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Entry is a rate-limiting step for viral infection in a *Drosophila melanogaster* model of pathogenesis

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Abstract

The identification of host factors that control susceptibility to infection has been hampered by a lack of amenable genetic systems. We established an *in vivo* model to determine the host factors that control pathogenesis and identified viral entry as a rate-limiting step for infection. We infected *Drosophila melanogaster* cells and adults with drosophila C virus and found that the clathrin-mediated endocytotic pathway is essential for both infection and pathogenesis. Heterozygosity for mutations in genes involved in endocytosis is sufficient to protect flies from pathogenicity, indicating the exquisite sensitivity and dependency of the virus on this pathway. Thus, this virus model provides a sensitive and efficient approach for identifying components required for pathogenesis.

The host-virus relationship is a dynamic and evolving process in which the virus minimizes its visibility while the host tries to prevent and eradicate infection. Initially, the virus must enter its target cells and migrate to the appropriate intracellular compartment for viral replication. Subsequently, the infection spreads to additional susceptible cells and hosts. To overcome infection, the host must recognize the presence of the virus and eliminate it as efficiently as possible. Because viruses are obligate intracellular parasites with limited coding capacity, they co-opt host proteins and processes to complete their life cycle while simultaneously evading the immune system of the host[1,2]. Thus, the outcome of an infection depends on the effectiveness of the host's ability to overcome the viral pathogen in the face of the virus's ability to replicate and evade[3,4].

In this 'arms race' between host and parasite, it is not just in the parasite that stealth is selected. If a host is able to make itself invisible to its pathogen, it will have evaded infection. The study of resistance to endemic pathogens has led to the identification of previously unknown host factors that can be modulated to resist infection and are therefore reasonable targets for antimicrobial drugs. For example, in the avoidance of malaria there is a positive selection for mutations in many host factors involved in basic cellular processes,

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COMPETING INTERESTS STATEMENT

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including hemoglobin and glucose-6-phosphate dehydrogenase[5]. In addition, CCR5, a human immunodeficiency virus (HIV) coreceptor required for viral entry, is under negative selection in the human population because of the HIV epidemic[6]. Seemingly healthy individuals with mutant CCR5 are resistant to the virus because it is unable to enter some target cells and replicate in the host. Thus, the modulation of host pathways affects viral replication and hence host morbitity.

As seen in HIV, the early steps in the viral life cycle are important for replication and tropism and thus pathogenesis. In addition to extracellular receptors, intracellular factors can control outcomes. Poliomyelitis, the acute disease of the central nervous system caused by poliovirus, provides such an example. In most infected individuals the virus is cleared before infection of the central nervous system occurs. This tissue tropism is determined only in part by the presence of the receptor. In humans, many tissues including the liver express the receptor and are exposed to virus, but are not infected[7]. Moreover, transgenic mice expressing the human receptor can be infected, but only in a subset of expressing tissues. Therefore, additional intracellular host factors must control the specificity of infection and thus pathogenesis.

The identification of host factors involved in viral infections and pathogenesis has been difficult. This is partly because of the lack of ‘forward’ genetic systems amenable to viral infection as well as the lack of a suitable *in vivo* model for testing the interactions between the virus and host. Although some genetic studies in animals have been done, they have mainly relied on a candidate gene approach, whereby the interactions between a virus or viral mutants and known genes involved in immunity have been tested[8]. Other studies have taken advantage of naturally occurring mouse strain polymorphisms to identify important and previously unknown mediators of viral pathogenesis. This led, for example, to the identification of a specific natural killer cell receptor as a chief determinant for susceptibility to mouse cytomegalovirus[9]. Such work hints at the power of a ‘forward’ genetic model, although the models now available are unwieldy for large-scale analyses.

To generate a tractable genetic system amenable to viral infections, we established a model in *Drosophila melanogaster* based on infection with Drosophila C virus (DCV) that will provide insights into the mechanisms by which viruses infect and kill the host. DCV most closely resembles vertebrate picornaviruses, a large class of positive single-stranded nonenveloped RNA viruses that are responsible for a wide variety of diseases in humans and agriculture. For example, poliovirus and the foot-and-mouth disease virus belong to this family[10]. By studying virus-host interactions in *D. melanogaster*, we hope to identify host factors required for viral infection and the conserved mechanisms by which organisms combat viruses.

Here we demonstrate that DCV infects tissue culture cells using endocytosis as the mechanism of entry. By taking advantage of the large number of mutants available in *D. melanogaster*, we were able to establish that clathrin-mediated endocytosis is an essential host pathway required for viral infection and pathogenesis *in vivo*. Flies carrying mutations in genes involved in various aspects of receptor-mediated endocytosis at the plasma membrane showed resistance to viral infection. Specifically, we identified clathrin heavy

chain, α-Adaptin, abnormal wing discs, synaptotagmin and shibire as host factors required for viral infection. Subtle attenuation of the endocytic machinery had considerable protective effects. Because moderate inhibition of the pathway *in vivo* can attenuate viral growth while having no adverse effects on the host, this indicates that inhibition of viral entry by modulation of endocytosis might lead to new antiviral therapies.

RESULTS

DCV infection and entry *in vitro*

To establish the characteristics of DCV infection in *D. melanogaster*, we developed an assay to monitor infection *in vitro*. We generated infectious DCV (with a strain isolated originally in Charolles, France) by inoculating the *D. melanogaster* tissue culture cell line DL2 (ref. 11). DCV grew in the cells, as evidenced by cytopathic effect (CPE) and by the presence of infectious virions after biochemical purification from infected cells. CPE, in contrast to budding, is the mechanism of viral release used by many viruses, including mammalian picornaviruses[12]. We monitored CPE by flow cytometry and distinguished dead cells from live cells by staining with propidium iodide. We noted increased cell death in DL2 cells by 4 d after infection (Fig. 1a). We used a modified CPE assay to determine the titer of the purified virus, and found that the concentration of the purified virus was 1×10^9 to 1×10^{10} infectious units (i.u.)/μl (data not shown).

To monitor infection more directly, we generated polyclonal anti-bodies to DCV in chickens. Immunoblot analysis confirmed that the antibody recognized purified virions and cells infected with DCV but not uninfected controls (Fig. 1b). The sizes of the reactive bands were consistent with the molecular weights of the structural capsid proteins, between 28 and 38 kDa for DCV[13].

