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RACK1 controls IRES-mediated translation of viruses

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SUMMARY

Fighting viral infections is hampered by the scarcity of viral targets and their variability resulting in development of resistance. Viruses depend on cellular molecules for their life cycle, which are attractive alternative targets, provided that they are dispensable for normal cell functions. Using the model organism *Drosophila melanogaster*, we identify the ribosomal protein RACK1 as a cellular factor required for infection by internal ribosome entry site (IRES)-containing viruses. We further show that RACK1 is an essential determinant for hepatitis C virus translation and infection indicating that its function is conserved for distantly related human and fly viruses. Inhibition of RACK1 does not affect *Drosophila* or human cell viability and proliferation, and RACK1-silenced adult flies are viable, indicating that this protein is not essential for general translation. Our

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AUTHOR CONTRIBUTIONS

KM and LMH performed and analyzed most experiments in drosophila and Huh7.5.1 cells, respectively. CM and AG designed, performed or supervised, and analyzed the data for the experiments in flies and in Fig. 6. SM prepared Fig. S6, and AF analyzed the polysome microarray expression data. YV and JV identified RACK1 as a protein associating with AGO2 and R2D2 in DCV infected cells. FM designed, performed or supervised, and analyzed the data for the polysome and *in vitro* translation experiments. JAH, TFB, CS and JLI designed the experiments, analyzed the data and wrote the article.

SUPPLEMENTAL INFORMATION

Supplemental information includes Extended Experimental procedures, six Supplementary Figures and two Supplementary Tables. The microarray data presented in Figure 4H have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database under the accession number GSE60374.

findings demonstrate a specific function for RACK1 in selective mRNA translation and uncover a new target for the development of broad antiviral intervention.

INTRODUCTION

Viral infections are a significant threat for all living organisms. In humans, acute and chronic viral infections cause a wide spectrum of diseases, including life-threatening inflammation and cancer. A major challenge for the control of viral infections is that viruses, due to the small size of their genomes, offer few intrinsic targets either for recognition by the immune system or for inhibition by antiviral effector molecules. Furthermore, the error-prone viral polymerases allow RNA viruses to rapidly escape detection by the immune system and to resist the adverse effects of directly acting antiviral molecules. Significantly, viruses rely on numerous host factors for essential functions during their life cycle. These are not subject to rapid sequence changes and hence provide good alternative targets for antiviral therapy. Therefore, a central challenge is to identify cellular factors required for viral replication but dispensable for normal cell function.

RNA replication, transcription and translation are critical steps in the life cycle of RNA viruses, which involve interactions with host-cell molecules. In the model organism *Drosophila melanogaster*, the small interfering (si) RNA pathway targets viral RNAs (reviewed in (Ding, 2010)). In order to better characterize the contribution of the three core components of this pathway, Dicer-2, R2D2 and AGO2, we performed a proteomic analysis of the complexes assembling around these molecules in infected *Drosophil*a cells (in preparation). One protein copurifying with R2D2 and AGO2 in cells infected with the picorna-like *Drosophila* C virus (DCV) was the evolutionarily conserved ribosomal protein RACK1. The RACK1 protein has been extensively studied during the last two decades, and shown to be involved in different aspects of cell regulation. RACK1 is an adapter protein, interacting with a variety of signaling molecules (e.g. PKC, Src, MAPK) (Belozerov et al., 2014; Gibson, 2012; Long et al., 2014), and is a component of the 40S subunit of the ribosome (Coyle et al., 2009; Sengupta et al., 2004). RACK1 is thus ideally suited to connect signal transduction pathways to the regulation of translation (Nilsson et al., 2004). Indeed, RACK1 was found to interact with the initiation factor eIF6, which associates with the 60S subunit of the ribosome, and prevents its association with the 40S subunit. eIF6 phosphorylation by RACK1-assisted PKC triggers its release from the 60S subunit, thus promoting the formation of 80S active ribosomes (Ceci et al., 2003).

Here, we show that RACK1 is mandatory for DCV replication, but largely dispensable for cell viability and proliferation. We further demonstrate that RACK1 is required for internal ribosome entry site (IRES)-dependent translation in *Drosophila*, and in human hepatocytes, where this factor is an essential determinant of hepatitis C virus infection. By contrast, RACK1 is not required for 5′ cap-dependent translation. Collectively, our data unravel a specific function for ribosomal protein RACK1 in selective mRNA translation of fly and human viruses and uncover a previously undiscovered target for the development of broad antiviral intervention.

RESULTS

RACK1 is required for *Dicistroviridae* infection in *Drosophila*

In a proteomic analysis of the interactome of Dicer-2, R2D2 and AGO2 in virus infected cells, to be reported elsewhere, we identified 16 ribosomal proteins. To address the functional relevance of this finding, we systematically depleted these ribosomal proteins from S2 cells by RNAi, and tested DCV replication. Knockdown of most ribosomal genes affected cell viability or proliferation and did not yield interpretable results with regards to DCV infection (Figure 1A, B). Indeed, silencing of these genes may result in decreased ability of the cells either to support viral replication, or to control the infection. By contrast, depletion of RACK1 (Figure S1A) did not affect cell viability or proliferation in S2 cells (Figure 1B, C) or in two other cell lines (Figure S1B). However, it resulted in a significant decrease of DCV titer in infected cells (Figure 1A). Furthermore, RACK1 silencing did not affect replication of either FHV or VSV (Figure 2A, B), indicating that the RACK1-depleted cells are not only viable and able to proliferate, but can also support replication of other viruses. To test whether the effect of RACK1 was specific to DCV, or to the family to which it belongs, we infected S2 cells with Cricket Paralysis Virus (CrPV), another member of the *Dicistroviridae* family. Replication of CrPV was also strongly impaired when RACK1 was depleted (Figure 2B).

We next confirmed these findings *in vivo*. RACK1 null mutant flies are not viable, indicating that RACK1 exerts developmental functions (Kadrmas et al., 2007). In agreement with this finding, silencing RACK1 expression with a small hairpin (sh) RNA driven by the broadly active *actin5C* promoter was embryonic lethal. When the thermosensitive Gal80 system was used to express the shRNA only in adult flies, development occurred normally and the adult flies expressed significantly reduced levels of RACK1 at the permissive temperature of 29°C (Figure 2C). The reduced levels of RACK1 did not affect the viability of the flies, although it reduced longevity by 20% at this temperature. In addition, the eggs laid by RACK1-silenced females showed a phenotype similar to that of RACK1 mutants (Figure S1C) (Kadrmas et al., 2007). Thus, even though RACK1 is required during development, it appears to be largely dispensable in adult flies. As expected, when these flies were challenged with DCV, both viral RNA and capsid protein levels were markedly reduced at 1 and 2 days post-infection compared to controls (Figure 2D). Overall, our data indicate that replication of the *Dicistroviridae* DCV and CrPV requires the ribosomal factor RACK1, which is otherwise dispensable for the viability of S2 cells and adult flies.

