Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition

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The widespread class of RNA viruses that utilize internal ribosome entry sites (IRESs) for translation include poliovirus and Hepatitis C virus. To identify host factors required for IRES-dependent translation and viral replication, we performed a genome-wide RNAi screen in *Drosophila* cells infected with Drosophila C virus (DCV). We identified 66 ribosomal proteins that, when depleted, specifically inhibit DCV growth, but not a non-IRES-containing RNA virus. Moreover, treatment of flies with a translation inhibitor is protective in vivo. Finally, this increased sensitivity to ribosome levels also holds true for poliovirus infection of human cells, demonstrating the generality of these findings.

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Viruses are obligate intracellular pathogens and must perform certain steps in order to complete their life cycle, including entry into the cytoplasm of the host cell, uncoating, translation, replication, and virion assembly. Although all viruses must complete these steps, different families have developed radically different strategies to do so. As one consequence of their small genome size, all viruses require host-encoded proteins to replicate. The identification of host proteins and pathways coopted by viruses to complete their life cycles has given us insights into both host–pathogen interactions and into the normal function of the implicated cellular systems.

Not only do viruses need host factors to replicate, but they must also compete directly with the host cell for factors. For example, all viruses use the host-encoded translation machinery, including the ribosome, to translate their genomes (Bushell and Sarnow 2002). In order to successfully vie for host ribosomes and the limited pool of initiation factors, many viruses prevent host-encoded mRNAs from accessing ribosomes. Specifically, internal ribosome entry sites (IRES)-containing viruses have evolved a sophisticated translation mechanism that bypasses the requirement for the 5′ ^{m}Gppp cap (a characteristic of host mRNAs), and instead utilizes a highly structured RNA sequence, which allows cap-independent recruitment of ribosomes to the viral RNA (Hellen and Sarnow 2001). Although different IRESs use diverse mechanisms to bring ribosomes to the viral genome, they all bypass some steps required for the translation of host-encoded messages, which in turn allows viral and host messages to be modulated differentially (Pestova et al. 2001). For example, by inactivating the eIF-4F complex that is required for capped host mRNAs to load onto the ribosome, polioviruses are able to inhibit the translation of host messages without affecting IRES-dependent translation (Hellen and Sarnow 2001).

Because these viruses use this unique mechanism for translation, it has been hypothesized that specific inhibitors of IRES-dependent translation could be therapeutic for viral infections. However, such interventions have yet to materialize. The identification of those host factors that are required or limiting for IRES-dependent translation may allow more directed screens for small molecules that inhibit IRES function. That viral replication factors encoded by the host instead of the virus can be used as therapeutic targets has traditionally been unexplored. However the recent development of anti-CCR5 agents for treatment of HIV infection establishes the po-

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tential of this complementary approach (Shaheen and Collman 2004).

Despite the demonstrable value of information on host factors involved in viral infection, our ability to identify these factors has been hampered by the dearth of amenable forward genetic systems. *Drosophila* has been widely used as a model to study development, cell biology, and innate immunity due to its powerful genetics and conservation with vertebrate systems. The full-genome sequence of *Drosophila* along with the development of RNAi technology permits the systematic survey of the phenotypic consequences of transcript depletion. This tool, when applied in a global, unbiased way, provides an in vitro genetic system that can be used to study the effects of gene knockdown on any system that can be adapted to high-throughput screening. We have previously developed a genome-wide set of *Drosophila* double-stranded RNA (dsRNA) reagents (Boutros et al. 2004). Here we have extended the use of this methodology to identify host factors that are essential for growth and replication of Drosophila C virus (DCV).

DCV is an IRES-containing RNA virus that is a natural pathogen of *Drosophila* and can infect both flies and tissue culture cells, leading to lethality (Cherry and Perrimon 2004). This family of viruses includes many human pathogens including the picornaviruses poliovirus, Rhinovirus, and Hepatitis A virus as well as flaviviruses such as Hepatitis C virus (Knipe and Howley 2001). While these mammalian viruses have one IRES at the 5′ end of the genome, DCV along with other dicistroviridae have two IRESs (Johnson and Christian 1998). Despite this difference, these insect viruses share many physical and morphological properties with mammalian picornaviruses (King and Moore 1988; Johnson and Christian 1998; Tate et al. 1999). Moreover, where studied, the infectious cycle of DCV resembles that of pathogenic IRES-containing mammalian viruses (Scotti et al. 1981; Reavy and Moore 1983; Moore et al. 1985; King and Moore 1988). The recent use of DCV to identify a set of host factors required for viral entry found conserved proteins involved in clathrin-mediated endocytosis (Cherry and Perrimon 2004). Such experience suggested to us that the system is sensitive and has high conservation with mammalian viral biology. Consequently, we proposed, first, that this virus–host pair would be amenable to a genome-wide RNAi approach, and second, that any factors we discover to be required for DCV replication may also be important for the life cycle of related mammalian viruses.

