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Forward Genetic Screening In Human Haploid Cells To Identify Host Factors Required For Virus And Toxin Entry

Amber Michelle Riblett
University of Pennsylvania, amber.riblett@gmail.com

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Abstract
My dissertation research has focused on identifying host cellular factors required by the bunyavirus Rift Valley fever virus (RVFV), an RNA virus that causes disease in humans and animals, and the secreted Toxin B (TcdB) from Clostridium difficile, a bacterial pathogen that causes severe antibiotic-associated diarrheal disease. In 2015, the WHO named the ten emerging diseases most likely to cause severe outbreaks in the near future, and three are caused by bunyaviruses (including RVFV). Concern is likewise mounting about the increasing incidence, virulence, and antibiotic-resistance of C. difficile infection worldwide. A better understanding of the molecular details of the pathogenesis of these diseases is urgently needed in order to inform the development and application of therapeutic interventions. The data presented in this thesis summarize the results of two independent screening projects, each utilizing a strategy of forward genetic screening in a mutagenized human haploid cell library. Our RVFV screen identified a suite of enzymes involved in glycosaminoglycan biogenesis and transport, including several components of the cis-oligomeric Golgi (COG) complex. In addition, we identified the gene PTAR1, disruption of which led to RVFV resistance and reduced heparan sulfate surface levels. Biochemical and genetic approaches were utilized to show that both pathogenic and attenuated RVFV strains require GAGs for efficient infection in some cell types, with the block to infection being at the level of virion attachment. Our TcdB screen identified the Wiskott–Aldrich syndrome protein and SCAR homologue (WASH) complex as a host cellular factor supporting TcdB intoxication. Involvement of the WASH complex in TcdB entry was validated by pharmacologic inhibition of recycling endosomes and the use of mouse fibroblasts lacking a functional WASH complex due to genetic ablation of the core WASH1 gene. The host factors supporting TcdB internalization and transport are largely unknown, and our data help to elucidate the mechanism of intoxication of this important and poorly-characterized virulence factor.

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FORWARD GENETIC SCREENING IN HUMAN HAPLOID CELLS TO IDENTIFY HOST FACTORS REQUIRED FOR VIRUS AND TOXIN ENTRY

Amber M. Riblett

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Supervisor of Dissertation

_____

Robert W. Doms, M.D., Ph.D., Professor of Pathology and Laboratory Medicine

Graduate Group Chairperson

_____

Daniel S. Kessler, Ph. D., Associate Professor of Cell and Developmental Biology

Dissertation Committee

Stuart N. Isaacs, M.D., Associate Professor of Medicine

Michael S. Marks, Ph.D., Professor of Pathology and Laboratory Medicine

Susan R. Weiss, Ph.D., Professor of Microbiology

Matthew D. Weitzman, Ph.D., Associate Professor of Pathology and Laboratory Medicine

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ABSTRACT

FORWARD GENETIC SCREENING IN HUMAN HAPLOID CELLS

TO IDENTIFY HOST FACTORS REQUIRED FOR VIRUS AND TOXIN ENTRY

Amber M. Riblett

Dr. Robert W. Doms

My dissertation research has focused on identifying host cellular factors required by the bunyavirus Rift Valley fever virus (RVFV), an RNA virus that causes disease in humans and animals, and the secreted Toxin B (TcdB) from *Clostridium difficile*, a bacterial pathogen that causes severe antibiotic-associated diarrheal disease. In 2015, the WHO named the ten emerging diseases most likely to cause severe outbreaks in the near future, and three are caused by bunyaviruses (including RVFV). Concern is likewise mounting about the increasing incidence, virulence, and antibiotic-resistance of *C. difficile* infection worldwide. A better understanding of the molecular details of the pathogenesis of these diseases is urgently needed in order to inform the development and application of therapeutic interventions. The data presented in this thesis summarize the results of two independent screening projects, each utilizing a strategy of forward genetic screening in a mutagenized human haploid cell library. Our RVFV screen identified a suite of enzymes involved in glycosaminoglycan biogenesis and transport, including several components of the cis-oligomeric Golgi (COG) complex. In addition, we identified the gene *PTAR1*, disruption of which led to RVFV resistance and reduced heparan sulfate surface levels. Biochemical and genetic approaches were utilized to show that both pathogenic and attenuated RVFV strains require GAGs for efficient infection in some cell types, with the block to infection being at the level of virion

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CHAPTER 1: INTRODUCTION

INVESTIGATING HOST-PATHOGEN INTERACTIONS

Our bodies are constantly assailed by disease-causing microorganisms wielding a diverse arsenal of molecular weapons that they employ to gain entry into our tissues, break down our defenses, and reprogram our cellular machinery. The foot soldiers carrying out these attacks - such as viruses, bacteria, and their secreted toxins - rely on a large number of host cellular factors and pathways as they enter, traffic through, and exert various pathophysiological effects upon the host cell. From the initial interactions between the pathogen's surface proteins and the host cell's plasma membrane to the apoptosis, lysis, or takeover of the host cell, a pathogen uses its limited protein reservoir to co-opt the much more extensive machinery found within the host. A virus can use this cellular infrastructure to carry out its genome replication, assemble new virions, and move throughout the host cell. Bacterial toxins hitchhike along host endocytic pathways, rely on host cell proteases for cleavage, and trigger signaling cascades to modify host cell function in a way that benefits the bacteria. This manipulation of the resources offered by the host with which it has coevolved is a defining trait of our microscopic invaders, and identification of those host factors upon which the pathogen relies (such as cell surface receptors) has provided invaluable information about the lifecycles and mechanisms of action of the causative agents of many important human diseases.

Traditionally, relatively reductionist approaches have been taken to identify specific interactions between pathogen and host cell molecules. More recently, rapid advances in high-throughput screening technologies based upon small molecules, loss-

of-function libraries, and interactome characterization have informed our understanding of nearly every stage of host-pathogen interaction and identified targets for therapeutic intervention. The study of how pathogens co-opt cellular machinery has also yielded tremendous insight into the function of human biological pathways, such as the discovery of RNA splicing in adenovirus-infected cells that led to the subsequent understanding of this as a normal cellular function (1, 2). I was extremely interested in the power of high-throughput screening techniques to identify novel aspects of pathogen entry and infection, and also in the ability of such studies to teach us about fundamental cell biology. Of particular interest to me was the application of these screens to emerging and poorly-characterized diseases. My dissertation research has therefore focused on optimizing a forward genetic screening strategy to identify host cellular factors that are required by bunyaviruses, a family of RNA viruses that can cause disease in humans and animals, and the secreted Toxin B from *Clostridium difficile*, a bacterial pathogen that causes a severe antibiotic-associated diarrheal disease in humans.

VIRAL INFECTION OF HOST CELLS

Viruses that cause human disease package their DNA or RNA genomes into nucleocapsid complexes that are sometimes surrounded by a lipid bilayer membrane called the viral envelope. Structural proteins known as the viral glycoproteins stud the membranes of these enveloped viruses and are available to access and interact with the surface of the host cell. A myriad of other proteins, such as viral RNA-dependent RNA polymerases, reverse transcriptases, and matrix proteins may or may not be present inside of the virion, depending on the type of virus.

During the first step of virus entry, one or more viral proteins that are exposed (glycoproteins in the case of enveloped viruses) interact with attachment factors and receptors on the host cell surface. There is a tremendous diversity of both the type of host factors used by the virus during this stage of entry as well as the nature of the interactions that occur. Virions have been shown to interact with proteins, carbohydrates, and lipids – and these interactions vary greatly in their strength and duration [reviewed in (3)]. These components at the cell surface may serve as attachment factors that concentrate the virus particles in two dimensions, such as heparan sulfate proteoglycans that interact with the E2 glycoprotein of hepatitis C virus (HCV) during the initial binding of the virus to the cell (4). They may also induce conformational changes that allow the virus to directly fuse with the cell membrane. During entry of human immunodeficiency virus type 1 (HIV-1), for example, its glycoprotein Env first binds to the CD4 receptor, and this binding induces a conformational change that allows Env to bind its coreceptor (CCR5 or CXCR4), which leads to membrane fusion [reviewed in (5)]. Cell surface components may also function as entry receptors that facilitate uptake of the virus particle into the host cell's endocytic pathway, as is the case for GD1a-mediated uptake of polyomavirus into early endosomes (6-8). The interaction between the virus and cell surface proteins might also serve to transduce signals that in some way reprogram the cell to make it more susceptible to infection, such as the actin rearrangement triggered by tyrosine kinase activation following binding of simian virus 40 (SV40) to its receptor [reviewed in (3)].

To enter the cell, viruses are able to utilize a range of available existing endocytic pathways. Pinocytic uptake mechanisms, such as clathrin-mediated endocytosis, macropinocytosis, and caveolae-mediated endocytosis are the most commonly used,

but use of other pathways, such as phagocytosis, has been demonstrated, as well as variations on each of these pathways, and indication of entry via novel or uncharacterized pathways. Interestingly, it has also been found that some viruses are capable of entering host cells via multiple routes, often in a cell-dependent manner. For example, influenza A virus, which usually enters through clathrin-coated pits, can in some cases utilize macropinocytosis as an alternative entry pathway (9-11). Following their endocytosis, virions find themselves in the lumen of a primary endocytic vesicle, such as an early endosome, macropinosome, or a caveosome. From there, the virus must penetrate through its vacuole to deliver its genome into the host cell cytosol. For enveloped viruses, this process involves viral glycoprotein-mediated fusion of viral and vesicular membranes. Cues from the maturing endosomes, such as a lowering of pH, trigger conformational changes in the glycoproteins, which are then able to effect fusion of the membranes.

After gaining access to the cytosol, the virus needs to uncoat its genome in order to begin the process of replication. For RNA viruses, this typically takes place in the cytoplasm (often in intimate association with organelle membranes) whereas most DNA viruses replicate in the nucleus. As a general rule, viral capsids remain intact until they have trafficked to their site of replication. Viruses that replicate in the nucleus utilize many different strategies of gaining entry to this organelle. HSV-1 and adenovirus nucleocapsids traffic to the nucleus and then dock at the nuclear pore complex (NPC), where they uncoat and deliver their genomes directly into the nucleus (12-14). Some other DNA viruses move either fully or partially through the NPC before uncoating their genome. Regardless of the eventual site of replication, to reach it, the incoming virion (or trafficking capsid) is reliant upon the transport machinery of the cell. For example,

viruses have been shown to depend upon actin and related proteins (including the Arp2/3 complex and Rho GTPases) or movement along microtubules by dyneins and kinesins, as well as other accessory proteins that act to coordinate transport, such as Rab GTPases, tethering complexes, etc. [reviewed in (15)].

At the site of replication, many viruses induce the formation of virus factories, dramatic rearrangements of organelle-derived membranes that serve to facilitate the efficient replication of viral genomes and proteins. Membranes for virus factories have been shown to be derived from lysosomes, smooth and rough ER, the Golgi apparatus, and even autophagosomes. Virus factories can be formed by enveloped as well as non-enveloped viruses, can be located in many different subcellular locations, and can take a variety of shapes. For example, adenoviruses and polyomaviruses (both non-enveloped DNA viruses) set up aggresome-like virus factories in the nucleus, whereas flaviviruses and coronaviruses (enveloped RNA viruses) generate virus factories that take the shape of double-membrane vesicles in association with the ER. These virus factories can help to shield viral components from host cell immune detection, act as a scaffold for replication complexes, and recruit cellular factors such as mitochondria [reviewed in (16)]. Once a sufficient number of viral proteins and genome copies have been generated, they assemble into virions and are released from the infected cell. For enveloped viruses, this step involves the theft of host membrane at the site of budding with the assistance of recruited cellular membrane deformation machinery. As this deformation must occur in a “reverse topology,” such that the virion buds away from the cytoplasm, a number of enveloped viruses have been shown to recruit the host ESCRT (endosomal sorting complexes required for transport) proteins, the only cellular pathway yet identified that performs membrane deformation in this direction. The first described

and best characterized example of virus hijacking of ESCRT machinery for virion budding is the recruitment by retroviral Gag polyproteins of ESCRT and associated proteins to the plasma membrane by means of amino acid sequences that mimic those used by cellular adaptor proteins (17-20). Some enveloped viruses, such as influenza A and Semliki Forest virus, have evolved mechanisms of ESCRT-independent membrane deformation during virion release (21, 22). Viruses that bud intracellularly (for example, into the Golgi apparatus) must use the host cell's exocytic machinery to traffic in vesicles to the plasma membrane to be released. Other viruses spread within the infected host by means of cell-to-cell transmission, bypassing the need for release of infectious virions and cell-free dissemination.

Our ability to glean insight into the mechanistic detail of these later stages of virus replication has been hampered by a lack of assays to detect viral assembly and release that are amenable to the most commonly used high-throughput screening techniques. Traditionally, screening for cellular factors that impact virus replication has employed reporter genes or cytotoxicity as a read-out of viral infection. However, for most viruses, this places the temporal limit of phenotype detection at the stage of translation of viral proteins, and does not allow for the detection of meaningful and interesting defects in assembly or release of virions. For this reason, our understanding of the entry pathways utilized by many viruses is often more extensive than our understanding of the details of their assembly and release processes.

THE BUNYAVIRIDAE

Bunyaviruses are enveloped single-stranded negative-sense RNA viruses that comprise five genera: *Phlebovirus*, *Orthobunyavirus*, *Nairovirus*, *Tospovirus*, and

Hantavirus. Within these genera are more than 350 viruses, making the *Bunyaviridae* the largest family of RNA viruses in the world (23). Most of these viruses infect arthropods, although some can infect plants or rodents. Of the bunyaviruses that infect arthropods and rodents, a number can be borne by these vectors into human hosts where they cause a wide range of diseases. Notable human pathogens within the *Bunyaviridae* family include Crimean Congo Hemorrhagic Fever virus (CCHFV), a Nairovirus, which is endemic to regions of Africa, Europe, and Asia, and causes a febrile illness characterized by joint pain and vomiting that can progress to uncontrolled bleeding (24). The *Hantavirus* genus includes the causative agents of Hemorrhagic Fever with Renal Syndrome (HFRS) in Europe and Asia and Hantavirus pulmonary syndrome (HPS) in the Americas. The emergence and spread of newly-identified bunyaviruses, as well as important progress in recent years toward a more detailed understanding of bunyavirus structure and genetics, has renewed interest in this large and diverse family of viruses.

The bunyavirus that was the focus of much of my doctoral work is the phlebovirus Rift Valley fever virus (RVFV), named for the disease that was first described during an epidemic in 1931 at a sheep farm in the Rift Valley of Kenya (25, 26). Rift Valley fever is a viral zoonosis that passes into livestock and human populations via a number of mosquito vector species. In livestock, the disease is fatal to newborn animals and causes spontaneous abortions in pregnant animals; in humans it is a febrile illness which progresses, in approximately 8% of patients, to a severe disease characterized by encephalitis, retinitis, or hemorrhagic syndrome (27). Rift Valley fever remained confined to sub-Sahran Africa until 1977, when 18,000 people became ill during an outbreak in Egypt; in 2000, it spread beyond the African continent

into Saudi Arabia and Yemen (28). Because of the broad host tropism of this virus and the ease with which it moves into the dominant mosquito species in a given region, there is great concern that climate change will expand the geographical range of its current vectors, allowing the virus to move into naive populations (29, 30). This is particularly alarming as recent outbreaks have seen mortality rates increase - an outbreak in 2007 in Kenya reported a 29% case fatality rate, compared to the historical average of 0.5-1.0% mortality (28). As there are currently no FDA-approved vaccines or therapeutics, a better understanding of how the virus that causes Rift Valley fever replicates within its host will be of great benefit.

BUNYAVIRUS STRUCTURE AND ENTRY

The bunyavirus genome comprises a small (S), medium (M), and large (L) segment that encode, respectively, the nucleocapsid protein (N), the two glycoproteins (Gn and Gc), and the viral RNA-dependent RNA polymerase (L). Three nonstructural proteins are also encoded: two on the M segment, termed NSm1 and NSm2; and one on the S segment, termed NSs. These nonstructural proteins play important roles during pathogenesis, such as suppressing apoptosis and limiting the IFN-mediated antiviral response, but they are dispensable for growth in tissue culture (31). Bunyaviruses have broad tropism and enter mammalian cells via receptor-mediated endocytosis. Entry of RVFV into dendritic cells has been shown to be mediated by the C-type lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) but as this lectin is not expressed on most of the cell types that RVFV productively infects, the receptor (or receptors) used by the virus to enter other cells remains unidentified (32). It is also unclear precisely what cellular endocytic machinery is required for RVFV entry. A

study using non-replicating RVFV virus-like particles (VLPs) reported that the entry of these particles into BHK-21 and A549 cells was both dynamin- and clathrin-dependent (33). The same year, Harmon and colleagues demonstrated that the MP-12 attenuated vaccine strain of RVFV enters HepG2 and HeLa cells in a caveolae-mediated, clathrin-*independent* manner (34). Our lab has shown that MP-12 infection of 293T and *Drosophila* S2 cells was blocked by inhibitors of macropinocytosis (35).

Following endocytosis, the bunyavirus envelope membrane undergoes glycoprotein-driven fusion with the endosomal membrane. See Figure 1-1 for an overview of the bunyavirus replication cycle. The Gc glycoprotein of RVFV is a Class II fusion protein (36), and acidification of the endosomal compartment triggers a conformational change that exposes hydrophobic residues that insert into the host membrane and mediate fusion. For RVFV Gc, the pH threshold at which this occurs has been reported to be around 5.5 - 5.7, which is consistent with fusion from within the late endosome (33). However, Rab7, which is the Rab GTPase primarily responsible for late endosomal maturation, is not required for the entry of many bunyaviruses, including the nairovirus CCHFV (37, 38), the orthobunyavirus La Crosse virus (39), and the phlebovirus Uukuniemi virus (40).

Once membrane fusion has occurred, the ribonucleoproteins (RNPs), comprising the three genome segments encapsidated by the nucleocapsid protein N, are released into the cytoplasm. The viral RNA-dependent RNA polymerase then transcribes viral mRNAs, and viral proteins are translated in association with ER-derived membranes. Within the ER, the bunyavirus Gc and Gn proteins form heterodimers after which a Golgi localization signal within the Gn protein causes these heterodimers to traffic to the Golgi apparatus. Viral factories are formed that function as scaffolds for genome replication

and formation of new RNPs. These RNPs then traffic to the Golgi membranes where the Gc-Gn heterodimers have accumulated, and interaction between RNPs and Gc-Gn heterodimers is thought to trigger assembly and budding of bunyavirus virions into the Golgi membranes. The nascent virions traffic in exocytic vesicles from the TGN to the plasma membrane, where these vesicles fuse and release the virions. Evidence for most of these steps of the bunyavirus replication cycle consists of immunofluorescence or electron microscopy visualization of virus proteins or particles within infected cells, and the viral and host factors responsible for these processes remain largely undefined.

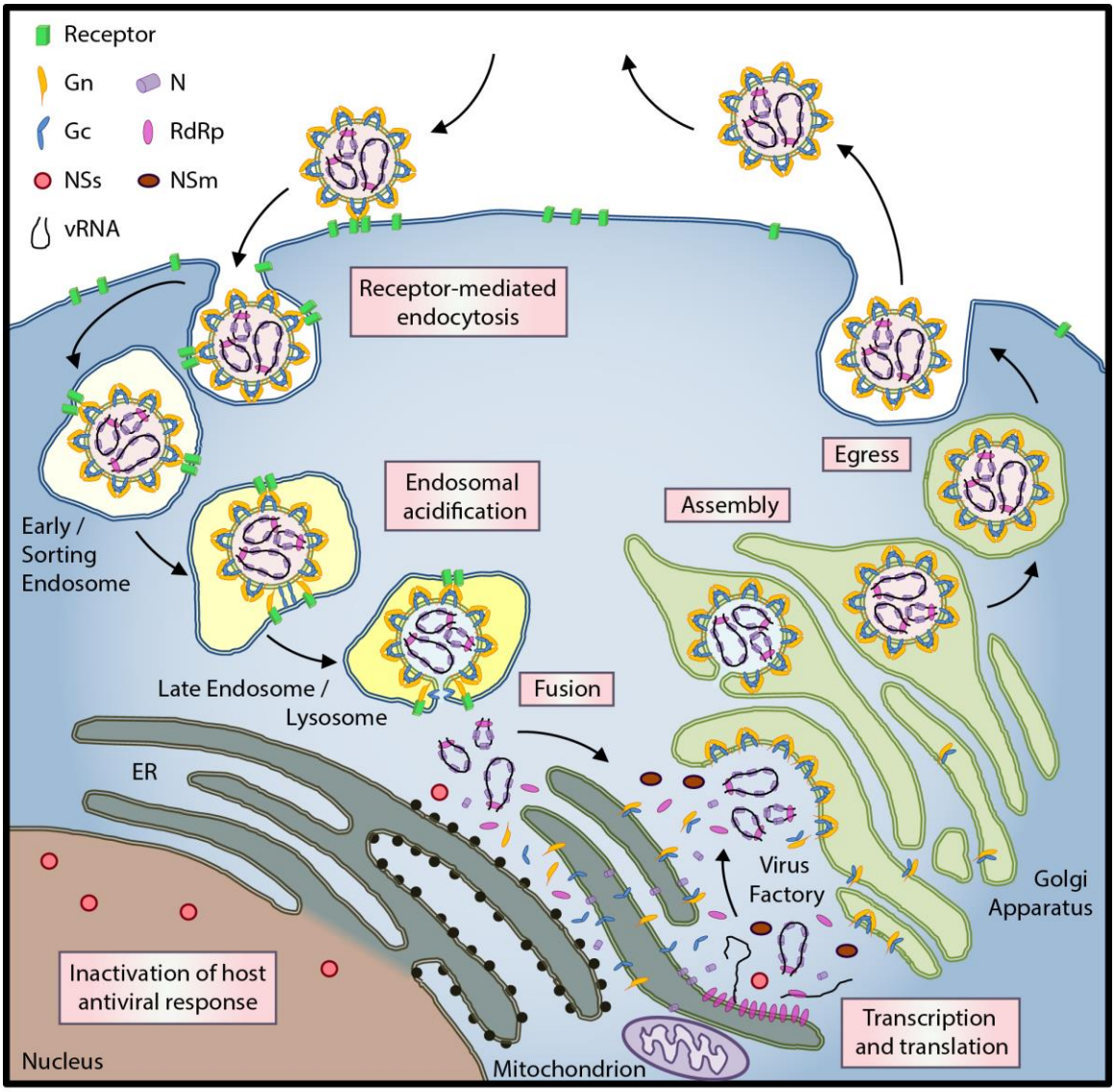

Figure 1-1. Bunyavirus replication cycle. Binding of the viral glycoprotein to a cellular receptor induces endocytosis and entry into an early endosome. Acidification of the endosome causes Gn and Gc to dissociate and RNPs are released following Gc-mediated fusion. Viral tubes form in close association with the Golgi apparatus, rough endoplasmic reticulum, and mitochondria and serve as a hub for viral transcription and translation. Gn-Gc heterodimers accumulate in the Golgi, the site of virus assembly and budding. Infectious virions traffic in vesicles to the plasma membrane, where they fuse and are released.