RACK1 is required for viral IRES-dependent translation

Our data indicate that RACK1 is required for a step of viral replication specific to *Dicistroviridae*. Whereas FHV and VSV use a canonical strategy of cap-dependent initiation of translation, DCV and CrPV RNA recruits the 40S ribosomal subunit through IRES sequences to initiate translation (Figure S2A). Furthermore, although initially identified as a scaffolding protein involved in protein kinase C signaling, RACK1 is now recognized as a component of the 40S subunit of the ribosome. This suggested to us that RACK1 was required for viral translation. We first verified that RACK1 is indeed required at the ribosome level for CrPV replication. We silenced RACK1 expression in a stable cell line

using an shRNA targeting the 5′ untranslated region (Figure S1D), and observed a marked decrease in CrPV replication (Figure S1E). Transfection of a vector expressing wild-type RACK1 restored CrPV replication in these cells (Figure 3A). By contrast, expression of mutant proteins unable to interact with either RpS17 (D108Y) (Kuroha et al., 2010) or 18S rRNA (R38D/K40E) (Coyle et al., 2009) did not rescue CrPV replication (Figure 3A). We conclude that RACK1 is required in the 40S ribosomal subunit for CrPV replication.

To confirm that RACK1 is involved in translation from *Dicistroviridae* RNAs, we tested whether its depletion affected translation of luciferase reporters placed under the control of the two IRES elements from CrPV (Figure 3B and Figure S2C). Translation of a 5′ cap-dependent RNA was not affected in the absence of RACK1, although it was affected when expression of eIF4E was knocked down. Translation from the CrPV 5′ IRES reporter was not reduced, and was even slightly increased, when eIF4E was silenced, suggesting that the 5′ IRES drives non canonical translation. Interestingly, a significant reduction of luciferase production was observed for the 5′ IRES reporter in RACK1 silenced cells (Figure 3B). Silencing of RACK1 did not affect the amount of the 5′ IRES reporter luciferase mRNA in the cells, indicating that RACK1 affects translation, rather than RNA stability (Figure S3). By contrast, translation driven by the intergenic (IGR) IRES (Jan and Sarnow, 2002; Spahn et al., 2004) was not affected by the level of RACK1 in the cells (Figure 3B). Polysome profiles from S2 cells and RACK1-silenced stable derivatives of these cells (Figure S1D) were similar, confirming that RACK1 does not affect significantly general translation (Figure 3C). Finally, we prepared cell-free translation extracts from control and RACK1-depleted S2 cells, and used them to monitor translation of *in vitro* transcribed, capped and IRES-dependent RNAs. Translation of the 5′ IRES reporter RNA was strongly reduced in the RACK1 depleted extract. By contrast, translation of the 5′ CAP and IGR IRES dependent reporters was not inhibited and was even slightly stimulated (Figure 3D). Overall, our data indicate that ribosomal RACK1 is required for IRES-dependent translation of *Dicistroviridae* both *ex vivo* and *in vitro*.

RACK1 is an essential host factor for HCV infection

RACK1 is an evolutionarily strongly conserved factor, and we asked whether it plays a role in the translation driven by the IRES of a mammalian virus. Hepatitis C virus (HCV), a major cause of liver disease and hepatocellular carcinoma, is a positive strand RNA virus member of the *Flaviviridae* family depending on a highly structured IRES for its translation (Figure S2) (Spahn et al., 2001). Transfection of an siRNA targeting RACK1 markedly reduced expression of the protein in Huh7.5.1 cells (Figure 4A), a human hepatocyte-derived cell line highly permissive for HCV infection (Lindenbach et al., 2005; Wakita et al., 2005). Infection of RACK1-depleted Huh7.5.1 cells by cell culture-derived HCV (Jc1 strain) was strongly and significantly reduced, as revealed both by immunodetection of the viral core protein (Figure 4A) and the focus forming assay performed by infection of naïve Huh7.5.1 cells with supernatants from infected and treated cells (Figure 4B). A similar inhibition of infection was observed for HCV Luc-Jc1 (Figure 4C), a well-characterized recombinant virus expressing a luciferase reporter (Figure S2B). Inhibition of RACK1 expression was as efficient as the silencing of the key HCV host factors CD81 (Koutsoudakis et al., 2007) and Cyclophilin A (CypA) (Kaul et al., 2009) (Figure 4A, B, C).

We next transiently depleted RACK1 in Huh7.5.1 cells replicating the reporter virus HCV Luc-Jc1, and observed a marked impairment of HCV replication (Figure 4D), demonstrating that RACK1 is required for HCV translation/replication rather than entry. HCV replication rebound observed after day 4 was due to progressive loss of RACK1 silencing leading to neosynthesis of RACK1 (Figure S4A).

To confirm that the inhibition of HCV replication is indeed mediated by the effect of RACK1 on IRES-mediated translation, we established stable cell lines expressing an $IRES_{HCV}$-luciferase reporter construct or a classical capped reporter gene (Figure S2C), and transfected these cells with RACK1-specific siRNAs. Silencing of RACK1 markedly and specifically decreased $IRES_{HCV}$-dependent translation, to a similar extent as an antiviral siRNA directed against the $IRES_{HCV}$ (Figure 4E). By contrast, silencing of ribosomal protein RPS3 inhibited translation from both IRES- and 5′ cap-dependent reporter constructs (Figure 4E). Similar results were obtained when *in vitro* transcribed reporter mRNAs were transfected into Huh7.5.1 cells, ruling out an effect of RACK1 on transcription of the $IRES_{HCV}$-luciferase reporter gene (Figure 4F).

Importantly, RACK1-specific siRNAs did not affect cell proliferation (Figure S4B) or viability, in contrast to silencing of the ribosomal protein RPS3 (Figure 4G). A genome-wide microarray analysis of polysomes prepared from control or RACK1-silenced human Huh7.5.1 cells revealed that the amount in polysomes of mRNAs for most genes, including house keeping genes and important hepatocyte specific genes such as albumin or lipoproteins, was not affected by RACK1 depletion (Figure 4H). Of note, silencing of RACK1 also did not affect the presence of 5′ terminal oligopyrimidine tract (TOP) mRNAs in polysomes (for details, see Supplemental information). This result suggests that translation of the large majority of mRNAs is not affected by the absence of RACK1 in human hepatocytes under normal culture conditions and confirms the results obtained in the model organism *Drosophila*.

