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Two missense mutations in *SALL4* in a patient with microphthalmia, coloboma, and optic nerve hypoplasia

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Abstract

To investigate the genetic etiology of anophthalmia and microphthalmia, we used exome sequencing in a Caucasian female with unilateral microphthalmia and coloboma, bilateral optic nerve hypoplasia, ventricular and atrial septal defects and growth delays. We found two sequence variants in *SALL4* - c.[575C>A], predicting p.(Ala192Glu), that was paternally inherited, and c.[2053G>C], predicting p.(Asp685His), that was maternally inherited. Haploinsufficiency for *SALL4* due to nonsense or frameshift mutations has been associated with acro-renal ocular syndrome that is characterized by eye defects including Duane anomaly and coloboma, in addition to radial ray malformations and renal abnormalities. Our report is the first description of structural eye defects associated with two missense variants in *SALL4* inherited in trans; the absence of reported findings in both parents suggests that both sequence variants are hypomorphic mutations and that both are needed for the ocular phenotype. *Sall4* is expressed in the developing lens and regulates *Bmp4*, leading us to speculate that altered *BMP4* expression was responsible for the eye defects, but we could not demonstrate altered *BMP4* expression *in vitro* after using small interfering RNAs (siRNAs) to reduce *SALL4* expression. We conclude that *SALL4* hypomorphic variants may influence eye development.

Keywords

Anophthalmia; BMP4; coloboma; microphthalmia; SALL4

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Introduction

The human Spalt family members, Sal-like proteins 1 to 4 (*SALL1*, *SALL2*, *SALL3* and *SALL4*), are transcription factors containing C2H2 double zinc fingers that have been implicated in developmental eye defects (Kohlhase et al., 2002a). Sequence variants interrupting function in *SALL4* cause acro-renal-ocular syndrome (AROS), characterized by eye defects including Duane anomaly and coloboma, radial ray malformations and renal abnormalities (Kohlhase et al., 2003; Borozdin et al., 2004a; Borozdin et al., 2004b; Kohlhase et al., 2005). *SALL4* variants also cause Okihiro syndrome, or Duane-radial ray syndrome (MIM 607323;Al-Baradie et al., 2002; Kohlhase et al., 2002b; Kohlhase et al., 2003). Loss of function for *SALL2* results in non-syndromic ocular coloboma affecting the iris and retina (Kelberman et al., 2014). Sequence variants in *SALL1* causes Townes-Brocks syndrome (TBS; MIM 107480; (O'Callaghan and Young, 1990; Kohlhase et al., 1998; Powell and Michaelis, 1999; Miller et al., 2012). Eye defects are rare with *SALL1* sequence variants (Miller et al., 2012), but unilateral chorioretinal coloboma with absent vision in the affected eye and anophthalmia and microphthalmia have been described (Botzenhart et al., 2005; Bardakjian et al., 2009).

Patients with AROS have displayed a range of structural eye defects, including microphthalmia, microcornea, cataract and colobomas involving the iris, choroid and optic nerve, in addition to nystagmus and strabismus (Becker et al., 2002; Kohlhase et al., 2003; Aalfs et al., 1996; Borozdin et al., 2004a; Borozdin et al., 2004b; Kohlhase et al., 2005). All of the *SALL4* sequence variants associated with eye disease have been nonsense, frameshift or deletion mutations and thus are likely associated with premature protein truncation and/or haploinsufficiency. We present a female with unilateral microphthalmia and coloboma, bilateral optic nerve hypoplasia, cardiac septal defects and growth delays who was found on whole exome sequencing to have two missense sequence variants in *SALL4*.

