ANNUAL REVIEWS

Annual Review of Virology

Viral Evolution Shaped by Host Proteostasis Networks

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Annu. Rev. Virol. 2023. 10:77–98

First published as a Review in Advance on April 18, 2023

The *Annual Review of Virology* is online at virology.annualreviews.org

https://doi.org/10.1146/annurev-virology-100220-112120

Keywords

viral adaptation, protein folding biophysics, stress response, chaperone, quality control, drug and immune system resistance

Abstract

Understanding the factors that shape viral evolution is critical for developing effective antiviral strategies, accurately predicting viral evolution, and preventing pandemics. One fundamental determinant of viral evolution is the interplay between viral protein biophysics and the host machineries that regulate protein folding and quality control. Most adaptive mutations in viruses are biophysically deleterious, resulting in a viral protein product with folding defects. In cells, protein folding is assisted by a dynamic system of chaperones and quality control processes known as the proteostasis network. Host proteostasis networks can determine the fates of viral proteins with biophysical defects, either by assisting with folding or by targeting them for degradation. In this review, we discuss and analyze new discoveries revealing that host proteostasis factors can profoundly shape the sequence space accessible to evolving viral proteins. We also discuss the many opportunities for research progress proffered by the proteostasis perspective on viral evolution and adaptation.

1. INTRODUCTION

Mutational tolerance: the extent to which a protein remains functional in spite of mutations

How are viral populations shaped by natural selection and genetic drift? What pressures drive viral adaptation? Can we predict or even alter the evolutionary trajectory of a virus? Answering these and other fundamental questions relevant to viral evolution is a key objective for multiple reasons. Viruses are obligate pathogens, with many viruses infecting plants and animals in ways that impose significant burdens on global health and the economy. Understanding the principles of virus evolution is crucial for developing effective antiviral strategies, advancing viral epidemiology, and predicting the emergence of new viral diseases. Moreover, viruses are powerful model systems for elucidating evolutionary mechanisms, owing to the high degree of control that can be exerted on their evolutionary process in a laboratory setting and the robust ability to test the reproducibility of evolution experiments (1). A thorough understanding of viral evolution provides key insights into evolutionary processes at work in cellular organisms.

With increasing interest in viruses as both pathogens and model systems, important discoveries have been made in the field of evolutionary virology. Research has focused heavily on genome-level mechanisms of viral evolution, including mutation rate (2, 3), recombination and reassortment (4, 5), and mutational robustness (6, 7). Much progress has also been made toward elucidating key selection pressures with therapeutic relevance, including receptor recognition, host antiviral response, and interhost transmission.

Despite major advances on these fronts, some of the most fundamental determinants of viral evolution have received relatively limited attention. A major example is viral protein folding. Like most other proteins, viral proteins must fold into their proper 3D structures to gain functional activity. Mutations that cause severe protein folding defects are purged, regardless of how adaptively beneficial the resulting phenotypes they would otherwise encode might be. This biophysical challenge is a key force directly shaping viral evolution at the molecular level (8).

That said, the biophysics of viral protein folding does not exist in a vacuum. Rather, the synthesis, folding, assembly, and quality control of viral proteins all occur inside host cells. It follows that host cell factors regulating these processes for endogenous proteins are likely to play a crucial role in assisting the folding of and defining the mutational tolerance of viral proteins. Indeed, cellular protein homeostasis, or proteostasis, is maintained by a complex network of chaperones and protein quality control factors termed the proteostasis network (9, 10). Many viruses are known to parasitize components of the host's proteostasis network during various stages of their replication cycles, further underscoring the potential importance of this network for viral evolution (11, 12).

In this review, we discuss evidence that host proteostasis factors shape the sequence space accessible to evolving viruses. In Section 2, we detail the underlying rationale for how protein folding can affect viral evolution. In Section 3, we briefly survey host proteostasis networks and explain how viral protein folding can be enabled by hijacking these proteostasis networks. In Section 4, we overview studies in nonvirus systems illustrating how proteostasis networks affect the evolution of endogenous proteins, providing a key foundation for more recent studies on viruses. In Section 5, we present emerging evidence that host proteostasis machineries directly affect the evolution of viral proteins. Finally, in Section 6, we outline additional topics in viral evolution where understanding the contribution of host proteostasis networks is essential.

Our main focus in this review is on how the biophysics of protein folding intersects with the composition and activities of metazoan proteostasis networks to shape the evolution of viral pathogens. We do not emphasize functions of host chaperones and quality control factors in higher-level phenotypes such as viral replication or infection, as excellent reviews on these subjects can be found elsewhere (11, 12). This review also does not emphasize the relationship between protein folding and genetic robustness (i.e., the invariance of phenotypes in the face of genetic variation) in viruses (13). While high mutational tolerance is closely related to enhanced genetic

robustness, the latter also depends on factors other than protein folding, such as transcription regulation and population size. Reviews on the evolutionary importance of genetic robustness, including in RNA viruses, are available elsewhere (7, 13). Finally, this review largely focuses on pathogenic metazoan viruses, touching only briefly on select other systems where relevant. Nevertheless, the principles described are applicable to any virus–host relationship.

2. FOLDING BIOPHYSICS SHAPES PROTEIN EVOLUTION

High mutation rates during genomic replication are a double-edged sword for viral fitness. Combined with large population sizes during infection and short generation times, mutations are the essential raw ingredient for viral evolution (2, 14, 15). Frequent mutation allows viruses to readily adapt to diverse selection pressures, including antiviral drugs and antibodies where resistance development can directly enhance the virus's pathogenicity. Despite these benefits, high mutation rates do come at a substantial cost. The majority of mutations result in negative effects on organismal fitness (16–18). Likewise in viruses, most single-nucleotide mutations are deleterious or lethal, regardless of the host species (19). Relatedly, many RNA viruses undergo particularly error-prone genomic replication, such that even a modest increase in their mutation rate leads to excessive accumulation of deleterious mutations and makes faithful replication impossible (20, 21).

A fundamental reason why most mutations exert negative fitness effects is that mutated sequences encode biophysically defective protein variants much more frequently than functionally improved variants (22). Mutations can cause protein folding defects in multiple ways. First, mutations can induce kinetic defects, where folding is decelerated or misfolding is accelerated (**Figure 1*a***). Second, mutations can be thermodynamically destabilizing, increasing the free energy of the native conformation and/or decreasing the free energy of unfolded or misfolded states, independent of the folding pathway (**Figure 1*b***). Third, mutations can increase the propensity for aggregation, where protein molecules accumulate in nonfunctional, often insoluble assemblies (**Figure 1*c***). Importantly, while substitutions that affect function are often clustered around a specific region (e.g., enzyme active sites or host antibody-targeted epitopes) (23), substitutions throughout the protein sequence can affect protein folding biophysics.

