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Rescue of inherited retinal degeneration associated with *Aipl1* defects

Cristy Ann Ku

Dissertation submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of

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In
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Keywords: inherited retinal degeneration, LCA, cone-rod dystrophy, AIPL1, gene therapy, AAV
Abstract

Rescue of inherited retinal degeneration associated with *Aipl1* defects

Cristy Ann Ku

Since groundbreaking clinical trials treating Leber congenital amaurosis (LCA) patients with *retinal pigmented epithelium-specific 65 kDa protein* (*Rpe65*) defects, which were the most successful gene therapy studies to date, much effort has been put into finding other candidate genes associated with inherited retinal degeneration to treat. Animal models with *Rpe65* defects demonstrate an unusual circumstance that greatly favors treatment, where dramatic vision loss is disproportionately greater than the ensuing slow photoreceptor degeneration. This pattern is also believed to occur in *Rpe65*-LCA patients, and stems from the primary defect in retinal pigmented epithelial (RPE) cells that subsequently perturbs photoreceptors. However, a majority of inherited retinal degenerations are associated with photoreceptor gene defects, and do not show this advantageous disparity between visual dysfunction and photoreceptor loss. To combat this, efforts have concentrated on improving photoreceptor transduction efficiency with viral vectors, and delivering gene replacement to slower progressing retinal dystrophies.

*Aryl hydrocarbon receptor interacting protein-like 1* (*Aipl1*) is mainly associated with the severe retinal degeneration, LCA. However, reports of *Aipl1* defects in patients showing slower progressing retinal disease, retinitis pigmentosa (RP) and cone-rod dystrophy (CORD), points to a subset of *Aipl1* patients as potential candidates for future AAV-mediated gene therapy trials. Multiple mouse models with *Aipl1* defects have been developed and contribute to the ease of conducting pre-clinical rescue studies to examine the rescue potential of *Aipl1*.

To investigate the rescue potential of *Aipl1* defects, we conducted AAV-mediated gene replacement studies in *Aipl1*-null mice, a model for *Aipl1*-LCA, the most rapidly progressing of any retinal degeneration mouse model. Our study examines the recent advancements in AAV viral vectors, using a combined self-complementary Y733F capsid mutant AAV8 (sc-Y733F-AAV8), to evaluate whether these advancements would provide functional benefits to a model of retinal degeneration. We found that the sc-Y733F-AAV8 viral vector significantly improved vision rescue in *Aipl1*-null mice as compared to conventional single-stranded AAV8, even when treatment was administered at postnatal day 10, after initiation of photoreceptor degeneration. From these studies, we also conclude that the *Aipl1*-null mouse provides a good model to test the functional benefits of ongoing and future advancements in AAV viral vectors.

In addition to LCA, *Aipl1* has been reported in patients with CORD and RP. We were particularly interested in a mutation, P351Δ12 hAipl1 because of its association with CORD and our ongoing interest in the role of *Aipl1* in cone photoreceptors. We generated a transgenic mouse model expressing P351Δ12 hAIPL1, which showed a cone-rod dystrophy phenotype similar to a case of *Aipl1*-CORD in a patient with this mutation. The mice demonstrated early and rapidly declining cone-mediated vision with slow progressing rod defects. Additionally, in our studies of double transgenic mice expressing both mutant P351Δ12 hAIPL1 and WT hAIPL1, we conclude that the P351Δ12 hAipl1 mutation does exert dominant effects that explain the autosomal dominant inheritance pattern observed in the *Aipl1* patients with this mutation.
Interestingly, despite the dominant phenotype, we were able to rescue cone dysfunction and degeneration following AAV-mediated gene delivery of WT hAIPL1. We believe this is due to the drastic overexpression of WT hAIPL1 that is achieved with AAV gene delivery. Further work with this novel model of Aipl1-CORD is needed to elucidate the dominant mechanism of the P351Δ12 hAIPL1 protein, which appear to behave differently in rods and cones, as rod dysfunction was not rescued with AAV-mediated overexpression of WT hAIPL1. With these successes in pre-clinical Aipl1 rescue studies, which have also been independently demonstrated by two other groups, we conclude that Aipl1 defects show great potential for movement into AAV-mediated gene therapy clinical trials.
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ad</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AIP1</td>
<td>Aryl hydrocarbon receptor interacting protein-like 1</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CC</td>
<td>Connecting cilia</td>
</tr>
<tr>
<td>CEP290</td>
<td>Centrosomal protein of 290 kDa</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CORD</td>
<td>Cone-rod dystrophy</td>
</tr>
<tr>
<td>CRB1</td>
<td>Crumbs homolog 1</td>
</tr>
<tr>
<td>Crx</td>
<td>Cone-rod homeobox</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
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<tr>
<td>GUCY2D</td>
<td>Guanylyl cyclase 2D</td>
</tr>
<tr>
<td>GUCY2E</td>
<td>Guanylyl cyclase 2E</td>
</tr>
<tr>
<td>h</td>
<td>Human</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>ILM</td>
<td>Inner limiting membrane</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IRD</td>
<td>Inherited retinal degeneration</td>
</tr>
<tr>
<td>IS</td>
<td>Inner segment</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeats</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>LCA</td>
<td>Leber congenital amaurosis</td>
</tr>
<tr>
<td>LRAT</td>
<td>Lecithin retinol acyltransferase</td>
</tr>
<tr>
<td>m</td>
<td>Mouse</td>
</tr>
<tr>
<td>MERTK</td>
<td>C-mer proto-oncogene receptor tyrosine kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OS</td>
<td>Outer segment</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>Post-injection</td>
</tr>
<tr>
<td>PDE6</td>
<td>Phosphodiesterase 6</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase-C</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
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PPIase  Peptidyl-prolyl cis-trans isomerase
RCS     Royal College of Surgeons (rat)
RGC     Retinal ganglion cell
RK      Rhodopsin kinase
RP      Retinitis pigmentosa
RPE     Retinal pigmented epithelium
RPE65   Retinal pigmented epithelium-specific 65 kDa protein
RPGRIP1 X-linked retinitis pigmentosa GTPase regulator-interacting protein 1
sc      Self-complementary (AAV)
ss      Single-stranded (AAV)
SEM     Standard error of the mean
TPLR    Transient pupillary light reflex
TPR     Tetracopeptide repeat
trs     Terminal resolution site
VEGF    Vascular endothelial growth factor
VP      Viral capsid protein
Chapter 1

Literature Review

Significance: Inherited retinal degenerations (IRD)

Hereditary retinal degeneration is a major cause of blindness affecting an estimated 1 in 3000 people (Wright et al., 2010). However, it is actually comprised of a diverse group of blinding diseases that exhibit a spectrum of presenting symptoms and severity, with varied onset and progression of vision loss. Leber congenital amaurosis (LCA) is the most severe of inherited retinal degenerations (IRD), characterized by severe visual impairment within the first few months of life and extinguished or severely reduced rod- and cone-mediated electroretinograms (ERGs) (Schappert-Kimmijser et al., 1959; Foxman et al., 1985). Relative to LCA, retinitis pigmentosa (RP) and cone-rod dystrophy (CORD) are less severe along the IRD spectrum, showing later onset and slower progression to complete vision loss. RP patients present with night blindness as the first visual impairment alongside normal visual acuity. In contrast to RP, CORD patients initially present with decreased visual acuity, central vision loss, and dyschromatopsia with progressive loss of peripheral, rod-mediated vision. Visual defects and subsequent retinal degeneration stem from improper splicing, nonsense, or missense genetic mutations translating altered, dysfunctional, or non-functional proteins whose normal function is essential in photoreceptors or retinal pigmented epithelium (RPE).

While historically untreatable, recent breakthroughs in pre-clinical and clinical gene replacement therapy trials have led to the potential to treat retinal dystrophies. Specifically, adeno-associated virus (AAV)-mediated gene therapy for LCA due to retina pigment epithelium-specific 65 kDa protein (Rpe65) gene defects showed therapeutic effects in phase I
clinical trials (Bainbridge et al., 2008; Maguire et al., 2008; Cideciyan et al., 2009; Jacobson et al., 2012) with ongoing phase II/III clinical trials (ClinicalTrials.gov, January 15, 2011). 

*Rpe65* clinical trials are one of the most successful examples of gene therapy to date in safety and efficacy, leading to a push for treating inherited retinal dystrophies linked to other causative genes. An inherent challenge, however, is the multitude of associated genes implicated within even a single inherited retinal degenerative disease, such as retinitis pigmentosa (RP) associated with defects in over 50 genes, and the more severe Leber congenital amaurosis (LCA) associated with defects in 16 genes (RetNet, http://www.sph.uth.tmc.edu, November 15, 2011). A majority of these are photoreceptor genes (RetNet), making targeting of photoreceptor defects an important next step in ocular gene therapy.

*Aipl1* is one photoreceptor gene demonstrating potential for entry into clinical trials. *Aipl1* defects are associated mainly with autosomal recessive LCA, making up 7% of LCA cases (Sohocki et al., 2000b). Additionally, *Aipl1* defects are associated with RP and CORD in select cases, pointing to a subset of *Aipl1* patients with a larger window of opportunity for treatment relative to *Aipl1*-LCA patients. Multiple *Aipl1* mouse models allow for pre-clinical testing of the rescue potential of *Aipl1* associated IRDs. Two independent *Aipl1*-null mouse models phenocopy LCA, and demonstrate the most rapid photoreceptor degeneration in any mouse model of retinal degeneration. *Aipl1*-null mice not only provide a uniquely suited model for testing the efficiency of viral vectors, but also proof-of-concept that less severe retinal degenerations may successfully be treated if the most severe of retinal degenerations is rescued. *Aipl1* hypomorph mice with a 75-80% reduction in protein levels, and our novel transgenic mouse expressing patient mutation P351Δ12 h*Aipl1* offer models of *Aipl1*-RP and -CORD, which may better predict the rescue capacity of *Aipl1* associated with less severe non-LCA IRDs.
Retinal and retinal pigmented epithelium (RPE) defects in IRD

The neural retina is a laminated structure with three cellular layers, the outer nuclear layer (ONL), the inner nuclear layer (INL), and the retinal ganglion cell (RGC) layer (Fig. 1A). Rod and cone photoreceptors, which make up the ONL of the neural retina, have three distinct cellular compartments (Fig. 1A). Photoreceptor outer segments (OS) are membranous discs that house essential phototransduction proteins. OS are shed and regenerated daily to negate the toxic effects of accumulated photo-oxidative products (Kevany and Palczewski 2010). Inner segments (IS) house the cellular machinery for synthesis of proteins, including outer segment proteins which are transported from the IS to OS via a connecting cilia (CC). The last photoreceptor compartment, the synaptic terminal, is the site of glutamate release which signals to secondary neurons in the INL. Visual transduction then propagates to the tertiary RGCs, the axons of which converge and form the optic nerve, feeding information to the visual cortex.

The non-neural RPE, located between photoreceptors and the vascular choroid (Fig. 1A), plays essential roles in the health and maintenance of photoreceptors. The RPE functions to absorb excess light, transport nutrients and ions to the retina from the choriocapillaries, and provide anti-oxidant and repair functions against high photo-oxidation occurring in the eye (Kevany and Palczewski 2010). Additionally, it serves two vital functions associated with retinal degeneration when awry: phagocytosis of shed photoreceptor outer segments; and production of visual retinoid 11-cis retinal, necessary for generating photoactivatable opsins (Fig. 1B).

Mutations in the RPE gene, Mertk (c-mer proto-oncogene receptor tyrosine kinase), lead to defective OS phagocytosis due to a lack of signaling for OS ingestion (Feng et al., 2002), and is associated with retinitis pigmentosa (RetNet) and modeled by the Royal College of Surgeons (RCS) rat. Defects in four RPE genes involved in the visual retinoid cycle are associated with
IRD (Fig. 1B, Fig. 2) (RetNet). Lrat (lecithin retinol acyltransferase), Rdh12 (retinal dehydrogenase 12), and Rpe65 (retinal pigmented epithelium-specific 65 kDa protein), the genetic mutation currently treated in gene therapy clinical trials, are all associated with both RP and LCA. Rdh5 (retinal dehydrogenase 5, or cis-RDH in Fig. 1B) is associated with CORD.

Photoreceptor gene defects are more numerous in their mechanisms of disease. Broad mechanisms of dysfunction include, but are not all-inclusive to the following: retinal development/retinal cell fates and structure, phototransduction cascade, photoreceptor OS structure, and protein transport across the connecting cilium (Fig. 2). Photoreceptor genes make up the majority of genetic defects, as compared to the four RPE genes, associated with IRD (Fig. 2). However, prior to recent advancements in AAV, transduction of the neural retina with viral vectors was highly inefficient. In contrast, early viral-mediated ocular gene therapy demonstrated robust RPE transduction. We first discuss the early developments in AAV-mediated gene delivery coupled with in vivo characterization of Rpe65 defects that led to the successful translation into Rpe65 clinical trials. Subsequently discussed are the recent advancements in viral vectors allowing for efficient photoreceptor transduction, and the potential for treatment of the photoreceptor gene, Aipl1.

Ocular viral gene therapy

Early ocular viral gene therapy studies, development of AAV

Attempts at ocular gene replacement therapy started in the mid 1990s, however, initial use of adenoviral (Ad) vectors resulted in inefficient transduction of neural retinal cells and limited duration of gene expression (Bennett et al., 1994; Jomary et al., 1994; Li, T. et al., 1994; Li, T. and Davidson 1995; Ali et al., 1997; Wright 1997). Even in an immune privileged site of
the eye, expression decreased by 3-4 weeks post-injection due to immune responses against viral proteins (Li, T. et al., 1994; Reichel et al., 1998). Re-administration of Ad accelerated loss of expression, whereas expression was prolonged in immunodeficient nude mice, further confirming that immunogenicity hinders effective use of Ad (Reichel et al., 1998). In contrast, early studies using recombinant adeno-associated viral (rAAV) vectors showed distinct advantages in greater safety, photoreceptor transduction and expression duration (Ali et al., 1996; Ali et al., 1997; Flannery et al., 1997; Jomary et al., 1997; Wright 1997).

Adeno-associated virus (AAV) is a linear single-stranded DNA virus belonging to the Parvoviridae family and the Dependovirus genus. As implied by its name, AAV was originally found as a contaminant in a preparation of simian adenovirus. There, it was characterized as replication defective, requiring a helper virus such as adenovirus to replicate (Atchison et al., 1965). AAV is non-pathogenic in humans and 40-80% of adults are seropositive for AAV without associated symptoms or disease (Sprecher-Goldberger et al., 1971). AAV shows low immunogenicity which is attributed to its lack of transduction and/or activation of antigen-presenting cells (APCs) (Jooss et al., 1998).

Cloning of the prototypic AAV2 into a bacterial plasmid was the first step of developing AAV as a vector and gene transduction system (Samulski et al., 1982; Hermonat and Muzyczka 1984). Recombinant AAV vectors devoid of all viral genes except for two inverted repeats (ITRs) between which the transgene of choice is cloned, further decreased immunogenicity by eliminating generation of wildtype AAV (Samulski et al., 1989). Rather, a helper plasmid which encodes AAV rep and cap genes necessary for AAV replication and viral capsid assembly, respectively, are supplied in trans alongside infection with helper Ad during rAAV vector production (Fig. 3), which are subsequently removed during purification.
Although wildtype AAV can integrate into the human genome at chromosome 19q13.3-qter (Kotin et al., 1991; Samulski et al., 1991), it requires AAV rep proteins which mediate complex formation at a sequence-specific integration site (Weitzman et al., 1994). Recombinant AAV vectors exist predominantly as non-integrated episomes in postmitotic cells (Muzychka et al., 1984; Duan et al., 1998; Schnepp et al., 2003; Penaud-Budloo et al., 2008; Schultz and Chamberlain 2008; Clark and Penaud-Budloo 2011) likely due to an absence of rep proteins in final rAAV vector preparations. Integration was however induced into double-stranded break sites following administration of DNA damaging agents (Miller et al., 2004).

As mentioned, adenovirus (Ad) is immunogenic and pathogenic, posing problems in production of AAV which required helper Ad for replication. Contaminating helper Ad in *in vivo* AAV-mediated gene delivery elicited cellular immune responses (Mccoy et al., 1995; Monahan et al., 1998). In addition to problematic *in vivo* humoral responses, presence of contaminating helper Ad hindered effective production of purified high-titer recombinant AAV.

**Production of high-titer purified AAV**

Several important developments have contributed to the capacity to produce high-titer purified AAV. First, greater understanding of the role of helper Ad in enhancement of transcription and translation of AAV *rep* and *cap* genes and second-strand synthesis during AAV replication (Jay et al., 1981; Richardson and Westphal 1984; Samulski and Shenk 1988; Chang et al., 1989; Janik et al., 1989; Ferrari et al., 1996), led to the development of a helper plasmid that encodes necessary Ad genes (Ferrari et al., 1997; Grimm et al., 1998; Xiao et al., 1998) (Fig. 3). This not only eliminated the need for infection with Ad and subsequent problems in purification of contaminating Ad, but also improved rAAV yields over 40-fold. This was attributed to a
controllable amount of helper Ad gene products, and freeing up of biochemical machinery since Ad helper plasmids do not lead to Ad virus packaging and replication (Xiao et al., 1998). Secondly, optimization of AAV rep and cap protein expression involved in replication and packaging improved yields (Grimm et al., 1998; Xiao et al., 1998; Collaco et al., 1999), as rep overexpression was found to limit capsid assembly (Fan and Dong 1997; Li, J. et al., 1997).

Lastly, novel purification methods such as heparin affinity chromatography for AAV2 (Clark et al., 1999), and iodixanol gradient centrifugation followed by anion-exchange chromatography for all AAV serotypes (Zolotukhin et al., 2002; De Backer et al., 2011) has replaced inefficient cesium chloride (CsCl) gradient centrifugation purification. Multiple CsCl gradient centrifugations needed for in vivo rAAV preparations led to low yields and lack of scalability. Moreover, aggregation of rAAV with Ad cellular proteins occurred in the presence of CsCl, and toxicity of CsCl necessitated extensive dialysis post-centrifugation (Burova and Ioffe 2005). Iodixanol centrifugation, in comparison, eliminated aggregation of macromolecules and did not require dialysis prior to subsequent chromatography purification (Zolotukhin et al., 1999). Most importantly, rAAV vector preparations showed superior viral infectivity following iodixanol compared to CsCl gradient purification (Zolotukhin et al., 1999).

**Transduction of retinal pigmented epithelial (RPE) cells**

The RPE is highly permissible for viral transduction which was achieved even in early studies using Ad (Jomary et al., 1994; Li, T. et al., 1994; Li, T. and Davidson 1995). However, immunogenicity and subsequent transient expression in RPE cells limited the in vivo use of adenoviral gene vectors (Li, T. et al., 1994; Reichel et al., 1998). The prototypic AAV2 showed efficient transduction and a greater safety profile than Ad (Ali et al., 1996; Grant et al., 1997).
More importantly, longevity of expression was demonstrated up to 11 weeks post-injection (Bennett et al., 1997).

