LETTERS

Discovery of insect and human dengue virus host factors

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Dengue fever is the most frequent arthropod-borne viral disease of humans, with almost half of the world's population at risk of infection[1]. The high prevalence, lack of an effective vaccine, and absence of specific treatment conspire to make dengue fever a global public health threat[1,2]. Given their compact genomes, dengue viruses (DENV-1–4) and other flaviviruses probably require an extensive number of host factors; however, only a limited number of human, and an even smaller number of insect host factors, have been identified[3–10]. Here we identify insect host factors required for DENV-2 propagation, by carrying out a genome-wide RNA interference screen in *Drosophila melanogaster* cells using a well-established 22,632 double-stranded RNA library. This screen identified 116 candidate dengue virus host factors (DVHFs). Although some were previously associated with flaviviruses (for example, V-ATPases and α-glucosidases)[3–5,7,9,10], most of the DVHFs were newly implicated in dengue virus propagation. The dipteran DVHFs had 82 readily recognizable human homologues and, using a targeted short-interfering-RNA screen, we showed that 42 of these are human DVHFs. This indicates notable conservation of required factors between dipteran and human hosts. This work suggests new approaches to control infection in the insect vector and the mammalian host.

DENV-1–4 are transmitted from one human host to another by mosquitoes of the *Aedes* genus, principally *Aedes aegypti* and *albopictus*[2]. Although there have been important efforts to sequence and annotate the genomes of these vectors[11–13], there is at present an unfortunate dearth of resources to carry out systematic functional genomics in *Aedes*. In contrast, there are robust materials and methods to do so in the related dipteran *Drosophila melanogaster*. To take advantage of these existing tools, DENV-2 New Guinea C (DEN2-NGC) was adapted by serial passage in D.Mel-2 cells, which are derived from S2 cells, over a period of 4 months (DEN2-S2; Supplementary Fig. 2). To identify DVHFs required for efficient propagation of DENV-2 in insect cells, we carried out a genome-wide RNA interference (RNAi) screen in *Drosophila* D.Mel-2 cells using the 22,632-double-stranded RNA (dsRNA) DRSC 2.0 library, designed and provided by the *Drosophila* RNAi Screening Center (www.flyrnai.org)[14]. The screen was performed in duplicate, assaying 45,264 infections, excluding controls. If either duplicate resulted in fewer than 12,500 cells per well, the dsRNA was excluded from further analysis—a criterion that excluded 2,343 dsRNAs. The remaining 20,224 dsRNAs were scored by their effect on infection, which was determined by measuring expression of envelope protein (Supplementary Fig. 3). Each pair of duplicate dsRNA was assigned a sum-rank score and those with scores expected with a frequency ≤ 0.065 by chance alone were selected for further analysis (Supplementary Fig. 4 and Methods). Of the 218 (1.1%) that met this criterion, we were able to readily resynthesize and rescreen 179 dsRNAs. We identified 118 dsRNAs, representing 116 unique DVHFs that inhibited infectivity by ≥ 1.5-fold with $P < 0.05$ (Supplementary Table 1).

The screen identified DVHFs previously known to be required for dengue and/or other flaviviral infections, such as an α-glucosidase and the V-ATPase proton pump (CG14476, VhaPPA1-1 and Vha14 in Fig. 1b, c)[3–5,9,10]. The effect on the V0 and V1 subunits of the V-ATPase provided strong evidence of a requirement for the holoenzyme. To obtain independent evidence for this, we tested the effect of bafilomycin, a specific V-ATPase inhibitor previously shown to inhibit flaviviruses[3,7,9], on DENV infection of C6/36 *Ae. albopictus* cells. Bafilomycin treatment induced a marked inhibition of both DEN2-S2 and DEN2-NGC replication in these mosquito cells (Supplementary Fig. 5). These data demonstrated the validity of the screen as they generalize the findings to a well-studied DENV2 and cells of the natural vector *Ae. albopictus*.

The vast majority of the DVHFs (111 out of 116) had not been previously identified as such. Extant annotation (FlyBase accession number FB2008_05) predicted diverse cellular functions for DVHFs. DnaJ-1 and CG3061 are predicted to be involved in the unfolded protein response, which is activated after DENV infection[15,16]. α-Adaptin, cnir, lqf, synaptogyrin, Syx4 and Syx13 are all involved in vesicular transport and endocytosis[17], which have been implicated in the entry and replication of a diverse group of viruses[18], including DENV[19]. The DVHF lqf interacts with the *Drosophila* homologue of human EPS15, which is required for West Nile Virus and DENV entry[19–21]. We posit that RNA-binding proteins, such as bol, Unr and CG5205, and the 3′–5′ exonuclease-like CG6744, assist in genome expression, replication and/or packaging (see later). Notably, the mosquito homologues of three DVHFs identified in our screen (pxb, H15 and Cyp6a19) were found to be differentially regulated after DENV infection in live mosquitoes[22].

