

Tuning the course of evolution on the biophysical fitness landscape of an RNA virus

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1 ABSTRACT

2 Predicting viral evolution remains a major challenge with profound implications for public
3 health. Viral evolutionary pathways are determined by the fitness landscape, which maps
4 viral genotype to fitness. However, a quantitative description of the landscape and the
5 evolutionary forces on it remain elusive. Here, we apply a biophysical fitness model based
6 on capsid folding stability and antibody binding affinity to predict the evolutionary
7 pathway of norovirus escaping a neutralizing antibody. The model is validated by
8 experimental evolution in bulk culture and in a drop-based microfluidics device, the
9 “Evolution Chip”, which propagates millions of independent viral sub-populations. We
10 demonstrate that along the axis of binding affinity, selection for escape variants and drift
11 due to random mutations have the same direction. However, along folding stability,
12 selection and drift are opposing forces whose balance is tuned by viral population size. Our
13 results demonstrate that predictable epistatic tradeoffs shape viral evolution.

14

15 INTRODUCTION

16 Responding to viral pandemics or to the emergence of new microbial pathogens is a major
17 challenge to public health¹. A critical component to this response is the prediction of the course
18 of microbial evolution. One approach to this prediction uses available genomic samples and
19 branching patterns of genealogical trees to statistically project future dominant strains².
20 However, this approach can only infer the likelihood that an existing viral strain will dominate,
21 and cannot predict emergence of novel strains that might become dominant due to *de novo*
22 beneficial mutations. It is these novel strains that are often the most virulent, posing the greatest
23 hazard to public health. Predicting the evolution of novel strains is the key to addressing this
24 challenge and requires knowledge of the relationship between mutations in the viral genome and
25 the fitness for individual organisms³. This relationship is the fitness landscape, which is a
26 complex, multidimensional function; however, this can rarely be quantitatively determined.
27 Nevertheless, it is essential for predicting selection of the most probable mutants. Moreover, the
28 fate of mutations is also a function of population structure⁴. In particular, population size
29 changes the balance between the impact of random mutations on fitness and that of selection⁵

1 and is thought to affect both the rate and direction of evolution⁶. Recent studies use microbial
2 fitness landscapes⁷⁻⁹ or population structure^{10,11} to predict the course of evolution, but to date,
3 none links these elements together. Without this link, further progress in predicting the course of
4 viral evolution is significantly hindered.

5 In this paper, we quantitatively determine a fitness landscape for an RNA virus subjected to the
6 environmental pressure of a neutralizing antibody, and use it to account for the evolution of the
7 virus under conditions that constrain population size. The experimentally measured fitness
8 landscape is correctly described by two biophysical parameters: the thermodynamics of folding
9 of the capsid protein and its binding to the antibody. We probe the evolution of a model
10 norovirus both in bulk, where population size is large, and in a microfluidic evolution chip which
11 uses small drops to perform millions of experiments¹²⁻¹⁶ that probe evolution in very small
12 population sizes. We show that the dynamics of viral adaptation is strongly dependent on
13 population size. These results can be quantitatively described by a theoretical framework that
14 combines protein biophysics and population genetics, providing the critical link between fitness
15 landscape and population structure that enables prediction of evolution.

16 We focus in this work on Murine Norovirus (MNV), a model for human RNA viruses which are
17 the major cause of epidemics in the world¹⁷⁻¹⁹. MNV is a non-enveloped RNA virus that consists
18 of 180 copies of the capsid protein assembled around a 7.5kb long positive-strand RNA genome.
19 It mutates at ~1 base per genome per replication cycle and produces ~10⁴ progenies in a single
20 cell infection, of which ~100 are infectious viral particles, or plaque forming units (pfu)¹².

21 RESULTS

22 Lab evolution of Norovirus in large and small population sizes

23 To study viral evolution, we propagate a viral isolate (MNV-1, denoted *wt*) in the presence of a
24 neutralizing antibody (mAb6.2,²⁰) that binds to the protruding domain (P-domain) of the capsid,
25 and prevents virus entry into the host cell^{21,22}. This set-up allows us to study the way the virus
26 evolves to adapt to a new environment. To investigate the dynamics of this escape from the
27 antibody, we sequence a 376 bp fragment of the genome encoding the outermost part of the P-
28 domain (residues 281 – 412 of VP1) and follow the frequency of 37,244 unique haplotypes

1 (Figure 1-figure supplement 1 and Table S1) observed over several passages, allowing us to
2 follow the evolution over several generations. First, we propagate *wt* in standard bulk culture
3 conditions, using $\sim 10^6$ virions per passage under Ab pressure (Figure 1A). The population is
4 initially dominated by the *wt* ($\sim 90\%$ of the population) with the rest of the viral quasi-species
5 consisting of single and double mutants (Table S1). After 2 passages the total number of
6 surviving viruses has decreased significantly due to the neutralizing effect of the Ab
7 (Figure 1-figure supplement 2); however, three single mutants E296K (A), D385G (B), T301I
8 (C) as well as their double mutants (AB, AC, BC) occur at higher frequencies than the other
9 haplotypes. By the fourth passage the triple mutant ABC, which first arises on passage 2, occurs
10 at a frequency even higher than the other mutant haplotypes (86%); moreover, the total number
11 of viruses increases to levels comparable to those observed after the first passage
12 (Figure 1-figure supplement 2). This suggests that ABC is an escape variant.

13 A central tenet of evolutionary theory is that the way organisms explore their fitness landscape
14 depends on the size of their population, which controls the balance between random mutational
15 drift (i.e., direction of *randomly arising* mutations) in the population and fitness driven selection
16 (i.e., direction of beneficial mutations)^{4,5}. This balance determines the most likely evolutionary
17 pathways on a given fitness landscape. Indeed, the population size may be particularly important
18 for noroviruses where a single viral particle is sufficient to infect the host animal²³; thus it is
19 possible that viruses propagate in very small populations as they adapt to a new environment
20 prior to the emergence of an epidemic.

21 The consequences of a smaller population size are captured by the S. Wright's shifting balance
22 theory⁶, which hypothesizes that evolution can proceed more efficiently in three phases: i) a
23 population is divided into subpopulations to weaken selection and increase drift, ii) each
24 subpopulation evolves independently, whereupon iii) the subpopulations are mixed back into a
25 large population and then all individuals compete. We can directly probe this hypothesis
26 experimentally by drastically reducing population size compared to typical laboratory bulk
27 cultures, which propagate $\sim 10^6$ to 10^8 viruses (Figure 1-figure supplement 2). To evolve
28 viruses in small population sizes we use a novel microfluidics system, the "Evolution Chip",
29 which propagates $\sim 10^6$ subpopulations of 1-10 infectious particles (pfu) in distinct and non-
30 mixing compartments (Figure 1B, Figure 1-figure supplement 3, Movie S1 and Movie S2).

1 The microfluidics system allows us to drastically reduce the population size without reducing the
 2 total number of viruses sampled, thereby maintaining the statistics comparable to that of a bulk
 3 experiment.

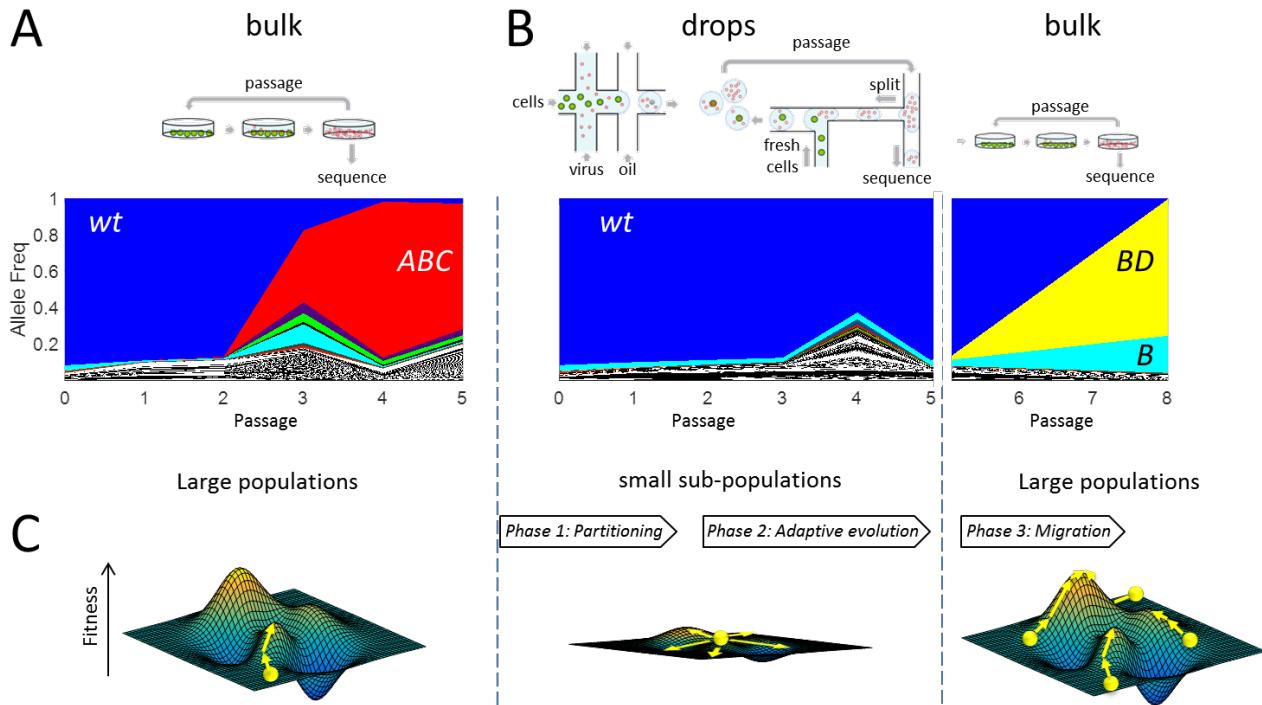


Figure 1. Wright's shifting balance theory (SBT) - Viral evolution in large and small population sizes.

A) Top: 10^6 viruses evolving against a neutralizing antibody in bulk by serial propagation. Bottom: The allele frequencies of 1,364 distinct P-domain haplotype sequences are plotted per passage (see also Figure S2). **B) Viral evolution in small populations.** Top: Experimental scheme of the three phases of Wright's SBT - Phase 1: 10^6 pico-liter drops are loaded with on average 1 virus and 2 host cells per drop. Phase 2: viruses evolve in drops for five passages. Phase 3: the emulsion is broken and all lineages evolve together in bulk for 3 additional passages (frequencies for passages 6 and 7 are interpolated from passages 5 and 8, see also Figure S3 and Movie S1, S2). Bottom: The allele frequencies of 620 distinct P-domain haplotypes from B (Phase 2, center panel, phase 3 right panel) are plotted per passage. (Figure S2). **C) Exploration of the fitness landscape for the three phases of SBT - phase 1 (left):** a previously adapted population (lower peak on left fitness landscape) is partitioned into smaller subpopulations. **phase 2 (middle):** small sub-populations adapt in isolated conditions where selection pressure is reduced, allowing an extended exploration of the fitness landscape. **phase 3 (right):** subpopulations migrate, mix and compete, evolving new and more fit variants that were explored in isolation (highest peak). Haplotype legend: A: E296K, B: D385G, C: T301I, D: A382V.

