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Hunting Viral Receptors Using Haploid Cells

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Abstract

Viruses have evolved intricate mechanisms to gain entry into the host cell. Identification of critical receptors has enabled insights into virus particle internalization, host and tissue tropism, and viral pathogenesis. In this review we discuss the most commonly employed methods for virus receptor discovery, specifically highlighting the use of forward genetic screens in human haploid cells. The ability to generate true knockout alleles at high saturation provides a sensitive means to study virus-host interactions. As an example, haploid genetic screens identified the lysosomal proteins, NPC1 and LAMP1, as intracellular receptors for Ebola virus and Lassa virus, respectively. From these studies emerges the notion that receptor usage by these viruses is highly dynamic involving a programmed switch from cell surface receptor to intracellular receptor. Broad application of genetic knockout approaches will chart functional landscapes of receptors and endocytic pathways hijacked by viruses.

Keywords

virus entry; receptor identification; genetic screens; Ebola virus; Lassa virus

Introduction

Viruses have developed unique mechanisms to breach the cell surface barrier in order to initiate a viral infection. They use elaborate tactics to enter the intracellular environment, commandeering host cell factors for this purpose. A functional entry receptor classically mediates the attachment of virus particles to the host cell surface and actively facilitates internalization. However, not all viruses follow this course. Some viruses make use of abundantly expressed surface proteins to initially attach to cells, but require a different receptor to enter cells. Other viruses depend upon co-receptors in addition to a primary entry receptor to facilitate virus entry. Despite the use of different entry strategies, virus-host receptor interactions are highly specific and are critical in establishing a viral infection.

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The identification of virus receptors expands our knowledge of virus entry and provides insights into the complexities of cellular, endocytic pathways. It also elucidates genetic determinants of viral infection in the host. Naturally occurring polymorphisms in receptor genes are likely to contribute to the susceptibility of individuals to viral infections. In human populations, positive natural selection of polymorphisms in viral receptor genes has been demonstrated for multiple viruses, underscoring their importance in viral pathogenesis ((1; 2)). More direct evidence comes from the strong protective effect against human immunodeficiency virus type-1 (HIV-1) infection in individuals carrying homozygous polymorphisms in C-C chemokine receptor type 5 (CCR5), a co-receptor for HIV-1 (3; 4).

Virus receptors are key determinants of tissue- and host-tropism. Receptor usage can determine cross-species transmissibility and act as a host barrier, preventing zoonotic infection by emerging viruses. This is beneficial in that it protects us from pathogenic animal viruses, however, it sometimes complicates the development of a small animal model due to receptor incompatibility. Receptor identification can therefore be valuable in creating susceptible animal models that allow us to further study viruses and test antiviral therapeutics. Poliovirus (PV) (5) and hepatitis C virus (HCV) (6) are prime examples of this phenomenon, in which knowledge of the receptor(s) was instrumental in the development of mouse models that recapitulated the complete viral life cycles.

Technological advances have produced powerful techniques that have improved sensitivity and precision in virus receptor identification, allowing us to understand some of the more complex virus entry mechanisms. The identification of several viral receptors has illuminated the sophisticated, molecular mechanisms by which viruses have evolved to gain access to the cell’s cytoplasm, and in so doing provided opportunities to further our understanding of viral pathogenesis. This review presents an overview of the experimental approaches frequently utilized to identify viral receptors, including biochemical and genetic strategies. The main focus is on genome-scale, mammalian, haploid genetic approaches that achieve complete knockout phenotypes, a technique with unique benefits in the study of virus-host interactions.

Biochemical and immunological approaches

Classical biochemical approaches exploit the high affinity interactions between the viral capsid and its cellular target in order to identify the virus receptor(s). The discovery of receptors for some of the world’s most pathogenic, emerging viruses including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and the New World arenaviruses viruses, Machupo virus and Junin virus, were made using their viral glycoproteins as ‘bait’ to isolate cellular binding partners (7–9) (Table 1). Coronaviruses like SARS-CoV and MERS-CoV derived their name from the typical crown-like appearance under electron microscopy. Their viral surface glycoproteins (appropriately named “spike” or ‘S’ proteins) are readily visible as protrusions from the viral particles and mediate the interaction between the viral particle and the cell. Hence, in an effort to identify the receptor for SARS-CoV, Li et al (7) fused part of the ectodomain of the viral S protein to the Fc domain of human immunoglobulin, and then performed an immunoprecipitation in the presence of a cell lysate from cells that are highly

susceptible to SARS-CoV. A cellular protein co-immunoprecipitated and was identified as carboxypeptidase angiotensin-converting enzyme 2 (ACE2) by mass spectrometry. ACE2, a membrane-associated enzyme, was found to mediate membrane fusion triggered by the viral S protein, facilitating viral entry. Many lines of evidence further established that ACE2 is the principal entry receptor utilized by SARS-CoV with significant implications for cross-species infections (reviewed in (10)). More recently a similar approach was used to identify dipeptidyl peptidase 4 (DPP4) as the functional receptor for MERS-CoV (8) within months after the initial emergence of the virus in Saudi Arabia in 2012 (11). These studies, amongst others, demonstrate the power of using the immunoprecipitation and mass spectrometry strategy for virus receptor identification. However, this approach requires prior knowledge of the virus's binding moiety in order to create the 'bait' molecule.

In the absence of this knowledge, other, more classical methods can be applied. One such approach involves screening large collections of monoclonal antibodies (mAbs) raised against cellular membrane proteins, to identify those mAbs that block or neutralize the infection of a specific virus in tissue culture. Perhaps the most well-known receptor identified through this neutralizing monoclonal antibody approach is CD4, a key receptor required for HIV-1 infection (12; 13). The restricted tropism of HIV-1 for immune cells prompted Dalgleish and colleagues (12) to determine if a selection of 155 well characterized mAbs (raised against human leukocyte differentiation antigens) would inhibit HIV-1 infection. Of those tested, all 14 anti-CD4 antibodies successfully blocked syncytia formation and HIV-1 (pseudotyped) infection in vitro. Together with a concurrent study by Klatzmann et al (13), this discovery made a tremendous impact in our understanding of HIV pathogenesis and allowed for further detailed studies on HIV entry. In the above case, the choice of antibodies was dictated by HIV tropism but the mAb approach has also been applied in a more unbiased manner. Random screening of over 2000 hybridomas from mice immunized with cell preparations of HeLa cells identified one mAb that blocked Rhinovirus infection (14). The specificity of this and other similar antibodies helped fortify the notion that despite the remarkable diversity in rhinovirus serotypes (~100 serotypes; (15)), the majority share the same receptor, intercellular cell adhesion molecule-1 (ICAM-1) (16; 17).

