Enhancement of alphavirus replication in mammalian cells at sub-physiological temperatures

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Word counts: Abstract: 290, author summary: 175, main text: 6656

10 Figures

Keywords: alphavirus; subgenomic replicon; mammalian cells; sub-physiological temperature; stress granules

Short title: Alphavirus replication enhanced at sub-physiological temperatures

Author contributions: JG: conceptualization, formal analysis, investigation, visualization and writing. MH: conceptualization, funding acquisition, supervision, visualization and writing.

Abstract

Chikungunya virus (CHIKV) is a re-emerging Alphavirus transmitted by Aedes mosquitoes and causing fever, rash and arthralgia. Currently there are no vaccines or antiviral agents against CHIKV, therefore it is important to understand the molecular details of CHIKV replication. In this regard, the function of the Alphavirus non-structural protein 3 (nsP3) remains enigmatic. Building on previous studies (Gao *et al*, 2019), we generated a panel of mutants in a conserved and surface exposed cluster in the nsP3 alphavirus unique domain (AUD) and tested their replication phenotype using a sub-genomic replicon (SGR) in mammalian and mosquito cells. We identified three mutants that replicated poorly in mammalian cells but showed no defect in mosquito cells. We further showed that these mutants were temperature-sensitive, rather than species-specific, as they exhibited no replication defect in mammalian cells at sub-physiological temperature (28°C). Similar effects were observed in the context of infectious CHIKV as well as a closely related virus: O’Nyong Nyong virus (ONNV). Intriguingly, this analysis also revealed that the wildtype SGR replicated much more efficiently at sub-physiological temperature as compared to 37°C. This was not due to impaired interferon responses as this enhancement was also observed in Vero cells. Neither was this due to a defect in the phosphorylation of eIF2 α as treatment with ISRIB, an inhibitor of global translation attenuation, did not compensate for replication defects at 37°C. However, we noticed significant differences between the sizes and numbers of virus-induced stress granules (SG) at physiological and sub-physiological temperatures. As cells in the periphery will be at sub-physiological temperatures, and these will be the first cells infected in the

mammalian host following a mosquito bite, we propose that alphaviruses have evolved mechanisms to limit antiviral responses in these cells to promote viral genome replication.

Author summary

Chikungunya virus (CHIKV) is a re-emerging arbovirus. It is transmitted by *Aedes* species of mosquitos and poses massive epidemic threats. Arboviruses are unique in that they must be able to replicate efficiently in both the mosquito vector and the mammalian host, and therefore at different temperatures. Importantly, the first cells infected in the mammalian host following a mosquito bite will be in the skin and therefore at sub-physiological temperature (lower than 37°C). Here we show that mutants within one of the CHIKV proteins (nsP3) were unable to replicate at 37°C but replicated efficiently in mammalian cells at a sub-physiological temperature (28°C). We also showed that the wildtype virus replicated more efficiently at 28°C in comparison to at 37°C in mammalian cells. We investigated the mechanism behind this phenomenon and showed that at sub-physiological temperatures the virus induced the formation of smaller and more numerous cytoplasmic stress granules. We propose that alphaviruses have evolved mechanisms to promote their replication in mammalian cells at sub-physiological temperatures to facilitate infection of mammals via a cutaneous mosquito bite.

Introduction

Chikungunya virus (CHIKV) belongs to the *alphavirus* genus in the *Togaviridae* family [1]. It is an arbovirus that is transmitted by mosquitoes and causes fever, rash and arthralgia with infrequent long-term morbidity and in rare cases mortality [2]. CHIKV was first isolated in Tanzania in 1952-1953 [3, 4], and has subsequently spread across the world, including Africa, Europe, Asia and America [5, 6]. CHIKV is mainly transmitted by the mosquito species *Aedes aegypti* and *Ae. albopictus*, the latter has become the main vector for virus spread in Asia [7]. Both field and laboratory experiments demonstrated that the survival rate of these two mosquito species are affected by lower and upper temperature thresholds [8]. Models and human cases showed transmission occurs between 18°C and 34°C with maximal transmission occurring in a range from 26-29°C [9].

CHIKV has a positive-sense, single-stranded RNA genome, 11.5 kb in length, which is both capped and polyadenylated (Fig 1A). It contains two open reading frames (ORFs), the first ORF encodes the non-structural proteins (nsP1-nsP4) and is translated directly from full-length genomic RNA. ORF2 encodes the structural proteins (Capsid, E1-E3 and 6K) and is translated from a sub-genomic RNA. nsP1 to nsP4 are required for the synthesis of positive and negative full-length genomic RNA as well as the sub-genomic RNA. Biochemical analysis indicated specific functions of the non-structural proteins: nsP1 shows methyl- and guanyl-transferase activities, nsP2 exhibits helicase and protease activities, nsP4 is the RNA-dependent RNA polymerase. The function of nsP3 remains poorly defined [10], although it is essential in genome replication. CHIKV nsP3 consists of three domains: a macro-domain at the N-terminus, the central alphavirus

unique domain (AUD) and the hypervariable domain (HVD) at the C-terminus (Fig 1A). The macro-domain exhibits RNA binding, ADP-ribose binding and ADP-ribosylhydrolase activities [11-13]. The HVD is highly phosphorylated and interacts with a number of host proteins [14]. Despite high levels of conservation across alphaviruses, AUD function remains elusive. In this context our recent work showed that the AUD binds to viral RNA and plays a role in virus assembly by stimulating production of the sub-genomic RNA [15].

Eukaryotic initiation factor 2 (eIF2) is a key regulator of protein synthesis in mammalian cells [16]. Phosphorylation of the eIF2 α -subunit by one of four kinases (HRI, GCN2, PKR and PERK) prevents guanine nucleotide exchange on eIF2 and the regeneration of the active GTP-bound state, resulting in global translation attenuation [17]. Most alphaviruses possess a translation enhancer element within the sub-genomic RNA open reading frame that has been shown to counteract the translational inhibition in the presence of high levels of eIF2 α phosphorylation [22, 23]. Although CHIKV does not contain this element, CHIKV nsP4 has been shown to suppress eIF2 α phosphorylation [24].

Translation attenuation via eIF2 α phosphorylation results in the accumulation of stalled 48S mRNPs into cytoplasmic, non-membranous aggregates termed stress granules (SG). Typical SG contain silent mRNAs, translation factors, small ribosomal subunits, and various mRNA-binding proteins. Despite varied compositions resulting from different stresses [25], certain components including the Ras GTP-activating protein-binding protein G3BP1/2, are required for formation of all SGs [26, 27]. Alphavirus infection induces SG formation during the early stages [28]. It is assumed that upon virus-induced stress, 48S mRNPs bound by G3BP1 and other components such as TIA-1 form small

core aggregates that grow into larger SGs [29]. SG are highly dynamic and exhibit rapid exchange of mRNPs [30]. They can grow to several μm in diameter in 20 min and can be disassembled completely within 60 mins following the cessation of environmental stress or the addition of polysome-stabilizing agents such as cycloheximide (CHX) [31]. The CHIKV nsP3 hypervariable domain has been shown to interact with G3BP1/2 and regulate their localization [32]. Recent work demonstrated that the composition and disassembly of SG was determined by the ADP-ribosylhydrolase activity within the CHIKV nsP3 macro-domain [33].