TOXIN ENTRY OVERVIEW

The strategies used by bacterial toxins to enter mammalian cells share many common themes with the entry of viruses. Many bacterial species that cause human disease secrete toxins that modify host cellular function to benefit the pathogen, and most of these secreted toxins must access the cytosol to interact with their host target. Common cellular targets of secreted bacterial toxins include those that regulate host protein synthesis, immunomodulatory functions, and cell morphology.

Some bacterial species have evolved dedicated secretion systems to inject toxins through both the bacterial membranes as well as the host plasma membrane and deliver them directly to the cytosol, such as *Helicobacter pylori*, which uses its type IV secretion system (a large needle-like structure expressed by some Gram-negative bacteria) to inject effector proteins and DNA into the host cell (41). Intracellular bacteria that reside in phagosomes or other compartments are able to instead secrete toxins into the cytosol via translocation across that compartment's membrane. A number of species of extracellular bacteria secrete toxins that enter the surrounding host cells independent of such systems, however. Many of these secreted toxins (such as *C. difficile* TcdB, diphtheria toxin, Shiga toxin, pertussis toxin, and cholera toxin) belong to the general class termed AB toxins, so named for their two-component structure. The A subunit is the active component that interferes with host cell function (typically enzymatically) and the B subunit is the binding component responsible for attachment and entry into the host cell. Stoichiometry varies amongst the AB toxins: some (like TcdB and diphtheria toxin) are single-chain toxins, while others are organized into AB_5

structures (Shiga toxin and cholera toxin), binary structures (anthrax toxin), or tripartite structures (cytolethal distending toxin) [reviewed in (42)].

For these secreted toxins, just as with infectious virions, the first step of gaining access to the host cell is to bind to the cell surface via attachment factors and/or receptors, and to then either directly enter by forming a pore in the plasma membrane or hitch a ride on its receptor and be endocytosed. Like viral fusion proteins, toxins rely on a "trigger" from the host cell in order to effect a change in the toxin that enables it to gain access to the host cytosol. This is often accomplished in the acidifying endosomes via proteolytic cleavage of the toxin and/or pH-induced conformational changes, which can then form a pore through the endoplasmic membrane. The other main route of entry is to "reverse traffic" through the host secretory pathway to gain access to the ER, where the toxin can take advantage of pre-existing protein channels in order to bypass the need for pore-forming capabilities (42). Those toxins that utilize retrograde transport through the Golgi into the ER lumen often have evolved domains with sequences similar to ER retrieval (KDEL) sequences in order to hitchhike along the cell's existing pathway for ER retrieval. Once they've arrived at the ER lumen, there are multiple strategies for escape that have been described. One is the use of the Sec61 translocon, a channel in the ER membrane that can function bidirectionally. Another strategy (used by cholera toxin) is to "disguise oneself" as a misfolded protein and be transported out of the ER via the ER-associated degradation (ERAD) machinery (43).

The specific cellular entry route of toxins varies depending upon the identity of the receptor to which the toxin has initially bound at the cell surface. Therefore, these toxins can enter through clathrin-mediated endocytosis (as anthrax toxin does after binding to its receptors), through multiple pathways, including caveolae-dependent and -

independent, as well as clathrin-dependent and -independent endocytic routes (as cholera toxin does after binding to its receptor, the ganglioside GM1), or even utilize all of the cell's available endocytic pathways, as ricin toxin does by binding to terminal galactose residues on a variety of glycoproteins and glycolipids (44–50). For those toxins that gain access to the cytosol by forming pores in endosomes, the pH change as the endosome is acidified can trigger proteolytic cleavage of the toxin and/or conformation changes within toxin subunits. These pore-forming domains of the toxins then form channels within the vesicle membrane, allowing the catalytic domains to pass through and access the cytosol. The conformation of these catalytically active domains of the toxin may change (back) within the more neutral pH of the cytosol.

Once bacterial toxins, via any of these mechanisms discussed, have gained access to the host cell cytosol, they efficiently target a huge variety of host cellular processes with the overall goal of increasing pathogenesis and survival of the bacteria. For example, pertussis toxin has been shown to modulate the host immune and inflammatory responses via mechanisms such as inhibition of chemokine release [reviewed in (51)]. Because the activity of these secreted toxins are often so critical to the pathology of their bacteria of origin, understanding (and blocking) their route of entry is a major objective of medical research and is key to our ability to effectively treat many bacterial diseases.

CLOSTRIDIUM DIFFICILE TOXIN B

The anaerobic bacterium *Clostridium difficile* causes severe antibiotic- and hospital-associated diarrhea and pseudomembranous colitis. *C. difficile* infection can lead to septic shock, perforation of the intestine, and toxic megacolon, and is fatal for

about 9% of patients (52). Rates of *C. difficile* colitis have been increasing: from 2005 to 2010 there was a 47% increase compared to the five years prior (53) and the CDC estimated in 2011 that there were 500,000 cases of *C. difficile* infection in the United States (54). Spore formation by the bacterium and worsening resistance to treatment have hindered sterilization, prevention, and treatment efforts.

C. difficile secretes two main virulence factors that are responsible for its pathogenesis: TcdA and TcdB, both large toxins of the general AB class organization. TcdB has been shown to be approximately 100-1000 times more cytotoxic than TcdA, and is believed to be responsible for most of the severe disease symptoms associated with *C. difficile* infection (55–57). TcdB is 270 kDa and comprises four domains, as schematized in Figure 1-2A. These four domains are a glucosyltransferase domain (located at the N-terminus of the protein), a cysteine protease domain (responsible for auto-cleavage), a translocation domain (including a hydrophobic region), and a receptor binding domain containing a combined repeat oligopeptides (CROP) region, which is involved in binding and attachment at the cell surface.

The current model of TcdB entry into mammalian cells (see Figure 1-2B) involves initial attachment at the cell surface (mediated by the receptor binding domain) followed by endocytosis. Acidification of endosomes is thought to induce a conformational change in the toxin, leading to pore formation and translocation of the glucosyltransferase and cysteine protease domains into the cytosol (58). Autocatalytic cleavage of the glucosyltransferase domain by the protease domain (in response to host cell cofactors) then allows the toxic glucosyltransferase domain to interact with its target Rho GTPases in the cytosol (59). TcdB has been shown specifically to glucosylate RhoA (at Thr-37), Rac1 (at Thr-35), and Cdc42 (at Thr-35), although this varies by

bacterial strain (60). Glucosylation of these Rho GTPases disrupts the numerous cell processes that they control, including cell polarity, vesicle trafficking, and microtubule and actin cytoskeletal regulation. TcdB-treated cells rapidly lose their mophology (round up) and eventually undergo apoptosis.

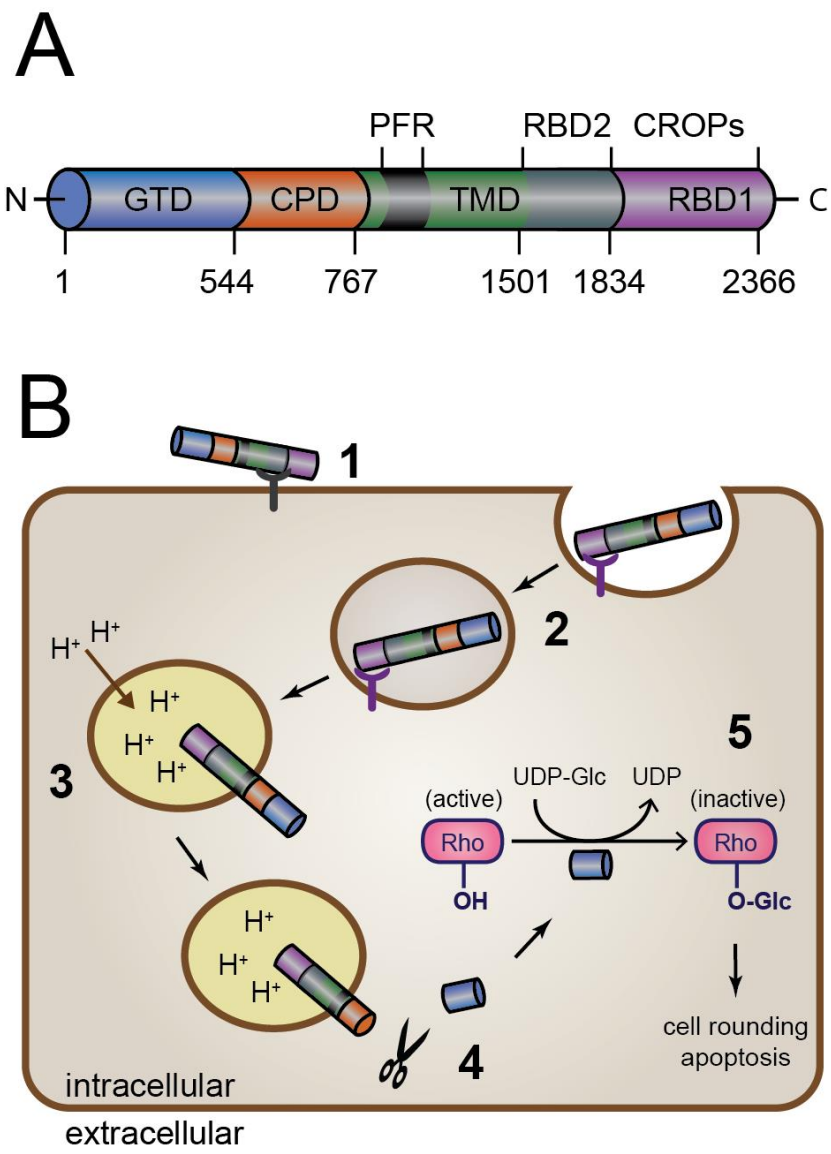

Figure 1-2. Structure and entry of TcdB toxin. (A) The structure of TcdB toxin. GTD: glucosyltransferase domain, CPD: cysteine protease domain, PFR: pore-forming region, TMD: translocation domain, RBD: receptor binding domain, CROPs: combined repetitive oligopeptides. (B) The intoxication process of TcdB: binding at the cell surface (1) is followed by receptor-mediated endocytosis (2). Acidification of the endosome induces a conformational change in the toxin that results in formation of a pore (3). Autocatalytic cleavage and release of the GTD (4) leads to glucosylation of Rho GTPases (5) which are thereby inactivated.

At the time of my TcdB screen, no receptors had been identified and the host factors involved in the early entry steps of the toxin were very poorly characterized. Toxin entry was shown to be blocked by pre-treatment with dynasore (a dynamin inhibitor) as well as expression of dominant-negative Eps15 (a component of clathrin-coated pits) and by pre-treatment with chlorpromazine (an inhibitor of clathrin-mediated endocytosis), but not by expression of plasmids encoding dominant-negative Caveolin-1, indicating that toxin entry is both dynamin- and clathrin-dependent (61). However, most published work looking at TcdB entry has focused on describing mutations or truncations of the toxin that block its activity. The readout for toxin entry / activity in most literature is a visual cell-rounding assay, or sometimes detection of glycosylation of Rac1 in cell lysates. A detailed understanding of the endocytic route of TcdB entry, its trafficking within the cell, its site of translocation, and the host factors upon which it relies during these processes is still badly needed.

SCREENING INTRODUCTION

High-throughput and unbiased screening techniques are a powerful tool for identifying host cellular factors that are required by viruses and toxins. In recent years, such screening techniques have been employed successfully to better characterize many different aspects of host-pathogen interactions. Here follows an overview of the primary screening strategies and examples of their use in identifying host factors impacting bunyavirus infection and toxin entry.

SMALL MOLECULE SCREENING

The lack of vaccines and therapeutics for many emerging viral and bacterial diseases has renewed interest in the screening of small molecule inhibitors, including the repurposing of clinically-approved pharmacologics. In 2016, Islam and colleagues used a high-throughput drug screen to identify compounds which potently inhibited RVFV infection, based upon a replication-competent recombinant virus lacking the NSs gene and bearing a fluorescent reporter (62). This study yielded six compounds (out of approximately 28,000 screened) that exhibited inhibitory activity at low concentrations with minimal cytotoxicity. Follow-up studies will be required to determine the mechanism of action of these compounds and their potential suitability as therapeutic agents against RVFV and perhaps other bunyaviruses. Bender *et al.* used the National Institutes of Health Clinical Collection as well as a library of non-FDA-approved bioactive compounds that are considered to be clinically safe in order to screen for an inhibitor of *C. difficile* TcdA and TcdB toxins (63). Their screen identified the compound ebselen (currently in clinical trials for unrelated conditions) as a potent inhibitor of the cysteine protease domain of both toxins, and verified in a mouse model that it decreases pathology of *C. difficile* infection.

Advances in inhibitor drug screening have also included methods to study the interactions between compounds that may be able to synergistically restrict viral infection. In 2012, Tan and colleagues described multiplex screening for interacting compounds (MuSIC), an analysis of all of the possible pairs of 1,000 commercially available compounds that were FDA-approved or clinically tested (64). The authors identify anti-inflammatory drugs as a group that synergistically enhanced anti-HIV activity

and informed drug-interaction network formation. Such screening methods may uncover previously uncharacterized therapeutic options within the pool of clinically-tested or -approved drugs.

BIOCHEMICAL APPROACHES

Valuable insight into the host-pathogen relationship can also be gleaned from interrogating physical interactions between pathogen and cellular proteins. The most widely-used applications for probing protein-protein interactions are yeast two-hybrid (Y2H) and affinity purification followed by mass spectrometry (AP/MS) techniques. Y2H screens utilize a reporter gene whose expression depends upon the activity of a transcription factor whose modular binding and activation domains have been fused, respectively, to bait and prey proteins. The protein of interest whose interacting partners are to be probed is the bait, and the prey proteins are typically libraries of proteins (or protein fragments) covering the genome of the organism of interest. These hybrid proteins are then introduced into cells, and if the bait and prey proteins interact, the binding and activation domains come into close enough proximity to reconstitute transcription factor activity and effect the expression of the reporter gene. During AP/MS, a bait protein of interest is pulled down via affinity for an antibody against either the protein itself or a tag to which it has been fused. One specialized type of such a tag is the two-part tag used in tandem affinity purification (TAP) techniques. The TAP tag comprises a Protein A tag and calmodulin binding peptide (CBP) tag separated by a recognition sequence that is specific to the Tobacco etch virus (TEV) protease. Protein complexes are purified by first capturing with the terminal Protein A tag, then using the TEV protease to cleave and release bound complexes and expose the CBP, followed by

a second affinity purification step of immobilization on calmodulin. This dual-affinity approach reduces the possibility of co-purification resulting from non-specific interactions.

Bunyavirus proteins have been used in protein-protein interaction screens to find interacting partners, particularly for the nonstructural protein NSs, which is known to be critical for viral defense against the host's type I interferon response. Leonard and colleagues performed yeast two-hybrid screening of a HeLa cDNA library using the BUNV NSs protein as bait (65). They identified MED8, a component of the Mediator complex, as a target of NSs during infection. Mediator is a key regulator of RNA polymerase II transcriptional activity, and the domain of NSs responsible for this MED8 interaction contains a motif that is highly conserved among orthobunyaviruses, suggesting that this interaction represents an important defense mechanism used by the virus to dismantle the host interferon response. In 2012, Rönnberg *et al.* used yeast two-hybrid screening with a mouse embryo cDNA library with the hantaviruses Puumala virus (PUUV) and Tula virus (TULV) NSs proteins as bait (66). From these two screens, 65 total host cellular proteins were identified as hantavirus interacting partners, with considerable overlap between the lists of partners for the two hantaviruses. This dataset provided insight into potential, previously-undescribed roles for NSs during infection, including regulation of apoptosis and interaction with proteins of the integrin complex.

An extensive survey of viral-host protein-protein interactions by Pichlmair and colleagues in 2012 used as bait a panel of 70 viral open reading frames (ORFs) selected for their roles in defending against the host innate immune response (67). The bunyavirus ORFs included in the panel were the NSs of RVFV, LACV, and Sandfly fever Sicilian virus (SFSV). These 70 viral ORFs were expressed within a HEK293 cell line

and then TAP followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify 579 interacting host proteins. Within these hits, there was an overrepresentation of proteins known to be involved in innate immunity, and specifically they noted an enrichment within the interacting partners of the negative-sense single-strand RNA for host proteins that may promote processing of viral RNA transcripts or prevent detection and degradation of these transcripts. In 2014, a follow-up study was published by Kainulainen *et al.* examining the interaction between RVFV NSs and the host F-box protein FBXO3 (68). FBXO3, which is a component of an E3 ubiquitin ligase, was shown to be recruited by NSs to effect the degradation of p62, a subunit of the general transcription factor TFIIH. Depletion of FBXO3 was unable to fully rescue interferon induction in RVFV-infected cells, did not affect the ability of NSs to degrade the interferon-induced antiviral effector dsRNA-dependent protein kinase R (PKR), and did not significantly impact viral replication. The authors therefore concluded that this FBXO3-mediated degradation of p62 is partially, though not completely, responsible for the ability of NSs to suppress the host interferon response. These findings highlight the capacity of protein-protein interaction studies for uncovering host factors that might not have been detected by gene-disruption or gene-depletion screening strategies, which usually depend upon robust viral replication or host cell survival phenotypes.

To my knowledge, no protein-protein interaction screens have been performed using any clostridial toxins. However, the *Helicobacter pylori* cytotoxin VacA was used as bait in a yeast two-hybrid screen to identify its interaction with the cellular protein receptor for activated C-kinase (RACK1), demonstrating the potential of such approaches to identify host protein interacting partners of bacterial toxins (69).

GENETIC APPROACHES

RNA interference (RNAi) technology was the first of a new generation of high-throughput screening approaches applied to the study of host-pathogen interactions. Examples of its use include the pioneering screens by Cherry, *et al.* to uncover a role for host organelle-reshaping and ribosomal proteins in Drosophila C virus replication (70, 71), a series of 2008 studies from multiple labs that identified many host factors necessary for human immunodeficiency virus (HIV)-1 replication (72–74), and the characterization in 2009 by Brass and colleagues of IFITM proteins as restriction factors for influenza, West Nile, and dengue viruses (75). For this screening technique, the incorporation of small interfering RNAs (siRNAs) into the RNA-induced silencing complex (RISC) effects the cleavage of target cellular mRNA and consequent knockdown of gene product expression. These siRNAs can be either directly introduced into the cell, or derived from supplied precursors: long double-stranded RNAs (dsRNAs) or short hairpin RNAs (shRNAs) that are then processed by cellular machinery. The availability of increasingly robust genome-wide libraries for RNAi screening has greatly increased its popularity as a high-throughput, unbiased screening platform.

Within the bunyavirus field, a 2013 RNAi screen by Hopkins *et al.* in Drosophila cells used dsRNAs targeting more than 13,000 genes, identifying 124 that restricted infection by the phlebovirus Rift Valley Fever virus (RVFV), with genes involved in DNA replication, the cell cycle, and mRNA metabolic processing being significantly enriched (76). Among these were the catalytic component of the mRNA decapping machinery (Dcp2) as well as two decapping activators, DDX6 and LSM7. Bunyaviruses “cap-snatch” by cleaving nucleotide sequences from the 5′ ends of host mRNAs in order to

prime the viral genome for transcription, and the authors showed that RVFV specifically cap-snatches the 5′ ends of Dcp2-targeted mRNAs, as did La Crosse virus (LACV), a member of the *Orthobunyavirus* genus. The year after, Meier and colleagues performed a screen using Uukuniemi virus (UUKV) in HeLa cells expressing the surface lectin CD209, which is an attachment factor for UUKV in dendritic cells (77). Two independent genome-wide siRNA libraries were used from two manufacturers: one library with four unpooled siRNAs per gene and one library with four unpooled siRNAs per gene. In both screens the v-SNARE VAMP3 was identified as a host factor required for the entry of UUKV. The importance of VAMP3 was also indicated by virtue of its being a target for the endogenous microRNA miR-142-3p, a microRNA identified as impacting infection after analysis of the seed sequences of the siRNAs used for screening. The authors examined incoming UUKV virions trafficking through the endocytic pathway and noted increasing colocalization of virions with VAMP3 as they moved within vesicles through the cytoplasm. At 20 min after internalization, maximum colocalization between UUKV virions and VAMP3 was observed within vesicles positive for lysosomal-associated membrane protein 1 (LAMP1), a marker for late endosomes and lysosomes. In VAMP3-depleted cells, incoming virions failed to reach these LAMP1-positive vesicles, indicating that their trafficking was arrested at an earlier endosomal compartment. These data informed our understanding of the host cellular machinery required for maturation of endosomal compartments and for the fusion of late-penetrating viruses within the acidic environment of late endosomes.

