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Review

# The importance of virion-incorporated cellular RNA-Binding Proteins in viral particle assembly and infectivity

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ABSTRACT

RNA is a central molecule in RNA virus biology due to its dual function as messenger and genome. However, the small number of proteins encoded by viral genomes is insufficient to enable virus infection. Hence, viruses hijack cellular RNA-binding proteins (RBPs) to aid replication and spread. In this review we discuss the 'knowns' and 'unknowns' regarding the contribution of host RBPs to the formation of viral particles and the initial steps of infection in the newly infected cell. Through comparison of the virion proteomes of ten different human RNA viruses, we confirm that a pool of cellular RBPs are typically incorporated into viral particles. We describe here illustrative examples supporting the important functions of these RBPs in viral particle formation and infectivity and we propose that the role of host RBPs in these steps can be broader than previously anticipated. Understanding how cellular RBPs regulate virus infection can lead to the discovery of novel therapeutic targets against viruses.

## 1. Introduction

Viruses are obligate intracellular pathogens that represent a threat to human health and the economy of countries. Viral genomes are typically small, encoding only a few proteins. To circumvent this limitation, viruses hijack host resources to complete their infection cycle. RNA is a central molecule in RNA virus biology since it functions not only as a messenger for protein synthesis (i.e. mRNA), but also as a genome. Since viruses cannot encode all the proteins necessary for viral (v)RNA replication/transcription, translation and packaging, viruses usurp cellular RNA-binding proteins (RBPs) [1–8]. On the other hand, cellular RBPs are also central to the antiviral defences [9,10]. The host cell senses viruses through specialised RBPs that recognise unusual signatures present in vRNAs, known as pathogen-associated molecular patterns (PAMPs), leading to the stimulation of interferon production and the activation of mechanisms to inhibit protein synthesis [11]. Cellular RBPs are thus fundamental components of the virus-host cell battlefield, either sustaining or restricting virus infection. Understanding how viruses interact with these cellular proteins can thus be instrumental for the development of novel antiviral therapies.

RBPs interact with RNA forming dynamic complexes known as ribonucleoproteins (RNPs), which are critical for mediating gene expression [12]. Recently, the repertoire of human cellular RBPs was increased dramatically to over 1500 proteins by the development of a proteome-wide approach known as RNA interactome capture (RIC) [13–15]. RIC employs ultraviolet (UV) crosslinking of 'zero' distance (<2 Å) protein-RNA interactions, followed by cell lysis under denaturing conditions, isolation of RBPs crosslinked to poly(A) RNA via oligo(dT) capture and quantitative mass spectrometry. Additionally, new methods based on organic-aqueous phase separation to detect also proteins bound to non-poly(A) RNAs have contributed to greatly extend the number of RBPs in the cell [16–18]. While a substantial proportion of these proteins interact with RNA using a defined set of well-characterised RNA-binding domains (RBDs), such as RNA-recognition motifs (RRMs), many RBPs lack them, suggesting the existence of unconventional modes of RNA binding [13–15]. Indeed,

*Abbreviations:* 4SU, 4-thiouridine; CLIP-seq, Crosslinking and immunoprecipitation followed by sequencing; DENV, Dengue virus; dsDNA, Double-stranded DNA; dsRNA, Double-stranded RNA; GO, Gene Ontology; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HIV-1, Human immunodeficiency virus 1; IAV, Influenza A virus; ivRBP, *In virion* RNA-binding protein; MeV, Measles virus; PAMP, Pathogen associated molecular pattern; pgRNA, Pre-genomic RNA; RBD, RNA-binding domain; RBP, RNA-binding protein; RIC, RNA-interactome capture; RNP, Ribonucleoprotein; RRM, RNA-recognition motif; RSV, Respiratory syncytial virus; RVFV, Rift Valley fever virus; SINV, Sindbis virus; vRNA, Viral RNA; vRNP, Viral ribonucleoprotein; VSV, Vesicular stomatitis virus; ZIKV, Zika virus.

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dozens of novel RBDs were later discovered using proteomic-based methods. These domains included enzymatic cores, protein-protein interaction surfaces and DNA-binding domains [19,20], as well as intrinsically disordered regions that emerged as a prominent mode of RNA binding [19,21].

Importantly, both classical and unconventional RBPs have been linked to virus infection and immunity, as reviewed before [5]. Moreover, analysis by comparative RIC of the 'RNA-binding proteome' of cells infected with the RNA virus Sindbis (SINV), revealed that the complement of cellular RBPs is pervasively remodelled upon infection [7]. Strikingly, many RBPs activated by SINV either sustain or repress infection, highlighting the critical roles of cellular RBPs as regulators of virus infection. Because SINV RNA is polyadenylated, RIC isolates both host and viral RNA. Indeed, many of the proteins enhanced by SINV infection were shown by orthogonal approaches to accumulate within the viral replication factories, suggesting a direct interplay with vRNA. However, RIC will also discover RBPs with differential RNA-binding activity that interact with cellular RNA instead of vRNA.