To dissect the cellular requirements for DCV replication, we undertook a genome-wide screen in tissue culture for host factors that, when lost, block viral infection. Using this strategy we identified the ribosome as limiting during infection. While the hypomorphic consequences of ribosomal protein depletion are tolerated, the cells become refractory to viral infection by DCV. In contrast, a non-IRES-containing virus is able to infect and replicate within these ribosome-depleted cells. This defect in the replication of DCV is mediated by a deficiency in translation from both IRESs present in DCV, suggesting that there may a general requirement for high levels of ribosomes for efficient translation from an IRES. Furthermore, the feeding of adult flies with a small molecule inhibitor of ribosomal function is protective in vivo. And most importantly, poliovirus infection was also attenuated by depletion of ribosomal proteins in human cells, demonstrating that attenuation of the translation apparatus may be a fruitful target for antiviral therapeutics.

Results

Genome-wide RNAi screen

To identify host factors required for viral infection in *Drosophila* cell culture we designed a sensitive and quantitative assay for viral replication that was responsive to RNAi. We used these conditions to perform a genome-wide screen of ~21,000 dsRNA covering 91% of the genes predicted by both the Berkeley *Drosophila* Genome Project (BDGP) and the Sanger Center (Boutros et al. 2004). dsRNAs are aliquoted into 384-well plates, with each plate containing controls. We used a dsRNA generated against viral sequences as a positive control as DCV is an RNA virus and therefore the viral genome is susceptible to RNAi mediated degredation. As a negative control we generated dsRNA against *gfp* because it is not present in our system. Cells were treated with dsRNA for 3 d and then were infected with DCV at a multiplicity of infection (MOI) of 1, and 24 h post-infection the cells were stained with a polyclonal FITC-conjugated anti-capsid antibody and a nuclear counterstain (Fig. 1A; Cherry and Perrimon 2004). Using this infection protocol and a positive control dsRNA against viral sequences we were able to inhibit infection in >99% of the cells, a reduction of >20-fold (Fig. 1B). We compared the fraction of infected cells from well to well, and a representative example of nine wells is shown (Fig. 1A). By visual inspection, we identified 210 dsRNA species that reduced the relative number of infected cells. In a secondary screen we resynthesized the dsRNAs identified in the primary screen and confirmed 112 genes that, when knocked down, reduced viral infection by >40% as assayed by antibody staining.

Attenuation of ribosome severely blocks viral replication

These genes fall into a number of functional categories (Fig. 1C). Surprisingly, the largest category identified genes that encode the structural components of the ribosome. We found 66 ribosomal protein genes that account for 57% of our candidate genes and 72% of total ribosomal genes present in RNAi set. These candidate gene products were evenly distributed among the different ribosomal subunits: We identified 74% of the ribosomal protein genes of the small subunit, 70% of the large subunit, and 67% of the acidic proteins (Supplementary Table 1). This suggests that general ribosome integrity is required for viral growth, and not some spe-

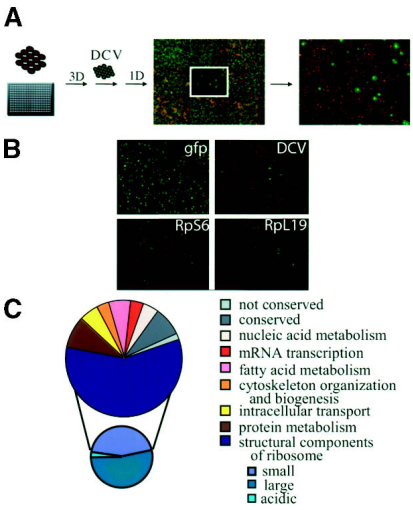

Figure 1. Genome-wide RNAi screen reveals that ribosomal protein levels are critical for DCV replication. (*A*) Schematic diagram of RNAi screen in pre-aliquoted 384-well plates. (*B*) Decreased viral antigen production post-dsRNA treatment with dsRNA against DCV, RpS6, or RpL19 as measured by the ratio of FITC-anti-DCV (green) versus Hoescht 33342 (red). (*C*) Frequency of encoded functional groups as curated by Gene Ontology (The FlyBase Consortium 2003) and manually assigned to representative categories for all verified candidates. The breakdown of the ribosomal components identified is shown.

cialized role for the individual proteins found in the screen.

dsRNA against ribosomal proteins led to a significant and reproducible reduction in viral infection as measured by immunofluorescence (Figs. 1B, 2A; data not shown). The average reduction observed across ribosomal proteins was eightfold. This reduction in the percentage of infected cells also resulted in reduced viral titers. Using an end-point titration assay, we found that RNAi against ribosomal proteins such as *RpS6* and *RpL19* resulted in a $>10^3$-fold reduction in virus production under conditions showing a 20-fold reduction in infection as measured by antibody staining (Fig. 2A). Taken together, these results show a severe inhibition of viral replication in response to depletion of the translation machinery.

