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1 **The NSs protein encoded by the virulent strain of Rift Valley fever virus targets the**
2 **expression of Abl2 and the actin cytoskeleton of the host affecting cell mobility, cell**
3 **shape and cell-cell adhesion**

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5 **Running title:** RVFV NSs protein targets the host's actin cytoskeleton

6

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Abstract

24 Rift Valley fever virus (RVFV) is a highly pathogenic zoonotic arbovirus endemic in many
25 African countries and the Arabian Peninsula. Animal infections cause high rates of mortality
26 and abortion among sheep, goats and cattle. In humans, an estimated 1-2% of RVFV
27 infections result in severe disease (encephalitis, hepatitis, retinitis) with a high rate of lethality
28 when associated to hemorrhagic fever. RVFV's NSs protein, which is RVFV's main factor of
29 virulence, counteracts the host innate antiviral response favoring viral replication and spread.
30 However, the mechanisms underlying RVFV-induced cytopathic effects and the role of NSs
31 in these alterations remain for the most undeciphered. In this work we have analyzed the
32 effects of NSs expression on actin cytoskeleton while conducting infections with the NSs
33 expressing virulent (ZH548) and attenuated (MP12) strains of RVFV and the non-NSs
34 expressing avirulent (ZH548 Δ NSs) strain as well as after the ectopic expression of NSs. In
35 macrophages, fibroblasts and hepatocytes NSs expression prevented the up-regulation of Abl2
36 (a major regulator of actin cytoskeleton) expression otherwise induced by avirulent infections
37 and identified here as part of the antiviral response. The presence of NSs was also linked to
38 and increased mobility of ZH548- as compared to ZH548 Δ NSs-infected fibroblasts and to
39 strong changes in cell morphology in non-migrating hepatocytes with reduction of
40 lamellipodia, cell spreading and dissolution of adherens junctions reminiscent of ZH548-
41 induced cytopathic effects observed *in vivo*. Finally, we show evidence of the presence of
42 NSs within long actin-rich structures associated to NSs dissemination from NSs expressing
43 towards non-NSs expressing cells.

44 **Importance.** Rift Valley fever virus (RVFV) is a dangerous human and animal pathogen that
45 was ranked in 2018 by the World Health Organization among the eight pathogens of most
46 concern likely to cause wide epidemics in the near future for which there is no, or insufficient,
47 countermeasures. The interest of this work resides in the fact that it addresses the question of

48 the mechanisms underlying RVFV-induced cytopathic effects that participate in RVFV's
49 pathogenicity. We demonstrate here that RVFV targets cell adhesion and actin cytoskeleton at
50 the transcriptional and cellular level, affecting cell mobility and inducing cell shape collapse
51 alongside with distortion of cell-cell adhesion. All these effects are susceptible to participate
52 in RVFV-induced pathogenicity, facilitate virulent RVFV dissemination and thus constitute
53 interesting potential targets in future development of antiviral therapeutic strategies that in the
54 case of RVFV, as several other emerging arboviruses, are presently lacking.

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Introduction

58 Rift Valley fever virus (RVFV) is a dangerous human and animal pathogen ranked in
59 2018 by the World Health Organization among the eight pathogens of most concern likely to
60 cause wide epidemics in the near future for which there is no, or insufficient, countermeasures
61 (<https://www.who.int/blueprint/priority-diseases/en/>). It is a mosquito-borne, zoonotic,
62 phlebovirus endemic throughout many African countries and the Arabian Peninsula (1).
63 Animal infections cause high rates of neonatal mortality and abortion mainly among sheep,
64 goats and cattle. Human infections lead to a wide range of clinical manifestations from mild
65 flue-like symptoms to severe illness such as encephalitis, retinitis and hepatitis, which can be
66 fatal when associated to hemorrhagic fever. An estimated 1-2% of human infections result in
67 severe disease often associated to high levels of mortality (1-3).

68 RVFV is an enveloped, segmented RNA virus of negative or ambisense polarity. It has
69 a tripartite single-stranded RNA genome consisting of the large (L) and medium (M)
70 segments of negative polarity and the small (S) segment of ambisense polarity. The L
71 segment encodes the RNA-dependent RNA polymerase, the M segment encodes two
72 glycoproteins as well as two non-structural NSm proteins and the S segment encodes the
73 nucleoprotein N with a negative polarity and the nonstructural NSs protein with a positive
74 polarity (2, 4, 5). The NSs protein that is produced shortly after infection (6) is not essential
75 for the viral cycle but is considered as the main virulence factor of RVFV. Indeed, the
76 virulent ZH548 strain that expresses NSs kills wild type mice whereas mutant viruses
77 carrying a NSs-deletion, either natural (Clone 13) or recombinant (ZH548 Δ NSs), are avirulent
78 and non-pathogenic for mice (7, 8). NSs is characterized by its capacity to accumulate in the
79 cell nucleus where it forms filament-like structures (9, 10) that include NSs itself as well as
80 several cellular transcription factors and co-factors (11, 12). NSs has been found associated to
81 specific cellular DNA sequences such as pericentromeric major satellite DNA sequences in

82 relation with chromosome cohesion defects (13) and the promoter region of the gene coding
83 for interferon-beta (IFN β), a cytokine that plays a major role in the innate antiviral response
84 of the host, maintaining the IFN β gene (*Ifnb1*) in a repressed state (12). Alongside with
85 inhibiting the expression of *Ifnb1* (7, 12, 14), NSs also counteracts the cellular innate immune
86 response by targeting kinase PKR for degradation (15). The inhibition of the host's innate
87 antiviral response directly affects, facilitating, viral replication and propagation. However, the
88 mechanisms, including the role of NSs, underlying the development of significant
89 ultrastructural changes such as cell shrinkage, rounding and loss of cell junctions that have
90 been observed *in vivo* in the liver after RVFV infection (16), potentially participating in
91 RVFV's pathogenicity, remain for the most to be identified.

92 In previous work, we have performed a genome-wide analysis of the interactions
93 between NSs and the host genome. For this, genome-wide chromatin immunoprecipitation
94 (ChIP) carried out using an anti-NSs antibody was combined with promoter sequence
95 microarray hybridization (ChIP-on-chip). The DNA immunoprecipitated in these experiments
96 was recovered from L929 murine fibroblastic cells either before or at different times post-
97 infection (p.i.) with the pathogenic, NSs-expressing ZH548 strain of RVFV (17). Several
98 cellular promoter regions were identified as significantly interacting with NSs and the
99 establishment of NSs interactions with these regions was often linked to the deregulation of
100 the expression of the corresponding genes. Among annotated NSs interacting genes were
101 present not only genes regulating innate immunity and inflammation but also genes regulating
102 cellular pathways potentially participating in RVFV's pathogenicity. The functional analysis
103 of the genes associated to these NSs-interacting regions, identified cell adhesion as the
104 biological process the most significantly enriched among cellular NSs-interacting genes (17).

105 In order to test the effect of RVFV infection and NSs protein in cell adhesion, we have
106 compared in this work the effects of infections with the NSs expressing strains either virulent

107 ZH548 (ZH) or attenuated (MP12) and the non-expressing NSs avirulent ZH548 Δ NSs (Δ NSs)
108 strain of RVFV on actin cytoskeleton and cell-cell adhesion at the transcriptional and cellular
109 level. We show here that the expression of the host's Abl2 protein, actin cytoskeleton
110 organization and cell-cell adhesion were differently affected after infection by NSs-
111 expressing and non-expressing strains of RVFV. Abl2 is a cytosolic protein encoded by the
112 *Abl2* gene (also known as *Arg* for Abelson related gene) that is considered as a major
113 regulator of actin cytoskeleton, regulating cell morphology and mobility as well as cell-cell
114 and cell-matrix adhesion (18-23). Within its sequence, Abl2 contains a tyrosine kinase
115 domain and two filamentous actin (F-actin) binding domains (20). Through its kinase domain
116 Abl2 regulates Rho GTPases resulting in a negative regulation of focal adhesion and stress
117 fiber formation, attenuating cell contractility and altering adhesion dynamics, that leads to the
118 negative regulation of cell migration (18) and the positive regulation of adherens junction
119 formation (21). Through its F-actin binding domains, Abl2 affects cell shape positively
120 regulating lamellipodia and membrane ruffles (20, 22). In this work we have identified the up
121 regulation of Abl2 expression as part of the cellular response meant to restrict virulence,
122 which was counteracted by the NSs protein following either infection by the ZH and MP12
123 strains of RVFV or the ectopic expression of NSs.

124 Δ NSs infection induced the activation of Abl2 expression that was correlated to a
125 diminution of cell migration. On the contrary, ZH infection prevented Abl2 up regulation in
126 fibroblast, macrophages and hepatocytes subsequently hampering the Δ NSs-induced
127 reduction of cell migration. Thus fibroblasts infected with virulent ZH strain migrated faster
128 than fibroblasts infected with the avirulent Δ NSs strain. In the case of non-migrating
129 hepatocytes, infection with ZH but not Δ NSs induced a strong collapse of cell shape and the
130 distortion of cell-cell adhesion. Similar effects on Abl2 expression and cell structure were
131 observed after infection with the NSs-expressing attenuated MP12 strain of RVFV as well as

132 following the ectopic expression of RVFV's NSs protein on hepatocytes where NSs was
133 identified as the viral factor responsible for the inhibition of the up regulation of Abl2
134 expression, capable also to autonomously affect the host's actin cytoskeleton. Interestingly,
135 NSs protein was found abundantly present within the cytoplasm of ZH- and MP12-infected
136 hepatocytes as well as within long actin-rich structures connecting neighboring cells. While
137 working with the attenuated MP12 strain at low multiplicity of infection, these NSs
138 containing actin-rich structures were found associated to the dissemination of NSs from
139 infected to non-infected cells.

140 We demonstrate here that infection with the virulent strain of RVFV targets cell
141 adhesion and actin cytoskeleton at the transcriptional and cellular level, affecting cell mobility
142 and inducing cell shape collapse alongside with distortion of adherens junctions. We propose
143 that the up regulation of Abl2 expression is part of the host's strategy to restrict virulence and
144 we discuss here how its counteraction by RVFV's NSs protein added to NSs effects on actin-
145 cytoskeleton and cell-cell adhesion alongside with the presence of NSs within intercellular
146 actin-rich structures could facilitate RVFV dissemination and pathogenicity.

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Results

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Avirulent and virulent strains of RVFV differently affected the expression of the gene coding for *Abl2*, a main regulator of actin cytoskeleton.

Infection with the virulent ZH548 (ZH) strain of RVFV is characterized by the presence of the non-structural NSs protein within the nucleus of infected cells, which is absent from cells infected with the avirulent ZH548 Δ NSs (Δ NSs) strain lacking NSs (Fig.1A). While, as expected, the structural N protein encoded by RVFV was expressed in cells infected by ZH as well Δ NSs.

