A host cell RNA-binding protein, Staufen1, has a role in hepatitis C virus replication before virus assembly

Samantha L. Blackham and Michael J. McGarvey

Faculty of Medicine, Imperial College London, London, UK

Staufen1 is a dsRNA-binding protein involved in the regulation of translation and the trafficking and degradation of cellular RNAs. Staufen1 has also been shown to stimulate translation of human immunodeficiency virus type 1 (HIV-1) RNA, regulate HIV-1 and influenza A virus assembly, and there is also indication that it can interact with hepatitis C virus (HCV) RNA. To investigate the role of Staufen1 in the HCV replication cycle, the effects of small interfering RNA knockdown of Staufen1 on HCV strain JFH-1 replication and the intracellular distribution of the Staufen1 protein during HCV infection were examined. Silencing Staufen1 in HCV-infected Huh7 cells reduced virus secretion by around 70%, intracellular HCV RNA levels by around 40%, and core and NS3 proteins by around 95 and 45%, respectively. Staufen1 appeared to be predominantly localized in the endoplasmic reticulum at the nuclear periphery in both uninfected and HCV-infected Huh7 cells. However, Staufen1 showed significant co-localization with NS3 and dsRNA, indicating that it may bind to replicating HCV RNA that is associated with the non-structural proteins. Staufen1 and HCV core protein localized very closely to one another during infection, but did not appear to overlap, indicating that Staufen1 may not bind to core protein or localize to the core-coated lipid droplets, suggesting that it may not be directly involved in HCV virus assembly. These findings indicate that Staufen1 is an important factor in HCV replication and that it might play a role early in the HCV replication cycle, e.g. in translation, replication or trafficking of the HCV genome, rather than in virion morphogenesis.

INTRODUCTION

The replication cycles for different RNA viruses are often similar with overlapping processes for virus replication and secretion (den Boon & Ahlquist, 2010). One example is the mechanism used by many viruses for genome replication, which involves the assembly of virus replication complexes inside a protective membranous web structure that is synthesized using cellular organelle membrane components (den Boon & Ahlquist, 2010). There is growing evidence to indicate that a common set of host factors may be used by viruses to assemble these structures and perform genome replication. For example, Arf GTPase proteins, which were originally shown to direct membrane reorganization during poliovirus replication (Belov et al., 2008), were recently implicated in the replication of the hepatitis C virus (HCV) genome and production of infectious virions (Matto et al., 2011). Furthermore, proteins regulating the 5′→3′ deadenylation-dependent mRNA decay pathway have been shown to regulate genome translation and replication of brome mosaic virus, which replicates in the yeast Saccharomyces cerevisiae model system (Mas et al., 2006). Mammalian homologues of these yeast proteins (LSm1–7, PatL1 and p54) have also been shown to regulate translation and replication of the HCV genome (Scheller et al., 2009).

These examples demonstrate how investigation of the host factors shown to be involved in the replication of other RNA viruses may facilitate the identification of novel factors involved in the replication of RNA viruses in which these mechanisms are less well understood. To facilitate the identification of novel host factors involved in the HCV replication cycle, host factors that have roles in replication cycles of other related RNA viruses can be examined. Examples of host factors with significant evidence that indicates they might be involved in the HCV replication cycle include: Rab GTPase proteins (Krishnan et al., 2007; Bruce et al., 2010) and RNA-binding proteins such as the T-cell restricted antigen-1 protein (TIA1) and TIA1 protein like-1 (TIAL1) (Li et al., 2002; Emara & Brinton, 2007; Yamasaki et al., 2007) and Staufen1 (Harris et al., 2006; Tingting et al., 2006).

