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Review

Transcription and replication mechanisms of *Bunyaviridae* and *Arenaviridae* L proteins

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ABSTRACT

Bunyaviridae and *Arenaviridae* virus families include an important number of highly pathogenic viruses for humans. They are enveloped viruses with negative stranded RNA genomes divided into three (bunyaviruses) or two (arenaviruses) segments. Each genome segment is coated by the viral nucleoproteins (NPs) and the polymerase (L protein) to form a functional ribonucleoprotein (RNP) complex. The viral RNP provides the necessary context on which the L protein carries out the biosynthetic processes of RNA replication and gene transcription. Decades of research have provided a good understanding of the molecular processes underlying RNA synthesis, both RNA replication and gene transcription, for these two families of viruses. In this review we will provide a global view of the common features, as well as differences, of the molecular biology of *Bunyaviridae* and *Arenaviridae*. We will also describe structures of protein and protein-RNA complexes so far determined for these viral families, mainly focusing on the L protein, and discuss their implications for understanding the mechanisms of viral RNA replication and gene transcription within the architecture of viral RNPs, also taking into account the cellular context in which these processes occur. Finally, we will discuss the implications of these structural findings for the development of antiviral drugs to treat human diseases caused by members of the *Bunyaviridae* and *Arenaviridae* families.

Contents

Abbreviations: aa, amino acid; BUNV, bunyamwera virus; CCHFV, crimean congo hemorrhagic fever virus; cRNA, complementary positive sense viral RNA; dsRNA, double stranded RNA; EM, electron microscopy; EN, endonuclease; FDA, United States Food and Drug Administration; GPC, glycoprotein precursor; HF, hemorrhagic fever; HFRS, hemorrhagic fever with renal syndrome; HIV, human immunodeficiency virus; IFN-I, type I interferon; IGR, intergenic region; JUNV, junin virus; LACV, la crosse virus; LASV, lassa virus; LCMV, lymphocytic choriomeningitis virus; LF, lassa fever; MACV, machupo virus; MG, mini genome; MHC, major histocompatibility complex; NCR, non-coding regions; NP, nucleoprotein; nsNSV, non-segmented negative stranded virus; NSV, negative strand virus; NW, new world; ORF, open reading frame; OTU, ovarian tumour; OW, old world; PDL-1, programmed death-ligand – 1; PIV3, parainfluenza virus 3; RdRpol, RNA dependent RNA polymerase; Rib, ribavirin; RING, really interesting new gene; RNP, ribonucleoprotein; RVFV, rift valley fever virus; S1P, site 1 protease; SARS, severe acute respiratory syndrome; SFTSV, severe fever with thrombocytopenia syndrome virus; siRNA, silencing RNA; sNSV, segmented negative stranded viruses; SSP, stable signal peptide; UTR, untranslated regions; vRNA, genomic negative sense viral RNA; wt, wild type.

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1. Introduction

Decades of research efforts aimed at the identification of new viral species have led to the isolation and characterization of a large number of viral strains belonging to *Bunyaviridae* and *Arenaviridae* families. These data have provided virologists with a detailed knowledge on their genome organization, as well as a large body of data on their proteins and RNA sequences.

Bunyaviridae "take their name from Bunyamwera virus (BUNV), which was originally isolated in 1943 from Aedes spp. mosquitoes during an investigation of yellow fever in the Semliki Forest, Uganda. During the following 25 years, several antigenically related viruses were identified in laboratories in South America, Africa and India, mostly owing to work that was funded by The Rockefeller Foundation" (Richard Elliott, 1954–2015) (Brennan et al., 2015; Elliott, 2014). Since the first reported bunyaviral disease in 1917 (Montgomery, 1917), the Nairobi Sheep Disease, more than 350 strains of *Bunyaviridae* have been isolated around the globe and classified into five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus*.

The first arenavirus identified was Lymphocytic Choriomeningitis Virus (LCMV), discovered in 1933 during the characterization of samples from an outbreak of St. Louis encephalitis. LCMV was found to cause aseptic meningitis and to be similar to an agent that cause chronic infections in mouse colonies. The name *Arenaviridae* was given after the sandy (latin, arenosus) appearance of thin sections of virions when examined by electron microscopy, which is thought to reflect the packaging of ribosomes into mature virions. By 1960, the *Arenaviridae* family was recognized to include a group of viruses that shared with LCMV common morphology, serology, biochemical features, and a natural history of establishing long-term chronic infections in their natural rodent hosts in the absence of noticeable clinical symptoms. Recently, the detection and characterization of a divergent group of arenaviruses in captive alethinophidian snakes has changed the view of arenaviruses exclusively infecting small mammals. To accommodate these findings, the *Arenaviridae* family includes now two genera: *Reptarenavirus* and *Mammarenavirus* (Radoshitzky et al., 2015). Because the still limited knowledge about the biology and protein structure and function of reptarenaviruses, this review would be restricted mammarenaviruses. We anticipate, however, that many of the structural and functional principles established for mammarenavirus proteins will be found to operate also within reptarenaviruses.

Members of the *Bunyaviridae* and *Arenaviridae* families are enveloped viruses with a segmented negative strand RNA genome consisting of three or two, respectively, segments and that share many essential aspects of their molecular and cell biology. A common feature of these two viral families is the use of a large multifunctional protein, L, which includes a central polymerase domain. L proteins direct both RNA replication and gene transcription of the bunya- and arenavirus genomes using similar mechanisms. Hence, the reason why this review covers both viral families. To frame the biological context in which these L proteins work, we have included information relevant to the central processes associated with the bunya- and arenavirus life cycles in infected cells. This review does not attempt to present a comprehensive and detailed discussion of the molecular and cell biology of these two viral families, which can be found in other recent reviews cited in this review, but rather our goal is to present an updated description of the transcription and replication mechanisms used by these two viral families on the light of recently reported high resolution structures for the bunyavirus La Crosse (LACV) L protein and related influenza trimeric polymerases.

2. The impact of bunya- and arenaviruses on human health

2.1. Bunyaviruses

Bunyavirus natural hosts are arthropods, animals and humans, only the bunyavirus from genus tospovirus infect plants. Certain bunyaviruses appear to infect only arthropods, indeed with the notable exception of hantaviruses, which are generally spread by rodents, bunyaviruses have arthropods as natural reservoirs and phylogenetic studies suggest an arthropod origin for this family of viruses (Marklewitz et al., 2015). Bunyavirus cause persistent infections in their arthropod or rodent reservoirs, in arthropods can be transmitted vertically by transovarian transmission or horizontally by venereal transmission and the transmission to humans and animals occurs by insect bites (ticks, mosquitoes or sandflies). Hantavirus are transmitted to humans mainly through aerosolized urine, faeces and saliva from infected rodents. Human to human transmission have been reported in very few cases for Andes virus (genus *Hantavirus*) (Padula et al., 1998) and is generally associated, like reported for Crimean Congo Hemorrhagic fever virus (CCHFV, genus *Nairovirus*) and Rift Valley Fever Virus (RVFV, genus *Phlebovirus*) (Kim et al., 2015; Martinez-Valdebenito et al., 2014; Vaheri and Vapalahti, 2011; van Eeden et al., 1985), to nosocomial infections of healthcare workers exposed to infected patients or slaughterhouse workers exposed to infected animals like sheep, goats and cattle, that can reach very high titers of virus in blood.

Human infections by bunyavirus can be severe and eventually fatal. New world hantaviruses (Andes Virus and Sin Nombre Virus) cause pulmonary syndrome while the Old word hantaviruses (Hantaan virus) cause hemorrhagic fever (HF) with renal syndrome reaching fatality rates of 50% and 1–15% respectively. Another relevant example is CCHFV, one of the most virulent and enigmatic bunyaviruses. In humans, CCHFV infection causes severe HF with mortality rates typically between 20 and 30% (Bente et al., 2013), this is particularly tragic taking into account that each year more than 1000 human cases are reported from countries of southeastern Europe and Turkey. In contrast, naturally infected animals do not appear to develop noticeable clinical symptoms, despite in viral endemic areas seropositivity can be detected in many mammalian species including bats (Hoogstraal, 1979). Phlebovirus infections (e.g. RVFV) or Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV)), cause fevers that can be mild but can also

result in severe HF, ocular disease, thrombocytopenia or encephalitis reaching fatality rates of 12–30% (Elliott and Brennan, 2014; Zhao et al., 2012).

Human bunyavirus infections are zoonotic and thereby their geographic incidence correlates with the distribution of the viral natural reservoirs. The continuous and increasing human impact on the environment is affecting the distribution and density of insects and animals through climate change (Clegg, 2009; Elliott, 2009) and other factors including changes in land use and human activities, like extensive farming, which can favour the spread and the outbreaks of emerging infectious diseases (Cleaveland et al., 2007). As a consequence, the geographical distribution of bunyavirus caused diseases is expanding as illustrated by the recent first lethal outbreak of CCHV in Spain (García Rada, 2016), indicating that CCHFV is expanding from eastern to western Europe. Other new pathogenic bunyavirus strains have emerged, including: SFTSV that emerged in China in 2009 (Yu et al., 2011) and expanded to Korea and Japan; Schmallenberg virus emerged in 2011 infecting cattle and quickly spread in western Europe (Elliott and Brennan, 2014; Hoffmann et al., 2012); and Toscana phlebovirus that was first isolated in 1971 in Italy and has expanded to surrounding countries (Spain, Greece, Portugal and Turkey) causing from asymptomatic infections to meningitis and meningoencephalitis (Charrel et al., 2005).

2.2. Arenaviruses

Arenaviruses cause chronic infections of rodents across the world, and human infections occur through mucosal exposure to aerosols or by direct contact of abraded skin with infectious materials (Buchmeier, 2007). Both viral and host factors contribute to a variable outcome of arenavirus infection, ranging from rapid virus clearance by the host defenses to chronic infection in the absence of clinical symptoms to severe acute disease (Buchmeier, 2007). Several arenaviruses cause HF disease in humans and pose important public health problems in their endemic regions (Bray, 2005; Geisbert and Jahrling, 2004). Thus, Lassa virus (LASV) infects several hundred thousand individuals yearly in West Africa resulting in a high number of Lassa fever (LF) cases associated with high morbidity and mortality (Richmond and Baglole, 2003). Notably, increased traveling has led to the importation of LF cases into non-endemic metropolitan areas (Freedman and Woodall, 1999; Isaacson, 2001). In addition, HF arenaviruses represent a credible bioterrorism threat (Borio et al., 2002). Moreover, evidence indicates that the worldwide-distributed LCMV is a neglected human pathogen of clinical significance (Fischer et al., 2006; Mets et al., 2000; Palacios et al., 2008; Peters, 2006). There are no FDA-licensed arenavirus vaccines and current anti-arenaviral therapy is limited to an off-label use of ribavirin, which is only partially effective and associated with significant side effects (Bausch et al., 2010). Therefore, there is a pressing need to develop effective strategies to combat human pathogenic arenaviruses, a task that will be facilitated by a better understanding of the arenavirus molecular and cell biology.

