Quantitative Proteomic Analysis of Host-virus Interactions Reveals a Role for Golgi Brefeldin A Resistance Factor 1 (GBF1) in Dengue Infection

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Dengue virus is considered to be the most important mosquito-borne virus worldwide and poses formidable economic and health care burdens on many tropical and subtropical countries. Dengue infection induces drastic rearrangement of host endoplasmic reticulum membranes into complex membranous structures housing replication complexes; the contribution(s) of host proteins and pathways to this process is poorly understood but is likely to be mediated by protein-protein interactions. We have developed an approach for obtaining high confidence protein-protein interaction data by employing affinity tags and quantitative proteomics, in the context of viral infection, followed by robust statistical analysis. Using this approach, we identified high confidence interactors of NS5, the viral polymerase, and NS3, the helicase/protease. Quantitative proteomics allowed us to exclude a large number of presumably nonspecific interactors from our data sets and imparted a high level of confidence to our resulting data sets. We identified 53 host proteins reproducibly associated with NS5 and 41 with NS3, with 13 of these candidates present in both data sets. The host factors identified have diverse functions, including retrograde Golgi-to-endoplasmic reticulum transport, biosynthesis of long-chain fatty-acyl-coenzyme As, and in the unfolded protein response. We selected GBF1, a guanine nucleotide exchange factor responsible for ARF activation, from the NS5 data set for follow up and functional validation. We show that GBF1 plays a critical role early in dengue infection that is independent of its role in the maintenance of Golgi structure. Importantly, the approach described here can be applied to virtually any organism/system as a tool for better understanding its molecular interactions. Molecular & Cellular Proteomics 13: 10.1074/mcp.M114.038984, 2836–2854, 2014.

Viruses modify the intracellular environment of infected host cells in a number of important ways, including subverting the antiviral response, reorganizing host membranes, and manipulating host signaling pathways to create an environment more favorable for infection. For example, some viral proteins co-opt host proteins to degrade host interferon signaling components, thus antagonizing the antiviral response (1, 2); other viral proteins recruit metabolic enzymes that are potentially involved in the biogenesis of replication complexes (RCs)1 (3); and some viral proteins interact with host regulatory proteins to block the cellular stress response (4). These examples illustrate only a few of the ways in which viral-host protein-protein interactions (PPIs) enable the viral life cycle and drive pathogenicity. Because of the limited coding capacity of many viral genomes, in particular RNA virus genomes, viral-host PPIs generally occur between a remarkably small number of viral proteins and a much larger number of host proteins (5). The study of these extensive interactions necessitates comprehensive and quantitative approaches.

1 The abbreviations used are: RC, replication complex; +ssRNA, positive-sense single-stranded RNA; ABC, ammonium bicarbonate; AcGFP, Aequorea coerelescens green fluorescent protein; AP, affinity purification; ARF, adenosine diphosphate ribosylation factor; ARF-GEF, ARF guanine nucleotide exchange factor; Arg10, Arginine-13C6-15N4; BFA, brefeldin A; cDNA, complementary DNA; coA, coenzyme A; COP, coatomer protein I; DENV, dengue virus; DMEM, Dulbecco’s Modified Eagle’s medium; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; FDR, false discovery rate; FPR, false positive rate; GCA, golgicide A; Glu-Fib, Glu-1-Fibrinopeptide B standard peptide; GO, gene ontology; HCV, hepatitis C virus; hiFBS, heat-inactivated fetal bovine serum; IC50, half maximal inhibitory concentration; ID, identification; IP, immunoprecipitation; IPI, international protein index; KD, knock-down; LDs, lipid droplets; LTO, linear trap quadrupole; Lys8, Lysine-13C6-15N2; MS/MS, tandem mass spectrometry; NS, nonstructural; I-DIFT, isotopic differentiation of interactions as random or targeted; PARP, poly(adenosine diphosphate-ribose) polymerase; PHK119, phosphatidylinositol 4-kinase IIα; PPI, protein-protein interaction; Pro6, Proline-13C5-15N2; ppm, parts per million; qPCR, quantitative real-time PCR; RT, room temperature; RQ, relative quantification; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; shRNA, short hairpin shRNA; SILAC, stable isotope labeling by amino acids in cell culture; TGN, trans-Golgi network; WT, wild type.
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the development and validation of which will potentially contribute to: 1) our understanding of the mechanisms by which viruses subvert cellular pathways to their own advantage; 2) our understanding of fundamental cell biology; 3) the choice of potential drug targets and the rational design of such drugs; and 4) our understanding of the host response to infection.

Dengue virus (DENV) is a positive-sense, single stranded RNA virus in the family Flaviviridae that is transmitted by the bite of an infected Aedes mosquito (6). DENV is an important emerging pathogen that is the causative agent of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome, diseases which cumulatively pose formidable economic and health care burdens in many tropical and subtropical countries worldwide (7). Recent estimates of the global burden of DENV infection have revealed that DENV infection is threefold more prevalent than previously estimated, with 400 million annual incidences worldwide (8). Moreover, development of an anti-DENV vaccine has been hindered by the existence of four antigenically distinct DENV serotypes (DENV-1, -2, -3, and -4), each of which is capable of producing the full spectrum of DENV-induced disease (9). DENV is also related to other flaviviruses that cause significant human disease, including yellow fever virus, West Nile virus, and Japanese encephalitis virus (10). Thus, insights into DENV biology may be applicable to other flaviviruses of medical importance.

The flavivirus genome encodes only three structural (C, pr/M, and E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), and is translated as a single polyprotein, which is later cleaved into the mature viral proteins (6). The three structural proteins, capsid (C), membrane (M), and envelope (E) comprise the virion, whereas the NS proteins are mainly responsible for carrying out genome replication in infected cells. Among the seven NS proteins, NS5 and NS3 are the two largest and most highly conserved proteins (11); moreover, each possesses multiple enzymatic activities. NS5 contains an RNA-dependent RNA polymerase domain as well as a nucleoside-2'-O-methyltransferase domain; both of these activities are essential for replication (12, 13). NS3, on the other hand, possesses an N-terminal serine protease domain, which is responsible for cleaving the viral polyprotein at several sites (along with its cofactor, NS2B) (14). The C-terminal domain of NS3 has 5' RNA triphosphatase, nucleoside triphosphatase, and helicase activities (15–17). NS5 and NS3 have been shown to interact in infected cells (18), most likely in the RC. The precise composition and biogenesis mechanisms of RCs are poorly understood, but likely involve host proteins as well as viral proteins. As with other viruses, DENV-host PPIs have been interrogated by a number of high-throughput yeast two-hybrid assays (19–31) and approaches coupling either affinity purification (AP), immunoprecipitation, or immunoaffinity purification (IP) with MS (32–35). These approaches have yielded a number of putative DENV-host PPIs; however, considering the large repertoire of interactions undertaken by other viruses (36–41), our knowledge of DENV-host PPIs is likely incomplete. One advantage of IP/MS approaches is their potential to comprehensively reveal bona fide time-resolved interactions from the environment of an infected cell; however, the extremely high sensitivity of modern mass spectrometers highlights the need to develop IP/MS workflows capable of reliably discriminating between genuine interactors and nonspecific contaminants (42). Here, we present a workflow incorporating immunoaffinity purification and quantitative proteomics from infected cells, followed by robust statistical analysis to identify high confidence interactors of virtually any protein of interest, and apply this workflow to DENV NS5 and NS3.

EXPERIMENTAL PROCEDURES

Cell Culture—Vero, HepG2, C6/36, and HEK293 cells were obtained from the American Type Culture Collection. HEK293-T cells were obtained from Thermo Scientific. All mammalian cells were maintained in high glucose Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with heat-inactivated fetal bovine serum (hFBS; Vero 5%, all others at 10%), nonessential amino acids (NEAA; Vero, HepG2, and HEK293), GlutaMAX (Invitrogen; HepG2, HEK293), and penicillin/streptomycin (P/S; 100 U/ml and 100 µg/ml, respectively). For tetracycline-inducible short hairpin RNA (shRNA) silencing experiments, tetracycline qualified hFBS (Thermo Scientific) was used. Mosquito C6/36 cells were propagated in Minimum Essential Medium (Cellgro) supplemented with 10% hFBS, NEAA, and P/S.

