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Defective viral genomes are key drivers of the virus–host interaction

Marco Vignuzzi[1] and Carolina B. López[2*]

Viruses survive often harsh host environments, yet we know little about the strategies they utilize to adapt and subsist given their limited genomic resources. We are beginning to appreciate the surprising versatility of viral genomes and how replication-competent and -defective virus variants can provide means for adaptation, immune escape and virus perpetuation. This Review summarizes current knowledge of the types of defective viral genomes generated during the replication of RNA viruses and the functions that they carry out. We highlight the universality and diversity of defective viral genomes during infections and discuss their predicted role in maintaining a fit virus population, their impact on human and animal health, and their potential to be harnessed as antiviral tools.

Viruses are remarkably resilient microorganisms able to adapt and survive in the complex host physiological and immune environment. Accumulating evidence demonstrates that viruses can generate modified forms of their genome during replication as tools to adapt to environmental challenges. While the standard genome encodes all the viral proteins required for sustaining viral replication, viral variants contain random mutations that can serve to enhance the ability of the virus to adapt to new conditions[1]. In addition, defective viral genomes (DVGs) and an expanding family of sub-viral particles (Box 1) that result from either small mutations or drastic truncations and modifications of the viral genome render the virus unable to complete a full replication cycle in the absence of a helper full-length virus to complement the functions lost.

DVGs were identified by Preben Von Magnus in the late 1940s as incomplete influenza viruses able to interfere with the replication of the wild-type virus[2]. More than two decades after their discovery, Alice Huang and David Baltimore coined the term defective interfering (DI) particles, or DIPs, to define viral particles that contain normal structural proteins but only a part of the viral genome. In addition, they stipulated that DIPs can only replicate in the presence of helper virus and that they interfere with the intracellular replication of non-defective homologous virus[3]. Huang and Baltimore theorized that DIPs play a critical role in determining viral pathogenesis. Follow-up studies using viruses grown to contain either a large content of DIPs, or depleted of them, revealed that DIPs reduced virulence in vivo[4,5], induced high levels of interferon (IFN) during infections in vitro[6–8], and promoted viral persistence in vitro[9–16] and in vivo[17,18]. However, despite their ubiquitous presence and important functions, the lack of appropriate technology to identify DIPs in infections in vivo led to a widespread belief that DIPs and their DVGs were largely a product of in vitro virus replication and were not relevant in natural viral infections[19,20]. By the late 1990s, the study of DVGs had slowed down drastically and was limited to their use as tools for studying viral replication or as potential antivirals.

Today, DVGs have been described in most RNA viruses (Table 1) and technological advances have contributed to establishing their role as *de facto* danger signals for triggering of antiviral immunity in many infections. In addition, we are beginning to appreciate their impact on the clinical outcome of natural infections and on the evolution of viruses. Moreover, we are witnessing rapid advancements in the understanding of the molecular mechanisms that regulate the generation of DVGs and those explaining their paradoxical roles in promoting antiviral immunity and viral persistence.

Here, we review evidence accumulated over more than half a century of observations on DVG generation and activity during RNA virus infections. We highlight recent advances that illustrate their critical impact on viral dynamics and evolution during both acute and long-term virus–host interactions. See Box 2 for a glossary of relevant terms.

Classes, types, structures and diversity

Different types of DVGs are defined by the type of genomic alterations present. Next generation sequencing (NGS) has revealed a large variety of DVG species present in some infections, and recent studies have begun to elucidate the distinct functions of these different DVGs.

Point mutations, hypermutations and frame shifts. While the first DVGs to be identified and distinguished from the wild-type full-length viral genome lacked large parts of the genome[21–25], there are a number of DVG types that do not involve drastic genomic alterations. Point mutations in RNA viruses can result in detrimental alterations because of the highly constrained nature of their genome organization. Indeed, a majority of randomly introduced mutations are either lethal or confer a significant fitness cost, as observed for vesicular stomatitis virus (VSV)[26], poliovirus[27] and influenza virus[28]. While it is inherently understood that detrimental mutations give rise to defective genomes, such genome types have historically not been considered DVGs. A genome harbouring a detrimental mutation in a structural protein could replicate, but not assemble properly; while a detrimental mutation in the replicase would yield a genome that can produce proper structural and assembly proteins, but could not replicate. Either of these genomes could potentially hijack the lacking functions from (and thus, interfere with) a full-length co-infecting virus. Indeed, studies where mutation rates are increased to perturb a viral population from viability to non-viability, revealed that the defective RNAs that appear prior to population

[1]Institut Pasteur, Viral Populations and Pathogenesis Unit, Centre National de la Recherche Scientifique UMR 3569, Paris, France. [2]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA. *e-mail: lopezca@vet.upenn.edu

Box 1 | The expanding family of sub-viral particles

In addition to DVGs, a growing number of viral particle variants and sub-viral agents have been discovered in viruses of plants, arthropods and mammals. These include viroids, satellite viruses, virophages and viral-like extracellular vesicles (VLVs). The definition of these entities seems, at times, blurry, due to their largely intersecting properties. In general, sub-viral agents, similar to DVGs, depend on complementation with the standard virus to replicate and spread. However, differences in their requirements for complementation, target virus and sequence identity with their helper virus are used to sub-classify them. Here, we provide current definitions of these sub-viral entities and highlight those aspects that differentiate them from DIPs and their DVGs.

Satellite viruses were originally described in plant viruses as linear or circular RNAs (200–1,800 nt long) that require a helper virus to propagate but are unrelated in sequence to the helper virus. Satellite viruses are generally dispensable for the replication of the helper virus, with some exceptions[192,193]. Satellite viruses differ from satellite RNAs in that they encode a protein that packages the satellite RNA into virions. Satellite RNAs can interfere with the replication of its helper virus and either attenuate or exacerbate disease. An example of a satellite virus is the satellite tobacco necrosis virus[194]. This positive-sense, single-stranded RNA satellite virus suppresses the replication of its helper virus and ameliorates tobacco necrosis virus symptoms[195].

