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SIPA1L3 identified by linkage analysis and whole-exome sequencing as a novel gene for autosomal recessive congenital cataract

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Congenital cataract (CC) is one of the most important causes for blindness or visual impairment in infancy. A substantial proportion of isolated CCs has monogenic causes. The disease is genetically heterogeneous, and all Mendelian modes of inheritance have been reported. We mapped a locus for isolated CC on 19p13.1–q13.2 in a distantly consanguineous German family with two sisters affected by dense white cataracts. Whole-exome sequencing identified a homozygous nonsense variant c.4489C>T (p.(R1497*)) in *SIPA1L3* (signal-induced proliferation-associated 1 like 3) in both affected children. *SIPA1L3* encodes a GTPase-activating protein (GAP), which interacts with small GTPases of the Rap family via its Rap-GAP-domain. The suggested role of Rap GTPases in cell growth, differentiation and organization of the cytoskeleton in the human lens, and lens-enriched expression of the murine ortholog gene *Sipa1l3* in embryonic mice indicates that this gene is crucial for early lens development. Our results provide evidence that sequence variants in human *SIPA1L3* cause autosomal recessive isolated CC and give new insight into the molecular pathogenesis underlying human cataracts.

INTRODUCTION

Congenital cataract (CC) is the most common ocular disease leading to blindness or severe visual impairment in children worldwide. The human lens is derived from epithelial cells, which differentiate into fiber cells to form the bulk of the lens. A single layer of epithelial cells covers the anterior surface of the lens and regulates most of its homeostatic functions. Any disturbance of lens development or homeostasis can result in cataract. CC may be isolated, associated with other anterior segment malformations of the eye, or other systemic findings as part of a syndrome.[1] In developed countries about one-third of isolated CCs are hereditary, and all three forms of Mendelian inheritance have been observed.[2] More than 20 different genes have been identified that cause either isolated congenital or isolated juvenile cataract (Table 1). They can be divided into four major groups: genes encoding (a) crystallins, (b) membrane proteins, (c) cytoskeletal proteins, and (d) transcription factors or other proteins regulating gene expression. Sequence variants in genes coding for crystallins (eg, *CRYAA*), the main structural proteins of the lens, account for ~50% of isolated familial cataracts.[3–12] Genes of group (b) encode gap junction proteins (*GJA3*, *GJA8)*, other cell junction proteins (*LIM2, TMEM114*), aquaporin water channels (*MIP*), and proteins involved in membrane-associated transport or signaling (eg, *CHMP4B*, *EPHA2*).[13–18] Genes of group (c) are essential for the synthesis of intermediate filament, and intermediate filament-like proteins (*VIM, BFSP1*, *BFSP2*).[19–21] Sequence variants in genes of group (d) are often associated with a syndromic phenotype and other ocular defects (eg, *PITX3*).[22–24] The remaining genes cannot be classified in the four major groups. They often encode proteins with unexpected functions, for example, those involved in lipid metabolism (*AGK*).[25] However, even more genes are expected to be involved in the etiology of CC. Their discovery could provide new insight into the underlying pathomechanisms.

We studied a consanguineous family with two sisters affected by isolated CC, indicating autosomal recessive inheritance by linkage analysis and whole-exome sequencing (WES).

MATERIALS AND METHODS

Patient ascertainment and DNA isolation

The two patients, their healthy sister and parents were seen at the Department of Ophthalmology of St Vincentius-Kliniken, Karlsruhe, Germany, and the Institute of Human Genetics, Heidelberg, Germany. Parents gave written consent for publication. The study adhered to the tenets of the Declaration of Helsinki and was approved by the local ethics committee. Genomic DNA of patients, their sister and parents was isolated from blood as previously described.[26]

Array analysis/SNP genotyping

Affymetrix CytoScan HD Oligo/SNP-Array was performed according to the manufacturer's instructions to genotype DNA of the affected individuals and both parents, and to exclude genomic imbalances in the affected patients. Arrays were scanned with the AffymetrixGeneChip Scanner 3000 7G and analyzed with Affymetrix Chromosome Analysis Suite software version 1.2 and

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[6]This work is dedicated to Herbert Steinbeisser who sadly passed away during the preparation of the manuscript.

