Seamless replacement of *Autographa californica* multiple nucleopolyhedrovirus *gp64* with each of five novel type II alphabaculovirus fusion sequences generates pseudotyped virus that fails to transduce mammalian cells

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*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), a member of the type I alphabaculoviruses, is able to transduce and deliver a functional gene to a range of non-host cells, including many mammalian lines and primary cells, a property mediated by the envelope fusion protein GP64. AcMNPV is non-cytopathic and inherently replication deficient in non-host cells. As such, AcMNPV represents a possible new class of gene therapy vector with potential future clinical utility. Whilst not a problem for *in vitro* gene delivery, the broad tropism displayed for non-host cells is less desirable in a gene therapy vector. The fusion protein *F* of type II alphabaculoviruses can substitute functionally for GP64, and such pseudotyped viruses display a severely impaired capacity for non-host-cell transduction. Thus, surface decoration of such an *F*-pseudotyped AcMNPV with cell-binding ligands may restore transduction competence and generate vectors with desirable cell-targeting characteristics. By seamlessly swapping the native *gp64* coding sequence with each of five sequences encoding different *F* proteins, a set of *F*-pseudotyped AcMNPV was generated. This report details their relative abilities both to functionally replace GP64 in viral growth and to transduce human Saos-2 and HeLa cells. All five supported viable infections in insect cell cultures and one, the *Mamestra configurata* NPV (MacoNPV) *F* pseudotype, could be amplified to titres close to those of native AcMNPV. In contrast, none was able to transduce the Saos-2 and HeLa cell lines. The robust support provided by MacoNPV *F* in virus production makes the corresponding pseudotype a viable scaffold to display surface ligands to direct selective mammalian cell targeting.

**INTRODUCTION**

The family *Baculoviridae* comprises large, rod-shaped, enveloped dsDNA viruses infective to insects belonging to the orders Lepidoptera, Hymenoptera and Diptera. Based on phylogenetic analysis of genome sequences, members of the family *Baculoviridae* are organized into four major genera: Alpha-, Beta-, Gamma- and Deltabaculovirus (Jehle et al., 2006), with the former being subdivided into type I and type II alphabaculoviruses based, in part, on their use of the different major envelope fusion proteins, GP64 and fusion (*F*), respectively (Pearson & Rohrmann, 2002). From a historical perspective, baculovirus species have been used both as effective biological pesticides (reviewed by Szewczyk et al., 2006) and, in the form of the prototype *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), as a popular gene-delivery vector for heterologous protein expression in cultured insect cells (reviewed by van Oers, 2011). Proteins expressed in such an expression system can be generated at high yield, correctly folded and with many of the post-translational modifications, such as glycosylation, associated with mammalian cells (van Oers, 2011). Some time...
after these applications were established, it was discovered that AcMNPV was also able to transduce and deliver a functional transgene to a range of mammalian cells both in vitro and in vivo (reviewed by Hürer & Hofmann, 2003). Demonstrated originally in primary hepatocytes and liver-derived cell lines (Hofmann et al., 1995; Boyce & Bucher, 1996), the range of cells transduced by AcMNPV has since grown (see Chen et al., 2011) and now includes several additional primary cell types, including hepatic stellate cells (Gao et al., 2002), neural cells (Sarkis et al., 2000) and pancreatic islet cells (Ma et al., 2000). This unexpected competence for non-host-cell transduction has prompted researchers to design AcMNPV-based vectors for a variety of novel applications including assay development in drug discovery (Kost et al., 2010), RNA interference (Nicholson et al., 2005; Ong et al., 2005), cancer (Wang & Balasundaram, 2010), gene therapy (Ghosh et al., 2004) and engineering of stem cells in tissue regeneration (Lin et al., 2010). A possible regulatory route towards the eventual clinical application of AcMNPV-based vectors has been discussed recently (Lesch et al., 2011).

