Arenaviridae exoribonuclease presents genomic RNA edition capacity.

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20 Abstract

21 The *Arenaviridae* is a large family of viruses causing both acute and persistent infections
 22 and causing significant public health concerns in afflicted regions. A “trademark” of infection is the
 23 quick and efficient immuno-suppression mediated in part by a 3’-5’ RNA exonuclease domain
 24 (ExoN) of the Nucleoprotein (NP). Mopeia virus, the eastern African counterpart of Lassa virus,
 25 carries such ExoN domain, but does not suppress the host innate immunity. We have recently
 26 reported the crystal structure of the Mopeia virus ExoN domain, which presents a conserved fold
 27 and active site. In the present study, we show that the ExoN activity rules out a direct link between
 28 ExoN activity and alteration of the host innate immunity. We found that the Arenavirus ExoN,
 29 however, is able to excise mis-incorporated bases present at the 3’-end of double stranded RNA.
 30 ExoN(-) arenaviruses cultured in cells dampened in innate immunity still replicated in spite of a
 31 significant reduction in the viral charge over several passages. The remaining ExoN(-) virus
 32 population showed an increased base substitution rate on a narrow nucleotide spectrum, linking the
 33 ExoN activity to genome editing. Since, the Arenavirus ExoN belongs to the same nuclease family
 34 as that of the nsp14 coronavirus ExoN ; which has been recently shown to promote viral RNA
 35 synthesis proofreading; we propose that Arenavirus ExoN is involved in a “limited RNA editing”
 36 mechanism mainly controlled by structural constraints and a low mutational/fitness ratio.

37 Author summary

38 Only *Arenaviridae* and *Coronaviridae* encode a 3’-5’ RNA exonuclease domain (ExoN) in
 39 their genome. This activity is either used to counteract the innate immunity response during viral
 40 infection or to ensure genome stability during replication. Mopeia virus (MOPV), the eastern
 41 African counterpart of Lassa virus, carries such ExoN domain, but does not suppress the host innate
 42 immunity. We studied MOPV ExoN activity both *in vitro* and *in cellula* to assess the role of ExoN

MOPV and found that the Arenaviral ExoN is fully active on dsRNA, and is able like the one of *Coronaviridae* to excise a mismatched base. We measured genetic stability and found evidence of a limited spectrum of RNA synthesis proofreading mechanism, together with a strongly impacted viral replication. We propose that the Arenaviral ExoN is involved in a functional check of the conserved RNA structures of the viral genome.

Introduction

Arenaviridae is a family of viruses that cause chronic infections of rodents and constitutes a reservoir of human pathogens across the world [1]. Already with a global distribution Lymphocytic choriomeningitis virus (LCMV) is the prototypic member of the family; it is one of the most studied virus and still an underestimated threat to human health [2–6]. In South America, Machupo, Guanarito, Junin, Sabia, and Chapare viruses are responsible for hemorrhagic fever [7] while in Africa Lujo [8] and Lassa viruses (LASV) constitutes a major public health concern [9–13]. Indeed, LASV is responsible for several hundred thousand infections per year alone [14]. It is a common endemic infection in West Africa (Sierra Leone, Guinea, Liberia, Nigeria) responsible for hearing loss, tremors and encephalitis [13,15]. Moreover, this endemic infection frequently spikes a high number of Lassa fever cases associated with significant mortality and high morbidity. The last episode started in February 2018, in the Niger delta region, presents a case fatality rate around 25 % [16]. This new epidemic reinforces the trends observed during the recent epidemics in Nigeria and Benin in January 2016 [17,18], indicating an increase in virulence, an expansion of spreading areas and the number of cases [19]. Humans become infected through contact with infected rodent excreta, tissues, or blood. Person-to-person transmission of Lassa fever can also occur particularly in the hospital environment in the absence of adequate infection control measures [20]. Until now, no licensed vaccine is available, and therapeutic options are limited to early administration of

67 ribavirin. Despite its public health significance, and recent major contributions [21–25],
68 *Arenaviridae* biology is still poorly understood.

69 *Arenaviridae* are negative-sense single-stranded RNA segmented viruses, with a genome
70 consisting of two segments L (~7.2 kb) and S (~3.4 kb). Each segment has an ambisense coding
71 mechanism, encoding two proteins in opposite orientation, separated by an intergenic region (IGR).
72 The L RNA segment encodes a large protein L (~200 kDa) and a small disordered protein Z (~ 11
73 kDa) [1]. L is a multi-domain protein including in its N-terminus an endonuclease domain followed
74 by a polymerase domain and in its C-terminus a cap binding like domain (for review [26]). Z, which
75 contains a RING finger motif, is a multifunction protein regulating the life cycle of the virus and
76 during budding assembles to form the matrix [27–29]. The S RNA encodes the precursor of mature
77 virion glycoprotein GP-C (75 kDa); that will give after post-translational cleavage GP-1 (40 to 46
78 kDa) and GP-2 (35 kDa) [30,31]; and nucleoprotein NP (~ 63 kDa) [25,32]. NP forms a polymer
79 protecting the genomic (and anti-genomic) RNA (RNA_v) [33]. L and NP together with RNA_v form
80 an active ribonucleic complex (RNP) for replication and transcription [34]. In addition to this
81 critical function, NP is involved in clearing off the cytoplasm of double stranded RNAs (dsRNA),
82 through its C-terminal exonuclease domain (ExoN) [25,32,35–37]. These dsRNAs are markers of
83 viral infection in the cell and are triggering host innate immunity response. Indeed, when dsRNA is
84 detected by proteins such as retinoic acid-inducible I (RIG-I) or melanoma differentiation-
85 associated 5 (MDA- 5), it initiates a signaling pathway that result in the translocation of interferon
86 (IFN) regulatory factor 3 (IRF-3) to the nucleus [38,39]. Then, IRF-3 activates the expression of
87 IFN- α/β , which initiates the antiviral response in the infected cells and primes neighboring cells for
88 a rapid response to viral invasion. From a modular (sequence and structure) perspective, all NP
89 presents a C-terminal ExoN domain (S1 Fig). The South Eastern African counterpart of Lassa Virus
90 is Mopeia virus (MOPV) [40], a non-pathogenic virus. The NP of these two viruses presents a high

91 sequence identity of about 73%, but contrary to Lassa virus, MOPV infection does not result in
 92 innate immunity suppression [41,42] leading us to suspect that the domain was not fully functional
 93 against $dsRNA$. Recent studies reported the structure of Mopeia ExoN domain [43] and evidence of
 94 an ExoN activity in NP of MOPV, essential for multiplication in antigen-presenting cells [44]. The
 95 observed fold conservation and activity raises questions about the biological role of the NP-exo
 96 MOPV, and whether it could be conserved for other functional or structural reasons [26]. In the
 97 DNA world, ExoNs are mainly involved in mechanisms of genome stability and error correction
 98 during or after DNA synthesis. Yet, in viral RNA world, the existence of ExoN is of rare occurrence
 99 as only two families of viruses possess a 3'-5' ExoN member of the DEDD super family:
 100 *Arenaviridae* and *Coronaviridae* [25,45–47]. The Coronavirus ExoN is part of the nsp14 protein,
 101 associated to the main replicative RNA-dependent polymerase nsp12. During RNA synthesis, nsp14
 102 belongs to the replication/transcription complex (RTC), excises mismatched bases occurring during
 103 processive RNA synthesis, and contributes to overall RNA synthesis fidelity [47–51].

