *Bmp7* (gift from E.J. Robertson), and *Msx2* (gift from Dr. R.E. Maxson, Jr). The numbers of embryos examined were as follows (numerical quantity for WT, *Smoc1<sup>TP/+</sup>*, and *Smoc1<sup>TP/TP</sup>*, respectively, shown in parentheses): *Msx2* (2, 1, 3) at E11.5; *Bmp2* (3, 0, 3), *Bmp7* (3, 0, 3), *Msx2* (3, 0, 3), and *Sox9* (2, 1, 3) at E12.5; *Bmp2* (1, 2, 3), *Bmp7* (2, 1, 3), *Msx2* (1, 2, 3), and *Sox9* (1, 3, 4) at E13.5. Stained embryos were cleared in glycerol to enable images to be produced with a VHX-1000 digital microscope (Keyence).

## Histology

Heads of embryos and newborns were fixed overnight in 4% paraformaldehyde in PBS at 4°C. These embryos were then washed in PBS. Frozen samples were serially sectioned at 16  $\mu$ m (E14.5) and 20  $\mu$ m (P0). The numbers of eyes examined (WT, *Smoc1<sup>TP/+</sup>*,

*Smoc1<sup>TP/TP</sup>*) were as follows: coronally sectioned at E14.5 (8, 10, 12), coronally sectioned at P0 (8, 10, 6), horizontally sectioned at P0 (2, 2, 4). For evaluation of ventral atrophy of the retina, only the coronally sectioned eyes were used. TB staining was performed according to standard protocols. Forelimbs of mice were fixed in 4% paraformaldehyde in PBS, decalcified in 10% EDTA, and embedded in paraffin. Forelimbs were serially sectioned at 4  $\mu$ m and stained with hematoxylin and eosin.

## Evaluation of Optic Nerve Diameter

The palatine and orbital bones were carefully removed to expose the optic chiasm and optic nerve. During the dissection process, 4% paraformaldehyde in PBS was frequently applied onto the gaps between the bone and optic nerve. Xylene cyanol was applied to enhance the outline of optic nerves at postnatal day 0 (P0). Photographs of optic nerves were taken with a VHX-1000 digital microscope, and the diameter was measured for right and left optic nerves with the bundled software included with the VHX-1000 instrument.

## Skeletal Staining

For skeletal preparations, mice were fixed in 99.5% ethanol after removal of the skin and viscera. Cartilage tissues were stained with 0.015% alcian blue and 20% acetic acid in 75% ethanol for three days at 37°C. After dehydration with 99.5% ethanol for three days, bones were stained with 0.002% alizarin red in 1% KOH. Then skeletons were cleared in 1% KOH for several weeks. For P14 mice, soft tissues were dissolved in 2% KOH before alizarin red staining.

## Nile Blue Staining

For the study of apoptosis of hindlimbs at E13.5 and E14.5, Nile blue (NB) staining was performed on the basis of a previously described protocol,<sup>23</sup> except that staining was performed at 37°C (not room temperature). Apoptosis was determined by NB-stained (deceased) cells. After rinsing in Tyrode solution, hindlimbs of control (WT and heterozygous littermates) and homozygous mice were evaluated. Photographs of dorsal aspects were taken with a VHX-1000 digital microscope. Experiments were repeated three times, and reproducible representative results are presented.

## Statistical Analysis

Statistical analyses were performed with the use of non-repeated-measures ANOVA followed by Dunnett's post hoc test. The results are given as mean  $\pm$  standard deviation, and the threshold p value for statistical significance was 0.01.

## Results

### Identification of Homozygous *SMOC1* Mutations

We have previously mapped the MLA locus to a 422 kb region at 10p11.23 by analyzing three families (one Japanese family [A] and two Lebanese families [B and C]). This region contained only one gene, *MPP7*, in which no mutations were found.<sup>12</sup> After a new Turkish family (X) was added to the analysis, the MLA locus was again searched by homozygosity mapping to the consanguineous families (X, B, and C) and haplotype mapping to family A for detection of compound-heterozygous mutations; however, we could not detect any common regions

among the four families. We then focused on identifying common regions in any three of the four families to allow for locus heterogeneity (Table S3).

A locus at 14q24.1-q24.2, which showed the highest LOD score (3.936) among the candidate regions larger than 2.0 Mb, was highlighted among families A, C, and X. This locus was analyzed with the use of additional microsatellite markers, and a 3.0 Mb region containing 24 genes was identified (Figures 1A and 1B). A total of 14 genes were sequenced, and homozygous mutations were found in *SMOC1*: c.718C>T (p.Gln240X) in family A, c.664+1G>A in family C, and c.378+1G>A in family X (Figures 1C and 1D). All of these homozygous mutations were cosegregated with the disease phenotype, and the parents of the individuals with these mutations were heterozygous carriers (Figure 1C). We could not find any mutations in *SMOC1* in family B, in which MLA is unlinked to the 14q24.1-q24.2 locus. Interestingly, in family A haplotypes of paternal and maternal alleles, each having the same mutation, are completely different (data not shown), suggesting that the same mutation may have occurred in separate events. The c.718C>T mutation was not detected in 289 healthy Japanese controls, including 100 Okinawa islanders. The other two mutations were not detected in ethnically matched controls (54 Lebanese and 99 Turkish subjects, respectively), nor in 289 Japanese controls. The two splice-donor-site mutations (c.664+1G>A and c.378+1G>A) are predicted to abolish a donor site, as predicted by ESE-finder, NetGene2, HSF2.4.1, SpliceView, and BDGP analysis (Table S4). Thus, the three mutations are likely to lead to a loss of functional *SMOC1*.