Viroids differ from satellite viruses in that they do not encode any protein, do not require helper virus for replication, and are not encapsidated. Viroids have a circular RNA genome (200–400 nt long) that is highly complementary and structured, and are adapted to carry out their complete life cycle as a result from interactions with the host cell machinery[196,197].

Virophages are 15–30 kbp long dsDNA viruses that normally produce icosahedral particles and parasitize from giant dsDNA viruses (mimiviruses and others) for propagation. Virophages infect the giant virus factory, thereby harming the giant virus[198]. The first virophage discovered, Sputnik, was found together with Acanthamoeba castellanii mamavirus and interferes with its propagation[199]. Several other virophages, as well as a large number of virophage candidates, have been identified since then[195,198,200]. Recent studies show that virophages could resemble *bona fide* DNA viruses[201] and their reclassification as their own viral family has been proposed[195].

VLVs are produced during viral infection and, in contrast to other extracellular vesicles, contain viral proteins and nucleic acids but lack capsid protein or viral genomes and, therefore, are not infectious. VLVs have been described in both RNA and DNA viruses, including herpes simplex virus-1, hepatitis C virus and Kaposi's sarcoma-associated herpes virus. VLVs play functional roles during viral infections by facilitating communication among cells and enhancing viral infection[202–204].

extinction interfere with replication of a wild-type viral genome[29,30]. Hypermutations and mutations leading to frame shifts also result in defective viruses[31] (Fig. 1a). One example is mutations introduced by the cellular protein APOBEC3G into pro-retroviruses[32]. APOBEC3G deaminates deoxycytidine nucleotides in the viral DNA, leading to hypermutations that interfere with the ability of the viral genome to integrate into the host genome and replicate. Interestingly, in opposition to an interfering activity for these mutated pro-viruses, it has been proposed that defective proviruses enhance fitness and that complementation among them drives viral persistence and pathogenesis[33,34].

Deletions. The genomic viral species most commonly referred to as DVGs are truncated viral genomes resulting from large internal deletions occurring during viral replication. Deletion DVGs tend to bear single large truncations that remove several or all essential genes required for self-propagation while retaining 5′ and 3′ ends as well as other RNA structural elements required for polymerase binding, replication and/or packaging[35–37]. Multiple variants also exist, including DVGs that can be described as 'mosaic' in that they appear to be the result of multiple recombination and rearrangement events, including deletions, insertions, duplications and even inversions of parts of the genome[38–40] (Fig. 1b).

Copy-backs. Copy-back and snap-back DVGs are rearranged genomes in which a sequence is duplicated in reverse complement to create theoretical stem-like structures (panhandle structures for copy-back DVGs or hairpin structures for snap-back DVGs)[41–43]. Copy-back DVGs have been reported in many negative-sense (ns) RNA viruses and are generated from the 5′ end of the genome in a process that produces DVGs with complementary 3′ and 5′ termini[44,45]. If the complementary region of the DVG comprises almost the entire sequence, the DVG can be further characterized as a snap-back DVG[43] (Fig. 1c). Copy-back DVGs are predicted to be generated when the viral RNA-dependent RNA polymerase (RdRP) detaches from the template and reattaches to the nascent strand, copying back the end of the genome[42,46].

DVG diversity during infection. Historically, it was thought that only certain viruses generated DVGs and that only one or a few distinct DVGs existed for any given virus. Most studies originally relied on high multiplicity of infection (MOI) passaging conditions that favoured the emergence of larger deletions (and thus, smaller DVGs) that could rapidly outcompete the longer full-length genomes owing to reduced replication times. Furthermore, these works relied on methods with low sensitivity of detection (such as visualization and purification on agarose gels) that would only isolate the most abundant DVGs. However, evidence for populations of distinct species of DVGs in a single infection has been reported since the 1980s[45,47]. We now know the diversity of DVGs to be much larger than initially appreciated and that certain DVGs are better than others at reaching high abundance, likely by retaining certain properties that confer replication advantage such as packaging signals and replication elements, or by acquiring the ability to interfere with the replication of other variants. NGS has revealed that DVGs are present in virtually any and every virus population, and that the diversity of DVGs is immense; however, the relative abundance of any given deletion or rearrangement varies, likely indicating that a variety of factors drive DVG generation and accumulation. The use of single-cell sequencing technology will allow precise quantification of the frequency and diversity of DVGs in an infected cell and will provide data on the distribution of DVG variants among a cell population.

Since most NGS alignment tools filter out reads with more than two mismatches, reads corresponding to deletion breakpoints or rearrangement junctions are rejected from a typical virus genome alignment. A re-analysis of rejected reads would identify these DVG hallmarks. With growing interest in this neglected part of the viral population, informatics tools such as ViReMa[48], DI-tector[49] and VODKA[50] are emerging. These tools re-examine reads that potentially harbour jumbled or rearranged viral sequences and have enabled a broader appreciation of DVG diversity. A current challenge with NGS data is to determine how best to differentiate between true DVGs and background error of NGS, how to quantify the relative abundance of any given DVG with respect to full-length

