**peLO Is Required for High Efficiency Viral Replication**

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**Abstract**

Viruses hijack host factors for their high speed protein synthesis, but information about these factors is largely unknown. In searching for genes that are involved in viral replication, we carried out a forward genetic screen for Drosophila mutants that are more resistant or sensitive to Drosophila C virus (DCV) infection-caused death, and found a virus-resistant line in which the expression of *peLO* gene was deficient. Our mechanistic studies excluded the viral resistance of *peLO* deficient flies resulting from the known Drosophila anti-viral pathways, and revealed that *peLO* deficiency limits the high level synthesis of the DCV capsid proteins but has no or very little effect on the expression of some other viral proteins, bulk cellular proteins, and transcribed exogenous genes. The restriction of replication of other types of viruses in *peLO* deficient flies was also observed, suggesting *peLO* is required for high level production of capsids of all kinds of viruses. We show that both *peLO* deficiency and high level DCV protein synthesis increase aberrant 80S ribosomes, and propose that the preferential requirement of *peLO* for high level synthesis of viral capsids is at least partly due to the role of *peLO* in dissociation of stalled 80S ribosomes and clearance of aberrant viral RNA and proteins. Our data demonstrated that *peLO* is a host factor that is required for high efficiency translation of viral capsids and targeting *peLO* could be a strategy for general inhibition of viral infection.

**Introduction**

Viruses are the most abundant intracellular pathogens on the earth. They can infect all living organisms and hijack their host factors for replication [1]. In order to withstand virus infections, their hosts have evolved multiple antiviral defense mechanisms [2,3,4]. For many years, scientists have been studying host-virus interactions in order to develop new and more effective strategies for the prevention and treatment of viral infection.

*Drosophila melanogaster* has been shown to be a powerful model system in studying host-pathogen interactions [5]. In addition to the widely appreciated achievement in studying antibacterial and antifungal immunity by using *Drosophila* [6], there is also growing understanding of *Drosophila*-virus interactions [7,8]. Several antiviral innate immunity pathways and their corresponding molecular mechanisms have been deciphered in *Drosophila*. RNA interference is the major antiviral pathway in *Drosophila* [9]. Similar to plants, *Drosophila* can generate both a local and a systemic antiviral RNAi response [10]. However, viruses can counteract host RNAi defense by expressing viral suppressors of RNAi (VSRs), such as FHV-B2, DCV-IA1, and Crpv-A [11,12,13,14]. Inducible gene expressions in response to viral infection also contribute to antiviral immunity, including the JAK-STAT pathway and the DExD/H-box helicase Dicer-2-mediated antiviral gene induction [15,16]. The Toll and IMD pathways are involved in restricting some specific viruses by mechanisms yet to be clarified [17,18]. Autophagy plays an important antiviral role against the vesicular stomatitis virus (VSV) in *Drosophila*, which is initiated by Toll-7 after its recognition of the VSV glycoprotein VSV-G [19,20]. It is apparent that some antiviral mechanisms are conserved between *Drosophila* and mammals but others are uniquely present in *Drosophila*.

Hijacking host cellular machineries for viral replication is another major part of host-virus interaction [1,21]. Studies in *Drosophila* have revealed that the clathrin-mediated endocytotic pathway is required for viral entry [22], some ribosomal proteins are involved in viral IRES-dependent translation [23], and the coat protein complex I (COPI) coatamer and fatty acid biosynthesis are required to form the intracellular vesicular compartment for viral replication [24]. All of these appear to be commonly involved in host-virus interaction of many different viruses. Targeting these events might be able to interfere with the replication of a broad panel of viruses; however, achieving the goal of virus inhibition without affecting normal cell function is a challenging task. Without exception, viruses have to use cellular protein translation machinery to synthesize their proteins. In many cases, viruses take over the hosts’ protein synthesis machinery to make huge amounts of viral proteins for their replication [25]. How can viruses so highly efficiently utilize the cellular system to synthesize their proteins is largely unknown. Information on the cellular factors that are required for high speed synthesis of viral proteins is very limited.
Author Summary

Viruses often can highly efficiently utilize the host system to make huge amounts of viral proteins for their replication; however, which host factors are needed by the viruses are largely unknown. We analyzed about one hundred Drosophila mutants and found that the pelo mutation in Drosophila inhibited Drosophila C virus (DCV) replication. We found pelo is specifically required for high efficiency synthesis of proteins of a number of viruses, suggesting that inhibition of pelo may mediate a general antiviral activity. We proposed that the function of pelo in quality control of protein synthesis is required for high efficiency viral protein synthesis. Our study presented here identifies a new host factor, pelo, that is specifically required for effective viral replication and it may be a new potential therapeutic target for broad-spectrum antiviral therapy.

