Defining host factors uniquely required for antibody-dependent enhancement of dengue virus infection

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Abstract

Defining host factors uniquely required for antibody-dependent enhancement of dengue virus infection

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Dengue virus (DENV) causes 100 million symptomatic cases annually and represents a growing public health threat as the geographic range of its vector mosquitoes expands. However, there are no treatments for dengue disease and limited vaccine options. The antibody response to DENV represents a major barrier in developing safe and effective clinical interventions; intermediate IgG antibody titers are correlated with more severe dengue disease. The prevailing theory behind this observation is a phenomenon known as antibody-dependent enhancement (ADE) of DENV, where DENV uses non-neutralizing IgG antibodies to enter target cells expressing Fc gamma receptors (FcgR). Beyond the requirement of Fc-FcgR interactions, functional requirements of ADE of DENV infection are unknown because existing studies are unable to establish cause and effect. However, functional requirements of DENV in the absence of antibodies infection have been robustly identified via genome-wide CRISPR

knockout screens. Therefore, I used genome-wide CRISPR knockout screening in a model amenable to efficient infection only via ADE to investigate the functional requirements of ADE.

By performing genome-wide and follow-up targeted CRISPR screens, I identified and validated novel candidate host factors specifically required for ADE. Specifically, I found that knockout of SV2B or TBC1D24, genes typically involved in vesicle trafficking and regulated secretion in the nervous system, reduced the level of infection via ADE, but not direct infection, and *trans*-complementation of these genes restored ADE efficiency to wild-type levels. Knockout of these genes reduced ADE efficiency in various contexts, including in assays using sera from DENV-experienced donors, fully infectious virus of each serotype, and multiple cell lines. Finally, knockout of these genes reduced binding of antibody-virion complexes to cells without affecting FcgR expression levels. Overall, these studies show SV2B and TBC1D24 are required for ADE of DENV infection and suggest a role for endocytic pathways typically involved in synaptic vesicle trafficking in this non-canonical viral uptake pathway. The findings described in this thesis are a first step toward increasing fundamental knowledge of the biology of this alternative infection route implicated in disease, which eventually could be exploited to inform therapeutic approaches.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This thesis would not be possible without an incredible community of people around me, both past and present.

On the scientific side, I want to thank all my mentors and lab mates through the years. This begins with my first mentor, Thai Tran, who nurtured and encouraged my growth early on. I was an eager high school student with a lot to learn, and Thai went the extra mile to keep science challenging and engaging, including designing scientific thought experiments for me. He also always kept the mood fun, including trying to get me to agree to let him aikido flip me in the tissue culture room. Thai implanted a love of science and research that has carried me to where I am, and I am grateful for his mentorship. I am also grateful for Colleen Ziegler, who took over as my mentor following Thai's departure from the lab. She continued to foster my growth and support me along my journey as a young scientist.

As an undergraduate student, I had an important mentor who I gained outside of the lab but nonetheless encouraged my scientific and intellectual growth, Joseph Foy. He challenged me to grow as a writer and thinker, and always encouraged me despite my worries, even calling me Dr. Belmont during visits to his office hours during my time as his student and beyond. I also want to thank a lab-based mentor who was important to my growth, Shiv Singh. He encouraged me to grow in my scientific capabilities, while also thinking about what my goals were and how they fit into my life.

I also want to extend deep gratitude for my time as an undergraduate student in the lab of Grant McFadden, who has continued to support my endeavors over time. I originally was only supposed to be a student for one summer, but the lab and I liked each other so much that I stayed on until it was time to move for graduate school. The lab was such a warm, welcoming, and encouraging environment to grow as a scientist. This is especially because of the fantastic mentorship I received there from Ana Lemos de Matos, who I see as the person who nurtured

and grew the love of science that Thai had sparked. She was there for me not just as a mentor, but also on a personal level during times of personal struggle. She saw me for who I was and supported me, including encouraging me to take care of myself. Ana was also the person who told me about Fred Hutchinson Cancer Center and inspired me to try to work here during graduate school, shaping my entire graduate experience. We shared many days in the culture hood together and many late-night conversations about life and careers in general. I wouldn't be the scientist I am without her; I am deeply grateful for her mentorship. While Ana was on maternity leave, another lab member stepped up to guide me, Lina Franco Achury. We also spent many hours together conducting experiments and trying to troubleshoot our protocols. She, too, nurtured my growth as a scientist and person and was also part of many of those late-night life and career conversations. Lina served as a secondary mentor to me, and I am very grateful for her. Finally, I would also like to thank Jazmin Galván Achi, who was another undergraduate student I worked closely alongside and mentored. Her enthusiasm was always infectious, and she and I shared many passions around social justice and its intersections with science. She was a fantastic support system, and I am grateful we had overlap in the lab, and even more grateful to have gotten to see what she has accomplished since. Overall, the McFadden lab was full of amazing people, and I could not have asked for a better place to grow.

My scientific journey was fundamentally shaped by my graduate experiences in the lab of Leslie Goo. Leslie has been such a fantastic mentor throughout the tumultuous process of a PhD. I had never been one to ask questions at a talk, but I knew when watching Leslie pitch her lab to the first-year MCB students that it was the work I wanted to dive into as a PhD student, and that I needed to ask questions after her talk. Once she and I met for the first time to discuss a potential rotation, it was clear that we were on the same page about many of our values in science, and that it would be very hard to find a better-suited mentor for myself. Going into graduate school, I knew I wanted to study host-virus interactions, and that I wanted to be in the lab of a newer faculty member who deeply valued mentorship: Leslie fit this description

perfectly. The first day of my rotation made it even clearer that I had found the place I wanted to do my PhD in, and trying to even think of doing another rotation was challenging. Leslie did a fantastic job of modeling and building a team of people who truly cared about one another and the world around them. The lab was supportive and collaborative, and its members were bright and innovative to back it up. Leslie always was understanding of me and my needs, and tried to solicit and respond to the feedback I and others gave. She gave us time to spotlight our values, identities, and cultures, and encouraged us to grow both as people and scientists. She was the exact mentor science needs more of, as was her lab. I am incredibly grateful for all of Leslie's mentorship, and for her belief in me, even when I didn't have that same confidence in myself.

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vent. Maya was the next person to join the lab after the initial wave, and I was fortunate enough to serve as her mentor. I was thoroughly impressed by how quickly she learned both the information and techniques in our lab, and knew she was going to go so far. We were so lucky that she stayed in our lab after her summer internship, and again when she stayed after she had graduated. It was such an honor to see her grow and become more confident as a scientist and person. Her sense of humor and personality also made our time together great. I am extremely grateful for Maya's help; she was instrumental in conducting the experiments outlined in this thesis work, and this thesis would not have happened without her help and brilliance. Caroline joined the lab as an MD-PhD student around the same time as Maya and was also an amazing co-student. She was incredibly brilliant, dedicated, and hard-working, yet remained humble, kind, and caring. Her willingness to be herself, her humor, her compassion, and her intelligence have always impressed me. Finally, Catiana was the most recent addition to our lab, and always added a positive energy to the lab. She always tried to connect with lab members, brighten our days, and help in as many ways as she could. Her kindness, authenticity, and humor made the lab a better place to be. Overall, the Goo crew consisted of so many amazing individuals who are as compassionate as they are brilliant, and I am so grateful to have worked alongside each of them. I am also grateful for their endless contributions to this thesis.

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have remained in touch even throughout my graduate studies, and I am grateful for their support. Each of the people and groups mentioned in this paragraph have such a special place in my heart, and I am deeply grateful for their presence in my life.

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Chapter 1

INTRODUCTION

Dengue virus (DENV) is the most prevalent arthropod-borne virus infecting humans globally, resulting in around 390 million infections annually [1]. Of these infections, around 100 million are clinically apparent, manifesting as illness ranging from a mild fever to a potentially fatal hemorrhagic disease [2–4]. Currently, 2.5 billion people live in endemic areas and are at risk of DENV infection [1]. Further, the public health threat of DENV continues to grow as climate change and global connectivity expand the geographic range of its mosquito vectors[5,6]. Despite this threat, there are no treatments for DENV disease beyond supportive care and only two vaccines have been approved; one has a suboptimal safety profile, and the other is not fully available due to safety and efficacy concerns [7–9]. Safe and effective vaccines and therapeutics for DENV are needed, but the complicated antibody response to DENV, which can at times be protective against infection and at other times enhancing of infection levels, represents a major barrier to their development. Therefore, there is a need for further research on the antibody response to DENV to identify therapeutics to avoid and fight severe disease outcomes, as well as to design vaccines with better safety profiles.

Flaviviruses

DENV is an arthropod-borne virus in the family *Flaviviridae* and genus *Flavivirus*. Flaviviruses are single positive-stranded RNA viruses, and generally are categorized as either insect-specific viruses, having no known vector, or transmitted between a tick or mosquito vector and vertebrate host [10]. Humans are one possible vertebrate host and can experience a range of pathologies from flavivirus infection, including fevers, encephalitis, hemorrhagic fevers,

vascular shock syndrome, hepatitis, jaundice, myalgia congenital abnormalities and fetal death, and acute flaccid paralysis [1,11–18]. Some viruses of concern include DENV, which is endemic to areas where around a quarter of the global population lives; West Nile and Zika viruses which have expanded globally and result in high infectivity and morbidity in humans; Yellow Fever Virus which has had repeated outbreaks and can cause hepatitis or renal failure; Japanese encephalitis virus which is endemic to areas where three billion people live; and a variety of other flaviviruses emerging globally including Usutu virus and tick-borne encephalitis virus [1,11–18].

In addition to causing similar pathologies, flaviviruses share a genome structure which consists of two untranslated regions and a single open reading frame. The 5' and 3' untranslated regions promote viral translation and replication through interactions with viral proteins and modulate host immunity [1,19], while the single open reading frame is translated as a single polyprotein that is later cleaved by host and viral proteases into ten proteins (Figure 1). This includes three structural proteins, which are capsid (C), precursor membrane/membrane (prM/M), and envelope (E). In addition to these structural proteins, flaviviruses also encode seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

From the 5' end of the genome, the first structural protein is the C protein, which is responsible for encapsidating and thus protecting the viral genome, as well as binding viral RNA and promoting viral proliferation [20–22]. C protein is synthesized as C-prM precursor that is cleaved first by NS3 and later by signal peptidase, which allows for polyprotein processing to be paused until replication has begun [20–24]. The second structural protein is prM, which aids in folding and maturation of the E protein and prevents premature fusion of the E protein to the host cell membrane during transport through the Golgi [25–30]. prM also heterodimerizes with E protein to retain the complex within the ER [25–30]. Ultimately, furin cleaves prM to generate a mature M protein; however, this process is not always efficient and can thus prevent E from forming homodimers parallel to the envelope as in a mature particle, leading instead to partially

mature or immature virion particles [31,32]. As partially mature or immature virions display different epitopes, prM can contribute to antibody recognition through exposing different epitopes; additionally, prM can be an antibody target. The third structural protein is E, which mediates receptor binding and membrane fusion [11,33–38]. The E protein is a major antibody target, and therefore is the primary determinant of neutralization on flavivirus particles [11,33–38]. On mature flavivirus particles, E protein forms homodimers parallel to the virus envelope and consists of three domains, referred to as DI-III, that are connected by hinges that allow for conformational changes. Specifically, the DI-DII and DI-DIII interfaces are pH-sensitive hinges [11,33–40]. The fusion peptide is in DII and is buried in a hydrophobic pocket of DI-DIII, while DIII is thought to be a receptor binder given it is frequently targeted by antibodies and has a role in determining tropism [11,33–38].

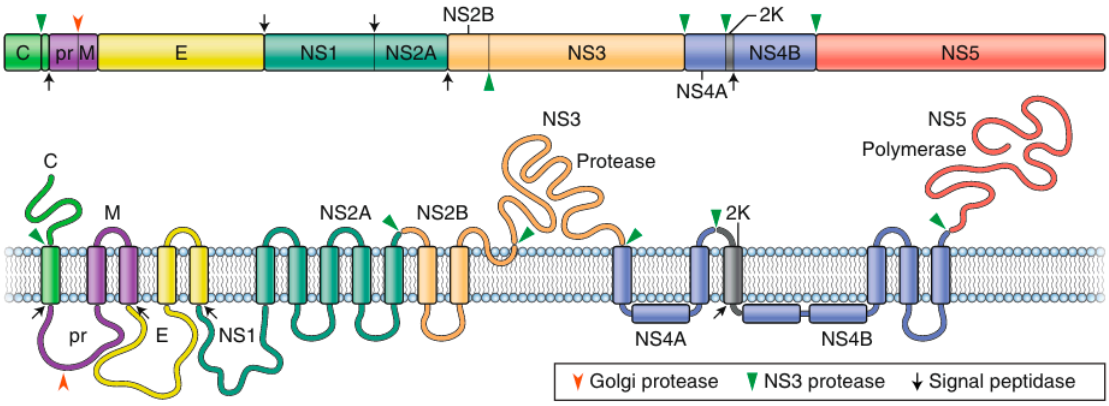

Figure 1.1. Flavivirus genome and polyprotein processing. The flavivirus single stranded positive RNA genome is organized as a single open reading frame translated as a polyprotein that is then cleaved by host and viral proteases into 3 structural (C, prM, E) and 7 non-structural proteins. The ten flaviviral proteins are shown as a polyprotein in the bottom half of the figure with proper membrane topology; protein segments below the membrane are in the ER, and segments above the membrane are in the cytosol. Image taken from Pierson and Diamond 2020, Figure 1[1]. Reproduced with permission, Springer Nature License #5837870321108.

The non-structural proteins, on the other hand, mostly play roles in mediating stages of the viral replication cycle and modulating innate immunity. The first nonstructural protein, NS1, exists in multiple forms; the secreted form is important for TLR activation and complement inhibition, while the intracellular form of NS1 participates in RNA replication and virus particle assembly [11,41–48]. Specifically, during viral replication, NS1 interacts with NS4A and NS4B to remodel the ER membrane to produce a structure known as the vesicle packet, while during particle assembly, NS1 interacts with prM and E [11,43–48].

Like NS1, NS2 and NS3 proteins have roles in viral replication, viral assembly, and antagonizing innate immunity. Specifically, NS2A recruits structural proteins and the 3' untranslated region of viral RNA to the virion assembly site [49–53]. NS2A also interacts with a complex formed by NS2B and NS3 to act as a serine protease that cleaves basic residues in viral proteins [49–53]. Beyond its role in the NS2B-NS3 complex, NS3 is also required for viral gene expression, encodes a nucleoside triphosphatase-RNA helicase that is required for RNA replication, and is involved in viral particle assembly [19,54–57].

Finally, NS5 primarily is involved in RNA synthesis and capping, though it also has some functions in antagonizing host immune responses. The RNA synthesis activity of NS5 is encoded by its C-terminal domain, which encodes the RNA-dependent RNA polymerase, while the capping activity is encoded by the N-terminal domain [58–61]. Both of these functions are accomplished through interactions with NS3, as NS3 assists in NS5's capping and synthesis activities, while NS5 assists with NS3's NTPase and RTPase activities [58–61]. The antagonistic activity toward the host immune response is primarily directed against the JAK/STAT pathway [58].

Flavivirus replication cycle

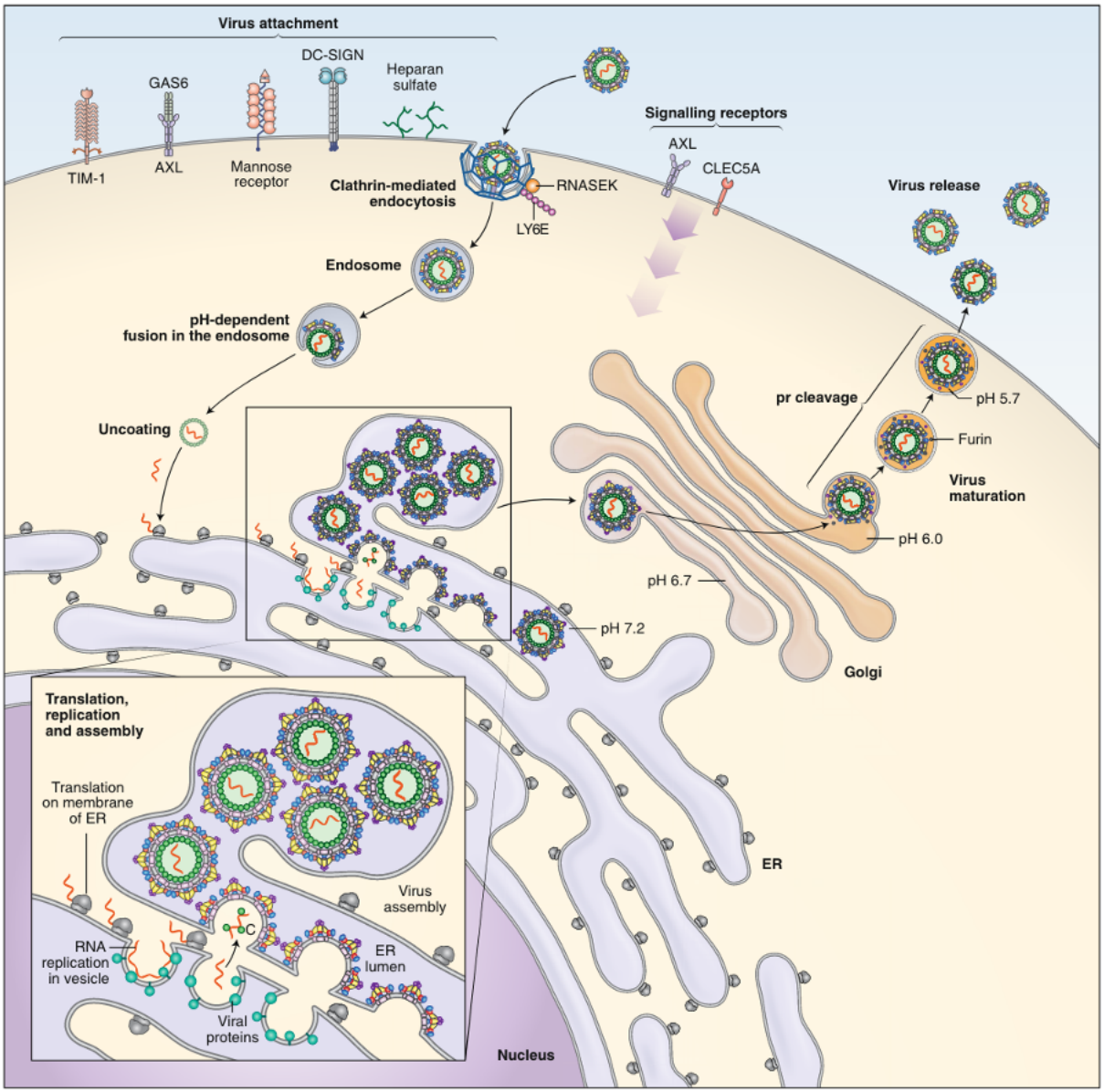

Figure 1.2 Flavivirus replication cycle. Flaviviruses begin their viral replication cycle when the virion binds to an attachment factor on the surface of the cell. Rather than one singular receptor, flaviviruses have been found to use one of many attachment factors to mediate entry, including those shown in the figure. Once bound to an attachment factor, the virion is then brought into the cell via clathrin-mediated endocytosis. The acidity of late endosomes triggers a conformational change in the envelope protein of the virion, allowing the viral and cell membranes to fuse. This also causes the viral genome to be released and travel to the ER, where it is translated and subsequently replicated. The

switch into replication is thought to be due to genome cyclization, which favors replication rather than translation [11] . Replication occurs within a replication organelle, which is a flavivirus infection-induced curvature in the ER membrane. Viral RNA then associates with structural proteins and buds into ER membranes. The immature virion is then trafficked through the acidic compartments within the trans-Golgi network, where it then goes through maturation, defined by furin cleavage of the pre-membrane protein, which then dissociates from the mature virion upon release from the cell. Image taken from Pierson and Diamond 2020, Figure 2 [1]. Reproduced with permission, Springer Nature License #5837870321108.