Table 1 | RNA virus families with described DVGs

Virus	Type of DVG	Known DVG functions	References
psRNA viruses			
Arteriviridae			
Porcine reproductive and respiratory syndrome virus	Deletion	UN	134
Equine arteritis virus	Deletion	Interference	40
Closteroviridae			
Citrus tristeza virus	Deletion	UN	135,136
Coronaviridae			
Berne virus	Deletion	Interference	137
Bovine coronavirus	Deletion	UN	138
Infectious bronchitis virus	Deletion	UN	139
Mouse hepatitis virus	Deletion	Interference/persistence	140,141
Transmissible gastroenteritis virus	Deletion	Interference	142
Flaviviridae			
Dengue virus	Deletion	Persistence	75, 143,144
Japanese encephalitis virus		Persistence	15,145
Hepatitis C virus	Deletion	Persistence	146,147
Murray Valley encephalitis virus	Deletion	Persistence	148
Tick-borne encephalitis virus	Deletion	Modulate virulence	149
West Nile virus	Deletion	Interference/persistence	82,150
Nepoviridae			
Tomato black ring virus	Deletion	Interference	151
Nodaviridae			
Flock House virus	Deletion	Modulate virulence	48
Picornaviridae			
Encephalomyocarditis virus	Deletion	Interference	152
Foot-and-mouth disease virus	Deletion	Interference	153
Mengo virus	Deletion	Interference	154
Polio virus	Deletion	Modulate virulence	24, 35, 124,155
Togaviridae			
Rubella virus		Persistence	156
Semliki Forest virus	Deletion	Interference/modulate virulence	4, 93, 106, 157,158
Sindbis virus	Deletion	Interference/IFN-induction	6, 63,159
Tombusviridae			
Cucumber necrosis virus	Deletion	Modulate virulence	160
Tomato bushy stunt virus	Deletion	Interference/modulate virulence	39
Turnip crinkle virus	Deletion	Interference/modulate virulence	161
nsRNA viruses			
Arenaviridae			
Lymphocytic choriomeningitis mammarenavirus	Deletion	Interference/modulate virulence/persistence	18, 162,163
Filoviridae			
Ebola virus	Deletion /copy-back	Persistence	74
Orthomyxoviridae			
Influenza virus	Deletion	Interference/IFN-induction/persistence/modulate virulence	2, 5, 10, 37, 51, 62, 97,164–170
Paramyxoviridae			
Human parainfluenza virus 3	Deletion /copy-back	Interference	112,171
Parainfluenza virus 5	Deletion/copy-back	IFN-induction	57
Measles virus	Deletion/copy-back	Interference/IFN-induction/persistence/modulate virulence	76, 90, 96, 100, 123, 172,173

Continued

Table 1 | RNA virus families with described DVGs (Continued)

Virus	Type of DVG	Known DVG functions	References
Mumps virus		Persistence/modulate virulence	9, 115,174
Sendai virus	Deletion/copy-back	Interference/IFN-induction/immune stimulation/ persistence/modulate virulence	7, 14, 23, 45, 54, 87–89,92, 104, 175,176
Peribunyaviridae			
Bunyamwera virus	Deletion	Interference	177
Phenuiviridae			
Rift Valley Fever virus (RVFV)	Deletion	Interference/modulate virulence	19
Toscana virus	Deletion	Interference	178
Pneumoviridae			
Human metapneumovirus	Copy-back	IFN-induction	52
Human respiratory syncytial virus	Deletion/copy-back	Interference/IFN-induction/persistence	55, 78,179
Rhabdoviridae			
Vesicular stomatitis virus	Deletion/copy-back	Interference/IFN-induction/persistence/modulate virulence	3, 8, 16, 21, 38, 42, 46, 80, 109,180–185
Rabies virus	Deletion	Interference/persistence	11,79
Tospoviridae			
Tomato spotted wilt virus	Deletion	Modulate virulence	186
dsRNA viruses			
Birnaviridae			
Infectious necrotic pancreatic virus	UN	Persistence	12
Partitiviridae			
Rosellinia necatrix virus	Deletion	Interference	187
Reoviridae			
Type 3 reovirus	Deletion	Interference	188
Wound tumor virus	Deletion	UN	189
Retroviruses			
Retroviridae			
Human immunodeficiency virus 1	Deletion/ hypermutation/frame shift	Persistence	34, 190,191

UN, unknown.

virus, and how best to normalize between samples and between sequencing runs—problems that are similar to transcriptome analysis of genetic isoforms. Furthermore, with increasing data sets and samples, it is becoming evident that DVG species are not necessarily the same if generated in different host and cell types, and the factors dictating these differences remain to be uncovered.

Mechanisms of DVG generation

DVGs have been long considered the result of stochastic mistakes introduced by the viral RNA polymerase that lacks proofreading activity. However, new evidence suggests that additional factors control DVG generation, opening the possibility of manipulating their generation for therapeutic purposes.

Random products or encoded in the viral genome? While DVGs are generated by many viruses, the molecular mechanisms that govern their generation are poorly understood. A predominant theory is that DVGs arise from random errors that occur during viral replication at high viral titers due to the combination of a lack of proofreading activity of the viral polymerase and the presence of lower fidelity variants that favour the generation of deletions. In support, analyses using deep sequencing approaches revealed that multiple species of DVGs are generated during infection. For example, in infections with Flock house virus, a positive-sense (ps)RNA virus, ClickSeq and nanopore sequencing identified a large and seemingly random population of deletion DVGs early after infection[48]. In addition, sequencing analysis of nasopharyngeal samples from influenza virus-infected humans or infections in vitro with human metapneumovirus or measles virus (MeV) revealed multiple DVG species in these infections[51–53]. However, different from deletion and point mutation DVGs, copy-back DVGs are frequently found in discrete dominant populations in an infected cell or tissue, and the same copy-back DVG seems to arise in independent infections with the same parental virus[54,55] or during infections with different virus strains[56]. The demonstration of hotspots for the generation of copy-back DVGs from respiratory syncytial virus (RSV) and the identification of specific nucleotides that determine where copy-back DVGs rejoin further demonstrate that the generation of copy-back DVGs is not completely random, but instead that specific sequences encoded in the viral genome direct or facilitate their formation[50]. In support, deep sequencing approaches have revealed discrete dominant DVG populations in infections with parainfluenza virus 5 and VSV[57,58]. Sequence similarities have been reported in the 5′ and 3′ regions flanking DVG deletion sites during influenza infection[51,59], suggesting that there is also some degree of conservation in the generation of deletion DVGs. These observations indicate that, at least

Box 2 | Glossary

Von Magnus particles. Defective influenza virus particles with the ability to interfere with the replication of homologous virus discovered by Preben Von Magnus in 1947.

