An optimized reverse genetics system suitable for efficient recovery of simian, human and murine-like rotaviruses

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ABSTRACT

An entirely plasmid-based reverse genetics (RG) system was recently developed for rotavirus (RV), opening new avenues for in-depth molecular dissection of RV biology, immunology, and pathogenesis. Several improvements to further optimize the RG efficiency have now been described. However, only a small number of individual RV strains have been recovered to date. None of the current methods have supported the recovery of murine RV, impeding the study of RV replication and pathogenesis in an in vivo suckling mouse model. Here, we describe useful modifications to the RG system that significantly improve rescue efficiency of multiple RV strains. In addition to the 11 RVA segment-specific (+)ssRNAs, a chimeric plasmid was transfected, from which the capping enzyme NP868R of African swine fever virus (ASFV) and the T7 RNA polymerase were expressed. Secondly, a genetically modified MA104 cell line was used in which several compounds of the innate immune were degraded. Using this RG system, we successfully recovered the simian RV RRV strain, the human RV CDC-9 strain, a reassortant between murine RV D6/2 and simian RV SA11 strains, and several reassortants and reporter RVs. All these recombinant RVs were rescued at a high efficiency (≥80% success rate) and could not be reliably rescued using several recently published RG strategies (<20%). This improved system represents an important tool and great potential for the rescue of other hard-to-recover RV strains such as low replicating attenuated vaccine candidates or low cell culture passage clinical isolates from humans or animals.

IMPORTANCE

Group A rotavirus (RV) remains as the single most important cause of severe acute gastroenteritis among infants and young children worldwide. An entirely plasmid-based reverse genetics (RG) system was recently developed opening new ways for in-depth molecular study of RV. Despite several improvements to further optimize the RG efficiency, it has been reported

that current strategies do not enable the rescue of all cultivatable RV strains. Here, we described helpful modification to the current strategies and established a tractable RG system for the rescue of the simian RRV strain, the human CDC-9 strain and a murine-like RV strain, which is suitable for both *in vitro* and *in vivo* studies. This improved RV reverse genetics system will facilitate study of RV biology in both *in vitro* and *in vivo* systems that will facilitate the improved design of RV vaccines, better antiviral therapies and expression vectors.

INTRODUCTION

Despite the introduction of multiple safe and effective rotavirus (RV) vaccines such as the widely licensed Rotarix and RotaTeq vaccines, species A RVs remain the single most important cause of severe acute gastroenteritis among infants and young children worldwide (1). RVs are responsible for between 128,000 and 215,000 deaths each year, primarily in developing countries (2). RVs belong to the *Reoviridae* family, comprising a variety of icosahedral, nonenveloped multi-segmented double-stranded (ds) RNA viruses. RVs have three concentric layers of protein that surround an RNA genome, which contains 11 dsRNA segments encoding six structural (VP1-VP4, VP6, VP7) and six nonstructural (NSP1-NSP6) proteins (3).

While our understanding of RV epidemiology, clinical course, pathophysiology, immunology and replication strategy has increased substantially over the last 40+ years, important questions about complex and multifaceted processes such as host-range restriction, determinants of virulence and immune correlates of protection are still poorly understood (3-6). However, due to the recent introduction of an efficient reverse genetics (RG) system for RVs (7), we now have a

70 powerful investigative tool to effectively explore these and other long standing questions
71 regarding RV biology. Kanai and collaborators first established an entirely plasmid-based RG
72 system for the simian RV SA11 strain (7). This virus was originally isolated in 1958 (8), is very
73 well adapted to cell culture, and has been used as a prototype strain in many RV studies over
74 the ensuing years (9). While it is capable of infecting a variety of animals including primates (10)
75 and mice (11), it is not highly pathogenic and does not spread efficiently from host-to-host in
76 experimental animal systems. In the Kanai RG system, 11 plasmids encoding each of the SA11
77 gene segments are co-transfected with helper plasmids encoding the reovirus fusion-associated
78 small transmembrane (p10 FAST) protein and the vaccinia virus capping enzymes into BHK-T7-
79 expressing cells. Following an MA104 cell overlay and inoculation of the mixed cell lysates from
80 the transfected BHK-T7 cells onto fresh MA104 cells, infectious RVs are recovered (7). After
81 this report, there have been several additional descriptions of the RG system adapted for the
82 rescue of two human RV strains: KU (12) and Odelia (13). The rescue of several recombinant
83 viruses based on an SA11 genetic backbone, some carrying heterologous RV gene segments
84 encoding VP4 and VP7 (14, 15), others carrying engineered segments 5 or 7 with associated
85 reporter genes have also been reported (7, 16-18). All these reports described some
86 modifications of the original rescue protocol that purported to further improve the efficiency
87 and/or utility of the original RG system.

88 The suckling mouse is currently the best-established and widely used small animal model
89 system for studying RV infection *in vivo* (4, 19, 20). This model faithfully recapitulates several
90 aspects of RV infection in human infants: suckling mice are highly susceptible to murine RV
91 infection and develop severe diarrhea following low titer inoculating doses of homologous
92 murine RVs, while immuno-competent adult mice, like adult humans are more resistant to RV
93 induced diarrheal disease. Of note, heterologous RVs (non-murine RV such as the SA11 strain)

can infect suckling and adult mice but replicate poorly, do not cause diarrhea when administered at low titers, and do not spread efficiently from host to host. In infant mice, cultured intestinal epithelial cells, and human organoid culture systems an important part of the host ability to restrict RV replication efficiently is mediated by innate immune response (21-23). Furthermore, characterization of the replication of murine and non-murine RV strains in the suckling murine host and in cultured cells has allowed the identification of several highly effective mechanisms that homologous viruses use to evade the host innate antiviral response (4, 20, 24-26).

