

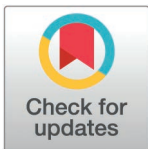
RESEARCH ARTICLE

Conserved non-coding RNA motifs influence the neuropathogenicity of Simbuviruses: Molecular dissection in the Schmallerberg virus model

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Abstract

The Simbu serogroup, part of the *Peribunyaviridae* family, includes arboviruses associated with febrile illnesses in humans and fetal congenital malformations due to viral neurotropism in ruminants. These viruses possess a tripartite, negative-sense RNA genome lacking the poly(A) tail. Notably, the 5' untranslated region (UTR) of the small (S) genomic segment contains conserved RNA elements, including a stem-loop (SL) structure and a sequence-based motif (GC signal) flanking the messenger RNA (mRNA) termination site. Although their functions remain unclear, their conservation and specific location suggest a potential role in mRNA transcription termination and translation initiation. A reverse genetics system for Schmallerberg virus (SBV) was used to create a viral recombinant library bearing deliberate mutations in both motifs. Replication kinetics, S segment transcription termination, and Nucleocapsid protein (N) abundance of rescued virus mutants were evaluated in mammalian and insect cell culture. Virulence was assessed in an immunocompetent mouse model. Characterization of the mutant viruses indicated that the SL structure is essential for viral production, with the stem length as a key feature; more than three complementary base pairs between the stem arms are necessary for replication. A shorter stem length impaired replicative fitness, N protein abundance and altered the mRNA to genomic RNA ratio. Point mutations in the GC signal disrupted proper mRNA termination, thereby limiting viral N protein synthesis and, thus, virion assembly. *In vivo*, attenuated viruses resulted in lower viral loads, reduced dissemination in mice brains, and improved survival rates compared to wild-type SBV. The GC signal mutants exhibited strong attenuation while still maintaining active transcription. Overall, these findings indicate that the SL and GC signal serve as *cis*-regulatory elements and are

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indirect determinants of SBV virulence, regulating viral replication and influencing neuropathogenesis.

Author summary

Understanding how viruses control their gene expression and cause disease is crucial for improving control strategies. Our research focuses on Schmallenberg virus, a ruminant pathogen. The virus belongs to a group of viruses that can cause congenital disorders when pregnant females transmit the virus to the developing fetal nervous system. We were interested in specific viral genome conserved features suspected to be crucial for the virus's life cycle. These include a structural element (stem-loop) and a sequence-based element, both found in similar viruses. Adopting a reverse genetics system we created modified versions of SBV to see how these elements impacted the virus infection in cells and mouse brains. Our findings showed that the mutant viruses produced fewer viral proteins and replicated poorly in cells. In mice, these altered viruses demonstrated reduced replication and spread in the brain, leading to mild disease and less mortality. Our results confirmed that the non-coding RNA structural element is essential for the virus to complete its life cycle, specifically the stem length being significant. The sequence-based element is also vital for properly processing viral genetic material and protein synthesis in the infected cells. These findings provide new insights into how these viruses operate and may identify potential targets for developing vaccines or improved strategies for controlling them.

Introduction

Viruses belonging to the Simbu serogroup, within the family *Peribunyaviridae*, are transmitted mainly by biting midges of the genus *Culicoides* spp. infecting a wide range of vertebrate hosts, often associated with veterinary and medical concerns [1]. Among significant simbuviruses infecting humans, Oropouche virus (OROV) was shown to re-emerge in Southern America in early 2024. While OROV was initially found to cause mainly an acute febrile illness, recent investigations have found fetal infections and adverse perinatal outcomes such as miscarriage, stillbirth, and congenital anomalies, including central nervous system (CNS) involvement and microcephaly [2–4].

Other affiliated simbuviruses can lead to transplacental transmission in ruminants, such as Schmallenberg (SBV), Akabane, Sathuperi, Aino, Shamonda, Peaton, and Shuni. Vertical transmission causes fetal malformations such as arthrogryposis, cerebellar hypoplasia, and hydranencephaly. Fetal infection is accompanied by histological changes, which include spongy degeneration and nonsuppurative encephalomyelitis [3,5–9]. Consequently, these ruminant simbuviruses can serve as relevant models for studying pathogenesis and infection mechanisms of human simbuviruses.

The genome of the *Peribunyaviridae* family consists of three negative single-stranded RNA segments: the large (L), the medium (M), and the small (S) [10]. The S segment encodes a non-structural protein (NSs) and the Nucleocapsid protein (N) in overlapping reading frames; the L segment encodes the viral polymerase (RdRp); and the M segment encodes a polyprotein precursor housing a non-structural protein (NSm) and two glycoproteins (Gn and Gc) [11–14]. The N and L proteins are essential for viral RNA (vRNA) transcription and replication. The RdRp cleaves capped host RNAs using its endonuclease domain to initiate mRNA transcription [15,16]. The N protein assists cap snatching, protects vRNA by encapsidating them into ribonucleoprotein complexes (RNP), and interacts with the other viral proteins during virion assembly in the cells [12].

Replication occurs in the cytoplasm and begins with the RdRp catalyzing primary mRNA transcription after RNPs release. This is followed by genomic RNA (gRNA) replication, where an intermediary transcript is generated, the complementary RNA (cRNA) [11]. The gRNA and the cRNA are complementary and of equal length, whereas the viral mRNAs display a shorter 3' end devoid of a poly(A) tail [16].

The genomic segments are flanked by 3' and 5' untranslated regions (UTRs), characterized by high variability in length and sequence across different orthobunyaviruses, even within the same serogroup [10]. UTRs serve as repositories for RNA motifs with distinct sequence patterns or structures. These motifs in turn offer binding sites for regulatory factors, including proteins or other RNAs of viral or cellular origin. These molecular interactions regulate gene expression by influencing mRNA stability, localization, and translation efficiency [17]. Orthobunyaviruses UTRs harbor a dual promoter for both transcription and replication, consisting of a panhandle structure hinging on the complementary nature of the 3' and 5' extremities of each segment [18]. Furthermore, these UTRs encompass an N protein encapsidation signal, and mRNA transcription termination signals that are essential for effectively synthesizing functional mRNA [19–22].

Previous studies have explored non-coding RNA motifs of the Bunyamwera virus (BUNV), located in the 5'UTR of the S segment gRNA, corresponding to the 3'UTR of the S segment derived cRNA and mRNA, revealing one stem-loop structure (SL) upstream and two termination signals downstream of the mRNA termination site [21–24]. In the first descriptions, a translation enhancer element was attributed to the upstream region of the SL, and transcription termination was attributed to a pentanucleotide sequence shared by both termination signals (5'-ACAGC-3') [21,23]. Thereupon, mRNA transcription termination was shown to be driven only by the proximal signal, indicating that the shared pentanucleotide is not crucial; instead, suggesting an alternative pentanucleotide (5'-GCUGC-3') described also in the *Phenuiviridae* family [22,24].

While the pioneering studies shed light on the strategies BUNV employs during its replicative cycle *in vitro* by scrambling or deleting large UTR sequences, they also left several gaps, particularly in terms of their precise sequence contributions, their relevance to viral pathogenesis, and their conservation trait among other orthobunyaviruses. Addressing the specific roles of non-coding RNA motifs in transcription termination and translation initiation within an independent poly(A) context is important for enhancing our fundamental knowledge of viral molecular biology and devising effective countermeasures.

To that end, SBV was picked as a model to perform a mutational analysis, targeting both the SL and the termination signal (GC signal). The study demonstrates the functional implications of both RNA motifs in SBV replication, mRNA transcription and termination, and translation in mammalian cells. Additionally, virulence and replication efficiency of engineered SBV recombinants were assessed using an immunocompetent mouse model, confirming the *in vitro* observations and the associated attenuated phenotypes.

Results

A SL structure and the GC dinucleotides, found in the S segment UTR, are conserved among several orthobunyaviruses

Mapping the mRNA termini using 3' RACE PCR, we previously identified an SL structure and a termination signal surrounding the mRNA termini in three simbuviruses infecting ruminants [16]. Simbuviruses are sorted into two clades A and B. While clade B gathers viral species infecting ruminants, the host range of clade A is more diversified and includes

humans [6]. Sequence alignment with other members of the Simbu serogroup revealed that both motifs were conserved in their placement within the UTR since SL and GC motifs were localized upstream and downstream of the termination site, respectively (Fig 1A). To expand upon this, we aligned the sequences of viral species related to the 18 serogroups gathered in the *Orthobunyavirus* genus. Both conserved motifs were identified in 7 serogroups: Simbu, Bunyamwera, California, Nyando, Gamboa, Tete, and Group C (Fig 1B).

More precisely, the nucleotide sequence of the SL motif varied between species. Nonetheless, sequence variation was found to maintain the motif structural foundation. Among all members of the clade B of the Simbu and in the two species of the Tete serogroup, the conserved SL structures are based on long stem arms composed of 9–12 complementary nucleotides forming the stem. In three simbuviruses species belonging to the clade A, notably OROV, and the remaining five serogroups, shorter stems are observed.

In contrast, the GC signal conserved across all the orthobunyaviruses analyzed comprises two GC dinucleotides separated by 1 to 7 nucleotides. The BUNV pentanucleotide signal described by Barr and colleagues (2006) overlaps the 5' GC dinucleotide of the termination signal evaluated in the current study (Fig 1B) [21].

Altogether, this analysis first revealed that a secondary RNA structure is maintained in the S segment UTR of several orthobunyaviruses, even if a high level of divergence occurred regarding the nucleotide composition. Second, a conserved localization of the SL structure and the GC signal was identified around the termination site of the mRNA in this large set of viral species.

Both non-coding RNA motifs impact the capability of rescuing infectious recombinant virus

Both simbuviruses RNA motifs were further characterized to determine their roles in viral replication fitness. The chosen reverse genetic system is based on a virulent strain of SBV (BH80/11–4). The model is adapted to mutagenesis using a three-plasmid-based system, in which each plasmid corresponds to one segment cRNA [25]. Deliberate mutations were introduced on the S plasmid targeting specific sequences in both non-coding RNA motifs.

Regarding the SL, the objective was to gradually decrease the complementarity between both stem arms until the structure was broken. In comparison, the WT-SBV SL structure is composed of a stem with nine complementary nucleotides, whereas SL7nt, SL5nt, and SL3nt constructs displayed stems formed by seven, five, and three complementary nucleotides, respectively. An unfold hairpin construct (SL5/3Δ) did not possess any complementarity, thus disrupting the SL formation. Another mutant was generated to keep the structure rather than the stem, meaning the five substitutions are counterbalanced on the opposite stem arm (SL5/3p). Further, two mutants were generated in which either stem arm contained the latter five substitutions, but without any counterbalance on the opposite stem arm (SL5p & SL3p). Finally, a single loop mutant was designed to evaluate any effects given by the loop region (SLloop).