In 2015, Yuan and colleagues transfected HeLa cells with a microRNA-adapted shRNA (shRNAmir) library and challenged them with TcdB toxin to screen for clones that were resistant to toxin-induced cell rounding (78). Their screen identified chondroitin

sulfate proteoglycan 4 (CSPG4) as a cell surface receptor that was capable of mediating internalization of TcdB in HeLa cells. Further, the authors demonstrated a direct interaction between the N-terminus of CSPG4 and the C-terminus of TcdB, at a region of the toxin's receptor-binding domain immediately adjacent to the CROP domain. Two important pieces of evidence from this study supported the conclusion, however, that an additional cellular receptor for TcdB exists. First, the resistance of CSPG4-/- HeLa cells to cell rounding was only observed at low concentrations of the toxin, and even slightly increasing the concentration of TcdB caused a loss of phenotype. Second, treatment of CSPG4 knockout mice with TcdB showed a decrease in plasma IL-8 levels (relative to WT mice) but no effect on animal survival. The authors suggested that the CROP domain was likely responsible for binding of another receptor.

The arrival of haploid screening in human cells, first described by Carette and colleagues in 2009, offered a loss-of-function forward genetic approach as a powerful alternative to traditional siRNA-based depletion screens (79, 80). In these screens, null alleles are generated in mammalian haploid cells using insertional mutagenesis, and the resulting cellular library is challenged by a selective agent such as a virus or toxin. Surviving cells, which presumably lack a gene required by the selective agent as a consequence of retroviral insertion, are pooled and deep sequencing is used to map the insertion sites of the mutagenizing lentivirus. Statistical analysis identifies the enrichment of insertion sites within the surviving (selected) population compared to the original mutant library, yielding a list of genes whose disruption confers a resistance phenotype. This approach identified the homotypic fusion and vacuole protein sorting (HOPS) tethering complex and the endo/lysosomal cholesterol transporter protein Nieman-Pick 1 (NPC1) as essential host factors for Ebola virus (EBOV) entry, and

uncovered the receptor-switching process of Lassa virus (LASV) as it engages first its α-dystroglycan receptor at the cell surface and then later its intracellular receptor, the lysosomal transmembrane protein LAMP1 (81–83). These studies have provided potential antiviral targets, as well as insight into the molecular determinants of host tropism, for these important human pathogens.

In 2014, our lab – in collaboration with the lab of Paul Bates – used a recombinant vesicular stomatitis virus (VSV), in which the Andes virus (ANDV) glycoproteins are expressed on the VSV core, to identify cellular host factors required for ANDV entry (84). This rVSV-ANDV was used to challenge a human haploid mutant library and multiple members of the sterol regulatory pathway were identified as impacting ANDV entry. This dependence upon cholesterol was validated using live wild-type ANDV, a member of the New World hantaviruses that are causative agents of hantavirus pulmonary syndrome (HPS). Cholesterol requirement during viral entry was verified through the use of Chinese hamster ovary (CHO) knockout cell lines, pharmacological inhibitors, siRNA depletion, and transcription activator-like effector nuclease (TALEN) disruption of members of the sterol regulatory pathway, as well as by direct depletion of cholesterol in the cellular membranes. Virus binding at the cell surface was unaffected, but an internalization defect was observed within cells that lack a functional sterol regulatory pathway. Interestingly, this exquisite dependence upon cholesterol is not shared by all members of the *Buyaviridae* family (unpublished data). The following year, Kleinfelter and colleagues independently confirmed these findings and extended the cholesterol-dependence phenotype to members of both the Old World and New World hantavirus clades (85). Cholesterol depletion was shown to significantly delay virus internalization, and to inhibit the ability of virions to fuse with cellular

membranes. This finding is intriguing, as the pH requirement for ANDV implicates it as a late-penetrating virus, but the liposome fusion results from Kleinfelter *et al.* suggest that ANDV may require a greater cholesterol concentration than what is present in the membranes of late endosomes. Detailed mechanistic studies will be needed to reconcile this, and to determine whether hantaviruses somehow modulate endosomal cholesterol composition, fuse specifically at cholesterol-rich microdomains, or whether cholesterol plays some other role during virus-membrane fusion.

Human haploid genetic screening was used in 2014 by Schorch and colleagues to examine the entry of *Clostridium perfringens* toxin TpeL, a recently-described glycosylating toxin that is structurally similar to TcdB but lacks the CROP domain (86). This screen identified low-density lipoprotein receptor-related protein 1 (LRP1) as a binding partner and endocytosis-mediating receptor for TpeL toxin. However, TcdB toxin lacking the CROP domain was capable of entering WT MEF cells and MEFs lacking LRP1 with equal efficacy. This indicate that the non-CROP region in the C-terminus receptor-binding domain of TcdB (which is approximately 50% similar to the corresponding region of TpeL) does not utilize LRP1 as a receptor for TcdB in this cell type.

The following year, LaFrance *et al.* generated a mutagenized library of Caco-2 cells using a retroviral gene-trap vector that confers resistance to neomycin (87). They challenged this library with TcdB and identified two clones with mutations in the polio virus receptor-like 3 (PVRL3) gene, a cell surface protein and member of the nectin family of adhesion molecules. Knockdown of PVRL3 in Caco-2 cells (by shRNA) conferred partial resistance to TcdB, as assayed by quantification of ATP to indicate cell viability. In HeLa cells, shRNA knockdown and CRISPR-mediated disruption of PVRL3

also increased cell viability after TcdB challenge, but it did not protect cells from the rounding activity of the toxin. Perplexingly, the authors did not show cell-rounding results in Caco-2 cells. They did look at protein expression levels (by Western blot) of both CSPG4 and PVRL3 in Caco-2 cells and HeLa cells, and found that CSPG4 is only expressed in HeLa cells, whereas PVRL3 is expressed in both cell types, though at a higher level in Caco-2 cells. The role of PVRL3 in TcdB entry of Caco-2 cells was further characterized by demonstration of direct binding of purified PVRL3 and TcdB proteins, as well as by increased cell ATP levels in the presence of TcdB treatment when the cells were pre-treated with anti-PVRL3 antibodies. PVRL3 and TcdB were also shown to colocalize in human colon explant tissue. These data indicate a role for PVRL3 in TcdB entry of colonic epithelial cells.

Just recently, a TcdB screen was published using a CRISPR sgRNA library introduced into HeLa cells that stably express the Cas9 endonuclease (88). The three top hits from this screen were UDP-glucose pyrophosphorylase (UGP2), an ezyme that produces the glucose required by TcdB for its glucosylation of target GTPases; CSPG4; and the protein frizzled class receptor 2 (FZD2), which is a member of the Frizzled family of Wnt signalling receptors. HeLa cell lines lacking FZD2 (via CRISPR-mediated disruption) were approximately 15-fold more resistant to TcdB lacking the CROP domain, but were not more resistant to full-length TcdB. However, when these cells were also disrupted at the loci encoding FZD1 and FZD7 proteins, the combined FZD1/2/7$^{-/-}$ HeLa cells were 10-fold more resistant to full-length TcdB than WT cells. The authors used truncation mutants of TcdB to show that CSPG4 functions as a CROP-dependent receptor, whereas Frizzled proteins act as CROP-independent receptors. Rescue experiments using introduction of CSPG4 and FZD2 as well as

competition experiments using pre-treatment of cells with the binding domains of these two proteins demonstrated that CSPG4 and Frizzled proteins act as non-competetive receptors for TcdB and that their relative contribution to toxin entry depends upon their cell-type-specific expression levels.

CONCLUSION

In December of 2015, the World Health Organization published its Workshop on Prioritization of Pathogens executive summary, which listed the emerging diseases most likely to cause severe outbreaks in the near future (89). Of the ten diseases named, three are caused by bunyaviruses: Rift Valley fever, Crimean-Congo hemorrhagic fever, and severe fever with thrombocytopenia syndrome. Concern is likewise mounting about the increasing incidence, virulence, and antibiotic-resistance of *C. difficile* infection worldwide (90). Within the United States, *C. difficile* infects half a million people annually, killing 29,000 each year, is the most common cause of nosocomial infections, and costs $4.8 billion dollars per year in healthcare expenses for acute care facilities, according to the CDC. A better understanding of the molecular details of the pathogenesis of these diseases is urgently needed in order to inform the development and application of therapeutic interventions. Interesting questions also remain to be answered about many of the fundamental cell biological processes involved in the entry of bunyaviruses and *C. difficile* Toxin B. For these reasons, my dissertation research applied high-throughput screening technology to interrogate the interactions of these fascinating and important pathogens with their mammalian host cells.

The data that follow in Chapters 2 and 3 summarize the results of two independent screening projects, each utilizing a strategy of forward genetic screening in

a mutagenized human haploid cell library. The first was performed with the vaccine strain of RVFV, and identifies glycosaminoglycans as a bunyavirus attachment factor on some cell types. The second screen was done by sequential challenge of the mutant library with TcdB, and indicates a role for an actin polymerization-regulating complex in the entry of this toxin.

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CHAPTER 2: A HAPLOID GENETIC SCREEN IDENTIFIES HEPARAN SULFATE PROTEOGLYCANS SUPPORTING RIFT VALLEY FEVER VIRUS INFECTION

ABSTRACT

Rift Valley fever virus (RVFV) causes recurrent insect-borne epizootics throughout the African continent, and infection of humans can lead to a lethal hemorrhagic fever syndrome. Deep mutagenesis of haploid human cells was used to identify host factors required for RVFV infection. This screen identified a suite of enzymes involved in glycosaminoglycan (GAG) biogenesis and transport, including several components of the cis-oligomeric Golgi (COG) complex, one of the central components of Golgi complex trafficking. In addition, disruption of the previously-uncharacterized gene *PTAR1* led to RVFV resistance as well as reduced heparan sulfate surface levels, consistent with recent observations that PTAR1-deficient cells exhibit altered Golgi complex morphology and glycosylation defects. A variety of biochemical and genetic approaches were utilized to show that both pathogenic and attenuated RVFV strains require GAGs for efficient infection on some, but not all, cell types, with the block to infection being at the level of virion attachment. Examination of other members of the Bunyaviridae family for GAG-dependent infection suggested that the interaction with GAGs is not universal among bunyaviruses, indicating that these viruses, as well as RVFV on certain cell types, employ additional unidentified virion attachment factors and/or receptors.

INTRODUCTION

Rift Valley fever virus (RVFV) is a member of the *Bunyaviridae* family of viruses that cause emerging infections that threaten both human and livestock populations on several continents (1). Bunyaviruses have a tripartite, negative-sense RNA genome and are frequently transmitted by insects (1). RVFV can be transmitted by mosquitoes or by exposure to infected tissues and body fluids and is considered endemic in much of Africa (2). In humans, RVFV can cause an acute fever leading to complications such as kidney failure and, in about 1% of cases, a lethal hemorrhagic fever (3, 4). In addition, RVFV spreads rapidly across infected herds of livestock and can cause significant mortality in infected animals (5, 6).

We took a genetic approach to identify host factors that are required for RVFV infection *in vitro* by employing an insertional mutagenesis screen using HapI cells, a human haploid cell line. By utilizing a retroviral gene trap, gene-inactivating insertion sites can be efficiently mapped with deep sequencing technology (7). This approach has successfully uncovered host factors required by a variety of pathogens, including viruses, bacteria, and bacterial toxins (8–12). When gene trap-mutagenized HapI cells were challenged with RVFV and the surviving cells were analyzed, there was an enrichment of sites of insertion into multiple genes involved in glycosaminoglycan (GAG) biosynthesis as well as genes for subunits of the cis-oligomeric Golgi (COG) complex and *PTAR1*. We confirmed the requirement for heparan sulfate during infection with RVFV isolates with a variety of genetic and biochemical perturbations, consistent with the findings from de Boer *et al.* (13). We now show that the dependency on heparan sulfate during RVFV infection is consistent across a representative panel of primary

RVFV isolates and, by employing vesicular stomatitis virus (VSV)-based pseudovirions, that utilization of GAGs by RVFV during infection occurs at the step of entry. We were able to identify, using a quantitative binding assay, virus attachment to be the specific entry step affected. However, the dependence of RVFV on GAGs for efficient infection was cell type dependent. Surfen (a small-molecule antagonist that binds to heparan sulfate) inhibited infection of HapI and SNB-19 cells by replication-competent RVFV, yet surfen did not impact infection of several other cell lines by RVFV, even though it efficiently blocked infection by herpes simplex virus 1 (HSV-1), a virus that depends upon heparan sulfate for efficient infection *in vitro*. Thus, while GAG interactions do significantly enhance RVFV infection in some contexts, other virus attachment factors must also exist and/or RVFV utilizes GAG structures that do not efficiently interact with surfen.

MATERIALS AND METHODS

Cells and viruses. HapI cells (7) and the derived mutant cell lines were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 10 units/ml penicillin, and 100 μg/ml streptomycin. HEK 293T, Vero E6, C6/36, L, and sog9 cells (a generous gift from Frank Tufaro) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 2 mM l-glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin. The following strains of RVFV were used in this study: MP-12, ZH-501, Kenya 9800523, and Kenya 2007002444. MP-12 was propagated in MRC-5 cells (at the University of Pennsylvania) or Vero E6 cells (at USAMRIID), while the ZH-501 and the Kenyan strains were propagated in Vero E6 cells. Viral titers on Vero E6 cells

were determined by plaque assay. Crimean-Congo hemorrhagic fever virus (CCHFV) strain IbAr10200 was propagated in CER cells, and viral titers on CER cells were determined. HSV-1 strain k-GFP (a generous gift from Nigel Fraser, University of Pennsylvania) was propagated in Vero E6 cells. Studies using RVFV ZH-501 were conducted in a biosafety level 3 laboratory at USAMRIID, whereas infections using the Kenyan RVFV strains and CCHFV were performed in a biosafety level 4 laboratory at USAMRIID. Appropriate safety protocols were followed, and personal protective equipment was worn while conducting experiments in the high-containment laboratories. The generation of *PTAR1*-deficient HapI cells was described before (14).

Insertional mutagenesis. HapI cells were mutagenized with a retroviral gene trap as described in reference 11 and exposed to strain MP-12. Surviving clones were expanded for genomic DNA isolation. Subsequently, gene trap insertion sites were amplified using an inverse PCR, submitted for parallel sequencing (Illumina HiSeq 2000), and aligned to the human genome (hg18) (10). Genes significantly enriched for gene-trap insertions compared to the sequences of an unselected control cell population were identified using a one-sided Fisher's exact test as described in reference 11.

RVFV pseudovirion production. To assess the specific role of GAGs in RVFV attachment and entry, as opposed to downstream replication events, we used a VSV pseudovirion system (15, 16) in which the VSV glycoprotein gene G was deleted from the viral genome (VSVΔG) and replaced with a reporter gene, either Renilla luciferase (VSVΔG-rLuc) or red fluorescent protein (VSVΔG-RFP). To generate VSVΔG pseudovirions possessing RVFV glycoproteins (or those of other viruses), the glycoproteins were provided in trans via an expression vector to cells transduced with the VSVΔG core. HEK 293T cells seeded in 10-cm2 plates were transfected with

pCAGGS RVFV ZH-548 M using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. This construct is codon optimized for expression in human cells and contains only the coding region of the M segment starting at the fourth ATG start codon, which omits the NSM coding region. At between 16 and 20 h after transfection, cells were transduced with VSVΔG pseudovirions bearing VSV G. After adsorption of pseudovirions for 1 h, cells were carefully rinsed four times with warm phosphate-buffered saline (PBS) containing calcium and magnesium, and then the medium was replaced with complete DMEM supplemented with 25 mM HEPES. Cell culture supernatants were collected 24 h later, clarified by low-speed centrifugation for 30 min at 4°C, filtered (pore size, 0.45 μm), and then aliquoted for storage at −80°C. Andes virus (ANDV) and Hantaan virus (HTNV) pseudovirions were generated in the same fashion.

Virus infections. To compare the ability of diverse RVFV strains or CCHFV to infect HapI cells and the derived mutant cell lines, we utilized a high-content imaging-based infection assay. Each cell line was seeded at a density of 1×10^4 cells per well in Greiner black well, clear-bottom 96-well plates. At 24 h after seeding of the cells, the culture medium was removed and the cells were infected with viruses diluted in complete IMDM. The virus inocula were not washed off and the plates were incubated at 37°C until approximately 18 to 20 h postinfection. At this point, the cell culture medium was removed from the cells and the plates were immersed in 10% neutral buffered formalin for 24 h to fix the cells and render virus noninfectious prior to removal from the high-containment laboratories. Prior to immunostaining for viral antigens, residual formalin was removed from the plates, and they were then rinsed extensively with phosphate-buffered saline (pH 7.4). The cells were permeabilized for 15 min with a

solution of 0.1% (vol/vol) Triton X-100 in PBS, and then the permeabilization buffer was rinsed away by additional PBS washes. The cells were blocked for at least 1 h using a 3% (wt/vol) solution of bovine serum albumin in PBS. Purified monoclonal antibodies specific for RVFV N (R3-1D8) or CCHFV N (9D5-1-1A) were diluted 1:1,000 in blocking buffer and then added to the cells for 1 h, followed by extensive washing with PBS. Anti-mouse immunoglobulin Alexa Fluor 568-labeled secondary antibody was diluted 1:2,000 in blocking buffer and then added to the cells for 1 h, followed by extensive washing in PBS. The cells were then counterstained with a solution of Hoechst 33342 (nuclei) and HCS CellMask deep red stain (total cell), each of which was diluted 1:10,000 in PBS. This counterstain solution was maintained on the plates during high-content imaging. Automated image acquisition was performed using an Operetta high-content imaging system. Three exposures (one for each of the fluorophores) in five separate fields were acquired in each well using a 20× air objective and a Peltier cooled 1.3-megapixel charge-coupled-device camera. The fluorophores were illuminated using a 300-W xenon arc light source and excitation (EX) and emission (EM) filters for the following: Alexa Fluor 568 (EX/EM), Hoechst 33342 (EX/EM), and HCS CellMask deep red (EX/EM). Image segmentation and analysis were performed using Harmony (version 3.0) software and standard scripts. These algorithms were used to first delineate nuclear and cell boundaries and then identify viral antigens by Alexa Fluor 568 staining. To calculate percent infection per image field, the number of cells exhibiting an Alexa Fluor 568 mean fluorescence intensity greater than the mean intensity for uninfected control wells was divided by the total cell number defined by Hoechst 33342 nuclear staining. For each well, the Harmony software reported the mean percent infection of the five fields. On average, 1,500 to 5,000 cells were analyzed per well. In each independent experiment,

at least 4 individual wells were analyzed for each cell line. Infections with VSVΔG-RFP pseudovirions or HSV-1 were carried out at a low volume for 1 h at 37°C, after which complete DMEM or IMDM was added to the wells. Twenty-four hours later, the cells were trypsinized, fixed in 2% paraformaldehyde and then analyzed for RFP (for VSVΔG pseudovirions) or green fluorescent protein (GFP) (for HSV-1) expression by fluorescence-activated cell sorting (FACSCalibur flow cytometer; BD Biosciences). For infections in the presence of surfen (5 μM; Sigma), dextran sulfate (5 μg/ml, 5 kDa; Sigma), or heparinase I (3 U/ml; Sigma), cells were pretreated for 1 h and, in the case of surfen and dextran sulfate, kept in the presence of drug for the duration of the infection. For soluble GAG competition experiments, heparin (10 and 100 μg/ml; Fisher BioReagents) and heparan sulfate (10 and 100 μg/ml; Iduron) were preincubated with HSV-1 or MP-12 at 25°C for 1 h. The virus and GAG solution was then allowed to adsorb onto cells for 1 h at 37°C, after which it was rinsed 3 times with PBS containing calcium and magnesium and cells were refed with fresh medium that did not contain either virus or GAGs. Infections were then harvested at 8 to 10 h postinfection (hpi), and percent infection was scored by flow cytometry, looking for either intracellular staining of the N protein (for MP-12) or expression of the GFP reporter protein (for HSV-1). For pseudovirion neutralization studies, RVFV and severe acute respiratory syndrome (SARS) coronavirus antisera (a generous gift from Stuart Nichol, Centers for Disease Control and Prevention) were preincubated with pseudovirions at the indicated dilutions for 30 min at 37°C. The linear range of the assay was determined by performing serial 10-fold dilutions of each virus stock on each target cell type and for each detection method used. Infection assays were typically linear over at least a 2-log-unit range of

virus dilutions, with the virus inoculum being adjusted to achieve infection levels of between 1 and 30%.

RVFV binding assay. Virus was diluted in DMEM (Gibco) and added to HapI cells and the derived mutant cell lines for 1 h at 37°C. The cells were then washed four times with PBS, and total RNA was isolated from the cells using a Qiagen RNeasy minikit. RNA was quantified by measuring the absorbance at 260 nm, and first-strand cDNA was generated from 1.5 μg of total RNA using a SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Primers specific to the MP-12 L segment (forward L segment primer 5′-TGAGAATTCCTGAGACACATGG-3′; reverse L segment primer 5′-ACTTCCTTGCATCATCTGATG-3′) were purchased from Invitrogen, and a 6-carboxyfluorescein/MGB probe specific to the MP-12 L segment with the sequence 5′-CAATGTAAGGGGCCTGTGTGGACTTGTG-3′ was purchased from Applied Biosystems. Reverse transcription-PCR (RT-PCR) was then performed using an ABI 7500 real-time PCR system (Applied Biosystems) with the following conditions: (i) denaturation at 95°C for 20 s and (ii) 40 cycles of PCR amplification with denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s. Data were analyzed using the ΔΔCT threshold cycle (CT) method by calculating the change in gene expression normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping gene (17).

Statistical analysis. Statistical significance was calculated using a two-tailed, one-sample t test by comparing the fold changes to the hypothetical value of 1 in Prism software (version 5.0a; GraphPad Software). P values were not reported for conditions where only two biological replicates were performed.

