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REVIEW

Ribonucleoprotein transport in Negative Strand RNA viruses

Cédric Diot¹ | Gina Cosentino¹ | Marie-Anne Rameix-Welti^{1,2}

¹Université Paris-Saclay – Université de Versailles St. Quentin, UMR 1173 (2I), INSERM, Montigny-le-Bretonneux, France

²Assistance Publique des Hôpitaux de Paris, Hôpital Ambroise Paré, Laboratoire de Microbiologie, DMU15, Versailles, France

Correspondence

Marie-Anne Rameix-Welti, Université Paris-Saclay, Université de Versailles St. Quentin, UMR 1173 (2I), INSERM, Montigny-le-Bretonneux, France; Assistance Publique des Hôpitaux de Paris, Hôpital Ambroise Paré, Laboratoire de Microbiologie, DMU15, Versailles, Fran.
Email: marie-anne.rameix-welti@uvsq.fr

Abstract

Negative-sense, single-stranded RNA (-ssRNA) viruses comprise some of the deadliest human pathogens (Ebola, rabies, influenza A viruses etc.). Developing therapeutic tools relies on a better understanding of their multiplication cycle. For these viruses, the genome replication and transcription activities most-often segregate in membrane-less environments called inclusion bodies (IBs) or viral factories. These “organelles” usually locate far from the cell surface from where new virions are released, and -ssRNA viruses do not encode for transport factors. The efficient trafficking of the genome progeny toward the cell surface is most often ensured by mechanisms co-opting the cellular machineries. In this review, for each -ssRNA viral family, we cover the methods employed to characterize these host-virus interactions, the strategies used by the viruses to promote the virus genome transport, and the current gaps in the literature. Finally, we highlight how Rab11 has emerged as a target of choice for the intracellular transport of -ssRNA virus genomes.

INTRODUCTION

Negative single-strand RNA viruses form the Group V of the Baltimore classification, based on their genomic organization: negative sense, single-stranded RNA genomes (-ssRNA). The negative sense, or antisense, genomic RNA requires to be transcribed into a complementary positive sense mRNA by the viral polymerase to enable viral protein expression. These viruses comprise multiple viral species of major concern for public health, such as rabies (RABV), Ebola (EBOV), measles (MeV), human respiratory syncytial (RSV), or influenza A and B viruses (IAV/IBV). -ssRNA viruses can be subdivided into non-segmented and segmented ones, depending whether the whole genetic information of the virus is present on a single or several RNA segments. A schematic of a -ssRNA virus particle is presented in Figure 1a, and the alternative family-specific naming of

virus proteins is listed in Figure 1b. These viruses possess an envelope derived from cellular membranes in which viral glycoprotein(s) are embedded. The inner side of the envelope is coated by the matrix protein, which participates in the virus assembly and budding during the multiplication cycle. The 10–15 kb viral genomic RNA is at the core of the virion in the form of ribonucleoprotein complexes or vRNP(s) whether the genomic RNA is segmented or not. vRNPs are comprised of the viral RNA encapsidated by a nucleoprotein (N or NP) further associated to an RNA-dependent RNA polymerase (RdRp, often called L) and its non-enzymatic cofactors involved in the viral RNA synthesis processes. vRNPs are the functional units supporting viral RNA synthesis. A schematic of the -ssRNA multiplication cycle is presented in Figure 1c. The entry steps lead to the release of the vRNPs in the cytoplasm of infected cells. The encapsidated genomic RNA are then used as a template by the polymerase and its cofactors for both replication of the genomic vRNA and transcription of viral mRNAs. For the vast majority of -ssRNA viruses these RNA synthesis processes cluster in cytosolic membrane-less

Abbreviations: ssRNA, negative-strand single stranded RNA; IB, inclusion body; LLPS, Liquid-Liquid Phase Separation; RE, recycling endosome; RNP, ribonucleoprotein.

