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Leber congenital amaurosis: Genes, proteins and disease mechanisms

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ABSTRACT

Leber congenital amaurosis (LCA) is the most severe retinal dystrophy causing blindness or severe visual impairment before the age of 1 year. Linkage analysis, homozygosity mapping and candidate gene analysis facilitated the identification of 14 genes mutated in patients with LCA and juvenile retinal degeneration, which together explain approximately 70% of the cases. Several of these genes have also been implicated in other non-syndromic or syndromic retinal diseases, such as retinitis pigmentosa and Joubert syndrome, respectively. *CEP290* (15%), *GUCY2D* (12%), and *CRB1* (10%) are the most frequently mutated LCA genes; one intronic *CEP290* mutation (p.Cys998X) is found in ~20% of all LCA patients from north-western Europe, although this frequency is lower in other populations. Despite the large degree of genetic and allelic heterogeneity, it is possible to identify the causative mutations in ~55% of LCA patients by employing a microarray-based, allele-specific primer extension analysis of all known DNA variants.

The LCA genes encode proteins with a wide variety of retinal functions, such as photoreceptor morphogenesis (CRB1, CRX), phototransduction (AIPL1, GUCY2D), vitamin A cycling (LRAT, RDH12, RPE65), guanine synthesis (IMPDH1), and outer segment phagocytosis (MERTK). Recently, several defects were identified that are likely to affect intra-photoreceptor ciliary transport processes (CEP290, LCA5, RPGRIP1, TULP1). As the eye represents an accessible and immune-privileged organ, it appears to be uniquely suitable for human gene replacement therapy. Rodent (Crb1, Lrat, Mertk, Rpe65, Rpgrip1), avian (Gucy2D) and canine (Rpe65) models for LCA and profound visual impairment have been successfully corrected employing adeno-associated virus or lentivirus-based gene therapy. Moreover, phase 1 clinical trials have been carried out in humans with RPE65 deficiencies. Apart from ethical considerations inherently linked to treating children, major obstacles for the treatment of LCA could be the putative developmental deficiencies in the visual cortex in persons blind from birth (amblyopia), the absence of sufficient numbers of viable photoreceptor or RPE cells in LCA patients, and the unknown and possibly toxic effects of overexpression of transduced genes. Future LCA research will focus on the identification of the remaining causal genes, the elucidation of the molecular mechanisms of disease in the retina, and the development of gene therapy approaches for different genetic subtypes of LCA.

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Abbreviations: aa, amino acid; ACHM, achromatopsia; ad, autosomal dominant; ar, autosomal recessive; CSNB, congenital stationary night blindness; ERG, electroretinogram; kDa, kiloDalton; KO, knockout; LCA, Leber congenital amaurosis; LP, light perception; MfERG, multifocal ERG; mo, month; NCL, neuronal ceroid lipofuscinosis; OCT, optical coherence tomography; ONL, outer nuclear layer; RPE, retinal pigment epithelium; RP, retinitis pigmentosa; we, week; xl, X-linked

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1. Introduction

Inherited sensory diseases are characterized by immense genetic and clinical heterogeneity, which poses great challenges for gene identification, mutation analysis, genetic counselling, and the development of therapies. The most severe form of inherited retinal blindness is Leber congenital amaurosis (LCA), which in most cases is inherited in an autosomal recessive (ar) manner. Research into the molecular causes of LCA in the past 12 years has revealed the underlying disease genes in ~70% of the cases. Fourteen genes involved in LCA and/or early onset retinal degeneration have been identified encoding proteins important in a wide variety of retinal developmental and physiological pathways. The *LRAT, MERTK*, *RPE65* and *TULP1* genes have been implicated in patients with early onset retinal degeneration partially overlapping LCA.

LCA serves as a model for all retinal dystrophies as recently therapeutic gene replacement trials with human subjects have commenced which represent the first attempts to treat inherited blindness. The purpose of this review is to summarize recent

developments in our understanding of this devastating disease at the clinical, molecular genetic, cellular, and protein level.

2. Phenotypic characteristics

2.1. Clinical characteristics

LCA represents a group of hereditary retinal diseases characterized and unified by the following constellation of four clinical features: severe and early visual loss, sensory nystagmus, amaurotic pupils, and absent electrical signals on electroretinogram (ERG) (Leber, 1869; Franceschetti and Dieterle, 1954). LCA presents very early in life, usually at around the age of 6 weeks, when parents note the oscillations of the eyes (nystagmus) or the absence of fixation. LCA is a rare retinal dystrophy, with a population frequency between 1/30,000 (Koenekoop, 2004) and 1/81,000 (Stone, 2007), but it accounts for $\geqslant$5% of all inherited retinopathies and approximately 20% of children attending schools for the blind (Koenekoop, 2004). Recognized and described for the first time in 1869 by Theodor Karl Gustav Leber, LCA was initially introduced as the congenital form of retinitis pigmentosa (RP) (Leber, 1869). Historically, the name LCA also became associated with congenital retinal blindness found in syndromes such as Joubert syndrome, peroxisomal diseases, Batten disease (neuronal ceroid lipofuscinosis, NCL) and others. Revisiting the diagnosis of LCA, Pinckers (1979) argued that what Leber had in mind with LCA is roughly the same disease group as what we now know as NCL. Most vision researchers currently consider LCA the most severe retinal dystrophy without major systemic features.

2.1.1. Visual function and longitudinal changes in visual function in LCA patients

Manifest visual function and visual acuity (VA) in LCA patients range widely, usually from 20/200 to light perception or even no-light perception. Visual acuities of 20/50 have been described in patients with *CRB1, LRAT,* and *RPE65* mutations, but acuities in that range do not appear to remain stable. Most patients with LCA have stable or relatively stable visual function, but some patients progress and lose visual function, while rarely LCA patients seem to improve. Three longitudinal studies of visual function in LCA patients were performed before the genome era, precluding useful genotype–phenotype correlations. Snellen visual acuity, grating acuities, dark-adapted visual thresholds, and flash visual evoked potentials were performed in a total of 90 LCA patients (Heher et al., 1992; Fulton et al., 1996; Brecelj and Stirn-Kranjc, 1999). Visual deterioration was observed in 15%, stability in 75%, and improvement in 10% of the patients. Koenekoop and coworkers found improvements in visual acuity, visual field, and cone ERG b-wave amplitudes in an LCA patient with a mutation in the *CRX* gene that was followed for 12 years (Koenekoop et al., 2001). LCA patients with *CRB1, LCA5,* and *RPE65* mutations show mild improvements in their visual function, but then decline after a period of stability (Yzer et al., 2003; Lorenz et al., 2000; Koenekoop, unpublished data). LCA patients with *CEP290* and *GUCY2D* mutations appear to have a very significant loss of vision, but then remain stable, while LCA patients who harbor *AIPL1* and *RPGRIP1* mutations have progressive loss of vision (Dharmaraj et al., 2000b; Koenekoop et al., 2007; Koenekoop, unpublished data). Other studies suggested that visual outcome is distinct and clinically recognizable in patients with mutations in *RPE65* versus those with *GUCY2D* mutations (Perrault et al., 1999; Lorenz et al., 2000). The first group is characterized by measurable acuities and nyctalopia, while the latter group is defined by poor vision, photoaversion and lack of nyctalopia.

2.1.2. Phenotypic variability

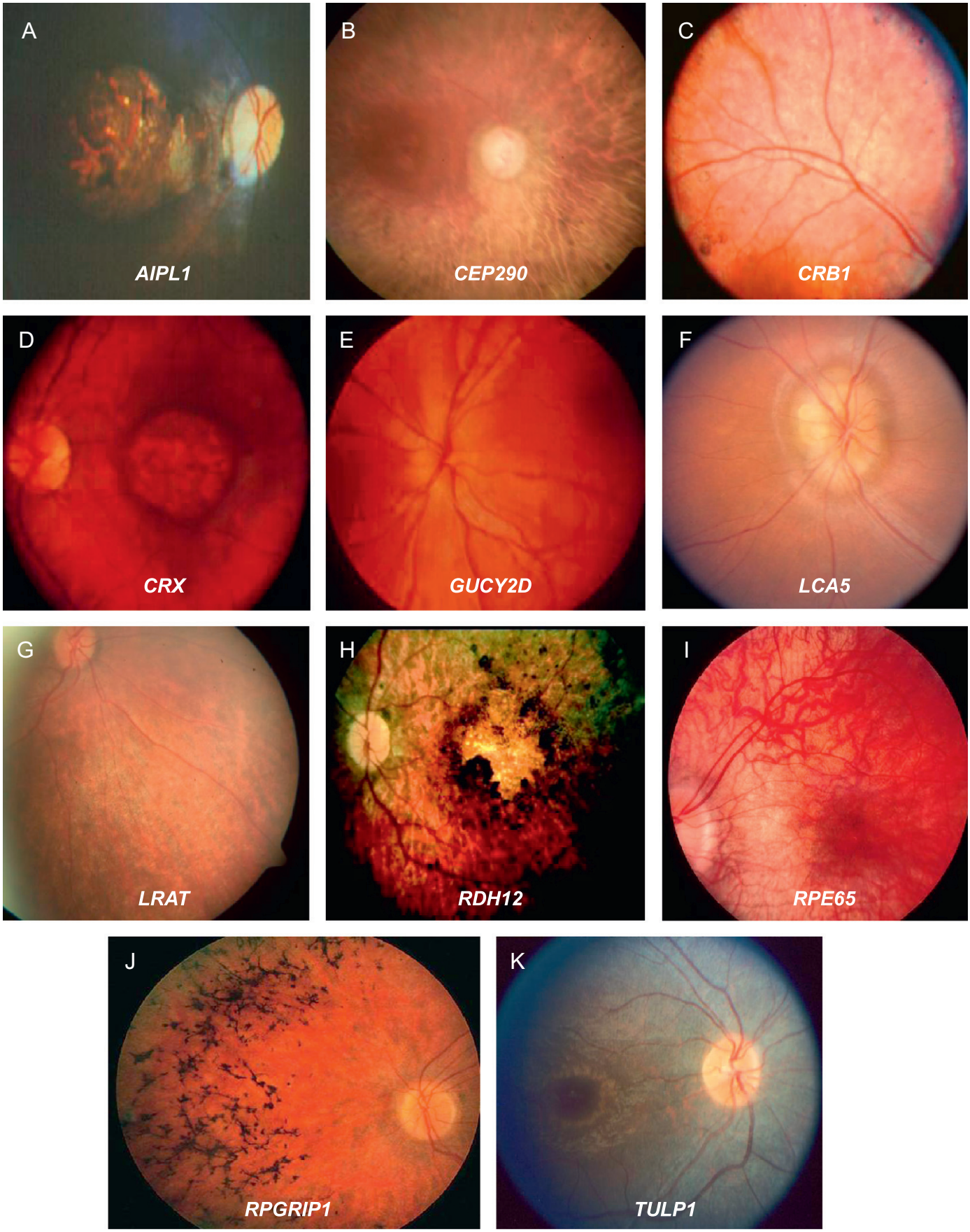
The phenotypic variability found in LCA patients is striking as heterogeneity is found in retinal appearance, refractive errors, photoaversion, nyctalopia and the oculodigital sign. Associated features such as keratoconus and cataracts, manifest visual function and longitudinal changes in visual function are also variable. The retinal appearances vary considerably (Fig. 1). The full phenotypic range of retinal aspects associated with LCA still needs to be determined and correlated with the various genotypes, but currently ranges from essentially normal retinal appearance, to mild retinal vessel attenuation, pseudopapilledema of the optic disc, maculopathy, macular coloboma, bone spicule pigmentation, nummular pigmentation, salt and pepper pigmentation, yellow confluent peripheral spots, white retinal spots, marbleized retinal changes, preserved para-arteriolar RPE (PPRPE) and Coats reaction (Fig. 1). It appears that gene-specific phenotypic features exist in LCA (Dharmaraj et al., 2004; Galvin et al., 2005; Koenekoop et al., 2007). These genotype–phenotype correlations are found in the retinal appearances and longitudinal changes in visual function (Koenekoop et al., 2007). Macular colobomas are a prominent and frequent retinal feature found in LCA, but the term is a misnomer as they are not developmental colobomas. They likely represent complete loss of retinal tissue in the fovea (Fig. 1). Colobomas are not found in all genetic types of LCA and may easily be confused with the scars of ocular toxoplasmosis.

2.2. Associated phenotypic features in LCA

LCA patients often have high refractive errors (from hyperopia to myopia) and most patients are high hyperopes (Heher et al., 1992), suggesting that congenital blindness significantly affects the emmetropization process or that the defective retinal genes also play a role in the determination of the size of the infant eye. It has been suggested that the degree of hyperopia may indicate the presence or absence of associated features in LCA (Wagner et al., 1985), but this association was not born out in subsequent studies (Dagi et al., 1990). Photoaversion (photophobia) can be a prominent feature in LCA (Dagi et al., 1990), as can nyctalopia (night blindness) (Lorenz et al., 2000), and these symptoms may be gene-specific (Perrault et al., 1999). The oculodigital sign of Franceschetti can be a very important and sometimes disturbing feature of LCA, although it is not pathognomonic for the disease. The sign consists of a repetitive, deep pushing of the knuckle or finger into the eye and socket. The exact molecular phenomenon is not clear but may be related to the production of phosphenes, which produce sparks of light that may satisfy the patients. The oculodigital sign may also be regarded as a repetitive stereotypic behavior, also known as blindism. The oculodigital phenomenon can be harmful as the deep set eyes of LCA patients may be caused by the persistent pushing, resulting in orbital fat atrophy. Deep set eyes (enophthalmos) may become a prominent facial feature of LCA patients. It has also been suggested that keratoconus, the thinning and ectasia of the cornea, is caused by the oculodigital phenomenon.

2.2.1. Keratoconus and cataracts

Keratoconus is often associated with LCA and may decrease visual function further. It is defined as a degenerative non-inflammatory disorder of the cornea with thinning and changes to a more cone-like shape to replace the gradual normal curvature. The etiology of keratoconus remains somewhat of a mystery, but genetic, environmental (rubbing) and toxic factors (from retinal cell death) have been postulated. Pathologically, keratoconus is characterized by progressive dissolution of Bowman's membrane


A
AIPL1
B
CEP290
C
CRB1
D
CRX
E
GUCY2D
F
LCA5
G
LRAT
H
RDH12
I
RPE65
J
RPGRIP1
K
TULP1

in between the corneal stroma and epithelium and increased activity by protease enzymes has been repeatedly found. Cataracts are often associated with LCA and with keratoconus, and may be associated with more severe phenotypes (Koenekoop, 2004). Visual function may be further compromised. The etiology of LCA-associated cataracts may also be a combination of genetic, environmental and toxic factors.

2.2.2. Mental retardation

Alström and Olson were the first to suggest the association of LCA with mental retardation and found that 17% of LCA patients suffered from mental retardation or seizures (Alström and Olson, 1957), while other investigators have reported between 26% and 52% of mental retardation in LCA (Schappert-Kimmijser et al., 1959; Dekaban, 1972; Vaizey et al., 1977). In the majority of these patients, CT scanning was not yet available, but in the studies where the brain was investigated (air encephalography), the majority of patients were found to have significant brain abnormalities, thereby excluding the diagnosis of LCA. More likely, these congenitally blind patients had a syndromic disease such as Batten disease/NCL or a peroxisomal disorder (see Section 2.4). In accordance with these findings, Nickel and Hoyt (1982) only found one child to have mental retardation in their series of 31 LCA patients. Presently, in LCA patients with the strict definition of congenital blindness, nystagmus, amaurotic pupils, absent ERG signals, and ar inheritance, whose genotype is known, no cases of mental retardation have been reported (Lotery et al., 2000; Dharmaraj et al., 2000b; Hanein et al., 2004). In another study, 6/40 families harboring *CEP290* mutations were found to have mental retardation (Perrault et al., 2007). In one family, one out of two LCA patients was mentally handicapped, while in another family only two out of five LCA patients were mentally retarded. Of the four remaining simplex LCA patients, two underwent MRI testing and were found to have the molar tooth sign, confirming the diagnosis of Joubert syndrome and excluding LCA. Lack of co-segregation of mental retardation with LCA in some families and an unsuspected diagnosis of Joubert syndrome in others, puts into question the correlation of mental handicap and LCA in this series.

2.2.3. Autism

Autism represents a brain developmental disorder characterized by impaired social interaction and communication, leading to conspicuous repetitive behavior. It is a very common disorder in the general pediatric population, with an estimated prevalence of 1–2 cases per 1000 children. The link between autism and LCA remains unclear and controversial. Rogers and Newhart-Larson (1989) found autism in 5 LCA patients but not in 5 congenitally blind patients from other causes, using the Reynell–Zinkin scale. They concluded that autism in LCA is attributable to a concurrent neurological disorder. Other investigators however studied 24 LCA children and found no autistic behavior in 20 of them, and reported mild autistic tendencies in 4 using the childhood autism rating scale (Fazzi et al., 2007). Our experience with LCA patients ($n = 600$) is similar in that there is no increased incidence of autism compared to the population of sighted children (R.K.K., personal communication).

