Parallel evolution and enhanced virulence upon *in vivo* passage of an RNA virus in *Drosophila melanogaster*

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Abstract

Virus evolution is strongly affected by antagonistic co-evolution of virus and host. Host immune pathways positively select for viruses that evade the immune response, which in turn may drive counter-adaptations in host immune genes. We investigated how host immune pressure shapes virus populations and virulence, using the fruit fly *Drosophila melanogaster* and one of its natural pathogens, Drosophila C virus (DCV), as a model. We performed an experimental evolution experiment in which DCV was serially passaged for 10 generations in three fly genotypes differing in their antiviral RNAi response, using wild-type flies and flies in which the endonuclease gene *Dicer-2* was either overexpressed or inactivated. We found that all evolved virus populations replicated more efficiently *in vivo* and were more virulent than the parental stock. The number of polymorphisms increased in all three host genotypes with passage number, which was most pronounced in *Dicer-2* knockout flies. Mutational analysis showed strong parallel evolution, as every lineage accumulated mutations in a specific region of the VP3 capsid protein in a host genotype-independent manner. The parental tyrosine at position 95 of VP3 was substituted with either one of five different amino acids in 14 out of 15 lineages. However, no consistent amino acid changes were observed in the viral RNAi suppressor gene 1A, nor elsewhere in the genome in any of the host backgrounds. Our study indicates that the RNAi response restricts the sequence space that can be explored by viral populations. Moreover, our study illustrates how evolution towards higher virulence can be a highly reproducible, yet unpredictable process.

Significance statement

The host immune response can have a major impact on the evolution of viruses. For instance, SARS-CoV-2 variants of concern have likely arisen in immunocompromised hosts. RNA interference (RNAi) is a potent antiviral immune-defense in insects, which we expect to likewise affect evolution of virus populations. To test this, we have either annulled or enhanced RNAi

activity in fruit flies and serially passaged virus populations on these hosts. Our experimental evolution study revealed that the immune response constrains the sequence space that a virus can explore, and that a single amino acid change in the viral capsid can have a large impact on virulence and adaptation to the environment.

Introduction

Viruses are ubiquitous parasites of cellular life and the most abundant biological entities on earth, predicted to appear in any replicator system (1–3). Virus-host coevolution is often understood as an arms race between host defense and viral counter-defense mechanisms. Viral evasion of the host immune system likely occurs in all host systems (4). For example, the interferon response, one of the most potent antiviral mechanisms in mammals, can be antagonized at the transcriptional, translational, or function level by many different viruses (5).

In insects, RNA interference (RNAi) plays a critical role in antiviral defense, as viral double stranded RNA (dsRNA) is processed by the endonuclease Dicer-2 (Dcr-2) into small interfering RNAs (siRNAs), serving as guides for the endonuclease Argonaute-2 (AGO2) to mediate viral RNA degradation (6). Several insect viruses have been shown to suppress this pathway, illustrating how immune pressure exerted by RNAi affects the genetic makeup of a virus (7, 8). For instance, the Drosophila C virus (DCV) 1A protein binds double-stranded RNA (dsRNA) and inhibits Dcr-2 nuclease activity (9), whereas proteins from two RNA viruses, the cricket paralysis virus 1A protein and the nora virus VP1 protein bind and inhibit AGO2 function (10–13).

Upon infection, RNA viruses create swarms of closely related within-host genotypes, often referred to as viral quasispecies (14), that contribute to the adaptability of a viral population to changing environments (15, 16). They are the result of the high mutation rate of the viral RNA dependent RNA polymerase (RdRP) and the large population sizes of RNA viruses, which can be as high as 10^12 infectious particles in an infected host (17, 18). Most mutations in this diverse virus population will be deleterious, some neutral, and very few beneficial (19, 20), and the frequency of these variants will fluctuate according to evolutionary forces, such as the positive and purifying selection imposed by the host immune system. New virus variants that are capable of escaping immune surveillance have an evolutionary advantage and will likely take over the

ancestral population. For instance, plants with a more restrictive immune system have been shown to drive faster evolution of turnip mosaic virus (21).

In this study, we assessed to what extent selection pressure by the immune system drives virus population evolution. We used as a model system the fruit fly *Drosophila melanogaster* infected with its natural pathogen DCV, a single-stranded positive-sense RNA virus of the *Dicistroviridae* family. The virus was serially passaged in wild-type flies and in fly mutants in which *Dicer-2* was either overexpressed or inactivated. Afterwards the evolved virus populations were characterized using next-generation sequencing (NGS) in combination with phenotypic assays for virus replication and host survival. We find that the increase in the number of polymorphisms during passaging was most pronounced in *Dicer-2* knockout flies. However, the RNAi response had little effect on the accumulation of specific amino acid changes in viral proteins. Instead, we observed parallel evolution of mutations in the VP3 capsid protein in all viral lineages that markedly enhanced viral replication and virulence.

Results

Experimental DCV evolution increases virulence

To study the effect of the host immune system on DCV evolution, we performed an experimental evolution experiment in which a parental stock was serially passaged over three fly genotypes with different RNAi activity (Figure 1A). Specifically, we used wild-type flies (w^{1118}, hereafter WT), flies with a frameshift mutation resulting in a premature stop codon in Dcr-2 protein (*Dcr-2*^{L811fsX} (22), hereafter *Dcr-2-KO*), and flies overexpressing a *Dcr-2* transgene controlled by the UAS upstream activation sequence and a *Tubulin-Gal4* driver (hereafter *Dcr-2-OE*). As expected (23, 24), *Dcr-2* expression correlated with survival after DCV infection, with *Dcr-2-KO* flies having the shortest life span and *Dcr-2-OE* flies the longest (Figure 1B).

For each host genotype, we allowed five DCV lineages to independently evolve during ten serial passages, with five independent lineages per genotype. To prevent population bottlenecks, flies were infected by intrathoracic injection with a high viral inoculum of 10^4 median tissue culture infectious doses ($TCID_{50}$), and virus titers were measured after each passage to normalize the inoculation of naive flies for the next passage (Figure 1C). With the exception of passage 3 and 5, where we observed significantly higher viral titers in *Dcr-2-KO* than in WT flies (*t*-test, $p < 0.05$, Figure 1C), the differences in viral titers between genotypes were generally small and not consistent along passages, likely because flies were harvested at the plateau of peak titers.

To examine the virulence of the evolved virus populations, we performed survival assays after inoculation of WT flies with the parental stock and virus stocks harvested after passage 1, 5 and 10 (P1, P5, P10; Figure 2A-D). Viruses from all lineages of all host genotypes induced higher mortality in WT flies when comparing P10 viruses with the parental stock (Log-rank test, $p < 0.0001$). Upon inoculation with 10^4 $TCID_{50}$ of virus stock, mean survival times were 5.5 days and 3.4 days for the parental stock and the P10-evolved virus populations (average of all genotypes and replicates), respectively. The mean survival time of WT flies decreased from P1 to P5 for

virus populations from all genotypes (paired *t*-test, $p < 0.005$), and it further decreased from P5 to P10 only in virus populations from WT flies (paired *t*-test, $p = 0.006$) (Figure 2D).

We next analyzed whether these survival phenotypes correlate with changes in replication kinetics of the evolved virus populations. To test this, we injected the parental, P1, and P10 virus stocks into WT flies and determined virus RNA levels at 12 hours post infection (hpi) (Figure 2E). We observed a large variation between replicates of genotypes at P1, with the parental stock accumulating 3.4e+05 RNA copies and the P1 viruses ranging from 4.2e+04 to 3.9e+07 RNA copies. Irrespective of the genotype, all lineages of the P10 virus populations reached higher viral RNA levels than the parental stock (paired *t*-test for each genotype, $p < 0.01$), with differences ranging from 2.8e+06 to 2.2e+08 RNA copies. This phenotype was maintained when the infection was performed orally (Supplementary Figure 1), indicating that our experimental evolution scheme did not lead to loss of infectivity via the presumed natural infection route. When injected into WT flies, P10 viruses evolved in *Dcr-2*-KO flies reached the highest RNA loads, compared to those evolved in WT and *Dcr2*-OE flies ($p = 0.0004$ and $p = 0.0319$, Tukey's HSD test for multiple pairwise comparison). Together, these results indicate that *in vivo* serial passage increased replication kinetics and virulence of virus populations.