The effect of RACK1 on viral translation is independent of the miRNA pathway

While this work was in progress, a role for RACK1 in miRNA function was reported in the plant *Arabidopsis thaliana* (Speth et al., 2013), the model organism *Caenorhabditis elegans* (Chu et al., 2014; Jannot et al., 2011) and humans (Otsuka et al., 2011). In light of the important impact of the cellular microRNA miR122 on HCV replication (Jopling et al., 2005), this suggested that RACK1 might operate on viral translation through the miRNA pathway. We first verified that RACK1 affects the miRNA pathway in *Drosophila*. Expression in S2 cells of two previously described miRNA reporters, *Par-6* and *nerfin-1* (Eulalio et al., 2007), was derepressed when RACK1 was silenced, indicating that in *Drosophila* as well, RACK1 is involved in miRNA function (Figure 5A, B). We note however that the derepression is much stronger for the miR1 reporter than for the miR9b reporter, suggesting that the role of RACK1 may be specific of a subset of miRNAs. By contrast, silencing of *Dcr-1* or *AGO1* derepressed equally well the two miR reporters (Figure 5A, B). We next tested whether miRNAs play a role in viral replication, by monitoring accumulation of viral RNAs in cells depleted of *Dcr-1* or *AGO1*. Silencing of *Dcr-1* had no effect on the viral RNA load of the four viruses tested (Figure 5C). Silencing

of *AGO1* did reduce to some extent CrPV and DCV RNA load. However, this reduction was variable in the case of DCV, and not to the extent of the reduction observed when *RACK1* was silenced for DCV and CrPV (Figure 5C). Thus, although the miRNA pathway may have a contribution in the replication of *Dicistroviridae*, our data suggest that the strong effect of RACK1 cannot be accounted for only by its effect on miRNA function. This was confirmed by the observation that silencing of *Dcr-1* or *AGO1* had no effect on translation driven by the $IRES_{CrPV\text{-}5'}$, unlike silencing of RACK1 (Figure 5D).

In mammalian hepatocytes, HCV translation depends on AGO2 and miR122 (Conrad et al., 2013; Roberts et al., 2011). As expected, transfection of Huh7.5.1 cells with a miR122 mimic increased HCV replication, while transfection of a miR122 inhibitor led to decreased viral replication (Figure 5E). Importantly, the impact of the miR122 mimic and the miR122 inhibitor on HCV replication did not depend on RACK1 (Figure 5E). To unambiguously determine whether the contribution of RACK1 to HCV translation was dependent on miR122, we used HEK-293T cells, which do not express miR122 ((Da Costa et al., 2012), Figure S5). Silencing of RACK1 expression efficiently repressed translation driven by the $IRES_{HCV}$ in these cells (Figure 5F). Finally, transduction of HEK-293T cells with an expression vector for miR122 did not affect the impact of RACK1 on HCV translation (Figure 5G), although miR122 was expressed and functional in these cells (Figure S5A, B). Collectively, these results indicate that RACK1 and miR122 regulate HCV translation by different mechanisms.

The eIF3j subunit is dispensable for cell viability, but important for CrPV and HCV replication

We next attempted to gain mechanistic insight on the role of RACK1 in viral translation. Previous cryo-electron microscopy studies have highlighted the interaction of the 40S subunit with the HCV IRES and, in spite of their low resolution, have suggested that binding of the HCV IRES triggers a pronounced conformational change in the small subunit of the ribosome (Spahn et al., 2001; 2004). HCV IRES has been also visualized on the 80S human ribosome and RACK1 localized in its vicinity (Boehringer et al., 2005; Sengupta et al., 2004). The recently elucidated crystal structure of the small subunit of the ribosome at 3.9Å (Rabl et al., 2011) allows to fit the crystal structure in the cryo-electron microscopy density. The picture obtained suggests that RACK1 is located in close proximity to the IRES of HCV in the region affected by the conformational change triggered upon $IRES_{HCV}$ binding (Figure S6A). By contrast, the $IRES_{CrPV\text{-}IGR}$, which does not depend on RACK1 (Figure 2C), interacts with a distinct site of the 40S subunit, directly contacting RpS25 (Figure S6B) (Fernandez et al., 2014; Koh et al., 2014; Schuler et al., 2006; Spahn et al., 2004). Although no direct contacts between RACK1 and $IRES_{HCV}$ could be observed, a recent study indicates that a peripheral domain of the translation initiation factor eIF3, which is required for $IRES_{HCV}$-dependent translation (Kieft, 2008), is in contact with RACK1 (Figure S6C) (Hashem et al., 2013a; Sun et al., 2013). This domain may be the functional link between RACK1 and $IRES_{HCV}$-dependent translation.

We asked whether some subunits of eIF3, such as eIF3c, which has been shown to interact with RACK1 in yeast, may be specifically involved in IRES-dependent translation, like

RACK1. We first tested in *Drosophila* S2 cells whether some subunits of the eIF3 complex are dispensable for cell viability in normal culture conditions. Out of the 14 genes encoding eIF3 components (the Drosophila genome contains two *eIF3g* paralogues, *CG8636* (*eIF3ga*) and *CG10881* (*eIF3gb*)), only two were not required for cell viability or proliferation (Figure 6A). One of these genes is *CG10881*, encoding eIF3gb, which is expressed specifically in testis (Chintapalli et al., 2007) and thus provides a useful negative control. The second gene is the *Drosophila* orthologue of *eIF3j* (Figure 6A). We next monitored CrPV replication in cells silenced for eIF3j or eIF3gb (*CG10881*). Although silencing of eIF3gb did not affect CrPV replication, silencing of eIF3j resulted in a significant reduction of CrPV replication (Figure 6B). Silencing of eIF3j, but not of *eIF3gb*, also affected translation of the $IRES_{CrPV5'}$-luciferase reporter, although not as strongly as silencing of RACK1 (Figure 6C). In Huh7.5.1 cells, silencing of eIF3c affected cell viability. By contrast, silencing of eIF3j only marginally affected cell viability (Figure 6D and (Wagner et al., 2014)). Interestingly however, it resulted in a moderate but significant decrease of HCV replication (Figure 6E). Altogether, these results suggest that the eIF3j subunit might participate in the observed effects of RACK1 on translation.

