Genetics inMedicine | ORIGINAL RESEARCH ARTICLE

Hypomorphic mutations identified in the candidate Leber congenital amaurosis gene *CLUAP1*

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Purpose: Leber congenital amaurosis (LCA) is an early-onset form of retinal degeneration. Six of the 22 known LCA genes encode photoreceptor ciliary proteins. Despite the identification of 22 LCA genes, the genetic basis of ~30% of LCA patients remains unknown. We sought to investigate the cause of disease in the remaining 30% by examining cilia-associated genes.

Methods: Whole-exome sequencing was performed on an LCA cohort of 212 unsolved probands previously screened for mutations in known retinal-disease genes. Immunohistochemistry using mouse retinas was used to confirm protein localization and zebrafish were used to perform rescue experiments.

Results: A homozygous nonsynonymous mutation was found in a single proband in *CLUAP1*, a gene required for ciliogenesis and cilia maintenance. *Cluap1* knockout zebrafish exhibit photoreceptor cell death as early as 5 days after fertilization, and rescue experiments revealed that our proband's mutation is significantly hypomorphic.

Conclusion: Consistent with the knowledge that *CLUAP1* plays an important role in cilia function and that cilia are critical to photoreceptor function, our results indicate that hypomorphic mutations in *CLUAP1* can result in dysfunctional photoreceptors without systemic abnormalities. This is the first report linking mutations in *CLUAP1* to human disease and establishes *CLUAP1* as a candidate LCA gene.

Genet Med advance online publication 28 January 2016

Key Words: ciliopathy; *CLUAP1*; early-onset retinal disease; Leber congenital amaurosis (LCA); photoreceptor connecting cilium

INTRODUCTION

Leber congenital amaurosis (LCA; http://www.omim.org/phenotypicSeries/PS204000) is the most severe form of nonsyndromic inherited retinal disease, with severe visual impairment within the first year of life. LCA is characterized by nystagmus and an extinguished electroretinogram. LCA affects between 1/30,000 and 1/80,000 individuals, differing by population, and accounts for 5% of all inherited retinal diseases.[1] As of 2015, 22 genes have been linked to LCA; however, conventional techniques for molecular diagnosis find mutations in these genes in only ~70% of LCA patients, highlighting the genetic heterogeneity of LCA.[1–5] Gene therapy in the retina of human LCA patients has made significant progress in restoring sight, emphasizing the importance of understanding the genetic etiology of every patient's disease to allow access to such technologies.[6] Genetic counseling based on a patient's molecular diagnosis also provides the opportunity for mutation screening in offspring and spouses. Discovering the cause of disease in the remaining ~30% of LCA patients is therefore critical to enable treatment for this devastating disease.

An absent electroretinogram, a hallmark of LCA, is indicative of malfunctioning photoreceptor cells, which initiate the recorded electrical signal and are responsible for the generation of the a-wave.[7] Every identified LCA disease gene has a role relevant to the proper functioning of photoreceptor cells.[1,8–14] Photoreceptors are remarkable cells with a highly specialized morphology designed to facilitate photon detection and transduction of visual information. The most striking feature of photoreceptors cells is the elongated outer segment, which contains stacks of membrane discs that house the transmembrane photosensitive opsin proteins that capture photons and activate the visual transduction cascade. The outer segment is linked to the inner segment of photoreceptor cells by a cilium called the connecting cilium, from which the outer segment develops; the outer segment in conjunction with the connecting cilium is considered a large modified primary cilium. The inner segment contains the

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Submitted 30 June 2015; accepted 4 December 2015; advance online publication 28 January 2016. doi:10.1038/gim.2015.205

cell's protein synthesis machinery; therefore, every protein with a role in the outer segment must be transported through the connecting cilium for proper function. The outer segment undergoes a massive rate of protein turnover to accomplish phototransduction, including 10% of the outer segment being shed every day. Shed discs are phagocytized by neighboring retinal pigmented epithelial cells and are then replaced through the voluminous protein transport needed for new disc formation.[15] The connecting cilium is therefore homologous to the transition zone of primary cilia found in other cell types and operates as a gatekeeper of protein trafficking to the outer segment.[16]

Dysfunction of the connecting cilium results in varying degrees of photoreceptor degeneration depending on which component of the connecting cilium is perturbed. In contrast to LCA cilia genes, mutations in *MAK*, which localizes to the connecting cilium, cause a form of retinal degeneration that has its onset at an average age of 20 years.[17] Highlighting the importance of cilia in retinal disease, ~15% of known retinal-disease genes are involved in connecting cilium function. Interestingly, this proportion increases to ~25% when considering only known LCA genes (*CEP290*, *IQCB1*, *LCA5*, *RPGRIP1*, *SPATA7*, *TULP1*), supporting the established notion that cilia are critical to early stages of photoreceptor cell development and maintenance.[9,18] Combining the knowledge that cilia genes are good retinal-disease gene candidates with the knowledge that cilia are important for early photoreceptor development leads to the conclusion that cilia genes are excellent candidate LCA genes.[19]