Ongoing accumulation of polymorphisms

We next investigated how the virus populations evolved through the serial passage experiment by next-generation sequencing (NGS). The viral genome was sequenced to high coverage (average coverage of 127,542, Supplementary Figure 2), allowing us to confidently call low-frequency mutations, as the median Phred sequencing quality score for the mutation calls was 39, corresponding to an error rate of 0.0001. In the evolutionary lineages, we observed increased numbers of polymorphic sites within the populations compared to the parental stock with a mean of 381 polymorphic sites per sample in passage 10, 116 more than in the parental stock (Figure 3A). We found between 179 and 499 SNVs per evolved population at a frequency below 1%, and

between 5 and 21 SNVs at an estimated frequency higher than 1% (Figure 3B). The number of polymorphic sites increased significantly through the serial passage in *Dcr-2-KO* and *Dcr-2-OE* flies (paired *t*-test, $p < 0.05$, Figure 3A). This increase was most pronounced in virus populations from *Dcr-2-KO* flies, which accumulated a mean of 444 polymorphic sites at passage 10 (Figure 3A), which was significantly different from virus populations from WT and *Dcr-2-OE* flies (*t*-test, $p < 0.02$). Yet, the vast majority of polymorphic sites are low-frequency mutations (median: 0.1%), and very few SNVs increased in frequency or reached fixation (Figure 3B).

We next analyzed virus population diversity over time and across host genotypes and found that the mean nucleotide diversity did not change significantly over time in any of the host genotypes, nor were there significant differences in population diversity between genotypes (mixed ANOVA, $p > 0.05$, Supplement Figure 3A). Mean nucleotide diversity was significantly higher in coding than in non-coding regions of the viral genome (mixed ANOVA, $p < 0.01$, Supplement Figure 3A). Specifically, genes encoding the viral VP3 protein displayed a significant increase in nucleotide diversity compared to the mean diversity of the whole coding region (paired *t*-test, $p < 0.05$, Supplementary Figure 3B).

Moderate host genotype-specific adaptation

We next assessed the shared and unique SNVs across genotypes, first considering SNVs with frequencies ≥ 0.01%, and found a similar number of unique SNVs between virus populations from WT and *Dcr-2-OE* flies (818 and 910, respectively), both having less SNVs than virus populations from *Dcr-2-KO* flies (1189 SNVs) (Figure 3C). 7.3% of the SNVs were shared between virus populations from all three fly genotypes, and it is noteworthy that almost all these (283 of 289, 97.9%) were non-synonymous mutations. When restricting the analysis to high-frequency SNVs (≥ 10%), the fraction of SNVs shared by the virus populations from all three fly genotypes increased to 21.3%, all of which were non-synonymous mutations (Figure 3D).

To understand if the proportions of shared and unique SNVs are the result of neutral evolution or if host-specific adaptation took place, we conducted a permutation test, as described by Bons et al. (25). Comparing the observed number of mutations unique to virus populations from *Dcr-2-KO* flies (131 mutations) to the expected number by random evolution (89 mutations expected from randomizations), 42 mutations are expected to result from virus adaptation, rather than neutral evolution ($p < 0.001$, Supplementary Figure 4). For the other host genotypes, we did not find significant differences between the expected and observed number of mutations. Together, these data indicate parallel evolution of shared SNVs resulting in amino acid changes as well as moderate host-genotype specific adaptation in the absence of a functional RNAi response.

Uridine deletions in homopolymeric tracts

To explore the evolution of the viral populations in more detail, we visualized the SNVs across the DCV genome in a heatmap (Figure 4A). We observed some SNVs that were already present in the parental stock and were retained in the viral populations throughout the experimental evolution in most of the lineages (nt 329, 504, 2686, 2736, 7082, 7458, 8089 and 8526), while others appeared *de novo* during the experiment in different lineages (e.g., nt 331, 1026, 4758, 6263 and 7557). Strikingly, we found a ubiquitous deletion of a uridine in a poly(uridine) tract at position 5765-5774, which gives rise to a frameshift and a premature stop codon in the *RdRP* gene. This deletion was also present in the parental stock and it was retained at low, yet fairly constant frequency through the experiment in all 15 lineages (around 4%, Supplementary Table 1). Likewise, another uridine deletion in a tract of 8 uridines at position 276-284 in the 5' untranslated region (UTR) occurred in all 15 lineages with a constant frequency of around 3% (Supplementary Table 1). To control for possible sequencing artifacts, we evaluated if the two uridine deletions were located at the edges of sequence reads containing them, as there is lower confidence in the base calling at read edges. However, in only 7% of reads the deletions were located in proximity to the read edges (within five nucleotides). Furthermore, the mean

sequencing quality score in the uridine tracks and the adjacent positions in reads with and without deletion is above 40 in both cases, corresponding to an error rate of 0.0001 (mean quality scores across both uridine tracks: 40.1; 10%-quantile: 38.2, 90%-quantile: 41.0; Supplementary Table 2). Further analysis suggested that such deletions also occur in other, published DCV NGS datasets, and we suggest that the viral RdRP is prone to slippage on homopolymeric uridine and that there is purifying selection against the occurrence of these tracts in RNA viruses (Supplementary text 1, Supplemental Table S3-S5).

Parallel evolution caused by positive selection on the capsid protein

Thirteen SNVs became dominant (frequency > 50%) after 10 passages, most of which changed the amino acid sequence of the capsid proteins in a host genotype-independent manner (Figure 3D and Figure 4B). Of note, two dominant synonymous SNVs appeared to be host genotype-specific, for 7118C became dominant only in WT flies (at frequency of 99% in one replicate and above 20% in other two) and 6845G in two lineages from *Dcr-2-OE* flies. However, the majority of the dominant SNVs were located at codon 95 of the capsid *VP3* gene (nt 7566-7567), where the tyrosine codon (Y95) in the parental stock was replaced in 14 out of 15 lineages (Figure 5A). The Y95 frequency declined below 60% in three out of five replicates of virus populations from *Dcr-2-OE* flies at P1, whereas the Y95 frequency remained above 90% in four and three replicates in *Dcr-2-KO* and WT flies, respectively, at P1. At P5, Y95 was undetectable in 12 out of 15 lineages, and at P10, Y95 was only retained in replicate D from *Dcr-2-OE* flies (at a frequency of 6%) and in replicate A from *Dcr-2-KO* flies, which was the only replicate in which Y95 remained dominant. Interestingly, in the latter replicate, two other capsid mutations, VP3 P92T and VP1 N114S, were fixated instead.

Strikingly, from all nine possible SNVs that can be formed from the parental UAU codon coding for Y95, five were found in the evolved virus populations (aspartic acid, asparagine, cysteine, serine and histidine). The emergence of these variants did not correlate with the host genotype in

which the virus populations had evolved. The remaining four possible codons were not detected, two of which being stop codons, one coding for a synonymous substitution, and one coding for phenylalanine, the amino acid most similar to tyrosine. Overall, these results suggest that the presence of a benzene ring at codon 95 of VP3 is detrimental to virus infection in flies. To confirm the selection forces statistically, we computed the population nucleotide diversity per synonymous (piS) and non-synonymous (piN) site for each protein separately. We find clear indication of positive selection (piN-piS > 0) in VP3 across all host genotypes, and significantly higher piN to piS values for all passages of populations evolved in *Dcr-2-KO* flies and for passage 1 populations evolved in WT flies (paired *t*-test, $p < 0.05$, Supplement Figure 5).

An exposed capsid amino acid is involved in fitness gain specific to the environment of replication

We hypothesized that the VP3 mutations were responsible for the observed fitness gains of the passaged virus populations (Figure 2E). To test this, we selected P10 lineages with one of the fixated mutations (frequency ≥ 90%), performed a serial dilution to remove minority variants, and analyzed growth kinetics in the *Drosophila* S2 cell line and in WT flies inoculated with a low dose (multiplicity of infection of 0.1 and 100 $TCID_{50}$, respectively). All variants efficiently replicated in S2 cells, but the parental stock showed higher replication kinetics than the other variants (Figure 5B). In contrast, the parental stock scarcely replicated in adult flies at this low inoculum, whereas the evolved variants replicated efficiently *in vivo* (Figure 5C).

Our results suggest that Y95 may be beneficial for DCV replication in cell culture, but detrimental *in vivo*. To investigate the effects of Y95 on the structural stability of the virion, we modeled the effect of the fixated mutations on the structure of the virus particle. We used the crystal structure of cricket paralysis virus (CrPV) (26), a related dicistrovirus which shares 60% sequence identity with the DCV capsid polyprotein, and 79% with the region of interest. Given the significant sequence identity between these two proteins, it is possible to model the variants in CrPV capsid

to predict their probable implications on the DCV capsid. The capsid polyprotein is a large assembly of 240 subunits, composed of copies of four proteins (VP1, VP2, VP3 and VP4) (Figure 6A). The region of interest is located in a loop close to the protein-protein interface between VP2 and VP3 (Figure 6A, 6C-6E). While this region is conserved across related dicistroviruses that share more than 50% sequence identity with the DCV capsid, the positions of interest in our study, i.e., 92 and 95, are relatively poorly conserved (Figure 6B). Moreover, given their location in the loop, the residues at these positions are solvent-exposed, suggesting that they may not be involved in maintaining the structural integrity of the capsid. Indeed, we do not find drastic differences in the predicted folding free energy of the capsid structure between the parental Y95 and its mutants (ranging from 0.71 kcal/mol to 3.33 kcal/mol; Supplementary Table 6), indicating that the observed fitness gains can not be explained by structural effects of the acquired mutations.

