Anthrax toxin receptor 1 is the cellular receptor for Seneca Valley virus

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Seneca Valley virus (SVV) is an oncolytic picornavirus with selective tropism for neuroendocrine cancers. It has shown promise as a cancer therapeutic in preclinical studies and early-phase clinical trials. Here, we have identified anthrax toxin receptor 1 (ANTXR1) as the receptor for SVV using genome-wide loss-of-function screens. ANTXR1 is necessary for permissivity in vitro and in vivo. However, robust SVV replication requires an additional innate immune defect. We found that SVV interacts directly and specifically with ANTXR1, that this interaction is required for SVV binding to permissive cells, and that ANTXR1 expression is necessary and sufficient for infection in cell lines with decreased expression of antiviral IFN genes at baseline. Finally, we identified the region of the SVV capsid that is responsible for receptor recognition using cryoelectron microscopy of the SVV-ANTXR1-Fc complex. These studies identify ANTXR1, a class of receptor that is shared by a mammalian virus and a bacterial toxin, as the cellular receptor for SVV.

Introduction

Seneca Valley virus (SVV) is the prototype member of the Senecavirus genus within the family Picornaviridae (1). SVV is a clade I strain of Senecavirus A (SVA) that has been shown to selectively infect and lyse cancers with neuroendocrine features, including a subset of small-cell lung cancer (SCLC) and pediatric neuroendocrine solid tumors (1–4). These cancers constitute a major cause of morbidity and mortality — SCLC alone is responsible for approximately 30,000 deaths annually in the US (5). Previous studies in preclinical mouse models and early-phase clinical trials confirmed the safety and potential efficacy of SVV as a novel cancer treatment, but clinical development of SVV has been hampered by a lack of understanding of host dependency factors (2, 3, 6–9).

Picornavirus tropism is restricted to permissive cells by the requirement for host expression of a viral receptor. Picornavirus receptors are typically immunoglobulin superfamily (IgSF) proteins that consist of a single transmembrane domain and 2–5 Ig domains (10). In all cases, the extreme amino-terminal Ig domain of the receptor interacts with the viral capsid in a canyon that surrounds the 5-fold axis. While IgSF receptors are most common, non-IgSF receptors and attachment factors are also documented and include transmembrane proteins as well as carbohydrates such as sialic acid and heparan sulfate (11, 12). Sialic acid acts as a receptor or attachment factor for a number of viruses and has been proposed as a component of the SVV receptor. Although enzymatic removal or blocking of sialic acid with lectins modestly reduces SVV infection in pediatric glioma models, the definitive identity of the SVV receptor has remained elusive (13).

Despite a requirement for receptor expression, this alone may not be sufficient for productive infection in all contexts. Mammalian viruses have varying degrees of sensitivity to innate cellular antiviral responses (14). The potency of cellular antiviral responses is similarly variable in normal and cancerous cells (14, 15). SCLC in particular has been shown to frequently lack key components of MHC class I antigen presentation as well as to have decreased expression of immune stimulatory cytokines (16–18). Both result in decreased tumor cell recognition and removal by the immune system. In the absence of innate immune defect, exogenous expression of the appropriate receptor may be insufficient for permissivity and is likely a shared requirement for successful replication of many oncolytic viruses (19).

In this study, we performed 2 genome-wide loss-of-function screens followed by gene expression analysis of publicly available cancer cell line data to identify essential host dependency factors of SVV infection.

