COPI Activity Coupled with Fatty Acid Biosynthesis Is Required for Viral Replication

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During infection by diverse viral families, RNA replication occurs on the surface of virally induced cytoplasmic membranes of cellular origin. How this process is regulated, and which cellular factors are required, has been unclear. Moreover, the host–pathogen interactions that facilitate the formation of this new compartment might represent critical determinants of viral pathogenesis, and their elucidation may lead to novel insights into the coordination of vesicular trafficking events during infection. Here we show that in *Drosophila* cells, Drosophila C virus remodels the Golgi apparatus and forms a novel vesicular compartment, on the surface of which viral RNA replication takes place. Using genome-wide RNA interference screening, we found that this step in the viral lifecycle requires at least two host encoded pathways: the coat protein complex I (COPI) coatamer and fatty acid biosynthesis. Our results integrate, clarify, and extend numerous observations concerning the cell biology of viral replication, allowing us to conclude that the coupling of new cellular membrane formation with the budding of these vesicles from the Golgi apparatus allows for the regulated generation of this new virogenic organelle, which is essential for viral replication. Additionally, because these pathways are also limiting in flies and in human cells infected with the related RNA virus poliovirus, they may represent novel targets for antiviral therapies.

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Introduction

Viruses, because of their small genome size, are dependent on a multitude of cellular factors to replicate within their hosts. Not only do they have to co-opt cellular factors in order to complete their replication cycle, but also they must efficiently and simultaneously coordinate many steps of their replication cycle using host-encoded machinery. This can require complicated compartmentalization of various steps in their lifecycle. For example, single-strand RNA viruses must simultaneously coordinate transcription, RNA replication, and RNA packaging activities using the same genomic RNA template.

One example of subcellular separation of these activities is the observation that all positive-strand RNA viruses, a group that includes poliovirus, undergo RNA replication in association with membranes of infected cells [1]. Depending on the specific virus, these membranes can be derived from a variety of sources within the host cell, including the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, chloroplasts, or from the endolysosomal compartment [2]. While the purpose of this compartmentalization has not been definitively established, several models have been discussed. One model suggests that this process provides a structural framework for replication, fixing the RNA replication machinery onto a confined two-dimensional space [3]. This compartmentalization of RNA replication may be important due to the fact that the viral RNA must be used for competing enzymatic activities: replication and transcription must both access templates using different machines. The separation of RNA templates into defined compartments may prevent interference between these processes. Another model postulates that the compartment may be generated by a cellular autophagic process. This may allow for the nonlytic release of virions [4], which in vivo could allow the virus to circumvent presentation by the immune system. Another possibility is that autophagy may play an antiviral role in clearing the cytoplasm of infectious virus. Therefore, the purpose of this targeted localization has yet to be understood.

Poliovirus, and picornaviridae in general (nonenveloped, positive-strand RNA viruses), do not use native organelle membranes for replication but instead actively induce the formation of a novel cytoplasmic vesicular compartment in infected cells [5,6]. During replication of these viruses, there is a massive rearrangement of intracellular membranes whereby the cytoplasm of the cell becomes densely packed with vesicles of nonuniform size [7]. Immunoelectron microscopy has revealed that the cytoplasmic surfaces of these vesicles are the sites of viral RNA replication and that a membrane compartment is essential for replication [8]. The mechanisms whereby these viruses induce this vesicular compartment and direct their RNA replication complex to

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Abbreviations: Arf, ADP-ribosylation factor; COPI, coat protein complex I; DCV, Drosophila C virus; dsRNA, double-strand RNA; ER, endoplasmic reticulum; GFP, green fluorescent protein; GO, Gene Ontology; RNAi, RNA interference; siRNA, small interfering RNA; SREBP, sterol regulatory element binding protein

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Synopsis

In order to successfully invade and replicate within their hosts, viruses hijack cellular factors. In the case of many RNA viruses, including a *Drosophila* picorna-like virus Drosophila C virus, they must undergo the essential step of genomic replication on the surface of cytoplasmic membranes. Specifically, for picornaviruses, these vesicles are induced in the infected cell, and the ontogeny and cellular factors required to form this compartment have been unclear. Circumstantial evidence has implicated coat protein complex I (COPI), COPII, and autophagy. Here, Cherry and colleagues present their findings using a genome-wide RNA interference screening approach using a picorna-like virus that COPI and fatty acid biosynthesis are critical host pathways required to generate this intracellular vesicular compartment. Furthermore, they show that loss of COPI, but not COPII, is protective both in adult flies and in human cells infected with the related picornavirus, poliovirus. These novel and exciting findings have broad-scale implications for picornavirus replication and for the potential use of these pathways as novel antiviral targets.

these specific intracellular membranes, and the cellular components involved in this process, are not established. Previous studies have implicated a number of different trafficking processes including coat protein complex I (COPI), COPII, and autophagy-mediated processes, with the membrane being derived from the ER via a COPII coatamer–mediated process or the Golgi via either COPI or autophagic vesicles [4,9–12]. Studies investigating how picorna and related viruses induce the formation of cellular membranes have used small molecule inhibitors such as Brefeldin A, which have pleiotropic effects, or have used steady-state localization experiments that are complicated by the fact that markers from many different intracellular organelles can be found residing on virus-induced membranes, making it difficult to assign a specific role for specific cellular components in the ontogeny of the vesicles.

To overcome some of these limitations, we used a genome-wide loss-of-function analysis to identify factors required for the generation of this compartment and viral RNA replication. To accomplish this, we studied Drosophila C virus (DCV), a natural pathogen of *Drosophila* that readily infects the cells and animals [13]. DCV is a dicistrovirus that is in many ways similar to picornaviruses such as poliovirus. Both are encoded by a single positive-strand RNA genome, with a genome-linked protein at the 5′ end and a poly adenosine tail at the 3′ end [14–16]. They also share many physical and morphological properties of the viral structural proteins including the requirement for virally encoded proteases for processing [17,18]. In addition, they are translated by a specialized, cap-independent, internal ribosome entry site (IRES) mechanism [19].

Using this *Drosophila* system allowed us to apply forward genetics to both the whole organism and cell culture to screen for host-encoded factors whose loss blocks viral replication. Recently, we conducted a genome-wide RNA interference (RNAi) screen and found over 100 genes required for efficient viral replication in tissue culture [20]. We have begun to validate the importance of these genes for virus replication by several approaches and to determine the stage in the viral lifecycle affected by the loss of each. Using this loss-of-function strategy, we found that DCV, like mammalian picornaviruses, induces a cytoplasmic vesicular compartment upon which viral RNA replication takes place. The formation of this structure was dependent on the cellular activity of COPI along with the generation of new membrane by the fatty acid biosynthetic pathway. Together, these host-encoded genes drive the formation of these vesicles for viral RNA replication. Moreover, animals mutant for fatty acid biosynthesis were attenuated for viral replication demonstrating that this pathway is required and limiting for infection both in vivo and in vitro. Importantly, we also found that COPI, but not COPII, was also required for poliovirus infection of human cells, demonstrating the generality of our findings.

Results

Gene Ontology Analysis Reveals Overrepresented Cellular Functions Necessary for Viral Replication

Genome-wide RNAi screening in *Drosophila* cells using high-throughput imaging identified 66 ribosomal proteins and 45 nonribosomal genes as required for DCV replication [20]. In our first study, we showed that knock-down of these ribosomal proteins blocked translation of IRES-containing viruses, including poliovirus, due to a requirement for higher levels of ribosomal function than for host messages, showing a unique sensitivity of this class of viruses to ribosome attenuation [20]. To identify roles for the remaining genes in viral replication, we analyzed Gene Ontology (GO) associations of the 45 nonribosomal genes required for DCV replication in *Drosophila* cells. This analysis showed that these genes fall into a small number of functional categories (Figure 1A). In addition, Table 1 lists the 45 genes, their effects on viral replication, GO category, and whether the double-strand RNA (dsRNA) amplicons identified have potential off-target effects as predicted by 21–base pair overlaps with other annotated genes. Based on the associations with Gene Ontology (GO) categories, some biological processes were significantly overrepresented. Specifically, statistical analysis revealed that vesicular trafficking processes were overrepresented while *Drosophila*-specific genes were underrepresented (30% of genome, 4% of set), suggesting that DCV selectively targets conserved features of the host cells, and not species-specific functions.