Many viruses require transit through an endocytic compartment for productive infection, whereas others do not and directly fuse with the plasma membrane. To test whether DCV travels through a vesicular compartment for entry, we took advantage of the fact that endocytosis is blocked at low temperature. Thus, by infecting cells with DCV at 4 °C we allowed extracellular binding but not internalization by endocytic vesicles. Next, we removed free virions by washing the cells and returned them to 25 °C. We did not detect this plasma membrane–bound fraction of DCV (Fig. 2a, B). After 3 h, we noted virions inside a vesicular compartment (Fig. 2a, C). This would not be expected if the viruses fused directly with the plasma membrane. Furthermore, longer incubation allowed the virus to escape from this compartment and gain entry into the cytoplasm to produce new viral antigens (Fig. 2a, D). Thus, the formation of vesicles containing viruses preceded the production of new viral antigens.

To directly demonstrate that the cytoplasmic staining was due to the production of new viral antigens, we treated cells before infection with cycloheximide. This treatment blocked the late production of new viral antigens in the cytoplasm (Fig. 2a, D) but not the vesicular staining (Supplementary Fig. 1 online). These data indicate that DCV traffics through a vesicular compartment for entry *in vitro*.

To further demonstrate a dependence of DCV on endocytosis for viral entry, we assayed whether viral replication was sensitive to bafilomycin A1 or valinomycin. Bafilomycin A1, a vacuolar (v)-ATPase inhibitor, blocks acidification of vacuolar endosomal compartments used extensively in mammalian systems to demonstrate the dependence of viral entry on acidification[14]. Inhibition of v-ATPase also blocks the movement of vesicles from the early endosomal compartment to the endosomal carrier vesicles, an early step in vesicular trafficking[15]. Valinomycin is an ionophore that causes the collapse of the membrane potential of all membranes in the cell, including the endosomal vesicles[16]. This also inhibits the v-ATPase, among other targets. We confirmed that these drugs affected vesicular trafficking in *D. melanogaster* cells by monitoring the uptake of 'LysoTracker', a dye that is selectively retained in acidified compartments. In the presence of valinomycin or bafilomycin A1, the cells were unable to efficiently internalize the dye, thereby demonstrating a defect in endocytosis (Supplementary Fig. 2 online).

To test the effects of these small molecules on viral replication, we pretreated DL2 cells with bafilomycin A1 or valinomycin and then added DCV. We washed the cells after 3 h to remove the drugs and free virus, and assayed them 20 h later with an immunofluorescence assay for the production of viral antigens. Both bafilomycin A1 and valinomycin inhibited viral antigen production (Fig. 2b). We used cycloheximide as a positive control for these experiments because it blocks the synthesis of new viral proteins. Quantification of three independent experiments showed that bafilomycin A1 inhibited viral growth by 98.6% ($P \leq 0.001$); valinomycin, by 100% ($P \leq 0.001$); and cycloheximide, by 99.9% ($P \leq 0.001$; Fig. 2c). These drugs had no effect on cellular viability in these conditions (data not shown). These results demonstrate a requirement for endocytosis in viral replication *in vitro*.

We next examined whether endocytosis is required to present the cellular receptor at the plasma membrane for viral binding and thus entry. We therefore tested whether the virus can enter cells in the presence of valinomycin, which blocks the movement of vesicles from the early endosomal compartment to the endosomal carrier vesicles. If the virus required endocytosis for entry, the virus would enter the cells and traffic to the early endosome but not gain entry to the cytoplasm to begin new replication. In contrast, if endocytosis were required to bring the receptor to the plasma membrane, no vesicular staining would be detected in these conditions. The virus was able to enter a vesicular compartment in the presence of valinomycin but did not express cytoplasmic antigen as in the untreated case, in which viral antigens are detected in the cytoplasm (Fig. 2a, G and H). This is consistent with a requirement for endocytosis in viral entry and not simply for the presentation of the cellular receptor to DCV.

Infection requires the endocytic machinery *in vivo*

To determine whether endocytosis is the mechanism for viral entry for DCV *in vivo* and, if so, to define essential components of this pathway, we microinjected the purified virus into wild-type flies. Infected flies succumbed to infection when injected with >100 i.u./μl of DCV (Fig. 3a and data not shown). Next, we tested whether flies that carry mutations in genes encoding various components involved in vesicular trafficking showed an altered susceptibility to DCV infection. We reasoned that flies deficient for components required for

viral entry should be resistant to DCV infection, and thus we specifically tested mutants deficient in the clathrin-mediated endocytosis pathway (clathrin heavy chain, α-Adaptin, abnormal wing discs, synaptotagmin)[17], vesicle coating (like AP180) and uncoating (hsc70–4 and cysteine string protein), vesicle fusion (syntaxin, synaptobrevin), the AP-3 adapter (orange, ruby, garnet), lysosomal transport (Vps homologs: deep orange and light), and late endosomal trafficking (hook)[18]. In addition, we also tested a class of eye color mutants (claret, lightoid and pink) on the assumption that they might encode additional trafficking proteins[19].

To analyze the requirement of these genes in DCV infection of adult flies, we tested flies with homozygous viable mutant back-grounds when available or otherwise, heterozygous flies. Flies with mutations in genes encoding components of the clathrin-mediated endocytosis machinery were clearly protected from lethal doses of virus. Flies with mutations in α-Adaptin ($\alpha\text{-}Ada^{3}/\alpha\text{-}Ada^{4}$) were completely protected, whereas flies on backgrounds with other mutations such as clathrin heavy chain ($chc^{4}/+$), abnormal wing discs (awd^{Kpn}/awd^{Kpn}) and synaptotagmin ($syt^{T77}/+$) showed significant resistance to infection (Fig. 3a,b and data not shown; $P < 0.05$ in >75% independent tests). There was resistance in some cases in heterozygous mutation backgrounds, indicating that the genetic back-grounds in the stocks were not responsible for the resistance to DCV. Furthermore, the observation that flies with heterozygous *α-Ada* ($\alpha\text{-}Ada^{3}/+$) as well as *awd* ($awd^{Kpn}/+$) mutant backgrounds were more resistant to infection than were wild-type flies demonstrates the exquisite sensitivity of DCV to the levels of these genes. That loss of even one copy of these genes (heterozygous mutants) had a detectable effect on the resistance of the flies to the virus but not on adult viability demonstrates that viral entry is a rate-limiting step for infection *in vivo*, and that specific components of the endocytic machinery are required for viral pathogenesis *in vivo*.

