A Functional Genomics Approach to Henipavirus Research: The Role of Nuclear Proteins, MicroRNAs and Immune Regulators in Infection and Disease

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Abstract Hendra and Nipah viruses (family *Paramyxoviridae*, genus *Henipavirus*) are zoonotic RNA viruses that cause lethal disease in humans and are designated as Biosafety Level 4 (BSL4) agents. Moreover, henipaviruses belong to the same group of viruses that cause disease more commonly in humans such as measles, mumps and respiratory syncytial virus. Due to the relatively recent emergence of the henipaviruses and the practical constraints of performing functional genomics studies at high levels of containment, our understanding of the henipavirus infection cycle is incomplete. In this chapter we describe recent loss-of-function (i.e. RNAi) functional genomics screens that shed light on the henipavirus–host interface at a genome-wide level. Further to this, we cross-reference RNAi results with studies probing host proteins targeted by henipavirus proteins, such as nuclear proteins and immune modulators. These functional genomics studies join a growing body of evidence demonstrating that nuclear and nucleolar host proteins play a crucial role in henipavirus infection. Furthermore these studies will underpin future efforts to define the role of nucleolar host–virus interactions in infection and disease.

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1   Introduction of Henipaviruses

1.1   Discovery and Classification

Paramyxoviruses (order *Mononegavirales*) are single-stranded RNA viruses of negative polarity that can cause diseases in humans (rabies, measles virus, mumps virus, respiratory syncytial virus, human parainfluenza virus, Ebola virus) and animals (Newcastle disease virus, canine distemper virus, borna disease virus). The family *Paramyxoviridae* is divided into two subfamilies (*Paramyxovirinae* and *Pneumovirinae*), with Hendra virus (HeV) being the foundation member of the genus *Henipavirus* in the subfamily *Paramyxovirinae*. The discovery of the HeV and Nipah virus (NiV) had a striking impact on our understanding of paramyxovirus biology. Henipaviruses have a much wider host range and a significantly larger genome than other paramyxoviruses, and to date are the only biosafety level (BSL)-4 agents within the family. With mortality rates of human infection between 50 and 100%, HeV and NiV are among the most deadly viruses known to infect humans.

HeV emerged in 1994 in the Brisbane suburb of Hendra, Queensland, Australia, where it caused an outbreak of severe respiratory disease in horses that led to the natural death or euthanasia of 14 out of 21 affected animals. Two people who had close contact with the infected horses were infected and one of these patients died

(Murray et al. 1995). Extensive sampling demonstrated that Australian mainland flying foxes (family *Pteropodidae*, genus *pteropus*) were seropositive for neutralising antibodies against HeV (Young et al. 1996), while the virus was subsequently isolated from flying fox uterine fluid and urine (Halpin et al. 2000), providing strong evidence for Australian mainland flying foxes as the HeV reservoir. Sporadic HeV incidents occurred in horses between 1994 and 2010, with 14 events identified. An alarming number of HeV incidents (34 in total) occurred between 2011 and 2013, with 18 of those occurring in 2011 alone, highlighting the unpredictable nature of HeV outbreaks. Seven human cases of HeV disease have been observed, four of which resulted in fatal disease. All recorded cases of HeV transmission to humans have occurred directly from affected horses. The horses are believed to have acquired HeV infection following direct exposure to secretions from flying foxes. More recently, the decline of reported human cases of HeV infection is potentially due to the development of a vaccine to inhibit HeV disease in horses (Middleton et al. 2014).

NiV was first identified during a disease outbreak on the west coast of Peninsular Malaysia in late 1998. Commercial pig farmers suffered disease characterised by febrile encephalitis that was linked to mild respiratory and neurological disease in pigs (Mohd Nor et al. 2000; From the Centers for Disease Control and Prevention 1999). Nucleotide sequencing demonstrated the virus was closely related to HeV, whilst fruit bats of the *Pteropodidae* family, *Pteropus* genus, were confirmed as the natural reservoir (Yob et al. 2001). Epidemiological evidence suggested that human infections were caused by transmission from pigs which likely had prior contact with fruit bats (Update: outbreak of Nipah virus–Malaysia and Singapore 1999). By mid-1999, cases of human infection were reported in Singapore, where abattoir workers developed NiV infection associated with contact with pigs imported from Malaysia. This initial outbreak of NiV in Malaysia resulted in 265 human cases reported with 105 deaths. Since 2001, NiV outbreaks have been reported almost every year in selected districts of Bangladesh (Hossain et al. 2008; Luby et al. 2009a). Unlike HeV, human-to-human transmission of NiV has been documented (Luby et al. 2009b), including in a hospital setting.

An increasing focus on flying foxes as viral reservoirs has led to the discovery of new henipaviruses. The genus was expanded in 2012 upon the isolation and characterisation of Cedar virus (CedPV), isolated from bat urine samples from a flying fox colony in Cedar Grove, South East Queensland. CedPV shows a remarkably similar genome organisation to HeV and NiV, antigenic cross-reactivity of the nucleocapsid protein between henipaviruses, and shares the same predominant entry receptor molecule, ephrin-B2 (Marsh et al. 2012). However, a critical difference between CedPV and HeV and NiV is that the CedPV P gene lacks coding capacity for the immune antagonising V protein, whilst the CedPV P protein shows an impaired capacity to bind and inhibit IFN signalling via signal transducer and activator of transcription (STAT)1 and STAT2 (Lieu et al. 2015). Accordingly, CedPV infection induces a robust type I interferon (IFN) response in human cells in vitro and does not cause clinical disease in ferret and guinea pig models of disease. Such findings highlight the importance of immune evasion in the context of henipavirus pathogenicity and demonstrate the diverse range of pathogenicity within the same genus.

