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ARTICLE

Specific tools for targeting and expression in Müller glial cells

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Despite their physiological roles, Müller glial cells are involved directly or indirectly in retinal disease pathogenesis and are an interesting target for therapeutic approaches for retinal diseases and regeneration such as *CRB1* inherited retinal dystrophies. In this study, we characterized the efficiency of adeno-associated virus (AAV) capsid variants and different promoters to drive protein expression in Müller glial cells. ShH10Y and AAV9 were the most powerful capsids to infect mouse Müller glial cells. Retinaldehyde-binding protein 1 (RLBP1) promoter was the most powerful promoter to transduce Müller glial cells. ShH10Y capsids and RLBP1 promoter targeted human Müller glial cells *in vitro*. We also developed and tested smaller promoters to express the large *CRB1* gene via AAV vectors. Minimal cytomegalovirus (CMV) promoter allowed expression of full-length CRB1 protein in Müller glial cells. In summary, ShH10Y and AAV9 capsids, and RLBP1 or minimal CMV promoters are of interest as specific tools to target and express in mouse or human Müller glial cells.

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INTRODUCTION

During vertebrate retina development, from a common pool of retinal progenitor cells, six types of neurons and the Müller glial cells are generated. While their somas reside in the inner nuclear layer, Müller glial cells extend from their basal end feet facing the vitreous and forming the inner limiting membrane, to their apical microvilli in the subretinal space. They provide structural, nutritional, homeostatic, osmotic, metabolic, and growth factor support to all six types of neurons and interact with photoreceptor cells to establish adherens junctions at the outer limiting membrane.[1,2] Despite their active role in the retinal signaling activity of neurons, Müller glial cells interact with the retinal blood vessels that participate in the establishment of the blood–retina barrier and regulate the retinal blood flow and are directly involved in the vision cycle of photopigments via the expression of the retinaldehyde-binding protein 1 (RLBP1).[1,3] In the last two decades, Müller glial cells are shown to reside as potential retinal progenitor cells in the adult retina.[2,4,5] In zebrafish and chick, following injury, the Müller glial cells can dedifferentiate, proliferate, and give rise to the six types of neurons to replace the lost neurons in the damaged area, whereas in mammals, this potency is limited.

Müller glial cells play a neuroprotective and/or deleterious role in response to retinal injury, stress, or degeneration via active gliosis.[1] Many diseases such as diabetes, macular edema, proliferative vitreoretinopathies, or ischemia directly affect their physiology leading to swelling, to proliferation, and eventually to death of the Müller glial cells. Most of the genes causing inherited retinal dystrophies affect mainly photoreceptor cells, but also Müller glial and retinal pigment epithelium (RPE). Mutations in the *RLBP1* and *CRB1* genes expressed in Müller glial cells have been reported to cause retinal dystrophies.[6–11] Therefore, Müller glial cells are an interesting target for new therapeutic approaches for retinal disease and regeneration *in vivo*.

Adeno-associated virus (AAV) vectors are the most powerful and safest viral vectors as over 20 approved clinical trials are ongoing in humans and also for retinal gene transfer.[12] Previously, we and others have shown that AAV6 capsids are the most efficient variant to drive expression in murine Müller glial cells via the intravitreal route.[13,14] AAV capsids have been engineered to enhance glial gene delivery, and the AAV6-derived capsid variant ShH10 was shown to transduce mainly Müller glial cells in the rat retina.[15,16] ShH10 viral vector-mediated–specific secretion of the growth factor glial cell-derived neurotrophic factor from Müller glial cells delays photoreceptor degeneration in a rat model of retinal dystrophy.[17] Selective loss of Müller glial cells using an optogenetic photo-inducible toxic protein leads to structural and retinal activity impairment in wild-type mice.[18] Using lentiviral vectors, several ubiquitous (cytomegalovirus (CMV), CAG) and specific (CD44, glial fibrillary acidic protein) promoters have been tested in rat retinas, and the highest expression level in Müller glial cells was obtained with a CD44 promoter.[19] Using AAV vectors, the glial fibrillary acidic protein promoter showed restricted expression in ciliary neurotrophic factor-activated Müller glial cells,[14] and engineered glial fibrillary acidic protein promoter with several hypoxia-responsive elements has been developed to induce expression under hypoxic condition.[20] So far, no strong, constitutive, and Müller glial cell–specific promoter has been reported using AAV vectors.

In this study, we analyzed the transduction profile of AAV6, ShH10, ShH10Y, and AAV9 via the intravitreal and subretinal route in mouse retinas and human retina cultures. We tested the potency of CD44

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and RLBP1 Müller glial cell–specific promoters and engineered small minimal CD44 and CMV promoters to efficiently transduce Müller glial cells. We showed that ShH10Y via the intravitreal route and AAV9 via the subretinal route showed the best efficiency to transduce mouse Müller glial cells. CD44 and CMV promoters using ShH10Y capsids drove expression mainly in Müller glial cells, whereas the RLBP1 promoter mediated high expression specifically in Müller glial cells via the intravitreal route. AAV ShH10 and ShH10Y capsids with CMV or RLBP1 promoters mediate green fluorescent protein (GFP) expression in human Müller glial cells *in vitro*. Finally, we showed that only a small minimal CMV promoter and the small human G protein–coupled receptor kinase 1 (GRK1) promoter drive efficient expression of full-length CRB1 protein in Müller glial or photoreceptors, respectively.

RESULTS

ShH10Y and AAV9 capsids efficiently transduce mouse Müller glial cells

We studied the transduction and expression profiles of AAV6, ShH10, and ShH10Y, (ShH10Y carries an additional tyrosine to phenylalanine mutation to enhance its transduction efficiency).[21] We intravitreally injected *Crb1*$^{-/-}$ retinas with 10^8, 10^9, 10^{10} genome copies (gc) of CMV-*GFP* transgene packaged in the different AAV serotypes. We analyzed the GFP expression by scanning laser ophthalmoscopy (SLO) and immunohistochemistry using cell type–specific immunomarkers in retinal sections. At 10^8 gc, the ShH10Y variant showed enhanced ability to transduce Müller glial cells relative to the unmodified ShH10 and AAV6 capsids (49 ± 6, 24 ± 3, and 21 ± 3%, respectively; Figure 1d), the total number of GFP-positive cells per millimeter was not different (28 ± 4, 29 ± 6, and 26 ± 3%, respectively). At one log unit higher titer (10^9 gc), ShH10Y also showed the highest percentage of transduced mouse Müller glial cells, whereas ShH10 and AAV6 transduced less efficiently (66 ± 4, 23 ± 2, and 17 ± 6%, respectively; Figure 1e), the total number of infected cells per millimeter was not different (49 ± 2, 47 ± 10, and 51 ± 2, respectively). At 10^{10} gc, the cell types infected by AAV6 were primarily around the blood vessels as shown previously,[14] whereas both ShH10 and ShH10Y showed a broader transduction pattern (Figure 1a–c). 62 ± 2% of the cells transduced by ShH10Y were Müller glial cells, whereas significantly less Müller glial cells were transduced with ShH10 (39 ± 1%) or AAV6 (18 ± 1%) (Figure 1a–c,f).