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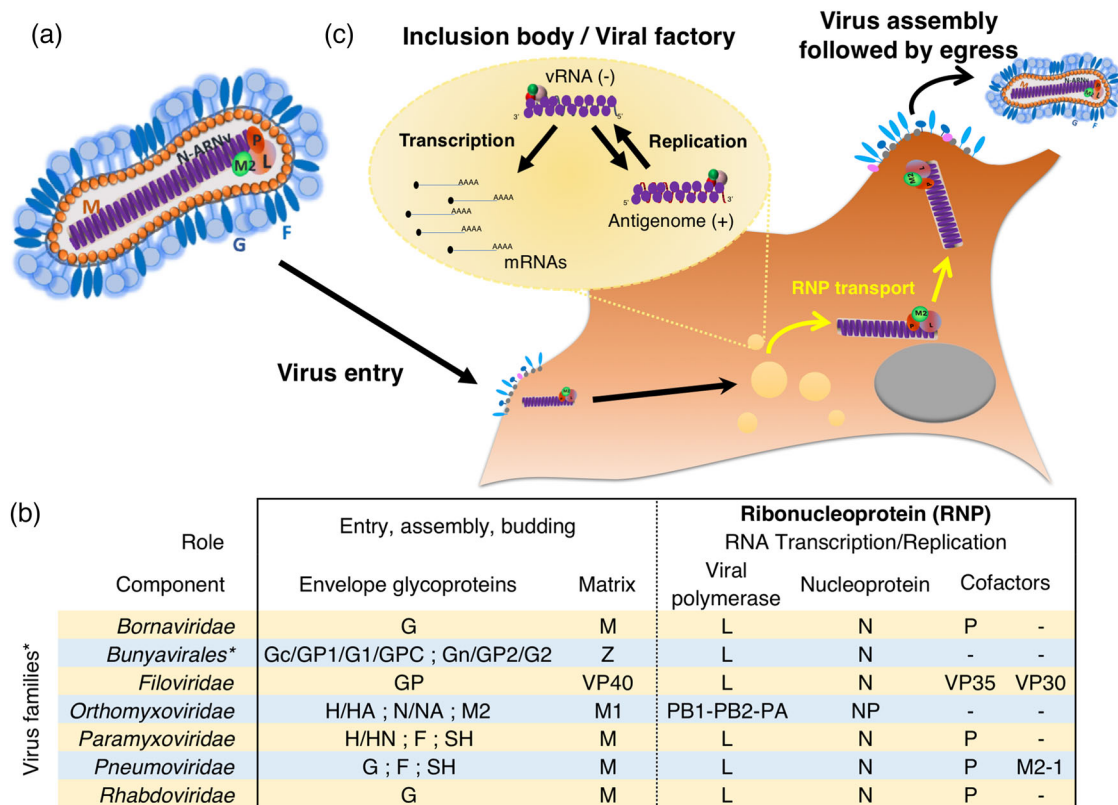


FIGURE 1 Blueprint of a -ssRNA virus replication cycle. (a) Schematic of a virus particle. Respiratory syncytial virus is used as a virus prototype. Surface glycoproteins (blue) are embedded in the envelope derived from cellular membranes. The matrix proteins (orange) form a lattice under the envelope. The encapsidated viral genomic RNA (purple) associated with the viral polymerase (light brown) and its cofactor (red and green) is at the center of the virion. (b) Table presenting family*-specific names of virus components. *Exception is made for the *Bunyavirales* order, formally referred as a virus family (*Bunyaviridae*), and for which the taxonomy has been recently reorganized. (c) Following entry, virus ribonucleoproteins (RNPs) are released in the cytosol where the virus protein expression starts. The RNPs serve as a template for both the transcription and replication of the virus genome. These RNA synthesis processes are segregated from the cytosolic content and occur in membrane-less compartments most often called inclusion bodies or viral factories. Upon replication, new RNPs are assembled, and then transported to the membrane. This is where new virions are assembled and released to infect a new cell. Elements on the cartoon are not scaled to the size of the cell.

compartments, most often called inclusion bodies (IBs) (Cifuentes-Muñoz et al., 2017; Dolnik et al., 2015; Heinrich et al., 2010; Lahaye et al., 2009; Rincheval et al., 2017). These IBs concentrate viral RNAs and proteins and some cellular proteins. Assembly of the neosynthesized genome into functional RNPs is also believed to occur in the IBs. The virions then assemble at or near the plasma membrane and bud releasing infectious virions in the extracellular compartment. Of note, *Orthomyxoviridae* and *Bornaviridae* are particular in the way that the virus transcription/replication occurs in the nucleus of the infected cells.

At the scale of the cell, vRNPs are gigantic complexes ($>10^4$ kDa) which cannot efficiently diffuse through the cytosolic content (Luby-Phelps, 1999). Yet, viral factories usually locate far from the cell surface where RNPs exit the infected cell into new virions and these viruses do not encode for any active transport component. To overcome the challenge of the RNP transport from the viral factories to the plasma membrane, viruses

evolved strategies aimed at hijacking cellular transport machineries. Indeed, cells have specialized machineries to ensure highly regulated transportation of various cargos across the cytoplasm. Cargos generally transit on the cytoskeleton in association to ATP-dependent fiber-specific molecular motors: kinesins and dyneins for the microtubules, and myosins for the actin polymers. The specific directionality of the transport is ensured by the motor dependency to the fiber polarity: anterograde transports are ensured by kinesins and most myosins, and retrograde transports are ensured by dyneins and Myosin IV. Given their importance in the intracellular transport, those two fibers are prime targets for viruses when it comes to organize the (long-)distance transport of RNPs.

