Title: Entangled adaptive landscapes facilitate the evolution of gene regulation by exaptation

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Abstract

Exaptation, the co-option of existing traits for new functions, is a central process in Darwinian evolution. However, the molecular changes leading to exaptations remain unclear. Here, we investigated the potential of bacterial transcription factor binding sites (TFBSs) to evolve exaptively for the three global *E. coli* transcription factors (TFs) CRP, Fis, and IHF. Using a massively parallel reporter assay, we mapped three combinatorially complete adaptive landscapes, encompassing all intermediate sequences between three pairs of strong TFBSs for each TF. Our results revealed that these landscapes are smooth and navigable, with a monotonic relationship between mutations and their impact on gene regulation. Starting from a strong TFBS for one of our TFs, Darwinian evolution can create a strong TFBS for another TF through a small number of individually adaptive mutations. Notably, most intermediate genotypes are prone to transcriptional crosstalk – gene regulation mediated by both TFs. Because our landscapes are smooth, Darwinian evolution can also easily create TFBSs that show such crosstalk whenever it is adaptive. Our study presents the first in vivo evidence that new TFBSs can evolve exaptively through multiple small and adaptive mutational steps. It also highlights the importance of regulatory crosstalk for the diversification of bacterial gene regulation.

Keywords: adaptive landscapes, navigability, gene regulation, bacteria, massively parallel reporter assay

38 **Introduction:**

39 Exaptation – the repurposing of an existing trait for a new function – is an important route
40 towards new traits in evolution. This process spans various biological levels, from the
41 organismal level—like bird feathers, initially thermoregulatory structures that became
42 indispensable for flight [1,2] — to the molecular level [3,4], such as the evolution of eye crystallins
43 from metabolic enzymes [5,6].

44

45 However, both the molecular changes and the adaptive value of evolutionary intermediates
46 during exaptive evolution remain largely unknown. Transcriptional regulation is an important
47 system to study exaptation due to its central role in life and evolution[7–10]. In transcriptional
48 regulation, a transcription factor protein (TF) binds a DNA sequence called a transcription
49 factor binding site (TFBS), which leads to activation or repression of a nearby gene's
50 transcription [11–13]. Through exaptation, a TFBS initially recognized by one TF can evolve to be
51 recognized by another TF. For example, during the evolution of vertebrate αA-crystallin, a
52 TFBS called a heat shock element was co-opted as a TFBS for the TF Pax6 to drive crystallin
53 expression in the eye lens [5]. Another example is the co-option of regulatory regions of genes
54 from transposable elements in the development of the mammalian uterus[14 15,16].

55

56 To understand the origin of such exaptations in molecular detail requires an understanding of
57 the adaptive landscapes on which TFBSs evolve[17,18]. An adaptive landscape is an analogue to
58 a physical landscape, in which individual locations correspond to a genotype, such as the DNA
59 sequence of a TFBS. The elevation at each location corresponds to a quantitative phenotype,
60 such as the ability of a TFBS to regulate gene expression, which depends on how strongly the
61 TF binds the TFBS.

62

The evolutionary process by which a strong ancestral TFBS for some TF (TF1) becomes exapted as a strong TFBS for another TF (TF2) usually requires an evolutionary path that involves multiple nucleotide changes. We call an adaptive landscape with evolutionary paths that correspond to transitions between TFBSs (or other traits) an *exaptation landscape*. A fundamental question about such a landscape is whether it harbors evolutionary paths towards an exapted trait (TFBS) that are *accessible* to natural selection. These are paths in which each mutational step is favored by natural selection, for example because it increases the strength of gene regulation by a new TF.

Any evolutionary path to a new TFBS may also harbor intermediate TFBSs that can mediate gene regulation through both TF1 and TF2, a phenomenon called transcriptional crosstalk [19–22]. Theoretical estimates suggest that 1-10% of all prokaryotic genes and 10-50% of eukaryotic genes may be subject to crosstalk [21] and its adaptive role is still debated [19–22]. Unfortunately, no pertinent empirical data is available.

