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A spontaneous mutation in *Srebf2* leads to cataracts and persistent skin wounds in the lens opacity 13 (*lop13*) mouse

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Abstract

Lens opacity 13 (lop13) is a spontaneous, autosomal recessive mouse mutant that exhibits nuclear cataracts. Histological analysis revealed swollen lens fiber cells and the presence of bladder cells within the lens cortex, as well as morgagnian globules and liquefied material at the lens posterior. At three months of age, in addition to cataracts, *lop13* mice also develop persistent skin wounds. Linkage analysis assigned the *lop13* locus to a 1.1 Mb region on mouse Chr. 15, encompassing 19 candidate genes. Sequence analysis identified a C3112T mutation in exon 18 of Sterol Regulatory Element Binding-Transcription Factor 2 (*Srebf2*) resulting in the R1038C substitution of a highly conserved arginine within the *Srebf2* regulatory domain. *Srebf2* belongs to a family of membrane bound basic-helix-loop-helix leucine zipper transcription factors that control the expression of genes involved in the biosynthesis and uptake of cholesterol and fatty acids. The lack of complementation observed in *Srebf2^{lop13/GT}* compound heterozygotes carrying the *Srebf2* gene trapped allele (*Srebf2^{GT}*), provides genetic evidence that the identified C3112T substitution in *Srebf2* is responsible for the *lop13* phenotype. Gas chromatography analysis identified lower levels of cholesterol in the *lop13* brain, liver and lens when compared to wild-type mice. These findings suggest that *lop13* is a hypomorphic mutation in *Srebf2*. As such, the *lop13* mouse presents an invaluable *in vivo* model for studying the contribution of *Srebf2* and cholesterol to maintaining the homeostasis of the lens and skin.

Introduction

Ocular lens transparency is essential for transmitting and focusing light onto the retina. Cataracts are defined as the loss of lens transparency and are considered clinically relevant if they significantly affect visual acuity. Although treatable with surgery, cataracts remain the leading cause of blindness worldwide, affecting approximately 18 million people (Foster and Resnikoff 2005). Age-related cataracts are the most common form of cataracts, in which the onset is after the age of forty (Congdon et al. 2004). As a result of population growth and aging worldwide, it has been predicted that by 2020 the number of individuals affected with cataracts would double from that observed in 2004 (Resnikoff et al. 2004).

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The development of age-related cataracts has been associated with environmental factors such as exposure to UV light, smoking, diabetes, myopia, nutrition, obesity, HDL cholesterol levels and corticosteroid use (Abraham et al. 2006; DeBlack 2003; Hodge et al. 1995; Taylor 1999; West and Valmadrid 1995). In addition, twin studies established a significant genetic component contributing to age-related cataracts (Hammond et al. 2001a; Hammond et al. 2001b; Hammond et al. 2000). It has been postulated that as many as 40 genes might be involved in cataract formation (Hejtmancik and Kantorow 2004). Allelic variations in *EPHA2*, *GJA8*, *GALT*, *SLC16A12*, *HSF4*, *GALK1*, *FTL* and *CRYAA* have been associated with the formation of age-related cataracts in some populations (Bhagyalaxmi et al. 2009; Faniello et al. 2009; Jun et al. 2009; Karas et al. 2003; Liu et al., 2011; Okano et al. 2001; Shi et al. 2008; Shiels et al., 2010; Shiels et al. 2008; Zuercher et al., 2010). However, the identified variations in these genes contribute to only a small proportion of all cases of age-related cataracts.

Congenital cataracts, although only a small portion of all cataract cases, have contributed a great deal to our knowledge about the molecular etiology of cataracts (Francis and Moore 2004; Graw 2004). To date, at least 39 cataract loci have been mapped and mutations have been identified in 26 genes (Hejtmancik 2008; Shiels et al. 2010; Shiels and Hejtmancik 2007). The genes and mutations associated with Mendelian forms of congenital cataracts have been recently reviewed (Hejtmancik 2008; Shiels and Hejtmancik 2007). The majority of mutations were identified in crystallins, a family of abundantly expressed structural proteins that are essential for transparency of the lens. In addition, mutations in gap junction proteins, lens membrane proteins, beaded filament structural proteins, and transcription factors have also been identified as contributing to Mendelian forms of congenital cataracts (Hejtmancik 2008). Although great progress has been made, the genetic basis of numerous Mendelian cataract loci remains unknown. At least 16 orphan cataract loci have been mapped onto a chromosome where the underlining genes and mutations remain unknown (Shiels et al. 2010).

Although congenital cataracts often occur as an isolated defect, the loss of lens transparency has been associated with syndromic diseases presenting clinically with additional ocular defects or with other systemic abnormalities. Congenital cataracts associated with syndromes that include only ocular defects often occur with varying defects of the anterior segment, optic nerve, retina and vitreous body. Mutations in the transcription factors *FOXE3*, *PAX6*, *PITX3*, *MAF* and *VSX2* have been associated with ocular syndromes that include cataracts, microphthalmia, secondary glaucoma and anterior segment defects (Gould and John 2002; Shiels et al., 2010). Congenital cataracts have also been associated with severe systemic disorders, including neurologic and metabolic disorders, with abnormalities of numerous organs. These syndromic cataracts are known to be caused by various genetic defects including single gene defects, chromosomal abnormalities, triplet repeat disorders and mitochondrial gene defects (Shiels et al. 2010). However, as with age-related and congenital cataracts, numerous genes associated with syndromic cataracts remain unknown.

In our quest for the identification of novel genes essential for lens transparency, we evaluated the lens opacity 13 (*lop13*) mouse, a spontaneous autosomal recessive mutation previously reported to exhibit nuclear cataracts between 3-4 weeks of age (Varnum 1981). In addition to cataracts, we show that *lop13* mice develop previously unreported persistent skin wounds. The initial linkage analysis assigned the *lop13* locus to mouse Chr. 15 (Chang et al. 2005). The results from this study show that a mutation in the sterol regulatory element binding transcription factor 2 (*Srebf2*) gene is responsible for the *lop13* phenotype. The role of *Srebf2* has been previously established as a transcriptional regulator of genes involved in *de novo* cholesterol biosynthesis, as well as genes associated with endocytic cholesterol

uptake (Horton et al. 2002). The role of *Srebf2* during the development and/or homeostasis of the lens and skin had never been evaluated.

