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Engineering attenuated virus vaccines by controlling replication fidelity

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Long-lasting protection against viral infection is best achieved by vaccination with attenuated viruses. Obtaining stably attenuated vaccine strains has traditionally been an empirical process, which greatly restricts the number of effective vaccines for viral diseases. Here we describe a rational approach for engineering stably attenuated viruses that can serve as safe and effective vaccines. Our approach exploits the observation that restricting viral population diversity by increasing replication fidelity greatly reduces viral tissue tropism and pathogenicity. We show that poliovirus variants with reduced genetic diversity elicit a protective immune response in an animal model of infection. Indeed, these novel vaccine candidates are comparable in efficacy to the currently available Sabin type 1 vaccine strain, but have the added advantage of being more stable, as their increased replication fidelity prevents reversion to the pathogenic wild-type phenotype. We propose that restricting viral quasispecies diversity provides a general approach for the rational design of stable, attenuated vaccines for a wide variety of viruses.

Vaccines remain the most effective means for combating viral diseases, yet only a handful of safe and effective vaccines are currently available. Over the years, three major vaccination strategies have been developed[1]. The oldest and most effective approach is immunization with a live attenuated virus. When available, this approach is ideal, because it typically elicits lifelong protective immune responses. Additionally, the low production and delivery costs of these vaccines often facilitate massive vaccination efforts. However, attenuated viruses carry the risk of reversion to the pathogenic phenotype, and some live vaccines result in virus shedding by the vaccinee into the environment, which can be a potential risk to an unvaccinated community. The other two strategies rely on immunization with killed, inactivated virus or recombinant viral proteins or subunits. These approaches, as well as the related nucleic acid–based vaccines, rely on producing an immune response to viral proteins in the absence of infection, and are thus safer and applicable to wider range of viruses. Unfortunately, these vaccines are often less effective that the attenuated virus vaccines. They usually require more inoculations (boosters) to induce and sustain a strong protective response and are less effective in generating adequate cellular immune responses[1–3]. The success of attenuated virus vaccines may stem from their ability to effectively activate several branches of the immune system, including innate and acquired (humoral and cellular) immunity. Furthermore, attenuated live vaccines often replicate in relevant tissues, such as mucosal-associated tissues, and are able to elicit both local and systemic protective immune responses.

Live attenuated virus vaccines have aided in the control of a number of diseases caused by RNA viruses, such as poliomyelitis, measles, mumps, rabies, rubella and yellow fever. Notably, despite the success of such vaccines, we lack a general and rational strategy to attenuate viruses for vaccine production. Historically, attenuated vaccines have been obtained by repeated passage of the viral pathogen through nonhuman cell lines to select for adaptive mutations that reduce its overall fitness, replication competence and pathogenicity in the human host. As a result of this empirical approach, the molecular basis of attenuation remains unclear for most vaccines and appears to be different for each vaccine strain. Of note, the intrinsically high mutation frequencies of RNA viruses, together with the selective pressures exerted by the environment to enhance viral fitness, often result in the reversion of vaccine strains to the wild-type pathogenic phenotype, which leads to infrequent but severe vaccine-associated disease.

The molecular basis of the high mutation frequency of RNA viruses lies in the high error rates of viral RNA polymerases, estimated to be 1×10^{-3} to 1×10^{-5} errors per nucleotide site per round of replication[4]. High mutation frequencies are thought to aid the virus in rapid evolution and adaptation. Notably, increasing the fidelity of a viral RNA polymerase restricts the genetic diversity of the viral population and leads to an attenuated virus phenotype in an animal model of infection[5]. We hypothesized that this approach could provide a rational strategy for designing attenuated vaccines. As a proof of concept for this strategy, we engineered poliovirus type 1 Mahoney variants with increased RNA polymerase fidelities and show that these engineered viruses are highly attenuated and genetically stable and elicit a long-lasting protective immunity.

RESULTS

Engineering viruses with increased replication fidelity

A poliovirus variant (G64S) carrying a higher-fidelity polymerase was previously identified in a screen for resistance to ribavirin, a base analog that induces lethal mutagenesis when misincorporated by the

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viral RNA polymerase[5–7]. Analysis of the viral RNA-dependent RNA polymerase (RDRP) of this variant identified a fidelity checkpoint consisting of a kinetic pause at the conformational change step preceding phosphoryl transfer and phosphodiester bond linkage formation[6]. Residue Asp238 in the RNA polymerase (**Fig. 1a**) interacts with the 2′ OH group of the incoming nucleotide substrate to facilitate a key accommodation step of the incoming nucleotide into the catalytic site of the polymerase[8]. The crucial spatial positioning of Asp238 is determined by a hydrogen bond network formed by Gly1, Gly64, Ala239 and Leu241 (**Fig. 1a**).

Genetic analyses identified a key role for Gly64 in this fidelity checkpoint[6,7,9]. To explore the dynamic range of RNA polymerase fidelity, we considered whether other amino acid replacements at position 64 might alter polymerase fidelity to different degrees.

Of the 19 amino acid alternatives to glycine, six substitutions did not produce viable virus (**Fig. 1** and **Supplementary Fig. 1a** online), whereas 13 others allowed for virus replication. However, eight viruses were unstable, and their engineered codon at position 64 spontaneously changed to encode other amino acids within the initial rounds of replication. Only five small amino acid alternatives to glycine were viable and stable through five serial passages: valine, alanine, serine, threonine and leucine (**Supplementary Fig. 1a**,**b**).

We next examined the susceptibility of each viable position 64 variant virus to ribavirin. We determined virus production by growing each variant in the presence of increasing concentrations of ribavirin (**Fig. 1b**). As previously reported, viruses encoding the G64S variant were resistant to high concentrations of ribavirin (400 μM or higher), whereas wild-type poliovirus was susceptible[5,7]. Viruses expressing two newly identified polymerase variants, G64A and G64T, were also highly resistant to ribavirin, whereas viruses encoding G64V or G64L polymerases seemed almost as susceptible to mutagen as wild-type virus.

