Evolutionary potential of transcription factors for gene regulatory rewiring

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SUMMARY

Gene regulatory networks evolve through rewiring of individual components, that is, through changes in regulatory connections. However, the mechanistic basis of regulatory rewiring is poorly understood. Using a canonical gene regulatory system, we quantify the properties of transcription factors that determine the evolutionary potential for rewiring of regulatory connections: robustness, tunability, evolvability. *In vivo* repression measurements of two repressors at mutated operator sites reveal their contrasting evolutionary potential: while robustness and evolvability were positively correlated, both were in trade-off with tunability. Epistatic interactions between adjacent operators alleviated this trade-off. A thermodynamic model explains how the differences in robustness, tunability and evolvability arise from biophysical characteristics of repressor-DNA binding. The model also uncovers that the energy matrix, which describes how mutations affect repressor-DNA binding, encodes crucial information about the evolutionary potential of a repressor. The biophysical determinants of evolutionary potential for regulatory rewiring constitute a mechanistic framework for understanding network evolution.

From the seminal discovery of repression and activation as the basic mechanisms of gene regulation[1,2], a fundamental picture has emerged, where individual regulatory components — promoters and transcription factors (TFs) — are interconnected into gene regulatory networks (GRNs): global structures that determine cellular gene expression patterns. However, a mechanistic understanding of how GRNs evolve is still lacking. GRN evolution can be studied at two opposing levels of organization: (i)

global emerging features of GRNs, such as functional redundancy, which can promote changes in network structure[3] or (ii) local rewiring, which leads to the formation of new regulatory connections within GRNs[4]. The principles of GRN evolution have been primarily studied globally, at the level of entire networks, through comparative genomic analyses[4,5] or *in silico*[6,7], in order to understand how global network features determine evolutionary properties like robustness[8] (phenotypic persistence in the face of mutation), tunability[9] (changes in gene expression levels), and evolvability[10] (capacity to acquire new regulatory connections). Yet, GRN structures can change solely through making and breaking of connections at the molecular level, that is, through local rewiring of individual components[11-16]. However, how characteristics of individual regulatory components impact GRN evolution by determining robustness, tunability and evolvability is unknown.

Local network rewiring, i.e. changes in the binding specificity of a TF, involves loss of binding, gain of binding and modifications in the strength of binding, which occur either through mutations in TFs or in DNA-binding sites of TFs (operators). Most experimental studies on network rewiring focused on mutations in proteins[17] or on the consequences of gene duplication events[18-20], showing that TF divergence affects GRN evolution[21]. However, in contrast to mutations in operators[22-24], mutational pathways of TFs are thought to be heavily constrained by epistasis between amino acids[25], the high frequency of deleterious mutations[26] and the strong pleiotropic effects of TFs[27], suggesting that operators are superior targets for modifying existing and acquiring novel network connections.

In contrast to previous studies on promoter evolution, which considered promoters independently of the associated TFs[24,28-30], we want to understand how the properties of a TF determine its evolutionary interactions with operator sites. To achieve this, we define the *evolutionary potential for local rewiring* with respect to point mutations in an operator, thus characterizing the evolutionary potential for an individual network component that does not itself change: the repressor. We combine three distinct properties, which have been previously used to describe network rewiring[11,31,32], to define the evolutionary potential of a repressor as the ability (i) to withstand operator mutations (*robustness*), (ii) to modify the strength of binding to existing operators (*tunability*), and (iii) to acquire binding to new operators (*evolvability*) (Fig.1a). Using two of the best understood prokaryotic repressors - Lambda CI and P22 C2 - we study how characteristics of individual TFs determine the evolutionary potential for regulatory rewiring.

RESULTS

Experimental system for quantitative measurements of evolutionary potential

We used homologous[33] elements of the bacteriophage Lambda and P22 genetic switches[34,35]. Specifically, we used Lambda CI and P22 C2 repressors, along with their respective P_R promoter regions. The P_R promoter region consists of RNA Polymerase (RNAP) binding sites and two operators, O_{R1} and O_{R2}, which regulate P_R expression through cooperative repressor binding (Fig.1b). We experimentally studied changes in gene expression, and hence binding of the repressors, along the mutational path between the two promoters by directionally mutating the operator sequence of one repressor to that of the other (Fig.1c). Throughout, we refer to systems containing

matching (non-matching) repressors and promoters as *cognate* (non-cognate) (Fig.1b). We created a library of O_{R1} operator mutants by selecting all base pairs known to have large impact on repressor binding[36,37], and that differed between Lambda and P22 O_{R1} sequences, resulting in six mutated positions (Fig.1d, Supplementary Table 1).Subsequently, we also investigated mutations in O_{R2}, even though repressor binding to this operator is considered to have only a minor direct impact on P_R repression[34]. All mutants were cloned into a very low copy number plasmid[38] and fluorescence as a proxy for P_R expression levels was measured in the presence and absence of repressor. This setup, which measures binding of two repressors along the mutational path between the two operators, allowed us to study in a comparative manner how the evolutionary potential for regulatory rewiring depends on repressors themselves.

Evolutionary potential of repressors

To characterize the evolutionary potential of the two repressors, we experimentally measured their robustness, tunability and evolvability in terms of how repressor binding is affected by operator mutations. Robustness and tunability were quantified on the cognate promoter background. *Robustness* was the fraction of cognate operator mutants that maintained at least 90% repression. *Tunability* was the standard deviation in repression levels when repression was reduced but not completely lost (90-10%). From these definitions, it does not follow that robustness and tunability are necessarily negatively correlated: the expression variability (tunability) generated by non-robust mutations can be either large or small.

Evolvability was the fraction of non-cognate operator mutants that could be repressed to at least 10%.

Lambda CI and P22 C2 have drastically different evolutionary potential (Fig.2a), in spite of their shared ancestry[33]. These differences are particularly evident when considering the relationship between repression and the number of mutations in the operator (Fig.2b). The high Lambda CI robustness to up to three mutations is surprising, since the O_{R1} site is almost fully conserved across at least twelve different lambdoid phages[39]. As this site is part of a complex promoter region in the phage, it could be conserved due to binding of RNAP or the second repressor in the switch (Cro). In contrast to Lambda CI, one to three mutations in the P22 cognate O_{R1} site led to a wide range of repression (0-100%).