#### ***Smoc1* Expression in the Developing Eye and Limb in Mice**

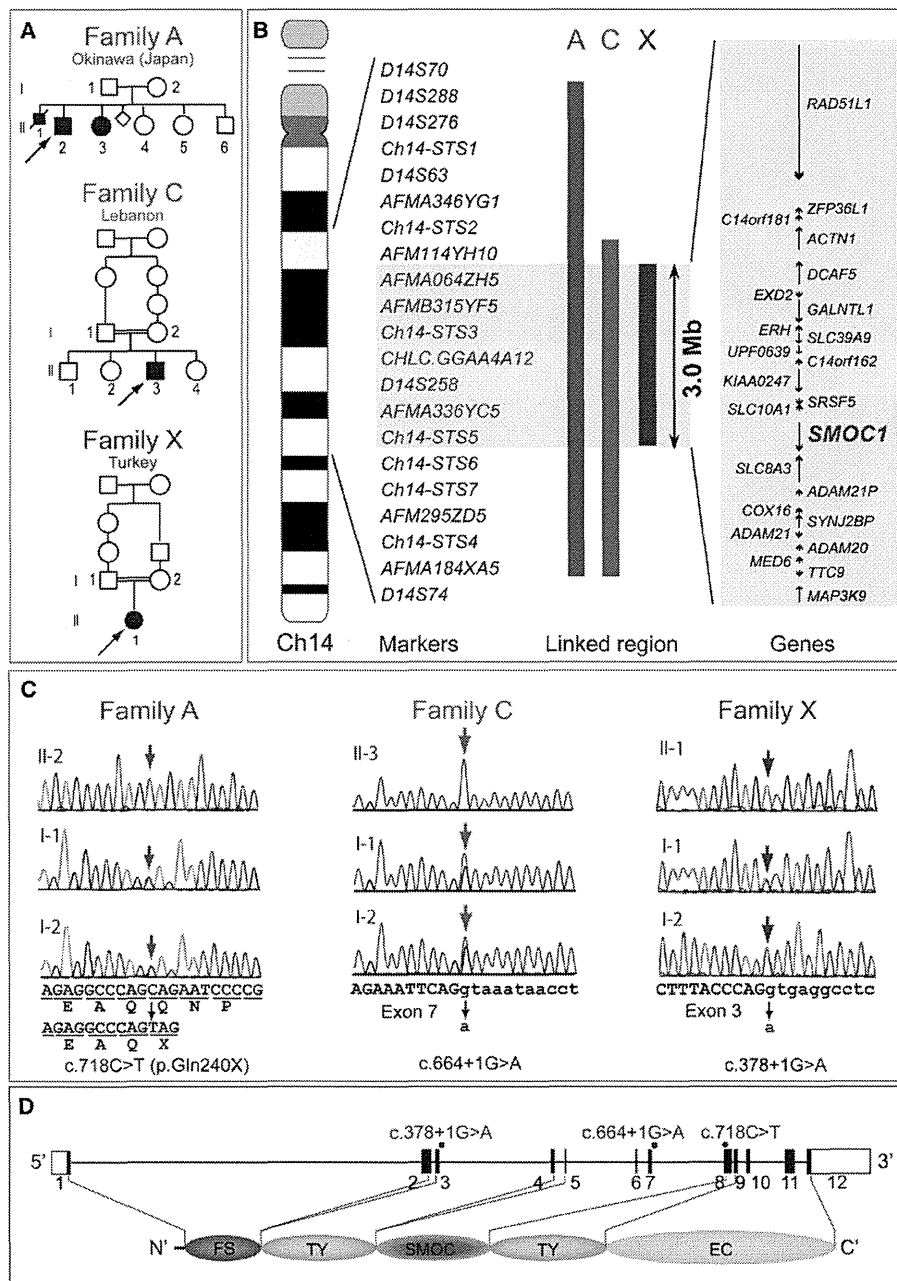
For the examination of *Smoc1* expression in the developing eye and limb, whole-mount *in situ* hybridization of mouse embryos was performed. *Smoc1* was expressed in the forebrain, midbrain, hindbrain, pharyngeal arch, somites, and forelimb buds at E9.5 (Figure 2A). At E10.5, *Smoc1* expression was observed in the optic stalk (Figure 2B), and at E11.5, expression was localized to the closure site of the optic cup (Figure 2C). Expression of *Smoc1* in developing limbs between E10.5 and E11.5 was observed in both dorsal and ventral regions, with a broader pattern of expression in dorsal regions, but expression was not detected in the most anterior, posterior, and distal parts of limb buds (Figures 2D and 2E). Expression coinciding with chondrogenic condensation was observed at E12.5 (Figure 2F), and expression then became restricted to future synovial joint regions at E13.5 (Figure 2G). This dynamic expression suggests that *Smoc1* plays a critical role in ocular and limb development.

#### **Ocular and Limb Anomalies in *Smoc1* Null Mice**

To investigate the pathological basis of MLA due to the loss of *SMOC1* function, we obtained *Smoc1* mutant

mice, PV384.<sup>17</sup> PV384 mice possess gene-trap insertions in the *Smoc1* locus and in three other loci. After PV384 mice were bred with C57BL/6J or ICR mice, we obtained three independent lines (no. 1 to no. 3), each with a sole insertion in intron 1 of *Smoc1* (Figure S1). We mainly analyzed line 1, but we confirmed similar phenotypes in lines 2 and 3. Heterozygous mutant mice (*Smoc1*<sup>TP/+</sup>) were healthy and fertile. Homozygous mice (*Smoc1*<sup>TP/TP</sup>) were null mutants, as they showed no native transcript of *Smoc1* (Figure S1E). Homozygous mice were viable at P0; however, they did not survive beyond the first 3 wks of life (Figure 3B). Their growth was retarded in comparison to WT and heterozygous littermates at P0 and P14 (Figures 3A and 3C). Developmental defects in eyes and optic nerves were evident at E14.5. Homozygous mice had relatively small eyes, and histological examinations revealed aplasia or hypoplasia of optic nerves (in 10 of 12 optic nerves), atrophy of the anteroventral part of the retina (in 11 of 12 eyes), and extension of the retinal pigmented epithelium (RPE) to the optic nerve (in 10 of 12 eyes) (Figures 3D–3I). These abnormalities were also observed at P0 (aplasia or hypoplasia of optic nerves [in 7 of 10 optic nerves], retinal atrophy [in 6 of 6 eyes], and RPE extension [in 3 of 6 eyes with identifiable optic nerves]) (Figures 3J–3M). WT or heterozygous littermates did not show any such abnormalities, except that a few eyes of heterozygous mice showed extension of the RPE at E14.5, but not at P0 (in 2 of 10 and 0 of 12 eyes, respectively). Toluidine blue (TB) staining showed ganglion cell layers that were thinned and irregular to varying degrees in homozygous mice, suggesting a reduced number of retinal ganglion cells (Figures 3J–3K'). Thus, *Smoc1* is required for axon sprouting, elongation, or maintenance of retinal ganglion cells.<sup>24</sup> Hypoplasia of optic nerves was further quantitatively confirmed by macroscopic examination: the average diameter of optic nerves of homozygous mice was significantly smaller than that of WT and heterozygous littermates at P0 and P14 (Figures 3L–3Q). These data clearly demonstrate that loss of *Smoc1* in mice affects development of the body, retina, and optic nerves, in a manner similar to that seen in MLA patients.<sup>3,4</sup>

Newborn homozygous mice could be readily identified by their hindlimb syndactyly and pes valgus, whereas no abnormalities were observed in WT and heterozygous pups (Figure 4 and Table 1). Interestingly, the severity of syndactyly varied between mouse lines: line 1 exclusively showed soft tissue syndactyly, whereas line 2 frequently showed four digits (Figures 4F and 4J). Skeletal preparations with alcian blue and alizarin red revealed that the foot with four digits had four phalanx and five metatarsals with fusion to each other (Figure 4K). Thus the *Smoc1* null mutation resulted in a spectrum of phenotypes, from soft tissue syndactyly to four fused digits, probably due to different genetic backgrounds. Bowed tibiae and hypoplastic fibulae were also consistently observed in homozygous mice (Figures 4H and 4L). The articulation between



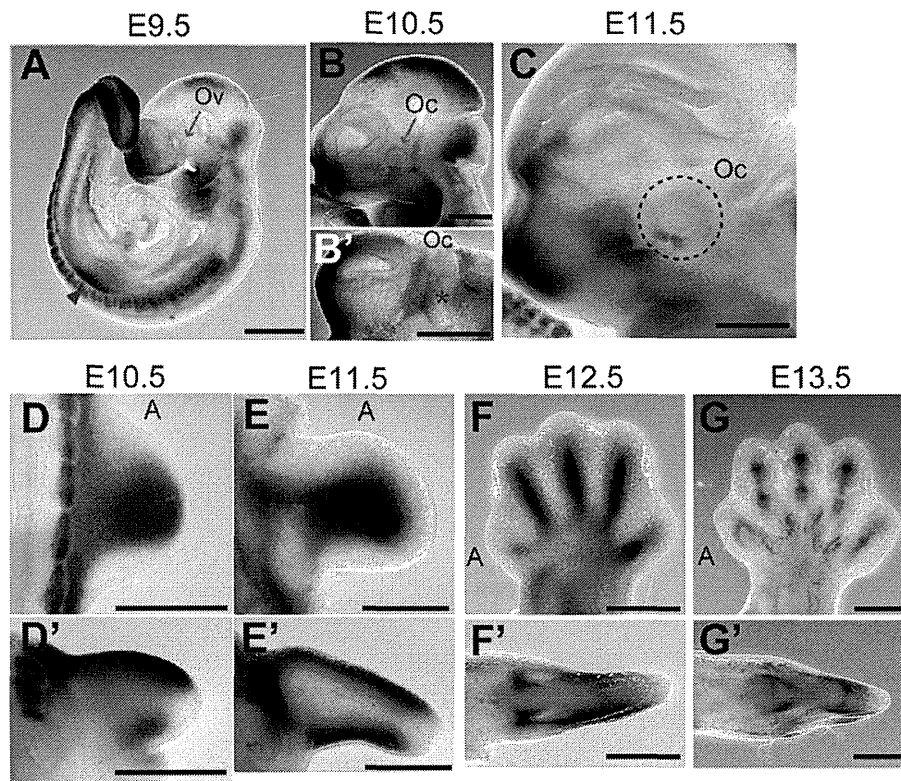
**Figure 1. Genetic Analysis of Three Families with Members Affected by Microphthalmia with Limb Anomalies**

(A) Pedigrees of the three families.

