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CRISPR-Csy4-Mediated Editing of Rotavirus Double-Stranded RNA Genome

Highlights

- CRISPR-Csy4 fused with NSP5 can cleave rotavirus (+) ssRNA inside viroplasms
- Csy4-cleaved (+)ssRNA replication intermediates are repaired as edited viral dsRNA
- Csy4 editing allows detection of products of secondary transcription
- Secondary transcription is the main source of rotavirus proteins in infected cells

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In Brief

Papa et al. engineer the CRISPR-Csy4 nuclease to localize into rotavirus viral factories and cleave (+)ssRNA replication intermediates, producing edits of the targeted dsRNA genome segment. This allows for the detection of secondary transcription-derived proteins made by the newly assembled viruses, demonstrating that they largely contribute to overall viral protein production.
CRISPR-Csy4-Mediated Editing of Rotavirus Double-Stranded RNA Genome

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SUMMARY

CRISPR-nucleases have been widely applied for editing cellular and viral genomes, but nuclease-mediated genome editing of double-stranded RNA (dsRNA) viruses has not yet been reported. Here, by engineering CRISPR-Csy4 nuclease to localize to rotavirus viral factories, we achieve the nuclease-mediated genome editing of rotavirus, an important human and livestock pathogen with a multisegmented dsRNA genome. Rotavirus replication intermediates cleaved by Csy4 is edited through the formation of precise deletions in the targeted genome segments in a single replication cycle. Using CRISPR-Csy4-mediated editing of rotavirus genome, we label the products of rotavirus secondary transcription made by newly assembled viral particles during rotavirus replication, demonstrating that this step largely contributes to the overall production of viral proteins. We anticipate that the nuclease-mediated cleavage of dsRNA virus genomes will promote an advanced level of understanding of viral replication and host-pathogen interactions, also offering opportunities to develop therapeutics.

INTRODUCTION

Prokaryotes have evolved an anti-viral defense mechanism based on CRISPR loci. These regions express pre-CRISPR RNAs (pre-crRNAs) containing short virus-derived sequences. These transcripts are processed to generate crRNAs, which are used to guide CRISPR nucleases to cleave foreign nucleic acids (Jinek et al., 2012). The six known types of CRISPR-Cas systems have different mechanisms of crRNA maturation (Makarova et al., 2018).

The CRISPR-Cas type I and type III (and likely type IV) systems use an endoribonuclease of the Cas6 superfamily to cleave an invariant portion of the pre-crRNAs to generate the mature crRNAs (Murugan et al., 2017; Özcan et al., 2019). Among them, Csy4/Cas6f of Pseudomonas aeruginosa type I-F CRISPR systems (Makarova et al., 2018), is a well-characterized small (21 kDa) and highly specific single-turnover RNA endoribonuclease, processing pre-crRNAs in a 28-nucleotide (nt) sequence (Cy28) to generate the mature crRNAs. Csy4 specifically binds a 16-nt RNA hairpin within Cy28 with very high affinity (KD = 50 pM) and cleaves directly downstream of the five-base-pair stem element (Haurwitz et al., 2010; Sternberg et al., 2012; Lee et al., 2013). Csy4 has already been applied to cleave several engineered Cy28-containing RNAs for biotechnological applications including a model human immunodeficiency virus (HIV) (Guo et al., 2015; Nissim et al., 2014; Lee et al., 2013).

Here, we applied Csy4 to rotavirus (RV), an important animal and human pathogen of the Reoviridae family with a multisegmented dsRNA genome, which replicates in cytoplasmic viral factories named viroplasms (Desselberger, 2014; Eichwald et al., 2004). Protein access to viroplasms is mainly restricted to some viral proteins and is regulated in a still unrecognized way (Eichwald et al., 2012). Exogenous non-viral proteins and exogenous viral RNAs are usually confined outside viroplasms (Campagna et al., 2005; Silvestri et al., 2004). In the past, we succeeded to localize EGFP and mCherry into viroplasms by fusing them to either of the RV non-structural proteins NSP5 or NSP2 (Eichwald et al., 2004; Papa et al., 2019). Here, we used these two viral proteins to shuttle CRISPR-Csy4 nuclease to RV viroplasms and to obtain the nuclease-mediated site-specific genome editing of a dsRNA virus. Using different recombinant RVs (rRVs) carrying the Csy4 target sequence in diverse positions and viral segments, we report that nuclease cleavage of viral positive single-stranded RNA (+ssRNA) within viroplasms results in small sequence-dependent deletions of the targeted genomic segment (gs) in a single replication cycle.
Using RV genome editing, we labeled products of RV secondary transcription, demonstrating the main role of secondary transcription in the overall production of viral proteins.

RESULTS

Targeting CRISPR-Csy4 to RV Viroplasms

To investigate the effect of Csy4 cleavage of rRV (+)ssRNAs, we first generated different SV5-tagged Csy4 stable MA104 cell lines. MA104 cells are widely used to study RV replication (Wu et al., 2017). Csy4 was expressed at lower levels (MA-Csy4) than Csy4-H29A (MA-Csy4-H29A) (Figure S1A), a catalytically inactive variant that preserves strong substrate binding affinity (Haurwitz et al., 2010; Sternberg et al., 2012). Csy4-H29A showed diffuse cytoplasmic distribution with no viroplasm localization in rRV-infected cells (Figure S1B). As RV NSP5 and NSP2 localize to viroplasms (Eichwald et al., 2004), we fused them to SV5-tagged Csy4 or Csy4-H29A to generate the MA104 cell lines named MA-NSP5-Csy4, MA-NSP2-Csy4, and MA-NSP5-Csy4-H29A (Figure S1C). Both NSP5-Csy4 and NSP5-Csy4-H29A fusion proteins showed higher expression than the NSP2-Csy4 chimera (Figure S1C). All three fusion proteins localized to viroplasms upon RV infection, whereas they showed a diffuse cytoplasmic localization in non-infected cells (Figures S1D–S1F). In MA-NSP5-Csy4 and MA-NSP5-Csy4-H29A cells, the number and size of viroplasms, the production of viral proteins (VP2 and NSP5), and viral progeny yield did not differ from those of parental MA104 cells (Figures S2A–S2D).

