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Retinal transduction profiles by high-capacity viral vectors

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Abstract

Retinal gene therapy with adeno-associated viral (AAV) vectors is safe and effective in humans. However, the limited cargo capacity of AAV prevents their use for therapy of those inherited retinopathies (IRs) due to mutations in large (>5kb) genes. Viral vectors derived from Adenovirus (Ad), Lentivirus (LV) and Herpesvirus (HV) can package large DNA sequences but do not target efficiently retinal photoreceptors (PRs) where the majority of genes responsible for IRs are expressed.

Here, we have evaluated the mouse retinal transduction profiles of vectors derived from 16 different Ad serotypes, 7 LV pseudotypes, and from a bovine HV. Most of the vectors tested transduced efficiently the retinal pigment epithelium (RPE). We found that LV-GP64 tends to transduce more PRs than the canonical LV-VSVG albeit this was restricted to a narrow region. We observed more extensive PR transduction with HdAd1, 2 and 5/F35++ than with LV, although none of them outperformed the canonical HdAd5 or matched the extension of PR transduction achieved with AAV2/8.

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INTRODUCTION

Gene therapy has great potential for treatment for many inherited retinopathies (IRs), which are mostly monogenic blinding diseases. Viral vectors based on adeno-associated viruses (AAV) have demonstrated to be safe and efficient in clinical trials for Leber Congenital Amaurosis type 2 (LCA2) (reviewed in [1]). LCA2 is an IR due to mutations in *RPE65*, a gene expressed in the retinal pigment epithelium (RPE)[1]. Most of the genes mutated in IRs are expressed in photoreceptors (PRs)[2]. Notably, AAV serotypes 5, 7, 8 and 9 target PRs of various species efficiently[3-7]. However, the cloning capacity of AAV vectors is close to its wild-type genome length (~4.7kb) and this represents a limitation for gene therapies of several IRs due to defects in larger genes, such as Usher1B (USH1B, OMIM entry #276900), Stargardt disease 1 (STGD1, OMIM entry #248200), and LCA10 (OMIM entry #611755). Moreover, AAV limited capacity may preclude the delivery of cassettes with large promoters that maintain transgene expression levels close to physiological as well as cell type specificity.

Viral vectors that transduce post-mitotic cells with transgene capacity higher than AAVs include adenovirus (Ad, with a wild-type genome length of ~36kb), lentivirus (LV, with a wild-type genome length of ~9kb), and herpesvirus (HV, with a wild-type genome length of ~150kb). The retinal transduction potential of each of them has been evaluated *in vivo* in animal models: recombinant vectors based on Ad5, the most studied Ad serotype in the context of gene therapy, transduce the RPE primarily when injected subretinally in the mouse retina[8-10]. LV vectors are enveloped and have been pseudotyped predominantly with the vesicular stomatitis viral glycoprotein (VSVG) since it confers tropism for a broad range of tissues[11]. The LV-VSVG whether based on the human immunodeficiency virus (HIV-1)[12-15], simian (SIV)[16-17] or feline immunodeficiency viruses (FIV)[18-19] transduces the RPE mainly when delivered in adult retinas. LV-VSVG vectors based on the equine infectious anaemia virus (EIAV) have been reported to transduce PRs efficiently[20]. However, LV transduction appears mostly limited to PRs in the newborn retina[21-22] which has a less compact structure than the adult retina[23-24]. Only a few reports are available on retinal transduction mediated by HV vectors based on Herpes simplex 1 (HSV-1) which is mostly limited to the RPE following subretinal injection[25]. Thus, none of the high-capacity viral vectors tested so far transduce PRs efficiently.

Here, we aimed at identifying high-capacity Ad, LV and HV vectors with more robust PR transduction efficiency than those described so far.

Evolution has modeled the innate ability of viruses to deliver genes to a cell. Ads are divided into subgroups (A to F) and recognize different cellular receptors[26]. In addition, since Ad entry requires high-affinity binding to cell receptors via the knob portion of the fiber, the Ad capsid can be genetically engineered to exchange surface proteins such as the knob (K) or fiber (F) among different serotypes or subgroups, potentially giving rise to modified

tropism. Previous works reported that a modified Ad5 (Ad5ΔRGD[27-28]) and Ad5-based vectors containing heterologous fibers (Ad5/F37[29] and Ad5/F35[30-31]) are efficient for PR targeting. In this work, in addition to naturally-occurring heterologous Ad capsids, we have analyzed PR transduction by 8 vectors based on Ad5 mutant capsids[32-38] (Table 1).

On the other side, heterologous glycoproteins[11] can be easily exchanged among enveloped LV thus generating pseudotyped LV vectors. These may have different transduction properties than vectors with the native envelope glycoproteins. Indeed, it has been shown that HIV may be redirected to the endocytic pathway from its normal route of entry when pseudotyped with VSVG[39]. Thus, we have tested 7 HIV-1 based LV vectors pseudotyped with various non-native envelope glycoproteins (Table 1), seeking for improved PR transduction.

HV vectors present the highest cloning capacity and naturally infect neurons. They are enveloped, but the attachment and entry processes are complex and not fully understood yet, resulting in few pseudotypes available[40]. Here we have evaluated the ability of a naturally-occurring bovine herpes virus (Table 1), which belongs to a different subfamily (gammaherpesviridae) than HSV-1 (alphaherpesviridae) that was previously investigated in the retina[25].

RESULTS

We evaluated a total of 16 Ad vectors (8 derived from human and chimpanzee naturally-occurring Ad and 8 genetically-modified Ad vectors) for PR transduction after subretinal delivery in the adult mouse retina (see Table 1). The ubiquitous cytomegalovirus (CMV) promoter was included in all vectors to investigate broad cellular tropism on retinal histological sections. The vectors contained either EGFP or β-galactosidase (lacZ) as reporter genes, and were subretinally injected in pigmented C57BL/6 or in albino CD-1 or BALB/c mice. Ad vectors were injected at doses ranging from 10^6 to 10^9 viral particles (vp)/eye. Depending on the availability, we used either first-generation (Ad, E1/E3-deleted) or helper-dependent adenoviral vectors (HdAd, deleted of all viral genes and containing only the *cis* sequences required for vector genome encapsidation[41]), as indicated in Table 1. Because we were interested in vector tropism and eyes were harvested 3-14 days after vector injection, HdAd and Ad can be directly compared for transduction efficiency. This comparison is possible given the rapid onset of transgene expression of Ad vectors, in contrast to AAV vectors. Retinal sections were analyzed for direct EGFP fluorescence or after X-gal staining. We tested the serotype 1 HdAd (HdAd1), serotype 2 HdAd (HdAd2), and serotype 6 Ad (Ad6) vectors from subgroup C that recognize the CAR receptor for initial attachment, followed by internalization through an interaction between the RGD motif in the Ad penton base with $\alpha_v\beta_{3\text{-}5}$ integrins[26]. The genetically modified HdAd5/F35, HdAd5/F35++, Ad5/F3 and HdAd5/K3 contain fiber or knobs from serotypes reported to recognize the cellular CD46 receptor[26]. Vectors derived from Chimpanzee viruses were classified into subgroup B (AdC1 and ChAd30) or subgroup E (ChAd7 and 63) based on sequence alignments of the hypervariable regions of the hexon gene[42]. Notably, the Ad receptors CAR, α_v-integrin and CD46 are expressed by PRs[30]

Consistent with previous published studies[8-10], we found that Ad5 targets mostly the RPE and Müller cells along with some PRs following subretinal injection (Figure 1 and Figure 2). Similar to Ad5, most of the Ad vectors tested targeted efficiently the RPE and some PRs (Figure 1 and Figure 2). However, HdAd1, HdAd2 and HdAd5/F35++ transduced the outer nuclear layer (ONL), which contains PRs, at levels that in some eyes appeared higher and more extensive than with HdAd5 (see Figure 1 and Table 1 for the vector descriptions). No β-galactosidase staining was detected in the retinas of control PBS-injected animals (data not shown).