Table 1 Genes involved in isolated congenital, infantile or juvenile cataract

Gene	*Inheritance*	*Other phenotypes*	*References*
Crystallins			
CRYAA	AD, AR	Microcornea, macrocephaly, coloboma, glaucoma, macular hypoplasia	8,52–54
CRYAB	AD, AR	Myopathia, cardiomyopathy	3,55–57
CRYBA1	AD	—	6
CRYBA2	AD	—	52
CRYBA4	AD, AR	Microcornea, microphthalmie	4,58
CRYBB1	AD, AR	Glaucoma, microcornea	9,52,59
CRYBB2	AD, AR	Microcornea, nystagmus	7,60,61
CRYBB3	AD, AR	Microcornea	2,10
CRYGC	AD	Microphthalmia/microcornea, glaucoma, corneal opacity, nystagmus, peripupillar iris atrophy	5,52,62
CRYGD	AD	Microcornea	11,63
CRYGS	AD	—	12
Membrane proteins			
CHMP4B	AD	—	18
EPHA2	AD, AR	—	16,17,64
GJA3	AD	—	65
GJA8	AD, AR		66,67
LIM2	AR	—	15
MIP	AD	—	13
TMEM114	AD	Unilateral absent kidney	14
WFS1	AD	—	68
Cytoskeletal proteins			
BFSP1	AR	—	21
BFSP2	AD, AR	—	19,69,70
VIM	AD	—	20
Transcription factors/gene-regulating proteins			
HSF4	AD, AR	—	71,72
PITX3	AD, AR	Optic nerve defects, anterior segment dysgenesis, microphthalmie, neurologic impairment	24,73,74
TDRD7	AR	—	75
Others			
FYCO1	AR	—	76
GCNT2	AR	—	77
AGK	AR	Sengers syndrome	25,78
UNC45B	AD	—	79

Abbreviations: AD, autosomal dominant; AR, autosomal recessive.
Genes are listed if human isolated cataract has been reported at least once. Some genes cause isolated cataract but have also been described in association with other ocular or non-ocular findings given in column 3. Genes associated with age-related cataracts are not included. The listed genes were covered with a median exon coverage of 118× among the four samples, only one exon in *LIM2* had a coverage of <15× in both patients.

Annotation Net Affx Build 32. Analysis for genomic imbalances was done at a resolution of 100 kb. Interpretation was based on human reference sequence GRCh37/hg19, February 2009.

Statistical linkage analysis

Genome-wide parametric linkage analysis with SNP genotypes was performed using ALOHOMORA and MERLIN software,[27,28] assuming affected family members were homozygous at a putative disease locus for an autosomal recessive disease allele inherited from a common ancestor. After performing several data quality checks, SNP marker with minor allele frequency (MAF) 0.15 and a minimum distance of 100 000 bp were selected to ensure low linkage disequilibrium between the markers.

Whole-exome sequencing

We performed WES on the patients (I.2 and I.3) and their parents (II.1 and II.2). Exome capturing was done using Agilent SureSelect Human All Exon V4 (without UTR). The samples were sequenced to an average base coverage of 145× on targeted regions, with 99.038% of the targeted bases having at least 10× coverage. For each sample, the raw reads were mapped to the hs37d5 (hg19+decoy sequences) reference genome using BWA 0.6.2[29] and PCR duplicates were marked by Picard (http://picard.sourceforge.net/). SNVs and short indels were called using SAMtools[30] and Platypus,[31] respectively, and annotated using ANNOVAR.[32] In-house Perl scripts were used to filter variants according to the following criteria: minimum coverage of 10 reads, a minimum QUAL score of 20 for SNVs, and 'PASS' for the built-in filters from Platypus for Indels. Variants with >1% MAF in the 1000 genome project[33] were considered as common alleles and removed from the candidate list. An in-house database with 79 exomes whose phenotype are different from the CC, were used to filter the sequencing artifacts and common alleles. Variants were removed if they were present with the same genotype in the in-house database. Using the ANNOVAR annotations, coding variants were defined as missense or nonsense variants, indels overlapping exonic regions, and variants ±2 base around the intron–exon junction (splice sites). Variants reported in this study

have been submitted to the LOVD database (http://databases.lovd.nl/shared/genes, IDs 00025020, 00025118, 00025124, 00025125).