Staufen is a dsRNA-binding protein originally identified in the fruit fly Drosophila melanogaster, where it is involved in the trafficking and translation of cellular mRNAs during embryonic development. In mammalian cells, two homologues of Staufen (Staufen1 and Staufen2) have been identified with 51% homology at the amino acid level. These proteins have been shown to bind specific motifs on cellular mRNA molecules to facilitate the formation of ribonucleoprotein complexes that control mRNA trafficking, translation and degradation (Furic et al., 2008). Although
the two mammalian Staufen homologues are thought to have similar intracellular functional roles, their expression patterns differ significantly, as Staufen1 is expressed ubiquitously, while Staufen2 expression is restricted to neurons (Furic et al., 2008; Maher-Laporte et al., 2010). In addition, several different protein isoforms have been observed for both Staufen homologue genes as a result of alternative splicing. These include the 55 and 63 kDa isoforms generated for Staufen1 and the 52, 56, 59 and 62 kDa isoforms generated for Staufen2. These isoforms have been shown to differ in their mRNA-binding specificity and are thought to form distinct ribonucleoprotein complexes in cells (Furic et al., 2008).

As a result of the role that Staufen proteins play in the trafficking, translation and degradation of mRNA molecules, Staufen1 and -2 have been shown to localize to the rough endoplasmic reticulum (ER) in ribonucleoprotein complexes associated with ribosomes. The only exception is the Staufen2 62 kDa isoform, which is found in ribosome-free ribonucleoprotein complexes (Furic et al., 2008). Staufen1 and -2 have also been observed to traffic between the nucleus and the cytosol to control the localization of newly synthesized mRNA (Miki et al., 2005). To enable the trafficking function of Staufen proteins, Staufen1 has been shown to contain a tubulin-binding domain and is able to form complexes with cytoskeletal filament proteins (tubulin and actin), cytoskeletal control proteins (CDC42 and RAC1) and cytoskeletal motor proteins (myosin and kinesin) (Villacé et al., 2004). Staufen2 has also been shown to traffic along microtubule filaments towards the dendrites of neurons in conjunction with mRNA molecules and can bind directly to α- and β-tubulin (Maher-Laporte et al., 2010).

Alongside the role of Staufen1 and -2 in mRNA trafficking, Staufen1 has also been shown to play a major role in the translation and degradation of cellular mRNA molecules. Binding of Staufen1 to the 3′-UTR of cellular mRNAs targets them for degradation through a process termed ‘Staufen-mediated decay’ (Kim et al., 2005). This relies on the recruitment of the up-frameneshift suppressor 1 protein (UPF1), a cellular RNA/DNA-dependent ATPase and helicase involved in both the processes of nonsense-mediated decay, which degrades mRNAs containing incorrect translation termination sequences and Staufen-mediated decay. Conversely, the binding of Staufen1 to the 5′-UTR of cellular mRNAs enhances the efficiency of their translation (Dugré-Brisson et al., 2005). Staufen1 is able to increase further the translational activity of a cell through its ability to control the balance between polysomes, which perform cellular mRNA translation, and stress granules, which translationally repress cellular mRNAs during times of cellular stress (Abrahamyan et al., 2010).

Staufen1 has been implicated in the replication cycles of several RNA viruses, including human immunodeficiency virus type 1 (HIV-1) and influenza A virus (IAV). In the HIV-1 replication cycle, Staufen1 has been shown to bind to the HIV-1 Gag precursor protein (Pr55Gag) and the HIV-1 RNA genome and is present in the HIV-1 virion. These interactions facilitate multimerization of the Gag precursor protein and enable encapsidation of the HIV-1 RNA during the assembly of nascent HIV-1 particles (Abrahamyan et al., 2010; Milev et al., 2010). As part of its role in virus assembly, Staufen1 has been shown to relocate, in conjunction with the HIV-1 Gag protein and HIV-1 RNA, to lipid raft domains of the plasma membrane where HIV-1 assembly takes place (Milev et al., 2010). Staufen1 has also been shown to play a separate role in the HIV-1 replication cycle controlling the rate at which HIV-1 Gag protein is translated (Abrahamyan et al., 2010). In the replication cycle of IAV, Staufen1 has been detected as part of a viral ribonucleoprotein complex containing viral RNA, viral polymerase and nucleoprotein. Its presence in this complex is thought to facilitate encapsidation of the viral RNA genome into nascent virus particles, as silencing Staufen1 expression reduces influenza virus particle production without affecting virus replication or the level of virus protein expression. An additional interaction has been identified between Staufen1 and the IAV non-structural protein NS1, although the precise function of this interaction is not clear at present (de Lucas et al., 2010). Alongside the findings that Staufen1 is involved in the replication cycle of both HIV-1 and IAV, other studies have found evidence that Staufen1 could bind to the 3′-UTR of the HCV genome and the 5′-UTR of the negative HCV RNA strand (Harris et al., 2006; Tingting et al., 2006). This indicates that Staufen1 may also be involved in the HCV replication cycle and may be required for processes such as genome replication or encapsidation during the assembly of nascent virus particles. Using Staufen1 expression knockdown and HCV–Staufen1 co-localization studies we provide evidence that Staufen1 may play a role in HCV replication before the stage of genome encapsidation.