104 Having noted the structural and functional relatedness of *Arenaviridae* ExoN to the
 105 *Coronaviridae* ExoN, we engaged into mechanistic studies of the MOPV ExoN. Here, we present a
 106 detailed characterization of the activity of NP-exo MOPV including substrate specificity and ion
 107 dependency, compared to the one of LCMV. We show that the *Arenaviridae* ExoN is active on 3'
 108 mismatched $dsRNA$ substrate mimicking a stalled RNA synthesis intermediate, in a remarkable
 109 substrate requirement similarity to Coronavirus nsp14. We report, however, that a mutated NP-exo
 110 MOPV abrogating the ExoN activity does not lead to an overall higher mutation rate in the
 111 surviving viruses, but rather drastically reduces the number of infectious viruses while increasing
 112 the release of non-infectious material from infected cells. Interestingly, few nucleotide substitution
 113 types appear to be significantly increased in the ExoN(-), establishing that ExoN is active on its
 114 own genomic RNA. All together these results confer potentially significant roles to the ExoN

115 domain in the *Arenaviridae* life cycle.

116 **Material and Methods**

117 **Cloning, mutagenesis, protein production and purification**

118 cDNA corresponding to NP ExoN domain of : MOPV (residues 365-570 - P19239), LCMV
119 (residues 357-559 - NP_694852) were cloned into the pETG20A expression vector using the
120 Gateway® method (Invitrogen), which adds a cleavable thioredoxin-hexahistidine tag at the N-
121 terminus. The integrity of the DNA construct was verified by DNA sequencing. The sequences of
122 the primers used to sub cloned each domain were:

123 LCMV forward:

124 GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGTTTAAGCT
125 ACAGCCAGACAATGCTTTTAAA ,

126 LCMV reverse:

127 GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTATGTCACATCATTGTTGGCCTCTA ,

128 MOPV forward:

129 GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGTTTAACCT
130 ACTCTCAGACAATGGA,

131 MOPV reverse:

132 GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTACAGGACAACCTCTGGGA.

133

134 Plasmids were used to transformed *E.coli* strain C2566 (NEB) protease-deficient and carrying
135 pRARE plasmid (Novagen). Bacteria were grown in LB medium (AthenaES) at 37°C to an
136 OD_{600nm} of 0.5. Expression was induced with 0.5 mM IPTG, and bacteria were grown shaking at

137 210 rpm overnight at 17°C in presence of 100μM of ZnCl₂. Bacteria were pelleted, frozen, and
138 stored at -80°C.

139 The three domains were purified at 4°C. Frozen pellet were melted on ice, resuspended in lysis
140 buffer (20mM HEPES pH7.5, 300 NaCl, 5 mM imidazole, 5% glycerol, 0,1 mg/ml lysozyme and
141 50 μg Dnase), sonicated, and the lysate was cleared by centrifugation at 20,000 rpm for 30 min.
142 Each protein was first purified by metal affinity chromatography using 2ml of His purTM cobalt
143 column (Thermo Scientific). The tag was removed by cleavage with TEV protease followed by
144 purification on a second cobalt affinity chromatography. Proteins were further purified by gel
145 filtration using superdex 75 column (GE Healthcare) in 20 mM HEPES pH 7.5, 300 mM NaCl, 2
146 mM MgCl₂ and 5% glycerol.

147 Mutants were generated for each domain by introducing single point mutations using the Quick
148 change site-directed mutagenesis kit (Stratagene). The primer sequences used for mutagenesis are
149 listed in the supplementary S1 Table. The presence of *ad hoc* mutations and the integrity of the
150 complete coding region of each mutant were confirmed by sequencing. All the mutants were
151 expressed and purified following the established protocol.

152 **RNA labeling and preparation**

153 Synthetic RNAs used in this study were purchased from Dharmacon or Biomers (HPLC
154 grade). They are listed in S2 Table and their predicted structures are shown in S2 Fig. All sens RNA
155 strands were labeled at their 5' end with [γ-³²P] ATP using protein nucleotide kinase (NEB)
156 according to the manufacturer's instructions. For experiments involving overhang mismatched
157 dsRNA, the dsRNAs were generated by annealing an anti-sense RNA strand containing 3'-phosphate
158 modifications with its 5'-radioactively labeled sens RNA strand. The annealing condition was,
159 heating at 70 °C for 10 min and then cooling down to room temperature (with a primer/template

ratio of 1.2:1).

Exonuclease activity assay.

Reactions were carried out in a buffer containing 20 mM Tris-Hcl, 5 mM MnCl_2 (unless specified by 5 mM of: MgCl_2 , CaCl_2 , or ZnCl_2) and 5 mM DTT. Standard reactions contained 0,25 μM of protein (NP-exo MOPV or LCMV or mutants) and 1,25 μM of radiolabeled RNA substrate. After incubated at 37°C, the reactions were quenched at intervals between 0 and 30 minutes by the addition of an equal volume of loading buffer (formamide containing 10mM EDTA). The products were heated at 70°C for 5 minutes, rapidly cooled on ice for 3 minutes followed by separation in a 20% poly-acrylamide gel containing 8 M urea and buffered with 0,5X Tris-borate-EDTA. Gels were exposed overnight to a phosphor screen and then visualized with a phosphoimager FLA-3000 (Fuji). Total RNA degradation products were quantified using Image Guage (Fuji), the speed of cleavage determined and graphs plotted using GraphPad PRISM version 6.0. Experiments were carried out at least in triplicate and only representative gels are shown.

Thermal shift assay.

A real-time PCR set-up (Bio-rad) was used to monitor the thermal unfolding of the ExoN domain of NP -MOPV or LCMV alone or in the presence of different divalent ions Mn^{2+} , Mg^{2+} , Ca^{2+} and Zn^{2+} . Proteins were equilibrated in a buffer containing 20 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol. All reactions were set up in a final volume of 25 μl in a 96-well plate with total protein concentration of 1,8 mg/ml, 1x SYPRO Orange and incubated with or without 5 mM of metal ions. The PCR plates were sealed with optical sealing tape (Bio-rad) and incubated in the PCR machine for 2 minutes at 20°C followed by 0,2°C increments to a final temperature of 95°C. Thermal denaturation was monitored using SYPRO Orange (Life Technologies) and the fluorescent

intensities were measured at 490 nm excitation and 530 nm emission wavelengths. The unfolding of proteins was monitored by following the increase in fluorescence of the probe as it binds to exposed hydrophobic regions of the denatured protein. The T_m was then calculated as the mid-log of the transition phase of the fluorescence curve using the Boltzmann equation. All measurements were performed in triplicates.