Cloning and characterization of additional AAV serotypes led to development of pseudotyped AAVs, where rAAV vectors express AAV2 rep proteins with cap proteins of a different AAV serotype. All AAV serotypes (AAV1-9) developed thus far have demonstrated efficient RPE transduction, with AAV2, 4, and 6 primarily transducing RPE over neural retina (Lebherz et al., 2008; Beltran 2009; Boye, S. E. 2012). Ultimately, ease of RPE transduction led to specific targeting of Rpe65 LCA patients for clinical trials and contributed to the success of these trials.

**Gene therapy for Rpe65 LCA: From pre-clinical to clinical trials**

RPE65 plays a role in the production of visual chromophore 11-cis retinal necessary for regeneration of photoactivatable rhodopsin (Redmond et al., 1998). Specifically, RPE65, an isomerase present in the retinal pigmented epithelium, converts all-trans retinyl esters into 11-cis retinyl esters (Fig. 1B). In LCA2, the primary defect is in RPE65, leading to dysfunctional rhodopsin and phototransduction signaling. Photoreceptors show subsequent but delayed degeneration, relative to early visual dysfunction in Rpe65-null mice and dogs. In Rpe65-null mice, initial morphological abnormalities are observed at 7-15 weeks of age with outer segment disorganization. Slow progressive loss of photoreceptors ensues with 70% of ONL remaining at 6-7 months, 50% of the ONL at 12-18 months, and 30% at 24 months of age (Redmond et al., 1998; Gouras et al., 2002; Cideciyan 2010). Although significant photoreceptor loss does not occur until about 12-18 months of age, ERG responses are abolished at 10 weeks of age (Redmond et al., 1998). A similar trend is present in the Rpe65 Briard dog, where a naturally
occurring homozygous 4-bp frameshift deletion was found. This mutation introduces a premature stop codon that truncates two-thirds of the protein resulting in a nonfunctional protein (Veske et al., 1999). At 5 weeks of age, Rpe65 dogs demonstrate abolished scotopic ERG responses and low photopic responses despite a near normal retina with some outer segment disorganization (Narfstrom et al., 2003). Photoreceptor cell loss begins at 7-18 weeks of age and is complete by 7 years of age in the peripheral retina (Wrigstad et al., 1992; Wrigstad et al., 1994; Hernandez et al., 2010). These results show that in young Rpe65 mice and dogs, photoreceptor loss does not correlate with the observed level of visual function loss (Cideciyan 2010).

This observation was confirmed with a mathematical model that predicts photoreceptor function proportional to the product of the number of surviving photoreceptors and the length of their outer segments in retinal degeneration (Machida et al., 2000), or approximately as the loss of light sensitivity (in linear units) proportional to the square of ONL thinning (Jacobson et al., 2005). In line with Rpe65 animal models, in vivo optical coherence tomography (OCT) studies of Rpe65 LCA patients show areas of partially retained photoreceptors averaging about 60-65% of normal photoreceptor thickness, which is greater than expected for the level of dysfunction at both the fovea and rod-rich regions (Jacobson et al., 2005; Cideciyan 2010). In comparison, OCT data from non-Rpe65 retinal degeneration patients demonstrated that photoreceptor loss was in line with loss of visual function (Jacobson et al., 2007b; Jacobson et al., 2008a). This characteristic of Rpe65 defects provides a therapeutic window of opportunity that led to successful vision rescue in Rpe65 animal models and indicated Rpe65 LCA patients as good candidates for gene therapy clinical trials.

Vision restoration was achieved with multiple AAV serotypes in the Rpe65-null mouse. Treatment with AAV1 at 2 months of age or earlier led to a high success rate and restoration of
ERG a- and b-waves, whereas treatment at 1.5 to 2 years of age led to a low success rate and restoration only of b-waves (Jacobson et al., 2005). *In utero* AAV1 treatment led to equal or greater rescue of ERG responses than treatment at 2 months of age (Dejneka et al., 2004). These studies indicate that early intervention benefits vision rescue even in *Rpe65* slow degeneration. Rescue of *Rpe65*-null mice was also achieved with AAV2 (Bennicelli et al., 2008), the serotype primarily used in rescue of the *Rpe65* canine model. Fully functional vision rescue was achieved in the *Rpe65* canine model as measured through 11-*cis* retinal regeneration, ERGs, pupillary light responses, cortical activity, and vision-guided navigation following treatment at 3-5 months of age (Acland et al., 2001; Aguirre et al., 2007), with stability of rescue up to 3 years post-injection (Acland et al., 2005). Safety of AAV2-*Rpe65* in nonhuman primates was also demonstrated prior to advancement into clinical trials (Jacobson et al., 2006).

Three independent phase I clinical trials demonstrated patient safety following delivery of AAV2-RPE65 to young adult *Rpe65*-LCA patients, with no significant occurrence of humoral responses nor presence of viral vectors in saliva, tears, or peripheral blood (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008). More importantly, the studies demonstrated treatment efficacy in multiple measurements of visual function. In one study, 3 of 3 patients showed improved visual acuity that lasted at least 1.5 years post-treatment (Maguire et al., 2008; Maguire et al., 2009). Despite unimproved visual acuity in the other two studies, improvements in vision were observed through more sensitive visual function assays. Treated eyes demonstrated improved light sensitivity as measured through full-field sensitivity tests (Hauswirth et al., 2008; Maguire et al., 2009), a psychophysical test designed to assay vision in patients with severe vision loss (Roman et al., 2007). Secondly, transient pupillary light reflex (TPLR) responses which demonstrate transmission of visual information from the retina to the
brainstem, were elicited at lower light intensities post-treatment than at baseline, demonstrating enhanced light sensitivity (Cidecian et al., 2008; Maguire et al., 2008, 2009). Three year post-treatment follow-up showed that patients also interestingly developed a pseudofovea, an extrafoveal area with improved visual acuity and light sensitivity that therefore functioned as a central fixation locus (Jacobson et al., 2012).

**Advancements in AAV for photoreceptor targeting**

**Rapid and efficient transduction of photoreceptors**

Although first generation AAV2 provide high transduction of retinal pigmented epithelium in Rpe65 clinical trials, AAV2 shows limited transduction of photoreceptors (Grant et al., 1997; Ali et al., 1998). Greater photoreceptor transduction has been achieved with AAV5, 7, and 8 over AAV2 (Yang et al., 2002; Allocca et al., 2007; Lebherz et al., 2008). The standout pseudotype for photoreceptor targeting has been AAV8 following its isolation from the rhesus monkey (Gao et al., 2002). AAV8 demonstrated greater photoreceptor transduction at early post-injection times, as rapid as 5 days post-injection compared to 1-2 weeks post-injection in AAV2 and 5. Additionally, at the plateau of expression, AAV8 provided significantly higher photoreceptor transduction than AAV5 (Natkunarajah et al., 2008). AAV7 demonstrated slightly higher or similar photoreceptor transduction to AAV8 at 4-5 weeks post-injection (Allocca et al., 2007; Lebherz et al., 2008), although early photoreceptor transduction has not been examined. The robust and rapid kinetics of photoreceptor transduction has set AAV8 apart from other recent AAV serotypes, providing an essential characteristic for rapid retinal degeneration often seen with photoreceptor gene defects. While AAV7 and 8 provide a clear advantage in photoreceptor tropism, the route of viral particle binding and entry has not been elucidated for
these serotypes. In contrast, it is known that AAV2 binds heparan sulfate and AAV5 sialic acid as the primary receptor for attachment (Summerford and Samulski 1998; Walters et al., 2001). Elucidating the receptor for AAV7 and 8 may shed light on the high photoreceptor tropism and potential methods to enhance photoreceptor targeting.

Early onset of expression is also achieved with self-complementary AAV (scAAV), which forms a double-stranded DNA (dsDNA) conformation that bypasses second-strand synthesis required by conventional single-stranded AAV (ssAAV) prior to transcription. scAAV is generated by mutating the terminal resolution site (trs) in the 5’ ITR in the wildtype AAV construct, which leads to read-through and formation of a dimeric inverted repeat structure with two complete ITRs at the ends and a mutated ITR in the middle during replication (Mccarty et al., 2001; Mccarty et al., 2003; Mccarty 2008) (Fig. 4). Expression initiates as early as post-injection (pi) day 2-3 with scAAV8 and scAAV2, as compared to pi day 5-7 in ssAAV8 and pi day 14 in ssAAV2 (Yokoi et al., 2007; Natkunarajah et al., 2008). In addition, scAAV provided higher transduction efficiency over ssAAV for AAV2, 5, and 8, at 5 weeks post-injection when maximal transduction has occurred (Yokoi et al., 2007; Natkunarajah et al., 2008). The increased efficiency of scAAV is partially attributed to degradation of ssAAV during the single-stranded phase. Further characterization of ss- and scAAV processing is needed however, as opposing studies report a transient instability of both ssAAV and scAAV following cell entry (Mccarty 2008).

Another advancement that improves stability of viral particles upon cell entry is AAV capsid mutations. Studies examining intracellular trafficking of rAAV revealed that ubiquitination and proteasomal degradation of viral particles was one route of decreased transduction efficiency (Yan et al., 2002; Zhong et al., 2007; Zhong et al., 2008a). Since
ubiquitination of cellular and viral proteins is often modulated through phosphorylation of serine and tyrosine residues, mutagenesis of surface-exposed tyrosine residues of capsid proteins were reasoned to protect against viral particle degradation. As hypothesized, tyrosine capsid mutants showed greater transduction over their wildtype capsid counterparts (Petrš-Silva et al., 2009; Ryals et al., 2011). Recently developed threonine capsid mutations are also showing similar effects to tyrosine mutations (Boye, S.E. 2012).

**Current limitations in AAV-mediated gene therapy**

Despite advantages in the safety and efficacy of AAV, some limitations remain with its use. The greatest hindrance is the limitation in transgene size of 4.7 kb for single-stranded AAV and 2.3 kb for self-complementary AAV. This size restriction, which must include both gene promoter and transgene, stems from the natural size of the wildtype AAV genome between two inverted terminal repeats (ITRs). It prevents delivery of LCA gene *Cep290* (7.4 kb), and scAAV packaging of *Crb1* (4.2 kb), *Gucy2d* (3.3 kb), and *Rpgrip1* (3.8 kb). Attempts at pushing beyond the packaging capacity showed that above 5.2 kb, vector genomes were truncated at the 5’ end (Dong et al., 2010; Wu, Z. et al., 2010). A slight improvement to 6.0 kb was obtained by eliminating the VP2 capsid protein, one of three capsid proteins normally needed for viral encapsulation (Grieger and Samulski 2005). In contrast to these studies, one group demonstrated intact viral genome packaging and *in vivo* transduction of an 8.9 kb transgene with AAV5 (Allocca et al., 2008). Despite this controversy, the packaging capacity of AAV is in stark contrast to the 36 kb capacity of Ad (Beltran 2009).

Another limitation in AAV retinal gene therapy is the cumbersome and delicate administration of viral solution into the subretinal space (Fig. 5), which is actually a non-
physiological potential space between the retina and RPE. In patients, the procedure requires a vitrectomy followed by a puncture through the retina (retinotomy). In addition to transient detachment and local damage of the retina, disadvantages of subretinal injection into patients with retinal degeneration are two-fold. First, non-uniform degeneration makes it difficult to identify the best area to introduce the virus. Secondly, the degenerating retina is extremely fragile and susceptible to retinal tears (Maguire et al., 2008).

In contrast, intravitreal injection (Fig. 5) is a safe and relatively easy outpatient procedure routinely conducted to deliver anti-VEGF drugs to wet AMD patients. Although transduction of retinal ganglion cells following intravitreal injections have been demonstrated with AAV2, 8, and 9 (Harvey et al., 2002; Lebherz et al., 2008; Giove et al., 2010; Yin et al., 2011), efficient transduction of outer retinal photoreceptors is hindered by the inner limiting membrane (ILM) (Dalkara et al., 2009). The ILM is a basement membrane composed of various extracellular matrix proteins, several of which have been found to mediate AAV2, 8, and 9 binding and accumulation at the vitreoretinal junction (Akache et al., 2006; Kolstad et al., 2010). The ILM is essential in retinal development but is dispensable in adults, as its removal is commonly conducted in macular hole surgeries. Importantly, this barrier was partially overcome with co-administration of proteolytic enzymes with AAV, a potential therapeutic application for transducing the outer retina via an intravitreal injection (Dalkara et al., 2009). However, further progress is needed to improve upon the efficiency of outer retinal transduction achieved in this study. Another advancement found to transduce photoreceptor following intravitreal delivery is AAV triple tyrosine capsid mutant, which interestingly transduced foveal cone photoreceptors in non-human primates (Boye, S.E. 2012). This is a particularly desirable development as foveal
thinning was a complication in several Rpe65 LCA patients receiving viral gene therapy, likely arising from foveal detachment following subretinal injections (Jacobson et al., 2012).

**Targeting photoreceptor gene defects: Aipl1 as a candidate gene**

**Overview**

Since Rpe65 LCA clinical trials, there has been a push to target additional genetic defects associated with inherited retinal degeneration. Recent and continuing advancements in AAV vectors improved photoreceptor transduction, allowing for correction of photoreceptor defects. Treatment of defects in Aipl1, a chaperone protein for the phototransduction protein phosphodiesterase 6 (PDE6), shows potential for entry into clinical trials. Although Aipl1 is mainly associated with LCA, the most severe of IRDs, it has also been linked to CORD and RP which show later onset and slower progression of vision loss (Fig. 7), indicating a subset of Aipl1 patients who can benefit from gene replacement therapy.

Multiple Aipl1 mouse models exist allowing for pre-clinical gene therapy studies. The Aipl1-null mouse models Aipl1-LCA, whereas the Aipl1 hypomorph mouse, with a 75-80% reduction in protein levels, models a slow progressing retinal degeneration. Additionally, our novel P351Δ12 Aipl1 mutant mouse phenocopies Aipl1-CORD. Successful vision rescue following AAV-mediated gene replacement was achieved in several of these mouse models, showing the potential to correct IRDs associated with Aipl1 defects.
Aryl hydrocarbon receptor interacting protein-like 1 (AIPL1) defects

Aryl hydrocarbon receptor interacting protein-like 1 (AIPL1) is a 384 aa protein found in the pineal gland and retina, where it is specifically expressed in early and mature rod and cone photoreceptors (Van Der Spuy et al., 2003; Kirschman et al., 2010). AIPL1 is a member of the immunophilin FK506 binding protein (FKBP) family. Similar to several multi-domain immunophilins, AIPL1 contains an N-terminal peptidyl-prolyl cis-trans isomerase (PPIase)-like domain and three C-terminal tetratricopeptide repeat (TPR) domains. Primate AIPL1 isoforms also contain a C-terminal proline-rich region referred to as the hinge region, an area of unknown function or significance (Fig. 6A).

Aipl1-null and hypomorphic mice, which express 20-25% of wildtype AIPL1 levels (Liu et al., 2004), show a specific and dramatic reduction of all rod PDE subunits despite unaltered mRNA levels (Liu et al., 2004; Ramamurthy et al., 2004), indicating the role of AIPL1 in post-transcriptional processing of rod PDE6 holoenzyme. Further work has elucidated that AIPL1 is required for the stability of rod PDE6 subunits and assembly of the PDE6αβγ2 complex, and that AIPL1 specifically interacts with the α catalytic subunit (Kolandaivelu et al., 2009). The interaction between AIPL1 and PDEα subunit is not surprising due to yeast two-hybrid studies showing the interaction of AIPL1 with farnesylated proteins (Ramamurthy et al., 2003). However, whether AIPL1 acts to facilitate the farnesylation reaction of PDEα, folding of PDEα or oligomerization of PDE6αβγ2 remains unknown, all of which may enhance PDE6 stability and assembly. The site of PDEα interaction for AIPL1 chaperone activity has also yet to be elucidated. The role of AIPL1 in cone photoreceptors is currently being elucidated, as loss of AIPL1 leads to rapid rod and cone degeneration associated with LCA and modeled in the Aipl1-null mouse. Loss of cone photoreceptors is not simply secondary to massive rod loss, as
demonstrated by the cone-specific elimination of AIPL1 leading to loss of photopic ERGs and cone death (Kirschman et al., 2010).

Defects in Aipl1 are associated with autosomal recessive Leber congenital amaurosis (LCA4), in approximately 7% of LCA cases (Sohocki et al., 2000b). Several reports also link Aipl1 defects to autosomal dominant cone-rod dystrophy (adCORD), juvenile retinitis pigmentosa (RP), non-early onset RP, and later onset retinal degeneration (Sohocki et al., 2000b; Vallespin et al., 2007; Walia et al., 2010; Jacobson et al., 2011) (Fig. 6C, Fig. 7).

LCA is the most severe of retinal degenerations, with severe visual impairment within the first few months of life and absent or severely reduced rod- and cone-mediated ERGs. RP patients typically present with night blindness as the first visual impairment alongside normal visual acuity, with somewhat varied progression and severity. Although clear diagnostic criteria are not universally agreed upon, juvenile, early- or childhood-onset RP typically denote symptoms initiating from 1 year to 10 years of age, whereas non-early or late-onset RP typically refers to initiation after 10 years of age. CORD patients present with impaired central vision and decreased visual acuity with progressive loss of peripheral vision, but retain measurable ERGs for several years. The case of Aipl1 associated with later onset retinal degeneration was described with slower onset and progression than LCA or juvenile RP (Jacobson et al., 2011). These cases of non-LCA IRDs associated with Aipl1 (Fig. 6C) indicate a larger window of opportunity for treatment relative to Aipl1-LCA. Additionally, evidence of ERG responses and photoreceptor survival in some Aipl1 patients demonstrate salvageable photoreceptor cells (Fig. 6C) (Jacobson et al., 2011; Pennesi et al., 2011; Testa et al., 2011).

The most common Aipl1 defect in over 60% of Aipl1-LCA cases is truncation mutation W278X (Dharmaraj et al., 2004; Hanein et al., 2004) leading to an unstable and degraded protein.
(Ramamurthy et al., 2003), which is mimicked in the Aipl1-null mouse model. However, point mutations and small base pair deletions in the PPIase-like and TPR domains of AIPL1 are also prevalent in the patient population. Several point mutations such as A197P and C239R retain functionality in vitro in interacting with and processing farnesylated proteins despite compromised folding and stability (Ramamurthy et al., 2003). Whether some mutations translate functional or partially functional protein is an important clinically relevant question. The Aipl1 hypomorphic mouse mimics such a hypothetical state. In addition, a reported patient mutation P351Δ12 in the C-terminal proline-rich hinge region retains partial functionality in vivo as demonstrated in our hAipl1 transgenic mouse model (Chapter 3). Although the rarity of Aipl1 cases severely limits any genotype-phenotype correlations that can be made, reports of Aipl1-CORD and -RP (Fig. 6C), combined with the idea that some mutations may retain functionality, indicate the potential of a subset of Aipl1 patients who may benefit from gene therapy.