Several approaches have been developed to elucidate the composition of viral ribonucleoproteins (vRNPs). Initially, studies on influenza A virus (IAV) and vesicular stomatitis virus (VSV) focused on isolating viral RNA polymerase complexes, as these should reflect the composition of replicating vRNPs to some extent [22–24]. While useful, these datasets were biased towards direct protein-protein interactions. More recently, different groups have employed diverse approaches to capture vRNA together with its interacting proteins, which are then detected by mass spectrometry. All these methods start by 'freezing' protein-vRNA complexes. This can be achieved by UV protein-RNA crosslinking exploiting the excitability of natural bases upon irradiation with UV light at 254 nm [2]. Alternatively, photoactivatable nucleotides, such as 4-thiouridine (4SU), can be incorporated into nascent vRNA, and protein-RNA crosslinking is achieved by irradiation with 365 nm UV light [1,3]. UV crosslinking only promotes covalent bonds between proteins and RNAs placed at 'zero' distances, thus displaying high specificity with the cost of low efficiency. Another approach typically used to immobilise protein-RNA interactions is formaldehyde crosslinking [4,6,8]. While more efficient than UV, formaldehyde also induces covalent bonds between proteins, which promotes the isolation of indirect binders through protein-protein bridges. Once protein-RNA complexes are 'frozen', the second step is to isolate the vRNA together with its covalently bound proteins. Typically, vRNA is captured with antisense DNA probe(s). This approach substantially enriches for vRNA [2,4,8], although can still co-purify RNAs in a non-specific fashion through the formation of partial hybrids with non-target sequences. If vRNA is labelled with 4SU (see above), it can alternatively be isolated by biotinylation of the sulfhydryl group in the 4SU base, coupled with streptavidin pull down [6]. Since exposed cysteines on protein surfaces can also be biotinylated, it is not surprising that this approach identifies a large proportion of cellular proteins lacking RNA-binding activity. Collectively, these methods have been applied to several virus models including dengue virus (DENV) [2,3,8], Zika virus (ZIKV) [8], SINV [6], poliovirus [1] and human immunodeficiency virus 1 (HIV-1) [4], representing important advances towards understanding vRNP composition. The interactomes between host proteins and incoming viral particles have been recently unravelled using a novel technique named VIR-CLASP [25]. VIR-CLASP uses 4SU to label the genomic RNA in infected cells, which is later incorporated into viral particles. Viruses released to the supernatant harbouring 4SU-labelled genomes are then used in very high multiplicity to infect new cells. Infection is followed by crosslinking with UV light at 365 nm, solid-phase capture of protein-RNA complexes and protein identification by mass spectrometry. VIR-CLASP has been successfully applied to Chikungunya virus or IAV and have revealed important regulators of the initial steps of the infection cycles of these viruses [25]. Despite their strengths and limitations, all these studies agree that both classical and unconventional RBPs engage with vRNA and play critical roles in infection.

In general, infection cycle of RNA viruses consist of three main phases (Fig. 1): (1) attachment of the viral particle to the cell and entry; (2) viral genome replication and expression; and (3) assembly and egress of the virus progeny [26]. The viral cycle begins when a virus binds to specific surface receptors and enters a host cell. Once inside the cell, the genomic RNA of positive-stranded viruses engages directly with the host protein synthesis machinery to produce the viral proteins required for replication. Conversely, negative-stranded RNA viruses typically carry the transcription machinery with them into the newly infected cell. Retroviral RNA must be reverse transcribed into DNA, which is then imported into the nucleus and integrated into the host chromosome to be transcribed by the cellular RNA polymerase II. The next stage involves the synthesis of the components (i.e. viral structural proteins and genomes) required to assemble the progeny viral particles that leave the producer cell to infect a new one. Virtually all these stages can be regulated by cellular RBPs (Fig. 1), although most work so far has focused on viral replication and protein synthesis [27–32]. However, new discoveries have highlighted the importance of cellular RBPs in the assembly of viral particles as well as in the very initial steps of infection, when the RNA genome is delivered into a newly infected cell. These steps are the focus of the present review.

One of the main open questions is how vRNA is recognised specifically by viral proteins with limited, if not non-existent, specificity for vRNA during the assembly of viral particles [33,34], and whether high-specificity cellular RBPs may cooperate with viral proteins to recognise the vRNA. Moreover, vRNA is generally highly structured [35,36]. Thus, it is plausible that cellular RBPs with helicase and chaperone activities facilitate the binding of capsid/nucleocapsid proteins across the vRNA to enable RNA packaging into virions. Immediately upon entry into the cell, vRNA must either be translated (positive-stranded RNA viruses), transcribed and replicated (negative-stranded RNA viruses) or reverse transcribed (retroviruses) without alerting the host immune defences, especially in the initial steps of infection where the virus is more vulnerable. How viruses achieve this remains largely unknown; however, recent evidence supports the idea that in certain cases such as HIV-1, some of the lifecycle of the vRNA takes place inside the 'protective walls' of the capsid shell [37–39]. Another interesting idea is whether vRNAs carry the necessary cellular components from the producer cell to maximise the efficiency of the subsequent initial steps of infection. Different proteomic studies have identified hundreds of cellular factors within the particles of several RNA viruses [40–55], many of which are RBPs. Here, we discuss the 'knowns' and 'unknowns' of the roles that virion-incorporated cellular RBPs could play in the assembly of viral particles and the early steps of infection in the new host cell.

### 1.1. *Which cellular RBPs are incorporated into viral particles?*

In the last two decades, the composition of the particles of several RNA viruses has been elucidated by proteomics. However, no work has currently focused on identifying the scope of cellular RBPs that are present in virions. To gain insights into this question, we compiled the proteomes of particles from ten different human RNA viruses (Fig. 2) [40–55]. Upon building the superset of host proteins present in virions (Table S1A), we cross-referenced it to the complement of experimentally determined human RBPs [15] as well as Gene Ontology (GO) terms and protein domains related to RNA binding. The resulting *in virion* RBPs (ivRBPs) are listed in Table S1B, including the dataset in which each protein was reported. We discover that virions contain many RBPs (Fig. S1A), 58 % of which harbour known RBDs, while the other 42 % were just recently classified as RBPs by RIC studies and their modes of RNA binding remain unknown [15].

Analysis of GO biological process terms (Fig. S1B) and protein-protein interaction networks (Fig. S2) indicate that ivRBPs can potentially participate in a wide variety of processes, e.g. transport, exocytosis, response to stress, immune system pathways, cytoskeleton