Sanger sequencing

Validation of the *SIPA1L3* sequence variant was performed by Sanger sequencing. Exon 17 and adjacent intron boundaries of the *SIPA1L3* gene (RefSeq NM_015073.1, exon numbering according to Ensembl database transcript ENST00000222345 (http://www.ensembl.org)) were sequenced from affected individuals (I.2 and I.3) and both parents (II.1 and II.2) using Big Dye Terminator V1.1 cycle sequencing kit and ABI 3130xl genetic analyzer. Sanger sequencing was also performed on genomic DNA from the healthy sister (I.1), to exclude homozygosity for the *SIPA1L3* nonsense variant. Primer sequences and PCR conditions are available on request.

cDNA analysis of SIPA1L3

RNA was isolated form peripheral blood of patient I.2 and healthy control persons as previously described.[34] cDNA was synthesized through reverse transcription (RevertAid H Minus Reverse Transcriptase, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

Different regions of the *SIPA1L3* cDNA were amplified by PCR. Sequence of primers and PCR conditions are available on request. PCR products were assayed on an agarose gel.

RESULTS

Patient reports

The parents were of German ancestry and consanguineous (fourth-degree cousins, II.1 and II.2, Figure 1a). Their first child was a healthy girl (I.1, Figure 1a) with normal development and without ocular problems. In the second child (I.2, Figure 1a), bilateral dense white cataracts were diagnosed at age 2 weeks. Blood and urine analyses for metabolic diseases were normal. The third child (I.3, Figure 1a) was diagnosed with bilateral dense white cataracts soon after birth. Pregnancy and birth history of all three children were unremarkable, there was no history of intrauterine infections or maternal medication. Both affected children were treated by cataract surgery, and aphakia was corrected with contact lenses. On last follow-up at age 4 years (I.2) and 2 years (I.3), both children showed a normal psychomotor development, normal growth, and no dysmorphic signs. Ophthalmologic examination of both parents did not show any ocular abnormalities.

Array analysis showed no significant copy number aberrations

The affected children (I.2 and I.3) were screened for CNVs (copy number variants). A paternally inherited duplication chr6.hg19: g.57237993_57641858dup was detected in patient I.2, but not in her affected sister (I.3). In both children no other CNVs (deletions or duplications not previously listed in the Database of Genomic Variants (http://dgv.tcag.ca/)) were found across the genome.

Genotyping showed a common haplotype block on chromosome 19p13.11–q13.2

Linkage analysis demonstrated genome-wide significant evidence of linkage of disease to a locus on chromosome 19p13.11–q13.2 (ranging from 16 743 209 to 39 150 199 bp, between SNP markers rs12461484 and rs7351086). The locus showed a maximal odds ratio lod-score of 3.3 (Figure 1b). The identified haplotype block on chromosome 19p13.11–q13.2 contained 271 protein coding genes in GENCODE version 19 (http://www.gencodegenes.org/), but none of the previously identified genes for isolated autosomal recessive CC.

WES revealed a homozygous nonsense variant in *SIPA1L3*

Subsequent WES of the patients and their parents showed only three coding variants (in the genes *CYFIP1*, *NPHS1*, and *SIPA1L3*), which were homozygous in both affected siblings (I.2 and I.3) and heterozygous in their parents (II.1 and II.2), and therefore consistent with an autosomal recessive disease model in a consanguineous family. The variant in *CYFIP1* (c.2707G>A; p.(G903S), *RefSeq NM_014608.3*) is listed in the dbSNP database (rs139635799, http://www.ncbi.nlm.nih.gov/SNP,[35]) and in the Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/,[36]) with a MAF of 0.592% and is therefore unlikely to be the cause of the cataract. This variant is not located within the linkage region on chromosome 19.