Materials and methods

Mice

The *lop13* and C3A.BLiA-*Pde6b*^{+/J} mice were obtained from Jackson Laboratory. C3A.BLiA-*Pde6b*^{+/J} is a *Pde6b*⁽⁺⁾ strain of C3H/HeJ (<http://jaxmice.jax.org/strain/001912.html>). Both C3A.BLiA-*Pde6b*^{+/J} and *lop13* strains exhibited normal breeding patterns. The *Srebf2* gene trap (GT) mouse was generated from a trapped embryonic stem (ES) cell line YTA054 (EI183084.1) (Stryke et al. 2003). The mutant allele was designated as *Srebf2*^{GT}. The YTA054 cells were injected into blastocysts from C57BL/6J mice and implanted into pseudopregnant C57/BL6 females. Germ line transmission was indicated by coat color, and heterozygote mutant animals were identified by PCR genotyping. The chimeric males were outcrossed to C57BL/6J females and progeny were genotyped for the presence of the trapped allele. *Srebf2*^{GT/+} mice were maintained on 129P2/OlaHsd and C57BL/6J mixed backgrounds. *Srebf2*^{GT/+} to *Srebf2*^{GT/+} breedings generated 51 mice from 9 litters and the progeny were genotyped. For complementation studies, *Srebf2*^{GT/+} to *lop13/lop13* breedings generated 37 mice from 12 litters and the progeny were genotyped. Mendelian segregation for all breedings was evaluated using the χ^2 test.

PCR Genotyping

2 mm tail clippings were collected and DNA was purified via boiling the tail sample with 50 mM NaOH, neutralizing the reaction with 1 M Tris pH 8.0 and centrifugation of the sample. Initially, PCR was carried out under the following conditions: 94°C for 3 minutes, (94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds) X 30 cycles in addition to 7 minutes at 72°C using primers: TTA TCG ATG AGC GTG GTG GTT ATG C and GCG CGT ACA TCG GGC AAA TAA TAT C that annealed in the β -galactosidase gene within the pGT0Lxf gene trap vector. To determine the exact genomic site of the trap insertion, we utilized Long Range-PCR (LR-PCR) with primers: TTC CAA GGA TCA GCT CAG GTC AC and TCT TTG AGC ACC AGA GGA CAT CC using the LR-PCR kit (Qiagen) following the manufacturer's protocol. The PCR conditions were: 93°C for 3 minutes, (93°C for 15 seconds, 62°C for 30 seconds and 68°C for 5 minutes) X 35 cycles. PCR products were electrophoresed, gel purified and sequenced as described previously (Talamas et al. 2006). Once the pGT0Lxf insertion site was cloned, genotyping of the wild-type *Srebf2* and *Srebf2*^{GT} allele was performed using the same forward primer ATC TAA CTG GTC CCC TGG AGC AC and reverse TGG GGA GGG AAT GAA GTA CTG ACT to amplify *Srebf2* intron 1 of the wild-type allele or reverse CAT ACA GTC CTC TTC ACA TCC ATG C to amplify the region of *Srebf2* intron 1 harboring the gene trap.

Clinical evaluation and histology

For clinical evaluation, mouse eyes were dilated with 1% Atropine Sulfate (Bausch & Lomb) and examined with a Topcon SL-D8Z slit lamp biomicroscope with a Nikon SLR-based Photo Slit Lamp imaging system. For histological analysis, eyes from eight week old animals were fixed in Zinc-formalin, or Davidson's solution, embedded in paraffin and sectioned to 4 μ m thickness as previously described (Hassemer et al. 2010). Skin wounds were imaged with a RetCam II (Clarity Medical Systems). Skin samples from mouse backs, as well as whole eye globes containing the eyelids were collected from wild-type and *lop13* mice and fixed in Davidson's solution, embedded in paraffin and sectioned to 4 μ m thickness. Following H&E staining, sections were mounted and photographed with a Nikon DS-Fi1 camera on a Nikon Eclipse 80i microscope.

Linkage Analysis

The *lop13* locus has been maintained on the congenic 129/sv-ter background. For linkage studies, *lop13* mice were outcrossed to C3A.BLiA-*Pde6b*⁺/J. The resulting F1 progeny were backcrossed to *lop13* mice to generate 326 F2 progeny. At four weeks of age, F2 progeny were clinically evaluated for the presence of cataracts as described above. Following phenotyping, F2 progeny were euthanized and tissues were collected. Genomic DNA was purified from collected tissues as previously described (Talamas et al. 2006). Initially, 25 F2 progeny were genotyped as previously described (Talamas et al. 2006) with polymorphic microsatellites selected across Chr. 15: *D15Mit86*, *D15Mit209*, *D15Mit105*, *D15Mit28*, *D15Mit260*, *D15Mit118*, *D15Mit33*, *D15Mit107*, *D15Mit108*, *D15Mit161* and *D15Mit14*. Microsatellite alleles were scored following electrophoresis. Linkage data was analyzed with MapManager QTX (<http://www.mapmanager.org/mmQTX.html>). An additional 119 progeny were mapped with *D15Mit29*, *D15Mit28*, *D15Mit260*, *D15Mit118*, *D15Mit33*, *D15Mit107*, *D15Mit108*, and *D15Mit161* only. Further fine mapping analysis was performed on an additional 182 F2 progeny using microsatellites *D15Mit33* and *D15Mit118*. The chromosomal break points were further genotyped in the five recombinant animals with SNPs we identified in *Csdc2* and *Serhl*, *ss325997172* and *ss32997173* respectively. The PCR conditions for *ss325997172* were: 95°C for 3 minutes, (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 sec) X 30 cycles, 72°C for 7 minutes using primers: GCT GGC TTC AAA CTG TCC TTT CA and GGT TGA AGG ACT GAG TGG GTG AC. The PCR conditions for *ss32997173* were: 95°C for 3 minutes, (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds) X 30 cycles, 72°C for 7 minutes using primers: CGC TAA AAC TGG GGC TAA GGA GA and ACT GAG GCA CAG GAA AGG GAG AA. The PCR products were electrophoresed, gel extracted and sequenced as described previously (Talamas et al. 2006).