With the aim to investigate the effect of RVFV infection on the expression of genes regulating cell adhesion, the main biological function of the host represented among NSs interacting promoter regions (17), the expression profile of 12 out of the 96 NSs-interacting genes associated with cell adhesion was analyzed in this work by quantitative qPCR (Fig.1B). The expression rates of these 12 genes, chosen because described as expressed in many different cell types, were analyzed with respect to three reference genes, in three different cell lines (fibroblasts, hepatocytes, macrophages) under conditions of either non-infection (NI) or at two different times after infection with either the ZH or the Δ NSs strain of RVFV.

In Fig.1B are shown the ZH/ Δ NSs expression ratios for the 12 genes tested. The expression of all genes excepting *Cdc42*, *Psen1* and *Pvr11* displayed a minimum of 2 fold differences in at least one cell line with the expression of the *Abl2* gene being negatively affected in all the cell lines and at all the time points tested (Fig.1B). As compared to NI conditions, *Abl2* gene expression was enhanced after Δ NSs- but not ZH-infection in the three cell lines tested (Fig. 1C) with consistent results observed also at the protein level (Fig.1D and E). These results suggested a potential role for *Abl2* in the innate antiviral response, as its

171 expression was induced after Δ NSs that induces the innate antiviral response whereas it was
172 blocked after ZH infection that counteracts the innate antiviral response.

173

174 ***Abl2* behaves as a gene associated to the cellular innate antiviral response independent**
175 **of interferon-beta signaling.**

176 In order to consolidate the potential link between *Abl2* expression and the innate
177 antiviral response, *Abl2* expression was further analyzed after infection of L929 cells
178 (corresponding to the murine fibroblasts used in Fig.1) with the Newcastle Disease Virus
179 (NDV). NDV is a *Paramyxoviridae* that, such as the Δ NSs strain of RVFV, is a good inducer
180 of the innate antiviral response in murine cells (24, 25). The expression level of the *Abl2* gene
181 was measured throughout the time course of infection by RT-qPCR alongside with the
182 expression of genes related to the IFN β response, a main actor of the innate antiviral response
183 (26, 27), such as the *Ifnb1* gene coding for IFN β and the *Oas1b* and *Irf7* genes corresponding
184 to two well characterized Interferon β Stimulated Genes (ISGs) (Fig.2A).

185 NDV infection of L929 cells induced the expression not only of the *Ifnb1*, *Oas1b* and
186 *Irf7* genes as expected, but also that of the *Abl2* gene (Fig.2A) as previously observed after
187 Δ NSs infection (Fig.1C). Again, the effects observed at the transcriptional level were also
188 observed at the protein level (Fig.2B and C). While the kinetics of expression of the ISGs
189 *Oas1b* and *Irf7* were, as expected, delayed compared to that of the *Ifnb1* gene, the kinetics of
190 the expression of the *Abl2* gene resembled more that of the *Ifnb1* gene suggesting that the
191 virus-induced activation of the expression of *Abl2* would be independent of IFN β . The role of
192 IFN β signaling on NDV-induced *Abl2* expression was tested using an anti-IFNAR antibody
193 that, by blocking the IFN β receptor IFNAR, antagonizes the IFN β response, neutralizing the
194 expression of ISGs (28). As expected, a dose-dependent inhibition of the expression of *Oas1b*
195 and *Irf7* was observed in the presence of the anti-IFNAR antibody (Fig.2D). However, no

196 effect was detected on the expression of the *Abl2* gene under the same conditions indicating
197 that although *Abl2* behaved as a gene associated to the innate antiviral response, its expression
198 in the context of NDV infection appeared independent of IFN β signaling.

199

200 **Activation of *Abl2* expression in the context of Δ NSs but not ZH infection was correlated**
201 **with the negative regulation of cell migration.**

202 In order to investigate the potential implication of the opposite effects of Δ NSs and
203 ZH infection on *Abl2* expression, we compared the effects of these two strains of RVFV on
204 cell migration that is negatively regulated by *Abl2* (18, 19, 23).

205 Wound healing assays (scratch tests) were carried out on monolayers of confluent
206 L929 fibroblasts, which are fast migrating cells, either NI or infected by the Δ NSs or ZH
207 strains of RVFV. The coverage of the open (scratched) cell-free area by migrating fibroblasts
208 was assessed over time using bright-field imaging. The % of open, cell-free, area was
209 determined by an automated quantitative analysis using the TScratch software (29).
210 Wounding, which corresponded to T=0 h, was performed 24 h after plating the cells on glass
211 cover slips and immediately before infection. Cell counting performed at the beginning (T=0
212 h) and the end (T=20 h) of the assay indicated that confluent cells did not divide during the
213 time lapse of the experiment (data not shown).

214 Under these experimental conditions, NI L929 fibroblasts reached complete coverage
215 of the open area at 20 h after wounding (Fig.3A). TScratch analysis of the % of open area
216 showed that infection with the Δ NSs and ZH strains of RVFV differently affected cell
217 migration (Fig.3B). Infection with the Δ NSs strain significantly inhibited cell migration with
218 Δ NSs-infected cells displaying significantly higher % of open area than NI cells starting 15h
219 p.i. (Fig.3B). Infection with the ZH strain hampered the inhibitory effect of Δ NSs on cell
220 migration. ZH-infected cells migrated faster than Δ NSs-infected cells with ZH-infected cells

221 displaying significant lower % of open area than Δ NSs-infected cells starting 15 h p.i.
222 (Fig.3B). The inhibitory effect of the Δ NSs strain of RVFV on cell migration was also
223 observed after NDV infection with NDV-infected cells displaying significantly higher % of
224 open area than NI cells starting 15 h p.i. (Fig.3C). Overall these results were in agreement
225 with the role of Abl2 as a negative regulator of cell migration (18) since Δ NSs- and NDV-
226 infected cells that expressed the highest amount of Abl2 corresponded to the slowest
227 migrating cells displaying the highest % of open area.

228 In order to question the role of Abl2 on the capacity to slow down cell migration in the
229 context of viral infection we used imatinib, which is an inhibitor of the Abl2 kinase activity
230 that has been previously used to counteract Abl2-dependent inhibition of cell migration (30).
231 The % of wound area under NI and NDV-infected conditions, in the presence or absence of
232 imatinib, was measured 15 h after wounding with 100% corresponding to the open wound
233 area under NI conditions in the absence of imatinib (Fig.3D). As previously observed, in the
234 absence of imatinib, the % of open wound area was significantly higher after NDV infection
235 than in NI cells (Fig.3D) in agreement with previous NDV-dependent reduction of cell
236 migration. In the presence of imatinib, the capacity of NDV infection to negatively affect cell
237 migration was significantly diminished (Fig.3D) indicative of a role for Abl2 kinase activity
238 on NDV-induced inhibition of cell migration.

239

240 **Virulent RVFV infection affected cell morphology and adherens junctions**

241 While through its kinase activity Abl2 negatively regulates cell migration in link with
242 the attenuation of cell contractility and focal adhesion dynamics (18), it positively regulates
243 cell spreading through its two F-actin binding domains promoting the formation of actin-rich
244 structures at the lamellipodia (22, 31, 32). The impact of Δ NSs and ZH infection on cell
245 morphology was analyzed independently of cell migration by fluorescence and confocal

246 microscopy using phalloidin as a marker of F-actin. Experiments were carried out on
247 hepatocytes (AML12 cell line) that constitute one of the main cell types targeted during
248 RVFV infection with liver damage in association with hemorrhagic fever being the most
249 frequent cause of death in humans (1-3). Under our experimental conditions AML12 cells did
250 not display migratory capacity neither before nor after infection with either Δ NSs or ZH
251 strains of RVFV (data not shown) thus allowing the analysis of cell structure independently of
252 cell migration. Despite the propensity of AML12 cells to adhere to each other, isolated cells
253 were identified that were used here to analyze effects on cell structure (Fig.4A-D). Important
254 changes in cell morphology were consistently observed after infection with the ZH strain with
255 respect to NI and Δ NSs-infected cells for which representative images are shown in Fig.4A.
256 While NI and Δ NSs-infected cells displayed significant cell spreading, ZH-infected cells
257 displayed a reduction of cell spreading, lamellipodia and membrane ruffles, and a
258 disorganization of actin fibers visible from 8h p.i. (Fig.4A). Also, long F-actin-rich structures
259 (indicated by an arrow in Fig.4A) were often seen present among isolated ZH-infected cells.
260 ImageJ software was used to quantify changes in cell shape and area based on phalloidin
261 staining of NI, Δ NSs- and ZH-infected cells. Original images were transformed into binary
262 images (Fig.4B) and the outlines of the binary images were used to measure cell area (Fig.4C)
263 and circularity (Fig.4D). Even though not statistically significant, a trend towards an
264 enhancement of cell area was observed 18h p.i. under conditions of Δ NSs infection with
265 respect to NI and ZH infection (Fig.4C) this being in agreement with the up-regulation of
266 Abl2 expression observed in Δ NSs-infected with respect to NI and ZH-infected cells
267 (Fig.1C). In addition, a significant increase in cell circularity was observed after ZH infection
268 at 18h p.i. with respect not only to Δ NSs infected cells but also NI cells (Fig.4D).

269 AML12 cells have a strong propensity to establish cell-cell contacts forming adherens
270 junctions that contain β -catenin. Abl2 has been described to positively regulate β -catenin

271 stability (333) and maintain adherens junctions (19, 21). In agreement with distinct effects on
272 Abl2 expression, infections with Δ NSs and ZH strains differently affected the stability of β -
273 catenin containing cell junctions (Fig. 4E). At late times after infection (18h p.i.), Δ NSs-
274 infected cells appeared to sustain β -catenin containing cell junctions, while a systematic
275 dissolution of β -catenin containing cell junctions was observed after infection with ZH
276 (Fig.4E). At earlier (8 h) times p.i., disruption of β -catenin containing adherens junctions was
277 evidenced among ZH-infected cells with accumulated NSs (Fig.4F). Dissolution of adherens
278 junctions among ZH-infected AML12 cells resulted in loosening of cell-to-cell contact,
279 visible after F-actin labeling in ZH-infected NSs-expressing with respect to NI cells (Fig. 4G)
280 with the strongest effect observed among the cells displaying the highest amount of NSs
281 (indicated by white arrows in Fig.4G).

282 Interestingly, while analyzing the distribution of Abl2 in ZH-infected cells with
283 respect to NSs protein we observed a co-localization of Abl2 with NSs that started to be
284 visible at 8h p.i. (indicated by arrows in Fig.4H) and was clearly enhanced 24 h p.i. (Fig.4H).
285 Even though not visible among all NSs expressing cells, Abl2 co-localization with NSs
286 appeared associated to dissolution of adherens junctions (24 h p.i.) (Fig.4H).