Effect of RNA polymerase fidelity on population diversity

We next assessed the effect of the position 64 mutations affecting the fidelity checkpoint on the overall diversity of the viral population. We determined the average mutation frequencies of each virus population by direct sequencing of individual viral genomes using two different approaches.

We first examined the mutation frequency of viable viruses within a population by sequencing viral RNA isolated from individual plaques[5]. In accordance with previous findings, wild-type poliovirus was highly diverse, having on average 1.89 mutations per genome (**Fig. 1c**, left), whereas the G64S variant restricted the viral population diversity to only 0.34 mutations per genome. Mutant polymerases G64A and G64T also restricted viral population diversity (0.56 and 0.59 mutations per genome, respectively). The G64V and G64L modifications resulted in a modest but significant reduction in viral diversity to mutation frequencies of 1.03 and 1.07 mutations per genome ($P < 0.01$ by ANOVA and Tukey HSD). Notably, the mutation frequencies observed for viruses carrying these polymerase variants correlated with their degrees of resistance to ribavirin (**Fig. 1d**).

The second approach to measuring genetic diversity in viral populations analyzed genomes that were extracted from virions, RT-PCR–amplified and then cloned into plasmids; thereby bypassing the plaque-purification step that probably limited the previous analysis to viable, replication-competent genomes. Thus, we expected that a larger average number of mutations per genome would be detected,

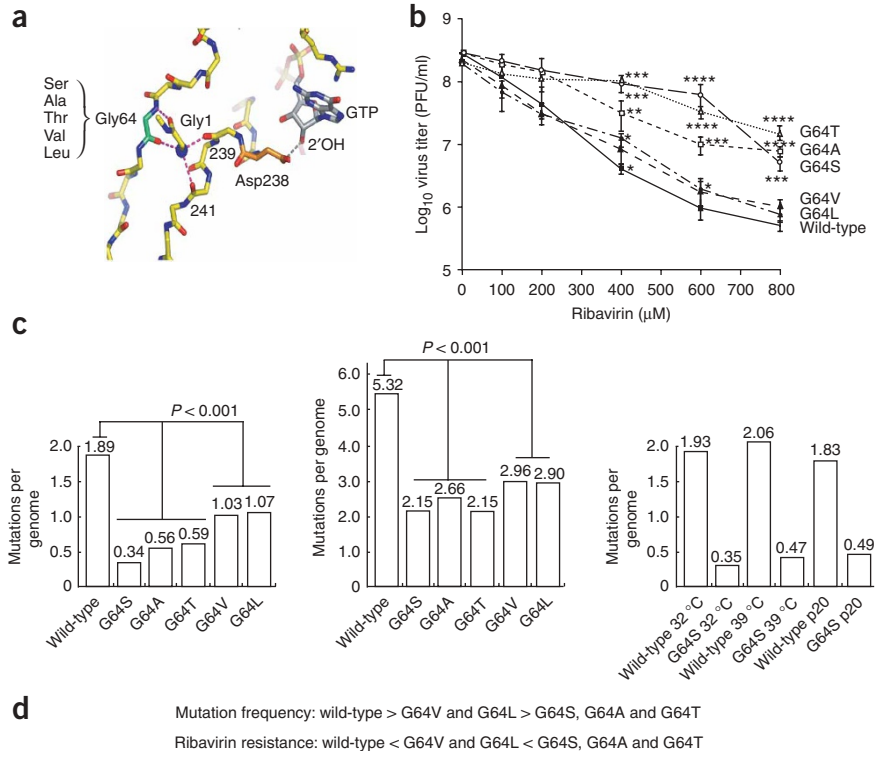

Figure 1 Design of high-fidelity polymerase variants of poliovirus. (**a**) Structural model of the extended hydrogen bond network within the poliovirus RDRP. Gly64 participates in a tetrahedral hydrogen bond network alongside residues 1, 239 and 241, which facilitate the interaction of Asp238 with the 2′ OH group of an incoming nucleotide. This interaction is the recently identified fidelity checkpoint[6]. Substitution of Gly64 with other residues is expected to alter the position of Asp238, decreasing the polymerase's intrinsic error rate. (**b**) Production of wild-type poliovirus (solid line) or position 64 variants (broken lines) in the presence of ribavirin (0–800 μM). Curves represent the triplicate average of three independent experiments ± s.d.; values for all high-fidelity virus titers at 400 μM ribavirin and above are significantly higher than those of wild-type virus (****$P < 0.0005$, ***$P < 0.001$, **$P < 0.005$ and *$P < 0.01$, Student's two-tailed unpaired t-test; $n = 6$ per time point). (**c**) The genetic diversity of a given virus population is represented as the average number of changes from the parental consensus sequence (40–100 genomes of each population were analyzed). Left, 24–48 plaque-purified viruses from each of 5 viral populations were sequenced. Middle, total RNA extracted from the virus stocks was used to produce cDNA and cloned into bacterial plasmids, and 50–100 individual plasmids per population were sequenced. Right, plaque-purified virus populations of wild-type or G64S virus grown for three passages at either 32° or 39 °C, or passaged 20 times; 20–24 viruses per population were sequenced. Values for wild-type virus; the group comprising G64S, G64A and G64T virus and the group comprising G64V and G64L virus are significantly different from each other ($P < 0.001$, ANOVA and Tukey HSD). (**d**) Representation of the hierarchy in mutation frequency shown in **c** and its correlation with RNA mutagen resistance shown in **b**.

because genomes containing multiple and/or lethal mutations would also be analyzed. Indeed, the wild-type virus population contained an average of 5.32 mutations per genome (**Fig. 1c**, middle). Consistent with the trends observed for the plaque-purification method, both the G64S and the G46T variants had the lowest value of 2.15 mutations per genome, followed by the G64A, G64L and G64V variants with values of 2.66, 2.90 and 2.96 mutations per genome, respectively.

