Forward and reverse genetics strategies for improving oncolytic reoviruses

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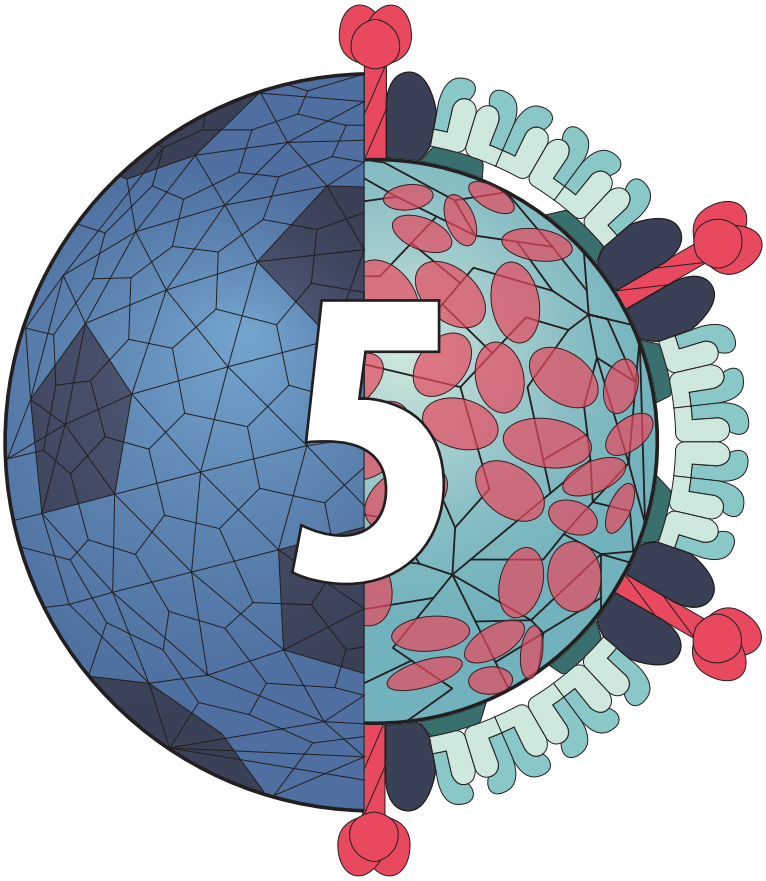
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REPLICATING REOVIRUSES WITH A TRANSGENE REPLACING THE CODONS FOR THE HEAD DOMAIN OF THE VIRAL SPIKE

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Gene Ther. 2015 Mar;22(3):267-79

ABSTRACT

The capacity to modify the reovirus genome facilitates generation of new therapeutic reoviruses. We describe a method for generating replication-competent reoviruses carrying an heterologous transgene. The strategy is based on the expanded-tropism reovirus mutant *jin-3*, which can infect cells independent of the reovirus receptor JAM-A. *Jin-3* harbours a mutation in the S1 segment resulting in a G196R substitution in the tail of the spike protein σ1. The use of the *jin-3* tail-encoding S1 segment allows replacing the codons for the JAM-A-binding head domain by up to 522 nt of foreign sequences, without exceeding the size of the wild-type S1 segment. We inserted the codons for the porcine teschovirus-1 2A element fused with those encoding the fluorescent protein iLOV. Replicating rS1His-2A-iLOV reoviruses were generated by co-transfection of expression plasmids for all reovirus segments. These reoviruses contain the S1His-2A-iLOV segment in absence of the wild-type S1 segment. Density-gradient centrifugation confirmed the association of the σ1-tail fragment with the capsid. Both JAM-A positive and JAM-A negative cells exposed to the rS1His-2A-iLOV reoviruses exhibited iLOV fluorescence, confirming the *jin-3* derived expanded-tropism phenotype. These data demonstrated the feasibility of generating decapitated replication-competent T3D reoviruses carrying a heterologous transgene.

INTRODUCTION

Mammalian orthoreoviruses are non-enveloped viruses with a segmented double-stranded RNA (dsRNA) genome. The name Reovirus is an acronym of Respiratory and Enteric Orphan virus. So far the virus has not been associated with severe pathology in humans, hence its designation as an orphan virus. In cell culture reovirus infection initiates a lytic replication cycle. The reovirus preferentially infects and lyses transformed cells. This has been attributed to the stimulating effects of the active RAS-signaling on virus uncoating, replication, egress, and the induction of apoptosis[1-4]. The reovirus' preference for transformed cells and the absence of pathology in humans have led to the development of reovirus Type 3 Dearing as a viral oncolytic agent for clinical use in a number of cancer types[5,6]. The results of the clinical trials published so far demonstrate that the reovirus-administrations are well tolerated. Moreover, evidence of anti-tumour efficacy has been acquired. Nonetheless, the antitumour efficacy could be improved. This may be accomplished by increasing the infection efficiency in tumour cells and by increasing the oncolytic potency of the virus. Here we describe an approach to generate replication-competent expanded-tropism reoviruses that harbour a heterologous transgene.

The mammalian reovirus genome consists of ten double stranded RNA segments, making genetic modification of the reovirus genome especially difficult. The development of plasmid-based reverse genetic systems for mammalian reoviruses facilitated the development of reverse genetics approaches[7]. However, there is limited information available regarding locations in the genome that could accommodate the insertion of new protein coding genes without compromising the virus' capacity for autonomous replication. So far, small exogenous sequences have been successfully added to the genome segments S1, S3, M1 and L1[8-10]. Insertion in these locations increases the size of the genome segments and this may compromise the genomic stability of the modified reoviruses. Others report the replacement of the open reading frame (ORF) from some of the segments by heterologous genes, but this yields replication-incompetent reoviruses that require trans-complementation of the deleted functions for propagation in helper cells[7,11]. Here, we present the generation of replication-competent reoviruses carrying the sequence encoding the fluorescent protein iLOV within the S1 segment.

In our approach we employed a feature of the *jin* mutants of the Type 3 Dearing reovirus. The *jin* mutants were obtained upon serial passaging of the T3D reovirus on glioblastoma cells that lack expression of the high-affinity reovirus receptor Junction-Adhesion Molecule A (JAM-A)[12]. The absence of JAM-A expression makes these cells

non-permissive for *wild-type (wt)* reovirus infection in monolayer cultures[13]. The *jin* mutants could productively infect a variety of JAM-A negative cells. All *jin* mutants harbour mutations in the S1 segment close to the region previously identified as the sialic acid-binding domain in the tail of the trimeric spike protein σ1[14,15]. This suggests that the *jin* mutants do not require the presence of the head domain for cell entry.

We used the S1 segment of reovirus *jin-3,* which harbours only one point mutation that results in a G196R change in the tail of the σ1-spike. To reduce the size of the S1 segment, the σ1 protein was truncated after amino acid 252. This allows inserting transgenes up to a length of 470 nucleotides (nt) without exceeding the length of the wild-type S1 segment. The ORF of the nonstructural protein σ1s, which entirely overlaps the σ1 ORF, but is read from another reading frame, is left intact. Although in different field isolates of T3 reoviruses the amino acid sequence of the σ1s is more heterogeneous than that of σ1, some regions are conserved[16]. The role of σ1s in the reovirus replication cycle has not yet been fully elucidated, but evidence suggests that it is involved in virulence and apoptosis[17] and is required for induction of G2/M cell cycle arrest in some cell lines[18,19].

In our study we have used the small fluorescent protein iLOV as the reporter[20]. The 13 kDa iLOV protein is evolved from the light, oxygen or voltage-sensing domain of the blue-light receptor phototropin 2 of *Arabidopsis thaliana*. The protein contains the flavin mononucleotide as chromophore. The small size (~330 nt) of the synthetic codon-optimized gene encoding the iLOV fluorescent protein makes it a suitable reporter for our approach.

Here we show how a synthetic transgene-containing segment can be incorporated in the genome of a mammalian reovirus without compromising the replicative capacity of the resulting recombinant virus. Furthermore we demonstrate that the codons of the head domain of the spike protein σ1 can be replaced by a small transgene, and that the transgene is expressed. The reoviruses carrying the σ1-tail of *jin-3* and the transgene on the S1 segment retained their capacity to infect JAM-A deficient cells. This technique may facilitate the development of new replication competent-recombinant reoviruses armed with a therapeutic transgene for oncolytic virus therapy. In addition this approach may be exploited for the generation of new reovirus-based life-virus vaccines.

RESULTS

Generation of the rS1His-2A-iLOV reoviruses

The *jin* reoviruses infect cells through an interaction between the tail domain of the reovirus spike protein and sialic-acid residues at the cell surface. This observation, together with the notion that the head domain is not essential for trimerization of σ1 molecules[21], led us to postulate that in *jin* viruses the head domain of the spike protein could be replaced by transgene sequences. To test this hypothesis, the codons for the head domain of σ1 were replaced by the iLOV-coding region in the S1 segment of *jin-3*. The trypsin cleavage site, which is present in the tail domain of the σ1 protein of the *wt*-T3D reovirus (*wt*-Reo) was retained in our modified variant (figure 1). Trypsin cleavage occurs after the arginine residue at position 245 of the T3D-σ1 protein[22]. This cleavage is important in the formation of Infectious Subvirion Particles (ISVPs) and can be of importance during the proteolytic disassembly[23].

The modified S1 segment encodes only the tail of σ1 (amino acids 1 - 252), followed by a 6xHistag, the porcine teschovirus-1 2A sequence (2A), and the iLOV coding sequence. The ORF is 1176 nt long (ORF of S1-*wt* is 1377 nt) and is followed by 198 nt of the 3' region of S1. The latter region contains sequences reported to be involved in encapsidation[24]. Translation of the modified S1 ORF generates two distinct proteins that are separated upon the ribosome skipping induced by the 2A peptide: the σ1-His protein and the iLOV fluorescent reporter protein (figure 1).

To generate the recombinant reovirus, plasmid pBT7-S1His-2A- iLOV encompassing the modified S1 segment, preceded by a T7 RNA polymerase dependent promoter and tailed by the hepatitis delta virus ribozyme, was transfected into BSR-T7 cells together with four plasmids encoding the other nine segments, essentially as described[25]. Virus was harvested at 48 hours post-transfection (P0). Part of the yield was used to infect 911scFvHis cells (P1). The presence of the 6xHistag in the capsid allows the modified virus to use the membrane-bound single-chain Fv recognising the His tag (scFVHis) as an artificial receptor[8]. Clear cytopathic effects (CPE) became apparent at 10-days post infection, suggesting the presence of infectious viruses (figure 2a). Upon harvesting, the virus was used to infect cultures of 911 cells in a plaque assay experiment. The plaques formed by rS1His-2A-iLOV viruses were smaller than those of *wt* reoviruses, and the borders are not clearly defined. The plaque morphology was similar to those of the *jin-3* viruses (data not shown). To determine the titers of the freeze-thaw batches of rS1His-2A-iLOV viruses we used the $TCID_{50}$ (50% tissue culture infective dose) method on 911 cells. On average the yields are lower compared to the *wt*-T3D reovirus and around 4 x 10^6 Infection Units per ml (IU_{911}/ml).