(B) Linkage analysis with SNPs and microsatellite markers on chromosome 14. From left to right: chromosome ideogram, genetic markers, linked regions of the three families, and genes mapped to the shortest overlapping linked region (between *AFM114YH10* and *Ch14-STS6* [UCSC coordinates, Feb. 2009: chromosome 14: 68,388,190–71,347,908 bp]).

(C) Sequences of mutations identified in each family. Affected patients in family A have a homozygous nonsense mutation (c.718C>T). Patients in families C and X have distinct homozygous splice-donor site mutations (c.664+1G>A and c.378+1G>A, respectively). For all mutations, parents of affected patients are heterozygous carriers, without exception. Sequences of the exon and intron are presented in upper and lower cases, respectively.

(D) At the top is a depiction of a schematic representation of *SMOC1* consisting of 12 exons (UTR and coding exons are indicated by open and filled rectangles, respectively). The locations of three mutations are indicated by red dots. At the bottom, the functional domains of *SMOC1* are depicted. Abbreviations are as follows: FS, the follistatin-like domain; TY, the thyroglobulin-like domain; SMOC, the domain unique to *SMOC*; and EC, the extracellular calcium-binding domain.



**Figure 2. *Smoc1* Expression in Mouse Embryos**

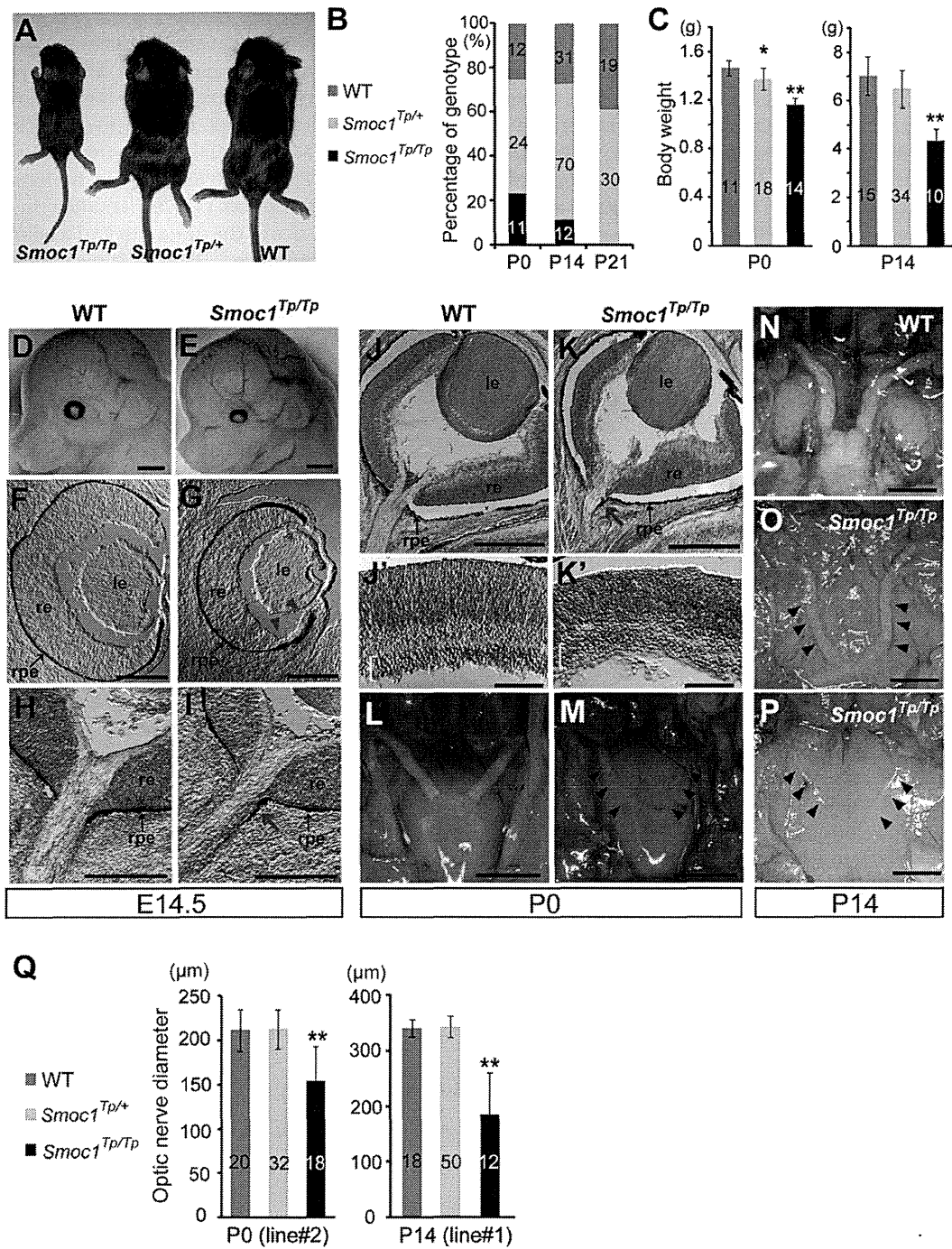
Lateral views of embryos (A–C) and a ventral view of the left part of the head (B', lateral view is shown at the top). (A) At E9.5, *Smoc1* was expressed in the forebrain, midbrain, hindbrain, pharyngeal arch, somites, and forelimb buds (magenta arrowhead), but not in the optic vesicle (Ov, blue arrow). (B and B') Expression in the optic stalk became evident at E10.5 (magenta asterisks), but was not evident in the optic cup (Oc, blue arrow). (C) Expression was restricted to the closure site of the optic cup (dashed circle) at E11.5. (D–G) Dorsal and (D'–G') posterior view of the right hindlimbs (dorsal view is shown at the top in D'–G'). The anterior side is indicated by an A. (D and D') At E10.5, *Smoc1* was more widely expressed in the dorsal part of the limb bud than in the ventral part. *Smoc1* expression is undetected in the most anterior, posterior, and distal parts of the limb bud. (E and E') At E11.5, ventral expression was broader than that in the previous stage. (F and F') At E12.5, expression was detected in areas consistent with chondrogenic condensation. (G and G') At E13.5, *Smoc1* expression became restricted to future joint regions. Scale bar represents 500  $\mu$ m.

tibia/fibula and calcanea of homozygous mice appeared malpositioned (Figures 4G and 4K), which might contribute to pes valgus. At P14, soft tissue syndactyly was also evident in most forelimbs of homozygous mice (Figures 4M–4O). Moreover, hindlimbs of homozygous mice showed synostosis between the 4<sup>th</sup> and 5<sup>th</sup> metatarsals (Figure 4T), which is observed in both the hands and the feet of MLA patients. Thus, many limb anomalies of MLA patients were recapitulated in *Smoc1* null mice (Table S1).