At the non-cognate site, even introduction of single point mutations in P22 O_{R1} led to repression of at least 35% by Lambda CI (Fig.2c). Gain of binding to the non-cognate site was much less frequent for P22 C2, and, except for one mutant, the range of repression was 0-20%, markedly lower than the 10-90% of Lambda CI (Fig.2c).

Overall, Lambda CI had higher robustness as well as evolvability, suggesting that a repressor that is more robust to mutations in its cognate operator might also more readily acquire novel binding sites. At the same time, P22 C2 was more tunable, indicating a trade-off between robustness and tunability. The consistently stronger binding of Lambda CI compared to P22 C2 suggests that the evolutionary potential for regulatory rewiring is a property of the repressor, not of the operator.

Thermodynamic model of evolutionary potential

In order to expand on the experimental findings and identify how evolutionary potential depends on the biophysical system parameters, we used a thermodynamic model of gene regulation[40,41] (Fig.3a). While experimentally we determined the general trends underlying the evolutionary potential of the two repressors by introducing mutations in a directional manner, we used the model to comprehensively explore all possible mutations in the six selected O_{R1} positions.

The model — for which all parameter values except repressor concentrations were taken from literature (Supplementary Table 3, Supplementary Fig.1) — accurately reproduced experimental observations in cognate mutants (Supplementary Fig.2). The poor model fit to non-cognate mutants is not surprising, as the model assumption of independent contribution of each position to the overall binding energy is known to be violated when mutated far away from the wild type sequence[42]. Nevertheless, the use of the model is justified because: the model performs comparably for both repressors (Supplementary Fig.2), it provides a lower bound for the experimentally measured non-cognate repression, and only modest improvements are achievable by accounting for dinucleotide dependencies[43,44].

We simulated binding to all possible mutants at the six chosen positions (4095) and quantified the evolutionary potential of repressors: for tunability and evolvability we used the same definitions as in the experiments (Fig.3b,c), but calculated them separately for each mutant class. We used a standard definition to quantify robustness in our simulations[8] (see Methods), which we could not apply to the

158 experimental measurements due to the insufficient number of mutants connected
159 by single mutations. Importantly, applying the experimental definition of robustness
160 to the simulations identified consistent differences in robustness (51.9% for Lambda
161 CI and 0.3% for P22 C2). Overall, model simulations corroborated the experimentally
162 determined differences in the evolutionary potential of the two repressors: Lambda
163 CI was more robust and more evolvable than P22 C2, but less tunable for up to three
164 mutations (Fig.3d).
165
166 To confirm that the observed differences in the evolutionary potential did not arise
167 from the specific operator sites used in this study, we simulated evolvability of both
168 repressors to 10^6 random operators. We found that Lambda CI bound a consistently
169 higher portion of random sites (Supplementary Fig.3) irrespective of repressor and
170 RNAP concentration, further supporting the view that evolutionary potential is a
171 property of the repressor, not the operator.
172
173 The thermodynamic model identifies several system parameters that affect the
174 evolutionary potential of a repressor (Fig.3a): (i) intra-cellular conditions, i.e.
175 concentrations of repressor and RNAP, (ii) interactions arising from the promoter
176 architecture, which in our system enable cooperative repressor binding, and (iii)
177 intrinsic binding characteristics of the repressor itself. Repressor-specific binding
178 characteristics are captured in the total binding energy, E_{tot}, which is determined by
179 the strength of repressor binding to its wild type operator (called 'offset', or E_{WT}), to
180 which the effect of each mutation on binding is added, as defined by the 'energy
181 matrix' (E_{seq}), so that $E_{tot} = E_{WT} + E_{seq}$. Hence, the 'offset' captures the overall

propensity of a repressor to bind cognate DNA, while the 'energy matrix' describes how operator mutations affect repressor binding.

Repressor and RNAP concentrations, as well as binding cooperativity, influence robustness, tunability and evolvability to different degrees, though not always in a straightforward manner (Fig.4a; Supplementary Fig.4, 5, 6). As such, the evolutionary potential for rewiring depends on intra-cellular conditions that change with cellular physiology[45], and on the promoter architecture that can determine binding cooperativity. Experimental measurements of relative repressor concentrations revealed 3.8 to 5.5-fold higher intracellular Lambda CI levels (Supplementary Fig.1). Reassuringly, the difference in evolutionary potential between repressors was consistently identified across a range of repressor and RNAP concentrations, making the model results largely independent of uncertainty in these parameters (Supplementary Fig.7).

Biophysical determinants of evolutionary potential

We asked if it was possible to reconcile the differences in the evolutionary potential between Lambda CI and P22 C2 by swapping their model parameters. Specifically, we calculated robustness and tunability for one repressor after swapping either repressor concentration or cooperativity with the parameter values of the other repressor. For evolvability, we only swapped repressor concentration, since the absence of a cognate O_{R2} site prevented cooperative binding.

Swapping either repressor concentration or cooperativity between Lambda CI and P22 C2 decreased the differences in robustness and evolvability, but still left a disparity in robustness, tunability and evolvability of at least 50% (Fig.4b). Therefore, intrinsic binding characteristics of repressors - the offset and the energy matrix - crucially determine their evolutionary potential, as previously found for the regulation of the *lac* promoter[46]. When we swapped the offset between the two repressors, we found that the effect was comparable to the effects of swapping either repressor concentration or cooperativity. Notably, swapping all three parameters did not lead to a full reconciliation between the two repressors (Fig.4b), indicating that the energy matrices accounted for the remaining differences of at least 30% (except for robustness when swapping from P22 C2 to Lambda CI).

To better understand the mechanism by which intrinsic binding characteristics of a repressor (offset and energy matrix) determine the differences in the evolutionary potential, we developed an intuitive and generic description of robustness, tunability and evolvability based on the sigmoidal curve relating repressor binding energy to repression (Fig.5a). The formulas in Figure 5a describe the evolutionary potential in terms of the offset and the energy matrix, rather than using the full thermodynamic model. Robustness is the average number of mutational steps needed to lose 50% of repression. Evolvability is the average number of mutational steps necessary to gain 50% of repression starting from a given random sequence. Tunability is the ease of generating variation in gene expression levels, i.e. the variation in repression around the half-repression point, defined in relation to the distance between this point and the cognate operator (Fig.5a).