Clusterin-associated protein 1, or *CLUAP1*, is required for ciliogenesis and localizes to the base and tip of cilia *in vitro* in mouse embryonic fibroblasts.[20] CLUAP1 associates with the intraflagellar transport (IFT) complex B group of proteins and undergoes IFT in both invertebrates and vertebrates.[21,22] *Cluap1*$^{-/-}$ mice exhibit midgestation lethality due to developmental complications attributed to their lack of primary cilia.[20] Interestingly, zebrafish with abolished *cluap1* expression also display premature mortality due to nonexistent ciliogenesis but can survive until at least 11 days postfertilization (dpf), allowing the examination of retinal tissue. Photoreceptor defects are apparent as early as 3 dpf, the same time point when wild-type zebrafish photoreceptors are undergoing outer segment formation, and rhodopsin in the mutant fish is mislocalized in the photoreceptor layer, an indication of aberrant IFT.[22] Aberrant IFT precedes cell death in animal models of LCA caused by mutations in all six known LCA cilia genes.[9,23–27] The retinas of *cluap1*$^{-/-}$ zebrafish lack photoreceptor cells by 5 dpf, whereas the remaining cell layers are intact.[28] The CLUAP1 protein contains two major domains, an N-terminal calponin homology-like domain and a C-terminal coiled-coil domain. Both domains are highly conserved from zebrafish to humans, and homologous domains can be found in many microtubule binding proteins.[29] The two major isoforms of *CLUAP1* are expressed in the human retina at moderate levels similar to IFT protein transcripts.[30]

Based on this evidence, we concluded that *CLUAP1* is a cilia gene important for photoreceptor outer segment formation in vertebrates and therefore an excellent candidate LCA disease gene. In this study of 212 unsettled LCA patients, we found a single proband homozygous for a nonsynonymous amino acid substitution in *CLUAP1*. Rescue experiments using zebrafish as a model system proved that our mutant allele is hypomorphic.

MATERIALS AND METHODS

Patient recruitment, clinical diagnosis, and DNA preparation

Proband MOGL3628 was recruited and diagnosed with LCA at the Montreal Children's Hospital at the McGill University Health Centre. This patient was diagnosed with LCA after we found nystagmus and very poor visual fixation at 6 weeks of age. An electroretinogram was nondetectable and the retina appeared normal. There were no systemic abnormalities. This study was approved by the McGill University Health Centre Research Institute Research Ethics Board and adhered to the tenets of the Declaration of Helsinki. Blood samples were collected from the proband and both parents after obtaining informed consent; DNA was extracted using the Qiagen blood genomic DNA extraction kit (Qiagen, Valencia, CA).

Whole-exome sequencing and next-generation sequencing data processing

One microgram of total patient DNA was sheared into 300- to 500-bp fragments. Fragments were end-repaired and a 3′ adenosine base added. Illumina Y-shaped adapters (Illumina, San Diego, CA) were ligated to DNA fragments, and 10 cycles of PCR were used to amplify the library with a unique barcode. Library DNA concentration was quantified using the Life Technologies picogreen assay (Life Technologies, Carlsbad, CA), and the library was pooled with five other libraries. Three micrograms of pooled DNA were capture-enriched using the NimbleGen SeqCap EZ Human Exome Library v2.0 Hybridization and Wash Kit (NimbleGen, Madison, WI). Exome-captured libraries were quantified and multiplex sequenced on an Illumina HiSeq 2000.

Next-generation sequencing reads were mapped to the hg19 human reference genome using BWA-MEM, duplicate reads were removed using Picard, local realignments were performed using GATK, and variants were called using Atlas2. A population frequency threshold of 0.5% was used to filter out common variants that occur too frequently to be the cause of a rare Mendelian disease. Four next-generation sequencing cohort databases were used to determine allele frequencies. The functional consequence of the remaining rare variants was annotated using ANNOVAR, and dbNSFP was used to compile *in silico* predictions about the deleteriousness of nonsynonymous variants. UGENE was used to perform the multiple sequence alignment using the MUSCLE alignment algorithm.

Please see the **Supplementary Methods** online for references and details.

Sanger sequencing

Sanger sequencing was used to confirm the authenticity of the variant identified by next-generation sequencing, to confirm the variant properly segregated in the proband's parents, and to

screen five additional LCA probands for mutations in *CLUAP1*. A PCR primer pair was designed for each exon of interest. After PCR amplification, the amplicons were sequenced on an Applied Biosystems 3730XL or 3500XL Genetic Analyzer (Applied Biosystems, Foster City, CA).

