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

Joe Rainger1, Ellen van Beusekom2, Jacqueline K. Ramsay3, Lisa McKe4, Lihadh Al-Gazali5, Rosanna Pallotta4, Anita Saponari6, Peter Brannen1, Malcolm Fisher1, Harris Morrison1, Louise Bicknell1, Philippe Gautier7, Paul Perry7, Kishan Sokhi8,15, David Sexton1, Tanya M. Bardakjian6, Adele S. Schneider6, Nursel Elcioglu8, Rainer Koenig9, Andre Mégarbané10, C. Nur Semerci11, Ayeshra Khan12, Saemah Zafar12, Raoul Hennekam13, Sérgio B. Sousa14, Lina Ramos14, Livia Garavelli15, Andrea Superti Furga16, Anita Wischmeijer15, Ian J. Jackson1, Gabriele Gillesen-Kaesbach17, Han G. Brunner2, Dagmar Wieczorek18, Hans van Bokhoven2, David R. FitzPatrick1*

1 Medical Research Council Human Genetics Unit, Institute of Genetics and Molecular Medicine, Western General Hospital, Edinburgh, United Kingdom, 2 Department of Human Genetics, Institute for Genetic and Metabolic Disorders and Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, 3 Departments of Paediatrics, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain, United Arab Emirates, 4 Regional Service for Diagnosis, Prevention, and Care of Birth Defects, Department of Medicine and Aging Sciences, Section of Preventive and Social Pediatrics, G. D’Annunzio University, Chieti, Italy, 5 Department of Orthopaedics and Trauma, University of Edinburgh, Royal Infirmary of Edinburgh, Little France, Edinburgh, United Kingdom, 6 Division of Genetics, Department of Pediatrics, Albert Einstein Medical Center, Philadelphia, Pennsylvania, United States of America, 7 Department of Pediatric Genetics, Marmara University Hospital, Istanbul, Turkey, 8 Ege University, Medical Faculty, Department of Pediatrics, Izmir, Turkey, 9 Institut für Humangenetik der Johann Wolfgang Goethe Universität, Frankfurt, Germany, 10 Unité de Génétique Médicale, Faculté de Médecine, Université Saint Joseph, Beirut, Lebanon, 11 Department of Medical Genetics, School of Medicine, Pammukale University, Denizli, Turkey, 12 Al-Shifa Trust Eye Hospital, Rawalpindi, Pakistan, 13 Department of Pediatrics and Department of Translational Genetics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands, 14 Serviço Genética Médica, Hospital Pediátrico de Coimbra, Portugal, 15 Department of Clinical Genetics, S. Maria Nuova Hospital, Reggio Emilia, Italy, 16 Department of Pediatrics, University of Lausanne, Switzerland, 17 Institut für Humangenetik, Universität zu Lübeck, Lübeck, Germany, 18 Institut für Humangenetik, Universitätsklinikum Essen, Essen, Germany

Abstract

Ophthalmal-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 targeted pre-conditional 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 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 targeted pre-conditional 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 (Smoc1tm1atm1a). Eye malformations, most commonly coloboma, and cleft palate occur in a significant proportion of Smoc1tm1atm1a embryos and pups. Thus partial loss of Smoc-1 results in a convincing phenocopy of the human disease. SMOC-1 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.


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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 tract 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. The mother had similar limb involvement but with normal eyes and the other siblings were reported as normal. In the second family, the proband was a girl with significant learning disability, bilateral anophthalmia, short fingers bilaterally and postaxial oligodactyly with four toes on both feet. Her younger sister was normal. 32 further cases of OAS in 23 different families have been reported [10–29]. Of the reported definite cases; 32/35 (91.4%) had anophthalmia (4:28 for unilateral/bilateral), 29 (82.9%) had lower limb postaxial oligodactyly, 20 (57.1%) had 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, BMP4A, BMP6B, BMP10 and BMP15 (BMP1 does not encode a growth factor but a tollloid-like protease[30]). BMPs are secreted into the extracellular space where they bind to BMP type I serine-threonine kinase cell surface receptors encoded by BMPRIA and BMPRIIB. The presence of BMP type I receptors appears sufficient for BMP binding, but a BMP type II receptor (encoded by BMPR2, 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-SMAD complex then translocates to the nucleus where its function, 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 antagonist 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 twist-gastrulation [35]) bind to bmp in the extracellular space and prevent binding to the bmp type I receptor. The ventral signaling centre (VSC) secretes bmp4 and bmp7 but also bmrp (bmp-binding endothelial regulator) [36] and sizzled, which inhibits tolldox-like-1, a zinc metalloproteinase that efficiently cleaves chordin [37]. The VSC also producesambi (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].