Discussion

In this study, we have explored the effect of immune pressure on virus evolution using *Drosophila* as a model host. We passaged DCV in flies differing in Dcr-2 activity, hypothesizing that variations in RNAi immune pressure would affect virus evolution at a phenotypic and genotypic level, e.g., by removing selection from the viral 1A protein that interferes with the host RNAi pathway. However, the effect of the RNAi machinery on DCV evolution was moderate. No specific polymorphisms accumulated in the DCV 1A gene in virus populations from *Dcr-2-KO* flies, nor in the other genotypes. However, we did observe that virus populations from *Dcr-2-KO* flies accumulated more polymorphisms than *Dcr-2* expressing flies, in line with previous observations (27), suggesting that the absence of immune pressure releases mutational constraints, allowing virus populations to further explore the mutational landscape.

Even though we observed some host genotype-specific effects on virus evolution, the strongest evolutionary pattern was shared among virus populations from all host genotypes, suggesting parallel and reproducible evolution. The predictability and reproducibility of evolutionary trajectories across independent populations has been a long-standing problem (28). Examples of parallel evolution exist, e.g., a mutation giving rise to antibiotic resistance was observed in multiple isolates of *Escherichia coli* independently *(29)*, and specific drug resistance-associated mutations appeared repeatedly in human immunodeficiency virus-1 (HIV-1) in individuals receiving antiretroviral therapy (30–32). Likewise, the same subset of mutations has arisen independently in HIV-1 in two different cell lines during a long-term experimental evolution experiment (33). In contrast, in the long-term evolutionary study of Lenski *et al. (34)*, the phenotype allowing *E. coli* to use citrate in aerobic conditions occurred in multiple independent lineages, but the underlying molecular mechanism was different (35). In our experiment, VP3 gene mutations occurred in parallel in all virus lineages, concomitant with a fitness gain and higher virulence *in vivo*. These

mutations were fixated in 14 out of 15 lineages independently, indicating that loss of Y95 is a highly reproducible, yet unpredictable process, as five different variants have taken over the population.

Another instance of polymorphisms occurring in all lineages are deletions of a single uridine in homopolymeric tracts of at least 6 uridines in the 5' UTR and in the RdRp gene. We observed that these uridine deletions also occurred in NGS data of DCV from other studies, and we propose that they are caused by RdRP slippage on homopolymeric uridine tracts. In contrast, thymidine deletions did not occur in NGS data from a DNA virus, herpes simplex virus 2, likely as a result of the proofreading activity of its viral DNA polymerase. In agreement, *E. coli* strains lacking mismatch repair mechanisms show increased numbers of deletions in simple sequences repeats (36).

It is well described that insertions and deletions preferentially occur in homopolymeric tracts in viral genomes (37). Some viruses make use of this, such as viruses of the *Potyviridae* family that use transcriptional slippage to insert an adenosine in a specific GAAAAAA sequence in viral transcripts to induce a frameshift and expression of an alternative viral protein (38). Likewise, the measles and Sendai viruses undergo a slippage event that adds an extra guanidine in transcripts coding for the P protein, creating a frameshift transcript that codes for the V protein involved in antagonizing host immunity (39). However, transcriptional slippage also occurs in picornaviruses on homopolymeric stretches of more than six uridines and these sequences are underrepresented in their genome, suggesting that there are also fitness costs associated with poly(uridine) tracts (40). The deletion in the *RdRP* gene introduces a premature stop codon in the RdRP and likely results in the production of defective viral genomes (DVGs). DVGs have been reported in many virus families and may affect pathogenesis via different mechanisms, like interfering with antiviral immunity or facilitating viral persistence (reviewed in (41)). Whether DCV likewise produces DVGs and their involvement in the infection cycle remains to be established.

We found that the parental stock replicated at low levels in flies receiving a low inoculum, but that it efficiently replicated in cell culture. This suggests that the parental virus is unfit because it lacks a basic function that is essential *in vivo*, but not in cell culture. Considering that the virus replicates efficiently in cells, we expect cell entry not to be impaired. Using structural models, we predicted that Y95 substitutions do not affect virus particle stability and that the residue is solvent exposed. We hypothesize that the observed phenotype is due to immune recognition of the parental stock containing Y95 in VP3, and escape thereof by mutants in VP3. This scenario is not unanticipated, as a single nucleotide substitution has been found to mediate immune evasion before. For example, blood clearance of alphaviruses like chikungunya virus is mediated mainly by resident macrophages in the liver, but a single amino acid substitution in the E2 glycoprotein disrupts this process and increases virus dissemination (42). We speculate that the parental virus is recognized by an immune receptor to mediate virus clearance, thus explaining the differences between *in vitro* versus *in vivo* replication.

Materials and Methods

Fly strains and husbandry

Flies were maintained at 25 °C in standard fly food. Eggs were treated with bleach and, subsequently, flies were treated with tetracycline for two fly generations, as described before (43). Absence of Nora virus, Drosophila C virus, Drosophila X virus and Cricket paralysis virus was confirmed by RT-PCR using random hexamers for cDNA synthesis and the following primers for PCR:

NoraFor ATGGCGCCAGTTAGTGCAGACCT;

NoraRev CCTGTTGTTCCAGTTGGGTTCGA;

DCVFor AAAATTTCGTTTTAGCCCAGAA;

DCVRev TTGGTTGTACGTCAAAATCTGAG;

DXVFor TCGGAAGAACCAAAAGGATG;

DXVRev GTCCTCTCCACGCACTCTTC;

CrPVFor ACGAGGAAGCAACTCAAGGA;

CrPVRev GAGCCCGCTGAGATGTAAAG.

Absence of *Wolbachia* in the fly stocks was confirmed by PCR using primers wspFor TTTGCAAGTGAAACAGAAGG, wspRev GCTTTGCTGGCAAAATGG. Isogenized *Dicer-2-KO (Dcr-2^{L811fsX})* have been described before (27). Flies overexpressing *Dicer-2* were generated by crossing *UAS-Dicer-2* virgin females (VDRC ID 60008, (44)) with *Tubulin-Gal4*/*TM3 Sb* male flies and flies lacking the *Sb* marker were selected from the F1 offspring. *w^{1118}* flies were used as wild-type. The flies used for the serial passage and the RNA extractions were 2-5 day-old females.

Virus stock and titration

The parental DCV stock (Charolles strain) was produced in *Drosophila* S2 cells, which were maintained in Schneider's medium supplemented with 10% fetal calf serum and 50 U/ml penicillin

and 50 µg/ml streptomycin (Gibco) medium at 27 °C. The stock was bottlenecked through serial dilutions, by selecting the highest dilution that still showed cytopathic effects. Subsequently, a T25 flask of confluent S2 cells containing five ml of medium was infected with the bottlenecked virus. After 72 hours, the supernatant was collected, centrifuged at 300 *g* for 5 min, aliquoted, and stored at -80 °C. Titers were determined by end-point dilution in 96-well plates, as described before (43), calculated with the Reed-Muench method, and expressed as median tissue culture infectious dose ($TCID_{50}$).

Experimental evolution

Females of each genotype were intrathoracically inoculated with 10,000 $TCID_{50}$ of DCV in 50 nl of 10 mM Tris-HCl, pH 7.3 using a Nanoject II microinjector (Drummond Scientific). For each of the five replicate lineages per genotype, forty flies were inoculated. For the first passage, flies were inoculated with the parental stock and from P1 onwards, all 15 lineages were kept independently. At each passage of the experimental evolution experiment, flies were harvested for preparation of virus stocks and RNA isolation. Pools of 10 flies were homogenized in 220 µl of PBS, using 1 mm silica beads in a Precellys homogenizer two times for 10 seconds, centrifuged at 16,000 *g* for 10 min to discard fly debris, and the supernatant was transferred to fresh tube, aliquoted, stored at -80°C, and titrated. Pools of five flies were homogenized in 1 ml of RNA-Solv (Omega) using 1 mm silica beads in a Precellys homogenizer for RNA extraction and NGS library preparation.

Virus infections and survival assays

To study virulence, 10,000 $TCID_{50}$ of all lineages from P1, P5, P10 and the parental DCV stock were injected separately into 35 female w^{1118} flies. Survival was checked daily and the curves were calculated using the Kaplan-Meier estimator. Early replication was assessed after inoculation of 2-5 day-old female w^{1118} 1000 $TCID_{50}$ of all lineages of P1, P10 or the parental

stock. Three pools of five flies per lineage were collected at 12 hpi and processed for RT-qPCR. For the oral infections, 30 female w^{1118} were inoculated with 1000 $TCID_{50}$ by intrathoracic injection, placed in tubes for three days, after which these flies were discarded and 30 naive female w^{1118} were added to the contaminated vial. Three pools of five flies were collected at 48 hpi and processed for RT-qPCR.