Pre-clinical rescue studies of Aipl1 defects

AAV-mediated gene replacement studies have successfully rescued vision in Aipl1 hypomorphic mice (Tan et al., 2009; Sun et al., 2010) which express 20-25% of wildtype levels of mAIPL1. Aipl1 hypomorphic mice show slow degeneration, with photoreceptor cell loss beginning at 3 months of age and reduced to half of the normal outer nuclear layer (ONL) thickness by 8 to 10 months of age (Liu et al., 2004). In AAV-rescue studies, Aipl1 hypomorph degeneration was expedited with light damage (Tan et al., 2009), with the 50% reduction in ONL thickness accelerated to 6.25 months of age. A second strategy was used in Sun et al., 2010, where Aipl1 hypomorphs were crossed with Aipl1-null mice to generate F1 “Aipl1 hypomorph” offspring, which exhibit a 75% reduction in ONL thickness by 11 months of age. These Aipl1 hypomorph
mouse models were successfully treated with single-stranded AAV2 (Tan et al., 2009) and AAV5 (Sun et al., 2010).

In contrast, AAV2 and AAV5 were unable to rescue photoreceptor structure and function in Aipl1-null mice (Sun et al., 2010), further confirming the subpar efficiency of AAV2 and 5 in photoreceptor targeting. Two independent Aipl1-null models recapitulate severe LCA with early visual defects accompanied by rapid degeneration, although one model demonstrates 13% of surviving photoreceptors in the peripheral retina at P60 (Dyer et al., 2004) whereas a second shows complete photoreceptor loss by P24 (Ramamurthy et al., 2004). Vision rescue was achieved in the former Aipl1-null model with AAV8 (Tan et al., 2009; Sun et al., 2010). However, more recent developments in AAV may improve vision rescue effects in the Aipl1-null mouse, which requires rapid and robust transduction to combat the extremely rapid retinal degeneration beginning at P8 and complete by P24.

Objective

The combination of recent developments in AAV increase the capability to rescue rapid retinal degeneration associated with photoreceptor gene defects. One such photoreceptor defect is Aipl1, in which our Aipl1-null mouse model shows the most rapid retinal degeneration of all mouse models. Study 1 is proof-of-principle that advancements in AAV significantly improve photoreceptor transduction over first generation AAV vectors, which were sufficient for slow retinal degeneration. Vision rescue achieved in Aipl1-null mice using self-complementary capsid mutant AAV8 is compared to first generation single-stranded AAV8 through functional, morphological, and biochemical assays. In addition, clinical reports of Aipl1 patients with relatively slower forms of retinal degeneration relative to LCA indicate that a population of Aipl1
patients may potentially benefit from gene replacement therapy. Study 2 provides a mouse model of an Aipl1 patient mutation showing a cone-rod dystrophy phenotype, demonstrating that Aipl1 mutations are potentially amenable to gene replacement therapy. Chapter 4 concludes with a summary of findings and the implications of gene replacement therapy for inherited retinal degeneration associated with Aipl1.
Fig. 1. The neural retina and RPE. (a) The laminated neural retina is made up of three cellular layers: the outer nuclear layer (ONL) is rod and cone photoreceptor nuclei; inner nuclear layer (INL) is composed of bipolar, horizontal, and amacrine cells; and the retinal ganglion cell (RGC) layer of which the axons form the optic nerve. The retinal pigmented epithelium (RPE) is located between photoreceptor outer segments (OS) and the vascular choroid, and is essential in the visual retinoid cycle. (b) The visual retinoid cycle involves enzymes in both the RPE and PR for regeneration of 11-cis retinal that upon photoisomerization, leads to activation of opsin and initiation of visual signal transduction. Abbreviations: ATP-binding cassette transporter, retina-specific (ABCR), retinol dehydrogenase (RDH), interphotoreceptor retinoid binding protein (IRBP), cellular retinoid binding protein (CRBP), lecithin retinol acyltransferase (LRAT), retinal pigment epithelium-specific protein, 65 kDa (RPE65), cellular retinaldehyde binding protein
(CRALBP), retinol binding protein 4 (RBP4). Adapted from (Besch et al., 2003; Kuksa et al., 2003).
Fig. 2. Genes associated with inherited retinal degeneration (IRD). Each IRD is associated with defects in numerous genes, some of which are also associated with other IRDs (in overlapping areas). LCA, the most severe of IRDs, has over 16 associated genes, with recent defects in Cabp4 (2010), Iqcb1 (2011), and Kcnj13 (2011) reported. Apart from these select few with unknown function, most genes associated with LCA have known mechanisms of dysfunction mainly in phototransduction (navy font), ciliary transport (pink), and visual retinoid cycle (maroon). Other important classes of dysfunction are retinal development and structure.
(dark green), photoreceptor OS structure (purple), and ion channels (light green). RP, on the other hand, is linked to over 50 genes, in which many have unknown functions. Genetic testing is not routinely conducted in CORD patients and it is predicted that many genes remain unidentified. Symbols: genes associated with autosomal dominant disease are *adLCA, “adRP, ^adCORD. Adapted from (Berger et al., 2010).
Fig. 3. Production of AAV. Original AAV production involved co-transfection of a plasmid with the transgene of choice cloned between two 145-bp inverted terminal repeats (ITRs) and a mini plasmid encoding necessary AAV rep and cap genes, alongside adenovirus (Ad) infection into 293 cells. Subsequent purification of Ad particles from rAAV preparations was required with this method. Understanding of the role of Ad genes in AAV replication substituted Ad infection with a helper plasmid encoding Ad genes E1a, E1b, E2a, and VA. Adapted from (Goncalves 2005).
Fig. 4. Single-stranded and self-complementary AAV. Replication of AAV particles initiates at the 3’ ITR (step 1) which acts as a primer for host DNA polymerase. Following elongation, the 5’ ITR is duplicated (step 2), and this duplex ITR is isomerized to a double-hairpin conformation (step 3) to create a new primer site for re-initiation of DNA synthesis (step 4). While the 3’ ITR is elongated and daughter strand displaced (ssAAV genome) (step 5m, 6m), rep 78/68 binds and nicks at the terminal resolution site (trs) of the ITR before being reached by DNA elongation occurring at the other end. Following elongation at the opposite end, the resultant duplex monomer (top structure following step 6m) recreates the template for isomerization in step 3. scAAV genomes are produced through deletion of one trs site from one ITR, such that the rep protein no longer creates the essential nick. The replication complex copies through the hairpin,
producing a double-stranded inverted repeat, with a wildtype ITR at both ends and a mutated ITR in the middle (structure following step 6d). The dimeric inverted repeat undergoes normal replication initiating at any of the wildtype ITRs (steps 7d-9d), with displacement and generation of the scAAV genome, which is an inverted repeat with a complete ITR at both ends and a mutated ITR in the middle. Adapted from (Mccarty 2008).
Fig. 5. **Subretinal and intravitreal routes of intraocular gene delivery.** Subretinal administration deposits viral solution between the retinal pigmented epithelium (RPE) and photoreceptors, ideal for transduction of either of these cell types. However, a puncture through the retina is required in subretinal injections leading to local damage and temporary local retinal detachment. Intravitreal injections deposit viral solution in the vitreal space behind the lens and does not cause retinal damage or detachment. Adapted from (Colella et al., 2009).
Fig. 6. **Aipl1 domains and function.** *Aipl1* (a) contains a FKBP-like domain, 3 tetracopeptide repeat (TPR) domains, and a C-terminal region present only in primate isoforms. (b) Three different patient mutations (*orange*, *blue*, *red arrows* and *underline*) disrupt a repeating PxxP motif (*black underline*) present in the C-terminal proline-rich hinge region (*grey boxes*). (c) *Aipl1* has been sporadically associated with inherited retinal degenerations outside of LCA, two of such cases associated with hinge region mutation, P351Δ12.
Fig. 7. Spectrum of IRD associated with Aipl1. Aipl1 is associated mainly with Leber congenital amaurosis (LCA), a rapid rod and cone dystrophy on the most severe end of the spectrum of inherited retinal degenerations (IRD), with severe visual deficits and loss of ERGs within the first few months of life. Additionally, Aipl1 mutations were found in patients with cone-rod dystrophy (CORD) and juvenile early onset-retinitis pigmentosa (RP) in a few isolated cases. CORD and RP show later onset and slower progression, with cone- and rod-mediated visual deficits, respectively, presenting as the first visual symptoms.
Chapter 2

Gene replacement therapy for Aipl1-null mice, a model of severe Leber congenital amaurosis

Abstract

Defects in the photoreceptor specific gene *aryl hydrocarbon receptor interacting protein-like 1 (Aipl1)* are associated with Leber congenital amaurosis (LCA), a childhood blinding disease with early-onset retinal degeneration and vision loss. Furthermore, *Aipl1* defects are characterized at the most severe end of the LCA spectrum. The rapid photoreceptor degeneration and vision loss observed in the LCA patient population is mimicked in a mouse model lacking AIPL1. Using this model, we evaluated if gene replacement therapy using recent advancements in adeno-associated viral vectors (AAV) provides advantages in preventing rapid retinal degeneration. Specifically, we demonstrated that the novel self-complementary Y733F capsid mutant AAV2/8 (sc-Y733F-AAV2/8) provided greater preservation of photoreceptors and functional vision in *Aipl1* null mice compared to single-stranded AAV2/8. The benefits of sc-Y733F-AAV were evident following viral administration during the active phase of retinal degeneration, where only sc-Y733F-AAV treatment achieved functional vision rescue. This result was likely due to higher and earlier onset of *Aipl1* expression. Based on our studies, we conclude that the sc-Y733F-AAV2/8 viral vector, to date, achieves the best rescue for rapid retinal degeneration in *Aipl1* null mice. Our results provide important considerations for viral vectors to be used in future gene therapy clinical trials targeting a wider severity spectrum of inherited retinal dystrophies.
Introduction

Inherited retinal dystrophies are a large group of clinically and genetically diverse conditions estimated to affect 1 in 3000 people (Daiger et al., 2007; Sun et al., 2010). The earliest and most severe form is Leber congenital amaurosis (LCA), which is estimated to account for ≥ 5% of inherited retinopathies (Schappert-Kimmijser et al., 1959; Koenekoop 2004). LCA is diagnosed with clinical findings of severely impaired visual function shortly after birth, sluggish pupillary responses, nystagmus, and an absent or markedly reduced electroretinogram (ERG). Patients with LCA may also exhibit fundus changes, photophobia, the oculodigital sign, keratoconus, and cataract (Schappert-Kimmijser et al., 1959; Foxman et al., 1985). To date, LCA is associated with mutations in at least 15 different genes expressed in either photoreceptor or retinal pigment epithelium (RPE) cells (Human Gene Mutation Database, http://www.hgmd.org, April 12, 2011). Clinical heterogeneity within LCA had long been recognized (Noble and Carr 1978; Foxman et al., 1985; Schroeder et al., 1987; Fulton et al., 1996), but more recent genotype-phenotype correlation studies showed subtle but important differences in fundus appearance, visual acuity, and disease progression (Perrault et al., 1999; Hanein et al., 2004; Galvin et al., 2005).

Greater understanding of the molecular basis of genetic defects in inherited retinopathies coupled with developments in viral-mediated gene delivery have led to potential therapies for some retinal dystrophies, which were a previously untreatable group of diseases. Defects in retinal pigment epithelium-specific protein 65 kDa (RPE65) are associated with autosomal recessive retinitis pigmentosa and LCA. In multiple animal models, mutations in RPE65 lead to vision loss but photoreceptor neurons develop normally and survive for an extended period (Wrigstad et al., 1994; Redmond et al., 1998; Pang et al., 2005). This characteristic of RPE65
mutations was crucial in successful preclinical trials in multiple animal models (Acland et al., 2001; Chen et al., 2006; Pang et al., 2006; Bennicelli et al., 2008). Although patients with RPE65 mutations exhibit greater photoreceptor degeneration than animal models, optical coherence tomography (OCT) demonstrated preservation of the photoreceptor cell layer that is greater than predicted from the level of visual dysfunction (Jacobson et al., 2005). These findings indicated that there is a window of time for therapeutic intervention, which made RPE65 patients an ideal initial target for gene therapy trials. Landmark clinical trials which delivered exogenous RPE65 gene using the adeno-associated virus 2 (AAV2), showed visual improvements in patients (Bainbridge et al., 2008; Cideciyan et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008; Cideciyan et al., 2009; Simonelli et al., 2010), and are paving the way for gene therapy for inherited retinal degenerative diseases associated with other genetic defects.

On the other end of the disease spectrum are mutations linked to LCA that cause rapid photoreceptor degeneration. The degeneration is pronounced when associated with defects in *Aryl hydrocarbon receptor interacting protein-like 1 (Aipl1)*. AIPL1 is a 386 amino acid protein that belongs to the FK506 binding protein (FKBP) family of chaperone proteins (Sohocki et al., 2000a), and is expressed early and specifically in photoreceptors (Hendrickson et al., 2008). AIPL1 is crucial for the stability of rod phosphodiesterase 6 (PDE6), an enzyme that is involved in cyclic GMP phototransduction signaling (Liu et al., 2004; Ramamurthy et al., 2004; Kolandaivelu et al., 2009). Although the role in cone photoreceptors is not fully understood, AIPL1 is required for cone function and survival (Kirschman et al., 2010). In the *Aipl1* null mouse, both rod and cone electroretinogram (ERG) responses are absent and complete photoreceptor cell loss occurs by 3 weeks of age (Ramamurthy et al., 2004). Patients with *Aipl1* mutations, implicated in 5-7% of LCA cases and several incidences of juvenile retinitis...
pigmentosa and cone-rod dystrophy (Sohocki et al., 2000b), have a stable but drastically reduced visual acuity by the first decade in life, ranging from 20/200 to no light perception (Galvin et al., 2005; Simonelli et al., 2007; Walia et al., 2010). The level of photoreceptor survival in patients has not been extensively examined, but two recent studies found photoreceptors in OCT scans of patients with mutations in \textit{Aipl1} (Jacobson et al., 2011; Testa et al., 2011). While the window of therapeutic opportunity is smaller in \textit{Aipl1} defects due to rapid photoreceptor degeneration, these findings highlight the possibility that patients with \textit{Aipl1} defects could benefit from early gene replacement therapy.

Two important proof-of-concept studies used adeno-associated viral (AAV)-mediated gene replacement therapy in the \textit{Aipl1} hypomorphic and null mouse (Tan et al., 2009; Sun et al., 2010). These were the first studies to show that \textit{Aipl1} replacement could restore functional rod and cone PDE6 and prevent the loss of photoreceptors. Since these two studies, advancements in virology resulted in AAV vectors with increased transduction efficiency and shortened onset of expression. The first development was self-complementary AAV, which produces a double stranded gene vector that is more stable and eliminates the need for second-strand DNA synthesis (Mccarty et al., 2001). These characteristics conferred both earlier onset and increased transduction efficiency compared to single-stranded AAV (Natkunarajah et al., 2008; Petersen-Jones et al., 2009; Kong et al., 2010). The second development was mutations of surface-exposed tyrosine residues on the viral capsid. The resultant decrease in capsid tyrosine phosphorylation reduces ubiquitination of viral particles and subsequently improves nuclear transport and transduction efficiency (Zhong et al., 2007; Zhong et al., 2008b).

The aim of this study was to utilize these developments in AAV vectors in order to maximize therapeutic effects of gene replacement therapy for rapid retinal degeneration.
Specifically, we assessed the efficiency of gene replacement therapy using single-stranded AAV2/8 (ssAAV) or the novel self-complementary Y733F tyrosine capsid mutant AAV2/8 (scAAV) delivering the human Aipl1 gene. In these studies, we first assessed the onset and efficiency of gene expression achieved with each virus in wild-type retina. We further evaluated the level of vision rescue that ssAAV or scAAV provides to Aipl1-/- mouse, a model of rapid retinal degeneration, following treatment at an early and later stage of photoreceptor degeneration.
Materials and Methods

Animals

*Aipl1* knockout mice were generated and characterized previously (Ramamurthy et al., 2004). *Aipl1* knockout mice and wild-type used in this study are in a mixed C57Bl/129sv background. Mice were maintained under 12-h light / 12-h dark light cycles. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at West Virginia University.

Generation of recombinant AAV vectors

Full-length human *Aipl1* was amplified from human retinal cDNA using *Aipl1* specific primers and cloned into the multiple cloning site of the parental pAAV-RKp-zsGreen vector to generate the pAAV-RKp-*Aipl1* plasmid, which was used to produce the ssAAV2/8-RKp-*Aipl1* viral vector as described in Sun et al (Sun et al., 2010). RKp-*Aipl1* was amplified from pAAV-RKp-*Aipl1* with primers that engineered flanking Kpn-I and Eag-I sites, and cloned into the self-complementary AAV packaging vector, to generate the SC-RKp-*Aipl1* plasmid. This plasmid was used to produce the scAAV2/8-Y733F-RKp-*Aipl1* viral vector. The self-complementary-Y733F-AAV2/8 pseudotyped viral vector was generated as described previously, using the AAV8 capsid protein with Y733F surface-exposed tyrosine residue mutation (Zolotukhin et al., 2002; Zhong et al., 2008b). All polymerase chain reaction (PCR) amplifications were performed with Phusion GC DNA polymerase master mix (Finnymes, Thermo Scientific, Lafayette, CO, USA), following manufacturer instructions for cycling conditions. All plasmids were sequenced with the Big Dye Terminator ready reaction kit (Perkin Elmer, Waltham, MA, USA).
Subretinal injection

*Aipl1-* mice injected at postnatal day 2 (P2) were anesthetized by chilling on ice. P10 *Aipl1-* mice and P14 wild-type mice were placed under general anesthesia with an intraperitoneal injection of ketamine (90 mg kg⁻¹) / xylazine (9 mg kg⁻¹) and received a drop of dilation solution, consisting of a 1:1 mixture of tropicamide (1%, Alcon, Fort Worth, TX, USA) : phenylephrine hydrochloride (2.5%, Bausch and Lomb, Tampa, FL, USA). For injection into mice prior to eyelid opening, an incision was made along the natural future eyelid line with surgical vannas scissors. A small incision was then made in the sclera just outside the pupillary margin with a 36-gauge needle. Viral solution was injected into the subretinal space using a pulled glass pipette attached to a pneumatic injector (MINJ-PD, Tritech Research, Los Angeles, CA, USA) using a transcorneal approach (Matsuda and Cepko 2004). Self-complementary and single-stranded AAV solutions were titer matched to 1.2×10¹³ viral particles per ml by diluting with phosphate buffered saline (PBS). Each animal received 1.0 µl of AAV in the right eye, and no injection in the untreated contralateral eye. Visualization during injection was aided by the addition of 0.1% fluorescein (100 mg ml⁻¹ AK-FLUOR, Alcon, Fort Worth, TX, USA) to the viral solutions.

