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Reassortment in segmented RNA viruses: mechanisms and outcomes

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Abstract

Segmented RNA viruses are widespread in nature and include important human, animal and plant pathogens, such as influenza viruses and rotaviruses. Although the origin of RNA virus genome segmentation remains elusive, a major consequence of this genome structure is the capacity for reassortment to occur during co-infection, whereby segments are exchanged among different viral strains. Therefore, reassortment can create viral progeny that contain genes that are derived from more than one parent, potentially conferring important fitness advantages or disadvantages to the progeny virus. However, for segmented RNA viruses that package their multiple genome segments into a single virion particle, reassortment also requires genetic compatibility between parental strains, which occurs in the form of conserved packaging signals, and the maintenance of RNA and protein interactions. In this Review, we discuss recent studies that examined the mechanisms and outcomes of reassortment for three well-studied viral families — *Cystoviridae*, *Orthomyxoviridae* and *Reoviridae* — and discuss how these findings provide new perspectives on the replication and evolution of segmented RNA viruses.

Viruses that maintain their genomes as several distinct RNA molecules are called segmented RNA viruses. They are ubiquitous in nature, infecting a wide variety of animals, plants and bacteria. To date, 11 different segmented RNA virus families have been described in the literature: *Arenaviridae*, *Birnaviridae*, *Bromoviridae*, *Bunyaviridae*, *Chrysoviridae*, *Closteroviridae*, *Cystoviridae*, *Orthomyxoviridae*, *Partitiviridae*, *Picobirnaviridae* and

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Reoviridae (TABLE 1). These families and their type species can differ considerably in their virion structure, genome architecture, replication cycle, host tropism and pathology. For example, some segmented RNA virus species, such as influenza A virus and rotavirus A, are associated with substantial human disease and serious economic burdens to society[1–3]. Other segmented RNA virus species affect humans indirectly through the infection of domestic animals and crops (for example, bluetongue virus, infectious bursal disease virus and tomato spotted wilt virus)[4–6]. However, a shared feature of all segmented RNA viruses is their capacity to exchange genome segments *in toto* during co-infection through a process called reassortment. Specifically, when two or more viruses infect a single host cell, they can package each other's genome segments into a nascent virion, thereby producing hybrid progeny (FIG. 1a). For multipartite viruses in the *Bromoviridae*, *Chrysoviridae*, *Partitiviridae* and *Picobirnaviridae* families, which incorporate their genome segments into several independent virus particles (TABLE 1), reassortment is stochastic and creates virions that have a random mix of genome segments from each parent. By contrast, for viruses that package their genome segments into a single virion, such as species in the *Cystoviridae*, *Orthomyxoviridae* and *Reoviridae* families, reassortment generally results in segment replacement, such that one co-infecting virus incorporates the genome segment (or segments) of another co-infecting virus in place of its own. In this case, genetic exchange requires the conservation of intricate assortment signals and preservation of the RNA–RNA and/or RNA–protein interactions that mediate genome packaging. For this reason, strain-specific differences in the sequences or structures of homologous RNAs and/or in the packaging proteins of co-infecting parent viruses can severely restrict the generation of reassortant progeny during co-infection. Moreover, for reassortants to selectively emerge at appreciable levels in the viral population, they must have a genomic composition that confers at least some modest advantage to viral fitness.

Conceptually, reassortment shares some features with sexual reproduction in eukaryotes, whereby chromosomes are segregated during meiosis and combined in various ways during gamete fusion (FIG. 1b). Sexual reproduction is argued to be evolutionarily advantageous to eukaryotes, in part, because it purges deleterious mutations and increases population-level genetic diversity, which is a prerequisite to evolution by natural selection[7–9]. Thus, by analogy to sexual reproduction, one theory posits that the reassortment capacity of segmented RNA viruses contributes to the maintenance of this genome structure[10–13]. However, rapidly evolving viral populations have more opportunities to remove unfit mutations than eukaryotes, and reassortment is clearly not necessary for the evolutionary success of the numerous non-segmented viruses. Therefore, it is possible that reassortment is a by-product of genome segmentation, rather than a key evolutionary driver of such a genome structure. Indeed, the evolution of genome segmentation may have been driven by other advantages that are conferred by this arrangement, such as the control of gene expression[14], increased coding potential of the genome[15] and enhanced stability of such virus particles[16]. Regardless of the original drivers of segmentation, the capacity of important human pathogens such as influenza A viruses and rotavirus A strains to reassort has important implications for their ongoing evolution and impact on global health.

The mechanisms and outcomes of reassortment can differ from recombination, which is another form of genetic exchange that occurs readily for some non-segmented RNA viruses,

particularly those with positive-sense RNA ((+)RNA) genomes[17] (FIG. 1c). During recombination, the viral polymerase begins copying the RNA template of one parental strain, and it then switches templates mid-synthesis to use that of a different parental strain. Therefore, the result of recombination is the generation of chimeric RNA molecules that contain regions of nucleotide sequence derived from each parent. Unlike reassortment, during which entire genes (or sets of genes) are exchanged by the swapping of segments, recombination can occur nearly anywhere in the RNA genome, even in the middle of a gene. Therefore, recombination can result in the formation of non-functional chimeric fusion proteins, whereas reassortment cannot[17]. In other words, reassortment is a mechanism that maintains the ORF of a gene and, consequently, maintains protein integrity, whereas recombination typically introduces changes in ORFs and their encoded proteins. Recombination has rarely been reported for segmented RNA viruses[18–21], and some of the detected recombination events may be the result of sequencing artefacts[22]. The lack of robust recombination among segmented RNA viruses is likely to be a reflection of their biology, specifically related to the manner in which their polymerases transcribe and replicate the genome segments in the absence of template switching. A detailed discussion of the mechanism, origins and evolutionary consequences of recombination in RNA viruses compared with reassortment is provided in REF. 23.

In this Review, we focus on the mechanisms and outcomes of reassortment for non-multipartite, segmented RNA viruses in the well-studied *Cystoviridae*, *Orthomyxoviridae* and *Reoviridae* families. In particular, we describe the strategies that are used by these viruses to ensure efficient incorporation of their genome segments into nascent virions, and we discuss how genetic incompatibilities during segment assortment and packaging can directly restrict the generation of reassortants during co-infection. We also highlight recent experimental and comparative genomic studies that elucidate the possible selection pressures that promote or temper the emergence of reassortant viruses in the population. Our goal is to provide new perspectives on the replication and evolution of segmented RNA viruses, which may in turn stimulate the development of measures for the prevention of disease.

Genome segment assortment and packaging

Cystoviridae

The *Cystoviridae* family is composed of segmented double-stranded RNA (dsRNA) viruses that infect Gram-negative bacteria[20]. The type species for this family is *Pseudomonas* phage φ6 (hereafter referred to as φ6), an extensively researched bacteriophage that primarily replicates within the various pathovars of the plant pathogen *Pseudomonas syringae*. Since its discovery in the early 1970s[24], φ6 has been used as a tractable model system to test evolutionary hypotheses within controlled laboratory settings and to uncover mechanisms of virus biology. Additional *Cystoviridae* family members have been found at various geographical locations around the world, which suggests that these viruses are widespread in nature[25–27].