None of the other mutations in trafficking genes that we tested (*or*, *g*, *rb*, *dor*, *lt*, *hk*, *lap*, *hsc70–4*, *csp*, *syb*, *nsyb*, *syx*, *com*, *ltd*, *ca*, *pn* or *p)* conferred DCV resistance ($P > 0.05$), indicating that they are dispensable for viral replication. However, because some genes were tested as heterozygous mutatations (*lap*, *hsc70–4*, *csp*, *syb*, *nsyb* and *syx*), we cannot exclude the possibility that a 50% reduction in the level of these genes is not sufficient to affect DCV susceptibility. In addition, the molecular nature of some of these alleles is not known, such that some mutations may be associated with residual gene activity.

To further demonstrate that these genes were required for viral entry, we tested for the production of actively replicating virus and viral antigens *in vivo*. First, we assayed infected flies for the production of viral antigens at various time points after infection by immunoblot analysis. Whereas viral proteins were detectable in wild-type flies infected with DCV by 48 h after infection, the α-Adaptin mutants had no detectable immunoreactive bands, even at 72 h after infection (Fig. 3c). The virus-mediated mortality correlates well with the detection of viral antigens. For example, flies that succumbed at a later time point (such as $chc^{4}/+$ flies) produced detectable viral antigens later in the time course. We also found a decrease in viral antigen production in *awd* (awd^{kpn}/awd^{kpn}) and *syt* ($syt^{T77}/+$) mutants (data not shown). These data reinforce the idea that these endocytosis genes are required for viral entry and that decreasing the efficiency of the endocytic machinery can attenuate the infection.

Next, we examined the distribution of viral antigens in infected flies using immunofluorescence techniques. The tissue tropism of viruses affects the pathogenic consequences of infection. Immunostaining allowed us to assess whether the mutant proteins were affecting viral replication in all or a subset of susceptible tissues. Immunofluorescence analysis of wild-type flies showed that the antibody did not recognize uninfected tissues and that the virus infected a subset of tissues, indicating that not all cell types may be able to support viral replication[20]. The restricted tissue expression may reflect the distribution of the receptor used by the virus for entry. The infected tissues included the fat body (Fig. 3d), the outer sheath of the egg chamber and sheaths surrounding individual ovarioles (Fig. 3d), visceral muscles along the midgut, and a subset of somatic muscles (data not shown). In contrast to the wide-spread infection of the fat body and egg chambers in infected wild-type flies, very few infected α-Ada^{3}/α-Ada^{4} flies showed positive immunore-activity in the fat body and mutant egg chambers (Fig. 3d). Similarly, there was vastly reduced immunostaining in *awd* (awd^{kpn}/awd^{Kpn}), *syt* (syt^{T77}/+) and *chc* (chc^{4}/+) mutant backgrounds (data not shown). In these mutants, all cell types normally infected were similarly protected from DCV replication. Moreover, the lack of staining in the mutant backgrounds demonstrates the exquisite sensitivity of this immunostaining assay for DCV infection. Altogether, the *in vivo* analysis of mutant flies demonstrated the general, essential and quantitative involvement of endocytosis in viral entry.

Autonomous requirement for endocytosis *in vivo*

To further characterize the requirements for endocytosis in viral entry, we tested whether there was an autonomous requirement for endocytosis in infection. For this we used a mosaic analysis *in vivo* by blocking endocytosis specifically in the fat body but not other tissues such as the egg chamber; both of these are normally infected by DCV. We took advantage of a dominant negative form of dynamin to specifically inactivate endocytosis in a tissue-specific way. Dynamin, encoded by the shibire gene, is the GTPase required for the fission of endocytic vesicles from the plasma membrane and thus should be required for endocytosis and entry in this system[21–23]. We were unable to use temperature-sensitive shibire mutants because of the lethality associated with paralysis at the nonpermissive temperature. Instead we took advantage of the Gal4-UAS system, in which a transgene containing UAS transcription factor sites is expressed when the transcriptional activator Gal4 is expressed in a tissue-specific way. We generated mosaic flies that express a dominant negative form of dynamin (*UAS-shi*^{K44A}) only in the adult female fat body with a transgenic line that expresses Gal4 with a yolk promoter (*yolk-Gal4* driver[24,25]). Using this strategy we found that the infected female flies did not have detectable viral antigen production in their fat body (n = 15; Fig. 4), whereas the other tissues normally infected by DCV, such as the egg chamber, were positive by immunofluorescence (Fig. 4, data not shown). Thus, our results demonstrate that endocytosis is required autonomously for viral infection *in vivo* and that this pathway is amenable to inhibition.

DISCUSSION

We have developed a *D. melanogaster* paradigm to facilitate the genetic analysis of the mechanism(s) underlying host-virus interactions and the factors controlling susceptibility.

We used the ability of the picorna-like virus DCV to infect both tissue culture cells and flies to demonstrate that DCV traffics through a vesicular compartment. Furthermore, the infection of flies carrying mutations many genes involved in clathrin-mediated endocytosis (*chc*, *α-Ada* and *awd)* allowed us to demonstrate that attenuation of the endocytic machinery could protect *D. melanogaster* from infection by DCV. This *in vivo* analysis of viral entry has led to the identification of specific factors that regulate infection. Because flies that are heterozygous for mutations in components of the endocytic pathway were protected from DCV-induced lethality, our results emphasize the sensitivity of viral infections to modulation of endocytosis *in vivo*, thus demonstrating that viral entry is a rate-limiting step in the infection process. Our analyses establish the power of the *D. melanogaster* model in demonstrating essential pathways co-opted by viruses for infection and pathogenesis.

We chose DCV as our model virus for several reasons. DCV is an endemic *D. melanogaster* pathogen and thus has coevolved with its host, thereby allowing a dissection of the interactions between a virus and its natural host[26,27]. Flies isolated in different parts of the world have varying susceptibility to this virus; strains of flies from Japan are highly resistant to DCV[28]. DCV isolated in Charolles, France, kills flies in 3 d, whereas DCV isolated from the French Antilles kill adults in 10 d (ref. 29). Infection of susceptible strains results in overt pathology and death. DCV is associated with sporadic laboratory population decreases, indicating that there is a fine balance between the virus and host for survival[30]. However, the natural route of infection is unclear. There are conflicting reports as to whether ingestion of DCV leads to mortality[28,31]. Therefore, we used an injection protocol to ensure reproducible infection and lethality. Molecular characterization of DCV has classified this virus as a member of the picorna-like virus family, although recently it has been characterized as a dicistroviridae[32]. Given the similarity of DCV to the widespread picornaviruses, this genetically tractable system may have direct biological relevance to human and animal picornaviruses. This has been clearly demonstrated for Toll-like receptors in antibacterial responses in *D. melanogaster*.