DISCUSSION

A new function for RACK1 in IRES-dependent translation

Our data reveal a new function for RACK1 in specific mRNA translation. Indeed, silencing RACK1 expression does not affect viability of *Drosophila* S2 or human Huh7.5.1 cells in tissue culture, indicating that formation of active ribosomes is not strictly dependent on RACK1. *In vivo* as well, translation can occur in the absence of RACK1, as lethality in *RACK1* mutant animals does not occur before larval stages for *Drosophila* and gastrulation in mice (Kadrmas et al., 2007; Volta et al., 2013). In agreement with this observation, translation of a 5′ cap-dependent reporter was not affected in the absence of RACK1 in *Drosophila* and human cells. Nevertheless, the fact that *RACK1* mutant animals cannot complete their development suggests that this protein is required for the translation of some cellular mRNAs, in addition to viral IRES-containing RNAs. Interestingly, previous studies have highlighted the role of another protein from the 40S subunit of the ribosome, RpS25, in IRES-dependent translation (Landry et al., 2009). Performed on yeast and mammalian tissue-culture cells with IRES reporter assays, these experiments concluded that RpS25 is essential for the activity of two viral IRES, $IRES_{HCV}$ and $IRES_{CrPV\text{-}IGR}$. The mechanism used by RpS25 and RACK1 to promote translation is probably different because (i) RpS25 is required for $IRES_{CrPV\text{-}IGR}$, unlike RACK1; and (ii) structural data place RpS25 at a distance from RACK1 on the 40S subunit of the ribosome, providing an explanation for its importance on the activity of the $IRES_{CrPV\text{-}IGR}$. Several other ribosomal proteins (e.g. RpL38, RpL40) were recently proposed to be involved in specific translation of some 5′ cap-dependent mRNAs (Kondrashov et al., 2011; Lee et al., 2013), indicating that transcript-specific regulation can occur in the absence of IRES elements. Our data lend support to an evolving picture of the eukaryotic ribosome, which includes structurally peripheral components such as RACK1 involved in the modulation of translation of specific mRNAs (reviewed in (Xue and Barna, 2012)). They have implications for the development of new

antivirals, and raise questions on the mechanism underlying the role of RACK1 in IRES-dependent translation.

RACK1 as a target for broad antiviral intervention

Our results open interesting therapeutic perspectives for a broad range of viral infections including chronic hepatitis C, a major cause of liver cirrhosis and cancer. Because HCV translation initiates viral genome neosynthesis via the formation of the replication complex, RACK1-mediated translation is a crucial step in virus propagation. Thus, RACK1 is a novel host target for antiviral therapy, which is complementary to interferon-based therapies or direct-acting antivirals (DAAs). DAAs have achieved high response rates with cure in late-stage clinical trials, but high costs will limit their broad access. In addition, certain patient groups (e.g. genotype 3, renal failure, hepatic decompensation, liver transplantation) will need complementary approaches (Chung and Baumert, 2014; Liang and Ghany, 2013).

The low variability of host factors targeted by host-targeted antivirals (HTAs) results in a high genetic barrier to resistance (Nathan, 2012). Indeed, HTAs effectively inhibit HCV escape variants (Fofana et al., 2010; Lupberger et al., 2011), as well as DAA-resistant virus (Xiao et al., 2014a). Furthermore, their complementary mechanism of action results in synergy with DAAs (Xiao et al., 2014b). Given that HTAs interfere with host targets, one theoretical caveat is the possibly greater risk of cellular toxicity as compared to DAAs. Interestingly, our data obtained in cell culture models did not reveal any major toxicity linked to RACK1 inhibition. Thus, our proof-of-concept studies in state-of-the-art cell culture models open a highly attractive and innovative perspective to develop small molecules targeting RACK1. RACK1 inhibitors may also be of interest for treatment of infection of many other human or animal viruses using 5′ cap-independent mechanisms for the translation of their RNAs.

Mechanistic insight on the role of RACK1 in IRES-dependent translation

While this work was in preparation, several reports described a role for RACK1 in miRNA function. However, our data in *Drosophila* and human cells indicate that the role of RACK1 in IRES-dependent translation does not involve small regulatory RNAs. Nevertheless, the connection between RACK1 and AGO proteins is intriguing, and suggests that RACK1 may participate in a checkpoint for the control of the translation of specific mRNAs by miRNAs or siRNAs.

The ribosome code or filter hypothesis posits that some ribosomal proteins have evolved to mediate translation of specific mRNAs (Mauro and Edelman, 2002; Topisirovic and Sonenberg, 2011; Xue and Barna, 2012). A central unresolved issue of this hypothesis is the nature of the *cis*-acting elements defining a possible “ribosome code”. In the case of RACK1, these *cis*-acting elements include viral IRES. Interestingly, the $IRES_{CrPV\text{-}IGR}$ is active in the absence of RACK1, unlike the $IRES_{CrPV\text{-}5'}$ or the $IRES_{HCV}$. This $IRES_{CrPV\text{-}IGR}$ (class I IRES) is capable on its own, without any initiation factors, of binding directly the 40S subunit and of recruiting the 60S subunit to form an active 80S ribosome, thus bypassing the loading of the initiator methionyl-tRNAi (Jan and Sarnow, 2002; Pestova et al., 2004). By contrast, the function of $IRES_{HCV}$ (class II IRES) requires two canonical

eIFs, eIF2 and 3, as well as Met-tRNAi (Kieft, 2008). This suggests that the effect of RACK1 on translation initiation may require one of these factors. Interestingly, the eIF3 complex binds to the 40S ribosomal subunit, and to the $IRES_{HCV}$ (e.g. (Kieft et al., 2001)). Furthermore, RACK1 was shown to associate with one of the eIF3 subunits in order to assemble a translation pre-initiation complex in yeast (Hashem et al., 2013a; Kouba et al., 2012).

Although our understanding of the molecular structure of the core of the 13 subunits eIF3 complex has progressed remarkably in recent years (e.g.(Hashem et al., 2013b; Sun et al., 2011)), the role of the non-core subunits remains essentially untested in animals. Interestingly, the subunit eIF3e in the yeast *Schizosaccharomyces pombe* is involved in translation of a selected set of RNAs (Sha et al., 2009; Zhou et al., 2005). More recently, one of the two *eIF3h* genes present in zebrafish, *eIF3ha*, was shown to encode a factor specifically targeting crystalline isoform mRNAs for translation during lens development (Choudhuri et al., 2013). Our data indicate that, like RACK1, the subunit eIF3j is not required for cell viability in *Drosophila*, but is required for CrPV replication and $IRES_{CrPV5'}$ driven translation. This raises the possibility that RACK1 and eIF3j act together in translation of a specific subset of mRNAs.

Several observations support a role for eIF3j in selective mRNA translation. First, it is located in the decoding center of the 40S ribosomal subunit, where it can regulate access to the mRNA binding cleft (Fraser et al., 2007; 2009). Second, it is located at the periphery of the eIF3 complex, often in sub-stoichiometric quantities, indicating that it can undergo regulated cycles of association and dissociation (Hinnebusch, 2006; Miyamoto et al., 2005; Sha et al., 2009). Third, experiments in *S. pombe* and human cells indicate that it can be regulated post-translationally by phosphorylation (Sha et al., 2009) or caspase-mediated C-terminal truncation (Bushell et al., 2000). Altogether, this suggests that RACK1 may act as a scaffold recruiting an enzyme modifying eIF3j in order to allow access of the entry channel of the 40S subunit to IRES-containing mRNAs. In a way, such a scenario would be reminiscent of the recently described role of another eIF3 subunit, eIF3e, which controls the recruitment of the kinase Mnk1 to phosphorylate eIF4E, thus promoting selective mRNA translation in human cells (Walsh and Mohr, 2014).