Mutations can be tolerated only as long as the resultant protein can properly fold and retain sufficient functional activity to support viral replication. For example, a viral surface protein

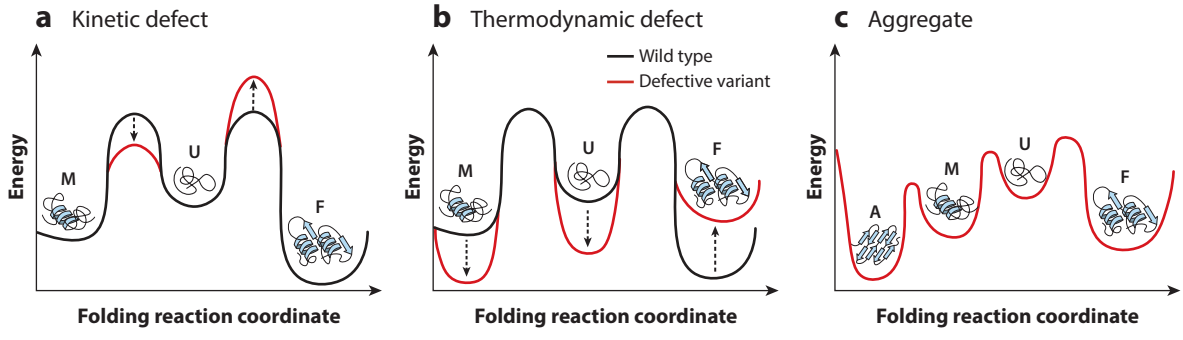


Figure 1

Examples of protein folding defects. (*a*) Kinetic defects can increase the energy required to attain the native state or reduce the energy needed to attain a misfolded/unfolded state. For example, in this figure, the protein favors a misfolded (M) conformation and/or is trapped in its unfolded (U) conformation owing to a kinetic defect. (*b*) Thermodynamic defects can increase the energy of the native folded (F) state. They can also decrease the energy of a misfolded/unfolded state, making the native state relatively less stable. (*c*) A variant that is more aggregation prone might have a partially misfolded state, a low kinetic barrier that can lead preferentially to an aggregated (A) state.

variant that cannot fold, and therefore cannot mediate host–virus membrane fusion during infection, will be selected against regardless of how antibody-resistant it otherwise would be. Hence, protein folding biophysics is a necessary and foundational constraint on viral evolution (23–26). This constraint is particularly important for adaptation because mutations that encode the nonconservative amino acid substitutes required for most adaptive functions are, as a general rule, more biophysically deleterious than mutations that encode conservative substitutions (27).

Stability: the free energy difference (ΔG) between the denatured state and the native state of a protein

Considerable evidence supports the notion that this biophysical challenge critically shapes the evolution of viruses. For example, intrinsically disordered protein regions tolerate nonsynonymous mutations better than ordered regions during potyvirus evolution, implying that protein folding functions as a purifying selection pressure (28). Selection to maintain proper protein folding is also a critical determinant of recombination patterns in begomoviruses (29). In particular, considerable research has focused on how protein thermodynamic stability plays a crucial role in the evolution of many viruses. Seminal simulations by Wylie & Shakhnovich (8) predicted that thermodynamic stability is the major factor defining the distribution of fitness effects of mutations in stochastically evolving asexual viruses, and it was also shown that viral protein instability can enhance host-range evolvability (30). Thermodynamic stability was experimentally shown to be a major constraining factor for the evolution of human immunodeficiency virus 1 (HIV-1) Pol protein, both for drug-resistant sequences and for immune response-escape mutations (31). Stability-mediated epistasis also enables fixation of deleterious mutations in influenza A nucleoprotein (NP) that are likely to be important for viral immune escape (32). Similarly, the stability of influenza hemagglutinin (HA) is implicated in determining the fitness of various lineages (33), and the stability of murine norovirus 1 capsid protein is one of two major factors that determine viral fitness landscapes in norovirus (34).

3. THE VIRAL HOST CELL'S PROTEOSTASIS NETWORK

3.1. Cellular Protein Folding Relies on the Proteostasis Network

If protein folding biophysics shapes protein evolution, then what shapes protein folding biophysics in cells? Although a protein's primary sequence determines its folding landscape in isolation, protein folding in vivo also critically depends on the cellular milieu in which the folding occurs. Viral proteins fold inside host cells, where extensive proteostasis networks play a central role in determining whether and how a protein will successfully navigate its folding landscape (11).

3.2. Organization of the Cellular Proteostasis Network

Cellular protein folding challenges are addressed by an intricate system of chaperones and quality control factors that work to shepherd nascent proteins into functional conformations and to refold or remove misfolded proteins. In this section, we briefly summarize how proteostasis network components maintain proper protein folding and how these networks are regulated.

3.2.1. How proteostasis is maintained. The proteostasis network ensures proper protein folding via two main pathways (35, 36) (**Figure 2*a***). First, proteostasis network components promote proteostasis by making the protein folding landscape less rugged (37). For example, chaperones bind to client proteins, often in unfolded or intermediate folding states. These chaperones then assist folding by shepherding client proteins into functional conformations and preventing misfolding or aggregation during the folding process without being present in the final structure (38) (**Figure 2*a***). The most widely studied metazoan chaperones include heat shock proteins (HSPs), such as the Hsp90 and Hsp70/Hsp40 systems. There are also unique classes of chaperones such