Adopting these generic definitions results in simple analytical expressions (Fig. 5a), which show that robustness and evolvability are positively correlated through the number of mutations that separate the given random sequence from the cognate operator. This correlation holds true as long as: (i) the average mutational effect size (m) is relatively small and similar between repressors – a reasonable assumption if the scale of m is set by the energetics of hydrogen bonds (1-3 kcal/mol)[47], which can be tested by obtaining energy matrices for other repressors; and (ii) the energy matrix is a fixed property of a repressor, meaning that m stays constant when mutating towards a random non-cognate site. Tunability, on the other hand, is in a trade-off with robustness, although the dependence of tunability on the standard deviation of mutational effects suggests that this relationship can be adjusted to some extent.

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Applying these generic definitions to the systems used in this study, we observe higher robustness and evolvability, but lower tunability for Lambda CI (Fig.5a). To illustrate that these generic definitions are in accordance with the binding landscape obtained through model simulations, we used the simplest model setup where repressors bind only a single operator site and repressor concentrations are the same. We selected three operator sequences for each repressor - the cognate (E_{WT}), the non-cognate ($E_{non\text{-}cognate}$), and the weakest binding (E_{max}) sequence - computed their binding energies, and positioned them on the sigmoidal repression curve.

The consistently stronger binding of Lambda CI to all three types of operators (Fig.5b) arises from its lower offset (-13.2 kcal/mol, compared to -12 kcal/mol for P22 C2) and smaller average mutational effect size (1.23kcal/mol, compared to 2.43kcal/mol for P22 C2). Positioning the mean binding energy of each mutant class (Fig. 2) on the sigmoidal curve (hence not using the full model but only the offset and the energy matrix) allowed accurate predictions of the experimental measurements, at least for cognate sites (Supplementary Fig.8). Therefore, the lower offset of Lambda CI places it further away from the slope of the repression curve (Fig.5b), resulting in higher robustness, but lower tunability. Similarly, Lambda CI binds the non-cognate operator, all of its mutants, and even the operator sequence with weakest possible binding more strongly (Fig.5b), illustrating that, on average, Lambda CI binding a random sequence will be closer to the rise of the sigmoidal curve and hence, more evolvable.

Role of inter-operator epistasis

We investigated experimentally if promoter architecture — the existence of multiple operator sites — can affect the observed trade-off between robustness/evolvability and tunability. We first tested the effects of mutating four residues in the Lambda cognate O_{R2} (Supplementary Table 4). The effects of mutations in O_{R2} on repression (Fig.6a) were modest (75-100% repression), but less robust than mutations in O_{R1} (comparing Fig.6a to Fig.2b top panel), despite the supposedly weaker influence of O_{R2} on repression[34].

We tested for interactions between mutations in two operators (inter-operator epistasis) by creating a cognate library with mutations in both O_{R1} and O_{R2}. Because the trade-off between high robustness and low tunability was observed only in Lambda CI, we focused only on inter-operator epistasis in the cognate Lambda system. We randomly selected three neutral O_{R1} mutants, and combined each with eight randomly selected O_{R2} mutants (Supplementary Table 1,4). We observed a wider spectrum of repression values (40-80%), and hence higher tunability, among these mutants (Fig.6b) compared to mutations in individual operators (Supplementary Table 5). This meant that mutations in O_{R2} exacerbate the effects of phenotypically neutral O_{R1} mutations, indicating pervasive inter-operator epistasis (Supplementary Table 6). Inter-operator epistasis arising from multiple mutations in both operators could not be captured by the thermodynamic model (Supplementary Fig.9), which is in contrast to a previous study where we introduced only a single point mutation into each operator[48]. However, the findings we report here are in line with studies showing that the presence of multiple operators can obstruct sequence-based predictions of gene expression[49].

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Inter-operator epistasis alleviated the trade-off between robustness and tunability for Lambda CI in O_{R1}, likely by effectively modifying cooperative repressor binding. This role of inter-operator epistasis could be specific to operators that are functionally connected through cooperative binding, and might be different for redundant operators. Our results suggest that for cooperative binding, additional operators can facilitate network rewiring, as inter-operator epistasis helps generate expression level diversity, while maintaining robustness to the existing operators.

DISCUSSION

The principles that govern gene regulatory evolution, which have been studied primarily from a global network perspective, remain poorly understood. Here, we identify the biophysical mechanisms that determine the evolutionary potential of transcription factors for rewiring of regulatory network connections. Specifically, we provide an analytical expression (Fig. 5a) that, under reasonable assumptions, correlates robustness, tunability and evolvability (as defined in this study). Indeed, we experimentally observed these correlations for two closely related repressors: Lambda CI is more robust and at the same time more evolvable, while P22 C2 is more tunable. These differences in mutational effects likely arise from differences in specific DNA binding mechanisms[50]: while the binding specificity of Lambda CI is mostly based on direct contacts between operator bases and amino acid residues[36], the affinity of P22 C2 relies strongly on the local DNA conformation[37,51]. The nonlinear relationship between binding energy and repression, which is inherent to the thermodynamic model[52] (Fig.3), captures the differences in robustness, tunability and evolvability, explaining how the intrinsic binding characteristics of a repressor determine its evolutionary potential for regulatory rewiring (Fig.5a). The model does so by representing the evolutionary potential for each repressor through its total binding energy (offset E_{WT} plus energy matrix E_{seq}) and the average effect size of mutations (given by the energy matrix). Typically, energy matrices are used to determine and predict binding of TFs to a given DNA sequence[53]. However, our

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findings imply that the composition of the energy matrix crucially determines not only the current regulatory structure, but also the potential of the repressor to contribute to GRN evolution through making and breaking of individual connections. It is worth noting that while we only considered steady state expression levels, operator mutations could also affect expression dynamics, which might be subject to different constraints.

The *in vivo* positive correlation between robustness and evolvability is surprising, as molecular systems that are more persistent in the face of mutational pressure are generally assumed to be less likely to acquire novel functions[54]. Previous theoretical studies attempted to resolve this paradox by describing how robustness and evolvability 'emerge' as properties of existing networks[3,8,55,56], but so far, direct experimental approaches have been missing. We experimentally resolve this apparent paradox by showing that local mechanisms of TF-DNA binding intrinsically correlate robustness and evolvability in a positive manner. In fact, this positive correlation can be explained through an analytical expression that shows how robustness and evolvability are connected through the mutational distance between the cognate operator and a random DNA sequence (Fig.5a). As such, a more promiscuous TF is simultaneously more robust and more evolvable, retaining cognate binding more easily while facilitating acquisition of novel operator sites. The positive correlation between robustness and evolvability can facilitate GRN evolution[19] by enabling a neutral network of genotypes, throughout which mutations have small phenotypic consequences[3,8]. Lambda CI is known to be promiscuous, showing nonspecific binding across the *E. coli* genome[57] and to non-cognate phage

operators[58]. Thus, a Lambda CI-like TF has a higher potential to become a global regulator, whereas a P22 C2-like TF would be more suited as a local regulator, since its easy loss of binding could facilitate rewiring by reducing detrimental crosstalk[59]. However, the same biophysical mechanisms can impose a trade-off between evolvability and tunability, thus constraining the range of expression levels that can be achieved by a promiscuous TF at a single operator.

