

# Chikungunya virus non-structural protein 2-mediated host shut-off disables the unfolded protein response

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The unfolded protein response (UPR) is a cellular defence mechanism against high concentrations of misfolded protein in the endoplasmic reticulum (ER). In the presence of misfolded proteins, ER-transmembrane proteins PERK and IRE1 $\alpha$  become activated. PERK phosphorylates eIF2 $\alpha$  leading to a general inhibition of cellular translation, whilst the expression of transcription factor ATF4 is upregulated. Active IRE1 $\alpha$  splices out an intron from XBP1 mRNA, to produce a potent transcription factor. Activation of the UPR increases the production of several proteins involved in protein folding, degradation and apoptosis. Here, we demonstrated that transient expression of chikungunya virus (CHIKV) (family *Togaviridae*, genus *Alphavirus*) envelope glycoproteins induced the UPR and that CHIKV infection resulted in the phosphorylation of eIF2 $\alpha$  and partial splicing of XBP1 mRNA. However, infection with CHIKV did not increase the expression of ATF4 and known UPR target genes (GRP78/BiP, GRP94 and CHOP). Moreover, nuclear XBP1 was not observed during CHIKV infection. Even upon stimulation with tunicamycin, the UPR was efficiently inhibited in CHIKV-infected cells. Individual expression of CHIKV non-structural proteins (nsPs) revealed that nsP2 alone was sufficient to inhibit the UPR. Mutations that rendered nsP2 unable to cause host-cell shut-off prevented nsP2-mediated inhibition of the UPR. This indicates that initial UPR induction takes place in the ER but that expression of functional UPR transcription factors and target genes is efficiently inhibited by CHIKV nsP2.

Received 2 October 2014

Accepted 11 November 2014

## INTRODUCTION

Many newly translated proteins, including those of viral origin, are translocated into the endoplasmic reticulum (ER) for post-translational modifications (e.g. glycosylation) and proper folding, before being secreted or transported to various cellular organelles. Several stimuli can disrupt the homeostasis in the ER, including viral infections that result in high expression of viral glycoproteins (He, 2006). An increase in the concentration of unfolded and misfolded proteins in the ER lumen results in ER stress. To cope with ER stress, eukaryotic cells have the ability to sense unfolded protein levels and to regulate the transcriptional and translational machinery to reduce general protein synthesis and increase the protein-folding capacity of the ER. The mechanisms by which cells respond to ER stress are collectively called the unfolded protein response (UPR). The initial responses of the UPR aim to reduce ER stress and aid in cell survival. However, prolonged activation of the UPR results in the induction of apoptosis (Kohnno, 2010; Ron & Walter, 2007; Zhang & Kaufman, 2008). The UPR is

initiated when Ca<sup>2+</sup>-dissociated heavy-chain binding protein (BiP), also known as glucose-regulated protein 78 (GRP78), dissociates from three distinct ER-transmembrane UPR sensors to bind misfolded proteins in the ER lumen (Bertolotti *et al.*, 2000; Gething, 1999).

Dissociation of GRP78/BiP from dsRNA-dependent protein kinase (PKR)-like ER kinase (PERK) allows PERK to phosphorylate the  $\alpha$ -subunit of eukaryotic translation initiation factor-2 (eIF2 $\alpha$ ). Phosphorylation renders eIF2 $\alpha$  unable to be recycled back into its active GTP-bound state, reducing the general level of translation and thereby reducing the protein load in the ER lumen (Harding *et al.*, 2002). Under conditions of eIF2 $\alpha$  phosphorylation, activating transcription factor 4 (ATF4) is selectively translated (Vattem & Wek, 2004). ATF4 causes upregulation of UPR target gene transcription, including redox and metabolism regulatory proteins and pro-apoptotic protein DNA damage-inducible protein C/EBP-homologous protein 10 (CHOP or GADD153) (Harding *et al.*, 2000).

Dissociation of GRP78/BiP from inositol-requiring 1 $\alpha$  (IRE1 $\alpha$ ) results in the oligomerization and *trans*-auto-phosphorylation of the kinase domain of IRE1 $\alpha$ , which activates its cytoplasmic RNase activity. This results in the removal of a 26 bp intron from X-box-binding protein 1 (XBP1) mRNA, allowing translation of the full-length transcription factor (Yoshida *et al.*, 2006). In the nucleus, XBP1 promotes the transcription of genes that increase (i) protein-folding capacity, (ii) chaperone protein entry to the ER, and (iii) ER-associated degradation (Lee *et al.*, 2003; Shaffer *et al.*, 2004; Yamamoto *et al.*, 2004).

The third unfolded protein sensor is activating transcription factor 6 (ATF-6). After the ER-luminal domain of ATF-6 has sensed the unfolded protein load and disassociates from GRP78/BiP, ATF-6 is cleaved and acts as a potent transcription factor for the expression of many ER chaperones as well as XBP1 (Haze *et al.*, 1999; Yoshida *et al.*, 2001). The three arms of the UPR form a highly cross-linked network, as specific interactions between ATF-6 and XBP1 have been reported and the transcription of CHOP and GRP78/BiP can be induced via all three arms of the UPR (Takayanagi *et al.*, 2013; Yamamoto *et al.*, 2007, 2004).

To facilitate their replication, viruses manipulate many processes within their host cells. Enveloped viruses often rely on the ER for the maturation of their glycoproteins and transport of the glycoproteins to the plasma membrane. Many viruses inhibit, modulate or exploit arms of the UPR. For instance, human cytomegalovirus and Semliki Forest virus (SFV) induce XBP1 mRNA splicing (Barry *et al.*, 2010; Isler *et al.*, 2005), whereas hepatitis C virus and herpes simplex virus induce ATF-6 cleavage (Burnett *et al.*, 2012; Tardif *et al.*, 2002). West Nile virus infection initiates both XBP1 mRNA splicing and ATF-6 cleavage (Ambrose & Mackenzie, 2011). The induction of the IRE1 $\alpha$  and/or ATF-6 arms of the UPR may help to maintain ER homeostasis by increasing protein-folding capacity, thereby facilitating viral glycoprotein maturation and host-cell survival. In contrast, most viruses block activation of the PERK pathway and the downstream phosphorylation of eIF2 $\alpha$ , thereby avoiding translational inhibition and the subsequent induction of apoptosis via CHOP (Ambrose & Mackenzie, 2011; Burnett *et al.*, 2012; Groskreutz *et al.*, 2010).