Murine RVs have proven difficult to adapt to cell culture without losing virulence. In addition, in general, when adapted they replicate poorly in cell culture, in the range of 1-5X10^5 plaque forming units (PFUs)/mL (27, 28) making them difficult to rescue using the current RG strategies. Many human RV isolates also replicate less robustly than a variety of animal strains and even some animal RV strains have been difficult to rescue despite their robust replication ability. For these reasons, we sought to develop a modified, more reproducible and efficient RG protocol for RV, which relies on the inclusion of a recently described “Chimeric Cytoplasmic Capping-Prone Phage Polymerase” (C3P3-G1) (29) as well as a genetically modified MA104 cell line. This new protocol enables a more efficient recovery of some human, simian and murine-like RV strains that had previously proven difficult to rescue using current RG strategies.

RESULTS

Generation and characterization of an IRF3 and STAT1 defective MA104 cell line.

Interferons (IFNs) are key components of the innate host defense against many viruses including RV (4, 24, 30-32). Although MA104 cells are believed to have a blunted IFN response and this feature plus other characteristics has made them a highly permissive cell substrate for

RV propagation (33), we reasoned that disarming IFN signaling in MA104 cells might enhance RV replication and RG recovery rates. We took advantage of the parainfluenza virus 5 (PIV5, previously SiV5) V protein and the bovine viral diarrhea virus (BVDV) N protease, which target the signal transducer and activator of transcription 1 (STAT1) (34, 35) and the interferon regulatory factor 3, also known as IRF3 (36, 37) respectively for degradation. In addition to the V protein's ability to prevent the antiviral response by degrading STAT1, its ability to disarm the RNA sensing pathway by disrupting the RIG-I and MDA-5 activation has been reported (38, 39). Of note, RIG-I and MDA-5 are known to mount an early interferon response to RV infection (40). We expressed PIV5 V and BVDV N proteins, either individually or in combination in MA104 cells, as previously described (41). Protein levels of STAT1 and IRF3 were examined by western blot to confirm functionality-expression of N and V proteins in MA104 stable cell lines (dual-expressing cells designated as MA104 N*V cells) (Fig 1A).

We next treated the MA104 N*V cells with IFN-α to assess their ability to respond to exogenous type I IFNs (IFN-I) (Fig 1B). Wild-type (wt) MA104 cells responded to IFN-α treatment, with rapid phosphorylation of STAT1 (tyrosine 701), followed by induction of canonical interferon-stimulated genes (ISGs) including the Myxovirus resistance protein 1 (MX1) and the interferon-induced transmembrane protein 3 (IFITM3) proteins (Fig 1B). In contrast, there was a complete loss of STAT1 phosphorylation in MA104 N*V cells (Fig 1B). In addition, neither IFITM3 nor MX1 levels increased following IFN-α treatment (Fig 1B). Collectively, these data indicated that the MA104 N*V cells have a diminished response to IFN-α.

Inhibition of IRF3 and STAT1 responses in MA104 cells enhances the replication of several RV strains.

We next examined the ability of several human and animal RV strains to replicate in the wtMA104 as compared to MA104 N, V, or N*V cells. All 5 RV strains tested replicated to significantly higher virus titers in the MA104 N*V cells (Fig 2A). While some RV strains (e.g.

RRV and SA11) showed only modest, but significant ($p \leq 0.05$) increases, the human CDC-9 strain, a new RV vaccine candidate strain (42), had an almost 10-fold enhancement in viral yield in the MA104 N*V cells (Fig 2A). For two murine RV strains (ETD and D6/2), we also observed significant increases in viral titers (~5-fold). Taken together, these findings demonstrate that the MA104 N*V cells have lower levels of endogenous IRF3 and STAT1 and these lower levels are associated with significantly enhanced replication capacity of selected human, simian and murine RV strains.

In addition to virus titers, we compared RV mRNA levels in wtMA104 and MA104 N*V infected cells, with or without IFN-α pretreatment (Fig 2B). No difference was observed in RV NSP5 mRNA levels in MA104 N*V cells in the presence or absence of IFN-α. In contrast, the replication of an IFN sensitive RV strain (such as UK bovine RV (40) was decreased by 1 Log_{10} post IFN-α stimulation of wtMA104 cells (Fig 2B). Interestingly, we found that, for the human CDC-9 strain, pre-treatment of wtMA104 cells with IFN-α (Fig 2B) did not suppress RV replication. Nevertheless, the level of CDC-9 mRNA was higher (~10X) in the STAT1/IRF3 modified MA104 N*V cells as compared to wtMA104 (Fig 2B). Based on these findings, we hypothesized that the modified MA104 N*V cells may be a better cell substrate than wtMA104 cells to enhance the RG recovery of some RV strains.

The MA104 N*V cell line enhances the RG recovery of the human rCDC-9 RV.