Given the key role that rewiring of local regulatory connections plays in changing GRN structure, the scarcity of direct experimental approaches studying the mechanisms of rewiring is striking. Our work provides a mechanistic link between the biophysics of TF-DNA binding and GRN evolution. Epistatic interactions, which emerge through the presence of multiple operators and alleviate the trade-off between tunability and robustness/evolvability, can prevent a straightforward prediction of how local rewiring properties determine global network evolution. Moreover, the binding landscape for regulatory rewiring we describe is based purely on biophysical characteristics that connect genotype (mutations) to phenotype (gene expression levels), which will be further shaped by selection forces acting on this landscape[29,30,60]. By integrating biophysical models with the existing molecular knowledge of regulatory elements, our work provides the first steps towards a quantitative mechanistic framework for understanding gene regulatory network evolution.

METHODS

Strains and plasmids

The experimental system is based on the 'genetic switches' of the bacteriophages Lambda and P22, which have similar regulatory architecture and substantial structural homology due to shared ancestry[33]; specifically we use the P_R promoter system. We constructed a template plasmid consisting of two parts that are separated by 500 random base pairs and a terminator sequence (represented by a hairpin structure in Fig.1b): an inducible repressor gene on one strand and a regulatory region controlling a fluorescence marker on the other strand. Either Lambda CI or P22 C2 were placed after an inducible P_{TET} promoter. The fluorescent protein gene *venus-yfp*[61] was placed under the control of the P_R regulatory promoter region, containing an RNAP binding site as well as two operators, O_{R1} and O_{R2}, either from Lambda or P22. Specifically, for Lambda P_R we used the region from -60bp upstream of the transcriptional start site to +9bp downstream. To our knowledge the specific location of the transcriptional start site for P22 P_R has not been defined. Therefore, upstream of O_{R2} and downstream of O_{R1} we used the wild type P22 sequence that was of the same bp length as the analogous Lambda P_R regions. This meant that we used the wild type P22 sequence from -65bp upstream up to the start codon of *cro*. O_{R1} more strongly binds the repressor and is in direct overlap with the RNAP binding site (-10). O_{R2} has a weaker affinity for the repressor, and assists in repression mainly through cooperative binding between two repressor dimers[62]. Downstream of the phage sequences both promoter regions contain the same ribosomal binding site in front of the reporter gene. These parts were cloned in all four combinations (cognate combinations: Lambda *cI* with Lambda P_R, and P22 *c2* with P22 P_R; non-cognate combinations: Lambda *cI* with P22 P_R, and P22 *c2* with Lambda P_R) into a low copy number plasmid (pZS*) containing a kanamycin

resistance marker[38]. The TL17 terminator sequences followed the repressor genes, and the T1 terminator the *venus-yfp* (Fig.1b). The plasmid libraries were then transformed into MG1655 derived *E. coli* cells (strain BW27785, CGSC#: 7881)[63].

Construction of mutant O_{R1} libraries

We created a library of mutants in O_{R1} by selecting six base pairs that were found to be most important for the binding of either of the two repressors[36,37], and that differed between Lambda and P22 O_{R1} sequences. This was done by aligning the O_{R1} sites from Lambda and P22 wild type operators (according to homology, not symmetry) and comparing the corresponding base pairs in the operator sites. The six base pairs that were most important for repressor binding and that differed between the two operators were substituted by the base pairs of the non-cognate O_{R1} in both directions: starting with wild type Lambda O_{R1} and mutating it to be more similar to P22 O_{R1}; as well as starting with wild type P22 O_{R1} and mutating it to be more similar to Lambda. We generated all six single mutants, four double, five triple, four quadruple, three quintuple, and the sextuple mutant. For mutating Lambda O_{R1} from cognate to non-cognate, ten additional mutants were constructed that did not contain mutations in base pairs overlapping the -10 binding region of RNAP: two double, two triple, two quadruple, three quintuple, and another sextuple mutant. For the quintuple and sextuple mutants an additional base pair was chosen, that was linked to high affinity binding of Lambda CI (Supplementary Table 1). The additional double and triple mutants were also created for the P22 non-cognate library. O_{R1} operator libraries were constructed by synthesizing oligos of 73bp length (Sigma Aldrich), carrying wild type O_{R2} and mutated O_{R1} (Supplementary Table 1), and

418 cloning them into the experimental system plasmid backbone (Fig.1b). Clones
419 carrying correct mutants were confirmed through Sanger sequencing.

420

421 We also tried to construct promoter regions containing cognate O_{R1} and non-cognate
422 O_{R2}. As both operators contain parts of the RNAP binding site, we did not obtain
423 fluorescence expression in the absence of CI from these promoters even when we
424 varied the spacing between the operators. This is possibly due to factors other than
425 sequence-dependent binding energy playing a role in the regulatory context of these
426 promoters[49].

427

428 *Fluorescence assays*

429 We measured fluorescence of all O_{R1} mutants (Lambda and P22 cognate and non-
430 cognate systems), both in the presence and in the absence of the inducer aTc. Three
431 biological replicates of each mutant of the library were grown at 37°C overnight in
432 M9 media, supplemented with 0.1% casamino acids, 0.2% glucose, 30μg/ml
433 kanamycin, and either without or with 15ng/ml aTc. Overnight cultures were diluted
434 1,000X, grown to OD_{600} of approximately 0.05, and their fluorescence measured in a
435 Bio-Tek Synergy H1 platereader. All replicate measurements were randomized across
436 multiple 96-well plates. All measured mutants had fluorescence levels significantly
437 above the detection limit of the plate reader, resulting in measurements at least 1.5
438 fold greater than the non-fluorescent control.

