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Emerging zoonotic viruses: new lessons on receptor and entry mechanisms

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Viruses enter the host cell by binding cellular receptors that allow appropriate delivery of the viral genome. Although the horizontal propagation of viruses feeds the continuous emergence of novel pathogenic viruses, the genetic variation of cellular receptors can represent a challenging barrier. The SARS coronavirus, henipaviruses and filoviruses are zoonotic RNA viruses that use bats as their reservoir. Their lethality for man has fostered extensive research both on the cellular receptors they use and their entry pathways. These studies have allowed new insights into the diversity of the molecular mechanisms underlying both virus entry and pathogenesis.

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Mankind is under a permanent threat from novel pathogens qualified as emerging [1,2••,3]. Here I review the receptors and mode of entry of three emerging zoonotic viruses, responsible for rare but deadly diseases, whose natural reservoir is the bat: severe acute respiratory syndrome coronavirus (SARS-CoV), Hendra (HeV), Nipah (NiV), Ebola (EboV), and Marburg (MarV) viruses.

SARS-CoV: a dangerous affinity

SARS-CoV has a ~30 kb positive RNA genome and the crown-like shape typical of the *Coronavidae*. A regular array of viral spike glycoprotein (S) trimers constitutes the viral envelope. S mediates binding to the cellular receptor Angiotensin Converting Enzyme 2 (ACE2) [4••], and ensures the viral-cell membrane fusion that allows virus entry.

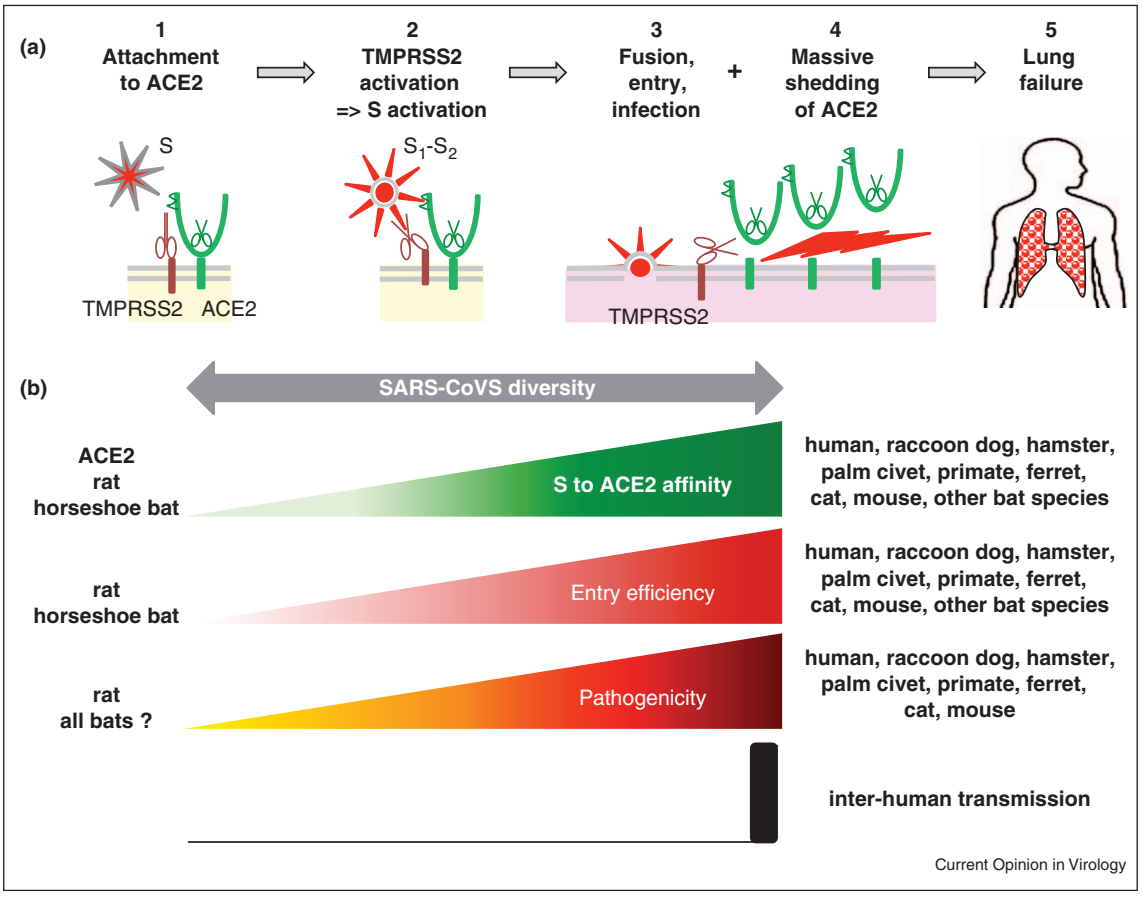
As an ectometalloprotease with monocarboxypeptidase activity, ACE2 cleaves the vasoconstrictor Angiotensin II octapeptide into the vasodilatator Ang1-7 heptapeptide. ACE2 protects the heart, lung and kidney from deleterious vasoconstriction and prevents the onset of an acute respiratory distress syndrome [4••,5–8]. The tissue distribution of ACE2 (pneumocytes I and II, lung epithelium progenitor cells, small intestine enterocytes, kidney, heart cardiomyocytes and endothelium) mostly correlates with the known replication sites of SARS-CoV, and could explain the poor lung repair following SARS infection [4••,9].