A commonly used biochemical tool employed to hunt for virus receptors is the virus overlay protein blot assay (VOPBA). This technique involves electrophoresis of purified fractions of cell membrane proteins from a susceptible cell line, followed by blotting the separated proteins onto a nitrocellulose membrane, and then "probing" the blotted membrane with intact virus (18). Sequential column chromatography of the purified cell membrane fractions or mass spectrometry analysis is then used to identify the putative receptors. A constraint of VOPBA is that the denaturing conditions of SDS-PAGE on host proteins can affect whether virus particles will bind to the cellular receptor on the blot. However, if the receptor polypeptide that interacts with the virus is exposed correctly, the receptor can be identified. This was aptly demonstrated by the discoveries of α-dystroglycan (α-DG) as the receptor of lymphcytic choriomeningitis virus (LCMV) and Lassa virus (19), and nucleolin as the respiratory syncytial virus (RSV) receptor (20).

Genetics-based screens

Genetic screens have revolutionized the means by which we can study virus-host interactions, dramatically enhancing our capacity to identify essential cellular factors involved in virus propagation, and allowing the decipherment of complex molecular mechanisms that viruses use in a successful infection. The types of screens are comprised of gain-of-function and loss-of-function strategies, described in more detail below.

Complementary DNA libraries

A particularly effective gain-of-function approach to identify viral receptors is to transduce a complementary DNA (cDNA) library derived from a susceptible cell line into an otherwise non-permissive cell line. Only those cells that express a functional receptor will be productively infected, allowing for subsequent isolation and identification. In pioneering studies, the poliovirus receptor was identified by a genetic gain-of-function approach involving genomic DNA transfections and screening of cDNA libraries by in situ hybridization (21). Studies using cDNA expression libraries played an integral role in unravelling the many players involved in the elaborate mechanism of entry of HCV. An initial report in 1998 identified CD81, a tetraspanin family member, as the critical entry receptor of HCV (22). A cDNA expression library screening approach indirectly assessed virus-binding efficiency by using HCV's major envelope protein (E2) as a probe. Later studies showed that while CD81 is required for entry, it was not sufficient to mediate efficient internalization (23). Moreover, there was a poor correlation between CD81 expression and HCV infectivity of susceptible versus non-permissive cell lines (24; 25). In 2002, another receptor, scavenger receptor type B class 1 (SR-B1), was found to be important for HCV entry through a series of biochemical analyses including immunoprecipitation (26). New evidence now suggests that the HCV sites required for binding to CD81 are only exposed after HCV-SR-B1 interaction (27), and that HCV-CD81 binding results in a glycoprotein conformational change (28), allowing lateral movement of the virus to areas of cell contact (29). Intriguingly, even with the expression of CD81 and SR-B1, there were still some cell lines resistant to HCV entry. Evans et al (30) delved further into solving the HCV entry puzzle, by performing a cyclic, lentivirus-based repackaging screen of a cDNA library derived from the highly HCV-permissive Huh-7.5 (hepatocarcinoma) cell line. An HCV-non-permissive, SR-B1^{+} CD81^{+} cell line (293 human embryonic kidney cells) was then transfected with the latter library, and infected with HIV-1 particles pseudotyped with HCV glycoproteins. Claudin-1, a tight junction protein, was found to contribute to the later stages of the HCV entry process, acting downstream of CD81 binding (30). Another junction-associated protein, occludin, was also identified as a critical factor required for HCV to enter cells, again using a cyclic, retrovirus-based repackaging cDNA library screen in an HCV-non permissive mouse fibroblast cell line (NIH3T3 cells) overexpressing CD81, SR-B1 and claudin-1 (31). Since this study, the transferrin receptor (32), epidermal growth factor receptor and ephrin receptor A2 (33) have all been implicated as proteins that contribute to efficacious HCV entry and internalization. While the mechanism of HCV entry is incredibly complex and is still not fully understood (reviewed in (34; 35), these studies have greatly contributed to our understanding of how HCV is internalized. Other examples that highlight how cDNA expression library strategies have

been utilized to identify important viral receptors are mentioned in Table 1, and include the discoveries of junction adhesion molecule-1 (JAM1) and signalling lymphocytic activation molecule family member 1 (SLAMF1) for reovirus and measles virus respectively (36; 37).

Although a robust technique, there are some drawbacks to using cDNA library-based screens. Firstly, the cDNA library created is biased by the non-uniform distribution of mRNAs isolated at the time of purification. This therefore limits the opportunity to identify a receptor if its expression level is low. Secondly, the approach relies on the availability of cell lines that are non-permissive to viral infection or attachment. This can impede the identification of receptor(s) for viruses with wide tissue tropism.

Microarray data and Bioinformatics

Differential expression analysis has been utilized extensively to study the modulation of host gene expression during a virus infection. A recent study employed the wealth of gene expression data present in databases in an innovative way to identify a new measles receptor (38). Although SLAMF1 acts as a receptor in macrophages and dendritic cells, the receptor in epithelial cells was not known. Muhlebach et al (38) found several potential receptor candidates in respiratory epithelial cell lines after comparative bioinformatics analyses of susceptible versus non-permissive cell lines, with selection based on high expression ratios and biological characteristics. These candidates were cloned and transfected into a non-permissive cell line followed by MV infection to determine which candidate(s) could render cells susceptible to MV. The adherence junction protein, Nectin-4, emerged as the only candidate able to do so, engaging MV in primary respiratory epithelial cells as a means to exit the host by crossing the airway passage barrier after propagation in lymphoid tissues. A similar differential expression approach was used to identify T-cell Ig and mucin domain 1 (TIM-1) as a cell surface receptor for Ebola virus (39). Although it is currently not a common technique to identify virus receptors, the growth and improved accessibility of databases containing gene expression data could accelerate this line of research.

