The LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus

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Vesicular stomatitis virus (VSV) exhibits a remarkably robust and pantropic infectivity, mediated by its coat protein, VSV-G. Using this property, recombinant forms of VSV and VSV-G-pseudotyped viral vectors are being developed for gene therapy, vaccination, and viral oncolysis and are extensively used for gene transduction in vivo and in vitro. The broad tropism of VSV suggests that it enters cells through a highly ubiquitous receptor, whose identity has so far remained elusive. Here we show that the LDL receptor (LDLR) serves as the major entry port of VSV and of VSV-G-pseudotyped lentiviral vectors in human and mouse cells, whereas other LDLR family members serve as alternative receptors. The widespread expression of LDLR family members accounts for the pantropism of VSV and for the broad applicability of VSV-G-pseudotyped viral vectors for gene transduction.

receptor-associated protein | virus entry | sLDLR

Soluble LDLR Inhibits VSV Infectivity by Binding to VSV. Initially we confirmed our previously reported observation that sLDLR inhibits VSV infectivity (28); to this end, we used highly purified (Fig. 1A, Inset) recombinant human sLDLR, consisting of seven cysteine-rich repeats, which correspond to the ligand-binding domain of LDLR (30). Recombinant sLDLR inhibited the VSV-triggered cytopathic effect in human epithelial WISH cells in a dose-dependent manner, with an IC50 of 55 ng/ml (~0.4 nM; Fig. 1A). Similar results were obtained with mandin darby bovine kidney (MDBK) cells, and mouse L cells (Fig. 1B). Exposure of cells to as little as 0.1 multiplicity of infection (MOI) of VSV for only 5 min was sufficient to trigger a complete cytopathic effect at 17 h after infection (Fig. 1C, well “V”), indicating that the majority of the cell lysis was due to secondary infection by the VSV progeny. Addition of sLDLR before or concomitantly with VSV completely blocked the VSV-triggered cytopathic effect, whereas its addition 5–10 min after VSV challenge partly inhibited only the secondary infection, resulting in a plaque-like appearance (Fig. 1C). In contrast, removal of sLDLR before virus challenge resulted in a near complete cytopathic effect (Fig. 1C, well “R”). These results indicated that to exert its antiviral effects, sLDLR must be present both at the early stages of the viral infection and at later stages, to also inhibit secondary infection by viral progeny. To test whether sLDLR inhibits the initial binding of VSV to cells, we exposed WISH cells to VSV for 15 min in the absence or presence of sLDLR, then washed the cells and measured cell--associated VSV by quantitative and by semiquantitative RT-PCR of VSV RNA. We found that sLDLR inhibited VSV binding to cells in a dose-dependent manner, at both 4 °C and 37 °C (Fig. 1D, Inset).

The inhibition of virus–cell binding mediated by sLDLR suggested that sLDLR inhibits VSV infectivity by binding to either the virus or to a putative cellular VSV receptor. To test the

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immobilized sLDLR in PBS with or without CaCl2 (1 mM). The products of VSV RNA, isolated after similar experiments, performed at 4 °C spikes, quantitative analysis of its binding to immobilized sLDLR indicated the VSV entry port. On the basis of increased binding of LDL to LDLR is Ca2+ ions. (Fig. 1A) Survival ± SD of WISH cells as determined by Neutral red staining after treatment with sLDLR and challenge by VSV at the indicated MOI, n = 3. (Inset) SDS/PAGE of sLDLR (10 μg). Molecular mass markers (kDa) are shown on the right lane. (B) Surviving WISH cells, bovine MDBK cells, and murine L cells after treatment with sLDLR were then washed and brought to 37 °C for 1 h to allow internalization of the bound Dil-LDL. VSV inhibited binding of Dil-LDL to the FS-11 fibroblasts, as determined by Neutral red staining after treatment with sLDLR and challenge by VSV at the indicated MOI. (Upper) Immunoblotting of VSV-G after coimmunoprecipitation of a solubilized VSV-sLDLR complex with the following antibodies (lanes): 1, mAb 28.28 anti-LDLR; 2, mAb C7 anti-LDLR; 3, isotype control mAb; 4, no antibody. (Lower) Immunoblotting of VSVG-G-LV in the presence or absence of sLDLR (5 μg/mL). Nuclei were counterstained with Hoechst 33258 (blue). (Insets) Enlarged magnifications. (I) Average ± SD EGFP expression in cultures transfected as shown in H. ***p < 0.003, n = 4. N.S., not significant (P = 0.5252), n = 4.

possible binding of sLDLR to VSV, we used surface plasmon resonance. Binding of LDL to LDLR is Ca2+ ions. (31) Similarly, we found that VSV effectively bound to immobilized sLDLR in PBS, but only in the presence of Ca2+ (Fig. 1E). Because the VSV envelope contains 400–500 trimeric VSV-G spikes, quantitative analysis of its binding to immobilized sLDLR reflects avidity rather than affinity. Dose–response binding of VSV to immobilized sLDLR gave a dissociation constant (Kd) of 10–11 M, indicating a very high avidity (Fig. S1). VSV-G-pseudotyped lentiviral vectors (VSV-G-LV) share with VSV only their receptor-interacting component, VSV-G, and hence can be used for measuring the avidity of VSV-G to sLDLR. To this end we immobilized VSV-G-LV to the sensor chip and analyzed binding of increasing sLDLR concentrations in the presence of Ca2+. As expected, the affinity of a single sLDLR molecule interacting with VSV-G (Kd = 10–8 M; Fig. 1F) was lower than the avidity measured by VSV binding to immobilized sLDLR. In a control experiment we tested binding of sLDLR to immobilized lymphocytic choriomeningitis virus-pseudotyped lentivector (LCMV-LV), which differs from VSV-G-LV only in its coat protein. sLDLR did not bind to the immobilized LCMV-LV. The high avidity of the VSV binding to sLDLR and the dependence of the binding on Ca2+ strongly supported the specificity and physiological relevance of this in vitro interaction. Further evidence for the interaction between the ligand-binding domain of LDLR and VSV-G was obtained by communoprecipitation. sLDLR was added to a suspension of VSV and then immunoprecipitated with protein-G-bound anti-LDLR mAb 28.28 (32), anti-LDLR mAb C7, an isotype-matched control mAb, or no antibody. SDS/PAGE and immunoblotting with anti-VSV-G and anti-LDLR antibodies revealed that sLDLR was specifically bound to VSV-G (Fig. 1G).