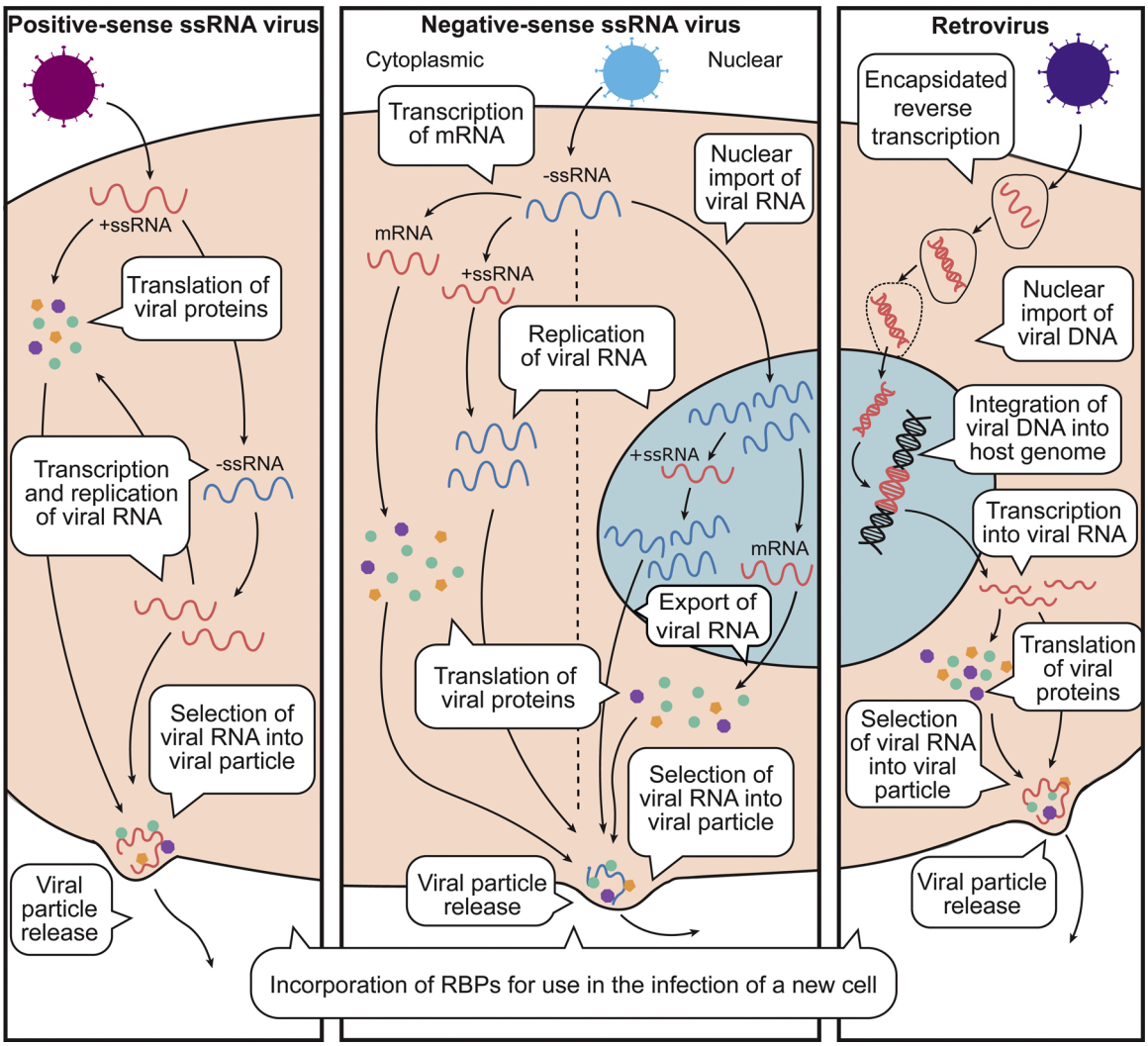


**Fig. 1. Infection cycles of positive-sense single-stranded (ss)RNA viruses, negative-sense ssRNA viruses and retroviruses.** Each panel shows a highly simplified version since the infection cycles of individual viruses within each group are very diverse. Steps during which cellular RNA-binding proteins could be involved are highlighted in the speech bubbles.

organization, translation, RNA processing, RNA stability or protein folding. However, the main limitation of the cited proteomic analyses is that budding of the viral particles results in the uptake of a portion of the cytoplasm and plasma membrane. Hence, it is challenging to discriminate between abundant proteins randomly incorporated into virions and with no function in virus biology, from those with an active regulatory role. We reasoned that host proteins with a true function in infection are likely to be identified in multiple datasets and across different virus species and cell lines (Fig. S3). Strikingly, we noticed that 14 ivRBPs are commonly detected in the particles of different viruses (at least 5 viruses out of 10, Table S1B). For example, cofilin 1 (CFL1) was identified in 8 of the 10 viruses studied here. CFL1 is a regulator of actin cytoskeleton reorganisation and has been shown to play a crucial role in Measles virus (MeV) RNP formation for vRNA synthesis [56], IAV assembly and budding [57] and the production of infectious respiratory syncytial virus (RSV) [58]. However, whether the RNA-binding activity of CFL1 plays a role in infection requires further investigation. Moreover, the importance of CFL1 incorporation into particles should also be further tested functionally, since it could also be due to passive uptake with actin, which is often present in viral particles. Many ivRBPs such as annexins, heat shock family proteins (HSP), peptidylprolyl isomerase A (PPIA – also cyclophilin A), eukaryotic translation elongation factors (EEF), heterogeneous nuclear ribonucleoproteins (HNRNP) or poly(rC) binding protein 1 (PCBP1), have been linked to infection in multiple ways (Fig. S2), and here we show that they are incorporated in the particles of several viruses (Table S1B). Whether these proteins are incorporated passively due to high abundance or through convergent, virus-driven active mechanisms such as specific interactions with viral proteins or RNA, requires further investigation. However, PPIA illustrates well why the second option should not be underestimated, as it interacts specifically with the capsid of HIV and this interaction is critical for capsid core stability [59,60]. Whether PPIA RNA-binding activity plays a role on this recruitment remains unknown. Many ribosomal proteins are also present in the superset of ivRBPs, together with RNA helicases that aid the unwinding of RNA (Fig. S2). Interestingly, several ivRBPs are restricted to the virions of a certain superclass of viruses. For example, heat shock protein 90 alpha (HSP90AA1), transgelin 2 (TAGLN2) and HNRNPD are found exclusively in negative-stranded RNA viruses. Nevertheless, it is important to note that all these virion proteomes have been generated using different i) virion isolation methods, ii) proteomic approaches and iii) data analyses. These divergences are noticeable when comparing datasets generated in closely related viruses or even the same virus (Fig. S3). For example, each of the six HIV-1 virion proteomes that we examined identified a distinct number of proteins (Fig. 2), reflecting differences in virion isolation stringency, proteomic depth and/or statistical thresholds. The analysis of virion proteomes is