287

288 **The NSs-expressing MP12 strain of RVFV affected Abl2 expression and cell morphology** 289 **similarly to the ZH strain**

290 In order to further confirm the main role of NSs in targeting the actin-cytoskeleton of
291 the host at the transcriptional and cellular level, we carried out infections with RVFV's MP12
292 strain that even though attenuated by several mutations in its S, M and L segments still
293 encodes for a functional NSs protein (34, 35). The NSs protein encoded by the MP12 strain
294 formed filamentous structures in the nuclei of AML12-infected cells (Fig. 5A) similarly to the
295 ZH-encoded NSs protein (Fig.4). The NSs-dependent inhibition of IFN β gene expression is a

296 major trait observed after ZH infection (7, 8, 12, 23). Comparison of IFN β expression in NI,
297 Δ NSs-, ZH- and MP12-infected AML12 cells indicated that, as in the case of ZH infection,
298 MP12 infection strongly inhibited the expression of the IFN β gene otherwise induced by
299 Δ NSs infection (Fig.5B). Alongside with measuring IFN β expression, we also measured the
300 level of Abl2 mRNA in NI, Δ NSs-, ZH- and MP12-infected cells (Fig.5C). The significant
301 Δ NSs-dependent induction of Abl2 expression previously observed in AML12 cells (Fig.1C),
302 and reproduced here, was significantly hampered not only after infection with the ZH strain in
303 agreement with our previous observations but also after MP12 infection (Fig.5C).
304 Consistently, as in ZH-infected cells, the expression of NSs in MP12-infected cells was
305 correlated with a strong alteration of cell shape and spreading (Fig. 5D) and with a loosening
306 of adherens junctions (Fig.5E) for which representative images are shown here. Results
307 obtained after MP12 infection at low multiplicity of infection (MOI), confirmed the
308 correlation between NSs expression and disruption of β -catenin rich adherens junctions (as
309 indicated by a white arrow in Fig.5E) as compared to non-NSs expressing cells. Overall, the
310 results obtained, which are in agreement with previously reported functional similarity
311 between MP12 and ZH encoded NSs proteins (34), confirmed the capacity of NSs protein to
312 affect actin cytoskeleton hampering the up-regulation of Abl2 expression and affecting cell
313 shape and cell-to-cell adhesion.

314

315 **ZH and MP12 encoded NSs proteins are found present within long actin-rich structures**
316 **in association with intercellular NSs dissemination**

317 AML12 cells infected with MP12 displayed not only nuclear but also a cytoplasmic
318 distribution of NSs. In Fig.5E, the cytoplasmic staining of NSs extended more than 30 μ ms
319 away from the nucleus of the corresponding NSs-expressing cell, reaching towards non-NSs
320 expressing cells through weakened adherens junctions (as indicated by a grey arrow in

321 Fig.5E). Cytoplasmic NSs protein was also detected in non-infected cells (indicated by an
322 arrow in Fig.5F), suggesting a dispersion from neighboring infected cells expressing N and
323 NSs favored by disrupted adherens junctions.

324 The presence of cytoplasmic NSs aggregates was a consistent trait observed not only
325 among MP12-infected cells but also among ZH-infected cells that persisted throughout
326 infection and remained visible 18h p.i. (Fig.6A). Among ZH-infected cells, NSs protein was
327 very often visible within F-actin-rich extensions as indicated by white arrows in
328 representative Fig.6A. Occasionally, these actin-rich extensions extended over a long distance
329 interconnecting cells more than 100 μ m apart (Fig. 6B), terminating in an apparent delivery
330 process of the NSs protein into the cell body of a neighboring cell (Fig.6B, insets) suggesting
331 a cell-to-cell spreading of NSs.

332 In order to further investigate the capacity of NSs to disseminate from NSs-expressing
333 to non-NSs expressing cells, we carried out infections with the attenuated strain of MP12 at a
334 low MOI. Viral replication and the % of NSs positive cells were jointly analyzed throughout
335 infection. Viral replication was determined by measuring the relative expression of N and
336 NSs RNAs by RT-qPCR and the percentage of NSs expressing cells was analyzed after
337 fluorescent labeling of cells with Hoechst and anti-NSs antibodies. In order to carry out a
338 random analysis of NSs+ cells, cells were counted according to Hoechst staining before
339 further analyze for NSs staining. Measurements were done throughout two independent
340 infections with each infection carried out with a different batch of MP12 RVFV (Figs.6C and
341 D). Even though differences in the levels of N and NSs expression and in the percentage of
342 NSs expressing cells were observed between the two infections, the values evolved according
343 to the same pattern (Figs.6C and D). MP12 RVFV stopped replicating 24 p.i. with the relative
344 expression of N and NSs RNAs remaining constant (infection#1) or slightly diminishing
345 (infection#2) between 24 and 48h p.i. (Fig.6C). The percentage of NSs positive cells was

346 proportional to the amount of N and NSs RNA between the two infections as well as between
347 8 and 24 h p.i. for each infections. However, notwithstanding a stop in the increase of N and
348 NSs RNA expression, the percentage of NSs positive cells continued to increase between 24
349 and 48 h p.i. in both infections (Fig.6D). MP12 infection has been shown to induce cell cycle
350 arrest (36), similarly to what we have previously shown in the case of ZH infection (13), thus
351 the increase of NSs+ positive cells observed between 24 and 48 h p.i. suggested the existence
352 of a cell-cycle independent mechanism of dissemination of NSs from NSs-expressing towards
353 non-NSs expressing cells.

354 The distribution of NSs+ cells was further analyzed among microscope field of views
355 specifically selected for NSs staining. In Fig.6E are shown representative images of fields of
356 views selected for NSs staining at 8, 24 and 48 h p.i. in the case of infection#1. At 8 h p.i. no
357 more than one NSs+ cell was observed within a field, at 24 h p.i. clusters of NSs+ cells
358 appeared (indicated by a white arrow in Fig.6E) with an average of 11.6 % of NSs+ cells
359 present *per* field. The clustered distribution of NSs+ cells was further enhanced at 48h p.i. so
360 that an average of 58.4 % of NSs+ cells were present *per* field selected for NSs staining
361 (Fig.6E). Yet, when counting NSs+ cells in a random selection of field views, only an average
362 of 3.25 % of NSs+ cells at 24h, with several fields displaying none, rising to 7.6 % *per*
363 random field at 48h p.i. was determined (Fig.6D).

364 A cytoplasmic spreading of NSs more than 30 μ m away from the cell's nucleus was
365 again observed 24 h p.i. (indicated by a grey arrow in Fig.6E). Also, a gradient-like
366 distribution of NSs was observed 48 h p.i. (indicated by a triangle in Fig.6E), suggesting a
367 from cell to cell spreading of NSs. In order to further confirm the spreading of NSs from
368 NSs+ towards non-NSs expressing cells, we looked for NSs dissemination events under
369 conditions for which only a small percentage NSs+ cell were detected in a field view, such as
370 was the case during infection#1 of AML12 cells by MP12 at low MOI 24h p.i.. Under these

371 conditions, NSs dissemination events were indeed identified such as the one shown in Fig.6F
372 where an infected, NSs-expressing cell (indicated by an arrow) bridges two non-infected,
373 non-NSs expressing cells through long actin-rich NSs-containing structures that ended up in a
374 NSs delivery process (Fig.6F). Even though the N protein of RVFV was also visible within
375 actin rich extensions, only NSs was delivered to non-infected cells as observed in previous
376 Fig.5F.

377 RVFV's N protein was consistently found present within long actin-rich structures in
378 the context of ZH infection but to a lesser extent following MP12 infection at low MOI
379 (Fig.6G). Since NSs protein was similarly present within long actin rich extensions in the
380 context of ZH and low MOI MP12 infections, the differential staining observed here for
381 RVFV's N protein in long actin rich extensions, for the most not co-localizing with NSs,
382 suggested that the N protein of RVFV was not necessary for NSs to be present within these
383 structures.

384

385 **The ectopic expression of NSs protein mimics ZH infection**

386 The presence of the non-structural NSs protein within intercellular actin-rich
387 protrusions raised the question of the potential capacity of NSs to convey virulent phenotypes
388 autonomously, independently of other viral factors.

389 The capacity of the ectopically expressed NSs protein to induce ZH-like phenotypes
390 was tested by transfecting non-infected AML12 cells with either a plasmid carrying the
391 cDNA coding for NSs (pCI-NSs) or the empty vector (pCI). Twenty-four hours after
392 transfection, the expression of NSs RNA was detected in AML12 cells transfected with pCI-
393 NSs and not in pCI-transfected cells (Fig.7A). The expression of NSs RNA was calculated
394 here with respect to three reference genes (*Ppib*, *Utp6c* and *Hprt1*) for which the
395 corresponding expression rates did not vary after transfection with either pCI-NSs or pCI.

396 Indicative that at least 24 h after its expression, NSs did not significantly affected the
397 transcriptional capacity of AML12 cells. This lack of effect on the transcriptional capacity of
398 the host observed here 24 h after NSs expression is in agreement with our previous
399 observations in ZH-infected L929 cells (17) and with more recently reported results in MP12-
400 infected HEK293 cells (37). Notwithstanding variations observed from one transfection assay
401 to another, 10-30 % of the pCI-NSs transfected cells were identified at the single cell level as
402 expressing NSs protein by immunofluorescence using anti-NSs antibody. In these cells, the
403 ectopically expressed NSs protein formed nuclear filamentous structures (Fig.7B) resembling
404 those observed after infection with the virulent ZH strain of RVFV. However, compared to
405 ZH- and MP12-infected AML12 cells, the ectopically expressed NSs protein displayed an
406 exclusively nuclear distribution with no NSs detected outside the nucleus, suggesting that a
407 viral component other than NSs could be required to induce a cytoplasmic NSs distribution in
408 AML12 cells.

409 The effect of the ectopically expressed NSs protein on actin cytoskeleton and cell
410 morphology was analyzed by fluorescence and confocal microscopy using phalloidin to label
411 F-actin alongside with anti-NSs antibodies. Cells transfected with the empty vector (Fig. 7C,
412 pCI) displayed a morphology and an actin cytoskeleton organization similar to that of non-
413 transfected, non-infected AML12 cells (Fig. 4A). Among AML12 cells transfected with pCI-
414 NSs (Fig. 7C, pCI-NSs), those cells that ectopically expressed NSs (indicated by arrowheads
415 in Fig.7C) displayed a strong reduction of cell spreading and a disorganization of F-actin
416 fibers similarly to ZH- and MP12-infected AML12 cells (Figs. 4A and 5D). Therefore the
417 non-structural NSs protein alone, in the absence of any other RVFV component, was able to
418 induce changes in cell morphology similar to those induced by the virulent ZH strain. The
419 effect of the ectopically expressed NSs protein on the expression of the *Abl2* gene was also
420 analyzed. No significant changes in *Abl2* gene expression were observed among cells

421 transfected with pCI-NSs as compared to pCI (Fig.7D). This was similar to the lack of effect
422 of ZH and MP12 infection on the level of *Abl2* expression displayed by NI AML12 cells
423 (Figs.1C and 5C respectively) and further confirmed the absence of effect of NSs expression
424 on the host transcriptional capacity. However, the ectopic expression of NSs was capable to
425 significantly inhibit the expression of the *Abl2* gene induced after infection with NDV
426 (Fig.7D), similarly to the previously observed capacity of ZH and MP12 infection to prevent
427 the increase of *Abl2* gene expression otherwise induced by Δ NSs (Figs.1C and 5C
428 respectively), further confirming the capacity of NSs protein to mimic ZH-induced effects
429 independently of any other protein encoded by RVFV.

