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Christian K Pfaller
Mayo Clinic

Roberto Cattaneo
Mayo Clinic

Matthias J. Schnell
Thomas Jefferson University, Matthias.Schnell@jefferson.edu

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Reverse genetics of *Mononegavirales*: How they work, new vaccines, and new cancer therapeutics

Christian K. Pfaller[a], **Roberto Cattaneo**[a,*], and **Matthias J. Schnell**[b,c,**]

[a]Department of Molecular Medicine, Mayo Clinic, Rochester, MN 55905, USA

[b]Department of Microbiology and Immunology, Philadelphia, PA 19107, USA

[c]Jefferson Vaccine Center, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA

Abstract

The order *Mononegavirales* includes five families: Bornaviridae, Filoviridae, Nyamaviridae, Paramyxoviridae, and *Rhabdoviridae*. The genome of these viruses is one molecule of negative-sense single strand RNA coding for five to ten genes in a conserved order. The RNA is not infectious until packaged by the nucleocapsid protein and transcribed by the polymerase and co-factors. Reverse genetics approaches have answered fundamental questions about the biology of *Mononegavirales*. The lack of icosahedral symmetry and modular organization in the genome of these viruses has facilitated engineering of viruses expressing fluorescent proteins, and these fluorescent proteins have provided important insights about the molecular and cellular basis of tissue tropism and pathogenesis. Studies have assessed the relevance for virulence of different receptors and the interactions with cellular proteins governing the innate immune responses. Research has also analyzed the mechanisms of attenuation. Based on these findings, ongoing clinical trials are exploring new live attenuated vaccines and the use of viruses re-engineered as cancer therapeutics.

Keywords

Mononegavirales; Reverse genetics; Tropism; Attenuation; Pathology; Receptors; Tropism; Innate immunity; Vaccines; Cancer therapy

Mononegavirales: non-segmented negative strand RNA viruses

Non-segmented negative strand RNA viruses (NS-NSVs) are a large group of different viruses found both in animals and plants. NS-NSVs include several major human, animal, and plant pathogens that have a great impact on human health and are commercially very important. The order of *Mononegavirales* contains 5 families: Bornaviridae, Filoviridae, Nyamiviridae, Paramyxoviridae, and *Rhabdoviridae* (Taxonomy, 2013) (Fig. 1).

*Corresponding author. Cattaneo.Roberto@mayo.edu (R. Cattaneo) . **Corresponding author at: Department of Microbiology and Immunology, Philadelphia, PA 19107, USA. Matthias.schnell@jefferson.edu (M.J. Schnell). .

The *Bornaviridae* family includes only one genus *Bornavirus*. Borna disease virus (BDV) causes severe neurobehavioral changes in horses and sheep; there are conflicting reports about BDV infecting humans and causing disease (for review (Kinnunen et al., 2013)). Interestingly, BDV replicates in the nucleus of the infected cell. Nevertheless, BDV has the typical genome organization of a NS-NSV (Fig. 2) with a nucleoprotein (N), polymerase cofactor (X/P), matrix protein (M), surface glycoprotein (G) and polymerase (L).

The family *Filoviridae* was named after the Latin noun *filum* meaning “thread” because of their long “thread-like” virions of up to 800–1200 nm. The genome organization generally follows the pattern of other NS-NSV wherein VP35 is the polymerase cofactor and VP40 is the matrix protein (Fig. 2). Ebola virus (EBOV) recently caused a large outbreak in West Africa with thousands of deaths and worldwide repercussions. The *Filoviridae* family was reclassified in 2014 into three genera (*Ebolavirus*, *Marburgvirus*, and *Cuevavirus*) with the former two of them being most important for human disease (Kuhn et al., 2014).

The new family *Nyamiviridae* (Taxonomy, 2013) contains the single genus *Nyavirus* with the two species Nyamanini virus (NYMV) and Midway virus, which were isolated from insects and birds (Mihindukulasuriya et al., 2009). Like BDV, NYMV replicates in the nucleus (Herrel et al., 2012).

The *Paramyxoviridae* family is large and divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The latter includes two genera, *Pneumovirus*, which includes human Respiratory syncytial virus (RSV), an important human pathogen discussed in more detail below. The other genus is *Metapneumovirus*, which includes the important pathogen human Metapneumovirus (hMPV). Interestingly, hMPV and RSV do cause a very similar disease. hMPV was isolated for the first time in 2001, but nevertheless might be the second most important virus for lower respiratory tract infection in humans after RSV (for review (Falsey, 2008)). An unusual feature for *Pneumovirinae* compared to other NS-NSVs is that they encode two nonstructural proteins (NS1 and NS2), which are located upstream of the nucleoprotein within the genome (Fig. 2) and interfere with the host innate immunity.

The other subfamily, *Paramyxovirinae* is divided into seven genera, two of them including several viruses of great importance for human and animal health. Typically, viruses of the *Paramyxovirinae* subfamily encode six or seven genes. Viruses are divided into different genera depending on two characteristics: (1) expression of one or two additional proteins (called V and C) from their P gene; and (2) neuraminidase activity of the attachment glycoprotein (hemagglutinin, H or hemagglutinin-neuraminidase, HN) (Lamb and Parks, 2013). A fairly newly discovered fish-infecting paramyxovirus, Atlantic salmon paramyxovirus, established the new genus *Aquaparamyxovirus*. Avian paramyxoviruses, among them the species prototype Newcastle disease virus (NDV) form the genus *Avulavirus*. Fer-de-Lance paramyxovirus is a reptile virus and represents the genus *Ferlavirus*. The two viruses in the *Henipavirus* genus, Hendra (HeV) and Nipah virus (NiV) are emerging infectious pathogens naturally harbored by bats, but highly pathogenic when transmitted to human or other mammals, causing respiratory or neurological diseases. Distinct from other viruses in the subfamily, the attachment protein is not called H, but simply glycoprotein (G). The genus *Morbillivirus* consists of closely related, but highly

host-specific viruses including Measles virus (MeV), Canine distemper virus (CDV), and Rinderpest virus (RPV). Although infecting epithelial tissues of their host, a common feature of these viruses is lymphotropism and virus-induced immunosuppression. The attachment protein H does not have neuraminidase activity. The genus *Respirovirus* includes several parainfluenza viruses, which cause flu-like respiratory diseases. The murine prototype, Sendai virus (SeV), is among the most important model paramyxoviruses. Parainfluenza viruses (PIVs) type 1 and 3 are human pathogens, which are similarly problematic as RSV and hMPV from the *Pneumovirinae*. The seventh genus, *Rubulavirus* consists of a second group of parainfluenza viruses, among them the important human pathogens Mumps virus (MuV), human Parainfluenza viruses type 2 and 4, and Parainfluenza virus type 5, formerly known as Simian virus 5 (SV5). Although pathogenesis of these viruses is similar to respiroviruses, they are grouped into a distinct genus, because they do not express a C protein and encode a small hydrophobic membrane protein (SH).

The *Rhaboviridae* family contains both animal and plant viruses, which are divided into 11 genera with 71 species. The best know species infecting human and animals is Rabies virus (RABV) of the *Lyssavirus* genus, which contains 13 other species causing a rabies-like disease in most mammals. Whereas classical RABV does cause 99% of human cases, the other species are important because the current RABV does not protect against several of them (Evans et al., 2012). The other well-known member is vesicular stomatitis virus (VSV), which is “the model” rhabdovirus because it has been widely used to study NS-NSV molecular virology and biochemistry. Both *Lyssavirus* and *Vesiculovirus* have only the five genes defining the basic NS-NSV genome organization (Fig. 2).

NS-NSVs have very specific or broad host tropism, depending on the virus. For example, RABV has a wide host range and can infect most mammals. On the other hand, MeV infects only certain primates. While NS-NSVs have different host(s) and tissue tropism and come in different shapes and sizes, their genomes are similarly organized and they have, with a few interesting variations, a similar replication process.

Replication of NS-NSVs

The genome of all NSVs is a single-stranded RNA of negative polarity, which can be one molecule (NS-NSV) or multiple segments for segmented negative-strand RNA viruses (S-NSV). Fig. 3 shows the replication cycle of RABV. The mode of replication and specific elements required for replication and transcription are conserved for all NSVs. The ribonucleoprotein (RNP) is the functional template for replication and transcription. As the name suggests, RNP is RNA associated with a protein. Both the minus-strand genome and the plus-strand anti-genome are encapsidated into the nucleoprotein (N or NP); the naked RNA is not infectious (Conzelmann, 2004). Encapsidation follows a common mechanism for all NSV (Green et al., 2014). This is an important feature of NSVs because such a RNP-template must be formed when a new virus is created *de novo* from cDNA. This encapsidation step has been a major challenge to the generation of NSVs from cDNA (see below). In order to begin replication, NSV need a complex composed of the viral polymerase (L), the catalytic enzyme, and the non-catalytic phospoprotein (P), in addition to the RNP (Conzelmann, 2004).

