Elongin C contributes to RNA polymerase II degradation by the interferon antagonist NSs of La Crosse orthobunyavirus

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Abstract

Mosquito-borne La Crosse virus (LACV; genus Orthobunyavirus, family Peribunyaviridae, order Bunyavirales) causes up to 100 annual cases of severe meningoencephalitis in children and young adults in the United States. A major virulence factor of LACV is the non-structural protein NSs which inhibits host cell mRNA synthesis to prevent the induction of antiviral type I interferons (IFN-alpha/beta). To achieve this host transcriptional shutoff, LACV NSs drives the proteasomal degradation of RPB1, the large subunit of mammalian RNA polymerase II. Here, we show that NSs acts in a surprisingly rapid manner, as RPB1 degradation was commencing already at 1 hour post infection. The RPB1 degradation was partially dependent on the cellular E3 ubiquitin ligase subunit Elongin C. Consequently, removal of Elongin C, but also of the subunits Elongin A or B by siRNA transfection partially rescued general RNAP II transcription and IFN-beta mRNA synthesis from the blockade by NSs. In line with these results, LACV NSs was found to trigger the redistribution of Elongin C out of nucleolar speckles, which however is an epiphenomenon rather than part of the NSs mechanism. Our study also shows that the molecular phenotype of LACV NSs is different from RNA polymerase II inhibitors like alpha-amanitin or Rift Valley fever virus NSs, indicating that LACV is unique in involving the Elongin complex to shut off host transcription and IFN response.
Significance

The mosquito-borne La Crosse virus (LACV; genus Orthobunyavirus, family Peribunyaviridae, order Bunyavirales) is prevalent in the United States and can cause severe childhood meningoencephalitis. Its main virulence factor, the non-structural protein NSs, is a strong inhibitor of the antiviral type I interferon (IFN) system. NSs acts by imposing a global host mRNA synthesis shutoff, mediated by NSs-driven proteasomal degradation of the RPB1 subunit of RNA polymerase II. Here, we show that RPB1 degradation commences as early as 1 hour post infection, and identify the E3 ubiquitin ligase subunit Elongin C (and its binding partners Elongin A and B) as an NSs cofactor involved in RPB1 degradation and in suppression of global as well as IFN-related mRNA synthesis.
Introduction

Members of the mosquito-borne Orthobunyaviruses, present all over the world, are getting increased awareness as a threat to human and animal health. The African type species Bunyamwera virus (BUNV), that per se causes a mild febrile illness in humans, has shown potential to convert into a hemorrhagic fever virus, called Ngari (1, 2). The Asian-australian Akabane virus as well as the Schmallenberg virus (SBV) that has recently spread all over Western Europe, are causing stillbirths, abortions and congenital malformations in large numbers of ruminants (3, 4). Recurrent epidemics of debilitating fever due to Oropouche virus (OROV) infections, raging since over 60 years, have affected more than half a million people in Latin America (5, 6). Also Maguari-like viruses, associated with febrile illness, are infecting humans all over South America (7). Members of the Maputta serogroup are responsible for epidemics of an acute polyarthritis-like disease in Papua-New Guinea and Australia (8). La Crosse virus (LACV) is the causative agent of a severe meningoencephalitis that mostly (but not exclusively) affects children and young adults in the United States (9-12). Per year, up to maximally 100 cases require hospitalization or even intensive care, exceeding West Nile virus in numbers of pediatric neuroinvasive arboviral infection (13). A substantial proportion of patients are suffering from long-lasting neurological problems (11). As most infections, especially of adults, are however mild or inapparent, the number of subclinical infections was estimated to be around 300,000 annually (14).

The group of orthobunyaviruses is taxonomically defined as a genus within the family Peribunyaviridae, order Bunyavirales (15). The pleomorphic virions are enveloped and have a diameter of approximately 100 nm. As typical for bunyaviruses (16), their genome consists of three segments of negative-strand RNA that are named L (large; ca. 7000 nt), M (medium; ca. 4500 nt) and S (small; ca 950 nt). The L segment encodes the RNA-dependent RNA polymerase (RdRP), the M segments encodes a polyprotein that is processed to the envelope glycoproteins Gn, NSm (non-structural, M segment) and Gc, and the S segments encodes the nucleocapsid protein N and the non-structural protein NSs. All genomic segments are encapsidated by N protein and contain noncoding
regions at their 5’ and 3’ ends that have the potential to anneal to a so-called “panhandle structure”
due to partial sequence complementarities. The panhandle sequences are bound by the L RdRP and
constitute the promoter for viral mRNA transcription and genome replication (17).