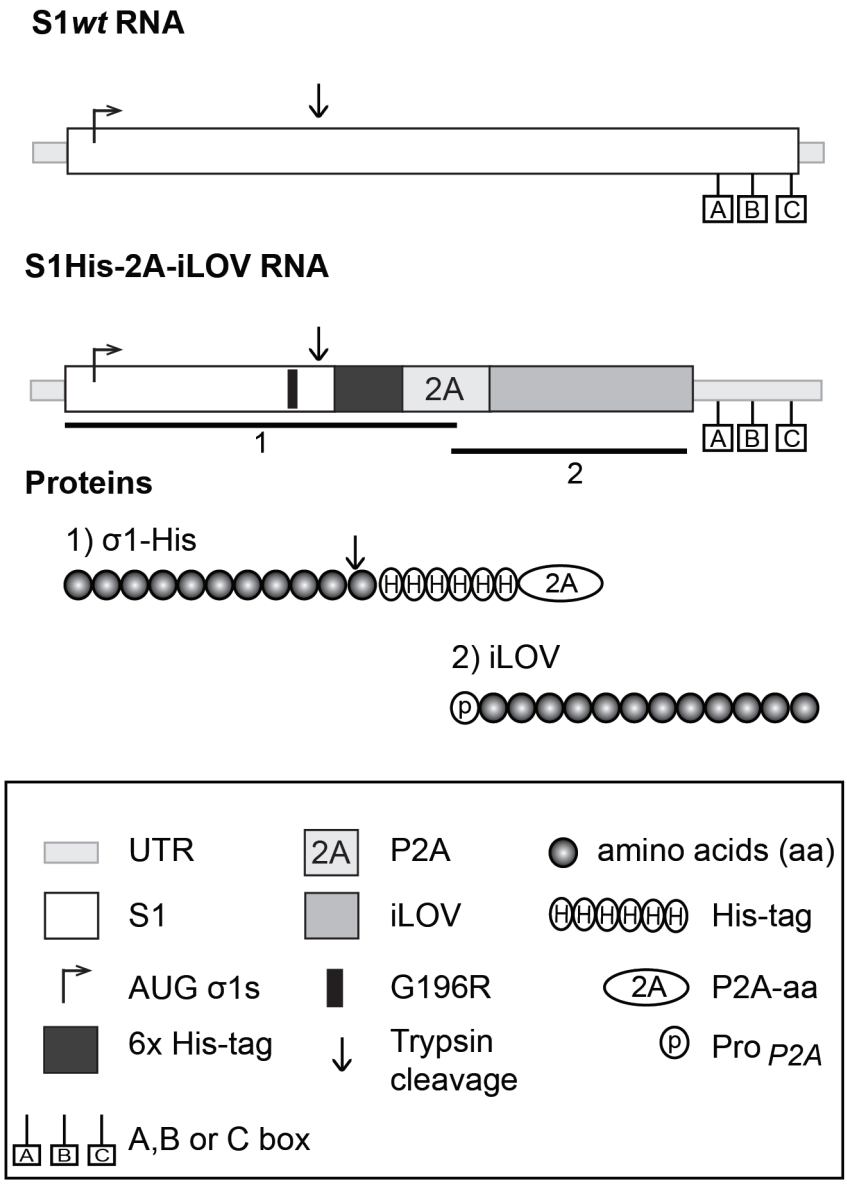

Figure 1. Schematic representation of S1-*wt* and S1His-2A-iLOV RNA. The length of the S1His-2A-iLOV and the *wt*-S1 segment is 1386 nt and 1416 nt, respectively. Two proteins are synthesized from the S1His-2A-iLOV ORF: 1) the σ1-His that contains some additional amino acids derived from the 2A peptide, and 2) the iLOV protein that contains an additional proline at the N-terminus as result of processing of the 2A sequence. (The drawing is not depicted in scale with regard to sizes of the molecules.) UTR, untranslated region.

For the rS1His-2A-iLOV viruses the yields per cell in three independent CsCl-density gradient purified virus batches ranged between 5.2 x 10^3 and 1.0 x 10^4 physical particles per cell, which is 10 – 30 fold lower than the yields obtained for *wt* and *jin-1* viruses. The iLOV-derived fluorescent signal was detectable by fluorescence microscopy (figure 2b). The fluorescence was quantified by flow cytometry. For this, 911scFvHis cells were either mock infected or infected with an early passage (P2) rS1His-2A-iLOV at a multiplicity of infection (MOI_{911}) of 0.4 IU_{911}/cell. Fluorescence was analysed by flow cytometry at 72 hours post-infection (figure 2c). In the rS1His-2A-iLOV infected cultures, 57% of the cells exhibited the iLOV fluorescence. These data demonstrate that the replacement of the codons for the head domain of the reovirus spike protein σ1 by an iLOV coding fragment yields infectious and replicating reoviruses.

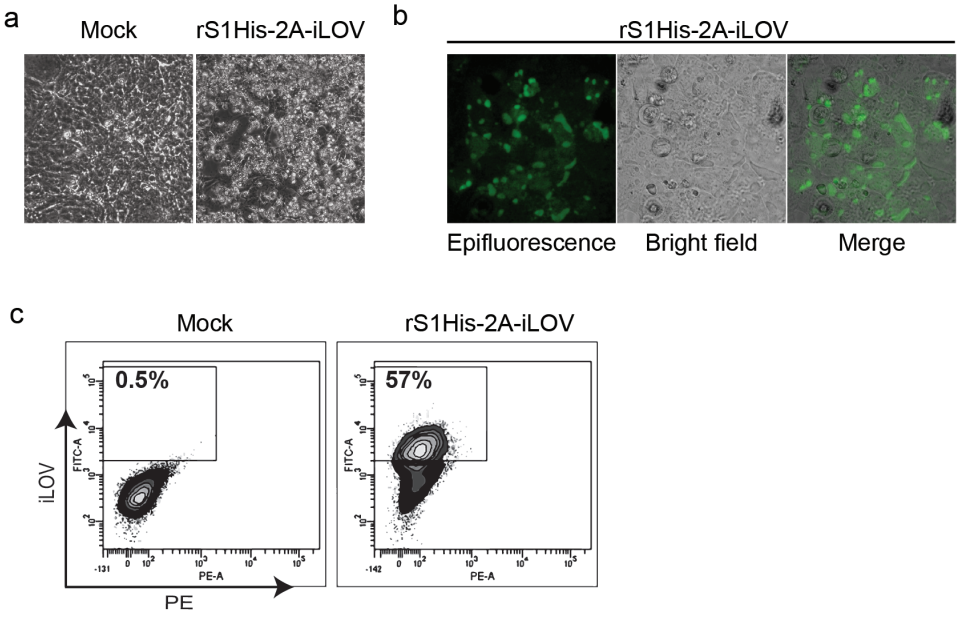

Figure 2. The recombinant S1His-2A-iLOV reovirus replicates in cells and is infectious. (a) Morphological appearance of mock infected 911scFvHis cells and 911scFvHis cells infected with an early passage of rS1His-2A-iLOV. **(b)** Detection of iLOV positive 911scFvHis cells infected with an early passage rS1His-2A-iLOV virus (MOI_{911} ~ 0.3). Bright-field and fluorescence photographs were taken using an Olympus CKX41 microscope. **(c)** Detection of iLOV-positive 911scFvHis cells infected with rS1His-2A-iLOV by flow cytometry at 72 hours post infection. 911scFvHis cells were either mock infected or infected with an early passage of rS1His-2A-iLOV (MOI_{911} ~ 0.4). The iLOV signal is plotted on the Y-axis (FITC-A) against the PE-A signal on the X-axis.

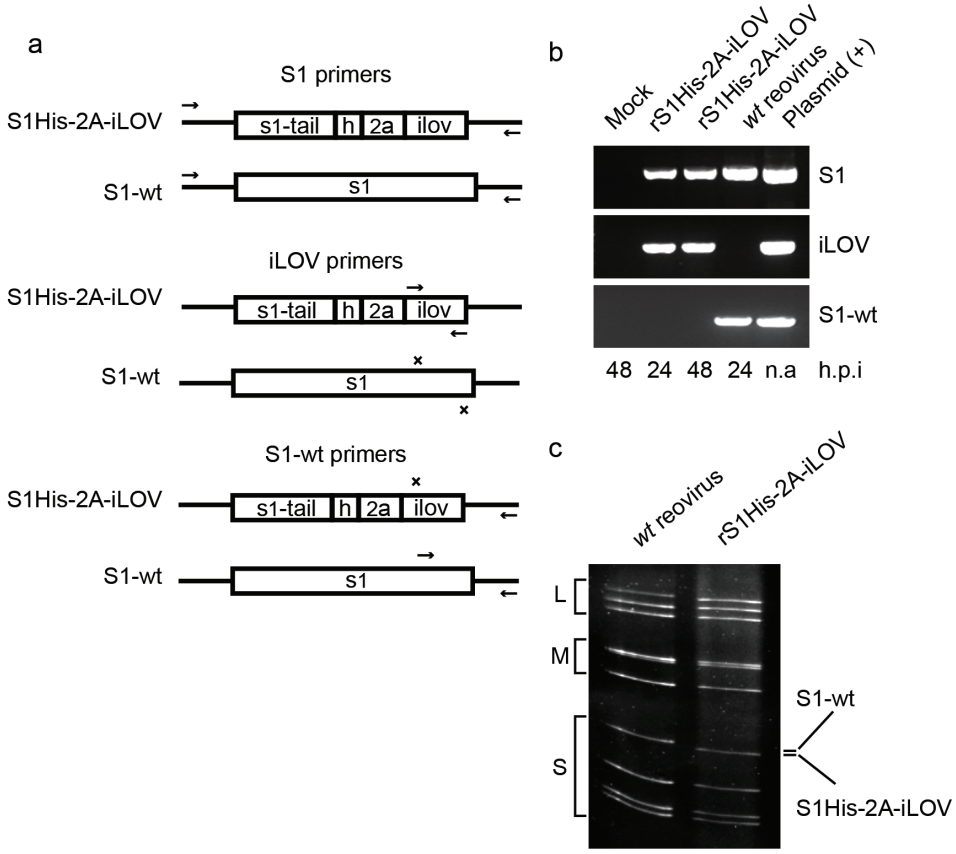

Figure 3. Detection of S1His-2A-iLOV RNA in infected 911scFvHis cells. (a) Schematic representation of binding sites for the primers used in the RT-PCR procedure. The primer-binding sites are indicated by arrows. The crosses denote non-binding sites. PCR products formed with S1 primers (S1For and S1endR) are 1416 bp (S1 derived from *wt* Reovirus) or 1386 bp (S1 derived from rS1His-2A-iLOV) in length. The PCR product generated with iLOV primers (iLOVFor and iLOVRev) is 303 bp and with S1-wt primers (S1testFor and S1endR) is 354 bp. **(b)** RT-PCR to detect S1 RNA, iLOV or absence of the S1 head domain in 911scFvHis cells infected with passage 1 of the rS1His-2A-iLOV reovirus, at 24 and 48 hours post infection (h.p.i). Controls are RNA isolated from either uninfected (mock; negative) or *wt* reovirus infected 911scFvHis cells, resp. 48 or 24 hours post infection. The positive DNA controls are plasmids pBACT7S1His-2A-iLOV in case of S1 and iLOV PCR and pBACT7S1T3D for the S1-*wt* PCR. Time post infection is not applicable (n.a.) for the PCR controls. **(c)** RNA electrophoresis of *wt* reovirus and rS1His-2A-iLOV virus. RNA was isolated from infected 911scFvHis cells, analysed by 12% PAGE-SDS and detected with Sybr-Gold. L, M and S and specifically S1 RNA segments are indicated.