The ectopeptidase Type II transmembrane protease serine subfamily member 2 (TMPRSS2) was recently identified as a companion molecule of ACE2 [10•,11•,12•]. TMPRSS2 is detected on the epithelium of the small intestine and respiratory tract, that is the major cell targets of SARS-CoV, but not on the endothelium, which is refractory to SARS-CoV infection [13,14]. TMPRSS2 and ACE2 physically interact [10•]. Only a few S proteins get cleaved by TMPRSS2 to allow a pH- and cathepsin-independent efficient entry of SARS-CoV [10•,12•]. TMPRSS2 cleaves S protein at sites distinct from those ascribed to trypsin and cathepsin L [12•]. Upon contact of ACE2 with S protein, ACE2 is also cleaved by TMPRSS2 [10•]. When expressed on opposing membranes, SARS-CoV S and the ACE2 + TMPRSS2 complex induce intercellular fusion [11•]. However, newly expressed S proteins escape cleavage by TMPRSS2 allowing the production of virions decorated with uncleaved S [10•,11•,12•], possibly because the tripartite association is prevented intracellularly.

The present model of virus entry predicts the following (Figure 1a): SARS-CoV S protein binds to the ACE2 receptor via the concave $S^{424–494}$ region of the receptor binding site (RBD) that cradles over 17 nm^2 of the outer surface of the N-terminal lobe of the ACE peptidase domain, that is outside the enzymatic site [15••]. This activates TMPRSS2 to cleave a few S proteins into fusion-competent S1-S2 homodimers [10•,11•,12•,16], which immediately undergo typical class I fusion protein structural changes [17] which permit the viral envelope to fuse with the plasma membrane. S1-S2 heterodimers are probably too unstable to be incorporated into infectious virus particles [16,18]. Moreover, activated TMPRSS2, and possibly ADAM17/TACE (TNFα converting enzyme) [4••,19], concomitantly cleave ACE2. This results in the massive shedding of ACE2 ectodomains [10•], probably due to amplification of the constitutive pathway [5].

ACE2 shedding is not required for SARS-COV entry [5,20], but is probably responsible for the associated major lung failure. Indeed, soluble S both induces ACE2 shed-