Despite recent advances, the process by which RNPs are transported to the plasma membrane remains relatively poorly understood (yellow in Figure 1c). This is mainly due to the nature of viral cycles, which should be seen as continuums rather than series of disconnected

steps: successive events are deeply intertwined and laborious to disentangle. A study case on RABV import, the causative agent of rabies highlights those difficulties. The negative impact of anti-actin and -microtubule drugs on the RABV multiplication is known since the 1980s (Ceccaldi et al., 1989; Lycke & Tsiang, 1987). But it was not clear from the published datasets what step(s) of the viral cycle was disrupted, and especially what type of fibers was involved in the transportation of vRNPs. In fact, approaches targeting the cytoskeleton heavily depend on the RABV post-infection time window. For instance, anti-microtubule drugs used at different timings can generate seemingly incompatible results (Xu et al., 2021; Zan et al., 2017). This is because cytoskeleton fibers support different stage-specific processes of the virus replication cycle. Actin is first required very early in the virus cycle, for the clathrin-mediated endocytosis of the virus by the cell (Piccinotti et al., 2013). Here, actin fibers coordinate the numerous factors mediating the endocytosis, and passively mitigate the local tension applied on the invaginated membrane (Kaksonen et al., 2006). While remaining intact within in the endosomal vesicles, the internalized viral particles travel along the microtubules towards the soma (Le Blanc et al., 2005; Xu et al., 2015). It is only then that the virus envelope and endosome membrane fuse to release the viral RNPs (Piccinotti & Whelan, 2016). Physical interactions between the RNP components and subunits of microtubule-associated motors then promote the virus transcription (Bauer et al., 2015; Tan et al., 2007). How can one disentangle these processes, and assess the transport of RNPs?

In this manuscript, we review the current understanding of RNP trafficking, for each of the main viral families harboring a -ssRNA genome. After a brief description of the methodologies employed, the literature is individually discussed for each viral family. Finally, using our recent findings on RSV, we highlight how Rab11 has emerged as a cofactor of choice for the RNP transport of several -ssRNA viruses.

Studying viral RNP transport

Studying directly RNP transport requires the ability to characterize directed motions and mostly relies on imaging methodologies. Two methods of choice, relatively simple in their design, have emerged from the literature. Ideally, authors generate recombinant viruses expressing a fluorescently tagged RNP (Figure 2, Method A). Noteworthy engineering recombinant viruses from reverse-genetics systems (i.e., plasmids) is a delicate and laborious task for -ssRNA viruses. Moreover, fusing fluorescent tags to a RNP component without perturbing its multiple functions is particularly difficult. Authors alternatively (transiently) express an individual fluorescent RNP component in cells subsequently infected

with a wild-type virus strain (Figure 2, Method B). In addition to preserving the native structure of the virus genome, this approach allows for titrating the expression levels and detrimental fitness of the tagged-protein. For both methodologies, the incorporation of the fluorescent protein in the neo-synthesized RNPs enables live-visualization of the RNPs.

More elaborate techniques have been combined to live-imaging for studying RNP transport in the IAV fields. For instance, RNPs were tracked in cells transfected with an antibody specifically directed against the RNP-containing NP (Momose et al., 2011), or using affinity RNA aptamers tags (Chiu et al., 2022). In spite of their potential biasing effects on the velocity of the objects tracked, these methods allow for detecting RNPs in contexts where fusion-proteins are detrimental for the virus replication.

A consequent literature has also been generated by imaging from fixed samples which limit the interpretations in the sense that motions must be inferred from static subcellular localization patterns. This type of samples confers a more versatile catalogue of techniques and imaging targets. For instance, detecting genomic RNA in live-imaging remains inaccessible for most viruses while established procedures have been developed for their static detection by Fluorescence In Situ Hybridization (FISH) for individual RNA sequence types (Jo et al., 2010; Rincheval et al., 2017), in multiplexed configurations (Lakdawala et al., 2014), or at single-molecule level (Chou & Lionnet, 2018). Using fixed samples also allows for using higher-resolution imaging technologies, such as electron microscopy (Alenquer et al., 2019; De Castro Martin et al., 2017).

The extreme relevance of anti-cytoskeleton compounds when studying intracellular transport is worth mentioning in this section. As such, drugs depolymerizing and stabilizing the actin (Cytochalasins and Phalloidin) and microtubule (Nocodazole and Paclitaxel) are almost systematically tested in the literature covered in this review. These drugs provide invaluable tools to study the direct involvement of a cytoskeleton fiber type in RNP transport, especially when observations are made immediately following the addition of the compound. The cytoskeleton however influences virtually all cellular processes and cytoskeletal perturbations may indirectly disrupt viral RNP transport and viral multiplication. Especially in longer term drug exposure contexts, RNP transport phenotypes must be confirmed by complimentary approaches.

The actin-dependent transport of filovirus RNPs

Filoviruses are best known for comprising the Marburg (MARV) and Ebola (EBOV) viruses, which have caused ravaging epidemics in Africa since the first reported