Beyond similar protein functions, flaviviruses also share a similar replication cycle, which is shown in Figure 2. Cell surface proteins absolutely required for flavivirus entry have not been identified. Instead, a variety of attachment factors have been described. Attachment factors include a variety of C-type lectins which are highly expressed on myeloid-derived cells, such as DC-SIGN on dendritic cells or mannose receptor, and phosphatidylserine receptors, normally involved in phagocytosis [1,11,62–65]. Additionally, heparan sulfates, which are anionic polysaccharides on cell surface proteins, can serve as attachment factors [66–68]. Phosphatidylserine often becomes enriched on viral envelopes which allows for them to be directly bound by receptors including in the T-cell immunoglobulin domain and mucin (TIM) domain family or the Tyro3, Axl, and Mertk (TAM) family [1,11,62,65,69]. Thus, factors like TIM-1 or Axl and its natural ligand Gas6 can also serve as attachment factors [65,69].

Following attachment, flaviviruses are brought into the cell via clathrin-mediated endocytosis and delivered to early or intermediate endosomes that later mature into late endosomes, which have a lower pH level [1,11,62]. During endosomal trafficking, this late endosome acidity triggers a conformational change in the envelope protein of the virion, causing the E-DII fusion loop to penetrate the host cell membrane and the E protein to fold into a fusogenic trimer [1,11,62,70]. This conformational change also triggers uncoating of the viral genome, which is delivered into the cytosol and transported to the ER membrane for translation

[1,11,62]. Translation relies on structures within the 5' and 3' noncoding regions and is cap-dependent. The polyprotein is cleaved co- and posttranslationally by a combination of host signal peptidases (which cleave C/prM, prM/E, E/NS1, and 2K/NS4B), NS2B-3 (which cleaves NS2A/NS2B, NS2B/3, NS3/NS4A, NS4A/2K, and NS4B/NS5), and an unknown peptidase (which cleaves NS1/2A) [1,11,62].

Following translation, the switch from genome translation to replication is mediated by genome cyclization [1,11,62,71,72]. During replication, negative-strand RNA is synthesized first to serve as a template for synthesizing positive-strand genomes. Genome replication and viral assembly are spatially coordinated via virus-induced ER membrane invaginations that make up a structure known as the flavivirus replication organelle [1,11,62,73–75]. Assembly of flavivirus particles begins with C protein dimers associating with genomic RNA and budding into ER membranes on which prM-E proteins associate [1,11,62,73,75]. Replication organelle pores are near assembly sites, which is thought to promote the linking between replication and assembly [1,11,62,73,75]. Viral RNA is recruited into budding virions and transported through the trans-Golgi network; the low pH triggers prM-E to undergo a conformational change [1,11,62]. The immature virion continues to mature as it is trafficked across the trans-Golgi network, where furin cleaves off the virion's pre-membrane protein; the pr component then dissociates from the mature virion upon release from the cell at the cell membrane [1,11,62].

The process of prM cleavage is inefficient, impacting virion structure and the corresponding antibody response. Complete cleavage of the pr component of prM results in E proteins that lay flat against the virion surface for a smooth virion (shown in Figure 3A and 3B), while incomplete prM cleavage results in E proteins that form heterotrimeric spikes (shown in Figure 3C and 3D) [32]. These differences in the arrangement of E proteins on the virion surface result in altered interactions with antibodies; uncleaved pr on incompletely mature virions exposes the otherwise cryptic E protein fusion loop, which is a target of poorly neutralizing antibodies with a high potential for mediating ADE. [32]. Further, antibodies can target prM itself;

these antibodies are primarily non-neutralizing [32]. Therefore, cleavage of prM can contribute to ADE of DENV which will be further described later in this introduction.

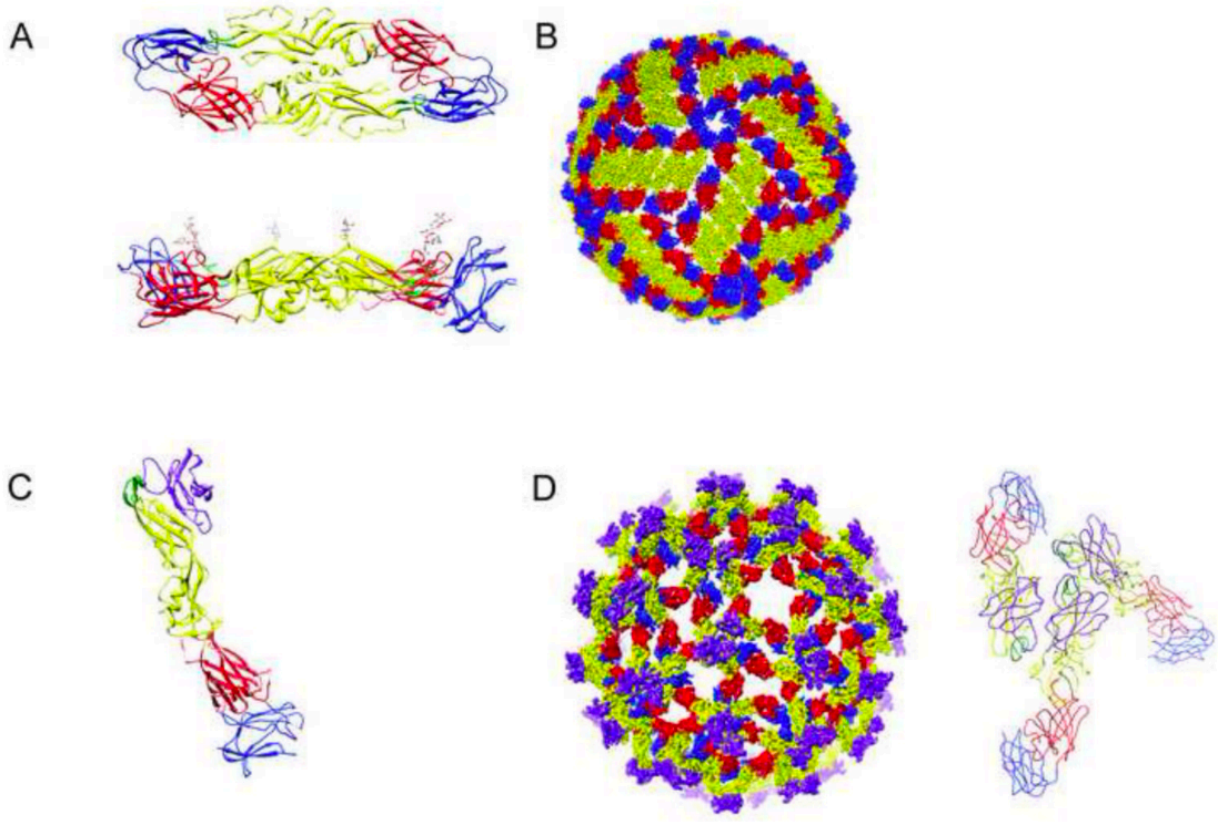

Figure 1.3. Structure of flavivirus virions and the organization of their E proteins. (A) The E protein is shown in its configuration during complete prM cleavage when the virion is mature, with domain III in blue, fusion loop in green, domain II in yellow, and domain I in red. **(B)** A mature flavivirus virion is shown, with 30 rafts of three antiparallel E protein dimers in a flat-lying herringbone pattern. This is the configuration of the virion during complete prM cleavage and virion maturation. **(C)** The E protein is shown in its configuration during incomplete prM cleavage when the virion is immature. Coloration is the same as in **(A)**, except prM is shown here, in purple. **(D)** An immature flavivirus virion is shown with arrangement of sixty prM-E heterotrimeric spikes. Image taken from Pierson and Diamond 2012, Figure 1 [32]. Reproduced with permission, Elsevier License #5837870827909.

Dengue

DENV is vectored by *Aedes* mosquitoes and undergoes a sylvatic cycle, meaning it occurs in wild animals, and a human transmission cycle. *Aedes aegypti and Aedes albopictus* are the primary vectors in the human transmission cycle, while arboreal *Aedes* mosquitoes are the main vectors for the sylvatic transmission cycle [76]. Sylvatic dengue strains are mainly hosted by non-human primates, and the emergence of distinct human strains likely occurred following ecological separation from sylvatic strains [76]. Indeed, evolutionary relationships between sylvatic strains and current DENV serotypes (DENV types defined by their different antigenic profiles, discussed more below) suggest the latter likely evolved independently after large urban populations were established through multiple spillover events [77].

Dengue serotypes

There are four circulating DENV serotypes, which are grouped according to serological cross-reactivity. Across serotypes, viruses differ by around 25-40% in their E protein amino acid sequence [6,78]. Within serotypes, DENV is further divided into genotypes, which are no more than 6% divergent in their nucleotide sequence and differ by around 3% in their E protein amino acid sequence [6,78]. Serotypes were first described after the observation that primary DENV infection with one type of DENV led to full protection against future infections of the same type, but only partial and temporary protection against future infections of different types [79–81]. This was then supported by *in vitro* neutralization experiments, as well as observations that antibody responses became more type-specifically protective over time and that antibodies to a heterologous DENV type were associated with enhanced infection or severe disease *in vivo* or clinically, respectively [82,83]. The different serological responses to the four serotypes have posed a challenge to developing safe and effective antibody-based therapeutics and vaccines for DENV. Within-serotype antigenic variation can also impact efficiency of antibody and serum

neutralization, and reinfection with homologous serotypes is also possible due to this variation [84–88].

Antiviral functions of antibodies

Antibodies are made up of two main fragments: the fragment that is antigen-binding (Fab) recognizes an epitope, and the fragment that is crystallizable (Fc) interacts with Fc receptors (FcR) on cells to mediate downstream effector functions. Different antibody types bind to different FcRs; for instance, IgG antibodies bind to FcgRs. In humans, there are a few FcgRs– FcgRI (FcgRIa/b/c), FcgRII (FcgRIIa/b/c), and FcgRIII (FcgRIIIa/b) [89–91]. All of the FcgRs are considered activating, meaning that they elicit immune functions such as antibody-dependent cytotoxicity, except for FcgRIIb, which provides negative feedback and inhibits FcgR activation [89–91].

Immune complexes crosslink with the FcgRs, which initiate downstream signaling pathways [89–91]. Activating receptors signal through immunoreceptor tyrosine based activating motifs (ITAMs) [89–91]. For activating receptors, this includes tyrosine phosphorylation of ITAMs by SRC kinases, which recruits SYK family kinases [89–91]. Downstream targets like phosphoinositide 3-kinase and others are then activated, creating docking sites for Bruton's tyrosine kinase and phospholipase C gamma [89–91]. These docking events then activate and lead to an intracellular calcium flux which triggers further downstream signals [89–91]. Downstream signals ultimately lead to a variety of effector functions, including antibody-dependent cellular cytotoxicity (ADCC), oxidative bursts, antibody-dependent cellular phagocytosis (ADCP), and cytokine release [89–92]. ADCC occurs when antibodies coat antigens and activate effector cells to bind the complex to a FcgR [89–92]. In an oxidative burst, FcgR crosslinking assembles the NADPH-dependent oxidase complex and promotes reactive oxygen species [89–92]. ADCP results from when IgG-opsonized virions are degraded in phagosomes

and digested by lysosomal enzymes [89–92]. During cytokine release, transcriptional activation occurs and drives the expression and release of pro-inflammatory cytokines [89–92].

On the other hand, the inhibitory receptor FcgRIIb has a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) [89–91]. Crosslinking of FcgRIIb recruits phosphatases to the ITIM domain, which hydrolyze phosphoinositide 3-kinase, meaning that downstream targets Bruton's tyrosine kinase and phospholipase C gamma are unable to be activated [89–91]. This inhibition is vital to modulating the antiviral functions of antibodies. However, these activating and inhibitory responses can instead be hijacked by viruses, such as DENV, to cause antibodies to play proviral functions during infection.

Proviral functions of antibodies

During DENV infection, a process known as antibody dependent enhancement (ADE) of infection can sometimes occur where antibody-bound virions are thought to be brought into the cell via interactions between the Fc portion of the antibody and the FcgR on the host cell.

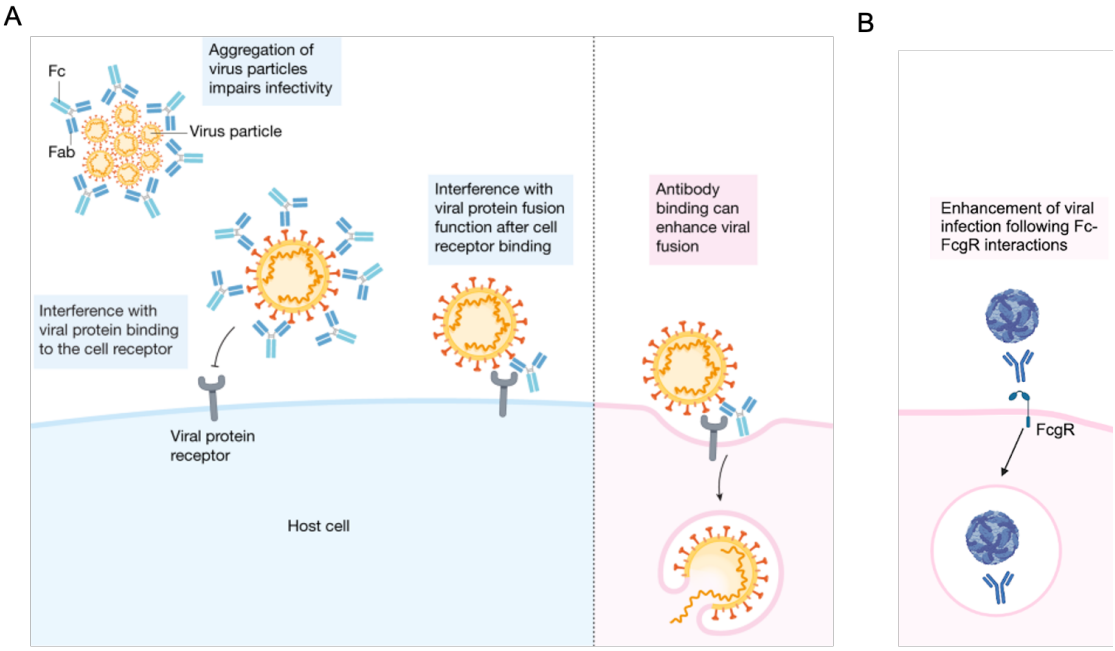

Figure 1.4. Antibody functions: neutralization, effector, or enhancement. (A) Antibodies can play a neutralizing role against infection by aggregating and surrounding viral particles, preventing viral

binding to host cell receptors, or blocking viral protein fusion following binding, as shown on the left side. Image is from Arvin et al 2020 [93], reproduced with permission, Spring Nature License #5837871064481. **(B)** Alternatively, antibodies can enhance viral infection through ADE, a process by which interactions between the Fc portion of the antibody and the FcgR bring the antibody-virion complex into the cell. Image was created with Biorender.com.

ADE is a phenomenon that can be observed *in vitro* for many viruses, but rarely has clinical implications, with the major exception being DENV (discussed more below). ADE was first observed when Murray Valley encephalitis virus (a flavivirus) or Ross river virus (an alphavirus) infected cells pre-incubated with antisera at a greater rate than infected cells treated with virus alone [94]. In respiratory syncytial virus (RSV) or influenza, some studies have reported correlations between cross-reactive poorly neutralizing antibodies and worse disease outcomes [93,95]. For instance, some children who were immunized against RSV had higher rates of hospitalization after developing non-neutralizing antibodies. However, the disease stemmed from the antibodies activating the complement pathway in the lungs and inducing lung tissue damage rather than enhancement of infection by antibodies [96]. In influenza, some studies showed a higher disease risk in individuals who had been vaccinated against influenza, citing cross-reactive poorly neutralizing antibodies, while other studies found cross-reactive antibodies to be protective [93,95]. However, overall in cases of both RSV and influenza, wider surveillance of disease suggests no association between antibodies and enhanced disease in humans [93,95]. In RSV, prophylactic monoclonal antibody treatment has been successful and morbidity of RSV does not correlate with changing antibody titers, while in influenza, cross-reactive antibodies largely protect against infection with other influenza strains [93,95]. Therefore, while ADE can often be observed *in vitro* with various viruses, it is much rarer to identify its relevance in human disease.