Protein translation is a tightly controlled cellular process, and also is a part of the checking mechanism that eliminates aberrant transcripts and proteins [26]. The pelo-Hbs1 complex (also known as Dom34-Hbs1) recognizes stalled ribosomes caused by defective mRNAs as well as rRNAs and promotes ribosomal subunit dissociation and the release of peptidyl-tRNA [27,28]. Therefore, the complex participates in quality control for non-go decay (NGD) and non-stop decay (NSD) [29,30,31]. ABCE1, a conserved member of the ATP-binding cassette (ABC) family of proteins, is involved in pelo-Hbs1 mediated disassembly of the ribosome in mammalian cells [32]. Recent work has shown that Dom34-Hbs1 is also required for nonstop protein clearance from translators for normal organelle protein influx [33]. pelo is a highly conserved gene from yeast to human. It contains three eRF1 (euarkyotetic translation termination factor 1) domains, X-ray structural analysis of yeast Dom34 and the archaea homolog Pelota reveals that the structure of pelo is similar to eRF1 except for its N-terminal domain [34,35]. Deletion of Dom34 in yeast led to delayed progression through the G1 phase of the cell cycle, aberrant meiosis, and an altered polyribosome profile [36,37]. In Drosophila, pelo is required for meiotic cell division and controls germ-line stem cell self-renewal [38,39]. The mammalian homolog of pelo may be required for progression of the mitotic cell cycle and pelo deficient mice are embryonic lethal [40].

Drosophila C virus (DCV) is the best studied and a relatively simple Drosophila virus. It belongs to the Dicistroviridae family. It is a non-enveloped RNA virus and its capsid is composed of the three major proteins VP1 (33 kDa), VP2 (29 kDa), VP3 (28 kDa), and two minor proteins, VP0 (37.7 kDa) and VP4 (8.5 kDa), with VP0 as a precursor of VP3 and VP4 [41]. DCV contains only one single positive-strand RNA genome which is polyadenylated and with a genome-linked protein at the 5’ end [42,43]. DCV replicates rapidly after injection into adult flies and causes host death in as few as 3 days making it an ideal pathogen for performing a death screen [44]. In an effort to search for host factors that are required for the viral replication, we carried out a forward genetic screen for Drosophila mutants that are more resistant or sensitive to DCV infection-caused death. As a result, we found that pelo deficiency can mediate DCV resistance. Further characterization revealed that pelo is not involved in the known antiviral pathways in Drosophila. Our mechanistic studies showed that pelo is required for high efficiency synthesis of DCV capsid proteins and thus DCV replication; the function of pelo in the dissociation of stalled 80S ribosomes is at least part of the underlying mechanism that allows for high speed synthesis of DCV capsid proteins.

Results

pelo deficiency in Drosophila mediates resistance to DCV infection-caused death

To identify genes that are involved in host-virus interactions, we screened about 100 P-element insertion fly lines, generated by mobilizing a P [Mar-UAS.6.11] transposon to random autosomal sites from the X chromosome, for their sensitivity to DCV-induced death. The mutants were infected by septic injury with DCV to identify lines that are more resistant or sensitive to DCV infection than wild-type. The survival curve of a group of mutant lines is shown as an example of the screen (Figure S1). Two virus-resistant lines were screened out and we were able to identify the P-element insertion site in one of the lines numbered R32. The resistance to DCV induced death of R32 is shown in Figure 1A. The P-element insertion site of R32 is in the 5’UTR of the pelo gene and thus may disrupt pelo expression (Figure 1B). Since the genes of Ps3-C1, hoip, and CG31710 are also located near the P-element insertion site, we used qRT-PCR to determine the mRNA expression levels of these and the pelo genes. We found that the expression of pelo is reduced while the other three genes are not changed in the R32 line (Figure 1C). We then generated a specific polyclonal antibody to pelo and used it to confirm that the protein level of pelo was really reduced in R32 (Figure 1C).

R32 fly is healthy but male-sterile, which is consistent with a previously reported pelo deficient line called pelo1 [38,39]. pelo1 has a P-element inserted in the third intron of pelo (Figure 1B), which disrupts pelo protein expression (Figure 1D). To collect more evidence for or against the role of pelo in DCV sensitivity, we compared the sensitivity of pelo1 and its control line to DCV infection. pelo1 is resistant to DCV infection (Figure 1D), supporting the conclusion obtained from the R32 line that pelo deficiency causes DCV resistance. In order to better study the role of pelo in DCV sensitivity, we generated a pelo knockout line by deleting exons 1 and 2 (Figure 1B), which completely eliminated pelo protein expression (Figure 1E). Consistently, the pelo1-/− line had DCV resistant phenotype (Figure 1E). To unambiguously demonstrate that pelo deficiency causes DCV resistance in Drosophila, we rescued the expression of pelo in pelo1-/− flies by using a UAS-pelo transgene and a ubiquitous da-GAL4 driver (Figure 1F). The rescued line became more sensitive to DCV infection and thus had a phenotype similar to wild-type flies (Figure 1F). It is reported that bacterial symbiont Wolbachia increases resistance of Drosophila to RNA viral infections [43]. To examine whether Wolbachia infection influences our experiments, we measured Wolbachia infection status in our fly lines. All the fly lines we used except pelo1 are infected with Wolbachia (Figure S2A) and the infection levels are almost the same (Figure S2B), suggesting that the DCV resistant phenotype in pelo deficient flies is not caused by Wolbachia infection. Together, these data demonstrated that pelo deficiency results in DCV resistance in Drosophila.