In principle, polymerase activity and fidelity can be altered by growth at suboptimal temperatures. To examine this possibility, we grew wild-type and G64S virus at 32 °C or 39 °C. Passage 3 populations were plaque-purified and sequenced. We observed no significant difference between the sequence diversities of wild-type and G64S virus grown at either temperature (**Fig. 1c**). We next determined whether the sequence diversity in higher-fidelity populations increases over a more extensive number of replication cycles. Wild-type and G64S viruses were passaged 20 times at a low multiplicity of infection (MOI = 0.1), representing approximately 40–60 replication cycles. Even after prolonged passaging, G64S virus retained a restricted genetic diversity. The data extend previous observations that genetic diversity of a higher-fidelity virus population quickly reaches equilibrium and maintains restricted diversity over time[5,6].

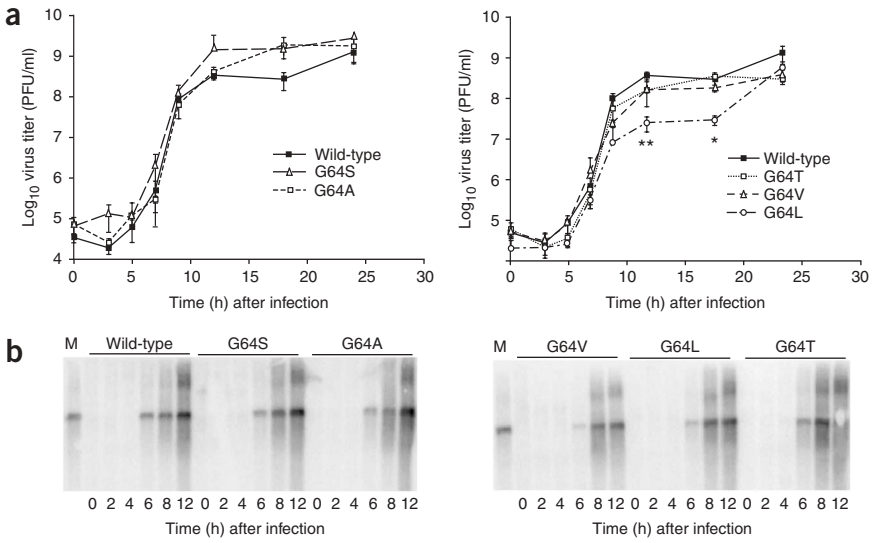

Figure 2 Modulation of viral RNA polymerase fidelity does not negatively affect replication kinetics. (**a**) One-step growth curves of wild-type virus or high-fidelity variants. HeLa cells were infected with each virus at different time points after infection, and viral progeny was titered by standard plaque assay. The replication kinetics of each variant were not significantly different from wild type except where indicated (**$P < 0.005$ and *$P < 0.05$, unpaired two-tailed Student's *t*-test, $n = 3$ per time point). Results are represented in two panels for clarity but are representative of a single experiment. Viral growth is shown as titers of viral progeny as a function of time after infection. The mean ± s.d. of three independent experiments is shown. (**b**) Northern blot analysis of viral RNA production as a function of time. Total cytoplasmic RNA from cells infected with each virus variant was extracted at time points indicated, purified and subjected to northern blot analysis with a radiolabeled probe specific for the poliovirus genome. M, *in vitro*–transcribed poliovirus RNA as marker. Northern blot shown is representative of two independent experiments.

Our results show that polymerase fidelity is not a fixed trait in a given virus and can be altered by simple substitutions of amino acids participating in fidelity checkpoints. Notably, the evolutionary pressure to modulate the error rates in viral polymerases may allow for fine-tuning through a larger dynamic range than previously anticipated.

Effect of fidelity checkpoint mutations on viral replication

We next examined the replication kinetics of these Gly64 polymerase mutants in HeLa cells (**Fig. 2a**). All viral variants showed growth rates and final yields comparable to those of wild-type virus (**Fig. 2a**). G64A and G64S viruses consistently grew to slightly higher final yields with the same kinetics as wild-type virus (**Fig. 2a**). G64L virus was slightly compromised in replication, but eventually reached wild-type titers (**Fig. 2a**). Northern blot analysis of viral RNA showed no significant defects in viral RNA accumulation for any of the variants (**Fig. 2b**). Of note, sequencing data indicated that all the viruses maintained their engineered position-64 substitution (**Supplementary Fig. 1b**). The observation that higher-fidelity polymerase variants replicate with wild-type kinetics has important implications for their vaccine potential, as a robust infection will induce stronger immune responses and should favor stability of the vaccine strain.

Increasing fidelity restrains virus adaptation

By conventional attenuation, live virus vaccines generally have a fitness defect with respect to the wild-type virus. Low fitness levels combined with the high mutation rates of RNA virus replication promote the acquisition of mutations that increase viral fitness and pathogenesis. The emerging higher-fitness viruses can quickly dominate the population and pose a threat both to the vaccinee and to unvaccinated individuals in the vaccinee's vicinity (http://www.polioeradication.org/vaccines.asp). For example, more than 80% of the virus shed by vaccinees who receive the Sabin-3 oral poliovirus vaccine (OPV) strain corresponds to revertants with second-site attenuation-suppressor mutations and pathogenic characteristics[10–14]. An attractive aspect of exploiting high–replication-fidelity virus to develop an attenuated vaccine is that, unlike other forms of attenuation, this approach would actually reduce the ability of the vaccine strain to mutate and evolve toward more pathogenic forms.