The following supplements are available for Figure 1:

1 **Figure 1-figure supplement 1. Overview of the experimental set-up**

2 **A)** RAW 264.7 cells exposed to MNV-1 virus are infected and viral progenies are released into
3 the supernatant. To passage viruses, a small volume fraction of the supernatant is transferred to
4 a new sample of fresh cells. The viral genomes in the remaining supernatant are prepared for
5 sequencing. **B)** The 7.5kb long MNV-1 genome includes 4 main reading frames with the capsid
6 protein gene (VP1) residing in the second frame. For our purposes, we sequence a 376bp long
7 fragment in VP1 that encodes part of the protruding domain (P domain) of the capsid protein. **C)**
8 *Left:* Cryo-EM structure of MNV capsid, adapted from ²¹. *Right:* Model of MNV made by fitting
9 the P-domain of MNV-1 ²⁴ and the shell domain from the Norwalk virus structure²⁵ into the
10 cryo-EM density of MNV-1 ²².

11 **Figure 1-figure supplement 2. Allele frequency and viral RNA concentration from**
12 **experimental viral evolution in bulk and drops**

13 **A)** The allele frequencies of 1,364 distinct P-domain haplotypes of the evolution in bulk
14 (Figure 1A) are plotted per passage, with the y-axis plotted in log-scale to show the polyclonal
15 structure of the viral quasi-species. **B)** The allele frequencies of 620 distinct P-domain
16 haplotypes of the evolution in drops (Figure 1B) are plotted per passage, with the y-axis plotted
17 in log-scale to show the polyclonal structure of the viral quasi-species. We note that in (A) and
18 (B), similar to primary Figure 1, dominant haplotypes are individually color coded but do not
19 appear due to log transformation of Allele frequency. **C, D)** The concentration of viral RNA in the
20 experimental samples is plotted per passage for both the evolution in bulk (C) and that in drops
21 (D).

22 **Figure 1-figure supplement 3. “Evolution Chip”: a system for evolving virus in very small**
23 **populations**

24 *Left:* Virus and host cells co-encapsulation. *Middle:* Drop incubation. *Right:* Drop passaging. All
25 scale bars are 100 μm .

26 **Table S1.** Complete list of MNV-1 variants from deep sequencing

27 **Movie S1.** Encapsulation of virus and cells in drops

28 **Movie S2.** “Evolution Chip” for serial passaging in drops

1 In stark contrast with the bulk experiments, amplification and hence growth of potential escape
2 variants that sweep the population is precluded when each variant is confined in a single drop
3 with just two host cells; as a result, the *wt* remains the dominant fraction of the observed viruses
4 through all passages. Potential escape viruses are present, but are in complete isolation from each
5 other, at population sizes of just a single infection event per generation. This microfluidic
6 experiment (**Figure 1B**) implements the first two phases of the shifting balance theory⁶: i)
7 partitioning the population in drops to weaken selection and increase genetic drift and ii)
8 evolving the sub-populations without competition between drops. However, the absence of
9 competition precludes detection of potential escape variants. To overcome this, we break the
10 emulsion after five passages in the evolution chip, mix the contents of all the drops and
11 propagate the sample in the presence of Ab under bulk conditions for three additional passages
12²⁶. This enables new escape variants that evolved in isolation to take over the integrated
13 population and facilitates their identification, isolation and characterization. This also
14 implements the third phase of the shifting balance theory: iii) mixing the subpopulations back
15 into a large population. Remarkably, after mixing, a double mutant, D385G-A382V (*BD*),
16 sweeps the population; (**Figure 1B**) this is in sharp contrast with the standard bulk cultures
17 where the triple mutant *ABC* sweeps the population.

18 Next, we determine if the escapees in drops are more fit than the escapees in bulk. To address
19 this question, we engineered the mutations into the infectious clone and recovered mutant
20 viruses. Next, we performed head-to-head competition of *wt*, *BD*, and *ABC* variants. We show in
21 **Figure 2** the frequency of each of the three clones at the end of the competition. Indeed, *BD* and
22 *ABC* are true escape variants since they outcompete *wt* under neutralizing antibody. However,
23 without antibody, *wt* is more fit than both *BD* and *ABC*, which explains the observation that
24 neither *BD* nor *ABC* spontaneously arise in serial passaging without Ab. More importantly, in the
25 competition between *BD* and *ABC*, we find that the escapee from droplets is more fit compared
26 to the escapee from bulk (Figure 2). Despite being more fit, *BD* is not detected in any of the bulk
27 serial passaging under neutralizing Ab, while *ABC* was observed thrice. We hypothesize that the
28 initial acquisition of the mutation D (enroute to *BD*) is limited in large populations, because
29 epistatic interactions dictate that the virus traverse a low fitness regime, before climbing up a
30 local fitness peak. Indeed, such unlikely pathways on the landscape can be accessed if selection
31 is weakened by decreasing population size as Wright originally envisioned.

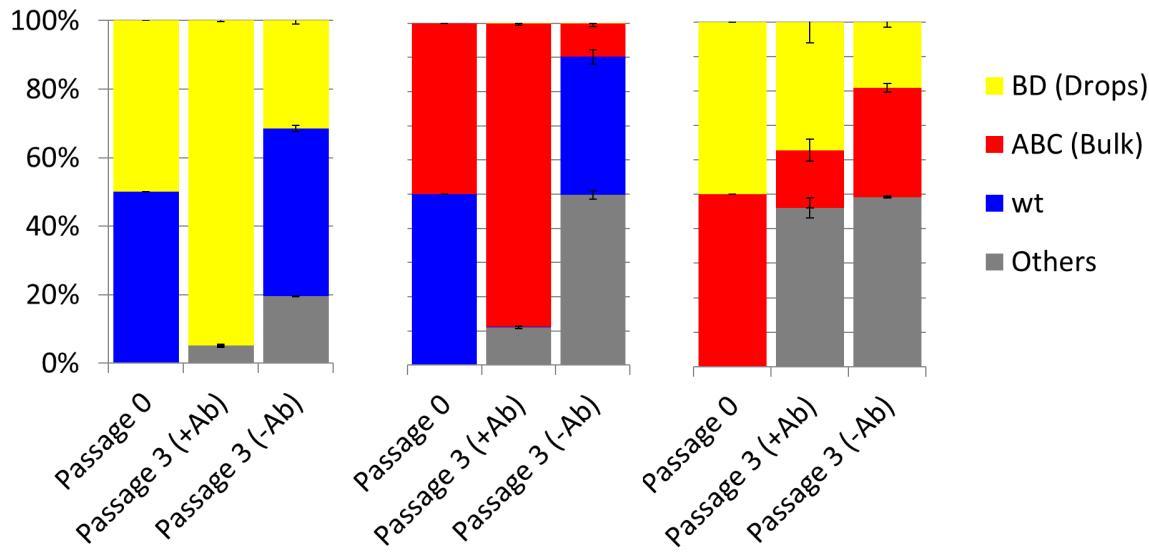


Figure 2. Allele frequency of the clone after 3 passages in competition assays. To perform pairwise competition of the clones, we mixed equal titers of the clone, propagate them for 3 passages, and then perform deep sequencing. Error bars are S.E. of 3 biological replicates for each measurement. See also Table S2.

The following supplement is available for Figure 2:

Table S2. Analysis for head to head competitions

Fitness landscape of norovirus escaping an antibody is projected onto the biophysical properties of its capsid domain

These results provide strong qualitative support to Wright's shifting balance theory showing that the evolutionary dynamics and outcome dramatically depend on the population structure. To understand these dynamics quantitatively we must first develop a tractable model for the fitness landscape of the virus. In general, fitness is expected to be a complex function of multiple traits. Instead we focus on the dependence of viral fitness in the presence of a neutralizing antibody on two biophysical properties of the P-domain: The folding energy, which is a measure of stability, and the binding affinity to the antibody, which is a measure of neutralization. While the importance of binding affinity to antibody is apparent, the universal importance of protein folding stability for bacterial and viral fitness was also shown^{8,27}. This choice of variables is further supported by the fact that all the mutations of the dominant escape variants we observe in

- 1 our experiments are located within the binding site between the P-domain and the Ab, as shown
- 2 by mapping the mutations on the 3D structure of the *wt* P-domain in **Figure 3A**.

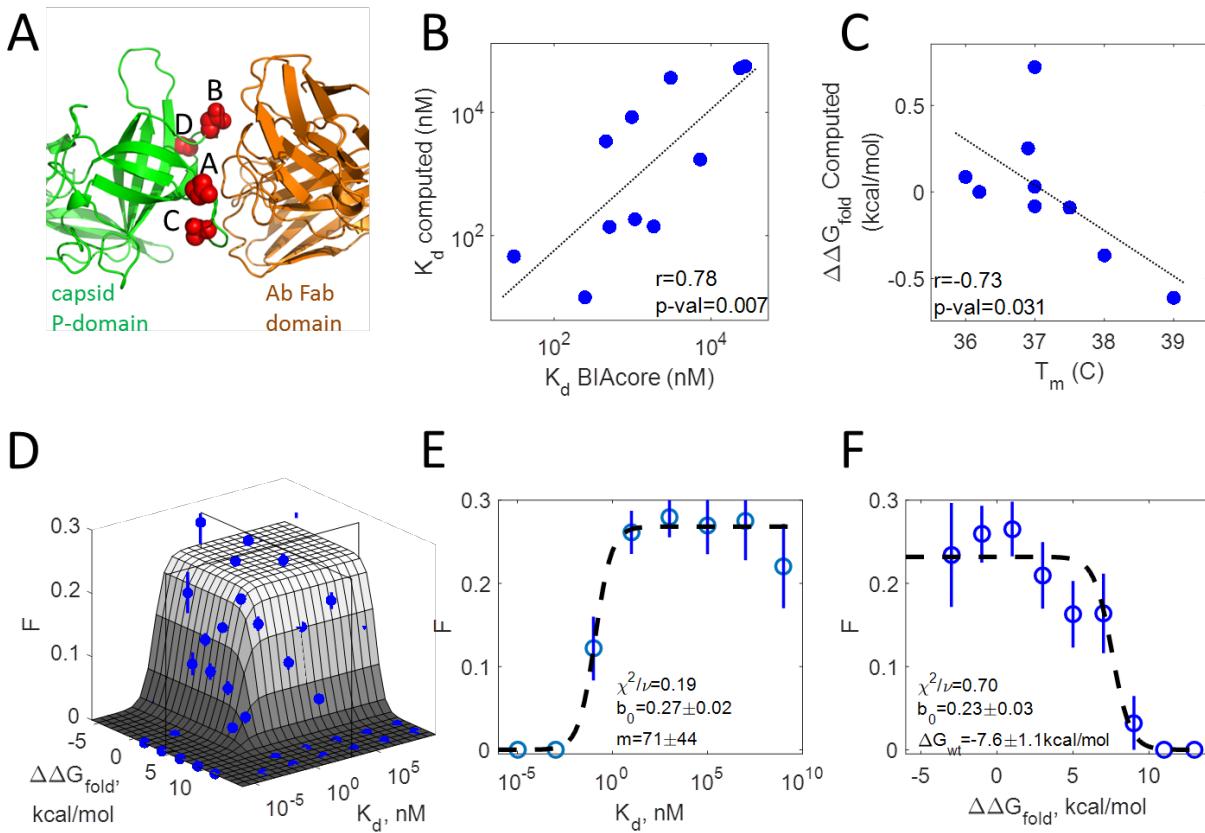


Figure 3. Fitness landscape of norovirus escaping a neutralizing antibody

A) The P-domain Antibody complex structure. The SNPs of all dominant P-domain variants (red circles) are located on the docking site of the P-domain-antibody complex (PDB ID: 3LQE). **B)** A high correlation exists between Ab dissociation constant K_d that was experimentally measured using surface plasmon resonance (SPR) and the one computed from force field calculations **C)** The anti-correlation between the experimentally measured P-Domain melting temperature (T_m) and the folding stability computed from force field calculations. Two outlier variants were excluded from the analysis. **D)** A 3D plot of the probability of infection F averaged over 2,076 distinct haplotypes binned according to their dissociation constant K_d and folding stability ΔG_{fold} (blue points) overlaid with the theoretical fit according to Eq. 1 (gray surface). Cross sections (black frames) mark the regions used for the projections in B and C. **E)** The probability of infection for all haplotypes with $\Delta G_{fold} < 4.5\text{Kcal/mol}$ (cross section parallel to K_d axis in A) is projected on the K_d - F plane, binned according to their K_d (blue points) and overlaid with the theoretical fit to Eq. 1 (dashed line). **F)** The probability of infection for all haplotypes with $K_d > 10^3\text{nM}$ (cross section parallel to ΔG_{fold} axis in A) is projected on the ΔG_{fold} - F plane, binned according to their ΔG_{fold} (blue points) and overlaid with the theoretical fit to Eq. 1 (dashed line).

is determined from deep sequencing lysates of *in vitro* experiments in the presence of neutralizing antibody. K_d and ΔG_{fold} are estimated from mapping the haplotype mutations to the 3D structure of the capsid P-domain in complex with the neutralizing antibody.