2.2.4. Olfactory dysfunction

Previously unrecognized and not self-reported is the olfactory dysfunction in LCA patients with *CEP290* mutations, but not in LCA or RP patients with mutations in *RPGRIP1* and *RPGR* (McEwen et al., 2007). It was also established that olfactory dysfunction is not due to ciliary absence or a structural defect, as is the case in Bardet-Biedl syndrome (BBS)-associated olfactory dysfunction, but due to mislocalized olfactory cilium G-proteins (McEwen et al., 2007). Olfactory dysfunction was also found in *CEP290* mutation carriers.

2.3. Clinically overlapping non-syndromic eye diseases

For the pediatric ophthalmologist, the clinical presentation of LCA represents a complex dilemma, as many non-syndromic and syndromic ocular diseases with an "LCA-like ocular phenotype" present in a very similar fashion, and the clinical overlap can be striking and confusing. Retrospective diagnostic re-evaluation studies have led to surprising numbers of diagnostic "errors" (Lambert et al., 1989). The underlying pathology, molecular defects, visual prognosis, inheritance, and future treatments of these overlapping entities are entirely different than those of LCA. It is imperative to separate these overlapping eye diseases from LCA. Rapid and accurate clinical diagnostics is therefore essential. The ocular entities that overlap with LCA because of similar early onset visual loss, nystagmus and initial retinal appearance are complete and incomplete achromatopsia (ACHM), complete and incomplete congenital stationary night blindness (CSNB), albinism, and optic nerve hypoplasia.

2.3.1. Albinism

Albinism represents a group of non-progressive X-linked or ar retinal dystrophies with significant clinical overlap with LCA, as the patients present with poor vision and nystagmus at around 6 weeks of age. Albinism is characterized by hypo-pigmentation of the hair, skin and eye, foveal hypoplasia, and increased decussation at the optic chiasm. Genetically heterogeneous, with currently 14 genes known, albinism gene products participate in the production, metabolism or distribution of melanin (Zuhlke

Fig. 1. Color photographs of the retinas of 11 LCA patients with known genotypes, in alphabetical order from left to right and up and down (A–K). All LCA patients have recessive mutations except the patient with a heterozygous *CRX* mutation. (A) This 8-year-old black female LCA patient with p.Thr124Ile/p.Pro376Ser ***AIPL1*** compound heterozygous missense mutations has a prominent macular coloboma, retinal arteriolar narrowing and optic disc pallor. (B) This 40-year-old French-Canadian male LCA patient with homozygous p.Cys998X ***CEP290*** nonsense mutations has marked choroidal sclerosis, pale optic discs, barely visible retinal vessels, and relative preservation of the posterior pole. (C) A 10-year-old female with preserved para-arteriolar RPE (PPRPE) and nummular pigment changes harbors a common homozygous p.Cys948Tyr ***CRB1*** missense mutation. (D) A 10-year-old female with a prominent maculopathy and relatively normal appearing vessels and optic disc has a heterozygous p.Pro9fs frameshift mutation in ***CRX***. (E) This 25-year-old female LCA patient with relatively normal retinal appearance, including retinal color, retinal vessel caliber, and optic disc appearance, has compound heterozygous p.Leu954Pro/p.Ser981fs missense and frameshift mutations in ***GUCY2D***. (F) A 25-year-old female with LCA with prominent optic disc drusen, improving visual acuities, relatively well preserved retinal aspects and mild vessel narrowing, has a large homozygous g.19612-18015del1598 deletion encompassing ***LCA5*** exon 1. (G) This 10-year-old male has 20/100 acuities, nystagmus and nyctalopia. The retinal appearance shows a normal optic disc, mild vessel narrowing and very mild salt and pepper RPE changes. We found a homozygous p.Met73fs frameshift mutation in ***LRAT***. (H) This 8-year-old male patient with LCA has a rapidly progressive disease with 20/400 acuities, 5o visual fields, nystagmus, nyctalopia, a prominent maculopathy, and a homozygous p.Cys285Tyr missense mutation in ***RDH12***. (I) A 20-year-old male patient with LCA and 20/80 acuities, mild nystagmus, nyctalopia, characteristic RPE translucency and relatively normal vessel caliber and optic disc. We found a homozygous p.Tyr368His mutation in ***RPE65***. (J) A 10-year-old girl with light perception vision, mild autism, +5.00 D hyperopia, retinal pigment degeneration with bone spicules, vessel dragging and a homozygous p.Arg89X nonsense mutation in ***RPGRIP1***. (K) A 15-year-old girl presented with nyctalopia at age 8, 20/200 acuities, nystagmus, a perifoveal yellow annular ring, mild pigmentary changes and a homozygous c.718+2T>C splice-site mutation in ***TULP1***. No fundus photos are shown of LCA patients with the rare genotypes *IMPDH1, RD3* or *MERTK1*.

et al., 2007). Albinism can be distinguished from LCA by an ERG, which is normal or supernormal in the albino patient, while non-detectable in LCA.

2.3.2. Optic nerve hypoplasia

Optic nerve hypoplasia (ONH) is an important cause of pediatric visual morbidity and is often associated with structural brain abnormalities in the Desmorsier syndrome, also known as septo-optic dysplasia. Septo-optic dysplasia comprises the triad of ONH, pituitary gland hypoplasia, and midline structural brain defects (such as agenesis of the septum pellucidum or thinning of the corpus callosum). ONH is relatively common and may be on the rise, as it has been associated with young maternal age, smoking and fetal alcohol syndrome. Patients present with congenital visual loss and nystagmus, a normal appearing retina and a small optic nerve (which may not be obvious). To complicate matters, Janaky and coworkers described ERG abnormalities in ONH, possibly as a result of trans-synaptic degeneration beyond the ganglion cell layer (Janaky et al., 1994). The ERG abnormalities were confirmed in another study, although most patients with ONH have normal ERGs (Cibis and Fitzgerald, 1994). ONH is a sporadic disease, with no obvious Mendelian inheritance. Supranormal regression of ganglion cell axons in the optic nerve, and not primary failure of development, may be the principal disease mechanism of ONH. This is confirmed by histopathology, which reveals a lack of degenerated neurons in ONH and may point to axonal loss due to apoptosis during retinal development. Mutations have been found in Hesx-1, a protein involved in regulation of normal forebrain and eye development (Rainbow et al., 2005). In mice, loss of netrin-1, a protein involved in optic ganglion cell axon guidance at the optic nerve, leads to ONH (Deiner et al., 1997).

2.3.3. Complete achromatopsia (rod monochromatism)

Patients suffering from complete ACHM present with very early onset visual loss, nystagmus, photophobia and an essentially normal appearing retina. At the age of 6 weeks, complete ACHM is indistinguishable from LCA without performing an ERG or genetic test. ERG testing demonstrates an absence of cone responses and normal rod responses, unlike the ERG findings of LCA, which shows non-detectable cone and rod responses. One problem is that the ERG is technically difficult in this age group, photophobia makes the ERG more difficult, and most importantly, the ERG of a 6-week-old infant is not fully developed (Fulton et al., 2003; Hansen and Fulton, 2005). Older children with ACHM typically develop 20/200 VA and have complete absence of color vision. The retinal appearance remains normal in most cases and histologically, cone-like structures have been identified in the photoreceptor layer (Harrison et al., 1960; Falls et al., 1965). OCT studies show a normal thickness of the foveal ONL (Varsanyi et al., 2005). ACHM is a stationary retinal dystrophy. It is reasonable to postulate that in infancy, ACHM is a retinal dysfunction and that photoreceptor death is not the mechanism of the cone ERG loss.

In humans, three ACHM genes are currently known, each of which encode phototransduction proteins (Kohl et al., 2000, 2002; Wissinger et al., 2001). Mutations in cyclic nucleotide-gated channel α-3 or β-3 (*CNGA3* and *CNGB3*) and guanine nucleotide α-transducin (*GNAT2*) genes can give rise to this phenotype, but there are no discernable phenotypic differences between the genotypes (Varsanyi et al., 2005).

2.3.4. Congenital stationary night blindness

Congenital stationary night blindness (CSNB) represents a relatively common group of stationary retinal dystrophies with non-progressive nyctalopia, decreased visual acuity and nystagmus. Autosomal dominant (ad), ar and X-linked (xl) recessive forms have been described. At the molecular level, CSNB is a neuronal transmission disease with defects between photoreceptor and bipolar cells. The ar and xl forms of CSNB exhibit very significant clinical overlap with LCA, such that young infants with these types of CSNB may easily be confused with LCA on initial presentation (Weleber and Tongue, 1987). ERG recordings may show important differences to separate the two groups of diseases, but ERGs are technically difficult in infants, ERG parameters are not fully developed until 1 year of age, and one form of xlCSNB may reveal almost non-detectable ERG recordings.

XlCSNB is divided into two types. Complete CSNB is characterized by complete loss of the rod b-wave and oscillatory potentials, but normal cone a-waves, and is caused by mutations in the *NYX* gene encoding nyctalopin (Bech-Hansen et al., 2000). Incomplete CSNB exhibits a reduced rod b-wave, a substantially reduced cone a-wave and 30 Hz flicker ERG response. Incomplete CSNB is due to mutations in the calcium gated channel gene *CACNA1F* (Bech-Hansen et al., 1998). ArCSNB may be caused by mutations in the metabotopic glutamate receptor 6 gene *GRM6* and the phenotype resembles complete CSNB, but shows a distinct scotopic 15 Hz flicker ERG pattern (Zeitz et al., 2005). A second form of arCSNB is very similar to incomplete CSNB and is caused by mutations in the calcium binding protein-4 gene *CABP4* which is present at high density in the synaptic terminals of photoreceptors (Zeitz et al., 2006).

2.4. Clinically overlapping syndromic eye diseases

An "LCA-like ocular phenotype" can be found in several syndromic diseases, which may initially present without the systemic features but may dominate the phenotype later in life. The most important are Alström syndrome, Batten disease, Joubert syndrome, peroxisomal diseases, and Senior-Løken syndrome (SLSN).

2.4.1. Alström syndrome

Alström syndrome presents in infancy like LCA with early central visual loss and nystagmus, but later children develop the full syndrome with retinal degeneration, hearing loss, obesity and diabetes mellitus. Although there is overlap with BBS, Alström patients do not develop mental handicaps, polydactyly or hypogonadism. An evaluation of 182 Alström patients by Marshall and coworkers, led to the identification of additional phenotyping features, including cardiac (dilated cardiomyopathy), urological, gastrointestinal, pulmonary and neurobehavioral abnormalities (seizures and developmental delays) (Marshall et al., 2005). On initial evaluation, the phenotype may be dominated by ocular features, as the systemic features progress later. The *ALMS1* gene is mutated in Alström syndrome. It is ubiquitously expressed and the encoded protein localizes to centrosomes and ciliary basal bodies (Collin et al., 2002). *ALMS1* mutations do not interfere with the formation of cilia. However, mutant alleles are associated with age-dependent loss of primary cilia, suggesting that the Alström syndrome phenotype results from impaired cilia function rather than abnormal cilia development (Collin et al., 2002).

2.4.2. Batten disease

NCL is a group of pediatric neurodegenerative diseases known collectively as Batten disease. Two clinical forms of NCL overlap significantly with LCA. Congenital NCL is characterized by early developmental regression, visual loss, nystagmus and epilepsy in addition to the name-giving accumulation of autofluorescent liposomal storage material, lipofuscin. The gene for congenital NCL is *CTSD*, which encodes cathepsin D, and the cerebral and

retinal phenotype is caused by deficiency of this enzyme (Steinfeld et al., 2006). The infantile-onset NCL (Santavuori disease) is clinically characterized by normal development, visual failure, speech and motor deterioration, and seizures which appear between the ages of 6 and 24 months. Mutations in *PPT1* cause the infantile form of NCL (Vesa et al., 1995). PPT1 is an enzyme that removes fatty acids during lysosomal degradation.

2.4.3. Cerebello-oculo-renal syndromes

Cerebello-oculo-renal syndromes (CORS) are a group of partly overlapping syndromes that can affect the brain, eye and/or kidney. SLSN is an ar disease with the main features of nephronophthisis and retinal degeneration, varying from RP to LCA. Joubert syndrome (JBTS), also named Joubert syndrome and related disorders (JSRD) by hypoplasia of the cerebellar vermis with the characteristic neuroradiologic 'molar tooth sign' and accompanying neurological symptoms, including dysregulation of breathing pattern and developmental delay. JBTS can include a severe, early onset retinal dystrophy and nephronophthisis. Meckel (or Meckel-Gruber) syndrome (MKS) is characterized by renal cysts and variably associated features including developmental anomalies of the central nervous system (typically encephalocele), hepatic ductal dysplasia and cysts, and polydactyly. MKS is usually embryonic lethal. Mutations in several centrosomal and ciliary proteins have been associated with these syndromes (for a recent review, see Hildebrandt and Zhou, 2007).

Mutations in the *CEP290* (*NPHP6*) gene can cause SLSN, JBTS, BBS, and MKS (Sayer et al., 2006; Valente et al., 2006; Baala et al., 2007; Frank et al., 2007; Helou et al., 2007; Leitch et al., 2008), and have also been found to be a frequent cause of LCA (den Hollander et al., 2006). In one report, several LCA patients with *CEP290* mutations presented neurological symptoms that are characteristic of JBTS (Perrault et al., 2007).

2.4.4. Peroxisomal diseases

Peroxisomal diseases, also known as peroxisome biogenesis disorders (PBD), can be divided into three phenotypic classes: Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. All may phenotypically overlap with the ocular phenotype of LCA. PBDs form a spectrum of overlapping phenotypes with the most severe being the Zellweger syndrome and the least severe represented by infantile Refsum disease. Although the ocular phenotypes of PBDs overlap with LCA, the systemic features of cerebro-hepato-renal syndrome dominate the phenotype. Patients with PBD often present with retinal dystrophy, sensorineural deafness, dysmorphic features, developmental delay, hepatomegaly, and early death. The PBDs are caused by mutations in one of 12 different *PEX* genes which encode peroxins involved in peroxisome biogenesis (Okumoto et al., 1998). Peroxisomes are ubiquitous eukaryotic cell organelles, whose enzymes are involved in lipid metabolism.

2.5. Retinal function and structure

2.5.1. Electroretinogram

Historically, the first measurements of visual function in LCA were performed by electroretinogram (ERG) measurements. In short, ERGs measure the mass electrical response of the entire retina and the measurements can be separated into photopic (cone) and scotopic (rod) components, and can distinguish first-order neuron function (a-wave from photoreceptors) from second-order neuron function (b-wave from bipolar and Müller cells). ERG responses are non-detectable in LCA patients and are one of the basic features in the diagnosis, although a flat ERG tracing does not mean that the photoreceptors are dead or have disappeared. Because of the lack of ERG information in LCA patients and in an attempt to understand gene-specific retinal dysfunctions in LCA, heterozygous carriers of LCA mutations were analysed using ERG. Cone ERG dysfunction was found in *GUCY2D* mutation heterozygotes (Koenekoop et al., 2002), rod ERG dysfunction in *AIPL1* mutation heterozygotes (Dharmaraj et al., 2004), and rod and cone ERG dysfunctions in *RPGRIP1* mutation heterozygotes (R.K.K., unpublished results). Multifocal ERG analysis is powerful in detecting regional retinal dysfunction in carriers with heterozygous LCA mutations, such as in *CRB1* (Yzer et al., 2006a).

2.5.2. Autofluorescence measurements

Fundus autofluorescence Autofluorescence measures lipofuscin accumulation in the RPE which is related to shed photoreceptor disc segments. It allows for the visualization of disease-specific distributions of lipofuscin in the RPE, often not (yet) visible on ophthalmoscopy. Autofluorescence images can be used in children to differentiate hereditary retinal diseases. Lorenz et al. (2004) found absent or minimal autofluorescence in all LCA patients with compound heterozygous or homozygous *RPE65* mutations, while autofluorescence was normal in the heterozygous parents and in LCA patients without mutations in *RPE65*. AF was found to be normal in LCA patients with *GUCY2D* mutations.