Here, we explored the impact of chikungunya virus (CHIKV) on the UPR. CHIKV is a re-emerging mosquito-transmitted member of the genus *Alphavirus* (family *Togaviridae*) that causes sporadic epidemics of primarily rheumatic disease, with the largest epidemic ever recorded starting in 2004 in Africa and spreading across Asia (Suhrbier *et al.*, 2012), recently reaching Oceania (Horwood *et al.*, 2013) and affecting millions of people. The recent explosive outbreak in the Caribbean (Leparc-Goffart *et al.*, 2014) was the first in the western hemisphere and has even reached the USA (McCarthy, 2014). The structural proteins of CHIKV are expressed as a polyprotein that results from translation of the

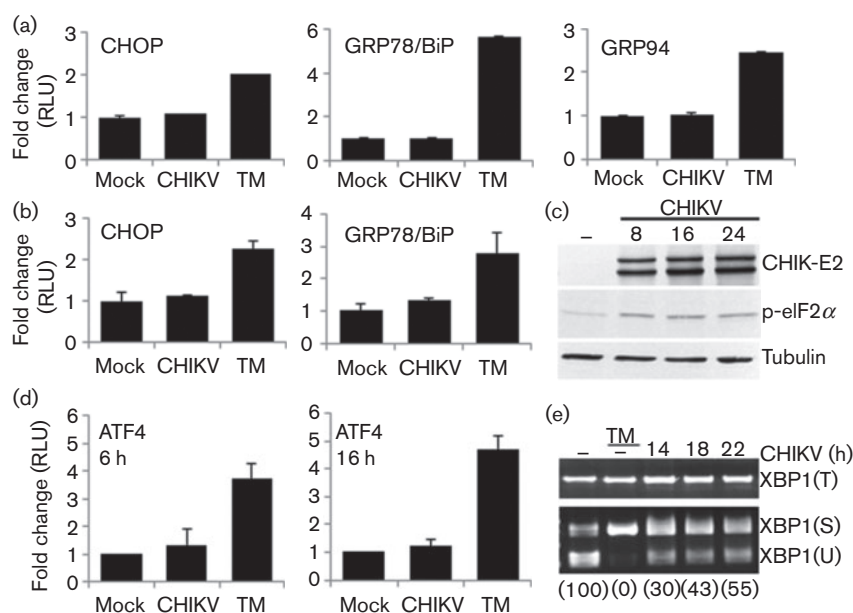
viral subgenomic RNA. After autocatalytic cleavage of the capsid protein, the remaining polyprotein translocates to the ER. In the ER, the polyprotein is cleaved by host signalases into precursors E2 and E1, which are *N*-linked glycosylated before being transported to the Golgi network (Jose *et al.*, 2009; Metz *et al.*, 2011). We investigated whether CHIKV envelope glycoproteins induced the UPR and whether CHIKV replication affected UPR target gene expression and the expression of ATF4. Furthermore, we analysed the activation and localization of XBP1 and concluded that host-cell shut-off, mediated by viral non-structural protein 2 (nsP2), was responsible for modulating the UPR.

## RESULTS

### Effect of CHIKV infection on UPR activation

CHIKV replication in cell culture is relatively fast and cytopathic, with the production of progeny virus and clear expression of structural proteins from 6 h post-infection (p.i.) (Scholte *et al.*, 2013). To determine whether CHIKV infection leads to the activation of the UPR, the induction of three well-known ER stress-related proteins, CHOP, GRP78/BiP and GRP94 (Chang *et al.*, 1989), was investigated. Vero cells were co-transfected with a plasmid containing the promoter region and 5'-untranslated region (UTR) of one of these target genes upstream of a firefly luciferase (Fluc) gene and a plasmid constitutively expressing *Renilla* luciferase (Rluc). Given that CHIKV infection induces host shut-off and reduces RNA polymerase II-driven transcription (Akhrymuk *et al.*, 2012; Gorchakov *et al.*, 2005), we studied the induction of UPR reporter genes using a luciferase-based assay in which all Fluc values were normalized for constitutive Rluc expression. One day after transfection, cells were infected with CHIKV at an m.o.i. of 15. At 16 h p.i., the induction of three UPR target genes was measured (Fig. 1a). As a positive control, uninfected cells were treated with tunicamycin (TM) for 6 h. TM is a microbial toxin, commonly used to induce the UPR by blocking *N*-linked glycosylation. Treatment with TM resulted in a 2-, 6- and 2.5-fold upregulation of the CHOP, GRP78/BiP and GRP94 reporters, respectively. In contrast, CHIKV infection did not upregulate the activity of any of these UPR reporters (Fig. 1a). Because prolonged CHIKV infection induces host shut-off, we studied the UPR at an earlier time point as well. However, at 6 h after CHIKV infection, additional expression of the UPR reporter genes was also not induced (Fig. 1b).

Both PKR and PERK have been reported to be activated early during alphavirus infection (Gorchakov *et al.*, 2004; Nivitchanyong *et al.*, 2009; Rathore *et al.*, 2013; Ventoso *et al.*, 2006; White *et al.*, 2011). To confirm the activation of the PERK–eIF2 $\alpha$ –ATF4 branch of the UPR we analysed the phosphorylation status of eIF2 $\alpha$  (Fig. 1c). A time-course experiment indicated that CHIKV infection in Vero cells resulted in a marked increase in p-eIF2 $\alpha$  within 8 h p.i. The phosphorylation of eIF2 $\alpha$  was concurrent with the



**Fig. 1.** CHIKV infection does not activate the UPR. (a, b, d) Vero cells were co-transfected with plasmids constitutively expressing Rluc and either CHOP-, GRP78/BiP- or GRP94-Fluc (a, b) or ATF4 (d) reporter plasmids. Cells were not infected (mock) or infected with CHIKV (m.o.i. 15). The UPR was induced with tunicamycin (TM; 5 µg ml<sup>-1</sup>) for 6 h. Cells were assayed for dual-luciferase expression at 16 (a, d) or 6 (b, d) h p.i. Values are depicted as fold change of Fluc normalized for Rluc relative to the untreated mock. Error bars represent SD. (c) Vero cells infected with CHIKV (m.o.i. 10) for 8, 16 and 24 h were harvested and immunostained for CHIKV E2, phosphorylated eIF2-α (p-eIF2α) and β-tubulin. (e) Vero cells were not infected (mock) or infected with CHIKV (m.o.i. 10). Total RNA was harvested and XBP1 mRNA was reverse transcribed and PCR amplified. Samples were split into two, from which one subset was digested with *Pst*I. Both digested and undigested amplicons were loaded on a 1.5% agarose gel. Numbers within parentheses are the band intensity of the XBP1(U), normalized to XBP1(T) and relative to mock. S, spliced; T, total; U, unspliced; RLU, relative light units.