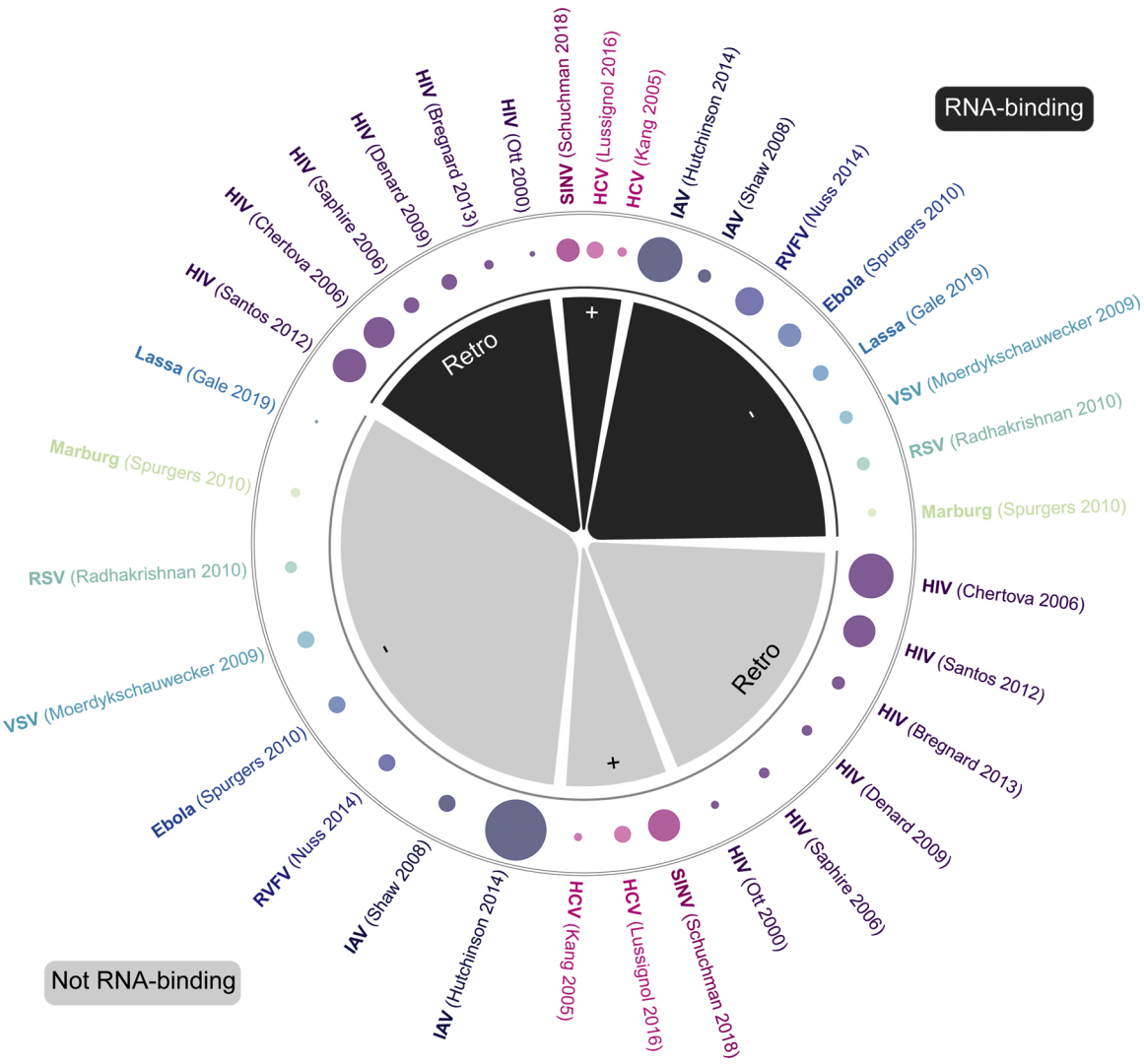


**Fig. 2. An overview of virion proteome datasets from human RNA viruses.** The inner circle shows the relative number of proteins for retroviruses (Retro), positive-sense ssRNA viruses (+), and negative-sense ssRNA viruses (−). The outer circle shows the relative sizes of individual datasets. A large proportion of the proteins detected in virions have been indicated to be RNA binders (in black) either based on RNA-interactome capture experiments, Gene Ontology terms, or protein domain composition.

complicated by potential contamination with cellular proteins, either within extracellular vesicles that copurify with viral particles or via nonspecific interactions with the virus exterior. Most of the viruses used in our metanalysis were purified on sucrose cushions or density gradients, and some lack additional purification steps [41,43,47,48,50,52]. Few of the latter studies showed that the vast majority of their samples were intact viral particles using transmission electron microscopy. Interestingly, several studies incorporated further purification strategies: (1) treatment of viral particles with proteases such as subtilisin [40,44] or proteinase K [45,46] to remove non-specific binders and vesicles due to an alteration in their density; (2) CD45 immunoaffinity depletion as CD45 is present on microvesicles but not in HIV particles [42]; (3) solubilisation of lipid bilayers for isolation of HIV cores by including a detergent layer on the sucrose density gradient [49]; (4) haemadsorption to and elution from red blood cells to select for IAV particles with receptor binding and cleavage activity, provided by haemagglutinin and neuraminidase [51]; (5) sequential affinity purification with a heparin column to select for HCV particles containing the viral E2 protein, followed by antibody- or tag-mediated capture of envelope-containing particles [53]. By focusing on proteins identified in multiple datasets, we expect to minimise technical differences between datasets, as well as biological factors, such as the incidence of randomly incorporated proteins (see above). However, passive incorporation due to abundance cannot be excluded a priori, and we recommend interested readers to test the abundance of their candidates in proteomic analyses of whole cell lysates, if available. Table S1 highlights many of these robustly identified ivRBPs and we discuss below several ivRBPs with well-known roles in infection, and others that remain poorly characterised.

## 2. Do cellular RBPs participate in viral particle assembly?

### 2.1. vRNA capture and trafficking to the plasma membrane

The cytoplasm of the cell is a hostile environment for vRNA due to the presence of antiviral sensors. These cytoplasmic immune surveillance factors are mostly specialised RBPs that detect viral double-stranded (ds)RNA, under-methylated cap structures or triphosphate 5′ ends, which are common signatures present in vRNA [61–64]. The recognition of vRNA as ‘foreign’ nucleic acid can trigger the antiviral response and direct the RNA decay machinery towards the vRNA [65,66]. Hence, it is crucial for vRNAs to effectively engage with cellular RBPs to increase their stability and translatability [67].

