Quantitative proteomic analysis reveals unfolded protein response involved in severe fever with thrombocytopenia syndrome virus infection

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Abstract
Severe fever with thrombocytopenia syndrome (SFTS) is an emerging, highly pathogenic, infectious disease caused by infection with a newly discovered tick-borne phlebovirus, SFTS virus (SFTSV). Limited information on the molecular mechanism of SFTSV infection and pathogenesis impedes the development of effective vaccines and drugs for SFTS prevention and treatment. In this study, an isobaric tag for relative and absolute quantification (iTRAQ)-based quantitative proteomic analysis of SFTSV-infected HEK 293 cells was performed to explore dynamic host cellular protein responses towards SFTSV infection. A total of 433 out of 5,606 host proteins involved in different biological processes were differentially regulated by SFTSV infection. The proteomic results highlighted a potential role of endoplasmic reticular stress-triggered unfolded protein response (UPR) in SFTSV infection. Further functional studies confirmed that all three major branches of the UPR, including the PRKR-like endoplasmic reticulum kinase (PERK), the activating transcription factor-6 (ATF6) and the inositol-requiring protein-1 (IRE1)-X-box-binding protein 1 (XBP1) pathways, were activated by SFTSV. However, only the former two pathways play a crucial role in SFTSV infection. Furthermore, expression of SFTSV glycoprotein (GP) alone was sufficient to stimulate the UPR, while suppression of PERK and ATF6 notably decreased GP expression. Interestingly, two other newly discovered phleboviruses, Heartland virus (HRTV) and Guertu virus (GTV), also stimulated the UPR, suggesting a common mechanism shared by these genetically related phleboviruses. This study provides a global view to our knowledge on how host cells respond to SFTSV infection and highlights that host cell UPR plays an important role in phlebovirus infection.
Importance

Severe fever with thrombocytopenia syndrome virus (SFTSV) is an emerging tick-borne bunyavirus that causes severe fever with thrombocytopenia syndrome in humans, with a mortality rate reaching up to 30% in some outbreaks. There are currently no FDA-approved vaccines or specific antivirals available against SFTSV. To comprehensively understand the molecular interactions occurring between SFTSV and the host cell, we exploit quantitative proteomic approach to investigate the dynamic host cellular responses to SFTSV infection. The results highlight multiple biological processes being regulated by SFTSV infection. Among these, we focused on exploration of the mechanism of how SFTSV infection stimulates the host cell’s unfolded protein response (UPR) and identified the UPR as a common feature shared by SFTSV-related new emerging phleboviruses. This study, for the first time to our knowledge, provides a global map for host cellular responses to SFTSV infection and highlighted potential host targets for further research.
Introduction

Severe fever with thrombocytopenia syndrome virus (SFTSV) is an emerging tick-borne virus that causes severe fever with thrombocytopenia syndrome (SFTS). Since the first report in China in 2009, SFTSV has spread over China, South Korea and Japan, with a mortality rate reaching up to 30% (1-4). SFTSV is a novel member of the genus Phlebovirus, family Phenuiviridae, order Bunyavirales (https://talk.ictvonline.org/taxonomy). Recently, a succession of other novel emerging phleboviruses that are closely related to SFTSV, including Heartland virus (HRTV, identified in USA in 2012) (5), Hunter island group virus (HRGV, identified in Australia in 2014) (6) and Guertu virus (GTV, identified in China in 2018) (7), have been reported, highlighting their potential threats to public health. Currently, there are no therapeutics or US Food and Drug Administration (FDA)-approved vaccines to combat infections of SFTSV and these related viruses.

SFTSV is an enveloped virus with a tripartite, single-stranded, negative-sense RNA genome comprising large (L), middle (M) and small (S) segments. The L segment encodes an RNA-dependent RNA polymerase (RdRP), while the M segment encodes glycoproteins Gn and Gc, which form a heterodimer on the surface of the virus particle to mediate viral entry and egress. The S segment employs an ambisense strategy to encode nucleoprotein (NP) and nonstructural protein (NSs). SFTSV infection is initiated by virus binding to cell attachment factors, including C-type lectins and nonmuscle myosin heavy chain IIA, followed by internalization of virions into clathrin-mediated endocytosis (8). After the release of viral ribonucleoprotein in the cytoplasm, replication and transcription of viral genomes start. The assembly and release of SFTSV progeny virions occur at the Golgi apparatus and Golgi-derived
vesicles. To establish successful infection, SFTSV must manipulate host proteins to favor its own replication. However, there lacks a comprehensive understanding of the molecular interactions occurring between SFTSV and host cells (9).

Virus infection induces different stress responses in host cells. The endoplasmic reticulum (ER) stress response is a highly conserved mechanism that may arise from accumulation of misfolded or unfolded proteins, depletion of ER membranes for virus assembly and release, competition with host proteins for modifications by viral glycoproteins, etc. (10). To relieve ER stress and re-establish protein folding homeostasis, a series of intracellular protein quality control signaling pathways known as the unfolded protein response (UPR) are activated. The UPR induces cellular transcriptional and translational responses, resulting in global inhibition of protein synthesis to reduce protein overload, up-regulation of molecular chaperones to promote protein folding, as well as activation of ER-associated degradation (ERAD) to eliminate unfolded proteins from the ER (11). The UPR is regulated by three main signaling branches, namely the PRKR-like endoplasmic reticulum kinase (PERK)(12), the activating transcription factor-6 (ATF6) (13) and the inositol-requiring protein-1 (IRE1)-X-box-binding protein 1 (XBP1) (14) pathways. Many viruses, including both enveloped viruses (herpesviruses, flaviviruses, coronaviruses, arenaviruses, etc.) and non-enveloped viruses (coxsackievirus) can trigger ER stress and the UPR during their infections. In many cases, activation of the UPR is required for efficient virus replication (15, 16). For example, the arenavirus lymphocytic choriomeningitis virus, which is also a negative-sense, single-stranded RNA virus with a segmented genome, activates the ATF6 pathway for optimal virus multiplication during acute infection (17). In contrast, a recent report showed that an alphacoronavirus transmissible
gastroenteritis virus (TGEV) infection induced ER stress and triggered the UPR, and ER stress negatively regulated TGEV replication (18). Activation of the UPR also contributes to virus pathogenesis. Another recent study indicated that Zika virus (ZIKV) infection triggered the UPR in the cerebral cortex of infected postmortem human fetuses, which disturbed normal neurogenesis and contributed to ZIKV-associated microcephaly (19). It has been reported that Tula hantavirus triggers pro-apoptotic signals of ER stress in Vero E6 cells (20). However, the detailed interactions between bunyaviruses and three main signaling branches of the UPR have not been reported yet.

In this study, to systematically identify host proteins involved in SFTSV-host interactions, the dynamic host cellular responses to SFTSV infection were investigated by isobaric tag for relative and absolute quantification (iTRAQ)-based quantitative proteomic analysis. iTRAQ is an isobaric labeling method employed in quantitative proteomics by tandem mass spectrometry for the identification and quantitation of proteins from different sources in a single experiment (20, 21). Human embryonic kidney (HEK 293) 293 cells are highly permissive to SFTSV infection and in vivo model showed that SFTSV replicated and caused pathological changes or lesions in kidney cells in mice and macaques (22, 23). In addition, a wide variety of functional studies of SFTSV have been performed in this cell line, we decided to choose HEK 293 cells for proteomic study (7, 24, 25). Our results provide a global map showing how host cells respond to SFTSV infection and highlight multiple biological processes being regulated by SFTSV infection. Among these, we focused on exploration of the mechanism of how SFTSV infection stimulates host cell UPR and, in turn, how the three classical pathways of UPR affect SFTSV infection.
Results

Global host cellular protein responses to SFTSV infection revealed by quantitative proteomic analysis

Before quantitative proteomic analysis, the growth kinetics of SFTSV in HEK 293 cells was monitored by measuring viral titer. Briefly, HEK 293 cells were infected with SFTSV at a multiplicity of infectivity (MOI) of 5 and the viral titers were measured with end point dilution assays (EPDAs). As shown in Fig. 1A, the replication rate of SFTSV increased over the time from 6 to 48 h post-infection (h p.i.), while after 48 h p.i., the replication of SFTSV entered into stationary phase. The highest virus titer could reach to $\sim 1 \times 10^8$ tissue culture infectious dose 50 (TCID$_{50}$) units/cell at 48 h p.i., which is consistent with a recent report (7). MTT assay showed that SFTSV had no significant effect on cell viability before 48 h p.i. (Fig. 1B). Therefore, samples collected at 6, 12, 24 and 48 h p.i. were used for iTRAQ-based quantitative proteomic analysis (Fig. 1B). As illustrated in Fig. 1C, the extracted proteins from SFTSV infected- or mock infected cells were subjected to trypsin digestion, and further labeled with different iTRAQ reagents. Then peptides were mixed at a ratio of 1, subjected to SCX fractionation and LC-MS/MS analysis. Three independent biological replicates were performed at all the four time points.