NS-NSV transcription begins when the viral polymerase recognizes the 3′ end of the genome and transcribes a short leader RNA followed by the viral genes. These are flanked by conserved sequences that signal to the polymerase complex to start or stop (Fig. 3) (Whelan et al., 2004). Transcription is not always successfully reinitiated after each stop sequence; this lack of polymerase reinitiation results in a 3′-5′ transcription gradient. For example, the VSV transcript levels are reduced by about 20% at each gene junction (Iverson and Rose, 1981), and similar observations have been reported for MeV and other NS-NSVs (Cattaneo et al., 1987).

The polymerase complex may switch to replication mode as function of the amount of N and/or P protein available. In replication mode, the transcription start-and-stop signals are ignored and full-length NP-encapsidated anti-genomic RNA is synthesized (Barik and Banerjee, 1992; Gupta and Banerjee, 1997). The antigenomic RNP serves as template for the production of more genomic RNPs. The matrix (M) protein organizes the assembly of these genomic RNPs and the surface glycoprotein(s) and controls transcription (Whelan et al., 2004).

Reverse genetics: how did it all begin?

The foundations for RNA virus reverse genetics were laid in 1978 by the observation that full-length cDNA copies of bacteriophage Qbeta RNA cloned into a plasmid form plaques after transfection of *E. coli* (Taniguchi et al., 1978). Three years later a similar observation was reported for the eukaryotic Poliovirus (Racaniello and Baltimore, 1981). While the subsequent development of reverse genetics for many more positive strand RNA viruses was relatively straightforward, the complex mechanisms of NSV replication initially represented a difficult barrier to overcome. However, in 1989 the Palese group showed that a marker gene (chloramphenicol acetyl transferase, CAT) can be temporarily expressed by influenza virus (Luytjes et al., 1989). Luytjes et al. inserted the CAT open reading between the genomic 3′ and 5′ ends of a synthetic viral RNA, utilized purified NP and PBA, PB1, and PB2 protein to create a synthetic RNP genome segment *de novo*, transfected it, and detected CAT activity after infection with standard virus.

When attempts to recover infectious virus by encapsidating NS-NSV genomes *in vitro* and transferring them into virus-infected cells failed, focus shifted to short artificial RNA derived from the 3′ and 5′ end of the genome containing a marker gene (CAT) or to short, naturally-occurring genomes of defective interfering (DI) viral particles. Park and Krystal established a system to create a synthetic DI-RNA for SeV. They fused the T7 RNA polymerase promoter 5′ to the viral genome and linearized the plasmid to generate the 3′ genome end (Park et al., 1991). After *in vitro* transcription, this synthetic RNA was transfected into cells, and CAT activity was detected after helper virus infection. Collins and colleagues reported the recovery of a RSV-derived DI with almost 50% of the genome length (Collins et al., 1993).

In 1992, the Wertz group utilized a hepatitis delta virus (HDV)-derived ribozyme to create an exact 3′ end of the RNA instead of just linearizing the plasmid encoding the genomic RNA. In combination with the expression of the viral replication proteins through the

vaccinia virus T7-RNA polymerase expression system (Fuerst et al., 1987), this resulted in efficient and reproducible recovery of the synthetic RNP (Pattnaik et al., 1992). Analogously, the Conzelmann group used a similar system to recover several artificial RABV genomes expressing two different marker genes (Conzelmann and Schnell, 1994). Importantly, recovery efficiency of these synthetic model RNAs was inversely proportional to genome size (Conzelmann and Schnell, 1994).

A positive approach for the recovery of a negative sense RNA virus

The new approach that allowed the first successful recovery of a NS-NSV (Schnell et al., 1994) was based on the use of anti-genomic (plus strand) rather than the genomic (minus strand) RNA that was previously used for experiments based on synthetic DI genomes. Use of plus-strand RNA appeared counterintuitive, but Schnell et al. were concerned that simultaneous expression of naked negative sense genomic RNA and positive strand mRNAs would result in hybridization and generation of double strand RNA, inducing interferon while also reducing successful encapsidation. Indeed, RABV was rescued from a positive strand cDNA (antigenome), but not when the negative strand genome was transcribed (Schnell et al., 1994).

The Rose (Lawson et al., 1995) and Wertz (Whelan et al., 1995) laboratories then used variants of the positive approach to successfully recover genetically marked recombinant VSV and several other groups recovered recombinant viruses from the families *Paramyxoviridae* and *Filoviridae* using similar or slightly modified systems as for RABV (Table 1 and references therein). Several groups attempted virus recovery using both the anti-genomic and the genomic RNA, but only the anti-genomic, positive strand RNA worked. There has been one exception: the Nagai group recovered recombinant SeV by expression of the negative strand genome. However, rescue efficiency from anti-genomic RNA was about 100-fold lower than from genomic RNA (Kato et al., 1996).

Alternative recovery systems

Stable cell lines expressing the viral replication proteins have been used as alternative virus recovery systems. The Billeter group recovered the first paramyxovirus in 1995 by using a cell line stably expressing MeV N, P and T7 RNA polymerase (Radecke et al., 1995). After transfection of a plasmid expressing the MeV anti-genome and a plasmid expressing MeV L, infectious genetically marked MeV was rescued with high efficiency (Radecke et al., 1995). Buchholz et al. generated a BHK-derived cell line stably expressing T7 RNA polymerase and recovered a slow, replication-impaired BRSV mutant (Buchholz et al., 1999). This indicated that the system was very efficient and remediated the cytolytic and inhibitory effects of vaccina virus. A key parameter for efficient virus rescue is the synthesis of genomes with correct 5′ and 3′ ends. T7-dependent transcription of viral antigenomes added three non-virus G bases. While these bases were removed during replication, their presence interfered with efficient RNP formation. To address this problem, Le Mercier et al. introduced a hammer-head ribozyme (HamRz) after the GGG of the T7 promoter (Le Mercier et al., 2002), which created an exact 5′ end, improving rescue efficiency. McGettigan et al. introduced the sequence of HamRz into the RABV vaccine vector SPBN,

which allowed recovery of a RABV-based vaccine construct (McGettigan et al., 2003). Analogously, for HeV and NiV, the introduction of a HamRz in front the 5′ antigenome greatly improved recovery efficiency (Yun et al., 2014). Moreover, use of a more efficient HDV ribozyme to cleave the correct 3′ genome end improved the recovery frequency of RABV 100-fold (Ghanem et al., 2012).

In summary, the recovery of NS-NSVs has come a long way. The recovery frequency for RABV was initially ~1 focus forming unit (ffu) for 10^7 cell in the vaccinia-based system (Schnell et al., 1994). It improved by a factor of 10 on T7 cells when the 3′ ends were cleaved with a core HDV ribozyme. Precise and efficient cleavage of both ends yielded 1 ffu for 10^4 cells (Ghanem et al., 2012). However, there is room for improvement. If efficiency of the recovery improved, it would become possible to obtain even viruses with certain classes of lethal mutations, *e.g.* virus particles could be recovered but would not be able to reinfect or replicate. However for such approaches to become possible, another new key finding, such as the use of the antigenome and the creation of the exact 3′ and 5′ end of the genome, will be required.

Better understanding of the life cycle of NS-NSV by reverse genetics

While *Mononegavirales*, especially VSV, have contributed greatly to our understanding of molecular and cell biology, their life cycle in natural hosts is less well understood. For example, before the advent of reverse genetics, very little was known about where viruses replicate immediately after contagion, which made it impossible to understand the series of events leading to disease. One of the motivations for the development of reverse genetics was to apply new experimental approaches to study tissue tropism and pathogenesis.

Fluorescent reporters expressed by recombinant viruses now provide a simple way to follow their spread through the host organism and to identify target tissues and cell types infected. RABV is now a widely used tool to study neuroanatomy and neuronal connection. Whereas such study was initially performed with naturally-occurring RABV strains (Kelly and Strick, 2000), reverse genetics opened new possibilities utilizing vector with marker and modified tropism (Wickersham et al., 2013).