The following figure supplements are available for Figure 3:

1 **Figure 3-figure supplement 1. Distribution of viral fitness estimated from deep**
2 **sequencing**

3 **A)** Distribution of 3,770 growth rates calculated from the first 3 passages of all evolution
4 experiments. **B)** The number of haplotypes that were binned in each 2D bin of **Figure 3D** is
5 color coded with a logarithmic gray scale representation.

6 **Figure 3-figure supplement 2. Fitness landscape of MNV-1 in the absence of a**
7 **neutralizing antibody**

8 **A)** A 3D plot of the probability of infection (z-axis) averaged over ~2,000 distinct haplotypes
9 binned according to their dissociation constant K_d and folding stability $\Delta\Delta G_{fold}$ (blue error bars)
10 overlaid with the theoretical fit according to **Equation 1** (gray surface). Cross sections (black
11 boxes) border the regions used for the projections in B and C. **B)** The probability of infection for
12 all haplotypes with $\Delta\Delta G_{fold} < 8 \text{ kcal/mol}$ (cross section parallel to K_d axis in A) projected on
13 the K_d -z plane, binned according to their K_d (blue error bars) and overlaid with the theoretical
14 fit according to **Equation 1** (dashed line). **(C)** The probability of infection for all haplotypes is
15 projected on the $\Delta\Delta G_{fold}$ -z plane, binned according to their $\Delta\Delta G_{fold}$ (blue error bars) and
16 overlaid with the theoretical fit according to **Equation 1** (dashed line). Probability of infection is
17 determined from deep sequencing lysates of *in vitro* experiments in the absence of neutralizing
18 antibody K_d and $\Delta\Delta G_{fold}$ are estimated from mapping the haplotypes' mutations to the 3D
19 structure of the capsid P-domain in complex with the neutralizing antibody.

20 To calculate the folding energy of the P-domain and its binding affinity to the antibody for each
21 haplotype sequence, we use force field calculations based on the structural mapping in
22 **Figure 3A**^{26,28} to determine the change in folding energy $\Delta\Delta G_{fold}$ between the mutant and the *wt*;
23 from this we determine ΔG_{fold} of the mutant by adding the folding energy of the *wt*, ΔG_{wt} . We
24 also determine the change in binding energy, $\Delta\Delta G_{bind}$, between the mutated P-domain-Ab
25 complex and the *wt*; from this we determine the dissociation constant $K_d = K_0 \exp(\beta\Delta\Delta G_{bind})$
26 where K_0 is the dissociation constant of the *wt*. We test the accuracy of our calculations by
27 comparing the calculated biophysical properties of the escape haplotypes to experimentally

1 measured properties. To accomplish this, we express and purify the P-domain of each
2 haplotype²⁹ and measure its binding to the Ab to extract K_d ; we also measure the melting
3 temperature of the P-domain, T_m , which correlates inversely to ΔG_{fold} (**Table S3** and ref.³⁰). The
4 measured values of the biophysical properties of the dominant escape haplotypes correlate
5 strongly with the calculated values of the same haplotypes, as shown in **Figure 3B-C**.
6 Importantly, we reverse engineer the escape viruses with their haplotype sequences on the
7 background of the *wt* for the rest of the virus and confirm that the observed mutations in the P-
8 domain are directly responsible for their increase in fitness both *in vitro* (**Figure 4A** and **Figure**
9 **4-figure supplement 1A**) and *in vivo* in mice (**Figure 4B** and **Figure 4-figure supplement 1B**);
10 thus, our biophysical variables are relevant for viral fitness inside the real animal host.

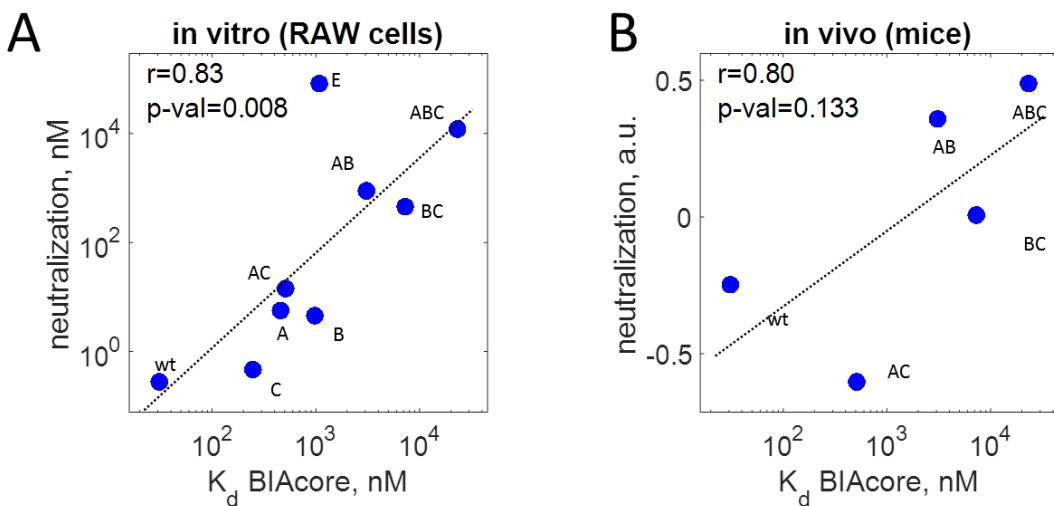


Figure 4. MNV-1 neutralization versus binding affinity of the P-domain to neutralizing antibody

A) *in vitro* neutralization of dominant haplotypes correlates to their K_d and the average ratio between them is ~ 120 , in good agreement with the modeled value of $m \approx 70$. E: L386F **B)** *in vivo* neutralization of dominant haplotypes in mice correlates to their K_d .

The following figure supplement is available for Figure 4:

11 **Figure 4-figure supplement 1. MNV-1 neutralization versus folding stability of the P-**
12 **domain**

13 **(A)** MNV-1 neutralization in RAW cells correlates with T_m . Using reverse engineering, we
14 introduce the dominant SNPs (**Figure 3A**) on the background of *wt* MNV-1 and measure their
15 effective neutralization (see *Methods*). **(B)** MNV-1 neutralization in mice weakly correlates with
16 T_m (see *Methods*).

1 The biophysical fitness landscape describes the dependence of viral fitness in the presence of a
2 neutralizing antibody on ΔG_{fold} and $1/(mK_d)$, where the parameter m accounts for the multiple
3 binding sites of the capsid. To formulate the viral fitness we assume that the *wt* P-domain occurs
4 in three specific states: folded and unbound, folded and bound, and unfolded (which is always
5 unbound). The virus infects only when the P-domain is folded and unbound, hence, we can
6 express the viral infectivity F at a given concentration of antibody $[Ab]$ as³¹:

7

$$F = b_0 \frac{e^{-\beta \Delta G_{fold}}}{1 + e^{-\beta \Delta G_{fold}} + \frac{[Ab]}{mK_d} e^{-\beta \Delta G_{fold}}} , \quad (\text{Equation 1})$$

8 where the numerator is the Boltzmann probability of being folded and unbound and the
9 denominator is the partition function that sums over the probability of all three states, and
10 $\beta=1/k_B T$ where k_B is the Boltzmann constant and T is the temperature. The function F has two
11 regimes as shown by the surface in **Figure 3D**. For low binding affinities and stable P-domain
12 structures, viruses are expected to infect host cells at some fixed probability, $0 < b_0 < 1$,
13 determined by the average effect of all remaining viral properties on the infection process, and
14 $F=b_0$. By contrast, when the binding to the Ab is strong or when the P-domain is unstable, the
15 virus cannot infect its host and $F=0$.

16 To compare the model to experiment, we use sequencing data to determine the growth rate of
17 each virus from the change in genome haplotype frequencies between successive generations⁹.
18 The growth rates distribute into two distinct groups with 87% of haplotypes exhibiting little or
19 no growth and the rest exhibiting considerably larger growth. We take the first group to be non-
20 infective, and take the second group to be infective (**Figure 3-figure supplement 1A**). For each
21 haplotype sequence, we map the mutations to the 3D structure of the *wt* P-domain²⁸ and use Eris
22 force field calculations to determine the change in folding energy $\Delta \Delta G_{fold}$ between the mutant
23 and the *wt*; from this we determine stability of the mutant $\Delta G_{fold}=\Delta G_{fold,wt}+\Delta \Delta G_{fold}$, where ΔG_{wt} is
24 the folding energy of the *wt*. We also determine the change in binding energy, $\Delta \Delta G_{bind}$, between
25 the mutated P-domain-Ab complex and the *wt*; from this we determine the dissociation constant
26 $K_d=K_0 e^{\beta \Delta \Delta G_{bind}}$ where K_0 is the dissociation constant of the *wt*. We bin the haplotypes using
27 $\Delta \Delta G_{fold}$ and K_d and calculate F for each bin from the fraction of infective haplotypes. This

1 binning exploits the large number of unique haplotypes to reduce the effects of errors in the
2 calculations and of contributions from other biophysical properties. We fit the model by varying
3 the three unknown parameters, b_0 , ΔG_{wt} and the multiplier m .

4 We obtain excellent agreement between the model and the data, as shown by the dashed line in
5 **Figure 3E-F**. The infectivity of haplotypes is zero at low K_d or high ΔG_{fold} , while at high K_d and
6 low ΔG_{fold} , the landscape plateaus at $F \sim 0.25$ independent of either of the biophysical coordinates.
7 The value of $1/m \approx 1.4\%$ obtained from the fit reflects the fact that only about 3 of the 180 P-
8 domains on the capsid have to be blocked by the Ab to prevent infection (**Figure 3E** and ref. ¹²).
9 The value of $F \sim 0.25$ at the plateau is significantly less than the expected value of $b_0 = 1$; this
10 points to the role of factors not included in the model, such as the interaction of the capsid with
11 the host-cell receptor, in successful infection. In the absence of the neutralizing antibody, we do
12 not expect the fitness landscape to depend on K_d , which is indeed the case (**Figure 3-figure**
13 **supplement 2**). Altogether, these results demonstrate that binding and folding energies are very
14 good predictors of viral extinction; however they are less successful in predicting infectivity.
15 This suggests that antibody escape and folding stability of the capsid protein are necessary but
16 not sufficient for viral infection.