RNA interference-based screening technology

The discovery and application of RNA interference (RNAi) has allowed for loss-of-function genetics in mammalian cells. In high-throughput genome-wide screens for host factors, a plethora of candidate genes that may play roles in the life cycle of different viruses, including HIV-1 (40), have been identified. Specifically regarding the identification of receptors, a genome-wide RNAi screen for the Sindbis alphavirus performed in Drosophila cells (41) revealed important entry factors. From the 9 transmembrane genes identified in the screen, Drosophila’s natural resistance-associated macrophage protein (dNRAMP), was chosen for functional validation because it was conserved, ubiquitously expressed and plasma membrane-associated. Like dNRAMP, a human homolog, NRAMP2, was capable of mediating binding and entry of Sindbis virus in human cells, suggesting that Sindbis uses a conserved receptor to establish infection across disparate hosts.

RNAi technology has yielded insights into virus-host interactions on a large-scale as comprehensively described by Cherry (42). However, partial gene depletion and the potential of off-target effects are limitations of this approach (43; 44).

Haploid genetic screening approach

The diploid nature of somatic mammalian cells makes it impractical to perform forward genetics due to the inefficiency of creating true genetic knock-outs (i.e. deleterious mutations in both alleles of a gene). In contrast, yeast models are pragmatic and valuable genetic tools to answer questions relating to eukaryotic biology, primarily due to the ability to set up crosses and the capacity of yeast to exist in haploid form during its natural life cycle. The haploid form allows the study of recessive genetic phenotypes that are often masked in diploid cells (45). Yeast models have been applied to understanding host-pathogen interactions. The discovery that yeast supported the translation, transcription and RNA replication of the alpha-virus-like plant virus, brome mosaic virus (BMV) (46), paved the way for the use of yeast genetics to dissect virus-host interactions. Later, Kushner et al identified almost 100 host genes involved in BMV replication using an ordered array of yeast deletion mutant strains covering ~80% of yeast genes (47). Amongst other findings, yeast genetics uncovered that host mRNA decapping factors play an important role in translating BMV RNAs and recruiting them from translation to RNA replication (48; 49). Furthermore, Lee and colleagues found that genes involved in unsaturated fatty acid synthesis are required for replication of BMV (50). This approach yielded valuable insights into RNA replication of BMV as well as two other viruses, also shown to replicate in yeast (tomato bushy stunt virus (51), and flockhouse virus (52)). However, this approach cannot be used to probe virus entry and, more importantly, the majority of human viruses do not replicate in yeast, restricting its broad application.

Haploid mammalian cells

Mammalian somatic cells consist of diploid genomes with pairs of chromosomes, allowing for the masking of any dysfunctional or lethal mutations found on individual alleles that may occur during development. Diploidy ensures the fitness of individuals, however it limits our ability to study mammalian gene function, given the difficulty of specifically manipulating the same gene on both alleles (Figure 1a). In an effort to overcome this, Guo and colleagues made use of Bloom's syndrome protein (Blm)-deficient embryonic stem cells, which display a high rate of mitotic recombination (53). In these cells it is possible to make a genome-wide library of homozygous mutant cells generated from heterozygous mutations. However, these libraries still contain an abundance of heterozygous mutations, reducing the efficiency of this method.

Clearly, haploid human cells would greatly facilitate somatic cell genetics. Human cancer cell lines, perhaps the most used "model organisms", display chromosome aberrancies ranging from near-haploid to hyperdiploid. In 1995, Anderson et al established a heterogeneous cell line called KBM-7 from the bone marrow of a chronic myeloid leukaemia patient (54), that was a mix of near-haploid and hyperdiploid cells. A stable, near-haploid sub-clone line was later derived from early passages of this cell line (55), potentially facilitating somatic cell genetics to study biological processes relevant to human disease.

KBM-7 cells are of a hematopoietic lineage, non-adherent and haploid for all chromosomes except Chromosome 8, which is present in two copies. In an attempt to reprogram KBM-7 cells into near-haploid induced pluripotent cells, the transcription reprogramming factors,

OCT4, SOX2, c-MYC, and KLF4 (56), were expressed in KBM-7 cells (57). Although this did not lead to full pluripotency, the derived cell line lost typical blood cell markers, became adherent and also lost the second copy of chromosome 8. This newly generated cell line was named HAP1 and fortuitously proved susceptible to infection by a wider variety of viruses than its predecessor KBM-7 (57; 58). More detailed analysis using single-nucleotide polymorphism array data revealed that HAP1 cells are still not fully haploid as they retain two copies of a fragment of chromosome 15, one of which is fused to chromosome 19. A final modification to the HAP1 cells was performed recently to render a new cell line (engineered-haploid or eHAP cells) that is completely haploid (59). This involved the use of clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) technology (60; 61) to excise the duplicated 30 mega-base fragment that encompassed 330 genes, creating an entirely haploid cell line. The new cell line has the potential to be an even more potent tool for screening purposes.

In addition to human haploid cells, two groups individually derived haploid mouse embryonic stem (ES) cells from parthenogenic mouse blastocysts (62; 63). The resulting haploid ES cell population was enriched via multiple rounds of fluorescence-activated cell sorting (FACS), exhibited a typical mouse ES cell colony morphology, and could be used in forward genetic screens. These cells produce viable and fertile progenies after intracytoplasmic injection into mature oocytes, allowing new tools for the generation of genetic models for recessive traits (64–66). Haploid ES cells have since been generated from rat and monkey (67; 68). To date, these cells have not been used for purposes of identifying virus receptors, however they have been applied to studying toxins (62) and chemotherapeutic compounds (69; 70), and have the potential for utilization in virus-host interaction studies (Table 2).

Implementation of haploid genetic screens

Similar to forward genetic screens in model organisms such as yeast, the mammalian haploid screening approach allows genome-scale cell libraries (containing null alleles in practically all non-essential genes) to be generated using a form of mutagenesis (71; 72). This is followed by phenotypic selection, PCR-based mapping of the sites of mutation in the selected population, and determination of significance of enrichment compared to an unselected control dataset (Figure 1b and c).