pelo deficiency inhibits DCV replication in adult flies and Drosophila S2 cells

To characterize the pelo deficiency-mediated antiviral effect, we measured the viral titer at different time points in the virus-infected wild-type and pelo1-/− flies before their death and found that the titer in the mutant flies were dramatically reduced compared to wild-type flies (Figure 2A). Consistent with the lower viral titer in pelo1-/− flies (Figure 2A), the amounts of viral RNA and capsid proteins were also reduced in pelo1-/− flies (Figures 2B and 2C). The antibody of DCV was produced by inoculation of rabbit with the purified virus, so that it can recognize all the viral capsid proteins. Based on molecular weight and mass spectrum
analysis described later, we knew that the upper band is VP0 and the lower band is the mixture of VP1, VP2, and VP3. We also could detect a weak band of a molecular mass of 20 KD when in long exposure but not the 8.5 KD band corresponding to VP4 (data not shown). This decreased viral load correlated with the increased survival rate of the pelo mutant at later stages of DCV infection (Figure 1E). Thus, pelo deficiency-caused resistance to DCV infection-induced death is due to the fact that pelo deficiency limits DCV replication.

In order to study pelo deficiency-mediated resistance to DCV infection at the cellular level, we evaluated whether Drosophila S2 cells can be used in this study. pelo expression can be effectively knocked-down by RNAi in S2 cells (Figure 2E). pelo knockdown S2 cells have apparently normal morphology and do not have any noticeable changes in cell proliferation when compared with mock treated or control RNAi (dsGFP) treated S2 cells (Figure S3). We infected the control and pelo knockdown S2 cells with DCV and measured the viral RNA by qRT-PCR and the viral proteins by antibodies against viral capsid proteins. As shown in Figures 2D and 2E, both viral RNA and capsid proteins in pelo knockdown cells were much less than that in control cells. Thus the S2 cell line is a suitable culture cell system for studying the mechanism of pelo deficiency-mediated inhibition of DCV replication.

pelo is not involved in the known Drosophila antiviral mechanisms

There are several antiviral mechanisms in Drosophila, including RNAi, autophagy, antiviral gene expression regulated by Dicer-2, JAK-STAT, and NF-KB [8]. Since reported studies showed that the NF-KB pathway and autophagy can restrict certain viruses but not DCV in Drosophila [17,18,19], we explored whether pelo can work as a negative regulator of the other antiviral mechanisms.
Because RNAi is the major defense mechanism against viral infection in Drosophila, we first tested whether loss of pelo affects siRNA-mediated gene silencing. S2 cells were co-transfected with Firefly and Renilla luciferase reporters and then treated with dsRNA targeting the Firefly luciferase. RNAi efficiency was measured by the decrease in Firefly luciferase activity relative to that of the control Renilla luciferase. We found that pelo deficiency had no effect on dsRNA-mediated inhibition of Firefly luciferase expression (Figure 3A). It is known that the induction of Vago by DCV infection is dependent on the Dicer-2 pathway [16] and we found that pelo deficiency did not further up-regulate Vago in DCV infected flies (Figures 3B), indicating that pelo deficiency did not affect Dicer-2 pathway. DCV-induced JAK-STAT activation can be measured by the expression of its target gene vir-1 [15]. The induction of vir-1 by DCV in pelo+/− flies was not enhanced but even reduced when compared with wild-type flies (Figure 3C). However, when pelo knockdown S2 cells were used, we did not detect any effect of pelo deficiency on DCV induced expression of vir-1 (Figure S4). While we do not know why there is difference between fly and S2 cells in JAK-STAT activation, these data still excluded the possibility that DCV resistance in pelo deficient flies or cells is due to an enhancement of JAK-STAT activation.

Figure 2. pelo deficiency inhibits viral replication. (A–C) Flies were challenged with DCV and then three pools of ten flies were collected at the indicated time points post-infection. The viral titer was determined by end-point dilution (A). The accumulation of DCV RNA was measured by qRT-PCR. Data represent the mean ± SD of triplicates (B). The accumulation of DCV capsid proteins was measured by immunoblotting with anti-DCV antibody (C). (D and E) S2 cells were untreated (Mock) or treated with dsRNAs against GFP (dsGFP) or pelo (dspelo) for 6 days and then infected with DCV (MOI = 0.1). The accumulation of DCV RNA (D) or capsid proteins (E) was measured as described in B and C, respectively.

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pelo deficiency selectively inhibits expression of DCV capsid proteins

The process of viral replication includes attachment, penetration, uncoating, biosynthesis of viral nucleic acids and proteins, assembly, and release [46]. To understand how pelo deficiency inhibits DCV replication, we ought to determine which step of DCV replication is affected by pelo. Since DCV is a positive-sense RNA virus, inhibition of protein synthesis by cycloheximide should block viral replication but not affect DCV entry. We therefore infected S2 cells with DCV in the presence of cycloheximide and analyzed the internalized DCV genomic RNA by qRT-PCR. The levels of DCV RNA in the control and pelo knockdown S2 cells were the same (Figure 3D), indicating that loss of pelo did not affect the efficiency of DCV entry.

Then we wanted to analyze the biosynthesis of viral nucleic acids and proteins. We measured the RNA level of DCV by qRT-PCR and labeled DCV-infected S2 cells with 35S-Met at different time points post-infection. We found that at 6 hour post-infection, there is no significant difference in viral RNA amounts between control and pelo knockdown S2 cells but the newly synthesized viral protein is less in pelo knockdown cells (Figure S5), suggesting that pelo plays a promoting role in viral protein synthesis.