2.5.3. Optical coherence tomography

Optical coherence tomography (OCT), is a noncontact, non-invasive imaging technique used to obtain high resolution cross-sectional images of the retinal architecture. OCT is analogous to ultrasound B-scan imaging but light rather than sound waves are used in order to obtain a much higher longitudinal resolution of approximately 10 μm in the retina. OCT is clinically useful for imaging selected retinal dystrophies and the retinal response to the genetic lesion. Jacobson and coworkers determined the outer nuclear layer (ONL) and retinal laminae thickness in many LCA patients with *RPE65* mutations (Jacobson et al., 2007a). At the fovea, all 11 analysed patients with *RPE65* mutations had measurable ONL layers and 50% had normal foveal thickness. They revealed a peak of ONL thickness at around 3–5 mm from the fovea for several patients. This ONL peak corresponds to the well known high rod density ring in normals. These data clearly show that retinal dystrophy patients with *RPE65* mutations despite their advanced ages (11–53) and with advanced visual loss, have viable photoreceptors. However, cone photoreceptor loss occurred from 3 years onwards which gradually increased (Jacobson et al., 2007a). Residual cone photoreceptor structure and function persisted for decades. RPE pigmentation was within normal limits up to the third decade despite the partial loss of cones. After the third decade, the RPE showed demelanization and the visual acuity consistently worsened.

OCT studies of LCA patients with *CRB1* mutations revealed a lack of normal lamination, the retinal thickness was increased, but the ONL layer at the central fovea was reduced (Jacobson et al., 2003). The retinas of patients with *GUCY2D* mutations were found to contain intact cone photoreceptors at age 7 years (Milam et al., 2003). Also, ONL measurements in a patient with *RPGRIP1* mutations were normal at the foveal center, and rapidly declined to immeasurable with increasing eccentricity from the fovea (Jacobson et al., 2007b). In contrast, LCA patients with *RDH12* mutations had thick retinas, while others were thin, and all had the same disorganized pattern which is remarkably different from *RPE65*-associated disease (Jacobson et al., 2007c). LCA patients with *CEP290* defects unexpectedly retained photoreceptors in the cone-rich fovea, despite severe visual loss, and the visual brain

pathways remained anatomically intact (Cideciyan et al., 2007). LCA patients with mutations in the remaining genes (*AIPL1, CRX, IMPDH1, LCA5,* and *RD3*) have not yet been analysed. Despite retinal remodelling in LCA, OCT studies suggest viable photoreceptors which appear to remain until relatively late in the disease process.

2.5.4. Vascular diameter

The caliber of the retinal arterioles is an indicator of the overall metabolic status of the retina (Grunwald et al., 1996). In advanced retinal degeneration, the metabolic activity of the retina is low and subsequently the retinal arterioles are very thin. It is possible to perform image analysis to measure the diameter of the retinal arterioles in the fundus photographs of children with LCA (Hansen et al., 2008). This method may be very helpful to determine treatment effects in longitudinal treatment trials.

2.5.5. Dark-adapted visual thresholds

In addition to ERG measurements, another important method to assess visual function is to measure retinal sensitivity using psychophysical testing. ERG signals may be non-detectable while threshold measurements may be possible. Rod and cone threshold abnormalities can be measured by perimetric testing with both the Tübinger perimeter (Oculus, Germany) and the Humphrey Field Analyzer (Allergan Humphrey, San Leandro, CA). Dark-adapted thresholds can be determined with 500 and 656 nm test stimuli with a diameter of 1.7° and duration of 500 ms, using the Tübinger perimeter, at various retinal locations along the horizontal meridien.

Static visual fields can also be obtained by using the 10-2 standard protocol of the Humphrey field analyzer with a 0.43° diameter test stimulus and background luminance of 1.07 log cd/m^2. Various measurements at 2° intervals can be obtained from 10° nasal to 10° temporal along the horizontal meridian. In children, measurement of the dark-adapted visual threshold (DAT) using a preferential looking procedure has been used to obtain useful retinal sensitivity measurements (Fulton and Hansen, 1983).

2.5.6. Post-mortem retina pathology

Thirteen pathological specimens have been described in the literature that most likely represent LCA. It is important to keep in mind that all globes (except one) were obtained from adults with LCA, and likely underwent extensive secondary retinal changes from retinal remodeling and re-wiring. Unfortunately, most of these cases have not been genotyped, precluding crucial histopathology-genotype correlations. However it may be possible to recognize at least three possible disease categories (Koenekoop, 2004).

Seven cases most likely represent *degenerations* (Aubineau, 1903; Sorsby and Williams, 1960; Kroll and Kuwabara, 1964; Francois and Hanssens, 1969; Flanders et al., 1984; Sullivan et al., 1994; Porto et al., 2002; discussed in Koenekoop, 2004) based on the fact that the photoreceptor, inner retinal layer, and RPE layer showed extensive atrophy, dropout, and/or gliosis. It is also possible that these cases represent *aplasias* or *dysfunctions* with a consequent, superimposed *degeneration*. Only one globe of an infant with LCA and *RPE65* mutations has been published and the pathology suggests a degenerative process (Porto et al., 2002).

Three of the cases appear to represent *aplasias*, based on the complete absence of the photoreceptor layer or the presence of a very unusual looking, primitive cuboidal cells in this layer (Vrabec, 1951; Babel, 1962; Gillespie, 1966; discussed in Koenekoop, 2004), but none of these cases has been genotyped.

Finally, three cases may represent the *biochemical dysfunction (dysplasia)* category of disease, because almost the entire retina, including the inner layers, the photoreceptor layer, and the RPE appear intact, despite the fact that these globes came from adults or young adults who are blind from LCA (Horsten and Winkelman, 1960; Babel, 1962; Milam et al., 2003; discussed in Koenekoop, 2004).

Despite photoreceptor changes in the ONL, the inner retina was essentially normal in appearance and architecture based on microscopic histological evaluation in six out of the 13 globes (Aubineau, 1903; Horsten and Winkelman, 1960; Babel, 1962; Kroll and Kuwabara, 1964; Flanders et al., 1984). These findings bode well for cell replacements and visual prosthetics in the future. One very important and illustrative case of an LCA patient with *GUCY2D* mutations was found to have relatively intact retinas with viable appearing cone photoreceptors (Milam et al., 2003).

Heegaard et al. (2003) described an unusual vascular morphology in an enucleated eye from a 22-year-old LCA patient. The patient and an affected sibling carried a paternal p.His82Tyr *AIPL1* variant which was not found in healthy controls, and a maternal p.His90Asp *AIPL1* polymorphism. In a highly unusual manner, the vascular system attenuated towards the retinal periphery, and stopped 4 mm from the ora serrata (Heegaard et al., 2003). In this unusual case, it was mainly retinal venules that showed dilatations, in particular in the posterior pole. In the mid-peripheral retina, the density of capillaries of the arterioles was significantly decreased. The surviving photoreceptors were cone-dominated. Transfection of the p.His82Tyr *AIPL1* mutant in neuroblastoma cells revealed a normal subcellular localization and solubility, but an increased ability of AIPL1 to redistribute GFP-NUB1 to the cytoplasm and resolve NUB1 fragment inclusion formation (van der Spuy and Cheetham, 2004). The absence of a second *AIPL1* allele suggests that it could be located outside the open reading frame or constitutes a heterozygous deletion refractory to DNA PCR detection. Alternatively, the identification of an *AIPL1* variant was a chance finding or the inheritance pattern is more complex and other genes are involved. As only the *AIPL1, CRX, GUCY2D*, and *RPE65* genes have been analysed in this patient, pathology might be caused by mutations in another LCA gene.

3. LCA genes and mutations

3.1. Gene-identification strategies

LCA is a heterogeneous disease, caused by mutations in 14 identified genes and an unknown number yet to be discovered genes. The 14 LCA genes have been identified by various methods, including classical linkage analysis, identity-by-descent mapping, and the candidate gene approach. Linkage analysis with microsatellite markers has been a laborious gene-identification method in the past. The availability of single nucleotide polymorphism (SNP) microarrays now enables rapid and relatively cheap linkage analysis in only a few days (Sellick et al., 2004). SNP microarrays containing up to 900,000 SNPs (Affymetrix 5.0 array) are currently available, but genotyping of 6000 (Illumina) or 10,000 (Affymetrix) SNPs is sufficient for genome-wide linkage analysis in families exhibiting a Mendelian inheritance pattern. Classical linkage analysis, however, requires the availability of relatively large families. Linkage data from smaller families can only be combined if there is no genetic heterogeneity. For ar LCA, families with at least 6 affected individuals are required to obtain significant linkage. In consanguineous families 3–4 affected individuals can be sufficient for significant linkage analysis, depending on the degree of consanguinity and the number of

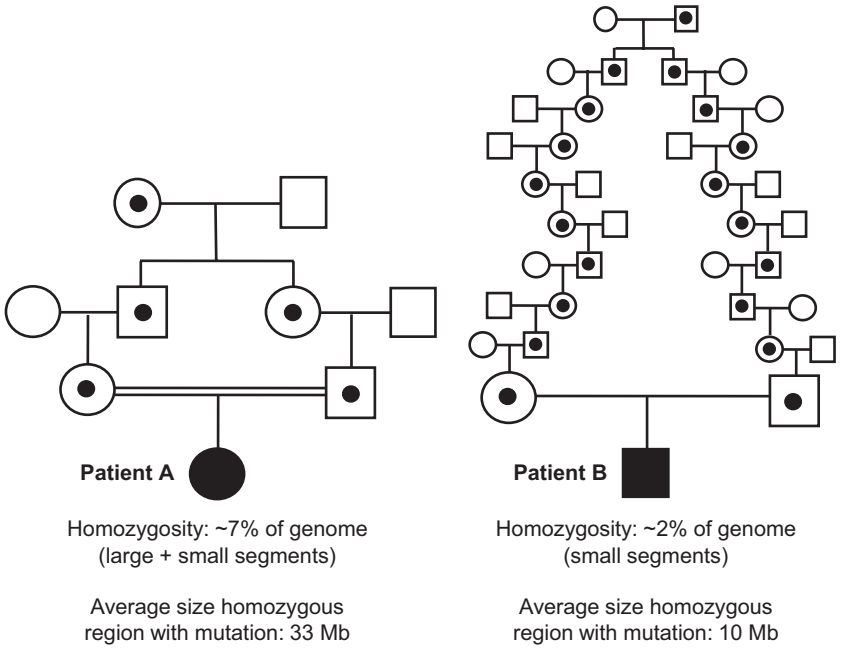


Fig. 2. Principle of identity-by-descent mapping. In the left pedigree, a typical consanguineous family (first cousin marriage), each affected individual theoretically is homozygous for 1/16 part (~200 Mb) of the genome. The causative mutation is inherited from the great grandmother to both parents of the patient. In the right pedigree, a common ancestor living 10 generations ago carries the pathologic mutation. As a rule of thumb, a causative mutation in an affected individual in the *N*th generation will be located in a homozygous chromosomal region of 100/*N* Mb, in patient A on average 33 Mb and in patient B on average 10 Mb. A patient in a consanguineous pedigree typically will contain more than 10 homozygous chromosomal segments that are larger than 5 Mb; a patient from a non-consanguineous family typically will carry one or two homozygous segments that are larger than 5 Mb, but often are smaller.

consanguinity loops in the family. The *AIPL1*, *GUCY2D* and *RDH12* genes have been identified by classical linkage analysis in relatively large consanguineous and non-consanguineous families (Perrault et al., 1996; Sohocki et al., 2000a; Janecke et al., 2004). In addition, two loci (LCA9 on 1p36, and LCA3 on 14q24) have been mapped by linkage analysis, but the underlying genes have not yet been identified (Stockton et al., 1998; Keen et al., 2003).

The causative gene can be mapped effectively in consanguineous families by identity-by-descent (IBD) mapping. In this approach, chromosomal regions are searched that are inherited from both parents through a common ancestor. This approach can be very powerful in small families that are not large enough for linkage analysis, including isolated patients of consanguineous and non-consanguineous matings (Fig. 2) (den Hollander et al., 2007b). For cases from outbred populations, the homozygous regions are expected to be smaller than in cases from inbred populations, and therefore high-density SNP arrays should be used ($\geqslant$250 K). As a rule of thumb, the chromosomal region containing a homozygous mutation inherited from a common ancestor of the patient's parents, measures 100 cM/N, where *N* is the number of generations between the common ancestor and the patient (Woods et al., 2006).

IBD mapping recently led to the identification of both the *CEP290* and *LCA5* genes (den Hollander et al., 2006, 2007a). The *CEP290* gene was a strong candidate gene in one of the shared homozygous regions since it was previously found to be mutated in the *rd16* mouse (Chang et al., 2006). Mutation analysis in the French Canadian family identified an intronic mutation, c.2991+1655A>G (p.Cys998X), that results in the insertion of a cryptic exon in the mRNA and introduces a premature stop codon in the CEP290 protein. Strikingly, this mutation was found in 20% of LCA patients, rendering it one of the most important causes of LCA to date (den Hollander et al., 2006).

While the LCA5 locus was identified by linkage analysis in a family of the Old Order River Brethren (Dharmaraj et al., 2000a), the gene was ultimately identified by an IBD mapping approach in one consanguineous and one non-consanguineous patient, as these two patients showed a region of IBD at the LCA5 locus (den Hollander et al., 2007a).

Another method that has been used to identify LCA genes is the candidate gene approach. Since LCA only affects the retina, genes that are expressed preferentially in the retina or that encode proteins that have an important function in the retina, have been considered candidate genes for LCA. Many LCA patients and many candidate genes need to be screened in order to identify new genes. Mutation analysis of such candidate genes in patients with LCA or juvenile retinal dystrophies identified causative mutations in the *LRAT* (Thompson et al., 2001), *RPE65* (Gu et al., 1997; Marlhens et al., 1997; Morimura et al., 1998) and *RPGRIP1* (Dryja et al., 2001; Gerber et al., 2001) genes. The *CRB1* (Lotery et al., 2001; den Hollander et al., 2001a), *CRX* (Freund et al., 1998) and *IMPDH1* (Bowne et al., 2002) genes were considered candidate genes for LCA since they were previously implicated in other forms of retinal degeneration (Freund et al., 1997; Swain et al., 1997; den Hollander et al., 1999; Bowne et al., 2002).

Animal models with retinal dystrophies have also been instrumental to identify genes for LCA or early onset retinal dystrophy. The *MERTK* and *RD3* genes were considered candidate genes for retinal dystrophies since they were found to be implicated in the RCS rat and the *rd3* mouse, respectively (Gal et al., 2000; Nandrot et al., 2000; Friedman et al., 2006).

Though nearly all identified LCA genes are expressed preferentially or specifically in the retina, the recent identification of two LCA genes, *CEP290* and *LCA5*, which are both ubiquitously expressed, highlights the important role of ciliary proteins in the pathogenesis of LCA. An online database that can be very helpful in identifying new LCA genes is the Ciliary Proteome Database (Gherman et al., 2006). This database contains ciliary and basal body proteins that were identified in 10 independent studies using various experimental approaches, ranging from mass

Table 1
Frequency of mutations in the 14 LCA genes

Study	AIPL1	CEP290	CRB1	CRX	GUCY2D	IMPDH1	LCA5	LRAT	MERTK	RD3	RDH12	RPE65	RPGRIP1	TULP1
Dharmaraj et al. (2000b)				2/100	6/100							3/100		
Lotery et al. (2000)				5/176	11/176							12/176		
Sitorus et al. (2003)	1/21				0/21							2/21		
Hanein et al. (2004)	6/179		18/179	1/179	38/179							11/179	8/179	3/179
Booij et al. (2005)			4/35		4/35							2/35	2/35	
Zernant et al. (2005)	16/205		11/205	3/205	24/205							5/205	10/205	
Yzer et al. (2006b)	3/58		9/58	0/58	6/58			0/58	0/58		0/58	1/58	0/58	
Simonelli et al. (2007)	3/95	4/95	7/95	0/95	5/95			0/95	0/95			8/95	0/95	
Vallespin et al. (2007a)	1/42		10/42	0/42	0/42			0/42	0/42			0/42	3/42	
Henderson et al. (2007)	4/59		8/59	1/59	9/59			0/59	0/59			2/59	3/59	
Vallespin et al. (2007b)		4/49												
Sohocki et al. (2000b)	11/188													
den Hollander et al. (2001a)			7/52											
Lotery et al. (2001)			21/233											
Perrault et al. (2000)					24/118									
Morimura et al. (1998)												7/45		
Thompson et al. (2000)												20/228		
Simovich et al. (2001)												8/98		
Dryja et al. (2001)													3/57	
Gerber et al. (2001)													8/142	
den Hollander et al. (2006)		16/76												
Perrault et al. (2007)		38/192												
Bowne et al. (2006b)						2/24								
den Hollander et al. (2007a)							2/93							
Gerber et al. (2007)							3/179							
Thompson et al. (2001)								3/267						
Senechal et al. (2006)								1/216						
Sweeney et al. (2007)								0/82						
Gal et al. (2000)									3/328					
Tada et al. (2006)									1/96					
Tschernutter et al. (2006)									1/96					
Friedman et al. (2006)										1/881				
Janecke et al. (2004)											3/89			
Perrault et al. (2004)											8/110			
Thompson et al. (2005)											22/1011			
Sun et al. (2007)											2/36			
Hagstrom et al. (1998)														2/536
Paloma et al. (2000)														1/49
Overall mutation frequency	45/847 (5.3%)	62/412 (15.0%)	95/958 (9.9%)	12/914 (1.0%)	127/1088 (11.7%)	2/24 (8.3%)	5/272 (1.8%)	4/819 (0.5%)	5/774 (0.6%)	1/881 (0.1%)	35/1304 (2.7%)	81/1341 (6.0%)	37/872 (4.2%)	6/764 (0.8%)

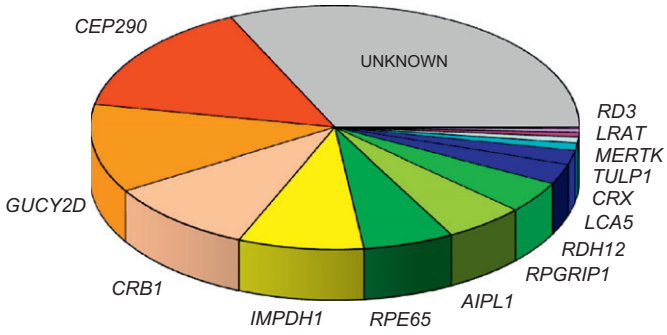


Fig. 3. Prevalence of LCA-associated mutations for the 14 causative genes (also see Table 1). *CEP290* (15%), *GUCY2D* (12%) and *CRB1* (10%) are the most frequently mutated genes. Mutations in approximately 30% of all cases remain to be identified.

spectrometry to comparative genomics. Proteins that were recently identified in a proteomic analysis of the mouse photoreceptor sensory cilium complex (Liu et al., 2007) have also been added to the Ciliary Proteome Database, increasing its value for identifying new LCA genes.