439

Fluorescence values were normalized by OD_{600} values (in RFU=Relative Fluorescence Units) and averaged over three replicates. Repression values were calculated as a normalized ratio between the measured fluorescence with and without the repressor:

$$Percent\ repression = \left(1 - \frac{RFU_{repressor}}{RFU_{no\ repressor}}\right) * 100.$$

Standard errors of the mean repression values were calculated using error propagation in order to account for the inherent variability in the fluorescence measurements. The fluorescence levels measured in the absence of repressor were comparable across all Lambda operator mutants, as well as all P22 operator mutants (Supplementary Table 2). This means that the reported differences in percent repression arose mainly from changes in repressor binding, rather than alterations to the RNAP binding site. Moreover, our simulations showed that changes in RNAP concentration, which correlates with the strength of RNAP binding, do not change the qualitative pattern of binding for the two repressors. Interestingly, when compared to P22 wild type O_{R1}, all of the P22 cognate O_{R1} operator mutants showed increased expression levels in the absence of repressor. Lambda P_R is a stronger promoter than P22 P_R, and introducing mutations in the operator region of P22 P_R increased promoter strength by making it more similar to Lambda P_R.

Direct comparisons between the *in vivo* effects of operator mutations on gene expression level that we measured, and the previous published studies of the same operators[36,37] were hindered by the *in vitro* nature of previous studies. All previous studies of Lambda P_R and P22 P_R mutants relied on biochemical filter binding assays,

463 which do not account for cooperativity between the two sites, and as such do not
464 necessarily translate quantitatively into gene expression levels. As such, comparisons
465 between published and our data are possible only through a modeling framework,
466 such as the one we utilize (see Materials and Methods section 'Thermodynamic
467 model of repression at the P_R promoter').

468

469 For the experimental data, the evolutionary properties were calculated in the
470 following way: robustness and tunability of the repressors were evaluated on the
471 cognate operator mutants. Robustness for the experimental data was calculated as
472 the percent of mutants for which >90% of the wild type repression was retained.
473 Tunability was calculated as the standard deviation in repression levels for mutants
474 that exhibited between 10% and 90% of the wild type repression. On the cognate
475 background, mutants that were repressed less than 10% were considered neither
476 robust nor tunable. Evolvability was calculated as the portion of non-cognate
477 mutants that were repressed to more than 10%.

478

479 Cellular concentrations of the two repressors were determined using Western blots.
480 Lambda CI and P22 C2 were cloned with a His-Tag or an HA-Tag, respectively, at their
481 carboxy-terminal end. Rat and rabbit primary antibodies (Roche and Thermo Fisher,
482 respectively) in combination with Goat anti-rat and anti-rabbit secondary antibodies
483 (Thermo Fisher) were used to detect them. Samples were processed once at full
484 concentration and once at 2-fold dilution. The obtained bands from gel
485 electrophoresis were normalized by a household gene and normalized

concentrations between the two repressors were compared as ($\frac{concentration_{Lambda\ CI}}{conce}_{P22\ CI}$). Lambda CI was present in excess over P22 C2: 3.8-fold for full concentration samples and 5.5-fold for diluted samples. We also tested variation in repressor levels by measuring fluorescence from the P_{TET} promoter on the same plasmid construct as used in the library measurements for 6 replicates either without or with 15ng/ml aTc and found only minor variability (without aTc: 3.6% CV, with aTc: 2% CV) that cannot explain the experimentally observed differences between the repressors.

Thermodynamic model of repression at the P_R promoter

The model is based on previously described thermodynamic approaches[40,41], which rely on several assumptions: (i) TF binding to DNA takes place at thermodynamic equilibrium; (ii) gene expression can be equated with the probability of binding of participating proteins (in our case RNAP and repressor); and (iii), the contribution of each base pair in the operator to binding is additive. The probability of a gene being expressed is derived by summing the Boltzmann weights over all promoter occupancy states where RNAP is bound. Boltzmann weights are given by $w_i=[N]* e^{(E_{tot}-\mu)}$, where E_{tot} is the energy of a certain configuration, N is the molecule concentration (in μM), and μ is the chemical potential. E_{tot}, the total binding energy, is composed of the offset (E_{WT}), which is the energy of binding to a reference (wild type) sequence; and the binding energy derived for a specific sequence from the energy matrix of the binding protein $E_{seq}=\sum^{l}_{i=1} \epsilon_i(a_i)$, where l is the length of the sequence, a_i the specific nucleotide at position i, and ϵ_i the energy

contribution due to the energy matrix of the specific nucleotide a at position i. Total binding energy is therefore E_{tot}= E_{WT}+ E_{seq}. Binding energies and chemical potential are given in *kcal/mol*. In our model system, there are two operator sites (O_{R1} and O_{R2}) that can each be occupied by a repressor dimer, and binding to each operator site is affected by the strength of cooperative binding between them. The probability of the gene being expressed is then given by the sum of all states conducive to promoter expression (RNAP bound) normalized by the sum over all possible states:

$$Gene\ expression = \frac{1}{1 + \frac{K_p}{[RNAP]} * \frac{\left(1 + 2\frac{[R]}{K_R} + \left(\frac{[R]}{K_R}\right)^2 e^{\omega}\right)}{\left(1 + \frac{[R]}{K_R}\right)}}$$

, where $K_x = e^{(E_{tot,x} - \mu)}$ represents the effective equilibrium dissociation constant (relative to the genomic background) – which is the concentration for half-maximal occupation of the site - of, either RNAP (K_P) or the repressor (K_R). Please note that we account for concentration-specific effects separately and μ incorporates only non-specific background binding and other unspecific cellular effects. The probability of transcription factor (TF)–DNA binding is of the form[22]: $p_i = \frac{[TF_i]/K_i}{1 + [TF_i]/K_i}$. Based on Garland (2002), we can assume that K_x is individually tunable for each binding site. [R] is the concentration of repressor dimers, which is the effective concentration, as repressors only bind as dimers and, as we assume fast dimerization[64], this corresponds to half of the total monomer concentration in the cell. [RNAP] is the concentration of RNAP, and ω is the cooperativity energy value, describing the strength of interaction between two repressor dimers. All concentrations and dissociation constants are given in units of μM. The calculated gene expression value is a relative measure, with

1 indicating full expression and 0 no expression. Percent repression was then calculated using the formula:

$$Percent\ repression = \left(1 - \frac{gene\ expression_{repressor}}{gene\ expression_{no\ repressor}}\right) * 100.$$

In the 'main model', which is used throughout the study, RNAP competes with repressor binding at O_{R1}, and repressor binding to O_{R1} is increased by cooperative binding of a second dimer to O_{R2}. Therefore, the following scenarios are possible: (i) the promoter can be bound by neither protein; (ii) RNAP can be bound either alone or together with repressor at O_{R2}; or (iii) repressor bound to O_{R1} keeps RNAP from binding, either by binding on its own or cooperatively together with another repressor at O_{R2}. The corresponding formula was taken from Bintu et al., 2005 (Case 4). We also considered an 'alternative model' where O_{R2} binding impedes RNAP binding as well (Bintu et al., 2005; Case 6), but as the main model always gave a better fit to experimental data, we utilized only the main model throughout.