Virus and Infections—DENV-2 stocks of MON601, a laboratory-derived clone of the DENV-2 New Guinea C strain (43), were generated by transfecting Vero cells with in vitro-transcribed RNA transcripts and then passaging supernatants twice in C6/36 mosquito cells. Stocks were concentrated ~10-fold by centrifugation in 100-kDa cutoff Millipore filter units (Amicon, Bilerica, MA) and titers were determined by plaque assays on Vero cells. For DENV infections, cells were generally washed with Dulbecco’s Phosphate-Buffered Saline (DPBS) and then infected for 1 h. Viral inoculum was prepared in low-serum (2% hFBS) C6/36 maintenance medium with 25 mM HEPES. After infection, inoculum was removed and replaced with fresh maintenance medium. To assess DENV internalization, virus was adsorbed to cells for 1 h at 4 °C to prevent internalization before replacing the inoculum with medium containing either DMSO or glicidic A (GCA). Cells were then shifted to 37 °C to allow synchronous virus internalization. Intracellular RNA was measured at 30 min, a time point at which the majority of bound DENV virions have been shown to be internalized (44). To ensure that only internalized virus, as opposed to adsorbed virus, was quantitated, cells were washed several times with PBS and incubated with an alkaline, high-salt solution at 0 °C (45).

Plasmids—For expression of C-terminally Aequorea coerelescens green fluorescent protein (AcGFP)-tagged NS3 or NS5 in mammalian cells, the appropriate coding sequences were amplified from pDWS601 (43) using oligos 5′–GTCGACGACCACATGGCTGGACT-ATTGTCGGAGTGC-3′ and 5′–GGATCCCTCCTCCCTCTTCTC-CAAGCTGAAACTC-3′ (NS3) or 5′–GTCGACGACCACATGGCAACT- GGCAACATAGGAGAG-3′ (NS5). Forward oligos introduced three in-frame linker glycine residues prior to the AcGFP (hereafter referred to as GFP) coding sequence. PCR products were subcloned into the BamHI and
Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)— Heavy HEK293-T cells were generated by passaging in SILAC DMEM (Thermo Fisher) supplemented with 0.398 mM heavy L-arginine HCl (15Cu, 15Nj) and 0.798 mM heavy L-lysine·2HCl (15C0, 15Nj) (Cambridge Isotope Laboratories or Sigma-Aldrich), 10% dialyzed hiFBS (Cellgro or Thermo Scientific; heat-inactivated in-house), P/S, NEAA, GlutaMAX, and 200 µg/ml L-proline (Sigma-Aldrich). In parallel, light HEK293-T cells were generated by growing in SILAC DMEM supplemented as above except with the substitution of light L-arginine and L-lysine (Fisher Scientific). After five passages in SILAC medium, percent incorporation of heavy amino acids was verified by MS to be >95% (supplemental Fig. S1). A proline titration experiment was also performed to determine the minimum amount of supplemented proline to prevent conversion of heavy arginine to heavy proline (supplemental Fig. S2).

Isotopic Differentiation of Interactions as Random or Targeted (I-DIRT) Immunoaffinity Purifications—The I-DIRT technique was performed essentially as described previously (42). Briefly, heavy and light SILAC HEK293-T cells were seeded into 15 cm² plates at 50,000 cells/cm² in antibiotic-free SILAC medium. For each I-DIRT IP, one or two 15 cm² plates were seeded with heavy cells and one or two 15 cm² plates were seeded with light cells; thus, each I-DIRT IP was performed from either two (three NS3 reps and two NS5 reps) or four (one NS3 rep and one NS5 rep) 15 cm² plates. The next day, light cells were transfected with constructs driving the expression of GFP, NS5-GFP, or NS3-GFP alone, with the modification that SILAC DMEM (Thermo Fisher) supplemented with 0.398 mM heavy L-arginine HCl and 0.798 mM heavy L-lysine·2HCl (Cambridge Isotope Laboratories or Sigma-Aldrich), 10% dialyzed hiFBS (Cellgro or Thermo Scientific) supplemented with 0.398 mM heavy L-arginine HCl and 0.798 mM heavy L-lysine·2HCl (Cambridge Isotope Laboratories or Sigma-Aldrich), 10% dialyzed hiFBS (Cellgro or Thermo Scientific; heat-inactivated in-house), P/S, NEAA, GlutaMAX, and 200 µg/ml L-proline (Sigma-Aldrich). In parallel, light HEK293-T cells were generated by growing in SILAC DMEM supplemented as above except with the substitution of light L-arginine and L-lysine (Fisher Scientific). After five passages in SILAC medium, percent incorporation of heavy amino acids was verified by MS to be >95% (supplemental Fig. S1). A proline titration experiment was also performed to determine the minimum amount of supplemented proline to prevent conversion of heavy arginine to heavy proline (supplemental Fig. S2).

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Mass Spectrometry— Gel lanes were divided into 6–10 slices which were diced into cubes and washed twice with destain solution [25 mM ammonium bicarbonate (ABC) in 50% ACN] for 30 min at 37 °C. Samples were then reduced with 10 mM DTT in 100 mM ABC for 30 min at 60 °C and then alkylated with 55 mM iodoacetamide in 100 mM ABC for 1 h at RT. Gel pieces were washed twice more in destain for 15 min at 37 °C and dehydrated by incubating in ACN for 15 min at RT. Subsequently, gel pieces were rehydrated in 12.5 ng/µl trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI) in 50 mM ABC for 10 min at RT. Gel pieces were then covered with additional 50 mM ABC as needed and incubated overnight at 37 °C. To extract peptides, gel pieces were incubated in 1% TFA solution for 5 min at RT. Extracted peptides were dried in a Speedvac and then adjusted to pH < 3 prior to clean up on a C18 spin column (Waters Sep-Pak Vac 1 cc, 100 mg). Peptides were resuspended in 20 µl loading buffer (0.1% formic acid, 1% ACN) prior to MS analysis. For one of the NS3-GFP samples, in-solution rather than in-gel digestion was performed. For the in-solution digest, samples were eluted with 65 µl 0.5% Rapigest (Waters, Milford, MA) in 50 mM ABC for 15 min at RT. 35 µl of this elution was added to 65 µl 50 mM ABC, and samples were reduced with 5 mM DTT at 60 °C for 30 min. Samples were cooled to RT, and iodoacetamide was added to 15 mM. Samples were incubated for 30 min in the dark and peptides were trypsin-digested as above. Samples were cleaned on C18 columns as above. One NS5-GFP sample was also digested in solution but yielded a low number of protein identifications (IDs) and is not included in this analysis.

Generally, peptides were loaded onto prepacked fused silica trap columns (Integra Frit Capillary, New Objective, Woburn, MA) in solvent A (0.1% formic acid in H2O) and eluted with an increasing percentage of solvent B (0.1% formic acid in ACN) in 75, 105, or 135 min gradients. The 75 min gradient of solvent B (in solvent A) was: 3% constant, 0–5 min; 3–25%, 5–35 min; 25–35%, 35–40 min; 35–80%, 40–45 min; 80% constant, 45–50 min; 80–35%, 50–55 min; 35–35%, 55–57 min; 35% constant, 57–75 min. The 105 min gradient of solvent B (in solvent A) was: 3% constant, 0–5 min; 3–25%, 5–65 min; 25–35%, 65–70 min; 35–80%, 70–75 min; 80% constant, 75–80 min; 80–35%, 80–85 min; 35–35%, 85–87 min; 35% constant, 87–105 min. The 135 min gradient of solvent B (in solvent A) was as follows: 3% constant, 0–5 min; 3–25%, 5–95 min; 25–35%, 95–100 min; 35–80%, 100–105 min; 80% constant, 105–110 min; 80–35%, 110–115 min; 35–35%, 115–117 min; 35% constant, 117–135 min. constant, 18 min. Peptides were eluted onto a fused silica capillary column (PicoFrit, New Objective) packed with 10 µm pore size Proteo Pep II C18 resin. Flow rates were held constant at 300 nL/min. Mass spectrometry was performed on either a Thermo Linear Trap Quadrupole (LTQ) Velos Orbitrap furnished with an auto-sampler and Eksigent ultra-high pressure nanopump, or a Thermo LTQ Orbitrap Classic equipped with an Agilent NanoHPLC system. Mass spectrometers were operated in a data-dependent mode: Full MS scans were performed at 60,000 resolution and were followed by ten subsequent tandem mass spectrometry (MS/MS) scans in which the most intense ion from the MS scan, the second most intense ion from the MS scan, and so on were chosen to undergo CID. CID fragmentation was achieved with a normalized collision energy of 35%, an activation Q of 0.250, and an activation time of either 30 msec (Orbitrap Classic) or 10 msec (Orbitrap Velos). MS scans were obtained in profile mode; MS/MS scans in centroid mode. The LTQ-Orbitrap settings used were: MS m/z mass range, either 400–1800 or 375–1800 (Orbitrap Classic) or 400–2000 (Orbitrap Velos); MS/MS minimum signal required, 500 (Orbitrap Classic) or 1000 (Orbitrap Velos); isolation width, 2 (Orbitrap Classic and Velos); default charge state, 2 (Orbitrap Classic and Velos). In order to select only the 2+, 3+, and 4+ ions for CID fragmentation, the following modes were enabled: FT master scan...
preview mode, charge state screening, monoisotopic precursor selec-
tion, non-peptide monoisotopic recognition, and charge state re-
jection of unassigned charge states and 1+ ions. Dynamic exclusion
was enabled in all runs, with an exclusion list size of 500 and an
exclusion duration of either 60 s (Orbitrap Velos) or 20 s (Orbitrap
Classic) applied.

