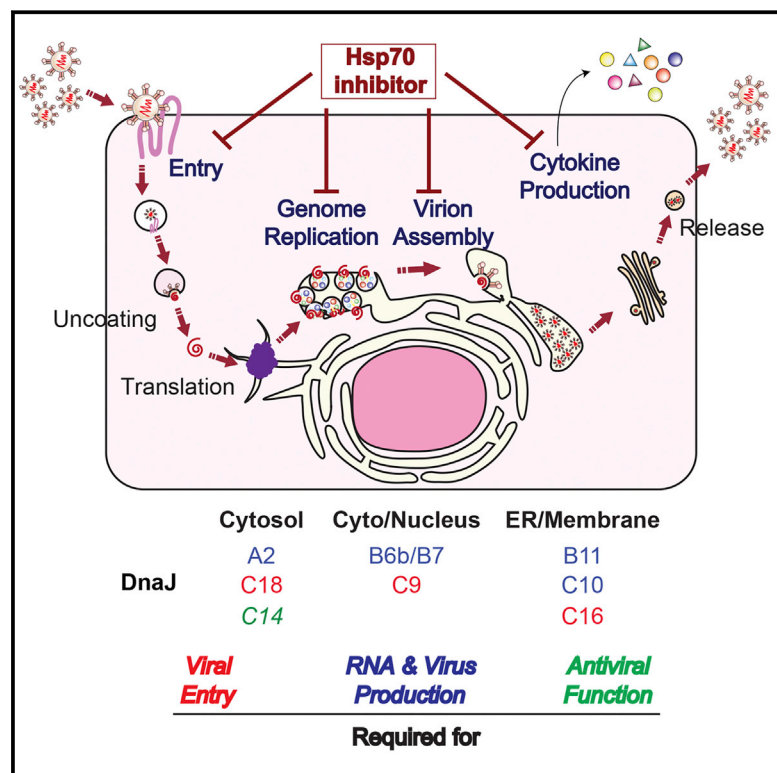# Cell

Defining Hsp70 Subnetworks in Dengue Virus Replication Reveals Key Vulnerability in Flavivirus Infection

Graphical Abstract

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In Brief

Dengue virus infects almost 400 million people annually with no treatment or vaccine. Hsp70/DnaJ chaperone network components are found to be required at distinct steps of dengue viral life cycle, with compounds that allosterically modulate Hsp70 showing potent antiviral activity against dengue and other flavivirus pathogens.

Highlights

- The Hsp70 chaperone network mediates distinct steps of the dengue virus life cycle

- DENV cycle requires Hsp70 for viral entry, RNA replication, and virion production

- Hsp70 function at each step of DENV cycle is specified by different DNAJ proteins

- Drug inhibitor of Hsp70 potently blocks DENV infection in human and mosquito cells

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Defining Hsp70 Subnetworks in Dengue Virus Replication Reveals Key Vulnerability in Flavivirus Infection

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SUMMARY

Viral protein homeostasis depends entirely on the machinery of the infected cell. Accordingly, viruses can illuminate the interplay between cellular proteostasis components and their distinct substrates. Here, we define how the Hsp70 chaperone network mediates the dengue virus life cycle. Cytosolic Hsp70 isoforms are required at distinct steps of the viral cycle, including entry, RNA replication, and virion biogenesis. Hsp70 function at each step is specified by nine distinct DNAJ cofactors. Of these, DnaJB11 relocalizes to virus-induced replication complexes to promote RNA synthesis, while DnaJB6 associates with capsid protein and facilitates virion biogenesis. Importantly, an allosteric Hsp70 inhibitor, JG40, potently blocks infection of different dengue serotypes in human primary blood cells without eliciting viral resistance or exerting toxicity to the host cells. JG40 also blocks replication of other medically-important flaviviruses including yellow fever, West Nile and Japanese encephalitis viruses. Thus, targeting host Hsp70 subnetworks provides a path for broad-spectrum antivirals.

INTRODUCTION

A third of the world population is at risk of infection with the mosquito-borne dengue virus (DENV) (Bhatt et al., 2013; Shepard et al., 2014), with an estimated 390 million infections annually (Bhatt et al., 2013). Any of four serotypes causes a range of severe diseases; dengue fever (DF) is a debilitating acute flu-like illness, while dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), with about 500,000 cases annually, are life-threatening diseases typified by vascular leakage and circulatory shock (Halstead, 2007)(Bhatt et al., 2013). DENV's major in vivo targets in humans are myeloid cells, including dendritic cells (DCs) and macrophages (Schmid et al., 2014). The dysregulated overproduction of cytokines and chemokines during DENV infection is thought to contribute to the increased vascular permeability, disruption of the coagulation system and shock associated with DHF/DSS (Rothman, 2011). Despite its burden on global health, no specific antivirals or vaccines are licensed for human use (Lim et al., 2013).

DENV belongs to the genus *Flavivirus* of the family *Flaviviridae*, comprising other clinically relevant arthropod-borne viruses such as yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV) (Lindenbach et al., 2007). Flaviviruses have a capped positive-sense single-stranded RNA genome of ~11 kb that encodes a single polyprotein, which is co- and post-translationally cleaved by host and viral proteases into three structural (capsid, prM, and E) and seven non-structural (NS1, NS2A/B, NS3, NS4A/B, and NS5) proteins (Apte-Sengupta et al., 2014; Lindenbach et al., 2007). Capsid encapsidates the genomic RNA and is then enveloped by membranes containing prM and E to produce progeny virions (Perera and Kuhn, 2008). The non-structural proteins are involved in viral replication and modulate the host cell environment by, for example, remodeling cellular membranes or inhibiting immune responses (Rothman, 2011; Rodriguez-Madoz et al., 2010).

Like all RNA viruses, flaviviruses are dependent on the host cell machinery for replication. With only ten proteins, DENV completely remodels the cell and generates an ER-derived membranous web (Junjhon et al., 2014; Welsch et al., 2009), where viral replication complexes are assembled. DENV proteins also associate with lipid droplets (LD) (Figure 1A) (Samsa et al., 2009). The ability of such a small genome to remodel the cell is linked to the structural and functional complexity of DENV proteins (Perera and Kuhn, 2008). This, in turn, presents a challenge to viral protein folding and assembly. Indeed, many viruses depend on host molecular chaperones for folding and proteostasis (Mayer, 2005; Nagy and Pogany, 2012). Chaperones are reported to facilitate flavivirus replication; ER chaperones associate with DENV E (Limjindaporn et al., 2009), and Hsp70 protects JEV NS3 and NS5 from degradation (Ye et al., 2013). However, the interplay between chaperone networks and the viral life cycle remains unclear. The structural complexity and accumulation to high levels of viral proteins may make viruses hyper-dependent on chaperones, and vulnerable to their inhibition (Neckers and Tatu, 2008; Geller et al., 2012).
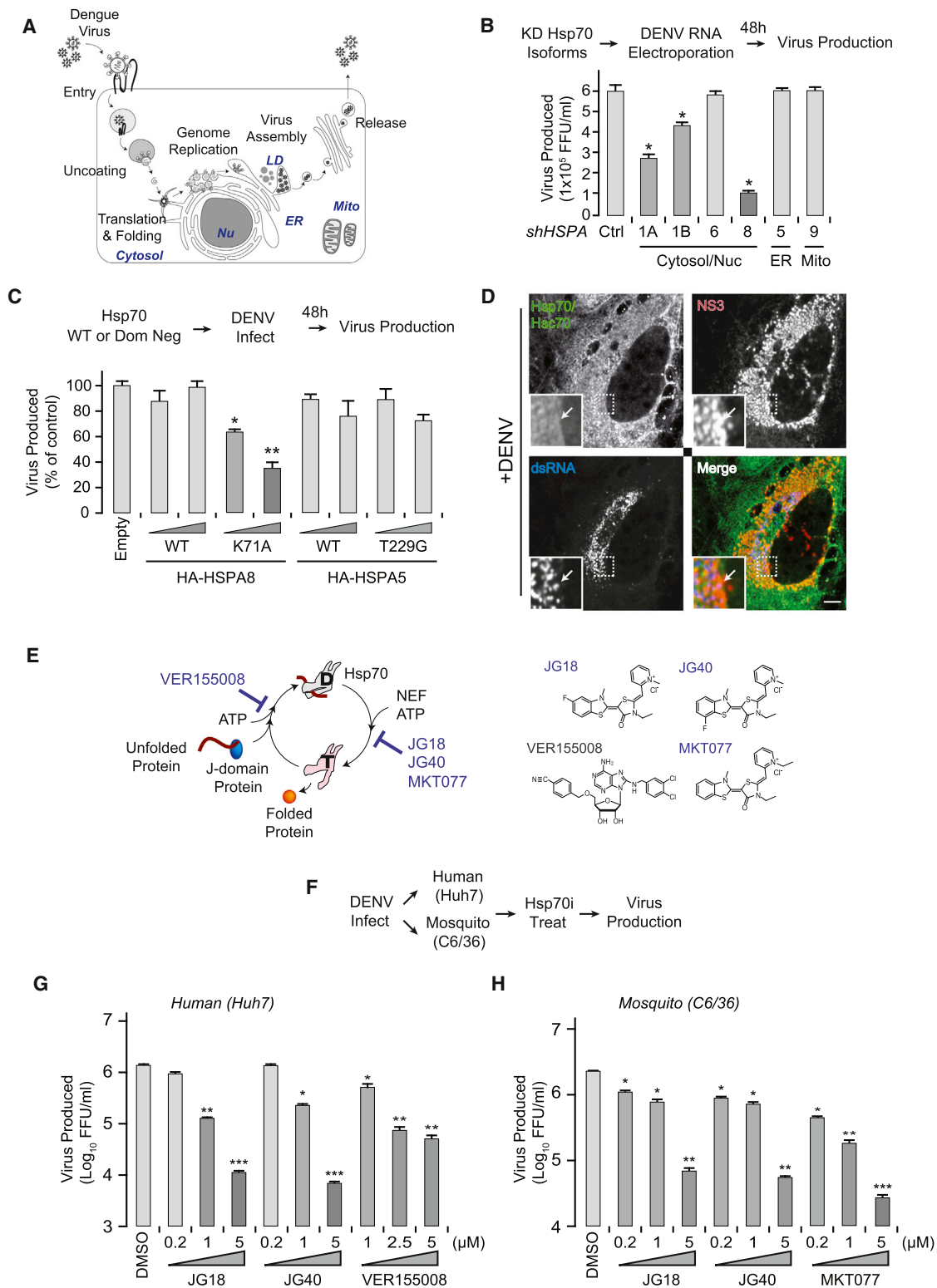
Figure 1. Cytosolic Hsp70 Isoforms Are Required for DENV Life Cycle

(A) DENV life cycle highlighting major steps and their subcellular localization. Nu, nucleus; LD, lipid droplet; Mito, mitochondria, ER; endoplasmic reticulum.

