Hendra and Nipah viruses: different and dangerous

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Abstract | Hendra virus and Nipah virus are highly pathogenic paramyxoviruses that have recently emerged from flying foxes to cause serious disease outbreaks in humans and livestock in Australia, Malaysia, Singapore and Bangladesh. Their unique genetic constitution, high virulence and wide host range set them apart from other paramyxoviruses. These features led to their classification into the new genus Henipavirus within the family *Paramyxoviridae* and to their designation as Biosafety Level 4 pathogens. This review provides an overview of henipaviruses and the types of infection they cause, and describes how studies on the structure and function of henipavirus proteins expressed from cloned genes have provided insights into the unique biological properties of these emerging human pathogens.

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Hendra virus (HeV) and Nipah virus (NiV) join a growing list of viruses for which bats have been implicated as the natural host, a list that started with rabies virus in 1934 (REF. 1) and the most recent additions to which were severe acute respiratory syndrome (SARS)-like coronaviruses in 2005 (REFS 2,3). Bats are classified in the order Chiroptera (from the Greek 'cheiros', hand, and 'pteros', wing), and it is within the genus *Pteropus* in the family Pteropodidae, or Old World fruit bats, that we find the natural hosts of HeV and NiV. Pteropid bats are commonly referred to as flying foxes[4] (FIG. 1).

HeV and NiV are not the only paramyxoviruses likely to have a bat origin. Menangle virus, which caused a reproductive disease in an Australian piggery in 1998, also seems to have *Pteropus* species as its natural hosts[5,6], and Tioman virus was found in the urine of the Malaysian flying fox *Pteropus hypomelanus*[7]. Bat parainfluenza virus was isolated in India from a member of the *Rousettus* genus within the family Pteropodidae[8]. Last, Mapuera virus was isolated in Brazil from a bat of the *Sturnira* genus in the family Phyllostomidae (New World leaf-nosed bats)[9]. The genetic constitution of HeV and NiV and their zoonotic potential, high virulence and wide host range set them apart from other paramyxoviruses. This review provides an overview of the henipaviruses and summarizes recent molecular analyses of the structure and function of henipavirus proteins that have contributed greatly to our understanding of these unique pathogens.

The genus Henipavirus

Paramyxoviruses are classified in two subfamilies, *Paramyxovirinae* and *Pneumovirinae* (BOX 1). The virions and nucleocapsids of HeV and NiV display morphological features that are typical of members of the subfamily *Paramyxovirinae* (FIG. 2a,b), and their genetic organization resembles that found in viruses in the Respirovirus and Morbillivirus genera in this subfamily[10] (FIG. 2c).

Several features distinguish henipaviruses from other paramyxoviruses. Genetic attributes include the unique, genus-specific 3′ leader and 5′ trailer sequences, which function as promoters for transcription and replication of genomic RNA, respectively[11,12], and the presence of the sequence GDNE in a highly conserved catalytic site in the transcriptase protein, instead of the GDNQ sequence that is found in almost all other non-segmented negative-strand RNA viruses[12,13]. Interestingly, one of the most prominent features that differentiates henipaviruses from other paramyxoviruses is the length of the viral genome, which at 18,234 nucleotides (nt) for HeV and 18,246 nt for NiV is approximately 2,700 nt (15%) longer than others in the family[12,13]. But, as far as genome length is concerned, the henipaviruses are not alone, and the genomes of the as-yet-unclassified paramyxoviruses Tupaia virus[14] and J virus[15] are 300 nt shorter and >700 nt longer, respectively, than those of henipaviruses. However, henipaviruses remain unique in having their 'extra' genomic nucleotides in the form of long untranslated regions, mostly at the 3′ end in five of the six transcription units, the exception being the L gene[10–12] (FIG. 2c). Despite the extra length

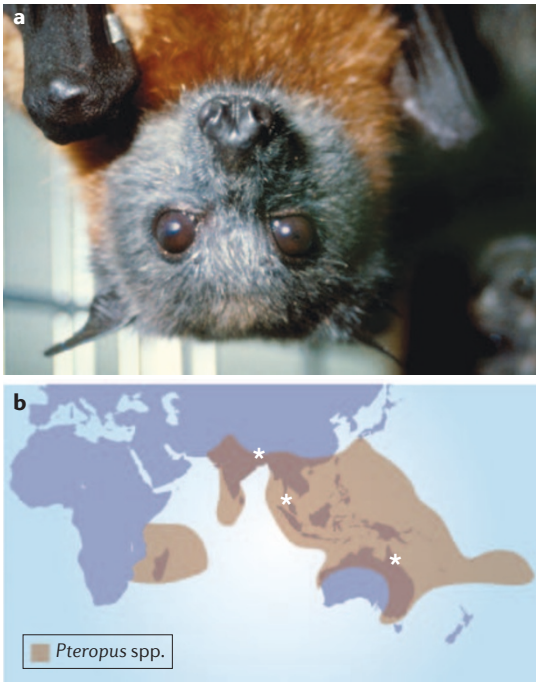


Figure 1 | **Flying foxes, their distribution and the locations of disease outbreaks caused by Hendra virus and Nipah virus. a** | *Pteropus poliocephalus* is an Australian flying fox and member of the family Pteropodidae, one of 18 bat families in the order Chiroptera. There are four *Pteropus* species in Australia[4]. **b** | Sixty-five *Pteropus* species are distributed from Madagascar through the Indian subcontinent to south-eastern Asia and Australia and as far east as the Cook Islands[4]. Some *Pteropus* species are among the largest of all bats, weighing as much as 1.2 kg and displaying a wing span of up to 1.7 m. *Pteropus* species are unique because they lack the complex neural and behavioural mechanisms required for echolocation that characterize the vast majority of bat species. Instead, they have large eyes and they navigate visually, feeding mainly on fruit and flowers, which they locate by smell. The sites of disease outbreaks caused by henipaviruses are indicated. Map modified with permission from REF. 4 © (2002) University of New South Wales Press.

of the henipavirus genome, all proteins except one, the phosphoprotein (P), are approximately the same size in the Respirovirus, Morbillivirus and Henipavirus genera; the henipavirus P protein is approximately 100 to 200 amino acids longer than cognate respirovirus and rubulavirus proteins[13,16].

Henipaviruses also have unique biological features. They are the only zoonotic paramyxoviruses and are highly pathogenic. Although there are no data on the total number of people infected with NiV during an outbreak in Bangladesh in 2004, 75% of patients that were identified as having NiV-associated illness on the basis of positive serology or on epidemiological grounds died[17]. The range of species that are susceptible to henipaviruses is also quite remarkable. In addition to at least three pteropid species, NiV infects five terrestrial species in four mammalian orders[18–23]. Experimental henipavirus infections extend the number of susceptible terrestrial orders to five by including the Rodentia[24,25]. This exceeds the extensive host range of canine distemper virus, a morbillivirus that naturally infects many species in the order Carnivora, such as dogs, ferrets, raccoons and lions, but can also experimentally infect hamsters and pigs (order Rodentia and Artiodactyla, respectively)[26,27].