The broad range of non-host cells transduced by AcMNPV, together with the ability of the fusion protein GP64 to functionally replace fusion proteins from lentivirus (Schauber et al., 2004) and human respiratory syncytial virus (Oomens & Wertz, 2004), indicate that GP64 acts as a promiscuous fusion protein. Whilst such lack of selectivity is not relevant for AcMNPV-mediated gene delivery to cultured mammalian cells, it is less desirable if developing AcMNPV as a potential gene therapy vector because of unwanted bystander effects. Thus, it would be advantageous if AcMNPV-based vectors could be engineered with selective cell tropisms whilst retaining facile amplification to high titre in insect cell culture and the capacity to deliver a functional cargo to non-host cells. AcMNPV GP64 is necessary for viral attachment to (Heffernon et al., 1999), membrane fusion during entry into (Blissard & Wenz, 1992; Kingsley et al., 1999) and subsequent budding from (Monsma et al., 1996; Oomens & Blissard, 1999) host insect cells. In addition, GP64 plays a critical role in mammalian cell transduction (Tani et al., 2001). A number of studies (Table 1) have demonstrated that the majority of type II alphabaculoviral F proteins, although apparently binding to a different insect cell receptor (Westenberg et al., 2007), can substitute functionally for GP64 by supporting AcMNPV production in culture, albeit with reduced titres. In contrast, F-pseudotyped Agp64 AcMNPV vectors are severely compromised for mammalian cell transduction (Liang et al., 2005; Westenberg et al., 2007, 2010; Yu et al., 2009), confirming the key role that GP64 plays in non-host-cell transduction. However, the ability of F proteins to support Agp64 AcMNPV in cultured host-cell entry and budding, plus the observation that they promote membrane fusion in an in vitro assay (Lung et al., 2002), indicate that the failure of F-pseudotyped viruses to transduce mammalian cells may result from a block at virus attachment or internalization rather than endosomal escape. Thus, as others have discussed (Yu et al., 2009), decorating the surface of F-pseudotyped Agp64 AcMNPV vectors with, for example, cell-binding ligands or peptides represents a possible path towards generating AcMNPV-based gene therapy vectors with transductional targeting characteristics.

We recently developed (Westenberg et al., 2010) a modified counter-selection recombineering approach that enables us to undertake robust, seamless and unrestricted modification of the DNA sequence of the AcMNPV replicon bMON14272 (Luckow et al., 1993). This modified approach was necessary to overcome, during the counter-selection step, unwanted intra-molecular rearrangements that can occur between pairs of homologous regions present in the bacmid sequence (Westenberg et al., 2010). We are employing the technique as part of a strategy to generate AcMNPV-based vectors designed to deliver therapeutic genes and/or dsRNAs selectively to hepatic stellate cells in the context of liver fibrotic disease. Our initial aim is to identify, by replacing gp64 with a range of

### Table 1. F-pseudotyped Agp64-AcMNPV

<table>
<thead>
<tr>
<th>F source*</th>
<th>Locus†</th>
<th>Promoter‡</th>
<th>Agp64 rescue§</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeMNPV</td>
<td>polh</td>
<td>gp64</td>
<td>Yes</td>
<td>Lung et al. (2002); Yu et al. (2009)</td>
</tr>
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<td>SeMNPV</td>
<td>gp64</td>
<td>gp64</td>
<td>Yes</td>
<td>Westenberg et al. (2010)</td>
</tr>
<tr>
<td>AdhoNPV</td>
<td>gp64</td>
<td>gp64</td>
<td>Yes</td>
<td>Westenberg et al. (2010)</td>
</tr>
<tr>
<td>LdMNPV</td>
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<td>gp64</td>
<td>Yes</td>
<td>Lung et al. (2002)</td>
</tr>
<tr>
<td>HearNPV</td>
<td>polh</td>
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<td>Yes</td>
<td>Long et al. (2006a)</td>
</tr>
<tr>
<td>AgseGV</td>
<td>polh</td>
<td>gp64</td>
<td>Yes</td>
<td>Yin et al. (2008)</td>
</tr>
<tr>
<td>PlxyGV</td>
<td>polh</td>
<td>gp64</td>
<td>No</td>
<td>Lung et al. (2002)</td>
</tr>
</tbody>
</table>

*Source of F protein CDS. Type II alphabaculoviruses: SeMNPV, Spodoptera exigua MNPV; AdhoNPV, Adoxophyes honmai NPV; LdMNPV, Lymantria dispar MNPV; HearNPV, Helicoverpa armigera NPV. Betabaculoviruses: AgseGV, Agrotis segetum granulovirus; PlxyGV, Plutella xylostella GV.

†AcMNPV locus containing F CDS.

