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Loss of the BMP Antagonist, SMOC-1, Causes Ophthalmo-Acromelic (Waardenburg Anophthalmia) Syndrome in Humans and Mice

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Abstract

Ophthalmo-acromelic syndrome (OAS), also known as Waardenburg Anophthalmia syndrome, is defined by the combination of eye malformations, most commonly bilateral anophthalmia, with post-axial oligosyndactyly. Homozygosity mapping and subsequent targeted mutation analysis of a locus on 14q24.2 identified homozygous mutations in SMOC1 (SPARC-related modular calcium binding 1) in eight unrelated families. Four of these mutations are nonsense, two frame-shift, and two missense. The missense mutations are both in the second Thyroglobulin Type-1 (Tg1) domain of the protein. The orthologous gene in the mouse, Smoc1, shows site- and stage-specific expression during eye, limb, craniofacial, and somite development. We also report a pre-conditioned gene-trap mutation of Smoc1 (Smoc1tm1a) that reduces mRNA to ~10% of wild-type levels. This gene-trap results in highly penetrant hindlimb post-axial oligosyndactyly in homozygous mutant animals (Smoc1tm1a/Smoc1tm1a). Eye malformations, most commonly coloboma, and cleft palate occur in a significant proportion of Smoc1tm1a/Smoc1tm1a embryos and pups. Thus partial loss of Smoc1 results in a convincing phenocopy of the human disease. SMOC1 is one of the two mammalian paralogs of SPARC-related modular calcium binding 1 that reduces mRNA to ~10% of wild-type levels. This gene-trap results in highly penetrant hindlimb post-axial oligosyndactyly in homozygous mutant animals (Smoc1tm1a/Smoc1tm1a). Eye malformations, most commonly coloboma, and cleft palate occur in a significant proportion of Smoc1tm1a/Smoc1tm1a embryos and pups. Thus partial loss of Smoc1 results in a convincing phenocopy of the human disease. SMOC1 is one of the two mammalian paralogs of Drosophila Pentagone, an inhibitor of decapentaplegic. The orthologous gene in Xenopus laevis, Smoc-1, also functions as a Bone Morphogenic Protein (BMP) antagonist in early embryogenesis. Loss of BMP antagonism during mammalian development provides a plausible explanation for both the limb and eye phenotype in humans and mice.

Introduction

Congenital absence of an eye (here termed anophthalmia) is a rare malformation in humans with a live birth prevalence of less than 1 in 10,000 [1]. Identifiable single gene disorders account for ~25% of bilateral anophthalmia. The known genetic causes include compound heterozygous mutations in PAX6, de novo heterozygous loss-of-function mutations in SOX2 [2–4], inherited or de novo heterozygous loss-of-function mutations in OTX2 [5,6], homozygous loss-of-function mutations in STRA6 [7] and possibly inherited, heterozygous loss-of-function mutations in BMP4 [8]. In most cases of anophthalmia no eye is visible on clinical
Author Summary

Ophthalmo-acromelic syndrome (OAS) is a rare congenital genetic disorder involving complete absence of the eyes and limb malformations, with missing or fused bones in the feet and hands. In this paper we report the identification of genetic changes to both copies of the SMOC1 gene as the cause of most cases of OAS. We have identified eight different mutations in this gene in unrelated individuals, and six of these mutations are predicted to completely abolish SMOC1 function. We have also genetically disrupted the mouse Smoc1 gene to produce only 10% of normal levels. These animals, called Smoc1<sup>tm1a/tm1a</sup> mice, have similar hindlimb malformations to those seen in the limbs of human OAS patients, resulting in missing toes in some mice and fusion of toes in others. Smoc1<sup>tm1a/tm1a</sup> embryos and pups also have eye malformations but these are milder than those seen in human cases, perhaps because, unlike the human cases, the mice still have some residual function of the gene. We suggest that the normal function of SMOC1 may be to regulate an important class of growth factors, called Bone Morphogenetic Proteins (BMPs), which are essential for normal embryonic development.

examination but optic nerves, chiasm and optic tracts remnants are visible on magnetic resonance imaging. Absence of the eye with ipsilateral absence of optic nerves, chiasm and optic tracts is termed true anophthalmia and is taken to suggest very early failure of ocular development.

Ophthalmo-acromelic syndrome (OAS), also known as Waardenburg anophthalmia syndrome, is one of the most frequently reported causes of true anophthalmia, which occurs in association with a distinctive pattern of distal limb anomalies (Figure 1a–1c). OAS is an autosomal recessive disorder (MIM #206920) first reported 50 years ago by Waardenburg in two unrelated families [9]. This original report illustrated the phenotypic spectrum associated with this disorder. The first family was a sibship of nine and consisted of two sisters with unilateral anophthalmia, one of whom had coloboma in her contralateral eye. Both had bilateral synostosis of the 4th and 5th metacarpals and bilateral postaxial four-toe oligodactyly with soft tissue syndactyly. A brother had synostosis of the 4th and 5th metacarpals and bilateral postaxial synostosis of the 4th and 5th metacarpals and bilateral postaxial metacarpals and bilateral postaxial four-toe oligodactyly with fusion of metacarpals 4–5 and 13 (37.1%) had learning disability. Other recurrent features included oro-facial clefts (4/35) and perinatal or early postnatal death (10/35) in the 25 families. At the point of submission of this paper, very little was known of the molecular basis of OAS. A locus on 10p11.23 with a reported LOD score ≥3 had been suggested on the basis of linkage analysis in three unrelated families. However, no pathogenic mutations could be identified in any of the genes in the linkage interval [17].