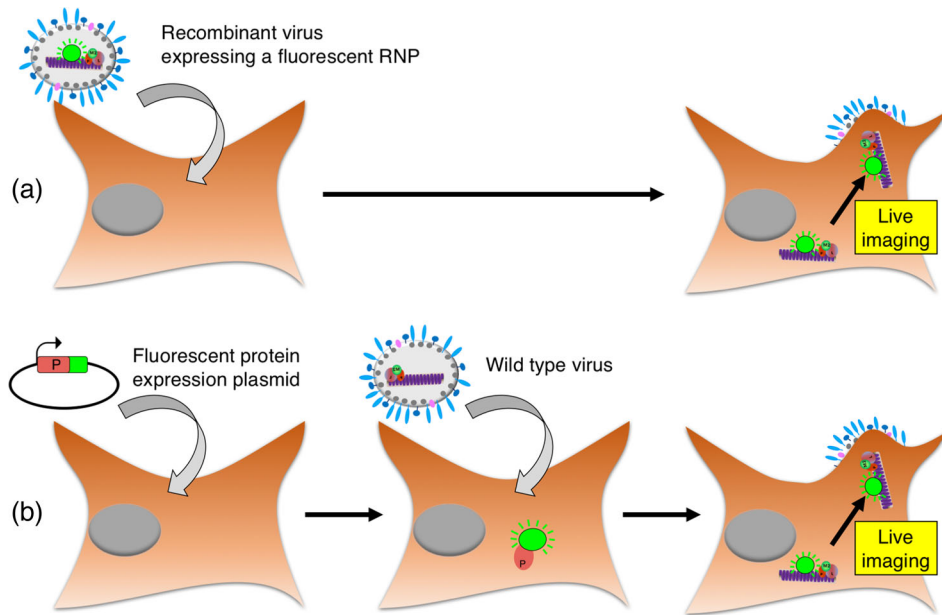


FIGURE 2 Schematic of the optimal strategies to study virus RNP transport. (a) Cells are infected with a recombinant virus expressing a fluorescent RNP component. (b) Cells expressing a fluorescent RNP component are infected with a wild-type virus strain. In both scenario, the fluorescent proteins are incorporated in the neo-synthesized RNPs and can then be tracked by live-microscopy.

outbreak in 1976. Other members of the family have been identified, but neither their replication cycle nor their pathological potential has been robustly investigated.

The transport of filovirus RNPs was assessed in two consecutive studies, using the same approach (Figure 2, Method A). Cells expressing a fluorescent EBOV or MARV RNP component, the VP30, were infected with a wild-type virus, and used for live-cell imaging (Schudt et al., 2013, 2015). Transport events of RNPs were tracked from the IBs to the filopodia, which are virus induced extensions of the cellular membrane and where filovirus egress occurs. These movements were strongly impaired in cells treated with drugs impairing the actin fibers' stability, while Nocodazole had no effect. RNPs transport is thus actin-dependent and microtubule-independent (Schudt et al., 2013, 2015). Transport of the matrix protein (VP40) was also found to solely rely on the actin-network (Adu-Gyamfi et al., 2012). To assess whether the matrix protein (VP40) could be part of the RNP transport, the authors generated a recombinant virus expressing a fluorescent VP40, and infected cells expressing the fluorescent VP30 (Figure 2, Method A+B). They found that whereas RNPs and VP40 use the same fibers to travel in the cell, and whereas a pool of VP40 locates at the IBs, RNPs emanating from IBs were not associated to VP40 during their transport (Schudt et al., 2013). In a later study based on co-transfection of EBOV protein, it was found that N, VP35 (phosphoprotein) and VP24 (inhibitor of viral RNA synthesis) were necessary and sufficient to ensure pseudo-RNP transportation. The requirement of VP24 for transport could

ensure transport of RNPs not involved in viral RNA synthesis (Takamatsu et al., 2018). Their precise role remains to be explored.

The actin-dependent transport of RNPs seems motorized by two processes, which depend on the stage of the transport process. At early stages, RNPs emanating from the IBs are accompanied by the formation of actin comets, that are WAVE-Rac1-Arp2/3 dependent, which suggests an active role of actin nucleation in the transport process (Grikscheit et al., 2020; Schudt et al., 2015). At late stages, once located near the membrane, RNPs associate to VP40, where the complex co-opts the Myosin-10 to navigate (Berg & Cheney, 2002; Schudt et al., 2013). It is only then that egress occurs, thereby engaging several actin cofactors such as IQAP1, and for which additional roles in transport events have not been studied in depth (Lu et al., 2013).

Rhabdoviruses: VSV model and long-distance transport of RABV-RNPs

Rhabdoviruses comprise ~200 species, and infect various hosts. Yet, the current rhabdovirus knowledge was mainly derived from two members: rabies (RABV), known for its historical human burden, and vesicular stomatitis virus (VSV) which has become a model of choice owing to its wider tropism and biosafety.

The transport of neosynthesized VSV RNPs was studied by the Pattnaik group using a VSV strain encoding for a fluorescent RNP component (Das et al., 2006). Time-lapse imaging revealed RNP trajectories from the

nucleus periphery toward the cellular membranes in close association with mitochondria. Nocodazole treatment of infected cells resulted in a drastic shift of fluorescent signals from a diffuse polarized pattern in untreated cells, to very large aggregates surrounding the nucleus. Microtubules involvement in RNP transport was further supported by the observation of an obvious association of neosynthesized RNPs with stained microtubules. However, the sole involvement of microtubules in RNP transport remains unclear. First because the anti-MT treatments carried out in this manuscript were performed before and maintained along the infection. This approach can affect the earliest steps of the viral cycle and therefore bias observations on everything that follows. Second because anti-actin drugs were not tested, while actin is crucial for the mobility of mitochondria (Morris & Hollenbeck, 1995). In line with this uncertainty, it was reported that when anti-actin drugs are used at 1 hpi, after the initial steps of the multiplication cycle, the mobility of the VSV RNPs is greatly reduced (Yacovone et al., 2016).