Antibody-dependent enhancement of dengue infection

While many viruses that exhibit ADE *in vitro* often do not show clinical relevance in human disease, DENV is an exception. In DENV infection, epidemiological studies have shown that pre-existing antibodies from a prior exposure to one DENV serotype are associated with increased likelihood of severe disease following subsequent infection with a different serotype [4,17,97–99]. Additionally, severe disease is more prevalent in infants born to DENV-immune parents following waning of in-utero passively transferred IgG levels [98]. The clinical relevance of this phenomenon is attributed to a few factors. First, there are multiple serotypes of DENV that are antigenically distinct from each other. Second, one prior infection with a given serotype does not confer immunity against other serotypes. Finally, DENV's primary target cells are FcgR-expressing cells such as myeloid cells, dendritic cells, and macrophages. The theory behind this clinically observed phenomenon is that virions bound to non-neutralizing IgG can hijack an alternative FcgR-mediated entry pathway to enhance dengue infection, known as ADE (Figure 3).

Whether antibodies enhance or neutralize infection is determined by the stoichiometry of antibody binding to virions. This is evident when DENV is used to infect FcgR+ cell lines such as K562 or U937 [100,101], which are poorly permissive to infection in the absence of IgG antibodies. In these cells, infection is generally neutralized at high antibody concentrations, while at intermediate antibody concentrations, infection is largely enhanced (illustrated in Figure 4). Further, concentration-dependence observed *in vitro* correlates with clinical observations that the risk of severe dengue disease is highest within an intermediate range of antibody concentrations [4,97,99]. Infant cases of dengue also demonstrate a dependence on antibody concentrations, as the greatest portion of severe dengue cases is observed when parentally-derived neutralizing antibody titers wane [98].

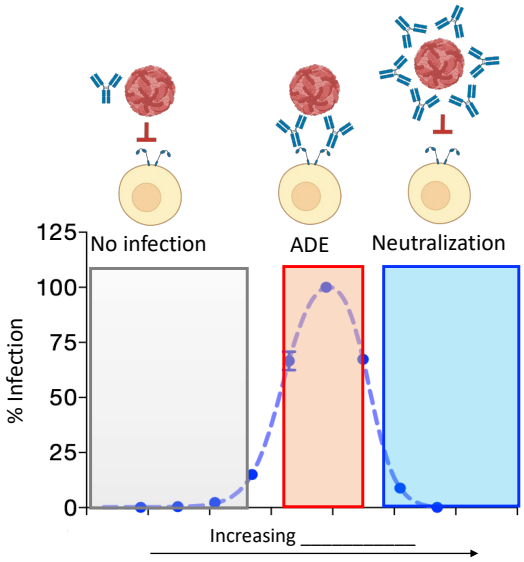

Figure 1.5. Stoichiometric dependence of ADE. The stoichiometric dependence of ADE is evident when DENV is used to infect K562 cells, which express FcgRIIa and are poorly permissive to infection in the absence of IgG antibodies. Thus, at low antibody concentrations, no infection is observed. At high antibody concentrations, antibodies can neutralize DENV. However, at intermediate levels of antibody concentration, enhancement of infection is observed.

ADE is hypothesized to be due to extrinsic and intrinsic mechanisms. Extrinsic ADE refers to the mechanisms by which the level of infection increases due to the presence of antibodies, whether by increasing the amount of infected cells or the infection rate of cells compared to the infection caused by the virus alone [94]. Intrinsic ADE, on the other hand, refers to the intracellular mechanisms that contribute to severity of infection and is thought to have a larger contribution to increasing viral production. Indeed, when interferon signaling is blocked, extrinsic ADE alone results in only 3-fold higher viral production compared to direct DENV infection, while intrinsic ADE (which includes intact interferon signaling) results in a 100-fold increase in viral production compared to direct DENV [102]. When ADE of DENV occurs *in vitro*, ligation of activating FcgRs (including FcgRI, FcgRIIa, and FcgRIIa) leads to suppression of cell signaling pathways that ultimately downregulate antiviral responses in ADE-infected cells [94].

For instance, activation of SARM and TANK blocks toll-like receptors 3,4, and 7 and ultimately suppresses type I interferon [94]. Transcriptomic studies have also indicated that individuals undergoing severe dengue disease have gene expression indicative of antiviral response suppression, such as downregulation of interferon and upregulation of IL10 compared to milder disease [94].Therefore, both the extrinsic and intrinsic mechanisms leveraged by ADE contribute to levels of DENV infection.

DENV thus represents an example of a virus that has both *in vitro* and epidemiological evidence to suggest ADE of infection. ADE has further been shown *in vitro* to be a concentration-dependent process that uses both extrinsic and intrinsic mechanisms to enhance viral infection and disease severity, characteristics that can also be modulated by other determinants further described below.

Determinants of severity of dengue disease and of antibody-dependent enhancement of dengue infection

Viral factors including serotype determine disease severity, though this varies across different locations of study. For instance, studies in Thailand showed secondary DENV2 infection was associated with higher risk of severe disease than secondary DENV1 or DENV3 infection, though secondary DENV3 infections also had a high risk of severe disease [103]. In Cuba, severe disease was most associated with DENV1 or DENV2 primary infection followed by DENV1 or DENV3 infection, while DENV2 followed by DENV3 had low severity [103]. This can be further complicated by the genotypes of the viruses themselves. For instance, a primary DENV1 infection followed by a secondary DENV2 infection has led to severe disease in some locales but not others due to amino acid differences in the envelope protein and untranslated regions of the virus [103,104]. More recent studies in Nicaragua have shown that a primary infection with ZIKV can also be associated with increased disease risk of DENV in a serotype-dependent manner: ZIKV increases disease severity risk with DENV4 but not DENV1 [99]. This

study also showed that pre-existing antibodies increased risk of severe disease following a secondary infection with DENV2 and DENV4, decreased risk of severe disease following DENV1 secondary infection, and had concentration-dependent effects on risk of disease severity following DENV3 infection [99].

Another factor that determines severity of disease and susceptibility to ADE of DENV is antibody isotype. Previously, studies on the roles of different immunoglobulin isotypes in dengue have focused on IgM and IgG; IgM responses were seen to precede DENV-reactive IgG, which reached higher titers during secondary infection [105]. Further, IgG antibodies were exclusively thought to mediate neutralization or enhancement of infection. However, recent studies from our lab and Wegman et al have identified the importance of IgA in DENV infections. IgA was initially observed to be the dominant antibody expressed during acute primary DENV infection (considered to be milder in disease), and was a smaller fraction in secondary dengue (associated with more severe disease) [105]. The Goo lab identified that a strong candidate for a broadly neutralizing antibody against DENV was natively an IgA antibody [106] that caused no ADE across a range of antibody concentrations [106]. Even further, when tested in combination with the IgG form, the IgA form of the antibody reduced the level of ADE caused by the antibody's IgG form proportional to ratio of IgA to IgG concentration. [106] These observations were further supported in another study which showed that IgA could not mediate ADE of infection and did not affect production of pro-inflammatory cytokines by primary human macrophages [105,107]. Together, these works suggest that antibody isotype dictates the ability of the antibody to mediate ADE *in vitro*.

Another antibody-related factor that impacts dengue disease severity and ADE potential includes antibody glycosylation status, particularly afucosylation, and antibody orientation. Afucosylation is the absence of fucose groups in the Fc region of the antibody, which increases antibody binding due to lower steric hindrance. Afucosylation of at least 10% of Fc glycans on antibodies has been linked to an elevated risk of symptomatic dengue infections in infants [108].

Further, afucosylation has been linked to more severe dengue disease during secondary DENV infection [109]. This is likely thought to be because afucosylation of Fcs increases affinity for FcgRIIIa, which enhances DENV replication in FcgRIIIa+ monocytes [108]. Further, the orientation of the antibody relative to the virion impacts its ability to trigger ADE. For instance, antibodies that are perpendicular to the virus surface are correlated with higher levels of ADE since the Fc region of the antibody is fully exposed for binding by FcgR [110]. On the other hand, antibodies that lay flat to the virus surface sterically hinder interactions between the Fc portion of the antibody and FcgR [111].

Finally, the viral protein and specific epitope targeted by the antibody impacts the severity of ADE of infection. Generally, antibodies to epitopes that are not well-exposed produce cross-reactive but poorly neutralizing antibodies (those prone to ADE), while antibodies to well-exposed epitopes produce potently neutralizing antibodies (not prone to ADE). Many weakly neutralizing (and thus prone to ADE) antibodies are to the fusion loop alone of the E protein as it is not well-exposed, or to prM as fully immature virions with prM are not infectious on their own but become infectious when complexed with an anti-prM antibody [112–116]. This is because the antibody response is defined by a stoichiometric threshold. The greater number of accessible epitopes the antibody can engage with, the higher chance there is that the virion can be neutralized. If instead an antibody targets a poorly accessible epitope, then there is less potential for neutralization, and instead higher potential for ADE of infection. [117].

Vaccines against dengue virus

The complicated antibody response to DENV has been a major barrier in creating a safe and effective dengue vaccine. Since ADE occurs at intermediate antibody concentrations of non-neutralizing IgG antibodies, vaccine candidates must trigger a balanced and lasting neutralizing antibody response to all four serotypes across a range of antibody concentrations.

These challenges are illustrated by the first licensed vaccine against DENV, a chimeric tetravalent live attenuated vaccine called Dengvaxia. As a tetravalent vaccine, Dengvaxia is comprised of the prM and E proteins of the four DENV serotypes combined with yellow fever nonstructural genes. Individuals receiving Dengvaxia who were previously DENV seronegative had the highest rate of hospitalization [118], suggesting the vaccine has a suboptimal efficacy and safety profile presumably due to an imbalanced polyclonal neutralizing antibody response to all four serotypes, thereby mimicking a round of infection. Therefore, DENV-naïve vaccine recipients were predisposed to more severe disease upon subsequent infection, while DENV-experienced vaccine recipients were protected since subsequent infections beyond a second heterologous infection induce more potent and broad neutralizing antibody responses [119–124]. Vaccine efficacy also varied among the four serotypes, with an efficacy of 63% against DENV1, 39% against DENV2, 75% against DENV3, and 74% against DENV4 [125,126]. Though multiple factors contributed to poor vaccine efficacy, one important factor is amino acid similarity between the strain used in the vaccine and the disease-causing strain encountered by the vaccine recipient [125–127] .

An additional live attenuated vaccine against DENV has been developed, called TAK-003. TAK-003 contains four DENV strains that use a DENV2 backbone instead of yellow fever virus [128]. So far, it has shown protection against DENV regardless of serostatus of vaccine recipients, though its overall efficacy (73%) seems to decline over time following vaccination [129,130]. However, more recent studies with longer follow-up times have shown that efficacy of protection against different serotypes varies in dengue-experienced versus dengue-naive recipients differs: the former are protected against all four serotypes, while the latter are protected against DENV1 and DENV2 only [131]. TAK-003 has been approved for use in individuals in some countries regardless of previous dengue infection history; however, as longer-term follow-up data becomes available, this will need to be re-evaluated, particularly in light of the potentially imbalanced protection observed in dengue-naive recipients [131].

A third DENV candidate vaccine that is farther along the development pipeline is TV003/TV005, which is also a live attenuated tetravalent vaccine. However, its components include three wild-type dengue viral strains of DENV1, 3, and 4 that have attenuating deletions, and a fourth component, which consists of DENV-2 prM and E genes substituted into the attenuated DENV4 backbone [132,133]. Initial testing of TV003 showed success, as it induced a range of seroconversion from 64% in DENV2 to 100% in DENV4; TV005 boosted the response against DENV2 to 84% [7,134,135]. The vaccine was then licensed in Brazil and tested, showing a balanced neutralizing antibody response again, this time all with all four serotypes above 78% seroconversion regardless of prior DENV exposure [7,135]. The balanced antibody response in both DENV-experienced and DENV-naive recipients is promising, as this indicates minimal risk of ADE. However, further testing is needed to confirm this, especially over time as antibody titers wane and drop to potentially sub-neutralizing concentrations which may instead promote enhanced disease.

Other vaccine candidates have been in development, spanning many formats including live-attenuated, inactivated, DNA, subunit, heterologous prime/boost, virus-like particles, and viral vectors [133,136]. However, these other vaccine candidates need further testing to determine their level of protection against all four serotypes [133,136,137]. Therefore, the imbalanced antibody response and the implicated ADE of infection remains a challenge among current vaccine candidates, highlighting the importance of further understanding of ADE and its role in vaccination outcomes.

Prior studies on ADE mechanisms

Prior studies contribute to our understanding of ADE but do not reveal its functional requirements. *In vivo* models of DENV fail to capture key aspects of pathogenesis or immunity [138]. Wild-type mice can restrict DENV through STAT2 and thus do not show signs of DENV disease, meaning mouse models must use mice that are immunocompromised or humanized,

or use DENV strains that are mouse-adapted [139]. However, the mice in these models often exhibit symptoms and antibody responses that differ from those observed in human disease [140–144]. Non-human primate models, an alternative to mouse models, have also shown limitations. While DENV can replicate in non-human primates, they do not experience clinical disease [145,146]. Therefore, lack of strong *in vivo* models of pathogenesis has led the field to rely on *in vitro* studies.

Many *in vitro* studies have focused on the impacts of ADE on the expression of different genes, particularly immune cytokines. Overall, it is thought that ADE upregulates expression of anti-inflammatory cytokines and downregulates expression of pro-inflammatory cytokines and anti-viral responses [101,147–154]. Additionally, ADE has also been found to induce changes in cell morphology such as membrane ruffling and extension of protrusions following actin redistribution; these changes have also been linked to PI3K [155]. However, these studies often have contradictory results and study only a subset of genes, rather than look comprehensively across the genome.

Other approaches to understand ADE have often compared gene and protein expression levels in serum from individuals experiencing mild versus severe cases of dengue disease. These studies identified the previously described correlation between antibody afucosylation and severe dengue disease [108,109], and have also identified changes in gene expression correlated with mild versus severe disease [156]. Studies on sera from people infected with examine the full transcriptome rather than just a subset of genes; however, it is impossible to determine the mechanism by which cells were infected, therefore making it unclear whether the observed effects are due to ADE.

These previous approaches to study ADE of DENV infection increased our understanding of ADE of DENV infection but have key limitations. In addition to the limitations described above, these studies determine effects on gene expression following infection rather than functional requirements for ADE of DENV infection to occur. Therefore, a comprehensive,

unbiased approach to determine host factors functionally required for ADE of DENV infection is lacking.

CRISPR Knockout Screening

Genome-wide CRISPR knockout screens have proven to be an invaluable tool in deciphering the functionally required host factors involved in viral infections, including in flaviviruses. While many prior genetic manipulations allowed for gene disruption, these techniques often resulted in a knockdown rather than complete knockout. However, CRISPR-Cas editing technology can efficiently induce complete knockouts in almost any cell type [157]. This results in stronger phenotypic changes and allows for screens that use CRISPR to have a higher ratio of truly enriched genes to false positives [157]. Multiple CRISPR knockout screens performed across several different groups have identified similar genes and pathways as vital host dependency factors. Two screens using DENV2 strain 16681 both identified endoplasmic-reticulum-associated genes involved in various functions– genes involved in ER translocation (such as SSR1-3, SEC61A1), genes in the ER-associated degradation pathway (such as EMC1-7), and genes in the oligosaccharyltransferase complex (such as STT3A, STT3B, OSTC, and OST4) were identified in both screens [158,159]. Later screens identified additional ER-associated genes, such as DPM1 and DPM3 [160], as well as genes such as TMEM41B with no prior role in DENV infection [161]. Each of these screens led to the validation of the roles of these genes in DENV infection both within and across studies, indicating the utility and robustness of genome-wide CRISPR knockout screens as a tool to identify the functional requirements of a viral infection.

CRISPR knockout screens address many limitations of prior approaches to study ADE: they yield highly consistent and reproducible results, can be used to survey across the entire genome in an unbiased manner, and can measure what genes are functionally required for the process being studied. However, prior CRISPR knockout screens performed to identify host

factors required for DENV infection used cells that could be infected with DENV in either the presence or absence of antibodies, meaning that it there is no way to determine whether the identified host factors play ADE-specific roles in DENV infection. Therefore, CRISPR knockout screening performed in a cell type that is poorly permissive to DENV infection in the absence of antibodies is needed to determine host factors specifically required for ADE of DENV infection.

Goals of this thesis

My overall goal of this thesis work was to identify and characterize host factors required for ADE, but not direct infection, of DENV. First, I will describe the CRISPR knockout screening and other methodologies used throughout this thesis (Chapter II). Next, I identify ADE-specific candidate host factors using genome-wide CRISPR knockout screening followed by a targeted sub-library screen. I then validate two candidate host factors identified in our screen in dose-dependent ADE assays using loss- and gain-of-function cell lines and investigate how broadly required these host factors are for dengue infection. Then, I study how these host factors mediate ADE of DENV. Finally, in Chapter III, I will discuss further studies stemming from this thesis work, and the implications of this work for ADE biology and design of clinical interventions for DENV. This thesis identifies two novel host factors required for ADE of DENV infection *in vitro*, providing insights into the fundamental mechanisms of an alternative viral uptake pathway.

CHAPTER 2

FUNCTIONAL GENOMICS SCREENS REVEAL A ROLE FOR TBC1D24 AND SV2B IN ANTIBODY-DEPENDENT ENHANCEMENT OF DENGUE VIRUS INFECTION

Text in this chapter is adapted from the following manuscript:

Belmont L, Contreras M, Cartwright-Acar CH, Marceau CD, Agrawal A, Levoir LM, Lubow J, Goo L. Functional genomics screens reveal a role for TBC1D24 and SV2B in antibody-dependent enhancement of dengue virus infection. *bioRxiv* 2024.04.26.591029; doi:10.1101/2024.04.26.591029

Abstract

Dengue virus (DENV) can hijack non-neutralizing IgG antibodies to facilitate its uptake into target cells expressing Fc gamma receptors (FcgR) - a process known as antibody-dependent enhancement (ADE) of infection. Beyond a requirement for FcgR, host dependency factors for this non-canonical infection route remain unknown. To identify cellular factors exclusively required for ADE, here, we performed CRISPR knockout screens in an *in vitro* system permissive to infection only in the presence of IgG antibodies. Validating our approach, a top hit was FcgRIIa, which facilitates binding and internalization of IgG-bound DENV but is not required for canonical infection. Additionally, we identified host factors with no previously described role in DENV infection, including TBC1D24 and SV2B, both of which have known functions in regulated secretion. Using genetic knockout and *trans*-complemented cells, we validated a functional requirement for these host factors in ADE assays performed with monoclonal antibodies and polyclonal sera in multiple cell lines and using all four DENV serotypes. We show that knockout of TBC1D24 or SV2B impaired binding of IgG-DENV complexes to cells without affecting FcgRIIa expression levels. Thus, we identify cellular factors beyond FcgR that

are required for ADE of DENV infection. Our findings represent a first step towards advancing fundamental knowledge behind the biology of ADE that can ultimately be exploited to inform vaccination and therapeutic approaches.