1.2 Natural Reservoir and Other Novel Henipaviruses

In addition to these three viruses, the henipavirus genus is likely to be expanded in the future to accommodate the discovery and characterisation of emerging viruses from bats and other reservoirs. West African fruit bats harbour neutralising antibodies against HeV and NiV in particular, demonstrating a wider geographical range for henipaviruses not limited to pteropid bats (Hayman et al. 2008). Furthermore, a novel henipa-like virus, Mojiang paramyxovirus, was isolated from rats in the Yunnan Province of China in 2012 and may have caused fatal disease in three individuals (Wu et al. 2014). Alarmingly, a recent study looking at bat and human serum samples from Cameroon found that 3–4% of human samples were seropositive for henipaviruses, and that this was almost exclusively among individuals who reported butchering bat meat, providing the first evidence of human henipavirus spillover infections in Africa (Pernet et al. 2014).

2 Functional Genomics Analysis of Henipavirus Infection

2.1 Platforms for Functional Genomics and Challenges for Studying BSL-4 Pathogens

There are currently no licensed therapies to treat human cases of henipavirus infection. Therefore, gaining a deeper understanding of host pathways exploited by henipaviruses for infection may identify targets for new antiviral therapies. Viruses rely on the cell host machinery for completion of their infection cycle and therefore have adapted to interact with or exploit host molecules. Retroviruses, most DNA viruses, and many orthomyxoviruses replicate their genomes in the host nucleus. Conversely, most positive-sense single-stranded viruses such as picornaviruses and flaviviruses and negative-sense, single-stranded viruses such as filoviruses, rhabdoviruses, and paramyxoviruses are perceived as cytoplasmic viruses and therefore are believed to not have a nuclear stage in their life cycle, replicating their genome entirely in the cytoplasm (Lamb and Parks 2007). However, proteins of some of these viruses can traffic into nuclear compartments during infection (Peeples 1988; Yoshida et al. 1976; Ghildyal et al. 2003; Monaghan et al. 2014; Wang et al. 2010) and this movement is sometimes critical for efficient infection (Wang et al. 2010). This evidence indicates that the host nucleus may play a significant role in the infection cycle of henipaviruses and that the dynamics of virus–host interactions that occur in the nuclear compartments is an understudied area of molecular biology and virology. Furthermore, since important discoveries in cell biology often follow studies of how viruses exploit normal host machinery, investigations into these nuclear interactions may reveal interesting novel insights into the cell biology of the mammalian nucleus. With this in mind, functional genomics provides a powerful and unbiased approach to study these biological questions.

Functional genomics refers to the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by sequenced genomes (Hieter and Boguski 1997). A wide range of laboratory techniques can be considered as functional genomics, including genome interaction mapping (at the DNA level), microarrays, transcriptomics and serial analysis of gene expression (SAGE) (at the RNA level), yeast 2 hybrid systems and affinity chromatography and mass spectrometry (at the protein level) and loss-of-function studies such as mutational studies, RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR) studies. Functional genomics has demonstrated much power in its ability to dissect the dynamic interplay between host and viral factors during a virus infection, paving the way for novel drug targets. For instance, a haploid genetic screen resulted in the discovery of the once elusive entry receptor for Ebola virus (Carette et al. 2011). There have been many full- or partial-genome RNAi screens of host–virus interactions, including orthomyxoviruses (Brass et al. 2009; Hao et al. 2008; Karlas et al. 2010; Konig et al. 2010; Shapira et al. 2009), retroviruses (Zhou et al. 2008; Konig et al. 2008; Brass et al. 2008) and flaviviruses (Ang et al. 2010; Sessions et al. 2009). Until recently, such information was lacking for henipaviruses, and perhaps surprisingly, for paramyxoviruses generally.

Functional genomics screens can be technically challenging, laborious and involve the use of robotics and advanced imaging equipment. Consequently there are technical and practical challenges to performing high-throughput screens at higher levels of containment. HeV and NiV are classified at BSL-4 agents due to their association with lethal human disease and the absence of preventive measures and effective treatments to combat infections. BSL-4 facilities feature additional precautions to protect workers from infections and prevent exposure, such as infectious work being conducted within class II biosafety cabinets, limited access by secure, locked doors, HEPA filtration of laboratory air, and additional primary containment (positive pressure air suits or class III biosafety cabinets). Due to these limitations, previous genome-wide screens for BSL-4 viruses used surrogate viruses, such as pseudotyped particles, and have been performed under BSL-2 conditions (Kouznetsova et al. 2015; Kleinfelter et al. 2015).

2.2 Functional Genomics Studies on Henipaviruses

Functional genomics have been employed to study henipavirus infection. For instance, the entry receptor of HeV and NiV, ephrin-B2, was identified by microarray analysis of infection-permissive and infection-resistant cell lines (Bonaparte et al. 2005). Transcriptomics and proteomics have been utilised to uncover key differences in cellular responses to HeV infection in HeV disease-susceptible (human) and disease-resistant (bat) cells, and suggest that activation of apoptosis pathways via the innate immune pathway may contribute to the tolerance of henipaviruses by flying foxes (Wynne et al. 2014). Here we largely

focus on findings from two recent RNAi screens to identify protein-coding genes and host-encoded microRNAs impacting the henipavirus infection cycle in human cells. Not only can these findings be compared to published RNAi screens of host–virus interactions, but the identification of host genes required for infection (as opposed to those that are merely differentially expressed during infection) may deliver new targets for the development of antiviral therapies.

The large number of HeV incidents in Australia from 2011 to 2013 prompted researchers at our laboratory to establish the capability to perform genome-wide RNAi screens at BSL-4. Central to this work was the development of a recombinant HeV expressing the renilla luciferase construct, which allowed for high throughput and rapid measurement of virus infection (Marsh et al. 2013). This recombinant virus was shown to be lethal in the ferret model of henipavirus disease and exhibited a pathogenesis profile comparable to the wild-type virus. Functional genomics at high containment also required the establishment of protocols and/or safe work procedures for the operation and decontamination of liquid handling robots.