EXPERIMENTAL PROCEDURES

Silencing candidate gene expression by RNAi and screening

DsRNAs targeting the candidate genes were designed using the E-RNAi algorithm (http://www.dkfz.de/signaling/e-rnai3/). Knock-down in *Drosophila* S2 cells was performed in 96-well plates using the bathing method, and cells were challenged with virus 4 days later. Viral load was determined by qRT-PCR. Alternatively, infected cells were fixed and labeled with anti-capsid antibodies for immunofluorescence analysis using the InCELL1000 Analyzer workstation (GE LifeSciences). Image data processing was performed using the InCELL Analyzer software. See Extended Experimental procedures in Supplemental material for more details.

Preparation of cell-free extract for *in vitro* translation

In vitro translation competent extracts were prepared from control or RACK1-silenced S2 cells as described in (Wakiyama et al., 2005). Briefly, cells were resuspended in lysis solution [40 mM Hepes–KOH (pH 8), 100 mM potassium acetate, 1 mM magnesium acetate, and 1 mM dithiothreitol] at a cell density of approximately 10^9 mL^{-1} and were placed in the Cell Disruption Bomb (Parr Instrument Company). The homogenate produced upon the pressure release was cleared by centrifugations at 4°C, and creatine kinase was added at 0.24 mg.mL $^{-1}$ of lysate, before storage in aliquotes at −80°C. Reporter mRNAs were synthesized by transcription *in vitro* using recombinant T7 RNA polymerase. A non-functional cap (ApppG) (New England Biolabs) was added at the 5′ end of the IRES monocistronic reporter mRNAs to protect them from degradation. Cap-dependent translation was measured with a Renilla Luciferase reporter mRNA that was capped with the ScriptCap m7G capping system (Epicentre Biotechnologies). *In vitro* translation was performed as previously described (Wakiyama et al., 2005) and under sub-saturating conditions to avoid substrate titration.

HCV infection and replication assays

Huh7.5.1 human hepatoma cells were infected with cell culture-derived HCV (HCVcc strains Jc1 and Luc-Jc1, half-maximal tissue culture infectious dose (TCID50 10^4 mL^{-1} for both viruses)) as described (Lupberger et al., 2011; Pietschmann et al., 2006). Two days before infection, gene silencing was performed by reverse transfection with 10 nM of siRNA (Silence®Select siRNA, Ambion) specific for RACK1, CD81, Cyclophilin A, HCV IRES or a nonspecific control siRNA. Viral infection and RACK1 depletion were analyzed by western blotting and quantified by counting of focus forming units (ffu)/mL following immunostaining using a HCV core-specific antibody (mAbC7-50, Affinity BioReagents, CO) or by luciferase reporter gene expression in cell lysates 3 days post infection. For HCV replication experiments, Huh7.5.1 cells were electroporated with HCV Luc-Jc1 RNA (Koutsoudakis et al., 2007). Three days later, cells were reverse transfected with siRNAs.

Supplementary Material

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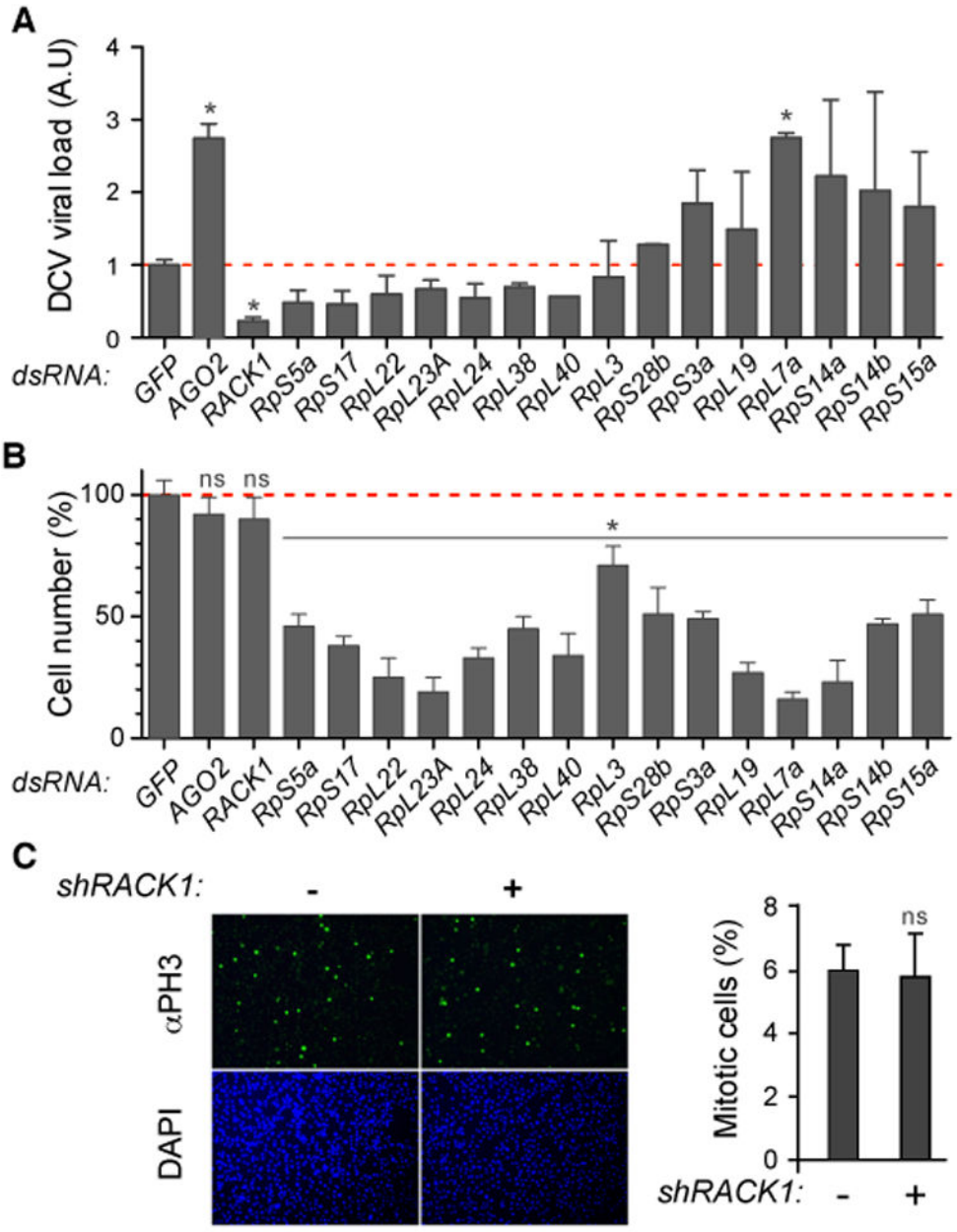

Figure 1. RACK1 is required for DCV replication, but not for viability or proliferation in *Drosophila* cells

(A, B) Quantification of DCV viral RNA levels by qRT-PCR (A) and of cell numbers as estimated by DAPI staining (B) in cells treated with the indicated dsRNAs to induce silencing. Cells treated with a dsRNA corresponding to GFP and AGO2 sequences are used as a reference and a control, respectively. (C) S2 cells stably transfected with a metallothionein promoter driven vector expressing a shRNA targeting the 5′ UTR from the *RACK1* gene were treated or not with CuSO4 for three days, stained with DAPI and an anti-phospho H3 antibody (left panels) and counted (right panel). Data represent the mean and s.e.m. of at least three independent experiments. ns: non significant; * $p<0.05$. See also Figure S1 and Table S1.