To analyze replication of the different capsid variants, P10 viruses lineages with the variants fixated at a frequency ≥ 90% were bottlenecked by selecting the last well showing cytopathic effect of and end-point dilution, titrated, and Sanger sequenced to confirm the presence of the mutation. 100 w^{1118} female flies were inoculated with 100 $TCID_{50}$ of each viral stock, and three pools of five flies per variant were collected at the given time points and processed for RT-qPCR.

RT-qPCR

Total RNA was extracted, reverse transcribed using the TaqMan reverse transcription kit (Thermo Fisher) using oligo (dT) as a primer and quantitative PCR analysis was performed using the GoTaq qPCR SYBR mastermix (Promega) according to the manufacturers' recommendations on a LightCycler 480 instrument (Roche). DCV was quantified using primers DCV For TCAAGAAAAGTTGCGTGGGT and DCVRev CAGAGCGTCCTTGGAGAGTG, and expression was normalized to expression of *Ribosomal protein 49*, amplified using primers Rp49For ATGACCATCCGCCCAGCATAC and Rp49Rev CTGCATGAGCAGGACCTCCA. A standard curve of 10-fold dilutions of plasmid pAc5.1-V5-His-A (Invitrogen) containing the DCV ORF-2 sequence was used to convert Ct values to genome copy numbers.

RNA extraction, cDNA synthesis and NGS library preparation

Total RNA was isolated using RNA-Solv (Omega Bio-tek), and reverse transcribed using Superscript IV (Thermo fisher) and oligo (dT) as a primer, following the manufacturers'

instructions. Viral cDNA was PCR amplified using CloneAmp HiFi (TaKaRa) in four overlapping amplicons of about 2.2 kb, using the following primers:

For1 TGTACACACGGCTTTTAGGTAGA;

Rev1 GGAAAAGTGTTGCAAGAGCGA;

For2 TGTTCTTCGGGAAATGGGGA;

Rev2 TGCCTGTCCCACACGAATAG;

For3 GTTACGCGTGTCCTTTGACG;

Rev3 AACTTCCGTACCAACGCTCA;

For4 AAAGTTGCGTGGGTTTGTGG;

Rev4 CGTGTAAGCAGGGCAGATAGT).

Amplicons were purified with the NucleoSpin kit (MACHEREY-NAGEL), sheared in a Bioruptor Pico (Diagenode) by 12 cycles of 30 sec of sonication and 30 sec of cooling in 1.5 ml Bioruptor tubes (Diagenode), mixed in equimolar proportions, and used to prepare the library with the Next ultra II DNA library prep kit (E7645, NEB) using 1 µg of RNA per sample as input. Samples were multiplexed with barcodes (E7335, E7500, E7710, E7730, NEB), and the libraries were sequenced on an Illumina HiSeq 4000 sequencer as paired-end 100 base reads by the GenomEast platform, a member of the 'France Genomique' consortium. Image analysis and base calling were performed using RTA version 2.7.7 and bcl2fastq version 2.20.0.422.

NGS data processing

NGS data were analyzed using a custom computational workflow (Supplementary Figure 6). For processing the raw read data, the bioinformatics pipeline V-pipe (45) was integrated into our workflow. BWA-MEM (46) was used to align reads from the parental stock to the DCV EB reference strain that contains UTR sequences (GenBank accession number: NC_001834.1). Bcftools was used to generate a consensus sequence from the parental sequence, which was then used to align the raw reads from the evolutionary samples from passage 1, 5, and 10 using

BWA-MEM. Mutations with respect to the parental stock consensus sequence were called using ShoRAH (47) with default parameters (posterior threshold of 0.9, alpha 0.1 and shift 3). The posterior threshold is chosen conservative to make sure only mutations of high confidence are included in the subsequent analysis. Moreover, we applied the Strand Bias Filter (48) to filter out further variation of low confidence. Phred sequencing quality scores for mutation calls were accessed using pysamstats (https://github.com/alimanfoo/pysamstats). Further, to evaluate the differences of our parental stock to its ancestor, the Charolles strain, the parental stock sample was processed with respect to the Charolles reference sequence (GenBank Accession Number: MK645242.1). An adapted version of vcf_annotator (https://github.com/rpetit3/vcf-annotator) was used to annotate non-synonymous mutations. Population nucleotide diversity and number of polymorphisms were computed considering only mutations with a minimum frequency of 0.0001. SNPGenie's within-pool analysis was used in the default settings and minimum frequency 0.0001 (49) to compute the population nucleotide diversity per synonymous (piS) and non-synonymous (piN) site.

Permutation test

A permutation test as described before (50) was used to test for host genotype-specific adaptation. Mutations that reached the frequency threshold of 0.0001 in at least one evolutionary line were considered for this analysis. For each mutation, the number of lineages containing the mutation were counted. Then, each mutation occurrence was randomly reallocated to a lineage by using the number of mutations that occurred in each lineage as weights. In this manner, 1000 samples were drawn. The observed number of mutations that were unique or shared among genotypes was then compared to the number expected from the permutation samples, and a two-sided test was used to assess the difference. For each category, *p*-values were computed as $p =$

$1 - 2\left|\frac{1}{n_s}\sum_{i=1}^{n_s} \quad 1_{\{x_i > x_{obs}\}} - 0.5\right|$ where n_s is the number of samples drawn, x_i is the i-th random realization and x_{abs} is the observed number of mutations in the respective category.

Randomization test

A randomization test was performed to test whether the observed number of FF encoded as 'TTTTTT' was different from the expected number based on random mixing of the TTT and TTC codons. For each viral genome, we counted the total number of FF occurrences, FF encoded by TTTTTT (n_{TTTTTT}), and the occurrences of the TTC and TTT codon were counted. For each viral genome, $n_s = 1000$ samples were drawn, by randomly pairing the TTC and TTT codons based on their codon frequencies. To test whether the number TTTTTT encodings from the randomization exceeds the observed number, a two-sided test was performed. *P*-values were computed as $p = 1 - 2\left|\frac{1}{n_s}\sum_{i=1}^{n_s} \quad 1_{\{x_i > n_{TTTTTT}\}} - 0.5\right|$ where x_i denotes the number of TTTTTT realizations in the i-th randomization.

Quality analysis of deletion calls

To evaluate the quality of reads supporting the two uridine deletions at positions 276-284 and 5765-5774, an additional quality control was performed on the alignment files of all samples. The mean position-wise base calling quality scores were assessed in the homopolymeric regions of the two deletions and the values were compared between reads with deletions to the values of reads without the deletions. Next, the location of the deletions on the reads containing them were evaluated. In particular, we quantified the proportion of reads where the deletion track was located within a range of five nucleotides from the read edges.

Capsid structural modeling

In the absence of structural information of the DCV capsid protein, the crystal structure of the capsid of cricket paralysis virus (CrPV; PDB: 1B35) was used to model the impact of mutations on capsid structure. CrPV capsid is a large assembly of 240 subunits, containing copies of four proteins- VP1, VP2, VP3 and VP4. Mutations of interest, located at sites 92 and 95 in VP3, were modeled for all VP3 subunits in the capsid assembly. Structural models were constructed for each mutation using the functions RepairPDB and BuildModel from the FoldX suite (v.5.0) with five iterations of sidechain rotamer adjustments. For each mutant constructed, the average differences in capsid structural stabilities, i.e., free energy of protein folding (kcal/mol), between the parental amino acid and the mutants were calculated. To assess sequence conservation of the residues at positions 92 and 95 in the DCV capsid polyprotein (NP_044946.1), a multiple sequence alignment of closely related homologues (>50% sequence identity), identified using a BLAST search, was generated. These homologs included proteins from Warroolaba Creek virus (QIJ25856.1), Rondani's wasp virus 1 (QXL90737.1), Cricket paralysis virus (QXV86620.1), Hypsignathus monstrous dicistrovirus (AZR39356.1), Millport beadlet anemone dicistro-like virus 1 (ASM93983.1), Maanshan Dicis tick virus 1 (UYL95313.1), Nilaparvata lugens C virus (AIY53986.1), Arma chinensis virus 1 (UVJ69137.1), Caledonia beadlet anemone dicistro-like virus 2 (ASM93985.1), Anopheles C virus (YP_009252205.1), Guiyang dicistrovirus 2 (UHK03031.1), Apis dicistrovirus (YP_009388500.1), as well as other, uncharacterized members of the *Cripavirus* genus (ULF99862.1) and the *Dicistroviridae* family (QJI52080.1, AKA63264.1, URG14534.1, ULF99814.1).