In this study we sought to investigate the function of AUD in CHIKV replication in cells from both the vertebrate host and the mosquito vector. Three AUD mutants (W220A, D249A and Y324A) replicated poorly in mammalian cells but efficiently in mosquito cells, and we demonstrate that this phenotype was temperature-dependent rather than species-specific. Importantly, we also demonstrated that a wildtype CHIKV sub-genomic replicon (SGR) replicated more efficiently in mammalian cells at a sub-physiological temperature. Although phosphorylation of eIF2 α was suppressed at lower temperature, it was not the key factor regulating the replication phenotypes observed. Using the related O'Nyong Nyong virus (ONNV) we revealed that at sub-physiological temperatures formation of SG in response to infection was altered. This provided a potential explanation for the enhanced genome replication at sub-physiological temperature.

Results

Construction and analysis of the AUD mutants

To identify conserved residues within the CHIKV AUD across the *Alphavirus* genus, a variety of AUD amino acid sequences from both Old and New World alphaviruses were aligned (S1A Fig). As the nsP2/nsP3 protein structure of Sindbis virus (SINV) has been determined [34], and the AUD sequences between SINV and CHIKV share high homology (118 out of 243 residues are identical), a panel of conserved and surface exposed residues in the AUD were selected for mutation based on the SINV nsP2/nsP3 protein structure [34] (Fig 1B and S1B Fig). Seven residues were chosen for further study as they were absolutely conserved or in other cases substituted by residues with similar physical characteristics. Y324 (nsP3 amino acid numbering) was selected as a control as it is located at the end of AUD and conserved among alphaviruses. These mutations were introduced into a CHIKV dual luciferase sub-genomic replicon (SGR-D-Luc) (Fig 1A), which was derived from the ECSA strain LR2006 OPY1 isolate [35, 36]. In this construct renilla luciferase (R-Luc) is fused within the nsP3 hypervariable domain in ORF1, and firefly luciferase (FF-Luc) replaces the structural proteins in ORF2. The activity of R-Luc is thus a marker of ORF1 translation from the full-length RNA: at early time points R-Luc levels reflect transfection efficiency and input RNA translation, at later times R-Luc is also dependent upon genome replication. As ORF2 is translated from a sub-genomic RNA (sgRNA) which is templated from the negative strand replicative intermediate, FF-Luc expression is dependent upon genomic replication but will also reflect translation from the

sgRNA. A polymerase-inactive mutant (GDD-GAA in nsP4) was used as a negative control.

The AUD mutants in SGR-D-Luc exhibited different replication phenotypes in mammalian and mosquito cells

CHIKV mainly induces arthritis and joint pain [2] and infects muscle cells. We therefore chose to investigate the effects of the AUD mutants on CHIKV genome replication initially in C2C12, an immortalized mouse myoblast cell line widely used in the study of CHIKV [37]. As expected, wildtype (WT) SGR-D-Luc showed a very high level of replication in C2C12 cells, with FF-Luc levels peaking at 12 h post transfection (h.p.t) and increasing over 1000-fold between 4-12 h.p.t. (Fig 2A). AUD mutants T218A, M219Q, P221A, C246A and A251Q exhibited similar levels of replication as compared to WT, however, W220A, D249A and Y324A showed significant lower levels of replication compared to WT (Fig 2A).

As CHIKV is transmitted by mosquitoes we then evaluated the replication capacities of the AUD mutants in C6/36 and U4.4 cells, both derived from *Ae. albopictus* mosquitoes. Importantly, C6/36 cells harbor a frameshift mutation in the Dicer-2 (Dcr2) gene, which leads to the translation of a truncated Dcr2 protein and a defect in the RNAi response [38]. Consistent with this, although WT SGR-D-Luc showed robust replication in both cell lines, C6/36 supported higher levels of replication than U4.4 cells (Fig 2B). Interestingly, unlike the replication defects observed in C2C12 cells, all AUD mutants showed similar levels of replication compared to WT in both U4.4 and C6/36 cells (Fig 2B).

The striking phenotypic differences between mammalian and mosquito cell lines for the three AUD mutants (W220A, D249A and Y324A) led us to investigate further. A simple explanation might be that these mutants had reverted to WT in mosquito cells as we had previously observed this for the AUD mutant R243A/K245A [15]. This mutant replicated poorly in mammalian cells but efficiently in mosquito cells, and this was explained by the fact that R243A/K245A had reverted to WT in mosquito cells allowing efficient replication. To test this assumption, total RNA from U4.4 and C6/36 cells at 72 h.p.t was extracted, amplified by RT-PCR and sequenced. Surprisingly, none of the AUD mutants had reverted to WT, and all three mutants retained the same sequence as the input RNA (Fig 2C). The results demonstrated that the ability of these three AUD mutants to replicate in mosquito cells was not due to reversion.

To confirm that the phenotype of the three AUD mutants was not specific to C2C12 cells we further investigated this in other mammalian cells. These included RD (a human rhabdomyosarcoma cell line), two human hepatoma cell lines, Huh7 and Huh7.5 and BHK-21 hamster kidney fibroblast cells. Huh7.5 are derived from Huh7, and due to a mutation in one allele of the retinoic acid-inducible gene I (RIG-I) [39], a cytosolic pattern recognition receptor responsible for the type 1 interferon (IFN) response, Huh7.5 cells are defective in innate immunity. Reassuringly, all three AUD mutants showed replication defects compared to WT in all these cell lines (Fig 2D), although the magnitude of the defective phenotypes varied between different cell lines. In particular the defective phenotypes were less apparent in BHK-21 cells, this is consistent with our observations of other mutants in nsP3 [40]. We conclude that the three residues W220, D249 and

Y324 within the CHIKV nsP3 AUD play a role in genome replication in mammalian cells but not in mosquito cells.

The AUD mutants exhibit a temperature dependent phenotype

We reasoned that a simple explanation for the different phenotypes in mammalian and mosquito cells might be the temperature difference, as mammalian cells are cultured at 37°C whereas mosquito cells are cultured at 28°C [41]. To test this, the replication of WT and mutant SGR-D-Luc were analysed in C2C12 cells incubated at 37°C or 28°C. Surprisingly, the replication of the three mutants was indistinguishable from WT at 28°C (Fig 3A), in addition we noted that for WT SGR-D-Luc, replication rates were similar at both temperatures up to 12 h.p.t. However, at 28°C replication continued to increase (approximately 100-fold) from 12 to 24 h.p.t, whereas at 37°C it peaked at 12 h.p.t. It should be noted that the optimal temperature for FF-Luc activity is between 22-28°C [42], so to confirm that this observation reflected a bona fide effect on genome replication, rather than increased activity of the FF-Luc reporter at the lower temperature, translation of CHIKV SGR at the two temperatures was quantified by western blotting for nsP1 expression (Fig 3B). Reassuringly, none of the mutants exhibited detectable nsP1 at 37°C whereas a low level of nsP1 was detectable for the WT at this temperature. In contrast WT and all mutants exhibited high levels of nsP1 at 28°C, consistent with the elevated levels of replication and translation at this temperature. In addition, we saw the same phenotype when levels of R-Luc were measured (S2 Fig).