DIPs. Defective interfering particles. Viral particles containing a fraction of the viral genome able to replicate only in the presence of helper virus and interfere with the intracellular replication of non-defective homologous virus.

DVGs. Defective viral genomes. Viral genomes with defective ability to replicate in the absence of a co-infecting standard virus. Viral genomes can become defective due to mutations, deletions or a variety of gene rearrangements.

RdRP. RNA-dependent RNA polymerase. Viral enzyme that copies the viral RNA during viral replication.

MOI. Multiplicity of infection. Ratio of infectious virus to target cells.

TIPs. Therapeutic interfering particles. Synthetic DVGs with strong interfering activity proposed as therapeutics to outcompete standard viruses.

in some infections, DVG generation is not a completely stochastic process and, instead, virus-encoded sequences favour the production and/or amplification of predominant DVGs. It remains to be determined whether conservation is a property of certain DVG types and which specific sequences and/or RNA structures lead to DVG generation in these conditions.

Role of viral proteins. A number of viral proteins are implicated in the generation of DVGs, the best studied being the viral RdRP. Engineered viral polymerases with a decreased fidelity and an increased mutation rate produce highly attenuated viruses[60,61] which, in many instances, correlate with enhanced production of DVGs[62–65] (Fig. 2a). Although the mechanism mediating DVG generation by mutant RdRP remains speculative, a few models are emerging. For example, in viruses with low-fidelity RdRP, such as Sindbis virus or tombusvirus, an increased production of DVGs correlates with an enhanced rate of viral RNA recombination[63,65]. In addition, during influenza virus infection, variations in the elongation capacity of the polymerase associate with differential generation of DVGs[64]. A role for the polymerase multimerization activity was also recently proposed as a driver of DVG generation during influenza virus infection[62].

Viral proteins implicated in the regulation of viral transcription and replication are also associated with the generation of DVGs. Mutations in the influenza virus nuclear export protein (NEP, also known as NS2), which regulates the synthesis of complementary RNA, result in enhanced DVG production[66]. Similarly, deletion or mutations of the paramyxovirus C proteins that regulate template switching from antigenomic to genomic replication result in enhanced copy-back DVG production[53,56] (Fig. 2a). Curiously, in infections with influenza virus lacking NEP or paramyxovirus lacking C, DVG production is observed in conditions that normally restrict the generation of DVGs, such as infections at low MOI. It is possible that the enhanced production of copy-back DVGs in these situations is related to higher template availability or to an indirect effect of viral polymerase activity, but the precise mechanism remains to be established. In addition, a single amino acid mutation in the Sendai virus (SeV) nucleoprotein that reduced the density of the ribonucleoprotein associates with DVG generation[67] (Fig. 2b). Lastly, in lymphocytic choriomeningitis mammarenavirus (LCMV) infection, the PPXY domain encoded within the matrix protein drives the production of viral particles containing defective genomes, but not those containing standard genomes[68] (Fig. 2c).

Replication-driven template-switching. RNA recombination is a major driver of deletion DVG formation. A predominant model proposes that sequences at the break point or structural signals in the template RNA promote the replicase to switch to the acceptor RNA and resume synthesis (Fig. 2d). Replicase-driven recombination was proven in biochemical assays using RdRPs of a large number of RNA viruses[69–71]. Variations of this model include the forced template switch mechanism in which the replicase switches template after encountering the 5′ end of the template generating head-to-tail RNA dimers. If the templates are DVGs, these would lead to head-to-tail DVG dimers. The 5′ end could also be modified by endo- or exo-nucleases, leading to new versions of these genomes[72].

RNA editing as a driver of DVG diversity. Editing of viral RNA leads to hypermutations and can result in the generation of DVGs, as reported in persistent measles infections[31]. In addition, a high rate of viral adenine-to-guanine (or uracil-to-cytosine) RNA editing by adenosine deaminase acting on RNA occurs in DVGs from VSV, human metapneumovirus and MeV[38,52,53]. In some cases, RNA editing regulates the immunostimulatory potential of DVGs[53]. However, the impact of DVG editing on viral replication seems to be virus-specific[73]. Whether DVGs have a higher rate of editing than the standard viral genome, as well as the impact of editing generated diversity in DVG evolution and selection, remains to be determined.

Roles in pathogenesis

DVGs have three well-described functions that relate to their role in pathogenesis: interference with standard viral replication, immunostimulation and establishment of viral persistence (Fig. 3a).

Interference with viral replication and viral production. DVGs were discovered during the search for factors responsible for reduced infectivity of influenza virus after passages at high titers[2]. DVGs with the ability to interfere with the replication of their parental virus both in vitro and in vivo are found in most positive and negative-sense (ns)RNA viruses[54,74–82]. DVGs accumulate at higher rates than full-length viral genomes in co-infected cells due to their shortened length and, in the case of copy-back species, their highly efficient flanking trailer promoters[83,84]. A predominant theory for how DVGs interfere with the replication of standard virus is based on the observed competition between defective and full-length viral genomes for viral components needed for replication (Fig. 3b). As DVGs accumulate to high levels, it is predicted that they can directly interfere with helper virus replication by monopolizing the viral polymerase and/or competing for structural proteins[84,85].

It should be noted that while most evidence supports the notion that the shortest DVGs with largest deletions are best able to outcompete full-length virus because their much smaller size can be more rapidly replicated, most of these studies consist of high MOI cell culture conditions which favour competition dynamics based on replication kinetics. The question arises as to whether a longer deletion DVG that retains more coding sequence, yet contains mutated proteins (presumably replicating faster than full-length genomes, yet slower than the shortest DVGs), could be a better competitor of wild-type virus in certain conditions because it additionally expresses defective proteins that, in turn, interfere with wild-type proteins (for example, in multi-component structures such as capsids or replicases).