The φ6 virion consists of an outer lipid envelope surrounding a nucleocapsid shell and an icosahedral procapsid core[28]. Within the core reside three dsRNA genome segments, totalling >13 kb in length and encoding 13 viral proteins[25] (FIG. 2a). The segments each

contain several ORFs that are flanked by 5′ and 3′ UTRs, and they are named according to their sizes: small (S; 2.9 kb), medium (M; 4.1 kb) and large (L; 6.4 kb). During the replication cycle of φ6, dsRNA genome segments are transcribed into (+)RNA molecules by viral polymerases[29]. In addition to acting as templates for protein synthesis, these (+)RNAs are the form of the φ6 genome that is incorporated into nascent particles[25] (FIG. 2b). Using an *in vitro* packaging system, it was shown that φ6 (+)RNAs are inserted individually and sequentially into a pre-formed procapsid core through an entry portal at one five-fold icosahedral axis[30,31] (FIG. 2b). The empty core initially displays only the binding site for the S (+)RNA segment, leading to its recruitment and packaging. Thereafter, a conformational change occurs in the core that reveals a binding site for the M (+)RNA segment[32]. Again, only after packaging of the M segment is the binding site for the L (+)RNA segment revealed. It was also demonstrated through *in vitro* assays that the *cis*-acting RNA sequence and structural elements that are crucial for packaging are located in the 5′ UTRs[33] (FIG. 2a). A 5′-terminal 18 bp sequence is shared among the S, M and L segments and enables φ6 to distinguish between viral RNAs and host RNAs. The gene-specific packaging signals that differentiate S, M and L segments during packaging are located ~200 bp downstream of the 18 bp conserved sequence. Following encapsidation of all three φ6 (+)RNAs, the procapsid core expands, thereby triggering the core-associated viral polymerases to convert the (+)RNAs into dsRNA genome segments through a single round of negative-sense RNA ((−) RNA) synthesis[20]. Additional virion morphogenesis, including the acquisition of an outer envelope, leads to the production of fully infectious φ6 particles.

It is predicted, albeit not experimentally demonstrated, that the vast majority of nascent φ6 virions that are produced during the viral life cycle contain all three genome segments. This prediction is based on the observations that (+)RNA packaging is sequential and inter-segmentally dependent, and that the three φ6 genome segments are present in equimolar amounts at the viral population level. Nevertheless, it has been shown that φ6 packaging can be drastically manipulated *in vitro*, yielding particles with more or fewer than three genome segments or with rearranged genome segments[34–36]. For example, one study created a φ6 mutant that did not package the S segment owing to an amino acid substitution in one of its core proteins[35]. This mutant still efficiently packaged and replicated the M and L segments, thereby propagating a virus that contains two segments in a non-lytic carrier state in the bacterial host. Furthermore, it was shown that the entire φ6 genome can be concatenated into a single RNA molecule and still produce a viable mutant with only moderate replication defects[36]. The observation that non-segmented variants of φ6 can be created in the laboratory but do not emerge at detectable levels in nature suggests that genome segmentation provides a fitness advantage.

Orthomyxoviridae

The *Orthomyxoviridae* family of segmented (−)RNA viruses consists of six different genera, three of which (Influenzavirus A, Influenzavirus B and Influenzavirus C) cause respiratory disease in humans[37]. Of these three genera, Influenzavirus A (consisting of a single species, influenza A virus) imparts the largest medical and economic burdens; seasonal epidemics of strains of influenza A virus account for 27,000–55,000 deaths each year in the United States

alone, with an annual cost of US$87.1 billion to the healthcare system[2,38]. Influenza A viruses can also cause pandemics, the most severe of which occurred in 1918–1919 and is estimated to have killed 20–50 million people globally[39]. In addition to infecting humans, influenza A viruses are endemic in several other animal species, including pigs, dogs, horses, bats and birds, which provide natural reservoirs for viral evolution[40].

Influenza A viruses exist as pleomorphic, enveloped virion particles, each encasing a genome of eight (−)RNA molecules (FIG. 2c). The individual genome segments of an influenza A virus range in size from 0.9–2.3 kb in length, and the total genome length is approximately 13.5 kb (REF. 41). Each (−)RNA contains 13 ORFs, in the antisense orientation, which are flanked by 3′ and 5′ UTRs. Altogether, an influenza A virus encodes at least 13 proteins in its eight genome segments. The termini of the viral (−)RNA consist of highly conserved 12–13 bp sequences that can partially anneal with each other in *cis* so that the molecules fold over and form a corkscrew shape (FIG. 2c). Multiple copies of the viral nucleocapsid protein (NP) bind to the length of each (−)RNA, and a heterotrimeric polymerase complex is attached to the end where the 3′ and 5′ termini connect. Thus, the eight influenza A virus genome segments are packaged into virions as eight distinct ribonucleoprotein (RNP) complexes[42,43] (FIG. 2c).

The manner in which influenza A viruses package each of their eight genome segments has not yet been fully resolved. However, the available data are most consistent with the notion that this is a selective, non-random process that it is mediated by interactions between the (−)RNA molecules themselves[44,45] (FIG. 2d). Individual RNPs are assembled in the nucleus, and they must then translocate to the plasma membrane, where they are incorporated into a budding enveloped virion. Using fluorescence *in situ* hybridization, it was shown that the RNPs are exported from the nucleus as subcomplexes, which further assort into a supramolecular complex that contains all eight RNPs while trafficking to budding sites[46–48]. Additional studies support the idea that the subcomplexes consist of specific pairs of (−)RNAs that directly engage each other and that the supramolecular complex is formed through an elaborate interaction network between the (−)RNAs of the subcomplexes[49–52]. Although an *in vitro* packaging system is lacking for influenza A viruses, studies of defective-interfering RNAs have shed light on which regions of the viral genome are crucial for the assortment process. Specifically, defective-interfering RNAs have been engineered to contain large deletions in the central ORFs but to maintain the extreme ORF termini as well as the 3′ and 5′ UTRs of the genome segments, and such RNAs are capable of competing with full-length segments for packaging[53]. This indicates that the segment-specific assortment signals are located within ~300 bp from the termini of the (−)RNA molecules (FIG. 2c). Furthermore, several studies have used reverse genetics approaches to engineer viruses that encapsidate reporter genes, thereby defining those nucleotides that are crucial for the packaging of each (−)RNA segment into a virion[44]. However, how these sequence elements are recognized in the context of the RNP is unclear. One possibility is that some regions of the (−)RNA termini lack NP, enabling them to adopt local secondary or tertiary structures and to mediate RNA–RNA interactions.

It was originally proposed that the packaging efficiency for influenza A viruses was very high and that most nascent virions contained a full complement of all eight RNPs[44,45]. This

theory was supported by structural analysis of individual virions using thin-section electron microscopy and electron tomography[43,52,54]. However, influenza A virions can be engineered in the laboratory to contain more or fewer than eight genome segments, which suggests that some level of inefficiency is tolerated[55,56]. Furthermore, additional studies have demonstrated that when cells are infected at a low multiplicity, most fail to express at least one of the viral proteins[57], providing evidence for a model of influenza A virus packaging that is less than perfect. This result suggests that the gene encoding the protein that failed to be expressed was defective or missing altogether in these semi-infectious particles. Moreover, the efficiency of segment packaging was found to vary between virus strains and to be influenced by mutations in specific viral proteins[57,58]. Finally, semi-infectious particles are estimated to outnumber complete particles by 6/1 (REF. 58), and they readily participate in reassortment events during co-infection with complete particles[59]. Thus, further studies aimed at elucidating the effect of semi-infectious particles on the long-term evolution of influenza A viruses are warranted.