Structure and sequence analysis

Structure and sequence comparison of Arenavirus exonuclease with other viral exonuclease.

Structure similarities were search with PDBeFold [52] using the MOPV exonuclease domain as a search model (PDB : 5LRP). Corresponding sequences were aligned based on structure comparison using Expresso [53]. Figures were generated with the programs ESPript-ENDscript [54], WebLogo server [55] and UCSF chimera [56].

Sequence retrieval of mammarenavirus L and analysis.

All annotated complete protein sequences of Mammarenaviruses L protein were downloaded from NCBI. The dataset of 559 sequences was manually curated using Jalview [57] in order to remove Identical, mis-annotated or complete sequences with undefined amino acid (X). The remaining 395 sequences were aligned using MUSCLE [58] constituting the *Mammarenavirus* (MAMV) dataset. From this latter two dataset are being derived the Mopeia virus dataset of 12 sequences, and the Lassa Virus dataset of 277 sequences, as being the closest homologue of Mopeia virus with a large number of sequences. Amino acid composition (%) for position in the sequence corresponding to the Mopeia emerging mutant were calculated with Jalview and represented using WebLogo [55] for the three datasets.

203 **Viral infection and genome sequencing.**

204 The MOPV strain AN21366 (JN561684 and JN561685) was used to establish the reverse
205 genetics system for MOPV. The detailed procedures for virus rescue, production, titration and
206 infection of Vero E6 cells (VERO C1008 [Vero 76, clone E6, Vero E6] ATCC® CRL-1586™
207 (ATCC, LGC Standards, Molsheim, France) are described in [44]. In brief, the recombinant NP-exo
208 WT and mutant (D390A/G393A) MOPV were used to infect Vero E6 cells using a MOI of 0.01.
209 Supernatants were collected four days post infection. Viral production/titration/infection were
210 repeated iteratively for 10 times. For viral titration, the presence of the viruses was revealed by
211 immunostaining in infected cells with a polyclonal rabbit antibody that recognizes the MOPV Z
212 protein (Agrobio, France) and a phosphatase alkaline-conjugated polyclonal goat anti-rabbit
213 antibody (Sigma) and 1-Step NBT/BCIP substrate (Thermo Fisher scientific, Waltham, MA).
214 Results are expressed in Focus Forming Unit per mL (FFU/mL). For RNA quantification, viral
215 RNA were extracted from cell culture supernatants (Qiagen, Courtaboeuf, France) and
216 quantification was performed with the EuroBioGreen qPCR Mix Lo-ROX (Eurobio, Les Ulis,
217 France), using an in house developed assay with 5'-CTTTCCCCTGGCGTGTCA-3' and 5'-
218 GAATTTTGAAGGCTGCCTTGA-3' primers. Deep sequencing analysis of viral genomes was
219 performed as described in [44].

220 **Results**

221 **The MOPV NP-exo exhibits a metal-dependent 3'-5' ExoN activity.**

222 The ExoN domain of NP-MOPV (NP-exo MOPV) was incubated with a 5' radiolabeled 22
223 nt RNA hairpin (HP4, S2A Fig) whose 3'-end is base-paired into a double stranded RNA. The
224 reaction was stopped at intervals of 0.1 , 5 and 30 minutes (Fig 1A). In the presence of Mg²⁺, NP-

225 exo MOPV is able to cleave this stable RNA hairpin (DG=-14.7 kcal/mole) predominantly down to
 226 a 18-mer product. After removing the 1st 4 nucleotides, degradation stops at the loop region. A
 227 similar experiment in the presence of Mn²⁺ allows further degradation into the loop, whereas ExoN
 228 is inactive in the presence of either Zn²⁺ or Ca²⁺. The laddering degradation pattern visualized on the
 229 gel, together with radio-label quantification indicate that it acts in the 3'-5' direction (S2B Fig).
 230 Visual examination of degradation kinetics shows, a band-product accumulation prior to G
 231 nucleotides, indicating that the latter are slower to remove than Cs. We also tested the ExoN activity
 232 of the ExoN domain of NP-LCMV (NP-exo LCMV) (Fig 1A) on the same RNA substrate. Both
 233 proteins cleave the RNA following a similar degradation pattern and comparable kinetics (S2B Fig
 234 and S3 Table). The two ExoNs exhibit their highest activity in the presence of Mn²⁺, followed by
 235 Mg²⁺ and they are inactive in the presence of either Ca²⁺ or Zn²⁺ or EDTA (Fig 1A). These results
 236 show that the nature of the ions is altering the ExoN activity and its associated pattern of
 237 degradation can be greatly modulated by the nature of the metal ion co-factor.

238 **Stabilizing effect by ion cofactor is not correlatable to NP-exo activity.**

239 To study the effect of divalent metal binding on the stability of NP-exo MOPV, we measured
 240 the change in melting temperature (T_m) by a Thermal Shift Assay (TSA) in the presence of 5 mM
 241 of several metal ions. NP-exo MOPV without metal ions has a T_m of 49.4 °C. Positive T_m shifts
 242 are observed in the presence of MnCl₂ (+16.5 °C), MgCl₂ (+4.9 °C), CaCl₂ (+7.5 °C) and a slight
 243 negative shift in ZnCl₂ (-2.2 °C) (Fig 2). Simultaneously we compared the effect of these ions on
 244 the stability of NP-exo LCMV (Fig 2). The T_m value for NP-exo LCMV without metal ions is 50.6
 245 °C and increases in the presence of MnCl₂ (+13.6°C), MgCl₂ (+6 °C), CaCl₂ (+7.67 °C) but
 246 decreases with ZnCl₂ (-10.3 °C). These results indicate that the ion stabilization pattern for each
 247 ExoN is unique nevertheless, a similar stabilization trend is observed with MnCl₂ inducing highest

248 stability in all ExoNs. It is also worth noting that CaCl_2 which inhibits the 3'-5' ExoN activity is a
249 better stabilizer than MgCl_2 .

250 Our results show that stability and activity are uncoupled : lowering the energy of the domain is not
251 key for activation. Rather the nature of the ion plays a key role : the small radius and higher
252 coordination of Mn^{2+} over Mg^{2+} allow a higher number of water molecules available for being
253 activated for the nucleophilic attack. On the contrary, Ca^{2+} with a larger radius slightly deforms the
254 catalytic site [43] and increases the distances with the substrate, thus impairing the reaction.