Activity of Csy4 and Csy4 Fusion Variants

RNA cleavage activity of the Csy4 variants was evaluated using an EGFP reporter plasmid having the Cy28 target sequence located immediately after the ATG initiation codon (Figure S2E), and the cleavage of the reporter transcript was measured as reduction of EGFP fluorescence (Borchardt et al., 2015). In MA-NSP5-Csy4 cells, EGFP expression was abolished (a 20-fold decrease) compared to cells expressing the Csy4-H29A mutant and the parental MA104 (Figure S2E), and a 4-fold decrease in EGFP fluorescence was observed in MA-NSP2-Csy4 and MA-NSP5-C4 cells (Figure S2E). Expression levels of control EGFP lacking the Csy4 activity (Figure S2E). Genome editing of gs5 dsRNA required Csy4 localization to viroplasms, as infection of MA-NSP5-Csy4 cells, with the nuclease not targeted to viroplasms, did not affect the migration pattern of the newly made gs5 (Figures 1B and 1C). These data suggest that RV genome editing requires cleavage of the (+)ssRNA transcript by Csy4 chimeras. At 16 hpi in MA-NSP5-Csy4 cells, the newly produced rRV-gs5 dsRNAs showed that 80% of gs5 migrated as a shorter segment (Figures 1B and 1C) and further passages resulted in complete gs5 editing (Figures S3E and S3F). In contrast, infection of the parental MA104 or MA-NSP5-Csy4-H29A did not show any difference in the dsRNA migration patterns (Figures 1B and 1C). The gs5 deletion was less frequent (20% of the total gs5) in MA-NSP2-Csy4 cells (Figure S3B and S3C), consistently with their lower Csy4 activity (Figure S2E).

Viroplasm-Targeted Csy4 Nuclease Mediates Editing of RV gs5

The RV genome segment 5 (gs5) includes a single open reading frame (ORF) expressing the 60-kDa non-structural protein NSP1, which antagonizes the interferon response (Barro and Patton, 2007; Davis and Patton, 2017) but is dispensable for virus replication in cultured cells (Kanai et al., 2017; Komoto et al., 2018). We used the recently developed fully tractable RV reverse genetics system (Komoto et al., 2018; Papa et al., 2019) to generate a rRV (SA11 strain) containing a modified gs5 (rRV-gs5*). The engineered gs5* encoded NSP1 C-terminally tagged with SV5 and had the Cy28 sequence in the (+)ssRNA strand between the STOP codon and the 3’ untranslated region (3’ UTR) (Figure 1A). The genomic dsRNA migration profile of rRV-gs5* showed the expected increase in size of gs5 (gs5*, red arrow), also confirmed by sequencing, and packaging of all the other genome segments (Figures S3A and S3D). rRV-gs5*-infected MA104 cells produced the 60-kDa SV5-tagged NSP1 protein and similar hyper-phosphorylation of the RV essential protein NSP5 (Figure S3B; Eichwald et al., 2004; Papa et al., 2019). rRV-gs5* showed minimal changes in viral replication at early hours post-infection (hpi) compared to rRV-wild type (rRV-WT), even though a slightly reduced replication fitness was present at late hpi (Figure S3C). rRV-gs5* was designed to investigate the fate of virus replication upon cleavage of the (+)ssRNA transcript by Csy4 chimeras. At 16 hpi in MA-NSP5-Csy4 cells, the newly produced rRV-gs5* dsRNAs showed that 80% of gs5* migrated as a shorter segment (Figures 1B and 1C) and further passages resulted in complete gs5* editing (Figures S3E and S3F). In contrast, infection of the parental MA104 or MA-NSP5-Csy4-H29A did not show any difference in the dsRNA migration patterns (Figures 1B and 1C). The gs5* deletion was less frequent (20% of the total gs5*) in MA-NSP2-Csy4 cells (Figures 1B and 1C), consistently with their lower Csy4 activity (Figure S2E).

Figure 1. CRISPR-Csy4 Editing of RV gs5

(A) Scheme of (+)ssRNA of WT gs5 and gs5*, whose sequence is shown below; red arrowhead indicates Csy4 cleavage site. The 16-nt Csy4 binding site in Cy28 is highlighted in blue.

(B and C) Electrophoretic pattern (B) and quantification (C) of dsRNA genome of rRV-WT or rRV-gs5* derived from indicated cells at 16 hpi. Red arrow, gs5*; red asterisks, edited gs5*. Genomic segments are indicated on the left. For (C), data are means ± SEM.

(D) Sequence of edited gs5*ΔΔ27 and gs5*Δ42.

(e) RT-PCR products of gs5*ΔΔ27 (174 bp) and gs5*ΔΔ27 (159 bp) and gs11 (as control) from rRV-gs5*-infected cells at 7 hpi.

