It has emerged that DDX3 plays a role in antiviral innate immunity. However, the exact mechanism by which DDX3 functions in antiviral innate immunity remains to be determined. We found that the expression of the protein activator of the interferon-induced protein kinase (PACT) was regulated by DDX3 in human cells. PACT acts as a cellular activator of retinoic acid-inducible gene-I-like receptors in the sensing of viral RNAs. DDX3 facilitated the translation of PACT mRNA that may contain a structured 5′ UTR. Knockdown of DDX3 decreased the viral RNA detection sensitivity of the cells. PACT partially rescued defects of interferon-β1 and chemokine (C-C motif) ligand 5/RANTES (regulated on activation normal T cell expressed and secreted) induction in DDX3-knockdown HEK293 cells. Therefore, DDX3 may participate in antiviral innate immunity, at least in part, by translational control of PACT. Moreover, we show that overexpression of the hepatitis C virus (HCV) core protein inhibited the translation of a reporter mRNA harboring the PACT 5′ UTR. The HCV core protein was associated and colocalized with DDX3 in cytoplasmic stress granules, suggesting that the HCV core may abrogate the function of DDX3 by sequestering DDX3 in stress granules. The perturbation of DDX3 by viral proteins delineates a critical role for DDX3 in antiviral host defense.
DDX3 regulates PACT translation

DDX3 was recently reported to be a transcriptional regulator of the IFN-β promoter [32], a signal transducer downstream of TANK-binding kinase 1 (TBK1), as well as IκB kinase ε (IKKe) [29,31], and a viral RNA sensor through interactions with RIG-I and MAVS [41]. However, the cause of such multiple acts of DDX3 remains ambiguous. DDX3 has been identified as a phosphorylation target of TBK1 [32]. Phosphorylated DDX3 can be recruited to the IFN-β promoter, suggesting that DDX3 acts as a transcriptional regulator [32]. Concurrently, DDX3 was shown to interact with IKKe as a scaffold protein, which facilitates the phosphorylation of IRF3/7 by IKKe and leads to IFN-β induction [29,31]. Furthermore, DDX3 interacts with MAVS [41], an adaptor triggering RIG-I-mediated type I IFN induction, suggesting that DDX3 acts as a sensor of viral RNAs and placed DDX3 upstream of TBK1/IKKe. However, the exact mechanism by which DDX3 participates in antiviral innate immunity is still not fully understood.

PACT is a double-stranded RNA (dsRNA)-binding protein, which functions as a cellular activator of RIG-I to facilitate the sensing of viral RNAs [42]. Some viruses avoid immune detection by direct targeting of PACT [43–47]. The perturbation of PACT-induced activation of RIG-I by a variety of viral proteins suggests a critical role for PACT in antiviral host defense. In the present study, we show that DDX3 was required for efficient translation of PACT mRNA in human cells. Knockdown of DDX3 resulted in impaired antiviral innate immune responses. PACT partially rescued defects of IFN-β and chemokine (C-C motif) ligand 5 (CCL5)/RANTES (regulated on activation normal T cell expressed and secreted) induction in DDX3-knockdown cells. Taken together, it is shown that DDX3 may participate in antiviral innate immunity, at least in part, by translational control of PACT.

**Results**

**DDX3 regulates the expression of PACT**

We previously performed polysome fractionation of DDX3-depleted HeLa cell extracts followed by cDNA microarray analysis of cellular mRNAs whose translation was impeded [15]. Among these mRNA targets of DDX3, we found that several candidates are involved in antiviral innate immunity, including PACT. To confirm whether PACT is regulated by DDX3, we examined the expression of PACT in DDX3-knockdown cells and mock-treated cells. Immunoblotting analysis
showed that the protein level of DDX3 was significantly reduced in HeLa and HEK293 cells transduced with either of DDX3-targeting shRNAs (Fig. 1A,B, lanes 2–3) of which shDDX3-2 was more effective than shDDX3-1 in inhibiting DDX3 expression. Cyclin E1 has been identified as a translational target of DDX3 and thus serves as a positive control [15]. As observed with cyclin E1, the level of PACT protein considerably declined in DDX3-knockdown cells (Fig. 1A,B), suggesting that DDX3 may control the expression of PACT protein. We also evaluated PACT and cyclin E1 protein levels in DDX3-overexpressing HeLa cells. Both PACT and cyclin E1 protein levels were up-regulated by DDX3 in a dose-dependent manner (Fig. 1C). The results confirmed our notion that PACT is regulated by DDX3.