We also observed the same results in two other mammalian cell lines, Huh7 and Vero (Fig 3C, D). Vero are African Green Monkey kidney epithelial cells and are IFN-deficient

thus unable to mount an IFN response following virus infection (although they possess IFN receptors and can respond to exogenous IFN). Our data suggest that the enhanced replication at 28°C is not due to the previously documented reduced efficiency of the IFN response at sub-physiological temperatures [43].

We conclude that the three AUD mutants (W220A, D249A and Y324A) are temperature sensitive, exhibiting no defective phenotype in mammalian cells at a sub-physiological temperature. However, we also note the novel observation that replication of WT CHIKV SGR is significantly enhanced at sub-physiological temperature.

Enhanced replication at sub-physiological temperature is not dependent on the nature of the nsP3-fused reporter protein and is alphavirus specific.

As the SGR-D-Luc construct contains two reporters, we considered it prudent to confirm that the phenotype we observed was not influenced by these reporters. To test this, two alternative CHIKV SGRs were utilised, namely SGR-nsP3-mCh-FF-Luc (in which the R-Luc reporter was replaced with mCherry) and SGR-FF-Luc (lacking R-Luc and thus expressing WT unfused nsP3) (Fig 4A). These three SGRs were assayed in three cell types, C2C12, Huh7 and Vero cells at the two temperatures (28°C or 37°C). All three showed enhanced levels of replication at 28°C compared to 37°C (Fig 4B-D). As described previously (Fig 3A), this effect was most apparent between 12-24 h.p.t.

We reasoned that the enhancement of genome replication at sub-physiological temperature might be a common feature in other arboviruses that would share a mode of transmission from mosquitoes to humans via a peripheral bite. We therefore tested

replication of mini-genome constructs derived from Bunyamwera virus (BUNV), the type species of the bunyaviridae (negative-sense, single-stranded RNA viruses) [44] and an SGR derived from Zika virus, a positive-sense, single-stranded RNA virus in the Flaviviridae family [45]. As shown in Fig 4E and F, both the BUNV mini-genome [46] and the Zika virus SGR [47] showed similar levels of genome replication at physiological and sub-physiological temperatures (Fig 4F), indicating that this phenotype is specific to alphaviruses.

Overall, data from three CHIKV SGRs at different temperatures confirmed that enhanced genome replication at sub-physiological temperature is not dependent on the nature of the nsP3-fused reporter protein and is specific to alphavirus.

Temperature sensitive AUD mutants retain the phenotype in the context of infectious CHIKV and O'Nyong Nyong virus (ONNV).

As we had previously identified a novel function of the CHIKV AUD in promoting virus assembly, we wished to determine whether the phenotypes we had observed in the context of CHIKV SGR were also seen in infectious virus. The three AUD mutations (W220A, D249A and Y324A) were therefore generated in the backbone of the ICRES-CHIKV infectious clone [36]. In vitro transcribed RNA for each construct was transfected into C2C12 cells and released virus were harvested at 48 h post infection (h.p.i) and titrated by plaque assay on BHK-21 cells. As shown in Fig 5A, the three mutants produced significantly less infectious virus than WT at 37°C (approx. 10^4 reduction in titre). At 28°C, unlike the SGR data where the mutants were indistinguishable from WT, the mutants exhibited significantly reduced titres although this phenotype was less

pronounced than at 37°C (between 5-50 fold reduction). Sequence analysis of ICRES-CHIKV AUD mutants at 28°C or 37°C indicated that no reversion had occurred at either temperature (data not shown). However, unlike the marked differences in genome replication observed using SGRs, there was only a modest increase (2-fold) in virus production at 28°C compared to at 37°C. This suggested that the higher level of genome replication at 28°C did not lead to a concomitant increase in assembly and release of infectious virus.

We also extended this analysis to a closely related virus, ONNV, which can be manipulated at BSL2. We exploited an infectious clone of ONNV in which the fluorescent reporter ZsGreen is expressed on a sgRNA driven from a second copy of the sub-genomic promoter (ONNV-2SG-ZsGreen) (Fig 5B) (kind gift from Prof. Andres Merits, University of Tartu). This enables rapid assessment of genome replication by quantification of ZsGreen fluorescence per cell, as well as simultaneous measurement of infectious virus production by quantification of ZsGreen positive cells. It should be noted that although expression of ZsGreen is dependent on genome replication as production of the sgRNA is templated from the negative strand, the levels of ZsGreen expression will be representative of both replication and translation of the sgRNA. We also observed that the three AUD mutants exhibited a temperature dependent replication phenotype in the context of ONNV (Fig 5C). At 28°C the level of ZsGreen expression per cell was indistinguishable between WT and the three AUD mutants, whereas at 37°C the mutants showed a significant reduction in ZsGreen expression per cell. Interestingly, in contrast to the CHIKV results, sequence analysis revealed that at 37°C, W220A and D249A had reverted to WT and Y324A exhibited two compensatory pseudorevertant mutations (nsP2

R768Q and nsP3 C173Y) (data not shown), however, no reversion was observed for the AUD mutants at 28°C. Interestingly, we noticed a significant difference in virus production with plaque assays conducted at different temperatures. Due to the fact that the AUD mutants had reverted at 37°C, there were no significant differences in virus production at 37°C when the virus was titrated at either 37°C or 28°C (Fig 5D). In contrast, when viruses were produced at 28°C and titrated at 28°C, the AUD mutants exhibited virus titres indistinguishable from WT, whereas when titrated at 37°C they showed significant reduction in virus titres compared to WT. These data confirm that mutant viruses produced at 28°C retained the growth defects at 37°C.

Temperature shift assay reveals that early events in SGR-D-Luc replication are not enhanced at sub-physiological temperature

Previous studies have investigated temperature-sensitive mutants of alphaviruses by use of a temperature shift assay [48-50]. To further explore the role of temperature in controlling CHIKV genome replication, a temperature shift assay using WT SGR-D-Luc was conducted in C2C12 cells. WT SGR-D-Luc RNA was transfected into C2C12 cells and incubated either at 37°C or 28°C for 12 h, with and without a subsequent temperature shift from 12-24 h.p.t (28°C to 37°C (designated S*) or 37°C to 28°C (designated S#)) (Fig 6A). As seen previously (Fig 3A), replication of WT SGR-D-Luc (as assessed by FLuc levels) up to 12 h.p.t proceeded at broadly similar rates at both temperatures (Fig 6B), and between 12-24 h.p.t replication increased ~100-fold at 28°C but did not increase at 37°C. However, if the temperature was shifted from 28°C to 37°C at 12 h.p.t (S*), the replication increased only ~20-fold between 12-24 h.p.t (Fig. 6B, left graph). The opposite effect was seen when transfected cells were first incubated at 37°C: replication reduced