We also evaluated the impact of sLDLR on EGFP expression after transduction of cells with an EGFP-encoding VSV-G-LV. Figs. 1 H and I show that sLDLR completely blocked transduction of newborn human FS-11 foreskin fibroblasts by EGF-encoding VSV-G-LV. In contrast, sLDLR did not inhibit transduction of the cells with an EGF-encoding LCMV-LV, which differs from VSV-G-LV only by its coat protein. Taken together, these results indicate that sLDLR inhibits VSV infectivity by binding to VSV-G.

**LDLR Is the Major VSV Receptor in Human Cells.** The fact that sLDLR bound VSV at high avidity and inhibited its infectivity indicated that sLDLR masked VSV constituents essential for its interaction with a cellular receptor, prompting us to examine whether LDLR serves as the VSV entry port. On the basis of increased binding of radiolabeled VSV to trypsin-treated cells, earlier studies concluded that the VSV receptor was unlikely to be a protein (22, 33). To examine this conclusion more rigorously, we tested trypsin-treated cells for their resistance to VSV infection. We exposed these cells in suspension to trypsin/EDTA or to EDTA alone for 30 min, then washed the cells three times with medium containing 10% (vol/vol) FBS to block residual trypsin activity, as described previously (22). We then challenged the cell suspensions with VSV, washed the cells, plated them, and incubated them for 17 h. The EDTA-treated cells were completely lysed by VSV, whereas the trypsin-treated cells were fully resistant to VSV infection (Fig. 24, Upper). Plaque assays of the culture supernatants revealed ~500-fold lower VSV yields in the trypsin-treated cultures (Fig. 24, Lower). These results indicate that a cell surface protein is essential for VSV infectivity, probably serving as a VSV receptor.

We then examined whether VSV and LDL, the physiological LDLR ligand, compete for binding to LDLR. FS-11 fibroblasts were incubated with increasing concentrations of VSV, followed by fluorescently labeled LDL (Dil-LDL) (4 h, 4 °C). The cultures were then washed and brought to 37 °C for 1 h to allow internalization of the bound Dil-LDL. VSV inhibited binding of Dil-LDL to the FS-11 fibroblasts in a dose-dependent manner (Fig. 28). No uptake was seen when Dil-LDL alone was similarly incubated with the LDLR-deficient (34) GM701 fibroblasts (Fig. 2C). Similarly, VSV inhibited Dil-LDL binding to FS-11 fibroblasts, as determined by flow cytometry (Fig. 2F). These results indicate that VSV and LDL share LDLR as their common receptor. However, as we reported previously (28), LDLR-deficient
Those of the LDLR (35). Because sLDLR completely blocked the ligand-binding domain of all LDLR family members contains other LDLR family members serve as alternative VSV entry ports.

Fig. 2. VSV and LDL share a common cell surface receptor. (A) Surviving WISH epithelial cells, pretreated with trypsin-EDTA or EDTA, washed and challenged with VSV (0.015 MOI, 15 min). Figure is representative of six replicates. VSV yield (Lower) was determined by a plaque assay of the culture supernatants. \( P < 0.03, n = 3. \) (B) Internalized Dil-LDL (red) in FS-11 fibroblasts after binding (1.67 μg/mL, 4 h, 4 °C) in the presence of the indicated VSV MOI. The cultures were then washed, and bound Dil-LDL was allowed to internalize (1 h, 37 °C). (Insets) Higher magnifications. (C) Upper) Immunoblot of LDLR in WT FS-11 fibroblasts and LDLR-deficient GM701 fibroblasts. (Lower) Lack of Dil-LDL uptake by LDLR-deficient GM701 fibroblasts. (D) Flow cytometry of FS-11 fibroblasts treated with Dil-LDL as in A in the absence or presence of VSV (MOI = 2000). \( n = 3. \) (E) LDLR-deficient GM701 fibroblasts untreated or treated with sLDLR (1 μg/mL) and challenged with VSV (MOI = 1).

To obtain further evidence that LDLR is a VSV receptor, we used mAbs raised against epitopes within the ligand-binding domain of human LDLR (32). Because LDLR-deficient cells were still susceptible to VSV infection (Fig. 2E), we resorted to limited infection, thereby rendering the cell surface receptor the rate-limiting component. We incubated WISH cells with anti-LDLR mAbs for 30 min at 4 °C, followed by VSV challenge (MOI = 0.05, 4 °C, 1 h). The cultures were washed and then incubated for 17 h at 37 °C in the presence of the same antibodies. mAb 29.8, directed against class A cysteine-rich repeat 3 of the LDLR ligand-binding domain, almost completely inhibited the VSV-triggered cytopathic effect in WISH cells, whereas mAb 28.28, directed against repeat 6, did not inhibit VSV infectivity (Fig. 3A). Using the same infection protocol revealed that mAb 29.8 almost completely inhibited the VSV-triggered cytopathic effect in WT FS-11 fibroblasts but not in the LDLR-deficient GM701 fibroblasts (Fig. 3B). These experiments indicate that LDLR is the major VSV receptor in human cells, and VSV requires cysteine-rich repeat 3 of the LDLR ligand-binding domain to infect human cells; furthermore, it is likely that VSV uses alternative entry port(s) in the LDLR-deficient cells.