One notable group of genes within the vesicle trafficking category included five of the seven COPI coatamer proteins: *alphaCOP, betaCOP, beta'COP, gammaCOP,* and *zetaCOP* (Figure 1B, $p < 0.001$ for enrichment relative to representation in the genome, by Fisher exact test). To confirm that loss of COPI was responsible for the phenotype and to determine whether the two COPI genes (*deltaCOP* and *epsilonCOP* [*CG9543*]) not identified in the screen were erroneously missed (i.e., false negatives), we synthesized additional dsRNAs for the seven COPI coatamer genes and tested them for their role in DCV replication. These analyses revealed that dsRNAs against all COPI components except *epsilonCOP* blocked DCV replication, although some differences in the extent of the effect were observed, possibly reflecting variability in the efficiency of the gene silencing mediated by the dsRNAs (Figure 1B). Thus, we can conclude that *deltaCOP* was a false negative in the primary screen. However, neither of the dsRNAs directed against *epsilonCOP* had an effect on DCV replication. RT-PCR analysis demonstrated that *epsilonCOP* mRNA was depleted, demonstrating that the dsRNA was functional (Figure S1).

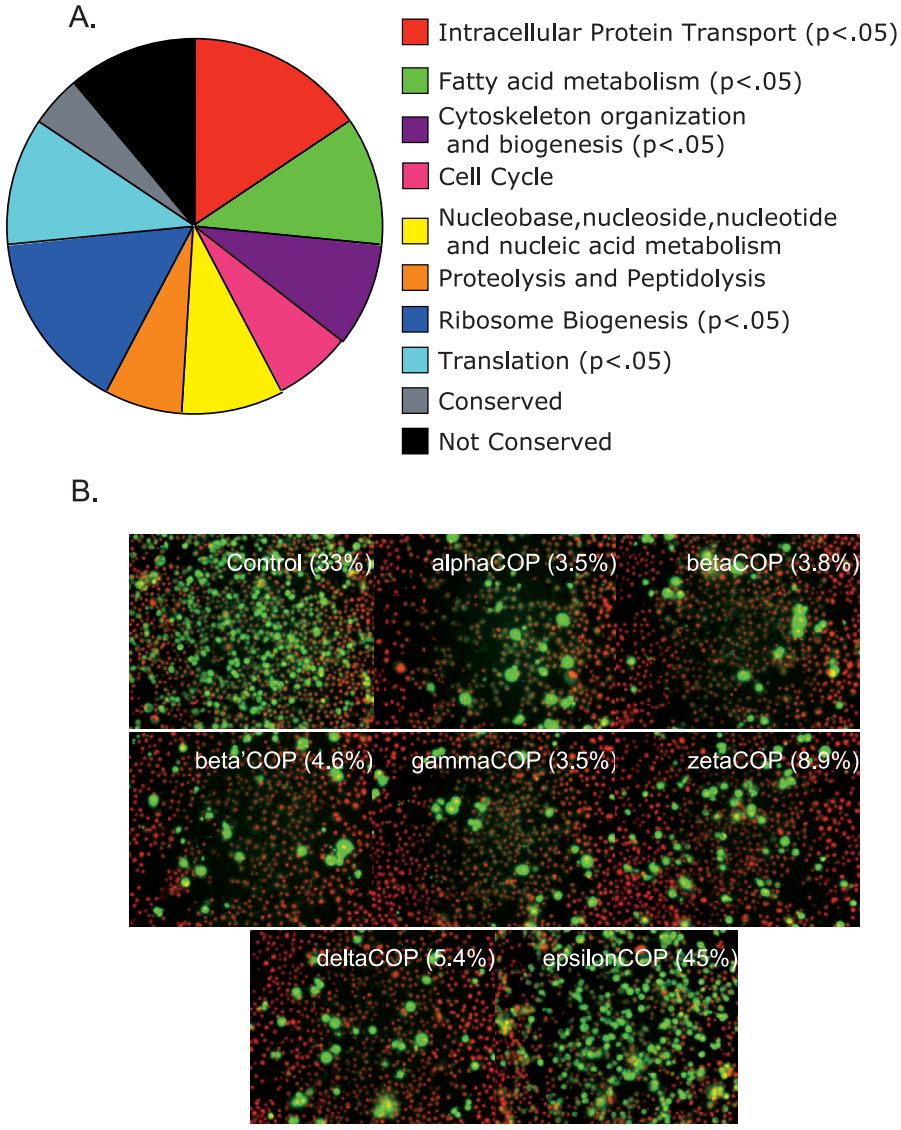

Figure 1. COPI Coatamer Complex Is Required for Viral Replication

(A) Frequency of encoded functional groups as curated by GO (The FlyBase Consortium) and manually assigned to representative categories for all verified candidates. Categories that are overrepresented with $p < 0.05$ are indicated.

(B) Decreased viral replication post dsRNA treatment with dsRNA against *alphaCOP, betaCOP, beta'COP, gammaCOP, deltaCOP,* and *zetaCOP* as compared to dsRNA treatment with GFP or *epsilonCOP*. Images were quantified as the percentage of infected cells (FITC-anti DCV [green]) divided by (Hoescht 33342 [red]).

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However, the protein may be long-lived—something we could not assess due to the absence of antibodies directed against either this or the other *Drosophila* COPI proteins. Nevertheless, these data suggest that it may have an unknown homolog which can substitute for its function. It should be noted that in yeast only six of the seven coatamer components are essential—only *epsilonCOP* is dispensible [21]. This suggests that *epsilonCOP* may be dispensable for COPI function in both yeast and *Drosophila*.

COPI, but Not Secretion, Is Required for DCV Replication

Two vesicular coat complexes, COPI and COPII, are required for trafficking between the ER and Golgi [22]. Formation of transport vesicles is dependent on the recruitment of these cytosolic coat proteins to the surface of the donor compartment membrane from which they bud. Whereas the COPII coatamer is required for the anterograde transport of proteins from the ER to the Golgi, the COPI coatamer is required for retrograde transport of recycled proteins and membrane from the Golgi to the ER [23]. Blocking either the COPI or the COPII coatamer pathways blocks protein secretion, as observed in yeast mutants for the orthologous genes and in studies using RNAi screening for genes required for general secretion [24]. However, we only identified COPI-associated genes and none of the COPII coatamer components (Sec13, Sec31, Sec23, and Sec24 [*CG1472*]) in our screen, suggesting that either the COPII coatamer proteins are refractory to RNAi-mediated depletion in our cells or DCV replication specifically requires COPI function but not COPII.