To confirm that HdAd1, HdAd2 and HdAd5/F35++ transduce adult mouse PR efficiently as it appears using the CMV-lacZ expression cassette, we produced HdAd vectors containing the PR-specific rhodopsin (Rho) promoter which drives the expression of the EGFP reporter gene. The HdAd vectors were all injected subretinally in C57BL/6 mice at a dose of 5.5×10^8 genome copies (GC)/eye, and compared to HdAd5 and AAV2/8, containing the same expression cassette and injected at the same dose. Eyes were harvested 14 days or one month later and retinas cryosectioned for further histological analysis. HdAd1, HdAd2 and HdAd5/F35++ showed an extension of PR transduction which was lower than with HdAd5, which was similar to that of AAV2/8 (see Figure 3a). The pattern of PR transduction mediated by HdAd1 and 2 was patchy, as observed in a previous work using a different Ad vector containing a Rho-EGFP cassette[28]. HdAd5-mediated PR transduction appeared patchy in some eyes, and continuous in others. In Figure 3a and b, the four best transduced retinas with each serotype are shown. HdAd1 and 2 seemed to transduce PRs more intensely than HdAd5, although along a narrower area.

LV vectors have a larger cloning capacity compared to AAVs. However, VSVG-pseudotyped LV vectors efficiently transduced newborn[14-15] but not adult PRs[12-15]. Here, we have evaluated six HIV-1-based vectors pseudotyped with various envelopes (Table 1) and compared them to the more studied VSVG. In previous works, the mokola pseudotype was shown to transduce RPE[13,43] after subretinal delivery, and RRV to transduce the RPE of Sprague-Dawley rats[44].

LCMV and GP64 pseudotyped vectors transduced the RPE of Wistar rats, although the vectors were based on a simian (SIV) and bovine immunodeficiency viruses (BIV), respectively[45-46]. To our knowledge, neither the EbolaΔO nor the JSRV envelopes had been previously tested in the retina. All vectors contained the CMV-EGFP expression cassette, they were injected subretinally in C57BL/6 mice and retinas were harvested 14 days later. The injected doses range between 2×10^3 and 1×10^5 transducing units (TU)/eye (Figure 4) and they were the maximum allowed for each viral preparation given that 1 μl is the maximum volume that can be delivered subretinally in adult mice[47]. All vectors, excluding JSRV, transduced the RPE (Figure 4). The baculovirus GP64-pseudotyped LV (6×10^4 TU/eye) appeared to drive a more robust PR transduction (Figure 4, see inset) than the standard VSVG (1×10^5 TU/eye), which transduced PRs in one eye out of five (not shown). However, the transduction was restricted to a narrow area. To evaluate whether LV-GP64 drives PR transduction to levels that can be attractive for therapeutic applications, we generated LV-GP64 vectors containing the Rho-EGFP expression cassette. C57BL/6 retinas were subretinally injected with 4.5×10^5 GC/eye of either LV-GP64 or -VSVG vectors for

comparison and analyzed at 2 weeks post-injection. No EGFP fluorescence was observed with either vector (not shown).

Among the various high-capacity vectors available, we finally evaluated the herpevirus-based BoHV-4-CMV-EGFP. At 2, 7 and 28 days after subretinal injection of 8×10^6 GC/eye, BoHV-4-CMV-EGFP resulted in efficient RPE but modest PR transduction in adult C57BL/6 retinas (Figure 5). To properly evaluate the specific PR transduction of BoHV-4 vectors, we produced one containing the Rho-EGFP cassette and we injected it subretinally in C57BL/6 mice at a dose of 2.5×10^8 GC/eye. AAV2/8 Rho-EGFP was injected in contralateral eyes at 5×10^8 GC/eye for comparison. Retinal cryosections analyzed one month later by fluorescence microscopy showed that only scattered PRs were transduced (Figure 5) by BoHV-4-Rho-EGFP in contrast to the wide transduction achieved with the AAV2/8 vector.

To test the longevity of mouse retinal transduction mediated by high-capacity viral vectors, we injected subretinally C57BL/6 mice with vectors expressing EGFP under the control of the CMV promoter to monitor transgene expression *in vivo* by non-invasive fluorescent fundoscopy. Fluorescence was detected for 20 months in HdAd5-injected retinas (Figure 6), which almost equals the mouse lifespan and is the most sustained expression reported thus far in the retina for this vector. EGFP expression was maintained for up to: i. 14 months post-injection in the eyes injected with LV-VSVG (Figure 6), -GP64, -RRV or -EbolaΔO (not shown); ii. 4 months post-injection in those containing BoHV-4. These were the latest time points analyzed (Figure 6).

To investigate the safety of subretinal delivery of high-capacity viral vectors, we monitored retinal electrical activity by Ganzfeld electroretinogram (ERG) (Figure 7) before sacrificing the animals at the time points of the last ophthalmoscopy (shown in Figure 6). The maximum b-wave amplitude values in retinas that were injected with HdAd5, LV-VSVG, -RRV, -EbolaΔO, -GP64 or BoHV-4 are shown in Figure 7, and compared with the maximum b-wave amplitude of PBS-injected or non-injected controls of similar age. The amplitude of the b-waves were not significantly lower in eyes injected with viral vectors than in control eyes.

DISCUSSION

In the present work, we compared a large series of 24 different high-capacity viral vectors for their efficiency at transducing the adult mouse retina following subretinal delivery. Finding an efficient vector, capable of carrying large genes, would be a significant step forward for the development of gene therapy approaches for retinal blinding conditions due to defects in large genes such as STGD1, LCA10, and USH1B and for which no therapy is currently available.

We investigated 16 different Ad vectors and found that HdAd5, 1, 2 and 5/F35++ transduced PRs. AdC1, Ad5/F3 and HdAd5/6 targeted the RPE exclusively, and the rest of the tested vectors transduced RPE along with some PRs. Previous reports had shown that Ad5/F35[30-31] and Ad5ΔRGD[27-28] are efficient for PR targeting. However, in our hands

these vectors did not perform better than Ad5 in terms of transduction efficiency when containing the ubiquitous CMV promoter. The PR transduction levels of HdAd1, HdAd2 and HdAd5/F35++ appeared higher than those of HdAd5 when using the CMV-lacZ cassette. Even if HdAd 1, 2 and 5 belong to the same C subgroup and recognize CAR receptor for initial binding, it has been reported that subgenus C Ads have differences in the fiber knob region[48], which could account for different affinities to a target receptor. Differences in transduction capabilities among subgroup C Ads were also found in liver gene transfer studies where Ad2 was found to be a weaker transducer than Ad5[49]. Ad2 retinal transduction had been studied previously, showing β-galactosidase expression in RPE and ganglion cells[50], but not in PRs. This different outcome could be explained by the short 24 hour post-injection time-point that was evaluated by the authors whereas in our experiments PR expression was analyzed four days after injection. However, the use of HdAd1, HdAd2, and HdAd5/F35++ vectors containing the Rho-EGFP cassette resulted in levels of ONL transduction which were overall similar to the most commonly used HdAd5. The transduction differences observed using the CMV-lacZ and the Rho-EGFP expression cassettes could be due to the presence of “pseudotransduction” after injection of lacZ-expressing vectors. Some β-galactosidase enzyme may diffuse from the RPE to the inter-photoreceptor matrix (IPM) and to the ONL, as previously suggested[13, 19, 51].

Among the various LV pseudotypes tested, LV-GP64 appeared to have a greater PR transduction efficiency than LV-VSVG. However this was limited to a narrow area. The interaction of GP64 with phospholipids on the cell surface was first reported to play a role in baculovirus infection into mammalian cells[52] and lately, binding to heparan sulfate was also found to be involved[53]. Both pathways may have played a role in LV-GP64-mediated retinal transduction. In our case LV-Mokola failed to transduce PRs as it has been previously shown [13, 43]. In the case of LV-LCMV, even if the RPE was transduced it is possible that we have underestimated its PR transduction properties since its infectious titer could not be assessed on HT-1080 cells, the target cells used to titer the LV vectors used in this study.