Immunohistochemistry

In vivo immunohistochemistry was performed on adult mouse retinal sections. Anti-CLUAP1 and anti-acetylated α-tubulin were used for primary antibodies, and 4′,6-diamidino-2-phenylindole was used as a counterstain. *In vitro* immunohistochemistry was performed on hTERT-RPE1 cells transiently transfected to overexpress human FLAG-tagged *CLUAP1* under control of a CMV promoter. *CLUAP1* cDNA was mutagenized to recreate the proband's mutation. Anti-FLAG tag and anti-acetylated α-tubulin were used for primary antibodies, and 4′,6-diamidino-2-phenylindole was used as a counterstain.

Please see the **Supplementary Methods** online for experimental details, reagent sources, and reagent concentrations.

Zebrafish functional experiments

The proband's mutation was recreated at the homologous zebrafish cDNA residue. Capped mRNA was amplified, template DNA was degraded, and mRNA was purified. Embryos were lysed according to a previously published protocol.[31] Day 0 embryos were lysed at 8–9 hours postfertilization (hpf), and day 1 embryos were lysed at 24 hpf. Western blotting was performed using anti-GFP. Anti-β-tubulin was used as a loading control. Zebrafish rescue experiments were performed by injecting embryos from *cluap1*$^{-/+}$ incrosses at the one-cell stage with varying concentrations of zebrafish wild-type and mutant *cluap1* mRNA tagged with *GFP*. *GFP* mRNA was injected as a negative control. An average of 96.5 embryos was injected per allele per concentration and for controls. The percentage of phenotypic zebrafish was quantified at 3 dpf. Rescue experiments were performed twice; therefore, reported data are the average of two experiments. *P* values were obtained using a Student *t*-test. Error bars represent the standard deviation of the two independent experiments.

RESULTS

Whole-exome sequencing identifies homozygous variants in *CLUAP1* as candidate pathogenic mutations in an LCA proband

Saudi Arabian proband MOGL3628, currently age 5 years, exhibited severe visual function limited to light perception by 6 weeks of age, accompanied by nystagmus, the oculodigital sign, and an extinguished electroretinogram. The proband's fundi appeared relatively normal (**Supplementary Figure S1** online). Whole-exome sequencing (WES) identified 349 rare protein-altering variants (**Supplementary Table S1** online). No causal mutations were found among the eight variants located in known retinal-disease genes (**Supplementary Table S2** online), so a list of all potentially biallelic variants was created assuming the recessive inheritance pattern usually seen in LCA patients (**Supplementary Table S3** online). Given the importance of cilia-associated genes in retinal disease, a list of cilia genes (**Supplementary Methods** online) was compared with the genes present in the biallelic variant list, and the cilia gene *CLUAP1* was identified as containing a homozygous nonsynonymous mutation that prompted further analysis of this proband. All 35 potentially biallelic variants in 21 genes were subsequently subjected to a systematic candidate disease gene prioritization strategy to exclude or prioritize each gene for further testing. Both gene-level and variant-level information were leveraged during the prioritization process, and the primary reasons for gene exclusion or prioritization are listed in **Supplementary Table S3** online. A description of the process is found in the **Supplementary Methods**. The mutations in *CLUAP1* were deemed the most likely candidates for the cause of disease; therefore, their authenticity was validated and proper segregation of the mutations was confirmed by Sanger sequencing (**Figure 1a**).

CLUAP1 has two isoforms that are both expressed in the human retina: a long isoform (NM_015041, NP_055856), which encodes 413 amino acids, and a short isoform (NM_024793, NP_079069), which encodes 247 amino acids. The mutation occurs in the eighth exon of *CLUAP1* and affects the cDNA sequence of both isoforms, c.817C>T long and c.319C>T short (**Table 1**). This mutation results in a leucine-to-phenylalanine substitution, p.L273F long and p.L107F short (**Table 1**), which is predicted to either be damaging or have a functional impact by 11/12 of the *in silico* prediction algorithms queried in dbNSFP (**Table 1**). The mutation is extraordinarily rare—it is found in the Exome Aggregation Consortium database at a frequency of only 1/121,086 chromosomes or 0.0008%, although Saudi Arabians are underrepresented in the examined databases and the single individual seen to be a carrier for the allele is of reported European descent (**Table 1**).

Our proband's mutation falls in CLUAP1's coiled-coil domain, one of two characterized functional domains in the CLUAP1 protein. Both the coiled-coil and calponin homology–like domains have been reported to be involved in microtubule binding activity, as shown in IFT proteins with homologous domains. The coiled-coil domain is also associated with facilitating protein–protein interactions, and it is within this region that our proband's mutation lies (**Figure 1b**). A protein multiple sequence alignment of human CLUAP1 and its orthologs reveals that the affected leucine residue is conserved in all sighted organisms, including the fruit fly *Drosophila melanogaster* and the body louse *Pediculus humanus corporis*, whose protein sequences display much less overall conservation compared with analyzed vertebrates. Like CLUAP1, the worm ortholog DYF-3 has been shown to be required for neuronal sensory cilium formation in *Caenorhabditis elegans*, but the specific residue is not conserved in either worm aligned (**Figure 1c**).[32]