RESULTS

Effect of targeting Staufen1 mRNA on HCV replication and secretion

To investigate whether Staufen1 has a role in HCV replication and secretion, three small interfering RNA (siRNA) molecules (siSTAU1-1, siSTAU1-2 and siSTAU1-3) were used to target the expression of Staufen1. Knockdown of these targets had significant effects on HCV strain JFH-1 replication and secretion. Silencing Staufen1 with these individual siRNAs consistently reduced Staufen1 mRNA levels by around 90 % and intracellular HCV RNA levels by 30–40 %, and reduced the titres of secreted HCV virus by 65–70 % (Fig. 1).

Quantification of HCV protein abundance following Staufen1 knockdow

To assess the effect of Staufen1 silencing on HCV protein expression, Western blotting was used to determine HCV
core and NS3 protein abundance in HCV-infected Huh7 cells transfected with the three Staufen1 siRNAs. Quantification of protein abundance was performed to determine the fold change in HCV core and NS3 proteins as a result of Staufen1 knockdown (Fig. 2a). Silencing the expression of Staufen1 with the individual siRNAs caused a reduction in the abundance of both HCV core and NS3 proteins. The fold reduction of core protein caused by Staufen1 silencing was much greater than the fold reduction observed for the NS3 protein. For example, a 95% reduction was observed in core protein abundance with the siSTAU1-1 siRNA compared with a 45% reduction observed in NS3 abundance. These differences were most significant with the siSTAU1-1 and siSTAU1-2 siRNA molecules (Fig. 2a, b).

**Intracellular localization of Staufen1 during HCV infection**

To explore further the role of Staufen1 in the HCV replication cycle, the intracellular localization of the Staufen1 protein during HCV infection was investigated.

Staufen1 antibody detected a protein that localized predominantly at the nuclear periphery (Fig. 3a), most likely in the ER where Staufen1 is known to localize (Wickham et al., 1999). Confirmation that the staining is specific to Staufen1 was carried out in Huh7 cells transfected with either Staufen1 siRNAs or a non-targeting siRNA and stained for the Staufen1 protein. A large reduction was observed in the intensity of Staufen1 staining in Huh7 cells transfected with the Staufen1 siRNAs compared with cells transfected with the negative control siRNA (Fig. 3b), indicating that the antibody specifically stains the Staufen1 protein.

To investigate whether HCV infection altered the localization of Staufen1, Huh7 cells were infected with HCV for 48 h and co-stained for Staufen1 and HCV core protein. When comparing the Staufen1 staining observed for the HCV-infected Huh7 cells (core-positive cells) and the uninfected Huh7 cells (core-negative cells), no alterations in Staufen1 localization were observed (Fig. 4a).