Vectors derived from herpesvirus are interesting for PR targeting because they naturally infect neurons[11]. Studies with HSV-1 containing the CMV-EGFP expression cassette showed that only the RPE was transduced in most cases[25] although in one report PR outer segments were also found to be transduced[51]. Here, we evaluated whether the vector based on the bovine BoHV-4, isolated in Europe from respiratory and ocular diseases[54], could have an innate tropism for PRs. This vector is not pathogenic, it is unlikely to be oncogenic, and can infect several cell types from different animal species[55-56]. Here, we show that BoHV-4-based CMV-EGFP vectors efficiently target RPE and some PRs as early as 2 days post injection and up to one month post injection (the latest time point evaluated) (Figure 5). When PR-specific Rho-EGFP cassette was used, very rare PRs were positive for transgene expression (Figure 5). It is possible that the strong transcriptional activity of the CMV promoter is requested for PR transduction by a BoHV-4-based vector.

The large capacity vector platforms we have used result in long-term transgene expression in the retina. We show that EGFP expression mediated by HdAd5 is present by fundus ophthalmoscopy for 20 months after injection (Figure 6), while first generation Ad5 transduction shut s off at 3 months (not shown). Retinal transduction mediated by HdAd5

was previously found to be sustained for up to 12 months[57]. Here, we report a duration that largely exceeds prior studies (20 months) that is close to the lifespan of a mouse and is consistent with previous studies showing multi-year HdAd-mediated expression in the liver of animal models[58]. Consistent with previous studies[59], we also show that both LV-VSVG and -GP64 express EGFP long-term in the mouse retina for up to 14 months post-injection. *In vivo* retinal transduction mediated by HSV-1 vectors has been transient with loss of transgene expression by 6 weeks post-injection[25], representing a drawback for their applications in genetic diseases requiring long term expression of the therapeutic gene. In contrast, we show that subretinal delivery of BoHV-4-based vectors results in stable EGFP fluorescence detectable up to 4 months post-injection by ophthalmoscopy. This finding is the first report of long-term retinal transduction mediated by an HV vector.

Subretinal injection of HdAd5, LV-VSVG, -GP64, -RRV- and EbolaΔO or BoHV-4 was not associated with significant reduction of the ERG b-wave amplitude when compared to control eyes (see Figure 7). However, additional studies including a careful histopathological examination are required to rule out potential toxicity resulting from subretinal delivery of high-capacity vectors. It is somehow puzzling that none of the high-capacity vectors investigated significantly outperforms the others, despite the wide difference in their capsid proteins. In the case of Ad, we have tested capsids recognizing different receptors, covering almost all the reported repertoire (CAR, $\alpha_v\beta_{3\text{-}5}$ integrins, CD46, and others) which are localized in the adult mouse ONL[30]; yet we were not able to detect levels of PR transduction dramatically higher than those achieved with the canonical HdAd5. The larger size of the high-capacity particle we have investigated compared to AAV might play a role. Indeed, several AAV serotypes (AAV5, AAV7, AAV8 and AAV9) efficiently transduce PRs[4], and while the diameter of an AAV particle is around 25nm[60], the Ad, LV and HV viral vector particles all have diameters >80nm[61-63]. Thus, diffusion of larger viral particles towards PRs may be limited by anatomical barriers, which might prevent their interaction with their cognate receptors[64-65]. In the retina, hindrance may be exerted by the IPM and the outer limiting membrane (OLM), which may limit access to PR bodies from the subretinal space where the large vectors are injected. This is indirectly suggested by the higher levels of PR transduction observed for both Ad and LV vectors in the developing mouse retinas where these barriers may be more permissive to the vectors. Indeed, Ad5 transduces efficiently PRs when delivered to neonatal retinas[8, 24] and LV-VSVG transduced[14-15] and rescued[21, 66] PR degeneration when administered to neonatal but not to mature mouse PRs[12, 14-15]. As a matter of fact, the IPM was found to be an effective barrier that impaired LV-VSVG from transducing the adult PR layer[23]. Notably, a LV vector based on the equine infectious anemia virus (EIAV), which was originally shown to transduce[67-68] and rescue neonatal mouse PRs affected by STGD1[22] has been recently reported to transduce adult rabbit and macaque PRs[20] with satisfactory safety profile. However, thus far there is no evidence that these levels of adult PR transduction are therapeutically relevant. The two clinical trials which are currently being carried out for both Usher1B (NCT01505062, NCT02065011) and STGD1 (NCT01367444, NCT01736592) using EIAV vectors will help to understand whether this class of vectors effectively transduces adult human PRs. Weakened physical barriers due to PR loss are observed in animal models of retinal degeneration such as *rd* and *Rho*−/−[24, 69]. PR transduction

mediated by Ad5 and LV-VSVG was shown to be more efficient in *rd* than in wild type retinas[15, 24], while more Müller cells were transduced by LV-Mokola in *Rho*−/− than in control retinas[69]. These results suggest that disruption of anatomical barriers may improve Ad- and LV-mediated penetration and thus transduction[24, 69], or as well indicate that retinal remodeling during disease may modify the tropism of high-capacity vectors[69]. A recent work carried out in adult mouse retinal explants, which lack physical barriers since they are compromised during the process of explantation, have shown that LV-VSVG is not able to transduce mouse PR[70]. Surprisingly, when a human retinal explant was transduced with LV-VSVG, limited PR transduction was observed, which suggests that transduction may also be species-dependent [70].

In summary, among the 24 different high-capacity viral vectors investigated, we found HdAd1, 2 and 5/F35++ to transduce mouse PRs with similar efficiencies to HdAd5, but with an overall PR transduction efficiency that appears lower than the one of AAV2/8 that remains the golden standard among naturally-occurring AAV serotypes for inherited PR diseases. Recently, effective gene transfer to mouse PRs has been shown with dual AAV vectors which significantly expand AAV cargo capacity in the retina[71-72]. In the future, it will be interesting to compare side-by-side the PR transduction efficiency of high-capacity HdAd-based vectors to that of dual AAV vectors, which allow efficient PR transduction although at levels which are lower than those obtained with normal size AAV vectors[71-72].

MATERIALS AND METHODS

First Generation Ad vectors

Chimpanzee ChAd7, ChAd30 and ChAd63 preAd plasmid were obtained as described previously[42]. Ad5, AdC1 and Ad6 plasmids were constructed by following the same procedure as described in[42] starting from wild type viruses obtained from ATCC. These plasmids were first digested with PmeI to release the viral ITRs. Then, 3-5×10^6 HEK293/PER.C6 cells were transfected with 10 μg of Ad viral plasmids using Lipofectamine (Invitrogen). This generated recombinant Ad vectors which were then expanded using 2×10^9 cells. Purification was performed by two-step cesium chloride gradient[42]. The Ad5, Ad6, AdC1, ChAd7, ChAd30 and ChAd63 vectors containing the CMV-EGFP expression cassette were provided by S. C.

Ad5/F2+pK[36], Ad5/F2+RGD[36] and Ad5ΔRGD[37] viruses were constructed using a combination of conventional cloning and bacterial RecA-mediated recombination. Ad5-, Ad5/F2+pK- and Ad5/F2+RGD-CMV-lacZ plasmids were digested with PacI and recovered as viruses in 293 cells. Virus were propagated, purified and titered as described[36]. R. J. P provided these vectors. Ad5ΔRGD[37] was transfected and recovered as a virus in 293 cells, banded in CsCl gradients and collected, dialyzed, and aliquoted as described[37]. This vector was provided by D. M. S.

The Tigem Adenoviral Vector Core (P.P. and N.B.P.) expanded in 293 cells seed vectors provided by other collaborators, which included Ad5-CMV-lacZ, Ad5ΔRGD-CMV-EGFP, Ad5/F2+pK-CMV-lacZ and Ad5/F2+RGD-CMV-lacZ.

The E1-deleted Ad vector FGAd5F3-CMV-LacZ[34] was a generous gift from Dr. David T. Curiel, Washington University, St. Louis, MO, USA (and provided to us by P. N.).