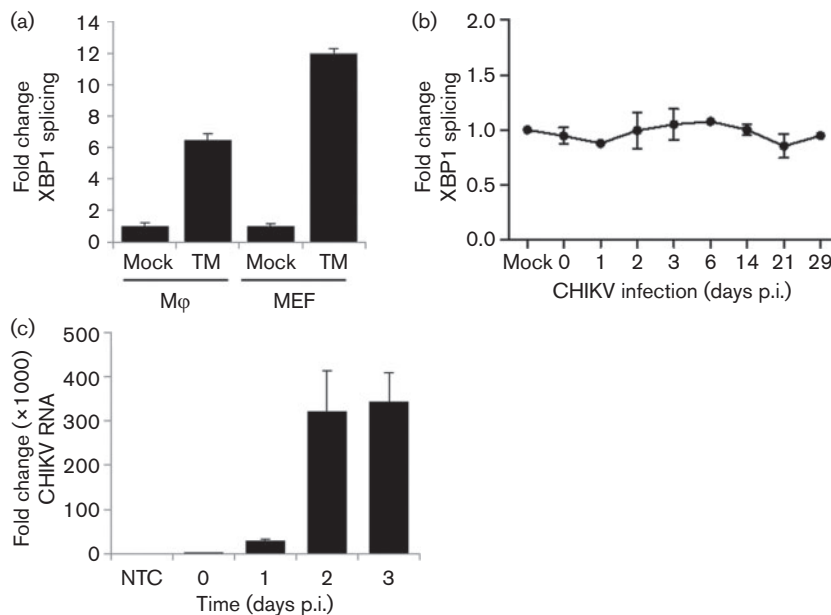
expression of CHIKV envelope proteins, as indicated by the presence of E2 and precursor E3E2 (Fig. 1c). Phosphorylation of eIF2α limits general translation and selectively upregulates the translation of transcription factor ATF4 (Vattem & Wek, 2004). A plasmid containing the promoter and 5'UTR of ATF4 upstream of a Fluc gene was transfected into Vero cells concurrently with a plasmid that constitutively expresses Rluc. Treatment with TM resulted in a respective 4- and 5-fold upregulation of the ATF4 reporter, whereas CHIKV infection for 6 or 16 h did not induce ATF4 reporter activity (Fig. 1d), indicating that the expression of viral glycoproteins and the phosphorylation of eIF2α by CHIKV infection do not effectively stimulate the PERK arm of the UPR.

The IRE1α-XBP1 arm of the UPR has been shown to be activated during infection with other viruses (Ambrose & Mackenzie, 2011; Barry *et al.*, 2010; Isler *et al.*, 2005). To investigate whether CHIKV infection induced XBP1 mRNA splicing, Vero cells were mock infected, infected with CHIKV or treated with TM in a time-course experiment. In uninfected cells, most of the XBP1 mRNA remained in its unspliced form (U) (Fig. 1e, lane 1). When treated with TM, nearly all XBP1 mRNA was spliced (S) (Fig. 1e, lane 2). During CHIKV infection, part of the

XBP1 mRNA was spliced and some remained unspliced between 14 and 22 h p.i. (Fig. 1e, lanes 3–5). When the amount of XBP1(U) mRNA was quantified and normalized for total XBP1(T), the relative amount of XBP1(U) during CHIKV infection was 30–55% of the mock-untreated and TM-treated controls (Fig. 1e). These data suggested that CHIKV infection results in partial XBP1 activation; however, a substantial amount of XBP1 remains in its inactive unspliced form.

### CHIKV infection does not induce XBP1 splicing in mice

Next, we analysed XBP1 mRNA splicing *in vivo* in an adult WT mouse model of CHIKV arthritis. To increase the sensitivity of the XBP1 splicing assay, we designed a semi-quantitative reverse transcriptase PCR assay to measure the relative levels of spliced and unspliced XBP1 mRNA. To validate the XBP1 mRNA splicing assay in murine cells, XBP1 mRNA splicing was measured in murine splenic macrophages and mouse embryonic fibroblasts (MEFs). Treatment with TM resulted in a respective 6- and 12-fold increase in mouse embryonic macrophages and MEFs (Fig. 2a). In the *in vivo* experiment, C57BL/6 mice were either



**Fig. 2.** XBP1 mRNA splicing in murine cells and in a mouse model. (a) Isolated murine splenic macrophages (Mφ) and MEFs were mock treated or TM treated. (b) Mouse feet were infected with CHIKV and harvested at the indicated days p.i. Values are depicted as fold induction of spliced divided by unspliced XBP1 mRNA, normalized to RPL13A and relative to the mock infection. Error bars represent 1 SD. (c) CHIKV RNA copies harvested from infected mouse feet at the indicated days post-inoculation.

mock infected or infected with CHIKV as described previously (Gardner *et al.*, 2010). Tissue samples from mouse feet were collected at the indicated days p.i. and XBP1 mRNA splicing was measured (Fig. 2b). The infected mouse feet displayed all the signs of CHIKV-specific inflammation, with maximum foot swelling at day 6 p.i. and ample CHIKV RNA replication in the first 3 days p.i. (Gardner *et al.*, 2010; Metz *et al.*, 2013; Poo *et al.*, 2014; Rudd *et al.*, 2012) (Fig. 2c and data not shown). However, XBP1 mRNA splicing could not be detected in these tissues (Fig. 2b). Taken together, these results indicated that, although CHIKV infection did induce XBP1 mRNA splicing in Vero cells *in vitro*, XBP1 splicing remained undetectable in mouse feet during an *in vivo* infection.