430 The capacity of the ectopically expressed NSs protein to mimic the effects of ZH
431 infection on Δ NSs-induced cellular gene expression was further analyzed for genes
432 participating in the IFN β response and the coagulation cascade whose expressions have been
433 previously reported to be affected after ZH with respect to Δ NSs infection (17, 24). As shown
434 in Fig. 7D, the ectopic expression of NSs in AML12 cells significantly inhibited the NDV-
435 induced activation of the expression of the genes related to the IFN β response (such as the
436 *Ifnb1* gene coding for IFN β and the two ISGs *Irf7* and *Oas1b*) while it activated the
437 expression of the *F10* gene coding for coagulation factor F10, as it was previously observed
438 after infection with the ZH strain with respect to Δ NSs-infected cells (17).

439 Overall these results demonstrated that the NSs protein encoded by RVFV had the
440 capacity to autonomously induce phenotypes similar to those observed after infection with the
441 pathogenic virulent ZH strain of RVFV, raising the question of the potential deleterious
442 effects that could be conveyed by the intercellular spreading of NSs protein.

443

Discussion

443

444 We show here evidence that the viral NSs protein, which is the major factor of
445 virulence of RVFV, affects the actin cytoskeleton of the host at the transcriptional and cellular
446 level. At the transcriptional level, NSs expression counteracted the up-regulation of Abl2
447 expression, a major regulator of actin cytoskeleton that in this work was found associated to
448 the cellular response to a viral infection. At the cellular level, the presence of NSs was
449 associated to strong changes in cell morphology that were reminiscent of the effects on cell
450 and tissue structures observed after ZH infection *in vivo* (16).

451 Besides a major role for NSs on cell structure and actin cytoskeleton organization, we
452 describe here for the first time an effect of RVFV's NSs-expressing ZH strain on cell mobility
453 with ZH- migrating faster than Δ NSs-infected cells. In the case of influenza virus infection,
454 restriction of cell mobility was found to be highly protective (38). Similarly, we hypothesize
455 that the reduction of cell mobility induced here after avirulent Δ NSs infection in correlation
456 with an up-regulation of Abl2 expression would be necessary to limit viral dissemination by
457 migrating innate immune cells such as macrophages, dendritic cells, neutrophils and
458 microglia that are infected by RVFV *in vitro* and/or *in vivo* (39, 40). Hampering reduction of
459 cell mobility, as observed here following virulent ZH infection, could favor virulence and
460 pathogenicity. In agreement with this hypothesis, a recent work has shown a major role for
461 the migration into the central nervous of innate immune cells carrying a viral antigen in lethal
462 RVFV encephalitis in Lewis rats (40).

463 Abl2 has been shown to negatively regulate cell mobility in link with the negative
464 regulation of cell contractility and focal adhesion dynamics resulting from Abl2 regulation of
465 the activity of Rho GTPases (18-20). Also, Abl2 positively regulates cell-cell adhesion (in
466 link with its negative effect on cell contractility and the stabilization of β -catenin) (19, 21, 33)
467 and cell spreading in link with its capacity to directly interact with F-actin (22, 31, 32). The

468 effects on cell mobility observed after Δ NSs infection with respect to NI and ZH-infected
469 cells were indeed those expected for an up-regulation of Abl2 expression. Also in agreement
470 with Δ NSs-induced up-regulation of Abl2 expression, a trend towards cell spreading and
471 consolidation of adherens junctions was observed in Δ NSs- with respect to non-infected cells.
472 If the effects of Δ NSs infection on cell morphology remained weak, it is probably because NI
473 AML12 cells expressed Abl2, displayed substantial cell spreading and consistent formation of
474 β -catenin rich adherens junctions before Δ NSs infection, independently of an up-regulation of
475 Abl2 expression. Therefore, no more than limited effects on AML12 cell morphology with no
476 further significant enhancement of cell spreading and cell-cell adhesion, are expected
477 following Δ NS-induced up-regulation of Abl2.

478 In the case of either ZH and MP12 infection or ectopic expression of NSs, the
479 morphological changes induced in link with NSs expression resembled those expected for
480 Abl2 deficient cells. However, these changes were not always correlated with differences in
481 Abl2 expression. In fact, strong morphological differences were observed not only between
482 Δ NSs- and ZH- or MP12-infected cells but also between ZH- and non-infected cells while
483 both displayed similar levels of Abl2 expression. However, it is plausible that the association
484 of Abl2 with NSs filaments highlighted in this work could impact Abl2's capacity to interact
485 with the actin cytoskeleton and thus participate in the establishment of the NSs-dependent
486 morphological changes resembling Abl2 deficient phenotypes.

487 Nonetheless, even though NSs-dependent effects on cell mobility and cell morphology
488 resembled those expected for Abl2 deficient phenotypes, the direct link between the targeting
489 of Abl2 by NSs and NSs-dependent effects on actin cytoskeleton shown in this work remains
490 presently to be demonstrated.

491 The presence of long intercellular actin-rich NSs-containing structures, associated to
492 the dissemination of NSs protein, was another feature of ZH- and MP12-infected AML12

493 cells. These structures are reminiscent of the actin-rich intercellular connections that have
494 been described to facilitate virus spreading to neighboring cells, as in the case of influenza A
495 virus (41), HIV-1 (42) or lymphocytic choriomeningitis virus (LCMV) (43). Cell-to-cell
496 transmission of the LCMV through actin-rich intercellular structures was facilitated by virus-
497 induced enhanced cell migration (43), an interesting feature with respect to the capacity of ZH
498 infection to hamper negative regulation of cell mobility. Actin-rich intercellular structures
499 have also been described to facilitate the dissemination of prion and prion-like proteins (44,
500 45). *In vivo*, only a very small fraction of the host cells are primary infected cells and the
501 efficiency of the host's antiviral response largely depends on the paracrine IFN β signaling
502 triggered by the small number of cells initially infected (46). NSs was proven here to be able
503 to affect the host's IFN β response alone, independently of any other viral compound.
504 Therefore, the dissemination of NSs from NSs-expressing to non-NSs expressing cells that we
505 have shown here to occur at a low MOI between neighboring cells through disrupted
506 adherens junctions or long actin rich structures, could have strong negative effects on the
507 capacity of the host to stop viral dissemination *in vivo*.

508 The presence of NSs within actin-rich extensions and NSs dissemination was observed
509 here in ZH- and MP12-infected AML12 cells that displayed a dual nuclear/cytoplasmic
510 distribution of NSs but was not observed in cells ectopically expressing NSs that displayed
511 only a nuclear NSs distribution. A recent work by Li *et al.* (10) has demonstrated an
512 important role *in vivo* for the dual nuclear and cytoplasmic distribution of NSs in RVFV's
513 virulence and pathogenicity. In order to question the possible role of the subcellular
514 distribution of NSs protein in regulating intercellular NSs spreading, it would be necessary to
515 analyze the effect of the different rRVFVs established by Li *et al.* (10) on NSs dissemination.
516 Li *et al.* (10) have described cytoplasmic versus nuclear distribution of NSs in relation with
517 specific regions of NSs. This suggests a role for NSs structure and/or NSs interaction with

518 another protein in the regulation of the nuclear/cytoplasmic distribution of NSs. The absence
519 of cytoplasmic NSs among cells ectopically expressing NSs suggested a role for another viral
520 component in this process.

521 At the transcriptional level, NSs altered not only the expression of Abl2 but also that
522 of other genes involved in cell adhesion. For instance, we showed here (Fig.1) that, compared
523 to Δ NSs, ZH infection negatively impacted *Lgals3bp* gene expression that codes for an
524 extracellular matrix protein promoting integrin-mediated cell-matrix and cell-cell adhesion
525 (47, 48). Also, while analyzing alterations in the host transcriptome following infection with
526 the ZH strain of RVFV with respect to non-infected cells, Pinkham et al. (49) identified the
527 Interleukin-linked kinase (ILK) pathway as one of the main host's pathways altered after
528 infection. ILK connects the extracellular matrix to actin cytoskeleton by interacting with the
529 cytoplasmic domain of β integrins regulating several functions related to actin cytoskeleton
530 organization. Interestingly, the cytoplasmic tail of β 3 integrin has been shown to directly
531 interacts with Abl2 (22) thus connecting Abl2 with ILK signaling, further establishing cell
532 adhesion as a main biological processes targeted by RVFV.

533 Overall we have shown here that infection with the virulent ZH strain of RVFV
534 prevented the up-regulation of the expression of Abl2, counteracted Δ NSs-induced inhibition
535 of cell migration, affected cell shape and induced the dissolution of adherens junctions. These
536 effects were shown here to be dependent on the expression of NSs that itself was shown to be
537 present within long actin-rich structures involved in the dissemination of NSs to neighboring,
538 non-NSs expressing cells. These NSs-dependent effects on the host's actin cytoskeleton are
539 susceptible to participate in RVFV-induced pathogenicity by facilitating virulent RVFV
540 dissemination and thus could constitute interesting potential targets in future development of
541 antiviral therapeutic strategies that in the case of RVFV, as several other emerging
542 arboviruses, are presently lacking.

543

Materials and Methods

543

544 **Virus and cells**

545 Stocks of RVFV ZH548, ZH548 Δ NSs and MP12 were produced under BSL3 conditions by
546 infecting Vero cells at m.o.i. of 10^{-3} and harvesting the medium at 72 hr p.i. Cell lines were
547 cultured at 37 °C under 5% CO₂ in medium supplemented with 10 μ g/mL
548 penicillin/streptomycin (Gibco, Thermo Scientific) and 10% (v/v) fetal calf serum (FCS,
549 Gibco). Murine fibroblastic L929 (ATCC) cells were grown in MEM medium (Gibco).
550 Murine hepatocyte, alpha mouse liver 12, AML12 cells (ATCC ref. CRL-2254) were cultured
551 in DMEM-F12 medium (Gibco) supplemented with 0.005 mg/mL insulin, 0.005 mg/mL
552 transferrin, 5 ng/mL selenium (Sigma) and 40 ng/mL dexamethasone (Gibco, Thermo
553 Scientific). Non-infected cells (NI) correspond to mock-infected cells that were treated as
554 infected cells except that no virus was added. When indicated, antibody directed against the
555 mouse IFN α /b receptor 1 (clone MAR1-5A3 from BD Biosciences n°561183) was added
556 directly to the media 15 h before infection and kept in the media during the entire time of
557 infection. All infections were carried out at an MOI=5 unless otherwise indicated.