Candidate gene analysis

For the exon and intron/exon junction analysis, primers were designed to anneal 50 bp downstream from intron/exon junctions, targets were PCR amplified from genomic DNA and sequenced as previously described for exon sequencing (Talamas et al. 2006). For cDNA analysis and *Sreb2* expression, RNA was purified from the spleens or eyes from wild-type and *lop13/lop13* mice, reverse transcribed and amplified as previously described (Talamas et al. 2006). DNASTar software was utilized for comparative analysis of the sequences (Madison, WI.). The list of primer sequences is summarized in Table S1.

Cholesterol analysis

Livers (n=7), brains (n=8), and lenses (n=4) from wild-type and *lop13* mice were individually homogenized in a Dounce homogenizer in 1.0 mL of PBS. A 1:1:0.5 mixture of chloroform:methanol:water was utilized to extract the lipids. The organic phases were combined, washed once with 0.5 mL of KCl (1M) followed by 0.5 mL of water, dried at 40°C under N₂ gas and stored at -80°C. The cholesterol analysis was performed as previously described (Ryu et al. 2006) with modifications. Briefly, an equivalent of 0.1 mg of lipid was used per sample. Cholesterol-d6 (4.366 nmoles) (C/D/N Isotopes Inc, Pointe-Claire) was added to each sample prior to derivatization. Each lipid extract was dried under nitrogen and re-dissolved in pyridine (70 uL) and the sample derivatized by addition of a silylation reagent (30 uL) such as *N*-trimethylsilyl-*N*-methyltrifluoroacetamide with trimethylchlorosilane (MSTFA+1% TMCS) and incubated at 50°C for 1 h. GC-MS analysis of the silylated extracts was performed on an Agilent model 6890N GC coupled to an Agilent model 5975N quadrupole mass selective detector (MSD). Separation was achieved on a HP-5MS fused silica capillary column (5% phenyl methyl siloxane; column length, 60 m; internal diameter, 0.25 mm; film thickness, 0.25 µm). The GC operating conditions were as follows: initial temperature of 150°C, increasing to 300°C a rate of 25°C/min, then from

300 to 325°C at a rate of 3°C/min with a final isothermal hold at 325°C for 5 min (total run duration is 19.33 minutes). Helium was used as carrier gas at an initial flow of 1.0 mL/min. The samples (1 µL) were injected in a split mode with an Agilent 7683 autosampler. The temperature of the injector and the mass spectrometry transfer line was set at 280°C. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV ionization energy, scanning for selective ions for each metabolite: cholesterol (*m/z* 368 and 458) and cholesterol-d6 (*m/z* 374 and 464). Data were acquired and processed with Agilent Chemstation software, AMDIS (www.amdis.net), and MET-IDEA (Broeckling et al. 2006).

The serum cholesterol analysis was performed as previously described (Garber et al. 2000). Briefly, wild-type (*n*=4) and *lop13* (*n*=4) mice were fasted for 8 hours prior to the blood draw. Blood was collected from the submandibular vein and serum was collected following centrifugation at 13,000 rpm for 10 minutes. Cholesterol levels were subsequently analyzed via FPLC using the Infinity Total Cholesterol reagent (Fisher).

Results

lop13 phenotype

Clinical evaluation of *lop13* eyes at postnatal day 28 (P28) confirmed the presence of bilateral nuclear cataracts (data not shown) as previously reported for *lop13* mice (Varnum 1981). Between 4-10 weeks of age, the *lop13* nuclear cataracts progressed to mature cataracts (Figure 1B); by three months of age, all *lop13* mice exhibited hypermature cataracts (data not shown). The histological analysis of *lop13* lenses confirmed the clinical findings. Lens epithelial cells at the anterior of the lens were normal (data not shown). Within the lens cortex and at the lens posterior, (Figures 1D and 1F) abnormalities of the lens fiber cells, such as swollen cortical fibers and bladder cells (Cogan 1962) were noted. Liquefied lens material and proteinaceous morgagnian globules (Cogan 1962) associated with the degeneration of lens fibers were also present at the posterior poles (Figure 1F).

In addition to the cataract phenotype, around 3 months of age, *lop13* mice developed persistent wounds of the skin. The wounds were observed in the areas surrounding the eye (Figure 2B) and the skin on the back of the neck (not shown). Once wounds developed, they did not heal. Histological analysis of *lop13* skin, prior to the wound formation, did not identify any difference in the skin morphology between wild-type and *lop13* mice (Figures 2C and 2D).

