GenBank accession number		
YP_001648053.1	AAP92340.1	AGT77289.1
CAA30136.1	ABF00945.1	AAW33516.1
AET14262.1	ABF00940.1	YP_002213831.1
CAA09497.1	AGT75584.1	AGT75888.1
CAD91889.1	AP_000576.1	AAZ15249.2
ACY41087.1	AGT76272.1	AAZ13824.2
ABO48392.1	AAW33476.1	AGT76147.1
NP_044849.1	ADQ38371.1	AGT77111.1
EPY75987.1	AGT77732.1	AGT75671.1
YP_001648062.1	AET87303.1	AFQ34339.1
XP_004185906.1	AAW33432.1	AET87262.1
EMH76195.1	AFH58030.1	AET87221.1
CAA38621.1	ACV41281.1	AFQ34378.1
XP_001735501.1	ACU57037.1	AGV32761.1
XP_654477.1	ACO81791.1	AP_000266.1
EMD48635.1	AAW33115.1	CAC08221.2
EKE43021.1	AGF90825.1	AAW33337.2
CAA37450.1	AFQ34496.1	AAW33246.2
YP_002004529.1	AFQ34457.1	AAT97530.2
P03680.1	NP_073685.1	AAS16276.1
CAA37451.1	AGT76234.1	YP_006272954.1
P06950.1	AAR89955.1	YP_068023.1
NP_690635.1	AGT76890.1	P05664.1
ACH57069.1	AGT77245.1	AP_000304.1
P19894.1	AGT76978.1	P87503.1
CAJ57275.1	AFV96276.1	0905196A
P33538.1	AFQ34417.1	AAS10360.1
1XHX_A	AET87180.1	AAS10432.1
CAA36327.1	AET87139.1	AAS10396.1
EPR79322.1	AAW33386.2	AAW33204.1
AAN62492.1	AP_000539.1	AGT76666.1
YP_002213842.1	ABB17778.1	ABK35035.1
P22374.1	AFQ34300.1	AFA46720.1

Table S1. 99 TP-DNAP1 homologs generated via protein BLAST (Altschul et al, 1990), Related to Figure 1

TP-DNAP1	mKate2 fluorescence (a.u.)	Average *leu2 (Q180*)* copy number per cell	Ratio
w.t.	1455.5	68.9	0.047
	1650.14	88.1	0.053
I777K	1192.83	54.1	0.045
	1064.79	45.8	0.043
I777K, L900S	601.91	22.5	0.037
	617.55	38.6	0.063
L474W, L640Y,	298.97	8.63	0.029
I777K	180.05	11.1	0.062
L477V, L640Y,	288.24	16.9	0.058
I777K, W814N	233.35	15.6	0.067

Table S3. Calibration curve of qPCR-determined p1 copy number to p1-encoded mKate2 fluorescence, Related to Figure 1 and STAR Methods

Five TP-DNAP1 variants were chosen to represent the range of p1 copy numbers observed across all experiments. OR-Y24 strains containing these TP-DNAP1s were grown in biological duplicates. Each biological duplicate was expanded in technical triplicates for mKate2 fluorescence measurements. In parallel, biological duplicates were expanded in single large volume cultures and subject to DNA extraction and qPCR measurements (see STAR Methods). Data shown are mean fluorescence in arbitrary units (a.u.) and qPCR-determined p1 copy numbers. The ratio of copy number to mKate2 fluorescence is shown for each sample. The linear regression has low background and a strong fit ($y = 0.048x + 0.206$, $r^2 = 0.954$).

