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To cite this article: Sílvia Vale-Costa & Maria João Amorim (2016): Clustering of Rab11 vesicles in influenza A virus infected cells creates hotspots containing the 8 viral ribonucleoproteins, Small GTPases, DOI: [10.1080/21541248.2016.1199190](https://doi.org/10.1080/21541248.2016.1199190)

To link to this article: <http://dx.doi.org/10.1080/21541248.2016.1199190>



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Accepted author version posted online: 23 Jun 2016.
Published online: 23 Jun 2016.



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Clustering of Rab11 vesicles in influenza A virus infected cells creates hotspots containing the 8 viral ribonucleoproteins

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ABSTRACT

Influenza A virus is an important human pathogen causative of yearly epidemics and occasional pandemics. The ability to replicate within the host cell is a determinant of virulence, amplifying viral numbers for host-to-host transmission. This process requires multiple rounds of entering permissive cells, replication, and virion assembly at the plasma membrane, the site of viral budding and release. The assembly of influenza A virus involves packaging of several viral (and host) proteins and of a segmented genome, composed of 8 distinct RNAs in the form of viral ribonucleoproteins (vRNPs). The selective assembly of the 8-segment core remains one of the most interesting unresolved problems in virology. The recycling endosome regulatory GTPase Rab11 was shown to contribute to the process, by transporting vRNPs to the periphery, giving rise to enlarged cytosolic puncta rich in Rab11 and the 8 vRNPs. We recently reported that vRNP hotspots were formed of clustered vesicles harbouring protruding electron-dense structures that resembled vRNPs. Mechanistically, vRNP hotspots were formed as vRNPs outcompeted the cognate effectors of Rab11, the Rab11-Family-Interacting-Proteins (FIPs) for binding, and as a consequence impair recycling sorting at an unknown step. Here, we speculate on the impact that such impairment might have in host immunity, membrane architecture and viral assembly.

ARTICLE HISTORY

Received 8 May 2016
Revised 24 May 2016
Accepted 26 May 2016

KEYWORDS

correlative light and electron microscopy; influenza A virus assembly; Rab11 GTPase; Rab11 family interacting proteins (FIPs); recycling endosome; segmented genome; viral ribonucleoproteins (vRNPs)

Introduction

Influenza A virus (IAV) contains a segmented genome of negative sense-RNA and encodes up to 18 identified proteins.¹ The genome is divided into 8 distinct independent replication units composed of different RNAs in the form of viral ribonucleoproteins, through encapsidation by the viral heterotrimeric RNA-dependent-RNA-polymerase (RdRp)² and nucleoprotein (NP).³ Segmentation allows the formation of a genetic diverse pool of genomes each composed of 8-segments, on account of an error prone RdRp, which might give rise to antigenic drift. It also permits genome mixing of 2 parental strains in natural co-infections and emergence of novel viral reassortants. There is a vast pool of influenza subtypes, given the high genetic variability of the antigenic viral transmembrane proteins (hemagglutinin and neuraminidase). These subtypes can be found dispersed but restricted to a series of hosts (humans, pigs, domestic poultry, and horses, among others) and be perpetuated in their natural reservoir, the wild waterfowl.⁴ The mechanisms described for attaining genomic diversity might contribute to overcome host species barriers, allowing interspecies

transmission, acquisition of drug resistant traits and/or epitope replacement, a process known as antigenic shift that has been correlated with pandemic outbreaks in humans. Hence, in-depth understanding of the mechanisms sustaining the assembly of influenza A segmented genome is of importance to human health. Formation of the 8-segment genomic core has been subject of much interesting research. A hierarchical network of RNA-RNA interactions has been proposed by many research groups to sustain formation of the genomic core and this has been extensively reviewed elsewhere.^{5,6} Recently, one protein has been shown to be required for this complex formation. The regulator of the recycling endosome, the GTPase Rab11, was proposed to transport vRNPs to the cell surface,^{7–10} by a process that originated enlarged structures containing Rab11 and the distinct vRNPs, as observed by confocal microscopy (Fig. 1). These structures, located beneath the plasma membrane—the site of viral assembly—place pools of different vRNPs in close contact. These results led to the proposal of a model (Model 1 in Fig. 1) in which Rab11 vesicular transport facilitates the association of the 8 different viral RNAs while on