For random insertional mutagenesis, the haploid cells are transduced with a retroviral gene-trap vector (71). This vector contains a strong splice acceptor site, an efficient polyadenylation signal and a marker gene (usually *GFP*). These elements ensure transcriptional termination when inserted in intronic (or exonic) regions resulting in inactivation of the gene. Due to the haploid nature of the cells, gene disruption with the retroviral vector results in the generation of null mutants. It is important to note that insertion by the retroviral gene trap is not entirely random and has a clear bias for insertion around active promoters (73). This bias is advantageous for forward genetic screens because integration near the 5’end of genes leads to complete knockouts and the preference for transcribed genes reduces noise caused by integration outside genes. A comparison with gene expression data and the mapping of insertions sites from roughly 1% of mutagenized

cells showed that approximately 98 % of the expressed genes contained insertions. The mutation rate of genes that are classified as “marginally expressed” is only slightly lower with ~90% of these genes containing insertions (72). The collective data inferred that the full library comprised of mutations in nearly all genes, including those that are lowly expressed. A similar retroviral-based mutagenesis strategy in haploid mouse ES cells showed comparable mutation rates (62).

Other forms of mutagenesis include transposon-based insertional mutagenesis, which has been successfully employed in haploid ES cells (63; 70; 74), and chemical mutagenesis. The latter relies on a chemical mutagen such as N-ethyl-N-nitrosourea (ENU), and has the potential to efficiently, and with reduced bias, mutagenize the haploid genome (75). In addition, it can create alleles with unique properties such as thermo-sensitive alleles, active site mutants or partially functional mutants. The site of mutation is not marked by a common element, as is the case with insertional mutagenesis, thus PCR-based methods cannot be applied to map mutation sites created by chemical mutagens. Although this is a major drawback, rapid advances currently being made in deep sequencing have the potential to overcome this hurdle.

Once the haploid genome has been mutagenized, selection involves exposure of the genome-scale library to a selection agent based on lethality or reporter gene expression. This leads to a positive selection for those cells that carry loss-of-function mutations in genes important for the biological process under study. To comprehensively map the insertions sites in the phenotypically-selected population, the sites of insertion are specifically amplified, deep sequenced and mapped to the genome. The number of independent insertions present in the selected population is determined for each gene and compared with the mutation frequency of the particular gene in the control population that has not undergone selection. A significance of enrichment is calculated allowing for the identification of candidate genes that are important for the biological process being evaluated. The versatility of the approach has been demonstrated by the identification of critical genes involved in anti-cancer drug action, ER-associated degradation, pathogen manipulation of the host immune system, phosphatidylserine exposure during apoptosis and bacterial and viral infection (see Table 2 and references herein).

Genetic dissection of host-pathogen interactions

Application of the genetic screening technology in human haploid cells has focused on host-pathogen interactions. These interactions can be modelled in human tissue culture systems, are often species-specific, and complete knockout helps in revealing their phenotype. When using bacterial toxins or cytopathic viruses, phenotypic-enrichment is conveniently achieved by selecting for mutants that survive the lethal insult. The use of reporter-based selection strategies has also recently been explored, and offers the potential to study the effects of biological or chemical agents that do not cause cell death (76).

Using the haploid genetic screening approach in human haploid, KBM-7 cells, an initial screen was performed to find host factors critical to diphtheria toxicity (71; 72). The diphtheria receptor, heparan-binding EGF-like growth factor receptor (HBEGF) was identified as the most significant hit in the screen, reinforcing the notion that cellular

receptors act as major rate limiting factors. The other significant hits were the diphthamide biosynthesis genes DPH1, DPH2, DPH4 and DPH5. Diphthamide is a unique posttranslational modification on a residue present only in eukaryotic elongation factor 2 (eEF2). This diphthamide residue is ribosylated by diphtheria toxin, causing inactivation of eEF2, and ultimately cell death. The biosynthetic pathway that converts histidine into diphthamide has been extensively studied using yeast genetic screens, initially leading to the identification of five genes (named DPH1-5) required for the formation of diphthamide ((77) and references herein). In addition to the genes known to play a role diphtheria toxicity, a previously uncharacterized gene (WDR85) was also identified in the haploid screen. WDR85 contains domains that often mediate protein-protein interactions.

WDR85 (later renamed to DPH7) was shown to be required for efficient ribosylation of eEF2 in human cells. Unlike eEF2 isolated from wild type cells, eEF2 isolated from WDR85 mutant cells showed a strong association with DPH5. Subsequent studies with the yeast homolog of DPH7 confirmed this observation and demonstrated an unexpected role of DPH7 in catalysing demethylation of a previously unknown intermediate, leading to a revised scheme of the diphthamide biosynthesis pathway (78–80). Thus, genetic screens in human haploid cells provides the opportunity to discover novel factors involved in host-pathogen interactions, even in biological systems that have been extensively scrutinized in genetically tractable model organisms.

Several haploid genetic screens investigating host factors that interact with bacterial toxins demonstrated that this screening approach is well suited to identify receptors important for entry. Novel candidate receptors were found for different members of the cytolethal distending toxin (CDT) family. One of these candidates with a predicted membrane localization (TMEM181), was shown to bind in vitro to purified *E. coli*–derived CDT and was rate limiting for intoxication, indicative of a putative receptor (71). Another novel toxin receptor was identified in a screen to study the host factors involved in toxicity caused by the *Clostridium difficile* transferase (81). Lipolysis-stimulated lipoprotein receptor (LSR) was the strongest significantly enriched gene from the latter screen. Purified ectodomain of LSR directly bound toxin, and in a competitive inhibition assay, prevented intoxication of VERO cells. Gain-of-function studies in non-permissive cell lines showed heightened sensitivity to toxin treatment and increased cell surface binding of toxin when LSR was overexpressed. After its identification as the cellular receptor for *C. difficile* transferase, LSR was found to also act as the receptor for two other toxins secreted by members of the *Clostridium* genus: *C. perfringens* iota toxin and *C. spiroforme* toxin (82). Additionally, a similar genetic approach in HAP1 cells identified low-density lipoprotein receptor-related protein 1 (LRP1) as a host cell receptor of *C. perfringens* TpeL toxin (83). The (re)discovery of host receptors for viruses such as influenza and reovirus further exemplified the utility of the haploid genetic approach to hunt down receptors, and initiated the interest in exploring virus entry (72).