#### Reduced Interdigital Apoptosis and Disturbed BMP Signaling

Among the various abnormalities caused by loss of *Smoc1* function, we focused on soft tissue syndactyly, which was commonly observed in both fore- and hindlimbs of null mutants. It is possible that the syndactyly is caused by failed apoptotic regression of the interdigital mesenchyme. To examine this hypothesis, hindlimbs were stained with NB sulfate at E13.5 and E14.5, the time

when interdigital apoptosis is most evident. In control embryos (WT and heterozygous littermates), NB-stained apoptotic cells were identified in the interdigital mesenchyme, where regression of the interdigital webbing occurs in the distal region (Figures 5A and 5C). By contrast, the number of apoptotic cells in the mesenchyme between digits 2 and 3 and digits 3 and 4 was dramatically reduced in homozygous mice at E13.5 and E14.5, along with persistent webbing in the distal region (Figures 5B and 5D, magenta asterisk). BMP signaling is involved in apoptosis of the interdigital mesenchyme.<sup>25,26</sup> *Bmp2*, *Bmp7*, and *Msx2*, a direct target of BMP signaling, were strongly expressed in the interdigital mesenchyme of control hindlimbs at both E12.5 and E13.5. However, the expression of these three genes was profoundly reduced and perturbed in hindlimbs of homozygous mice (Figures 5E–5J). These data suggest that inhibition of apoptosis is spatiotemporally correlated to reduced and/or disturbed expression of genes involved in BMP signaling in the interdigital mesenchyme.



**Figure 3. Growth and Ocular Phenotypes of *Smoc1* Null Mice**

(A) Representative *Smoc1*<sup>Tp/Tp</sup> mouse, showing a small body in comparison to *Smoc1*<sup>Tp/+</sup> and WT littermates.

(B) Genotypes of living pups during the first 3 wk of life.

(C) Body weight of pups of each genotype at P0 (left panel) and P14 (right panel).

(D and E) Relatively small eyes were evident in *Smoc1*<sup>Tp/Tp</sup> mice in comparison to WT mice.

(F–K') Coronal sections of eyes at E14.5 (F–I) and P0 (J–K') with TB staining (H, I, and J–K'). (F–I) Atrophy of the anteroventral part of the retina (G, magenta arrowheads, dorsal view shown at the top), hypoplastic optic nerve, and extension of the RPE to the optic nerve (I, magenta arrow) in *Smoc1*<sup>Tp/Tp</sup> mice at E14.5. (J and K) Hypoplastic optic nerve and RPE extension in *Smoc1*<sup>Tp/Tp</sup> mice at P0 (K, magenta arrow). Note that sections in which optic nerves appeared most thick are presented in (H–K). (J'–K') In higher-magnification views of (J and K), a thinned and irregular ganglion cell layer (white brackets) was observed in *Smoc1*<sup>Tp/Tp</sup> mice. Abbreviations are as follows: le, lens; re, retina; rpe, retinal pigmented epithelium.

(L–P) Ventral views of the brain showing optic nerves at P0 (L and M) and P14 (N–P), showing various degrees of optic nerve hypoplasia.



## Discussion

In a previous report, we performed parametric linkage analysis with three families (families A, B, and C) and found 16 loci showing a LOD score ( $\theta = 0.000$ ) higher than 3.0. Additional microsatellite markers highlighted only one locus, 10p11.23.<sup>12</sup> However, no mutations were found in the candidate gene *MPP7*.<sup>12</sup> By recruiting a new family (family X) to this study, we successfully found homozygous mutations in *SMOC1* in families A, C, and X. In family B, no *SMOC1* mutations were found, indicating the genetic heterogeneity in MLA. Patients with *SMOC1* mutations and *Smoc1* null mice showed similar limb anomalies, such as oligodactyly, syndactyly, synostosis of 4<sup>th</sup> and 5<sup>th</sup> metacarpals, hypoplasia of fibula, and bowed tibia. Oligodactyly, syndactyly, and synostosis of 4<sup>th</sup> and 5<sup>th</sup> metacarpals are common in MLA patients.<sup>2-4</sup> However, hypoplastic fibula and bowed tibia are less common in patients with MLA, as four out of 34 MLA patients showed these anomalies in the previous report.<sup>3</sup> Although one patient with a *SMOC1* mutation from family C did not show bowed tibia and hypoplastic fibula, these anomalies could be features specific to *SMOC1* mutations. Further *SMOC1* analysis of other MLA patients should delineate the phenotypic consequences caused by *SMOC1* mutations.

Accumulating evidence suggests that BMP signaling plays crucial roles in early eye vesicle and limb patterning, skeletal formation, and apoptosis of the interdigital mesenchyme,<sup>25-29</sup> and mutations involving BMP signaling cause human malformations including ocular, limb, and skeletal anomalies.<sup>7,30-33</sup> Here, we present genetic evidence that *SMOC1* is essential for ocular and limb development in humans and mice. Furthermore, *Xenopus smoc* can inhibit BMP signaling,<sup>11</sup> suggesting that *SMOC1/Smoc1* can also modulate BMP signaling in humans and mice. Indeed, we observed reduced and/or disturbed expression of genes involved in BMP signaling in the interdigital mesenchyme in *Smoc1* null mice, and limb and ocular abnormalities associated with loss of *Smoc1* function are consistent with phenotypic consequences of disturbed BMP signaling. Conditional inactivation of *Bmp2* in the limb showed 3/4 syndactyly, and a similar deficiency of both *Bmp2* and *Bmp7* resulted in malformed fibulae in mice.<sup>25</sup> Moreover, mice deficient in *Fmn1*, a repressor of BMP signaling, showed four digits, fused metatarsal bones, and an absence of fibulae in the hindlimbs,<sup>34</sup> suggesting the importance of altered BMP signaling in these features. Concerning ocular phenotypes, haploinsufficiency of mouse *Bmp4* resulted in a decreased number of ganglion layer cells and absence of the optic nerve similar to *Smoc1* null mice,<sup>35</sup> indicating that altered BMP signaling

is also involved in the ocular phenotype. Interestingly, knockdown experiments of *smoc* by antisense morpholino in *Xenopus* showed absence or severe deformity of the eye and other anterior structures, which were accompanied by aberrant expression of *otx2*, *tbx2* in the eye field.<sup>11</sup> Mutations of *OTX2* (MIM 600037) cause microphthalmia, syndromic 5 (MCOPS5 [MIM 610125]) in humans.<sup>36</sup> Moreover, targeted disruption of *Tbx2* resulted in a marked reduction in the size of the optic cup and a failure of optic nerve formation in mice.<sup>37</sup> Thus, it is possible that loss of *SMOC1* function could alter the expression of *OTX2* and *TBX2* (MIM 600747) by disturbing BMP signaling in human developing eyes.

It is unknown how the loss of functional *SMOC1*, a BMP antagonist, leads to reduced expression of genes involved in BMP signaling in the interdigital mesenchyme in *Smoc1* null mice. In the case of *Fmn1*-deficient mice, the loss of the repressor of BMP signaling resulted in downregulation of *Fgf4* and *Shh* and in upregulation of *Gremlin* expression at E10.5, and absence of apoptosis of the interdigital mesenchyme between the two middle digits at E13.5.<sup>34</sup> Thus, there is a possibility that loss of *SMOC1* could cause the imbalance among BMP, SHH, and FGF signaling, which would subsequently lead to reduced and/or disturbed expression of genes involved in BMP signaling in the interdigital mesenchyme. In fact, we observed reduced expression of *Msx2* in the progressive zone of hindlimbs at E11.5 (Figure S2). Moreover, expression of *Sox9*, the initial cartilage condensation marker, showed abnormal limb patterning, suggesting that *SMOC1* may affect BMP signaling even at early stages of limb development (Figure S3). Further examinations are required for understanding spatial and temporal actions of *SMOC1/Smoc1* protein during limb development.

In conclusion, our data demonstrate that *SMOC1/Smoc1* is an essential player in both ocular and limb development in humans and mice and give further support to the crucial roles of BMP signaling in these systems.