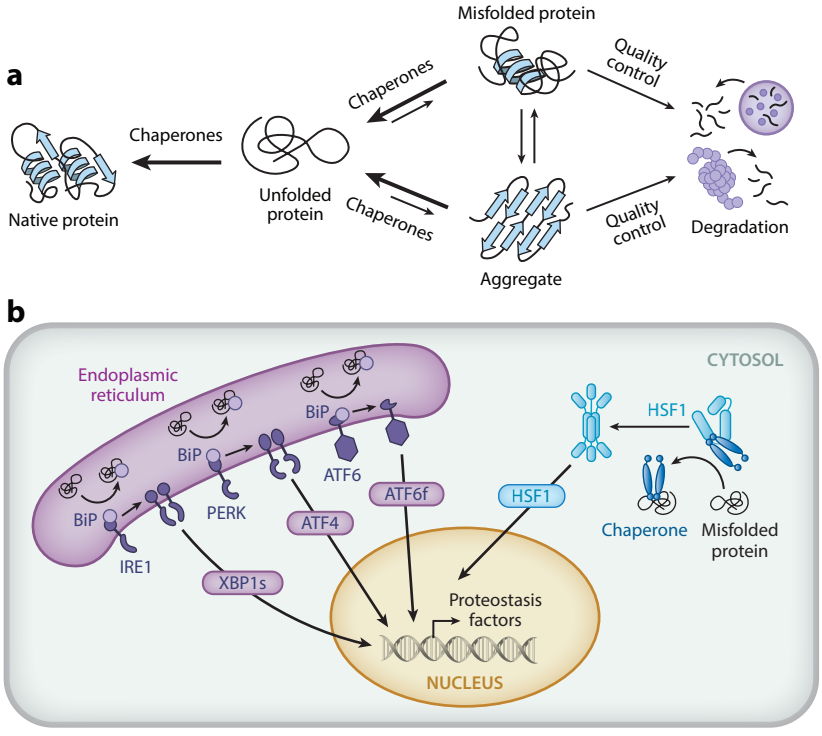


Figure 2

Organization of the cellular proteostasis network. (*a*) Proteins must fold into their native conformations to function but may instead misfold or aggregate. Chaperones can promote protein folding and/or prevent and correct misfolding and aggregation. Proteins with unresolved defects are usually identified and targeted for proteasomal or lysosomal degradation by quality control factors. (*b*) Proteostasis networks are dynamically regulated via transcriptional stress response pathways. In metazoan cells, these pathways are specific to different subcellular compartments. Abbreviations: ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; ATF6f, activating transcription factor 6 fragment; BiP, binding immunoglobulin protein; HSF1, heat shock factor 1; IRE1, inositol requiring enzyme 1; PERK, PRK-like endoplasmic reticulum kinase; XBP1s, X-box binding protein 1 spliced.

as lectin-based chaperones (e.g., calnexin, calreticulin) that specifically assist with glycoprotein folding in the secretory pathway, and ring-shaped chaperonins (e.g., TRiC and the bacterial chaperonin GroEL/ES system) that provide an encapsulated environment for folding. In addition to chaperones, folding enzymes often directly catalyze key steps in protein folding, such as prolyl *cis-trans* isomerization (peptidyl prolyl isomerases) and disulfide isomerization (protein disulfide isomerases). For simplicity in this review, we use the overarching term chaperones to refer to classically defined chaperones, chaperonins, and folding enzymes as a group—although differences in the types of viral protein folding problems they can solve are certainly meaningful.

Second, proteostasis network components promote proteostasis by ensuring the timely degradation of aberrantly folded or aggregated proteins and/or preventing trafficking of incompletely folded or misfolded proteins (**Figure 2*a***). Such components of the proteostasis network are often termed quality control factors. For example, some chaperones can triage misfolded proteins for ubiquitination by E1/E2/E3 ubiquitin ligases, which target terminally misfolded proteins for proteasomal degradation (39). Other quality control factors can also recognize

misfolded proteins in a specific cellular compartment [e.g., the endoplasmic reticulum (ER)] and retrotranslocate them to the cytosol for ubiquitination and degradation (e.g., ER-associated degradation) (40). Alternatively, some quality control factors can mediate lysosomal degradation of proteins by either recruiting autophagic machinery to aggregated proteins or directly translocating the aberrant proteins to the lysosome (41). Although the ubiquitin-proteasome system and the autophagy-lysosome system often degrade soluble misfolded proteins and large insoluble aggregates, respectively, the substrate specificity is not absolute and the two systems can complement each other.

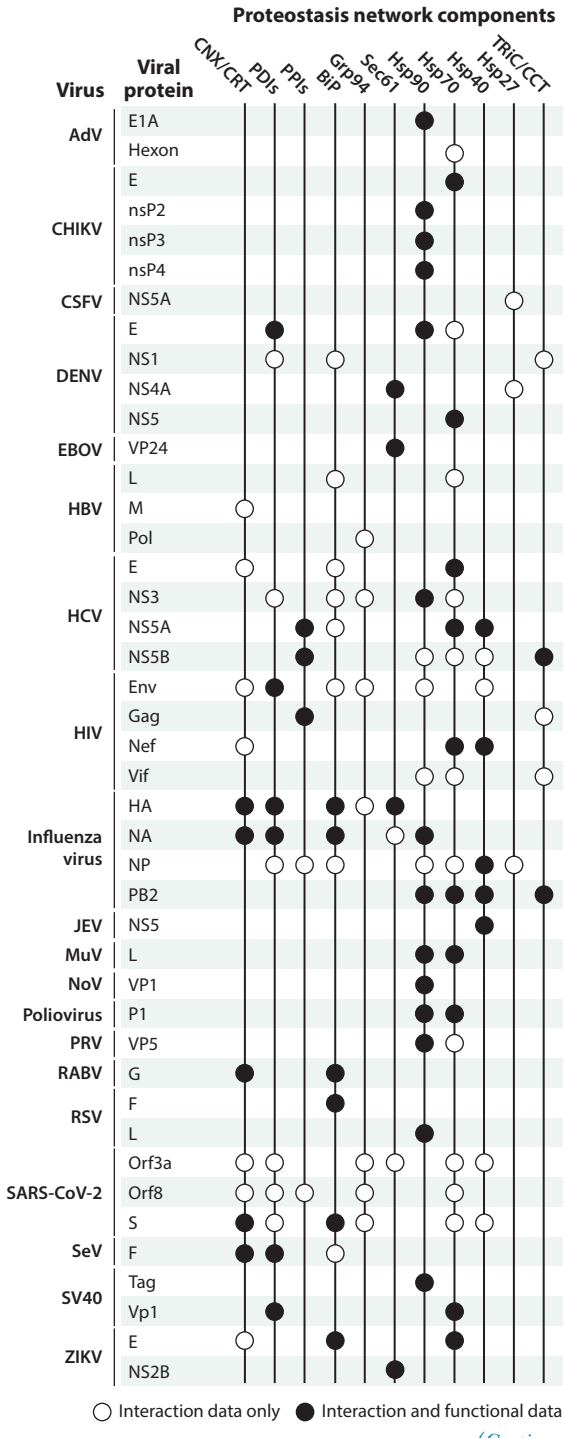
3.2.2. How proteostasis networks are regulated. Proteostasis networks are dynamic, responding in real time to diverse protein folding stresses, such as heat shock, oxidative stress, or increase in translational load during viral infection (11, 36, 42, 43). This responsiveness is mediated by transcriptional stress response pathways, which, in metazoan cells, are specific to subcellular compartments (**Figure 2*b***). Specialized proteins within each compartment function as sensors of misfolded or aggregated proteins. Their activation leads to induction of transcription factors that regulate the expression of numerous genes encoding proteostasis factors (together with global translation attenuation). In this review, we focus on stress response pathways in the cytosol and ER. ER proteostasis factors often interact with viral membrane glycoproteins, while cytosolic proteostasis factors mostly interact with soluble viral proteins (11, 44).

