

Quasispecies Dynamics of RNA Viruses

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Abstract RNA viruses, such as human immunodeficiency virus, hepatitis C virus, influenza virus, and poliovirus replicate with very high mutation rates and exhibit very high genetic diversity. The extremely high genetic diversity of RNA virus populations originates that they replicate as complex mutant spectra known as viral quasispecies. The quasispecies dynamics of RNA viruses are closely related to viral pathogenesis and disease, and antiviral treatment strategies. Over the past several decades, the quasispecies concept has been expanded to provide an adequate framework to explain complex behavior of RNA virus populations. Recently, the quasispecies concept has been used to study other complex biological systems, such as tumor cells, bacteria, and prions. Here, we focus on some questions regarding viral and theoretical quasispecies concepts, as well as more practical aspects connected to pathogenesis and resistance to antiviral treatments. A better knowledge of virus diversification and evolution may be critical in preventing and treating the spread of pathogenic viruses.

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1 Introduction

RNA viruses are important pathogens of humans, animals, and plants. This group of viruses exhibits rapid evolution and high variability, which have important implications for the control and spread of viral diseases. The high mutation rates of RNA viruses allow them to escape host defenses and therapeutic interventions with antivirals or vaccines. These highly mutable entities can also quickly adapt to new environments and ecological changes, as evidenced by the emergence and reemergence of viral infections from animal reservoirs, including human immunodeficiency virus (HIV), SARS, influenza, West Nile fever, Ebola, and dengue fever, among others.

RNA viruses form complex distributions of closely related but nonidentical genomes that are subjected to a continuous process of genetic variation, competition, and selection (Fig. 1). These so-called viral quasispecies have been described in vivo through the analysis of molecular and biological clones isolated from viral populations, and more recently using ultradeep sequencing techniques. The viral quasispecies was first documented with bacteriophage Q β , during replication in its *Escherichia coli* host (Domingo et al. 1978); it was later confirmed for many RNA viruses,

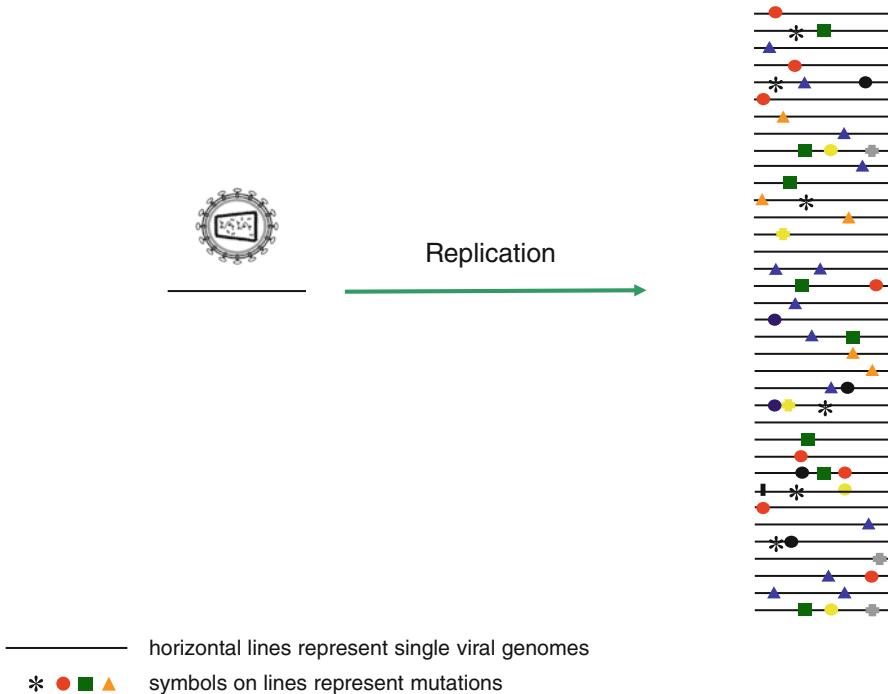


Fig. 1 Schematic representation of a viral quasispecies. Viral genomes are represented as horizontal lines, and mutations as symbols in the lines. Upon infection with an RNA virus—even with a single particle, as depicted here—viral replication leads to a mutant spectrum of related genomes, termed quasispecies

including animal viruses (Sobrino et al. 1983) and important human pathogens such as influenza virus (Lopez-Galindez et al. 1985), HIV type 1 (–1) (Meyerhans et al. 1989), human hepatitis C virus (HCV) (Martell et al. 1992), and poliovirus (Vignuzzi et al. 2006), as well as for plant viruses and viroids (Ambros et al. 1999). The term quasispecies was first used by Eigen and Schuster to theoretically describe the type of population structure proposed to have mediated the self-reproduction, self-organization, and adaptability of primitive replicons during the early stages of the development of life on Earth (Eigen 1971; Eigen and Schuster 1977). They described the self-reproducing entity not as a single molecule but as a “swarm” or “cloud” of variant reproductive molecules with a numerical distribution governed by an equation; Eigen and Schuster referred to this distribution as “quasispecies” (Eigen and Schuster 1977).

Experimental work performed by virologists has shown that the classic genetic concepts of wild-type and mutant may not be applicable to molecular viral elements; in particular, the idea of individuality does not relate to single, replicative RNA molecules, but instead must be applied in terms of a “swarm,” “cloud,” or quasispecies (Fig. 1). Virologists currently use the term quasispecies to refer to distributions of non-identical but related genomes that are subjected to a continuous process of genetic variation, competition, and selection; in this concept, the “swarms” or “clouds” of genomes, rather than individual genomes, function as units of selection (Lauring and Andino 2010; Mas et al. 2010; Ojosnegros et al. 2011; Perales et al. 2010). This means that the evolution of individual viral genomes is decisively influenced by the mutant spectrum surrounding them and that, unavoidably, a group of individuals must be selected. Experimental work has demonstrated that the evolvability of individual viral genomes is constrained by the distribution of its mutational neighbors (Burch and Chao 2000; de la Torre and Holland 1990). Due to their high mutation rates, rapid generation time, and short genomes, RNA viruses are an excellent and simple tool for using experimental virology to explore and challenge population genetics and system biology concepts, including fitness variations (Chao 1990; Holland et al. 1991; Martinez et al. 1991), Muller’s ratchet theory (Chao 1990), the Red Queen hypothesis (Clarke et al. 1994), epistasis (Bonhoeffer et al. 2004; Sanjuan et al. 2004), etc.

In this chapter, we describe how viral quasispecies are generated and how they impact viral evolution, pathogenesis, and treatment. We also show how the quasispecies concept can be extended to other fast-evolving entities, such as cancer cells, bacteria, or prions.

2 Generation of RNA Virus Diversity

Unlike eukaryotic DNA polymerases, RNA viruses lack proofreading activity; thus, the error rate during replication has been estimated at 10^{-4} to 10^{-5} mutations per nucleotide during each cycle (Table 1) (Domingo et al. 2006). If one assumes that 10^9 to 10^{12} viral particles are present at any given time in an acutely infected organism, these must be the product of at least 10^7 to 10^8 replication cycles. Given the length

Table 1 Important parameters that influence variability and adaptability of RNA virus populations

Average number of mutations per genome within the viral population of an infected individual	Generally averages 1–100 (more in some cases) mutations per genome
Mutation rate	Estimated at between 10^{-4} to 10^{-5} mutations per nucleotide per cycle of replication
Genome length	3 to 32 kb
Virus population size and fecundity	Variable, but an acutely infected organism may harbor 10^9 – 10^{12} viral particles at any given time
Mutations needed for a phenotypic change	Many recorded adaptive changes depend on one or a few mutations

of the RNA virus genome (approximately 10,000 nucleotides), it is likely that every possible single point mutation (10^4) and many double mutations will occur by the time the population reaches the size of many natural virus populations. In contrast, the total number of possible single mutations for a mammalian genome is about 10^{10} , well above the population size of mammalian species. In RNA viruses, although specific combinations of multiple mutations may be rare, it is clear that the degree of potential genetic change drives their diversification in response to selective pressures of host immune responses or antiviral therapies (Table 1).

Theoretical work predicts the existence of a limiting value of error or mutation rate—termed the “error threshold”—that must not be surpassed if the wild-type is to be kept stable (Eigen 1971, 2002). It has been suggested that mutation rates for RNA viruses are close to the error threshold, and can be forced into error catastrophe by a moderate increase in mutation rate. Pioneer studies demonstrated that mutagenesis by a variety of chemical mutagens conferred only 1.1 – to 2.8-fold increases in mutation frequencies at defined single base sites in vesicular stomatitis virus and poliovirus (Holland et al. 1990). These results suggested that a high mutation rate is an adaptive trait of RNA viruses and that RNA virus genomes are unable to tolerate many additional mutations without a loss of viability. Studies on HIV-1, lymphocytic choriomeningitis virus, and foot and mouth disease virus have led to similar conclusions (Grande-Perez et al. 2002; Loeb et al. 1999; Sierra et al. 2000). This concept of the error threshold opened a new paradigm for how to fight viruses, not by inhibiting their replication but rather by favoring it with an increased rate of mutation (Fig. 2). Several studies in cell culture and in vivo have supported lethal mutagenesis as a viable antiviral strategy (Lauring and Andino 2010), and a clinical trial was recently reported in which a mutagenic pyrimidine analog was administered to HIV-1 infected patients (Mullins et al. 2011).