DCV contains two open reading frames (ORFs). ORF1 encodes a ~200 kDa polyprotein that includes the domains of helicase, protease, and RNA-dependent RNA polymerase. ORF2 encodes a ~100 kDa polyprotein which is subsequently cleaved into the capsid proteins [47]. There is no subgenomic RNA transcription during the DCV lifecycle and translation of both ORFs proceeds from the genomic RNA [48]. To further analyze DCV protein synthesis, we labeled DCV-infected S2 cells with 35S-Met at 6 hours post-infection for 30 min. 7 proteins were detected in DCV-infected cells, but not in non-infected cells, and were named DCV-1 to 7 (Figure 4A). In order to make sure that these proteins were all viral proteins and not the host proteins induced by viral infection, we used actinomycin D, which can inhibit host transcription and thus inhibit protein synthesis, with the exception of DCV proteins. As expected, actinomycin D treatment greatly reduced the amount of host proteins but had no effect on DCV-1.
to 7 (Figure S6A), demonstrating that all these proteins were encoded by viral RNA. *pelo* deficiency reduced the expression of DCV-2, DCV-4, DCV-5, DCV-6, and DCV-7, but had very little to no effect on the expression of DCV-1 and DCV-3 (Figures 4A and 4B). Analysis of viral RNA in the control and *pelo* knockdown cells revealed no significant difference at 6 hours post-infection (Figure 4C). These results suggested that *pelo* regulates the expression of some but not all DCV proteins at the translational level.

The molecular weights of DCV-4 to 6 correspond to those of DCV capsid proteins [41]. More convincingly, antibodies against DCV capsid proteins recognized protein bands corresponding to the sizes of DCV-4 to 7 (Figure 2C and data not shown). The levels of these proteins are much higher than the levels of DCV-1 to 3, supporting the prediction that DCV-4 to 7 are in fact capsid proteins. To determine the identities of these proteins, we cut their bands from the Coomassie blue-stained SDS-PAGE gel and did mass spectrometry analysis (Table S1). Identified peptides of DCV-1 were located in the 975–1759 aa of ORF1, including the protease and RdRp domains. Peptides of DCV-3 were in the region of 297–679 aa, which includes the helicase domain in ORF1. Peptides of DCV-4 were in the 291–623 aa of ORF2, which corresponds to VP0. Because we could not separate DCV-5 and 6 well, the identified peptides of these two protein bands were almost the same. The peptides were distributed along the whole sequence of ORF2, consistent with the prediction that they are the mixture of VP1, VP2, and VP3. Peptides of DCV-7 were in the 647–850 aa of ORF2, indicating that DCV-7 is also encoded by ORF2. Interestingly, we could not find the corresponding band of ORF2. Interestingly, we could not find the corresponding band of DCV-2 in the Coomassie blue-stained gel despite its 35S-Met labeling level being equal to that of DCV-1 and DCV-3. We then analyzed the stability of the DCV proteins by a pulse-chase experiment and found that DCV-2, but not the other DCV proteins, had very short half-life (Figures S6B and S6C). *pelo* knockdown did not affect the turnover of these DCV proteins. The short half-life of DCV-2 explains why it is 35S-visible but Coomassie-invisible, because quick turnover make it rich in 35S-Met labeling (newly synthesized proteins) but its total amount is low. Because DCV-2 has almost equal 35S-Met labeling intensity as DCV-1 and DCV-3 but its half-life is much shorter than theirs, the instantaneous expression of DCV-2 should be higher than DCV-1 and DCV-3. Thus, DCV-2 may not be an ORF1 protein, but an ORF2 protein. Based on its size, DCV-2 cannot be the entire polyprotein of ORF2 or VP0. Since inhibition of either proteasomes or lysosomes by MG132 or chloroquine, respectively, could not increase the half-life of DCV-2 (data not shown), we predict that DCV-2 is an unprocessed precursor or intermediate product of capsids.

*pelo* deficiency apparently does not influence the synthesis of most, if not all kinds of cellular proteins in S2 cells based on the 35S-Met labeling experiments (Figures 4A, 4D and S6). The levels of proteins expressed by transiently transfected plasmids in the control and *pelo* knockdown cells were the same or almost the same (Figure 4E). Taken together, these data suggested that *pelo* is selectively involved in the expression of some DCV proteins such as capsids.

*pelo* does not target IRES or the termination sequence of ORF2

Since capsid proteins of DCV are encoded by ORF2, *pelo* may regulate the translation of DCV ORF2. The translation initiations of DCV ORFs are mediated by two different internal ribosomal entry sites (IRES). We constructed bicistronic luciferase reporters containing either the DCV IRES1 or IRES2 (Figure 5A), and neither of these two IRES-dependent translations was influenced by the depletion of *pelo* (Figure 5B). Because the structure of *pelo* is similar to eRF1 and *pelo* has functions in promoting ribosomal shunt dissociation, we tested whether the termination sequence of ORF2 is regulated by *pelo*. We fused termination regions of ORF1 or ORF2 to Firefly luciferase reporters (Figure 5C), and found that the expression of both reporters was not influenced by *pelo* knockdown (Figure 5D). Our reporter studies suggested that *pelo* does not function in the regulation of either IRES dependent translational initiation of ORF2 or termination of ORF2.