3 The Reliance of Henipavirus Infection on Nuclear and Nucleolar Proteins

3.1 Genome-Wide RNAi Screening

A genome-wide analysis of host protein-coding genes required for henipavirus infection involved a primary screen assaying 18,120 protein-coding genes, followed by a secondary deconvolution screen and a tertiary screen determining whether screen results obtained using recombinant HeV could be recapitulated using wild-type HeV and NiV (Deffrasnes et al. 2016). Applying a robust Z score normalisation method often used to interpret siRNA screen results (Birmingham et al. 2009; Zhang et al. 2006), 585 and 630 genes were identified that promoted or suppressed HeV infection, respectively, without adversely impacting cell numbers. At the completion of the primary screen, 200 proviral genes were selected based on rank for the secondary deconvolution screen. By this measure, 20 high- and 46 medium-confidence genes (>2 standard deviations from mean mock values for 4/4 or 3/4, or 2/4 siRNAs, respectively) were identified as being required for HeV infection. The apparent reliance of henipavirus infection on the nuclear or nucleolar host proteins was particularly striking, as over 40% of high confidence hits localise in the nucleus or nucleolus, with many involved in ribosome biogenesis (Table 1).

The nucleus is the site of gene expression and DNA transcription into mRNA, and houses the early steps of the RNAi pathway. The nucleus is separated from the cell cytoplasm by the nuclear envelop which contains nuclear pores and import/export proteins allowing the passage of small molecules such as mRNA. Nuclear import/export proteins such as XPO1 and KPNA3, which are required for

Table 1 Nuclear or nucleolar host protein-coding genes required for HeV infection

Entrez gene	Function
DDX10	Pre-rRNA cleavage and component of the U3 small subunit processome
EIF2S3	Recruitment of methionyl-tRNA to the 40S ribosomal subunit, initiation factor
ESF1	RPL & RPS family member, pre-rRNA processing
FBL	Pre-ribosome processing, chemical modification of pre-rRNA
GTPBP4	Ribosome subunit assembly
KPNA3	Nuclear export/import
IMP4	Pre-rRNA cleavage and component of the U3 small subunit processome
MRPL12	Structural constituent of the mitochondrial ribosome
POLR3E	rDNA transcription
PWP2	RNA binding and snoRNA binding
RPL13A	Pre-ribosome processing, modification of pre-rRNA
RPL7A	Component of the 60S ribosomal subunit
SP7	Transcriptional regulation
XPO1	Nuclear export

trafficking of larger molecules like proteins, were identified by RNAi screen as required for henipavirus infection (Deffrasnes et al. 2016).

The nucleolus is a highly dynamic structure and has increasingly been shown to play a critical role in virus–host interactions (Rawlinson and Moseley 2015; Xu et al. 2016). The nucleolus contains three regions composed of the fibrillar centre (FC) in the middle, surrounded by the dense fibrillary component (DFC) and the granular component (GC). This membrane-less structure contains a high concentration of proteins and RNAs and is the site of ribosomal RNA (rRNA) synthesis and ribosome production but is also a multifunctional structure in eukaryotic cells. Cell cycle progression, stress response, genetic silencing, regulation of apoptosis, cell migration and invasion are all functions associated with the nucleolus or partly regulated in this compartment (Rawlinson and Moseley 2015; Xu et al. 2016; Pederson 2010).

3.2 Fibrillarin and Its Role in Henipavirus Infection

Fibrillarin is the main nucleolar protein responsible for the chemical modification of ribosomal RNA (rRNA). This 34–38 kDa 2′-O-methyltransferase transfers methyl groups from its substrate, the S-adenosylmethionine (SAM), to the 2-hydroxyl groups of ribose target in rRNA. Fibrillarin has also been shown to methylate glutamine residue 104 of the human histone H2A, weakening its binding to the FACT (facilitator of chromatin transcription) complex and impacting chromatin remodelling and rDNA transcription by RNA Pol I (Tessarz et al. 2013), which points at an additional role for fibrillarin in ribosome biogenesis and translation.

Fibrillarin itself is methylated on several arginine residues by protein arginine N-methyltransferase 1 (PRMT1), which is thought to influence its activity (Rodriguez-Corona et al. 2015).

Expression levels of fibrillarin have been shown to be regulated by p53 through direct binding to fibrillarin intron 1. Abnormal levels of fibrillarin have been detected in p53-inactivated cancer cells and a decrease in p53 levels has been associated with an increase in fibrillarin expression, and conversely an increase in p53 expression results in decreased fibrillarin expression (Marcel et al. 2013). High levels of fibrillarin lead to changes in the rRNA methylation pattern, diminished translation fidelity and increase in IRES-mediated translation of some cancer genes. Moreover, ribosome biogenesis is often dysregulated and over-activated in cancer cells that have a decreased or absent p53 expression (Marcel et al. 2013).

In its N-terminal region, fibrillarin contains a glycine- and arginine-rich region (the GAR domain) enabling interaction with cellular and viral proteins, and acting as a nucleolar retention signal. Its C-terminal region (MTase) contains multiple RNA-binding domains, a catalytic site allowing for fibrillarin methyltransferase function, and is the site for NOP56/58 interaction. Fibrillarin is a part of at least one nucleolar ribonucleoprotein (snoRNP) complex comprising the NOP56, NOP58 and 15.5 K nucleolar proteins. X-ray data have suggested that the methylation of rRNA requires the formation of this complex with involvement of four fibrillarin molecules interacting with different regions of the target rRNAs. The yeast equivalent of fibrillarin, NOP1, has been more extensively studied than the human counterpart but fibrillarin is a well-conserved protein in most organisms, reinforcing the notion that all post-transcriptional processes involving fibrillarin such as chemical modification (methylation) of rRNA, pre-rRNA cleavage and ribosome assembly are essential for proper cellular functioning (Rodriguez-Corona et al. 2015).