Here, we experimentally map the exaptation landscape of TFBSs for three pairs of prokaryotic TFs in *Escherichia coli* [11,23,24], namely CRP (*cAMP receptor protein*[25]), Fis (*Factor for Inversion Stimulation*[26]) and IHF (*Integration Host Factor*) [27]. These TFs come from distinct families [28], have different biological roles, and are known as global regulators because they regulate hundreds of genes [11,23 23,29].

We ask for all three pairs of these TFs (CRP-Fis, CRP-IHF, Fis-IHF) whether it is possible for a strong TFBS recognized by one TF to evolve into a strong TFBS for another TF through accessible mutational paths, i.e., paths on which each step increases regulation by the new TF. We called such strong TFBSs "wild-type" sequences (WT_{TF}). Using a combinatorially

complete library[30] comprising all possible mutant intermediates between wild-type binding site pairs, we measured the strength of gene regulation conferred by each intermediate through a green fluorescent protein (GFP) reporter system.

We show that all three TF exaptation landscapes are smooth. Each has two global peaks of strong TFBSs, one for each TF, which facilitates exaptation through adaptive evolution. We also show that all intermediate TFBSs between the wild-type sequences for each pair of TFs are subject to crosstalk. That is, they can mediate regulation by both TFs to some extent. This extensive crosstalk can hinder or facilitate the exaptation of prokaryotic TFBSs, depending on its adaptive value.

100 **Results**

101

102 **A sort-seq approach for mapping exaptation landscapes**

103 To measure how strongly the TFBSs we study regulate gene expression, we redesigned a
104 plasmid-based system from a previous study [31] (**Figure 1a, Supplementary Figures S1-S2,**
105 **Supplementary Methods 2-3**). This system allowed us to express our TFs in a controlled
106 manner, and to quantify the ability of a TFBS to be recognized by the TF through its effects on
107 the regulation of the GFP reporter gene (**Figures 1b-c**). For brevity, we refer to this ability as
108 the strength of a TFBS or its *regulation strength*.

109

110 To study the evolution of new TFBSs on three exaptation landscapes, we performed the
111 following procedure for each of our three pairs of *E. coli* TFs. We started from strong TFBSs
112 for each TF (named WT_{CRP}, WT_{Fis}, WT_{IHF}) in each TF pair, and aligned these reference TFBSs
113 to determine the smallest number of nucleotide changes needed to convert one into the other
114 (**Figure 1d, top panel, Supplementary Figure S3, Supplementary Tables S2-S3**). We then
115 synthesized a library of DNA sequences containing each sequence on each mutational path
116 between these strong binding sequences (**Figure 1d, bottom panel**).

117

118 Subsequently, we measured the regulation strengths of each sequence in this library to both
119 TFs. In other words, each sequence becomes associated with two quantities, reflecting its
120 ability to mediate gene expression regulation by each of the two TFs. To this end, we employed
121 a massively parallel reporter assay called "sort-seq"[31] (**Figure 1e**, **see Methods**,
122 **Supplementary Methods 7.2**). The landscapes we studied for TF pairs CRP-Fis, CRP-IHF,

and Fis-IHF comprised 256, 128, and 128 distinct TFBSs, respectively (**Figure 1f, Supplementary Figure S3 Supplementary Table S3**).