**Database Searching and Analysis**—Peak lists were generated from
raw files with MaxQuant v1.2.2.5 (48) according to the standard
workflow (49). The integrated Andromeda peptide search engine (50)
(configured with AndromedaConfig v1.2.2.4) was used to search
MS/MS spectra first against the human International Protein Index
(IPI) database (v3.68; 87,083 entries actually searched) for initial mass
recalibration; subsequently, the main search was performed against a
concatenated custom database (174,828 protein entries actually
searched) containing the human IPI database (v3.69; 87,152 entries),
bovine and porcine trypsin (ref NP_001107199.1 and sp P00761.1),
the 10 mature DENV proteins plus the DENV 2K peptide (obtained
from gb|AAC59275.1), the AceGFp sequence (gb|AAN41637.1), com-
mon contaminants (248 entries, provided by MaxQuant), and reverse
counterparts of all of the above entries. Custom entries were com-
plied in Microsoft Word and the reverse counterparts were generated
by MaxQuant. Database species restriction to Homo sapiens (and
custom entries as appropriate) is justified because only proteins
obtained from lysates of DENV-infected human cells were analyzed in
this work. For assessing peptide-to-MS/MS spectrum matches,
Andromeda scores were determined by MaxQuant. The average
Andromeda peptide identification score was 135 (median 126), with only
4.1% having an Andromeda score below 60. The minimum score was
set to 0 (default setting). This threshold was justified by the observa-
tion that it yielded a false positive rate of < 5% (on the protein level)
in the entire data set, calculated as follows: First, we assumed that the
false positive rates (FPRs) in the reverse and forward databases were
equal. Using this assumption, the number of false positives in the
forward database can be doubled (as an estimate for the number of
false positives in the forward database), and this number divided by
the number of forward hits. This calculation yielded an overall FPR of 4.6%.

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The minimum number of SILAC labels that included 95% of all the input proteins for each individual
experiment (supplemental Fig. S3). Proteins that were immunoaffinity
enriched from these mixed lysates and had light to heavy ratios that
exceeded the threshold in each biological replicate were defined as
described as specific interactors.

**Network Analysis**—Interaction networks were constructed from
large-scale interaction and functional association databases with
GeneMANIA (51). High confidence NS5 and NS3 data sets (passing the
cutoff threshold in 3/3 experiments) were entered into GeneMANIA with
the following settings: (1) all networks enabled, (2) equal weighting by
network, and (3) 20 related genes displayed. GeneMANIA performed
a false discovery rate (FDR)-corrected hypergeometric test for gene on-
tology (GO) category enrichment in the input data set, compared with the
background set of GO annotations in the entire *Homo sapiens*
gene. Significantly enriched GO categories, and their associated q
values, are reported. For each GO category enriched in the interaction
network, the number of genes in the interaction network annotated in
that category, as well as the number of total genes in the *Homo sapiens*
genome annotated with the same GO category, are also reported.

**Antibodies and Reagents**—Commercially available antibodies were
obtained from the following sources: rabbit polyclonal anti-GBF1
(N-term) from GeneTex, Irvine, CA; rabbit polyclonal anti-BIG1, anti-
CAD, and anti-XPOT from Bethyl Laboratories, s; Montgomery, TX;
rabbit polyclonal anti-ZW10, anti-HSP90α, anti-SLIRP, anti-HSP47
(SERPINF1), anti-IPO4, and anti-HSP27 from Abcam (Cambridge,
MA); mouse monoclonal anti-HSP90β and anti-HSP70 from Abcam;
rabbit polyclonal anti-HEATR2 from Bioz; and mouse monoclonal anti-GM130 from BD Biosciences. Rabbit polyclonal antibodies
against GFP have already been described (47). Alexa Fluor 488-
conjugated goat anti-mouse antibodies were obtained from Invit-
gen. Rabbit polyclonal antibodies against the methyltransferase
domain of NS5 (residues 1–295) and helicase domain of NS3 (res-
ides 171–618), recombinantly produced as GST-fusion proteins in
*E. coli*, were raised in New Zealand White rabbits by R&R Research,
LLC and affinity purified. Brefeldin A (BFA) and GCA were obtained
from Sigma-Aldrich St. Louis, MO.

**Reciprocal Immunoprecipitation/Immunoblot Validation**—HepG2
cells were seeded at 70,000 cells/cm² 1 day before DENV infection
(moi = 1). At 24 h post-infection, cells were lysed in the same buffer as
used in the I-DIRT experiments, with the exception that PMSF was
omitted. Antibodies were cross-linked to Protein G Dynabeads (25
μl/immunoprecipitation) as before. The amount of antibody used per
immunoprecipitation varied in accordance with the manufacturer’s in-
structions for each antibody; generally, 5–15 μg/immunoprecipitation
was used. Rabbit IgG (Sigma) and mouse IgG (Bethyl Laboratories)
were at 1 μg/μl in PBS. For each experiment, the amount of IgG
cross-linked to the control beads was equal to the highest amount
of antibody used in that particular experiment. Approximately 100 μg
lysat was used in each immunoprecipitation. Lysates were rotated with
the appropriate antibodies, cross-linked to Protein G Dynabeads, for 10
min at RT. Beads were then washed three times with either PBS or
PBS/0.05% Tween-20. Bound proteins were eluted with 35 μl 1×
lithium dodecyl sulfate loading buffer and resolved on NuPAGE
gels as above. Proteins were transferred to nitrocellulose membrane and
immunoblotted with the designated antibodies.

**Quantitative Real-Time PCR (qPCR)**—Intracellular viral and cellular
RNA was obtained using the RNeasy Mini Kit (Qiagen). Generally, 1 μg of RNA was used for complementary DNA (cDNA) synthesis using
the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). For
qPCR reactions, PerfeCTa SYBR Green FastMix Low ROX (Quanta
Biosciences) was used on an Applied Biosystems 7500 Real-Time
PCR System. Primers were generally obtained from q PrimerDepot
(52) or PrimerBank (53) and were as follows: CAD, 5′-ATTGGGAGC-
TGATGGAG-3′ and 5′-CAAGCCGCACACAGGCT-3′; DENV
NS2A, 5′-ACCTGGGAAAGGATGGTATG-3′ and 5′-ATGGTCCT-
CTGGTATGTTGCTG-5′ (54); DHOR7, 5′-ATTGTCGAAGGCGC-
GAG-3′ and 5′-CTTGGATGGCGTTCTGTCGTA-3′; DPM1, 5′-TCATAGAT-

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GATGGAAGCCCA-3' and 5'-CCCAACCTTTTCTCTGGG-3'; GFB1, 5'-CTGTCGCTAGTTTGGCC-3' and 5'-TCCAGAAAAGGCTCAAGG-3'; H3SPD1, 5'-CTACCTGCCTACCCGCTTCA-3' and 5'-CAACGCTA-3; MTOR, 5'-CAAGCAGATGCAGAAGCT-3' and 5'-TCCGCTGCTGAGTTTATGA-3'; RPL13A, 5'-CCTGGAGGAGAAGAGAAAAGA-3' and 5'-TTGAGGACCTCTGTGTATTGTGGAACA-3'; SERPINH1, 5'-AGATGAGATGCTGTCG-3' and 5'-CATGGCCACGAGGGTTT-3'; SPTLC1, 5'-AGAGTGCGACAAGAAGCCTC-3' and 5'-ACACCAACCTTTTCTCTGGG-3'; ZW10, 5'-TTGAAATATCCATCACCATATTCTGA-3' and 5'-AGTCTCAGTGTCAGGGCA-3'.