The viral particles (vp)/mL titer of the first generation Ad vectors, obtained by measuring the absorbance at 260nm following virion lysis and correction for vector genome size, was provided for each vector by our collaborators.

Helper-dependent Ad vectors

All HdAd vectors were produced in 116 cells as previously described[41]. The helper virus AdNG163[73] was used to produce HdAd5-CMV-lacZ and HdAd5-Rho-EGFP-WPRE-bGHpA. The helper virus Ad2LC8cCARP[74] was used to produce HdAd2-CMV-LacZ and HdAd2-Rho-EGFP-WPRE-bGHpA. The helper virus Ad1LC8cCEVS-1[75] was used to produce HdAd1-CMV-LacZ and HdAd1-Rho-EGFP-WPRE-bGHpA. The helper virus AdHPBGF35[33] was used to produce HdAd5F35-CMV-LacZ. The helper virus AdNG163-5F35++ (modified as described in [35]) was used to produce HdAd5F35++CMV-LacZ and HdAd5F35++-Rho-EGFP-WPRE-bGHpA. The helper virus Ad5/3-NG163[32] was used to produce HdAd5K3-CMV-LacZ. The helper virus AdNG177[38] was used to produce HdAd5/6-CMV-LacZ. The HdAd vectors were provided by P. N. The Tigem Adenoviral Vector Core supplied HdAd5-CMV-lacZ and expanded HdAd5-Rho-EGFP-WPRE-bGHpA.

For the generation of HdAd-Rho-EGFP-WPRE-bGHpA vectors, the Rho-EGFP-WPRE-bGHpA cassette was PCR-amplified from the pAAV2.1-Rho-EGFP[4] plasmid using primers with AscI restriction enzyme site at each end. The primer sequences are: Fw 5′-GGC GCG CCC TAG CAG ATC TTC CCC ACC TAG CC-3′ and Rv 5′-GGC GCG CCT CCC CAG CAT GCC TGC TAT TG-3′. The PCR product was inserted in pCR®2.1-TOPO® (Invitrogen, S.R.L., Milan, Italy), sequenced and then sent to Eurofins MWG Operon (Ebersberg, Germany) that performed the subcloning in the adenoviral pΔ25.3E4 plasmid (derived from pΔ28E4[76], and generously provided by the Tigem Adenoviral Vector Core). For HdAd-Rho-EGFP-WPRE-bGHpA vectors, titers in genome copies (GC)/mL were determined by PCR quantification using TaqMan (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). The FAM-labeled TaqMan probe used was bGH Prb 5′-TCC CCC GTG CCT TCC TTG ACC-3′ and the primers were bGH Fwd 5′-TCT AGT TGC CAG CCA TCT GTT GT-3′ and bGH Rev 5′-TGG GAG TGG CAC CTT CCA-3′.

LV vectors

The HIV-1-based lentiviral vectors used for this work were produced through a quadruple transfection of a shuttle and two backbone plasmids (pCMV/HIV *gag-pol*, pCMV/Rev, and pHIVψeGFP)[77] as well as an envelope plasmid that changed each time to generate each of the different 7 pseudotypes (see Table 1). The DNA was transfected into 15-cm diameter dishes of 80% confluent HEK293T cells using calcium phosphate transfection. After 12 h the cells were washed, and fresh medium was added (DMEM, 2% [vol/vol] FBS, 1% [vol/vol] Pen-Strep). Supernatants were collected at 24, 36, 48, 60, and 72 h posttransfection and frozen at −80°C. The supernatants were thawed, filtered through a 0.45-μm-pore-size filter, and pelleted by a 16-h centrifugation step (7,700 × *g* at 4°C in a Sorvall GSA rotor). The viral pellet was resuspended in DMEM for an approximate 200-fold concentration, and the

virus was stored at −80°C until use. Titers were calculated on HT-1080 cells (ATCC) and given in transducing units (TU)/mL.

The Rho-EGFP lentiviral plasmid was generated as follows: Rho-EGFP was amplified by PCR from pAAV2.1-Rho-EGFP[4] using the primer Fw 5′-AAT TCA ATT GCT AGC AGA TCT TCC CCA CCT AGC CAC-3′ which contained the MfeI restriction enzyme site and the primer Rv 5′-TTA ACT CGA GCT TGT ACA GCT CGT CCA TGC CG-3′ that contained the XhoI site. The PCR product was subcloned in pCR®2.1-TOPO® (Invitrogen, S.R.L., Milan, Italy) and sequenced. The Rho-EGFP fragment was then digested with MfeI/XhoI, purified and ligated with the LV plasmid backbone. The backbone consisted of the pSMPUW Universal Lentiviral Expression Vector (Promoterless) VPK211 (Cell Biolabs, CA, USA) digested with EcoRI/SalI (sharing compatible cohesive ends with MfeI and XhoI of the insert, respectively). The obtained LV-Rho-EGFP plasmid was then sequenced and sent to the Gene Transfer Vector Core (University of Iowa, IA, USA) for production of LV vectors pseudotyped with either VSVG or GP64 envelope glycoproteins. Physical titers (GC/mL) of vectors with the Rho-EGFP cassette were obtained with the Lentivirus qPCR Titer Kit (Applied Biological Materials Inc, Richmond, BC, Canada). Transducing/infectious titers on HT1080 cells could not be calculated since the Rho promoter is inactive in this cell type. The LV vectors were provided by B. L. D.

BoHV-4 vectors

BoHV-4-A[78], BoHV-4-A-EGFPΔTK[55] and BoHV-4-A-Rho-EGFP-WPREΔTK were propagated by infecting confluent monolayers of Madin Darby Bovine Kidney [(MDBK) ATCC, CCL-22], bovine embryo kidney [(BEK) provided by Dr. M. Ferrari, Istituto Zooprofilattico Sperimentale, Brescia, Italy; (BS CL-94)] or BEK expressing cre recombinase (BEK*cre*)[78] at a multiplicity of infection (M.O.I.) of 0.5 $TCID_{50}$ (50% tissue culture infectious dose) per cell and maintained in minimal essential medium (MEM; SIGMA) with 2% FBS for 2 h. The medium was then removed and replaced with fresh MEM containing 10% FBS. When approximately 90% of the cell monolayer exhibited cytopathic effect (CPE) (72 h post infection), the virus was prepared by freezing and thawing cells three times and pelleting the virions through 30% sucrose, as described previously[79]. Virus pellets were resuspended in cold MEM without FBS. $TCID_{50}$ were determined by limited dilution in MDBK cells. The physical titer of the CMV-EGFP vector was calculated as GC/mL by RT-PCR with a probe recognizing EGFP. The sequence of the FAM-labeled TaqMan probe used is: 5′-TTC AAG TCC GCC ATG CCC GAA-3′ and that of the primers is: Fw 5′-CCA CAT GAA GCA GCA CGA CTT-3′; Rv 5′-GGT GCG CTC CTG GAC GTA-3′.