As a result, a total of 5,606 host proteins were quantified (Table S1). The ratio distributions of all quantified proteins were profiled (Fig. 1D). Among the identified host proteins, 25 were up-regulated and 33 were down-regulated at 6 h p.i.; 23 were up-regulated and 34 were down-regulated at 12 h p.i.; 26 were up-regulated and 37 were down-regulated at 24 h p.i.; 282 were up-regulated and 19 were down-regulated...
at 48 h p.i. Differentially expressed proteins were significantly enriched at 48 h p.i., suggesting that SFTSV replication had a profound impact on host cells at this time point. Further examination of these proteins indicated that 26 host proteins were differentially regulated at two time points. Seven host proteins were differentially regulated at three time points, including Cytochrome c oxidase assembly protein COX16 homolog (COX16), FUN14 domain-containing protein 1 (FUNDCl), E3 ubiquitin-protein ligase TRIM21 (TRIM21), Vacuolar protein sorting-associated protein 13D (VPS13D), Endoplasmic reticulum chaperone BiP (GRP78), Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2), and Zinc finger and BTB domain-containing protein 7A (ZBTB7A), while fibronectin (FN1) was up-regulated at all the four time points (Table S2). To find out the possible relationship between virus infection with host cellular responses, we further determined virus infection ratio and the expression patterns of the major viral proteins (the nucleocapsid protein (NP) and the glycoprotein (GP)). Approximately 49.98%, 94.37%, 99.96% and 100% cells were infected (Fig. 1E, the grey columns) and approximately 3, 7, 8 and 10-fold increase in NP and GP protein levels (Fig. 1E, the blue and red lines) at 6, 12, 24, and 48 h p.i., respectively. Therefore, compared to the kinetics of virus infection and viral protein expression, host protein changes to SFTSV infection seemed to be delayed and modest.

To validate our MS data, quantitative RT-PCR analysis to determine the transcription levels of eight randomly selected host proteins was performed. Although the fold changes quantified by quantitative RT-PCR and MS were not identical, their change tendencies were similar. As shown in Fig. 1F, quantitative RT-PCR data indicated that genes encoding FN1, Golgi integral membrane protein 4 (GOLIM), transcription
factor AP-1 (JUN), E3 ubiquitin-protein ligase RNF25 (RNF25), transcriptional enhancer factor TEF-1 (TEAD1), TSC22 domain family protein 4 (TSC22D4) and vesicle-associated membrane protein-associated protein A (VAPA) were up-regulated, while the gene encoding serine/threonine-protein kinase RIO2 (RIOK2) was first down-regulated and then recovered as a result of SFTSV infection, which was consistent with our MS data (Table S1).

To determine which biological processes were regulated during the SFTSV infection process, gene ontology (GO) analysis was performed. As shown in Fig. 2, at 6 h p.i., only the process “response to wounding” was over-represented (Fig. 2A), while at 12 h p.i., “positive regulation of fibroblast proliferation”, “protein retention in Golgi apparatus” and “innate immune response” were over-represented (Fig. 2B). At 24 h p.i., five biological processes were found to be up-regulated and two of them (“ATF6-mediated unfold protein response” and “positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress”) belong to ER-stress response (Fig. 2C). More biological processes were over-represented at 48 h p.i., and again “response to endoplasmic reticulum stress” was up-regulated and six hits from the category are significantly enriched (Fig. 2D).

These data revealed a prominent cluster of up-regulated host genes linked to endoplasmic reticulum stress (ER)-stress and highlighted that ER stress response was triggered by SFTSV infection. Since the role of the UPR in bunyavirus life cycle hasn’t been thoroughly depicted, we therefore decided to perform an in-depth analysis on this biological process.
UPR is activated by SFTSV infection

One consequence of activating ER stress is the up-regulation of genes involved in protein folding, such as ER chaperones, as represented by GRP78 and glucose related protein 94 (GRP94), and isomerases, including protein disulfide isomerase A3 and A4 (PDIA3 and PDIA4). Therefore, Western blot and quantitative RT-PCR were performed to determine whether these ER chaperones were regulated during SFTSV infection. As shown in Fig. 3A, intracellular protein levels of GRP78 were up-regulated at 12, 24 and 48 h p.i., while as a control the intracellular protein levels of a cytosolic chaperone, HSP90AB1, was not up-regulated. We further found that mRNA levels of GRP78/GRP94 as well as PDIA3/PDIA4 increased after SFTSV infection (Fig. 3B), confirming that ER stress was activated by SFTSV infection in HEK 293 cells. To better reflect the situation of virus infection in vivo, mouse peripheral blood mononuclear cells (PBMCs) were infected with SFTSV at an MOI of 5 and subjected to quantitative RT-PCR analysis. We found intracellular mRNA levels of GRP78, GRP94, PDIA3 and PDIA4 elevated significantly in SFTSV infected PBMCs, indicating that ER stress response was activated as a result of SFTSV infection in PBMCs (Fig. 3B).

UPR is a cellular adaptive response for restoring ER homeostasis in response to ER stress (11). Here, we detected the effects of SFTSV infection on three branches of the UPR, including the PERK, ATF6 and IRE1 pathways. PERK is an ER-localized kinase whose lumenal domain senses an excess of unfolded proteins that enter the ER (26). Here, Western blot analysis of HEK 293 cell extracts indicated PERK was phosphorylated at 48 h p.i. in SFTSV-infected cells (Fig. 3C), suggesting that PERK
was activated. Activated PERK will further phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF2α), and thus attenuate global protein synthesis. We therefore investigated the phosphorylation state of eIF2α during SFTSV infection over a 48-h time course, and found a significant increase in the levels of phosphorylated eIF2α, but not the total eIF2α proteins at 48 h p.i. in SFTSV-infected cells compared with the mock-infected group (Fig. 3C), suggesting that the PERK pathway is activated upon SFTSV infection. Next, the activation of the ATF6 signaling pathway during SFTSV infection was examined. ATF6 is constitutively expressed as a 90 kDa protein (namely ATF6 p90), and upon ER stress, ATF6 is cleaved to an N-terminal 50 kDa protein (namely ATF6 p50). Western blot analysis showed that ATF6 was decreased at 48 h p.i. (Fig. 3C), suggesting that the ATF6 signaling pathway was activated at the late phase of SFTSV infection. Viral glycoprotein (GP) was used to indicate successful infection (Fig. 3C). Finally, the activation of the IRE1-XBP1 pathway was examined. Activation of IRE1 causes post-transcriptional cleavage of the XBP1 mRNA (unspliced XBP1) that produces the spliced form of XBP1 mRNA, which encodes the transcriptionally active form of the XBP1 gene. Similarly, HEK 293 cells were mock treated or infected with SFTSV over a 48-h time course. Both unspliced XBP1 and spliced XBP1 were amplified by RT-PCR. As shown in Fig. 3D, spliced XBP1 was detected at 24 and 48 h p.i. in SFTSV-infected cells but not in mock-infected cells, suggesting that the IRE1 pathway was also activated by SFTSV at these time points. The above results demonstrated that SFTSV infection could activate all three classical branches of the UPR.

PERK and ATF6 signaling pathways play critical roles in SFTSV infection processes
The up-regulation of the UPR in SFTSV-infected cells implied that the UPR might play a role in viral replication. Thus, we explored the roles of three branches of the UPR in the SFTSV replication process by RNA interference (RNAi). Briefly, HEK 293 cells were transfected with small interfering RNA (siRNA) targeting PERK, ATF6 and XBP1, or scrambled siRNA, and the knockdown efficiency was detected at 24 h post-transfection (p.t.) by quantitative RT-PCR (Fig. 4A). All siRNAs used could reduce intracellular mRNA levels of XBP1, ATF6 and PERK significantly without cytopathic effects (Fig. 4A and B). Twenty-four hours after siRNA transfection, cells were infected with SFTSV at an MOI of 1 and were also collected at 48 h p.i. Quantitative RT-PCR results showed that the intracellular levels of viral RNA (L and M segment) were lower in ATF6- or PERK-knockdown cells, but not in XBP1-knockdown cells (Fig. 4C), indicating that depletion of ATF6 or PERK inhibited SFTSV replication. The supernatant was also collected at 48 h p.i., and viral titers were determined. As shown in Fig. 4D, SFTSV titers were significantly decreased in ATF6- or PERK-knockdown cells, but not in XBP1 silenced cells, which is similar to the result of Fig. 3C. The above results indicated that two of the three main branches of the UPR, the ATF6 and PERK pathways, facilitated SFTSV infection process.