Electroretinographic (ERG) analysis

Mice were dark-adapted overnight prior to testing. Eyes were topically dilated with a 1:1 mixture of tropicamide: phenylephrine hydrochloride. For ERG testing, mice were placed on a heated platform with continuous flow of isoflurane anesthesia through a nose cone (1.5% isoflurane with 2.5 liters per min (lpm) oxygen flow rate). A reference electrode was placed subcutaneously in the scalp. ERG responses were recorded from both eyes with silver wire electrodes placed on each cornea, with contact being made with a drop of hypromellose solution (2% hypromellose in PBS) (Gonioscopic Prism Solution, Wilson Ophthalmic, Mustang, OK, USA). Rod dominated
responses were elicited in the dark with flashes of LED white light at increasing flash intensities. Light-adapted cone responses were elicited in the presence of a 41 cd/m² rod-desensitizing white background light with 2.5 cd · s/m² LED white light flashes. ERGs were performed on the UTAS Visual Diagnostic System with BigShot Ganzfeld with UBA-4200 amplifier and interface, and EMWIN 9.0.0 software (LKC Technologies, Gaithersburg, MD, USA).

**Vision-dependent behavioral assay**

Morris water maze assay to test for visual dependent behavior was performed as described in Pang et al (Pang et al., 2008). Mice were tested in a dim-lit room at 1.5 to 2.0 lux. Briefly, the task involved swimming in a 4 foot diameter water pool to an escape platform marked with a flag as a visual guidance cue. The time to reach the escape platform was recorded. The escape platform was washed between each trial to eliminate non-vision guided cues. Mice were trained to this task for 3 days, consisting of 5 trials each day. During this training period, the escape platform was stationary and the drop-off location of the mouse differed for each trial. On the 4th training day, the escape platform was moved to a different location in each trial. On the test day, day 5, the escape platform was also moved to a different location in each trial. However, to control for differences in swimming distances, the platform locations were kept constant for every mouse tested across all treatment groups. The reported times were taken from this final test day as group average times.

**Immunoblotting, immunohistology, and morphometric analysis**

Mice were euthanized by CO₂ inhalation and eyes were enucleated. For immunoblots, flash frozen retinal samples dissected from enucleated eyes were sonicated in 6 M urea buffer (6 M urea, 4% sodium dodecyl sulfate (SDS), 0.5 M Tris pH 6.8, 10 mg/ml DTT). Protein concentrations were estimated using a NanoDrop spectrophotometer (Thermo Scientific). 100 µg
total protein samples were resolved on 10% resolving SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride membranes (Immunobilon-FL, Millipore, Billerica, MA, USA). Membranes were blocked with blocking buffer (Rockland Inc., Gilbertsville, PA, USA) for 30 min at room temperature, and incubated with the indicated primary antibodies. Blots were washed in PBST (Phosphate buffered saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4·7H_2O, 1.4 mM KH_2PO_4, with 0.1% Tween-20) twice for 20 min and incubated in secondary antibody, goat anti-rabbit IRDye 680 (LI-COR Biosciences, Lincoln, NE, USA) for 30 min at room temperature. After 3 washes with PBST, membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and bands quantified using Odyssey Infrared Imaging System software.

For immunohistochemical studies, eyes were immersed in 4% paraformaldehyde fixative for 15 min prior to removal of the anterior segments and lens. Eyecups were fixed for an additional 4 h, washed in PBS (Phosphate buffered saline) for 20 min, incubated in 10% sucrose/PBS for 1 h at room temperature, and transferred to 20% sucrose/PBS for overnight incubation at 4°C. Eyes were then incubated in 1:1 mixture of 20% sucrose in PBS:OCT (Cryo Optimal Cutting Temperature Compound, Sakura, Torrance, CA, USA) for 1 h, and flash frozen in OCT. Cryosectioning was performed with a Leica CM1850 Cryostat, and serial retinal sections of 14 µm thickness were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Retinal sections mounted on slides were washed with 1X PBSTx (1X PBS with 0.1% TritonX-100), and incubated for 1 hr in blocking buffer (1X PBS with 5% goat sera, 0.5% TritonX-100, 0.05% sodium azide). Retinal sections were incubated with primary antibody for 2-3 hrs, followed by three 10 min washes with 1X PBSTx before incubation with secondary antibody (Alexa Fluor-488 or Alexa-Fluor 568, Invitrogen, Carlesbad, CA, USA) for
30 min. The nuclear marker, 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen, 1:5000 dilution) was added for 10 min, and washed with 1X PBSTx before mounting with Prolong anti-fade reagent (Invitrogen) and coverslipping. Slides were viewed and imaged on a laser scanning confocal (Zeiss LSM 510) on a Zeiss LSM AxioImager Z1 microscope. Outer nuclear layer cell quantification was conducted on retinal sections from 3 different animals with the highest ERG responses in each treatment group. Serial sections for each retina were examined to determine the area with greatest photoreceptor preservation, which typically showed a uniform preservation in 40-50% of serial sections. Confocal images of DAPI stained nuclei were taken at five fixed locations of a section, to control for variability across the eyecup. Confocal images were analyzed using ImageJ software (NIH, Bethesda, MD) to determine the photoreceptor cell count, which was reported as an average of the 15 measurements. Each measurement spanned approximately 200 µm in width of the retina.

For retinal wholemounts, eyes were enucleated with the orientation maintained by puncturing at the dorsal position, and fixed in 4% paraformaldehyde for 15 min. The anterior segments were removed to create an eyecup, and a vertical incision was created at the most dorsal position. The retina was carefully removed from the eyecup and fixed for an additional 6-12 hrs at 4°C. The retina was washed twice for 30 min prior to incubation in primary antibody for 12-24 hrs at 4°C. Removal of excess primary antibody was conducted with two 30 min washes, followed by incubation in secondary antibody for 12-24 hrs at 4°C, washed twice for 20 min, and mounted and coverslipped. Wholemounts were viewed and imaged on an Olympus AX70 Provis microscope with an Optronics Microfire color CCD camera (Optronics, Golenta, CA, USA), using Stereo Investigator (MBF Bioscience, Milliston, VT, USA) and PictureFrame (Optronics, Golenta, CA, USA) software. Quantification of wholemounts was conducted using
ImageJ software to analyze 20х objective wholemount images taken with the same exposure time. Each data point, expressed in fluorescent arbitrary units, was an average of 9 measurements obtained from 3 injected retinas with 3 images for each retina.

The following primary antibodies were used at the indicated dilutions: Human AIPL1 (1:1000) (Ramamurthy et al., 2004), PDE6αβγ (MOE) (1:2000) (CytoSignal Inc., Irvine, CA, USA), PDE6β (1:1000) (Affinity BioReagents, Golden, CO, USA), cone PDEα’ (1:1000) (Kirschman et al., 2010), rod opsin (4D2) (1:2000) (gift from Dr. Robert Molday, University of British Columbia, Vancouver, British Columbia, Canada), red/green opsin (1:1000) (Chemicon, Millipore, Billerica, MA, USA), PKCα (1:2000) (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Rhodamine peanut agglutinin (PNA) (1:500) (Vector Laboratories, Inc., Burlingame, CA, USA) and DAPI (1:5000) (4’6-diamidino-2-phenylindole, Invitrogen) were also used in immunocytochemistry.

**Histologic, semithin sections, and transmission electron microscopy**

For hematoxylin and eosin stained images, enucleated whole eyes were fixed in Alcoholic Z-fix (Excalibur Pathology) for 48 hrs at room temperature. Serial sections of paraffin embedded fixed whole eyes were mounted on slides (Excalibur Pathology). Images were collected on an Olympus AX70 Provis microscope using PictureFrame software (Optronics, Golenta, CA, USA).

For light and transmission electron microscopy, enucleated eyes were lightly fixed in freshly prepared fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.5) for 30 min. The anterior segment and lens were removed and eye cups were returned to fixative for 48 hours at room temperature, prior to dissection into 6-8 wedge-shaped pieces. Wedges were dehydrated in a graded ethanol series, then embedded in Polybed 812 (PolySciences, Inc., Warrington, PA, USA). Semi-thin (1 μm) sections were collected onto glass
slides, stained with toluidine blue, and visualized using a Zeiss Axioimager 2 microscope equipped with EC Plan-Neofluar 40x (N.A. 0.75) and 100x (1.3 N.A.) objectives to identify best rescued areas. Thin sections (ca. 80 nm) from selected wedges were collected onto nickel grids, stained with 2% uranyl acetate and lead citrate, and imaged using an FEI Morgagni transmission electron microscope at 80 kV.

**Statistical analyses**

Statistical analyses were conducted on GraphPad Prism4 software (GraphPad Software, Inc., La Jolla, CA, USA), using one-tailed unpaired *t*-tests for two group comparisons, and ANOVA followed by Tukey-Kramer HSD post-hoc tests for multiple group comparisons.
Results

Self-complementary AAV capsid mutant induces earlier and higher expression of hAIPL1 in retina

Self-complementary Y733F tyrosine capsid mutant AAV2/8 combines two recent developments in AAV vectors shown to increase transduction efficiency and decrease expression latency of reporter genes (Natkunarajah et al., 2008; Petersen-Jones et al., 2009; Kong et al., 2010). To compare the onset and levels of expression of single-stranded AAV and self-complementary capsid mutant AAV, we injected ssAAV-RKp-hAipl1 (henceforth referred to as ssAAV) or scAAV-Y733F-RKp-hAipl1 (henceforth referred to as scAAV) into postnatal day 14 (P14) wild-type retina. Viral titers were matched between ssAAV and scAAV for all experiments throughout this study. The vectors expressed the human Aipl1 gene under the short rhodopsin kinase promoter (RKp), which is active in both rod and cone photoreceptor cells as early as P2 (Young et al., 2003; Young et al., 2005; Khani et al., 2007). Expression of exogenously delivered human AIPL1 was investigated through immunofluorescent staining of whole mounted injected eyes with an antibody that specifically recognizes the human isoform of AIPL1. Eyes injected with ssAAV exhibited low AIPL1 expression in sparse areas beginning at post-injection (pi) day 3, with progressive increases at pi day 5 and 7. Expression of AIPL1 began to plateau between pi day 7 and 14 (Fig. 1A). In comparison, eyes injected with scAAV exhibited expression of AIPL1 in larger areas beginning at pi day 2 and uniformly high expression at pi day 3. Expression of AIPL1 increased steadily and uniformly at pi day 5, 7, and 14 (Fig. 1A). Fluorescent counts quantifying AIPL1 expression were conducted on three treated retina per post-injection time point. Mean fluorescent counts were significantly higher in scAAV treated eyes than ssAAV treated eyes at pi day 3, 5, and 14 (p < 0.0005) (Fig. 1B). scAAV treated retina had a four-fold
higher mean fluorescent count than ssAAV at pi day 3, and a two-fold difference at pi day 5 and pi day 14. Differences in AIPL1 expression were particularly evident at earlier time points, with expression levels of scAAV at pi 3 greater than levels of ssAAV at pi 5.

**Early administration of AAV-mediated gene replacement therapy efficiently rescues photoreceptor function in Aipl1-/− mice**

* Aipl1-/− mice exhibit rapid and severe photoreceptor degeneration (Dyer et al., 2004; Ramamurthy et al., 2004), with initiation of cell death at postnatal day 8 (P8) (Supplementary Fig. 1). In addition, photoreceptor cells in these mice are not functional at any age tested (Ramamurthy et al., 2004). Our goal was to compare the efficacy of ssAAV and scAAV in preventing cell death and in rescuing visual function. Therefore, we injected AAV-hAipl1 subretinally into one eye of Aipl1-/− mice at postnatal day 2 (P2). At this early stage of retinal development, rod photoreceptors in mice are beginning to form. Electroretinograms (ERGs), a measure of light dependent electrical responses from photoreceptor cells and downstream neurons, were conducted to evaluate photoreceptor function after treatment with either ssAAV or scAAV. The $a$-wave in ERG correlates with photoreceptor activity, while the $b$-wave arises mainly due to downstream bipolar cell activity. Representative ERG recordings at P30 under dark-adapted (Fig. 2A) and light-adapted conditions (Fig. 2B), showed restoration of scotopic and photopic ERG responses in ssAAV and scAAV treated eyes compared to a complete absence of response in the contralateral untreated eye. The scAAV treated eye showed greater $a$- and $b$-wave amplitudes and oscillatory potentials than the ssAAV treated eye at all dark-adapted scotopic flash intensities of -3.6, -1.6, -0.6, and 0.4 log (cd · s/m²) flashes. At a flash intensity of -0.6 log (cd · s/m²), the photoreceptor responses ($a$-wave amplitudes) of individual mice in each
treatment group showed intergroup variability (Fig. 2C). However, the mean group average for scAAV was statistically higher than for ssAAV treated mice (p = 0.027; ssAAV treated mean = 49.5 ± 8.7 µV, n = 10; scAAV treated mean = 79.5 ± 11.6 µV, n = 10). This difference in group means of \( a \)-wave amplitudes was statistically significant at every scotopic flash intensity recorded (p values indicated in figure, ranging from p = 0.017 to p = 0.027) (Fig. 2D).

**Early administration of AAV-mediated gene replacement therapy restores rod and cone PDE6 expression and slows photoreceptor degeneration in \( Aipl1^{-/-} \) mice**

To evaluate the effects of \( Aipl1 \) replacement on photoreceptor survival, we examined the retinal morphology in AAV treated and untreated control eyes. Immunocytochemical and morphometric analyses were conducted at P65 after a single injection at P2. As expected, treated eyes showed expression of hAIPL1 in photoreceptor inner segments and outer plexiform layer (Kirschman et al., 2010; Sun et al., 2010) (Fig. 3A). Concomitant with the expression of hAIPL1, rod and cone PDE6 was observed along with rod and cone opsins, indicating the presence of intact rod and cone outer segments (Fig 3A). ssAAV treated eyes showed 4-5 rows of photoreceptor cell nuclei compared to a complete loss of photoreceptors in the contralateral untreated eye. scAAV treated retina had 5-6 rows of photoreceptor nuclei (Fig. 3A, and Supplementary Fig. 2). To quantify the observed differences in outer nuclear layer thickness, photoreceptor cell nuclei counts were conducted on retina from each treatment group. The mean number of photoreceptor cell nuclei of scAAV treated retina was significantly greater than ssAAV treated retina by 45% (p = 0.005; ssAAV mean = 125.92 ± 14.43; scAAV mean = 182.85 ± 13.42) (Fig. 3B). In agreement with our ERG and morphology results, western blots showed that retina from scAAV treated animals expressed higher levels of AIPL1 and rod PDE6 than
ssAAV treated animals (Fig. 3C). This trend was also observed when protein levels were examined at P35, where all scAAV treated animals expressed higher levels of AIPL1 and rod PDE6 (Supplementary Fig. 3) at this earlier post-injection time point.

**Early administration of AAV-mediated gene replacement therapy preserves photoreceptor ultrastructure**

The effectiveness of AAV-mediated gene replacement therapy on preserving photoreceptor ultrastructure was evaluated through light and transmission electron microscopy. Semi-thin sections showed scAAV treated retina with longer inner and outer segments compared to ssAAV treated retina (Fig. 4A). At best, outer segments in ssAAV treated retina were 40-50% of the length of outer segments in an age-matched wild-type retina, whereas scAAV treated retina were 50-60% of wild-type retina (Fig 4A). Additionally, semi-thin sections showed similar findings to immunohistochemical studies in outer nuclear layer thickness, with ssAAV treated retina with 4-5 rows of nuclei and scAAV treated retina with 5-6 rows (Fig. 4A).

Ultrastructurally, both ssAAV and scAAV treated retina displayed intact photoreceptor outer segments with normally organized and densely stacked disc membranes, similar to wild-type retina (Fig. 4B).

**Early administration of AAV-mediated gene replacement therapy restores visually guided behavior in Aipl1-/- mice**

To assess whether the observed rescue of electrophysiological responses and retinal morphology result in improvements in visually guided behavior, P2 AAV treated mice were tested with the Morris water maze at P60. The Morris water maze, adapted from Pang et al.
(Pang et al., 2008), tested for the ability of the mice to visually locate an escape platform. Under dim light conditions, untreated wild-type $Aipl1^{+/+}$ mice took 3.45 ± 0.44 sec ($n = 7$) to locate the platform (Fig. 5). In comparison, untreated $Aipl1^{-/-}$ mice located the platform in 24.09 ± 2.72 sec ($n = 8$), which was significantly greater than wild-type mice ($p < 0.001$). $Aipl1^{-/-}$ mice treated with ssAAV found the platform in 6.88 ± 0.87 sec ($n = 6$), and scAAV treated mice took 7.82 ± 0.81 sec ($n = 7$), both of which were significantly less than untreated $Aipl1^{-/-}$ mice ($p < 0.001$). There was no statistical difference between ssAAV treated and wild-type mice, between scAAV treated and wild-type mice, or between ssAAV and scAAV treated mice ($p > 0.05$ for all comparisons, Analysis of Variance (ANOVA) with Tukey-Kramer Honestly Significant Difference (HSD) statistical analysis).

**Gene replacement therapy with self-complementary AAV capsid mutant effectively rescues rod and cone function in $Aipl1^{-/-}$ mice with active photoreceptor degeneration**

Our experiments comparing transduction kinetics of ssAAV and scAAV in wild-type retina demonstrated higher expression of $AIPL1$ at earlier time points with scAAV treatment. We hypothesized that this characteristic of scAAV is important in maximizing rescue of photoreceptors during active degeneration in $Aipl1^{-/-}$ mice, since these mice have a rapid rate of degeneration. To determine the efficiency of ssAAV and scAAV treatment in actively degenerating retina, viral injections were administered at postnatal day 10 (P10). At this stage of retinal development, photoreceptor cell death is evident in $Aipl1^{-/-}$ mice (Supplementary Fig. 1). ERG recordings were conducted at P30 to examine the effects of the delayed ssAAV and scAAV treatment on photoreceptor function. Representative ERG traces showed robust scotopic and photopic responses elicited from scAAV treated animals compared to weak responses from
ssAAV treated animals (Fig. 6A, B). The photoreceptor response (a-wave amplitudes) of individual mice in each treatment group showed all scAAV treated animals with higher a-wave amplitudes than ssAAV treated animals, and a significant difference in group means (p = 0.0006; ssAAV treated mean = 4.22 ± 2.17 µV, n = 9; scAAV treated mean = 47.67 ± 10.73 µV, n = 9) (Fig. 6C), under dark-adapted conditions with a flash intensity of -0.6 log (cd · s/m²).