The ability of Staufen1 to interact with HCV proteins and HCV RNA was also investigated during HCV infection. Huh7 cells infected with HCV for 48 h were co-stained for Staufen1 and HCV core protein (Fig. 4b), Staufen1 and HCV NS3 protein (Fig. 4c), and Staufen1 and dsRNA (representing the replicating viral genome) (Fig. 4d). Although Staufen1 and the HCV core proteins localized very closely to one another during infection, the two proteins did not appear to overlap, indicating that Staufen1 may not localize to the core-coated lipid droplets (Fig. 4b). When comparing the localization of Staufen1, NS3 and dsRNA, significant co-localization of Staufen1 was observed with NS3 (overlap coefficient, 0.590) and dsRNA (overlap coefficient, 0.685), although there were still some areas of the cell where Staufen1 was found separately from both NS3 and dsRNA (Fig. 4c, d).
DISCUSSION

Staining of HCV-infected Huh7 cells for the Staufen1 protein demonstrated that the cellular localization of Staufen1 does not appear to alter significantly during HCV infection (Fig. 4a). Further investigation of Staufen1 localization during infection also indicated that Staufen1 does not appear to co-localize with the HCV core protein, but may partially co-localize with the HCV NS3 protein and the dsRNA replicative intermediate of the HCV genome (Fig. 4b–d). These findings suggest that Staufen1 may localize to the HCV replication complexes that contain the HCV NS3 protein and dsRNA intermediate, but not the core-coated lipid droplets that participate in virus assembly. This indicates that Staufen1 may facilitate the processes involved in replication, translation or trafficking of the HCV genome, but not the process of nucleocapsid assembly and supports the observation that Staufen1 knockdown reduced the extracellular virus titres and the intracellular abundance of HCV RNA and proteins present in HCV-infected cells (Figs 1 and 2). It also supports the observation that Staufen1 interacts with the 3′-UTR of the HCV genome and the 5′-UTR of the negative RNA strand, which are present in HCV replication complexes as the replicative intermediate form of the HCV genome (Harris et al., 2006; Tingting et al., 2006). Together these findings indicate that the function of Staufen1 in the HCV replication cycle may be distinct from the function observed for Staufen1 in the replication cycles of HIV and IAV, where it has been shown to co-localize with virus nucleocapsid proteins to facilitate nucleocapsid formation (Abrahamyan et al., 2010; de Lucas et al., 2010) and has been shown to relocalize to virus assembly sites as a result of infection (Milev et al., 2010).

Although the co-localization data support the observation that Staufen1 silencing reduced HCV replication and secretion, it does not explain why the rate of secretion was reduced more significantly than the rate of replication in the HCV-infected cells (Fig. 1). These findings initially indicated that Staufen1 is more likely to be required for the process of virion particle formation than the process of HCV genome replication. However, the lack of co-localization observed between Staufen1 and the HCV core protein suggests that Staufen1 is unlikely to be present at the site of nucleocapsid formation or during the budding and secretion of nascent virus particles. One possible explanation that may account for the findings observed in both the siRNA
knockdown assay and co-localization study is that Staufen1 may be required for an early stage of the virus assembly process that is not associated with the HCV core protein. For example, Staufen1 may facilitate the switch from genome replication to assembly within the HCV replication complex, as part of a larger ribonucleoprotein complex, which may also contain host factors required for the processes of HCV genome replication or translation. Therefore, knockdown of Staufen1 may disrupt the correct formation of this ribonucleoprotein complex and may alter the binding of other essential HCV cofactors required for a switch from replication to assembly. Alternatively, Staufen1 may be required for trafficking of the HCV genome to the lipid droplet surface for initiation of nucleocapsid formation. This possibility is supported by recent evidence which shows that Staufen1 interacts with ESCRT (endosomal sorting

Fig. 4. Intracellular localization of Staufen1 during JFH-1 infection. Staining of Staufen1 (green) and HCV core (red) (a), HCV core (red) (b), NS3 (red) (c), and dsRNA (red) (d). Cell nuclei were also stained with DAPI (blue). Magnification: ×60 (a); ×100 (b–d).
complex required for transport components) to play a key role in HIV-1 genomic RNA trafficking prior to virus assembly and disruption of this interaction reduced virus production but not genomic viral RNA (Ghoujal et al., 2012).

These type of effects could explain how Staufen1 silencing was able to reduce the extracellular virus levels (around 70% reduction) much more significantly than the intracellular levels of HCV RNA (around 40% reduction) in the HCV-infected Huh7 (Fig. 1).