In eukaryotes, ribosome biogenesis involves numerous nucleolar proteins and accessory factors, around 80 ribosomal proteins, many small nucleolar RNAs (snoRNAs), three RNA polymerases (RNA polymerase I, II and III) and four different species of rRNAs. The process of assembly of elongation-competent 80S ribosomes is divided into three major steps: (1) ribosomal DNA (rDNA) transcription into precursor rRNAs (pre-rRNAs), (2) processing of pre-RNAs into mature rRNAs, and then (3) assembly of rRNAs with ribosomal proteins into functional ribosomes. In the nucleolus, the RNA polymerase I (RNA Pol I) is responsible for transcribing the 18S, 5.8S and 28S rRNA from a single polycistronic pre-rRNA, while RNA pol III transcribes the 5S rRNA in the nucleus (Xue and Barna 2012). The pre-RNAs are then cleaved and modified during the pre-rRNA processing phase. All ribosomal proteins (RP) are transcribed in the cytoplasm by RNA Pol II and then translated before migrating to the nucleolus. These RP, along with nucleolar proteins such as fibrillarin and RPL13A, are responsible for modifying the rRNAs (ribose 2′-O-methylation, pseudouridylation, etc.) with the activity of more than 100 snoRNAs guiding the process in a site-specific manner. The main nucleolar protein involved in rRNA modification is fibrillarin, which methylates more than 100 sites essential for ribosome biogenesis

and stability. Although these post-transcriptional modifications are crucial for ribosome functions, their roles are not yet fully understood. In eukaryotes, the large 60S subunit of ribosomes is made of the 5S, 5.8S, and 28S rRNA along with multiple large subunit ribosomal proteins (RPL), while the small 40S subunit is made of the 18S rRNA along with multiple small subunit ribosomal proteins (RPS). The two subunits are assembled in the nucleolus into the 80S ribosomes before being transferred into the cytoplasm.

Deffrasnes and colleagues showed that siRNA-mediated knockdown of fibrillarin expression dramatically reduced HeV protein production and viral genome replication but did not impact viral fusion, and that fibrillarin catalytic activity was essential to henipavirus infection. On the other hand, overexpression experiment did not lead to an increase in viral titers, suggesting that a simple reduction or increase in overall ribosome production is unlikely to explain the reliance of henipaviruses on fibrillarin activity (Deffrasnes et al. 2016).

3.3 Modulation of Translation in Henipavirus Infection

The requirement of fibrillarin and several other proteins from the ribosomal biogenesis pathway for henipavirus infection points a reliance on translation for efficient infection. However, while we tend to view ribosomes as homogenous, new studies reveal a more heterogeneous nature of ribosomes due to differences in the ribosomal proteins recruited, post-translational modifications of rRNA and rRNA composition. Moreover, ribosomal proteins have been found to have additional functions outside of their primary roles in ribosomes and to be involved in other nucleolar functions such as regulation of cell proliferation, tumorigenesis and DNA damage response (Xu et al. 2016; Xue and Barna 2012; Au and Jan 2014).

In eukaryotes, most messenger RNA (mRNA) harbour a 5′ 7-methylguanosine cap structure and a 3′ poly(A) tail, which are both required for canonical, cap-dependent translation. A cap-independent translation mechanism also utilised by a subset of host proteins is called Internal Ribosome Entry Site (IRES)-mediated translation. It is believed that most genes translated via an IRES are related to stress response, cell proliferation, cell death/survival, and that IRES-mediated translation happens when the canonical cap-dependent translation is inhibited either by the host reaction to environmental factors, damage, stress or infections. However, a group recently suggested that thousands of human genes are translated via this cap-independent mechanism, representing a 50-fold increase in the number of sequences previously associated with this translation pathway (Weingarten-Gabbay et al. 2016).

Recently a new type of translation has been described in vesicular stomatitis virus (VSV)-infected cells. This non-canonical cap-dependent protein translation involves the ribosomal protein RPL40 acting as a constituent of the large subunit of ribosomal complexes and suggests a novel ribosome-specialised translation initiation pathway benefiting viral mRNA translation (Lee et al. 2012). Translations of

viral proteins from several other mononegaviruses, including the paramyxoviruses measles virus (MeV) and Newcastle disease virus (NDV), and a subset of cellular transcripts, are also RPL40-dependent.

How henipavirus mRNAs are translated is not fully understood. Whilst the RPL40-dependent form of cap-dependent translation remains to be characterised in detail, one could speculate that fibrillarin, like RPL40, acts a novel initiation factor for henipavirus mRNAs. The fact that depleting cells of fibrillarin did not impact synthesis of influenza A viral proteins (which occurs via the canonical cap-dependent pathway) suggests that henipavirus mRNA translation occurs via a non-canonical pathway, perhaps used by a subset of cellular transcripts. Such a concept would allow henipavirus protein synthesis to proceed in an environment where viruses may induce cellular translation shutdown in order to suppress host antiviral immune responses. There are several reports of paramyxoviruses blocking canonical translation pathways, including the MeV N protein binding to the eukaryotic initiation factor 3 (eIF3-p40) (Sato et al. 2007), whilst the P and V proteins of simian virus 5 (SV5) limit activation of the double-stranded RNA (dsRNA)-dependent protein kinase (PKR) to limit both host and viral protein translation (Gainey et al. 2008). Similar to SV5, siRNA-mediated depletion of PKR results in increased HeV growth (robust Z score 1.46), consistent with the notion that shutdown of host protein translation inhibits henipavirus infection.

If future studies do indeed demonstrate a role of fibrillarin in influencing the synthesis of ribosome subtypes required for viral protein translation, this may explain the targeting of fibrillarin by several viral proteins. Fibrillarin binds the HeV matrix (M) protein during the early stages of infection, whilst the HIV-Tat protein has been reported to bind fibrillarin and U3 snoRNA, both required for pre-rRNA processing, and this interaction reduces the pool of cytoplasmic ribosomes (Ponti et al. 2008). Intriguingly, the nucleoprotein of porcine reproductive and respiratory syndrome virus, the non-structural protein 1 (NS1) of a H3N2 influenza virus (Melen et al. 2012) and the non-structural protein 3b of the severe acute respiratory syndrome coronavirus (Yuan et al. 2005) all bind and co-localise with fibrillarin in the nucleolus; however, the reasons for this binding are yet to be determined.