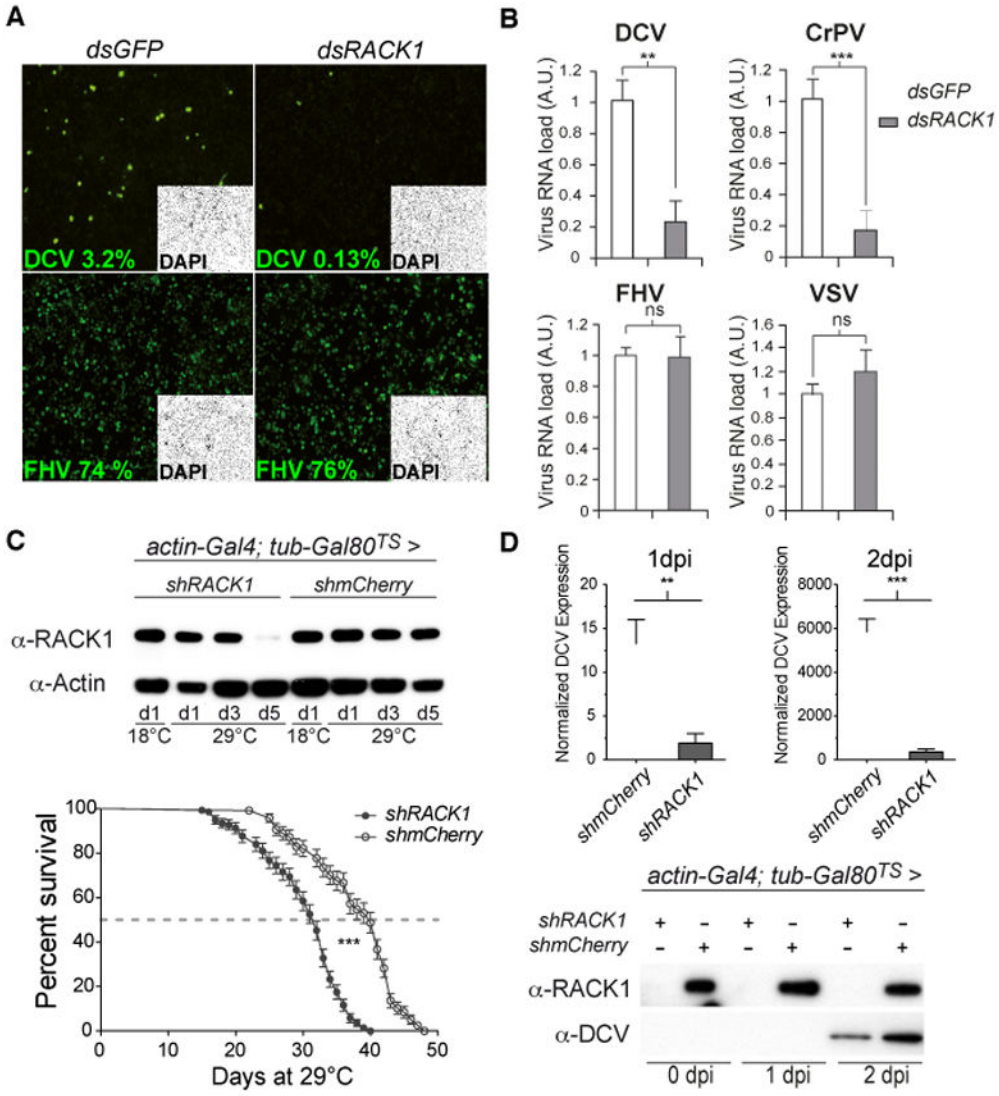

Figure 2. RACK1 is required for replication of DCV and CrPV, but not FHV and VSV

(A, B) S2 cells were treated with either control (*GFP*) or *RACK1* dsRNA for 4 days, before challenge with DCV, FHV, VSV or CrPV. Viral infection was monitored by immunofluorescence (A) and qRT-PCR (B) 16h or, in the case of VSV, 48h later using antibodies recognizing capsid proteins. The percentage of infected cells is indicated for each virus in panel A. (C) Silencing of *RACK1* expression in transgenic flies expressing a shRNA targeting the 5′ UTR from the *RACK1* gene, using the Gal4-UAS system and the broadly expressed actin-Gal4 driver controlled by the thermosensitive (TS) tub-Gal80 repressor. A shRNA targeting the mCherry protein was used as a control. The life span of RACK1 depleted flies is shown in the bottom graph. (D) *RACK1* silenced flies infected by DCV after 5 days at 29°C show a decrease of the viral RNA and protein, as indicated by qRT-PCR (upper panel) and western blot. Data represent the mean and s.e.m. from at least three independent experiments. *ns*: non significant; dpi: days post-infection; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. See also Figures S1, S2.