The prototypic arenavirus LCMV provides investigators with a highly tractable experimental system to investigate virus-host interactions and associated disease, including zoonotic events at the root of many emerging viral infections of humans. Studies using LCMV have led to major concepts in virology and immunology that apply universally to other viral infections, including virus-induced immunopathology and MHC restriction (Oldstone, 2002; Zinkernagel, 2002), and the contribution to viral persistence of negative immune regulators like PDL-1 (Barber et al., 2006). Age, immune status and genetic background of the mouse, as well as route of infection, strain and viral dose influence the outcome of LCMV infection of the mouse (Buchmeier, 2007; Oldstone, 2002; Zinkernagel, 2002), which facilitates the identification and characterization of both host and viral determinants of the outcome of infection.

3. Bunya- and arenavirus genome organization and proteins

3.1. Bunyaviruses

Bunyaviruses have a genome consisting of three strands of negative-sense, single-strand RNA, designated according to their size as L (large), M (medium) and S (small) (Elliott, 2014). The basic set of genes encoded by all bunyaviruses encompasses the multifunctional RNA-dependent RNA polymerase (RdRpol), named L protein, in the L segment, the two glycoproteins Gn and Gc in the M segment, and the nucleocapsid protein (NP) in the S segment. Additional non-structural proteins can be coded within the M and S segments, named NSm and NSs respectively, following different coding strategies (Fig. 1). Except for hantavirus, *bunyaviruses* code in the M segment the NSm protein as an insertion between the two glycoproteins which are cleaved by cellular proteases during the polyprotein maturation (orthobunya-, nairo- and phlebovirus) or by having the coding ORF with the inverse polarity (tospoviruses) at the 5′ of the genomic fragment. Bunyavirus transcription is carried out using the negative sense viral genome as template, the mRNAs of NSV proteins coded in the inverse polarity (positive) need to be transcribed using the positive sense viral cRNA as template, this implicates that the viral transcription promoters need to be functional in both vRNA and cRNA. The strategy of inverse polarity is used in the S segment for the NSs by tospovirus, phlebovirus and arenaviruses (see below). With the exception of Anopheles A, Anopheles B, and Tete serogroups, the orthobuyaviruses code for an NSs protein by overlapping the ORF with the NP (Mohamed et al., 2009). Bunyavirus NSs play an eminent role in inhibiting the interferon response (Ly and Ikegami, 2016; Schoen and Weber, 2015) and in plants (for tospovirus) interferes the plant antiviral RNA silencing (Hedil and Kormelink, 2016). NSm proteins instead are integral membrane proteins with an unclear general role among bunyavirus. NSm has been associated to viral assembly in BUNV (Shi et al., 2006), and been shown to be nonessential for virus replication in Oropuche virus (Tilston-Lunel et al., 2016) and reported to inhibit apoptosis in RVFV (Won et al., 2007).

Each of the viral RNAs (vRNAs) contains non-coding sequences (NCRs) at their 5′-and 3′-termini ends, also known as untranslated regions (UTRs), which play an essential role as promoters of genome transcription and replication (see below), these regions include sequences involved in genome packaging and transcription termination signals. The most characteristic feature of the 5′ and 3′ UTRs is the high conservation and complementarity of their nucleotides at the genome boundaries (In Fig. 1 we can see the standard sequences found in each genus). These regions have been studied for orthobunyavirus in two independent studies determining that the essential features to be functional are the specific sequence for most of the first eleven nucleotides and the 3′-5′ complementarity for the next four nucleotides (Barr and Wertz, 2004; Kohl et al., 2004). Looking at the conserved sequences of each genus we realize that have in common at least eight nucleotides with near to perfect Watson-Crick complementary (Fig. 1). Based on cross linking experiments these complementary regions were proposed to base pair in the RNPs forming panhandled genomes, this idea was reinforced by the circular nature of bunyaviral RNPs and have been extensively repeated in the bibliography (Raju and Kolakofsky, 1989). Strikingly structural and functional studies on the LACV L protein and its interaction with the viral promoter clashed with this hypothesis by showing a specific L protein – promoter interaction but only if both 5′ and 3′ genomic ends are single

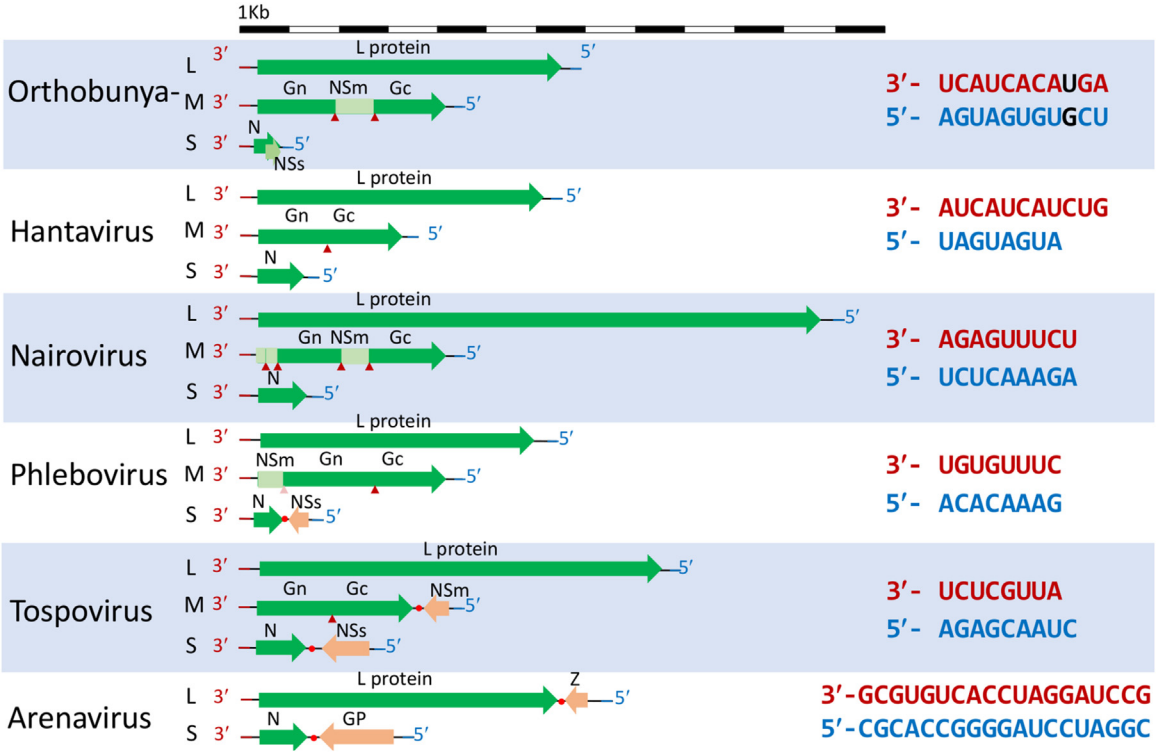

Fig. 1. Genome structure of *Bunyaviridae* and *Arenaviridae*. The genome segment for the five genus of the *Bunyaviridae* and *Arenaviridae* families are shown. The size of each genome segment is indicated by the upper 1Kb rule. The regions containing the ORFs are indicated by green arrows for those proteins whose mRNA is transcribed from the vRNA and orange for those transcribed from the cRNA. The red triangles indicate the location of the protease cleavage sites within the indicated polyproteins and the red spots indicate the location of transcription termination signals. The conserved 3′ (red) and 5′ (blue) termini vRNA sequences are indicated on the right, and show the Watson-Crick base pairing complementary. Conserved non complementary bases are written in black.

stranded (see below) and not as dsRNA, consequently the hypothesis of dsRNA ended genomes have been updated (Kolakofsky, 2016). The NCRs extend beyond the promoter being very variable in length between bunyavirus ranging from 14 and 44 nucleotides for hantavirus Oropouche virus 5′ and 3′ respectively and 169 and 226 nucleotides for orthobunyavirus bunyamwera serogroup (Elliott and Blakqori, 2011). The termination signals, as well as packaging signals, are presumably associated to RNA secondary structures as stem loops (Albariño et al., 2007; Ikegami et al., 2007; Osborne and Elliott, 2000), this needs to be compatible with the mechanism of vRNA coating by the NPs, which tends to maintain the RNA as single stranded (Reguera et al., 2014). Some of these loops may emerge from the RNPs and should be free of NPs in order to be accessible to the polymerase or other putative factors controlling transcription termination, RNP packing or virus budding.

3.2. Arenaviruses

Arenaviruses are enveloped viruses with a bi-segmented negative-stranded RNA genome. Each genome segment, L (large, 7.3 kb) and S (small, 3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by a non-coding intergenic region (IGR). The S RNA encodes the viral NP and the glycoprotein precursor (GPC) that is co-translationally cleaved by the signal peptidase to generate a 58-amino acid stable signal peptide (SSP) and post-translationally processed by the site 1 protease (S1P) to generate the mature virion surface glycoproteins GP1 and GP2 that together with SSP form the GP complex that mediates virus receptor recognition and cell entry (Beyer et al., 2003; Pinschewer et al., 2003). The SSP has been implicated in different aspects of the trafficking and function of the viral envelope glycoproteins (Saunders et al., 2007; York et al., 2004; York and Nunberg, 2009, 2007, 2006). GP-1 mediates virus interaction with host cell surface receptors and is located at the top of the spike, away from the membrane, and is held in place by ionic interactions with the N-terminus of the transmembrane GP-2. The arenavirus RING finger protein Z is a structural component of the virion that has been shown to be the arenavirus counterpart of the M protein found in many other NS enveloped RNA viruses that plays a critical role in assembly and cell release of mature infectious virions (Perez and de la Torre, 2003; Strecker et al., 2003; Urata et al., 2006). The L RNA encodes the viral RNA dependent RNA polymerase (L protein), and the small RING finger protein Z, that has functions of a bona fide matrix protein (see below) (Perez et al., 2003; Strecker et al., 2003).

Arenavirus genomes exhibit high degree of sequence conservation at their 3′-termini and similarly to other viruses with segmented negative strand (sNS) RNA genomes, they Exhibit 5′- and 3′- complementary at their L and S genome segments that are predicted to form panhandle structures. There are significant differences in sequence and predicted folded structure between the S and L IGR, but among isolates and strains of the same arenavirus species the S, as well as L, IGR sequences are highly conserved.