The cytosolic stress response, known as the heat shock response (HSR), is regulated by the transcription factor heat shock factor 1 (HSF1). A current model posits that HSF1 is inhibited by chaperones in its inactive monomeric state. Accumulation of aberrantly folded or unfolded proteins titrates away the chaperones, allowing HSF1 to form a trimer that can function as a transcriptional activator (45).

The ER stress response, known as the unfolded protein response (UPR), is regulated via three transcription factors: X-box binding protein 1 spliced (XBP1s), activating transcription factor 6 fragment (ATF6f), and activating transcription factor 4 (ATF4) (43, 46). The ER's Hsp70 chaperone binding immunoglobulin protein (BiP) plays a key role in inducing these transcription factors, as it is titrated off the ER luminal domains of inositol-requiring enzyme 1 (IRE1) (resulting in XBP1s production), protein kinase R–like endoplasmic reticulum kinase (PERK) (resulting in ATF4 production), and the full-length form of ATF6 (leading to cleavage into ATF6f). These three transcription factors lead to upregulation of partially overlapping, yet distinctive, gene sets that can enhance ER proteostasis (47, 48).

3.3. The Proteostasis Paradigm for Viral Evolution

Viruses are minimalistic pathogens that generally lack autonomous proteostasis machineries. Instead, viruses rely heavily on their host's proteostasis machineries for the synthesis, folding, co- and post-translational modification, trafficking, and quality control of their proteins (11, 49–53). Numerous viral proteins have been established to critically rely on host chaperones to fold and assemble, with select examples shown in **Figure 3**. Many viral proteins are also degraded via the host's quality control pathways (54). A few examples include the nonstructural proteins of Japanese encephalitis virus and dengue virus (55), HIV viral infectivity factor (56), sindbis virus RNA polymerase (57), and various hepatitis virus proteins (58–62) (see the sidebar titled Proviral Functions of Quality Control Pathways). Moreover, even when a wild-type viral protein is not known to interact extensively with host proteostasis factors, such interactions are likely to be enhanced if the viral protein acquires biophysically deleterious mutations (63).



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Select examples of viral proteins whose proper folding likely depends on host proteostasis network components. White circles indicate the existence of data showing that the viral protein and the host proteostasis network component interact (e.g., coimmunoprecipitation). Black circles indicate that, in addition to interaction data, there are functional data further supporting that the protein folding depends on the proteostasis network component (e.g., the steady-state level of the viral protein decreases upon chaperone inhibition). Related references are provided in the **Supplemental Material**. Abbreviations: AdV, adenovirus; BiP, binding immunoglobulin protein; CHIKV, chikungunya virus; CNX, Calnexin; CRT, calreticulin; CSFV, classical swine fever virus; DENV, dengue virus; EBOV, Ebola virus; Grp94, glucose-regulated protein 94; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; Hsp, heat shock protein; JEV, Japanese encephalitis virus; MuV, mumps virus; NoV, norovirus; PDI, protein disulfide isomerase; PPI, peptidyl prolyl isomerase; PRV, pseudorabies virus; RABV, rabies virus; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SeV, Sendai virus; SV40, simian virus 40; ZIKV, Zika virus.

Supplemental Material >

PROVIRAL FUNCTIONS OF QUALITY CONTROL PATHWAYS

Many viruses exploit host quality control pathways for proviral purposes. For example, some viruses hijack the ubiquitin-proteasome system to regulate transcriptional activities and viral budding, maintain proper ratios of viral proteins, and target cellular antiviral proteins for degradation (51, 142, 143). Some viruses also use autophagy machineries to selectively degrade cellular factors associated with the inflammation response and to assist with viral release. Autophagosomes have also, in some cases, been proposed to serve as a membrane platform for viral replication complexes (54, 144).

It follows that the composition and activities of host proteostasis networks can determine whether a viral protein will properly fold and become functional, or get degraded via the quality control pathway. This interplay can be especially important when a mutation gives rise to a viral protein with folding defects, as most mutations do. How host proteostasis networks might affect viral evolution can be visualized by plotting a theoretical viral protein's mutational change versus the protein's ability to access a functional, folded state (25, 36, 37) (**Figure 4*a***). The virus becomes more fit as it adapts to a new environment by acquiring functionally beneficial mutations, but those same mutations often compromise the relevant viral protein's foldability. If the mutation results in a protein variant with severe folding defects, the resulting protein will be purged via purifying selection, no matter how advantageous the new function this variant would otherwise confer. This sequence space accessible to a viral protein, which is limited by protein foldability, can potentially be expanded or restricted by changes in the composition and activities of the host proteostasis networks that help viral proteins navigate these biophysical challenges. For example, elevated levels of chaperones that promote folding could expand the accessible sequence space by assisting the folding of biophysically defective viral protein variants (**Figure 4*b***). On the other hand, enhanced levels of quality control factors could restrict the accessible sequence space by more rapidly targeting viral proteins with folding defects to degradation (**Figure 4*c***). Thus, by directly shaping the sequence space an evolving protein can access, host proteostasis networks function as a crucial force determining the evolutionary trajectories available to a virus (64–68).

4. CELLULAR PROTEOSTASIS NETWORKS CAN SHAPE THE EVOLUTION OF ENDOGENOUS PROTEINS

The concepts undergirding the proteostasis paradigm for viral evolution are founded on groundbreaking work showing that cellular proteostasis factors can shape the evolution of endogenous