3.2. LCA mutations

To date, more than 400 mutations have been identified in the 14 LCA and juvenile RP genes. The inheritance pattern of LCA in most cases is ar, and due to restricted family size results in many isolated cases. However, a small number of dominant cases have been reported. Dominantly inherited LCA has been associated with a 12-bp deletion in the *CRX* gene (Sohocki et al., 1998). In addition, two *de novo* frameshift mutations in *CRX* have been described in two unrelated LCA patients (Freund et al., 1998), and two *de novo* missense mutations in the *IMPDH1* gene have been detected in two other unrelated patients (Bowne et al., 2006b). All four patients were isolated cases, and therefore a dominant mode of inheritance could not be confirmed. The mutations were not present in the patient's parents, indicating that they arose as new mutations, or were perhaps the result of a germ-line mosaicism. The finding of single *de novo* mutations indicates that not all isolated LCA cases are recessive.

A systematic mutation analysis of all known LCA genes in large LCA cohorts has not been performed, but a comprehensive analysis of one or more LCA genes in patient cohorts has been carried out (Dharmaraj et al., 2000b; Lotery et al., 2000; Sitorus et al., 2003; Hanein et al., 2004; Booij et al., 2005) and others have analyzed patient groups for all known mutations in the known LCA genes using the LCA mutation chip (see Section 4.4) (Zernant et al., 2005; Yzer et al., 2006b; Henderson et al., 2007; Simonelli et al., 2007; Vallespin et al., 2007a). The percentages of patients with mutations in the known LCA genes are shown in Table 1. Together, mutations in these 14 genes account for approximately 70% of all LCA cases. The most frequently mutated genes are *CEP290* (15%), *GUCY2D* (12%) and *CRB1* (10%) (Fig. 3).

Several founder mutations have been identified that are frequent in certain populations but absent in others. The intronic *CEP290* mutation p.Cys998X was found in 20% of LCA patients of worldwide origin in two reports (den Hollander et al., 2006; Perrault et al., 2007). All patients that carried this mutation were of European ancestry. The mutation was found less frequently in a group of Italian LCA patients (4%; Simonelli et al., 2007) and Spanish LCA patients (8%; Vallespin et al., 2007b), suggesting that it is an ancient mutation that arose in the north of Europe several centuries ago. Another, less frequent, founder mutation (p.Lys1575X) in the *CEP290* gene was detected in the north of France (Perrault et al., 2007). Mutations in the *GUCY2D* gene are more frequently found in patients from Mediterranean countries than in patients of worldwide origin (Hanein et al., 2002). However, the *GUCY2D* mutation p.Arg768Trp was found relatively frequent in north-western Europe (Yzer et al., 2006b), and the c.2943delG mutation represents an ancient founder mutation in the Finnish population (Hanein et al., 2002). The most frequent *CRB1* allele, p.Cys948Tyr, is found in patients of worldwide origin (den Hollander et al., 1999). The p.Trp278X mutation in the *AIPL1* gene accounts for approximately half of all *AIPL1* alleles, and may represent a founder mutation in the Pakistani population (Sohocki et al., 2000a, b).

Can genotype–phenotype correlations be discerned for LCA? Certain retinal appearances (Fig. 1) and changes in longitudinal aspects of visual function appear to be gene-specific (Koenekoop et al, 2007). For example, a PPRPE appearance of the retina is strongly suggestive of *CRB1* mutations. Transient visual function improvements are suggestive of *RPE65* mutations, and a relatively preserved retinal appearance suggests *GUCY2D* mutations. Genotype–phenotype correlations such as these found in LCA appear to be much scarcer in the much more prevalent RP disease phenotype. It is tempting to speculate that this important difference is due to the fact that LCA is a developmental retinal dystrophy, which may arrest at different time points depending on the gene defect (giving rise to phenotypic differences), while RP is an "acquired" retinal dystrophy and inexorably leads to slow but progressive cell death. The mechanistic cause for this difference is currently not known and explanations await further investigations.

3.3. Complex inheritance and modifier genes

Digenic inheritance was established for RP by Kajiwara et al. (1994). The p.Leu185Pro mutation in *peripherin/RDS* causes RP only in patients who also carry a mutation in the *ROM1* gene. Two *ROM1* mutations have been decribed to cause digenic RP when coinherited with the p.Leu185Pro mutation in *peripherin/RDS*: a null mutation and a p.Gly113Glu missense mutation (Kajiwara et al., 1994; Dryja et al., 1997). This digenic inheritance of double heterozygous *peripherin/RDS* and *ROM1* mutations is relatively rare, accounting for 0.5–3% of cases of presumed ad RP in a population of predominantly European origin (Dryja et al., 1997; Sullivan et al., 2006).

The existence of genetic modifiers is strongly suspected in *Crb1* mutant mice, where the photoreceptor degeneration varied strongly with the genetic background (Mehalow et al., 2003). This might explain why patients with *CRB1* mutations can either present early onset RP or LCA, while no clearcut genotype–phenotype correlation could be established based on the type of identified *CRB1* mutations (den Hollander et al., 2004).

The variable expression of *CEP290* null mutations, ranging from non-syndromic LCA, Joubert syndrome to a severe developmental disorder (MKS), suggests an important influence of other genetic factors (den Hollander et al., 2006; Sayer et al., 2006; Valente et al., 2006; Baala et al., 2007; Frank et al., 2007; Perrault et al., 2007). Triallelic inheritance has been recognized in families with BBS, a multisystemic disorder characterized by postaxial polydactyly, progressive retinal dystrophy, obesity, hypogonadism, renal dysfunction, and mental retardation (Katsanis et al., 2001). In other BBS families an epistatic effect has been described, which means that a more severe phenotype is seen in patients who carry a third mutation in a second BBS gene (Badano et al., 2003). Interestingly, Leitch et al. (2008) recently identified a homozygous *CEP290* nonsense mutation (p.Glu1903X), together

with a complex *MKS3* allele (p.Gly218Ala; p.Ser320Cys), in a patient with BBS, thereby expanding the *CEP290* disease spectrum. Functional proof that the *MKS3* mutations interact with *CEP290* defects was provided using morpholinos against *cep290* and *mks3* that showed a synergistic effect on the aberrant development of zebrafish embryos (Leitch et al., 2008).

A systematic analysis of all the known mutations in all the known LCA genes identified a higher number of LCA patients that carry a third allele than what would be expected by chance only (Zernant et al., 2005). Family members who carry a third allele in a second gene presented a more severe phenotype than family members who do not carry the third allele (Silva et al., 2004; Zernant et al., 2005).

It has been shown that a sequence variation in the *Rpe65* gene (p.Met450Leu) can act as a genetic modifier in inherited retinal degenerations in mice, presumably by regulating the daily rate of photon absorption through modulation of rhodopsin regeneration kinetics. Increased absorption of photons and light sensitivity appear to accelerate retinal degeneration via an apoptotic cascade (Samardzija et al., 2006).

Mice that carry the recessive mutation *tub* develop neurosensory defects including retinal and cochlear degeneration. A genome-wide linkage scan for modifiers of the retinal phenotype identified linkage on mouse chromosome 11 (the locus was named motr1, for Modifier of Tubby Retinal degeneration 1), and two regions of suggestive linkage on chromosomes 2 and 8 (Ikeda et al., 2002a). The underlying genes that cause the epistatic effect remain to be identified.

3.4. Molecular diagnosis

Genetic testing of LCA patients is crucial for genetic counseling of affected families. A molecular diagnosis can confirm the clinical diagnosis, distinguish LCA from other retinal diseases, and can give a patient a more accurate visual prognosis based on genotype–phenotype correlations that have been established for several LCA genes. Establishing a molecular diagnosis has become even more important since recent studies have shown that mutations in the LCA gene *CEP290* can also cause severe systemic abnormalities in JBTS, BBS or MKS (Sayer et al., 2006; Valente et al., 2006; Baala et al., 2007; Frank et al., 2007; Leitch et al., 2008). Genetic testing is also a prerequisite to enroll patients into clinical trials that will be tailored to counteract specific gene defects. Promising results have been reported for gene replacement therapy and pharmacological intervention in model organisms of LCA caused by *RPE65* mutations, and clinical trials in LCA patients are in progress (Bainbridge et al., 2008; Maguire et al., 2008).

Establishing a molecular diagnosis for LCA is hampered by the heterogeneous nature of the disease. Systematic sequence analysis or mutation scanning (e.g. via single strand conformation polymorphism, denaturing high-pressure liquid chromatography, or denaturing gradient gel electrophoresis) of all the known LCA genes would require the analysis of over 200 exons spanning 35,000 basepairs of DNA, which would be an expensive and laborious exercise. A cost-effective test that can overcome this problem is the LCA mutation chip (Asper Ophthalmics, Estonia), a microarray that contains all known mutations in the known LCA genes (Zernant et al., 2005). The test is based on the allele-specific primer extension analysis, is available for the entire scientific community, and is continuously updated with new mutations in the known genes and mutations that will be identified in new LCA genes. The LCA mutation chip is now estimated to identify the causative disease gene in approximately 55% of new LCA patients, but the effectivity can vary between populations (Yzer et al., 2006b; Henderson et al., 2007; Simonelli et al., 2007; Vallespin et al., 2007a).

A more comprehensive, but also more expensive, alternative is the re-sequencing chip for early onset retinal degenerations (Mandal et al., 2005). This microarray allows the re-sequencing of 11 genes involved in early onset retinal degenerations, and therefore also allows the detection of new mutations. However, the chip only covers two of the LCA genes (*CRB1* and *RPE65*), and would therefore be less effective to determine a molecular diagnosis in LCA patients. Moreover, heterozygous deletions and duplications larger than 1 bp are not detected.

The Carver lab (University of Iowa, Iowa City) performs genetic testing using a "mutation detection probability distribution", which is based on the fact that the occurrence of mutations varies significantly (Stone, 2007). With this approach, the regions of genes that have shown to contain the most frequent the mutations in the past are screened first in new LCA patients. This can significantly reduce the costs of genetic testing, since the most common alleles are detected early in the testing process.

One problem that is encountered during genetic testing, is to determine whether a new variant is pathogenic or not, which can particularly be challenging for amino acid (aa) variants, or sequence variants that do not lead to aa changes. One characteristic of pathogenic variants is that they cosegregate with the phenotype in the family. Affected siblings should carry both mutant alleles; non-affected siblings should carry no or one mutant allele, and non-affected parents should be heterozygous carriers, unless a variant has arisen *de novo*. The Carver lab uses a scoring system that divides the pathogenic potential into categories, ranging from 0 (extremely unlikely to cause disease) to 3 (pathogenicity is strongly supported) (Stone, 2003). Besides checking SNP databases and testing control individuals for a new variant to determine the allele frequency in the normal population, several tools exist that can be helpful to predict pathogenicity, including the Blosum 62 substitution matrix (Henikoff and Henikoff, 1992), PolyPhen (Ramensky et al., 2002), SIFT (Ng and Henikoff, 2003), PMut (Ferrer-Costa et al., 2005), and homology modeling (Krieger et al., 2003). In addition, one should check whether new variants can introduce or interfere with a splice site (Reese et al., 1997), or interrupt an exonic splice enhancer (Cartegni et al., 2003).

4. LCA disease mechanisms

4.1. Phototransduction (AIPL1, GUCY2D)

4.1.1. AIPL1

The aryl hydrocarbon receptor protein-like 1 (AIPL1) is a 384-aa protein that shares 49% identity with the human aryl hydrocarbon receptor interacting protein (AIP). A tetratricopeptide repeat (TPR) domain comprising three TRP motifs is conserved in AIP and AIPL1 (Fig. 4). The TPR motif is a degenerate, 34-residue sequence comprising a pair of anti-parallel α-helices. TPR domains function as molecular scaffolds mediating protein interactions. A primate-specific poly-proline-rich sequence of 56 aa with unknown function is present at the C-terminus in human AIPL1. At early stages *AIPL1* is expressed in the central and peripheral retina, which coincides with rod and cone photoreceptor development (van der Spuy et al., 2003). However, in the adult retina, expression is restricted to rod photoreceptors (van der Spuy et al., 2002) (Fig. 5).

AIPL1 mutations are grouped into three classes. The class I missense mutations (e.g. p.Met79Thr, p.Cys89Arg) are located in the N-terminus; the class II missense and stop mutations

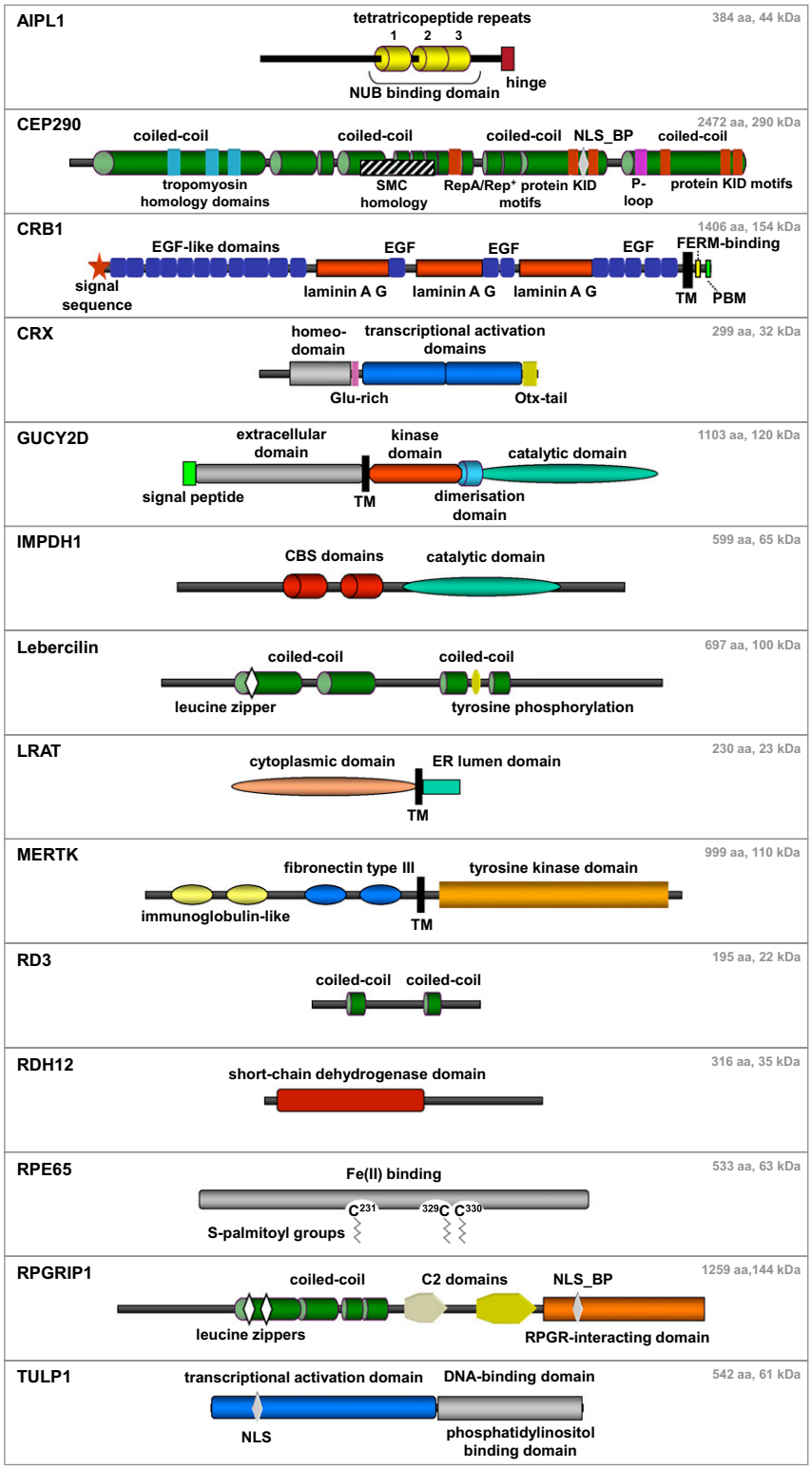


Fig. 4. LCA protein domain structures. The proteins are not drawn to scale. Polypeptide length and theoretical molecular weight are indicated in the right-upper corners. CBS, cystathionine beta synthase; EGF, epidermal growth factor; ER, endoplasmatic reticulum; FERM, 4.1, ezrin, radixin, moesin; Glu, glutamic acid; NLS, nuclear localization signal; NLS_BP, bipartite nuclear localization signal; PBM, PDZ binding motif; SMC, structural maintenance of chromosomes; TM, transmembrane.