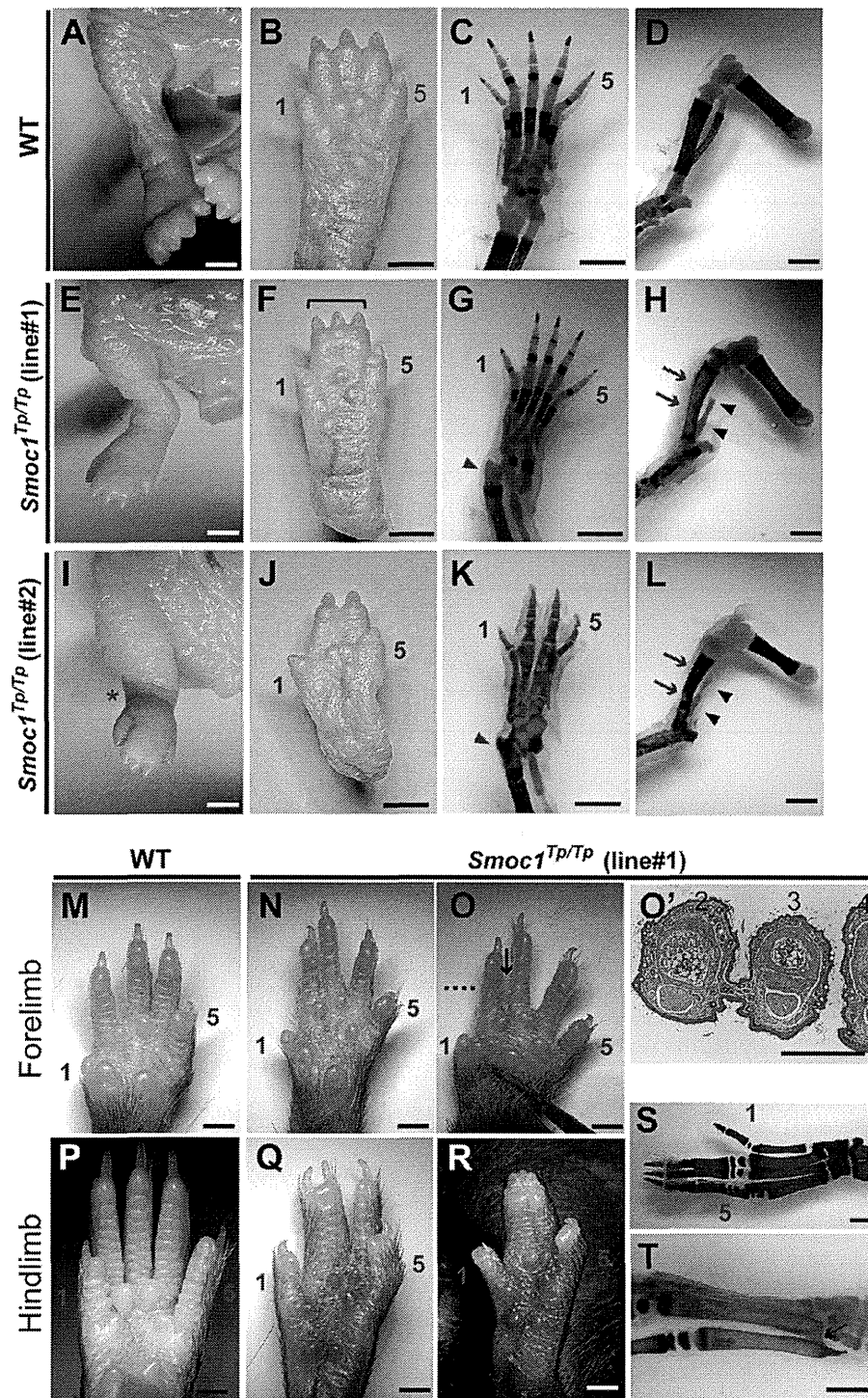
## Supplemental Data

Supplemental Data include three figures and four tables and can be found with this article online at <http://www.cell.com/AJHG/>.

## Acknowledgments

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(Q) Optic nerve diameter. Optic nerves were significantly hypoplastic in *Smoc1<sup>Tp/Tp</sup>* mice in comparison to WT and *Smoc1<sup>Tp/+</sup>* littermates. The numbers of pups (B and C) or eyes (Q) corresponding to each genotype are indicated within bars. Error bars indicate standard deviation: \* $p < 0.01$ , compared with WT. \*\* $p < 0.01$ , compared with WT and *Smoc1<sup>Tp/+</sup>*. Scale bars represent 1 mm (D, E, and L-P), 200  $\mu$ m (F-I), 500  $\mu$ m (J and K), and 100  $\mu$ m (J' and K').



**Figure 4. Limb Phenotypes of *Smoc1* Null Mice**

Limbs of WT (A–D, M, and P) and *Smoc1<sup>Tp/Tp</sup>* mice (E–L, N–O', and Q–T) at P0 (A–L) and P14 (M–T). Digit identities are indicated by the numbers 1 (thumb, anterior) and 5 (little finger, posterior). Skeletal staining with alcian blue and alizarin red is presented (C, D, G, H, K, L, S, and T). *Smoc1<sup>Tp/Tp</sup>* mice showed pes valgus (E and I), soft tissue syndactyly (F and G), and four digits with metatarsal fusion (J and K). Malposition of the articulation between the tibia/fibula and the calcanea (G and K, magenta arrowheads), bowed tibia (magenta arrows), and hypoplastic fibula (arrowheads) of *Smoc1<sup>Tp/Tp</sup>* mice (H and L) were observed. 2/3 soft tissue syndactyly (N) and 2/3 webbing (O) were evident in forelimbs of *Smoc1<sup>Tp/Tp</sup>* mice. (O') A transverse section taken at the level indicated by the dashed line in (O) showed 2/3 webbing. 2/3 syndactyly (Q), 2/3/4 syndactyly (R), synostosis between the 2<sup>nd</sup> and 3<sup>rd</sup> proximal phalanx and metatarsals (S), and synostosis between the 4<sup>th</sup> and 5<sup>th</sup> metatarsals (T, arrow), observed in the hindlimbs of *Smoc1<sup>Tp/Tp</sup>* mice. Scale bars represent 1 mm (A–O and P–T) or 500  $\mu$ m (O').

**Table 1. Limb Abnormalities in *Smoc1*<sup>Tp/Tp</sup> Mutants**

Genotype	Talipes Valgus (No. of Affected/ Total No. of Pups)	Forelimb Abnormalities (No. of Limbs)	Hindlimb Syndactyly (No. of Limbs)				Other External Abnormalities (No. of Pups)	4 <sup>th</sup> and 5 <sup>th</sup> Metatarsal Fusion (No. of Affected/Total No. of Limbs)
			None	2/3 <sup>a</sup>	3/4 <sup>b</sup>	2/3/4 <sup>c</sup>		
<b>Postnatal Day 0</b>								
<i>Smoc1</i> <sup>Tp/+</sup> (line 1, CS7BL/6J)	0/42	0	84	0	0	0	0	
<i>Smoc1</i> <sup>Tp/+</sup> (line 2, ICR mixed)	0/38	0	76	0	0	0	0	
<i>Smoc1</i> <sup>Tp/Tp</sup> (line 1, CS7BL/6J)	10/10	0	3	0	3	12	2	
<i>Smoc1</i> <sup>Tp/Tp</sup> (line 2, ICR mixed)	13/17	1 <sup>d</sup>	1	1	9	4	19	cleft palate (3)
<b>Postnatal Day 14</b>								
<i>Smoc1</i> <sup>Tp/+</sup> (line 1, CS7BL/6J)	0/70	0	140	0	0	0	0	
<i>Smoc1</i> <sup>Tp/Tp</sup> (line 1, CS7BL/6J)	11/11	18 <sup>e</sup>	2	7	3	8	2	hypoplastic thumbs (5)

<sup>a</sup> Syndactyly between the 2<sup>nd</sup> and 3<sup>rd</sup> digits.  
<sup>b</sup> Syndactyly between the 3<sup>rd</sup> and 4<sup>th</sup> digits.  
<sup>c</sup> Syndactyly between the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> digits.  
<sup>d</sup> 2/3 soft tissue syndactyly.  
<sup>e</sup> Eleven limbs showed 2/3 webbing, four limbs showed 2/3 soft tissue syndactyly, and one limb showed 3/4 syndactyly.  
<sup>f</sup> Based on examination of skeletal preparations.