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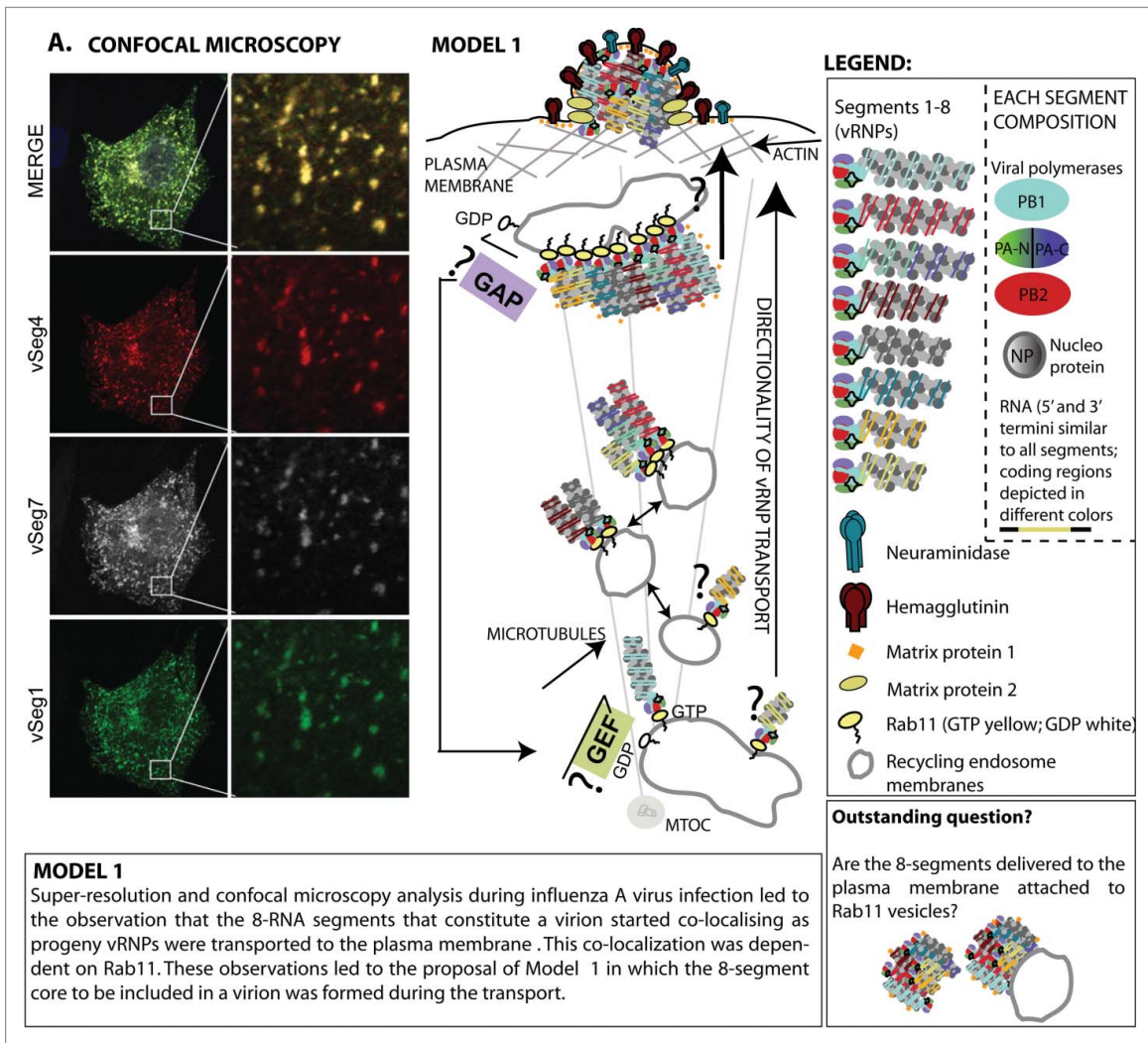


Figure 1. Proposed model for assembly of 8-vRNPs on route to the plasma membrane using a Rab11-dependent pathway.

route to the plasma membrane.^{11,12} In agreement, super-resolution studies have shown that the level of co-localization of the different vRNPs significantly increase when segments associate with active Rab11.¹¹ Besides the outstanding question stated in Figure 1, related with delivery of the assembled genomic core from Rab11 vesicles to the plasma membrane, molecular details on how vRNPs are transported on Rab11 were still lacking.