For pathogenicity studies, we will focus here on the morbilliviruses MeV and CDV. How these viruses cause immunosuppression has been a key question (Schneider-Schaulies and Schneider-Schaulies, 2009). Until recently textbooks suggested that morbilliviruses replicated in the epithelia in the airways immediately after contagion, and that the infection eventually somehow caused immunosuppression. But in 2000, researchers identified a protein expressed on the surface of immune cells, the signaling lymphocyte activation molecule (SLAM), as the primary receptor for MeV (Tatsuo et al., 2000). One year later it was observed that MeV entered well-differentiated primary airways epithelia much more efficiently from the basolateral side than from the apical side (Sinn et al., 2002). Taken together, these two observations suggested that MeV and the other morbilliviruses may take advantage of SLAM-expressing alveolar macrophages to traverse the respiratory epithelium immediately after contagion, allowing rapid spread in lymphatic organs and causing immunosuppression.

This hypothesis was tested in the ferret model with a new CDV expressing the green fluorescent protein (GFP). Indeed it was documented that this virus replicated briskly in local lymph nodes and primary lymphatic tissues before spreading to airways epithelia (von Messling et al., 2004). This sequence of events was confirmed through the infection of macaques with GFP-expressing MeV able to enter cells through either SLAM or through the epithelial receptor nectin-4 (Lemon et al., 2011; Leonard et al., 2010, 2008; Muhlebach et al., 2011). Thus immunosuppression occurs at least in part due to rapid, very efficient virus replication in primary and secondary immune organs. These studies, which have fundamentally altered our understanding of morbillivirus pathogenesis, have been made possible by reverse genetics techniques.

Virulence: innate immunity control proteins

Viruses must control the innate immune response to replicate efficiently in a host. The *Mononegavirales* do this by targeting both the interferon (IFN) induction and IFN signaling pathways, as discussed in recent reviews (Gerlier and Lyles, 2011; Goodbourn and Randall, 2009; Parks and Alexander-Miller, 2013; Ramachandran and Horvath, 2009; Rieder and Conzelmann, 2009; Schneider et al., 2014).

Analysis of the mechanisms by which individual viruses counteract the host innate immune response has progressed rapidly in recent years. It has become evident that even viruses with small genomes counteract the interferon system by interacting with several of its components. For example, viruses of the *Paramyxovirinae* subfamily express either one or both types of accessory proteins named V and C. These proteins are dispensable for virus spread in certain transformed cell lines. However, their deletion leads to strong attenuation in natural hosts, where infection induces adaptive immune responses of similar magnitude as those of wild type infections (Devaux et al., 2008; Kato et al., 1997; von Messling et al., 2006).

The V proteins of paramyxoviruses inhibit innate immune responses by binding to the cytoplasmic double-stranded RNA (dsRNA)-receptor melanoma differentiation-associated protein 5 (mda5), as well as the signal transducers and activators of transcription 1 and 2 (STAT1, STAT2) (Parks and Alexander-Miller, 2013). Mapping of the interaction sites of each of these cellular proteins on the V protein of Morbilliviruses has allowed a new approach to the assessment of the relevance of individual viral proteins for the interactions with specific hosts. A recombinant MeV that was unable to antagonize STAT1 function was generated, and its virulence was analyzed in rhesus monkeys (Devaux et al., 2011). This recombinant virus could not control inflammation and was attenuated in rhesus monkeys. On the other hand, when essentially the same study based on STAT1 interactions abrogation was performed with CDV, it resulted in a virus that had minimal effects on CDV pathogenesis in ferrets (Svitek et al., 2014). This finding indicated STAT1 inhibition is insufficient to disrupt the innate immune response *in vivo*. On the other hand, in this virus-host pair, the mda5 and STAT2 interactions played an essential role in pathogenesis, and their ablation completely attenuated the virus (Svitek et al., 2014). Thus, even if reverse genetics is allowing a systematic approach to the study of the relevance of different innate

immunity interactions for virulence, the results are not always predictable. Nevertheless, this knowledge is essential for the rational design of attenuated vaccines.

Developing new vaccines against NS-NSV: rational attenuation

The ability to recover NS-NSVs opened new areas of research. First of all, researchers were now able to add specific mutations or exchange genes to modify the viral genome, and they were able to analyze how such modifications affect the viral life cycle and pathogenicity of the respective virus. Moreover, such manipulation allowed researchers to modify the virus in such a way that new “designer vaccines” could be generated. Additionally, researchers started to focus on using NS-NSVs as vectors for immunization against infectious diseases by expressing foreign antigens (for review see (Bukreyev et al., 2006)). Both of these areas, namely the generation of novel and improved NS-NSV vaccines as well the use of NS-NSVs as vaccines for other pathogens grew incredibly fast in number during the past few years. As a result, we will not be able to give a complete overview for all of the approaches, but rather we will give some examples of such approaches.

While the MeV live attenuated vaccine, now in use for more than 50 years is very safe and efficacious (Griffin and Pan, 2009; Katz, 2009), long standing attempts to develop vaccines against other *Mononegavirales* have been less successful. In particular, RSV is notable for a historic vaccine failure in the 1960s involving a formalin-inactivated vaccine that primed for enhanced disease in RSV naïve recipients. Live vaccines candidates have been shown to be free from this complication (Collins et al., 2013). However, early efforts to develop vaccines through the classic methods of serial cold-passage yielded vaccine candidates that either were not attenuated in young infants or had unacceptable adverse effects (Karron et al., 2013).

A reverse genetics system for producing infectious RSV developed in 1995 (Collins et al., 1995) was the basis for the production of all the current attenuated vaccine candidates. Reverse genetics allowed first, to directly identify and characterize attenuating mutations in existing attenuated strains, and second, to produce novel mutations based on functional knowledge. Attenuating mutations can be combined to produce live-attenuated candidate vaccines with a range of phenotypes and properties. However, the resulting levels of attenuation cannot be predicted precisely (Karron et al., 2013, 2005). The best candidate for a live-attenuated RSV vaccine (Phase I clinical trial NCT01893554, Table 2) so far combines the described attenuation through temperature-sensitive mutations with additional deletion of the NS2 gene (Luongo et al., 2013), which is a major IFN antagonist of RSV (Bossert et al., 2003; Lo et al., 2005; Spann et al., 2005).

A different approach currently used to generate a live-attenuated vaccine for RSV is codon-pair de-optimization of the genome sequence (Le Nouen et al., 2014). Existing frequent codons in open reading frames are replaced by alternative rare codons, resulting in a highly temperature sensitive mutant RSV (Le Nouen et al., 2014). More targeted codon de-optimization of both NS genes resulted in a genetically stable virus that was attenuated and induced high levels of neutralizing antibodies in a mouse model (Meng et al., 2014).

EBOV and MARV of the *Filoviridae* family target the innate immune response through multiple mechanisms. The major IFN antagonist is the phosphoprotein VP35, which sequesters dsRNA (Cardenas et al., 2006) and blocks the phosphorylation of IRF3 (Basler et al., 2003). Recombinant mouse-adapted EBOV expressing VP35 with a single point mutation making it unable to block IRF3 activation is highly attenuated in a mouse model (Hartman et al., 2008) and therefore might represent a good candidate for vaccine development. The IFN signaling pathway is blocked by the matrix protein VP40 of MARV, which inhibits phosphorylation of STAT1/STAT2 (Valmas et al., 2010), or by the minor matrix protein VP24 of EBOV, which blocks karyopherin-α-mediated nuclear import of STAT1/STAT2 (Reid et al., 2006). How these findings can be implicated in the development of highly attenuated vaccines needs to be determined.

In vivo attenuation of the neurotropic RABV can be achieved by targeting its IFN antagonist, the P protein (Rieder and Conzelmann, 2009). It inhibits the activation of IRF3 (Brzozka et al., 2005) and STATs (Brzozka et al., 2006; Vidy et al., 2005). Since P is an essential cofactor of the viral polymerase, it cannot be deleted from the virus. However, P expression can be minimized by moving the gene behind the L gene (Brzozka et al., 2005) or by introducing internal ribosome entry site (IRES) elements from positive strand RNA viruses of the *Picornaviridae* family (Marschalek et al., 2009). The resulting viruses have proven high attenuation in mice (Marschalek et al., 2009; Rieder et al., 2011), but whether they protect against lethal challenge still needs to be tested. RABVs expressing P that is unable to inhibit IRF3 activation are attenuated in wt mice, but not in $IFNAR^{ko}$ mice (Rieder et al., 2011). One of these viruses tested as a vaccine candidate successfully protected foxes from RABV challenge, although neutralizing antibody titers were lower than with standard vaccine; the same virus failed to induce sufficient levels of neutralizing antibodies and protection in skunks (Vos et al., 2011). RABV expressing a mutant form of P (W265G/M287V) unable to interact with STAT1 was completely attenuated in ddY mice (Wiltzer et al., 2014). When infected intracranially with this virus, mice only developed mild symptoms and recovered completely.