Results

Genome-wide loss-of-function screens identify ANTXR1 as essential for SVV infection. The pooled genome-scale CRISPR knockout (GeCKO) v2 human single-guide RNA (sgRNA) library targets over 19,000 genes within the human genome and has the ability to efficiently knock out genes using the Cas9 DNA nuclease (20–24). Because of the high efficiency of gene disruption in haploid cells, we performed a genome-wide screen in one of the only known human haploid cell lines, HAPI, which we found to be permissive to SVV at relatively high MOI (refs. 25, 26, and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI93472DS1). HAPI-Cas9
cells were transduced with pooled GeCKO lentivirus library and challenged with an SVV infection at an MOI of 1,000 virus particle/cell (vp/cell), which resulted in more than 90% cell death (Figure 1A and Supplemental Figure 1A). Genomic DNA was extracted from the expanded surviving cell population and subsequently analyzed by high-throughput sequencing to determine changes in sgRNA representation in comparison with controls. Representation of nontargeting control sgRNAs was maintained from the plasmid pools until the end of the screen; however, notable changes were observed in targeting sgRNAs, reflecting loss of sgRNAs that target essential genes (Supplemental Figure 1, B–E). The most significantly enriched sgRNAs in the SVV selected pool were found to target the \( \text{ANTXR1} \) gene, which encodes anthrax toxin receptor 1 (27). \( \text{ANTXR1} \) and the testis expressed 2 gene (\( \text{TEX2} \)) were the only genes with multiple sgRNAs significantly enriched in the SVV-resistant sample (Figure 1B). Highly enriched sgRNAs were individually tested for the ability to confer resistance to SVV in HAPI cells (Figure 1C). We observed 6 sgRNAs targeting 3 different genes that conferred SVV resistance after gene knockout in HAPI cells, including 3 independent sgRNAs targeting the \( \text{ANTXR1} \) gene. Both enriched sgRNAs targeting the \( \text{TEX2} \) gene conferred resistance as well as 1 sgRNA targeting the nuclear receptor gene \( \text{NR2C2} \).

To validate our results in a cell line of immediate relevance to neuroendocrine cancers, we repeated the GeCKO screen in the highly SVV-permissive H446 SCLC cell line. GeCKO lentivirus-transduced H446-Cas9 cells were challenged with SVV at an MOI of 1 vp/cell, which resulted in more than 99% cell death. The percentage of surviving cells after SVV infection was much lower than in the HAPI screen, allowing isolation of individual cell colonies, instead of a pooled population. Genomic DNA from each colony was extracted, and individual sgRNAs were identified by Sanger sequencing (Table 1). In 23 of 25 resistant colonies (92%), sgRNAs targeting \( \text{ANTXR1} \) were present, and comprised 3 independent sgRNAs targeting \( \text{ANTXR1} \). Each sgRNA identified in the H446 screen was tested individually in a secondary screen of parental H446 cells for the ability to confer SVV resistance (Figure 1D). All 3 \( \text{ANTXR1} \)-targeting sgRNAs identified in the screen were able to confer resistance to SVV; however, no other candidate sgRNAs altered SVV permissivity in parental H446 cells.

\( \text{ANTXR1} \) is necessary for permissivity in neuroendocrine cancer cell lines. We assessed the genomic sequence of \( \text{ANTXR1} \) in clones isolated from the H446 screen and found that all 5 \( \text{ANTXR1-KO} \) clones contained insertions or deletions (indels) in exon 2 of the \( \text{ANTXR1} \) gene. These indels would lead to a frameshift and premature stop codon, predicted to result in a truncated \( \text{ANTXR1} \) protein (Table 2). We also confirmed the loss of SVV permissivity in \( \text{ANTXR1-KO} \) clones using a cell viability assay with parental H446 cells and nonpermissive A549 cells as positive and negative controls, respectively (Figure 2A). After a 72-hour incubation period with SVV, we observed a significant loss of viability with increasing MOI of SVV in parental H446 cells. All \( \text{ANTXR1-KO} \) lines as
Some viruses are able to increase viral spread into bystander cells without the use of their canonical receptor, but it is currently unknown whether SVV has this capability (29). We further examined whether bystander cells lacking ANTXR1 expression could be infected by neighboring cells through cell-cell spread in a mixed cell population containing both parental and ANTXR1-KO cells. We first created an H446 ANTXR1-KO clone that stably expressed the mCherry fluorescent protein (ANTXR1-KO mCherry). We then cocultured parental H446 and ANTXR1-KO mCherry cells at a 1:1 cell number ratio and challenged cells with SVV-GFP using pure parental and ANTXR1 mCherry cells at a 1:1 cell number ratio and challenged cells with SVV-GFP using pure parental and ANTXR1 mCherry). We then cocultured parental H446 and ANTXR1-KO mCherry SVV-001 cohort were significantly associated with permissivity (P = 0.0023, Fisher’s exact test). Most strikingly, none of the 20 permissive cell lines lacked expression of ANTXR1, supporting the hypothesis that ANTXR1 is a required host dependency factor for SVV infection.