(F) Relative frequencies of gs5*ΔΔ27 and gs5*ΔΔ24 (45 clones for MA-NSP5-Csy4 and 30 clones for MA-NSP2-Csy4).
gs5* editing outcomes, with the Δ42 deletion slightly more frequent (55% NSP5-Csy4, 60% NSP2-Csy4) than the Δ27 (45% NSP5-Csy4, 40% NSP2-Csy4) (Figure 1F).

The 3′ end of both deletions was always compatible with the Csy4 cleavage site (Figure 1D), consistent with Csy4 nuclease activity. In both cases, a G nucleotide was present immediately upstream of the deletions. The two newly edited rRVs gs5*-Δ42 and gs5*-Δ27 were independently packaged into newly made viral particles and were not further mutated after 6 consecutive passages in MA104 cells, as confirmed by sequencing (Figures S3H and S3I). Consistent with the Csy4 high specificity (Haurwitz et al., 2012, 2010; Stemberg et al., 2012; Lee et al., 2013), deletions were observed exclusively in the rRV gs5* containing the Csy4 target sequence (Figure 1B; Figure S3H) and rRV-WT equally replicated in cells with active or inactive Csy4 fusions, suggesting a lack of Csy4 off-target editing of rRV double-stranded genome segments (Figure S3G).

To investigate whether gs5* editing was dependent on the position of the nuclease target sequence, we generated rRV-gs5*/284 carrying, similarly to gs5*, the same Cy28 sequence located in this case 284 nts (instead of 95) upstream of the 3′ end (Figure S4A). As NSP1 is dispensable for viral replication in RV-infected cells (Komoto et al., 2018), NSP1 truncation did not affect rescue of the rRV-gs5*284.

The dsRNA profile and sequencing showed that 95% of gs5*/284 was edited in MA-NSP5-Csy4 cells but not in parental MA104 or MA-NSP5-Csy4-H29A cells (Figures S4B and S4C). The edited gs5*/284 segments contained, with similar frequency, the same Δ42 (66%) and Δ27 (34%) deletions observed for gs5* (Figure S4D); these findings ruled out that the Cy28 position affects the editing outcome.

CRISPR-Csy4 Editing of RV gs7 and gs10 and Multiplex Editing

To investigate editing events in other genome segments, we first obtained a rRV with a modified gs7 (rRV-gs7*), in which NSP3 was C-terminally SV5 tagged and the Cy28 sequence inserted 28 nt from Csy4 cleavage site (Figure 1D), consistent with Csy4 nuclease activity. In both cases, a G nucleotide was present immediately upstream of the deletions. The two newly edited rRVs gs5*-Δ42 and gs5*-Δ27 were independently packaged into newly made viral particles and were not further mutated after 6 consecutive passages in MA104 cells, as confirmed by sequencing (Figures S3H and S3I). Consistent with the Csy4 high specificity (Haurwitz et al., 2012, 2010; Stemberg et al., 2012; Lee et al., 2013), deletions were observed exclusively in the rRV gs5* containing the Csy4 target sequence (Figure 1B; Figure S3H) and rRV-WT equally replicated in cells with active or inactive Csy4 fusions, suggesting a lack of Csy4 off-target editing of rRV double-stranded genome segments (Figure S3G).

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The dsRNA profile and sequencing showed that 95% of gs5*/284 was edited in MA-NSP5-Csy4 cells but not in parental MA104 or MA-NSP5-Csy4-H29A cells (Figures S4B and S4C). The edited gs5*/284 segments contained, with similar frequency, the same Δ42 (66%) and Δ27 (34%) deletions observed for gs5* (Figure S4D); these findings ruled out that the Cy28 position affects the editing outcome.

Csy4-Mediated Editing to Study RV Secondary Transcription

A hallmark of RV biology is the transcriptional activity of intermediate viral particles (Desselberger, 2014). Mature RVs are triple layered particles (TLPs) that after entry lose the outer layer and become transcriptionally active double layered particles (DLPs). DLPs generate transcripts used as templates for the dsRNA synthesis and as mRNAs to produce viral proteins. During virus replication, newly made DLPs are assembled within viroplasms and are supposed to start a new wave of transcription called secondary transcription before budding into the endoplasmic reticulum (ER) and maturing into TLPs (Estes and Greenberg, 2013).

To what degree secondary transcription and the consequent translation contribute to the overall production of viral proteins remains unknown (López et al., 2005). Csy4 editing of the RV genome is an approach to study secondary transcription by monitoring the expression of edited genome segments in a single replication cycle. Upon transcription, the newly generated edited segments produce mRNAs different from the one of the original infective particles.

To detect proteins produced exclusively by edited mRNA, we engineered rRV-gs5*–HA, containing a short 23-nt Csy4 target
Figure 3. Csy4-Mediated Detection of Secondary Transcription

(A) Scheme of gs5*-HA (+)ssRNA, showing the in-frame Δ36 deletion producing NSP1-HA and the Δ21 deletion. C-terminal amino acid sequence and expected molecular masses of NSP1-SV5 and NSP1-SV5-HA are shown. Arrowhead indicates the Csy4 cleavage site.