**DDX3 regulates PACT expression at the protein level**

To examine whether DDX3 affects the expression level of PACT mRNA, we detected both protein and mRNA expression levels in DDX3-knockdown cells and mock-treated cells. Immunoblotting analysis showed that knockdown of DDX3 in HeLa cells by either DDX3-targeting shRNA caused an apparent reduction in the level of PACT protein (Fig. 2A), although the level of PACT mRNA was not affected by DDX3 knockdown (Fig. 2B). Quantitative immunoblotting analysis showed that the PACT protein levels were significantly reduced by up to 47% and 72% by shDDX3-1 and shDDX3-2, respectively (Fig. 2C). PACT mRNA levels were further measured by quantitative real-time RT-PCR and normalized to the levels of β-actin mRNA. However, there was no significant change in the PACT mRNA level in DDX3-knockdown cells compared to mock-treated cells (Fig. 2C). Because DDX3 only affects PACT expression at the protein level, it is possible that DDX3 regulates the translation of PACT mRNA.

**DDX3 is required for efficient translation of PACT mRNA**

To confirm whether DDX3 affects the translation of PACT mRNA, polysome distribution of PACT mRNA was evaluated using a sucrose gradient
sedimentation and the mRNA level was analyzed by quantitative real-time RT-PCR in DDX3-knockdown and mock-treated HeLa cells. Knockdown of DDX3 by shDDX3-1 and shDDX3-2 did not cause a detectable change in the polysome profile (Fig. 3A, top), which is consistent with the view that DDX3 is dispensable for general mRNA translation [14,15]. Accordingly, no visible change was observed in different density gradient fractions of 18S and 28S ribosomal RNAs (Fig. 3A, bottom). However, the distribution of PACT mRNA in polysome fractions was evidently changed in DDX3-knockdown HeLa cells compared to mock-treated cells (Fig. 3A, middle). Knockdown of DDX3 by shDDX3-1 and shDDX3-2 resulted in an accumulation of PACT and cyclin E1 mRNAs in the 40S ribosomal fraction (Fig. 3A, fraction 3), indicating a stalled translation initiation complex. By contrast, polysome distribution of β-actin mRNA was not significantly affected by DDX3 knockdown.

The distribution of an mRNA within the polysome fractions is reflective of its translational efficiency. Quantitative analysis of mRNAs by real-time RT-PCR showed that approximately 40% of PACT mRNA was associated with polysomes in mock-treated cells, whereas only 24% (shDDX3-1) and 16% (shDDX3-2) of PACT mRNA remained associated with polysomes in DDX3-knockdown cells (Fig. 3B), suggesting that the translational efficiency of PACT but not β-actin mRNA was decreased by DDX3 knockdown (Fig. 3B). The translational efficiency of cyclin E1 mRNA was also down-regulated in DDX3-knockdown cells (Fig. 3B). Therefore, we conclude that DDX3 is required for efficient translation of PACT and cyclin E1 mRNAs in HeLa cells.

**DDX3 facilitates the translation of PACT mRNA via its 5’ UTR**

We have previously proposed that DDX3 is required for the translation of mRNAs that contain a long or structured 5’ UTR [13–15]. The 201 nucleotides of 5’ UTR of PACT mRNA contain a GC-rich (74.6% GC) sequence and are predicted to form a stable secondary structure...
with an estimated free energy of $-109.50$ kcal mol$^{-1}$ (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Fig. 4A).