Expression of SFTSV GP up-regulates the UPR

To explore how SFTSV triggers the UPR, the effect of expression of individual SFTSV proteins on the UPR was examined. HEK 293 cells were transfected with empty vector, or vectors expressing different SFTSV proteins or GFP as a control. At 48 h p.t., the expression of viral proteins and GFP in HEK 293 cells was confirmed by Western blots (Fig. 5A). Then, the intracellular protein levels of GRP78 and GRP94
were further determined with Western blot analysis. As shown in Fig. 5A, GRP78 and
GRP94 increased only in cells expressing GP (the full length glycoprotein, containing
both Gn and Gc) or cells treated with tunicamycin (Tm), a reported inducer of the
UPR, but not in cells expressing other viral proteins, including NP, NSs and RdRP, or
the control GFP. We also detected intracellular mRNA levels of *GRP78* and *GRP94* in
HEK 293 cells by quantitative RT-PCR. As shown in Fig. 5B, intracellular mRNA
levels of *GRP78* and *GRP94* were significantly elevated in GP-expressing cells. We
further found that compared to other viral proteins or empty vector control, GP could
activate the expression of GRP78 in a dose-dependent manner (Fig. 5C). The above
results suggested that the SFTSV GP alone can activate the UPR.

**PERK and ATF6 signaling pathways are important for maintaining intracellular
levels of GP**

We further investigated the roles of the three major UPR signaling pathways in
determining the expression levels of individual viral proteins. In the case of SFTSV
infection, intracellular expression levels of NP, NSs, RdRP and GP were lower in
PERK- or ATF6-knockdown cells but not in XBP1-knockdown cells (Fig. 6A), which
was in accordance with the above results that knockdown of ATF6 or PERK led to
reduced virus replication and production (Fig. 4B and C). Moreover, the intracellular
protein levels of GP decreased much more significantly than those of other viral
proteins when the ATF6 or PERK signaling pathways were impaired (Fig. 6A),
suggesting that GP was specifically down-regulated. To further investigate the impact
of the ATF6 and PERK pathways on GP expression, HEK 293 cells were firstly
transfected with target siRNAs or scrambled siRNA, and then transfected with
plasmids expressing GP or NSs as a control at 24 h post siRNA transfection.
Forty-eight hours post plasmid transfection, HEK 293 cells were collected and the intracellular levels of the two viral proteins were detected by Western blots. As shown in Fig. 6B, knockdown of ATF6 or PERK resulted in decreased protein levels of intracellular GP, but not NSs. In contrast, neither GP nor NSs expression was influenced by knockdown of XBP1. This result further confirmed that the proper expression of GP either in the condition of SFTSV infection or alone is dependent on the ATF6 and PERK pathways.

**UPR signaling is also activated by infection with other closely related phleboviruses**

We next explored whether activation of the UPR is a unique feature for SFTSV infection or a common mechanism shared by some genetically closely related phleboviruses. As mentioned earlier, HRTV and GTV are two newly identified tick-borne phleboviruses and are phylogenetically closely related to SFTSV (5, 7). We investigated the UPR responses in HEK 293 cells, which are also permissive to both HRTV and GTV infection (Fig. 7A) (7). The infection rates represented by the expression of NP (Fig. 7A) and the virus titers at different time points (Fig. 7B) showed that among the three viruses, the infectivity of GTV is the highest, while HTRV is the lowest in HEK 293 cells (P < 0.001). In GTV-infected HEK 293 cells, GRP78 was up-regulated, suggesting that the UPR was activated (Fig. 7C). We found that decrease of ATF6 p90 protein (Fig. 7C) and splicing of XBP1 mRNA began at 12 h p.i. (Fig. 7E), while the phosphorylation level of eIF2α protein increased obviously at 48 h p.i. (Fig. 7C), indicating that GTV can activate all three branches of the UPR. Similarly, in HRTV-infected HEK 293 cells, an increase in phosphorylation level of eIF2α protein began to be detected at 48 h p.i., while a decrease in ATF6 p90 protein
began to be detected at 24 h p.i. (Fig. 7D), and a weak increase in the spliced XBP1 mRNA was detected at 12 h p.i. (Fig. 7F), suggesting that infection of HRTV could also activate the three branches of the UPR. The above data suggested that activation of the UPR may represent a common feature for phleboviruses, at least for SFTSV-related virus groups.

Discussion

Newly emerging phleboviruses, such as SFTSV, HRTV, HRGV, etc., pose a serious threat to public health. Currently, there are no FDA-approved drugs or vaccines to combat phlebovirus infection, and this is in part due to a lack of comprehensive understanding of the molecular interactions occurring between phleboviruses and host cells. Although loss of function based screenings at a whole-genome scale have been performed on different phleboviruses, including Rift Valley fever virus (RVFV) (9, 27), Uukuniemi virus (UUKV) (28) and SFTSV (9, 24), a global map showing how phleboviruses regulate and manipulate host biological processes for viral infection is still unavailable.

In this study, to identify host proteins involved in the SFTSV replication process and decipher how virus infection affects biological processes of host cells, a quantitative proteomic analysis of SFTSV-infected cells was performed at 6, 12, 24 and 48 h p.i. A total of 5,606 host proteins were quantified, with 433 being differentially regulated, accounting for 7.7% of the quantified host proteins (Table S2). Among these, 310 up-regulated proteins were identified, with ~90% being enriched at the late stage of infection (48 h p.i.). Among these, only FN1 was up-regulated cross all time points, while 7 proteins were up-regulated cross three time points, 12 proteins were
up-regulated at two time points (Table S2). The other 123 proteins were found to be down-regulated, and they were not enriched at any infection time-point (Fig. 1C).

Although a relatively high MOI was used in this study, we didn’t observe obvious cytopathic effect (CPE) or apoptosis in HEK 293 cells over a 48-h infection course (Fig. 1B). A previous report also demonstrated that no cell death or apoptosis was induced by SFTSV in monocytes THP-1 cells (29). Our proteomic data reflected that SFTSV infection cause a modest influence on host protein levels, which may partially explain the mild CPE caused by this virus.

Previous large-scale analyses of phlebovirus and host interactions via a “loss of function” strategy identified hundreds of host proteins that could affect phlebovirus infection (9, 27, 28, 30). Among these proteins, 44 were identified as being differentially regulated by SFTSV infection in this study (Table S3), suggesting that these proteins may play roles in the phlebovirus life cycle by changing protein levels via local synthesis and degradation. Among these proteins, two have been functionally characterized in other phleboviruses. ER chaperone GRP78 was up-regulated in our study, and a previous siRNA screening study indicated that knockdown of GRP78 can inhibit the replication of UUKV (28); meanwhile, knockdown of NF-kappa-B essential modulator (NEMO), another up-regulated protein identified in our study, facilitated the replication of UUKV (28).