Studies investigating the processes involved in the trafficking of the HCV genome to the lipid droplet surface for nucleocapsid formation have demonstrated that the HCV genome may be transported in a complex that contains NS3 alongside other HCV proteins (e.g. NS2, NS3A, P7, E1 and E2) (Ma et al., 2011). It is possible that this complex may also contain the Staufen1 protein and may be responsible for the co-localization observed between Staufen1 and NS3 during HCV infection (Fig. 4c). The NS3 helicase domain was also recently shown to interact directly with the HCV core protein; this provides further evidence to indicate that NS3-containing ribonucleoprotein complexes may play an essential role in the trafficking of the HCV genome to the lipid droplet surface for initiation of virus particle assembly (Mousseau et al., 2011).

Alongside the observations in this study that suggest a potential role for Staufen1 in the regulation of HCV genome replication, translation and trafficking, a recent study investigating HCV endocytosis indicated that Staufen1 may be required during HCV particle entry (Coller et al., 2009). The intracellular localization observed for Staufen1 during HCV infection indicates that Staufen1 is unlikely to play a role in the process of E1/E2-mediated cellular entry (Fig. 4). Instead, Staufen1 may facilitate actin and microtubule filament trafficking of the pseudo-particle RNA following endocytosis or translation of the RNA at the ER. It is also possible that the role of Staufen1 in the regulation of host translation may regulate the expression of a key host factor involved in the process of endocytosis (Kim et al., 2007). Further investigation should determine whether Staufen1 knockdown significantly alters the rate of HCV genome replication and translation using replication-deficient HCV variants and subgenomic replicon-expressing cells. In addition, further analysis of interactions between Staufen1 and HCV proteins and RNA, and the role of Staufen1 in HCV RNA trafficking should help to elucidate the processes involved in the replication and encapsidation of the HCV genome.

**METHODS**

**Cell culture and HCV infection.** Huh7.5 and Huh7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Invitrogen) at 37 °C, 5% CO₂. Full-length JFH-1 virus was produced using Huh7.5 cells transfected with RNA transcribed from the pJFH-1 plasmid (Wakita et al., 2005) as described previously (Blackham et al., 2010). JFH-1 HCV was subsequently used to infect Huh7 cells (m.o.i.=3) for 48 h (Blackham et al., 2010). Cell viability was confirmed for all of the experiments described by using an alamarBlue assay (Invitrogen).

**siRNA knockdown.** Huh7 cells (1 × 10⁵) were seeded in 24-well plates and transfected, using Lipofectamine 2000 (Invitrogen) with 25 nM Staufen1-specific siRNAs (Ambion): STAU1-1 (5’-GAAGCAUUUGUAUCUCU[dt][dT]-3’), STAU1-2 (5’-GGACUGAAUAAAGAGGA[dt][dT]-3’ and STAU1-3 (5’-GGCGUGAGGUAAGUCAA[dt][dT]-3’) and 25 nM of a negative-control non-targeting siRNA (Blackham et al., 2010). After 8 h, the transfection medium was replaced with growth medium. The transfected cells were incubated for 48 h and infected with JFH-1 at an m.o.i. of 3. Four hours after infection, the cells were washed and incubated with growth medium for another 48 h. Intracellular RNA was extracted using TRIzol reagent, treated with DNase and used directly in quantitative reverse transcription-PCR (qRT-PCR) for quantification of target mRNA and JFH-1 HCV RNA (Blackham et al., 2010). The efficiency of knockdown of Staufen1 siRNAs (STAU1-1, STAU1-2 and STAU1-3) was greater than 90%.