While ANTXR1 expression appears to be a requirement for SVV permissivity, the CCLE data set suggests that it is not sufficient: 42 of 62 ANTXR1-expressing cell lines analyzed for permissivity (67.7%) are nonpermissive, corresponding to a low positive predictive value of 0.32. We were therefore prompted to identify predictive gene expression differences between ANTXR1-expressing permissive and nonpermissive classes. We used competitive gene set enrichment to identify significantly differentially expressed gene sets from the Reactome database (32, 33). We identified 7 gene sets, all of which were significantly downregulated in permissive cell lines expressing ANTXR1 (Figure 4B). The most significant gene set was INTERFERON_ALPHA_BETA_SIGNALING, in which 34 of 44 genes (77%) were significantly downregulated in permissive cell lines. The enrichment for this gene set (q = 0.0046) can be visualized in Figure 3C. A sample-wise analysis of gene expression was performed to see whether the gene set enrichment we observed was driven by cell lines derived from a particular tumor histology (ref. 34 and Figure 3B). We found that lack of expression of these gene sets was enriched among SCLC and neuroblastoma cell lines (Figure 3D). To determine whether this expression signature was operant in vivo, we performed a similar analysis of human tumor xenograft data from the Pedi-
AntXR1 leads to the loss of SVV permissivity. (A) Three of the H446 AntXR1-KO cell lines were challenged with increasing MOIs of SVV for 72 hours. Cell viability was determined via AlamarBlue. Parental H446 cells and the nonpermissive NSCLC cell line A549 were used as positive and negative controls, respectively. Each data point represents the average of n = 6 replicates with error bars representing SD. (B) Permissive SCLC and pediatric cancer cell lines were transduced with an sgRNA targeting AntXR1. Parental (light gray) and AntXR1-KO (black) cells were challenged with SVV-GFP and analyzed by flow cytometry. Each bar represents the average of n = 3 replicates with error bars representing SD. Unpaired 2-sided t tests were used to determine statistical significance. (C) Parental H446 cells (top), H446 AntXR1-KO mCherry cells (bottom), or 1:1 mixture of parental/AntXR1-KO mCherry cells (middle) were challenged with SVV-GFP. White arrows indicate adjacent SVV-GFP–infected parental H446 and uninfected AntXR1-KO mCherry cells. Scale bars: 100 μm. Images representative of 3 independent experiments. (D) Parental H446 tumors (light green/dark green), H446 AntXR1-KO mCherry tumors (pink/red), and 1:1 mixture of parental/AntXR1-KO mCherry tumors (light purple/dark purple) were challenged with WT SVV-001 or PBS vehicle. Tumor volumes were measured every other day. Each data point corresponds to the average of n = 4–5 tumors with error bars representing SD. A 2-way ANOVA test with multiple comparisons was used to determine statistical significance. ****P ≤ 0.0001.
Re-expression of ANTXR1 rescues SVV permissivity. To test the specificity of the ANTXR1 sgRNAs, we evaluated whether exogenous re-expression of ANTXR1 could rescue permissivity to SVV in ANTXR1-KO cells. We cotransfected 3 H446 ANTXR1-KO lines with an ANTXR1-HA expression plasmid and an mCherry fluorescent protein expression plasmid and challenged the cells with SVV-GFP 16 hours after transfection (Figure 4A and Supplemental Figure 3). H446 ANTXR1-KO or DMS79 were significantly impaired in viral production compared with parental H446 cells. These results suggest that high baseline expression of IFN pathway components in ANTXR1-expressing cells can attenuate an SVV infection.