In particular, the molecular mechanisms underlying Rab11 remodeling, leading to the formation of the enlarged structures have been poorly characterized and their ultrastructural nature was not (until recently) available. Whether Rab11 enlarged structures result from coalesced vesicles or vesicles that fuse and hence increase in size, represents 2 distinct molecular processes and involves different factors. Their discrimination provides important clues on the process of assembly of influenza A segmented genome. In relation to the molecular players involved in the transport of vRNPs to the cell surface, 2 points are pertinent. The first is to identify how vRNPs

bind to Rab11 vesicles. The viral polymerase PB2, constituent of RdRp on vRNPs, was shown to promote this binding, but it is still unclear whether binding is direct.^{7,10} The second is to understand what happens to host recycling at a molecular level once vRNPs attach to Rab11 vesicles. Answering the second point would benefit from the development of robust, quantitative methods to compare amounts of factors bound to the recycling endosome that are either involved in sorting or transported by vesicles in infected versus healthy cells, as well as determining variations in the kinetics of the process. An exhaustive analysis would require an in-depth knowledge of the molecules governing the trafficking of recycling in host cells from vesicular biogenesis until fusion with acceptor membranes, and defining the cargo they transport. Although the complete characterization of the protein/lipid networks involved in each step (under distinct stimuli) is far from complete, there has been a lot of progress in characterizing the molecular mechanisms involved in host recycling that will be explored below.

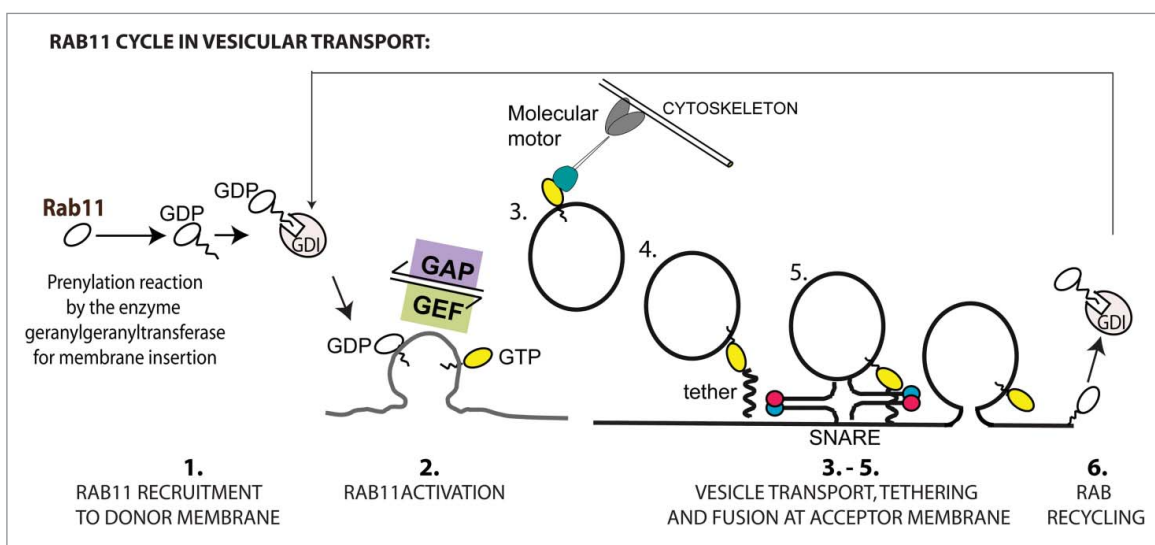


Figure 2. Rab11 cycle in vesicular transport (steps in Rab11 cycle are numbered from 1 to 6). Figure adapted from ref. 13.

The information provided shows that at least a partial comparative analysis of the infected vs. the healthy cell is attainable and should be informative regarding how infection alters recycling to modulate cellular architecture, and/or innate responses to infection.

Rab11-dependent vesicular trafficking in host cells

The recycling endosome or endocytic recycling compartment (ERC) is defined by the presence of Rab11 and its effectors. Rab11 regulates recycling from the ERC and of specific cargo from the TGN to the plasma membrane. It comprises the products of 3 genes, Rab11a, Rab11b and Rab25 (reviewed in ref. 13). Rab11a (and to a minor extent Rab11b), but not Rab25, have been implicated in IAV life-cycle,^{7-10,14} and for this reason an overview on Rab11a regulated vesicular transport will be provided. The sequential steps involved in Rab11a-mediated transport to the plasma membrane in uninfected cells are depicted in Figure 2 and have been detailed by us elsewhere.¹³ Importantly, the list of molecular interactions required for each of these steps is far from complete and has been described in refs. 15, 16. Here, we provide a comprehensive description, at the molecular level, highlighting identified players involved in transporting cargo to the cell surface on Rab11 vesicles.