For BoHV-4-A-Rho-EGFP-WPREΔTK cloning, Rho-EGFP-WPRE-bGHpA was excised from the pAAV2.1-Rho-EGFP plasmid[4] with NheI/XhoI, blunted with T4 DNA polymerase and sub-cloned in a SmaI digested pINT2 shuttle vector[55], a plasmid vector containing two BoHV-4 TK gene sequences, to obtain pTK-Rho-EGFP-WPRE-TK. Next, TK-Rho-EGFP-WPRE-TK was cut out from the plasmid backbone and electroporated in SW102 *E. coli* containing the pBAC-BoHV-4-A-KanaGalKΔTK[78]. pBAC-BoHV-4-A-KanaGalKΔTK is a BoHV-4 genome clone coming from a non-pathogenic strain of BoHV-4 isolated from the

milk cell fraction of an healthy cow, whose genome was cloned as a Bacterial Artificial Chromosome (BAC), pBAC-BoHV-4-A; and where its TK locus was targeted with a KanaGalK selectable cassette[78]. TK-Rho-EGFP-WPRE-TK electroporated *E. coli* containing the pBAC-BoHV-4-A-KanaGalKΔTK were first heat-induced, then negatively selected on deoxygalactose minimal plates. The resulting clones were additionally negatively selected with medium containing kanamycin. Then, to obtain pBAC-BoHV-4-A-Rho-EGFP-WPREΔTK, the retargeting was performed to the same site to replace the KanaGalK cassette with the TK-Rho-EGFP-WPRE-TK cassette. Retargeted clones (pBAC-BoHV-4-A-Rho-EGFP-WPRE ΔTK) were distinguished from the un-retargeted control clone (pBAC-BoHV-4-A-KanaGalKΔTK), by HindIII digestion. The selected clones' stability was assessed by serially passaging over 25 days and analysis by HindIII restriction enzyme digestion. Infectious BoHV-4-A-Rho-EGFP-WPREΔTK virus was reconstituted in both BEK and BEK*cre* cells[78], which enabled the depletion of the floxed BAC cassette from pBAC-BoHV-4-A-Rho-EGFP-WPRE ΔTK. Furthermore, BoHV-4-A-Rho-EGFP-WPREΔTK, BoHV-4-A-EGFPΔTK and BoHV-4-A were compared in terms of replication and no differences were observed among them. The Rho-EGFP vector physical titer was calculated by RT-PCR with a probe recognizing the bGHpA sequence, as explained in the Materials and Methods section for the Ad vectors. All BoHV-4 vectors were provided by G. D.

AAV Vectors

AAV2/8 vectors were produced by the TIGEM AAV Vector Core (Napoli, Italy) using the pAAV2.1-CMV-EGFP[80] and -Rho-EGFP[4] expression plasmids, and the packaging pAAV2/8 plasmid[81]. Vectors were produced by triple transfection of 293 cells followed by cesium chloride purification[43]. For each viral preparation, physical titers (GC/mL) were determined by both PCR quantification using TaqMan (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and dot-blot analysis[82].

Animal Procedures and Vector Administration

Ethics Statement—All studies on mice were conducted in strict accordance with: i. the institutional guidelines for animal research; ii. the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research; iii. the Italian Ministry of Health regulation for animal procedures. All procedures on mice were submitted to the Italian Ministry of Health; Department of Public Health, Animal Health, Nutrition and Food Safety on October 17th, 2011. The Ministry of Health approved the procedures by silence/consent, as per article 7 of the 116/92 Ministerial Decree. Surgery was performed under anesthesia and all efforts were made to minimize suffering.

Mice—Four to five week-old male C57BL/6, CD-1 or BALB/c mice (Harlan, S. Pietro al Natisone, Italy) were anesthetized with an intraperitoneal injection of 2 mL/100 g body weight of avertin [1.25% w/v of 2,2,2-tribromoethanol and 2.5% v/v of 2-methyl-2-butanol (Sigma-Aldrich, Milan, Italy)][83], then viral vectors were delivered subretinally via a trans-scleral trans-choroidal approach as described[47]. A volume of 1 μl of viral vectors diluted in phosphate buffered saline (PBS) was delivered subretinally.

Histological Analysis

Mice were sacrificed and their eyeballs were harvested and fixed overnight by immersion in 2% or 4% paraformaldehyde. Before harvest, the temporal aspect of the sclerae was marked by cautery to orient the eyes with respect to the injection site at the moment of the inclusion. The eyeballs were cut so that the lens and vitreous could be removed leaving the eyecup intact. Mice eyecups were infiltrated with 30% sucrose for cryopreservation and embedded in tissue freezing medium (O.C.T. matrix, Kaltek, Padua, Italy). For each eye, 150 to 200 serial sections (10 μm-thick) were cut along the horizontal plane and the sections were progressively distributed on 10 slides so that each slide contained 15 to 20 sections, each section representing the whole eye at different levels. The sections were stained with 49,69-diamidino-2-phenylindole (Vectashield, Vector Lab Inc., Peterborough, UK) if the vector contained EGFP as a reporter protein. If the viral vector contained lacZ as the reporter gene, the cryosections were treated for X-gal staining (see below). EGFP and X-gal were monitored with a Leica DM 5000B microscope at various magnifications.

X-gal staining

Cryosections were fixed in 0.5% glutaraldehyde in PBS+ buffer (1 mM CaCl2 and 0.5 mM MgCl2 in PBS [pH 7.4]) for 10 minutes at room temperature. After fixation, three 5-minute rinses were performed with PBS+ buffer. Sections were stained using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM MgCl2 and 0.5 mg/mL X-gal powder in PBS+ buffer) at room temperature with protection from light. The reaction was stopped when staining was observed in the sample cryosections, and were analyzed only if the negative control (cryosections from non-injected or PBS-injected retinas) were X-gal negative. After they were X-gal stained, the sections were counterstained with eosin, dehydrated and coverslipped.

Fundus Photography

Fundus imaging was performed from 4 days to 20 months post viral injection (PI) using a Topcon TRC-50IX retinal camera connected to a charge-coupled-device NikonD1H digital camera (Topcon Medical System). Mice ocular fundi were photographed using a 300 W flash with a fluorescein filter, after dilating the pupils with a drop of tropicamide 1% (Visufarma, Roma, Italy),.

Electroretinogram Recordings

Electrophysiological recordings in mice were performed as detailed[84].

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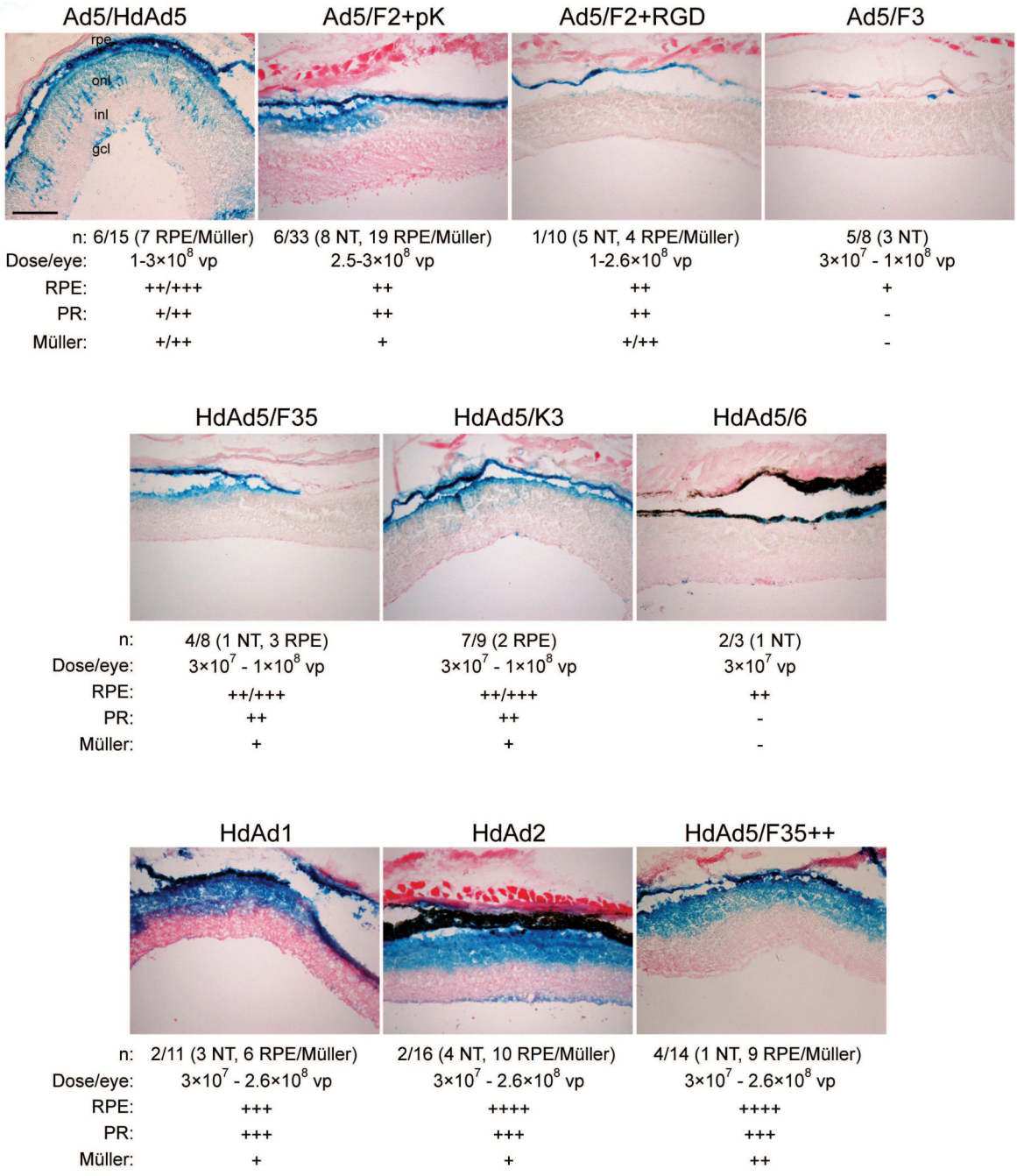