Regarding the GC signal, the approach intended to modify the GC dinucleotides organized in tandem repeats with alternative dinucleotides. In the GCTT construct, both GC were replaced with TT dinucleotide, while in the GCCG construct, both GC were replaced with CG dinucleotide. Eventually, a mutant was established to evaluate the contribution of the non-conserved trinucleotide present between the GC tandem repeats (GCmid) (Fig 2A).

The reverse genetic assays first revealed that no infectious virus could be isolated or propagated from two co-transfection assays involving the SL5/3Δ, SL5p, SL3p (absence of SL structure), and SL3nt constructs. No cytopathic effect (CPE) was observed in BHK-21 cells, even after repeating the assay five times, nor after five blind successive passages. Regarding the mutants that were effectively rescued, CPE was observed at specific time points following the BHK-21 infection according to the viral genotypes (Fig 2A). It was concluded that SL mutants only replicate if the stem arms are composed of more than three complementary nucleotides. Three viruses obtained from constructs providing an intact stem length, namely rSLloop-, rGCmid-, and rSL-5/3p-SBV, behave similarly to the rWT-SBV since the corresponding recombinants induced evident CPE at 48 h.p.i. CPE was delayed about 24 h for viruses rescued from the four remaining constructs (rSL7nt-, rSL5nt-, rGCTT-, and rGCCG-SBV). Indeed, CPE was not observed before 72 h.p.i.

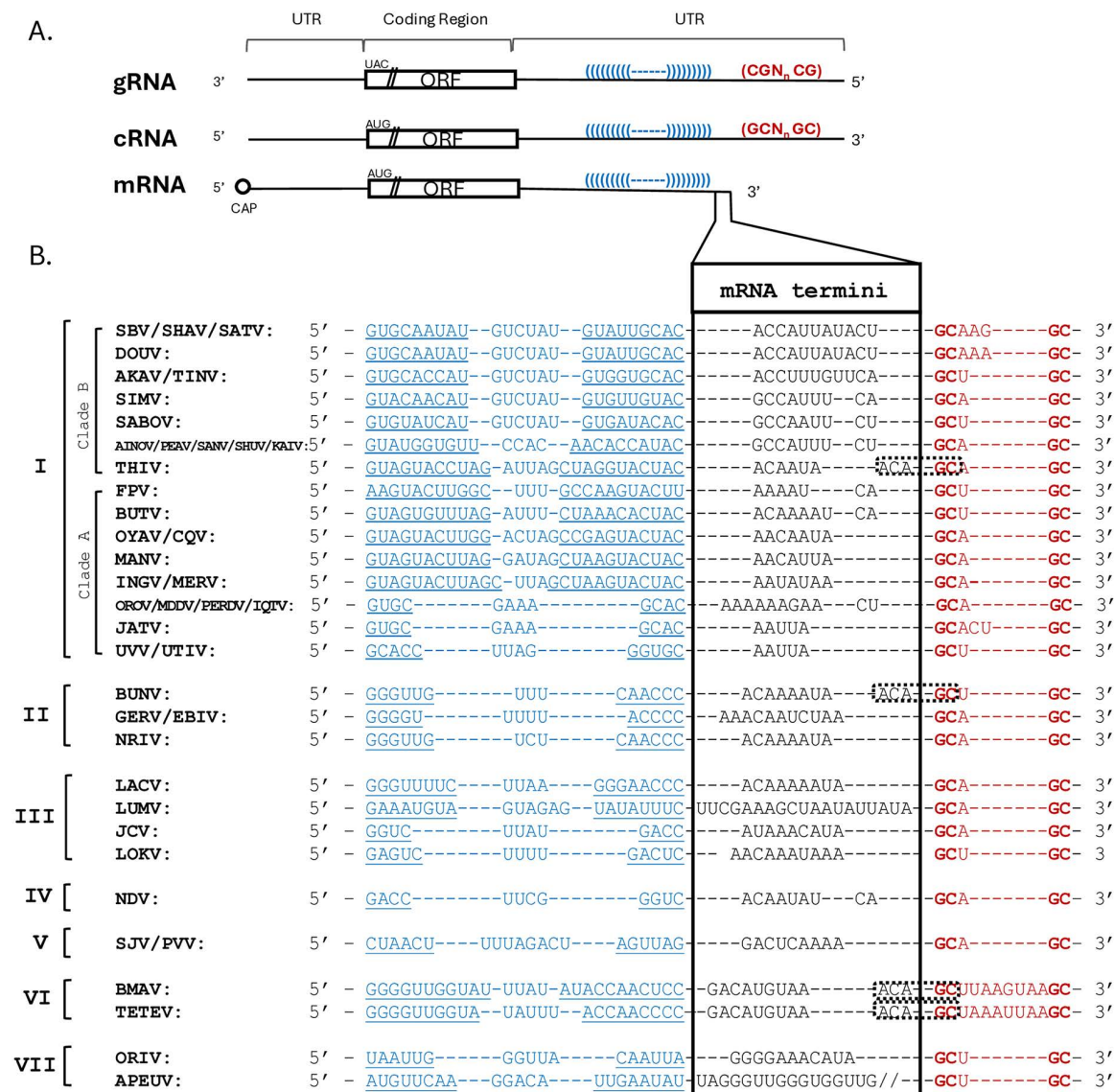


Fig 1. Conserved motifs in the S segment 3'UTRs of orthobunyaviruses. **A)** Schematic representation of the three SBV S segment RNA transcripts. The UTRs of the gRNA, cRNA and mRNA contain conserved motifs that are depicted. The SL structure is shown in blue and in the Vienna format, and the termination signal sequence is shown in red. **B)** Multiple sequence alignment of representative *Orthobunyavirus* genus members bearing both conserved elements. The SL sequences are shown in blue, and the stem arms are underlined. The termination signals are displayed in red and the GC conserved nucleotides of the termination signal are in bold. The mRNA termini highlighted in a black box indicates the region where mRNA transcription terminates relative to full-length cRNA sequences. The dashed boxes are indicating the pentanucleotide initially reported by Barr et al. 2006 [21]. Roman numerals indicate the serogroups: I, Simbu; II, Bunyamwera; III, California; IV, Nyando; V, Gamboa; VI, Tete; VII, Group C. In serogroup I (Simbu), sequences are categorized into clades A and B. The amino acid similarity for the S segment is 59.7% to 71.9% between clades, supported by > 97% bootstrap values across genomic segments [6]. SBV, Schmallenberg virus; SHAV, Shamonda virus; SATV, Sathuperi virus; DOUV, Douglas virus; AKAV, Akabane virus; TINV, Tinaroo virus; SIMV, Simbu virus; SABOV, Sabo virus; AINOV, Aino virus; PEAV, Peaton virus; SANV, Sango virus; SHUV, Shuni virus; KAIV, Kaikalur virus; THIV, Thimiri virus; FPV, Facey's Paddock virus; BUTV, Buttonwillow virus; OYAV, Oya virus; CQV, Cat Que virus; MANV, Manzanilla virus; INGV, Ingwavuma; MERV, mermet virus; OROV, Oropouche virus; MDV, Madre de Dios virus; PERDV, Perdoes virus; IQIV, Iquitos virus; JATV, Jatobal virus; UUV, Utinga virus; UTIV, Utinga virus; BUNV, Bunyamwera virus; EBIV, Ebinur Lake virus; GERV, Germinston virus; NRIV, Ngari virus; LACV, La Crosse virus; LUMV, Lumbo virus; JCV, Jamestown Canyon virus; LOKV, Lokern virus; NDV, Nyando virus; SJV, San Juan virus; PVV, Pueblo Viejo virus; BMAV, Batama virus; TETEV, Tete virus; ORIV, Oriboca virus; APEUV, Apeu virus.

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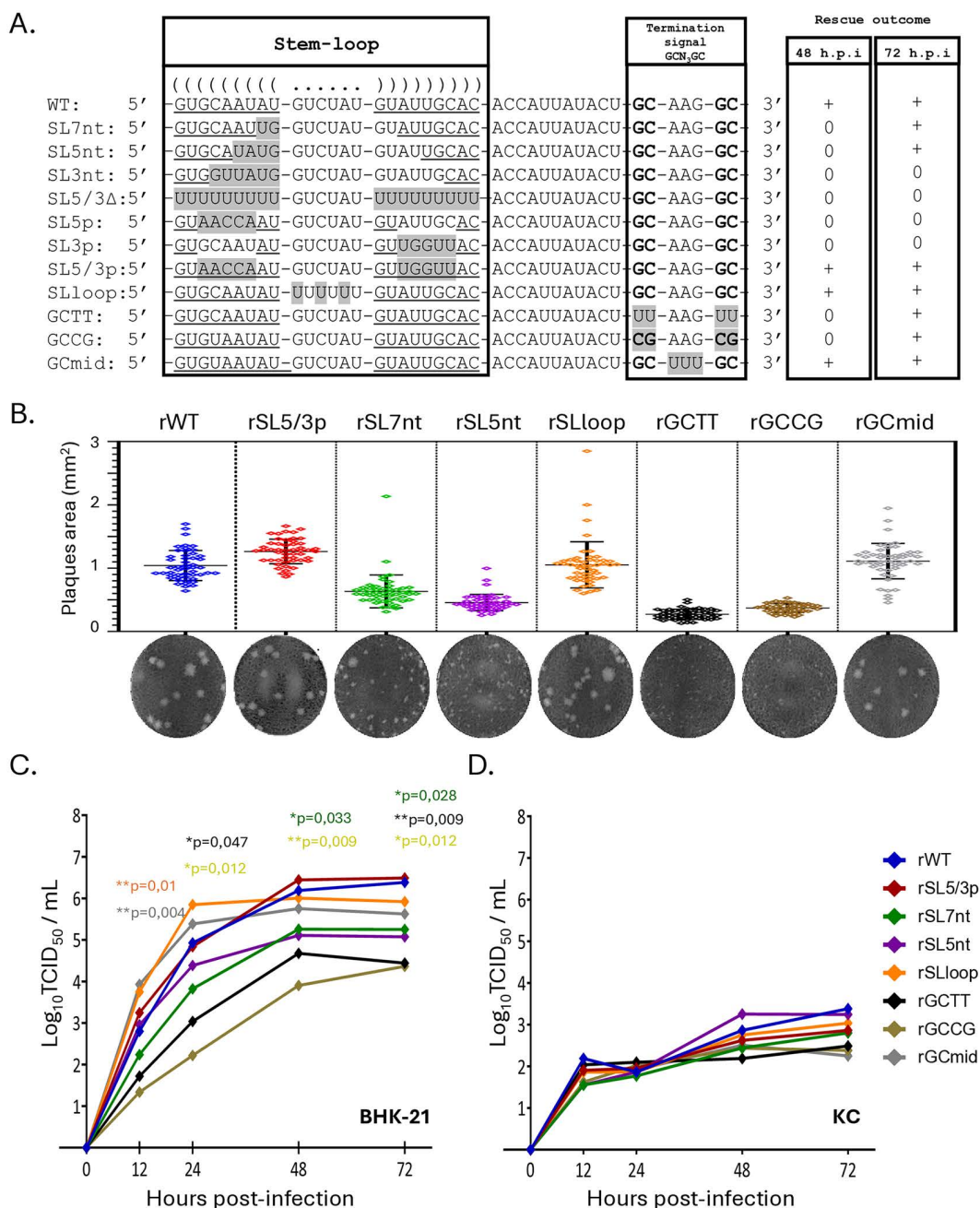


Fig 2. *In vitro* viral infectivity and replication efficiency of the rSBV library. **A)** Graphic representation of the different mutations introduced among the SBV S segment conserved motifs. Highlighted nucleotides correspond to the introduced substitutions. Underlined bases represent complementary nucleotides in the stem arms. Conserved nucleotides of the termination signal are in bold. **B)** Viral plaque phenotype comparison and area raw data of the different rSBV. Plaque assays in BHK21 cells were fixed at 72 h.p.i. in 4% PFA and stained with crystal violet. Representative images are shown out of an n=3. The mean and the SD are plotted. Comparative growth kinetics analysis in **C)** BHK-21 cells and **D)** KC cells infected with a multiplicity of infection (MOI) of 0.002. Culture supernatants were collected at the time points indicated. Viral titers normalized to time 0. The reported values are means from three independent experiments. Statistical differences were evaluated using a two-way ANOVA followed by Dunnett multiple comparisons. Asterisks indicate the level of significance when compared against the rWT-SBV (*p<.05; **p<.01; ***p<.001).