Figure 3. ANTXR1 expression is significantly associated with permissivity. (A) Scaled log$_2$ ANTXR1 gene expression of permissive (top), nonpermissive (middle), and all (1,037) cell lines in the CCLE (bottom). ANTXR1 expression was significantly associated with permissivity ($P = 0.0023$, Fisher’s exact test). Inset table summarises the findings of cell lines confirmed to be permissive (P) or nonpermissive (NP) and their ANTXR1 expression status as ANTXR1+ (ANTXR1+) or nonexpressing (ANTXR1–). (B) An enrichment score was calculated for each cell line tested and for each of the 7 significant gene sets. Enrichment scores are highly correlated between the gene sets given their overlapping gene constituents. Permissivity is indicated in the column label. (C) An enrichment barcode plot depicting the negative enrichment of type I IFN signaling genes in permissive cell lines ($q = 0.0046$). The t statistic for each gene in the set is plotted as a vertical black bar. Significantly upregulated genes fall within the red region, while significantly downregulated genes fall within the blue region. (D) Sample-wise enrichment scores were calculated for the top enriched gene set and plotted based on the histology of the tumor of origin. SCLC and neuroblastoma stand out as lacking genes involved in type I IFN signaling. (E) Receiver operating characteristics of ANTXR1 (AUC = 0.66), IFN enrichment score (AUC = 0.80), or a combination of the 2 predictors (AUC = 0.89).

Type and SVV permissivity. Interestingly, these cell lines are enriched for downregulation of antiviral IFN signaling genes, potentially explaining our prior observations (Supplemental Figure 2C).

We further sought to test whether an SVV infection in ANTXR1-expressing cells could be affected by the presence of an active IFN pathway. We performed 1-step single growth kinetic assays with SVV-GFP (MOI = 0.1 vp/cell) in parental H446, H446 ANTXR1-KO, and DMS79 cells, which express ANTXR1 but have higher gene expression of the IFN pathway components (Supplemental Figure 2D). H446 ANTXR1-KO or DMS79 were significantly impaired in viral production compared with parental H446 cells. These results suggest that high baseline expression of IFN pathway components in ANTXR1-expressing cells can attenuate an SVV infection.
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LX22cl ANTXR1-KO lines with the ANTXR1-HA and mCherry expression plasmids, and subsequently incubated the cells with SVV-GFP. Cells were analyzed by flow cytometry and gated to select for transfected cells (mCherry+). Compared with parental mCherry+GFP+ cells, we observed a significant decrease in the mCherry+GFP+ population in ANTXR1-KO H446 and LX22cl cells that was rescued upon transfection with the ANTXR1-HA expression plasmid (Figure 4B). Expression of the ANTXR1-HA fusion protein was confirmed in each ANTXR1-HA–transfected cell line by immunoblot using an HA tag–specific antibody (Supplemental Figure 3 and Figure 4C). Re-expression of ANTXR1 protein in ANTXR1-KO cell lines is sufficient to rescue SVV permissivity.

Exogenous expression of ANTXR1 is sufficient to induce SVV permissivity. We next sought to determine whether expression of ANTXR1 protein was sufficient to increase the permissivity of the nonpermissive SCLC cell lines H69 and H146, which do not express the gene. After transduction with a doxycycline-inducible ANTXR1-HA expression lentivirus, we incubated parental and ANTXR1-expressing H69 and H146 cells in the presence or absence of 1 μg/ml doxycycline for 72 hours, challenged with SVV-GFP, and analyzed by flow cytometry (Figure 4D). Parental H69 and H146 cells and ANTXR1-transduced cells, in the absence of doxycycline, showed GFP− populations under 1.5%, as expected. Upon doxycycline treatment, both ANTXR1-transduced H69 and H146 cells showed a significant increase in SVV-GFP–infected cells to 7.46% ± 0.17% and 18.3% ± 0.20%, respectively. Expression of the ANTXR1-HA protein in the doxycycline-induced cells was confirmed by Western blot (Figure 4E). These data confirm that exogenous expression of ANTXR1 is sufficient to induce permissivity in SVV-resistant SCLC cell lines that lack endogenous ANTXR1.