Follow-up work on cells continuously exposed to anti-MT drugs revealed that the cell surface localization of the matrix protein M is, in contrast to RNPs, microtubule-independent (Das et al., 2006, 2009). This supports a model in which M and RNP transportations are disconnected.

As mentioned above VSV is naturally pantropic and mostly studied using common cell types. The conservation of the genome organization allows to some extent for extrapolating on the RABV biology. But given the neurotropic circulation of RABV, which involves very-long-range transports, the relevance of the VSV model is unclear.

In neurons, RABV spreads from the axon to the cell body (soma), then to the cell dendrites where RABV buds at pre-synaptic terminals and infects the next naïve neuron. This cycle is iteratively repeated until reaching the central nervous system (Ugolini 1995). In contrast to the axon-to-soma transport (i.e., the import of RNPs), the soma-to-dendrite transport step has not been characterized yet. Given the importance of microtubule transport for the neuronal transport, it is assumed that RNPs are transported along this fiber. Indirect clues, such as a physical association of L and P microtubule motors tend to support this model (Bauer et al., 2015; Tan et al., 2007). Surprisingly however, no ultimate demonstration of a retrograde microtubule-dependent transport of neosynthesized RNPs has been reported yet.

Perhaps even more surprising, such a microtubule-dependent transport has been demonstrated in the opposite, anterograde, direction (from the soma along the axon), using neurons grown in polarizing chambers (Bauer et al., 2014). In these cells, RNPs were co-transported with secretory vesicles where the envelope glycoprotein G is embedded (Bauer et al., 2014). It

remains now to be shown whether or not the transport of neosynthesized RNPs is mechanistically similar in the retrograde direction.

A limited literature on BDV and bunyaviruses transport processes

The *bornaviridae* is the only viral family harboring a non-segmented -ssRNA and exhibiting nuclear replication/transcription activities. Its prototype is the Borna Disease Virus (BDV). Alike what is seen with other *MNVs*, these RNA synthesis processes are segregated from the cell in actual IBs (Hirai et al., 2021; Matsumoto et al., 2012). This virus is understudied and the mechanisms by which RNPs navigate within the cell are unknown. Yet, transport processes are fundamental for BDV regarding its neurotropism and trans-synaptic cell-to-cell mode of transmission, because it involves very-long-distance axonal shuttling of RNPs. The exit from the nucleus is unclear. Several putative Nuclear Export Signals (NES) have been detected on RNP component (notably N and P), which could allow the nuclear export of RNPs through the CRM1 pathway. However, their accessibility in the context of RNP arrangements remains to be demonstrated. Alike what was found for IAVs (see *Orthomyxoviridae* section), the matrix protein M exhibits a nuclear location and harbors a NES. It is tempting to speculate that BDV could use an IAV-like mechanism for the export of nascent RNPs (Honda & Tomonaga, 2013). The cytosolic transport of RNPs is not well understood either but it is suspected to rely on the microtubule fibers for two major reasons. First, actin destabilization has no effect on cell-to-cell transmission of the virus (Charlier et al., 2013). Second, axonal transport is known to heavily rely on microtubules (Maday et al., 2014). Perhaps anecdotally but reinforcing the suspicion of the microtubule involvement, the human factor EBLN1, an evolutionary maintained BDV-endogenized gene, has recently been characterized for its critical importance in the microtubule organization (Myers et al., 2016). To what extent and how is still to be discovered.

Bunyavirales order include more than 10 viral families. Despite comprising dozens of zoonotic pathogens, several aspects of the Bunyavirus replication have been neglected and the core knowledge regarding this order is still fragmented. The *Bunyavirales* genome is segmented into three negative-strand RNA molecules. Some families composing this virus order present an atypical feature, with one or two of these segments encoding for ambisense genes (illustratively: 5'→←3'). For these, the mRNA transcription of the 3' gene is initiated at the 3' end of the segment. Aside from this particularity, the bunyavirus replication somewhat displays features usually found in +ssRNA viruses. First, the genome is replicated in viral factories near the endoplasmic reticulum or Golgi apparatus endomembranes (De

Castro & Risco, 2014). Second, and perhaps most importantly for this review, a large proportion of bunyaviruses is assumed to bud in the lumen of these neighboring organelles. For these, the RNP transport conundrum nearly becomes anecdotal. Plasma membrane budding has however been observed for some bunyaviruses (Goldsmith et al., 1995; Ravkov et al., 1997), raising questions about how their RNPs are transported.