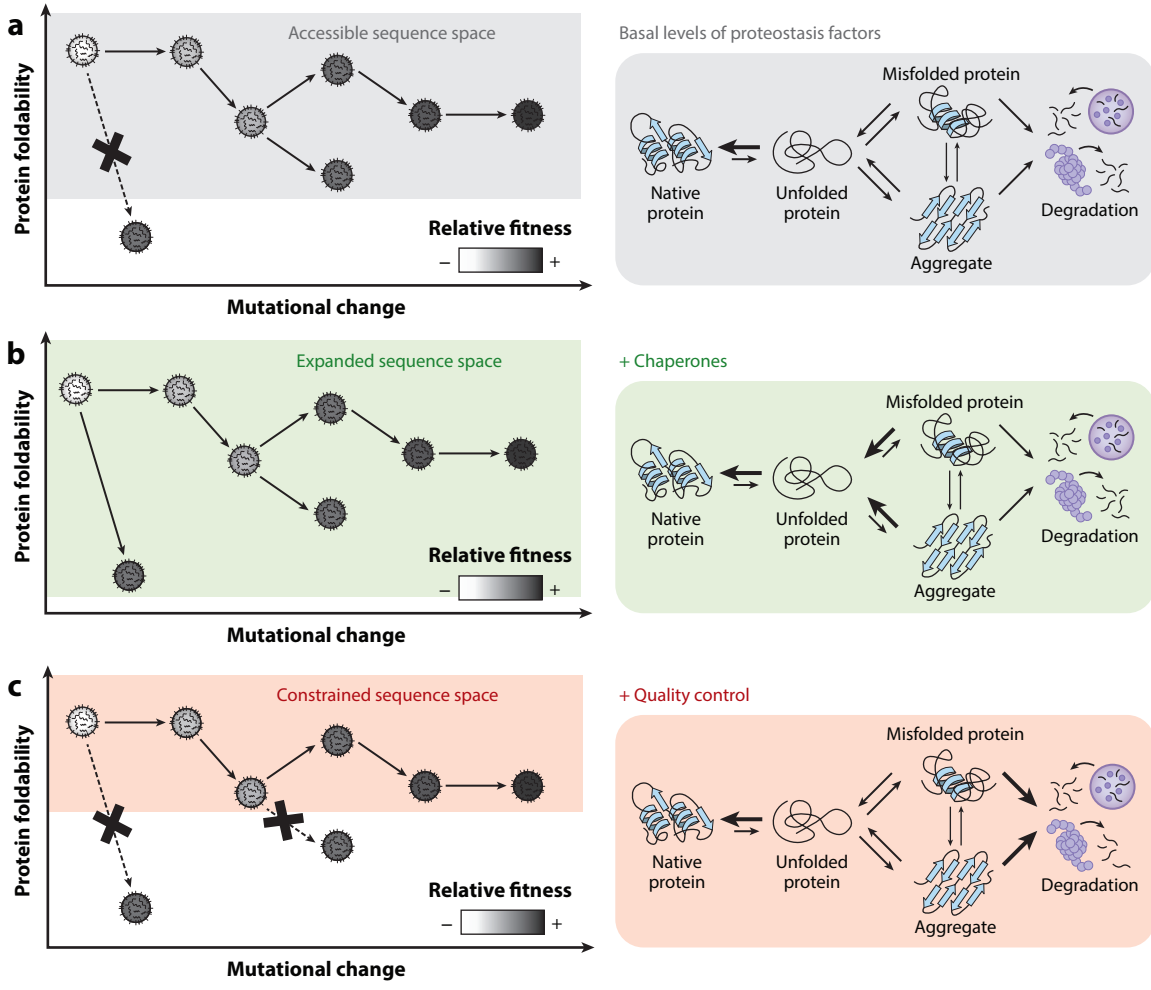


Figure 4

Proteostasis networks can influence the sequence space accessible to client proteins and thereby shape their evolution (25). (*left*) Viruses can sustain adaptive mutations (*x*-axis) that influence their relative fitness (gradient scale). However, if a mutation results in a protein variant with severe folding defects (*y*-axis), the virus harboring that protein variant is not viable (marked by an X in the trajectory), regardless of the potential fitness benefits the mutation would otherwise confer. The extent to which a viral protein can successfully fold thus determines the sequence space accessible to any given viral protein (shaded area of the graph). (*right*) The protein folding landscape can change depending on the compositions and activities of the proteostasis networks. (*a*) Hypothetical accessible sequence space at basal levels of chaperones and quality control factors. (*b*) When chaperones are upregulated, more protein variants can fold into their native conformations and the accessible sequence space expands. (*c*) When quality control factors are increased, more protein variants are targeted for degradation and the accessible sequence space constricts.

client proteins (64–68). Lindquist and others (69–74) pioneered the idea that the Hsp90 chaperone can affect cognate client protein evolution. Specifically, they demonstrated a key role for Hsp90 in modulating the phenotypic variation of diverse organisms by interacting with endogenous proteins (69–74). In some of these studies, Hsp90 was observed to muffle the phenotypic effects of mutations, potentially by allowing defective protein variants to fold successfully. Hence, novel phenotypes can be uncovered when Hsp90 is inhibited (69, 72, 74). In other cases, the potential

buffering effect of Hsp90 makes certain adaptively beneficial mutations accessible only when Hsp90 is active and available (70). Nevertheless, the exact molecular mechanism of how Hsp90 affects evolution at the organismal level was not clearly identified in these earlier studies. For example, the observed phenomena could be due to a primary effect (i.e., a direct role for Hsp90 in folding the evolving client protein) or a secondary effect (Hsp90 perturbation altering the folding or behavior of other cellular proteins that then affect the activity of the evolving protein).

Building on this paradigm, several other chaperones were identified to affect the evolution of endogenous client proteins. In *Escherichia coli*, overexpression of DnaK, an *E. coli* homolog of Hsp70, increases tolerance to nonsynonymous mutations (75). GroEL/ES overexpression can enhance organismal fitness upon accumulation of random mutations (76) and promote genetic variation and client protein evolution (77) under specific conditions (78, 79) or reduce genetic diversity by purging deleterious mutations (80). In *Saccharomyces cerevisiae*, genes encoding chaperone clients diverge faster and accumulate more genetic diversity than do genes for nonclients (81). Additional computational work has further supported the idea that chaperone-mediated protein folding can affect the evolutionary dynamics of endogenous client proteins (82–84).

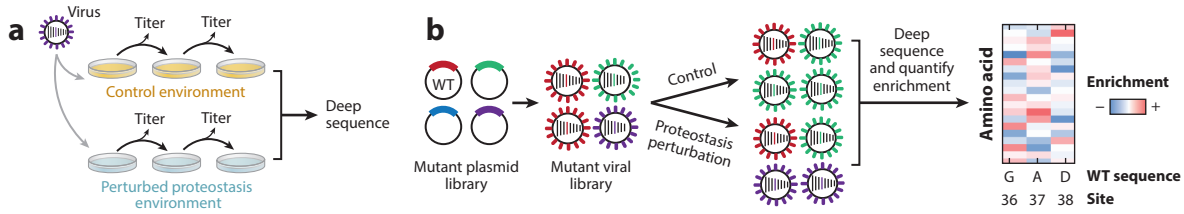
Although there are fewer studies focused on quality control factors, such factors can also alter the sequence space accessible to endogenous proteins. In particular, the *E. coli* quality control factor Lon protease affects the mutations accessible to the metabolic enzyme dihydrofolate reductase (DHFR) (85). For example, trimethoprim-resistant mutations with low in vivo stability are more fit when Lon is deleted (86). On the other hand, many advantageous point mutations in DHFR become inaccessible upon reintroduction of Lon in an *E. coli* strain that naturally does not express Lon (87). Lon can also affect the magnitude and direction of epistatic mutations in DHFR (88).

5. HOST PROTEOSTASIS NETWORKS SHAPE VIRAL EVOLUTION

Building on foundational studies that established that (*a*) folding biophysics constrains what mutations are tolerated, (*b*) many viral proteins rely on host proteostasis networks to fold, and (*c*) proteostasis factors shape the evolution of endogenous proteins, studies emerging in the 2010s began to reveal that host proteostasis networks can shape the evolution of viral proteins.