(legend continued on next page)
sequence (Cy23) (Sternberg et al., 2012), and the hemagglutinin (HA)-tag coding sequence downstream of the NSP1-SV5 STOP codon (Figure 3A). gs5*-HA was designed to express HA-tagged isoforms of NSP1-SV5 after in-frame deletions of at least 27 nts, which eliminate the STOP codon (Figure 3A). In MA-NSP5-Csy4 cells, rRV-gs5*-HA was edited to 36- and 21-nt deletions, with a total editing efficiency of 80% at 16 hpi (Figures 3B–3D). The gs5*-HA Δ36 transcript was detected from 5 hpi and became more abundant (63% versus 36%) than the non-edited gs5*-HA at 9 hpi (Figure 3E). The Δ36 editing was expected to result in a 61.4-kDa HA-tagged NSP1-SV5 protein, whereas the Δ21 and the non-edited gs5*-HA encoded a 60.4-kDa NSP1-SV5 isoform (Figure 3A), Western blot analysis detected the HA-tagged NSP1-SV5 in MA-NSP5-Csy4 cells from 7 hpi (Figure 3F) but not in MA-NSP5-Csy4-H29A cells, in which gs5*-HA was not edited (Figures 3B, 3C, and 3F). As only newly made particles can contain an edited gs5*-HA, expression of NSP1-SV5-HA allowed monitoring translation of the newly produced transcripts derived from secondary transcription. Time course analysis using an anti-SV5 antibody, which recognizes both HA-edited and non-edited NSP1, showed that from 5 to 9 hpi, production of NSP1-SV5-HA progressively increased, becoming predominant (73%) at 9 hpi (Figures 3G and 3H). The contribution of the secondary transcription is likely underestimated because of the incomplete editing of gs5*-HA and the generation of the not HA-labeled Δ21 isoform. Encouraged by this result, we produced rRV-gs5*-HA-EGFP* (Figure 4A), which would express EGFP only upon Csy4-mediated in-frame deletion of at least 27 nts in the engineered gs5. The two G nucleotides upstream Cy23 involved in the formation of the Δ21 and Δ27 deletions were mutated to A. The size of gs5*-HA-EGFP* was too large to detect the edited segment by dsRNA gel migration (Figure S6A), but by Sanger sequencing decomposition (Brinkman et al., 2014), the total editing efficiency was 9.6% and the only significant editing event was the in-frame Δ36 deletion (Figure S6B). Cytotluorimetric analysis (Figure 4B) and confocal fluorescence images (Figures 4C and 4D; Figure S6C) of NSP1-EGFP spots (Murphy and Arnold, 2019) showed 15% of EGFP-positive cells in rRV-infected MA-NSP5-Csy4 at 12 hpi. EGFP was detected only in cells sustaining viral replication (anti-NSP2) (Figure 4C) and was compromised upon inhibition of viroplasm formation by the proteasome inhibitor MG132 (Contin et al., 2011; Figures 4E and 4F; Figure S6E). EGFP-positive cells were absent in control-infected MA-NSP5-Csy4-H29A (Figure S6F). Following the kinetics of NSP1-EGFP by live-cell imaging, NSP1-EGFP was detected from 6 hpi and increased, reaching a plateau in the number of florescent cells from 13 hpi (Figures 4D and 4F; Video S1). Similar results were obtained scoring the total number of green spots or average number of NSP1-EGFP spots per fluorescent cell (Figure 4E; Figure S6D). Taken together, our data highlight the importance of secondary transcription in RV replication.

**DISCUSSION**

CRISPR nucleases have been exploited to edit several viral DNA genomes or their DNA replication intermediates, including those of hepatitis B virus, herpesviruses, human papilloma virus, HIV-1, and retroviruses (de Buhr and Lebbink, 2018; Chen et al., 2018; van Diemen et al., 2016; Schiwon et al., 2018; Wang et al., 2016a, 2016b; Yoshiha et al., 2019).

Although RNA targeting nucleases, such as CRISPR-Cas13 and CRISPR-Csy4, and Francisella novicida Cas9 have been used to disrupt viral ssRNAs of lymphocytic choriomeningitis vi-

ruses, influenza A virus, vesicular stomatitis virus, hepatitis C virus, HIV-1, and, lately, SARS-CoV-2 (Abbott et al., 2020; Freije et al., 2019; Guo et al., 2015; Price et al., 2015), there are no reports showing the use of CRISPR nucleases or other homing nucleases for targeting and editing viruses with a dsRNA genome. Among them, the peculiar replication mechanism of RV in hardly accessible viral factories poses several challenges for the effective deployment of nucleic acid editing tools (Silvestri et al., 2004). In addition, there are no known repair mechanisms for a cleaved RV genome nor sequence-specific programmable CRISPR nucleases targeted to dsRNA published yet.

We paved the way for successful nuclease-mediated genome editing of dsRNA viruses by targeting the small and highly specific P. aeruginosa CRISPR-Csy4 to cleave the (+)ssRNA intermediates formed within viroplasms, during RV dsRNA genome replication (Borodavka et al., 2017; Patton et al., 2006). This was obtained by fusing Csy4 to the viroplasm-localizing viral proteins NSP5 and NSP2 (Eichwald et al., 2004, 2012; Fabbretti et al., 1999). Three of the 11 RV gss (gs5, gs7, and gs10) were engineered to contain the Csy4 target sequence in different locations. Csy4 activity produced discrete deletions in the RV genome, ranging from 21 to 45 nt with efficiency up to 95% in a single round of infection, increasing to 100% in subsequent rounds. Csy4 was very effective also for multiplex editing of at least three different RV gss (gs5, gs7, and gs10), offering the opportunity to simultaneously induce in vivo editing isoforms in different viral genes. Editing was observed exclusively on genome segments carrying the target sequence, indicating that Csy4 fusions with NSP5 or NSP2 had no obvious off-target activity to the WT RV genome.

The presence of editing only with viroplasm-localized Csy4 suggests that it occurs within viroplasms and involves the RNA

**B** Electrophoretic pattern of rRV-gs5*-HA genome replicated in the indicated cells at 16 hpi. Red arrow and asterisk indicate, respectively, non-edited and edited gs5*-HA.