Discovery of novel virus receptors

The first haploid genetic screen to identify a previously unknown virus receptor investigated the cellular entry of Ebola virus. As a model for Ebola virus entry, replication-competent

vesicular stomatitis virus (VSV) carrying the Ebola virus glycoprotein (rVSV-GP-EboV) was used (84–87). Cellular entry was dependent on the provided glycoprotein, while the cytolytic nature of VSV ensured stringent selection. Similar VSV-based Ebola vaccines have been used extensively to study virus entry and are under development as vaccine candidates (84; 88). To identify genes important for Ebola virus entry, mutagenized HAP1 cells were exposed to rVSV-GP-EboV (57). Most of the significantly enriched genes that were identified in the screen encoded genes with known functions in endosomal/lysosomal trafficking, including all six members of the HOPS complex that mediate endosome maturation and fusion to the lysosome (89). Cells that carried knockout mutations in the HOPS components, VPS11 or VPS33, were shown to be resistant to rVSV-GP-EboV but not wild-type VSV or eight other cytolytic viruses. These other viruses included influenza A virus which is known to travel through the endocytic route and enters the cytosol from late endosomes. This suggested that Ebola virus entry is dependent on the establishment of a different late endosomal / lysosomal compartment (LE/Lys) compared to influenza A. A subsequent study confirmed this notion and showed that trafficking to LE/Lys is a crucial rate-defining step for Ebola virus entry (90).

Interestingly, the single most significant hit in the haploid genetic screen for Ebola virus was Niemann–Pick C1 (NPC1), encoding a cholesterol transporter that is localized in LE/Lys. This receptor was critically required for Ebola infection, independent of its cholesterol transporting function. NPC1-loss led to the accumulation of viral particles in intracellular structures and prevented fusion of the viral membrane with the endosomal membrane, indicating that NPC1 is required for Ebola virus to release its RNA genome into the cytosol. Validation experiments with wild type Ebola virus confirmed the importance and specificity of NPC1 in the Ebola life cycle in different cell types including human fibroblasts from patients who lack functional alleles in the NPC1 gene, and human peripheral blood monocyte-derived dendritic cells. NPC1-knockout mice, in contrast to wild-type mice, were resistant to lethal challenge of mouse-adapted Ebola and Marburg virus (57).

In an independent study, NPC1 was identified as critical for Ebola virus infection through a chemical screening approach (91). It was shown that a potent, antiviral drug targeted NPC1, and that this drug interferes with Ebola virus glycoprotein binding to NPC1. Further research reinforced the concept that NPC1 acts as an internal receptor for Ebola by fine-mapping the interaction domains, showing a dependence of the Ebola-NPC1 interaction on proteolytic cleavage of the viral glycoprotein (via cathepsin proteases), and demonstrating that human NPC1 allows infection of otherwise non-permissive reptilian cells (92). Together, these studies suggest a hypothetical model (Figure 2a) in which Ebola virus is internalized and travels through the cell via an endocytic pathway. As the endosome matures, resident cathepsin proteases cleave a heavily glycosylated domain from the surface glycoprotein of the virus. Endosome-lysosome fusion, mediated by the HOPS complex, then allows the virus particle to interact with NPC1. We speculate that this interaction triggers fusion of the viral lipid membrane with the host endosomal membrane allowing viral RNA release into the cytosol.

The haploid genetic screen elucidated a unique entry mechanism for Ebola virus and implicated an unlikely candidate for a receptor, due to its unusual subcellular localization.

Since this study, other filoviruses including Marburg virus and Lloviu virus have been shown to use of NPC1 as a receptor, suggesting a universal function of NPC1 for filoviruses (93). The identification of NPC1 along with other essential genes involved in Ebola virus internalization emphasizes the robustness and sensitivity of the haploid genetic screening approach to detect essential host factors in an unbiased fashion.

This is further confirmed in a recent study of Lassa virus, an Old World arenavirus that causes Lassa fever, a severe viral hemorrhagic disease. Jae et al (58) performed a genome scale haploid genetic screen for host factors critical for Lassa virus entry using VSV pseudotyped with Lassa glycoprotein. Although α-DG has long been accepted as the cell surface receptor of Lassa virus (19), host tropism of the virus is not fully explained by α-DG expression. Chicken cells, for example, express functional α-DG yet are resistant to experimental Lassa virus infection (94).

With this knowledge, the haploid screen (58) was set up in both wild type HAP1 cells and HAP1 cells lacking α-DG. Lysosomal-associated membrane protein 1 (LAMP1), an abundant protein component of the lysosomal membrane, was found to be an essential factor for Lassa virus entry. In vitro binding experiments showed that at a neutral pH, Lassa virus glycoprotein was tightly bound to α-DG as expected. However, when the pH was lowered to 5.5 (lysosomal pH), a receptor switch occurred whereby Lassa virus glycoprotein lost binding to α-DG and instead now strongly associated with LAMP1. Experimental re-routing of LAMP1 to the cell surface using a previously described mutant (95) concurrent with expression of Lassa glycoprotein triggered massive pH-induced syncytia, suggesting that LAMP1 interaction is imperative for promoting membrane fusion between the viral membrane and endosomal membrane.

LAMP1 is a heavily glycosylated protein, but during Lassa virus infection only 1 of the 11 N-linked glycosylation sites is indispensable for entry. Strikingly, this glycosylation site is conserved amongst species sensitive to Lassa but absent in birds, presenting a host barrier for inter-species transmission. In vivo relevance of the above observations was demonstrated by showing that LAMP1 knockout mice are resistant to wild-type Lassa virus. Overall, this study suggests a model (Figure 2b) where Lassa virus is first incorporated into the endocytic pathway by interaction with its cell surface receptor α-DG. It then traffics along the endocytic route in an increasingly acidic environment. In the lysosomal compartment, Lassa virus disengages from α-DG and interacts with its intracellular receptor, LAMP1. This interaction triggers membrane fusion and cytosolic release.