Materials and Methods

Clinical Report

Ethical approval was obtained from the Committee on Human Research at the University of California, San Francisco and the Institutional Review Board at Einstein Medical Center. The proband was delivered after an uneventful pregnancy. Two small septal defects, including a membranous muscular ventricular septal defect (VSD) and atrial septal defect (ASD) were detected in the neonatal period, but surgery was not required. At 3 weeks of age, her right eye was noted to be smaller than her left eye. At 5 months of age, she was diagnosed with microcornea, microphthalmia, lenticular opacities and amblyopia with an afferent pupillary defect, all involving the right eye. She had bilateral optic nerve hypoplasia that was more severe on the right. A magnetic resonance imaging scan of the brain showed asymmetry of the ocular globes and optic nerves, but the extra-ocular muscles were symmetric. At age 33 months, she had full extraocular motility without nystagmus, but did have an afferent pupillary defect of her right eye.

At 5 years of age, growth parameters were in the normal range (height 25–50th centile, weight 50th centile and head circumference 10–25th centile). There were no limb

defects.When last reviewed at 8 years of age, she had speech therapy and vision therapy, but her cognitive development and general health were normal. Prior investigations included a normal karyotype and array comparative genomic hybridization (array CGH). She also had a normal renal ultrasound scan and normal hearing evaluation.

Both parents had no significant findings on external examination, by report. The proband's paternal grandmother had macular degeneration that was diagnosed in adult life and her paternal grandfather had history of cataract surgery and type 2 diabetes mellitus. There was no known consanguinity.

Exome Sequencing

This patient and her biological parents underwent exome sequencing as part of a larger study involving patients with microphthalmia, anophthalmia and coloboma (MAC; Slavotinek et al., 2015). Libraries were prepared and sequenced on a HiSeq4000 (Illumina, San Diego, CA, USA) according to previously published methods (Slavotinek et al., 2015). Variants were assessed for deleteriousness using Sorting Intolerant from Tolerant (SIFT) and PolyPhen-2 databases and mutations that had a SIFT score <0.05 or a PolyPhen-2 score >0.909 were retained. Variants were excluded from consideration if they were not in exons, were present in the database of single nucleotide polymorphisms (dbSNP135), or had a minor allele frequency of greater than 0.01 in the 1000 Genomes database. We also excluded candidate genes in a published list of common false positive genes to rule out genes that are likely to have mutations due to their length or inherently variable nature (Fuentes Fajardo et al., 2012). Selected variants were verified using Sanger sequencing using previous methods (Slavotinek et al., 2013).

Cell Culture and Small Interfering RNA (siRNA) to Reduce SALL4 Expression

HEK293T cells and NT2 cells (UCSF cell culture facility) were cultured according to standard methods on 6-well plates at a density of 5×10^4 cells per well. Cells were transiently transfected in six-well dishes with small interfering (si)RNAs using Lipofectamine® 3000 (Life Technologies, Grand Island, NY). We transfected siRNA (Silencer® Select s32817; Life Technologies, Grand Island, NY) targeting exon 2 of *SALL4* at base pair (bp) 557 (NM_020436.3) at a final concentration of 30 nM. At 48 hours, RNA was obtained and cDNA was prepared. Silencer® Select GAPDH Positive Control siRNA was used as a positive control. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) using gene-specific primers (Table S1) was run on an 7500 RT-PCR machine (Applied Biosystems, Foster City, CA) and data was analyzed according to the $^{\Delta\Delta}$Ct method using negative control siRNA and human *GAPDH* as internal controls. Results were normalized to expression levels in cells transfected with control siRNA and statistical comparisons between the expression in cells transfected with siRNA for *SALL4* and expression in cells transfected with siRNA for *GAPDH* were performed with an unpaired *t*-test.