The currently used VSV for reverse genetics is already attenuated compared to wild-type VSV even though the mechanism has not been identified yet (Publicover et al., 2004). One of the earliest rational approaches to attenuated VSV was the reduction of viral growth by the deletion of the cytoplasmic domain of the VSV G from 29 amino acids (aa) to 9 aa or 1 aa, respectively (Publicover et al., 2004; Roberts et al., 1998).

Another approach to VSV attenuation was by rearrangement of the viral genes. Because transcription for NS-NSVs is progressively reduced from 3′ to 5′ (see above), the change of the gene order alters the protein expression levels and therefore reduces viral replication (Wertz et al., 1998). As expected, VSV modified by gene rearrangement were greatly attenuated *in vitro* and *in vivo* (Wertz et al., 1998).

The ability to create new vaccines by targeted attenuating changes of the viral genome was also shown very early on for RABV. After intensive screening for pathogenicity markers, the Dietzschold and Schnell laboratories showed that the RABV G protein as well as the level of replication were major factors for RABV pathogenicity (Faber et al., 2007, 2004;

McKenna et al., 2004; Pulmanausahakul et al., 2008). Based on these studies, candidate new RABV vaccines were generated by introducing attenuating mutations into the G protein in RABV vaccine strains (Gomme et al., 2011). However there are still major concerns about using live vaccines in humans, and this is especially true for RABV. So other approaches, such as gene deletion, have been used to create replication-deficient (Cenna et al., 2009; Gomme et al., 2010) and highly replication-impaired RABV vaccines and vectors (McGettigan et al., 2014).

Novel viral vectors based on NS-NSVs for immunizations against other pathogens

Vaccines are one of the greatest achievements in medicine, as they protect us from infectious diseases caused by natural pathogens. One to two inoculations of an attenuated pathogen elicit humoral and cellular immune responses. Through reverse genetics specific mutations with predictable phenotypes can be introduced into wild type or attenuated virus strains, and their effects on the viral life cycle and pathogenicity can be verified. Based on these modifications, new vectors for immunization against multiple infectious diseases are being developed (for review see (Bukreyev et al., 2006)).

RABV and VSV were not only the first NS-NSVs recovered from cDNA, they were also the first NS-NSV to be developed as potential vectors. In the case of RABV, Mebatsion et al. showed that the CAT marker gene can be expressed by RABV and is stable over more than 25 passages (Mebatsion et al., 1996). Similar findings were made simultaneously for VSV, indicating not only stable expression of CAT but also that it did not did not affect the viral life cycle. Moreover Schnell et al. showed that the minimal transcription start/stop found within the VSV genome was sufficient to express a foreign gene (Schnell et al., 1996). Research on several other NS-NSV expressing marker genes followed, and the researchers showed similar results, particularly the highly stable expression.

Stable foreign gene expression by NS-NSV vectors may seem surprising considering the high mutation rate of RNA polymerases in general (for review (Lauring et al., 2013)). However, the helical nucleocapsids of NS-NSV form open structures that can grow in length. These open structures do not impose the limitations inherent in the icosahedral symmetry constraining the cargo volume, and thus the genome length, of most positive strand viruses. Another source of genomic instability, genetic recombination, is minimized by the fact that the genomic RNA of NS-NSV is always encapsidated, rather than naked as the genome of plus-strand RNA viruses. Indeed, several studies have indicated that recombination for NS-NSV is a very rare event, and so far it has only been described for RSV (Collins et al., 2008; Spann et al., 2003).

Different NS-NSV-based vectors are now used as vaccine vectors to express protein of other pathogens for immunization. Based on its ease of use, probably the most utilized vaccine vector is VSV that has been developed for influenza virus (Roberts et al., 1998), RSV (Kahn et al., 1999), human papilloma virus (Reuter et al., 2002), and henipaviruses (Kurup et al., 2014), to just name a few. Most developed are the VSV vaccine vectors against human immunodeficiency virus type 1 (HIV-1) and EBOV.

For HIV-1, studies showed that VSV induces strong immune responses in mice and in nonhuman primates (NHPs). However, as with other HIV-1 vaccine approaches, not all animals were protected when a highly pathogenic challenge virus was used (Ramsburg et al., 2004). These results were similar to those seen for the RABV-based vector, where the immune responses were potent in mice (Lawrence et al., 2013) and NHPs and the SIVmac251 challenge virus was controlled, but the RABV-based vector did not protect from a highly pathogenic challenge virus (Faul et al., 2009). Nevertheless, a highly attenuated form of the live-viral VSV vector is currently being tested in a phase I clinical trial for HIV-AIDS (NCT01438606, Table 2). Other approaches for HIV-1 vaccines are based on NS-NSV vectors such as MeV (Lorin et al., 2004) (NCT01320176, Table 2) or NDV (Carnero et al., 2009). These vectors are desirable due to their proven efficacy and safety profiles (MeV) (del Valle et al., 2007), or because they are replication-deficient in mammals (NDV). Both MeV and NDV are currently in different stages of development as the search for an effective HIV-1 vaccine continues.

At least six recently initiated clinical studies are assessing the efficacy of NS-NSV-based vectors as vaccines against EBOV (Table 2; for review (Marzi and Feldmann, 2014)). This effort is urgent due to the current public health crisis in West Africa. Multiple NHP studies have proven that a recombinant VSV that has been deleted of its own G protein and is instead expressing EBOV glycoprotein (GP) is efficient for preventing the disease (VSVΔG-ZEBOV, Table 2). A different approach is used for the RABV vector, which contains EBOV GP in addition to RABV G. Because both proteins are incorporated into the RABV virions, this vaccine can use an inactivated form of the virus, and therefore it should be a very safe vaccine against EBOV and RABV (Blaney et al., 2013) infections, which are both a problem in West Africa.

Beside these two rhabdoviral vectors, human parainfluenza virus type 3 (hPIV3) (Bukreyev et al., 2007) and the other paramyxovirus NDV (DiNapoli et al., 2010) expressing EBOV GP are being developed as potential EBOV vaccines. The life cycle of these viruses should allow intranasal or oral application, which is an advantage, and both of these viral vectors might be safer than other live-viral vectors. However, concerns include preexisting immunity for hPIV3 (a common human cold virus) and the lack of a strong anti-EBOV immune response. As for all such live vectors, the challenge with these is to achieve a balance between pathogenicity and immunogenicity.

Since the major target for NS-NSV antibodies is the glycoprotein or glycoproteins, these have been exchanged to circumvent vector-specific neutralizing immune responses. For VSV, the Rose laboratory used the G protein of the New Jersey strain to boost HIV-immunity elicited by a vector using the G protein from the non-cross-reactive VSV serotype Indiana (Haglund et al., 2002). Importantly, the filovirus GPs can functionally replace VSV G, a fact that is being used to develop Ebola vaccines (review see (Marzi and Feldmann, 2014)). Even if interference against successive immunization with vaccines containing different GPs has not been documented, vector-induced cytotoxic T-cells directed against the other VSV proteins may eventually affect vector efficiency.

On the other hand, because an inactivated vaccine does result in infection, multiple applications of an inactivated NS-NSV may not induce specific cytotoxic T-cells that interfere with repeated vaccination. This has been confirmed for RABV: multiple immunizations with inactivated virions containing foreign antigens are possible in the presence of vector immunity (Hudacek et al., 2014; Papaneri et al., 2012).

In summary, different NS-NSV vectors hold great potential for the development of new vaccines. It is important to note that certain advantages and disadvantages exist for each of them. These include concerns regarding vector pathogenicity for live, replication-competent vectors, and the potential need for multiple inoculations for replication-deficient or inactivated vaccines. To develop multiple NS-NSV vector platforms is advisable because multiple immunizations are not possible for most live viral vectors due the vector-directed immune response that is induced after the first application.