SMOC1 is encoded by the human gene SMOC1 (SPARC related modular calcium binding f) and was initially characterised as a basement 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- terminal 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, XSMOC-1, 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 <i>in situ</i> 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 <i>Smoc1</i> also shows significant overlap with the human disease. SMOC-1 and SMOC-2 appear to be the two vertebrate paralogs of the <i>Drusophila</i> protein Pentagone that has recently been shown to function as an antagonist of Decapentaplegic (Dpp) signaling <i>in vivo</i>
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 Z = 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

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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 SNPChip 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

Figure 2. Mutation analysis. (a) Family pedigrees and associated SMOC1 mutations identified. The pedigree for Family 1 is representative and shows segregation of a homozygous SMOC1 mutation (c.911delG; p.Asp305SerfsX59) in affected individuals with both parents (and all unaffected sibs) being heterozygous carriers. (b) Schematic of the SMOC1 gene (top) and predicted protein (below), illustrating the exon positions for all eight mutations identified in the OAS families. Coding exons are coloured black and numbered, UTRs are brown, protein domains are labeled with amino acid residue numbers. Red arrowheads indicate the position of the mutations in the peptide. Red asterisks highlight the missense changes, which are located in the second thyroglobulin domain thought to be involved in the control of proteolytic degradation (n.t.- Sample not tested).

doi:10.1371/journal.pgen.1002114.g002
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<td>–</td>
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<td>Yes</td>
<td>Yes</td>
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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 Smoc1tm1a/tm1a 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 Smoc1tm1a/tm1a and Smoc1tm1a/+ genotypes, but with less
intense staining in Smoc1tm1a/+ 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 1st pharyngeal
mesenchyme (Figure 3g). The only major difference
between the missense mutation cases and those with null
mutations, is it reasonable to speculate that inhibition of a
developmentally expressed peptidase, possibly a cysteine cathepsin,
may be the non-redundant developmental function of 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 Smoc1tm1a/tm1a homozygous mice was
very similar to the lower limb phenotype in human OAS cases.
The combination of ossese synaectly and oligodactly 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 hedghog (Shh) [47,48] and
BMP4 & BMP7 signaling proteins [49,50]. A significant
proportion of the Smoc1tm1a/+ homozygous mice have clef
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 mice 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 Smoc-
1 function could partially rescue the ocular phenotype in the mice.

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 homoygous. 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. However, both residues show complete conservation
with the second Tg1 in both mouse and Xenopus tropicalis Smoc-1
and Thr283 is conserved in the second Tg1 in Drosophila
Pentagone (see Figure S3). The mutation of Arg278 to Cys may
disrupt the highly conserved pattern of disulphide bonding within
the second Tg1 [41]. Given that there is no obvious difference
between the missense mutation cases and those with null
mutations, it is reasonable to speculate that inhibition of a
developmentally expressed peptidase, possibly a cysteine cathepsin,
may be the non-redundant developmental function of SMOC-
1.
Figure 3. A targeted Smoc1 mutation caused an ophthalmo-acromelic-like phenotype in mice. (a) OPT representation of wild type (WT) Smoc1 expression at embryonic day (E) 9.5 (green represents Smoc1 expression); 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 frontal nasal processes, as well as extending caudally in the neural tube. (c) In E10.5 Smoc1tm1a/tm1a 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, the 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 Smoc1tm1a/tm1a embryo in the developing eye showing that expression was restricted to ventral regions of the presumptive optic stalk (POS). (f) 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 Smoc1tm1a/tm1a eye showing an optic fissure closure defect (arrowhead) consistent with coloboma (scale bars = 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-j) Pictograms of sections through E14.5 heads showing a failure in palatal shelf (PS) fusion in the developing palate in the Smoc1tm1a/tm1a 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 Smoc1tm1a/tm1a littermate (k) had hindlimb oligodactyly affecting the axial digits, with only 4 digits present. (l) Skeletal preparation of P0 Smoc1tm1a/tm1a hindlimb with osseous fusion of the phalanges of digits 3–4 (red arrow). doi:10.1371/journal.pgen.1002114.g003