(e.g. p.Trp88X, p.Q163X, p.Ala197Pro, p.Cys239Arg, and p.Trp278X) are located in the TPR motifs, and the class III mutations are small in-frame deletions located in the C-terminus (Fig. 4). The class I and II mutations are associated with ar LCA; the class III mutations appear to be associated with ad cone-rod dystrophy and juvenile RP (Sohocki et al., 2000b).

Yeast two-hybrid (YTH) screening using AIPL1 as bait identified NEDD8 Ultimate Buster 1 (NUB1) as an interacting protein (Akey

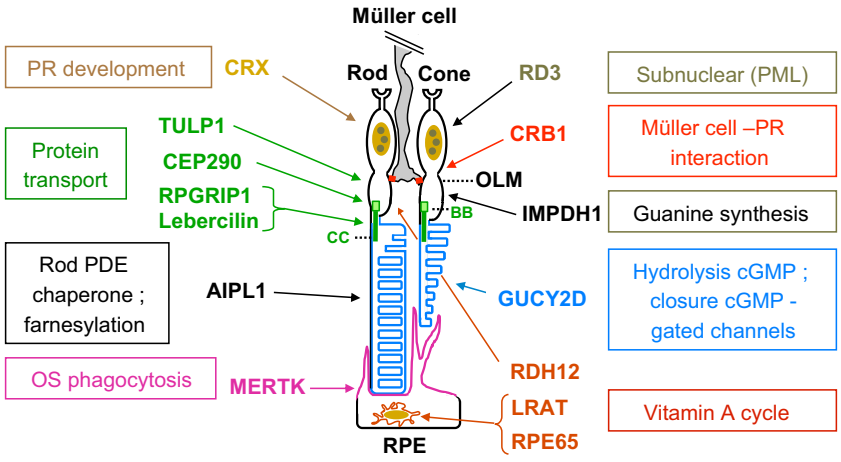


Fig. 5. Spatial expression of LCA genes that can be grouped according to nine different retinal functions. AIPL1 is predominantly located in rod photoreceptor outer segments. CRB1 is located in the cell membrane of Müller cells. CEP290 resides in the basal bodies. IMPDH1 and RDH12 are both predominantly located in rod and cone photoreceptor inner segments. LRAT and RPE65 are located in the membranes of the endoplasmatic reticulum of RPE cells. BB, basal body; CC, connecting cilium; promyelocytic leukemia; OLM, outer limiting membrane; OS, outer segments; PR, photoreceptor.

et al., 2002), which is thought to be involved in cell cycle progression by downregulating NEDD8 expression. The NUB1 binding domain of AIPL1 consists of aa 181–330. The AIPL1 binding domain of NUB1 consists of aa 569–584, overlapping with the NEDD8 binding site at aa 536–584. The p.Ala197Pro, p.Cys239Arg, and p.Trp278X mutations disrupt AIPL1-NUB1 binding (Kanaya et al., 2004).

In another YTH screening AIPL1 was found to interact with several farnesylated proteins (Ramamurthy et al., 2003). Protein analysis of a mouse with an Aipl1 knockdown (with hypomorphic mutation; see Table 2) revealed that all subunits of the rod cGMP phoshodiesterase show significantly reduced levels before noticeable retinopathy that starts at a later time point (Liu et al., 2004). All LCA mutations disrupt AIPL1 binding to farnesylated proteins. A comparable result was reported using a complete knockout of Aipl1 (Ramamurthy et al., 2004). Though ~10% of cGMP-PDE protein was detected, its enzymatic activity was absent. AIPL1 acts as a specialized chaperone by enhancing the farnesylation of cGMP-PDE-α, promoting cGMP-PDE folding, and assembling the subunit complex. *Aipl*$^{-/-}$ mice (Dyer et al., 2004) resemble *rd/rd* mice (Bowes et al., 1990) since both show reduced cGMP hydrolysis activity and elevated cGMP, causing rapid degeneration of photoreceptor cells. Interestingly, despite the similarities in disease mechanisms caused by both mutations in *AIPL1* and in *PDE6A* or *PDE6B*, as genetic defects in all these genes lead to markedly elevated and toxic levels of cGMP which both cause retinal degeneration, the resulting phenotypes are distinct. In humans, *AIPL1* mutations lead to the early onset and severe LCA, while *PDE6A* or *PDE6B* mutations lead to the later onset and initially much less severe arRP. The reason for this important difference is currently not know, but warrants further investigations.

4.1.2. GUCY2D (RetGC-1)

GUCY2D encodes a 1103 aa (120 kD) membrane guanylate cyclase (Fig. 4), RetGC-1, the enzyme involved in the resynthesis of cGMP required for the recovery of the dark state after phototransduction. *GUCY2D* is expressed specifically in the retina, where it localizes to the nuclei and inner segments of rod and cone photoreceptors. Many patients carry *GUCY2D* protein truncating mutations on both alleles, which are expected to result in the total absence of cyclase activity (Perrault et al., 1996, 1999). Functional analysis of LCA mutations in vitro showed that missense mutations in the catalytic domain result in complete inability to hydrolyze GTP to cGMP (Duda et al., 1999a, b; Rozet et al., 2001). Some missense mutations in the extracellular domain do not affect catalytic activity and likely result in misfolding of the mutant protein and subsequent degradation in the endoplasmatic reticulum (Rozet et al., 2001). However, a missense mutation affecting the start codon, as well as the p.Cys105Tyr and p.Leu325Pro mutations reduce the cyclase activity for as yet unknown reasons (Tucker et al., 2004).

Complete loss-of-function of RetGC-1 in LCA hinders the restoration of the basal levels of cGMP of cone and rod photoreceptor cells, leading to a situation equivalent to constant light exposure during photoreceptor development (Perrault et al., 2000). Two LCA-associated *GUCY2D* missense mutations in the catalytic domain (p.Pro858Ser, p.Leu954Pro; Fig. 4) were shown to result in severely reduced guanylyl cyclase activities (Tucker et al., 2004). An active catalytic site is formed from two head-to-tail oriented catalytic domain monomers (Liu et al., 1997; Tucker et al., 1998). Co-transfection of HEK293 cells with wild-type and p.Pro858Ser or p.Leu954Pro mutant RetGC-1 resulted in a 50% decrease of GCAP-2 stimulated cyclase activity, suggesting that heterodimers are inactive or poorly active (Tucker et al., 2004). This dominant-negative effect for recessive mutations could be shown in heterozygous carriers of the p.Leu954Pro mutation, who showed cone ERG abnormalities (Koenekoop, 2004). This is consistent with the findings that the expression of *GUCY2D* is much higher in cones than in rods (Dizhoor et al., 1994; Liu et al., 1994) and that Gucy2d KO mice develop a cone dystrophy (Yang et al., 1999).

4.2. Retinoid cycle (RDH12, LRAT, RPE65)

Photoactivation of rhodopsin and cone pigments causes isomerization of 11-*cis*-retinal to all-*trans*-retinal, which is recycled in a pathway termed the visual (retinoid) cycle. Three genes encoding proteins that play important roles in the visual cycle (Fig. 6) were found to be mutated in patients with LCA or juvenile ar retinal dystrophy, i.e. *LRAT*, *RDH12*, and *RPE65* (Morimura et al., 1998; Thompson et al., 2001; Janecke et al., 2004).

4.2.1. RDH12

RDH12 is a 316 aa (35 kD) member of a subfamily of four retinol dehydrogenases (RDH11-14). *RDH12* is expressed in the

mouse and human photoreceptor inner segments and ONL (Haeseleer et al., 2002; Jacobson et al., 2007c; Kurth et al., 2007). In the visual cycle, RDH12 can catalyze reduction of all-*trans*-retinal and 11-*cis*-retinal to their corresponding retinols. In vitro studies suggest that decreased 11-*cis*-retinal production due to disruption of the visual cycle (McBee et al., 2001; Lamb and Pugh, 2004) can be a cause of the degeneration caused by *RDH12* mutations.

Deletion of *Rdh12* in mice does not result in retinal degeneration as seen in humans with *RDH12* mutations, but does slow the kinetics of all-*trans*-retinal reduction, delaying dark adaptation. However, accelerated 11-*cis*-retinal production and increased susceptibility to light-induced photoreceptor apoptosis were also observed in $Rdh12^{-/-}$ mice, suggesting that RDH12 plays a unique, nonredundant role in the photoreceptor inner segments to regulate the flow of retinoids in the eye (Maeda et al., 2006). In the $Rdh12^{-/-}$ mice, all-*trans*-retinal in the inner segments cannot be quickly cleared and might trigger light-induced retinal degeneration. It is probably essential to clear the inner segments of toxic all-*trans*-retinal. By supplying chromophore to rhodopsin, accelerated 11-*cis*-retinal production in $Rdh12^{-/-}$ mice can also contribute to the aberrant accumulation of all-*trans*-retinal in the inner segments under intense light illumination. Thus, severe visual impairments of individuals with null mutations in *RDH12* may be caused by light damage.

The finding that $Rdh12^{-/-}$ mice retain a nearly normal visual cycle can be explained in two ways. The first is that RDH12 activity is not essential for regeneration of the 11-*cis*-retinal chromophore in mice or humans, and instead functions in another as yet unrecognized aspect of photoreceptor physiology. The absence of a rapid retinal degeneration in $Rdh12^{-/-}$ mice could also be due to species differences related to retinal physiology, compensation of *Rdh12* loss of function by another RDH enzyme, or disease onset linked to time in years rather than relative age. Expression of *RDH12* in photoreceptor inner, but not outer, segments requires that reduction of all-*trans*-retinal occurs at a site distant from its release from bleached photopigments, potentially involving a novel trafficking mechanism.

RDH12 is an NADP(+)-dependent oxidoreductase that in vitro catalyzes the reduction of all-*trans*-retinaldehyde to all-*trans*-retinol or the oxidation of retinol to retinaldehyde depending on substrate and cofactor availability. Although bi-directional in vitro, in living cells RDH12 acts exclusively as a retinaldehyde reductase, shifting the retinoid homeostasis toward the increased levels of retinol and decreased levels of bioactive retinoic acid (Lee et al., 2007). The retinaldehyde reductase activity of RDH12 protects the cells from retinaldehyde-induced cell death, especially at high retinaldehyde concentrations, and this protective effect correlates with the lower levels of retinoic acid in *RDH12*-expressing cells. Disease-associated mutants of RDH12, p.Thr49Met and p.Ile51Asn, exhibit significant residual activity in vitro, but are unable to control retinoic acid levels in the cells because of their dramatically reduced affinity for NADPH and much lower protein expression levels. These results suggest that RDH12 acts as a regulator of retinoic acid biosynthesis and protects photoreceptors against overproduction of retinoic acid from all-*trans*-retinaldehyde, which diffuses into the inner segments of photoreceptors from illuminated rhodopsin. These results are consistent with the observation that Rdh12 null mice are highly susceptible to light-induced retinal apoptosis in cone and rod photoreceptors.

4.2.2. LRAT and RPE65

The retinal pigment epithelium (RPE) is a monolayer of cells apposed to the outer surface of the retinal photoreceptor cells and is involved in many aspects of photoreceptor cell maintenance including the retinoid visual cycling (Fig. 6), photoreceptor outer segment disc phagocytosis and recycling. Two key components of the visual cycle are LRAT and RPE65.

LRAT is a 230-aa polypeptide (26 kD) that catalyzes the synthesis of retinyl esters, thereby drawing retinal from the circulation to storage depots such as lipid droplets of hepatic stellate cells and the retinosome structures in the RPE (Imanishi et al., 2004a, b). LRAT is localized to the membrane of the endoplasmic reticulum and assumes a single membrane-spanning topology with an N-terminal cytoplasmic/C-terminal luminal orientation (Moise et al., 2007). In eukaryotic cells, the C-terminal transmembrane domain is essential for the activity and endoplasmic reticulum membrane targeting of LRAT. In contrast, the N-terminal hydrophobic region is not required for endoplasmic reticulum membrane targeting or enzymatic activity, and its aa sequence is not conserved in other species.

RPE65 is an abundant RPE-expressed 63-kDa (533 aa) microsomal protein with isomerase activity (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005). Isomerohydrolase activity assays showed that the enzymatic activity of RPE65 requires LRAT coexpression. This observation is consistent with the finding that all-*trans* retinyl esters are the substrate for the isomerohydrolase reaction (Gollapalli and Rando, 2003; Moiseyev et al., 2003). RPE65 is homologous to β-carotene 15,15′-monooxygenases in mammals, which catalyze the initial step in biosynthesis of vitamin A (Redmond et al., 2001). The molecular structure of apo-carotene oxygenase, resolved by X-ray diffraction analysis, is a seven-bladed-propeller, and a similar structure is predicted for RPE65 (Guo et al., 2007). It was suggested that RPE65 associates with membranes through *S*-palmitoylation of residues Cys^{231}, Cys^{329}, and Cys^{330} by LRAT (Xue et al., 2004). However, employing $Lrat^{-/-}$ mice, Jin et al. (2007) have recently shown that LRAT is not required for isomerase activity beyond synthesis of retinyl-ester substrate, and that the association of RPE65 with membranes is neither dependent upon LRAT nor the result of *S*-palmitoylation. The affinity of RPE65 for membranes is probably an intrinsic feature of this protein. It is not clear whether LRAT and RPE65 are physically linked to form an enzymatic complex or whether they are simply located in cis in the same membrane.

RPE65 shares 4 histidine residues with members of the carotenoid oxygenases superfamily, which are involved in iron binding and essential for its catalytic activity (Poliakov et al., 2005). It was recently shown that RPE65 is an iron(II)-dependent isomerohydrolase and that RPE65 binds an iron ion with a stochiometry of ~0.8 (Moiseyev et al., 2006).

Mice lacking Rpe65 are unable to synthesize and recycle rhodopsin, due to a block in the regeneration of 11-*cis*-retinal. In the absence of RPE65, retinyl esters accumulate in the RPE cells, but the cause of death of the RPE cells is likely light-independent signalling of the constitutively active rhodopsin, which is not bound to 11-*cis*-retinal (Woodruff et al., 2007).

There are conflicting publications with respect to the site of expression of *RPE65* in the neural retina as *Rpe65* was found to be expressed in cone photoreceptors (Znoiko et al., 2002), or not (Hemati et al., 2005). Immunocytochemistry in the macaque localized RPE65 to the central retina; immunoblotting revealed a higher concentration in the central retina (Jacobson et al., 2007a). Early cone photoreceptor loss in LCA patients with *RPE65* mutations suggests that RPE65 is involved in visual chromophore production and is important for cones. While early cone loss was evident, residual retained cone structure and function for many years in humans support the speculation that alternative pathways are critical for cone photoreceptor survival. Other confirmation of the presence of RPE65 in cones came from studies by

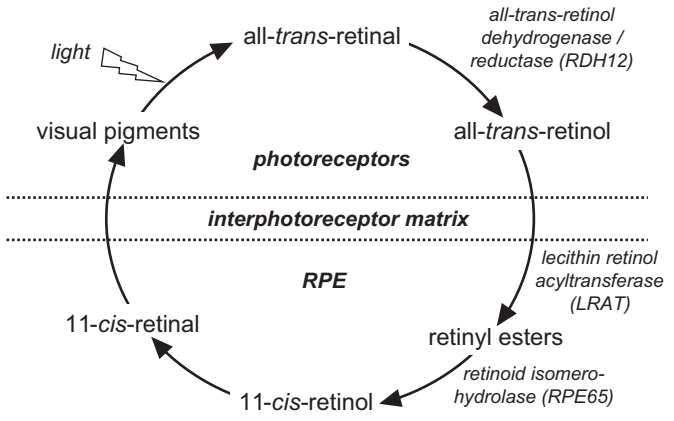


Fig. 6. Visual cycle illustrating the enzymatic conversion of all-*trans*-retinal to 11-*cis*-retinal using RDH12 in photoreceptors, and the intimately connected LRAT and RPE65 in the RPE.