To better understand how SFTSV infection affects biological processes of host cells, a cellular response map was created, in which regulated proteins are sorted and aligned according to their biological functions. As shown in Fig. 8A, many biological processes and protein complexes are apparently regulated as a result of SFTSV
infection. These include 1) **Host proteins that may be involved in SFTSV replication cycle.** For example, syntaxins (STX3 and 7), the small Rab GTPases (RabL3, 1A, 32 and 35), VAPA/VAPB are well-known cellular factors that participate vesicle trafficking, membrane fusion, protein complex assembly. Poly(c) binding proteins (PCBP) 1 and 2 facilitate viral replication of EV71, porcine reproductive and respiratory syndrome virus (31, 32). 2) **TLR signaling pathway.** Although TLRs are generally expressed at low levels in HEK 293 cells, multiple modulators of the TLR signaling pathway were up-regulated in SFTSV-infected cells, including NEMO, TRADD and JUN (Fig. 8A and Table S2), suggesting the TLR signaling pathway may be activated in SFTSV-infected HEK 293 cells. In SFTSV-infected patients, elevated pro-inflammation cytokines, including IL-1β, IL18 and RANTES, were observed, suggesting SFTSV infection activates the production of pro-inflammation cytokines (33). A recent study of SFTSV-infected mice indicated that the TLR signaling pathway is essential for the production of IFN-I and inflammatory cytokines in vivo, and enhanced production of multiple inflammatory cytokines and chemokines may trigger the lethal SFTS (34). 3) **Ubiquitin system.** Multiple host proteins involved in the ubiquitin system were differentially regulated after SFTSV infection (Fig. 8A), including E3 ubiquitin ligase TRIM11 (tripartite motif-containing protein 11), TRIM21, both of which were up-regulated at 48 h p.i. (Table S2). Another protein involved in the ubiquitin system, OTULIN (OTU domain-containing de-ubiquitinase with linear linkage specificity), was up-regulated. OTULIN is an essential negative regulator of inflammation (35, 36). 4) **ER-stress.** GO analysis indicated that proteins involved in ER stress and the UPR were also up-regulated at both 24 and 48 h p.i. Particularly GRP78, the most notable marker and important chaperon of UPR, was up-regulated at 12, 24 and 48 h p.i., suggesting the UPR was apparently induced by
Then, we performed an in-depth investigation of the interactions between SFTSV and the three classical branches of host UPR, including PERK, ATF6 and IRE1. The result showed that SFTSV infection could activate all the three branches of UPR (Fig. 3), however, only PERK- and ATF6 pathways were found to play important roles in SFTSV infection (Fig. 4). PERK can sense an excess of unfolded proteins in the ER, and subsequently phosphorylates the eIF2α, attenuating global protein synthesis (26). Multiple viruses have been reported that can affect eIF2α phosphorylation, including human cytomegalovirus (HCMV) (37), dengue virus (DENV) (26), West Nile virus (WNV) (38), ZIKV (39), Junín virus (JUNV) and Machupo virus (MACV) (40). WNV (16, 38), CSFV (15) and TGEV (18) can activate PERK-mediated eIF2α phosphorylation (26). However, the activation of eIF2α phosphorylation upon TGEV infection inhibited TGEV replication by suppressing protein translation and promoting IFN-I production (18). Although eIF2α phosphorylation was induced by SFTSV (Fig. 3C), we did not detect a significant decrease in the overall protein levels. Therefore, the mechanism on how SFTSV promotes productive infection via phosphorylation of eIF2α and the capacity of SFTSV to translate in an eIF2α-independent manner are currently unclear. In addition, although PERK-eIF2α pathway was activated by SFTSV infection (Fig. 3C), we could not exclude the possibility that phosphorylation of eIF2α may occur in the context of the UPR by other kinases, such as heme-regulated inhibitor (HRI) that responds to heme deprivation, general control non-derepressible-2 (GCN2) that responds to amino acid deprivation, and protein kinase R (PKR), which is activated by double-stranded RNA (10, 41). Activation of the ATF6 pathway targets ATF6 from the ER to the Golgi,
where it is proteolytically cleaved (42), and ATF6 further translocates into the nucleus to activate the expression of ER chaperones (10) (Fig. 8B). WNV\textsubscript{KUN} infection can activate the ATF6 pathway to facilitate replication and immune evasion (16). Acute lymphocytic choriomeningitis (LCMV) infection selectively induces the ATF6 pathway, which is likely beneficial for virus replication and cell viability (17). Flaviviruses have evolved to activate the IRE1-XBP1 arm of the UPR, and this may expand both the time and space available for flavivirus replication (43). Here, we found that knockdown of key UPR sensors, PERK or ATF6, reduced viral titers (Fig. 4). Potentially the UPR may extend the life-span of an infected cell, thereby increasing progeny virus.

We also investigated whether activation of the UPR is a common mechanism shared by the SFTSV-related phleboviruses, HRTV and GTV. Distinct effects on UPR induction have been observed in genetically related viruses. For example, in the cases of alphaviruses, Semliki Forest virus (SFV) infection can activate the UPR (44), while Chikungunya virus (CHIKV) infection suppresses the UPR (45). Differing phenomena have been found even for the same virus. For example, infection with WNV NY-99 strain activates all three pathways of the UPR (38), while infection with the WNV\textsubscript{KUN} strain activates the ATF6 and XBP-1 pathways but not the PERK pathway (16), and this may be due to differences in the viral strains and/or cell lines used. Our results suggest that activation of ER-stress and UPR is not confined to SFTSV infection, but seems to be conserved in some genetically related viruses, although the detailed roles of host UPR in the HRTV and GTV life cycles need further investigation. However, we also noticed slight differences in the extent and (or) time course of UPR activation by SFTSV, HRTV or GTV, and this may be due to the
different cell sensitivity to the three distinct phleboviruses (Fig, 7A and B), resulting in different viral replication and protein expression levels.

Virus surface glycoproteins or membrane proteins have been reported as one of the inducers of the UPR in some viruses, such as the G protein of LCMV (17), spike protein of severe acute respiratory syndrome associated coronavirus (SARS-CoV) (46), and hydrophobic proteins of flaviviruses (16, 43, 47). Here, we identified GP, but not the other viral proteins (RdRP, NP or NSs) of SFTSV as the inducer of the UPR (Fig 5). Activation of the UPR by SFTSV GP may be caused by accumulation of unfolded or misfolded proteins in the ER, since GP alone localizes in the ER as well as in the ER-Golgi complex and is responsible for recruiting RdRP and NP into these compartments during virus infection (48). Aggregated GP may modify membrane permeability of the ER, in turn altering ion homeostasis (10, 49). In addition, viral glycoproteins generally contain abundant post-translational modifications (PTMs), such as glycosylation and disulfide-bond formation, and are closely associated with ER stress and the UPR, probably via competing with host protein for modifications (50). Crystal structure information indicated that SFTSV GP contains five N-glycosylation sites and is stabilized by 21 disulfide bonds (51, 52). The PTMs in SFTSV GP may be one of the inducers triggering ER stress and the UPR, which needs further study. As concluded in Fig. 8B, SFTSV infection produces large amounts of GP in the ER, which induces ER stress and activates all three main branches of the UPR. The ATF6 and PERK pathways are important for maintaining intracellular protein levels (Fig. 6) and probably correct folding of GP; thus, these may benefit SFTSV proliferation.
Taken together, this study, for the first time to our knowledge, provides a global map for host cellular responses to SFTSV infection. By examining differentially regulated host proteins, we demonstrated that SFTSV infection could induce the UPR, which further favored SFTSV replication. Furthermore, a critical role of SFTSV GP in activation of the UPR was elucidated, and activation of ER stress and the UPR was implicated as a common feature shared by SFTSV-related phleboviruses, at least HRTV and GTV. In addition, many other important host biological processes highlighted by this research provide potential host targets for basic research as well as anti-SFTSV drug development.

Materials and Methods

Cells and viruses

HEK 293 cells and Vero cells were obtained from the China Center for General Virus Culture Collection (CCGVCC) and grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C with 5% CO₂. SFTSV WCH-2011/HN/China/isolate 97 (53), Heartland virus isolate Patient1 (5) and GTV strain DXM (7) were obtained from CCGVCC and propagated in Vero cells in a biosafety level 3 (BSL-3) laboratory.

Viral infectivity analysis

To analyze the one-step growth curve of SFTSV in HEK 293 cells, SFTSV was inoculated in HEK 293 cells at an MOI of 5 TCID₅₀ units/cell. After 1 h of attachment, the supernatant was replaced with fresh cell culture medium. The tissue culture supernatant of the infected HEK 293 cells was collected at 3, 6, 12, 24, 36, 48
and 72 h p.i., and virus titers were determined by an EPDA (monitoring the expression of NP by immunofluorescence microscopy) as previously described (54).

To compare the infectivity of SFTSV, GTV and HRTV, the three viruses were used to infect HEK 293 cells at an MOI of 5 TCID$_{50}$ units/cell, virus titers were determined at 6, 12, 24, 48 h p.i. by EPDAs. Infection experiment was performed in triplicate and virus titers were analyzed using two-way analysis of variance (2-ANOVA) (SPSS Inc., 2003) with virus type and time as factors. If significant effects were found, difference of titers between two viruses was evaluated by Fisher’s LSD tests.

**iTRAQ labeling and LC-MS/MS analysis**

HEK 293 cells were infected with SFTSV at an MOI of 5 or mock infected. At 6, 12, 24, 48 h p.i., cells were harvested and the extracted proteins were reduced with 10 mM DTT, alkylated with 40 mM iodoacetamide before being digested with trypsin (Promega). The digested peptides were desalted with a SepPak C18 cartridge (Waters) and dried by SpeedVac (Thermo). Three independent biological replicates were performed.