(B) Depletion of cytosolic Hsp70 isoforms reduces DENV replication. In vitro transcribed DENV RNA introduced by electroporation; 48 hr post-infection (hpi), supernatants were harvested and extracellular virus titrated by focus forming assay (FFA). Ctrl: shRNA against luciferase as a control.

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Pharmacological chaperone inhibitors are emerging as promising cancer therapies, particularly Hsp90 inhibitors (Neckers and Tatu, 2008; Trepel et al., 2010). A number of viruses are also sensitive to Hsp90 inhibitors (Geller et al., 2012), without evolving drug-resistant viral escape variants (Geller et al., 2013; Geller et al., 2007). Since drug resistance is a major stumbling block in antiviral treatment (De Clercq, 2007), the potential inability of viruses to become chaperone-independent makes chaperones attractive antiviral drug targets.

We have a limited understanding of the chaperone network architecture and its relationship to the organization of the cell (Hartl et al., 2011). One hallmark of the evolution of the chaperone machinery is the progressive diversification of chaperone isoforms and cofactors (Powers and Balch, 2013). In bacteria there is one Hsp70 chaperone and one Hsp70 cofactor, DnaJ, while in humans there are 13 Hsp70s and over 45 DNAJ-like proteins (Kampinga and Craig, 2010; Powers and Balch, 2013). This diversification may allow more nuanced regulation of chaperone activity in an increasingly complex proteome. Understanding how chaperone systems facilitate a complex phenotype like viral infection could provide a window into the functional specialization of chaperone isoforms. Here, we address the role of Hsp70 chaperones and their DnaJ cofactors in the DENV life cycle. We find that cytosolic Hsp70s, assisted by distinctly localized DnaJ's, are required at multiple steps of the viral life cycle. Importantly, Hsp70 provides a susceptible node for antiviral Hsp70 inhibitors, with negligible toxicity and no resistance. Hsp70 inhibitors block the replication of diverse DENV serotypes and other flaviviruses (WNV, YFV, TBEV). The multifaceted roles of Hsp70 subnetworks in flavivirus infection may provide the basis for broad-spectrum, resistance-free antivirals for a range of human diseases.

RESULTS

Hsp70 Is Required for DENV Infection of Human and Mosquito Cells

To explore the involvement of Hsp70 in the DENV lifecycle (Figure 1A), we established six Hsp70 isoform-specific shRNA knockdown (KD) cell lines (Figure S1A) and confirmed reduction of mRNA (50%–80%, Figure S1A) and protein (50%–95%, Figure S1B). While reductions in some Hsp70 isoforms upregulated others, Hsp90 immunoblot confirmed that a general stress response was not induced (Figure S1B). Depletion of cytosolic HSPA1A, 1B and 8, (also called Hsp70-1A, 1B and Hsc70, respectively) significantly reduced DENV infectious particle production (Figure 1B). In contrast, depletion of ER-resident Hsp70 HSPA5 (BiP) or mitochondrial Hsp70 HSPA9 had no effect on DENV (Figure 1B). KD of all cytosolic isoforms caused similar re-

ductions in viral RNA (vRNA) levels (Figure S1C) but HSPA8 had the most dramatic effect on virion production (Figure 1B). Furthermore, overexpression of dominant-negative cytosolic Hsc70, mutated in the ATPase active site (HSPA8 K71A) (O'Brien et al., 1996), significantly and dose dependently reduced vRNA replication and virion production (Figure 1C, S1D). In contrast, dominant-negative BiP (HSPA5 T229G) had no effect. Immunofluorescence analysis showed that cytosolic Hsp70s, while ubiquitous, were concentrated in viral replication complexes containing double-stranded RNA (dsRNA) and NS3 (Figure 1D; see S1E for uninfected control).

To overcome the confounding effects of functional redundancy between Hsp70 isoforms we employed a chemical biology approach. Hsp70 is a weak ATPase regulated by several sets of cellular cofactors (Figure 1E) (Hartl et al., 2011). DnaJ-like proteins contain a J-domain that binds Hsp70 and promotes ATP hydrolysis, stabilizing the substrate-Hsp70 complex. Many DnaJ homologs also have domains that help deliver substrate proteins to Hsp70. Nucleotide exchange factors (NEFs) promote the release of Hsp70-bound substrates. Chemical modulators that interfere with these enzymatic activities and protein-protein interactions have been developed (Assimon et al., 2013). One class of molecules, exemplified by VER155008, competes with ATP for Hsp70 binding (Williamson et al., 2009), while MKT077 and its analogs target Hsp70-NEF interactions (Figure 1E) (Assimon et al., 2013; Li et al., 2015; Li et al., 2013). We used both VER155008 and MKT077 and two improved MKT077 analogs, JG18 and JG40 (Figure 1E). Of note, MKT077 analogs act similarly to the Hsp70 mutant K71A, since both stabilize Hsp70-substrate interactions by blocking NEF binding (Wang et al., 2013). The binding site of MKT077 analogs is known and conserved across eukaryotes and all Hsp70 isoforms (Assimon et al., 2013), thus the compounds function in both natural DENV hosts, humans, and mosquitoes (Figures 1F–1H). Indeed, JG18, JG40, and VER155008 dose dependently blocked DENV propagation in human (Huh7) cells (Figure 1G), while MKT077, JG18, and JG40 suppressed DENV in mosquito (C6/36) cells (Figure 1H). The fact that these chemically distinct compounds block DENV propagation is strong evidence for a role for Hsp70 in DENV replication. Furthermore, the structurally similar but inactive JG18 and JG40 derivatives JG19 and JG28 did not suppress DENV propagation (Figure S1F). Thus, both genetic (Hsc70 K71A) and chemical (JG40, JG18) disruption of Hsp70-NEF function inhibited DENV replication, demonstrating the selectivity of the compounds and supporting a role for Hsp70 in DENV replication.

We next focused on the second-generation, metabolically stable MKT077 analogs, JG18 and JG40 (Li et al., 2013). A concern

(C) Overexpression of dominant-negative cytosolic Hsp70 but not ER Hsp70 BiP reduces DENV propagation. Wild-type (WT) or dominant negatives (K71A or T229G) of cytosolic (HSPA8) or ER (BiP) Hsp70 were transduced into Huh7 cells. Transfected cells were infected with DENV2 at MOI 0.5 and extracellular virus quantified 36 hpi by FFA.

(D) Hsc70/Hsp70 co-localizes with DENV replication compartments. Huh7 cells infected with DENV2 were stained at 48 hpi with a pan cytosolic Hsc70/Hsp70 antibody (green), dsRNA (blue) and NS3 (red), marking viral replication sites. Scale bar, 10 μm

(E) Hsp70 chaperone and ATPase cycle is regulated by co-chaperone cofactors and targeted by inhibitors. Hsp70 inhibitors used here: VER15508 blocks ATP binding to Hsp70. JG18, JG40 and MKT077 block binding of Hsp70 to Nucleotide Exchange Factors (NEF).