255 **NP-exo MOPV catalytic residues compared to NP-exo LCMV.**

256 We mutated each catalytic residue to alanine in order to assess their respective contribution
257 in the conserved DEDDh catalytic motif, and tested them for ExoN activity. For the NP-exo MOPV
258 mutants D390A, E392A and D534A, the 3'-5' ExoN activity is completely abolished whereas
259 D467A and H529A are still able to slowly excise up to two nucleotides. For the NP-exo LCMV
260 mutants, a slightly different result is observed. D382A and E384A show a complete loss of activity,
261 D459A excises two nucleotides but more efficiently than D467A of NP-exo MOPV as judged by the
262 diminution of the 22 nts band-product. The H517A also shows residual activity while the D522A is
263 able to degrade almost the total amount of 22 nt dsRNA up to 20/19 nts (Fig 1A). We compared the
264 efficacy of cleavage between the wild type NP-exo MOPV and NP-exo LCMV to their
265 corresponding mutants D467A and D459A respectively. Our kinetic experiment indicates that the
266 initial excision rate of NP-exo MOPV and NP-exo LCMV wild types are similar, the rate of D459A
267 of LCMV decreases to about half that of the wild-type, and that of MOPV D467A is significantly
268 affected (S3 Fig).

269

270 **NP-exo MOPV _{ds}RNA substrate specificity.**

271 In order to investigate the substrate requirement for NP-exo MOPV, and NP-exo LCMV ,
 272 we tested their activities on different RNA substrates HP4, A30 (poly A) and LE19. All these single
 273 stranded RNA (_{ss}RNA) forms several types of secondary structures RNA, which were predicted
 274 using Mfold server [59] (S2A Fig). The ExoN assay confirms and extends findings shown in Fig 1,
 275 *i.e* that NP-exo MOPV and NP-exo LCMV cleave RNA substrates whose 3' ends are engaged into a
 276 double stranded structure (Fig 3), consistent with a strict specific double stranded RNA
 277 requirement. It is particularly striking in the case of LE19 : at time 0, we observed the 3 species of
 278 secondary structures (migration for type A : 19 , B: 18 and C : 17 nucleotides respectively) and with
 279 time the top band-product disappears to the profit of an RNA of 17 nucleotides. We observed that
 280 NP-exo seems to be partly active on small secondary structure _{ds}RNA but is inactive on _{ss}RNA (Fig
 281 3).

282
 283 As the NP-exo MOPV presents similar *in vitro* behavior to other Arenavirus NP-exo, we
 284 conclude that the ExoN activity *per se* is not responsible for immune suppression, and that the latter
 285 is mediated by elements embedded in the domain itself. It was thus of interest to better characterize
 286 the substrate specificity of the NP-exo in order to disclose its role in arenavirus replication.

287 **NP-exo is able to excise a _{ds}RNA 3'-end mismatch.**

288 We measured NP-exo MOPV and NP-exo LCMV's ability to cleave different _{ds}RNA
 289 substrates. Because the key enzyme in the innate immune response; the protein kinase RNA-
 290 activated (PKR); is induced by the presence of _{ds}RNA, we made use of a perfectly annealed _{ds}RNA,
 291 as well as several potential RNA substrates such as those mimicking an erroneous replication
 292 product with one, two or three mismatched nucleotides at the 3'-end. To that end, a 40-mer RNA

293 template (RT1) blocked in 3'-end with a phosphate group was annealed to a radiolabeled RNA
 294 carrying zero (RL2*) or one (RL3*), two (RL4*) or three (RL5*) non-complementary nucleotides
 295 at its 3'-end as shown in Fig 4A. Fig 4B shows that both enzymes are strict $_{ds}$ RNA ExoNs, with the
 296 interesting specific ability to digest substrates carrying a single 3'-terminal mismatch (RL3*/RT1).
 297 Quantification of total product (Fig 4C) shows a comparable hydrolytic activity between the
 298 perfectly annealed $_{ds}$ RNA and a single 3'-terminal mismatch. The cleavage efficiency, however,
 299 drastically drops with the number of unpaired bases at the 3'-end.

300 **The arenavirus NP-exo domain is structurally and functionally similar to the Coronavirus** 301 **RNA 3'-mismatch excising ExoN.**

302 The overall fold of NP-exo MOPV is homologous to that of the other arenavirus ExoNs
 303 [43]. Structural comparison reveals that the structure of NP-exo MOPV is very similar to that of
 304 LASV, LCMV and Tacaribe virus (TCRV) structures with overall r.m.s.d of ~ 1 Å or less, while the
 305 residues of the catalytic site are perfectly superimposed. Indeed, the four conserved catalytic
 306 residues (D390 E392 D467 H529 D534) from NP-exo MOPV are located at virtually identical
 307 positions as those of the other three ExoNs with only minor differences in their orientations. The Zn
 308 coordinating residues (E400, C507, H510 and C530) which are highly conserved in arenaviruses are
 309 also oriented in an identical manner in all four structures.

310 A fold similarity search retrieved three ExoN of various origin, namely *Arenaviridae*,
 311 *Coronaviridae*, and a human histone mRNA 3'-ExoNs (S4A Fig). Not only the catalytic core of all
 312 these enzymes is conserved (S4B Fig), but also they all possess the ability to remove few unpaired
 313 nucleotides in the 3'-to-5' direction. We analyzed comparatively the NP-exo MOPV structure and
 314 the nsp14 SARS-CoV protein (Fig 5) which also possesses a 3'-exoribonuclease activity able to
 315 excise 3'-end mismatch on $_{ds}$ RNA [47]. The comparison of their topology and of their active site

shows that secondary structure elements belonging to the catalytic core are arranged in a similar manner. Our results suggest a common origin of the *Coronaviridae* Nsp14 and *Arenaviridae* NP ExoNs.

The arenavirus NP-exo domain affects viral replication but is not involved in genome stability.

To assess the possible role of the NP-exo activity in viral replication and/or genome stability, we passaged iteratively 10 times in Vero E6 cells at a MOI of 0.01 a NP-exo defective virus (NP-exo(-))carrying the D390A/G393A mutants as well as a NP-exo WT recombinant MOPV. We first quantified both the infectious titers (Fig 6A, left axis) and the NP RNA viral loads (Fig 6A, right axis) of the cell culture supernatants. Our results showed that the infectious viral titers of both viruses followed a parallel trend, the NP-exo(-) always presenting a 40 (minimum, passage 4) to 190 (maximum, passage 6) fold decrease in titer compared to the NP-exo WT MOPV. From passages 1 to 5/6, infectious titers continuously decreased (down to a 5 fold for the WT and down a 90 fold for the mutant compared to passage 1) before a rebound from passage 6 to 8/9 to titers similar to those of passage 2 followed by another general decrease at passage 10. The viral loads of NP-exo WT and NP-exo(-) MOPV described the same trends as the infectious viral titers albeit with less pronounced variations. The viral load for the WT virus remained stable along the passages with a maximum 3 fold difference while the NP-exo(-) virus had a maximum 13 fold difference. We also calculated the RNA/FFU ratio for both viruses (Fig 6B). On average, the NP-exo WT and NP-exo mutant viruses respectively presented one infectious particle for 3600 and 23000 NP RNA copies, respectively. Interestingly, the maximum ratio was reached at passage 5 for both viruses with 1 FFU for 11300 copies for the WT NP-exo and 1 FFU for 119600 copies for the NP-exo(-). Therefore, the suppression of the NP-exo activity of MOPV promoted both a reduction of the infectious titer and an increased amount of non-infectious material released from infected cells.