‡Promoter used to drive F CDS.

§Functionally substitutes for GP64.
sequences corresponding to the F-encoding ORFs from different type II alphabaculoviruses, an F-pseudotyped Δgp64 vector that can be cultured to high titre and lacks the promiscuous mammalian cell transduction characteristics of native AcMNPV. The envelope surface of this candidate F-pseudotyped vector will then be decorated with cell-binding ligands designed to provide selective cell targeting. Using this method, we previously reported (Westenberg et al., 2010) the seamless replacement of gp64 with F coding sequences (CDSs) from SeMNPV and AdhoNPV generating F-pseudotyped Δgp64 AcMNPV viruses that had lost the capacity to deliver a GFP transgene to mammalian cells. In the current study, we describe the construction of new Δgp64 AcMNPV vectors pseudotyped with five additional type II alphabaculovirus F proteins and report on their respective abilities in supporting virus production and the delivery of a reporter to mammalian cells.

RESULTS AND DISCUSSION

As a guide to F CDS selection, we undertook a molecular phylogenetic analysis of all type II alphabaculoviral F protein sequences available at the time and, by visual examination of the resulting phylogram (Fig. 1), selected the following five previously uncharacterized species that covered the major evolutionary nodes: ChchNPV (van Oers et al., 2005), SfMNPV (Harrison et al., 2008), SpltNPV strain G2 (Pang et al., 2001), MacoNPV-A (Li et al., 2002) and AgseNPV (Jakubowska et al., 2006). Using our modified recombineering protocol (Westenberg et al., 2010), the F CDSs were PCR amplified and, following addition of extended terminal sequences homologous to the gp64 5′ and 3′ flanking regions, via an intermediate subcloning step, each sequence was introduced into a Δgp64-bMON14272 bacmid (Fig. 2). This approach to generate F-pseudotyped AcMNPV resulted in the F CDS being placed in the equivalent genomic context as gp64 and differs from the more traditional method of utilizing Tn7-mediated transposition to insert sequences into the polyhedrin (polh) locus (Luckow et al., 1993). PCR and restriction enzyme analyses of all final bacmid sequences (Fig. S1, available in JGV Online) confirmed that our modified protocol, which employs terminal homology arms significantly longer (~600 bp) than the usually sufficient 50 bp, was both efficient – for each F pseudotype, at least three of five clones examined contained the desired F sequence (data not shown) – and, importantly, generated products free of the intra-molecular deletions that can otherwise commonly occur in the repetitive bMON14272 target during counter-selection recombineering (Fig. S1) (Westenberg et al., 2010). The resulting Δgp64>F bacmids and the control Δgp64 bacmid were each subsequently fitted, by standard Tn7 transposition, with a GFP reporter sequence driven by a hybrid cytomegalovirus (CMV)–p10 promoter (Westenberg et al., 2010), enabling facile visual monitoring of both Sf9 insect cell infection and mammalian cell transduction.

A transfection/infection assay (Fig. 3a, b) confirmed the ability of all the F pseudotypes to mediate viable infections from transfected Sf9 cells, confirming that all the F proteins investigated in this present study could functionally replace GP64. Thus, to date, it appears that the ability to functionally replace GP64 may be a universal feature common to all F proteins from type II alphabaculoviruses (Table 1). In contrast, of the two F proteins investigated...
from betabaculoviruses, only the fusion protein from AgseGV was demonstrated to be functionally analogous to GP64 (Yin et al., 2008) (Table 1).

We used a semi-quantitative assay as a means of directly comparing the ability of each of the five F-pseudotyped viruses generated in this study, plus two generated previously and the Δgp64–gp64 rescue vector (Westenberg et al., 2010), to functionally replace GP64. All viruses were amplified in S9 cells under equivalent conditions of culture and m.o.i., and titres were determined at 5 days post-infection (p.i.). All F-pseudotyped bacmids and the gp64 rescue bacmid were able to support virus production in culture (Table S1) with titres ranging from 1.9 × 10^6 to 1.5 × 10^9 p.f.u. ml^-1 for the ChchNPV (vMW023) and MacoNPV (vMW021) F pseudotypes, respectively. In comparison, the AcMNPV GP64 rescue virus (vMW033) was amplified to ~10^9 p.f.u. ml^-1. Thus, although the titre was tenfold less than the GP64 rescue virus, the MacoNPV F-pseudotyped virus amplified to a useable concentration and this pseudotype has become our working vector for further development. Subsequently, in order to generate sufficiently concentrated virus stocks to enable mammalian cell transduction at virus:cell ratios close to 100, additional amplifications, with empirical combinations of starting cell densities and m.o.i. (data not shown), were carried out for those pseudotypes, such as ChchNPV F, that produced low titres in the first, controlled amplification. These provided working virus stocks for all F pseudotypes of ≥2.0 × 10^7 p.f.u. ml^-1 apart from ChchNPV and SpltNPV, which remained difficult to amplify to titres above those first recorded (Table S1).