Pharmacological Inhibition of COPI-mediated Transport—For drug inhibition studies, infections were performed as normal except that medium added after infection contained drugs at the following concentrations: GCA, 10 μM; BFA, 5 μg/ml; or DMSO at an equivalent concentration. GCA and BFA stocks were made up in DMSO and stored at −20 °C. For some infections, cells were incubated with viral inoculum at 4 °C for the duration of the infection to allow binding but prevent internalization; after inoculum removal (defined as t = 0), cells were shifted to 37 °C to allow internalization and the progression of infection. For quantifying viral internalization, surface-associated (i.e. uninternalized) virions were removed from cells via an alkaline high-salt wash (pH 9.5, 1 μM NaCl) as in (45).

shRNA-Mediated Gene Silencing—Nonreplicating lentiviral vectors for shRNA-mediated silencing of the following host genes were obtained from Thermo Scientific (gene, clone ID, and mature antisense sequence: CAD, V3THS_336915, 5'-TCTGTTCATTCTGGACCA-3'; DHCR7, V2THS_225786, 5'-TATTTAGAAGAATTAATG; DPM1, V2THS_16574, 5'-TATAACATGCTGTTGTC-3'; GFB1, V2THS_23644, 5'-TTCTGAATAACCTCATGGG-3'; H3SPD1, V2THS_380887, 5'-AAAGCAGTTTCACACACT-3'; MTOR, V2THS_401532, 5'-TATTCTGATACAACATG-3'; SERPINH1, V3THS_397092, 5'-TGTAGTGCTGTGCTGT-3'; SPTLC1, V3THS_404423, 5'-TAAACATCAGTTTACACT-3'; ZW10, V2THS_36690, 5'-ACACCAACCTTTTCTCTGGG-3').

Nonnonsilencing TRIPZ and GIPZ lentiviral control vectors were also obtained from Thermo Scientific. To generate nonreplicating lentivirus stocks, lentiviral gene silencing constructs were cotransfected with pMD2.G and psPAX2 vectors (Addgene plasmid 12259 and Addgene plasmid 12260, respectively; deposited by Dr. Didier Trono) into packaging HEK 293-T cells. Cell supernatants containing nonreplicating lentiviral vectors were harvested, filtered through 0.2 μm syringe filters, and stored at −80 °C prior to transductions or used immediately.

For shRNA-mediated knockdown (KD) studies, HepG2 cells were transiently transfected with each GFP-tagged viral construct and subsequently infected with DENV. Thus, interactions of GFP-tagged viral proteins within live infected cells were restricted and then infected with DENV. Thus, interactions of GFP-tagged viral proteins within live infected cells were restricted and then infected with DENV. Thus, interactions of GFP-tagged viral proteins within live infected cells were restricted and then infected with DENV. Thus, interactions of GFP-tagged viral proteins within live infected cells were restricted and then infected with DENV.

RESULTS

High Confidence NS5 and NS3 Interactors—In our analysis we strove to reduce background, exclude contaminant proteins, and identify bona fide interactions by: 1) isolating complexes from infected cells; 2) appending affinity tags to the viral baits (NS5 and NS3), thereby allowing tag-only IPs to be performed; and 3) incorporating an isotopic labeling-based approach, the I-DIR T technique (42), to discriminate high affinity prelysis interactions from nonspecific postlysis interactions. Despite numerous strategies and tags, introduction of affinity tags into the viral genome failed to yield viable recombining virus, suggesting that the virus does not readily tolerate insertions into either NS5 or NS3 coding sequences (data not shown). As an alternative approach, we used transient ectopic expression of tagged viral proteins during viral infection. Cells transfected with each GFP-tagged viral construct and subsequently infected with DENV produced approximately equal levels of ectopically expressed fusion protein and virally expressed untagged protein (supplemental Fig. S4A, S4B). NS5 is known to shuttle between the nucleus and the cytoplasm (55, 56); therefore, we also confirmed that NS5-GFP exhibited a similar subcellular localization to that of native NS5 in DENV-infected cells (supplemental Fig. S4C).

The I-DIR T approach is a powerful technique for discriminating interactions occurring prelysis (i.e. in live infected cells) from those occurring postlysis (i.e. nonspecific interactions). Our workflow for obtaining protein complexes, including our incorporation of the I-DIR T technique, is detailed in Fig. 1A. In brief, metabolically light cells were transfected with constructs driving the expression of NS5-GFP, NS3-GFP, or GFP and then infected with DENV. Thus, interactions of GFP-tagged viral proteins within live infected cells were restricted to exclusively light proteins. In parallel, metabolically heavy cells were mock-transfected and subsequently infected with X-100/PBS for 10 min at RT, blocked in 10% BSA/PBS for 30 min, and incubated in primary antibodies (anti-GM130) for at least 2 h. Cells were washed 3 times in 3% BSA/PBS and incubated with secondary antibodies (Alexa Fluor 488-conjugated anti-mouse) for 45 min. After three washes with PBS, slides were mounted with Slow Fade Gold DAPI (Invitrogen), sealed, and visualized on a Delta Vision RT microscope equipped with a CoolSnap camera and an Olympus lens (40x).

Statistical Analysis—Differences between continuous variables were analyzed with the unpaired t test (two-tailed, assuming unequal variance) to evaluate their statistical significance, defined as p < 0.05. To determine significant differences in qPCR experiments, t-tests were performed on the values of 2ΔΔCT, where ΔΔCT represents the difference in cycle thresholds between the gene of interest and RPL13A (internal control gene) for each condition, unless otherwise stated. All t test calculations were performed in Microsoft Excel. qPCR NS2A data are expressed as fold relative to RPL13A data, as calculated by Applied Biosystems 7500 software (comparative ΔΔCT method). IC50 values were obtained by fitting dose-response inhibition curves to the log10[GCA] values and relative amounts of NS2A using GraphPad Prism (GraphPad Software, Inc., San Diego, CA), with the least squares (ordinary) fit option enabled.
DENV. At 24 h post-transfection, light and heavy cell lysates were made and mixed 1:1 prior to rapid IPs with anti-GFP antibodies cross-linked to Protein G Dynabeads; immunoblots showing the efficiencies of isolation are shown in supplemental Fig. S4D. In this approach, proteins acquiring their association with the viral bait within infected cells should be primarily light, whereas contaminants/nonspecific interactors that associate postlysis should be detectable by an increased heavy to light ratio. To analyze the initial distributions of protein ratios, aliquots of mixed lysate IP inputs were saved for each experiment. After washing the beads, GFP-tagged viral baits and associated viral and cellular proteins were eluted and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie staining of the gel revealed that no proteins appeared to bind to the GFP negative control; in contrast, a large number of proteins were associated with NS3-GFP and/or NS5-GFP (Fig. 1B). We refrained from increasing the stringency of our IP buffer further, as increased stringency could also result in the loss of more weakly bound, yet nevertheless specific, members of each protein complex. Instead, we relied on the I-DIRT method to address interaction specificity; in fact, one of the key strengths of the I-DIRT approach is that it allows IPs to be performed in relatively nonstringent conditions while still allowing the discrimination of specific from nonspecific interactors (42).

In-gel trypsin digestion was performed prior to LC-MS/MS analysis to identify and quantify the heavy to light ratios of the proteins associated with the tagged viral baits in each of the three independent IPs, as well as for the proteins present in the mixed lysate inputs of the IPs. Isotope pattern assembly into SILAC pairs, peak detection, and protein heavy to light ratio quantification were performed with MaxQuant (48, 49).

MaxQuant calculates protein heavy to light ratios as the median of all peptide ratios assigned to a distinct protein or