Introduction

The complicated antibody response to the four circulating serotypes of dengue virus (DENV1-4) represents a major barrier to the development of safe and effective vaccines and therapeutics. Specifically, primary infection with one DENV serotype does not confer durable immunity against infection by the other three serotypes. Instead, the biggest risk factor for dengue disease is secondary infection with another DENV serotype in the presence of pre-existing DENV-specific IgG antibodies from a prior exposure [4,17,97–99]. The prevailing theory behind this phenomenon of antibody-dependent enhancement (ADE) is that non-neutralizing IgG antibodies facilitate virus uptake into target cells through Fc-Fc gamma receptor (FcgR) interactions[162].

In vitro studies have established that antibody-mediated neutralization and enhancement of infection depends on IgG concentration [117]. This is evident in the infectivity curve observed in FcgR+ cell lines (such as K562 and U937) widely used to study ADE as they are permissive to infection only in the presence of DENV-reactive IgG [100,101]. In these cells, peak enhancement of infection occurs at intermediate antibody levels; higher antibody concentrations neutralize infection, while lower concentrations do not enhance infection. Importantly, the risk of severe dengue disease risk in humans is also highest within a narrow range of intermediate titers of pre-existing antibodies to DENV[4,97,99].

Canonical DENV infection in the absence of antibodies is predominantly initiated via classical clathrin-dependent endocytosis following direct virion interaction with cellular attachment factors [163–166]. In contrast, efficient uptake of IgG-opsonized DENV is dependent on intact signaling of FcgRs such as FcgRIIa [100,167], an activating FcgR expressed on relevant

DENV target cells *in vivo* [101,102,168–172]. Live-cell imaging and single-particle tracking studies found that actin-mediated plasma membrane protrusions facilitated the uptake of IgG-opsonized but not 'naked' DENV particles [155], suggesting that unique entry factors are important for ADE. Additionally, IgG-dependent uptake of DENV particles can increase not only the number of infected cells, but also the viral output per infected cell [162], suggesting that DENV-host interactions downstream of viral entry also contribute to distinct infection outcomes observed under ADE and non-ADE conditions. This is unsurprising given that Fc-FcgR signaling regulates many cellular processes, including endocytosis, cell proliferation and maturation, and innate immunity [91].

Beyond dependence on antibody concentration and the role of FcgR in initiating uptake of IgG-bound virions, the functional requirements for DENV infection via ADE are still unknown. This knowledge gap persists largely because *in vivo* models fail to fully capture DENV immunity and pathogenesis [173–175]. Our limited understanding of potential ADE mechanisms comes from *in vitro* studies that do not establish cause and effect. Most previous studies selectively investigated the expression of specific cytokines in response to IgG-mediated entry of DENV [101,147–154]. Conversely, unbiased transcriptomic profiling of whole blood or peripheral blood mononuclear cells have identified differentially expressed genes in individuals with mild versus severe dengue disease [108,109,148], but these studies cannot establish whether the observed profiles are specific to antibody-mediated infection. Although a recent study showed that DENV infection via ADE uniquely altered the expression of multiple host genes [176], their direct functional contribution to ADE was not explored. Thus, existing studies have only indirectly suggested potential ADE mechanisms.

A comprehensive analysis of which host factors are functionally required for ADE is lacking. Genome-wide CRISPR knockout screens have enabled high-throughput, unbiased, and reproducible discovery of viral host dependency factors [157]. Such screens of direct (non-ADE) DENV infection in the absence of antibodies performed independently by multiple researchers

using multiple cell lines and viral strains have identified highly concordant host dependency factors [158–161,177]. However, as these prior screens were performed in the context of non-IgG-mediated DENV infection, they could not identify host factors uniquely required for ADE. Here, to advance mechanistic understanding of ADE beyond existing descriptive studies, we performed a CRISPR/Cas9-based genome-wide and follow-up targeted knockout screens in K562 cells, which are permissive to efficient infection only via ADE [167]. This approach was designed to reveal host factors exclusively required for ADE mechanisms. Validating this approach, our screens identify candidate ADE-specific host factors with no previously defined role in DENV infection, including TBC1D24 and SV2B, both of which are essential in trafficking specialized recycling endosomes during regulated secretion [178,179]. We validated a functional role for TBC1D24 and SV2B in promoting ADE of all four DENV serotypes, with monoclonal antibodies and polyclonal sera, and in multiple cell lines. Further, we show that knockout of TBC1D24 or SV2B reduced the efficiency of binding of IgG-DENV complexes to cells despite maintaining similar levels of FcgRIIa expression to unedited cells.

Thus, we identify for the first time host factors beyond FcgR that are required for efficient ADE of DENV infection. Our screen represents a novel discovery tool for cellular factors and processes uniquely subverted by DENV during ADE that can be exploited to significantly advance our understanding of ADE mechanisms.

Materials and Methods

2.1. Cell lines

K562 cells (Cat# CCL-243, ATCC), U937 cells (provided by Taia Wang, Stanford University), and Raji cells stably expressing DCSIGNR (Raji-DCSIGNR) (provided by Ted Pierson, NIAID, NIH, Bethesda, MD) were maintained in RPMI 1640 supplemented with GlutaMAX (Cat# 72400-047; ThermoFisher Scientific), 7% fetal bovine serum (FBS) (Cat# 26140079, lot 2358194RP, ThermoFisher Scientific) and 100 U/mL penicillin-streptomycin (Cat#

15140–122; ThermoFisher Scientific). HEK-293T/17 cells (Cat# CRL-11268, ATCC) were maintained in DMEM (Cat #11965118; ThermoFisher Scientific) supplemented with 7% FBS and 100 U/mL penicillin-streptomycin. C6/36 cells (Cat # CRL-1660, ATCC) were maintained in EMEM (Cat # 30-2003, ATCC) supplemented with 10% FBS. TZM-bl cells (provided by Michael Emerman, Fred Hutchinson Cancer Center, Seattle, WA) were maintained in DMEM (Cat #11965118; ThermoFisher Scientific) supplemented with 7% FBS and 100 U/mL penicillin-streptomycin.

K562-DCSIGN cells were generated by lentiviral transduction. A plasmid expressing DCSIGN (Genbank Accession NM_021155.4) fused to BFP in a lentiviral vector was synthesized (VB221014-1121vtg, VectorBuilder) and used to transduce K562 cells as described below in "Lentiviral production and transduction." Transduced cells were stained using anti-DCSIGN antibody (Cat #330105, Biolegend) and cell populations highly expressing both DCSIGN and BFP were bulk sorted (Sony MA900).

C6/36 cells were maintained at 30°C in 5% CO2; all other cell lines were maintained at 37°C in 5% CO2.

2.2. Viruses

DENV1 UIS 998 (isolated in 2007, Cat# NR-49713), DENV2 US/BID-V594/2006 (isolated in 2006, Cat# NR-43280), DENV3/US/BID- V1043/2006 (isolated in 2006, Cat# NR-43282), and DENV4 strain UIS497 (isolated in 2004, Cat# NR-49724) were obtained from BEI Resources (Manassas, VA) and propagated on C6/36 cells. Virus-containing supernatant from days 3 to 8 post-infection was pooled, centrifuged at 500 x *g* for 5 min, filtered through a 0.45 µm Steriflip filter (Cat# SE1M003M00, Millipore-Sigma), and stored at -80°C until use. DENV2 S16803 reporter virus particles (DENV2-GFP) (Cat # RVP-201) [180] were purchased from Integral Molecular, Inc (Philadelphia, PA). Infectious titers of viral stocks were determined by infecting Raji-DCSIGNR cells with 2-fold serial dilutions of virus. Cells infected with fully

infectious virus were fixed and permeabilized using BD cytofix/cytoperm (Cat #554717, BD Biosciences) according to the manufacturer's instructions, stained with APC-conjugated E-protein-specific antibody, 4G2 for 30 minutes at 4°C and washed in cytoperm/wash buffer twice before quantification of APC+ cells by flow cytometry (Intellicyt iQue Screener PLUS, Sartorius AG). Antibody 4G2 was isolated and purified by the Fred Hutchinson Cancer Center Antibody Technology core following expansion of the hybridoma D1-4G2-4-15 (Cat# HB-112, ATCC) and purification of IgG from culture supernatant. Purified 4G2 was conjugated to APC via the Lighting-Link APC-conjugation kit (Cat# ab201807, Abcam) according to manufacturer's instructions. Cells infected with DENV2-GFP were fixed with 2% paraformaldehyde and GFP+ cells quantified by flow cytometry (Intellicyt iQue Screener PLUS, Sartorius AG).

2.3. *Lentiviral production and transduction*

Lentivirus was produced in HEK-293T/17 cells by co-transfection of lentiviral plasmids with psPAX2 (Cat# 12259, Addgene) and pMD2.G (Cat# 12259, Addgene) at a mass ratio of 2:1.33:1, respectively, using the Lipofectamine™ 3000 Transfection Reagent (Cat# L3000001, ThermoFisher Scientific). Supernatants were collected 48 hours post-transfection, passed through a 0.22 μM filter, and either stored at -80°C or immediately used to transduce cells. Target cells were seeded in 6-well plates at a density of 2e5 cells per well in 2 mL RPMI 1640 with 7% FBS and 1% penicillin-streptomycin the day prior to transduction. On the day of transduction, cells were pelleted and resuspended in 250 μL lentivirus and 8 μg/mL DEAE-Dextran (Cat# D9885, Sigma) in a total volume of 1.7 mL, followed by spinoculation at 1000 x *g* for 2h at 30°C. Medium on spinoculated cells was aspirated and replaced with 2 mL fresh RPMI formulated as above. After incubation at 37°C for 24h, cell culture medium was replaced again. After at least 3 days since spinoculation, transduced cells were either bulk sorted by FACS (Sony MA900) or subjected to drug selection, depending on lentivirus vector marker.

2.4. Genome-wide and targeted CRISPR screens

K562-Cas9-Blast cells (provided by Andreas Puschnik, Chan Zuckerberg Biohub, San Francisco) were generated by transduction with lentiCas9-Blast (Cat# 52962, Addgene) and selection with blasticidin as described previously [158]. Approximately 200 million K562-Cas9-Blast cells were transduced with each GeCKOv2 library A or B [181] (Addgene #1000000048 or #1000000049, respectively) at a MOI of 0.3 in the presence of 10 μg/mL protamine sulphate. Three days post-transduction, cells were selected in 1 μg/mL puromycin. Sixty four million mutagenized cells for each library (A and B) were infected with DENV2-GFP at an MOI of 24 in the presence of 1.25 μg/mL anti-DENV2 mouse antibody DV2-70 [182] (provided by Michael Diamond, Washington University, St. Louis, MO) by spinoculation at 930 x g for 2 hours at 30°C, and then incubated at at 37°C for two days. Next, to isolate ADE-resistant cell populations, GFP-negative cells were bulk sorted (Sony SH800) and allowed to recover and multiply for five days at 37°C prior to re-infection by ADE under the above conditions. After three total rounds of ADE and bulk sorting, genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (Cat #51185, Qiagen), and sgRNA sequences were amplified and prepared for next-generation sequencing via the NextSeq platform (Illumina). The enrichment of each sgRNA in the selected cells relative to the unselected libraries cultured and harvested in parallel was calculated using MAGeCK [183].

The custom targeted library against the top 500 highest-ranking candidates from the genome-wide screen was designed using CHOPCHOP v3 and Guides [184,185]. We used six guides per gene, plus 200 non-targeting control (NTC) guides (**Table S2**). Pooled gRNAs were synthesized (Twist Biosciences, South San Francisco, CA) and cloned into lentiCRISPRv2 (Cat# 52961, Addgene), packaged into lentivirus as described above, and titered on TZM-bl cells, as previously described [186]. Approximately 6.4 million K562 cells were transduced with

the targeted lentivirus library at an MOI of 0.5 (500-fold coverage) in the presence of 8 μg/mL of DEAE-Dextran by spinoculation at 930 x g for 2 hours at 30°C. The following day, cells were selected for two weeks using 1 μg/mL of puromycin. Mutagenized cells (3.2 million) were subjected to three rounds of infection via ADE and sorting, and subsequently prepared for next-generation sequencing (Illumina MiSeq) and analysis as described above for the genome-wide screen. Targeted screens were performed in biological triplicate on three independently mutagenized cell library populations.

2.5. Generation of clonal KO cell lines

The following individual KO cell lines were generated by nucleofection of Cas9-sgRNA ribonucleoprotein complexes (RNPs): K562 (TBC1D24 KO), U937 (TBC1D24 KO and SV2B KO), and K562-DCSIGN (NTC, TBC1D24 KO, FcgRIIa KO). RNPs were assembled by combining 6 μL of 30 pmol/μL of a pre-made mixture of three equimolar sgRNAs (Synthego Gene KO Kit v2), 1 μL of 20 uM Cas9-NLS protein (Synthego), and 18 μL of SF Cell Line Nucleofector solution (Lonza V4XC-2032). Complexes were mixed and incubated for 10 min at room temperature prior to addition to 5 μL of cells (1e5 for U937, 2e5 for K562). This mixture was transferred to a 16-well nucleocuvette for nucleofection with an Amaxa 4D Nucleofector (Lonza) according to manufacturer's protocol using pulse code FF-120 for K562 cells or EP-100 for U937 cells. Following nucleofection, cells were incubated for 10 minutes at room temperature, and then transferred into a 24-well plate in a total volume of 500 μl for recovery. After 24 hours, the medium on the cells was replaced. Seventy two hours post-nucleofection, single clones were isolated via limiting dilution.

SV2B K562 KO, FcgRIIa K562 KO, and SV2B K562-DCSIGN KO cell lines were generated via nucleofection with top-ranking sgRNAs (**Table S4**) cloned into px458 (Cat# 48138, Addgene). One million cells resuspended in 100 μL of SF Cell Line Nucleofector solution

(Lonza V4XC-2012) were electroporated with 2 μg plasmid using pulse code FF-120 (Lonza Amaxa 4D Nucleofector). Cells were incubated for 10 minutes at room temperatue, resuspended in 500 μL of RPMI with 7% FBS and 1% penicillin-streptomycin, and then moved into a 6-well plate for recovery at 37°*C.* After 48 hours, GFP-positive single cells were sorted into 96-well plates (Sony SH800).

For genotyping, genomic DNA was isolated (QuickExtract, Cat #QE0905T, Lucigen) and the gRNA-targeted site was amplified by PCR (primers listed in **Table S4**) and Sanger sequenced. Reads were aligned to reference sequences obtained from parental unedited (WT) cells and analyzed for the presence of indel mutations (Geneious Prime 2020.1.2). Mixed traces from heterozygous clones were deconvolved using ICE analysis [187].
FcgRIIa KO U937 cells have been previously described [108] and were provided by Taia Wang (Stanford University).

2.6. Generation of trans-complemented cell lines

Lentiviral transduction as described above was used to generate *trans*-complemented KO lines. The following cDNA constructs were obtained: SV2B (Cat #OHu12014D, GenScript) and TBC1D24 (Cat #OHu21983, GenScript). The cDNA of interest was amplified by PCR using primers (**Table S4)** with overhangs that allow directional cloning into EcoRV-linearized pHIVdTomato (#21374, Addgene) using the 2X Gibson Assembly Kit (Cat # E2611S, New England Biolabs). Assembled constructs were confirmed by whole plasmid sequencing (Plasmidsaurus, Eugene, OR). KO cells were transduced with lentiviral preparations to deliver the gene of interest and then bulk sorted (Sony MA900) based on high dTomato expression.

2.7. Validation ADE assays

Dose-dependent ADE assays were performed with fully infectious versions of DENV1-4 or single-round infectious DENV2-GFP reporter virus particles. Viral stocks were diluted to 5-10% final infectivity (determined on Raji-DCSIGNR cells as described above) and incubated with 5-fold serial dilutions of monoclonal antibody or polyclonal sera for 1 hr at room temperature before addition of 2e5 (in a 384-well plate) or 3.33e5 (in a 96-well plate) K562 or U937 cells, respectively. DV2-70 mouse monoclonal antibody was provided by Michael Diamond (Washington University, St. Louis, MO) [182] and human monoclonal J9 IgG was recombinantly produced as previously described [188]. Human convalescent serum samples from three independent DENV-immune donors were obtained from BEI Resources (Cat #NR-50229, NR-50232, NR-50231). Following incubation at 37°C for 2 days, cells were processed according to the protocol described above (section "Viruses") and infection was quantified by flow cytometry (Intellicyt iQue Screener PLUS, Sartorius AG).

2.8. Direct infection of K562-DCSIGN cells

Two hundred thousand cells in 20 μL complete RPMI were infected with an equal volume of DENV2-GFP at a MOI of 24 and added in duplicate to a 384-well plate by spinoculation for 2 hours at 33°C and 1000 x*g*. Cells were resuspended and incubated at 37°C for 2 days prior to quantification of infected cells by flow cytometry as described above.