Identification of non-receptor host factors required for viral entry

Besides direct identification of key receptors, the haploid genetic screens also pinpoint critical dependencies for entry such as posttranslational modifications, cholesterol biosynthesis and endosome/lysosome function. For Lassa virus, the haploid genetic screen revealed a suite of proteins involved in the biosynthesis of the dystroglycan moiety that is installed on the α-DG protein (Figure 2b). In this case, Lassa virus served as a sensitive probe for this posttranslational modification and consequently several of the genes identified overlapped with all known genes that cause Mendelian dystroglycanopathies when mutated (96). The robustness of this approach also allowed identification of novel genes (including

TMEM5 and SGK196) that subsequently were shown to explain a subset of patients with the Walker-Warburg syndrome dystroglycanopathy (96; 97). In an approach similar to the Ebola and Lassa virus screens, a haploid genetic screen was performed to identify host factors important for entry of a new world hantavirus (Andes virus) (98). The most significantly enriched genes included all four members of the major cellular sterol regulatory pathway (SREBF2, SCAP, S1P and S2P), firmly implicating cholesterol synthesis and uptake as critical for Andes virus infection. The results were confirmed in other cell types and pharmacological inhibition of SP1 also blocked Andes virus glycoprotein-mediated infectivity. Identification of the Achilles heel in Andes virus infection may have therapeutic implications for development of antiviral drugs because clinically approved regulators of sterol synthesis are available.

It is worth noting that the host cell factors required for virus entry varied significantly amongst the three viruses above, despite their common utilization of endocytic pathways; Lassa virus required several glycosylation factors, Ebola virus required host factors involved in proper maturation and fusion of endosomes and lysosomes, and Andes virus required cholesterol biosynthesis factors (98). Technical variation between the screens is minimal because all used the same cell type and the same recombinant VSV backbone. This indicates that viruses of different families have evolved to use specialized endocytic entry routes making them sensitive to disruption of different subsets of genes.

Collectively, the above-mentioned studies demonstrate that haploid genetic screens provide a versatile and unbiased way to study host-virus interactions. Identification of viral receptors not only illuminates critical pathogenesis mechanisms, it also contributes significantly to our understanding of how viruses have evolved to exploit defined molecular components of cellular biological processes. However, some limitations exist. These screens are confined to the use of a restricted set of haploid cell lines (human cell lines (KBM-7, HAP1), mouse, rat and monkey embryonic stem cells). A broad range of phenotypes can be studied in these haploid cells (see table 2) and findings validate well in other cell types or in small rodent models. However, certain phenotypes cannot be studied because they require the use of cell lines derived from particular tissues. For example HCV exclusively infects hepatocytes. Another consideration is that although complete knockout generally gives strong and reproducible phenotypes, genes that are essential for growth in a tissue culture dish cannot be functionally probed. These genes are likely enriched for ribosomal subunits, RNA splicing, DNA transcription and other housekeeping genes. Their importance for maintenance of basic cellular functions complicates analysis using any loss-off-function approach.

Intracellular virus receptors: escape from the belly of the beast

A striking feature of the receptors identified for Ebola and Lassa virus is that the interaction does not occur at the cell surface, as is the case for many other functional receptors, but instead occurs intracellularly in the endocytic compartments. The interaction is “programmed” to occur after a conformational switch triggered by cathepsin cleavage (for Ebola virus) or low pH (for Lassa virus). It is likely beneficial to delay this receptor-virus interaction until after entering the endocytic pathway to prevent premature exposure of

conserved receptor-binding domains on the virus particle, as these are often the target for extracellular, neutralizing antibodies (99–101). Initial binding and entry into the cell is not dictated by these intracellular receptors. Indeed, other cell surface receptors have been identified that are important for this step: α-DG for Lassa virus and multiple plasma membrane surface-expressed proteins (e.g., C-type lectins, DC-SIGN, integrins, TIM-1, Axl) for Ebola virus ((102) and references herein). These attachment receptors appear to display some redundancy in terms of their necessity in a viral infection. In the absence of α-DG, cells become more dependent on heparan sulphate biosynthesis for Lassa virus entry (58), and cells that do not express DC-SIGN or TIM-1 are still permissive for Ebola virus infection (39). In contrast, the intracellular receptors, NPC1 and LAMP1, are critically required for entry into multiple cell types and in mouse models for Ebola and Lassa virus infection respectively (57; 58). Other studies lend support to an emerging notion that endocytic receptors play crucial roles in pathogen entry through specific interactions that are not initiated at the cell surface. For example, after Shiga toxin recognizes its cell surface receptor, the glycosphingolipid globotriaosyl ceramide, it travels via retrograde transport through the Golgi apparatus and to the endoplasmic reticulum before being released into the cytoplasm (103). This release was convincingly shown in a recent study to be dependent on a Golgi-resident protein, GPP130, which acts as an intracellular receptor that binds directly to the B-subunit of shiga toxin in the exit process (104). Importantly, manganese treatment, which induces degradation of GPP130, potently inhibited lethal Shiga toxicity in tissue culture and in vivo, inferring that identification of intracellular receptors may lead to treatments for pathogen infections. This is particularly relevant in light of the Ebola outbreak of 2014 in West Africa. The World Health Organization (WHO) estimated over 21,000 cases due to this epidemic, with as many as 8,000 deaths (105). Currently, there is no approved antiviral treatment or vaccine against Ebola (106). Small molecule inhibitors of NPC1 show promise in tissue culture models for Filovirus infection, perhaps providing a host target amendable to pharmaceutical inhibition (57; 91; 107; 108).

Perspectives

Emerging and re-emerging viruses threaten global human health on a continuous basis, necessitating flexible and rapid methods to assess what host factors are requirements for infection. Haploid genetic screens add a comprehensive, flexible and time- and cost-effective approach to identify critical host factors that complement existing biochemical and genetic approaches. Complete gene knockout rather than knockdown of protein levels results in strong phenotypes allowing for stringent screens. In addition to this, sensitivity of the screens is ensured by the high rate of mutagenesis per gene (often exceeding a hundred independent insertion events), which reduces background noise. Genome-scale knockout approaches probe host-factor requirements in an unbiased fashion, and since entry by a viral receptor is a distinct rate-limiting step in viral infections, these genes often surface as prominent hits in genetic screens. The examples mentioned above show that new (intracellular) receptors can be identified even for viruses that have been studied extensively and have attachment factors that are well characterized. Applying these approaches to a multitude of different viruses will provide a functional landscape of endocytic routes that viral entry relies on. This will require a concerted effort to test a broad range of viruses from

different families. The recent development of CRISPR/Cas9 genome editing technology (reviewed in (109; 110)) will allow knockout screens to be performed in a wider range of cell lines. Questions remain. Would viruses from different families have evolved to use common endocytic routes? Will these routes be amendable to pharmaceutical inhibition to create broad-spectrum antiviral drugs? Will the screens reveal that other viruses show dependencies that are not related to viral entry, but rather viral translation of viral genome replication?