The susceptibility of humans, the high virulence of the viruses and the absence of therapeutic modalities and vaccines have led to the classification of HeV and NiV as Biosafety Level 4 (BSL4) pathogens. Globally, only a limited number of laboratories have appropriate facilities for growth and handling of BSL4 pathogens. Among these, even fewer have facilities in which animals, particularly large animals, can be infected with HeV or NiV and in which workers can be protected in plastic suits, supplied with breathing air. This has limited the number of investigations into the interaction of henipaviruses with their natural hosts, susceptible livestock and laboratory animal species. However, issues of personal safety and biocontainment are minimized when working with cloned henipavirus genes in eukaryotic expressions systems, and it is from such studies that most of our current knowledge on the structure and function of henipavirus proteins has been obtained. Before discussing the molecular observations that shed light on the unusual biological properties of these emerging pathogens, let us first summarize the emergence of HeV and NiV in a timeline (TIMELINE) and briefly review the clinical and pathological outcomes of virus infection.

Henipavirus infections

Henipavirus infections are characterized by their systemic nature, with evidence of infection in multiple organ systems. The outcome of infection differs significantly in terrestrial and chiropteran hosts (BOX 2). In terrestrial species, both HeV and NiV display a predominantly respiratory or neurological tropism, depending on the host. Infections can be associated with high morbidity and case-fatality rates, such as in HeV infection of horses and NiV and HeV infections of people, or lower morbidity and mortality rates, best represented by NiV infection of pigs[20,22,28–30]. HeV-infected horses develop acute, febrile respiratory disease that is sometimes accompanied by facial swelling, ataxia and, terminally, copious frothy nasal discharge[31]. Respiratory signs also predominate in NiV infection of pigs, especially in young animals that develop fever, nasal discharge, rapid and laboured respiration and a notable, harsh and non-productive cough, giving rise to the name 'barking-pig-disease'[22]. Nevertheless, neither HeV nor NiV cause a solely respiratory syndrome in horses and pigs, respectively. A proportion of convalescent horses have re-presented with neurological signs, and clinical signs consistent with multifocal neurological disease have also been observed in growing pigs, together with sudden death in mature animals[22].

In humans, symptomatic NiV infection has mainly taken the form of severe acute encephalitis. Many NiV-infected patients have reduced levels of consciousness at presentation and signs consistent with brain-stem involvement[32]. However, up to 25% of cases also exhibited respiratory signs[30,33]. Infection with NiV can also take a more

Zoonotic
A zoonotic infection is an infection of animals that can be transmitted to humans.

Biosafety Level 4
(BSL4). BSL4 is the highest safety rating for laboratories, used for handling agents that pose a high risk of life-threatening disease and for which there is no vaccine or therapy. Other BSL4 agents include Ebola virus and Marburg virus.

chronic course, with serious neurological disease occurring late (in excess of 4 years) following a non-encephalitic or asymptomatic infection[34,35]. The recurrence of neurological manifestations has also been noted in patients who had previously recovered from acute encephalitis (relapsed encephalitis)[34]. Cases of relapsed encephalitis presented from several months to nearly 2 years after the initial infection and, interestingly, two further cases of relapsed encephalitis were observed in the autumn of 2003, some 4 years after initial infection[35]. Taken together, there is nearly a 10% incidence rate of late encephalitic manifestation, with a mortality rate of 18%. So, with both NiV and HeV, a prolonged period of infection is possible before the manifestation of serious neurological disease. Viral antigen was found in neurons in patients who died of late-onset encephalitis[34], raising questions about the underlying mechanisms that allow these viruses to escape immunological clearance for such an extended period.

Many fewer cases of human HeV infection have been recorded, and the associated disease syndrome is correspondingly less well defined. Affected patients have had influenza-type symptoms, and fatal HeV encephalitis has been described in one patient more than a year after a self-limiting episode of meningitis that was, in retrospect, also attributed to HeV infection[29,36].

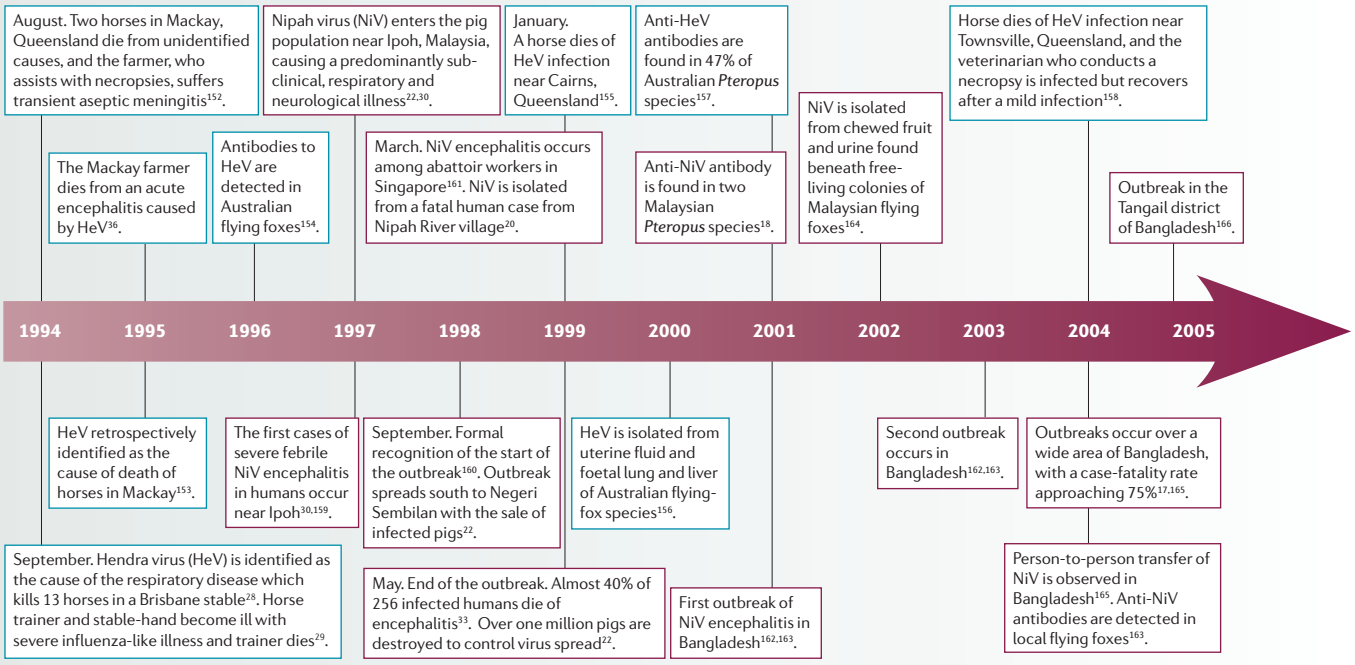
Molecular insights into henipavirus biology

The molecular basis of paramyxovirus virulence, host range and cell tropism is determined to a significant degree by the cell-attachment (G) and fusion (F) proteins, which determine host range and cell tropism by virtue of their roles in binding to cell receptors and fusing the virus and host-cell membranes; and by the products of the P gene, which modulate virulence by abrogating the cellular interferon (IFN) response. Recent studies on the structure and function of henipavirus proteins expressed from cloned genes have shown that the henipavirus G and F glycoproteins and the P protein also influence host range, cell tropism and virulence, but do so in ways that are both surprising and unique.

The henipavirus G protein. Unlike other members of the *Paramyxoviridae*, which have a limited host range, henipaviruses naturally infect flying foxes, horses, pigs, cats, dogs and humans, and experimental investigations have extended this host range to include guinea pigs and hamsters. The susceptibility of several cultured cell types was noted during the initial attempts to isolated HeV from clinical samples[28]. The broad species tropism of henipaviruses is also reflected in an *in vitro* fusion assay in which henipavirus F and G proteins that were expressed on the surface of effector cells by vaccinia virus facilitated fusion with adjacent target cells from a range of species, including rabbit, monkey and mouse[37]. The fact that an identical pattern of target-cell susceptibility was observed in fusion assays using HeV and NiV glycoproteins indicated that both viruses used the same cell receptor[38]. These observations indicate that henipavirus receptors are ubiquitously expressed.