Reoviridae

The *Reoviridae* family of segmented dsRNA viruses includes several clinically and economically important human, animal and plant pathogens, such as rotaviruses, bluetongue viruses and rice dwarf viruses[1,4,60]. Rotaviruses are well-studied members of the *Reoviridae* family because they cause life-threatening gastroenteritis in infants and young children. Before the worldwide introduction of two vaccines in 2006, strains of the rotavirus A species were estimated to have killed ~450,000 children each year[1,61]. Strains of rotavirus A also infect numerous mammalian and avian species, including pigs, cows, horses, rabbits, cats, dogs, mice and birds, which are reservoirs for viral evolution. Ongoing epidemiological surveillance data also show that strains from the divergent species rotavirus B and rotavirus C are important causes of morbidity and mortality in pigs and cows[62–65], and that they may be underappreciated causes of disease in humans[66–69].

The rotavirus A virion is a non-enveloped, triple-layered particle that encloses a dsRNA genome of 11 segments[70]. The viral genome segments range in size from 0.5–3.3 kb, and the genome as a whole totals 23.0 kb (FIG. 2e). The segments are each organized as a central ORF flanked by 5′ and 3′ UTRs. In general, each gene is monocistronic, encoding a single viral protein. An additional ORF has been described in one segment for some rotavirus strains[71], enabling the expression of up to 12 proteins in total. The assortment and packaging process of rotaviruses is very poorly understood because the field lacks both *in vitro* packaging assays and efficient reverse genetics methods. Nevertheless, the available data suggest that this process shares aspects of both φ6 and influenza A virus assortment and packaging[72] (FIG. 2f). For example, as for φ6, the dsRNA genome segments of rotavirus A are transcribed by viral polymerases into (+)RNA molecules, which are the form of the genome that is assorted and packaged into nascent virion particles[73–76]. However, unlike the φ6 genome, the rotavirus A (+)RNAs are not inserted one by one into a pre-formed core. Instead, it is hypothesized that rotavirus A genome assortment occurs in a manner that is similar to influenza A virus genome assortment. In particular, it is thought that the 11 distinct (+)RNAs engage each other through *cis*-acting RNA elements to form a supramolecular complex that is encapsidated by the core shell protein during early virion

assembly (FIG. 2f). The 5′ and 3′ UTR sequences differ for each of the 11 (+)RNAs, but these sequences are highly conserved among homologous gene segments from different strains of rotavirus A. The segment-specific packaging signals for rotavirus A (+)RNAs are predicted to reside within these 5′ and 3′ termini. *In silico* analyses of nucleotide sequences from strains of rotavirus A have identified several putative RNA structural elements in these terminal regions that may represent assortment signals[77,78]. For strains of the bluetongue virus and mammalian orthoreovirus species, two other *Reoviridae* family members, some packaging signals have been identified with the help of *in vitro* assembly and reverse genetics; they involve the 5′ and 3′ UTRs, and include some coding sequences[79–83]. Therefore, the location of the packaging signals for rotavirus A strains and other *Reoviridae* family members may be similar to the location of the influenza A virus signals (FIG. 2f). During or immediately following their packaging into a core assembly intermediate, rotavirus A (+)RNAs are converted into dsRNAs by viral polymerases[70]. The nascent core assembly intermediate then undergoes additional morphogenesis to become an infectious triple-layered, non-enveloped particle.

The efficiency of genome packaging is poorly understood for the *Reoviridae* family, members of which have 9, 10, 11 or 12 dsRNA genome segments. The observation that no family members have 13 or more segments may be a reflection of the packaging process and the icosahedral capsid architecture. Specifically, each of the 12 fivefold axes of the inner core shell is predicted to have space for only one dedicated polymerase complex and one associated genome segment[84]. However, it is unclear why some *Reoviridae* family members package fewer than 12 segments, thereby leaving one or more fivefold vertices unoccupied. For example, strains of rotavirus A have 11 genome segments, which are present in equimolar amounts at the population level. Moreover, variants of rotavirus A with partially duplicated genome segments and/or foreign sequence insertions have been isolated or engineered[85–87]. This suggests that these viruses can accommodate extra nucleic acid and, theoretically, that they may be able to package additional segments. That being said, there have been no reports of strains of rotavirus A that contain an extra copy of a genome segment, nor have there been reports of variants that lack one or more genome segments. This particular observation is intriguing, given that some strains do not express the accessory protein NSP1 owing to spontaneous deletions or mutations in the NSP1-coding genome segment[88,89]. For these strains, the defective NSP1-coding genome segment is still efficiently packaged into nascent virions, which suggests that the (+)RNA molecule itself is crucial for viral replication, perhaps during assortment. Therefore, we hypothesize that rotaviruses and other members of the *Reoviridae* family use an all-or-none packaging mechanism similar to that of φ6. More specifically, we predict that the full complement of (+) RNA segments must be incorporated into a core assembly intermediate for genome replication to take place. In this model, each packaged (+)RNA would have a dedicated polymerase that acts only on the specific associated segment but that functions in concert with the ten other polymerases to simultaneously replicate the dsRNA genome. Future investigations into the details of rotavirus assortment, packaging and genome replication are warranted, but such investigations may require the development of robust *in vitro* assays.

Generation of reassortants during co-infection

Given the exquisite selective packaging mechanisms for *Cystoviridae*, *Orthomyxoviridae* and *Reoviridae* family members, it is no surprise that successful reassortment between two parental strains during co-infection requires a high degree of genetic compatibility. More specifically, the capacity of one parental strain to package the genome segment of another requires the maintenance of intricate RNA–RNA and/or protein–RNA interactions. Indeed, there has been no description of reassortment occurring between segmented RNA viruses that belong to different families (for example, an *Orthomyxoviridae* member and a *Reoviridae* member); these viruses are simply too divergent to participate in genetic exchange. Even for more closely related viruses within the same genus, subtle differences in viral RNAs and proteins can temper the efficiency with which reassortants are generated during co-infection. It is likely that molecular failures at the level of segment assortment and packaging are a major reason for why the frequency of reassortants is lower than expected following experimental co-infections in the laboratory setting.