Upon accumulation of viral RNA and proteins, these components

must travel to the virus assembly areas and, in some instances, vRNA trafficking has been proposed to be mediated by the cytoskeleton [68–73]. The role of protein chaperones in the trafficking of vRNPs is becoming increasingly evident. Many cellular chaperones and peptidyl-prolyl cis/trans isomerases (immunophilins) are hijacked by viruses to enable viral replication and translation by folding viral proteins [74,75]. Interestingly, new proteome-wide approaches have revealed that both chaperones and immunophilins interact with RNA *in vivo* and exert critical functions in RNA metabolism, including regulatory roles within the spliceosome, control of transcription and mRNA stability and ribonucleoprotein remodeling [13–15]. Moreover, their RNA-binding surfaces have recently been revealed, suggesting that their ability to act on proteins and bind to RNA is interconnected [19]. RIC analysis of SINV-infected cells revealed that the RNA-binding activity of several protein chaperones and immunophilins is enhanced upon infection and that their functional perturbation severely impairs viral fitness [7]. The importance of host chaperones in infection is well illustrated by HIV-1 RNPs. Viral genomic RNA interacts with staufen double-stranded RNA-binding protein 1 (STAU1) in the cytoplasm forming HIV-1-dependent STAU1-RNPs [76–78]. Compositional characterisation of these RNP complexes revealed the presence of the chaperones HSPD1 (HSP60), HSPA1 (HSP70) and HSPA8 (HSC70) 78]. It has been proposed that the stress-inducible chaperone HSP70 and its constitutive form, HSC70, interact with nascent HIV-1 Gag-vRNA complexes and hold them in an assembly-competent conformation during their transport towards the plasma membrane [79]. Strikingly, these proteins are also present within HIV-1 virions (Fig. S2C), suggesting that they associate with vRNA at different stages of the infection cycle 78]. The participation of HSC70 in viral particle assembly is also illustrated by hepatitis C virus (HCV) infection. HSC70 interacts with HCV RNA and colocalises with the HCV capsid and E2 proteins at assembly sites [80]. Knockdown of HSC70 [80] or abrogation of protein activity with allosteric inhibitors [81] decreases viral particle production, but has no effect on viral replication. Whether direct binding of HSC70 to the HCV RNA genome contributes to the formation of infective virions is still unclear. The roles of cellular RNA-binding chaperones, therefore, may span replication, translation, trafficking and virion assembly (Fig. S2).

Cellular RBPs are also thought to contribute to capture the vRNA, amongst all the cellular RNAs in the cytoplasm, to be packaged into the virion. Several viruses, such as the bacteriophage MS2, encode capsid proteins with high specificity and affinity for stem loops present within the vRNA [82]. However, other viruses, such as HIV-1, do not encode for any protein with high specificity and affinity for the vRNA. Hence, how these viruses select and package their vRNA into virions against cellular counterparts remains poorly understood. For example, it was believed that the HIV-1 nucleocapsid subunit of the viral polyprotein Gag recognises vRNA from the pool of cellular RNAs by a unique binding event at a *cis*-acting packaging element in the 5′ leader of the vRNA [83]. However, a recent study using CLIP-seq (crosslinking and immunoprecipitation followed by sequencing) discovered that, in addition to the binding site in the 5′ leader, Gag interacts in the cytoplasm with other discrete sites across the HIV-1 RNA [34], placed at the Rev recognition element (RRE) and 3′ UTR. However, when the vRNA reaches the plasma membrane, this binding pattern changes dramatically, and Gag covers virtually the whole vRNA. How Gag switches from selective to non-selective binding is not understood. Since many cellular RBPs interact with HIV-1 RNA and Gag [4,78,84], it is tempting to speculate that they might cooperate with HIV-1 nucleocapsid to recognise HIV-1 genome in the cytoplasm of the infected cell. In agreement, it has been proposed that STAU1 assists Gag interaction with HIV-1 RNA [85]. The resulting RNP is transported through the cytoplasm to the plasma membrane where STAU1 facilitates the multimerization of Gag on the vRNA [86]. This multimerization step enables the formation of immature virus particles, where the HIV-1 genomic RNA acts as a nucleation site for assembly [87,88]. Interestingly, STAU1 only modulates Gag assembly once the tail of Gag has anchored into the plasma membrane suggesting that STAU1 may play a role in ensuring the RNP is delivered to the membrane [86]. STAU2 has also been shown to promote the activity of HIV-1 Rev protein in exporting vRNA from the nucleus, along with DExH-box helicase 9 (DHX9) and ArfGAP with FG repeats 1 (AGFG1) [89–91]. Together, these studies suggest that Staufen proteins play crucial roles in the transport of HIV-1 RNPs from the nucleus to the plasma membrane for viral particle formation. Additional studies should be carried out to determine if other cellular RBPs cooperate with viral capsids and nucleocapsids to recognise the vRNA and to enable vRNP trafficking.

### 2.2. Release of viral particles from the plasma membrane

The cellular endosomal sorting complex required for transport (ESCRT) is a large multi-component machinery comprised of five complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and Vps4, which assemble sequentially in a multi-step manner following ubiquitination of ESCRT-0 [92]. The ESCRT machinery is hijacked for viral particle release across most types of viruses including retroviruses, filoviruses, arenaviruses, paramyxoviruses, rhabdoviruses, flaviviruses, reoviruses and picornaviruses [93]. Viruses can either hijack ESCRT for assembly and budding at the plasma membrane or for budding at endosomal membranes into the cytoplasm. ESCRT is typically involved in the budding of vesicles from the endosomal membrane into the endosomal compartment. However, ESCRT proteins can also perform a “reverse topology” membrane fission. ESCRT is the only cellular machinery with such ability and this is perhaps why so many viruses have evolved to exploit it [93].

ESCRT-I/II essentially function to recruit the ESCRT-III protein CHMP4 (charged multivesicular body protein 4) to membranes where the formation of ESCRT-III filaments is then promoted. The formation of ESCRT-III filaments drives the budding of the membrane, but it is not understood exactly how this is achieved. Programmed cell death 6 interacting protein (PDCD6IP or ALIX) recruits CHMP4 and the ESCRT-III complex to the plasma membrane. Several studies have shown that many viral structural proteins initiate ESCRT driven budding from the membrane by recruiting ALIX. Examples include Gag of HIV-1 [94], the accessory C protein of human parainfluenza type 1 [95], Ebola virus VP40 [96], pX of hepatitis A virus [97] and the NS3 protein of DENV [98] and yellow fever virus [99].