To ascertain additional patients with *CLUAP1* mutations, we corresponded with our LCA expert clinician–scientist collaborator (R.K.K.), who was able to identify five additional unsolved LCA probands for whom homozygosity mapping

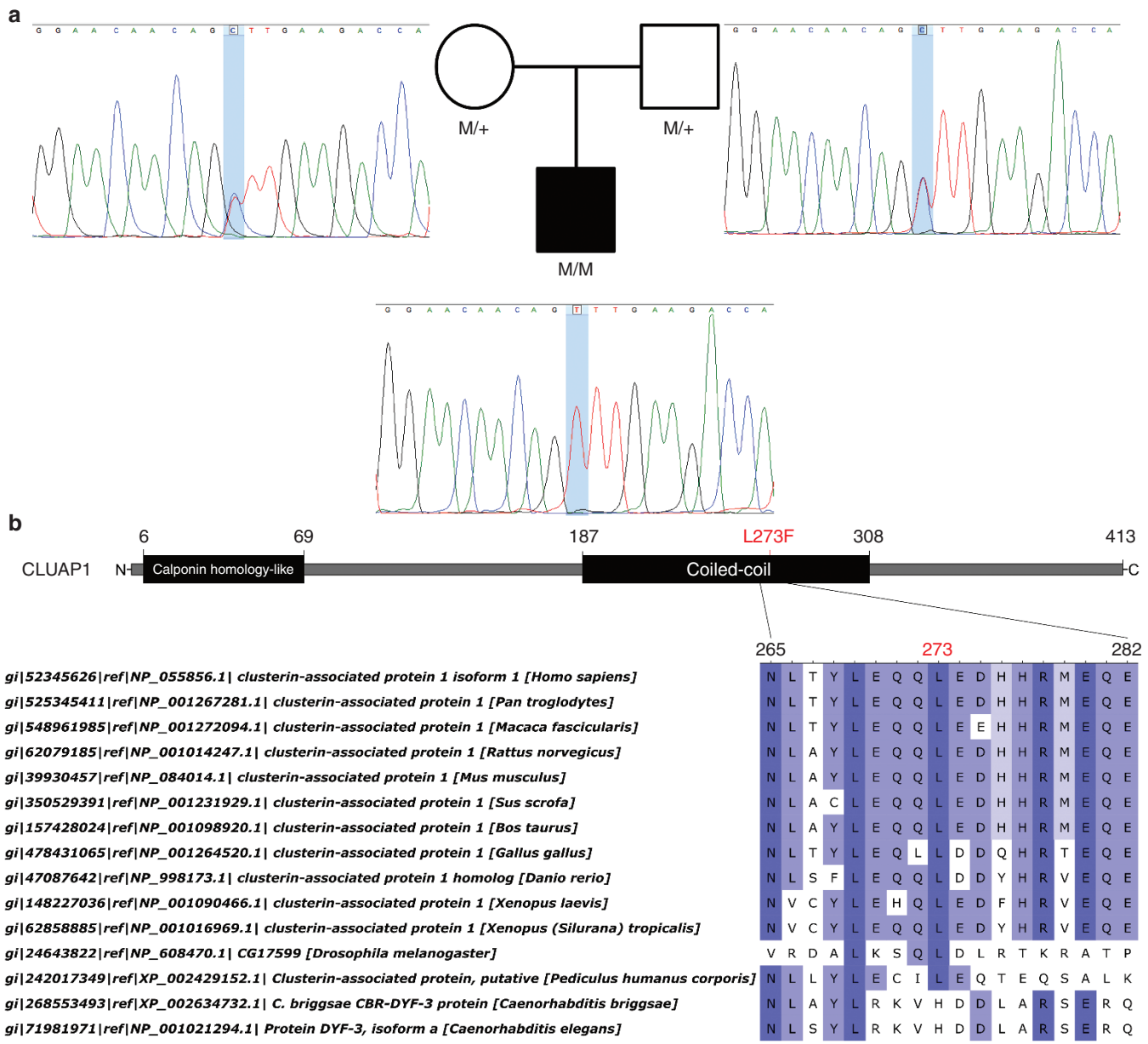

Figure 1 Pedigree, Sanger sequencing traces, and 2D CLUAP1 protein structure with an multiple sequence alignment of the affected residue. (**a**) The proband's pedigree and Sanger sequencing traces showing proper segregation of the mutant allele. (**b**) A 2D graphical representation of the long isoform of CLUAP1 containing two characterized functional domains with the location of the proband's mutation displayed in red. A protein multiple sequence alignment surrounding the amino acid affected by the proband's mutation showing the leucine at position 273 is conserved in sighted organisms. Aligned columns are colored a darker blue with increasing conservation at that position.

had mapped a significant homozygous interval surrounding and containing *CLUAP1*. Direct Sanger sequencing of the *CLUAP1* gene in these individuals did not identify any candidate mutations. We additionally contacted members of the European Retinal Disease Consortium, of which R.K.K. is a member, and indicated our interest in *CLUAP1* via GeneMatcher, a component of the Matchmaker Exchange network, but we were unable to identify potential collaborators who had retinal-disease patients with *CLUAP1* mutations.