Bone morphogenetic proteins (BMPs) account for 10 of the 33 members of the transforming growth factor beta (TGFβ) superfamily of peptide growth factors in humans and are encoded by the genes BMP2, BMP4, BMP5, BMP6, BMP7, BMP9, BMP10 and BMP15 (BMP1 does not encode a growth factor but a tolloid-like protease[30]). BMPs are secreted into the extracellular space where they bind to BMP type 1 serine-threonine kinase cell surface receptors encoded by BMPR1A and BMPR1B. The presence of BMP type I receptors appears sufficient for BMP binding, but a BMP type II receptor (encoded by ACVR2A and ACVR2B) is required for phosphorylation of the BMP type I receptors, endocytosis and activation of the signal transduction cascade [31]. The intracellular domain of the activated BMP type I receptors in turn phosphorylates a Ser-Ser-X-Ser (SSXS) motif at the C-terminal end of one of three homologous protein products of the human genes SMAD1, SMAD3 and SMAD9. Phosphorylated SMAD1/5/9 (pSMAD1/5/9 known as regulatory- or R-SMADs) then bind to the co-SMAD encoded by SMAD4. The co-SMAD/ R-SMA complex then translocates to the nucleus where it functions as a transcription factor mediating the activation of target genes [32]. It has recently become clear that BMP signaling can also directly induce the activation of the MAPK pathway [33].

The formation of BMP signaling gradients is used extensively throughout vertebrate embryonic development. The formation and maintenance of stable developmental gradients appears to require multiple mechanisms to balance agonistic and antagonistic effects on BMP signaling. The complexity of the system is demonstrated by the molecular basis of dorsal and ventral signaling centres in the gastrula of Xenopus laevis embryos [34]. The dorsal signaling centre (DSC; Spemann’s organizer) has the general effect of antagonizing the Bmp gradient from the ventral signaling centre. The DSC secretes noggin and chordin, which (together with twisted-gastrulation [35]) bind to bmp in the extracellular space and prevents binding to the bmp type I receptor. The ventral signaling centre (VSC) secretes bm4p and bmp7 but also bmper (bmp-binding endothelial regulator) [36] and sizzled, which inhibits tolloid-like 1, a zinc metalloproteinase that efficiently cleaves chordin [37]. The VSC also produces bambi (bmp and activin membrane-bound inhibitor), a bmp receptor that lacks the catalytic intracellular domain and thus acts dominant-negatively to inhibit bmp signaling [38].

SMOC-1 is encoded by the human gene SMOC1 (SPARC related modular calcium binding f) and was initially characterised as a basal membrane protein with significant homology to BM-40 (also known as SPARC and osteonectin) [39]. The domain structure of the SMOC-1 peptide and the close homolog SMOC-2 [40], is evolutionarily conserved [41] and consists from N- to C-terminus of a follistatin-like domain, two thyroglobulin type I (Tg1) domains and an EF-hand calcium-binding domain. The ortholog of SMOC-1 in Xenopus laevis, XSSMC1, has been shown to function as a BMP antagonist. Uniquely among the known peptide BMP antagonists, SMOC-1 was able to antagonize BMP activity in the presence of a constitutively active BMP receptor. The molecular basis of this antagonism is not yet clear but may function by stimulating MAPK-mediated phosphorylation of the linker (i.e. non-SSXS) region of the R-SMAD proteins [42].

We report the identification of a locus for OAS on 14q24.2 with subsequent identification of homozygous, predicted loss-of-function mutations in the SMOC1 gene in eight out of fourteen unrelated families with OAS. Whole mount in situ hybridisation (WISH) combined with optical projection tomography (OPT) shows site- and stage-specific developmental expression of the orthologous mouse gene, Smoc1, in embryonic limb bud and craniofacial structures. The phenotype associated with homozygosity for a targeted “pre-conditional” gene-trap mouse mutation of Smoc1 also shows significant overlap with the human disease. SMOC-1 and SMOC-2 appear to be the two vertebrate paralogs of the Drosophila protein Pentagone that has recently been shown to function as an antagonist of Decapentaplegic (Dpp) signaling in vivo.
We discuss the potential role for SMOC-1 in modulating BMP signaling during eye and limb development.

**Results**

**Mapping and Mutation Analysis**

A locus for OAS at 14q24.2 was identified using autozygosity mapping with 10K SNP chip data from multiple, apparently unrelated consanguineous pedigrees. Affected individuals from eight of the fourteen families showed tracks of 20 homozygous SNPs in a row at this locus (Dataset S1). This locus was confirmed with multipoint linkage analysis using data from three of these families giving a combined LOD score of 5.3 (Figure 1d). The critical region was narrowed to ~1 Mb using microsatellite markers in four families (Figure 1e). To identify the causative gene, all coding exons for each gene in the critical region were sequenced (Figure 1f).

Potentially deleterious mutations were identified in only one gene: **SMOC1**, and independent homozygous **SMOC1** mutations were found in eight out of fourteen families (Figure 2a, Table 1). Of these, 6 mutations predicted complete loss of protein function; 4 are nonsense mutations and 2 are single base deletions or

[33].

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**Figure 1. Mapping ophthalmo-acromelic syndrome.** Clinical photographs (a,b) and radiographs (c) of patient R14C12 showing bilateral anophthalmia, in association with bilateral postaxial oligodactyly and cutaneous syndactyly of 2nd & 3rd toes. (d) Multipoint linkage analysis using 10K SNP chip data from families 1–3 showing a significant LOD score of Z = 5.3 at 14q22.3–24.2, a region also identified by autozygosity mapping (see Table 1). (e) Microsatellite marker analysis for affected individuals in Families 1–3 and Family 9 showing region of homozygosity, but no common haplotype. (f) The microsatellite data refined the OAS candidate interval to Chr14:69.89–71.26 Mb which is shown diagrammatically with the 22 annotated genes that were sequenced in this study (UCSC assembly GRCh37).

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insertions resulting in a frameshift. Two different missense changes were identified, both are in the C-terminal region of the second thyroglobulin type I domain of SMOC-1 (Figure 2b). No mutations were identified in sequence analysis of the SMOC1 coding region in 190 healthy blood donors. No mutations in SMOC1 could be identified in 6 of the 14 families. There were no obvious phenotypic differences between the cases with and without SMOC1 mutations; all have classical OAS. SNP and microsatellite data on two of the six families without detectable mutations showed large regions of homozygosity across the 14q24 region containing SMOC1. This suggests that we have missed a mutation within the transcription unit or that there may be a regulatory mutation impairing SMOC1 transcription. In the four remaining families no plausible locus could be identified using homozygosity mapping.