Ribosomal proteins are reduced upon loss of a single component

Consistent with the assumption that dsRNAs targeting the ribosome affect the complement of ribosomal proteins in the cell, we found that RNAi against *RpS6* leads to a reduction in RpS6 protein levels (Fig. 2B). Moreover, RNAi against other ribosomal proteins identified in the screen such as *RpL19* also led to a reduction in RpS6 levels (Fig. 2B) while RNAi against *RpS25* (a gene not identified in the screen) did not reduce RpS6 levels. This coregulation of ribosomal protein levels was specific because the relative abundance of alpha-tubulin was not significantly affected in cells that were substantially depleted for RpS6 (Fig. 2B). Additionally, analysis of total proteins by Coomassie stain also demonstrated that there were minimal effects on global protein levels (data not shown).

Given that the previous experiment only tested the steady-state levels of host protein synthesis under these hypomorphic conditions we also tested whether general host translation is affected using an S^{35} methionine-pulse post-RNAi. We treated cells with dsRNA against either a control or *RpS6* or *RpL19* and pulsed the cells for 1 h. We found that there was a less than twofold decrease in host protein translation under these conditions while cycloheximide treatment decreases incorporation by >10-fold (Fig. 2C). This is in contrast to the >10-fold decrease observed in DCV replication (Fig. 2A). Therefore, there is a differential effect on DCV replication over the general effect on host protein translation.

To determine whether this depletion of ribosomal proteins was at the level of protein or RNA we analyzed total RNA levels of *RpL19* in cells depleted for different ribosomal proteins or a *gfp* control. While we observed a loss in RpS6 protein levels in cells treated with dsRNA against *RpL19*, we observed no effect on *RpS6* RNA levels (Fig. 2D). Likewise, the *RpL19* levels and species present were markedly changed upon treatment with dsRNA against *RpL19* and not when the cells were treated with other dsRNAs (Fig. 2D). This demonstrates the specificity of dsRNA-mediated depletion of host mRNAs.

This suggests that the loss of a single ribosomal protein can lead to a specific decrease in other ribosomal protein levels in the absence of an overt effect on the cell's protein complement. That levels of ribosomal proteins are affected in *trans* by the loss of one component is, to our knowledge, a novel finding.

Hypomorphic depletion of ribosome is tolerated by cells

That there are only small global effects on the cells, as measured by S^{35} methionine incorporation or alpha-tubulin levels, is consistent with our findings that RNAi against these ribosomal proteins did not lead to an increase in cell death as measured by membrane permeability (Fig. 2E). The average percent cell death in untreated cells was 6.9 ± 1.5 and 7.5 ± 1.4 in cells treated with dsRNA against the 66 ribosomal proteins identified. Moreover, the average cell number in the untreated case was 1390 ± 167 compared with 1250 ± 203 in the ribosomal dsRNA-treated samples. In contrast, treatment with dsRNA against the gene *thread*, an inhibitor of apoptosis, led to a dramatic increase in cell death (Fig. 2E; Boutros et al. 2004). Our data are in agreement with Boutros et al. (2004), who found that there was on the order of a twofold reduction in ATP levels post-RNAi against ribosomal proteins while we observed a twofold reduction in S^{35} incorporation using different cell lines at different time points post RNAi treatment. Taken to-