Statistical tests

Two-sided *t*-test on log10-transformed DCV titers for each passage to test for significant pairwise differences among the viral titers in the different genotypes. The mean survival time of WT flies upon infection with virus populations from the different passages were compared across passages using the paired two-sided *t*-test. Mortality of WT flies infected with P10 viruses and the

parental stock viruses was compared using the log-rank test. For each genotype, the viral RNA levels were compared to the parental stock using the lower-tailed paired *t*-test with a significance level of 0.01. Viral RNA levels at passage 10 were compared using Tukey's HSD test for multiple comparison. For each genotype, the increase of polymorphic sites across passages was tested for significance using the paired two-sided *t*-test. To compare differences in the number of polymorphic sites among the host genotypes at passage 10, two-sided *t*-tests were used. To test for significant changes in viral nucleotide diversity within passages and between fly genotypes, a mixed ANOVA was used with passages 1, 5 and 10 as within-subject factor and fly genotype as between-subject factor. To compare the mean nucleotide diversity between coding and non-coding regions, a mixed ANOVA was used with passages as the within-subject factor and coding or non-coding region as the between-subject factor. Two-sided paired t-tests were used to compare piN and piS values per gene, passage and genotype. Two-sided *t*-tests were used to compare viral RNA loads between the different VP3 variants at 96 hpi in S2 cells and WT flies.

Data availability

All raw NGS data produced during the experimental evolution study has been deposited in NCBI BioProject under the accession number PRJNA993483.

Code availability

The data processing pipeline can be found at https://github.com/cbg-ethz/DCV-Evolution-Data-Processing.

Acknowledgements

We thank members of the laboratory for discussions and Carla Saleh (Pasteur Institute) for providing *Dicer-2* knockout flies. This work was financially supported by the European Union's

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Figure legends

Figure 1. Experimental evolution of DCV in flies differing in their RNAi response. (**A**) A parental stock of DCV Charolles strain was serially passaged in wild-type flies (WT), *Dicer-2* knockout flies (*Dcr-2-KO*), and flies overexpressing *Dicer-2* (*Dcr-2-OE*), with five biological replicates per genotype, during ten fly generations. Flies were inoculated by intrathoracic injections and titers were determined for every passage. Evolved virus populations were characterized phenotypically using growth curves and survival assays and genotypically by next-generation sequencing. (**B**) Survival curves of WT, *Dcr-2-KO* and *Dcr-2-OE* flies inoculated with 1,000 $TCID_{50}$ of DCV. Two to five-day-old female WT flies were inoculated intrathoracically (n = 30) with the parental stock and survival was monitored daily. (**C**) Viral titers of all lineages at each viral passage. $TCID_{50}$, median tissue culture infectious dose. Bars represent means and SD of the five viral lineages. Asterisks indicate $p < 0.05$, t-test.

Figure 2. Increased virulence of evolved virus populations independent of the host genotype. (**A**–**C**) Survival curves of WT flies inoculated with 10,000 $TCID_{50}$ of the parental DCV stock and virus populations at passage 1, 5 or 10 from the indicated genotypes. Two to five-day-old female WT flies (n = 30) were inoculated intrathoracically and survival was monitored daily. Results are shown as means of the host survival for all five lineages for virus populations from WT (**A**), *Dcr-2-OE* (**B**) and *Dcr-2-KO* flies (**C**). (**D**) Difference of the mean survival time for each virus stock relative to the parental stock. Dashed lines connect the values of each replicate. Asterisks indicate $p < 0.05$, paired *t*-test. (**E**) Early replication kinetics of parental and evolved virus populations. 1,000 $TCID_{50}$ of each virus stock were injected into WT flies and three groups of five flies each were collected at 12 hours post infection (hpi). Viral RNA copy numbers were quantified by RT-qPCR. The dashed line shows the RNA copies of the parental stock (P0). Results are shown as means and SD of three replicates. Viral RNA loads were compared to the

load of the parental stock with a paired *t*-test. Viral RNA levels at passage 10 were compared using Tukey's HSD test for multiple comparison. *P*-values are indicated with asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

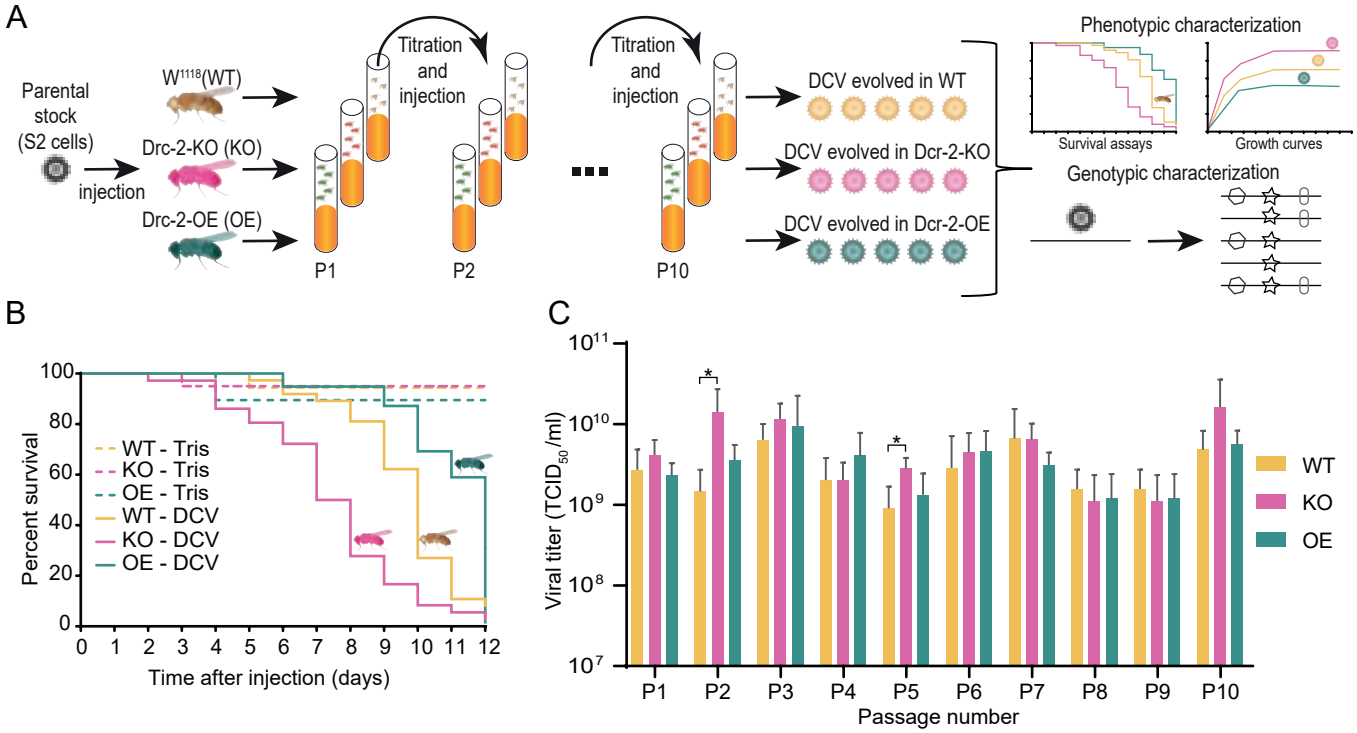
Figure 3. Increasing genetic variation during serial passage. (A) Number of polymorphic sites of the virus populations from the three host genotypes across passages. Data are shown as means and SEM across the five lineages for each genotype. The dashed line marks the number of polymorphic sites in the parental stock. **(B)** Frequencies of single-nucleotide variants (SNVs) in the indicated bins for virus populations in WT, *Dicer-2-OE* and *Dicer-2-KO* flies. Error bars indicate 95% confidence intervals based on the bootstrap distribution. **(C–D)** Venn diagram of the number of unique and shared SNVs between virus populations from WT, *Dicer-2-OE* and *Dicer-2-KO* flies. Per genotype, SNVs from all replicates and passages with a minimum observed frequency of 0.01% (C) or 10% (D) were combined. The ratios in (C) indicate the number of non-synonymous mutations to all mutations. Asterisks in (D) indicate non-synonymous mutations.