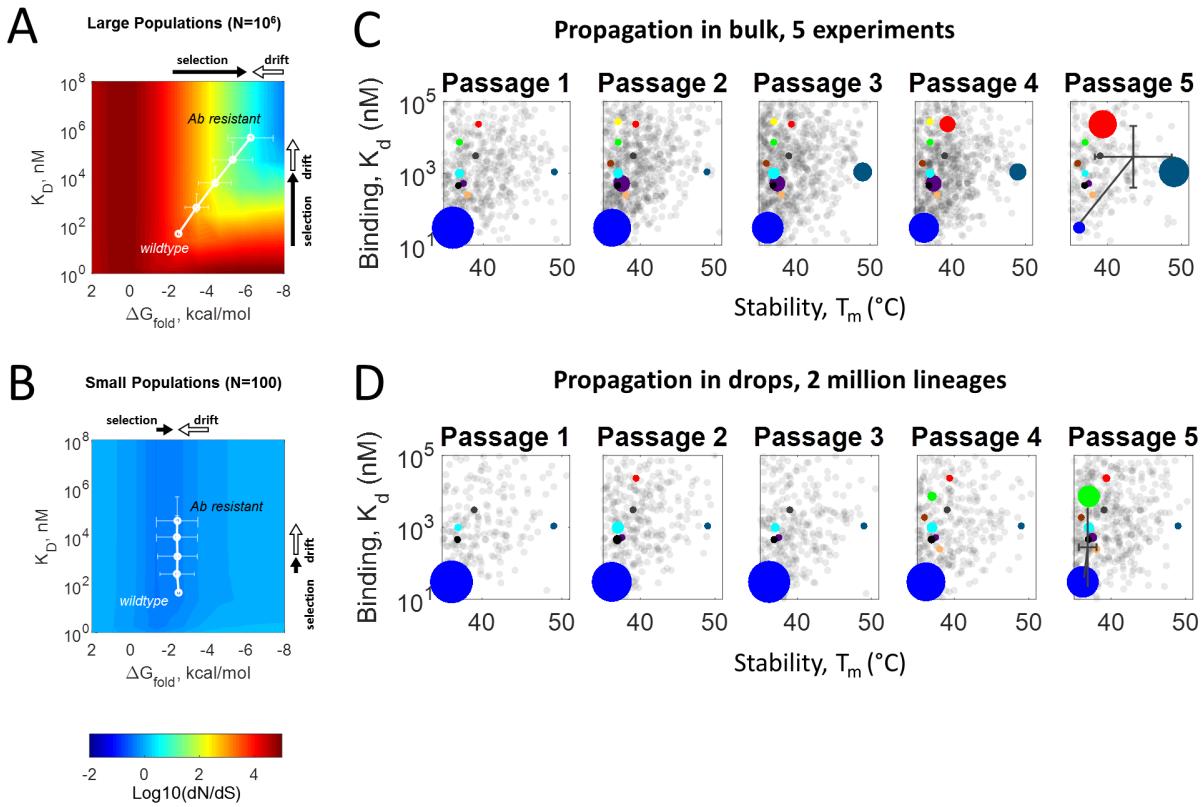
17 **Most likely pathways on the fitness landscape predicted by protein biophysics and**
18 **population genetics**

19 To determine how the virus evolves on the fitness landscape, we use population genetics theory
20 and calculate the ratio dN/dS , where dN is the rate of non-synonymous evolutionary rate and dS
21 is the synonymous evolutionary rate ³² (For greater details, see section on Balance between
22 selection and mutational drift on the fitness landscape in the Materials and Methods). As a rule,
23 mutants affect the population more in regions where non-synonymous mutations are beneficial
24 (high dN/dS), driving it down the gradient of dN/dS towards regions where non-synonymous
25 mutations are deleterious (low dN/dS) and thus do not affect the population. The dN/dS ratio is a
26 function of population size and of the fitness effect of a mutation or the selection coefficient
27 $s = (F_{mutant} - F_{wildtype}) / F_{wildtype}$ ^{32,33}. Using the fitness landscape from **Equation 1**, we calculate the
28 dN/dS ratio on the viral fitness landscape and how it depends on population size (see Methods

1 and^{34,35} for details). In large population sizes, dN/dS exhibits a strong gradient towards high K_d
2 and high folding stability (**Figure 5A**). Consequently, a *wt* population that is initially unstable
3 and neutralized by Ab is expected to evolve resistance by increasing both K_d and stability.
4 However, in small population sizes the gradient of dN/dS is directed only towards high K_d and
5 the same initially unstable population will evolve resistance to Ab without increasing folding
6 stability (**Figure 5B**).

7 The effect of population size on the course of evolution can be explained by analyzing the
8 balance between selection and mutational drift, the two forces driving evolution. The direction of
9 drift and selection along the trait of folding stability and binding affinity to the antibody can be
10 inferred from protein engineering and systematic studies on effects of random mutations on
11 proteins. Along the axis of folding stability ΔG_{fold} , beneficial mutations increase folding stability,
12 but random de novo mutations in proteins tend to decrease stability³⁶⁻³⁹. Thus, selection and drift
13 act in opposite directions (**Figure 5A-B**, arrows), leading to mutation-selection balance. Along
14 the axis of binding affinity K_d , beneficial mutations for the virus lead to escape from Ab (towards
15 high K_d), and random mutations on protein interfaces perturb binding (also towards high K_d).
16 Thus, selection and drift act along the same direction (**Figure 5A-B**, arrows).

17 To validate the difference in expected pathways to antibody resistance based on population size,
18 we simulate the trajectory for 4 sequential fixations of a single mutation starting from the
19 position of the *wt* virus population on the viral fitness landscape (see³¹ for details). For large N ,
20 the simulations show that the norovirus population increases both its folding stability and
21 dissociation constant, K_d , as it escapes the neutralizing antibody, following the trajectory shown
22 by the solid points in **Figure 5A-B** (see also **Figure 5-figure supplement 1** for simulations in
23 the polyclonal regime). After 4 sequential single mutations the model shows a rise of 5 orders of
24 magnitude in K_d and an increase of 3kcal/mol in ΔG_{fold} towards stabilization corresponding to a
25 change of 9°C in T_m . On the other hand, for small N the simulations show that the norovirus
26 population increases its K_d to escape the antibody but exhibits only a small change in folding
27 stability of less than 1kcal/mol, corresponding to an increase in T_m of about 2°C, as shown by the
28 trajectory in **Figure 5B** (see also **Figure 5-figure supplement 1**).



1

Figure 5. Most likely pathways on the fitness landscape predicted by protein biophysics and population genetics. **A,B)** Average stringency of selection for several population sizes (see Text and). For large population sizes, the increase in K_d is strongly coupled to the increase in T_m . However, for small population sizes, the selection for K_d is decoupled from the selection for folding stability. The white lines are the predicted trajectories from forward evolutionary simulations of an MNV population escaping an Ab, but with a P-domain which is unstable. Each trajectory is the average of 1000 independent simulations. The direction of selection (black arrows) is towards greater folding stability and weaker affinity to the antibody. Selection is strong when the P-domain is unstable and/or is tightly bound to the Ab. Selection pressure is approximately zero when the fitness landscape is flat (neutral). Along the direction of folding stability, most random mutations are destabilizing which lead to a mutational drift (white arrows) towards protein destabilization. Along binding affinity axis, most random mutations perturb the protein-protein interaction that leads to a mutational drift towards weaker binding. **C,D)** Density plots of all haplotypes grouped according to passages. Color circles denote dominant haplotypes whose biophysical properties were measured, while the remaining gray circles denote haplotypes whose biophysical properties were calculated. The size of the circle represents the allele frequency of each haplotype. Gray cross at passage 5 denotes mean and s.d. of all haplotypes.

The following figure supplement is available for Figure 5:

1 **Figure 5-figure supplement 1. Simulations of evolutionary dynamics on the fitness
2 landscape with a polyclonal viral population**

3 The average folding stability and K_d is shown for 5 simulated passages of populations of 10 and
4 10^6 viruses. The simulation was repeated once for the large population and 1,000 times for the
5 small population and the average of all repeats are shown (SSE error bars are not visible
6 at<0.1%). For this simulation we used the initial conditions: ΔG_{fold} =-2.5kcal/mol, K_d =46 nM,
7 $[Ab]$ =8 nM and μ_r =0.1.

8

9 To compare the theory to experiment we plot the position of viral haplotypes evolving in
10 5 independent bulk passaging experiments as a function of K_d and T_m , denoting their frequencies
11 by the size of the circles for each passage. To that end we include the calculated biophysical
12 values K_d and T_m (gray haplotypes), using the correlation curve in **Figure 3B** to relate between
13 ΔG_{fold} and T_m , as well as the biophysically measured values for select haplotypes (colored
14 symbols). There is a clear trajectory as the intermediate variants evolve, having increasingly
15 weaker affinities and higher T_m , with the escape variants at passage 5 ultimately having the
16 weakest affinity, with an overall average of K_d ~3,000 nM and the highest P-domain stability
17 with an average of T_m ~43.5°C, as shown in **Figure 5C**. We also plot the position of viral
18 haplotypes evolving in ~2 million independent drop passaging experiments. We observe a clear
19 trajectory of increasingly weaker affinities while maintaining original T_m , with the escape
20 variants at passage 5 ultimately having an overall average of K_d ~300 nM and T_m ~36.8°C, as
21 shown in **Figure 5D**. The direction of these trajectories parallels that of the simulated
22 trajectories, supporting our choice of these explicit biophysical properties as valid and useful
23 coordinates for the fitness landscape. This also provides us a theoretical framework with which
24 to interpret new experimental data and to test fundamental concepts of evolutionary theory.

25 **DISCUSSION**

26 It is a central concept in evolutionary biology theory that the population size determines the
27 balance between two evolutionary forces, selection and mutational drift^{5,34}. The results
28 presented here provide direct experimental evidence in support of this central concept. Moreover,
29 the role of Wright's shifting balance theory in real evolution has been contentious and
30 controversial because of lack of direct experimental demonstration⁴⁰. Using the segregation in

1 the Evolution Chip, we show that isolated viral populations starting at a fitness valley in the
2 presence of a neutralizing Ab are able to explore unlikely pathways on the adaptive fitness
3 landscape and, following migration, shift the whole population to new fitness peaks (**Figure 1C**);
4 this is a direct demonstration of the shifting balance theory.

5 The ability to change the course of evolution using the Evolution Chip and to predict its direction
6 on the biophysical fitness landscape with the parameters of protein stability and binding may be
7 helpful in addressing pandemics. These biophysical parameters are more generally applicable to
8 other viruses; for example, both binding ⁴¹ and folding stability ⁸ are relevant traits for the
9 evolution of an influenza virus in the presence of a neutralizing antibody. Prediction of future
10 diversification of circulating viral mutants is key to developing and potentially fielding an
11 effective vaccine prior to, or in the early stages of a pandemic. The methodology presented here
12 may assist in proactive exploration of viral diversification to inform selection of novel viruses
13 prior to their natural emergence, for developing viral therapeutics.

14
15 **MATERIALS AND METHODS**
16

17 Microfluidic devices:

18 We fabricate polydimethylsiloxane (PDMS) devices using photolithography and coat them with
19 fluorophilic Aquapel (Rider, MA, USA) to prevent wetting of drops on the channel walls.
20 Electrodes are fabricated on chip using low melting temperature solder ⁴². The designs used to
21 fabricate the devices are available in ACAD format (**Supplementary File S1**). We use OEM
22 syringe pumps (KD Scientific, MA, USA) to drive the fluidics and a fast camera (HiSpec1,
23 Fastec Imaging, USA) to image encapsulation and drop fusion.