5.1. Hsp90 Can Rescue Defective HIV Protein Variants

Early studies focused on specific, important substitutions in viral proteins that reduce fitness and used a high-throughput approach to identify host chaperones that can rescue the viruses carrying these mutations. Joshi & Stoddart (89) investigated mutations in HIV protease that make it resistant to the antiviral drug ritonavir but also result in decreased proteolytic activity. HIV viruses carrying these protease variants exhibit reduced infectivity owing to incomplete cleavage of capsid proteins (which are substrates of protease), but their replication can be rescued by T cell activation (90). To identify specific host factor(s) responsible for viral rescue, the authors generated a complementary DNA library from activated T cells and used a genetic screen and validation experiments to show that HSP90AB1 overexpression rescued protease-mutant virus. Interestingly, pharmacologic inhibition of Hsp90 restricted the replication of both wild-type and protease-mutant viruses, but the IC_{50} was sevenfold lower for the protease-mutant virus, suggesting that Hsp90 plays a particularly important role in determining the accessibility of ritonavir-resistant mutations. In a follow-up study, Joshi and colleagues (91) further tested whether HSP90AB1 expression could rescue HIV with various capsid protein mutations that reduce infectivity. Similar to observations with protease-mutant virus, HSP90AB1 expression rescued HIV harboring any of the tested capsid protein variants but with varying effect sizes. These results were among the first to suggest that host chaperone levels and activities may be able to alter the relative fitness of different viral variants.



c

	Advantages	Disadvantages
Serial passaging	• Can be used to detect epistatic mutations • Mimics evolution in nature	• Limited in number of variants that can be tested • Subject to stochasticity of evolution
DMS	• Tests almost all single amino acid variants • Identifies mutations that could be evolutionarily (dis)favorable	• Difficult to detect epistatic mutations • Does not mimic evolution in nature

Figure 5

Methods for studying the evolution of viral proteins in the context of proteostasis perturbation in a laboratory environment include (*a*) serial passaging and (*b*) DMS. (*c*) There are advantages and disadvantages to both methods, depending on the type of information one wishes to obtain from the experiment. Abbreviations: DMS, deep mutational scanning; WT, wild type.

5.2. Host Proteostasis Factors Shape Viral Evolutionary Trajectories

Concrete evidence that host proteostasis networks shape the evolution of viruses was provided via long-term serial passaging experiments with influenza and poliovirus (65, 68). In these serial passaging experiments, cells with altered proteostasis environments were created using small molecule chaperone inhibitors or via chemical genetic control of proteostasis network-regulating transcription factors (36, 92, 93) (**Figure 5*a***). The cells were then infected with viruses and, after a defined growth period, the resulting viral pools were titered and used to infect fresh cells displaying the same proteostasis environment as the first passage. This process was iterated for multiple passages, and the final (and sometimes intermediate) viral populations were collected and sequenced to investigate the identity and frequency of mutations accumulated.

Using serial passaging of influenza, Phillips et al. (65) showed that the mutational trajectories of viral proteins strongly depend on the composition and activities of the host's proteostasis network. In this experiment, influenza evolution was studied in three distinctive host proteostasis environments. First, a constitutively active form of HSF1 was inducibly expressed using destabilizing domain technology (93), resulting in upregulation of the cytosolic and nuclear proteostasis machineries—especially chaperones. Second, the small molecule STA9090 (94) was used to pharmacologically inhibit Hsp90, resulting in loss of a key host chaperone's activity (see the sidebar titled Pharmacologic Inhibition of Hsp90). Third, cells with unperturbed proteostasis environments were used.

Influenza A serial passaging under these conditions resulted in a rate of adaptation, assessed as the frequency at which nonsynonymous mutations became fixed, that was significantly lower when Hsp90 was inhibited than the rate in the other two environments. On the other hand, the rate of fixation for synonymous mutations did not significantly depend on the host cell proteostasis environments. To decrease the effect of evolutionary stochasticity across replicates, Phillips et al. (65) additionally analyzed the mutational trajectories of variants present in at least 20% of the founder virus in all experimental replicates. They observed that different high-frequency variants were fixed when HSF1 was activated versus when Hsp90 was inhibited. The relative fitness of select variants in distinctive proteostasis environments was validated via a head-to-head competition with the wild-type virus. In addition, deep sequencing of the influenza polymerase proteins

PHARMACOLOGIC INHIBITION OF Hsp90

While small molecule inhibitors of heat shock protein 90 (Hsp90) are valuable for their high dosability and rapid reversibility, they can lead to problematic pleiotropic effects (36). A major issue is induction of a compensatory stress response via heat shock factor 1 (HSF1) activation, which leads to dramatically increased levels of heat shock response (HSR)-regulated chaperones, including Hsp90 (105). For experiments involving Hsp90 inhibition, great care must be taken to identify small molecule concentrations that do not activate the HSR yet still successfully inhibit Hsp90. Researchers should note that, for some small molecules and some cell types, an acceptable dose range may not exist. Another alternative is the use of chemical genetic HSF1 inhibition in tandem with Hsp90 inhibitor treatment to acutely prevent the compensatory HSR (105).

PA and PB1 showed that certain protein regions accumulated a high density of nonsynonymous mutations only when HSF1 was activated.

Using serial passaging of poliovirus, Geller et al. (68) also showed that Hsp90 plays a critical role in viral evolution. In this study, the Hsp90 inhibitor geldanamycin was used to compare poliovirus evolution in normal versus Hsp90-inhibited cells. The authors focused on how the two proteostasis environments affect the evolution of P1 capsid precursor protein, which is the only poliovirus protein known to interact with Hsp90 (95). The global mutation rate of the entire viral genome, as well as the mutation rate of the P1 gene itself, was unaffected by Hsp90 inhibition. Nonetheless, Hsp90 inhibition was shown to increase the mutational diversity present in P1 and also to alter the available escape mutations on P1 when poliovirus was challenged with neutralizing antibodies.

To illuminate molecular details of this phenomenon, Geller et al. (68) deployed several computational techniques. Their results suggest that Hsp90 inhibition actually increased the frequency of destabilizing variants in P1, but that these variants have significantly lower hydrophobicity and may be less prone to aggregation. This result implies that Hsp90 may mediate a trade-off between P1 stability and aggregation during evolution, as higher hydrophobicity at the core stabilizes the protein but could increase aggregation propensity. Also noteworthy, Hsp90 inhibition during poliovirus serial passaging led to the emergence of synonymous mutations with rare codons in P1 that slow down the local rate of translation, potentially further assisting P1 folding (96).