ANTXR1 interacts directly with SVV. As ANTXR1 is a transmembrane protein and required for SVV infection in various permissive SCLC cell lines, we sought to determine whether ANTXR1 interacts directly with SVV. We used an ANTXR1-Fc chimera or a control isotype IgG1-Fc protein for coimmunoprecipitation (co-IP) studies. After incubation of Fc-bead complexes with SVV, all bound proteins were eluted and analyzed by Western blot using SVV rabbit antisera (Figure 5A). In all serially diluted ANTXR1-Fc samples incubated with SVV, we observed viral protein bands as well as a decrease in intensity of the bands corresponding to a
We did not detect any SVV protein bands in samples incubated with the IgG 1-Fc isotype control or samples not incubated with SVV. After confirming a direct interaction, we repeated the ANTXR1-Fc chimera co-IP studies in the presence of increasing amounts of sodium chloride (NaCl) to investigate the strength of the interaction in vitro under high ionic strength (Figure 5B). The intensity of viral protein bands did not change significantly with increasing salt concentration up to 2 M NaCl. As ANTXR1 has high sequence similarity to the high-affinity anthrax receptor, ANTXR2, we performed the co-IP with the ANTXR2-Fc chimeric protein using the ANTXR1-Fc protein as a positive control (27). We did not observe any bands corresponding to viral proteins in ANTXR2-Fc samples incubated with SVV, indicating the absence of an interaction between the extracellular domain of ANTXR2 and SVV (Figure 5C). Bands corresponding to viral protein were observed only in ANTXR1-Fc samples. Additionally, we investigated which region of the ANTXR1 extracellular domain was essential for the interaction with SVV and was therefore essential for rescue of SVV permissivity in ANTXR1-KO cells. We created an N-terminal deletion series of ANTXR1-HA expression plasmids that deleted increasing regions of the extracellular domain sequence of the ANTXR1 protein while preserving the signal peptide sequence (Supplemental Figure 4A). We then tested the ability of the truncated expression plasmids to decrease of bound ANTXR1-Fc protein. We did not detect any SVV protein bands in samples incubated with the IgG-Fc isotype control or samples not incubated with SVV. After confirming a direct interaction, we repeated the ANTXR1-Fc chimera co-IP studies in the presence of increasing amounts of sodium chloride (NaCl) to investigate the strength of the interaction in vitro under high ionic strength (Figure 5B). The intensity of viral protein bands did not change significantly with increasing salt concentration up to 2 M NaCl. As ANTXR1 has high sequence similarity to the high-affinity anthrax receptor, ANTXR2, we performed the co-IP with the ANTXR2-Fc chimeric protein using the ANTXR1-Fc protein as a positive control (27). We did not observe any bands corresponding to viral proteins in ANTXR2-Fc samples incubated with SVV, indicating the absence of an interaction between the extracellular domain of ANTXR2 and SVV (Figure 5C). Bands corresponding to viral protein were observed only in ANTXR1-Fc samples. Our results indicate that ANTXR1, and not ANTXR2, can directly interact with SVV in a high-affinity and stable interaction. Additionally, we investigated which region of the ANTXR1 extracellular domain was essential for the interaction with SVV and was therefore essential for rescue of SVV permissivity in ANTXR1-KO cells. We created an N-terminal deletion series of ANTXR1-HA expression plasmids that deleted increasing regions of the extracellular domain sequence of the ANTXR1 protein while preserving the signal peptide sequence (Supplemental Figure 4A). We then tested the ability of the truncated expression plasmids to...
rescue SVV permissivity in one of the H446 ANTXR1-KO clones (Supplemental Figure 4B). Unlike full-length ANTXR1 protein, all ANTXR1 truncations were unable to rescue SVV permissivity when challenged with SVV-GFP. We confirmed full-length and truncated ANTXR1-HA protein expression via Western blot (Supplemental Figure 4C). These results suggest there are required residues for the interaction of SVV and ANTXR1 located in the extreme N-terminal region of the ANTXR1 protein.