Since the MA104 N*V cell line supported higher levels of replication for several RV strains, we next assessed their ability to enhance RG rescue efficiency. Using the RG system described by Komoto (12) as a reference, MA104 N*V cells enabled an efficient rescue of the human CDC-9 strain (10 rescues in 10 attempts). In contrast, when wtMA104 cells were used instead, only 3 of 10 attempts resulted in the recovery of replication competent rCDC-9 virus. Additionally, the hard-to-rescue simian RRV strain was not rescued using wtMA104 cells (0 out of 6 attempts), but when MA104 N*V cells were used, rRRV could be rescued at low efficiency

(1 out of 3 attempts). These findings indicate that at least for some RV strains, the use of modified MA104 N*V cells improves the efficiency of the RV RG system.

RNA capping enzyme and T7 polymerase fusion protein further increases RV RG efficiency.

With MA104 N*V cells, we were able to rescue rRRV but at a low efficiency. In an attempt to further improve the RG system, we next added to the system an engineered chimeric protein (C3P3-G1) consisting of the African swine fever virus NP868R capping enzyme and the T7 DNA-dependent RNA polymerase (29). An earlier version of this plasmid had previously been shown to enhance the reovirus RG system success rate by approximately 100-fold (43). Such an increase in viral titer was explained by the capping of mRNA produced with this system, which enhances protein expression as well as assembly and RNA incorporation into reovirus virions (43). The inclusion of a cytomegalovirus support plasmid for the African swine fever virus NP868R capping enzyme in a modified RVA RG system has been reported (16). With this NP868R-based system, some recombinant rotavirus with a genetically modified segment 7 dsRNA were successfully rescued (16, 18). Nevertheless, the rescue efficiency of murine-like RV, such rD6/2 like (1) (see below) did not show an improvement using this NP868R-based system.

We next tested the rescue of the simian RRV strain using the modified Komoto RG system with or without C3P3-G1 supplementation at a 2:1 ratio along with the other 11 RRV plasmids. Inclusion of C3P3-G1 substantially increased the efficiency of rRRV rescue from 0/6 to 3/3. Hence, simply including the C3P3-G1 plasmid to the RV RG protocol significantly ($p \leq 0.05$) increased the efficiency of rRRV recovery in wtMA104 cells.

An RG system for the recovery of recombinant murine-like RVs.

So far, we have shown that either MA104 N*V cells or C3P3-G1 plasmid alone can significantly enhance RG rescue of a human or a simian RV strain. To test for potential synergy,

193 we attempted the RG rescue of a previously well-characterized, cultivatable and murine virulent
194 reassortment RV (designated D6/2) derived from a mouse pup co-infected with the non-cell
195 culture adapted EW strain of murine RV and the RRV strain of simian RV (20, 24). This
196 reassortant contains 10 of 11 murine EW strain genes and the 4th gene encoding VP4 from
197 RRV. The D6/2 strain induces diarrhea in suckling pups, transmits between littermates, and
198 replicates moderately well in cell culture (24). However, despite numerous attempts (>10), D6/2
199 could not been successfully rescued. We postulated, based on previous genetic analysis (24)
200 and monoresssortants between SA11 and D6/2 (Supplementary Figure 1), that substituting
201 SA11 genes 1 and 10 into a molecular D6/2-based recombinant murine RV might possess a
202 similar virulence phenotype as the naturally occurring murine D6/2 reassortant but be more
203 amenable to RG rescue.

204 Rescue of a rD6/2 like (1) RV; genes 2,3,5,6,7,8,9,11 from the parental wt non-cultivatable
205 murine EW parental strain, gene 4 from RRV and genes 1 and 10 from SA11, was carried out
206 as described by the Komoto RG system with modifications, or by including the C3P3-G1, or by
207 replacing wtMA104 cells with the MA104 N*V cells, or by using both modifications together. We
208 found that, although the addition of the C3P3-G1 plasmid alone, or the substitution of the
209 MA104 N*V cells alone, boosted recovery efficiencies, rescue was most efficient, when both,
210 the C3P3-G1 plasmid and the modified cell line were used together (Table 1). Based on these
211 results, we propose a new system for the rescue of murine-like RV and other hard-to-rescue RV
212 strains, as described in detail in the Material and Methods section and is summarized in Figure
213 3.

214 **Multiple reporter rRVs were rescued using the optimized RG system.**

215 To provide additional proof-of-concept that this optimized RV RG protocol provides major
216 advantages over other current systems (in modified versions), we directly compared the rescue
217 efficiency of this enhanced system to those described by either Komoto *et al*. or by Kanai *et al*.

(7, 12) with modifications. For this purpose, we tested the rescue efficiency of all recombinant RVs described above, including the simian RRV strain, the human CDC-9 strain, and the rD6/2 like (1) RV. We also included a few more genetically modified rRVs, such as a GFP expressing RRV (GFP and NSP3 separated by a P2A element on gene segment 7. Fig 5A) and a mono-reassortment of VP4 derived from the bovine RV UK strain on the human CDC-9 backbone (rCDC-9/UK_VP4). These particular RVs had not been consistently “rescuable” by us or others (personal communications) using standard RG strategies and served as additional examples to test whether the improved system allowed the efficient rescue of recombinant RVs bearing heterologous gene segments or engineered reporter genes.