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### Web Resources

The URLs for data presented herein are as follows:

BDGP, <http://www.fruitfly.org/>

ESEfinder 3.0, <http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/>

HSF2.4.1, <http://www.umd.be/HSF/>

NetGene2, <http://www.cbs.dtu.dk/services/NetGene2/>

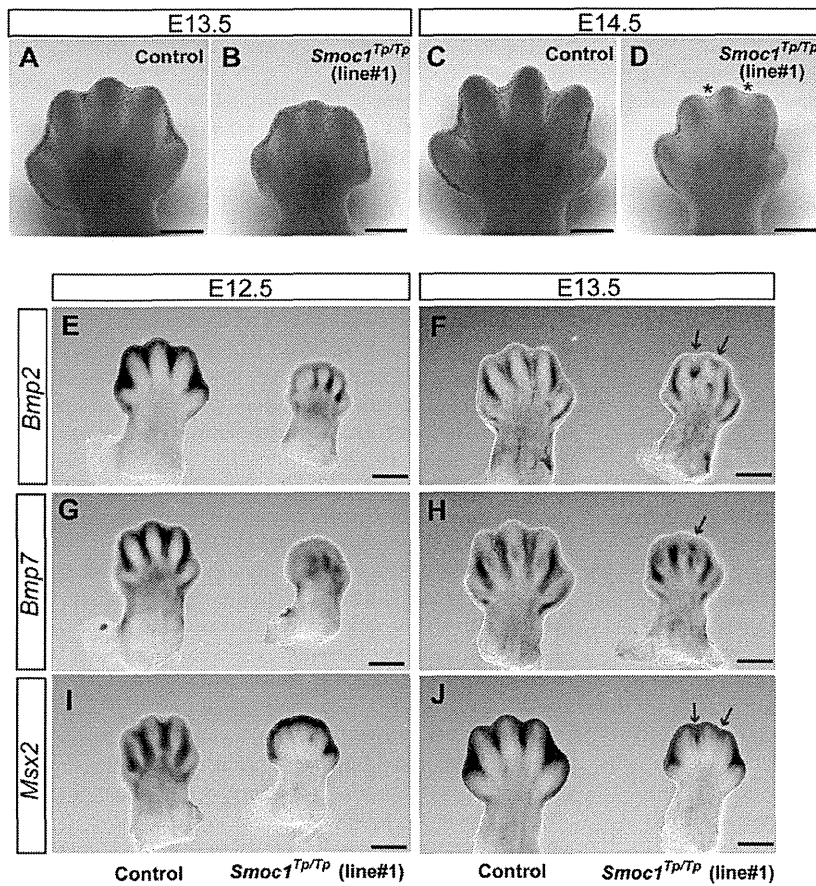
Online Mendelian Inheritance in Man, <http://www.ncbi.nlm.nih.gov/Omim>

UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway>

SpliceView, <http://zeus2.itb.cnr.it/~webgene/wwwspliceview.html>

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**Figure 5. Reduced Apoptosis and Altered BMP Signaling in the Interdigital Mesenchyme of *Smoc1* Null Mice**

(A–D) NB staining of left hindlimbs at E13.5 (A and B) and E14.5 (C and D). In comparison to control embryos (WT and *Smoc1*<sup>Tp/+</sup> littermates) (A and C), the number of NB-stained apoptotic cells in the interdigital mesenchyme of *Smoc1*<sup>Tp/Tp</sup> mice was dramatically reduced between digits 2 and 3 and digits 3 and 4 at both E13.5 and E14.5, and the webbing remained at a distal level (B and D, magenta asterisk).

(E–J) Whole-mount in situ hybridization of right hindlimbs at E12.5 (E, G, and I) and E13.5 (F, H, and J). At E12.5, interdigital expression of *Bmp2*, *Bmp7*, and *Msx2* was profoundly delayed in the hindlimbs of *Smoc1*<sup>Tp/Tp</sup> mice, and their expression in the interdigital mesenchyme was apparently perturbed, even at E13.5 (magenta arrows). Scale bar represents 500  $\mu$ m.

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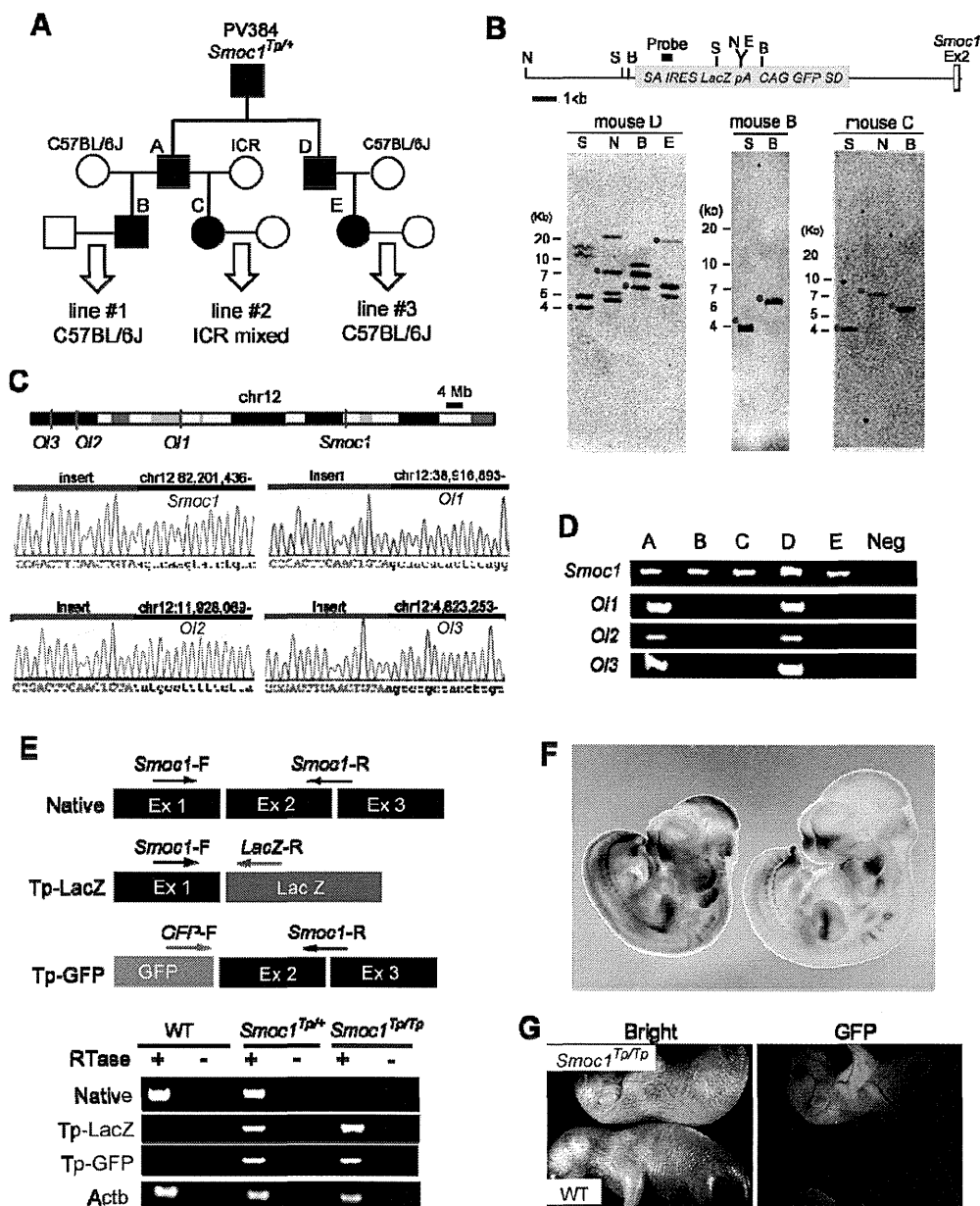
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Supplemental Data