Other LDLR Family Members Serve as Alternative VSV Entry Ports.

The ligand-binding domain of all LDLR family members contains multiple, class A cysteine-rich repeats, structurally homologous to those of the LDLR (35). Because sLDLR completely blocked VSV infectivity even in LDLR-deficient cells (Fig. 2E), we hypothesized that such additional family members could serve as the alternative VSV entry routes. Receptor-associated protein (RAP) is a common chaperone of all LDLR family members (35). When added exogenously, RAP completely blocks ligand binding to all LDLR family members with the exception of LDLR itself (36). Indeed, preincubation of cells with RAP inhibited the VSV-triggered cytopathic effect in LDLR-deficient GM701 fibroblasts but not in LDLR-expressing WT FS-11 fibroblasts (Fig. 3C). Similarly, measuring virus yields 7 h after infection revealed that LDLR-deficient GM701 fibroblasts were significantly less susceptible to VSV infection compared with WT fibroblasts (Fig. 3D). Importantly, RAP further attenuated VSV expression in the LDLR-deficient fibroblasts but not in the WT cells (Fig. 3D).

We then studied the impact of blocking all LDLR family members on VSV infectivity by combining RAP and anti-LDLR antibodies. We preincubated WISH cells either with the neutralizing or the nonneutralizing anti-LDLR mAbs, 29.8 and 28.28, in the absence or presence of RAP at 37 °C and then challenged the cells with VSV. RAP alone provided little protection from VSV infection, and nonneutralizing mAb 28.28 provided no protection, whereas anti-LDLR mAb 29.8 provided limited but significant protection. However, the combination of RAP and mAb 29.8, which blocks all LDLR family members, completely inhibited VSV infection (Fig. 3E).

We then studied the role of the LDLR family members in VSV uptake. WT and LDLR-deficient fibroblasts were incubated with VSV at conditions leading to internalization of at least two-thirds of cultures treated with VSV at MOI = 1 (Fig. 3F).

Fig. 3. LDLR and its family members are the major and the alternative VSV receptors, respectively. (A) Crystal violet-stained WISH cells, untreated (Ctrl.) or treated with anti-LDLR mAbs (30 min, 4 °C) and then subjected to limited infection by VSV (MOI = 0.05, 4 °C, 1 h). (B) Crystal violet-stained cultures of WT (FS-11) and LDLR-deficient (GM701) fibroblasts, either untreated (Control) or treated with isotype control mAb or anti-LDLR mAb 29.8 (12.5 μg/mL each), followed by VSV as in A. (C) Crystal violet-stained cultures of WT FS-11 fibroblasts and LDLR-deficient GM701 fibroblasts, treated with RAP (100 nM, 30 min, 37 °C) alone, VSV (MOI = 1) alone, or RAP followed by VSV. (D) Plaque assay of culture supernatants from WT FS-11 fibroblasts and LDLR-deficient GM701 fibroblasts (50,000 cells per well) preincubated (30 min, 37 °C) in DMEM-10 or in DMEM-10 + RAP (100 nM), then challenged with VSV (0.5 MOI, 30 min, 37 °C), washed three times, and incubated in DMEM-10 (0.1 mL, 37 °C, 7 h). ***P < 0.001, n = 4. (E) Crystal violet-stained WISH cells grown to confluence in 96-well plates, incubated (30 min, 37 °C) with the indicated combinations of RAP (200 nM), neutralizing anti-LDLR mAb 29.8, and nonneutralizing anti-LDLR mAb 28.2 (50 μg/mL each); cells were then challenged with VSV at the indicated MOI. Cell viability (bar plot) was determined by reading the OD540 of cultures treated with VSV at MOI = 0.06. ***P < 0.002, n = 4.
of the bound VSV (37). The cultures were then washed, immuno-
nostained with anti-VSV-G, and VSV foci were counted. Com-
pared with the WT FS-11 fibroblasts, the LDLR-deficient GM701
fibroblasts internalized significantly less VSV (Figs. 4 A and C).
This result confirmed that LDLR has a major role in VSV in-
ternalization. Furthermore, neutralizing mAb 29.8 but not the non-
neutralizing mAb 28.28 significantly inhibited VSV binding
and subsequent internalization into the WT fibroblasts (P < 0.05),
whereas the combination of mAb 29.8 and RAP, which blocks all
LDLR family members, completely abolished VSV binding and
subsequent internalization to these cells (Figs. 4 B and C). Hence,
we concluded that LDLR and its other family members mediate
VSV entry into human cells.

LDLR and Its Family Members Mediate Transduction by VSV-G-
Pseudotyped Lentiviral Vectors. VSV and the frequently used
VSV-G-LVs share VSV-G as their common coat protein, prompting us
to study the role of LDLR and its family members in cell transduction by an EGFP-encoding VSV-G-LV. After trans-
duction, WT FS-11 fibroblasts expressed significantly higher levels
of EGFP compared with LDLR-deficient fibroblasts (Fig. 5 A and B).
To demonstrate that the reduced EGFP expression in the
LDLR-deficient fibroblasts was due to lack of LDLR and not due to
other inherent difference between these two cell types, we per-
tformed two control experiments. First we transduced both the WT
and the LDLR-deficient fibroblasts with EGFP-encoding VSV-G-
LV in the presence of polybrene, an agent rendering virus entry
receptor-independent (38). Under these conditions, the level of
EGFP expression in the WT and the LDLR-deficient GM701
fibroblasts was comparable (Fig. 5 A and B). Furthermore, trans-
duction with another lentiviral vector, EGFP-encoding LCMV-LV,
which differs from VSV-G-LV only in its coat protein, gave very
similar levels of EGFP expression in the WT and LDLR-deficient
fibroblasts (Fig. 5 A and C). These two control experiments con-
firmed that the reduced level of EGFP expression observed in the
GM701 fibroblasts after transduction with VSV-G-LV was due to
their lack of LDLR expression.