To assess the genetic stability of the high-fidelity viruses, we developed an assay in which any of a number of mutations would confer a strong adaptive advantage to the virus (**Fig. 3**). For this assay, we introduced a target site complementary to the host endogenous let-7 micro RNA (miRNA) into the 5′ noncoding region of wild-type and high-fidelity variants (**Fig. 3a**). When grown in cell lines that express the let-7 miRNA, the corresponding site within the viral genome is targeted by the host gene-silencing machinery and the viral genome is degraded[15]. Any mutation in the let-7 sequence will give rise to a virus that can no longer be targeted by the gene-silencing machinery. In this system, the virus still replicates at low levels while being subjected to purifying selection, such that escape mutants are greatly favored. Thus, this system mimics the selective conditions faced by attenuated viruses during vaccination.

HeLa cells transfected with the let-7(+) virus bearing a wild-type polymerase almost immediately gave rise to viruses that had mutations in the let-7 target sequence at a number of different sites

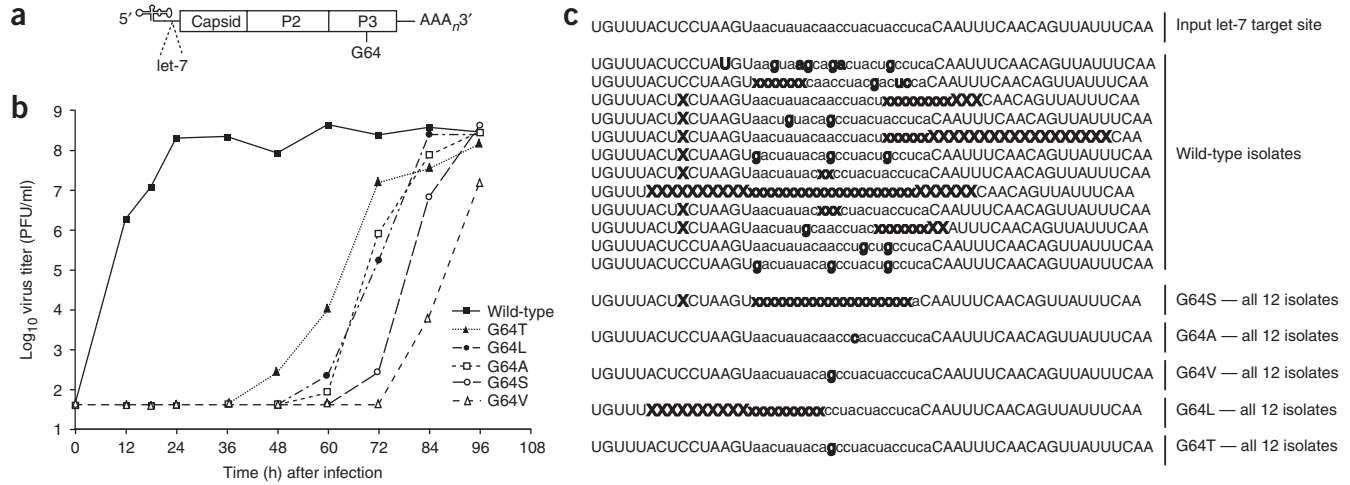

Figure 3 High-fidelity variants are less likely to revert or mutate toward higher-fitness genotypes. (**a**) Schematic of let-7–poliovirus constructs. The 22-nucleotide let-7 miRNA target sequence is inserted into the 5′ untranslated region of poliovirus bearing wild-type or higher-fidelity polymerases. Genomes bearing the let-7 target sequence will be targeted by the gene-silencing machinery in HeLa cells unless the let-7 target is mutated at one or more nucleotides. (**b**) HeLa cells were transfected with RNAs encoding infectious wild-type or higher-fidelity virus variants. At different time points after infection, the emergence of replication-competent, higher-fitness viruses resulting from mutation of let-7 target sequences was scored by plaque assay. The graph of viral titers as a function of time after transfection is representative of two independent experiments. (**c**) Sequences of the let-7 target site of individual escape mutants from each population. The input (transfected) RNA sequence is shown. Capital letters indicate poliovirus sequences; lower-case letters correspond to the inserted let-7 miRNA target sequence. Lettering in bold indicates mutations and deletions (x). For each population, 12 individual viruses were plaque-purified and sequenced.

(**Fig. 3c**), and virus titers increased exponentially within the first hours after transfection (**Fig. 3b**). In contrast, all high-fidelity variants were severely compromised in their ability to replicate until 96 h after transfection. Notably, sequencing of the let-7 miRNA target site of 12 individual clones from each population revealed that virus with wild-type polymerase escaped let-7 control through a diverse set of alterations of the target site, including multiple deletions and/or mutations (**Fig. 3c**). In contrast, escape mutants in the higher-fidelity populations arose from single escape events, as all 12 clones analyzed from any given population contained the exact same mutation within the let-7 target site (**Fig. 3c**). Thus, although increasing replication fidelity does not impair the replication rate of the virus under normal growth conditions, it markedly compromises the virus's ability to mutate toward a higher-fitness genotype, even under conditions in which selection would strongly favor viral emergence.