2.9. RT-qPCR infection assays

DENV2-GFP stocks at an MOI of 24 were incubated with 80 ng/mL J9 antibody for 1 hour at 4°C before addition to 3.33e5 K562 cells in duplicate wells of a 96-well plate on ice; all components were at an equal volume of 33 μL. Virus/antibody complexes were allowed to bind to cells for 1 hr at 4°C, followed by wash steps with 1X PBS to remove unbound virus/antibody complexes. Next, cells were either immediately (0 h time point) harvested for quantitative PCR

or incubated at 37°C for 15 min to trigger internalization. Following three wash steps in 1X PBS, cells were treated with 400 ng/μL proteinase K at 37°C for 45 minutes to remove non-internalized complexes. Following three wash steps with 1X PBS, cells were further incubated at 37°C for 2h, 6h, or 24h prior to lysis for quantitative PCR (QuantStudio™ 5 Real-Time PCR System, 96-well, Applied Biosystems™) using the Power SYBR™ Green Cells-to-CT™ Kit (Cat #4402954, Invitrogen) per manufacturer instructions. Data were analyzed using ABI QuantStudio 5 (Applied Biosystems). All viral RNA levels were normalized to 18S levels, and subsequently to control WT cells at 0 hours post-infection. Universal DENV primer and 18S primer sequences [189] can be found in **Table S4**.

2.10. Replicon assays

One million K562 cells were electroporated with 3 μg of DENV2-luciferase replicon [158] (provided by Jan Carette, Stanford University) in 100 μL SF Cell Line Nucleofector solution (Lonza V4XC-2012) using pulse code FF-120 (Amaxa 4D Nucleofector, Lonza). Cells were incubated for 10 minutes at room temperature following nucleofection, resuspended in 500 μL of RPMI with 7% FBS, then distributed into a 48-well plate (250 μL per well) and incubated at *37*°C. At each time point, cells were lysed using the Renilla-Glo® Luciferase Assay System (Cat #E2710, Promega) according to manufacturer suggestions, and frozen at -20°C. Samples from each timepoint were then concurrently processed using the Renilla-Glo® Luciferase Assay System according to manufacturer instructions, and analyzed on a plate reader (Infinite M1000 Pro, Tecan).

2.11. Determining FcgRIIa receptor expression

To assess surface FcgRIIa expression, 2e5 cells per cell type and stain were washed in FACS wash (FW, 2% FBS in 1x PBS) and resuspended in 50 μL anti-CD32-FITC (Cat #

60012.FI, StemCell) or isotype control (Cat # 11-4732-81, ThermoFisher Scientific), and incubated at 4°C for 20 min. FW was then added and a wash with FW was performed. Cells were then fixed in 2% PFA for 20 minutes, spun down, and resuspended in PBS at 4°C until acquisition. To assess total FcgRIIa expression, 2e5 per cell type and stain cells were fixed using Cytofix (Cat #554655, BD) for 20 minutes at 4°C cells before addition of perm/wash buffer (Cat #554723, BD) followed by two washes in perm/wash. Cells were then resuspended in either 50 μL anti-CD32-FITC or isotype control, and incubated at 4°C for 20 min. Cells were then washed with perm/wash prior to resuspension in PBS at 4°C until acquisition. Samples were analyzed via flow cytometry (Sony ID7000), and data were analyzed using FlowJo 10.9.0.

2.12. Statistical analysis

Area under the curve analysis, and paired and unpaired t-tests adjusted via Benjamini-Hochberg method were performed using GraphPad Prism 10.

Results

3.1. Genome-wide and targeted CRISPR screens identify host factors uniquely required for ADE.

Our screening strategy is outlined in **Figure 2.1A**. To comprehensively identify candidate ADE-specific host dependency factors, we first generated a genome-wide knockout (KO) library of K562 cells. As mentioned, these cells are widely used to study ADE because they express FcgRIIa and are only permissive to DENV infection in the presence of IgG antibodies [167]. We infected the KO library with single-round infectious reporter virus particles of DENV2 strain S16803 (DENV2-GFP) [180] pre-complexed with DV2-70, a mouse DENV2-specific IgG antibody [182] under optimized conditions that achieved stringent selection pressure (>95% infection). To maximize signal-to-noise ratio, we performed three rounds of infection and used fluorescence-activated cell sorting (FACS) to isolate cells resistant to ADE and thus likely had host

dependency factors knocked out. Specifically, after each round of infection via ADE, live, GFP-negative cells were sorted, allowed to recover and multiply, and then re-infected by ADE to ensure that lack of infection was due to gene KO instead of stochastic effects. We deep sequenced genomic DNA from the virus-selected cell population and used MAGeCK [183] to compare sgRNA enrichment relative to the uninfected KO library cultured and harvested in parallel.

As shown in **Figure 2.1B**, many highly-enriched hits include known host dependency factors identified in previous genome-wide screens in the context of direct (non-ADE) DENV infection [158–161,177]. In fact, the gene with the highest MAGeCK score by far was STT3A, which was identified in previous direct infection CRISPR screens and is required for efficient replication of DENV and other mosquito-borne flaviviruses [158–161,177]. This finding was unsurprising as we expect some shared features of direct infection and ADE. Interestingly, HELZ2, a previously described host factor that *restricts* direct flavivirus infection, was also enriched [190,191]. Validating our approach, FcgRIIa, a known ADE-specific host factor and the only activating FcgR expressed on K562 cells [167], was among the top hits. We also identified candidate ADE-specific genes like SV2B with no previously described role in DENV infection. In **Figure 2.1C**, we highlight that these novel genes were similarly enriched as FcgRIIa and interspersed among known direct infection host dependency factors identified in previous screens [158–161,177]. The full list of hits from the genome-wide screen is available in **Table S1**.

Although the enrichment of FcgRIIa indicated that our genome-wide screen was functioning as intended, many top hits were those identified in previous CRISPR screens in the context of direct infection [158–161,177] (**Figure 2.1B**). Therefore, to further enrich the most critical ADE-specific cellular factors, we performed a follow-up targeted screen using a custom sgRNA sub-library against the 500 highest-ranking genes from our genome-wide screen (**Tables S2-3**). To exclude potential off-target effects, we designed new sgRNA sequences distinct from those used in the initial genome-wide screen. As with the initial screen, K562 KO sub-libraries were

infected with DENV2-GFP pre-complexed with DV2-70 IgG, followed by FACS of cells rendered resistant to ADE in three successive rounds. Unlike in the original genome-wide screen, the top hit in this targeted screen was our positive control, FcgRIIa (**Figure 2.1D**). Additionally, genes with no previously defined role in DENV infection dominated the top hits. These results demonstrate increased stringency in the targeted screen for identifying candidate ADE-specific factors over the genome-wide screen (compare **Figure 2.1D** to **Figures 2.1B-C**).

After FcgRIIa, the second top-ranking hit in the targeted screen was TBC1D24, which contains a Tre2/Bub2/Cdc16 (TBC) domain common to Rab-GTPase-activating proteins [192], and a TLDc domain with a putative function in oxidative stress resistance [193]. TBC1D24 is involved in recycling clathrin-independent endocytosis cargo proteins and in synaptic endocytic vesicle trafficking [179,194]. Interestingly, SV2B, a gene that scored highly in our genome-wide, but not targeted, screen (**Figure 2.1B-D**), is known to have related functions [178,195–199]. Specifically, SV2B is one of three paralogs of the SV2 family of integral membrane glycoproteins that regulate synaptic vesicle function via its role in trafficking synaptotagmin, a calcium sensor protein for exocytosis [199,200]. To our knowledge, neither TBC1D24 nor SV2B has a previously described role in virus infection. In fact, the top 23 hits of the targeted screen were dominated by novel candidate ADE-specific factors (**Figure 2.1E**). Like TBC1D24 and SV2B, some but not all high-ranking hits have known functions in the nervous system. These include TUBB2A, a microtubule component known to interact with KIF1a, which is required for synaptic vesicle transport [201,202]; DNAJC6, a heat-shock protein involved in neuronal clathrin-mediated endocytosis [203]; and HMX3, a transcription factor involved in neuronal cell specification [204].

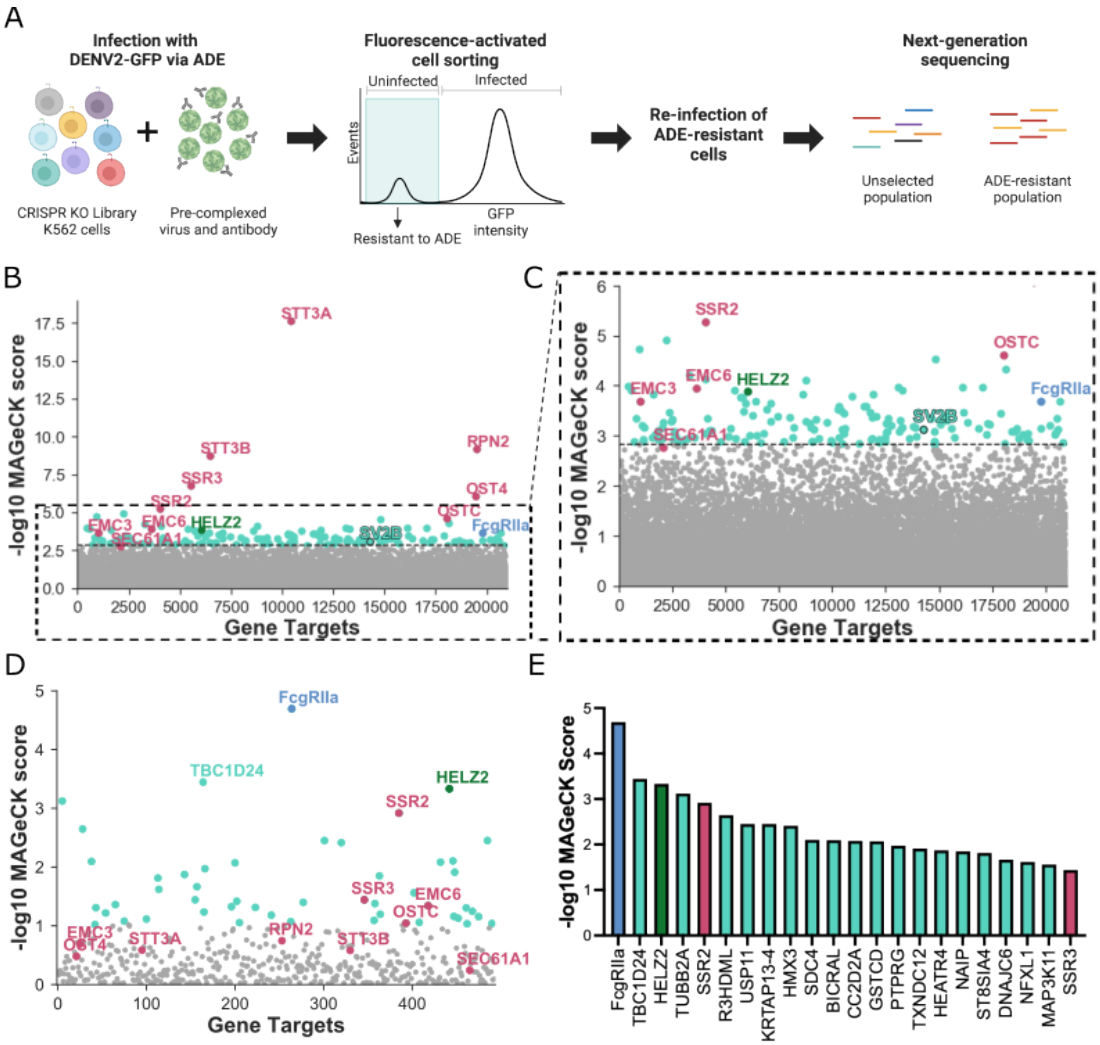

Figure 2.1. Genome-wide and targeted CRISPR knockout screens in human cells identify candidate host factors required for ADE of DENV infection**. (A)** Schematic for CRISPR-based genome-wide and targeted knockout screens. K562 cell mutant libraries were generated using either the GeCKOv2 sgRNA library or a compressed library targeting 500 genes for genome-wide or targeted screens, respectively. In both screens, cell libraries were infected via ADE with single-round infectious DENV2 expressing a GFP reporter (DENV2-GFP) pre-complexed with anti-DENV2 monoclonal IgG antibody. ADE-resistant cells were isolated via fluorescence-activated cell sorting, and re-infected with DENV2-GFP via ADE following recovery and expansion. After three iterative rounds of infection and sorting, we used deep sequencing and MAGeCK analysis to quantify the enrichment of sgRNAs in the infected cell population relative to the corresponding control uninfected population. **(B)** Gene enrichment in genome-wide CRISPR screen. Y-

axis displays MAGeCK score in the infected cell population; x-axis displays gene targets arranged randomly. Light blue: FcgRIIa, a known host factor required for ADE. Maroon: pro-viral host factors identified in previous genome-wide screens in the context of direct DENV infection [158–161,177]. Green: HELZ2, a previously identified host factor that restricts direct infection [190,191]. Teal: candidate novel ADE-specific host factors, including SV2B. The dotted line intersects SEC61A1, the lowest-ranking of validated host dependency factors for direct DENV infection identified in previous screens [205–207]; all genes below this point are shown in gray. **(C)** A zoomed-in view of graph shown in (**B)**, highlighting the enrichment of candidate novel ADE-specific host factors clustered around FcgRIIa. Color scheme is similar to (**B**). **(D)** Gene enrichment in targeted CRISPR screen. Color scheme is similar to (**B**), except that genes depicted in gray represent the bottom 90% ranking genes. **(E)** MAGeCK scores of top 23 genes enriched in the targeted screen. Color scheme is similar to (**B**). Panel (**A**) was created with Biorender.com.

3.2. Functional validation of TBC1D24 and SV2B as host dependency factors for ADE

We focused on validating the functional role of TBC1D24, the top-scoring gene in our targeted screen after FcgRIIa. We first generated TBC1D24 and FcgRIIa K562 KO clones and confirmed disruption of gene targets by PCR amplification and Sanger sequencing. FcgRIIa KO clone contained a frameshifting deletion (**Figure S1**) while TBC1D24 KO clone contained a large deletion at the beginning of exon 2, which is the first coding exon and encodes the TBC domain [208,209] (**Figure S2**). Next, we performed ADE dose-dependent assays using DENV2-GFP pre-complexed with mouse DV2-70 IgG, the same mouse antibody used in our screens. As expected, FcgRIIa KO largely abolished ADE (85% average reduction in area under the curve (AUC) compared to WT, **Figure S4**). Remarkably, TBC1D24 KO reduced ADE efficiency to similar levels as FcgRIIa KO (83% average reduction in AUC, **Figure S4A**).

Given the marked reduction of ADE efficiency due to TBC1D24 KO, we next investigated the role of SV2B, a host factor that shares a similar function as TBC1D24 in synaptic vesicle trafficking [178,195–199] and that was enriched in our genome-wide screen (**Figures 2.1B-C**). We generated a SV2B KO clone and confirmed a frameshifting deletion (**Figure S3)**. Dose-

response ADE assays performed with this K562 SV2B-KO clone revealed a 70% reduction in AUC compared to WT (**Figure S4B**), thus demonstrating a functional role for SV2B in ADE. To rule out a mouse antibody-specific artifact, we next performed the above ADE assays using a broadly reactive human monoclonal anti-DENV IgG antibody, J9 [210]. In these assays, TBC1D24 KO (**Figure 2.2A**) and SV2B KO (**Figure 2.2B**) each resulted in a ~50% reduction in AUC compared to WT cells. Notably, ADE efficiency was rescued in KO cells *trans*-complemented with the gene of interest, but not with empty vector (**Figures 2.2A-B**), demonstrating that reduction in ADE efficiency was specifically due to KO of TBC1D24 or SV2B. Together, these experiments establish a functional role for TBC1D24 and SV2B in ADE.

Next, to confirm that the roles of TBC1D24 and SV2B are unique to IgG-mediated infection, we generated corresponding clonal KO lines in K562 cells engineered to express DCSIGN (K562-DCSIGN), a cellular attachment factor that permits direct DENV infection in the absence of IgG antibodies [211]. Following confirmation of target allele disruption (**Figures S5-S7**), we infected K562-DCSIGN KO clones with DENV2-GFP in the absence of antibodies. Due to substantial inter-clonal heterogeneity [212] even among non-targeting controls (NTC) (**Figure 2.2C**), we analyzed at least four KO clones per gene to mitigate clonal artifacts. FcgRIIa-KO, TBC1D24-KO, and SV2B-KO K562-DCSIGN clones reduced the efficiency of direct infection to relatively similar levels compared to the unedited (WT) K562-DCSIGN cell pool (median reduction of 33%, 27%, 24%, respectively, **Figure 2.2C**). As FcgRIIa is required for IgG-mediated, but not direct DENV infection, these results suggest that TBC1D24 and SV2B have minimal roles in direct (non-ADE) DENV infection.