Fig. 4. LDLR and its family members mediate VSV internalization by human
fibroblasts. (A) Internalized VSV in WT FS-11 fibroblasts and LDLR-deficient
GM701 fibroblasts after incubation with VSV (MOI = 500, 4 min, 37 °C) and
washing three times with PBS. The cultures were then fixed and stained with
anti-VSV-G (red). (B) Internalized VSV in WT FS-11 fibroblasts preincubated
with the indicated combinations of RAP and anti-LDLR mAbs (30 min, 37 °C),
followed by VSV as in A. (C) VSV foci in A and B were counted in fields
containing at least 30 cells. **P < 0.01; *P < 0.05 (compared with FS-11
challenged with VSV only, leftmost bar); n = 3.

To further confirm the role of LDLR in VSV-G-LV entry to cells, we rescued LDLR expression in the LDLR-deficient GM701
fibroblasts by polybrene-assisted transduction with an LDLR-
encoding VSV-G-LV. After rescue, the GM701 cells expressed
LDLR, as determined by immunoblotting (Fig. 5 D), and became
significantly more responsive to transduction with the EGFP-
encoding VSV-G-LV in the absence of polybrene (Fig. 5 E and F).
In a reciprocal experiment, knockdown of LDLR by specific
siRNA and not by scrambled, nontargeting control siRNA signifi-
cantly attenuated the transduction of FS-11 fibroblasts by VSV-G-
LV, whereas it had no significant effect on transduction of the cells
by LCMV-LV (Fig. S2). This study further confirmed that the
reduced transduction by VSV-G-LV observed in the LDLR-
deficient cells was due to lack of LDLR and not due to other
inherent differences between the WT FS-11 fibroblasts and the
LDLR-deficient GM701 cells.

We then studied whether other LDLR family members enable
transduction of cells by VSV-G-LV. As was the case with VSV
infection (Fig. 3 C–E), RAP further attenuated the transduction of
the LDLR-deficient GM701 fibroblasts by VSV-G-LV, indicating
that in addition to LDLR, other LDLR family members enabled the
residual transduction observed in the LDLR-deficient fibro-
basts (Fig. 6 A and B). In parallel, we found that similarly to hu-
man cells, LDLR-deficient murine embryonic fibroblasts (MEFs)
were significantly less susceptible to transduction by VSV-G-LV
compared with their WT counterparts, and RAP further attenu-
ated the VSV-G-LV-mediated transduction of the LDLR-
deficient MEFs. Unlike human fibroblasts, RAP significantly re-
duced VSV infectivity of WT MEFs (Fig. 6 C and D), suggesting
a more substantial role of the other LDLR family members in
VSV infection of mouse cells.

Taken together, our results demonstrate that LDLR is the
major entry port of both VSV and VSV-G-LVs in human and
mouse cells, whereas other LDLR family members serve as
alternative receptors. The complete protection from VSV infection obtained by blocking all LDLR family members identifies these receptors as the only possible VSV entry ports into human cells.

Discussion

In this study we provide several lines of evidence establishing LDLR as the major entry port of VSV and VSV-G-LV, including the high affinity and calcium ion dependence of VSV binding to soluble LDLR, the competition between VSV and LDLR for receptor binding, the inhibition of VSV internalization and infectivity by mAbs to the ligand-binding domain of LDLR, and the crucial role of LDLR in cell transduction by a VSV-G-LV. On the basis of binding of radiolabeled VSV to protease-treated cells, earlier studies proposed that the VSV receptor is not a protein (22, 24, 33). In contrast, our finding that such trypsin-treated cells resist VSV infection indicates that the VSV receptor is a protein. Two earlier studies indirectly support the role of LDLR as the major VSV receptor. Binding of VSV to MDCK epithelial cells is 100 times more prevalent at the basolateral membrane compared with their apical surface (39). Independently, it was shown that LDLR is expressed 100 times more efficiently on the basolateral surface of these MDCK cells (40).

The fact that LDLR-deficient fibroblasts were susceptible to VSV infection suggested the possible existence of alternative, albeit less-efficient virus entry routes. All LDLR family members contain conserved class A repeats in their ligand-binding domains, which is the same structural motif that we have identified as the VSV-binding epitope in LDLR. The ability of RAP, which blocks all LDLR family members except LDLR, to attenuate VSV infection of the LDLR-deficient fibroblasts indicates that the alternative VSV receptor is another member of the LDLR family. One possible candidate is LRP1, which is overexpressed in GM701 cells (41), possibly explaining why these fibroblasts were highly susceptible to limited VSV infection, whereas WT fibroblasts in which LDLR was blocked by a specific monoclonal antibody were fully protected under the same VSV challenge (Fig. 3B, Lower). Our observations that a combination of monoclonal anti-LDLR antibody and RAP abolished VSV binding and internalization and completely protected human cells from VSV infection (Figs. 3E and 4) indicate that VSV enters and infects human and mouse cells only through members of the LDLR family. LDLR family members are ubiquitously expressed in all cell types and across the animal kingdom (42), thereby providing the basis for the remarkable pantropism of VSV. Interestingly, however, we found that sLDLR did not inhibit infection of insect SF6 cells. Although the insect lipophorin receptor and mammalian LDLR are structurally highly similar, their mode of action is quite different. Whereas LDLR releases its cargo in the endosome, lipophorin remains associated with its receptor and is eventually resorbed (43). Hence VSV probably infects insect cells by other means.