Expression Analysis of Smoc1
As a first step to determining the likely developmental role of SMOC1 we undertook developmental expression analysis of the orthologous gene Smoc1 in whole mouse embryos. WISH analysis with an antisense riboprobe specific to Smoc1 and optical projection tomography (OPT) were used to create a 3D representation of both the anatomy and colorimetric staining. We found site- and stage-specific expression of Smoc1 at all stages examined (Figure 3a–3b; Video S1; Video S2). Staining in the limb bud was particularly interesting with expression seen first in
### Table 1. Clinical features and mutations in affected individuals with Ophthalmo-Acromelic Syndrome.

<table>
<thead>
<tr>
<th>FAMILY</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected Case</td>
<td>R14A9</td>
<td>R14C12</td>
<td>R1SH11</td>
<td>R23H3</td>
<td>17715</td>
<td>18177</td>
<td>15124</td>
<td>20384</td>
</tr>
<tr>
<td>Age assessed</td>
<td>13 Yr</td>
<td>9 Yr</td>
<td>6 Mo</td>
<td>7 Mo</td>
<td>18 Yr</td>
<td>40 Yr</td>
<td>11 Yr</td>
<td>14 Yr</td>
</tr>
<tr>
<td>Sex (Ratio)</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
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<td>Lebanese</td>
<td>Lebanese</td>
<td>Gypsy</td>
<td>Pakistani</td>
<td>Turkish</td>
<td>Calabrian</td>
<td>Puerto Rican</td>
<td>Turkish Sicilian</td>
</tr>
<tr>
<td>Consanguinity</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ocular defect</td>
<td>None</td>
<td>BA</td>
<td>BA</td>
<td>UA</td>
<td>BA</td>
<td>BA</td>
<td>BA</td>
<td>BA</td>
</tr>
<tr>
<td>Optic nerve/tract/chiasm present on scan?</td>
<td>Unknown</td>
<td>Remnants of optic nerve</td>
<td>Unknown</td>
<td>Absent</td>
<td>Absent</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Absent</td>
</tr>
<tr>
<td>Upper limb</td>
<td>cut synd</td>
<td>cut synd, hypoppl 5th finger</td>
<td>--</td>
<td>bilat 4/5 metacarpal fusion</td>
<td>--</td>
<td>bilat 4/5 metacarpal fusion, camptodactyly</td>
<td>bilat 4/5 metacarpal fusion</td>
<td>contractures of fingers</td>
</tr>
<tr>
<td>Lower Limb</td>
<td>cut synd 3–5</td>
<td>bilat missing postaxial ray cut synd 2–4 right, 2/3 left</td>
<td>bilat missing postaxial ray</td>
<td>bilat missing postaxial ray</td>
<td>bilat missing postaxial ray</td>
<td>bilat missing postaxial ray &amp; cut synd 2/3</td>
<td>Right fusion 4/5 metatarsal &amp; phalanx, cut synd 2–5</td>
<td>short 5th metacarpals</td>
</tr>
<tr>
<td>Other Limb/Skeletal Defect</td>
<td>Bowed tibia</td>
<td>Contractures of elbows, Coxa valga</td>
<td>TEV, bowed tibia</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Craniofacial</td>
<td>--</td>
<td>Cleft palate</td>
<td>--</td>
<td>Pierre Robin Sequence</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Other defects</td>
<td>Horseshoe kidney, hypospadias</td>
<td>Horseshoe kidney, mental retardation</td>
<td>Horseshoe kidney</td>
<td>Severe mental retardation, epilepsy, cryptorchidism</td>
<td>Severe mental retardation</td>
<td>Horseshoe kidney</td>
<td>Severe mental retardation</td>
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<td>Coding change</td>
<td>c.911delG</td>
<td>c.911delG</td>
<td>c.395dupA</td>
<td>c.212C&gt;T</td>
<td>c.1109C&gt;T</td>
<td>c.224C&gt;T</td>
<td>c.848C&gt;A</td>
<td>c.848C&gt;A</td>
</tr>
<tr>
<td>exon</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>11</td>
<td>2</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
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<td>Frameshift 2</td>
<td>Frameshift 2</td>
<td>Frameshift 1</td>
<td>Nonsense 1</td>
<td>Nonsense 2</td>
<td>Nonsense 3</td>
<td>Nonsense 4</td>
<td>Missense 1</td>
</tr>
<tr>
<td>IBD</td>
<td>14q24.2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Yr = years; Mo = months; F = Female; M = Male; UA/BA = Unilateral/Bilateral anophthalmia; IBD = Identity by Descent; Cut synd = cutaneous syndactyly; TEV = talipes equinovarus; 2/3 = second and third digits; 3–5 = third, fourth and fifth digits; 2–4 = second third and fourth digits; 2–5 = second, third, fourth and fifth digits; 4/5 = fourth and fifth digits; bilat = bilateral. 

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the very early limb bud anlage from 9.5 dpc (Figure 3a). At 10.5 dpc the limb expression distinctly localised to both the dorsal and ventral surfaces of the forelimb, but was predominantly dorsal in the hindlimb bud (Figure 3b, Figure S1). Strong expression was seen in the developing pharyngeal arches and the frontonasal region with low-level expression in the ectoderm overlying the developing optic vesicle (Figure S1). Using WISH and OPT no clear expression of Smoc1 was detected in the optic vesicle itself. There was clear expression in the developing somites at E9.5 and E10.5 (Figure 3a, 3b). At E9.5 there was also staining in the hindbrain (Figure 3a) and at E10.5 strong staining in the dorsal neural tube (Figure 3b).

**Targeted Mutation in Mouse Smoc1**

In order to determine if the non-redundant developmental role of SMOC1 is evolutionarily conserved we obtained mice with a targeted Pre-Conditional mutation in Smoc1 containing a LacZ reporter allele created as part of the EUCOMM project [43]. The integrated location and the details of the targeting construct are shown in Figure S1d. The Smoc1<sup>tm1a/tm1a</sup> mice have ~10% of wild-type levels of Smoc1 mRNA during development (Figure S1f), presumably as a result of splicing across the gene-trap insertion.