### CHIKV glycoproteins stimulate the UPR

As we observed signs of induction during the early steps of the UPR but no upregulation of the UPR target proteins in response to CHIKV infection, we determined whether overexpression of the CHIKV envelope proteins outside the context of virus replication activates the UPR. Vero cells were transfected with a plasmid expressing either EGFP or the CHIKV envelope glycoproteins (E3E26KE1) under the control of a cytomegalovirus promoter. The expression of CHIKV envelope glycoproteins was confirmed by immunofluorescence microscopy (Fig. 3a). To measure the effects of the CHIKV envelope glycoproteins on UPR target gene expression and the PERK arm of the UPR, GRP78/BiP-Fluc and ATF4-Fluc reporter plasmids were co-transfected with constitutive Rluc and either an EGFP-expressing plasmid or the plasmid expressing the CHIKV envelope glycoproteins. After 24 h, the overexpression of CHIKV envelope proteins resulted in a moderate but consistent upregulation of both ATF4 and GRP78/BiP

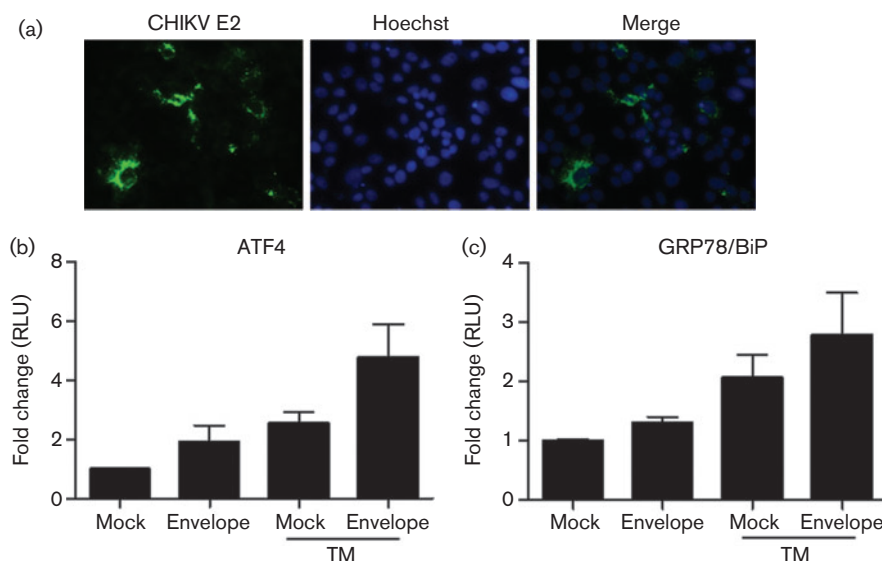
expression (Fig. 3b, c). Treatment with TM at 24 h post-transfection resulted in enhancement of ATF4 and GRP78/BiP expression compared with TM treatment or CHIKV envelope expression alone (Fig. 3b, c). These data suggested that CHIKV envelope protein expression results in UPR activation.

### CHIKV replication prevents effective activation of the UPR

As the expression of viral envelope glycoproteins, in the absence of viral RNA replication and non-structural proteins (nsP1–4), induced the UPR, we investigated whether the lack of UPR activation during CHIKV infection was the result of active inhibition by the virus. Vero cells were either mock infected or infected with CHIKV, and 4, 8 or 12 h later, they were treated with TM (10 h) (Fig. 4a). The UPR target reporters CHOP, GRP94 and GRP78/BiP were induced by TM in the mock-infected cells. However, this induction was reduced in infected cells that were treated with TM at 4 h p.i. and was completely absent in infected cells that were treated with TM at 8 or 12 h p.i. (Fig. 4b). In a similar experiment, TM-mediated induction of ATF4 was also completely inhibited when cells were infected with CHIKV 12 h prior to TM treatment (Fig. 4c).

In a time-course experiment (Fig. 4a), UPR induction using TM resulted in the splicing of almost the entire XBP1 mRNA pool (Fig. 4d, lane 2). Treatment with TM at 4 h p.i. with CHIKV also resulted in the splicing of all XBP1 mRNA (Fig. 4d, lane 3). However, TM treatment at 8 or 12 h after CHIKV infection no longer induced complete XBP1 mRNA splicing (Fig. 4d, lanes 4 and 5). Interestingly, CHIKV infection with (Fig. 4d) and without (Fig. 1d) TM treatment resulted in a gradual increase in the total amount





**Fig. 3.** Overexpression of CHIKV envelope glycoproteins induces the UPR. Vero cells were transfected with plasmids expressing either EGFP (mock) or CHIKV envelope proteins (E2) together with constitutively expressing RLuc and either ATF4–Fluc or GRP78–Fluc reporter plasmids. At 24 h post-transfection, cells were fixed, permeabilized and immunostained for CHIKV E2 (a) or lysed in passive lysis buffer and assayed for dual-luciferase activity (b, c). In (a), nuclei were stained with Hoechst dye; a merged image is shown on the right. Values in (b) and (c) are depicted as means of three individual experiments. Error bars represent 1 SEM. RLU, relative light units.

of unspliced XBP1 mRNA during these experiments. Next, we determined the subcellular localization of XBP1 protein to investigate whether the spliced XBP1 mRNA was translated in the functional XBP1 transcription factor in CHIKV-infected cells. When uninfected Vero cells were treated with TM (6 h) and immunostained for XBP1, XBP1 localized to the nucleus as expected (Fig. 4e). In contrast, during CHIKV infection, XBP1 remained predominantly cytoplasmic, even when the cells were treated with TM (Fig. 4e). Taken together, these results demonstrated that CHIKV infection inhibits the functional expression and/or subcellular localization of transcription factors ATF4 and XBP1 respectively, and effectively prevents the induction of UPR target genes.