**In situ cell staining**

**Immunofluorescent staining of Staufen1 in untreated Huh7 cells.** Huh7 cells (1 × 10⁵) were seeded onto coverslips in a 24-well tissue culture plate. After 24 h, the cells were fixed in 4% (w/v) paraformaldehyde, permeabilized and incubated for 1 h at room temperature in 1% (w/v) BSA/PBS. The cells were then stained for Staufen1 using a rabbit anti-Staufen1 antibody (Abcam), diluted 1:200 in 1% BSA/PBS. Detection of the bound primary antibody was performed using an Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen), diluted 1:1000 in 1% BSA/PBS. The cells were washed in PBS for 5 min (three times) and incubated in a humidity chamber with 20 µg DAPI ml⁻¹ (Invitrogen) for 10 min. The cells were washed three times with PBS, once with water and mounted using Mowiol 4-88 mounting medium (Sigma) (Milev et al., 2010). Core protein staining was analysed using a Nikon Eclipse TE2000S fluorescence microscope.

**Immunofluorescent staining of Staufen1 following STAU1 gene knockdown.** Huh7 cells (1 × 10⁵) were seeded in a 24-well tissue culture plate and transfected with 50 nM of Staufen1 siRNAs (25 nM siSTAU1-1 + 25 nM siSTAU1-2) and 50 nM of a negative control non-targeting siRNA using Lipofectamine 2000. At 48 h post-transfection, the cells were stained for the Staufen1 protein. Images were captured using a Nikon Eclipse TE2000S fluorescence microscope. The same exposure time and gain settings were used for image acquisition to allow accurate comparisons.

**Immunofluorescent staining after HCV infection.** Huh7 cells (1 × 10⁵) were seeded onto coverslips in a 24-well tissue culture plate. The following day, the cells were infected with JFH-1 HCV at an m.o.i. of 0.5 and incubated for 48 h. The cells were stained for Staufen1 as described above; HCV core using a mouse anti-HCV core antibody (clone C7-50; Abcam) diluted 1:400 in 1% BSA/PBS; HCV NS3 using a mouse anti-NS3 antibody (Virogen) diluted 1:200 in 1% BSA/PBS; and dsRNA using a mouse anti-dsRNA antibody (Scicons) diluted 1:200 in 1% BSA/PBS. Images were captured using a Nikon Eclipse TE2000S fluorescence microscope. Quantitative assessment of co-localization between Staufen1 and core, NS3 and dsRNA was performed by calculating the overlap coefficient (ranging from 0, minimum co-localization, to 1.00, maximum co-localization) of replicate analyses using ImageJ (NIH).

**qRT-PCR.** qRT-PCR was performed using a One-Step Quantitect RT-PCR SYBR Green kit (Qiagen). Reactions (10 µl) were performed using 250 ng RNA, 0.1 µl reverse transcriptase, 1 µM reverse primer, 1 µM forward primer, 5 µl 2 × Quantitect SYBR Green RT-PCR.
mastermix and nuclease-free water (primers: HCV forward 5'-TCTG-CGGAAACCGGTGAGTA-3' and reverse 5'TCAGGCAGTACCA-AGGC-3'; Staufen1 forward 5'-GCTCACTAGACACATTGGG-3' and reverse 5'-GTTCAACGCTGATGAGGAAGC-3'). The comparative Ct method was used to calculate fold change in HCV RNA and target mRNA abundance, with glyceraldehyde 3-phosphate dehydrogenase as the normalizing control gene. No template controls were used in all PCRs. The levels of intracellular RNA in HCV-infected cells treated with siRNAs were determined by qRT-PCR. The data represent the mean (±SD) of three independent experiments, each containing repeat siRNA transfections performed in duplicate (n=6). Calculation of statistical significance for qRT-PCR data were performed using a two-tailed unpaired t-test with GraphPad Prism software. Data points with a P value of <0.05 were considered statistically significant.