**C** Quantification of gs5*-HA editing; means ± SEM (n = 3).

**D** Frequency of Δ21 and Δ36 deletions (36 clones) from MA-NSP5-Csy4 cells at 9 hpi.

**E** Transcript-specific RT-PCR of gs5*-HA (213 bp) and gs5*-HA-Δ36 (177 bp) from MA-NSP5-Csy4 cells at the indicated hpi. The % Δ36 transcripts are means of n = 3 experiments.

**F** Western blot time course of NSP1-SV5-HA (detected with anti-HA) and VP2 from the indicated cells infected with rRV-gs5*-HA. β-actin was used as control.

**G** is as in (F) using anti-SV5. Red and black asterisks indicate HA-tagged and non-tagged NSP1-SV5, respectively.

**H** Relative expression of the two NSP1 isoforms as in (G); means ± SEM (n = 3).
The recent development of different reverse genetics protocols for RV represents a powerful tool to investigate virus biology (Kanai et al., 2017; Komoto et al., 2018; Papa et al., 2019). The nuclease-mediated editing of dsRNA virus could overcome current limitations of reverse genetics systems or be combined with them for new applications, for example, producing many edited virus variants unachievable directly by reverse genetics.

Overall, our data represent the description of nuclease-mediated editing of a dsRNA virus genome. The in vivo generation of recombinant RVs with edited genomes paves the way for harnessing this tool to study different aspects of viral replication, as well as its use for the identification of therapeutic drugs targeting the assembly of DLPs or other pathways associated with secondary transcription.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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Figure 4. Csy4-Mediated Live-Cell Imaging of RV Secondary Transcription and Translation

(A) Scheme of the gsk5*-HA-EGFP* (+)ssRNA showing the 136 deletion required for EGFP expression.
(B) The % of EGFP-positive cells non-infected or infected with RV-gsk5*-HA-EGFP* (MOI, 5) determined by cyttofluorimetry at 12 hpi.
(C) Immunofluorescence of cells infected as in (B). Infected cells and viroplasms were detected with anti-NSP2 (red). Scale bar, 13 μm.
(D) Time course of EGFP fluorescence from 5:40 to 9:20 hpi of RV-gsk5*-HA-EGFP infected MA-NSP5-Csy4 cells. Scale bar, 20 μm. In (C) and (D), nuclei stained with 4',6-diamidin-2-fenilindolo (DAPI) (blue).
(E) Live-cell imaging quantification of the total number of NSP1-EGFP spots in MA-NSP5-Csy4 cells (+/− MG132) and MA-NSP5-Csy4-H29A cells infected with RV-gsk5*-HA-EGFP* (MOI, 5).
(F) Percentage of EGFP fluorescent cells (all cells scoring a number of NSP1-EGFP positive spots of ≥1) as in (E).
Supplemental Information

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.108205.

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Author Contributions

Experiments were mainly conducted by G. Papa with the help of L.V. Live-cell imaging analysis was carried out by G. Papa, L.B., E.S., and M.G. O.R.B., G. Papa, and G. Petris planned the project and wrote the manuscript. All authors read, corrected, and approved the final manuscript.

Declaration of interests

The authors declare no competing interests.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| anti-SV5 mAb | Life Technologies | Cat# R960-25; RRID:AB_2556564 |
| anti-HA mAb clone HA-7 | Sigma-Aldrich | Cat# H3663; RRID:AB_262051 |
| anti-NSP5 guinea pig serum | Contin et al., 2010 | N/A |
| anti-NSP5 roTag mAb | Petris et al., 2014 | N/A |
| anti-VP2 guinea pig serum | Eichwald et al., 2004 | N/A |
| HRP-conjugated goat anti-guinea pig | Jackson ImmunoResearch | Cat# 106-035-003; RRID:AB_2337402 |
| HRP-goat anti-mouse IgG | KPL | Cat# 5220-0341 |
| HRP-conjugated anti-GAPDH mAb clone sc-47724 | Santa Cruz Biotechnology | Cat# sc-47724; RRID:AB_627678 |
| Mouse HRP-conjugated anti-actin mAb clone AC-15 | Sigma-Aldrich | Cat# A1978; RRID:AB_476692 |
| **Bacterial and Virus Strains** | | |
| rRV-wt | This paper | N/A |
| rRV-gs5* | This paper | N/A |
| rRV-gs7* | This paper | N/A |
| rRV-gs10* | This paper | N/A |
| rRV-gs5*-HA | This paper | N/A |
| rRV-gs5*/284 | This paper | N/A |
| rRV-gs5*-HA-EGFP* | This paper | N/A |
| rRV-gs5*-GA | This paper | N/A |
| E.coli DH5α | Thermo Fisher Scientific | Cat# 18265017 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| RNAzol | Sigma-Aldrich | Cat# R4533 |
| **Experimental Models: Cell Lines** | | |
| MA104 | ATCC | Cat# CRL-2378.1 |
| MA-NSP5-Csy4 | This paper | N/A |
| MA-NSP5-Csy4-H29A | This paper | N/A |
| MA-Csy4 | This paper | N/A |
| MA-Csy4-H29A | This paper | N/A |
| MA-NSP2-Csy4 | This paper | N/A |
| MA-NSP2-mCherry | Papa et. al, 2019 | N/A |
| BHK-T7 | Naoto Ito | N/A |
| **Oligonucleotides** | | |
| Primers for plasmid construction, see Table S1 | This paper | N/A |
| **Recombinant DNA** | | |
| pT7-VP1-SA11 | Kanai et al., 2017 | N/A |
| pT7-VP2-SA11 | Kanai et al., 2017 | N/A |
| pT7-VP3-SA11 | Kanai et al., 2017 | N/A |
| pT7-VP4-SA11 | Kanai et al., 2017 | N/A |
| pT7-VP7-SA11 | Kanai et al., 2017 | N/A |
| pT7-NSP1-SA11 | Kanai et al., 2017 | N/A |
| pT7-NSP2-SA11 | Kanai et al., 2017 | N/A |
| pT7-NSP3-SA11 | Kanai et al., 2017 | N/A |
| pT7-NSP4-SA11 | Kanai et al., 2017 | N/A |
| pT7-NSP5-SA11 | Kanai et al., 2017 | N/A |