To characterize this DCV system, we first analyzed the consequences of infection by DCV *in vitro*. We found that infection by DCV caused host cell disintegration, presumably from viral release. Little is known about the mechanisms underlying this CPE. Some evidence exists that picornaviruses inhibit apoptotic mechanisms early in infection, thus prolonging replication in cells and allowing them to multiply in metabolically active cells[33]. It is also likely that the virus uses specific mechanisms to lyse the cells, but those processes have been elusive.

Immunofluorescence analysis of infected flies at late time points after infection (72 h) showed that the cells infected early in the infection, such as those of the fat body, were intact (data not shown). Because the infected cells remained viable, the virions may have exited these cells by another, less destructive pathway, in contrast to the *in vitro* observations. Poliovirus uses host cell disintegration for release in fibroblasts but can exit polarized epithelial cells from the apical membrane by a less destructive but unresolved mechanism[34]. Likewise, another picornavirus, coxsackievirus, replicates cytolytically *in vitro* but replicates noncytolytically in the heart[35]. This indicates that there may be mechanisms *in vivo* for repressing DCV-mediated cytotoxicity, necessitating an alternative mechanism for viral exit.

This also emphasizes the need to analyze these processes *in vivo* rather than relying on *in vitro* analyses.

Viral entry is one step at which some viruses 'hijack' the cellular trafficking apparatus to cross into the cytoplasm from internal cellular compartments[36,17]. There are at least two mechanisms by which viruses enter cells after binding to their cellular receptor. Our *in vitro* experiments showed that DCV traffics through a vesicular compartment, which is kinetically consistent with involvement of endocytosis in viral entry. This observation was also supported by our ability to prevent DCV infection with drugs that inhibit endocytosis. Previous *in vitro* studies of mammalian viruses have suggested some members of the picornavirus family can fuse within the endosomal compartment (such as rhinovirus), whereas others may fuse at the plasma membrane (such as poliovirus); moreover, many have provided conflicting results[37,38]. These studies, mostly based on the use of dominant negative constructs and small-molecule inhibitors of endocytosis, have been difficult to interpret[39]. Moreover, they used tissue culture cell lines that often do not mimic the *in vivo* situation. Even in cases in which it is apparent that a virus requires endocytosis, the specific host factors that are necessary have not been established. Furthermore, whether this step in the viral life cycle is amenable to inhibition for antiviral therapies is also unresolved. Because binding and entry are the first steps in the life cycle of viral pathogenesis in the infection of new hosts, the elucidation of the host factors required for this process would aid our understanding of pathogenesis.

To assess the involvement of these processes *in vivo*, we developed a system to test adult viable flies with mutations in various genes required at different steps in vesicular trafficking. This allowed us to determine if DCV required components of vesicular trafficking for pathogenesis, which genes were necessary and whether DCV was sensitive to subtle alterations in dosage of these pathways.

Using this system we found that flies with mutations in specific components of clathrin-mediated endocytosis, including α-Adaptin and clathrin heavy chain, were resistant to infection, and had decreased production of viral antigens *in vivo*. Moreover, mosaic analysis of a dominant negative form of shibire (dynamin) showed that endocytosis is required autonomously for DCV infection. This experiment demonstrates that an additional component of the endocytic machinery is required for viral entry and that the effect was specific to the fat body and not due to some general change in the biology of the flies. We also identified abnormal wing discs (nucleoside diphosphate kinase), a gene that has recently been linked to endocytosis through its genetic interaction with shibire (dynamin)[40]. Only those flies with mutations in genes involved in endocytosis at the plasma membrane but not those genes required for later stages of trafficking were affected in their susceptibility to infection. These data are inconsistent with a simple requirement for trafficking of the viral receptor to the plasma membrane. Instead, they indicate that components of the endocytic machinery are required for viral entry. Given this and the *in vitro* evidence, we suggest that the virus binds to its cell surface receptor, is captured in clathrin-coated vesicles, traffics through the endocytic pathway and then escapes from this pathway at an early stage of the endocytosis pathway.

Along with the canonical genes required for endocytosis, we found a function for synaptotagmin in this process. Synaptotagmin is a vesicular trafficking protein that is involved in both exocytosis and endocytosis in the fly nervous system[41,42]. It contains an N-terminal transmembrane segment followed by a cytoplasmic tail made up of two calcium-phospholipid binding domains. Although the domains are structurally similar, only the second domain is linked to endocytosis because of its direct binding to the AP-2 adapter protein[43]. Moreover, specific cargo proteins (proteins carried in the vesicles) strengthen the interaction of synaptotagmin with AP-2, indicating involvement of synaptotagmin in coupling cargo at the plasma membrane directly to the endocytic machinery[44]. Further, a requirement for synaptotagmin in endocytosis of non-neuronal cells has been demonstrated in mammalian systems but not in the fly[45,46]. That we found a protective effect in synaptotagmin mutants, as measured by a delay in mortality and a decrease in the production of viral antigens, indicates that synaptotagmin is involved in general clathrin-mediated endocytosis in the fly. Flies with a mutated allele of the gene encoding synaptotagmin that produces a deletion in the C-terminal C2B domain, which interacts with AP-2 ($syt^{4D1}/+$), is also more resistant to infection, further supporting the idea of involvement of synaptotagmin in general endocytosis (data not shown)[41]. Moreover, that the fat body expression of dominant negative dynamin demonstrated a tissue-autonomous requirement for endocytosis in viral entry indicates that these endocytosis genes are not simply required for synaptic transmission for their effect on viral pathogenesis.

The sensitivity of DCV pathogenesis to alterations in the dosage of components of the endocytic machinery was unexpected. Even the removal of one copy of these genes had a detectable effect on the resistance of the flies to the virus but not on viability. That the flies were healthy but susceptible indicates that mutations in these genes are specifically affecting viral pathogenesis and not some nonspecific process. Previous studies in tissue culture models were unable to demonstrate this sensitivity. Two different models can explain this *in vivo* sensitivity. Perhaps viral entry *in vivo* is an inherently inefficient process because of low receptor expression or simply because of a low-affinity interaction of the capsids with cells. Alternatively, entry may be efficient but there may be mechanisms *in vivo* to inactivate the virions, such as proteases present within the fly hemolymph that may proteolyze the capsids, resulting in defective virions that cannot bind and enter the host cells. This would indicate that there is a competition *in vivo* for entry versus inactivation of the virus.