The increased non-functional 80S monoribosomes in *pelo* deficient cells may limit ribosome availability and contribute to the inhibition of DCV protein synthesis

Polysome profile analysis is frequently used to monitor the efficiency of translation [49]. We used it to examine whether there was any difference in the efficiency of DCV protein synthesis between control and *pelo* knockdown S2 cells. Cells were mock infected or infected with DCV for 6 hours. The 6 hours was chosen because at this time point the difference in DCV protein, but not DCV RNA, begins to appear between wild-type and *pelo* deficient cells (Figure 5S). Cell lysates were resolved on a 10–30% continuous sucrose gradient (Figure 6A). Knockdown of *pelo* increased the amount of 80S monoribosomes, which is consistent with the result obtained by studying Dom34 deletion in yeast [37]. However, the distribution of DCV RNAs in ribosome profiling fractions is about the same in wild-type and *pelo* knockdown cells (Figure 6B). DCV infection also leads to increase of 80S monoribosomes in wild-type S2 cells over the time of infection (Figures 6A and S7). DCV infection does not affect the level of 80S monoribosomes in *pelo* deficient cells much (Figure 6A), due to the already high level of 80S monoribosome and ineffective replication of DCV in *pelo* deficient S2 cells.

The 80S monoribosomes could associate with mRNA and also could be free. We extracted the total RNAs from 80S and polysome fractions prepared from non-DCV infected cells. The amount of RNA in the 80S fraction from the *pelo*-deficient cells was about three times of that from the control cells, while the quantities of RNA in the polysome fraction were the same in both the control and *pelo* knockdown cells (Figure 6C). Because rRNA is the major component of the total RNA extracted from these fractions, this data confirms that *pelo*-deficient cells have more 80S monoribosomes. We then analyzed the amount of mRNA associated with 80S monoribosomes and polysomes. The samples with equal amounts of total RNA, which indicated that the RNA samples were extracted from an equal number of ribosomes, were hybridized with digoxigenin (DIG)-labeled oligo (dT) probes to detect mRNA. DIG was detected with alkaline phosphatase labeled anti-DIG antibody by chemiluminescence (Figure 6D). The amount of Poly (A) RNA in the 80S fraction of the *pelo*-deficient cells was less than that of the control cells, whereas poly (A) RNA amounts in the polysome fraction were not affected by *pelo* knockdown. The same result was obtained by reverse transcribing total RNA with oligo (dT) in the presence of [α-32P] dTTP (Figure 6E). The level of incorporated [α-32P] dTTP in cDNAs should correlate with the level of mRNA. Based on these data, we concluded that the increased portion of 80S monoribosomes in *pelo*-deficient S2 cells was primarily contributed to by mRNA free ribosomes. By using the same approach, we determined that DCV infection-induced 80S monoribosomes in wild-type S2 cells are also mainly poly (A) RNA free ribosomes (Figure 6F).

Since Dom34 forms a complex with HBS1 to function [29,50], we knocked down HBS1 in S2 cells and found that DCV
replication was indeed inhibited (Figure S8). It has been well established recently that Dom34-HBS1 plays an essential role in the quality control of protein translation by dissociation of stalled ribosomes at the 3' end of aberrant mRNAs. The increase of 80S monoribosomes in pelo-deficient cells is most likely to be a result of those stalled ribosomes being processed to monomers. The increase in non-translating 80S ribosomes in DCV infected cells suggests that high level DCV protein synthesis produces more incidents of stalled ribosomes. Inefficient recycling of stalled 80S ribosomes in DCV RNA should result in the synthesis of truncated and mutated viral proteins and also could reduce the translation efficiency of viral RNA. Impairment of the dissociation of

Figure 4. pelo regulates the syntheses of some specific DCV proteins. (A) Cells pretreated with the indicated dsRNAs were either uninfected (−) or infected (+) with DCV (MOI=10) for 6 hours and then labeled with 35S-Met for 30 min. Labeled proteins were analyzed by Bis-Tris SDS-PAGE followed by autoradiography. The proteins that appeared after DCV infection are indicated by arrows and named DCV-1 to DCV-7. One 35S-Met labeled host cell protein between 37 and 50 KDa was used as loading control for protein analysis. (B) The quantities of DCV-1 to DCV-7 were determined by densitometry and normalized to mock control. The value of mock control was set at 1, and all other samples were normalized to it. (C) qRT-PCR analysis of DCV genomic RNA in different cells after 6 hours of DCV infection. Results were normalized to mock control and represent the mean ± SD of triplicates. (D) The quantities of metabolically labeled proteins (15–250 KD) were determined as decreased in B. (E) Immunoblot analysis of Flag-Firefly or Myc-Renilla expression in cells pretreated with indicated dsRNAs.