Although ALIX has largely been studied from a proteo-centric perspective, RIC studies have revealed that it is endowed with RNA-binding activity [15]. In addition, ALIX has recently been implicated in the recruitment of cellular RNAs to extracellular vesicles [100]. Importantly, the interaction of HIV-1 Gag with ALIX relies on the presence of vRNA. The N-terminal Bro1 domain and the central V-shaped domains of ALIX interact with HIV-1 nucleocapsid and p6 domains of Gag respectively [101,102]. Interestingly, the interaction between ALIX Bro1 domain and HIV-1 nucleocapsid is disrupted by RNase treatment, suggesting that the vRNA molecule forms a bridge between the two proteins. Both ALIX Bro1 and nucleocapsid are highly positively charged and are believed to establish electrostatic interactions with the phosphate backbone of the vRNA. The recruitment of ALIX by vRNA raises the question of whether ALIX is recruited to the HIV-1 RNPs in the cytoplasm or in the plasma membrane as the virus assembles 102]. Additionally, proteomic-based compositional analysis of the ZIKV and DENV RNPs revealed that ALIX also interacts with these vRNAs [8], although the exact role of these interactions remain unexplored.

Another class of unconventional RBPs implicated in virus release is the Annexin protein family. Annexins are calcium-dependent membrane binding proteins that carry out a diverse range of functions. They can reversibly associate with components of the cytoskeleton or with regulatory proteins and RNAs that mediate stress-induced intra- and intercellular signalling [103]. Annexin A2 (ANXA2) is a multifunctional, ubiquitously expressed protein with roles in membrane domain organisation, membrane fusion, vesicle aggregation and exocytosis [104]. It

has been shown to play a role in cell attachment and entry or replication of enterovirus, RSV, HCV and IAV, amongst other viruses [104]. However, we still lack a molecular understanding of the relationship between ANXA2 RNA-binding activity and its regulatory roles in infection. ANXA2 was shown to be involved in the formation of the HCV replication complex and can bind both HCV RNA, in a sequence-specific manner, and non-structural protein NS5B forming a ternary complex [105]. The silencing of ANXA2 has no effect on vRNA levels suggesting that it does not influence replication. Instead it significantly reduces the number of produced viral particles indicating that ANXA2 plays a role in HCV virion formation or release, although the mechanism by which this is achieved remains unknown [106]. ANXA2 has also been shown to be involved in virion assembly of MeV through recruitment of the viral matrix protein to the plasma membrane [107]. ANXA2 and other members of the annexin family have been identified within the viral particles of Ebola and Marburg virus [48], HIV-1 [42], IAV [44], VSV [45], Rift Valley fever virus [52] and RSV [47] (Table S1B). Together, these data suggest that ANXA proteins may be involved in the formation and release of particles of a broad range of viruses. However, the mechanisms of action of ANXA proteins and their RNA-binding activity in infection remains largely uncharacterised.

The compositional analysis of different virions has also revealed the presence of some members of the ADP ribosylation factor (ARF) GTPase family, suggesting a role in viral particle formation and/or release [55]. This is a RAS-related subfamily fundamental for the regulation of vesicle formation, trafficking and docking at target membranes, and has been implicated in the infection cycles of many pathogens [108]. ARFs have been involved in the recruitment of HIV-1 RNPs to the plasma membrane and the release of HIV-1 particles [109], and ARF1 regulates HIV-1 trafficking to the virological synapse [110]. Additionally, ARF1 is necessary for HCV RNA replication and production of infectious particles [111]. The possibility that the RNA-binding activity of ARF1 facilitates the localisation of vRNPs at the sites of viral particle assembly and egress calls further investigation.

## 3. Do cellular RBPs facilitate the initial steps of infection?

### 3.1. Translation initiation

After entry into the host cell, most positive-stranded RNA viruses release the genomic mRNA into the cytoplasm to be immediately translated into the viral replicase complex by the cellular translation machinery (Fig. 1). This is the case for SINV, a representative member of the Alphavirus genus [112]. It was recently described that SINV infection of mammalian cells produce two subpopulations of infectious viral particles. One of them, known as 'heavy' viral particles, exhibits an enhanced translation of the vRNA once inside the newly infected cell [113]. Only one homogeneous population of virions is released from the other natural host of SINV, mosquito, with an infectivity that matches that of 'heavy' viral particles. Authors showed that virion-incorporated host-derived factors, including the ribosomal components RPS14, RPS18 and 18S ribosomal RNA, and the cellular RNA binding motif protein 3 (RBM3), are responsible for vRNA superior translation. RBM3 has been shown to promote translation in different contexts [114] and its incorporation into SINV particles was recently confirmed [54], together with other ribosomal proteins and many cellular RBPs (Table S1C). Interestingly, while SINV particles with increased infectivity can be produced in either mammalian or mosquito cells, the enhanced vRNA translation only occurs in a newly infected mammalian cell, but not in a mosquito cell [113]. This striking phenomenon is recapitulated in animal models and in other alphaviruses [115]. This suggests that RBPs pre-loaded in the viral particle may interact specifically with mammalian proteins controlling translation initiation. The identity of these mammalian proteins remains unknown and uncovering them will be a challenge. However, recent work has shown that the mammalian ribosome establishes interactions with hundreds of cellular RBPs, which represent potential regulators of translation [116]. As many of these ribosome interactors are indeed found inside virions (Table S1), we anticipate that they may accompany the vRNA into the newly infected cell. Once the vRNA is released into the cytoplasm, these proteins may facilitate translation initiation by interacting with the ribosome. To what extent virion composition determines translation of incoming viral genomes awaits mechanistic characterisation. Moreover, whether this phenomenon can be extended to other RNA viruses beyond SINV remains to be explored.