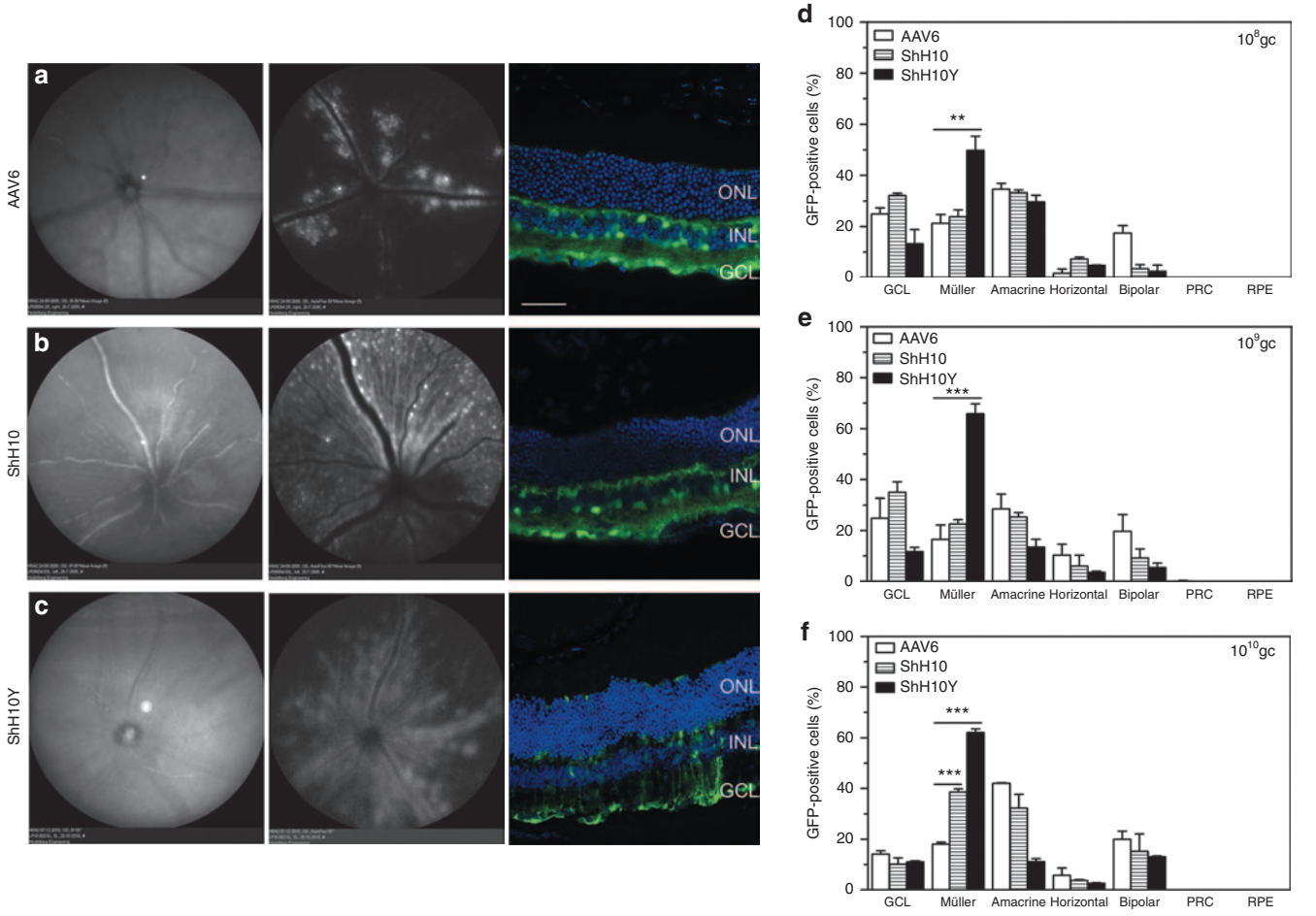

Figure 1 AAV6, ShH10, and ShH10Y tropism following intravitreal injection in adult murine retina. *In vivo* scanning laser ophthalmoscopy at 830 nm (left panel) for native fundus images and at 488 nm for green fluorescent protein (GFP) fluorescence images (middle panel) of *Crb1*$^{-/-}$ mice 3 weeks after intravitreal injection of 10^{10} genome copies (gc) of CMV-*GFP* with (**a**) AAV6 capsids and AAV6-derived capsids (**b**) ShH10 and (**c**) ShH10Y showed more widespread expression of GFP with ShH10 and ShH10Y capsids than AAV6, which localized mainly along the blood vessels. A representative transverse retinal section (right panel) revealed more GFP-positive Müller glial cells with ShH10Y capsids. Transduction profiles of three retinas injected with CMV-*GFP* vectors packaged in AAV6, ShH10, and ShH10Y capsids at (**d**) 10^8, (**e**) 10^9, and (**f**) 10^{10} gc showed that ShH10Y is the most powerful capsid to transduce mouse Müller glial cells even at low doses. Data are presented as mean ± SEM and $n = 3$/AAV/dose. $^{**}P < 0.01$, $^{***}P < 0.001$. Bar = 50 µm (**a**–**c**). AAV, adeno-associated virus; CMV, cytomegalovirus; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRC, photoreceptor cell; RPE, retinal pigment epithelium.

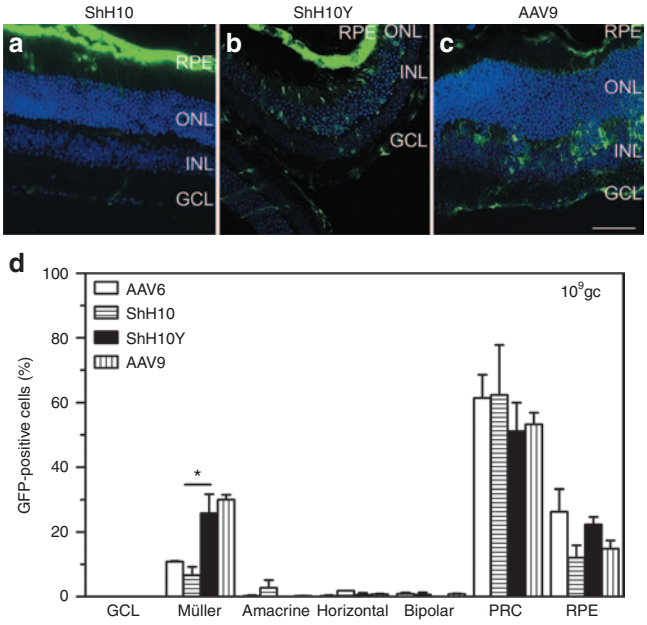

Figure 2 ShH10, ShH10Y, and AAV9 tropism following subretinal injection in adult murine retina. Representative transverse retinal section of *Crb1*$^{-/-}$ mice 3 weeks after subretinal injection of 10^9 genome copies (gc) of CMV-*GFP* with (**a**) ShH10, (**b**) ShH10Y, and (**c**) AAV9 capsids showed more GFP-positive cells with AAV9 capsids. (**d**) Transduction profiles of three retinas injected with CMV-*GFP* vectors packaged in AAV6, ShH10, ShH10Y, and AAV9 revealed that ShH10Y and AAV9 were the most powerful capsids to transduce mouse Müller glial cells via the subretinal route in contrast to AAV6 and ShH10. Data are presented as mean ± SEM and $n = 3$/AAV/dose. *$P < 0.05$. Bar: 50 µm (**a**–**c**). AAV, adeno-associated virus; CMV, cytomegalovirus; GCL, ganglion cell layer; GFP, green fluorescent protein; INL, inner nuclear layer; ONL, outer nuclear layer; PRC, photoreceptor cell; RPE, retinal pigment epithelium.

The number of GFP-positive cells per millimeter was not significantly different (152 ± 25, 123 ± 44, and 147 ± 17, respectively). In summary, intravitreally injected ShH10Y vectors showed higher transduction efficiency for mouse Müller glial cells than AAV6 or ShH10 vectors.

The AAV9 capsid has been shown to efficiently transduce many retinal cells including photoreceptors and Müller glial cells via the subretinal route.[22,23] Intravitreal injection of AAV9-CMV-*GFP* resulted in transduction of few ganglion cells (data not shown). We compared the subretinal transduction efficiency of AAV9 with AAV6,[14] ShH10, and ShH10Y (Figure 2). The percentage of GFP-positive Müller glial cells produced by AAV9 and ShH10Y injection was higher and similar (30 ± 2 and 26 ± 6%, respectively) relative to AAV6 and ShH10 vectors (11 ± 1 and 7 ± 3%, respectively). However, AAV9 transduced more mouse retinal cells than ShH10Y (483 ± 171 versus 100 ± 33 GFP-positive cells per millimeter). In summary, AAV9 and ShH10Y are potent capsids to transduce mouse Müller glial cells via the subretinal route.

The ShH10Y-CD44 promoter drives low levels of expression in Müller glial cells

The 1.8-kb mouse full-length CD44 promoter[24] was subcloned into the AAV plasmid and packaged in ShH10Y capsids. Three weeks after intravitreal injection of ShH10Y containing the full-length CD44-*GFP*, SLO imaging showed weak GFP expression along blood vessels with occasional brighter spots of GFP expression (Figure 3a). Transverse retinal sections showed weak GFP expression in the inner nuclear and ganglion cell layers. Cell-specific markers verified that GFP-positive cells were primarily Müller glial cells (Figure 3b,c), with a few GFP-positive amacrine, ganglion cells, or astrocytes (Figure 3c). When injected into the subretinal space, ShH10Y full-length CD44-*GFP* vectors generated low-level GFP expression primarily in Müller glial cells but also in RPE and photoreceptor cells at the site of injection (see Supplementary Figure S1a). Interestingly, we found increased GFP expression in areas associated with gliosis, ectopic localization of Müller glial nuclei, and presence of activated microglia cells (see Supplementary Figure S1b–d,e,g), compared with unaffected areas (Figure 3a–d and Supplementary Figure S1f).