CLUAP1 is localized at the connecting cilium of photoreceptor cells

Although CLUAP1 is proposed to be a ciliary protein based on functional studies, its precise localization in the retina is unknown. Given our proband's phenotype, we hypothesized that CLUAP1 is likely to be expressed in photoreceptor cells. To test this, we performed immunohistochemistry on sections of mouse retinas using an anti-CLUAP1 antibody as well as anti-acetylated α-tubulin and 4′,6-diamidino-2-phenylindole. CLUAP1 localizes to the connecting cilium of photoreceptor

cells located between the inner and outer segment layers of the retina (Figure 2). An inconsistent CLUAP1 signal is also seen in certain areas of the outer nuclear layer, but this may be the result of nonspecific antibody binding.

The proband's missense mutation negatively impacts CLUAP1 function

To investigate whether the proband's mutation affects CLUAP1 function by altering its localization, we performed immunofluorescence on hTERT-RPE1 cells transiently overexpressing either FLAG-CLUAP1 or FLAG-CLUAP1^{Mut}. CLUAP1 harboring the proband's mutation was observed to localize to the base of cilia in a manner similar to that of the wild type (**Supplementary Figure S2** online). To determine whether the proband's mutation is detrimental to CLUAP1 function *in vivo*, mutagenesis of wild-type zebrafish *cluap1* cDNA was performed to generate a cDNA construct containing a mutation at the zebrafish residue that is homologous to the human variant. Taking advantage of the *cluap1* knockout zebrafish model, which shows developmental defects typical for a ciliopathy, a functional analysis of the mutant *cluap1* was performed using rescue experiments. As described in the Materials and Methods section, GFP-tagged wild-type and mutant mRNA were injected into zebrafish embryos resulting from *cluap1*$^{+/-}$ incrosses.

To investigate whether the mutation affects *cluap1* expression, we first performed western blots using embryo lysates. Mutant Cluap1 was expressed at levels similar to those of wild type, leading to the conclusion that the mutation does not affect protein expression or degradation (Figure 3a). To assess the dysfunction of our mutant Cluap1, we screened for the ventral spine-curvature feature present in *cluap1*$^{-/-}$ knockout zebrafish at an early age. The percentage of phenotypic embryos was compared with the expected ~25% observed after control injections of *GFP*. To determine whether mutant Cluap1 could rescue the phenotype of *cluap1*$^{-/-}$ knockout zebrafish, low and high concentrations (1 and 130 pg/nl) of mutant mRNA were tested along with those of wild type. At the lower concentration of 1 pg/nl, no significant rescue effect was observed from mutant *cluap1* mRNA–injected embryos (25.7% phenotypic progeny, $P = 0.118$, Figure 3b and **Supplementary Table S4**), whereas wild-type *cluap1* mRNA displayed a borderline significant rescue (9.1% phenotypic progeny, $P = 0.053$, Figure 3b and **Supplementary Table S4**). At the higher concentration of 130 pg/nl, mutant *cluap1* performed similarly to wild type and

Table 1 Mutation information

Genome-level consequence		Gene		Gene-level consequence	
NC_000016.9:g.3573261C>T		*CLUAP1*		NM_024793:c.319C>T:p.L107F NM_015041:c.817C>T:p.L273F	
***In silico* nonsynonymous mutation deleteriousness prediction algorithms and corresponding predictions**					
SIFT	Polyphen2 HDIV	Polyphen2 HVAR	LRT	MutationTaster	MutationAssessor
Damaging	Damaging	Damaging	Damaging	Damaging	Medium impact
FATHMM	MetaSVM	MetaLR	VEST3	PROVEAN	CADD
Tolerated	Damaging	Damaging	Damaging	Damaging	Damaging
Frequency of variant in large-scale population studies (mutant allele count/total alleles)					
1000 Genomes Project	Human Genetic Variation Database			Exome Aggregation Consortium	CHARGE
0 (0/5,008)	0 (0/2,416)			0.0008% (1/121,086)	0 (0/21,880)