Paramyxoviruses fall into two broad categories, depending on whether or not their attachment glycoprotein has domains that bind red blood cells and release terminal *N*-acetyl neuraminic acid residues from

Timeline | **Emergence of henipaviruses**

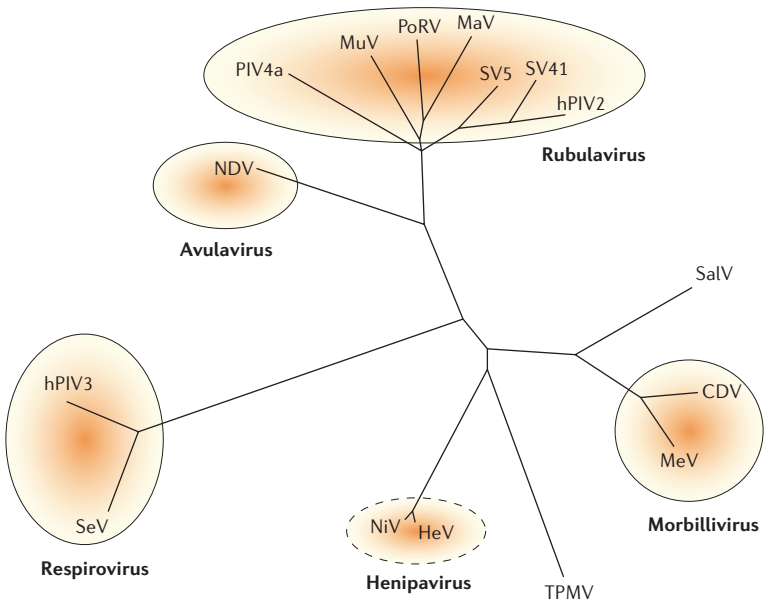


The emergence of Hendra virus and Nipah virus is detailed in boxes outlined in turquoise and purple, respectively.

Box 1 | **Classification of henipaviruses**

Viruses in the family *Paramyxoviridae* are classified in two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The latter subfamily contains two genera, Pneumovirus and Metapneumovirus. The number of genera in the *Paramyxovirinae* was increased in 2002 from three (Respirovirus, Morbillivirus and Rubulavirus) to five by the addition of two new genera, Avulavirus and Henipavirus[133]. The Avulavirus genus contains avian paramyxoviruses that were previously classified in the Rubulavirus genus, and the Henipavirus genus was created to accommodate Hendra virus and Nipah virus.

The phylogenetic tree shown here is based on an alignment of the deduced amino-acid sequence of the N gene of selected *Paramyxovirinae* subfamily members using the Neighbour-Joining method (see the genome organization of henipaviruses in FIG. 2). Viruses are grouped according to genus and abbreviated as follows. Morbillivirus genus: MeV (measles virus), CDV (canine distemper virus); Henipavirus genus: HeV (Hendra virus), NiV (Nipah virus); Respirovirus genus: SeV (Sendai virus), hPIV3 (human parainfluenza virus 3); Avulavirus genus: NDV (Newcastle disease virus); Rubulavirus genus: hPIV2 (human parainfluenza virus 2), MaV (Mapuera virus), MuV (mumps virus), PIV4a (parainfluenza virus 4a), PoRV (porcine rubulavirus), SV5 (simian parainfluenza virus 5), SV41 (simian parainfluenza virus 41); and unclassified viruses SalV (Salem virus) and TPMV (Tupaia paramyxovirus).



carbohydrate moieties. The presence of such haemagglutination and neuraminidase activities in avulaviruses and rubulaviruses correlates with their binding to *N*-acetyl neuraminic acid in cell-surface glycoprotein and glycolipid receptors[39]. However, although measles virus (MeV), a morbillivirus, displays haemagglutination activity, it binds to cells by a sialic-acid-independent mechanism[40]. The cell-surface-expressed proteins CD46 and SLAM (CD150) have been shown to act as MeV receptors, with SLAM now regarded as a universal morbillivirus receptor[41–44]. By contrast, HeV and NiV resemble members of the *Pneumovirinae* in possessing a third class of attachment protein, G, which displays neither haemagglutination nor neuraminidase activities. However, the G protein of HeV and NiV is structurally unrelated to the cognate pneumovirus protein[45]. These early observations indicated that henipaviruses, like morbilliviruses, might use cell-surface proteins as receptors in a process that does not require *N*-acetyl neuraminic acid[11,28,45,46]. Indeed, the susceptibility of target cells to HeV-mediated and NiV-mediated fusion could be destroyed by protease treatment[37].

The attachment proteins of paramyxoviruses are type II membrane glycoproteins consisting of a cytoplasmic tail, a transmembrane region, which anchors the protein to the viral envelope, a stalk and a globular head, which is composed of six protein sheets organized in a propeller-shaped structure[47,48]. Although the HeV G attachment protein has low amino-acid-sequence homology with attachment proteins from other paramyxoviruses, its globular head retains the propeller shape predicted for members of the family, and the location of neutralizing epitopes resembles that observed for other members of the *Paramyxovirinae*[45,49]. Soluble forms of HeV and NiV G proteins (sG), generated by replacing the cytoplasmic tail and transmembrane domains with an immunoglobulin κ leader sequence, retain biological activity[50,51]. They also retain an oligomeric structure, and bind to cells that are susceptible to henipavirus infection but fail to attach to infection-resistant cells, and as immunogens they elicit a potent crossreactive neutralizing antibody response against infectious HeV and NiV[50].

Preincubation of cells with HeV sG resulted in dose-dependent inhibition of both HeV and NiV infection, probably by blocking viral receptor engagement[50], and not surprisingly, sG proteins had key roles in determining and confirming the recent identification of the henipavirus cell receptor. NiV sG fused to the Fc region of human IgG1 was used to immunoprecipitate the receptor from the plasma membrane of cells that were permissive for NiV F-mediated and G-mediated fusion. The receptor was identified as ephrin B2 by mass spectrometry[51]. In another approach, microarray analysis was used to identify mRNA sequences that are expressed in henipavirus-susceptible cells but not in cells refractory to henipavirus infection[52]. From a list of genes encoding predicted membrane-localized proteins found only in susceptible cells, only one — encoding ephrin B2 — could render resistant cells susceptible to fusion as well as infection not only by NiV but also by HeV[52]. Henipavirus infection of ephrin-B2-expressing susceptible cells was blocked by soluble recombinant ephrin B2 (REF. 52), and the ability of ephrin B2 to serve as a receptor for virus was confirmed by showing that the sG protein of HeV and NiV bound to ephrin B2 *in vitro* with high affinity[52].