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 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 oligodactyly by altering the BMP gradient within the limb bud and thus alter antero-posterior 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 mutations have 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 uniform 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, smo1, in zebrafish embryos. Expression was evident in the brain, choroid fissure and pharyngeal arches. “Knock-down” experiments using a morpholino targeted to smo1 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 homozygosity 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>Smoc1tm1a/tm1a</th>
<th>Smoc1tm1a/Smoc1tm1a</th>
<th>Smoc1+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total analysed</strong></td>
<td>12 (26%)</td>
<td>21 (45.7%)</td>
<td>13 (28.3%)</td>
</tr>
<tr>
<td><strong>Eye phenotype</strong></td>
<td>5 (n = 9); 55.6%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hind limb phenotype</strong></td>
<td>11 (91.7%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Cleft palate</strong></td>
<td>4 (33.3%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Missing fibula</strong></td>
<td>6 (50%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*3 confirmed Smoc1tm1a/tm1a animals were embedded in paraffin before eye phenotype was established.

[doi:10.1371/journal.pgen.1002114.002]
ExhaleAR 1 with data subjected to multipoint linkage analysis using ALLOHOMORA and Allegro (V1.2c). Gene data in the candidate interval were retrieved from the Ensembl Human genome (GRCh37 assembly; http://www.ensembl.org/Homo_sapiens/index.html). Microsatellites containing tri- or tetraneucleotide repeats (Table 3) were identified from the UCSC browser (http://genome.ucsc.edu/index.html) and PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). FAM fluorescent labels were placed at the 3’-end of the forward primer. All microsatellites were tested for informativeness for each family.

**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 single step of 72°C×10 minutes. Prehybridisation was performed using Applied Biosystems 3130/3170 Genetic Analysers. Genbank sequences were sequenced using Applied Biosystems 3130/3170 Genetic Analysers. Genbank sequences were downloaded from NCBI build 37.2 and mutation analysis was performed with Mutation Surveyor Software (SoftGenetics LLC, PA, USA) or Sequencher 4.8 (GeneCodes Corp. MI, USA).

**In Situ Hybridisations**

To generate a DNA template for production of Smoc1 riboprobe, PCR was performed from mouse genomic DNA targeting the 3’ untranslated region (UTR) of the Smoc1 gene using primers with T3 and T7 RNA polymerase sites at the 5’ ends of the forward and reverse primers respectively (underlined) (Smoc1 Forward 5’- AATTAACCCTCACTAAAGGCGTGTGTGGTTTGTTTAATACGACTCACTATAG-3’, Smoc1 Reverse 5’- TAATACGACTCACTATAG-GTAGACTGCCAAGGGATCTGG-3’). Digoxigenin (DIG) labelled (Roche) sense and anti-sense riboprobes were generated by in vitro transcription using T7 RNA polymerase, sense and antisense riboprobes were generated by in vitro transcription using T3 and T7 RNA polymerase respectively. Whole-mount in situ hybridization to mouse embryos at 9.5 & 10.5 days post-coitum (dpc or E9.5/E10.5) was carried out as previously described [62].Briefly embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, proteinase K (10 µg/ml) (Roche) treated for 15–35 minutes, depending on the stage then washed twice in 0.1 M triethanolamine, washed in phosphate-buffered saline (PBS) -Tween (0.1%) and refixed in 4% PFA/0.2% glutaraldehyde for 20 minutes. Prehybridisation was performed for 2 hours and hybridisation for 40 hours at 60°C in hybridisation buffer containing each DIG labelled probe. Washes were in 2× hybridisation buffer for 10 minutes, 3×2×SSC ×0.1% Tween 20 for 20 minutes, 3×0.2×SSC ×0.1% Tween 20 for 30 minutes, all at 60°C. At room temperature the embryos were washed in 1 M Malic Acid with 2% BMB (Boehringer Mannheim blocking reagent) +20% heat-treated lamb serum solution for 2 hours and then in the same buffer containing a 1/2000 dilution of anti-DIG antibody coupled to alkaline phosphatase (Roche) overnight at 4°C. Embryos were washed 3×5 minutes and 3×1 hour in MAB. Colour detection was performed in 2 ml of BM purple precipitating solution (Roche).