Gene Ontology annotation (GATHER, www.gather.genome.duke.edu/) of DVHFs indicated a surprisingly high number (22) of nuclear proteins. Although DENV gene products are known to transit through the nucleus during the course of infection[23–26], it is also possible that DENV infection relocalizes many of these factors to the cytoplasm. Also notable was the large number of gene products predicted to be membrane-associated: 17 with the plasma membrane, and ten with intracellular membranes (endoplasmic reticulum, Golgi, vesicles and vacuole-like organelles) (Fig. 1d). The detection of many

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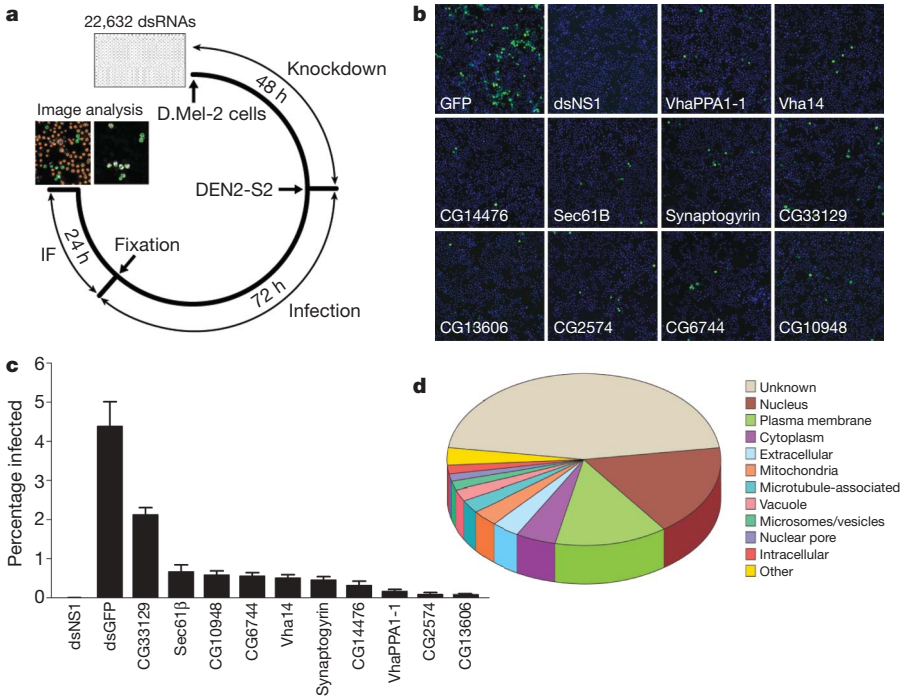

Figure 1 | Genome-wide RNAi screen for dipteran DVHFs. A total of 22,632 dsRNAs were assayed in duplicate for their effect on DEN2-S2 viral gene expression in D.Mel-2 cells. **a**, Schematic of the experimental protocol used in the screen. IF, immunofluorescence. **b**, Representative images of dsRNA-treated D.Mel-2 cells at ×20 magnification with nuclei staining (blue) and dengue E protein staining (green). **c**, The percentage of DEN2-S2-infected cells is indicated for controls and selected DVHFs. Error bars represent s.e.m. of ≥six independent observations. **d**, Cellular localization of the 116 DVHFs identified in the *Drosophila* screen according to Gene Ontology cellular component annotation (FlyBase accession FB2008_05).

membrane-bound gene products is fully consistent with the observations that viral infections cause remodelling of cellular membranes[27].