The entire multiplication cycle of bunyaviruses takes place in the cytoplasm. After entering the host
cell via clathrin-mediated endocytosis (18, 19) and subsequent low pH-driven membrane fusion,
mRNAs are transcribed from the incoming genome RNA nucleocapsids by L RdRP (“primary
transcription”). The transcription is primed by 12 to 18 nt long, 5’-capped oligonucleotides that had
been cleaved from host mRNA by an endonuclease activity residing in the N terminus of L (20). After
translation of the viral proteins, the viral genome RNA (vRNA) is replicated via a positive-sense,
encapsidated full-length intermediate, the copy RNA (cRNA). The newly generated vRNA
nucleocapsids can give rise to more mRNAs produced by secondary transcription, or become
packaged by peptidase-processed Gn/Gc on Golgi membranes and leave the cell via the exocytosis
pathway (19, 21, 22).

For orthobunyaviruses, the S segment-encoded protein NSs is a major determinant of pathogenicity,
acting as an antagonist of the antiviral type I interferon (IFN) response (19, 23). IFNs are cytokines
that become produced upon virus detection by host cells, and stimulate the expression of genes
(ISGs) for proteins with antiviral activity (24, 25). In the case of LACV, infection is detected by the IFN
system via the RIG-I/MAVS virus sensor axis (26-28). RIG-I, a cytoplasmic RNA helicase (29, 30) is
capable of recognizing the panhandle RNA of bunyaviruses, even if packaged by nucleocapsids (31,
32). RIG-I-mediated panhandle detection activates the transcription factor IRF-3, leading to the
production of IFN-α/β mRNA (33, 34). It is known that orthobunyavirus multiplication is affected by IFN
(35-38), and several ISGs were shown to be involved in this antiviral activity (36, 39-42). To
counteract the IFN/ISG induction, the orthobunyavirus NSs, however, massively and rapidly inhibits
cellular mRNA transcription, leaving the host unable to appropriately respond to the infection (35,
43). We have previously shown that the NSs of the orthobunyaviruses BUNV and LACV are directly
interfering with mRNA synthesis by the cellular RNA polymerase II (RNAP II) (28, 44). While BUNV NSs
is reducing the mRNA elongation-relevant phosphorylation of the C-terminal serine 2 residue (part of the 52 times repeated heptapeptide motif in the C-terminal domain CTD) (44), LACV NSs additionally degrades the large subunit (RPB1) of RNAP II (28). An RPB1-degradative activity was also described for the NSs of SBV (43, 45). Interestingly, the effect of LACV NSs on RPB1 has strong similarities with parts of the DNA damage response (DDR), not only in terms of RPB1 degradation, but also by activation of other DDR markers (28).

The impact of orthobunyavirus NSs on IFN induction and its biological relevance are well established (19). Mechanistically, however, far less is known. Here, we further investigated the effect NSs has on the cells and on RNAP II, and identified Elongin C and the Elongin complex as a host factor involved in NSs action.
Results

LACV NSs rapidly reduces RNAP IIo

To become transcriptionally active, the 260 kDa large subunit RPB1 of RNAP II gets hyperphosphorylated at the 52 heptad repeat sequences that are situated at the C-terminal domain (CTD) (46, 47). This results in a gain of molecular weight and hence in a band shift on immunoblots. The NSs proteins of both the orthobunyavirus type species BUNV and of LACV trigger the disappearance of the high molecular weight hyperphosphorylated RNAP II, termed IIo, and with some delay also of the lighter non-phosphorylated, transcriptionally inactive form (IIa) (28, 44). Moreover, the RPB1 CTD has two major phosphorylation sites within each of the 52 heptad repeats (consensus sequence YSPTSPS), serine 2 and serine 5. CTD-serine 5 phosphorylation is a hallmark of promoter-bound RNAP II, whereas CTD-serine 2 phosphorylation indicates transcriptional elongation (46). Both BUNV and LACV were shown to preferentially affect CTD-serine 2 phosphorylation, indicating specific inhibition of host mRNA elongation (28, 44).

Previously, we demonstrated the degradation of the hyperphosphorylated RNAP IIo by NS-expressing wt LACV in a time course experiment that started at 5 h p.i. (28). Meanwhile, however, it has become clear that the host cell elicits IFN induction even earlier, upon detection of the nucleocapsid-borne dsRNA panhandle by RIG-I immediately after virus entry (32). Moreover, RT-qPCR analyses demonstrated that mRNA transcription by LACV is detectable as early as 1 h p.i. (data not shown). To address this immediate-early step of infection, we conducted a time course experiment in human HuH-7 cells (infected at an MOI of 10) that covered 1 h p.i. to 4 h p.i.. Fig. 1A shows that even at 1 h p.i. there is a reduced RPB1 IIo signal in wt LACV-infected cells, whereas infection with an NSs-deleted LACV (LACVdelNSs) had no such effect. These differences in RPB1 IIo levels are not due to potential differences in viral replication, as demonstrated by comparisons of the immunoblot signal quantifications for RPB1 IIo and LACV N at 3 h p.i. and 4 h p.i. (Fig. 1B and C). Curiously, the phosphorylation states of either RPB1 CTD-serine 2 or 5 are not severely diminished at these time
points (in contrast to longer infections (28, 44)), probably due to short-term activation under infection, as seen for del delNSs virus. Interestingly, application of α-amanitin, a pharmaceutical transcription inhibitor known to induce RPB1 degradation (48), showed a slightly slower effect than wt LACV. α-amanitin was given at the same time as the virus, but unlike NSs which first has to be expressed, the full inhibitor dose is present right from the start. Similar results were obtained with the global inhibitor of DNA-dependent RNA polymerases, Actinomycin D (data not shown). The finding that LACV NSs is on a par with a chemical RNAP II inhibitor underscores the surprisingly fast and efficient destruction of RPB1 by LACV NSs.