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Sequence analysis of the viral stocks of the rWT-SBV and the other seven infectious rSBV viruses confirmed the presence of deliberate mutations. Subsequent *in vitro* and *in vivo* characterization were carried out using these viral stocks. After 10 successive passages, no undesired mutations, nor reversion of the introduced mutations were observed, indicating the stability of the rescued SBV recombinants.

Both motifs are determinants for viral infectivity and replication capacity in mammalian cells but not in insect cells

The biological properties of the whole set of rSBV viruses were assessed *in vitro*. At first, plaque sizes were measured as previous studies in different bunyaviruses found that attenuated viruses are associated with small plaque phenotype in mammalian cells [26]. Plaque size analysis revealed significant variations (Fig 2B). Three recombinant SBV induced large plaque areas that did not differ from rWT-SBV plaque areas, namely rSL5/3p-, rSLloop- and rGCmid-SBV. This result indicated that neither the sequence of the stem nor the loop or the in-between signal nucleotides influence the viral phenotype. Decreasing the complementarity of the stem (rSL7nt- and rSL5nt-SBV) was associated with significant plaque shrinkage relative to the rWT-SBV. Eventually, the difference was more pronounced for the recombinant viruses bearing mutations in the GC dinucleotides (rGCTT-, and rGCCG-SBV)(Fig 2B). Detailed analysis of plaque size data from three independent assays is reported in S1 Fig.

Next, we investigated the replication kinetics of the recombinant viruses in comparison with the rWT-SBV in BHK-21 cells (Fig 2C). Viral concentrations were determined from extracellular virus harvested at several times post-infection. Significant differences in the replication rate were detected as early as 12 h.p.i in comparison to rWT-SBV. The difference between the rSBV mutants (rSLloop- & rGCmid-SBV) and the rWT-SBV virus disappeared from 24 h.p.i until the last sampling time point. Viral titers reached a plateau from 48 h.p.i, except for rGCCG-SBV. End-point readings obtained at 72 h.p.i exhibited three profiles with (i) three SBV mutants (rSLloop-, rGCmid- & rSL5/3p-SBV) yielding the viral titer of the rWT-SBV, (ii) two rSBV mutants with decreased stem length (rSL7nt- and rSL5nt-SBV) presenting a 10-fold reduction of the titer, and (iii) two rSBV with mutated GC dinucleotides (rGCTT- and rGCCG-SBV) showing a 100-fold reduction (Fig 2C). Collectively, significant differences were observed according to the mutations introduced in the SL structure or in the GC signal when infectious viral load was assessed over time. Such pronounced trend was not noticed when assessing total vRNA amplification for the S, M, and L segments over time (S2A Fig). However, significant differences were observed for the M and the L segments at late sampling points for the rSBV with shorter stem length or mutated GC dinucleotides.

Assessment of the viral titer was also carried out in KC cells representative of SBV vector. The comparative analysis of the kinetic curves between cell lines showed that the rWT-SBV viral titer yield at 72 h.p.i. was 3 log₁₀ lower in insect cells compared to the one obtained in mammalian cells (Fig 2C-2D). KC growth kinetics exhibited no statistical differences between rWT-SBV and any other mutant, suggesting that the RNA motifs might confer a host-dependent replicative attribute. In addition, the shape of the curve differed as two growth phases were observed: the first one taking place during the first 12 h.p.i. characterized by a steeper slope followed by an initial plateau until 24 h.p.i. The second growth phase starts after 24 h.p.i. with a weaker slope. The absence of difference between rWT-SBV and the tested recombinants was confirmed by measuring total vRNA yields over time through RT-qPCR (S2B Fig). Overall, these cells are less permissive to SBV infection revealed by an absence of lytic CPE, lower viral titers, and slower accumulation of total vRNA transcripts over time.

Both motifs impact the N protein abundance, mRNA termination and the RNA transcription/replication dynamics

Previous studies divulged that the N protein is the most abundant viral protein, and it has regulatory functions for genome replication and transcription initiation. Therefore, synthesizing the N protein is a rate-limiting step during the viral replication cycle [27]. To assess if the candidate RNA motifs affect the N protein production, total protein extracts were prepared

from infected cells at 24 h.p.i. and the N protein abundance was measured by immunoblotting (Fig 3). Significant reduction was observed from rSL5nt-, rGCTT-, and rGCCG-SBV compared to rWT-SBV.

From the previous western blot experiment, several mutants exhibited lower levels of N protein while keeping the three genomic vRNA levels similar to rWT-SBV (S2B in S2 Fig). This uncoupling between RNA synthesis and protein production indicates that the motifs may influence vRNA termination instead of the amount of vRNA. To evaluate the contribution of the tested RNA motifs in processing shortened and mature mRNA, we performed 3' RACE-PCR followed by sequencing to map termination sites among the positive S segment transcripts. Two main RNA profiles were identified in BHK-21 cells: a larger product corresponding to the full length of the cRNA (839 nt) and a smaller product consistent with a mature mRNA molecule (788 nt), terminated with preference after the CA dinucleotide of the 5' -ACCAU- 3' sequence, immediately after the SL (Fig 4). The smaller product was identified in 57% of the clones analyzed of rWT-SBV.

Among the SL rSBV mutants, viruses bearing structural SL homologs (rSL5/3P- and rSLloop-SBV) displayed a similar RNA profile to rWT-SBV (Fig 4A). The decreased base-pairing interactions in the stem reduced the proportion of mRNA transcripts (Fig 4B). For instance, 20% and 0% of clones corresponded to mRNA for rSL7nt- and rSL5nt-SBV, respectively. Furthermore, rGCTT-, and rGCCG-SBV mRNA proportion is <4% indicating that proper termination or processing were dampened by these mutations (Fig 4C). These observations suggest that the reduced accumulation of mRNA during replication in the *in vitro* attenuated recombinants is associated with a hindered N protein accumulation.

The decreased levels of the N protein, despite consistent total RNA levels, indicate that these motifs may regulate not only the efficiency of termination but also the overall temporal dynamics of vRNA synthesis. Considering that both mRNA and cRNA are positive-sense transcripts, we hypothesize that mutations impacting termination could also affect the balance between transcription (mRNA production) and genome replication (cRNA and gRNA synthesis).

Strand-specific RT-qPCR analysis measuring the ratio of positive-sense RNA (mRNA plus cRNA) against negative-sense RNA (gRNA) over time, revealed different temporal patterns of vRNA synthesis (Fig 4). It is important to note that we are unable to differentiate between positive sense vRNAs since the mRNA sequence is entirely included within

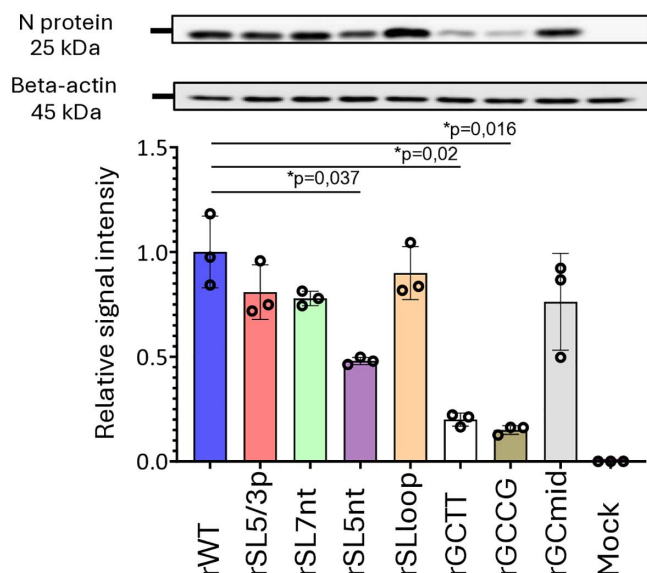


Fig 3. Comparative N protein abundance among the different rSBV. Western blot analysis and relative quantification. BHK-21 cells were infected with an MOI of 0.002. Cell lysates were collected at 24 h.p.i. Beta-actin was used as the loading control. Error bars are the SD of three independent experiments. Student's t-test analysis were performed to compare against the rWT-SBV. Asterisks indicate the level of significance (* $p < .05$; ** $p < .01$; *** $p < .001$).