Additionally, the difference in group means between ssAAV and scAAV treatment was significant for a-wave and b-wave amplitudes recorded under dark-adapted conditions, at all flash intensities of -3.6, -1.6, -0.6, and 0.4 log (cd · s/m²) (p values indicated in figure, ranging from p = 0.003 to p = 0.00001) (Fig 6D, E). A similar trend was observed with light-adapted ERG responses, a reflection of cone photoreceptor activity. A statistically significant difference in average b-wave amplitudes elicited under light-adapted conditions with a 0.4 log (cd · s/m²) flash intensity was observed (p = 0.014; ssAAV treated mean = 1.22 ± 1.22 µV, n = 9; scAAV treated mean = 20.44 ± 7.86 µV, n = 9) (Fig. 6F), with robust cone responses in scAAV treated animals. While ERG responses of ssAAV treated eyes were significantly lower than scAAV treated eyes, they were significantly higher than contralateral untreated eyes at all flash intensities (p < 0.05) (data not shown).

**Gene replacement therapy with self-complementary AAV capsid mutant slows rod and cone degeneration in Aipl1/- mice with active photoreceptor degeneration**

In P10 treated mice, the significant difference in ERG responses between ssAAV and scAAV already observed at P30 led us to conduct behavioral assays and morphological analyses at P35. Additionally, we wanted to accurately examine the behavioral and morphological correlates of the low ERG responses elicited from ssAAV treated mice at P30. Morphological
analyses were conducted to assess preservation of photoreceptors following delayed AAV-mediated gene replacement in *Aipl1/-/-* mice at P10. Retina from scAAV treated animals maintained 5-6 rows of photoreceptor cell nuclei (Fig. 7A, and Supplementary Fig. 2). In comparison, retina from ssAAV treated animals showed 2-3 rows of photoreceptor cell nuclei. These findings were supported by our cell counting results demonstrating the presence of significantly greater numbers of photoreceptor cell nuclei in retina from scAAV than ssAAV treated animals (*p* = 0.0000001; ssAAV mean = 76.90 ± 11.99; scAAV mean = 275.50 ± 21.71) (Fig. 7B).

Immunostaining for photoreceptor outer segment proteins such as rod and cone opsins revealed sparse and short outer segments in retina from ssAAV treated animals, a pattern also observed with immunostaining for rod and cone PDE6 (Fig. 7A). In contrast, retina from animals treated with scAAV displayed denser and longer rod and cone outer segments (Fig. 7A). Coincident with the presence of photoreceptor outer segments, retina from animals treated with scAAV exhibited strong expression of AIPL1 in inner segments, and rod and cone PDE6 in respective photoreceptor outer segments (Fig. 7A). Our immunofluorescence results are supported by immunoblotting experiments demonstrating higher levels of hAIPL1 and rod PDE6 in the majority of retina from scAAV treated animals (lanes 6-10) compared to ssAAV treated animals (lanes 1-5) (Fig. 7C). The amount of rod PDE6 in scAAV treated retina varied from 10-40% of wild-type levels. In comparison, the amount of rod PDE6 in ssAAV treated retinas was negligible, with 9% of wild-type rod PDE6 levels at best (lane 5).
Gene replacement therapy with self-complementary AAV capsid mutant rescues visually guided behavior in Aipl1-/− mice with active photoreceptor degeneration

To evaluate visually guided behavior following treatment at postnatal day 10 (P10), mice were tested with the Morris water maze at P35 (Fig. 8). Similar to our previous results, Aipl1+/+ wild-type mice took 4.38 ± 0.45 sec (n = 6) to locate the escape platform, whereas Aipl1-/− mice needed 25.20 ± 6.52 sec (n = 5) (p < 0.01). ssAAV treated mice took 24.00 ± 3.51 sec (n = 8), which was significantly different from wild-type (p < 0.01), but not from Aipl1-/− mice (p > 0.05). These results indicate that the visually guided behavior in P10 ssAAV treated mice was not improved from untreated Aipl1-/− mice. In contrast, the average time of scAAV treated mice to find the platform was 6.69 ± 1.30 sec (n = 7), which was significantly less than ssAAV treated mice and Aipl1-/− mice (p < 0.01), but not significantly different from Aipl1+/+ mice (p > 0.05), indicating that treatment with scAAV at P10 restored visually dependent behavior to wild-type levels. Similar results were observed when a comparison was conducted between ssAAV treated mice demonstrating ERG responses and scAAV treated mice, where the average time of ssAAV treated mice (n = 4, 17.87 ± 1.82 sec) was significantly different than scAAV treated mice (n = 7, 6.69 ± 1.30 sec) (p < 0.01), but not significantly different from Aipl1-/− mice (n = 5, 25.20 ±6.52 sec) (p > 0.05) (ANOVA with Tukey-Kramer HSD statistical analysis).
Discussion

In this study, we demonstrated that the faster and greater transduction efficiency of sc-Y733F-AAV2/8 over ssAAV2/8 led to greater preservation of visual function in AAV-mediated gene replacement therapy of Aipl1 deficient mice, a model of severe Leber congenital amaurosis. This is the first reported study to use sc-Y733F-AAV2/8 for rescue of vision in a model of rapid retinal degeneration. Self-complementary AAV, shown to decrease expression latency and increase transduction efficiency, is generally thought to have limited use due to the halved gene packaging capacity of single-stranded AAV. However, it has been shown that efficient packaging and expression of genes up to 3.3 kb may be possible (Wu, J. et al., 2007), extending the utility of self complementary AAV. Additionally, the advent of capsid tyrosine mutations provides a simple way to increase viral efficiency without placing additional size restrictions. While mutations of surface-exposed tyrosine residues on viral capsids decrease ubiquitination which largely improves transduction efficiency, the effect on expression latency remains unclear.

Interestingly, we observed differences between ssAAV and sc-Y733F-AAV (scAAV) treatments in Aipl1-/- mice treated at P2, when photoreceptors are not yet fully developed and no sign of degeneration is present. The aim of treating early was to provide maximal rescue of photoreceptors. Despite early treatment, we observed consistently lower ERG responses and photoreceptor cell survival in ssAAV treated compared to scAAV treated Aipl1-/- retina. However, the magnitude of differences between the two treatment groups did not translate into a difference in visually guided behavior under our lighting conditions. The observed differences between the two treatment groups were likely due to the lower levels of AIPL1 expression achieved with ssAAV, as demonstrated by our studies in wild-type retina. In contrast, the higher AIPL1 expression with scAAV treatment provided greater ERG responses and photoreceptor
survival. In addition to these benefits in preservation of photoreceptor structure and function, viral vectors that achieve higher expression have a practical advantage, allowing for administration of lower viral titers which is especially important in reducing potential adverse effects. The use of the scAAV viral vector, therefore, may be warranted and beneficial in gene replacement therapy clinical trials.

The functional advantage of scAAV was most striking following P10 administration, when the Aipl1 deficient photoreceptors are undergoing active degeneration. Late treatment with ssAAV achieved minimal photoreceptor cell survival and low ERG responses in a fraction of the treated animals, with no evidence of vision guided behavior. In contrast, scAAV treated animals showed greater photoreceptor cell survival, with concomitant ERG responses and vision guided behavior. Following late scAAV treatment, ERG responses persisted until the last time point tested at P60 (Supplementary Fig. 4). The preservation of vision achieved with both early and late administration of scAAV treatment demonstrates stability of vision rescue. To summarize, in a clinically relevant scenario of active photoreceptor degeneration, scAAV treatment showed clear benefits over ssAAV in vision rescue.

The advantages in vision rescue with scAAV treatment were likely a result of the early robust expression of hAIPL1 observed in our comparisons of transduction efficiency in wild-type retina. It took an additional 3-4 days for expression levels of AIPL1 from ssAAV treatment to reach a level attained by that of scAAV treatment. We speculate that the faster expression of AIPL1 with scAAV translates into quicker restoration of functional PDE6 and stabilization of cGMP levels, and subsequently greater preservation of outer segments. This proved particularly critical in vision rescue in mice where photoreceptor degeneration has initiated. Additionally, the higher expression achieved with scAAV contributed to the improved vision rescue. It is known
that \textit{Aipl1} hypomorphic mice, which express 20-30\% of wild-type AIPL1 levels, show abnormal photoreceptor function and slow degeneration (Liu et al., 2004). In contrast, \textit{Aipl1} heterozygous mice, with 50\% of protein, show no signs of photoreceptor degeneration or dysfunction (Ramamurthy et al., 2004). This indicates that a certain threshold level of AIPL1 is necessary for normal photoreceptor function and survival. The lower expression of AIPL1 achieved with ssAAV treatment in \textit{Aipl1}--/- mice may lead to a number of photoreceptors that do not cross this threshold, resulting in lower photoreceptor survival and subsequent lower ERG responses. Higher AIPL1 expression levels achieved with scAAV treatment leads to a greater probability that this threshold is surpassed in a larger number of photoreceptors, hence achieving greater photoreceptor survival and restoration of vision.

The demonstrated functional advantages of second generation AAV vectors, such as sc-Y733F-AAV2/8, are important in the future progress of AAV-mediated gene replacement therapy clinical trials. There is an impetus to broaden gene therapy clinical trials to treat a wider severity spectrum of inherited retinal dystrophies. Recent clinical studies report cases of adolescent and adult patients with genetic defects associated with rapid retinal degeneration, who show varying extents of ONL preservation and inner and outer segment integrity as observed with optical coherence tomography (OCT). This has been observed in patients with defects in \textit{CEP290} (Cideciyan et al., 2007; Pasadhika et al., 2010), \textit{GUCY2D} (Pasadhika et al., 2010), \textit{RPGRIP1} (Jacobson et al., 2007), and \textit{AIPL1} (Jacobson et al., 2011; Testa et al., 2011). These studies show that patients with genetic defects associated with rapid retinal degeneration may potentially benefit from AAV-mediated gene replacement therapy. The improved transduction onset and efficiency of second generation AAV vectors, such as sc-Y733F-AAV2/8, provides a larger therapeutic range for treating a spectrum of genetic defects with varying rates of disease.
progression. Additionally, the longevity of such residual photoreceptors cannot be predicted for each patient, and the best practical option is to provide the most rapid acting and efficient treatment possible. It remains to be examined whether infants and young children afflicted with LCA have a substantial amount of photoreceptors that can be salvaged with early gene therapy treatment. The aim of gene therapy here would be prevention of massive loss of retinal neurons. Our results demonstrated that sc-Y733F-AAV2/8 also provided functional benefits over ssAAV2/8 in early treatment of rapid retinal degeneration. Additional concurrent progress in viral-mediated gene delivery and clinical characterization is necessary to advance into clinical trials, but emerging evidence in both areas show promising prospects for gene replacement therapy to successfully treat a wider spectrum of genetic defects and severities of retinal dystrophies.
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Conflict of Interest Statement

William W. Hauswirth and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC, Inc.) that might, in the future, commercialize some aspects of this work.
Fig. 1. scAAV provides earlier onset and higher expression compared to ssAAV following subretinal injection into wild-type retina. (A) Wholemount images from injected retina at indicated post-injection (pi) times. All images were taken with the same exposure time at x20 magnification. Scale bar, 100 µm. (B) Quantitative analysis of AIPL1 expression in wholemount images, expressed in arbitrary units of fluorescence (AU). Except for pi 7, statistically significant differences in units of fluorescence between ssAAV and scAAV were observed. Error bars, ±SEM.
Fig. 2. Light-dependent photoreceptor responses from Aipl1−/− mice following early AAV treatment. One eye of Aipl1−/− mice was injected at P02 with the indicated AAV and ERGs were recorded at P30. ERG responses from (A) dark- and (B) light-adapted conditions were measured at the indicated log (cd · s/m²) flash intensities. Traces from a contralateral untreated
eye and an age-matched wild-type mouse are shown for comparison. (C) Dark-adapted photoresponses from Aipl1-/- treated mice at the flash intensity of -0.6 (log cd · s/m²). Each data point represents the measured a-wave amplitude of an individual treated mouse. The group average of scAAV treated mice was significantly higher than the group average of ssAAV treated mice (p ≤ 0.027). (D) Comparison of mean rod photoreceptor response (a-wave amplitude) between ss and scAAV treatments over increasing flash intensities. Average a-wave amplitudes from the scAAV treated mice were significantly greater than the ssAAV treated mice at all intensities (p ≤ 0.027). Error bars, ±SEM.
Fig. 3. Restoration of photoreceptor morphology and PDE6 expression in *Aipl1*/*-* retina following early AAV treatment. *Aipl1*/*-* mice were treated at P2 and retinas were collected at P60 for immunocytochemistry and immunoblots. (A) Confocal images of retinal sections stained with indicated antibodies. Peanut agglutinin (PNA) (red) which stains the cone sheath is a cone marker. Cell nuclei were stained with DAPI (blue). Images were taken at x63 magnification. Scale bar, 20 µm. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer. (B) Photoreceptor cell nuclei quantification of ssAAV and scAAV treated retina using retinal sections stained with DAPI. scAAV treatment showed significantly higher ONL cell count. **p = 0.005**. (C) Western blot analysis of hAIPL1, Rod PDE6αβ, and PKCα in retinal homogenates from various genotypes of mice.
cell counts than ssAAV treated retina (p ≤ 0.005). Error bars, ±SEM. (C) Western blots of retinal extracts from ssAAV and scAAV treated animals with indicated antibodies. Untreated Aipl1-/-(lane 5), wild-type (lane 6), and transgenic hAipl1 (lane 7) retina served as controls. PKCα expressed in bipolar cells, is a loading control.
Fig. 4

(A) Semithin light micrographs of P35 Aipl1−/− retina following treatment at P2 with ssAAV or scAAV. Semithin sections from a P35 Aipl1+/+ retina are shown for comparison. Scale bar, 20 µm. RPE, retinal pigmented epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer. (B) Electron micrographs at x2200 (top panel) and x8900 (bottom panel) magnification. RPE, retinal pigmented epithelium; MV, microvilli; OS, outer segment; IS, inner segment. Scale bars, 5 µm (top panel) and 1 µm (bottom panel).
**Fig. 5.** Vision-dependent behavior is restored in *Aipl1*−/− mice following early ss- or sc-AAV treatment. Mice received treatment at P2 and were tested at P60 with the Morris water maze. Age-matched untreated *Aipl1*−/− and *Aipl1*+/+ groups served as controls. The time to reach the escape platform was recorded and reported as group averages. Treatment with both AAVs restored vision-dependent behavior to wild-type *Aipl1*+/+ levels (p > 0.05), which were significantly different from untreated *Aipl1*−/− mice (***p < 0.001, ANOVA with Tukey-Kramer HSD post-hoc test). Error bars, ±SEM.
Fig. 6. Light-dependent photoresponses in Aipl1-/- mice following late administration of AAV-mediated gene replacement therapy. ERG responses were recorded at P30, following...
injection at P10. ERG tracings were recorded under (A) dark-adapted and (B) light-adapted conditions at the indicated flash intensities. (C) A-wave amplitudes of individual mice in ssAAV and scAAV treated groups, derived from ERG recordings at -0.6 (log cd · s/m²) flash intensity. All scAAV treated eyes showed higher a-wave amplitudes than ssAAV treated eyes. Additionally, the group average of scAAV treated mice was significantly higher than the group average of ssAAV treated mice (p ≤ 0.0006). Quantitative analysis of mean (D) a-wave and (E) b-wave amplitudes showed significantly higher amplitudes in the scAAV treated group than the ssAAV treated group across all flash intensities under dark-adapted conditions (p ≤ 0.003). (F) Quantitative analysis of mean b-wave amplitudes from ERGs recorded under light-adapted conditions. Higher photopic responses were elicited from scAAV treated eyes compared to ssAAV treated eyes (p ≤ 0.014). Error bars, ±SEM.
Fig. 7. Photoreceptor morphology of Aipl1-/- retina following late administration of AAV-mediated gene replacement. Aipl1-/- mice were treated at P10 and retinas were collected at P35 for immunocytochemistry. (A) Confocal images of retinal sections stained with indicated antibodies. Peanut agglutinin (PNA) (red) is used as a cone marker. Cell nuclei are stained with
DAPI (blue). Images were taken at x63 magnification. Scale bar, 20 µm. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer. (B) Quantification of photoreceptor cell nuclei showed significantly greater nuclei in scAAV compared to ssAAV treated retina (p ≤ 0.0000001). Error bars, ±SEM. (C) Western blots of ssAAV (lanes 1-5) and scAAV (lanes 6-10) treated retina. Untreated Aipl1-/- (lane 11), wild-type (lane 12), and transgenic hAipl1 (lane 13) retina serve as controls. PKCa expressed in bipolar cells, is a loading control.
Fig. 8. Vision-dependent behavior is restored to *Aipl1*-/- mice exclusively by scAAV treatment. Mice were tested at P35 after AAV administration at P10 with the Morris water maze assay. The mean time of scAAV treated mice was similar to wild-type *Aipl1*+/+ mice (p > 0.05), and both were significantly lower than untreated *Aipl1*-/- and ssAAV treated mice (*p < 0.01 at group indicated comparisons, ANOVA with Tukey HSD). The mean time of ssAAV treated mice was not significantly different from untreated *Aipl1*-/- mice (p > 0.05). Error bars, ±SEM.
Supplementary Fig. 1.

Rate of retinal degeneration and vision loss in the *Aipl1*−/− mouse.

(A) Immunostaining of *Aipl1*+/+ and *Aipl1*−/− retina at postnatal day 2 (P2), P8, P10, and P35 with propidium iodide, a marker of cell death. Arrowheads indicate photoreceptor cell death in *Aipl1*−/− retina, which began at P8 and was clearly evident at P10. Images were taken at x40 magnification. Scale bar, 20 µm. ONL, outer nuclear layer; INL, inner nuclear layer.
Supplementary Fig. 2. Photoreceptor rescue spanned the retina following both early and late administration of scAAV. Hematoxylin and eosin (H&E) sections of Aipl1−/− retina at P35 following scAAV treatment at (A) P2, and (B) P10. An untreated contralateral eye is shown for
comparison. Images of the eyecup were taken at x10 and photomerged, and at x40 magnification for inset images. Scale bar, 20 µm.
Supplementary Fig. 3

Supplementary Fig. 3. Protein expression at P35 following early administration of ssAAV and scAAV. Western blots of retina from Aipl1/- mice at P35 following treatment at P2, probed with the indicated antibodies. Representative samples from three ssAAV (lanes 1-3) and scAAV (lanes 4-6) treated mice are shown. Untreated Aipl1/- (lane 7), wild-type (lane 8), and transgenic hAipl1 (lane 9) served as controls. PKCα expressed in bipolar cells, is a loading control.
Supplementary Fig. 4. Longitudinal ERG responses recorded from a P10 treated scAAV mouse. ERG recordings from a representative individual mouse at (A) P30 and (B) P60 show maintenance of dark-adapted and light-adapted photoresponses, achieved with late administration of scAAV treatment. Dark-adapted ERG responses were elicited with the indicated flash intensities of -3.6, -1.6, -0.6, and 0.4 log (cd · s/m²). Light-adapted ERG responses were elicited with a flash intensity of 0.4 log (cd · s/m²).
Chapter 3

P351Δ12 Aipl1 mutation: a model of cone-rod dystrophy

Abstract

Defects in aryl hydrocarbon receptor interacting protein-like1 (Aipl1) are typically associated with the most severe of inherited retinal degenerations, Leber congenital amaurosis (LCA). Pre-clinical rescue studies of Aipl1-null mice, a model for LCA that has the most rapid retinal degeneration of any mouse model, have mainly served as proof-of-principle to demonstrate that even the most severe of retinal dystrophies can be rescued. However, reports of Aipl1 defects associated with less severe IRDs, such as retinitis pigmentosa (RP) and cone-rod dystrophy (CORD), highlight the potential for Aipl1 to be a candidate gene for future AAV-mediated gene therapy clinical trials. Specifically, the P351Δ12 hAipl1 mutation, a defect in the C-terminal primate-specific ‘hinge’ region of AIPL1, has been linked to autosomal dominant CORD and juvenile RP. Autosomal dominant retinal degeneration has not been otherwise associated with Aipl1, and is important to evaluate since it indicates that AAV-mediated gene replacement may not be helpful despite the slower degeneration associated with CORD and RP. Other mutations have also been reported in the AIPL1 hinge region, although the function of this primate-specific region remains unknown.