Figure 1



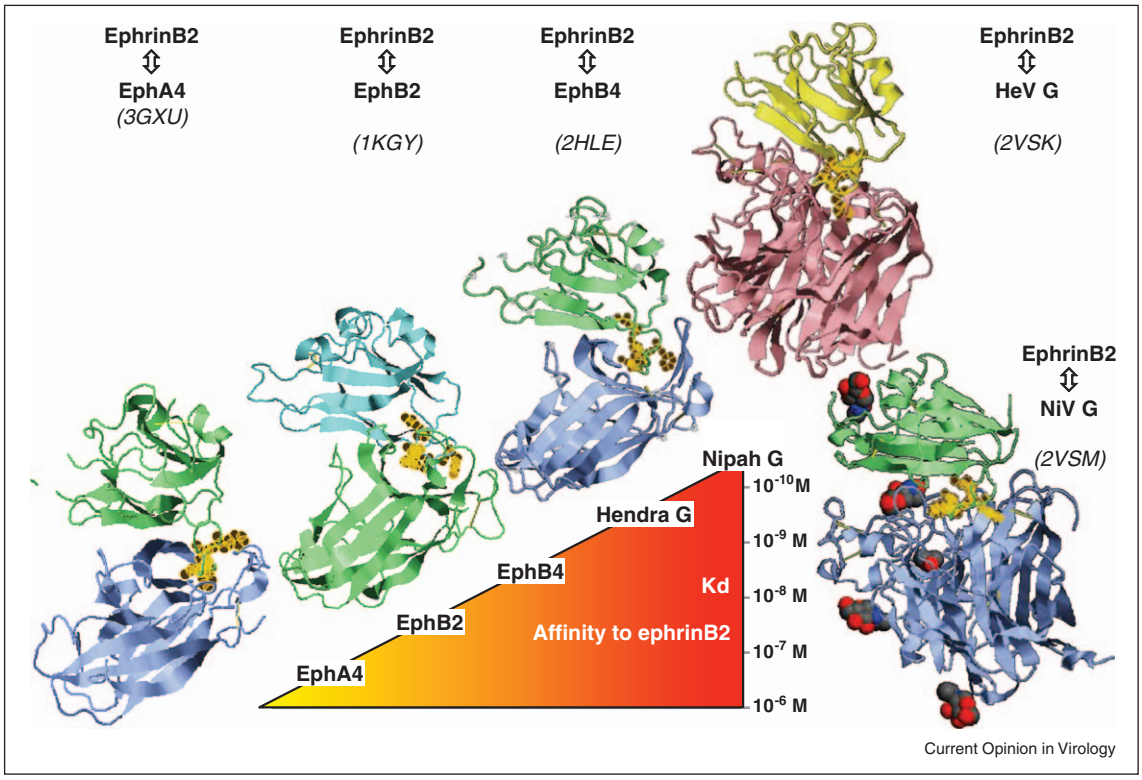
(a) Entry steps of SARS-CoV and pathogenic consequences, with (1) virus attachment to the cell via binding of S to ACE2, (2) activation of TMPRSS2 ectoprotease that leads to the cleavage and activation of virus S envelope protein into the fusion competent S1–S2 heterodimer, (3) fusion of the virus envelope with the plasma membrane to deliver the nucleocapsid into the cytoplasm and allow virus replication, (4) proteolytic cleavage by TMPRSS2 (and/or ADAM17/TACE) ectoprotease with shedding of the ectodomain of the majority of ACE2 molecules independently of their use by SARS-CoV leading to (5) lung failure. **(b)** Relationship between the affinity of SARS-CoV S protein with the ACE2 receptor, entry efficiency, pathogenicity and inter-human transmission.

ding and worsens the clinical signs of SARS [21]. In humans and according to virus strain diversity, S/ACE2 affinity correlates with the efficacy of virus entry, the level of ACE2 cleavage and the intensity of the pathology. Furthermore, the highest affinity is associated with inter-human transmission [20,22••] (Figure 1b). Antibodies targeting the S binding site on ACE2 strongly inhibit viral infection [23]. The correlation between S/ACE2 affinity and SARS-CoV pathogenicity extends to the host range for other mammal species (except bats) in determining whether a particular ACE2 protein can act as a receptor for SARS-CoV or not (including among bats) (Figure 2b) [23–27]. Consequently, the accuracy of an animal model of human infection critically depends on the capacity to mimic the affinity between the human ACE2 and viral S protein. In mammals, except bats, SARS-CoV induces a 'toxic-like' syndrome by triggering a devastating massive cleavage of the cellular receptor used for entry, the molecular basis of which remains to be documented. Why bats do not exhibit clinical signs of infection by SARS-CoV remains puzzling.

Henipaviruses: a universal receptor?