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non-translating 80S ribosomes should reduce the availability of ribosomes for protein translation. It is believed that the relationship between free ribosome concentration and the rate of peptide formation follows the Michaelis-Menten equation [51] (Figure S9). Based on the Michaelis-Menten equation, the reduction of free ribosome concentration has a more inhibitory effect on the production of quickly synthesized proteins than on the production of slowly formed proteins (Figure S9). In the case of DCV, a single viral RNA encodes capsid proteins DCV-4 to 7 and non-capsid proteins DCV-1 and 3. Since a large amount of capsid proteins is required for efficient DCV replication, reduced free ribosomes in \textit{pelo} knockdown cells are likely to be responsible for the phenomenon in which \textit{pelo} knockdown had much more of an effect on the protein synthesis of DCV-4 to 7 than on that of DCV-1 and 3 (Figure 4).

High speed synthesis of capsid proteins is required for the replication of many different viruses. In order to further evaluate the notion that the availability of free ribosomes in \textit{pelo}-deficient flies limits viral replication, we examined whether \textit{pelo} deficiency can influence the replication of three other viruses, single-stranded RNA virus \textit{Cricket Paralysis Virus} (Crpv), double-stranded RNA virus \textit{Drosophila X virus} (DXV), and a large dsDNA virus \textit{invertebrate iridescent virus 6} (IIV6). We used qRT-PCR as described by published studies [15,17,52] to measure the corresponding RNA or DNA levels of each virus in the infected flies, and found that the replications of these viruses were all slower in \textit{pelo}~\textsuperscript{−/−} flies (Figure 6G). We also analyzed Crpv, DXV, and IIV6 at different time points after infection in wild-type and \textit{pelo}~\textsuperscript{−/−} flies and measured viral titers in flies at 3 days post-infection (Figure S10). The data support the conclusion that the replications of these three different viruses are suppressed in \textit{pelo}~\textsuperscript{−/−} flies. The \textit{pelo} deficiency selectively resists viral infection as \textit{pelo}~\textsuperscript{−/−} flies are not resistant but even more sensitive to bacterial infection (data not shown). These data support the idea that the defect in the recycling of stalled ribosomes in \textit{pelo}-deficient cells limits viral replication and implies that \textit{pelo} deficiency mediates a general antiviral activity.

**Discussion**

Through a forward genetic screen for \textit{Drosophila} mutants with increased or decreased susceptibility to DCV-induced death, we have identified a virus-resistant line in which the \textit{pelo} gene was mutated. We further demonstrated, by using this and other \textit{pelo}-deficient \textit{Drosophila} lines, and by rescuing \textit{pelo} expression in \textit{pelo}~\textsuperscript{−/−} flies, that \textit{pelo} deficiency led to resistance to DCV infection.
**A**

- Graph showing OD$_{260nm}$ values for different treatments.

**B**

- Graph showing total DCV RNA percentage for different treatments.

**C**

- Bar graph comparing normalized RNA quantities for dsGFP and dspelo in 80S and polysome.

**D**

- Bar graph comparing normalized densitometric values of DIG-labeled probes for dsGFP and dspelo.

**E**

- Graph showing $^{32}$P radioactivity for 80S and polysome.

**F**

- Bar graphs comparing normalized RNA quantities and densitometric values of DIG-labeled probes for Uninf and DCV treatments.

**G**

- Bar graph showing fold change in expression for Crpv, DXV, and IIV6 for wt and pelo$^{-}$.
Figure 6. The increased non-functional 80S monoribosomes in pelo-deficient cells may limit free ribosome availability for high level viral protein synthesis. (A) Cells pretreated with the indicated dsRNAs were either uninfected (Uninf) or infected with DCV for 6 hours (MOI = 10) followed by polysome profile analysis. (B) qRT-PCR analysis of DCV RNA in individual fractions of sucrose gradients. The distribution of DCV RNA among the fractions is shown as a percentage of the total DCV RNA. (C) Total RNA of the 80S fraction and one of the polysome fractions were extracted. RNA quantities are shown as their relative ratios compared to that of 80S in dsGFP-treated control cells. (D) Equal amounts of RNA from each sample were reverse transcribed with oligo (dT) in the presence of [α-32P] dTTP. The resulting cDNAs were recovered by using NucAway spin column. The radioactivity was measured with liquid scintillation counting. Results are the mean ± SD of triplicates. (E) Cells were uninfected (Uninf) or infected with DCV for 12 hours (MOI = 30) and then were followed by polysome profile analysis. Total RNA of 80S fractions of each sample were extracted and then analyzed as described in C and D. (G) pelo is generally involved in the replication of different viruses. wt and pelo -/− flies were infected with Crpv, DVX, or IIV6 for 72 hours. RNA was extracted from Crpv and DVX infected flies, and DNA was isolated from IIV6 infected flies. qRT-PCR or qPCR was used to analyze the amounts of these three different viruses. Results were normalized to wt and shown as the relative values. Data are the mean ± SD of triplicates.

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previously dipped into the viral solution (DCV, 5 × 10^{11} TCID_{50}/ml; Crpv, 4 × 10^{7} TCID_{50}/ml; DXV, 6 × 10^{7} TCID_{50}/ml; IIV6, 7 × 10^{10} TCID_{50}/ml) and only the very tip of the needle is inserted into the fly. The infected flies were monitored for mortality or collected at indicated time points for different assays. Viral titers were determined in S2 cell culture and calculated according to the end-point method of Reed and Muench [56].