We sought to examine the effects of the P351Δ12 hAipl1 mutation by generating a transgenic mouse with this mutation. As compared to a transgenic line expressing wildtype hAipl1 under the same mCrx promoter construct, mutant P351Δ12 hAipl1 mice demonstrated a CORD phenotype with early defects in cone-mediated vision and subsequent degeneration. The mCrx promoter is active in both rods and cones as early as embryonic day 12.5 (E12.5). A
dominant phenotype was observed in double transgenic animals expressing both mutant P351Δ12 hAIPL1 and WT hAIPL1, in agreement with the autosomal dominant inheritance observed in patients with the P351Δ12 hAIPL1 mutation. Interestingly, we were able to rescue cone-mediated vision in P351Δ12 hAIPL1 mice following AAV-mediated gene delivery of WT hAIPL1 despite the dominant phenotype of the P351Δ12 mutation. Our transgenic P351Δ12 hAIPL1 mouse offers a novel model of Aipl1-CORD, with distinct defects from both the Aipl1-null mouse mimicking LCA and the Aipl1-hypomorphic mice mimicking a slow progressing RP.
Introduction

Inherited retinal degeneration (IRD) is a group of blinding disorders affecting approximately 1 in 3000 people (Daiger et al., 2007). Multiple forms of inherited retinal degenerations exist with varied onset, symptoms, severity, and progression of disease. Leber congenital amaurosis (LCA) shows the earliest onset and greatest severity of inherited retinal dystrophies, presenting in early infancy with severe visual impairment and absent or markedly reduced rod and cone-mediated electroretinograms (ERG). Other IRDs such as retinitis pigmentosa (RP) and cone-rod dystrophy (CORD) show a greater variability in onset and rate of disease progression. Additionally, while LCA patients show severe defects early on in both rod- and cone-mediated vision, RP patients present with night blindness as the first visual impairment alongside normal visual acuity. In contrast, CORD patients initially present with decreased visual acuity, central vision loss, and dyschromatopsia, with progressive loss in peripheral, rod-mediated vision. Successful clinical trials of gene replacement therapy improving visual function in LCA patients with defects in *retinal pigmented epithelium-specific 65 kDa protein (Rpe65)* has garnered attention to finding another candidate gene for treatment. Outside of the unusual circumstance of *Rpe65*-LCA, where patients show significant visual defects disproportionate to slow photoreceptor loss (Jacobson et al., 2005; Cideciyan 2010), the slower disease progression of CORD and RP may indicate a larger therapeutic window in patients.

One such candidate gene for future AAV-mediated gene replacement is *aryl hydrocarbon receptor interacting protein-like 1 (Aipl1)*. Although defects in *Aipl1* are mainly associated with autosomal recessive LCA, sporadic reports have linked *Aipl1* to less severe IRDs of CORD, juvenile RP, non-early onset RP, and later onset retinal degeneration (Sohocki et al., 2000b; Vallespin et al., 2007; Walia et al., 2010; Jacobson et al., 2011). These cases point to a subset of
*Aipl1* patients who may be better candidates for gene replacement therapy due to a slower disease progression. Several non-LCA *Aipl1* cases are linked to defects in the C-terminal primate-specific proline-rich region of *Aipl1* (Fig. 1A, *grey box*), specifically mutations P351Δ12 (cDNA del1053-1064) and A336Δ2 (cDNA del1008-1009) (Sohocki et al., 2000a; Sohocki et al., 2000b). P351Δ12 *hAipl1* has been associated with autosomal dominant CORD and juvenile RP. Autosomal dominant disease has not been otherwise reported with *Aipl1*, and is important to examine because it indicates a dominant negative mechanism of dysfunction differing from the loss of function observed with truncation mutation W278X. Autosomal dominance is also clinically important to examine as it potentially indicates that AAV-mediated gene replacement would not be helpful for the P351Δ12 *hAipl1* mutation, despite a slower disease progression. Lastly, it may shed light on the function of the C-terminal primate-specific proline-rich hinge region of human *Aipl1*. The function of the hinge region in *hAipl1* remains unknown, although the additional size provided by this 56 aa region has been advantageously used to differentiate mouse and exogenously introduced human *Aipl1* in transgenic and AAV-mediated rescue studies (Tan et al., 2009; Kirschman et al., 2010; Sun et al., 2010).

The aim of this study was to examine the effects of the patient mutation, P351Δ12 *hAipl1*. We sought to characterize the visual dysfunction associated with this mutation, to evaluate whether this mutation leads to retinal degeneration in a dominant fashion, and to attempt to rescue the visual dysfunction with AAV-mediated gene replacement. To do so, we generated transgenic mice expressing this hinge region mutation alongside control mice expressing wildtype *hAipl1*, both expressed under the mouse *Crx* (*cone-rod homeobox*) promoter.
Materials and Methods

Animals

All experiments involving animals described in this study were approved by the Institutional Animal Care and Use Committee at West Virginia University (WVU). Mice were maintained under 12-h light / 12-h dark light cycles with food and water provided ad libitum.

Generation of transgenic hAipl1 mice

Full length hAipl1 was amplified from a human retinal cDNA library and engineered by polymerase chain reactions (PCR) with an N-terminal Flag epitope tag and cloned under the 2.3 kb mCrx promoter (Furukawa et al., 2002) using a mCrx-LacZ vector generously provided by Dr. Takahisa Furukawa (Osaka Bioscience Institute, Osaka, Japan). The P351Δ12 hAipl1 mutation was introduced through amplification with reverse primer 5’ CAGCTCTGCAGATGGTGCTGTGGGTGGCTCTGTGGATGACTGTGC 3’, introducing a 12 bp deletion at nucleotide position 1053-1064, and also cloned under the mCrx promoter. All PCR reactions were performed with Phusion GC DNA polymerase master mix (Finnymes, Thermo Scientific, Lafayette, CO, USA), following manufacturer instructions for cycling conditions and the sequence verified using Big Dye Terminator ready reaction kit (Perkin Elmer, Waltham, MA, USA). mCrx-hAipl1 (wildtype) and mCrx-P351Δ12 hAipl1 (mutant) was digested, purified using the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA), and injected into the pronuclei of oocytes from superovulated FVB/N females (WVU Transgenic Animal Core Facilities) followed by implantation into pseudopregnant females. Wildtype- and mutant P351Δ12 hAipl1 transgenic founders were identified through PCR primers amplifying an 750 bp fragment spanning the 3’ region of the mCrx promoter and 5’ region of hAipl1, using forward primer 5’ CTGGTTGCGAGGCAAGAGT 3’ and reverse primer 5’
GTCCGCTCCTCATCACATTT 3’. Founders were backcrossed into wildtype 129sv mice to overcome the Pde6brd1 mutation present in the FVB strain, and further backcrossed with Aipl1 knockout mice in a mixed C57Bl/129sv background described previously (Ramamurthy et al., 2004; Ku et al., 2011).

**Electroretinographic (ERG) analysis**

Mice were dark-adapted overnight prior to testing. Eyes were topically dilated with a 1:1 mixture of tropicamide: phenylephrine hydrochloride. For ERG testing, mice were placed on a heated platform with continuous flow of isoflurane anesthesia through a nose cone (1.5% isoflurane with 2.5 liters per minute (lpm) oxygen flow rate). A reference electrode was placed subcutaneously in the scalp. ERG responses were recorded from both eyes with silver wire electrodes placed on each cornea, with contact being made with a drop of hypromellose solution (2% hypromellose in PBS) (Gonioscopic Prism Solution, Wilson Ophthalmic, Mustang, OK, USA). Rod dominated responses were elicited in the dark with flashes of LED white light at increasing flash intensities. Light-adapted cone responses were elicited with white light flashes in the presence of a 30 cd/m² rod-desensitizing white background light. ERGs were performed on the UTAS Visual Diagnostic System with BigShot Ganzfeld with UBA-4200 amplifier and interface, and EMWIN 9.0.0 software (LKC Technologies, Gaithersburg, MD, USA).

**Optokinetic responses (OKR)**

Analysis of mouse optokinetic responses were examined using the OptoMotry system (CerebralMechanics, Inc., Lethbridge, Alberta, Canada). Briefly, the system consists of a square array of four computer monitors and an elevated platform on which a freely moving mouse is placed. The mouse is observed from above through a camera mounted at the top. The system was also adapted for measuring optokinetic responses at scotopic light levels, by surrounding the
platform with a large cylinder of neutral density filters (E-Colour #211 0.9 ND, Rosco Laboratories, Inc., Glendale, CA, USA), and observing the mouse using NightVision camera mode (Sony, San Diego, CA, USA) under a circular array of 6 infrared lights (OSRAM Opto Semiconductors, Inc., Sunnyvale, CA, USA), as described previously (Kolesnikov et al., 2010). The computer monitors form a virtual cylinder of rotating sine-wave vertical gratings which randomly rotates clockwise or counterclockwise as controlled by the OptoMotry computer program. Mice reflexively respond to the rotating gratings by turning their head in the corresponding direction of the grating. The observer registers the mouse behavior through the two-alternative forced-choice protocol (Umino et al., 2006), picking either clockwise or counterclockwise, and the computer program changes the grating spatial frequency (in the visual acuity mode) using the random staircase paradigm, until the threshold is reached at 70% of the trials (Umino et al., 2008). Visual acuity was defined as the threshold for spatial frequency of gratings and measured at the optimal speed (Sp) of 12.0 deg/s (Umino et al., 2008). In this mode, spatial frequency (Fs) was randomly changed by the computer protocol, starting from 0.042 cyc/deg, until threshold or maximal spatial frequency was determined. Temporal frequency (Ft) was automatically adjusted by the computer program based on the equation, Ft = Sp x Fs. Lastly, the OptoMotry apparatus can measure threshold spatial frequency from each eye independently, in a freely moving mouse without any suturing (Douglas et al., 2005). This is because the optokinetic tracking response is elicited when motion is in the temporal-to-nasal direction of the visual field. Thus, counterclockwise grating rotation will drive tracking through the right eye and elicit a counterclockwise optokinetic response; whereas clockwise grating rotation drives tracking through the left eye. This property of the apparatus was taken advantage of to
independently measure treated and untreated eyes in mice receiving AAV-mediated gene delivery.

**Immunoblotting, immunohistology, and morphometric analysis**

Mice were euthanized by CO₂ inhalation and eyes were enucleated. For immunoblots, flash frozen retinal samples dissected from enucleated eyes were sonicated in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, with protease inhibitors and 10 mg/ml dithiothreitol (DTT)). Protein concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). 150 µg total protein samples were resolved on a 8.7 cm long 10% polyacrylamide SDS-PAGE resolving gel (Criterion Midi format, Bio-Rad, Hercules, CA, USA), or on a longer format 16 cm long 9% polyacrylamide SDS-PAGE resolving gel where specified (Hoefer, Inc., San Francisco, CA, USA), and transferred onto polyvinylidene difluoride membranes (Immunobilon-FL, Millipore, Billerica, MA, USA). Membranes were blocked with blocking buffer (Rockland Inc., Gilbertsville, PA, USA) for 30 min at room temperature, and incubated with the indicated primary antibodies. Blots were washed in PBST (PBS with 0.1% Tween-20) twice for 20 min and incubated in secondary antibody, goat anti-rabbit or goat anti-mouse IRDye 680 (LI-COR Biosciences, Lincoln, NE, USA) for 30 min at room temperature. After 3 washes with PBST, membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and bands quantified using Odyssey Infrared Imaging System software.

For immunohistochemical studies, eyes were immersed in 4% paraformaldehyde fixative for 15 min prior to removal of the anterior segments and lens. Eyecups were fixed for an additional 2 h, washed in PBS for 20 min, incubated in 10% sucrose/PBS for 1 h at room temperature, and transferred to 20% sucrose/PBS for overnight incubation at 4°C. Eyes were
then incubated in 1:1 mixture of 20% sucrose in PBS:OCT (Cryo Optimal Cutting Temperature Compound, Sakura, Torrance, CA, USA) for 1 h, and flash frozen in OCT. Cryosectioning was performed with a Leica CM1850 Cryostat, and serial retinal sections of 14 µm thickness were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Retinal sections mounted on slides were washed with PBSTx (PBS with 0.1% TritonX-100), and incubated for 1 hr in blocking buffer (PBS with 5% goat sera, 0.5% TritonX-100, 0.05% sodium azide). Retinal sections were incubated with primary antibody for 2-3 hrs, followed by three 10 min washes with PBSTx before incubation with secondary antibody (Alexa Fluor-488 or Alexa-Fluor 568, Invitrogen, Carlesbad, CA, USA) for 30 min. The nuclear marker, 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen, 1:5000 dilution) was added for 10 min, and washed with PBSTx before mounting with Prolong anti-fade reagent (Invitrogen) and coverslipping. Slides were viewed and imaged on a laser scanning confocal (Zeiss LSM 510) on a Zeiss LSM AxioImager Z1 microscope.

The following primary antibodies were used at the indicated dilutions: Human AIPL1 (1:2500) (Ramamurthy et al., 2004), human and mouse AIPL1 (1:2500) (gift from Dr. Tiansen Li, National Eye Institute, Bethesda, MD), PDE6αβγ (MOE) (1:2000) (CytoSignal Inc., Irvine, CA, USA), PDE6β (1:1000) (Affinity BioReagents, Golden, CO, USA), cone PDEα’ (1:1000) (Kirschman et al., 2010), mouse cone arrestin (1:1000) (gift from Dr. Wolfgang Baehr, University of Utah, Salt Lake City, UT), guanylyl cyclase-E (GC-E) (gift from Dr. Baehr), red/green opsin (1:1000) (Chemicon, Millipore, Billerica, MA, USA). Rhodamine peanut agglutinin (PNA) (1:500) (Vector Laboratories, Inc., Burlingame, CA, USA) and DAPI (1:5000) (4’,6-diamidino-2-phenylindole, Invitrogen, Grand Island, NY, USA) were also used in immunocytochemistry.
Histologic, semithin sections, and transmission electron microscopy

For light and transmission electron microscopy, mice underwent transcardial fixation perfusion (Tissue Processing and Analysis Core, WVU) prior to retinal sample collection. Briefly, mice were deeply anesthetized with ketamine/xylazine (100 mg/kg ketamine, 10 mg/kg xylazine) prior to perfusion. The animal was perfused through the ascending aorta with a cannula attached to a perfusion pump (Harvard Apparatus, Holliston, MA, USA) with a pressure-relieving incision made in the right atrium. A vascular rinse was conducted prior to perfusion fixation (1% paraformaldehyde, 1.25% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.5). Enucleated eyes were further fixed (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.5) for 30 min prior to dissection and removal of the anterior segment and lens, and extensively fixed for 48 hours at room temperature. The fixed eyecup was dissected into 6-8 wedge-shaped pieces. Wedges were dehydrated in a graded ethanol series, then embedded in Polybed 812 (PolySciences, Inc., Warrington, PA, USA). Semi-thin (1 μm) sections were collected onto glass slides, stained with toluidine blue, and visualized using a Zeiss Axioimager 2 microscope equipped with EC Plan-Neofluar 40x (N.A. 0.75) and 100x (1.3 N.A.) objectives to identify best rescued areas. Thin sections (ca. 80 nm) from selected wedges were collected onto nickel grids, stained with 2% uranyl acetate and lead citrate, and imaged using an FEI Morgagni transmission electron microscope at 80 kV.

Generation of recombinant AAV vectors

Full-length human *Aipl1* was amplified from pAAV-RKp-\textit{hAipl1} plasmid (Sun et al., 2010) with primers that engineered flanking Kpn-I and Eag-I sites, and cloned into the scAAV packaging vector (University of Florida, Retinal Gene Therapy Group), to generate the sc-RKp-\textit{hAipl1} plasmid. All PCR amplifications were performed with Phusion GC DNA polymerase master mix.
(Finnymes, Thermo Scientific, Lafayette, CO, USA), following manufacturer instructions for cycling conditions. All plasmids were sequenced with the Big Dye Terminator ready reaction kit (Perkin Elmer, Waltham, MA, USA). The sc-RKp-hAipl1 plasmid was used to produce the self-complementary AAV8 Y733F surface-exposed tyrosine residue capsid mutant (scAAV2/8-Y733F-RKp-hAipl1) viral vector described previously (Ku et al., 2011) with a viral titer of $1.2 \times 10^{13}$ viral particles per mL.

**Subretinal injection**

Transgenic mice injected at postnatal day 10 (P10) and P15 were placed under general anesthesia with an intraperitoneal injection of ketamine (90 mg kg$^{-1}$) / xylazine (9 mg kg$^{-1}$) and received a drop of topical anesthetic, proparacaine hydrochloride (0.5%, Akorn, Lake Forest, IL, USA), followed by a drop of dilation solution, consisting of a 1:1 mixture of tropicamide (1%, Alcon, Fort Worth, TX, USA) : phenylephrine hydrochloride (2.5%, Bausch and Lomb, Tampa, FL, USA). For injection into mice prior to eyelid opening, an incision was made along the natural future eyelid line with surgical vannas scissors. A small incision was then made in the sclera just outside the pupillary margin with a 36-gauge needle. Viral solution was injected into the subretinal space using a pulled glass pipette attached to a pneumatic injector (MINJ-PD, Tritech Research, Los Angeles, CA, USA) through a transcorneal approach (Matsuda and Cepko 2004). Each animal received 1.25 µl of AAV in the right eye, and no injection in the untreated contralateral eye. Visualization during injection was aided by the addition of 0.1% fluorescein (100 mg ml$^{-1}$ AK-FLUOR, Alcon, Fort Worth, TX, USA) to the viral solutions.