Wenzel et al. (2007) who found that the retinas of $Nrl^{-/-}$ mice (with cone only retinas) contained elevated levels of Rpe65. Ablation of Rpe65 in $Nrl^{-/-}$ and $Rho^{-/-}$ mice (also cone only retinas) led to the absence of 11-*cis*-retinal.

Another source of controversy has centered around the cellular origin of the residual visual function in *Rpe65* knockout mice. By selective impairment of either rod function ($Rho^{-/-}$ mice) or cone function ($Cnga3^{-/-}$ mice) in $Rpe65^{-/-}$ mice, Seeliger et al. (2001) found that the ERGs of the $Rpe65^{-/-}$ and $Rpe65^{-/-}Cnga3^{-/-}$ mice were almost identical, while they found no ERG response in the $Rpe65^{-/-}Rho^{-/-}$ mice. Therefore, the rod system is the source of residual vision in Rpe65 deficient mice.

Microarray experiments and cone density analyses in the $Rpe65^{-/-}$ mouse revealed that certain cone-specific genes are downregulated and cones die very early in the disease process, while downregulation of rod-specific mRNAs was not observed and rods survived at early stages. Cone death occurred throughout the retina but was more significant in the center than in the periphery (Znoiko et al., 2005; Cottet et al, 2006). This suggests that the absence of 11-*cis* intermediates may be responsible for the cone degeneration.

As cones are an important component of human vision, it needs to be evaluated whether RPE cell-specific adeno-associated virus transfections are adequate to rescue RPE cells as well as, cones and rods, or whether cone-specific AAV types need to be incorporated in the therapeutic regimens (see: Section 6.1).

Low levels of cGMP mimic the light adapted state of the retina and lead to the LCA phenotype. In LCA patients with *GUCY2D* mutations, the phenotype is likely the result of lack of GUCY2D enzyme, which leads to the inability to produce cGMP. In LCA patients with *RPE65* mutations, the unbound opsin (without the 11-*cis*-retinal) causes a constitutively activated phototransduction cascade, resulting in rod cGMP PDEβ activation and hydrolysis of cGMP, leading to a similar paucity of cGMP as in *GUCY2D* patients. Interestingly, despite these similarities in presumed pathophysiological mechanisms, the resulting phenotypes are divergent, to the point where LCA patients with *GUCY2D* mutations are clinically distinguishable from patients with *RPE65* mutations. LCA patients with *GUCY2D* mutations have severe VA loss, relatively normal retinas but do not experience nyctalopia. Patients with *RPE65* mutations have striking nyctalopia, transient improvements in VA, and a characteristic retinal appearance with RPE translucency and salt and pepper changes. These differences are currently unexplained.

4.3. Photoreceptor development and structure (CRX, CRB1)

4.3.1. CRX

The Cone-rod homeobox gene *CRX* is a member of the highly conserved orthodenticle-related (otx) gene family, and encodes a 299-aa homeobox transcription factor with a predicted mass of 32 kDa (Fig. 4) (Furukawa et al., 1997). CRX is related to the products of other homeobox genes like *PAX6*, *SIX3*, *CHX10/VSX2* and *RX/RAX*, which play crucial roles at various stages of eye development (Chow and Lang, 2001). CRX is the earliest expressed photoreceptor marker in the retina, and is also expressed in pinealocytes in the pineal gland and regulates photoentrainment (Furukawa et al., 1999) and expression of genes involved in synthesizing the circadian hormone melatonin in mice (Li et al., 1998). CRX is essential for the differentiation and maintenance of photoreceptor cells. It acts synergistically with the eye-specific transcription factors neural leucine-zipper (NRL) and homeobox protein RX in the transactivation of photoreceptor-specific genes, regulating the high-level of expression of photoreceptor outer segment proteins such as rhodopsin, IRBP, β-PDE and arrestin (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997; Kimura et al., 2000; Mitton et al., 2000). Absence of Crx in $Crx^{-/-}$ mice prevents outer segment biogenesis, and therefore abrogates the phototransduction pathway in both rods and cones (Furukawa et al., 1997). Recently, CRX was found to interact with different histone acetyl-transferases, suggesting that a possible mechanism for CRX-mediated transcriptional activation is to recruit histone acetyl-transferases to photoreceptor gene chromatin for histone acetylation, thereby inducing and maintaining appropriate chromatin configurations for transcription (Peng and Chen, 2007).

4.3.2. CRB1

CRB1 is homologous to the Drosophila transmembrane protein Crumbs (den Hollander et al., 1999, 2001b). Drosophila Crumbs is required for maintenance of apico-basal cell polarity and adherens junction in embryonic epithelia (Tepass et al., 1990), and has a similar function in adult fly retina (Izaddoost et al., 2002). For epithelial cells and neurons like photoreceptors, separation of their apical and basal compartments is critical for correct functioning in cell-to-cell adhesion, intercellular signaling, directional transport of (secreted) molecules and correct tissue formation. Crumbs molecules are found in many species, ranging from invertebrates to mammals (Richard et al., 2006). In mice and humans, three Crumbs orthologues are detected: CRB1/Crb1, CRB2/Crb2 and CRB3/Crb3. *Crb1* is only expressed in the brain and retina (den Hollander et al., 2002), whereas *CRB2/Crb2* is also expressed in the kidney, RPE/choroid and at low levels in heart, lung and placenta (van den Hurk et al., 2005). *Crb3* is ubiquitously expressed in different (epithelial derived) tissues, including the retina (Makarova et al., 2003). In zebrafish, there are five Crumbs isoforms due to a genome duplication event in teleost fish: crb1, crb2a/ome, crb2b, crb3a and crb3b (Omori and Malicki, 2006). Alternative splicing of the gene encoding human CRB1 results in two isoforms, a 1376 aa extracellular protein with a predicted size of 151 kDa that is possibly secreted, and a 1406 aa transmembrane protein with a predicted size of 154 kDa (den Hollander et al., 2001b). Only the latter contains a 37 aa cytoplasmic domain, while both extracellular domains contain a signal peptide, 19 EGF-like domains, and three laminin A G-like domains (Fig. 4). CRBs migrate at a larger molecular weight than predicted due to post-translational modifications. The extracellular domain of CRB1 contains conserved N-linked glycosylation motifs, likely to be responsible for its N-glycosylation (Kantardzhieva et al., 2005). Whether or not carbohydrate binding proteins such as lectins

have affinity for Crumbs is not known, since no binding partners for the extracellular domain have been identified so far.

The intracellular domain of CRB1 has a highly conserved role in organizing a macromolecular protein scaffold, as reviewed by (Richard et al., 2006). This scaffold is located at the subapical region, a region just apical to the adherens junctions at the outer limiting membrane in the retina (van de Pavert et al., 2004; Kantardzhieva et al., 2005, 2006). Adherens junctions are the sites where the cytoplasmic face of the plasma membrane is attached to actin filaments. They are found in photoreceptors and Müller cells, as well as in epithelial cells, where they separate the apical and basolateral membrane. Immuno-electron microscopy in murine retinas revealed that Crb1 immunoreactivity is mainly present at the subapical region in Müller glia cells (Fig. 5), whereas CRB2 and CRB3 (and the scaffold members PATJ, MPP5 and MUPP1) are present both in photoreceptors and Müller glia cells (van Rossum et al., 2006). Loss of Crb1 in Müller glia cells of *Crb1*$^{-/-}$ mice resulted in an irregular number and size of their apical villi, suggesting that Crb1 is required to regulate number and size of these Müller glia cell villi (van de Pavert et al., 2007b).

CRB1-associated LCA is suggested to be caused by a developmental defect in the retina, since LCA patients with mutations in *CRB1* have a thickened retina and lack the distinct layering of a fully developed adult retina, resembling a more immature normal retina (Jacobson et al., 2003). Retinal patterning defects have also been observed in the zebrafish retina associated with mutations in different members of the Crumbs complex, including *ome* (oko meduzy) (Malicki and Driever, 1999), encoding Crb2a (Omori and Malicki, 2006).

4.4. Transport across the photoreceptor connecting cilium (TULP1, RPGRIP1, CEP290, Lebercilin)

4.4.1. TULP1

TULP1 is a member of the Tubby-like protein (TULP) family, consisting of 4 proteins in vertebrates (TUB, TULP1, TULP2, and TULP3) (Ikeda et al., 1999, 2002b). These proteins have an important role in the development and function of the central nervous system, as reviewed elsewhere (Ikeda et al., 2002b; Carroll et al., 2004). The 542-aa (61 kDa) TULP1 protein contains a C-terminal "tubby domain" of 240 aa that is conserved among the TULP family, and contains a phosphatidylinositol-binding region that may anchor the protein to the cell membrane (Fig. 4) (Xi et al., 2005). Its N-terminal half contains a nuclear localization signal and transcriptional activation activity, and the tubby domain exhibits DNA-binding activity which indicates a potential transcription factor activity (Boggon et al., 1999).

The gene is predominantly expressed in the photoreceptors of the retina (North et al., 1997; Ikeda et al., 1999), which was confirmed by the detection of the protein using a specific antibody (Hagstrom et al., 1999; Ikeda et al., 1999; Milam et al., 2000), and absence of the protein in photoreceptor-less *rd/rd* mice (Hagstrom et al., 1999). The protein was also detected at lower levels in the brain, in the paraventricular nuclei of the hypothalamus (Ikeda et al., 2000). However, *Tulp1*$^{-/-}$ mice only showed a retinal degeneration phenotype, and brain anomalies were not detected, indicating that its function in the brain is not crucial. In the retina, the protein localizes predominantly to the photoreceptor cells, both rods and cones (Hagstrom et al., 1999; Ikeda et al., 2000). In agreement with this, absence of Tulp1 was found to severely affect both light- and dark-adapted ERG responses (Hagstrom et al., 1999). Within the photoreceptor cells, TULP1 is most abundant in the inner segments, but could also consistently be detected in the perinuclear and synaptic regions (Hagstrom et al., 1999, 2001; Ikeda et al., 2000; Milam et al., 2000; Xi et al., 2005, 2007), and was mentioned to also be present in the connecting cilia (Hagstrom et al., 2001). In contrast to its suggested function as a transcription factor, no significant signal could be detected in the photoreceptor nuclei (Hagstrom et al., 2001). Overexpression of the protein in cell lines however revealed localization of a subset of the protein to the nucleoli (Xi et al., 2005) . This may indicate that the levels of TULP1 in the nuclei are too low to be detected, or its epitope is masked in the nucleus. Information about gene targets of TULP1 as a transcription factor remains elusive. TULP1 was also identified in retinal neuroblasts as early as 8 fetal weeks, suggesting an important role of TULP1 in retinal differentiation (Milam et al., 2000). This is in line with the early onset of the retinal degeneration in LCA. A low-level staining of the differentiating, as well as adult ganglion cells may also be important in that respect (Milam et al., 2000).

Absence of Tulp1 in knockout mice also induced mislocalization of rhodopsin to the plasma membrane of the inner segments, and budding into extracellular vesicles (Hagstrom et al., 2001). This is suggestive of a role of TULP1 in rhodopsin transport from the inner to the outer segment, without revealing its exact function in this complex process. Finally, immunoprecipitation experiments using anti-TULP1, followed by mass spectrometry have revealed interaction of TULP1 with F-actin (Xi et al., 2005) and, very recently, with dynamin-1 (Xi et al., 2007). The interaction with F-actin supports the hypothetical role in opsin transport across the cellular subcortical actin cytoskeleton from the inner segment through the connecting cilium to the outer segment. The binding to the neuronal-specific GTPase dynamin-1 is in line with this hypothesis. Given the importance of this GTPase in vesicle formation, trafficking and recycling, it strengthens the putative link with vesicular protein transport, and adds to the picture a suggestive role in protein trafficking to the synapse (Xi et al., 2007).

4.4.2. RPGRIP1

The RP GTPase Regulator interacting protein 1 (RPGRIP1) directly binds to the RP GTPase regulator (RPGR) with its C-terminal RPGR interacting domain (RID) (Boylan and Wright, 2000; Roepman et al., 2000). RPGR is mutated in the majority of patients with X-linked RP. Disease-associated missense mutations in the RCC1-like domain of RPGR disrupted the interaction between RPGRIP1 and RPGR, suggesting that this defect could underly the pathogenesis of RP (Roepman et al., 2000). LCA-associated mutations in the RID of RPGRIP1 could lead to a gain- and loss-of-binding to RPGR (Lu et al., 2005). Different splice variants of RPGRIP1 have been identified, the largest encoding a protein of 1259 aa with a predicted molecular weight of 144 kDa (Lu and Ferreira, 2005). RPGRIP1 contains two coiled-coil domains that are homologous to those found in proteins involved in vesicular trafficking (Fig. 4) (Boylan and Wright, 2000; Roepman et al., 2000; Hong et al., 2001), and a bipartite nuclear localization signal that could facilitate shuttling to the nucleus of some isoforms (Roepman et al., 2005). Also, translocation to the nucleus has been described after limited proteolytic cleavage, supporting distinct and spatiotemporal subcellular-specific roles to RPGRIP1 (Lu and Ferreira, 2005). The central region of RPGRIP1 contains two C2 domains, a truncated N-terminal C2-domain and a more conserved C-terminal C2-domain. Most LCA-associated missense mutations were identified in the latter, a putative Ca^{2+}-binding C2 domain (Roepman et al., 2005).

Localization of RPGRIP1 at other subcellular sites in the retina has also been reported (Roepman et al., 2000; Mavlyutov et al., 2002; Lu and Ferreira, 2005), including a prominent localization at the connecting cilium (Hong et al., 2001; Zhao et al., 2003). In addition, a YTH screen also identified interaction of RPGRIP1 with

nephrocystin-4, encoded by *NPHP4*. Nephrocystin-4 also localizes to cilia, and *NPHP4* mutations can lead to nephronophthisis (NPHP, juvenile kidney failure) and to Senior-Løken syndrome (SLSN, nephronophthisis with RP) (Mollet et al., 2002). The RPGRIP1-nephrocystin-4 interaction is disrupted either by mutations in RPGRIP1, found in patients with LCA, or by mutations in NPHP4, found in patients with nephronophthisis or SLSN (Roepman et al., 2005). This provides evidence for the involvement of this disrupted interaction in the retinal dystrophy of both SLSN and LCA patients. In light of these findings, a presumed function of RPGRIP1 in the retina could be to anchor a protein scaffold at the connecting cilium, linking a network of proteins that are crucial for retinal function. Studies of the retina of *Rpgrip1*$^{-/-}$ and *Rpgr*$^{-/-}$ mice showed that RPGRIP1 localized to the connecting cilium without RPGR, but not vice versa, indeed indicating that RPGR is dependent on RPGRIP1 to be anchored to the connecting cilium (Zhao et al., 2003).

4.4.3. CEP290

CEP290 is a centrosomal protein of 2472 aa with a molecular weight of 290 kDa. It was first identified in a proteomic analysis of the human centrosome (Andersen et al., 2003). Expression of the *CEP290* gene was severely reduced in *Crx*$^{-/-}$ mice and *Nrl*$^{-/-}$ mice, thus pinpointing it as the candidate gene harboring the causal mutation of the *rd16* mouse (Chang et al., 2006). The protein is strongly conserved throughout evolution, and contains several predicted motifs, including 13 coiled-coil domains (Fig. 4) (Sayer et al., 2006). A bipartite nuclear localization signal would explain its partial localization to the nucleus, where it binds to and activates transcription factor ATF4 (Sayer et al., 2006). Besides its localization to the centrosome of dividing cells and to the nucleus, the protein localizes to the basal bodies at the base of the cilia in many different cell types, including the photoreceptor connecting cilium (Chang et al., 2006; Sayer et al., 2006; Valente et al., 2006). Using immunoprecipitation from retinal extracts followed by western blotting, it was demonstrated that CEP290 exists in the same complex with several microtubule-based transport proteins, including RPGR (Chang et al., 2006). Many of these proteins were also detected in similar co-immunoprecipitation experiments with RPGR (Khanna et al., 2005). The dynamics of this complex, including the direct interactions of the protein members, remain to be identified. In this respect it is interesting that much more of the ΔCEP290 mutant protein in the *rd16* mouse is pulled down by RPGR upon immunoprecipitation with the anti-RPGR_ORF15^{CP} antibody than the much more abundantly expressed wild-type CEP290 protein. This could indicate a close relationship of both proteins. This relationship requires further analysis, as RPGR shows a different ciliary localization at the ciliary axoneme (Khanna et al., 2005), compared to the basal body localization of CEP290. The redistribution of RPGR, rhodopsin and arrestin in the *rd16* retinas suggests a function of CEP290 in microtubule-associated transport across the cilium. The finding that overexpression of the dynein motor subunit p50-dynamitin does not influence the localization of CEP290 in cells indicates that this transport is not dynein dependent. The presence of a C-terminal myosin-tail homology domain may provide a structural backbone to the myosin motor instead (Chang et al., 2006), although this remains to be identified.