The variants affecting *NPHS1* and *SIPA1L3* were located in the linkage region of chromosome 19 (17.9–39.2 Mbp). The variant in

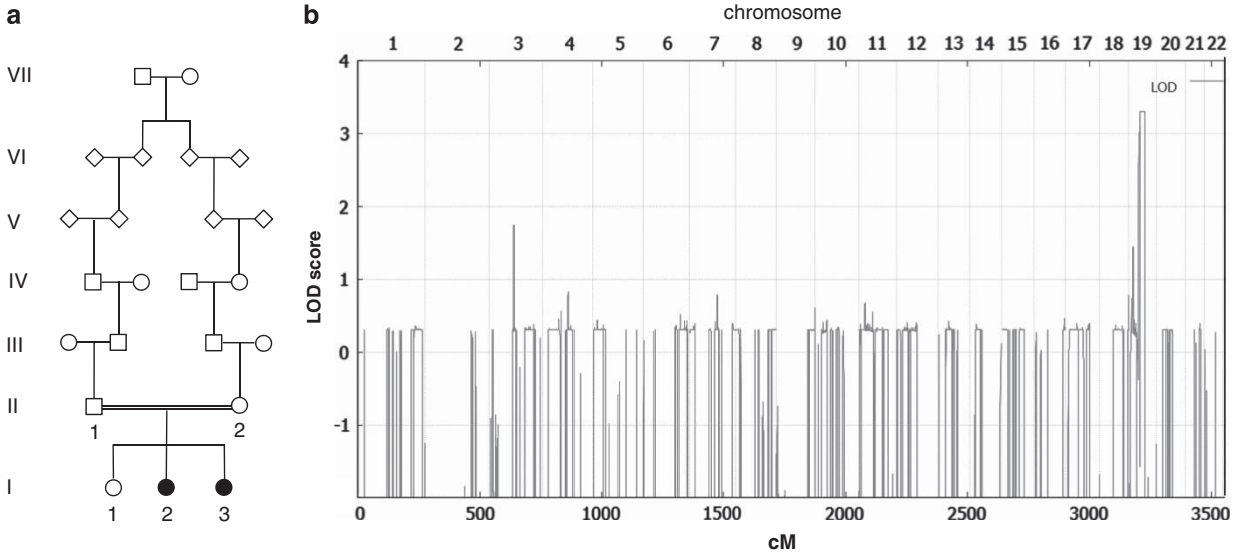

Figure 1 (**a**) The predigree of the family: extended family history showed distant consanguinity of the parents. (**b**) Linkage analysis: LOD-score distribution relative to chromosomal location with the highest LOD-score region on chromosome 19 (40.2–66.0 cM, corresponding to 17.0–39.2 Mbp). The lower peak on chromosome 3 does not reach a level of genome-wide significance.

NPHS1 (c.791C>G; p.(P264R), *RefSeq NM_004646.3*) is suggested to be a benign variant based on its allele frequency (MAF of ~1% according to the dbSNP database (rs34982899), and MAF of 1.5698% in the EVS) and on prediction by Mutation Taster (http://www.mutationtaster.org)[37] and SIFT analysis (http://sift.jcvi.org/).[38] In addition, sequence variants in *NPHS1* affecting gene function cause the severe phenotype of autosomal recessive congenital nephrotic syndrome of the Finnish type, which can clinically be excluded in our patients.

The variant in *SIPA1L3* (c.4489C>T; p.(R1497)*, *RefSeq NM_015073.1)* is absent in the 1000 genome project[39] and in the EVS, suggesting it is extremely rare in the population. It is predicted to introduce a premature stop codon in exon 17 at amino acid 1497 (p.(R1497*)). The homozygous *SIPA1L3* variant was validated by Sanger sequencing in both affected children. Both parents were confirmed to be heterozygous carriers (Figure 2a). Sanger sequencing on genomic DNA from the healthy sister (I.1) excluded homozygosity for the *SIPA1L3* variant (data not shown).

The *SIPA1L3* variant does not result in nonsense-mediated mRNA decay (NMD)

We were able to amplify cDNA of two different regions of *SIPA1L3* in patient I.2 (exons 11–13 and 16–18, Figure 2d), showing that *SIPA1L3* mRNA was not subject to degradation by NMD. This indicates that the *SIPA1L3* variant results in premature termination of translation and in the synthesis of a truncated protein lacking the last 284 amino acids.