In addition to mutations made by viral polymerases, other mechanisms are implicated in the generation of mutant clouds. RNA recombination and reassortment both create genetic diversity in RNA viruses; these processes are mechanistically different, but both require that two or more viruses infect the same host cell. Recombination can occur in all RNA viruses, irrespective of whether their

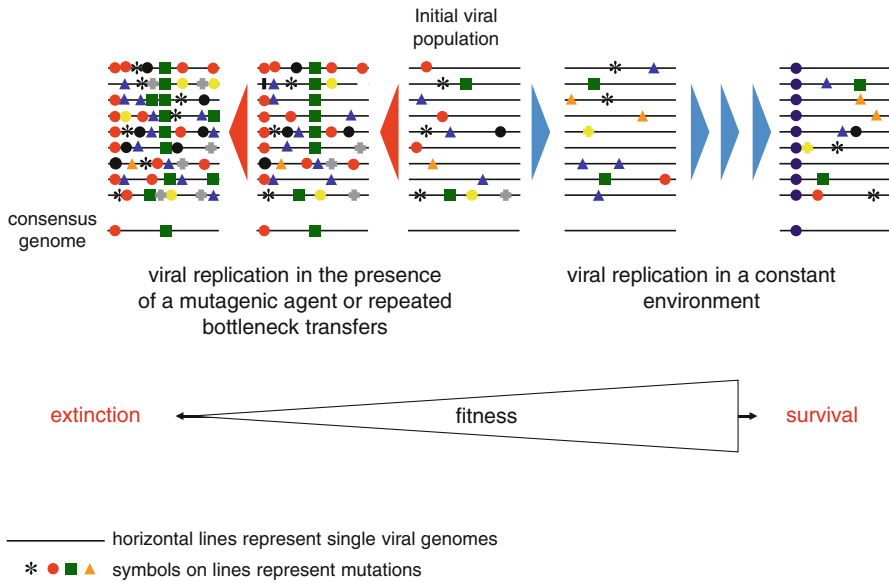


Fig. 2 Effect of elevated mutation rates on viral fitness and survival. A simplified view of quasispecies dynamics and fitness change is shown. Unrestricted replication (*blue arrowhead on the right, with multiple passages indicated by several arrowheads*) results in fitness gain, as depicted by the triangle at the bottom. Fitness gain can occur with or without variation of the consensus sequence. In contrast, replication in the presence of mutagen or repeated bottleneck transfers (*red arrowhead on the left*) results in accumulation of mutations that modify the consensus sequences, and decreased fitness. This figure is based on previously published data (Domingo et al. 2006)

genomes are composed of single or multiple segments. The process corresponds to the formation of chimeric molecules from parental genomes of mixed origin. A widely accepted model of RNA recombination is “copy choice” recombination (Lai 1992a, b), in which the RNA polymerase in RNA viruses (and reverse transcriptase in retroviruses) switches from one RNA molecule to another during synthesis, while remaining bound to the nascent nucleic acid chain, generating an RNA molecule with mixed ancestry. Reassortment is restricted to viruses that possess segmented genomes, and involves packaging of segments with different ancestry into a single virion. An important example of reassortment occurs in the influenza A virus; reassortment of different gene segments encoding influenza envelope or surface proteins, hemagglutinin (HA) and neuraminidase (NA), is associated with evasion of host immunity and sometimes with the occurrence of epidemics (Lindstrom et al. 2004).

RNA recombination and reassortment occur at highly variable frequencies in RNA viruses. The frequency of recombination varies in positive single-stranded RNA viruses, occurring at high levels in some groups, but far less frequently in other families such as the Flaviviridae, most notably HCV (Morel et al. 2011), in which only occasional instances have been reported. Recombination seems to

consistently occur less frequently in negative single-stranded RNA viruses, although some of them can still undergo reassortment (e.g., influenza A virus). Recombination occurs frequently in some retroviruses, most notably HIV.

HIV recombines at exceedingly high rates (Jung et al. 2002), approximately one order of magnitude more frequently than in simple gamma retroviruses, such as murine leukemia virus and spleen necrosis virus. The HIV-1 recombination rate has been precisely calculated to be 1.38×10^{-4} per site and generation (Shriner et al. 2004); therefore, the recombination rate for HIV-1 is approximately five-fold greater than the point substitution rate of 3.4×10^{-5} mutations per bp per cycle (Mansky and Temin 1995). Given the dynamics of HIV-1 turnover in vivo and a recombination rate of approximately three crossovers per cycle, some genome lineages from a 15-year-old infection may have experienced as many crossovers as base mutations in the genome. It has been proposed that recombination coupled with mutation profoundly influences HIV evolution, giving it a non-clonal and transient nature in vivo (Meyerhans et al. 2003). One example of the adaptive potential of HIV-1 recombination is the fact that multidrug-resistant HIV-1 variants can exist in cells as defective quasispecies, and can be rescued by superinfection with other defective HIV-1 variants (Quan et al. 2009). This phenomenon is most likely attributable to recombination during second rounds of infection, and suggests that defective HIV-1 variants may constitute part of the HIV-1 reservoir (Li et al. 1991). Lower recombination rates have been estimated for HCV, with a recombinant frequency normalized to a crossover range of one nucleotide of around 4×10^{-8} per site per generation (Reiter et al. 2011). However, due to the rapid virus turnover and the large number of HCV-infected liver cells in vivo, it is expected that recombination will be of biological importance when strong selection pressures are operative (Morel et al. 2011).

Host cell ssDNA cytidine deaminases (APOBEC3) are another source of HIV diversity. These cytidine deaminases can extinguish HIV-1 infectivity by incorporating into the virus particles; the subsequent cytosine deaminase activity attacks the nascent viral cDNA during reverse transcription, causing lethal mutagenesis. It has been recently demonstrated that APOBEC3G can also induce sublethal mutagenesis, which maintains virus infectivity and contributes to HIV-1 variation (Sadler et al. 2010). Mutation by host cell APOBEC3 deaminases is not restricted to retroviruses. Hepadnaviruses, such as hepatitis B virus (HBV), are also vulnerable to mutation by APOBEC3 (Suspene et al. 2005). Although the mutant spectrum resulting from APOBEC3 editing is highly deleterious, a small fraction of lightly APOBEC3G-edited genomes can impact HBV replication in vivo, and possibly contribute to immune escape (Vartanian et al. 2010). APOBEC3 can also reduce viral infectivity and increase the mutation frequency of negative-strand RNA viruses, such as measles (MV), mumps, and respiratory syncytial virus (Fehrholz et al. 2011).

The restriction factor cellular adenosine deaminase acting on RNA (ADAR1) catalyzes the conversion of adenosine (A) to inosine (I) on double-stranded RNA substrates (Samuel 2001), thereby introducing A-to-G mutations; this action inhibits replication of MV, as well as Newcastle disease virus, Sendai virus, and influenza virus (Ward et al. 2011). It is tempting to speculate that ADAR1 functions as a host

restriction factor of RNA viruses, analogous to the role of APOBEC3. It is possible that the extensive hypermutations of the matrix (M) gene of MV seen *in vivo* are the result of the known dispensability of the M protein for viral replication (Young and Rall 2009), with the M gene sequences representing viral decoy targets for hypermutation. However, hypermutations are also observed to a lesser extent in the fusion (F) and hemagglutinin (H) genes. One serious complication of MV infection is persistent central nervous system infection, known as subacute sclerosing panencephalitis (SSPE), that occurs at a frequency of 4–11 per 100,000 cases of MV infection. SSPE is a progressive, fatal neurodegenerative disease with the characteristic feature of MV replication in neurons (Griffin 2007). Interestingly, biased hypermutations play a direct role in the pathogenesis of SSPE by facilitating significantly prolonged MV persistence within the CNS, as opposed to mere accumulation. Significant A-to-G substitutions have also been seen in the viral M gene sequences of influenza A virus recovered from wild-type animals (Tenoever et al. 2007). This alternative source for generating mutant clouds has the potential to play a role in viral evolution, pathogenesis, immune escape, and drug resistance.