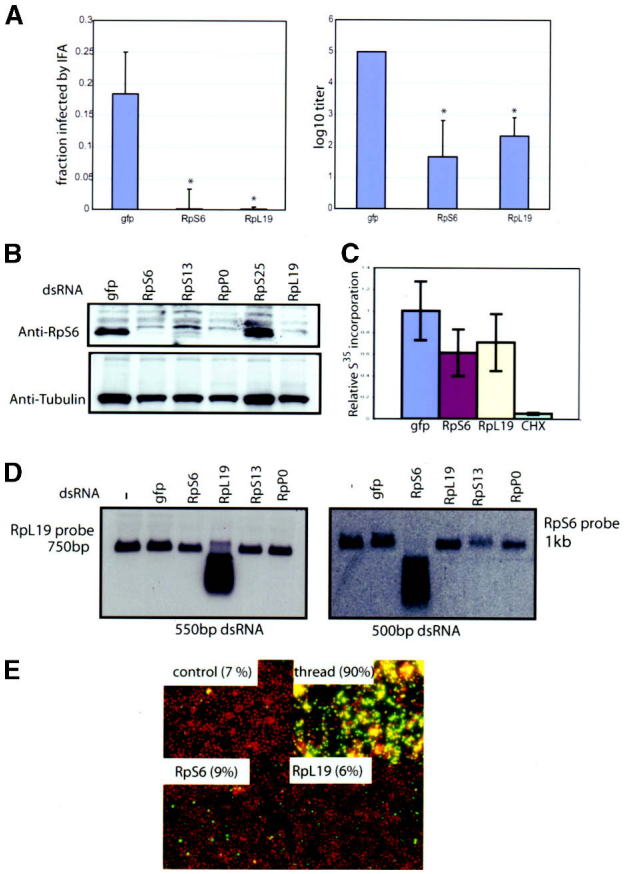

Figure 2. RNAi against ribosomal proteins leads to a tolerated depletion in the ribosome and protection from DCV infection. (*A*) Comparison of the effect of dsRNA treatment on viral replication as measured by immunofluorescence or viral titers released at 24 h post-infection. Results for averaged triplicate experiment where the error bars represent one standard deviation and asterisks denote $p < 0.05$ by *t*-test. (*B*) Western blot analysis of dsRNA-treated cell lysates probed with anti-RpS6 (*top* panel) or anti-tubulin (*bottom* panel). (*C*) Host protein synthesis is reduced by less than twofold as observed by pulse-labeling cells with S^{35} methionine for 1 h post-dsRNA treatment with either gfp, RpS6, or RpL19 while cycloheximide (CHX) reduces incorporation by >10-fold. Extracts were analyzed by trichloroacetic acid precipitation and quantitation (results for averaged triplicate samples from three independent experiments; error bars represent one standard deviation). (*D*) Northern blot analysis of dsRNA-treated total RNA probed with RpS6 or RpL19, respectively. (*E*) Fluorescence microscopy of cells treated with dsRNA. No significant change in cell number or cell death is evident by comparing the number of nuclei (Hoechst 33342-red) or the fraction of Sytox (green)-labeled nuclei to total nuclei (red) after depletion of ribosomal proteins by RNAi. dsRNA treatment to *thread* results in a significant increase in cell death. Percent of dead cells is shown for each treatment.

gether, we have found that although ribosomal protein levels were substantially depleted, there was not a significant increase in cell death or a decrease in cell number. These results suggest that RNAi against the ribosome leads to a hypomorphic phenotype that is tolerated by the cells under these conditions.

VSV replication is unaffected by dsRNA against the ribosome

Given that translation is fundamental to cellular function, one possibility is that, while the cells remain viable, they are not able to support viral replication for independent reasons. For example, in mammals, a general anti-viral response involves the global inhibition of host translation by preventing translation of both cellular and viral mRNAs (Samuel 2001). Clearly the overall abundance of host proteins or tubulin was not largely affected by the various RNAi treatments. To more rigorously examine global effects on viral replication, we tested whether Vesicular Stomatitis virus (VSV), an unrelated enveloped negative-stranded RNA virus that has both mammalian and insect hosts, was able to replicate in cells depleted for these ribosomal proteins (Knipe and Howley 2001). Using recombinant VSV that expresses Gfp upon replication at an MOI of 1, we found that loss of ribosomal proteins had a negligible effect on viral infection, whereas treatment with dsRNA against *Rab5*, a protein required for endocytosis and viral entry, protected the cells from VSV infection (Fig. 3A; Sieczkarski and Whittaker 2003). This finding suggests that ribosome-depleted cells can support new translation of mRNAs and viral infection by a non-IRES-containing virus.

Ribosomal depletion specifically blocks IRES-dependent translation

Therefore, the specific requirement of DCV for wild-type levels of ribosomal proteins suggests that DCV may have a unique requirement for the cellular translation apparatus. One specialized feature of DCV is that its mRNA is not translated by a 5′ cap-dependent translation mechanism as are most eukaryotic and VSV mRNAs, but by an internal ribosomes entry mechanism that is employed by various viral and a few cellular mRNAs (Wilson et al. 2000; Hellen and Sarnow 2001; Knipe and Howley 2001). Internal ribosome entry is mediated by an RNA element that is usually hundreds of nucleotides in length (Hellen and Sarnow 2001). While mammalian picornaviruses have one IRES element that directs the synthesis of one polyprotein, DCV along with other dicistroviridae have two IRESs depicted in Figure 3B (Johnson and Christian 1998). While different IRESs appear to differ in the details of their activity, they are all predicted to be highly structured RNAs that must bypass requirements for some initiation factors and bring the viral RNA to the ribosome (Pestova et al. 2001). During an infection, only one or a few viral RNAs gain access to the cytoplasm and must be translated before they are degraded in order for infection to initiate. We postulate that the reason that DCV replication is sensitive to levels of ribosomes in the cell is because the IRES(s) encoded in the viral genome require high levels of the translation machinery in order to access the ribosomes and thus be efficiently translated. If the viral genome competes inefficiently with cellular messages for translation, we would expect that decreasing the ratio of ribosomes to host messages would