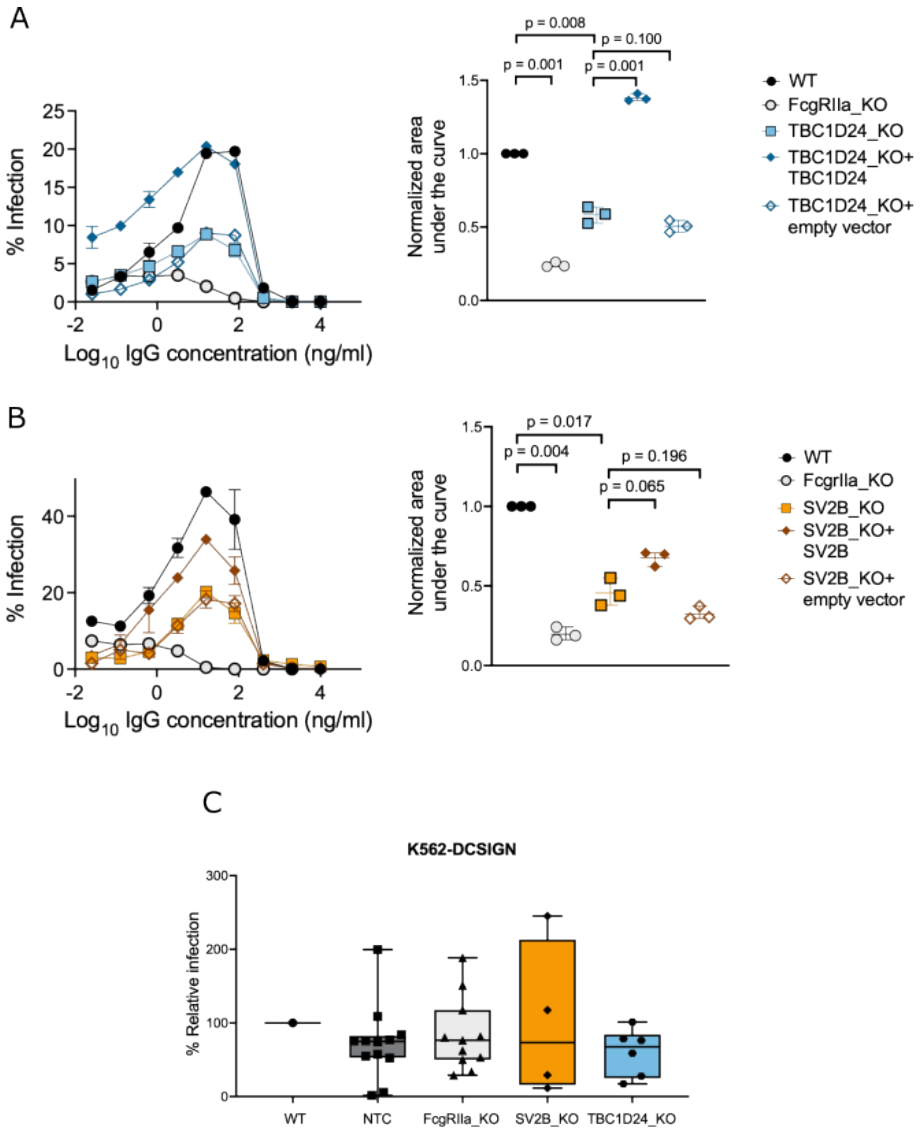

Figure 2.2. TBC1D24 and SV2B are required for efficient ADE but not direct infection. (**A-B**) (Left) Representative dose-response ADE curves for K562 (**A**) TBC1D24 KO clone (**B**) SV2B KO clone, and genetically *trans*-complemented K562 KO cells infected with DENV2-GFP in the presence of serially diluted human anti-DENV IgG monoclonal antibody J9 [[210]]. In each experiment, K562 WT cell pool and a FcgRIIa KO clone was included as a control. Data points and error bars represent the mean and range of infection in duplicate wells, respectively. Graphs shown are representative of at least three independent experiments. (Right) Quantification of area under the curve normalized to unmutagenized (WT) K562 cells from three independent dose-response ADE experiments, each represented as a data point.

Horizontal lines and error bars indicate mean and standard deviation, respectively. P-values shown are from multiple paired student's t-tests adjusted using the Benjamini-Hochberg method. (**C**) Efficiency of DENV2-GFP infection of the indicated K562-DCSIGN cells in the absence of IgG antibodies. Shown are percentage infections for individual KO clones (data points) normalized to unmutagenized (WT) K562-DCSIGN cell pool, median (horizontal line within box), 25th to 75th percentile (box), and minimum and maximum (whiskers). Data are representative of two independent experiments, each performed in duplicate wells. Comparisons of direct infection efficiency of each KO cell line to WT were not statistically significant ($p > 0.05$) as determined by multiple unpaired student's t-tests adjusted using the Benjamini-Hochberg method.

3.3. TBC1D24 and SV2B are required for efficient ADE in multiple contexts

We evaluated the role of TBC1D24 and SV2B in mediating ADE in various settings. First, to extend our validation studies with monoclonal antibodies above, we performed ADE assays in K562 cells using DENV2-GFP in the presence of serially diluted convalescent sera from three different DENV-immune donors (**Figure 2.3A**). TBC1D24 KO ablated ADE mediated by all three serum samples to similar levels seen with FcgRIIa KO control cells. SV2B KO also reduced ADE efficiency compared to WT cells, though its effect was more moderate compared to KO of TBC1D24 or FcgRIIa.

Next, to extend our findings with single-round DENV2-GFP, we performed ADE assays in K562 cells using fully infectious versions of all four DENV serotypes pre-complexed with J9 human monoclonal IgG (**Figure 2.3B**). ADE of all four DENV serotypes was abrogated in both TBC1D24-KO and FcgRIIa-KO cells. In contrast, SV2B KO reduced ADE of DENV1-4 to varying extents. Specifically, the strongest ADE reduction was observed for DENV3, and the weakest

for DENV2 (~80% and ~30% reduction in peak infection, respectively). SV2B KO reduced peak enhancement of infection of both DENV1 and DENV4 by ~50%.

Finally, we investigated the requirement for TBC1D24 and SV2B for efficient ADE in U937 cells (**Figure 2.3C-D**). Like K562 cells, U937 cells are commonly used to study ADE due to their susceptibility to efficient DENV infection only in the presence of IgG antibodies [[100,101]]. However, unlike K562 cells, U937 cells also express FcgRI in addition to FcgRIIa; both FcgRs are known to mediate ADE [[108]]. We tested four independent U937 KO clones each for TBC1D24 and SV2B (**Figure S8-9**) and included a previously generated U937 FcgRIIa KO clone [[108]] as a control in dose-response ADE assays. While KO of FcgRIIa abolished ADE in K562 cells (**Figures 2.3A-B**), it only partially reduced ADE efficiency in U937 cells (**Figures 2.3C-D**). The remaining ADE activity observed in FcgRIIa KO U937 is likely mediated by intact FcgRI expressed on these cells [[108]]. Notably, the reduction in ADE efficiency observed in SV2B-KO (**Figure 2.3C**) and TBC1D24-KO (**Figure 2.3D**) clones were comparable to, or in some cases (for TBC1D24 KO clones 3 and 4, **Figure 2.3D**), stronger than FcgRIIa KO in U937 cells.

Our combined results above show that the functional role of TBC1D24 and SV2B in ADE of DENV infection is not limited to a specific antibody, DENV serotype, or cell line.

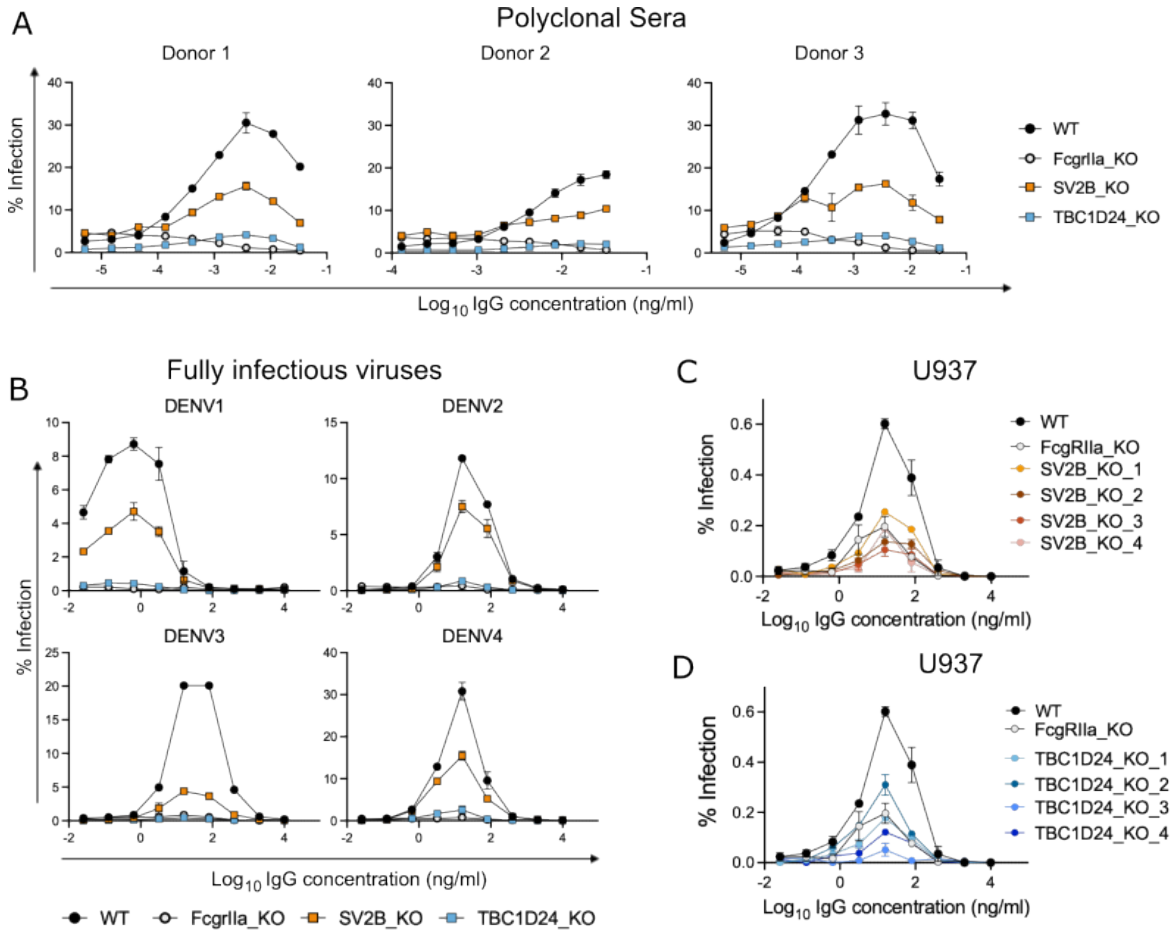

Figure 2.3. The role of TBC1D24 and SV2B in ADE is not limited to a single DENV serotype, antibody, or cell line. (**A**) DENV2-GFP was incubated with serial dilutions of convalescent serum from three independent DENV-immune donors prior to infection of the indicated K562 cells. Data points represent the mean and the error bars represent the range of infection in duplicate wells, respectively. Graphs shown are representative of two independent experiments. (**B**) Fully infectious DENV1-4 particles were incubated with serial dilutions of anti-DENV monoclonal IgG antibody (J9) prior to infection of the indicated K562 cells. Data points represent the mean and the error bars represent the range of infection in duplicate wells, respectively. Graphs shown are representative of two independent experiments. **(C-D)** DENV2-GFP was incubated with J9 monoclonal antibody prior to infection of clonal U937 cells with a KO in (**C**) SV2B or **(D)** TBC1D24. Data points represent the mean and the error bars represent the range of infection in duplicate wells. Data shown is representative of 4 independent experiments, each performed

in duplicate wells. In each experiment, WT U937 cell pool and a U937 FcgRIIa KO clone were included as controls.

3.4. *TBC1D24 and SV2B facilitate binding of DENV-IgG complexes to cells*

To determine at which step of the viral replication cycle TBC1D24 and SV2B are required during ADE, we first assessed the efficiency of binding and internalization of DENV2-IgG complexes into gene KO clones relative to WT K562 cells. Specifically, we measured cell-associated viral RNA levels by qRT-PCR either immediately following incubation of cells with IgG-virion complexes at 4°C (0h, binding), or following additional incubation at 37°C (internalization) for various time points. As expected, FcgRIIa KO cells substantially reduced cell-associated viral RNA levels at the initial timepoint, which corresponds to binding of IgG-DENV complexes to cells (79% reduction relative to WT, **Figure 2.4A**). Cell-associated viral RNA levels in TBC1D24-KO and SV2B-KO cells were also reduced at this initial timepoint (50% reduction for each compared to WT) (**Figure 2.4A**), indicating that TBC1D24 and SV2B promote binding of IgG-DENV complexes to cells.

Next, to confirm an impact on early infection steps, we performed an established luciferase replicon assay by electroporating DENV2-luciferase RNA into K562 cells to bypass entry [158]. Reporter gene activity over the first 10-12 h reflects translation of the input viral positive-stranded RNA genome while subsequent increases in signal are due to viral genome replication [158]. As a control, we included WT cells electroporated with replication-deficient mutant DENV (DENV GDD). We also included clonal K562 cells with a KO in STT3A, a host factor required for DENV RNA replication [158]. As expected, luminescence activity in replication-impaired controls (STT3A KO cells or WT cells electroporated with DENV GDD) were comparable to WT at early time points but were impaired beginning 23 hours post-electroporation (**Figure 2.4B**), indicating inhibition of viral genome replication, but not

translation. Also as expected, FcgRIIa KO cells had no impact on viral genome translation or replication (**Figure 2.4B**). Reporter gene expression in TBC1D24 KO and SV2B KO cells mirrored that in WT and FcgRIIa KO cells across all time points, indicating limited effects on viral genome translation and replication (**Figure 2.4B**). Together, these results demonstrate that TBC1D24 and SV2B act on early stages of ADE, starting from binding of IgG-DENV complexes to cells.

Because we observed an impact on binding of IgG-DENV complexes to K562 cells, we asked whether KO of TBC1D24 or SV2B impaired expression of FcgRIIa, the sole FcgR expressed on these cells. Total and surface FcgRIIa expression was comparable between KO and unmutagenized WT K562 cells, as assessed by both median fluorescence intensity values (**Figure 2.4C**) and distribution of FcgRIIa expression across the cell population (**Figure 2.4D**). These results indicate that reduction in binding efficiency of DENV-IgG in TBC1D24-KO and SV2B-KO cells was not due to overt defects in FcgRIIa expression.

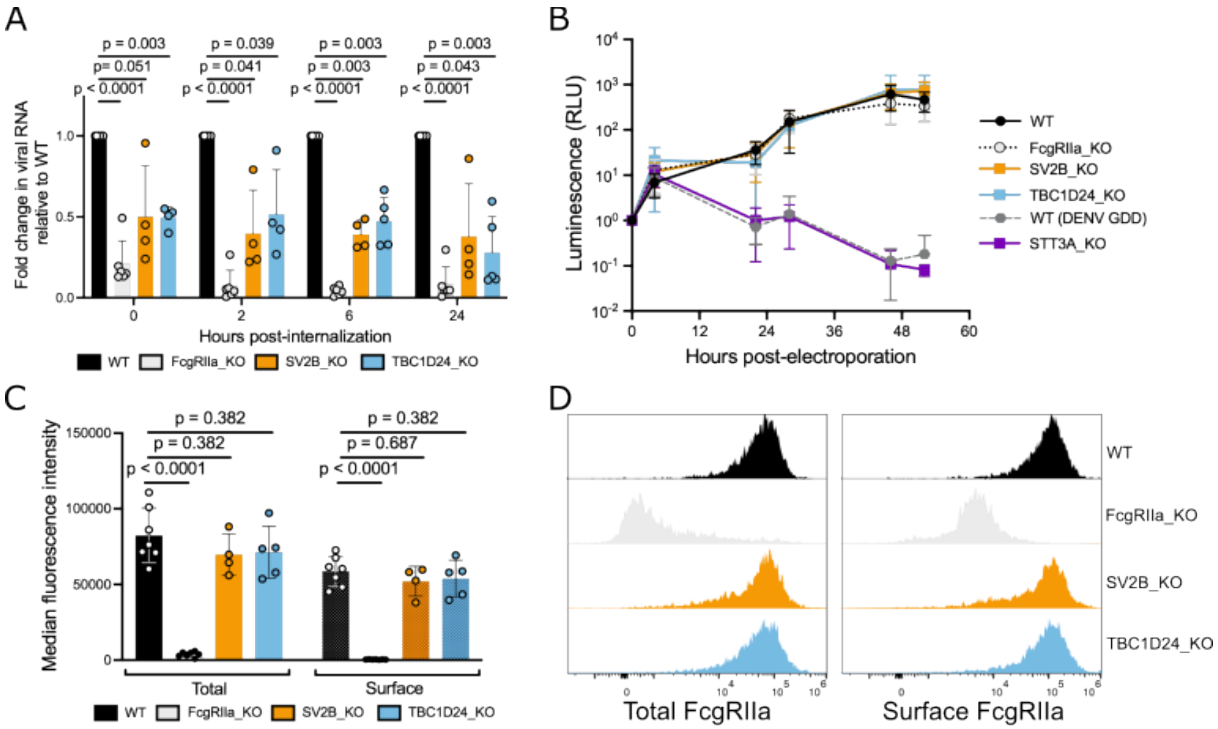

Figure 2.4. TBC1D24 and SV2B mediate efficient binding of DENV-IgG complexes to cells. (**A**) Quantitative RT-PCR of DENV RNA from cell-surface and internalized virions in K562 cells. DENV2-GFP pre-complexed with J9 monoclonal antibody were added to cells on ice for one hour. Cells were then washed to remove unbound virions and harvested either immediately (0h) or following additional incubation at 37C for the indicated time points (x-axis). Bars represent the mean normalized to WT at each time point from at least four independent experiments (data points) performed in duplicate wells and error bars show the standard deviation. **(B)** Relative luminescence of the indicated K562 cells electroporated with Renilla luciferase-expressing DENV2 replicon and lysed at indicated time points. Values for each cell line were normalized to the corresponding 0-hour time point to account for differences in electroporation efficiencies. Data points show the mean of four independent experiments, and error bars show the standard deviation. **(C)** Median fluorescence intensity of FcgRIIa expression in permeabilized (total expression) or unpermeabilized (surface expression) K562 cells. Bars represent the mean from at least four independent experiments (data points) and error bars show the standard deviation. **(D)** Histograms of FcgRIIa expression from a representative experiment of the data shown in (**C**). For (**A**) and (**C**), p-values shown are from multiple unpaired student's t-tests adjusted using the Benjamini-Hochberg method.

Discussion

By performing unbiased genome-wide and follow-up targeted CRISPR knockout screens, we identify for the first time candidate host dependency factors beyond FcgR that are exclusively required for ADE. Of these novel factors, we validated a functional role for TBC1D24 and SV2B in mediating efficient ADE of all four serotypes of DENV, using both monoclonal antibodies and polyclonal sera, and in multiple cell lines.