slightly between 12-24 h.p.t at 37°C, however, when temperature was shifted from 37°C to 28°C at 12 h.p.t, replication increased 5-fold between 12-24 h.p.t (Fig. 6B, right graph). Importantly, both cell growth assessed by quantification of viable cells (Fig 6C), and metabolic activity assessed by MTT assay (Fig 6D) were higher at 37°C compared to 28°C. Temperature shifts elicited the expected changes in both cell number and metabolic activity. As expected therefore, these data confirmed that the enhanced replication observed at sub-physiological temperature cannot be explained by increased cell growth and metabolic activity. In fact we observed the opposite, virus genome replication was elevated when cell growth and metabolic activity was dampened at the lower temperature. These data were also confirmed by Western blotting for nsP1 (Fig 6E), demonstrating that translation of the 5' ORF was also enhanced at the sub-physiological temperature. At this stage we also asked whether the replication level was correlated with the level of phosphorylated eIF2α (P-eIF2α), given that this is a critical factor in restricting translation during cell stress. However, there appeared to be little correlation between replication and levels of P-eIF2α (Fig 6C), with the exception that when the temperature was shifted from 37°C to 28°C at 12 h.p.t. (S#), P-eIF2α levels reduced, coincident with an increase in replication (Fig 6B, right hand graph).

Inhibition of eIF2α phosphorylation failed to recover replication defects of the AUD mutants at 37°C

To follow up on the possible role of eIF2α phosphorylation in the enhancement of genome replication at sub-physiological temperature, we exploited the well-characterised compound ISRIB which reverses the effects of eIF2α phosphorylation [51, 52]. As shown

in Fig 7A, replication of both WT and W220A SGR-D-Luc was enhanced 5-fold by 200 nM ISRIB at 28°C, suggesting that eIF2α phosphorylation may make a minor contribution to the higher level of replication at this sub-physiological temperature. However, at 37°C ISRIB treatment resulted in a 2-fold increase in replication for WT SGR-D-Luc but had no effect on W220A SGR-D-Luc (Fig 7A). ISRIB was titrated over a 100-fold concentration (200 nM – 20 μM) and the stimulatory effect was apparent at the lowest concentration with no cytopathic effect (S3 Fig). Similar results were observed in the context of ONNV infection (Fig 7B): ISRIB enhanced replication of both WT and W220A at 28°C, but at 37°C only WT was enhanced. These data indicated that pharmacological inhibition of the effects of eIF2α phosphorylation by ISRIB was able to stimulate alphavirus genome replication at 28°C, however, ISRIB treatment of WT at 37°C only modestly enhanced replication and did not recapitulate the differences observed at the sub-physiological temperature (eg Fig 3). In addition, ISRIB treatment did not restore the replication ability of the W220A mutant at the non-permissive temperature.

Alterations in stress granule formation in response to ONNV infection at physiological and sub-physiological temperatures

In a further attempt to shed light on the reasons why alphavirus genome replication was enhanced in mammalian cells at sub-physiological temperature we turned our attention to stress granules (SG). These are large cytoplasmic foci that are nucleated by the aggregation of 48S mRNPs, which accumulate following stress-induced translation arrest [53]. SG contain aggregates of stalled, translationally silent mRNAs, initiation factors, small ribosomal subunits, and mRNA binding proteins [54]. Although SG have been shown to be antiviral, paradoxically alphavirus infection has been reported to induce SG

and nsP3 has been shown to interact with SG components, in particular G3BP1/2, to modulate their composition [32, 55]. SG formation can be induced by treatment with sodium arsenite (NaAsO_2) which results in oxidative stress, and they can be disassembled by CHX treatment. We considered that SG formation might be relevant in this investigation as it had been previously shown that NaAsO_2 was only able to induce SG at 37°C but not 28°C [29]. We confirmed that this was also the case in our hands (Fig 8A, B), as no G3BP1-positive SG were observed following NaAsO_2 treatment at 28°C.

We proceeded to analyse G3BP1-positive SG in cells infected with ONNV-ZsGreen at both temperatures. Both the number and size of SG were determined in 10 cells and compared to those induced by NaAsO_2 . ZsGreen positivity enabled us to identify infected cells. In contrast to the inability of NaAsO_2 to induce SG at 28°C, ONNV infection was able to induce SG at both temperatures. Interestingly, the G3BP1-positive SG induced by ONNV infection differed significantly in both size and number at the two temperatures (Fig 9A, B). We observed that at 28°C the average cross-sectional area of SG was significantly smaller than at 37°C (mean $0.5 \mu\text{m}^2$ compared to $\sim 1.6 \mu\text{m}^2$), although both of these were also significantly smaller than those induced by NaAsO_2 at 37°C (mean $\sim 4.5 \mu\text{m}^2$). In addition, CHX treatment reduced the size of ONNV-induced SG from a mean of $1.6 \mu\text{m}^2$ to $\sim 0.8 \mu\text{m}^2$ at 37°C, whereas at 28°C CHX treatment had no effect on SG size. In contrast, ONNV infection at 28°C resulted in a larger number of SG compared to at 37°C, and infection at both temperatures resulted in more SG than after NaAsO_2 treatment (Fig. 9B).

We also analysed the presence of dsRNA in ONNV-infected cells as a direct indicator of genome replication using the dsRNA-specific antibody, J2. Consistent with our previous

results using CHIKV SGR (Fig 3), we saw a higher level of dsRNA in cells infected at 28°C compared to 37°C (Fig. 9C), indicating elevated genome replication at the sub-physiological temperature. However, quantification of ZsGreen in individual cells revealed lower levels at 28°C compared to at 37°C, further evidence that the enhanced levels of genome replication seen at the sub-physiological temperature did not directly correlate with increased translation from the sgRNA.

The difference in the sizes of SG induced by either NaAsO₂ or ONNV at the two temperatures led us to ask whether there were any gross differences in their composition. Cells treated with NaAsO₂ or infected with ONNV were therefore analysed by indirect immunofluorescence for two other well-characterised SG components: the mRNA binding protein T-cell-restricted antigen 1 (TIA-1) and the translation initiation factor eIF4G [56]. As shown in Fig 10A/B, SG induced by NaAsO₂ at 37°C contained both TIA-1 and eIF4G, and consistent with the lack of G3BP1-positive SG at 28°C, neither TIA-1 or eIF4G accumulated into SG at 28°C. In contrast, following ONNV infection TIA-1 positive SG were observed at both temperatures but eIF4G did not accumulate into SG at either temperature. These data are consistent with the previous observations [33] that SG induced by alphavirus infection are compositionally distinct from those induced by other cellular stresses, however they do not shed any light on the different seen in ONNV-induced SG at the two temperatures.