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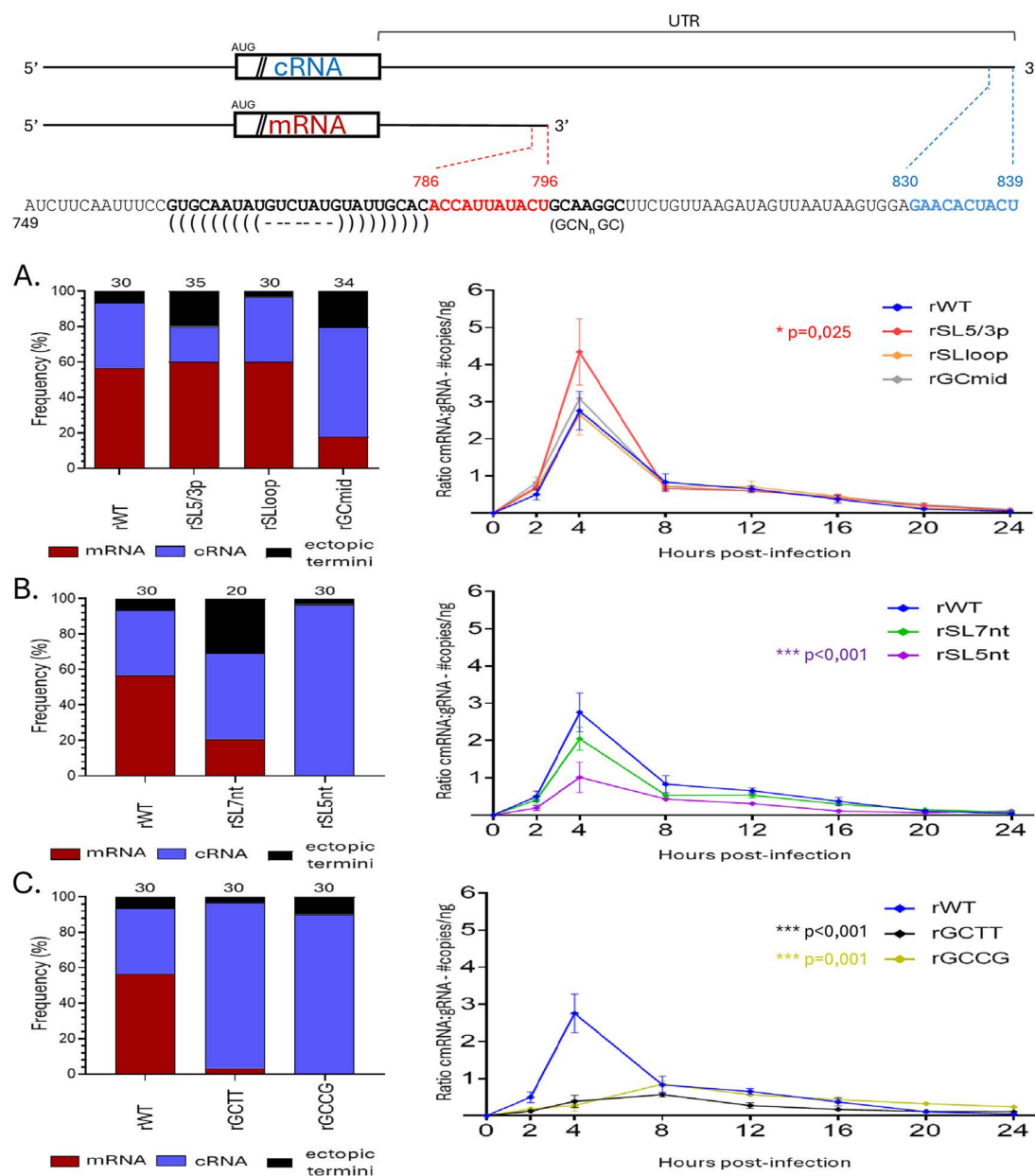


Fig 4. SBV S segment termination profiles and viral RNA synthesis dynamics. The WT SBV cRNA and mRNA 3'UTR sequence is presented on the top panel. The SL structure is noted in the Vienna format below the corresponding sequence. Nucleotides in bold indicate the RNA motifs. Nucleotides in red represent the mRNA termini and the blue nucleotides the cRNA termini. Termination profiles (left panels) and transcription/ replication dynamics (right panels) are compared between rWT-SBV and mutants with **A)** equivalent fitness, **B)** attenuated phenotypes due to reduced stem length, and **C)** attenuated phenotypes due to GC signal substitutions. The termination profiles were evaluated from samples collected at 72 h.p.i derived from BHK-21 cells infected with an MOI of 0.002. The 3' RACE method detects mRNA termination sites through adapter-primed RT-PCR followed by cloning and Sanger sequencing of the 3' ends. Numbers above the bars indicate the clones sequenced per virus. The stacked bar graphs show the proportions of clones corresponding to cRNA, mRNA, or transcripts with ectopic termination sites. The vRNA transcription/replication dynamics were determined by RT-qPCR in BHK-21 cells infected at a MOI of 0.02, with cell lysates collected over 24 hours. The positive-to-negative RNA ratios are plotted over time. Total positive-sense vRNA includes both mRNA and cRNA, and the negative-sense only gRNA. RNA titers normalized to time 0. The data presented are the mean $\pm$ SEM from three independent experiments. Statistical significance was assessed by one-way ANOVA based on area under the curve. Asterisks indicate the level of significance when compared against the rWT-SBV (* $p < .05$; ** $p < .01$; *** $p < .001$).

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the cRNA. The rWT-SBV displayed a biphasic pattern, in which transcription is dominant in early time points peaking at 4 h.p.i. followed by a shift from transcription to replication between 4–8 h.p.i. as gRNA synthesis increased (Fig 4A). The rSL5nt-SBV reached a lower peak at 4 h.p.i. (Fig 4B). On the other hand, rGCTT- and rGCCG-SBV exhibited the lowest transcriptional peaks and delayed timing for the shift from transcription to replication (8–12 h.p.i.) (Fig 4C). However, the replication phase appeared similar to rWT-SBV on later time points.

Both motifs influence SBV replication and therefore virulence in a mouse infection model

To study rSBV mutants *in vivo*, we first determined the minimal viral dose required to induce disease in suckling mice systematically (S3 Fig). To that end, groups (n = 12) were administered via the intracranial (I.C.) route with three doses (200, 400, and 800 PFU/mice) of rSBV-WT and one group as a sham-inoculated control. Highest doses (400 and 800 PFU) induced clinical signs from 5 days post-infection (d.p.i), and lethality in more than 95% of the infected animals, while low dose inoculum gave rise to clinical signs from 8 dpi and induced a fatal outcome in 80% of animals (S3A in S3 Fig). The clinical signs caused by rWT-SBV included weight loss, gait changes, fore limb monoparesis, hind limb paralysis, complete paralysis, or sudden death. These observations and additional behavior parameters were compiled to calculate a litter health score (S3C in S3 Fig and S1 Table), which was further used to evaluate the series of rSBV. In lethal cases, brain tissues were harvested, examined, and prepared for histopathological evaluation. Gross brain lesions were barely detected through necropsies and mainly consisted of focal meningeal hemorrhages. No mortality, symptoms, or histopathological changes were noticed in the sham-inoculated groups of any experiments, indicating that the disease is due to the viral infection and not the inoculation procedure itself. Since mice infected with 400 and 800 PFU showed similar outcomes in terms of disease onset, body weight, histopathological lesion and lethality, we decided to administer the dose at 400 PFU that systematically induced an efficient mice brain infection and to enable identification of subtle clinical changes associated to an impairment of rSBV replicative abilities.

Based on these settings, an experiment was designed to assess the pathogenicity of the SBV mutants in comparison with rWT-SBV. rSL5/3p-, rSLloop-, and rGCmid-SBV induced 95% to 100% mortality rate within 6 – 8 d.p.i, similarly to rWT-SBV. The other four recombinant SBV, namely rSL7nt- and rSL5nt-, rGCTT- and rGCCG-SBV, proved to be attenuated with mortality rates of 75%, 10%, 10%, and 0%, respectively, at 15 d.p.i (Fig 5A). Two of the attenuated viruses (rSL7nt- and rSL5nt-SBV) induced a decrease of weight gain starting from 9 d.p.i until the end of the experiment (Fig 5B). Moreover, rSL7nt-SBV differed from the rWT-SBV in terms of symptoms, consistent with a vestibular syndrome (head tilt and circling), as well as a delayed disease onset and long duration, beginning from day 7 to day 13 post-infection (Fig 5C). The attenuated phenotype was even more pronounced in animals infected with rSL5nt-SBV, as evidenced by the weight gain, the clinical scores, and the disease onset during the observation period (Fig 5B–5C).

Histopathological analysis at 5 d.p.i. was performed using predetermined criteria based on tissue lesions (inflammation, neuronal injury, hemorrhages) and severity scores (S2 Table and Fig 6A). SBV-induced lesions were distributed across different regions of the encephalon following unilateral I.C injection into the ventricle. The brainstem was the most affected region, followed by the midbrain, the thalamus, and the cerebellum (Fig 6B). Total histopathological score matched the clinical score and mortality rates, with the highest scores induced by rWT-, rSL5/3p-, rSLloop-, and rGCmid-SBV. The two recombinants, rSL7nt- and rGCCG-SBV, induced mild lesions in a restricted number of brain regions. No histopathological changes could be identified following the infection with rSL5nt- and rGCTT-SBV. Altogether, the virulence pattern of the tested recombinants in an *in vivo* setting reflected the *in vitro* phenotypes.

Besides assessing the neuropathogenicity of the whole series of recombinants, the production of infectious viral particles was measured in mice brain homogenates on the one hand, and by measuring vRNA production on the other hand (Fig 7A–7B). Infectious virus production mirrored the virulence level of the SBV mutants. While recombinant SBV with intact SL structure (rWT-, rSL5/3p-, rSLloop) or intact GC dinucleotides (rGCmid) showed a gradual increase of the viral production peaking at 5 d.p.i, attenuated viruses displayed decreased and delayed virus production. Viral loads found in

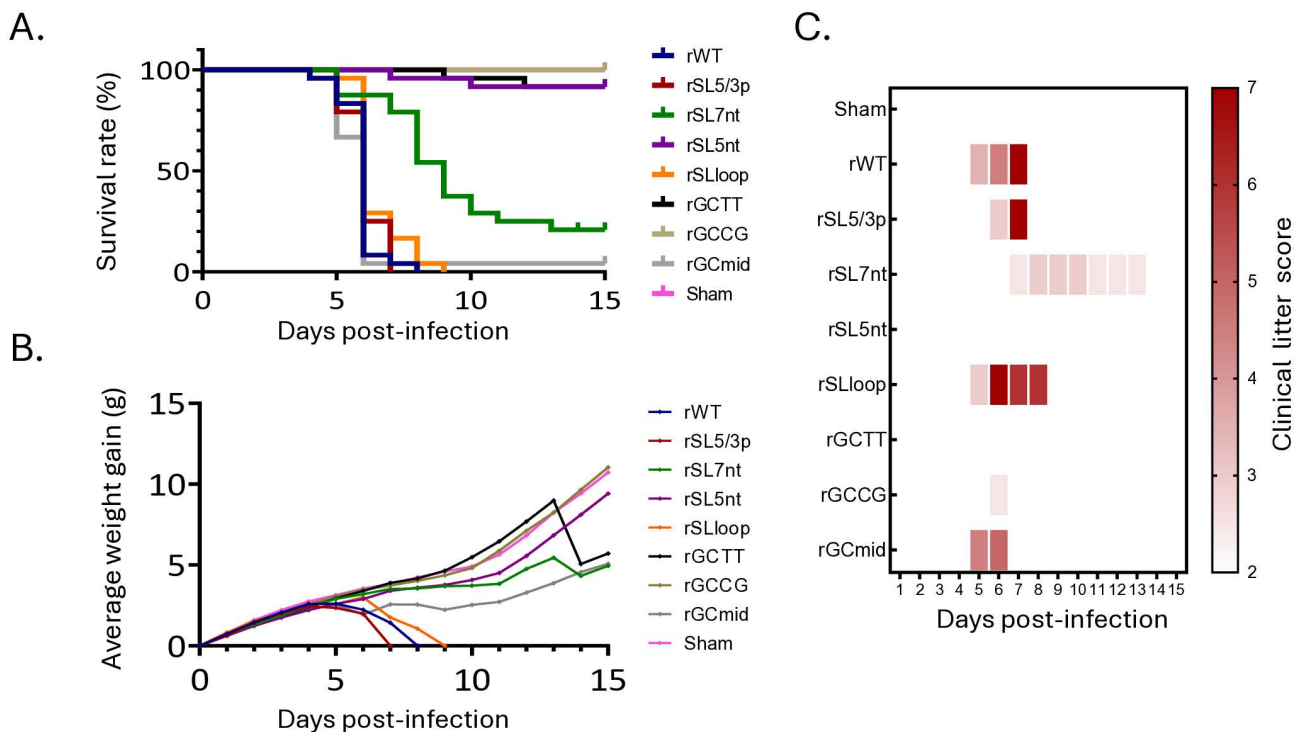


Fig 5. Health-related outcomes in a mouse infection model. Comparison of **A)** survival rate, **B)** average pup weight gain and **C)** clinical score of pups inoculated I.C at PND7 with 400 PFU. All the groups (n = 12) were monitored for 15 days. Survival rates were plotted as Kaplan-Meier graphs. Weights represent average pup weight, calculated by dividing total litter weight by number of survival pups. Weight data was normalized to day 0 base-line (set to 0g) to show pup average weight change over the infection course. The data presented are the mean from two independent experiments.