RESULTS

An insertional mutagenesis screen for RVFV host factors in a human haploid cell line. To identify the host factors needed for RVFV infection, 1×10^8 HapI cells were mutagenized using a retroviral gene trap vector (11). Subsequently, mutagenized cells were infected with the cytotoxic RVFV MP-12 strain and the surviving cells were expanded as a polyclonal cell population. Following isolation of genomic DNA, gene trap insertion sites were sequenced and aligned to the human genome. Subsequently, the retroviral insertions within genes in the virus-resistant population were counted and compared to the number of insertions within the same gene in an unselected cell population (11). Genes significantly enriched ($P < 0.001$) for insertions in the virus-selected cell population were identified (Fig. 2-1A). These contain multiple genes encoding enzymes required for synthesis of glycosaminoglycans (refer to Fig. 2-1B), including the four enzymes needed for the tetrasaccharide linkage region (XYLT2, B4GALT7, B3GAT3, and B3GALT6) (18–23), two enzymes involved in proteoglycan chain elongation (EXT1 and EXT2) (24), and the enzyme that catalyzes both N-deacetylation and N-sulfation during the biosynthesis of heparan sulfate (NDST1) (25). Genes required for the synthesis (*UXS1*, *UGDH*) or transport (*SLC35B2*) of critical moieties for heparan sulfate chain formation (26–28) were also enriched in cells resistant to RVFV infection (Fig. 2-1A and B). In addition to genes directly involved in heparan sulfate biosynthesis, several subunits of the conserved oligomeric Golgi (COG) complex (*COG1*, *COG2*, *COG3*, *COG4*, *COG5*, *COG7*, *COG8*) (29) were identified from the screen. It is known that perturbation of the COG complex attenuates O-linked glycosylation by impairing Golgi complex function (29, 30). Another hit in this screen

encoded UNC50, a Golgi complex-resident transmembrane protein that plays a role in nicotinic acetylcholine receptor trafficking in Caenorhabditis elegans (31). Finally, this screen identified the gene for prenyltransferase alpha subunit repeat containing 1 (*PTAR1*) to be important for RVFV infection. PTAR1 was previously shown to affect glycosylation (11), possibly by influencing vesicular trafficking through prenylation of Rab GTPases (14, 32). Although genes involved in vesicular trafficking could represent more direct interactions with RVFV, the overlap of these results with those from a screen performed for cell surface GAG expression (11) suggests a function for these genes in the presentation of glycans at the cell surface.

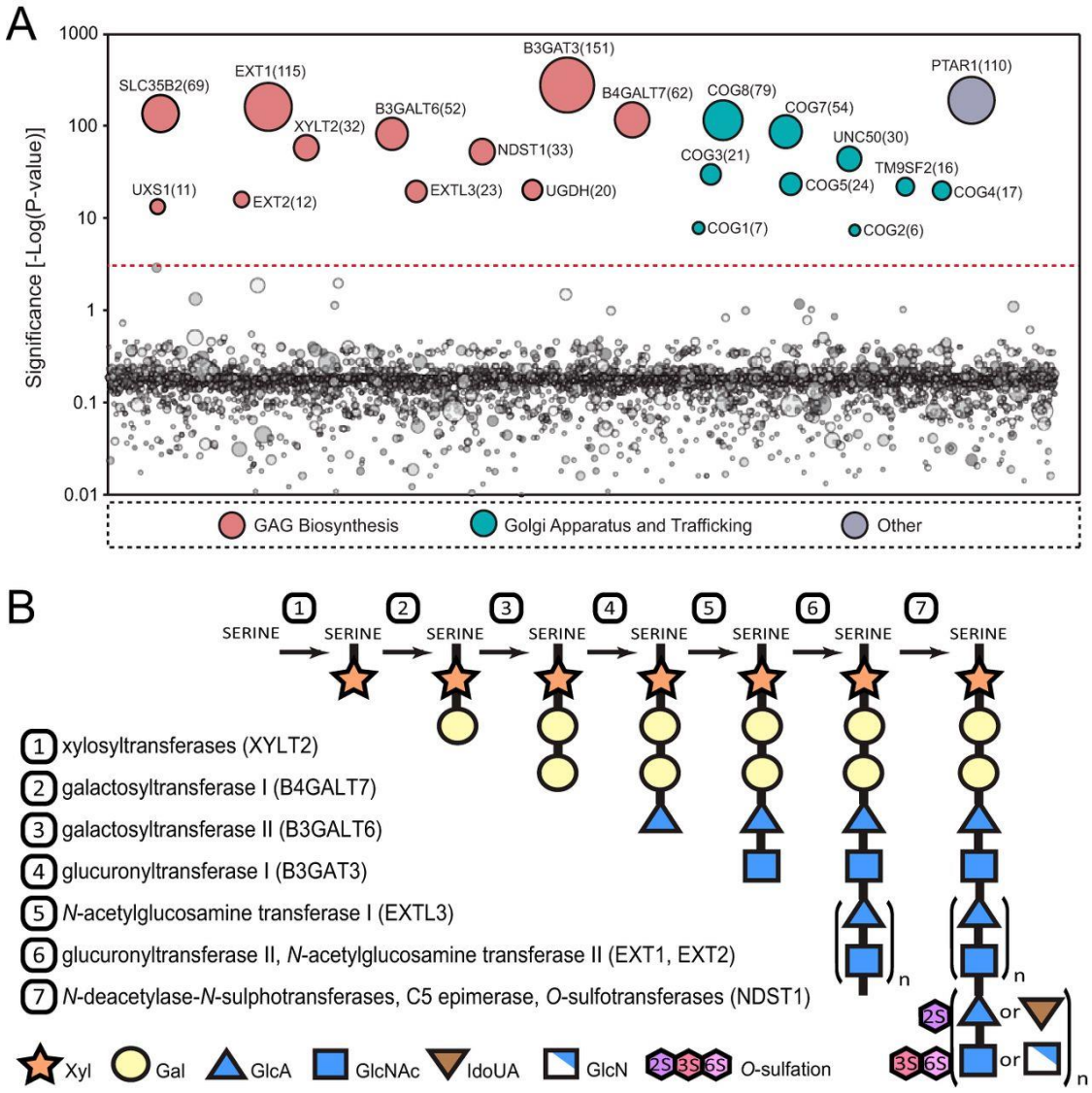

Figure 2-1. Human haploid mutagenesis screen for RVFV host factors. (A) Plot showing genes enriched in the virus-selected population compared with their levels in an unselected population. Each circle demarks a gene, with its y-axis coordinate representing the false discovery rate-corrected P value and its area reflecting the number of identified unique gene trap integrations. Genes that are significantly enriched in the virus-selected population ($P < 0.001$) are colored and horizontally grouped on the basis of their function. (B) Overview of heparan sulfate synthesis. Xyl: xylose, Gal: galactose, GlcA: glucuronic acid, IdoUA: iduronic acid, GlcN: *N*-acetylglucosamine. Genes involved in heparan sulfate synthesis that were significantly enriched in our RVFV screen are shown in parentheses.

GAGs are important for RVFV infection. Because the majority of genes identified in our screen pertained to GAG synthesis, we first focused on elucidating the role of GAGs during RVFV infection. We were able to obtain single-cell clones of gene-trapped *B3GAT3* (B3GAT3GT) and *B4GALT7* (B4GALT7GT) and exposed these cells to the MP-12 strain of RVFV. As shown in Fig. 2-2A, these cells were markedly resistant to MP-12 infection. Importantly, reintroduction of the respective cDNAs completely restored sensitivity to virus infection, indicating that the observed resistance phenotype can be solely attributed to the gene-trapped loci (Fig. 2-2A). To determine whether the synthesis of the O-linked tetrasaccharide linker was required for RVFV infection, we produced a B3GAT3GT cell line stably expressing an enzymatically inactive point mutant of GlcAT-I (D194A/D195A) (33). As with the B3GAT3GT cells stably expressing an empty vector construct, introduction of this enzymatically inactive form of GlcAT-I into B3GAT3GT cells did not rescue MP-12 infection (Fig. 2-2B). B4GALT7 encodes the β-1,4-galactosyltransferase GalT-I, which catalyzes the enzymatic step immediately upstream of the β-1,3-galactosyltransferase reaction in the synthesis of the GAG linker (Fig. 2-1B). As with the B3GAT3GT cell panel, MP-12 infection also required a catalytically active form of GalT-I (Fig. 2-2B), further suggesting that RVFV is dependent upon GAGs for efficient infection.

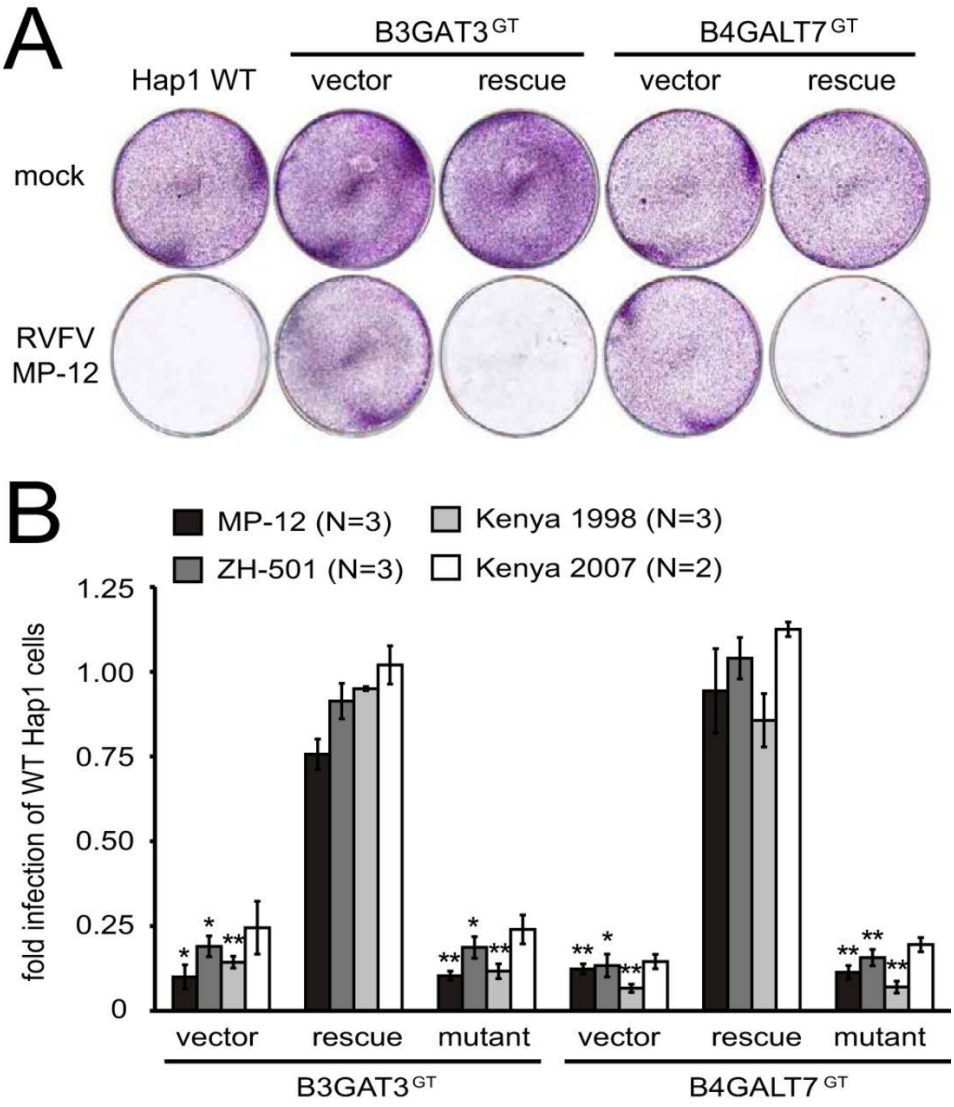

Figure 2-2. GAGs are important for RVFV infection. (A) Expression of the B3GAT3 and B4GALT7 gene products is required for strain MP-12 infection. B3GAT3 and B4GALT7 gene-trapped (GT) HapI cells transfected and selected to stably express empty vector (vector) or wild-type (WT) protein (rescue) were infected with MP-12, and surviving cells were stained with crystal violet. (B) GAGs are important for diverse strains of RVFV. B3GAT3GT and B4GALT7GT HapI cells that stably express the empty vector (vector), the wild-type protein (rescue), or a catalytically inactive point mutant (mutant) were infected with the MP-12, ZH-501, Kenya 980052 (Kenya 1998), and Kenya 2007002444 (Kenya 2007) strains of RVFV, and the percentage of infected cells was normalized to the percentage of infected parental HapI cells. Bars indicate SEMs (n = 3 for MP-12, ZH-501, and Kenya 980052; n = 2 for Kenya 2007002444). *, $P < 0.005$; **, $P < 0.001$.

For some viruses, the requirement for GAGs for infection of cells in culture is a trait acquired during *in vitro* passaging, often leading to attenuation (34–36). To determine whether this was the case with RVFV, we infected the B3GAT3GT and B4GALT7GT cell panels with three pathogenic strains of RVFV: ZH-501, Kenya 9800523 (1998), and Kenya 2007002444 (2007). We found that infection by these primary RVFV strains was also strongly inhibited in cells lacking functional GlcAT-I and GalT-I. Infection was rescued by expression of the wild-type construct but not the enzymatically inactive constructs (Fig. 2-2B). The dependence of primary RVFV strains upon these enzymes indicates that the requirement of GAGs for viral infection is not due to cell culture adaption or attenuation.

To further test the hypothesis that RVFV infection requires GAGs, we used various GAG perturbants. The small molecule surfen binds to negatively charged GAG species on the cell surface (37). Infection of HapI cells in the presence of surfen led to a 10-fold reduction of MP-12 infection but not vesicular stomatitis virus (VSV) infection (Fig. 2-3A). Infection of the HapI cells by herpes simplex virus 1 (HSV-1), which is known to utilize heparan sulfate for attachment, was decreased to levels close to background levels by the addition of surfen. Enzymatic removal of cellular heparan sulfate with heparinase also greatly attenuated MP-12 infection (Fig. 2-3A). Since GAGs are highly negatively charged, nonspecific electrostatic effects could facilitate the interaction between RVFV surface glycoproteins and cellular GAGs. To address this issue, we infected HapI cells in the presence of dextran sulfate, a biologically inert, negatively charged carbohydrate polymer. In contrast to HSV-1, the presence of dextran sulfate had little impact on MP-12 infection (Fig. 2-3A), suggesting that the interaction with cellular GAGs has some degree of specificity.

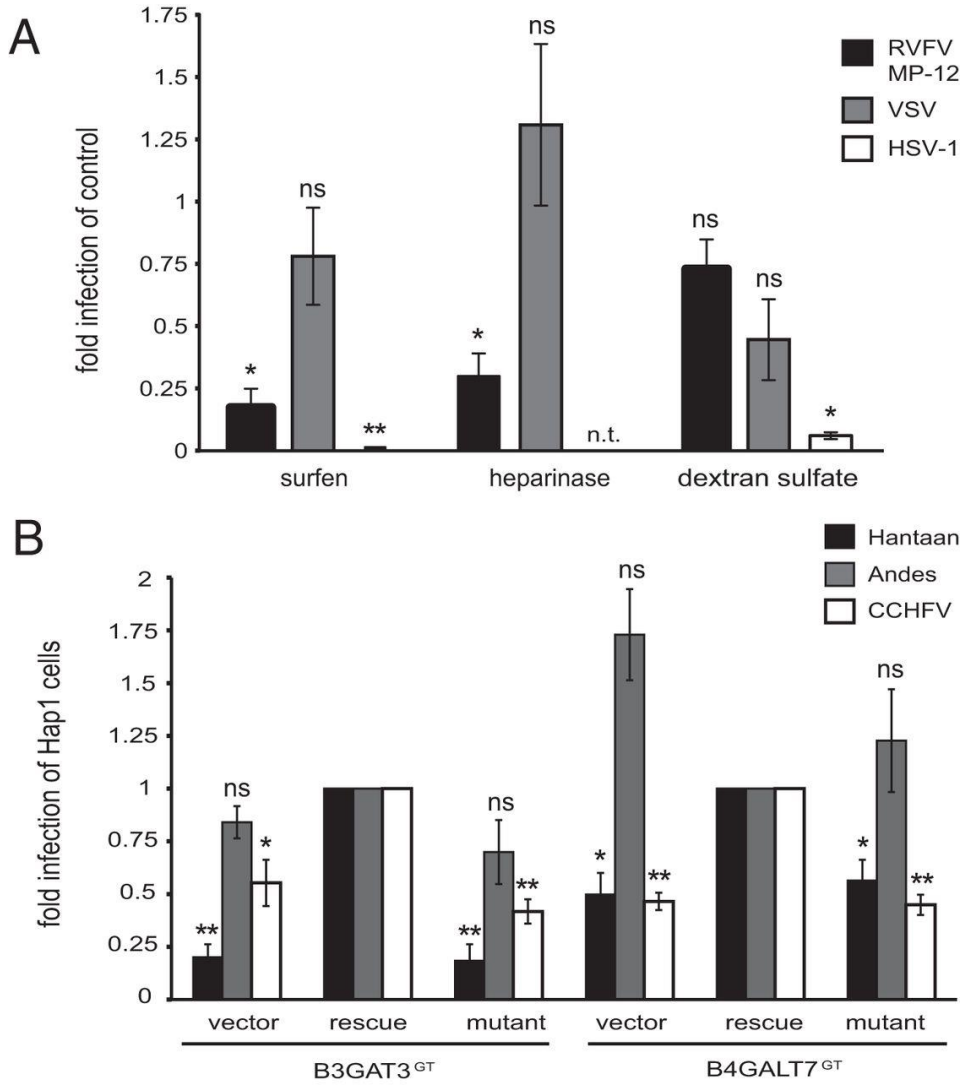

Figure 2-3. Perturbants of GAGs and the requirement of GAGs among bunyaviruses. (A) GAG perturbants reduce the MP-12 infectivity of HapI cells. HapI cells were pretreated with the small molecule surfen (5 μM), heparinase I (3 U/ml), or dextran sulfate (50 μg/ml) and infected with MP-12, VSV, or HSV-1. The percentage of infected cells was normalized to the percentage of infected cells treated with the vehicle control (dimethyl sulfoxide for surfen, PBS for heparinase I, and water for dextran sulfate). Bars indicate SEMs (n = 3). *, $P < 0.005$; **, $P < 0.0001$; ns, no significant difference; n.t., not tested. (B) Role of GAGs for various members of the Bunyaviridae family. B3GAT3GT and B4GALT7GT cells (see Fig. 2 legend) were infected with either Hantaan or Andes virus pseudovirions or replication-competent CCHFV. The percentage of infected cells was normalized to the percentage of infected wild-type cells rescued with each protein. Bars indicate SEMs (n = 3). *, $P < 0.05$; **, $P < 0.005$; ns, no significant difference.

Differential requirement for GAGs among Bunyaviridae family members. To examine whether the interaction of RVFV with GAGs was unique among bunyaviruses, we infected the B3GAT3GT and B4GALT7GT cell panels with pathogenic, replication-competent Crimean-Congo hemorrhagic fever virus (CCHFV) and VSV pseudovirions bearing the Andes or Hantaan virus glycoproteins. CCHFV is a member of the Nairovirus genus, and both Andes and Hantaan viruses are members of the Hantavirus genus, which are further subdivided into New World (Andes virus) and Old World (Hantaan virus) hantaviruses (38, 39). Interestingly, Hantaan virus pseudovirions required catalytically active GlcAT-I and GalT-I for efficient infection of HapI cells, while Andes virus pseudovirions did not (Fig. 2-3B). Infection with CCHFV was reduced 2-fold when B3GAT3 or B4GALT7 were absent (Fig. 2-3B). Thus, the role of GAGs during infection by other members of the *Bunyaviridae* family varies.

RVFV utilizes at least one surfen-resistant cellular factor *in vitro*. We next sought to characterize the role of GAGs during MP-12 infection of different cell lines using surfen as an inhibitor of GAG function. We observed that surfen inhibited MP-12 infection in SNB-19 cells, a glioblastoma cell line, but did not inhibit MP-12 infection in HEK 293T or mouse L cells, a mouse epithelium-derived cell line (Fig. 2-4A), or in Vero cells (data not shown). As a positive control for surfen activity, infection by HSV-1 was strongly inhibited in all cells (Fig. 2-4A). As an alternative means of examining GAG utilization in L cells, we also tested MP-12 infection in sog9 cells, which are clonal isolates of L cells that are defective in the EXT1 gene (40). EXT1 is responsible for polymerizing disaccharide subunits from the nascent tetrasaccharide linker and was identified in our screen as being important for RVFV infection of HapI cells (Fig. 2-1B). In contrast to infection by HSV-1, infection by MP-12 was unaffected by the loss of GAGs

in sog9 cells (Fig. 2-4B). To further examine the variance of this GAG-dependent phenotype across cell types, we preincubated RVFV or HSV-1 with either heparin, heparan sulfate, chondroitin sulfate, or dextran sulfate for 1 h prior to infection of a panel of cell lines, including HEK 293T, A549, HeLa, Vero, and (with RVFV only) C6/36 cells, in addition to the HapI cells. Heparin and heparan sulfate inhibited infection of both RVFV and HSV-1 on HEK 293T, A549, HapI, and HeLa cells by at least 2-fold but not on Vero cells, an African green monkey cell line, or of C6/36 cells, an *Aedes albopictus* mosquito cell line (Fig. 2-4C). Similar results were obtained with dextran sulfate, whereas preincubation with chondroitin sulfate had only a very modest effect on the four human cell lines and no effect on the Vero and C6/36 cells (data not shown). Since the composition of GAGs varies between cell types, this suggests that the GAG species that facilitate RVFV infection may not be ubiquitously expressed. Alternatively, as is the case with HSV-1, another entry factor may also be able to compensate for the lack of GAGs on some cell types (41). An endocytosis-mediating receptor(s) for RVFV has not been identified, and these data suggest that multiple entry factors are likely involved in RVFV infection and that their relative importance may vary between cell types.