For influenza viruses, the compatibility of packaging signals in the form of conserved RNA–RNA interactions is a primary determinant that dictates the reassortment potential for any two co-infecting parental strains[44] (FIG. 3a). A remarkable example of this was provided by the demonstration that the reassortment restriction between influenza A viruses and influenza B viruses can be overcome, at least for the genome segment encoding haemagglutinin (HA), simply by using reverse genetics to swap the packaging signals[90]. However, it is important to note that studying the molecular determinants of reassortment restriction using reverse genetics does not fully recapitulate restrictions during co-infection because such studies do not take into account the important role of competition among homologous segments. In support of this idea, it was shown that although reverse genetics can create all possible reassortants between an avian and a human influenza A virus strain, such hybrid progeny are not readily produced during co-infection[91]. The reason for this discrepancy is related to the fact that the human influenza A virus RNAs interact suboptimally with those from avian strains (FIG. 3a). In other words, low-affinity interactions between the non-cognate RNAs (that is, those that are derived from different parental viruses) are readily outcompeted by the optimal, higher-affinity interactions between cognate RNAs (that is, those that are derived from the same parental virus). In addition to influencing the overall frequency of reassortants for influenza A viruses, subtle differences in RNA–RNA interactions during assortment and packaging also affect the constellation of genome segments in any resulting hybrid progeny. In fact, it has long been observed that some segments are preferentially packaged together such that the genotypes of influenza A virus reassortants are not random. For example, the segment-specific RNA–RNA interactions that occur during assortment have been shown to differ from strain to strain, suggesting that only reassortants that co-package interacting segments would maintain the supramolecular network and produce viable progeny[49]. Furthermore, in the absence of segment mismatch, influenza A viruses reassort with high frequency, demonstrating that there are few extrinsic barriers to exchange[92].

The genetic limitations on the capacity to create reassortants during co-infection may be less stringent for the *Cystoviridae* family than for the *Orthomyxoviridae* family. In fact, isolates

from the *Cystoviridae* family with a high level of sequence divergence are able to reassort with φ6 in the laboratory setting and in nature[26,93]. However, *in vitro* packaging assays suggest that there may be some direct restrictions to genetic exchange. For example, it was shown that *Pseudomonas* phage φ13 (hereafter referred to as φ13) can efficiently package the φ6 M (+)RNA segment, even though this segment carries a packaging signal very divergent from the φ13 packaging signal[94]. By contrast, φ6 was incapable of packaging the M (+)RNA segment of φ13 unless the φ6 packaging sequences were appended to the molecule[94]. This result for these members of the *Cystoviridae* family is similar to the reports for influenza A viruses and suggests that even if genetic exchange can occur, not all combinations of genome segments are tolerated, and restrictions to reassortment may be strain specific.

Similar to the restriction on reassortment between influenza A viruses and influenza B viruses, strains of rotavirus A are incapable of reassorting with strains of other rotavirus species following experimental co-infection of cells or animals. However, there seem to be restrictions that prevent successful genetic exchange even in strains of rotavirus A, which are closely related, as the frequency of reassortants in a given population of progeny is usually much lower than the frequency predicted based on chance alone[95]. Similar observations have been made for other members of the *Reoviridae* family, including mammalian orthoreoviruses[96,97] and bluetongue viruses[98]. However, for all members of the family *Reoviridae*, it remains to be tested whether reassortants are simply not generated during co-infections, or whether they are generated but do not emerge in the population because they are less fit than their parental strains (see below).

An interesting aspect of the replication cycles of members of the *Reoviridae* and *Cystoviridae* families, and a factor that may influence the generation of hybrid progeny during co-infection, is that genome replication (that is, dsRNA synthesis) occurs following segment assortment and packaging. Thus, for a reassortant progeny to be generated, the viral polymerase of one strain must be capable of replicating the packaged (+)RNAs of a different parental strain. For rotaviruses, the polymerase recognizes the (+)RNA template by a sequence-specific interaction with seven nucleotides that are located at the 3′ end of the molecule[99]. Rotavirus A, rotavirus C, rotavirus D and rotavirus F strains have a similar seven-nucleotide sequence (UGUGACC or UGUGGCU), which differs substantially from that of rotavirus B, rotavirus G and rotavirus H strains (AAAACCC, AAGACCC or UAUACCC)[100]. Therefore, the polymerases of rotavirus A, C, D and F strains would not be able to efficiently bind to and replicate the (+)RNA templates of rotavirus B, G and H strains, and vice versa (FIG. 3b). Similar strain-determined template specificities were found for the polymerases of *Cystoviridae* members φ6, φ13 and *Pseudomonas* phage φ8 (REF. 101). In light of this, suboptimal protein–RNA interactions during assortment, packaging and replication are expected to influence the generation of reassortants for viruses in the *Cystoviridae* and *Reoviridae* families.

Emergence of reassortants in nature

The increased capacity for whole-genome sequencing has facilitated new approaches that have revealed the importance of reassortment in the emergence of viruses with novel

phenotypes (FIG. 4), including those that are associated with outbreaks. Large-scale comparative genomics studies of *Cystoviridae*, *Orthomyxoviridae* and *Reoviridae* family members in various hosts have detected numerous reassortants in viral populations[26,102–114]. For example, reassortment can lead to the creation of more-fit variants that outcompete previously circulating strains, and such cases are extremely well documented for influenza A viruses. Several influenza A viruses endemic in swine or birds have been successfully transmitted to humans, and in many cases, reassortment has been instrumental in the major evolutionary transition that is required for this transmission to humans. This is illustrated by the 1957 'Asian' and 1968 'Hong Kong' pandemics, which were both associated with reassortant viruses comprising both human and avian virus genome segments. Similarly, the 2009 pandemic resulted from a reassortment event between highly divergent North American swine viruses and Eurasian swine viruses. In all three pandemics, the reassortment event resulted in novel human viruses that carried divergent genes encoding HA and neuraminidase (NA) derived from the animal viruses; these human viruses express HA and NA antigens that are not well recognized by human adaptive immune responses (FIG. 4a). Reassortment among co-circulating human strains of the same HA–NA subtype is also important for the evolution and emergence of seasonal strains of influenza A virus[115], including those that are antigenically novel[106], those with enhanced transmissibility[105] and those that are resistant to antiviral drugs[116].

Although reassortment can provide fitness advantages to the progeny virus if that progeny acquires a beneficial allele, reassortment can alternatively confer fitness costs if it uncouples a set of alleles that operate best when kept together (FIG. 4b). For example, reassortment has the potential to unlink RNAs or their encoded proteins that interact functionally during the viral replication cycle. As a consequence, a reassortant might exhibit suboptimal RNA–RNA, protein–RNA and/or protein–protein interactions during its *de novo* replication cycle, thereby making it less able to propagate and spread (that is, less fit) than the non-reassortant parental strains. The observation that reassortment can lead to attenuated viruses with reduced replicative fitness in this way has fostered the development of vaccine strains for influenza A viruses and rotavirus A (BOX 1).

Box 1

Reassortant viruses as live-attenuated vaccine strains

The capacity of influenza viruses and rotaviruses to generate functional new variants through reassortment has been harnessed to produce highly effective vaccines that stimulate immune responses without causing disease. The vaccines contain reassortants generated in the laboratory that combine the immunogenic surface proteins from field strains within the genetic backbones of specific laboratory-adapted 'master donor' strains that exhibit desired properties, such as high titre growth or attenuation. For example, in the Unites States, the seasonal influenza immunization programme is anchored by two types of vaccines, an inactivated influenza vaccine (IIV) and a live-attenuated influenza vaccine (LAIV; called FluMist)[128,129]. Both vaccines are quadrivalent formulations that consist of two strains of influenza A virus (H3N2 and H1N1) and two strains of influenza B virus (Yamagata and Victoria lineages). During vaccine production, 6/2 reassortants are

generated; these contain the 6 internal genome segments from the laboratory-adapted master donor strain and the 2 haemagglutinin (HA)-encoding and neuraminidase (NA)-encoding gene segments from the field isolates. These vaccines are modified bi-annually on the basis of genetic and antigenic analyses of the dominant circulating global strains. However, the extensive lead time that is required to produce and evaluate candidate vaccine strains occasionally results in mismatches between vaccine strains and field strains, which results in reduced vaccine effectiveness. In the future, the use of reverse genetics to directly engineer reassortant vaccine candidates may shorten this lead time and reduce mismatches. Moreover, the directed introduction of growth-restricting mutations into field isolates through reverse genetics may bypass the need to create reassortants and could enable the rapid production of new live-attenuated vaccines that more closely match circulating strains.