Fold-change of p1 copy number over w.t.		
	G410 (w.t.)	G410H
w.t.	1.0 ± 0.27	1.53 ± 0.33
N423R	0.59 ± 0.11	1.03 ± 0.21
N423Q	0.44 ± 0.22	0.93 ± 0.18
L640A	0.069 ± 0.015	1.19 ± 0.22
N423R, L640A	0.092 ± 0.056	0.24 ± 0.082

Table S5. Mutation G410H broadly increases activity of TP-DNAP1 variants, Related to Figure 1

Mutation G410H was added to several low activity TP-DNAP1s. Mutants were transformed into OR-Y24 and subject to p1 copy number measurements. Data shown are mean fold-change ± standard deviation for biological triplicates, calculated using equation (5).2 of Frishman, 1975.

Index of extinct population in 96-well tray		
Original experiment	Revival experiment 1	Revival experiment 2
1	51	39
9	63	51
32	66	63
33	84	88
34		90
40		92
42		
49		
50		
55		
57		
90		

Table S6. Stochastic extinction in revival experiments of PfDHFR evolution, Related to Figure 3

Indices of populations that went extinct during the 90-replicate *Pf*DHFR evolution experiment and during revival experiments. In the revival experiments, cultures were inoculated from glycerol stocks of passage 5, at which point all 90 populations grew robustly. Cultures were revived in SC media supplemented with 2.5 mM pyrimethamine and passaged with the same protocol used for the original evolution experiment.

Approach	*Orthogonal DNA replication*	*Repurposing of the bacteriophage life cycle*	*Targeted in vivo mutagenesis*	*Rounds of multiplexed genome mutagenesis*	*Rounds of ex vivo mutagenesis and in vivo selection*
Systems	OrthoRep	Phage assisted continuous evolution (PACE)	CRISPR-guided DNA polymerases*, Pol I/ColE1-based systems**, In vivo continuous evolution (ICE), cytidine deaminase-based systems, TaGTEAM	MAGE-based systems, Cas9-based multiplexed genome editing systems	Traditional directed evolution methods, compartmentalized partenered replication (CPR)***
Level of continuousness	Indefinitely continuous. Mutagenesis is enforced because it is coupled to replication of the target gene that is being selected for during evolution.	In principle, indefinitely continuous. However, it is limited by frequent occurrence of contaminants/cheaters where the engineered M13 phage obtains the pIII gene encoded in the selection host. (pIII is the basis for selection.) It is also tedious to implement for long periods of time due to the constant influx of mutagneic *E. coli* required.	These systems risk breaking down due to the possibility of mutations in the *cis* elements that recruit mutagenesis machinery. Several systems cease mutagenesis within a few generations.	These are primarily genome engineering methods but they can be used for step-wise directed evolution as well. They are limited by the number of labor-intensive rounds that can be performed.	These are step-wise methodologies and are limited by the number of labor-intensive rounds that can be performed.
Mutagenesis rates achieved	Genes of interest are mutated *in vivo* at ~10^{-5} substitutions per-base. Future DNAP engineering should yield variants with error rates up to ~10^{-3} and higher.	Genes of interest are mutated in *E. coli* at ~10^{-3} substitutions per-base.	Mutation rates vary across systems, but are generally high (>10^{-5} substitutions per-base).*CRISPR-guided DNA polymerases induce exceptionally high mutation rates (10^{-4} - 10^{-3} substitutions per base) for short target regions.	Mutagenesis can be controlled via oligo synthesis, so mutation rates can be as high as desired, but the sequence space that can be accessed is bottlenecked by DNA transformation efficiency. Furthermore, mutations can't accumulate close to each other because oligos need preprogrammed constant binding sites.	Mutagenesis is typically performed via error-prone PCR or site saturation mutagenesis, so mutation rates can be as high as desired, but the sequence space that can be accessed is bottlenecked by DNA transformation efficiency.
Targeting	Completely orthogonal (at least 100,000-fold mutational targeting).	Mutations exclusively accumulate in the genes encoded in the bacteriophage genome, because the host *E. coli* cells are continuously diluted away.	These systems suffer from moderate to severe off-target mutagenesis.	Mutations are mostly targeted to genes of interest, but off-target mutations may occasionally occur, especially in MAGE strains that disable mismatch repair.	Complete mutational targeting to genes of interest.
Range of available selections	All growth-based selections and non-growth based selections like FACS, droplet sorting, cell surface antibody display, etc.	Selectable phenotypes must be coupled to gene expression.	All growth-based selections and non-growth based selections like FACS, droplet sorting, cell surface antibody display, etc.	All growth-based selections and non-growth based selections like FACS, droplet sorting, cell surface antibody display, etc.	All growth-based selections and non-growth based selections like FACS, droplet sorting, cell surface antibody display, etc.
Scalability	Can be used for hundreds of parallel evolution experiments.	Generally limited to <10 parallel experiments.	Can be used for hundreds of parallel evolution experiments.	Generally limited to <10 parallel experiments.	Generally limited to <10 parallel experiments.
Ease of use	Requires only a simple strain construction step, followed by serial passaging under selective conditions.	Requires integration of genes into the bacteriohpage genome, followed by continuous evolution in a turbidostat setup.	Requires only a simple strain construction step, followed by serial passaging under selective conditions.	Requires step-wise rounds of mutation, transformation, selection, and DNA extraction.	Requires step-wise rounds of mutation, transformation, selection, and DNA extraction. CPR additionally requires an emulsification step.
Number of genes that can be simultatenously evolved	<10 genes	<10 genes	Depending on the system, up to hundreds of genes	Up to hundreds of genes	<10 genes
Host organisms used so far	Yeast	Currently bacteriophage M13 in *E. coli*.	Depending on the system, these methods have been established in *E. coli*, yeast, and mammalian cells.	Depending on the system, these methods have been established in *E. coli* and yeast.	*E. coli*, yeast, and mammalian cells.
Additional notes			**Although the Pol I/ColE1-based systems couple mutagenesis to DNA replication, they quickly cease mutagenesis due to high levels of genome-wide mutagenesis.		***CPR requires selection to be coupled to gene expression.
References	Ravikumar et al., 2014; This work	Badran and Liu, 2015; Esvelt et al., 2011	Camps et al., 2003; Crook et al., 2016; Fabret et al., 2000; Finney-Manchester and Maheshri, 2013; Halperin et al., 2018; Hess et al., 2016; Ma et al., 2016; Moore et al., 2018	Barbieri et al., 2017; DiCarlo et al., 2013; Jakočiūnas et al., 2018; Wang et al., 2009	Ellefson et al., 2014; Packer and Liu, 2015;