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**Fig. 1.** Workflow for identifying high confidence interactors of DENV NS5 and NS3 and typical I-DIRT immunoaffinity purification (IP). A, Schematic diagram of workflow, including incorporation of the I-DIRT technique. Metabolically heavy cells were generated by passaging in medium containing exclusively heavy lysine and arginine (Lys8 and Arg10) until incorporation was verified to be >95%. In parallel, metabolically light cells were maintained in identical medium, with the exception that light lysine and arginine were substituted for their isotopically heavy counterparts. For I-DIRT IPs, only light cells were transfected with constructs driving the expression of GFP-tagged viral baits, or the GFP tag alone as an additional control. In parallel, heavy cells were mock transfected. As soon as transfection mixtures could be removed, all cells (heavy and light) were infected with DENV. At 24 h post-transfection (19 h postinfection), cell lysates were made and their protein contents quantitated. For the I-DIRT IPs, equal protein amounts of heavy and light lysates were thoroughly mixed, and an aliquot was reserved for determination of protein ratio inputs. Then, rapid IPs of tagged protein complexes were performed using anti-GFP antibodies, cross-linked to Protein G Dynabeads, and the heavy/light lysate mixtures. After washing the beads, proteins associated with the GFP-tagged viral baits were eluted and resolved by SDS-PAGE. In-gel trypsin digestion and C18 clean-up were then performed in preparation for LC-MS/MS. After database searching and analysis, the cumulative distributions of the light to heavy ratios in the mixed lysate inputs were used to identify the cutoff threshold ratio that included 95% of the input proteins. In our analysis, high confidence interactors were considered to be proteins that passed this cutoff in each of three independent experiments. B, Typical I-DIRT immunoaffinity purification. Mixed lysate inputs and proteins associated with GFP-tagged viral complexes, obtained in a typical I-DIRT IP, were resolved by SDS-PAGE and stained with Coomassie dye. Lanes 1–3, 0.1% of mixed lysate inputs: lane 1, GFP input; lane 2, NS5-GFP input; lane 3, NS3-GFP input. Lanes 4–6, 67% of eluates: lane 4, GFP IP; lane 5, NS5-GFP IP; and lane 6, NS3-GFP IP. Asterisks designate GFP and the GFP-tagged viral baits.
protein group; program settings were adjusted so that only unique peptides and razor peptides (i.e. peptides shared by different proteins of a group) were used for protein quantification. Minimum thresholds required for data to be processed included at least one heavy to light ratio count for each protein group, although data were filtered postanalysis to require at least two ratio counts in each biological replicate. Acceptable peptide and protein FDRs were set to 1% each. Complete quantification results for protein groups, sometimes containing multiple isoforms and/or multiple individual members of a group detected in each IP, are shown in supplemental Table S1. In this study, we did not attempt to unambiguously identify which member of a protein group was present; for clarity, “protein groups” will be referred to as “proteins” hereafter. All peptides matching to all members of a family, even when a single peptide was found in multiple proteins, are reported in supplemental Table S1C. Proteins associated with tagged viral baits were classified as high confidence interactors if their light to heavy ratios were higher than the ratio corresponding to 95% of the proteins in the mixed lysate cumulative distributions for each experiment (supplemental Fig. S3). For determining light to heavy ratio cutoffs, the light to heavy ratios of all proteins identified in the mixed lysate inputs were determined. Of these, 13 outlier proteins (out of 1463 total IDs) were excluded from analysis because the standard deviation (S.D.) of \[\log_2(\text{heavy to light ratios of all redundant quantifiable peptides}) \times 100\] (defined as ratio heavy to light variability by MaxQuant) exceeded 100%. Each independent replicate thus yielded its own unique cutoff value, specific to its mixed lysate input; biological reproducibility was addressed by requiring candidates to pass this cutoff value in each of three biological replicates. Analytical reproducibility was addressed by running the Glu-1-Fibrinopeptide B standard peptide (Glu-Fib, amino acid sequence: EGVNDNEEGFFSAR, monoisotopic m/z = 1770.68) at least between every six runs, and usually between every two to three runs. The Glu-Fib +2 ion (m/z = 785.8421) was monitored and verified to be within 3 ppm error for all standards. For each set of IPs (NS5-GFP, NS3-GFP, or GFP), the ratio counts used for quantitation of each protein, average light to heavy ratios of each protein, and light to heavy ratio SDs for each protein are given in supplemental Table S1. For the GFP control, no host proteins passed this selection (data not shown). For NS5, 53 host proteins passed; for NS3, 41 host proteins passed (Fig. 2 and supplemental Table S2). Of these host proteins, 13 were shared by both NS5 and NS3. To determine how many of these specific interactors had appeared in negative controls from unrelated pull-down experiments, the resultant list of 81 unique interactors of NS5, NS3, or NS5 and NS3 was uploaded into the Contaminant Repository for Affinity Purification database (57) (http://www.crapome.org; supplemental Table S3).

Validation of Interactions by Reciprocal-Coimmunoprecipitation—To ensure that the interactions undertaken by the GFP-tagged viral proteins were also undertaken by virally expressed NS5 and/or NS3, we performed reciprocal coimmunoprecipitations with a panel of antibodies against interactors in our data sets using lysates from DENV-infected cells. We chose human hepatocellular carcinoma HepG2...
Identification of High Confidence DENV-host Interactions

cells because the liver is believed to be a major target organ of DENV (58) and detailed ultrastructural studies of DENV RCs have been carried out in a similar hepatocellular line (59). We obtained antibodies against 19 high confidence interactors of NS5 and/or NS3; seven of these antibodies were unsuccessful in immunoprecipitating their target antigens and/or immunoblotted their target antigens (anti-HSPA8, anti-XPO2, anti-XPO7, anti-SURF4, anti-DHCR7, anti-UBXD8, and anti-HSPD1) and were thus excluded from further analysis. The 12 remaining candidates were involved in diverse processes, including membrane trafficking between the ER and the Golgi (GBF1 and ZW10), nuclear transport (IPO4 and XPOT), the unfolded protein response (SERPINH1, HSP70, HSP27, HSP90α, and HSP90β), transcriptional regulation (SLIRP), dynein transport (HEATR2), and nucleotide biosynthesis (CAD). Target proteins were immunoprecipitated out of lysates from DENV-infected HepG2 cells, and the eluates were immunoblotted for the target proteins and either NS5 or NS3. Immunoblot analysis of the eluates validated the interactions revealed by I-DIRT: anti-GBF1, -SLIRP, -CAD, -XPO7, and -HSP90α antibodies specifically co-immunoprecipitated NS5 (Fig. 3A), and anti-IPO4, -HSP27, -SERPINH1, -ZW10, -HSP90β, and -HSP70 antibodies similarly coimmunoprecipitated NS3 (Fig. 3B). Of the interactors tested, only HEATR2 yielded inconclusive results and thus could not be validated by this approach; however, we also note that anti-HEATR2 antibodies immunoprecipitated HEATR2 with variable efficiency (data not shown). Thus, we validated 5/5 NS5 interactors, and 6/7 NS3 interactors, using this approach, indicating that our GFP-tagged viral proteins undertake many of the same interactions as the corresponding virally expressed proteins.

Functional Validation of Interactions by shRNA-Mediated Knockdown—To determine the extent to which the high confidence interactors identified by I-DIRT play a role in DENV infection, we used shRNA to silence a panel of host genes and then determined the effect on DENV infection. The genes in our panel included interactors of both NS5-GFP and NS3-GFP (CAD, DHCR7), only NS5-GFP (MTOR, SPTLC1), and only NS3-GFP (DM1, HSPD1, SERPINH1, and ZW10) (supplemental Tables S1 and S2). HepG2 cells were transduced with nonreplicating lentiviral vectors harboring shRNA targeting one of the host genes. After doxycycline-mediated induction of shRNA expression, cells were infected with DENV and intracellular RNA was harvested at 24 h postinfection. The efficiency of DENV replication (Fig. 3C) and the extent of host gene silencing (Fig. 3D) in each KD cell line were determined by qPCR. The three host genes whose silencing affected DENV infection to the greatest extent were DHCR7, SERPINH1, and ZW10 (mean ± S.D.: 48 ± 14%, 52 ± 13%, and 68 ± 3% inhibition, respectively).

NS5-GFP and NS3-GFP Interaction Networks—Components of NS5-GFP complexes included some expected host proteins, such as exportin-1 (CRM1), a known interactor of NS5 that mediates its nuclear export (56), and fatty acid synthase (FASN), an enzyme involved in fatty acid biosynthesis that has been shown to relocalize to RCs in DENV-infected cells (3). To gain additional insight into possible functional connections between the proteins present in the NS5-GFP and/or NS3-GFP complexes, we generated interaction networks with GeneMANIA (51) to identify significantly enriched (q < 0.05) GO annotations (Fig. 4). As expected, the interaction networks of NS5-GFP and NS3-GFP exhibited some similarities and also notable differences. Lipid metabolism was well represented in both interaction networks, with fatty-acyl-coenzyme A (CoA) biosynthesis (q < 0.05, NS5 and NS3), fatty-acyl-CoA metabolism (q < 0.05, NS3) and long-chain fatty-acyl-CoA metabolism (q < 0.05, NS5 and NS3) all significantly enriched (Fig. 4 and supplemental Table S4). This observation is consistent with studies indicating that DENV subverts the fatty acid biosynthetic pathway for RC biogenesis (3). Both interaction networks were also enriched in transmembrane transporter activity for carboxylic acids and organic acids (Fig. 4 and supplemental Table S4).