3 Quasispecies, Viral Disease, and Pathogenesis

Whether RNA virus genomic diversity affects viral pathogenesis is one of the most intriguing topics within the field of RNA virus evolution. Characterization of virulence determinants of pathogenic agents is of utmost relevance for designing disease-control strategies. Typically, virulence determination has been attributed to nucleotide changes in specific genomic regions. For instance, in the type 3 vaccine strain, P3/Sabin, a uridine residue at nucleotide 472 in the 5′ noncoding region, and a phenylalanine at amino acid 91 of capsid protein VP3 have been identified as contributing to reduced poliovirus neurovirulence (Minor et al. 1989). All three Sabin vaccine strains contain strong attenuation determinants. However, more recent work has shown that other factors, such as quasispecies diversity, can determine the pathogenic potential of a viral population; in these cases, pathogenicity will be determined by the “quasispecies” and not by the “individual”. Poliovirus carrying a high-fidelity polymerase replicates at wild-type levels but generates less genomic diversity (Pfeiffer and Kirkegaard 2003, 2005; Vignuzzi et al. 2006), which leads to a loss of neurotropism and an attenuated pathogenic phenotype. Importantly, expanding the quasispecies diversity of the high-fidelity virus population by chemical mutagenesis prior to infection restored neurotropism and pathogenesis (Vignuzzi et al. 2006). These results indicate that complementation between quasispecies members provides viral populations with a greater capacity to evolve and adapt to new environments and challenges during infection—indicating selection at the population (quasispecies) level rather than on individual mutants. Consequently, viral pathogenesis would be modulated by the proportion of attenuated and virulent genomes, and their interactions. This conclusion challenges the evolutionary biology dogma in which individuals are the ultimate target of selection.

Similar results have been obtained with chikungunya virus (CHIKV), a mosquito-borne virus that has caused outbreaks in humans since the eighteenth century and that, since 2004, has appeared in Africa, Indian Ocean islands, Southeast Asia, Italy, and France (Powers and Logue 2007). Serial passage of CHIKV in ribavirin or fluorouracil resulted in the selection of a mutagen-resistant variant with a single amino acid change (C483Y) in the RNA polymerase gene that increases replication fidelity. This unique arbovirus fidelity variant increases replication fidelity and generates populations with reduced genetic diversity. In mosquitoes, high-fidelity CHIKV produces lower infection and dissemination titers than wild-type. In newborn mice, high-fidelity CHIKV produces truncated viremias and lower organ titers. These results indicate again that increased replication fidelity and reduced genetic diversity negatively impact arbovirus fitness in invertebrate and vertebrate hosts (Coffey et al. 2011). Mutant high-fidelity RNA viruses, coupled with other attenuating mutations, could be useful for developing genetically stable live virus vaccines (Vignuzzi et al. 2008).

Viral genetic diversity is important for the survival of the viral population as a whole in the presence of selective pressures favoring mutations that yield beneficial phenotypes. These mutants are expected to survive and act as founders for the next generation. However, high mutation rates are also observed in RNA viruses that infect bacteria and thus do not face an adaptive immune response, suggesting that the high mutation rate of RNA viruses cannot completely be ascribed to a specific life history (Belshaw et al. 2008). Similarly, it has been provocatively proposed that HIV-1 variation (a paradigm of viral diversity) is essentially the result of “its lifestyle rather than a perverse predilection for error” (Wain-Hobson 1996). Although the HIV-1 mutation rate is an order of magnitude lower than that of influenza A virus, the extent of variation encountered during the 5- to 10-year course of a single individual HIV-1 infection is greater than the 1-year global genetic drift of influenza A (Korber et al. 2001). This enormous genetic diversification of HIV-1 has inevitably led to a search for links between HIV-1 variation and pathogenesis. It has been suggested that following infection, de novo generation of variants is necessary for the onset of AIDS (Nowak et al. 1991; Nowak and McMichael 1995). Genetic diversity in the HIV-1 envelope from typical patients and infected children has been correlated with disease stages (Ganeshan et al. 1997; Shankarappa et al. 1999). HIV-1 can use two chemokine receptors, CCR5 and CXCR4, as coreceptors for viral entry, and uses the CCR5 coreceptor in approximately 90% of primary infections. However, a substantial proportion of individuals develop viruses that use the CXCR4 co-receptor, which is associated with an accelerated T CD4+ cell decline and a more rapid progression to AIDS (Koot et al. 1993). Cytotoxic T lymphocytes (CTLs) that kill infected target cells play an important role in the control of HIV-1 during the acute and chronic phases of an HIV-1 infection (Ogg et al. 1998). The most documented CTL-escape mechanism is acquisition of amino acid substitutions within the CTL epitope and/or its flanking regions. These changes reduce the ability of viral peptide to bind to HLA class I molecules, and lead to impaired T-cell receptor recognition, and defective epitope generation (Ogg et al. 1998). A small number of people demonstrate sustained ability to control HIV-1 replication without

therapy. Such individuals, referred to as HIV controllers, typically maintain stable CD4+ cell counts, do not develop clinical disease, and are less likely to transmit HIV to others (Deeks and Walker 2007). Genome-wide association analysis in a multiethnic cohort of HIV-1 controllers and progressors has demonstrated that the nature of the HLA-viral peptide interaction is the major factor modulating durable control of HIV infection (Pereyra et al. 2010). Viral fitness cost precludes the emergence of variants within the CTL epitopes recognized by controllers' HLAs, indicating that variation allows evasion of immune surveillance and therefore contributes to pathogenesis (Phillips et al. 1991).

4 Quasispecies and Virus Treatment

One of the most important practical consequences of the viral quasispecies concept is its impact on antiviral therapies. Diversification of RNA virus populations clearly drives antiviral therapy response. An important example of the high adaptability of RNA viruses is the high frequency of mutant viruses with one or a few amino acid substitutions that confer reduced sensitivity to antiviral inhibitors. This general phenomenon has been documented for many viruses over the past several decades, and has made it very difficult to treat several viral diseases (Briones et al. 2006). The best example of adaptive selection is the HIV-1 virus mutants that are resistant to antiretroviral inhibitors. All currently available classes of antiretroviral therapy (reverse transcriptase, fusion, co-receptor antagonists, and integrase inhibitors) exert selective pressure for target gene mutations that confer high-level drug resistance (Johnson et al. 2011). The capacity of novel compounds to exert selective pressure for a mutation is now used as evidence of anti-HIV-1 activity. Experimental studies of HIV-1 populations have demonstrated the existence of many resistant mutants in HIV-1 populations before they have been exposed to the inhibitors (Najera et al. 1995). These resistant mutants may exist at very low frequencies in the naive viral population, but then selectively multiply in the presence of the inhibitor. The relative fitness values of wild-type and resistant mutants in the absence and presence of the inhibitor determine the kinetics and degree of dominance of resistant mutants (Coffin 1995).

Like HIV, other RNA viruses can also evade antiviral treatments, including influenza virus, HCV, and HBV. HBV is a DNA virus, but its DNA replicates through a genomic RNA intermediate and utilizes a virally encoded reverse transcriptase. Consequently, a significant amount of diversity, similar to that seen in RNA viruses, occurs in the sequences of HBV isolates. Until recently, monotherapies or sequential treatments with nucleoside analogues were widely used to treat chronic HBV infection. Not surprisingly, this approach has resulted in the generation of multidrug-resistant viruses (Locarnini and Warner 2007). Current treatment of chronic HCV infection is based on the combination of pegylated interferon- α and ribavirin; this regimen eradicates the virus in up to 80% of patients infected with genotypes 2 or 3, but in only 40–50% of patients infected with HCV genotype 1 (Pawlotsky 2011).

Studies of recently developed direct-acting antiviral molecules against HCV have shown that administration of these drugs alone may lead to the selection of resistant viruses, raising concerns that resistance may undermine therapy based on direct-acting antivirals (Pawlotsky 2011). Two HCV NS3 protease inhibitors, telaprevir and boceprevir, have already been approved for HCV infection treatment, and several other drugs that are directed against different HCV proteins are in phase II and III of clinical development. As expected, resistant mutants to telaprevir and boceprevir preexist in HCV populations before they have been exposed to the inhibitors (Bartels et al. 2008; Cubero et al. 2008; Franco et al. 2011). Mathematical modeling suggests that at least three direct-acting antiviral molecules should be used (Rong et al. 2010), but the final number will depend on their modes of action and the likelihood that HCV variants bearing substitutions in different regions of the genome conferring resistance to the different classes of drugs are present in the same strain (Pawlotsky 2011). HCV shares many properties with HIV; both are highly variable viruses with quasispecies distribution, large viral populations, and very rapid turnover in the individual patient. Fortunately, unlike HIV, the HCV replicative cycle is exclusively cytoplasmic, with no host genome integration or episomal persistence in infected cells; therefore, HCV infection is intrinsically curable, but the development of antiviral resistance in chronic viral infections like HIV, HCV, or HBV can thwart the success of future treatments. For instance, the development of resistances to first generation HCV NS3 protease inhibitors, boceprevir and telaprevir, may compromise the treatment success of the next generation of NS3 inhibitors, now in clinical development. Moreover, resistant viruses can be transmitted, compromising the efficacy of new antivirals at the population level. Viral quasispecies are endowed with memory of their past intra-host evolutionary history, maintained in the form of minority variants (Briones et al. 2006; Briones and Domingo 2008). These variants can reemerge and become a major quasispecies variant if the quasispecies is subjected to selective pressures. This is particularly relevant in antiviral treatment because minority memory drug-resistant variants can quickly expand under drug selection pressure. One example of the key role of minority HIV-1 variants is the fact that women who receive intrapartum nevirapine monotherapy are less likely to exhibit virologic suppression after 6 months of postpartum treatment with a nevirapine-containing regimen (Jourdain et al. 2004). RNA viruses can escape from antiviral activity through mutations in the target viral gene itself, causing decreased affinity to the inhibitor and leading to resistance. These changes also affect the phenotype of the targeted protein, and consequently decrease the replication capacity of the virus. Continuous replication of these viruses may result in the acquisition of compensatory changes, which can fixate the drug-resistant variant in the viral population and increase viral fitness (Martinez-Picado et al. 1999; Nijhuis et al. 1999). Therefore, since the frequency of a variant in a quasispecies depends on the relative fitness of that particular variant, memory genomes that are maintained after drug discontinuation will be present at a higher frequency than in the original population.