Furthermore, recent studies have demonstrated that DVGs and full-length viral genomes dominate in different cells during infection,

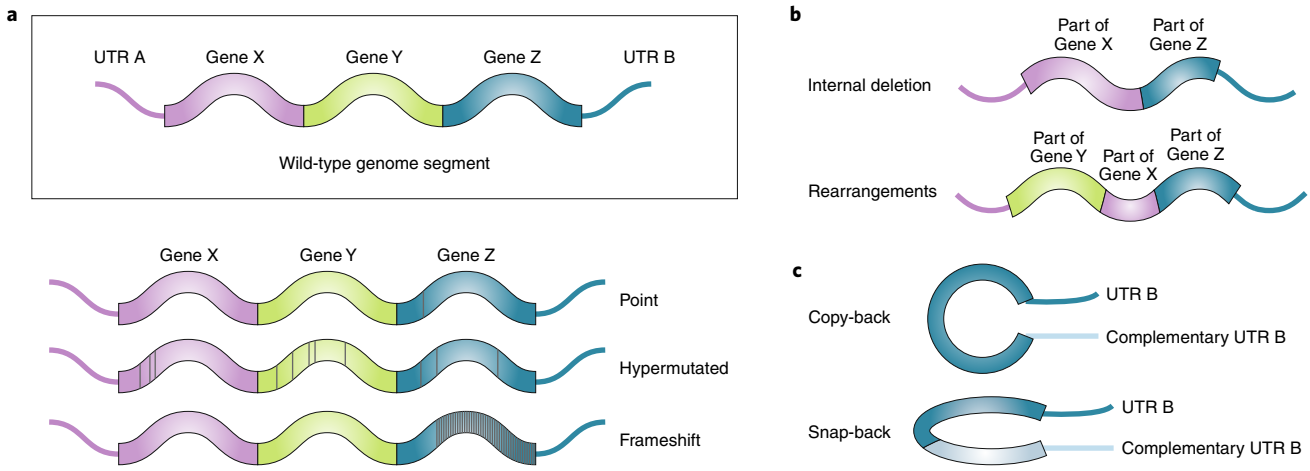

Fig. 1 | Classes of DVGs. a, Mutations in the viral genomes can lead to the generation of DVGs. These mutations can be single point mutations, hypermutations or frameshift mutations that alter virus replication, viral protein expression and/or viral protein function. **b**, Deletion-type DVGs occur when the polymerase skips part of the genome during replication and generates a truncated version of the genome. Deletion DVGs could also involve genomic rearrangements and gene duplication. Deletion generally results in lost or altered expression of one or more genes. **c**, Snap-back and copy-back DVGs are generated in nsRNA viruses when a sequence is duplicated in reverse complement to create theoretical panhandle structures for copy-back DVGs or hairpin structures for snap-back DVGs. Complementary sequence duplication occurs when the polymerase is released from the template strand and reattaches back to the nascent strand, copying back the end of the nascent genome. They can also occur from the polymerase copying back another complementary genome bound in *trans*. Most described snap-back and copy-back DVGs are generated from the 5′ end of the genome with the complementary end containing a duplication of the unstranslated region (UTR). Various degrees of complementation within Gene Z in the illustrated model can be found in copy-back DVGs. These types of genomes are not transcribed but can be replicated by the viral polymerase.

conferring distinct functions to different infected cells[86,87]. While cells dominated with full-length viral genomes are the predominant producers of viral particles containing either full-length or defective genomes, cells enriched in DVGs do not produce many particles of any species[86]. These data highlight the need to consider the single cell versus population level impacts of DVGs during interference.

Triggers of antiviral immunity. DVGs, especially those of the copy-back type, strongly induce the expression of type I and III IFNs, tumour necrosis factor (TNF), interleukin (IL)-6, IL-1β and other pro-inflammatory cytokines, and are the primary stimuli of antiviral immunity in many infections[6–8,52,54,55,88–90] (Fig. 3c). In addition, DVG stimulation optimizes the antigen presentation capacity of antigen-presenting cells[91,92]. Accumulating evidence indicates that the immunostimulatory activity of DVGs is maintained in vivo and during natural infections in humans. Increased survival of infected mice in infections containing DVGs have been reported for multiple viruses[5,19,93]. In mice infected with the respiratory viruses SeV, influenza or RSV, IFNs and pro-inflammatory cytokines are strongly induced only after DVGs have accumulated to detectable levels[54,55]. Detection of DVGs in respiratory secretions of children infected with RSV correlates with expression of antiviral genes[55], and highly pathogenic influenza virus isolates that fail to induce potent antiviral responses in humans have an impaired ability to generate DVGs[62].

Immunostimulatory DVGs can be recognized by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). While TLR signalling is not essential for production of type I IFNs in vitro in response to DVGs, RLR signalling is required[55,88,94–98]. SeV copy-back DVGs are stronger immunostimulators than deletion DVGs[89] and are among the strongest known inducers of the antiviral response. Therefore, much of what we know of DVGs immunostimulatory activity is based on the study of SeV copy-back DVGs. Copy-back DVG RNA binds RIG-I[94,97,99] and DVGs from SeV, MeV and RSV viruses strongly stimulate RIG-I-dependent signalling[55,92,95]. RIG-I is triggered by binding of 5′ di- or triphosphates present on uncapped RNA or short regions of double-stranded (ds)RNA. Phosphatase treatment suppresses the ability of in vitro-transcribed copy-back DVGs to trigger IFN production following transfection supporting the role of RIG-I in DVG sensing[91,94,96]. SeV DVGs can also associate with melanoma differentiation-associated protein 5 (MDA5)[88,94], but the role for MDA5 in sensing other DVGs is less clear[99,100]. Other cellular proteins can also associate with DVGs. For example, MeV DVGs associate with the dsRNA binding protein named protein activator of the interferon-induced protein kinase to optimally activate RIG-I[96].