Regarding the differences between the two networks, the most highly enriched process in the NS5-GFP interaction network was Golgi-to-endoplasmic reticulum (ER) retrograde vesicle-mediated transport (q < 0.001). Retrograde transport from the Golgi to the ER is mediated by coatomer protein I (COPI)-coated transport vesicles (60). This transport pathway is potentially of interest because DENV RCs are assembled on remodeled host ER membranes through mechanisms that are poorly understood (59). An important difference between NS5 and NS3 is their subcellular localization. Although both are present to some extent on RCs on ER-derived membranes (59), NS5 also undergoes both nuclear import (55) and export (56). As might be expected from the nuclear localization of NS5, both the nuclear pore and the nuclear envelope were significantly enriched (q < 0.02) in the NS5-GFP interaction network (Fig. 4A and supplemental Table S4). In contrast, the NS3-GFP interaction network was most enriched in protein folding and the unfolded protein response (q < 0.001 for both; Fig. 4B and Supplemental Table S4). Interestingly, the NS3-GFP interaction network was also significantly enriched for the viral infectious cycle, the viral reproductive process, and major histocompatibility complex class I protein binding (q < 0.01 for all; Fig. 4B and Supplemental Table S4). Complete lists of significantly enriched GO annotations (q < 0.05) for each data set are provided in Supplemental Table S4.

A Functional Role for GBF1 Early in DENV Infection—Based on the significant enrichment of Golgi-to-ER retrograde transport in the NS5-GFP interaction network (Fig. 4) and the potential role this pathway could have in the generation of ER-derived DENV RCs, we proceeded by focusing on the NS5 interactor, GBF1. GBF1 is a large (> 200 kDa) multidomain protein best known for its role in COPI-mediated transport, where it activates ARFs (adenosine diphosphate ribosylation factor proteins) through its ARF guanine nucleotide exchange factor (ARF-GEF) activity (61). We began by treating DENV-infected cells with two
pharmacological inhibitors that block ARF activation, BFA and GCA, and determining the effect on DENV infection. These two drugs have different specificities: BFA targets multiple ARF-GEFs, including GBF1 (62), whereas GCA specifically targets GBF1 (63). We infected cells with DENV and then incubated cells for 16 h in the presence of BFA (5 μg/ml), GCA (10 μM), or...
vehicle alone. The levels of intracellular DENV RNA were then quantified by qPCR. Treatment with either compound reduced the level of intracellular DENV RNA ~10-fold (Fig. 5A); importantly, the drug concentrations in these experiments were not significantly toxic to cells at the incubation times; used (supplemental Fig. S5). Because BFA and GCA inhibited DENV infection to a similar extent (Fig. 5A), we used the GBF1-specific compound, GCA, for all subsequent inhibition experiments. To exclude the possibility that GCA-mediated inhibition of DENV infection was caused by pleiotropic effects, we overexpressed either wild-type GBF1 (GBF1WT), or a GCA-insensitive version harboring an M832L mutation (GBF1M832L) (63), in DENV-infected cells. Overexpression of GBF1WT partially rescued DENV infection in the presence of
GBF1 plays an essential and specific role early in DENV infection. A, Pharmacological inhibition of GBF1 inhibits DENV infection. HepG2 or HEK293 cells were infected with DENV for 1 h (moi/1000) and then treated with DMSO, BFA (5 μg/ml), or GCA (10 μM). After 16 h, the levels of DENV NS2A were quantified by qPCR and normalized to the internal control RPL13A. The amount of DENV RNA in DMSO-treated cells was set to 1. Data are expressed as means ± S.D. of relative quantification values of NS2A (n = 3 for HepG2; n = 4 for HEK293). B, GCA-mediated inhibition of DENV infection is rescued on the RNA level by overexpression of GBF1WT and a drug-insensitive version thereof (GBF1M832L). HEK 293-T cells were transfected with pCI, pCI-GBF1WT, or pCI-GBF1M832L. At 24 h post-transfection, cells were reseeded into 6-well plates. The following day, cells in representative wells were counted to ensure that infections were set up at the same moi. After infection with DENV (moi = 1–2), cells were incubated in medium with either DMSO or GCA (10 μM) for 16 h. DENV RNA was then quantified as in A. For each condition (pCI, pCI-GBF1WT, or pCI-GBF1M832L), the relative quantification value of DENV NS2A in DMSO-treated cells was set to 1. Results are expressed as means ± S.D. of relative quantification values of NS2A (n = 4). *p < 0.05. C, GCA-mediated inhibition of DENV infection is rescued on the protein level by overexpression of GBF1WT and a drug-insensitive version thereof (GBF1M832L). Experiments were performed as in B, except that equal amounts of protein in cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then probed with anti-GBF1, anti-NS5, and anti-tubulin antibodies. D, NS5 interacts with GBF1, but not BIG1. HepG2 cells were infected with DENV (moi = 2) for 1 h, and cell lysates were prepared at 24 h postinfection. Immunoprecipitations were performed with the following antibodies: rabbit IgG (control), rabbit polyclonal anti-BIG1, and rabbit polyclonal anti-GBF1. Coimmunoprecipitated proteins were analyzed by immunoblotting the eluates with anti-NS5, anti-BIG1, and anti-GBF1 antibodies. Inputs and eluates shown in the same horizontal row are from the same film exposure. A representative result from three independent experiments is shown. E, Golgicide A does not inhibit DENV internalization. HepG2 cells were infected with DENV (moi = 5–10) and then infected with DENV (moi = 3) for 1 h at 4 °C to allow virion adsorption. After 1 h, viral inoculum was removed and replaced with prewarmed medium with either DMSO or GCA (10 μM). After a 30 min incubation to allow virus internalization, bound (i.e., uninternalized) virus was removed via several washes with PBS and a 2 min incubation with a high-salt, high-pH solution at 0 °C. Intracellular DENV RNA was then quantified as in A. Data are expressed as means ± S.D. (n = 3). F, DENV replication is most sensitive to GCA at early time points in infection. HepG2 cells were prechilled at 4 °C and then infected with DENV (moi = 5–10). Virus was adsorbed for 1 h at 4 °C, and cells were washed to remove virus input. Inoculum was replaced with prewarmed medium (24, 36, and 48 h samples) or medium containing DMSO or GCA (10 μM) (12 h samples). For the 12 h sample, cells were incubated 12 h in the presence of DMSO or GCA, and then intracellular RNA was harvested. When the 12 h sample was harvested, the cells in the 24 h sample were washed to remove extracellular virus, and medium with either DMSO or GCA was added (24 h sample). The cells in the 36 h sample were washed with PBS and a 2 min incubation with a high-salt, high-pH solution at 0 °C. Intracellular DENV RNA was then quantified as in A. The normalized level of NS2A in DMSO-treated cells at 12 h was set to 1 for each experiment, and levels of NS2A in all other conditions are expressed relative to this value. Data are expressed as means ± S.D. (n = 4). *p < 0.05. n.s., not significant. RQ, relative quantification.
GCA, as demonstrated by the increased levels of both DENV RNA (Fig. 5B, \( p = 0.049 \)) and NSS protein (Fig. 5C). Moreover, overexpression of GBF1\textsubscript{M832L} rescued DENV infection to an even greater extent than GBF1\textsubscript{WT} (Fig. 5B, \( p = 0.008 \)) (Fig. 5C). Cumulatively, these data suggest that GCA-mediated inhibition of DENV infection results from the specific effect of GCA on GBF1, because DENV infection in the presence of GCA can be rescued by overexpression of its target molecule.

As an additional control for the specificity of the NS5-GBF1 interaction, we investigated whether NS5 interacted with BIG1, a related ARF-GEF with a similar domain structure and molecular weight (64). GBF1 and BIG1 were immunoprecipitated from a lysate of DENV-infected HepG2 cells, and the presence of coimmunoprecipitated NS5 was analyzed by immunoblotting. GBF1 was efficiently immunoprecipitated from the DENV-infected cell lysate, and NS5 was clearly associated with GBF1 (Fig. 5D). Similarly, BIG1 was efficiently immunoprecipitated out of the lysate; however, only a trace amount of NS5 was associated with BIG1 (Fig. 5D). NS5 was also absent from the IgG control. These data further indicate that NS5 specifically interacts with GBF1.

DENV virions rely wholly on clathrin-mediated endocytosis for uptake into cells (44). Although GCA has been shown to have no effect on clathrin-mediated endocytosis (63), we confirmed that GCA did not interfere with virus entry in our system. Quantitation of intracellular DENV RNA at 30 min postinfection confirmed that GCA did not interfere with virus entry in our system and molecular weight (64). GBF1 and BIG1 were immunoprecipitated from a lysate of DENV-infected HepG2 cells, and the presence of coimmunoprecipitated NS5 was analyzed by immunoblotting. GBF1 was efficiently immunoprecipitated from the DENV-infected cell lysate, and NS5 was clearly associated with GBF1 (Fig. 5D). Similarly, BIG1 was efficiently immunoprecipitated out of the lysate; however, only a trace amount of NS5 was associated with BIG1 (Fig. 5D). NS5 was also absent from the IgG control. These data further indicate that NS5 specifically interacts with GBF1.