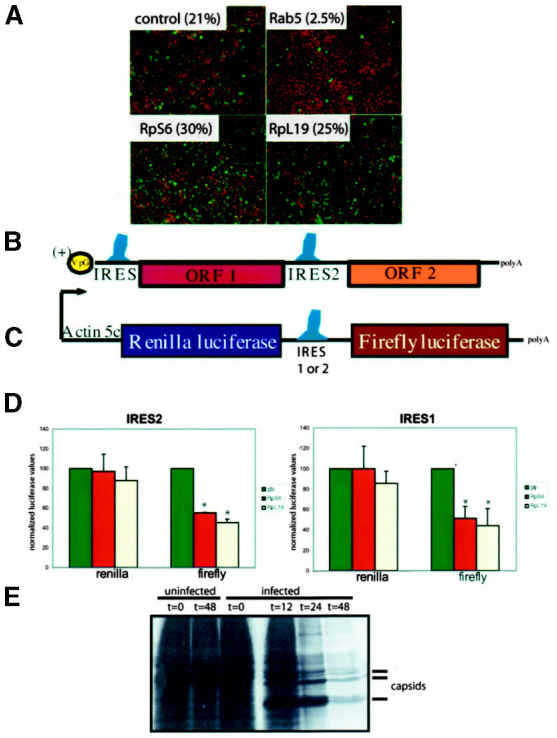

Figure 3. Depletion of the ribosome affects IRES-dependent translation but not 5′-cap dependent translation. (*A*) Fluorescence microscopy of cells treated with dsRNA and subsequently infected with VSV-gfp. dsRNA against ribosomal proteins has no effect on the percent infection by comparing gfp expression (green) to total nuclei stained with Hoechst 33342 (red). dsRNA treatment against *Rab5* results in a significant protection from infection. Percent infected cells are shown for each treatment. (*B*) Schematic diagram of the DCV genome describing the location of the two viral IRESs. (*C*) Schematic diagram of the bicistronic vector used to determine whether depletion of ribosomal proteins has an effect on translation from a 5′-capped message (*Renilla* luciferase) or a DCV IRES (firefly luciferase). (*D*) dsRNA treatment against ribosomal proteins RpS6 or RpL19 results in a significant effect on translation from DCV IRES1 and IRES2 as measured by firefly luciferase, but not on the translation of the 5′-capped message as measured by *Renilla* luciferase (results for averaged triplicate experiments where error bars represent one standard deviation and asterisks denote $p < 0.05$ by *t*-test). Cells treated with Gfp dsRNA are set to 1, and all other dsRNA treatments are normalized to that condition. (*E*) DCV continues to be translated while host synthesis is repressed as observed by pulse-labeling cells with S^{35} methionine for 2 h at the indicated times post-infection with DCV or mock infected (uninfected). Soluble extracts were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

lead to a defect in viral translation. Thus one would predict that a level of ribosomal attenuation would exist whereby cells are viable but unable to support IRES-dependent translation. Consistent with this notion, we found that cell entry was unaffected under conditions of either cycloheximide treatment or dsRNA against *RpS6* or *RpL19* (Cherry and Perrimon 2004; data not shown).

To test whether the defect in viral replication was indeed due to a direct effect on translation from the viral IRESs we used a bicistronic vector system (Johannes et al. 1999). The first cistron directs the cap-dependent translation of *Renilla* luciferase, while the second cistron mediates the cap-independent, IRES-dependent translation of firefly luciferase (Fig. 3C). The relative production of these two reporters allows us to assess the effects of ribosomal protein depletion on these two activities. We tested both the 5′ and internal IRESs present in DCV and found that depletion of ribosomal proteins by RNAi led to a significant and specific decrease in IRES-dependent translation but not in cap-dependent translation (Fig. 3D). This reduction was observed for both IRESs present in DCV suggesting a general defect in IRES function resulting from ribosomal depletion.

Based on analogy to picornaviruses, one would predict that DCV infection results in inhibition of host translation by a mechanism to which the virus itself is insensitive. To examine this, we infected cells with DCV and pulse-labeled with S^{35} methionine at different times post-infection. By 12 h post-infection, translation from host mRNAs is inhibited while viral RNA continues to be translated (Fig. 3E). The levels of viral mRNA translation are low compared to other picornaviruses as was also observed with another member of the dicistroviridae, cricket paralysis virus (Wilson et al. 2000). It is possible that the *Drosophila* cells used lack some factors that modulate the DCV-IRES. Also, the translation of both host and viral RNA is inhibited at late time points in DCV-infected cells. That DCV represses host translation similar to mammalian IRES-containing viruses is tied to the hypothesis that IRES-dependent translation is inefficient compared to cap-dependent translation, and is thus sensitive to competitive inhibition by the latter. Consequently these viruses have developed the means to eliminate competition upon successful infection by preventing host messages from being translated.

Drug treatment in vivo attenuates infection

Given that attenuation of ribosome function protects cultured cells from DCV infection, we tested whether the general translation inhibitor Hygromycin B could protect flies from DCV infection (Brodersen et al. 2000). We fed adult flies serial dilutions of sublethal doses of the aminoglycoside eukaryotic ribosomal inhibitor Hygromycin B and then challenged them with DCV. We monitored the flies' susceptibility to infection and found that the drug causes a dose-dependent inhibition of virus-induced lethality. At concentrations between ~17.5 and 280 μM we observed protection from DCV infection in the absence of drug toxicity (Fig. 4A). We also tested another inhibitor of host translation, neomycin, and observed similar effects (data not shown). This demonstrates that there is a level of translational attenuation that can be tolerated by organisms and is sufficient to confer some protection from viral infection. These data suggest that general translation inhibitors may present a novel avenue for anti-viral therapeutics.