DVG-induced RLR signalling is not simply a result of higher viral RNA content in the infected cells, as increasing the amount of DVG-deficient virus does not increase the IFN response[54,88,92]. Importantly, efficient sensing of copy-back DVGs occurs even in the presence of virus-encoded antagonists of the cellular sensing pathways[88,92]. These observations suggest that unique features of DVGs favour their detection during infection. The predicted long dsRNA stretch formed by the reverse complementary ends of copy-back DVGs was thought to be a critical factor in their immunostimulatory activity[8,99,101]. However, recent evidence demonstrates the existence of additional features in DVGs that have a larger impact on their immunostimulatory potential. Structural modelling identified a 44 nucleotide (nt)-long stem-loop motif ($DVG_{70–114}$) in a SeV DVG-546, a well-characterized and potent immunostimulatory copy-back DVG, which was absent in the SeV genome and spans the unique junction formed between the genomic break and rejoin points that form this copy-back DVG. Deletion of $DVG_{70–114}$ reduces the DVG immunostimulatory activity, while introduction of the motif into an immunologically inert RNA improves its ability to induce the expression of type I IFNs and IFN-stimulated genes[94]. $DVG_{70–114}$ acts in concert with the 5′-triphosphate motif to activate RIG-I and allows for enhanced RLR polymerization, a marker of activation[94]. $DVG_{70–114}$ is active in the context of SeV infection, as recombinant viruses carrying DVGs that lack this motif significantly

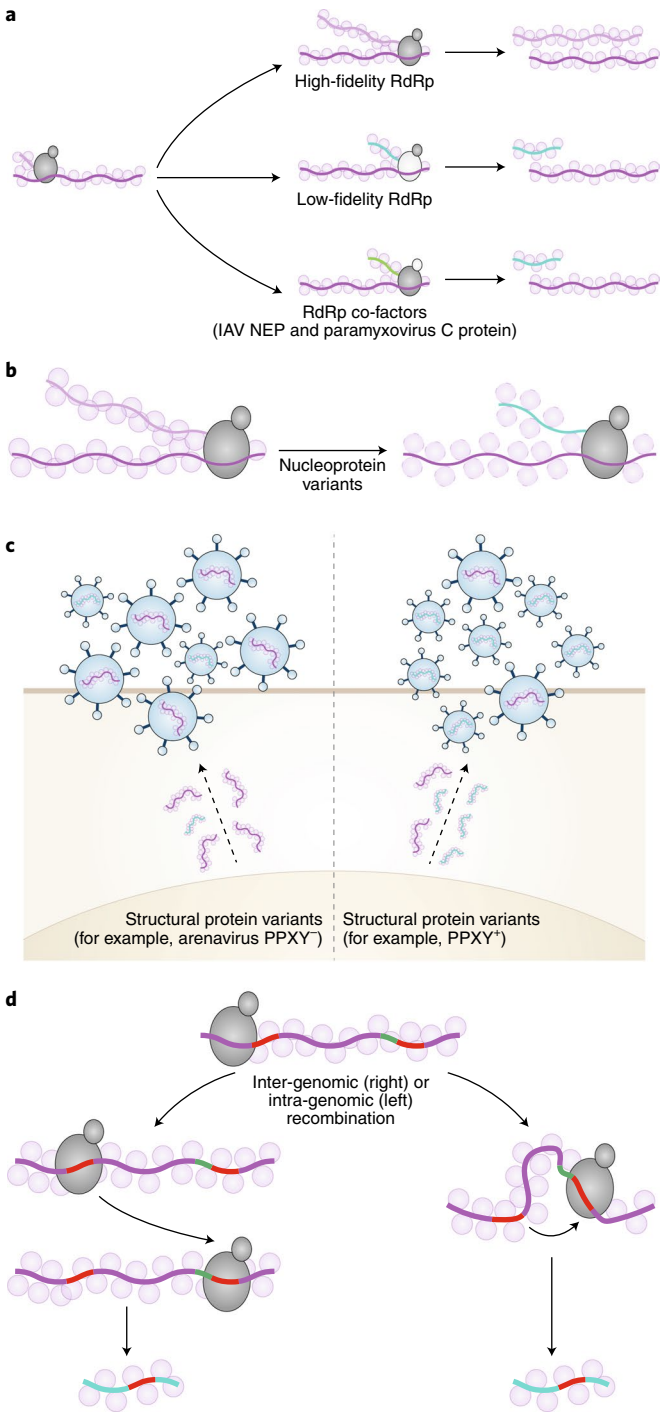

Fig. 2 | Mechanisms of DVG generation. a, Variations in the viral RdRp fidelity due to mutations or effects of virus-encoded co-factors, such as the influenza A virus (IAV) NEP or the paramyxovirus C protein, can favour the generation of DVGs. **b**, Variants of the nucleoprotein with altered binding to viral RNA can promote DVG generation. **c**, Variants of structural proteins, such as the PPXY domain in the matrix protein of arenaviruses, can lead to DVG generation. **d**, Inter- and intra-recombination events using homologous sequences (red) can lead to the formation of deletion DVGs.

lose immunostimulatory potential[94]. Failure to detect dsRNA through immunostaining during SeV infection[102] and normal immunostimulatory activity following disruption of the complementarity between the 5′ and 3′ ends of copy-back SeV DVGs[94] further support the notion that the predicted long complementary ends of copy-back DVGs have a minor role in the onset of anti-viral immunity. It remains to be determined when and how $DVG_{70\text{-}114}$ is exposed during infection, and whether similar motifs are present in other highly immunostimulatory DVGs.

DVG immunostimulation is also an important factor in the modulation of infections in insects. Similar to the RIG-I and MDA5 PRRs in mammals, insects sense viral RNA through the protein Dicer-2. This protein processes the viral RNA into small interfering RNAs that protect insects from re-infection with the same virus[103]. DVGs are the primary targets for Dicer-2 (ref. [103]). These observations indicate that the immunostimulatory activity of DVGs is widespread and may have a significant impact on the spread of pathogens within and across species.