NP, the most abundant viral polypeptide both in infected cells and virions, is the main structural component of the viral RNP responsible for directing the biosynthetic processes of RNA replication and gene transcription of the viral genome. NP exhibits also a type I interferon (IFN-I) counteracting activity that initial mutation-function studies mapped to the C-terminus of NP (Martinez-Sobrido et al., 2009, 2007, 2006). Crystallographic studies identified distinct N- and C-terminal domains within NP of the

Old World arenaviruses LASV (Brunotte et al., 2011a,b; Hastie et al., 2011; Qi et al., 2010) and LCMV (West et al., 2014), and similar findings were subsequently documented for the New World arenaviruses Junin (Zhang et al., 2013) and Tacaribe (Jiang et al., 2013). The N-terminal domain was proposed to have potential cap-binding activity that could provide the host-derived primers to initiate transcription by the virus polymerase (Qi et al., 2010), However, subsequent studies showed that this putative NP cap-binding domain corresponded to the NP RNA binding site and thereby the cap-binding activity of NP remains to be proven (Hastie et al., 2011). In contrast, the C-terminal domain has a folding that mimics that of the DEDDH family of 3′- 5′ exoribonucleases like the one associated with SARS Coronavirus nsp14 protein (Eckerle et al., 2010). Functional studies confirmed the 3′ – 5′ exoribonuclease activity of LASV NP, which was proposed to be critical for the anti-IFN activity of NP but dispensable for the role of NP on replication and transcription of the viral genome. This assertion, however, needs to be carefully evaluated as LCMV with a mutant NP lacking the 3′- 5′ exoribonuclease had a large decrease in fitness during its replication in IFN-deficient Vero cells (Huang et al., 2015; Martinez-Sobrido et al., 2009).

The arenavirus L protein has a central region that conserved motifs characteristically found in the RdRpols (Fig. 5, see below) of negative strand RNA viruses (NSVs), whereas the N-and C-terminal parts did not exhibit clear protein signatures including RNA helicases or methyltransferases involved in RNA capping (Vieth et al., 2004). Mutation-function studies of the LASV L protein showed that residues critical for function were located both within and outside the predicted polymerase domain and identified specific amino acid residues that were required for RNA transcription, but not replication, mediated by the LASV polymerase in cell-based minigenome (MG) assays (Hass et al., 2008; Lehmann et al., 2014; Lelke et al., 2010). Interestingly, mutations of evolutionary conserved acidic (Asp and Glu) and basic (Lys and Arg) residues within the LASV L protein identified residues that played a critical role in mRNA synthesis without significantly affecting RNA replication (Lelke et al., 2010). Acidic and basic residues can contribute to catalytic and binding sites for endoribonucleases and capping enzymes, and bioinformatics analysis revealed that the N-terminus of LASV L protein had a distant similarity to type II endonucleases (ENs) (Lelke et al., 2010). Moreover, the crystal structure of the N-terminal region of LCMV uncovered an EN domain of similar structure to the EN domains of the influenza PA and La Crosse L proteins (Morin et al., 2010). Likewise, a high-resolution crystal structure of the N-terminal region of LASV L protein revealed high structural homology with the one determined for the EN domain present in the N-terminal region of LCMV L (Wallat et al., 2014). Biochemical and functional studies have shown that LASV L protein is organized into three distinct structural domains and that at specific amino acid positions LASV L can be split into an N-and C-parts that are able to functionally trans-complement each other (Brunotte et al., 2011a,b). In addition, electron microscopy characterization of a functional Machupo (MACV) L protein has revealed a core ring-domain decorated by appendages, which likely reflects a modular organization of the arenavirus polymerase (Kranzusch et al., 2010; Kranzusch and Whelan, 2012).

Arenavirus the L protein function is regulated by the Z protein, a small (~11 kDa) RING finger protein that mediates several distinct virus–virus and virus-host protein–protein interactions that contribute to its role as a bona fide matrix protein (Matrix) that regulates viral assembly and budding, as well as a key actor in regulating viral RNA synthesis and counteracting some components of the host innate immune response to infection. The Z protein is encoded by an ORF with genome polarity and transcription of its mRNA starts at the 5′ end of the L RNA anti-genomic segment (Fig. 1). Thus, the transcription of Z mRNA occurs once the L anti-genomic RNP species have been produced and not during the first transcriptional rounds of the infection. Z expression levels are regulated and differences in its expression between early and late phases of the virus life cycle likely reflect different roles played by Z at different stages of infection. Low expression levels of Z early during the virus life cycle can facilitate its role as regulator of viral RNA replication and gene transcription (Garcin et al., 1993), through direct interaction with the L protein (Kranzusch and Whelan, 2011), whereas increased Z expression levels a later times during the arenavirus life cycle will promote virus assembly and budding (Perez et al., 2003; Strecker et al., 2003; Urata et al., 2006).

4. Bunyavirus and arenavirus cell entry and delivery of the viral RNP into the cell cytoplasm

Bunyaviruses can use different receptors for cell entry, including DC-SIGN, also used by other viruses such HIV or HCV, L-SIGN or glycosaminoglycans such heparan sulphate (Albornoz et al., 2016). The interaction between the cellular receptor and the bunyavirus surface glycoprotein triggers endocytosis and membrane fusion in a clathrine dependent manner, which leads to the release of the bunyavirus RNP into the cellular cytoplasm, where transcription and replication take place. The arrangement of the glycoproteins in their surface is variable showing from pleomorphic arrangement (*e.g.* Bunyamwera and LACV orthobunyavirus) to icosahedral symmetry (*e.g.* RVFV and Uukuniemi phlebovirus, with T = 12 triangulation) (Bowden et al., 2013; Överby et al., 2008). The glycoproteins interact with the RNPs NPs and the RNA probably giving stability to the particles, an important aspect for virus transmission that could be studied in bunyaviruses as has been done for non-enveloped spherical viruses (Reguera et al., 2004). The specific interactions between the viral glycoproteins and the RNPs are also determinant for the packing of RNPs into viral particles during virus maturation, which takes place generally in viral factories at the Golgi apparatus (Pettersson and Melin, 1996; Salanueva et al., 2003).

Consistent with a broad host range and cell type tropism, a highly conserved and widely expressed cell surface protein, alpha-dystroglycan (aDG) has been identified as a main receptor for LCMV, LASV and several other arenaviruses (Cao et al., 1998; Kunz et al., 2002). However, many arenaviruses appear to be able to use alternative receptors (Kunz et al., 2004), and human transferrin receptor 1 was identified as a cellular receptor used for entry of the New World HF arenaviruses Junin (JUNV) and MACV (Radoshitzky et al., 2007). Arenavirus cell entry is via receptor-mediated endocytosis. The acidic environment of the late endosome facilitates a pH-dependent conformational change in the GPC complex that induces a GP-2 mediated fusion step between viral and cell membranes (Eschli et al., 2006), which releases the viral RNP into the cytoplasm and subsequent onset of viral RNA synthesis.

The tripartite and bipartite segmented genomes of bunya- and arenaviruses are never found as free RNA species, but rather always assembled into RNPs containing genome RNA species tightly interacting with the viral NPs, and at least one molecule of the L protein per RNP complex. The RNPs are carried from cell to cell in enveloped virions of an average diameter of between 80 and 160 nm. To be infectious, bunya- and arenaviruses need to include at least one copy of each genome segment into the mature virion progeny. Fluorescence In Situ Hybridization (FISH) experiments performed using RVFV infected cells and virions showed heterogeneity in segment composition of mature bunyavirus virions, suggesting that packaging is less selective that the one observed for the related orthomyxoviruses with eight genome RNA segments (Schreur and Kortekaas, 2016).

5. Bunya- and arenavirus RNA replication and gene transcription

Because of the negative polarity of the bunya- and arenavirus vRNA, once in the cytoplasm the RNPs need first to transcribe their mRNAs as a necessary step to produce the viral proteins for next replicative rounds. This is the essential reason why they, as all NSVs, need to carry all the viral transcriptional machinery (RNPs) in the viral particles. Transcription initiation is carried out through a unique mechanism shared by all sNSV called cap-snatching by which a short fragment of 5′-capped primers are stolen by the L protein machinery from the host cell mRNAs to prime the synthesis of viral mRNAs. Bunya- and arenavirus polymerases differ on the length of the 5′-capped primers they use to prime transcription, which are 12–18 and 4–5 long for bunyaviruses and arenaviruses, respectively. Bunyavirus transcription is coupled to translation (Barr, 2007) suggesting that a coordination, perhaps including specific interactions, may occur between the RNPs and the cellular translational machinery. In contrast, in cells infected with the arenavirus Tacaribe transcription of the NP mRNA could be detected early on in the presence of inhibitors of protein synthesis (Franze-Fernández et al., 1987). Transcription termination occurs at specific, conserved sequences (termination signals) without the addition of a poly-A tails. Bunyavirus and arenaviruses carry out replication in two steps: first the *de novo* (primer independent) synthesis of an intermediate RNA with positive polarity (cRNA) and second, using this as template, the synthesis of the new generation of genomic vRNA with negative polarity. Both processes are deeply coupled to the RNP assembly and NP and L protein availability, therefore the NP concentration in the infected cells could influence the replication versus transcription activity of the L protein.

The RNPs of many bunya- and arenaviruses have been characterised by negative staining electron microscopy in viruses, infected cells and isolated RNPs. A common aspect is their assembly in flexible "pearl necklace" like circular assemblies (Talmon et al., 1987; Young and Howard, 1983), some bunyavirus, as hantavirus could have rigid RNPs, at least at some steps of the infection (Goldsmith et al., 1995). For orthobunya- and phleboviruses, the flexibility is achieved by the NP – NP interactions that, mediated by flexibly hanging C- and N-terminal extensions, can adopt several protein–protein geometries (Reguera et al., 2014). This would allow to adopt different degrees of RNP compaction at different steps of the viral cycle (Reguera et al., 2013) (*e.g.* a relaxed form for the vRNA and cRNA reading during replication and transcription and a compacted form for virus budding).

Arenavirus polymerases need NP and L as minimal viral trans-acting factor required for efficient RNA synthesis (Hass et al., 2004; Lee et al., 2000; Lee and de la Torre, 2002; Lopez et al., 2001). Genetic and biochemical evidence indicated that oligomerization of L is required for the activity of the arenavirus polymerase (Sanchez and de la Torre, 2005). Consistent with this finding biochemical and MG-based functional studies have shown that LASV L protein contains both N- and C-termini sites that mediate L-L interaction (Brunotte et al., 2011a,b).