The modified Kanai and the Komoto protocols rescue recombinant SA11 very efficiently (Table 1). However, rescue frequency of the recombinant human CDC-9 strain was very low using either the Kanai or Komoto modified protocols (0/2 and 0/8 respectively) and a recombinant RRV strain was either not isolated (0/2) or isolated only rarely (1/6) using the modified Kanai or Komoto protocols respectively. Similarly, neither of these modified RG protocols were particularly efficient for the rD6/2 like (1) RV, when compared to the improved protocol, which included both the MA104 N*V cells and the C3P3-G1 plasmid (Table 1). In all comparisons, these rescue improvements were significantly more efficient than the other modified protocols ($P < 0.01$).

To validate the genomic RNA migration patterns of the rescued recombinant RVs, the dsRNA genomes were isolated and examined by RNA polyacrylamide gel electrophoresis (PAGE). The dsRNA genome profiles for all recovered recombinant RVs using the optimized RG system are shown in Fig 4. The dsRNA migration patterns between wt and recombinant viruses were identical for all the RV strains recovered. For the rD6/2 like (1) RV, the genome profiles confirmed that segments 1 and 10 originated from SA11 and the remaining nine from the D6/2 RV. The mono-reassortment containing bovine RV UK strain VP4 on the CDC-9 backbone showed the same migration dsRNA pattern as wtCDC-9, except for segment 4, which

co-migrated with UK segment 4. Finally, RNA-PAGE was used to identify the modified segment 7 from rRRV-GFP (Fig 4 and 5C), which was additionally confirmed by sequence analysis (data not shown). Altogether, these findings corroborate the identities of all the recombinant RVs rescued and document the enhanced efficiency of the improved RG protocol for a wide variety of RV strains that had proven difficult to rescue using conventional protocols.

The genetic stability of the rescued recombinant RVs was assessed (Fig 5B and 5C) by 5X serial passage (p1-p5) in wtMA104 cells and the recombinant progenies from each passage were titrated by a standard focus forming assay. Virus titers generally increased in the first two passages (Fig 5B), and then remained stable. The multi-step growth kinetics (Fig 5D) of recombinant RVs and their plaque sizes (Fig 5E) in wtMA104 cells were also examined and were not statistically different from their parental strains. Interestingly, the rD6/2 like (1) RV, which, unlike the D6/2 prototype, harbored 2 genes from SA11, did not show statistically significant differences in its *in vitro* growth characteristics compared to the D6/2 parental strain. But the rRRV-GFP virus that carries an engineered segment 7, although it showed a similar growth curve to the wtRRV, formed smaller plaques than its parental strain (Fig 5E). The same phenotype was also observed for other recombinant RVs carrying fluorescent reporters such as rSA11-GFP, rSA11-mCherry (17) and rSA11-UnaG (16). GFP signals were exclusively observed in rRRV-GFP infected RV antigen VP6 positive cells (Fig 5F). This rRRV-GFP virus was stable over 8 passages in wtMA104 cells (Fig 5C).

Recombinant RVs replicate in the intestine and cause diarrhea *in vivo*

Finally, to determine whether rRRV and rD6/2 like (1) RV are able to infect mice as their wtRV counterparts do, the replication, spread, and pathogenesis of these recombinant RV were studied in an *in vivo* mouse model (44). Litters of 4 to 5-day-old mice were orally inoculated with doses of $1X10^4$ PFUs of rD6/2 like (1) or D6/2, or $1X10^7$ PFUs of rRRV or wtRRV. Assessment

of diarrhea by standard diarrhea scores and intestinal replication as measured by fecal RV shedding by RT-qPCR (45) were monitored. We observed virtually identical fecal shedding and diarrhea curves of wtRRV and rRRV following infection (Fig 6A, 6B).

As is shown in Fig 6C and 6D, shedding curves between rD6/2 like (1) vs D6/2 did not show significant difference. Further, we found that the rD6/2 like (1) RV induce diarrhea and can spread just as the D6/2 does, since we observed that the non-infected pups (mock) kept in the same cage as RV infected pups developed diarrhea on 3 to 4 dpi (Fig 6D) and their feces, collected at day 5-8 pi, was positive for mRNA NSP5 as detected by RT-qPCR. In order to further characterize the infection in the mouse pups, the dsRNA migration pattern of rD6/2-like (1) RV was analyzed. RV was isolated from stools collected between day 2-4 of rD6/2-like (1) RV mice-infected and after 3 serial passages on MA104 cells, a portion of the extracted dsRNA from the cells was electrophorized (Fig 6E). Taken together, these results support the conclusion that recombinant RVs exhibit the same *in vivo* phenotype as their corresponding wtRV counterparts. As anticipated and consistent with previous characterizations (20, 24, 25) rD6/2-like (1) was able to efficiently infect mice, induce diarrhea, and spread to uninfected litter mates in a manner similar or identical to D6/2 RV.