(F–H) Hsp70 inhibitors suppress DENV propagation in human and mosquito cells in a dose dependent manner. (F) Experimental design: Huh7 (G) and C6/36 (H) cells infected with DENV2 at MOI 0.5 for 1 hr, and inhibitors added for 36 hr (Huh7 cells) or 48 hr (C6/36 cells). Extracellular virus: determined by FFA. All data are expressed as means ± SD of three independent experiments each carried out in triplicate. *p < 0.01, **p < 0.005.
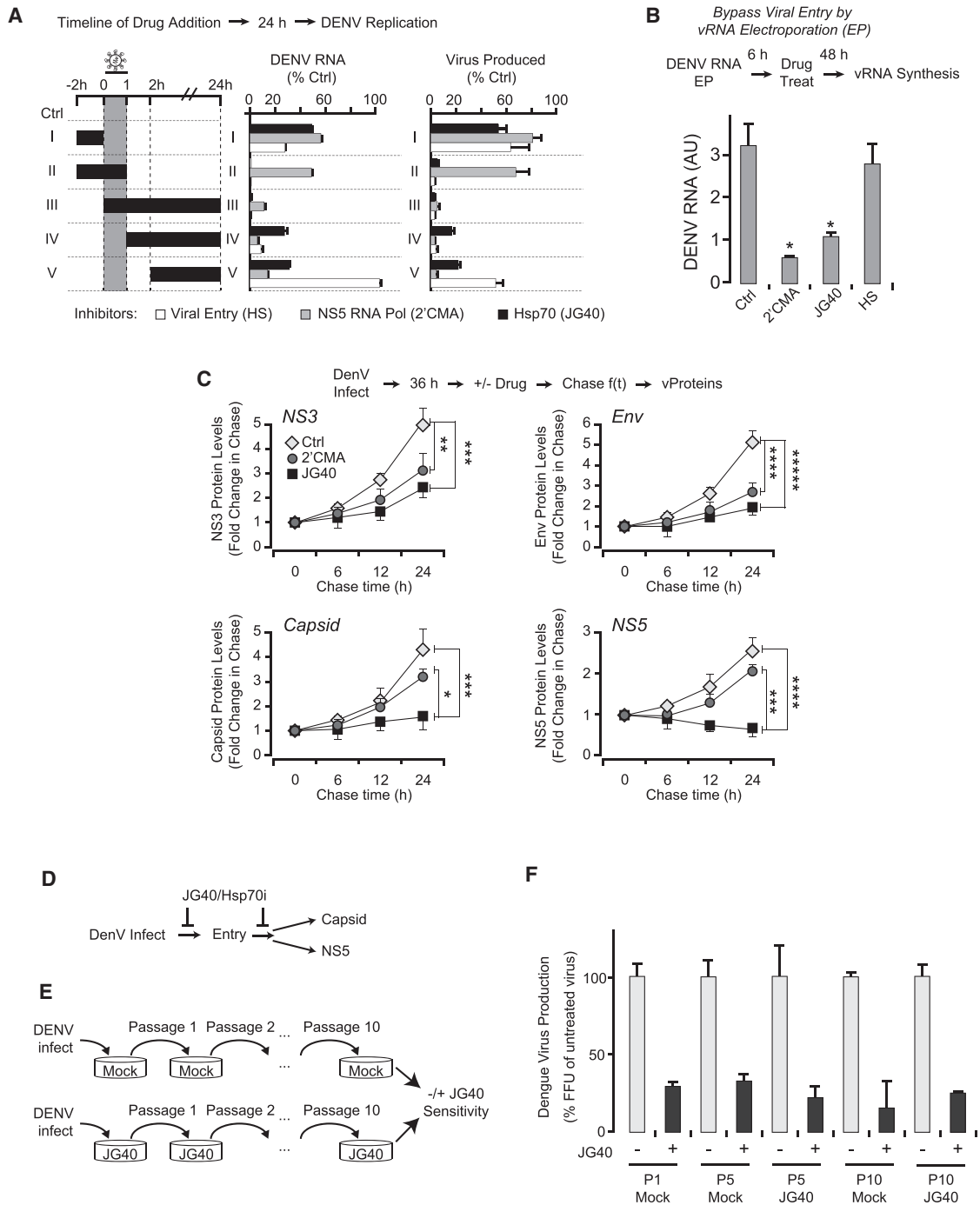
Figure 2. Hsp70s Facilitate Multiple Steps in the DENV Viral Life Cycle

(A) Hsp70 inhibition blocks viral entry and post-entry steps. Time course of drug-addition experiment vis-a-vis DENV infection compared JG40 (Hsp70i); entry inhibitor heparin (HP) and NS5 polymerase inhibitor (2′CMA). Huh7 cells infected with DENV2 at MOI 0.5. Intracellular DENV RNA and extracellular viral production: measured by qRT-PCR and FFA, respectively.

(B) JG40-treatment suppresses DENV replication post-entry. DENV RNA electroporated into Huh7 cells and inhibitors added 6 hpi. After 48 hr, intracellular vRNA was quantified by qRT-PCR.

(C) Hsp70 inhibition decreases DENV capsid and NS5 protein levels. Huh7 cells infected with DENV2 at MOI 0.5 for 36 hr. Inhibitors were then added and cells harvested as indicated. The levels of viral and host proteins during treatment were assessed by immunoblot using IR fluorescence on a Li-Cor Odyssey System (see Fig.S2F-S2G). (D) Hsp70 functions at different steps in the DENV lifecycle suggesting low chances of viral resistance to the drug.

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with Hsp70 inhibitors is that they may block essential chaperone functions, leading to host cell toxicity. However, allosteric inhibitors of the Hsp70-NEF complex are surprisingly non-toxic in normal fibroblasts and animals (Assimon et al., 2013; Li et al., 2015; Li et al., 2013), likely because they leave other chaperone functions intact. Consistent with the idea that viral infection creates a hyper-dependence on distinct chaperone activities compared to host proteostasis, neither JG18 nor JG40 was toxic to host cells at concentrations that inhibit viral replication, as measured by the complementary MTT and LDH release assays (Figure S1G).

Hsp70 Acts at Multiple Steps in the DENV Infectious Cycle

The activity of Hsp70 inhibitors on DENV replication was comparable to a previously described NS5 polymerase inhibitor, 2′C-methyladenosine (2′CMA; Figures S2A–S2E). Hsp70 inhibitors and 2′CMA suppressed vRNA synthesis (Figures S2A and S2B) and viral protein expression (vProtein; Figure S2C) in human and mosquito cells. To dissect which steps in the viral life cycle require Hsp70 in human cells, we performed an order-of-addition experiment using JG40, 2′CMA, and the entry inhibitor heparin (HP). As expected, HP only blocked vRNA replication and viral production (Figure 2A, series I-IV) when added prior to or concurrently with DENV infection (Figure 2A, series V). In contrast, 2′CMA was effective only when added concurrently with or after infection (Figure 2A, series III-V). JG40 inhibits DENV production and vRNA replication in any of these treatment regimes, indicating that Hsp70 is required both at entry and for post-entry steps (Figure 2A, series I-V). To bypass viral entry, we directly electroporated in vitro transcribed genomic vRNA into Huh7 cells in the presence of these various compounds. As expected, HP no longer inhibited viral replication, but both 2′CMA and JG40 inhibited vRNA production to a similar extent (Figure 2B). Thus, Hsp70 is required for both entry and post-entry steps of the viral life cycle.

A drug-chase experiment in infected cells next examined whether Hsp70 inhibitors specifically affect individual DENV proteins. Huh7 cells were infected with DENV for 36 hr; then either 2′CMA, JG40, or vehicle were added and the level of viral RNA and proteins examined during a 24 hr time course. Both 2′CMA and JG40 blocked vRNA production to a similar degree, and no new vRNA was synthesized during the chase (Figure S2A, 4F). Both drugs also decreased the expression of cytosolic and ER viral proteins (Figure 2C, S2F and S2G; see Experimental Procedures). All DENV proteins are translated in an equimolar ratio as a single polyprotein, but their steady-state levels are also determined by their half-life. For a given protein, the ratio between 2′CMA and JG40 treatment should reveal which proteins are further destabilized by Hsp70 inhibition, a hallmark of an Hsp70 substrate. The levels of cytosolic NS3 and membrane-bound E, prM, NS2B, and NS4B all decreased to a similar extent

upon 2′CMA or JG40 treatment (Figure 2C, S2G). In contrast, JG40 disproportionately reduced the levels of NS5 and capsid (Figure 2C, S2G). This is not due to positional effects on translation, since the capsid is the most N-terminal protein in the polyprotein and NS5 the most C-terminal. Thus, Hsp70 is required for folding and/or stabilization of capsid and NS5, suggesting these proteins are Hsp70 substrates (Figure 2D).

Dengue Virus Cannot Develop Resistance to Small Molecule Inhibitors of Hsp70

The mutational plasticity of viruses allows them to escape from most antiviral drugs targeting viral and even host factors (Dowd et al., 2014; Lauring et al., 2013). The spread of resistant viruses then renders drugs completely or partially ineffective. To determine if DENV can escape treatment with Hsp70 inhibitors, we serially passaged DENV in the presence of 2′CMA (Figure S2D) or 3 μM JG40 (Figure 1G). At each passage, the drug-sensitivity of the untreated and compound-passaged virus was tested in the presence of JG40 or 2′CMA (Figure 2E, S2H). Even after ten passages in the presence of JG40, the virus remained as sensitive to JG40 as untreated or parental virus (Figure 2F) while virus passaged in 2′CMA developed significant resistance against 2′CMA by passage 10 (Figure S2H). Similar results were obtained in at least three independent biological replicates (data not shown). These results, together with the low toxicity of JG40 in human cells, suggest Hsp70 inhibitors could provide an antiviral treatment against DENV replication that exhibit reduced emergence of drug resistant variants.

Cytosolic Hsp70 Is Required for NS5 Stability and Its Polymerase Function

To examine whether the observed decrease in NS5 upon Hsp70 inhibition is due to proteasomal degradation, cells infected with DENV for 36 hr were treated with JG40 for an additional 12 hr with or without the proteasome inhibitor MG132 (Figure 3A). Indeed, the JG40-induced reduction in NS5 was abrogated by proteasome inhibition (Figure 3A). Furthermore, Hsp70 appears to act directly on NS5 in the absence of other viral proteins, as transfected HA-NS5 was also degraded upon addition of JG40 in a proteasome-dependent manner (Figure 3B). NS5 also co-immunoprecipitated with Hsp70 (Figure 3C). Even though MG132 abrogated the JG40-induced NS5 reduction (Figures 3A and 3B), restoring NS5 levels did not rescue vRNA synthesis or virus production (Figure 3D). Since MG132 does not block DENV replication under the conditions of the experiment, it appears that Hsp70 is not only required for NS5 stability, but also for the acquisition of its folded, functional state. To test this, we isolated active crude replication complexes (RCs) from DENV-infected cells. These RCs produce vRNA in an NS5-dependent manner (Figure 3E), allowing us to examine the effect of JG40 on the activity of NS5 that has already folded and assembled into the large multi-component replication complex.

(E) Serial passaging of DENV in JG40 to test development of drug resistance. At each passage, Huh7 cells were infected with DENV2 at an MOI of 0.5 in the presence or absence of JG40. Virus produced in each passage served as input in the next passage in the absence (Mock) or presence (JG40) of drug.

(F) DENV remains JG40-sensitive following serial passage in the presence of JG40. JG40 sensitivity for the indicated virus passages obtained as above. Extracellular virus produced in the presence or absence of JG40 was quantified by FFU assay 36 hpi.