Rab11 is translated in the cytoplasm and requires prenylation for attachment to ERC membranes. Prenylation renders Rab11 prone to aggregation, thus requiring association with guanine dissociation inhibitor factors (GDI) for cytosolic solubility. Rab11 is recruited to ERC membranes (step 1) that, as any other membrane of the eukaryote cell, has a precise composition or identity,¹⁷ including the presence of guanine exchange factors (GEFs) that promote GDP-GTP exchange on Rab11 thereby “activating” this GTPase (step 2). When active, Rab11 suffers a conformational change and acquires affinity to a series of effectors. Some effectors cooperatively work to transport cargo along cytoskeletal tracks (step 3), by associating with a series of identified molecular motors shown in Table 1. Specifically, binding of some molecular motors to Rab11 vesicles was shown to require adaptor proteins. The adaptors include the Rab11-family-interacting-proteins (FIPs), composed by 5 identified members (numbered 1 to 5) and, in addition, some of the members contain distinct isoforms.^{18,19} For convenience, in Table 1 is indicated which FIP is required for each molecular motor binding to Rab11 vesicles. Depending on the molecular motor type, cargo can be transported either on microtubules using dyneins (toward the MTOC) and kinesins (away from MTOC) or on actin using myosins. However, this is not the case for

Table 1. Modulators and effectors identified in Rab11 cycle in vesicular transport. Table from ref. 13.

Recruitment / activation	Transport	Tethering	Fusion	Inactivation / Recycling
Crag ²²	Myosin Vb (FIP2) ²³⁻²⁷	Rab11b ²⁸	SNAP25 ²⁹	Evi5 ³⁰
REI-1 ³¹	KIF5a and KIF3 (Kinesin II) (FIP5) ³²	Sec15 (exocyst) ³³⁻³⁶	SYN4 ³⁷	TBC1D9B ³⁸
PIP4KIII ³⁹	KIF13A ²⁰	Munc 13-4 ⁴⁰	VAMP8 ⁴¹	Cholesterol ⁴²⁻⁴⁴
	DLIC1/2* (FIP3) ⁴⁵			

*Binding of this effector to Rab11 has been shown to be indirect via Rab11-Family interacting proteins (FIPs)

binding of all motors to Rab11 vesicles, with at least one molecular motor, KIF13A, found to bind Rab11 independently of FIP adaptors as demonstrated by yeast-2 hybrid and fluorescent resonance energy transfer studies.²⁰ Rab11 vesicles while on route, search, dock (step 4) and then fuse (step 5) with acceptor membranes. These processes are also controlled by Rab11 effectors that operate downstream vesicular movement. Docking involves tethering to acceptor membranes. Fusion requires both the matching of proteins called soluble NSF (N-ethylmaleimide sensitive fusion proteins) attachment receptors (SNAREs) that are located on vesicles and their acceptor membranes, as well as energy. Energy is supplied in the form of ATP and is produced by synaptosomal-associated proteins (SNAP).²¹ Finally, Rab11 is switched off by GTPase activating proteins (GAPs), for ending of its activity, and is recycled back to the original membrane (step 6). A list of all identified Rab11 effectors involved in steps 1 to 6 is shown in Table 1, but readers are remitted to ref. 13 and indicated papers for further information regarding these factors.

Rab11-dependent vesicular trafficking in IAV infected cells

The redistribution of Rab11 observed upon IAV infection was recently shown by Vale-Costa et al.⁴⁶ to be caused by the binding of vRNPs to Rab11 vesicles, rather than by a byproduct of viral gene expression. Although this work still did not unequivocally show that the binding between vRNP and Rab11 was direct, it made important contributions in characterizing the consequences of vRNP binding to Rab11 vesicular trafficking. First, it showed that the efficiency in host recycling significantly decreased as infection progressed. Second, using an artificial system for targeting Rab11 to the mitochondria and pull down assays, this work confirmed previous reports on a competition model between vRNP and FIPs for Rab11 binding.¹⁰ Importantly, exogenous expression of a domain shared by all FIPs, required for Rab11 binding but unable to recruit the cognate molecular motors of FIPs, was able to mimic the appearance of enlarged Rab11 structures in uninfected cells, as observed by confocal microscopy. This strongly indicates that there is a causal relationship between FIP-mediated motor recruitment and redistribution of Rab11. These results provide the molecular basis underlying a viral-induced mechanism that culminates in increased colocalization of all the vRNPs. Third, Vale-Costa et al.⁴⁶ characterized alterations to Rab11-positive membranes during infection at an ultrastructural level.