Modulation of replication fidelity strongly attenuates viruses

We next evaluated the effect of restricting population diversity on viral pathogenesis in infected mice[5]. We initially determined the 50% lethal dose (LD_{50}) for each replication-fidelity variant in cPVR transgenic mice expressing the human poliovirus receptor (**Fig. 4a** and **Table 1**). After intramuscular injection, wild-type poliovirus quickly invaded the central nervous system, resulting in paralysis and death within 5 d (**Fig. 4a** and **Table 1**). The LD_{50} of wild-type poliovirus is 1.2×10^6

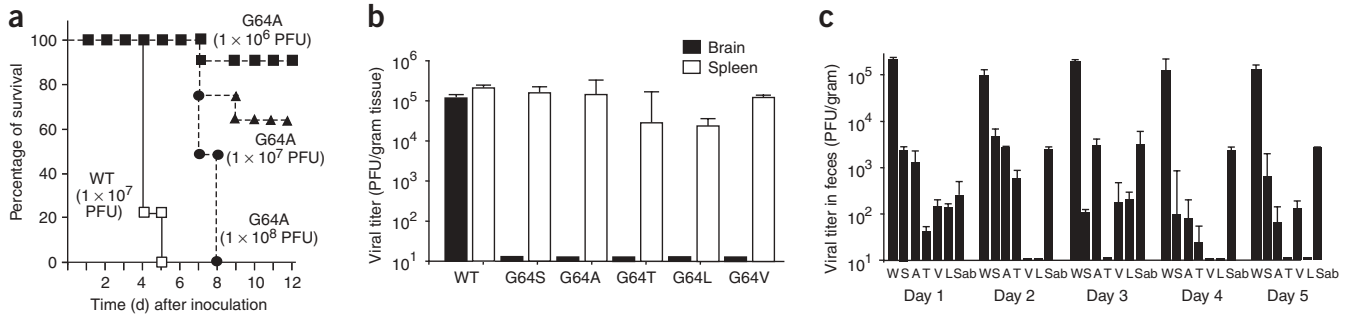

Figure 4 Modulation of replication fidelity strongly attenuates viruses and restricts tissue tropism and viral shedding. (**a**) High-fidelity variants are less virulent and show delayed onset of symptoms at even the highest doses. cPVR mice were infected with serial dilutions of wild-type virus (WT) or high-fidelity variants and monitored for the onset of disease and mortality. Depicted are the survival curves of mice receiving different doses of G64A virus, which was found to be the least attenuated of all viruses, and WT virus. (**b**) Tissue tropism of high-fidelity variants is restricted. cPVR mice were infected with 0.1LD_{50} of each virus variant, and, on each day after infection, mice from each group were killed to determined the viral titers in either brain or spleen homogenates. The graph depicts the viral titers (mean ± s.d., $n = 5$) present in the brains of mice on day 5, when viral titers were at their peak. (**c**) High-fidelity variants have reduced shedding. cPVR mice were infected with 0.1LD_{50} of each virus (Wild-type, W; G64S, S; G64A, A; G64T, T; G64V, V; G64L, L and Sabin 1, Sab) and shedding of virus from feces was collected and quantified daily ($n = 6$ per group). Mean viral titers ± s.d. are shown.

Table 1 High-fidelity variants are 40–300 times less virulent than wild-type poliovirus

Virus	LD_{50} (PFU)
Wild-type	1.2×10^6
G64S	3.9×10^8
G64A	4.9×10^7
G64T	$>1.8 \times 10^8$
G64V	$>1.8 \times 10^8$
G64L	$>1.8 \times 10^8$
Sabin 1	$>1.0 \times 10^8$

All LD_{50} values for higher-fidelity viruses are significantly different from the wild type ($P < 0.001$, Kaplan-Meier test; $n = 12$ per group per dose).

infectious particles (plaque-forming units, PFU; **Table 1**). In comparison, all high-fidelity viruses were strongly attenuated, with LD_{50} values that were 40–300-fold higher than those achieved with wild-type virus (**Fig. 4a** and **Table 1**) and with a marked delay in the onset of symptoms at even the highest doses (**Fig. 4a** and **Supplementary Fig. 2** online). The LD_{50} values of these high-fidelity mutants exceeded those of the currently used live attenuated Sabin vaccine strains of poliovirus[16]. Notably, all of the alterations at position 64 prevented the establishment of infection in the central nervous system while allowing wild-type levels of viral replication in non-neuronal tissues (**Fig. 4b**). This is in contrast with the Sabin vaccine strains, whose attenuation stems from reduced overall fitness[17]. The decreased fitness of Sabin strains, associated with reduced replication and translation rates in tissue culture, favors the selection of revertants with wild-type pathogenic characteristics[13–15]. We hypothesized that generating a more robustly replicating vaccine strain might lower the risk of reversion. Indeed, replication of high-fidelity variants in mice over the 5-d period after infection did not result in reversion of the mutation at position 64 (**Supplementary Fig. 1b**).

To further examine the stability of the high-fidelity replication variants in infected mice, we performed mouse-to-mouse passages of the G64S variant. Mice were infected intramuscularly with 1×10^8 PFU of G64S virus. Five days after infection, the virus was isolated from several tissues, pooled together and reinoculated into naive mice. This procedure was repeated five times, with 25 total days of replication in mice. Notably, after passage 5 (p5), the mouse-passaged G64S virus population did not show evidence of increased pathogenesis. Indeed, 1×10^8 PFU of G64S-p5 did not paralyze any of the mice inspected ($n = 10$) or produce other symptoms after intramuscular reinoculation, whereas 1×10^8 PFU of wild-type virus resulted in 100% death by 5 d after infection (data not shown). Sequencing of the G64S-p5 virus showed that the serine at position 64 remained stable in the mouse model and that no additional mutations were introduced and fixed in the higher-fidelity population (**Supplementary Fig. 1b**).

Restricted tissue tropism and viral shedding

Previous observations have indicated that G64S virus cannot enter and/or establish a productive infection in the central nervous system[5,18]. We thus examined the tissue tropism of all the new high-fidelity replication variants generated in course of this study. All replication-fidelity variants accumulated at wild-type levels in the spleens of infected mice (**Fig. 4b**), indicating that these vaccine candidates replicate efficiently in non-neuronal tissues. However, whereas wild-type poliovirus was present in the brains of all mice examined after 2 d of infection and reached maximal levels at 4 and 5 d after infection (**Fig. 4b**), we never detected the attenuated fidelity variants in the brains of infected mice (**Fig. 4b** and data not shown).