Energy values for binding to mutated sequences were calculated for RNAP and repressor binding using the respective energy matrices by adding up the individual relative contributions of each base pair and adding an offset. The offset is the energy of binding of the repressor to the wild type sequence, which was added because the energy matrix calculates only energy differences relative to wild type binding. Binding energy matrices were based on Sarai & Takeda (1989) for Lambda CI, on Hilchey et al. (1997) for P22 C2 - which were both determined biochemically - and,

552 for RNAP, on an ongoing work on RNAP binding to Lambda P_R within the group. Wild
553 type binding affinities of Lambda CI to both operators (offset) were taken from Vilar
554 (2010). Other model parameters were taken from the following sources: binding
555 cooperativity and nonspecific binding energy were adopted from Hermsen et al.
556 (2006); wild type binding affinities for both operators were obtained from Hilchey et
557 al. (1997) for P22 repressor; and binding energy and concentration for RNAP were
558 taken from Santillan & Mackey (2004)[65]. Promoter strength for both Lambda P_R and
559 P22 P_R was based on previously published values for the Lambda P_L promoter[66], but
560 we also found that the results were not sensitive to this parameter. Repressor dimer
561 concentrations were the only parameters that were fitted to the data by means of a
562 Monte Carlo algorithm. The algorithm used simulated annealing to find the optimal
563 parameter values minimizing the squared difference between the predicted and
564 observed percent repression between the data and the model. The fitted difference
565 in concentration values between the two repressors is slightly lower than found
566 experimentally (Supplementary Fig.1). We tested the model for concentration values
567 from 0- to 7-fold difference, and always found the same trends in the evolutionary
568 potential (Supplementary Fig.7). Note that standard experimental measures cannot
569 provide effective TF concentrations (i.e. proteins that are free to bind at the target
570 site), especially when two TFs are not equally promiscuous, as these measures
571 cannot distinguish free and non-specifically bound proteins. Because of this, and
572 because the overall differences in evolutionary potential did not depend on
573 variations in repressor concentration parameters, we used repressor concentrations
574 determined by the best model fit, and not those we experimentally measured. All
575 parameter values used in the model are shown in Supplementary Table 3.

In order to verify the fit of our model to the experimental data, linear regression was performed between the data obtained experimentally (see *Fluorescence assays*) and the prediction of repression values produced through the thermodynamic model. Matlab R2015a software was used to calculate the regression, R squared and P-values for the O_{R1} library (Supplementary Fig.2). The model accurately reproduced experimental observations in cognate mutants, but did not fit non-cognate mutant measurements (Supplementary Fig.2). The lack of fit to non-cognate mutants is not surprising, as thermodynamic models assume an independent contribution of each position, which does not hold when mutated far away from the wild type operator sequence[42,67]. Nevertheless, because the model provided a lower bound on the experimentally measured non-cognate repression levels (Supplementary Fig.2), we used it to explore parameters affecting repression at non-cognate sites as well.

Robustness

Robustness was calculated for repressors binding to cognate mutants only if they retained more than 20% repression. We counted the number of robust neighbors for each operator, where 'robust neighbor' refers to an operator sequence that is exactly one mutation away from the reference and exhibits more than 90% repression of the reference repression value. Specifically, starting from the wild type, each mutant (above the 20% repression threshold) was taken as a reference and repression of all other mutants that are exactly one mutation away was calculated. The relative count of robust neighbors was averaged for each reference operator

and the mean was taken over each mutant class. This procedure was repeated with different values for cooperativity (1,3,5,7 kcal/mol), repressor concentration (1,3,5,7 μM) and RNAP concentration (1,3,5,7 μM). We tested if the results were sensitive to the percent repression thresholds by calculating robustness for 80% and 95% thresholds, and found no qualitative differences. For comparison with the experimental data and the definition of robustness used there, we also calculated robustness as the percent of all mutants for which >90% of the wild type repression was retained.

Tunability

Tunability was determined for repressor binding to cognate mutants with repression values between 10% and 90%, as the standard deviation over those mutants for each mutant class. Tunability was calculated for different values of cooperativity (1,3,5,7 kcal/mol), repressor concentration (1,3,5,7 μM) and RNAP concentration (1,3,5,7 μM). We tested if the results were sensitive to the percent repression thresholds by calculating tunability for 5% and 20% lower, as well as 80% and 95% upper threshold bound, and found no qualitative differences.

Evolvability

Evolvability was calculated for repressor binding to non-cognate mutants exceeding a threshold of 10% repression. For each mutant class the number of mutants above the threshold was counted and averaged. This procedure was repeated with different values for cooperativity (1,3,5,7 kcal/mol), repressor concentration (1,3,5,7

622 μM) and RNAP concentration (1,3,5,7 μM). We tested if the results were sensitive to
623 the percent repression thresholds by calculating evolvability for 5% and 20%
624 thresholds, and found no qualitative differences.

625 *Evolvability on random operators*

626 The promoter region for the random sequence library was based on the *lac*
627 operon[68], because the binding sites for RNAP and repressor do not overlap in this
628 system, thereby avoiding unwanted modifications of RNAP binding by an
629 introduction of a random operator. Binding affinities for RNAP were calculated for
630 this system using the energy matrix from Kinney et al., 2010. For the operator sites,
631 1,000,000 random 17bp-long sequences for Lambda CI, and 18bp-long sequences for
632 P22 C2 were created in Matlab R2015a. The 1bp difference in the length of the sites
633 used for the two repressors corresponds to the actual length of their respective
634 cognate operator sites. Binding affinities to these operators were calculated for
635 Lambda and P22 repressors using their energy matrices.