**Soluble ANTXR1-Fc chimera blocks SVV infection in vitro.** We next sought to determine whether the interaction between the SVV and ANTXR1-Fc or ANTXR2-Fc chimera could attenuate a cellular SVV infection. We incubated SVV-GFP with the ANTXR1-Fc, ANTXR1-Fc, or IgG1-Fc protein before an overnight incubation with parental H446 cells and subsequent analysis by fluorescence microscopy (Figure 5D). Cells incubated with SVV-GFP and IgG1-Fc or ANTXR2-Fc protein showed high levels of GFP+ cells indicative of a productive SVV infection. Cells incubated with SVV-GFP and ANTXR1-Fc protein showed no detectable GFP+ cells, indicating a substantial lack of SVV-GFP infection in these cells. These results demonstrate that only exogenous ANTXR1 protein and not ANTXR2 protein is able to block a cellular SVV-GFP infection, and further support ANTXR1 as the primary cellular receptor for SVV.

**Loss of ANTXR1 protein expression abrogates SVV binding to permissive cells.** We sought to determine whether ANTXR1-KO cells had lost the ability to bind SVV. We also assessed the potential role of TEX2, another candidate from the HAP1 screen, in binding SVV. We incubated parental, ANTXR1-KO, and TEX2-KO H446 cells with WT SVV labeled with the fluorophore Cy5 (SVV-Cy5) and analyzed the cells by flow cytometry using the nonpermissive SCLC cell line DMS114 as a negative control for SVV binding (Figure 5E). Parental H446 incubated with SVV-Cy5 showed a high level of fluorescence (mean fluorescence [MF] = 2,373) compared with DMS114 cells incubated with SVV-Cy5 (MF = 425). TEX2-KO H446 cells showed a similar fluorescence profile to parental H446 cells (MF = 2,233), indicating there was no loss of SVV binding ability corresponding to loss of TEX2 protein expression. In contrast, ANTXR1-KO H446 cells showed a markedly diminished fluorescence profile similar to that of the negative control line, DMS114 (MF = 358). Loss of SVV binding was observed only in ANTXR1-KO cells, indicating that not only does ANTXR1 bind directly to SVV based on co-IP data, but it is the major binding determinant for the virus in intact cells.

**Cryoelectron microscopy of capsid-receptor complex.** We analyzed the complex of SVV bound to the ANTXR1-Fc chimeric protein by cryoelectron microscopy (cryo-EM). Picornaviruses have an icosahedral capsid formed by 60 copies of a protomer consisting of 3 major capsid proteins, VP1, VP2, and VP3, and a fourth much smaller protein, VP4, positioned in the interior of the capsid. Copies of VP1 are assembled around the 5-fold axis, while VP2 and VP3 alternate around the 3-fold axis (36). Our reconstruction matches the existing atomic model of the virus (37) when filtered to 14.5 Å resolution (Figure 5F). Additionally, the map displays the receptor subdomains distributed radially around the 5-fold axis, in crown-like geometry similar to other picornaviruses, such as poliovirus (38), rhinovirus (39), or coxsackieviruses (40). The map revealed the receptor binding quasiperpendicular to the capsid close to the center of the protomer, making contact with all 3 major capsid proteins and centered around the “puff” loop of VP2.

**Discussion**

ANTXR1 functions as 1 of the 2 receptors for the *Bacillus anthracis* toxin (27). SVV is unique among known viruses in using ANTXR1, or any related protein, as a primary receptor. In contrast to a number of previously identified receptors of other picornaviruses, ANTXR1 is not a member of the immunoglobulin superfamily (IgSF) of receptors (10). Although ANTXR1 shares common features to the IgSF receptors in being a single-pass transmembrane glycoprotein, it may be unique in its role as a receptor to both a mammalian virus and a bacterial toxin.

Interestingly, ANTXR1 was initially discovered as a tumor endothelial marker and is also known as TEM8 (41). We show that ANTXR1 is frequently expressed on the surface of tumor cells compared with normal cells. Efforts to develop a therapeutic antibody targeting ANTXR1 expressed in tumor endothelium have been hampered by cross-reactivity of the antibody to ANTXR2. The exquisite selectivity of SVV for ANTXR1 and the medium-resolution cryo-EM map described here may inform future therapeutic development in the antibody space for both antiangiogenic purposes and a potentially novel target for select neuroendocrine cancers.