Small promoters are required to fit large genes such as full-length 4.2-kb *CRB1* complementary DNA (cDNA) within the 4.7-kb AAV packaging limit to allow efficient packaging and expression.[10,25] It was shown that a 0.4-kb CD44 promoter was sufficient to drive significant levels of gene expression in vascular smooth muscle cells.[26] Therefore, we tested the 0.4-kb minimal CD44 promoter and analyzed its pattern of expression in the retina. Three weeks after intravitreal injection of ShH10Y-minimal CD44-*GFP*, no GFP was visible on SLO imaging (Figure 3e). Immunohistochemistry against GFP showed few GFP-positive cells per eye and GFP expression just above detection levels. Most of the GFP-positive cells were Müller glial cells (Figure 3f,g). Truncation of the promoter did not affect the transduction profile of the promoter but drastically reduced the number of GFP-positive cells per millimeter (113 ± 11 for full-length CD44 versus 2 ± 1 for minimal CD44; Figure 3h). We conclude that the AAV-minimal or full-length CD44 promoters are not appropriate to express CRB1 protein.

The ShH10Y-RLBP1 promoter mediates high levels of expression in Müller glia cells

A 2.8-kb fragment of the human RLBP1 promoter, which is highly conserved between human and mice,[27] was synthesized and subcloned into an AAV2 plasmid. At 3 weeks postintravitreal injection of ShH10Y-RLBP1-*GFP*, SLO revealed strong GFP expression mainly along the blood vessels and around the optic nerve (Figure 4a). The RLBP1 promoter restricts expression to Müller glial cells, which was confirmed by specific immunomarkers (Figure 4b–c). No colocalization of GFP was detected with cell-specific markers for amacrine and ganglion cells, cones, or photoreceptor cells (Figure 4d–f).

When delivered subretinally, ShH10Y-RLBP1-*GFP* vectors showed on SLO a typical pattern of GFP expression (Figure 5a). Transverse sections revealed strong GFP expression restricted to RPE and Müller glial cells (Figure 5b–c). Endogenous staining for RLBP1 protein (RPE and Müller glial cells)[28] showed strong colocalization with GFP (Figure 5d), whereas no colocalization was found with markers for other cell types (Figure 5e–g). In summary, when applied subretinally, the AAV9 RLBP1 vector drives expression in RPE and Müller glia cells, when delivered into the vitreous, the ShH10Y-RLBP1 promoter restricts expression to Müller glia cells.

Transduction of the human retina

We tested the potency of ShH10 and ShH10Y capsids and RLBP1 promoter to transduce and express GFP in human Müller glial cells. For this purpose, application of 10^{10} gc of ShH10-CMV-*GFP*, ShH10Y-CMV-*GFP*, and ShH10Y-RLBP1-*GFP* was performed on cultures of human retinal explants. After 7 days in culture, transverse retinal sections revealed GFP expression in human Müller glial cells independently of the capsids or promoter used (Figure 6a,c,d). Retinas injected with CMV-*GFP* vectors showed many transduced photoreceptors at the site of injection, whereas no transduced photoreceptors were detected with RLBP1-*GFP* (Figure 6b).

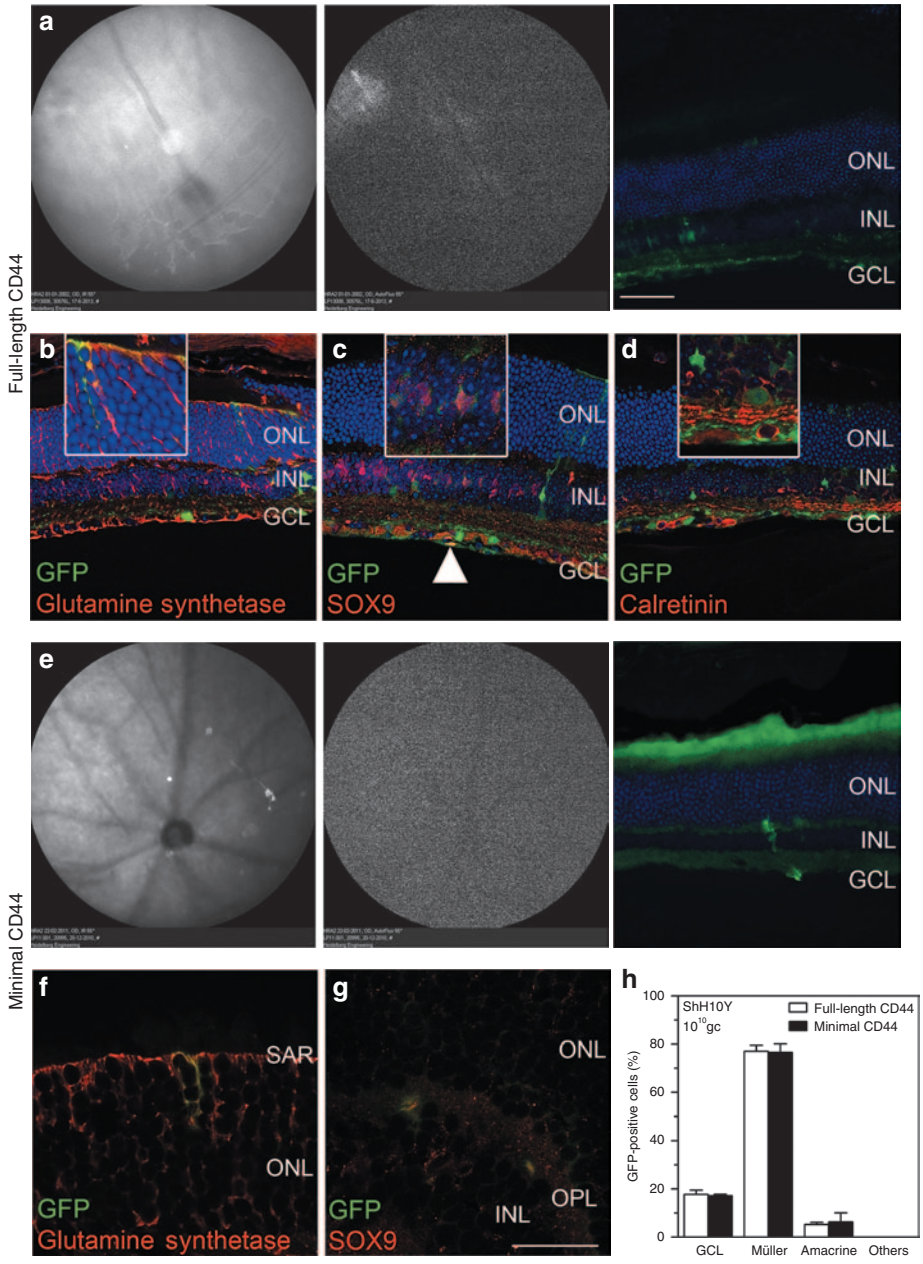

Figure 3 CD44 promoters drive low green fluorescent protein (GFP) expression mainly in Müller glial cells. *In vivo* scanning laser ophthalmoscopy (SLO) at 830 nm (left panel) for native fundus images and at 488 nm for GFP fluorescence (middle panel) of *Crb1*$^{-/-}$ mice 3 weeks after intravitreal injection of 10^{10} genome copies (gc) of (**a**) AAV2/ShH10Y full-length CD44-*GFP* showed low GFP expression mainly along the blood vessels, and a representative transverse retinal section (right panel) showed low levels of GFP expression mainly in Müller glial cells. Confocal imaging of immunostaining of (**b**) glutamine synthetase, (**c**) SOX9, and (**d**) calretinin showed GFP expression mainly in (**b–c**) Müller glial cells, (**d**) fewer amacrine and ganglion cells, and (**c**) astrocytes (white arrowhead). *In vivo* SLO at 830 nm (left panel) for native fundus images and at 488 nm for GFP fluorescence (middle panel) of *Crb1*$^{-/-}$ mice 3 weeks after intravitreal injection of (**e**) 10^{10} gc of AAV2/ShH10Y-minimal CD44-*GFP* revealed no detectable GFP, and a representative transverse retinal section (right panel) showed fewer GFP-positive cells expressing low levels of GFP. Confocal imaging of immunostaining of (**f**) glutamine synthetase and (**g**) SOX9 showed that most of the GFP-positive cells were Müller glial cells. (**h**) Transduction profiles of four retinas injected with AAV2/ShH10Y full-length or minimal CD44-*GFP* at 10^{10} gc revealed that up to 75% of the GFP-positive cells were Müller glial cells. Data are presented as mean ± SEM and $n = 4$/promoter. Bar: 50 µm (**a**–**e**), 25 µm (**f**,**g**). AAV, adeno-associated virus; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; SAR, subapical region.