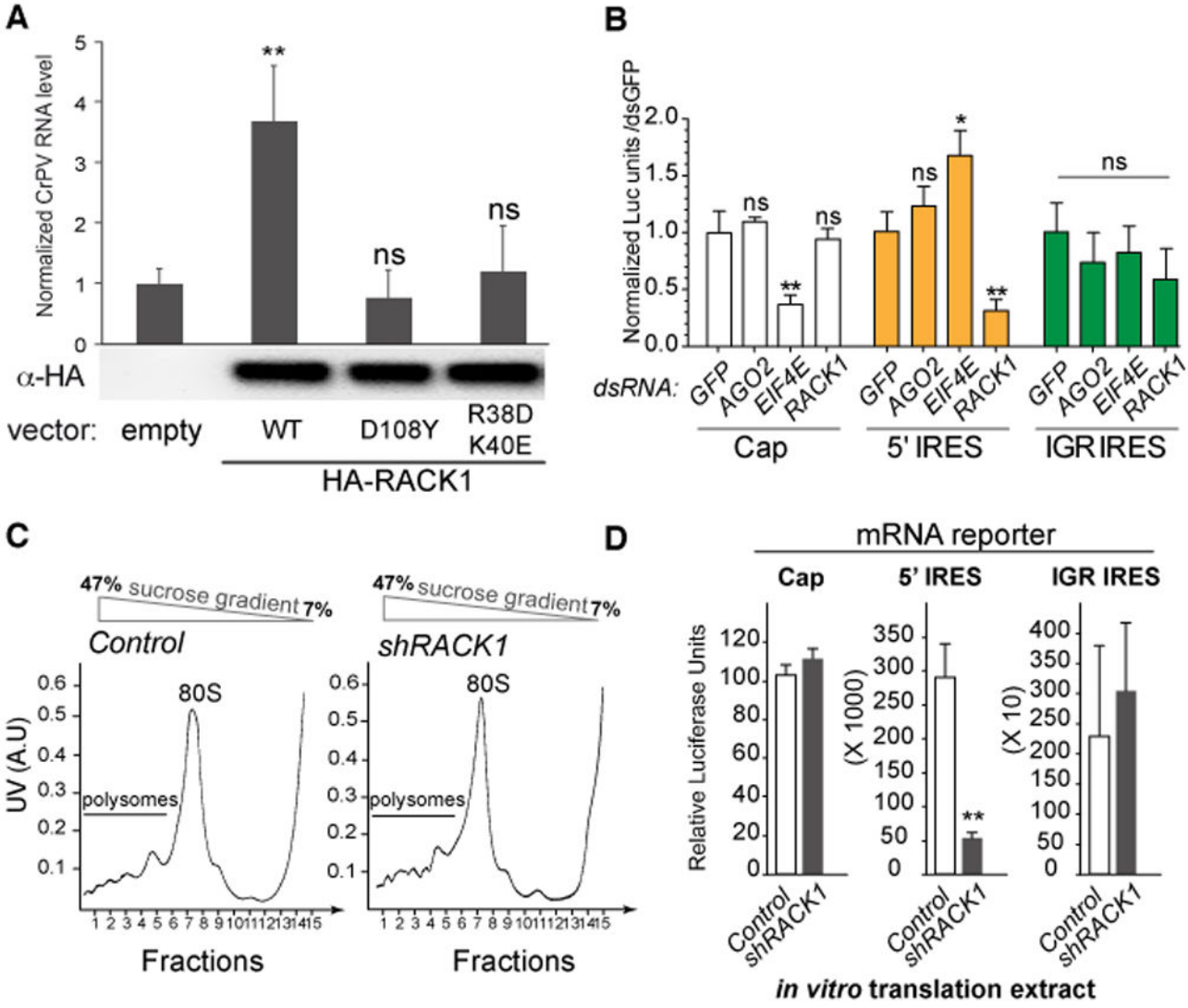

Figure 3. The ribosomal protein RACK1 is required for IRES-mediated translation

(A) Stable S2 transformants expressing a shRNA targeting the 5′ UTR of *RACK1* were transfected with vectors expressing three versions of RACK1 (WT, D108Y or R38D/K40E). Expression of the transfected RACK1 was monitored by western blot using an antibody recognizing the N-terminal tag HA. The cells were infected with CrPV for 16h, and viral RNA loads were determined by qRT-PCR. Data represent the mean and s.e.m. from three independent experiments. (B) RACK1 is required for translation regulated by the 5′ IRES, but not the intergenic (IGR) IRES, of CrPV. S2 cells were treated with dsRNAs corresponding to *GFP* (control), *AGO2*, *eIF4E* or *RACK1* for 3 days, before transfection of the indicated Luciferase reporters (5′CAP, $IRES_{CrPV\text{-}IGR}$ or $IRES_{CrPV\text{-}5'}$; see Fig. S2). Luciferase activity was monitored 48h later. The ratio of the activity of the IRES-dependent luciferase and the 5′ cap-dependent luciferase is plotted and normalized to the control for the three reporters. Data represent the mean and s.d. from six independent experiments. (C) Polysome profiles from S2 cells expressing or not a shRNA targeting the 5′ UTR of

RACK1. The position of the peaks corresponding to the 80S ribosomes and the polysomes are indicated. (D) *In vitro* translation of capped and IRES-dependent reporters using cell free extracts prepared from control or RACK1-silenced S2 cells. Data represent the mean and s.d. from three independent experiments. ns: non significant, * $p<0.05$, ** $p<0.01$. See also Figures S2, S3.

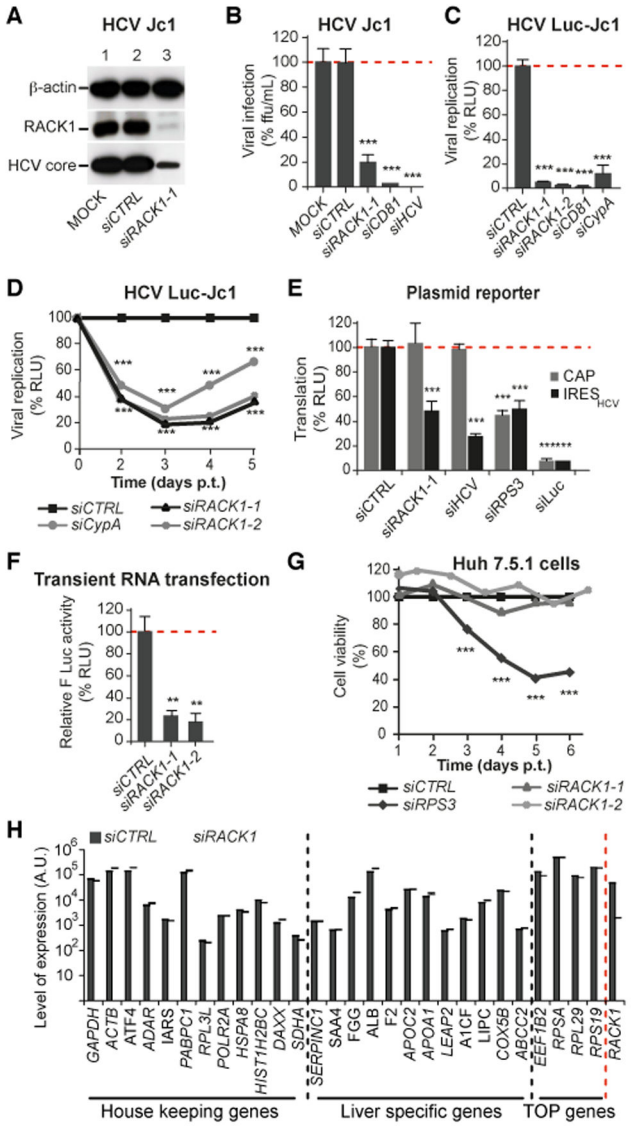

Figure 4. RACK1 is a specific host-factor required for IRES-mediated translation of HCV