Discussion

This study started with the intention of building on our previous work which identified a role for two residues (P247 and V248) in the function of the CHIKV AUD [15]. Alanine substitution of these residues (termed the PV mutant) disrupted the binding of the AUD to the sub-genomic promoter, reducing levels of structural proteins with concomitant effects on virus assembly. In this study we generated a series of mutations in surface exposed and conserved residues proximal to P247 and V248 to further investigate the phenotype. Surprisingly, most of these mutations had no effect on genome replication, although we cannot rule out an effect on virus assembly as this was not evaluated in this study. However, our experiments did demonstrate a role in genome replication for three residues W220, D249 and Y324, interestingly these residues are distal from each other (Fig 1B). Indeed, Y324A was chosen as a control, being defined as the last residue in the AUD [34]. The phenotypes of the 3 mutants were observed in all mammalian cells tested although the magnitude of the defects varied somewhat. Intriguingly, in BHK-21 cells the mutants appeared to be better tolerated, as seen previously for both PV [15] and mutations in the nsP3 macrodomain [40]. Our study took a surprising turn when we observed that the 3 mutants exhibited no defective phenotype in mosquito cells and also when assayed in mammalian cells at sub-physiological temperatures, defining these as temperature-sensitive (ts) mutants. This analysis also revealed that the wildtype CHIKV replicated more efficiently at sub-physiological temperatures in mammalian cells and we focused our attentions on this intriguing observation.

As 28°C is a very low temperature for the growth of mammalian cells, we also tested the phenotype of W220A at an intermediate temperature, 32°C, likely to be better tolerated

by cells in culture. Reassuringly, W220A replicated as well as WT at this temperature and WT was itself more efficient than at 37°C (S4 Fig). It should be noted that ts mutations in alphavirus nsP3 have been previously identified, for example in Sindbis virus F312S and A268V both resulted in a defect in negative-strand RNA synthesis at the non-permissive temperature (40°C) [57]. In addition, a macro-domain mutant A68G had a partial phenotype. These data point to a critical role of nsP3, and the AUD in particular, in genome replication. Further biochemical analysis of these ts mutants in nsP3 may shed light on the specific role of nsP3.

In considering why alphavirus genome replication in mammalian cells might be enhanced at sub-physiological temperatures, we reasoned that the first cells infected via a mosquito bite would be in the periphery and thus not at 37°C. It would be reasonable to expect that other arboviruses would also exhibit the same differential, however, that was not the case as both BUNV and ZIKV replicated to similar levels at 28°C and 37°C. This suggested that this phenotype would be driven by alphavirus specific processes, and we noted that both the nsP3 macrodomain and the AUD do not have functional homologues in either bunyaviruses or flaviviruses. Analysis of the timescale of genome replication at different temperatures also revealed that the phenotype was most apparent after 12 h.p.t. In most cases, replication rates up to 12 h.p.t. did not differ between the two temperatures (eg see Fig 3A), however between 12-24 h.p.t. replication continued to increase at 28°C, but plateaued at 37°C. One interpretation for this observation is that this reflects an aspect of the cellular response to virus genome replication that is perhaps ablated or blunted at the sub-physiological temperature. An obvious candidate would be the type 1 IFN response, which has indeed been shown to be reduced at lower temperatures promoting

CHIKV arthritis in a mouse model [58]. However, our data suggest that this is not the explanation for our findings as we observed the same phenotype in Vero and Huh7.5 cells, both of which are defective in aspects of the IFN response.

An alternative possibility was that the host cell translational shutoff mediated by the phosphorylation of eIF2 α was impaired at sub-physiological temperatures. However, we were unable to determine any specific correlation between eIF2 α phosphorylation and genome replication (eg see Fig 6C). In addition, treatment with ISRIB, a small molecule that reverses the effects of eIF2 α phosphorylation [52], failed to recapitulate the effect of the lower temperature on replication of wildtype CHIKV or ONNV at 37°C, and did not permit the replication of W220A at 37°C. We therefore turned our attention to stress granules (SG), cytoplasmic protein-mRNA aggregates formed in response to stress and believed to play an antiviral role. The link between alphavirus infection and SG formation is confusing, as alphaviruses induce formation of SG that differ in composition from canonical SG induced by other stressors, yet two key components of SG, GTPase-activating protein (SH3 domain)-binding proteins 1 and 2 (G3BP1/2), are critical for alphavirus genome replication [27].

As seen previously [29], treatment with NaAsO₂ (which induces oxidative stress) did not result in SG formation at 28°C, suggesting that there was a defect in this process at the sub-physiological temperature. However, alphavirus (ONNV) infection did induce SG formation at both temperatures – the composition of these SG was comparable (Fig 10) but both their size and quantity were different (Fig 9 B/C). Specifically, at 28°C SG were smaller, more numerous and were not disassembled by CHX treatment. To attempt to understand why the formation of smaller and more numerous SG might promote genome

replication, we calculated the mean surface area and volume of SG per cell. To do this we assumed that SG are spherical and used the following parameters: at 28°C the mean cross-sectional area of SG was 0.5 μm^2 (radius = 0.4 μm) and the mean number per cell were 65, at 37°C the mean cross-sectional area of SG was 1.6 μm^2 (radius = 0.7 μm) and the mean number per cell were 29 (Fig 9B). Using these parameters at 28°C the mean surface area of SG was 123 μm^2 per cell and the mean volume was 1.6 μm^3 per cell. At 37°C the mean surface area of SG was 178 μm^2 per cell and the mean volume was 41.6 μm^3 per cell. Therefore, the volume of SG at 37°C was 26-fold greater than at 28°C. One possible interpretation of this is that the smaller volume of SG at the sub-physiological temperature limited the sequestration of proteins such as G3BP1/2 that are required for genome replication, allowing that process to continue. Why the process of alphavirus-driven SG formation should differ at different temperatures remains to be determined, however, importantly the ADP-ribosylhydrolase activity of the nsP3 macro-domain has recently been shown to play a key role in SG formation, disassembly and composition [33]. It is possible that the enzymatic activity and/or substrate specificity of the macrodomain could be modulated by temperature. Although the AUD *per se* has not been directly shown to play a role in SG formation, it is flanked by the macro-domain and HVD, both of which now have been implicated in SG formation. It is thus possible that all three domains of nsP3 coordinate to control SG formation and enable genome replication.

In conclusion, our data have revealed a hitherto unrecognized enhancement of alphavirus genome replication in mammalian cells at sub-physiological temperature that may play a role in the early stages of transmission of virus to the mammalian host via a mosquito bite. Our observations point to a critical role of the AUD in this difference, and our ongoing

work seeks to understand the effects of temperature on the nsp3 interactions with host cell proteins using a proteomic approach.

Material and methods

Sequence alignment

AUD amino acid sequences in a variety of alphaviruses (Sindbis, Semiliki, Ross River, Venezuelan, Eastern and Western Equine Encephalitis, ONNV and CHIKV) were obtained from NCBI and aligned by Clustal Omega. The conserved AUD residues were identified by Pymol and their locations were predicated from the Sindbis nsP2/3 protein structure (PDB ID code 4GUA) [15].