4 Viral Targets Within the Host Cell Nucleus

4.1 Role of the M Protein in the Henipavirus in Infection Cycle

Many negative strand viruses encode viral proteins that localise in the nucleus and/or nucleolus at some point in their infection cycle [reviewed in (Rawlinson and Moseley 2015; Hiscox 2003; Oksayan et al. 2012; Flather and Semler 2015; Watkinson and Lee 2016)]. Within the *Paramyxoviridae*, nuclear localisation of matrix (M) protein has previously been described for NDV (Peeples 1988), Sendai

virus (SeV) (Yoshida et al. 1976), human respiratory syncytial virus (Ghildyal et al. 2003), HeV (Monaghan et al. 2014) and NiV (Wang et al. 2010). During the early stages of henipavirus infection or when expressed ectopically (Monaghan et al. 2014; Wang et al. 2010), the HeV and NiV M proteins traffic through the nucleolus to the cytoplasm. It has been recently shown that nuclear traffic is required for the henipavirus M protein to coordinate viral budding. The henipavirus M protein is a structural protein that mediates viral assembly and budding (Liljeroos and Butcher 2012; Takimoto and Portner 2004; Eaton et al. 2007). Indeed, for both HeV-M and NiV-M, overexpression of these proteins alone is sufficient to trigger viral-like particles (VLPs) that bud into the supernatant. Wang and colleagues (2010) demonstrated that mutation of NiV-M nuclear localisation signals (NLS) or nuclear export signals (NES) blocks nuclear/cytoplasmic traffic and impairs viral budding. Furthermore, a highly conserved lysine residue in the NLS (K258) serves two functions: its positive charge mediates NiV-M nuclear import, while is also a potential site for monoubiquitination which regulates NiV-M nuclear export. Mutation of K258 or the treatment of cells with proteasome inhibitors such as MG132 inhibits both NiV-M budding and NiV infection. Consistent with this work, siRNA-mediated knockdown of the protein ubiquitin D (UBD) inhibits HeV and NiV infection (Deffrasnes et al. 2016).

4.2 The Impact of M-Binding Host Proteins on Hendra Virus Infection

This raises the question: do henipavirus M proteins traffic through the nucleolus for other reasons? The multi-faceted roles of paramyxovirus proteins in replication-specific roles and various cellular processes, particularly immune evasion, would suggest so. To explore whether M binds to host proteins associated with infection efficiency, results from the genome-wide RNAi HeV screen were cross-referenced against a proteomics study by Pentecost and colleagues cataloguing host proteins that bind HeV-M and NiV-M, among other paramyxovirus M proteins (Pentecost et al. 2015). That study revealed that the henipavirus M interactome spans hundreds of host proteins, with interactions with nuclear pore complex proteins, nuclear transport receptors and nucleolar proteins particularly prevalent. Interestingly, NiV-M and HeV-M interactomes show notable overlap to other paramyxovirus M proteins, including SeV and NDV, with over 60% of the proteins found in any single interactome also found in the interactomes of one or more of the other three viruses (Pentecost et al. 2015). Whilst the binding of fibrillarin to HeV-M was demonstrated by co-immunoprecipitation assays (Deffrasnes et al. 2016) and this was not observed by proteomics, interactions were observed between HeV-M and numerous nucleolar proteins such as NOP58 (Pentecost et al. 2015) which forms a complex with fibrillarin, supporting a functional interaction between fibrillarin and HeV-M.

The relative HeV growth (presented as robust Z scores) in cells depleted of the 389 HeV-M-binding host proteins is shown in Fig. 1a. Of the 327 candidates assayed, HeV-M binds to 22 protein-coding genes that have a large impact (robust Z score ≤ -2 or ≥ 2) on HeV infection, roughly evenly distributed between proviral (12) and antiviral (10) candidates. Designating all candidates genes with Z scores <0 as proviral and genes with Z scores >0 as antiviral, host proteins that bind HeV-M appears to be pro- and antiviral at approximately equal ratios with a slight enrichment of proviral genes (174 proviral candidates vs. 146 antiviral candidates).

An assessment of whether the relative abundance of HeV-M–host protein interactions indicated a likelihood of that host protein adopting a proviral or antiviral function was also carried out (Fig. 1b). The relative abundance of host proteins within the proteomics dataset is represented as the normalised spectral abundance factor (NSAF), with higher NSAF values presenting more abundant interactions. Plotting NSAF values against robust Z scores demonstrates that host proteins that bind HeV-M with high abundance (NSAFe5 scores between 250 and 938) were more proviral (11 candidates) than antiviral (4 candidates, Z score sums: proviral 13.9, antiviral 2.6). These candidates are listed in Table 2 and include several ribosomal proteins, further implicating M in host translation.

5 Host Proteins Targeted by Henipaviruses for Immune Evasion

5.1 Immune Modulating Function of P-Encoded Proteins

Akin to fibrillarin, the critical role of host molecules in henipavirus infection and pathogenesis can be inferred by their specific targeting by viral proteins. This is particularly true in the context of immune evasion, as the innate antiviral immune response is a known target for several henipavirus proteins.