New *Mononegavirales* for oncolytic therapy

The concept of virotherapy originates from the observation of occasional tumor regressions after natural viral infections (Kelly and Russell, 2007). While early virotherapy clinical trials performed decades ago were poorly controlled, the advent of reverse genetics allowed researchers to operate with viruses for which replication and gene expression could be easily monitored. Current virotherapy clinical trials are based on viruses of nine different families, including *Paramyxoviridae* and *Rhabdoviridae* within the order *Mononegavirales* (Miest and Cattaneo, 2014). In these trials, therapeutic efficacy is assessed by well-defined biological end points, host immunity is documented, and vectors and clinical trial protocols are continuously improved (Liu et al., 2007; Russell et al., 2012). MeV and MuV infections, among others, have occasionally been associated with cancer remissions (Kelly and Russell, 2007). For example, a so-called “spontaneous” complete regression of a large retro-orbital Burkitt’s lymphoma tumor was documented after acute measles infection (Bluming and Ziegler, 1971), and spontaneous cases of lymphoma remissions after acute measles have been documented in hospitals of three continents (Kelly and Russell, 2007). MeV and MuV, as the other “oncolytic” viruses, replicate preferentially in cancer cells because these accumulate mutations in innate immunity and cell cycle control proteins. Most virus strains used in current clinical trials are further targeted for selective replication in cancer cells through genetic modifications (Cattaneo et al., 2008).

Rather than attempting to cover all pre-clinical activities ongoing with recombinant *Mononegavirales*, we discuss here in some depth three viruses that are already in cancer clinical trials (Table 2): two genetically modified MeV and one VSV (*Rhabdoviridae* family). As mentioned in the “vaccine” section, the modular nature of *Mononegavirales* genomes, in combination with lack of icosahedral symmetry in viral particles, allows stable expression of additional proteins, including those used for tracking or arming the recombinant oncolytic viruses.

MV-CEA: development and validation

Approval of any new drug, including recombinant viruses, for patient delivery in clinical trials is preceded by detailed FDA review of its manufacturing process, as well as

comprehensive toxicology and biodistribution studies. A key first step for the development of MeV-based cancer clinical trials was the generation of viruses for which distribution and replication throughout the body can be monitored. Two approaches were taken to facilitate infection monitoring. In the first one, the non-immunogenic soluble form of the carcinoembryonic antigen (CEA) was expressed from an additional transcription unit (Peng et al., 2002). The replication of this virus (MV-CEA, Table 2, top line) can be easily documented by measuring CEA blood concentration by using an available clinical kit.

To support a phase I trial of intraperitoneal administration of MV-CEA in patients with recurrent ovarian cancer, biodistribution, toxicity, and efficacy studies were performed. Biodistribution was characterized in MeV-sensitive Ifnar^{ko}-CD46Ge transgenic mice (Mrkic et al., 1998). This analysis revealed that MV-CEA administered into the peritoneal cavity efficiently infected peritoneal macrophages and these trafficked to abdominal draining lymph nodes, as well as to the marginal zone of the spleen (Peng et al., 2003). Toxicology studies were performed by intraperitoneal administration of large doses of MV-CEA in the same model. These studies were essentially negative, with no significant toxicity encountered at any dose level. Efficacy studies, which included dose–response analyses in an intraperitoneal ovarian cancer xenograft model, allowed researchers to correlate the different kinetic profiles of CEA expression with the different therapeutic outcomes (Peng et al., 2006).

Based on these studies a clinical trial was planned to determine the maximum tolerated dose of intraperitoneal administration of MV-CEA (Table 2; clinicaltrials.gov identifier: NCT00408590). The trial foresaw treatment of groups of three patients with 10-times increasing doses of virus (10^3–10^9 infectious units), for a total of 21 patients. Because of the requirement to completely evaluate the results obtained with a group of patients before proceeding to the next one, the trial needed about 5 years to be completed. At the end of the trial, it was concluded that the intraperitoneal treatment with MV-CEA was well tolerated even at the highest doses. Interestingly, median survival of patients on study was 12 months, comparing favorably to an expected median survival of 6 months in this patient population (Galanis et al., 2010). Since the presence of neutralizing antibodies was suspected as being the major limitation for efficacy, a follow up phase II clinical trial is planned to addresses this limitation by delivering the virus through cell carriers. In this protocol (Table 2; clinicaltrials.gov identifier: NCT02068794), patients with recurrent ovarian cancer are being treated with mesenchymal stem cells infected with the other recombinant virus MV-NIS.

MV-NIS: monitoring the spread of oncolytic viruses in patients over time

Towards providing anatomical information about the location of virus-infected cells in cancer patients, *in vivo* spread of an oncolytic virus should be monitored noninvasively over time. To achieve this goal a recombinant virus coding for the human thyroidal natrium iodine symporter (NIS) was generated (Dingli et al., 2004). NIS is a channel protein that transports iodine, and its expression in the thyroid has been exploited for more than 50 years in clinical practice for thyroid imaging with ^{123}I, or thyroid ablation with ^{131}I.

MV-NIS infected cells can concentrate radioactive iodine from the bloodstream, enabling noninvasive single photon emission computed tomography imaging of infection using ^{123}I or technetium. This approach has been used for high resolution monitoring of viral replication in pre-clinical models (Miest et al., 2013). MV-NIS has also been used to enhance the therapeutic potency of measles virotherapy by timed administration of ^{131}I (Dingli et al., 2004). Phase I clinical trials using MV-NIS have been initiated for ovarian cancer, myeloma, mesothelioma, and head and neck cancer (Table 2; clinicaltrials.gov identifier: NCT00408590, NCT00450814, NCT01503177 and NCT01846091).

As MV-CEA, clinical grade MV-NIS was manufactured in a dedicated facility while adhering to the principles of Good Manufacturing Practice (GMP). Since the intravenous delivery of up to 10^{11} infectious units was foreseen, a new process was developed for the manufacture of high titer virus stocks. This resulted in the production of pure and homogeneous MV-NIS at a concentration of 10^9 infectious units/ml. Pre-clinical efficacy studies were conducted in SCID mice bearing subcutaneous myeloma xenografts. Pre-clinical pharmacology and toxicology studies were conducted in MeV-susceptible $Ifnar^{ko}$-CD46Ge transgenic mice, and in MeV-naïve squirrel monkeys (Myers et al., 2007).

Multiple myeloma was selected as target for the first systemically delivered oncolytic vitotherapy clinical protocol because most patients have strongly reduced antibody titers to many infectious agents, including MeV. The multiple myeloma phase I clinical trial (NCT00450814) had a standard cohorts-of-3 design with a first dose level of 10^6 infectious units of MV-NIS, increasing by 10-fold dose increments to a maximum feasible dose of 10^{11} infectious units. At the highest dose, the virus was infused into a superficial arm vein in 100 mL of normal saline over 60 min (Russell et al., 2014).

Even if all eligible patients had relapsing myeloma refractory to approved therapies, Russell et al. reported success: the first two measles seronegative patients treated at the highest dose responded to therapy, and one experienced durable complete remission at all disease sites. Tumor targeting was clearly documented by NIS-mediated radioiodine uptake in virus-infected plasmacytomas. Toxicities resolved within the first week after therapy (Russell et al., 2014). This was the first well-documented remission from disseminated cancer after systemic virotherapy. Based on this success, this clinical trial is being expanded at the highest virus dose. The target group will include patients with minimal if any measles-neutralizing antibodies.

VSV-IFNβ: attenuating toxicity by interferon expression

A genetically modified VSV producing interferon-β (VSV-IFNβ) is the third recombinant virus of the Mononegavirales order approved as experimental cancer therapeutic. A phase I clinical trial to evaluate its safety in patients with liver cancer is recruiting (Table 2; clinicaltrials.gov identifier: NCT01628640), and a clinical trial for head and neck cancer is in preparation (Kurisetty et al., 2014). Several years ago it was shown that VSV induces potent *in vitro* and *in vivo* tumor cytotoxic effects, and its oncolytic efficacy was documented in a number of xenograft and syngeneic models (Barber, 2005). However,

VSV-induced neurotoxicity initially limited the clinical development efforts with this agent (Johnson et al., 2007).

To attenuate toxicity, a recombinant VSV that carries the gene encoding interferon-β was developed. This virus showed an improved safety profile while keeping its oncolytic potency (Obuchi et al., 2003; Willmon et al., 2009). The primary purpose of the current phase clinical trial of liver cancer is to evaluate the safety of VSV-IFNβ. Although the primary goal of any phase I study is to evaluate safety, patients may benefit clinically by having shrinkage or stabilization of their tumor or reduction in their cancer related symptoms, as observed in some of the MeV-based clinical protocols.

Next generation oncolytic viruses: enhancing efficacy

Viruses currently used in cancer clinical trials are safe at the highest doses achievable by today's manufacturing processes: adverse events beside fever and general flu-like symptoms are rare (Liu et al., 2007). No transmission of an oncolytic virus from treated patients to carers or other contacts has been noted, although shedding has been documented in the urinary and respiratory tract (Galanis et al., 2010). While safety was consistently shown, efficacy is limited. Thus the current key challenge is to develop more effective oncolytic viruses that replicate with greater efficiency and specificity.