To test whether DVHFs were required for propagation of DENV in the vector mosquito *Ae. aegypti*, we tested the effect of depleting mosquito homologues of lola (NCBI accession AAEL009212), CG10320, a putative NADH dehydrogenase (AAEL007054), and Cyp6a19 (AAEL009124) using an established method of RNAi-mediated gene silencing[28]. A dsRNA targeting AAEL009212 reduced the DEN2-NGC capacity to infect the midgut tissue at 7 days after ingestion of infected blood (Fig. 2). The effect of DVHF gene silencing on DENV infection was probably underestimated because of an aberrantly low titre of one group of control green fluorescent protein (GFP)-dsRNA-injected mosquitoes (Fig. 2). A dsRNA targeting AAEL009124 did not affect infectivity. Although inhibition of infectivity after depletion of AAEL007054 was not statistically significant, exclusion of the point that appears to be an outlier leads to a reduction in infectivity that approaches significance and suggests that this gene product could be a DVHF in *Ae. aegypti* (Fig. 2). Given the complex spatio-temporal dynamics of DENV infection in the mosquito, the fact that the mosquitoes are genetically polymorphic, the inherent variability of blood meal infections[29], and the uncertainty of achieving gene product depletion in the appropriate tissue and time after dsRNA injection, it was remarkable to obtain inhibition with these DVHFs. These data, together with those obtained earlier with *Ae. albopictus* cells, validate the use of the *Drosophila* screen to identify dipteran DVHFs.

The 116 DVHFs had 82 readily identifiable human homologues, which we targeted with a library of short interfering RNAs (siRNAs; Fig. 3a). We supplemented the library with siRNAs targeting gene products that were functionally associated with the V-ATPase but had not scored as DVHFs in the D.Mel-2 screen—for instance, V-ATPase accessory proteins not found in insects. Of the 82 homologues of the dipteran DVHFs, 42 (51%) scored as human DVHFs (Fig. 3b, c and Supplementary Table 2). The notably high number of dipteran DVHFs that were also required for infection of human cells further validates the screen, and provides, to our knowledge, the first evidence for widespread conservation of flavivirus–host interactions between invertebrates and vertebrates (Supplementary Fig. 6 and Supplementary Table 3).

Knockdown of DVHFs in HuH-7 cells significantly reduced the formation of DEN2-NGC infectious particles (Fig. 3d). Knockdown of DVHFs predicted to be involved in entry, post-translational modifications and transcription accounted for most of those that resulted in greater than tenfold inhibition of viral propagation. To ascertain

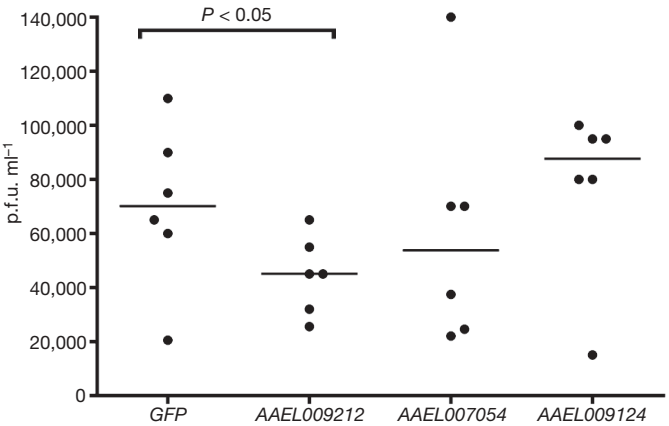

Figure 2 | Injection of *Ae. aegypti* mosquitoes with dsRNA targeting a DVHF inhibits dengue virus propagation. Four-day-old female mosquitoes were injected with dsRNAs targeting *GFP*, *AAEL009212*, *AAEL007054* or *AAEL009124*. Three days after injection, mosquitoes were fed on a DEN2-NGC-supplemented blood meal. Seven days later, 30 mosquitoes for each condition were randomly sorted into six groups of five, their midguts were removed, homogenized and titred. Data points indicate the combined titre of five dsRNA-treated mosquito midguts. Lines indicate the median value of the six biological replicates. Significance at < 0.05 level was determined using a one-sided Student's *t*-test of viral titres (dsDVHF versus dsGFP). p.f.u., plaque-forming units.