S1His-2A-iLOV RNA is present in the recombinant reoviruses

To confirm the presence of the modified S1His-2A-iLOV segment in the recombinant reoviruses, RT-PCR was performed using total RNA isolated from rS1His-2A-iLOV-infected 911scFvHis cells at 24 and 48 hours post-infection. A schematic overview of the primers and their binding sites is represented in figure 3a. The S1 primer pair amplified the *wt* S1 segment as well as the iLOV-containing segment. The length of the PCR product of the iLOV-containing S1 segment almost equals the length of the *wt-T3D*-S1 segment (figure 3b) and the segment is present in both isolations (24 and 48 hour). The iLOV-specific PCR product is detectable in both isolates but not in the *wt*-Reo sample. As expected the PCR primers used to detect the region encoding the head domain of σ1 only amplified a fragment in the RNA isolated from *wt* reovirus infected cells but not in the samples from rS1His-2A-iLOV virus infected cells. Sequence analysis of the amplified S1His-2A-iLOV PCR product showed no additional mutations within the segment (data not shown).

To further confirm the presence of replicating reoviruses, total RNA was isolated from cells infected with either *wt*-Reo or with the rS1His-2A-iLOV reovirus, and used for poly-acrylamide gel electrophoresis (PAGE) (figure 3c). The ten individual segments could be detected, further confirming the presence of replicating reoviruses. The modified S1 segment is slightly smaller than the wild-type S1 segment, resulting in a marginally increased electrophoretic mobility. From these data we conclude that the S1His-2A-iLOV segment is packaged in the reovirus particles and that the *wt* S1 segment is absent from these reovirus preparations. These data demonstrate that the rS1His-2A-iLOV reoviruses replicate independent of viral helper functions.

The σ1-His protein is detectable in 911scFvHis cells infected with the recombinant reovirus and is incorporated in the rS1His-2A-iLOV particles

To verify that the inclusion of the porcine teschovirus-1 2A sequence yields a truncated σ1 protein in the rS1His-2A-iLOV infected cells, 911scFvHis cells were exposed to rS1His-2A-iLOV virus at an MOI_{911} of 2. At 24 and 48 hours post infection the cells were harvested and protein lysates of the infected cells were analysed by western-blotting. The presence of truncated σ1 was detected by immunoblotting using the polyclonal rabbit antibody S1R3 directed against a σ1-derived synthetic peptide. The σ1-His protein is detectable already 24 hours after infection. A specific band of approximately 30 kDa is seen only in lysates prepared from the rS1His-2A-iLOV infected cells. This is in agreement with the calculated molecular mass of the truncated σ1-His protein. In

contrast, in the lysates from the cells infected with the *wt* reovirus show a band that corresponds to the calculated molecular mass of *wt*-σ1 (approximately 49 kDa) (figure 4a).

To confirm the presence of the truncated σ1-His protein in the virus capsid, rS1His-2A-iLOV particles were produced and purified by CsCl density gradient centrifugation. Protein lysates from these purified particles were used for western blot analysis. As a control, purified *wt*-Reo was included. Again a band was detected migrating at an apparent molecular mass of 30 kDa only in the rS1His-2A-iLOV particles (figure 4b) and this band was also detected with the antibody directed against the 2A peptide. The σ3 is used as a loading control. Taken together, our data demonstrate that the 2A sequence yields separate σ1 and iLOV proteins. In addition, our data show that at least part of the truncated σ1 protein is associated with the reovirus capsid.

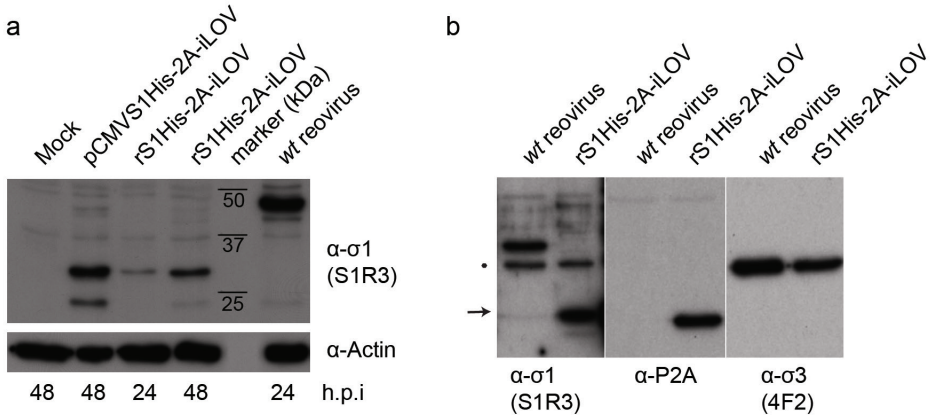

Figure 4 Detection of σ1-His in infected 911scFvHis cells and CsCl purified particles. (a) 911scFvHis cells were infected with early passage rS1His-2A-iLOV. Lysates of infected cells were made 24 hr or 48 hr post infection (h.p.i) and analysed by Western blotting. As a positive control for σ1His protein, cells transfected with pCMVS1His-2A-iLOV are used and lysates were made 48 hr post transfection. Cells infected with *wt*-reovirus (24 h.p.i) are taken along to visualize the difference in size; predicted size of *wt* σ1 is about 49 kDa and σ1-His after P2A processing is about 30 kDa. σ1 is detected with S1R3, polyclonal rabbit antibody directed against N-term of σ1. As a loading control a β-actin antibody is used. **(b)** Approximately $5*10^9$ particles of CsCl purified *wt* Reovirus or σ1-His containing rS1His-2A-iLOV reovirus was loaded onto 12% SDS-PAGE gels. S1R3 polyclonal antibody against N-term of σ1 was used for detection of both, the *wt*-σ1 and the σ1-His protein (arrow). The smaller σ1-His protein is also detected with an antibody directed against the P2A peptide. The dot indicates a background band, probably cross reactivity with σ3. As an indication of the amount of particles that are loaded on gel, σ3 is detected with monoclonal antibody 4F2.

rS1His-2A-iLOV virus can infect cells independently of the artificial scFvHis receptor and the natural JAM-A receptor.

So far, the rS1His-2A-iLOV reovirus was propagated in cells that contain the scFvHis as an artificial receptor for the modified virus. To test whether the rS1His-2A-iLOV virus can infect cells independently of this scFvHis receptor, cultures of 911 cells were exposed to the rS1His-2A-iLOV virus at an MOI_{911} of 0.4 and analyzed by flow cytometry for iLOV expression at 72 hours post infection (figure 5a). Approximately 46% of the 911 cells exhibited iLOV fluorescence. This demonstrates that the rS1His-2A-iLOV recombinant reovirus can also infect cells in absence of the artificial scFvHis receptor. To further test if the infection is independent of the reovirus receptor JAM-A, cultures of the JAM-A-deficient U118MG cells were exposed to rS1His-2A-iLOV at an MOI_{911} of 0.4 (figure 5a). Approximately 21% of the U118MG cells exhibit iLOV fluorescence. This demonstrates that the rS1His-2A-iLOV virus does require neither expression of scFvHis nor JAM-A as receptor for entry into cells.

To test the cytolytic activity of the rS1His-2A-iLOV reovirus, monolayer cultures of several cell lines that resist *wt*-Reo infection were exposed to rS1His-2A-iLOV, or *wt*-Reo at an MOI_{911} of 1, or mock infected. Four days post infection, cells were inspected visually for signs of CPE and micrographs were taken with an original magnification of 200x (figure 5b). Subsequently the viability of the cultures was established on the same day (figure 5c). In parallel, cultures of 911 cells were included to demonstrate that both *wt-T3D* reovirus (*wt*-Reo) and the modified rS1His-2A-iLOV virus induce cell death in JAM-A positive, permissive cells (figure 5b and c). In monolayer cultures of JAM-A deficient U-118MG cells, only the rS1His-2A-iLOV reoviruses, but not *wt*-Reos, induce CPE, resulting in a reduced viability (figure 5b and c). The U251 cell line has a low JAM-A expression (data not shown) and as a result is weakly permissive to *wt*-Reo. In contrast to *wt*-Reo, the rS1His-2A-iLOV reovirus induces marked cell death in the U251 cells. Finally, the chicken hepatoma cell line LMH does not express a functional JAM-A receptor and therefore resists *wt*-Reo infection. It is however sensitive to rS1His-2A-iLOV (figure 5c).

The *wt*-Reo induces apoptosis in many transformed cells[3]. The viral spike protein σ1 has been implicated in the induction of the programmed cell death[26]. To study whether the rS1His-2A-iLOV viruses, which lack the head domain of the σ1 spike protein, induce apoptosis we measured the activity of the caspases 3/7 upon reoviruses exposure in 911 cells. The cells were exposed to rS1His-2A-iLOV and, as controls, to *wt*-T3D and *jin-3* reoviruses at an MOI_{911} of 1. Caspase-3/7 activity was measured 24 or 48 hours post infection (figure 5d). In the 911 cells infected with rS1His-2A-iLOV the induction