All data are expressed as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001, *****p < 0.0005.
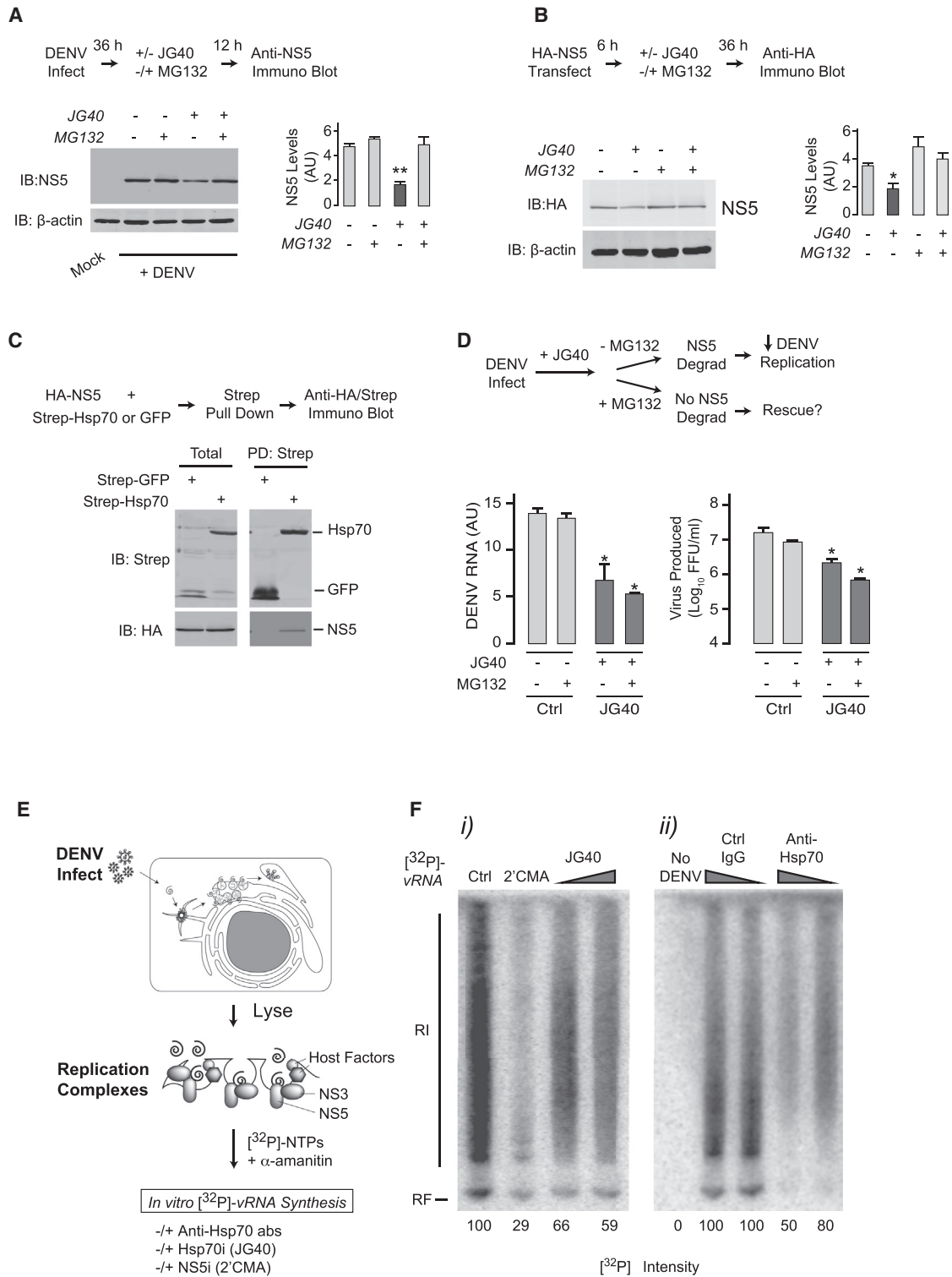
Figure 3. Hsp70 Required for Polymerase NS5 Biogenesis and Function

(A) Hsp70 inhibition leads to proteasomal degradation of NS5. Huh7 cells were infected with DENV2 at MOI 0.5. At 36 hpi, JG40 was added with or without proteasome inhibitor MG132 for an additional 12 hr. Immunoblot analysis (left panel) and quantification (right panel) show that JG40-induced NS5 decrease is rescued by MG132.

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α-amanitin was added to inhibit host RNA polymerase, and DENV vRNA synthesis monitored by incorporation of radioactive ^{32}P-nucleotides into dsRNA duplexes (replicative form; RF) and replication intermediates (RI) (Figure 3F). No radioactive species were detected using uninfected cells (no DENV, Figure 3F, ii), confirming their dependence on DENV RCs. As expected, the NS5 inhibitor 2′CMA blocked production of vRNA species (Figure 3F, i). Of note, JG40 also caused a reduction in vRNA synthesis (Figure 3F, i), and incubating the RCs with anti-Hsp70 antibodies blocked vRNA production in a concentration-dependent manner (Figure 3F, ii). We conclude that the large 100 kDa protein NS5 is a direct substrate of Hsp70, which is required for both stabilizing NS5 and for continued NS5 activity after replication complexes assemble.

Hsp70 Associates with Capsid Protein and Promotes Virus Production

A parallel analysis of the role of Hsp70 in capsid stability showed MG132 did not fully restore the drop in capsid levels observed with JG40 (Figure 4A). Since capsid associates with LDs (Samsa et al., 2009) and vesicles, we considered lysosomal degradation as an alternative degradation route. Despite the confounding fact that the lysosome inhibitor concanamycin A (CM) reduces overall DENV replication (not shown), co-incubation of JG40 with CM did partially restore capsid levels. We also observed an additive effect of blocking both proteasomal and lysosomal degradation (Figure 4A). JG40 also reduced levels of transfected HA-capsid, circumventing the effect of these inhibitors on DENV replication (Figure 4B). This reduction was partially abrogated by independent treatment with MG132 and CM, and additively blocked when both pathways were inhibited (Figure 4B). Thus, JG40 induces capsid degradation via proteasomal and lysosomal routes.

Hsp70 also associated with capsid in co-immunoprecipitation experiments (Figure 4C). Immunofluorescence analysis demonstrated that, as reported (Balinsky et al., 2013; Samsa et al., 2009), some of the capsid pool is directed to nucleoli, and in the cytoplasm capsid associates with LDs and nearby vesicles, forming puncta and ring-like structures distinct from sites containing viral dsRNA (Figures 4D and S3). We therefore questioned whether Hsp70 is required for the assembly of viral particles. 2′CMA or JG40 were used to inhibit vRNA production 36 hpi to compare the effect of the drugs on encapsidation of preexisting vRNA (Figure 4E). Both inhibitors rapidly blocked vRNA synthesis to a similar extent (Figure 4F), but had very different effects on the accumulation of intracellular (Figure 4G) and extracellular

infectious virus (Figure 4H). Cells treated with 2′CMA continue to produce viral particles and secrete them into the medium for at least 24 hr post-treatment. This suggests that by 36 hpi, DENV-infected cells contain sufficient vRNA and proteins to support the assembly of new virus particles. In contrast, JG40 treatment did reduce intracellular and extracellular virus accumulation, suggesting Hsp70 plays an additional role in virion biogenesis.

Selected J-Domain Proteins Are Required for DENV Replication

How and why chaperones select their substrates in vivo is an important and poorly understood question. Substrate selection and stable binding to Hsp70 is thought to rely on DNAJ proteins (Figure 5A), which in humans constitute the largest and most diverse sub-group of chaperones. All DNAJs contain a J-domain and additional domains confer distinct subcellular localization and recruitment to specific complexes. Depending on their additional domains, DNAJs are classified into three types: type I, also called DNAJA; Type II, or DNAJB; and Type III, or DNAJC (Kampinga and Craig, 2010). The diversification of J-domain proteins likely underlies the multifaceted regulation of Hsp70, however, few examples exist of the division of labor among different DnaJs.

To test if the distinct function of Hsp70 in the DENV life cycle is driven by different DnaJ proteins, we conducted a comprehensive screen using two or three shRNAs per J-domain protein in human cells. Thirty-five of the KD cells were viable (Figure 5A, S4A, S4B), and KD of DNAJs affecting DENV replication was confirmed by qRT-PCR and immunoblot (Figures 5B and 5C). DENV infection was significantly reduced upon depletion of eight DNAJ proteins (Figure 5A, S4A), and enhanced upon depletion of the known DENV restriction factor DnaJC14 (Yi et al., 2011). Following electroporation of vRNA, four of the DNAJ proteins no longer inhibited infectious virion production, suggesting they participate in viral entry (Figure 5D-F). In contrast, five DNAJs still inhibited DENV particle production (Figure 5D) and vRNA synthesis (Figure 5E), which we independently confirmed using enzymatically-produced esiRNAs (Figure S4C). The distinction between DNAJs required in entry versus post-entry steps was also confirmed by immunoblot for viral proteins following vRNA electroporation (Figure 5F). The DNAJ proteins required for DENV replication exhibit distinct domain structures and subcellular localizations (Figure S4D) (Kampinga and Craig, 2010). Those involved in entry include DnaJC9, reportedly nuclear localized, as well as the membrane-anchored DnaJC16

(B) Hsp70 inhibition directly leads to proteasomal degradation of NS5. 293T cells transfected with HA-NS5 were treated with JG40 with or without proteasome inhibitor MG132 for 36 hr and analyzed as in (A).

(C) Hsp70 physically associates with NS5. Strep-tagged Hsp70 or a GFP control were expressed together with HA-tagged NS5 in 293T cells, and their association assessed by coimmunoprecipitation and immunoblot. Total: represents 20% of the IP input.

(D) Rescue of NS5 levels by MG132 is not enough to restore DENV RNA replication and viral progeny production. NS5 degradation was blocked as in Figure 3A, and levels of intracellular viral RNA and extracellular virus production measured by qRT-PCR (left panel) and FFA (right panel), respectively.