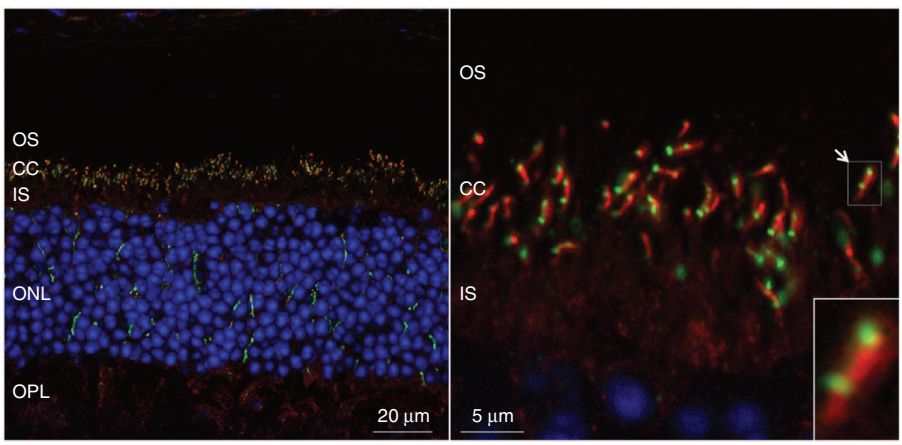

Figure 2 CLUAP1 localizes to the connecting cilium in adult mouse retinas. Mouse immunohistochemistry staining using anti-CLUAP1 (green), anti-acetylated α-tubulin (red), and 4′,6-diamidino-2-phenylindole (blue). CLUAP1 can be seen to localize to specific puncti between the inner segment (IS) and outer segment (OS) layers of photoreceptors cells. CLUAP1 localization overlaps with the tip and base of the acetylated α-tubulin staining corresponding to the tip and base of connecting cilia (CC). ONL, outer nuclear layer; OPL, outer plexiform layer.