**Smoc1 Targeted Mouse**

The Smoc1 pre-conditional knockout mouse (EUCOMM Project 48154; strain C57BL/6N-A-Smoc1<sup>tm1a(EUCOMM)Wtsi</sup>) referred to as Smoc1<sup>fl/o</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/hgt/report/gene_report/project_id = 48154 and in Figure S1. Genotypes were confirmed using the Smoc1 forward primer 5’-GGTCCTGCTGGTGGTCCGGCTGCTG-3’ (positioned at mChr12:82,236,250 bp) and Smoc1 reverse primer 5’-CCTGCTGCTCAACAGTTTCCCGC-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’-TTAGTCCTCCACCCCTTTCTCC-3’ to PCR mixes as multiplex reactions produced a targeted-allele specific amplicon of 250 bp. Early 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 collected for production of Smoc1 riboprobe, PCR was performed from mouse genomic DNA as described above with WT and Smoc1<sup>fl/o</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) using an ABI-HT7900 SDS instrument (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>
<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>D1456790 (67906158–67906384)</td>
<td>CTGACATTTTGGGAAAAGG</td>
<td>GTGACTGCTGCGTCTGAGG</td>
<td>320 bp (tetra)</td>
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<tr>
<td>D1456889 (6889253–68892381)</td>
<td>TCTGGAGGACCTAGAGGAG</td>
<td>CCCCGAGCAAAGCTGC</td>
<td>300 bp (tetra)</td>
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<tr>
<td>D1456921 (69216842–69217056)</td>
<td>CAGCTACTCTACCGCTTC</td>
<td>ACACCTGCTGCTGCTAAC</td>
<td>200 bp (tetra)</td>
</tr>
<tr>
<td>D1456964 (67949225–67949499)</td>
<td>GCCCTTGAAGATGTTT</td>
<td>GATAGCAGCTGCTCACCC</td>
<td>250 bp (tetra)</td>
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<tr>
<td>D145258 (70583011–70583186)</td>
<td>TCACAGTCGCTGAGCA</td>
<td>CTAATAATGGGAGGAG</td>
<td>170 bp (dI)</td>
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<tr>
<td>D1456968 (7086661–70869493)</td>
<td>CTCCATAGGACAACAGCTCC</td>
<td>GGCAGAAATTCGGCTGAGC</td>
<td>285 bp (tetra)</td>
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<tr>
<td>D1457023 (71168266–71168625)</td>
<td>AGGGTTATTGAGGAAAGG</td>
<td>GAGCATGAGGAGGAGG</td>
<td>330 bp (tri)</td>
</tr>
<tr>
<td>D1457033 (71262900–71260458)</td>
<td>TGAGCCCACTAGTCCAC</td>
<td>AGTCAGGCACTAGTCCAC</td>
<td>270 bp (tetra)</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pgen.1002114.t003

SMOC1 Mutations Cause Ophthalmo-Acromelic Syndrome
inclusion of either Smoc1 forward 5′-GGATGGTCTTCCATGACA-
CAGG-3′ and reverse 5′-TCAGTCTCATGCAGACAG-3′ primers or Hprt forward 5′-CTGGTGAAAAAGGCCTCCTG-3′ and reverse 5′-CAAG-
GGCATAACAGACACAG-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 eosin. For skeletal preparations the animals were dehydrated in 95% ethanol for 24 hours; followed by 72 hours in 100% acetic; 3 days in stain solution (1 part 0.3% alcan 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; 3 days in 1% KOH/50% 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 Smoc1

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 Smoc1

Figure S3 Alignment of human SMOC1 Thyroglobulin type-1 (Tg1) domains with Tgl domains from mouse Smoc-1, Xenopus tropicalis Smoc-1 and Drosophila melanogaster Pentagone. Alignment of the Tg1-1 and Tg1-2 domains from mouse Smoc-1 and human SMOC1 with the Tg1-2 domains from Xenopus tropicalis Smoc-1 and Drosophila melanogaster Pentagone. The
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 the peptide; Tg1-2 = second Tg1 in the peptide; hSMOC1 = human SMOC1; mSmoc1 = mouse Smoc1; xtSmoc1 = Xenopus tropicalis Smoc1; dmPent = Drosophila melanogaster Pentagone protein; Q9b4F8 etc are UniProt accession numbers.

(DOC)

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 Arakçu 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 HGB H-B DRF. Performed the experiments: JR LA-G EvB JKR LM PB MF HM PG PP KS DS. Analyzed the data: JR DW H-B DRF. Contributed reagents/materials/analysis tools: IJJ DW GGK LG ASF AW AK SZ NE RP AS.

References

9. Smoc1; dmPent = Drosophila melanogaster Pentagone protein; Q9b4F8 etc are UniProt accession numbers.

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