A major challenge for live, attenuated vaccine development is to achieve high immunogenicity while minimizing viral shedding by the vaccinee into the environment. Indeed, shedding of OPV-derived polioviruses is a major impediment to the Global Polio Eradication Initiative. We thus evaluated viral shedding by mice inoculated intravenously with the high–replication-fidelity virus variants. Of note, all Gly64 variants presented a marked reduction of two to three orders of magnitude in viral shedding with respect to the wild type (**Fig. 4c**). It thus appears that restricting population diversity by increasing replication fidelity decreases virus dissemination, potentially reducing the risk of virus release into the environment.

Replication-fidelity viruses are highly immunogenic

We next determined the immunogenic potential of the high–replication-fidelity viruses. Sera collected from immunized mice

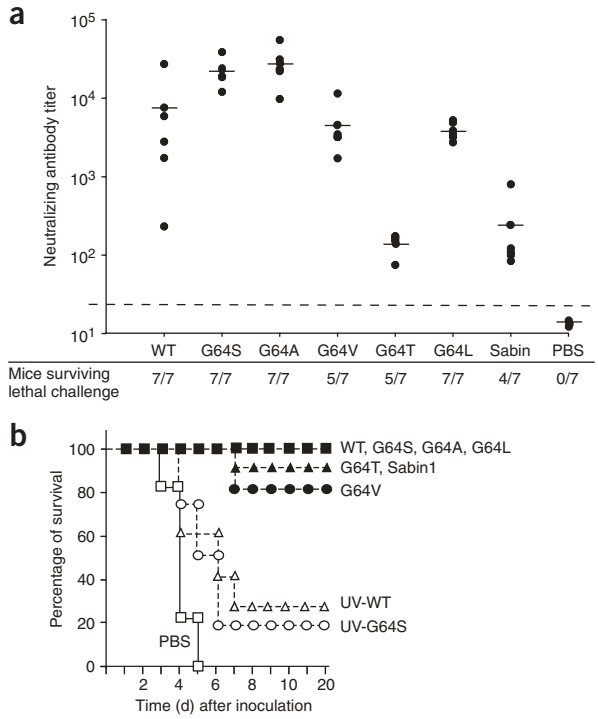

Figure 5 High-fidelity vaccine candidates induce high levels of neutralizing antibody and confer long-lasting immunity against lethal challenge. (**a**) cPVR mice were immunized with 1×10^6 PFU of the high-fidelity vaccine candidates, wild-type poliovirus, Sabin vaccine strain or PBS. At 4 weeks after immunization, serum was collected from mice and neutralizing antibody titers were determined. Neutralizing antibody titers (reciprocal of the serum dilution able to neutralize 100 $TCID_{50}$ of wild-type poliovirus) are shown for individual mice (solid circles) and as group means (barred circles). Titers for PBS-immunized mice were below the detection level of the assay (indicated by dashed line). The number of mice protected in each group after lethal intraperitoneal infection with $5LD_{50}$ of wild-type poliovirus is indicated below the *x*-axis. (**b**) Immunization of mice with high-fidelity vaccine candidates confers long-lasting immunity to lethal infection. cPVR mice received a single immunization with $0.1LD_{50}$ of high-fidelity vaccine candidates, wild-type poliovirus, Sabin 1 vaccine strain, 1×10^7 UV-inactivated wild-type or G64S virus, or PBS. Six months after infection, mice were challenged with $10LD_{50}$ of wild-type virus by intramuscular injection. Survival curves for mice immunized with wild-type and high-fidelity variants are significantly higher than those for mice immunized with control PBS and UV-inactivated virus ($P < 0.001$, Kaplan-Meier test).

4 weeks after infection revealed that all high-fidelity variants induced poliovirus-specific neutralizing antibodies (**Fig. 5a**). Large amounts of neutralizing antibody were elicited by the G64S and G64A variants, with values significantly higher than those elicited by wild-type poliovirus ($P < 0.01$, unpaired two-tailed *t*-test) and the Sabin strain ($P < 0.001$). The amounts of neutralizing antibody obtained with G64L and G64V variant vaccination were also higher than those obtained with Sabin vaccine strain ($P < 0.001$) and similar to those from a wild-type virus infection. Although G64T virus and the current Sabin vaccine strain induced neutralizing antibody over background (PBS, saline inoculation control), the induction was two orders of magnitude lower than that obtained by inoculation with the G64L and G64V variants (**Fig. 5a**). We thus conclude that, at least in this mouse model, the high-fidelity replication variants are equivalent or superior immunogens compared to the current Sabin 1 vaccine strain.

We next challenged the vaccinated mice with a lethal ($5LD_{50}$) intraperitoneal inoculation of wild-type poliovirus one month after immunization. Protection of mice from lethal infection correlated with the neutralizing antibody abundance. Thus, whereas all mice immunized with wild-type, G64S, G64A and G64L candidates were protected (**Fig. 5a**), mice vaccinated with viruses that had elicited lower amounts of neutralizing antibodies, G64V, G64T and Sabin 1 vaccine strain, were only partially protected (four or five of seven mice protected). Nonetheless, these mice showed a delayed onset of symptoms and death when compared to PBS-immunized control mice, which were fully susceptible to poliovirus-induced disease (0 of 7 control mice survived).

As a more stringent test, we allowed for a 6-month interval between a single-dose vaccination with each vaccine candidate and a subsequent challenge with a lethal amount ($10LD_{50}$) of wild-type poliovirus administered by intramuscular injection. By this route, the virus quickly accesses the central nervous system by axonal retrograde transport[19,20] and produces rapid signs of paralysis and death within 3–5 d. Notably, the majority of the mice immunized with the high-fidelity variants were protected from paralysis and death, whereas control mice receiving UV-inactivated virus (wild-type or G64S) were not protected (**Fig. 5b**). These results indicate that all fidelity-variant vaccine candidates induce long-lived immunity from a single immunization performed 6 months before challenge.