DISCUSSION

We identified a nonsense variant (c.4489C>T; p.(R1497*)) in the gene *SIPA1L3* (signal-induced proliferation-associated 1 like 3) in a consanguineous family with autosomal recessive CC using a combined strategy of linkage analysis with WES.

In the present family with parents who were 4th degree cousins, a single region with significant linkage could be identified (19p12.11–q13.2, lod-score: 3.3). This illustrates that, in particular, in case of distant consanguinity linkage analysis may help to bring down the

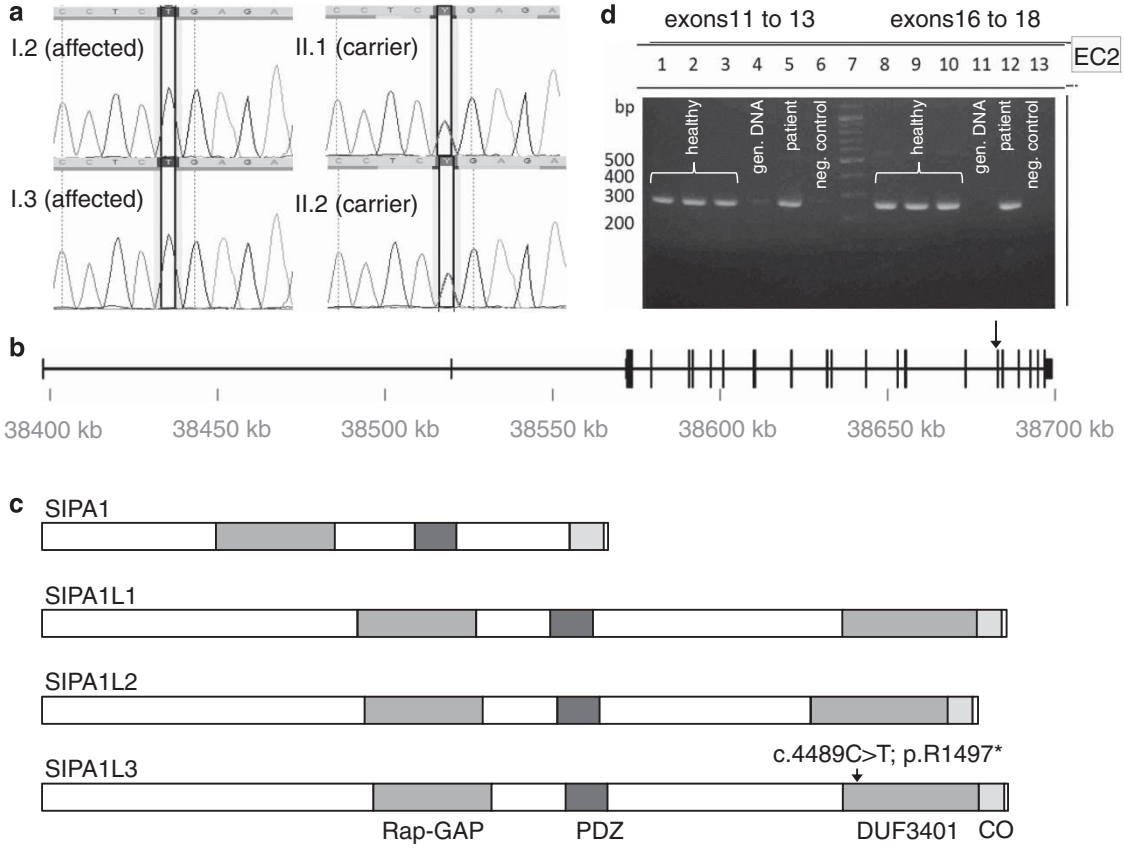

Figure 2 (**a**) Sequence analysis by Sanger sequencing confirmed homozygosity for the nonsense mutation c.4489C>T (p.R1497*) in exon 17 of *SIPAL3* in the two affected siblings (II.2 and II.3) and heterozygosity in their healthy parents (I.1 and I.2). (**b**) Exon/intron structure of *SIPA1L3*. Exons are shown as vertical lines. Exon numbering is according to ensemble transcript ENST00000222345 (http://www.ensembl.org). The *SIPA1L3* variant c.4489C>T (marked by an arrow) is located in exon 17 of the full-length mRNA (NM_015073.1, transcript ENST00000222345), which comprises 22 exons. The first two exons are non-coding exons. The Ensemble database lists 12 putative transcripts, but only the full-length transcript (ENST00000222345) is known to be protein coding. (**c**) Schematic domain structure of SIPA1L3 protein and its paralogs SIPA1, SIPA1L1 and SIPA1L2. The location of the *SIPA1L3* mutation c.4489C>T (p.R1497*) is shown by an arrow. Conserved protein domains of SIPA1, SIPA1L1, SIPA1L2 and SIPA1L3 are a Rap GTPase-activating protein (Rap-GAP) domain, a PDZ domain, and coiled-coil (CO) domain. The domain of unknown function (DUF3401) is similar between SIPA1L1, SIPA1L2 and SIPA1L3 but not present in SIPA1. (**c** and **d**) cDNA analysis of *SIPA1L3*. Amplification of exons 11–13 and 16–18 of *SIPA1L3* cDNA, respectively. 1–3 and 8–10: cDNA of healthy control persons; 4 and 11: genomic DNA of healthy control person; 5 and 12: cDNA of the patient; 6 and 13: negative control; 7: marker (100 bp ladder).