Cell culture

Mammalian cells RD, Huh7, Huh7.5, Vero and BHK-21 cells were in DMEM supplied with 10% Fetal Calf Serum (FCS), 0.5 mM non-essential amino acids and penicillin-streptomycin (100 units/mL). C2C12 cells were supplied with the same components apart from 20% FCS. All mammalian cells were maintained at 37°C or 28°C with 5% CO₂. RD cells were obtained from Nicola Stonehouse (University of Leeds), Huh7 cells were obtained from John McLauchlan (Centre for Virus Research, Glasgow), Huh7.5 cells were obtained from Charles Rice (Rockefeller University, New York), BHK-21 cells were obtained from John Barr (University of Leeds), C2C12 cells were obtained from Michelle Peckham (University of Leeds). U4.4 and C6/36 cell lines (*Ae. albopictus*) were obtained from Susan Jacobs (The Pirbright Institute) and were cultured in Leibovitz's L-15 media

containing 10% FCS, 10% tryptose phosphate broth and penicillin-streptomycin (100 units/mL), at 28°C without CO₂.

Plasmid constructs

SGR-D-Luc was used as a template for site-directed mutagenesis using specific primers to generate each of the AUD mutations. The AUD mutated fragments were excised and ligated into the SGR-D-Luc backbone. AUD mutated fragments were also ligated into the infectious ICRES-CHIKV clone [36]. For construction of the ONNV AUD mutants, a fragment including the AUD coding sequence was excised from the ONNV-2SG-ZsGreen (donated by Prof. Andres Merits, University of Tartu), and ligated into pcDNA3.1 to generate pcDNA3.1-ONNV-AUD. This was used as a template for site-directed mutagenesis using specific primers to generate ONNV AUD mutations. The ONNV AUD mutated fragments were excised from pcDNA3.1-ONNV-AUD and ligated into the ONNV-2SG-ZsGreen backbone. SGR-nsP3-mCh-FF-Luc was generated by SpeI digestion of the FF-Luc fragment from SGR-D-Luc and insertion of the mCherry coding sequence. SGR-FF-Luc was constructed by SpeI digestion of the FF-Luc fragment from SGR-D-Luc and self-ligation.

Transfection and Dual-luciferase Assay

Capped RNAs of WT and mutant SGR-D-Luc, ICRES-CHIKV, ONNV-2SG-ZsGreen, or WT SGR-nsP3-mCh-FF-Luc, WT SGR-FF-Luc were synthesized and purified using the mMACHINE™ SP6 Transcription Kit (Thermo Fisher Scientific). In brief, 250 ng of each of the RNAs was transfected into 5 x 10⁴ cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cells were harvested at 4, 12,

24 or 48 h.p.t. and both FF-Luc and R-Luc activities were measured using the Dual-luciferase Kit (Promega) according to the manufacturer's instruction. The experiments were repeated three times independently.

Sequence analysis

Total RNA from transfected or infected cells was extracted by Trizol, followed by reverse transcription to generate cDNA using the SuperScript IV Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. The expected DNA fragments of CHIKV or ONNV genome were amplified by PCR using specific primers (Primer sequences available upon request). Then all PCR products were sequenced.

Temperature shift assay

C2C12 cells were transfected with WT SGR-D-Luc RNA and incubated at 28°C or 37°C, with and without a temperature shift from 12-24 h.p.t (from 28°C to 37°C (designated S*) or 37°C to 28°C (designated S#)). Cells were harvested at 4, 12 and 24 h.p.t. All the cells were then lysed and measured using a dual luciferase kit (Promega), nsP1 expression were also detected by western blotting. Along with the temperature shift assay, cell growth number at 37°C or 28°C were counted by trypsin treatment and dilution into 1 ml of DMEM and cell metabolic activity at two temperatures was assessed by MTT assay.

MTT assay

C2C12 cells were seeded into 96 well plates. The next day, cells were mock transfected and incubated at 37°C or 28°C. At 4, 12 or 24 h.p.t, cell media were removed and replaced with 150 µl phenol-red free DMEM media and 30 µl 6 mM MTT. Cells were incubated at

37°C for 2 h before media were removed and replaced with 100 µl DMSO. Then cells were covered with foil and agitated until all MTT granules had dissolved, prior to reading absorbance at 570 nm.

Plaque assay

As described above, cell supernatants were harvested 48 h.p.i and stored at -80°C. Supernatants were thawed, serially diluted and applied to monolayers of BHK-21 cells for 1 h at 37°C. The inocula were aspirated and cells were overlaid with 0.8% methylcellulose for 72 h at 37°C, 96 h at 28°C for ONNV infection, and 48 h at 37°C for ICRES-CHIKV. Plaques were either visualized by EVOS microscopy (ONNV) or staining with 0.05% crystal violet for 30 min (CHIKV).

ISRIB assay

C2C12 cells were transfected with WT SGR-D-Luc or mock transfected, and 200 nM ISRIB was added to cells at 2 h.p.t. Cells were then harvested at 4, 12, 24 h.p.t. and dual luciferase activities were measured. For ONNV infection, C2C12 cells were infected with WT and W220A ONNV at an MOI of 0.1 for 1 h at 37°C, washed with PBS 3 times and replaced with complete media containing 200 nM ISRIB prior to incubation at 28°C or 37°C. Levels of nsP1, eIF2α, P-eIF2α were detected by western blotting.

Western blotting

Cells were lysed in 1X passive lysis buffer (PLB) or 1X Glasgow Lysis Buffer (GLB) supplemented with protease inhibitors and phosphatase inhibitors (Roche Diagnostics, specific for detection of P-eIF2α), protein samples were quantified by BCA assay (Pierce)

according to manufacturer's instruction. 10 or 20 µg of each protein samples was heat denatured at 95°C for 5 min and separated by SDS-PAGE, proteins were then transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 50% (v/v) Odyssey blocking buffer (Li-Cor) diluted in 1X Tris-buffered saline tween-20 (TBST) (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4). The membrane was then incubated with primary antibodies (1:1000 dilution) at 4°C overnight and the next day incubated with fluorescently labelled anti-mouse (700 nm) and anti-rabbit (800 nm) secondary antibodies for 1 h at room temperature (RT). Membranes were then imaged on a Li-Cor Odyssey Sa Imager (Li-cor).

Immunofluorescence assay

RD cells were seeded into 12 well plates with coverslips. The next day, cells were infected with WT ONNV at an MOI of 5 by incubating inoculum at 37°C for 1 h and then replaced with complete media. Mock infected cells were treated with 0.5 mM NaAsO₂ for 1 h at 7 h.p.i, then cells were either fixed by 4% formaldehyde or treated with 100 µg/ml CHX for 45 min and then fixed by 4% formaldehyde. Cells infected with WT ONNV were also treated with 100 µg/ml CHX for 45 min at 8 h.p.i and then fixed with 4% formaldehyde. Cells were washed with PBS, permeabilized with ice-cold methanol at -20 °C for 10 min and blocked with 2% BSA for 1 h. The cells were incubated with primary antibody to G3BP1 (Abcam) (1:200), eIF4G (Thermo) (1:100 dilution) or TIA (Thermo) (1:100 dilution), or a combination of G3BP (Proteintech) (1:100) and P-eIF2α (Thermo Fisher) (1:100) at 4°C overnight. The next day, cells were washed with DEPC-PBS and incubated with J2 dsRNA antibody (Scicons) (1:100 dilution) at RT for 1 h. All cells were stained with Alexa Fluor-594 chicken anti-rabbit and Alexa Fluor-647 goat anti-mouse secondary antibodies

(1:500 dilution) at RT for 1 h. Cells were mounted onto glass slides with Prolong Gold antifade reagent (Invitrogen) containing 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) and sealed with nail varnish. Confocal microscopy images were acquired using a Zeiss LSM880 upright microscope. Post-acquisition analysis of images was performed using Zen software (Zen version 2018 black edition 2.3, Zeiss), or Fiji imageJ (v1.49) software.