4.4.4. Lebercilin

The *LCA5* gene is almost ubiquitously expressed during early embryonic development, while at later stages its expression shifts towards ciliated tissues (den Hollander et al., 2007a). The *LCA5* gene product lebercilin (697 aa; apparent molecular weight 100 kDa) was found to be present in the ciliary proteome database (Gherman et al., 2006) and localization to cilia of cultured cells and to the photoreceptor connecting cilia was confirmed using anti-lebercilin antibodies in immunohistochemistry and immuno-EM analyses (den Hollander et al., 2007a). Lebercilin contains four coiled-coil domains, a leucine-zipper motif, and a putative tyrosine phosphorylation signal (Fig. 4). The function of lebercilin was further unraveled by expression of GFP-tagged recombinant proteins in ciliated as well as non-ciliated cells. In non-ciliated cells, the protein localized to the mother centriole of the centrosome and to microtubules. An increased expression of recombinant lebercilin in some cells induced microtubule bundling, indicating a role of the protein in microtubule dynamics. In ciliated cells, lebercilin localized to the basal bodies and transition zone of the cilia when the protein expression was low (den Hollander et al., 2007a). Upon increased expression, the protein also decorated the full ciliary axoneme and microtubule cytoskeleton of the cells. Tandem affinity purification of lebercilin revealed interesting details of the link between lebercilin and ciliary and centrosomal function. Among the 24 proteins that could be identified to associate with lebercilin, either directly or in a complex, four functional groups could be distinguished: adaptor proteins, cytoskeletal proteins, cellular signalling proteins and (co-) chaperones. The associated pathways could be disrupted by mutations in *LCA5*, although the exact pathologic mechanism is not yet known.

With these four ciliary proteins, the role of disrupted ciliary processes in the molecular pathogenesis of LCA is emphasized, pinpointing a growing group of LCA subtypes as ciliopathies.

4.5. Miscellaneous (IMPDH1, MERTK, RD3)

4.5.1. IMPDH1

Mutations in the *IMPDH1* gene were initially identified in ad RP (Bowne et al., 2002; Kennan et al., 2002). In these studies, the gene was found to be downregulated in the retinas of *Crx*$^{-/-}$ (Bowne et al., 2002) and *Rho*$^{-/-}$ mice (Kennan et al., 2002). *IMPDH1* is widely expressed in many tissues, but expression levels are higher in the retina than in all other tissues tested, in particular in the inner segments (Bowne et al., 2006a). In addition, several unique retinal isoforms have been detected, which were the result of alternative splicing and alternate start sites of translation. This might explain the retina-specific phenotype associated with *IMPDH1* mutations (Bowne et al., 2006a).

IMPDH1 functions as a tetramer composed of four identical subunits, which catalyzes the rate-limiting step of *de novo* guanine synthesis. It converts inosine monophosphate into xanthosine monophosphate with the reduction of NAD. The adRP-associated mutations p.Arg224Pro and p.Asp226Asn are not located in the catalytic domain (Fig. 4), and do not reduce the IMPDH1 enzyme activity (Aherne et al., 2004; Mortimer and Hedstrom, 2005). One study suggests that these mutations cause misfolding and aggregation of the protein (Aherne et al., 2004), but this was not confirmed in another study (Mortimer and Hedstrom, 2005). Both mutations are located in the second cystathionine beta synthase (CBS) domain of IMPDH1 (Fig. 4). Although the role of the CBS domains in IMPDH1 is not yet known, it was shown that it can bind single-stranded nucleic acids and therefore might play a role in transcription, translation, post-translational modification, localization or other aspects of RNA metabolism (McLean et al., 2004). *IMPDH1* mutations associated with adRP and *de novo* LCA significantly reduce the nucleic acid binding affinity and specificity (Mortimer and Hedstrom, 2005; Bowne et al., 2006b). Although the disease mechanism associated with *IMPDH1* mutations is not yet understood, it might involve a perturbation in RNA metabolism in photoreceptor cells.

4.5.2. MERTK

A mutation in the *MERTK* gene was initially identified in the Royal College of Surgeons (RCS) rat, a classical model of retinal degeneration in which the RPE fails to phagocytose shed outer segments (D'Cruz et al., 2000; Nandrot et al., 2000). The *MERTK* gene is expressed in several tissues, and strongly in RPE and macrophages, two cell types that are capable of phagocytosis. *MERTK* encodes a receptor tyrosine kinase that is a member of a family of at least three receptor tyrosine kinases, which also includes Axl and Tyro3. The members of the Mer-family exhibit a similar extracellular domain structure that is composed of two immunoglobulin-like domains followed by two fibronectin type-III repeats, a transmembrane domain and an intracellular tyrosine kinase domain (Fig. 4).

MERTK is an integral component of the RPE phagocytic process in cell culture, in which it functions to trigger ingestion of bound outer segments (Feng et al., 2002). The phagocytosis of outer segments depends on activation of MERTK by its ligands, Gas6 and protein S (Hall et al., 2001, 2005). The intracellular tyrosine kinase domain of MERTK interacts with the SH2 domain of the guanine exchange factor (GEF) Vav1 (Mahajan and Earp, 2003). MERTK activation leads to tyrosine phosphorylation and release of Vav1. This activates the GEF activity of Vav1, which could presumably lead to cytoskeletal remodeling and phagocytosis. Internalization signals are presumably amplified by an intracellular signaling mechanism between MERTK and $\alpha V\beta 5$ integrin, a vibronectin receptor which is present at the RPE-outer segment interface (Finnemann, 2003; Wu et al., 2005). Nearly all *MERTK* mutations identified in patients with early onset retinal dystrophy represent loss-of-function alleles that are predicted to result in truncated *MERTK* protein lacking the intracellular tyrosine kinase domain (Gal et al., 2000; McHenry et al., 2004; Tschernutter et al., 2006). Only one missense mutation, p.Arg844Cys, has been identified, which presumably causes loss of function due to decreased protein stability (McHenry et al., 2004).

4.5.3. RD3

The mouse *Rd3* gene is preferentially expressed in the retina, and exhibits an increasing expression during early postnatal development (Friedman et al., 2006). The RD3 protein has a predicted molecular mass of 22 kDa, and contains two putative coiled-coil domains which might serve as protein-interaction sites (Fig. 4). In transfected COS-1 cells, RD3 shows subnuclear localization adjacent to premyelocytic leukemia gene product (PML) bodies. PML bodies are implicated in diverse biological functions, including DNA repair, antiviral response, apoptosis, proteolysis, gene regulation, and tumor suppression. The precise role of RD3 in the retina and its disease mechanism remain to be elucidated.

5. Animal models for LCA

5.1. Mouse models for LCA

In Table 2, we summarize 13 mouse models with a retinal dystrophy that to varying degrees mimic human LCA. We have indicated which exons were naturally mutated or targeted by homologous recombination, the methodology used, the ages at which rods and cones start to degenerate, whether light damage plays a role in the retinal dystrophy, other morphological retinal features, and whether the mouse model recapitulates human LCA. All human LCA genes have orthologues in mouse. Four mouse LCA genes (*Cep290-rd16, Crb1-rd8, Rd3-rd3*, *Rpe65-rd12)* have been found to contain naturally occurring mutations. The others, except for the recently identified *LCA5* gene, have been targeted by homologous recombination (Table 2). A relatively mild mutation was introduced in the *Aipl1* gene by the insertion of a *neomycin* gene in intron 2 (Liu et al., 2004). To study the effect of a p.Cys250Trp missense mutation previously identified in patients with RP type 12 (RP with preserved para-arteriolar RPE), this variant was introduced into the mouse *Crb1* gene (van de Pavert et al., 2007a).

Tissue-specific induction of gene disruption of *Lrat* and *Rdh12* was achieved in recombinant mice. Through homologous recombination, loxP elements were introduced on both sides of exon 1 and exons 1–3, respectively (Kurth et al., 2007; Ruiz et al., 2007). Crossing these mice with mice carrying a Cre recombinase-encoding gene driven by a tissue-specific promotors resulted in RPE and photoreceptor-specific ablation of these genes, respectively (Kurth et al., 2007; Ruiz et al., 2007).

Though the *Impdh1* gene has been targeted, the eyes of these mice have not yet been investigated (Gu et al., 2003).

Except for the *Crb1*, *Gucy2D* and *Rdh12* knock-outs, disruption of the respective LCA genes led to severe retinal degeneration, although the differences in retinal development and structure between man and mouse make a direct comparison very difficult. Mice lack a cone-dominated fovea, which implies that mutations in cone-expressed genes may have a different phenotypic outcome.

Mice are nocturnal animals which may result in a very different vulnerability to light-induced retinal degeneration. Thus far, light was shown to increase the structural abnormalities in *Crb1* and *Rdh12* knockout mice, but not in *Aipl1*$^{-/-}$ mice (van de Pavert et al., 2004; Ramamurthy et al., 2004; Maeda et al., 2006; van de Pavert et al., 2007a, b). Other mouse models were not tested in a similar manner but as light accelerates progression of retinal degeneration in many animal models of RP, it can be expected that this is also the case for other LCA mouse models.

A sequence variant in the *Rpe65* gene (Rpe65-450Leu or Rpe65-450Met) can act as a modulator of light-damage susceptibility in mice by influencing the kinetics of rhodopsin regeneration and thus by modulating the photon absorption (Samardzija et al., 2006). Depending on exposure duration and light intensity applied, white fluorescent light induces photoreceptor apoptosis and retinal degeneration in wild-type mice by the activation of one of two known molecular pathways. These pathways depend on activation of the transcription factor c-Fos/AP-1 or on phototransduction activity. Retinal degeneration was reduced in Rho^{P23H} transgenic mice expressing the Rpe65-450Met variant. These mice retained more rhodopsin than transgenic mice expressing the Rpe65-450Leu variant. In addition, lack of phototransduction slowed retinal degeneration whereas ablation of c-Fos had no effect. Thus, sequence variations in the *Rpe65* gene can act as genetic modifiers in inherited retinal degeneration, presumably by regulating the daily rate of photon absorption through the modulation of rhodopsin regeneration kinetics. Increased absorption of photons and/or light sensitivity appears to accelerate retinal degeneration via an apoptotic cascade which involves phototransduction but not c-Fos.

5.2. Feline, canine, and avian models for retinal dystrophies

5.2.1. Cat model for CEP290-associated retinal dystrophy

A subpopulation of Abyssinian cats shows an ar form of retinal degeneration closely resembling human RP (Jacobson et al., 1989; Narfstrom and Nilsson, 1989). Affected cats have normal vision at birth but develop early funduscopic changes at the age of 1.5–2 years (Narfstrom and Nilsson, 1985). At the age of 7 months, ERG

Table 2
Overview of all natural and man-made mouse models for LCA

Gene	Mutated exons	Method	Degeneration of rods (age)	Degeneration of cones (age)	Light induced dystrophy	Other morphological or functional abnormalities	Recapitulates human LCA	References
Aipl1	1–2	KO	+++ (7 we)	+ (7 we)		Müller cell activation	Yes	Dyer et al. (2004)
	2–5	KO	+++ (3 we)	+++ (3 we)	No		Yes	Ramamurthy et al. (2004)
	intron 2	KD	+ (8 mo)			Müller cell activation	No	Liu et al. (2004)
Cep290	del35-39	Natural (rd16)	+++ (4 we)	+++ (4 we)		*Δ*Cep290 associates stronger with RPGR	Yes	Chang et al. (2006)
Crb1	3481delC	Natural (rd8)	+	+		Focal pseudorosettes inferior nasal	No	Mehalow et al. (2003)
	1	KO	+ (3–9 mo)	+ (3–9 mo)	Yes	Focal pseudorosettes inferior temporal	No	van de Pavert et al. (2004, 2007b)
	3 (C249W)	KI	+ (24 mo)	+ (24 mo)	Yes	Focal pseudorosettes	No	van de Pavert et al. (2007a)
Crx	2–4	KO (−/−)	+++ (2 we)	+++ (2 we)		Circadian entrainment affected	Yes	Furukawa et al. (1999)
		KO (+/−)	ERG↓ (2 mo)	ERG↓↓ (2mo)		Inner retinal abnorm.	Yes	Pignatelli et al. (2004)
						Synaptogenesis defect		Morrow et al. (2005)
Gucy2D	5	KO	–	+			No	Yang et al. (1999)
			ERG↓ (1 mo)	ERG↓↓ (1 mo)				
			ERG↓ (5 mo)	ERG absent (5 mo)				
Impdh1	9	KO	?	?			?	Gu et al. (2003)
Lrat	1	KO	++ (6–8 we)	?		ROS reduced 35% in length (6–8 we)	Yes	Batten et al. (2004)
			ERG↓↓ (8 we)	ERG↓↓ (8 we)				
	1	RPE KO				Rpe65 ↓; Rgr ↓↓	Yes	Ruiz et al. (2007)
Mertk	?	KO	+++ (7 we)	+ (7 we)		Pyknotic nuclei in 71% of PR cells	Yes	Duncan et al. (2003) Camenisch et al. (1999)
Rdh12	1–3	KO	+/− (1 yr)	+/− (1 yr)	Yes	Inner segments defect	No	Maeda et al. (2006)
	1–3	KO	+/− (7 mo)	+/− (7 mo)			No	Kurth et al. (2007)
Rd3	3 (R107X)	Natural (rd3)	++ (32 d)	+++ (32 d)			Yes	Friedman et al. (2006) Chang et al. (1993) Linberg et al. (2005)
Rpe65	1–3	KO Natural (rd12)	++ (15 we)	–		Cones preserved	Yes	Redmond et al. (1998) Pang et al. (2005)
Rpgrip1	Intron 14 (insertion)	KO	+++ (2–3 mo)	+++ (2–3 mo)		Connecting cilium normal	Yes	Zhao et al. (2003)
Tulp1	8–9	KO	+++ (4 we)	+++ (4 we)		No hearing defects	Yes	Ikeda et al. (2000)

ERG↓, ↓↓, electroretinogram amplitudes reduced, severely reduced; KD, knock-down; KO, knock-out; mo, months; Rpe65 ↓, Rpe65 expression reduced; Rgr ↓↓, Rgr expression severely reduced; ROS, rod outer segments; −absent, +/− subtle ,+ moderate, ++ severe, +++ full degeneration; we, weeks.

a-wave amplitudes are reduced (Kang Derwent et al., 2006). At first morphological changes are seen in rod outer segments from age 5–8 months. Lamellar discs are disorganized and disrupted, with vacuoles present at the base of outer segments. With progression of the disease, rods degenerate. Cones are similarly involved later in the disease process and complete photoreceptor degeneration and blindness are observed at the end stage, usually at the age of 3–5 years (Narfstrom, 1985). An intronic mutation (IVS50+9T>G [c.6960+9G>T]) was identified that creates a strong canonical splice (GT) donor site 4 bp upstream of the wild-type splice donor site that consists of a non-canonical (GC) sequence (Menotti-Raymond et al., 2007). The mRNA isolated from mutant cat retina and RPE contained a 4-bp insertion, which created a frameshift resulting in two missense codons, followed by a stop codon. Although it is very well possible that nonsense-mediated decay results in a reduced amount of *CEP290* mRNA, the predicted protein lacks the last 159 aa (p.Gln2320fsX3). It is intriguing that both the most frequent human and cat *CEP290* mutations create new splice sites and are associated with non-syndromic forms of retinal degeneration, LCA and RP respectively. The frequent human *CEP290* intron mutation c.2991+1655A>G (p.Cys998X) only leads to missplicing in ~50% of the mRNA, rendering it a hypomorphic mutation (den Hollander et al., 2006). Possibly, the p.Gln2320fsX3 mutation in Abyssinian cats, due to it's location at the carboxy terminus, also represents a hypomorphic mutation.