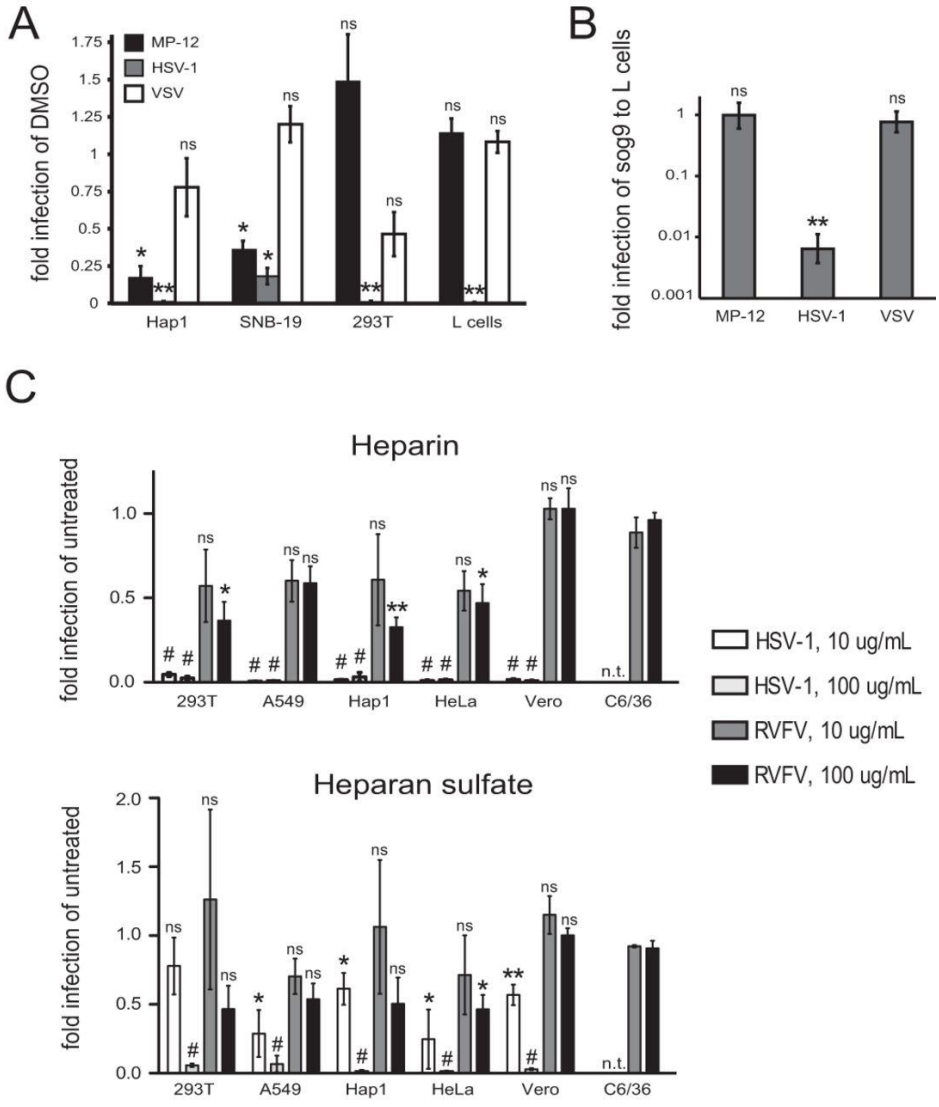

Figure 2-4. Examination of GAG utilization by MP-12 during infection of various cell lines. (A) MP-12 infection is resistant to surfen in some cell lines. Various cell lines were infected with MP-12, HSV-1, and VSV in the presence of either dimethyl sulfoxide (DMSO) or 5 μM surfen. The percentage of infected cells was normalized to the percentage of infected cells in the dimethyl sulfoxide-treated group. (B) L and sog9 cells were infected with MP-12, HSV-1, and VSV. The percentage of infected sog9 cells was normalized to the percentage of infected L cells. Bars indicate SEMs (n = 3). *, $P < 0.01$; **, $P < 0.0001$; ns, no significant difference. (C) Preincubation of HSV-1 or RVFV (MP-12 strain) with either soluble heparin or heparan sulfate prior to infection of HEK 293T, A549, HapI, HeLa, Vero, or C6/36 cells. The concentrations listed to the right of the graphs refer to the concentration of GAG species used during preincubation. Bars indicate SEMs (n = 3 for all cells except C6/36 cells, for which n = 2). *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$; ns, no significant difference.

GAGs are important for RVFV entry and binding. Based on the results of the blocking experiments with surfen and the fact that many viruses utilize GAGs for cellular attachment, we hypothesized that GAGs facilitate efficient entry by enhancing binding of RVFV to HapI cells. To examine this, we took advantage of the VSV pseudovirion system that has been successfully employed for other members of the *Bunyaviridae* family (16). The RVFV surface glycoproteins GN and GC are provided in trans to replication-incompetent vesicular stomatitis virus lacking its glycoprotein (VSVΔG). To validate the antigenic specificity of RVFV pseudovirions, we pretreated RVFV pseudovirions with an antiserum against RVFV or the severe acute respiratory syndrome (SARS) virus as a control. Infection by RVFV pseudovirions but not those bearing the VSV G protein was inhibited in the presence of the RVFV antisera (Fig. 2-5A). Infection by RVFV pseudovirions was also sensitive to lysosomotropic agents (data not shown), consistent with the requirement for acidic endosomal pH for infection with RVFV and other members of the Bunyaviridae family (42–45). We then infected the B3GAT3GT and B4GALT7GT cell panels with both RVFV and VSV pseudovirions that express red fluorescent protein (RFP). As with replication-competent RVFV, infection with RVFV pseudovirions required catalytically active GlcAT-I and GalT-I (Fig. 2-5B). In contrast, infection with pseudovirions bearing the VSV G protein was relatively unaffected, thus directly implicating GAGs in RVFV entry.

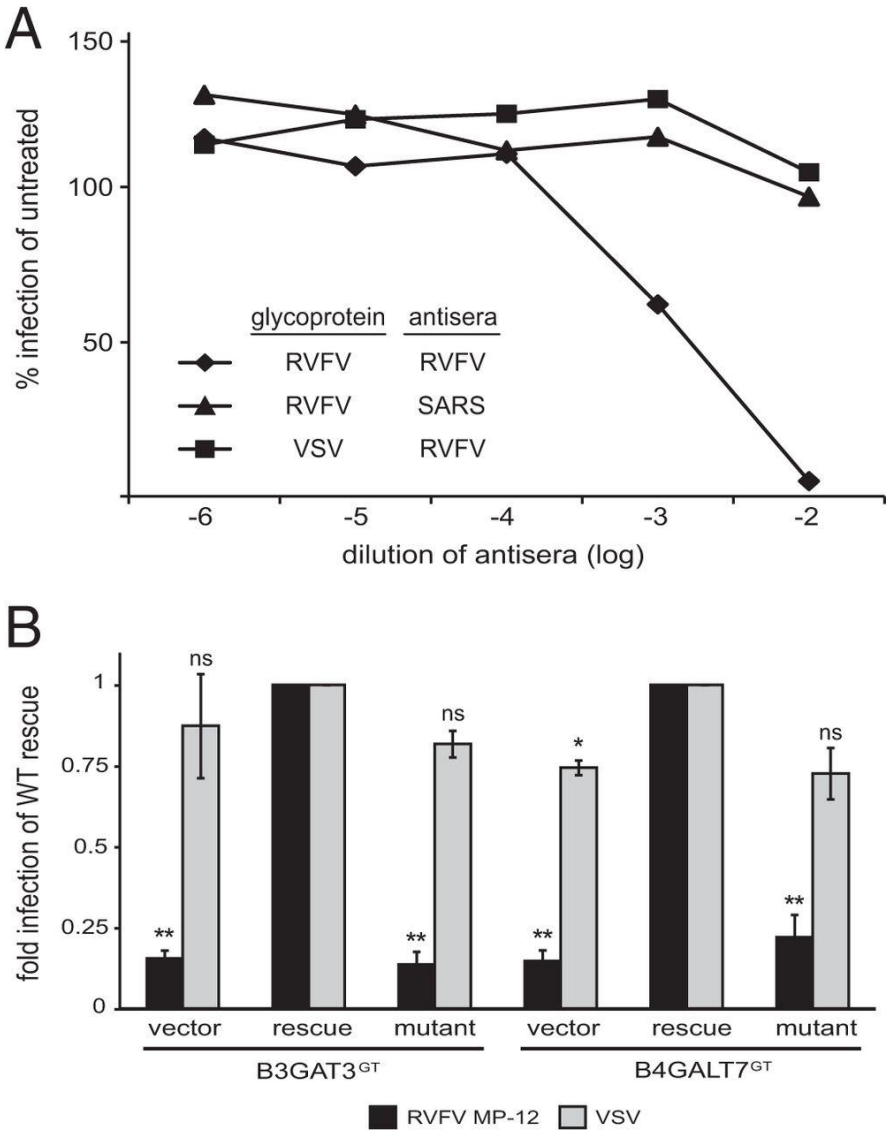

Figure 2-5. Cellular GAGs are important for RVFV entry. (A) Validation of RVFV pseudovirions. RVFV and VSV pseudovirions were preincubated with antiserum against either RVFV or the SARS virus before infection of Vero E6 cells. Infection values are normalized to the viral inoculum used with untreated cells. (B) B3GAT3GT and B4GALT7GT HapI cells that stably express the empty vector, the wild-type protein, or a catalytically inactive point mutant were infected with either RVFV or VSV pseudovirions. The percentage of infected cells was normalized to the percentage of infected wild-type cells rescued with each protein. Bars indicate SEMs (n = 3). *, $P < 0.01$; **, $P < 0.005$; ns, no significant difference.

To measure RVFV virion binding, we employed a quantitative reverse transcription-PCR (qRT-PCR) assay that detects RVFV L gene copies. We first confirmed the linear range of our assay by diluting MP-12 on HapI cells and measuring relative MP-12 binding and found that virus binding increased linearly with virus input over a 3-log-unit range (data not shown). When this assay was applied to the B3GAT3GT and B4GALT7GT cell panels, MP-12 binding strongly correlated with the presence of catalytically active GlcAT-I and GalT-I (Fig. 2-6). To confirm the role of GAGs in facilitating RVFV binding, we also measured the effect of surfen on RVFV binding. Consistent with its role in infection, surfen also blocked RVFV binding to a similar degree (Fig. 2-6). Taking these data together, we conclude that the deficiency in RVFV infection in the absence of GAGs is due to a defect at the level of virion attachment.

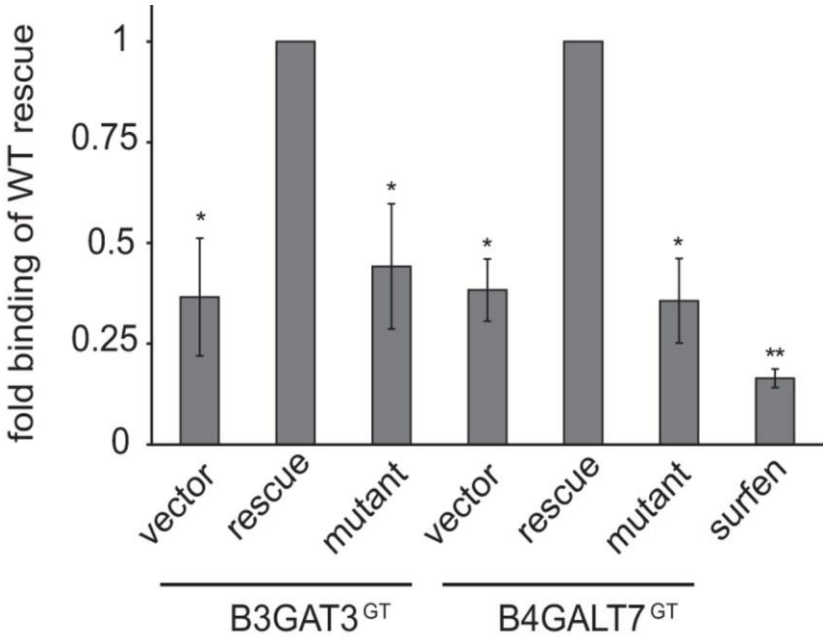

Figure 2-6. Cellular GAGs are important for RVFV binding. B3GAT3GT and B4GALT7GT HapI cells that stably express the empty vector, the wild-type protein, or a catalytically inactive point mutant were incubated with MP-12 for 1 h at 37°C, and the amount of cell-associated MP-12 was normalized to the number of wild-type cells rescued with each protein. The amount of cell-associated MP-12 on wild-type HapI cells was also measured in the presence of dimethyl sulfoxide or surfen and normalized to that for dimethyl sulfoxide-treated samples. Bars indicate SEMs (n = 5 for HapI gene-trapped mutant cells, n = 3 for surfen-treated cells). *, $P < 0.05$; **, $P < 0.001$; ns, no significant difference.

PTAR1 deficiency attenuates heparan sulfate expression and confers resistance to RVFV infection. HapI cells lacking a functional PTAR1 (14) were largely resistant to RVFV infection, and this phenotype could be corrected by reintroduction of wild-type *PTAR1* cDNA (Fig. 2-7A), indicating that the virus resistance phenotype was caused by the loss of PTAR1. In line with previous observations (11, 14), PTAR1-deficient cells showed a marked decrease in cell surface heparan sulfate abundance, as measured by flow cytometry (Fig. 2-7B). Similar to the virus resistance phenotype, heparan sulfate deficiency, too, could be corrected by complementation with wild-type *PTAR1* cDNA (Fig. 2-7A and B). Considering the requirement of heparan sulfate for RVFV infection, it seems plausible that improper presentation of heparan sulfate at the cell surface is responsible for the observed virus resistance of PTAR1-deficient cells. Thus, our screen has identified host factors required for RVFV infection. These factors are involved in various steps of the heparan sulfate biosynthesis pathway and include PTAR1, which constitutes a novel RVFV host factor affecting heparan sulfate expression at the cell surface.

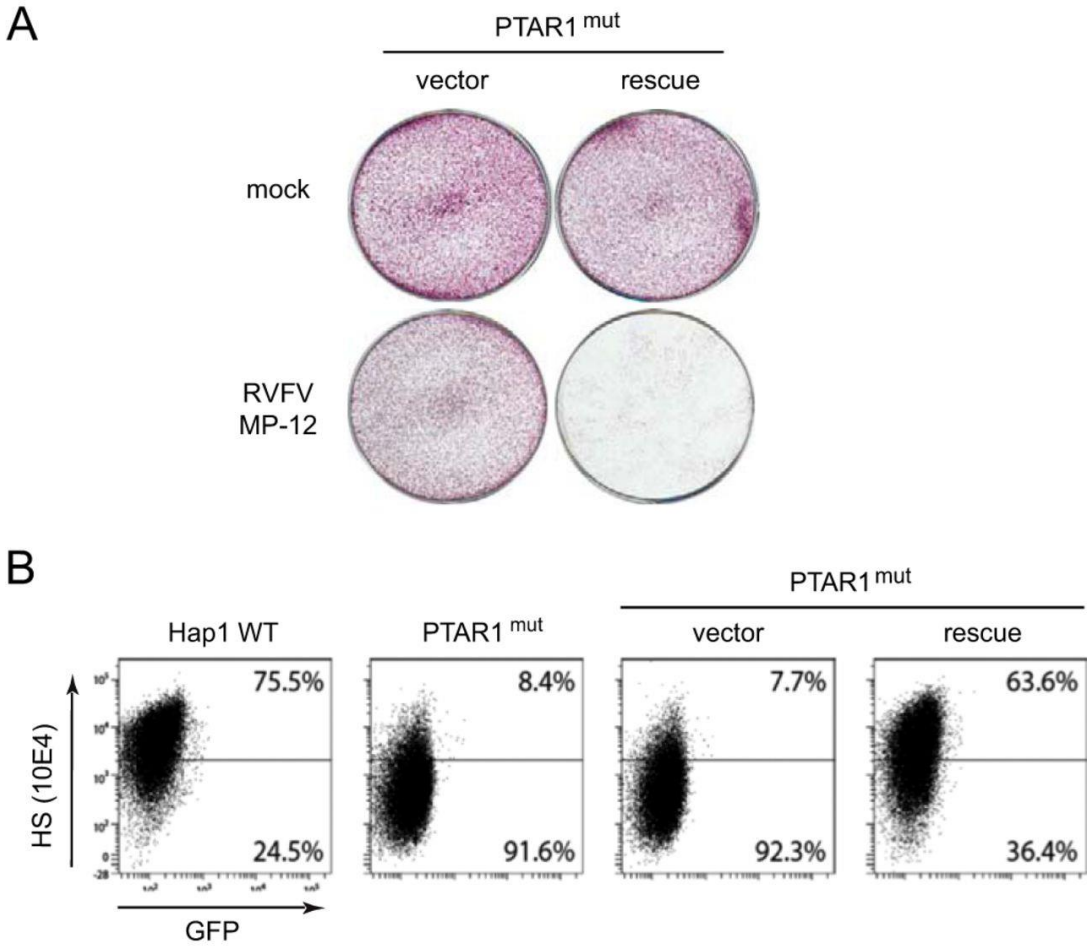

Figure 2-7. The loss of PTAR1 renders cells resistant to RVFV infection and leads to decreased heparan sulfate levels on the cell surface. (A) Infection of wild-type and PTAR1 mutant (PTAR1mut) cells with MP-12. Surviving cells were visualized by crystal violet staining. (B) Flow cytometric analysis of heparan sulfate (HS) levels, using a specific antibody (10E4), on the surfaces of nonpermeabilized wild-type cells, PTAR1 mutant cells, and PTAR1 mutant cells complemented with either an empty vector or PTAR1 cDNA. The percentage of cells above and below a signal threshold (horizontal line) is indicated. In line with previous observations (11, 14), the loss of PTAR1 reduces the levels of heparan sulfate present on the cell surface, and these levels can be corrected by introduction of PTAR1 cDNA.

DISCUSSION

Cell surface carbohydrates can affect virus entry at the stage of virion attachment, but the importance of this interaction varies among viruses and cells. For example, sialic acid is thought to be sufficient for influenza virus attachment and entry, while the role of GAGs during HSV-1 entry is more complex (41, 46, 47). The herpesviruses are thought to first engage heparan sulfate on the surface of cells before engaging specific receptors (48). Heparan sulfate greatly facilitates HSV-1 attachment and infection under many conditions but is not essential for infection in all contexts (41, 49). For example, CHO cell mutants deficient in GAG synthesis can be rendered permissive by expressing either of the HSV-1 entry receptors nectin-1 (PVRL1) or HVEM (TNFRSF14) (50). The expression levels of viral receptors can therefore determine whether GAGs are required for efficient viral entry.

The cellular receptor(s) for RVFV is currently not known, and since a nonpermissive cell line is yet to be described, it is possible that more than one molecule may serve as a receptor for RVFV. The C-type lectin DC-SIGN has been shown to promote the binding and internalization of RVFV on dermal dendritic cells, although this protein is not expressed in most of the tissues which the virus has been shown to infect (51). A genome-wide RNA interference screen performed by Hopkins and colleagues did not identify glycosaminoglycans among their list of genes that impacted RVFV infection (52). We have shown that several perturbations of GAGs inhibited RVFV entry and attachment on some cell types, but the relative contribution of other RVFV entry factors remains unknown. Because we observed differential sensitivities of RVFV to surfen, it is possible that the requirement for GAGs across cell types is a function of the relative

expression levels of an uncharacterized RVFV receptor(s), GAG structures to which surfen binds inefficiently, or unidentified attachment factors. Indeed, the composition of cellular GAGs between cells is highly variable (53). While heparan sulfate is the best-studied variant, there are at least four other species, each consisting of a unique disaccharide unit. Several enzymes are involved in modifying the different glycan side chains following polymerization. For example, HSV-1 interacts with 3-O-sulfated heparan sulfate, which is catalyzed by the 3-O-sulfotransferase family of enzymes (49). Our data suggest that RVFV may require a specific enzymatic variant of a GAG species or cellular proteoglycan. Further work is needed to elucidate the role of specific GAG-modifying enzymes and cellular glycoproteins during RVFV infection.

Heparan sulfate has previously been implicated as playing a role in RVFV infection. A study by de Boer *et al.* employed a replication-incompetent virus-like particle (VLP) system and found that CHO cells with genetic deficiencies in GAG synthesis were highly resistant, though not immune, to RVFV infection (13). This is in line with our observation that HapI cells incapable of producing GAGs are approximately 10-fold more refractory to RVFV infection than their parental (wild-type) HapI cells. Infection by Toscana virus, another member of the genus *Phlebovirus* of the family *Bunyaviridae*, has been shown to be inhibited by bovine lactoferrin through competition for GAGs on the cell surface (54). These results and our finding that the importance of GAGs and heparan sulfate for RVFV infection exhibited cell type dependence suggest that these molecules serve as virus attachment factors that can enhance but that are not absolutely required for virus infection and therefore do not represent indispensable viral receptors.

By employing RVFV-VSVΔG pseudovirions and an RVFV binding assay, we definitively linked GAGs to RVFV entry and, more specifically, to virus binding. It remains

to be determined whether the impact of heparan sulfate on RVFV infection of some cell types reflects the inefficiencies of cell-free virus attachment *in vitro* or whether these interactions are important *in vivo* as well, though the fact that primary RVFV strains behaved similarly to the MP-12 vaccine strain shows that these interactions are not the result of *in vitro* virus adaptation. Interestingly, the tissue tropism of adeno-associated virus 2 (AAV2) to the liver and kidney, organs in which RVFV also establishes productive infection, is exquisitely linked to interactions with GAGs (55–58). Infections with RVFV in pregnant livestock are devastating, and pathological studies of infected pregnant livestock reported extremely high virus titers in the placenta, an organ whose cells express high levels of surface GAGs (59, 60). Interactions with placental GAGs may explain the mechanism by which RVFV localizes to the placenta from the bloodstream.

The haploid genetic screen utilized here identified multiple genes involved in GAG synthesis or transport, including *PTAR1*. Whereas we cannot formally exclude the possibility that PTAR1 affects virus susceptibility by other means, it is most likely also involved in mediating GAG-dependent viral entry. Cells deficient for PTAR1 displayed decreased levels of heparan sulfate at their cell surface, which is in agreement with the observations obtained with cells with *PTAR1* mutations in previous genetic screens (11, 14). Additional experiments examining the precise role of PTAR1 in heparan sulfate biogenesis and trafficking are needed to shed light on the mechanism of PTAR1-dependent RVFV infection. Finally, the ability of this screening approach to identify additional host factors that are important for RVFV infection may be enhanced by employing cell types where virus attachment occurs in a GAG-independent manner.