Using the LacZ reporter in targeted animals, we were able to further assess the developmental expression of Smoc1 (Figure 3c, 3d, and 3g). Spatial and temporal expression data were similar between Smoc1<sup>+/+</sup> and Smoc1<sup>tm1a/tm1a</sup> genotypes, but with less intense staining in Smoc1<sup>+/+</sup> mice (data not shown). The X-gal staining at E10.5 of both whole mount embryos and cryosections was consistent with the wild type OPT data. Cryosections through the maxillary-mandibular hinge region of the 1<sup>st</sup> pharyngeal (branchial) arch at E10.5 showed striking regional specificity of gene activation (Figure 3g). The expression was present within the mesenchyme or neural crest of both components of the first arch but is most intense in the sub-epithelial mesenchyme within the hinge region. Sharp boundaries of expression are evident in the subepithelial mesenchyme (Figure 3g). The only major difference between the riboprobe WISH analysis and the X-gal staining was the strong expression identified within the developing eye seen in the latter but not the former (Figure 3c). This staining was particularly strong at E10.5 within the ventral aspect of the developing optic nerve (Figure 3d).

Further phenotypic analysis of mutant animals showed that limb malformations were present in a high proportion of embryos and pups (Table 2). Oligodactyly (+/- fibular agenesis) or osseous syndactyly of both hindlimbs was present in 11/12 of Smoc1<sup>tm1a/tm1a</sup> animals (Figure 3j–3k and Figure S1). Smoc1<sup>tm1a/tm1a</sup> embryos and neonates were also smaller than littermates but did not appear to have any other major malformations (Figure S2). To date all Smoc1<sup>tm1a/tm1a</sup> animals allowed to litter have died at or soon after birth. A proportion of this perinatal lethality is likely related to the presence of cleft palate in a significant proportion (4 of 12) of the Smoc1<sup>tm1a/tm1a</sup> animals (Figure 3i). However it is not clear what accounts for the mortality in the non-cleft cases. Interestingly, unexplained perinatal lethality is reported in human OAS cases, being predominantly iris and retinal coloboma. This may relate to the difference in mutation type between the mouse and human cases: most human mutations are apparently null, whereas the mouse were less severe than those seen in human OAS cases, being predominantly iris and retinal coloboma. This may relate to the difference in mutation type between the mouse and human cases: most human mutations are apparently null, whereas the mouse line had 10% of normal Smoc1 transcript levels present, most likely due to splicing across the gene trap. This level of Smoc1 function could partially rescue the ocular phenotype in the mice. Analysis of the phenotype associated with a null allele in mice that was detected in the C-terminal region of thyroglobulin which appear to function as peptidase inhibitors, specifically inhibitors of cysteine cathepsins [44,45]. Neither Arg278 or Thr283 show identity at the equivalent residue within the first Tg1 in human SMOC1. Interestingly, Cathepsin H has been shown to be involved in Bmp4 degradation during lung development [46]. It is also possible that a mutation resulting in constitutive activation of the target peptidase could phenocopy SMOC1 mutations.

The expression analysis and targeted partial gene inactivation in mouse embryos strongly supports the critical and non-redundant developmental role of SMOC1 suggested by the human genetic analysis and that this role is conserved across evolutionary time. The hindlimb phenotype in Smoc1<sup>tm1a/tm1a</sup> homozygous mice was very similar to the lower limb phenotype in human OAS cases. The combination of osseous syndactyly and oligodactyly suggest that the mechanisms controlling digit number within the limb bud are significantly impaired. The control of digit number is critically dependent on correct dosage of sonic hedgehog (Shh) [47,48] and BMP4 & BMP7 signaling proteins [49,50]. A significant proportion of the Smoc1<sup>tm1a/tm1a</sup> homozygous mice have cleft palate, a feature common to human OAS and consistent with the high level of expression of Smoc1 that was detected in the developing first pharyngeal arch. The eye malformations seen in the mouse were less severe than those seen in human OAS cases, being primarily iris and retinal coloboma. This may relate to the difference in mutation type between the mouse and human cases: most human mutations are apparently null, whereas the mouse line had 10% of normal Smoc1 transcript levels present, most likely due to splicing across the gene trap. This level of Smoc1 function could partially rescue the ocular phenotype in the mice. Analysis of the phenotype associated with a null allele in mice that we are currently making will answer this question. We will also screen a cohort of human patients to determine if hypomorphic mutations in SMOC1 may cause coloboma.

The formation of precise gradients of diffusible ligands is required during embryogenesis both for patterning - the formation of complex tissue structures from apparently homogenous populations of multipotent cells - and to control growth to achieve correct final organ size with appropriate symmetry of paired structures. BMPs represent an important class of diffusible ligands with roles in both patterning and control of organ size [51,52].

**Discussion**

We report compelling evidence that loss-of-function mutations in SMOC1 cause a significant subset of OAS cases. No non-synonymous changes were found in any region of the SMOC1 coding region in the 190 control individuals that were fully sequenced but we identified eight mutations within OAS families that are all different and homozygous. Six of these mutations (four nonsense and two frame-shift) are very likely to result in severe or complete abrogation of protein function. The two missense mutations (p.Arg278Cys & p Thr283Asn) we have identified are both located in the second thyroglobulin type-1 domain (Tg1) of SMOC-1. Tg1 domains are cysteine-rich motifs that were first identified in the C-terminal region of thyroglobulin which appear to function as peptidase inhibitors, specifically inhibitors of cysteine cathepsins [44,45]. Neither Arg278 or Thr283 show identity at the equivalent residue within the first Tg1 in human SMOC-1. Interestingly, Cathepsin H has been shown to be involved in Bmp4 degradation during lung development [46]. It is also possible that a mutation resulting in constitutive activation of the target peptidase could phenocopy SMOC1 mutations.