Statistical analysis

Statistical analysis was performed using the unpaired Student's t tests (two-tailed). Data in bar graphs represent statistically significant differences and displayed as the means $\pm$ S.E. of three experimental replicates (n = 3).

Acknowledgments

We thank Prof. Andres Merits (Institute of Technology, University of Tartu, Estonia) for the CHIKV SGRs, ICRES constructs, ONNV-2SG-ZsGreen construct and antibody to nsP1. We thank Dr. Andrew Tuplin for the Zika virus replicon and Dr John Barr for the BUNV mini-genome construct. We thank Drs. Niluka Goonawardane and Ruth Hughes for help in Fiji imageJ analysis. We also thank Dr Sreenivasan Ponnambalam for access to the EVOS microscope.

Funding

This work was supported by a Wellcome Investigator Award to MH (grant number 096670). JG was supported by a University of Leeds/China Scholarship Council PhD

studentship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figure legends

Figure 1. (A) Structure of the CHIKV genome and nsP3. The positive-sense RNA is capped and polyadenylated and templates translation of the 5'ORF into the four non-structural (ns) proteins which transcribe a negative-sense intermediate. Transcription of a sub-genomic RNA is then initiated from the sg-prom and acts as a template for translation of the 3'ORF encoding the structural proteins. nsP3 comprises 3 domains: Macro-domain, AUD (alphavirus unique domain), HVD (hypervariable domain). The structure of the CHIKV-Dluc subgenomic replicon (SGR) is shown. R-Luc (Renilla luciferase) is expressed as a fusion within nsP3, FF-Luc (firefly luciferase) is expressed from the sg-prom. **B. Three-dimensional structure of the SINV AUD [34].** Surface exposed and conserved residues targeted for mutagenesis are highlighted in the corresponding colours.

Figure 2. Replication of CHIKV AUD mutants in mammalian and mosquito cells. C2C12 (A), C6/36 and U4.4 (B), RD, Huh7, Huh7.5 and BHK-21 cells (D) were transfected with wildtype (WT) and mutant SGR-D-Luc and cells were harvested at indicated time points. FF-Luc activities of WT and mutants were measured and normalized to their respective 4 h values and thus represent fold-change compared to those 4 h values. (GAA: nsP4 polymerase inactive mutant). Significant differences denoted by ** (P<0.01), *** (P<0.001), **** (P<0.0001), compared to WT are only shown for the 12 h time point for clarity. Data are displayed as the means ±S.E. of three

experimental replicates. (C) RT-PCR and sequence analysis of SGR-D-Luc W220A, D249A and Y324A in U4.4 and C6/36 cells. RNAs of AUD mutants were extracted 72 h post transfection (h.p.t.), followed by RT-PCR and sequence analysis.

Figure 3. Replication and translation of CHIKV AUD mutants in mammalian cells at 28°C or 37°C. C2C12 (A), Huh7 (C) and Vero cells (D) were transfected with WT and mutant SGR-D-Luc RNAs and cultured at 28°C or 37°C. Cells were harvested at indicated time points. FF-Luc activities of WT and mutants in C2C12 and Huh7 cells were measured and normalized to their respective 4 h values and value represent fold-change compared to the 4 h values. FF-Luc activities in Vero cells were normalized to 4 h R-Luc activities. (GAA: nsP4 polymerase inactive mutant). Significant differences denoted by ** ($P<0.01$), *** ($P<0.001$) **** ($P<0.0001$), compared to WT are only shown for the 12 h time point for clarity. Data are displayed as the means $\pm$ S.E. of three experimental replicates. (B) Protein expression of nsP1 for the WT and mutant SGR-D-Luc at 28°C or 37°C. Cells were lysed at 24 h.p.t., 10 μ g of each sample was loaded and analysed by western blot. Actin was also detected by western blot as a loading control.

Figure 4. Replication of CHIKV SGRs, Bunyamwera (BUNV) mini-genome and Zika virus SGR at 28°C or 37°C. (A) Schematic of three CHIKV SGRs. CHIKV-nsP3-mCherry-FF-Luc-SGR was constructed in which the R-Luc reporter was replaced with mCherry. CHIKV-FF-Luc-SGR was constructed by deleting R-Luc and thus expressing WT unfused nsP3. C2C12 (B), Huh7 (C) and Vero (D) cells were transfected with the three CHIKV SGRs and incubated at 28°C or 37°C. Cells were harvested at indicated time points. FF-Luc activities of WT and mutants were measured and normalized to their respective 4 h values and thus represent fold-change compared to those 4 h values (GAA:

nsP4 polymerase inactive mutant). Significant differences denoted by ns ($P>0.05$), * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) **** ($P<0.0001$), are only shown for the 12 h time point for clarity. Data are displayed as the means $\pm$ S.E. of three experimental replicates. Replication of BUNV mini-genome (**E**) and Zika virus SGR (**F**) at 28°C or 37°C. Cells were collected at indicated time points and R-Luc or Nano luciferase (N-Luc) activities were measured and normalized to their respective 4 h values.

Figure 5. Phenotype of WT and AUD mutants in the context of ICRES-CHIKV and

ONNV at 28°C or 37°C. (A) Phenotype of WT and AUD mutants in ICRES-CHIKV production at 28°C or 37°C. C2C12 cells were transfected with WT and mutant ICRES-CHIKV RNA and incubated at 28°C or 37°C. Supernatants were collected 48 h post infection (h.p.i.) from both temperatures and titrated at 37°C by plaque assay. **(B)** Schematic of ONNV-2SG-ZsGreen. ZsGreen was inserted after nsP4 and expressed by a duplicated sub-genomic promoter (sg-prom). **(C)** Normalized ZsGreen expression of WT and mutant ONNV at 48 h.p.t at 28°C or 37°C. C2C12 cells were transfected with WT and mutant ONNV RNAs and incubated at 28°C or 37°C. Cells were fixed with 4% formaldehyde at 48 h.p.t. and ZsGreen expression were scanned and normalized by EVOS microscopy. *** ($P<0.001$) **** ($P<0.0001$), compared to WT at their respective temperature. Data are displayed as the means $\pm$ S.E. of three experimental replicates. **(D)** Virus production of WT and mutant ONNV harvested from 28°C or 37°C. Supernatants of WT and mutant ONNV collected from cells at 28°C or 37°C at 48 h.p.i were titrated by plaque assay on BHK-21 cells at 37°C or 28°C. BHK-21 cells were fixed with 4% formaldehyde at 72 h.p.i at 37°C or at 96 h.p.i at 28°C. Infectivity was determined by enumerating ZsGreen-positive cells using EVOS microscopy.