Genetic mapping of the *lop13* locus

An earlier report established linkage of the *lop13* locus to mouse Chr. 15 without any additional information regarding the genetic position of *lop13* on Chr. 15 (Chang et al. 2005). To further map the *lop13* locus, we generated 326 F2 backcross progeny that were clinically evaluated for cataracts. The analysis identified 46.3% (151/326) of the mice affected with cataracts and 53.7% (175/326) of the mice with clinically normal lenses. The χ^2 analysis confirmed that the expected Mendelian ratios were observed. To confirm linkage to Chr. 15, we initially genotyped 25 F2 progeny with 12 polymorphic microsatellites spread across Chr. 15. The analysis confirmed linkage of the *lop13* locus to Chr. 15 (LOD>10) and assigned *lop13* as being between microsatellites *D15Mit29* and *D15Mit161* (data not shown). We genotyped an additional 119 F2 backcross mice with microsatellite markers between *D15Mit29* and *D15Mit161*. The analysis mapped the *lop13* locus as being between microsatellites *D15Mit118* and *D15Mit33* (Figure 3A). An additional 182 F2 progeny were genotyped with *D15Mit118* and *D15Mit33*, which led to the identification of three more recombinant animals between these two microsatellites (data not shown). To further map the chromosomal break points in the five recombinant animals, we searched for additional

polymorphic markers within the genomic region between *D15Mit118* and *D15Mit33*. However, no other microsatellites polymorphic between C3A.BLiA-*Pde6b*^{+/J} and 129/sv-ter were identified. The SNP database analysis (<http://www.ncbi.nlm.nih.gov/SNP/MouseSNP.cgi>) identified *rs8248376* and *rs4230909* as polymorphic SNPs within the genomic region between *D15Mit118* and *D15Mit33*. However, *rs8248376* and *rs4230909* were not informative in our cross (data not shown) precluding further mapping of the chromosomal break points with established markers.

The evaluation of the mouse genome (Build 37/mm9 assembly) established that the physical distance between *D15Mit118* and *D15Mit33* is 1.8 Mb (Figure 3C) encompassing 29 candidate genes (<http://genome.ucsc.edu/cgi-bin/hgGateway>). We evaluated the NCBI (<http://www.ncbi.nlm.nih.gov/gene/>) and MGI databases (<http://www.informatics.jax.org/>) to identify if any genes from the *lop13* critical region were associated with cataracts and/or skin phenotypes in humans or mice. None of the candidate genes within this region had been previously identified as being associated with cataract formation or were identified as playing a role in the development or homeostasis of the lens and skin. Thus, we initiated sequencing of the 29 candidate genes. Our sequence analysis of the *Csdc2* gene identified an SNP that was termed *ss325997172*, exhibiting polymorphisms between the C3A.BLiA-*Pde6b*^{+/J} strain (“C” allele) and the 129/sv-ter strain (“A” allele) in this locus (Figure 3B). In the C3A.BLiA-*Pde6b*^{+/J} strain, *ss325997172* results in a nonsynonymous substitution of L7S, whereas the sequence from *lop13* mice matched the *Csdc2* (NM_145473) reference sequence. Similarly, our sequence analysis identified an SNP in the 5’ UTR region of the *Serhl* gene (nucleotide 82930685) termed *ss325997173* showing polymorphism between the C3A.BLiA-*Pde6b*^{+/J} (“G” allele) and the 129/sv-ter strain genotyped (“C” allele) in this locus. These two novel informative SNPs between the C3A.BLiA-*Pde6b*^{+/J} and the 129/sv-ter strains provided an opportunity to further narrow down the *lop13* candidate region. The chromosomal break points from the two recombinants established the *lop13* critical region as being between *ss325997172* and *ss325997173* (data not shown), a region of to 1.1 Mb (Figure 3C) encompassing 19 candidate genes (<http://genome.ucsc.edu/cgi-bin/hgGateway>).

The *Srebf2*^{C3112T} mutation

Sequence analysis identified a C3112T substitution in exon 18 of *Srebf2* (Figure 4A). *Srebf2* was reported to be a ubiquitously expressed transcription factor in embryonic and adult tissues (Miserez et al. 1997) although expression of *Srebf2* was never evaluated in the lens. RT-PCR analysis revealed expression of *Srebf2* in the embryonic and postnatal lens (Figure 4B). Thus, we proceeded with further evaluation of *Srebf2* as a candidate gene. All affected *lop13* mice exhibited the presence of the “T” allele at *Srebf2* nucleotide 3112. This mutation results in an R1038C substitution within the regulatory domain of the *Srebf2* protein (Figure 4C). The R1038 amino acid is highly conserved, suggesting an imperative function across different species (Figure 4D). To evaluate if the R1038C substitution was potentially a rare polymorphism, the mouse SNP database was evaluated (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=20788). No SNPs were identified in *Srebf2* exon 18. In addition, the genotype of the *Srebf2* C3112 locus was independently evaluated in the C57BL/6J, C3H/HeJ, CAST/EiJ, RIIS/J and DBA/2J strains. All strains exhibited the presence of the “C” allele at *Srebf2* nucleotide 3112, indicating that R1038 was present in all strains. This suggested that the identified *Srebf2*^{R1038C} substitution in *lop13* is most likely not a rare polymorphism.

In addition to the ORF sequences of *Srebf2*, there is a sequence for the microRNA *Mir33* within intron 16 of *Srebf2* (NR_029804.1). Sequence analysis of the entire *Srebf2* intron 16 encompassing the *Mir33* sequence did not reveal any difference between wild-type and *lop13* mice.

Complementation studies

To provide genetic evidence that the disease causing mutation in *lop13* mice was in the *Srebf2* gene, we initiated complementation studies. *Srebf2* knock-out mice die between E7-8 (Horton et al. 2002). The *Srebf2*^{+/-} line had not been maintained and *Srebf2*^{-/-} knock-out ES cells are no longer available (J. Goldstein *personal communication*). Instead, we took advantage of gene-trapped stem cells. The YTA054 ES cells contain the pGT0Lxf gene trap vector inserted into *Srebf2* intron 1