There are two licensed classes of anti-influenza drugs: M2 ion channel blockers (amino-adamantines: amantadine and rimantadine) and NA inhibitors (oseltamivir and zanamivir); however, the 2009 H1N1 pandemic viruses, including the earliest

isolate, are already amino-adamantine-resistant (Dawood et al. 2009). In contrast, most of the currently circulating pandemic viruses are susceptible to NA inhibitors (Itoh et al. 2009); therefore, pandemic influenza patients are treated with NA inhibitors in many countries. Studies with seasonal H1N1, H3N2, and highly pathogenic avian H5N1 viruses revealed that single amino acid substitutions at several positions in or around the NA active site confer resistance to viruses against NA inhibitors. One study detected the NA H274Y substitution in sporadic cases of oseltamivir-treated and – untreated patients infected with 2009 H1N1 pandemic viruses (Leung et al. 2009). Importantly, viruses with the NA H274Y substitution were comparable to their oseltamivir-sensitive counterparts in their pathogenicity and transmissibility in animal models (Kiso et al. 2010). Again, it seems unrealistic that antiviral monotherapy could stop an RNA virus.

Mounting evidence shows that single-stranded DNA viruses (all with genomes smaller than ~13 kb) evolve at rates approaching those observed in their RNA counterparts (Duffy et al. 2008), suggesting that combination therapy may also be considered for the treatment of some DNA viruses. Single-stranded viral DNA replication mechanisms are generally less prone to proofreading, and isolated single-stranded DNA seems to be resistant to mismatch repair. The first precise estimates for the rate of single-stranded DNA virus evolution came from a study on canine parvovirus (CPV-2), in which a substitution rate of approximately 10^{-4} substitution/site per year was estimated (Lopez-Bueno et al. 2006; Shackelton et al. 2005). This value is within the range observed in RNA viruses (Domingo et al. 2006).

In recent years, several cellular factors have been identified in some viruses (e.g., HIV, HCV, and HBV) that are closely involved with the virus replication cycle, and that can be targeted to prevent virus spread. The genetic barrier for viral escape may be much higher when cellular factors are targeted; virus adaptation to alternative cellular co-factors is expected to be more complicated or even impossible when no alternative cellular functions are available. Targeting cellular functions is obviously not without danger. The use of host gene targets requires careful selection; knock-down of cellular factors essential for virus replication may also be detrimental to the cell and the host. The recent availability of CCR5 antagonists has raised concern that genetic, biological, or chemical CCR5 knockout—although beneficial against some pathogens (e.g., HIV-1)—could be deleterious for host processes involved in pathogen response (Telenti 2009). Targeting cellular factors requires extensive toxicity studies, but in the case of CCR5, we know that the protein does not fulfill an essential function in human physiology (Liu et al. 1996). Unfortunately, targeting cellular viral cofactors does not preclude the emergence of drug-resistant viruses. Viral resistance to CCR5 antagonists (maraviroc) has been extensively observed (Libre et al. 2010). HIV-1 can selectively express variants of the envelope protein that either exhibit higher CD4 receptor affinity (Agrawal-Gamse et al. 2009) or recognize the inhibitor-bound CCR5 complex (Westby et al. 2007). Such drug pressure can also raise the possibility of viral escape by triggering a switch to CXCR4 as an alternative receptor; such CXCR4-using HIV-1 variants may be more pathogenic (Nedellec et al. 2011). Likewise, cyclophylin inhibitors—promising potent HCV

inhibitors that are now in late clinical trials, and that target a host protein (cyclophilin peptidyl-prolyl cis-trans isomerase activity)—can drive the selection of HCV resistant viruses with amino acid substitutions in the viral proteins NS2 and NS5 (Pawlotsky 2011).

The emergence of resistant virus variants poses a serious medical problem. Consequently, different strategies have been developed to counteract viral escape. Over a decade of experience with HIV antiretroviral therapy has taught us that it is unrealistic to try to target RNA viruses with only one antiviral agent because the virus will rapidly develop resistance. Large population sizes, high replication rates, and high error rates of RNA viruses provide the basis for mutation, and rapid growth of escape variants that are likely present before therapy begins. To counteract this situation, antiviral therapies now involve co-administration of multiple antivirals targeting different viral proteins or targeting only one viral protein but through different mechanisms of action. This strategy can reduce the emergence of single-resistant viruses, as exemplified with the multiple anti-HIV drug combination approach, known as highly active antiretroviral therapy (HAART) (Ho 1995). The clinical success of HAART warrants the use of a similar strategy to counteract viral escape during treatment of other RNA virus infections.

5 Quasispecies Theory and Non-viral Biological Systems

Cancer cells display uncontrolled growth, invasion of adjacent tissues, and sometimes metastasis. To achieve these properties, cells alter their genetic information through DNA point mutations, chromosomal rearrangements, and/or epigenetic changes. Mutations in cellular DNA are more frequent in tumor cells, and microsatellite and chromosomal instability have also been associated with cancer. Furthermore, cancer cells may show a mutator phenotype that increases the probability of achieving the most advantageous mutation combination for tumor growth (Bielas et al. 2006; Loeb 2001). Deamination cell machinery, like APOBEC, has been recently associated with this mutator phenotype (Vartanian et al. 2008); it has been hypothesized that recurrent low-level mutation by APOBEC3A could catalyze the transition from a healthy genome to a cancer genome (Suspene et al. 2011). Mutations in about 300 genes have been related to cancer (Futreal et al. 2004), which are located predominantly in protein kinase domains and in domains of proteins involved in DNA binding and transcriptional regulation (Futreal et al. 2004). Other mutations have been described in cancer cells (Futreal et al. 2004; Greenman et al. 2007), although a majority could be acting as accompanying mutations. Through the use of high-throughput sequencing technologies (ultra-deep sequencing), it has been discovered that every tumor harbors high-frequency mutations—usually mutations resulting in the gain of function of an oncogene or the loss of a tumor suppressor—accompanied by a complex combination of low-frequency mutations (Chin et al. 2011). Mutations are thought to drive the global cancer phenotype, and their characteristics resemble those of viral quasispecies, with the presence of a

dominant clone accompanied by a “cloud” of minor forms. There is tremendous complexity and heterogeneity in the pattern of mutations in tumors of different origins.

In 1976, it was proposed that cancer was a complex evolutionary system that showed high heterogeneity and clonal evolution (Nowell 1976). This seminal description of cancer as an evolutionary process predicted clonal expansions, individual variations in response to interventions, and therapeutic resistance. Cancer is in fact a complex biological system that evolves through mutations and epigenetic changes, following Darwinian principles of competition and selection. This selection operates in the entire body, at the level of cellular clones that can survive and evade control signals. Some cancer studies have been based in an evolutionary and ecological context (Maley and Forrest 2000; Merlo et al. 2006). Clonal diversity in cancer cells is a factor for predicting progression in an esophageal adenocarcinoma cancer model (Maley et al. 2006). Theoretical studies have correlated cancer with genetic instability (Gonzalez-Garcia et al. 2002; Maley and Forrest 2000), with quasispecies models of minimal replicators (Brumer et al. 2006; Sole et al. 2003; Tannenbaum et al. 2006), and even with incursions into error catastrophe (Sole and Deisboeck 2004). These studies reveal the high genetic heterogeneity of tumor cells as the source of adaptation used by cancer to fight against the immune system, become resistant to different treatments, invade adjacent tissues, and sometimes metastasize and invade other organs. Using mathematical models, it has been proposed that tumors, in contrast to viral quasispecies, benefit from a highly stable component: cancer stem cells (Sole et al. 2008). Sole et al. (2008) argued that tumors manifest two components; the more variable component exploits phenotypes that allow the tumor to grow and survive, while cancer stem cells exist as a lesser but more robust component and act as a reservoir of stability. This strategy would work as life insurance for a tumor, allowing cancer cell progeny to mutate beyond the limits established for normal cell types.