To investigate the molecular mechanism by which DDX3 functions in the translation of PACT mRNA, we established a firefly luciferase (Fluc) reporter containing the 5' UTR of PACT mRNA and performed a dual-luciferase reporter assay. A Renilla luciferase (Rluc) reporter with an unstructured 5' UTR was co-transfected with the Fluc reporter as an internal control. Changes in the translation of reporter mRNAs were assessed by the relative Fluc/Rluc activity in DDX3-knockdown cells compared to mock-treated cells. A significant reduction (23.3% and 41.8% by shDDX3-1 and shDDX3-2, respectively) of the relative Fluc/Rluc activity was observed in DDX3-knockdown cells without changing the level of the reporter mRNAs.

Fig. 3. Knockdown of DDX3 inhibits translation initiation of PACT mRNA. (A) HeLa cells were transduced with the empty lentiviral vector (pLKO.1) or the pLKO.1 vector expressing DDX3 shRNAs (shDDX3-1 and shDDX3-2). Cells were harvested for analysis at 3 days post-transduction. Cytoplasmic extracts were loaded on a linear 15–40% sucrose gradient ultracentrifugation. After centrifugation, the polysome profile was plotted by A$_{254}$ values (top). Total RNA was extracted from each fraction for analysis. The purified RNA was resolved on a 1% formaldehyde/agarose gel, and rRNAs were visualized by ethidium bromide staining (bottom). The levels of mRNA were analyzed by quantitative real-time RT-PCR using specific primers for PACT, cyclin E1 and $\beta$-actin mRNAs (middle). (B) Translational efficiency of $\beta$-actin, PACT and cyclin E1 mRNAs in mock-treated (pLKO.1) and DDX3-knockdown (shDDX3-1 and shDDX3-2) HeLa cells was calculated and shown as a percentage. The bar graph shows the changes of translational efficiency as the mean ± SEM of at least three independent experiments (ns, not significant; ***P < 0.001, *P < 0.05).
The above data suggest that DDX3 participates in translational control of PACT mRNA, at least in part, via the structured PACT 5' UTR.

Because PACT functions in antiviral innate immunity, DDX3-depleted cells may have compromised antiviral capacity. We therefore addressed the effects of DDX3 knockdown on the type I IFN and inflammatory chemokine induction. To mimic viral infection, we first stimulated HEK293 cells with the synthetic analog of dsRNA poly(I:C), as well as the HCV PAMP RNA, a polyU/UC sequence in the 3' region of the HCV genome. Transfected poly(I:C) and the HCV PAMP RNA function as ligands for cytoplasmic RLRs to induce antiviral innate immune response [36]. As expected, poly(I:C) induced IFN-β1 and CCL5/RANTES expression in a dose-dependent manner, and this was impaired in DDX3-knockdown HEK293 cells (Fig. 5A, lanes 1–6). Similar results were obtained by transfection with the HCV PAMP RNA into HEK293 cells to induce IFN-β1 and CCL5/RANTES expression (Fig. 5A, lanes 7–8). To mimic bacterial infection, we also stimulated HEK293 cells with lipopolysaccharide (LPS), a major constituent of the cell wall of most Gram-negative bacteria. LPS-induced IFN-β1 and CCL5/RANTES expression was also impaired in DDX3-knockdown HEK293 cells (Fig. 5A, lanes 9–10). By contrast, expression of the cytokine TNF-α was not significantly affected by DDX3 knockdown in HEK293 cells (Fig. 5A). This result supported a role of DDX3 in innate immunity.

Next, we evaluated whether PACT can rescue the defect of impaired IFN-β1 and CCL5/RANTES induction caused by DDX3 knockdown. Immunoblotting analysis showed that knockdown of DDX3 by shDDX3-2 down-regulated PACT expression in HEK293 cells (Fig. 5B, lane 3). Transient expression