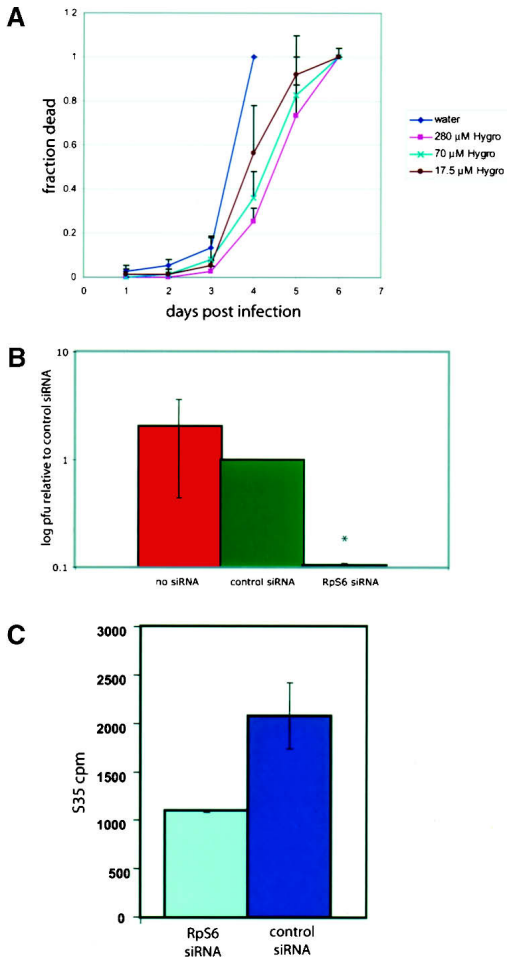

Figure 4. Inhibition of ribosomal function protects from infection in vivo and from poliovirus infection. (*A*) Adult wild-type flies (male, Canton-S) fed serial dilutions of Hygromycin B (280–17.5 μM) and then challenged with DCV were monitored daily for mortality. Error bars represent one standard deviation; results from a triplicate representative experiment averaged. (*B*) Poliovirus infection of Hela cells pretransfected with siRNAs against RpS6 results in inhibition of viral replication. Control siRNA pretreatment is set to 1 and the relative production of poliovirus is shown for four independent experiments where error bars represent one standard deviation and asteriks denote $p < 0.05$. (*C*) Host protein synthesis is reduced twofold as observed by pulse-labeling cells with S^{35} methionine for 20 min post-siRNA treatment with RpS6. Extracts were analyzed by trichloroacetic acid precipitation and quantitation (results for two independent experiments averaged; error bars represent each value).

Poliovirus infection is also sensitive to levels of translation apparatus

Because attenuation of the ribosome inhibited translation from both IRESs present in DCV, we hypothesized that this effect may be generalizable to mammalian IRES-containing viruses such as poliovirus. The two IRESs present in DCV are quite different from one another and are thought to use different mechanisms for translation initiation (Pestova et al. 2001). To test whether attenuation of the ribosome could also protect human cells from an IRES-containing virus, we infected cultured human cells pretreated with an siRNA against *RpS6* with poliovirus at an MOI of 4. We observed a 10-fold reduction of viral titers in cells transfected with an siRNA against *RpS6* compared to a control siRNA (Fig. 4B). Similar results were obtained with siRNAs against *RpP0* and *RpL19* and at an MOI of 16 (data not shown), suggesting that knock-down of several ribosomal proteins can protect an infected cell, even if it is invaded by more than one infectious virus. Under these conditions where there is a 10-fold reduction in viral replication, there is only a twofold reduction in host translation as measured by S^{35} incorporation (Fig. 4C). Moreover, reduction in viral yield was not due to loss in cell density or viability in cells treated with *RpS6* siRNA (Supplementary Table 2).

Discussion

These findings demonstrate that both insect and mammalian IRES-containing RNA viruses are unusually sensitive to levels of the ribosomal machinery. Consistent with our observation that attenuation of the ribosome can be tolerated by cells, it has been reported that many yeast ribosomal subunits are dispensable for growth, and likewise conditional mutation of *RpS6* in the mouse liver has no effect on hepatocyte viability or translation even though there is a reduction in the production of 40S ribosomal complexes (Volarevic et al. 2000; Giaever et al. 2002). Moreover, zebrafish carrying heterozygous mutations in a number of ribosomal proteins are predisposed to cancer (Amsterdam et al. 2004). How the loss of ribosomal proteins leads to different phenotypic consequences is presently unclear. However, the fact that ribosomal inhibition can be tolerated in vitro, together with the fact that pharmacologic inhibition of translation protects from DCV infection in vivo, suggests a novel approach to viral inhibition. Whereas poliovirus had until recently been considered conquered, the recent poliomyelitis outbreak in West Africa gives new urgency to the search for therapeutics for this disease. Furthermore, given that additional IRES-dependent viruses, including Hepatitis C virus and rhinovirus, are widespread human pathogens that are also currently without efficacious treatments, our data suggest that modulating host ribosome activity could be a promising new direction for antiviral drug discovery.