An ShH10Y-minimal CMV promoter showed increased tropism for Müller glial cells

A shorter and engineered version of the CMV promoter (0.26 kb) was designed with enhancer elements from the full-length CMV promoter.[29,30] Three weeks after intravitreal injection of ShH10Y-minimal CMV-*GFP*, GFP was visible on SLO imaging along and between the blood vessels (Figure 7a). GFP-positive cells were mainly Müller glial cells (Figure 7b), with a few bipolar (Figure 7c) or amacrine cells (Figure 7d) and very few horizontal cells (Figure 7e). The minimal CMV promoter slightly modified the cell-specific expression profile, as more Müller glial cells were GFP positive (79 ± 3% for minimal CMV versus 64 ± 3% for full-length CMV; Figure 3f).

When injected subretinally, AAV2/9-minimal CMV-*GFP* showed many GFP-positive cells similar to the full-length CMV

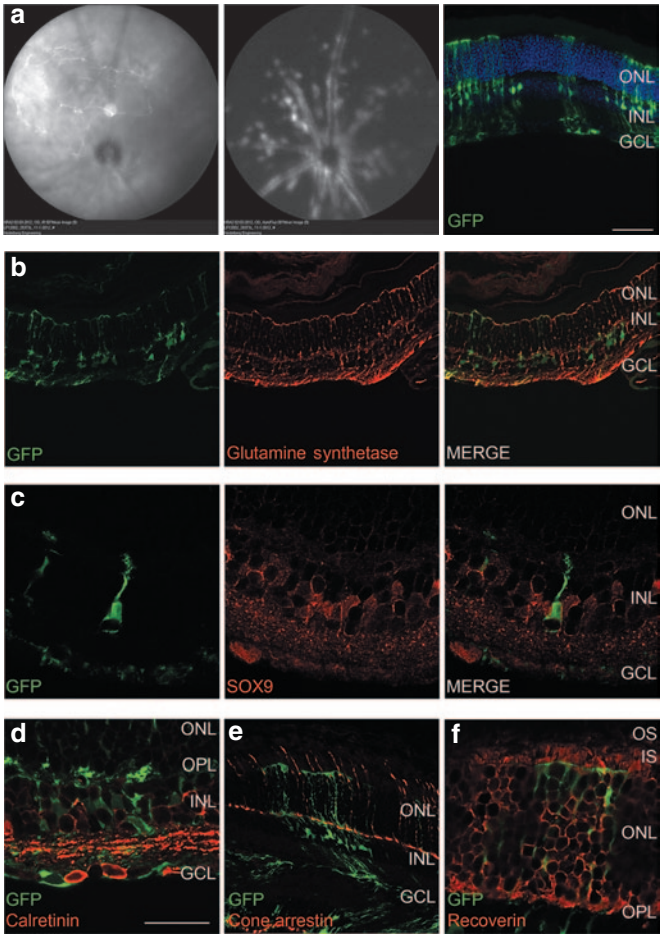

Figure 4 The RLBP1 promoter drives strong green fluorescent protein (GFP) expression specifically in Müller glial cells via the intravitreal route. *In vivo* scanning laser ophthalmoscopy at 830 nm (left panel) for native fundus images and at 488 nm for GFP fluorescence (middle panel) of four *Crb1*$^{-/-}$ mice 3 weeks after intravitreal injection of 10^9 genome copies of (**a**) AAV2/ShH10Y-RLBP1-*GFP* showed high GFP expression mainly along the blood vessels, and representative transverse retinal section (right panel) revealed that only Müller glial cells expressed GFP. Confocal imaging of GFP-positive cells coimmunostained with (**b**) glutamine synthetase, (**c**) SOX9, (**d**) calretinin (a marker for amacrine cells), (**e**) cone arrestin (a marker for cones), and (**f**) recoverin (a marker for photoreceptors) showed only colocalization of GFP with glutamine synthetase and SOX9, two specific markers for Müller glial cells. $n = 4$. Bar = 50 µm (**a**,**b**,**e**), 25 µm (**c**,**d**,**f**). AAV, adeno-associated virus; GCL, ganglion cell layer; INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; RLBP1, retinaldehyde binding protein 1.

promoter (see Supplementary Figure S2a) at 3 weeks postinjection. However, in contrast to the full-length CMV promoter, the minimal CMV promoter mediated decreased levels of GFP expression in the RPE (Figure 2c and Supplementary Figure S2a). The AAV-minimal CMV vector transduced Müller glial cells (see Supplementary Figure S2b), cone (see Supplementary Figure S2e) and rod photoreceptor cells (see Supplementary Figure S2e), and only few bipolar (see Supplementary Figure S2c) and amacrine cells (see Supplementary Figure S2d). The ShH10Y-minimal CMV vector showed a reduced number of GFP-positive RPE cells (5 ± 2% for minimal CMV versus 12 ± 1% for full-length CMV; see Supplementary Figure S2f).

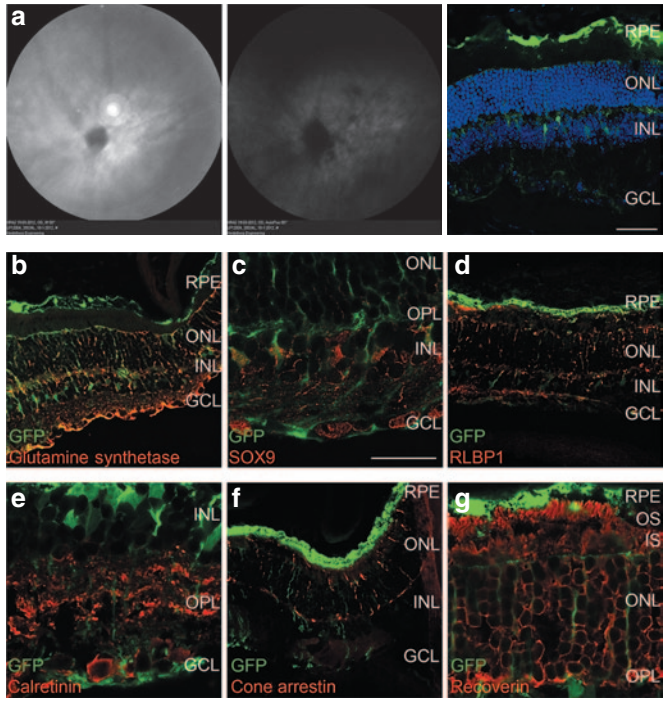

Figure 5 The RLBP1 promoter drives strong green fluorescent protein (GFP) expression in Müller glial cells and retinal pigment epithelium (RPE) via the subretinal route. *In vivo* scanning laser ophthalmoscopy at 830 nm (left panel) for native fundus images and at 488 nm for GFP fluorescence (middle panel) of four *Crb1*$^{-/-}$ mice 3 weeks after subretinal injection of 10^9 genome copies of (**a**) AAV2/ShH10Y-RLBP1-*GFP* showed a typical pattern of GFP expression following subretinal injection, and a representative transverse retinal section (right panel) revealed that only Müller glial and RPE cells expressed GFP. Confocal imaging of GFP-positive cells coimmunostained with (**b**) glutamine synthetase, (**c**) SOX9, (**d**) native RLBP1 protein (Müller glial and RPE cells), (**e**) calretinin (a marker for amacrine cells), (**f**) cone arrestin (a marker for cones), and (**g**) recoverin (a marker for photoreceptors) showed only colocalization of GFP with glutamine synthetase, SOX9, and RLBP1. $n = 4$. Bar = 50 µm (**a**,**b**,**e**), 25 µm (**c**,**d**,**f**). AAV, adeno-associated virus; GCL, ganglion cell layer; INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; RLBP1, retinaldehyde binding protein 1; RPE, retinal pigment epithelium.

Minimal promoters showed efficient expression of full-length *CRB1*

Several studies have demonstrated that AAV viruses have a limited packaging capacity of 4.7–5.2 kb of transgenic DNA depending on the serotype, larger transgenes showed 3′ or 5′ deletions and/or significant reductions in the titer.[31–34] We attempted to express a full-length *CRB1* cDNA (4.2 kb) with a 5.23-kb AAV vector that included a full-length CMV promoter, short synthetic polyadenylation sequences, and AAV2 inverted terminal repeat sequences. We found that CRB1 protein was barely detectable upon subretinal injection of AAV2/5-CMV-*hCRB1* vector and was undetectable from CMV-*hCRB1* vector packaged with AAV6, ShH10, or ShH10Y capsids (see Supplementary Figure S3a). However, the human *CRB1* transcript was detected in retinas transduced with AAV6, ShH10, or ShH10Y capsids (see Supplementary Figure S3b). Using human embryonic kidney 293T cell lines, we showed that CRB1 and GFP proteins were efficiently expressed upon transfection and infection, respectively, whereas almost no CRB1 protein was detectable after infection (see Supplementary Figure S3c). Therefore, we suggest that the use of full-length CMV promoter and *CRB1* gene exceeds the AAV packaging limitation.