TBC1D24 and SV2B have established functions in trafficking specialized recycling endosomes relevant for neurotransmission ([178,179]). To our knowledge, a role for TBC1D24 in viral infection had not been described prior to our study. However, other TBC proteins, namely TBC1D16 and TBC1D20, have been shown to display antiviral and proviral activities, respectively [[213,214]]. Beyond its well defined role in synaptic vesicle trafficking, SV2 acts as the receptor for botulinum toxin [[215]]. Additionally, Sindbis virus infection upregulates expression of homologs of mammalian SV2 in *Aedes aegypti* mosquitoes [[216]], which are the primary vectors for DENV. Combined with our findings here, these studies suggest that viruses can subvert host factors involved in regulated secretion, unexpectedly including those that traditionally mediate neurotransmission. Indeed, Zika virus, a flavivirus closely related to DENV, enhances the expression of synaptotagmin-9 protein, a calcium sensor in neuroendocrine cells, and alters its subcellular localization [[217,218]]. Moreover, TMEM41B, which is involved in synaptic transmission in motor circuit neurons [[219,220]], is a host dependency factor for infection by multiple flaviviruses [[161]] and coronaviruses [[221–224]]. As described above, in the context of ADE of DENV infection, our targeted screens also identify other top hits with links to synaptic processes (**Figure 2.1E**), though their roles in ADE remain to be functionally validated. Nevertheless, their enrichment suggests that non-canonical IgG-mediated DENV entry [[155]] could exploit unconventional endocytic pathways described for synaptic processes [[225]].

Although the efficiency of ADE of DENV2-GFP mediated by monoclonal IgG was impaired to a similar extent by KO of either TBC1D24 or SV2B (**Figures 2.2A-B**), the former had a broader and more substantial impact in ADE assays using all four DENV serotypes and in the presence of polyclonal sera (**Figures 2.3A-B**). This finding implies a more critical role for TBC1D24 in ADE that is robust to assay conditions and may partly explain why SV2B was enriched in the genome-wide screen, but not the more stringent follow-up targeted screen. An alternative explanation is that in addition to its requirement for efficient ADE, SV2B may play a minor role in direct infection. Although the median reduction in direct infection efficiency of SV2B KO clones was comparable to control FcgRIIa KO clones, we note that two of four SV2B KO clones displayed a substantial reduction in direct infection efficiency compared to WT K562-DCSIGN cells. Further studies using additional clones will be required to clarify the relative contribution of SV2B to ADE and direct infection. As TBC1D24 and SV2B have shared functions in vesicle trafficking, it is also possible that they have partially redundant roles in ADE. This hypothesis can be tested in future studies examining whether ADE efficiency in SV2B KO cells can be rescued by overexpression of TBC1D24 and vice versa.

We found that KO of TBC1D24 and SV2B impaired efficient binding of IgG-bound DENV to K562 cells. Given the established role of FcgRIIa in mediating binding and internalization of IgG-DENV complexes [167], we were surprised that KO of TBC1D24 or SV2B minimally impacted FcgRIIa expression. As FcgRIIa association with lipid rafts has been shown to be important for ligand binding activity [226–228], it is possible that TBC1D24 and SV2B instead regulate the composition of cell membranes and/or trafficking of FcgRIIa to specific membrane microenvironments. Another possibility is that these host factors are involved in actin-driven cell membrane protrusions that have been implicated as a novel FcgR-dependent mechanism to capture IgG-bound DENV particles [155].

Another unexpected finding is the high enrichment of HELZ2 in both genome-wide and targeted screens. HELZ2 is an interferon-stimulated helicase and nuclear factor coactivator

[190,191,229] with previously described *antiviral* activity in the context of direct DENV and to a lesser extent, Zika virus, infection [190,191]. Thus, HELZ2 may be among a growing set of interferon-stimulated genes with both antiviral and proviral functions [190,230]. HELZ2 appears to exert its anti-DENV activity by modulating host lipid metabolism following direct infection [190]. This finding raises a possible link between HELZ2 and one of the above hypothetical *proviral* mechanisms of TBC1D24 and SV2B in the context of ADE. There are two human isoforms of HELZ2, of which the longer isoform appears to exhibit higher interferon responsiveness [190,191]. Although both isoforms are targeted by the most enriched sgRNAs in our targeted screen, it remains to be determined whether the apparent proviral activity of HELZ2 in the context of ADE is isoform-dependent.

One limitation of our study is that although we validated the requirement of TBC1D24 and SV2B for efficient ADE in multiple cell lines, we were unable to confirm our findings in primary cells due to difficulty in maintaining cell viability following CRISPR editing and subsequent infection via ADE. Another limitation is that we were unable to detect TBC1D24 and SV2B in unedited WT cells via western blotting so we could not confirm loss of protein expression in KO cells. Notably, expression of cellular factors at levels insufficient for protein detection can nevertheless affect virus infection, as demonstrated for the alphavirus receptor, MXRA8 [231], and the interferon stimulated gene, LY6E [232]. Moreover, our ability to rescue ADE efficiency in KO cells via *trans*-complementation with the gene of interest supports a specific functional requirement for these host factors (**Figures 2.2A-B**). It is possible that infection with DENV via ADE upregulates the otherwise limited endogenous expression of TBC1D24 and SV2B proteins in non-neuronal cells.

In summary, our screen highlights features shared between direct infection and ADE, and those exclusively required during ADE. Among the latter, we demonstrated a functional role for TBC1D24 and SV2B in promoting efficient ADE of DENV infection in multiple contexts. TBC1D24 and SV2B were not enriched in previous genome-scale screens that reproducibly

identified host dependency factors for direct flavivirus infection [158–161,177] and have no known roles in virus infection in general. In the absence of a biologically relevant *in vivo* model that can recapitulate dengue disease and immunity, this *in vitro* study is a key step in advancing our limited knowledge surrounding the biology of ADE of DENV. Further validation and mechanistic studies of TBC1D24, SV2B, and other screen hits can also lay the foundation for discovering host proteins and pathways that can be targeted by antiviral drugs to thwart dengue disease. Of note, SV2 proteins are the target of existing anti-epileptic drugs, some of which are FDA-approved [233–236]. It would be interesting to test the ability of these drugs to disrupt ADE processes.

Chapter 3

CONCLUSIONS AND FUTURE DIRECTIONS

Through CRISPR knockout screening and loss- and gain-of-function validation studies, we identified SV2B and TBC1D24 as host factors broadly required specifically for ADE in the presence of sera from multiple dengue-experienced donors, with all four DENV serotypes, and in multiple cell lines. Additionally, we showed that SV2B and TBC1D24 mediate binding of antibody-virion complexes without altering FcgRIIa expression levels. By identifying host factors beyond FcgR that are required for ADE, this work represents an important step in advancing our understanding of ADE biology and potential pathways exploited by DENV during this non-canonical infection pathway.

Both SV2B and TBC1D24 have established roles in neurotransmission through regulated secretion and trafficking specialized recycling endosomes [178,179], suggesting DENV can hijack endocytic pathways traditionally involved in synaptic processes during ADE. This could be explored in further studies on these genes and their canonical interacting partners during ADE of DENV, described below in the section entitled “What viral and/or cellular proteins do SV2B and TBC1D24 interact with?”. Additionally, top candidate host factors enriched in our targeted screen but not studied further also have roles linked to the nervous system and synaptic processes, such as TUBB2A (encodes a beta-tubulin that interacts with a required factor for synaptic vesicle transport, KIF1A), DNAJC6 (a heat-shock protein that acts in clathrin-mediated endocytosis in neurons), and HMX3 (involved in cell specification of neurons) [201–204]. Validating these host factors through similar experiments as performed in this thesis, described in the section entitled “What additional host factors and cell pathways mediate ADE of DENV infection?”, would also further develop the potential connection between ADE of DENV and these nervous system pathways.

Other top hits from the targeted screen not further studied in this thesis implicate potential roles of other cellular pathways. This includes genes like R3HDML, a predicted serine protease inhibitor; USP11, a downregulator of NFkB signaling and a deubiquitylator; MAP3K11, which phosphorylates MAP2Ks involved in the JNK, ERK, and p38 pathways; SDC4, a heparan sulfate proteoglycan; and BICRAL, a gene involved in chromatin remodeling [237–241]. As these additional genes mediate gene regulation and transcriptional activation, perhaps these roles could be more related to the gene expression changes associated with intrinsic ADE. This hypothesis could also extend to HELZ2, a top-scoring gene in both our genome-wide and targeted screens that is a helicase and nuclear factor coactivator [190,191,229]. To test this hypothesis, we could knockout each of these genes in cell lines and assay for the protein and gene expression of an array of pro- and anti-inflammatory cytokines (ie IL-6, IL-10, IL-12, TNFa, IFNs) in knockout versus wild-type cells at baseline and during ADE of infection through immunoblotting and qPCR, respectively. Alternatively, HELZ2 modulates host lipid metabolism following direct DENV infection [190], which could link HELZ2 with a hypothetical mechanism of action for TBC1D24 and SV2B in ADE discussed in Chapter 2. For this hypothesis, we could assay for lipid metabolism in cell lines with knockout of HELZ2, TBC1D24, or SV2B compared to wild-type cells, such as through bioluminescent assays that detect glycerol, triglyceride, cholesterol, and cholesterol ester levels. Altered levels of any of these lipids in knockout cells compared to wild-type cells would suggest these host factors indeed modulate lipid metabolism, warranting further studies to characterize this defect, including immunofluorescent staining and confocal microscopy to study localization of the identified lipid(s).

Further characterization of the roles of SV2B and TBC1D24 in ADE of DENV

While we tested the role of SV2B and TBC1D24 in two different cell lines widely used to study ADE of DENV, we were unable to successfully test the role of these genes using primary

cells. Primary cells are derived from the cell type that would be naturally infected, allowing us to confirm the relevance and role of SV2B and TBC1D24 during ADE of infection in conditions more similar to those encountered during natural infection. As DENV targets monocytes, macrophages, and dendritic cells in humans, we attempted to isolate monocyte-derived macrophages from whole blood samples for subsequent editing and ADE assays. However, following isolation and CRISPR editing of monocytes, we observed limited cell viability and low editing efficiency. Future efforts should focus on titrating the ideal conditions for increasing cell viability and editing efficiency, including reaction quantities for nucleofection of sgRNAs, cell density for plating following editing, and method of isolating monocytes. Once conditions have been optimized, infection via ADE can be performed using polyclonal serum from DENV-experienced individuals and fully infectious DENV of all four serotypes in primary monocyte-derived macrophages with knockouts of either SV2B, TBC1D24, or FcgRIIa. This model would more closely mimic conditions observed in natural infection and would therefore be more insightful in determining whether SV2B and TBC1D24 can mediate ADE in a more clinically-relevant model.

Further, we were unable to detect protein expression of SV2B or TBC1D24 in our cell models by Western blot. This could be because these proteins are expressed at low levels at baseline but are upregulated upon DENV infection via ADE. To test this, we would perform ADE of DENV infection and collect lysates from infected cells (wild-type versus knock-out of SV2B or TBC1D24) at different time points for subsequent immunoblotting. If protein expression of these host factors is still undetectable, this could indicate low endogenous protein levels. This is not a unique phenomenon, as cellular host factors expressed at undetectable levels can mediate virus infection, such as in the cases of MXRA8 and LY6E [[231,232]]. In this case, detection methods with a higher level of sensitivity would be required. Performing mass spectrometry with isotopically-labeled peptides is a method of protein detection with higher sensitivity, which could allow us to quantify smaller amounts of protein present. Alternatively, CRISPR-based activation

could be used to enhance expression of SV2B and TBC1D24 for detection via immunoblotting and/or mass spectrometry.

While we showed that SV2B and TBC1D24 mediate binding of antibody-virion complexes, it is unclear whether they do so through the same means or differently. TBC1D24 or SV2B KO led to a reduction, rather than full ablation, of infection via ADE. As these genes play similar roles in vesicle trafficking, it would be worthwhile to test whether these genes have a redundant role in ADE. To test this, we would cross-complement these genes (ie over-expressing SV2B in a TBC1D24 KO and over-expressing TBC1D24 in an SV2B KO) and create a double knockout line; each line would then be tested in a dose-response ADE assay. If their roles are redundant, one would expect cross-complementation to restore infection levels, while double KO would have a stronger reduction in infection levels.

What viral and/or cellular proteins do SV2B and TBC1D24 interact with?

Identifying other host or viral factors that SV2B and TBC1D24 interact with would shed further light on how they mediate ADE and what factors and pathways are used in ADE of DENV infection. To identify these proteins, we would perform co-immunoprecipitation (co-IP) followed by mass spectrometry on infected cells. Any identified proteins would then be validated first by repeating the co-IP and immunoblotting against the specific interactor. If protein levels are undetectable, I could generate lines that overexpress SV2B or TBC1D24 at different levels to titrate the optimal level of SV2B or TBC1D24 expression to perform the co-IP and mass spectrometry with minimal off-target effects. This would allow for an unbiased approach to identify interacting partners of SV2B and TBC1D24 during ADE of DENV.

Once interactors are identified, double knockouts of SV2B or TBC1D24 and their respective identified interactor(s) would be generated and tested in a dose-response ADE assay. Since SV2B or TBC1D24 knockout led to only a reduction in efficiency of ADE of DENV rather than a full ablation, it is likely that knocking out SV2B or TBC1D24 and their interactors

would lead to an even stronger reduction, or potentially ablation, in infection levels. Subsequently, we could perform immunolabeling of SV2B, TBC1D24, FcgR, and any interacting proteins followed by confocal microscopy. This would allow us to confirm visually that the results of the co-IP indicate co-localization, while also allowing us to visualize whether the complexes formed by SV2B, TBC1D24, and/or their interactors co-localize with FcgR to mediate binding of virus-antibody complexes. If I am unable to successfully immunolabel SV2B or TBC1D24, I could instead use the aforementioned overexpression lines, or generate fluorescently-tagged mutants of these host factors for use in microscopy. To further characterize how SV2B, TBC1D24, and their interactors mediate binding of antibody-virion complexes, we could perform single-particle tracking of fluorescently-labeled DENV during infection via ADE in cells with various genetics backgrounds (ie, wild type or KO of SV2B, TBC1D24, and/or their interactors) to visualize the trajectory and localization of antibody-virion complexes, especially during early steps of binding.

SV2B and TBC1D24 are known to interact with other proteins in their canonical functions; testing whether these interacting proteins are involved in mediating ADE would also be vital for characterizing how their canonical functions differ from or overlap with their role in ADE of DENV as well as what other genes mediate ADE. For instance, SV2B traffics synaptotagmin to sense calcium and regulate exocytosis, while TBC1D24 activates Rab35 to regulate release of neurotransmitters [[179,199,200]]. To test whether these canonical interacting proteins are expressed in our cells of interest, we first will measure gene and protein expression via qRT-PCR and immunoblotting. To then characterize whether these proteins have intact interactions with SV2B or TBC1D24, we would perform co-IP followed by immunoblotting; the subcellular localizations of interactions would then be characterized via confocal microscopy on immunolabeled samples. Finally, if these known interactors still interact with SV2B or TBC1D24 during ADE, we would then test their essentiality in ADE of DENV by generating KO cells and performing dose-response ADE assays.

Are SV2B and TBC1D24 mediators of FcgR signaling more broadly?

Since SV2B and TBC1D24 mediate binding of antibody-virion complexes during ADE of DENV infection, it would be interesting to delineate further their involvement with FcgR signaling more broadly. First, we can test how KO of SV2B or TBC1D24 impacts the internalization of labeled beads that are similar in size to, smaller than, or larger than antibody-virion complexes. This would allow us to gauge whether the role of these genes is specific to internalizing antibody-virion complexes in ADE, or whether their roles are also more broadly applicable to internalization of similarly and/or differently sized particles. Downstream of this, we could also assay for different FcgR functions in the presence versus absence of SV2B or TBC1D24. Following cross-linking of FcgRIIa, phosphorylation of the ITAMs in the receptor occurs which activates SYK and SRC kinases and the protein kinase C pathway [91]. This then triggers an influx of calcium ions in the cell and actin remodeling to allow for phagocytosis; these steps can ultimately activate transcription factors like p38 and JNK which can lead to release of cytokines [91]. To measure these cellular activities, a variety of read-outs could be used, such as immunoblotting for ITAM phosphorylation or protein kinase expression levels, measuring calcium ion concentrations, and visualizing actin remodeling via confocal microscopy. To gauge whether SV2B or TBC1D24 are required for these FcgR functions during ADE and more broadly, we could compare these readouts in the wild type, SV2B KO, or TBC1D24 KO cells during both ADE of DENV and during FcgR signaling in the absence of DENV (accomplished using an anti-FcgRIIa antibody in uninfected cells).

Reduced binding of antibody-virion complexes to FcgRs can be indicative of improper FcgR conformation on the cell membrane, which could be caused by altered receptor trafficking or altered membrane lipid composition. To gauge whether FcgRIIa trafficking is altered, imaging of fluorescently-tagged FcgRIIa and immunolabelled cellular compartments in cells that are wild type or have a KO of SV2B or TBC1D24 over a time course will be informative. Additional

immunolabels could be attached to proteins associated with the intracellular trafficking of FcgRIIa, such as EEA-1, Syntaxin 5, and TI-VAMP [242], or those associated with ubiquitination [243]. As FcgRIIa can be localized to lipid raft domains upon IgG-mediated cross-linking [226–228], it would be interesting to test whether SV2B or TBC1D24 alter the membrane lipid composition. To address this question, confocal laser scanning microscopy could be used on fluorescently-tagged or immunolabeled proteins and lipids (such as FcgRIIa and cholesterol) in fixed cells to provide an initial visualization of how FcgRIIa is distributed in the plasma membrane relative to lipid components such as cholesterol-rich lipid rafts [244]. Further resolution could be gained with single dye tracing, which uses fluorescence microscopy in combination with small single fluorophore dyes attached to a target molecule and enables molecular characterization of domains in the membrane, such as how different phospholipids diffuse or how membrane proteins move [244]. Thus, we could visualize membrane lipid domains and how FcgRIIa moves throughout them, which could be compared across cells that are wild-type or have a knockout of SV2B or TBC1D24.

What additional host factors and cell pathways mediate ADE of DENV infection?