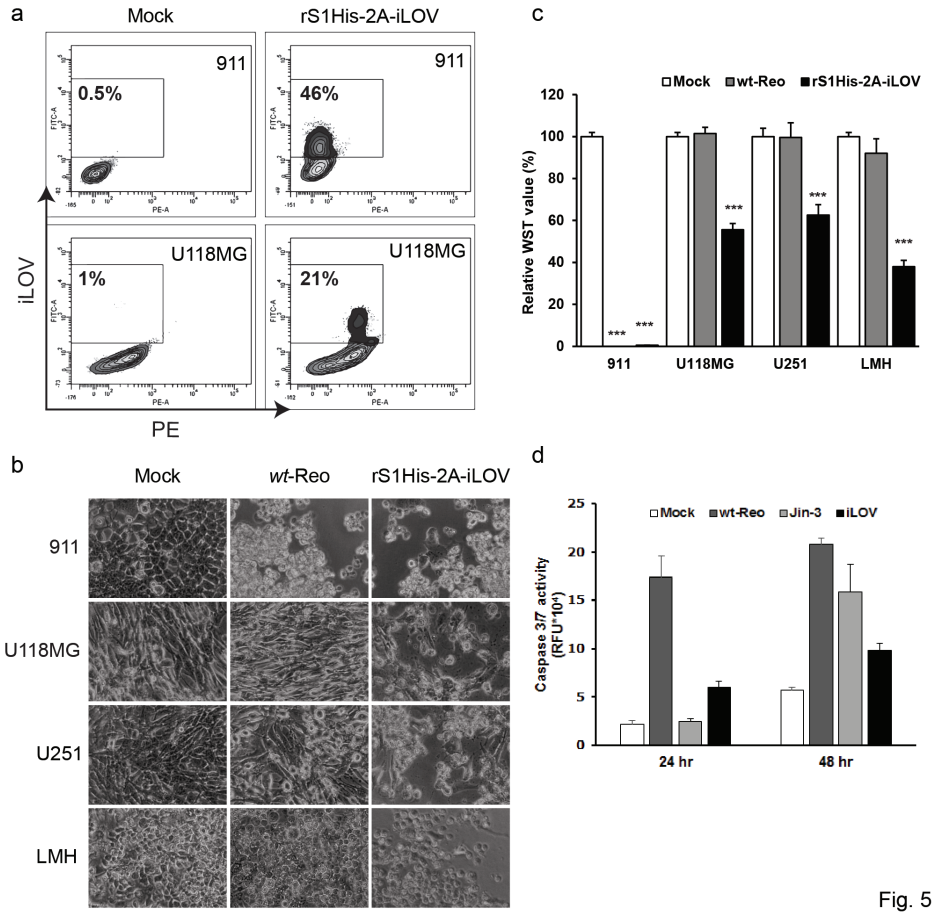

Fig. 5

Figure 5. rS1His-2A-iLOV reovirus infection is independent of the scFvHis receptor and Junction Adhesion Molecule-A . **(a)** 911 cells and U118MG cells were mock infected or infected with an early passage rS1His-2A-iLOV reovirus (MOI_{911} ~ 0.4). Cells were analysed by flow cytometry 72 hours post infection. iLOV signal is plotted on the Y-axis (FITC-A) against the PE-A signal on the X-axis. **(b)** CPE induction in the cell lines upon mock infection or upon infection with *wt*-T3D reovirus or rS1His-2A-iLOV (MOI_{911} ~1). Micrographs were taken 4 days post infection, prior to WST-1 assay. **(c)** Cell viability, as measured by WST-1 assay, of the various cell lines upon mock infection or infection with *wt*-T3D reovirus or rS1His-2A-iLOV (MOI_{911} ~1). Relative WST values are calculated as percentages of OD_{450} values of the infected cells over the mock treated cells. The error bars represent the SD (n=4). *** P<0.0001 and * P<0.01. **(d)** Capsase-3/7 activity in mock-infected culture or cell cultures (911) infected with *wt*-T3D reovirus, *jin-3,* and rS1His-2A-iLOV at an MOI_{911} ~ 5. Caspase 3/7 activity was measured 24 and 48 hours post infection. The error bars represent the SD (n=4). OD, optical density.

of caspase-3/7 activity is evident at 24 hours and 48 hours post infection, although in a lesser extent than upon *wt*-Reo infection. Also in cell exposed to *jin-3* caspase-3/7 activity is induced albeit somewhat delayed.
Taken together, these results demonstrate that the rS1His-2A-iLOV reovirus is cytotoxic in JAM-A deficient as well as in JAM-A-expressing cell lines. In 911 cells an increase in caspase-3/7 activity is detected upon rS1His-2A-iLOV infection, suggesting that these viruses can exert their cytolytic activity by the induction of classic apoptosis.

Lec2 cells are less sensitive for rS1His-2A-iLOV infection than the parental CHO cells

The JAM-A independent cell entry of rS1His-2A-iLOV is possible by the interaction of the *jin-3* derived sialic-acid binding region in the tail region of σ1. To confirm that sialic-acids are involved in binding of rS1His-2A-iLOV to cells, we exposed Lec2 cells to this virus. Lec2 cells are Chinese Hamster Ovary mutant cells that have a defect in the translocation of CMP-sialic-acid into the Golgi compartment causing a 90% reduction of sialic acids on their cell surface[27]. To verify this phenotype in Lec2 cells we exposed Lec2 and normal CHO cells to FITC-A-conjugated Wheat Germ Agglutinin (WGA-FITC) and measure the binding of WGA-FITC to the cells by flow cytometry (figure 6a). The FITC signal of WGA-FITC exposed CHO cells is stronger than in Lec2 cells (Median FITC-A value in WGA-FITC CHO cells is 18349 and for WGA-FITC-exposed Lec2 cells 2012), confirming the sialic acids-deficient phenotype of the Lec2 cells.
Next we assessed the sensitivity of Lec2 cells to infection with reovirus rS1His-2A-iLOV. Lec2, CHO and 911 cells were mock infected, infected with rS1His-2A-iLOV, or with *jin-3* at an MOI_{911} of 0.5 and 48 hours post infection the iLOV-positive cells were detected by flow cytometry (figure 6b). As expected exposure to *jin-3* did not result in detectable green fluorescence. This confirms the absence of autofluorescence upon infection with reovirus . In Lec2 cells infected with rS1His-2A-iLOV only 14% of the cells are iLOV positive, while the CHO and 911 cells infected with rS1His-2A-iLOV contain over 70% of iLOV positive cells. These data demonstrate that the reduction of the sialic-acids concentration on the surface of the Lec2 cells correlates with a decreased sensitivity to rS1His-2A-iLOV virus infection.

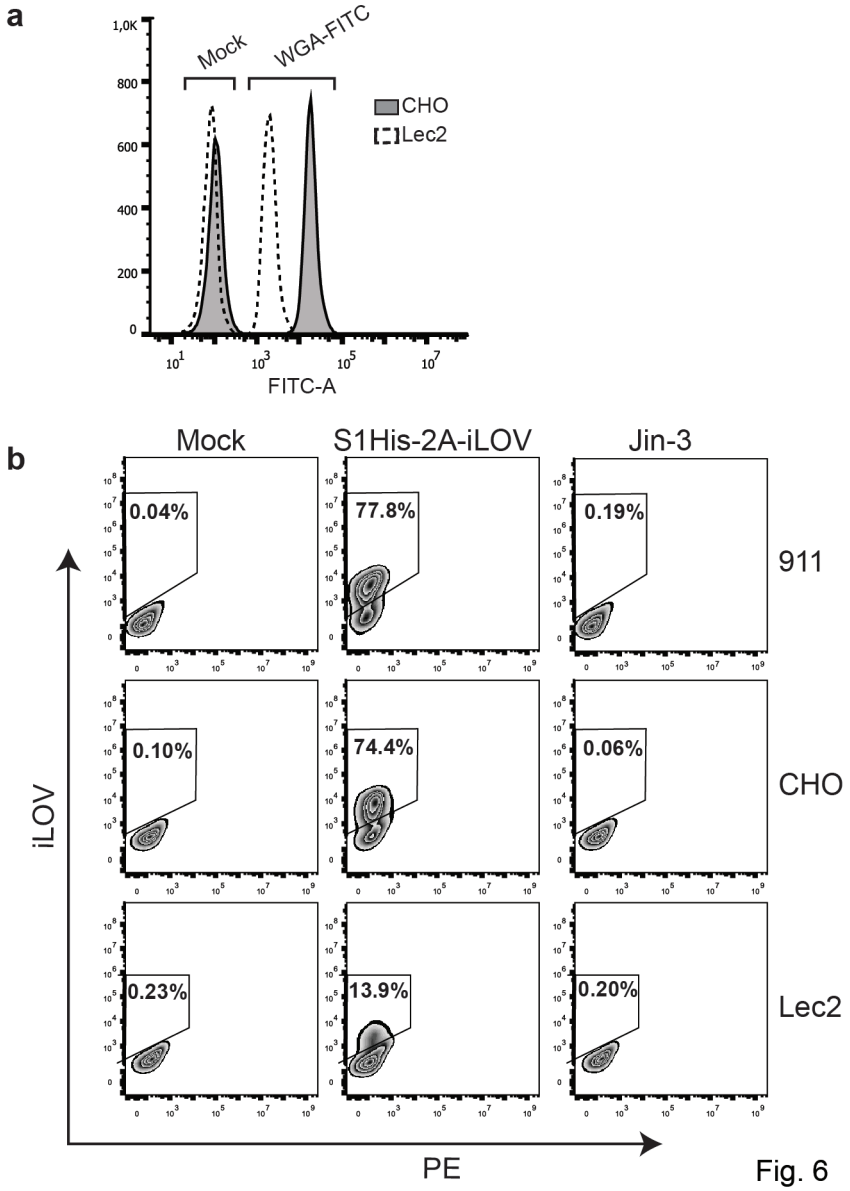

Figure 6. The fraction of iLOV-positive cells upon reovirus rS1His-2A-iLOV exposure of Lec2 and CHO cells. (a) Lec2 cells and CHO cells were exposed to WGA-FITC and WGA-binding was evaluated by flow cytometry analysis. Lec2 cells have a ~90% reduction in WGA-FITC binding. **(b)** Flow cytometry analysis of 911, CHO, and Lec2 cells following infection with rS1His-2A-iLOV, *jin-3* (MOI_{911}~0.5) or mock infected cells, 48 hours post infection. iLOV signal is plotted on the Y-axis against the PE-A signal on the X-axis. Gates for iLOV positive cells are set per cell line.

rS1His-2A-iLOV reoviruses retained the preferential cytolytic activity in tumour cells

To verify that the preferential cytolysis of transformed cells is maintained for rS1His-2A-iLOV, cultures of diploid human skin fibroblasts (VH10 cells) were used as a model. VH10 cells were exposed to rS1His-2A-iLOV, and *jin-3* reoviruses or mock infected. Compared to the 911-control infection (same as in figure 6b) only a few iLOV positive cells (3.19%) were detected in the VH10-infected cells (figure 7a). To further demonstrate that the VH10 cells resist rS1His-2A-iLOV-induced cell death, cells were exposed to rS1His-2A-iLOV and *jin-3* reoviruses at an MOI_{911}~5, or mock infected. Viability was measured four days post infection. As a positive control, 911 cells were included in the assay (figure 7b). Whereas in 911 cells both reoviruses induce significant cell death in the monolayer cultures, the viability of the VH10 cell cultures is not affected. These results confirm that similar to the *jin-3 and wt*-T3D reoviruses, the rS1His-2A-iLOV viruses do not infect and lyse normal diploid skin fibroblasts.

Characterization of two deletion mutants that lost the iLOV gene

In two batches of the rS1His-2A-iLOV reoviruses, derived from independent transfection experiments, we noted the loss of transgene expression and the appearance of deletion mutants upon prolonged passaging. The viruses had been propagated for 10 rounds on 911 cells (Experiment 1) and 911scFvHis cells (Experiment 2). RT-PCR analysis revealed the presence of deletion mutants in the rS1His-2A-iLOV stocks (data not shown). This was confirmed by the gel electrophoresis of RNA isolated from infected cells (figure 8a). Already at passage 3 of the reovirus on 911 cells, the deleted segment could be seen as an aberrant RNA segment migrating faster than the smallest S segment. On gel, both the full-length S1His-2A-iLOV segment and the S1 deletion segment are visible, suggesting a heterogeneous population of viruses. In RNA isolated from passage 10 only the S1 deletion segment could be detected, suggesting that this deletion mutant overgrew the population.

In a batch of rS1His-2A-iLOV reovirus propagated on 911scFvHis cells a deletion mutant was observed after 10 passages with an even smaller S1 segment. Sequence analyses of PCR products of the deletion mutants revealed that in both mutants the iLOV encoding region had been deleted resulting in S1 segments of 1001 nt (*dl1,* S1His-2A-iLOVΔ*nt 877-1261*) and 922 nt (*dl2,* S1His-2A-iLOVΔ*nt 751-1214*) in length, respectively (figure 8b). In *dl2*, the 6xHis-tag and the 2A sequence had also been deleted. Interestingly, in *dl2* the A-box, and in *dl1* the A-box and the B-box, which both have been implicated

in encapsidation of reovirus RNA into the viral capsid[24], were deleted, while the C-box element had been retained in both mutants. This suggests that the A and B boxes are dispensable for packaging the S1 segment RNA into the viral capsid.