(A–C) Huh7.5.1 cells were transfected with siRNAs either control (siCTRL) or targeting RACK1 (siRACK1-1 or -2), CD81 (siCD81), Cyclophylin A (siCypA), or HCV IRES (siHCV) before infection three days later with HCV Jc1 (A, B), or HCV Luc-Jc1 (C). Viral infection was monitored 3 days post-infection, by immunoblotting using antibodies recognizing HCV core protein (A); by counting foci forming units (ffu/ml) (B); or by quantifying luciferase activity (C). (D) HCV Luc-Jc1 replicating cells were transfected with siCTRL, two different siRNAs targeting RACK1 or siCypA, and replication was monitored during 5 days by luciferase activity quantification. (E) Huh7.5.1. cell lines stably expressing an IRES ($IRES_{HCV}$-Luc) or a 5′ cap (CTRL-Luc) dependent luciferase reporter gene were transfected with siCTRL, siRACK1, siHCV, siRPS3 or siLuc. Translation was monitored 72h later by luciferase activity quantification. (F) Huh7.5.1 cells were transfected with the indicated siRNAs and, 72 h later with *in vitro* transcribed $IRES_{HCV}$ or 5′ cap dependent luciferase mRNAs. Luciferase activity was monitored 5h later. (G) Cell viability of

Huh7.5.1 cells silenced with the indicated siRNAs was measured during 5 days using MTT assay. ** $p<0.01$; *** $p<0.005$. (H) Quantification of representative mRNAs in polysomes prepared from Huh7.5.1 cells transfected with siCTRL or siRACK1. Gene expression levels, shown in arbitrary units, was determined by hybridization on genome wide microarrays, and represent the mean +/− s.d. of 4 individual samples. Each sample was analyzed individually. See also Figures S2, S4 and Table S2.

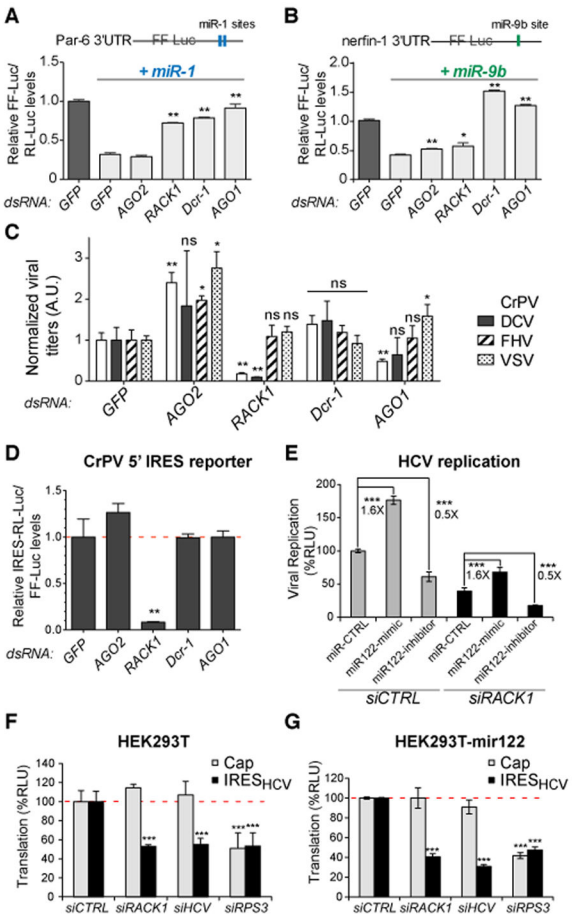

Figure 5. The effect of RACK1 on viral translation is independent of the miRNA pathway

(A, B) RACK1 is required for miR1 and miR9b silencing. The structure of the *Par-6 3′UTR* and *nerfin-1 3′UTR* reporter constructs is represented on top, and the luciferase activity in cells silenced for the indicated genes is shown below. (C) Effect of the depletion of *AGO1*, *Dcr-1* and *RACK1* on replication in *Drosophila* S2 cells of CrPV, DCV, FHV and VSV. Cells were transfected with the indicated dsRNAs, and infected four days later. Viral RNA was extracted 24hpi, and quantified by qRT-PCR. (D) Silencing of *AGO1* or *Dcr-1* does not affect the activity of a Luciferase reporter gene controlled by the $IRES_{CrPV\text{-}5'}$ in *Drosophila* S2 cells. (E) A miR122 mimic and a miR122 inhibitor affect HCV replication similarly in control or *RACK1*-silenced Huh7.5.1 hepatocytes. (F–G) Silencing of *RACK1* affects the activity of the $IRES_{HCV}$-luciferase reporter in miR122 deficient (F) and stably transfected miR122 expressing (G) HEK-293T cells, respectively. Data represent the mean and s.e.m. of at least three independent experiments. ns: non significant; * p<0.05; ** p<0.01, *** p<0.001. See also Figure S5.

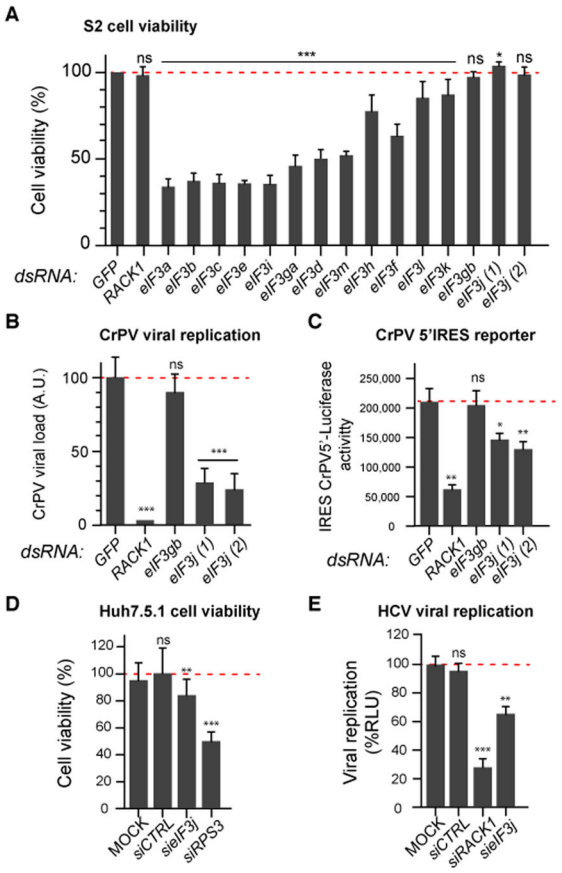

Figure 6. eIF3j is required for CrPV and HCV replication, but not for cell viability

(A) Quantification by the MTS assay of the number of viable cells 5 days after treatment of S2 cells with the indicated dsRNAs. Two different dsRNA preparations, targeting different regions of the gene, were used for *eIF3j*. (B) Quantification by qRT-PCR of CrPV viral RNA levels in S2 cells treated with the indicated dsRNAs. (C) Activity of the $IRES_{CrPV5'}$ in S2 cells silenced for the indicated genes. (D) Quantification of Huh7.5.1 cell viability after silencing of the indicated genes. (E) Quantification of HCV replication in Huh7.5.1 cells transfected with the indicated siRNAs. See also Figure S6.