NiV and HeV constitute the *Henipavirus* genus of the *Paramyxoviridae* family and are responsible for fatal respiratory and neurological diseases. Their non-segmented negative strand RNA genomes code for two envelope glycoproteins. The fusion protein F is synthesized as a precursor maturated into a functional F1–F2 heterodimer by cathepsin L via a clathrin-mediated recycling endosomal pathway [28,29]. The attachment protein G is a tetramer consisting of two disulfide bridged dimers. Like the *morbillivirus* H and *parainfluenzae* HN proteins, its C-terminal globular head is folded into a six β-sheet blade propeller surmounting a stalk, transmembrane region and cytosolic tail. The sugar-free β1–β6 dimer interface is

Figure 2



Crystal structure of ephrinB2 in complex with EphA4, EphB2, EphB4, HeV and NiV G showing nearly identical binding modes and affinity ranking of ligands. Structures (PDB identification codes indicated in parenthesis) were drawn using FirstGlance in Jmol (http://molvis.sdsc.edu/fgij/). Phe120, Asn123, Trp125 and Leu129 of the F–G loop of ephrinB2 are decorated with golden circles for easier perception.

conserved. The two heads rotate relative to each other by 0°, 63° and 30–40° for henipavirus G, measles virus H and HN, respectively, while the buried area is 9–10 nm^2 for G and H against 18 nm^2 for HN [30]. The sugars of G are poor in the DC-SIGN ligand oligomannose but rich in the sugar moiety recognized by LESCtin, a lectin specifically expressed on the sinusoidal endothelial cells of lymph node and liver [31].

G protein attaches to the cellular receptors ephrinB2 and ephrinB3 [32]. Ephrins are ligands of Eph receptor tyrosine kinases involved in cell homeostasis [33•]. EphB4 and its ligand ephrinB2 are expressed on the endothelial cells of veinules and small arteries, respectively [34]. Their reciprocal trans-endocytosis acts as a repulsive signal during vasculogenesis [33•]. EphrinB2 is also expressed in the smooth muscle cells of vessels [34]. The tissue distribution of ephrinB3 is restricted to the spinal cord and the *corpus callosum*. [35]. EphrinB2/B3 expression correlates with the tropism of NiV in infected humans [32].

Ephrin binding sites on NiV and HeV G map to the top of the globular head over the β5 and β6 blades [36••], that is away from the center and side of the propeller, where sialic acid and protein receptors bind parainfluenza HN proteins and measles H, respectively [37]. Astonishingly, both the physiological ligands of ephrinB2, EphB2, EphB4 and EphA4, and the G proteins of NiV and HeV bind the same site (F_{113}–K_{131}) on the G–H loop [30,31,36••,38,39] (Figure 2). However, the henipavirus G proteins exhibit the highest affinity to ephrinB2 [38,40•].

EphrinB2 from mammal species, including human, horse, mouse, pig, cat, dog, and bat, can act as efficient cellular receptor for NiV. Indeed, the G–H loop FTIKFQE(-F,Y)SPN**LW**G(L,H)EF sequence is highly conserved between ephrinB2 and ephrinB3 including in those from the distantly related species zebrafish and chicken as supported by successful replication of NiV in chicken embryo [41]. Bronchial epithelium gets infected in the pig and cat [42–44] but not in human [45] suggesting ectopic expression of ephrinB2/3 in the former species. A **LW**/YM substitution prevents ephrinB1 from acting as a receptor for NiV and HeV and binding to EphB4 and EphB2 receptor [35,46,47]. Correlatively, (i) all ligands compete with each other, (ii) the ephrinB2/3 binding site on NiV-G is exquisitely neutralized by specific antibodies *in vitro* and *in vivo* [48,49•] and (iii) there is a cross-protection between NiV and HeV [50]. The affinity of