Results

Exome Sequencing

Data regarding coverage of the proband's exome is provided in Table S2. Variant analysis demonstrated two missense substitutions in exon 2 of *SALL4* (NM_020436.3) - c.[575C>A], predicting p.(Ala192Glu) and c.[2053G>C], predicting p.(Asp685His) (Table 1). Both sequence variants were confirmed using Sanger sequencing (Fig. S1); c.[575C>A] was paternally inherited, and c.[2053G>C] was maternally inherited. Both variants were predicted to be damaging with three software programs (Table 1). Mutation Taster predicted that p.(Ala192Glu) would alter splicing, with an increased donor splice site (wildtype: 0.58 and mutant: 0.99) for exon intron site CAAG/gtgg, and creation of a new splice site. The alternative splicing was predicted to cause loss of all of the double zinc fingers in this protein, but there was no RNA from the patient to verify aberrant splicing. p.(Ala192Gln) was present at a low frequency (6/121,266) in the ExAC control database, but p.(Asp685His) was not present (Table 1). We assessed species conservation for both variants and p.Ala192 was highly conserved in different species, whereas p.Asp685 was less conserved (Fig. S2A and Fig. S2B; Table S3). Reanalysis of the .BAM files for this patient did not show any evidence of a large structural rearrangement, such as a chromosome translocation, affecting *SALL4* (data not shown).

We did not find any other sequence variants in published gene lists for MAC (Deml et al., 2014; Prokudin et al., 2014) that were predicted to alter function and did not find any other cause for the eye defects in this child.

Cell Culture and Small Interfering RNA (siRNA) to Reduce SALL4 Expression

The mean expression +/− standard deviation for *BMP4*, *SOX2* and *OTX2* genes after *SALL4* knockdown are shown for HEK293T (Fig. 1) and NT2 cells (Fig. 2). We found no significant difference in *BMP4* or *OTX2* expression after reduction of *SALL4* in HEK293T cells. However, *SOX2* showed a significant increase in expression after siRNA for *SALL4* in HEK293T cells (Fig. 1), but not in NT2 cells (Table S4).

Discussion

We report a female patient with microphthalmia, colobomas and optic nerve hypoplasia, VSD, ASD and growth delays who was found to have two missense substitutions in *SALL4* that were inherited in trans and predicted to affect function. Her parents each carried one variant and were not reported to have eye defects. As *SALL4* mutations have been associated with ocular defects in AROS (Table 2), we consider that inheritance of both of these sequence variants is likely to account for her eye malformations and that the inheritance pattern in this family is most consistent with compound heterozygosity for two hypomorphic mutations that cause birth defects. Variants in *SALL4* typically result in an altered reading frame with premature protein truncation and nonsense-mediated decay with the phenotype resulting from haploinsufficiency (Borozdin et al., 2004a, Kohlhase et al., 2005; Borozdin et al 2007). As both parents were not reported to have any clinical findings, it is probable that both of the missense variants do not significantly affect function in the

heterozygous state. Non- penetrance for *SALL4* variants has been infrequently demonstrated and was found in 1/69 (1.4%) people with a pathogenic *SALL4* sequence variant (Kohlhase et al., 2015).

Missense variants in *SALL4* are rare and only one has previously been described. A five year old child with type III Duane anomaly, deep-set eyes with partial strabismus of right eye, hypotelorism, a single central upper incisor, pituitary hypoplasia with short stature, growth hormone deficiency and an empty sella, had c.[2663A>G], predicting p.(His888Arg), that was inherited from the child's father and paternal grandmother with type I Duane anomaly, hypoplasia of the thenar eminences and double-rowed maxillary central incisors (Miertus et al., 2006; Kohlhase et al., 2015). The variant p.(His888Arg) alters a histidine residue critical for zinc finger structure in the most carboxy terminal double zinc finger of SALL4 and was predicted to lead to increased DNA-binding affinity of the domain and altered zinc finger binding (Miertus et al., 2006).

Sall4 can act either as a transcriptional repressor or activator. The *SALL4* promoter is strongly activated by TCF4E/LEF1 and regulated by canonical Wnt signaling (Boehm et al., 2006). *Sall4* interacts with *Tbx5* during limb and heart development in mice and fish (Boehm et al., 2007) and regulates *Bmp4* expression in a *Sall4* null mouse (Sakaki-Yumoto et al., 2006). *Sall4* also interacts with *Nanog*, a homeodomain transcription factor that can sustain pluripotency in murine ES cells (Wu et al., 2006). Finally, *Sall4* represses *pou5f3* during neurogenesis, enabling the neural plate to respond to inductive signals from Fgf, retinoic acid and Wnt (Young et al., 2014).