The results from the RNA analyses were further verified at the protein level in 911scFvHis-cell lysates from cells infected with the reovirus batches containing the deletion mutants (figure 8c). In passage 2 of both batches and passage 10 of the first batch, the σ1 protein contains the 2A element, whereas 2A is no longer detectable in passage 10 of batch 2. As expected the truncated σ1 protein migrates at an apparent molecular mass of 26 kDa instead of 30 kDa.

Taken together our data indicate that it is possible to insert the iLOV coding region in the S1 segment. However care should be taken to test the integrity of the transgene containing segment if the recombinant viruses are passaged to high passage number. Notably, apart from these deletions no other mutations were found in the S1 segment. This suggests that the sequence encoding the N-terminal 252 amino acids of the *jin-3* σ1 is sufficient for cell entry.

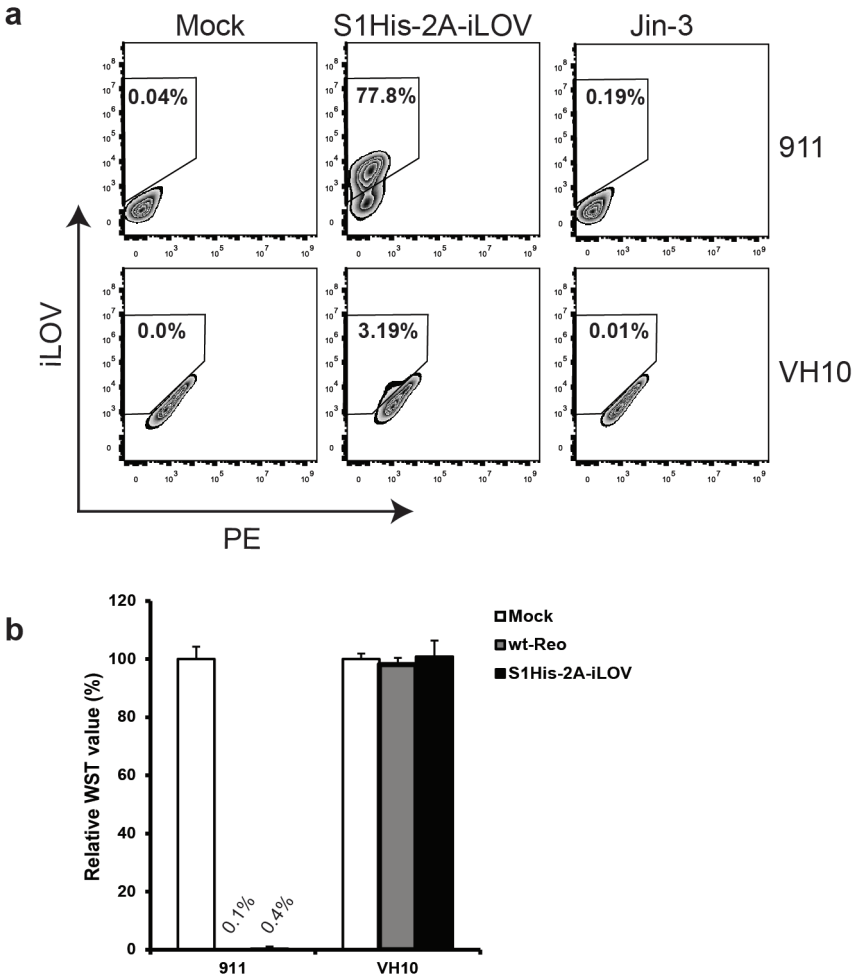

Figure 7. Detection of iLOV positive cells and viability of VH10 cells exposed to rS1His-2A-iLOV. (a) Flow cytometry analysis of 911 and VH10 cells exposed to rS1His-2A-iLOV, *jin-3* (MOI_{911}~0.5) or mock treated cells, 48 hours post infection. The panel of 911 cells is copied from fig 6 b (VH10 samples were included in the same FACS experiment). iLOV signal is plotted on the Y axis against the PE-A signal on the X-axis. Gates for iLOV positive cells are set per cell line. **(b)** Cell viability, as measured by the WST-1 assay, four days post infection with rS1His-2A-iLOV, *jin-3* (MOI_{911}~1) or mock infected. Relative WST values are calculated as percentages of OD_{450} values of the infected cells over the mock treated cells. The error bars represent the SD (n=4). OD, optical density.

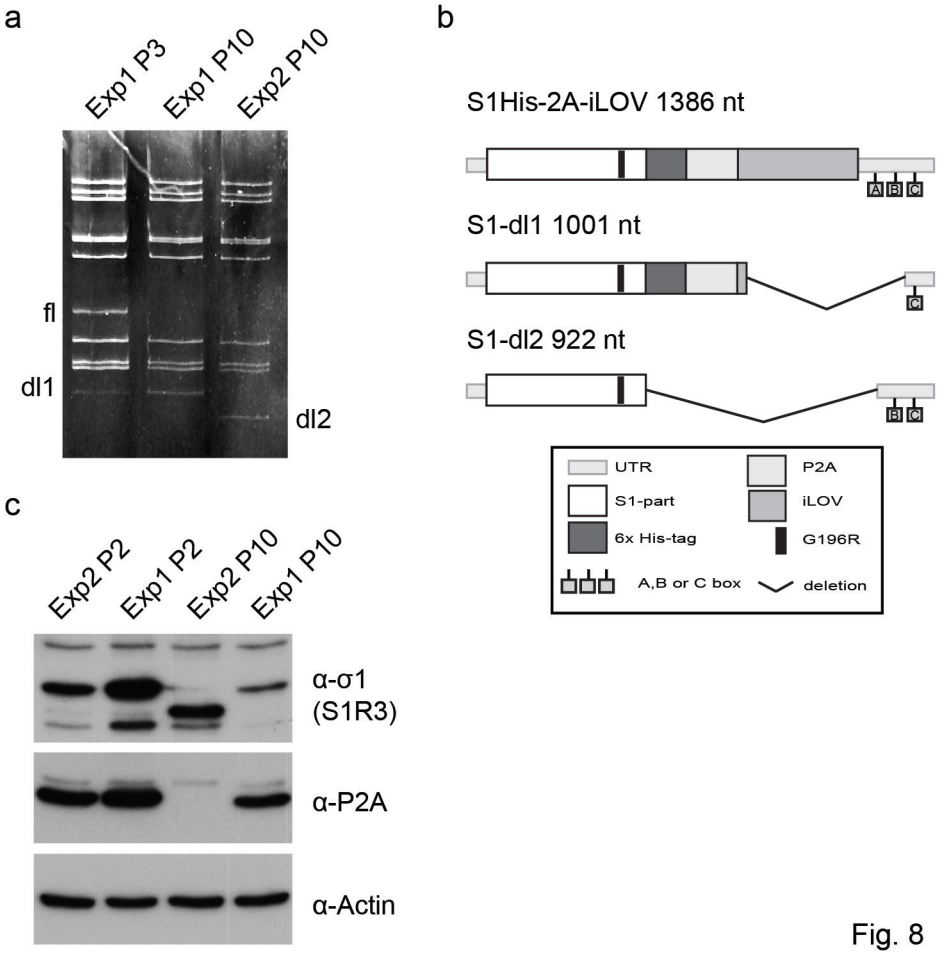

Fig. 8

Figure 8. Deletion mutants are detected in two batches of the rS1His-2A-iLOV virus. (a) RNA electrophoresis of 3 rS1His-2A-iLOV isolates from 2 independent experiments. In P3 of experiment 1 (Exp1) the deletion in the S1 segment (dl1) is already present next to the full-length (fl) S1His-2A-iLOV segment, while in P10 only S1-dl1 is present. Size of S1-dl1 is 1001 bp. Size of S1-dl2, derived from second experiment (Exp2) after 10 passages in 911scFvHis cells is 922 bp. **(b)** Schematic overview of the two S1-deletion mutants compared to the full length S1His-2A-iLOV. **(c)** Detection of σ1 protein in infected 911scFvHis cells in two independent batches of rS1His-2A-iLOV reoviruses containing deletion mutants. Lysates were made 48 hours after infection and equal amounts of protein were loaded on 12% PAGE-SDS gels and detected with antibodies directed against σ1 (S1R3), P2A (ABS31) or actin. The predicted size of σ1 in the deletion mutant (S1-dl2) is about 26 kD.

DISCUSSION

Our data suggest that the S1 segment encoding σ1 is suitable for insertion of a transgene encoding a heterologous protein. We demonstrate that the S1 segment can harbour heterologous sequences that encode the fluorescent protein iLOV. In this construct, the iLOV-encoding sequence was preceded by the codons for the porcine teschovirus-1 2A peptide. Upon translation this yields separate proteins from the fused open reading frame. To reduce the size of the transgene-containing S1 segment, the sequences encoding the head-domain of σ1 were deleted. The head domain harbours the amino acids that interact with the JAM-A receptor. The head-domain could be removed by employing the σ1 sequences from the *jin-3* mutant[12]. This mutant is one of a series of reovirus T3D mutants that can infect cells in a JAM-A receptor-independent fashion. The mutations in these *jin*-mutants lead to amino-acid alterations close to the sialic-acid binding site in the σ1 protein, which is located in the tail domain of the spike[14,15,26].
To release the S1-His protein from the iLOV protein, the porcine teschovirus-1 2A element was introduced. The length of the fragment encoding the 2A peptide is only 66 nt, and it also includes the codons for a GSG-linker to improve the efficiency[28,29]. The 2A mediated separation is highly efficient and results in reovirus particles that do not contain the iLOV protein.
The S1His-2A-iLOV RNA was found to be packaged in the reovirus particles in 911 and 911scFvHis cells and transferred to target cells as was evident from RT-PCR analyses. The truncated σ1-His protein fragment was detected in infected cells and in CsCl-gradient purified reovirus particles.
The utility of the σ1 head domain-coding region as a locale for the transgene was further supported by the observation that an attenuated T3D reovirus derived from persistently infected HT1080 cells carries a mutation in S1 which resulted in a stop codon near the codons encoding the trypsin-sensitive site at amino acids 245 of the spike protein. This mutation results in truncation of the σ1 protein leading to attenuation of the virus upon inoculation of immune-deficient mice[30]. While the size of the S1 segment in this isolate was not altered, the σ1 protein lacks the head domain. This mutant reovirus replicated efficiently in L929 cells. As in our experiment the truncated σ1 protein was detected by immunoblots in purified particles produced on of L929 cells. The truncated protein consisted of the first 251 amino acids of σ1, and still contained the sialic-acid binding domain and the open reading frame encoding the σ1S protein[15,31,32].
In our modified S1 segment, the last 195 nucleotides of wild-type S1 segment were left intact. This region has been predicted to contain three cis-acting sequences required for packaging of the reovirus plus-strand RNA in the core; A, B and C-boxes[24]. In a

deletion mutant characterized in our study we found that the A and the B-box were deleted. This suggests that the A and B box are dispensable, reducing the length of the region required for packaging to only 36 nt. The 5'-end of the S1 segment has been left intact. In our initial experiments, we tried to include the ORF encoding the *Discosoma sp.* red fluorescent protein (DsRed). This ORF is 678 nt in length, and was placed in-frame, downstream of the 6xHis-tag and the porcine teschovirus-1 2A element. Although we noticed the formation of replicating reoviruses, none of these transferred an intact copy of the DsRed transgene, as was evident from the complete absence of red fluorescence in the infected cells, despite the presence of marked cytopathicity. In this construct the total length of the modified S1 segment was 1722 nt, and we speculated that our inability to generate the recombinant dsRed-gene containing reoviruses was due to the larger size of the S1-dsRed segment. So far, limited data is available on the factors that restricts the packaging capacity of foreign sequences into the reovirus genome. In a recent study the insertion of long unmodified Simian Immunodeficiency Virus derived gag sequences in the reovirus M1 and L1 segments were not compatible with replication[9]. The failure to recover the recombinant reoviruses with the longer gag inserts was explained by the formation and stability of the RNA secondary structures of the inserted gag sequence. Upon reduction of the stability of the predicted RNA structure by wobble-mutagenesis, infectious recombinant reoviruses with the longer gag inserts could be obtained.