**Quantification of HCV secretion.** To investigate the effect of siRNA transfection on JFH-1 virus secretion, the titres of JFH-1 virus in the medium collected at 48 h post-infection were quantified. Huh7 cells were seeded in 96-well plates at a density of 1 x 10^4. The following day, growth medium was removed from the cells and was replaced with 100 µl of the medium collected for each transfection reaction, which had been diluted 1:100 in DMEM. At 48 h post-infection, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and incubated for 1 h at room temperature with a mouse anti-HCV core antibody (clone C7-50) diluted 1:400 in 1% BSA/PBS. The cells were then washed for 5 min (three times) in PBS and incubated for 1 h at room temperature with Alexa Fluor 594-conjugated donkey anti-mouse polyclonal antibody (Invitrogen) diluted 1:1000 in 1% BSA/PBS. The cells were washed 5 min (three times) in PBS and mounted using Mowiol mounting medium. Core staining was visualized using a Nikon EclipseTE2000S fluorescence microscope. The number of core-positive foci was counted for each sample. The foci numbers counted for wells infected with medium from target-specific siRNA transfections were compared with the foci numbers counted for wells infected with medium from the non-targeting siRNA transfections. Each medium sample was analysed in duplicate.

**Western blot analysis during JFH-1 infection.** Huh7 cells were seeded in a six-well tissue culture plate at a density of 2.5 x 10^5. The following day, the cells were either infected with JFH-1 at an m.o.i. of 3, or mock infected with an equal volume of concentrated conditioned growth medium (Blackham et al., 2010). At 48 h post-infection, the cells were washed in PBS and harvested in 50 µl 0.5% Triton X-100 lysis buffer [20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM EDTA and 1 x protease inhibitors (100 x stock; Pierce Biosciences)]. Cell lysates were incubated at 4°C for 30 min, centrifugation was performed in a Sorvall T2000 microcentrifuge at 14 000 g for 20 min and the supernatant was collected and stored at −20°C. Protein quantification was performed using Bradford reagent (Sigma), and 50 µg of each protein lysate was run on a 10% (w/v) polyacrylamide gel in 1 x Tris/glycine running buffer (25 mM Tris base, 190 mM glycine and 0.1% SDS, pH 8.3). The gel was transferred onto nitrocellulose membrane in 1 x Tris/ glycine running buffer containing 20% (v/v) methanol. Transfer was performed at 100 V for 60 min at 4°C. The membrane was blocked using 4% (w/v) skimmed milk powder/PBS with 0.1% Tween 20 (PBST) for 1 h at room temperature. The membrane was probed using 5 ml primary antibody diluted in 4% (w/v) BSA/PBST and incubated overnight at 4°C. The primary antibodies used were: rabbit anti-Staufen1 antibody (Abcam); mouse anti-HCV core antibody, diluted 1:1000 (clone C7-50); mouse anti-HCV NS3 antibody, diluted 1:1000 (Virogen); and mouse anti-z-tubulin antibody, diluted 1:10 000 (clone B512; Sigma). Following incubation with primary antibody, the membrane was washed in 25 ml PBST for 10 min, three times. For detection of the bound primary antibody, the membrane was probed for 1 h at room temperature with either 5 ml HRP-conjugated goat anti-rabbit antibody (Millipore) diluted 1:2000 in 4% BSA/PBST to detect Staufen1, or 5 ml HRP-conjugated goat anti-mouse polyclonal antibody (Millipore) diluted 1:2000 in 4% BSA/PBST to detect core, NS3 or z-tubulin,. The membrane was washed in 25 ml PBST for 10 min, three times, and developed using a chemiluminescent ECL substrate (Bioscience Supersignal West Pico Substrate; Pierce) for 5 min. The chemiluminescent signal was detected by X-ray film. Quantification of Western blot protein bands was performed using ImageJ. The mean intensity of each protein band was quantified and normalized using the band intensities quantified for the z-tubulin protein. Fold change in protein abundance represents mean fold change (and replicate range) calculated using repeat band intensity quantifications of duplicate protein bands. Fold changes were calculated by comparing the normalized band intensities quantified for the JFH-1 HCV-infected samples with those quantified for the mock-infected samples at each time point. P values were calculated using a two-tailed unpaired t-test with GraphPad Prism software.

**ACKNOWLEDGEMENTS**

We thank Dr Takaji Wakita, Tokyo Metropolitan Institute for Neuroscience, for the kind gift of JFH-1.

**REFERENCES**


mRNAs associated with Staufen1- and Staufen2-containing ribonucleoprotein complexes. RNA 14, 324–335.