LCMV mRNAs have 4 or 5 non-templated nucleotides and a cap structure at their 5′-ends, which are obtained from cellular mRNAs via a cap-snatching mechanism whose details remain to be determined, but a functional EN domain within the N-terminal region of the arenavirus L polymerase plays a critical role in this process. Transcription termination of subgenomic non-polyadenylated viral mRNAs were mapped to multiple sites within the distal side of the IGR (Meyer et al., 2002), and the IGR was shown to act as a bona fide transcription termination signal for the virus polymerase (Pinschewer et al., 2005). The NP and L coding regions are transcribed into a genomic complementary mRNA, whereas the GPC and Z coding regions are not translated directly from genomic RNA, but rather from genomic sense mRNAs that are transcribed using as templates the corresponding antigenome RNA species, which also function as replicative intermediates. The 5′-end of arenavirus genome and antigenome RNA species contain a non-templated G residue that has been proposed to reflect a prime-and-realign mechanism for RNA replication mediated by the virus polymerase (Garcin and Kolakofsky, 1992, 1990; Raju et al., 1990).

Mutation-function analysis of the genome 5′-and 3′-termini using the LCMV and LASV MG-based assays indicated that the activity of the arenavirus genomic promoter requires both sequence specificity within the highly conserved 3′-terminal 19 nucleotides of arenavirus genomes, and the integrity of the predicted panhandle structure formed via sequence complementarity between the 5′- and 3′-termini of viral genome RNAs (Hass et al., 2006; Perez and de la Torre, 2002). These studies revealed that arenavirus RNA replication and transcription are regulated in a coordinated manner. Likewise, MG-based assays provided direct experimental confirmation that intracellular levels of NP do not determine the balance between virus RNA replication and transcription (Pinschewer et al., 2003), a finding conceptually similar to that reported for the paramyxovirus RSV (Fearns et al., 1997).

Cell-based MG systems for different arenaviruses including LCMV, LASV and TCRV have shown that Z protein is not required for RNA replication and gene transcription mediated by the virus L polymerase, but rather Z exhibited a dose-dependent inhibitory effect on both RNA biosynthetic processes mediated by the arenavirus polymerase complex (Cornu and de la Torre, 2001; Hass et al., 2004; Lee et al., 2000; Lopez et al., 2001). Consistent with these findings, studies using in vitro reconstitution of RNA synthesis directed by the L polymerase of MACV have provided evidence that Z, via direct interaction with L, is able to lock the L polymerase in a promoter-bound, catalytically inactive state (Kranzusch and Whelan, 2011). However, the mechanisms by which Z exerts this action to downregulate viral RNA synthesis and promote virus assembly remain little understood. The determination of high resolution structures of the arenavirus L proteins in complex with their Z proteins will facilitate the elucidation of this question.

The development of arenavirus reverse genetics systems has provided investigators with a novel and powerful approach to investigate the viral cis-acting sequences and proteins, both viral and cellular, that control cell entry, RNA replication, gene expression, assembly and budding of arenaviruses (Cheng et al., 2013; Emonet et al., 2011; Ortiz-Riano et al., 2013). Likewise, the ability to rescue infectious arenaviruses with predetermined mutations in their genomes and examine their phenotypes both in cultured cells and *in vivo*. This approach should facilitate to identify and functionally characterize novel arenavirus L-host cell protein interactions that may contribute to different outcomes of arenavirus infection.

6. Structure of L Proteins

The structure of the LACV bunyavirus polymerase reported by Gerlach et al., (2015); provides the first high resolution structural insight for a sNSV L protein and its astonishing similarity with the influenza heterotrimeric polymerase strongly suggests that L proteins for bunya- and arenavirus share a common structural architecture implicating similar mechanisms of action with possibly genus or species specific variations. In the central region of the polypeptide chain the L proteins have the RdRpol subdomains (palm, fingers and thumb) where the A B C D E and F (also named pre-A) motifs reside (Fig. 2). The RdRpol N-terminus is extended by a 490 aa long domain named PA-C like domain due to the extraordinary similarity that has with the influenza C-terminal domain of the PA polymerase subunit. This domain is further extended by a linker region and together conform one side of the protein core. The

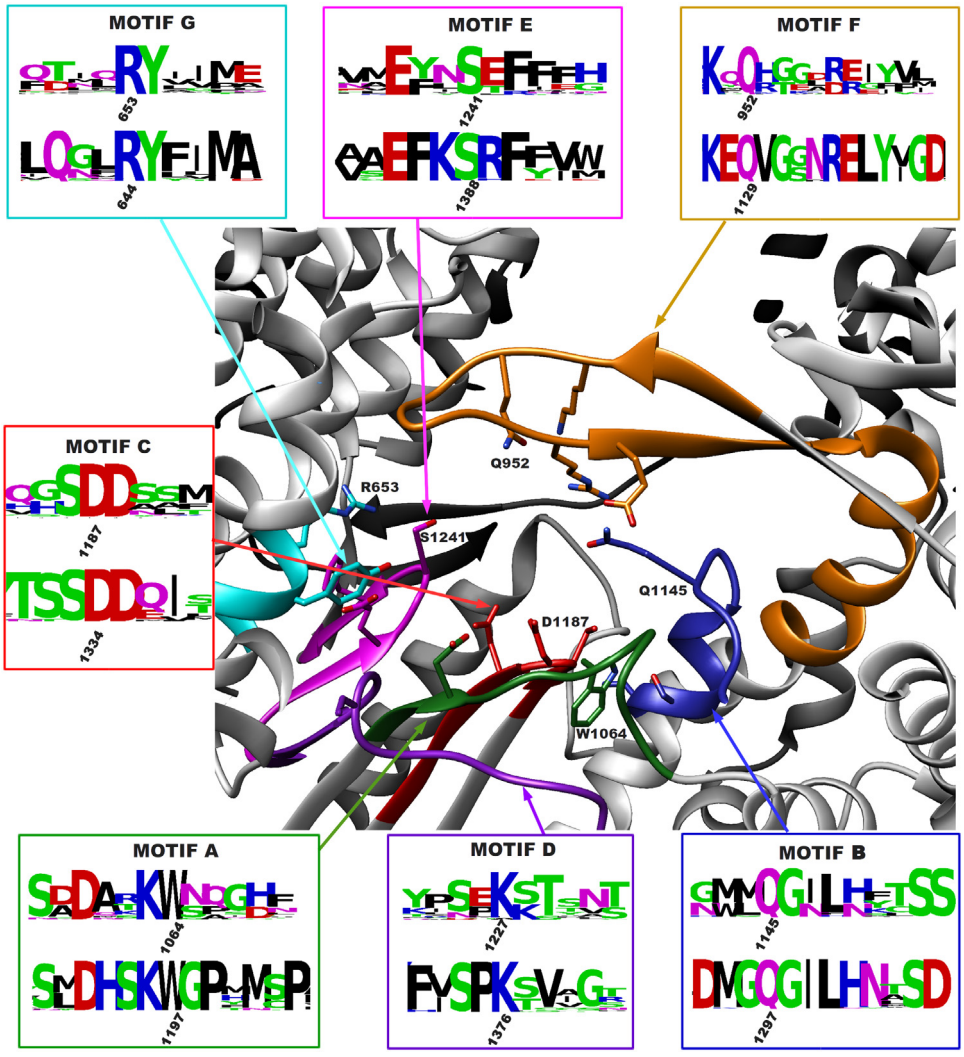

Fig. 2. Polymerase active site motifs of *Bunyaviridae* and *Arenaviridae* L proteins. Structure of the core of the polymerase domain (PDB code: 5amr) of LACV with the five motifs: G (cyan), F (orange), A (green),B (blue),C (red),D (purple), E (pink). Surrounding the structure are presented the weblogos of each motif, on the top are the *Bunyaviridae* motifs (numbering based on LACV) and in the bottom the *Arenaviridae* motifs (numbering based on LASV). Despite similarities in the catalytic site and surrounding areas, the overall broad divergence of the L protein sequences prevents to derive a reasonable structural model of *Arenaviridae* polymerase based on the crystal structured determined for LACV polymerase.

C-terminus of the RdRpol is extended by several protein domains (bridge, thumb ring and lid). C- and N- extensions close the RdRpol like a cage, a globular core where the access is very restricted and controlled (Fig. 3). This kind of caged polymerases product of N- and C-terminal extensions of the RdRpol domain, whether by elongating the polypeptide chain (*e.g.* bunyavirus) or by adding protein subunits in complex (*e.g.* Influenza), are found in non segmented NSV and dsRNA virus polymerases (Reguera et al., 2016a).

Despite the fact that *Bunyaviridae* and *Arenaviridae* exhibit broad genetic divergence, their L proteins, as well as those from other related sNSV like tenuivirus or emaravirus, share common essential aspects. The large L proteins are variable in size ranging from 2000 to 3950 aa in length (*Reptarenavirus* and *Nairovirus* genus respectively). Despite these differences, all maintain the active site conserved motifs in the central part of the polypeptide chain (Fig. 2). The determination of the structures of the LACV and Influenza polymerases allowed by structural alignments the identification of two new conserved motifs for sNSV named motif G and H that are located near to the RdRpol catalytic motifs in the structure and whose contributions to polymerase function remain to be experimentally determined (Fig. 2). The motif G is the only not belonging to the conserved RdRpol subdomains. It is deployed in the active site from the N-terminal extension of the cage (the PA-C like domain) (see LACV L protein scheme in Fig. 4 and Gerlach et al., 2015). The conserved arginine of motif G is positioned close to the priming NTP position in the active site. The conservation of this arginine residue within motif G was noticed early based on sequence alignments, but only the recent structural data have revealed its association to the polymerase active site (Gerlach et al., 2015; Müller et al., 1994).