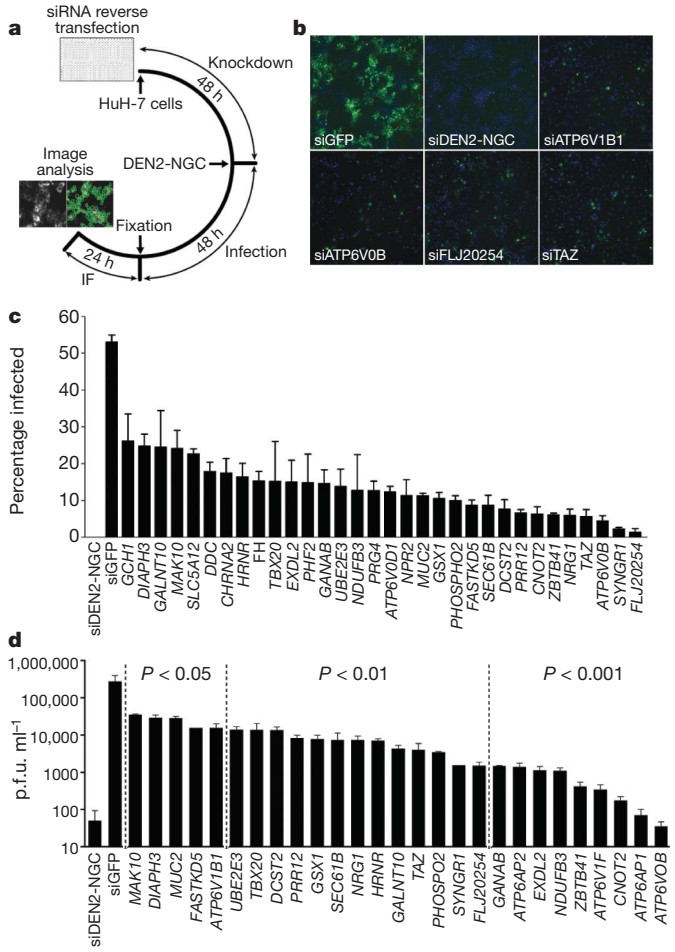

Figure 3 | Screen for human DVHFs. siRNAs targeting 82 human DVHF homologues were screened in HuH-7 cells for their ability to inhibit DEN2-NGC. **a**, Schematic of the experimental protocol. **b**, Representative images of siRNA-treated HuH-7 cells at ×10 magnification with nuclei staining (blue) and dengue E protein staining (green). **c**, The percentage of DEN2-NGC-infected cells is indicated for controls and selected DVHFs. **d**, Viral propagation after treatment with control or DVHF siRNAs was measured 72 h after DEN2-NGC infection and plotted on a logarithmic scale. Error bars in **c** and **d** indicate s.e.m. of three independent observations.

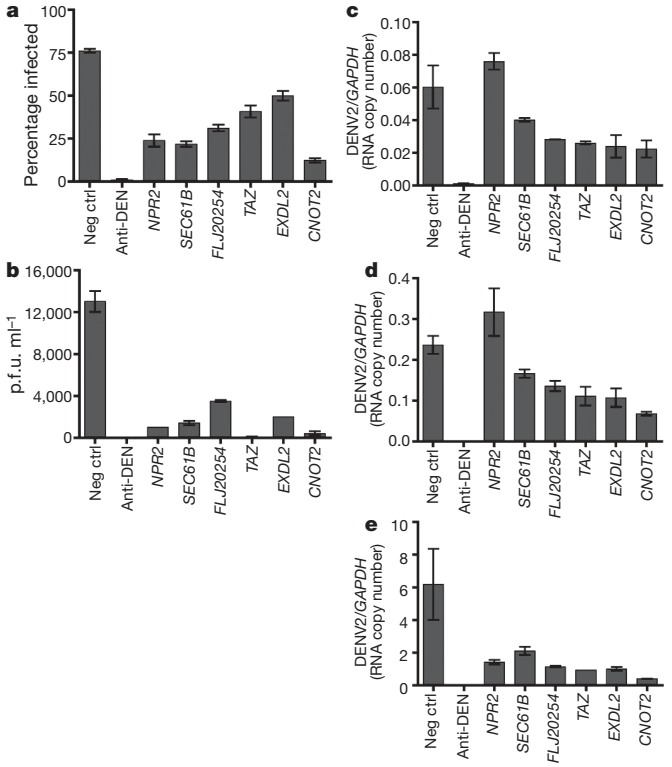

Figure 4 | Analysis of DENV RNA accumulation after DVHF knockdown. **a**, The percentage of DENV2-NGC-infected cells 48 h after infection (multiplicity of infection (MOI) ~ 1.4) is indicated for controls and for six DVHFs. Neg ctrl, negative control. Error bars represent the s.d. of six replicates. **b**, Viral propagation 48 h after infection was calculated for controls and six DVHFs. Error bars represent the range of duplicates. **c**–**e**, Viral RNA accumulation was measured by RT–qPCR at 18 h (**c**), 24 h (**d**) and 48 h (**e**) after infection, and normalized to *GAPDH*. Values represent the average of median qPCR measurements. Error bars represent the range of duplicates.

whether or not the DVHFs were required for other viral infections, we determined whether knockdown of DVHFs led to alterations in the gene expression for yellow fever virus (17D vaccine strain), another flavivirus, and Coxsackie B3 (strain 20; CB3), an enterovirus. Of six DVHFs that showed ≥twofold inhibition of DEN2-NGC E protein expression by at least two independent siRNAs, only one (FLJ20254) scored by the same criteria as required for yellow fever virus and three (CNOT2, FLJ20254 and TAZ) as required for CB3 gene expression (Supplementary Table 4). This suggests some shared host factors among these RNA viruses, but also points to the existence of dengue-specific host factors.