Whether this sensitivity to entry *in vivo* is a general phenomenon or specific to DCV–*D. melanogaster* interactions remains to be examined. Mice with two mutant synaptotagmin homologs, but not other components of the clathrin-mediated endocytosis pathway, are available[47,48]. Viral challenge of mice with mutant clathrin, AP-2, dynamin and nucleotide diphosphate kinase will demonstrate whether the sensitivity of DCV to endocytosis mutants will hold true in higher organisms infected with other viruses.

Although it is apparent that there is a strong selection for pathogen fitness, the host-pathogen interaction also enforces selection for evasion on the host. This process is more difficult to observe in the hosts, as they have longer reproductive cycles and lower rates of mutations. However, in the case of malaria, which has been endemic in Africa for centuries, examples of genetic selection against this pathogen in humans exist[5]. The resistance to endemic

pathogens has led to the identification of previously unknown host factors that can be modulated to resist infection and therefore are reasonable targets for antimicrobial drugs. As with the positive selection of people with genetic variants resistant to malaria, genes enabling viral entry may be under negative selection in *D. melanogaster*. Whereas endocytosis is certainly necessary for life, its attenuation may be survivable and in some cases may be advantageous. The modest modulation of endocytosis that we showed here is sufficient to protect *D. melanogaster* from infection, indicating that the natural endocytic activity is very close to a threshold. The fact that the amount of expression of many endocytosis genes in flies is so close to the threshold of nonpermissivity for DCV infection may well be evidence of an escalating selection by viruses for ever lower amounts of endocytosis. Further studies with natural pathogens to study viral-host interactions will allow identification of additional host pathways and processes that are under selection in populations for the evasion of infection.

METHODS

Fly stocks.

All flies were maintained on standard medium at 24 °C. The following mutant strains were used in this study: clathrin heavy chain (chc^4), α-Adaptin (α-Ada^3, α-Ada^4), nucleoside diphosphate kinase (awd^{K-pn}), shibire (*UAS-shi.K44A*), AP3-σ (orange: or^1), AP3-δ (garnet: g^1, g^2), AP3-β (ruby: rb^1), Vps18p (deep orange: dor^8, dor^1), Vps41p (light: lt^1), hook (hk^1), likeAP180 (lap^1), hsc70–4 ($Hsc70\text{–}4^{03550}$), cysteine string protein (Csp^{03988}), synaptobrevin (Syb^{k07703}), neuronal synaptobrevin (syb^{I18}), syntaxin (syx^{L266}), NEM-sensitive fusion protein (com^{ST53}, com^{191}), synaptotagmin (syt^{T77}, syt^{AD1}), *claret* (ca^1), prune (pn^1), lightoid (ltd^1), pink (p^p, p^{snb}), Yolk protein1-Gal4 (*yp1–64*) and wild-type (*WT*) Oregon R (*OreR*). Alleles are described in the drosophila database (http://flybase.bio.indiana.edu/).

Infections.

Flies 2–4 d old were used for viral challenge. Glass needles ‘pulled’ as for microinjection into embryos were loaded with the inoculum (1×10^5 i.u./μl and connected to an Eppendorf microinjection setup. We injected approximately 200 nl into the anterior ventral abdomen near the dorsal-ventral boundary. Flies were injected and were subsequently maintained at 24 °C. Groups of 25 or more flies were challenged, monitored for mortality and assigned scores daily. Statistical significance was determined with a log rank test. Only results of $P < 0.05$ in >75% of independent tests were considered significant in mutants that were affected.

CPE assays.

DL2 cells (a gift from P. Christian, CSIRO Entomology, Canberra, Australia) were maintained and propagated as described[11]. Cells inoculated at a multiplicity of infection of one were assayed for cell death by flow cytometry (Beckton Dickenson) 4 d later by staining with propidium iodide (5 μg/ml). Cellular debris were electronically ‘gated out’ of the analysis. To determine the viral concentration, DL2 cells were plated at 30% confluence on tissue culture plastic and inoculated with serial dilutions of the virus. At 4 d after infection, the cells were stained for 10 min at room temperature with Hoescht 33342 (1:1,000 dilution;

Molecular Probes) and Sytox green (1:5,000 dilution; Molecular Probes). The cells were washed twice with PBS and analyzed with an automated Nikon microscope and Metamorph analysis software to determine the minimum concentration of virus that resulted in CPE. This was defined as 1 i.u.

Immunoblot analysis.

For antibody production, virus purified as described above was injected into chickens (Covance). Antibody was purified from eggs following the manufacturer's protocol (Promega). Adult flies or cells were lysed in radioimmunoprecipitation assay buffer supplemented with a protease inhibitor 'cocktail' (Boehringer). Samples were separated by 12% SDS-PAGE and blotted (Immobilon P). Membranes were incubated with antibody to DCV (anti-DCV; 1:50,000 dilution) followed by anti-chicken–horseradish peroxidase (1:200,000 dilution; Jackson Immunochemicals). Blots were visualized with ECL (Amersham).

Immunofluorescence assays.

Flies were dissected in 4% formaldehyde in PBS and fixed for 20 min. Flies were washed in PBS plus 0.1% Triton (PBST), blocked in 3% BSA in PBST and then incubated overnight at 4 °C in anti-DCV (1:5,000 dilution) in PBST. After the membrane was washed, anti-chicken–fluorescein isothiocyanate (1:400 dilution; Jackson Immunochemicals) 3% BSA in PBST was added and samples were incubated overnight at 4 °C. Samples were washed again and mounted in glycerol and Antifade (Molecular Probes). Samples were counterstained with Alexa Fluor 568–phalloidin (1:200 dilution; Molecular Probes) and analyzed with a Leica confocal microscope.

For endocytosis experiments, 3×10^5 cells were plated and placed at 4 °C. Virions were added (1×10^8) and samples were incubated for 3 h at 4 °C. Cells were washed and brought to 25 °C. Cells were washed in PBS and then fixed with 4% formaldehyde in PBS for 10 min at room temperature. Cells were washed twice with PBST, blocked with 3% BSA in PBST. Anti-DCV (1:5,000 dilution) was followed by anti-chicken–fluorescein isothiocyanate (1:200 dilution; Jackson Laboratories) and Hoescht 33342 (1:1000 dilution; Molecular Probes). Cells were analyzed with a Leica confocal microscope.