(A, B and C) Data are expressed as mean ± SD of three independent experiments. *p < 0.05. **p < 0.01

(E) In vitro RNA replication assay to test the effect of Hsp70 inhibition on activity of preassembled, folded DENV replication complexes. Crude replication complexes were harvested from infected cells 48 hpi, and processed as described.

(F) Hsp70 is required for NS5 polymerase activity. [^{32}P]-vRNA synthesized in vitro in isolated replication complexes in the presence of NS5 or Hsp70 inhibitors (i) or following addition of control or anti-Hsp70 antibody (ii). No vRNA is synthesized in extracts from uninfected cells. The data shown are representative of three independent experiments.
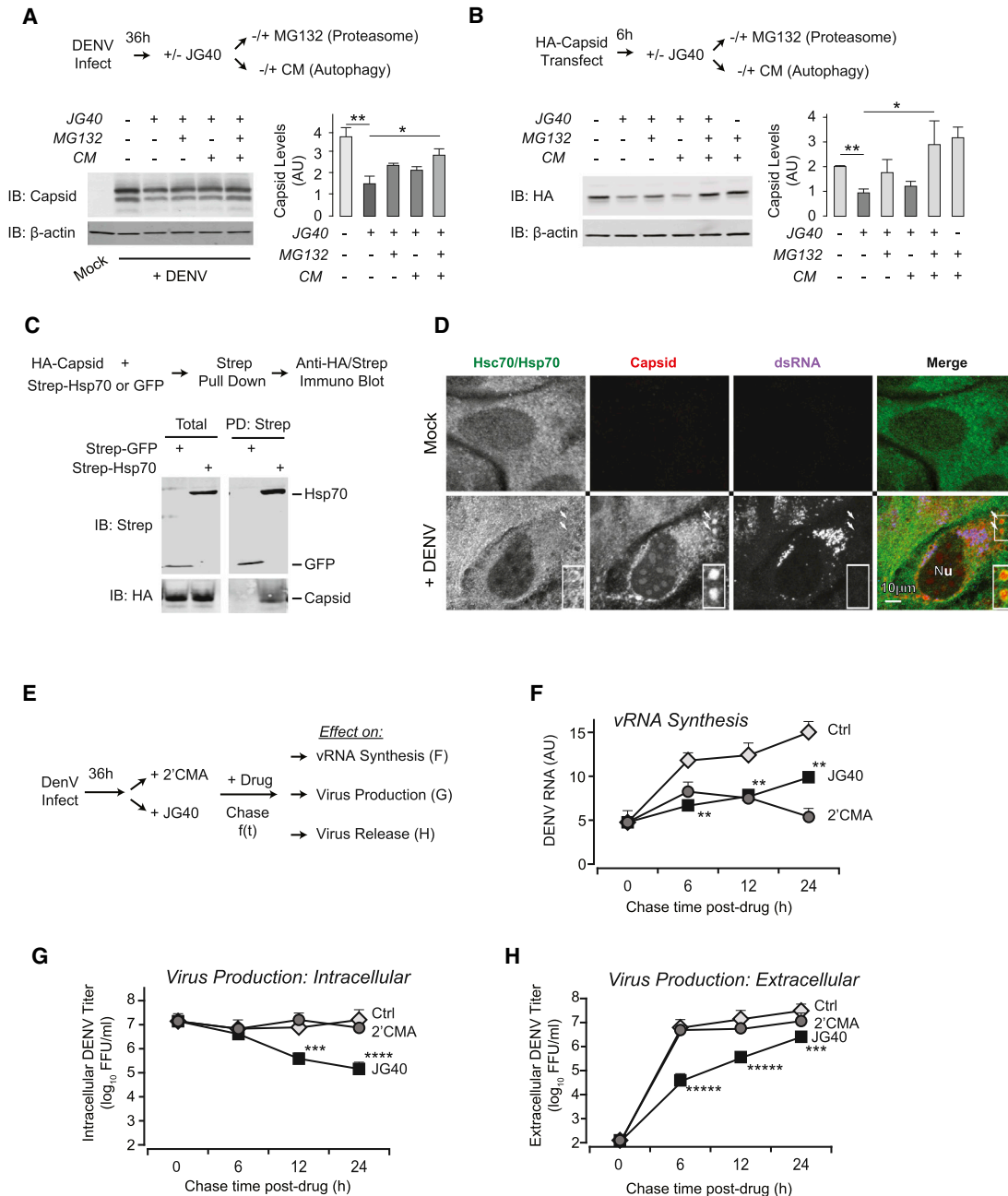
Figure 4. Hsp70 Interacts with Capsid and Participates in Virion Production

(A and B) JG40 treatment leads to capsid degradation through proteasome- and autophagy-dependent pathways. (A) Huh7 cells were infected with DENV2 at MOI 0.5. At 36 hpi, JG40 was added with or without proteasome inhibitor MG132 and/or lysosomal inhibitor CM for an additional 12 hr. Immunoblot analysis (left panel) and quantification (right panel) show that the JG40-induced decrease in NS5 is rescued by MG132.

(B) 293T cells expressing HA-capsid were treated with JG40 with or without MG132 and/or CM for 36 hr and analyzed as in (A).

(C) Hsp70 physically associates with capsid. Strep-tagged Hsp70 or a GFP control were expressed together with HA-tagged capsid in 293T cells, and their association assessed by coimmunoprecipitation and immunoblot.

(D) Hsp70 co-localizes with capsid in infected cells. Huh7 cells infected with DENV2 were immunostained for Hsc70/Hsp70 (green), dsRNA (blue) and capsid (red) 48 hpi. Insets: ring-shaped structures formed by capsid due to its association with LD and vesicles. Scale bar, 10 μm

(E–H) Hsp70 is required for infectious viral particle production. (E) Scheme of drug-chase experiment comparing the effect of the NS5 inhibitor (2′CMA) and the Hsp70i JG40 on vRNA production and virion production. Huh7 cells infected with DENV2 at MOI 0.5 were treated at 36 hpi with 3 μM 2′CMA or 3 μM JG40 and levels of intracellular DENV RNA was measured by qRT-PCR (F); intra- and extracellular virion levels (G and H) were measured by FFA. Data are expressed as mean ± SD of three independent experiments.

*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001, *****p < 0.0005.
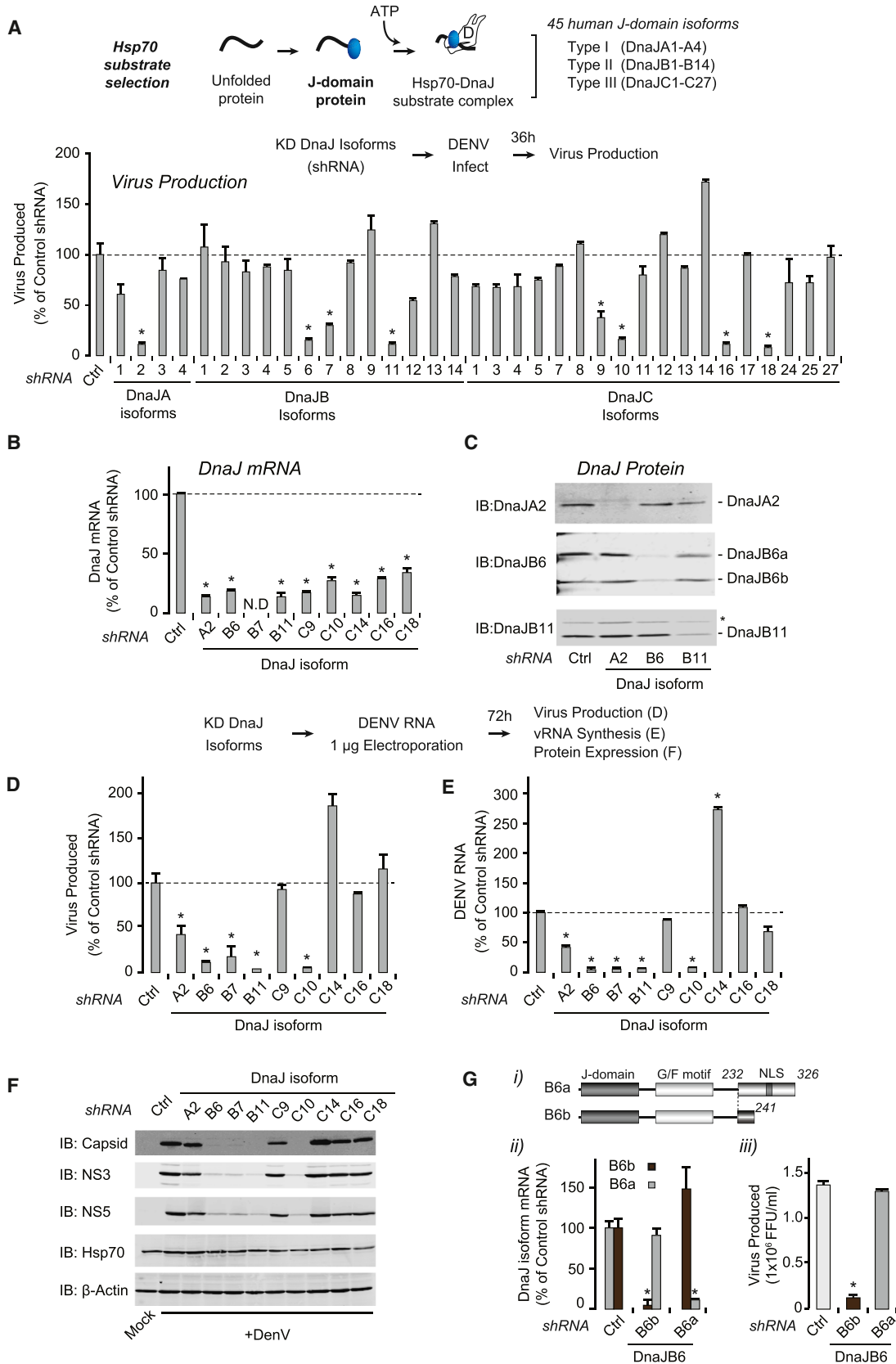
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and DnaJC18, which expose the J-domain into the cytosol. Post-entry steps require DnaJA2, DnaJB6, DnaJB7, DnaJB11, and DnaJC10. The cytosolic DnaJA2 significantly inhibited virus production and vRNA synthesis, with only a minor impact on viral proteins. The closely related DnaJA1 and DnaJA3 had little or no effect on DENV replication, indicating that related isoforms can have different substrate specificities. DnaJC10, a lumenal ER chaperone (Kampinga and Craig, 2010), may participate in folding ER-bound DENV proteins, or alternatively, host proteins required for DENV replication. Membrane-anchored DnaJB11 is also ER localized (Kampinga and Craig, 2010). DnaJB6 exists as two alternatively spliced isoforms: DnaJ6Ba is predominantly nuclear and DnaJB6b is nuclear/cytoplasmic (Figure 5G, i). We used shRNAs to selectively KD these splice-variants (Figure 5G, ii, S4E). DENV RNA replication, protein expression, and virion production were all significantly reduced in DnaJB6b KD cells but unaffected by DnaJB6a KD (Figures 5G, iii, S4E and S4F). Thus, closely related DnaJs, and even splice-variants, have divergent specificities and functions. We conclude that the distributed action of DNAJ cofactors localized in distinct cellular compartments orchestrates the many processes leading to productive DENV infection.