For human strains of rotavirus A, two live-attenuated vaccines are widely used globally: the monovalent Rotarix vaccine (GlaxoSmithKline) and the pentavalent RotaTeq vaccine (Merck). The RotaTeq vaccine is composed of five bovine–human reassortant strains (10/1 or 9/2) that contain 9 or 10 internal bovine rotavirus genes (from strain WC3), the human virus VP7 coding genes with rotavirus G1, G2, G3 and G4 genotype specificities, and the human virus VP4 coding gene with a strain P[8] genotype specificity[130]. The attenuated phenotype that is conferred by the reassortant gene constellation of the RotaTeq vaccine strains enables them to induce intestinal mucosal immunity without causing disease. Interestingly, although rotaviruses and influenza A viruses are both segmented viruses that use reassortment to advance their evolution and diversity, the pace of antigenic change among circulating influenza viruses has necessitated frequent adjustments of the IIV and LAIV vaccine formulations, whereas the rotavirus reassortant vaccine has remained effective for nearly 10 years without change[61,128].

Importantly, in some cases, the failure to detect reassortants following experimental co-infection might reflect the poor fitness of hybrid progeny caused by mismatched alleles, rather than restrictions on the actual generation of the reassortant during co-infection. For example, for influenza A viruses, uncoupling of the three polymerase-coding genes (*PA*, *PB1* and *PB2*) by reassortment can lead to the formation of viruses with a diminished capacity for RNA synthesis[117–119]. Essentially, the polymerase proteins of some non-cognate strains are not able to effectively interact to form a functional enzyme complex (proteins of human viruses do not interact with proteins of avian viruses, for instance). Similar observations have been made for rotavirus replicase complex proteins, whereby the subunits of rotavirus A and rotavirus C strains cannot functionally substitute for each other[120–122]. Furthermore, comparative genomics studies also support the notion that inter-segmental RNA or protein co-adaptation tempers reassortment among co-circulating strains. For example, a mutual information-based algorithm was used to define amino acid residues that co-varied in multi-sequence alignments of proteins from rotavirus A strains[112]. The data revealed a vast network of interconnected amino acids in various viral proteins, some of which are not known to physically interact with each other. Thus, reassortment may also be limited by the selective constraints that are placed on functionally co-adapted, albeit non-interacting, proteins. However, it is also important to mention that less-fit reassortants with

mismatched-allele constellations can acquire corrective mutations that restore interaction interfaces between non-cognate (that is, not co-adapted) proteins (FIG. 4c). In fact, it has been shown that low-fitness influenza A virus reassortants can accumulate fitness-restoring mutations in functionally interacting proteins if the reassortants are serially passaged in the laboratory[117,123–126]. There is also increasing evidence to support the notion that reassortment events cause a temporary increase in the rate of amino acid changes for influenza A viruses as the viral proteins adapt to a new genetic environment[127]. To date, there have been no studies that address the role of co-adaptive changes influencing RNA–RNA interactions and, in turn, reassortment for segmented RNA viruses, but this remains an important area for future investigation.

These observations for influenza A viruses and strains of rotavirus A regarding allele combinations are in contrast to those for φ6 and members of the *Cystoviridae* family in general. As mentioned above, even members of the *Cystoviridae* family with a high level of sequence divergence were found to undergo frequent reassortment in nature[26]. It is interesting to speculate that perhaps members of the *Cystoviridae* family are not subjected to the same purifying selection pressures that are imposed on influenza A viruses and strains of rotavirus A following reassortment, maybe simply because of the way the genes are organized within the segments. In particular, the genes that encode interacting proteins of φ6 are usually located on the same segment (for example, all procapsid proteins are encoded by the L segment); therefore, reassortment would not uncouple functionally interacting alleles. Thus, φ6 represents an ideal experimental system for further investigation of the genetic linkages among genome segments for members of the *Cystoviridae* family and the effect of those linkages on the frequency of reassortment.

Outlook

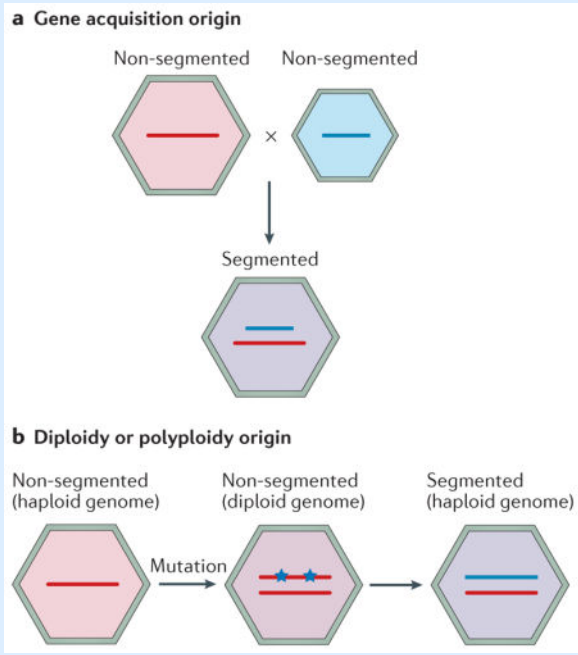
In summary, segmented RNA viruses include some of the most important human, animal and plant pathogens in recent history. The biological mechanism that originally produced these viruses from non-segmented precursors remains unknown (BOX 2), but one of the most apparent consequences of this genome structure is the generation of novel reassortant progeny during co-infection. Reverse genetics and other experimental advances have increased our ability to investigate the molecular constraints on reassortment under controlled experimental conditions. Moreover, recent advances in genome-sequencing technologies have furthered our understanding of segmented RNA virus diversity and have shed light on the frequency of reassortants in natural populations. Importantly, for influenza A viruses and rotaviruses, these discoveries have shown how reassortment contributes to viral pathogenic and zoonotic potentials, and have enabled the generation of live-attenuated reassortant vaccines. However, some key outstanding questions remain unanswered and should be the focus of future research endeavours. How do influenza viruses and rotaviruses selectively package their genome segments? What are the relative contributions of failed RNA–RNA, protein–RNA and protein–protein interactions to reassortment restriction between any given strains? Are there virus-extrinsic barriers to reassortment within the infected host or within the environment? What are the biological and temporal dynamics that are required to achieve robust reassortment? What is the contribution of semi-infectious particles or defective-interfering particles to viral replication, reassortment and evolution?

Do some individual genome segments evolve biased packaging so that they are over-represented in the reassortant progeny (that is, are there ‘selfish genes’)? The answers to these questions are expected not only to inform disease prevention and control strategies, but also to shed light on our basic understanding of organismal evolution.