The polymerase motifs are surrounded by large protein regions with no sequence similarity and genus-wise very variable in size from which no structural or functional information could be inferred by aminoacid sequence alignments. The landmark represented by the determination of the atomic structure of the LACV polymerase by X-ray crystallography and cryoelectron microscopy uncovered large protein stretches flanking the conserved polymerase domain building a cage structure wherein the RdRpol reaction occurs (Gerlach et al., 2015) (Fig. 3). Four independent channels give access to a central chamber inside the cage with space to harbour a small stretch of dsRNA. Based on homology with other viral polymerases, these channels could be assigned to NTP entry, template entry, template exit and product exit channels. This

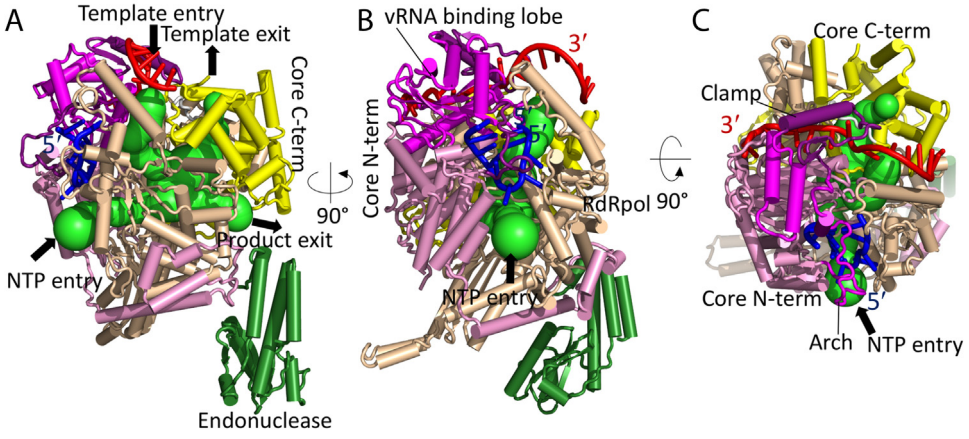

Fig. 3. LACV L protein crystal structure. Three views of the structure of the L protein first 1750 aa residues are shown in complex with viral RNAs represented in cartoons with the RdRpol domain in wheat, the core N-terminal extension in pink (within the vRNA binding lobe highlighted in violet), the core C-terminal extension in yellow and the hanging EN in green. A more detailed description of the structure and domains within the core C-terminal and N-terminal extensions is found in Gerlach et al. (2015). The vRNAs are coloured in red (3′) and blue (5′). The inner cavities are represented by green spheres as described in Gerlach et al. (2015). A, view of the polymerase clearly showing the four tunnels for the template exit and entry, the NTP entry and the product exit, converging in a central cavity where the RNA synthesis occurs. Core C-terminal and the RdRpol appear delimiting the cavity and tunnels. The cap-snatching EN is hanging from the core attached through a flexible link. B, rotating 90° the structure to the right is shown the NTP entrance close to the 5′ RNA binding site, the RdRpol and core N-terminal extensions appear delimiting the cavities and the NTP entry site. In violet is shown the vRNA binding lobe. C, rotating again the structure 90° towards the top is shown the vRNA binding lobe which is binding the 3′ RNA in one side, with a clamp insertion trapping the RNA, and 5′ RNA in the other, with an arch insertion partially covering the RNA.

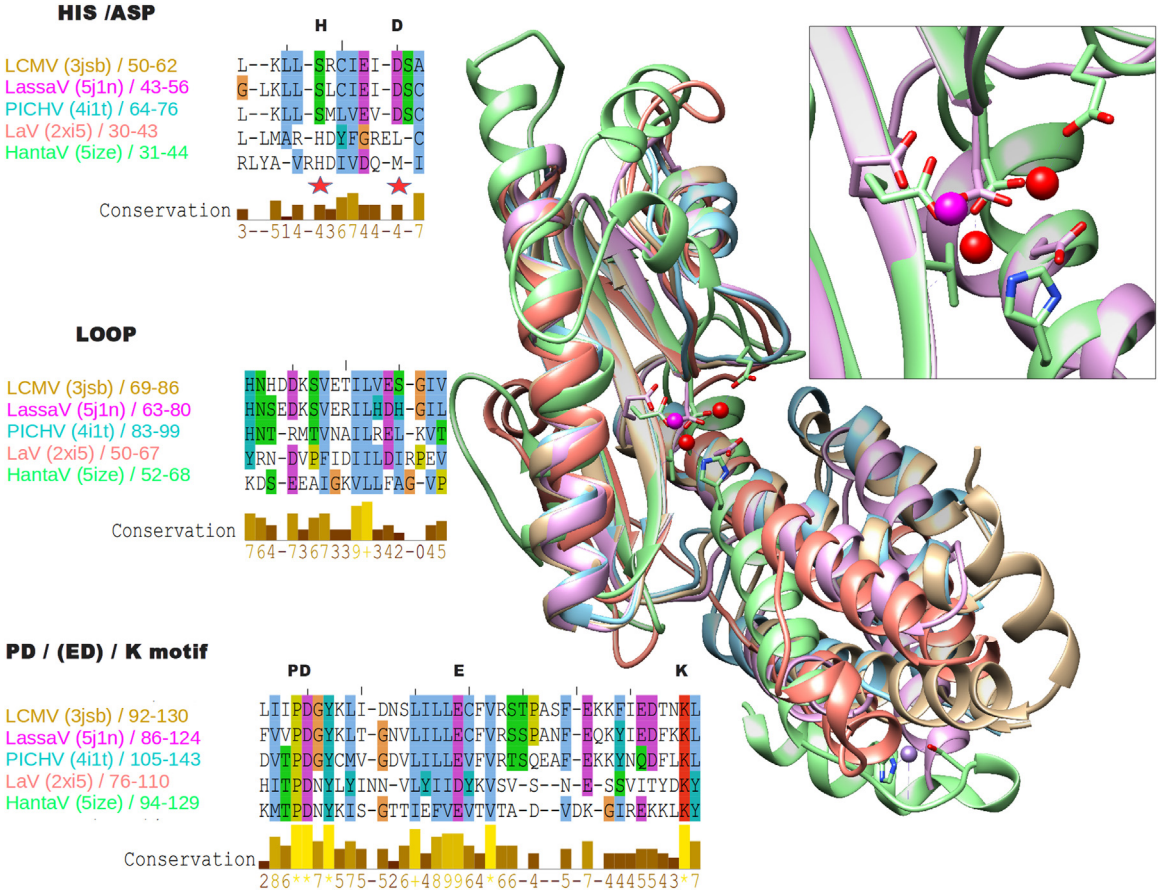

Fig. 4. Structural alignment of the L proteins cap-snatching endonucleases. The crystal structures of cap-snatching ENs belonging to mammarenavirus, orthobunyavirus and hantavirus. LCMV (PDB code: 3jsb, light brown); LASV (PDB code: 5j1n, pink); PICV (PDB code: 4i1t, cyan); LACV (PDB code: 2xi5, salmon); Hantaan virus (PDB code: 5ize, light green). Structures are represented in cartoon and residues of the catalytic site of LASV and Hantaan virus are shown in sticks with their respective manganese ions (coloured magenta and red respectively). The three conserved regions (His/Asp; LOOP; PD(ED)K motif) are shown on the side with Clustal color code scheme. Conserved residues are shown in bar diagrams under the alignment. For the His/Asp motif top letters and bottom red stars locate the key residues of the motif. For the PD(ED)K motif top letters locate the key residues of the motif. Side caption show a zoomed view of the catalytic site.

channels distribution indicates that the vRNA, cRNA and mRNAs are synthetized and sorted from the nascent dsRNA in two independent strands before exiting the cage, explaining how sNSVs are able to produce their genomes as single stranded. The close proximity of the template entry and exit channels also explains how the template can be read within RNP assemblies with a minimal disruption and possibly without the need of any RNP disassembly. The crystal structure of the related influenza heterotrimeric polymerase has the same overall architecture and cavity distribution confirming that it is a common feature for all sNSV, including *Bunyaviridae* and *Arenaviridae* L proteins. This feature is not unique for sNSV, as the polymerases of dsRNA viruses adopt a similar cage architecture with the four channels, a finding that supports the evolutionary relationship between these two groups of viruses and the relevance of these cage shaped polymerases in RNA virus evolution (Reguera et al., 2016a).

Another conserved feature between the L proteins from *Bunyaviridae* and *Orthomyxoviridae* families is the allosteric regulation of RNA synthesis by the 5′-end of the vRNA that folds as a hairpin and allosterically binds to the polymerase surface inducing the active conformation of the RdRpol active site. This interaction is rather folding specific and allows for certain degree of sequence variability as long as the folding and key interacting bases in the loop are preserved. The structure of the LACV polymerase also supports the specific recognition of the viral RNA, showing the extended 3′-end vRNA binding with high affinity and in a sequence specific manner to a RNA binding site on the surface of the polymerase. This binding site is different to the one observed in Influenza, suggesting differences among sNSV in the 3′ RNA binding site within their polymerases, which may provide also a main mechanism for the genome-specific recognition by the different polymerases. The need for the polymerase to recognize the same specific sequence in the vRNA and cRNA can explain the strict complementarity of 5′ and 3′ genomic ends since it ensures that the same sequences are maintained at the 3′-end of vRNA and cRNA species. The results derived from structural studies on bunyavirus L polymerase are consistent with those obtained from the structure of the vRNA – L protein complex for the arenavirus Machupo (MACV), where the 3′ vRNA end was shown to bind the L protein as single stranded and in a sequence specific manner (Kranzusch et al., 2010). In the case of arenaviruses, the Z protein plays a central role on the regulation of the polymerase activity by allowing the interaction of L protein – vRNA but inhibiting RNA synthesis, likely by stabilising the L protein – RNA complex in a pre-initiation state (Kranzusch and Whelan, 2011).

Crystallographic and CryoEM structures show that the N-terminus of the LACV L protein, corresponding to the EN responsible for cap-snatching, is flexibly hanging from the central core. This peripheral extension of the L proteins has also been observed for the MACV L protein, and described in detail by high resolution structures for the polymerases of influenza and VSV, and were associated in all the structures to the transcriptional processes of cap-snatching for sNSV and capping for nsNSV (Chou et al., 2016; Kranzusch et al., 2010; Pflug et al., 2014; Reguera et al., 2016a). These peripheral extensions are in all these polymerases located at the opposite side of the template RNA entry and exit channels showing a protein compartmentalization where the RNA reading occurs in one side and the product RNA in the other. This has interesting implications, *e.g.* the cap snatching EN would not have access to the vRNA template, which enters the L protein at the opposite side, when is detached from the NPs for entering the L protein.