GCA overexpression of GBF1\textsubscript{M832L} rescued DENV infection to an even greater extent than GBF1\textsubscript{WT} (Fig. 5B, \( p = 0.008 \)) (Fig. 5C). Cumulatively, these data suggest that GCA-mediated inhibition of DENV infection results from the specific effect of GCA on GBF1, because DENV infection in the presence of GCA can be rescued by overexpression of its target molecule.

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To further test the hypothesis that GCA-mediated inhibition of DENV infection is caused by a specific effect of GCA on GBF1, we investigated whether reducing the level of GBF1 would affect the ability of GCA to inhibit DENV infection. We performed dose-response experiments to determine the half maximal inhibitory concentration (IC\textsubscript{50}) of GCA to inhibit DENV infection in control versus GBF1\textsubscript{KD} cells. The IC\textsubscript{50} value was \( \sim 50 \)-fold higher in control cells compared with GBF1\textsubscript{KD} cells (Fig. 6B; \( 6.4 \mu M \pm 3.7 \mu M \) versus \( 0.13 \mu M \pm 0.11 \mu M \), respectively; \( p = 0.031 \)). These data demonstrate that reducing the level of GBF1 sensitizes DENV infection of cells to GCA, indicating that KD of GBF1 and GCA inhibit DENV infection in a concerted manner. This observation reinforces the idea that GBF1 is a specific host factor used by DENV, and supports a proviral role for GBF1.

The Golgi apparatus has been shown to be completely dispersed in cells treated with 10 \( \mu M \) GCA (63). To explore a potential pleiotropic effect on DENV infection caused by this disruption of the Golgi apparatus, we examined whether GCA inhibition of DENV infection could be uncoupled from its effects on Golgi morphology. To this end, we titrated the minimum amount of GCA required to disrupt Golgi apparatus structure and compared this with the minimum amount of GCA needed to inhibit DENV infection. Wild-type cells were treated with various concentrations of GCA (or DMSO) for 16 h, fixed, and immunolabeled with anti-GM130 antibodies (cis-Golgi marker). As shown in Fig. 6C, DMSO-treated cells exhibited a tight, compact Golgi structure (left panel), whereas the Golgi apparatus was completely dispersed in cells treated with 1 \( \mu M \) GCA (right panel). The extent of Golgi dispersal in cells treated with 1 \( \mu M \) GCA was the same as that in cells treated with 10 \( \mu M \) GCA (data not shown). Thus, 1 \( \mu M \) GCA is sufficient to fully disrupt Golgi morphology (Fig. 6C), but not sufficient to inhibit DENV infection (Fig. 6B), indicating that GCA-mediated Golgi disruption by itself does not inhibit DENV infection.
Fig. 6. DENV infection of GBF1-KD cells is sensitized to GCA in a manner independent of Golgi disruption. A, DENV infection in GBF1-KD HepG2 cells. HepG2 cells were transduced with nonreplicating lentiviral vectors harboring either nonsilencing control shRNA, or shRNA targeting GBF1. Stable cell lines were obtained with puromycin selection (1.5 μg/ml) and were generated fresh for each experiment. After induction of shRNA expression with doxycycline (2 μg/ml for 48 h), the cells in at least two representative wells of each cell line were detached by trypsinization and counted at least in duplicate to ensure that infections were set up at the same moi. Cell viabilities were also determined by trypan blue exclusion and determined to be 95% at the time of infection in all experiments (data not shown). Cells were then infected with DENV (moi = 1) and incubated for 16 h, after which time intracellular RNA was extracted and/or cell lysates were made. After synthesis of cDNA, the amounts of DENV NS2A and GBF1 were quantified by qPCR and normalized to the internal control RPL13A. The relative quantification amount of either NS2A or GBF1 in the nonsilencing control was then set to 1. Data are expressed as relative quantification means ± S.D. (n = 3 for each experiment). Although relative quantification results are shown, t-tests were performed on the 2^-ΔΔCt values, as described under “Experimental Procedures.” Left panel: DENV infection in GBF1-KD cells versus DENV infection in control cells. Horizontal bars represent means; individual experiments are shown with black triangles. Vertical error bars represent ± S.D. (n = 3). n.s. = not significant. Right lower panel: Extent of GBF1 KD in GBF1-KD cells. Means ± S.D. (n = 3) are shown for the cells used in the left panel. Upper right panel: Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then probed with anti-GBF1 antibodies. n.s., not significant; RQ, relative quantification. B, GBF1-KD cells are sensitized to GCA-mediated inhibition of DENV...
In this study, we presented and validated an approach for obtaining high confidence PPI data for virtually any protein of interest. This approach incorporates 1) a shotgun LC-MS/MS approach; 2) an isotopic labeling strategy to eliminate postlysis contaminants; 3) the use of tagged proteins in the context of wild-type infection; and 4) a rigorous statistical approach enabling high confidence interactors to be selected using a cutoff threshold specific to each biological replicate. To the best of our knowledge, only three previous studies, none of which focused on DENV, have combined metabolic labeling with immunoaffinity purification- and MS-based proteomics workflows aimed at identifying viral-host PPIs (67–69). Although similar to the workflows that were employed in (68, 69), our approach has two important differences: 1) the I-DIRT approach allows contaminants to be discriminated from bona fide interactors prior to enrichment, thus avoiding potential artifacts resulting from the enrichment process (42); and 2) our analysis of the IP inputs allows an appropriate light to heavy ratio cutoff threshold to be chosen specifically for each individual IP. We anticipate that this approach will be a useful tool in the study of virus-host interactions, a central aspect to the study of all viruses and indeed all pathogens.

We focused on DENV NS5 and NS3, two proteins essential for DENV replication. We stress that although a large number of proteins were associated with NS5 and/or NS3 as determined by Coomassie staining (Fig. 1B), and indeed a large number of total protein IDs were obtained per IP (857 and 879 on average, respectively), only 53 high confidence NS5 interactors, 52 of which were novel, and 41 high confidence NS3 interactors, all of which were novel, passed our I-DIRT threshold in each of three independent experiments. This finding clearly illustrates the power of the I-DIRT approach to exclude nonspecific associations from the final data sets. However, we also recognize that the complete repertoire of PPIs may not have been identified by our approach because: 1) our tagged viral baits were not expressed in the context of the entire DENV polyprotein, 2) virally expressed NSS was also present, and 3) the I-DIRT approach was unable to discriminate between contaminants and rapidly exchanging bona fide interactors. Regarding point 1), the cleavage of NSS from a polyprotein precursor has been shown to affect its ability to interact with some proteins (1); these interactions may not have been identified by our ectopic expression approach. Regarding point 2, the binding site for a certain interaction may be occupied by the virally expressed protein, leading to competition for a given interaction between the ectopically expressed and virally expressed proteins. Finally, regarding point 3, the I-DIRT approach is unable to discriminate between a nonspecific postlysis contaminant and an interacting protein that undergoes rapid dissociation and reassociation. Additional time-course experiments, in principle, should help the identification of such dynamic interactors. In these experiments, the duration of the IP would be varied to allow sampling of the light to heavy ratios of the components of the protein complex over time. A stably associating contaminant protein should exhibit a constant light to heavy ratio over time. On the other hand, a dynamically interacting bona fide interactor would be expected to initially have a high light to heavy ratio, because the association was acquired pre-lysis. However, such a rapidly exchanging protein is expected to display a decrease in its light to heavy ratio over time until equilibrium is reached. Despite these caveats of our approach, the frequency with which we validated interactions by reciprocal-co-immunoprecipitation (11/12; Fig. 3) gives us great confidence that although our interactor data sets are not exhaustively comprehensive, they are likely to consist of bona fide interactors. This conclusion is based on the power of the I-DIRT approach to allow us to filter for interactions that must have taken place before cell lysis and the fact that the GFP alone control did not.
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yield any interactions. Furthermore, our data sets also contain functionally relevant interactors, as evidenced by the gene silencing data that indicate at least two NS3 interactors (out of four tested) and one NS3/NS5 interactor (out of two tested) are important in DENV infection (SERPINH1, ZW10, and DHC7, respectively; Fig. 3C). Interestingly, pharmacological inhibition of CAD, the other NS3/NS5 interactor tested by shRNA silencing, has been shown to potentiate inhibit DENV replication (70). We speculate that the difference between the potent effect observed on DENV infection after pharmacological inhibition of CAD and the moderate effect we observed on DENV infection after shRNA-mediated silencing of CAD is caused by incomplete knockdown (Fig. 3D).