(<http://www.genetrap.org/cgi-bin/annotation.py?cellline=YTA054>). The pGT0Lxf gene trap vector contains a splice acceptor sequence upstream of a Beta-geo (β-galactosidase/neomycin phosphotransferase II fusion) reporter gene resulting in a fusion mRNA joining *Srebf2* exon1 to the Beta-geo coding sequence, predicted to generate a non-functioning fusion protein (<http://www.genetrap.org/cgi-bin/annotation.py?cellline=YTA054>). We set out to determine the precise genomic location of the pGT0Lxf gene trap insertion. LR-PCR analysis identified pGT0Lxf trap presence following nucleotide 81994703 in *Srebf2* intron 1 (Figure 5B). RT-PCR analysis of *Srebf2*^{GT/+} mice confirmed the presence of the fused *Srebf2*^{GT} transcript (Figure 5D). The *Srebf2*^{GT/+} mice were clinically evaluated for cataracts and did not differ from wild-type mice (data now shown). In order to obtain *Srebf2*^{GT/GT} mice, we set up *Srebf2*^{GT/+} X *Srebf2*^{GT/+} breedings and generated 51 mice from 9 different litters. Genotyping of the progeny at P0.5 identified 15 *Srebf2*^{+/+} and 36 *Srebf2*^{GT/+} mice with the absence of any *Srebf2*^{GT/GT} mice, suggesting embryonic lethality *Srebf2*^{GT/GT} mice. Thus, for complementation studies we set up *Srebf2*^{GT/+} X *lop13/lop13* breedings. This cross generated 37 mice from 12 litters. We clinically evaluated all complementation progeny. At six weeks of age, 12/37 mice exhibited cataracts similar to the cataracts observed in *lop13* mice (Figure 6B) and 25/37 had normal lenses (Figure 6A). Genotyping revealed that all mice exhibiting cataracts were compound heterozygote *Srebf2*^{lop13/GT} and animals that were clinically normal genotyped as *Srebf2*^{lop13/+}. The χ^2 analysis identified deviation from the expected Mendelian segregation ($p < 0.05$) suggesting embryonic lethality of some *Srebf2*^{lop13/GT} animals. Histological analysis confirmed the presence of cataracts in *Srebf2*^{lop13/GT} mice with the presence of swollen fibers and bladder cells at the cortex and liquefied lens material and morganian globules at the lens posterior (Figures 6D and 6F), similar in appearance to the cataract phenotype observed in *lop13* lenses. The *Srebf2*^{lop13/GT} mice also developed persistent wounds of the skin surrounding the eyes and back of the neck as observed in the *lop13* mice (data not shown). The lack of complementation in compound heterozygote *Srebf2*^{lop13/GT} mice indicates that the C3112T substitution in *Srebf2* is the mutation responsible for the *lop13* phenotype.

Cholesterol analysis

We next examined whether the *Srebf2*^{C3112T} mutation altered cholesterol levels in *lop13* tissues. Lipids from the brain, liver and lenses were extracted from *lop13* and wild-type mice and the cholesterol levels were analyzed by gas chromatography. A significant decrease in cholesterol levels was observed in the *lop13* brain as compared to the wild-type brain ($p < 0.05$), with the *lop13* brain containing an average of 219.25 ($\pm$ 34.3) μ mol of cholesterol/g of lipid and the wild-type brain containing 368.6 ($\pm$ 49) μ mol of cholesterol/g of lipid (Figure 7A). The average level of cholesterol in the *lop13* liver was 24.4 ($\pm$ 5.6) μ mol of cholesterol/g of lipid and 91.9 ($\pm$ 16.5) μ mol of cholesterol/g of lipid the wild-type liver, resulting in a significant decrease of cholesterol in the *lop13* liver ($p < 0.005$) (Figure 7B). A significant decrease ($p < 0.05$) in cholesterol levels was also observed in the *lop13* lens containing an average of 1.6 ($\pm$ 0.19) μ mol of cholesterol/g of lipid, compared to the wild-type lens containing 3.2 ($\pm$ 0.43) μ mol of cholesterol/g of lipid (Figure 7C). In contrast, there was no difference in serum cholesterol levels between the wild-type and *lop13* mice (Figure 7D).

Discussion

In this study, we identified a C3112T mutation in *Srebf2* exon 18 as being responsible for the *lop13* phenotype. *Srebf2* belongs to a family of membrane bound basic-helix-loop-helix leucine zipper transcription factors that control the expression of genes involved in the biosynthesis and uptake of cholesterol and fatty acids (Ikonen 2008; Rawson 2003). When cholesterol is abundant within a cell, the *Srebf2* precursor resides in the ER membrane bound to SREBF chaperone (Scap) via the regulatory domain (Goldstein et al. 2006; Ikonen 2008). Cellular cholesterol binds to Scap, altering its conformation so that insulin induced gene (*Insig*) binds to the Scap/*Srebf2* complex and retains the complex in the ER (Ikonen 2008; Radhakrishnan et al. 2007). In the absence of cholesterol within the cell, the lack of cholesterol binding to Scap renders the protein incapable of binding to *Insig* (Sun et al. 2007). The Scap/*Srebf2* complex is no longer retained in the ER and gets translocated to the golgi where site-1 and site-2 proteases cleave the hydrophobic loop of *Srebf2*, releasing the transcriptionally active N-terminal fragment to be transported to the nucleus (Goldstein et al. 2006; Ikonen 2008). The N-terminal *Srebf2* fragment can then bind to an E-box inverted DNA repeat (5'-CANNTG-3') sequence, as well as the direct DNA repeat of the sterol binding element (SRE) (5/TCACNCCAC-3') (Ikonen 2008; Raghov et al. 2008).

In mice, a null allele of the *Srebf2* gene results in embryonic lethality (Horton et al. 2002) indicating an essential role of *Srebf2* for embryogenesis. In humans polymorphisms in *SREBP2* have been identified as being associated with polygenic and familial hypercholesterolemia (Duan et al. 2005; Miserez et al. 2002; Muller and Miserez 2002). A mutation in *SREBF2* in a mutant CHO cell line was identified that resulted in a truncated gene product and resistance to transcriptional repression by 25-hydroxycholesterol. Consequently, the mutation resulted in the constitutive transactivation of *SREBF2* targets, independent of levels of sterols within the cell (Yang et al. 1994). These studies collectively suggest that mutations in *Srebf2* can result in a spectrum of phenotypes depending on the degree to which *Srebf2* transcriptional activity is affected and ultimately the levels of cholesterol within the cell.