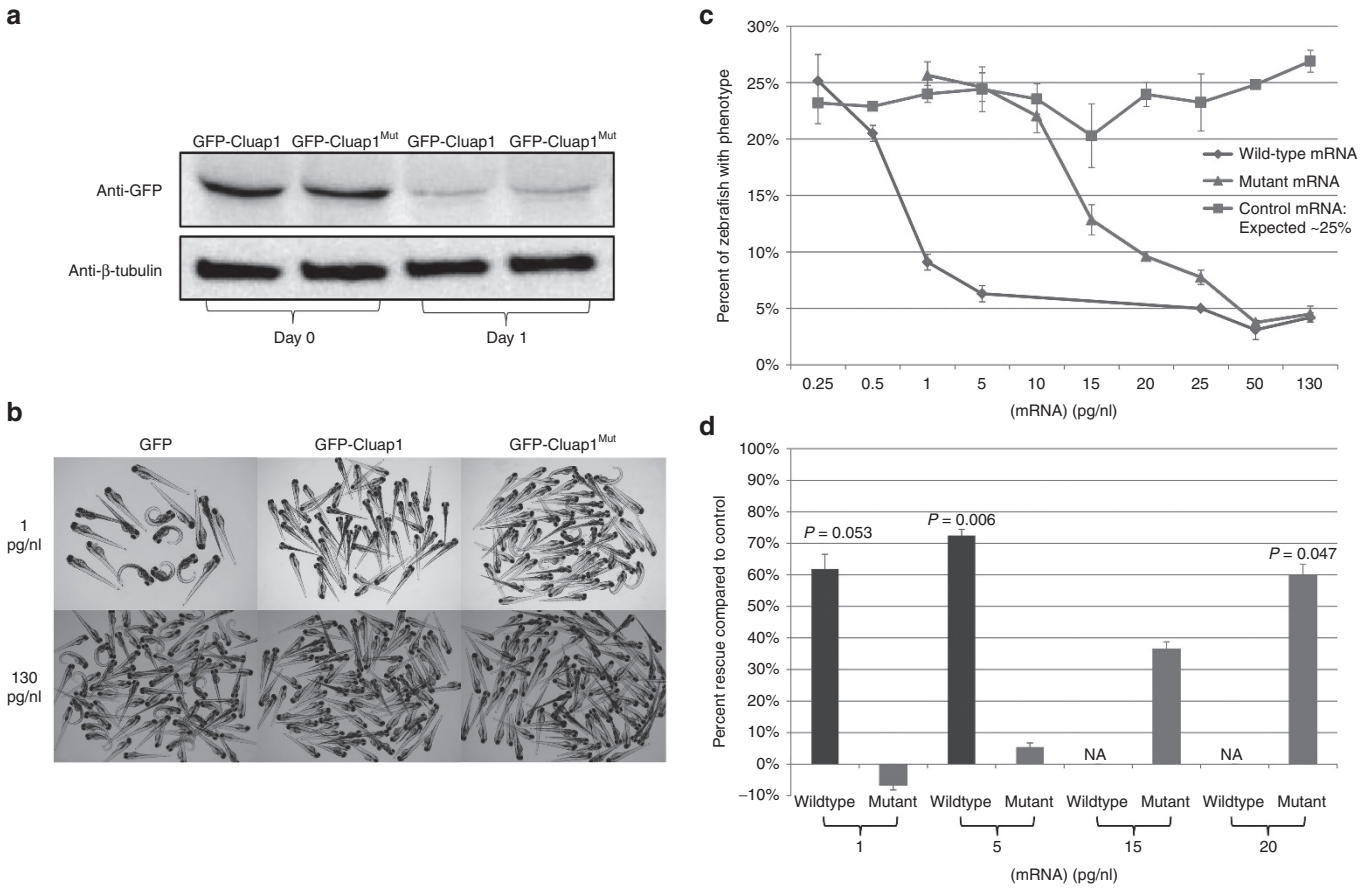

Figure 3 Mutant Cluap1 is expressed at levels similar to those of wild type but requires 20 times the amount to achieve an equivalent rescue effect. (**a**) Zebrafish embryos injected with 25 pg/nl *GFP-cluap1* or *GFP-cluap1*^{Mut} mRNA express similar amounts of Cluap1 as verified by western blotting. (**b**) Embryos resulting from *cluap1*$^{-/+}$ incrosses injected with control *GFP* mRNA exhibit the expected 25% phenotypic proportion. At a low concentration (1 pg/nl), wild-type *cluap1* mRNA noticeably rescues the spine-curvature feature of *cluap1*$^{-/-}$ zebrafish embryos, whereas mutant *cluap1* mRNA does not. At a high concentration (130 pg/nl), mutant *cluap1* mRNA rescues the spine-curvature feature comparable to wild-type *cluap1* mRNA, revealing a dose-dependent response. (**c**) Dose-response curves of rescue experiments. Zebrafish injected with control *GFP* mRNA (boxes) showed approximately the expected 25% Mendelian ratio. Zebrafish injected with *GFP-cluap1* mRNA (circles) reached a borderline significant ($P = 0.053$) rescue at 1 pg/nl. Zebrafish injected with *GFP-cluap1*^{Mut} mRNA (triangles) significantly ($P = 0.047$) rescued the mutant phenotype at a concentration of 20 pg/nl. (**d**) The percentage of progeny rescued as compared with control injections at the same concentration highlights the 20-fold increase in the amount of mutant *cluap1* mRNA required to achieve a level of rescue significance comparable to wild-type *cluap1*. Errors bars in **c** and **d** represent the SD from two independent experiments.

exhibited a clearly significant rescue effect (4.5% phenotypic progeny, $P = 0.007$, **Figure 3b** and **Supplementary Table S4**).

Because a high concentration of mutant *cluap1* rescued the spine-curvature feature, we next quantified the functionality of the mutant protein by varying the concentration of *cluap1* mRNA injections from 0.25 to 130 pg/nl. Graphing the dose–response curve of these rescue experiments revealed that the proband's mutation does indeed interfere with Cluap1 function. Although wild-type *cluap1* mRNA can reach a borderline significant rescue effect at the previously mentioned concentration of 1 pg/nl, mutant *cluap1* mRNA requires a concentration of 20 pg/nl to achieve a similar level of rescue significance (9.6% phenotypic progeny, $P = 0.047$, **Figure 3c** and **Supplementary Table S4** online). To visualize these data in an alternative form, we compared the percentage of progeny expected to be phenotypic based on control injections to the percentage actually observed after mutant and wild-type *cluap1* mRNA injections to determine the percentage of progeny that were rescued, which highlights the hypomorphic nature of our proband's allele (**Figure 3d**). Based on these functional rescue assays, Cluap1 containing our proband's mutation has as little as 5% of its remaining activity, as judged from the 20-fold increase in mutant Cluap1 concentration required to achieve a rescue significance comparable to that of wild type.

DISCUSSION

Because *CLUAP1* is required for photoreceptor function in other vertebrates, and because our LCA proband's allele is an extremely hypomorphic *CLUAP1* allele, we conclude that *CLUAP1* is very likely to be necessary for photoreceptor function in humans. Therefore, this is the first report linking hypomorphic mutations in *CLUAP1* to human disease and establishes *CLUAP1* as a candidate LCA gene. Identifying *CLUAP1* as a novel candidate LCA gene is important both for understanding

human biology and for unlocking the future molecular diagnosis of patients affected by mutations in this gene. *CLUAP1* can now be studied in the context of being a human ciliopathy gene, and establishing a new candidate disease gene allows for faster and less expensive molecular diagnosis of future patients because geneticists can target the *CLUAP1* locus for examination. *CLUAP1* mutations appear to be a rare cause of LCA, so further studies screening additional patient cohorts are needed to determine the true proportion of LCA patients with *CLUAP1* mutations.