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infected mouse brains with rSL7nt- and rSL5nt-SBV were 10-fold and 10000-fold lower than the rWT-SBV, respectively. In addition, new progeny virus production appeared delayed in the brains of mice infected with these two recombinant viruses.

Regarding rGCTT- and rGCCG-SBV, hampered replication was observed, as evidenced by the impossibility of detecting infectious particles. However, before the 1 d.p.i, there was a 100-fold increase in the S segment vRNA level. Afterwards, the RNA curves remained steady from 1 to 14 d.p.i, indicating active yet limited transcription. A similar trend was seen when the L segment RNA load was assessed (S4 Fig).

SL and GC motifs impact N protein abundance and processing of the mRNA 3' extremities in the brain of SBV-infected mice

Through immunolabelling in the mouse brains at 5 d.p.i, a semiquantitative approach assessed the N protein abundance by estimating the immunoreactive cells (Fig 7C). The rWT-SBV condition obtained the highest scores across all the anatomical regions evaluated, with particularly intense labeling of the N protein observed in the midbrain, thalamus, and the brainstem (Fig 7D). In contrast, brains from mice infected with rSL5nt-, rGCTT-, and rGCCG-SBV never displayed labeled cells in any replicates. The conditions rSL5/3p-, rSLloop-, and rGCmid-SBV, despite having the same disease outcome as the rWT, exhibited significantly lower immunoreactivity, mainly in the thalamus, midbrain, and cerebellum, indicating reduced viral spreading. Strikingly, rSL7nt-SBV obtained higher scores than highly virulent recombinants in two compartments of the encephalon, namely the cerebellum and brainstem.

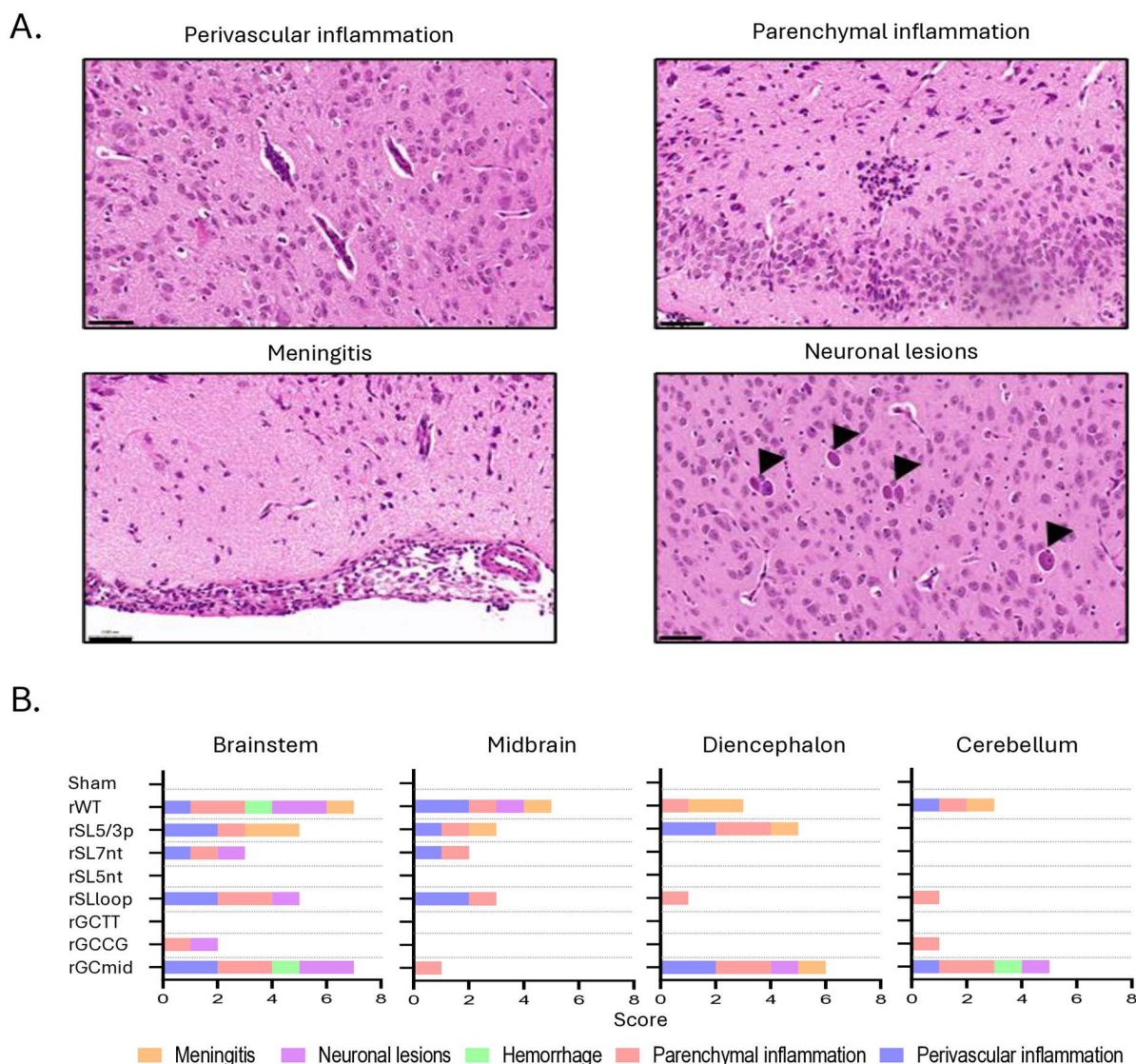


Fig 6. Pathogenic properties of SBV recombinant viruses. Histological examination of infected brain parasagittal H&E sections at 5 d.p.i. **A)** Representative examples of the four tissue lesions scored in the semiquantitative assessment. Inflammatory infiltrates consist of lympho-histiocytic cells invading the meningeal layers, perivascular spaces, or the brain parenchyma. Neuronal lesions are characterized by perikaryons with intense cytoplasmic and nuclear eosinophilia (arrowheads)(right bottom panel). Scale bars=0.05 mm. **B)** The cumulative histopathological scores for each region-of-interest are shown according to the rSBV viruses. Lesions were scored from 0 to 2 according to the tissues lesions severity in a blind manner.

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Eventually, the characterization of the SBV mutants was completed by determining RNA termination patterns of the S segment from the mouse brains infected at 5 d.p.i (Fig 8). The 3'UTR of the S segment positive transcripts revealed two distinct profiles, as found *in vitro*, with a larger product corresponding to cRNA and a truncated product consistent with mRNA. Strikingly, the smaller product was the predominant profile identified from *in vivo* infected samples. For the three viruses with intact SL structure (rWT-, rSL5/3P-, and rSLloop-SBV), 90% to 100% of the transcripts showed termination sites localized between SL and GC motifs (Fig 8A). The mouse brains infected with rSL7nt-, rSL5nt-, and rGCmid-SBV displayed a decreased proportion of shortened RNAs of about 82%, 66%, and 86%, respectively (Fig 8A-8B). In infected