Ephrin B2 is a member of a family of cell-surface glycoprotein ligands that bind to ephrin (Eph) receptors, a large family of receptor tyrosine kinases[53,54]. Although initially identified in vertebrates as regulators of axon path finding and neuronal cell migration, Eph receptors and ephrins have been found in arthropods, nematodes and even sponges[54]. This indicates a primordial function for these molecules, and they are now known to mediate cell-to-cell communication and regulate cell attachment and repulsion. Eph receptors and ephrins have key roles during development, especially in the nervous and vascular systems[55,56]. Ephrin B2 is found in neurons, smooth muscle, arterial endothelial cells and capillaries[55,57–59]. The structure of the Eph B2 receptor, ephrin B2 and the complex that they form has been determined by X-ray crystallography[60]. Ephrin B2 is glycosylated but the side chain is short, containing a single mannose residue and two *N*-acetyl glucosamine residues, and lacks sialic acid[61].

Type II membrane glycoproteins
Transmembrane glycoproteins with a cytoplasmic N terminus.

Fc region
The region of an antibody that is responsible for binding to antibody receptors (FcR) on cells and the C1q component of complement.

Tunica media
All blood vessels, except capillaries, comprise three layers surrounding a central lumen: the outer tunica adventitia, the inner tunica intima and the middle tunica media. The tunica media is composed predominantly of smooth muscle and also contains autonomic nerves.

Type I membrane protein
A single-pass transmembrane protein that contains an N-terminal lumenal domain with carbohydrate moieties and a C-terminal cytoplasmic domain.

trans-Golgi network
The area of the Golgi where secretory and membrane proteins are sorted to their final destination.

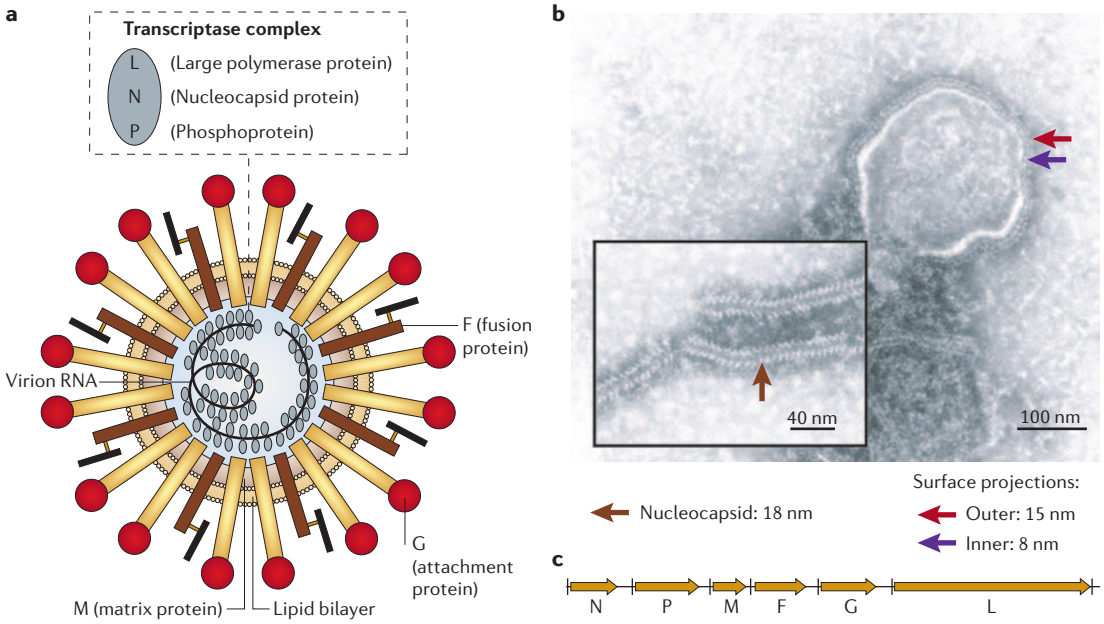


Figure 2 | **Structure of henipaviruses and their genomes. a** | A schematic representation of henipavirus structure. Henipaviruses, like other paramyxoviruses, contain a linear ribonucleoprotein (RNP) core consisting of a single-stranded genomic RNA molecule of negative polarity to which nucleocapsid proteins (N) are tightly bound in a ratio of one N for every six nucleotides[127,139]. The RNP also contains smaller numbers of the phosphoprotein (P) and the large (L) polymerase protein, both of which are required to transcribe genomic RNA into mRNA and anti-genome RNA. The RNP core is surrounded by an envelope from which two spikes protrude; one is the receptor-binding glycoprotein (G) and the other the fusion (F) protein. The G and F proteins are arranged as homotetramers and homotrimers, respectively. The matrix protein (M) which underlies the viral envelope is important in determining virion architecture and is released from the RNP core on its entry into cells. **b** | Electron micrograph of Hendra virus (HeV). The ultrastructural characteristics of HeV and Nipah virus have been reviewed[140]. **c** | The henipavirus genome. The negative-sense genomic RNA is presented in the 3′ to 5′ orientation. The open reading frames indicated by the yellow arrows encode the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein or attachment protein (G) and large protein (L) or RNA polymerase, in the order 3′-N-P-M-F-G-L-5′. The vertical lines represent gene start and stop signals. Note the long untranslated 3′ regions in all genes except the L gene. All genes except the P gene are monocistronic. The P gene of henipaviruses encodes not only the P protein, but also V, C and W proteins (BOX 3). Genomic RNA in RNPs is transcribed by the viral polymerase which associates with the RNP at the 3′ terminus and sequentially generates discrete mRNAs from each of the viral genes. The mRNAs are not produced in equimolar amounts and there is a transcription gradient from the N to the L gene, with significant attenuation at the M–F and G–L gene junctions of HeV, a pattern of attenuation more closely resembling that observed in Sendai virus than in measles virus[141–143].

The identification of ephrin B2 as the cell receptor for both HeV and NiV and the widespread occurrence of the molecule in vertebrates, particularly in arterial, but not venous, endothelial cells, in the smooth muscle of the tunica media and in neurons, provide an explanation for the wide host range of henipaviruses and the systemic nature of the infections they cause[51,52].

The henipavirus F protein. Paramyxoviruses contain two membrane glycoproteins: the G glycoprotein, which is required for cell attachment, and the F glycoprotein, which is required for the fusion of the viral and host-cell membranes (FIG. 2). Following virus attachment to a permissive host cell, fusion occurs at neutral pH, delivering the viral genetic material into the cytoplasm. In a related process, cells expressing these glycoproteins on their surfaces can fuse with other receptor-bearing cells, resulting in the formation of multinucleated giant cells (syncytia), a hallmark of the paramyxovirus cytopathic effect. The paramyxovirus F glycoproteins are class I fusion proteins with two α-helical domains, referred to as heptad repeats, that are involved in the formation of a trimer-of-hairpins structure or six-helix bundle during or immediately following fusion[62–64]. Peptide sequences that correspond to either heptad repeat of the F protein of several paramyxoviruses, including HeV and NiV, have been shown to be potent inhibitors of fusion[65–69]. Indeed, HeV and NiV peptide-fusion inhibitors have been shown effective in blocking live virus infection *in vitro*, and might offer an exploitable therapeutic avenue[69].