Distinct Roles of DnaJB11 and DnaJB6b in DENV Replication

We next focused on DnaJB11 and DnaJB6b, since their KD resulted in strong post-entry effects. To distinguish between a role in vRNA synthesis and viral particle assembly, we electroporated an excess of vRNA into cells depleted of DnaJB6b or DnaJB11. This incoming vRNA should provide enough template for viral protein synthesis and biogenesis, reducing the requirement for vRNA synthesis (Figure 6A). Depletion of DnaJB6b and DnaJB11 resulted in comparable reductions in vProtein (Figure 6A, i) and vRNA (Figure 6A, ii). However, viral particle production was virtually unaffected in DnaJB11-depleted cells, suggesting that significant reductions in vRNA and vProtein levels can have only modest effects on the output of viral particles. In contrast, DnaJB6b depletion significantly reduced infectious particle release (Figure 6A, iii). Therefore, DnaJB6b participates in viral particle biogenesis, while DnaJB11 is not required if sufficient vRNA and vProteins are present.

The observed reduction in viral infectivity upon DnaJB6 KD could be due to reduced particle production or impaired particle maturation. These possibilities make distinct predictions as to how DnaJB6 KD affects the ratio of infectious versus total virions (Figure S5A). To address this, we quantified vRNA genomes from equivalent amounts of focus forming units (FFUs) from the super-

natants of infected DnaJB6 KD or control cells. A reduction in infectivity would predict that more viral particles, i.e., more RNA genomes, will be required for the same number of FFU in DnaJB6 KD cells. This was not the case (Figure S5A), confirming that DnaJB6 is required for viral particle production itself.

Immunofluorescence analysis using confocal microscopy and super-resolution *s*tochastic *o*ptical *r*econstruction *m*icroscopy (STORM) (Figures 6B, 6C, and S5B) indicated that DnaJB11 is distributed throughout the cell, co-localizing with the ER, in uninfected cells. Upon infection, DnaJB11 was additionally enriched in dsRNA-containing DENV replication complexes (Figures 6B, 6C, and S5B,). Immunoprecipitation of DnaJB11 followed by RT-PCR analysis (Figure 6D) confirmed that both positive and negative vRNA strands associate with DnaJB11, probably in DENV replication complexes.

Since DnaJB6b is cytosolic and facilitates viral particle biogenesis, we tested its association with capsid, the cytosolic component of virions. DnaJB6b co-immunoprecipitated with transfected HA-capsid (Figure 6E). In DENV-infected cells, capsid was distributed throughout the cell and displayed a previously-described association with LDs (white arrow, shown in inset) and other vesicular structures (Figure 6F) (Samsa et al., 2009). While DnaJB6 localized to the cytosol and nucleus in uninfected cells (Figures 6F and S5C), DnaJB6 co-localized with capsid on vesicular structures and on the surface of LDs after infection, and the proportion of nuclear DnaJB6 was slightly reduced. These results suggest that DnaJB6 associates with capsid to facilitate viral particle biogenesis. To test whether DnaJB6b acts only through Hsp70, we employed two mutations known to disrupt different aspects of DnaJB6b activity (Figure 6G, i). The Hsp70 recruitment ability of DNAJB6 was disrupted by replacing its J-domain HPD motif with an AAA sequence. DnaJB6 is mutated in inherited myofibrillar myopathy and limb-girdle muscular dystrophy (Stein et al., 2014), and we also tested the effect of a disease-linked DnaJB6b mutation on DENV replication (Figures 6G and S5D, F93L). In DnaJ-depleted cells, only wild-type (WT) DnaJB6b fully restored viral production (Figure 6G, i–iii, and S5D). However, the HPD-AAA mutation, but not the F93L mutation, had a modest effect in restoring vRNA synthesis (Figure 6G, ii). Thus, the interaction of DnaJB6b with Hsp70 is important to promote viral particle biogenesis, though DnaJB6b may also possess intrinsic chaperone activity.

JG40 Inhibits the Replication of Divergent Flaviviruses in Human Dendritic Cells

Primary human monocyte-derived dendritic cells (MDDCs) are an established ex vivo model system for DENV (Figure 7A) (Aguirre


Figure 5. A Subset of Human DNAJ Co-chaperones Is Required for the DENV Life Cycle

(A) Screen for human DNAJ involvement in DENV replication. Forty-five human DNAJs were tested for effect on DENV by KD using three different shRNAs. Indicated DNAJ-KD cells were established as for Hsp70s in Figure 1, and infected with DENV2 for 36 hr. Extracellular virus production was measured by FFA. Ctrl: shRNA against luciferase.

(B and C) Validation of KD of different DNAJ isoforms by assessing mRNA levels (B) and DNAJ protein (C).

(D–F) Identification of DNAJs involved in post-entry stages of the DENV life cycle. DENV vRNA was electroporated into DNAJ-KD cells. Extracellular viral infectivity (D); intracellular viral RNA (E) and viral proteins (F) were measured after 72 hr, by FFA, qRT-PCR and immunoblotting, respectively.

(G) Isoform DnaJB6b is required for DENV propagation. (i) Splicing creates DnaJB6 variants. KD cells were established for each variant, and infected with DENV2 for 36 hr. The levels of each variant transcript (ii) and extracellular viral production (iii) were measured by qRT-PCR with variant-specific primers and FFA, respectively. Data are representative of three independent experiments and expressed as mean ± SD of triplicates. *p < 0.01.
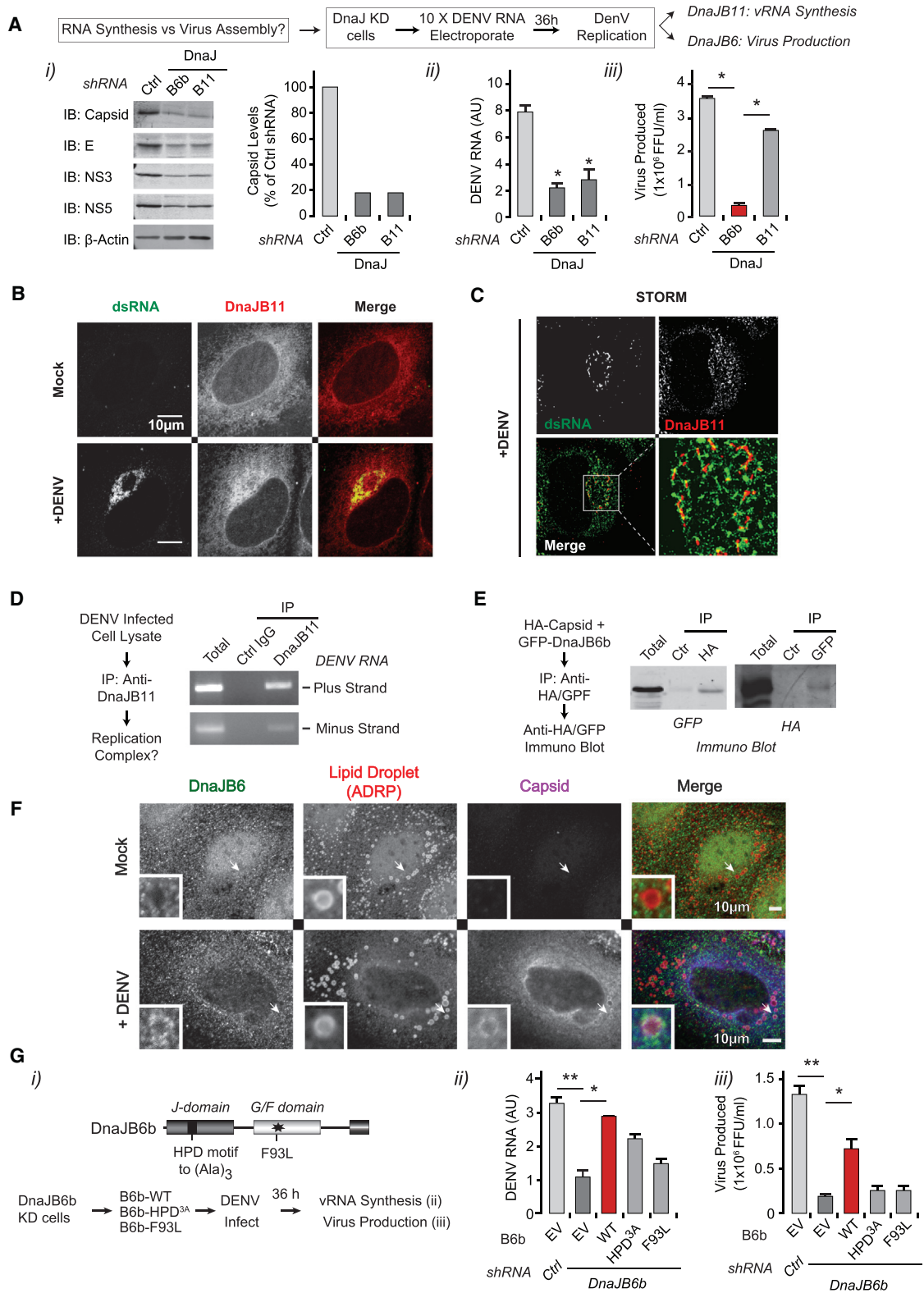
Figure 6. Spatial and Functional Diversity of DNAJs in DENV Propagation