Towards improving cancer specificity of *Mononegavirales*, three types of targeting are possible: particles can be activated through cancer-specific proteases, cell entry can be re-directed through cancer-specific cell surface proteins, and microRNA down-regulated in cancer cells can be exploited. To improve efficacy viruses are armed through the expression of either prodrug convertases that can activate cancer therapeutics, or ion channels that enable radiosensitization, or immunostimulatory cytokines that induce antitumor immunity (Miest and Cattaneo, 2014). To provide shielding from neutralizing antibodies different envelopes are used sequentially. We discuss here selected examples of recombinant viruses that illustrate different targeting or arming principles, or their combination towards a specific cancer treatment.

Targeted viruses

The principle of cancer-specific *in situ* activation through proteases was established with the *Paramyxoviridae* SeV (Kinoh et al., 2004) and MeV (Springfeld et al., 2006). This approach is based on the modification of their fusion proteins, which require protease cleavage for activation. Cleavage was made dependent on a matrix metalloprotease, MMP-2, which recognizes and cleaves a specific hexapeptide sequence. MMP are zinc-dependent endopeptidases that promote tumor progression by cleaving the extracellular matrix, and are up-regulated in almost every type of human cancer (Egeblad and Werb, 2002).

A recombinant MeV was generated with a sequence recognized by MMP-2 engineered into the fusion protein. This virus was unable to propagate unless it was added to cells expressing MMP-2. In mice, the virus retained full oncolytic activity when inoculated into MMP-positive subcutaneous cancers, but unlike the wild-type virus, it did not kill susceptible mice after intracranial inoculation (Springfeld et al., 2006). Thus the MMP-2 cleavable virus is

safer than its standard precursor. While safety is not an issue in current clinical trials, future one may consider more aggressive dosing. In these cases, enhanced tumor specificity by MMP-selective activation may maintain an ideal safety profile.

The principle of cancer-specific cell entry was also developed with *Paramyxoviridae*. In the envelope of these viruses, receptor attachment and fusion functions are separated on two proteins. In contrast, a single protein of other *Mononegavirales* families performs both functions. Among the *Paramyxoviridae*, targeting of the MeV envelope is most advanced. The MeV attachment protein (hemagglutinin, H) interacts with different receptors: the primary receptor signaling lymphocyte activation molecule (SLAM, CD150) is used for initial spread in lymphatic organs (Ferreira et al., 2010; Tatsuo et al., 2000), whereas the adherens junction protein nectin-4 is subsequently used to gain access to the upper airways epithelium and exit the host (Muhlebach et al., 2011). In addition, the vaccine strain has gained the ability to use the ubiquitous membrane cofactor protein (MCP, CD46). The footprints of all three receptors on H have been characterized structurally and functionally (Mateo et al., 2014).

In 2000 MeV cell entry was targeted to designated receptors simply by adding small specificity determinants to the H-protein (Schneider et al., 2000). It was then demonstrated that even larger single chain antibodies could be used to target viral entry in cultivated cells (Hammond et al., 2001), as well as in xenografts set in immunodeficient mice (Bucheit et al., 2003). Availability of single chain antibodies against almost every cancer-relevant cell surface protein allowed testing of many potential entry targets. Indeed MeV-based re-targeting is versatile: many re-targeted viruses have been generated and shown to be effective in different animal models of oncolysis (Nakamura et al., 2005).

On the other hand, cell entry targeting may not be necessary for most cancer applications: MV-CEA and MV-NIS can enter cells through CD46, which is over-expressed in many cancer types (Russell and Peng, 2009). In view of this fact, and of the complex regulatory requirements and large investments necessary to produce clinical grade viruses, specifically entry re-targeted strains have not yet reached production stage. However, recent results questioned whether CD46-dependent entry always favors efficient oncolysis: no direct correlation between CD46 expression levels and therapeutic efficacy was observed in clinical trials, and oncolytic ablation of certain lymphoma xenografts occurred only when cell entry occurred through SLAM (Miest et al., 2013). Thus, entry targeting may soon be re-prioritized.

Based on positive results with other virus types, the principle of negative post-entry targeting was established in the two *Mononegavirales* VSV and MeV (Edge et al., 2008; Leber et al., 2011). In particular, since neuron-specific microRNA-7 is downregulated in gliomas but highly expressed in normal brain tissue, a microRNA-sensitive MeV containing target sites for this microRNA was engineered. Even though highly attenuated in presence of microRNA-7, this virus retained full efficacy against glioblastoma xenografts. Furthermore, microRNA-mediated inhibition protected transgenic mice susceptible to MeV infection from a potentially lethal intracerebral challenge. Importantly, endogenous microRNA-7 expression in primary human brain resections tightly restricted replication and spread of

microRNA-sensitive virus. Since the three targeting mechanisms discussed above are based on different principles, they can be combined.

Armed viruses, and the pathway to clinical translation

While multiple targeting layers will yield viruses that replicate very selectively within tumors, the main limitation of current clinical trials is efficacy. Cancer therapy efficacy can be enhanced by the combination of different treatment modalities. Indeed, no single drug or treatment will cure cancer, and most therapeutic regimens are based on combinations of drugs, radiation, and surgery to maximize patient survival. Recombinant viruses armed with specific genes may perform even better by integrating different components of current cancer therapy regimens (Ottolino-Perry et al., 2010). For example, judiciously timed administration of ^{131}I can be used to enhance the therapeutic potency of virotherapy (Dingli et al., 2004).

A second example of this integrative approach is an armed and targeted virus for lymphoma treatment. This virus was generated as an enhancer of FCR, a front-line treatment for certain forms of non-Hodgkin lymphoma. The FCR regimen is based on cycles of treatment with fludarabine phosphate, cyclophosphamide, and the anti-CD20 antibody Rituxan. As an alternative to Rituxan, a CD20-targeted measles virus was considered. This virus was armed with the prodrug convertase purine nucleotide phosphorylase, which converts fludarabine phosphate to a highly diffusible substance that is capable of efficiently killing bystander cells. The CD20-targeted and convertase-armed virus was shown to synergize with fludarabine to achieve oncolytic efficacy after systemic inoculation in a mantle cell lymphoma xenograft model (Ungerechts et al., 2007). Precise timing of cyclophosphamide, virus, and fludarabine administration was shown to increase the window of therapeutic opportunity (Ungerechts et al., 2010).

Finally, we note that the original MeV infectious cDNA, from which MV-CEA and MV-NIS were derived, accidentally accumulated mutations in the innate immunity control proteins that further attenuate viruses derived from it (Devaux et al., 2011, 2007). While this over-attenuation may have contributed to the safety of MN-CEA and MV-NIS, it may also have impacted their clinical efficacy.