For drug treatments, 1×10^4 cells in 100 μl were seeded into 96-well plates 24 h before addition of the drug. Drugs were added to the cells, and 15 min later 1×10^5 virions were added. Then, 3 h later the wells were washed and fresh media was added. Plates were analyzed 20 h later as described above, except with an automated Nikon microscope and Metamorph analysis software. Bafilomycin A1 (0.02 μg/ml), valinomycin (0.01 mg/ml) and cycloheximide (0.01 mg/ml) were obtained from Sigma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Alcami A & Koszinowski UH Viral mechanisms of immune evasion. Immunol. Today 21, 447–455 (2000). [PubMed: 10953097]
2. Ploegh HL Viral strategies of immune evasion. Science 280, 248–253 (1998). [PubMed: 9535648]
3. Biron CA Initial and innate responses to viral infections—pattern setting in immunity or disease. Curr. Opin. Microbiol 2, 374–381 (1999). [PubMed: 10458991]
4. Guidotti LG & Chisari FV Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol 19, 65–91 (2001). [PubMed: 11244031]
5. Cooke GS & Hill AVS Genetics of susceptibility to human infectious disease. Nat. Rev. Genet 2, 967–977 (2001). [PubMed: 11733749]
6. Samson M et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 382, 722–725 (1996). [PubMed: 8751444]
7. Nomoto A, Koike S & Aoki J Tissue tropism and species specificity of poliovirus infection. Trends Microbiol 2, 47–51 (1994). [PubMed: 8162441]
8. Goodbourn S, Didcock L & Randall RE Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol 81, 2341–2364 (2000). [PubMed: 10993923]
9. Lee SH et al. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. Nat. Genet 28, 42–45 (2001). [PubMed: 11326273]
10. Rueckert RR in Fields Virology (eds. Fields BN, Knipe DM & Howley PM) 609–654 (Lippincott-Raven, Philadelphia, 1996).
11. Johnson KN & Christian PD Molecular characterization of Drosophila C virus isolates. J. Invertebr. Pathol 73, 248–254 (1999). [PubMed: 10222177]
12. Fields BN, Knipe DM & Howley PM Virology (Lippincott-Raven, Philadelphia, 2001).
13. Moore NF, Pullin JS, Crump WA & Plus N The proteins expressed by different isolates of Drosophila C virus. Arch. Virol 74, 21–30 (1982). [PubMed: 6297428]
14. Klasse PJ, Bron R & Marsh M Mechanisms of enveloped virus entry into animal cells. Adv. Drug Deliv. Rev 34, 65–91 (1998). [PubMed: 10837671]
15. Bayer N et al. Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection. J. Virol 72, 9645–9655 (1998). [PubMed: 9811698]
16. Carrasco L Picornavirus inhibitors. Pharmacol. Ther 64, 215–290 (1994). [PubMed: 7533301]
17. Takei K & Haucke V Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol 11, 385–391 (2001). [PubMed: 11514193]
18. Narayanan R & Ramaswami M Endocytosis in Drosophila: progress, possibilities, prognostications. Exp. Cell Res 271, 28–35 (2001). [PubMed: 11697879]
19. Lloyd V, Ramaswami M & Kramer H Not just pretty eyes: Drosophila eye-colour mutations and lysosomal delivery. Trends Cell Biol 8, 257–259 (1998). [PubMed: 9714595]
20. Lautie-Harivel N & Thomas-Orillard M Location of Drosophila C virus target organs in Drosophila host population by an immunofluorescence technique. Biol. Cell 69, 35–39 (1990). [PubMed: 2261574]
21. Chen MS et al. Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. Nature 351, 583–586 (1991). [PubMed: 1828536]
22. van der Bliek AM & Meyerowitz EM Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature 351, 411–414 (1991). [PubMed: 1674590]

23. Damke H, Binns DD, Ueda H, Schmid SL & Baba T Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages. Mol. Biol. Cell 12, 2578–2589 (2001). [PubMed: 11553700]
24. Moline MM, Southern C & Bejsovec A Directionality of wingless protein transport influences epidermal patterning in the Drosophila embryo. Development 126, 4375–4384 (1999). [PubMed: 10477304]
25. Vidal S et al. Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kB-dependent innate immune responses. Genes Dev 15, 1900–1912 (2001). [PubMed: 11485985]
26. Jousset FX, Plus N, Croizier G & Thomas M [Existence in Drosophila of 2 groups of picornavirus with different biological and serological properties]. C R Acad. Sci. Hebd. Seances Acad. Sci. D 275, 3043–3046 (1972). [PubMed: 4631976]
27. Plus N, Croizier G, Jousset FX & David J Picornaviruses of laboratory and wild Drosophila melanogaster: geographical distribution and serotypic composition. Ann. Microbiol. (Paris) 126, 107–117 (1975). [PubMed: 811144]
28. Thomas-Orillard M, Jeune B & Cusset G Drosophila-host genetic control of susceptibility to Drosophila C virus. Genetics 140, 1289–1295 (1995). [PubMed: 7498770]
29. Plus N, Croizier G, Reinganum C & Scott PD Cricket paralysis virus and drosophila C virus: serological analysis and comparison of capsid polypeptides and host range. J. Invertebr. Pathol 31, 296–302 (1978). [PubMed: 98592]
30. Miller LK & Ball AL The Insect Viruses (eds. Fraenkel-Conrat H & Wangner RR) (Plenum, New York, 1998).
31. Gomariz-Zilber E, Poras M & Thomas-Orillard M Drosophila C virus: experimental study of infectious yields and underlying pathology in Drosophila melanogaster laboratory populations. J. Invertebr. Pathol 65, 243–247 (1995). [PubMed: 7745279]
32. Johnson KN & Christian PD 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 (1998). [PubMed: 9460942]
33. Koyama AH et al. Suppression of apoptotic and necrotic cell death by poliovirus. J. Gen. Virol 82, 2965–2972 (2001). [PubMed: 11714972]
34. Tucker SP, Thornton CL, Wimmer E & Compans RW Vectorial release of poliovirus from polarized human intestinal epithelial cells. J. Virol 67, 4274–4282 (1993). [PubMed: 8389927]
35. Kandolf R, Selinka H & Klingel K in Molecular Biology of Picornaviruses (eds. Semler BL & Wimmer E) 405–413 (ASM Press, Washington, DC, 2002).
36. Mellman I Endocytosis and molecular sorting. Annu. Rev. Cell. Dev. Biol 12, 575–625 (1996). [PubMed: 8970738]
37. DeTulleo L & Kirchhausen T The clathrin endocytic pathway in viral infection. EMBO J 17, 4585–4593 (1998). [PubMed: 9707418]
38. Marsh M & Pelchen-Matthews A Endocytosis in viral replication. Traffic 1, 525–532 (2000). [PubMed: 11208139]
39. Damke H, Baba T, van der Bliek AM & Schmid SL Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. J. Cell Biol 131, 69–80 (1995). [PubMed: 7559787]
40. Krishnan KS et al. Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling. Neuron 30, 197–210 (2001). [PubMed: 11343655]
41. DiAntonio A, Parfitt KD & Schwarz TL Synaptic transmission persists in synaptotagmin mutants of Drosophila. Cell 73, 1281–1290 (1993). [PubMed: 8100740]
42. Littleton JT, Stern M, Perin M & Bellen HJ Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. Proc. Natl. Acad. Sci. USA 91, 10888–10892 (1994). [PubMed: 7971978]
43. Littleton JT et al. Synaptotagmin mutants reveal essential functions for the C2B domain in Ca^{2+}-triggered fusion and recycling of synaptic vesicles in vivo. J. Neurosci 21, 1421–1433 (2001). [PubMed: 11222632]