Using correlative light and electron microscopy, where Rab11 or NP were individually tagged with GFP, this work

showed that IAV infection induced clustering, rather than fusion and enlargement, of vesicles. These clusters, found scattered throughout the cytoplasm and near the apical plasma membrane by electron microscopy, were composed of heterogeneous vesicles in size and harboured coiled-coil string-like structures attached to their exterior that spatially matched vRNP or Rab11 confocal staining. Vesicular clustering is in agreement with impairment in vesicular sorting, which was also elucidated in this work. In addition, areas positive for Rab11 and vRNPs by confocal microscopy contained U-shaped and double membrane vesicles. The current resolution of the technique does not allow to unequivocally state that these are Rab11 vesicles, and future work should address this. However, the increase in the numbers of U-shaped and double vesicles observed at later time points of infection might be an indication that Rab11-positive vesicles suffer rearrangements by mechanisms other than impaired sorting. Overall, the formation of Rab11-dependent vRNP hotspots showed in the Model 2 of Figure 3 could, nevertheless, facilitate the assembly of the genomic core proposed by others.^{9,11,12} However, so far, the functional significance of vesicular clustering awaits experimental support, as with the microscopy techniques currently available it has not been possible to resolve assembled sets with 8-segments in the cytoplasm of IAV infected cells.

Future perspectives

Whether Rab11 is directly involved in promoting the assembly of IAV segmented genome remains unclear, with many unresolved questions. Clearly the most pressing outstanding issues relate with how/where the 8-vRNPs assemble and the delivery of vRNPs from Rab11 vesicles to the plasma membrane (highlighted in Fig. 3). The results in ref. 46 and reported by other groups^{9,47} suggest that vRNPs might be released from Rab11 vesicles before reaching the plasma membrane. In this regard, it is pertinent to identify host factors mediating their delivery from Rab11 agglomerates. Assembly of the genomic core on Rab11 vesicles deserves revisiting with appropriate set of approaches and is still a fundamental open question. Even though Rab11 *per se* does not seem to mediate interactions between the different vRNPs, it might be crucial by placing them in close contact and thus facilitate the assembly of the genomic core. Vale-Costa et al.⁴⁶ proposed a mechanistic model explaining at a molecular level the formation of the vRNP hotspots, constituted of clustered vesicles harboring protruding vRNPs (Model 2, Fig. 3). Binding of vRNPs to Rab11 vesicles competes with binding of FIPs and leads to an impairment of recycling sorting. Whether impairment operates at the level of transport, tethering or fusion of vesicles to the plasma membrane remains to be