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**Fig. 4.** DDX3 facilitates the translation of reporter mRNAs containing the PACT 5' UTR. (A) Schematic representation of the firefly luciferase (Fluc) reporter (pFL-SV40) with the human PACT 5' UTR (201 nucleotides), for which the predicted secondary structure is shown. (B) HeLa cells were transduced with the empty lentiviral vector (pLKO.1) or the pLKO.1 vector expressing DDX3 shRNAs (shDDX3-1 and shDDX3-2). After 48 h, HeLa cells were co-transfected with the pFL-SV40 reporter containing the PACT 5' UTR and the control pRL-SV40 vector encoding the Renilla luciferase (Rluc). Cells were lysed for analysis at 24 h post-transfection. For each transfectant, the Fluc activity was normalized to that of the Rluc control. The bar graph shows the relative Fluc/Rluc activities in DDX3-knockdown cells compared to mock-treated cells (top). Fluc and Rluc mRNAs were analyzed by quantitative real-time RT-PCR (bottom). Data are shown as the mean ± SEM from at least three independent experiments (***P < 0.001).
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Fig. 5. PACT partially rescued the antiviral innate immune defects caused by DDX3 knockdown. HEK293 cells were transduced with the empty lentiviral vector (pLKO.1) or the pLKO.1 vector expressing DDX3 shRNA (shDDX3-2) for subsequent functional assessment. (A) At 3 days post-transduction, HEK293 cells were transfected with poly(I:C) or the HCV PAMP RNA or treated with bacterial LPS for 4 h at different concentrations as indicated (µg·mL⁻¹). Total RNA extracted from HEK293 cells was analyzed by conventional RT-PCR using specific primers for IFN-β1, CCL5/RANTES, TNF-α, and β-actin mRNAs. PCR products were resolved by 1.5% agarose gel electrophoresis. (B) At 48 h post-transduction, cells were further transfected with the pcDNA3.1 vector or the FLAG-tagged PACT-expressing pcDNA3.1 vector (PACT-FLAG) to assess whether PACT can be rescued in DDX3-knockdown cells. Cell lysates were subjected to immunoblotting analysis using antibodies against DDX3, α-tubulin and PACT. Detection of α-tubulin was used as a loading control. (C) Under a similar experimental setting to that described in (B), HEK293 transfectants at 3 days post-transduction were further transfected with 0.1 µg·mL⁻¹ of poly(I:C) or 0.2 µg·mL⁻¹ of the HCV PAMP RNA, or treated with 0.1 µg·mL⁻¹ of bacterial LPS for 4 h to induce antiviral innate immune response. Total RNA extracted from HEK293 cells was analyzed by quantitative real-time RT-PCR using specific primers for IFN-β1, CCL5/RANTES, TNF-α and β-actin mRNAs. The bar graph shows the relative mRNA levels of IFN-β1, CCL5/RANTES and TNF-α normalized to β-actin as the mean ± SEM from at least three independent experiments (***P < 0.001, **P < 0.01; ns, not significant).

of FLAG-tagged PACT restored PACT level in DDX3-knockdown HEK293 cells (Fig. 5B, lane 4). To test whether PACT can rescue the defect of IFN-β1 and CCL5/RANTES induction in DDX3-knockdown cells, we transfected poly(I:C) or the HCV PAMP RNA into HEK293 cells to induce antiviral innate immunity. The production of IFN-β1, CCL5/RANTES and TNF-α mRNAs was measured by quantitative real-time RT-PCR using β-actin for normalization (Fig. 5C). Knockdown of DDX3 showed an approximate 80% reduction in the IFN-β1 and CCL5/RANTES induction in HEK293 cells transfected with poly(I:C) or the HCV PAMP RNA, whereas PACT partially rescued their expression (Fig. 5C, lanes 3–4). By contrast, transient expression of FLAG-tagged PACT did not rescue impaired IFN-β1 and CCL5/RANTES induction in DDX3-knockdown HEK293 cells treated with bacterial LPS (Fig. 5C, lanes 3–4). These results indicated that DDX3 participates in antiviral immune response, at least partly, through translational control of PACT protein expression.
HCV core protein binds to DDX3 and abrogates its function in translational control