24

25 Reagents

26 For the inert carrier oil we use HFE-7500 (3M, USA) with 1% w/w of a block co-polymer
27 surfactant of perfluorinated polyethers (PFPE) and polyethyleneglycol (PEG)⁴³. A compatible
28 surfactant is available commercially (008-FluoroSurfactant, Ran Biotechnologies, USA). To
29 separate the emulsion, we use a commercially available demulsifier (1H,1H,2H,2H-perfluoro-1-
30 octanol, CAS # 647-42-7). Other chemicals were purchased from Sigma Aldrich (St. Louis,
31 MO).

1 Clones and Antibodies

2 The plaque-purified MNV-1 clone (GV/MNV-1/2002/USA) MNV-1.CW3⁴⁴ (referred herein as
3 *wt*) was used at passage 6 for in vitro passaging experiments. Recombinant MNV-1 viruses
4 containing P-domain point mutants E296K (*A*), D385G (*B*), T301I (*C*), A382V (*D*), L386F (*E*),
5 E296K-T301I (*AC*), E296K-D385G (*AB*), T301I-D385G (*BC*), A382V-D385G (*BD*) and
6 E296K-T301I-D385G (*ABC*) were generated as previously described²⁰. The isotype control IgG
7 directed against Coxsackievirus B4 (CV) (clone 204-4) was purchased from ATCC (HB 185).
8 Anti-MNV-1 monoclonal antibody (mAb) A6.2 (IgG2a) was grown in Bioreactor CELLline CL
9 1000 flasks (Sigma-Aldrich) following the manufacturer's recommendations. Antibodies were
10 purified over a HiTrap protein A column (GE Healthcare) according to the manufacturer's
11 instruction, dialyzed against PBS, and stored at -20 °C.

12

13 MNV-1 P-domain mutant expression and purification

14 All recombinant proteins was expressed and purified as previously reported²¹ with some
15 modifications. Briefly, the P domain of MNV-1 (residues 225 to 541) was cloned into a pUC57
16 expression vector with NH2-terminal 6-histidine tag. The protein was expressed overnight at
17 20°C in Escherichia coli. The cells were subsequently lysed, and the protein was obtained from
18 the supernatant, two steps purification by Ni column and by gel filtration on a Superdex 75
19 column (GE Healthcare). The proteins were dialyzed overnight at 4°C against a phosphate buffer
20 (pH=7.8 and 20 mM NaCl).

21

22 Folding stability measurement by thermofluorescence

23 Thermal denaturation was carried out using melt-curve module of BioRad CFX96, and Sypro
24 Orange dye as a probe for unfolding as described earlier⁴⁵. The dye was added to the final
25 concentration of 5× in a 25 µl reaction volume containing 4 µM of protein in 10 mM sodium
26 phosphate buffer pH 7.8. To control for concentration dependence, we also performed the
27 experiment using 2 µM concentration of protein. The T_m is estimated as the extremum of the
28 derivative of the fluorescence signal. We report the average T_m of eight technical repeats, four for
29 4 µM of protein and another four for 2 µM of protein.

30

31 Binding kinetics by surface plasmon resonance

32 Realtime biomolecular analysis was performed by surface plasmon resonance (SPR) using a
33 BIACore 3000 instrument equipped with nitrilotriacetic acid (NTA) sensor chip. Purified

1 monoclonal antibody A6.2 was immobilized on the surface while the MNV-1 P-domain variants
2 were the analytes. To perform single kinetic measurement, we (1) Quickinjected 20 μ L of buffer
3 (10 mM sodium phosphate buffer pH 7.8 with 0.005% surfactant to prevent minimizing non-
4 specific interaction); (2) Kinjected 50 μ L of protein with 100 s dissociation; (3) Quickinjected 10
5 μ L of regeneration buffer (pH 2.0); and (4) Quickinjected 20 μ L of phosphate buffer. For each
6 MNV variant, we performed kinetic measurements with concentrations in the range of 0.5 to 100
7 μ M. Data analysis was conducted with BIAevaluation package. Curve fittings were done with
8 the 1:1 Langmuir binding model.

9

10 Cell Culture

11 RAW 264.7 (murine macrophage) cells were purchased from ATCC (Manassas, VA) and
12 maintained as described previously^{21,46}. Adherent cell culture medium (RAW medium) contains
13 Dulbecco's Modified Eagle's Medium, 4 mM L-glutamine, 100 μ g/mL penicillin, 100 μ g/mL
14 streptomycin, 10 mM HEPES, 10% heat-inactivated fetal bovine serum. The RAW 264.7 were
15 adapted to suspension culture in spinner flasks for these experiments, for compatibility with
16 drop-based microfluidics. Suspension cell culture medium (Suspension Medium) contains
17 adherent RAW medium supplemented with sodium bicarbonate (7.5%). BSRT7 (BHK cells
18 expressing T7 polymerase) cells were cultured in DMEM supplemented with 10% heat-
19 inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml
20 streptomycin⁴⁷. For all viral infection experiments, suspension RAW 264.7 cells were suspended
21 in Enhanced Suspension Medium (ESM) comprised of Suspension Medium supplemented with
22 15% optiprep.

23

24 Viral evolution in bulk

25 RAW 264.7 suspension cells are centrifuged for 5 min at 3,000 rpm and re-suspended in fresh
26 medium at a concentration of 6×10^6 cells/mL. In antibody neutralization experiments, virus is
27 first incubated for 30 min at 37°C with mAb A6.2 in 200 μ L PBS prior to dilution into ESM at
28 4×10^6 pfu/mL, such that the final Ab concentration is 8.57 nM. One mL of cell suspension and 1
29 mL of virus suspension are mixed in a single well of a 12-well dish containing a sterile stir bar
30 and incubated on stir plate in a 37°C incubator, 5% CO₂, for 24 hrs. To passage viral progeny to
31 the next generation (the progeny of the first inoculum is considered passage 0 (P0)), 250 μ L of
32 the supernatant from cell lysates of the previous generation is supplemented to 2 mL of a fresh
33 suspension of 2×10^6 RAW cells/mL in ESM in a 12-well dish containing a sterile stir bar and
34 incubated on the stir plate in a 37°C incubator, 5% CO₂, for 24 hrs. In antibody neutralization
35 experiments, 8.57 nM mAb A6.2 is supplemented to the fresh suspension of RAW cells prior to

1 passaging. Cell lysates were harvested by 2 rounds of freeze/thaw and centrifugation for 5 min at
2 5,000 x g.

3

4 Viral evolution in drops

5 To evolve viruses in small population sizes we use a novel microfluidics system, the “Evolution
6 Chip”, which propagates $\sim 10^6$ subpopulations of 1-10 infectious particles (pfu) in distinct and
7 non-mixing compartments. We encapsulate ~ 2 host cells and ~ 1 pfu in 100 μm diameter aqueous
8 drops in inert oil at a rate of millions per hour. The resultant emulsion is incubated under
9 physiological conditions for one viral life cycle (~ 24 hours). Successful replication of the virus
10 leads to the death of the host cell and the release of viral progeny within the drop. Thus, to
11 enable continued passage, each drop is split and $\sim 10\%$ of its volume, containing viral progeny
12 from the previous generation, is merged with a new drop containing a fresh host cell for the next
13 generation.

14 Suspension-adapted RAW 264.7 cells were centrifuged for 5 min at 3,000 rpm, re-suspended in
15 ESM at 8×10^6 cells/mL. The suspension of cells is co-flowed at a 1:1 ratio with MNV-1 virus
16 diluted into ESM at 4×10^6 pfu/mL. The two aqueous phases - cell suspension and buffer - meet
17 immediately before passing through the microfluidic drop making junction so that they only mix
18 inside the 100 μm drops containing them (**Supplementary Movie 1**). For the continuous phase
19 dispersing the drops, we use HFE-7500 Oil with 1% surfactant. Typical flow rates are 8 mL/hr
20 for the oil and 2 mL/hr for cells and virus. Drops were collected in 1.5 mL tubes and incubated at
21 37°C, in 5% CO₂. Following 18-24 hr incubation at 37°C, drops are re-injected into the
22 “evolution-chip” microfluidic device where $\sim 10\%$ of their volume is split off and fused with
23 freshly formed drops containing freshly prepared suspension of RAW cells in ESM. The
24 synchronization in the device ensures that in >95% of cases, the split from one drop fuses
25 with exactly one newly formed drop, enabling the viral lineages to propagate in isolation.
26 Typical flow rates are 4 mL/hr for the oil, 1 mL/hr for the re-injected drops, and 1 mL/hr for the
27 fresh cells. The newly formed drops are collected in 1.5 mL tubes and incubated at 37°C, in 5%
28 CO₂, while the content of the old, split drops are extracted for analysis (The progeny of the first
29 encapsulation is considered passage 0 (P0)). In antibody neutralization experiments, 8.57 nM
30 mAb A6.2 is supplemented to the fresh suspension of RAW cells prior to re-injection of drops.

31

32 Head to head competitions between viral strains

33 Next, we determine if the escapees in drops are more fit than the escapees in bulk. To address
34 this question, we performed head-to-head competition of *wt*, *BD*, and *ABC* strains. We mixed
35 equal viral titers of each pair of strains, passaged them for 3 rounds and then performed deep

1 sequencing. The competition was performed under two conditions, with and without neutralizing
2 antibody, and over three replicates. The sequencing results of the competitions are given in
3 **Table S2.**

4 RAW 264.7 suspension cells are centrifuged for 5 min at 3,000 rpm and 2 mL of a fresh
5 suspensions of 1×10^6 RAW cells/mL in RAW medium is dispensed in 6-well plates. In antibody
6 neutralization experiments, virus is first incubated for 30 min at 37°C with mAb A6.2 in 200 μ L
7 PBS prior to adding it into the wells, such that the final Ab concentration is 0.08 nM. Cells and
8 virus are incubated in a 37°C incubator, 5% CO₂, for 48 hrs. To passage viral progeny to the next
9 generation (the progeny of the first inoculum is considered passage 0 (P0)), 200 μ L of the
10 supernatant from cell lysates of the previous generation is supplemented to 2 mL of a fresh
11 suspension of 1×10^6 RAW cells/mL in RAW medium in a 6-well dish and incubated in a 37°C
12 incubator, 5% CO₂, for 24 hrs. In antibody neutralization experiments, 0.08 nM mAb A6.2 is
13 supplemented to the fresh suspension of RAW cells prior to passaging. Cell lysates were
14 harvested by 2 rounds of freeze/thaw and centrifugation for 5 min at 5000 x g.

15 In-vitro neutralization measurements

16 To measure neutralization in-vitro we followed Fischer et. al ¹² to obtain a neutralization curve
17 which we then fit by the equation $y = 1 / \left(1 + \frac{k_d}{X}\right)$ where y is the viral titer, X is the *Ab*
18 concentration and k_d is the fitting parameter.

19 In-vivo neutralization measurements

20 Mouse studies were performed in accordance with local and federal guidelines as outlined in the
21 “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health.
22 Protocols were approved by the University of Michigan Committee on Use and Care of Animals
23 (UCUCA Number: 09710). Viral strains were neutralized in STAT^{-/-} mice injected with 500ug
24 mAb A6.2 and compared to their infection in mice injected with an isotype as described in ⁴⁸.
25 The decrease in viral titers was first standardized for each tissue across viral strains, before the
26 average over all tissues within each strain was taken as its final neutralization score. The study
27 was performed in biological triplicates. No randomization or blinding was used.