The expression analysis and targeted partial gene inactivation in mouse embryos strongly supports the critical and non-redundant developmental role of SMOC-1 suggested by the human genetic analysis and that this role is conserved across evolutionary time. The hindlimb phenotype in Smoc1<sup>tm1a/tm1a</sup> homozygous mice was very similar to the lower limb phenotype in human OAS cases. The combination of osseous syndactyly and oligodactyly suggest that the mechanisms controlling digit number within the limb bud are significantly impaired. The control of digit number is critically dependent on correct dosage of sonic hedgehog (Shh) [47,48] and BMP4 & BMP7 signaling proteins [49,50]. A significant proportion of the Smoc1<sup>tm1a/tm1a</sup> homozygous mice have cleft palate, a feature common to human OAS and consistent with the high level of expression of Smoc1 that was detected in the developing first pharyngeal arch. The eye malformations seen in the mouse were less severe than those seen in human OAS cases, being predominantly iris and retinal coloboma. This may relate to the difference in mutation type between the mouse and human cases: most human mutations are apparently null, whereas the mouse line had 10% of normal Smoc1 transcript levels present, most likely due to splicing across the gene trap. This level of Smoc1 function could partially rescue the ocular phenotype in the mice. Analysis of the phenotype associated with a null allele in mice that we are currently making will answer this question. We will also screen a cohort of human patients to determine if hypomorphic mutations in SMOC1 may cause coloboma.

The formation of precise gradients of diffusible ligands is required during embryogenesis both for patterning - the formation of complex tissue structures from apparently homogenous populations of multipotent cells - and to control growth to achieve correct final organ size with appropriate symmetry of paired structures. BMPs represent an important class of diffusible ligands with roles in both patterning and control of organ size [51,52].
Figure 3. A targeted \textit{Smoc1} mutation caused an ophthalmo-acromelic-like phenotype in mice. (a) OPT representation of wild type (WT) \textit{Smoc1} expression at embryonic day (E) 9.5 (green represents \textit{Smoc1} expression); \textit{Smoc1} is expressed in the pharyngeal (branchial) arches (BA), the
rostral neural tube (NT), in the anlage of the forelimbs (FL), the fronto-nasal region (FN), and in the somites (S). (b) At E10.5, expression is maintained in the branchial arches, somites and in the frontonal nasal processes, as well as extending caudally in the neural tube. (c) In E10.5 Smoc1 mutant embryos, β-galactosidase activity was observed in tissues consistent with the OPT analysis of WT Smoc1 expression: in the dorsal hindlimbs; in the medial regions of dorsal and ventral forelimbs, branchial arches, in the frontonasal processes, and in the somites. In addition, strong signal was observed in the eye region (scale bar = 500 µm). (d) X-gal stained sagittal sections of a representative E10.5 Smoc1 mutant embryo in the developing eye showing that expression was restricted to ventral regions of the presumptive optic stalk (POS). (e) Examination of optic nerve morphology identified an extension of the RPE into the dorsal optic nerve in mutant animals compared to control. (f) Photographs of Smoc1 mutant eye showing an optic fissure closure defect (arrowhead) consistent with coloboma (scale bar = 100 µm). (g) Expression in the 1st branchial arch mesenchyme was distributed in proximal regions and absent from distal areas, with positive signal also seen in the epithelial cells at the hinge region between maxillary (MX) and mandibular (MD) components (arrowheads) (scale bar = 100 µm). (h) X-gal stained sagittal sections of sections through E14.5 heads showing a failure in palatal shelf (PS) fusion in the developing palate in the Smoc1 mutant embryo (i) compared to the fully fused WT littermate (h). (j,k) Surface rendered visualization of OPT reconstructions of hindlimbs at E14.5. (j) WT embryo with normal arrangement of 5 digits in the hindlimb whereas the Smoc1 mutant littermate (k) had hindlimb odontogically affecting the axial digits, with only 4 digits present. (l) Skeletal preparation of P0 Smoc1 mutant hindlimb with osseous fusion of the phalanges of digits 3–4 (red arrow).

Much of what we know about the formation, maintenance and function of BMP gradients derived from studies of *Drosophila* Decapentaplegic (Dpp) [53]. BMPs are considered to be the mammalian paralogs of Dpp. SMOC-1 and its close homolog SMOC-2 appear to be the mammalian paralogs of *Drosophila* Pentagone (Pent) [54]. Pent functions as an *in vivo* antagonist of Dpp by preventing receptor endocytosis close to its source thus allowing gradients to form over wider distances within the wing imaginal discs. Lack of Pent results in a very steep and narrow gradient of Dpp signaling, which in turn causes a relative deficiency of Dpp further from the source. In *Drosophila* the control of cell proliferation within the wing imaginal disc is dependent on Dpp signaling [55]. In the chick it has been shown that the level of cell proliferation within the limb bud must be precisely specified to in order to result in sufficient antero-posterior expansion to form the correct digit number [48]. If a similar mechanism exists in vertebrates then it may be that SMOC1/Smoc1 mutations could cause odontogically by altering the BMP gradient within the limb bud and thus alter anteroposterior expansion. Although the molecular basis of the developmental pathology associated with OAS remains to be elucidated, support for SMOC-1 mediated BMP antagonism as a component is provided by human and mouse genetic data that indicate the importance of BMP signalling in both limb [56,57] and eye [8,58,59] development. Interestingly, heterozygous BMP4 signalling in both limb [56,57] and eye [8,58,59] has been associated with microphthalmia, microcornea, coloboma, retinal dystrophy, and tilted optic disc [8]. In addition, BMP4 mutations are also associated with digital anomalies (polydactyly) and cleft lip/palate [60]. The partial overlap between the OAS phenotype and the phenotypes associated with BMP4 disruptions may reflect a functional relationship between SMOC-1 and BMP4.

Following submission of this paper two other groups have identified SMOC1 mutations as a cause of OAS [10,61]. One group studied five affected individuals from four unrelated OAS families [61]. They identified the locus on 14q24 and then found SMOC1 mutations in three out of four of the families. Interestingly these were the same families in whom linkage to 10p11.23 had been previously reported by the same group [17]. The SMOC1 mutations were all homozygous and plausibly loss of function (one nonsense and two 3’ splice site mutations). This group also reported the phenotype in homozygous mice with Sleeping beauty transposon-induced gene trap mutations of *Smoc1*. The expression analysis and limb phenotype of the mice are very similar to that reported here. Their homozygous mice also showed unexplained early lethality. Interestingly their mice had small eyes but they do not report coloboma. The optic nerves were shown to be significantly narrower than non-homozygous animals and they also showed extension of the RPE into the optic nerve. The second paper reports linkage to 14q24 and identification of a 3’ splice site mutation in *SMOC1* in a single multiplex family with OAS [10]. This group also reports developmental expression analysis of the orthologous gene, *smoc1*, in zebrafish embryos. Expression was evident in the brain, choroid fissure and pharyngeal arches. “Knock-down” experiments using a morpholino targeted to *smoc1* resulted in microphthalmia and brain abnormalities in the injected embryos. Taken together these papers strongly support loss of *SMOC-1* function as the major cause of OAS and that this protein has a conserved non-redundant function during ocular and limb development.