**SMOC1 Is Essential for Ocular and Limb Development**

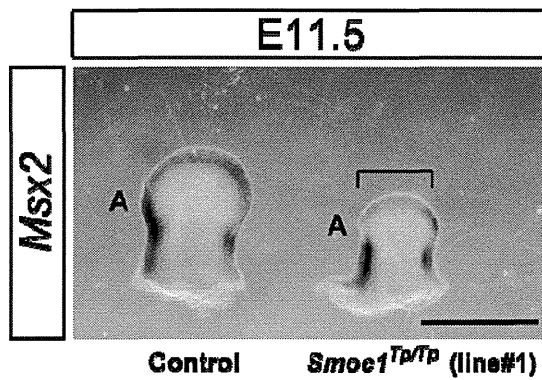
**in Humans and Mice**

Ippei Okada, Haruka Hamanoue, Koji Terada, Takaya Tohma, Andre Megarbane, Eliane Chouery, Joelle Abou Ghoch, Nadine Jalkh, Ozgur Cogulu, Ferda Ozkinay, Kyoji Horie, Junji Takeda, Tatsuya Furuichi, Shiro Ikegawa, Kiyomi Nishiyama, Satoko Miyatake, Akira Nishimura, Takeshi Mizuguchi, Norio Niikawa, Fumiki Hirahara, Tadashi Kaname, Koh-ichiro Yoshiura, Yoshinori Tsurusaki, Hiroshi Doi, Noriko Miyake, Takahisa Furukawa, Naomichi Matsumoto, and Hiroto Saito



## Figure S1. Characterization of PV384 (*Smoc1* Mutant) Mice

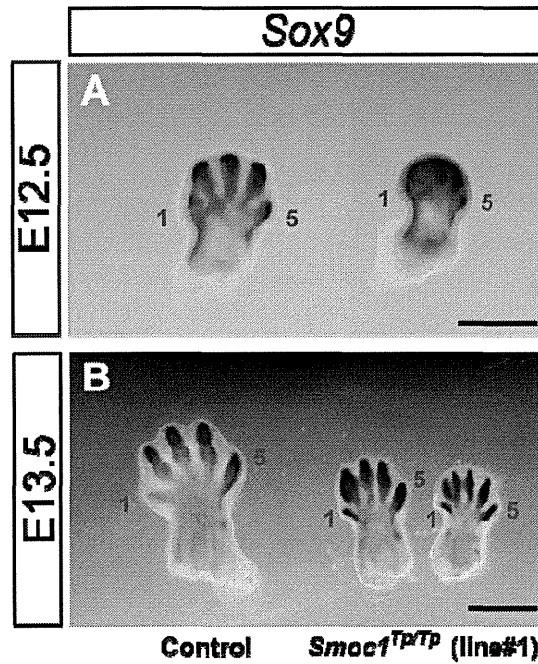
(A) Schematic representation of PV384 mouse lines. Mice heterozygous for a *Smoc1* gene-trap insertion are indicated by filled symbols. (B) Southern hybridization analysis of PV384 mice. (Top) A partial restriction map (N, *NdeI*; S, *SacI*; Bg, *BglII*; E, *EcoRI*) and the position of the probe for Southern hybridization are indicated. SA, splice acceptor; IRES, internal ribosome entry site; pA, poly(A); GFP, green fluorescent protein; SD, splice donor. (Bottom) Southern hybridization of the probe on the genomic DNA of mice D, B and C. While mouse D showed three to four bands, mice B and C showed only one band corresponding to the DNA fragment containing the *Smoc1* locus (red dots). (C) Identification of the other three loci containing gene-trap insertions (*O11* to *O13*). Mouse chromosome 12 ideogram and the four loci are indicated with red bars (top). Electropherogram of flanking genomic sequences are shown (bottom). (D) PCR genotyping to detect gene-trap insertions at four different loci. Note that the three lines (#1 to #3) were derived from mice (B, C and E) which had a single insertion at the *Smoc1* locus. Neg, no template PCR. (E) Confirmation by RT-PCR of native *Smoc1*, promoter-trapped (Tp-LacZ) and poly(A)-trapped (Tp-GFP) transcripts. The native *Smoc1* transcript was detected in WT and *Smoc1*<sup>Tp/+</sup> embryos, but was undetected in *Smoc1*<sup>Tp/Tp</sup> embryos, indicating that *Smoc1*<sup>Tp/Tp</sup> is null for *Smoc1*. Promoter-trapped and poly(A)-trapped transcripts were detected in both *Smoc1*<sup>Tp/+</sup> and *Smoc1*<sup>Tp/Tp</sup> mice.  $\beta$ -actin (*Actb*) was used as an internal control. (F) LacZ staining of heterozygous embryos (right) shows a similar pattern to that of *Smoc1* expression (left) in the limbs, optic nerve, pharyngeal arches and somites.  $\beta$ -galactosidase activity in whole embryos was detected as previously described (Hogan, B.L., Beddington, R., Constantini, F. & Lacy, E. *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd edn., (Cold Spring Harbor Laboratory Press, New York, 1994). (G) Comparable bright-field and fluorescence photographs of GFP-positive (top, *Smoc1*<sup>Tp/Tp</sup>) and GFP-negative (bottom, WT) newborns



**Figure S2. Reduced Expression of *Msx2* in Hindlimbs of *Smoc1* Mutant Mice**

Whole mount *in situ* hybridization at E11.5. Dorsal view of the right hindlimbs is presented. Anterior side is indicated by A. Expression of *Msx2* was reduced in progressive zone of hindlimbs of *Smoc1*<sup>Tr/Tr</sup> mice (bracket). Scale bar, 1 mm.





**Figure S3. Delayed and Altered Expression of *Sox9* in Hindlimbs of *Smoc1* Mutant Mice**

Whole mount *in situ* hybridization of right hindlimbs at E12.5 (A) and E13.5 (B). Future digit identities are indicated by numbers 1 (thumb, anterior) and 5 (little finger, posterior). (A) Expression of *Sox9* at E12.5 was delayed in hindlimbs of *Smoc1<sup>Tp/Tp</sup>* mice compared with that of control mice, suggesting a delay of limb development. (B) Compared with control mice (left), expression of *Sox9* in hindlimbs of *Smoc1<sup>Tp/Tp</sup>* mice at E13.5 showed abnormally thick cartilage condensation in future digit 2 (middle) or extra numbers of cartilage condensation (right), suggesting limb patterning defects. Scale bar, 1 mm.