For iTRAQ labeling, 100 μg of peptides from SFTSV- or mock-infected cells was resuspended in iTRAQ dissolution buffer, and then different iTRAQ reagents (SCIEX) were added. After being incubated in room temperature for 1 h, equal amounts of labeled peptides were mixed and desalted with SepPak C18 cartridge (Waters). The mixed peptides were fractionated using strong cation exchange as previously described (55). The fractionated peptides were dried by SpeedVac and stored at
LC-MS/MS analysis was performed on a quadrupole-time of flight LC/MS/MS mass spectrometer (TripleTOF 5600+, SCIEX) equipped with a nanospray source. Peptides were first loaded onto a C18 trap column (Agilent Technologies) and then eluted into a C18 analytical column (Eksigent). For MS/MS analysis, each scan cycle consisted of one full-scan mass spectrum followed by 20 MS/MS events. Mass spectra were extracted by Peakview v2.0 (SCIEX).

**MS data analysis**

Three independent biological replicates were performed, and peptides from three biological replications were analyzed by LC-MS/MS independently. MS spectra were submitted to ProteinPilot v5.0.1 (SCIEX) to perform peptide identification and quantification. The UniProt_Human database was used. Search parameters were as follows: Sample Type: iTRAQ 8plex (Peptide labeled); Cysteine Alkylation: Iodoacetamide; Digestion: Trypsin; Miss cleavages tolerance: 2; Fixed modification: carbamidomethyl Cys; Variable modification: none; MS1 initial mass error tolerance value: 0.05 Dalton; MS2 initial mass error tolerance value: 0.1 Dalton; Instrument: TripleTOF 5600. The false discovery rate (FDR) analysis in ProteinPilot uses a “decoy database searching” strategy, and the FDRs of ProteinPilot search results were all set as lower than 1% at the protein level, and only peptides with confidence score > 95% were used. In each replicate, protein ratio was calculated from the weighted average ratios of each peptide, with peptide intensity as the weight. The protein ratio values used for bioinformatics analysis were the weighted averages of the three biological replicates, while the P value for protein ratio was calculated and further
corrected with multiple Bonferroni correction (Table S1). The cutoff for differentially regulated proteins was set as described in a previous study (56, 57). Briefly, the Gaussian distribution of protein ratios was analyzed, and values deviating from the mean of the normally distributed data by 3.3 standard deviations were considered as cutoff values. Only proteins meeting the following two criteria were considered as being differentially regulated: 1) ratios > up-regulated or < down-regulated cutoff values; 2) corrected $P$ value for protein ratio < 0.05.

**Gene ontology (GO) analysis**

To perform GO analysis, differentially regulated proteins were submitted to DAVID (https://david.ncifcrf.gov/) (58), with all quantified proteins in this study being set as the background. Proteins were classified into different categories based on their roles in biological processes, and a statistical over-representation test was performed. $P$ values were assessed with a binomial test and corrected for multiple testing using a Bonferroni procedure. Only categories with a $P$ value < 0.05 were considered as over- or under-represented.

**Western blot analysis**

Mouse monoclonal antibodies against β-actin (ProteinTech, China) and GRP78 (HuaBio, China), and rabbit polyclonal antibodies against PERK, ATF6, XBP1, HSP90AB1 (ProteinTech, China), and eIF2α and phospho-eIF2α (Ser51) (Cell Signaling Technology) were purchased from the indicated manufacturers.

Rabbit or mouse sera against SFTSV NSs (anti-NSs), NP (anti-NP), GP (anti-Gn), RdRP (anti-RdRP) and HRTV NP (anti-HNP) were used to probe the corresponding
proteins expressed in HEK 293 cells (59). The antibody against STFSV GP (anti-Gn) was used to detect GTV GP, since GTV GP share 79.4% aa identity with SFTSV GP (7). For Western blot analysis, protein samples were subjected to SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). After being blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20, the membrane was probed with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected by an enhanced chemiluminescence kit (Thermo Fisher Scientific).

**PBMCs preparation**

Mouse PBMCs were isolated from blood samples by density gradient centrifugation method using Histopaque (Sigma). Briefly, the blood was layered on LSM medium gently in the ratio of 1:1 and subjected to centrifugation at 100 g for 30 minutes. The white layer representing PBMCs was aspirated out gently and transferred aseptically into sterile centrifuge tubes. The suspension of cells was then washed and cultured in DMEM supplemented with 20 mM of L-Glutamine and 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

**Quantitative real-time PCR (quantitative RT-PCR)**

Cells were harvested, and total cellular mRNA was extracted using TRIzol reagent (Promega). mRNA was then subjected to reverse transcription using a Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Quantitative RT PCR was performed by using specific primers for targeting genes with SYBR Premix Ex Taq™ (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System.
**XBP1 splicing assay**

SFTSV-, HRTV- and GTV-infected or mock-infected cells were harvested at different time points, and total cellular RNA was extracted with TRIzol reagent (Promega). cDNA was synthesized using a MMLV reverse transcriptase (Promega). XBP1 cDNA was amplified using primers (5’-CATGGCCTTGTAGTTG-3’ and 5’-CTGGGTCCACCAAGTTGT-3’) containing the IRE1 splicing site (17). PCR products of ~270 bp and ~244 bp, representing unspliced and spliced XBP1, respectively, were separated on 2% agarose gels.

**siRNA transfection**

2 × 10^5 HEK 293 cells pre-seeded in 24-well plates were transfected with 20 pmol of siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. In a parallel experiment, scrambled siRNA was included as a control. Twenty-four h p.t., cells were collected and subjected to further analyses. All siRNA oligonucleotides used in the study were synthesized by GenePharma (Suzhou, China), and the sequences were as follows (5’-3’): siPERK#1: GUGUGCUAGCAACCUCUAA; siPERK#2: GGAACGACCUGAAGCUAUAA; siATF6#1: GCAGCAACCAUUUCAGGUU; siATF6#2: CCACCCAUAACAGACACCAC; siXBP1#1: GCCUGUCUGUACUUCAUCAA; siXBP1#2: AGAUCGAAGGAGGCUCGAAU.

**Plasmid transfection**

HEK 293 cells (2 × 10^5) pre-seeded in 24-well plates were transfected with plasmids
encoding SFTSV NP, NSs, GP and RdRP genes, respectively, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In the parallel experiments, empty vector and (or) plasmid expressing EGFP were included as controls. Twenty-four h p.t., cells were collected and subjected to further analyses.

### MTT assay

HEK 293 cells were infected with SFTSV (MOI = 5) and harvested at 24, 48 and 72 h p.i., or HEK 293 cells were treated with the desired concentrations of siRNAs for 24 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich) was added at a final concentration of 5 mg/ml. The cells were incubated at 37°C for 4 h, and the supernatant was removed. Then, 50 μl of DMSO was added to each well, and the emitted light at 492 nm was measured with a Thermo Multiskan enzyme-linked immunosorbent assay (ELISA) reader (Thermo, Waltham, MA).

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### References

1. Cyranoski D. 2018. East Asia braces for surge in deadly tick-borne virus. Nature 556:282-283.
2. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, Zhang L, Zhang QF, Popov VL, Li C, Qu J, Li Q, Zhang YP, Hai R, Wu W, Wang Q, Zhan FX, Wang XJ, Kan B, Wang SW, Wan KL, Jing HQ, Lu JX, Yin WW, Zhou H, Guan XH, Liu JF, Ren J, Wang H, Zhao Z, Song JD, He JR, Wang JS, Fu XP, Sun LN, Dong XP, Feng ZJ, Yang WZ, Hong T, Zhang Y, Walker DH, Wang Y, Li DX. 2011. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med 364:1523-1532.

3. Kim KH, Yi J, Kim G, Choi SJ, Jun KI, Kim NH, Choe PG, Kim NJ, Lee JK, Oh MD. 2013. Severe fever with thrombocytopenia syndrome, South Korea, 2012. Emerg Infect Dis 19:1892-1894.

4. Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, Kamei T, Honda M, Ninomiya D, Sakai T, Senba T, Kaneyuki S, Sakaguchi S, Satoh A, Hosokawa T, Kawabe Y, Uemura T, Katayama Y, Miyahara M, Ijuin M, Doi K, Okuda M, Umeki K, Saito T, Fukushima K, Nakajima K, Yoshihara T, Tani H, Fukushima S, Ozuma M, Ogata M, Shimojima M, Nakajima N, Katano H, Fukumoto H, Sato Y, Hasegawa H, Yamagishi T, Oishi K, Kurane I, Morikawa M, Ijuin M, Doi K, Okuda T, Umeki K, Saito T, Fukushima K, Nakajima K, Yoshihara T, Tani H, Fukushima S, Ozuma M, Ogata M, Shimojima M, Nakajima N, Katano H, Fukumoto H, Sato Y, Hasegawa H, Yamagishi T, Oishi K, Kurane I, Morikawa M, Ijuin M. 2014. The first identification and retrospective study of Severe Fever with Thrombocytopenia Syndrome in Japan. J Infect Dis 209:816-827.