28

29 Measurement of infectious virus titer

30 Viral titer was determined by either plaque assay, as described previously⁴⁹, or by TCID50 assay,
31 as described previously ⁵⁰. TCID 50% infectivity was translated to plaque forming unit (pfu)
32 using the conversion of 0.7 TCID50 units to 1 pfu.

1 Quantification of viral genome

2 Viral RNA isolated from experimental samples by MagMAX Viral RNA Isolation Kit (Life
3 Technologies) was evaluated by qPCR using MNV-1-specific primer/probe sequences (Forward:
4 GTGCGAACACAGAGAACG, Reverse: CGGGCTGAGCTTCCTGC, and probe:
5 FAM/CTAGTGTCTCCTTGGAGCACCTA/TAMARA)²¹. Takara One Step reagent
6 (Mountain View, CA) was used to measure the genome copy of viral samples. qRT-PCR was
7 performed on ABI real time PCR machine (OneStep Plus) using the following thermal cycling
8 parameters: 5 min at 42° C and 10 sec at 95° C, 40 cycles of 5 sec at 95° C and 34 sec at 60° C.
9 Titered MNV-1 viral stock and PicoGreen (quBit)-quantitated pT7 MNV 3'RZ plasmid⁵⁰ were
10 used as standards for pfu/mL and genome copies/mL analysis, respectively.

11

12 Amplicon preparation and sequencing

13 Viral RNA was isolated using the MagMax viral RNA kit (Life Technologies), then subjected to
14 DNase digest using DNAfree Turbo DNase (Life Technologies). SuperScript III reverse
15 transcriptase (Life Technologies) was then used to convert vRNA to first strand DNA with
16 specific primers tiling the entirety of MNV-1 at ~1kb increments using the following primers:

17 AGCCGATCACAGGCTCCTGGC

18 CCATGTTGGATAAGAGGGCTGGC

19 ACGCACTTCCTCAACTCAGCCG

20 GGCCATGCTGATCCTGGCCA

21 CCACCAGGATGCCATCCGAGA

22 GTCGACATCAGCGCGTGGTATGA

23 CAACAGGGTGGGCACCACGTC

24 CAACAACAGGGCTCTCAGCATAAACCAAG

25 Library preparation for Illumina sequencing on the miSeq platform was carried out in accordance
26 with Illumina tech note 15044223 Rev. A using KAPA HotStart HiFi DNA polymerase 2x
27 master mix (KAPA Biotechnologies), with primers for MNV-1 capsid amplification
28 corresponding to MNV-1 VP1 nucleotides 848-1275 (Forward:
29 TCGTCGGCAGCGTCAGATGTATAAGAGACAGGTTCATGGGTGTCCTGCTT,
30 Reverse:
31 GTCTCGTGGGCTCGGAGATGTATAAGAGACAGGGGGAGAAAGGGACCAATT;

1 gene specific portion in bold). After purification with Ampure XP (Beckman Genomics),
2 samples were quantified using Qubit high sensitivity reagents (Life Technologies), then pooled
3 in equimolar ratios assuming specific amplification during adapter addition. Final pools of up to
4 96 libraries were quantified using high sensitivity Bioanalyzer reagents (Agilent) prior to
5 sequencing 2X250 bp long paired end reads of the amplicons with miSeq (v2 chemistry, miSeq
6 control software v2.3.0.3). Samples were supplemented with 10% phiX control library
7 (Illumina). All reagents were used according to manufacturer's recommendations, and obtained
8 from Life Technologies unless otherwise noted.

9

10 Amplicon sequencing analysis

11 Paired end reads were obtained from the MiSeq after going through the MiSeq Reporter
12 Generate FASTQ Workflow, which demultiplexes the raw data by MID pair (removing adapter
13 sequences in the process), and creates a separate pair of output files for each paired-end sample.
14 These paired-end reads are aligned to the MNV-1 reference sequence (RefSeq: NC_008311)
15 using bowtie2 (<http://bowtie-bio.sourceforge.net/bowtie2/>) and only reads that include the
16 amplicon range (nucleotides 5918 to 6293 of MNV-1) without insertions and deletions are
17 analyzed. SNPs in each of the remaining reads are saved for further analysis together with their
18 Illumina reported quality. SNPs in overlapping regions of the paired end reads are called using
19 the read with the higher quality. If the call in both forward and reverse reads match, the quality
20 of SNP is taken as the sum of qualities, otherwise, the difference between the higher and lower
21 quality is taken. Sequences with more than 20 substitutions were discarded and the remaining
22 reads were then clustered into unique candidate haplotypes and tested for statistical significance
23 using a hypothesis test. The null hypothesis presumed that a haplotype candidate is generated by
24 sequencing errors in the reading of one of its "potential ancestors". Potential ancestors are
25 haplotypes that are both "nearest neighbors" - have the smallest hamming distance to the
26 candidate - and are more frequent - have more reads than the candidate. We used a simple
27 generative model using a base-calling substitution rate equal to the lowest read quality recorded
28 for any of the relevant substitutions in the candidate reads and then calculated the probability that
29 the observed candidate was derived from this distribution (see next section on Determination of
30 Significance).

31 Determination of significance from sequencing

32 Initially, the most abundant haplotype is assigned P-value=0. Haplotypes are tested in
33 descending order of their frequency. For each haplotype, a group of "potential ancestor
34 haplotypes" was defined as those haplotypes that are both "nearest neighbors" - have the smallest
35 hamming distance to it - and are more frequent - have more reads than this haplotype. The single
36 event probability that a copy of the candidate haplotype was generated while sequencing a copy
37 of each potential ancestor is calculated using the quality of reads of the nucleotides that differ

1 between the two haplotypes: the score for each substitution address was calculated as the lowest
2 quality over all reads sequenced in that address for the candidate haplotype. The final score was
3 calculated from the product of all single substitution scores. The probability P_{ij} that a candidate
4 haplotype i was generated by sequencing errors in the reading of a potential ancestor j was
5 determined using the complementary cumulative density (CCDF) of a binomial function, with
6 the number of trials equal to the frequency of the potential ancestor, the number of successes
7 equal to the frequency of the candidate haplotype and the success rate taken as the single event
8 probability. This probability is calibrated according to the probability of ancestor P_j so that
9 $P'_{ij} = 1 - (1 - P_{ij})(1 - P_j)$. Finally, the P-value of haplotype i is calculated as the maximum
10 probability over all potential ancestors: $P_i = \max\{P'_{ij}\}$.

11

12 Calculation of probability of infection P_{infect} from growth rates

13 To obtain the fitness landscape in **Figure 3**, we define the probability of infection of each
14 haplotype as $P_{inf} = \Theta(\rho - 10^{-3})$, where ρ is the growth rate of the haplotype calculated as described
15 below. Applying this threshold is motivated by the fact that the distribution of growth rates is
16 bimodal – most haplotypes are either infective to some extent ($\rho \geq 0.1$) or not ($\rho = 0$) with less
17 than 1% of growth rates fall within the intermediate region (see
18 **Figure 3-figure supplement 1A**). Consequently, the setting of the threshold is insensitive in the
19 region (0,0.1).

20

21 Calculation of growth rates from haplotype frequencies

22 We calculate growth rates by comparing between haplotype frequencies of consecutive
23 generations. To avoid sequencing errors, we only consider frequencies with $P\text{-value} \leq 10^{-4}$.
24 Additionally, since our growth rates are relative to that of the wildtype wt , we only use data from
25 the first 2 passages of evolution, where the wt is still the most abundant haplotype.

26 For each pair of consecutive generations that were sequenced, we calculate the growth rate ρ_i of
27 all haplotypes that were detected in the samples using a simple linear model:

28 $n'_i = n_i p_i$ (Equation 2)

29 where n_i and n'_i are the copy numbers of haplotype i in the first and second generation
30 respectively. We ignore mutation rates since mutation rates of MNV ($\sim 10^{-4}$) are smaller than the
31 sequencing errors ($\sim 10^{-3}$). Assuming unbiased sampling of original copies during sequencing, we
32 substitute:

$$n_i = f_i \cdot \Sigma n \quad (\text{Equation 3})$$

$$n_i' = f_i \cdot \Sigma n' = f_i \cdot \Sigma (n_i \cdot \rho_i) = f_i \cdot \Sigma (f_i \cdot \rho_i \cdot \Sigma n)$$

3 where f_i and f'_i are the frequency of haplotype i measured by sequencing the first and the second
 4 sample respectively. When we use these substitutions in the original equation (1) we get:

$$5 \quad f_i' \Sigma (f_i \cdot \rho_i \Sigma n) = f_i \cdot \rho_i \Sigma n \quad (\text{Equation 4})$$

$$f_i \cdot \Sigma(f_i \cdot \rho_i) = f_i \cdot \rho_i$$

7 the resulting set of linear equations, one per haplotype, is a homogenous system:

$$8 \quad AP = 0 \quad (\text{Equation 5})$$

9 where the parameter matrix is $A_{ij} = f_i'f_j - \delta_{ij}$ and the growth rates vector is $P_j = \rho_j$.

10 The non-trivial solution solves for all growth rates, assuming a growth rate of 1 for the wild type.

11 Since the same haplotype may exist in more than one pair of consecutive generations, multiple
12 growth rates can be assigned to the same haplotype, in which case the average growth rate is
13 calculated for this haplotype. The distribution of all growth rates is plotted in
14 **Figure 3-figure supplement 1A.**

15

16 Viral fitness function based on thermodynamics of protein folding and binding

17 We assume that the fitness F is proportional to fraction of MNV-1 P-domain that are folded and
 18 free from the antibody. If we further assume that the MNV-1 P-domain exists in three states—
 19 folded unbound, folded bound and unfolded (always unbound)—then we can express fitness F
 20 as

$$F \sim P_{\text{inf}} = \frac{e^{-\beta \Delta G_{fold}}}{1 + \left(\frac{[Ab]}{mC_0} \right) e^{-\beta \Delta G_{bind}} e^{-\beta \Delta G_{fold}} + e^{-\beta \Delta G_{fold}}} \quad (21)$$

$$= \frac{1}{1 + \left(\frac{[Ab]}{mK_d} \right) + e^{\beta \Delta G_{fold}}} \quad (\text{Equation 6})$$

where ΔG_{fold} and ΔG_{bind} are the free energies of folding and binding, $[Ab]$ is the antibody concentration, C_0 is the standard reference concentration and $\beta=1/k_B T$. The parameter

1 m accounts for the added entropy due to the fact that any of the 180 P-domains on the viral
 2 capsid have a similar contribution to the neutralization of the capsid.

3 We note that this formulation takes into account the known experimental fact that binding
 4 induces folding. Specifically, the total fraction of folded proteins is the sum of proteins that are
 5 folded and bound to the *Ab* and proteins that are folded and free, and then normalized by total
 6 number of proteins in solution:

7

$$8 P_{folded} = \frac{\left(\frac{[Ab]}{mK_d}\right)e^{-\Delta G_f} + e^{-\Delta G_f}}{1 + \left(\frac{[Ab]}{mK_d}\right)e^{-\Delta G_f} + e^{-\Delta G_f}} \quad (\text{Equation 7})$$

9 Indeed, $P_f \rightarrow 1$ when $[Ab] \rightarrow \infty$ or $K_d \rightarrow 0$. That is, when there is a sufficient amount of
 10 antibody or when the binding to the Ab is very tight, all P-domains will eventually bind to the
 11 antibody and be folded.