44. Haucke V & De Camilli P AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. Science 285, 1268–1271 (1999). [PubMed: 10455054]

45. von Poser C et al. Synaptotagmin regulation of coated pit assembly. J. Biol. Chem 275, 30916–30924 (2000). [PubMed: 10906143]

46. Haucke V, Wenk MR, Chapman ER, Farsad K & De Camilli P Dual interaction of synaptotagmin with m2- and a-adaptin facilitates clathrin-coated pit nucleation. EMBO J 19, 6011–6019 (2000). [PubMed: 11080148]

47. Ferguson GD, Anagnostaras SG, Silva AJ & Herschman HR Deficits in memory and motor performance in synaptotagmin IV mutant mice. Proc. Natl. Acad. Sci. USA 97, 5598–5603 (2000). [PubMed: 10792055]

48. Fernandez-Chacon R et al. Synaptotagmin I functions as a calcium regulator of release probability. Nature 410, 41–49 (2001). [PubMed: 11242035]

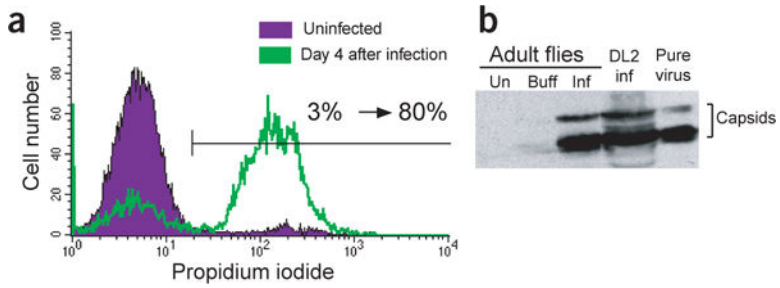

Figure 1.

DCV is infectious *in vitro*. (**a**) Flow cytometry of DL2 cells stained with propidium iodide at 4 d after infection (unshaded) or after being left uninfected (shaded). Cellular debris were electronically 'gated out' of the analysis. The percentage of dead cells before and after infection is calculated from the electronically gated regions shown (bracketed line). The shift from 3% to 80% dead cells indicates substantial cytopathic effect. (**b**) Immunoblot probed with anti-DCV of lysates from adult flies left uninfected (Un), 48 h after buffer injection (Buff) or at 48 h after infection (Inf). Infected DL2 cells (DL2 inf) and purified virions (Pure virus) are also shown. Lysates were quantified and normalized before loading, excluding the purified virions.

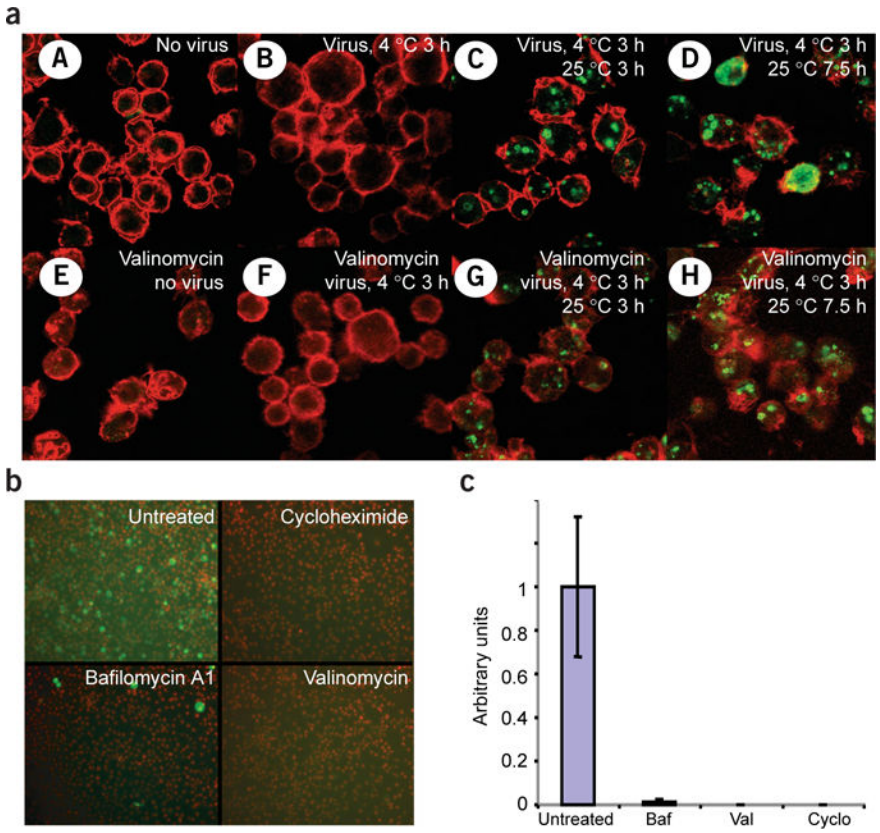

Figure 2.

DCV traffics through a vesicular compartment and requires a functional endocytic apparatus *in vitro*. (**a**) Time course of infection monitored by immunofluorescence. Cells are uninfected (A), infected for 3 h at 4 °C to allow surface binding but no endocytosis (B), infected for 3 h at 4 °C followed by 3 h at 25 °C to release the block to endocytosis and monitor viral trafficking (C), and infected for 3 h at 4 °C followed by 7.5 h at 25 °C to monitor the initiation of new viral replication (D). Panels E–H are the same time course and conditions as in A–D except valinomycin was added 15 min before $t = 0$. Green, anti-DCV; red, Alexa Fluor-568–phalloidin. (**b**,**c**) Immunofluorescence assay of DL2 cells infected with DCV at 24 h after infection shows that cells treated with bafilomycin A1 (Baf), valinomycin (Val) or cycloheximide (Cyclo), have much less immunostaining than untreated cells (Untreated; no drugs). All nuclei are labeled in red (Hoescht 33342) and infected cells, in green (anti-DCV). This is quantified in **c** for three sites per well in three independent experiments. Data are presented relative to the mean fractional infection seen in untreated cells (0.52 ± 0.16).