Prior work described by Liu et al. suggested that sialic acid could be a component of the SVV receptor in pediatric glioma models (13). As sialic acids can be found on a number of different carrier molecules, enzymatic removal of sialic acids and competitive blocking by lectins may potentially affect diverse cellular processes or occlude a portion of the virus/receptor binding interface, altering SVV permissivity. Conversely, the data presented here identify ANTXR1, a receptor that is both necessary and sufficient for SVV permissivity in both SCLC and pediatric cancer cells.

Our gene expression analysis suggests that beyond expression of ANTXR1, cancer cell defects in the innate immune response are important determinants of successful SVV replication. We have previously shown that SVV has a preference for the variant subtype of SCLC, with permissivity correlated to a high *NEUROD1/ASCL1* gene expression ratio (3). Although these neurogenic transcription factors were not identified in our screens, we have shown that *NEUROD1* expression can be used as a correlative indicator of innate immune response pathway activity. These features, together with ANTXR1 expression, may define a category of cancers particularly amenable to treatment with SVV. An improved understanding of how cellular innate immune response pathways dictate permissivity could identify synergistic combination strategies with therapeutic agents targeting these pathways in cancer cells.

The identification of the receptor for a virus is an important first step in understanding its tropism. For SVV, this discovery is additionally germane, as ANTXR1 expression will facilitate the identification of patients who could potentially benefit from SVV virotherapy. SCLC is a highly aggressive and nearly universally lethal cancer, for which few tractable therapeutic targets have been identified. Many large-scale clinical trials, conducted without biomarker selection, have failed to advance the standard of care for this disease (42, 43). Carefully defined biomarkers that would focus novel therapeutic studies on the potentially responsive subset of patients could change this field. The identification of ANTXR1, and possibly suppressed innate immunity, as selection criteria will help define the structure of our subsequent clinical trials.
Methods

**Human GeCKO v2 library screens.** The Human GeCKO v2 library was obtained as 2 half libraries (libraries A and B) in the lentivirus backbone (Addgene plasmid 52962) as a gift from Feng Zhang (Broad Institute, Massachusetts Institute of Technology, Cambridge, MA, USA). The Memorial Sloan Kettering Cancer Center RNAi core facility amplified the pooled libraries by electroporation of Endura
electrocompetent cells (Lucigen) as described previously (20, 21). All cell line and virus information is described in the Supplemental Methods. DNA libraries were used to create lentivirus, transduced into H446-Cas9 and HAP1-Cas9 cells, and challenged with SVV-001 as reported in Supplemental Methods. All methods performed to identify and test each sgRNA for importance in SVV permissivity are described in Supplemental Methods.

**Cell viability assays and analysis.** Twenty-four hours before infection, cells (5.0 × 10^3) were seeded into black opaque 96-well plates (Corning) in 100 μl media. Plates were infected with serial dilutions of SVV from an MOI of 5,000 vp/cell to 5.0 × 10^{-5} vp/cell and incubated at 37°C in OptiMEM. The nonpermissive SCLC cell line DMS114 was used as a negative control. Cells were then processed and analyzed via flow cytometry as described in Supplemental Methods.

**SVV-GFP binding experiments.** SVV was incubated with the amine-reactive Cy5 dye (GE Healthcare) in sodium carbonate buffer (pH 9.3) for 1 hour at room temperature. Excess dye was removed by filtration through gel filtration columns (GE Healthcare) in HEPES buffer. Virus aliquots were stored at −80°C. Parental, ANTXR1-KO, and TEx2-KO H446 cells were incubated with SVV-Cy5 for 30 minutes at 37°C in OptiMEM. Western blot analysis or challenged with SVV-GFP for flow cytometry, catalog 13901) as a loading control. Immunoblotting was performed using HRP-conjugated secondary antibodies (Cell Signaling) and detection by chemiluminescence (GE Life Sciences).