5. McMullan LK, Folk SM, Kelly AJ, MacNeil A, Goldsmith CS, Metcalfe MG, Batten BC, Albarino CG, Zaki SR, Rollin PE, Nicholson WL, Nichol ST. 2012. A new phlebovirus associated with severe febrile illness in Missouri. N Engl J Med 367:834-841.

6. Gauci PJ, McAllister J, Mitchell IR, St George TD, Cybinski DH, Davis SS, Gubala AJ. 2015. Hunter Island Group Phlebovirus in Ticks, Australia. Emerg Infect Dis 21:2246-2248.

7. Shen S, Duan X, Wang B, Zhu L, Zhang Y, Zhang J, Wang J, Liu T, Kou C, Liu D, Lv C, Zhang L, Chang C, Su Z, Tang S, Qiao J, Moming A, Wang C, Abudurexiti A, Wang H, Hu Z, Zhang Y, Sun S, Deng F. 2018. A novel tick-borne phlebovirus, closely related to severe fever with thrombocytopenia syndrome virus and Heartland virus, is a potential pathogen. Emerg Microbes Infect doi: 10.1038/s41426-018-0093-2

8. Hofmann H, Li X, Zhang X, Liu W, Kuhl A, Kaup F, Soldan SS, Gonzalez-Scarano F, Weber F, He Y, Pohlmann S. 2013. Severe fever with thrombocytopenia virus glycoproteins are targeted by neutralizing antibodies and can use Dc-SIGN as a receptor for pH-dependent entry into human and animal cell lines. J Virol 87:4384-4394.

9. Hopkins KC, McLane LM, Maqbool T, Panda D, Gordesky-Gold B, Cherry S. 2013. A genome-wide RNAi screen reveals that mRNA decapping restricts bunyaviral replication by limiting the pools of Dep2-accessible targets for cap-snatching. Genes Dev 27:1511-1525.

10. Jheng JR, Ho JY, Horng JT. 2014. ER stress, autophagy, and RNA viruses. Front Microbiol 5:388.

11. Chan SW. 2014. Unfolded protein response in hepatitis C virus infection. Front Microbiol 5:233.

12. Sood R, Porter AC, Ma K, Quilliam LA, Wek RC. 2000. Pancreatic eukaryotic initiation factor-2alpha kinase (PEK) homologues in humans, Drosophila melanogaster and Caenorhabditis elegans that mediate translational control in response to endoplasmic reticulum stress. Biochem J 346 Pt 2:281-293.
13. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. 1999. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10:3787-3799.

14. Tirasophon W, Welihinda AA, Kaufman RJ. 1998. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endo ribonuclease (Ire1p) in mammalian cells. Genes Dev 12:1812-1824.

15. He W, Xu H, Gou H, Yuan J, Liao J, Chen Y, Fan S, Xie B, Deng S, Zhang Y, Chen J, Zhao M. 2017. CSFV Infection Up-Regulates the Unfolded Protein Response to Promote Its Replication. Front Microbiol 8:2129.

16. Ambrose RL, Mackenzie JM. 2011. West Nile virus differentially modulates the unfolded protein response to facilitate replication and immune evasion. J Virol 85:2722-2732.

17. Pasqual G, Burri DJ, Pasquato A, de la Torre JC, Kunz S. 2011. Role of the host cell's unfolded protein response in arenavirus infection. J Virol 85:1662-1670.

18. Xue M, Fu F, Ma Y, Zhang X, Li L, Feng L, Liu P. 2018. The PERK Arm of the Unfolded Protein Response Negatively Regulates Transmissible Gastroenteritis Virus Replication by Suppressing Protein Translation and Promoting Type I IFN Production. J Virol. doi: 10.1128/JVI.00431-18.

19. Gladwyn-Ng I, Cordon-Barris L, Alfano C, Creppe C, Couderc T, Morelli G, Thelen N, America M, Bessieres B, Encha-Razavi F, Bonniere M, Suzuki IK, Flaman M, Vanderhaeghen P, Thiry M, Lecuit M, Nguyen L. 2017. Stress-induced unfolded protein response contributes to Zika virus-associated microcephaly. Nat Neurosci. 21(1):63-71.

20. Li XD, Lankinen H, Putkuri N, Vapalahti O, Vaheri A. 2005. Tula hantavirus triggers pro-apoptotic signals of ER stress in Vero E6 cells. Virology 333:180-189.

21. Zieske L.R. 2006. A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. J Exp Bot 57:1501-1508.

22. Liu Y, Wu B, Paessler S, Walker DH, Tesh RB, Yu XJ. 2014. The pathogenesis of severe fever with thrombocytopenia syndrome virus infection in alpha/beta interferon knockout mice: insights into the pathologic mechanisms of a new viral hemorrhagic fever. J Virol 88:1781-1786.

23. Jin C, Jiang H, Liang M, Han Y, Gu W, Zhang F, Zhu H, Wu W, Chen T, Li C, Zhang W, Zhang Q, Qu J, Wei Q, Qin C, Li D. 2015. SFTS virus infection in nonhuman primates. J Infect Dis 211:915-925.

24. Drake MJ, Brennan B, Briley K, Jr., Bart SM, Sherman E, Szemiel AM, Minutillo M, Bushman FD, Bates P. 2017. A role for glycolipid biosynthesis in severe fever with thrombocytopenia syndrome virus entry. PLoS Pathog 13:e1006316.

25. Ning YJ, Wang M, Deng M, Shen S, Liu W, Cao WC, Deng F, Wang YY, Hu Z, Wang H. 2014. Viral suppression of innate immunity via spatial isolation of TBK1/IKKepton from mitochondrial antiviral platform. J Mol Cell Biol 6:324-337.

26. Pena J, Harris E. 2011. Dengue virus modulates the unfolded protein response in a time-dependent manner. J Biol Chem 286:14226-14236.

27. Harmon B, Bird SW, Schudel BR, Hatch AV, Rasley A, Negrete OA. 2016. A Genome-Wide RNA Interference Screen Identifies a Role for Wnt/beta-Catenin Signaling during Rift Valley Fever Virus Infection. J Virol 90:7084-7097.

28. Meier R, Franceschini A, Horvath P, Tetard M, Mancini R, von Mering C, Helenius A,
Lozach PY. 2014. Genome-wide small interfering RNA screens reveal VAMP3 as a novel host factor required for Uukuniemi virus late penetration. J Virol 88:8565-8578.

Qu B, Qi X, Wu X, Liang M, Li C, Cardona CJ, Xu W, Tang F, Li Z, Wu B, Powell K, Wegner M, Li D, Xing Z. 2012. Suppression of the interferon and NF-kappaB responses by severe fever with thrombocytopenia syndrome virus. J Virol 86:8388-8401.

Riblett AM, Blomen VA, Jae LT, Altamura LA, Doms RW, Brummelkamp TR, Wojcechowskyj JA. 2015. A Haplotype Genetic Screen Identifies Heparan Sulfate Proteoglycans Supporting Rift Valley Fever Virus Infection. J Virol 90:1414-1423.

Luo Z, Dong X, Li Y, Zhang Q, Kim C, Song Y, Kang L, Liu Y, Wu K, Wu J. 2014. PolyC-binding protein 1 interacts with 5'-untranslated region of enterovirus 71 RNA in membrane-associated complex to facilitate viral replication. PLoS One 9:e87491.

Beura LK, Dinh PX, Osorio FA, Pattnaik AK. 2011. Cellular poly(c) binding proteins 1 and 2 interact with porcine reproductive and respiratory syndrome virus nonstructural protein 1beta and support viral replication. J Virol 85:12939-12949.

Sun Y, Jin C, Zhan F, Wang X, Liang M, Zhang Q, Ding S, Guan X, Huo X, Li C, Qu J, Wang Q, Zhang S, Zhang Y, Wang S, Xu A, Bi Z, Li D. 2012. Host cytokine storm is associated with disease severity of severe fever with thrombocytopenia syndrome. J Infect Dis 206:1085-1094.

Yamada S, Shimojima M, Narita R, Tsukamoto Y, Kato H, Saijo M, Fujita T. 2018. RLRs and TLRs Signaling Pathways Cause Aberrant Production of Inflammatory Cytokines/Chemokines in an SFTSV Infection Mouse Model. J Virol. doi: 10.1128/JVI.02246-17.