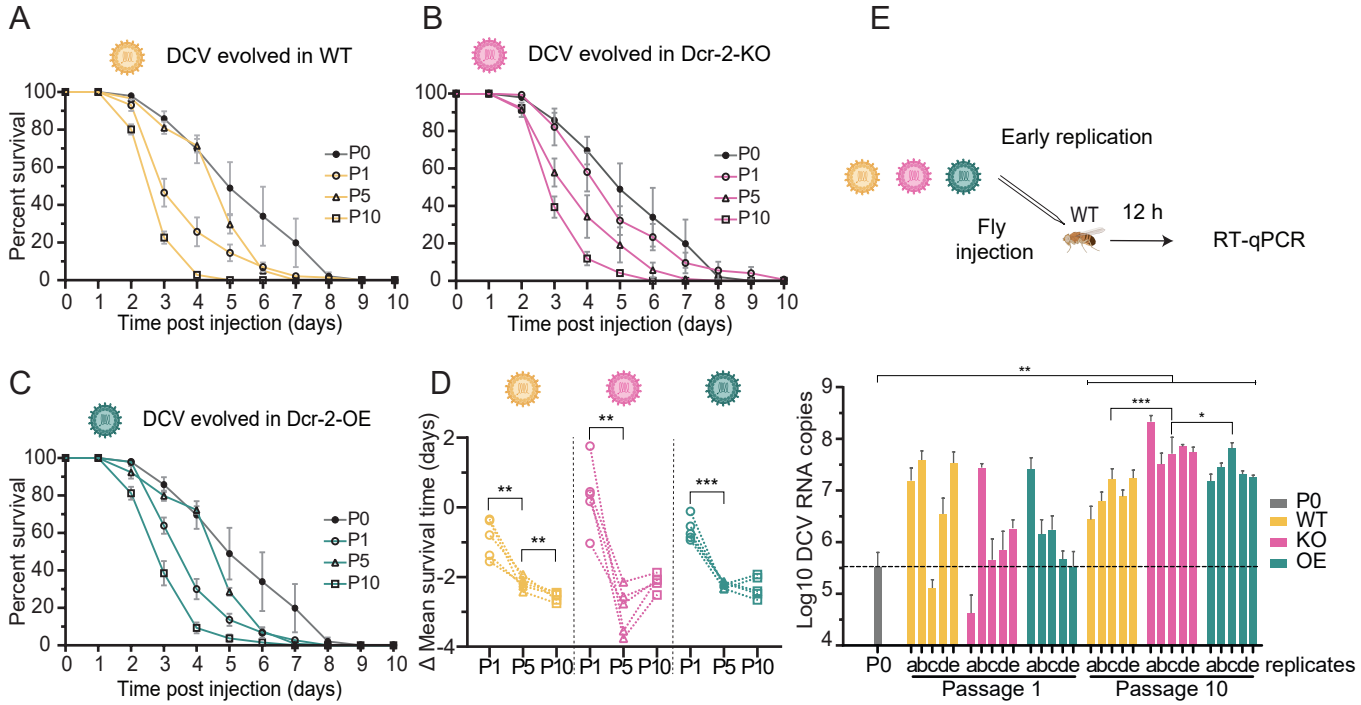
Figure 4. Parallel evolution of virus lineages. Heatmap of SNV frequencies of all SNVs **(A)** or non-synonymous SNVs **(B)** for virus populations from WT, *Dicer-2-OE* and *Dicer-2-KO* flies. The x-axis represents genome positions relative to the DCV EB reference strain (NC_001834.1). Only SNVs that occurred in at least one sample with an observed frequency ≥ 0.01 were taken into account. Deletions were excluded from the analysis in panel (B). Schematic representations of the DCV genome are shown below the heatmaps.

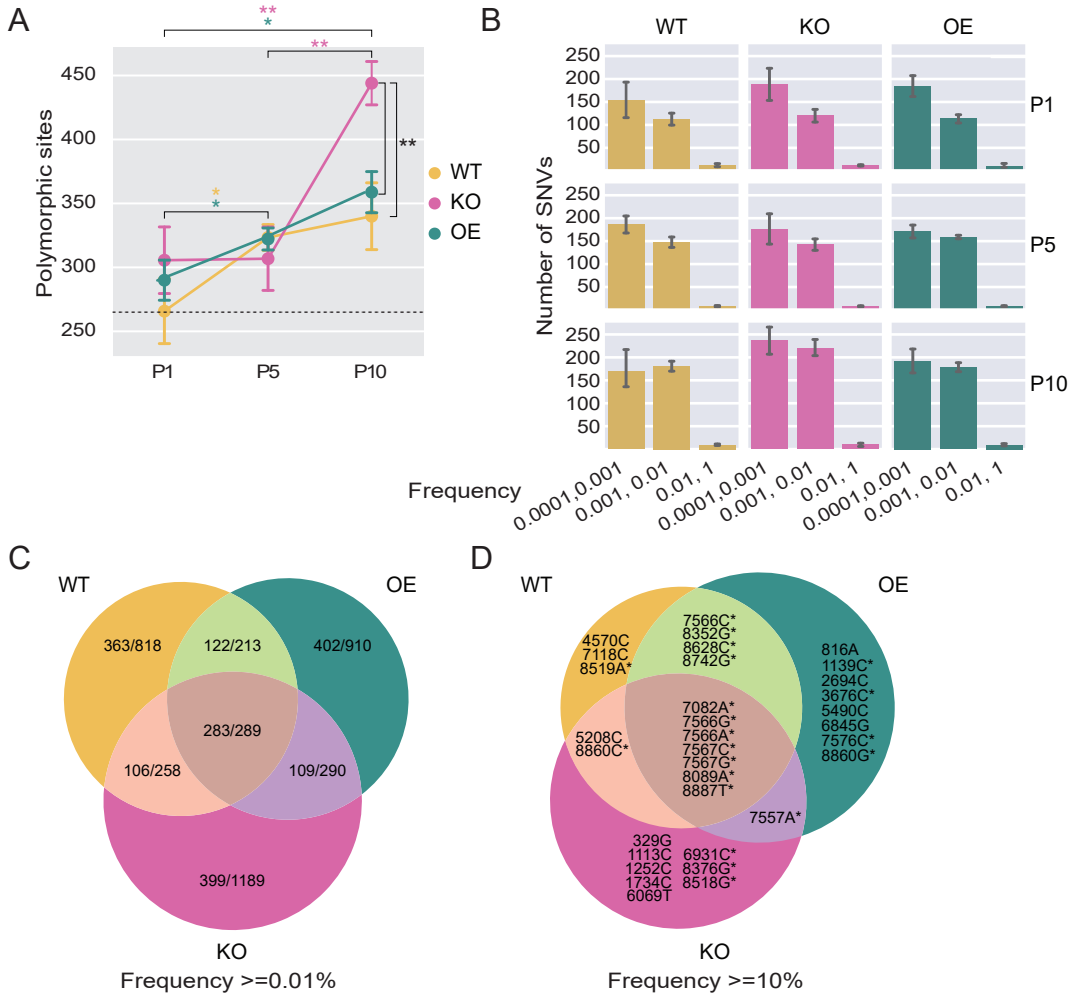
Figure 5. A capsid mutation increases *in vivo* viral fitness. (A) Polymorphism frequency of the codon coding for amino acid 95 of VP3 in DCV populations obtained during experimental evolution in the indicated genotype and lineage. UAU represents the parental codon. One variant retained the 95Y residue, but contained a substitution in its vicinity (P92T). **(B)** Growth curves of

individual variants in WT flies after inoculation with 100 $TCID_{50}$ of each variant. Three pools of five flies were collected per time point for viral RNA quantification by RT-qPCR. Viral RNA levels are normalized to the 0 hpi time point and presented as geometric mean and SD. **(C)** Growth curves of individual variants in S2 cells infected at MOI of 0.1. Three wells were collected per time point for viral RNA quantification by RT-qPCR. Viral RNA levels are normalized to the 0 hpi time point and presented as geometric mean and SD. Differences between the parental and evolved lineages RNA levels were analyzed by a paired *t*-test. *P*-values are indicated with asterisks: **, $p < 0.05$; ***, $p < 0.001$*

Figure 6. Structural analysis of mutations in the capsid. (A) The complete structure of CrPV capsid assembly (PDB: 1B35) is illustrated with VP1 (blue), VP2 (green), VP3 (yellow) and VP4 (red). For simplicity, a five-fold face of the capsid is shown in cartoon representation with Tyr95 and Pro92 highlighted as spheres. **(B)** Multiple sequence alignment of the homologues closely related to VP3 of the DCV capsid is shown for the region of interest alone. The accession numbers of the homologues are followed by their respective sequence identities shared with the DCV reference sequence (NP_044946.1). Completely conserved residues are highlighted in red blocks, while columns with relatively poor conservation have no annotation. **(C)** The amino acids surrounding parental Y95 and P92 within a distance of 6Å are shown in sticks, which are located in a solvent-exposed loop connecting two beta strands. **(D)** A similar view shows S95 in place of Y95, typically found in DCV reference sequences in databases. **(E)** A marginally stabilizing mutant P92T (-1.79 kcal/mol) is shown. Proximity of the loop to VP2 (green) at a distance of 4.7Å is depicted in panels (C–E).