Box 2

Possible origins of RNA virus genome segmentation

It is unknown how an ancestral non-segmented RNA virus underwent genome segmentation in the first place, but different theories have been proposed to explain the origin of segmented RNA viruses. One theory posits that genome segmentation may have arisen following the accidental merging of RNA genomes from two different viruses (see the figure, part **a**). This theory is supported by a recent analysis of Jingmen tick virus (a taxonomically unclassified segmented RNA virus), as two of the four positive-sense RNA ((+)RNA) genome segments are genetically related to those of flaviviruses, whereas the other two are completely unique, which suggests that they were acquired independently from a still-unidentified parental ancestor[131]. It is possible that the acquisition of a novel RNA virus genome provided the Jingmen tick virus ancestor an evolutionary advantage over parental strains that lacked such extra genome segments. Alternatively, genome segmentation could have arisen as a downstream consequence of diploidy or polyploidy, whereby the precursor non-segmented RNA virus may have randomly packaged more than one copy of its genome into a nascent virion (see the figure, part **b**). Diploidy and polyploidy are argued to be evolutionarily advantageous in complex organisms because they buffer against the effects of deleterious mutations. Accumulation of mutations over time may have enabled the ‘duplicate’ genome to encode new proteins, evolving into a new genome segment[132]. Indeed, diploidy and polyploidy have been documented for measles viruses and Ebola viruses, which normally have single-stranded negative-sense RNA ((−)RNA) genomes[133–135]. Moreover, diploidy is a hallmark of the RNA genome structure of several retroviruses, including HIV. As diploidy and polyploidy require that there are few restrictions on the amount of nucleic acid that can be packaged into a virus particle, such genomes may have evolved more easily for enveloped viruses than for non-enveloped viruses with stringent capsid sizes.



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Glossary

Segmented RNA viruses
Viruses in which the genome consists of more than one RNA molecule (that is, segments). The genome segments can be packaged within a single virion particle or into separate particles

Type species
A representative viral strain that is studied to understand the biology of an entire viral genus or family

Reassortment
A process of genetic exchange whereby two or more parental viruses co-infect a single host cell and exchange genome segments. The outcome is the formation of hybrid viral progeny with genome segments derived from multiple parental strains

Assortment
The mechanism by which a segmented virus packages one of each genome segment into a virion particle

Viral fitness

The capacity of an individual virus to generate infectious progeny, relative to other virus genotypes in the population

Pathovars
Bacterial strains with the same or similar characteristics

***In vitro* packaging system**
A simplified experimental system in which viral genome segments are incorporated into a virion particle; this occurs in a test tube and outside the context of an infected host cell

Defective-interfering RNAs
Spontaneously generated mutant RNA molecules that usually contain large gene deletions but maintain sequences that are crucial for their replication and packaging. These RNAs reduce the fitness of full-length viruses during cellular co-infection

HA–NA subtype
A binomial system of classification for influenza A viruses that is based on the neutralizing antibody response to the virion structural proteins haemagglutinin (HA) and neuraminidase (NA)

Diploidy or polyploidy, In virology
when an individual virus encapsidates two (diploidy) or more (polyploidy) copies of the genome into a single virus particle

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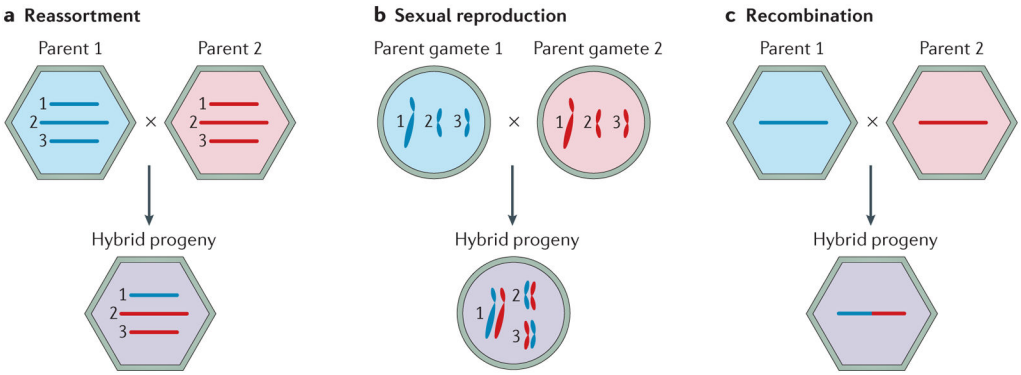

Figure 1. Reassortment, sexual reproduction and recombination

a | Reassortment in non-multipartite RNA viruses. Two virus particles are shown, each with a full complement of three viral genome segments. Following reassortment, hybrid progeny can be formed that contain segments derived from both parents. **b** | Sexual reproduction. Two parent gamete cells are shown, each with a haploid genome of three chromosomal segments. Following sex between the two parents, a hybrid diploid progeny is produced that contains one copy of each chromosome from each parent. **c** | Recombination in non-segmented, single-stranded RNA viruses. Following recombination between two virus particles, chimeric genomes are produced that have regions derived from each parent.