The highly variable replication rate of cancer cells carries straightforward clinical implications. The mutant “cloud” generated during cancer cell replication allows the tumor to face diverse challenges, including the immune system and treatment.

Cancer must be treated with therapies that can overcome mutator or suppressor genotypes, but even the most potent anticancer drugs may fail when administered individually (Luo et al. 2009). Highly active anticancer treatments or orthogonal therapy (the equivalent of HAART used in HIV-1 therapy) may be more adequate cancer therapy. Also in a homology to the treatment of HIV-1, sequential administration of anticancer compounds can lead to treatment failure. Concurrent administration of these therapies can increase the threshold of emergence for mutations conferring treatment resistance, i.e., such treatment can increase the number of mutations required to reduce drug activity (Luo et al. 2009). Orthogonal cancer therapies act synergistically when they attack a cancer in at least two different ways, such that a suppressor mutation against the first therapy cannot suppress the second therapy and vice versa. Because cancer is a compilation of very different diseases, orthogonal therapy will vary depending on tumor genotype and possibly patient genotype; it is also necessary to pay close attention to the treatment effects because

cancer therapies, with their DNA-damaging nature, could increase the mutation rate. As an additional parallelism with RNA viruses, lethal mutagenesis has been proposed as a novel therapeutic approach for the treatment of solid tumors (Fox and Loeb 2010) (Fig. 2).

It is now recognized that bacteria very frequently do not exist as solitary cells, but instead as colonial organisms that exploit elaborate systems of intercellular communication to facilitate their adaptation to changing environmental conditions. The social behavior of bacteria resembles the heterogeneity described for RNA virus populations. Social behaviors related to antibiotic production, virulence, motility, or biofilm formation have been extensively described (Rumbaugh et al. 2009). A good example of bacteria social behavior is the biofilm, which can be simply defined as communities of microorganisms living on surfaces and encased within an extracellular polymeric slime matrix (Costerton et al. 1978). A more complex definition would incorporate terms such as structural heterogeneity, genetic diversity, and complex community interactions (Stoodley et al. 2002). These organic super-structures have important clinical implications as infectious agents (Costerton et al. 1987, 1999), as well as in terms of antibiotic resistance. The form of antibiotic resistance exhibited by biofilms seems to differ from the innate resistance conferred to individual bacterial cells by plasmids, transposons, and mutations (Costerton et al. 1999). It has been proposed that biofilm communities, rather than individuals, are the target of evolutionary selection (Caldwell and Costerton 1996), and that biofilm antibiotic resistance is due to an altered chemical microenvironment or a subpopulation of microorganisms within the biofilm that forms a unique and highly protected, phenotypic state, with cell differentiation similar to that seen in spore formation (Stewart and Costerton 2001). Multiple resistance mechanisms can act together; thus, to be clinically effective, anti-biofilm therapies may have to simultaneously target more than one mechanism, similar to orthogonal cancer therapies or multiple antiretroviral drug approaches.

Prions are non-genetic macromolecular systems that can also display heterogeneity regarding features that are important to their biological function. Prions are the infectious agents responsible for a variety of neurodegenerative disorders, including scrapie in sheep, bovine spongiform encephalopathy in cattle, and new variant Creutzfeldt-Jacob disease and kuru in humans. The principal, if not only, component of the prion is PrP^{Sc}, a β -sheet-rich conformer of the prion protein PrP. PrP^{Sc} propagates by eliciting conversion of PrP^C (the physiological form of PrP) into a likeness of itself. The seeding hypothesis posits that PrP^C is in equilibrium with PrP^{Sc} or a PrP^{Sc} precursor, with the equilibrium largely in favor of PrP^C; PrP^{Sc} is only stabilized when it forms an aggregate (or seed) containing a critical number of monomers, after which, monomer addition ensues rapidly (Jarrett and Lansbury 1993). Prions exist as distinct strains that can be characterized by their incubation time and the neuropathology they elicit in a particular host (Bruce et al. 1992). Many different strains can be propagated indefinitely in hosts that are homozygous for the PrP gene; the protein-only hypothesis assumes that each strain is associated with a different conformer of PrP^{Sc} (Bessen and Marsh 1992; Peretz et al. 2001; Telling et al. 1996). The recent discovery of fungal prions that are not associated with disease suggests that prions may constitute a new and

widespread regulatory mechanism maintained through evolution (Jarosz et al. 2010; Tuite and Serio 2010; Tyedmers et al. 2008). Similar to viral quasispecies, prions cloned by end-point dilution in cell culture can gradually become heterogeneous by accumulating protein-folding mutants (Li et al. 2010). Importantly, selective pressures have been shown to result in the emergence of variants, including drug-resistant mutants (Ghaemmaghami et al. 2009; Li et al. 2010; Mahal et al. 2010), indicating that not only nucleic acid-based systems can show high population heterogeneity and experience selective events. A protein is defined by a primary structure, but can be folded in different ways, each one associated with a different phenotype that can be selected and further propagated. Prion populations show high population size and conformation heterogeneity; recent results suggest that such heterogeneity may underlie selection and propagation capacity, which is typical Darwinian behavior. It is still largely unknown whether a population of this type evolves as a sum of its components or only as molecular individualities (Straub and Thirumalai 2011). Protein conformation is the final result of multiple amino acid-amino acid interactions, which are themselves subjected to molecular fluctuations such as ionization and ionic interaction, or hydrophobic contacts dependent on torsion angles of bonds that are also subjected to thermal fluctuations. Thus, it is not unexpected that a collection of related but non-identical conformations exist in populations of proteins, or that environmental factors may favor some conformations over others. The environment may also dictate the presence of minority conformations at different frequencies. Transitions among related conformation states in prions became apparent because they have the capacity to produce disease. These observations open new prospects for research on the molecular mechanisms of protein aggregation, and whether a specific conformation variant can nucleate the conversion of additional representatives to form mutant aggregates (Bernacki and Murphy 2009).

6 Concluding Remarks

The quasispecies concept has provided a framework to understand RNA virus populations and to develop therapeutic strategies that successfully combat deadly virus pandemics (e.g., HIV-AIDS, HCV). The theoretical and experimental development of the quasispecies concept has challenged our view of Darwinian evolution. Dynamic distributions of genomes appear to be subject to genetic variation, competition, and selection, and may be able to serve as therapeutic targets rather than targeting individuals. The challenge remains to determine how the study of quasispecies will improve the development of new antiviral, antibacterial, anticancer, or antiprion strategies.

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References

- Agrawal-Gamse C, Lee FH, Haggarty B, Jordan AP, Yi Y, Lee B, Collman RG, Hoxie JA, Doms RW, Laakso MM (2009) Adaptive mutations in a human immunodeficiency virus type 1 envelope protein with a truncated V3 loop restore function by improving interactions with CD4. *J Virol* 83(21):11005–11015
- Ambros S, Hernandez C, Flores R (1999) Rapid generation of genetic heterogeneity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host. *J Gen Virol* 80:2239–2252
- Bartels DJ, Zhou Y, Zhang EZ, Marcial M, Byrn RA, Pfeiffer T, Tigges AM, Adiwijaya BS, Lin C, Kwong AD, Kieffer TL (2008) Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A Protease inhibitors in treatment-naive subjects. *J Infect Dis* 198(6):800–807
- Belshaw R, Gardner A, Rambaut A, Pybus OG (2008) Pacing a small cage: mutation and RNA viruses. *Trends Ecol Evol* 23(4):188–193
- Bernacki JP, Murphy RM (2009) Model discrimination and mechanistic interpretation of kinetic data in protein aggregation studies. *Biophys J* 96(7):2871–2887
- Bessen RA, Marsh RF (1992) Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *J Virol* 66(4):2096–2101
- Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA (2006) Human cancers express a mutator phenotype. *Proc Natl Acad Sci USA* 103(48):18238–18242
- Bonhoeffer S, Chappey C, Parkin NT, Whitcomb JM, Petropoulos CJ (2004) Evidence for positive epistasis in HIV-1. *Science* 306(5701):1547–1550
- Briones C, Domingo E (2008) Minority report: hidden memory genomes in HIV-1 quasispecies and possible clinical implications. *AIDS Rev* 10(2):93–109
- Briones C, de Vicente A, Molina-Paris C, Domingo E (2006) Minority memory genomes can influence the evolution of HIV-1 quasispecies in vivo. *Gene* 384:129–138
- Bruce ME, Fraser H, McBride PA, Scott JR, Dickinson AG (1992) The basis of strain variation in scrapie. In: Prusiner SB, Collinge J, Powell J, Anderton BB (eds) *Prion diseases of humans and animals*. Ellis Horwood, New York/London, pp 497–508
- Brumer Y, Michor F, Shakhnovich EI (2006) Genetic instability and the quasispecies model. *J Theor Biol* 241(2):216–222
- Burch CL, Chao L (2000) Evolvability of an RNA virus is determined by its mutational neighbourhood. *Nature* 406(6796):625–628
- Caldwell DE, Costerton JW (1996) Are bacterial biofilms constrained to Darwin's concept of evolution through natural selection? *Microbiologia* 12(3):347–358
- Chao L (1990) Fitness of RNA virus decreased by Muller's ratchet. *Nature* 348(6300):454–455
- Chin L, Hahn WC, Getz G, Meyerson M (2011) Making sense of cancer genomic data. *Genes Dev* 25(6):534–555
- Clarke DK, Duarte EA, Elena SF, Moya A, Domingo E, Holland J (1994) The red queen reigns in the kingdom of RNA viruses. *Proc Natl Acad Sci USA* 91(11):4821–4824
- Coffey LL, Beeharry Y, Borderia AV, Blanc H, Vignuzzi M (2011) Arbovirus high fidelity variant loses fitness in mosquitoes and mice. *Proc Natl Acad Sci USA* 108(38):16038–16043
- Coffin JM (1995) HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* 267(5197):483–489
- Costerton JW, Geesey GG, Cheng KJ (1978) How bacteria stick. *Sci Am* 238(1):86–95
- Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 41:435–464
- Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418):1318–1322
- Cubero M, Esteban JI, Otero T, Sauleda S, Bes M, Esteban R, Guardia J, Quer J (2008) Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. *Virology* 370(2):237–245

- Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 360(25):2605–2615
- de la Torre JC, Holland JJ (1990) RNA virus quasispecies populations can suppress vastly superior mutant progeny. *J Virol* 64(12):6278–6281
- Deeks SG, Walker BD (2007) Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity* 27(3):406–416
- Domingo E, Sabo D, Taniguchi T, Weissmann C (1978) Nucleotide sequence heterogeneity of an RNA phage population. *Cell* 13(4):735–744
- Domingo E, Martin V, Perales C, Grande-Perez A, Garcia-Arriaza J, Arias A (2006) Viruses as quasispecies: biological implications. *Curr Top Microbiol Immunol* 299:51–82
- Duffy S, Shackelton LA, Holmes EC (2008) Rates of evolutionary change in viruses: patterns and determinants. *Nat Rev Genet* 9(4):267–276
- Eigen M (1971) Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften* 58(10):465–523
- Eigen M (2002) Error catastrophe and antiviral strategy. *Proc Natl Acad Sci USA* 99(21):13374–13376
- Eigen M, Schuster P (1977) The hypercycle a principle of natural self-organization. Part a: emergence of the hypercycle. *Naturwissenschaften* 64(11):541–565
- Fehrholz M, Kendl S, Prifert C, Weissbrich B, Lemon K, Rennick L, Duprex PW, Rima BK, Koning FA, Holmes RK, Malim MH, Schneider-Schaulies J (2011) The innate antiviral factor APOBEC3G targets replication of measles, mumps, and respiratory syncytial virus. *J Gen Virol* 93(3):565–576
- Fox EJ, Loeb LA (2010) Lethal mutagenesis: targeting the mutator phenotype in cancer. *Semin Cancer Biol* 20(5):353–359
- Franco S, Bellido R, Aparicio E, Canete N, Garcia-Retortillo M, Sola R, Tural C, Clotet B, Paredes R, Martinez MA (2011) Natural prevalence of HCV minority variants that are highly resistant to NS3/4A protease inhibitors. *J Viral Hepat* 18(10):e578–e582
- Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R, Rahman N, Stratton MR (2004) A census of human cancer genes. *Nat Rev Cancer* 4(3):177–183
- Ganeshan S, Dickover RE, Korber BT, Bryson YJ, Wolinsky SM (1997) Human immunodeficiency virus type 1 genetic evolution in children with different rates of development of disease. *J Virol* 71(1):663–677
- Ghaemmaghami S, Ahn M, Lessard P, Giles K, Legname G, DeArmond SJ, Prusiner SB (2009) Continuous quinacrine treatment results in the formation of drug-resistant prions. *PLoS Pathog* 5(11):e1000673
- Gonzalez-Garcia I, Sole RV, Costa J (2002) Metapopulation dynamics and spatial heterogeneity in cancer. *Proc Natl Acad Sci USA* 99(20):13085–13089
- Grande-Perez A, Sierra S, Castro MG, Domingo E, Lowenstein PR (2002) Molecular indetermination in the transition to error catastrophe: systematic elimination of lymphocytic choriomeningitis virus through mutagenesis does not correlate linearly with large increases in mutant spectrum complexity. *Proc Natl Acad Sci USA* 99(20):12938–12943
- Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR (2007) Patterns of somatic mutation in human cancer genomes. *Nature* 446(7132):153–158
- Griffin DE (2007) Measles virus. In: Knipe DM, Howley PM (eds) *Fields virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1551–1585
- Ho DD (1995) Time to hit HIV, early and hard. *N Engl J Med* 333(7):450–451

- Holland JJ, Domingo E, de la Torre JC, Steinhauer DA (1990) Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. *J Virol* 64(8):3960–3962
- Holland JJ, de la Torre JC, Clarke DK, Duarte E (1991) Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. *J Virol* 65(6):2960–2967
- Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, Sakabe S, Imai M, Hatta Y, Watanabe S, Li C, Yamada S, Fujii K, Murakami S, Imai H, Kakugawa S, Ito M, Takano R, Iwatsuki-Horimoto K, Shimojima M, Horimoto T, Goto H, Takahashi K, Makino A, Ishigaki H, Nakayama M, Okamatsu M, Warshauer D, Shult PA, Saito R, Suzuki H, Furuta Y, Yamashita M, Mitamura K, Nakano K, Nakamura M, Brockman-Schneider R, Mitamura H, Yamazaki M, Sugaya N, Suresh M, Ozawa M, Neumann G, Gern J, Kida H, Ogasawara K, Kawaoka Y (2009) In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 460(7258):1021–1025
- Jarosz DF, Taipale M, Lindquist S (2010) Protein homeostasis and the phenotypic manifestation of genetic diversity: principles and mechanisms. *Annu Rev Genet* 44:189–216
- Jarrett JT, Lansbury PT Jr (1993) Seeding “one-dimensional crystallization” of amyloid: a pathogenic mechanism in Alzheimer’s disease and scrapie? *Cell* 73(6):1055–1058
- Johnson VA, Calvez V, Gunthard HF, Paredes R, Pillay D, Shafer R, Wensing AM, Richman DD (2011) 2011 Update of the drug resistance mutations in HIV-1. *Top Antivir Med* 19(4):156–164
- Jourdain G, Ngo-Giang-Huong N, Le Coeur S, Bowonwatanuwong C, Kantipong P, Leechanachai P, Ariyadej S, Leenasirimakul P, Hammer S, Lallemand M (2004) Intrapartum exposure to nevirapine and subsequent maternal responses to nevirapine-based antiretroviral therapy. *N Engl J Med* 351(3):229–240
- Jung A, Maier R, Vartanian JP, Bocharov G, Jung V, Fischer U, Meese E, Wain-Hobson S, Meyerhans A (2002) Multiply infected spleen cells in HIV patients. *Nature* 418(6894):144
- Kiso M, Shinya K, Shimojima M, Takano R, Takahashi K, Katsura H, Kakugawa S, Le MT, Yamashita M, Furuta Y, Ozawa M, Kawaoka Y (2010) Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses. *PLoS Pathog* 6(8):e1001079
- Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, Coutinho RA, Miedema F, Schellekens PT, Tersmette M (1993) Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Ann Intern Med* 118(9):681–688
- Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V (2001) Evolutionary and immunological implications of contemporary HIV-1 variation. *Br Med Bull* 58:19–42
- Lai MM (1992a) Genetic recombination in RNA viruses. *Curr Top Microbiol Immunol* 176:21–32
- Lai MM (1992b) RNA recombination in animal and plant viruses. *Microbiol Rev* 56(1):61–79
- Lauring AS, Andino R (2010) Quasispecies theory and the behavior of RNA viruses. *PLoS Pathog* 6(7):e1001005
- Leung TW, Tai AL, Cheng PK, Kong MS, Lim W (2009) Detection of an oseltamivir-resistant pandemic influenza A/H1N1 virus in Hong Kong. *J Clin Virol* 46(3):298–299
- Li Y, Kappes JC, Conway JA, Price RW, Shaw GM, Hahn BH (1991) Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J Virol* 65(8):3973–3985
- Li J, Browning S, Mahal SP, Oelschlegel AM, Weissmann C (2010) Darwinian evolution of prions in cell culture. *Science* 327(5967):869–872
- Lindstrom SE, Cox NJ, Klimov A (2004) Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957–1972: evidence for genetic divergence and multiple reassortment events. *Virology* 328(1):101–119
- Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86(3):367–377
- Llibre JM, Schapiro JM, Clotet B (2010) Clinical implications of genotypic resistance to the newer antiretroviral drugs in HIV-1-infected patients with virological failure. *Clin Infect Dis* 50(6):872–881