A
Parental stock (S2 cells)
injection
W1118(WT)
Drc-2-KO (KO)
Drc-2-OE (OE)
Titration and injection
P1
P2
P10
DCV evolved in WT
DCV evolved in Dcr-2-KO
DCV evolved in Dcr-2-OE
Phenotypic characterization
Survival assays
Growth curves
Genotypic characterization
B
Percent survival
Time after injection (days)
WT - Tris
KO - Tris
OE - Tris
WT - DCV
KO - DCV
OE - DCV
C
Viral titer (TCID50/ml)
Passage number
WT
KO
OE


A
DCV evolved in WT
Percent survival
Time post injection (days)
P0
P1
P5
P10
B
DCV evolved in Dcr-2-KO
Percent survival
Time post injection (days)
P0
P1
P5
P10
E
Early replication
Fly injection
WT
12 h
RT-qPCR
C
DCV evolved in Dcr-2-OE
Percent survival
Time post injection (days)
P0
P1
P5
P10
D
Δ Mean survival time (days)
P1 P5 P10 P1 P5 P10 P1 P5 P10
Log10 DCV RNA copies
P0
WT
KO
OE
P0 abcde abcde abcde abcde abcde abcde replicates
Passage 1
Passage 10


A
Polymorphic sites
450
400
350
300
250
P1
P5
P10
WT
KO
OE
B
WT
KO
OE
Number of SNVs
P1
P5
P10
Frequency
0.0001,0.001
0.001, 0.01
0.01, 1
C
WT
OE
363/818
122/213
402/910
283/289
106/258
109/290
399/1189
KO
Frequency >=0.01%
D
WT
OE
4570C
7118C
8519A*
7566C*
8352G*
8628C*
8742G*
816A
1139C*
2694C
3676C*
5490C
6845G
7576C*
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8860C*
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7567G*
8089A*
8887T*
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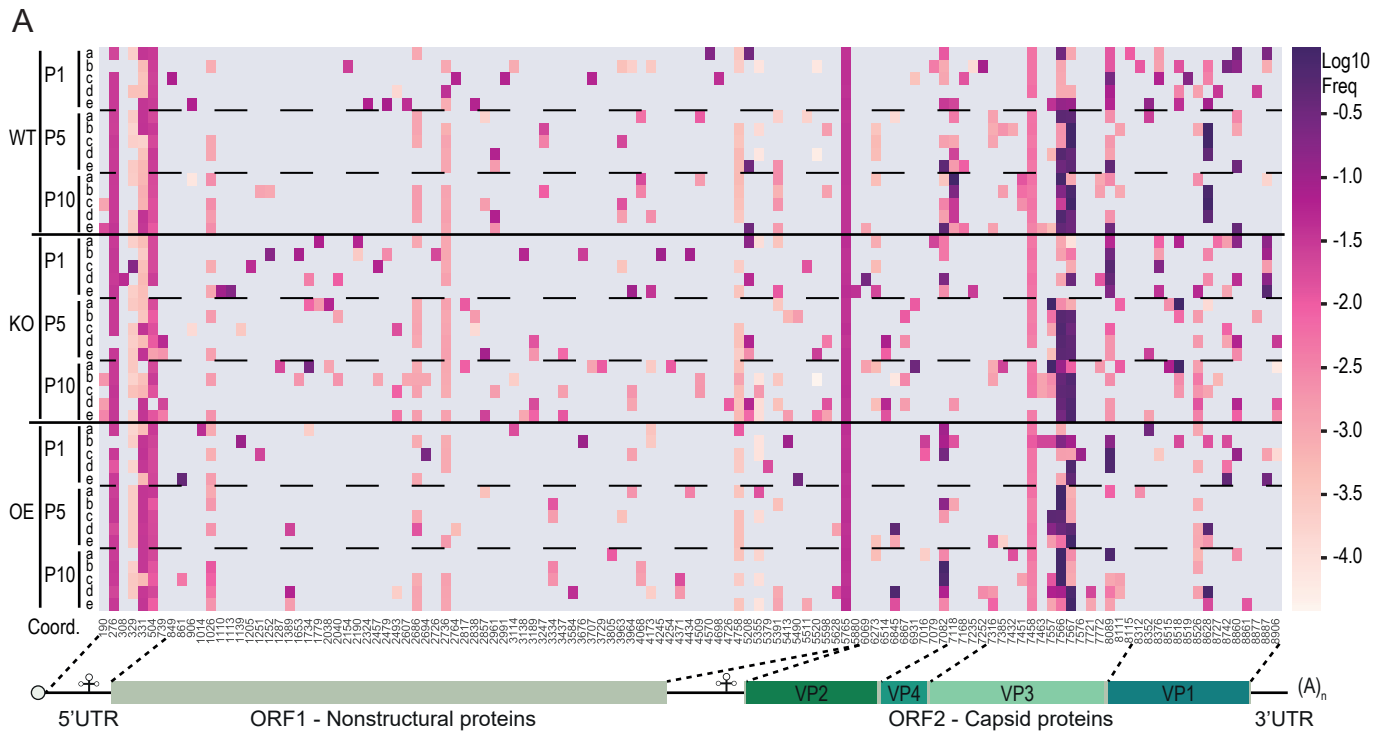
8518G*
KO
Frequency >=10%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WT
KO
OE
P1
P5
P10
a
b
c
d
e
Log10
Freq
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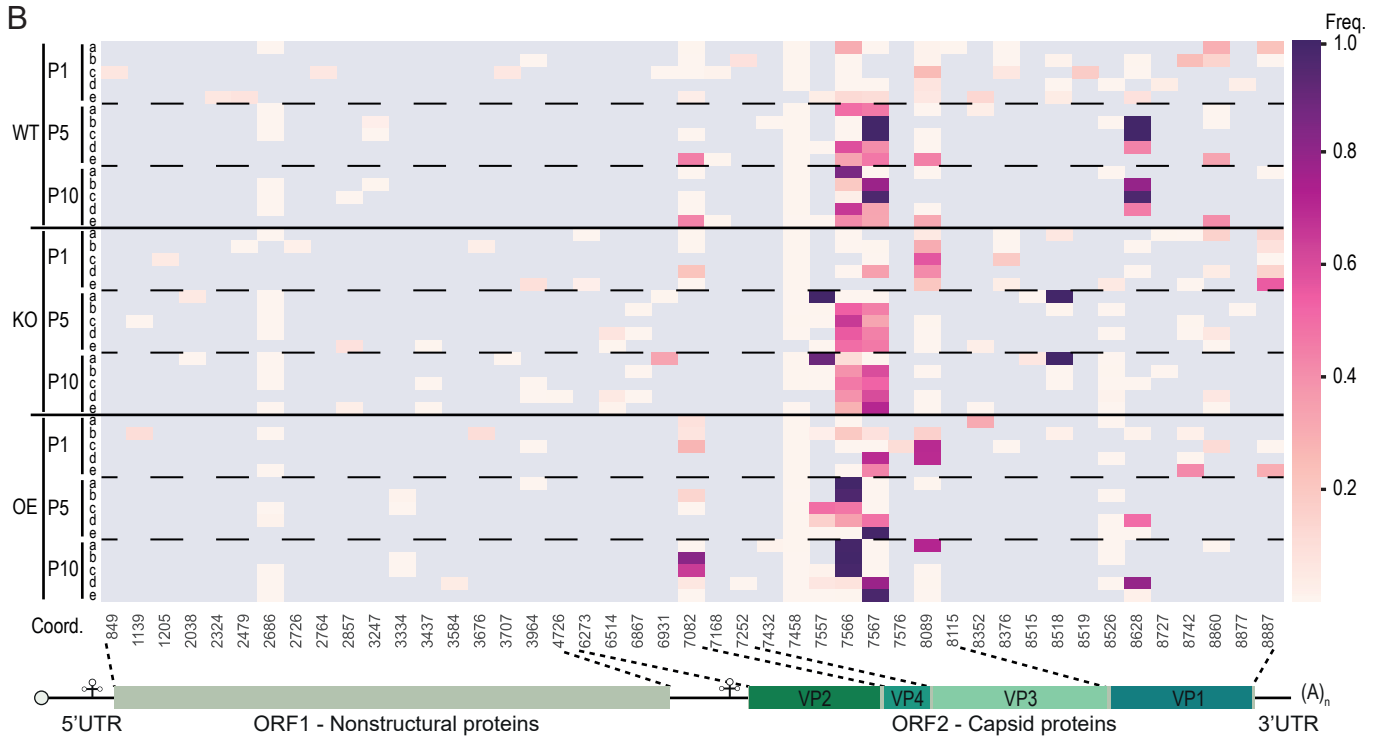
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
Coord.
5'UTR
ORF1 - Nonstructural proteins
VP2
VP4
VP3
VP1
ORF2 - Capsid proteins
3'UTR
(A)n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P1
P5
P10
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b
c
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e
Freq.
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0.4
0.2
Coord.
849
1139
1205
2038
2324
2479
2686
2726
2764
2857
3247
3334
3437
3584
3676
3707
3964
4726
6273
6514
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6931
7082
7168
7252
7432
7458
7557
7566
7567
7576
8089
8115
8352
8376
8515
8518
8519
8526
8628
8727
8742
8860
8877
8887
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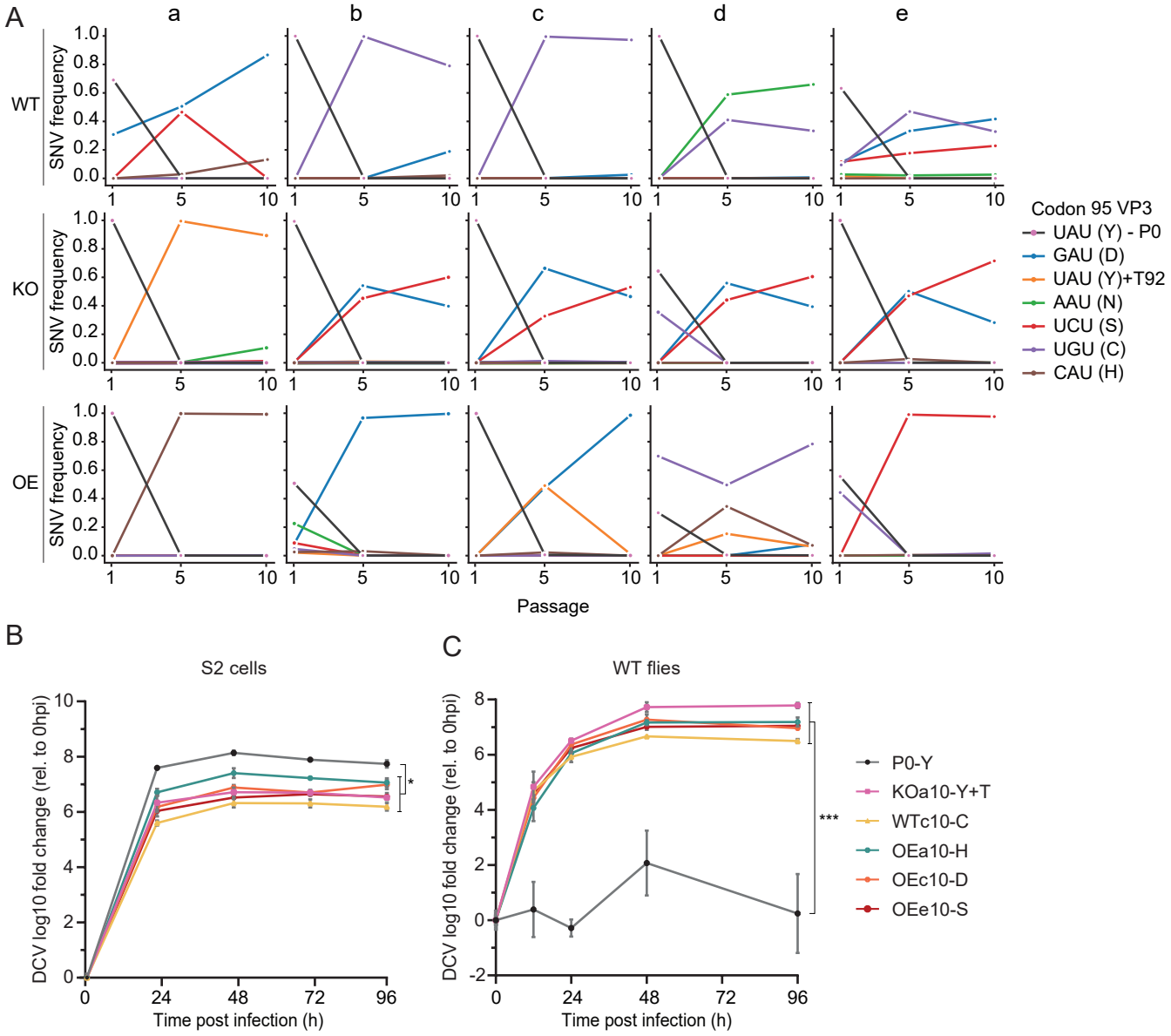
ORF1 - Nonstructural proteins
VP2
VP4
VP3
VP1
ORF2 - Capsid proteins
3'UTR
(A)n