339 We next investigated the genomic stability of these two viruses at passage 1 and 10 through deep
 340 sequencing analysis. We almost reached a complete coverage of the MOPV genomes except for the
 341 5' and 3' end and most of the intergenic region of both segments. The tandem repeated and
 342 complementary sequences promote strong secondary structures in the intergenic regions and may
 343 explain the lack of reads observed for this region for both viruses.

344 The presence of WT NP sequences detected at passage one for both viruses likely originated from
 345 the plasmid expressing the WT NP ORF used for rescuing the virus (data not shown). To make sure
 346 our results matched a standard threshold usually observed with the presence of an internal control of
 347 sequencing, we considerate a 5% cutoff as a significant read percentage for the effective presence of
 348 a mutation (minimum mean coverage of 2636 reads for the L segment of the NP-exo(-) virus and
 349 maximum mean coverage of 12610 reads for the S segment of the NP-exo WT virus). As shown in
 350 Table 1 & S4, mutations targeting either the ORFs or the UTR/IGR regions are already present in
 351 both segments of the NP-exo WT and NP-exo(-) viruses (2.90 and 2.15 mutations/kb respectively)
 352 as early as passage one in VeroE6 cells after the virus rescue in BHKT7 cells. The overall mutation
 353 rate slightly increases comparatively at passage ten with 3.93 mutations/kb for the NPexo WT virus
 354 and 3.36 mutations/kb for the NP-exo(-) virus. Theses results indicate that NP ExoNs does not
 355 affect the overall genome stability but affects the viral replication.

356 **The arenavirus NP-exo domain is active on its own genome.**

357 We observe that the overall mutation rate is stable between NP-exo WT and NP-exo(-) MOPV, yet
 358 we also notice that the frequency of these mutations has changed. We observe at passage 10 a
 359 comparable number of mutations along the S segment but a decrease of ~ 22 % on the L segment of
 360 the mutant together with an increase of their occurrence frequencies. For both, these mutations
 361 appears along the entire L segment at an average frequency of ~ 10.1 % (comprised between 5 to 24

362 %) for the WT and of ~ 16.6 % (comprised between 5 to 93 %) for the mutant, in particular the
363 substitution of C to T. While for the S segment mutations appear along the entire for the WT and
364 clustered for the mutant at respective frequencies of 14.6 % and 19.5 %.

365 Among all mutations recorded, only a few were present for either both viruses and/or at the two
366 different passages. Indeed, three mutations in the S segment and one mutation in the L segment are
367 present for both viruses at passages one and ten and likely represent stable quasi-species (Table S4,
368 and Table 1 yellow highlight).

369 Three mutations in the L segment are commonly found for the two viruses only at passage 10 (Table
370 1, orange highlight). Interestingly, three non-synonymous mutations in the L-polymerase ORF
371 (S184L, S1021P and L1477S, Table 1, red highlight) became majority for the NP-exo(-) virus at
372 passage 10.

373 To ascertain the trend observed in our genomic sequencing data, we investigated the natural
374 occurrence of theses mutations in *Mammarenavirus* (MAMV) using bioinformatics. The pre-
375 supposed being that if these mutations appear randomly they should be significantly (> 5 %)
376 represented in the general population of MAMV and LASV.

377 From the three subsets of sequences generated; *i.e* MAMV, LASV and MOPV; we observed that the
378 three mutations S184L, S1021P and L1477S appeared in conserved regions in all three subsets, that
379 none of the three mutants were reported in MOPV subset and finally that the three amino acid are
380 subject to diverse selective pressure (Fig 7). Indeed, serine at the position 184 represents the
381 majority of the observed amino acid variants while the specific mutation S184L represents only 1 %
382 of the total observed sequences in the LASV or MAMV subsets. This observation indicates that the
383 mutation is viable but most likely costly to be maintained by the virus. On the other hand, at
384 position 1021, the serine observed in MOPV subset does not represent the majority of the observed
385 amino acid in the other subsets, the proline is by far the most frequent amino acids found (Fig 7). In

386 LASV, the serine subset represents only 3% of the observed amino acids at this position while 10%
 387 in the MAMV subset (Fig 7). This observation indicates that this position in MOPV is an oddity
 388 compared to the others. It seems that the natural tendency in the L protein is to have a proline rather
 389 than a serine at this position. The fact that we observe that particular reversion in the NP-Exo(-)
 390 could indicate that for L MOPV there is a constraint at this particular position. Finally, at position
 391 1477, the amino acid found at this position for the three subsets is a leucine (Fig 7), indicating that,
 392 that particular position is under a high selective pressure. The mutation L1477S was never observed
 393 in any subsets, this mutation can be interpreted as unlikely and therefore considered as direct
 394 consequence of the NP-Exo(-).
 395 Our results show that compared to the WT virus, the abrogation of the NP-exo activity did not
 396 increase the mutation rate found in the MOPV genomic sequences present in the cell culture
 397 supernatant, but the emergence of the three mutants of rare occurrence are the direct consequence of
 398 the NP-Exo(-), which have relaxed the control over certain position implying a direct effect of the
 399 MOPV exonuclease on its own genomic RNA.

400

401 Discussion

402 The paradigm of *Arenaviridae* NP ExoN states that it is involved in innate immunity
 403 suppression [37,60–62], through degradation of dsRNAs which would otherwise stimulate the innate
 404 immunity response. Several reports have demonstrated that NP is responsible for the degradation of
 405 these dsRNAs using the 3'-5' ExoN located at its C-terminus [25,32,35,63]. This ExoN comprises a
 406 DEDDh catalytic motif that is completely conserved across the *Arenaviridae* [35] implying this
 407 activity may be a general feature of arenavirus NPs. The ExoN domain is conserved within the
 408 family regardless of both the virus pathogenic potential and its ability to suppress efficiently type I
 409 IFN, as previously reported for TCRV and MOPV [42,60,64]. MOPV is the closest counterpart of

410 LASV and presents a 73% NP sequence identity with LASV. During LASV infections, the virus
411 targets mainly macrophages (MP) and dendritic (DC) cells [65], and infections are characterized by
412 high viremia and generalized immune suppression supposedly due to innate immune inhibition by
413 the ExoN domain. Both MOPV and LASV induces strong type I IFN responses in MP and
414 moderately in DC, but contrarily to LASV which abrogates this response, MOPV does not [42,64].