The *Srebf2*^{C3112T} mutation identified in *lop13* mice results in a R1038C substitution of an evolutionarily conserved arginine residue within the *Srebf2* regulatory domain. Our results showed significantly reduced levels of cholesterol in *lop13* brains, livers and lenses. Our hypothesis is that R1038C alters the *Srebf2* regulatory domain's ability to efficiently interact with Scap, and consequently reduces the levels of the *Srebf2* precursor that is translocated to the golgi. Alternatively, the R1038C substitution might result in a conformational change of *Srebf2* that compromises the efficacy of the site-1 or site-2 or both proteases. These mechanisms would alter the levels of the transcriptionally active *Srebf2* N-terminal fragment in the nucleus and result in reduced transactivation of *Srebf2* downstream targets. This is further supported by our results showing that the levels of circulating cholesterol in *lop13* mice did not differ from wild-type mice. *Srebf2* is the principal transcriptional regulator of genes essential for the cholesterol synthesis and endocytic cholesterol uptake (Ikonen 2008; Raghov et al. 2008). Thus, the *Srebf*^{R1038C} hypomorphic function would consequently decrease both the rate of *de novo* cholesterol synthesis and the rate of endocytic cholesterol uptake.

In humans, mutations in seven out of 25 genes involved in the endogenous cholesterol synthesis pathway result in clinically distinct metabolic malformation syndromes. These syndromes include varying abnormalities of the brain, face, lens, lung, heart, liver, skeleton and skin (Herman 2003; Kelley and Herman 2001; Nwokoro et al. 2001). Cataracts and skin abnormalities were observed in some patients affected with metabolic malformation syndromes (Herman 2003; Kelley and Herman 2001). The mechanisms by which mutations

in different genes of the cholesterol synthesis pathway have resulted in variable and overlapping phenotypes has not yet been elucidated. It has been hypothesized that the accumulation of various sterol precursors in addition to the deficiency of cholesterol might result in the disruption of normal development (Nwokoro et al. 2001). The variation in clinical phenotypes might be dependent upon the specific precursor from the cholesterol synthesis pathway that is accumulating. However, the reduction of tissue cholesterol without the accumulation of cholesterol precursors, has been associated with cataracts in Shumiya rats (Mori et al. 2006). These rats carry hypomorphic mutations in lanosterol synthase and farnesyl diphosphate farnesyl transferase 1, resulting in 57% of the cholesterol levels detected in wild-type lenses (Mori et al. 2006). The results from our study also support that reduced levels of cholesterol in *lop13* tissues plays a role in the cataract and skin phenotype. However, we have not yet investigated if cholesterol precursors may also be accumulating in *lop13* tissues, and therefore participating in the formation of cataracts and skin abnormalities observed in *lop13* mice.