Table 1. Nonribosomal Genes Required for DCV Replication

Gene Name	Identifier	DRSC Amplicons	Unigene Accession Numbers	Category	Fold-Decrease	Human Homolog	Off-Targets (21 bp)	Off-Targets (19 bp)
Dp	FBgn0011763	DRSC07402	Dm.6412	Cell cycle	1.8	TFDP2	0	0
CycE	FBgn0010382	DRSC03296	Dm.3509	Cell cycle	1.4	CCNE1	0	0
cdc2	FBgn0004106	DRSC03504	Dm.3187	Cell cycle	1.4	CDC2	0	0
CG6962	FBgn0037958	DRSC16153	Dm.1159	Conserved[a]	2.4	FLJ20297	0	2
Fax	FBgn0014163	DRSC11345	Dm.6723	Conserved[a]	3.1	C6orf168	0	0
TER94	FBgn0024923	DRSC07560	Dm.2968	Cytoskeleton organization and biogenesis	3.8	VCP	0	1
Map205	FBgn0002645	DRSC16732	Dm.1892	Cytoskeleton organization and biogenesis	4.4		0	1
Tektin-C	FBgn0035638	DRSC09741	Dm.5022	Cytoskeleton organization and biogenesis	4.9	TEKT1	0	1
Arpc3B	FBgn0030818	DRSC20126	Dm.19161	Cytoskeleton organization and biogenesis	7		0	1
Bgm	FBgn0027348	DRSC03495	Dm.20327	Fatty acid metabolism	1.6	BGR	0	1
CG11198	FBgn0033246	DRSC07249	Dm.11366	Fatty acid metabolism	7.2	ACACA	0	0
CG5844	FBgn0038049	DRSC15890	Dm.1171	Fatty acid metabolism	8.4		0	0
CG3523	FBgn0027571	DRSC00268	Dm.7216	Fatty acid metabolism	10.6	FASN	0	5
HLH106	FBgn0015234	DRSC11182	Dm.7945	Fatty acid metabolism	38.2	SREBF1	0	0
ZetaCOP	FBgn0040512	DRSC11412	Dm.20098	Intracellular protein transport	1.8	COPZ1	0	0
GammaCOP	FBgn0028968	DRSC16955	Dm.1895	Intracellular protein transport	3.1	COPG	0	0
Rab5	FBgn0014010	DRSC00777	Dm.6724	Intracellular protein transport	3.3	RAB5C	0	2
BetaCop	FBgn0008635	DRSC20312	Dm.2883	Intracellular protein transport	3.4	COPB	0	1
AlphaCop	FBgn0025725	DRSC08706	Dm.2217	Intracellular protein transport	3.8	COPA	0	0
beta'Cop	FBgn0025724	DRSC03492	Dm.2980	Intracellular protein transport	6.5	COPB2	0	1
Rack1	FBgn0020618	DRSC03405	Dm.6830	Intracellular protein transport	11.2	GNB2L1	0	1
CG7835	FBgn0030879	DRSC20058	Dm.3606	Not conserved[b]	1.4		14[c]	85[c]
DRSC01393		DRSC01393	None	Not conserved[b]	2.8		0	0
DRSC05108		DRSC05108	None	Not conserved[b]	4.6		2	3
CG4585	FBgn0025335	DRSC04475	Dm.3875	Not conserved[b]	5.2		0	0
CG9300	FBgn0036886	DRSC11064	Dm.999	Not conserved[b]	8.4		0	0
CG17209	FBgn0030687	DRSC19766	Dm.14839	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	1.7	POLR3A	0	0
Ercc1	FBgn0028434	DRSC07424	None	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	1.8	ERCC1	0	0
CG1814	FBgn0033426	DRSC06711	Dm.8006	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	2.8	TU12B1-TY	0	0
CG3436	FBgn0031229	DRSC00605	Dm.1591	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	7.6	HPRP8BP	0	0
Prosalpha7	FBgn0023175	DRSC07516	Dm.2041	Proteolysis and peptidolysis	1.8	PSMA1	0	1
Ubi-p63E	FBgn0003943	DRSC22016	Dm.2641	Proteolysis and peptidolysis	3.7	UBC	0	0
CG11700	FBgn0029856	DRSC17794	Dm.26471	Proteolysis and peptidolysis	13.9	UBB	3[d]	3[d]
lp259	FBgn0025366	DRSC03352	Dm.4835	Ribosome biogenesis	2.9	TINP1	0	0
cg8801	FBgn0028473	DRSC05954	Dm.6389	Ribosome biogenesis	2.9	GTPBP4	0	0
CG12050	FBgn0032915	DRSC02152	Dm.1478	Ribosome biogenesis	3.5	FLJ12519	0	1
Pit	FBgn0025140	DRSC22798	Dm.1273	Ribosome biogenesis	4.8	DDX18	0	0
CG14210	FBgn0031040	DRSC19566	Dm.6071	Ribosome biogenesis	6	MGC2574	1	5
CG5651	FBgn0035946	DRSC10533	Dm.7778	Ribosome biogenesis	12.3	ABCE1	0	0
CG4364	FBgn0032138	DRSC02742	Dm.372	Ribosome biogenesis	2.1	PES1	0	2
Trip1	FBgn0015834	DRSC03464	Dm.23184, Dm.2654	Translation	2.9	EIF3S2	0	0
eIF-1A	FBgn0026250	DRSC16937	Dm.1640	Translation	3.2	EIF1AX	0	0
EIF3-S10	FBgn0037249	DRSC12339	Dm.5844	Translation	6.4	eIF3-S10	0	1
ERF1	FBgn0036974	DRSC11779	Dm.2095	Translation	8	ETF1	0	1
Su(var)3–9	FBgn0003600	DRSC13081	Dm.3299	Translation	26	EIF2S3	0	0

[a]Conserved = has orthologs outside of insects, but does not fit into above GO categories.
[b]Not conserved = has no ortholog outside of insects.
[c]Likely has off-target effects.
[d]The amplicon has homology to homologs.
Gene name and FlyBase identifier are listed along with GO category. Fold-decrease represents the average decrease in percent infection compared to control treatment. The closest human homolog is indicated. The number of exact 21-bp targets in the Drosophila genome that are not the intended target.
The off-target genes are homologs of the intended target. Genes listed as conserved have orthologs in Homologene but are not within the GO categories listed. Genes listed as not conserved do not have orthologs outside of insects in Homologene.
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To rule out a role for COPII in DCV replication, we modified a microscopy-based assay to quantitatively monitor secretion of a plasma membrane reporter protein, Delta, to test whether the dsRNAs directed against COPII components were effective [25]. Using *Drosophila* cells that were stably transformed with an inducible membrane-bound form of Delta (DeltaNdeMyc cells [26]), we were able to assess the effects of different dsRNAs on the secretory pathway using flow cytometry. We pretreated cells with dsRNAs for 3 d and then induced the transgene with the addition of copper

sulfate for 2 h. Cell surface levels of Delta were measured by staining unpermeabilized cells with an antibody that recognizes extracellular Delta. Using this assay on control cells, we detected a large increase in surface Delta expression as compared to uninduced cells (Figure 2A, compare green and purple). Treatment of the cells with dsRNA against COPI *(betaCOP)* or COPII *(sec23)* components significantly decreased the extracellular levels of Delta as expected (Figure 2A). While dsRNA treatment against *sec23* did not block secretion as much as knockdown of *betaCOP,* treatment with dsRNA against Syntaxin 5 *(Syx5),* a t-SNARE required at the Golgi for secretion [27], was able to block secretion to a similar extent as COPI. Despite this, treatment with dsRNA against *sec23* or *Syx5* had no effect on viral replication (unpublished data). These data suggest that DCV replication does not require a functional secretory pathway per se but instead specifically requires COPI (but not COPII) coatamer function.

COPI Is Required Downstream of Entry

We next sought to determine which step in the viral lifecycle requires COPI function, and so we first determined whether depletion of COPI by dsRNA treatment affected DCV entry in tissue culture cells. We had previously determined that viral entry requires clathrin-mediated endocytosis and thus did not anticipate a requirement for COPI during entry. Nevertheless, we tested whether pretreatment with dsRNAs against control (GFP [green fluorescent protein]), COPI *(betaCOP),* or COPII *(sec23)* affected viral entry. To do this, the cells were pretreated with dsRNAs for 3 d and, then incubated at 4 °C to block endocytosis. Next, virions were added at a multiplicity of infection of 10 and allowed to bind to the surface of these dsRNA-treated cells. After 1 h of binding, the cells were washed to remove unbound virions and then returned to 25 °C to allow endocytosis to resume. After 3 h, the cells were immunostained to visualize virions during entry through the endocytic compartment. Using this assay, and quantitation with automated image analysis, we found that depletion of COPI (or COPII) did not block viral entry (Figure 2B).

To verify that this assay is sensitive to genes required for DCV entry, we tested the effect of Rab5, another cellular factor identified in our screen. Rab5 encodes a small GTPase required for endocytosis, and as such should be required for DCV uptake [28]. Indeed, we found that treatment with Rab5 dsRNA resulted in a 4-fold decrease in viral entry (Figure 2B). We did not identify other genes involved in clathrin-mediated endocytosis in this screen, suggesting that genes such as clathrin may be difficult to deplete in the *Drosophila* cell lines used. However, our RNAi screen was able to uncover a component of the endocytic apparatus required for viral entry (Rab5) and further underscores the role for COPI as required for a step in the viral lifecycle postentry.

DCV Induces a Cytoplasmic Vesicular Compartment that Is the Site of RNA Replication

All positive-strand RNA viruses undergo RNA replication on the surface of intracellular membranes [1]. Picornaviruses, which are similar in many respects to DCV, induce the formation of cytoplasmic vesicles of nonuniform size, and it is on the surface of these vesicles that viral RNA replication occurs [5–7]. We used ultrastructural analysis to test whether DCV might induce a similar vesicular compartment and found that, indeed, vesicles were induced in the cytoplasm of infected cells but not in the uninfected control cells (compare Figure 3A to Figure 3B). While the vesicles were nonuniform in size, they averaged 115 nm in diameter.