F proteins are type I membrane proteins, and biologically active F consists of two disulphide-linked subunits, F_1 and F_2, that are generated by the proteolytic cleavage of a precursor known as F_0 (REF. 70). Most paramyxovirus F precursors are cleaved by furin, a ubiquitous, calcium (Ca^{2+})-dependent protease that is localized in the *trans*-Golgi network of many eukaryotic cells[71]. The minimum sequence requirement for efficient processing by furin *in vitro* is RXXR[72], a sequence that is highly conserved in paramyxovirus F proteins. Viruses with this sequence,

Clathrin
A structural protein that polymerizes into polyhedral lattices to form a membrane coat around vesicles involved in membrane transport in both the endocytic and biosynthetic pathways.

such as MeV, cause systemic infections after initial infection of the respiratory tract. By contrast, a small number of paramyxovirus F proteins are proteolytically cleaved by extracellular trypsin-like proteases that recognize a single basic residue at the cleavage site. Cleavage *in vivo* is achieved by trypsin-like proteases such as tryptase Clara and miniplasmin that have limited distributions and, as a result, viruses like Sendai virus (SeV) remain localized in the respiratory tract[73,74]. In view of the fact that henipaviruses generate systemic infections, it was surprising to find that the henipavirus F-protein cleavage site does not contain multiple basic residues. The cleavage site in the HeV F protein contains a single basic residue, lysine, in the sequence VGDVKLAG[75]. In NiV, the lysine is replaced by arginine[11]. A role for furin in cleavage was excluded when it was shown that LoVo cells, human colon-carcinoma cells that lack furin, support the replication of HeV and permit cleavage of the NiV F protein[75,76]. The involvement of an enzyme with specificity for single basic residues was also ruled out by the fact that activation of the HeV and NiV F proteins in cell culture did not require exogenous trypsin, a requirement for SeV replication *in vitro*[11,38,75].

Studies using a range of protease inhibitors in conjunction with conditions that block the movement of glycoproteins through the secretory pathway indicated that the HeV F protein is cleaved in the secretory vesicles that bud from the *trans*-Golgi network[76]. However, more recent studies on the NiV F protein have shown that cleavage is not mediated in vesicles during transport along the secretory pathway, but only after endocytosis of the protein[77,78]. The 45-amino-acid cytoplasmic tail of henipavirus F proteins contains an endocytosis signal, which directs the protein expressed on the cell surface into clathrin-coated vesicles for ongoing transport to other cellular compartments[79]. Removal of the signal not only abrogates endocytosis of the F protein but, remarkably, also prevents its cleavage into F_1 and F_2 (REFS 77,78). This observation provides an explanation for the finding that, although removal of the endocytosis signal causes an increase in the concentration of cell-surface-expressed NiV F, it also caused a decrease in the size of NiV glycoprotein-induced syncytia[79] because the F protein remained in an uncleaved form.

The subcellular location, specificity, sensitivity to pH and decreased requirement for Ca^{2+} indicate that the protease responsible for processing henipavirus F proteins differs from proteases that have been previously implicated in the maturation of viral proteins[76–78]. Furthermore, the failure to abrogate cleavage by replacing the arginine of the NiV F-protein cleavage site with a non-polar residue was particularly surprising, and contrasts with the absolute need for a basic residue or residues in the F proteins of all other paramyxoviruses[80]. Recently, it was shown that the lysosomal cysteine protease, cathepsin L, is responsible for the proteolytic cleavage of the HeV F protein[81].

The henipavirus P gene products. The IFN system is one of the first lines of innate immune defence against infection in mammals, and is designed to limit the spread of microorganisms from the source of infection[82–85]. There are two types of IFN. Type I IFNs are produced in response to virus and bacterial infection and comprise a family of related IFN-α proteins and IFN-β. The type II IFN, IFN-γ, is synthesized only by certain cells of the immune system. Here, we focus solely on the antiviral type I IFN response.

The transcriptional activation of type I IFN-α/β genes is a complex, bi-phasic process. The first phase, IFN induction, occurs in cells soon after infection (FIG. 3) and leads to the synthesis of IFN-β and a subset of IFN-α proteins[84,86]. The IFN induction pathway can be activated by double-stranded (ds)RNA[87] or by virus infection, in which viral components other than dsRNA might be responsible[88]. For the sake of simplicity, we will refer here to the process by which IFN is induced as the dsRNA-signalling pathway. In the second phase, IFN signalling (FIG. 4), the IFNs that are induced as a result of virus infection bind to type-I-IFN receptors on the surface of both infected and uninfected cells, and activate hundreds of IFN-inducible genes, some of which have antiviral activity[82–84].

Box 2 | **Henipavirus infection in flying foxes**

Despite the high prevalence of antibodies to henipaviruses, particularly in Australian pteropids, neither Hendra virus (HeV) nor Nipah virus (NiV) has been associated with any naturally occurring disease of flying foxes. The subclinical nature of HeV infection of pteropids has been confirmed by experimental infection of several species of Australian flying foxes[134,135].
A comparison of the pathology observed in henipavirus-infected chiropteran and terrestrial mammals provides some insights into the different clinical outcomes of infection. The predominant lesion in natural and experimental henipavirus infection of terrestrial animals, including humans, is systemic vasculitis, which affects smaller vessels in many organs, with clinical symptoms arising predominantly from infection of the lung and/or the central nervous system[21,136,137]. Viral antigen is detected in syncytial cells in vascular endothelium and, in the case of NiV infection, in bronchial and alveolar epithelium. Henipaviruses are readily recovered from nasopharyngeal secretions, urine and internal organs including lung and brain[21,138]. By contrast, infection of flying foxes with doses of HeV consistently shown to be lethal in horses generated only sporadic vasculitis in the lung, spleen, meninges, kidney and gastrointestinal tract, and only in a proportion of infected bats[134,135]. Viral antigen is detected in the tunica media rather than endothelial cells. In infected pregnant flying foxes, antigen is observed in similar locations and in the placenta[135].

Two observations might explain the lack of systemic disease in flying foxes. First, the presence of antigen in the tunica media rather than endothelial cells indicates that the latter might be spared from infection, therefore reducing the clinical effects associated with vasculitis. Second, the striking reduction in the level of antigen in flying foxes compared to horses and cats indicates that factors not found in terrestrial mammals that limit the ability of HeV to replicate could be at play in flying foxes. Indeed, after experimental infection of flying foxes with HeV, only half the animals show a rise in antibody titre, which is often low and sometimes of short duration (<3 weeks). Despite rigorous sampling regimes, virus has been isolated only infrequently, and where isolation was successful, positive sources included urine and the foetus, heart, placenta, kidney and spleen of two pregnant bats[134,135].