The HCV core protein interacts with DDX3 [48–50] and interferes with DDX3-mediated IFN-β induction [30]. However, the molecular mechanism underlying the impaired IFN-β induction by the HCV core protein has not yet been fully defined. We assumed that the HCV core protein may bind to DDX3 and abrogate its function in antiviral innate immunity through translational control of PACT. To address this possibility, we examined the effects of HCV core on the translation of a reporter mRNA containing the PACT 5' UTR by a dual-luciferase reporter assay. Overexpression of GFP-tagged HCV core, a matured form of HCV core, inhibited PACT translation by direct binding and redistribution of DDX3 into cytoplasmic SGs. (A) HeLa cells were co-transfected with the pFL-SV40 reporter containing the PACT 5' UTR and the control pRL-SV40 vector in combination with the pEGFP-N1 vector encoding GFP or HCV core-GFP. Cells were lysed for analysis at 48 h post-transfection. For each transfectant, the Fluc activity was normalized to that of the Rluc control. The bar graph shows the relative Fluc/Rluc activities in HCV core-overexpressing (HCV core-GFP) cells compared to control (GFP) cells (top). Fluc and Rluc mRNAs were analyzed by quantitative real-time RT-PCR (bottom). Data are shown as the mean ± SEM from at least three independent experiments (***P < 0.001; ns, not significant). (B) HEK293 cells were transfected with the pEGFP-N1 vector encoding GFP or HCV core-GFP. Cells were lysed for analysis at 48 h post-transfection. Immunoprecipitation was performed using anti-GFP antibody coupled to protein A sepharose beads. Immunoprecipitates were treated with 1 μg/mL RNase A at 37 °C for 30 min. Bound proteins were eluted and subjected to immunoblotting with anti-DDX3 antibody (top) or anti-GFP antibody (bottom). Ig H represents the immunoglobulin heavy chain. (C) HeLa cells were transfected with the pEGFP-N1 vector encoding HCV core-GFP for 24 h. Immunofluorescent staining of HeLa cells was carried out using antibodies against DDX3, eIF4A, HuR and nucleolin (NCL). Co-localization of HCV core-GFP (green) and DDX3, eIF4A or HuR (red) in cytoplasmic SGs was observed under a fluorescence microscope.
of HCV core from the HCV-1b genotype [51], reduced the relative Fluc/Rluc activity without changing the level of the reporter mRNAs (Fig. 6A). We also demonstrated that GFP-tagged HCV core associates with endogenous DDX3 in HEK293 cells by immunoprecipitation (Fig. 6B). This suggests that the HCV core protein may inhibit the translation of PACT mRNA by binding to DDX3. According to our previous study [14], DDX3 is predominantly localized in the cytoplasm and recruited to cytoplasmic stress granules (SGs) under stress conditions. We also observed that GFP-tagged HCV core was mostly colocalized with DDX3 in cytoplasmic SGs (Fig. 6C), which accumulate stalled translation initiation complexes. Consistent with this notion, colocalization of GFP-tagged HCV core with SG marker proteins eIF4A and HuR was observed in cytoplasmic SGs (Fig. 6C), suggesting that the HCV core protein may inhibit the function of DDX3 in antiviral innate immunity through a translational mechanism. In addition to cytoplasmic SGs, colocalization of GFP-tagged HCV core with nucleolin (NCL) in the nucleolus was also observed (Fig. 6C).

In the present study, we propose a model of DDX3-mediated translational control during HCV infection (Fig. 7). HCV genomic RNAs can stimulate a RIG-I-mediated antiviral innate immune response in the presence of PACT during viral replication [36]. However, the HCV core protein interferes with the antiviral innate immune response by abrogating DDX3-mediated translational activation of PACT. Loss of the PACT protein attenuates the detection sensitivity of RIG-I to the HCV genomic RNAs and, in turn, affects IFN-β induction in HCV-infected cells (Fig. 7). Taken together, HCV can evade antiviral innate immunity through the DDX3/PACT axis to establish persistent or chronic infection.

**Discussion**

Previous studies have indicated that DDX3 is involved in antiviral innate immunity [31,32], although the underlying molecular mechanisms are still not fully understood. In the present study, we show, for the first time, that DDX3 regulates PACT expression by promoting the translation of PACT mRNA, which likely harbors a structured 5′UTR (Figs 1–4). Our results thus provide another layer of evidence indicating that DDX3 participates in antiviral innate immunity through regulation of PACT protein level. The accumulation of PACT mRNA in the fractions containing 40S ribosomal subunits in DDX3-knockdown cells (Fig. 3) supports the view that DDX3 participates in ribosome scanning of PACT mRNA during translation initiation.