(A) Introduction of excess DENV RNA by electroporation rescues DENV production in DnaJB11-KD, but not in DnaJB6-KD cells. Intracellular proteins *(i)*, viral RNA *(ii)*, and extracellular viral infectivity *(iii)* were measured by immunoblot, qRT-PCR, and FFA, respectively, at 36 hpi. *p < 0.05

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et al., 2012; Rodriguez-Madoz et al., 2010), allowing us to examine the therapeutic potential of Hsp70 inhibitors (Figures 7 and S6). JG40 dose-dependently inhibited DENV2 replication, to undetectable levels at 5 μM (Figure 7C) but did not reduce MDDC viability at these concentrations (Figure 7B). JG40 also reduced vRNA accumulation (Figure S6A). JG40's higher potency in primary MDDCs compared to Huh7 cells is interesting, since transformed cells have disregulated chaperone activity, making primary cells more responsive to chaperone inhibition (Trepel et al., 2010). The therapeutic window for antiviral use of chaperone modulators is also greater in primary cells (Geller et al., 2013; Geller et al., 2007). JG40 also inhibited DENV4, the most divergent of the four DENV serotypes (Figures 7C and S6A). We next examined the antiviral effects of JG40 on the flaviviruses WNV (strain Kunjin; KUNV), YFV, and the TBEV model Langat virus (LGTV). All three viruses were dose-dependently inhibited by JG40 (Figures 7D, S6A, and S6B), and in all cases infectious virus was undetectable with 5 μM JG40 (Figure 7D). JG40 is therefore a broad spectrum antiviral active against distantly related flaviviruses, many of which have limited treatment options.

Effects of JG40 on Cytokine and Chemokine Induction in DENV-Infected MDDCs

An ideal dengue therapy should attenuate the overproduction of cytokines and chemokines associated with DHF/DSS as well as viral replication. We therefore measured the effect of JG40 on relevant cytokines and chemokines produced during DENV2 or DENV4 infection in MDDCs (Figures 7E and S6C). The pro-inflammatory cytokines TNF-α, IL-6, and IL-1β induce fever and may cause vascular permeability during DENV infection; the chemokine RANTES is involved in leukocyte recruitment, and type I interferon (IFN) is involved in viral clearance but also contributes to the "flu-like" symptoms of dengue disease (Charo and Ransohoff, 2006; Rothman, 2011). As we showed previously, DENV2 does not induce type I IFN, and transiently induces pro-inflammatory cytokines and chemokines (Figure S6C) (Aguirre et al., 2012; Rodriguez-Madoz et al., 2010). In contrast, DENV4 induced higher levels of chemokines and pro-inflammatory cytokines (Figures 7E and S6C), perhaps due to intrinsic viral differences or due to higher replication levels (Figure 7C). JG40 dose-dependently reduced the induction of IFN-α, TNF-α, RANTES, IL-1β, and IL-6 in DENV4-infected MDDCs (Figure 7E). However, macrophage inflammatory protein 1β (MIP-1β), involved in leukocyte recruitment (Charo and Ransohoff, 2006), was unaffected (Figure S6C), indicating that JG40 does not grossly alter the ability of DCs to induce and secrete proteins.

In principle, the effects of JG40 on cytokine and chemokine production may stem from the inhibitor's effects on viral replication, and the resultant reduction in immunostimulatory pathogen-associated molecular patterns (PAMPs). However, when we directly stimulated MDDCs with the dsRNA mimic poly(I:C) without viral infection, JG40 still reduced TNF-α and RANTES induction, while IFN-α was unaffected (Figures 7F and S6D). Thus, JG40 directly modulates the production of a subset of cytokines independently of its effects on viral replication.

DISCUSSION

Due to their small genomes, RNA viruses rely on the host machinery to support their life cycle, providing a window into fundamental cellular processes. Here we examined the role of the complex Hsp70 chaperone network, central to protein homeostasis, in DENV replication. We find that distinct Hsp70 isoforms and their DnaJ cofactors orchestrate the proteostatic control of specific steps in the DENV life cycle. Distinct steps in DENV replication require different DNAJ Hsp70-cofactors localized to distinct cellular compartments. Our data illustrate how a non-specific chaperone like Hsp70 acquires functional and subcellular specificity through the action of DNAJ cofactors. Furthermore, compounds targeting Hsp70 appear to be promising antivirals, for several reasons. First, due to the dependence on Hsp70 at various steps of the viral life cycle, DENV cannot escape these drugs. Second, because these compounds allosterically modulate Hsp70 rather than fully block its activity, they exhibit negligible toxicity at concentrations that completely block virus production. This favorable therapeutic window likely arises from the chaperone-hyperdependence of the virus. Most excitingly, these compounds are effective against different DENV serotypes and diverse flaviviruses. Importantly, they also decrease the virally-induced release of inflammatory cytokines that contribute to severe dengue disease. The effectiveness of antivirals is severely limited by the emergence and spread of resistance (Lipsitch et al., 2007). However, so far no viral resistance has been observed for three unrelated viruses treated with inhibitors of two different chaperones, Hsp90 and Hsp70. This unique property of targeting viral proteostasis may close a gap in antiviral drug development.

Multiple DNAJs Facilitate Distinct Steps of the DENV Replication Cycle

The Hsp70 network has become progressively diversified in evolution (Powers and Balch, 2013). Our work on one simple
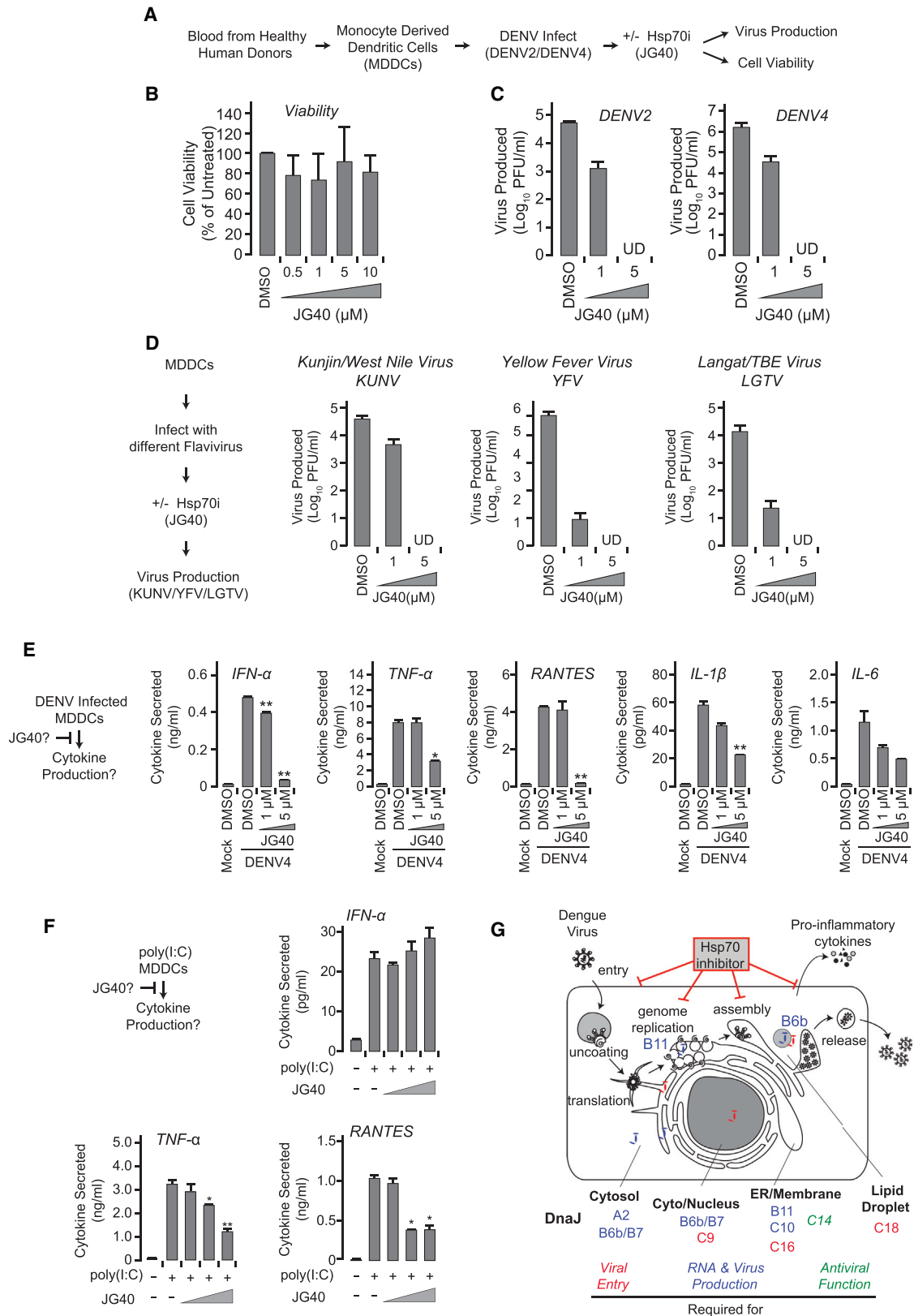

(B and C) DnaJB11 relocalizes to DENV replication sites. Uninfected Huh7 cells or cells infected with DENV2 for 48 hr were immunostained for DnaJB11 (red) and dsRNA (green) and imaged using confocal microscopy (B) and super-resolution microscopy by STORM (C). Scale bar, 10 μm

(D) DnaJB11 physically associates with DENV vRNA. DENV-infected cell lysates were subjected to immunoprecipitation with DnaJB11 antibodies. vRNA was extracted and positive strand and negative strand detected by RT-PCR.