Finally, in six families with typical OAS we could not identify SMOC1 mutations, including the original family described by Waardenburg in 1935 [9]. In two of these six families, affected individuals show homozgyosity over the region of 14q24.2 suggesting that we have significant limitations in our current SMOC1 mutation analysis strategy. However, four families showed no apparent autozygosity around *SMOC1*, suggesting the likely existence of other OAS loci. Identifying causative genes at other loci is likely to help elucidate the embryopathology and is an active area of our future work.

### Materials and Methods

**Patient Recruitment and Ethics Approval**

All patient related work was carried out with full written consent of the families. Details of the mutation positive cases are provided in Table 1. The informed consent process was reviewed and approved following consideration by national ethical committee systems in the UK and the Netherlands. Several of the cases have been previously published. [16,19,22,23,26]

### Mapping and Linkage Analysis

Patient, parental and unaffected sib genomic DNA samples were run on Affymetrix GeneChip Human Mapping 10K Arrays (Xba131) and autozygosity mapping was performed using

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**Table 2. Phenotypes identified in Smoc1-targeted mice.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Smoc1+/tm1a/tm1a</th>
<th>Smoc1+/tm1a</th>
<th>Smoc1+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total analysed</td>
<td>12 (26%)</td>
<td>21 (45.7%)</td>
<td>13 (28.3%)</td>
</tr>
<tr>
<td>Eye phenotype</td>
<td>5 (n=9*)</td>
<td>55.6%</td>
<td>0</td>
</tr>
<tr>
<td>Hind limb phenotype</td>
<td>11 (91.7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cleft palate</td>
<td>3 (33.3%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Missing fibula</td>
<td>6 (50%)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*3 confirmed Smoc1+/tm1a animals were embedded in paraffin before eye phenotype was established.

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SMOC1 Mutations Cause Ophthalmo-Acromelic Syndrome

PCR and Sequence Analysis

Genomic DNA samples were Whole Genome Amplified (WGA) using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) according to the manufacturer’s guidelines. For PCR, 50 ng WGA template DNA was amplified with 0.2 μM primers and 2x Custom ReddyMix (Thermo Scientific) in H2O to 25 μL. Cycle conditions: 95°C×3 minutes; and 35×cycles of 95°C×1 minute, 56°C×45 seconds, and 72°C×1 minute; followed by a single step of 72°C×10 minutes. All mutations were confirmed by resequencing using non-WGA genomic DNA with identical reaction conditions. Sequencing was performed using Applied Biosystems 3130/3170 Genetic Analysers.

Smoc1 Targeted Mouse

The Smoc1 pre-conditional knockout mouse (EUCOMM Project 48154; strain C57BL/6N-A-Smoc1tm1a(EUCOMM)WTSI, referred to as Smoc1<sup>fl/fl</sup> in this manuscript) was generated by the International knockOut Mouse Consortium (IKMC) under UK Home Office Project License 60/3785 (IJ Jackson, MRC Human Genetics Unit). Details of the allele and targeting strategy can be found at: http://www.eucomm.org/htgt/report/gene_report?project_id = 48154 and in Figure S1. Genotypes were confirmed using the Smoc1 forward primer 5’-GGTTCTGCTGAGGTAGCTTG- 3’ (positioned at mChr12:82,236,250 bp) and Smoc1 reverse primer 5’-CCTCATCTCCAACTTCTCTC-3’ (positioned at mChr12:82,236,907 bp) which flank the targeted endogenous exon and produce a wild-type PCR amplicon of 658 bp. Adding the targeting cassette specific primer 5’-TTAGTCCCAACCCCTTCTCTC-3’ to PCR mixes as multiplex reactions produced a targeted-allele specific amplicon of 250 bp. Ear-tip DNA was extracted by incubating in 50 μL of 25 mM NaOH 0.2 mM EDTA solution at 95°C for 1 hour, then adding 50 μL of 40 mM Trizma. 1 μL of template was used for PCR reactions with 0.2 μM primers and 2x Custom ReddyMix (Thermo Scientific) in H2O to 50 μL. Cycle conditions: 95°C×3 minutes; and 31×cycles of 95°C×45 seconds, 56°C×40 seconds, and 72°C×1 minute; followed by a single step of 72°C×10 minutes. Products were run on 1% TBE agarose gels. For qRT-PCR, mouse hind limbs at stage 10.5 dpc were dissected and mRNA extracted using TRIzol (Invitrogen) according to the manufactures instructions. Mice were genotyped by PCR using genomic DNA as described above with WT and Smoc1<sup>fl/fl</sup> samples carried on for testing. Samples were DNaseI treated and then cycled using Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems) with the following conditions: 48°C×30 minutes, 95°C×10 minutes, followed by 40 cycles of 95°C×15 seconds and 60°C×1 minute. Each 10 μl reaction contained 0.08 μl of RT Enzyme Mix; 5 μl of RT-PCR Mix; 1 μl RNA sample and 1.92 μl ddH2O, with the

Table 3. Microsatellite repeat marker PCR and primer properties.