Damgaard RB, Walker JA, Marco-Casanova P, Morgan NV, Titheradge HL, Elliott PR, McHale D, Maher ER, McKenzie AN, Komander D. 2016. The Deubiquitinase OTULIN Is an Essential Negative Regulator of Inflammation and Autoimmunity. Cell 166:1215-1230 e1220.

Hrdinka M, Fiil BK, Zucca M, Leske D, Bagola K, Yabal M, Elliott PR, Damgaard RB, Komander D, Jost PJ, Gyrd-Hansen M. 2016. CYLD Limits Lys63- and Met1-Linked Ubiquitin at Receptor Complexes to Regulate Innate Immune Signaling. Cell Rep 14:2846-2858.

Isler JA, Skalet AH, Alwine JC. 2005. Human cytomegalovirus infection activates and regulates the unfolded protein response. J Virol 79:6890-6899.

Medigeshi GR, Lancaster AM, Hirsch AJ, Briese T, Lipkin WI, Delfilippis V, Fruh K, Mason PW, Nikolich-Zugich J, Nelson JA. 2007. West Nile virus infection activates the unfolded protein response, leading to CHOP induction and apoptosis. J Virol 81:10849-10860.

Hou S, Kumar A, Xu Z, Airo AM, Stryapunina I, Wong CP, Branton W, Tchesnokov E, Gotte M, Power C, Hobman TC. 2017. Zika virus hijacks stress granule proteins and modulates the host stress response. J Virol. doi: 10.1128/JVI.00474-17.

Huang C, Kolokoltsova OA, Mateer EJ, Koma T, Paessler S. 2017. Highly pathogenic New World arenavirus infection activates the pattern recognition receptor PKR without attenuating virus replication in human cells. J Virol. doi: 10.1128/JVI.01090-17.

Chaveroux C, Sarcinelli C, Barbet V, Belfeki S, Barthelaix A, Ferraro-Peyret C, Lebecque S, Renno T, Bruhat A, Fafournoux P, Manie SN. 2016. Nutrient shortage triggers the hexosamine biosynthetic pathway via the GCN2-ATF4 signalling pathway. Sci Rep
ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell 6:1355-1364.

Yu CY, Hsu YW, Liao CL, Lin YL. 2006. Flavivirus infection activates the XBP1 pathway of the unfolded protein response to cope with endoplasmic reticulum stress. J Virol 80:11868-11880.

Barry G, Fragkoudis R, Ferguson MC, Lulla A, Merits A, Kohl A, Fazakerley JK. 2010. Semliki forest virus-induced endoplasmic reticulum stress accelerates apoptotic death of mammalian cells. J Virol 84:7369-7377.

Fros JJ, Major LD, Scholte FEM, Gardner J, van Hemert MJ, Suhrbier A, Pijlman GP. 2015. Chikungunya virus non-structural protein 2-mediated host shut-off disables the unfolded protein response. J Gen Virol 96:580-589.

Barry G, Fragkoudis R, Ferguson MC, Lulla A, Merits A, Kohl A, Fazakerley JK. 2010. Semliki forest virus-induced endoplasmic reticulum stress accelerates apoptotic death of mammalian cells. J Virol 84:7369-7377.

Lundu T, Tsuda Y, Ito R, Shimizu K, Kobayashi S, Yoshii K, Yoshimatsu K, Arikawa J, Kariwa H. 2018. Targeting of severe fever with thrombocytopenia syndrome virus structural proteins to the ERGIC (endoplasmic reticulum Golgi intermediate compartment) and Golgi complex. Biomed Res 39:27-38.

Gingell D. 1973. Membrane permeability change by aggregation of mobile glycoprotein units. J Theor Biol 38:677-679.

Bhandary B, Marahatta A, Kim HR, Chae HJ. 2012. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. Int J Mol Sci 14:434-456.

Halldorsson S, Behrens AJ, Harlos K, Huiskonen JT, Elliott RM, Crispin M, Brennan B, Bowden TA. 2016. Structure of a phleboviral envelope glycoprotein reveals a consolidated model of membrane fusion. Proc Natl Acad Sci U S A 113:7154-7159.

Wu Y, Zhu Y, Gao F, Jiao Y, Oladejo BO, Chai Y, Bi Y, Lu S, Dong M, Zhang C, Huang G, Wong G, Li N, Zhang Y, Li Y, Feng WH, Shi Y, Liang M, Zhang R, Qi J, Gao GF. 2017. Structures of phlebovirus glycoprotein Gn and identification of a neutralizing antibody epitope. Proc Natl Acad Sci U S A 114:E7564-E7573.

Lam TT, Liu W, Bowden TA, Cui N, Zhuang L, Liu K, Zhang YY, Cao WC, Pybus OG. 2013. Evolutionary and molecular analysis of the emergent severe fever with thrombocytopenia syndrome virus. Epidemics 5:1-10.

Zhang Y, Shen S, Fang Y, Liu J, Su Z, Liang J, Zhang Z, Wu Q, Wang C, Abudurexiti A, Hu Z, Zhang Y, Deng F. 2018. Isolation, Characterization, and Phylogenetic Analysis of Two New Crimean-Congo Hemorrhagic Fever Virus Strains from the Northern Region of Xinjiang Province, China. Virol Sin 33:74-86.

Chai F, Li HY, Wang W, Zhu XJ, Li Y, Wang S, Guo L, Zhang LK, Xiao G. 2015. Subcellular quantitative proteomic analysis reveals host proteins involved in human cytomegalovirus infection. Biochimica et biophysica acta 1854:967-978.
56. Emmott E, Goodfellow I. 2014. Identification of protein interaction partners in mammalian cells using SILAC-immunoprecipitation quantitative proteomics. J Vis Exp:51656.

57. Zhang LK, Lin T, Zhu SL, Xianyu LZ, Lu SY. 2015. Global quantitative proteomic analysis of human glioma cells profiled host protein expression in response to enterovirus type 71 infection. Proteomics 15:3784-3796.

58. Huang da W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44-57.

59. Ning YJ, Feng K, Min YQ, Cao WC, Wang M, Deng F, Hu Z, Wang H. 2015. Disruption of Type I Interferon Signaling by NSs Protein of Severe Fever with Thrombocytopenia Syndrome Virus via Hijacking STAT2 and STAT1 into Inclusion Bodies. J Virol. 89:4227-4236.
Figure legends

Fig 1. Quantitative proteomics analysis of SFTSV-infected HEK 293 cells. (A) Kinetics of SFTSV replication in HEK 293 cells. HEK 293 cells were infected with SFTSV at an MOI of 5, the supernatants were harvested at the indicated time points and the virus titers were measured by TCID\textsubscript{50}. All experiments were performed at least three times, and values represent means ± SDs from three replicates. (B) HEK 293 cells were infected with SFTSV at an MOI of 5, and at the indicated time points HEK 293 cells were harvested and subjected to MTT assay to measure cell viability. (C) Workflow for iTRAQ-based quantitative proteomic analysis of SFTSV-infected HEK 293 cells. (D) Volcano plot showing log\textsubscript{2} fold change plotted against –log\textsubscript{2} adjusted P value for SFTSV-infected cells versus mock-treated cells at different times p.i. (E) Kinetic of viral protein ratio and infection ratio of SFTSV infected HEK 293 cells. Viral protein ratio was measured by MS. Infection ratio was measured by detecting NP positive cells versus all cells detected. (F) Validation of MS results using quantitative RT-PCR. HEK 293 cells were infected with SFTSV at an MOI of 5 or mock infected. At the indicated time intervals, cells were harvested and intracellular mRNAs were extracted and subjected to reverse transcription. The intracellular RNA levels of the corresponding proteins were measured with quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin and actin were chosen as internal controls. Intracellular RNA levels at each time point of SFTSV infection were normalized to those in the mock-infected cells. The experiments were repeated twice. Bars in (A) and (D) represent standard deviations. M: mock treated; V: SFTSV infected; N: number of proteins quantified; up: up-regulated protein; down: down-regulated protein; FN1: fibronectin; GOLIM4: Golgi integral membrane protein 4; JUN: transcription factor AP-1; RIOK2: serine/threonine protein kinase RIO2;
RNF25: E3 ubiquitin-protein ligase RNF25; TEAD1: transcriptional enhancer factor 875
TEF-1; TSC22D4: TSC22 domain family protein 4; VAPA: vesicle-associated
membrane protein-associated protein A.

**Fig 2. Gene ontology analysis of regulated proteins based on biological processes.**
Differentially regulated proteins at each time point were subjected to DAVID,
respectively. Regulated proteins were grouped based on their roles in biological
processes, and a statistical over-representation test was performed to determine which
biological process was over-represented by differentially regulated proteins. Only
biological processes over-represented by differentially regulated proteins at 6 h p.i.
(A), 12 h p.i. (B), 24 h p.i. (C) and 48 h p.i. (D) were considered as being regulated by
SFTSV infection. The categories labeled in red in (C) and (D) are UPR-related
pathways.