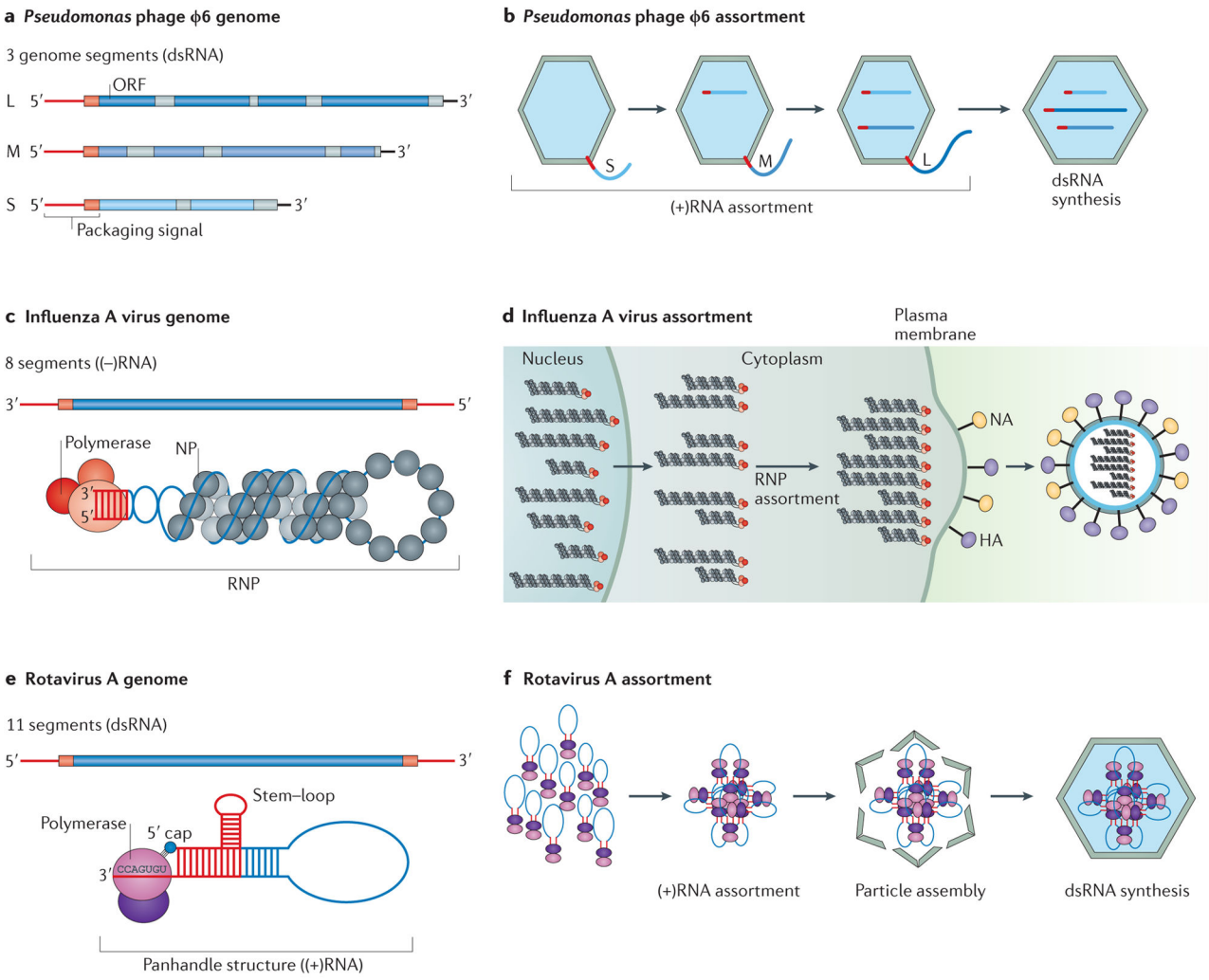

Figure 2. *Pseudomonas* phage φ6, influenza A virus and rotavirus genome organization and assortment

a | The *Pseudomonas* phage φ6 genome consists of three double-stranded RNA (dsRNA) segments: small (S), medium (M) and large (L). Blue indicates ORFs, and grey represents intergenic regions; lines at the 5′ and 3′ termini represent UTRs. Sequences that are known to be important for the selective packaging of φ6 single-stranded positive-sense RNA ((+)RNA) replication intermediates are shown in red. **b** | A model of φ6 genome segment assortment and packaging. φ6 (+)RNAs are packaged sequentially. Initially, the procapsid has a binding site only for the S (+)RNA segment, enabling it to be inserted. Following the packaging of the S segment, a binding site for the M (+)RNA segment is revealed in the procapsid, enabling that segment to be inserted. Finally, a binding site for the L (+)RNA segment is revealed, the segment is inserted, and the entire complement of φ6 (+)RNAs are encapsidated. Following packaging of all three (+)RNA segments, the procapsid core expands, which triggers the conversion of the three (+)RNAs into double-stranded RNA (dsRNA) genome segments by viral polymerases. **c** | The influenza A virus genome comprises eight negative-sense RNA ((−)RNA) segments. A representative segment is shown as a linear (−)RNA molecule (top) and as a ribonucleoprotein (RNP; bottom), in

which the (−)RNA is bound by a heterotrimeric polymerase complex and nucleocapsid protein (NP). The ORF, UTRs and sequences that are important for selective genome packaging are coloured as in part **a. d** | A model of genome segment assortment and packaging in influenza A viruses. Eight influenza A virus RNPs are synthesized in the nucleus and individually exported into the cytosol, where they pair up with each other. While en route to the plasma membrane, the eight RNPs form a supramolecular complex that is encapsidated by a lipid envelope during budding to form the virion. **e** | The genome of rotavirus A is composed of 11 dsRNA segments, one of which is shown as a (+)RNA precursor in linear form (top) and folded into a putative panhandle shape (bottom). The ORF, UTRs and sequences that are important for selective packaging are coloured as in part **a** and part **b**. A polymerase–capping enzyme complex is thought to be bound to the 3′-terminal UGUGACC sequence. A putative stem–loop structure may act as an assortment and/or packaging signal. **f** | A model of genome segment assortment and packaging in rotaviruses. The 11 (+)RNAs, each with a bound polymerase–capping enzyme complex, are thought to pair up and eventually form a supramolecular complex that is encapsidated by a forming virion particle. During or immediately after encapsidation, the (+)RNAs are converted into dsRNA genome segments by their dedicated polymerase. The polymerases function while tethered to the viral capsid (not shown).

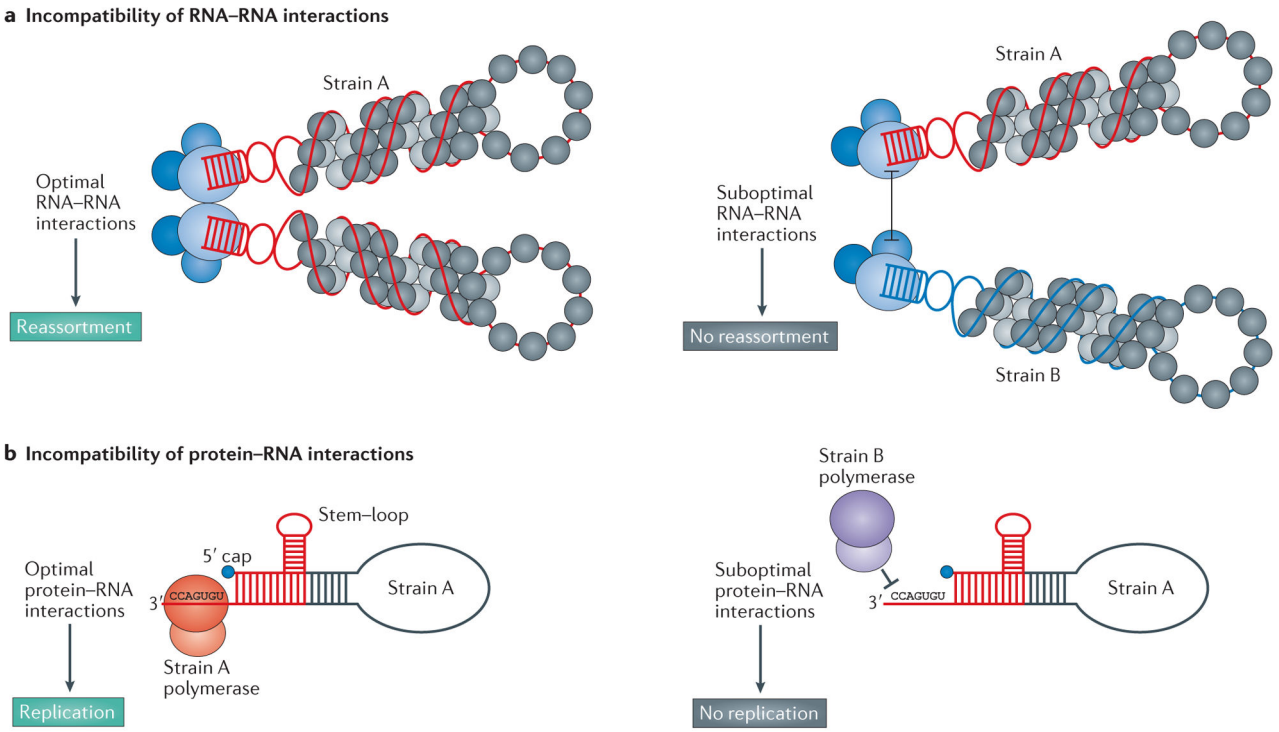

Figure 3. Direct restrictions on the generation of reassortants

a | Incompatibility of RNA–RNA interactions. Two influenza A virus genome segments are shown as ribonucleoproteins (RNPs), each derived from a parent strain (strain A is shown in red and strain B is shown in blue). If the packaging signals are compatible (left), the RNA molecules can interact, which leads to co-packaging and reassortment. However, if the packaging signals are not compatible, the RNA molecules will interact suboptimally, thereby preventing their co-packaging. **b** | Incompatibility of protein–RNA interactions. A rotavirus A positive-sense RNA ((+)RNA) molecule from one strain may be recognized only by the polymerase from that same strain. If the polymerase in the virion is from a different strain and is unable to recognize the (+)RNA molecule, replication does not occur, thus restricting the generation of reassortants.