<table>
<thead>
<tr>
<th>Microsatellite (Chr14: bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Approx size (Repeat type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D14565790 (67906158–67906384)</td>
<td>CTGACATTTGAGAAAAGG</td>
<td>GGTACTGCTGAGGTCTGGA</td>
<td>320 bp (tetra)</td>
</tr>
<tr>
<td>D14565889 (68892553–68892831)</td>
<td>TCTCGAGCACTGAGAAGAGGA</td>
<td>CCCAGGCAAAAGTGAGA</td>
<td>300 bp (tetra)</td>
</tr>
<tr>
<td>D14565921 (69217056)</td>
<td>CAGCTACTTCCACCCCTTC</td>
<td>ACCATCTGGCTCAAGAAC</td>
<td>200 bp (tetra)</td>
</tr>
<tr>
<td>D14565969 (67949449)</td>
<td>CGCCCCTTGAAATGATTTT</td>
<td>GATGACCACCTGCTACCA</td>
<td>250 bp (tetra)</td>
</tr>
<tr>
<td>D145258 (70583116–70583186)</td>
<td>TCATCTGCTGGAGAAG</td>
<td>CTAAATAGTGCGGAGCTG</td>
<td>170 bp (di)</td>
</tr>
<tr>
<td>D1456985 (70768661–70768943)</td>
<td>CCTGAAGAAACCCAGTCTCC</td>
<td>GGCAGAAAATCTGCTAGAAG</td>
<td>285 bp (tetra)</td>
</tr>
<tr>
<td>D1457023 (71168626–71168625)</td>
<td>ACGGTTTACCGGACAGGAAG</td>
<td>GCAGTACAGGAGGAGGATG</td>
<td>330 bp (tri)</td>
</tr>
<tr>
<td>D1457033 (71260458)</td>
<td>TGAGCCCGAGATTCAGAC</td>
<td>AGTCCTCCGACATGGCACCAG</td>
<td>270 bp (tetra)</td>
</tr>
</tbody>
</table>

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inclusion of either Smoc1 forward 5'-GGATGTTCTCCCTTGAACAGGG-3' and reverse 5'- TCAGCTCCTCGGAACAGCAGG-3' primers or Hprt forward 5'- CTGTTGAAAGGACCTCTGCG-3' and reverse 5'-CAAGGCTGATGACAGCACAA-3' primers. Each reaction was performed in triplicate in optical reaction plates (384-well, Applied Biosystems), and RNA samples were also run without RT Enzyme Mix for negative controls with the same reaction conditions.

**Histological and Histochemical Analysis**

Embryos were fixed in 4% PFA; dehydrated through graded alcohol series and xylene; and embedded in paraffin. Microtome sections were cut at 6 μm and rehydrated through ethanol series and stained with haematoxylin and cosin. For skeletal preparations the animals were dehydrated in 95% ethanol for 24 hours; followed by 72 hours in 100% acetone; 3 days in stain solution (1 part 0.3% alcian blue in 70% ethanol; 1 part 0.1% alizarin red in 95% ethanol; and 1 part acetic acid, in 17 parts 70% ethanol); followed by 3 days clearing in 1% KOH; 3 days in 1% KOH/30% glycerol; and two 24 hour periods in 1% KOH/50% glycerol; in 1% KOH/70% glycerol and were analysed in 100% glycerol. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed as follows; targeted mouse embryos were dissected at 10.5 dpc and rinsed in PBS, then fixed for 1 hour in 4% PFA at 4°C, rinsed again in PBS and then washed for 3x 20 minutes in detergent wash (2 mM MgCl2, 0.1% Sodium Deoxycholate, 0.02% NP-40 [Igepal CA 650]), in PBS). Detection was performed in β-galactosidase stain (0.085% NaCl, 5 mM K3 [Fe(CN)6], 5 mM K4 [Fe(CN)6], 200 μl/ml X-gal [Promega], in detergent wash), followed by a brief final stain fixation in 4% PFA for 30 minutes for cryosection analysis. Embryos were dissected and fixed as above, then incubated overnight in 20% sucrose/PBS at 4°C, transferred into OCT solution and frozen embedded on dry ice. Sections of 25 μm thickness were cut at −20°C, air dried and rinsed briefly in PBS. X-gal staining was then performed as described above.

**Optical Projection Tomography**

For optical projection tomography (OPT) analysis In Situ stained embryos were mounted in 1% agarose, dehydrated in methanol and then cleared overnight in BABB solution (1 part Benzyl Alcohol: 2 parts Benzyl Benzoate). Samples were then imaged using a Bioptons OPT Scanner 3001 (Bioptions, UK) using brightfield analysis to detect tissue autofluorescence for capture of anatomical and signal data (wavelengths: excitation at 425 nm, emission: 475 nm). The resulting data were reconstructed using Bioptions proprietary software (Bioptions, MRC Technology, Edinburgh, UK), then automatically thresholded to remove background and finally merged into a single 3D image output using Bioptions Viewer software.

**Supporting Information**

**Figure S1** Expression of Smoc1 in the limb buds and eye, and phenotype in Smoc1tm1a/tm1a/tm1a limbs. (a–c) Magnified images and digital section of the same OPT reconstruction of a wild-type E10.5 embryo shown in Figure 2 with green staining representing Smoc1 expression. (a) Smoc1 expression is seen in both the dorsal and ventral surface of the forelimb buds. (b) In the hindlimb expression is predominantly dorsal and with small region of ventral expression. (c) Digital section showing symmetrical low-level expression of Smoc1 in the surface ectoderm overlaying the optic vesicles (OV). (d) Diagram illustrating the gene targeting for the EUCOMM pre-conditional Smoc1 knockout mouse at the genomic level (top) with the relative positions of RT-PCR primers indicated. Targeting cassette (bottom) is specific for exon 4 of Smoc1. Positions of genotyping PCR primers are indicated (GT-F & -R2, blue, are endogenous-gene specific, GT-R1 is cassette-specific). Note that the targeting cassette has the β-galactosidase ORF inserted downstream of an EN2 splice acceptor site (EN2 SA) and internal ribosomal entry site (IREs), allowing for reporter expression in successfully Smoc1-targeted animals (pA is Poly(A) tail). The cassette also features FRT and Cre-recombinase (loxP) sequences for future gene manipulation. Ensemble exon identifiers are given. A table of the phenotypes associated with the targeted allele is available in Table 2 of the main manuscript. (e) Agarose gel indicating each allele identified by genotyping PCR primers. A = phenotypically wild type animal, B = littermate with hindlimb oligodactyly, Wt = wild type unrelated mouse DNA sample. Primer pair 1 (GT-F & GT-R2) amplified wild type allele only, Primer pair 2 (GT-F & GT-R1) amplified the targeted allele only, multiplex reaction (GT-F & GT-R2 & GT-R1) identified both WT and mutant alleles and revealed animal B as a Smoc1 homozygote (Smoc1tm1a/tm1a) and A as a heterozygote (Smoc1tm1a/tm1a). Multiplex reactions were then used as standard for genotyping Smoc1 status in analysed animals. (f) Quantitative RT-PCR analysis of dissected hindlimb tissue from E10.5 wild type and homozygote animals revealed normal organization and retinal cell-layer lamination in Smoc1tm1a/tm1a mutant eyes. Abbreviations: c, cornea; ln, lens; r, retina; gc, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigmented epithelium (Scale bars: a & b = 250 μm; c, d & e = 100 μm). (g) Maximum width measurements were taken for optic nerves (on) of Smoc1tm1a/tm1a mutants (n = 17) and controls (n = 17) from paraffin-embedded sections. This plot shows the significantly smaller mean diameter of the optic nerve in mutants compared to control animals with the error bars representing the non-overlapping of 95% confidence limits; 125.7 μm (118.2–133.3) and 147.0 μm (139.6–154.5) respectively. (h) Transverse digital sections from OPT scans of E14.5 WT and Smoc1tm1a/tm1a embryos showing an apparently normal size and shape of developing kidneys (arrows). Scale bar = 1 mm. (i) Whole mount X-gal stained Smoc1tm1a/tm1a embryo at E14.5. This embryo was dissected to reveal the developing kidneys. No X-gal positive cells can be seen in the kidneys (LK, left kidney; RK, right kidney) or bladder (B). Stain is clearly seen in both adrenal glands (arrowheads) and in tissue adjacent to the developing vertebral (V). Scale bar = 500 μm. (j) Sagittal cryosection of 25 μm thickness counterstained with eosin showing strong X-gal staining in the tissue between the developing vertebral bodies (VB). (k) E14.5 stage left kidney seen in i. No stain positive cells can be seen within the kidney (KID) but a cluster of positively staining cells are seen along the posterior surface of the developing kidney. Scale bar = 100 μm.