Table S7. Comparison of directed evolution systems, Related to Figure 1

Substitution	L474W, L640Y, I777K, W814N	V574F, I777K, L900S	L477V, L640Y, I777K, W814N
A:T→T:A (transversion)	<1.2%	<1%	<1.1%
A:T→C:G (transversion)	<1.2%	<1%	<1.1%
A:T→G:C (transition)	65.2%	69%	73.1%
G:C→T:A (transversion)	<1.2%	1%	<1.1%
G:C→C:G (transversion)	1.2%	<1%	2.2%
G:C→A:T (transition)	29.9%	26.8%	21.3%

Table S8. Substitution mutation preferences of highly error-prone TP-DNAP1s, Related to Figure 1

All substitution preferences except for the G:C→A:T transition were measured from reversion of *leu2 (538C>T, 540A>G)*. The substitution rate of G:C→A:T was determined from fluctuation tests of *ura3 (278A>G)*. The per-base-normalized G:C→A:T substitution rates of TP-DNAP1 (L474W, L640Y, I777K, W814N), TP-DNAP1 (V574F, I777K, L900S), and TP-DNAP1 (L477V, L640Y, I777K, W814N) are 6.41×10^{-6} s.p.b, 1.73×10^{-5} s.p.b, and 1.77×10^{-5} s.p.b, respectively. These rates were incorporated in proportion to the individual error rates calculated for each of the other five substitutions (see STAR Methods for details). Data shown are normalized percentages of each substitution mutation.