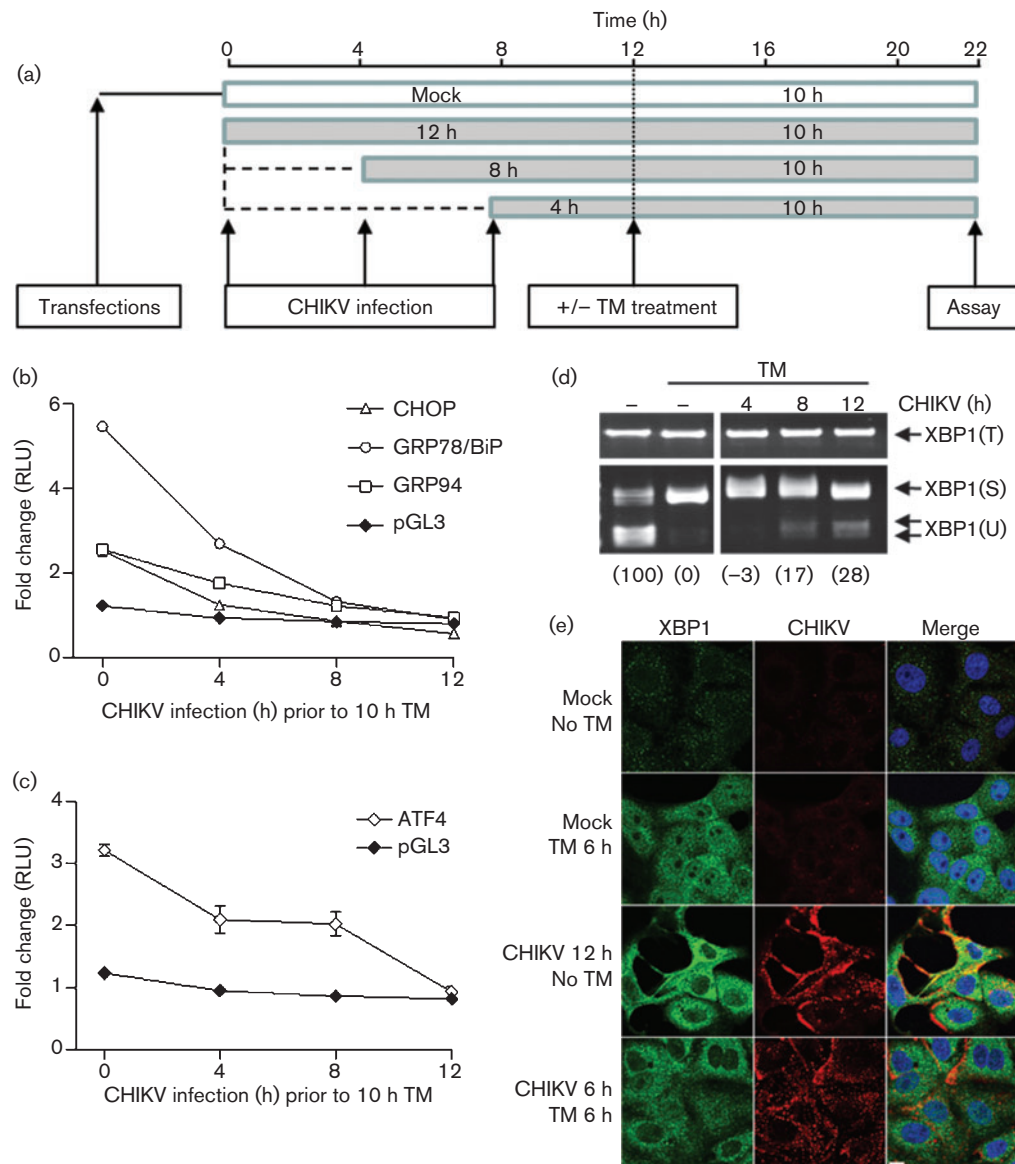
### nsP2 inhibits the upregulation of UPR target genes

Overexpression of CHIKV glycoproteins activated the UPR, but CHIKV infection did not (Figs 1–4), suggesting that one or more of the CHIKV nsPs are able to inhibit activation of the UPR. Plasmids expressing individual CHIKV nsPs (Fros *et al.*, 2010, 2013) were co-transfected with UPR–Fluc reporter plasmids and a plasmid constitutively expressing RLuc. At 16 h post-transfection, the cells were treated with TM. CHIKV nsP2 clearly inhibited the TM-mediated induction of both ATF4 and GRP78/BiP reporters (Fig. 5a, b). As CHIKV nsP2 is a multifunctional protein, with clear roles in host shut-off (Akhrymuk *et al.*, 2012; Bouraï *et al.*, 2012), nsP2 mutants that do not inhibit

cellular protein synthesis were analysed for their ability to inhibit TM-mediated UPR induction. Mutations in the nuclear localization site (KR649) or proline 718, both known to abolish host transcriptional shut-off activity (Fros *et al.*, 2013), rendered nsP2 unable to inhibit TM-induced ATF4 and GRP78/BiP reporter activity (Fig. 5c, d). These data suggested that CHIKV infection inhibits the UPR via nsP2-mediated host shut-off.