LDLR family proteins are endocytosed and recycle back to the membrane every 10 min, irrespective of ligand binding (44), and hence are ideal virus entry ports. It is therefore not surprising that in addition to VSV, several other unrelated viruses have been suggested to use these receptors as their ports of cellular entry (45–47). Of particular interest are the minor group common cold virus (46) and hepatitis C virus (48), which much like VSV use LDLR as well as other LDLR family members for cell entry. Similar to any other ligand, once internalized, LDLR must dissociate from its receptor. The endosomal lumen is characterized by low pH and low concentration of calcium ions; both these features are required for β-VLDL release from LDLR (49). Our finding that Ca\(^{2+}\) is essential for binding of VSV to immobilized sLDLR in vitro suggests that calcium ion depletion might also facilitate VSV release from its receptor after internalization.

In recent years high-throughput genome-wide screens became the method of choice for deciphering gene function. However, such screens may fail in cases of genetic redundancy, and the VSV receptor is a good case in point. A recent study using genome-wide RNAi screen identified 173 host genes essential for completion of the VSV replication cycle, but it did not detect the VSV receptor despite its obviously essential role (50). Recently it was demonstrated that the endoplasmic reticulum chaperone gp96 (endoplasm or GRP94) is essential for VSV binding to cells and for their subsequent infection (27). This chaperone is a constituent of a multiprotein complex, required for protein folding in the endoplasmic reticulum (51). Grp78, another component of this multiprotein complex, was reported to interact with LDLR (52). In preliminary studies we found that knockdown of gp96 disrupted the glycosylation of LDLR, manifested by reduced apparent molecular mass in SDS/PAGE. It is therefore likely that processing of other LDLR family members, which serve as VSV receptors, also requires gp96, thereby explaining its critical role in VSV infectivity.

The identification of the VSV receptor is of significant clinical importance because recombinant VSV and VSV-G-pseudotyped viral vectors are being developed for viral oncolysis, for vaccination, and for gene therapy. Up-regulation of LDLR in vivo [e.g., by pretreatment with statins (53)] might increase the efficacy of such vectors. Furthermore, liver cells and certain tumor cells, which express high levels of LDLR (54), might be the preferred targets of VSV-G-based gene therapy as well as VSV-G-based viral oncolysis.

Materials and Methods

LDLR-deficient human GM701 fibroblasts were from the Coriell Institute. Human FS-11 foreskin fibroblasts were kindly provided by M. Revel. VSV (Indiana Strain) and all other cell types were from ATCC. Cells were grown in media containing 10% (vol/vol) FBS (MEM-10 or DMEM-10). VSV was propagated in WISH cells, purified by gradient centrifugation, and plaque-assayed. sLDLR\(_{25-213}\) was produced in CHO cells and purified to homogeneity. VSV cytopathic effects were evaluated 17 h after VSV challenge. Plaque assays, flow cytometry, preparation of lentiviral vectors, transduction of cells, RT-PCR, quantitative PCR, surface plasmon resonance, knockdown of LDLR mRNA, immunoblotting, and all other methods were performed according to published procedures or as recommended by the various manufacturers. Trypsin digestion was performed using cell culture grade trypsin/EDTA on cells in suspension. Residual trypsin activity was blocked by 3× washing of the cells in DMEM-10 before VSV challenge. Image analysis and counting of nuclei, plaques, and VSV foci was performed using the Imagej program (National Institutes of Health). Fluorescence intensities and internalized VSV foci were normalized to the number of nuclei/fields containing at least 30 nuclei. Statistical analysis was performed using the unpaired Student t test of the KaleidaGraph program on at least three independent replicates. Details can be found in SI Materials and Methods.
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Supporting Information

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SI Materials and Methods

Cells and Reagents. Human amniotic WISH cells (CCL 25), mandarin darby bovine kidney cells (CCL22), L-929 (CCL-1), HEK 293T cells (CRL-11268), and vesicular stomatitis virus (VSV) (Indiana Strain) were obtained from ATCC. Human FS-11 foreskin fibroblasts (1) were kindly provided by M. Revel (Weizmann Institute, Rehovot, Israel). Human LDL receptor (LDLR)-deficient GM701 fibroblasts isolated from a homozygous familial hypercholesterolemia patient were obtained from the Coriell Institute (Camden, NJ). WISH and MDBK cells were grown in MEM supplemented with 10% (vol/vol) FBS, 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (MEM-10) at 37 °C in 5% (vol/vol) CO₂ in humid air. HEK 293T, L cells, FS-11, and GM701 cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (DMEM-10) at 37 °C in 8% (vol/vol) CO₂ in humid air. Human sLDLR (soluble form of LDLR), corresponding to codons 25–313, encompassing the seven class A cysteine-rich repeats, was produced in CHO cells and purified to homogeneity. It was kindly provided by Inter-Lab, Yavne, Israel. Monoclonal anti-LDLR antibody C7 (catalog #MABS26) was from Millipore. Monoclonal anti LDLR antibodies 29.8 and 28.28 were kindly provided by Inter-Lab. Monoclonal anti-VSV-G (VSV surface glycoprotein) antibody clone P5D4 was from Chemicon (Millipore, catalog #AP124B). Cy3-labeled streptavidin (catalog #016-160-084) was from Jackson ImmunoResearch. HRP-rabbit polyclonal anti-mouse antibody conjugate (catalog #ab6728) was from Abcam. Biotinylated anti-human LDLR antibody (catalog #BAF2148) and streptavidin HRP (catalog #89088) were from R&D Systems.