415 Our functional study demonstrates that the ExoN structure, substrate specificity, and
416 mechanism is indeed conserved across the family. It is also clear from the structure-ion analysis
417 [43], that toying with the catalytic ion leads to slight structural changes which impact dramatically
418 the activity. Although NP-exo MOPV, NP-exo LCMV have similar cleavage patterns, mutation
419 analysis of the DEDDh motif reveals that the exact residues critical for 3'-5' ExoN vary between
420 both domains. For NP-exo MOPV, D390A, E392A and D534A (D389A, E391A and D533A LASV
421 equivalents) completely abolishes 3'-5' ExoN activity consistent with results from *in vitro* studies on
422 LASV [35], while D467A and H529A (D466A and H529A LASV equivalents) retains some
423 residual activity (see below). For NP-exo LCMV, a previous study by Martínez-Sobrido and
424 collaborators [61], correlated innate immunity suppression to ExoN mutants, postulating a direct
425 involvement of the ExoN activity. We observed that mutant D382A completely loses ExoN activity
426 consistently with results from reverse genetic studies [61]. A noticeable difference concern the
427 mutant E384A, that was shown to have no effect and be dispensable for ExoN activity [61], is
428 rather shown critical in our *in vitro* study and consistent with the structural data as E384 (equivalent
429 to MOPV E392) is involved in binding one of the catalytic ion (S4 Fig). Under our conditions,
430 D459A and H517A still retains their ability to cleave two nucleotides meanwhile D522A shows a
431 significant activity leading to the removal of two to three nucleotides. Theses latter three mutants
432 were not reported before but the analysis of the structure of NP-exo MOPV confirms that major
433 features such as fold, and the two ion binding sites (catalytic and structural) are conserved within

the *Arenaviridae* [25,32,35,36,63]. Residues D390, E392, D534 of NP-exo MOPV directly coordinate the catalytic ion. Mutation of these residues logically alter ion binding and thus leads to complete loss of catalytic activity. The residual activity observed for D522A of NP-exo LCMV is rather difficult to explain as the two structures present no clear differences in the ion binding mode. The only noticeable difference is about the hydrophobic environment that may compensate the faulty metal-binding site in the case of NP-exo LCMV. The residual activity observed for D467 (D459 of LCMV) is rather difficult to explain as the two structures present no clear differences, yet that residue is very likely involved in the cleavage mechanism. Indeed, the kinetic experiments comparing the WT NP-exo MOPV and NP-exo LCMV to corresponding mutants show that the first event of the hydrolysis is comparable between NP-exo MOPV and NP-exo LCMV, while mutation of the aspartate reduces drastically the hydrolysis kinetics for NP-exo MOPV but only moderately for NP-exo LCMV (S3 Fig). These differences suggest that the aspartate (respectively D467, D459) is involved in the structural set-up of the active site for positioning the ion responsible for the nucleophilic attack. The general mechanism for RNA hydrolysis is a two metal ion mechanism described by Steitz and Steitz [66]. It involves metal A and B positioned ~ 4 Å apart each other and across the target phosphodiester bond. Metal ion A facilitates the formation of the attacking nucleophile. This is followed by the formation of a penta-covalent intermediate which is stabilized by both metal ions. Metal ion B then eases the exit of the leaving group. With the help of the LASV structures of Jiang and collaborator [36], we made an attempt to reconstitute a model of the general mechanism for RNA hydrolysis (S5A Fig). In NP-exo MOPV structure (and all the others) only the metal B is visible. The site receiving the other catalytic metal A is partly created with interaction of the RNA and residues D390 and D467 (S5B Fig). The interaction between the ion in position A and D467 is mediated through a water molecule. Therefore, this might explain why this mutant retained a partial residual activity.

458 As it was shown for TCRV, the ExoN domain of MOPV *in vitro* is endowed with full ExoN
459 activity and obeys to the same structural and energetic constraints as those of other *Arenaviridae*
460 ExoNs [36,43]. Therefore MOPV ExoN activity alone is not *per se* responsible for the differences
461 in innate immunity suppression between MOPV and LASV. Rather, the presence of the ExoN
462 activity may serve other purposes in the viral life cycle, which might be connected directly or
463 indirectly to innate immunity.

464 In particular, previous studies on LCMV and PICV suggested that altering the NP ExoN also
465 impacts replication, irrespective to the IFN status of the host cell [61,67]. The structural relatedness
466 with the Coronavirus ExoN and its implication in viral replication prompted us to investigate to
467 which extend the arenavirus ExoN domain is able to excise unpaired nucleotides. Our enzyme
468 activity assays demonstrate that NP-exo MOPV and NP-exo LCMV can efficiently and selectively
469 cleave a _{ds}RNA mimicking an erroneous replication product carrying one 3'-mismatched nucleotide
470 (Fig 4). Our analysis confirms that despite additional inserted structural elements, the two domains
471 belong to the same Ribonuclease H-like superfamily. In the case of *Coronaviridae*, several studies
472 have pointed to a main role of the ExoN domain of nsp14 in RNA proofreading [47–50] to maintain
473 genome stability. Structural comparison between ExoN domain of nsp14 and MOPV shows
474 conservation of active site and main fold (Fig 5) suggesting that they have a distant but common
475 origin. Recent work by Becares and colleagues have shown that nsp14 of Coronavirus is also
476 involved in innate immunity modulation [68]. Therefore, these data show that at least in
477 *Coronaviridae* the 3'-5' ExoN activity is not exclusively assigned to a specific role but is involved in
478 different aspect of the viral life cycle. Our study present clear evidence that much like the
479 coronavirus nsp14 [47], the *Arenaviridae* NP ExoN excises a 3'-end mismatch _{ds}RNA *in vitro*, and
480 based on previous report that this activity is directly connected to RNA replication [61,67].

481 From our data, the MOPV polymerase exhibits an average error rate estimated around 3

482 mutations / kb. This means that the polymerase was able to incorporate a mismatch and then
 483 elongate it. The error rate does not change between the WT and the mutant, which is consistent with
 484 the fact that we did not altered the polymerase. For both, these mutations appears along the entire L
 485 segment at an average frequency of ~10.1 % (comprised between 5 to 24 %) for the WT and of
 486 ~16.6 % (comprised between 5 to 93 %) for the mutant. The fact that unlikely mutants have become
 487 prevalent, as observed at passage 10, reflect that the control over certain type of mutation have been
 488 abolished, thus implicating that ExoN is active on its own genomic RNA. In this study, it is not the
 489 particular set of mutations that is relevant but rather that a set of unlikely mutations have emerged.
 490 The bias inferred by impairment of the ExoN, together with the biochemistry presented here is
 491 consistent with the idea that ExoN is involved in a mismatch excision system.