Although, basal skin cells are capable of endocytic cholesterol uptake from the circulation, most of the cholesterol in the epidermis is synthesized via *de novo* synthesis (Feingold 2009). The adult ocular lens is nonvascularized and fulfills its cholesterol needs solely via *de novo* synthesis (Cenedella 1983). These findings suggest an essential role of *de novo* cholesterol synthesis for the normal function of the lens and skin. This is further supported by studies where treatments of animals with some pharmacological agents that inhibit enzymes from the cholesterol synthesis pathway resulted in cataracts and defects in the barrier function of the skin (Cenedella 1996; Feingold 2009). Therefore, hypomorphic *Srebf^{R1038C}* function likely causes the *lop13* cataracts and skin wounds due to a decreased rate of *de novo* cholesterol synthesis. However, the precise role of *de novo* cholesterol synthesis in these tissues remains unknown. In the lens, almost all of the cholesterol is confined to the lens fiber cell plasma membranes. These membranes are unusual, as they contain the highest molar ratio of cholesterol to phospholipid of any known membrane (Li et al. 1985). Defects in the structure of *lop13* lens fiber cells further supports an essential role of cholesterol for the lens fiber cells. In the skin, cholesterol is a major lipid class in the stratum corneum that serves as the principal barrier against chemical, microbial and mechanical forces. The absence of any cellular defects in *lop13* skin prior to the wound formation suggests that cholesterol may be critical for wound repair. In addition, the expression of *Srebf2* and the rate of epidermal cholesterol synthesis are increased during repair of the permeability barrier of the skin (Feingold 2009). Future studies of animal models with cholesterol biosynthesis defects will likely yield insight into the specific mechanism(s) in which cholesterol is imperative in these tissues. The *lop13* mouse is an excellent resource for evaluating the role of *Srebf2* and *de novo* cholesterol synthesis in maintaining the homeostasis of the lens and skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Abraham AG, Condon NG, Gower E West. The new epidemiology of cataract. *Ophthalmol Clin North Am.* 2006; 19:415–425. [PubMed: 17067897]
- Bhagyalaxmi SG, Srinivas P, Barton KA, Kumar KR, Vidyavathi M, Petrash JM, Reddy G Bhanuprakash, Padma T. A novel mutation (F71L) in alphaA-crystallin with defective chaperone-like function associated with age-related cataract. *Biochim Biophys Acta.* 2009; 1792:974–981. [PubMed: 19595763]
- Broeckling CD, Reddy IR, Duran AL, Zhao X, Sumner LW. MET-IDEA: data extraction tool for mass spectrometry-based metabolomics. *Anal Chem.* 2006; 78:4334–4341. [PubMed: 16808440]
- Cenedella RJ. Source of cholesterol for the ocular lens, studied with U18666A: a cataract-producing inhibitor of lipid metabolism. *Exp Eye Res.* 1983; 37:33–43. [PubMed: 6873203]
- Cenedella RJ. Cholesterol and cataracts. *Surv Ophthalmol.* 1996; 40:320–337. [PubMed: 8658343]
- Chang B, Hawes NL, Hurd RE, Wang J, Howell D, Davisson MT, Roderick TH, Nusinowitz S, Heckenlively JR. Mouse models of ocular diseases. *Vis Neurosci.* 2005; 22:587–593. [PubMed: 16332269]
- Cogan DG. Anatomy of lens and pathology of cataracts. *Exp Eye Res.* 1962; 1:291–295. [PubMed: 14021964]
- Congdon N, Vingerling JR, Klein BE, West S, Friedman DS, Kempen J, O'Colmain B, Wu SY, Taylor HR. Prevalence of cataract and pseudophakia/aphakia among adults in the United States. *Arch Ophthalmol.* 2004; 122:487–494. [PubMed: 15078665]
- DeBlack SS. Cigarette smoking as a risk factor for cataract and age-related macular degeneration: a review of the literature. *Optometry.* 2003; 74:99–110. [PubMed: 12585737]
- Duan X, Zhu W, Li Y, Zhang Z, Zhao Y, Dao J, Xiao Y. The effect of sterol regulatory element-binding protein 2 polymorphism on the serum lipid in northern Chinese subjects. *J Lipid Res.* 2005; 46:252–257. [PubMed: 15547298]
- Faniello MC, Di Sanzo M, Quaresima B, Nistico A, Fregola A, Grosso M, Cuda G, Costanzo F. Bilateral cataract in a subject carrying a C to A transition in the L ferritin promoter region. *Clin Biochem.* 2009; 42:911–914. [PubMed: 19254706]
- Feingold KR. The outer frontier: the importance of lipid metabolism in the skin. *J Lipid Res.* 2009; 50(Suppl):S417–422. [PubMed: 18980941]
- Foster A, Resnikoff S. The impact of Vision 2020 on global blindness. *Eye (Lond).* 2005; 19:1133–1135. [PubMed: 16304595]
- Francis PJ, Moore AT. Genetics of childhood cataract. *Curr Opin Ophthalmol.* 2004; 15:10–15. [PubMed: 14743013]
- Garber DW, Kulkarni KR, Anantharamaiah GM. A sensitive and convenient method for lipoprotein profile analysis of individual mouse plasma samples. *J Lipid Res.* 2000; 41:1020–1026. [PubMed: 10828095]
- Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. *Cell.* 2006; 124:35–46. [PubMed: 16413480]
- Gould DB, John SW. Anterior segment dysgenesis and the developmental glaucomas are complex traits. *Hum Mol Genet.* 2002; 11:1185–1193. [PubMed: 12015278]
- Graw J. Congenital hereditary cataracts. *Int J Dev Biol.* 2004; 48:1031–1044. [PubMed: 15558493]
- Hammond CJ, Duncan DD, Snieder H, de Lange M, West SK, Spector TD, Gilbert CE. The heritability of age-related cortical cataract: the twin eye study. *Invest Ophthalmol Vis Sci.* 2001a; 42:601–605. [PubMed: 11222516]
- Hammond CJ, Snieder H, Gilbert CE, Spector TD. Genes and environment in refractive error: the twin eye study. *Invest Ophthalmol Vis Sci.* 2001b; 42:1232–1236. [PubMed: 11328732]
- Hammond CJ, Snieder H, Spector TD, Gilbert CE. Genetic and environmental factors in age-related nuclear cataracts in monozygotic and dizygotic twins. *N Engl J Med.* 2000; 342:1786–1790. [PubMed: 10853001]
- Hassemer EL, Le Gall SM, Liegel R, McNally M, Chang B, Zeiss CJ, Dubielzig RD, Horiuchi K, Kimura T, Okada Y, Blobel CP, Sidjanin DJ. The waved with open eyelids (woe) locus is a hypomorphic mouse mutation in Adam17. *Genetics.* 2010; 185:245–255. [PubMed: 20194968]