**Cells, RNAi and transfection**

*Drosophila* S2 cells (ATCC) were cultured at 25°C in SF900-II SFM medium (Gibco), dsRNA targeting *pelo* (nucleotides 304 bp–3938 bp), *HBS1* (nucleotides 706–2119 bp), *Firefly* (nucleotides 1144–1650 bp), and *GFP* (nucleotides 65–559 bp) were generated from T7-promoter-flanked PCR products by in vitro transcription using T7 transcription kit (Promega). RNAi treatments were performed as previously described [57]. In brief, S2 cells were incubated with SF900-II SFM medium containing 10 μg/ml dsRNA and then were replenished with fresh dsRNA-containing medium daily for 6 days.

For transfection, S2 cells were incubated in Schneider’s medium (Lonza) supplemented with 10% FBS, and transfections were performed by the Calcium Phosphate precipitation method. After 2–3 h of incubation, the cells were washed three times with PBS and then starved with methionine-free culture medium for 15 min. After methionine starvation, cells were incubated with 0.2 μCi/ml 35S-Methionine in methionine-free medium for 30 min, washed three times with PBS and lysed with Laemmli sample buffer. Labeled proteins were then recovered by using NucAway spin column (Ambion) while removing the salts and unincorporated [35S] Met for 30 min, washed three times with PBS and then lysed (time point 0) or chased for the indicated time with excess cold methionine.

**Metabolic labeling**

Cells were washed three times with PBS and then starved with methionine-free culture medium for 15 min. After methionine starvation, cells were incubated with 0.2 μCi/ml 35S-Methionine in methionine-free medium for 30 min, washed three times with PBS and lysed with Laemmli sample buffer. Labeled proteins were recovered by using NucAway spin column (Ambion) while removing the salts and unincorporated [35S] Met for 30 min, washed three times with PBS and then lysed (time point 0) or chased for the indicated time with excess cold methionine.

For viral infection, 2–4 days old flies of the stated genotype were infected by septic injury with the indicated virus as described [55]. Briefly, the thorax of the fly was pricked with a thin needle previously dipped into the viral solution (DCV, 5 × 10^{11} TCID_{50}/ml; Crpv, 4 × 10^{7} TCID_{50}/ml; DXV, 6 × 10^{7} TCID_{50}/ml; IIV6, 7 × 10^{10} TCID_{50}/ml) and only the very tip of the needle is inserted into the fly. The infected flies were monitored for mortality or collected at indicated time points for different assays. Viral titers were determined in S2 cell culture and calculated according to the end-point method of Reed and Muench [56].

**Polysome analysis**

Cells were incubated with 100 μg/ml cycloheximide for 10 min and then washed twice with cold PBS containing cycloheximide (100 μg/ml). 5 × 10^{6} cells were lysed with 1 ml polysome lysis buffer (10 mM HEPES-KOH pH 7.4, 5 mM MgCl2, 150 mM KCl, 0.5% NP-40, 0.5 mM DTT, 100 μg/ml cycloheximide, 100 U/ml RNAsin RNase inhibitor [Promega], and EDTA-free protease inhibitor cocktail Complete [Roche]). Cell debris was removed by centrifuging for 10 min at 16,000 g. The cytoplasmic supernatant was layered onto 10 ml of 10–50% continuous sucrose gradient and centrifuged at 4°C for 2 h at 36,000 rpm in a Beckman SW41 rotor. Then 0.4-ml fractions were collected from the top of the gradient and the polysome profile was monitored by RNA absorbance at 260 nm.

RNA of each fraction was extracted by using RNAiso Plus (Takara) and the distribution of DCV RNA was measured by quantitative RT-PCR. For the slot blot assay, RNA was incubated with digoxigenin (DIG)-labeled oligo (dT) probes. After hybridization, samples were applied to the nylon membrane by using a Bio-Dot apparatus (Bio-rad) and fixed by UV crosslinking. Following the washing and blocking steps, the membrane was probed with anti-DIG antibody that was coupled to alkaline phosphatase (Roche), and then detected by incubating with the chemiluminescent substrate CDP-star and exposing the blot to X-ray film. The quantities of DIG-labeled probes were determined by densitometric analysis. For radioactive assay, equal amounts of RNA from each sample were reverse transcribed with oligo (dT) in the presence of [α-32P] dTTP. The resulting cDNAs were recovered by using NucAway spin column (Ambion) while removing the salts and uncincorporated [α-32P] dTTP. The radioactivity was measured with liquid scintillation counting (Beckman).

**Statistical analysis**

Statistical analysis was performed using the unpaired two-tailed Student’s t-test with the Prism GraphPad software. P<0.05 was considered significantly different.

**Accession numbers**

NCBI gene ID numbers for *Drosophila melanogaster* genes mentioned in the text. *pelo* (34286); *HBS1* (117365); *Pka-C1* (318907); *haup* (44173); *CG31710* (318907); *vir-1* (34652); *vago* (32040).

Genebank accession numbers for the genome sequences of the viruses. *Drosophila C virus* (DCV), NC_001834; *Drosophila x virus* (DXV), NC_004177, NC_004169; *Cricket paralysis virus* (Crvp), NC_003924; *Invertebrate iridescent virus 6* (IIV6), NC_003038.
Supporting Information

Figure S1 Screen for Drosophila mutants with increased or decreased susceptibility to DCV-induced death. 2–4 days old flies were injected with DCV and then monitored for mortality. y w was used as a genetic background control. 60 flies of each line were used. About 100 mutant fly lines were screened and ten lines were shown.