DISCUSSION

In this study, we made several significant modifications of the current RV RG system and evaluated whether these modifications improved rescue efficiency for certain RV strains. By adding the recently described C3P3-G1 plasmid (29) along with using genetically modified MA104 N*V cells with reduced capacity to mount an antiviral IFN response, we developed a more efficient and consistently successful (≥80%) RV RG protocol that allowed the recovery of several recombinant RVs that current RG strategies did not efficiently permit. The precise mechanisms by which the use of C3P3-G1 and of the modified MA104 N*V cell line allowed enhanced rescue efficiency are not known. Although the full relevance of the CAP structure to

294 the RV replication cycle is not well understood, the enhanced capping activity provided for the
295 C3P3-G1 system seems to be useful. On the other hand, although BHK-T7 constitutively
296 expresses T7 polymerase, an increased amount of this polymerase provided by transient C3P3-
297 G1 transfection, could also be responsible for higher levels of pT7-RV plasmid transcription. The
298 disrupted IFN-I response at several levels in the modified MA104 N*V seems likely to be
299 involved in the capacity of this line to facility the RV rescue. If so, this benefit could be strain
300 specific, since the ability of different RV strains to effectively counter the IFN response at
301 different levels is documented (4, 31).

302 Our findings indicate that the rescued RVs are genetically stable and showed similar replication
303 phenotypes to their corresponding wtRV parents. In addition, we determined that an RRV
304 carrying a GFP reporter and a human CDC-9 harboring a heterologous UK VP4 segment can
305 be efficiently recued and remain genetically stable.

306 We established a tractable RG system for the rescue of the simian RRV strain, a prototype
307 simian RV used as an experimental model for numerous *in vivo* and *in vitro* studies
308 (4, 19, 20, 24). With this improved system, the reliable rescue of recombinant RVs based on an
309 RRV genetic background is now feasible. In addition, data from suckling mice infection with
310 wtRRV vs rRRV, demonstrate that rRRV is capable of infecting mice and producing diarrhea in
311 a manner similar or wtRRV. These rRRV or RRV genetic background viruses, can now be used
312 in future studies to better understand the genetic basis of systemic RV spread (24, 46), RV
313 associated biliary disease (47-49), and heterotypic immunity (50).

314 We also described the rescue via RG of the CDC-9 human RV strain, currently being evaluated
315 as a potential inactivated human RV vaccine candidate. This strain was first isolated from fecal
316 specimens and then adapted to grow in Vero cells and it has been shown to be safe,
317 immunogenic, and effective at inducing immunity against severe RV disease in several animal
318 models (42, 51). The role of the individual RV proteins as contributors to the protective efficacy

of CDC-9 can now be directly examined in relevant animal models using selected monoreassortants (Supplementary Figure 1). Notwithstanding some initial difficulties, we were able to rescue a recombinant murine-like RV (reassortment RV: genes 2,3,5,6,7,8,9,11 from the parental wt murine EW strain, gene 4 from RRV and genes 1 and 10 from SA11) which is called rD6/2 like (1). Prior publications from our lab demonstrated that the host-range restricted murine RV replication phenotype in the mouse intestine is primarily attributed to gene segments 4 and 5 and does not involve genes 1 and 10 (24). An as we expected this *bona fide* murine RV was able to efficiently infect mice, produce diarrhea and spread to uninfected litter mates similar to as a murine RV.

The new RG capability will now permit us to study the mechanisms and viral determinants of host range restriction, tissue tropism and systemic spread in mice in much greater detail and depth and we plan to actively pursue these areas in future studies.

MATERIALS AND METHODS

Cell culture and viruses: The *Cercopithecus aethiops* epithelial cell line MA104 (ATCC CRL-2378) was grown in Medium 199 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 I.U. penicillin/mL, 100 µg/mL streptomycin and 0.292 mg/mL L-glutamine (complete medium). A Baby Hamster Kidney fibroblast cell line stably expressing T7 RNA polymerase BHK-T7, was kindly provided by Dr. Ursula Buchholz (Laboratory of Infectious Diseases, NIAID, NIH, USA) and previously described (52). This cell line was cultured in complete (10% FBS, 100 I.U. penicillin/mL, 100 µg/mL streptomycin and 0.292 mg/mL of L-glutamine) Dulbecco's modified Eagle's medium (DMEM) and 0.2 µg/mL of G-418 (Promega) was added to the complete medium at every other passage. MA104*N, MA104*V and MA104 N*V stable cell lines were generated from MA104 cells as described previously (41) and cultured in complete medium 199 in the presence of puromycin (5 µg/mL), blasticidin (5 µg/mL)

and puromycin (3 µg/mL) + blasticidin (3 µg/mL) respectively. Both antibiotics were purchased from InvivoGen, San Diego, CA. Cells were stimulated with human IFN-α A/D (800 UI/mL) for 30 min or 16 h for either western blot or RV replication studies, respectively.

The wtRV strains used in this study include simian RRV (G3P[3]) (53), and SA11(G3P[2]) (53), human CDC-9 P50 (G1P[8]) (42), bovine UK (G6P[5]) (53), and the murine reassortant D6/2. These and other recombinant RVs were propagated in wtMA104 cells as described (54). Prior to infection, all RV inocula were activated with 5 µg/mL of trypsin (Gibco Life Technologies, Carlsbad, CA) for 30 min at 37°C.