**Statistical analyses**

Statistical analyses were conducted on GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA, USA), using two-tailed unpaired $t$-tests for two group comparisons, and ANOVA with
Bonferroni correction for multiple group comparisons.
Results

Generation of wildtype- and mutant P351Δ12 hAIPL1 mice

To examine the effects of the P351Δ12 hAIPL1 mutation, a 12 bp, 4 aa in-frame deletion in the C-terminal proline-rich region of hAIPL1 (Fig. 1A), transgenic P351Δ12 hAIPL1 mice were generated. A control transgenic line expressing wildtype hAIPL1 (designated WT hAIPL1) was generated alongside the transgenic mutant line. Both wildtype and mutant transgenes were driven by the 2.3 kb mcrx promoter (Fig. 1B), active beginning at embryonic day 12.5 (E12.5) in retinal progenitor cells (Furukawa et al., 2002). We chose the mcrx promoter due to its early activity and because it drives expression in both rod and cone photoreceptors (Furukawa et al., 2002), an important property since cone cell death occurs in the absence of Aipl1 in a previous transgenic model expressing hAIPL1 (Kirschman et al., 2010) driven by Nrl (neural retina-specific leucine zipper) (pNrl-hAIPL1), a known rod-specific promoter (Akimoto et al., 2006; Kirschman et al., 2010; Kautzmann et al., 2011). Transgenic WT hAIPL1 mice were backcrossed into mouse Aipl1-null mice to generate WT hAIPL1 (mAipl1+/−) (designated as WT hAIPL1 (+/-)) and WT hAIPL1 (mAipl1−/−) (designated as WT hAIPL1 (-/-)) littermates. The same breeding scheme was conducted with the experimental P351Δ12 hAIPL1 transgenic mice. Mice from both lines were normal, healthy, and fertile, with no gross morphological abnormalities. All comparisons subsequently made are between control WT hAIPL1 and experimental P351Δ12 hAIPL1 in a mAipl1-null background (WT hAIPL1 (mAipl1−/−) and P351Δ12 hAIPL1 (mAipl1−/−)) unless otherwise explicitly stated.

Protein expression of P351Δ12 hAIPL1 was comparable to WT hAIPL1 (Fig. 2). To note, the mobility of mutant P351Δ12 hAIPL1 was shifted, running slightly below WT hAIPL1 in immunoblots. Additionally, with the flag-epitope tag, both WT and P351Δ12 hAIPL1 run slightly
higher than non-tagged hAIPL1. Expression of wildtype and mutant hAIPL1 is greater than 50% of endogenous mAIPL1 levels, as quantified through the use of an AIPL1 antibody that recognizes human and mouse AIPL1 equally (gift from Dr. Tiansen Li). This is equivalent to mAIPL1 heterozygous mice (mAIPL1+/−) which show no visual dysfunction or retinal degeneration (Ramamurthy et al., 2004).

P351Δ12 hAIPL1 mice show drastically reduced photopic responses and reduced scotopic responses at an early age

Electroretinography (ERG) was performed at P15, shortly after eyelid opening in mice, to evaluate whether the P351Δ12 hAIPL1 mutation leads to visual deficits. P351Δ12 hAIPL1 (mAIPL1−/−) mice had markedly reduced photopic, cone-mediated ERG responses, of approximately 40% of ERG responses from WT hAIPL1 (mAIPL1−/−) mice (p < 0.0005 at all light intensity comparisons; log 0.4 WT hAIPL1 mean = 73.20 ± 6.96 µV, n=5; log 0.4 P351Δ12 hAIPL1 mean = 30.50 ± 4.94 µV, n=4; log 0.7 WT hAIPL1 mean = 95.80 ± 6.02 µV, n=5; log 0.7 P351Δ12 hAIPL1 mean = 42.50 ± 7.00 µV, n=4; log 0.9 WT hAIPL1 mean = 110.0 ± 6.00 µV, n=5; log 0.9 P351Δ12 hAIPL1 mean = 47.00 ± 10.31 µV, n=4) (Fig. 3A, C). Scotopic, rod-mediated ERG responses were also reduced, although less drastically, to approximately 50-70% of WT hAIPL1 ERG levels, with greater reduction at lower flash intensities (Fig. 3B, C) (p values as indicated, ranging from p < 0.05 to p < 0.0005). We therefore examined the intensity-response relationship from scotopic ERG measurements and found that rods from mutant P351Δ12 hAIPL1 mice had a higher half-saturating light intensity compared to WT hAIPL1 (Fig. 3D), indicating reduced sensitivity.
**P351Δ12 hAipl1 mice show accelerated decline of photopic responses and gradual decline of scotopic responses**

To evaluate whether the visual defect was progressive or stationary, we measured ERG responses in animals of increasing age. P351Δ12 hAipl1 mice showed a rapid decline in photopic cone-mediated ERG responses decreasing to approximately 10% by P60. The 10% of WT hAipl1 photopic response comes from several P351Δ12 hAipl1 mice that showed small phototopic responses, whereas the other half of the mutant mice had completely abolished photopic ERG responses. This trend was similar at P90-P120, resulting in approximately 5% of WT hAipl1 photopic response.

Scotopic, rod-mediated ERG responses also decreased albeit at a much slower rate (Fig. 4, dashed line). The largest decline in scotopic response in P351Δ12 hAipl1 mice was observed between P60 and P90, where 49% of WT hAipl1 response decreased to 37% and remained stable until P360 (P360 data not shown).

**P351Δ12 hAipl1 mice show decreased visual behavior**

To determine whether decreased ERG responses affected functional vision in mutant P351Δ12 hAipl1 mice, we performed behavioral tests based on the ability of the mouse to reflexively track computer-generated sinusoidal gratings (Douglas et al., 2005). Visual acuity is evaluated by measuring the threshold (maximal) spatial frequency of the gratings that elicits an optokinetic response from the mouse. Measurements are conducted under photopic and scotopic conditions (Kolesnikov et al., 2010). At P30, P351Δ12 hAipl1 mice showed significantly lower optokinetic responses under photopic conditions (Fig. 5A, photopic left) \( p = 0.0184 \); WT mean = 0.5339 ± 0.03541 cyc/deg, \( n=5 \); P351Δ12 mean = 0.3298 ± 0.0045 cyc/deg, \( n=2 \). Under
scotopic conditions, mutant mice showed a trend toward lower optokinetic responses than wildtype, however, a significant decrease was observed at only one scotopic background light intensity (Fig. 5A, scotopic right) \( (p = 0.036; \text{WT mean} = 0.4048 \pm 0.02497 \text{ cyc/deg, n=5}; \text{P351}\Delta12 \text{ mean} = 0.2882 \pm 0.022 \text{ cyc/deg, n=2}) \).

Functional vision further decreased at P60, where the maximal spatial frequency that P351\(\Delta12\) \(hAipl1\) mice respond to optokinetically was lower than that of WT \(hAipl1\) mice, under all scotopic background light intensities (Fig. 5B, scotopic right) \( (p\ \text{values as indicated, ranging from } p < 0.05 \text{ to } p < 0.0005) \). Although significantly decreased, some optokinetic response could still be elicited under scotopic conditions. This differed from measurements under photopic conditions, where mutant P351\(\Delta12\) \(hAipl1\) mice showed no reliable optokinetic response (Fig. 5B, photopic left) \( (p = 0.0006; \text{WT mean} = 0.4087 \pm 0.04430 \text{ cyc/deg, n=6}; \text{P351}\Delta12 \text{ mean} = 0.1052 \pm 0.0004861 \text{ cyc/deg, n=4}) \). Additionally, we confirmed that photopic conditions used were specifically measuring cone-mediated visual behavior with \(cpfl1\) mutant mice (generously provided Dr. Bo Chang, Jackson Labs), which lack photopic ERG responses due to a spontaneous mutation in cone phosphodiesterase 6 (cPDE6). Adult \(cpfl1\) mice showed no reliable optokinetic response under photopic conditions (data not shown).

**Cone photoreceptor cell death and cone outer segment disorganization in P351\(\Delta12\) \(hAipl1\) mice.**

Because of the observed defects in photopic vision, we examined cone outer segment morphology and cone photoreceptor cell death. At P17, early photoreceptor cell death was observed in retinal sections from P351\(\Delta12\) \(hAipl1\) mice through propidium iodide (Fig. 6A, propidium iodide red), where strong staining indicate dead cells. A majority of dead cells were
located at the top edge of the outer nuclear layer (ONL) where cone cell nuclei typically reside. Some propidium iodide positive cells co-localized with red/green opsin (Fig. 6A, green), whereas non-co-localizing dead cells likely indicates blue cone cell death. Despite the observed cone cell death at P17, remaining cones showed intact cone outer segments of relatively comparable length to WT hAipl1 cones, as indicated by R/G opsin staining (Fig. 6A, R/G opsin, green, and Supplemental Fig. 1). However, by P35, cone arrestin staining (Fig. 6B, cArr red) showed cone outer segment shortening (white arrows indicate cone outer segments).

**P351Δ12 hAipl1 mice show decreased cone phosphodiesterase 6 and rod transducin α**

Through immunocytochemistry, we observed that mutant P351Δ12 hAipl1 mice show decreased expression of cone PDEα’ early on at P15 (Fig. 7A), which was confirmed with semi-quantitative immunoblotting (Fig. 7B). This decrease was specific to cone PDEα’, as levels of cone transducin α, another cone outer segment protein, were similar in mutant P351Δ12 compared to wildtype hAipl1 mice (Fig. 7B). There is a possible decrease in GC-E in cones as observed through immunocytochemistry at P15 (data not shown) and P35 (Fig. 4B); however, this was not observed in immunoblots (Fig. 7B) due to the expression of GC-E in both rods and cones, where 97% of photoreceptors are rods in the mouse retina. Lastly, we observed a slight decrease in levels of rod transducin α (Fig. 7B), which may account for the decreased rod sensitivity and defects in rod-mediated vision observed at P15 (Fig. 2B, C, D).

**Gene delivery of WT hAipl1 into mutant P351Δ12 hAipl1 mice**

To address whether the P351Δ12 hAipl1 mutation acts in a dominant fashion, we introduced WT hAipl1 into mutant mice through two means. First, we generated double
transgenic mice by crossing WT hAipl1 with P351Δ12 hAipl1 mice, which were both flag-tagged
at the N-terminus of AIPL1 (Fig. 1B). Double transgenic mice which co-express WT and
P351Δ12 hAIPL1 (blue and red waveforms) continue to show a defect in cone-mediated ERG
responses, as compared to a littermate expressing WT hAIPL1 (black waveforms) (Fig. 8A, B).
Rod-mediated scotopic ERG responses were also decreased in double transgenic mice as
compared to their WT hAipl1 littermate, although ERG responses were slightly greater than
previously observed in single transgenic P351Δ12 hAipl1 mice. However, a direct comparison
was not possible as no single transgenic P351Δ12 hAipl1 mice were found in the litters analyzed.
This may be explained by the observed protein levels where, in our single transgenic mice,
mutant P351Δ12 hAIPL1 was slightly less (< 2-fold) than WT hAIPL1 in each respective
transgenic line, whereas the amount of P351Δ12 hAIPL1 was approximately 5-fold less than the
co-expressed WT hAIPL1 in double transgenic positive mice.

The second method expressed a non-tagged WT hAIPL1 via AAV-mediated gene
delivery into transgenic mutant P351Δ12 hAipl1 mice, using a previously published scAAV8-
Y733F-RKp-hAipl1 viral vector (Ku et al., 2011). We first administered gene delivery at P15 and
observed rescue of photopic cone-mediated ERG responses (p = 0.0401; treated mean = 30.67 ±
3.844 µV, n=3; untreated mean = 6.33 ± 6.33 µV, n=3), but not scotopic rod-mediated responses.
We then administered gene delivery at P10 to ensure that lack of rod-mediated rescue was not
due to early rod photoreceptor loss. However, P10 gene delivery of WT hAipl1 also did not
improve rod-mediated ERG responses, although cone-mediated rescue was improved by earlier
gene delivery at P10 over P15 (p = 0.0235; P10 treated mean = 62.0 ± 8.386 µV, n=3; P15
treated mean = 30.67 ± 3.844 µV, n=3) (Fig. 9A, B). Rescue effects on cone-mediated ERG
responses following one AAV treatment administered either at P10 or P15 were observed until
P130, the last tested age. Additionally, photopic ERG responses in rescued eyes did not change longitudinally between P30, the first ERG testing date, and P130, the last ERG age tested. Cone-mediated visual behavior as measured through optokinetic responses was greater in treated eyes as compared to untreated eyes (p = 0.0105; treated eye mean = 0.4278 ± 0.09206 cyc/deg, n=4; untreated eye mean = 0.09625 ± 0.06172 cyc/deg, n=4) (Fig. 10).

To resolve the difference we see in introduction of WT hAIPL1 through AAV-mediated gene delivery as compared to genetic manipulation, protein expression of WT hAIPL1 was compared between the two methods. We observed a drastically greater expression of WT hAIPL1 in AAV-treated eyes as compared to double transgenic mice (Fig. 11). Although the WT hAIPL1 band could not be fully resolved from the Flag P351Δ12 hAIPL1 band in AAV-treated eyes, we estimate (non-tagged) WT hAIPL1 to be in at least 25-fold excess of mutant P351Δ12 hAIPL1, whereas Flag WT hAIPL1 in double transgenic mice are about 5-fold excess of Flag P351Δ12 hAIPL1 (Fig. 11).
Discussion

Our studies demonstrate that P351Δ12 hAipl1, a rare mutation reported in two probands, is associated with retinal degeneration of a CORD phenotype. The patients, however, had discordant diagnoses; one with CORD and the other with juvenile RP, although both were reported to have likely inherited the mutation in an autosomal dominant manner (Sohocki et al., 2000b). Our transgenic P351Δ12 hAipl1 mouse model shows an early loss of cone-mediated vision and subsequent cone photoreceptor degeneration. We attribute cone defects to decreased cone PDE6α’, which despite lower expression levels, appeared to localize correctly prior to later cone outer segment disorganization and degeneration. Additionally, we observed decreased expression of guanylyl cyclase-E in cone photoreceptors through immunohistochemistry; however, due to the expression of GC-E in both rods and cones and the overwhelming number of rods in retina compared to cones, we were unable to confirm this in semi-quantitative immunoblots. Additionally, mutant mice showed early subtle defects in rod-mediated vision, particularly with decreased sensitivity, which may be associated with the observed decrease in rod transducin α. Despite this finding however, rod-mediated vision decreased at a very slow rate, plateauing around P120.

Interestingly, through our double transgenic experiments, we observed that the P351Δ12 hAipl1 mutation acted in a dominant fashion in that cone-mediated vision could not be rescued with genetic re-introduction of WT hAipl1. Expression levels of WT hAIPL1 and P351Δ12 hAIPL1 in double transgenic positive mice were relatively comparable. This mimicks a heterozygous state of P351Δ12 hAipl1, similar to what would occur in the reported P351Δ12-CORD and RP patients. Therefore, our mouse model which shows a CORD phenotype, confirms the dysfunction associated with the P351Δ12 hAipl1 mutation even in a heterozygous
state. The varying phenotypes between patients with this mutation remains unresolved, however, we speculate that it may be due to differences in ratios of WT to mutant AIPL1 protein in rods and cones.

Despite the autosomal dominant inheritance pattern in patients and the dominant-like phenotype observed in our double transgenic animals, cone-mediated vision was improved following AAV-mediated gene replacement of WT hAipl1 in mutant P351Δ12 hAipl1 mice. This is likely due to the high protein expression of WT hAIPL1 achieved in treated animals. Treated mutant mice however, did not show any appreciable improvement in scotopic rod-mediated vision, despite the overwhelming amount of WT hAIPL1 protein expressed. This may be due to local retinal damage from AAV injections that negates any improvement that can be seen overall. Alternatively, it may suggest that the P351Δ12 hAIPL1 acts in a dominant negative fashion which cannot be overcome with excess WT hAIPL1 in rods, and implicates a difference in Aipl1 function in rods and cones that may be attributable to the AIPL1 hinge region.

The function of the AIPL1 hinge region remains in question; however, our model clearly shows detrimental effects of mutations in this region. It is possible that the dominant negative effects of the P351Δ12 hAipl1 mutation are exerted through this C-terminal primate-specific region, as transgenic positive P351Δ12 hAipl1 in a mAipl1 heterozygous background (P351Δ12 hAipl1 (mAipl1+/−)) show no visual dysfunction (Supplementary Fig. 2). However, whether the dominant negative effects stem from a negative interaction between wildtype and P351Δ12 hAIPL1, or alternatively, mutant P351Δ12 hAIPL1 negatively interacting with other essential photoreceptor proteins remains to be determined.
Multiple pre-clinical AAV-mediated rescue studies of defects in *aryl hydrocarbon receptor interacting protein like-1* (*Aipl1*) have been recently conducted on previously reported *Aipl1* mouse models (Tan et al., 2009; Sun et al., 2010; Ku et al., 2011). *Aipl1*-null mice which show the most severe and rapidly progressing degeneration of all retinal dystrophy models, mimicks truncation mutation W278X, the most prevalent mutation in *Aipl1*-LCA patients (Sohocki et al., 2000a; Sohocki et al., 2000b; Dharmaraj et al., 2004; Testa et al., 2011). Successful rescue of *Aipl1*-null mice provided proof-of-principle demonstrating that if the most severe of retinal degenerations is rescued, other *Aipl1* defects may potentially be treated (Tan et al., 2009; Sun et al., 2010; Ku et al., 2011). However, the practical clinical target would likely be a less severely implicated *Aipl1* mutation. Our novel transgenic P351Δ12 *hAipl1* mouse offers a model to study *Aipl1*-CORD patients, and while these patients are rare, may benefit from gene replacement therapy.
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Figures

Fig. 1

(A) The P351Δ12 hAIPL1 mutation interrupts a putative PxxP motif in the C-terminal proline-rich hinge region of hAIPL1. (A) The 12 bp deletion (blue arrow, underline, and box) results in a 4 amino-acid in-frame deletion, eliminating 1 of 5 potential PxxP motifs (black underline) present in the C-terminal proline-rich, primate-specific region (grey box) of hAIpl1. (B) Transgenic mice were generated expressing either N-terminal Flag-tagged wildtype hAIpl1 (control transgenic line) or Flag-tagged P351Δ12 hAIpl1 (experimental line) under the 2.3 kb mCrx promoter.

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Fig. 2. Expression of Flag-P351Δ12 hAIPL1 is comparable to Flag-wildtype (WT) hAIPL1.