DISCUSSION

Here we show that restricting the quasispecies diversity of a viral population provides a new approach to engineering live attenuated vaccines for RNA viruses. These new vaccine strains have a substantial reduction in pathogenicity yet elicit long-lasting protective immunity from lethal challenges with pathogenic viruses. One of the strategic advantages of this approach is that attenuation of these viruses stems from enhancing their replication fidelity rather than decreasing their overall fitness. As a result, these vaccine strains are genetically stable and less likely to mutate and revert to a pathogenic wild-type phenotype, one of the major problems associated with the live attenuated vaccines currently used. Furthermore, high-fidelity viruses have an impaired ability to spread through the organism, which both limits their pathogenicity and greatly decreases shedding of the live virus into the environment. Another advantage of this virus-attenuation approach is that despite the viruses' reduced ability to spread through an organism, they maintain their replicative capacity in certain peripheral tissues, thus resulting in a robust immune response.

We propose that restricting the population diversity of RNA viruses by enhancing their replication fidelity may constitute a general strategy for vaccine development that circumvents the limitations of the current approaches to develop attenuated vaccines, which largely rely on a process of trial and error. Recent observations indicate that for several RNA viruses, pathogenicity is linked to genome diversity[18,21–31]. The current study thus provides a rational approach for viral attenuation that may be applicable to a broader range of RNA viruses.

Our proposed strategy to develop attenuated viral vaccines presents a key advantage over current approaches, which are based on the identification of viruses with an overall lower fitness. In contrast to these approaches, our strategy produces attenuated viruses with robust replication characteristics (**Fig. 2** and **Fig. 4c**). This important feature has the twofold advantage of promoting an effective immune response and reducing the chance of the attenuated virus being outcompeted by a fitter, more pathogenic virus, should it arise during replication[32].

Evolutionary fitness and population diversity result from the interplay of mutation rates and selective pressures[33,34]. Here we developed a strategy to tune the mutation rate of an RNA virus. Our results show that amino acid position 64 of the poliovirus RNA polymerase, which is central to the modulation of replication fidelity, can be mutated to any of the five smallest amino acids without a noticeable effect on the viral replication cycle. Our data demonstrate that poliovirus RNA polymerase fidelity can be fine-tuned and modulated without affecting replication (**Fig. 2**). However, restricting the diversity of an RNA virus by increasing its fidelity compromises the ability of the virus to adapt to changing environments (**Fig. 3**), escape antiviral immunity and survive population bottlenecks. These findings are consistent with the idea that RNA viruses have evolved suboptimal viral polymerase fidelity to permit rapid evolution and adaptation.

Our findings may have immediate practical implications for the prevention of diseases caused by picornaviruses in general and enteroviruses in particular. Both the structure and the intrinsic high error rate of viral RDRP appear to be well conserved among all picornaviruses[35]. The polymerase residues in the hydrogen bond network modulating replication fidelity are particularly well conserved, indicating that our approach for modulating replication fidelity in this virus family may be generally applicable to creating live vaccines for a large number of pathogenic picornaviruses for which no vaccines are available[36].

Less is known about the mechanisms that control replication fidelity in other virus families. However, it should be possible to isolate high-fidelity variants of these viruses by taking advantage of the general susceptibility of RNA viruses to mutagens such as ribavirin or 5-fluorouracil[37,38]. Indeed, this strategy has been successfully used to identify a high-fidelity polymerase mutant of foot and mouth disease virus[10]. Applying such selection strategies to other viruses may not only provide novel structural and mechanistic insights into polymerase fidelity, but also uncover new candidates for attenuated vaccine strains for diseases that remain refractory to vaccine development.

Although it is not known how an increase in polymerase fidelity would affect the safety of a vaccine within large-scale vaccination programs, it is possible that the information described here may help to improve the stability and safety of current attenuated live-poliovirus vaccines by combining well-established attenuating mutations of Sabin strains with replication-fidelity determinants.

Our finding that high-fidelity polioviruses show high immunogenicity without reverting to wild-type phenotype or shedding into the environment may provide a new and important tool for the polio

eradication program, which has recently stalled owing to several drawbacks inherent to the currently used OPV[17,32]. Indeed, the recent roadblock in the poliovirus eradication program has led to a debate about the most appropriate vaccination protocol to achieve the goal of eradication[39–42]. In developed countries with no incidence of wild-type poliovirus–associated paralytic poliomyelitis, use of the OPV is avoided in favor of the safer but less effective inactivated polio virus (IPV). In developing countries, however, the use of the expensive IPV presents many logistical problems that are compounded by the fact that IPV is thought to be ineffective at inducing immunity at the gut mucosal surface. As a result, it provides individual protection against polio paralysis but, unlike OPV, is less effective in preventing the spread of wild-type circulating poliovirus[43,44].

In light of these events, there is a renewed interest in new or improved poliovirus vaccines. Two other approaches have been recently proposed. In one approach, viral RNA translation efficiency is compromised by the introduction of synonymous rare codons[45,46]. In a second approach, the 5′ internal ribosomal entry site structure is modified to stabilize attenuation determinants[47] or is exchanged in its entirety for that of human rhinovirus type 2 (ref. 48). These approaches, together with the strategy described here, can be used for the production of either live attenuated vaccine candidates or killed IPV vaccine, as producing IPV from wild-type virus would represent a considerable risk during the late stages of a poliovirus eradication campaign.

Although the mouse model used here recapitulates many major aspects of human polio disease and demonstrates the safety and immunogenicity of high-fidelity replication viruses, there are a number of differences between the model and human disease, most notably the fact that it is not possible to infect the mice orally. However, the promise of these preclinical animal studies is encouraging for further testing in the virus's natural hosts. These future studies should determine whether these high-fidelity polio vaccine strains could indeed protect humans while reducing virus dissemination and shedding into the environment.