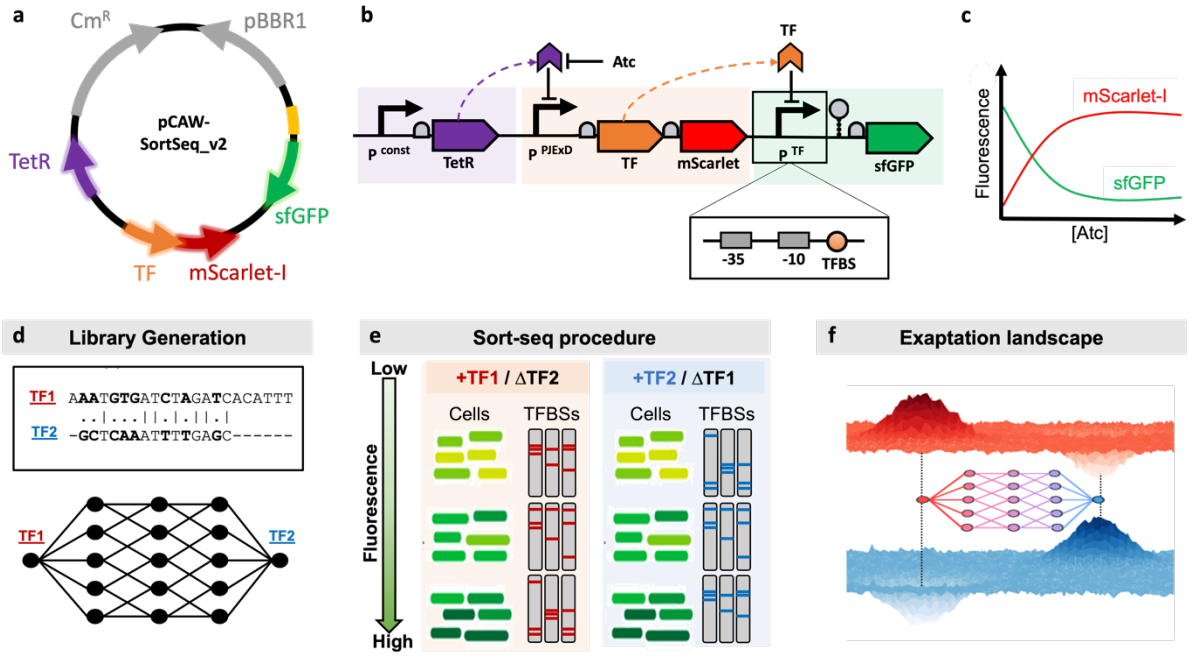

Figure 1. Experimental workflow. a. The plasmid system. We redesigned a previously developed plasmid[31] (**Supplementary Methods 2-3**) that encodes a chloramphenicol resistance gene as a selective marker (CmR, grey, right-oriented arrow) and features a broad-host low-copy replication origin (pBBR1, grey, left-oriented arrow). **b. The regulatory modules.** The plasmid harbors three modules interconnected through regulatory interactions. The first module comprises the constitutively expressed *tetR* gene (purple). The TetR regulator controls the second regulatory module (red box) through the *plTetO1* promoter[32]. This second module encompasses a bicistronic operon, comprising both the TF of interest (orange) and a reporter *mScarlet-I* gene[33] (red). In the third module (green box in b), the TF interacts with its cognate TFBS or a variant from the appropriate library. This interaction results in the transcriptional repression of the fluorescent reporter gene *sfgfp*[34] through steric hindrance, blocking RNA polymerase activity. The addition of anhydrotetracycline (Atc) alleviates repression by TetR, prompting the expression of the TF of interest, which leads to repression of *sfgfp*. To safeguard against the unintended effects of 5'- untranslated regions (UTRs) created by some TFBS library variants on the expression of the *sfgfp* mRNA, we used the RiboJ[35,36] transcriptional insulator (hairpin structure, in grey). Ribosome binding sites (RBSs) are highlighted as grey semi-circles. **c. Basic reporter dynamics.** As the TetR inducer (Atc) levels increase, more TetR regulator is sequestered from its binding site, allowing the expression of the TF and the mScarlet-I reporter (red). Increasing TF concentration causes a decrease in green fluorescence, because the TF causes increasing repression of the sfGFP reporter. **d. Library generation.** Alignment of two strong-affinity ("wild-type") TFBSs for individual TFs, indicating positions (dots) at which the two aligned sites differ from each other. We created a binding site library by varying the nucleotides at all nucleotide positions that differ between the two sites. The library comprises 2^n sequences, where n is the number of positions at which the sites differ (See **Supplementary Methods 4, Supplementary Table S3** and