**Table S1. Phenotypes of *SMOCl/Smoc1* Mutations in Humans and Mice**

	A-II-2	A-II-3	C-II-3	X-II-1	<i>Smoc1</i> <sup>TrAp</sup> mice
<b>origin</b>	Okinawa, Japan	Okinawa, Japan	Lebanon	Turkey	
<b>consanguinity</b>	–	–	+	+	
<b>sex</b>	male	female	male	female	
<b>ocular abnormality</b>	+	+	+	+	+
anophthalmia	bilateral	bilateral	bilateral	bilateral	small eye
loss of optic nerve (CT)	bilateral	bilateral	bilateral	nc	(aplasia/hypoplasia)
loss of optic tract (CT)	+	+	–	nc	ND
<b>upper limb abnormality</b>	+	+	+	+ <sup>a</sup>	+
syndactyly	–	–	–	+	+
metacarpal synostosis	4th and 5th fingers	4th and 5th fingers	–	4th and 5th fingers	–
hypoplasia	–	–	–	5th finger	–
coalition of capitate and hamate	–	–	–	+	–
clinodactyly	+	–	+	+	–
camptodactyly	+	–	+	–	–
simian crease	+	+	–	+	ND
<b>lower limb abnormality</b>	+	+	+	+ <sup>b</sup>	+
oligodactyly / syndactyly / polydactyly	bilateral oligodactyly	bilateral oligodactyly	bilateral syndactyly	bilateral oligodactyly	syndactyly
metatarsal synostosis	+	+	–	–	+
bowed tibia	+	(mild)	–	–	+
hypoplastic fibula	+	(mild)	–	+	+
abnormal cleavage between toes	1st and 2nd toes	1st and 2nd toes	1st and 2nd toes	–	–
dermal syndactyly	2nd and 3rd toes	2nd and 3rd toes	2nd to 5th toes	–	+
pes valgus	+	–	–	–	+
<b>other</b>					
congenital malformation of palate	–	–	–	+(high arched)	+(cleft palate, in line#2)
failure to thrive	+	+	+	+	+(growth retardation)
developmental retardation	DQ=10	DQ=15	+	–	ND
cryptorchidism	right		nc		ND
sacral dimple	nc	nc	nc	+	ND
<b><i>SMOCl</i> mutation</b>	<b>c.718C&gt;T</b>	<b>c.718C&gt;T</b>	<b>c.664+1G&gt;A</b>	<b>c.378+1G&gt;A</b>	<b>gene trapping</b>

CT, computed tomography; DQ, developmental quotient

nc, not confirmed ND, not determined

a, 5th metacarpal in the left hand is absent

b, distal phalanges of the 4th toe on both feet are absent

**Table S2. Marker Primers for Chromosome 14 Mapping**

<b>Marker</b>	<b>Forward (5' &gt; 3')</b>	<b>Reverse (5' &gt; 3')</b>	<b>Fluorescence</b>	<b>Product size (bp)</b>
<i>D14S70</i>	ATCAATTTGCTAGTTTGGCA	AGCTAATGACTTAGACACGTTGT	VIC	214
<i>D14S288</i>	AGCTAGACTCTGCCATAAACA	TGGAGACAGGAACAACACAC	NED	203
<i>D14S276</i>	TGCTTTACCAAGTGCATCAC	AGCTCAGAATCTAGGCCCT	NED	90
<i>Ch14-STS1</i>	GCCCTGGAGCATCTTGTAGT	GTTTCAGGTTTGGCCATGAG	FAM	162
<i>D14S63</i>	GGCCAGGTTTCAATCAGTTT	GCCAGAGAGCCACACTGTAT	VIC	205
<i>AFMA346YG1</i>	AAGAGACTGACATAGCCAGTT	CCGAGATACAAACATGGA	NED	112
<i>Ch14-STS2</i>	TTTTCATATTTTTGAGAGTTTTAGG	GCTGGCGAAAAGACAAGATT	NED	288
<i>AFM114YH10</i>	TGTTCTAGTTGATGTGAGACTT	TATTTGAGGACCTGCTGTAA	FAM	216
<i>AFMA064ZH5</i>	TGGATTGTTTGCTCTCAGAT	TAATGTCACTGCCTGGGA	FAM	261
<i>AFMB315YF5</i>	CTGGGCAGTGA CTCTAGGAGAC	GGGAATACAGTGTCCAATGACC	VIC	196
<i>Ch14-STS3</i>	TGCTTCAAACCTTGCCTCTT	CCCTGCTTTGTACCTCTTC	VIC	243
<i>CHLC.GGAA4A12</i>	GCCGAAAGAAAGAAAAAAGG	CGAATGCATACTTGCTGTTG	VIC	120
<i>D14S258</i>	TCACTGCATCTGGAAGCAC	CTAACTAAATGGCGAGCATTGAG	FAM	176
<i>AFMA336YC5</i>	AGATTTTGGATGTATCAGGC	CAGAAGCAATAGGATGGATG	NED	168
<i>Ch14-STS5</i>	TTATGCAACCATAGCCTTTGC	GAGGTTGAGCAAGACCCTGT	NED	201
<i>Ch14-STS6</i>	CCCACATCCAACACTGAGAA	CCTTCCCTCTGTGTCCTCAC	VIC	215
<i>Ch14-STS7</i>	CTCCCTTGATGTGTGAAGCA	TTTTCAACACCACCACCAGA	NED	218
<i>AFM295ZD5</i>	TTGCTTTCACTCCCCATT	TGCACTTGAAGATTCAGATAAGG	FAM	152
<i>Ch14-STS4</i>	GGCCAACATGATGAAACCC	AAGGCTCAGCAAGAAGAACTC	FAM	355
<i>AFM184XA5</i>	GACTGAGGCTCAAGGATTGC	CTTCCACTAATGGCGAGGAA	VIC	250
<i>D14S74</i>	CCTGTACCACTACCTGAGTTGAGT	CTTTGGCTGCCCGAAA	VIC	304

**Table S3. Common Candidate Regions in Any Three of the Four Families**

Chr	Physical position	size	SNP numbers	LOD scores					
				A	B	C	X	All families 3 families	
5	44228425-45740067	1,511,643	17	0.852	1.164	-2.935	1.453	0.534	3.469
5	57974102-58367038	392,937	19	0.852	1.075	-0.474	1.453	2.907	3.380
5	61832737-62244988	412,252	13	0.852	1.150	-0.478	1.453	2.978	3.455
6	8431193-8722149	290,957	21	0.977	1.041	1.683	-0.847	2.854	3.701
6	25928376-27047713	1,119,338	10	0.977	1.176	1.790	-0.845	3.098	3.943
6	33478496-34613887	1,135,392	12	0.977	0.929	1.582	-0.843	2.645	3.488
6	123015089-123893054	877,966	17	0.977	1.177	-1.714	1.414	1.854	3.568
7	9174771-9431031	256,261	18	0.977	1.174	1.804	-6.996	-3.041	3.955
7	14738170-14997102	258,933	13	0.977	0.947	1.698	-0.845	2.776	3.621
10	16851432-17381572	530,141	18	0.977	1.183	-6.438	1.454	-2.825	3.613
10	17704372-24780906	7,076,535	151	0.977	1.183	-27.00	1.454	-23.40	3.613
10	28006811-28197289	190,479	6	0.977	1.183	Inf	1.454	Inf	3.613
10	28305685-28541472	235,788	14	0.977	1.183	1.829	1.454	5.442	
10	28633450-29361379	727,930	56	0.977	1.183	1.829	-3.742	0.247	3.988
11	48058313-48987539	929,227	6	-8.150	1.103	1.828	1.343	-3.876	4.275
12	43151728-43514937	363,210	22	-4.025	1.183	1.626	1.162	-0.054	3.971
14	68275342-71054478	2,779,137	63	0.977	-2.713	1.828	1.131	1.223	3.936
14	71220216-71295001	74,786	2	0.977	0.437	1.828	0.015	3.257	
14	71412340-71658253	245,914	6	0.977	0.437	1.828	-6.865	-3.623	3.243
15	58696863-58853363	156,501	10	Inf	0.817	1.798	1.450	Inf	4.065

Gray highlighted: previous candidate region on 10p12.33-p11.23 (Hamanoue, H. et al., Am J Med Genet A, 2009)

Green highlighted: the region analyzed in this study