5.3. Proteostasis Factors Can Alter Viral Protein Mutational Tolerance

Serial passaging is advantageous because it mimics natural viral evolution, allowing researchers to investigate in a controlled manner how different host environments lead to different evolutionary trajectories, including the emergence of epistatic mutations (65, 68, 97). Nevertheless, the approach is limited because generation and quantitation of genotypic diversity rely solely on errors introduced during genomic replication. This feature limits the diversity of mutations that can be assessed, which, together with variant bottlenecking during passaging, can make the observed results highly subject to the stochasticity of evolution.

In a complementary approach, more recent studies have adopted deep mutational scanning (DMS) (64, 66, 67, 98–102) to quantitatively assess the mutational profile of viral proteins in diverse host proteostasis environments. In typical viral DMS experiments, a mutant plasmid library that encodes nearly all possible single amino acid substitutions for a given viral gene is used to generate a mutant viral library (**Figure 5*b***). The viral library is then passaged in cells in the presence and the absence of proteostasis perturbation to allow for batch competition. Finally, the gene encoding the protein of interest is deep sequenced, and the sequencing data are used to quantify the

relative frequency of each substitution. Although DMS does not directly mimic natural evolution in the manner that serial passaging does, it substantially increases the number of variants that can be reproducibly assessed and provides quantitative insight into what mutations are evolutionarily favored or disfavored in different proteostasis environments (**Figure 5*c***).

DMS of influenza A NP showed that the host's HSF1-regulated proteostasis machinery assists influenza to evade the innate immune system (64). In human cells, NP experiences selection pressure from the innate immune system—especially the human innate immune factor Myxovirus A protein (103). In particular, Pro283 is one of the key substitutions in NP that enabled Myxovirus A resistance during the 1918 influenza pandemic and is nearly universally conserved in major modern human influenza strains (104). Phillips, Ponomarenko, and colleagues (64) performed DMS on NP in cells where HSF1 was inhibited using a chemical genetic tool (105) versus in cells with no proteostasis perturbation, at both a permissive (37°C) and a febrile (39°C) temperature. They observed that Pro283 is disfavored at 39°C and that this effect became more prominent when NP folding and stability are further challenged by HSF1 inhibition (64). Molecular dynamics simulations and a thermal aggregation experiment showed that Pro283 NP is biophysically defective, supporting the hypothesis that HSF1-regulated proteostasis factors directly assist the folding (or prevent nonfunctional aggregation) of Pro283 NP. This result demonstrates that the host's proteostasis network can play a critical role in determining the fitness of an innate immune escape mutation of NP, representing a previously unknown viral adaptation strategy.

Similar to cytosolic chaperones affecting the sequence space of cytosolic proteins, it is equally possible that host ER chaperones can determine the sequence space of viral proteins that traverse the secretory pathway. Indeed, DMS of influenza A HA showed that stress-independent, small molecule-mediated upregulation of the XBP1s transcription factor (36, 47, 48, 93, 106) of the UPR enhances the mutational tolerance of HA (66). Moreover, the sites with enhanced mutational tolerance correlate with the sites that experience decreased mutational tolerance upon temperature increase. This differential effect of temperature versus XBP1s upregulation is particularly prominent at antigenic sites on HA, making it interesting to speculate that ER chaperones shape influenza A adaption by buffering thermally unstable mutations important for antibody escape.

Contrastingly, Yoon, Nekongo, and colleagues (67) used DMS to show that XBP1s upregulation globally reduces the mutational tolerance of HIV envelope (Env) protein. Conserved regions of Env, where substitutions are likely to be more biophysically deleterious, exhibit larger depletion of mutations upon XBP1s activation than do variable regions. Env variants that are strongly selected against upon XBP1s upregulation also display trafficking defects and have higher predicted $\Delta\Delta G$ than the variants that are selected for. These results suggest that Env variants with biophysical defects are preferentially targeted for degradation rather than having their folding enhanced by XBP1s-regulated factors, although conclusive evidence is not yet available. Importantly, and opposite to the global trend, some sites on Env displayed an increase in mutational tolerance upon XBP1s upregulation, including an antibody binding site and a fusion peptide inhibitor (antiviral peptides that inhibit the fusion of HIV and the cellular membrane) binding site. This observation indicates that, while XBP1s-regulated proteostasis factors overall decrease the genotypic diversity accessible to Env, they may still sometimes assist viral adaptation depending on the additional selection pressures present (e.g., neutralizing antibodies or antiviral drugs).

5.4. Important Gaps in Knowledge

These recent studies establish that host proteostasis networks can shape the accessible sequence space of evolving viral proteins. Nevertheless, such results have only begun to emerge. Many important gaps in knowledge still exist, providing ample fodder for future work.

HIJACKING CHAPERONES TO AUGMENT LABORATORY-DIRECTED EVOLUTION

A particularly impactful generalization of these principles may be found in the directed evolution field. Modern directed evolution platforms increasingly involve campaigns carried out inside cells or living organisms (145–150). Such platforms provide an opportunity to modulate relevant proteostasis network mechanisms to expand the mutational landscapes that can be explored in target proteins. We may be able to take lessons learned from the proteostasis paradigm in viral evolution to turbocharge these human efforts to evolve better biotechnologies in cell-based contexts.

First, there is no in vivo example showing that host proteostasis factors influence viral evolution. While the available in vitro experiments are important foundational studies, the cell lines used in these experiments may not accurately represent the proteostasis networks that viral proteins interact with in vivo. Proteostasis networks vary across cell types, tissues, and host organisms (107, 108), and they also differ between normal cells and cancer cells (109). Although there are many practical and biosafety constraints associated with in vivo studies, the use of immortalized cell lines or cell types that are different from those that a virus infects in nature can inaccurately or only partially capture the interplay between viral evolution and the host proteostasis factors. Additional in vivo experiments in complex host organisms would significantly enhance our understanding of this interplay.

Second, published work in this field is currently limited to only a few RNA viruses—HIV, influenza, and poliovirus—and often to only a few specific proteins within those viruses. On the host side, available studies are mostly limited to testing the consequences of transcription factor perturbation, such that additional experiments are required to attribute observed phenomena to a specific chaperone or quality control factor. Further investigations into the effect of proteostasis networks on the evolution of additional viruses and viral proteins, particularly DNA viruses and other classes of viral proteins, as well as studies on how specific chaperones and quality control factors shape viral evolution, will be important for elucidating the generalizability of these phenomena (see the sidebar titled Hijacking Chaperones to Augment Laboratory-Directed Evolution).