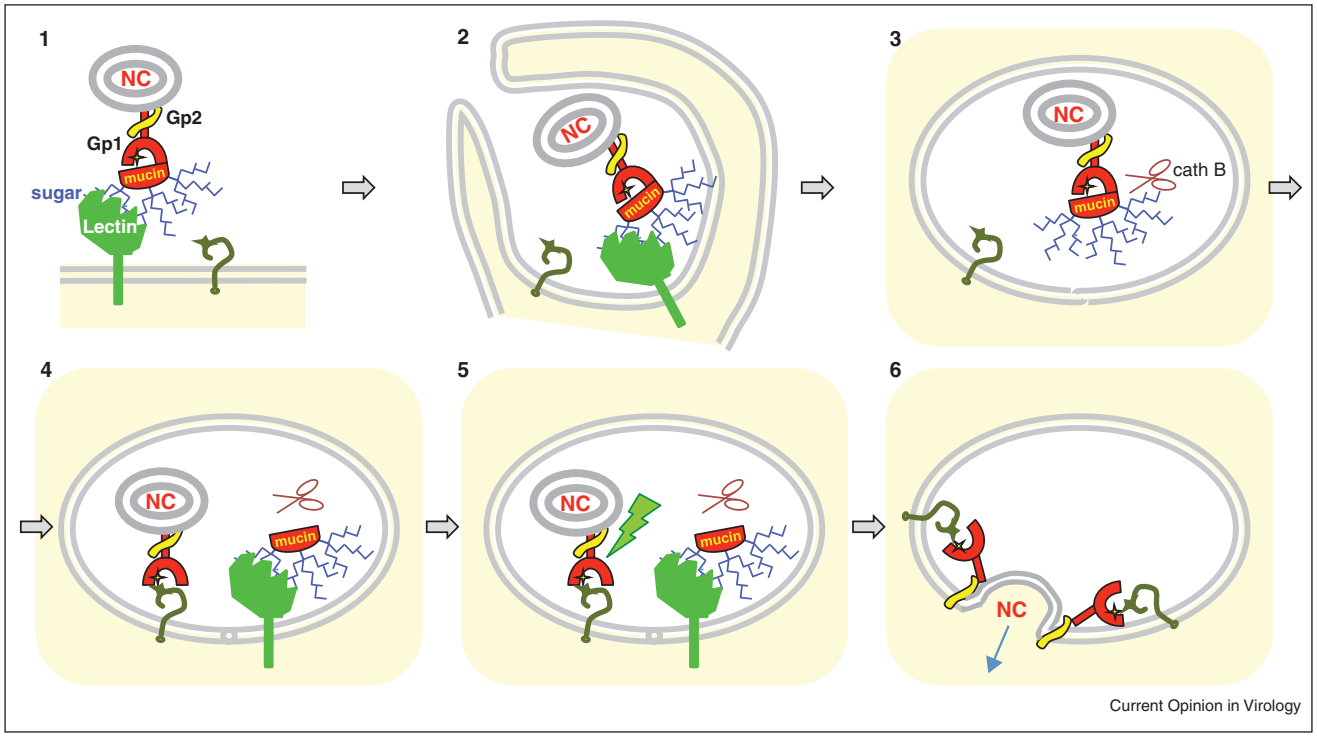
NiV-G and HeV-G for ephrinB2/B3 correlates with the efficiency of virus entry [48,51].

Interestingly, whereas NiV G and F induce fusion of cells expressing ephrinB2/B3, NiV preferentially enters after internalization via macropinocytosis [52••], though acidic pH is not required [52••,53]. Virus entry, but not membrane fusion, is inoperative when the cytosolic tail of ephrinB2 has its PDZ-binding motif deleted or Tyr_{304} mutated [52••]. These two motifs recruit Grb4 and the P21-activated kinase 1 (Pak1)/CdC42/Rac1 complex that govern macropinocytosis [33•,54,55,56•,57–59]. The need for macropinocytosis while the fusion machinery is operative at the cell surface is puzzling. Macropinocytosis occurs very rapidly upon contact [60] and could be faster than the fusion step but then macropinocytosis inhibitors would not be expected to prevent virus entry. Several hypotheses can be proposed: (i) fusion requires a specific Ca^{++} [61] and/or Na^{+} ionic environment as documented for Semliki Forest virus [62]. (ii) NiV replication requires a specific conditioning of the cytoplasm induced by contacting ephrinB2/B3. (iii) The nucleocapsid needs to reach a particular cytoplasmic location deeper in the cells, more favorable for viral polymerase activity. The latter would not be unprecedented since forced rerouting of virus normally entering by fusion at the cell surface into the endocytic pathway results in hampered infectivity as shown for pseudotyped measles virus and lentivectors [63–65].

Filoviruses: an elusive receptor

The *Filoviridae* EboV and MarV cause severe hemorrhagic fevers and septic-like shock in humans [66]. Their non-segmented negative RNA genomes code for the envelope glycoprotein GP which ensures both attachment to a (still elusive) cellular receptor and membrane fusion. GP is cleaved by a furin-like protease into mature GP1–GP2 heterodimers [67]. Curiously, mutation of the furin-cleavage site does not abolish GP-mediated virus entry due to alternative cleavage [68]. GP is heavily glycosylated with sugar moieties recognized by LESCtin and DC-SIGN/R lectins that can enhance but not mediate infection [69–72]. This high glycan content shields MHC class I and β-integrin from antibody recognition [73,74], a finding that explains the previously reported apparent downregulation of the latter [75].