We also showed that DDX3 is required for IFN-β1 and CCL5/RANTES induction when cells were transfected with viral RNAs (Fig. 5), supporting its role in antiviral innate immunity. Our results indicate that the impairment of innate immunity observed in DDX3-knockdown cells was likely a result of the down-regulation of PACT. PACT can partially rescue the impaired innate immunity caused by DDX3 depletion (Fig. 5). In agreement with a previous study [41], we suggest that DDX3 functions in the sensing of viral infection. Many viruses have evolved various strategies for evading detection by both innate and adaptive immune responses. HCV can utilize the core protein to counteract the antiviral effects of DDX3. We provide evidence that HCV core results in a decrease in the translation of reporter mRNA containing the PACT 5′UTR (Fig. 6). The interaction between HCV core and DDX3 appears to play an important role in attenuating antiviral immune responses. The redistribution
of DDX3 into cytoplasmic SGs was frequently observed in HCV core-overexpressing cells (Fig. 6). Thus, the HCV core protein may inhibit DDX3 function by direct binding and/or sequestration into SGs to interfere with the expression of PACT, which is required for activation of RIG-I-mediated virus detection (Fig. 7). In addition to HCV core, vaccinia virus protein K7 [31] and hepatitis B virus polymerase [40] proteins also abrogate IFN induction by targeting DDX3. It is likely that these viruses have developed a common strategy for evading or suppressing the host’s antiviral innate immunity. Notably, PACT also serves as a target of a variety of viruses. It has been reported that viral proteins, including the influenza A virus NS1 protein [44], the Ebola virus VP35 protein [43,46], the herpes simplex virus US11 protein [45] and the middle east respiratory syndrome coronavirus 4a protein [47], can abrogate RIG-I-mediated IFN induction by direct interaction with the PACT. By contrast, DDX3-mediated translational control of PACT expression is a novel strategy for counteracting antiviral innate immunity.

Notably, PACT also serves as an activator of the protein kinase R (PKR) [52,53], which is activated by viral dsRNAs to inhibit translation in infected cells. Activated PKR is able to phosphorylate the eukaryotic translation initiation factor eIF2α, thereby inhibiting translation initiation of cellular and viral mRNAs. PACT-mediated activation of PKR plays a key role in host defense against viral infections. It is obvious that DDX3 can modulate PKR activity through translational control of PACT. Moreover, PACT also functions as a binding partner of Dicer in RNA interference (RNAi) [54], which is a gene silencing process triggered by dsRNAs [55,56]. RNAi also plays an antiviral role in infected plants [57], invertebrates [58] and mammals [59]. Dicer requires PACT to facilitate substrate specificity during small interfering RNA and microRNA biogenesis. The loss of PACT strongly reduces the accumulation of mature microRNA [60]. Therefore, it would be interesting to study the importance of DDX3-mediated translational control of PACT in the RNAi pathway.

**Experimental procedures**

**Cell culture and transfection**

HeLa and HEK293 cells were grown at 37 °C in DMEM supplemented with 10% FBS, 100 U·mL⁻¹ penicillin, and 100 μg·mL⁻¹ streptomycin. Cell transfection was performed using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA), essentially in accordance with the manufacturer’s instructions.

**Plasmid constructs**

The plasmid expressing FLAG-tagged DDX3 has been described previously [4]. For the in vivo translation assay, the PACT 5’ UTR fragment obtained by RT-PCR using total RNA from HEK293 cells as the template was inserted into an unique HindIII site upstream of the firefly luciferase coding region in the pFL-SV40 vector [14]. The control reporter pRL-SV40 (Promega, Madison, WI, USA) has been described previously [14]. To generate a construct encoding the HCV PAMP RNA, the HCV PAMP fragment (223 nucleotides) obtained by PCR using HCV subgenomic replicon HCV-EV71I-Luc [61] as the template was inserted into yT&A cloning vector (Yeastern Biotech, Taipei, Taiwan). To generate a construct expressing FLAG-tagged PACT protein, the ORF of PACT obtained by RT-PCR using total RNA from HEK293 cells as the template was inserted into pcDNA3.1 (Life Technologies). To generate a construct expressing HCV core-GFP fusion protein, the HCV core DNA fragment was subcloned from pFS-HCVc174 [51] into NgoI and XhoI sites of pEGFP-N1 (Clontech, Palo Alto, CA, USA). All constructs were confirmed by direct sequencing.