Figure 6. Temperature shift assay of WT SGR-D-Luc. (A) Schematic of temperature shift assay. Transfected cells were incubated at either 28°C or 37°C prior to a temperature shift from 12-24 h.p.t. (28°C to 37°C (designated 24 h S#) and 37°C to 28°C (designated 24 h S*) respectively). Controls maintained the same temperature from 0-24 h.p.t. (B) Replication of WT SGR-D-Luc at 28°C or 37°C. WT SGR-D-Luc RNA was transfected into C2C12 cells and incubated at 28°C or 37°C according to the schematic in (A) FF-Luc activities were measured and normalized to their respective 4 h values. Significant differences denoted by ** (P<0.01), **** (P<0.0001), compared to replication at 12 h.p.t. Data are displayed as the means ±S.E. of three experimental replicates. Cell numbers were enumerated (C) and cell metabolic activity (D) determined by MTT assay at 28°C or 37°C. (E) Protein expression of nsP1 at 28°C or 37°C. Cells were lysed at 24 h.p.t. and 10 µg of each sample was analysed by western blot. Actin was also detected by western blot as a loading control.

Figure 7. ISRIB treatment did not recover the replication phenotype of AUD mutants. (A) Replication of SGR-D-Luc W220A was not recovered by ISRIB treatment at 37°C. C2C12 cells were transfected with SGR-D-Luc WT or W220A RNA and incubated at 28°C or 37°C. Cells were then harvested at indicated time points. FF-Luc activities were measured and normalized to their respective 4 h values and thus represent fold-change compared to those 4 h values (GAA: nsP4 polymerase inactive mutant). Significant differences denoted by ns (P>0.05), ** (P<0.01), compared to replication at 24 h.p.t. without ISRIB at the respective temperatures. Data are displayed as the means ±S.E. of three experimental replicates. (B) Replication of ONNV W220A was not recovered by ISRIB at 37°C. C2C12 cells were infected with ONNV WT and W220A at MOI 0.1 and

incubated at 28°C or 37°C. Cells were fixed at 48 h.p.i. and ZsGreen intensities were quantified by EVOS microscopy. Significant differences denoted by ** ($P<0.01$), *** ($P<0.001$), **** ($P<0.0001$), compared to replication 48 h.p.i. without ISRIB at the respective temperatures.

Figure 8. Stress granule (SG) formation in uninfected cells at 28°C or 37°C. (A)

RD cells were incubated at 28°C or 37°C. Cells were either mock treated or treated with 0.5 mM sodium arsenite (NaAsO_2) for 1 h. Cells were either fixed by 4% paraformaldehyde or treated with 100 $\mu\text{g/ml}$ cycloheximide (CHX) (on cells treated with NaAsO_2) for 45 min prior to fixation. Cells were then stained with G3BP antibody (red). **(B)** Quantification of G3BP protein expression from mock, NaAsO_2 and NaAsO_2 + CHX treated cells at different temperatures. Values were determined for 10 cells and calculated by Fiji ImageJ. **(C)** The number of SG were determined for 10 cells.

Figure 9. Different sizes and numbers of SG induced by NaAsO_2 and ONNV at 28°C

or 37°C. (A) RD cells were mock infected or infected with ONNV WT at MOI 5 and incubated at 28°C or 37°C. 7 h later, mock infected cells were then treated with 0.5 mM NaAsO_2 for 1 h. Cells were either fixed with 4% paraformaldehyde or treated with 100 $\mu\text{g/ml}$ CHX (on cells treated with NaAsO_2) for 45 min prior to fixation. Cells were then stained with G3BP1 antibody (red) or dsRNA antibody J2 (gray). Scale bar 1 μm . **(B)** Quantification of the sizes of SG induced by NaAsO_2 and virus at 28°C or 37°C. The sizes of SG were determined for 10 cells and calculated by Fiji ImageJ. Significant differences denoted by **** ($P<0.0001$), compared with the sizes of virus-induced SG at 37°C. **(C)** Quantification of the overall dsRNA, G3BP1, ZsGreen and dsRNA/ZsGreen levels at 28°C or 37°C. Values were determined for 10 cells and calculated by Fiji ImageJ.

Significant differences denoted by ** ($P < 0.01$), **** ($P < 0.0001$), compared their respective protein expression at 37°C.

Figure 10. TIA-1 but not eIF4G is a component of virus-induced SG at both 28°C and 37°C. RD cells were either mock infected (A) or infected with ONNV WT (B) at MOI 5 and incubated at 28°C or 37°C. 7 h later, mock infected cells were treated with 0.5 mM NaAsO₂ for 1 h. Cells were fixed with 4% paraformaldehyde and stained with TIA-1 antibody, eIF4G antibody (red) or dsRNA antibody J2 (gray) as indicated .

Supporting information

S1 Figure. (A) Sequence alignment of AUD from multiple alphaviruses indicating key mutated residues. (B) Ribbon structure of SINV AUD (PDB ID code 4GUA) with the mutants shown in different colors [34]. Image was constructed using PyMol.

S2 Figure. R-Luc activities of WT and mutant SGR-D-Luc in mammalian cells at 28°C or 37°C. The detailed transfection procedure was shown in Figure 3. R-Luc activities of WT and mutant SGR-D-Luc were measured and normalized to their respective 4 h values and thus represent fold-change compared to their respective 4 h values. (GAA: nsP4 polymerase inactive mutant).

S3 Figure. ISRIB titration. (A) Replication of WT SGR-D-Luc with different concentrations of ISRIB. C2C12 cells were transfected with WT SGR-D-Luc and incubated at 28°C or 37°C. Different concentrations of ISRIB were added 2 h.p.t at 37°C. Cells were harvested at indicated time points and FLuc activities were measured. (B) Cell viability of different concentrations of ISRIB by MTT assay. Along with the transfection,

921 different concentrations of ISRIB were added to mock transfected cells, followed by MTT
922 assay.

923 **S4 Figure. Replication of WT and W220A SGR-D-Luc or ONNV with or without ISRIB**
924 **at 32°C. (A)** Replication of WT and W220A SGR-D-Luc with or without ISRIB at 32°C.
925 C2C12 cells were transfected with WT or W220A SGR-D-Luc RNA and incubated at 32°C.
926 Cells were treated with 200 nM ISRIB at 2 h.p.t and collected at indicated time points and
927 FF-Luc activities from each time point were normalized to their respective 4 h value. **(B)**
928 Replication of WT and W220A ONNV with or without ISRIB at 32°C. C2C12 cells were
929 infected with WT or W220A ONNV at an MOI of 0.1 and incubated at 32°C. Cells were
930 treated with 200 nM ISRIB at 0 h.p.t and were fixed by 4% paraformaldehyde at 48 h.p.i.
931 ZsGreen expression were measured by EVOS microscopy.

Figure 1

Figure 2

Figure 3

Figure 4

A ICRES CHIKV titre

B ONNV-ZsGreen

C

D ONNV-ZsGreen virus titre

Figure 5

A Schematic of temperature shift experiment

B

C Cell growth

D MTT assay

E

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10