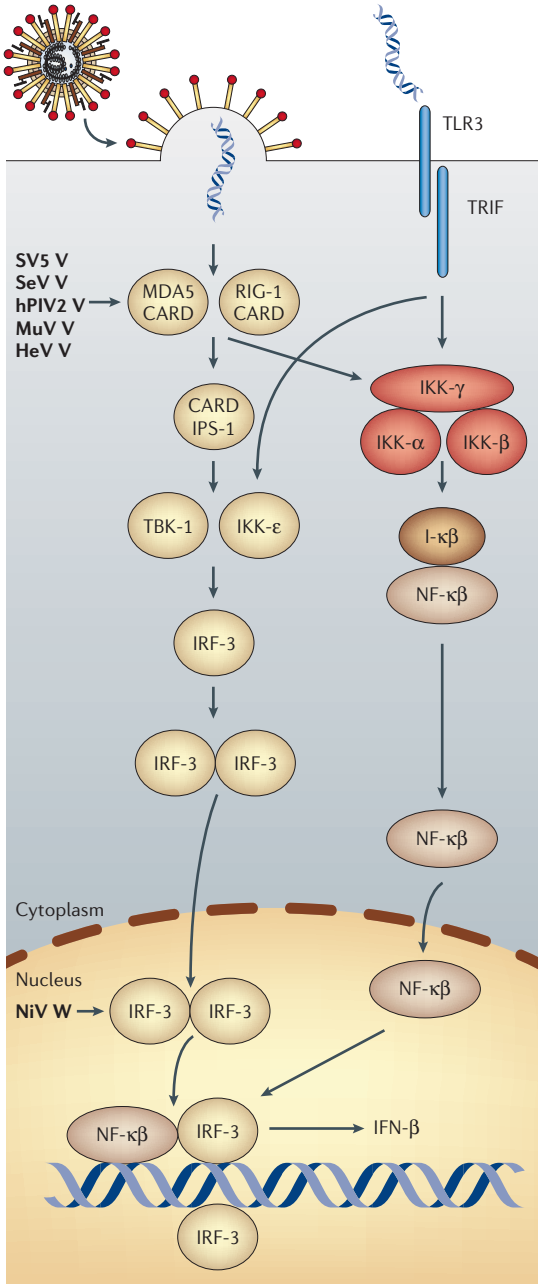


Figure 3 | **Interferon (IFN) induction or double-stranded (ds)RNA signalling.** The innate immune system depends on the ability of cells to detect the presence of unique, pathogen-specific molecules. The molecule considered most likely to be seen as foreign by virus-infected cells and activate the innate immune system is dsRNA, generated as a result of virus infection[87]. Several cellular sensors detect the dsRNA signal and respond by activating pre-existing transcription factors such as IFN-regulatory factor 3 (IRF-3) and the general transcription factor nuclear factor (NF)-κB[83,97,144–146]. Activated IRF-3 and NF-κB are redistributed to the nucleus, where they cooperate with other transcriptional activators to induce transcription of the interferon (IFN)-α/β genes. In one pathway, the sensor is an intracellular RNA helicase encoded by the retinoic inducible gene-1 (*RIG1*) or the melanoma differentiation-associated gene 5 (*MDA5*)[102,147]. RIG-1 and MDA5 proteins are DExD/H-box RNA helicases that unwind dsRNA by virtue of their intrinsic ATPase activity. They also contain caspase-recruitment domains (CARD). The binding of dsRNA to the helicase has been hypothesized to result in the activation of the ATPase leading to conformational changes in CARD[102]. An activated CARD acts as an interface between signalling molecules, and the CARD of RIG-1 and MDA5 has been shown to interact with the CARD-like domain of a protein called IFN-β promoter stimulator 1 (IPS-1)[148] to transmit a signal downstream, resulting in the phosphorylation of IRF-3 by the kinases TANK-binding kinase 1 (TBK-1) and IκB kinase ε (IKK-ε)[149,150]. Activated IRF-3 dimerizes and is translocated to the nucleus. Intracellular dsRNA signalling through RNA helicases also activates NF-κB. The inhibitor of NF-κB, I-κB, is phosphorylated by an activated member of the IKK complex, and I-κB is destroyed in proteasomes. NF-κB is therefore released and translocated to the nucleus. A second IFN-induction pathway, likely to be activated after the helicase-dependant pathway, uses Toll-like receptor 3 (TLR3), which probably detects dsRNA released from virus-infected cells[151]. Signalling through TLR3 is mediated by an intracellular adaptor protein called TRIF, which signals two protein-kinase complexes, TBK-1–IKK-ε and IKK-α– IKK-β, and leads to the activation of both IRF-3 and NF-κB. The sites where henipavirus and other paramyxoviruses are known to interfere with dsRNA signalling and the viral proteins responsible are indicated (see text). HeV, Hendra virus; hPIV2, human parainfluenza virus 2; MuV, mumps virus; NiV, Nipah virus; SeV, Sendai virus; SV5, simian parainfluenza virus 5.

Over millions of years of co-evolution, almost all viruses have evolved ways to evade the IFN-induced antiviral responses of their hosts[83,85,89,90]. These mechanisms include the inhibition of host-cell transcription and translation and the consequent failure to synthesize IFN, inhibition of dsRNA-signalling and IFN-signalling pathways, and antagonizing the IFN-induced antiviral effector proteins. The anti-IFN activities of paramyxoviruses are encoded by the viral NS1 and NS2 genes in respiratory syncytial virus, a member of the *Pneumovirinae* subfamily[91,92], and by the P gene in the *Paramyxovirinae*. Products of the P gene inhibit both dsRNA signalling[93–96] and IFN signalling[97–99]. The anti-IFN strategies used by paramyxoviruses vary, both between genera and among viruses within a specific genus. This is primarily because the P gene and its encoded proteins are both organized and expressed in a genus-specific manner (BOX 3). Recent analyses have revealed the unique way in which the P-gene products of HeV and NiV act as virulence determinants by antagonizing the IFN response of infected cells.

Inhibition of dsRNA signalling. In the henipaviruses, dsRNA signalling is inhibited not only by the accessory V protein, as observed for rubulaviruses and respiroviruses[93–95,100,101], but surprisingly also by the accessory W protein (FIG. 3). The V protein of HeV targets the helicase encoded by the melanoma differentiation-associated gene 5 (*MDA5*), like its rubulavirus and respirovirus counterparts[102]. Whether the

Nuclear-localization signal
A positively charged region of a protein that is responsible for directing its transport through nuclear-membrane pores and into the nucleus.

Proteasomes
Most of the degradation of cytosolic and nuclear proteins in eukaryotic cells is catalysed by multi-subunit proteases known as proteasomes. Targeting of proteins to proteasomes most often occurs through the attachment of multiple ubiquitin tags.

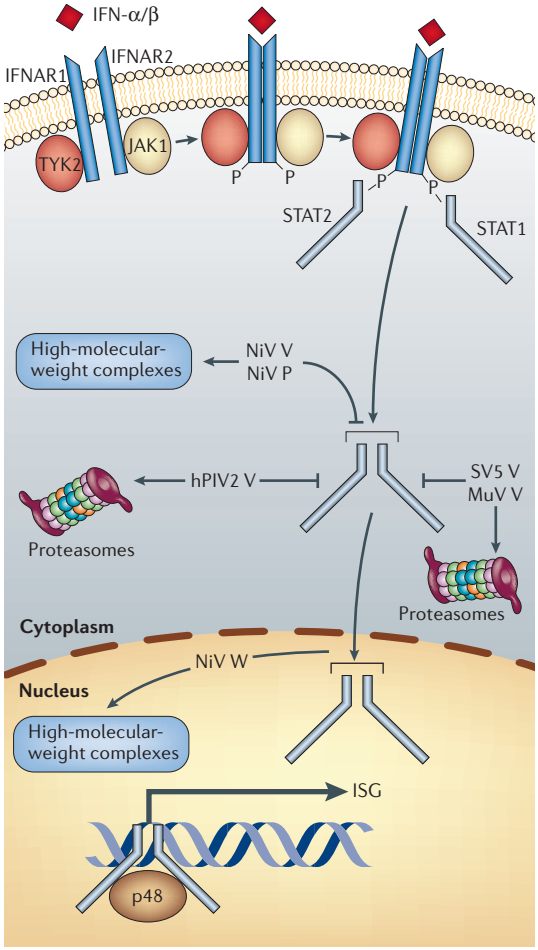


Figure 4 | **Interferon (IFN) signalling.** Type I IFN (IFN-β and a subset of IFN-α) induced as a result of virus infection activates IFN-inducible genes using the Jak-STAT pathway, a signalling pathway shared by many cytokines and growth factors that use members of the Janus tyrosine kinase family (TYK2 and JAK1) and a family of proteins called signal transducers and activators of transcription (STAT)[83,84,90,97]. Type I IFN binds to two heterologous receptor subunits (IFNAR1 and IFNAR2) on the cell surface, and their dimerization leads to the activation of TYK2 and JAK1 tyrosine kinases bound to IFNAR1 and IFNAR2, respectively. TYK2 and JAK1 cross-activate each other and phosphorylate STATs. STAT1 and STAT2 form heterodimers and translocate to the nucleus where, in association with a DNA-binding protein p48 in a complex called IFN-stimulated gene factor 3 (ISGF3), they activate the transcription of genes containing IFN-stimulated response elements within their promoters. Antiviral activity and the additional, profound effects that IFNs have on cellular physiology are mediated by hundreds of IFN-induced proteins. The best characterized antiviral IFN-inducible gene products include dsRNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetases (2-5A) and RNase L and Mx proteins, which inhibit virus replication in various ways[82]. Paramyxoviruses block IFN signalling by targeting specific components of the Jak-STAT pathway (see text).