Bunyaviridae and *Arenaviridae* cap-snatching ENs were first identified within the amino terminus of the bunyavirus LACV and the arenavirus LCMV L proteins (Morin et al., 2010; Reguera et al., 2010). Their crystallographic structures have a common basic bilobular fold consisting in a central beta sheet lobe surrounded by alpha helixes connected to an alpha bundle lobe (Fig. 4). The active site is located between the lobes, where two catalytic manganese ions are highly coordinated by the catalytic residues that constitute the conserved motif PD. (E/D)XK. This motif defines this family of ENs, and can be found in the amino terminus of near all sNSV L proteins with the notable exception of nairovirus, including Crimean Congo Hemorrhagic Fever Virus (CCHFV), which after the EN motif have an additional extension of ≈600 aa at N-terminus harbouring the OTU domain (see below). Additional structures of cap-snatching ENs were reported for hantaviruses and Lassa virus (Fernández-García et al., 2016; Reguera et al., 2016b; Wallat et al., 2014), which together with *Orthomyxoviridae* family ENs (Dias et al., 2009; Guilligay et al., 2014; Yuan et al., 2009) have generated a broad perspective of their structure and function. The PD. (E/D)XK motif has been also shown to be essential for transcription for phlebovirus (RVFV) and nairovirus (CCHFV) by mutagenesis in MG systems (Devignot et al., 2015; Klemm et al., 2013). A recent comparative study has proposed the classification of the cap-snatching ENs in two groups attending to the presence or absence of a catalytic histidine; the ENs having the catalytic histidine (His +), with H.PD. (E/D)XK motif, predominant in *Bunyaviridae* and *Orthomyxoviridae* families, show a high in vitro EN activity when isolated. A second group lacking or substituting the His by a glutamic acid (His-), predominant in *Arenaviridae*, has barely detectable EN activity. These huge differences in activity can be explained by structural differences that for arenavirus ENs would favour the non-canonical binding of the catalytic metal ions. Since in the infection context both kind of ENs are actively performing cap-snatching, these differences in *in vitro* activity can reflect a highly controlled process of cap-snatching with different regulatory mechanisms in *Arena-* and *Bunyaviridae* L proteins, the firsts needing the activation of the EN for transcription in the context of the full length L protein. This classification of ENs on His+ (active) His- (barely active) have important implications for the development of antiviral drugs targeting cap-snatching ENs (Reguera et al., 2016b). In addition to the cap-snatching these ENs could play other roles in the infection. We can find an example in the Andes virus L protein, where the highly active EN is able to supress the mRNA levels and protein expression in mammalian cells (Heinemann et al., 2013).

Another crucial aspect of the cap-snatching process is the recognition of the mRNA 5′ capped structures. This is achieved in *Orthomyxoviridae* by a cap-binding domain located in the PB2 polymerase subunit (Guilligay et al., 2008; Pflug et al., 2014), and predicted to reside at the C-terminus of *Arena-* and *Bunyaviridae* L proteins (Gerlach et al., 2015; Reguera et al., 2010). The available LACV polymerase structure lacks this region, and thereby further structural and functional studies are needed to confirm the presence of cap-binding domains to gain a better understanding of cap-snatching mechanism in *Arena- and Bunyaviridae* L proteins, as have been achieved for Influenza polymerases (Reich et al., 2014; Thierry et al., 2016; see the review in this special issue Pflug et al., 2017).

The L proteins of the *Nairovirus* genus of the *Bunyaviridae* family, including Crimean Congo Hemorrhagic Virus (CCHFV) have additional extensions or insertions inside the basic architecture shared between sNSV L proteins. Based on the common architecture between NSV and the conserved EN and RdRpol motifs, it appears that nairovirus L proteins extend their N-terminus beyond the cap-snatching EN by ≈ 600 aa (Fig. 5). This extension, similarly to the ENs of influenza and LACV, is probably hanging from the central polymerase core and includes at its very N-terminus a conserved ovarian tumour (OTU) domain. The OTU domain protease activity counteracts the host innate immune response by deconjugating ISG15 and Poly-Ubiquitin conjugates, targeting a specific motif conserved in both conjugates (RLRLGG) (Frias-Staheli et al., 2007). The structures of the CCHFV OTU domain in complex with

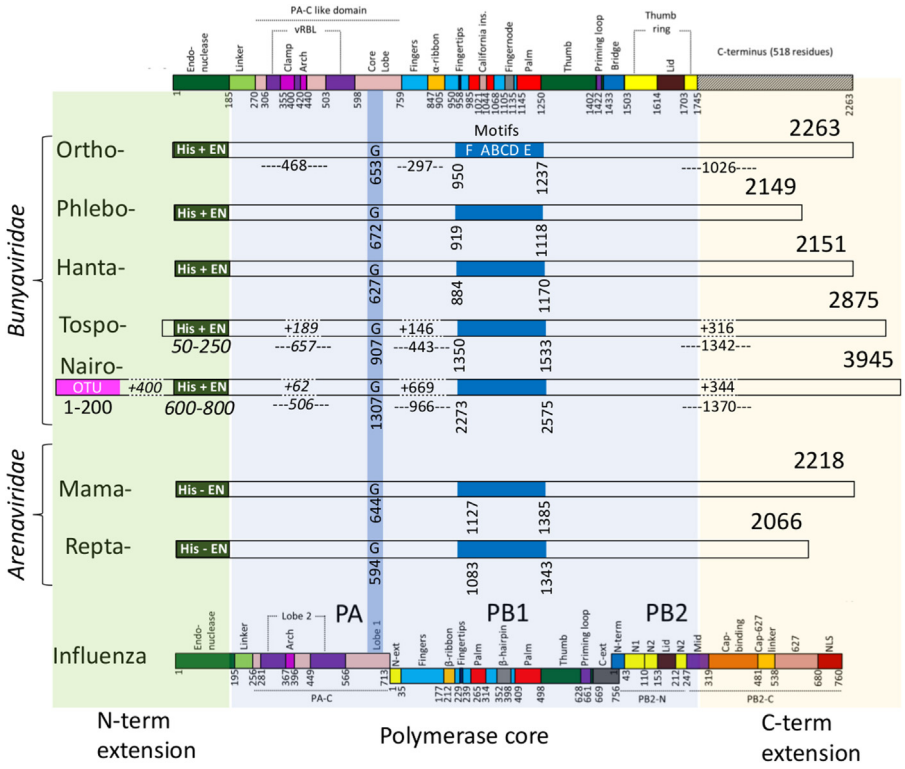

Fig. 5. Structural organization of L proteins. Schematic superposition of L proteins belonging to each genus of the *Bunyaviridae* and *Arenaviridae* families. The structural organization of the LACV L protein (top) and influenza heterotrimeric polymerase (bottom) derived from their respective crystal structures is represented like in Gerlach et al., 2015 and Pflug et al., 2014. The polymerases are aligned with respect to the conserved polymerase motifs shown in Fig. 3 and the conserved PD. (E/D)XK motif. The residue numbers for the polymerases correspond to LACV (orthobunyavirus), RVFV (phlebovirus), Hantaan (hantavirus), Tomato spotted wilt virus (tospovirus), CCHFV (nairovirus), Lassa (mammarenavirus) and Golden gate virus (reptarenavirus). The length (amino acid residues) for each polymerase is indicated in their upper right side. The distances between conserved motifs and characterised domains are indicated for LACV (Gerlach et al., 2015) and the tospo- and nairovirus indicating the length of the insertions within the dashed lines stretches. Since the EN domains have not been isolated yet for tospo- and nairovirus the distances between the ENs and polymerase motifs or OTU domain are estimated values taking into account the conserved PD. (E/D)XK motif and the approximate size of EN domains (200aa) and are written in italics. The scheme illustrates the overall architecture of L proteins and the predicted positions of the more relevant insertions for the larger tospo- and nairovirus polymerases.

ISG15 and Ubiquitin show at the atomic level the specific recognition of the RLRLGG motif in the protease active site cavity (James et al., 2011). The function and structure of the ≈400 aa residues between the OTU domain and the EN still need to be elucidated.

Between the EN and the RdRpol subdomains is found the PA-C like domain connected to the EN domain by a linker that buttresses the polymerase core and positions the EN at the edge of the product exit channel, where the cap-snatching takes place. Compared to other bunya- and arenaviruses, nairovirus and tospovirus L proteins have a larger PA-C like domain. Using as reference motif G, we can extrapolate that tospovirus have an insertion about 190 aa long in the viral RNA binding lobe area and CCHFV have an even bigger insertion, about 650 aa long, occurring between the PA-C like and the polymerase fingers subdomain. The structural consequences could range from an enlargement of the polymerase core to the insertion of extra domains with possibly new functions. Since in LACV L protein the PA-C like domain include the viral RNA binding lobe, engaged on 3′ promoter recognition and allosteric regulation of the 5′ RNA through specific insertions (the clamp and the arch, see Fig. 3) the RNA binding is one of the polymerase functions susceptible to be affected by this enlargement in tospovirus. At the C-terminus, where the cap-binding domain is predicted to be, nairovirus and tospovirus L proteins have additional extensions of ≈325 and 390 aa respectively. Again, this could cause the enlargement of the polymerase core or could reflect the addition of new functional domains. The extra functions associated with the complexity of the nairovirus L proteins represent novel targets for therapeutic drugs, which represent an unmet need to combat the devastating effects of CCHFV infections of humans.

7. Structure and function of the mammarenavirus Z protein

Comparison of mammarenavirus Z proteins aa sequences shows in overall a modest degree of conservation (below the 20%), this similarity is even lower when the Z sequences of the recently identified reptarenaviruses are included in the alignments. There are however three regions within Z that exhibit a higher degree of conservation (Fig. 6): i) a myristoylation site marked by a G motif conserved at the N-terminus, critical for membrane anchoring (Strecker et al., 2006, 2003) and interaction of with other viral proteins (Capul et al., 2011). ii) A central RING domain (Borden and Freemont, 1996), binding two zinc ions through 3 conserved motifs (Ring 1–3). iii) A C-terminal part harbours one (or more) proline rich regions forming the late motif. Late motifs have been identified in the matrix proteins of various enveloped RNA viruses and in the Gag proteins of a number of retroviruses. They mediate protein–protein interactions between viral proteins and components of the endosomal sorting complexes required for transport (ESCRT), which mainly constitute the vacuolar protein sorting (VPS) pathway (Freed, 2002). Both old world (OW) and new world (NW) mammarenavirus contain a highly conserved YxxL motif located in central RING domain (Ring 2) which is not present in reptarenavirus. Furthermore, all mammarenavirus Z proteins carry P[T/S]AP- and PPPY-type late domains in their C-terminal parts while reptarenavirus Z proteins carry a shorter PEP or are depleted of late domain (i.e. CAS and Hans Kompis viruses).