- Hejtmancik JF. Congenital cataracts and their molecular genetics. *Semin Cell Dev Biol.* 2008; 19:134–149. [PubMed: 18035564]
- Hejtmancik JF, Kantorow M. Molecular genetics of age-related cataract. *Exp Eye Res.* 2004; 79:3–9. [PubMed: 15183095]
- Herman GE. Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes. *Hum Mol Genet.* 2003 12 Spec No 1, R75–88.
- Hodge WG, Whitcher JP, Satariano W. Risk factors for age-related cataracts. *Epidemiol Rev.* 1995; 17:336–346. [PubMed: 8654515]
- Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest.* 2002; 109:1125–1131. [PubMed: 11994399]
- Ikonen E. Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol.* 2008; 9:125–138. [PubMed: 18216769]
- Jun G, Guo H, Klein BE, Klein R, Wang JJ, Mitchell P, Miao H, Lee KE, Joshi T, Buck M, Chugha P, Bardenstein D, Klein AP, Bailey-Wilson JE, Gong X, Spector TD, Andrew T, Hammond CJ, Elston RC, Iyengar SK, Wang B. EPHA2 is associated with age-related cortical cataract in mice and humans. *PLoS Genet.* 2009; 5:e1000584. [PubMed: 19649315]
- Karas N, Gobec L, Pfeifer V, Mlinar B, Battelino T, Lukac-Bajalo J. Mutations in galactose-1-phosphate uridyltransferase gene in patients with idiopathic presenile cataract. *J Inherit Metab Dis.* 2003; 26:699–704. [PubMed: 14707519]
- Kelley RI, Herman GE. Inborn errors of sterol biosynthesis. *Annu Rev Genomics Hum Genet.* 2001; 2:299–341. [PubMed: 11701653]
- Li LK, So L, Spector A. Membrane cholesterol and phospholipid in consecutive concentric sections of human lenses. *J Lipid Res.* 1985; 26:600–609. [PubMed: 4020298]
- Liu Y, Ke M, Yan M, Guo S, Mothobi ME, Chen Q, Zheng F. Association between gap junction protein- α 8 polymorphisms and age-related cataract. *Mol Biol Rep.* 2011; 38:1301–1307. [PubMed: 20582632]
- Miserez AR, Cao G, Probst LC, Hobbs HH. Structure of the human gene encoding sterol regulatory element binding protein 2 (SREBF2). *Genomics.* 1997; 40:31–40. [PubMed: 9070916]
- Miserez AR, Muller PY, Barella L, Barella S, Staehelin HB, Leitersdorf E, Kark JD, Friedlander Y. Sterol-regulatory element-binding protein (SREBP)-2 contributes to polygenic hypercholesterolaemia. *Atherosclerosis.* 2002; 164:15–26. [PubMed: 12119189]
- Mori M, Li G, Abe I, Nakayama J, Guo Z, Sawashita J, Ugawa T, Nishizono S, Serikawa T, Higuchi K, Shumiya S. Lanosterol synthase mutations cause cholesterol deficiency-associated cataracts in the Shumiya cataract rat. *J Clin Invest.* 2006; 116:395–404. [PubMed: 16440058]
- Muller PY, Miserez AR. Identification of mutations in the gene encoding sterol regulatory element binding protein (SREBP)-2 in hypercholesterolaemic subjects. *J Med Genet.* 2002; 39:271–275. [PubMed: 11950857]
- Nwokoro NA, Wassif CA, Porter FD. Genetic disorders of cholesterol biosynthesis in mice and humans. *Mol Genet Metab.* 2001; 74:105–119. [PubMed: 11592808]
- Okano Y, Asada M, Fujimoto A, Ohtake A, Murayama K, Hsiao KJ, Choeh K, Yang Y, Cao Q, Reichardt JK, Niihira S, Imamura T, Yamano T. A genetic factor for age-related cataract: identification and characterization of a novel galactokinase variant, “Osaka,” in Asians. *Am J Hum Genet.* 2001; 68:1036–1042. [PubMed: 11231902]
- Radhakrishnan A, Ikeda Y, Kwon HJ, Brown MS, Goldstein JL. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc Natl Acad Sci U S A.* 2007; 104:6511–6518. [PubMed: 17428920]
- Raghow R, Yellaturu C, Deng X, Park EA, Elam MB. SREBPs: the crossroads of physiological and pathological lipid homeostasis. *Trends Endocrinol Metab.* 2008; 19:65–73. [PubMed: 18291668]
- Rawson RB. The SREBP pathway—insights from Insigs and insects. *Nat Rev Mol Cell Biol.* 2003; 4:631–640. [PubMed: 12923525]
- Resnikoff S, Pascolini D, Etya’ale D, Kocur I, Pararajasegaram R, Pokharel GP, Mariotti SP. Global data on visual impairment in the year 2002. *Bull World Health Organ.* 2004; 82:844–851. [PubMed: 15640920]

- Ryu HK, Jung BH, Kim KM, Yoo EA, Woo JT, Chung BC. Determination of cholesterol in human hair using gas chromatography-mass spectrometry. *Biomed Chromatogr.* 2006; 20:999–1003. [PubMed: 16544269]
- Shi Y, Shi X, Jin Y, Miao A, Bu L, He J, Jiang H, Lu Y, Kong X, Hu L. Mutation screening of HSF4 in 150 age-related cataract patients. *Mol Vis.* 2008; 14:1850–1855. [PubMed: 18941546]
- Shiels A, Bennett TM, Hejtmancik JF. Cat-Map: putting cataract on the map. *Mol Vis.* 16:2007–2015. [PubMed: 21042563]
- Shiels A, Bennett TM, Hejtmancik JF. Cat-Map: putting cataract on the map. *Mol Vis.* 2010; 16:2007–2015. [PubMed: 21042563]
- Shiels A, Bennett TM, Knopf HL, Maraini G, Li A, Jiao X, Hejtmancik JF. The EPHA2 gene is associated with cataracts linked to chromosome 1p. *Mol Vis.* 2008; 14:2042–2055. [PubMed: 19005574]
- Shiels A, Hejtmancik JF. Genetic origins of cataract. *Arch Ophthalmol.* 2007; 125:165–173. [PubMed: 17296892]
- Stryke D, Kawamoto M, Huang CC, Johns SJ, King LA, Harper CA, Meng EC, Lee RE, Yee A, L'Italien L, Chuang PT, Young SG, Skarnes WC, Babbitt PC, Ferrin TE. BayGenomics: a resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res.* 2003; 31:278–281. [PubMed: 12520002]
- Sun LP, Seemann J, Goldstein JL, Brown MS. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. *Proc Natl Acad Sci U S A.* 2007; 104:6519–6526. [PubMed: 17428919]
- Talamas E, Jackson L, Koeberl M, Jackson T, McElwee JL, Hawes NL, Chang B, Jablonski MM, Sidjanin DJ. Early transposable element insertion in intron 9 of the Hsf4 gene results in autosomal recessive cataracts in *lop11* and *ldis1* mice. *Genomics.* 2006; 88:44–51. [PubMed: 16595169]
- Taylor HR. Epidemiology of age-related cataract. *Eye.* 1999; 13(Pt 3b):445–448. [PubMed: 10627822]
- Varnum DS. Nuclear cataract (nuc). *Mouse News Letter.* 1981; 64:59.
- West SK, Valmadrid CT. Epidemiology of risk factors for age-related cataract. *Surv Ophthalmol.* 1995; 39:323–334. [PubMed: 7725232]
- Yang J, Sato R, Goldstein JL, Brown MS. Sterol-resistant transcription in CHO cells caused by gene rearrangement that truncates SREBP-2. *Genes Dev.* 1994; 8:1910–1919. [PubMed: 7958866]
- Zuercher J, Neidhardt J, Magyar I, Labs S, Moore AT, Tanner FC, Waseem N, Schorderet DF, Munier FL, Bhattacharya S, Berger W, Kloeckener-Gruissem B. Alterations of the 5'untranslated region of SLC16A12 lead to age-related cataract. *Invest Ophthalmol Vis Sci.* 2010; 51:3354–3361. [PubMed: 20181839]

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