Third, while it is evident that proteostasis factors can define the relative fitness of different viral protein variants, the molecular mechanism is, at this point, largely left unclear. Most studies lack information showing whether the observed effects are primary or secondary. For example, only a small number of the studies in which viral protein mutational landscapes have been shown to depend on host proteostasis factors are accompanied by experimental work demonstrating actual biophysical defects in select protein variants (64, 67, 68). To the best of our knowledge, no studies to date have tested whether these defective variants actually result in increased interactions with host proteostasis factors, or whether such interaction has a functional consequence. Moreover, there is little information available on how a specific protein folding defect will change whether the protein variant is triaged to chaperones for folding assistance or to quality control factors for degradation. An in-depth understanding of the molecular mechanisms of the interplay between host proteostasis networks and viral evolution will enhance our ability to explain and also predict the pleiotropic outcomes of proteostasis network perturbation.

Fourth, how proteostasis factors might affect synonymous mutations remains largely unexplored. So far, only the poliovirus study observed any effect at all on synonymous mutations upon Hsp90 inhibition (68). Synonymous mutations do not change the amino acid sequence of a protein, but they can still affect protein folding (110). Codon choice puts RNA molecules in distinct fitness landscapes because mutations lead to different amino acid substitutions depending on the

original codon. For example, poliovirus variants with different synonymous substitutions in the viral capsid protein exhibit differences in mutational robustness (111). Moreover, rare codons can decrease the translation rate and allow for independent folding of protein domains and/or may ensure proper interactions with chaperones (96, 112). It will be important for future studies to elucidate whether and how host proteostasis networks affect the accessible synonymous mutations, and what protein folding consequences those mutations have.

6. SELECTED TOPICS IN VIROLOGY WHERE THE PROTEOSTASIS PARADIGM MATTERS

Beyond fundamental understanding, why does the proteostasis paradigm for viral evolution matter? In this section, we briefly highlight a few of the important topics in virology where it is critical, going forward, to understand the contribution of host proteostasis networks.

6.1. Perturbation of Proteostasis Networks as Therapeutic Adjuvants

The strong dependence of viruses on host proteostasis factors has led to the hypothesis that chaperone inhibition could itself be a host-targeted antiviral strategy. Indeed, several studies have explored this possibility and even shown that there can be high barriers to viruses acquiring resistance to host chaperone modulators (95, 113–118). Alternatively, and arguably even more promising, chaperone inhibitors could potentially be coadministered with other, virus-targeted, antiviral drugs to decrease the likelihood of resistance. For example, telaprevir, an antiviral drug against hepatitis C virus that inhibits DnaK, lowers the frequency of resistance against rifampicin when the two drugs are coadministered in *Mycobacterium smegmatis* (119). Moreover, some drug-resistance mutations confer resistance by disrupting the biophysical properties of the viral protein in which they occur, and chaperone inhibitors may make those mutations particularly inaccessible (120, 121). Thus, perturbation of host proteostasis networks, such as via pharmacologic inhibition of chaperones, may provide therapeutic adjuvants for use alongside antiviral drugs (or antiviral antibodies) to prevent or delay resistance onset.

6.2. Evolution of Antigenic Escape Variants

Many viral proteins are targeted by neutralizing antibodies and must constantly undergo antigenic evolution. Existing work suggests that host proteostasis factors may play a key role in defining what mutational trajectories are available for antigenic escape of viruses (66–68). In-depth studies on this topic may have particularly important implications for future antibody and vaccine design strategies.

6.3. Mutational Error Catastrophe

RNA viruses mutate on the edge of mutational error catastrophe, meaning that they mutate at such a high rate that even modest enhancements to their mutation rates can lead to a rapid loss of viral viability, at least partly owing to production of biophysically deleterious protein variants (122–129). Several small molecules have been developed to target RNA viruses and cause error catastrophe, some for therapeutic applications (122–125, 130). It would be both fundamentally and translationally useful to investigate how alteration of global host proteostasis networks, or of individual network components, influences the susceptibility of an RNA virus to mutational error catastrophe.

6.4. Viral Host Switching

Viral host switch: evolutionary change of a virus to infect a new host species; also termed zoonotic transmission if the host switch occurs between animals and humans

RNA chaperones: a class of nucleic acid binding proteins that can mitigate RNA misfolding problems without being present in the final folded structure

Viruses face many adaptive challenges during viral host switch (131). A key challenge is maintaining proper protein folding in the context of their new host's proteostasis machineries. Understanding the role of host proteostasis machineries during host switch may help us answer interesting questions, including but not limited to the following: (*a*) Some viruses may strictly require specific cognate host chaperones, a phenomenon that has indeed been observed in the bacteriophage space (132, 133). How does this feature limit their host switching capacity? (*b*) Is there a causal relationship between bats serving as viral reservoirs and their tissues expressing high levels of HSPs (134, 135)? (*c*) Can we predict zoonotic transmission based on how conserved proteostasis network components are across different organisms? (*d*) Do cellular proteostasis network compositions, which vary in a cell type–specific manner even within a given host, shape the cell tropism of a virus?

6.5. RNA Structure and RNA Chaperones

Just like proteins, RNA structures exist in a dynamic equilibrium between diverse conformational ensembles, and RNA molecules also face unique folding challenges (136, 137). Proper maintenance of viral RNA structure (or structural flexibility) is required for successful viral replication, especially for RNA viruses (138, 139), and it is known that both virus-encoded and host cell RNA chaperones play diverse roles in viral life cycles (140). It is possible that host RNA chaperones shape the accessible sequence space, at the nucleotide level, for viral RNA molecules and thereby shape viral evolution, in analogy to the interplay between host chaperones and viral proteins.

7. CONCLUSIONS

Cellular proteostasis machineries are sophisticated actors that affect the folding biophysics of viral proteins. When a virus acquires biophysically deleterious mutations, host proteostasis machineries can determine the fate of the resulting protein variant. This phenomenon then shapes the evolutionary trajectories available to a virus at the molecular level. By studying the interplay between host proteostasis machineries and viruses, we learn about an underappreciated force that crafts how viral diversity manifests, which is a key component of understanding and predicting virus evolution.

To date, only a limited number of virus–host systems have been studied in this field. Nevertheless, these studies clearly show that the virus–host proteostasis network interaction is of tremendous importance for understanding virus evolution in general, as well as for developing better therapeutics and predicting viral evolution. Future studies may seek to establish an exhaustive understanding of virus–proteostasis network interactions from across the biosphere, as well as how these interactions impact viral adaptation. In the long run, the proteostasis paradigm should be implemented into our broader understanding of viral drug and immune system resistance, viral host switching and pandemics, and viral success on Earth (141).

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation (NSF) CAREER award 1652390 (to M.D.S.) and National Institutes of Health grants R35GM136354 (to M.D.S.) and R01AI168166

(to M.D.S. and C.B.O.). J.Y. was supported by a Kwanjeong overseas scholarship and a Massachusetts Institute of Technology (MIT) Mathworks graduate fellowship. C.B.O. also acknowledges support from NSF Division of Environmental Biology grant 2142719 and from the Martin Luther King, Jr. Visiting Professors and Scholars Program at MIT.

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