To determine whether we had identified DVHFs that affected both early and late steps in the viral life cycle, and to gain some early mechanistic insights we examined viral RNA accumulation in HuH-7 cells treated with siRNAs targeting *NPR2*, *SEC61B*, *FLJ20254* (also known as TMEM214), *TAZ*, *EXDL2* and *CNOT2* transcripts. As expected, knockdown of these DVHFs reduced both the number of cells expressing DENV E protein and the titre of infectious virus recovered 48 h after infection with DEN2-NGC (Fig. 4a, b). Consistent with this, we noted a decrease in viral transcripts measured by quantitative PCR with reverse transcription (RT–qPCR) (Fig. 4e). Accumulation of viral transcripts at 18 and 24 h after infection was clearly decreased in cells depleted of *FLJ20254*, *TAZ*, *EXDL2* and *CNOT2* (Fig. 4c, d), indicating that these DVHFs act on steps required for the accumulation of RNA (for example, early events). In contrast, knockdown of *NPR2* did not result in lower RNA levels at early times after infection, indicating that this DVHF acts at steps downstream of RNA accumulation. Given its location in the plasma membrane[30], it is likely that NPR2 is involved in the assembly or exit of DENV.

It is difficult to draw definitive conclusions for the 40 human homologues of dipteran DVHFs that did not recapitulate the effect on DENV infection (Supplementary Fig. 6). It is likely that some of these failed merely because their knockdown in human cells was ineffective. Nonetheless, these 40 genes that did not score in the human screen are not enriched in the aforementioned processes (for example, endocytosis), but show highly significant enrichment for genes involved in immunity, suggesting that many represent dipteran-specific DVHFs.

The availability of resources to carry out en masse analysis in *D. melanogaster* and human cells permitted us to perform a study that extended the known list of DVHFs by several fold. Given the likelihood of some false positives and the certainty of false negatives, this work represents an incomplete first version of what eventually will be a comprehensive DVHF list. Nonetheless, this study could lead to new targets for vector intervention. Furthermore, the information uncovered here should be used to explore the contribution of human DVHFs to disease severity and their potential in the treatment of dengue fever, and related illnesses such as West Nile encephalitis/fever and yellow fever.

METHODS SUMMARY

The 22,632 dsRNA collection of the DRSC has been previously described[14]. The DEN2-S2 virus used in the primary screen was isolated via serial passage of

DEN2-NGC (a gift from A. de Silva) in D.Mel-2 cells (Invitrogen). The experimental schedule for the primary and secondary screens is outlined in Fig. 1a. The human screen was performed with HuH-7 cells treated independently with two siRNAs (Qiagen) for each gene product, and with DEN2-NGC according to the schedule outlined in Fig. 3a. Infected cells were labelled with the anti-E, 4G2 primary antibody (isolated from the DI-4G2-4-15 hybridoma (American Type Culture Collection) and Alexa-488 anti-mouse secondary (Invitrogen), and counterstained with Hoescht 33342 (Sigma). Imaging and analysis of infection were carried out with a Cellomics ArrayScan Vti HCS machine.

Selection of candidates from the primary screen was done using a nonparametric approach, the sum-rank algorithm, to produce an appropriate summary statistic of each dsRNA tested in duplicate. In brief, within each plate wells were ranked by the percentage of infected cells, with the well with the lowest percentage infected cells given rank = 1. For each dsRNA tested in duplicate, we calculated a sum rank (SR) statistic using the formula: SR = rank on plate1 + rank on plate2. Sum ranks at either extreme are less likely to be observed by random chance. The number of times a given sum rank is expected to occur near the lower extreme (SR = 2) for a single pair of duplicate plates is given by: $E[SR] = (SR - 1)/(\text{number of valid wells})$, in which E denotes expectation. $E[SR]$ scores below 0.065 were used to select potential targets (218 dsRNAs) for further analysis (see Supplementary Fig. 4).

Standard experimental procedures were used for all other experiments. See Supplementary Information for more detail.

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Author Contributions All authors contributed to the strategy and implementation of the work.

Author Information Complete list of hits and dsRNA sequence information are available at the DRSC website (http://www.flyrnai.org). Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.L.P. (pears016@mc.duke.edu) or M.A.G.-B. (garci001@mc.duke.edu).