558

559 **Antibodies**

560 Primary antibodies used for immunofluorescence and/or Western blot were mouse anti-NSs
561 and rabbit anti-N polyclonal antibodies raised against the entire NSs or N protein of RVFV
562 respectively (9); anti- β -catenin monoclonal antibody (BD Transduction laboratories
563 Cat#610154); anti-Abl2 (anti-Arg ref 07-262) from Merck Millipore and anti-GAPDH (ref
564 3781) from ProSci. Secondary antibodies used for immunofluorescence were Alexa 488
565 fluor-conjugated chicken anti-mouse from Invitrogen (A21200) and Alexa 555 fluor-
566 conjugated donkey anti-rabbit from Invitrogen (A31572). Secondary antibodies used for

567 Western blot were ECL Mouse IgG, HRP-linked whole Ab (NA931) and ECL Rabbit IgG,
568 HRP-linked whole Ab (NA934) from GE Healthcare Life Sciences.

569

570 **Immunofluorescence.**

571 Cells grown in 6-well plates on coverslips were fixed with 3.7 % formaldehyde in phosphate-
572 buffered saline (PBS) for 15 min and permeabilized with 1 % Triton X-100 in PBS for 20
573 min. The cells were incubated for 1 h at room temperature with the corresponding primary
574 antibodies diluted in PBS-5% bovine serum albumin. After washing with PBS the cells were
575 next incubated for 45 min at room temperature with the corresponding secondary antibodies
576 or phalloïdin-FITC (Sigma P5282).

577

578 **Image analysis and quantification.**

579 Samples were analyzed at room temperature by confocal laser scanning microscopy using a
580 AxioImager Z2 (Zeiss LSM710 confocal system). This system is equipped with a 63× lens,
581 1.4-numerical-aperture oil immersion lens (Plan Neofluor). For oil immersion microscopy, we
582 used oil with refractive index of 1.518 (Zeiss). Images were captured in the z-axis
583 corresponding to the optical axis of the microscope at 0.37 μm intervals with the z-axis going
584 through the image planes. LSM Zen imaging software was used for image capture. The
585 images were analyzed by the LSM5 Image browser or Image J software. All images are
586 representative of a minimum of two independent infections for all conditions described in this
587 work. The Analyze Particle Plugin of Image J was use to analyze and quantify cell shape and
588 area.

589

590 **Cell transfection.**

591 Plasmid pCI-NSs used for heterologous NSs protein expression contains the cDNA sequence
592 of NSs from ZH548 RVFV strain (50). Twenty-four hours before transfection, AML12 cells
593 were plated in 6 well plates at a density of 2×10^5 cells per well. Mix A containing 2 μ g of
594 plasmid diluted in 50 μ l of low fetal bovine serum OptiMEM media was added to mix B
595 containing 5 μ l of Lipofectamine 2000 (ref 11668-027, Invitrogen) diluted in 195 μ l of
596 OptiMEM and incubated at room temperature for 20 min before being dispensed into the
597 well. Transfected cells were further incubated as previously described for 24 h before
598 treatment.

599

600 **Scratch wound healing assay.**

601 Murine fibroblast L929 cells were plated on coverslips in 6-well plates at a density of 600.000
602 cells/well and cultured as previously described for 24 h to reach full confluence before
603 scratch. Cell monolayers were scratched with a P10 pipette tip, incubated in fresh MEM
604 medium supplemented with 10% FBS and treated as indicated in Fig.3. After treatment cells
605 were fixed in 3.7 % formaldehyde for 20 min. Images were collected at indicated times under
606 a wild field microscope and quantitatively analyzed using the T-Scratch software (30).

607

608 **RT-qPCR**

609 Total RNA was extracted using Tri Reagent (Sigma) according to the manufacturer's
610 protocol. 1 μ g of total RNA was reversely transcribed using High Capacity cDNA Reverse
611 Transcription Kit (Applied Biosystems) according to the manufacturers' recommendations
612 using Random Primers. qPCR was performed using SYBR Green (Thermo Scientific)
613 reagents: 95°C 15 minutes, then 40 cycles at 95°C 15 seconds, 60°C 30 seconds, 72°C 15
614 seconds, followed by a dissociation step. Relative quantification of mRNA expression was
615 calculated using the $\Delta\Delta C_T$ method using three reference genes among Ppib, Hprt1, Utp6c and

616 Rplp0. For fold changes lower than 1 (translating a repression of the gene's expression),
617 values were set such that a (1/2) fold change corresponds to -2, a (1/4) corresponds to -4 and so
618 on. Sequences (5'-3') of primers used for RT-qPCR analysis were as follows= *Abl2* F:
619 GAGCCACCGTTTTACATTGTGA and R: CTCGCCCACTAGGCAGTTC; *Cdc42* F:
620 CTTCTTCGGTTCTGGAGGCT and R: GGGATCTGAAGGCTGTCAAG; *Fblim1*
621 F: TAGCCGTGAGTGAGGAAGTG and R: CAGAGAGTGAGGCATTGGTCT; *Hprt1* F:
622 TCCTCCTCAGACCGCTTTT and R: CCTGGTTCATCATCGCTAATC; *Ifnb1* F:
623 ATGAACAACAGGTGGATCCTCC and R: AGGAGCTCCTGACATTTCCGAA; *Irf7* F:
624 CAGCGAGTGCTGTTTGGAGAC and R: AAGTTCGTACACCTTATGCGG; *Lgals3bp* F:
625 TGCTGGTTCAGGGACTCAA and R: CCACCGGCCTCTGTAGAAGA; *Lpp* F:
626 GCAACCAAGAAGTCCGCAAC and R: GCCGTAGTATAGGAGGCTGGA; *N* protein of
627 RVFV F: AAGGCAAAGCAACTGTGGAG and R: CAGTGACAGGAAGCCACTCA; *NSs*
628 protein of RVFV F: GCACCTCCACCAGCGAAGCC and R:
629 CCAGTGAGGGTTCTCCAAGAGGC; *Oas1b* F: GAGGTGCCGACGGAGGT and R:
630 TCCAGATGAAGTCTTCCCAAAG; *Pkp2* F: GCCGAGTGTGGCTACATCC and R:
631 CTGCTGGTTCGGTGAAGGTT; *Ppib* F: GGAGATGGCACAGGAGGAAA and R:
632 CCGTAGTGCTTCAGTTTGAAGTTCT; *Psen1* F: ATACCTGCACCTTTGTCCTACT and
633 R: GCTCAGGGTTGTCAAGTCTCT; *Ptprf* F: CTGCTCTCGTGATGCTTGGTT and R:
634 ATCCACGTAATTCGAGGCTTG; *Pvr11* F: GACTCCATGTATGGCTTCATCG and R:
635 CACTCGTTTCTCGTAGGGAGG; *Rplp0* F: CACTGGTCTAGGACCCGAGAAG and R:
636 GGTGCCTCTGGAGATTTTCGk; *Thbs1* F: GGGGAGATAACGGTGTGTTTG and R:
637 CGGGGATCAGGTTGGCATT; *Utp6c* F: TTTCGGTTGAGTTTTTCAGGA and R:
638 CCCTCAGGTTTACCATCTTGC; *Vezt* F: AACCCCGAACACTTGGGATG and R:
639 GAAGGCTCGGCTATTGGCTG; *Vtn* F: CCCCTGAGGCCCTTTTTTCATA and R:
640 CAAAGCTCGTCACACTGACA.

641

642 **Western blot**

643 Total protein extracts were prepared from cells grown in 6-well plates in 250 μ l/well
644 of RIPA lysis buffer to containing phosphatase inhibitor PhoSTOP (Roche, 04 906 856 001)
645 and protease inhibitor (Roche, 04 693 132 001). After centrifugation at 14 000 rpm for 10 min
646 at 4°C, lysates were heated in Laemmli buffer and loaded in NuPAGE 4-12 % sodium
647 dodecyl sulfate-polyacrylamide precast gels (Life Technologie). Loaded proteins were
648 transferred on PolyVinylidene diFluoride (PVDF) membrane. Membranes were incubated
649 overnight at 4°C with primaty antibodies, washed with TBS-T (Tris Base Sodium Tween 0.1
650 %) and incubated with the corresponding secondary antibodies for 1 h at room temperature.
651 Imager ImageQuant LAS4000 was used for chemiluminescent protein detection. Relative
652 quantification of proteins was carried out using Image J software.

653

653

Declarations

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Figure Legends

823 **Figure 1. Infection with RVFV affects the expression of genes related to cell adhesion**
824 **including Abl2 expression.** (A) Murine L929 fibroblasts either non-infected (NI) or infected
825 with the virulent ZH548 (ZH) or the avirulent ZH548 Δ NSs (Δ NSs) strains of RVFV were
826 labeled 15 h p.i. using a DNA intercalating agent to visualize the nucleus (Hoechst, in blue),
827 an antibody directed against the structural N protein of RVFV (N, in red) or an antibody
828 directed against the non-structural NSs protein of RVFV (NSs, in green). (B, C) The
829 expression of 12 genes associated with cell adhesion, previously identified to interact with the
830 NSs protein of RVFV (17), was measured in macrophages (RAW cell line), hepatocytes
831 (AML12 cell line) and fibroblasts (L929 cell line) either non-infected (NI) or at the indicated
832 times after infection with ZH or Δ NSs strains. Purified RNAs were analyzed by RT-qPCR
833 using Biomark with primers specific for each gene of interest. Relative quantification of
834 mRNA expression was calculated with respect to three reference genes corresponding to
835 *Hmbs*, *Ppib* and *Hprt1* in the case of RAW cells; *Hprt1*, *Ppib*, and *Rplp0* in the case of
836 AML12 cells; *Ppib*, *Rplp0* and *Utp6c* in the case of L929 cells. In (B) the fold change in ZH-
837 infected cells was calculated with respect to Δ NSs-infected cells. In (C) fold induction was
838 calculated with respect to non(mock)-infected (NI) cells. Results correspond to the average of
839 n (independent experiments)=3 in the case of AML12 and L929 cells for all conditions tested
840 (except L929 ZH 16h for which n=2) and n=2 in the case of RAW cells for all conditions
841 tested. Data are means \pm s.d. One-way ANOVA Tukey's multiple comparison test. In (B), the
842 expression level of certain genes could not be tested since the corresponding mRNA remained
843 not detectable (ND). (D, E) Abl2 protein level was analyzed by Western blot in extracts from
844 L929 cells non-infected (NI) or infected by the Δ NSs or ZH strains of RVFV. (E)
845 Quantification using ImageJ of n (independent experiments)=3 in the case of ZH and Δ NSs
846 15 and 20 h p.i. and n=6 for NI 15 and 20 h. Data are means \pm s.d. One-way ANOVA test

847 with Bonferroni correction. P-value <0.0001 (****), <0.001 (***), < 0.01 (**) and <0.05 (*);
848 ns= not significant. Images correspond to a single confocal section. Bar=10 μ m.