Having generated working viral stocks, we then investigated whether any of the F pseudotypes were able to transduce either of the human cell lines Saos-2 and HeLa, which are readily transduced by native AcMNPV. Following incubation of Saos-2 cells with SeMNPV, AdhoNPV, MacoNPV or AgseNPV F-pseudotyped virus, fluorescence microscopy identified only very occasional, discrete GFP-expressing cells in each case (Fig. 3d). Apart from these isolated cells, no other GFP-expressing Saos-2 cells were observed. In HeLa cells, the situation was even more marked, as extensive searching failed to observe even occasional GFP-expressing cells after incubation with any of the F-pseudotyped viruses (Fig. 3f). As expected, the GP64 rescue vector transduced both Saos-2 and HeLa cells with efficiencies that were routinely >90% (Fig. 3 and data not shown). Therefore, although all the F proteins tested here were able to substitute functionally for GP64 and support virus generation, the loss of the native envelope fusion protein essentially abolished the capacity for non-host-cell transduction, confirming the key role that GP64 plays in this process.

A number of studies have reported on investigations into the attachment and entry mechanisms involved during mammalian cell transduction with native GP64-equipped AcMNPV. Whilst early studies indicated roles for simple electrostatic interactions (Duisit et al., 1999) and phospholipids (Tani et al., 2001), later reports proposed the involvement of clathrin-mediated endocytosis (Long et al., 2006b) and macropinocytosis (Matilainen et al., 2005). More recently (Laakkonen et al., 2009), it has been suggested that neither of these latter mechanisms is significantly involved and that, instead, entry of AcMNPV into mammalian cells is mediated via a phagocytosis-like uptake process involving large plasma membranous invaginations associated with raft structures. Interestingly, experimental evidence has also been provided (Dong et al., 2010), demonstrating that incubation at low pH promotes AcMNPV uptake into both host S9 and non-host mammalian cells via an endosome-independent pathway.
involving direct membrane fusion at the cell surface. In contrast to these studies involving AcMNPV, there is little, if any, data available that sheds light on the block in mammalian cell transduction observed with F-pseudotyped AcMNPV that we, and others, have reported. As a first step towards this, we undertook a preliminary capsid immunolabelling experiment to investigate the fate of MacoNPV F-pseudotyped AcMNPV following incubation with Saos-2 cells (Fig. 4). Soon after incubation, immunostaining at the cellular periphery was observed with both the MacoNPV F-pseudotyped virus and a gp64 rescue virus, indicating that the former bound to the cell membrane. The lack of subsequent GFP expression in the cells bound by this pseudotype suggested a block at some subsequent point in the route normally followed by GP64-equipped virus. Further experiments are required to expand and extend this observation including the use of additional pseudotypes and cell lines and investigations to examine the effect of lowering the pH during cell–virus incubation.

The modified counter-selection recombineering method described by us previously (Westenberg et al., 2010) that enables precise and seamless modification of the AcMNPV genome has been further validated by the work detailed in the present report. The optimized methodology will enable others to undertake targeted modifications to the AcMNPV genome ranging from the types of seamless CDS swaps reported here down to subtle, single base-pair deletions,
insertions or changes. We are employing this approach to furnish the surface of MacoNPV F-pseudotyped AcMNPV with cell-binding ligands designed to provide desired cell-targeting properties. If successful, such a strategy would represent an advance towards creating potentially clinically useful AcMNPV-based gene therapy vectors.