The interaction of primary pathogenic RVFV isolates with GAGs suggests that this interaction might be an attractive pharmacological target in humans or other

animals. Heparan sulfate has indeed been shown to be important in human papillomavirus infection of mouse female genital tracts (61), and administering anti-heparan sulfate peptides as a prophylactic eye drop was shown to inhibit the spread of HSV-1 in the mouse cornea (62, 63). Although we need to further characterize the exact role of GAGs during RVFV infection *in vitro* and *in vivo*, our current study suggests that disruption of virus-GAG interactions could be a viable antiviral therapy or prophylactic measure.

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CHAPTER 3: THE WASH COMPLEX IS REQUIRED FOR INTOXICATION OF CLOSTRIDIUM DIFFICILE TOXIN B

ABSTRACT

Clostridium difficile infection causes a severe diarrheal disease that is increasing in incidence and becoming more resistant to treatment. Its secreted virulence factor Toxin B (TcdB) is internalized into host cells, where it inactivates Rho GTPases and causes significant cytotoxicity. The host factors supporting TcdB internalization and transport are largely unknown. We report here a forward genetic screen in human haploid cells that identified the Wiskott–Aldrich syndrome protein and SCAR homologue (WASH) complex as a host cellular factor supporting TcdB intoxication. The involvement of the WASH complex in TcdB entry was validated by pharmacologic inhibition of recycling endosomes and the use of mouse fibroblasts lacking a functional WASH complex due to genetic ablation of the core *WASH1* gene. Our data help to elucidate the mechanism of intoxication of this important and poorly-characterized virulence factor.

INTRODUCTION

The anaerobic bacterium *Clostridium difficile* causes severe antibiotic- and hospital-associated diarrhea and pseudomembranous colitis. *C. difficile* infection can lead to septic shock, perforation of the intestine, and toxic megacolon, and is fatal for about 9% of patients (1). In the United States, the CDC estimates 500,000 cases of *C. difficile* infection annually (2) and rates of *C. difficile* colitis have been increasing: from 2005 to 2010 there was a 47% increase compared to the five years prior (3). Spore

formation by the bacterium and worsening resistance to treatment have hindered sterilization, prevention, and treatment efforts.

C. difficile secretes two large multidomain toxins, TcdA and TcdB, that are the virulence factors primarily responsible for its pathogenesis. TcdB has been shown to be approximately 100-1000 times more cytotoxic than TcdA, and is believed to cause most of the severe disease symptoms associated with *C. difficile* infection (4-6). TcdB is 270 kDa and comprises the following domains: a glucosyltransferase domain (GTD) located at the N-terminus of the protein, a cysteine protease domain (CPD) responsible for auto-cleavage, a translocation domain (TMD) that includes a pore-forming region (PFR), a receptor binding domain (RBD) containing a combined repeat oligopeptides (CROP) region involved in binding and attachment at the cell surface, and newly-identified secondary RBD adjacent to the first. See Figure 3-1A for a schematic of the TcdB domain organization.

After binding to the cell surface via either of the RBDs, the toxin is internalized into an endosome. Acidification of endosomes is thought to induce a conformational change in the toxin, leading to pore formation and translocation of the CPD and GTD into the cytosol (7). The CPD then cleaves the GTD, freeing it to interact with its target Rho GTPases in the cytosol. TcdB has been shown specifically to glucosylate RhoA, Rac1, and Cdc42, although this varies by bacterial strain (8). Glucosylation of these Rho GTPases disrupts the numerous cell processes that they control, including cell polarity, vesicle trafficking, and microtubule and actin cytoskeletal regulation. It is this inactivation of the Rho GTPases that are thought to be responsible for the cytotoxic effects of TcdB. The current model of the TcdB intoxication is shown in Figure 3-1B.

Recent studies have demonstrated that chondroitin sulfate proteoglycan 4 (CSPG4) and Frizzled proteins can act as non-competitive receptors for TcdB but that their relative contributions to toxin entry depend upon their cell-type-specific expression levels (9,10). The host factors involved in the remaining entry steps of the toxin have not been well characterized. TcdB entry was shown to be blocked by pre-treatment with dynasore or chlorpromazine, but not by expression of plasmids encoding dominant-negative Cav-1 or Eps15, indicating that toxin entry is both dynamin- and clathrin-dependent (11). The endocytic route utilized by TcdB as it traffics within the cell, its site of translocation, and the host factors upon which it relies during these processes have yet to be described.

To identify host factors required for intoxication by TcdB, we have employed an unbiased forward genetic screening strategy using human haploid cells. We report here a role for the Wiskott–Aldrich syndrome protein and SCAR homologue (WASH) complex, which mediates membrane-cytoskeleton interactions and is important for cargo sorting at endosomes, in the entry of TcdB toxin. These data help to elucidate the mechanisms of intracellular transport of TcdB in mammalian cells.

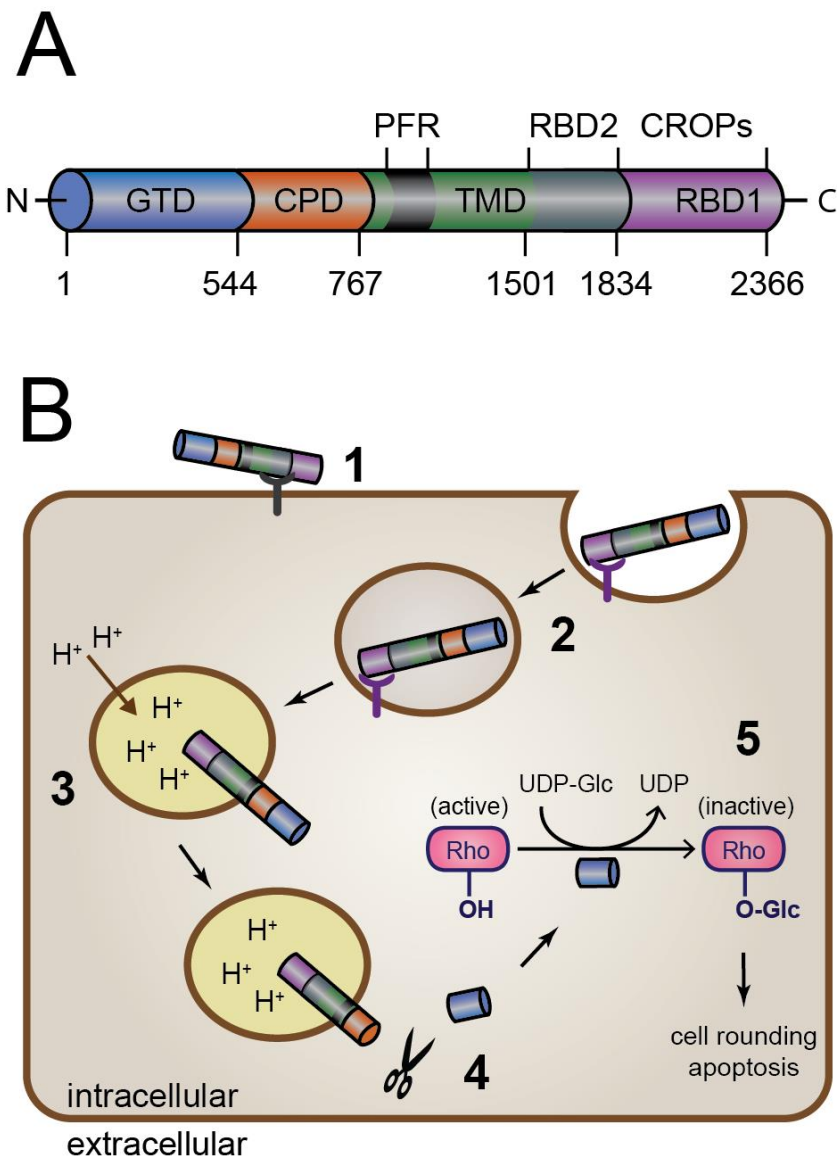

Figure 3-1. TcdB entry and structure. (A) The structure of TcdB toxin. GTD: glucosyltransferase domain, CPD: cysteine protease domain, PFR: pore-forming region, TMD: translocation domain, RBD: receptor binding domain, CROPs: combined repetitive oligopeptides. (B) The intoxication process of TcdB: binding at the cell surface (1) is followed by receptor-mediated endocytosis (2). Acidification of the endosome induces a conformational change in the toxin that results in formation of a pore (3). Autocatalytic cleavage and release of the GTD (4) leads to glucosylation of Rho GTPases (5) which are thereby inactivated.

MATERIALS AND METHODS

Haploid mutant library generation and screening. Approximately $1x10^9$ HAP1 cells were mutagenized as previously described (12). Briefly, HAP1 cells were enriched for haploid status and then mutagenized with a retroviral pLentiET gene trap virus. Parental (WT) HAP1 cells ($3x10^6$ cells total) and the mutant library ($8x10^7$ cells total) were challenged in parallel with TcdB at a concentration of 0.5 nM. Three sequential toxin selections were performed. After the first and second selections, surviving cells were allowed to expand for approximately 8 days before being trypsinized and re-seeded at 30% confluency prior to subsequent challenge. After the third selection, all WT HAP1 cells were dead, and the surviving cells from the mutant library were allowed to recover for 10 days and then pooled for genomic DNA isolation.

Integration site mapping. DNA from the mutant library as well as the TcdB-selected population was isolated and gene trap insertion – host junction sites were amplified and then submitted for sequencing on either 454 or Illumina platforms, followed by alignment to the human genome (hg18). Significance of enrichment of the integration sites identified within the TcdB-selected pool relative to the unselected library was calculated using one-sided Fisher's exact test.

Cell culture. *WASH1*$^{flox/flox}$ and *WASH1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were a kind gift from Dan Billadeau (Mayo Clinic) and were generated as described in (13). Vero C1008 cells and HeLa cells were obtained from ATCC. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented by 10% fetal bovine serum (FBS).

Reagents and antibodies. Toxin B (TcdB) from *Clostridium difficile* was purchased from List Biological Laboratories, catalog number 155L. Primaquine was purchased from Sigma-Aldrich, catalog number 160393. The following antibodies were used: chicken anti-TcdB (List Biological Laboratories 754A), mouse anti-Rac1 clone 23A8 (EMD Millipore 05-389), mouse anti-Rac1 clone 102 (BD Biosciences 610650), rabbit anti-FAM21C (Abcam 184131), rabbit anti-EEA1 (Cell Signaling Technology 2411S), rabbit anti-LRP1 (Abcam 92544), rabbit anti-CSPG4 (Abcam 139406), and rabbit anti-FZD7 (Abcam ab51049).

Cell-rounding assay. Cells were plated at 25% confluency 15-20 hr prior to treatment with TcdB. Toxin was diluted in DMEM + 10% FBS and applied to cells at the indicated concentrations. After 6 hours, cells were imaged on a Nikon Eclipse TE300 inverted microscope and scored by visualization of rounded cells (minimum of 100 cells per image). Percent cell rounding was calculated for each sample as follows: [(number rounded cells / number of total cells analyzed) * 100].

Immunoblotting. Cells were trypsinized and cell pellets were rinsed with PBS, flash frozen, and then resuspended in 2X lysis buffer (0.1M Tris with 20% glycerol and 5% SDS). Lysates were heated at 95°C for 5 min, passed through a 28-guage needle 3 times, heated again at 95°C for 5 min, and total protein was quantified using the Pierce BCA Protein Assay Kit. Samples were run through 4-12% Bis-Tris gradient gels at 100V in 1X MES buffer, and then transferred to PVDF membranes using the iBlot 2 Dry Blotting System. Membranes were blocked in a milk solution for 1 hr at room temperature and then probed with primary antibodies (diluted in blocking buffer) overnight at 4°C. Membranes were then washed and probed with HRP-conjugated secondary antibodies. HRP was detected using SuperSignal West Pico or Femto

Chemiluminiscent Substrate (Fisher Scientific 34095 or 34080) and developed on the GE Amersham Imager 600.

RESULTS

An insertional mutagenesis screen in human haploid cells identifies host cell factors required for TcdB toxin. To identify host factors used by TcdB, we employed an unbiased forward genetic screening strategy in the human haploid cell line HAP1, which was derived from KBM7 cells (14). Approximately one billion HAP1 cells were mutagenized with a lentiviral gene trap vector to generate a library of loss-of-function clones harboring inactivating viral insertions. This library, as well as parental (unmutagenized) HAP1 cells, was thrice challenged with TcdB toxin at 0.5 nM concentration. After the third challenge, there were no surviving cells within the parental population, and the surviving clones from the mutant library were pooled for DNA sequencing and mapping of the lentiviral integration sites. A profile of insertion sites within the selected population was determined and compared to that of the original mutant library. We determined the integration-site containing genes that were significantly (p value < 0.05) enriched within the selected cells relative to the library to yield a hit list of genes whose disruption confers a resistance phenotype, as shown in Figure 3-2.

Among these hits, we identified LDL receptor related protein 1 (LRP1), which was recently reported to be a receptor for the *Clostridium perfringens* toxin TpeL, a closely-related clostridial glycosylating toxin that lacks the CROPs region (15). The authors examined the entry of CROP-deficient truncated *C. difficile* TcdB toxin into MEFs that lack LRP1, and saw no difference compared to parental (wild-type) MEFs, so

there has not yet been any evidence that LRP1 acts as either a receptor or attachment factor for TcdB, although this may depend on cell type. We also identified *WASHC5* (*KIAA1096*) and *WASHC4* (*KIAA1033*), which encode the proteins Strumpellin (also called WASHC5) and Strumpellin and WASH-interacting protein (SWIP, also called WASHC4), two of the five core members of the WASH complex. Additional hits included *COMMD8*, *COMMD10*, *C16orf62*, and *CCDC63*, all of which encode members of the COMMD/CCDC22/CCDC93 (CCC) complex, a recently-identified multisubunit protein complex that interacts with the WASH complex and participates in cargo transport at endosomes (16). The screening hits also included multiple genes encoding proteins that have established or putative roles in membrane trafficking and endocytosis.

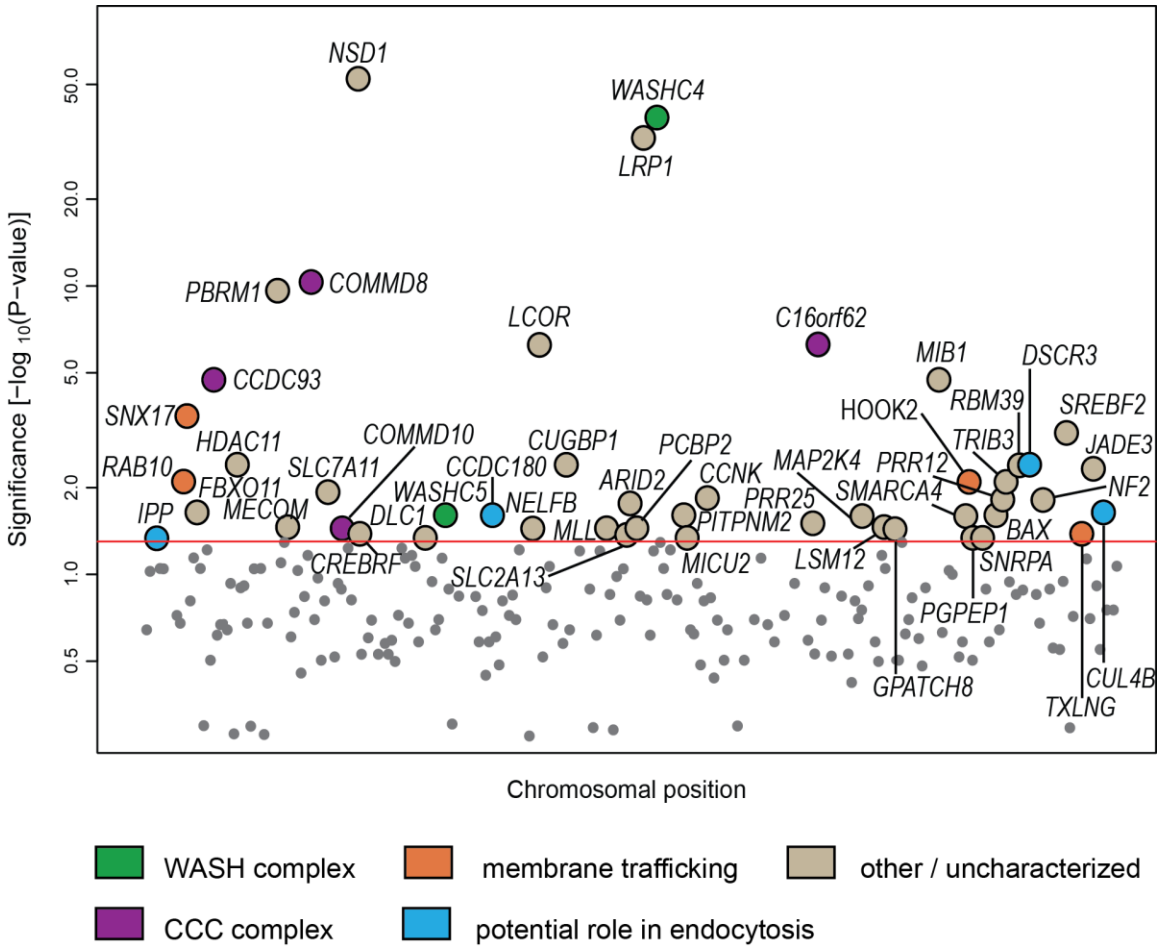

Figure 3-2. Human haploid screen for TcdB host factors. Genes whose disruption was significantly enriched (*p* value > 0.05 indicated by red line) in the TcdB-selected population relative to the unselected mutant library. Each circle denotes a gene, with y-axis position showing the false discovery rate-corrected *p* value, and distribution along the x-axis corresponding to chromosomal position. Circle colors indicate genes encoding members of the Wiskott–Aldrich syndrome protein and SCAR homologue (WASH) complex, the COMMD/CCDC22/CCDC93 (CCC) complex, or the function of the gene product.

Because our screen identified multiple members of the WASH complex as well as the interacting CCC complex, and because the host machinery required for transport of TcdB through the cellular endocytic pathway has not been well described, we decided to investigate the involvement of the WASH complex in TcdB entry. The WASH complex is a Type I nucleation-promoting factor that has been shown to interact with the retromer cargo-selective complex (CSC) at the endosomal membrane, where it activates the Arp2/3 complex in order to drive formation of branched actin patches that define microdomains of the tubular endosomal network. These actin patches are critical for cargo transport back to the plasma membrane as well as to the Golgi apparatus, and may also play a role in signaling processes [reviewed in (17)].

TcdB cytotoxicity is blocked by inhibition of recycling endosomes. An important role for the WASH complex has been defined during the process of endosome-to-cell surface recycling. Driven by its association with the retromer CSC and sorting nexin 27 (SNX27), the WASH complex can be recruited to sites of cargo that contain a PDZ domain-interacting motif, where its actin patch-forming activity is required for the recycling of these cargoes, such as β2 adrenergic receptors, from EEA1-positive endosomes back to the plasma membrane (18). We therefore used the recycling inhibitor primaquine to interrogate the involvement of this pathway during TcdB intoxication. Primaquine has been shown to interfere with the function of recycling endosomes in a manner that is independent of pH neutralization or osmotic swelling (19).

Vero cells (derived from African green monkey kidney) or HeLa cells were plated at approximately 25% confluency and then pre-treated for one hour with 100 uM primaquine prior to addition of TcdB toxin at 0.2 or 0.8 pM, or a vehicle control of

equivolumetric DMSO. Cell rounding was scored (at six hours post intoxication) as a read-out for TcdB activity. Figures 3-3A and 3-3B show the imaging and quantification, respectively, for primaquine's inhibition of TcdB-induced rounding in Vero cells, and Figures 3-3C and 3-3D show the same for HeLa cells. In both cell lines, pre-treatment with primaquine greatly decreased the cell-rounding phenotype, though this effect was more pronounced in the Vero cells, perhaps due to the fact that they are more sensitive to the effect of toxin (nearly all Vero cells rounded with treatment of 0.8 pM TcdB, compared to ~ 50% rounding of HeLa cells at the same concentration; Fig. 3-3C versus 3-3D). This supports a role for recycling endosomes in the trafficking of TcdB toxin.

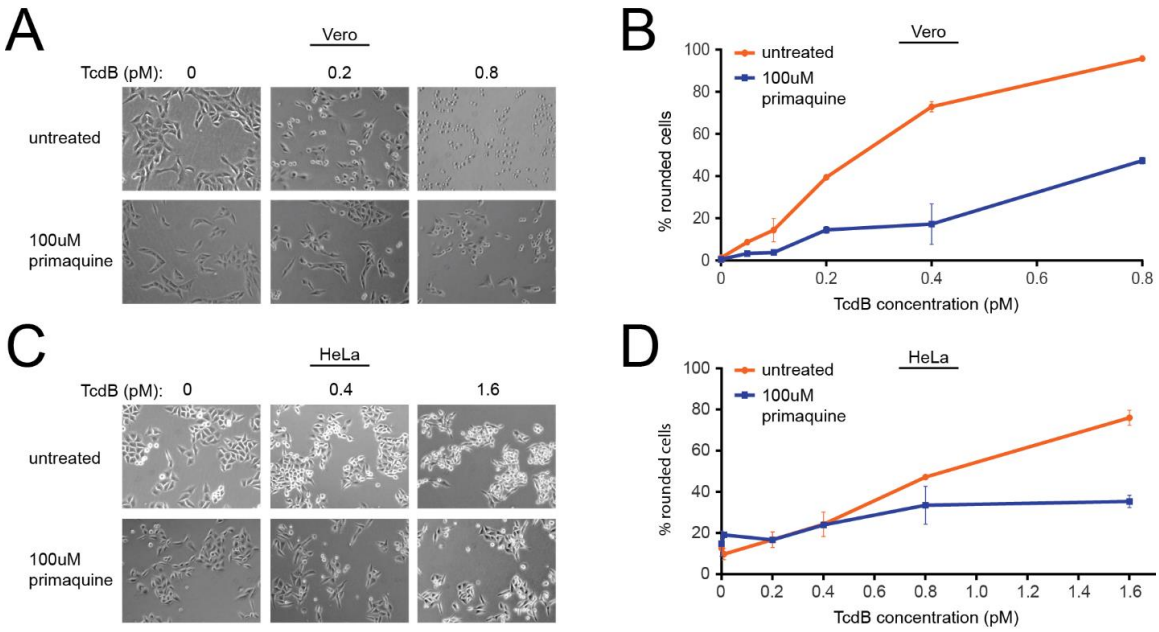

Figure 3-3. Primaquine treatment blocks cytotoxicity of TcdB toxin. Vero cells (A,B) and HeLa cells (C,D) were pre-treated with primaquine and then intoxicated with TcdB for six hours at the indicated concentrations. Brightfield imaging of treated cells (A,C) and quantification of cell rounding (B,D). Shown are representative images for selected concentrations; a minimum of 100 cells per sample were imaged and quantified; N=2 independent experiments.