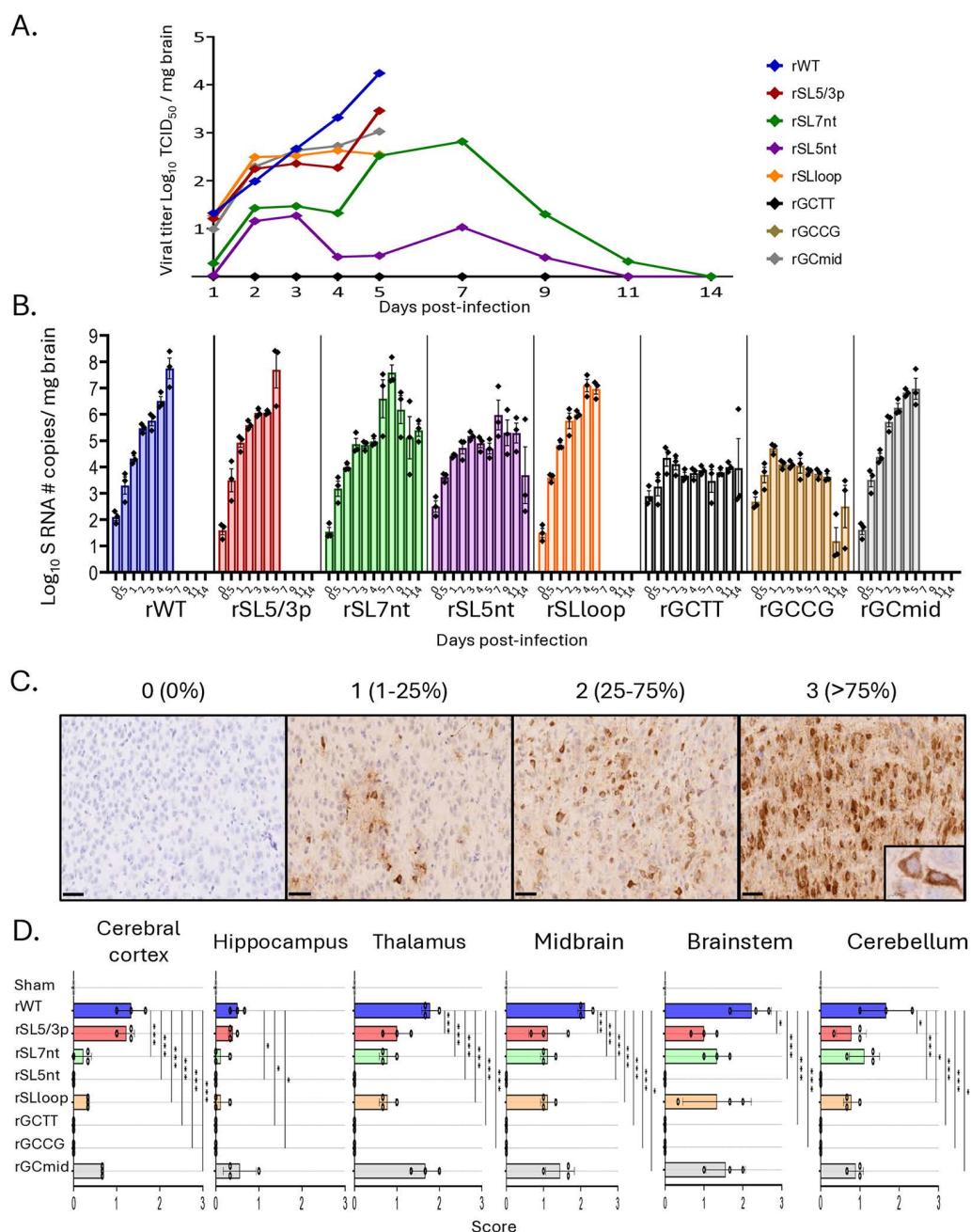


Fig 7. SBV replicative levels and N protein abundance in the mice brains. **A)** Infectious viral titers and **B)** viral S segment RNA load were measured on a daily basis, from the brains of three mice at each time point. Points represent the data of each replicate, and error bars represent the SEM. The data was collected from two independent experiments. **C)** Semiquantitative scoring criteria for IHC assessment. The scoring was based on the neuronal cytoplasmic labelling of the N protein (right corner inset), where a negative immunoreactivity is 0, $\leq 25\%$ of immunoreactive cells = 1, $\leq 75\%$ of immunoreactive cells = 2, and $>75\%$ of immunoreactive cells = 3. Parasagittal brain sections were analyzed at 5 d.p.i. The nuclei were counterstained with hematoxylin. Scale bars = 0.05 mm. **D)** Semiquantitative N protein expression across brain regions for each rSBV. Samples were assessed by three blind evaluators. The mean $\pm$ SD are plotted. Differences in means were analyzed using one-way ANOVA followed by Dunnett multiple comparisons. Asterisks indicate the level of significance when compared against the rWT-SBV (* $p < .05$; ** $p < .01$; *** $p < .001$).

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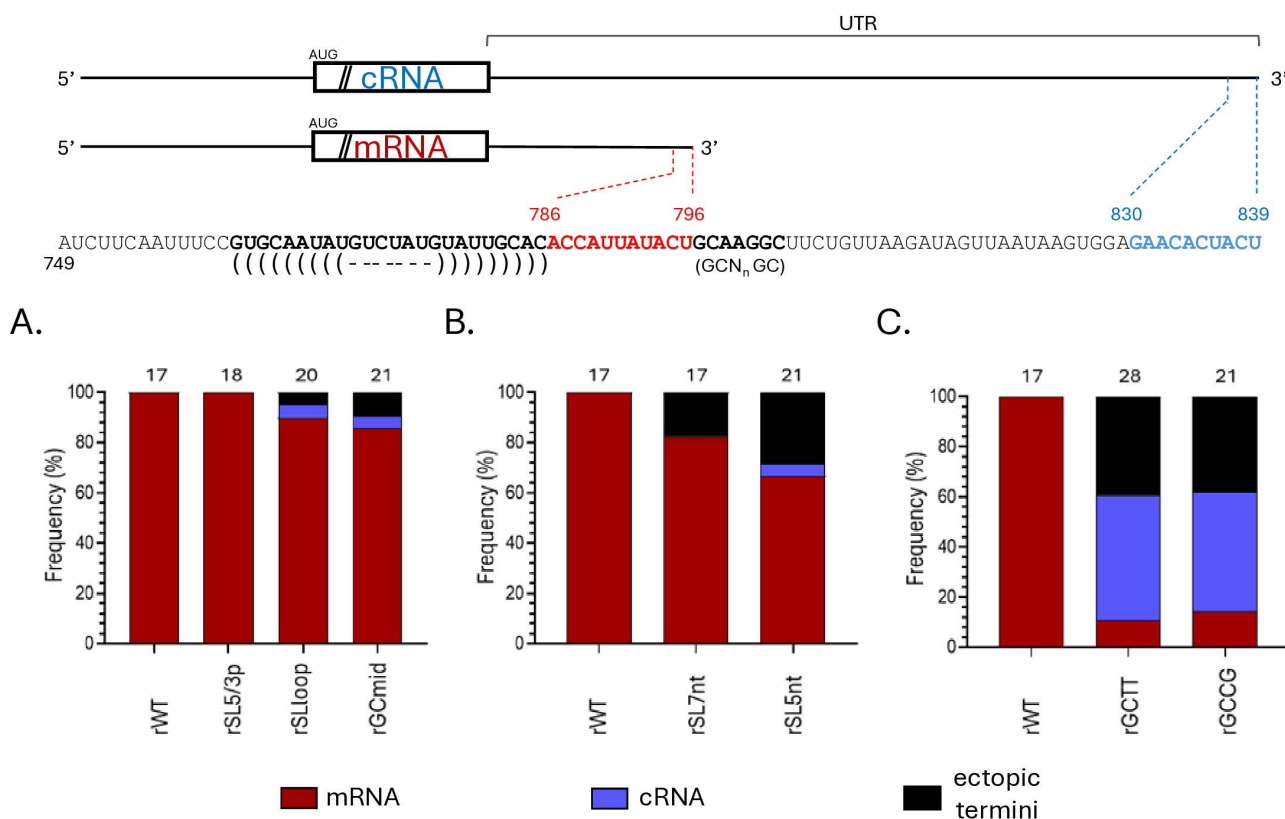


Fig 8. SBV S segment termination profiles in mice brains. The WT SBV cRNA and mRNA 3'UTR sequence is presented on the top panel. The SL structure is noted in the Vienna format below the corresponding sequence. Nucleotides in bold indicate the RNA motifs. Nucleotides in red represent the mRNA termini and the blue nucleotides the cRNA termini. Termination profiles are compared between rWT-SBV and mutants with **A)** equivalent fitness, **B)** attenuated phenotypes due to reduced stem length, and **C)** attenuated phenotypes due to GC signal substitutions. Mouse brains were collected at 5 d.p.i. The 3' RACE method detects mRNA termination sites through adapter-primed RT-PCR followed by cloning and Sanger sequencing of the 3' ends. Numbers above the bars indicate the clones sequenced per virus. The stacked bar graphs show the proportions of clones corresponding to cRNA, mRNA, or transcripts with ectopic termination sites.

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brain tissues with rGCTT- and rGCCG-SBV the mRNA profile was found in <15% of the clones evaluated. Instead, a higher proportion of transcript with ectopic termination sites were observed (Fig 8C).

Discussion

This study explored the functional implications of *cis*-regulatory elements in the 5' UTR of the SBV S segment gRNA or the 3'UTR S derived cRNA and mRNA, building upon previous work identifying novel mRNA termination mechanisms in simbuviruses [16]. Through mutational analysis, reverse genetics, and replicative assays, we confirmed the roles of the SL structure and GC signal in virion production, mRNA transcription and termination, the timing of the transition between RNA transcription and replication, N protein accumulation, and virulence. Our results demonstrated the indispensable nature of the SL structure for efficient virion production. Decreasing the stem length significantly impairs vRNA synthesis and protein expression. Thus, conferring replicative disadvantages *in vitro* and *in vivo*, highlighting the importance of the RNA structural motif in regulating several viral replicative cycle stages. In contrast, the termination signal was not essential for SBV, but we validated it as a trigger for mRNA transcription termination, with the conserved nucleotides playing an indispensable role. Interestingly, rSL5nt-SBV obtains a similar profile to the GC mutants, even

containing the standard GC signal, suggesting a synergistic function between both regulatory elements for transcription termination.

Several differences in methods may explain why our findings differ from those of earlier works regarding the motifs identified and their functions during the replicative cycle. Barr et al. (2006) employed a BUNV minireplicon system, while Blakqori et al. (2012) and our group used recombinant rescued viruses. Considering the later strategy no impact of the mutation can be attributed to the step depending on the T7 polymerase. Indeed, after the rSBV were rescued, all observed phenotypes and related sequences are the consequence of the viral RdRp processing. This includes the appropriate stoichiometric ratios and spatial distribution of L and N proteins expression, RNP assembly dynamics, and the temporal balance between RNA transcription and replication [21,22]. Moreover, the mutation strategies in earlier studies were based on scrambled sequences and deletions [21–23,28]. In contrast, the current research involved a detailed structural mutational analysis of the SL and the termination signal through point mutations.

Our study significantly expands existing understanding of S segment *cis*-regulatory elements by implementing an approach that includes kinetics in mammalian infection contexts and in midge cells, as it permits the observation of their functionality under varying selective pressures and host environments. Arboviruses need to balance replication efficiency with vector survival for successful transmission. The absence of significant differences between rWT-SBV and the other recombinant viruses in KC cells, along with significant attenuation in mammalian cells, indicates that these conserved RNA motifs might confer host-specific regulatory functions. Though the conservation of these RNA motifs suggests evolutionary advantage across host systems [29]. KC cells respond to SBV infection by producing 21 nt viral siRNAs through the exogenous RNA interference pathway, leading to reduced viral protein production and viral titers [30]. The biphasic growth kinetics observed in KC cells may reflect the establishment of persistent infection. In these cases, viral replication remains low, ensuring long-term infectivity throughout the vector's lifespan and possibly vertical transmission [31,32].

An observation across our experiments is the uncoupling between the impact of the motifs on N protein measurements and their impact on the 3' RNA termination profile. For example, rSL5nt yields 0% correctly terminated mRNA, but still produces N protein, though at a much lower level than rWT-SBV. This suggests potential technical limitations of the 3'RACE PCR, while effective for mapping the termination sites, may not capture all vRNA populations due to a limited sample size. Hence, the strand-specific RT-qPCR results aligned with the tendency observed at late time points of infection, where the amount of positive transcripts is mainly determined by the amount of cRNA, not of mRNA.

The mouse model further enables the evaluation of these elements' contributions to viral kinetics and pathogenic outcomes, which cannot be adequately modeled *in vitro*. Virulence is intricately tied to the virus capacity to infect permissive cells, manipulate cellular functions, evade immune surveillance, invade host cells, and induce tissue damage. Genetically engineered attenuated viruses exhibited a pronounced replicative deficiency in the current animal model. The dysfunction was associated with delayed disease onset and higher survival rates, potentially linked to increased mRNA misprocessing. All in all, the early transcription defects induce a misregulation of the composition of vRNA transcripts during SBV infection leading to decreased N protein accumulation and subsequently may impact viral genome packaging giving rise to an attenuate phenotype.