Other high-scoring genes from the targeted screen should be validated and characterized. Of the most interest is HELZ2, as this gene was highly enriched in both our genome-wide and targeted screens, which plays a known role in promoting direct DENV infection and is known to be a pro- or anti-viral factor more broadly. Other candidates of interest include high-ranking hits with functions tied to trafficking in the nervous system, which are thus similar to the canonical functions of SV2B and TBC1D24. TUBB2A was the highest-scoring of these genes, making it the most enticing candidate for further studies; TUBB2A is a microtubule component and known interactor of KIF1A which mediates synaptic vesicle transport [201,202]. Similarly, DNAJC6 and HMX3 would also be interesting candidates for follow-up studies, as they both function in the nervous system (as a heat-shock protein and a transcription factor,

respectively) [203,204]. For these genes and other top-scorers, the same validation process used on TBC1D24 and SV2B would be performed. We would start by generating KO lines in K562 cells for initial validation in dose-dependent ADE assays, followed up by *trans*-complementation. Once an initial role is found, KO lines in U937 would be generated and tested, along with testing fully infectious viruses of all serotypes and serum from dengue-experienced donors. Finally, the role in the viral replication cycle and effects on FcgR expression would be studied. Viral replication studies would include the binding and internalization qRT-PCR assays as well as replicon assays to measure translation and replication, as performed in Chapter II of this thesis, while flow cytometry could be used to assay FcgR expression levels as described in Chapter II of this thesis.

Implications for clinical interventions for DENV

SV2B and TBC1D24 require further exploration before they can be used as druggable targets to prevent ADE. Drugs targeting SV2 proteins with high affinity are already developed as antiepileptics [233–236]. Though the exact role of SV2B in epilepsy is unknown, padsevonil acts as an antiepileptic by binding with high affinity to all three isoforms of SV2 (A/B/C) and acting as an agonist for the $GABA_AR$ BZD site [236]. People with epilepsy from TBC1D24 mutations are often treated with antiepileptic drugs that are not TBC1D24-specific, such as valproate or phenytoin [245]. Future studies include performing ADE dose-dependent assays in the presence of varying levels of padsevonil, valproate, and/or phenytoin. If higher levels of these drugs show lower amounts of infection via ADE, this could warrant further testing *in vivo*, where humanized mice could be infected with DENV in the presence of anti-DENV serum and varying levels of padsevonil, valproate, and/or phenytoin. If mice have less severe symptoms or better survival in the presence of higher levels of these drugs, this could suggest these drugs may have utility in treating severe dengue disease. As current drugs do not specifically bind to SV2B or TBC1D24, drugs with these specific activity profiles could be developed. Further, SV2B or TBC1D24 may

have the potential to serve as biomarkers during assessment of disease escalation risk in an individual or of vaccine efficacy and safety. This potential must first be confirmed by examining whether SV2B or TBC1D24 expression is correlated with more severe disease through studies *in vivo* and on blood cells of cohorts of individuals with mild versus severe dengue.

Implications for the study of ADE of DENV

Studies on ADE of DENV infection have lacked a comprehensive and unbiased approach to measure functional requirements, rather than effects of, this non-canonical viral uptake pathway. Therefore, this thesis marks the first application of a genome-wide CRISPR knockout screen to study functional requirements of ADE of DENV. Further, this thesis represents the first time ADE-specific host factors beyond FcgRs have been identified. We also identified, but did not test, multiple other candidate host factors and pathways that may be exploited during ADE of DENV infection which warrant further study. Though *in vitro* studies have inherent limitations, this work represents an important first step into further understanding the biology of ADE. With further validation and studies on TBC1D24, SV2B, and other host factors identified in this thesis, insights into potential therapeutic targets for severe dengue disease can ultimately be developed.

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Appendix A:

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Supplemental Table 1: Output of MAGeCK analysis of genome-wide screen.

URL accession:

https://www.biorxiv.org/content/biorxiv/early/2024/04/27/2024.04.26.591029/DC1/embed/media-1.xls?download=true

Supplemental Table 2: Complete list of genes and guides used for targeted sub-library screen.

URL accession:

https://www.biorxiv.org/content/biorxiv/early/2024/04/27/2024.04.26.591029/DC2/embed/media-2.txt?download=true

Supplemental Table 3: Output of MAGeCK analysis of targeted sub-library screen.

URL accession:

https://www.biorxiv.org/content/biorxiv/early/2024/04/27/2024.04.26.591029/DC3/embed/media-3.xlsx?download=true

Supplemental Table 4: Sequences of sgRNA oligos and PCR primers.

URL accession:

https://www.biorxiv.org/content/biorxiv/early/2024/04/27/2024.04.26.591029/DC4/embed/media-4.xlsx?download=true

Figure S1

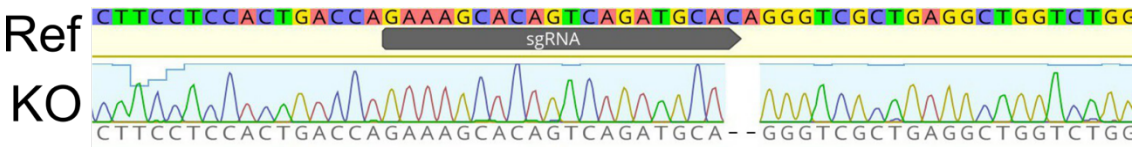

Fig S1: Genotyping of K562 FcgRIIa KO clone.

Sanger sequencing of locus targeted by gRNA in the K562 FcgRIIa KO clonal line. Traces were aligned to WT reference sequence ("Ref") to identify the 2 bp deletion.

Figure S2

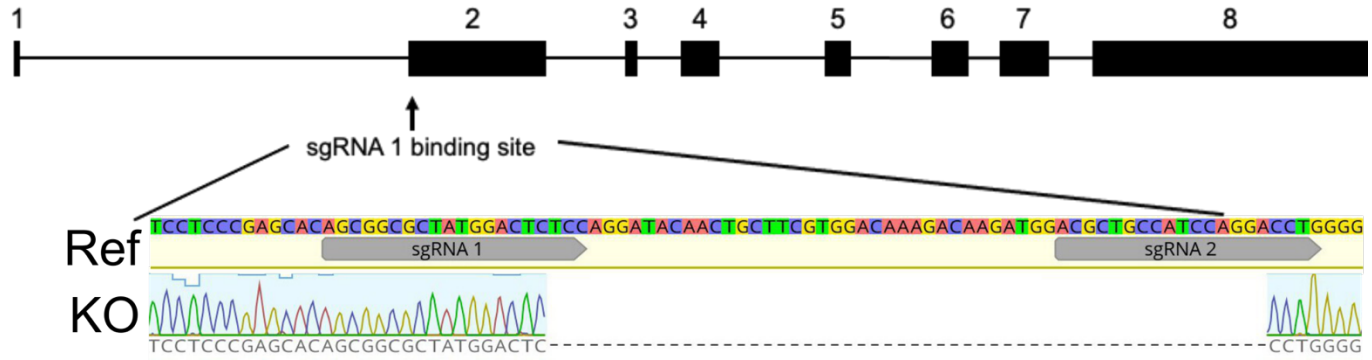

Fig S2: Genotyping of K562 TBC1D24 KO clone.

(Top) schematic of TBC1D24 exons (boxes) and introns (lines). (Bottom) Sanger sequencing of loci targeted by gRNA in the K562 TBC1D24 KO clonal cell line. Traces were aligned to WT reference sequence ("Ref") to identify the 54 bp deletion within exon 2.

Figure S3

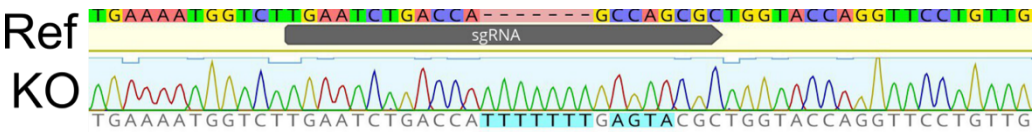

Fig S3: Genotyping of K562 SV2B KO clone.

Sanger sequencing of locus targeted by gRNA in the CRISPR-induced K562 SV2B KO clonal cell line.

Traces were aligned to WT reference sequence (“Ref”) to identify the 7 bp insertion and 4 bp missense.

Figure S4

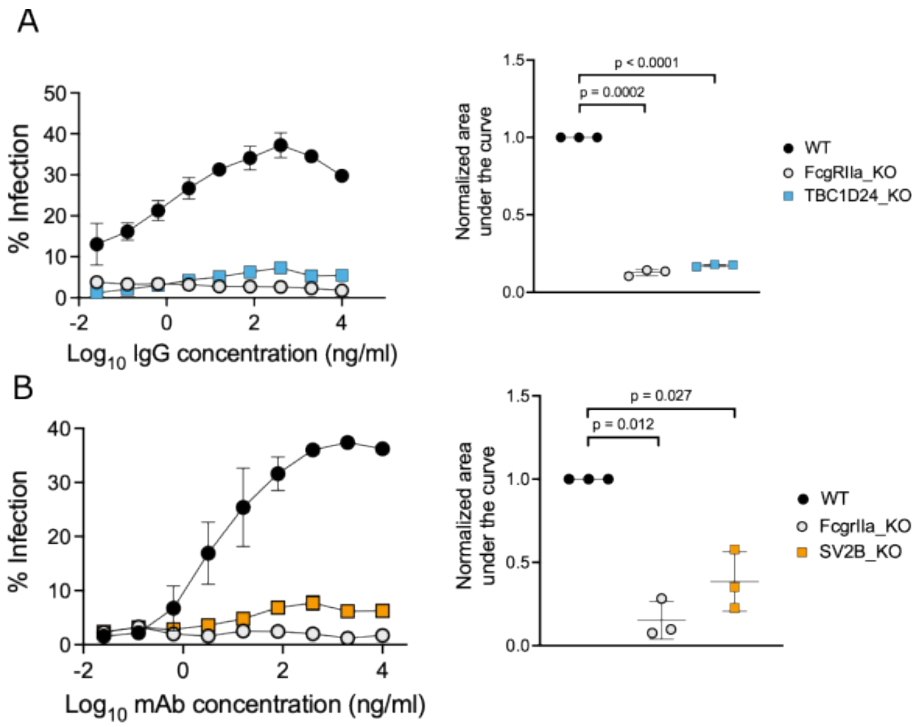

Fig S4: Functional validation of TBC1D24 and SV2B in ADE assays

A-B: (Left) The indicated K562 cells were infected via ADE using DENV2-GFP in the presence of serially diluted mouse anti-DENV IgG monoclonal antibody DV2-70 used in CRISPR screens. Data points represent the mean of three independent experiments normalized to the peak infection level of WT cells, and the error bars represent the standard deviation. (Right) Quantification of area under the curve normalized to WT K562 cells from three independent dose-response ADE experiments (data points), each performed in duplicate wells. Horizontal lines and error bars indicate mean and standard deviation, respectively. In each experiment, a FcgRIIa KO clone was included as a control. P-values shown are from multiple independent paired student's t-tests adjusted using the Benjamini-Hochberg method.

Figure S5

Clone	KO Score
KO_1	92
KO_2	nd
KO_3	97
KO_4	nd

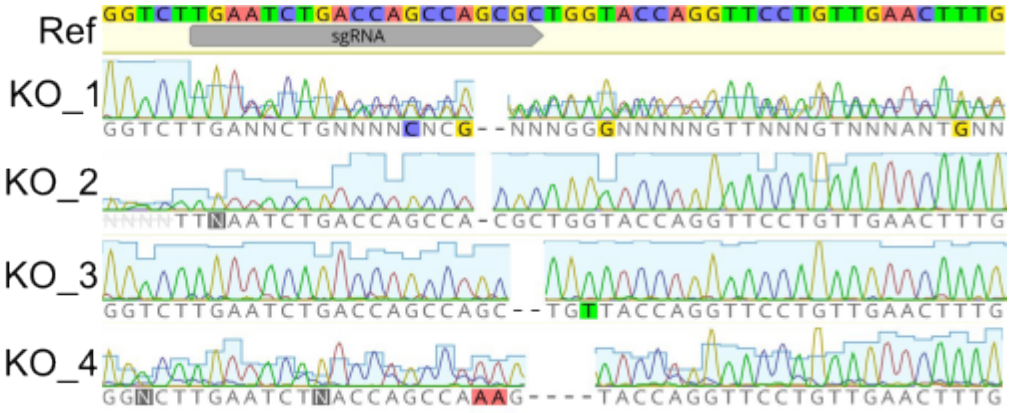

Fig S5: Genotyping of K562-DCSIGN SV2B KO clones.

Sanger sequencing of locus targeted by gRNA in the K562-DCSIGN SV2B KO clones. Traces were aligned to WT reference sequence to identify mutations. Heterogeneous mutations were deconvoluted using Inference of CRISPR Edits I (ICE; https://ice.synthego.com/#/). KO scores as determined by ICE are shown left; nd = not determined.

Figure S6

Clone	KO Score
KO_1	100
KO_2	92
KO_3	100
KO_4	93
KO_5	100
KO_6	100

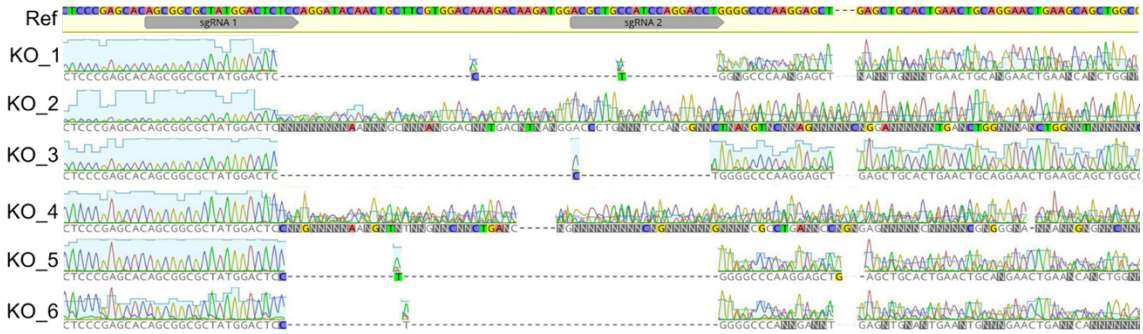

Fig S6: Genotyping of TBC1D24 KO K562-DCSIGN Cells.

Sanger sequencing of locus targeted by gRNA in the CRISPR-induced K562-DCSIGN TBC1D24 KO clones. Traces were aligned to WT reference sequence to identify mutations. Heterogeneous mutations were deconvoluted using ICE. KO scores as determined by ICE (https://ice.synthego.com/#/) are shown left.

Figure S7

Clone	KO Score
KO_1	100
KO_2	100
KO_3	100
KO_4	100
KO_5	95
KO_6	100
KO_7	100
KO_8	100
KO_9	96
KO_10	100
KO_11	100

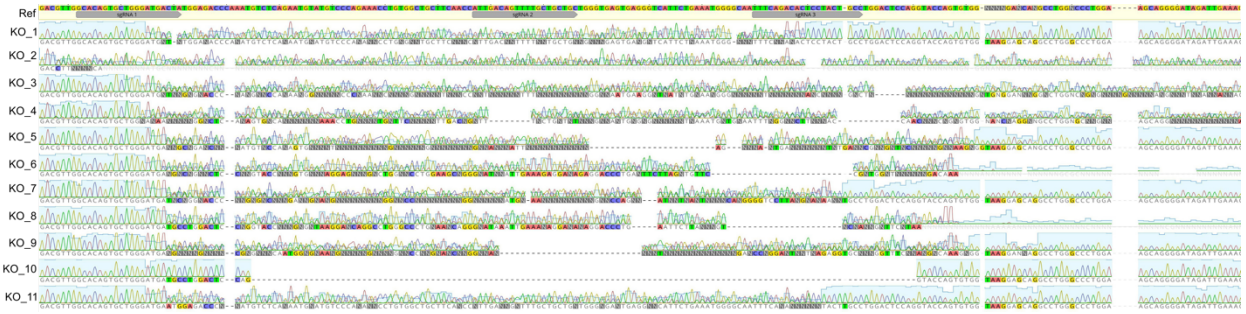

Fig S7: Genotyping of FcgRIIa KO K562-DCSIGN Cells.

Sanger sequencing of locus targeted by gRNA K562-DCSIGN FcgRIIA KO clones. Traces were aligned to WT reference sequence and deconvoluted using ICE (https://ice.synthego.com/#/). KO scores as determined by ICE are shown left.

Figure S8

Clone	KO Score
KO_1	100
KO_2	100
KO_3	100
KO_4	100

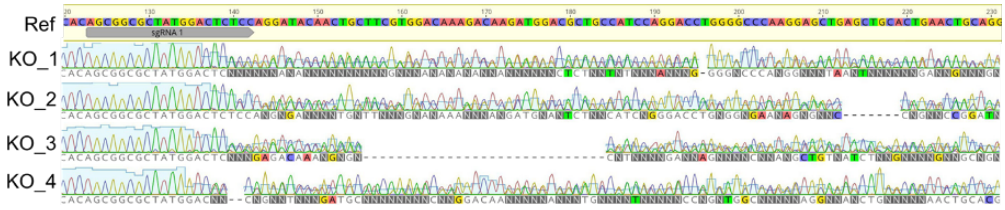

Fig S8: Genotyping of TBC1D24 KO U937 Cells.

Sanger sequencing of locus targeted by gRNA in U937 TBC1D24 KO clones. Traces were aligned to WT reference sequence and deconvoluted using ICE (https://ice.synthego.com/#/). KO scores as determined by ICE are shown left.

Figure S9

Clone	KO Score
KO_1	100
KO_2	100
KO_3	100
KO_4	100

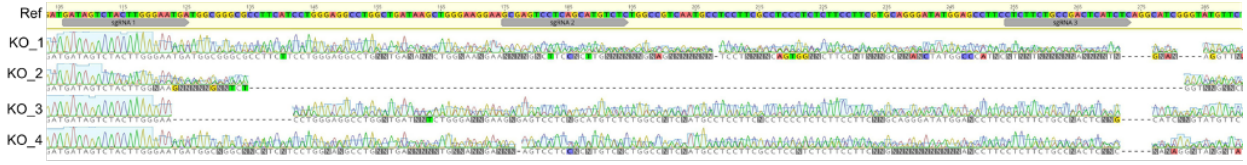

Fig S9: Genotyping of SV2B KO U937 Cells.

Sanger sequencing of locus targeted by gRNA in the CRISPR-induced U937 SV2B KO clones. Traces were aligned to WT reference sequence and deconvoluted using ICE (https://ice.synthego.com/#/). KO scores as determined by ICE are shown left.