- Locarnini S, Warner N (2007) Major causes of antiviral drug resistance and implications for treatment of hepatitis B virus mono-infection and coinfection with HIV. *Antivir Ther* 12(Suppl 3):H15–H23
- Loeb LA (2001) A mutator phenotype in cancer. *Cancer Res* 61(8):3230–3239
- Loeb LA, Essigmann JM, Kazazi F, Zhang J, Rose KD, Mullins JI (1999) Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc Natl Acad Sci USA* 96(4):1492–1497
- Lopez-Bueno A, Villarreal LP, Almendral JM (2006) Parvovirus variation for disease: a difference with RNA viruses? *Curr Top Microbiol Immunol* 299:349–370
- Lopez-Galindez C, Ortin J, Domingo E, del Rio L, Perez-Brena P, Najera R (1985) Heterogeneity among influenza H3N2 isolates recovered during an outbreak brief report. *Arch Virol* 85(1–2):139–144
- Luo J, Solimini NL, Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 136(5):823–837
- Mahal SP, Browning S, Li J, Saponitsky-Kroyter I, Weissmann C (2010) Transfer of a prion strain to different hosts leads to emergence of strain variants. *Proc Natl Acad Sci USA* 107(52):22653–22658
- Maley CC, Forrest S (2000) Exploring the relationship between neutral and selective mutations in cancer. *Artif Life* 6(4):325–345
- Maley CC, Galipeau PC, Finley JC, Wongsurawat VJ, Li X, Sanchez CA, Paulson TG, Blount PL, Risques RA, Rabinovitch PS, Reid BJ (2006) Genetic clonal diversity predicts progression to esophageal adenocarcinoma. *Nat Genet* 38(4):468–473
- Mansky LM, Temin HM (1995) Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* 69(8):5087–5094
- Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Esteban R, Guardia J, Gomez J (1992) Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* 66(5):3225–3229
- Martinez MA, Carrillo C, Gonzalez-Candelas F, Moya A, Domingo E, Sobrino F (1991) Fitness alteration of foot-and-mouth disease virus mutants: measurement of adaptability of viral quasispecies. *J Virol* 65(7):3954–3957
- Martinez-Picado J, Savara AV, Sutton L, D'Aquila RT (1999) Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J Virol* 73(5):3744–3752
- Mas A, Lopez-Galindez C, Cacho I, Gomez J, Martinez MA (2010) Unfinished stories on viral quasispecies and Darwinian views of evolution. *J Mol Biol* 397(4):865–877
- Merlo LM, Pepper JW, Reid BJ, Maley CC (2006) Cancer as an evolutionary and ecological process. *Nat Rev Cancer* 6(12):924–935
- Meyerhans A, Cheynier R, Albert J, Seth M, Kwok S, Sninsky J, Morfeldt-Manson L, Asjo B, Wain-Hobson S (1989) Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* 58(5):901–910
- Meyerhans A, Jung A, Maier R, Vartanian JP, Bocharov G, Wain-Hobson S (2003) The non-clonal and transitory nature of HIV in vivo. *Swiss Med Wkly* 133(33–34):451–454
- Minor PD, Dunn G, Evans DM, Magrath DI, John A, Howlett J, Phillips A, Westrop G, Wareham K, Almond JW et al (1989) The temperature sensitivity of the Sabin type 3 vaccine strain of poliovirus: molecular and structural effects of a mutation in the capsid protein VP3. *J Gen Virol* 70(Pt 5):1117–1123
- Morel V, Fournier C, Francois C, Brochot E, Helle F, Duverlie G, Castelain S (2011) Genetic recombination of the hepatitis C virus: clinical implications. *J Viral Hepat* 18(2):77–83
- Mullins JI, Heath L, Hughes JP, Kicha J, Styrchak S, Wong KG, Rao U, Hansen A, Harris KS, Laurent JP, Li D, Simpson JH, Essigmann JM, Loeb LA, Parkins J (2011) Mutation of HIV-1 genomes in a clinical population treated with the mutagenic nucleoside KP1461. *PLoS One* 6(1):e15135
- Najera I, Holguin A, Quinones-Mateu ME, Munoz-Fernandez MA, Najera R, Lopez-Galindez C, Domingo E (1995) Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. *J Virol* 69(1):23–31

- Nedellec R, Coetzer M, Lederman MM, Offord RE, Hartley O, Mosier DE (2011) Resistance to the CCR5 inhibitor 5P12-RANTES requires a difficult evolution from CCR5 to CXCR4 coreceptor use. *PLoS One* 6(7):e22020
- Nijhuis M, Schuurman R, de Jong D, Erickson J, Gustchina E, Albert J, Schipper P, Gulnik S, Boucher CA (1999) Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* 13(17):2349–2359
- Nowak MA, McMichael AJ (1995) How HIV defeats the immune system. *Sci Am* 273(2):58–65
- Nowak MA, Anderson RM, McLean AR, Wolfs TF, Goudsmit J, May RM (1991) Antigenic diversity thresholds and the development of AIDS. *Science* 254(5034):963–969
- Nowell PC (1976) The clonal evolution of tumor cell populations. *Science* 194(4260):23–28
- Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ (1998) Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279(5359):2103–2106
- Ojosnegros S, Perales C, Mas A, Domingo E (2011) Quasispecies as a matter of fact: viruses and beyond. *Virus Res* 162(1–2):203–215
- Pawlotsky JM (2011) Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology* 53(5):1742–1751
- Perales C, Lorenzo-Redondo R, Lopez-Galindez C, Angel Martinez M, Domingo E (2010) Mutant spectra in virus behavior. *Future Virol* 5(6):679–698
- Peretz D, Scott MR, Groth D, Williamson RA, Burton DR, Cohen FE, Prusiner SB (2001) Strain-specific relative conformational stability of the scrapie prion protein. *Protein Sci* 10(4):854–863
- Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI, Walker BD, Ripke S, Brumme CJ, Pulit SL, Carrington M, Kadie CM, Carlson JM, Heckerman D, Graham RR, Plenge RM, Deeks SG, Gianniny L, Crawford G, Sullivan J, Gonzalez E, Davies L, Camargo A, Moore JM, Beattie N, Gupta S, Crenshaw A, Burtt NP, Guiducci C, Gupta N, Gao X, Qi Y, Yuki Y, Piechocka-Trocha A, Cutrell E, Rosenberg R, Moss KL, Lemay P, O’Leary J, Schaefer T, Verma P, Toth I, Block B, Baker B, Rothchild A, Lian J, Proudfoot J, Alvino DM, Vine S, Addo MM, Allen TM, Altfeld M, Henn MR, Le Gall S, Streeck H, Haas DW, Kuritzkes DR, Robbins GK, Shafer RW, Gulick RM, Shikuma CM, Haubrich R, Riddler S, Sax PE, Daar ES, Ribaldo HJ, Agan B, Agarwal S, Ahern RL, Allen BL, Altidor S, Altschuler EL, Ambardar S, Anastos K, Anderson B, Anderson V, Andraday U, Antoniskis D, Bangsberg D, Barbaro D, Barrie W, Bartczak J, Barton S, Basden P, Basgoz N, Bazner S, Bellos NC, Benson AM, Berger J, Bernard NF, Bernard AM, Birch C, Bodner SJ, Bolan RK, Boudreaux ET, Bradley M, Braun JF, Brndjar JE, Brown SJ, Brown K, Brown ST, Burack J, Bush LM, Cafaro V, Campbell O, Campbell J, Carlson RH, Carmichael JK, Casey KK, Chambers ST, Chez N, Chirch LM, Cimoch PJ, Cohen D, Cohn LE, Conway B, Cooper DA, Cornelson B, Cox DT, Cristofano MV, Cuchural G Jr, Czartoski JL, Dahman JM, Daly JS, Davis BT, Davis K, Davod SM, DeJesus E, Dietz CA, Dunham E, Dunn ME, Ellerin TB, Eron JJ, Fangman JJ, Farel CE, Ferlazzo H, Fidler S, Fleenor-Ford A, Frankel R, Freedberg KA, French NK, Fuchs JD, Fuller JD, Gaberman J, Gallant JE, Gandhi RT, Garcia E, Garmon D, Gathe JC Jr, Gaultier CR, Gebre W, Gilman FD, Gilson I, Goepfert PA, Gottlieb MS, Goulston C, Groger RK, Gurley TD, Haber S, Hardwicke R, Hardy WD, Harrigan PR, Hawkins TN, Heath S, Hecht FM, Henry WK, Hladek M, Hoffman RP, Horton JM, Hsu RK, Huhn GD, Hunt P, Hupert MJ, Illeman ML, Jaeger H, Jellinger RM, John M, Johnson JA, Johnson KL, Johnson H, Johnson K, Joly J, Jordan WC, Kauffman CA, Khanlou H, Killian RK, Kim AY, Kim DD, Kinder CA, Kirchner JT, Kogelman L, Kojic EM, Korthuis PT, Kurisu W, Kwon DS, LaMar M, Lampiris H, Lanzafame M, Lederman MM, Lee DM, Lee JM, Lee MJ, Lee ET, Lemoine J, Levy JA, Llibre JM, Liguori MA, Little SJ, Liu AY, Lopez AJ, Loutfy MR, Loy D, Mohammed DY, Man A, Mansour MK, Marconi VC, Markowitz M, Marques R, Martin JR, Martin HL Jr, Mayer KH, McElrath MJ, McGhee TA, McGovern BH, McGowan K, McIntyre D, McLeod GX, Menezes P, Mesa G, Metroka CE, Meyer-Olson D, Miller AO, Montgomery K, Mounzer KC, Nagami EH, Nagin I, Nahass RG, Nelson MO, Nielsen C, Norene DL, O’Connor DH, Ojikutu BO, Okulicz J, Oladehin OO, Oldfield EC 3rd, Olender SA, Ostrowski M, Owen WF Jr, Pae E,