To our knowledge, the RNP transport for these “exceptions” was briefly interrogated in one only study focused Lassa virus (*Arenaviridae*), in cells transiently expressing a single virus protein. Authors found that the localization to the plasma membrane of the Z matrix protein was linked to TGN-originating endosomes through the subversion of the kinesin KIF13A, a motor for microtubule-dependent transport. The Z dependency to KIF13A was also found for the Machupo and Junin arenaviruses, which suggests that the process is broadly conserved within this family. In contrast, KIF13A expression has no effect on the localization of the nucleoprotein NP expressed alone (Fehling et al., 2013). Whether RNPs follow the behavior of individual NP is unclear, and further experiments are required to verify that this dataset is indicative of a non-coupled Z-RNP transport. This study echoes another publication on the Andes Virus (ANDV, *Hantaviridae*). In this work, Rab11 marked recycling endosomes (see next section), which motions can be motorized by the KIF13A, clearly co-localized with the NP during the infection, and Rab11 knock-down resulted in a 10-fold decrease of the virus multiplication (Rowe et al., 2008).

Rab11, a recurrent target of -ssRNA viruses

Rab proteins, for Ras-related in brain, are small GTPases which together with their interacting partners, regulate virtually every aspect of vesicular trafficking. Rab11 is one of dozen subfamilies. It includes Rab11a, Rab11b, and Rab25/Rab11c, and is increasingly recognized for its impact on viral RNPs trafficking. In short, Rab11a is a marker of recycling endosomes (RE) governing the slow-pace sorting and transport of endocytosis vesicles back to the apical plasma membrane (Grant & Donaldson, 2009). This protein has also been shown to control processes involving intracellular vesicles such as late endosomal trafficking and autophagy (Szatmári et al., 2014), or exocytosis (Urbé et al., 1993).

Rab11 acts via its partners from the Rab11 family interacting proteins (called Rab11-FIPs), which bridge Rab11 cargos to their targets such as cytoskeleton motors, fibers, and lipids. Those interactions drive the vesicles of interest to their destination, and consequently regulate the protein composition at the cellular membranes. They also confer to Rab11 a versatile traveling capacity

by allowing both actin and microtubule associated transports. As such Rab11 is placed at a critical place for the control of the polarity, the compartmentalization of the cell and all the subsequent physiological processes (Welz et al., 2014).

Influenza A viruses: The species for which the Rab11 involvement is best characterized

The transcription/replication process of the orthomyxoviruses, which comprise the influenza A viruses (IAV), is atypical among their kind, as it occurs in the nucleus of the infected cells. This feature involves a two-step process for the transport of the RNPs. First, RNPs exit the nucleus. For the sake of this review, we will not cover in depth this step for which the literature is rich. In brief, chromatin-adjacent neo-assembled RNPs are complexed with the matrix protein M1 and the Nuclear Export Protein NEP to exit the nucleus by subverting the CRM1-exportin pathway (Elton et al., 2001; Neumann et al., 2000; Watanabe et al., 2001).

The cytosolic journey of IAV RNPs has been extensively studied. A compelling body of work produced in the last ~15 years has demonstrated the central role of Rab11 in this process. The first evidence is the observation of a clear accumulation of RNPs at the MTOC (Momose et al., 2007), where Rab11 heavily co-localizes. And from there, fluorescent RNPs travel with Rab11, most likely primarily along microtubules (Amorim et al., 2011; Avilov et al., 2012; Eisfeld et al., 2011; Bruce et al., 2010). Anti-actin drugs also showed an effect on RNP movements, but the mechanism underlying this inhibition remains unclear (Avilov et al., 2012). Efforts have been put to characterize the mechanism of the Rab11 subversion at the molecular level, and it seems clear now that those transports are driven by a direct interaction of Rab11 with the C-ter domains of the PB2 subunit of the IAV trimeric polymerase complex (Amorim et al., 2011; Avilov et al., 2012; Veler et al., 2022).

For the longest time, these data were understood as a direct subversion by IAVs of the RE process, during which the RNPs would take advantage of the physiological trajectories of RE vesicles to reach the membrane. This model has been refined in recent publications. Upon IAV infection, Rab11 natural functioning is impaired (Bhagwat et al., 2020; Kawaguchi et al., 2015; Vale-costa et al., 2016), and the endoplasmic reticulum is heavily remodeled around the MTOC (De Castro Martin et al., 2017). This leads to a micro-environment where bio-condensates concentrating Rab11 and the RNPs form upon LLPS (Alenquer et al., 2019). These liquid organelles are then thought to act as a reservoir feeding the RNP transport line by engaging endoplasmic reticulum vesicles irregularly coated with Rab11 (De Castro Martin et al., 2017; Vale-Costa & Amorim, 2017).

Two fundamental questions remain unsolved. First, while the data support a model in which Rab11 associated RNPs primarily traffic along microtubules, anti-microtubule drugs modestly impair RNP transport and more generally the virus multiplication. Is there an alternative Rab11-independent pathway? Can Rab11 associated RNPs co-opt actin fibers when the microtubule network is unavailable? 2- It is unclear if traffic of RNPs relies on Rab11 up to the budding site, or if additional mechanisms are involved in the terminal stages of the transport. The overall involvement of Rab11 in the IAV multiplication cycle is discussed in more depth in the following review (Amorim, 2019).

The uncharacterized Rab11 associated transport of paramyxovirus RNPs

Paramyxoviridae comprises several viruses of human health concern, dispersed in several genera: morbilliviruses (Measles (MeV)), rubulaviruses (Mumps (MuV)), respiroviruses (Sendai and Human Parainfluenza (SeV and HPIVs)), or the emergent henipaviruses (Nipah and Hendra (NiV and HeV)). Several studies on the RNP transport in this viral family exhibit a striking resemblance both in terms of methods employed, and in terms of results generated.