Figure S2 Measuring the Wolbachia infection status of the fly lines used. (A) PCR amplification with primers for the Wolbachia specific genes wsp and wspB on DNA extracts of indicated fly lines. PCR amplification of mt 12S rRNA was used as a DNA extraction control. (B) The amounts of Wolbachia DNA in indicated fly lines were measured by qPCR. Results were normalized to y w and shown as the relative values. Data are the mean ± SD of triplicates.

Figure S3 Growth curve of cells pretreated with indicated dsRNAs. Cells pretreated with the indicated dsRNAs for 6 days were seeded at a density of 10^4 cells/ml (day 0) and then counted every day. Data are the mean ± SD of triplicates.

Figure S4 The expressions of JAK-STAT target gene after DCV infection in S2 cells. S2 cells were untreated (Mock) or treated with the indicated dsRNAs for 6 days and then infected with DCV (MOI = 0.1). The expressions of vir-l (A) and the accumulation of DCV RNA (B) were analyzed by qRT-PCR. Data are the mean ± SD of triplicates.

Figure S5 The biosynthesis of viral nucleic acids and proteins during the course of DCV infection. (A) Cells pretreated with the indicated dsRNAs were infected with DCV (MOI = 10) and then collected at different time points. The accumulation of DCV RNA was measured by qRT-PCR. Data are the mean ± SD of triplicates. (B) Cells were labeled with 35S-Met for 30 min at different time points post-infection. Labeled proteins were analyzed by Bis-Tris SDS-PAGE followed by autoradiography. (C) The accumulation of DCV RNA was measured by qRT-PCR. Data are the mean ± SD of triplicates.

Figure S6 DCV-1 to 7 are viral proteins and DCV-2 has high rate of turnover. (A) Cells pretreated with the indicated dsRNAs were either uninfected or infected with DCV (MOI = 10) for 2 hours and then actinomycin D was added to the medium to inhibit host mRNA transcription. 4 hours later, cells were labeled with 35S-Met for 30 min in the presence of actinomycin D or absence of actinomycin D. Labeled proteins were analyzed by Bis-Tris SDS-PAGE followed by autoradiography. (B and C) Uninfected (Uninf) cells or cells infected with DCV for 6 hours were labeled with 35S-Met for 30 min. Cells were washed three times with PBS and then lysed (time point 0) or chased for the indicated times with excess cold methionine. The stabilities of labeled proteins were detected by Bis-Tris SDS-PAGE, followed by autoradiography.

Figure S7 Polysome profile of DCV infected S2 cells. Cells were infected with DCV (MOI = 10) and then collected at different time points for polysome profile analysis. Lysates were layered on a 10–50% sucrose gradient and centrifuged. 0.3-ml fractions were collected and the polysome profile was monitored by RNA absorbance at 260 nm. Note that there is an extra peak in DCV-infected cells at 12 h post-infection, which is most likely from the packaged viruses.

Figure S8 Replication of DCV is slower in HBS1 knockdown S2 cells. (A) Cells pretreated with indicated dsRNAs were challenged with DCV and harvested at different time points post-infection. The accumulations of DCV capsid protein were measured by immunoblotting. (B) Knockdown efficiency of HBS1 was assessed using qRT-PCR. Results are the mean ± SD of triplicates.

Figure S9 Mathematical calculation of the effect of ribosome concentration on different-speed synthesized proteins. (A) Michaelis-Menten equation of formation of peptide. Vₐ: rate of peptide A formation in wild-type cells. [S]: concentration of free ribosome in wild-type cells. Vₐmax: maximum rate of peptide formation. Kₐ: Michaelis constant of peptide A formation. (B) Vₐmax: rate of peptide A formation in pelo⁻/⁻ cells. [S]: concentration of free ribosome in pelo⁻/⁻ cells. AS: [S]⁻¹. (C) Vₐ and Vₐmax: rate of B peptide formation in wild-type and pelo⁻/⁻ cells, respectively. Kₐ: Michaelis constant of peptide B formation. (D) The effect of decrease in free ribosome concentration [S] on the more quickly synthesized peptide A is greater than that on the more slowly synthesized peptide B.

Figure S10 pelo deficiency inhibits the replication of different type viruses. (A–C) wt and pelo⁻/⁻ flies were infected with virus and collected at the indicated time point. RNA was extracted from Crpv (A) and DXV (B) infected flies, and DNA was isolated from IIV6 (C) infected flies. qRT-PCR or qPCR was used to analyze the amounts of these three different viruses. (D) Flies were infected with indicated virus for 3 days. Three pools of ten flies were collected and homogenized. The viral titer in the homogenate was determined by end-point dilution. Data are the mean ± SD of triplicates.

Table S1 Peptides identified in mass spectrometry.

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Author Contributions

Conceived and designed the experiments: XW JH. Performed the experiments: XW WTH ST DM WC LL LT CQZ. Analyzed the data: XW YL FH JC JH. Wrote the paper: XW FH JH.

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