849 **Figure 2. *Abl2* behaves as a gene of the cellular innate antiviral response independent of**
850 **interferon-beta signaling. (A)** The expression of genes associated to the IFN β response,
851 coding for IFN β and for two ISGs (IRF7 and OAS1B), alongside with the gene coding for
852 *Abl2*, was measured by RT-qPCR in L929 cells either non-infected (NI) or at different times
853 after NDV infection. Relative expressions were determined with respect to three reference
854 genes (*Utp6c*, *Ppib*, *Hprt1*) and fold inductions were calculated with respect to non-infected
855 (NI) cells. Results correspond to the average of n (independent experiments)=3 for all
856 conditions tested. Data are means \pm s.d. Statistical analysis was carried out only for *Abl2* gene
857 expression using one-way ANOVA test with Dunnett correction with each NDV-infected
858 condition compared to non-infected conditions. **(B, C)** *Abl2* protein level was analyzed by
859 Western blot in extracts from L929 cells non-infected (NI) or infected by NDV. **(C)**
860 Quantification using ImageJ with n (independent experiments)=3 for NDV 8, 20 and 24 h p.i.
861 and n=10 for NI. Data are means \pm s.d. One-way ANOVA test with Dunnett correction and
862 each NDV-infected condition compared to non-infected condition. **(D)** The expression of
863 genes associated to the IFN β response, coding for IFN β and for two ISGs (IRF7 and
864 OAS1B), alongside with the gene coding for *Abl2*, was measured by RT-qPCR in L929 cells
865 collected 6 h p.i. with NDV, either not-treated or treated with increasing amounts of anti-
866 IFNAR antibody. Relative expressions were determined with respect to three reference genes
867 (*Utp6c*, *Ppib*, *Hprt1*) and fold inductions were calculated with respect to non-infected (NI)
868 cells considered as 100%. Results correspond to the average of n (independent
869 experiments)=4 for 0 μ g; n=2 for 0.5 μ g; n=3 for 1.5 and 5 μ g of anti-IFNAR antibody. Data
870 are means \pm s.d. One-way ANOVA test with Bonferroni correction for each gene separately.
871 P-value <0.001 (***), < 0.01 (**) and <0.05 (*).

872 **Figure 3. Infection with Δ NSs or NDV reduced cell migration.** (A) Representative bright-
873 field images of a monolayer of confluent non-infected (NI) L929 cells taken at different times
874 after introducing a scratch wound with a pipette tip. Images are shown before (original
875 images) and after automated analysis by TScratch program. Percentage of open areas as
876 determined by the TScratch program are indicated with 100% corresponding to the area of the
877 entire image. (B, C, D) Scratch-wound closure of L929 cells was monitored over time in non-
878 infected (NI) cells and after infection with Δ NSs or ZH (B) or NDV (C) or NDV 15h p.i. in
879 presence or absence of imatinib (D). Wound closure is expressed as the % of remaining open
880 area uncovered by cells as determined by the TScratch program with 100% corresponding in
881 (B, C) to the scratch area of non-infected (NI) cells at time point 0 h and in (D) to the scratch
882 area of non-infected cells at 15h in the absence of Imatinib. (B) For each condition (NI, Δ NSs
883 and ZH), results correspond to the average of n (number of images analyzed)=18 (from 3
884 independent experiments) for 0, 8, 15 and 20 h; n=12 (from 2 independent experiments) for
885 10h. (C) For each condition (NI, NDV), results correspond to the average of n (number of
886 images analyzed)=12 (from 2 independent experiments) for 0, 8, 15, 20 and 24 h. (D) Results
887 correspond to the average of n (number of images analyzed)=9 (from 3 independent
888 experiments). Data are means \pm s.d. One-way ANOVA test with Bonferroni correction for
889 each time point separately (B), for all samples (D) and Student's t-test in (C). P-value
890 <0.0001 (****), <0.001 (***), <0.01 (**) and <0.05 (*).

891 **Figure 4. Infection with the virulent ZH but not the avirulent Δ NSs strain affects cell**
892 **morphology and adherens junctions.** Murine hepatocytes (AML12 cell line) either non-
893 infected (NI) or infected with the avirulent Δ NSs or the virulent ZH strains of RVFV were
894 labeled 8 h (A, B, F and H) and 18 h (E, G and H) p.i. using a DNA intercalating agent to
895 visualize the nucleus (Hoechst, in blue), phalloidin to visualize filamentous actin (F-actin in
896 green or grey in A, B, G and H), an anti-NSs antibody (NSs, in red), an anti-beta-catenin

897 antibody (b-cat in green in **E** and **F**) and an anti-Abl2 (Abl2 in green in **H**). White arrows
898 indicate long F-actin-rich extensions in **A**, dissolution of adherens junctions in **F** and **G** and
899 co-localization of Abl2 with NSs in **H**. (**B**) The analyze particle function of ImageJ software
900 was used to transform the images into binary images and determine the cell outlines. (**C**, **D**)
901 Main parameters such as the cell area and perimeter were determined and cell shape was
902 analyzed by calculating the circularity= $4\pi \times (\text{area}/\text{perimeter}^2)$. Results, from a minimum of 2
903 independent experiments for each condition, correspond to the average of n (number of cells
904 analyzed)=70 for NI; n=11 for Δ NSs 8 h p.i.; n=18 for Δ NSs 18 h p.i. ; n=48 for ZH 8 h p.i.;
905 n=21 for ZH 18 h p.i.. Data are means \pm s.d. One-way ANOVA test with Dunnett correction
906 with Δ NSs and ZH compared to NI and Δ NSs compared to ZH. P-value <0.05 (*). Images
907 correspond to single confocal sections. Bar=10 μ m.

908 **Figure 5. Infection with the NSs-expressing MP12 of RVFV affects Abl2 expression and**
909 **cell morphology.** Murine AML12 hepatocytes infected with the MP12 strain of RVFV were
910 labeled 8 h (**D**) and 24 h (**A**, **E**, **F**) p.i. using a DNA intercalating agent to visualize the
911 nucleus (Hoechst, in grey or blue), antibodies directed against the non-structural NSs (in red)
912 or the N (in green) proteins of RVFV and phalloidin to visualize filamentous actin (F-actin in
913 green or grey). In **E** and **F** MP12 infection was carried out at a MOI=0.01. (**B**, **C**) The
914 expression of the genes coding for IFN β (**B**) and Abl2 (**C**) was measured by RT-qPCR in
915 AML12 cells either non-infected (NI) or at the indicated times after infection with Δ NSs, ZH
916 or MP12 strains. Relative expressions were determined with respect to *Rplp0* as reference
917 gene and fold inductions were calculated with respect to non-infected (NI) cells. Results
918 correspond to the average of n (independent experiments)=2. Data are means \pm s.d. One-way
919 ANOVA Tukey's multiple comparison test. (**E**) Grey arrow indicates NSs dissemination and
920 white arrow indicates dissolution of adherens junctions. (**F**) White arrow indicates NSs

921 dissemination. P-value <0.05 (*). In **E** and **F** images correspond to single confocal sections.
922 Bar=10 μ m.

923 **Figure 6. ZH and MP12 encoded NSs proteins are found present within long actin-rich**
924 **structures in association with intercellular dissemination.** Murine hepatocytes (AML12
925 cell line) infected with the NSs-expressing ZH and MP12 strains of RVFV at an MOI= 0.01
926 in the case of MP12 were labeled 8 h (**B**, **E** and **G**), 18 h (**A**), 24 h (**E** and **F**) and 48 h (**E**) p.i.
927 using a DNA intercalating agent to visualize the nucleus (Hoechst in grey or blue in **E**, **F** and
928 **G**), phalloidin to visualize filamentous actin (F-actin in green or grey in **A**, **B** and **F**), an anti-
929 NSs antibody (NSs, in red) and an anti-N antibody (N in green in **F** and **G**). White arrows
930 indicate NSs-containing actin-rich structures in **A**, NSs dissemination in **E** and infected, NSs-
931 expressing cells in **F**. White triangle indicates a gradient staining of NSs in **E**. (**C**) The
932 expression N and NSs RNAs was measured by RT-qPCR in AML12 cells either non-infected
933 (NI) or at the indicated times after infection with MP12 at an MOI= 0.01. Relative
934 expressions were determined with respect to *Rplp0*. (**D**) The percentage of cells displaying
935 NSs labeling was randomly determined at indicated times following MP12 infection at MOI=
936 0.01. In **A**, **B**, **F** and **G** images correspond to single confocal sections. Bar=10 μ m.

937

938 **Figure 7. The ectopic expression of NSs protein affects cell morphology and gene**
939 **expression mimicking ZH infection.** AML12 cells were transfected with a plasmid encoding
940 the NSs protein of RVFV (pCI-NSs) or with the empty vector (pCI). Twenty-four hours after
941 transfection, the presence of NSs mRNA was quantified by RT-qPCR (**A**) and the presence of
942 NSs protein was analyzed by fluorescence (**B**) using an anti-NSs antibody (NSs, in green) and
943 a DNA intercalating agent to visualize the nucleus (Hoechst, in blue). (**C**) Cell shape was
944 analyzed by fluorescence using phalloidin to visualize filamentous actin (F-actin, in red) and
945 a DNA intercalating agent to visualize the nucleus (Hoechst, in blue). (**D**) Twenty four hours