As with any genetic approach, partial redundancy in a pathway can obscure identification of important host factors. For example, in the above-mentioned screen for Lassa virus entry (58), the abundance of hits involved in the biosynthesis of the dystroglycan moiety complicated analysis of its other protein receptors. Only after genetically disabling α-DG (DAG1 knockout cells) and repeating the screen in this sensitized genetic background, did the intracellular receptor, LAMP1, rise to prominence. In the absence of α-DG, heparan sulphate served as an alternate attachment factor for Lassa virus. Thus, although the functional receptor is likely to be identified in wild type background, modifier screens such as the latter show potential in identifying partial redundant host pathways that are exploited by viruses to complete their life cycle. In this way, similar strategies that have been successfully used in classical genetic model systems such as Drosophila and yeast can further refine the forward genetic approach using haploid human cells. These refinements will sharpen the tools used in the hunt for viral receptors to the advancement of our understanding of virus biology.

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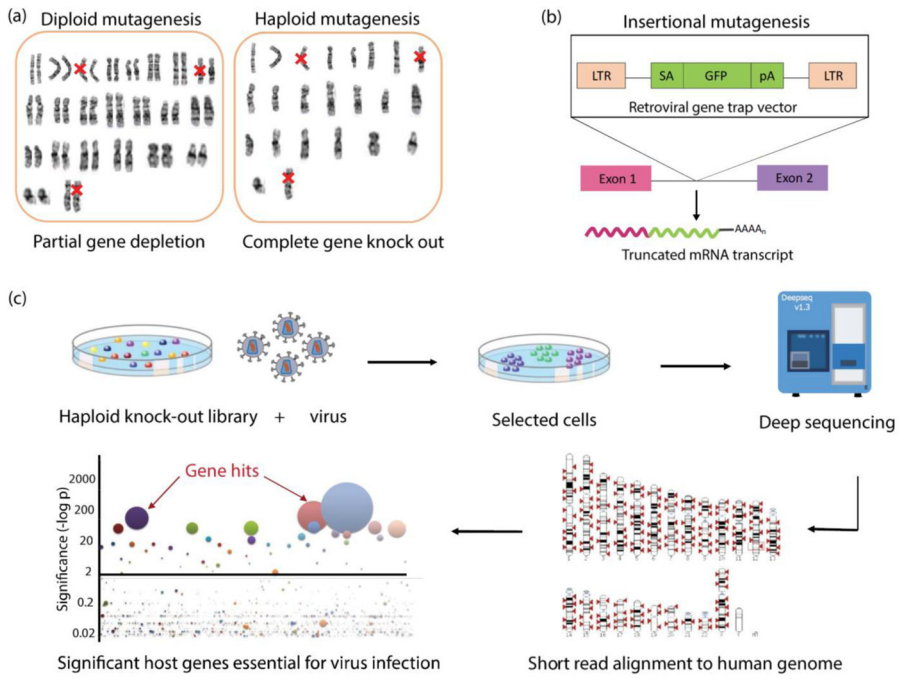

Figure 1.

Overview depicting the application of haploid cells in forward genetic screening approaches. (a) Mutagenesis of a diploid genome results in heterozygous mutations, which are often phenotypically masked by expression from the remaining wild type allele. In contrast, mutations in haploid cells allow complete genetic knock-out of the gene of interest. (b) In the human haploid genetic screening approaches, retroviral gene trap vectors carrying a splice acceptor site, reporter gene and polyadenylation signal, integrate randomly into the genome of exposed cells. When inserted into intronic (or exonic) regions, the vector disrupts transcription, creating a truncated mRNA transcript, which often results in genetic knock-out. (c) Schematic diagram depicting the steps involved in performing a haploid genetic screen. Haploid cells are randomly mutagenized using a retroviral gene trap vector to create a screening library of high complexity. Cells with mutations in genes that are critical to viral infection are phenotypically selected by infecting with a cytolytic virus of interest. Genomic DNA of the virus-resistant pool is used to PCR amplify DNA flanking the retroviral insertion sites. After deep sequencing and alignment to the human genome, the number of independently generated insertions per gene is counted. These counts are compared to a control dataset derived from cells that are not phenotypically selected. Statistical analysis yields a significance of enrichment value for each gene and these values are plotted to visualize host genes that are deemed essential for virus infection.

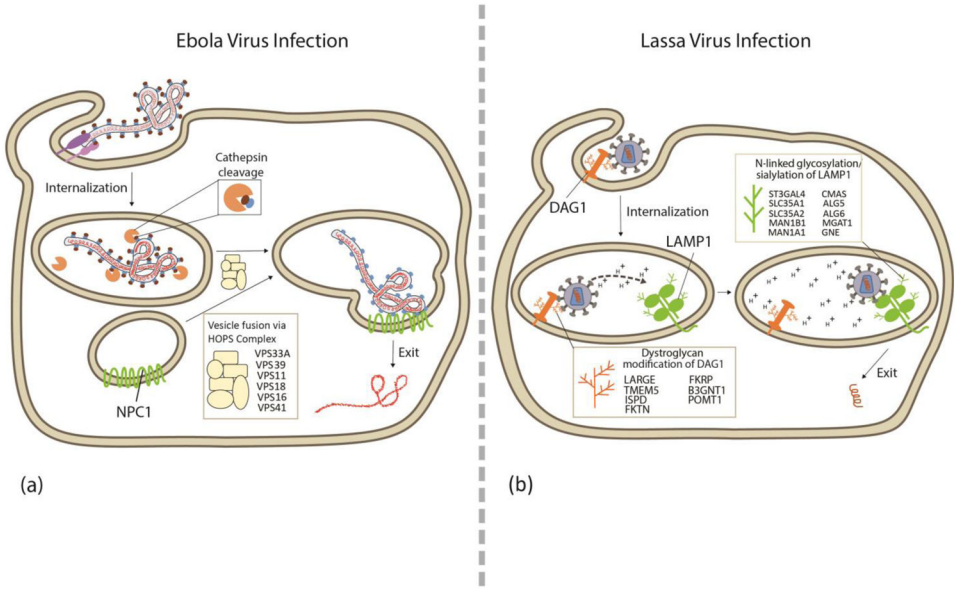

Figure 2.