number of genetic variants identified by high throughoutput sequencing. Within the linkage region, WES identified only two homozygous sequence variants (*NPHS1*: c.791C>G; p.(P264R) and *SIPA1L3*: c.4489C>T; p.(R1497*)) in the affected individuals. The variant in *NPHS1* was excluded from further considerations because of its frequency and predicted effect. The *SIPA1L3* variant c.4489C>T introduces a premature stop codon at amino acid 1497 p.(R1497*). *SIPA1L3* full-length mRNA (NM_015073.1) encodes 1781 amino acids. The nonsense variant was predicted to be disease causing by *in silico* analysis and expected to result either in NMD or a premature termination of translation. Expression analysis in patient 1 showed that *SIPA1L3* mRNA is not subject to NMD, indicating synthesis of a truncated protein lacking the last 284 amino acids.

This is the first variant in *SIPA1L3* that has been described in a human disease. *SIPA1L3* has been previously identified as a putative candidate gene for isolated CC in a study using mainly a mouse embryonic gene expression data set. *SIPA1L3* showed highly lens-enriched expression patterns at three key developmental time points from lens placode invagination to lens primary fiber cell differentiation.[40] The early expression of *Sipa1l3* in murine lens and the presence of a complete white CC, which represents the end stage of cataract formation, in the present family both indicate that sequence variants in *SIPA1L3* might have a very early effect on lens development, probably at the time of fiber cell differentiation.

SIPA1L3 falls within a previously mapped human cataract locus in which the causative gene had not been identified.[41] Sequencing of several candidate genes within this region (*MIP, LIM2, SIX5,* and *FTL*, but not *SIPA1L3*) in the affected family members failed to detect a sequence variant. However, in this family the cataract showed autosomal dominant inheritance, later onset and a cortical localization.[41] Other putative cataract genes in the previously mapped region for autosomal dominant cataract include *PRX, EML2, SPINT2,* and *PVRL2*.[40] The above candidate genes were covered in our exome sequence data and included for further data analysis but did not yield any interesting candidate variants. For the time being, it cannot be excluded that sequence variants in *SIPA1L3* may result in both autosomal dominant and autosomal recessive CC, probably with a more severe phenotype and earlier onset in homozygous individuals than in heterozygous ones.