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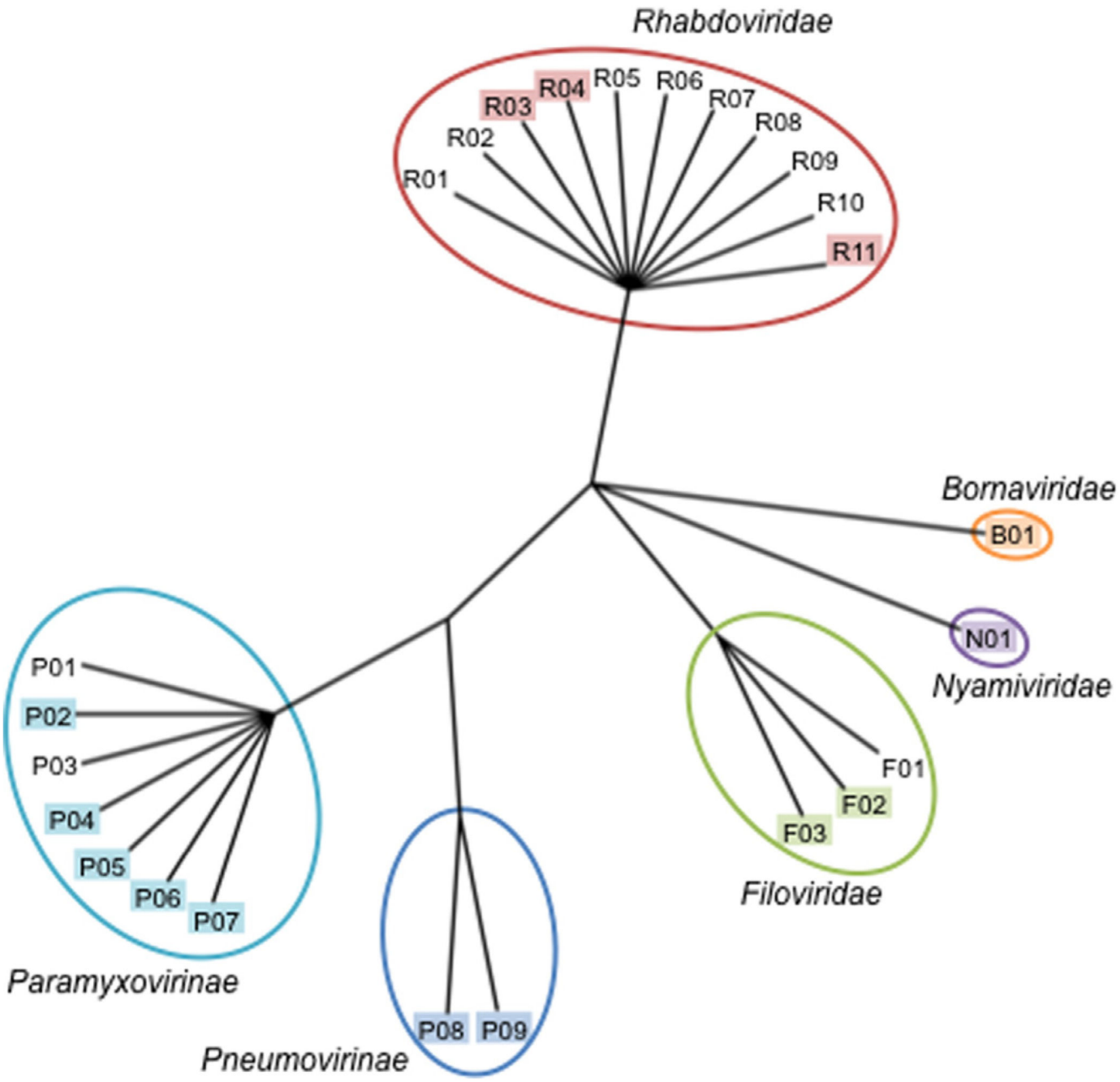

Fig. 1.

Phylogeny of the genera within the order Mononegavirales. Genera for which reverse genetics systems have been established for at least one species are highlighted. The phylogenetic tree was generated with phyloT (Letunic and Bork, 2007, 2011). Abbreviations: B01 – Bornavirus; F01 – Cuevavirus; F02 – Ebolavirus; F03 – Marburgvirus; N01 – Nyavirus; P01 – Aquaparamyxovirus; P02 – Avulavirus; P03 – Ferlavirus; P04 – Henipavirus; P05 – Morbillivirus; P06 – Respirovirus; P07 – Rubulavirus; P08 – Metapneumovirus; P09 – Pneumovirus; R01 – Cytorhabdovirus; R02 – Ephemerovirus; R03 – Lyssavirus; R04 – Novirhabdovirus; R05 – Nucleorhabdovirus; R06

– Perhabdovirus; R07 – Sigmavirus; R08 – Sprivivirus; R09 – Tibrovirus; R10 – Tupavirus; R11 – Vesiculovirus.

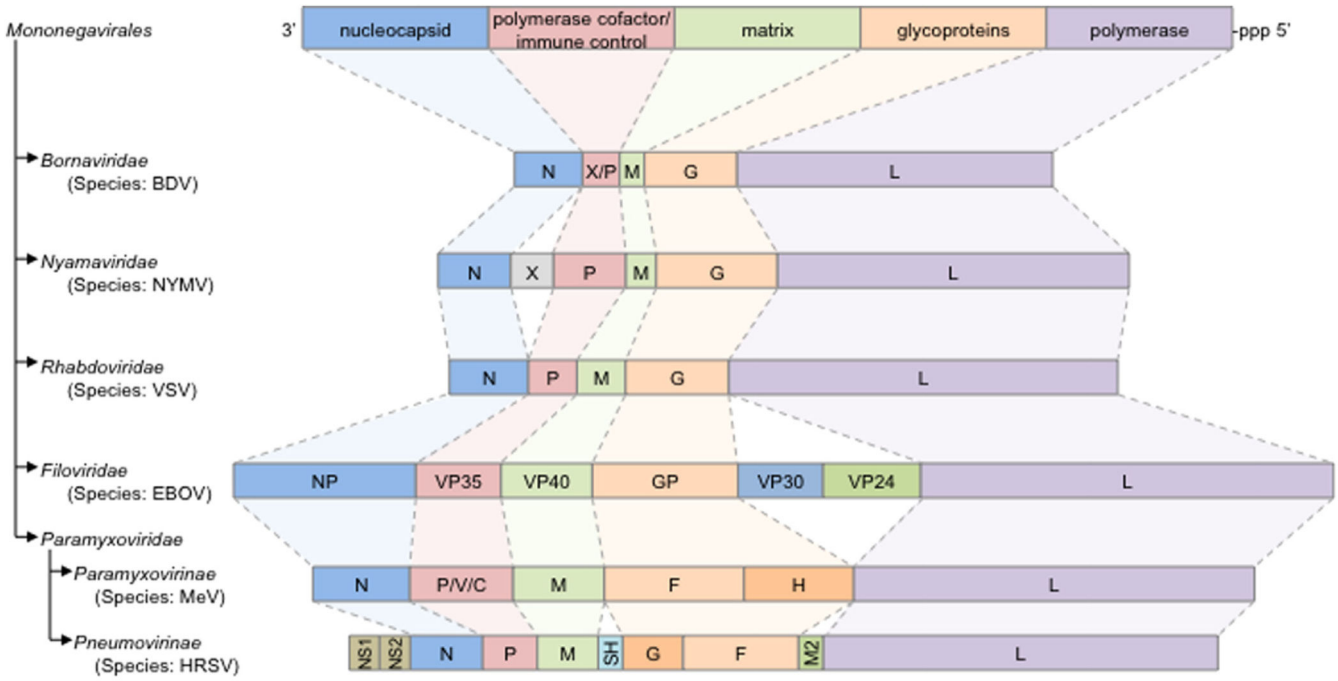

Fig. 2.

General genome organization of Mononegaviruses. Size of the genomes and individual genes is proportional to their length.