An Essential Role for GBF1 Early in DENV Infection—The NS5-GFP interaction network identified here was significantly enriched in proteins involved in retrograde Golgi-to-ER transport, including GBF1 (Fig. 4 and supplemental Table S4). Using primarily a combined pharmacological and genetic approach, we showed that GBF1 plays a proviral role in DENV infection (Figs. 5 and 6). We speculate that the discrepancy between the magnitudes of DENV inhibition by GCA treatment versus GBF1 KD (Fig. 5A versus Fig. 6A) is caused by incomplete GBF1 KD (Fig. 6A). GBF1 is an essential gene; thus, we were unable to induce shRNA expression any longer than 48 h, because at later time points, cells underwent apoptosis as indicated by the accumulation of a poly(adenosine diphosphate-ribose) polymerase (PARP) cleavage product (data not shown). This observation is consistent with a previous study in which silencing of GBF1 in HepG2 cells was shown to result in cell cycle arrest and extensive apoptotic cell death, as assessed by the presence of the PARP cleavage product, after 48 h (71). Nevertheless, our data suggest that the essential role of GBF1 in DENV infection 1) occurs early in infection and 2) is independent of the role of GBF1 in the maintenance of Golgi structure (Figs. 5 and 6).

What could this essential role of GBF1 be? The GCA-pulse experiments (Fig. 5F) support a model in which GBF1 is required early in DENV infection, perhaps at the stage of RC biogenesis, and in which the requirement for GBF1 disappears after RCs are established. This model is in agreement with studies performed with Kunjin virus showing that the later in infection that BFA is added, the lesser the extent to which virally induced membrane structures are disrupted (72). This model is also in agreement with studies showing that BFA inhibits hepatitis C virus (HCV) to a weaker extent as infection becomes established (73). These experiments also provide further support that GCA-mediated inhibition of DENV infection is not caused by any cellular toxicity of GCA, because GCA pulses of the same concentration and duration either had no effect, a moderate effect, or a strong effect on DENV infection depending only on the time postinfection that they were added.

Perhaps the best characterized function of GBF1 is to activate ARF proteins, which in turn recruit various effectors such as lipid-modifying enzymes (74). Thus, one possibility is that NS5 recruits GBF1 to DENV RCs, where GBF1-activated ARF indirectly influences the lipid composition of the surrounding membranes. Mass spectrometric profiling of a membrane fraction enriched in replication activity prepared from DENV-infected mosquito cells revealed that this fraction exhibits a distinct lipid profile, compared with the analogous membrane fraction obtained from cells exposed to ultraviolet light-inactivated DENV (75). Of the many types of lipids enriched in DENV replication membranes, bioactive sphingolipids such as ceramides are some of the most interesting. These wedge-shaped lipids can induce negative curvature in membranes, which has been speculated to be important for inducing the convoluted morphology of RCs (75). Another lipid that has been shown to be up-regulated in DENV-infected cells is sphingomyelin (75). The pathogenic bacterium Chlamydia trachomatis co-opts GBF1 and ARF1 to acquire sphingomyelin for its intracellular membranous compartments (76). Thus, another possibility is that NS5 interaction with GBF1 allows DENV to co-opt membrane trafficking pathways to obtain lipids required for RC biogenesis. This hypothesis is consistent with our finding that the requirement for GBF1 is strongest early in DENV infection, and disappears as infection progresses (i.e. after RCs have formed). We also note that ZW10, a validated interactor of NS3 whose knockdown inhibited DENV replication by ~70% (Fig. 3), is also involved in trafficking between the ER and the Golgi (77), further supporting the importance of this pathway in DENV infection.

Another possibility is that the essential role of GBF1 in DENV infection involves lipid droplets (LDs), which have been shown to play a critical role in the DENV lifecycle (78). Interestingly, an LD-binding domain was recently identified in GBF1 (79). Furthermore, GBF1 and other COPII-related proteins have been shown to be important for LD metabolism (80, 81); GBF1 interacts with adipose triglyceride lipase (ATGL), a lipolytic enzyme required for the metabolism of stored triglycerides, and delivers it to LDs (81, 82). The NS5-GFP interaction data set also contains another interactor of ATGL, UBXD8, which negatively regulates ATGL by dissociating its co-activator (83). Thus, NS5 interactions with regulators of LD metabolism may affect the balance between energy storage and utilization at LDs.

We do not yet know whether the interaction between GBF1 and NS5 is direct, or if it is bridged by other host factors; the domains of each protein that mediate the interaction are also unknown. Current work is focused on addressing these questions. We also did not observe, by indirect immunofluorescence, any dramatic differences in GBF1 localization in DENV-infected cells compared with mock-infected cells (data not shown). However, we expect that only a small proportion of the total cellular pool of GBF1 is recruited to DENV RCs. This is because mislocalization of the entire population of GBF1 in DENV-infected cells would presumably drastically affect the integrity of the secretory pathway, which is needed.
for mature virions to exit the cell (59, 84, 85). Furthermore, only a small proportion of the total population of DENV NS5 resides in RCs, with the rest localizing to the nucleus (55, 59).

**GBF1 and Other Viruses**—Most, if not all, positive-sense single-stranded RNA (+ssRNA) viruses are thought to require intracellular membranes to form vesicular structures that facilitate replication (86). Although BFA blocks multiple trafficking pathways and causes dispersal of various organelles, including the Golgi apparatus and endosomes (87, 88) it has surprisingly diverse effects on infection by +ssRNA viruses. Some +ssRNA viruses, such as HCV (73), poliovirus (89), and DENV (this study) are highly sensitive to BFA. However, BFA has little to no effect on infection by other +ssRNA viruses, including foot-and-mouth disease virus (90), encephalomyocarditis virus (91), feline calicivirus (even at concentrations as high as 0.1 mg/ml (92)) and murine norovirus (93). The latter is especially interesting, as murine norovirus RCs appear to be derived from cellular membranes originating from the ER (93).

The observation that BFA-mediated inhibition of infection is not a property common to all +ssRNA viruses argues against general toxicity and/or broadly inhibitory effects on cellular trafficking being responsible for BFA-mediated inhibition of DENV infection, and supports the idea that the specific targets of BFA are involved.

GBF1 has been shown to be involved in the replication of HCV (classified in the *Flaviviridae* family with DENV) (73), as well as diverse other viruses, including poliovirus (46, 94, 95), Coxackie B3 virus (related to poliovirus) (96), and mouse hepatitis coronavirus (97). However, these viruses do not all appear to usurp GBF1 in the same manner. To the best of our knowledge, no interactions between any HCV protein and GBF1 have been described. Rather, the involvement of GBF1 appears to be indirect: HCV NS5A binds phosphatidylinositol 4-kinase IIIα (PI4KIIIα) and stimulates its activity (98, 99); PI4KIIIα is essential for recruiting GBF1 to Golgi membranes (100). Thus, for HCV, GBF1 has been proposed to contribute to the production of inositol phospholipids, such as phosphatidylinositol 4-phosphate, at the viral RC (101). This local lipid production has been shown to also rely on other COP1 components, such as ARF1 (101). However, KD of PI4KIIIα has been shown to have no effect on DENV infection (98).

Two related enteroviruses, poliovirus and Coxackie B3 virus, express a protein termed 3A that has been shown to bind to the N-terminal portion of GBF1 and recruit it to viral RCs (96, 98, 102, 103). For the enteroviruses, GBF1 appears to play some role in viral RNA replication other than the remodeling of cellular membranes into replication structures (94, 103). Interestingly, the BFA sensitivity of poliovirus infection could be rescued by a truncated version of GBF1, lacking the Sec7 domain (46). We attempted to rescue the GCA infection by overexpression of a similar version of GBF1, truncated immediately after the HUS domain (residues 1–709), and did not observe any effect (data not shown). Thus, GBF1 likely plays a different role in DENV infection than it does in enterovirus infection. Future studies will aim at determining the contribution(s) of GBF1 and/or ARFs to DENV RC biogenesis, and investigating the contribution of the GBF1-NS5 interaction to DENV infection.

**Acknowledgments**—We thank Dr. David Jans (Monash University, Victoria, Australia) for providing the infectious DENV cDNA clone (pDVWS601) and Dr. Stephen Whitehead (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) for graciously providing us with DENV protocols.

* This project was supported by the National Institute of Health Technology Centers for Networks and Pathways. We acknowledge funding from the National Center for Research Resources (5U54-RR022220-07/NIH) and the National Institute of General Medical Science (8U54-GM103511-08/NIH and P50-GM076547/NIH). This work was also supported in part with federal funds from the National Science Foundation MRI grant No. 0923536, from the National Institutes of Health National Institute of General Medical Sciences under grant Nos. 2P50 GM076547/Center for Systems Biology, GM087221, S10RR027584.

[This article contains supplemental Figs. S1 to S5 and Tables S1 to S4.]

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