12

13 Fitness effects of mutations (selection coefficient) on the viral fitness landscape

14 Conceptually, evolution may be thought of as a hill-climbing process, whereby the rate of ascent
 15 is proportional to the effect of mutations on fitness. The fitness effect of a mutation is quantified
 16 by the selection coefficient $s = (F_{mut} - F_{wt})/F_{wt}$. The subscript “*mut*” and “*wt*” refers to mutant and
 17 wildtype, respectively. At the protein level, a non-synonymous mutation will change the folding
 18 and binding free energies as $\Delta G_{fold,mut} = \Delta G_{fold,wt} + \Delta \Delta G_{fold}$ and $\Delta G_{bind,mut} = \Delta G_{bind,wt} + \Delta \Delta G_{bind}$. For the
 19 fitness function (**Equation 1** and also **Equation 6**), we can derive a simple expression for the
 20 selection coefficient.

$$21 F_{mut} = \frac{1}{1 + \frac{[Ab]}{mC_0} e^{-\beta(\Delta G_{bind,wt} + \Delta \Delta G_{bind})} + e^{\beta(\Delta G_{fold,wt} + \Delta \Delta G_{fold})}} \quad (\text{Equation 8})$$

22 Consider the case when $\Delta \Delta G_{fold} \ll 1$ and $\Delta \Delta G_{bind} \ll 1$. Then, using the expansion $e^x \approx 1 + x$, when
 23 $x \ll 1$:

$$\begin{aligned}
 1 \quad F_{mut} &\approx \frac{1}{1 + \frac{[Ab]}{mC_0} e^{-\beta(\Delta G_{bind,wt})} \beta(1 - \Delta \Delta G_{bind}) + e^{\beta(\Delta G_{fold,wt})} \beta(1 + \Delta \Delta G_{fold})} \\
 2 \quad &\approx F_{wt} + F_{wt} \left(\beta \Delta \Delta G_{bind} \frac{[Ab]}{mC_0} e^{-(\Delta G_{bind,wt})} - \beta \Delta \Delta G_{fold} e^{(\Delta G_{fold})} \right)
 \end{aligned} \tag{Equation 9}$$

Thus, the selection coefficient is

$$\begin{aligned}
 3 \quad s &= \frac{F_{mut} - F_{wt}}{F_{wt}} \\
 &\approx \beta \Delta \Delta G_{bind} \frac{[Ab]}{mC_0} e^{-(\Delta G_{bind,wt})} - \beta \Delta \Delta G_{fold} e^{(\Delta G_{fold})} \\
 &= \beta \Delta \Delta G_{bind} \frac{[Ab]}{mK_{d,wt}} - \beta \Delta \Delta G_{fold} e^{(\Delta G_{fold,wt})}
 \end{aligned} \tag{Equation 10}$$

4
 5 From **Equation 10**, the magnitude of the selection coefficient of a given mutation depends on the
 6 wildtype folding stability ($\Delta G_{fold,wt}$) and binding affinity ($K_{d,wt}$); hence, there is strong epistasis
 7 on the fitness landscape. Moreover, because a single mutation can affect both folding and
 8 binding—mutations are pleiotropic in their effect to proteins—both terms in **Equation 10**
 9 contribute to the selection coefficient. This epistasis-pleiotropy link at the level of protein
 10 biophysics is the origin of the coupling between the selection for folding stability and binding.

11 The difference in sign for the two terms in **Equation 10** also denotes the opposite direction of
 12 selection for the two biophysical traits. Along folding stability, selection points towards more
 13 stable proteins (more negative values of ΔG_{fold}); along binding affinity, selection points towards
 14 weaker binding (larger values of K_d) (**Figure 5A**). By contrast, randomly occurring mutations
 15 tend to destabilize proteins and protein-protein complexes^{38,39}, rendering both $\Delta \Delta G_{fold}$ and
 16 $\Delta \Delta G_{bind}$ more positive. Thus, along folding stability, mutational drift points towards less stable
 17 proteins, opposite to the selective force; however, along binding affinity, drift also points
 18 towards weaker binding (**Figure 5A**), parallel to the selective force. We present a formal
 19 treatment of the balance between selection and drift in the next section.

20 Balance between selection and mutational drift on the fitness landscape: Theoretical analysis

21 We now formally combine the contribution of drift and selection and quantitatively determine
 22 the course MNV-1 evolution on the fitness landscape. We first limit ourselves to the simple
 23 monoclonal regime, where each generation is dominated by just one strain with a probability
 24 equal to its probability of fixation. To that end, we compare the probability of fixation of a non-

1 synonymous mutation that changes folding stability and binding with the probability of fixation
2 of a synonymous mutation, which we assume is neutral ($s=0$). We denote this ratio as ω . The
3 underlying motivation for this ratio is that when $\omega=1$, mutations are neutral at the level of fitness,
4 and mutational drift dominates. Specifically,⁵¹

$$5 \quad \omega = \frac{dN}{dS} = N_e P_{fix}(s, N_e) = N_e \left[\frac{1 - \exp(-2s)}{1 - \exp(-2N_e s)} \right] \quad (\text{Equation 11})$$

6 where $P_{fix}(s, N_e)$ is the probability of fixation⁴, N_e is the population size and s is the selection
7 coefficient (**Equation 10**). s is a function of the wildtype folding stability ΔG_{wt} and binding
8 affinity K_0 and the changes induced by the mutation to folding stability and binding $\Delta\Delta G_{bind}$ and
9 $\Delta\Delta G_{fold}$. Because the effect of random mutations on folding stability and binding, denoted by
10 $p(\Delta\Delta G_{fold})$ and $p(\Delta\Delta G_{bind})$, are well-characterized^{38,39}, we can derive the rate of protein evolution
11 averaged over all possible effect of random mutations on folding and binding, that is,

$$12 \quad \bar{\omega}(\Delta G_{fold,wt}; K_{d,wt}; N_e) = \iint ap(\Delta\Delta G_{fold})p(\Delta\Delta G_{bind})d(\Delta\Delta G_{fold})d(\Delta\Delta G_{bind}) \quad (\text{Equation 12})$$

13 Eq. S10 defines the average stringency of Darwinian selection as a function of background, and
14 hence the strength of epistatic interaction on the fitness landscape defined by protein folding and
15 binding.

16 We show in **Figure 5A-B** (colormap) the value of the integral over the range of $K_{d,wt}$ and $\Delta G_{fold,wt}$
17 values. Indeed, positive selection is strongest when the *wt* P-domain is unstable (low ΔG) or
18 tightly bound to the antibody. At high population sizes, purifying selection and positive
19 selection dominates in the regime of low folding stability and tight binding affinity (red colors in
20 **Figure 5A**). The prediction is that if the population starts in the unstable regime, it would
21 migrate away from the regime where selection is strong and towards the regime where it is more
22 neutral.

23

24 Simulations of evolutionary dynamics on the fitness landscape: Monoclonal regime

25 We complement our theoretical analysis above by performing explicit simulation of population
26 dynamics. This evolutionary dynamics allows for the occurrence of random mutation and then
27 selection. The algorithm is as follows:

28 *Initialization*

29 We start with a viral population of size N . Since the population is monoclonal, the entire
30 population is uniquely determined by single values of folding stability and binding affinity. That

1 is, the population is a single point defined by the ordered pair (ΔG_{fold} , K_d). For the simulation
2 shown in **Figure 5A-B**, the population starts at (-2.5 kcal/mol, 46 nM).

3 *Propagation*

- 4 1. A non-synonymous mutation occurs in the MNV-1 p-domain at a rate of $\mu_r = 0.1$ per
5 replication⁵². This mutation changes the folding and binding free energies as
6 $\Delta G_{fold,mut} = \Delta G_{fold,wt} + \Delta\Delta G_{fold}$ and $\Delta G_{bind,mut} = \Delta G_{bind,wt} + \Delta\Delta G_{bind}$. The value $\Delta\Delta G_{fold}$ is
7 drawn from a Gaussian distribution with mean 1 kcal/mol and s.d. 1.7 kcal/mol^{37,38}. The
8 value $\Delta\Delta G_{bind}$ is drawn from a Gaussian distribution with mean 1 kcal/mol and s.d.
9 2 kcal/mol^{37,38}. Consequently, $K_{d,mut} = K_{d,wt} e^{(\beta\Delta\Delta G_{bind})}$.
- 10 2. Calculate the fitness of the mutant F_{mut} using **Equation 1**.
- 11 3. Calculate the selection coefficient $s = (F_{mut} - F_{wt})/F_{wt}$.
- 12 4. Determine the probability of fixation⁴

$$P_{fix}(s, N_e) = \frac{1 - \exp(-2s)}{1 - \exp(-2N_e s)} \quad (\text{Equation 13})$$

- 14 5. Draw a random number r between 0 and 1. If $r <= P_{fix}$, the mutation is fixed and the
15 folding stabilities and binding affinities are updated; otherwise, keep the wildtype values.
16 6. Repeat steps 1-5 for a specified number of fixations (In **Figure 5A-B**, we ran simulations
17 until fixation of 4 amino acid substitutions).

18

19 Simulations of evolutionary dynamics on the fitness landscape: Polyclonal regime

20 The analyses in the previous section are performed in the monoclonal regime, where a closed
21 formula of the stringency of selection is tractable. However, a true viral quasi-species is
22 necessarily polyclonal because of the high mutation rate, thus, we also perform forward
23 polyclonal regime simulation, for which the closed form formula for the stringency of selection
24 is no longer accurate. Results are shown in **Figure 5-figure supplement 1**.

25 *Initialization*

26 We start with a viral population of size N . Each of the N virion in the population is characterized
27 by its folding stability and binding affinity. Thus, the entire population constitutes a distribution
28 of ΔG_{fold} and K_d values and migrates as a “cloud” on the fitness landscape. For the simulation
29 shown in Fig. S4, we start with a population of N randomly chosen variants from the sequenced
30 MNV-1 viral stock.

1 *Propagation*

2 1. Each of the N viruses has a chance to infect and replicate with a probability given by
3 the fitness function **Equation 1**.

4 2. Each replicating virus mutates at a rate of $\mu_r = 0.1$ per p-domain per replication⁵². If a
5 mutation occurs, the folding stability and binding affinity are updated similar to Step #1
6 in the monoclonal regime. The number of progenies is calibrated to N to maintain a
7 constant population size.

8 3. If no viruses are chosen for replication, the sample is extinct.

9 4. Repeat from steps 1-3 for desired number of generations. To compare the number of
10 generations to the number of fixations in the monoclonal simulations, we assume that
11 each fixation requires 4 generations.

12

13 **ACKNOWLEDGEMENTS**

14 The authors would like to acknowledge C. Wilke and J. Plotkin for very helpful comments. This
15 work was supported by Defense Advanced Research Projects Agency (DARPA) contract #
16 HR0011-11-C-0093, by the National Science Foundation (DMR-1310266 to DW and MCB-
17 1243837 to ES), NIH GM068670 (ES), by the Harvard Materials Research Science and
18 Engineering Center (DMR-1420570) and by the National Natural Science Foundation of China
19 (HZ, 81372496).