946 after transfection with plasmids pCI or pCI-NSs, transfected cells were either non-infected
947 (NI) or infected with NDV. Four hours after infection, RNA was collected and the expression
948 level of RNA coding for NSs, Abl2, IFN, Irf7, Oas1b and F10 was measured by RT-qPCR.
949 **(A, D)** Relative expressions were calculated with respect to three reference genes (*Ppib*,
950 *Utp6c*, *Hprt1*). In the case of the *Abl2*, *Ifnb1*, *Irf7* and *Oas1b* genes, the % of expression was
951 determined with respect to pCI+NDV conditions considered as 100%. **(A, D)** Results
952 correspond to an average of n (independent experiments)=3 except for the *Ifnb1* and *Oas1b*
953 genes for which n=4. Data are means \pm s.d. **(D)** Student's t-test. P-value <0.001 (***) , < 0.01
954 (**) and <0.05 (*). Images correspond to single confocal sections. Bar= 10 μ m.
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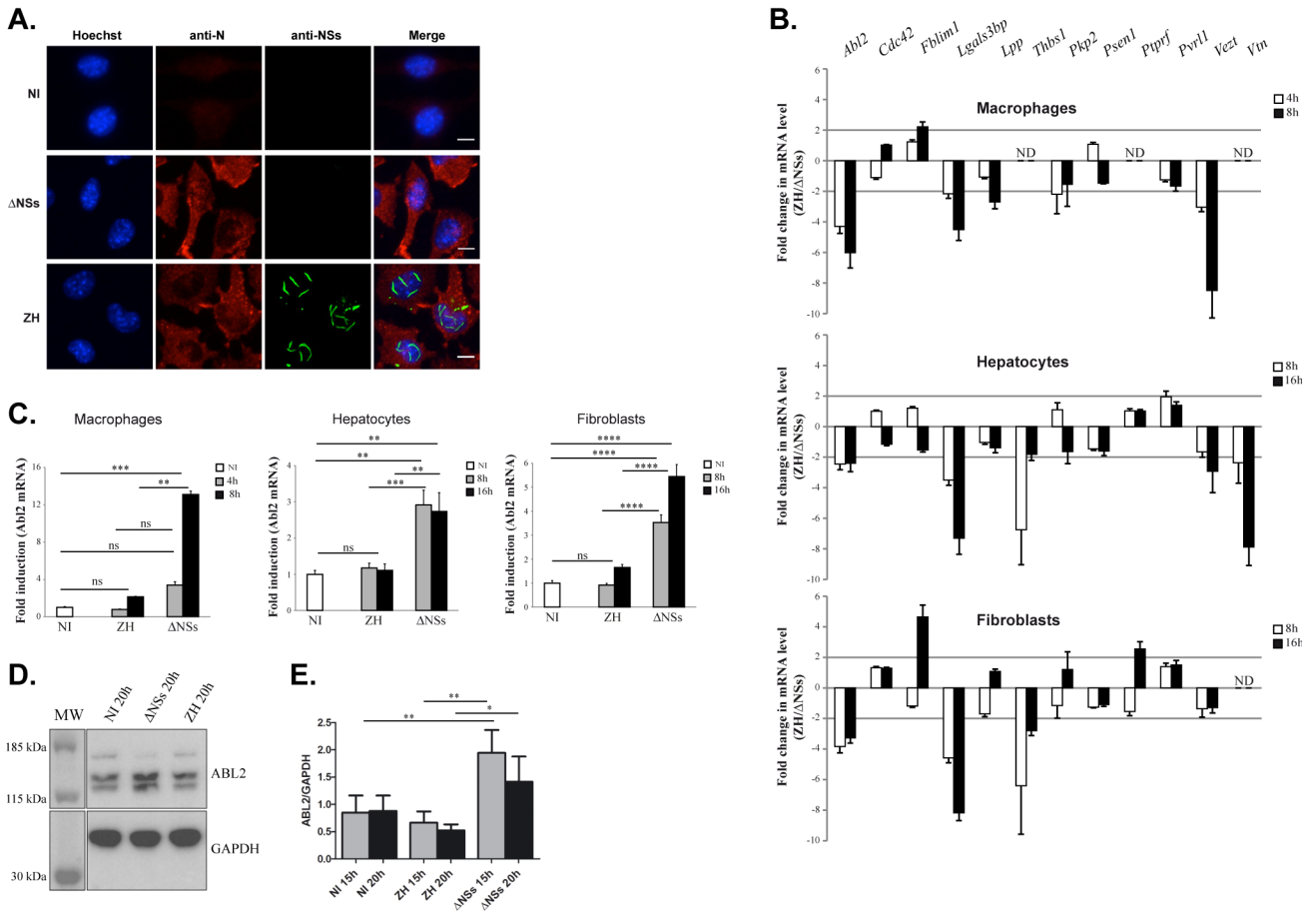


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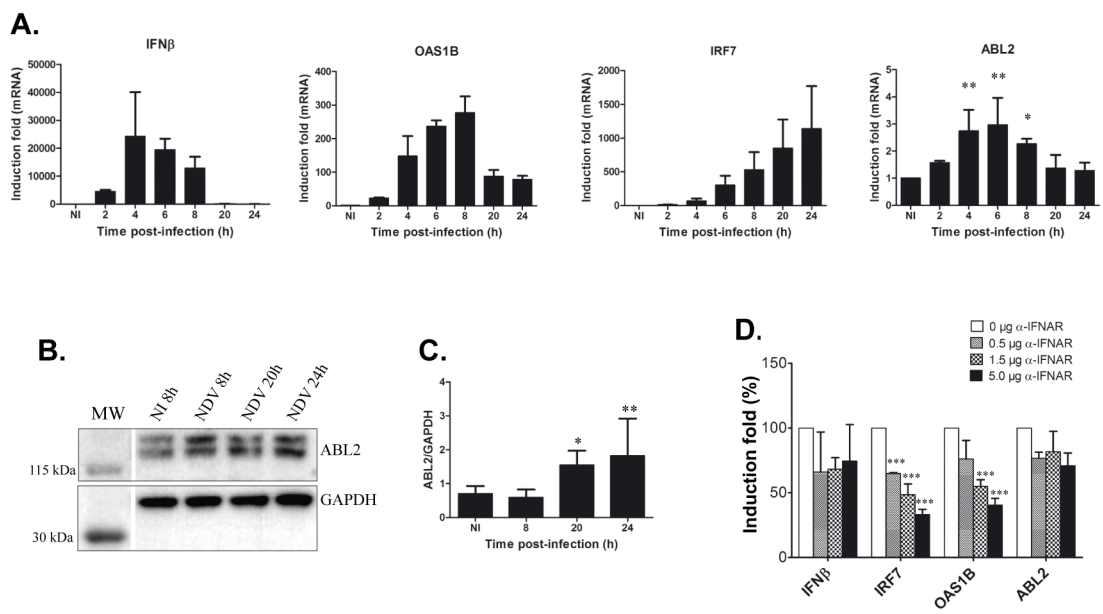


Figure 2

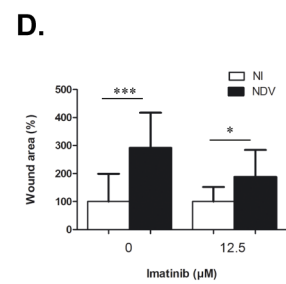
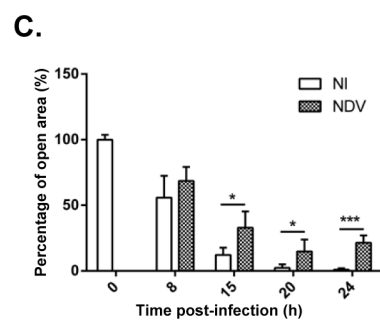
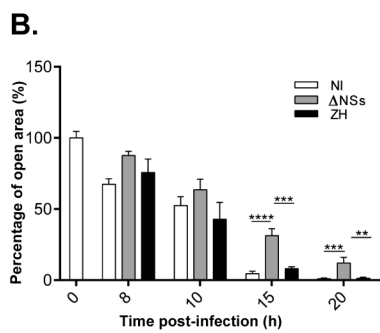
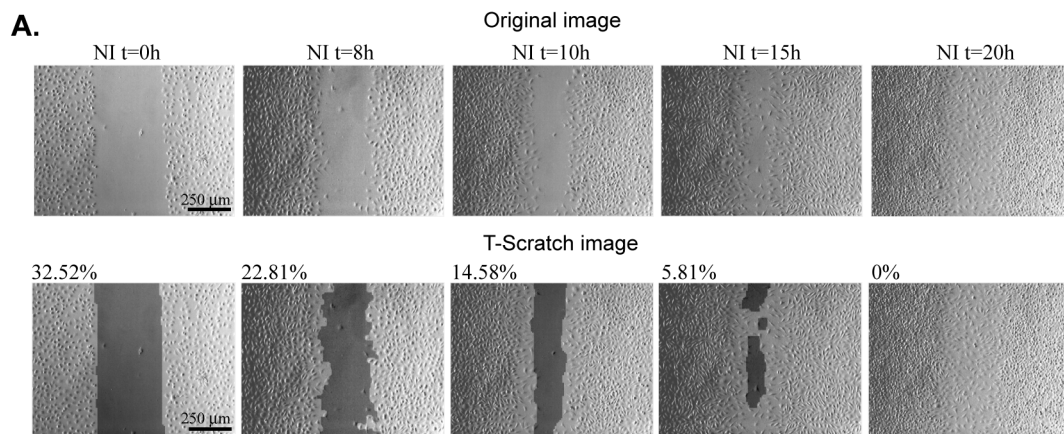