Assay of sLDLR Antiviral Activity. Cells (WISH and MDBK, 30,000 cells per 0.1 mL per well; L cells, 20,000 cells per 0.1 mL per well) in 96-well plates were incubated for 15 min with serially twofold-diluted sLDLR, starting at 8 μg/mL (214 nM). VSV [1 multiplicity of infection (MOI)] was then added, and after 17–24 h the cultures were stained with crystal violet [5% (wt/vol) in 66% (vol/vol) aqueous methanol] and photographed. For viability assays, WISH cells were similarly incubated with serially twofold-diluted sLDLR starting at 1 μg/mL and challenged with VSV. Cells remaining after VSV challenge were incubated with Neutral red (70 μg/mL in 0.1 mL DMEM-10 per well at 37 °C, 30 min), washed three times with PBS, extracted with a lysis buffer [28 mM trisodium citrate, 0.06% 10 N HCl, 48.9% (vol/vol) methanol, 0.1 mL], and OD₅₅₀ was determined.

Quantitative PCR of Cell-Associated VSV. WISH cells were plated in six-well plates (7.5 × 10⁵ cells per well, in 2 mL of MEM-10, 17 h). The plate was then cooled to 4 °C for 1 h, and sLDLR was then added at the indicated concentrations. After 15 min VSV (10 MOI) was added, and the cultures were kept at 4 °C for 4 h. The cells were then washed three times with cold PBS, and total RNA was prepared using the PureLink RNA Cultured Cell Kit from 5 Prime. cDNA was prepared from total RNA (1 μg) using High Capacity cDNA Reverse Transcriptase Kit (catalog #4368814, Applied Biosystems). Unique probes and gene-specific primer pair combinations for TATA box binding protein (TBP) mRNA, which served as a reference transcript, and target gene probes were designed (nucleotides 1146–1207 of VSV-G) using Roche ProbeFinder software version 2.32 (Roche Diagnostics). TBP cDNA samples were diluted 20-fold for use as working templates in quantitative RT-PCR (qRT-PCR). qRT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). qRT-PCR was carried out using a Roche LightCycler 480 Real-Time PCR System, and the reactions (10 μL final volume) contained 0.25 μM primers (Sigma) and 0.1 μM probe (Roche Diagnostics). The amplification program was as follows: an initial denaturation step at 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 1 min, and 40 °C for 30 s. The gene expression level was normalized to the TBP reference mRNA. The fold change in gene expression compared with mRNA from control cells was calculated using LightCycler software version 4.05. The results are represented as the mean ± SD of triplicate and displayed as histograms.

Semi quantitative RT-PCR of Cell-Associated VSV. WISH cells were plated in six-well plates as before. sLDLR (5 μg/mL, 133.5 nM) was added to the cells for 15 min before infection with VSV (0.2 MOI) for 10 min at 4 °C or at 37 °C. The cells were then washed 10 times with cold PBS to remove any unbound virus, cells were trypsin-digested, and viral RNA was extracted using the High Pure Viral Nucleic Acid Kit (catalog #11 858 874 001, Roche Diagnostics). RT-PCR was performed (M-MLV, catalog #M170A, Promega). For first-strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using random primers and 200 U of M-MLV reverse transcriptase in a Tris-HCl buffer (pH 8.3, 50 mM, 25 μL) containing 75 mM KCl, 3 mM MgCl₂, 500 μM dNTP, and 10 mM DTT. PCR was performed with the Red Load Taq master mix (Fermentas) with the designed primer pairs of VSV sequences (amplifying nucleotides 6096–6223 of the VSV genome). The amplification program was as follows: initial denaturation step at 95 °C, 15 min, followed by 31 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. PCR products were analyzed by gel electrophoresis.

Surface Plasmon Resonance. Analyses were performed using a BIAcore 3000 instrument (GE Healthcare). sLDLR (0.02 mg/mL) in sodium acetate buffer (15 mM, pH 3.8, 40 μL) was immobilized on a CM5 sensor chip according to the manufacturer’s instructions. VSV (molecular mass 2.66 × 10⁶ Da) was suspended at 10⁷ pfu/mL in PBS with or without CaCl₂ (1 mM) and passed over the immobilized sLDLR in the sensor chip. For measuring VSV avidity to sLDLR, the VSV suspension was passed on the sensor chip at the indicated concentrations. For measuring VSV-G affinity to sLDLR, a preparation of VSV-G-pseudotyped lentiviral vectors (VSV-G-LV) (15 μg/mL) was immobilized on a sensor chip, and sLDLR in PBS plus CaCl₂ was passed on the chip at the indicated concentrations. The dissociation constants were calculated using BIAevaluation 4.1 software according to the manufacturer’s instructions.

Communoprecipitation of VSV-G and sLDLR. Purified VSV (50 ng) and sLDLR (6 μg/mL) were incubated (2 h at room temperature). Protein-G Sepharose beads (100 μL) were incubated with 10 μg of anti-LDLR mAb 28.28 (2), anti-LDLR mAb C7, an isotype-matched control mAb, or no antibody (1 h at 4 °C). Then the antibodies-conjugated beads were washed, mixed with the VSV–sLDLR complex, and incubated for 2 h at 4 °C. Next the beads were washed with 0.1% BSA and 1 M NaCl in PBS to overcome nonspecific interactions. The bound proteins were eluted with a sample buffer and subjected to SDS/PAGE [7.5% (wt/vol) acrylamide]. Solubilized VSV was run in parallel to provide a VSV-G marker. Proteins were then transferred to a nitrocellulose membrane, and the membrane was immunoblotted with anti
VSV-G (Sigma, 9.8 mg/mL diluted 1:1,000) or the anti-LDLR mAb 29.8 followed by HRP-rabbit anti-mouse conjugate (Abcam, 2 mg/mL, 1:20,000) as a second antibody.