The molecular mechanism responsible for the eye defects associated with *SALL4* haploinsufficiency is unknown.We hypothesized that reduced levels of *SALL4* would alter regulation of *BMP4*, as homozygous null mice for *Sall4* showed significant reduction in *Bmp4* (Sakaki-Yumoto et al., 2006). However, no change in *BMP4* expression was found after siRNA targeting *SALL4 in vitro*, indicating that the decrease in *BMP4* may be context dependent. Instead, we found that reduced expression for *SALL4* resulted in upregulation of *SOX2* in HEK293T cells only (Table S4; Fig. 1). In *Danio rerio*, *sox2* and *sall4* are expressed in the cell nucleus and their expression patterns overlap (Thisse et al., 2001). Chip on chip analysis in W4 ES cells has confirmed an interaction between *Sall4* and *Sox2*, although reduced *Sall4* expression was associated with reduced *Sox2* expression, rather than upregulation (Yang et al., 2008). Haploinsufficiency for *SOX2* is strongly associated with MAC (Bardakjian et al., 2015), but the developmental effects of upregulation of *SOX2* have been less well studied and our siRNA experiments do not allow us to conclude that altered *SOX2* expression is relevant to the eye defects found with *SALL4* haploinsufficiency.

Conclusion

We report a female with unilateral microphthalmia and coloboma and optic nerve hypoplasia who had two missense substitutions in *SALL4* that were inherited in trans and predicted to disrupt function. In view of the previously described role of haploinsufficiency for *SALL4* in the pathogenesis of eye defects, we consider that these variants are most probably relevant to the ocular phenotype, although *SALL4* missense mutations have not been frequently

reported. A reduction in *SALL4* expression using siRNA was associated with increased *SOX2* expression in HEK293T cells, but not in NT2 cells, and further work is needed prior to concluding that dysregulation of *SOX2* is relevant to the mechanism for *SALL4* mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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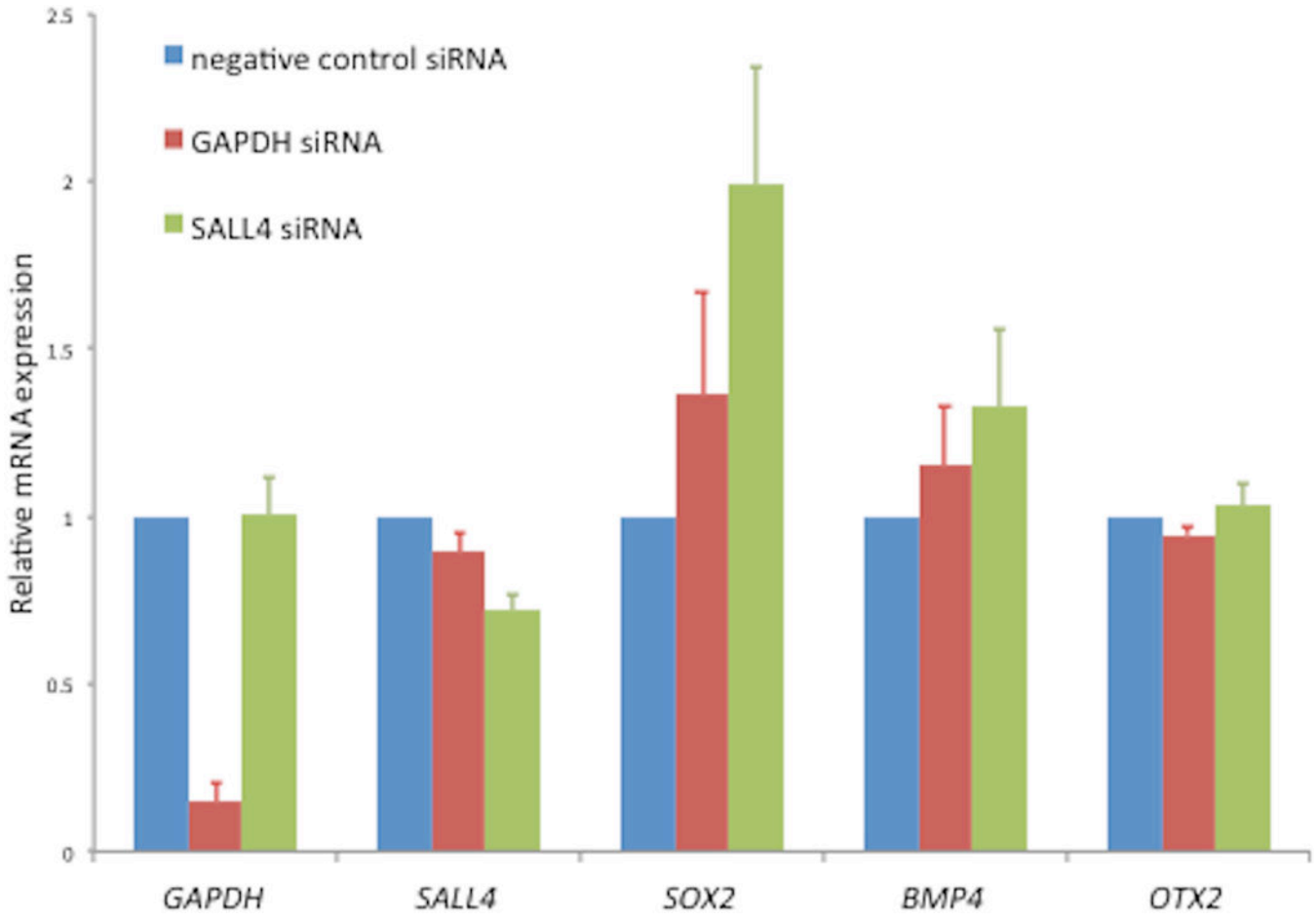