We next determined whether DCV uses these vesicles as a site for RNA replication. To this end, we generated an antibody against the DCV helicase, an integral component of the RNA replication machinery. We stained cells that were either uninfected or infected with DCV with the anti-helicase antibody and monitored localization using immunoelectron microscopy. We found that the DCV helicase was localized to the surface of these vesicles and did not stain the uninfected cells (Figure 3F–3G and unpublished data). Greater than 90% of the gold labeling was vesicle associated, demonstrating that the RNA replication machinery is indeed compartment bound. Importantly, the vesicles were detectable by 10 h postinfection but not at an earlier time point (unpublished data), consistent with our previous findings that new viral protein synthesis begins at approximately 7 h postinfection [13]. Because these viruses do not encapsidate the RNA polymerase, RNA replication cannot begin until the genomic RNA is translated to produce the viral factors required for RNA replication including the helicase. Altogether, our data demonstrate that the vesicles form postentry and posttranslation and are the site of RNA replication.

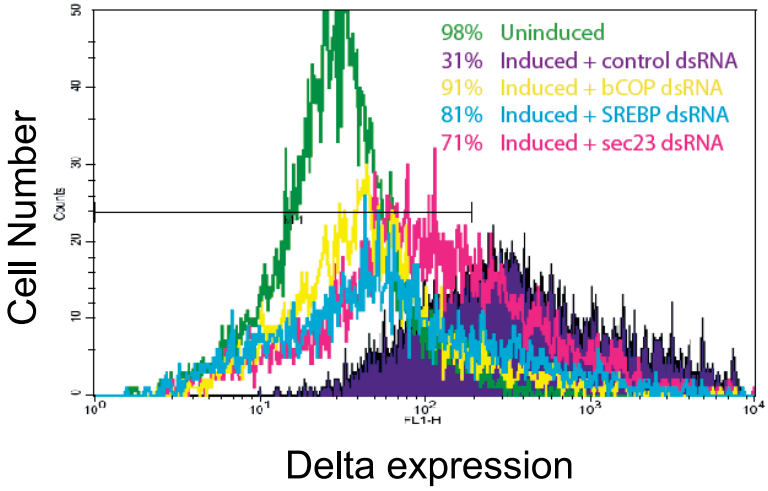
Vesicle Formation Is COPI Dependent

Because DCV induces a cytoplasmic vesicular compartment and requires the COPI coatamer for replication, we tested whether this compartment might be generated by the activity of the COPI complex. If the COPI coatamer directly forms these vesicles, then this might explain why DCV replication is dependent on COPI, but not COPII, function. Thus, we tested whether depletion of COPI affected the formation of DCV-dependent vesicles by treating cells with dsRNAs against COPI *(betaCOP),* COPII *(sec23),* or a control (GFP) followed by DCV infection. At 10 h postinfection, both control cells (Figure 3B) and COPII-treated cells (*sec23,* Figure 3D) were densely packed with the newly formed vesicles, despite the fact that dsRNA against *sec23* reduced transport of Delta to the cell surface. Randomly selected cells were quantitated in terms of the presence or absence of the characteristic cytoplasmic vesicles; 87% of control cells and 83% of *sec23*-depleted cells were filled with the vesicles. In contrast, the COPI-depleted cells had a 2.5-fold reduction in the percentage of cells that contained vesicles (34%, Figure 3C), suggesting that the COPI coatamer is required to generate this vesicular compartment during DCV replication.

DCV Replication Disrupts the Golgi Apparatus

Since the COPI coatamer normally buds vesicles from the Golgi apparatus [22,29], we reasoned that the virus-induced vesicular compartment might be generated by COPI-mediated budding of vesicles from the Golgi during DCV replication. Although the COPI machinery required for trafficking is conserved between yeast, mammals, and insects, there are striking differences in the morphologies of the Golgi apparatus. In *Drosophila,* the Golgi cisternae are not interconnected to form a single copy organelle that is typically juxtanuclear. Instead, the vesicles are stacked and dispersed throughout the cytoplasm, as is the case in plants and yeast [30, 31]. We tested whether the morphology of the

A.



B.

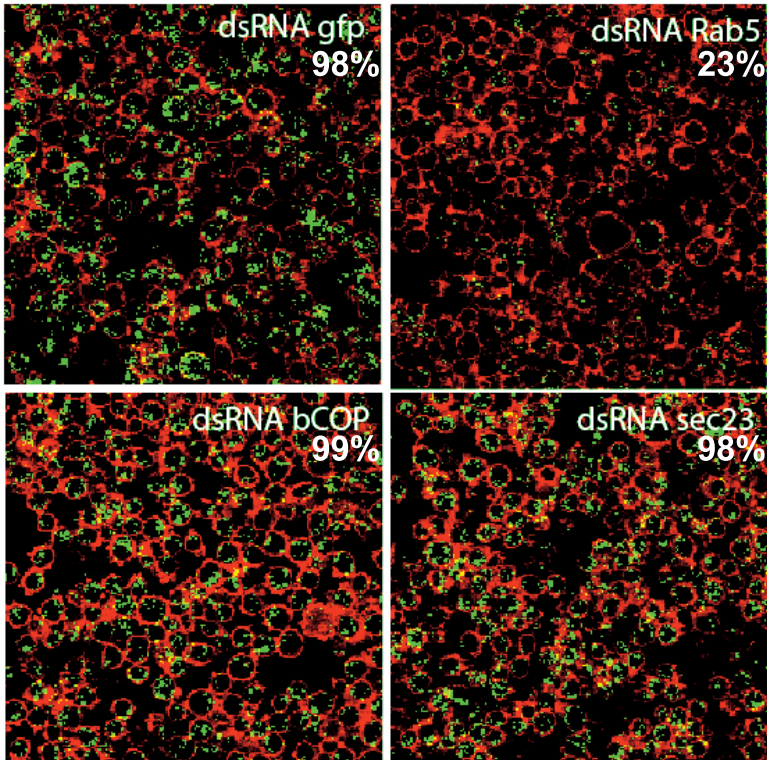


Figure 2. COPI Is Specifically Required for DCV Replication Postentry

(A) FACS analysis demonstrates that COPI *(betaCOP)* and COPII *(sec23)* are required for Delta secretion. DeltaWTNdeMYC cells were treated with dsRNA and subsequently Delta expression was induced for 2 h. Extracellular Delta expression was monitored by FACS. The bar was set such that 98% of the total uninduced cells were negative for Delta staining. Induction leads to a shift in the population to express surface Delta, such that only 31% of the cells remain negative. Under these conditions, COPI, COPII, and SREBP block surface staining.

(B) DCV entry requires endocytosis *(Rab5)* but not COPI *(bCOP)* or COPII *(sec23)* function. Cells were pretreated with dsRNA, infected at 4 °C to allow surface binding, followed by 3 h at 25 °C to release the block to endocytosis and monitor viral trafficking. Viral uptake was measured by determining the percentage of cells (red) that contained virus (green). Green, anti-DCV; red, Alexa-fluor-568-phalloidin.

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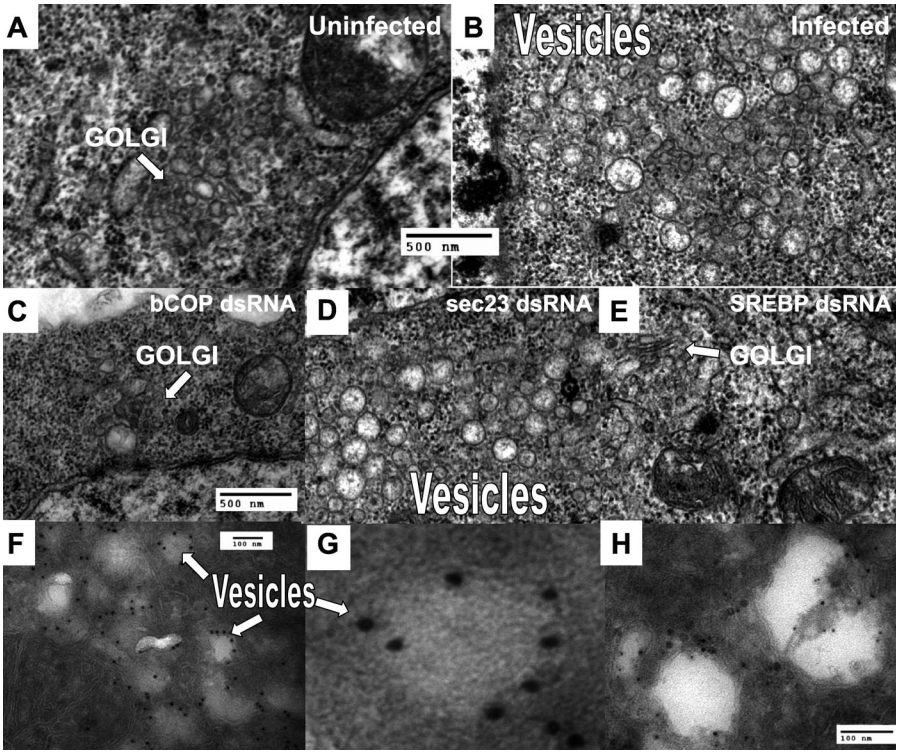

Figure 3. Ultrastructural Analysis Reveals Virus-Dependent Vesicular Compartment

(A) Uninfected cells with intact Golgi.