The WASH complex is required for TcdB cytotoxicity. To more directly test the involvement of the WASH complex in TcdB entry, we utilized *WASH1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) bearing a deletion within the *WASH1* gene that results in dysfunction of the entire WASH complex (13). In these *WASH1*$^{-/-}$ MEFs, the central WASH1 component of the complex (see Figure 3-4A) is depleted and expression of the other subunits is greatly decreased, leading to trafficking defects and a collapsed endosomal network (13).

We first sought to characterize the cell surface expression of proteins that might be involved in TcdB binding and attachment, to see whether changes in their levels might account for the decreased toxin entry. As the WASH complex is required for recycling of many proteins to the cell surface, it was possible that this type of indirect effect may have been responsible for the resistance phenotype of WASH-deficient clones in our screen, rather than a direct impact on toxin intracellular trafficking. We stained non-permeabilized cells with antibodies raised against CSPG4 and FZD7, two proteins that have been shown to act as receptors for TcdB in some cell types (9,10).

Fluorescence staining profiles of the *WASH1*$^{-/-}$ and *WASH1*$^{flox/flox}$ MEFs, shown in Figure 3-4B, show no obvious differences in expression of CSPG4 between the cell lines. Cell surface presentation of FZD7 and LRP1 was slightly increased in the *WASH1*$^{-/-}$ MEFs, indicating that these potential receptor or attachment factors are still present at the cell surface in this WASH-deficient cell line.

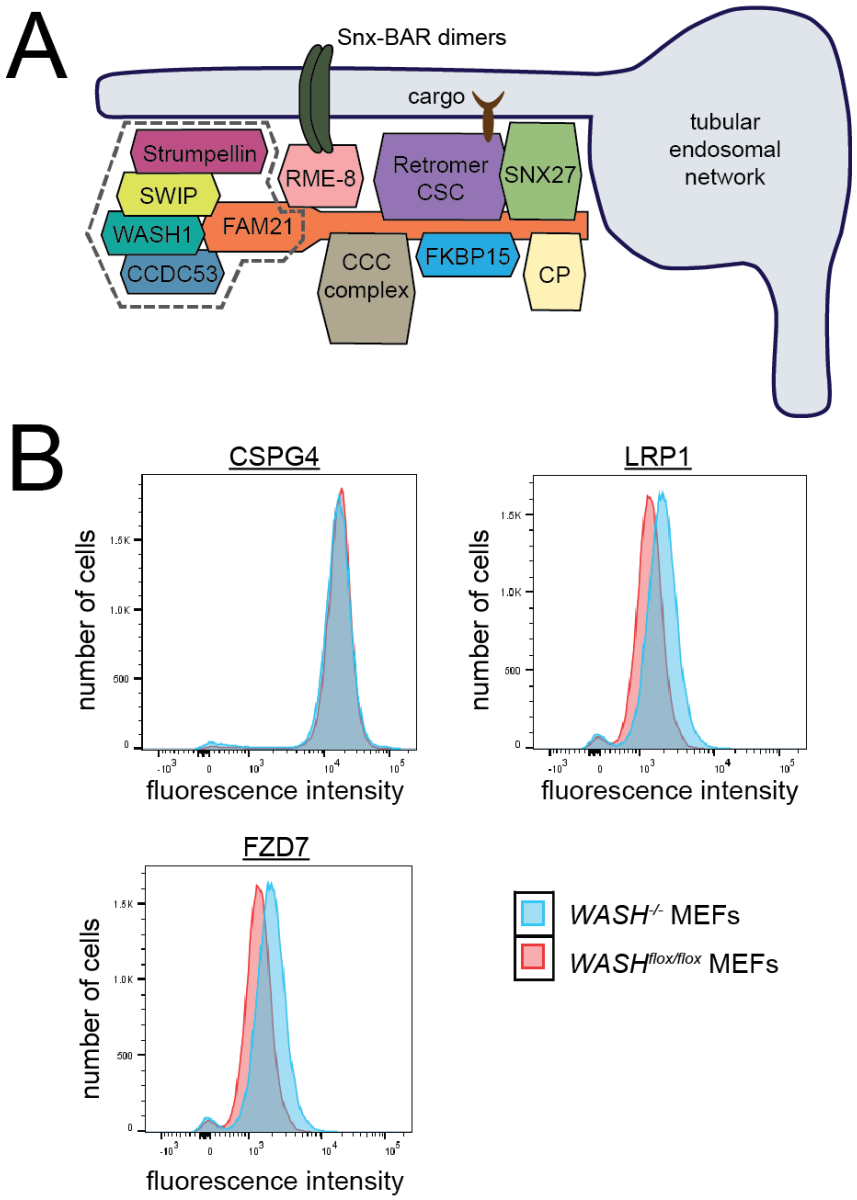

Figure 3-4. Characterization of WASH-deficient fibroblasts. (A) The WASH complex shown with its key interacting partners and adaptor proteins at the tubular endosomal network. WASH complex components: Strumpellin, SWIP, WASH1, CCDC53, and FAM21 (also called VPEF). Interacting partners and adaptor proteins: the retromer cargo-selective complex (CSC) comprising VPS26, VPS29, and VPS35, which mediates cargo selection; RME-8 (also called DNAJC13) that coordinates association of the WASH complex with the retromer sorting nexin (Snx-BAR) dimer; FKBP15 (also called WAFL), which interacts with the FAM21 tail; SNX27 that helps direct this

machinery to recycle its PDZ domain-interacting cargo via interaction with the CSC and FAM21; the COMMD/CCDC22/CCDC93 (CCC) complex, which is involved in endosomal sorting and interacts with FAM21; and the heterodimer capping protein (CP, also called CapZ) that caps the barbed ends of actin filaments. (B) Cell surface staining of *WASH1*$^{flox/flox}$ and *WASH1*$^{-/-}$ MEFs.

We then tested the susceptibility of the *WASH1*$^{flox/flox}$ and *WASH1*$^{-/-}$ MEFs to TcdB intoxication. The *WASH1*$^{flox/flox}$ and *WASH1*$^{-/-}$ cells were plated at approximately 25% confluency 15-20 hours prior to intoxication and then TcdB (at 0, 0.2, 0.4, 0.8, and 1.6 pM) or DMSO (as an equivolumentric vehicle control) was applied to the cells for 6 hours. The cells were then imaged (Figure 3-5A) and quantified for cell rounding (Figure 3-5B). Cytotoxicity of the toxin was decreased in the WASH-deficient MEFs approximately 2-fold, though not completely blocked. This resistance phenotype supports a role for the WASH complex in the entry of TcdB toxin into mammalian cells.

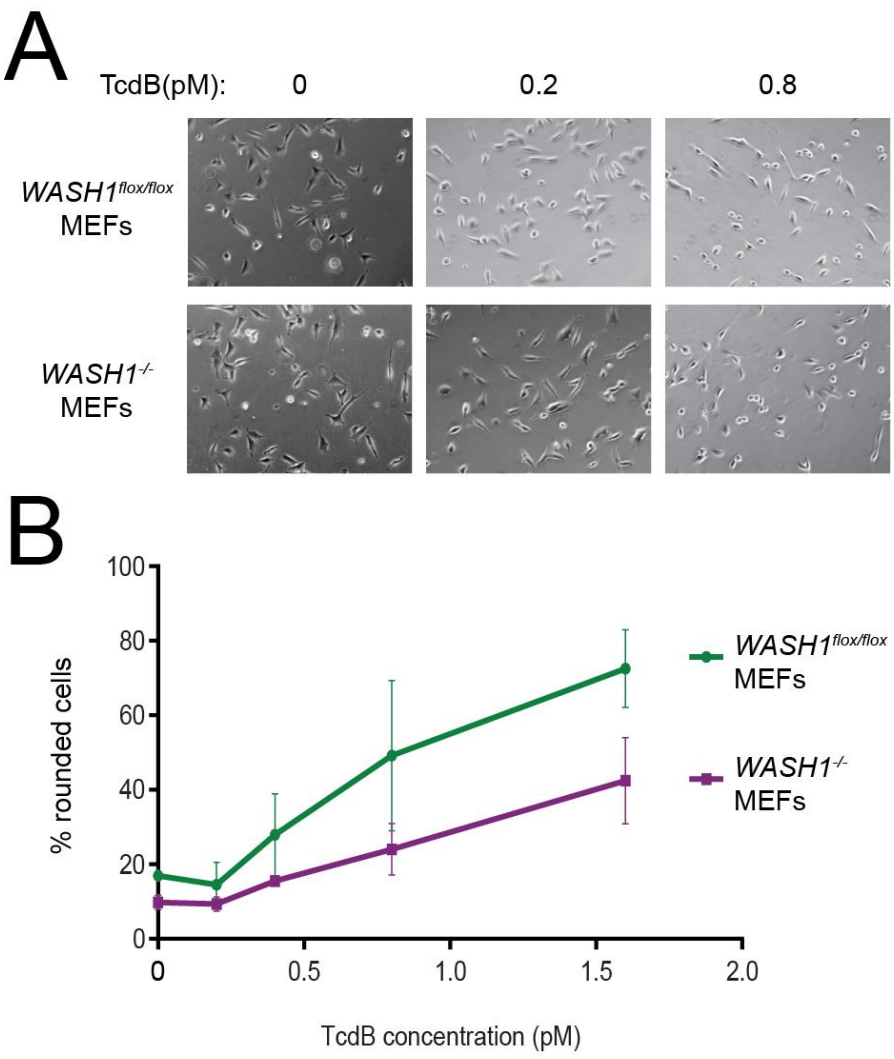

Figure 3-5. Cell-rounding of TcdB-treated *WASH*$^{flox/flox}$ and *WASH*$^{-/-}$ fibroblasts. (A) Representative images of cell-rounding activity of TcdB on *WASH*$^{flox/flox}$ and *WASH*$^{-/-}$ MEFs at selected concentrations. (B) Quantification of the percent cells rounded. A minimum of 100 cells per sample were imaged and quantified; N=2 independent experiments.

Rac1 glycosylation by TcdB in the absence of a functional WASH complex. To further probe the involvement of the WASH complex in TcdB intoxication, we treated *WASH*$^{flox/flox}$ and *WASH*$^{-/-}$ MEFs with the toxin and then lysed cells after 30 or 45 minutes of toxin treatment in order to examine the kinetics of TcdB glucosylation of its target GTPase Rac1. We took advantage of two mouse antibodies against Rac1: clone 102, which detects only non-glysocylated Rac1 protein, and clone 23A8, which detects both glycosylated and non-glycosylated Rac1 protein. Whole cell lysates were separated by SDS-PAGE gel electrophoresis, transferred to PVDF membranes, and then probed with the aforementioned antibodies. Western blots of these two timepoints are shown in Figure 3-6A and 3-6B. The gel analysis tool of ImageJ was used to quantify the density of bands and we then calculated the fraction of non-glycosylated Rac1 for each sample as [density of the band detected by the clone 102 antibody) / (density of band detected by clone 23A8 antibody)] and this fraction (normalized to untreated cells) is plotted in Figure 3-6C and 3-6D.

After 30 minutes of TcdB treatment at the lower concentration, the majority of cellular Rac1 in both the *WASH*$^{flox/flox}$ and the *WASH*$^{-/-}$ MEFs was still non-glycosylated, with a greater degree of Rac1 glycosylation by 0.02 pM TcdB than 0.005 pM, but levels were comparable between the WASH-deficient cells and the control cells. After 60 minutes of TcdB treatment, both concentrations of toxin we tested produced significantly more glycosylation of Rac1 compared to the 30-minute treatment. The degree of Rac1 glycosylation in the *WASH*$^{flox/flox}$ MEFs was slightly greater than that of the *WASH*$^{-/-}$ MEFs at this timepoint, but the phenotypic effect of WASH-deficiency was more modest than that observed with the cell-rounding assay. Interestingly, the concentrations of toxin we used for this assay (both of which were able to glycosylate significant fractions

of cellular Rac1 by 60 minutes) were equal to or less than 0.02 pM, a concentration of toxin which was only able to produce a cell-rounding phenotype in fewer than 20% of cells for both WASH-deficient and control MEFs. These data therefore indicate that glycosylation of Rac1 cannot necessarily be used as a read-out for the full spectrum of cytotoxic activity of the toxin, and that the WASH complex may be required during a stage of TcdB intoxication that only slightly impacts its ability to glycosylate Rac1.

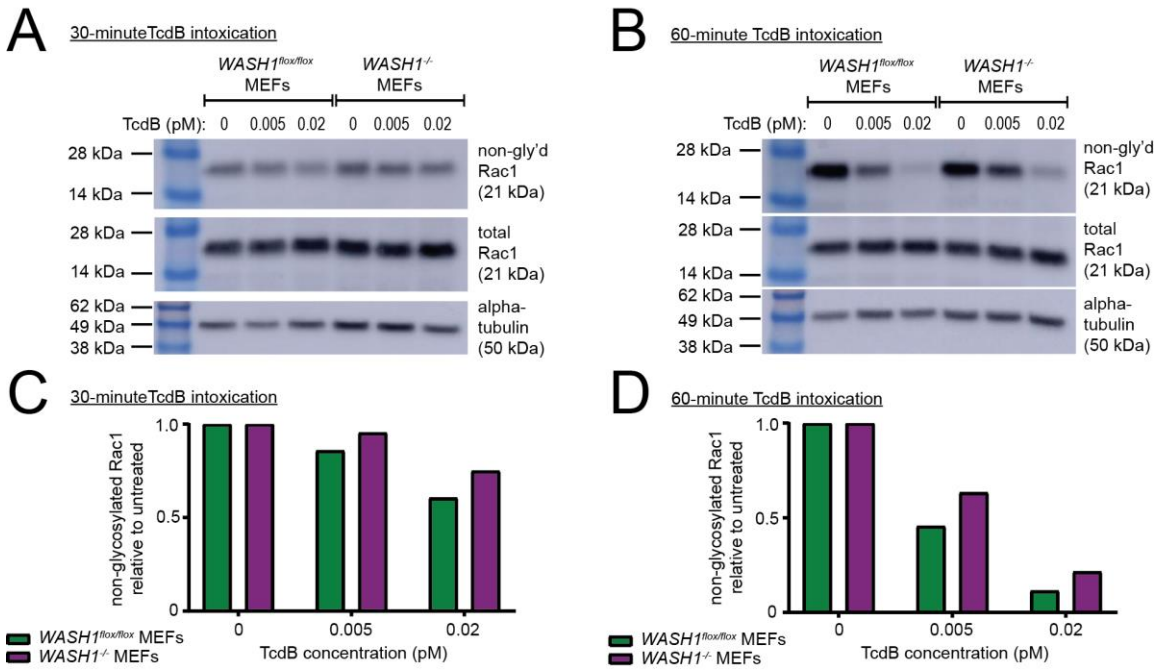

Figure 3-6. Western blot analysis of Rac1 glycosylation by TcdB in *WASH1*$^{flox/flox}$ and *WASH1*$^{-/-}$ fibroblasts. *WASH1*$^{flox/flox}$ and *WASH1*$^{-/-}$ MEFs were treated with TcdB for either 30 minutes (A,C) or 60 minutes (B,D) after which cells were lysed and subjected to immunoblot against either non-glycosylated Rac1 (using anti-Rac1 clone 102) or total Rac1 (using anti-Rac1 clone 23A8). Western blots (including a loading control, alpha-tubulin) are shown in (A) and (B) and quantification of the above Western blots (done in ImageJ) are shown in (C) and (D).

DISCUSSION

We report here a forward genetic screen in human haploid cells that identifies host factors involved in TcdB intoxication. These screening hits were enriched for genes encoding proteins involved in endocytosis and membrane trafficking, in particular the WASH complex and its interacting partner the CCC complex. We pharmacologically inhibited endosomal recycling to show that this process is required for TcdB intoxication of mammalian cells, and used WASH-deficient fibroblasts to show that TcdB cytotoxicity is diminished in the absense of a functional WASH complex. These fibroblast cell lines also showed a modest decrease in levels of glycosylation of Rac1, a target GTPase of TcdB toxin when it enters the cytoplasm, although the concentration of TcdB required to glycosylate the majority of cellular Rac1 was much lower than the concentration required to induce a cell-rounding phenotype in the majority of cells.

Since its initial characterization less than a decade ago, the WASH complex has emerged as a key regulator of multiple aspects of cargo sorting and membrane trafficking at endosomes (20,21). There are several possible ways in which the WASH complex may function to support TcdB intoxication. Its nucleation of actin branching at the tubular endosomal network has been shown to be involved in cargo recycling to the plasma membrane, as well as to the trans-Golgi network (TGN), depending upon its interactions with adapter proteins, particularly sorting nexins (18, 21-23). A role for the WASH complex has also been demonstrated in the maturation of endosomes and delivery of cargo to lysosomes (24). As the endocytic route used by TcdB toxin has not been fully characterized, it is possible that the WASH complex could be supporting TcdB entry at any of the aforementioned locations. Elucidation of the endosomal

compartments through which TcdB traffics will help to inform our understanding of its potential reliance upon the WASH complex.

Other hits from our screen are also suggestive of the involvement of particular arms of the host endocytic machinery in TcdB entry and therefore warrant investigation. SNX17, for example, has been shown to be important for cargo to escape a lysosomal fate, although the authors found that it did not co-precipitate WASH complex proteins (25). HOOK2 is a relatively uncharacterized microtubule-binding protein that's been shown to recruit cargo to the centrosome (26). We also did not directly test the involvement of the CCC complex, which interacts with the WASH complex and has been shown to regulate recycling of Notch, LDLR, and the copper transporter ATP7A (16, 27, 28). It would be very interesting to see whether the CCC complex may be acting to support TcdB entry in a manner that is either independent of its established partnership with the WASH complex, or whether it is able to partially rescue the trafficking defect that results from WASH complex dysfunction.

Our screen also identified the gene *FBXO11*, which encodes a member of the F-box protein family that interacts with the TGFβ signalling pathway. This gene was the top hit in the recent CRISPR screen that identified Frizzled proteins as TcdB receptors (10), suggesting quite strongly that it may be required for TcdB intoxication. One of our top hits was the gene *LRP1*, which encodes a receptor for the closely-related clostridial toxin TpeL. Although the authors concluded that LRP1 did not play a role in CROP-less TcdB entry, it may be worthwhile to re-examine the possibility that LRP1 supports TcdB entry in some way. Since the use of CSPG4 and Frizzled proteins as receptors for TcdB has been shown to depend upon the relative expression levels of these proteins at the

cell surface, it is certainly possible that LRP1 facilitates entry of TcdB in our screening cell line, HAP1, and not in the MEFs used by Schorch, *et al.* (15).

Together, our data indicate a novel role for the WASH complex in the intoxication of mammalian cells by the *C. difficile* virulence factor TcdB. This sheds light on the array of host cellular factors that are utilized by the toxin as it enters and traffics through the cell, and it furthers our understanding of the function of this recently-characterized nucleation-promoting machinery. Future work is needed to define the precise endocytic compartments occupied by TcdB within the cell, and to characterize the mechanism by which the host's cargo trafficking infrastructure interacts with and supports the entry of this clinically important toxin.

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CHAPTER 4: DISCUSSION AND FUTURE PERSPECTIVES

THE COMPLEMENTARITY OF DIVERSE SCREENING APPROACHES

High-throughput loss-of-function screening techniques have provided tremendous insights into host-pathogen interactions, and one of the most widely-used and efficacious of these has been the stalwart RNAi screening approach. RNAi screens have been performed in a wide range of cell lines, spanning multiple species, and have interrogated aspects of cell biology as diverse as organelle morphology and mechanisms of resistance to drug toxicity [reviewed in (1)]. As RNAi screening became more popular, though, it also became evident that the technology suffered from issues of reproducibility and a high rate of false discovery. Results of the three genome-wide siRNA screens performed with HIV in 2008 (2–4), each of which had generated a list of approximately 300 genes supporting HIV infection in 293T or HeLa-derived cells, were subjected to in-depth meta-analysis by Bushman and colleagues in 2009, who reported that the percentage of overlap in gene hits between any two of the three screens was 6% at most (5). Two genome-wide RNAi screens were performed in 2009 to uncover host factors required for hepatitis C virus (HCV) replication in human cells. Tai *et al.* (6), using an HCV subgenomic replicon, reported the identification of 96 genes that support HCV replication, and Li *et al.* (7), using infectious virus, then identified 262 genes impacting infection, only 15 of which overlapped with the previous screen's findings. In the last five years, two genome-wide RNAi screens using Sindbis virus (SINV) have been performed, one in Drosophila cells (8, 9), and one in human cells (10). The screen in Drosophila cells identified 57 genes supporting and 37 genes that restricted SINV

infection, while the screen in human cells identified 56 genes supporting and 62 genes restricting infection – but there was very little overlap between the genes identified [compare (10) Tables S2 and S3 with human homologues of (9) Table S1].