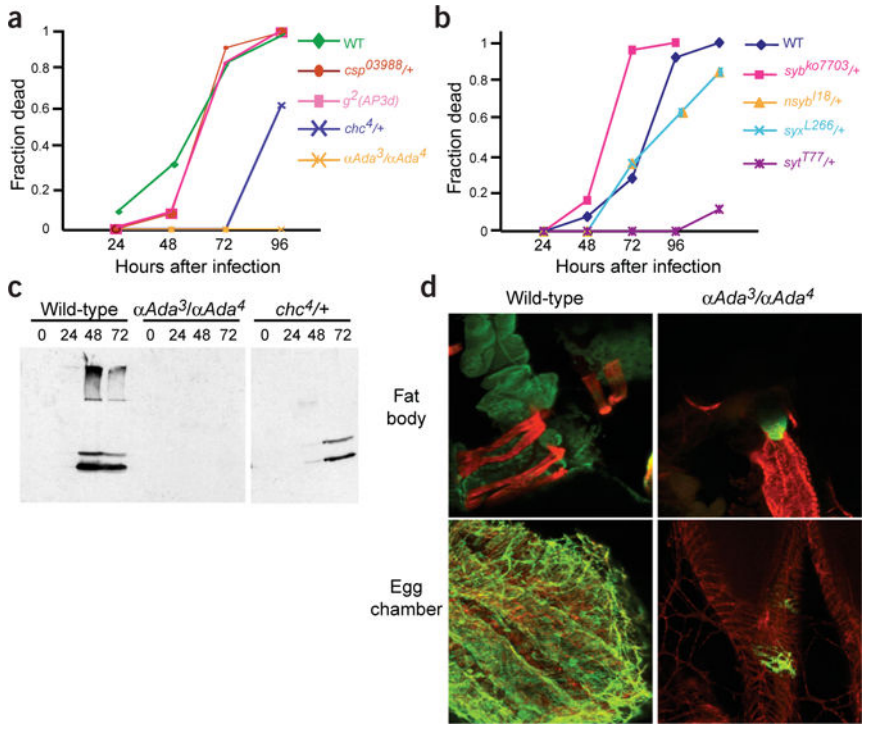

Figure 3.

Clathrin-mediated endocytosis is required for DCV infection. (**a**,**b**) The clathrin heavy chain (*chc4/+*), α-Adaptin (*α-Ada3/α-Ada4*), synaptotagmin (*sytT77/+*) and abnormal wing discs (awd^{K-pn}/awd^{K-pn}) mutants are more resistant to DCV infection than are wild-type flies. In contrast, other mutations that perturb vesicular trafficking, tested as homozygous (or^{1}, g^{2}, rb^{1}, dor^{8}, dor^{1}, lt^{1}, hk^{1}, com^{ST53}, com^{191}, ca^{1}, pn^{1}, ltd^{1}, p^{p} and p^{snb}) or heterozygous (lap^{1}, $hsc70\text{–}4^{03550}$, csp^{0398}, syb^{k07703}, $nsyb^{I18}$ and syx^{L266}) mutations, were not substantially different from wild-type (a representative experiment is shown with a subset of mutants). Groups of >25 flies were injected with DCV and monitored daily for mortality. (**c**) Immunoblot analysis shows that the delayed mortality correlates with the production of viral antigens. Wild-type, *α-Ada3/α-Ada4* or *chc4/+* flies were left uninjected (0) or were challenged with DCV, and protein lysates were generated (time points after infection, above lanes), normalized and probed with anti-DCV. (**d**) Immunofluorescence analysis of DCV-infected wild-type or *α-Ada3/α-Ada4* fat body and egg chambers shows vastly reduced immunostaining in the infected mutant tissues. Green, anti-DCV; red, Alexa Fluor-568–phalloidin.

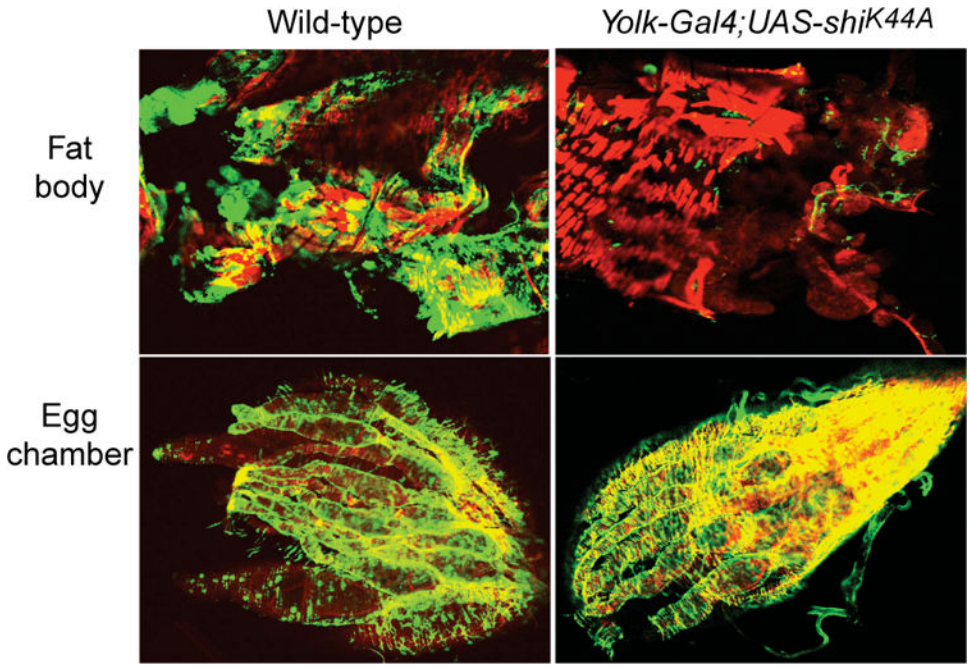

Figure 4.

Autonomous requirement for dynamin in viral entry. Immunofluorescence analysis of fat body and egg chambers of wild-type flies or mosaic flies (*yolk-Gal4/UAS-shi*^{dn}) shows that loss of dynamin activity in the fat body is sufficient to block the production of viral antigens but not in the egg chamber of the same fly. Green, anti-DCV; red, Alexa Fluor-568–phalloidin.