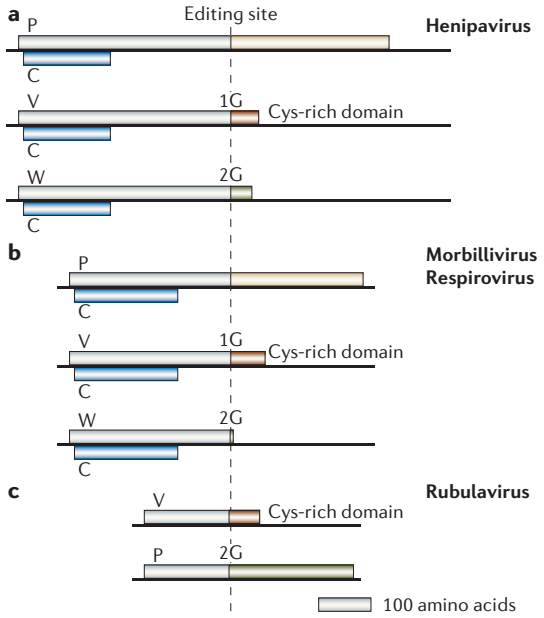
binding activity of the HeV V protein resides in the C-terminal, cysteine-rich terminal domain, like the respirovirus simian parainfluenza virus 5 (SV5), or the N-terminal domain, the site of henipavirus anti-IFN-signalling activity (see below), is not yet known. The NiV V protein blocked activation of IFN-regulatory factor 3 (IRF-3)-responsive promoters in response to intracellular dsRNA signalling, but did not inhibit activation in response to dsRNA signalling through Toll-like receptor 3 (TLR3)[103].

Although the V and W proteins share an extensive N-terminal component, the influence of their C-terminal domains can be seen in the fact that NiV V and W proteins exert antagonistic effects in different cellular compartments and target different steps in the dsRNA-signalling pathways. The W protein contains a nuclear-localization signal in the unique C-terminal domain — an unexpected observation for an RNA virus that replicates solely in the cytoplasm — and inhibits activation of IRF-3-responsive promoters in response to both intracellular dsRNA signalling and signalling through TLR3 (REFS 103–105).

Inhibition of IFN signalling. In uninfected cells, signal transducers and activators of transcription 1 (STAT1) is found in both the cytoplasm and the nucleus, whereas STAT2 seems to have a solely cytoplasmic distribution. However, following IFN treatment, both STAT proteins are located in the nucleus as components of a complex called IFN-stimulated gene factor 3 (ISGF3), which in turn activates transcription of IFN-responsive genes (FIG. 4). Paramyxoviruses inhibit IFN signalling by targeting the STAT proteins and preventing their translocation to the nucleus[83,85,89,90,97,99]. Not surprisingly, the henipaviruses also target STAT proteins, but they use a novel strategy that extends the methods used by paramyxoviruses to abrogate IFN signalling.

In rubulavirus-infected cells, targeted STAT proteins are directed to proteasomes following their polyubiquitylation by a virus-induced ubiquitin ligase, a multi-subunit complex that contains the V protein, cellular cofactors and the target STAT protein. Whereas SV5 targets STAT1 for proteasomal destruction, the anti-STAT activity of human parainfluenza virus 2 (hPIV2) is directed at STAT2 (REFS 101,106,107). In mumps virus (MuV)-infected cells, both STAT1 and STAT3 are eliminated, and STAT2 is left intact[108,109]. Although STAT1 is destroyed in SV5-infected cells, there is an absolute requirement for STAT2 in the ubiquitin-ligase complex[110,111]. In the case of hPIV2, where STAT2 is removed, STAT1 is required for ubiquitin-ligase activity[110,112]. Although the cysteine-rich C-terminal domain of the MuV and hPIV2 V proteins can antagonize IFN signalling when expressed alone, and recombinant hPIV2 and SV5 V proteins lacking the C-terminal domain fail to inhibit IFN signalling, the cysteine-rich domain is not solely responsible for the inhibitory activity[94,113]; regions within the N-terminal region of the V protein are also required[114,115].

Box 3 | **The henipavirus P gene**



The paramyxovirus P gene encodes several proteins by means of internal translation-initiation sites, overlapping reading frames and an unusual transcription process in which one or more non-templated G nucleotides are inserted at a conserved editing site, resulting in a shift of reading frame during translation[127]. The figure shows a schematic representation of mRNAs transcribed from the P gene of henipaviruses compared with those of morbilliviruses, respiroviruses and rubulaviruses. In henipaviruses (**a**) and respiroviruses and morbilliviruses (**b**), the unedited P-gene transcript encodes the P protein, and the V protein is generated by a separate transcript containing a single G nucleotide inserted at the editing site. Insertion of two G residues generates a transcript encoding a protein usually called W. V and W proteins share their amino termini with the P protein. Compared with morbilliviruses and rubulaviruses, henipaviruses have an N-terminal 100–200-amino-acid extension that might have evolved to better equip the viruses to antagonize the cellular interferon response (see text). The P, V and W proteins have unique C-terminal domains. In the P protein, this region is essential for viral RNA synthesis and contains sites for binding to the N and L proteins in ribonucleoproteins. The C-terminal domain of the V protein is highly conserved among paramyxoviruses and contains seven perfectly conserved cysteine residues. The C-terminal domain of the W protein is frequently short because of the presence of a stop codon soon after the editing site, but in henipaviruses the W-specific domain is 43 amino acids in length, compared with 55 for the V-protein C-terminal domain[10]. The P genes of henipaviruses, morbilliviruses and most respiroviruses contain a second short discrete overlapping reading frame upstream of the editing site, which in P, V and W mRNAs encodes the C protein. The structure of the P gene differs in rubulaviruses (**c**), where the primary transcript encodes the V protein, and transcripts with two G nucleotides inserted at the editing site generate the P protein. Note the long 3′ untranslated region of the henipavirus P gene RNAs.

By contrast, respiroviruses such as SeV and hPIV3 and morbilliviruses such as MeV use alternative strategies to block IFN signalling (FIG. 4). SeV and hPIV3 inhibit tyrosine phosphorylation of STAT1, STAT2 or TYK2, a process that requires the accessory C protein[95,100,101,116]. Several anti-IFN-signalling strategies have been proposed for MeV, all of which leave STAT proteins intact but prevent their translocation to the nucleus[117]. In MeV-infected cells, tyrosine phosphorylation of STAT1 and STAT2 is inhibited by the V protein and STAT1 is retained in a complex with the IFN receptor[118,119]. The MeV C protein has also been implicated as an inhibitor of IFN signalling, but its precise role has yet to be confirmed[118,119].