Facilitators of RNA virus persistence. DIPs facilitate the establishment of persistently infected cell cultures in a variety of RNA virus infections, including influenza, SeV, Ebola, mumps and Semliki Forest virus[10,13–15,74,104–107]. Such in vitro evidence is accompanied by studies in mice showing that infection with Semliki Forest virus or LCMV containing DIPs establish persistent infections[18,108], as well as one study reporting DVGs in the brain of human patients who had died due to post-MeV subacute sclerosing panencephalitis[17].

DIPs and full-length viruses cycle asynchronously in many persistent infections in vitro[109,110] and in vivo[19,109]. Cycling occurs in a predictable pattern and has been mathematically modelled using variations of the predator–prey model[111]. A theory to explain the asynchronous cycling of full-length viral genomes and DVGs during persistency was put forward in 1970 (ref. [3]). This theory, which is based on the interference effect of DVGs on the replication of the full-length genome, proposed the following: DIPs accumulate during virus replication until they reach high concentrations and become predominant. In this condition, DVGs interfere with the replication of the full-length viral genome by competing for essential replication machinery, driving a reduction in the full-length virus. During this process, some previously uninfected cells are infected by standard virus and re-initiate the cycle. Interestingly, in some persistent infections the amount of DIPs appears constant[112]. What drives these cyclic patterns in some viruses but not others, and whether host factors such as the infected cell type influences the cycling pattern, remain unknown.

Recent evidence indicates that the mechanisms involved in the establishment of persistence are more complex than simple intracellular competition for the replication machinery among different types of viral genomes[87]. Using RNA in situ fluorescent hybridization, Xu et al. showed that during infection with SeV or RSV containing DIPs, there is heterogeneity in the content of viral genomes in the infected population[87]. While some cells are enriched in DVGs, others are enriched in standard full-length viral genomes. The mechanisms for this heterogeneity are currently unknown, however, striking functional differences among these cell populations are beginning to emerge[86,87]. Cells enriched in DVGs engage the RLR sensing pathway and produce IFNs and other pro-inflammatory molecules, including TNF. In addition, these cells induce a pro-survival program, also dependent on signalling through the RLR pathway. These programs protect DVG-high cells from TNF-mediated death, while cells lacking DVGs die during infection. Surviving DVG-high cells can be propagated for months as a persistent infection. This mechanism provides an explanation for the paradoxical stimulation of both antiviral immunity and establishment of persistence by DVGs. It remains unclear how the enhanced survival of DVG-high cells leads to persistence, and how this survival fits into the cycling of DVG and standard virus observed in many infections. Cycling may occur at the intracellular level (where each infected cell goes through cycles of standard viral genome or DVG enrichment driven by competition and interference with the viral replication machinery) and/or at the population level, where

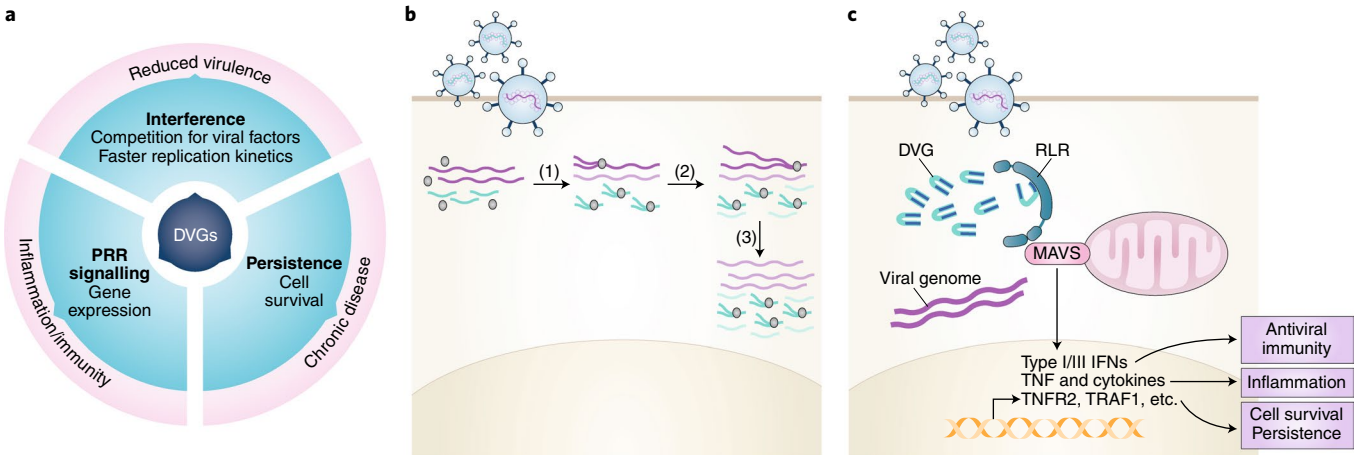

Fig. 3 | Functions and modes of actions of DVGs. a, Overview of the known effects of DVGs on the standard virus and host cells, as well as their impact on viral pathogenesis. **b**, Proposed mechanism of competition for viral products in cells containing several copies of standard virus and DVGs, resulting in 'interference'. (1) The viral polymerase replicates DVGs more efficiently than standard virus due to their shorter length and flanking trailer promoters. (2) These DVG properties lead to faster accumulation of DVGs in the infected cell. (3) DVGs eventually outcompete standard virus to become the predominant species and interfere with standard virus replication. **c**, Proposed mechanism for immunostimulation and cell survival induced by copy-back DVGs. Infected cells first detect DVGs through the RNA sensors RIG-I or MDA5, which signal through the adaptor protein MAVS for the production and secretion of type I and III IFNs pro-inflammatory cytokines and pro-survival proteins.

individual infected cells will determine the composition of the pool of standard virus or DIPs available for infection of new cells.