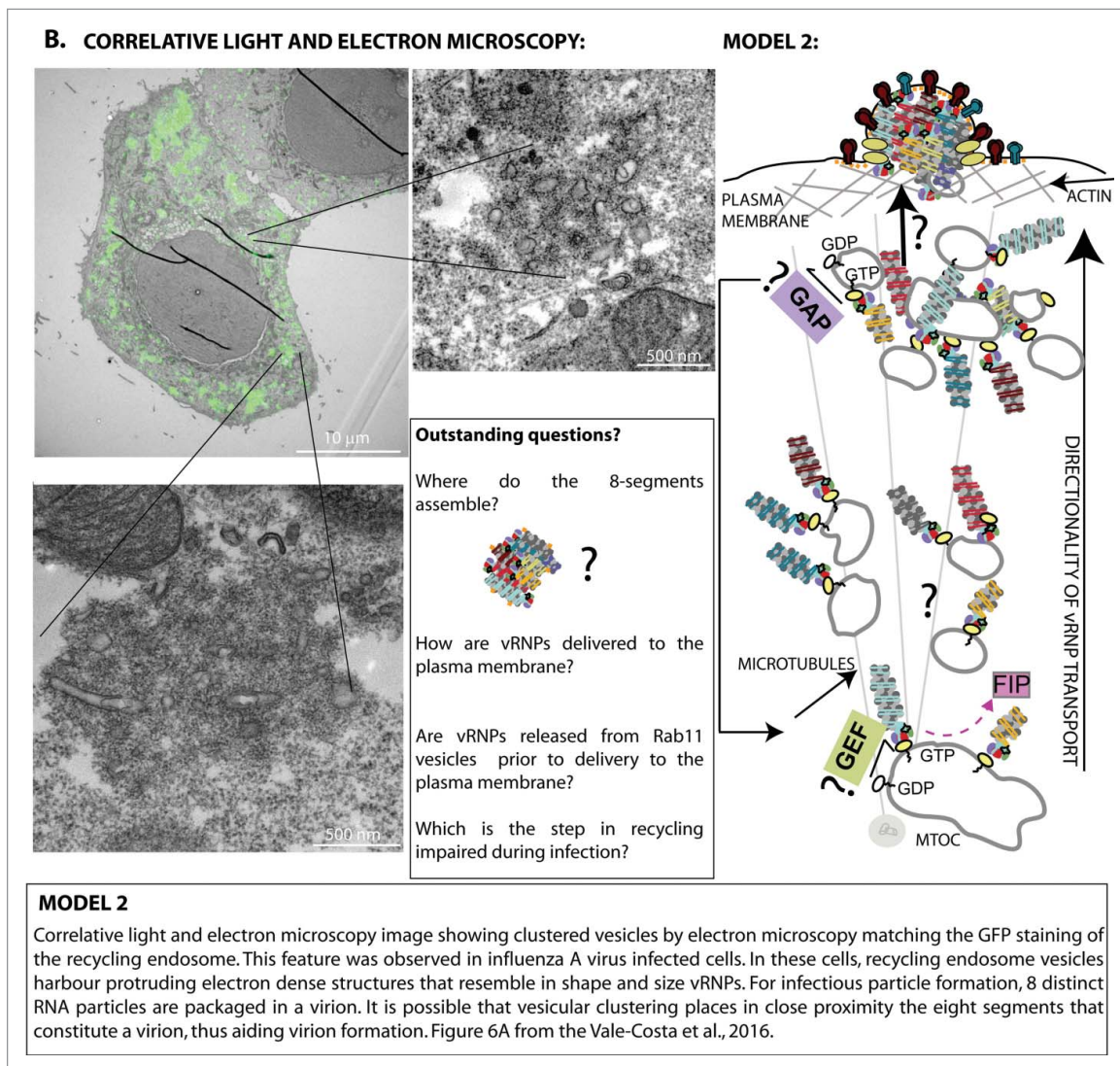


Figure 3. Proposed model for Rab11 vesicular impairment during IAV infection.

identified. Transport on microtubules does not seem to be impaired;^{7,8,10} however, no study has unequivocally compared Rab11 vesicular transport on microtubules of infected cells to that occurring in normal circumstances. Regardless of the transport efficiency, the molecular motor(s) involved in vRNP transport in Rab11 vesicles are not yet known. Given that vesicular transport involves a series of sequential processes where binding of a specific factor impacts in downstream steps, it is possible that inhibition of FIP binding affects several downstream events. Also, inhibiting FIP binding might only partially explain vesicular clustering with the contribution of other yet unidentified factors, including vRNP-vRNP interactions bound to take place and increase the cohesion of clustered vesicles. In addition, given the complexity in Rab11-mediated transport depicted in Figure 2, it is very unlikely that Rab11 will be the only factor in this pathway important for IAV infection.

In agreement, the levels of Rab11 were shown to increase in the membranes of infected cells, indicating that infection alters Rab11 regulation. In this regard, the identification of Rab11 GEFs and GAPs should provide invaluable information on mechanisms controlling IAV infection. Also, during IAV infection alterations in the levels of cholesterol in Rab11 vesicles were reported,⁴⁸ suggesting that the lipid metabolism is remodeled by infection. Interestingly, the presence of U-shaped and doubled membrane vesicles reported by Vale-Costa et al.,⁴⁶ might be a consequence of lipid reshaping in membranes. However, if these are indeed positive for Rab11, or the mechanisms by which they are formed remains to be understood. Regardless of their nature, the functional significance of vesicular clustering during infection remains uncharacterized. In other viral infections, namely positive sense RNA viruses, vesicular clustering has been associated with mechanisms of viral

assembly, escaping host innate immunity, and membrane recruitment (reviewed for example in ref. 49). Whether clustering plays any of these roles in IAV infections is currently being investigated.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

Funding is provided by the Fundação para a Ciência e a Tecnologia, Portugal, grant numbers SFRH/BPD/94204/2013, PTDC/IMI-MIC 1142 2012, and IF/00899/2013.

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