The initial core of the literature on MeV RNP transport first suggested a role of the matrix protein M, given its natural ability of M to localize at both the IBs and the membrane (Riedl et al., 2002). This was further supported by the fact that the accumulation of nucleoprotein N to the cellular membranes at terminal stages of the infection depends on a N–M interactions (Iwasaki et al., 2009). Since the role of M in the virus budding is actin-dependent, RNP transport was assumed to also rely on the same cytoskeleton fibers (Dietzel et al., 2013).

A pivotal study from 2013 challenged this model. The authors followed RNPs by live-imaging using a recombinant strain encoding for a fluorescently tagged polymerase (Figure 2, Method A). This virus allowed to characterize an intense concentration of RNPs at the MTOC, and RNP movements along the microtubules. Questions were then raised about the potential motors fueling these movements. The authors were inspired by this MTOC localization and previous studies in which virus transport processes were found to be dependent on endosomal pathways. They searched for a co-localization of the RNPs with the different types of endosomes and found a selective association of RNPs with Rab11 positive recycling endosomes. At a functional level, most gene inactivation approaches carried in classical cell lines, and using classical assays, led to marginal differences. In contrast, Rab11 appeared essential for an optimal virus growth in a polarized manner, via RNP transport toward the apical membrane (Nakatsu et al., 2013).

This biased interest toward a potential role of Rab11 is not unprecedented in the paramyxovirus field. Since the 2000s, Rab11 associated transport of RNPs for in SeV, MuV, or hPIV-1, has been selectively interrogated using recombinant viruses and/or a fluorescently tagged Rab11 protein, and similar results were obtained (Chambers & Takimoto, 2010; Katoh et al., 2015; Stone et al., 2016).

RSV, the most recently reported Rab11-dependent species

Two human respiratory pathogens belong to the *pneumoviridae*: Respiratory Syncytial Virus (RSV), and the human metapneumovirus (hMPV) for which the current understanding of the multiplication cycle is mostly derived from RSV. Alike what was observed for the viruses already mentioned, anti-cytoskeleton have been reported to impair the multiplication of RSV. But the datasets reported are unsuited to specifically argue about transport dynamics and mechanisms (Kallewaard et al., 2005).

Until very recently, the community was clueless about RSV RNP transport. To study this process, our group recently engineered a recombinant RSV strain encoding for a fluorescently tagged nucleoprotein N protein, and employed live-imaging strategies (Figure 2, Method A). We were able to record clear long-distance motions of RNPs, sometimes originating from the IBs, along the microtubules, and exhibiting an obvious directionality. Still using live-imaging methodologies, we observed a clear abolition of these movements on Nocodazole treated cells, while Cytochalasin D had no effect (Cosentino et al., 2022). Finally, we observed that these motions were physically associated to, and partially dependent on, the recycling endosome marker Rab11. Our imaging set-up unfortunately did not allow for a direct observation of the RNPs' entire lifetime.

Vanover et al. previously suggested that RNPs were recruited into intracellular vesicular extensions (called filaments in the original manuscript and renamed here for clarity) prior to assembly into filamentous virions at the plasma membrane. These vesicular extensions originated from recycling endosomes as they were decorated by surface recycled viral G protein and formed in a microtubule- and dynein-dependent manner (Vanover et al., 2017). Given the biological relevance of Rab11 in the recycling endosomal processes, it is tempting to speculate that our datasets captured the RNPs in transit to these extensions.

In the early 2000s, directional release of RSV progeny was shown to be impaired by dominant negative (DN) forms of Rab11-FIP1 and -FIP2. The phenotype observed were linked to defects in the budding process, in a Myosin-dependent manner. It is however possible that defects at earlier steps in the viral cycle could