Much of the reason for this lack of overlap between seemingly similar RNAi screens has been ascribed to the off-target effects of siRNAs and differences between technical aspects of the screening conditions. In a recent analysis of three genome-wide RNAi screens (one with UUKV and two with bacterial pathogens), Franceschini and colleagues concluded that the phenotypic effects of siRNA oligos were in fact predominantly due to off-target microRNA activity conferred by the seed region sequence, rather than the intended siRNA activity (11). They found significantly higher phenotypic correlations when siRNA oligos from different vendors were grouped by seed sequence (nucleotides 2-8) than when they were grouped by intended target (full-length complementarity of all 21 nucleotides). The authors confirmed these findings by designing custom oligos containing seed sequences predicted to impact infection that were flanked by arbitrary sequences outside of the seed region, and demonstrated that overexpression of known human microRNAs phenocopied the effect of siRNA oligos with corresponding seed sequences. These findings beg a reexamination of the raw data that have been generated by previous RNAi screens, as well as an attentive consideration of microRNA effects during analysis of any future screens. In addition to the off-target activities of the oligos themselves (which can cause both false positive and negative results), differing gene expression levels between cell types, variable efficiencies of transfection protocols, and discordance between knockdown timing and the half-life of the target protein can all contribute to a high false-negative rate. Recent improvements in both design and analysis of RNAi screens have sought to address

these problems, such as the Minimum Information About an RNAi Experiment (MIARE) reporting guidelines (http://miare.sourceforge.net) that have been established, and the utilization of the multiple orthologous RNAi reagents coupled with RNAi gene enrichment ranking (MORR-RIGER) method, which helps to reduce false negatives and filter off-target effects (12). For a detailed discussion about the factors impacting RNAi screen success, recent technical updates, and current design and analysis strategies, see reference (1) and the references therein.

Like RNAi, haploid screening is a forward genetic approach, allowing for discovery of novel host factors in the absence of a presumed or suspected mechanism of action. Although the technique is relatively new and comparatively few studies employing this approach to study virus-host interactions have been published, it is clear that haploid screening offers some important advantages over RNAi screening. A significant advantage is the fact that the insertional mutagenesis strategy employed to generate the haploid libraries usually results in complete disruption of the gene product, rather than the transient partial depletion that results from RNAi targeting. This in turn greatly increases the signal-to-noise ratio of the data that are obtained. Generation of many independent mutants within the library that each bear separate integrations into the same gene locus also allows for rigorous statistical analysis to identify genes whose absence was selected for within the surviving mutant pool. The fact that this selection is occurring in a cell line of human origin is also attractive because it increases the likelihood of finding biologically meaningful factors that participate in the host-pathogen interaction during the course of human disease.

It may be premature to attempt to evaluate the reproducibility of haploid genetic screens as published applications of this screening technique have utilized a diverse

array of pathogens, including Ebola virus, Lassa virus, RVFV, enterovirus D68, and adeno-associated virus serotype 2 (13–18). Diphtheria and anthrax toxins, *Clostridium perfringens* TpeL toxin, *Pseudomonas aeruginosa* exotoxin A, and *Staphylococcus aureus* α-toxin (19–22) have also been investigated with this approach. To our knowledge, ANDV is the only selective agent to have been used in two completely independent haploid genetic screens performed by different labs. The degree of overlap between these two screens, however, was striking. In the 2014 study by Petersen and colleagues, four genes encoding members of the sterol regulatory pathway (SREBF2, S1P, S2P, and SCAP) were enriched for disrupting integrations well above any other genes (23) and the 2015 screen performed by Kleinfelter *et al.* reported that these exact same four genes were also their top hits, and that three other genes involved in cholesterol biosynthesis (*LSS*, *SQLE*, and *ACAT2*) were the next most frequently disrupted (24). This identification of multiple members of a biological pathway has been seen in many of the aforementioned haploid screens, and it not only demonstrates the high level of mutagenesis coverage in the libraries that have been generated thus far, but it also increases the confidence that screening hits are biologically relevant.

The haploid screening technique is not without drawbacks. Due to the nature of disrupting mutagenesis in a haploid genetic background, this screening strategy is unlikely to identify host factors that are required for cell viability. Additionally, most haploid screens have relied upon cell death as a phenotypic read-out, a decision that greatly increases the throughput of the screen but that may prevent the identification of a gene whose disruption produces an intermediate phenotype in which virus infection is delayed or partially suppressed. We find it interesting that in a number of the published screens a single biological pathway is clearly identified by virtue of multiple retroviral

gene insertions to the near exclusion of other hits. In the two Andes virus haploid screens (23, 24), cells that survived the viral challenge almost invariably had one of several genes involved in cholesterol biosynthesis disrupted, and in the RVFV haploid cell screen we performed, genes contributing to glycosaminoglycan synthesis and Golgi complex function were mutated in the surviving pool almost to the exclusion of any other mutations. In contrast, RNAi screens often implicate several biological pathways as being important for viral replication, as did the RVFV RNAi screen published by Hopkins *et al.* Variables that could impact the results of haploid cell screens could include the multiplicity and timing of infection as well as the length of time cells are cultured after virus challenge. Finally, most haploid screens have utilized mutant libraries generated in the human haploid cells HAP1, a line derived from the KBM-7 chronic myeloid leukemia cell line, which restricts its use to pathogens that are capable of entering these cells, as well as introducing an element of complexity due to cell-type-specific variations that have been observed in entry mechanisms and pathway use of viruses and toxins.

Interrogating host-pathogen protein-protein interactions through Y2H, AP/MS, or proximity labeling makes it possible to identify host factors based upon the a priori association of a pathogen protein and a cellular protein within the biological context of the host cellular environment. Many of the common phenotypic read-outs used during viral screening techniques, such as production of a reporter protein or host cell death, have the distinct disadvantage of restricting host factor discovery to those which impact a specific subset of stages during the viral replication cycle. High-throughput screens to identify cellular factors required for viral assembly and egress, for example, have proven difficult to design, and screens to identify host factors required for viral infections have largely focused on the rate-limiting stages of entry and replication. Another important

advantage to protein-protein interaction screening is that it allows for the identification of host factors whose depletion or disruption may be cytotoxic, or even lethal. On the other hand, antibodies to affinity purify a pathogen protein are not always available, and the introduction of a tag or the precipitation conditions may perturb protein function or have other unforeseen consequences.

The use of multiple complementary screening techniques can serve to address and overcome the varying advantages and disadvantages presented by using each of the techniques on their own. Performing multiple screens in parallel can help eliminate false-positive hits, even if the differences between the screens are relatively subtle technical changes such as use of different viral strains, cell types, or siRNA libraries. With each new published screen, the pool of datasets available to draw from also increases, which will allow for valuable comparisons of one's screening results with the reported hits from other related screens.

HAPLOID GENETIC SCREENING: LESSONS LEARNED

Many factors influence the outcome of haploid screens, and during the projects described here, as well as other screens done in our lab or in collaboration with the Bates lab, I have learned some lessons about the design of these screens. The generation of the mutant library is the first point of strategy, as care should be taken to limit expansion of the cells after lentiviral mutagenesis. The reason for this is that mutations introduced by this process can have dramatic effects on the growth rate of the cells, and this leads to an outgrowth of fast-growing mutants and a loss of slow-growing mutants.

Another factor that greatly impacts screen design is the nature of the selective agent being used to challenge the library. I have used toxins, pseudoviruses, and live viruses for screening, and different screening strategies are required for each. Toxins and pseudoviruses are both replication-incompetent, meaning that multiple challenges may be necessary in order to obtain sufficient selection. Challenge dose must be carefully titrated ahead of time, in order to be able to screen efficiently (without needing to apply too many rounds of selection). Screening with live virus offers a mix of advantages and disadvantages. The primary advantage is that live virus screening allows you to probe all steps of the viral life cycle, as opposed to just the early entry events that are mediated by the viral glycoprotein (which is what screening with a pseudovirus does). Additionally, live viruses give you greater confidence that hits are biologically meaningful, and not an artifact of the pseudovirus structure. One major disadvantage to live virus screening, though, is the lack of control over multiplicity of infection (MOI) during the screen. For example, if you apply virus to your library of 10^9 mutant cells at an MOI of 0.1 plaque-forming units (PFU) / cell initially, you can expect to infect and kill ~ 10% of your cells. Then, if your virus replicates in 12 hours, with a burst size of 1000 new virions per infected cell, that means that by the time your screening plates have been allowed to sit in selection overnight, the MOI has changed from 0.1 PFU/cell to 1000 PFU/cell – quite a different challenge being encountered by the cells!

This idea of carefully controlling the MOI during the screen is important because if the MOI is too high (such as the MOI of 1000 PFU/cell that can result after one round of viral replication, as mentioned above) then you will lose intermediate phenotypes that may be biologically meaningful and interesting. A gene whose disruption renders that cell resistant to virus at a 10-fold higher level than wild-type cells, for example, could

have a meaningful impact during viral infection, but this cell would succumb to viral challenge at the MOI of 1000 PFU/cell, and would therefore not be pulled out as a hit in such a screen.

On the other hand, if your MOI (or challenge concentration, in the case of toxins) is too low, then the result is a decrease in the signal-to-noise ratio due to mutant cells that survive challenge in a non-specific manner (that is to say, they never actually encountered the selective agent). This is of particular concern due to the differences in growth rate among the cells in the mutant library. There are many genes (often well-described oncogenes) that encode nuclear proteins whose loss results in a hyperproliferative phenotype, and these genes have appeared as “hits” across multiple screens. Although I have not formally tested this, I suspect that the reason we pull these genes out as hits from unrelated screens is that their disruption increases the survival of these mutant cells in a manner unrelated to the selective agent, owing merely to their increased rate of replication (and resultant over-enrichment in the selected pool). This is supported by the fact that I have seen a greater representation of such genes among the hits from screens done with toxin (which lasted almost a month) than with screens done with live virus (which last a week at most) because the longer screening duration would allow for a greater over-representation of these hyperproliferative mutants. For this reason, it’s incredibly important to balance completeness of selection with minimizing the duration of selection.

RECENT ADVANCES IN GENETIC SCREENING TECHNIQUES

The hunt for host-pathogen interactions going forward will be greatly aided by many exciting developments in loss-of-function screening technology. In addition to the

human HAP1 cell line, haploid cell lines have been generated from fish, mouse, monkey, and rat embryonic stem cells (25–29). A fully haploid human cell line has also been derived by genome editing using the clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided endonuclease Cas9 to excise the fragment of Chromosome 15 that was integrated onto Chromosome 19 and was preventing the HAP1 cell line from being fully haploid (30). This updated cell line, termed eHAP, will likely replace the HAP1 line in the generation of new mutagenesis libraries.

CRISPR technology has now also been applied to high-throughput functional genomic screening. This DNA-editing technique was adapted from the type II CRISPR bacterial adaptive immune system in which the endonuclease Cas9 is recruited to the DNA of invading pathogens by two RNA components: a CRISPR RNA (crRNA) that contains a DNA fragment complementary to the foreign target, and a trans-activating CRISPR RNA (tracrRNA) which acts as a scaffold. The crRNA and tracrRNA can be fused to form a single guide RNA (sgRNA), greatly simplifying the process of synthesizing and delivering custom CRISPR/Cas9 machinery in order to disrupt a gene of interest. The Cas9-induced cleavage triggers the cell's double-strand break repair response, leading either to indel mutations, or (if supplied) the introduction of a sequence of interest. For a detailed technical review of CRISPR/Cas systems and their utility for genome engineering, see reference (31).

Generation of sgRNA libraries providing genome-wide targeting by CRISPR/Cas9 has opened the door to a new method of high-throughput screening to identify host factors required by pathogens. In one recent study, a CRISPR sgRNA library was used to identify genes required for the induction of cell death by West Nile virus (32). In another, the *Staphylococcus aureus* toxin α-hemolysin was used to

challenge a genome-wide CRISPR sgRNA library, and uncovered a role for sphingomyelin synthase 1 (SGSM1), which regulates lipid raft formation (33). Lentiviral vector delivery of the sgRNAs and the Cas9 endonuclease have been developed, and are being optimized for efficient delivery (34).

THE NEXT GENERATION OF BIOCHEMICAL SCREENING TECHNIQUES

To identify potential cellular interacting partners of viruses and toxins, it is now also possible to circumvent the requirement that proteins associate strongly enough with the bait protein that they can be pulled down by the (T)AP/MS techniques. Martell and colleagues introduced in 2012 a new genetically-encoded reporter molecule that can be used for both electron microscopy as well as proximity labeling followed by MS to detect nearby proteins (35, 36). The authors engineered a monomeric variant of ascorbate peroxidase, which they have termed APEX, that is active in all cellular compartments (including the cytosol), a major advantage over the horse radish peroxidase (HRP) tag typically used. This APEX tag can oxidize biotin-phenol (in the presence of a hydrogen peroxide catalyst) into phenoxyl radicals, and these short-lived radical species react with electron-rich amino acids present in proteins that are fewer than 20 nm away. This results in the biotin-labeling of endogenous proteins adjacent to the APEX-tagged protein of interest, and these can be identified by streptavidin purification followed by digestion and MS analysis. An improved version of this peroxidase, termed APEX2, was recently obtained by yeast display evolution and exhibits increased activity, stability, and sensitivity (37).

Another proximity-labeling approach developed in 2012 by Roux *et al.* is named proximity-dependent biotin identification (BioID) and it employs a promiscuous mutant of

the *E. coli* biotin ligase BirA fused to a bait protein of interest (38). As with the APEX labeling technique, neighboring proteins that have been biotinylated within the cell can be affinity purified and identified. BioID has been used to better characterize the constituents and architecture of the nuclear pore complex and to identify the interactome of the Ewing sarcoma fusion oncoprotein EWS-Fli-1 (39, 40). This approach has also been used to study host-pathogen interactions during bacterial and viral infection. Mojica and colleagues fused the BioID BirA to SINC, a type III secreted effector from *Chlamydia psittaci*, and showed that it targets the nuclear envelope of both infected and neighboring cells (41). In 2015, Le Sage *et al.* used HIV-1 Gag protein fused to BioID to identify 47 associated proteins that were biotinylated by the fusion protein when it was transfected into Jurkat cells (42). Two of the putative host factors identified, DDX17 and RPS6, were validated as interacting partners of Gag by co-immunoprecipitation experiments. A substantially smaller biotin ligase, BioID2, was recently described to have higher activity and to improve the function and localization of the resultant fusion protein (43). These new proximity-labeling technologies represent exciting additions to the screening toolbox.

NEW BUNYAVIRUS TECHNICAL RESOURCES

Recent advances in bunyavirus research have greatly expanded the options available for generating bunyavirus reporter systems to enable high-throughput or automated screening. Among orthobunyaviruses, a replication-competent recombinant BUNV has been generated bearing a fluorescent or V5 tag on either Gc or L, respectively (44, 45). In 2013, reverse genetics was described for Schmallenberg virus (SBV) and in 2015 a BHK cell line was developed that constitutively expressed the SBV

N protein and a minigenome system was described for Oropouche virus (46–48). Efficient reverse genetics has also now been established for Akabane virus, further expanding the options for bunyavirus screening approaches (49). For the phlebovirus RVFV, there exists both a reverse genetics toolset as well as a BHK replicon cell line expressing the S and L segments of the genome (50, 51). We and others have also utilized pseudovirion systems, described in (52) and (53), in order to screen for host factors required during entry of bunyaviruses. These pseudotyped virions can be used at the BSL2 level and allow for the convenient use of either cell death or a genetically-encoded reporter (e.g. luciferase or a fluorescent protein) to facilitate high-throughput, cell-based screening approaches.

EXPANDING CELLULAR TARGETS

In addition to the screening techniques focused on genes and proteins, there has been renewed interest in developing high-throughput approaches to identify metabolites and lipids that are involved in viral infection. Analysis with LC-MS can be used to quantify changes in the metabolomic profile of infected cells relative to uninfected cells, providing insight into pathogen alteration of host metabolism as well as yielding potential therapeutic targets. This approach was used to quantify the levels of known metabolites at different time points during infection with human cytomegalovirus (HCMV), herpes simplex virus type-1 (HSV-1), and influenza A (IAV), demonstrating each virus's ability to differentially remodel the host's metabolism during infection (54–56). In the case of HCMV and IAV, pharmacological inhibition of fatty acid biosynthesis was shown to effectively restrict viral replication, demonstrating the power of such screens to inform the development (or re-purposing) of therapeutics. In 2013, Morita and colleagues

tested a library of bioactive lipids for an effect on IAV replication, and observed potent inhibition with the lipid mediator protectin D1 (PD1) (57). Treatment with PD1 was able to protect against influenza in a mouse model, even if it was not supplied until severe disease had developed.

Another important aspect of host-pathogen dynamics that could be examined is that of interactions between RNA and proteins during viral infection. Yeast three-hybrid screening provides a powerful tool for identifying proteins that bind to a specific RNA sequence. This technique, first described by SenGupta and colleagues (58), detects RNA-protein interactions by utilizing two hybrid proteins whose proximity activates a reporter gene when both proteins bind to a hybrid RNA molecule. Yeast three-hybrid screening was used to identify human ribosomal proteins that bind to the 3' untranslated region of hepatitis C virus (HCV) using a human cDNA library as prey and the viral RNA sequence as bait (59). Covalent UV crosslinking during infection could also be used to capture and characterize the RNA-protein interactome in a manner similar to the technique described by Castello *et al.* in 2012 (60).

COMMON THEMES OF VIRUS AND TOXIN ENTRY

Haploid genetic screening, as well as most of the other screening modalities discussed in this thesis, can be used to probe for host factors utilized by a variety of pathogens. Although our lab historically has studied interactions between viruses and host cells, I decided to do a screen using *C. difficile*'s TcdB toxin because early entry events during virus and toxin invasion of host cells share many common themes.

The initial attachment of the bunyavirus glycoprotein to a cell-surface receptor, the internalization of the virion as a result of this interaction, and the entry of the virion

into the endocytic pathway of the cell are mechanistically similar to the binding and internalization of many bacterial toxins. Bunyavirus glycoproteins likewise mediate fusion of viral and cellular membranes within acidified endosomes, in a manner that echoes the pore formation activity of TcdB toxin. Indeed, many virus entry studies employ virus-like particles (VLPs), often containing only the glycoprotein, to examine early entry events. This highlights the parallels between glycoprotein-driven virus entry and entry of toxins.

Studies of virus entry and toxin entry can inform each other on both the host as well as the pathogen side. Characterization of the cellular endocytic machinery, for example, informs (and is informed by) our understanding of host factors required by the viruses and toxins that hitchhike within the endolysosomal pathway as they enter the cell. Many technical tools used for virus entry assays also have the potential to be applied to toxin entry studies. For example, the fusion of β-lactamase protein to HIV Vpu provides an excellent tool to assay fusion of the virus at the plasma membrane. In this assay, the cell is loaded with a fluorescent dye cleavable by the β-lactamase protein, and cleavage of the dye therefore indicates that the virion contents have gained access to the cytosol (aka fusion has occurred). I have adapted this assay in our lab to look at fusion of bunyavirus pseudovirions from within endosomes, and it could be nicely applied to study TcdB toxin pore formation. For this purpose, the β-lactamase protein would need to be fused to the N-terminus of the toxin, as it is the glucosyltransferase domain that is translocated through the endosomal membrane and then cleaved off into the cytosol.

Other classical virus entry assays could be useful when looking at entry of toxins, such as acid-bypass experiments in which the pH is lowered in order to drive fusion at

the plasma membrane and bypass the requirement for receptor-mediated endocytosis. Likewise, many of the reagents that we learn about when studying virus entry have also been used to look at toxin entry, especially those that inhibit endocytosis pathways. These tools include lysosomotropic agents; inhibitors of clathrin-mediated endocytosis, dynamin, macropinocytosis, and caveolae; expression of dominant-negative versions of cellular proteins such as Rab and Rho GTPases; and cytoskeletal drugs targeting actin and microtubules; as well as many others. Because viruses and toxins rely on much of the same cellular machinery to enter the host cell, studies looking at the early entry events of viruses and toxins are really not so philosophically disparate at all, especially in their roles as probes to uncover novel elements of these fundamental cell biology processes.

FUTURE PERSPECTIVES

The screens presented here demonstrate the utility of unbiased forward genetic screening to identify host cellular factors used by the bunyavirus Rift Valley fever virus (RVFV) and the large clostridial glucosylating toxin TcdB. The pathways hit by these screens include heparan sulfate biosynthesis, which is required for attachment of RVFV at the cell surface, and endosomal transport protein complexes, which play a role in the cytotoxicity of TcdB. Many important questions remain about the exact role of the WASH complex and the CCC complex in TcdB entry, as well as the possible significance of other hits from the TcdB screen such as SNX17, HOOK2, and RAB10. These factors all have demonstrated roles in the regulation of endosomal transport, but it is unclear how their known functions may (or may not) impact TcdB entry and trafficking. Toxin resistance phenotypes of mutations in these genes may be due to indirect effects,

such as loss of expression of a receptor at the cell surface. Since TcdB has been shown to use multiple receptors in a cell-type-dependent manner, it is possible that there exists an as-yet-unidentified proteinaceous receptor that plays a role in its entry into HAP1 cells, and whose expression (e.g. via recycling) is dependent upon the proteins and complexes that were hit in this screen. Preliminary immunofluorescence imaging studies suggest that there is a toxin trafficking defect in WASH-deficient cells, and that toxin may remain associated with EEA1-positive endosomes in the absense of a functional WASH complex, but this remains to be conclusively demonstrated. One intriguing possibility is that the WASH complex is required for pore formation of TcdB due to a role in lysosomal maturation, which has been observed in amoeba cells (61). Because an acidic pH triggers the conformational change in the toxin that allows for translocation of the glucosyltransferase and cysteine protease domains, a defect in endosomal acidification is a likely explanation for the cytotoxicity defect observed in WASH-deficient cells. Introduction of a fluorescent tag to track the subcellular localization of the glucosyltransferase domain of the toxin would be a logical next step in this study, and I am very interested in performing these and other follow-up experiments to see where this project will lead.

The haploid genetic screening techniques used for the studies presented in this thesis, as well as the other screening approaches discussed, are powerful tools for the investigation of host-pathogen interactions. As we grapple with emerging viral diseases and enter the age of antibiotic-resistance, it is becoming increasingly important to identify factors required by viruses and toxins as they invade the host cell. Dramatic innovations in recent years of high-throughput screening techniques promise to push

forward our understanding of how pathogens interact with their host cells, and will help us to develop targeted therapeutics.

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