**Flow cytometry analysis.** Parental and ANTXR1-KO cell lines were seeded in tissue culture–treated 6-well plates 24 hours before SVV-GFP infection. Cells were infected with SVV-GFP at the 50% tissue culture infective dose (TCID_{50}) for each cell line and incubated at 37°C for 6–16 hours with uninfected cells as controls. Cells were subsequently harvested, processed, and analyzed via flow cytometry on a BD LSR II Flow Cytometer (Becton Dickinson) as reported in Supplemental Methods. Additional gating and analysis were performed with FlowJo analysis software (Tree Star). Analyzed data and SDs were plotted using GraphPad Prism 6 software.

**Gene expression analysis.** Normalized gene expression data for cancer cell lines was downloaded from the Cancer Cell Line Encyclopedia (CCLE) or from the Pediatric Preclinical Testing Program (PPTP) (30, 35). Custom content descriptor files (CDFs) were used for both gene expression data sets. For CCLE microarray data, we used a CDF corresponding to ENTREZG v15. For PPTP microarray data, which include admixed mRNA of both human and mouse origin, we used a human-specific H-spec CDF (44). To determine the appropriate cutoff for cell lines expressing ANTXR1, local modes in the density distribution of ANTXR1 expression were identified, the lowest of which was designated as nonexpressed. The SD of this peak was then determined and an expression cutoff equal to 10 SDs above the mode was set, based on the work of Zilliox and Irizarry (31). Similar results were obtained using a Gaussian mixture model. Gene expression analysis was performed using the R statistical programing environment and the Bioconductor suite of tools. Differentially expressed genes were identified using LIMMA to fit a linear model.
to each gene and generate moderated t statistics using an empirical Bayes approach. Gene set enrichment analysis was performed using CAMERA, a purely competitive gene set testing approach (45). Sample-wise enrichment was determined using gene set variation analysis (34). Receiver operating characteristics were calculated using sample-wise enrichment score, ANTXR1 expression, or a combined predictor modeled using logistic regression as predictors.

**Cryoelectron microscopy.** Equal volumes of virions at 0.2 mg/ml and ANTXR1 at 1 mg/ml were mixed, giving a ratio of approximately 101 receptors per binding site. The samples were mixed and kept for 90 minutes at 37°C and transferred on ice for another 90 minutes. Specimens were prepared by application of 3 μl of purified virus on glow-discharged Quantifoil holey carbon grids (Quantifoil Micro Tools GmbH). The excess buffer was blotted and the grid was flash-plunged into liquid ethane using a Leica KF80 cryofixation device (C. Reichert OPTische Werke AG). Grids were loaded onto a Gatan 914 Cryoholder. Images were collected on a JEOL JEM2200FS microscope (JEOL Ltd.) operated at 200 kV using minimal dose conditions with an electron dose of approximately 30 electrons/Å². An in-column omega energy filter was used to improve image contrast by zero-loss filtering with a slit width of 25 eV. Automated data collection was carried out using SerialEM software. The micrographs were recorded at a defocus between 1 and 3 μm, on a 4k × 4k complementary metal oxide semiconductor camera (Tietz Video and Image Processing Systems) at a calibrated magnification of 50,000 corresponding to a pixel size of 3.12 Å.

One thousand seven hundred individual virus particles were selected from micrographs using E2BOXER software (46). Contrast transfer function (CTF) parameters were calculated using CTFFIND3 (47), and micrographs with poor CTF estimates were discarded. Orientation, classification, and refinement were done in Relion (48) using as initial reference a strongly low-pass version of the SVV atomic model (37). By calculating the Fourier shell correlation between 2 halves of the data set, the resolution of the map was estimated to be 14.5 Å. Similarly, a 34-Å reconstruction of the capsid without the receptor bound was calculated from 950 receptor particles. The reconstructed maps were visualized using Chimera (49).

**Statistics.** Two-way ANOVA tests with multiple comparisons were performed where applicable to determine statistical significance.

**Study approval.** All animal experiments and procedures were carried out under an animal protocol approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center, New York, NY, USA.

**Author contributions.** Authors contributed to this work in the following ways: designing research studies: LAM, LNB, MB, JTP, and CMR; conducting experiments: LAM, LNB, EEG, and JTP; acquiring data: LAM, LNB, and EGG; analyzing data: LAM, LNB, MB, and JTP; and writing the manuscript: LAM, MB, JTP, and CMR.

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