Besides LACV and related orthobunyaviruses, also Rift Valley fever virus (RVFV; genus Phlebovirus, family Phenuiviridae, order Bunyavirales) encodes an NSs that strongly inhibits RNAP II activity (49, 50). The two NSs proteins play the same biological role (IFN induction antagonism) and target the same cellular function (RNAP II mRNA transcription), but are unrelated with respect to size and amino acid sequence. Using recombinant RVFV encoding the NSs genes of either LACV or RVFV (both equipped with a Flag tag), we compared their RPB1-destructive activities, again in HuH-7 cells within the first 4 h of infection with an MOI of 10. As shown in Fig 2, the NSs of RVFV is expressed from 1 h p.i. on, but exhibited no RNAP II- destructive activity. RVFV-expressed LACV NSs, by contrast, becomes detectable at 3 h p.i. and diminishes RNAP IIo from this time point on. Thus, taken together, our data show an astonishingly rapid attack of RNAP II by LACV NSs, with an efficiency comparable to a pharmaceutical transcription blocker. Moreover, the RNAP II inhibition profile is different from the other established bunyaviral RNAP II inhibitor, RVFV NSs.

The Elongin complex is involved in NSs-mediated RNAP II suppression and IFN antagonism

The phenotype of RNAP II degradation by LACV NSs resembles the one occurring after DNA damage (28). When RNAP II translocation on DNA is stalled by genotoxic damages or chemical inhibitors like α-amanitin (46), the RPB I subunit becomes ubiquitinated and proteasomally degraded (51).
Elongin E3 ubiquitin ligase complex, consisting of the subunits Elongin A, B, and C, was shown to be involved in degradative polyubiquitination of mammalian RPB1 (52-55). In undamaged cells, however, Elongin A/B/C promotes RNAPII transcription (hence its name) by decreasing transient RNAP II pausing (56). We investigated a possible involvement of the Elongin complex in NSs action. First of all, we knocked down mRNAs of the individual Elongins, and monitored the effect under LACV infection. Human A459 cells were transfected with siRNA against Elongin A, B, or C, infected with wt LACV or LACVdelNSs (MOI 10), and lysed and immunoblotted 16 h later. Fig. 3A demonstrates that the siRNAs were strongly reducing the individual protein levels, and that Elongins B and C seem to stabilize each other, as the knockdown of either of these reduced levels of the other one. However, quantification of the immunoblot signals show that Elongin C is most reduced when knocked down by specific siRNA, whereas under conditions of Elongin B knockdown residual amounts of Elongin C are remaining (Fig. 3B). Regarding RNAP, levels of the Ilo fraction are still largely suppressed by LACV infection when Elongins were depleted by siRNAs (see Fig. 3A). However, quantification and statistics of the immunoblot signals revealed that RPB1 Ilo levels partially recovered under conditions of Elongin C knockdown (Fig. 3C). Elongin A or B knockdown, by contrast, had no such effect.

We also measured another hallmark of NSs action on RNAPII, the suppression of $\gamma$-actin intron RNA levels. Introns have a short half live (57), and the blockade of RNAP II elongation by LACV NSs dries out their supply within 4 h of infection (28). As shown in figure 3D, wt LACV infection suppressed $\gamma$-actin intron RNA down to approximately 3% of mock levels. Interestingly, siRNA-mediated removals of either Elongin A, B, or C were all able to rescue $\gamma$-actin intron RNA significantly and to a certain extent, but again the Elongin C knockdown reached the highest level. Compared to the control siRNA, Elongin C siRNA alleviated the wt LACV-triggered $\gamma$-actin intron RNA reduction from 3% to 19% of mock levels, i.e. by a factor of approximately 6.