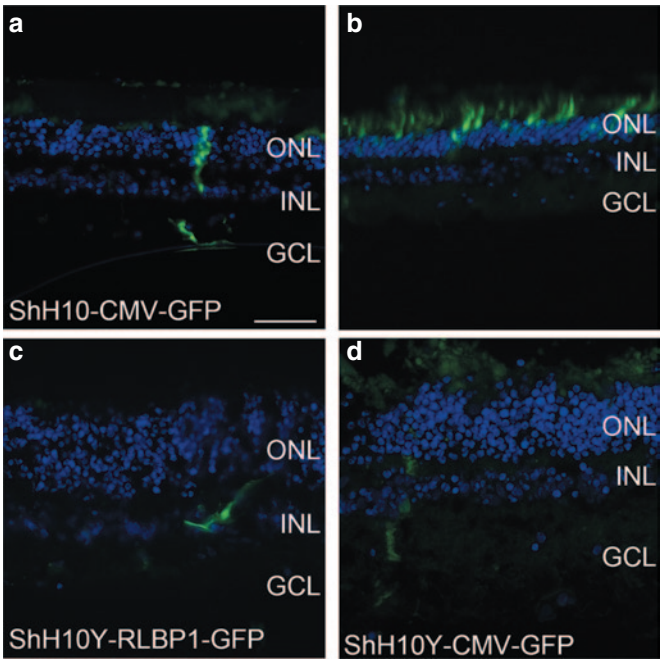

Figure 6 ShH10 and ShH10Y variants transduced human Müller glial cells *in vitro*. Transverse section of cultured retinas from donor eyes with 10^{10} genome copies of (**a**,**b**) AAV2/ShH10-CMV-*GFP*, (**c**) AAV2/ShH10Y-RLBP1-*GFP*, and (**d**) AAV2/ShH10Y-CMV-*GFP* revealed the presence of GFP-positive Müller glial cells with the three different vectors (**a**,**c**,**d**) and GFP-positive photoreceptors only with CMV promoter vectors. Bar: 50 µm (**a**–**c**). AAV, adeno-associated virus; CMV, cytomegalovirus; GFP, green fluorescent protein; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RLBP1, retinaldehyde binding protein 1.

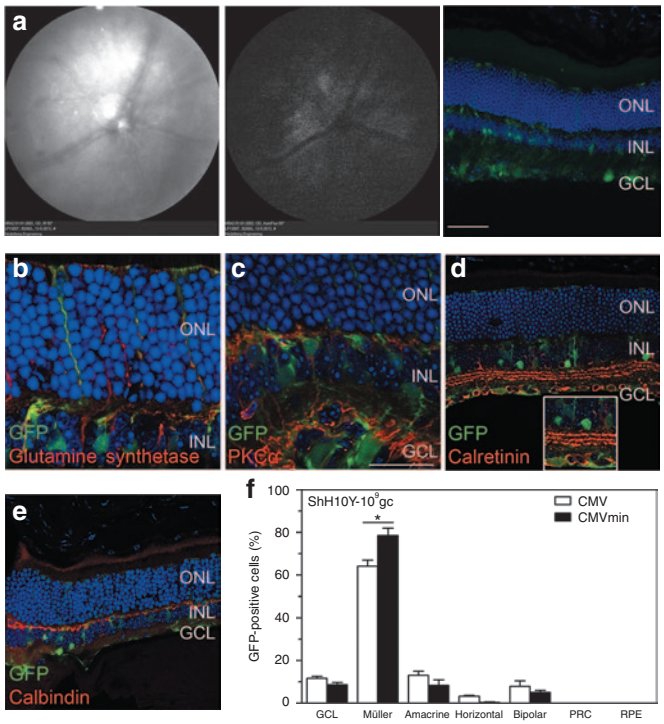

Figure 7 The minimal cytomegalovirus (CMV) promoter showed increased Müller glial cell restricted expression via the intravitreal route. *In vivo* scanning laser ophthalmoscopy at 830 nm (left panel) for native fundus images and at 488 nm for green fluorescent protein (GFP) fluorescence (middle panel) of *Crb1*$^{-/-}$ mice 3 weeks after intravitreal injection of 10^9 genome copies (gc) of (**a**) AAV2/ShH10Y-minimal CMV-*GFP* showed GFP expression along and between the blood vessels, and a representative transverse retinal section (right panel) revealed many GFP-positive Müller glial cells. Confocal imaging of immunostaining of (**b**) glutamine synthetase (a marker for Müller glial cells), (**c**) protein kinase C α (PKCα; a marker for bipolar cells), (**d**) calretinin (a marker for amacrine cells), and (**e**) calbindin (a marker for horizontal and amacrine cells) showed GFP expression in Müller glial, amacrine, and ganglion cells and barely in horizontal cells. (**f**) Transduction profiles of three retinas injected with AAV2/ShH10Y full-length or minimal CMV-*GFP* at 10^9 gc revealed that the minimal CMV promoter mediated increased Müller glial cell restricted expression compared with the native CMV promoter. Data are presented as mean ± SEM and $n = 3$/promoter. *$P < 0.05$. Bar = 50 µm (**a**,**d**,**e**), 25 µm (**b**–**c**). AAV, adeno-associated virus; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRC, photoreceptor cells; RPE, retinal pigment epithelium.

In order to obtain efficient and persistent expression of a functional CRB1 protein in Müller glial cells,[35] the AAV vector insert has to be within the 4.7- to 5.2-kb packaging limit, and this can be achieved by either shortening the promoter or the *CRB1* gene. The minimal CMV (Figure 7 and Supplementary Figure S2) and the human GRK1 promoter[36] (see Supplementary Figure S4a–b) were used to drive the expression of the full-length *CRB1* cDNA in Müller glial cells, photoreceptors, and RPE cells and only cones and rods, respectively. The full-length CMV promoter and an engineered 0.5-kb human rhodopsin promoter (see Supplementary Figure S4c–d) were used to express a naturally occurring 3.9-kb short variant of the *CRB1* cDNA, called *CRB1Δ* in Müller glial cells, photoreceptors and RPE cells and only rods, respectively. This transcript variant carries an in-frame deletion of exons 3–4 and is expressed in the human retina.[37] All four AAV-*CRB1* expression vectors mediated detectable levels of CRB1 protein in *Crb1*$^{-/-}$ retinas and expression at the subapical region near physiological levels (Figure 8). Using AAV2/9-minimal CMV-*CRB1*, full-length CRB1 protein was mainly found at the subapical region of Müller glia cells and photoreceptors and at the apical membrane of RPE cells (Figure 8c). Using AAV2/9-hGRK1-*CRB1*, full-length CRB1 protein was expressed in rod and cone photoreceptors and was mainly found at the subapical region or in inner segments and minimally in their somata (Figure 8d). Using AAV2/9-CMV-*CRB1Δ*, the short CRB1Δ protein was mainly found at the subapical region of Müller glia cells and photoreceptors but also at a low level of expression in the soma of photoreceptors and in RPE (Figure 8e). Using AAV2/9-RHO-*CRB1Δ*, the short CRB1Δ protein was specifically expressed in rod photoreceptors but likely overexpressed as the protein localized at the subapical region, in their inner segments and somata (Figure 8f).

However, using *CRB1* or *CRB1Δ* vectors, we found some potential toxicity associated with high levels of CRB1 around the site of injection (see Supplementary Figure S3d). Especially with AAV2/9-CMV-*CRB1Δ* vectors (CRB1Δ expression in RPE, Müller glial cells, and photoreceptors), large areas were affected with complete lack of photoreceptors, lack of Müller glial cells (see Supplementary Figure S3e–f), and the presence of large phagocytic vacuoles in CD11b-positive immune cells and/or microglias and T lymphocytes (see Supplementary Figure S3g–h).