(E) DnaJB6b associates with capsid in the absence of infection. GFP-tagged DnaJB6 or GFP were overexpressed together with HA-tagged capsid in 293T cells and association measured by immunoprecipitation and immunoblot.

(F) DnaJB6 co-localizes with capsid in infected cells. Uninfected Huh7 cells or cells infected with DENV2 for 48 hr were immunostained for DnaJB6 (green), ADRP (a marker of lipid droplets; red) and capsid (blue). Inset: magnification of capsid containing cellular structure. Scale bar, 10 μm

(G) Wild-Type DnaJB6b, but not its mutants can rescue the reduced DENV propagation in DnaJB6b KD cells. (i) DnaJB6b domain structure and position of HDP-AAA mutation, disrupting Hsp70 binding, and disease associated F39L mutation. DnaJB6b or its mutants were transfected into KD cells 48 hr prior to infection with DENV2 at MOI 0.5. At 36 hpi, intracellular viral RNA (ii) and extracellular virus production (iii) were measured as described. Data are representative of three independent experiments and expressed as mean ± SD of triplicate. *p < 0.05, **p < 0.01.

A

Blood from Healthy Human Donors → Monocyte Derived Dendritic Cells (MDDCs) → DENV Infect (DENV2/DENV4) → +/- Hsp70i (JG40) → Virus Production / Cell Viability

B

Viability — Cell Viability (% of Untreated) vs JG40 (µM): DMSO, 0.5, 1, 5, 10

C

DENV2 — Virus Produced (Log_{10} PFU/ml) vs JG40 (µM): DMSO, 1, 5 (UD)

DENV4 — Virus Produced (Log_{10} PFU/ml) vs JG40 (µM): DMSO, 1, 5 (UD)

D

MDDCs ↓ Infect with different Flavivirus ↓ +/- Hsp70i (JG40) ↓ Virus Production (KUNV/YFV/LGTV)

Kunjin/West Nile Virus KUNV — Virus Produced (Log_{10} PFU/ml) vs JG40(µM): DMSO, 1, 5 (UD)

Yellow Fever Virus YFV — Virus Produced (Log_{10} PFU/ml) vs JG40(µM): DMSO, 1, 5 (UD)

Langat/TBE Virus LGTV — Virus Produced (Log_{10} PFU/ml) vs JG40(µM): DMSO, 1, 5 (UD)

E

DENV Infected MDDCs — JG40? ⊣↓ Cytokine Production?

IFN-α, TNF-α, RANTES, IL-1β, IL-6 — Cytokine Secreted vs Mock, DMSO, DMSO, 1 µM, 5 µM JG40, DENV4

F

poly(I:C) MDDCs — JG40? ⊣↓ Cytokine Production?

IFN-α, TNF-α, RANTES — Cytokine Secreted vs poly(I:C), JG40

G

(legend on next page)

"organism" that uses the protein folding machinery provides insight into the division of labor and connectivity of the many DNAJs and Hsp70s in human cells. It is striking how many DNAJs are involved in viral replication. Nine DNAJs have strong effects upon depletion, while others, such as DNAJC25 or DNAJC13, have smaller but reproducible effects. As we observed strong effects with single-gene depletions, there must be limited redundancy among these DNAJ isoforms. Some DNAJs, e.g., DNAJB11, were reorganized upon infection (Figure 6) while others, e.g., DNAJA2, were not (data not shown). Future studies should clarify whether DENV actively remodels chaperone localization or whether the high levels of viral substrates concentrate their binding factors.

Our data indicate a role for Hsp70 and a subset of DNAJs in viral entry, and a direct role for Hsp70 and DNAJB11 in viral RNA synthesis and for Hsp70 and DNAJB6 in virion production. Hsp70 is not only required for folding and assembly but also for function of the active replication complex. Interestingly, DNAJB6 is important in the regulation of protein aggregation diseases (Hageman et al., 2010), suggesting this chaperone may function to regulate protein assembly processes. While these DNAJs have a direct role in viral proteostasis, at least some DNAJs may participate in host processes required by DENV.

Therapeutic Potential of Hsp70 Inhibitors as Pan-Flavivirus Antivirals

The Hsp70 inhibitor used here, JG40, caused over four logs reduction in DENV2 and DENV4 replication in MDDCs, with negligible toxicity to host cells. Since MDDCs closely model human physiology and disease, these reductions may more accurately reflect the dependence of DENV on the cellular protein folding machinery during human infection than cell lines. In addition, JG40 suppressed the production of proinflammatory cytokines and chemokines, suggesting that Hsp70 inhibitors might benefit patients both by directly inhibiting DENV replication, and by reducing the production of cytokines contributing to severe dengue disease. The comparison of JG40 with the specific NS5 inhibitor 2′CMA is informative for designing antiviral strategies. While 2′CMA is more specific and potent in inhibiting vRNA production, the infected cell makes sufficient precursors to continue new virus production long after inhibitor addition. In contrast, JG40 simultaneously inhibits replication of viral RNA and packaging of viral particles, increasing its effectiveness.

One exciting finding is that JG40 is effective against a diverse set of mosquito- and tick-borne flaviviruses that cause severe human disease (Figures 7 and S6). Our experimental design in primary human cells only measured post-entry effects, thus these compounds might be even more effective in vivo as viral entry would additionally be targeted. Future research should further compare the chaperone requirements of different flaviviruses at all stages of their life cycles.

Hsp70 inhibitors may represent a novel class of broadly-acting antivirals to treat diverse flavivirus infections for which there are no specific approved treatments. Considering their pan-flavivirus specificity alongside the lack of observed resistance, such compounds could become an important tool for reducing the worldwide human disease burden caused by these diverse viruses.

EXPERIMENTAL PROCEDURES

Infectious cDNA clone pD2/IC-30P-A of DENV2 (strain 16681) (from CDC [Butrapet et al., 2000]) was used to in vitro transcribe vRNA that was electroporated into Huh7 cells to produce infectious DENV2. Kunjin virus (KUNV) and Langat virus (LGTV) were kindly provided by Dr. Jean Lim (Icahn School of Medicine at Mount Sinai, NY, USA). DENV and yellow fever virus (YFV) stocks were prepared in Huh7 and C6/36 cells; KUNV and LGTV stocks were prepared on Vero cells. Virus titrations were carried out using the methylcellulose overlay method as described in Supplemental Experimental Procedures.

Cells were maintained at 37°C (Huh7) or 32°C (C6/36). MDDCs were generated from healthy human blood donors (New York Blood Center, NY, USA) as described (Rodriguez-Madoz et al., 2010). qRT-PCR quantification of purified RNA was performed using primers specified in Table S1. Transcripts were normalized to GAPDH or Rps11 mRNA (human) or 18S rRNA (mosquito). Viral genome equivalents were quantified using +ssRNA virus-specific standards. Each experiment was independently performed at least three times and each time was performed in triplicate.

Extracellular secreted cytokines were quantified using a customized multiplex ELISA (Millipore, Billerica, MA, USA) as per manufacturer's instructions.

Plasmid transfections, immunoprecipitations, immunoblots, isolation of DENV replication complexes, and in vitro DENV replication assay and other biochemical analyses were performed as described in the Supplemental Experimental Procedures. For immunofluorescence, cells cultured on glass slides were incubated with the relevant primary and secondary antibodies and imaged by confocal microscope and stochastic reconstruction microscopy (STORM) as described in Supplemental Experimental Procedures.

Statistical significance was determined using two-tailed Student's t test analysis for samples of equal variance, with sequentially rejective Bonferroni correction where appropriate. Significance levels are as stated in the figure legends.

Figure 7. JG40 Inhibits the Replication of DENV and Other Flaviviruses in Primary Human MDDCs and Modulates Cytokine Induction during DENV Infection

(A–C) JG40 potently inhibits different DENV serotypes at concentrations without toxicity to the host cell. (A) Effects of JG40 in primary human MDDCs at 36 hr post-treatment. (B) Host toxicity of JG40 was determined by LDH release into the culture supernatant. Differences are not statistically significant. (C) MDDCs infected with DENV2 or DENV4 at MOI 0.5 and subsequently treated with JG40 for 36 hr. The level of extracellular viral infectivity was measured by plaque assay. UD, undetectable. Data are the mean of three independent donors, expressed as mean ± SD.

(D) Broad Spectrum action of JG40 inhibiting the replication of diverse flaviviruses. MDDCs were infected with KUNV, YFV or LGTV at MOI 0.5 and subsequently treated with JG40 for 36 hr. The level of extracellular viral infectivity was measured by plaque assay. UD, undetectable. Data are the mean of three independent donors, expressed as mean ± SD.

(E) JG40 can suppress cytokine induction during DENV infection. Human MDDCs were infected with DENV4 (MOI 0.5) and subsequently treated with JG40 for 36 hr. Cytokine levels in the culture supernatant were measured by ELISA. *p < 0.05, **p < 0.01. One representative donor of three shown.

(F) JG40 directly affects the production or secretion of a subset of cytokines. Human MDDCs were treated with increasing amounts (0, 1, 5, or 10 μM) of JG40, and subsequently transfected with 5 μg/ml poly(I:C). Cytokine induction was measured by ELISA. *p < 0.05, **p < 0.01 (one-tailed Student's t test with Bonferroni correction). One representative donor of three shown.

(G) Role of the Hsp70/DNAJ network at various steps in the DENV life cycle, highlighting the therapeutic potential of Hsp70 inhibitors (see text for details).

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