The importance of cilia in many areas of the body and the importance of *CLUAP1* in cilia raise concerns about the clinical presentation of the presented proband and why syndromic features have not been observed. We considered addressing this question by sectioning the retinas of embryos rescued with mutant *cluap1* mRNA to verify that a retinal phenotype persists after the correction of spine curvature. Unfortunately, given the rapid decay of the injected mRNA and the exogenous protein being minimally detectable at 1 dpf (**Figure 3a**), it is unlikely that this approach would be informative because photoreceptor development is not complete until around 5 dpf. However, three possible explanations exist for this observation. The first is that our proband does indeed have a syndromic disease but additional features have yet to manifest due to the young age. This would not be surprising because multiple syndromic ciliopathies such as Joubert syndrome, Senior-Løken syndrome, and Alström syndrome are known to present a retinal-degeneration feature preceding complications in other tissues. These syndromes can in fact be misdiagnosed as LCA because of the lack of additional symptoms in an affected individual's initial clinical presentation.[1]

The second explanation is that the specific mutation that our proband harbors is detrimental only to a retina-specific function and therefore only a retinal phenotype is observed. The connecting cilium in photoreceptors is a unique form of cilia containing proteins not found in most other ciliated cell types in the body. The presence of retina-specific proteins at the connecting cilium means that proteins like CLUAP1 whose expression is not restricted to the retina conceivably have a purpose specialized to the connecting cilium involving interactions with retina-specific proteins. This explanation is supported by the fact that the proband's mutation causes a nonsynonymous amino acid substitution and that the mutation resides in CLUAP1's coiled-coil domain known to accommodate protein–protein interactions. The residue that our proband's mutation affects is not conserved in *Caenorhabditis elegans* and *Caenorhabditis briggsae*, implying that the residue is not essential for ciliogenesis but instead is important for some function specific to the organisms in which it is strictly conserved. The absence of eyes is one of the defining features separating these worms from the other species in the multiple sequence alignment; therefore, a safe hypothesis appears to be that the conserved leucine is required for a function involved in vision.

The third explanation is that our proband's mutation results in a general hypomorphic form of CLUAP1 and that the retina is more sensitive to this decrease in CLUAP1 function than other tissue types, possibly comparable to the ubiquitously expressed LCA gene *NMNAT1*.[8] The variability in phenotype caused by mutations in the same gene can range drastically, especially in retinal disease, depending on the exact nature of the patients' alleles. It is possible that a mutation residing in CLUAP1's calponin homology-like domain would confer a different phenotype impacting other tissues, and it is likely that the complete loss of function of CLUAP1 would not be compatible with human life as seen in other vertebrates. The function and expression of genes are also not always conserved across species, and human *CLUAP1* is not detected in the human kidney by northern blotting, which could explain the lack of kidney defects observed in the original zebrafish polycystic kidney disease screen.[33,34]

In 2008, known LCA genes were reported to account for 70% of LCA cases; based on our experience in 2015, this percentage can vary up to 75%, but for the most part it has changed unappreciatively despite the identification of an additional eight genes. At least six of the reported LCA disease genes, including the two most recently published genes, account for a remarkably small portion (~1%) of all LCA cases.[11–13,35–37] *CLUAP1* adds to this trend, accounting for 1/212 LCA patients in our cohort (0.47%). More than one-quarter of all LCA patients are waiting for molecular diagnoses, and these patients inspire two interesting hypotheses regarding future directions for studying the genetics of LCA. The first is the big-fish hypothesis, which can be summarized as saying that any one gene responsible for a significant proportion of patients' disease (a "big fish") has probably already been discovered at this stage of human genetics research. This means that only "little fish" are left, which is why we see new LCA disease genes accounting for a minor fraction of patients. It is therefore possible that the source of disease representing the 30% of unsolved LCA cases will comprise more than 25 novel disease genes, with each accounting for an ever smaller fraction of patients.

The second alternative hypothesis is that the cause of disease resides in known LCA genes but modern genotyping techniques cannot identify the causal variation. For most genetic disorders WES is widely considered the standard method for molecular diagnosis, but it is currently unsuitable for identifying potentially causative variations in introns, promoters, enhancers, and large structural variations. Even in regions captured by WES there are data that we do not fully know how to interpret, including variation in untranslated regions, synonymous variation, and intronic variation close to an exon but outside the 2-bp splicing consensus sequence. Understanding variation in these atypical regions is imperative in the research of human disease because groups may focus efforts on finding novel disease genes when the disease-causing mutations they seek are "right under their noses." The truth is most likely a combination of these two hypotheses, leaving numerous possibilities for the future of LCA research.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

We thank the proband and their family for participating in this study. This work is supported by the National Institutes of Health (grants R01-DK092808 and 1P30-DK090744 to Z. Sun and shared grant 1S10RR026550 to R.C.), the National Eye Institute (grants R01EY022356, R01EY018571, and P30EY002520 to R.C.), the Retina Research Foundation, and the Foundation Fighting Blindness (grant BR-GE-0613-0618-BCM to R.C.). R.K.K. acknowledges the Foundation Fighting Blindness (Canada), the Canadian Institutes of Health Research, and Fonds de Recherché Santé Quebéc et Réseau Vision, all of which supported this work. Z. Soens is supported by National Eye Institute training grant 5T32EY007001-38.

DISCLOSURE

The authors declare no conflict of interest.

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