DISCUSSION

In this study, we show that (i) ShH10Y and AAV9 are the most efficient capsids for transduction of mouse Müller glial cells via the intravitreal and subretinal routes, respectively. (ii) The combination of the ShH10Y capsid and RLBP1 promoter efficiently drives Müller glial cell–specific GFP expression. (iii) The combination of ShH10Y capsid with the CD44 promoter was inefficient at driving GFP expression, and this expression was not specific for Müller glial cells. (iv) The ShH10Y capsid and

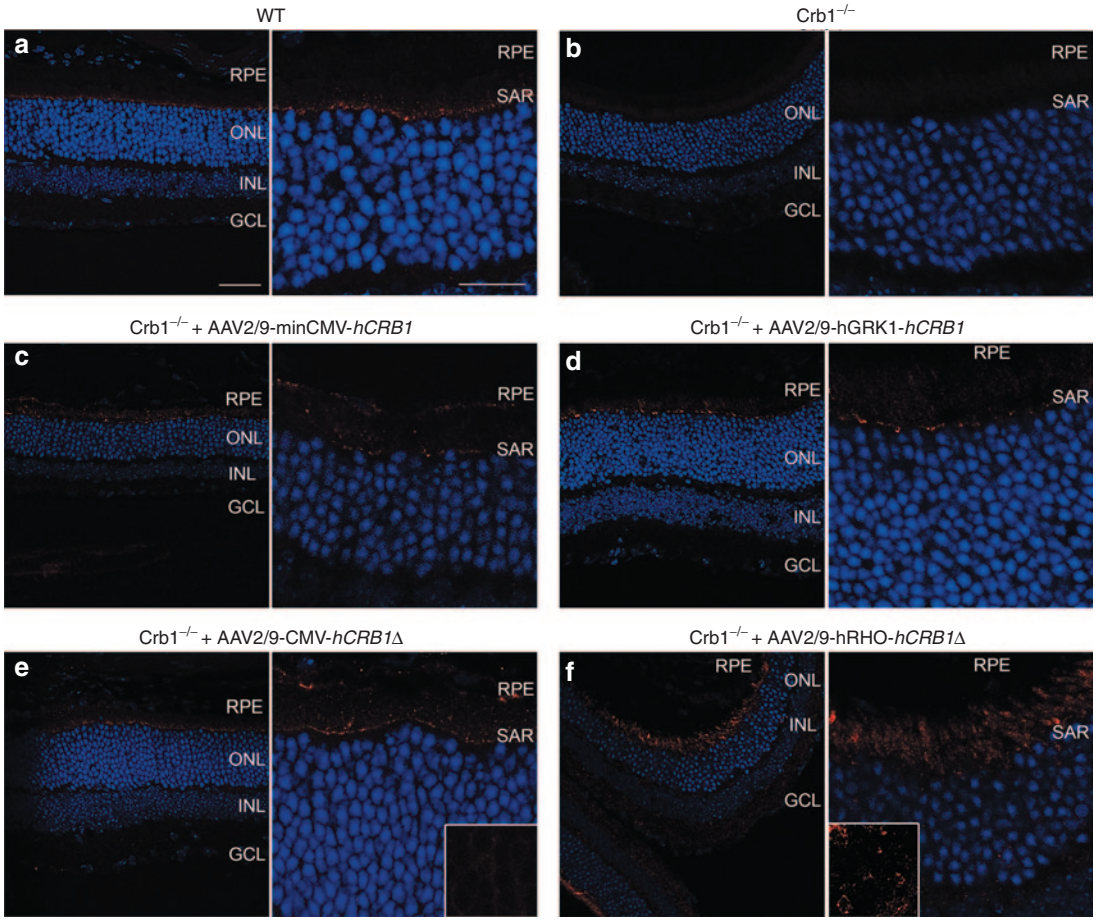

Figure 8 Minimal promoters lead to efficient full-length CRB1 expression. Immunohistochemistry against CRB1 showed strong staining at the subapical region (zoom of right panel in left panel) in (**a**) wild-type retinas in contrast to (**b**) *Crb1*$^{-/-}$ retinas. Three weeks postsubretinal injection of 5×10^9 genome copies (gc) of either (**c**) AAV2/9-minCMV-*hCRB1* or (**d**) AAV2/9-hGRK1-*hCRB1* in *Crb1*$^{-/-}$ retinas, CRB1 protein was detected at the subapical region and membrane-associated in the inner segment (IS) and barely in the soma of photoreceptors and at the apical membrane of the retinal pigment epithelium (RPE) with minimal CMV promoter. Three weeks postsubretinal injection of 5×10^9 gc of either (**e**) AAV2/9-CMV-*hCRB1Δ* (*CRB1* variant carrying an in-frame deletion of exons 3–4) or (**f**) AAV2/9-hRHO-*hCRB1Δ* in *Crb1*$^{-/-}$ retinas, CRB1 protein was detected at the subapical region and found membrane-associated and cytoplasmic in the ISs and somata of photoreceptors and at the apical membrane of the RPE with CMV promoter. $n = 4$/CRB1 variant. Bar = 50 µm (left panel), 25 µm (zoomed right panel). AAV, adeno-associated virus; GCL, ganglion cell layer; GRK1, G protein–coupled receptor kinase 1; INL, inner nuclear layer; ONL, outer nuclear layer; RHO, rhodopsin; SAR, subapical region.

minimal CMV promoter expression vector generated specific expression in Müller glial cells. (v) Both the ShH10Y-CMV or ShH10Y-RLBP1 expression vectors mediated GFP expression in Müller glial cells in cultured human retinas. (vi) The AAV-minimal CMV and human GRK1 expression vectors can be used to express full-length CRB1 protein in mouse Müller glial cells and photoreceptor cells. In conclusion, ShH10Y and AAV9 capsids in combination with the RLBP1 and minimal CMV promoters are valuable new tools to target gene expression to human and mouse Müller glial cells.

ShH10, a capsid variant derived from AAV6, showed a significant variation in its transduction profile between rat and mouse retinas. Whereas up to 94% of the GFP-positive cells were Müller glial cells in rat retinas,[16] only 39% were Müller glial cells in mouse retinas at the highest vector doses. Differences in transduction efficiency and tropism of the native AAV6 had been previously reported between rat and mouse retinas, and this difference may be due to differences in the mechanical barrier function of inner limiting membrane between the species.[14,16,38] In rat, AAV6 transduces few ganglion or inner nuclear layer cells,[16] whereas in mice, AAV6 capsids efficiently infect the retina along the major retinal blood vessels where the inner limiting membrane is thought to be thinner.[14] The receptor binding characteristics of AAV6 and AAV6-derived capsids may also be highly dependent on the protein composition of the inner limiting membrane barrier. Another reason that may explain the difference in tropism observed with application of ShH10 vectors in mouse is that this mutant was initially selected from a library of millions of variants using primary astrocytes from human adult cortex[15] and subsequently tested for its transduction characteristics on rat Müller glial cells *in vivo*.[16] The ShH10 variant with the additional Y445F mutation, which reduces the number of exposed tyrosine residues on the AAV surface and resultant ubiquitination and proteasome degradation, showed a threefold increase in efficiency for transduction of mouse Müller glial cells in comparison to AAV6, which was seen at low and high titers. ShH10 and ShH10Y capsids were able to infect Müller glial cells in human retina in culture similarly to AAV6 as described previously.[14]

The CD44 promoter, which has been shown to efficiently drive gene expression in Müller glial cells using a lentiviral vector in rats,[19] showed low levels of gene expression that was not restricted to Müller glial cells in mouse retina. Truncation of the CD44 promoter decreased its basal activity, a finding that is in contrast to the observations in vascular smooth muscle.[26] One interesting finding is that

in the degenerating areas due to the *Crb1*$^{-/-}$ phenotype and/or injury from the subretinal injection, increased transduction and/or expression was observed in areas with gliosis and activated microglia cells. It has been previously described that the CD44 promoter is upregulated in arterial wall injury *in vivo* and can be activated by the proinflammatory cytokine interleukin 1β.[39,40] We speculate that similar to smooth muscle cells, retinal injury and possibly proinflammatory cytokines (activated microglial and Müller glial cells) might increase the CD44 promoter activity in retina. As the injury-responsive element, activating protein 1 is conserved in the minimal CD44 promoter,[26] an interesting *in vivo* study using the full-length and the minimal CD44 promoters, and testing injection of different cytokines might reveal that the CD44 promoter in the retina reacts similar as in smooth muscle cells. Therefore, the ShH10Y-CD44-*GFP* virus might be an interesting tool to study inflammatory processes in the retina.