Use as antivirals and vaccine adjuvants

The strong interfering and immunostimulatory activities of DVGs make them attractive candidates for vaccine adjuvants and antivirals[113,114]. The ability of DIPs to interfere with the replication of standard viruses and diminish virus-associated disease has been extensively demonstrated in mice during infection with virus stocks containing DIPs[4,54,75,77,115], even when the vaccine contained standard virus inactivated by ultraviolet treatment[116]. A similar protection has been reported in ferrets vaccinated with an influenza vaccine containing only DIPs[117]. Synthetically engineered DIPs with strong interfering potential, or 'therapeutic interfering particles (TIPs)', have been more recently proposed as a possible strategy to control viral infections. TIPs would theoretically replicate faster than the wild-type virus and therefore outcompete the virus hindering spread and transmission. TIPs would have the advantage of being active only in organisms already infected with the wild-type virus due to their dependence on a helper virus to replicate[118,119]. Although still in the exploratory phase, it remains to be determined how TIPs would impact virus persistence, the generation of adaptive mutations and the generation of new infectious viruses by complementation.

Whether protection elicited by natural or synthetic DIPs is due to direct interference with the standard virus replication or through strong immunostimulatory ability of DVGs is unclear. SeV copy-back DVGs augment the antigen presentation capacity of mouse and human dendritic cells, resulting in enhanced activation of T cells[91]. In addition, experimental vaccines against influenza virus and RSV adjuvanted with in vitro-transcribed SeV DVGs delivered subcutaneously, intramuscularly or intranasally show improved antibody production and increased protection from virus challenge[91,120]. SeV DVG-derived oligonucleotides (DDOs) containing the immunostimulatory motif $DVG_{70–114}$ are effective adjuvants able to bias the humoral and cellular responses against inactivated virus and protein vaccines towards type I immune responses including antibodies of the IgG2a/c isotypes, Th1 $CD4^+$ T cells and cytotoxic $CD8^+$ T cells in mice[91,121]. DDOs also synergize with the emulsion antibody AddaVax and enhance its type I immunity-driving potential by mechanisms that depends on type I IFNs[121]. Notably, a DIP influenza vaccine conferred protection to an unrelated virus through stimulation of type I IFN production[122], suggesting that they can be used as prophylactic or therapeutic antivirals.

DVGs are present in live attenuated vaccines against polio, measles and influenza viruses[76,123–126]. However, their impact in the development of protective immunity and vaccine efficacy has not been formally assessed. Based on their interfering and immunostimulatory ability, it is speculated that DVGs may enhance the efficiency of the vaccine while enhancing safety of the virus by reducing its replication and spread. If correct, it would be important to carefully regulate the amount of DVGs in vaccine preparations to avoid complete interference and drastic reduction of the virus to a point of ineffectiveness.

Impact on viral evolution and dynamics

While recent NGS data reveal that hundreds of DVGs can arise within a single viral infection, the fact that a smaller subset of dominant DVGs are repeatedly detected in different samples[50] indicates that complex dynamics are at play within the viral population. These complexities include competition (and possibly compensation or cooperation) between different DVGs and positive selection of the best competitors that implicates their relative fitness in relation to the wild-type parental virus and other DVGs. Parameters such as replication fitness, packaging, immunoregulation and other traits determine the virus dynamics. It is important to stress that most events leading to DVG formation, including mutations, deletions, recombination and translocations, are either non-viable or deleterious to the virus. In addition, although hundreds or even thousands of different DVGs are generated during a virus infection, the vast majority of these will be lost during the population bottlenecks that occur in vivo, for example when crossing anatomical barriers or during transmission from host to host[127]. However, there are instances where these genomes could make it through bottlenecks, such as during infections of hosts that are immunosuppressed or have comorbidities where founding populations are increased. In addition, infections may occur with virions that have co-packaged wild-type genomes and DVGs[128] or virions may aggregate during infection enhancing co-transmission, as seen for VSV[129] and poliovirus[130]. Current

mathematical models have generally only considered one dominant DVG[131,132]; further development is required to incorporate the potential cooperation and competition among others.

While historically DVGs have been considered replication waste, an artifact of cell culture passaging conditions or simply a nuisance to laboratory experimentation, a renewed interest in this part of the viral population may reveal a more functional or biological relevance to their existence. Do DVGs exist for a reason? Why keep waste lying around? Given the notoriously high recombination and reassortment rates of some viruses, it is possible that for viruses that undergo recombination, DVGs can provide a repertoire of mutations that could feed back into the viable virus population to promote adaptation. It remains to be seen whether DVGs can provide a selective advantage to the virus, and whether the high MOI or localized co-infection conditions are biologically relevant outside of cell culture.

Recent work in insects suggests that DVGs, which are a preferred template for the generation of the viral DNA form of RNA viruses, provide additional substrate to help boost the RNA interference response that is responsible for viral persistence in insects[103]. Indeed, it was shown that a change in the amount of viral DNA generated during RNA virus infection in Drosophila, altered the persistence and kinetics of a wild-type RNA virus infection. The authors suggested that evolution has perhaps fine-tuned the production of DVGs to balance wild-type infection and promote persistence (and ultimately, transmission of viruses, including arboviruses in mosquitoes).

Additionally, a closer examination of DVG dynamics within viral populations can help better understand the biology of standard viruses. Indeed, in addition to distinguishing between the dispensable and indispensable nucleotide sequences, proteins and RNA structures, we can identify what viral components can operate in *cis* or in *trans*. A recent study of HCV DVGs, for example, identified novel *cis*-acting RNA structures that were required for replication and packaging[133].

Concluding remarks

Emerging technologies that allow the identification of defective and standard viruses in natural infections, as well as those allowing the establishment of specific associations of DVGs with functional outcomes, have been crucial in reviving the interest in studying DVGs. Recent work has provided new appreciation for DVG diversity and the potentially critical role of DVGs in defining the clinical outcome of infections; however, a number of questions remain unanswered: what are the molecular mechanisms driving the generation of DVGs? Can DVGs be harnessed for the control of virus pathogenesis and spread? How do alterations in the DVG population impact virus evolution and adaptation to new hosts? How do host factors impact DVG accumulation and activity? Further technological developments and interdisciplinary research will be required to obtain these answers.

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Competing interests

The authors declare no competing interests.

Additional information

Correspondence should be addressed to C.B.L.