**Preparation of VSV-G-LV and Lymphocytic Choriomeningitis Virus-Pseudotyped Lentiviral Vector.** EGFP expressing VSV-G pseudotyped lentiviral vectors were produced as described (3) but with the following modifications: HEK 293T cells in DMEM-10 (20 mL) were plated in a 15-cm dish precoated with 0.01% poly-d-lysine. The culture was transfected by a mixture of four expression vectors (30 μg DNA in 2 mL PBS in total, encoding VSV-G-Gag-Pol-Rev; TurboGFP at a 1:1:1:2 ratio, respectively (Invitrogen, except TurboGFP, which was from Sigma), using jetPEI (60 μg, Polysul Plus Transfection) as a transfection reagent. After 24 h, media were changed to DMEM-2 (15 mL). Culture medium containing the resulting viral vectors was collected at 24 and at 48 h and was concentrated 75-fold by ultrafiltration on a 10-kDa cutoff membrane (Centricon 10, Millipore). A plasmid encoding the lymphocytic choriomeningitis virus (LCMV) coat protein was kindly provided by A. Panet (Hebrew University, Jerusalem, Israel) and was used as above instead of the VSV-G plasmid for production of LCMV-pseudotyped lentiviral vector (LCMV-LV).

**Transduction of Cells.** Cells (20,000 per 0.3 mL DMEM-10 per well or 20,000 per 1 mL per well) were seeded in either eight-well μ-slides (ibidi) or in 24-well plates, respectively. After 17–24 h, indicated reagents (sLDLR, receptor-associated protein, etc.) were added, and after 30 min the cultures were transduced with VSV-G pseudotyped or LCMV-LV encoding EGFP. After 72 h nuclei were counterstained with Hoechst 33258, and the extent of EGFP expression was evaluated by fluorescence microscopy.

**Fluorescence Microscopy: EGFP and Hoechst 33258.** Cells (20,000 cells per 0.3 mL per well) were cultured in eight-well μ-slides or in 24-well plates for 2–4 d. Nuclei were stained with Hoechst 33258 (2 μg/mL in DMEM-10, 15 min). The cultures were observed by fluorescence microscopy (Olympus IX71) using excitation at wavelength <485 nm and emission of wavelength >530 nm for EGFP, wavelength <380 nm and >420 nm for Hoechst 33258.

**Image Analysis.** Images of Hoechst 33258-stained nuclei were selected by the Magic Wand tool of Adobe Photoshop, copied into a separate image, and counted by the analyze particle tool of the ImageJ program (www.imagej.nih.gov/ij/). The extent of EGFP expression (the green channel) in three to four fields was determined using the analyze-histogram tool of the ImageJ program. The total fluorescence (sum of pixel intensity on a scale of 0–255 times the pixel counts) was then normalized to the number of nuclei. Means ± SD of three to four separate measurements in fields containing at least 30 nuclei are represented.

**Trypsin Digestion of the Cell Surface VSV Receptor.** Human epithelial WISH cells were grown to confluence in T75 flasks, and the cultures were washed three times with PBS and then treated either with Trypsin/EDTA or EDTA alone for 30 min at 37 °C. The resulting cell suspensions were then washed three times with MEM-10 and suspended in MEM-10. VSV (MOI = 0.015) was then added for 15 min at 37 °C, and the cultures were then washed three times with MEM-10, suspended in MEM-10, and then plated at 5*10^5 cells/mL in 96-well plates (0.1 mL per well). The cultures were incubated for 17 h at 37 °C, culture supernatants were collected for plaque assay, and the cell cultures were stained with crystal violet and photomicrographed.

**Plaque Assay of VSV-G.** Human WISH cells (900,000 per well, 0.2 mL) were grown to confluence in six-well plates. Serially 10 times diluted samples containing VSV (0.1 mL) were added to the plates, and incubation continued for 30 min at room temperature. MEM-10 (2 mL) containing 0.6% agarose, preheated to 37 °C, was added to each well. The cultures were then incubated for 17 h at 37 °C or until plaques were microscopically visible. The agarose layer was then carefully removed, and the cultures were stained with crystal violet and photographed. Plaques were counted using the ImageJ program. Data are averages ± SD of three to four replicates.

**Competition Between VSV and Fluorescently Labeled LDL.** FS-11 cells (5,000 cells per 0.3 mL DMEM per well) in eight-well μ-slides (ibidi) were plated to 4 °C, and VSV was added at the indicated MOI for 5 min on ice. Fluorescently labeled LDL (Dil-LDL, 1.67 μg/mL) was added and incubation continued at 4 °C for 4 h. The cells were then washed three times with cold PBS and incubated at 37 °C for 1 h in DMEM. Nuclei were then stained with Hoechst 33258 as before, and the cultures were evaluated by fluorescence microscopy. Dil-LDL visualization was determined by excitation at <530 nm and emission >600 nm.

**Flow Cytometry.** FS-11 cells (50,000 cells per 1 mL DMEM per well) in 12-well plates were cooled to 4 °C, and VSV suspended in DMEM or medium alone was added (5 min, 4 °C), followed by Dil-LDL (4.8 μg/mL). Incubation continued at 4 °C for 4 h; the cells were then washed three times with cold PBS and then incubated in DMEM-10 at 37 °C for 1 h. The cultures were trypsin-digested, and Dil-LDL uptake was determined using flow cytometry (BD FACSAria III). Analysis was performed on a window that excluded aggregates, and 10,000 cells were measured. Dil-LDL excitation was at <530 nm and emission was at >600 nm.