The mouse model provided valuable insights into SBV neuropathogenicity, mirroring the histological findings of Varela and peers (2013) [33]. Notably, the rSLloop- retained rWT-SBV virulence and clinical presentation despite regional N distribution differences, whereas SL7nt brain dissemination is also altered, it exhibited reduced mortality and altered clinical symptoms, suggesting these observations reflect overall viral attenuation rather than the motifs mediating neurotropism. The observed regional differences in viral N protein distribution between mutants should be interpreted cautiously, given confounding factors inherent to the experimental model. The first two postnatal weeks of rodent brain development are characterized by diverse events, including neurogenesis, cellular migration, programmed cell death, and synaptogenesis within a rapidly expanding brain volume with significant regional heterogeneity [34]. Furthermore, traumatic brain injury can produce neuronal apoptosis with different interregional vulnerability, suggesting that even mild tissue disruption from

intracranial injection may create region-specific responses [35]. The absence of stereotactic guidance during viral inoculation can introduce additional variability.

The absence of poly(A) tails in viral transcripts, though uncommon among eukaryotic mRNAs, is not unprecedented in certain RNA viruses, as observed in Flaviviruses (e.g., DENV & WNV), Bromoviruses (e.g., Alfalfa mosaic virus & Brome mosaic virus), Tymovirus (e.g., Turnip yellow mosaic virus), and Reovirus [36–38]. The ambisense ssRNA *Arenaviridae* and *Phenuiviridae* intergenomic region structures contribute to this diversity [39]. The current *cis*-regulatory elements are conserved across different orthobunyavirus serogroups, which suggests that this element has been under strong selective pressure throughout the evolution of these viruses, potentially broadening the generalizability of the findings. Thus, exploring the functional implications of these elements across more viral species is essential.

Translation of BUNV-like mRNAs relied heavily on the presence of eIF4G but was independent of PABP, implying that the 3' UTR either binds directly to eIF4G or to some yet-to-be-identified cellular factor that interacts with eIF4G [23]. Identifying viral or cellular binding partners for these regulatory elements could shed light on the regulatory interactions governing viral gene expression and indirectly pathogenesis. Lately, studies on poly(A) independent models such as the Andes Orthohantavirus and Rotavirus have led to the identification of host RNA binding proteins (e.g., Mex3A and ATP5B) interacting with motifs in the 3'UTR, playing key roles in the viral replicative cycles [40,41]

The identified *cis*-regulatory elements may be RNA targets, opening new avenues for antiviral therapy development, including RNA-based therapeutics or live attenuated vaccines [42]. The current reverse genetic system has also been employed for live attenuated vaccine development by deleting non-structural protein genes (NSs and NSm) [43]. The genetic stability through 10 passages is remarkable. Despite a significant decrease in fitness, the lack of reversion or compensatory mutations suggests that restoring function would require multiple nucleotide changes (reversing 4–8 nt substitutions), which are probabilistically unlikely to occur simultaneously. Alternatively, the severely attenuated recombinants maintain sufficient replication to passage without extinction, but not enough rounds to select and amplify beneficial mutations, regardless of a high selection pressure. This genetic stability should be thoroughly addressed when searching for vaccine development [44].

Altogether, we have demonstrated the non-coding RNA motifs' roles in viral transcription, vRNA synthesis dynamics, 3' mRNA termination, viral load and indirectly neuropathogenesis. This study has expanded our knowledge of the mechanisms governing SBV gene expression, viral fitness, and pathogenesis, paving the way for future interdisciplinary collaborations and integrative approaches. These efforts aim to provide a comprehensive understanding of viral molecular interactions and facilitate the development of effective countermeasures against infections caused by members of the Simbu serogroup.

Materials and methods

Ethics statement

All the experimental protocols and handling procedures were conducted in compliance with the European Communities Council Directives for Animal Experiments (2010/63/EU and directive 86/609/EEC) and were reviewed and approved by the Animal Ethics Committee of the Namur University (Permit numbers: UN VA 22/384, UN VA 22/394, UN MU 23/406).

Cell and virus

BHK-21 fibroblasts (ATCC CCL-10) were maintained in Glasgow Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, tryptose phosphate broth (2.95 g/L), Penicillin (100 U/mL), and Streptomycin (100 µg/mL). BSRT-7 cells kindly provided by Dr. Vitour were employed on the reverse genetic system, as they constitutively express the T7 polymerase. BSRT-7 cells were supplemented with G418 (1 mg/ml) every five passages. Both cell lines were cultured at 37°C in an atmosphere containing 5% CO₂. KC cells, obtained from *Culicoides sonorensis*, were maintained in Schneider

medium (Gibco) supplemented with 10% FBS, amphotericin B (2.5 µg/ml) and gentamycin (25 µg/ml). KC cells were cultured at 25°C without additional CO₂.

The German BH80/11–4 strain was employed as the reference template (GenBank accession numbers S: HE649914, M: HE649913, L: HE649912). Each full-length cRNA S, M, L WT SBV segments were cloned inside pUC57 plasmids, kindly provided by Dr. Massimo Palmarini. Each viral sequence is flanked by a 5' T7 promoter and 3' Hepatitis d ribozyme and T7 terminator.

Rescue of recombinant viruses

Mutation of the WT S pUC57 3' UTR was achieved by site-directed mutagenesis, targeting different positions within the sequence of the two elements. To that end, a two-step PCR approach was performed with the specific primers ([S3 Table](#)). PCR assays were carried out with a high-fidelity DNA polymerase (Q5 - New England Biolabs Inc). The protocol encompasses a polymerase activation at 98°C for 30 s, followed by 35 cycles including a denaturation at 95°C for 15 s, annealing at 63 °C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR mixes were composed of Q5 reaction buffer, dNTPs 0.2mM each, 0.5µM primers, and 0.02u/µL Q5 polymerase. PCR amplicons were purified with the NucleoSpin gel and PCR clean-up kit (Macherey-Nagel). The overlapping PCR involves mixing the bisected segment amplicons, launching the first 5 cycles without primers, allowing them to anneal, and leading to the full-length amplicon to be amplified throughout the primed steps. After digestion with *Bam*HI and *Kpn*l, the mutated antigenomic inserts were ligated into a dephosphorylated pUC57 vector linearized with the same enzymes. After molecular cloning and amplification in *E. coli* TG1, plasmid DNA was purified by using NucleoBond Xtra Midi Kit (Macherey-Nagel). The plasmids were sequenced (Eurofins Genomics) to confirm the desired mutations and their proper localization within the vector.

A total of 5*10⁵ BSRT-7 cells were seeded on 6-wells plates the day before. Then, transfection of 1µg of each WT M and L pUC57 plasmid DNA and the corresponding S pUC57 was performed with Lipofectamine 2000 reagent according to the manufacturer's protocol (Thermo Scientific). After 5 days post-transfection, supernatants were collected and inoculated on BHK-21 cells. BHK-21 cells were monitored to evaluate the rise of cytopathic effect (CPE), reflecting the production of progeny recombinant virus, as previously described by R. M Elliott and collaborators (2013) [25]. Since no plaque picking was applied, the viral stocks obtained from the reverse genetic system do not represent populations deriving from a single clone. The viral working stocks were produced from the second passage in BHK-21 cells, then clarified by low-speed centrifugation, and aliquots were stored at –80°C until titration and further processing.

RNA analysis

All available sequences of the orthobunyaviruses [S 3'UTR](#) were extracted from the GenBank database (www.ncbi.nlm.nih.gov). A total of 47 sequences were obtained ([S4 Table](#)), and multiple sequence alignments were done in Geneious Prime 2020.0.3 software. The secondary RNA structure predictions were generated using the RNAfold server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>).

Viral plaques assay

Semiconfluent monolayers of BHK-21 cells in six-well plates were infected and overlaid with 0.35% SeaPlaque Agarose supplemented maintenance medium (Lonza). After 4 days of incubation at 37°C in 5% CO₂. Paraformaldehyde (4% final concentration) was added and incubated for 30 minutes to fixate the cells. Then, the overlay was removed before being counterstained with crystal violet solution. The assay was performed in three independent replicates. For each replicate, 50 randomly selected plaques of each rescued virus were measured using ImageJ software (<http://rsb.info.nih.gov/ij/>). The Kruskal-Wallis test was applied to compare the rWT-SBV area against the other recombinant viruses per replica.

Viral titration

Growth kinetics were assessed for all viable recombinant viruses compared to the rWT-SBV. BHK-21 and KC cells with 80% confluency were inoculated at a MOI of 0.002. Supernatants were recovered at 0, 24, 48, and 72 hours post-infection (h.p.i) and stored at -80°C . Centrifugation at 5000g for 5 min was applied during brain tissue preparation. The BHK-21 cell line was used to determine the viral titer by an end-point dilution assay of both growth kinetic experiments (BHK-21 and KC cells). The CPE was assessed and counted on 96-well plates (6 replicates by dilution), and the titers were displayed as 50% tissue culture infectious dose per mL ($\text{TCID}_{50}/\text{mL}$), according to the Reed and Muench method. The limit of detection corresponds to 20 $\text{TCID}_{50}/\text{mL}$.

RT-qPCR

RNA isolation was performed using the QIAmp Viral RNA kit (Qiagen), while RNA isolation from 30mg of brain homogenates was done using the NucleoSpin RNA kit (Macherey-Nagel), following the manufacturer's instructions. The specific primers and probes for each SBV genomic segment are reported in [S3 Table](#). The RT-qPCR assay was performed employing the Takyon One-Step No Rox Probe 5X MasterMix dTTP kit (Eurogentec). The thermal profile was 48°C for 10min preceding 1 step at 95°C for 3 min, followed by 40 cycles at 95°C for 15s, 58°C for 20s, and 72°C for 30s, on the thermocycler Biorad CFX96. For quantifying the genomic load, standard curves were performed by using serial dilutions (from 10^1 to 10^9 copies/ml) of the plasmids S, M, and L WT pUC57. The efficiency of the different RT-qPCR assays targeting S, M, and L segments was determined as 99.91%, 98.97%, and 98.19%, respectively.

Western blotting

Briefly, total cellular protein samples were prepared from cells rinsed with PBS (pH 7.4) and lysed on ice for 30 min in RIPA lysis buffer (150mM NaCl, 5mM EDTA, 50mM Tris HCL, 1% NP- 40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with 1mM phenylmethanesulfonyl fluoride, 1% triton X-100 (Merck), and 1X protease inhibitor cOmplete (Roche). Proteins were separated by electrophoresis in 10% polyacrylamide gels and afterward actively transferred to a nitrocellulose membrane (Whatman Protran BA85, GE Healthcare Life Sciences). The membranes were blocked by incubation with 5% Bovine serum albumin on Tris-saline buffer plus 0.1% Tween-20 and probed with the following primary antibodies: in-house rabbit polyclonal antiserum against the SBV N protein diluted 1:2000, and monoclonal mouse anti-beta-actin diluted 1:3000 (58169, Cell Signaling Technology). Peroxidase-labelled secondary antibodies against rabbit and mouse were used in a dilution of 1:2000. The immunoreactions of interest were detected using Pierce ECL Western Blotting substrate (Thermoscientific) and an ImageQuant LAS4000 camera system (GE Healthcare Life Sciences). Beta-actin was used to demonstrate equal protein loading. Image J software will be used to calculate the relative signal intensities for the SBV N protein relative to the loading control.