Figure 3

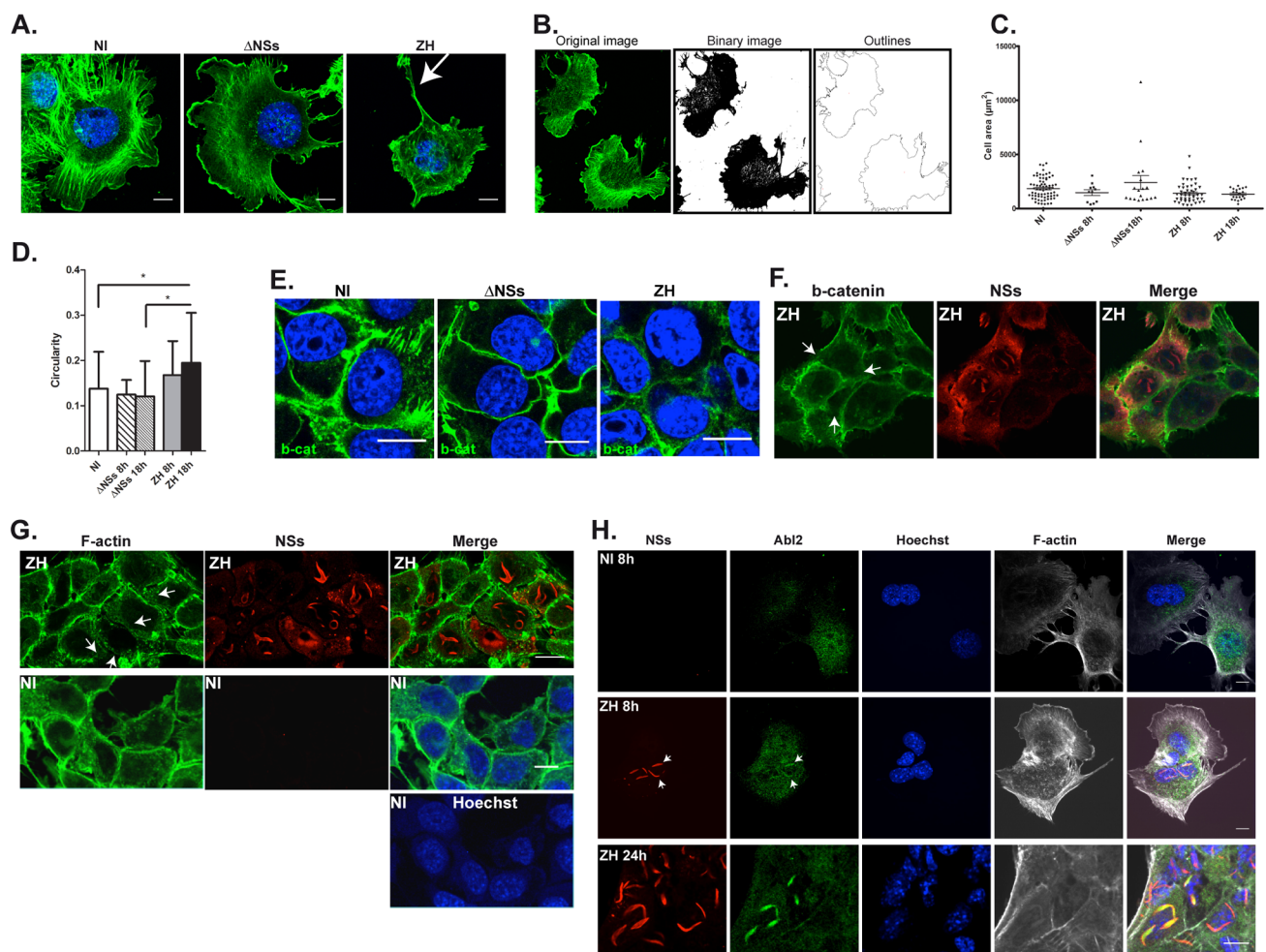


Figure 4

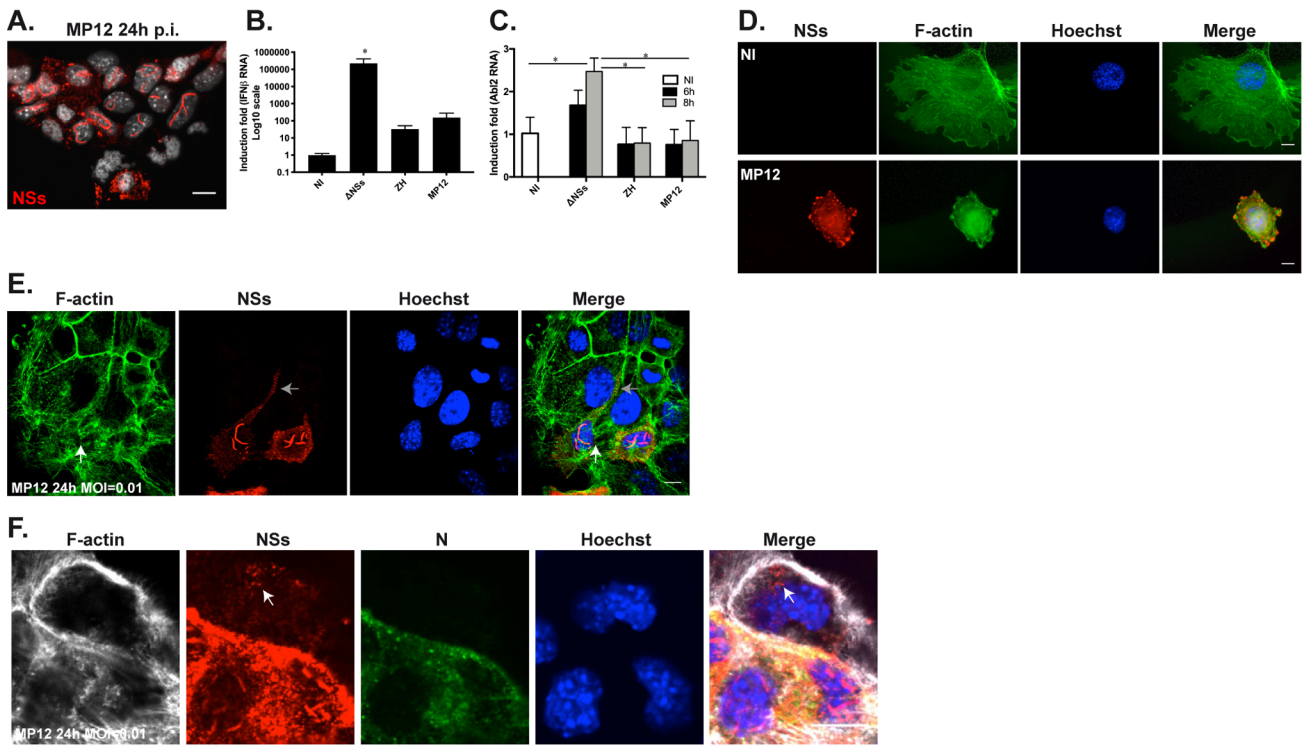


Figure 5

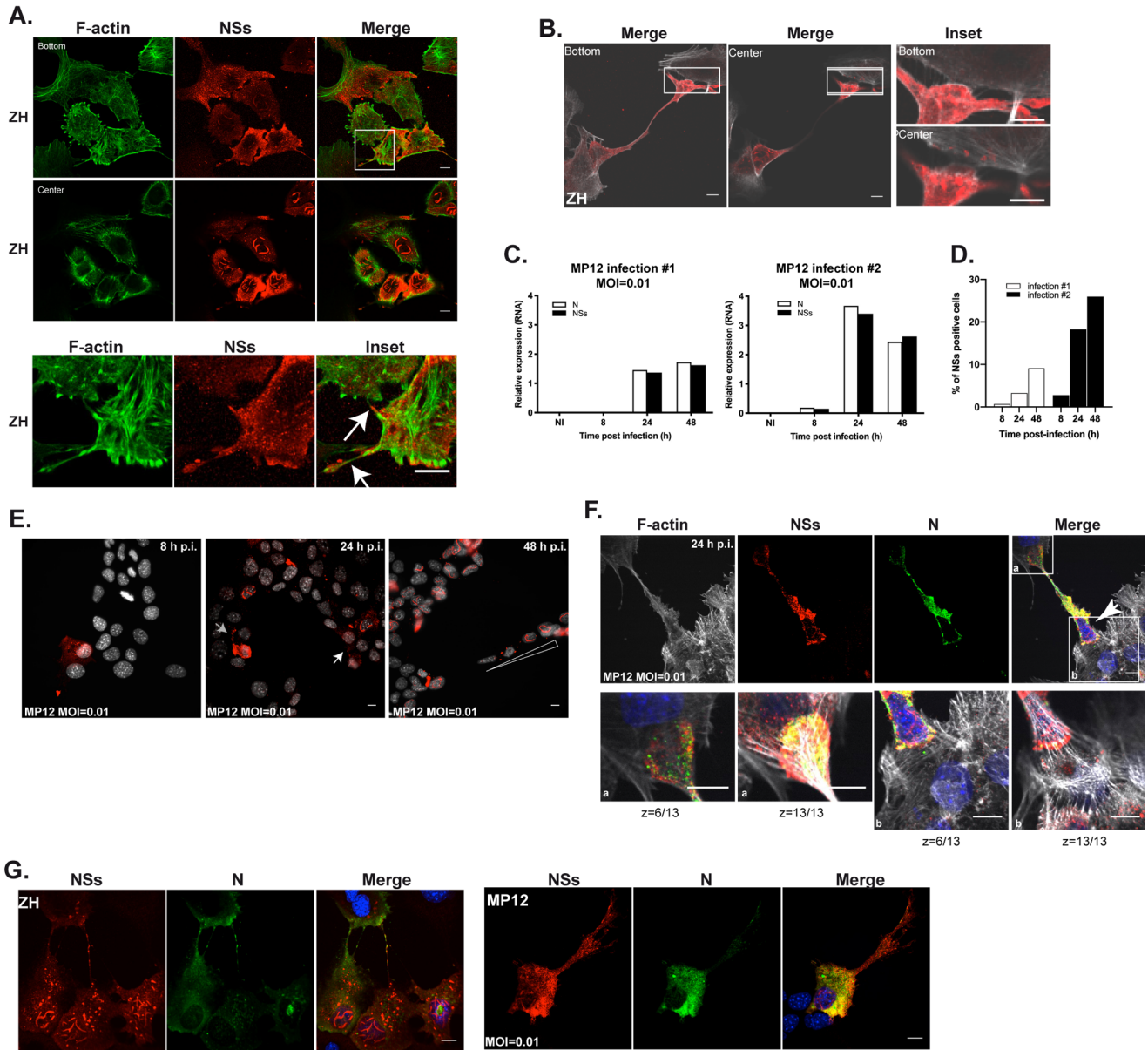


Figure 6

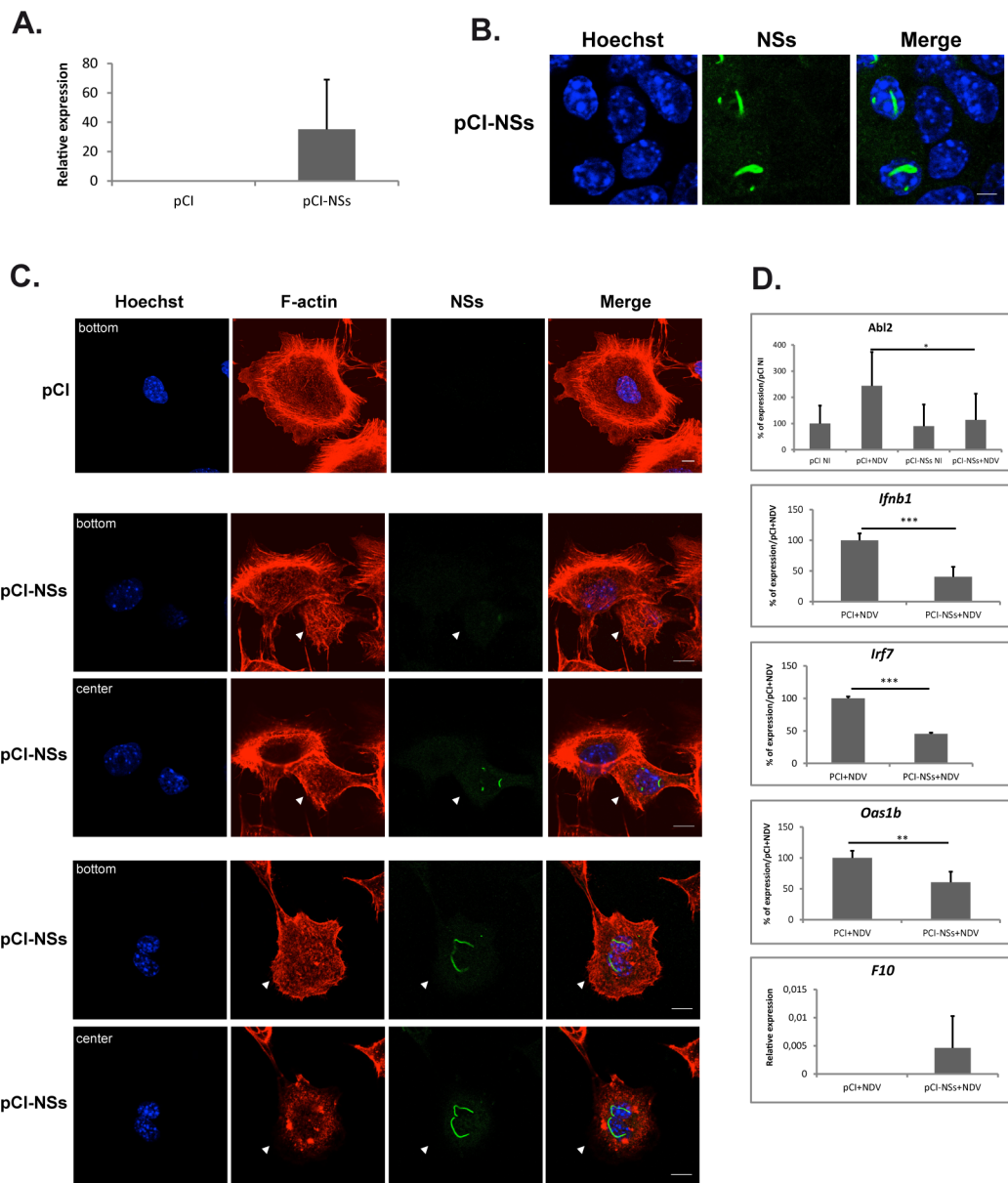


Figure 7