**Lentivirus-mediated RNAi knockdown**

All of the plasmids required for lentivirus production were provided by the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The two pLKO.1-shRNA vectors used to knockdown DDX3 were: TRCN0000000002 (shDDX3-1) and TRCN0000000004 (shDDX3-2). The control plasmid was TRCN0000231693 (shLuc). The transfection reagent Lipofectamine® 2000 (Life Technologies) was used for lentiviral production in 293T cells with a packaging construct (pCMV ΔR8.91), an envelope construct (pMD.G) and pLKO.1-shRNA. To knockdown endogenous DDX3, HeLa cells were transduced with shRNA-expressing lentivirus at a multiplicity of infection of 10 virus particles-cell⁻¹ in growth medium containing 8 μg·mL⁻¹ polybrene at 37 °C, 5% CO₂. After 24 h, puromycin (2 μg·mL⁻¹) was added to the medium for selecting infected cells. Cells were harvested 3 days post-transduction for analysis.

**Immunoblotting and immunoprecipitation**

For immunoblot analysis, proteins were transferred onto a PVDF Transfer Membrane (Perkin Elmer, Waltham, MA, USA). Protein blots were blocked with 3% skim milk in TBST buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20) at room temperature for 1 h. The primary antibodies used included affinity-purified rabbit anti-DDX3.
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(0.1 μg·mL⁻¹) [14], rabbit anti-α-tubulin (dilution 1:2000; Cell Signaling, Beverly, MA, USA), rabbit anti-PACT (dilution 1:1000; Cell Signaling), mouse anti-cyclin E (0.4 μg·mL⁻¹; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-GFP (0.2 μg·mL⁻¹; Abcam, Cambridge, MA, USA). Blots were incubated with primary antibodies in blocking buffer at room temperature for 2 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) for X-ray film exposure.

To immunoprecipitate HCV core-GFP, 2 μL of anti-GFP antibody (Abcam) were coupled to protein A-sepharose beads in NET-2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% NP-40). Transfected HEK293 cells (10⁶ cells) were lysed in NET-2 buffer containing 1% NP-40 and 1× protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). Cell lysates were incubated with the antibody-beads conjugate at 4 °C with gentle agitation for 4 h. The beads were washed four times with 1 mL of cold NET-2 buffer to remove unbound proteins. Immunoprecipitates were treated with 1 mg·mL⁻¹ RNase A at 37 °C for 30 min. Bound proteins were eluted with 1× SDS sample buffer and resolved by 10% SDS/PAGE.

RT-PCR and quantitative real-time PCR

RT-PCR was used to detect the mRNA expression level. Extracted total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s instructions. The resulting cDNA was subjected to conventional PCR or quantitative real-time PCR analysis. Conventional PCR was performed using GoTaq DNA polymerase (Promega) and the forward and reverse primers: PACT [forward primer (FP): 5'-CCAGGGAAACACC GATTCA-3', reverse primer (RP): 5'-GCACGTGTATTTGCACTCAGA-3'], β-actin (FP: 5'-GCCCTGAGGCACCTTCCA-3' and RP: 5'-C GGTGTTCCAGTCTACACTTTCA-3') and TBP (FP: 5'-TGTC TCGGGCCTGTATCATT-3' and RP: 5'-TGGAACACA TCCACTGACACA-3').