636

637 *Swapping model parameters of the two repressors and comparing evolutionary*
638 *properties*

639 We calculated robustness and tunability for Lambda CI after swapping the values for
640 repressor concentration, cooperativity, and offset with the respective values for P22
641 C2. The values were calculated separately for each mutant class (number of
642 mutations). We first swapped each parameter value individually, and then we
643 swapped all three parameters with the values of P22 C2. For evolvability, only the
644 values for repressor concentration and offset were swapped individually and

simultaneously. The same simulations were done for P22 C2 with Lambda CI parameters. For each evolutionary property, we used a linear regression to determine the R^2 value for the goodness of fit between the reference repressor with its wildtype parameter values, and the other repressor with the swapped parameter(s). Regression was carried out across the six mutant classes. The fact that swapping repressor concentrations did not reconcile the evolutionary potential of the two repressors provides further evidence that the experimentally observed differences in the evolutionary potential between the two repressors (Fig.2) could not be attributed solely to the measured differences in their intracellular concentrations (Supplementary Fig.1).

Relationship between binding energy and repression

The total binding energy (E_{tot}) is related to gene expression through:

Gene expression $= \frac{1}{1+[R]e^{E_{tot}-\mu}}$, with $E_{tot} = E_{WT} + E_{seq}$

, where μ describes the chemical potential of a repressor. The relationship between binding energy and repression is sigmoidal, with the position of the curve for a given repressor determined by μ and repressor concentration (which we set to 1 as we do not want to consider concentration effects here). The same chemical potential and repressor concentration was used for Lambda CI and P22 C2 and taken from Hermsen et al., 2006[69]. The positions of a certain operator sequence for a specific repressor on the curve are then given by the total binding energy, E_{tot}, with concentrations for the two repressors being the same. We wanted to develop generic definitions of robustness, tunability and evolvability as properties of only the

energy matrix and E_{WT}. The average effect size of one mutation (m) is determined by taking the average of the energy matrix for a given repressor (grand mean over the non-zero entries of the energy matrix, calculated in our example for the six mutated positions) and the deviation in mutational effects (σ) is calculated as standard deviation over all non-zero entries of the energy matrix. Robustness can then be defined as $Rob = \frac{E_{1/2} - E_{WT}}{m}$ and evolvability as $Evo = \frac{E_{1/2} - E_{random}}{m}$,where $E_{1/2}$ is the binding energy at half repression (50%) and E_{random} is the typical binding energy to a random sequence, which will be equal to non-specific binding above a certain number of mutations[42] and is from that point on independent of the energy matrix. Derivation shows that evolvability and robustness are correlated by the number of average mutations between the cognate operator binding energy and the binding energy of a random sequence (*#mut*), as m determines the positioning of E_{random} relative to E_{WT}: $Evo = \frac{E_{1/2} - E_{random}}{m} = \frac{E_{1/2} - (E_{WT} + \#mut * m)}{m} = Rob + \#mut$. This correlation depends critically on two assumptions. First, we assume that the typical mutational effect size (m) is relatively small compared to the offset (E_{WT}) and comparable between different repressors. We base this assumption on the notion that TF-DNA binding is determined by the strength of hydrogen bonds, which range between 1-3kcal/mol[47]. The second assumption is that the energy matrix is an intrinsic property of a repressor, meaning that it doesn't change depending on the DNA sequence that the repressor is binding to. In other words, we assume that m is constant across all binding sites, cognate and non-cognate. Tunability can be defined around $E_{1/2}$ as $Tun = (\sigma * \frac{d\,repression}{d\,binding\,affinity}|_{E_{1/2}})/Rob$,where $\frac{d\,repression}{d\,binding\,affinity}|_{E_{1/2}}$ gives the slope of the sigmoid curve at $E_{1/2}$. Positions on the curve for both

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repressors were calculated for binding to cognate operators, non-cognate operators and the operator with weakest possible binding (according to the energy matrix). Moreover, mean energy values for each mutant class were calculated from model simulations for the cognate and non-cognate operators and placed on the curve. Their locations on the curve provide mean repression values that were then compared to the experimental data through linear regression (Supplementary Fig.8). Matlab R2015a software was used to calculate the regression, R squared and P-values. The fit was similar to the one obtained using the full model (Supplementary Fig.2).

Lambda cognate O_{R2} mutant library

O_{R2} mutant operators were synthesized analogously to O_{R1} mutants. Based on the assumption that energy matrices between the two closely related operators are likely to be very similar, mutated base pairs in O_{R2} were chosen in positions corresponding to the mutations in O_{R1}. However, the last two were discarded as possibly interfering with RNAP binding (-35 region), leaving four base pairs for mutation (Fig.2b). Four single, six double, four triple and the quadruple mutant were constructed in the Lambda cognate system and measured as described previously. The fit between data and model was determined through linear regression (Supplementary Fig.9a).

Lambda cognate O_{R1} - O_{R2} mutant library

713 O_{R1}-O_{R2} mutant operators were synthesized analogously to O_{R1} mutants, but with one
714 to three mutations in O_{R1} and one to four mutations in O_{R2}. One single, one double
715 and one triple O_{R1} mutant, that showed no decrease in repression, were combined
716 with each of eight randomly selected O_{R2} mutants (two single, three double, two
717 triple, and the quadruple). O_{R1}-O_{R2} mutant operators were constructed in the
718 Lambda cognate system, as P22 C2 had very low robustness and hence no trade-off,
719 and measured as described previously. The fit between data and model was
720 determined through linear regression (Supplementary Fig.9b).

721

722 *Calculation of epistasis in O_{R1}-O_{R2}* mutants

723 We measured epistasis in two ways. First, through its effect on the tunability of the
724 system, where we considered that a given combination of O_{R1}-O_{R2} mutations is in
725 epistasis when the presence of mutations in both operators significantly increased
726 the variance in the observed gene expression levels, compared to the variance
727 achieved by mutations in O_{R1} alone. We compared the variance independently for
728 each mutant class (number of mutations). Second, we calculated epistasis between
729 mutations in the two operators as a deviation from the multiplicative expectation of
730 double mutant repression level based on single mutant effects:

731 $$epistasis = \frac{percent\ repression_{O_{R1}-O_{R2}}}{percent\ repression_{O_{R1}} * percent\ repressio\ \ _{O_{R2}}},$$

732 and conducted FDR-corrected two-tailed t-tests for each of the double mutants, to
733 determine if epistasis was significantly different from the null multiplicative
734 expectation (Supplementary Table 6).

DATA AND SOFTWARE AVAILABILITY

Experimental data that support the findings of this study have been deposited in IST DataRep and are publicly available at https://datarep.app.ist.ac.at/id/eprint/108.