Fig. 1. siRNA and qRT-PCR in HEK293T cells show that reduced *SALL4* expression results in up-regulation of *SOX2*, but not *BMP4*.

Genes amplified by gene-specific primers are indicated on the X-axis; relative mRNA expression is indicated on the Y-axis. Blue columns show data from wells transfected with negative control siRNA and expression for these wells has been normalized to 1 for comparison. Red columns show data from wells treated with 30 nM *GAPDH* siRNA treatment and green columns show data from wells treated with 30 nM *SALL4* siRNA (s32817). The first set of three columns confirms reduced expression of *GAPDH* after 30 nM *GAPDH* siRNA treatment (red column) using GAPDH-specific primers. The second set of columns confirms reduced expression of *SALL4* after 30 nM *SALL4* siRNA treatment (green column) using SALL4 specific primers. The set of columns relating to *SOX2* shows significantly increased *SOX2* expression after 30 nM *SALL4* siRNA treatment (green columns) compared to control siRNA; p value <0.05. The remaining sets of columns show no significant changes in *BMP4* and *OTX2* expression. All results are expressed as the mean ± standard deviation; sample size n = 4

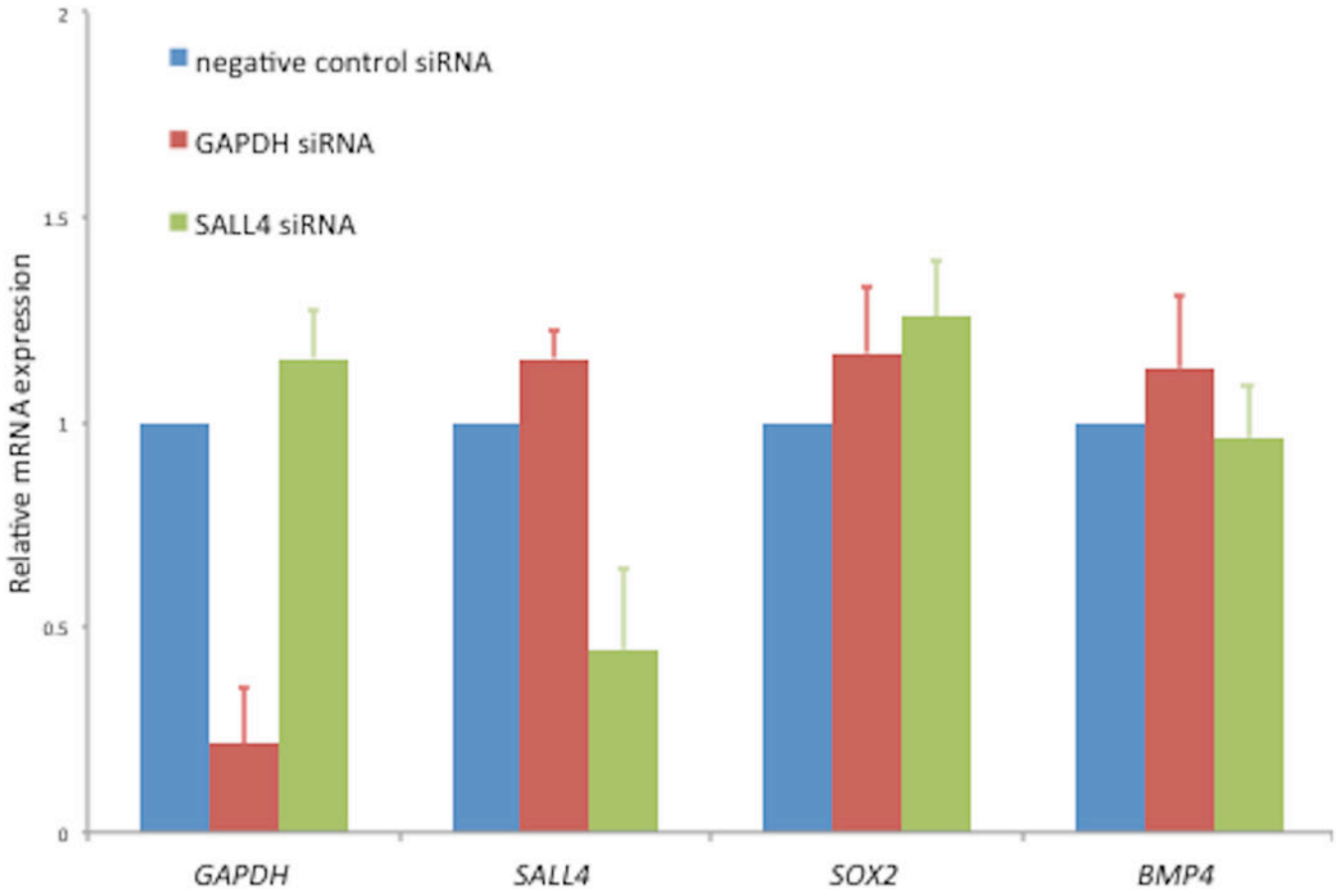

Fig. 2. siRNA and qRT-PCR in NT2 cells show that reduced *SALL4* expression does not results in up-regulation of *SOX2*.

Genes amplified by gene-specific primers are indicated on the X-axis; relative mRNA expression is indicated on the Y-axis. Blue columns show data from wells transfected with negative control siRNA and expression for these wells has been normalized to 1 for comparison. Red columns show data from wells treated with 30 nM *GAPDH* siRNA treatment and green columns show data from wells treated with 30 nM *SALL4* siRNA (s32817). The first set of three columns confirms reduced expression of *GAPDH* after 30 nM *GAPDH* siRNA treatment (red column) using GAPDH-specific primers. The second set of columns confirms reduced expression of SALL4 after 30 nM *SALL4* siRNA treatment (green column) using *SALL4* specific primers. The set of columns relating to *SOX2* does not show significantly increased *SOX2* expression after 30 nM *SALL4* siRNA treatment (green columns) compared to control siRNA (p value >0.05). The remaining set of columns shows no significant changes in *BMP4* expression. All results are expressed as the mean ± standard deviation; sample size n = 3

Table 1.