492 Although the presence of mismatch excision system is logically associated to very large
 493 genomes (~30 kb) in *Coronaviridae*, the presence of such activity, and potentially such RNA repair
 494 system, in *Arenaviridae* of intermediate genome size (~11 kb) remains puzzling. Our results shows
 495 a clear diminution of the viral titer for viruses depleted of ExoN activity, but no clear evidence of a
 496 drastic increase of mutation in the genome that would lead to catastrophic event. Then what is
 497 happening in these mutated viruses? One tentative explanation could be the ExoN is involved in : i)
 498 checking and maintaining the sequence integrity of the conserved genomic region at its extremities,
 499 and/or ii) the structural integrity of the Intergenic Region (IGR). Indeed, both regions have been
 500 previously reported as being critical for viral fitness: i) The conserved region (19 nucleotides)
 501 exhibit high degree of sequence conservation at the 3'-termini and is complementary to the 5' end of
 502 the genome (for review [26]). This sequence serves as a selective docking platform for the
 503 polymerase [69] for which 3'-end binds with high affinity and in a sequence specific manner [70].
 504 ii) Similarly, alteration of the IGR structure leads to reduce efficient transcription termination and
 505 viral assembly [71,72]. In such hypothesis, the impairment of the NP-exo activity leads to a

506 scenario in which the polymerase is able to incorporate a mismatch but is unable to elongate it,
 507 leading to a decrease of suitable genomic material to package, therefore without the ExoN control
 508 the number of functional RNP would be reduced and consistent with the loss of viral fitness
 509 observed here and else [61,67]. Therefore we propose that the Arenavirus ExoN is involved in a
 510 “limited proof-reading” mechanism driven by structural constraints rather than genomic stability.
 511 Another observation that concurs to the “limited proof-reading” mechanism is the difference of
 512 Ribavirin efficiency on *Arenaviridae* and *Coronaviridae*. Ribavirin is the only drug so far
 513 administered on large scale and having demonstrated a decrease of mortality rates up to 5%, if
 514 administered within the first 6 days of arenaviral illness [73]. On the other hand, Ribavirin is
 515 ineffective against coronaviruses [74,75], as nsp14 ExoN domain excises the nucleotide analogue
 516 [51]. It is likely, that for *Arenaviridae*, the ExoN activity involved in a “limited proof-reading”
 517 mechanism remains as a trace of its past common ancestor with *Coronaviridae*. The critical
 518 problem of genomic stability being solved, either by the conservation of the “original” function of
 519 the ExoN for *Coronaviridae*, or by genome segmentation for *Arenaviridae*.

520

521 As a conclusion we have shown that the MOPV ExoN is fully functional, behaving like
 522 other Arenaviral ExoN on $dsRNA$. We have demonstrated that Arenaviral ExoN are able to excise an
 523 RNA mismatch, and that is active on its own genomic RNA like its counterpart in *Coronaviridae*.
 524 Under the conditions used here, abrogation of ExoN activity does not impact genomic stability
 525 significantly. Our results suggest that the *Arenaviridae* RNA ExoN, like that of *Coronaviridae*, is at
 526 a crossroad between replication efficiency and innate immunity evasion in the infected cell.

527

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534

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537

538 **Figure legends**

539 **Fig 1. Comparison of the ExoN activity of NP-exo MOPV and NP-exo LCMV.** (A) Effect of
540 divalent cations on ExoN activity of NP-exo MOPV and NP-exo LCMV. RNA HP4 was incubated
541 with NP-exo MOPV or NP-exo LCMV for 0, 0.1, 5 and 30 minutes (min) in the presence of 5 mM
542 Mg^{2+} , Mn^{2+} , Zn^{2+} , Ca^{2+} , or EDTA. Digestion products were separated on a 20 % denaturing PAGE
543 and revealed by autoradiography. (B) Comparative mutational analysis of ExoN activity. The
544 DEDDh residues were mutated to alanine. Equal amounts of wild type (WT) or mutants of NP-exo
545 MOPV or NP-exo LCMV were incubated with HP4 for 0, 5 and 30 mins. Products were separated
546 on denaturing PAGE and visualized by autoradiography. NC indicates the substrate without
547 proteins. Sketch on the top of figure A illustrates the hairpin structure of HP4. On the side of each
548 gel is presented the migration size ladder in nucleotides (nts).

549 **Fig 2. Effect of divalent-Cation on thermal stability of NP-exo MOPV and NP-exo LCMV.** Bar
550 chart showing the shifts in melting temperatures of NP-exo MOPV and NP-exo LCMV measured in
551 the presence of 5 mM of the indicated ions by TSA.

552 **Fig 3. Comparison of the substrate on the ExoN activity of NP-exo MOPV and NP-exo LCMV.**
553 Comparative ExoN activity on three different RNA substrate ; A30 (ssRNA), LE19 (ssRNA forming
554 three types of secondary structures), HP4 (stable RNA hairpin). Equal amounts of each RNA
555 substrate were incubated with NP-exo MOPV or NP-exo LCMV for 0, 5, 30 minutes. Digestion
556 products were analyzed as in Fig1. On the side of each gel is presented the migration size ladder in

557 nucleotides (nts). A star highlight the enrichment of the band corresponding of a RNA of 17 nts
558 Type A RNA with degradation of 2 nucleotides.

559 **Fig 4. Time course hydrolysis of paired and mismatched 3'-end nucleotide base pair by NP-exo**
560 **MOPV and NP-exo LCMV.** (A) Schematic representation of dsRNA mimicking a replication
561 intermediate. RL2*/RT1, RL3*/RT1, RL4*/RT1 and RL5*/RT1 represent dsRNAs carrying either
562 zero, one, two and three non-complementary nucleotides respectively at their 3'-ends. (B) Equal
563 amounts of the dsRNA substrate (1,25 μ M) listed above were incubated in the absence or presence
564 of 0,25 μ M of NP-exo MOPV or NP-exo LCMV at 37 °C for 1, 2, 5 and 10 min. 0 is negative
565 control without proteins. A migration size ladder in nucleotides (nts) is presented on the side of the
566 gel. Digestion products were analyzed on 20% denaturing PAGE and visualized by
567 autoradiography. (C) Bar graph showing the total degradation product of substrate at various times.
568 Total degradation products were quantified using phosphorimager FLA-3000 (Fuji) and graphs
569 plotted using Graphpad. PRISM.

570 **Fig 5. Comparison of the ExoN domains : nsp14 SARScov and NP-exo MOPV.** Topology
571 diagrams of the (A) nsp14 SARScov and (B) NP-exo MOPV. The 5 β -strands that constitute the
572 central β -sheet are colored blue, pink, brown, yellow and green from the first to the fifth strand
573 respectively. The 4th and 6th α -helices of the DEDD motif are colored purple and orange
574 respectively. The uncolored spheres represent additional non-conserved secondary structures. The
575 location of the catalytic residues are indicated with red spheres. Extra domain insertions for each
576 ExoN are enclosed with in the large gray circles. The N and C terminals are indicated with the
577 uncolored squares. The topology diagrams indicates a similar fold and a conservation of the
578 catalytic core for both ExoNs. (C) Structural alignment of the active sites of nsp14 SARScov and
579 NP-exo MOPV. Color codes are same as in the topology diagrams except for secondary structures

580 colored cyan (NP-exo MOPV) or sandy brown (nsp14 SARScoV) which are not a part of the
581 catalytic fold. The superposition shows that catalytic residues are located at virtually identical
582 positions.