Material and methods

Cells and virus

Schneider line 2 cells were maintained as described (Cherry and Perrimon 2004). DCV was purified and titered as described (Cherry and Perrimon 2004).

An infectious cDNA clone of VSV was engineered to contain an additional transcriptional unit encoding gfp, generating VSV-gfp. The gfp ORF was amplified by PCR from pGreenlantern (Invitrogen) and inserted between conserved VSV gene-start end sequences engineered at the leader-N gene junction. VSV-gfp

was recovered, purified, and titered as described (Whelan et al. 1995).

dsRNA treatments

dsRNA was generated as described (Boutros et al. 2004). The cells were plated at 20,000 cells per well in 10 µL serum-free media into 384-well plates pre-aliquoted with 250 ng of dsRNA. One hour later 20 µL of complete media were added (Clemens et al. 2000). Cells were used 3 d later.

Viral infections

Three days post-dsRNA addition cells were infected with DCV at a MOI of 1. DCV was prepared as previously described (Cherry and Perrimon 2004). One day later the cells were fixed and stained with FITC-conjugated anti-capsid DCV and counterstained with Hoescht 33342 as described (Cherry and Perrimon 2004). Images were captured using an automated microscope as described and analyzed using Metamorph software (Kiger et al. 2003). For viral titers, supernatant was removed before fixing the cells and serial dilutions were used to infect cells. The highest dilution that resulted in positive infection by immunofluorescence was considered the titer of the sample.

Three days post-dsRNA cells were infected with VSV-gfp at a MOI of 1. Twenty-four hours later the cells were fixed and stained as above.

Protein analysis

Western blot analysis was performed as described (Cherry and Perrimon 2004) using using anti-RPS6 (Cell Signaling, 1:200) or anti-Tubulin (Sigma, 1:2000).

For total protein analysis cells were pulsed with S^{35} methionine (NEN) for 2 h at the indicated time points post-infection with DCV. Total cell lysates were prepared as above and run on a 10% SDS-Page gel and visualized using fluorography.

For S^{35} methionine incorporation cells treated with dsRNA or siRNAs were pulsed for 1 h or 20 min, respectively, with S^{35} methionine (NEN) and prepared as above. Total cell lysate was precipitated with 10% trichloroacetic acid and quantitated using a scintillation counter.

Northern blot analysis

Total RNA was extracted from dsRNA-treated cells using Trizol following the manufacturer's protocol. Ten micrograms RNA were run on a 1% gel and transferred to Hybond-N^+ nylon and a radiolabeled probe was generated from the PCR product used for dsRNA production. Hybridization was carried out with Church buffer as recommended.

Cell death analysis

Three days post-dsRNA cells were analyzed for cell death by staining with Sytox (Molecular Probes, 1:5000) and Hoescht 33342 for 10 min. The samples were washed twice in PBS before images were collected and analyzed.

Luciferase assays

IRES1 was cloned by PCR using primers 5′-AGAATTCTT TATATCGTGTGTACATATAAATA-3′ and 5′-CATGCCAT GGTTATCGTTAAGCGCAAGATC-3′ (nucleotides 1–796) and IRES2 using 5′-CATGCCATGGTTGTTTGAAAGTTAG CAGGTT-3′ and 5′-CGGAATTCTTAAGATGTGATCTTGC TT-3′ (nucleotides 6080–6282); these primers contain EcoRI and NcoI restriction sites that were used to clone these into the intercistronic region of a bicistronic luciferase reporter vector (c53/Delta EMCV) and subcloned into pACTpl to express in *Drosophila* cells. Cells (150,000) were cotransfected with 0.1 µg of bicistronic vector plus 0.1 µg dsRNA with Effectene (Qiagen) using the manufacturer's protocol. Five days post-transfection the cells were assayed using Dual-glo luciferase reagents (Promega) following the manufacturer's protocol and quantitated using an Analyst GT plate reader (Molecular Devices).

Feeding experiments

Groups of 25 flies were placed on standard media plus serial dilutions of Hygromycin B (17.5–280 µM; Calbiochem) 1 d prior to infection. They were infected with ~5×10^4 virions by injection and monitored daily (Cherry and Perrimon 2004).