Plasmids. The simian SA11 plasmid collection: pT7-VP1SA11, pT7-VP2SA11, pT7-VP3SA11, pT7-VP4SA11, pT7-VP6SA11, pT7-VP7SA11, pT7-NSP1SA11, pT7-NSP2SA11, pT7-NSP3SA11, pT7-NSP4SA11, and pT7-NSP5SA11 as well the three helper plasmids pCAG-D1R, pCAG-D12L and pCAG-FAST-p10 were originally made by Dr. Takeshi Kobayashi (Research Institute for Microbial Diseases, Osaka University, Japan) and obtained from Addgene (7). The whole murine pT7-D6/2 plasmid collection and the pT7-UKVP4 were commercially synthesized (GenScript USA Inc.) The complete simian pT7-RRV plasmid collection was originally constructed by Dr. Susana Lopez (UNAM, Mexico City, Mexico). The modified pT7-RRV-NSP3 (see Fig 5A) was engineered following a validated approach previously described (16, 18). The plasmid constructs for individual genes of RV CDC-9 strain at passage 11 in MA104 cells were provided by Dr. Baoming Jiang (CDC, Atlanta, USA) (55). The purification of all the plasmids was performed using QIAGEN Plasmid Miniprep kit per manufacturer's instructions. To validate all the new pT7-RV plasmids, a panel of SA11 × CDC-9 or RRV or D6/2 monoreassortant viruses on the strain SA11 genetic background were generated following the Komoto *et al* (12) procedure with the following modifications or the improved RG protocol (see below and Supplementary Figure 1).

367 **Generation of recombinant rotaviruses:** Recombinant RVs were generated as described by
368 Kanai *et al.* (7) or Komoto *et al.* (12) with some modifications. For Kanai protocol 7.5-8.5 ×
369 10^4 BHK-T7 cells were seeded in 12-well plates, 48 h after cells were cotransfected with 0.4 µg
370 of either RV rescue plasmid (1-fold), 0.0075 µg of pCAG-FAST, and 0.4 µg of each capping
371 enzyme expression plasmid using 2 µL of TransITLT1 (Mirus) transfection reagent per
372 microgram of plasmid DNA. After plasmid transfection, all the transfected cells were processed
373 as described below. The original Komoto protocol was modified as indicated: confluents
374 monolayers of BHK-T7 cells, seeded in 12-well plates (see conditions above) were transfected
375 with 0.4 µg of either RV rescue plasmid (1-fold), with 3-fold increased amounts of the two
376 plasmids carrying the NSP2 and NSP5 genes (1.2 µg/well) using 3 µL of TransIT-LTI
377 transfection reagent per µg of plasmid DNA, the transfected BHK-T7 cells were then processed
378 as described below.

379 The improved protocol is described in brief: using 1-well of a 12–well plate as a reference, 7.5
380 X10^4 BHK-T7 cells were resuspended in 1 mL of complete DMEM (10% heat inactivated SFB,
381 100 I.U/mL penicillin, 100 µg/mL streptomycin, 0.292 mg/mL) G418-free medium and seeded
382 into the well. Forty-eight hours later, the medium was replaced by 800 µL of fresh complete
383 DMEM medium, and then the sub-confluent BHK-T7 monolayer was transfected with the
384 corresponding transfection mix that contained 125 µL of prewarmed Opti-MEM, 400 ng each of
385 the 11 RVA pT7 plasmid, except pT7-NSP2 and pT7-NSP5 which were added at 1200 ng, and
386 800 ng of the plasmid pCMVScript-NP868R-(G4S)4-T7RNAP (C3P3-G1). As transfection
387 reagents, 14 µL of Trans IT-LTI (Mirus Bio LLC) were used. All the plasmids and transfection
388 reagents were mixed in a pipet by gently moving it up and down and then incubated at room
389 temperature for 20 min. After this time, the transfection mixture was added drop by drop to the
390 medium of BHK-T7 monolayers and then the cells were returned to 37°C. 16-18 hours later,
391 two washes with FBS-free medium were done after which 800 µL of serum-free DMEM was

added to the transfected-BHK-T7 cells. Twenty-four hours later, $5X10^4$ wtMA104 (modified Kanai and Komoto protocols) or MA104 N*V cells (improved protocol) in 200 µL of serum-free DMEM was added to the well, along with 0.5 µL/mL of porcine pancreatic type IX-S trypsin (Sigma-Aldrich). MA104 N*V and BHK-T7 cells were co-cultured for 72 hours, after which they were frozen and thawed three times. To remove cell debris, the lysate was centrifuged at 350 x g for 10 min at 4°C and then activated with 2.5 µg/mL of trypsin to infect a 3-day old monolayer of MA104 cells. After 1h of adsorption, the inocula were removed and 1 mL of serum-free 199 medium supplemented with 0.5 µg/mL of trypsin was placed on the cells. MA104 cells were incubated at 37°C for 5 days or until cytopathic effects were observed (passage 1). We defined as successfully rescue virus, when MA104 cells infected whit the corresponding RV rescued passage 1 were positive by immunostaining using an anti-DLPs antibody (see section of focus-forming assay).

Plaque and focus-forming assays: Culture supernatant or virus samples were serially diluted 2 or 10-fold and added to a monolayer of MA104 cells for 1 h at 37 °C. Samples were removed and replaced with 0.1% agarose (SeaKem® ME Agarose. Lonza) in M199 serum free medium supplement with 0.5 µg/ mL of trypsin. Cultures for plaque assay were incubated for 2-5 days at 37 °C, then fixed with 10% formaldehyde and stained with 1% crystal violet (Sigma-Aldrich) to visualize plaques. Cultures for focus forming assay were incubated for 16-18h at 37 °C, then fixed with 10% paraformaldehyde, permeabilized with 1% tween 20, stained with rabbit hyperimmune serum to rotavirus (anti-DLPs) produced in our lab and previously described (56) and anti-rabbit horseradish peroxidase antibody. Viral foci were stained with 3-3′-diaminobenzidine and DAB Chromogen kit (Dako) and enumerated visually.