583 **Fig. 6. Effect of inactivated ExoN viruses on viral fitness and genome stability.** (A) Iterative
584 passages of NP-exo WT and D390A/G393A recombinant MOPV in Vero E6 cells. *De novo* stocks
585 of both viruses, from passage 1 to passage 10, were used to infect cells for 4 days with MOI 0.01.
586 Samples of supernatants were collected for viral infectious titration and NP RNA copy
587 quantification. Results for the left Y axis represent the infectious viral titers (FFU/mL) and results
588 from the right Y axis represent the NP RNA load (NP RNA copies/mL) of the corresponding cell
589 culture supernatants. (B) Ratio calculation for NP RNA copy load over infectious viral titer (RNA
590 copies / FFU) for NP-exo WT and D390A/G393A recombinant MOPV for the ten passages
591 considered in (A).

592 **Fig. 7. Representation of statistical occurrence of an amino acid at specific position.**
593 Represented the WebLogo of the corresponding MOPV amino acids S184, S1021 L1477 in all
594 Mammarenavirus, LASSV and MOPV sub set of sequences. The position of the residue of interest
595 is indicated with a red star. Size of the residue is proportional to its probability of occurrence (0 – 1).
596 For clarity, on the side of the WebLogo is the detailed statistical occurrence of all amino acids
597 found in the deposited sequences.

598

599 **Supporting information**

600 **S1 Fig. Modular and organization of *Arenaviridae*' NP.** Schematic of the two domains
601 organization of *Arenaviridae*' NP, with its corresponding domains structures. N-terminal domain
602 corresponding to the nucleoprotein domain (PDB : 3T5N) and C-terminal domain corresponding to
603 the ExoN domain (PDB : 3Q7C) of LASV. Each domain is represented in ribbon and colored
604 following rainbow nomenclature from blue (N-terminus) to red (C-terminus). Flexible linker is
605 represented as green line.

606 **S2 Fig. Secondary structures adopted by RNA substrates used in the study.** (A) These
607 structures were predicted using Mfold server (<http://unafold.rna.albany.edu/?q=mfoldand>). The
608 minimum free energy of each structure is indicated below it. RNA HP4 is a stable RNA hairpin.
609 RNA LE19 presents 3 types of secondary structures. Type A : two double stranded nucleotides, type
610 B : long hanging 5' and short hanging 3' extremities, type C : long 5' 3' Hanging extremities. RNA
611 A30 long single stranded RNA without secondary structure. (B) Expected pattern of digestion based
612 on a dsRNA 3'5' ExoN activity for each type of tested RNA.

613 **S3 Fig. Kinetics of HP4 cleavage by NP-exo MOPV WT, its Mutant D467A, NP-exo LCMV**
614 **WT or the corresponding mutant D459A.** HP4 was incubated with equal concentration of NP-exo
615 MOPV WT, D467A , NP-exo LCMV WT or D459A for 0, 2, 4, 6, 8, 10 and 15 mins. Reactions
616 products were separated on 20 % denaturing PAGE and products revealed by autoradiography. RNA
617 cleavage was then quantified from this data and plotted as the percentage of product formed with
618 time.

619 **S4 Fig. Structural alignment of 3'-5' ExoN :** (A) Ribbon representation of the superposition of
 620 NP-exo Mopeia Virus (cyan pdb : 5LRP), nsp14 SARScoV Exo (beige pdb : 5C8S), NP-exo Lassa
 621 Virus (orange pdb : 4FVU), histone mRNA stem-loop by 3'-ExoN Homo Sapiens (green pdb
 622 1ZBH). All structures were retrieved by PDBeFold (<http://www.ebi.ac.uk/msd-srv/ssm>), all similar
 623 sequences were discarded. Overall fold is similar; in caption a zoom of the active site shows a quasi
 624 perfect superimposition of the DEDD motif. Green spheres are in the zoom represent Mg ions. (B)
 625 Weblogo of the active site derived from the structural alignment. Structure and sequence
 626 comparison lead to conclude to a common origin of the ExoNs.

627 **S5 Fig. A model of the mechanism of calcium inhibition of 3'-5' ExoN activity.** (A) The dsRNA
 628 from LASV NP-C structure (PBD code:4GV9) modeled into the MOPV Nexo-Mg structure.
 629 MOPV-Mg represented as cartoon (Helices in orange, β -strands in green and loops in cyan) and the
 630 dsRNA is shown as a stick model. The green balls represent magnesium atom. (B) An enlarged
 631 view of the active indicating the model positions of ions during cleavage mechanism. Calcium
 632 substitution of the magnesium ion mediates inhibition of 3'-5' ExoN activity as a result of its atomic
 633 radius, binding flexibility and poor activation of water. Ca atom is represented in grey.

634 **S1 Table. Primer sequences used for mutagenesis of the LCMV and MOPV.**

635 **S2 Table. Oligomers names and sequences.**

636 **S3 Table. Comparison of remaining sequence length function of time.**

637 **S4 Table. Observed S segment mutations of WT and ExoN(-) viruses at passages #1 and #10.**

Figure 1

A

B

Figure 2

Figure 3

Figure 4

Figure 5

A

B

C

Figure 6

A

B

Figure 7

Mammarenavirus

Lassa Virus

Mopeia Virus

	Passage #01					
	position*	ref	mut	region	residue	frequency %
	9	A	G	5'UTR		16,667
	12	A	G	5'UTR		12,121
	17	A	G	5'UTR		5,036
	742	A	T	Lpol	M229L	5,552
	2463	T	A	Lpol	F802L	5,583
	2518	T	C	Lpol	L822L	6,373
	6452	A	G	Lpol	E2132G	5,816
	6602	G	T	Lpol	R2182I	5,553
	6870	C	T	IGR		12,903
	6990	C	A	Z	R68M	7,550
	6992	T	G	Z	D67D	7,462

Passage #10					
position*	ref	mut	region	residue	frequency %
5	A	C	5'UTR		8,333
5	A	G	5'UTR		8,333
9	A	G	5'UTR		6,122
129	A	C	Lpol	K23N	6,522
608	C	T	Lpol	S184L	93,178
1456	A	T	Lpol	R467W	24,704
2079	T	C	Lpol	D674D	6,373
2233	G	A	Lpol	D726N	6,045
2427	G	A	Lpol	V790V	7,237
2622	T	C	Lpol	D855D	9,348
3118	T	C	Lpol	S1021P	58,105
3163	A	T	Lpol	T1036S	5,868
3624	T	C	Lpol	N1189N	8,228
4487	T	G	Lpol	L1477W	10,772
4500	T	A	Lpol	D1480D	5,134
4883	A	G	Lpol	L1477S	60,227
5239	G	A	Lpol	E1728K	8,367
6302	A	T	Lpol	D2082V	6,604
6567	A	T	Lpol	R2169R	9,287
6729	G	C	Lpol	R2225S	9,980
6769	T	G	Lpol	Stop-E	6,829
6772	G	A	IGR		9,596
6870	C	T	IGR		6,667
6965	G	T	Z	L76L	6,655
7032	T	C	Z	L54L	27,373

Table 1 : Observed mutation between WT and Mutant Exo (-) of segment L at passages #1 and #10.