The biologically relevant reason for LACV NSs to degrade transcribing RNAP II is suppression of IFN induction at the mRNA transcription level (35). When expression of the three Elongins in A549 cells was individually suppressed by siRNA transfection and IFN induction was measured, a similar picture...
as with the γ-actin intron RNA reduction emerged. Suppression of Elongin C rescued IFN-β mRNA induction in wt LACV-infected cells from around 400 fold to more than 7,000 fold (as compared to the approximately 70,000 fold measured for the delNSs virus), i.e. by more than 1 log₁₀ (Fig. 4A).

siRNAs against Elongin A or B, by contrast, rescued IFN-β induction in wt LACV-infected cells at a lower, but still significant level. ISG56 (IFIT1) is another virus-induced gene that plays a role in the immediate early antiviral state (58). Also ISG56 mRNA induction was rescued best in wt LACV-infected cells when Elongin C expression was impeded (Fig. 4B). Importantly, under these single-step growth conditions (MOI of 10, 16 h of infection) none of the Elongin knockdowns significantly influenced viral RNA levels, neither of wt LACV nor of LACVdelNSs (Fig. 4C). However, as some siRNAs did increase IFN and ISG56 mRNA levels in wt LACV-infected cells, we also tested the effect of the Elongin knockdowns in the IFN-competent A549 cells under multistep growth conditions (MOI 0.01), at an extended incubation time (24 h), and with virus titrations. Indeed, with this more sensitive setting we observed the expected difference between wt LACV and the LACVdelNSs virus (35), and that the knockdown of Elongins A or C (but not B) reduced the growth of both wtLACV and the LACVdelNSs virus (Fig. 4D). Thus, again Elongin C turned out as a factor exhibiting a clear impact on LACV.

Taken together, our siRNA experiments establish the Elongin A/B/C complex and especially Elongin C as a contributor to RPB1 degradation, RNAP II transcription shutoff, and IFN mRNA downregulation by LACV NSs.

**LACV NSs relocates Elongin C**

Using immunofluorescence analysis, we sought to observe the behaviour of the Elongins in LACV-infected cells. To better distinguish between primary effects caused by NSs and possible secondary effects caused by the NSs-mediated RPB1 degradation and block in host mRNA transcription, we treated the HuH-7 cells in parallel with the RNAP II inhibitor α-amanitin. In the immunofluorescence,
Elongin A exhibited in uninfected cells a nuclear signal with the tendency to form speckles (Fig. 5A). The nuclear pattern did not change in infected cells (MOI 1), independent of whether it was wt LACV or LACVdelNSs. The Elongin A speckles however diminished in signal strength and number when α-amanitin was applied. Elongin B produced a nuclear immunofluorescence signal that did not change by any of the treatments (Fig. 5B). Elongin C also exhibited a nuclear speckle pattern in uninfected cells and in cells infected with the LACVdelNSs virus (Fig. 6). In cells infected with wt LACV, however, the speckles were barely visible, whereas under α-amanitin treatment the speckles dissolved but the signal remained nuclear. Since on the other hand protein levels of all Elongins remained unchanged under infection (see Fig. 3), the diminished Elongin C signal in wt LACV-infected cells apparently derived from a relocalization and dilution throughout the cell.

Thus, infection with the NSs-expressing wt LACV converted the nuclear Elongin C speckles in a manner that was unique and clearly distinct from α-amanitin. The subcellular distribution of elongin A and B, by contrast, remained unchanged by infection although α-amanitin had an influence on Elongin A distribution. From the different phenotypes of infection and α-amanitin we conclude that the redistribution of Elongin C is specific for LACV NSs and not simply a consequence of its ability to block RNAP II.

Elongin C relocalization is a secondary effect of NSs action

The subnuclear distribution pattern of Elongin C in uninfected cells is reminiscent of nucleoli, the sites of ribosomal RNA synthesis by RNAP I and ribosome biogenesis (59). Indeed, using nucleolin as marker, we detected a clear and 1:1 overlap with the Elongin C signal in HuH-7 cells (Fig. 7). Both nucleolin and Elongin C remained unchanged in LACVdelNSs-infected cells, but became evenly distributed in the nucleus upon α-amanitin treatment. wt LACV-infected cells (MOI 1) exhibited an intermediate phenotype of nucleolin distribution, with still distinguishable but weaker nucleolar speckles, whereas the Elongin C signal became undetectable, as shown above. These results are in
line with our previous results showing that LACV NSs neither impedes RNAP I transcription nor nucleolar localization of RNAP I transcripts (28).

NSs seems to specifically disassemble the nucleolar Elongin C speckles without entirely destroying the structural integrity of the nucleoli. Since under NSs on one hand Elongin C becomes undetectable in immunofluorescence but on the other hand levels on immunoblots remain unchanged, we investigated the possibility of a redistribution to the cytoplasm. Leptomycin B (LMB) is an inhibitor of CRM1/exportin 1, the major nuclear export factor for proteins. Using LMB, we could indeed trap Elongin C in the nucleoli of HuH-7 cells infected with wt LACV at an MOI of 1 (although the signal was somewhat weaker than in mock infection) (Fig. 8A). This confirms our assumption of an NSs-driven redistribution out of the nucleus, either by expulsion or by cytoplasmic retention. Nonetheless, immunoblots of HuH-7 cells infected with viruses at an MOI of 10 show that blocking nuclear export by LMB could not relieve the destruction of RPB1 by NSs, in contrast to the proteasomal inhibitor MG132 (Fig. 8B). Also when α-amanitin was applied LMB was unable to entirely rescue RPB1 from proteasomal destruction.