5.2.2. Dog model for RPE65-associated retinal dystrophy

A subset of briard dogs show a visual impairment due to a homozygous 4-bp deletion in the *RPE65* gene, which results in a frameshift and a premature stopcodon (460_463delAAGA; p.Lys154fsX52) (Aguirre et al., 1998). Dogs show prominent RPE inclusions and slightly abnormal rod photoreceptor morphology early in life, and slowly progressive photoreceptor degeneration. Photoreceptors appeared quite normal in the periphery, but slightly disorganized at the posterior pole and equator. There was an uneven shortening of rod inner segments that caused the rod outer segments to have variable length and resulted in an increased prominence of the cones. ERG analysis revealed severely decreased rod and cone responses. Based on the relatively well preserved retinal architecture, it was initially thought that this

phenotype represented CSNB (Aguirre et al., 1998). The phenotype was changed to juvenile RP, when progressive ERG changes were noted.

5.2.3. Chicken model for GUCY2D-associated retinal dystrophy

The *GUCY2D* gene encodes the enzyme retinal guanylate cyclase (RetGC-1) in both photoreceptors though levels are higher in cones than in rods. As mentioned previously, GUCY2D replenishes cGMP, a second messenger that binds to cGMP-gated channel proteins and keeps them open in the dark. In a chicken strain with an ar form of retinal degeneration, a 22-kb deletion was found in the chicken *GUCY2D* orthologue, *GUCY1B*. This deletion encompasses exons 4–7, which, at the mRNA level, were replaced by a 81-bp fragment 89% identical to a reverse fragment from exon 9 (Semple-Rowland et al., 1998). The deletion/insertion does not disrupt the open reading frame, but, as a 214-aa segment encompassing the transmembrane domain is deleted in the predicted protein, it is presumed unstable and dysfunctional (Laura et al., 1996). One-day-old mutant chicken retinas show no degeneration; 7–10 days after hatch, a retinal dystrophy is apparent in the central retina and proceeds progressively towards the periphery (Ulshafer et al., 1984; Ulshafer and Allen, 1985). At 115 days, few cone photoreceptors remain in the central retina, and by 6–8 months, the photoreceptor cells are fully degenerated. Even before morphological abnormalities are noted, no ERG responses can be measured (Ulshafer et al., 1984). These chickens experience low levels of cGMP and thereby a permanent closure of the cGMP-gated cation channels, mimicking constant light stimulation.

6. Therapeutics for LCA

Because the eye is a relatively unique and sophisticated central nervous system appendage, it provides several distinct advantages for gene replacement. Unlike the brain and brainstem, the eye is easily accessible, and harbors a natural subretinal space, where the bolus of therapeutic solution can be placed relatively safely and without leakage into the systemic circulation. This space is also protected from surveillance mechanisms that normally counteract foreign substances. Finally, the eye and the visual system can be routinely and easily monitored for visual function and retinal structural changes after injections with noninvasive advanced technology, such as visual acuities, contrast sensitivity, fundus auto-fluorescence (FAF), dark-adapted visual thresholds, vascular diameters, pupillometry, ERG, multifocal ERG and OCT.

6.1. RPE65 gene therapy in mouse, canine, and man

Proof of principle for gene therapy comes from Briard dog studies, which are an excellent model for human LCA caused by *RPE65* mutations (Acland et al., 2001). After subretinal *RPE65* cDNA delivery to the blind Briard dogs with null mutations in *RPE65*, blue light simulated dark-adapted ERGs and cone flicker ERGs improved by 16% compared to the uninjected eyes. One injection covering approximately 25% of the canine retina was introduced subretinally containing an AAV viral particle construct. Higher order visual function was assessed by pupillometry, which revealed an improvement in pupillary constriction compared to the uninjected eye. Visual evoked cortical potentials were demonstrated in the treated eyes but not in the untreated ones. Finally, and most impressive was the qualitative behavioral assessments which showed photopic visual behavior in the treated dogs that was indistinguishable from the normally sighted dogs. In the dark, the treated dogs were able to avoid objects unlike the untreated dogs (Acland et al., 2001).

A second study was performed by the Acland group to address questions of longevity of the treatment effect, safety and repeatability. Irrespective of AAV vector pseudotypes, human or canine *RPE65* cDNA in the viral particle, driven by a constitutive or tissue-specific promoter, 23 of 26 eyes responded in a similar positive fashion to gene replacement by subretinal injection (Acland et al., 2005). Intravitreal injections did not work. Three years after injections, visual recovery remained stable. Serum immunoreactivity increased in all injected dogs, but did not interfere with success levels. Intraocular immunoreactivity levels increased slightly in a few dogs, but were not specific for RPE65. RT-PCR studies of brain, kidney, bone marrow and other tissues did not reveal extra-ocular expression of *RPE65* (Acland et al., 2005). Altering the serotype of the AAV virus to AAV4 containing a human *RPE65* promotor and cDNA in an attempt to specifically target RPE cells, Rolling et al. (2006) showed restoration of cone and rod ERG responses 15 days after injection, which is more rapid than previously established. Also, dogs injected after age 30 months did not show recovery, suggesting a time window of treatment success (Acland et al., 2005).

Lentiviral mediated *RPE65* gene replacement also restores visual function in Rpe65 deficient mice (Bemelmans et al., 2006). A positive correlation was found between transduced RPE area and ERG response, while specific immuno-histological studies revealed cone preservation after treatment. Vector dose appeared to be positively associated with ERG response, while a transition zone was documented where the transgene was not detected but cones were spared, suggesting a "distance" effect that may be useful for treating the fovea, without disturbing the subfoveal area with the injection.

Based on the successful studies described above, two groups embarked on a human *RPE65* replacement trial and tested visual function in human LCA before and after gene replacements. Maguire et al. (2008) injected adeno-associated viral (AAV) particles (1.5×10^{10} viral particles in 150 μl) containing AAV2.hRPE65v2 carrying a chicken β-actin promoter in the subretinal space creating a dome shaped retinal detachment starting superonasally to the macula in three LCA patients (age range 19–26 years). Two subjects carried homozygous missense (p.Glu106Lys) and one a predicted null mutation (p.Arg234Ter) in *RPE65*. After investigating vitreoretinal and systemic adverse effects, they measured pupillometry (which measures retinal sensitivity from the retina to the brainstem), nystagmus frequency (an indirect measure of fixation ability), ETDRS visual acuities (the gold standard of visual acuity measurements), Goldman visual fields (which measures peripheral visual field size), and the ability to navigate an obstacle course, before and after the surgery. Cone and rod a-wave (which measures photoreceptor responses) and b-wave (which measures bipolar and Müller cell responses) measurements by ERG were not yet performed. No intraocular or systemic inflammation was found. Four subjective and two objective measures of visual function improved after several weeks. After 2 weeks, patients self-reported improvement in their vision. This was confirmed by remarkable improvements in ETDRS acuities, ranging from 3 to 4.5 lines. One of the patients improved from hand motion vision to 20/710, which is an improvement of 45 letters (4.5 ETDRS lines) and clinically very significant. Visual fields are helpful in determining peripheral visual field size, although they are somewhat variable between visits. On average, the three patients had 80° fields before and 200° degree fields after gene replacement, which are also significant changes. Videos of the obstacle course showed improvements in confidence and time in the subjects. Objectively, nystagmus frequency was measured and improved, while pupillometry showed significant improvements in the ability of the pupil to constrict, which

reflects enhanced retinal sensitivity, and better transmission to the brainstem (Maguire et al., 2008).

Bainbridge et al. (2008) injected AAV2/2.hRPE65 (10^{11} viral particles in 1 ml) containing a human RPE65 promoter subretinally in three LCA patients with *RPE65* mutations ranging from 17–23 years of age. The resulting bleb included the fovea, for a retinal area of ~1/3 of the total retina. Patients with *RPE65* null mutations were excluded. Prednisolone PO was given as an anti-inflammatory agent. After the injections, they did not find evidence of disseminated virus or adverse ocular or systemic effects. Measurements included full field, flicker, pattern and multifocal ERGs, automated dark adapted and microperimetry and kinetic perimetry by Goldmann. Acuities were measured by ETDRS charts, while mobility was tested by an obstacle course.

At baseline, acuities in the Bainbridge study were slightly better than in the Maguire study. In one patient, ETDRS acuities improved in the study eye from 20/286 to 20/145 at 12 months, but because the control eye improved from 20/150 to 20/120, the investigators felt that the improvement was not significant. It is entirely possible that nystagmus dampening improved the acuities in the control eye, or RPE65 protein found its way to the control eye, as was suggested in the canine experiments by Narfstrom et al. (2003). However, the other two patients had no significant improvements in acuities, unlike the Maguire et al group. In one of the three patients, there was a very significant 14 dB (25 fold) improvement in retinal sensitivity measured by microperimetry, as well as a significant enhancement on dark adapted perimetry. This same patient was also found to have a dramatic improvement in mobility through the obstacle course, reducing the walk time from 77 s to 14 s. ERG measures for the three LCA patients were not significantly different from baseline (Bainbridge et al., 2008).

Both *RPE65* gene replacement studies show an exciting improvement in visual function in humans suffering from advanced stages of LCA. The viral dosages are considered low, and the LCA patients are considered old. In the second decade LCA patients with *RPE65* mutations are probably in a disease stage when photoreceptor apoptosis may already have commenced. Subsequently, one may predict that improvements may even be better in younger subjects using higher dosages. The treatments appear to have short term safety, but longer follow up is necessary, and safety evaluations need to continue for higher doses and for many years. Differences in the visual outcomes of LCA patients in the two studies may be due to differences in genotypes, differences in promoters driving the respective *RPE65* genes, and differences in acuities at baseline.

No changes in ERG measurements were found in either study, which may be due to the low signals on ERG testing characteristic of LCA. Acuity improvements found in both studies are most likely due to photoreceptor rescue, but future studies must prove photoreceptor responses on ERG after gene therapy, as was documented in the murine and canine *RPE65* models.

These two studies rank among the most profound advances in the field of human medicine, and illustrate the enormous possibilities of Genetic Medicine as gene discovery leads to gene and protein understanding, which in these beautiful studies led to gene replacement and photoreceptor rescue. They represent a paradigm shift in our management and insights of retinal dystrophies of all types, previously thought to be an untreatable group of human diseases. Also, these significant results go beyond the fields of vision and ophthalmology. Further work is now possible and feasible and must include younger LCA patients with mutations in *RPE65* and genes, after each genetic type has been properly studied from bench to bedside. Many questions remain concerning long term safety and efficacy, unilateral versus bilateral treatments, multiple treatments, and the efficacy time windows for genetic subtypes of LCA.

6.2. GUCY2D rescue in an avian model

Chickens with a deletion in the GUCY2D orthologue GUCY1B, lack RetGC-1, therefore lack cGMP, maintain the channels in the closed position and are blind at birth, making them an excellent model for human LCA. Blind chicks do not exhibit optokinetic nystagmus reflexes, representing compensatory head movement in response to a moving stimulus of alternating black and white vertical bars. They also do not show useful pecking behavior, and both cone and rod ERGs are non-detectable. On embryonic day 2 (E2), lentiviral particles packaged with bovine RetGC-1 cDNA, driven by a CMV promotor, were injected into the ventricular space of the neural tubes of seven GUCY1B defective chicks before hatching, and compared to wild-type and uninjected GUCY1B chicks (Williams et al., 2006). Optokinetic nystagmus reflexes were video recorded and are thought to assess peripheral vision. Volitional visual behavior in response to colored food particles, which assesses central visual acuity, was also recorded. Cone and rod ERGs were performed. RT-PCR and immunohistochemistry were performed after a study period of 6 weeks.

Optokinetic nystagmus reflexes and pecking behavior were significantly improved in the treated chicks. The ERG a-waves (representing photoreceptor responses) was improved by 6% (Williams et al., 2006). The lentiviral transgene, and RetGC-1 mRNA were found in the treated but not in the untreated animals. Histopathological analyses revealed an increase in the ONL thickness in the treated animals, and relative preservation of structure compared to untreated blind chicks, but the treatment did not prevent retinal degeneration. In this first study of replacement of an enzyme-encoding photoreceptor gene, the investigators were able to restore chick vision by transducing a relatively small fraction of the photoreceptor population.

6.3. RPGRIP1 gene therapy in a mouse model

The *RPGRIP1* gene encodes a protein that localizes to the connecting cilium linking the inner to the outer segment. It tethers its molecular partner RPGR, is involved in the regulation of protein trafficking across the cilium and is involved in outer segment disc morphogenesis. The *Rpgrip1*$^{-/-}$ mouse develops a severe retinal degeneration that commences on post natal day 15 (P15), and is very significant by 3 months of age (Pawlyk et al., 2005). AAV constructs with a murine opsin promoter and murine *Rpgrip1* cDNA were injected subretinally at P18-P20. Five months after injection, retinas injected with the *RPGRIP1* gene were found to contain both the RPGRIP1 and RPGR proteins in the connecting cilum between the outer segments and inner segments, illustrating that *RPGRIP1* was expressed properly in the correct location. Wild-type mice have 10–11 rows of nuclei in the ONL; untreated mice only have 1 row, while the mice treated by *RPGRIP1* replacement exhibited 4–5 rows of nuclei. Mislocalized rhodopsin molecules, were normally localized as a result of the gene replacement. ERG b-waves declined by 22% per month in the untreated eyes, while the treated eyes declined by 6% per month. Five months post injection, the mean b-wave amplitudes in the treated eyes were about 20% of the b-wave amplitudes in normal eyes (Pawlyk et al., 2005).

In summary, gene therapy of a RPE-expressed gene encoding a vitamin A cycle enzyme (*RPE65*), a photoreceptor gene encoding a phototransduction enzyme (*GUCY2D*) and a photoreceptor gene encoding a structural protein (*RPGRIP1*) have all been shown to be successful. Photoreceptor rescue and concomitant visual

improvement has been achieved in animal models with LCA. Gene therapy requires intact viable photoreceptors. Children with LCA have the most to gain by timely gene replacement, but the dilemma is that they also have the most to loose. Other critical issues and questions remaining for gene therapy in LCA are the optimal viral species, the optimal serotype, exact timing of the intervention, the dose and repeatability of treatment, the longevity of the therapeutic effect, ocular and non-ocular safety issues and the effects of amblyopia on visual recovery. LCA animal model treatment successes have very recently led to the first human LCA gene replacement trials in London, UK and Philadelphia, USA.

7. Future perspectives

The two ultimate goals of LCA research are to provide efficient and affordable molecular diagnostics and to design novel treatment regimes. To accomplish these goals, a multidisciplinary effort is required combining the strengths of genetics, molecular biology, proteomics, and ophthalmology.

To identify the remaining 30% of genetic causes of LCA, IBD mapping in both consanguineous and outbred families must be expanded by combining high-density SNP data obtained in very large LCA patient cohorts. Novel large-scale sequence analysis methods based on *in situ* PCR and minisequencing will enable the analysis of up to 4 million bps in one single experiment, allowing the mutation analysis of candidate genes in very large homozygous chromosomal regions. Mutation analysis based on APEX-microarray technology will improve gradually, but will never reach 100% efficiency as novel (“private”) mutations and copy-number variations will be missed. Novel sequencing techniques are still a magnitude too expensive for routine use, but the next generation sequencing technology can be expected to make comprehensive LCA gene analysis affordable. A complete mutation analysis of all putative retinal disease genes might also shed light on the enormous variability of disease expression in individuals with *CEP290* mutations (from the lethal Meckel-Gruber syndrome to isolated LCA), and probably will reveal other genetic networks in LCA and other associated diseases.

Clinical evaluations to document the entire spectrum of phenotypes associated with LCA mutations, must keep pace with the rapid discovery of new LCA genes. Ophthalmologists must develop, validate and utilize a genotype–phenotype classification system for LCA, both in patients and their parents, which can facilitate an efficient phenotype-based mutation analysis strategy. Detailed phenotyping will improve as OCT technology allows more detailed images of photoreceptors and their interactions with other retinal cells. New technology focussed on the assessment of photoreceptor viability, such as retinal glucose metabolism (PET scan), oxygen measurements, ATP and blood flow measurements, will enter the phenotyping arena.

Effective treatment regimes are only possible when a thorough knowledge of the normal function of the LCA proteins and their protein context has been achieved. The positive results of the phase 1 gene therapy trials for the *RPE65* gene provide a basis to expand this technology to other retinal disorders and likely serve as a model to treat other neurosensory diseases. However, overexpression of LCA proteins may have detrimental effects in gene rescuing attempts. Knowledge of the LCA disease mechanisms on a molecular level is required to enable an accurate analysis of the molecular events that are triggered by variation of gene expression. Recent technological advances that include microarray analysis of the expression of nearly all genes simultaneously, and proteomics techniques to identify the subcellular levels and content of protein complexes, provide promising possibilities to dissect the retinal pathways involved in LCA. To achieve a long-lasting treatment in as many patients with LCA as possible, a concerted effort is needed in all disciplines involved.

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