The henipavirus genome contains six transcriptional units, N, P, M, F, G and L, coding for nine proteins (Eaton et al. 2007). The P gene alone codes for at least four of the proteins: P, W, V and C (Eaton et al. 2006). All four of these proteins are involved in modification of the immune response in the host cell, through inhibition of the type I interferon (IFN) responses [reviewed in (Audsley and Moseley 2013)]. Intracellular detection of pathogen-associated molecular patterns (PAMPs) is mediated by membrane-bound Toll-like receptors (TLRs) or cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerisation domain containing (NOD)-like receptors (NLRs). Engagement of these receptors with their agonists results in the activation of complex signalling pathways culminating in the production of cytokines and anti-microbial compounds. A critical component of this response is the type I IFN system, which induces a local antiviral state upon detection of viruses or intracellular bacteria or molecules associated with their replication (Schoggins and Rice 2011).
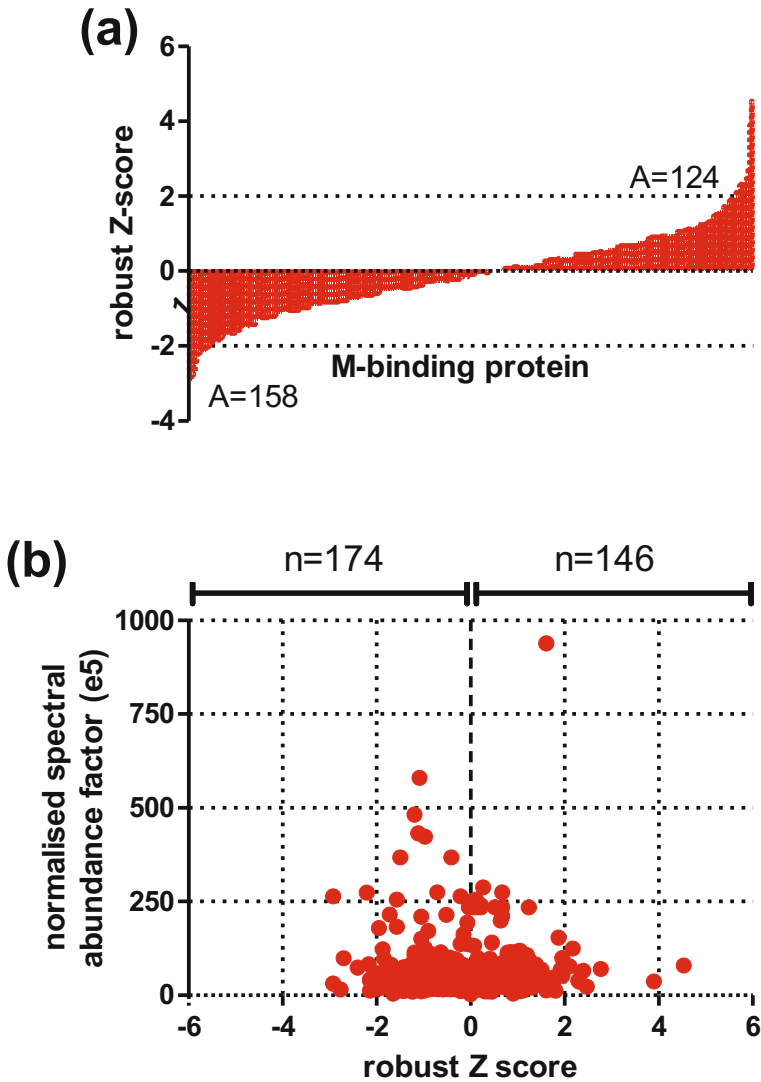
Fig. 1 The impact of HeV-M-binding host proteins on HeV infection. Cross-reference analysis of host proteins that bind HeV-M (Pentecost et al. 2015) and genome-wide analysis of host protein-coding genes associated with HeV infection (Deffrasnes et al. 2016). **a** Z scores resulting from growing HeV in cells depleted of HeV-M-binding proteins. Genes with Z scores <0 were designated proviral, while genes with Z scores >0 were designated antiviral. Values represent the sum of all the Z scores. It should be noted that 43 genes were excluded from analysis due to ambiguous gene identification listings in the proteomics study, whilst the silencing of 19 additional gene targets resulted in cell death that prevented the measurement of virus growth. **b** Plot of the Z score of HeV-M-binding proteins (x-axis) and relative abundance of HeV-M interactions, represented by normalised spectral abundance factor (y-axis)

Table 2 Robust Z score of HeV-M-binding host protein-coding genes

	Entrez gene ID	Relative abundance (NSAFe5)	Relative HeV infection (robust Z score)
Proviral	TUBAL3	580.35	−1.09
	RPL27A	482.31	−1.2
	RPL28	431.84	−1.12
	RPL38	422.59	−0.97
	ARF3	367.72	−1.5
	ARF1	367.72	−0.41
	ACTA1	274.63	−0.71
	NEDD8	273.90	−2.21
	RPL19	264.12	−2.92
	RPS27L	264.12	−0.22
	ACTA2	255.35	−1.57
Antiviral	TUBA8	938.83	1.61
	ARF4	287.60	0.26
	ACTC1	274.63	0.67
	ACTG2	255.35	0.11

Viral replication is typically detected by TLRs 3 and 7/8 in endosomal compartments (Alexopoulou et al. 2001; Lund et al. 2004), whilst RIG-I and/or melanoma differentiation-associated gene 5 (MDA5) recognise short or long viral dsRNA intermediates in the cytosol (Yoneyama et al. 2004; Triantafilou et al. 2012). TLR3 activates the TIR-domain-containing adapter-inducing IFN-β (TRIF) (Matsumoto et al. 2011), whilst RIG-I/MDA5 interact via their caspase recruitment domains (CARDs) with MAVS (mitochondrial activated signalling protein) (Seth et al. 2005) to induce signalling. Activation of TRIF or MAVS promotes recruitment of multiple cytosolic effectors, resulting in the phosphorylation and dimerisation of interferon regulatory factor (IRF) 3 or liberation of NF-κB from its inhibitory complex. These transcription factors then shuttle into the nucleus to form part of a large multiprotein complex that binds to the promoter region of IFN-β and initiates transcription (Honda and Taniguchi 2006).

The C-terminus of the HeV V protein binds and sequesters MDA5, thereby impairing IFN-β transcription in response to double-stranded RNA (Andrejeva et al. 2004). This binding appears to be conserved amongst most paramyxoviruses including NiV, SV5 and mumps virus (Childs et al. 2007). Intriguingly, RIG-I is not targeted by paramyxovirus V proteins, and perhaps consistent with this, the genome-wide RNAi screen suggested that depleting cells of MDA5 increased HeV infection (robust Z score 2.02), whilst targeting RIG-I had very little impact (Z score −0.37).