Immunoblots of P10 retinal lysates from transgenic WT \textit{hAipl1} and P351Δ12 \textit{hAipl1} mice probed with anti-AIPL1 that recognizes both mouse and human AIPL1 equally (gift from Dr. Tiansen Li). Equal amounts of protein were loaded for each sample (150 \(\mu\)g). mAIP1: mouse AIPL1, hAIPL1: human AIPL1, Tg: transgenic hAIPL1.
Fig. 3. Mice with \textit{P351\Delta12 hAipl1} mutation show an early dramatic decrease in photopic vision and defects in scotopic vision. Families of representative ERG responses from postnatal day 15 (P15) wildtype \textit{hAipl1} and mutant \textit{P351\Delta12 hAipl1} mice, as recorded under (A) photopic and (B) scotopic conditions at indicated log intensity (cd \cdot s / m^2) flashes. (C) Comparison of mean cone and rod photoreceptor responses (\textit{a}-wave amplitudes) between WT (\textit{white bar}; \textit{n}=5) and \textit{P351\Delta12 hAipl1} (\textit{grey bar}; \textit{n}=4). Average \textit{a}-wave amplitudes (\pm SEM) from WT \textit{hAipl1} mice were significantly different from \textit{P351\Delta12 hAipl1} mice at all photopic and scotopic flash at indicated scotopic flash intensities. \textit{P} values are indicated as follows: *\textit{P}<0.05, **\textit{P}<0.005, ***\textit{P}<0.0005. (D) Intensity-response relationship for scotopic \textit{a}-wave amplitude, where data was fitted with hyperbolic function (Fulton and Rushton 1978), \textbf{A} = A_{\text{max}} \left( \frac{l^n}{l^n + l_h^n} \right),
where $A_{\text{max}}$ is the maximal $a$-wave amplitude, $n$ is the Hill coefficient, and $I_h$ is the half-saturating light intensity. This curve fit yielded scotopic $a$-wave half-saturating light intensities of $0.03346 \pm 0.003360 \text{ cd} \cdot \text{s/ m}^2$ (WT $hAip1l$, $n=5$) and $0.05774 \pm 0.004798 \text{ cd} \cdot \text{s/ m}^2$ (P351Δ12 $hAip1l$, $n=4$). Data points are mean ± SEM.
Fig. 4. Accelerated loss of photopic ERG responses with slower decline in scotopic ERG responses in P351Δ12 hAipl1 mutant mice. ERG responses over time (from P15 to P120), as expressed as the ratio of (P351Δ12 hAipl1 / WT hAipl1) a-wave amplitudes, obtained from measurements taken with 2.5x10^{-2} cd · s / m² flashes under scotopic, rod-mediated conditions (dashed line) and 2.5 cd · s / m² flashes under photopic, cone-mediated conditions (solid line) (P15, n=4; P30, n=6; P60, n=5; P90, n=5; P120, n=5).
Fig. 5. P351Δ12 hAipl1 mice show impaired visual function. Mean threshold spatial frequencies (cyc/deg) of WT (n=5) and P351Δ12 hAipl1 (n=2) mice, obtained using OptoMotry, a virtual optokinetic stimuli apparatus (CerebralMechanics, Inc.) at (A) postnatal day 30 (P30), and (B) postnatal day 60 (P60). Multiple measurements were obtained across the indicated background light intensities under scotopic conditions. Bars and data points are mean ± SEM values. P values are indicated as follows: *P < 0.05, ** P < 0.005, ***P < 0.0005.
Fig. 6. Cone degeneration and cone outer segment shortening in mice with P351Δ12 hAipl1 mutation. Immunocytochemistry using indicated antibodies on retinal sections from WT hAipl1 and P351Δ12 hAipl1 mice. (A) Propidium iodide (red) was used as a marker of cell death,
showing photoreceptor cell death along cone nuclei stained with red/green (R/G) opsin (green) in retinal sections from postnatal day 17 (P17) mutant P351Δ12 hAipl1 (bottom two rows) but not WT hAipl1 (top row) mice. (B) Rod and cone outer segments are localized with guanylate cyclase (GCE, green) with specific localization of cone outer segments (white arrowheads) using the cone arrestin antibody (cArr, red) in postnatal day 35 (P35) WT hAipl1 (top row) and P351Δ12 hAipl1 mutant mice (bottom two rows) retinal sections. Cell nuclei are stained with DAPI (4’,6-diamidino-2-phenylindole, blue). COS: cone outer segment, CIS: cone inner segment, ONL: outer nuclear layer. Scale bar, 20 μm.
Fig. 7 P351Δ12 hAipl1 mutant mice show decreased cone phosphodiesterase 6. (A)

Immunostaining of retinal sections from P15 (top two rows) and P35 (bottom two rows) WT hAipl1 and P351Δ12 hAipl1 mice with antibody for cone phosphodiesterase α’ subunit (cPDE6α’, green), co-stained with cone marker, peanut agglutinin (PNA, red). Cell nuclei are stained with DAPI (4’,6-diamidino-2-phenylindole, blue). Scale bar, 20 µm. COS, cone outer
segment; CIS, cone inner segment; ONL, outer nuclear layer. (B) Immunoblots of retinal lysates from P35 WT hAipl1 and P35Δ12 hAipl1 mice probed with the indicated antibodies against cone photoreceptor proteins, cone PDE α’ and cone transducin α; and rod proteins, rod transducin α. Guanylyl cyclase (GC-E) is present in both cones and rods.
Fig. 8. Double transgenic mice expressing WT and P351Δ12 hAIPL1 show defects in cone-mediated vision. P351Δ12 hAipl1 mice were crossed with WT hAipl1 mice to generate offspring which are potentially double transgenic positive. (A) Co-expression of WT and mutant P351Δ12 hAIPL1 was evaluated by extensively running a 9% polyacrylamide SDS-PAGE resolving gel of 16 cm height. Controls (Control: Flag WT and Flag Mut lanes), are run on the same gel to show the expected size differences. (B) ERG responses from the corresponding individual mice shown in the immunoblot. Photopic and scotopic responses from a mouse expressing Flag WT hAIPL1 (Flag WT lane in immunoblot; black ERG waveforms), and two independent mice co-expressing Flag-WT and Flag-P351Δ12 hAIPL1 (2xFlag Tg 1, 2 in immunoblot; blue and red ERG waveforms).
Fig. 9. WT hAipl1 gene replacement into mutant P351Δ12 hAipl1 mice rescued cone- but not rod-mediated ERG responses. AAV-mediated gene delivery using scAAV8-hRK-hAipl1 viral vector, which introduced non-tagged WT hAipl1 into mutant P351Δ12 hAipl1 mice administered either at P10 or P15. Treated eyes showed improved (A) cone-mediated ERG responses, but not (B) rod-mediated ERG responses, when compared directly to its untreated eye. Measurements shown were obtained at P30 with 2.5 cd·s/m² flashes with 30 cd/m² rod-desensitizing white background light for photopic ERG responses, and 2.5x10⁻² cd·s/m² flashes for scotopic ERG responses. Bars and data points are mean ± SEM values. P values are indicated as follows: *P < 0.05, ** P < 0.005.
Fig. 10. Treated P351Δ12 hAipl1 mice showed cone-mediated visual function. Threshold spatial frequencies (cyc/deg) as measured using OptoMotry, from mutant P351Δ12 hAipl1 mice that received AAV-mediated gene delivery of WT hAipl1 in one eye (treated) whereas the opposite eye remained untreated (n=4). Data points are individual threshold spatial frequency values, with connecting lines indicating the untreated and treated eye from the same mouse. Bars show mean ± SEM values (cyc/deg) of untreated and treated groups respectively, which were significantly different (*P < 0.05).
Fig. 11

**Fig. 11. Gene introduction through AAV-mediated gene delivery shows drastically higher expression of WT hAipl1 than in double transgenic animals.** To differentiate flag-tagged WT, flag-tagged mutant P351Δ12 hAIPL1, and non-tagged WT hAIPL1, we extensively ran a 9% polyacrylamide SDS-PAGE resolving gel of 16 cm height. Controls are run on the same gel to show the expected size differences. Flag-tagged WT hAIPL1 (*Control: Flag WT* lane), flag-tagged P351Δ12 hAIPL1 (*Control: Flag Mut* lane), and non-tagged WT hAIPL1 (*Control: No tag WT* lane), show slight band shifts from highest to lowest in immunoblots, respectively. The control non-tagged WT hAIPL1 lane is from retinal lysate of *pNrl-hAipl1* transgenic mice (Kirschman et al., 2010). Double transgenic animals were generated by crossing Flag-WT *hAipl1* mice with Flag-P351Δ12 *hAipl1* mice, and retinal lysate from three separate animals are shown (*2xFlag Tg* lanes). Flag-P351Δ12 *hAipl1* mice were administered AAV-mediated gene delivery of non-tagged WT *hAipl1*, using a scAAV8-Y733F-*hAipl1* viral vector (Ku et al., 2011), and retinal lysate from three separate animals are shown (*AAV-treated 1, 2, 3* lanes). Retinal lysate from double transgenic mice were age-matched to AAV-treated mice aged P130, and equal protein (150 µg) was loaded for all lanes.
Supplementary Fig. 1. Cone outer segments are of relatively normal length at early ages in P351Δ12 hAipl1 mice. Immunostaining of retinal sections from P15 WT hAipl1 and P351Δ12 hAipl1 mice with red/green opsin (green) and peanut agglutinin (PNA, red) showing cone outer segments. Cell nuclei are stained with DAPI (4’,6-diamidino-2-phenylindole, blue). Scale bar, 20 µm. COS, cone outer segment; CIS, cone inner segment; ONL, outer nuclear layer.
**Supplementary Fig. 2**

Supplementary Fig. 2. Mice positive for P351Δ12 hAipl1 transgene in a mAipl1+/− background do not show visual dysfunction. (A) Photopic, cone-mediated, and (B) scotopic, rod-mediated ERG responses of mutant P351Δ12 hAipl1 in a mAipl1+/− background (P351Δ12 hAIP1+ mAIP1(+/−), *middle column*) mice are similar to control transgene negative mAipl1+/− mice (mAIP1(+/−), *left column*), in contrast to the absent photopic response and decreased scotopic responses from mutant P351Δ12 hAipl1 in a mAipl1−/− background (P351Δ12 hAIP1+ mAIP1(+/−), *right column*) mice, even at older ages tested (P170 shown). Photopic and scotopic measurements are taken with the indicated log intensity (cd · s / m²) flashes.
Chapter 4

General Discussion, Conclusion, and Future Work

Study 1: Gene therapy for Aipl1-null mice, a model of severe Leber congenital amaurosis

Recent advancements in AAV viral vectors were developed to treat retinal degeneration more rapid and severe than that seen with Rpe65-LCA. Developments in self-complementary AAV and viral capsid mutations demonstrated faster onset and greater transduction efficiency with fluorescent reporters (Natkunarajah et al., 2008; Zhong et al., 2008b; Petersen-Jones et al., 2009; Petrs-Silva et al., 2009; Kong et al., 2010; Petrs-Silva et al., 2011). We wanted to examine whether these improvements translate into functional benefits. In Aipl1-null mice, the most severe mouse model of retinal degeneration, we evaluated whether the combined self-complementary Y733F capsid mutant AAV2/8 (referred to as scAAV) viral vector, would enhance rescue over single-stranded AAV2/8 (referred to as ssAAV), the most efficient vector for photoreceptor targeting at that time. We observed clear functional benefits of using scAAV over ssAAV, particularly when treatment was administered later in disease progression at postnatal day 10 (P10) when photoreceptor cell death initiates in Aipl1-null mice. Early intervention at P2 also provided significantly greater ERG levels and morphology in scAAV-treated as compared to ssAAV-treated, although the difference was not to the same degree as that observed following P10 treatment (Ku et al., 2011).

Since our studies, AAV8-Y733F capsid mutant has been used to successfully rescue vision in other photoreceptor gene defects, Gucy2e (Boye, S.L. et al., 2011), Pde6brd10 (Pang et al., 2011), and Pde6anmf363 (Wert et al., 2012). Rescue studies of Gucy2e-null mice demonstrated functional benefits in cone- and rod-mediated ERG responses with AAV8-Y733F
over AAV5 viral vector (Boye, S.L. et al., 2011). However, it is unpredictable whether this effect would still hold up in a comparison with AAV8, since AAV8 was previously shown to be superior to AAV5 in rapid photoreceptor transduction (Allocca et al., 2007; Lebherz et al., 2008; Sun et al., 2010). The comparison between ssAAV8 and ssAAV8-Y733F would have been an important comparison since our studies had used a combined self-complementary Y733F capsid mutant viral vector, a development that cannot always be taken advantage of with the size limitation of scAAV. To our knowledge, no other AAV-mediated gene rescue studies for inherited retinal degeneration have compared the rescue efficacy achieved between viral vectors.

*Aipl1*-null mice offer a unique model to test the functional utility of future developments in viral vector efficiency as new advancements emerge, such as viral particles with multiple surface capsid mutations (Petrs-Silva et al., 2011; Ryals et al., 2011). Lastly, successful rescue of *Aipl1*-null mice demonstrated independently by multiple groups (Tan et al., 2009; Sun et al., 2010; Ku et al., 2011) provides proof-of-concept that even the most severe of retinal degenerations can be rescued if AAV-mediated gene replacement is provided prior to significant photoreceptor cell loss.

**Study 2: P351\(\Delta\)12 *Aipl1* mutation: a model of cone-rod dystrophy**

While *Aipl1* is mainly linked to LCA, *Aipl1*-CORD and -RP patients may more greatly benefit from AAV-mediated gene replacement since disease progression is prolonged in CORD and RP as compared to LCA. The P351\(\Delta\)12 *hAipl1* mutation has been associated with CORD and RP in two probands. We were interested in this mutation not for the sole purpose of pinpointing one *Aipl1* mutation to target with gene therapy, as this would make up a minority of *Aipl1* patients even if found in additional cases. Instead, the P351\(\Delta\)12 mutation brings up additional
questions, which we sought to answer by creating a transgenic mouse model expressing the mutant P351Δ12 hAipl1 protein.

Apart from the W278X truncation mutation shown to translate an unstable protein, it is unclear why some Aipl1 mutations cause LCA compared to other mutations causing less severe IRDs. While Aipl1-null mice phenocopies severe LCA and Aipl1-hypomorph mice phenocopies a very slowly progressing RP, there is no Aipl1 model that phenocopies CORD. With the association of P351Δ12 hAipl1 to CORD, this transgenic mouse model may offer some insight to the role of hAipl1 in cones. The P351Δ12 hAipl1 is also associated with autosomal dominant disease which we thought a priori would preclude it from benefitting from AAV-mediated gene replacement therapy. However, the autosomal dominant inheritance pattern brought up interesting possibilities in examining abnormal and normal interacting protein partners of hAipl1, since it signifies a possible dominant negative effect of the mutation.

We investigated the phenotype of mice expressing mutant P351Δ12 hAipl1 and observed visual defects mimicking cone-rod dystrophy (CORD). This is in agreement with a reported P351Δ12 hAipl1 patient, although a second patient had a discordant diagnosis of juvenile RP (Sohocki et al., 2000b). In our transgenic P351Δ12 hAipl1-CORD model, we attribute the observed cone defects to decreased cone phosphodiesterase 6 (cPDE6). In addition to dramatic cone defects, we observed early defects at postnatal day 15 in rod-mediated ERG responses and subsequent rod cell loss, which we attribute to decreased rod transducin. A reduction in rod transducin would produce subsequent defects in signal transduction, which is reflected in our observations of reduced sensitivity in rod-mediated vision in P351Δ12 hAipl1 mice.
P351Δ12 hAipl1 was specifically linked with autosomal dominant retinal degeneration, which has not been otherwise observed with Aipl1 defects. We confirmed autosomal dominant disease associated with the P351Δ12 mutation through double transgenic mice co-expressing WT and mutant P351Δ12 hAipl1, which continued to show cone and rod visual defects. However, drastic overexpression of WT hAipl1 with AAV-mediated gene delivery into P351Δ12 hAipl1 mice rescued cone, although not rod, defects. Further work is needed to examine the mechanism of dominant dysfunction with P351Δ12 hAipl1, which may differ in cones and rods as suggested by these rescue studies.

This also implicates that the normal role of Aipl1 differs in cones and rods, a question that is currently being investigated in a ‘cone-only’ retina lacking mAipl1 (Nrl⁻/⁻/Aipl1⁻/⁻ double knockout mice) (Kolandaivelu et al., manuscript in preparation). Nrl (neural retina-specific leucine zipper) is an important transcription factor specifying rod photoreceptor differentiation, therefore, all photoreceptors develop into cone-like cells in Nrl⁻/⁻ mice. In Nrl⁻/⁻/Aipl1⁻/⁻ mice, the lack of mAipl1 in cones leads to de-stabilized cPDE6 observed in pulse-chase experiments and a subsequent dramatic decrease in cPDE6 levels (Kolandaivelu et al., manuscript in preparation). These studies affirm the role of Aipl1 in cPDE6, but does not confirm nor exclude a dominant negative effect of the P351Δ12 hAipl1 mutation on cPDE6. However, P351Δ12 hAipl1 dysfunction in cones could potentially be examined through a comparison of interacting partners between Flag-WT hAipl1 and Flag-P351Δ12 hAipl1 in an Nrl⁻/⁻/Aipl1⁻/⁻ background in future studies.

In regards to the rod dysfunction observed in P351Δ12 hAipl1 mice, decreased rod transducin may stem from an active dominant negative effect of mutant P351Δ12 hAipl1 on this essential phototransduction protein. An alternative explanation is that mutant P351Δ12 hAipl1
shows lower stability or activity than WT hAIPL1, resulting in subpar functioning of AIPL1. This theoretical possibility would also have to assume functional differences between human and mouse AIPL1, since mAipl1-hypomorphic mice expressing 20-25% of normal mAIPL1 levels do not show defects in rod transducin (Liu et al., 2004).

A last question that remains to be examined is the role of the C-terminal primate-specific region in human AIPL1. In addition to ensuring that mutant P351Δ12 hAIPL1 effects were not due to mCrx promoter insufficiencies, another reason we generated a control WT hAipl1 transgene line was to examine interacting partners of human AIPL1 using the N-terminal flag-epitope tag. Initial attempts at flag co-immunoprecipitation were unsuccessful as background binding to flag agarose beads was high, however, use of flag magnetic beads may yield cleaner results. Although we were unable to directly answer this question in our current studies, our findings clearly indicate that mutations in this region lead to visual dysfunction and degeneration. Altogether, we conclude that our transgenic P351Δ12 hAipl1 mouse offers a novel model for Aipl1-CORD, which can be further used to evaluate the mechanism of dominant dysfunction, and that our WT hAipl1 line can be further used to examine the normal role of hAipl1 in cones.

**Summary:**

Our work demonstrates that visual dysfunction associated with Aipl1 defects can be significantly improved with AAV-mediated gene therapy, in both Aipl1-null mice modeling Aipl1-LCA and transgenic P351Δ12 hAipl1 mice modeling Aipl1-CORD. Although our work confirms that dominant disease is likely with a P351Δ12 mutation in a heterozygous state, overexpression of WT hAIPL1 with AAV-mediated therapy can rescue cone defects, and if
directly targeted to the macula, AAV treatment would still drastically help maintain visual function in patients. These studies indicate Aipl1 as a potential candidate for future AAV gene therapy clinical trials, provided that early intervention is administered to a patient with a slower progressing disease. Additionally, Aipl1 is small enough to be packaged within a self-complementary AAV vector with extremely rapid and efficient transduction, which provides an advantage of targeting Aipl1 over other genes with a similar predicted rapid progression of retinal degeneration.
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