To circumvent the problem of possible size constraints, we evaluated the use of the smaller reporter iLOV. The coding region of this reporter is only 333 nucleotides in length. With the S1His-2A-iLOV construct we could generate replicating reoviruses harbouring the iLOV ORF as a reporter gene. The iLOV signal could be detected in infected cells by fluorescence microscopy, but the signal was often weak and bleached rapidly. Nevertheless, the signal was detectable by flow cytometry. The iLOV sequence was based on a codon-optimized version of the iLOV protein sequence described in 2008[20]. A codon-optimized iLOV has been used to generate an infectious recombinant foot-and-mouth disease virus[33]. Here too, the fluorescent signal of iLOV was reported to be relatively weak. Recently, more stable iLOV variants were generated that provided a stronger signal in bacterial and mammalian cells[34].

We have used a similar strategy for the expression of a small viral protein fused to the 2A sequence. Viable recombinant viruses could be generated and our preliminary results demonstrate the absence of deletions in the virus preparation upon prolonged passaging (up to 7 passages) (data not shown). These data further suggest that our

head-replacement approach for expressing heterologous transgenes is feasible. It may be warranted that the integrity of the viruses should be tested regularly to avoid the presence of deletion mutants lacking the transgene product.

Based on the identification of the two viable S1-deletion mutants in our batches of rS1His-2A-iLOV reoviruses, the truncated S1 RNAs are effectively packaged into the reovirus cores. Smaller M1 deletion fragments were detected upon serial passages of reovirus Type 1 x Type 3 reassortants[35]. The smallest deletion mutant of M1 that was assembled into progeny viruses was approximately 350 nucleotides in length. The M1-deletion mutants are defective and rely on the presence of helper viruses that contain the full-length M1 to complement the deficiency. Based upon the analyses of different deletion mutants the authors concluded that the deletions do not occur randomly, instead they depend on the presence of particular sequence elements. In avian reoviruses, which are more distant members of the orthoreovirus genus[36], high MOI passaging yielded defective interfering (DI) particles containing S1-deletion mutants in which the residual S1 segment was approximately 400 nucleotides in length[37]. The increase in the presence of the S1-deletion segment coincided with a decrease in full-length S1 RNA in purified avian virus particles. In infected chick embryo fibroblasts (CEF), the presence of the DI particles decreases the viral yields. As with the mammalian M1-deletion mutants, the replication of these DI mutants in cells relies on the presence of viruses with the full-length S1. Our S1-deletion mutants are distinct from these DI particles by virtue of their capacity to replicate autonomously and hence independently of wild-type helper viruses. Therefore such mutants may pave the way for generating recombinant reoviruses with even larger replacements of the S1 segment by heterologous sequences. The replacement of the head domain allows the insertion transgenes up to 522 nucleotides in length without exceeding the size of the *wt* S1 segment. It remains to be established whether the occurrence of deletion mutants in reoviral vectors constitutes a limitation for their use as oncolytic agents in clinical applications. Whereas deletion mutants are detected, in our hands the frequency of their occurrence is low enough to prevent them from impeding the preclinical experiments with these transgene containing viruses. Stocks containing deletion mutants can just be discarded. In both cases the deletion mutants could be traced back to low passage number stocks, suggesting that they may have been produced already during the generation of the recombinant viruses. The methodology to generate these viruses involves the generation of non-polyadenylated RNA molecules in the cells using T7 polymerase and ribozymes and such transcripts are known to be instable. This is supported by the observation that the rS1His-2A-iLOV virus could be stably propagated for 10 passages after expansion of a clonal isolate (data not shown).

So far, the yields of the head-domain replacement reovirus vectors is 10 – 30-fold lower than those of the *wt*–T3D reoviruses on a per-cell basis. Several mechanisms could contribute to the reduced yields. The initial infections of the producer cells may be more asynchronous than with head-domain containing viruses, leading to reduced yields in single-step growth cultures. However, delaying the harvest did not compensate for this and did not normalize the yield (data not shown). Alternatively, the region of the *wt*–T3D S1 segment that was deleted in the rS1His-2A-iLOV virus may contain *cis*-acting elements that negatively affect the virus yields when deleted. So far we have found no evidence for such mechanism. Also it could be conceivable that the iLOV transgene contains sequences that inhibit efficient production of particles. This seems unlikely, since the mutants from which the iLOV transgene had been deleted did not yield higher titers. Also, reoviruses with other small transgenes did yield similar titers (data not shown). Prolonged passaging of the rS1His-2A-iLOV virus did not yield other mutations in S1 than the deletion mutants mentioned above. This suggests that the *jin-3* mutation leading to the G196R substitution is stable and does not evolve further to further enhance reovirus yields. Our current efforts to improve the yields employ reoviruses carrying therapeutic transgenes and involve bioselections.

The virus retained the capacity of the parental *jin-3* mutant to infect cells in the absence of the JAM-A receptor on the target cells, which is evident from the virus' capacity to infect JAM-A negative U118MG cells and chicken LMH cells in monolayer cultures The virus rS1His-2A-iLOV induces caspase 3/7 activity in 911 cells. This demonstrates that the induction of apoptosis by reovirus is independent of the virus' capacity to bind JAM-A. This is consistent with roles of either sialic-acid binding by the reovirus spike protein, or the σ1S protein in this process[18,26], as both of these functions are retained in the reovirus vectors described in our study.

The modification of the S1 segment in the rS1His-2A-iLOV does not result in a loss of preference for transformed cells. Neither rS1His-2A-iLOV virus nor the *jin-3* virus do not induce CPE or apoptosis in human diploid skin fibroblasts. This, together with the virus' capacity to induce cytopathic cell death makes the modification strategy particularly suited for arming oncolytic reoviruses with transgenes that encode immune stimulatory molecules, antigenic proteins, or anti-tumour polypeptides. This strategy may be suitable for generating replication-competent reoviruses carrying a transgene for use as a vaccine vector or for boosting the antitumour-efficacy of oncolytic reoviruses.

MATERIALS AND METHODS

Cell lines and viruses

The cell lines U118MG, U251, LMH, CHO, Lec2 (all obtained from ATCC) and 911 were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Life Technologies, Bleiswijk, The Netherlands), supplemented with 8% fetal bovine serum (FBS; Invitrogen) and with penicillin and streptomycin (pen-strep) as described[13,38]. LMH cells were cultured on dishes coated with 0.1% gelatin (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Lec2 cells were cultured in alpha-MEM, supplemented with 8% FBS and pen-strep. The 911scFvHis cells were generated by transduction of 911 cells with the lentivirus vector pLV-scFvHis-IRES-Neo as described before[8]. The resulting polyclonal cell line was cultured in DMEM containing 8% FBS, pen-strep and 200 µg/ml G418. The T7-RNA polymerase expressing cell line BSR-T7[39] was provided by K. Conzelman and cultured in DMEM, 8%FBS, pen-strep and 400 µg/ml G418. Normal foreskin fibroblasts (VH10), were provided by B. Klein[40] and cultured in high-glucose DMEM supplemented with 8% FBS and pen-strep. All cells are cultured in an atmosphere of 5% CO2 at 37°C.

The wild-type T3D virus strain R124 was isolated from reovirus T3D stock VR-824 from the American Type Culture Collection (stock VR-824) by two rounds of plaque purification and propagated on 911 cells. In text this virus is referred to as *wt*-T3D reovirus. The viruses were quantitated by plaque assay on 911 cells as described and virus concentrations and multiplicities of infection were defined on the bases of these titers, e.g. MOI_{911}[37].

Cloning of S1His-2A-iLOV in pBacT7 backbone

The S1His-2A-iLOV segment was designed *in silico* and a DNA copy was synthesized by Eurofins MWG Operon (Ebersberg, Germany). The total length of this synthetic segment is 1386 bp (Fig. 1). The segment sequence is assembled to contain the following features : *1)* nt 1 to 768 from the S1 segment of reovirus mutant *jin-3*; this includes the 5' UTR, entire σ1s ORF, and the first 252 amino acids of the *jin-3* σ1, including the codons for the G196R change near the sialic-acid binding domain[12]; *2)* the codons for a 6xHis-tag (18 bp) which is placed in frame with the σ1 part; *3)* the codons for the porcine teschovirus-1 2A sequence (66 bp + 3 additional bp); *4)* the iLOV-encoding sequence (333 bp) which was reverse translated and human-codon optimized from the amino acid sequence published by Chapman et al.[20] using the web-based Encor Biotechnology Inc. (http://www.encorbio.com/protocols/Codon.htm); *5)* the stop codon and 3' end of S1 segment from nt 1219 to 1416 of reovirus T3D, which include A-, B, and C-box

elements predicted for encapsidation of the reovirus ssRNA in the capsid[24,41], as well as the 3′ UTR. The resulting sequence was manually edited to remove or include specific restriction enzyme recognition sites in the designer DNA fragment.
The synthetic S1His-2A-iLOV fusion construct was inserted in a pBluescript plasmid by Eurofins MWG Operon (to generate pBSmwg-S1His-iLOV). The S1His-2A-iLOV part was further PCR amplified from this construct using forward primer T7S1For (5′-TAATACGACTCACTATAGCTATTGGTCGGATGGATCCTCGCCTACGT-3′) and reverse primer S1iLOVendR (5′-ACGTGATATCCCTCGCGATGAAAT-3′) with *Pfu* polymerase (Fermentas, FisherScientific, Landsmeer, The Netherlands). The underlined sequences are the parts complementary to the sequence in the pBSmwg-S1His-2A-iLOV construct. The PCR product was digested with *Sac*II and purified with SureClean (Bioline; GC Biotech BV, Alphen aan den Rijn, The Netherlands) according to the manual. The PCR product was inserted in the pBACT7 backbone of pBacT7-S1T3D[7]. Plasmid pBacT7-S1T3D was purchased at Addgene (plasmid 33282, www.addgene.org). The *wt* S1T3D was removed by digestion with *Sma*I and *Sac*II and the pBACT7 backbone was isolated from a 1% agarose gel and purified by JetSorb (Genomed; ITK Diagnostics BV, Uithoorn, Netherlands) according to the manufacturer's manual. The *Sac*II-digested S1His-2A-iLOV-containing PCR product was inserted in the *Sma*I and *Sac*II digested pBACT7 DNA with T4 DNA ligase (Fermentas, FisherScientific, Landsmeer, The Netherlands), resulting in construct pBT7-S1His-2A-iLOV.