## DISCUSSION

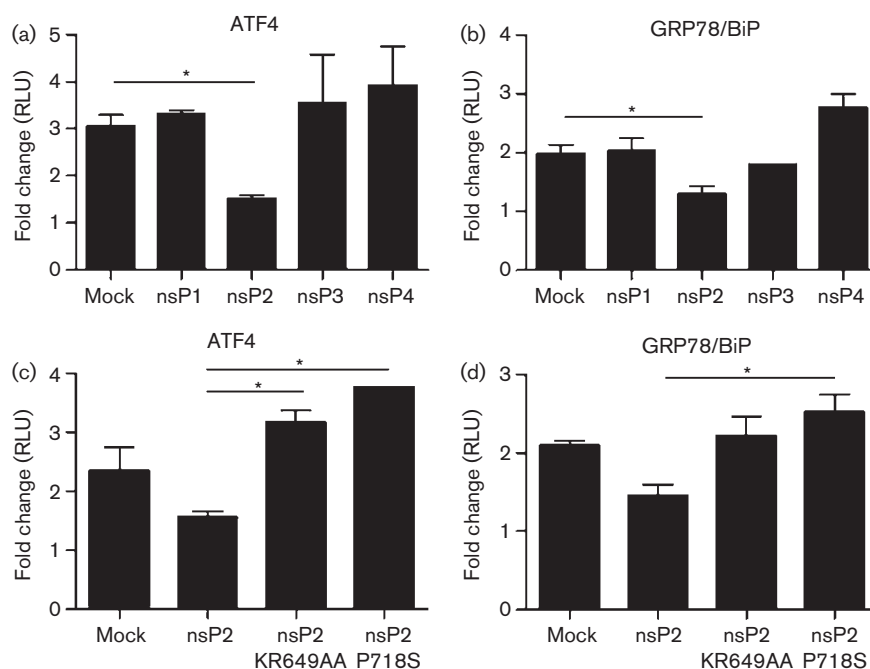
Alphavirus infection has been reported to induce several key components of the UPR. PERK is activated early during Sindbis virus (SINV) and CHIKV infections, and XBP1 mRNA splicing is initiated during SFV infection (Barry *et al.*, 2010; Nivitchanyong *et al.*, 2009; Rathore *et al.*, 2013). Here, we demonstrated that, although eIF2 $\alpha$  is phosphorylated and part of the XBP1 mRNA pool is spliced, XBP1 is not present in the nucleus, and the upregulation of ATF4 and UPR target genes is completely inhibited in CHIKV-infected cells. Transient expression studies of nsPs demonstrated that nsP2 is responsible for preventing an effective UPR. In addition to PERK, eIF2 $\alpha$  can be phosphorylated by a number of other kinases, including PKR, which recognizes (viral) dsRNA (Harding *et al.*, 2002). To allow the translation of viral proteins, most viruses prevent the phosphorylation of eIF2 $\alpha$  (Ambrose & Mackenzie, 2011; Burnett *et al.*, 2012; Groskreutz *et al.*, 2010). Although inhibition of eIF2 $\alpha$  phosphorylation via



**Fig. 4.** CHIKV replication constrains the UPR. (a) Schematic representation of the experiment. (b, c) Vero cells were co-transfected with plasmids constitutively expressing RLuc and UPR-Fluc reporter plasmids. Cells were not infected (mock) or infected for with CHIKV (m.o.i. 10) for 4, 8 or 12 h prior to an additional 10 h of TM (5 µg ml<sup>-1</sup>) stimulation. At the end of the time-course experiment, cells were lysed and luciferase activity was measured. Values are depicted as fold change of Fluc normalized for RLuc relative to the untreated mock. Error bars represent 1 sd. RLU, relative light units. (d) Vero cells were infected with CHIKV (m.o.i. 10) and stimulated with TM in an identical time-course experiment to that in (a). Total RNA was harvested and XBP1 mRNA was reverse transcribed and amplified, digested with *Pst*I and loaded on a 1.5% agarose gel. Values within parentheses show the band intensity of the XBP1(U) normalized for XBP1(T) and relative to mock. (e) Vero cells were either mock infected or infected with CHIKV LS3 (m.o.i. 5) and treated with TM as indicated. Cells were fixed and stained for XBP1 (green) and dsRNA (red) as a marker for CHIKV replication.

CHIKV nsP4 has been reported (Rathore *et al.*, 2013), we and others have found that alphaviruses are exceptional in allowing the phosphorylation of eIF2 $\alpha$  during productive infection (Gorchakov *et al.*, 2004; Nivitchanyong *et al.*, 2009; Ventoso *et al.*, 2006; White *et al.*, 2011). In our experiments, CHIKV infection resulted in the phosphorylation of

eIF2 $\alpha$  within 8 h p.i. (Fig. 1c). The translation of alphaviral structural proteins from their subgenomic messenger is unaffected by the phosphorylation of eIF2 $\alpha$ . A stable hairpin loop structure in the 26S promoter of the subgenomic mRNA from SINV was shown to stall the ribosome on the correct AUG, providing resistance to eIF2 $\alpha$  phosphorylation



**Fig. 5.** CHIKV nsP2-induced host shut-off inhibits the UPR. Vero cells were co-transfected with plasmids expressing individual CHIKV nsPs (a, b) and mutants thereof (c, d), UPR–Fluc reporter plasmids and a plasmid constitutively expressing Rluc. At 18 h post-transfection, cells were either induced with TM (6 h, 5  $\mu\text{g ml}^{-1}$ ) or mock treated and lysed, and luciferase activity was measured. Values are depicted as the mean of two to four independent experiments, relative to the corresponding non-induced samples. Error bars represent 1 SEM and an asterisk indicates a significant difference ( $P < 0.05$ ). RLU, relative light units.

(Ventoso *et al.*, 2006). Surprisingly, the translational shut-off during alphavirus infection has been shown to be independent of eIF2 $\alpha$  phosphorylation, indicative of an additional mechanism by which alphaviruses modulate the translational machinery of the host (Gorchakov *et al.*, 2004; White *et al.*, 2011). In agreement with this hypothesis, the phosphorylation of eIF2 $\alpha$  during CHIKV infection did not result in an increased expression of the eIF2 $\alpha$  phosphorylation-insensitive transcription factor ATF4 (Fig. 1d), nor did it upregulate the induction of the known UPR target genes GRP78/BiP, CHOP and GRP94 (Fig. 1a, b). In fact, when the UPR was induced with TM in the context of a CHIKV infection, the expression of UPR target gene reporters and transcription factor ATF4 was clearly inhibited (Fig. 4b–d).

SFV replication has been shown to induce the UPR only when the envelope proteins are expressed during RNA replication (Barry *et al.*, 2010), an observation consistent with our results showing the ability of CHIKV envelope proteins to induce GRP78/BiP and ATF4. The addition of TM to cells expressing the CHIKV envelope proteins resulted in enhanced induction of the UPR (Fig. 3b, c). These results indicate that CHIKV envelope proteins expressed outside the context of viral infection do induce the UPR, with the presence of these glycoproteins markedly increasing TM-mediated activation of the UPR. However, no stimulation of the UPR target genes was observed during CHIKV replication, not even when cells

were treated with TM (Figs 1 and 4). This clearly indicates that, despite the ability of the envelope proteins to activate the UPR, the induction of transcription factor ATF4 and UPR target genes is effectively inhibited during CHIKV infection.