(B) Vesicles were generated at 10 h postinfection throughout the cytoplasm of cells pretreated with dsRNA against GFP and infected with DCV.

(C–E) Cells were pretreated with dsRNA against COPI *(bCOP)* (C), COPII *(sec23)* (D), or *SREBP* (E), infected with DCV, and prepared for electron microscopy.

(F) Immunoelectron microscopy of *Drosophila* cells infected with DCV and the RNA replication machinery was visualized using anti-DCV helicase and a secondary antibody coupled to 10-nm gold particles. The surfaces of cytoplasmic vesicles (arrows) are stained.

(G) Higher-magnification view of DCV helicase–labeled vesicle.

(H) Immunoelectron microscopy of *Drosophila* cells infected with DCV and the RNA replication machinery was visualized using anti-DCV helicase and a secondary antibody coupled to 5-nm gold particles. The Golgi was visualized using an anti-Golgi antibody *(DG13)* and a secondary antibody coupled to 12-nm gold particles.

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Golgi was altered during DCV replication by infecting *Drosophila* cells with DCV and monitoring the structure of the Golgi using an antibody to a membrane-bound Golgi resident protein. Using confocal microscopy, we found, as has been observed by others, that the Golgi has a punctate morphology in uninfected *Drosophila* cells [30,32] (Figure 4A, uninfected cells). In contrast, the morphology of the Golgi was altered in the infected cells. The punctae seemed smaller and increased in number throughout the cytoplasm of the cell (Figure 4A, infected cells). This disruption resembles the large number of dispersed vesicles observed by electron microscopy. These observations were made using two different antibodies against the Golgi (anti-Golgi and DG13). Moreover, we co-stained infected cells with antibodies to DCV helicase and a Golgi marker (DG13) using immunoelectron microscopy. We found that the DCV-induced vesicles that are coated by the viral helicase (5-nm gold particles) are also positive for the Golgi marker (12 nm) (Figure 3H). This demonstrates that these vesicles are indeed derived from the Golgi apparatus.

Because Golgi morphology was disrupted by DCV during replication, and depletion of the COPI coatamer blocked DCV replication; we reasoned that the Golgi morphology of COPI-depleted cells might be altered. Indeed, treatment of cells with dsRNA to COPI *(betaCOP)* led to a decrease in Golgi staining (Figure 4B), which was distinct from the change in Golgi morphology observed upon infection (compare the signal levels in uninfected cells in Figure 4A and 4B). The absolute levels of Golgi markers were decreased in COPI-treated cells, whereas the signal was dispersed in DCV-infected cells as observed by confocal microscopy. In contrast, treatment with dsRNA to a ribosomal protein, RpS6, which blocks DCV translation, and thus replication at a different step in the lifecycle, had no effect on Golgi morphology (unpublished data). Moreover, depletion of *Syx5* (t-SNARE) or COPII *(sec23)* led to a loss in Golgi staining but had no effect on viral replication (Figure 4C and 4D). This demonstrates that loss of Golgi per se is not sufficient to block viral replication. Instead, the specific loss of COPI results in a loss of the Golgi, a defect in vesicle formation, and a block in viral replication. Together, this suggests that the vesicular compartment is formed by COPI coatamer-mediated disassembly from the Golgi during infection and that the Golgi disruption mediated by the loss of COPII does not block COPI access to the appropriate target for vesicle formation.

Fatty Acid Biosynthesis Is Required to Generate This Vesicular Compartment

The apparent requirement for the generation of a COPI-dependent vesicular compartment for DCV replication led us

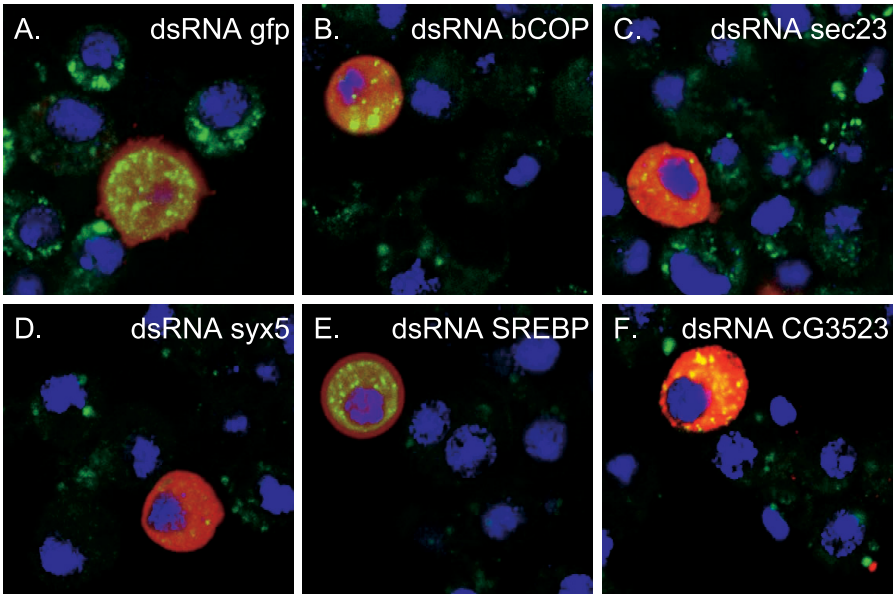

Figure 4. COPI-Dependent Golgi Disassembly in DCV Infected Cells

Confocal analysis of cells pretreated with the indicated dsRNA and infected with DCV.

(A) Golgi morphology of DCV-infected control cells (GFP) reveals that the normal punctate staining in uninfected cells is dispersed during viral replication.

(B–F) Loss of COPI *(bCOP)* (B), *SREBP* (E), or *CG3523* (F) but not COPII *(sec23)* (C) or *Syx5* (D) results in a decrease in viral infection. Note that the Golgi stain is reduced in uninfected COPI, COPII, *SREBP, CG2523,* and *Syx5,* but only the loss in COPI, *SREBP,* or *CG3523* results in a decrease in DCV replication. Green, anti-Golgi *(DG13);* red, anti-DCV; blue, Hoescht 33342.

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to hypothesize that there might be additional genes identified in our genome-wide RNAi screen that might also be required to generate this vesicular compartment. Therefore, we tested whether any of the other genes had a phenotypic effect on the morphology of the Golgi, and thus might be required for the generation of the vesicular compartment. We screened the 45 genes and found that while the majority had no effect on the Golgi as monitored by immunofluorescence microscopy, two additional genes led to a loss in Golgi staining similar to that seen on treatment with dsRNAs against the COPI coatamer components. Both of these *(HLH106* and *CG3523)* encode factors required for fatty acid biosynthesis (Figure 4F and unpublished data).

HLH106, also known as sterol regulatory element binding protein *(SREBP),* is the master transcriptional regulator of fatty acid metabolism, and directly controls the transcription of *CG3523* which encodes fatty acid synthase, the first rate-limiting enzymatic step in the pathway [33]. These results indicate that fatty acid biosynthesis is required both for DCV replication and for maintenance of the Golgi compartment. Additionally, we found that depletion of these factors had no effect on viral entry (unpublished data). Instead, depletion of these factors blocked the formation of the virus-induced vesicular compartment as measured by electron microscopy (Figure 3, unpublished data).

Our finding that fatty acid biosynthesis was required for the formation of this compartment is consistent with the observation that infected cells have a net increase in membrane due to the large number of cytoplasmic vesicles (2-fold increase in total membrane). Our results are also supported by the observation that cerulenin, a fatty acid synthase inhibitor, blocks positive-strand RNA virus (including poliovirus) replication in tissue culture [34]. While *SREBP* in mammals is cholesterol responsive and as such regulates both fatty acid metabolism and cholesterol biosynthesis, in insects *SREBP* responds to palmitate levels (*Drosophila* is a cholesterol auxotroph [35]). Nevertheless, *Drosophila SREBP* controls many of the same regulators of fatty acid biosynthesis, suggesting conserved functions in viral replication.