- Parsonnet J, Pavlatos AM, Perlmutter AM, Pierce MN, Pincus JM, Pisani L, Price LJ, Proia L, Prokesch RC, Pujet HC, Ramgopal M, Rathod A, Rausch M, Ravishankar J, Rhame FS, Richards CS, Richman DD, Rodes B, Rodriguez M, Rose RC 3rd, Rosenberg ES, Rosenthal D, Ross PE, Rubin DS, Rumbaugh E, Saenz L, Salvaggio MR, Sanchez WC, Sanjana VM, Santiago S, Schmidt W, Schuitemaker H, Sestak PM, Shalit P, Shay W, Shirvani VN, Silebi VI, Sizemore JM Jr, Skolnik PR, Sokol-Anderson M, Sosman JM, Stabile P, Stapleton JT, Starrett S, Stein F, Stellbrink HJ, Stermann FL, Stone VE, Stone DR, Tambussi G, Taplitz RA, Tedaldi EM, Theisen W, Torres R, Tosiello L, Tremblay C, Tribble MA, Trinh PD, Tsao A, Ueda P, Vaccaro A, Valadas E, Vanig TJ, Vecino I, Vega VM, Veikley W, Wade BH, Walworth C, Wanidworanun C, Ward DJ, Warner DA, Weber RD, Webster D, Weis S, Wheeler DA, White DJ, Wilkins E, Winston A, Wlodaver CG, van't Wout A, Wright DP, Yang OO, Yurdin DL, Zabukovic BW, Zachary KC, Zeeman B, Zhao M (2010) The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science* 330(6010):1551–1557
- Pfeiffer JK, Kirkegaard K (2003) A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. *Proc Natl Acad Sci USA* 100(12):7289–7294
- Pfeiffer JK, Kirkegaard K (2005) Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice. *PLoS Pathog* 1(2):e11
- Phillips RE, Rowland-Jones S, Nixon DF, Gotch FM, Edwards JP, Ogunlesi AO, Elvin JG, Rothbard JA, Bangham CR, Rizza CR et al (1991) Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* 354(6353):453–459
- Powers AM, Logue CH (2007) Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Gen Virol* 88(Pt 9):2363–2377
- Quan Y, Liang C, Brenner BG, Wainberg MA (2009) Multidrug-resistant variants of HIV type 1 (HIV-1) can exist in cells as defective quasispecies and be rescued by superinfection with other defective HIV-1 variants. *J Infect Dis* 200(9):1479–1483
- Reiter J, Perez-Vilaro G, Scheller N, Mina LB, Diez J, Meyerhans A (2011) Hepatitis C virus RNA recombination in cell culture. *J Hepatol* 55(4):777–783
- Rong L, Dahari H, Ribeiro RM, Perelson AS (2010) Rapid emergence of protease inhibitor resistance in hepatitis C virus. *Sci Transl Med* 2(30):30ra32
- Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, West SA (2009) Quorum sensing and the social evolution of bacterial virulence. *Curr Biol* 19(4):341–345
- Sadler HA, Stenglein MD, Harris RS, Mansky LM (2010) APOBEC3G contributes to HIV-1 variation through sublethal mutagenesis. *J Virol* 84(14):7396–7404
- Samuel CE (2001) Antiviral actions of interferons. *Clin Microbiol Rev* 14(4):778–809, table of contents
- Sanjuan R, Moya A, Elena SF (2004) The contribution of epistasis to the architecture of fitness in an RNA virus. *Proc Natl Acad Sci USA* 101(43):15376–15379
- Shackelton LA, Parrish CR, Truyen U, Holmes EC (2005) High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proc Natl Acad Sci USA* 102(2):379–384
- Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, Gupta P, Rinaldo CR, Learn GH, He X, Huang XL, Mullins JI (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. *J Virol* 73(12):10489–10502
- Shriner D, Rodrigo AG, Nickle DC, Mullins JI (2004) Pervasive genomic recombination of HIV-1 in vivo. *Genetics* 167(4):1573–1583
- Sierra S, Davila M, Lowenstein PR, Domingo E (2000) Response of foot-and-mouth disease virus to increased mutagenesis: influence of viral load and fitness in loss of infectivity. *J Virol* 74(18):8316–8323
- Sobrinho F, Davila M, Ortin J, Domingo E (1983) Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell culture. *Virology* 128(2):310–318
- Sole RV, Deisboeck TS (2004) An error catastrophe in cancer? *J Theor Biol* 228(1):47–54
- Sole RV, Fernandez P, Kauffman SA (2003) Adaptive walks in a gene network model of morphogenesis: insights into the Cambrian explosion. *Int J Dev Biol* 47(7–8):685–693

- Sole RV, Rodriguez-Caso C, Deisboeck TS, Saldana J (2008) Cancer stem cells as the engine of unstable tumor progression. *J Theor Biol* 253(4):629–637
- Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358(9276):135–138
- Stoodley P, Cargo R, Rupp CJ, Wilson S, Klapper I (2002) Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J Ind Microbiol Biotechnol* 29(6):361–367
- Straub JE, Thirumalai D (2011) Toward a molecular theory of early and late events in monomer to amyloid fibril formation. *Annu Rev Phys Chem* 62:437–463
- Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP (2005) Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. *Proc Natl Acad Sci USA* 102(23):8321–8326
- Suspene R, Aynaud MM, Guetard D, Henry M, Eckhoff G, Marchio A, Pineau P, Dejean A, Vartanian JP, Wain-Hobson S (2011) Somatic hypermutation of human mitochondrial and nuclear DNA by APOBEC3 cytidine deaminases, a pathway for DNA catabolism. *Proc Natl Acad Sci USA* 108(12):4858–4863
- Tannenbaum E, Sherley JL, Shakhnovich EI (2006) Semiconservative quasispecies equations for polysomic genomes: the haploid case. *J Theor Biol* 241(4):791–805
- Telenti A (2009) Safety concerns about CCR5 as an antiviral target. *Curr Opin HIV AIDS* 4(2):131–135
- Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, Mastrianni J, Lugaresi E, Gambetti P, Prusiner SB (1996) Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science* 274(5295):2079–2082
- Tenoever BR, Ng SL, Chua MA, McWhirter SM, Garcia-Sastre A, Maniatis T (2007) Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. *Science* 315(5816):1274–1278
- Tuite MF, Serio TR (2010) The prion hypothesis: from biological anomaly to basic regulatory mechanism. *Nat Rev Mol Cell Biol* 11(12):823–833
- Tyedmers J, Madariaga ML, Lindquist S (2008) Prion switching in response to environmental stress. *PLoS Biol* 6(11):e294
- Vartanian JP, Guetard D, Henry M, Wain-Hobson S (2008) Evidence for editing of human papillomavirus DNA by APOBEC3 in benign and precancerous lesions. *Science* 320(5873):230–233
- Vartanian JP, Henry M, Marchio A, Suspene R, Aynaud MM, Guetard D, Cervantes-Gonzalez M, Battiston C, Mazzaferro V, Pineau P, Dejean A, Wain-Hobson S (2010) Massive APOBEC3 editing of hepatitis B viral DNA in cirrhosis. *PLoS Pathog* 6(5):e1000928
- Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R (2006) Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* 439(7074):344–348
- Vignuzzi M, Wendt E, Andino R (2008) Engineering attenuated virus vaccines by controlling replication fidelity. *Nat Med* 14(2):154–161
- Wain-Hobson S (1996) Running the gamut of retroviral variation. *Trends Microbiol* 4(4):135–141
- Ward SV, George CX, Welch MJ, Liou LY, Hahn B, Lewicki H, de la Torre JC, Samuel CE, Oldstone MB (2011) RNA editing enzyme adenosine deaminase is a restriction factor for controlling measles virus replication that also is required for embryogenesis. *Proc Natl Acad Sci USA* 108(1):331–336
- Westby M, Smith-Burchnell C, Mori J, Lewis M, Mosley M, Stockdale M, Dorr P, Ciarabella G, Perros M (2007) Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J Virol* 81(5):2359–2371
- Young VA, Rall GF (2009) Making it to the synapse: measles virus spread in and among neurons. *Curr Top Microbiol Immunol* 330:3–30