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.

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. 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). In the last two decades, Müller glial cells are shown to reside as potential retinal progenitor cells in the adult retina. In zebrafish and chick, Müller glial cells extend from their basal end feet facing the vitreous and give rise to the six types of neurons to replace the lost neurons 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. 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. 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. 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. 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. 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. Selective loss of Müller glial cells using an optogenetic photo-inducible toxic protein leads to structural and retinal activity impairment in wild-type mice. 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. Using AAV vectors, the glial fibrillary acidic protein promoter showed restricted expression in ciliary neurotrophic factor-activated Müller glial cells, and engineered glial fibrillary acidic protein promoter with several hypoxia-responsive elements has been developed to induce expression under hypoxic condition. 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...
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 capsids 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.
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. 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)18 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).
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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 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 1010 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 (e) 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) 1010 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 1010 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.
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 S2c) 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 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 10⁸ 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.

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 10⁸ 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.24–34 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.
In order to obtain efficient and persistent expression of a functional CRB1 protein in Müller glial cells, 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 (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. 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).

Figure 6 ShH10 and ShH10Y variants transduced human Müller glial cells in vitro. Transverse section of cultured retinas from donor eyes with 1010 genome copies of (a,b) AAV2/ShH10-CMV-GFP, (c) AAV2/ShH10Y-RBPL1-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; 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 109 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 Cα (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 109 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.

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 microglia 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
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minimal CMV promoter expression vector generated specific expres-
sion in Müller glial cells. (v) Both the SH10Y-CMV or SH10Y-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,
SH10Y and AAV9 capsids in combination with the RLBP1 and mini-
mal 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, and
this difference may be due to differences in transduction efficiency
and tropism of the native AAV6 had been previously reported
between rat and mouse retinas. Differences in transduction efficiency
and tropism of the native AAV6 had been previously reported
between rat and mouse retinas.16 Wherein the inner limiting mem-
brane barrier function of inner limiting membrane between the species.

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 obser-
vations in vascular smooth muscle.20 One interesting finding is that

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.

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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.20,28 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 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.23–26 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 previously44,45 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 CMV-CRB1 cDNA, the woodchuck posttranscriptional regulatory element and the bovine growth hormone polyadenylation sequence and subcloned in pAAV2 via BglI restriction sites in pAAV2-coCRB1 vectors. pAAV2-coCRB1 vectors. The 0.31-kb human CRK1 promoter was synthesized at GenScript and subcloned using SpeI and XhoI restriction sites in pAAV2-GFP vectors and pAAV2-coCRB1 vectors. The 0.52-kb human RLBP1 promoter27 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 BglII 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,23,24 synthesized at GenScript and subcloned using SpeI and XhoI restriction sites in pAAV2-GFP and pAAV2-coCRB1 vectors. The 0.31-kb human GRK1 promoter was synthesized at GenScript and subcloned using SpeI and XhoI restriction sites in pAAV2-GFP vectors and pAAV2-CRB1 vectors. pAAV2-coCRB1 vectors 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 SpeI and XhoI restriction sites in pAAV2-GFP vectors and pAAV2-coCRB1 vectors. AAV2 stocks were generated and purified as previously described.44,46 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-ShH10Y445F16,17 or pAAV9 capsid plasmid into human helper plasmid pDP6 (Plasmid Factory, Bielefeld, Germany) or pHelper and pAAV9 vectors. The human CD44 promoters were amplified by polymerase chain reaction from CD44prm-mouse and human Müller glial cells and express relatively large genes such as CRB1.

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 anesthesia. The 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 SPM (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.47 Retinas were injected under the lifted inner limiting membrane with 1 µl of 1 × 1010 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).

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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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