LMB had no impact on wt LACV titers (data not shown) or the N protein expression (see Fig. 8B).

Thus, NSs removes Elongin C from the nucleus in a Crm1-dependent manner, but this mechanism seems a secondary phenomenon rather than an essential step in the attack of RNAP II. Altogether, our data show that the E3 ubiquitin ligase subunit Elongin C is involved in the NSs-mediated destruction of the RNAP II large subunit RPB1 and the suppression of IFN induction, and gets relocalized as a consequence.
Morphological changes of nucleoli were long known as a hallmark of orthobunyavirus infection (60). Moreover, recently a nucleolar localization sequence important for NSs action was described for the orthobunyavirus SBV (45). Our findings that (i) Elongin C co-localizes with nucleolin in uninfected cells, (ii) contributes to the NSs-mediated RNAP II degradation, and (iii) becomes redistributed in the presence of NSs, are in line with those earlier observations. Thus, Elongin C appears to be a player in the anti-IFN strategy of LACV, further underscored by the increase of IFN induction and RNAP II activity by more than 1 log₁₀ step in Elongin C-deficient cells that were infected with wt LACV.

The cellular Elongin complex consisting of Elongin A, B, and C is able to foster both the elongation of mRNA transcription by RNAP II (56) and (the B/C complex) the degradation of RPB1 under conditions of stress (53-55). These activities are congruent with the action of orthobunyavirus NSs, which targets exactly these activities, as it inhibits mRNA elongation and drives RPB1 degradation (28, 44).

Moreover, the fact that mostly Elongin C participated in these NSs activities is in accord with our immunofluorescence data which show its relocalisation by NSs, but not that of Elongin A and B. Moreover, Elongin C was the only Elongin complex subunit that localizes to the nucleoli, which in turn are reorganized by orthobunyaviral NSs proteins (45, 60).

Our attempts to robustly demonstrate a direct interaction between LACV NSs and Elongin C in transfected cells have failed (data not shown), in line with the absence of Elongin C in the LACV NSs interactome (61). Moreover, LACV NSs does not exhibit a discernible nucleolar localization, but is faintly distributed all over the nucleus (28), and the nucleolar localization sequence detected for SBV NSs is not conserved in LACV NSs (45). Thus, the effect of NSs on Elongin C in the nucleoli is most likely transient or indirect. Also the relocation of Elongin C might be a secondary effect and not causative, as nuclear export inhibition by LMB partially prevented Elongin C redistribution, but not RPB1 degradation by NSs. We therefore hypothesize that LACV NSs recruits an Elongin C-dependent
host cell pathway that leads to RPB1 degradation and inhibition of host mRNA transcription. The
relocalization of Elongin C is a morphological consequence of this process (but NSs-specific, since
RNAP II inhibition by α-amanitin has a different phenotype), but not necessarily causative. Why
Elongin A and B seem less important remains unclear. A slight but statistically significant rescue of
RNAP II transcription activity was, however, measurable also in those knockdowns.

Despite the significant impact of the Elongin C knockdown, it was not sufficient to entirely rescue
RNAP II and IFN induction from NSs-mediated destruction and blockade. This is comparable to the
observation with RVFV NSs and its cellular cofactor, the E3 ubiquitin ligase subunit FBXO3. Similar to
the situation with LACV NSs and Elongin C, IFN induction by wt RVFV is rescued by 1 log_{10} step in cells
depleted of FBXO3, but an NSs-deleted virus mutant still induced IFN-β by another log_{10} step (62).
RVFV NSs is known to counteract IFN induction by several mechanisms, namely (i) sequestration of
subunits p44 and XPB of the general RNAP II transcription factor TFIIH, (ii) destruction of the TFIIH
subunit p62 (mediated by mentioned FBXO3), and (iii) recruitment of the IFN promoter suppressor
SAP30 (49, 50). It is therefore conceivable that LACV NSs, despite having approximately just 1/3 of
the size of RVFV NSs, has similarly evolved several, independent mechanisms to destroy RNAP II and
suppress IFN induction, and the Elongin C-dependent branch is just one of them. Removing Elongin C
can help to uncover these additional anti-IFN strategies which may contribute to the degradation of
RPB1 and breakdown of RNAP II activity which is even faster than for RVFV NSs.