A
a
b
c
d
e
WT
KO
OE
SNV frequency
Passage
Codon 95 VP3
UAU (Y) - P0
GAU (D)
UAU (Y)+T92
AAU (N)
UCU (S)
UGU (C)
CAU (H)
B
S2 cells
DCV log10 fold change (rel. to 0hpi)
Time post infection (h)
C
WT flies
P0-Y
KOa10-Y+T
WTc10-C
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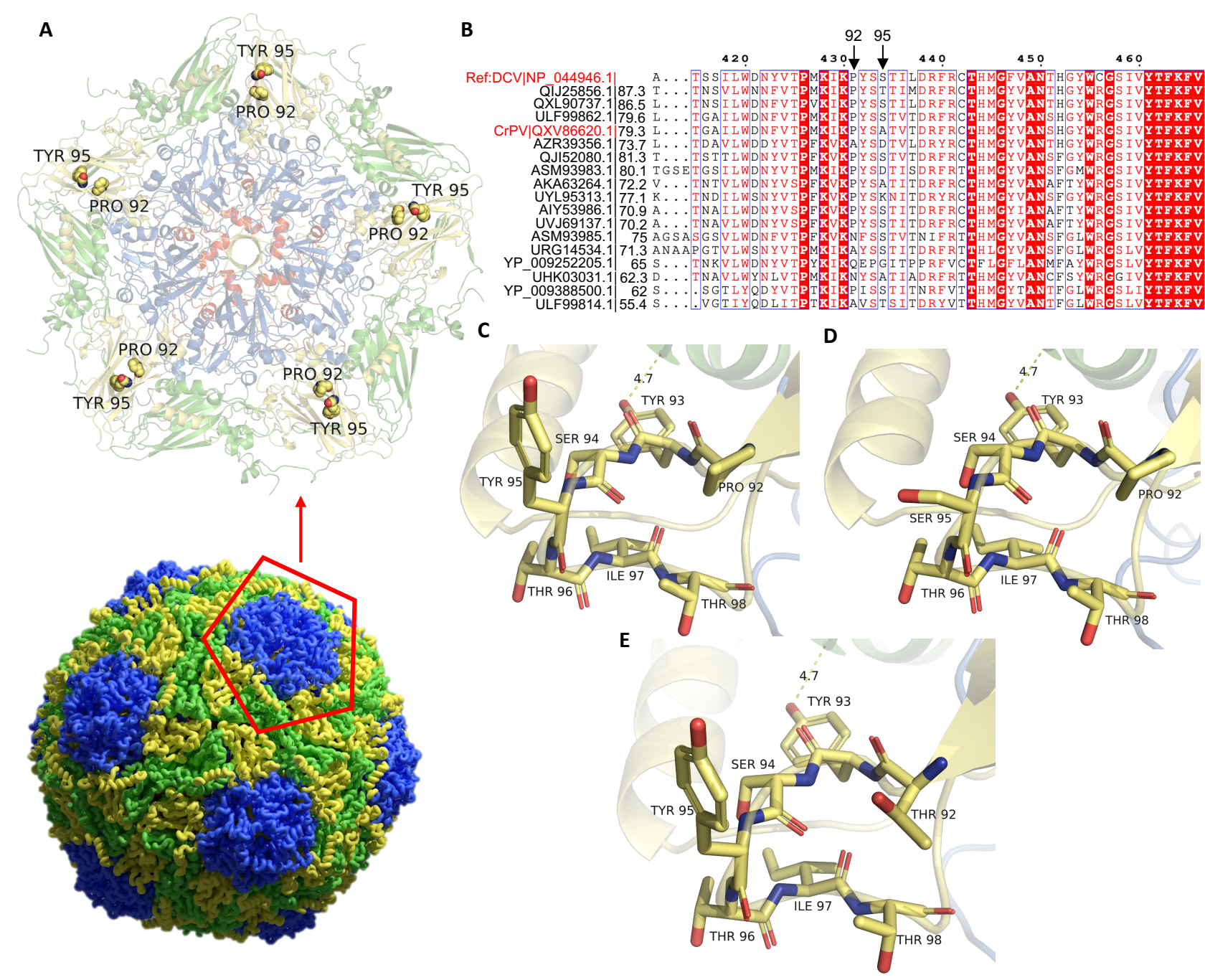
OEe10-S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THR 96
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