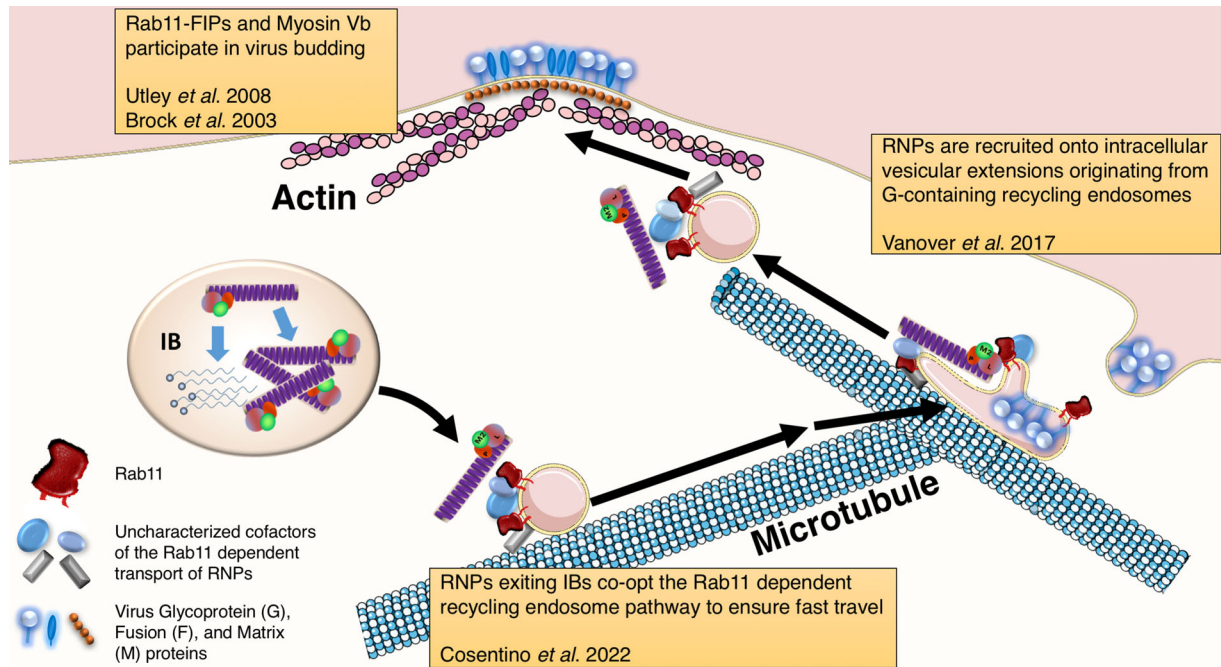


FIGURE 3 Model of RSV trafficking in RSV. RNPs produced in IBs would first be transported to the plasma membrane along microtubules in association to RE vesicles. Once near the cell surface, RNPs would be recruited to G-labeled RE vesicles forming intracellular vesicular extensions. Finally, RNPs associated RE vesicles would be transferred to the subcortical actin network prior to viral filament formation.

contribute to the results as the DN-Rab11-FIPs were expressed continuously during the RSV life cycle in these assays. Since Rab11-FIP1, FIP2 and the Myosin Vb mediate the interaction of Rab11 vesicles to the actin network, one might propose that after fast transportation along the microtubules Rab11 associated RNPs are transferred on actin network. These publications and our findings converge toward the importance of Rab11 in RSV multiplication (Brock *et al.*, 2003; Utley *et al.*, 2008), for which we propose a working model in Figure 3.

CONCLUSION

Negative RNA viruses have developed very different strategies to ensure the transport of their RNPs into the cell cytoplasm. Understanding this mechanism has long suffered from difficulties in applying live-imaging methodologies on infect-ed (-ious) samples. Yet, characterizing these transports could lead to identify new therapeutic targets or to original vaccine strategies. In spite of the recent multiplication of studies dedicated at characterizing RNP trafficking, the field is only emerging and there are more open questions than problems solved (See summary table in Figure S1). The depth of the *influenza*-Rab11 literature is somewhat of an exception in this field.

To ensure the rapid transportation of RNPs to the budding site, -ssRNA viruses heavily engage the cytoskeleton network. For most viruses, the viral and cellular proteins involved are poorly known, and even less char-

acterized at the structural level. The identity of molecular motors involved in this process is especially unclear. The human genome encodes for dozens of these motors. To what extent they can complement each other for the RNP-transport, and to what extent this phenomenon hinders the search of these cofactors should be explored in the future.

The IAV literature clearly demonstrates that the virus does not simply use the cellular transport network but rather shapes it for its own use. Aside from this example, the field unfortunately lacks studies exploring the virus-induced disturbances of the cellular transport networks. Future studies on these aspects are required to better illuminate the sophistication of the vRNP trafficking processes. This also represent a significant opportunity to better understand the balances involved in the physiological functioning of these cellular pathways.

While a large body of the -ssRNA phylogeny exhibits divergent subversion strategies, several virus families have converged toward the hijacking of the recycling endosome (*orthomyxoviridae*, *paramyxoviridae*, *pneumoviridae*). All these viruses naturally infect polarized respiratory epithelial cells with a basolateral and an apical pole. The recycling endosome is especially important in these cells as it is directed toward the apical membranes and maintains the polarization. Hijacking Rab11 may promote apical budding and thus advantage a regional spread of the virus progeny (Blau & Compans, 1995; Katoh *et al.*, 2015). This interpretation remains however limited by the cellular models used in most of

the literature: the implementation of live-imaging strategies and more generally the manipulation of polarized epithelia requires an exquisite expertise.

As illustrated along this review, our overall understanding of -ssRNA RNP trafficking remains relatively fragmented. But the available literature shows undeniable glimpses of an intense richness in terms of strategies, mechanisms, and cellular partners involved. While more work is required on a virus by virus basis, efforts are made by different laboratories to increment on this knowledge base, and the ad hoc imaging technologies are more and more accessible. In fact, this field is likely to be on the brink of major breakthroughs.

AUTHOR CONTRIBUTION

The manuscript was written by Cédric Diot, Gina Cosentino, and Marie-Anne Rameix-Welti.

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CONFLICT OF INTEREST

The authors have no competing interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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