Poliovirus infections

HeLa cells were cultured in antibiotic-free DMEM medium, supplemented with 10% FBS and L-glutamine. Seventy-five percent confluent cultures were transfected with 10 pmol RpS6 siRNA (Dharmacon) or randomized control siRNA in 100 µL transfection media consisting of OptiMEMI and Lipofectamine 2000 in an eight-chamber dish. After a 2-h incubation at 37°C, 200 µL media were added to each well to yield a final siRNA concentration of 100 nM in a final volume of 0.3 mL. After 45 h of transfection, the medium was removed and cells were infected with poliovirus at a MOI of 4 in a total volume of 100 µL. After a 30-min incubation at 37°C medium was added and cells were incubated for an additional 4 h at 37°C. Virus was harvested and viral yield was determined by plaque assay as described (Johnson and Sarnow 1991).

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References

Amsterdam, A., Sadler, K.C., Lai, K., Farrington, S., Bronson, R.T., Lees, J.A., and Hopkins, N. 2004. Many ribosomal protein genes are cancer genes in zebrafish. *PLoS Biol.* **2:** E139.

Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Consortium, H.F., Paro, R., and Perrimon, N. 2004. Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* **303:** 832–835.

Brodersen, D.E., Clemons Jr., W.M., Carter, A.P., Morgan-Warren, R.J., Wimberly, B.T., and Ramakrishnan, V. 2000. The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. *Cell* **103:** 1143–1154.

Bushell, M. and Sarnow, P. 2002. Hijacking the translation apparatus by RNA viruses. *J. Cell. Biol.* **158:** 395–399.

Cherry, S. and Perrimon, N. 2004. Entry is a rate-limiting step for viral infection in a *Drosophila melanogaster* model of pathogenesis. *Nat. Immunol.* **5:** 81–87.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. 2000. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci.* **97:** 6499–6503.

The FlyBase Consortium. 2003. The FlyBase database of the

Drosophila genome projects and community literature. *Nucleic Acids Res.* **31:** 172–175.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., et al. 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418:** 387–391.

Hellen, C.U. and Sarnow, P. 2001. Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes & Dev.* **15:** 1593–1612.

Johannes, G., Carter, M.S., Eisen, M.B., Brown, P.O., and Sarnow, P. 1999. Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. *Proc. Natl. Acad. Sci.* **96:** 13118–13123.

Johnson, K.N. and Christian, P.D. 1998. The novel genome organization of the insect picorna-like virus *Drosophila* C virus suggests this virus belongs to a previously undescribed virus family. *J. Gen. Virol.* **79:** 191–203.

Johnson, K.L. and Sarnow, P. 1991. Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus. *J. Virol.* **65:** 4341–4349.

Kiger, A., Baum, B., Jones, S., Jones, M., Coulson, A., Echeverri, C., and Perrimon, N. 2003. A functional genomic analysis of cell morphology using RNA interference. *J. Biol.* **2:** 27.

King, L.A. and Moore, N.F. 1988. Evidence for the presence of a genome linked protein in two insect picornaviruses, cricket paralysis virus and *Drosophila* C virus. *FEMS Lett.* **50:** 41.

Knipe, D.M. and Howley, P.M. 2001. *Fields' virology*. Lippincott-Raven, Philadelphia.

Moore, N.F., Reavy, B., and King, L.A. 1985. General characteristics, gene organization and expression of small RNA viruses of insects. *J. Gen. Virol.* **66:** 647–659.

Pestova, T.V., Kolupaeva, V.G., Lomakin, I.B., Pilipenko, E.V., Shatsky, I.N., Agol, V.I., and Hellen, C.U. 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proc. Natl. Acad. Sci.* **98:** 7029–7036.

Reavy, B. and Moore, N.F. 1983. Cell-free translation of *Drosophila* C virus RNA: Identification of a virus protease activity involved in capsid protein synthesis and further studies on in vitro processing of cricket paralysis virus specified proteins. *Arch. Virol.* **76:** 101–115.

Samuel, C.E. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.* **14:** 778–809.

Scotti, P.D., Longworth, J.F., Plus, N., Croizier, G., and Reinganum, C. 1981. The biology and ecology of strains of an insect small RNA virus complex. *Adv. Virus Res.* **26:** 117–143.

Shaheen, F. and Collman, R.G. 2004. Co-receptor antagonists as HIV-1 entry inhibitors. *Curr. Opin. Infect. Dis.* **17:** 7–16.

Sieczkarski, S.B. and Whittaker, G.R. 2003. Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. *Traffic* **4:** 333–343.

Tate, J., Liljas, L., Scotti, P., Christian, P., Lin, T., and Johnson, J.E. 1999. The crystal structure of cricket paralysis virus: The first view of a new virus family. *Nat. Struct. Biol.* **6:** 765–774.

Volarevic, S., Stewart, M.J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S.C., and Thomas, G. 2000. Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science* **288:** 2045–2047.

Whelan, S.P., Ball, L.A., Barr, J.N., and Wertz, G.T. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc. Natl. Acad. Sci.* **92:** 8388–8392.

Wilson, J.E., Powell, M.J., Hoover, S.E., and Sarnow, P. 2000. Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. *Mol. Cell. Biol.* **20:** 4990–4999.