An interesting side observation of our studies was the establishment of Elongin C (but not A or B) as a
nucleolar protein. We were able to locate Elongin A in a database of the nucleolar proteome (63), but
not Elongin C. We therefore hypothesize a transient and weak association, which is probably
disrupted upon cell lysis or nucleoli preparation. In support of this, LACV (and SBV) NSs disturbs the
nucleolar organization only slightly (and LACV NSs still allows RNAP I activity there (28)), but this
might be sufficient to release Elongin C. α-amanitin by contrast dissolves nucleoli entirely, and also
redistributes Elongin C.
In summary, we have shown that LACV NSs triggers the destruction of RNAP II in a surprisingly fast manner, starting at 1 h postinfection. This is in line with the earlier observation that the pattern recognition receptor RIG-I of the IFN immune system is capable of recognizing already the incoming LACV nucleocapsids, thus requiring an immediate viral counterreaction (32). The fast RNAP II degradation by NSs leads to general blockade of host mRNA production, and hence to an inability of the cell to produce IFN and alert the surrounding cells. RNAP II degradation is due to ubiquitination and proteasomal activity, and involves the host factor Elongin C that is expelled from the nucleoli during the course of these events. This fast and efficient way of shutting down the IFN response contributes to the virulence of LACV, and it will be interesting to see whether other pathogenic orthobunyaviruses are using the same mechanism.
Materials and Methods

Cells and Reagents.

Human HuH-7 and A549 cells, African green monkey Vero 76 and CV-1 were maintained in Dulbecco’s modified Eagle’s medium (Gibco, #21969035) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine (Gibco, #25030081), 50 U/ml penicillin, and 50 µg/ml streptomycin (Gibco, #15140122). The specific use of the respective cell line is indicated in the figure legends. The RPB1 specific inhibitor α-amanitin was purchased from AppliChem (A1485,0001), the nuclear export inhibitor Leptomycin B from USBiological (L1671-38B), the proteasomal inhibitor MG132 from Cayman Chemical (10012628), the NEDD8 inhibitor MLN4924 form Cayman Chemical (905579-51-3) and the p97 inhibitor NMS-873 form Selleck Chemicals (S7285).

Viruses and virus titration

Recombinant wild-type La Crosse virus (wt LACV) and LACV lacking NSs expression (LACVdelNSs) were rescued as described previously (64). Recombinant Rift Valley fever virus expressing a C-terminal 3×Flag tag NSs (rRVFVΔNSs::Flag-NSsRVFV) or a N-terminal 3×Flag tag LACV NSs (rRVFVΔNSs::Flag-NSsLACV) were rescued as described previously (65).

Both LACV (BSL-2) and RVFV (BSL-3) were propagated on Vero 76 cells by infecting with MOI 0.001 of the respective virus and cultivating the cells for three days. The supernatant was cleared by centrifugation and the virus titer determined by plaque test on CV-1 cells, using Avicell overlay (66). After 3 days of incubation the overlay was washed of using PBS and the cell layer stained with staining solution (0.75 % crystal violet, 3.75 % formaldehyde, 20 % ethanol, 1 % methanol) for 10 min. The amount of virus was then calculated as PFU/ml.
Immunoblot analysis

After washing the cells once with PBS, the cells were lysed in a 1:1 mixture of tissue protein extraction reagent (Thermo Scientific, 78510) supplemented with phosphatase (Calbiochem, #524625) and protease (Roche, #4693116001) inhibitors and 2X sample buffer (62.5 mM Tris from a 0.5 M stock [pH 6.8], 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol). The lysates were boiled for 10 min and then separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% (wt/vol) nonfat dry milk powder in TBS-T and probed with primary antibodies against the following targets: RPB1/Pol II (N-20) (Santa Cruz, sc-899, 1:500, rabbit, polyclonal), RPB1 CTD-pSer2/Ser2 α-CTD ((3E10), 1:500, rat, polyclonal), RPB1 CTD-pSer5/Ser5 α-CTD (3E8) (both antibodies a kind gift from Dirk Eick, Helmholtz-Zentrum Munich, Germany, 1:500, rat, polyclonal), anti-LACV N (kind gift from Georg Kochs, Institute of Virology, Freiburg, Germany, 1:15000, rabbit, polyclonal), anti-RVFV N (67), 1:2000, mouse, polyclonal), Elongin A/TCEB3/SIII/p110 (110 kDa) (Sigma Aldrich, #HPA005910, 1:500, rabbit, polyclonal), Elongin B (18 kDa) (Santa Cruz, sc-11447, 1:250, rabbit, polyclonal), Elongin C/SIII p15 (15 kDa) (BD Transduction Laboratories, #610761, 1:250, mouse, monoclonal), anti-ß-tubulin (Abcam, ab6046, 1:1000, rabbit, polyclonal), Anti-Flag (TM) (Sigma-Aldrich, #F7425, 1:3200, rabbit, polyclonal). As secondary antibodies were peroxidase-conjugated anti-mouse (Thermo Fisher, #0031430 1892913, 1:40000, goat, polyclonal), peroxidase-conjugated anti-rabbit IgG (Thermo Fisher, #0031460 1892914 NCI1460, 1:40000, goat, polyclonal) and were peroxidase-conjugated anti-rat (Jackson Immuno Research, #712-036-150, 1:40000, donkey, polyclonal) used. The western blot signals were detected on a Chemidoc (Biorad) using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, #34096).