Generation of reovirus rS1His-2A-iLOV

For generation of recombinant reoviruses we used the procedure described by Boehme et al.[42] with some modifications. Whereas the original article employs 10 plasmids for providing the full complement of reovirus segments[7], our system uses five plasmids. In addition to pBT7-S1His-2A-iLOV, we used the following plasmids which were all obtained from Addgene: pT7-L1T1L (plasmid 33286), pT7-L2-M3T3D (plasmid 33300), pT7-L3-M1T3D (plasmid 33301) and pT7-M2-S2-S3-S4T3D (plasmid 33302). For generating recombinant reoviruses the five plasmids were transfected into BSR-T7 cells using the TransIT-LT1 transfection reagent (Mirus; Sopachem BV, Ochten, Netherlands), according to the manufacturer's manual. Two µg of each of the plasmids was mixed and added to the cells with 30 µl TransIT reagent. Two days post-transfection the cells were harvested and lysed by three cycles of freeze-thawing. After pelleting the cell debris, the cleared supernatant containing the recombinant reoviruses was added to 911scFvHis cells in a 6-well plate. Upon the first appearance of cytopathic effects (CPE) the cells were harvested and the rS1His-2A-iLOV reovirus was released from the cells by three cycles of freeze-thawing. The cell debris was removed from the reovirus-

containing lysate by centrifugation (10' at 3000 x g), and stored at -20 °C until further use. The virus was routinely passaged by exposing fresh semi-confluent cultures to dilutions of the cleared lysates.

A medium-sized batch of cesium chloride-purified rS1His-2A-iLOV was prepared by loading the cleared freeze-thaw lysates onto a discontinuous CsCl gradient (1.45 g/cm^3 and 1.2 g/cm^3) in PBS. After centrifugation in a SW28 rotor at 95000 x g for 4 hours at 16 °C, the lower band, containing the infectious particles, was harvested and desalted in an Amicon® Ultra 100K device according to manufacturer's manual (Millipore, Merck Chemicals BV, Amsterdam, Netherlands). The CsCl-purified reovirus was recovered in reovirus storage buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl, 10mM MgCl2·6 H2O, 5% sucrose) was aliquoted and stored at -80 °C until use. The amount of particles was calculated based on the OD_{260} values[43].

Reverse transcriptase PCR and sequence analysis

Cells (911scFvHis, 1*10^5) in 24 well plates were infected with rS1His-2A-iLOV. Since we had no indication of the titer of the early passaged batch (P1 of rS1His-2A-iLOV) obtained in experiment 1, 1/20th part of the isolated reovirus was used for exposure to the cells. Total RNA was extracted with the Absolutely RNA miniprep kit (Stratagene, Agilent Technologies, Amstelveen, The Netherlands) from the infected cells according to the manual. cDNA was synthesised with the S1endR primer (5'- GATGAAATGCCCCAGTGC-3') and SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Bleiswijk, The Netherlands). In the PCR the following primers were used to detect 1) S1 (S1For: 5'- GCTATTGGTCGGATGGATCCTCG-3' and S1endR); 2) iLOV (forward primer: 5'- ATGATCGAGAAGAACTTCGTGATC-3' and reverse primer: 5'- CACGTGGTCGCTGCCGTCCAGCTG-3') and 3) S1Head part (S1testFor: 5'-GAGCATGTGGATAGGAATTG-3' and S1endR) with GoTaq polymerase (Promega, Leiden, The Netherlands). The positions of the primer binding sites are shown in figure 3B.

For sequence analysis *PFU* was used as the polymerase and the PCR products of the S1 segments (full-length in early passages and S1-dl1 and –dl2) were sent to our LGTC department (LUMC, Leiden, the Netherlands). Primers used in the sequence reactions: S1endR and S1For.

Western blot analysis

Cell lysates were prepared in Giordano Lysis Buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.1% Triton, 5 mM EDTA) supplemented with protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). Total amount of protein in the lysates was measured by Bradford assay (Biorad, Veenendaal, The Netherlands). Equal

amounts of lysate (30 µg) were loaded into the wells of a 12% polyacrylamide-SDS gel after addition of western sample buffer (final concentrations: 10% glycerol, 2% SDS, 50 mM Tris-HCl pH 6.8, 2.5% β-mercaptoethanol and 0.025% bromophenol blue).
For electrophoresis of CsCl purified reoviruses ~5*10^9 particles were mixed with the above-mentioned western sample buffer and added to a 12% polyacrylamide SDS gel. The proteins were transferred to Immobilon-P (Millipore, Etten-Leur, The Netherlands) and visualized using standard protocols. For every antibody a different polyacrylamide-SDS gel was used unless otherwise stated.
The antibodies used in this study were S1R3, a monospecific polyclonal rabbit serum directed against the synthetic peptide GLAELRVDHDNLVARV, representing amino acids 115-130 of the σ1-tail (generated by A. D. Lipińska, University of Gdańsk, Gdańsk, Poland). The peptide was glutaraldehyde-conjugated to glutathione S-transferase purified from the pGEX system, according to the manufacturer instructions (Amersham) and used to immunize rabbits. Mouse monoclonal antibody 4F2, directed against reovirus σ3 obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242[44]. Antibody (mouse) directed against β-Actin: ImmunO anti-Actin clone C4 (MP Biomedicals, Eindhoven, The Netherlands). And rabbit anti-P2A peptide serum ABS31 (Merck-Millipore, Amsterdam, The Netherlands).

Flow cytometry analysis

Cells (911scFvHis, 911 or U118-MG) were infected with rS1His-2A-iLOV (MOI_{911}'s are indicated in the text). Cells were detached from the 24-well plates with trypsin and fixed in 4% paraformaldehyde at 72 hours post infection. After three washes in FACS buffer (PBS with 0.5% BSA and 2 mM EDTA) cells were assayed in FACS buffer for iLOV presence on a BD LSRII flow cytometer. ILOV signal was measured by selecting the blue laser (excitation at 488 nm) with the green fluorescence channel (FITC A in the plots) and the yellow fluorescence channel for PE (negative) signal. For the detection of iLOV positive cells in relation to sialic-acid binding or normal diploid cells 911, CHO, Lec2 or VH10 cells were mock infected, infected with rS1His-2A-iLOV or *jin-3* as an additional control. Both viruses were added to cells with the same MOI_{911} (see text) and 48 hours post infection prepared for FACS analysis as described above. In both experiments, gates for iLOV positive cells were set per cell line, depending on the population of the negative mock infected cells. Data were analysed with FACSDiva software (BD Bioscience, Breda, The Netherlands).

Detection of WGA-FITC binding

CHO and Lec2 cells were transferred by trypsin treatment to tubes. Trypsin was inhibited by adding the cell suspension to DMEM containing 2% FBS. After one centrifugation step for 3 min. at 350 x g, cells were resuspended in PBS (mock) or WGA-FITC (1 µg/ml in PBS) to a final concentration of ~$7.5*10^5$ cells per 500 µl. Cells were placed at room temperature for 15 min. to initiate binding of WGA-FITC, followed by three washes with FACS buffer (3 min. at 350 x g) to remove excess of unattached WGA-FITC. Detection of FITC-positive cells was done in 500 µl FACS buffer on a BD LSRII flow cytometer. Data were analysed with FACSDiva software (BD Bioscience, Breda, The Netherlands).

Reovirus RNA electrophoresis

For reovirus RNA analyses the method used is adapted from a rotavirus protocol described by Navarro et al.[45] Cells in 6-well plates were infected with *wt*-Reo or rS1His-2A-iLOV reoviruses. Upon the first appearance of CPE, the cells were harvested from the medium and subjected to three cycles of freeze-thawing in 100 µl PBS containing 2% FBS. For the dsRNA isolation, SDS was added to the samples to a final concentration of 1%, boiled for 3 minutes, and placed on ice for at least 1 minute before 1 µl RNAse free DNAse (Fermentas, FisherScientific, Landsmeer, The Netherlands) was added. The samples were then incubated for 30 minutes at 37 °C. Subsequently Proteinase K (Fermentas, FisherScientific, Landsmeer, The Netherlands) was added to a final concentration of 2 mg/ml and left at 37° C for 2 hours. RNA was extracted by 1 volume of Trizol reagent (Ambion, Life Technolgies, Bleiswijk, The Netherlands) followed by isopropanol precipitation. RNA was taken up in 25 µl RNase free water. To separate the RNA segments 10 µl was used and loaded on a 12% polyacrylamide-SDS gel after addition of 2x RNA loading dye (Fermentas, FisherScientific, Landsmeer, The Netherlands). Electrophoresis was carried out with 20 mA for ~12 hours, before staining with SYBR gold (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) and photographed on a BioRad Gel Doc™ XR+ System (BioRad, Veenendaal, The Netherlands).

Viability Assay

WST-1 reagent (Roche, Woerden, The Netherlands) was used to assay the viability of cells after reovirus infections. Cells were grown in 96-well plates and mock infected or infected with *wt*-Reo or early passage (P2) rS1His-2A-iLOV reovirus with an MOI_{911} of 1, in quadruple. Four days post infection the WST-1 reagent was added to the wells according to the instructions. The mock infected cells were set to 100% viability. The

statistical significance was determined with GraphPad Prism v6 using the 2-way ANOVA with Dunnett's multiple comparison test. The results are considered significant for $P<0.05$.
For the viability assay with the VH10 cells and 911 cells as a control, cells were infected with rS1His-iLOV, *jin-3* (MOI_{911}~1) or uninfected in quadruplicate and four days post infection the viability of the cultures was determined by WST-assay.

Caspase-3/7 activity assay

For measuring caspase activity, the 911 cell line was cultured in 96-well plates ($1.5*10^4$ cells per well in quadruplicate) and mock infected, infected with reovirus containing lysates of *wt*-T3D reovirus, rS1His-2A-iLOV, or *jin-3* mutant reovirus at an MOI_{911}~5. Twenty four hours and 48 hours post infection caspase-3/7 activity was measured by the Caspase-Glo 3/7 detection kit (Promega, Leiden, The Netherlands).

Determination of rS1His-2A-iLOV by $TCID_{50}$ assay

One day prior to the assay, 911 cells were plated in 96-well plates. The next day cells were infected with 10-fold serially dilutions of rS1His-2A-iLOV virus, starting with a 100-fold dilution up to 10^{-9} in DMEM with 2% FBS. Of each dilution 50 µl/well was added to 10 wells containing 911 cells in 50 µl DMEM plus 2% FBS per well. The last 2 wells of each row were used as uninfected controls. After ~7 days, CPE was scored under a light microscope and titer was determined using the Reed-Müench method[46] and expressed as Infectious Units (IU_{911})/ml.

Nucleotide sequences

The sequence of the S1His-2A-iLOV segment is deposited in Genbank under accession number KJ806995. The GenBank ID's of our *wt*T3D (R124) segments are: R124 L1 GU991659; R124 L2 GU991660; R124 L3 GU991661; R124 M1 GU991662; R124 M2 GU991663; R124 M3 GU991664; R124 S1 GU991665; R124 S2 GU991666; R124 S3 GU991667; R124 S4 GU991668.
Genbank ID of *jin*-3 S1segment is: KJ806994.

Acknowledgements

We thank dr. Danijela Koppers-Lalic and Leithe Budel for helpful discussions and suggestions. Aitor Navarro is gratefully acknowledged for providing us the protocol for RNA electrophoresis and helpful comments.