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ACKNOWLEDGMENTS

We thank S. Abedon, R. Grah, K. Jain, C. Nizak, T. Paixão, M. Pleska, E. Reichhart and S. Sarikas for helpful discussions. We thank the anonymous reviewers for their insightful comments. This work was supported by the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007-2013) under REA grant agreement n° [291734] to M.L., and European Research Council under the Horizon 2020 Framework Programme (FP/2007-2013) / ERC Grant Agreement n. [648440] to J.P.B. C.I. is the recipient of a DOC Fellowship of the Austrian Academy of Sciences.

AUTHOR CONTRIBUTIONS

All authors conceived the study together. C.I. and M.L. designed and carried out the experiments and analyzed the data. C.I. wrote the code and ran the model. C.I. and M.L. wrote the initial draft of the manuscript and revised it together with G.T. J.P.B and C.C.G.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

Figure 1. Experimental investigation of evolutionary potential of a repressor. a) Mutations (indicated by 'x') in the cognate operator can either have no effect on repressor binding (**robust**); alter repressor binding (**tunable**); or remove repressor binding (not shown). Mutations in the non-cognate site can either have no effect on repressor binding (**not evolvable**); or lead to gain of repressor binding (**evolvable**). Together, robustness, tunability and evolvability describe the evolutionary potential for regulatory rewiring. **b)** The synthetic template consists of a repressor controlled by an inducible P_{tet} promoter, and a strong P_R promoter - containing two repressor operators (O_{R1} and O_{R2}) and the RNA Polymerase (RNAP) binding sites - that controls the expression of a fluorescence marker *venus-yfp*. **c**) An increasing number of mutations (blue) are introduced into the cognate operator (orange) of repressor A. The thickness of the blunt-ended arrows indicates the strength of repression. **d**) Homology alignment of Lambda and P22 O_{R1} and O_{R2}, showing mutated sites in bold. Arrows show O_{R1} base pairs that were exchanged. The dashed arrow marks an additional site that was used to construct four cognate Lambda mutants, as one of the original positions abolished RNAP binding (Supplementary Table 1).

Figure 2. Lambda CI and P22 C2 have different evolutionary potential. a) Robustness, tunability and evolvability of Lambda CI and P22 C2. **b)** Loss of binding was determined by mutating away from the cognate site, making it more similar to the non-cognate site. The dotted line shows the 90% repression threshold used to evaluate robustness. **c)** Gain of binding was determined by mutating away from the non-cognate site making it more similar to the cognate one. The dotted line shows the 10% repression threshold for evolvability. Expression levels in the absence of repressor are shown in Supplementary Table 2. Mutants that abolished RNAP binding are not shown, resulting in a different number of mutants in b) and c). Points show mean percent repression over three replicates, bars are standard errors of the mean. Lambda is orange, P22 is blue. Binding to the wild type cognate or non-cognate site is shown by a dark orange point.

Figure 3. Thermodynamic model of gene expression. a) Gene expression is determined by: intra-cellular concentration of (i) repressor, and (ii) RNAP; iii) cooperativity of binding between two repressor dimers; iv) binding energy to the wild type operator (offset E_{WT}); and v) additional contribution of each mutation to the binding energy (energy matrix). Negative (positive) entries in the energy matrix show mutations that decrease (increase) binding energy, and hence increase (decrease) repression. Zero values denote the wild type sequence. **b), c)** The sigmoidal relationship between binding energy and repression, determined by the thermodynamic model, provides quantitative definitions of robustness, tunability and evolvability. **d)** Comprehensive simulation of repression for all possible mutations in the six chosen positions in O_{R1}.

Figure 4. System parameters determine evolutionary potential. a) Correlation between each evolutionary property and a given system parameter: '+' indicates a positive correlation; '-' a negative correlation; '0' a negligible effect; and '*' a non-linear relationship. Lambda CI is orange, P22 C2 is blue. **b)** We swapped parameter values of repressor concentration, cooperativity and offset from one repressor to the other. 'Fraction of variance explained' (R^2) was calculated between the repressor with swapped parameter(s), and the other repressor with its original parameters. R^2 is shown as the grey portion of the pie charts: the fuller the pie chart, the more similar the evolutionary property between the two repressors. Starting from the original parameter values, each of the three parameters was swapped individually, and all three simultaneously.

Figure 5. Biophysical determinants of the evolutionary potential. a) Generic definitions of robustness, tunability and evolvability that utilize only the offset and the energy matrix. $Rob = \frac{E_{1/2}-E_{WT}}{m}$ and $Evo = \frac{E_{1/2}-E_{rando}}{m} = Rob + \#mut$, where $E_{1/2}$ is the binding energy at half repression (which equals the chemical potential, μ), E_{random} is the typical binding energy to a random sequence, m the average mutational effect size, and *#mut* the distance of the random sequence to the cognate operator in number of mutations (see Methods). Evolvability is negative as mutations towards $E_{1/2}$ improve binding. $Tun = (\sigma * \frac{d\,repression}{d\,binding\,affinity}|_{E_{1/2}})/Rob$, where σ is the standard deviation of the energy matrix and $\frac{d\,repression}{d\,binding\,affinity}|_{E_{1/2}}$ the slope of the sigmoid curve at $E_{1/2}$. The table shows the values for robustness, tunability and evolvability for the experimental systems (Fig.1b). Here, we calculated evolvability for the non-cognate sites of Lambda

CI and P22 C2. **b)** Locations of Lambda CI and P22 C2 binding to three categories of operators (E_{WT}, $E_{non\text{-}cognate}$, E_{max}) are indicated by large symbols on the sigmoidal curve relating binding energy and repression. Repressor concentrations are kept equal. Small symbols show mean energy values obtained through model simulations for different mutant classes (1 – single, 2 – double, etc) when mutating the cognate (crosses) or the non-cognate (circles) operators.

Figure 6. Inter-operator epistasis alleviates the trade-off between robustness and tunability. a) Homology alignment of Lambda and P22 O_{R2}, showing mutated sites in bold. Arrows show base pairs that were exchanged between the two operators (Supplementary Table 4). Loss of Lambda CI binding due to mutations in **b**) cognate O_{R2}; **c**) both cognate sites. Points are mean percent repression of three replicates, bars are standard errors of the mean. Plot symbols indicate O_{R2} mutant class. 'x' symbols correspond to the operator with the given O_{R1} mutation(s) and the wild type O_{R2} sequence (Fig.3b). One O_{R1}-O_{R2} mutant gave no measurable expression in the absence of repressor and is not shown.