Previous studies have shown that the IRE1 $\alpha$ –XBP1 arm of the UPR is activated during SFV infection (Barry *et al.*, 2010). In agreement with this finding, our *in vitro* experiments did show a moderate level of XBP1 mRNA splicing during CHIKV infection (Fig. 1e). A time-course experiment (Fig. 4d) revealed that at 4 h p.i., TM induction induced complete XBP1 mRNA splicing. Interestingly, XBP1 mRNA splicing upon TM induction at 8 h after CHIKV infection was partly inhibited. In addition, CHIKV infection of mice did not induce detectable levels of XBP1 mRNA splicing (Fig. 2b). Thus, although CHIKV infection activates the IRE1 $\alpha$ –XBP1 arm of the UPR in certain cell types, progressive CHIKV infection does severely limit the extent to which XBP1 mRNA is spliced. Immunostaining of XBP1 protein at 12 h after CHIKV infection showed that the nuclear accumulation of XBP1 was completely inhibited, even when cells were treated with TM (Fig. 4e). These data illustrate that, although some XBP1 mRNA splicing is induced by CHIKV infection in certain cell types, XBP1 proteins are absent from the nucleus of an infected cell.

Using a combination of independent PCR-based assays, dual-luciferase reporter systems, immunofluorescence and

Western blotting, we have shown that the IRE1 $\alpha$ -XBP1 and PERK arms of the UPR are effectively suppressed by CHIKV infection. Expression of individual CHIKV nsPs revealed that nsP2 is responsible for the inhibition of ATF4 and UPR target gene induction (Fig. 5a, b). nsP2 is a multifunctional protein with an active protease and helicase domain, and NTPase activity (Bourai *et al.*, 2012; Das *et al.*, 2014; Strauss & Strauss, 1994). In addition, it causes host-cell transcriptional shut-off via the degradation of a catalytic subunit of RNA polymerase II (Akhrymuk *et al.*, 2012), and also inhibits the JAK-STAT signalling pathway of the IFN response (Fros *et al.*, 2010, 2013). Here, we showed that point mutations in nsP2 that render the protein non-cytopathic by eliminating its function in host shut-off (KR649AA or P718S) (Akhrymuk *et al.*, 2012; Fros *et al.*, 2013) reversed the nsP2-mediated inhibition of the UPR (Fig. 5c, d). Alphaviruses carrying these mutations at homologous sites are effectively attenuated in cell culture (Dryga *et al.*, 1997; Tamm *et al.*, 2008), perhaps in part due to a failure to constrain the UPR. It would be informative to investigate how CHIKV replication and the UPR influence one another in light of these attenuating mutations. We postulate that the host-cell shut-off, which is governed by CHIKV nsP2, is responsible for the inhibition of the UPR, by preventing the upregulated expression of ATF4, active XBP1 and additional UPR target genes.

The diminished UPR activation during CHIKV infection suggests that antiviral effects, elicited by the UPR, could hamper CHIKV propagation. This provides a rationale for why CHIKV-induced host shut-off has evolved to also inhibit the UPR. In addition, immune responses directed against viral infections can be augmented by the UPR (reviewed by Smith, 2014). Replication of several RNA viruses, including SINV, was shown to be inhibited by a small-molecule deubiquitinase inhibitor that functions via activation of the UPR (Gonzalez-Hernandez *et al.*, 2014; Perry *et al.*, 2012). This indicates that alphaviruses are sensitive to the effects of the UPR and that activating the UPR may have potential as an antiviral strategy.

## METHODS

**Ethics statement.** All animals were handled in strict accordance with good animal practice as defined by the National Health and Medical Research Council of Australia. All animal work was approved by the QIMR Berghofer Medical Research Institute animal ethics committee.

**Cells and virus isolates.** Vero cells (ATCC CRL-1586) and MEFs from C57 BL/6 mice were cultured in RPMI 1640 (Gibco, Invitrogen) supplemented with 5% FCS at 37 °C and 5% CO<sub>2</sub>. Murine splenic macrophages were obtained by homogenizing a spleen in RPMI 1640 with 10% FCS. Tissue was pelleted and resuspended in 3 ml ACK lysis solution (150 mM NH<sub>4</sub>Cl, 1 mM Na<sub>2</sub>-EDTA, pH 7.3) for 3 min to remove any erythrocytes. Cells were washed and pelleted twice with RPMI 1640 with 10% FCS, before being plated and left to attach overnight. Medium was replaced by RPMI 1640 with 10% FCS containing 30% Langerhans cell conditioned medium (LCCM). Cells

were left to incubate for 3 days before being washed again with RPMI 1640 with 10% FCS and 30% LCCM. The CHIKV isolate (LR2006-OPY1) is a primary isolate from the recent outbreak in Reunion Island (Parola *et al.*, 2006), and CHIKV LS3 (Scholte *et al.*, 2013) is an infectious clone-derived virus.

**Reagents.** The UPR reporter constructs consisted of a pGL3 basic backbone with the promoter region of either GRP78/BiP [pGL3 basic GRP78P(−132)–luc] or GRP94 [pGL3 basic GRP94P(−363)–luc] or CHOP [pGL3 basic CHOP-luc] (kind gift from Dr K Mori, HSP Research Institute, Kyoto, Japan), or ATF-4 [CHOP11/cATF] (Addgene, Cambridge, USA). CHIKV (S27) envelope cassette was expressed from a pcDNA/DEST40 backbone (pcDNA-envelope) and the plasmids expressing individual nsPs have been described previously (Fros *et al.*, 2010, 2013). GeneJammer (Agilent Technologies) and Lipofectamine 2000 (Invitrogen) were used as transfection reagents, and TM (Sigma-Aldrich) was used to activate the UPR at a concentration of 5  $\mu$ g ml<sup>−1</sup>.