The henipaviruses, on the other hand, broaden the paramyxovirus STAT-targeting strategies by sequestering them in high-molecular-weight complexes[105,120,121] (FIG. 4). Remarkably, this activity does not reside in the cysteine-rich C-terminal domain of the V protein, but in an area upstream that is shared by the V, W and P proteins. This provides henipaviruses with a multi-pronged anti-IFN response in which STAT proteins are sequestered in complexes, consequently abrogating their biological activity[104,122]. The W protein is the most efficient, and the P protein the least efficient antagonist[105]. The V and W proteins of NiV showed IFN-antagonistic activity even when the C gene was mutated and when the domain downstream of the editing site was removed[122]. STAT tyrosine phosphorylation is also inhibited by all three NiV proteins[105,120]. The V and P proteins interact with STAT1 in the cytoplasm, whereas the W protein, armed with its nuclear-localization signal in the W-specific C-terminal domain, co-localizes with STAT1 in the nucleus[103,105].

The henipavirus P gene is larger than any of its paramyxovirus counterparts, and the encoded P, V and W proteins have an N-terminal extension of approximately 100–200 amino acids compared with cognate proteins in the subfamily[12,20] (BOX 3). The minimum domain required for IFN-antagonist activity and STAT1 binding maps to this region, between amino acids 50 and 150 (REF. 105). The NiV C protein also displays modest inhibition of IFN signalling, providing further depth to the multifaceted henipavirus strategy to abrogate IFN signalling, although the mechanism and target are unknown[122].

Not surprisingly, given their role as IFN antagonists, P-gene-encoded proteins of paramyxoviruses have been shown to be virulence and host-range determinants. The pathogenicity of SeV, MeV, hPIV3 and Newcastle disease virus (NDV) depends on virus inhibition of the IFN response, and mutations in proteins expressing anti-signalling activities alter the virus–host relationship in favour of the host[123–126]. It is worth noting that information on the function of the henipavirus W and C proteins was obtained with cells transiently overexpressing the proteins, and it is not known if they are

expressed in virus-infected cells. Immunoblot analysis using monospecific antibodies raised to the unique C-terminal region of the V protein confirms its presence in infected cells[10]. Any correlation between the virulence of henipaviruses and the wide range of anti-IFN strategies that they have acquired awaits the application of reverse genetics and the study of mutant-virus pathogenesis *in vivo*. Nevertheless, it can be speculated that the pathogenicity of henipaviruses in most terrestrial species, compared with their subclinical replication in Chiroptera, might be related to the ability of the virus to circumvent the host IFN response, and it will be of interest to determine the anti-IFN activities of the HeV and NiV P-gene products in chiropteran cells.

Conclusions

The routes by which henipaviruses emerged from obscurity in flying foxes to important pathogens in humans and livestock remain obscure. This is due primarily to our ignorance of virus ecology in bats. Although mechanisms of transmission from bat to livestock and human have been postulated, there are few data on which we can rely to develop models of virus transmission or risk-management strategies for control of diseases caused by henipaviruses in the future. The high virulence of henipaviruses, the absence of therapeutic intervention strategies and vaccines and their classification as BSL4 pathogens have undoubtedly impeded the rate at which information has been generated on the biology and pathogenesis of HeV and NiV. However, recent investigations into the structure and function of henipavirus proteins expressed by cloned P, F and G genes in cultured cells have provided valuable information on the nature of the relationship between henipaviruses and the cells they infect, and suggest explanations for the observed interaction between henipaviruses and their terrestrial hosts.

Two of the biological criteria that differentiate HeV and NiV from other paramyxoviruses are their wide host range and the virulence that they display in their hosts. The susceptibility to henipavirus infection of a range of mammalian species and the similarity in patterns of susceptibility to infection by HeV and NiV are now known to be due, at least in part, to the fact that both viruses use ephrin B2 as a cell receptor, a remarkably conserved surface glycoprotein of ancient lineage and widespread distribution among vertebrates. The widespread cellular distribution of ephrin B2, especially in vascular endothelial cells, also provides an explanation for one of the most frequently observed outcomes of henipavirus infection, namely systemic involvement of endothelial cells. However, it remains to be seen if ephrin B2 will be the universal henipavirus receptor used by all species and all naturally occurring HeV and NiV strains, or variants such as those implicated in the outbreaks of disease in Bangladesh where human-to-human transmission has been documented.

The recent molecular investigations have also revealed several other factors that probably contribute to virulence. The cleavage of the F protein by cathepsin L, a ubiquitous endosomal protease with a cleavage site that is unique among viral glycoproteins, facilitates virus dissemination *in vivo*. The widespread distribution of the protease among organs might also be crucial in the transmission of infectious virus within and between species. It is tempting to speculate that the virulence of henipaviruses is due at least in part to the multifaceted P-gene strategy that these viruses have developed to inhibit the IFN system, a strategy that is novel amongst the paramyxoviruses, having both cytoplasmic and nuclear components.

Several important questions have been raised by recent and varied *in vitro* studies on HeV and NiV. An appreciation of the factors that contribute to the virulence of henipaviruses in terrestrial hosts versus the outcome of virus infection of flying foxes might provide crucial clues. Is ephrin B2 the receptor in bats? In light of the highly conserved nature of murine and human ephrin B2 proteins, especially in the ectodomain, there will probably be significant homology in the ephrin B2 homologue from flying foxes. Does the ubiquity of cathepsin L, the F protease cleavage enzyme, extend to a range of flying-fox cells and tissues? Do henipaviruses use the same range of P-gene products to inhibit IFN in bats? If henipaviruses inhibit dsRNA signalling and IFN signalling in chiropteran cells, their limited replication observed in flying foxes could be due to other factors such as the nature, density and location of the bat cell receptors or the ability of the viral C protein to inhibit viral RNA synthesis in bat cells more effectively than has been observed in mammalian cells[127]. The C protein encoded by the respirovirus P gene has been shown to downregulate viral genome amplification[128,129] and transcription[127,130].

Alternatively, given the well known propensity of bats to tolerate infection with a wide range of viruses in the absence of clinical symptoms, more generic methods could be operative, such as the inhibition of virus replication by lectins such as mannose-binding protein and galectin-1 (REFS 131,132). Galectin-1, an endogenous lectin secreted by various cell types, has been shown to inhibit henipavirus envelope-glycoprotein-mediated cell fusion, probably by aberrantly oligomerizing NiV F and G glycoproteins[132]. In addition to this direct effect on virus replication, galectin-1 might also act indirectly to limit NiV replication because it enhances dendritic-cell production of proinflammatory cytokines such as interleukin 6 (IL-6), which has an essential role in the final differentiation of B cells into antibody-secreting cells[132]. The capacity of henipavirus P-gene products to abrogate the STAT-dependent IL-6-signalling pathway in terrestrial or chiropteran cells remains to be determined. The development of a range of anti-IFN strategies by henipaviruses might have evolved to maximize virus replication under conditions of restricted growth in bats. Finally, the ability to conduct these studies using recombinant molecular biological techniques on otherwise highly pathogenic and dangerous viruses has provided important information on the biology of HeV and NiV, which should prove exploitable in the near future and offer new or novel approaches in treating or preventing henipavirus infection.

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