METHODS

Phylogenetic analysis. Type II alphabaculovirus F and AcMNPV gp64 amino acid sequences were aligned using CLUSTAL W (Thompson et al., 1994), within MacVector sequence analysis software (MacVector Inc.), and the resulting multiple sequence alignment file was optimized by eye. Using this file as input, phylogenetic analysis (again within MacVector) was undertaken, via neighbour joining with systematic tie-breaking, and the resulting phylogenetic tree was rooted with GP64.

Microscopy. All bright-field and fluorescence microscopy was undertaken on an Olympus BX61 motorized microscope equipped with appropriate filter sets. Post-acquisition image analysis was performed with Cellsens (Olympus) and ImageJ (NIH) packages.

Plasmid construction and bacmid recombineering. Sequences encoding the F proteins of ChchNPV, SeMNPV, SpltNPV, MacoNPV-A and AgeseNPV were PCR amplified (Phusion; NEB) using 5 ng of the corresponding viral genomic DNA as template and the respective PAGE-purified oligonucleotide (ODN) pairs 6016a/6017a, 6018a, 6018a/6019a, 6019a/6020a (Table S2). Each resulting amplicon contained the individual F CDS flanked by 62 bp of S′ and 46 bp of 3′ sequence equivalent to regions immediately flanking the gp64 CDS of AcMNPV. The five amplicons were each cloned directly into SnaBI-linearized pMW009, the construction and utility of which has been described elsewhere (Westenberg et al., 2010), via an in vitro recombination system (In-Fusion; Clontech) according to the manufacturer’s protocol, generating plasmids pMW025 (ChchNPV F), pMW026 (SeMNPV F), pMW031 (SpltNPV F), pMW030 (MacoNPV F) and pMW032 (AgeseNPV F) (Table S3). Following DNA sequencing to confirm construct fidelity, the cloned sequences were released from their respective constructs by restriction with SmaI, generating F CDS-containing, recombineering-ready cassettes, which were gel purified and quantified by visualization against a DNA molecular mass ladder (2-log ladder; NEB). In addition, a control, Δgp64 SmaI restriction fragment, containing only 5′ and 3′ gp64 flanking sequences, was released from pMW009.

Each cassette, now equipped with ~600 bp flanking homology arms, was used to replace the rpsL-tet(A) counter-selection cassette (RT cassette) (Stavropoulos & Strathdee, 2001) in bacmid bMW009, in which the AcMNPV gp64 CDS has been directly replaced by the RT cassette, via counter-selection recombineering, as described previously (Westenberg et al., 2010). Briefly, aliquots (~0.1 ml) of bMW009-containing Escherichia coli MW001 cells (Westenberg et al., 2010), induced for phage λ Red activities and made electrocompliant, as described previously (Dolphin & Hope, 2006), were electroporated with one of the five F CDS-containing cassettes or the Δgp64, non-F CDS-containing control cassette (SmaI fragment of pMW009) (500 ng each), recovered in SOC medium without Mg2+ at 32 °C with shaking at 220 r.p.m. for 2.5 h and serially diluted (in M9 salts), and aliquots (50 μl) were spread on selective [containing 50 μg kanamycin (Kan) ml−1, 500 μg streptomycin (Sm) ml−1] or non-selective (containing 50 μg Kan ml−1) NSLB agar (Stavropoulos & Strathdee, 2001) plates and incubated at 32 °C for 48 h. For each cassette, five discrete SmR colonies were streaked onto fresh Kan/Sm-selective plates, incubated at 32 °C for 48 h and subjected to colony PCR to identify clones in which the RT cassette had been replaced with an F CDS. Subsequent restriction digestion analyses of the resulting PCR products were performed to confirm the integrity of the F CDS replacement sequence (Fig. S1). Single bacmid clones, containing the correctly inserted ChchNPV, SeMNPV, SpltNPV, MacoNPV or AgeseNPV F CDS or control sequence were named, respectively, bMW019, bMW018, bMW026, bMW017, bMW027 and bMW020 (Table S3). Bacmid DNAs were isolated and subjected to restriction mapping together with bMON14272, bMW009 and the previously generated bacmids bMW010, bMW028 and bMW025 (Westenberg et al., 2010) in which the native gp64 CDS was replaced by, respectively, SeMNPV F, AdhoNPV F and rescue AcMNPV gp64 CDS (Figs. S1 and S2). Each of the nine homologous regions (hr1, hr1a, hr2, hr2a, hr3, hr4a, hr4b, hr5a and hr5b) was PCR amplified with flanking ODNs, as described previously (Westenberg et al., 2010), and compared electrophoretically with equivalent PCR products amplified from bMON14272 (data not shown).