Figure 1. Mouse retinal transduction after subretinal delivery of adenoviral vectors

Ad vectors containing the CMV-lacZ cassette were delivered subretinally in adult CD-1, BALB/c or C57BL/6 mice. Retinas injected with Ad vectors were analyzed 4-14 days later. Magnification=20×; scale bar=100μm. The vector serotypes used are indicated above each panel. The n of retinas, the dose of each vector and the relative transduction observed in photoreceptors, retinal pigment epithelium and Müller cells are indicated below each panel. Abbreviations: rpe: retinal pigment epithelium; onl: outer nuclear layer; inl: inner nuclear

layer, gcl: ganglion cell layer; n: number of eyes; vp: viral particles; PR: photoreceptors; NT: not transduced.

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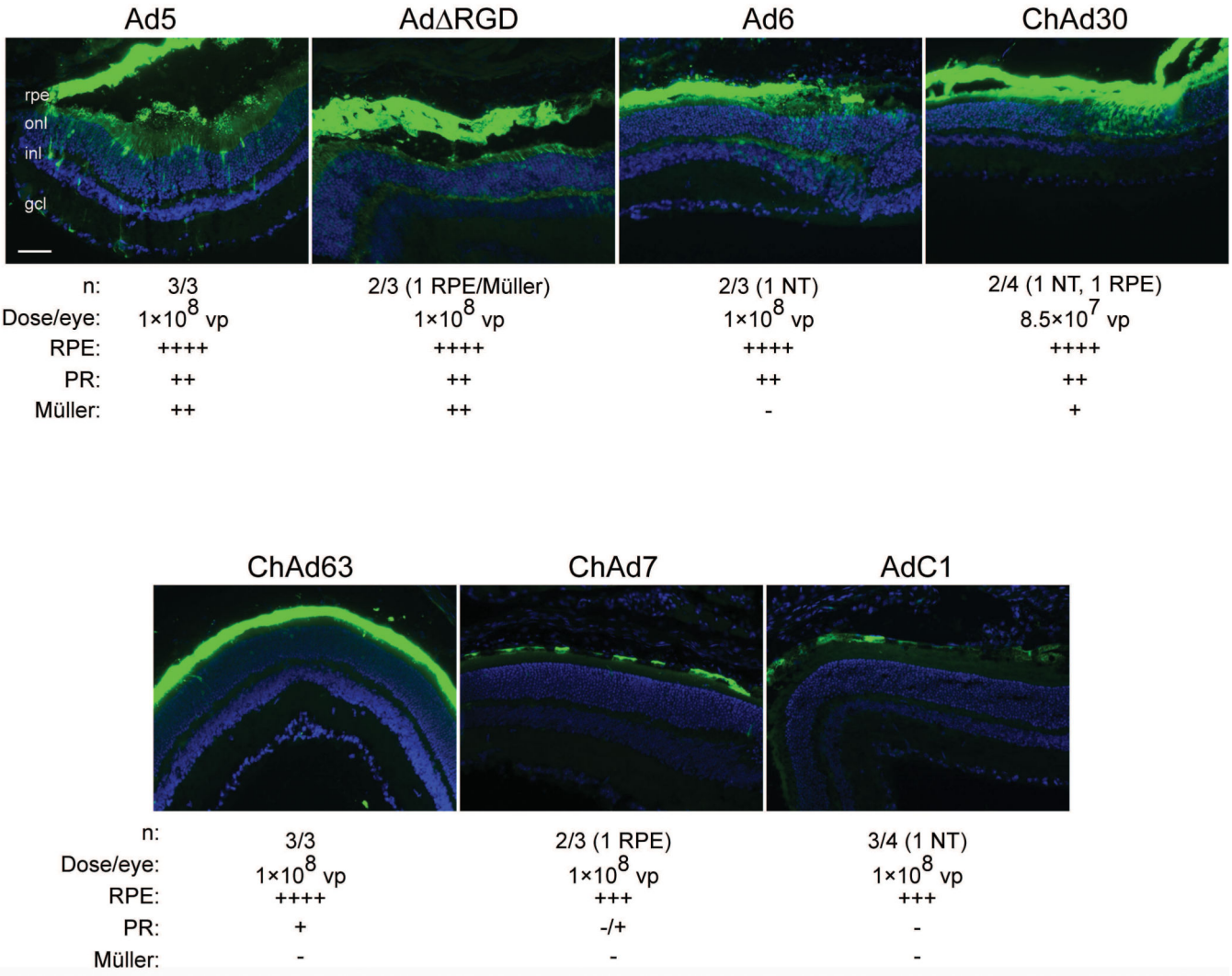

Figure 2. Mouse retinal transduction after subretinal delivery of adenoviral vectors

Ad vectors containing the CMV-EGFP cassette were delivered subretinally in adult C57BL/6 mice. Retinas were analyzed 3-6 days later. Magnification=20×; scale bar=50μm. The vector serotypes used are indicated above each panel. The n of retinas, the dose of each vector and the relative transduction observed in photoreceptors, retinal pigment epithelium and Müller cells are indicated below each panel. Abbreviations: rpe: retinal pigment epithelium; onl: outer nuclear layer; inl: inner nuclear layer, gcl: ganglion cell layer; n: number of eyes; vp: viral particles; PR: photoreceptors; NT: not transduced.