SREBP, COPI, and Fatty Acid Biosynthesis Are Limiting for Viral Replication in Animals

To test the dependence of DCV infection on host fatty acid biosynthesis in vivo, we infected *Drosophila SREBP* mutants with DCV. *SREBP*-null adults were generated by rescuing the larval lethality of the mutation with an RU486-inducible wild-type *SREBP* transgene that is expressed exclusively during larval development [36]. Withdrawal of RU486 results in adults that do not express the rescue construct and as such are null for *SREBP* as measured by Western blot analysis (Figure 5A). Therefore, *SREBP* is dispensable in adult flies. We challenged these mutants or their heterozygous siblings with DCV and monitored viral replication using two methods. First, we used immunoblot analysis to monitor viral capsid production as a function of time postinfection. Consistent with the cell-based results, *SREBP* mutant flies had reduced levels of viral antigen production as compared to heterozygous matched siblings normalized to cellular tubulin levels (Figure 5B). Second, we monitored viral RNA production by RT-PCR and found that loss of *SREBP* severely attenuated viral replication (Figure 5C). This demonstrates that the transcriptional master regulator of fatty acid biosynthesis, *SREBP,* is required and limiting for viral replication in animals.

We also found that there was a synthetic interaction between fatty acid biosynthesis and COPI activity in vivo.

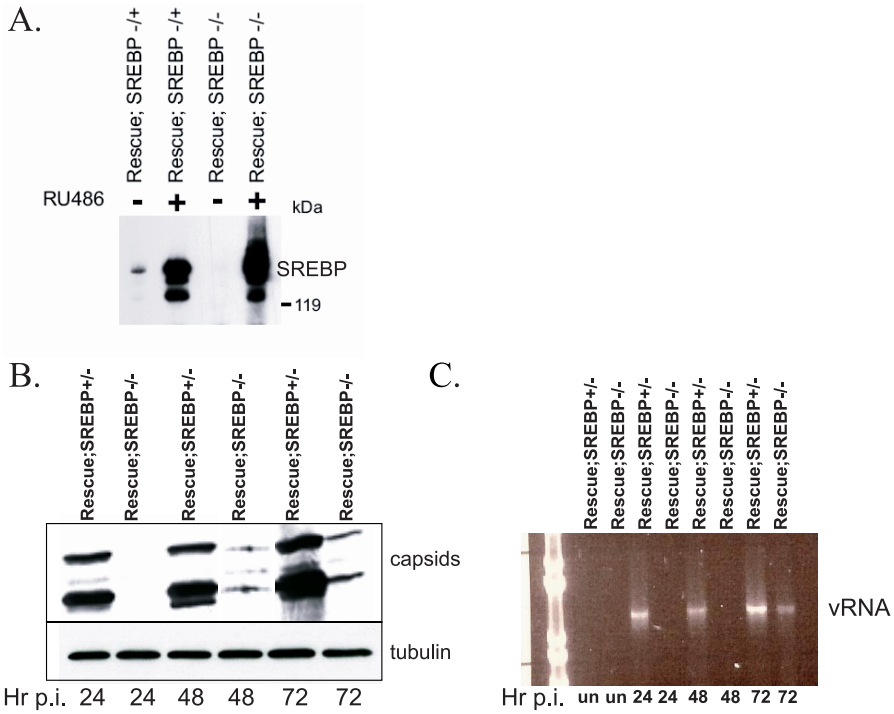

Figure 5. Attenuation of Fatty Acid Biosynthesis in Animals Is Protective

(A) *SREBP*-null flies were generated by rescuing the larval lethality using an inducible transgene and have no detectable SREBP protein as adults as measured by immunoblot probed with anti-*SREBP*.

(B) These *SREBP*-null flies are resistant to viral infection as measured by viral antigen production post infection. Heterozygous or homozygous *SREBP* mutant flies were challenged with DCV, and viral antigen production was measured as a function of time postinfection. Protein lysates were generated (hours postinfection indicated [Hr p.i.], normalized, and probed with anti-DCV or anti-tubulin for normalization.

(C) *SREBP*-null flies are resistant to viral infection as measured by viral RNA production postinfection. Heterozygous or homozygous *SREBP* mutant flies were challenged with DCV, and viral RNA production was measured by RT-PCR at the indicated time points postinfection (Hr p.i.).

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While heterozygous mutants of *gammaCOP* or fatty acid synthase *(CG3523)* were unaffected in their ability to support DCV replication, flies carrying mutant alleles for both *gammaCOP* and fatty acid synthase were attenuated in their ability to support DCV replication as measured by viral antigen production (Figure S2). Therefore, the pathways that were limiting in vitro for viral replication were also limiting in animals.

Poliovirus Infection Is Also Sensitive to Depletion of COPI but Not COPII

Because attenuation of COPI, but not COPII, blocked DCV replication, we hypothesized that this effect may be generalizable to mammalian picornaviruses such as poliovirus. To test whether attenuation of these pathways protected human cells from poliovirus replication, we infected human cells pretreated with small interfering RNAs (siRNAs) against control, *alphaCOP* or *sec23B* with poliovirus. We assayed infection by immunofluorescence staining of infected cells using an antibody against VP1, a capsid protein (Figure 6A). We quantitated these images and observed a significant reduction of viral infection in cells transfected with siRNAs against *alphaCOP* as compared to control siRNA or siRNA against *sec23B* (Figure 6B). To confirm that the siRNA against *sec23B* was functional, we measured the levels of *sec23B* and *alphaCOP* by RT-PCR. We found that treatment with either *sec23B* or *alphaCOP* siRNA led to a depletion of the cognate mRNA, demonstrating that *sec23B* is depleted yet is dispensable for poliovirus replication in human cells (Figure 6C). Therefore, these data suggest that poliovirus replication in human cells, like DCV replication in *Drosophila* cells, requires COPI, while COPII activity is dispensable.

Discussion

In this work, we have shown the dependence of DCV replication on an array of host-encoded factors. Using an unbiased loss-of-function screen, we identified a number of factors involved in different stages of the viral lifecycle. Moreover, because this RNAi methodology does not result in null alleles, but instead in hypomorphic phenotypes, we were able to identify the limiting components for viral replication in the cell. Interestingly, these host factors are significantly biased toward conserved genes as opposed to species-specific factors, demonstrating that DCV specifically, and perhaps viruses more generally, may selectively target conserved functions in the cell. This may help explain how some viruses can readily infect disparate hosts (mosquitoes, humans, etc.) and parsimoniously expand their range to new species. Moreover, because these required, but limiting, genes are essential for DCV replication in *Drosophila* cells, one may presume that related viruses may be dependent on similar cellular factors for replication. Indeed, we confirmed that poliovirus replication in mammalian cells is also dependent on COPI.

We found that DCV, like picornaviruses, induces the formation of a cytoplasmic vesicular compartment in