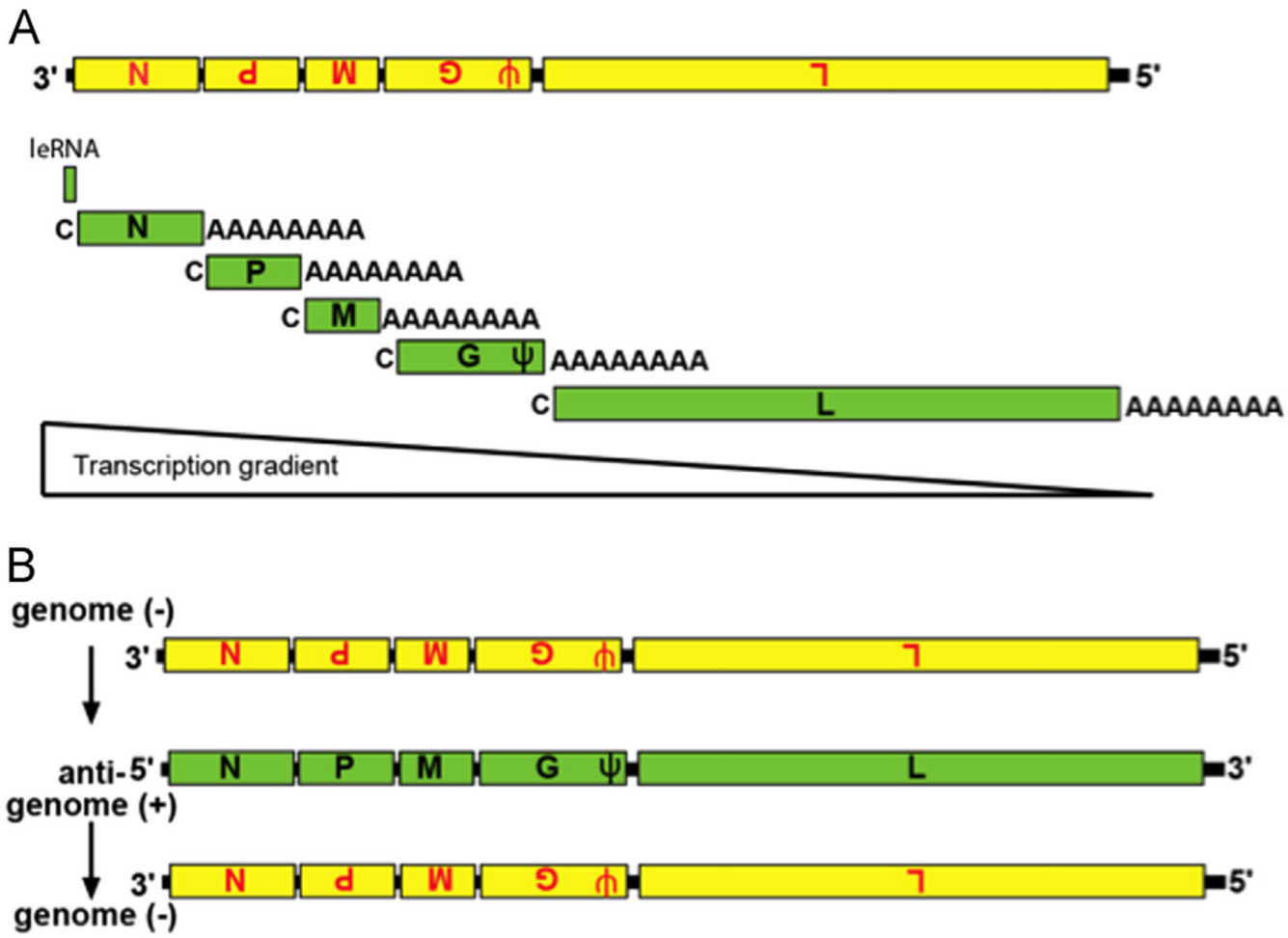

Fig. 3.

Transcription and replication of a NNSV shown for RABV. (A) The encapsidated negative-strand RNA (yellow) serves as a template for the polymerase complex. Transcription starts with a short uncapped leader RNA (leRNA) from the 3′ end of the genomic RNA; this is followed by the transcription of 5′ capped and polyadenylated mRNAs, which encode the viral proteins (green). The polymerase complex stops at a signal sequence, ignores the intergenic region (IGR) and restarts transcription at the transcription start signal sequence. Subsequent attempts at transcription by the polymerase complex are not always successful; therefore, attenuation of transcription occurs in the direction of 3′-5′ (transcription gradient). (B) During replication, the polymerase complex ignores the transcription start/stop signals within the RABV genome (yellow), rendering a full-length antigenomic RNA (green), which is also encapsidated. The antigenomic RNA is encapsidated into the N protein along with the genomic RNA. The synthesized antigenome serves then as a template for the synthesis of additional copies of genomic RNA (yellow).

Table 1

Rescue systems currently developed for *Mononegavirales*. References of the first published rescues of the respective species. N/A: No rescue system available as stated in the cited reference. —: No rescue system found in the literature.

Family	Subfamily	Genus	Species	Reverse Genetics established	Reference
Bornaviridae		B01 – *Bornavirus*	Borna disease virus	2005	Schneider et al. (2005)
Filoviridae		F01 – *Cuevavirus*	Lloviu cuevavirus	– –	
		F02 – *Ebolavirus*	Zaire ebolavirus	2001	Volchkov et al. (2001)
		F03 – *Marburgvirus*	Marburg marburgvirus	2006	Enterlein et al. (2006)
Nyamaviridae		N01 – *Nyavirus*	Nyamanini nyavirus	2013	Herrel et al. (2013)
Paramyxoviridae	*Paramyxovirinae*	P01 – *Aquaparamyxovirus*	Atlantic salmon paramyxovirus	– –	
		P02 – *Avulavirus*	Newcastle disease virus	1999	Romer-Oberdorfer et al. (1999)
		P03 – *Ferlavirus*	Fer-de-Lance paramyxovirus	– –	
		P04 – *Henipavirus*	Hendra virus	2013	Marsh et al. (2013)
			Nipah virus	2006	Yoneda et al. (2006)
		P05 – *Morbillivirus*	Canine distemper virus	2000	Gassen et al. (2000)
			Measles virus	1995	Radecke et al. (1995)
			Rinderpest virus	1997	Baron and Barrett (1997)
		P06 – *Respirovirus*	Human parainfluenza virus 1	2002	Newman et al. (2002)
			Human parainfluenza virus 3	1997	Hoffman and Banerjee (1997)
			Bovine parainfluenza virus 3	2000	Schmidt et al. (2000)
			Sendai virus	1995	Garcin et al. (1995)
		P07 – *Rubulavirus*	Human parainfluenza virus 2	2001	Kawano et al. (2001)
			Mumps virus	2000	Clarke et al. (2000)
			Parainfluenza virus 5	1997	He et al. (1997)
	Pneumovirinae	P08 – *Metapneumovirus*	Human metapneumovirus	2004	Biacchesi et al. (2004)
		P09 – *Pneumovirus*	Human respiratory syncytial virus	1995	Collins et al. (1995)
			Bovine respiratory syncytial virus	1999	Buchholz et al. (1999)
Rhabdoviridae		R01 – *Cytorhabdovirus*	Lettuce necrotic yellows virus	N/A	Kormelink et al. (2011)
		R02 – *Ephemerovirus*	Bovine ephemeral fever virus	– –	
		R03 – *Lyssavirus*	Rabies virus	1994	Schnell et al. (1994)
		R04 – *Novirhabdovirus*	Infectious hematopoietic necrosis virus	2000	Biacchesi et al. (2000)
			Viral hemorrhagic septicemia virus	2010	Ammayappan et al. (2011)
			Snakehead rhabdovirus	2000	Johnson et al. (2000)

Family	Subfamily	Genus	Species	Reverse Genetics established	Reference
		R05 – *Nucleorhabdovirus*	Potato yellow dwarf virus	N/A	Kormelink et al. (2011)
		R06 – *Perhabdovirus*	Perch rhabdovirus	– –	
		R07 – *Sigmavirus*	Drosophila melanogaster sigmavirus	– –	
		R08 – *Sprivivirus*	Spring viraemia of carp virus	– –	
		R09 – *Tibrovirus*	Tibrogargan virus	– –	
		R10 – *Tupavirus*	Tupaia virus	– –	
		R11 – *Vesiculovirus*	Vesicular stomatitis Indiana virus	1995	Lawson et al. (1995), Whelan et al. (1995)

Table 2

Selected *Mononegavirales* in clinical trials. Clinical trials can be found at https://clinicaltrials.gov/.

Virus strain	Purpose	Clinical trials
Respiratory syncytial virus RSV ΔNS2 Δ1313 I1314L	Live-attenuated vaccine	NCT01893554: Safety and immune response in infants and children (Phase I)
Respiratory syncytial virus RSV cps2	Live-attenuated vaccine	NCT01852266: Safety and immune response in RSV-seronegative infants and children (Phase I)
Measles virus MV1-F4	Vectored vaccine	NCT01320176: Safety and dose-response of MeV vector expressing HIV-1 clade B antigen in healthy adults (Phase I)
Vesicular stomatitis virus VSV-Indiana HIV gag	Vectored vaccine	NCT01438606: Safety and immunogenicity in healthy, HIV-1-uninfected adults (Phase I)
Vesicular stomatitis virus VSVΔG-ZEBOV (BPSC1001)	Vectored vaccine	NCT02280408: Safety and immunogenicity of prime-boost in healthy adults (Phase I)
		NCT02283099: Safety, Tolerability and Immunogenicity of a Single Ascending Dose (Phase I)
		NCT02287480: Safety and immunogenicity in healthy adults (Phase I)
		NCT02296983: Safety and immunogenicity in healthy adults in Kilifi, Kenya (Phase I)
		NCT02314923: Safety and immunogenicity in healthy adults (Phase I)
		NCT02344407: Safety and immunogenicity in healthy adults in Monrovia,
		Liberia; comparison with chimpanzee adenoviral vector ChAd3-EBO Z (Phase II)
Measles virus MV-CEA	Oncolysis: Tracking	NCT00408590: Ovarian cancer (Phase I) (Galanis et al., 2010)
		NCT00390299: Recurrent glioblastoma multiforme (Phase I)
Measles virus MV-NIS	Oncolysis: Tracking and Arming	NCT00450814: Recurrent or refractory multiple myeloma (Phase II) (Russell et al., 2014)
		NCT02068794: Recurrent ovarian cancer cell carriers (Phase II)
		NCT01503177: Malignant pleural mesothelioma (Phase I)
		NCT01846091: Recurrent or metastatic squamous cell carcinoma of the head and neck (Phase I)
Vesicular stomatitis virus VSV-IFNβ	Oncolysis: Selective Dis-Arming	NCT01628640: Hepatocellular carcinoma (Phase I)