### 3.2. Synthesis of vRNA

Particles of negative-stranded RNA viruses contain a viral RNA-dependent RNA polymerase and accessory proteins to transcribe the vRNA into mRNA upon entry into the host cell (Fig. 1). Later, this 'transcriptase' complex is modified to form a 'replicase' complex that synthesizes an intermediary positive-stranded RNA that serves as a template for producing new copies of the negative vRNA genome [117]. Thus, viral transcription and replication are two separate processes controlled by distinct vRNP complexes.

A representative example is VSV that forms two complexes composed of the viral RNA polymerase (L) and different viral and host proteins [22]. The VSV transcriptase complex contains the viral proteins L and P and the host proteins RNA guanylyltransferase and 5′-phosphatase (RNGTT) capping enzyme, EEF1A and HSP60 [22,118]. The last two proteins are important for the RNA polymerase activity of L and can be found within virions (Table S1B). In addition, PPIA is also bound to the vRNP complex inside purified VSV particles and is used for post-entry primary transcription [119]. Interestingly, the mentioned host RBPs are absent in the VSV replicase complex, which contains N, L and P viral proteins and synthesizes the plus strand 'antigenome' vRNA used as template for copying the viral genome [22]. This suggests that differential association of the RNA polymerase complex with host proteins may regulate the switching from transcription to replication. The cellular RBP HNRNPU interacts with the VSV leader RNA that is required for vRNA replication and inhibition of cellular transcription [120]. This protein is also packaged into VSV particles, but the potential association of virion-incorporated HNRNPU with the transcriptase or replicase complexes and its activity in the early steps of VSV infection is not well understood.

In IAV, the viral polymerase consists of three subunits: PB1, PB2 and PA. The cellular chaperone HSP90 is involved in the transport of PB1 and PB2 to the nucleus and modulates the interaction of PA with PB1 [121]. After binding of PA to PB1-PB2, HSP90 dissociates from the complex. In this way, authors suggested that HSP90 regulates the assembly of the mature trimeric polymerase complex. On the contrary, HSP90 was found to be associated with the trimeric polymerase complex in a different study [23]. HSP90 relocates to the nucleus after IAV infection [121,122], and this could reflect its interaction with newly synthesized polymerase subunits. Interestingly, HSP90 has been found inside purified IAV particles (as well as in other viruses, Table S1B), but the possibility that virion-incorporated HSP90 participates in transport of the incoming vRNP to the nucleus and/or the initial viral transcription has not been explored so far. *In vitro*, HSP90 stimulates IAV RNA synthesis by interacting with PB2 122], and binding of HSP90 to PB2 is increased during RNA synthesis [121]. Authors suggested that HSP90 may participate in the early steps of transcription elongation by dissociating the polymerase from the vRNP and stabilizing the different subunits during their transfer between RNA templates [121]. However, further investigation in the context of IAV-infected cells is required to elucidate the role of HSP90 and its recently described RNA-binding activity [19] in early IAV transcription.

### 3.3. Reverse transcription

It was believed that the capsid shell protecting HIV-1 RNA was

disassembled upon entry into the host cell, allowing viral RNA and proteins to associate with host RBPs in the cytoplasm of the newly infected cell to initiate reverse transcription. However, more than a decade of intensive work has challenged this model by discovering that reverse transcription takes place inside the capsid core [37–39,123–125], protected from the hostile intracellular environment, and uncoating occurs at the nuclear pore complex [126,127] or even inside the nucleus [128] (Fig. 1). Recently, it was shown that the presence of pores in the capsid hexamers allows deoxynucleotides to traverse the capsid shell to feed reverse transcription [38]. However, at only 8 Å wide, these pores are too small to allow proteins to pass through. Hence, host RBPs participating in reverse transcription must be present inside the capsid core prior to cell entry, likely being incorporated in the producer cell during the assembly of viral particles. The idea of bringing up key proteins from the producer cell is well characterised for negative stranded RNA viruses, which incorporate viral polymerase complexes including host factors within the viral particles to enable replication upon entry in the host cell (see above). Similarly, HIV-1 particles contain reverse transcriptase and integrase, two viral enzymes that are critical in both reverse transcription and pro-viral DNA integration into the host chromosome. Recently, integrase was shown to bind not only DNA but also specific structures in HIV-1 RNA within virions, and these interactions are critical for the correct localization of the vRNP inside mature virions [129]. Several studies have revealed that specific cellular ivRBPs either promote (e.g. UPF1 RNA helicase and ATPase [UPF1] [130], Y-box binding protein 1 [YBX1] [131], DHX9 [132], EEF1A [133,134], PPIA [135] and aminoacyl-tRNA synthetases [136]) or inhibit (e.g. Mov10 RISC complex RNA helicase [MOV10] [137], pyruvate kinase M1/2 [PKM] [138], enolase 1 [ENO1] [139]) reverse transcription (Table S1E). Nonetheless, the scope of cellular RBPs hijacked into HIV-1 particles and whether they play critical roles in these initial steps of infection inside the virion is not well defined yet.

As example, UPF1 is a cytosolic RNA helicase that plays crucial roles in HIV-1 infection and is incorporated into virions (Table S1E). Strikingly, reverse transcription fails if virions are generated in cells depleted of UPF1 or expressing an ATPase-defective UPF1 mutant [130]. Authors suggested that UPF1 could mediate the remodelling the vRNP to facilitate reverse transcription or, alternatively, promote the annealing of $tRNA^{Lys3}$ primer to the viral genome, as shown in other helicases such as DHX9 [130,140]. Conversely, recent evidence suggested that DHX9 participates in the elongation phase of reverse transcription but not in the annealing of $tRNA^{Lys3}$ to the vRNA [132]. Further research is required to elucidate the exact mechanism of action of UPF1, DHX9 and other cellular RBPs during HIV-1 reverse transcription.