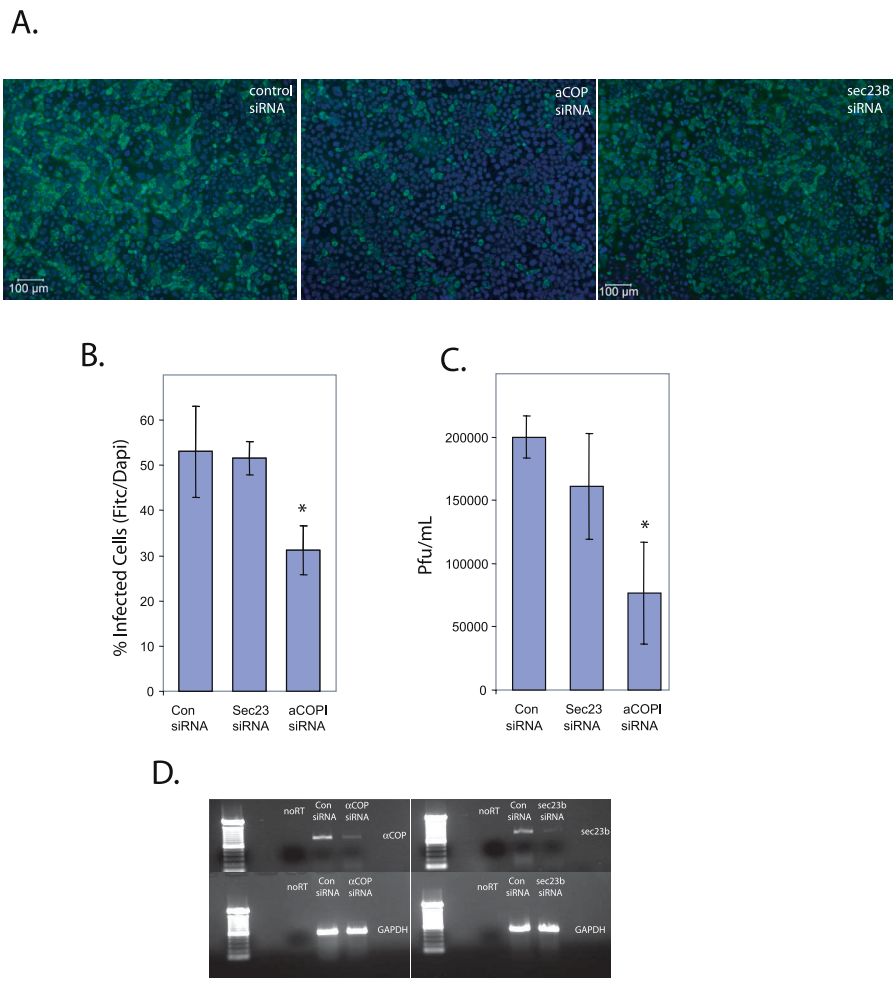

Figure 6. Attenuation of COPI but Not COPII Protects Human Cells from Poliovirus Infection

(A) Poliovirus infection of Caco-2 cells pretransfected with siRNAs against *alphaCOP* results in inhibition of viral replication but not control siRNA or siRNA against *sec23B* as measured by immunofluorescence analysis of infected cells (nuclei [blue] = DAPI, infected cells [green] = FITC-conjugated mouse anti-enterovirus VP1).

(B) Percent infection (FITC-positive cells/DAPI * 100) is shown for two independent experiments performed in triplicate where error bars represent one standard deviation. *$p < 0.05$.

(C) Plaque-forming units/mL (pfu/mL) are shown for the experiments performed in (B). *$p < 0.05$.

(D) RT-PCR on Caco-2 cells treated with siRNA against *alphaCOP* or *sec23B* demonstrates that treatment with either siRNA leads to a significant depletion of the cellular mRNA and amplification of GAPDH was used as loading control.

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infected cells and that this compartment is the site of viral RNA replication. Among the conserved genes whose disruption significantly reduced DCV replication were a group of vesicular trafficking genes including the COPI coatamer. Our data show that COPI was required downstream of viral entry and was essential for the production of this vesicular compartment. However, we cannot rule out the possibility that COPI is required postentry, prevesicle formation for a step in the viral lifecycle independent of its role in secretion. In contrast, our data clearly show that both COPII and autophagy were dispensable for both viral replication and vesicle formation. For autophagy, we directly tested the roles of Beclin-1, Atg5, Atg18, and Atg12 and found no defect in DCV replication (unpublished data). This is important because there has been much debate on the ontogeny of vesicles that arise during picornavirus replication. This is due, at least in part, to the experimental approaches used to determine the factors involved. For example, it is difficult to discern cause from effect in studies utilizing marker co-localization strategies, as many different cellular factors and compartments may be associated with the vesicles. By undertaking a loss-of-function study, we were able to identify specific factors required to form the vesicular compartment, rather than simply identifying factors found associated with the membranous compartment under steady state conditions. While it is possible that some of the vesicles associated with viral replication are generated via a COPII or an autophagic process, our data clearly demonstrate that these latter processes are not essential for bulk vesicle formation or for viral replication.

Because the COPI coatamer normally targets and buds from the Golgi and DCV-induced vesicles required COPI activity, we analyzed the state of the Golgi during infection. We found that normal Golgi morphology was disrupted during viral replication with many small punctae distributed throughout the cytoplasm of the cell, consistent with the disassembly of

the Golgi by the COPI coatamer and the formation of the vesicles used for RNA replication. We also demonstrated that a Golgi marker co-localizes with the RNA replication machinery at the DCV-induced vesicles. At present, we cannot determine if this compartment is formed directly by the conversion of the Golgi into a novel specific structure or whether membrane is derived from other cellular structures. This is in part because an intact Golgi was not required for vesicle formation, suggesting that the COPI coatamer targets a specific membrane component that remains accessible even if the Golgi is apparently disrupted by the loss of cellular factors including COPII or *Syx5*. This raises the possibility that DCV may derive membranes from other structures that are targets for COPI-dependent vesicle formation or that DCV may in some way redirect COPI to other sites. Possible explanations for the source of COPI-mediated membrane recruitment include the ER-intermediate compartment, as COPI has also been localized to this area, or the possible anterograde transport activity of the COPI coatamer. DCV, which is clearly dependent on COPI for the generation of its virogenic organelle and must in some way interdict normal COPI trafficking pathways, may prove to be a useful tool for dissecting COPI functions and the complex relationships between COPI, the Golgi, and the generation of vesicular structures upon which viral replication occurs.

Under normal circumstances, in order to drive the formation of COPI vesicles, the COPI coatamer complex is bound by the small GTPase ADP-ribosylation factor 1 (Arf1). These coatamer-GDP bound Arf1 complexes are activated by guanine nucleotide exchange factors to induce vesicle formation (reviewed in [37]). Our screen and additional experiments did not identify *Drosophila* Arf1, nor any of the other Arfs present in *Drosophila,* as being required for viral replication. This may be due to some functional redundancy between Arfs. Recent work on poliovirus demonstrated that a number of different Arfs were recruited to the poliovirus replication complexes [12]. Moreover, in a screen for factors involved in general secretion in *Drosophila* cells, two Arfs were essential [38]. Together, this may point to functional redundancy of Arfs under certain conditions in a variety of systems.

Once we identified the COPI complex as necessary for vesicle formation and Golgi maintenance, we reexamined the additional factors identified during the initial screen and found that suppression of fatty acid metabolism *(SREBP* and *CG3523)* decreased viral replication in vitro and in vivo and also prevented the formation of the vesicular compartment. Thus, we were able to assign a functional role for fatty acid metabolism in viral replication. This is perhaps not surprising since morphometric analysis of membrane profiles in DCV-infected cells indicated that there was a 2-fold increase in membrane surface area within 10 h of infection. Thus, virus infection leads to membrane redistribution involving both the Golgi and COPI as well as membrane expansion through de novo lipid biogenesis. Because the size of the virally induced vesicles (mean = 115 nm) was significantly larger than that of normal COPI vesicles (mean = 50 nm), they may be formed in part by active membrane biosynthesis at the budding site. Nevertheless, this coupling of fatty acid biosynthesis with COPI coatamer budding from the Golgi results in Golgi disassembly and the formation of a novel cytoplasmic vesicular compartment upon which viral RNA replication takes place.

More generally, it is not clear why it would be beneficial for a virus to generate a new membranous structure rather than use preexisting membranes for replication. Indeed, some viruses (e.g., flock house virus) use preexisting membranes for RNA replication instead of generating new structures [39]. Moreover, how viruses such as DCV coordinate and activate these cellular processes has yet to be shown, making this *Drosophila*-DCV system an ideal model both to identify cellular factors required for viral replication and to study how virus-dependent cellular structures and organelles are generated de novo from preexisting components within a cell.

Importantly, the work presented herein confirms the relevance of the cell culture model used to perform the initial RNAi screen by demonstrating a requirement in vivo for the machinery which produces the virogenic organelle. Moreover, we found that both COPI and fatty acid biosynthetic machinery were essential for viral replication in adult animals. This additional evidence supports the generality of the findings and is the first demonstration of the importance of the virus-induced membranous organelle for infection of the native host of a virus of this class. In addition, we extended our finding to the related picornavirus, poliovirus. Using a similar loss-of-function strategy in human cells, we found that, like DCV infection of *Drosophila* cells, poliovirus infection of human cells required COPI while COPII appeared dispensable. Therefore, our screen for host factors in this *Drosophila* model system provides insight into cellular genes required for viral replication in higher organisms.

More work will be necessary to identify the mechanistic aspects of vesicle formation and viral functions dependent thereon. Nevertheless, our results form a basis for investigation of virus–host interactions, the limiting cellular components required for the viral lifecycle, and potential antiviral targets in a system that is amenable to both forward and reverse genetic analysis and thus is uniquely suited to rapid and comprehensive dissection.