**Fig 3. SFTSV infection activates all three branches of the UPR. (A)**
SFTSV-infected HEK 293 cells were collected at the indicated time intervals, and
total proteins were extracted and subjected to Western blot analysis for GRP78/94. (B)
RNA samples from the above cells were extracted and subjected to reverse
transcription. Relative mRNA levels of the indicated proteins were measured with
quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin
and actin were chosen as internal controls. Intracellular RNA levels at each time point
of SFTSV infection were normalized to those in the mock-infected cells. All
experiments were performed at least three times, and values represent means ± SDs
from three replicates. *P < 0.05, **P < 0.01 by Student’s t-test. (C) SFTSV-infected
HEK 293 cells were collected at the indicated time intervals, and total proteins were
extracted and subjected to Western blot analysis for ATF6 p90, phos-eIF2α (Ser51), total eIF2α, PERK, Gn and the internal control actin. Red arrow: phosphorylated PERK; purple arrow: PERK. (D) RNA samples were also analyzed for spliced XBP1 mRNA by using reverse transcription PCR. The intensity of protein band was measured by Image J_v1.8.0. In each time point, protein intensity was first normalized to actin, and then normalized to the corresponding mock group.

Fig 4. Effects of UPR on SFTSV production. (A-B) Knockdown of targeted proteins by using RNA interference. HEK 293 cells were transfected with siRNAs against targeted host genes or scrambled siRNA (NC). Cells were collected at 48 h p.t., and total cellular RNA was extracted and subjected to reverse transcription. Intracellular RNA levels of ATF6, XBP1 and PERK were measured with quantitative RT-PCR (A). MTT analysis was performed to determine the cytopathic effects of the siRNAs. (B). (C-D) Effects of knockdown of host proteins on SFTSV production and replication. HEK 293 cells were transfected with siRNAs against targeted genes or scrambled siRNA (NC), and at 48 h p.t., cells were super-infected with SFTSV at an MOI of 1. At 48 h p.i., total cellular RNA was extracted, and SFTSV genomic RNA levels were measured with quantitative RT-PCR (C). The cell supernatant was collected and viral titer was measured by EPDAs (D). All experiments were performed in triplicate, and values represent means ± SDs from three replicates. *P < 0.05, **P < 0.01 by Student’s t-test.

Fig 5. Expression of SFTSV G proteins, but not other viral proteins, induces the cellular UPR. (A) HEK 293 cells were transfected with plasmids expressing viral proteins, or with GFP or empty vector as controls, and at 48 h p.t., cells were collected
and intracellular protein levels of GRP94, GRP78, viral proteins and the loading control actin were detected with Western blots. Tunicamycin (Tm), a reported inducer of the UPR, was used as a positive control. (B) HEK 293 cells were transfected with plasmids expressing viral proteins or GFP and intracellular RNA levels of GRP94 and GRP78 were detected with quantitative RT-PCR at 48 h p.t. (C) HEK 293 cells were transfected with increasing amounts of SFTSV protein-expressing plasmids, and empty vector as a control. At 48 h p.t., cells were collected and intracellular protein levels of GRP78, viral proteins and actin as a loading control were detected by Western blots. *P < 0.05, **P < 0.01 by Student’s t-test. The intensity of protein band was measured by Image J_v1.8.0. Protein intensity was first normalized to actin, and then further normalized to that of the empty vector transfected cells.

**Fig 6. Knockdown of PERK and ATF6 reduces intracellular levels of SFTSV GP.**

**(A)** HEK 293 cells were transfected with siRNAs against targeted host genes or scrambled siRNA (NC). At 48 h p.t., cells were super-infected with SFTSV at an MOI of 1 and then collected at 48 h p.i. Viral protein levels were analyzed by Western blots. **(B)** HEK 293 cells were co-transfected with plasmids expressing NSs or G proteins and siRNAs against targeted host genes or scrambled siRNA (NC). At 48 h p.t., cells were collected and viral-host proteins were subjected to Western blot analyses.

**Fig 7. GTV and HRTV infection activate three branches of the UPR.** HEK 293 cells were infected GTV, HRTV or SFTSV, and at indicated time intervals, **(A)** cells were fixed and intracellular level of NP was monitored by Immunofluorescence. **(B)** The supernatants of infected cells were also collected, and viral titer were measured with TCID\textsubscript{50}. All experiments were performed at least three times, and values
represent means ± SDs from three replicates. ***P < 0.001 by Fisher’s LSD tests.

GTV (C) or HRTV (E) infected HEK 293 cells were collected at the indicated time intervals, and total proteins were extracted and subjected to Western blot analyses for GRP78, ATF6 p90, phos-eIF2α (Ser51), total eIF2α, Gn, NP and the internal control actin. Anti-SFTSV Gn (anti-Gn) and anti-HRTV NP (anti-HNP) were used to detect GTV GP and HRTV NP, respectively. (D and F) RNA samples from the above cells were also analyzed for spliced XBP1 mRNA by using reverse transcription PCR. The intensity of protein band was measured by Image J_v1.8.0. In each time point, protein intensity was first normalized to actin, and then normalized to the corresponding mock group.

Fig 8. Proposed model for the UPR and other cellular responses regulated by SFTSV infection. (A) Global host cellular protein responses to SFTSV infection. According to the results of the quantitative proteomic analysis, proteins/protein complexes specifically regulated by SFTSV infection are sorted and aligned according to their biological functions. (B) Proposed interaction model between host UPR and SFTSV infection. SFTSV infection produces large amounts of unfolded GP in the ER, which activates all three main branches of the UPR. Among these, the ATF6 and PERK pathways facilitate proper folding of GP, and thus favor SFTSV replication.
**A**

|          | Mock | SFTSV | GTV | HRTV |
|----------|------|-------|-----|------|
| NP       | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| DAPI     | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| 6 h p.i. | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 12 h p.i.| ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| 24 h p.i.| ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
| 48 h p.i.| ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |

**B**

Viral titer

![Graph](image25.png)

**C**

|          | 6 h p.i. | 12 h p.i. | 24 h p.i. | 48 h p.i. |
|----------|----------|-----------|-----------|-----------|
| anti-GRP78 | (1.36)   | (1.20)    | (1.29)    | (1.55)    |
| anti-phos-eIF2a | (1.03) | (1.14) | (1.01) | (1.48) |
| anti-eIF2a | (1.15)    | (1.04)    | (0.98)    | (0.92)    |
| anti-ATF6 p90 | (1.13)   | (0.73)    | (0.58)    | (0.49)    |
| anti-Gn   | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) |
| anti-Actin| ![Image](image30.png) | ![Image](image31.png) | ![Image](image32.png) | ![Image](image33.png) |

**D**

|          | 6 h p.i. | 12 h p.i. | 24 h p.i. | 48 h p.i. |
|----------|----------|-----------|-----------|-----------|
| anti-GRP78 | (1.12)   | (0.93)    | (1.28)    | (1.40)    |
| anti-phos-eIF2a | (0.99) | (0.96) | (0.98) | (1.34) |
| anti-eIF2a | (1.05)    | (0.96)    | (0.99)    | (1.02)    |
| anti-ATF6 p90 | (1.11)   | (0.98)    | (0.78)    | (0.16)    |
| anti-HNP  | ![Image](image34.png) | ![Image](image35.png) | ![Image](image36.png) | ![Image](image37.png) |
| anti-Actin| ![Image](image38.png) | ![Image](image39.png) | ![Image](image40.png) | ![Image](image41.png) |

**E**

|          | 6 h p.i. | 12 h p.i. | 24 h p.i. | 48 h p.i. |
|----------|----------|-----------|-----------|-----------|
| unspliced-XBP1 | ![Image](image42.png) | ![Image](image43.png) | ![Image](image44.png) | ![Image](image45.png) |
| spliced-XBP1   | ![Image](image46.png) | ![Image](image47.png) | ![Image](image48.png) | ![Image](image49.png) |

**F**

|          | 6 h p.i. | 12 h p.i. | 24 h p.i. | 48 h p.i. |
|----------|----------|-----------|-----------|-----------|
| unspliced-XBP1 | ![Image](image50.png) | ![Image](image51.png) | ![Image](image52.png) | ![Image](image53.png) |
| spliced-XBP1   | ![Image](image54.png) | ![Image](image55.png) | ![Image](image56.png) | ![Image](image57.png) |

**Actin**

![Image](image58.png)