Similar to the NLR cytoplasmic antiviral immune responses, TLR3-dependent antiviral signalling is also inhibited by henipaviruses, with the W protein localising to the nucleus via the importin molecules KPNA3 and KPNA4 to block IRF3-responsive promoter activation by virus and intracellular dsRNA (Shaw et al.

2005). Transfecting NiV-W into cells in a dose-dependent manner sequesters inactive IRF3 in the nucleus, thus depleting the pool of available IRF3 for phosphorylation and activation. From the genome-wide screen, the impact of down-regulating TLR3 (Z score 1.16) and IRF3 (0.97) was a moderately antiviral phenotype.

5.2 Targeting of STAT by Henipaviruses

The best-characterised target of henipavirus immune evasion is the STAT proteins, critical signalling molecules in the context of type I IFN cytokine production conferring the antiviral state [reviewed in (Platanias 2005)]. The binding of type I IFN (IFN-α and IFN- β) and type II IFN (IFN-γ) to their respective receptor complexes leads to the phosphorylation and association of STAT1 and STAT2 heterodimers (for type I IFN signalling), or STAT1 homodimers (type II IFN). This prompts the formation of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) complexes that translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate transcription of IFN-stimulated genes (ISGs). Whilst there are hundreds, potentially thousands of ISGs that collectively confer antiviral immunity, very few ISGs have been functionally characterised in the context of henipavirus infection. One ISG, cholesterol 25 hydroxylase (CH25H), inhibits infection by NiV and a range of other RNA viruses by blocking membrane fusion between host and viral membranes (Liu et al. 2013a). Consistent with this observation, CH25H blocked HeV infection in the genome-wide RNAi screen (robust Z score 1.05).

Henipaviruses, like other paramyxoviruses, generate multiple alternative mRNAs from the P gene locus—P, V and W (Thomas et al. 1988). A fourth protein, C, is generated by alternate translation initiation site selection from all these mRNAs and does not share sequence homology to the other proteins. The P, V, and W proteins share 407 amino acids in their N termini and all three proteins bind to STAT1 and STAT2 via this N-terminal region (Ciancanelli et al. 2009; Rodriguez et al. 2004). Virus–host interactions in this context prevent STAT1/2 phosphorylation and activation, and lead to their sequestration in high molecular weight complexes (Rodriguez et al. 2003; Rodriguez et al. 2002; Shaw et al. 2004). Interestingly, the siRNA-mediated inhibition of STAT1 increased HeV infection in the genome-wide screen, but inhibition of STAT2 did not (robust Z scores of 1.01 and −0.67). This preliminary observation suggests that STAT1 activity may have a greater impact on henipavirus infection than STAT2, and may implicate type II in antiviral immunity against henipaviruses.

5.3   Novel Function of the M Protein in Immune Evasion

Although the role of henipavirus P gene products in immune evasion is well-established, a recent study demonstrates the surprising ability of NiV-M to antagonise the antiviral type I IFN response (Bharaj et al. 2016). The study by Bharaj and colleagues shows that NiV-M binds to and targets the E3-ubiquitin ligase TRIM6 for degradation. TRIM6 catalyses the synthesis of unanchored polyubiquitin chains that are used as a substrate for the activation of IkB kinase-e (IKKe), which phosphorylates IRF3 and activates IRF3-dependent transcription of type I IFN, and TNF-α. TRIM6 targeting by NiV-M occurs in the cytoplasm via an unknown mechanism not involving the proteasome or the lysosome, and requires nuclear/cytoplasmic trafficking of NiV-M. Similar to viral budding, this function of M is dependant on nuclear traffic, as K258 mutants of NiV-M do not target TRIM6 for degradation. The study expands our understanding of immune antagonism and highlights the potential purpose of henipavirus M protein nuclear trafficking.

6   The Impact of Host-Encoded MicroRNAs on Henipavirus Infection

6.1   Role of MicroRNAs in Viral Infection

MicroRNAs (miRNAs) are a class of small ($\sim$21–22 b.p.), single-stranded non-coding RNA molecules (Fayyad-Kazan et al. 2014; Neel and Lebrun 2013; Skalsky and Cullen 2010) involved in post-transcriptional gene regulation. MiRNAs function by binding to complementary sequences typically located in 3′ untranslated region (3′ UTR) of specific mRNA targets (Fayyad-Kazan et al. 2014; Neel and Lebrun 2013; Skalsky and Cullen 2010; Liu et al. 2013a). Depending on the degree of complementarity, this generally results in the suppression or degradation of target mRNA, thereby preventing encoded proteins from being translated (Fayyad-Kazan et al. 2014; Neel and Lebrun 2013; Skalsky and Cullen 2010). Although far less frequent, miRNA binding may also cause an increase in target mRNA translation and thus up-regulation of protein expression (Vasudevan et al. 2007).

In terms of target complementarity, miRNAs do not require perfect base pairing (tenOever 2013). As a result, one miRNA has the potential to regulate a surprisingly broad network of genes (Skalsky and Cullen 2010; Zhang et al. 2013), with certain miRNAs found to have binding sites located on several hundred different mRNA sequences (Guo and Steitz 2014). Despite the potential for widespread impacts, studies have described the effects of miRNA gene regulation on protein expression levels as generally 'subtle' (tenOever 2013) or 'typically relatively mild' (Selbach et al. 2008). This is due to the fact that, in general, miRNAs do not entirely silence but rather moderately repress translation and, hence, effectively fine tune rather than knock out gene expression (Baek et al. 2008).