Densitometry analysis
Western blot signals were quantified using the Image Lab software provided with the Chemidoc machine (BioRad). The intensity of the respective bands where measured using the volume tool, and the adjusted volume, accounting for background, was used for each band. The bands of interest were first normalized to the loading control (β-tubulin) of the respective sample and the normalized values were then compared to mock treated samples of the same experiment. The graphs presented depict fold compared to mock, with mock set to 100%.

**siRNA knock down of the Elongin complex components.**

Knockdowns of the indicated genes were performed 2 times by reverse transfection of small interfering RNAs (siRNAs). All siRNA were purchased from Qiagen: AllStar Negative Control siRNA (1027280), Elongin A (GeneSolution GS6924), Elongin B (GeneSolution GS6923) and Elongin C (GeneSolution GS6921). GeneSolution consists of four validated siRNAs against the respective transcript, that were mixed in equimolar ratios with each other. A final concentration of 50 nM for each siRNA solution was reverse transfected using Lipofectamine RNAiMAX (Invitrogen, 13778075) according to the manufacturer’s instructions. After four hours, the medium was changed and the cells incubated for three days. One day before the experiment, the cells were harvested, counted and equal amounts of cells were again reverse transfected as described above.

**Real-time RT-PCR**

Total cellular RNA was isolated with the RNeasy Minikit (Qiagen, 74104) eluted in 30 µl sterile H$_2$O, and 100 ng RNA of each sample were used as a template. For measuring total RNA amounts, cDNA synthesis was random primed using the QuantiTect Reverse Transcription kit (Qiagen, 205313). For measuring positive-strand-specific S segment RNA, cDNA synthesis was carried out using primer 5’ GACAGCGGCCCTAGGAGCCTGATGCGAAATTTCTG 3’ (reverse primer to amplify the N ORF of LACV S segment) and the PrimeScript RT Master Mix (Perfect Real Time) kit (Takara, RR036A). For
PCR, the QuantiTect SYBR green PCR kits (Qiagen, 204143) (random primed cDNA) or SYBR Premix Ex Taq (Tli RNase H Plus) (Takara, RR420A) (strand-specific cDNA) were used. Primers were either purchased from Qiagen: IFN-beta (QT00203763), IFIT1/ISG56 (QT00201012) and 18S RNA (QT00199367) or custom ordered for: γ-actin intron 3 (forward: 5’-GCTGTTCCAGCTCGTCCG-3’ and reverse: 5’-ATGCTCACACGCCAACATGC-3’) (68), LACV N RNA (forward: 5’-GGGTATATGACCTCTGCTG-3’ and reverse: 5’-GCTTCTCTCTGGCTTA-3’). The qPCR was carried out in a StepOne Real-Time PCR system (Applied Biosystems). All values obtained were normalized against the 18S RNA signal using the ddCT method (69).

Immunofluorescence analysis

Cells, grown on coverslips to 30–50% confluency, were either mock treated, infected with wt LACV or LACVSdelNSs or treated with α-amanitin for the indicated times. After fixing the cells with 3% paraformaldehyde they were permeabilized with 0.5% Triton X-100 in PBS. Unspecific staining was reduced by incubating the cells in blocking and staining solution (2% BSA, 5% glycerol, 0.2 % Tween-20 in PBS) for 30 min at room temperature. The following primary antibodies, diluted in blocking and staining solution, were used: rabbit polyclonal anti-LACV N (1:500), mouse monoclonal anti-LACV GC (1:400, kind gift from Francesco Gonzalez-Scarano, Perelman School of Medicine, Pennsylvania, USA), Elongin A (1:500, rabbit, Sigma Aldrich, HPA005910), Elongin B (1:100, rabbit, Santa Cruz, sc-11447), Elongin C (1:250, mouse, BD Transduction Laboratories, 610761), Nucleolin (1:1000, Abcam, ab22758). The primary antibodies were incubated on the cells at room temperature for 1 h, followed by three washes in PBS. As secondary antibody were either Alexa Fluor 488 Donkey anti-mouse IgG (1:200, Invitrogen/Molecular Probes, A21202), Alexa Fluor 555 Donkey anti-mouse IgG (1:200, Invitrogen/Molecular Probes, A31570), Alexa Fluor 555 Donkey anti-rabbit IgG (1:200, Invitrogen/Molecular Probes, A31572) or Alexa Fluor 488 Donkey anti-rabbit IgG (1:200, Invitrogen/Molecular Probes, A21206) used. The nuclei where visualized by staining the chromatin with 4’,6-diamidino-2-phenylindole (DAPI, 1:100) at the same time as secondary antibody incubation.
After 45 min of incubation the cells were again washed three times in PBS and once in H₂O and then mounted using Fluorsave solution (Calbiochem). Images were taken with an Apotome (Zeiss) at a magnification of ×63.