Interestingly, cellular RBPs have also been found in Hepatitis B virus (HBV) nucleocapsids. HBV is a 'gapped' DNA virus but initially a single-stranded RNA pre-genome (pgRNA) is packaged into viral particles. The pgRNA is reverse transcribed to a circular dsDNA within the viral capsid before the virus matures and is secreted from the cell [141]. The pgRNA is packaged through recognition of epsilon (ε) stem-loop structure in the 5′ terminus by HBV polymerase (Pol). It was shown that eukaryotic translation initiation factor (EIF)4E enables this recognition by forming an EIF4E-Pol-ε RNP complex that is incorporated into the nucleocapsid [142]. Reverse transcription of the pgRNA then requires HSP90 [143], which forms a RNP together with other cellular RBPs including HSC70 and DNAJA1 (HSP40) [144,145] to enable chaperone-mediated specific binding of HBV reverse transcriptase to pgRNA. How the RNA-binding activity of these proteins contribute to viral capsid assembly and/or reverse transcription remains poorly understood. Finally, other cellular ivRBPs such as DEAD-box helicase 3 X-linked (DDX3X) [145], apolipoprotein B mRNA editing enzyme catalytic subunit 3 G (APOBEC3 G) [146,147] and MOV10 [148] negatively regulate the very early steps of HBV reverse transcription. DDX3X interacts with HBV polymerase and requires ATPase but not helicase activity for minus-strand DNA synthesis inhibition [145]; APOBEC3 G acts via an unknown deamination-independent mechanism [147] that may involve direct binding to reverse transcriptase [146], while MOV10 binds directly to HBV RNA but not the viral polymerase [148]. However, the precise mechanisms by which these proteins affect HBV DNA synthesis still remain unclear.

## 4. Are antiviral RBPs incorporated into viral particles?

It is an evocative idea that cellular antiviral factors may be also packaged with the vRNA into virions in order to interfere with the initial steps of infection. Well-known antiviral RBPs such as zinc finger CCCH-type containing antiviral 1 (ZC3HAV1), APOBEC3C and APOBEC3F are detected in the proteome of purified SINV particles (Table S1C). However, studies exploring their role in the context of the viral particle or the early phase of SINV infection are non-existent to our knowledge. In addition, antiviral RBPs might be under-represented in the different virion proteomes analysed here (Table S1). This is most likely due to 1) the existence of sophisticated mechanisms to avoid the presence of restriction proteins inside virions, or 2) because the viral particles used in these proteomic studies were often produced in highly permissive cell lines with damaged interferon pathway. The first possibility has been widely studied in HIV-1, which excludes antiviral proteins from virions by different mechanisms. For example, serine incorporator 5 (SERINC5) and SERINC3 are relocalized from cell surface to endosomes by the accessory viral protein Nef [149,150]; YTH $N^6$-methyladenosine RNA binding protein 3 (YTHDF3) is cleaved by HIV-1 protease inside the viral particle [151], and APOBEC3 proteins are targeted for degradation by the viral protein Vif [152].

YTHDF3 has a strong affinity for $N^6$-methyladenosine ($m^6A$), a post-transcriptional RNA modification that has recently emerged as a key regulator of vRNA fate [153]. $m^6A$ can be found in the RNA of IAV, DENV, ZIKV, HCV, yellow fever virus, West Nile virus, Enterovirus 71 and HIV-1. In the case of HIV-1, $m^6A$ modification can influence different steps of the virus infection cycle including reverse transcription. Recently, YTHDF3 was found to be encapsidated into HIV-1 particles by interacting with nucleocapsid and negatively affected reverse transcription once the capsid core is delivered into the newly infected cell [151]. Interestingly, HIV-1 protease cleaves YTHDF3 into smaller fragments inside the virion, thus counteracting its antiviral activity. Other 'readers' of $m^6A$ RNA can be detected inside the viral particles of different viruses, including EIF3, insulin like growth factor 2 mRNA binding protein 1 (IGF2BP1) and HNRNPA2B1 (Table S1B). Further work is required to discover the potential role of $m^6A$-modified vRNA and ivRBPs in the early steps of infection.

One of the best studied cases of antiviral RBP acting inside virions is the APOBEC3 proteins [152]. These are cellular cytidine deaminases that catalyse the irreversible hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine. To exert their antiviral effects in HIV-1, these proteins are recruited into viral particles through binding to the vRNA and nucleocapsid. APOBEC3F, G and H have preference for G-rich and A-rich sequences which resemble the RNA-binding pattern of the HIV-1 nucleocapsid domain in Gag polypeptide, as determined by CLIP-Seq in cells and purified virions [34,154]. APOBEC3 proteins bind to a large proportion of cellular mRNAs in infected cells due to their low specificity. However, the G/A-rich sequence bias ensures that a proportion of APOBEC3 molecules interact with HIV-1 RNA [155] as well as with other retroviruses [156]. Once inside the viral particle, APOBEC3 proteins induce cytidine deamination of the reverse-transcribed DNA strand. Changes from C to U cause complementary G to A conversion during second strand synthesis and this hypermutation inhibits HIV-1 replication [152]. In addition, APOBEC3 proteins also restrict HIV-1 by a deamination-independent mechanism that consist of altering reverse transcription template switching frequency [157].

APOBEC3 G has also been shown to hinder replication of MeV, mumps virus and RSV [158]. However, it is unclear whether the deaminase activity is responsible for the inhibition of replication of

these viruses. APOBEC3 proteins, including APOBEC3C, F and H, also restrict replication of the human coronavirus NL63 via two mechanisms: cytidine deamination and binding to nucleocapsid protein [159]. APOBEC3C restricts ZIKV infection in a non-editing-dependent manner and is partially counteracted by a small subgenomic flavivirus RNA that sequesters it [160]. While APOBEC3s suppress the infection of a wide range of viruses by different, poorly understood mechanisms, it remains unknown if they are incorporated into these viral particles and exert their antiviral activity in the early steps of infection. This possibility deserves further investigation.

## 5. Conclusions

In summary, many cellular RBPs are hijacked by viruses to sustain infection and are involved in virtually all stages of their infection cycle. We have highlighted here many previously known and novel RBPs that are found inside virions from different human RNA viruses and depicted a clear gap in our understanding of their function in infection. Cellular ivRBPs may facilitate trafficking and selection of vRNA into assembling viral particles, protect the vRNA from the hostile cellular environment, or streamline the initial processes of vRNA metabolism upon entrance in a new host cell. Alternatively, some of these ivRBPs may function as part of the immune surveillance system of the cell and are incorporated into viral particles to interfere with infectivity. Whilst we have discussed several roles for ivRBPs in this review, much of these remain poorly characterised and mechanistic data is still missing. Future research must focus on deciphering the roles of ivRBPs and their newly described RNA-binding activities in virus infection. By broadening our understanding on these proteins, it will be possible to identify new targets with potential for host-based antiviral therapies.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.semcdb.2020.08.002.

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