The human RLBP1 promoter fragment showed a similar pattern of expression as the endogenous RLBP1 protein, restricted to RPE and Müller glial cells. However, this promoter is large (2.85 kb) and complex as the initiation codon is in exon 3.[27,41] A shortened RLBP1 promoter might be useful to obtain expression restricted to Müller glial cells and to package large genes such as *CRB1*. Several studies demonstrated that AAV viruses have a limited packaging and expression capacity of 4.7–5.2 kb of transgenic DNA depending on the serotype.[31–34] Here, we showed that the *CRB1* gene can be expressed in retinal cells using AAV vectors with short promoters such as a minimal CMV promoter or the human GRK1 promoter.

Over the last decades, Müller glial cells became an interesting target for therapeutic and regeneration studies. In this study, we characterized and provided new tools, which are combinations of ShH10Y capsids, RLBP1, or a minimal CMV promoter, to target mouse and human Müller glial cells and express relatively large genes such as *CRB1*.

MATERIALS AND METHODS

Generation and purification of the viral vectors

The pAAV2-*GFP* plasmids were generated previously[14,42] and consist of the flanking inverted terminal repeats of AAV2, the CMV promoter, the *eGFP* cDNA, the woodchuck posttranscriptional regulatory element, and the SV40 polyadenylation sequence. The pAAV2-*CRB1* plasmids consist of the flanking inverted terminal repeats of AAV2, the CMV promoter, the *CRB1* cDNA or codon optimized *CRB1* cDNA (pAAV2-co*CRB1*), and a 48-bp synthetic polyadenylation sequence. The murine full-length 1.8-kb[24] and the minimal 0.36-kb[26] CD44 promoters were amplified by polymerase chain reaction from CD44prm-pXP2 and subcloned using *Not* I and *Xho* I in pAAV2-*GFP* vectors. The human 2.85-kb RLBP1,[27] formally cellular retinaldehyde-binding protein (CRALBP), was synthesized at GenScript (Piscataway, NJ) with *eGFP* cDNA, the woodchuck posttranscriptional regulatory element and the bovine growth hormone polyadenylation sequence and subcloned in pAAV2 via *Bgl* II restriction sites. The minimal 0.26-kb CMV was assembled (sequence in Supplementary Materials and Methods) according to the minimal promoter and enhancer sequences previously described,[29,30] synthesized at GenScript and subcloned using *Spe* I and *Xho* I restriction sites in pAAV2-*GFP* and pAAV2-co*CRB1* vectors. The 0.31-kb human GRK1 promoter[36] was synthesized at GenScript and subcloned using *Spe* I and *Xho* I restriction sites in pAAV2-GFP vectors and pAAV2-coCRB1 vectors. pAAV2-co*CRB1Δ* were obtained by deletion of exons 3–4 (0.32 kb) of codon optimized *CRB1* cDNA. A 0.52-kb human rhodopsin promoter was designed, synthesized at GenScript, and subcloned using *Spe* I and *Xho* I restriction sites in pAAV2-*GFP* vectors and pAAV2-co*CRB1Δ* vectors.

AAV stocks were generated and purified as previously described.[14,16] Briefly, pAAV2-*GFP* transgene plasmids were cotransfected with the AAV2/6 helper plasmid pDP6 (Plasmid Factory, Bielefeld, Germany) or pHelper and pXX2-ShH10 or pXX2-ShH10Y445F[16,17] or pAAV9 capsid plasmid into human embryonic kidney 293T cells to generate AAV2/6, AAV2/9, AAV2/ShH10, and AAV2/ShH10Y viral particles. After DNase treatment, the lysates were ultracentrifuged onto an iodixanol density gradient (Sigma, St. Louis, MO). All viral titers were determined by quantitative polymerase chain reaction and all viral stocks with titers around 1×10^{13} gc per ml were stored at −80 °C.

Intravitreal and subretinal injections

Animal care and use of mice was in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research, and the protocols were approved by the Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW). *Crb1*$^{-/-}$ mice[11] were maintained on a 50% C57BL/6J and 50% 129/Ola genetic background and on a 12-hour day/night cycle and supplied with food and water *ad libitum*. One-month-old *Crb1*$^{-/-}$ mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine intraperitoneally, and the iris was dilated using phenylephrine (0.5% in saline). The mice were injected either subretinally or intravitreally as previously described[14] with 1 µl of 10^8, 10^9, and 10^{10} gc of each AAV. After injection, eyes were treated with chloramphenicol, and mice were kept on a heating pad until they fully recovered from anesthesia.

Scanning laser ophthalmoscopy

Three weeks after injection of the viral vector, the mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine intraperitoneally, and the iris was dilated using phenylephrine (0.5% in saline). *In vivo* SLO was performed using the Heidelberg Retina Angiograph 2 (HRA 2; Heidelberg Engineering, Heidelberg, Germany) at 830 nm for infrared-native fundus images and at 488 nm for GFP fluorescence images with a barrier filter at 500 nm.

Immunohistochemical analysis

The anaesthetized mice were sacrificed by cervical dislocation immediately after imaging. Enucleated eyes were fixed for 30 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and cryoprotected by subsequent incubations of 30 minutes in 5 and 30% sucrose in PBS. The eyes were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), frozen, and stored at −20 °C. Sections of 7–10 µm were generated using a Leica CM3050 cryostat (Leica Microsystems, Rijswijk, The Netherlands) and were either enclosed immediately in Vectashield DAPIHardSet mounting media (Vector Laboratories, Amsterdam, The Netherlands) or used for immunohistochemical analysis. Sections for immunohistochemistry were blocked for 1 hour in 10% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin in PBS, incubated in a moist chamber overnight (at 4 °C) or blocked for 2 hours at room temperature with appropriate primary antibodies (see Supplementary Table S1) diluted in 0.3% normal goat serum, 0.4 % Triton X-100, and 1% bovine serum albumin in PBS. After rinsing in PBS, the sections were incubated for 1 hour with Cy3-conjugated goat secondary antibodies anti-mouse or anti-rabbit and rinsed in PBS again. Sections were mounted in Vectashield HardSet 4′,6-diamidino-2-phenylindole (DAPI) mounting media. The GFP and the cyanine3 signal were visualized with a Leica DMRE fluorescence microscope for overview pictures and confocal SP5 (Leica Microsystems) for immunohistochemical analysis.

Quantification of the GFP-positive cells

Transduction profiles were analyzed by counting individual GFP-positive somata, defined by their typical morphology and/or with colabeling with specific marker for each cell type (see Supplementary Table S1) on the total number of GFP-positive cells. Müller glial cells have a well-defined morphology as they span the entire retina reaching the outer and inner limiting membranes, and they display lozenge-shaped nuclei in the middle of the inner nuclear layer. At least five different sections from each eye from three to four retinas were counted. The number of GFP-positive cells for each cell type was divided by the total number of cells to obtain the percentage of transduced cells.

In vitro transduction of the human retina

This study was performed in agreement with the declaration of Helsinki on the use of human material for research. Postmortem human donor eyes were acquired from the Euro Cornea Bank and were processed within 48 hours after death. Retinas from the middle periphery were dissected from the eye and cultured for 7 days with the photoreceptor layer facing down as previously described.[14] Retinas were injected under the lifted inner limiting membrane with 1 µl of 1×10^{10} gc of AAV2/ShH10-CMV-*GFP*, AAV2/ShH10Y-CMV-*GFP*, and AAV2/ShH10Y-RLBP1-*GFP*. After 1 week, the retinas were fixed for 30 minutes in 4% paraformaldehyde in PBS (pH 7.4), cryoprotected by subsequent incubations of 30 minutes in 5 and 30% sucrose in PBS, and frozen in Tissue-Tek. Sections of 10 µm were generated with a Leica CM3050 cryostat (Leica Microsystems) and were enclosed immediately in Vectashield DAPI HardSet mounting media (Vector Laboratories).