Figure 3



Model of filovirus entry. Virus binds to the cell surface via recognition of sugar moieties (blue branches) of GP1 by DC-SIGN or LECStin (light green) **(1)** to be immediately internalized by macropinocytosis **(2)** and migrate through the endocytic pathway **(3)** until the mucin-like domain (mucin) is cleaved off from GP1 by resident cathepsin B (cath B). This results in RBR (yellow star) being accessible for binding to the postulated receptor (dark green question mark) **(4)**. An additional activation event (disulfide bridge reduction?) occurs **(5)**. This triggers the conformational changes of GP2 (yellow) that mediates fusion of viral and endosomal membranes and ensures the delivery of the nucleocapsid (NC) into the cytosol (pale yellow background) where replication occurs **(6)**. Because the postulated receptor (question mark) is predicted to be expressed at the cell surface, it has been included in every step of virus internalization. Although not yet documented, endosome structures 4 to 6 may successively represent early, maturing, late and possibly endolysosomes, because of the ~1 h delay between the cell attachment and membrane fusion steps.

A cellular receptor of glycoprotein nature is predicted on the basis of saturable binding of soluble GP [76] and loss of binding after protease, periodate or tunicamycin treatment [76,77]. In infected animals, the virus disseminates in many tissues [66]. The EboV receptor is stocked in trans-Golgi network membranes in all cell types including the non-permissive T and B lymphocytes. It is exported to the cell surface upon cell adhesion and internalized via a microtubule-dependent and actin-dependent pathway, respectively [78•,79•]. EboV and MarV GP cross-compete for binding suggesting the use of a common receptor [76,80]. However, 3 out of 4 key lysines (at positions 114,115 and 140) defining the receptor binding region (RBR) of EboV GP1 [76] are not conserved in MarV GP1 [81••]. The structure of a soluble trimeric form of GP1–GP2 reveals a GP1-based chalice form, lined by the RBR. The fusion competent GP2 trimers cradle the chalice stem, with the internal fusion peptide flanked by two β-sheets. The RBR is mostly shielded by a glycan cap and a mucin-like domain [82••], the cleavage of which by cathepsins strongly enhances GP1–GP2 binding to the cell surface [76,83]. However, lowering the pH neither allows EboV entry at the cell surface, nor cell–cell fusion by mucin-deleted GP1–GP2, and the GP/receptor interaction is stable at acidic pH [76,77,84••].

In effect, EboV mostly enters by macropinocytosis with a requirement for lipid rafts, the Na^+/H^+ exchanger, Pak 1, Rac1, Rab5, Rab7, RhoC GTPase and the vacuole closure protein C-terminal binding protein 1 of E1A, CtBP/BARS [59,85••,86••,87–89]. Constitutive macropinocytosis in dendritic cells and macrophages fits with their permissiveness to EboV infection [90,91]. Activation of Ax1 enhances both macropinocytosis and EboV entry [92] although the latter may be mediated by serum Gas6, which was recently reported to mediate non-specific entry for several enveloped viruses [93].

The EboV (and MarV) entry process lasts for about 1 h [94,95] and can be schematized as follows (Figure 3): Firstly, (i) EboV attaches to the cells via the GP1/GP2 interaction with DC-SIGN/R and/or LECStin and is (ii) immediately internalized by constitutive and/or virus-contact-induced macropinocytosis. (iii) After ~30 min of endocytic trafficking, EboV reaches a late endosomal compartment, where (iv) the resident cathepsin B cleaves off the mucin-like domain [83,84••,96] to (v) expose GP1's RBR so that the putative receptor can be recruited; then, (vi) a late pH-dependent activation step of the mucin-deleted GP1/GP2 complex triggers the fusion activity of GP2, possibly via the reduction of a disulfide bridge [84••].

In conclusion, several lessons can be taken home. (i) Susceptibility to a disease can be driven by the affinity level between the viral attachment glycoprotein and its cellular receptor. (ii) Evolutionary conserved orthologs of a viral receptor can allow an extended host-range. (iii) A timely proteolytic activation of membrane fusion can occur only upon binding to the receptor. (iv) A viral glycoprotein may follow a complex maturation pathway during endosomal trafficking.

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