The structure of Z Lassa virus was solved in complex with eIF4E by NMR (Fig. 6) (Volpon et al., 2008). The structure of the complex shows that the N- (residue 1–29) and the C- terminal

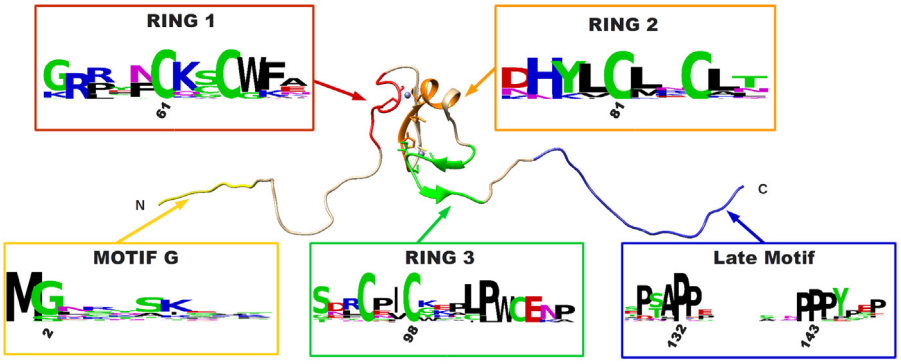

Fig. 6. NMR structure of Lassa virus Z protein. Z protein (PDB code: 2M1S) in ribbon surrounded by weblogos corresponding to motifs conserved across *Arenaviridae* family members. N-terminus and C-terminus are labelled N and C respectively. The protein has a central globular RING domain extended in both directions by disordered regions. The central RING domain has three conserved motifs (coloured in red, orange and green). The N-terminal region embed a conserved G motif (yellow) critical for myristoylation and membrane attachment, the C-terminal region contains proline rich late domain motifs (blue), which are variable in length and composition. Alignments of Z mamma- and reptarenavirus amino acid sequences was done by T-coffee including 37 Z protein sequences.

(residue 77–99) arms flanking the RING domain are disordered and only the central RING domain is structured. It is postulated that the disordered regions enable Z protein to recruit a variety of partners ranging from PML (promyelocytic leukemia protein), eIF4E (eukaryotic translation initiation factor 4E), PRH (proline-rich homeodomain protein), and ribosomal P proteins, RIG-I and caspases, but the how still needs to be characterised. The structure also shows the important residues of the RING 1 motif for the interaction with eIF4E (F60, K62, S63, W65) plus N68, K69, in Lassa virus numbering 30, 32, 33, 35, 38, 39 respectively) (Volpon et al., 2010). Interestingly, these residues are not strictly conserved among arenavirus not even within mammarenavirus, where conservation is only around F 32%, K 93%, S 64%, W 100% and N38%, K54%. Moreover analytical ultracentrifugation, size exclusion chromatography, and EM studies of LCMV Z protein indicate that eIF4E can interact with both oligomeric and monomeric forms of Z and that oligomeric forms regulate better eIF4E (Kentsis et al., 2002, 2001). Altogether, these data suggested that the structure probably represents only one particular binding mode. Indeed, a recent crystallographic study by Hastie and co-workers (Hastie et al., 2016) presents an alternative mode of association of the Lassa virus Z protein. Under crystallographic conditions, Z assembles in a dodecameric manner through a head to tail dimerization of the RING domain. This assembly introduces conformational changes thought to be required for a stable oligomerization leading to the viral matrix formation. Interestingly, the late part of RING 3 motif (Fig. 6: residues L102-P108, in Lassa virus numbering 71–77) seems to contribute to the assembly by connecting vertically the Z oligomers, which could explain how the matrix expend in the third dimension of space. Finally, that study shows that the disordered regions in N- and C-terminus play no role on the assembly of the oligomeric Z structure, which is consistent with the N-terminus being responsible for anchoring Z to the membrane, and the C-terminus mediating Z interaction with endosomal components required for moving the maturing virion toward the cell surface. These findings are consistent with Z playing a key role in virion assembly, which is further supported by EM studies indicating that Z bridges the vRNP with the viral envelope (Neuman et al., 2005). In addition the Z protein is also involved in the control of innate immunity, all pathogenic mammarenaviruses share an innate immune suppression mechanism that is based on the Z N-terminal binding to RIG-I-like receptors (RLRs) (Xing et al., 2015). Moreover, in the case of NW mammarenavirus and not the OW, Z protein also contributes in the suppression of type I IFN production by direct interaction with RIG-I (Fan et al., 2010).

The interactions and mechanisms by which Z protein contributes to the arenavirus's ability to counteract the host defence mechanisms are still little understood (reviewed in Fehling et al., 2012). The determination of high resolution structures of the arenavirus Z protein in complex with relevant Z- host cell interacting partners will help to understand the mechanisms underlying Z-mediated regulation of host cell factor functions to promote virus multiplication and how this may relate with the regulation of the L protein function.

8. Mechanisms of transcription and replication processes mediated by bunya- and arenavirus L polymerases

Bunya-and arenavirus L proteins specifically replicate and transcribe their viral genomes (vRNA) in the cellular cytoplasm. These biosynthetic processes require the negative sense RNA genome species to be coated by multiple copies of viral NP, which together with L protein assembled into a functional vRNP. This, in turn, requires that the vRNA, NP and L polymerase would undergo the appropriate coordinated interactions. The structure of LACV L protein has provided key information about how the polymerase interacts and is regulated by its genome. This, together with the channel distribution described above and the promoter sequence and base pairing requirements for transcription and replication, led to propose a model that describes all the necessary steps for vRNA replication (see Fig. 7 in Gerlach et al., 2015). This model has also raised many questions yet to be answered that we summarize in Fig. 7. The reported LACV L protein structure corresponds to a pre-initiation state where the vRNA still did not enter the polymerase (Fig. 7). At this stage the RNP could start transcription or replication, the main difference between both processes lies on the use of respectively cap-snatched cellular mRNA for priming the initiation or *de novo* initiation. Transcription in the related influenza virus polymerase is orchestrated by the cap-binding domain and the EN domain. The cap-binding domain in influenza virus polymerase is needed for the specific recognition of the capped RNA and also for orienting the snatched RNA towards the polymerase active site for transcription initiation (Reich et al., 2014). Similar requirements are expected for the L proteins, but the existence of a cap-binding activity for the L proteins still needs to be proven.

Transcription initiation has been proposed to occur by prime and realign for hanta- and orthobunyavirus based on the non-templated sequences consistently detected in viral mRNAs consisting in extra repeated triplets conserved at the genome ends (AUC AUC AUC for hantavirus, UCA UCA UCA for orthobunyavirus, see Fig. 1) (Garcin et al., 1995; Jin and Elliott, 1993). The prime and realign model proposed for the initiation of RNA replication by the arenavirus polymerase differs from the one proposed for

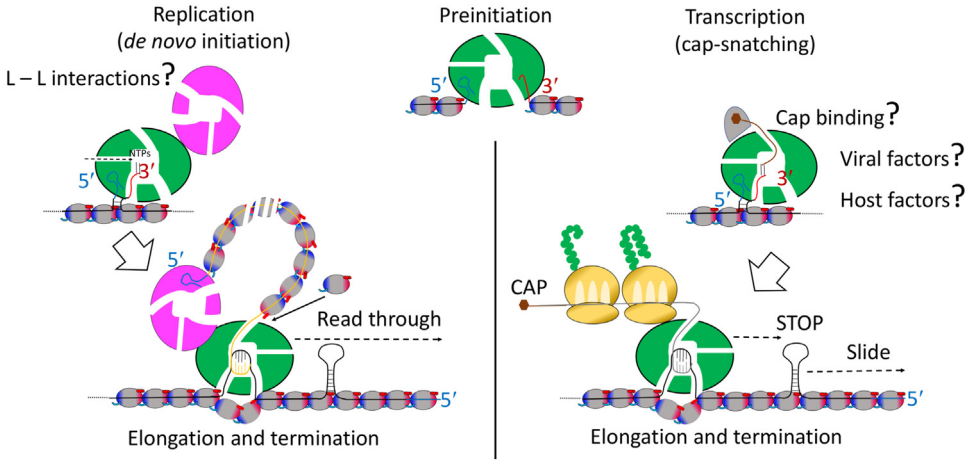

Fig. 7. Schematic representation of differences between transcription and replication processes carried out by the L proteins. From the preinitiation state the L protein starts transcription (right side) or replication (left side). Transcription initiation occurs by cap-snatching where RNA synthesis is primed by a capped cellular mRNA shortened to 12–18 or 4–5 nucleotides (for bunyaviruses and arenaviruses, respectively) by the EN (not shown in the scheme). In Influenza, the cap-binding domain is the responsible for directing the capped RNA towards the active site, the presence of a cap-binding domain in L proteins is still non proven. Likewise, it remains to be determined whether cellular factors are also necessary for transcription activation. The requirement of active translation for viral transcription elongation strongly suggest the coupling between the viral L protein and the host cell translational machinery in bunyaviruses. During transcription the polymerase stops at the transcription termination signal, because of its closed cage structure the polymerase cannot detach from the RNA template and would need to slide to the end of the genome to start another replicative or transcriptional round. The replication starts *de novo* (left side) and can need a helper polymerase that would interact with the nascent 5′ strand and coordinate the RNA synthesis with the RNP assembly. The L protein does not stop when reading the transcription termination signal and can continue the replication until the end of the genome to synthetize the complete cRNA.

bunyavirus transcription and assumes that arenavirus polymerases initiate, like many other viral RdRpols, RNA synthesis *de novo* only with GTP. Accordingly, arenavirus RNA initiation would take place from an internal templated cytidilate. Once the first phosphodiester bond has been formed, the pppGpC will slip backwards on the template and realign, creating a nascent chain whose 5′ end is at position −1 with respect to the template, before the polymerase resumes downstream synthesis. To maintain constant the length of the genome RNA despite addition of a non-templated nucleotide at the 5′-end, the polymerase terminates RNA synthesis by removing the last base at the 3′ end of the nascent chain. Arenavirus genome, and antigenome, sequence terminal complementarity combined with the prime and realign mechanism for replication initiation generates double-stranded RNA species with an overhanging 5'ppp nucleotide, a structure that has been shown to escape RIG-I recognition (Marq et al., 2010), as well as to act as an RIG-I decoy (Marq et al., 2011), and thereby prevent, or diminish, the subsequent RIG-I mediated interferon induction (Habjan et al., 2008; Wang et al., 2011). Therefore, the initiation mechanism of arenavirus RNA synthesis, and likely also hantavirus, seem to have evolved for evading the innate immune response triggered by RIG-I.

In addition to the differences between the mechanisms of initiation of gene transcription and RNA replication used by the L proteins, the mechanisms associated with transcription and replication termination events also exhibit differences. Transcription termination signals stops the RNA synthesis without the polyadenylation of the mRNA. The closed cage structure of the polymerase does not allow the detachment of the L protein from the template RNA, therefore the polymerase would be forced to slide until the 5′ end of the genome in order to start the next replicative or transcriptional round (Fig. 7). In contrast, during replication the transcription termination signal is replicated to produce the full cRNA species. This differential behaviour of the L protein when reading the transcription termination suggest that the transcription or replication modes of the L protein are maintained all along the RNA synthesis and not only during initiation.