**Figure S2** Phenotypic analysis and X-gal staining of Smoc1 mutant animals. (a & b) Low power magnification of H&E stained eyes revealed that mutant eyes were grossly normal but that overall eye size was reduced. (c & d) Higher magnification analysis revealed normal organization and retinal cell-layer lamination in Smoc1tm1a/tm1a mutant eyes. Abbreviations: c, cornea; ln, lens; r, retina; gc, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigmented epithelium (Scale bars: a & b = 250 μm; c, d & e = 100 μm). (e) Maximum width measurements were taken for optic nerves (on) of Smoc1tm1a/tm1a mutants (n = 17) and controls (n = 17) from paraffin-embedded sections. This plot shows the significantly smaller mean diameter of the optic nerve in mutants compared to control animals with the error bars representing the non-overlapping of 95% confidence limits; 125.7 μm (118.2–133.3) and 147.0 μm (139.6–154.5) respectively. (f) Transverse digital sections from OPT scans of E14.5 WT and Smoc1tm1a/tm1a embryos showing an apparently normal size and shape of developing kidneys (arrows). Scale bar = 1 mm. (g) Whole mount X-gal stained Smoc1tm1a/tm1a embryo at E14.5. This embryo was dissected to reveal the developing kidneys. No X-gal positive cells can be seen in the kidneys (LK, left kidney; RK, right kidney) or bladder (B). Stain is clearly seen in both adrenal glands (arrowheads) and in tissue adjacent to the developing vertebral (V). Scale bar = 500 μm. (h) Sagittal cryosection of 25 μm thickness counterstained with eosin showing strong X-gal staining in the tissue between the developing vertebral bodies (VB). (i) E14.5 stage left kidney seen in i. No stain positive cells can be seen within the kidney (KID) but a cluster of positively staining cells are seen within the adrenal (ADR). Scale bar = 100 μm.
.position of identical amino acid residues across all sequences is given by pink shading. The gray shading indicates the conservation of the positions in the Tg1-2 affected by the missense mutations and the nature and position of the mutations is shown in red text below. Key: Tg1 = Thyroglobulin type-1 domain; Tg1-1 = first Tg1 in hPep; Tg1-2 = second Tg1 in hPep; hSMOC1 = human SMOC1; mSmoc1 = mouse Smoc1; xtSmoc1 = Xenopus tropicalis Smoc1; dmPent = Drosophila melanogaster Pentagone protein; Q9hF8 etc are UniProt accession numbers.

(FIG)

Video S1 OPT analysis of Smoc1 expression in wild-type E9.5 mouse embryo. (MPG)

Video S2 OPT analysis of Smoc1 expression in wild-type E10.5 mouse embryo. (MPG)

Dataset S1 UCSC custom track for the hg18 genome build that represents the distribution of homozygous regions in the individuals affected with Ophthalmo-acromelic syndrome in our study. The homozygous regions are defined as the genomic coordinates encompassing 20 contiguous homozygous genotypic calls. The regions surrounding SMOC1 shows the multiple overlapping homozygous regions in unrelated families and provides a graphical representation of the linkage of OAS to this region of chromosome 14.

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We would like to express our thanks to the children and their families who gave their time and biological samples to make this research possible. We thank Drs Dariusz Michna and Cesare Rossi for help with Family 1 and Family 2 respectively. Dr. Nurten Arkarsu generously contributed genetic and clinical information on Family 7. We also acknowledge the help with sequencing from MRC HGU Technical Support.

Author Contributions
Conceived and designed the experiments: JR DW Hb-B DRF. Performed the experiments: JR LY-G ExB JK RLM PB HM FP KS DS. Analyzed the data: JR ExB LB Hb-B DRF. Contributed reagents/materials/analysis tools: IIJ DW AK SZ NE RP AS TM AB ASL AG BKB AM CNS RL SBDS. Wrote the paper: JR Hb-B DRF.

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Author Contributions
Conceived and designed the experiments: JR DW Hb-B DRF. Performed the experiments: JR LY-G ExB JK RLM PB HM FP KS DS. Analyzed the data: JR ExB LB Hb-B DRF. Contributed reagents/materials/analysis tools: IIJ DW AK SZ NE RP AS TM AB ASL AG BKB AM CNS RL SBDS. Wrote the paper: JR Hb-B DRF.

References