Immunoblot analysis. Cells were lysed in RIPA buffer [150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0] (Sigma-Aldrich) supplemented with

protease and inhibitor cocktails [1X] (Thermo Scientific™ Halt™ Protease and Phosphatase Inhibitor Cocktail.100X). Proteins in cell lysates were resolved in SDS-PAGE (Mini-PROTEAN® TGX™ Precast Gels [4-15%], Bio-Rad) and transferred to membranes (Nitrocellulose Membrane, 0.45 µm, Bio-Rad). The membranes were blocked by incubation with 5% BSA, 0.1% tween 20 in phosphate-buffered saline (PBS) for 1h at room temperature and with primary antibodies diluted in PBS containing 5% nonfat dry milk or 5% BSA, followed by incubation with secondary, species-specific, horseradish peroxidase-conjugated antibodies. The peroxidase activity was developed using the Clarity ECL substrate, Amersham Hyperfilm, and a STRUCTURIX X-ray film or Azure Imager, following the manufacturer's instructions. The blots were also probed with an anti-GAPDH antibody, which was used as a loading control.

The primaries antibodies and dilutions used were: IRF3 (CST, No. 4302, 1:1000), STAT1 (CST, No. 14994, 1:1000), Phospho-Stat1, Tyr701 (CST, No. 7649, 1:1000), IFITM3 (Proteintech, No. 11714-1-AP, 1:1000), Mx1 (SCT,No. 37849, 1:1000), GAPDH (Proteintech, No. 60004-1, 1:5000). As secondary antibodies: anti-rabbit (CST, No. 7074,1:5000) or anti-mouse (CST, No. 7076,1:5000) immunoglobulin G horseradish peroxidase-linked antibodies were used.

RNA gels. Viral dsRNA was extracted with TRIzol (Invitrogen) according the manufacturer's protocol and then mixed with Gel Loading Dye, Purple (6X), no SDS (New England Biolabs). Samples were subjected to PAGE (10%) for 2.5 h at 180 Volts and visualized by ethidium bromide staining (1 µg/mL) or 18h at 25 mA and silver stained using a previously described method (57).

Immunofluorescence analysis. MA104 cells were infected for 24h with rRRV-GFP at a MOI of 0.01 FFU, then fixed with 10% paraformaldehyde, permeabilized with 1% tween 20. The cells were incubated for 1 h at 37 ^{0}C temperature with an in-house rabbit anti-DLP antibody diluted at

1:1000. After, the cells were washed 3x with PBS and then incubated for 1h at 37 ^{0}C with chicken anti-Rabbit IgG, Alexa Fluor 594 (diluted 1:2000 in 0.2% FBS-PBS). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired under BZ-X Keyence fluorescence microscope.

Mice and RV infection. Wild type 129sv mice were originally purchased from the Jackson Laboratory and maintained as individual in house breeding colonies. 4-5 -day-old pups were orally inoculated with simian RRV (10^7 PFUs) or D6/2 (10^4 PFUs) or recombinant simian RV RRV strain (10^7 PFUs) or rD6/2 like (1) (10^4 PFUs) rescued using the reverse genetics method described above. Fecal specimens were collected on the indicated days post infection and subjected to a RT-qPCR based assay measuring RV gene NSP5 levels with standard curves to determine infectious virus particles per gram of stool samples as described (45). All mice were housed at the Veterinary Medical Unit of the Palo Alto VA Health Care System. All animal studies were approved by the Stanford Institutional Animal Care Committee.

Statistical analysis: All experiments, unless otherwise noted, have been repeated at least three times. The bar graphs are displayed as means ± SEM. Statistical significance were evaluated using GraphPad Prism 7.0. or the IBM SPSS Statistics Grad Pack 26.

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FIGURE LEGENDS

Figure 1*: MA104 N*V cell line characterization.* **(A)** Representative Immunoblot of cellular targets of N and V viral proteins in wild type (wt) and modified MA104 cells. Stable cell lines expressing N, V, or N and V proteins and wtMA104 cell lines were analyzed by immunoblot and the expression of STAT1 and IRF3 were detected with the indicated antibodies. GAPDH detection was used as a loading control. **(B)** wt and MA104 N*V cells were pretreated with or without 800 UI/mL of IFN-α for 30 min or 16 h and then processed for immunoblot. The expression of pSTAT1 (Tyr 701), IRF3, STAT1, IFITM3 and Mx1 was detected with the indicated antibodies. GAPDH detection was used as a loading control.

Figure 2: *MA104 N*V cells support higher levels of RV replication*. **(A)** MA104 and MA104 N*V cells were infected with the indicated RV strains at MOI of 1. At 24 hpi, cells were lysed, and virus titers determined by an immunoperoxidase focus-forming assay. **(B)** MA104 wt and N*V cells, pretreated with or without 800 UI/mL of IFN-α for 16 h were infected with indicated RV strains (MOI=0.01). At the indicated time points, total RNA was harvested and RV NSP5 mRNA levels were measured by RT-qPCR and normalized to GAPDH. The arithmetic means ± standard deviations from three (**A**) or two (**B**) independent experiments are shown. Statistical significance was evaluated by student's t-test. The asterisks indicate significant differences (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

Figure 3: Schematic representation of an improved reverse genetics system for RVs recovery including the use of N*V modified MA104 cells and the C3P3-G1 plasmid.