**Immunoblotting.** Cells in six-well plates were lysed with 0.1 mL per well of radio-immunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0), supplemented with Sigma protease inhibitors]. The lysates were centrifuged (10,000 × g, 10 min), and the supernatants containing the cellular proteins were collected. Protein concentration was determined using a BCA Protein assay reagent kit (Pierce) using BSA as a standard. Protein samples were boiled for 3 min in SDS/PAGE sample buffer containing 25 mM DTT, and the supernatants were resolved by gradient SDS/PAGE [7.5% (wt/vol) acrylamide]. Proteins were then transferred onto a nitrocellulose membrane and then incubated with the indicated antibodies. Second antibody conjugates were visualized by the Super Signal Detection Kit (Pierce). Human LDLR was detected by anti-LDLR mAb 29.8 (7 μg/mL) (2) or by biotinylated anti-human LDLR antibody (0.2 μg/mL).

**Blocking of VSV Infection with mAbs to LDLR.** Cells (30,000 cells per well in MEM-10) were plated in 96-well plates 17 h before the assay. Medium was replaced with MEM-1; the plates were then cooled to 4 °C, and antibodies were added to the cultures at the indicated concentrations for 30 min. VSV (MOI = 0.05) was then added and incubation continued at 4 °C for 1 h. The plates were then washed two times with antibody-containing MEM-1 and cultured at 37 °C for 17–24 h in the presence of the antibodies. The cultures were then stained with crystal violet as above, and the extent of protection from the cytopathic effect was determined microscopically.

**Measuring VSV Binding and Internalization.** FS-11 fibroblasts or GM701 fibroblasts (50,000 cells per well in DMEM-10) were seeded and grown to confluence on coverslips in 24-well plates. The cells were then preincubated with various combinations of receptor-associated protein (200 nM) and anti-LDLR mAbs 29.8 or 28.28 (50 μg/mL each) for 30 min at 37 °C. VSV (MOI = 500) was then added for 4 min at 37 °C; the cultures were washed three times with PBS and immediately fixed at room temperature with freshly prepared 3% (wt/vol) paraformaldehyde and 0.02% Triton X-100 (2 min) followed by 3% paraformaldehyde alone (20 min) and...
washed three times with PBS. The paraformaldehyde-generated Schiff bases were reduced with NaBH₄ (1 mg/mL, 20 min), washed, blocked with 0.1% BSA, incubated with anti-VSV-G (9.8 μg/mL, 1 h), biotinylated goat anti-mouse antibody (stock diluted 1:500, 1 h, room temperature), and Cy-3-labeled streptavidin (3 μg/mL, 30 min, room temperature). Nuclei were then stained with Hoechst 33258 (2 μg/mL in DMEM-10, 15 min). The cultures were observed by fluorescence microscopy (Olympus IX71) using excitation at wavelength <560 nm and emission of wavelength >580 nm for Cy-3, wavelength <380 nm and >420 nm for Hoechst 33258.

To calculate bound and internalized VSV foci, images were processed using Photoshop as follows: background was selected using the Magic Wand tool (tolerance = 40), the selection was inverted, and the resulting foci copied to a new image and saved as black and white images. Foci were then counted using ImageJ in triplicate fields, each having approximately 40 nuclei. Data are presented as foci/cell.

**Rescue of LDLR Expression in GM701 Cells.** An LDLR expression vector (product ID A0821, GeneCopoeia) was used for constructing LDLR-encoding VSV-G-LV as described above. GM701 cells (20,000 cells per 1 mL DMEM-10 per well) in 24-well plates were transduced with the LDLR-encoding VSV-G-LV in the presence of polybrene (8 μg/mL). Control GM701 cells were treated with polybrene alone. After 17 h, viral vectors and polybrene were replaced with medium lacking polybrene daily for 3 d. The cultures were then transduced with VSV-G-LV encoding GFP in the absence of polybrene. After 17 h, viral vectors were removed, and after an additional 48 h nuclei were counterstained with Hoechst 33258, and the extent of EGFP expression and nuclei counts were evaluated by fluorescence microscopy.

**Knockdown of LDLR mRNA.** FS-11 cells (200,000 cells per well in six-well plates) in MEM supplemented with 10% (vol/vol) lipoprotein-deficient FBS (2 mL) were transfected either with siRNA pools (ON-TARGETplus, Dharmacon RNAi Technologies) directed against human LDLR mRNA (NM_000527) or nontargeting scrambled control siRNA according to the manufacturer’s protocol. After 72 h cells were trypsin-digested, pooled from two wells, and then plated in 24-well plates (20,000 cells per 1 mL DMEM-10 per well) for lentiviral vector transduction as above, or in six-well plates (500,000 cells per 2 mL DMEM-10 per well) for immunoblotting of LDLR.


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**Fig. S1.** Dose–response of VSV binding to immobilized sLDLR by surface plasmon resonance in PBS containing 1 mM CaCl₂. sLDLR (0.02 mg/mL) was immobilized on a CM5 sensor chip, and VSV was passed at the indicated concentrations. The dissociation constant (K_d, 10⁻¹¹ M) was calculated using the BIAevaluation program. This value reflects the avidity of multiple VSV-G spikes toward the array of immobilized sLDLR molecules.
**Fig. S2.** LDLR knockdown diminishes transduction by VSV-G-LV. (A) WT F5-11 fibroblasts were transfected with control siRNA or with LDLR siRNA for 72 h. The cultures were then transduced with EGFP-encoding VSV-G-LV (upper four panels) or EGFP-encoding LCMV-LV (lower four panels). Nuclei were counterstained with Hoechst 33258. EGFP expression (green) and nuclei (blue) were visualized by fluorescence microscopy. (B) Immunoblot of LDLR in WT fibroblasts 72 h after knockdown with the indicated siRNA. (C) Average ± SD of EGFP expression shown in A. data were normalized to nuclei numbers. ***P < 0.0003, n = 4. N.S., not significant (P = 0.355, n = 3).