The protein encoded by *SIPA1L3* (signal induced proliferation associated protein 1 like 3, SIPA1L3, alias SPAL3) is conserved among different species (Supplementary Table S1). Human SIPA1L3 is predicted to contain a Rap GTPase-activating protein (Rap-GAP) domain, a PDZ domain, a domain of unknown function (DUF3401), and a C-terminal coiled-coil domain (Figure 2c). SIPA1L3 is closely related to SIPA1 (alias SPA1), SIPA1L1 (alias SPAL1), and SIPA1L2 (alias SPAL2) (Figure 2c, Supplementary Figure S2). The presence of a Rap-GAP domain indicates that SIPA1L3 is a GAP for small G-proteins of the Rap family. Binding of Rap to the Rap-GAP domain leads to conversion of Rap from its active, GTP-bound form to the inactive, GDP-bound form. This mechanism has been well studied for SIPA1 and SIPA1L1, which are negative regulators of Rap1 (RAP1A, Ras-related protein Rap-1A) and Rap2 (RAP2A).[42,43]

Rap1 and other small GTPases are suggested to be involved in important regulatory functions in the developing lens, possibly related to epithelial cell proliferation, fiber cell differentiation, gap junction formation, and cytoskeletal organization.[44] Interestingly, Rap1 controls integrin activation and integrin-mediated cell adhesion in different cell types. Integrins also regulate lens epithelial cell proliferation, survival and differentiation into fiber cells (for review see[45]).

The role of the PDZ domain, which is predicted to be required for protein–protein interactions, the C-terminal DUF3401 and the coiled-coil domain of SIPA1L3 is still unknown. *In silico* network analyses using Ingenuity software[46] showed an interaction between SIPA1L3 and 14-3-3ζ (YWHAZ), a small intracellular signaling molecule identified as one of the key proteins in human lens,[47] and also indicated an interaction between Rap1 and 14-3-3ζ. Gene expression data of rat lens indicated that several isoforms of 14-3-3 (including 14-3-3ζ) might be involved in cataract formation.[48] Furthermore, the PDZ domain of the SIPA1L3 homolog SIPA1L1 interacts with the renal aquaporine 2 water channel.[49] This might be of interest, because sequence variants in a gene for another aquaporine (aquaporine 0, *AQPO,* alias *MIP*) cause cataract formation.[13] In neuronal cells, an interaction between the PDZ domain of rat SIPA1L1 (SPAR) and the EphA4 receptor has been demonstrated.[50]

The *SIPA1L3* variant c.4489C>T; p.(R1497*) is predicted to result in a shortened protein, which includes the Rap-GAP and PDZ domain but lacks most parts of the DUF3401, the coiled-coil domain and the neighboring seven C-terminal amino acids (Figure 2). The exact function of the highly conserved C-terminal coiled-coil domain of SIPA1L3 has not been elucidated so far. Previous studies of the homolog SIPA1L1 have shown that its C-terminal part interacts with casein kinase I epsilon (CKIε), a protein involved in Wnt signaling. CKIε stimulates SIPA1L1 degradation, and alleviates SIPA1L1-mediated Rap1 inhibition.[51] If SIPA1L3 also interacts with CKIε, a C-terminal truncated SIPA1L3 protein might escape CKIε-induced degradation. Otherwise, it is possible that truncation of SIPA1L3 leads to aberrant protein folding, resulting in loss-of-function. We would expect that both mechanisms result in disturbance of SIPA1L3 downstream targets either by unphysiological inhibition of Rap (if the truncated SIPA1L3 escapes degradation) or overactivation of Rap signaling (if the truncating variant results in loss-of-function). We hypothesize that this mechanism disturbs the complex embryonic lens development processes in the present family, leading to early cataract formation. Further functional studies are however necessary to elucidate the exact function of SIPA1L3 and its protein–protein interactions in the human lens.

In summary, we could map an interval on chromosome 19p13.11–q13.2 for autosomal recessive CC and identified a homozygous nonsense variant (c.4489C>T; p.(R1497*)) in the gene *SIPA1L3*. There is convincing evidence, although no definite proof, that this variant is causative for the cataract in the present family. This is the first sequence variant in a GAP associated with human cataract formation. Further characterization of SIPA1L3 function, its putative interaction partners and its role in lens development, and mutation screening of *SIPA1L3* in additional cataract patients should provide insight into pathogenetic mechanism leading to cataract formation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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