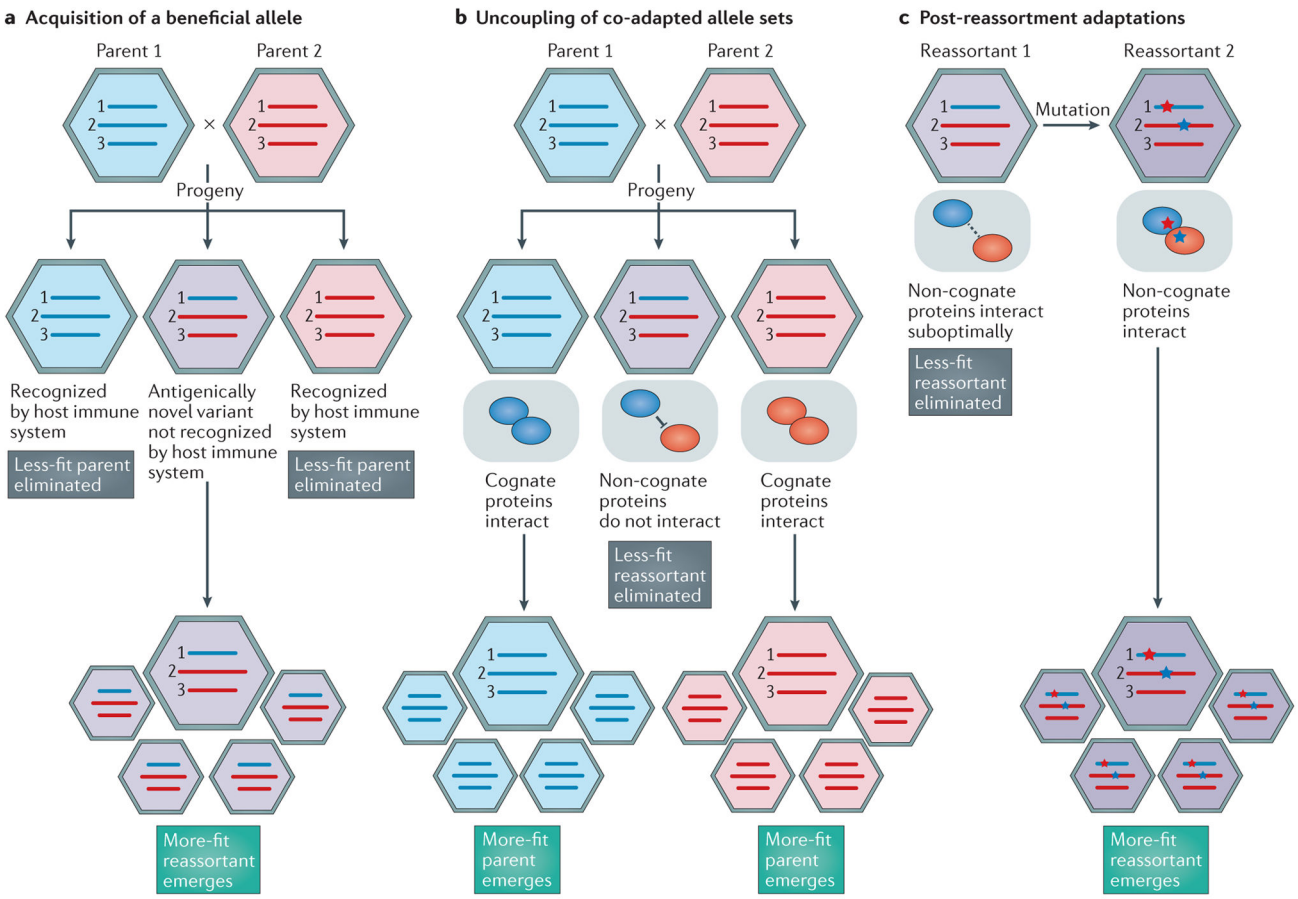

Figure 4. Fitness consequences of reassortment

a | Increase in viral fitness. Following reassortment, hybrid progeny can be formed that contain segments derived from both parents. In some cases, the new allelic combination confers phenotypic changes to the reassortant. For example, reassortment can produce an antigenically novel variant that is not recognized by the host immune system. This more-fit reassortant emerges in the host, whereas the less-fit parental strains are eliminated. **b** | Decrease in viral fitness. In some cases, reassortment can uncouple essential cognate protein sets that interact optimally when kept together. If non-cognate proteins do not interact, the reassortant would be less fit than parental strains and would therefore be eliminated from the population. **c** | Post-reassortment adaptations. A less-fit reassortant can accumulate mutations that restore the interaction interface between the non-cognate proteins. Such post-reassortment adaptive changes will enable the reassortant to regain fitness and emerge.

Table 1

Segmented RNA virus families: genome organization, type species and hosts

Family	Genome organization	Packaging	Type species of genera within family	Hosts
Arenaviridae	2 (−)RNA or ambisense * molecules	Single virion	Lymphocytic choriomeningitis mammarenavirus	Animals
Birnaviridae	2 dsRNA molecules	Single virion	Infectious bursal disease virus	Animals
Bromoviridae	3 (+)RNA molecules	Multipartite	Brome mosaic virus	Plants
Bunyaviridae	3 (−)RNA or ambisense molecules	Single virion	Rift Valley fever virus, Tomato spotted wilt virus	Animals and plants
Chrysoviridae	4 dsRNA molecules	Multipartite	Penicillium chrysogenum virus	Fungi
Closteroviridae	2 (+)RNA molecules	Multipartite	Lettuce infectious yellows virus	Plants
Cystoviridae‡	3 dsRNA molecules	Single virion	*Pseudomonas* phage φ6, *Pseudomonas* phage φ10, *Pseudomonas* phage φ13	Bacteria
Orthomyxoviridae‡	6–8 (−)RNA molecules	Single virion	Influenza A virus, Influenza B virus	Animals
Partitiviridae	2 dsRNA molecules	Multipartite	White clover cryptic virus 1	Plants, fungi and protozoa
Picobirnaviridae	2 dsRNA molecules	Multipartite	Human picobirnavirus	Animals
Reoviridae‡	8–12 dsRNA molecules	Single virion	Rotavirus A, Bluetongue virus	Animals and plants

dsRNA, double-stranded RNA; (+)RNA: positive-sense RNA; (−)RNA, negative-sense RNA.

*Ambisense refers to an RNA molecule that is positive-sense in some regions and negative-sense in other regions.

‡This Review focuses on these three families.