**Statistical analysis**

The quantitative data are presented as means ± standard deviations for three biological replicates. The statistical significance between two groups were examined by two-sided Student’s t test with P value of <0.05 considered as statistically significant.

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Figure legends

**Fig. 1: Rapid and specific RPB1 degradation by wt LACV.** HuH-7 cells were infected with wt LACV or LACVdelNSs viruses (MOI 10), or treated with α-amanitin (10 µg/ml). (A) Immunoblot analyses for the various RPB1 states and for viral N at 1 to 4 hrs post infection, using the antibodies indicated at the left. Immunoblot signals of RPB1 Ilo (B) and LACV N (C) were quantified and normalized to the tubulin signal. B and C show mean, SD and individual data points from 5 independent experiments. The non-parametric, one-tailed Wilcoxon’s paired signed-rank test was applied to test for a potential difference in signals for RPB1 Ilo and LACV N between wt LACV 3 hpi and the LACVdelNSs 3 and 4 hpi time points. * = p< 0.05; ns = non-significant. RT-qPCR analyses confirmed the similar replication rates and showed that comparable amounts of input virus were used in all cases (data not shown).

**Fig. 2: RPB1 degradation by LACV NSs expressed by RVFV.** HuH-7 cells were infected at an MOI of 10 with recombinant RVFV expressing either LACV NSs (rRVFVΔNSs::Flag-NSsLACV) or RVFV NSs (rRVFVΔNSs::Flag-NSsRVFV) and samples for immunoblot were taken at the indicated times p.i.. B and C show mean, SD and individual data points from 3 independent experiments, analyzed as described for Fig. 1.

**Fig. 3: Elongin C and RPB1 degradation.** A549 cells were transfected with siRNAs against the mRNAs for Elongin A, B, or C, or a negative control (CTRL). (A to D) Cells were mock infected or infected with wt LACV or LACVdelNSs (MOI 10) for 16 h. (A and B) The presence of RPB1, Elongin A,B, C, LACV N, and β-tubulin was monitored by immunoblotting (A), and the bands of Elongin C and RPB1 Ilo normalized to β-tubulin (B and C). (D) Amounts of γ-actin intron RNA were measured by RT-qPCR. B to D show mean, SD and individual data points from 5 independent experiments, analyzed as described for Fig. 1.
**Fig. 4: Elongin C and IFN induction.** The RNA samples used for figure 3E were also analyzed for the changes in mRNAs for IFN-β (A), ISG56 (B), and LACV S segment (C). (D) A549 cells were infected with of the respective viruses (MOI 0.01), and titers were measured at 24 h p.i..

**Fig. 5: Subcellular localization of Elongin A and B.** HuH-7 cells were either mock infected, infected with wt LACV, LACVdelNSs (MOI 1), or treated with α-amanitin (10 µg/ml) for 16 h. The cells were fixed, permeabilized and then stained against endogenous Elongin A (A) or Elongin B (B) and LACV GC protein, the nucleus was counterstained with DAPI.

**Fig. 6: Subcellular localization of Elongin C.** HuH-7 cells were infected or α-amanitin treated as indicated for figure 5, and then processed for immunofluorescence to stain against endogenous Elongin C and the LACV N protein.

**Fig. 7: Effect of LACV NSs on nucleolin.** HuH-7 cells were infected or α-amanitin treated as indicated for figure 5, and immunostained against endogenous Elongin C and nucleolin. The nucleus was counterstained with DAPI.

**Fig. 8: NSs-driven Elongin C redistribution and nuclear export.** (A) Immunofluorescence analysis. HuH-7 cells were pretreated with LMB (16 nM) for 1h and then infected with wt LACV (MOI 1). Untreated and mock infection controls were done in parallel. At 16 h p.i. cells were immunostained against Elongin C and LACV N, and the nucleus was counterstained with DAPI. (B) Immunoblot analysis. HuH-7 cells were pretreated with LMB as in (A) and then infected with wt LACV or LACVdel (MOI 10), or treated with α-amanitin. After removal of the inoculum 1 h later, medium was added containing either MG132 (10 µM) or LMB (16 nM) or the combination of the two. Untreated and mock infected controls were done in parallel. At 8 h.p.i. samples were analysed by immunoblot using the indicated antisera.
A

RPB1 mLo/mLa
CTD-pSer 2
CTD-pSer 5
LACV N
β-tubulin

h p.i. 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Mock wt LACV LACVdelNSs α-amanitin

B

RPB1 mLo/β-tubulin

Log10 fold over Mock

h p.i. 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
mock wt LACV LACVdelNSs α-amanitin

C

LACV N/β-tubulin

Log10 fold over Mock

h p.i. 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
mock wt LACV LACVdelNSs α-amanitin
Figure A: IFN-beta mRNA levels after treatment with different siRNAs.

Figure B: ISG56 mRNA levels after treatment with different siRNAs.

Figure C: LACV S segment RNA levels after treatment with different siRNAs.

Figure D: Virus titers after treatment with different siRNAs.