Statistical analysis

Statistical significance was calculated by using Student's *t*-test of three to four independent retinas. Values were expressed as mean ± SEM and $n = 3–4$ per condition. Values of $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered to be statistically significant. Calculations and graphs were generated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Bringmann, A, Pannicke, T, Grosche, J, Francke, M, Wiedemann, P, Skatchkov, SN *et al*. (2006). Müller cells in the healthy and diseased retina. *Prog Retin Eye Res* **25**: 397–424.
2 Reichenbach, A and Bringmann, A (2013). New functions of Müller cells. *Glia* **61**: 651–678.
3 Newman, EA (2004). Glial modulation of synaptic transmission in the retina. *Glia* **47**: 268–274.
4 Jadhav, AP, Roesch, K and Cepko, CL (2009). Development and neurogenic potential of Müller glial cells in the vertebrate retina. *Prog Retin Eye Res* **28**: 249–262.
5 Ahmad, I, Del Debbio, CB, Das, AV and Parameswaran, S (2011). Müller glia: a promising target for therapeutic regeneration. *Invest Ophthalmol Vis Sci* **52**: 5758–5764.
6 Hartong, DT, Berson, EL and Dryja, TP (2006). Retinitis pigmentosa. *Lancet* **368**: 1795–1809.
7 den Hollander, AI, Roepman, R, Koenekoop, RK and Cremers, FP (2008). Leber congenital amaurosis: genes, proteins and disease mechanisms. *Prog Retin Eye Res* **27**: 391–419.
8 Saari, JC, Nawrot, M, Kennedy, BN, Garwin, GG, Hurley, JB, Huang, J *et al*. (2001). Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation. *Neuron* **29**: 739–748.
9 Maw, MA, Kennedy, B, Knight, A, Bridges, R, Roth, KE, Mani, EJ *et al*. (1997). Mutation of the gene encoding cellular retinaldehyde-binding protein in autosomal recessive retinitis pigmentosa. *Nat Genet* **17**: 198–200.
10 den Hollander, AI, ten Brink, JB, de Kok, YJ, van Soest, S, van den Born, LI, van Driel, MA *et al*. (1999). Mutations in a human homologue of Drosophila crumbs cause retinitis pigmentosa (RP12). *Nat Genet* **23**: 217–221.
11 van de Pavert, SA, Kantardzhieva, A, Malysheva, A, Meuleman, J, Versteeg, I, Levelt, C, *et al*. (2004). Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure. *J Cell Sci* **117**: 4169–4177.
12 Henckaerts, E and Linden, RM (2010). Adeno-associated virus: a key to the human genome? *Future Virol* **5**: 555–574.
13 Hellström, M, Ruitenberg, MJ, Pollett, MA, Ehlert, EM, Twisk, J, Verhaagen, J *et al*. (2009). Cellular tropism and transduction properties of seven adeno-associated viral vector serotypes in adult retina after intravitreal injection. *Gene Ther* **16**: 521–532.
14 Aartsen, WM, van Cleef, KW, Pellissier, LP, Hoek, RM, Vos, RM, Blits, B *et al*. (2010). GFAP-driven GFP expression in activated mouse Müller glial cells aligning retinal blood vessels following intravitreal injection of AAV2/6 vectors. *PLoS ONE* **5**: e12387.
15 Koerber, JT, Klimczak, R, Jang, JH, Dalkara, D, Flannery, JG and Schaffer, DV (2009). Molecular evolution of adeno-associated virus for enhanced glial gene delivery. *Mol Ther* **17**: 2088–2095.
16 Klimczak, RR, Koerber, JT, Dalkara, D, Flannery, JG and Schaffer, DV (2009). A novel adeno-associated viral variant for efficient and selective intravitreal transduction of rat Müller cells. *PLoS ONE* **4**: e7467.
17 Dalkara, D, Kolstad, KD, Guerin, KI, Hoffmann, NV, Visel, M, Klimczak, RR *et al*. (2011). AAV mediated GDNF secretion from retinal glia slows down retinal degeneration in a rat model of retinitis pigmentosa. *Mol Ther* **19**: 1602–1608.
18 Byrne, LC, Khalid, F, Lee, T, Zin, EA, Greenberg, KP, Visel, M *et al*. (2013). AAV-mediated, optogenetic ablation of Müller Glia leads to structural and functional changes in the mouse retina. *PLoS ONE* **8**: e76075.
19 Greenberg, KP, Geller, SF, Schaffer, DV and Flannery, JG (2007). Targeted transgene expression in muller glia of normal and diseased retinas using lentiviral vectors. *Invest Ophthalmol Vis Sci* **48**: 1844–1852.
20 Prentice, HM, Biswal, MR, Dorey, CK and Blanks, JC (2011). Hypoxia-regulated retinal glial cell-specific promoter for potential gene therapy in disease. *Invest Ophthalmol Vis Sci* **52**: 8562–8570.
21 Petrs-Silva, H, Dinculescu, A, Li, Q, Min, SH, Chiodo, V, Pang, JJ *et al*. (2009). High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. *Mol Ther* **17**: 463–471.
22 Lei, B, Zhang, K, Yue, Y, Ghosh, A and Duan, D (2010). Adeno-associated virus serotype-9 mediated retinal outer plexiform layer transduction is mainly through the photoreceptors. *Adv Exp Med Biol* **664**: 671–678.
23 Gao, G, Vandenberghe, LH, Alvira, MR, Lu, Y, Calcedo, R, Zhou, X *et al*. (2004). Clades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol* **78**: 6381–6388.
24 Hebbard, L, Steffen, A, Zawadzki, V, Fieber, C, Howells, N, Moll, J *et al*. (2000). CD44 expression and regulation during mammary gland development and function. *J Cell Sci* **113 (Pt 14)**: 2619–2630.
25 Lipinski, DM, Thake, M and MacLaren, RE (2013). Clinical applications of retinal gene therapy. *Prog Retin Eye Res* **32**: 22–47.
26 Foster, LC, Wiesel, P, Huggins, GS, Pañares, R, Chin, MT, Pellacani, A *et al*. (2000). Role of activating protein-1 and high mobility group-I(Y) protein in the induction of CD44 gene expression by interleukin-1beta in vascular smooth muscle cells. *FASEB J* **14**: 368–378.
27 Vázquez-Chona, FR, Clark, AM and Levine, EM (2009). Rlbp1 promoter drives robust Müller glial GFP expression in transgenic mice. *Invest Ophthalmol Vis Sci* **50**: 3996–4003.
28 Bunt-Milam, AH and Saari, JC (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. *J Cell Biol* **97**: 703–712.
29 Stinski, MF and Roehr, TJ (1985). Activation of the major immediate early gene of human cytomegalovirus by cis-acting elements in the promoter-regulatory sequence and by virus-specific trans-acting components. *J Virol* **55**: 431–441.
30 Thomsen, DR, Stenberg, RM, Goins, WF and Stinski, MF (1984). Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *Proc Natl Acad Sci USA* **81**: 659–663.
31 Dong, B, Nakai, H and Xiao, W (2010). Characterization of genome integrity for oversized recombinant AAV vector. *Mol Ther* **18**: 87–92.
32 Lai, Y, Yue, Y and Duan, D (2010). Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. *Mol Ther* **18**: 75–79.
33 Wu, Z, Yang, H and Colosi, P (2010). Effect of genome size on AAV vector packaging. *Mol Ther* **18**: 80–86.
34 Hirsch, ML, Agbandje-McKenna, M and Samulski, RJ (2010). Little vector, big gene transduction: fragmented genome reassembly of adeno-associated virus. *Mol Ther* **18**: 6–8.
35 van Rossum, AG, Aartsen, WM, Meuleman, J, Klooster, J, Malysheva, A, Versteeg, I *et al*. (2006). Pals1/Mpp5 is required for correct localization of Crb1 at the subapical region in polarized Muller glia cells. *Hum Mol Genet* **15**: 2659–2672.
36 Khani, SC, Pawlyk, BS, Bulgakov, OV, Kasperek, E, Young, JE, Adamian, M *et al*. (2007). AAV-mediated expression targeting of rod and cone photoreceptors with a human rhodopsin kinase promoter. *Invest Ophthalmol Vis Sci* **48**: 3954–3961.
37 Pellissier, LP, Lundvig, DM, Tanimoto, N, Klooster, J, Vos, RM, Richard, F *et al*. (2014). CRB2 acts as a modifying factor of CRB1-related retinal dystrophies in mice. *Hum Mol Genet* e-pub ahead of print, 23 February 2014 (doi:10.1093/hmg/ddu089).
38 Dalkara, D, Kolstad, KD, Caporale, N, Visel, M, Klimczak, RR, Schaffer, DV *et al*. (2009). Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. *Mol Ther* **17**: 2096–2102.
39 Jain, M, He, Q, Lee, WS, Kashiki, S, Foster, LC, Tsai, JC *et al*. (1996). Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. *J Clin Invest* **97**: 596–603.
40 Foster, LC, Arkonac, BM, Sibinga, NE, Shi, C, Perrella, MA and Haber, E (1998). Regulation of CD44 gene expression by the proinflammatory cytokine interleukin-1beta in vascular smooth muscle cells. *J Biol Chem* **273**: 20341–20346.
41 Intres, R, Goldflam, S, Cook, JR and Crabb, JW (1994). Molecular cloning and structural analysis of the human gene encoding cellular retinaldehyde-binding protein. *J Biol Chem* **269**: 25411–25418.
42 Flannery, JG and Visel, M (2013). Adeno-associated viral vectors for gene therapy of inherited retinal degenerations. *Methods Mol Biol* **935**: 351–369.

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