Following transformation with the Tn7-transposase helper plasmid pMON124 (Luckow et al., 1993), bacmids bMW017, bMW018, bMW019, bMW026, bMW027 and bMW020 in E. coli MW001 received, via Tn7-mediated transposition (Bac-to-Bac; Invitrogen), the CMVpromP10promGFP dual insect/mammalian GFP reporter sequence from pMW005 (Westenberg et al., 2010), generating the respective bacmids bMW021, bMW022, bMW023, bMW034, bMW035 and bMW024 (Table S3).

Virus generation and mammalian cell transduction. S9 insect cells were passaged in shaking cultures (30 ml, 90 r.p.m., 28 °C) in serum-free medium (S9000 SFM; Invitrogen) supplemented with 2% (v/v) FBS. For virus generation, S9 cells (1.0 × 106) grown in monolayers were transfected (Cellfectin; Invitrogen) with bacmid DNA (~1 μg), cultured (5 days, 28 °C) and inspected for GFP expression by fluorescence microscopy. An aliquot (500 μl) of the clarified (10 min, 10000 g), filter-sterilized (0.45 μm) medium was used to infect a new batch of S9 cells (1.0 × 107). At 3 days p.i., cells were inspected for GFP expression. Subsequently, cells were passaged every 4–5 days until all cells were infected, at which point titres were
determined by an end-point dilution assay (O’Reilly et al., 1992). Quantitative determination of relative titre generation was performed by infecting S9 cells (1.0 × 10^6 cells ml^-1) in a shaking culture (30 ml, 90 r.p.m.) at an m.o.i. of 0.05. Cultures were harvested at 5 days p.i. and centrifuged (10 min, 1000 g) and the supernatants were filter sterilized. Titres were determined and the supernatants were stored at 4 °C. Individual bacmid constructs generated the corresponding ‘v’-prefixed virus (Table S3).

Soas-2 and HeLa cells were maintained (37 °C, 5% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% FBS. Cells were seeded (1.0 × 10⁵) in 24-well plates and, after incubation (24 h), the medium was replaced with 500 µl clarified filter-sterilized virus-containing S900II SFM resulting in virus:cell ratios of 50 (vMW022 and vMW023) or 100 (vMW011, VMW021, vMW033, vMW034, vMW035 and vMW036). After 1 h incubation at 28 °C with gentle shaking, the medium was aspirated, the cells were washed and fresh DMEM or Eagle’s minimal essential medium was added as appropriate. GFP expression was visualized in transduced cells by fluorescence microscopy (magnification ×20) at 48 h post-transduction.

**Immunohistochemistry.** Soas-2 cells (1.0 × 10⁵), seeded on poly-L-lysine-coated coverslips placed in the wells of a 24-well plate, were cultured for 14 h, as described above, cooled to 4 °C and incubated with vMW033 (Age64>gp64 rescue vector) or vMW021 (MacoNPV F-pseudotyped AcMNPV) for 1.5 h at 4 °C with gentle shaking at a virus:cell ratio of 500, after which they were washed twice with ice-cold PBS and fixed immediately (4% formaldehyde, room temperature, 20 min). After fixation, the cells were washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min, washed again three times with PBS and blocked with 10% goat serum in PBS for 2 h. The cells were then incubated for 14 h at 4 °C with an anti-VP39 mAb (Whitt & Manning, 1988) diluted 1:200 in 1% goat serum in PBS, washed three times with PBS and incubated for 1 h at 37 °C with Cy3-conjugated goat anti-mouse antibody (Catlag Laboratories) diluted 1:200 in 1% goat serum in PBS. As controls for specific antibody binding, mock-transduced cells, incubated with both antibodies, and transduced cells, incubated with the secondary antibody only, were used. Cells were finally washed twice with PBS, incubated for 30 min with Hoechst 33342 (10 µM in PBS; Sigma) and washed again twice with PBS. Coverslips were mounted in Fluorescence Mounting Medium (Dako) and the cells imaged (magnification ×100).

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