Deleterious sequence variants in *SALL4* a patient with microphthalmia, coloboma, and optic nerve hypoplasia.

Gene	Nucleotide	Amino acid alteration	Inheritance	SIFT[1]	PolyPhen-2[2]	Mutation taster[3]	ExAC browser[4]	Predicted effect
SALL4	c.575C>A	p.Ala192Gln	Pat.	D[5]; 0	PD[6]; 0.994 (sens. 0.69; spec.0.97)	DC[7]; 0.999	6/121,266; 0.00004948	Mutation
SALL4	c.2053G>C	p.Asn685His	Mat.	D; 0.01	PD; 0.983 (sens. 0.74; spec. 0.96)	DC; 0.999	Not present	Mutation

[1] SIFT = http://sift.jcvi.org

[2] Polyphen-2 = http://genetics.bwh.harvard.edu/pph2/

[3] Mutation Taster = http://www.mutationtaster.org

[4] ExAC Browser = http://exac.broadinstitute.org/

[5] D = Damaging

[6] PD = Probably damaging

[7] DC = Disease causing.

Table 2.

Phenotypic features of individuals with *SALL4* and *SALL1* mutations and structural eye defects in addition to Duane anomaly.

Family	Ocular findings	Other clinical features	*SALL4* mutation	Reference
Proband	Microphthalmia; coloboma; Bilateral optic nerve hypoplasia	Ventricular septal defect; atrial septal defect; growth delays	c.575C>A; p.Ala192Glu c.2053G>C; p.Asp685His	This paper
I-1 Mother	-	Absent thumbs; shortened forearms; L renal hypoplasia/pelvic kidney; conductive and sensorineural hearing loss	c.2593C>T; p.Arg865*	Becker et al., 2002; Kohlhase et al., 2003
II-1 Proband	Dysplastic optic discs; bilateral nystagmus; bilateral Duane anomaly	Absent thumbs/radii; shortened humeri; conductive hearing loss	c.2593C>T; p.Arg865*	Kohlhase et al., 2003
II-3 Proband	Cataract, iris and choroideal coloboma involving optic nerve, nystagmus, strabismus, bilateral microphthalmia and microcornea	L thumb aplasia; R thumb hypoplasia; crossed renal ectopia	c.2477delC; p.Pro826*fs*	Aalfs et al.,1996; Borozdin et al., 2004A
I-1 Mother	-	L thumb hypoplasia	Not tested	Borozdin et al., 2004A
II-4 Sister	-	Bilateral aplasia of thumbs, radii and ulnae; L humeral shortening	Not tested	Borozdin et al., 2004A
Family 3 Mother	L iris coloboma; L retinal coloboma; L Duane anomaly	R Triphalangeal thumb, short stature	c.1223_1226dupGACC; p.Phe410*fs*	Kohlhase et al., 2005
Family 3 Daughter	L iris coloboma; L retinal coloboma	Bilateral triphalangeal thumbs, short stature, hip dislocation, hypophyseal hypoplasia	c.1223_1226dupGACC; p.Phe410*fs*	Kohlhase et al., 2005
Family 2 1–2	R Duane anomaly	Bilateral absent thumbs and radii; ulna hypoplasia	Deletion exon 2 *SALL4*	Becker et al., 2002 Borozdin et al., 2004B
Family 2 II-1	R ‘morning glory’ optic disc; L dysplastic optic disc; L retinal coloboma; L Duane anomaly	Absent L thumb; hypoplastic R thumb; mild pelvicalyceal dilatation with grade I vesicoureteric reflux; moderate conductive hearing loss	Deletion exon 2 *SALL4;* Supernumerary Bisatellited marker derived from chromosome 22	Becker et al., 2002 Borozdin et al., 2004B
Family F	R chorioretinal coloboma with no vision in R eye	Imperforate anus, R preauricular tag, bilateral sensorineural hearing loss, long thumbs, polycystic kidneys and hypospadias	c.1145_1146insTA p.Leu383*fs*	Botzenhart et al., 2005