Figure 4: *Rotavirus dsRNA genomic profiles by RNA-PAGE*. Viral RNA extracted from MA104 cells infected with indicated RVs and then separated on a 10% polyacrylamide gel and stained by ethidium bromide. The position of segments of interest are marked with red asterisks. The dsRNA segment numbers are shown in the figure.

Figure 5: *Recovery of the recombinant RRV (rRRV) and the fluorescent RRV (rRRV-GFP)*. **(A)** pT7 organization of wild type NSP3 and GFP-NSP3 RRV, nucleotide positions are labeled. **(B)** Genetic stability of rRRV (blue) and rRRV-GFP (green). The recombinants rotaviruses were serially passaged 5 times in MA104 cells. Cells were harvest at day 5 post infection or when complete cytopathic effects were observed, the virus titer was determined by an immunoperoxidase focus forming assay. **(C)** Additionally, rRRV-GFP was passaged 8 times in MA104 cells, RNA viral from all passages (2-8) was extracted and separated on a 10% polyacrylamide gel and stained by ethidium bromide. The position of engineered segment 7 is marked with red arrowheads. The segment numbers are shown in the figure. **(D)** Replication kinetics of wtRRV, rRRV and rRRV-GFP. Monolayers of MA104 cells were infected with RVs at an MOI of 0.01 a in the presence of trypsin (0.5 µg/mL) and then were harvest at indicated times by freezing/thawing. The viral titers were determined by immunoperoxidase focus forming assay. Results are expressed as the mean viral titer from triplicate experiments. Error bars shown the SD. **(E)** Comparison of plaques size. Plaques were generated on MA104 monolayers and detected at 3 dpi by crystal violet staining. Representative photographs of viral plaques are shown. The sizes of at least 24 randomly selected plaques from 2 independent plaque assays were measured using the GraphPad Prism v7 and reported as area on relative units. Mean values and the standard deviation are shown. Statistical significance was evaluated by student's t-test. The asterisks indicate significant differences (*ns*: not significant, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). **(F)** Subcellular localization of GFP protein in rRRV-GFP infected cells. MA104 cells were infected with rRRV-GFP at MOI of 0.01. After 24 hpi, infected cells were fixed and visualized by fluorescence microscopy. Using anti-VP6 polyclonal antiserum and the Alexa Fluor 594 anti-rabbit IgG, nuclei were stained with DAPI. Scale bar: 50 µm.

Figure 6*: Characterization of rRVs in an in vivo mice model***.** Five-day-old 129sv mouse were orally inoculated with 10^7 PFUs of simian wtRRV or rRRV (**A-B**) or 10^4 PFUs of D6/2 or rD6/2 like (1) RV (**C-D**). Diarrhea was monitored from days 1 to 8 post infection and fecal specimens were collected on the indicated dpi and examined by RT-qPCR-based assay measuring RV gene NSP5 levels with standard curves as a measure of RV shedding per mg of stool. The numbers of mice in each group are indicated in parentheses. (**E**) dsRNA genomic profile from rD6/2 RV was confirmed by RNA-PAGE. The position of segments of interest are marked with red arrowheads. The segment numbers are shown in the figure.Statistical significance was evaluated by student's t-test. The asterisks indicate significant differences (*ns*: not significant *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

694 **TABLE LEGENDS**
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697 **Table 1:** Comparison of RG rescue frequencies for different RV strains and for modified RV
698 using Kanai♦, Komoto♦ and the improved RV system including MA104 N*V cells and the C3P3-
699 G1 plasmid. Statistical significance was evaluated by Chi square method. The asterisks indicate
700 significant differences (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).
701 [0] Numbers in parenthesis: the first number represents the number of successful rescues and the
702 second one the total number of attempts.
703 ♦Modified version.
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Table 1: Comparison of RG rescue frequencies for different RV strains and for modified RVs using Kanai♦, Komoto♦and the improved RV system including MA104 N*V cells and the C3P3-G1 plasmid. Statistical significance was evaluated by Chi square method. The asterisks indicate significant differences (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

Recombinant RV Strain		Rescue efficiency (%)		
		♦Kanai	♦Komoto	Improved
Human	rCDC-9	**0** (0/2)	**0** (0/8)	**100***** (5/5)
	rCDC-9/UK_VP4	**0** (0/2)	**0** (0/6)	**100***** (5/5)
Simian	rSA11	**100** (3/3)	**100** (3/3)	**100** (3/3)
	rRRV	**0** (0/3)	**16.7** (1/6)	**83.3**** (5/6)
	rRRV-GFP	**0** (0/3)	**16.7** (1/6)	**100***** (6/6)
Murine-like	SA11 X D6/2 reassortment [rD6/2 like (1)]	**0** (0/2)	**8.3** (1/12)	**80**** (4/5)

[0] Numbers in parenthesis: the first number represents the number of successful rescues and the second one the total number of attempts.

♦Modified version.