Haploid genetic screens reveal viral entry mechanisms via critical intracellular receptors. (a) Ebola virus can be internalized into the endocytic pathway via a number of cell surface receptors. As the endosome matures, resident cathepsin proteases initiate cleavage of a heavily glycosylated domain from the surface glycoprotein of Ebola. Endosome fusion, mediated by the HOPS complex, generates an NPC1 containing endosomal compartment where further cleavage occurs. Interaction of NPC1 then likely triggers fusion of the viral lipid membrane with the host endosomal membrane to allow viral RNA release into the cytosol. (b) Lassa virus is internalized into the endocytic pathway by its cell surface receptor alpha-dystroglycan (DAG1). Acidification of the endosome triggers a receptor switch (dashed arrow) where Lassa virus disengages from alpha-dystroglycan and engages with its intracellular receptor LAMP1. This interaction enables membrane fusion and cytosolic release of viral RNA. Correct dystroglycan glycosylation of DAG1 and N-linked glycosylation of LAMP1 is critical for viral entry as underscored by the near-complete identification of genes in distinct biosynthetic routes of these glycans. Every gene indicated by a gene symbol was found as a significant hit in the screens.

Table 1

An overview of experimental approaches used to identify receptors of viruses

Virus	Receptor	Method of identification	Reference
HIV-1	CD4	mAb	(12; 13)
Rhinovirus	ICAM-1	mAb	(16; 17)
Poliovirus	PVR	cDNA library	(21)
Echoviruses 6, 7, 12 and 21	DAF	mAb	(111)
Adenovirus-5/Coxsackie virus B	CAR	mAb/cDNA library	(112)
Hepatitis C	CD81	cDNA library	(22)
Lassa virus	α-DG	VOPBA	(19)
Measles	SLAM-F1	cDNA library	(37)
Reovirus	JAM1	cDNA library	(36)
Hepatitis C	SR-B1	IP	(26)
SARS	ACE2	IP & MS	(7)
New world arenaviruses	TFR1	IP & MS	(9)
Hepatitis C	Claudin-1	cDNA library	(30)
Hepatitis C	Occludin	cDNA library	(31)
Respiratory Syncytial virus	Nucleolin	VOPBA	(20)
Sindbis	nRAMP	siRNA screen	(41)
Measles	Nectin-4	Microarray/bioinformatics analyses	(38)
Ebola	TIM-1	Bioinformatic analyses	(39)
Ebola	NPC1	Haploid screen	(57)
Hepatitis B & D	NTCP	Advanced purification & MS	(113)
MERS	DPP4	IP & MS	(8)
Lassa virus	LAMP1	Haploid screen	(58)

Table 2

An overview of genetic screens performed in haploid mammalian cells

	Biological process	Selection agent	Cell system	Significant genes identified	Reference
Host pathogen interactions	Influenza virus infection	Influenza A	KBM7 cells	*CMAS, SLC35A2*	(71)
	Ebola virus infection	rVSV-GP-EboV*	HAP1 cells	*NPC1, HOPS* complex (*VPS33A, VPS11, VPS18)*	(57)
	Lassa virus infection	rVSV-GP-LassaV*	HAP1 cells	*LARGE, ISPD, FKTN, FKRP, POMT1, POMT2, DPM3, C3orf39, B3GNT1, SGK196, TMEM5, PTAR1, ST3GAL4, B3GALNT2*	(58)
	Reovirus infection	Reovirus	KBM7 cells	*JAM1*	(72)
	Herpesvirus immune modulation	KSHV-encoded K5 ubiquitin ligase	KBM7 cells	*PLP2*	(114)
	Cholera infection	Modified cholera toxin	KBM7 cells	*ST3GAL5, SLC35A2, B3GALT4, UGCG, ELF4*	(115)
	C. difficile transferase toxicity	*C. difficile* transferase	HAP1 cells	*LSR*	(81)
	Chlamydia trachomatis infection	C. trachomatis L2	HAP1 cells	*B3GAT3, B4GALT7, SLC35B2*	(116)
	Cytolethal distending toxin(CDT) toxicity	*E. coli* CDT	KBM7 cells	*TMEM181, SGMS1*	(71; 72)
	Ricin resistance	Ricin	Mouse haploid ESCs	*Gpr107*	(62)
Anti-cancer drug action	BCR-Abl tyrosine-kinase inhibition	Imatinib	KBM7 cells	*PTPN1, PTPN12, LZTR1*	(72)
	Nucleoside metabolic inhibition	Decitabine	KBM7 cells	*DCK*	(72)
	DNA repair inhibition	Olaparib	Mouse haploid ESCs	*Parp1*	(70)
	Therapeutic glycolysis inhibition	3-bromopyruvate	KBM7 cells	*SLC16A1, BSG*	(69)
	Chemotherapeutic small molecule cytotoxicity	YM155	HAP1 cells	*SLC35F2*	(117)
Other	TRAIL-dependent apoptosis	TRAIL	KBM7 cells	*CASP8, FADD*	(71)
	RIP3-kinase apoptosis pathway	RIP kinase 3 overexpression	HAP1 cells	*RIPK3, CASP8, FADD, c-FLIP, RIPK1, BID*	(118)
	NFκB negative regulators	NFκB reporter cell line	KBM7 cells	*CYLD, HEATR7A, LRRC8A, LRRC8D*	(119)
	Secretory vesicle transport	Brefeldin A	KBM7 cells	*ARF4, TRAPPC13*	(120)
	Tunicamycin entry	Tunicamycin	KBM7 cells	*MFSD2A*	(121)
	Exit from embryonic stem cell (ESC) self-renewal	Two GSK3 inhibitors (2i)	Mouse haploid ESCs	*Zfp706, Pum1*	(122)
	Mismatch repair pathway	Nucleotide analog 6-thioguanine (6-TG)	Mouse haploid ESCs	*Msh2*	(63)

* rVSV-GP-EboV or -LassaV – recombinant vesicular stomatitis virus pseudotyped with Ebola or Lassa virus glycoproteins