The role of miRNAs in the infection cycle of RNA viruses is becoming increasingly apparent. Certain miRNAs may promote virus replication by directly interacting with the viral genome or, alternatively, by down-regulating the expression of host genes that suppress virus infection (Skalsky and Cullen 2010; Roberts et al. 2011). Inhibiting specific 'proviral' miRNAs, therefore, may have a direct negative impact on the viral life cycle (Janssen et al. 2013) or alternatively render the intracellular environment unfavourable for virus replication (Stewart et al. 2013). In an example of the latter, miR-146a has been found to promote HeV infection by repressing ring finger protein 11, a negative regulator of NF-κB activity (Stewart et al. 2013). Furthermore, inhibiting miR-146a has been found to significantly reduce HeV replication in vitro (Stewart et al. 2013). On the other hand, miR-122 is an example of a miRNA that promotes hepatitis C virus (HCV) replication by directly interacting with the viral genome—this activity is the basis of the first miRNA inhibitor drug to enter phase II clinical trials (Janssen et al. 2013; Wilson and Sagan 2014).

6.2 *Host-Encoded MicroRNAs and Henipavirus Infection*

The functional genomics platform established as part of the screen of protein-coding genes associated with HeV infection was recently adapted to study the impact of host-encoded miRNAs on HeV growth (Foo et al. 2016). The screen involved the use of synthetic miRNA mimics and inhibitors targeting 834 microRNAs. Mimic and inhibitor screens identified 35 and 61 microRNAs, respectively, that promoted HeV infection, and 19 and 83 microRNAs, respectively, that inhibited virus infection. A major finding from this study was that all four members of the miR-181 family (-a to -d) promote infection by HeV and NiV. Infection promotion was primarily mediated via the ability of miR-181 to significantly enhance henipavirus-induced membrane fusion. Cell signalling receptors of ephrins, namely EphA5 and EphA7, were identified as novel negative regulators of henipavirus fusion. The expression of these receptors, as well as EphB4, was suppressed by miR-181 overexpression, suggesting that simultaneous inhibition of several Ephs by the miRNA contributes to enhanced infection and fusion. To our knowledge, this study represented the first evidence of a host-encoded miRNA promoting virus cell entry.

Previous studies have reported that members of the miR-181 family are involved in different aspects of immune regulation (Hutchison et al. 2013; Galicia et al. 2014; Zietara et al. 2013). Specifically, miR-181 has been found to play a central role in the regulation of B cell differentiation and T cell selection, maturation and sensitivity (Sun et al. 2014). For instance, induction of miR-181a has been found to occur at the CD4(+)–CD8(+) double-positive stage of T cell development, inhibiting the expression of CD69, Bcl-2 and T cell receptor—all involved in positive selection and T cell maturation (Neilson et al. 2007). In addition, miR-181c has been found to suppress CD4+ T cell activation by targeting interleukin 2 (IL-2) (Sun et al. 2014;

Xue et al. 2011). In addition, miR-181a expression levels have been shown to correlate with pro-inflammatory signals (e.g. IL-1β, IL-6 and TNF-α) in blood and various tissues of humans with chronic inflammation, as well as in the blood of LPS-treated mice (Xie et al. 2013). Consistent with the notion that miR-181 expression is immune-responsive, levels of miR-181 were up-regulated in the bio-fluids of ferrets and horses infected with HeV, suggesting that the host innate immune response may promote henipavirus spread and exacerbate disease severity.

The study of both miRNAs and protein-coding genes associated with HeV infection allows an assessment whether genes required for virus infection (i.e. proviral genes) are regulated by miRNAs that inhibit virus infection (i.e. antiviral microRNAs). Multiple members of the let-7 miRNA family inhibited HeV infection. There are 10 mature let-7 sequences in humans, with multiple roles described, including negative regulation of tumorigenesis (Shi et al. 2008; Esquela-Kerscher and Slack 2006). In a transcriptome-wide study in HeLa cells, genes significantly down-regulated by let-7b at either the mRNA level, protein level or both, included fourteen validated genes required for wild-type HeV infection, including *AKT1* (Selbach et al. 2008). Furthermore, six proviral genes contain putative let-7b binding sites in their 3′ UTR (*AKT1*, *C6Orf106*, *EIF2S3*, *HMGA1*, *IFITM3* and *SERPINH1*), as identified by DIANA-mirExTra (Alexiou et al. 2010). Collectively, these data suggest that let-7 miRNAs inhibit HeV by suppressing host proteins required for virus infection. Cross-referencing results from the protein-coding screen study showed that the majority of verified target genes for miR-181 and miR-17-92 miRNAs (proviral in the miRNA screen) were predominately antiviral, demonstrating a level of congruency between miRNA and protein-coding gene screens.

In contrast to let-7, all six members of the miRNA precursor miR-17 family (miR-17, -20a, -20b, -106a, -106b and -93), part of the oncogenic miR-17–92 polycistron, strongly promoted HeV infection. Interestingly, other miRNAs of the miR-17–92 cluster with distinct "seed" families (based on sequence identity at positions 2–7)—miR-18, miR-19 and miR-92) did not impact virus replication to a similar extent. The miR-17–92 cluster is a known oncogene locus—it is amplified in B cell lymphomas (Ota et al. 2004) and accelerates tumour development in a mouse B cell lymphoma model (He et al. 2005). Members of the miRNA precursor miR-17 family are expressed in almost all human tissues (Liang et al. 2007). In addition, miR-106a and -106b are expressed in peripheral blood mononuclear cells (PBMCs), platelets and exosomes derived from peripheral blood (Hunter et al. 2008).

7 Concluding Remarks

Henipaviruses are dangerous pathogens and control of disease caused by these viruses will critically rely on the development of new antiviral therapeutics and vaccination strategies. Currently, there is requirement for renewed research into the host immune responses to henipavirus infection and how competent immune responses may fight disease. A major challenge is to ascertain the molecular

mechanisms of virus replication and immunity associated with protection to infection. The improved knowledge of functional genomics approaches and immune response to viral infection means that we now have the tools to further progress our understanding and knowledge. Nevertheless, this must be implemented to develop advanced infection control approaches.

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