Materials and Methods

Cells, antibodies, and reagents. SL2 cells, DL2 cells, and DeltaWT-deMYC cells were grown and maintained as described previously [13,26], as were the production and purification of DCV [13]. Antibodies were obtained from the following sources: anti-DCV [13], anti-Golgi (Calbiochem, San Diego, California, United States), DG13 (anti-Golgi, gift of Vivek Malhotra, University of California San Diego), anti-Delta (C594.9B, Iowa Hybridoma Bank, University of Iowa, Iowa City, Iowa, United States), anti-tubulin (Sigma, St. Louis, Missouri, United States), and anti-dSREBP (IgG 3B, prepared as described [35]). Polyclonal anti-DCV helicase antibodies were generated in rabbits against a peptide corresponding to residues 483–498 of the replicase polyprotein within the helicase domain. Fluorescently labeled secondary antibodies against chicken were obtained from Jackson ImmunoResearch (West Grove, Pennsylvania, United States), and other secondary antibodies were from Molecular Probes (Eugene, Oregon, United States). Additional chemicals were obtained from Sigma.

RNAi. dsRNAs for RNAi were generated and used for RNAi for 3 d as described [20]. Amplicons used were generated by the DRSC and are described at http://flyrnai.org.

Secretion assay. DeltaWTdeMYC cells were treated with dsRNA and pulsed with 0.5 mM $CuSO_4$ for 2 h. All subsequent steps were performed at 4 °C. The cells were washed in FACS buffer (2% fetal calf serum, azide, PBS) and stained with anti-Delta (1:200) for 45 min. The cells were washed and stained with Alexa-488 anti-mouse for 45 min. Cells were analyzed on a FACScan flow cytometer with Cell Quest software (Becton-Dickinson, Palo Alto, California, United States), live cells were gated using forward scatter and side scatter, and propidium iodide was used to exclude dead cells.

Viral entry, infection, and immunofluorescence. Entry experiments were performed as described [13]. Cells were pretreated with dsRNA and placed at 4 °C. Virions were added (multiplicity of infection = 10) and incubated for 2 h. The cells were washed and incubated at room temperature for 3 h. Cells were then stained with anti-DCV as described and counterstained with Alexa-594 phalloidin (Invitrogen, Carlsbad, California, United States). For other experiments, cells were infected and stained as previously described [20]. Cells were imaged using automated microscopy [20] or confocal microscopy (Leica TCS SP2 AOBS) as indicated, and percent infection was measured. Image quantitation was performed using MetaMorph software (Molecular Devices, Sunnyvale, California, United States).

Electron microscopy. Cells were fixed on the dish in 2% PFA/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at RT, postfixed in 1% osmium tetroxide/1.5% potassium ferrocyanide in water for 30 min, and stained in 1% uranyl acetate in maleate buffer (pH 5.2) for 30 min at RT. After dehydration in a graded ethanol series, cells were removed from the dish in propyleneoxide and pelleted at 3,000 rpm for 3 min. Pellets were embedded in Epon. Ultrathin sections (approximately 80 to 90 nm) were mounted on copper grids, stained with 2% uranyl acetate in acetone followed by 0.2% lead citrate, and examined in a JEOL 1200EX transmission electron microscope, and images were recorded at a primary magnification of ×9,700.

For preparation of cryosections, cells were fixed in 4% paraformaldehyde (in 0.1 M sodium phosphate buffer [pH 7.4]) in a microfuge tube and pelleted for 3 min at 3,000 rpm. The supernatant was carefully removed, and fresh 4% paraformaldehyde was added. After 2-h fixation at room temperature, the cell pellets were washed with PBS containing 0.2 M glycine. Cell pellets were infiltrated with 2.3 M sucrose in PBS for 15 min and frozen in liquid nitrogen. Frozen samples were sectioned at −120 °C, transferred to formvar-carbon–coated copper grids, and floated on PBS. Gold labeling was carried out at room temperature on a piece of parafilm. All antibodies and Protein A-gold were diluted 1% BSA. Grids were floated on drops of 1% BSA for 10 min, transferred to 5-μl drops of primary antibody, and incubated for 30 min. The grids were then washed in PBS for a total of 15 min and transferred to drops of Protein A-gold or gold-labeled secondary antibody. Embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose. The grids were examined in a JEOL 1200EX transmission electron microscope, and images were recorded at a primary magnification of ×25,000 [40].

The total amount of membrane per cell was calculated by measuring the linear amount of membrane of similar sized sections of cells using MetaMorph software for uninfected ($n = 6$) and infected ($n = 6$) cells.

Fly stocks and viral infections. All flies were obtained from the Bloomington stock center unless stated otherwise and were maintained on standard medium at 24 °C. RU486-inducible Gal4 (S_1106) was used to rescue the larval lethality of the SREBP-null allele (189) by feeding larvae 200 μM RU486 which induced the expression of the UAS-SREBP rescue construct as described [36]. Following eclosion, adults were maintained on standard medium. The 4- to 5-d-old adults of the stated genotypes were inoculated with DCV as previously described [13].

Immunoblotting and RT-PCR. Flies were collected at the time points indicated. The flies were lysed in radioimmunoprecipitation buffer supplemented with a protease inhibitor cocktail (Boehringer-Ingelheim, Ingelheim, Germany). Samples were separated by 10% SDS-PAGE and blotted as previously described [13]. For RT-PCR, flies were lysed and total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer. cDNA was prepared using AMV reverse transcriptase (Invitrogen) and the virus-specific primer DCV2 and used for PCR with the DCV1 and DCV2 primers as described [41].

Poliovirus experiments. Poliovirus Sabin 2 was a kind gift from the Centers for Disease Control and Prevention (Atlanta, Georgia, United States). Virus was expanded by growth in HeLa cells and concentrated by ultracentrifugation through a sucrose cushion, and titers were determined by plaque assay on HeLa cells. For infections, Caco-2 cells were cultured as described [42] and transfected with 10 nM COPI, Sec23B, or control siRNAs (Dharmacon smart pools) using HiPerFect transfection reagent according to the manufacturer's protocol (Qiagen, Valencia, California, United States). Transfected cells were incubated with PV at a multiplicity of 1 PFU/cell in virus binding buffer (DMEM supplemented with 1 mM HEPES for 1 h at 4 °C). Following washing, virus infection was initiated by shifting cells to 37 °C in tissue culture medium for 7 h. Cells were fixed and permeabilized before staining with ice-cold methanol/acetone (3:1) for 5 min at room temperature. Cells were incubated with FITC-conjugated mouse anti-enterovirus VP1 (Ncl-Entero; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) for 1 h, washed, and mounted with Vectashield (Vector Laboratories, Burlingame, California, United States) containing DAPI. Images were captured with a confocal laser-scanning microscope (Leica, Exton, Pennsylvania, United States). Plaque-forming assays were performed in HeLa cells as described [42].

Statistical analysis. Statistical analyses for Figure 1 were performed using the Fisher exact test for small samples; the χ^2 test was used in situations were all counts are above five. Student's *t*-test was performed on the data from Figure 6.

Supporting Information

Figure S1. RNAi against *epsilonCOP* and *sec23* Leads to Depletion of mRNA

RT-PCR of cells treated with the indicated dsRNA. Total RNA was purified 3 d post treatment, used as a template for cDNA, and amplified using primers specific for the indicated genes. The amount of imput cDNA was varied to assess the linearity of the PCR conditions.

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Figure S2. DCV Replication Requires High Levels of COPI and Fatty Acid Synthase in Adults

The flies carrying a mutant allele of gammaCOP (gammaCop [S057302a]) and fatty acid synthase AU: Make CG3523 italic? If so, also gammaCOP(CG3523 [Df(2L)JS17]) are resistant to viral infection as measured by viral antigen production post infection. Flies heterozygous for each mutant or the compound mutants were challenged with DCV, and viral antigen production was measured by Western blot 24 h post infection. Protein lysates were generated and probed with anti-DCV or anti-tubulin for normalization.

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Author contributions. SC conceived, designed, and analyzed the experiments and wrote the manuscript. SC and HW performed the experiments. AK and RBR contributed reagents and data for Figure 5. CC generated the data for Figure 6. NP contributed to experimental design and the drafting of the manuscript.

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Competing interests. The authors have declared that no competing interests exist.

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