**XBP1 mRNA splicing.** Activation occurs via the removal of a 26 bp intron from XBP1 mRNA, which contains a *Pst*I restriction site. Total RNA extraction from cell cultures was performed using Trizol reagent (Invitrogen). Female C57BL/6 mice were inoculated with CHIKV LR2006-OPY1 (10<sup>4</sup> cell culture ID<sub>50</sub>) subcutaneously into the ventral side of each hind foot as described previously (Gardner *et al.*, 2010). For the extraction of total RNA from mouse feet, each foot was placed in a snaplock Eppendorf tube with 1.5 ml Trizol reagent and two 3 mm tungsten carbide beads (Qiagen). Tissue was homogenized at 25 Hz in a tissue lyser (Qiagen). Samples were centrifuged for 10 min at 12 000 r.p.m. at 4 °C using a small Eppendorf 5415 centrifuge with a 24 position rotor. The supernatant (1 ml) was used for further Trizol RNA extraction. To remove genomic DNA, all RNA samples were DNase treated (TURBO DNA-free; Applied Biosystems). The RNA from murine splenic macrophages and mouse feet was reverse transcribed using random primers. Murine RPL13A, spliced and unspliced XBP1, and CHIKV RNA were quantified by real-time PCR using platinum SYBR Green (Invitrogen), using a Rotor Gene RG-3000 (Corbett Research). Primers used in this assay were mXBP1 Fwd, mXBP1 U Rev and mXBP1 S Rev with the respective sequences: 5'-AAACAGAGTAGCAGCGCAGACTGC-3', 5'-GCTGCAGAGGTG-CACATAGTCTGA-3' and 5'-GCCTGCACCTGCTGCGGACTC-3'. Murine RPL13A was used as an internal control, with primers mRPL13A Fwd, 5'-GAGGTCGGGTGGAAGTACCA-3', and mRPL13A Rev, 5'-TGCATCTTGGCCTTTTCCTT-3'. CHIKV RNA was detected using primers 5'-AGCTCCGCGTCCTTTACC-3' (forward) and 5'-CAAATTGTCCTGCTTCTCTG-3' (reverse). mRNA from Vero cells was reverse transcribed (Superscript III; Invitrogen) using random hexamers (Roche), and XBP1 was PCR amplified using primers XBP1 F, 5'-CCGGAGCTGGGTATCTCAAAT-3', and XBP1 R, 5'-CCGTATC-CACAGTCACTGTAGCA-3'. Amplicons were digested with *Pst*I and loaded on an agarose gel for analysis (Ambrose & Mackenzie, 2011).

**Dual-luciferase assay.** One day after transfection with pGL-3 (Fluc) or the respective UPR reporter plasmids with pRL-SV40 (Rluc), Vero cells were infected and/or TM treated as indicated. At the end point of each experiment, cells were lysed in passive lysis buffer (Promega) and cellular debris was pelleted by centrifugation (6 min, 5000 r.p.m. Eppendorf 5415 centrifuge, as above). Fluc and Rluc luminescence was measured using a dual-luciferase reporter assay system (Promega). Supernatants (25–50  $\mu$ l) were transferred into a white Greiner F-bottom 96-well plate and scanned for luciferase luminescence in a POLARstar OPTIMA (BMG Labtech) plate reader. Significant differences ( $P < 0.05$ ) between two samples were tested using Student's *t*-test and significant differences between multiple samples with a Tukey honestly significant difference (HSD) test.

**Immunostaining.** Protein expression of CHIKV E2 and eIF2 $\alpha$  were analysed by SDS-PAGE. Mock-infected and infected cells were washed



once with PBS and lysed in RIPA buffer [PBS with 1 % NP-40, 0.5 % sodium deoxycholate and 0.1 % SDS, supplemented with Complete Protease Inhibitor Cocktail (Roche), 1 mM NaF and 1 mM Na<sub>3</sub>VO<sub>4</sub>] and clarified by centrifugation for 10 min at 13 000 r.p.m using an Eppendorf 5415 centrifuge, as above. After electrophoresis, denatured proteins were transferred to an Immobilon membrane (Millipore) for analysis by Western blotting. Membranes were blocked in 3 % skimmed milk in PBS with 0.05 % Tween 60 (PBST) for 1 h at room temperature. Membranes were washed three times for 5 min each with PBST and subsequently incubated for 1 h at room temperature with rabbit polyclonal anti-E2 (diluted 1:20 000; Metz *et al.*, 2011), anti-P-eIF2 $\alpha$  (diluted 1:500; Abcam) and anti- $\beta$ -tubulin (diluted 1:4000; Abcam) in PBST, respectively. Membranes were washed and treated with alkaline phosphatase-conjugated goat anti-rabbit IgG mAb (Sigma), diluted 1:3000 in PBST, for 45 min at room temperature. Membranes were washed twice for 5 min each with PBST and once for 10 min with AP buffer [100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris/HCl (pH 9.5), 0.1 % Tween 20]. Proteins were detected by nitro blue tetrazolium chloride/BCIP staining (Roche).

To determine the expression of CHIKV E2, Vero cells were transfected with pcDNA-envelope. After 24 h, the cells were washed with PBS and fixed with 4 % paraformaldehyde in PBS for 10 min at room temperature. The cells were washed twice with PBS and permeabilized with 0.1 % SDS in PBS for 10 min at room temperature. Samples were washed and incubated with PBS containing 5 % FBS and 1:5000-diluted rabbit anti-E2 polyclonal antibody, for 1 h at room temperature. Cells were washed three times with PBS and treated with 1:2000-diluted goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 h at room temperature. Finally, cells were washed and treated with 10  $\mu$ g Hoechst stain ml<sup>-1</sup> for 5 min at room temperature. Cells were analysed using a Zeiss Axio Observer Z1 m inverted microscope in combination with an X-Cite 120 series lamp.

The subcellular localization of XBP1 was determined with indirect immunofluorescence microscopy as described previously (Scholte *et al.*, 2013). dsRNA was detected using mouse mAb J2 (English & Scientific Consulting). Rabbit polyclonal antibody was used to detect XBP-1 (Santa Cruz Biotechnology). Primary antibodies were detected with Cy3- or Alexa Fluor 488-conjugated secondary antibodies (Jackson/Life technologies). Nuclei were visualized with Hoechst 33342. The coverslips were analysed using a Leica TCS SP5 confocal microscope and LAS AF Lite software (Leica).

## ACKNOWLEDGEMENTS

We thank Corinne Geertsema for technical support. This work is supported by the European 195 Community's Seventh Framework Programme (FP7 VECTORIE project number 261466) and NHMRC Australia, grant APP613622. AS and GPP should be considered joint last others.

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