Quantitative real-time PCR was performed using StepOnePlus™ Real-Time PCR Systems (Life Technologies) in accordance with the manufacturer’s instructions. The levels of mRNAs were detected with Fast SYBR® Green Master Mix (Applied Biosystems). In addition, quantitative real-time PCR of Fluc and Rluc was performed using the Custom TaqMan Assay Design Tool (Applied Biosystems). Quantitative analysis was performed by the measurement of C_t values during the exponential phase of amplification. Relative quantitation values were calculated using the 2⁻ΔΔC_t method.

Sucrose gradient sedimentation and polysome profiling

Cells were collected in cold PBS containing 100 μg·mL⁻¹ cycloheximide. All subsequent steps were performed at 4 °C. Cell pellets were resuspended in RSB-150 (10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂ and 150 mM NaCl) containing 100 μg·mL⁻¹ cycloheximide, 40 μg·mL⁻¹ digitonin (Calbiochem, San Diego, CA, USA), 20 U·mL⁻¹ RNAsin (Promega) and 1× protease inhibitor cocktail (Thermo Scientific). After incubation on ice for 5 min, cells were disrupted by passage through a 26-gauge needle five times. Cytoplasmic extracts were collected by centrifugation at 3000 g for 2 min, and clarified by further centrifugation at 17 000 g for 15 min. The samples were loaded on a linear 15-40% sucrose gradient and centrifuged at 178 000 g for 3 h. After centrifugation, total RNA was extracted from each fraction using phenol/chloroform extraction in the presence of 1% SDS and 0.25 mM NaCl, followed by ethanol precipitation. For polysome profile analysis, the gradients were monitored at 254 nm using an ISCO fractionation system (Teledyne ISCO Inc., Lincoln, NE, USA).

In vivo translation assay

DDX3-knockdown and mock-treated HeLa cells were transfected with a pFL-SV40 derived reporter (0.2 μg) and the control pRL-SV40 vector (0.2 μg). At 24 h post-transfection, cells were lysed in 1× Passive Lysis Buffer (Promega). For the HCV core overexpression assay, 1 μg of plasmids encoding GFP or HCV core-GFP were co-transfected with a pFL-SV40 derived reporter (0.2 μg) and the control pRL-SV40 vector (0.2 μg) into HeLa cells (2 × 10⁵ cells well⁻¹). At 48 h post-transfection, cells were lysed in 1× Passive Lysis Buffer (Promega). The activities of firefly luciferase and Renilla luciferase were measured using the Dual-Luciferase Reporter Assay System (Promega).

Immunofluorescent staining

DDX3-core-GFP was transiently expressed in HeLa cells for 24 h. Immunofluorescent staining of HeLa cells was performed as described previously [14]. Briefly, cells grown on cover slips were rinsed with cold PBS and fixed with 3% formaldehyde in PBS for 30 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min. After washing with PBS, cells were blocked with 1% BSA in PBS.
for 30 min and then incubated with the relevant primary antibodies in PBS at RT for 1 h. The primary antibodies used included mouse anti-DDX3 (0.4 μg·mL⁻¹; Santa Cruz Biotechnology), rabbit anti-eIF4A1 (1 μg·mL⁻¹; Abcam), mouse anti-HuR (1 μg·mL⁻¹; Santa Cruz Biotechnology) and mouse anti-NCL (0.4 μg·mL⁻¹; Santa Cruz Biotechnology). After washing with PBS twice, cells were incubated with appropriate secondary antibodies, including Alexa Fluor® 555 goat anti-mouse IgG (2 μg·mL⁻¹; Invitrogen) and Alexa Fluor® 555 goat anti-rabbit IgG (2 μg·mL⁻¹; Invitrogen), in PBS at RT for 1 h. After extensive washing with PBS, the specimens were mounted immediately and observed using an inverted fluorescence microscope (TE2000-U; Nikon Eclipse, Tokyo, Japan) equipped with a charge-coupled device camera.

Statistical analysis
All data are reported as the mean ± SE and were analyzed using PRISM, version 4.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed using an unpaired t-test. P < 0.05 was considered statistically significant.

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Author contributions
MCL performed most of the experiments. MCL and WYT planned the experiments. MCL analyzed the data. MCL, HSS, SWW and WYT wrote the paper.

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