REFERENCES

1. Marcato P, Shmulevitz M, Pan D, Stoltz D, Lee PWK. Ras Transformation Mediates Reovirus Oncolysis by Enhancing Virus Uncoating, Particle Infectivity, and Apoptosis-dependent Release. *Mol. Ther.* 2007; **15**(8)**:** 1522-1530.
2. Smakman N, Van Den Wollenberg DJM, Borel Rinkes IHM, Hoeben RC, Kranenburg O. Sensitization to apoptosis underlies KrasD12-dependent oncolysis of murine C26 colorectal carcinoma cells by reovirus T3D. *J. Virol.* 2005; **79**(23)**:** 14981-14985.
3. Tyler KL, Squier MK, Rodgers SE, Schneider BE, Oberhaus SM, Grdina TA *et al.* Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein sigma 1. *J. Virol.* 1995; **69**(11)**:** 6972-6979.
4. Tyler KL, Squier MK, Brown AL, Pike B, Willis D, Oberhaus SM *et al.* Linkage between reovirus-induced apoptosis and inhibition of cellular DNA synthesis: role of the S1 and M2 genes. *J. Virol.* 1996; **70**(11)**:** 7984-91.
5. Kyula JN, Roulstone V, Karapanagiotou EM, Melcher AA, Harrington KJ. Oncolytic reovirus type 3 (Dearing) as a novel therapy in head and neck cancer. *Expert Opin. Biol. Ther.* 2012; **12**(12)**:** 1669-1678.
6. Harrington KJ, Vile RG, Melcher a, Chester J, Pandha HS. Clinical trials with oncolytic reovirus: Moving beyond phase I into combinations with standard therapeutics. *Cytokine Growth Factor Rev.* 2010**:** 1-8.
7. Kobayashi T, Antar AA, Boehme KW, Danthi P, Eby EA, Guglielmi KM *et al.* A plasmid-based reverse genetics system for animal double-stranded RNA viruses. *Cell Host Microbe* 2007; **1**(2)**:** 147-57.
8. Van Den Wollenberg DJM, Van Den Hengel SK, Dautzenberg IJC, Cramer SJ, Kranenburg O, Hoeben RC. A strategy for genetic modification of the spike-encoding segment of human reovirus T3D for reovirus targeting. *Gene Ther.* 2008; **15**(24)**:** 1567-15678.
9. Demidenko AA, Blattman JN, Blattman NN, Greenberg PD, Nibert ML. Engineering recombinant reoviruses with tandem repeats and a tetravirus 2A-like element for exogenous polypeptide expression. *Proc. Natl. Acad. Sci. USA* 2013; **110**(20)**:** 1867-1876.
10. Brochu-Lafontaine V, Lemay G. Addition of exogenous polypeptides on the mammalian reovirus outer capsid using reverse genetics. *J. Virol. Methods* 2012; **179**(2)**:** 342-350.
11. Roner MR, Joklik WK. Reovirus reverse genetics: Incorporation of the CAT gene into the reovirus genome. *Proc. Natl. Acad. Sci. USA* 2001; **98**(14)**:** 8036-8041.
12. Van Den Wollenberg DJ, Dautzenberg IJ, Van Den Hengel SK, Cramer SJ, De Groot RJ, Hoeben RC. Isolation of reovirus T3D mutants capable of infecting human tumour cells independent of junction adhesion molecule-A. *PLoS ONE* 2012; **7**(10)**:** e48064.
13. Dautzenberg IJ, Van Den Wollenberg DJ, Van Den Hengel SK, Limpens RW, Barcena M, Koster AJ *et al.* Mammalian orthoreovirus T3D infects U-118 MG cell spheroids independent of junction adhesion molecule-A. *Gene Ther.* 2014; **21:** 609-617.

14. Barton ES, Connolly JL, Forrest JC, Chappell JD, Dermody TS. Utilization of sialic acid as a coreceptor enhances reovirus attachment by multistep adhesion strengthening. *J. Biol. Chem* 2001; **276:** 2200-2211.
15. Reiter DM, Frierson JM, Halvorson EE, Kobayashi T, Dermody TS, Stehle T. Crystal Structure of Reovirus Attachment Protein -s1 in Complex with Sialylated Oligosaccharides. *PLoS Pathog.* 2011; **7**(8)**:** e1002166-e1002166.
16. Dermody TS, Nibert ML, Bassel-Duby R, Fields BN. Sequence diversity in S1 genes and S1 translation products of 11 serotype 3 reovirus strains. *J. Virol.* 1990; **64**(10)**:** 4842-4850.
17. Hoyt CC, Richardson-Burns SM, Goody RJ, Robinson BA, DeBiasi RL, Tyler KL. Nonstructural Protein σ1s Is a Determinant of Reovirus Virulence and Influences the Kinetics and Severity of Apoptosis Induction in the Heart and Central Nervous System. *J. Virol.* 2005; **79**(5)**:** 2743-2753.
18. Poggioli GJ, Keefer C, Connolly JL, Dermody TS, Tyler KL. Reovirus-Induced G2/M Cell Cycle Arrest Requires ς1s and Occurs in the Absence of Apoptosis. *J. Virol.* 2000; **74**(20)**:** 9562-9570.
19. Boehme KW, Hammer K, Tollefson WC, Konopka-Anstadt JL, Kobayashi T, Dermody TS. Nonstructural Protein sigma1s Mediates Reovirus-Induced Cell Cycle Arrest and Apoptosis. *J. Virol.* 2013.
20. Chapman S, Faulkner C, Kaiserli E, Garcia-Mata C, Savenkov EI, Roberts AG *et al.* The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. *Proc. Natl. Acad. Sci. USA* 2008; **105**(50)**:** 20038-20043.
21. Leone G, Maybaum L, Lee PW. The reovirus cell attachment protein possesses two independently active trimerization domains: basis of dominant negative effects. *Cell* 1992; **71:** 479-488.
22. Chappell JD, Barton ES, Smith TH, Baer GS, Duong DT, Nibert ML *et al.* Cleavage susceptibility of reovirus attachment protein sigma1 during proteolytic disassembly of virions is determined by a sequence polymorphism in the sigma1 neck. *J. Virol.* 1998; **72**(10)**:** 8205-8213.
23. Alain T, Kim TS, Lun X, Liacini A, Schiff LA, Senger DL *et al.* Proteolytic disassembly is a critical determinant for reovirus oncolysis. *Mol. Ther.* 2007; **15:** 1512-1521.
24. Roner MR, Roehr J. The 3' sequences required for incorporation of an engineered ssRNA into the Reovirus genome. *Virol. J.* 2006; **3:** 1-11.
25. Kobayashi T, Ooms LS, Ikizler M, Chappell JD, Dermody TS. An improved reverse genetics system for mammalian orthoreoviruses. *Virology* 2010; **398**(2)**:** 194-200.
26. Connolly JL, Barton ES, Dermody TS. Reovirus binding to cell surface sialic acid potentiates virus-induced apoptosis. *J. Virol.* 2001; **75**(9)**:** 4029-4039.
27. Deutscher SL, Nuwayhid N, Stanley P, Briles EIB, Hirschberg CB. Translocation across golgi vesicle membranes: A CHO glycosylation mutant deficient in CMP-sialic acid transport. *Cell* 1984; **39**(2, Part 1)**:** 295-299.
28. Kim JH, Lee S-R, Li L-H, Park H-J, Park J-H, Lee KY *et al.* High Cleavage Efficiency of a 2A Peptide Derived from Porcine Teschovirus-1 in Human Cell Lines, Zebrafish and Mice. *PLoS One* 2011; **6**(4)**:** e18556.

29. Szymczak-Workman AL, Vignali KM, Vignali DA. Design and construction of 2A peptide-linked multicistronic vectors. *Cold Spring Harb. Protoc.* 2012; **2012**(2)**:** 199-204.
30. Kim M, Garant KA, zur Nieden NI, Alain T, Loken SD, Urbanski SJ *et al.* Attenuated reovirus displays oncolysis with reduced host toxicity. *Br. J. Cancer* 2011; **104**(2)**:** 290-9.
31. Chappell JD, Gunn VL, Wetzel JD, Baer GS, Dermody TS. Mutations in type 3 reovirus that determine binding to sialic acid are contained in the fibrous tail domain of viral attachment protein sigma1. *J. Virol.* 1997; **71**(3)**:** 1834-1841.
32. Chappell JD, Duong JL, Wright BW, Dermody TS. Identification of Carbohydrate-Binding Domains in the Attachment Proteins of Type 1 and Type 3 Reoviruses. *J. Virol.* 2000; **74**(18)**:** 8472-8479.
33. Seago J, Juleff N, Moffat K, Berryman S, Christie JM, Charleston B *et al.* An infectious recombinant foot-and-mouth disease virus expressing a fluorescent marker protein. *J. Gen. Virol.* 2013; **94**(7)**:** 1517-27.
34. Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, Pratt AJ *et al.* Structural tuning of the fluorescent protein iLOV for improved photostability. *J. Biol. Chem* 2012; **287**(26)**:** 22295-304.
35. Zou S, Brown EG. Identification of sequence elements containing signals for replication and encapsidation of the reovirus M1 genome segment. *Virology* 1992; **186**(2)**:** 377-88.
36. Bodelon G, Labrada L, Martinez-Costas J, Benavente J. The avian reovirus genome segment S1 is a functionally tricistronic gene that expresses one structural and two nonstructural proteins in infected cells. *Virology* 2001; **290**(2)**:** 181-91.
37. Ni Y, Kemp MC. Subgenomic S1 segments are packaged by avian reovirus defective interfering particles having an S1 segment deletion. *Virus Res.* 1994; **32**(3)**:** 329-42.
38. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, van Ormondt H, Hoeben RC *et al.* Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* 1996; **7:** 215-222.
39. Buchholz UJ, Finke S, Conzelmann KK. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J. Virol.* 1999; **73**(1)**:** 251-259.
40. Klein B, Pastink A, Odijk H, Westerveld A, van der Eb AJ. Transformation and immortalization of diploid xeroderma pigmentosum fibroblasts. *Exp. Cell Res.* 1990; **191**(2)**:** 256-62.
41. Roner MR, Steele BG. Features of the mammalian orthoreovirus 3 Dearing l1 single-stranded RNA that direct packaging and serotype restriction. *J. Gen. Virol.* 2007; **88**(12)**:** 3401-3412.
42. Boehme KW, Ikizler M, Kobayashi T, Dermody TS. Reverse genetics for mammalian reovirus. *Methods* 2011; **55**(2)**:** 109-13.
43. Transfiguracion J, Bernier A, Voyer R, Coelho H, Coffey M, Kamen A. Rapid and reliable quantification of reovirus type 3 by high performance liquid chromatography during manufacturing of Reolysin. *J. Pharm. Biomed. Anal.* 2008; **48**(3)**:** 598-605.
44. Virgin HW, Mann MA, Fields BN, Tyler KL. Monoclonal antibodies to reovirus reveal structure/function relationships between capsid proteins and genetics of susceptibility to antibody action. *J. Virol.* 1991; **65**(12)**:** 6772-6781.

45. Navarro A, Trask SD, Patton JT. Generation of genetically stable recombinant rotaviruses containing novel genome rearrangements and heterologous sequences by reverse genetics. *J. Virol.* 2013; **87**(11)**:** 6211-20.
46. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* 1938; **27**(3)**:** 493-497.