(Continued on next page)
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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pT7-gs5*            | This paper | N/A |
| pT7-gs5*-GA         | This paper | N/A |
| pT7-gs7*            | This paper | N/A |
| pT7-gs10*           | This paper | N/A |
| pT7-gs5*/284        | This paper | N/A |
| pT7-gs5*-HA         | This paper | N/A |
| pT7-gs5*-HA-EGFP*   | This paper | N/A |
| pPB-MCS             | Papa et. al. 2019 | N/A |
| pPB-NSP5-SV5-Csy4   | This paper | N/A |
| pPB-SV5-Csy4        | This paper | N/A |
| pPB-NSP5-SV5-Csy4-H29A | This paper | N/A |
| pPB-SV5-Csy4-H29A   | This paper | N/A |
| pPB-NSP2-SV5-Csy4   | This paper | N/A |

Software and Algorithms

- FlowJo Software: Becton, Dickinson and Company 2019 RRID:SCR_008520
- GraphPad Prism 7: GraphPad RRID:SCR_002798
- TIDE software: Brinkman et al., 2014 N/A
- Image Lab Software 6.0.1: Bio-Rad RRID:SCR_014210
- Columbus analysis software: PerkinElmer N/A

**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents, which may require a completed Materials Transfer Agreement, should be directed to and will be fulfilled by the Lead Contact, Oscar R. Burrone (burrone@icgeb.org).

**Materials Availability**
Processed data associated with this study are present in the paper. Other new material associated with this study are available from the lead author upon request.

**Data and Code Availability**
This study did not generate any unique datasets or code.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines**
MA104 (embryonic African green monkey kidney cells ATCC CRL-2378.1) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies) and 50 μg/ml gentamycin (Biochrom AG).

MA104-NSP5-Csy4 (MA-NSP5-Csy4), MA104-NSP5-Csy4-H29A (MA-NSP5-Csy4-H29A), MA104-Csy4 (MA-Csy4), MA104-Csy4-H29A (MA-Csy4-H29A), MA104-NSP2-Csy4 (MA-NSP2-Csy4), MA104-NSP2-mCherry (MA-NSP2-mCherry) stable transfectant cell lines were grown in DMEM containing 10% FBS, 50 μg/ml gentamycin and 5 μg/ml puromycin (Sigma-Aldrich).

BHK-T7 cells (Baby Hamster Kidney stably expressing T7 RNA polymerase) were cultured in Glasgow medium supplemented with 5% FBS, 10% Tryptose Phosphate Broth (TPB) (Sigma-Aldrich), 50 μg/ml gentamycin, 2% Non-Essential Amino Acids (NEAA) and 1% Glutamine.

**Plasmids**
RV plasmids pT7-VP1-SA11, pT7-VP2-SA11, pT7-VP3-SA11, pT7-VP4-SA11, pT7-VP6-SA11, pT7-VP7-SA11, pT7-NSP1-SA11, pT7-NSP2-SA11, pT7-NSP3-SA11, pT7-NSP4-SA11, and pT7-NSP5-SA11 (Kanai et al., 2017; Komoto et al., 2018) were used to rescue recombinant RVs by reverse genetics.

pPB-NSP5-SV5-Csy4 and pPB-SV5-Csy4 plasmids were obtained from a GenParts DNA fragment (Genscript) containing NSP5-SV5-Csy4 and SV5-Csy4 and inserted in the pPB-MCS vector (Papa et al., 2019) using BamHI-Xmal restriction enzymes sites.
pPB-NSP5-SV5-Csy4-H29A and pPB-SV5-Csy4-H29A plasmids carrying two nucleotides substitutions C82G and A83C in the Csy4 ORF were generated by QuikChange II Site-Directed Mutagenesis (Agilent Technologies) from the pPB-NSP5-SV5-Csy4 and pPB-SV5-Csy4 respectively using Csy4-H29A-FOR and Csy4-H29A-REV primers (Table S1).

A GenParts DNA fragment containing the NSP2-SV5-Csy4 was inserted into pPB-MCS vector using NotI-EcoRI restriction enzymes sites to obtain the pPB-NSP2-SV5-Csy4.

pEGFP-Cy28 was generated inserting Cy28 after the ATG into the pEGFP-C1 plasmid (Addgene) using the pEGFP-Cy28-FOR and pEGFP-Cy28-REV primers (Table S1) for QuikChange II Site-Directed Mutagenesis.

pT7-gs5* was generated inserting a GenParts DNA fragment into the pT7-NSP1-SA11 using PacI-BamHI restriction enzymes sites. This GenParts fragment contains the SV5 tag and Cy28 at position 1518 of the gs5. pT7-gs5*-GA was synthesized as above with a point mutation of G into an A at position 1580.

pT7-gs7* was generated inserting a GenParts DNA fragment into the pT7-NSP3-SA11 using SnaBI-SacI restriction enzymes sites. The GenParts fragment contains the SV5 tag and Cy28 at position 970 of the gs7.