Future studies should be aimed at solving the important question of identifying and functionally characterizing factors that promote the transcriptase versus replicase modes of the L protein. In both cases, allosteric regulation of 5′ RNA binding seems to be an essential requirement for the activation of the active site. Since the replication is coupled to the RNP assembly and the transcription is coupled to translation in bunyaviruses is reasonable to deduce that the protein environment associated to both processes could be playing a regulatory role. From the structural point of view the replication or transcriptional modes of the polymerase could translate in two different conformational states. For replication, the need of recruiting a new L protein and nucleoproteins candidates these two proteins as necessary replication enhancers and whether this happens through direct interactions with the L protein replicative mode or need the implication of other cellular factors still have to be addressed. For the switching to the L protein to the transcriptional mode, factors associated to the cellular translational machinery might be playing a role and could also be mediated through direct interactions. Therefore, a trustable characterization of host factors interacting with functional L proteins will provide some clues about which cellular factors could be playing a role in the L protein mode switch.

9. Development of new antiviral drugs targeting the activity viral L polymerase

Knowledge derived from structural and functional analysis of the tri-partite L-NP-RNA interaction can uncover novel possibilities for the development of antiviral drugs to combat human pathogenic bunya- and arenaviruses.

In the case of arenaviruses, LASV is the one with the highest impact in human health and evidence indicates that morbidity and mortality associated with LASV infection, and likely other HF arenavirus infections, is associated with, at least partly, the failure of the host's innate immune response to restrict virus replication and to facilitate the initiation of an effective adaptive immune response (McCormick and Fisher-Hoch, 2002). Accordingly, the extent of viremia is a highly predictive factor for the outcome of LF patients. This scenario suggests that therapeutic interventions resulting in reduced virus load, without achieving viral clearance, might suffice to promote the recovery of appropriate host defence responses to control virus multiplication and associated disease.

The nucleoside analogue ribavirin (Rib) (1-b-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to have prophylactic and therapeutic effects against several arenaviruses and bunyaviruses both in cultured cells and in vivo, as well as to reduce significantly both morbidity and mortality in humans associated with LASV infection (Andrei and De Clercq, 1993; Damonte and Coto, 2002), and experimentally in Machupo (Kilgore et al., 1997), Junin infections (McKee et al., 1988) and bunyavirus as hantavirus causing HFRS (McKee et al., 1988) if given early in the course of clinical disease. Rib is also an interesting scaffold to develop new therapeutic compounds as ETAR (1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole), shown to be effective particularly against hantavirus infections probably by having different mechanism of action (Chung et al., 2008). For some bunyavirus infections, as the caused by CCHFV or RVFV, Rib treatment have not a significant therapeutic effect (Bodur et al., 2011). Recently, favipiravir (T-705), a broad-spectrum RNA-dependent RNA polymerase inhibitor, has shown very promising results in guinea pig models of fatal Argentine HF disease caused by JUNV (Gowen et al., 2013) and LF caused by LASV (Safronetz et al., 2015). Rib has been shown also to act synergistically with other treatments as illustrated by its use together with T-705 to protect against RVFV phlebovirus (Scharton et al., 2014), and arenaviral (Westover et al., 2016) infections. Rib also enhanced the antiviral effect of silencing RNAs (siRNA) targeting the Hazara bunyavirus NP mRNAs in culture cells (Flusin et al., 2011), but the efficacy of Rib to treat bunyavirus human infections remains questionable.

The anti-arenaviral activity of Rib likely involves targeting different steps of arenavirus RNA replication and gene expression machinery. In addition, Rib can be used as substrate by the RdRpol of some riboviruses, including arenaviruses, leading to C to U and G to A transitions (Cameron and Castro, 2001; Crotty et al., 2000; Parker, 2005), and this mutagenic activity of Rib has been linked to its antiviral activity via lethal mutagenesis. The anti-arenavirus activity of Rib has been recreated in the LCMV (Ruiz-Jarabo et al., 2003) and LASV (Hass et al., 2004) MG systems, which provide investigators with excellent platforms to identify inhibitors of arenavirus replication and gene expression and to dissect their mechanisms of action. As predicted, the myristoylation inhibitor 2- hydroxymyristic acid (2-OHM) that was shown to inhibit Z-mediated arenavirus budding (Perez et al., 2004) did not affect MG driven RNA replication and expression, which further supports the feasibility of using cell-based MG rescue systems to identify drugs that interfere with specific steps of the arenavirus RNA replication and gene transcription processes directed by the arenavirus polymerase.

The above mentioned sequence and structural constraints of the arenavirus and bunyavirus genome promoters provide new potential targets for drugs capable of disrupting the interaction between the promoter and virus polymerase complexes, which would interfere with the essential biosynthetic processes of viral transcription and replication. The potential of RNA as target for therapeutic intervention is gaining great interest (Ellenbecker et al., 2012; Hermann, 2000; Sucheck and Shue, 2001; Sucheck and Wong, 2000). The function of many RNA molecules depends on defined three-dimensional structure. Small molecules may interfere with RNA functions by a number of mechanisms including inhibition of the formation of competent RNA-protein complexes at the level of the polymerase and the nucleoprotein. The study of small molecule RNA effectors has primarily focused on the aminoglycosides (Hermann, 2000; Sucheck and Shue, 2001), illustrated by the antibiotic activity associated with aminoglycoside targeting of bacterial 16S ribosomal RNA. During the last years there have been great progresses in developing aminoglycoside-based analogues (mimetics) to circumvent problems related to oral inactivity, toxicity, and weak binding of aminoglycosides (Sears and Wong, 2001; Ye and Zhang, 2002). Moreover, recent progress has allowed for the generation of complex combinatorial libraries of aminoglycosides that can be subjected to robust novel screening procedures (Hermann, 2000; Sears and Wong, 2001; Sucheck and Shue, 2001; Ye and Zhang, 2002). These developments are facilitating the identification of drugs that bind specifically to a variety of RNA folds, thus opening new ways to expand the existing repertoire of protein-targeted therapeutics. The potential of aminoglycosides as antiviral molecules has been illustrated by their ability to disrupt selectively the HIV-1 Rev-RRE (Zapp et al., 1993) and Tat-TAR (Wang et al., 1998) interactions. Similar approaches could be used to select aminoglycosides that interact with the arenavirus and bunyavirus promoters, and disrupt its activity, particularly for the reported secondary structure of the 5′ genome ends, a conserved feature between sNSVs responsible for the allosteric regulation of the L proteins (see above). The sequence conservation of the promoter of these two families of viruses predicts that aminoglycoside-based small molecules selected based on their activity against the promoter of a prototypic virus of each bunyavirus genus or arenavirus would be also extensive to other members of the genus or family. The interference of viral protein – RNA interactions have been addressed also by small RNA molecules (aptamers) or compounds (Suramin) that can block RNA binding sites of the nucleoproteins with potential antiviral effect. This have been explored for RVFV NP and similar approaches could be undertaken now towards the L protein – vRNA specific binding sites based on the available structures for the *in silico* design of interfering compounds and aptamers and using the high-throughput fluorescence-based methods available for the study of L protein vRNA interactions (Ellenbecker et al., 2015, 2014; Gerlach et al., 2015).

Mutation-function studies uncovered that many of the mutant L proteins exhibited a strong dominant negative phenotype under assay conditions where the wt and mutant L proteins did not compete for template RNA or other trans-acting proteins, viral or cellular, required for polymerase activity (Sanchez and de la Torre, 2005). These results were highly suggestive of L-L interaction being required for LCMV polymerase activity. Consistent with these genetic data, results from co-immunoprecipitation studies using L proteins containing two different tags, HA and Flag, provided biochemical evidence for L-L interaction (Sanchez and de la Torre, 2005). Intragenic complementation has been documented for the L genes of several NSV viruses, and direct L-L physical interaction, required for polymerase activity, has been demonstrated for the paramyxoviruses Sendai and parainfluenza virus 3 (PIV3) (Smallwood et al., 2002; Smallwood and Moyer, 2004). These results would suggest that disruption of this L-L interaction could have a potent inhibitory effect on the virus polymerase activity. Self-association of arenavirus NP is required for the formation of a functional vRNP. Therefore, disruption of NP-NP interaction represents another attractive anti-bunya-arenaviral strategy.

Cell-based MG rescue assays are attractive for identifying small molecules capable of disrupting L-L and NP-NP interactions, which would result in inhibition of the RNA biosynthetic processes of viral RNA replication and gene transcription directed by the functional vRNP. Protein-protein interactions have been generally regarded as difficult or refractory drug targets for small molecules due to a widely shared assumption that large protein interfaces are unlikely to be disturbed by small molecules. However, recent evidence suggests that the affinity between interacting proteins may be governed only by a minor part of the interface region. Targeting these highly "localized" surfaces may be sufficient for inhibiting protein–protein interactions. Notably, combinatorial libraries of small molecules generated via solution phase methodology have been uniquely successful in the identification of small molecules that inhibit protein–protein interactions on a series of significant biological targets (Boger, 2003; Boger et al., 1998). Similar approaches should be applicable to screens to identify

small molecule inhibitors of arenavirus and bunyavirus replication machinery.

10. Future perspectives

The reached milestone of having the first high resolution structure of a bunyaviral L protein opens a new exciting panorama for the coming years. Certainly, the structure of the C-terminus of the L proteins remains to be determined to confirm or not the presence of a cap binding domain. There is also the need to achieve different functional conformational states of the protein to better understand the replication and transcription processes as has been described for influenza polymerases (Thierry et al., 2016 and references therein) and to confirm or amend the different steps of replication proposed in Gerlach et al., (2015).

Nevertheless, the prove of concept that L proteins structures can be achieved by obtaining crystallisable stable L protein-RNA complexes will boost the structural characterization of other L proteins of highly pathogenic bunyavirus and arenavirus. This will provide a detailed description of the structural basis of common essential aspects for their replication and transcription as well as the specific features of each viral strain consequence of host adaption or related to differences of the RNP architecture. The consequences will range from allowing structure based site directed mutagenesis in transcription and replication *in vitro* or MG–based studies, or the structure based drug design of inhibitors against crucial interactions with the vRNA or the many catalytic activities of the L proteins from highly pathogenic virus.

Finally, another interesting aspect for future drug development and based on structural similarities found between L protein cap-snatching EN structures will be to test the cross reactivity of drugs targeting influenza polymerase cap-snatching machinery in arenavirus and bunyavirus polymerases. Indeed, a broad spectra antiviral would be the optimal solution to render available drugs for the treatment of such geographically dispersed outbreaks caused by bunya- and arenaviruses zoonotic diseases.

Acknowledgements

The cite we include in the introduction is to honour Dr. Richard Elliott, who recently, and much too early, passed away. His contributions to the bunyavirus field, product of his scientific excellence and commitment over decades of research, has been such that bunyavirus will be necessarily always associated to his name for decades to come.

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