METHODS

Viruses and plasmids. We used plasmid prib(+)XpAlong[49], containing the wild-type poliovirus type 1 Mahoney cDNA, to substitute the glycine-encoding codon 64 of the RDRP $3D^{pol}$ with all other amino acids using the Stratagene Quikchange XL kit. We produced virus stocks by electroporation of 20 μg of *in vitro*–transcribed RNA genomes, generated from the cDNA-bearing plasmids described above, into HeLa cells (1×10^7 cells/ml) in a 4-mm cuvette with the following pulse: 300 V, 24 Ω and 950 μF. We passaged the progeny five times further at a high MOI (MOI = 10) at 37 °C or 32 °C (Sabin strain). We performed three separate series of virus production for each codon change (**Supplementary Fig. 1**).

Genomic sequencing for mutational frequency. To assess population diversity, we used passage 5 viruses grown at 37 °C (**Fig. 1a**,**b**), passage 3 viruses grown at 32 °C or 39 °C (**Fig. 1c**) or passage 20 viruses grown at 37 °C (**Fig. 1c**). We determined sequence diversity by plaque assay as previously described[5]. Briefly, we performed RT-PCR on RNA extracted from plaque-purified viruses. We sequenced PCR products corresponding to nucleotides 300–3,300 (**Fig. 1a**,**c**). We sequenced 20–48 individual plaques per population for a total of 72,000–96,000 nucleotides per population to determine the average mutational differences per genome with respect to parental cDNA sequence. As an alternative method (**Fig. 1b**), we used RNA extracted from viral stock supernatants for RT-PCR amplification of nucleotides 300–3,300, subcloned the PCR product into pUC18 plasmid and sequenced 50–100 plasmids per population, for a total of 150,000–300,000 nucleotides. To ensure that enough starting genomic RNA was used (and that we were not amplifying only a handful of genomes), we also amplified a 1:100 dilution of the RNA by RT-PCR. If a similar, prominent PCR product resulted from this amplification, we could be confident that our starting RNA population was large enough to be representative of the total population.

One-step growth curve studies, northern blot analysis and ribavirin-sensitivity assay. These assays characterizing virus growth in tissue culture have been previously described[5]. For ribavirin resistance, we pretreated HeLa cells for 4 h with ribavirin (0–800 μM, Sigma), infected them at a low MOI of 0.01 and quantified progeny virus 48 h after infection by standard plaque assay. For one-step growth curves and northern blots, we infected HeLa cells at MOI = 10 and, at different time points, quantified progeny virus by plaque assay or extracted total cytoplasmic RNA for northern blot analysis. For the latter, we normalized RNA samples to rRNA content, hybridized them with a poliovirus-specific radiolabeled probe and quantified the RNA on a Storm phosphorimager (Molecular Dynamics).

Let-7 virus construction and assay. To construct let-7 viruses, we inserted *Bgl*II and *Eco*RI restriction site–flanked fragments containing the different polymerase coding sequences into the cDNA of the poliovirus genome containing the let-7(+) target sequence inserted at position 703 (ref. 15). We transcribed the RNA *in vitro* and transfected it into HeLa cells, which express the let-7 miRNA. At the indicated times, we froze the cells and isolated 12 individual viruses constituting let-7 escape mutants by plaque assay and then sequenced them.

Infection of susceptible mice. Protocols for the mouse studies described here were approved by the University of California–San Francisco Institutional Animal Care and Use Committee (IACUC). We used 8–12-week-old cPVR-transgenic mice expressing the poliovirus receptor in all experiments. We performed the following inoculations while the mice were under anesthesia: intramuscular (2×50 μl, one in each quadriceps), intraperitoneal (250 μl) and intravenous (100 μl, tail vein). We monitored the mice daily for the onset of paralysis and killed them if total paralysis was imminent. For determination of LD_{50} values by the Reed and Muench method, we infected 12 mice per group intramuscularly with serial dilutions of virus. For tissue tropism studies, we harvested whole organs (brain or spleen) or 40 fecal pellets from 5 mice that had been infected intravenously per group and homogenized the organs or pellets with an Ultraturrax T8 homogenizer (IKA Works). We titered tissue homogenates on HeLa cells by standard plaque assay. For the neutralization assay, we collected serum 4 weeks after the mice had been immunized with 1×10^6 PFU of virus intraperitoneally. We treated 100 times the 50% tissue-culture infectious dose ($TCID_{50}$) of wild-type poliovirus for 2 h with serial dilutions of sera (performed in octuplicate). We determined the serial dilution that completely neutralized $100TCID_{50}$ by incubating samples on HeLa cells in 96-well plates for 1 week. The neutralizing dilution was determined by the Reed and Muench method. The reciprocal of this dilution is the neutralizing antibody titer represented in the graph. For one set of protection studies (**Fig. 5a**), we challenged the mice immunized for neutralization assays via intraperitoneal injection with $5LD_{50}$ of wild-type poliovirus at 4 weeks after immunization. In another protection experiment (**Fig. 5b**), we immunized the mice with $0.1LD_{50}$ of wild-type virus, high-fidelity variant virus, Sabin 1 vaccine strain or with 1×10^7 PFU of UV-inactivated virus. Six months after immunization, we challenged the mice by intramuscular injection with $10LD_{50}$ of wild-type poliovirus.

Statistical analyses. We performed statistical analyses by the Student's paired or unpaired *t*-test or Mann-Whitney *U*-test. We rejected the null hypothesis at a *P* value of 0.05. Values are presented as means ± s.d. unless stated otherwise.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.V. and R.A. conceived the idea for the study; designed, directed and interpreted all of the experiments and prepared the manuscript. M.V. carried out most of the experiments. E.W. carried out the experiments presented in **Figure 1b**, **Figure 2a** and part of **Figure 2b**.

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