For the generation of pT7-gs5*/284, a GenParts DNA fragment containing the SV5 tag and Cy28 was inserted into pT7-NSP1-SA11 using MfeI-BamHI restriction enzymes sites. pT7-gs5*-HA was obtained inserting a GenParts DNA fragment including the SV5 tag, Cy23 and the HA tag in plasmid pT7-NSP1-SA11 using MfeI-BamHI restriction enzymes sites. pT7-gs5*-HA-EGFP* was generated cloning a GenParts DNA fragment into the pT7-NSP1-SA11 using MfeI-BamHI. The GenParts contains the SV5 tag, Cy23, the HA tag and the EGFP ORF upstream of the 3’UTR of gs5.

**METHOD DETAILS**

**Generation of stable cell lines**

MA-NSP2-mCherry cells were previously described (Papa et al., 2019). MA-NSP5-Csy4, MA-NSP5-Csy4-H29A, MA-Csy4, MA-Csy4-H29A, MA-NSP2-Csy4 were similarly generated by PiggyBac Technology (Papa et al., 2019; Yusa et al., 2011). Briefly, MA104 cells (10^5) were seeded in a 12 Multi-well plate, transfected the next day with the pCMV-HyPBase (0.5 μg) and lysed by freeze-thawing. 300 μl of the lysate was transferred to fresh MA104 wt cells, further cultured at 37°C for 4 days in FBS-free DMEM supplemented with 0.5 mg/ml puromycin for selection.

**Rescue of recombinant RVs from cloned cDNAs**

To rescue recombinant RV strain SA11 (rRV-wt), monolayers of BHK-T7 cells (4 x 10^5) cultured in 12-well plates were co-transfected using 2.5 μL of TransIT-LT1 transfection reagent (Mirus) per microgram of DNA plasmid. Each mixture comprised 0.8 μg of SA11 rescue plasmids: pT7-VP1, pT7-VP2, pT7-VP3, pT7-VP4, pT7-VP6, pT7-VP7, pT7-NSP1, pT7-NSP3, pT7-NSP4, and 2.4 μg of pT7-NSP2 and pT7-NSP5 (Kanai et al., 2017; Komoto et al., 2018). Furthermore 0.8 μg of pcDNA3-NSP2 and 0.8 μg of pcDNA3-NSP5, encoding NSP2 and NSP5 proteins, were co-transfected to increase rescue efficiency (Papa et al., 2019). To rescue recombinant RVs having modified genomic segments, pT7-gs5*, pT7-gs5*-GA, pT7-gs5*-284, pT7-gs5*-HA, pT7-gs5*-HA-EGFP*, pT7-gs7*, pT7-gs10* plasmids were used instead of pT7-NSP1-SA11, pT7-NSP3-SA11, pT7-NSP4-SA11 respectively.

**Replication kinetics of recombinant viruses**

MA104 cells were seeded into 24-well plates, infected with rRVs at MOI of 0.5 for multi-step growth curve experiments. Cells were harvested after 6, 12, 24, 36 hours post infection and lysed by freeze-thawing three times and activated with trypsin (1 mg/ml final concentration) (T0303-Sigma Aldrich) and lysed. 300 μL of the lysate was transferred to fresh MA104 wt cells, further cultured at 37°C for 4 days in FBS-free DMEM supplemented with 0.5 μg/ml trypsin until a clear cytopathic effect was visible. The modified genome segments of rescued recombinant rotaviruses were sequenced.

**EGFP-plasmid based transcript cleavage assay**

MA104, MA-NSP5-Csy4, MA-NSP5-Csy4-H29A, MA-NSP2-Csy4, MA-Csy4, MA-Csy4-H29A were seeded at a density of 2x10^5 in a 12 Multi-well plate and transfected with 0.5 μg of pEGFP-Cy28 or pEGFP plasmid using Lipofectamine 3000 following manufacturers' instructions.
instructions. The cells were imaged 24 hours post-transfection using a Nikon Eclipse (Ti-E, Nikon, Japan). The images were acquired and GFP expression was analyzed and quantified using ImageJ software.

**Western blot**

Samples from rRV-infected cells were run in reducing 10% or 7%–15% gradient SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and blocked in a 5% milk solution in PBS (PBS-milk) for 30 minutes. SV5-tagged proteins were detected using mouse anti-SV5 mAb (1:5000, Life Technologies); HA-tagged proteins were detected by anti-HA mAb (clone HA-7, 1:5000, Sigma-Aldrich); NSP5 and VP2 proteins were detected using anti-NSP5 guinea pig serum (1:5000) or anti-NSP5 roTag mAb and anti-VP2 guinea pig serum (1:2000) (Contin et al., 2010; Eichwald et al., 2004; Petris et al., 2014). As secondary antibodies were used HRP-conjugated goat anti-guinea pig (1:10000; Jackson ImmunoResearch) or goat anti-mouse IgG antibodies (1:10000; KPL).

Mouse HRP-conjugated anti-GAPDH mAb (clone sc-47724, Santa Cruz Biotechnology, 1:1000) and Mouse HRP-conjugated anti-actin mAb (clone AC-15, Sigma-Aldrich, 1:40000) were used as loading controls. Membranes were developed by Enhanced ChemiLuminescence System (Pierce ECL-Western blotting system, ThermoFisher-Pierce). Quantification analysis of bands were carried out using ImageLab 6.0.1 (Bio-Rad).