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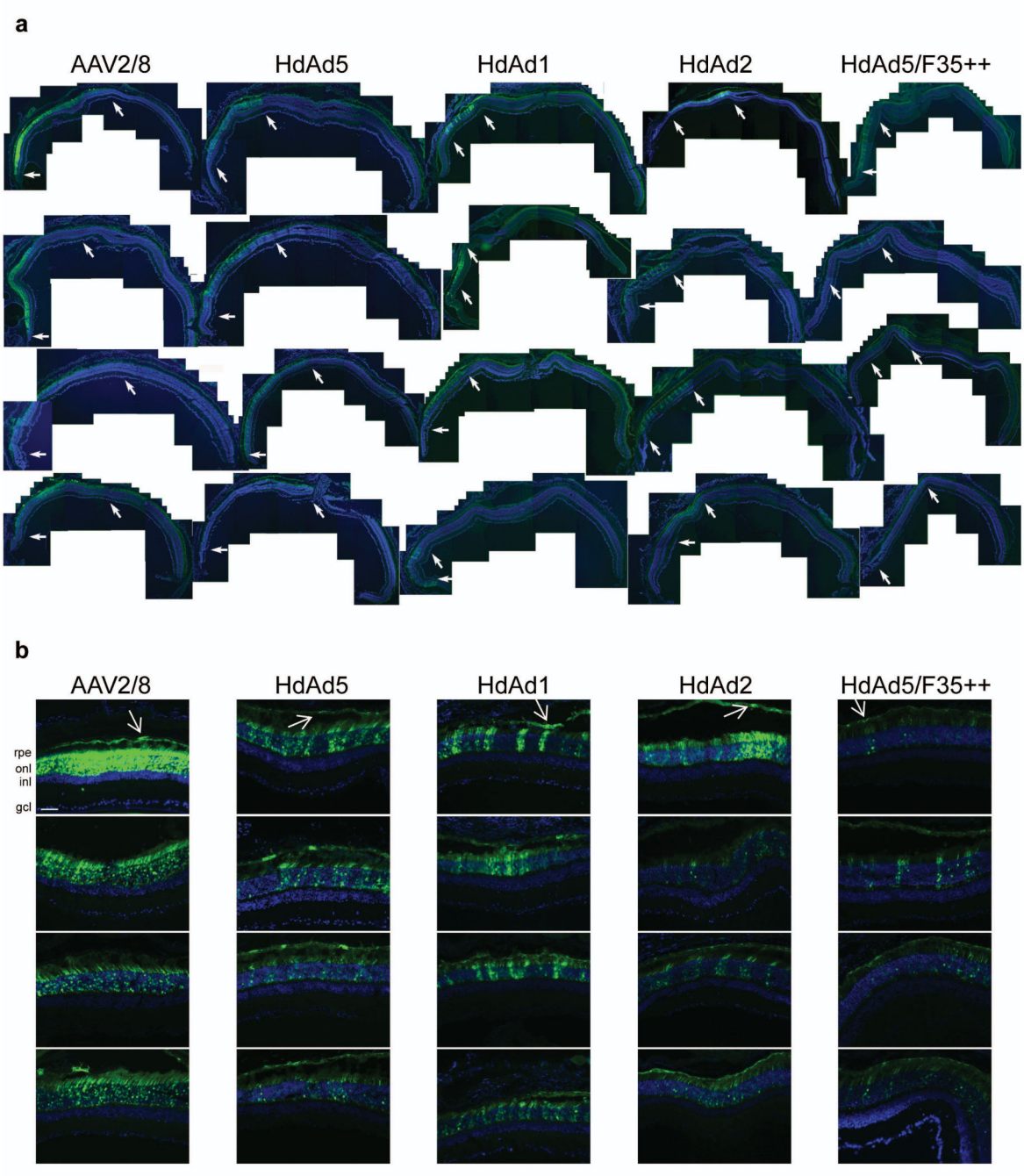

Figure 3. Mouse photoreceptor transduction after subretinal delivery of HdAd5, HdAd1, HdAd2, HdAd5/F35++ and AAV2/8

Vectors expressing EGFP from the PR-specific rhodopsin promoter where injected subretinally in adult C57BL/6 mice (5.5×10^8 GC/eye), and analyzed 14 days or one month post injection. **a)** Montage of whole retinal cross-sections from the four best transduced eyes for each serotype. The extension of PR transduction is indicated by arrows. The n of retinas showing a transduction pattern similar to those shown in the pictures are n=8/12 for AAV2/8, n=6/8 for HdAd5, n=8/9 for HdAd1, n=6/9 for HdAd2 and n=5/8 for HdAd5/F35++. In the remaining retinas, the ONL transduction was either significantly lower than in the

retinas shown in the pictures or undetectable. Magnification=10×. **b)** High-magnification images of the retinas shown in a). Magnification: 40×; scale bar=25μm. A faint, off-target EGFP signal is observed in the RPE of all retinas (arrows). Abbreviations: rpe: retinal pigment epithelium; onl: outer nuclear layer; inl: inner nuclear layer; gcl: ganglion cell layer.

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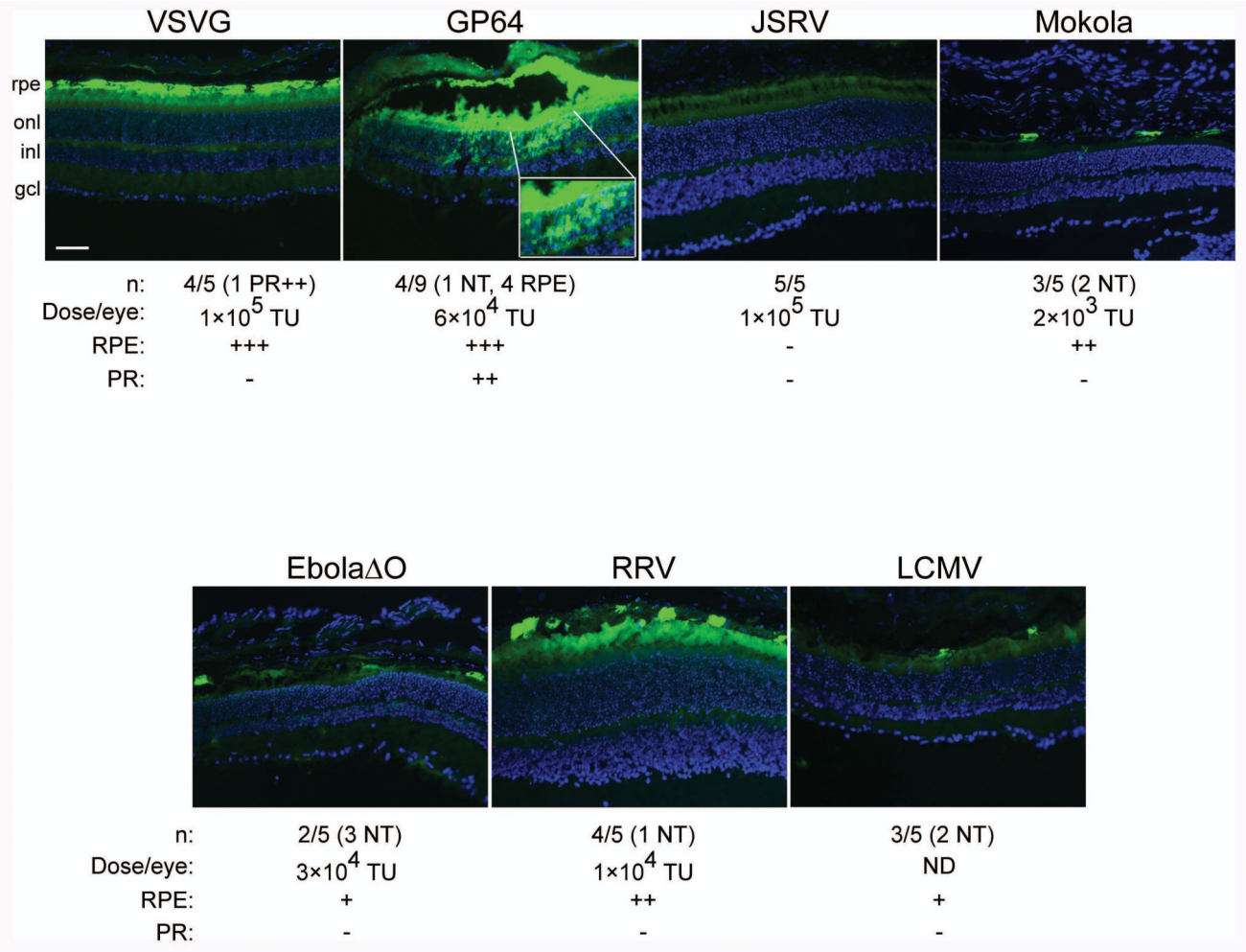

Figure 4. Mouse retinal transduction after subretinal administration of LV vectors

LV vectors containing the CMV-EGFP expression cassette were injected subretinally in adult C57BL/6 mice, and retinas were analyzed 2 weeks post injection. The dose used was the maximum possible for each LV vector based on the prep titers. The n of retinas, the dose of each vector and the relative transduction observed in photoreceptors and retinal pigment epithelium are indicated below each panel. RPE was the only target of most LV pseudotypes but LV-GP64, which transduced some photoreceptors (see inset). Abbreviations: rpe: retinal pigment epithelium; onl: outer nuclear layer; inl: inner nuclear layer; gcl: ganglion cell layer; PR: photoreceptors; TU: transducing units; NT: not transduced. Magnification=20×; scale bar=50μm.