20 **COMPETING INTERESTS**

21 The authors declare none.

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23

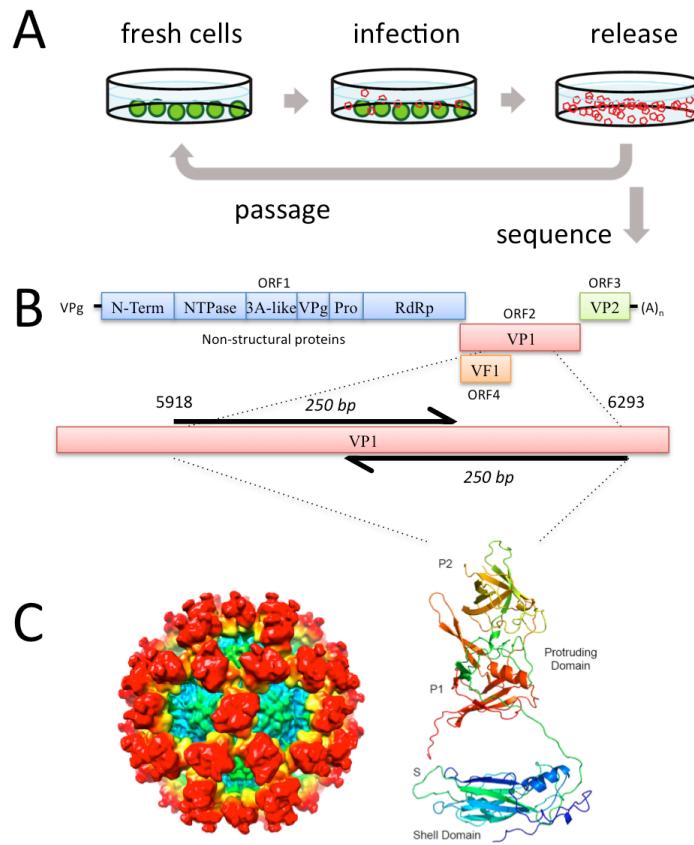


Figure 1-figure supplement 1.

1

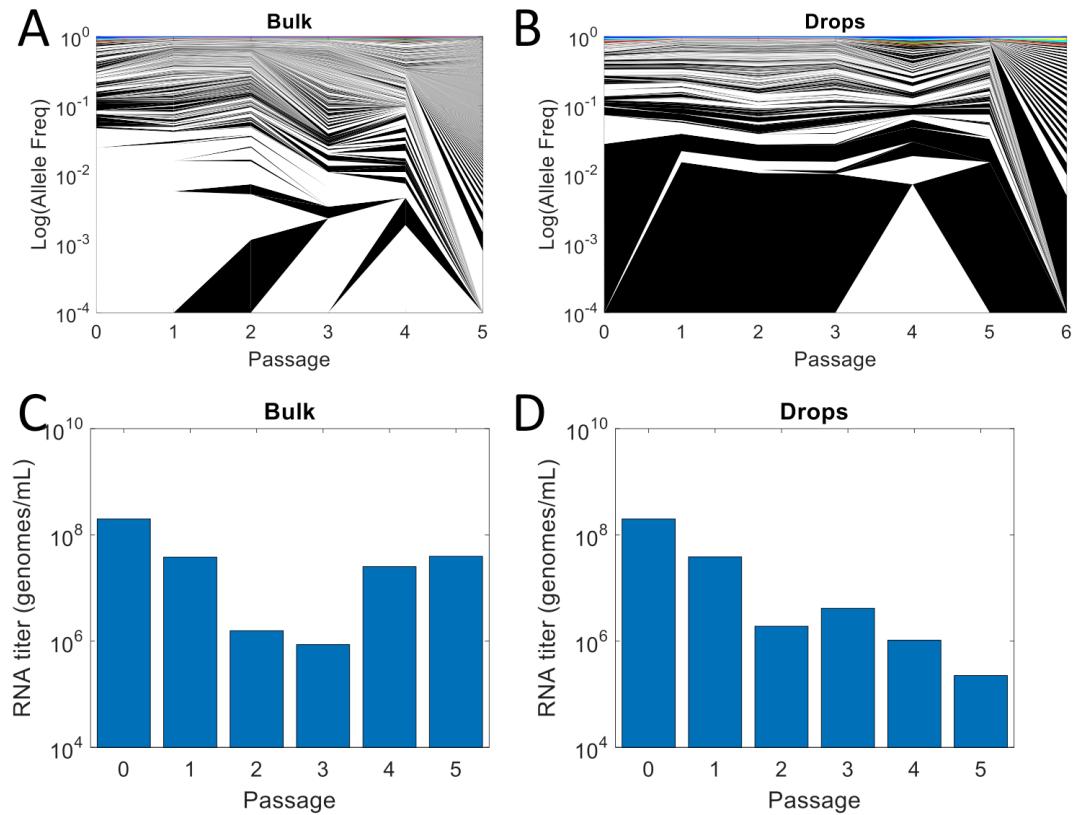


Figure 1-figure supplement 2.

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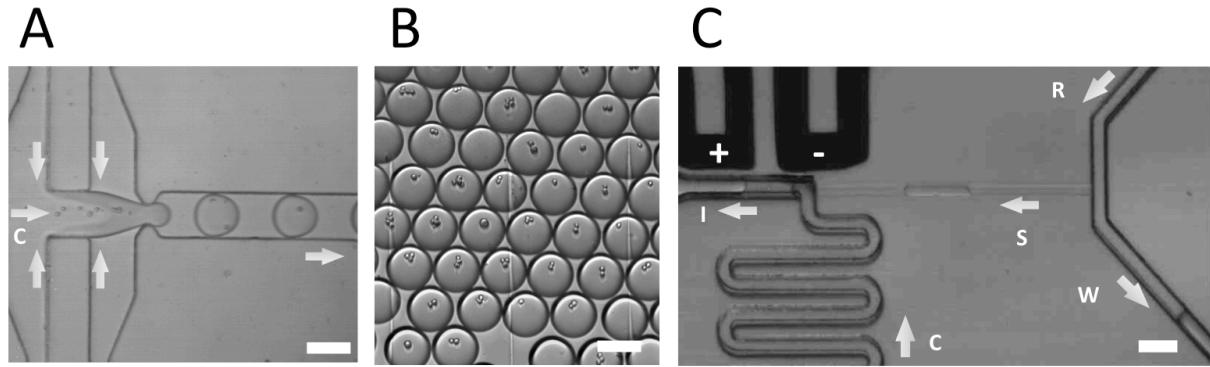


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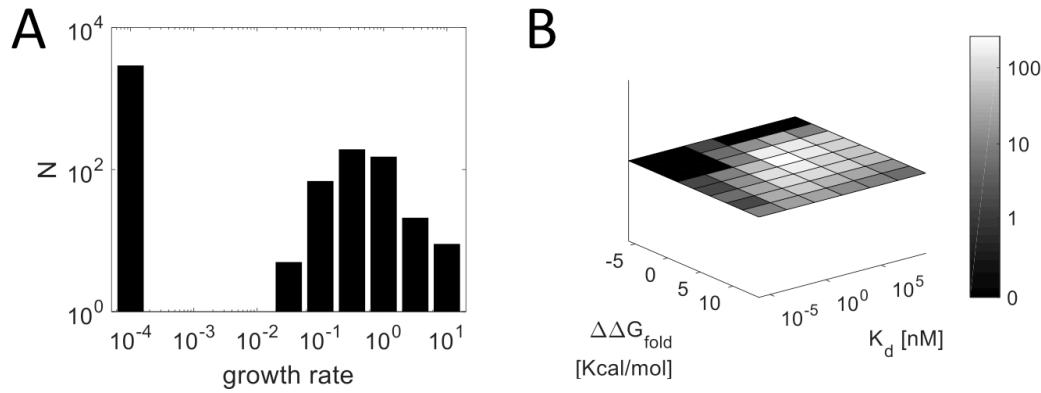


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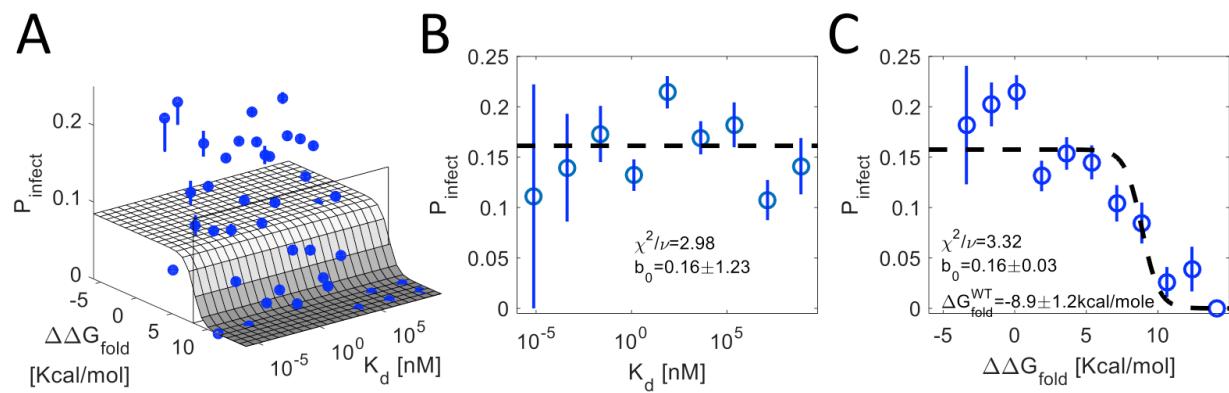


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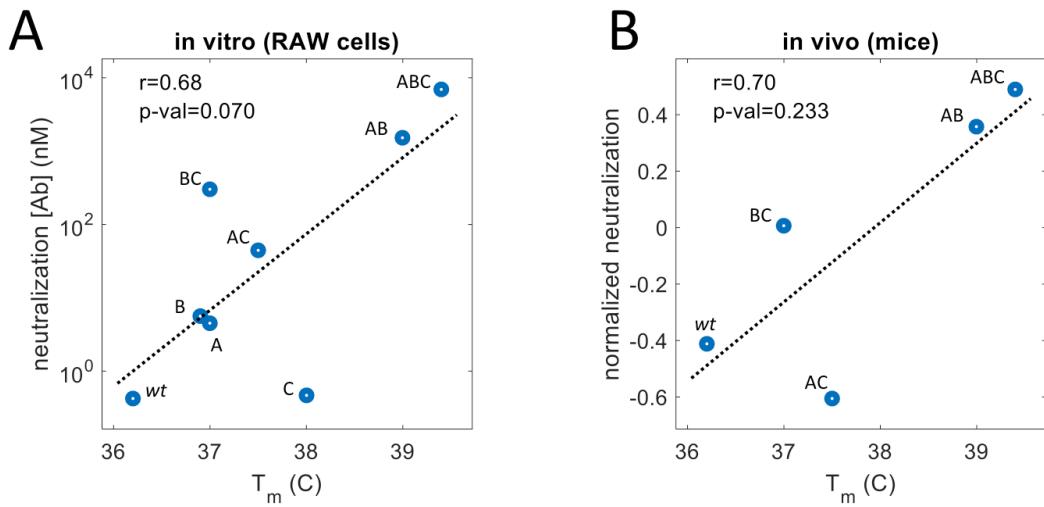


Figure 4-figure supplement 1.

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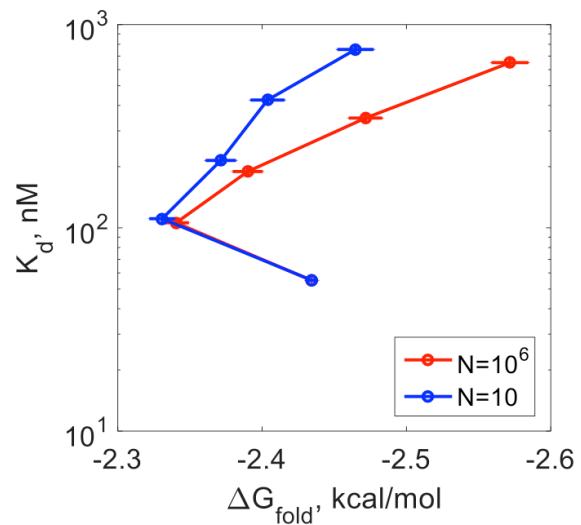


Figure 5-figure supplement 1.