3' RACE PCR

BHK-21 cells were infected with an MOI of 0.002. Supernatants were collected and clarified at 72 h.p.i. The viral RNA isolation was performed using the QIAmp Viral RNA kit (Qiagen), while RNA isolation from 30mg of brain homogenates (5 d.p.i.) was done using the NucleoSpin RNA kit (Macherey-Nagel) following the manufacturer's protocol. Poly (A) tailing of 5 μg of vRNA was performed using *E. coli* Poly(A) Polymerase (5 U - New England Biolabs Inc) and ATP (0.2 mM - New England Biolabs Inc). The poly-A-tailed vRNA was then purified with phenol:chloroform and followed by a reverse transcription using the GeneRacer OligodT primer (50 μM), and the SuperScript IV (Invitrogen). 3' Rapid amplification of cDNA ends (3' RACE) PCR was carried out using the GeneRacer Kit (Invitrogen). The first PCR was performed using 0.2 μM of gene-specific primer and 0.6 μM of 3'GeneRacer primer. The PCR products were subjected to a 1:10 dilution and used as template for the following nested PCR using 0.2 μM of the nested gene-specific

primer and 0.2 μ M of the 3'Nested GeneRacer primer ([S3 Table](#)). Both PCRs were performed using green GoTaq reaction buffer, dNTPs 0.2 mM of each, GoTaq DNA Polymerase 0.025 u/ μ L (Promega). All the primers are listed in the [S3 Table](#). The thermal profile of the reaction was as follows: 94°C for 3 min, then 35 cycles comprising 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. The final PCR products were gel purified, cloned into pGEM-T Easy Vector (Promega), and at least 15 randomly selected clones of each recombinant virus were sequenced by Sanger sequencing (Eurofins Genomics). Data analysis was carried out on Geneious Prime 2020.0.3

Strand-specific RT-qPCR

BHK-21 cells at 80% confluency were inoculated at a MOI of 0.02. After virus absorption at 37°C for 2 h, cells were rinsed with PBS (pH 7.4) and fresh medium was added. Cell lysates were recovered at 0, 2, 4, 8, 12, 16, 20, and 24 h.p.i. Virus-infected cells were lysed using the lysis buffer of the NucleoSpin RNA kit (Macherey-Nagel) and stored at –80°C until processing. Total RNA was extracted following the manufacturer's instructions and the concentration adjusted to 100ng/ μ L.

Strand-specific cDNA synthesis was performed using the Superscript IV First-Strand Synthesis system (Invitrogen) and specific RT primers for the negative, and the positive vRNA reported on the [S3 Table](#). Noteworthy, is the fact we cannot discriminate between positive sense vRNAs (cRNA and mRNA), as the mRNA sequence is completely contained in the cRNA. For each reaction, 1 μ g of total RNA was mixed with strand-specific RT primer (2 μ M), and dNTP mix (10 mM each). The mixture was incubated at 65°C for 5 min and then cooled to 4°C. After addition of the reaction buffer (5x), DTT(100mM), RNaseOUT (20 U) and Superscript IV reverse transcriptase (100 U), cDNA synthesis was performed at 55°C for 10 min, terminated at 80°C for 10 min, cooled to 4°C, and then treated with RNase H (2.5 U, New England Biolabs Inc) at 37°C for 30 min.

Quantitative PCR was performed using previously reported SBV S segment primers and probe (Fwd S qPCR, Probe S and Rev S qPCR), common to the three types of viral RNA, ([S3 Table](#)). The efficiency of the S segment qPCR was determined as 99.91%. Reactions used the Takyon No Rox Probe 5X MasterMix dTTP kit (Eurogentec) and 100 ng of specific cDNA. The thermal profile initiated 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s, on the thermocycler QuantStudio 1 system (ThermoFisher scientific).

Animals and experimental design

RjOri: SWISS breeders were sourced from a commercial laboratory (Janvier Labs, Le Genest-Saint-Isle, France). Litters were produced inside the rodent facilities of the Technology Platform of Life Sciences, University of Namur, Belgium. The study design and methodology, including the mouse model, age, inoculum volume, and intracranial injection procedures, were derived from previous studies that investigated the pathogenicity of SBV NSs lacking mutants [[33,45](#)]. Four studies were performed in 7 days old suckling mice. The intracranial injections were performed using microliter syringes and 33g needles (Hamilton Neuros syringe NRS 1705 RN) with a fixed volume of 10 μ L, administered 0.25mm lateral from the sagittal suture, 0.5mm rostral to the coronary suture, and 2mm beneath the skull entry site. Two independent biological replicates were performed in the study. The suckling mice that reached the humane endpoints were euthanized. The sham groups were inoculated with BHK-21 cell culture maintenance medium.

Study A was a dose-dependent assay. Litters of 12 pups were randomly distributed into four groups: a sham-inoculated group and three groups intracranially inoculated with rWT-SBV, testing different viral doses (200,400, and 800 PFU).

Study B consists of an interventional trial in which litters of 12 pups were randomly distributed into nine groups, testing the different rSBVs and comparing them with a sham-inoculated group. Moreover, the survival rate, clinical score, and weights were evaluated daily for 15 days. The mice that survived the disease progression were euthanized at the end of the study.

Study C was conceived as a growth kinetics experiment in which litters of 15 pups were randomly distributed into 9 groups: a sham-inoculated group and the others inoculated either with rWT-SBV or 7 different SBV recombinants. After the intracranial inoculation, 3 pups of each group were euthanized at each fixed time point (2, 12, 24, 48, 72, 96, and 120 h.p.i.). Brain samples were collected and stored until histology, immunohistochemistry, viral titration, and RT-qPCR were further analyzed. The pups who reached the humane endpoints outside the sampling time points were euthanized.

Finally, study D comprised five groups (n = 15), including a sham-inoculated group and four groups intracranially inoculated with the viruses, namely rSL7nt, rSL5nt, rGCTT, and rGCCG. The sampling points were 7, 9, 11, and 14 d.p.i. At each time point, 3 suckling mice were euthanized, and the brains were aseptically harvested to evaluate the viral titer and the genomic load.

Histology and Immunohistochemistry

Brains were harvested and post-fixed overnight in paraformaldehyde 4%. Brain hemispheres were then dehydrated, paraffin-embedded, and cut into 6- μ m serial sections. Several paraffin sections were dewaxed, rehydrated, and stained with hematoxylin and eosin and subsequently evaluated under a light microscope. Each slice was imaged with the Panoramic Desk II digital slide scanner (3DHistech Ltd.), using the SlideViewer software

For immunohistochemistry of the N protein, serial paraffin sections were dewaxed, rehydrated and subjected to heat-induced epitope retrieval in basic Dako buffer (Agilent) at 100 °C for 10 min. Endogenous peroxidase activity was abrogated with 3% H₂O₂ in methanol for 10 min. To avoid non-specific binding, a blocking step in 5% goat serum diluted in TBS was done over 30 min. Sections were then incubated in an in-house anti-N SBV protein rabbit polyclonal antiserum, diluted (1:3000) in TBS with 1% serum, overnight at 4 °C. The next day, biotinylated anti-rabbit IgG antibodies (1:100; Vectastain, ABC Kit, USA) were incubated on tissue slices for 1 h at room temperature. Sections were then incubated with a solution of peroxidase-bound streptavidin (1:100-Vectastain) for 45 min. Immunoreactivity was revealed using 3, 3'-diaminobenzidine (Dako, Glostrup, DK). Finally, sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Using the Cell Sens software, each slice was imaged with an Olympus BX63 microscope. Three blind evaluators performed a semiquantitative analysis, evaluating the quantity of immunoreactive cells per anatomical region.

Data analysis

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com). Differences in means were analyzed using a two-way ANOVA followed by Dunnett multiple comparisons. Quantitative results were presented as the mean $\pm$ standard error of the mean (SEM). The Kaplan-Meier survival curves were analyzed using the log-rank (Mantel-Cox) test. The viral phenotype was analyzed by using the Kruskal-Wallis non-parametric test. The N protein expression on cells was evaluated via a tailed, paired Student's t-test where a p-value <0.05 was considered statistically significant. The ratios cmRNA:gRNA were calculated for each time point. For statistical comparison between viral recombinants against rWT-SBV, the area under the curve was calculated and then the total area was analyzed by one-way ANOVA. The semiquantitative IHC analysis was performed via one-way ANOVA followed by Dunnett multiple comparisons. Unless otherwise specified, quantitative results were presented as the mean $\pm$ standard deviation (SD) of three independent experiments.

Supporting information

S1 Fig. rSBV plaques area comparative analysis Kruskal-Wallis ranks plot of three independent replicates at 72 h.p.i on infected BHK-21 cells. Box and whisker plots displaying the statistical distribution of each data set. Asterisks indicated the level of significance compare against the rWT-SBV (*p < .05; **p < .01; *p < .001). (TIF)**

S2 Fig. *In vitro* RNA load kinetics of the rSBV library. A) BHK-21 cells and B) KC cells were infected with a MOI of 0.002. Culture supernatants were collected at the indicated times points. RT-qPCR analysis of total vRNA segments S (top panel), M (middle panel), and L (bottom panel). The report values are means from three independent experiments. Error bars represent the SEM. Differences in means were analyzed using a two-way ANOVA followed by Dunnett multiple comparisons. Asterisks indicated the level of significance compare against rWT-SBV (* $p < .05$; ** $p < .01$; *** $p < .001$).

(TIF)

S3 Fig. *In vivo* dose-dependent trial of rWT-SBV. Comparison of A) survival rate, B) average pup weight gain and C) clinical score of pups inoculated I.C at PND7 with rWT-SBV using three different doses. All the groups ($n = 12$) were monitored for 15 days. Survival rates were plotted as Kaplan-Meier graphs. Weights represent average pup weight, calculated by dividing total litter weight by number of survival pups. Weight data was normalized to day 0 baseline (set to 0g) to show pup average weight change over the infection course. The data presented are the mean $\pm$ SEM from two independent experiments.

(TIF)

S4 Fig. *In vivo* RNA load kinetics of the rSBV library. Quantitative RT-PCR analysis of vRNA segment L. In mouse brains infected with 400 PFU of the different rSBV. Samples were collected during a 14 days period. The reported values are means from three independent experiments. The error bars represent the SEM.

(TIF)

S1 Table. Litter clinical score parameters.

(XLSX)

S2 Table. Histopathological scoring.

(XLSX)

S3 Table. List of oligonucleotides.

(XLSX)

S4 Table. *Orthobunyavirus* genus members bearing both RNA motifs; GenBank accession numbers.

(XLSX)

S5 Table. Numerical data of the experiment performed in BHK-21 cells.

(XLSX)

S6 Table. Numerical data of the experiments performed in Swiss mice.

(XLSX)

S7 Table. Numerical data of the experiments performed in KC cells.

(XLSX)

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