**Immunofluorescence**

Immunofluorescence experiments were performed using µ-Slide 8 Well Chamber Slide-well (iBidi GmbH). Cells were washed three times with PBS, treated with paraformaldehyde 3.7% in PBS for 15 minutes, cells were then incubated with 0.1% Triton X-100 (Sigma) in PBS for 5 minutes followed by incubation with 1% BSA for 30 minutes. For the detection of proteins, cells were incubated with primary antibody diluted in 1% PBS-BSA for 1 hour, washed three times with PBS and then incubated with secondary antibody for 45 minutes. Nuclei were then stained with ProLong Diamond Antifade Mountant with DAPI (Thermo Scientific) and the slides were imaged using a confocal setup (Zeiss Airyscan equipped with a 63x, NA = 1.3 objective). Antibodies were used at the following dilutions: mouse anti-SV5 mAb (1:1000); anti-NSP5 guinea pig serum 1:1000; anti-NSP2 guinea pig serum 1:200 (Contin et al., 2010; Eichwald et al., 2004); Alexa Fluor 488-conjugated anti-mouse, 1:500 (Life Technologies), and TRITC-conjugated anti-guinea pig, 1:500 (Life Technologies). Quantification analysis were performed using ImageJ software.

**Electrophoresis of viral dsRNA genomes**

Total RNA was extracted with RnaZol® (Sigma-Aldrich) from cells infected at MOI of 5 and lysed at 16 hours post infection. The RNA was run on a 10% poly-acrylamide denaturing gels (PAGE) for 2 hours at 180 Volts. The gel was than stained with ethidium bromide (1 µg/ml) and the dsRNA pattern was visualized using the ChemiDoc Imaging System (Gray and Desselberger, 2000). Quantification analysis of bands were carried out using ImageLab 6.0.1.

**dsRNA extraction from polyacrylamide gels**

The bands of interest from a polyacrylamide gel were cut, crushed with a pipette and placed in 1.5 mL tubes. 300 µL of elution buffer (0.5 M NH4OAc, 0.1% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) was added, mixed for 2 hours and then centrifuged 3 times at 5000 g for 1 minute to pellet the acrylamide residues. The RNA was precipitated with 1 volume of 3M NaOAc pH 5.2 and 3 volumes of 100% EtOH, centrifuged at 16000 g for 30 minutes, washed twice with 2 volumes of 70% EtOH, and resuspended in RNase free water. The dsRNA was denaturated, retro-transcribed, PCR amplified and cloned into a pcDNA 3.1 plasmid (Invitrogen) for sequencing.

**Cytofluorimetry**

MA-NSP5-Csy4 and MA-NSP5-Csy4-H29A were infected with rRV-gs5*-HA-EGFP* at MOI of 5 and collected 12 hours post infection. Cells were trypsinised, washed twice with PBS, centrifuged for 5 minutes at 200 g and resuspended in PBS. EGFP fluorescence was analyzed using a FACS Calibur cytofluorimeter (BD Biosciences).

**Live Cells imaging**

MA-NSP5-Csy4 and MA-NSP5-Csy4-H29A cells (2×10³) were seeded into CellCarrier-96 Ultra Microplates and infected with rRV-gs5*-HA-EGFP* at MOI of 5. After 1 hour, cells were stained with DAPI and live-cell imaging was started. Cells were imaged at 40x magnification (Olympus 40x NA 0.95) with a PerkinElmer Operetta High content microscope under controlled environmental conditions (37°C, 5% CO2). Image acquisition was performed with intervals of 20 minutes for a total of 12 hours. Image were analyzed using Columbus analysis software (PerkinElmer) and total cell number, number of EGFP positive spots per cell and % of EGFP positive cells (number of NSP1-EGFP positive spots ≥ 1) were calculated for each time point at single cell level.

**Sequencing of edited RV genomic segments**

Extracted RNA was subjected to RT-PCR using NSP1-FOR and NSP1-REV primers for gs5* and gs5*HA. gs10* and gs7* were amplified using gs10*-FOR, gs10*-REV, gs7*-FOR, gs7*-REV respectively (Table S1). All primers contain HindIII and XhoI restriction enzyme sites at the 5’ and 3’ end, respectively. PCR products were either sequenced and analyzed using the TIDE software.
(Brinkman et al., 2014) or gel purified before HindIII-XhoI digestion and cloning into pcDNA 3.1 plasmid. Colonies-derived PCR products were gel purified and sequenced.

**PCR using splicing primers**
Detection of specific edited RNAs, such as gs5*-Δ42 or gs5*-Δ27 was carried out by RT/PCR with primers gs5*-FOR and gs5*-Δ42-REV or gs5*-FOR and gs5*-Δ27-REV, respectively (Table S1). PCR amplification of gs5*-HA (213 bp) and gs5*-HA-Δ36 (177 bp) was performed using gs5*-HA-FOR and gs5*-HA-REV primers (Table S1). The latter allows specific amplification of only gs5*-HA and gs5*-HA-Δ36. PCR products were run on 2.5% agarose gel in TBE 1X (45 mM Tris-borate/1 mM EDTA) for 20 minutes at 120V. Quantification analysis of bands were carried out using ImageLab 6.0.1.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical Analysis**
Unless otherwise indicated, statistical analyses were performed with the Student’s t test using GraphPad Prism 7 software (GraphPad). The number of experiments or (biological) replicates (n) used for the statistical evaluation of each experiment is indicated in the corresponding figure legends. The data are plotted as a mean ± SD or SEM as indicated.