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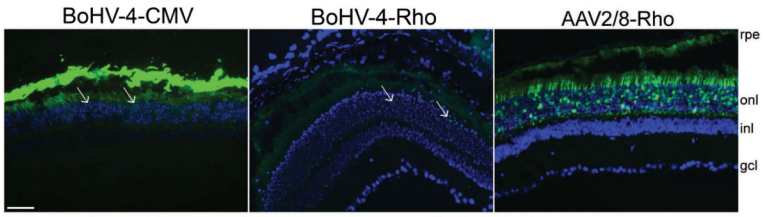

Figure 5. Mouse retinal transduction mediated by BoHV-4

The BoHV-4-CMV-EGFP vector was injected subretinally (8×10^6 GC/eye) in adult C57BL/6 mice (left panel). Histological analyses performed one month post-injection revealed that both the RPE and some photoreceptors (arrows) were targeted (n=2/4 retinas with a transduction pattern similar to that shown in the picture, the remaining retinas show fewer transduced photoreceptors). The BoHV-4 vector containing the photoreceptor-specific Rho-EGFP expression cassette was injected subretinally (2.5×10^8 GC/eye, middle panel, n=10/10) in adult C57BL/6 mice. AAV2/8 containing the same expression cassette was injected alongside at 5×10^8 GC/eye (right panel, n=4/4), showing that AAV2/8 targets PRs more efficiently than BoHV-4. Abbreviations: rpe: retinal pigment epithelium; onl: outer nuclear layer; inl: inner nuclear layer, gcl: ganglion cell layer. Magnification=20×; scale bar=50μm.

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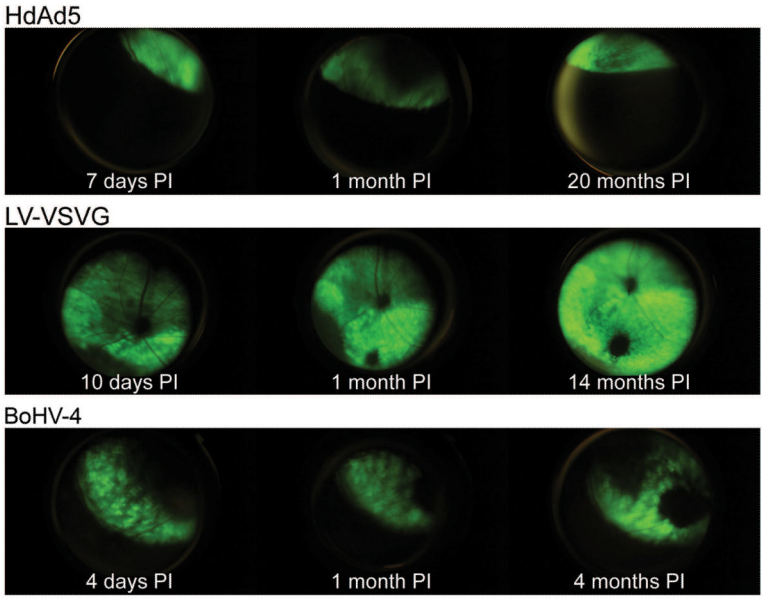

Figure 6. Long term transgene expression following subretinal delivery of HdAd5, LV and BoHV-4 vectors

Non-invasive fluorescent fundoscopies were obtained at various time points after subretinal injection of HdAd5-, LV-VSVG- or BoHV-4-CMV-EGFP in adult C57BL/6 mice. The fundus photographs show EGFP expression along time in a representative eye injected with HdAd5 (3×10^8 vp/eye, n=2/3 eyes showing a fluorescent signal at the fundoscopy similar to the image), LV-VSVG (1×10^5 TU/eye, n=2/2) and BoHV-4 (8×10^6 GC/eye, n=16/16). PI: post-injection.

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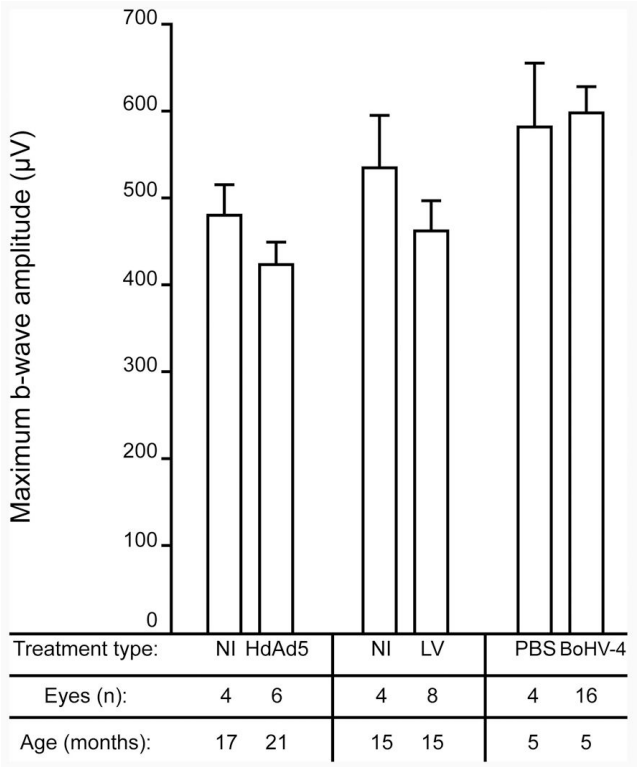

Figure 7. ERG responses following subretinal injection of HdAd5, LV or BoHV-4

Ganzfeld electroretinogram (ERG) maximum b-wave amplitudes (±SE) in retinas injected with: HdAd5-, LV- or BoHV-4-CMV-EGFP vectors. The b-wave ERG values of eyes injected with HdAd5 at 1-3×10^8 vp/eye were compared to those of non-injected C57BL/6 mice of similar age. LV-VSVG, -RRV, -EbolaΔO, -GP64 were pooled together in the LV column (n=2 eyes for each LV pseudotype). LV were injected at 1×10^4-1×10^5 TU/eye and the ERG values were compared to those of non-injected C57BL/6 mice. ERG values of eyes that received BoHV-4 at 8×10^6 GC/eye were compared to those of eyes injected with PBS at the same age. Abbreviations: NI: not injected.

Table 1

Viral vectors based on Adenovirus (Ad and HdAd), Lentivirus (LV) or Herpesvirus (HV) evaluated in this study

	Vector	Subgroup	Reference
Ad naturally-occurring n=8	HdAd1	C	75
	HdAd2	C	74
	HdAd5	C	73
	Ad6	C	ATCC® VR-6™
	AdC1	B	ATCC® VR-20™
	ChAd7	E	42
	ChAd30	B	42
	ChAd63	E	42
Ad genetically-modified n=8		**External surface protein**	
	HdAd5/F35	Ad35 fiber cloned in an Ad5 context	33
	HdAd5/F35++	Mutant Ad35 fiber with increased affinity for the CD46 ligand in an Ad5 context	35 and this publication
	Ad5/F2+pK	Heptalysin tract incorporation in the H-I loop of Ad2 fiber cloned in an Ad5 context	36
	Ad5/F2+RGD	RGD-motif incorporation in the H-I loop of Ad2 fiber cloned in an Ad5 context	36
	Ad5ΔRGD	RGD-motif deleted from penton base proteins of Ad5 capsid	37
	Ad5/F3	Fiber from Ad3 cloned in an Ad5 context	Generous gift from David T. Curiel, 34
	HdAd5/K3	Ad3 knob cloned in an Ad5 context	32
	HdAd5/6	Ad6 hexon hypervariable regions in Ad5 capsid	38
LV n=7	VSVG	Vesicular Stomatitis Virus Glycoprotein	85
	JSRV	Jaagsiekte Sheep Retrovirus	86
	RRV	Ross River Virus	87
	EBOLAΔO	Ebola mucin domain-deleted glycoprotein	77
	LCMV	Lymphocytic Choriomeningitis Virus	85
	MOKOLA	Mokola virus envelope glycoprotein	13
	GP64	Baculovirus GP64 envelope glyprotein	88
HV, n=1	BoHV-4	Bovine herpesvirus type 4	55