Supplementary Figure S3 for library details). **e. Transformation of the reporter system harboring the library into two distinct bacterial strains.** One strain expresses TF1 (+TF1) but not TF2 (ΔTF2), and the other TF2 (+TF2) but not TF1(ΔTF1). We then performed fluorescence-activated cell sorting (FACS) independently for each strain, separating cells (green rectangles) into specific bins according to their fluorescence. High-throughput sequencing of the library variants from each bin reveals the mutational differences in each variant relative to the reference TFBS, indicated as red and blue bars inside grey cylinders (TFBS), corresponding to data from the two TF-producing strains. The strength of regulation (binding) conveyed by each binding site is measured from the site's read count across bins (**Supplementary Methods 7.2**). **f. Mapping an Exaptation landscape.** Each TFBS is characterized by two regulation strengths, one for each TF. The regulation strength of each genotype defines an adaptive landscape for each TF (shown as the central-colored network, with the color gradient representing the regulation strengths for TF1, red, and TF2, blue). These can be interpreted as two entangled adaptive landscapes (red and blue above and below the network), where a single sequence is mapped to two phenotypes.

TFBS libraries for pairs of global regulators vary widely in their regulation strengths

Because each of the TFs we studied is also encoded in the *E. coli* genome, their simultaneous expression from a plasmid and the genome would not allow us to discriminate their individual regulation strengths for sequences in our library. We thus performed our experiments in *E. coli* strains in which the chromosomal copy of one TF was deleted (Δ*crp*, Δ*fis*, or Δ*ihf*, see **Supplementary Table S4**). By doing so, we were able to isolate the effect of one TF from that of the other TF in a pair. We observed no substantial growth differences between the deletion strains (**Supplementary Figure S4**).

To validate our reporter system, we studied the regulatory strength of our three wild-type TFBSs (WT_{CRP}, WT_{Fis}, WT_{IHF}, **see Supplementary Table S2**) in the presence of their cognate TFs (**Supplementary Figure S5**). We found that each wild-type TFBS can indeed strongly regulate expression through its cognate TF (**Supplementary Figure S5**). More importantly, we found negligible crosstalk between the wild-type TFBSs (**Supplementary Figure S5**).

After these preliminary experiments, we characterized the interactions between each TF pair and their respective libraries (**Figure 2**). As an example, to characterize the CRP-Fis library,

185 we studied this library in two reporter plasmids and two *E. coli* strains, one plasmid encoding
186 CRP (pCRP) in a Δ*fis* strain in which Fis is not expressed chromosomally (pCRP-Δ*fis*, **Figure**
187 **2a**), the other encoding Fis (pFis) in a Δ*crp* strain in which CRP is not expressed
188 chromosomally (pFis-Δ*crp*, **Figure 2a**). We applied the same strategy to each library (**Figures**
189 **2a-c, Methods**).
190
191 Sort-seq (**see Methods, Supplementary Methods 5, Supplementary Figures S6-S8**) allowed
192 us to characterize how strongly each TFBS variant in each of our libraries can regulate gene
193 expression in response to each of the two appropriate TFs (**Figures 2d-f**). Briefly, we
194 characterized the strength of regulation conveyed by each TFBS variant by averaging the
195 sequencing read counts of each sequence across fluorescence bins and normalizing them by
196 their respective wild-type regulatory strength (**Supplementary Methods 7.2**). The Pearson's
197 correlation coefficient R for pairwise associations of sequencing read counts for replicates of
198 each library ranged from 0.923 to 0.992 (**Supplementary Figures S9-S11**), indicating a high
199 technical reproducibility. The distribution of these scores is approximately normal
200 (**Supplementary Figure S12**), with many sequences regulating expression with intermediate
201 strength by both TFs, and few sequences regulating expression strongly by only one TF.
202
203 We further validated these observations by selecting ten sequences from each landscape
204 (**Supplementary Tables S6-S8**) that cover a wide range of measured regulatory strengths, and
205 individually measured their effects on GFP expression in the presence of either TF1 or TF2.
206 The correlation between these direct measurements and sort-seq data is strong and nearly linear
207 for all pairs of TFs, with Pearson's correlation coefficients R ranging from -0.71 to -0.81
208 (**Methods, Supplementary Figure S13**).
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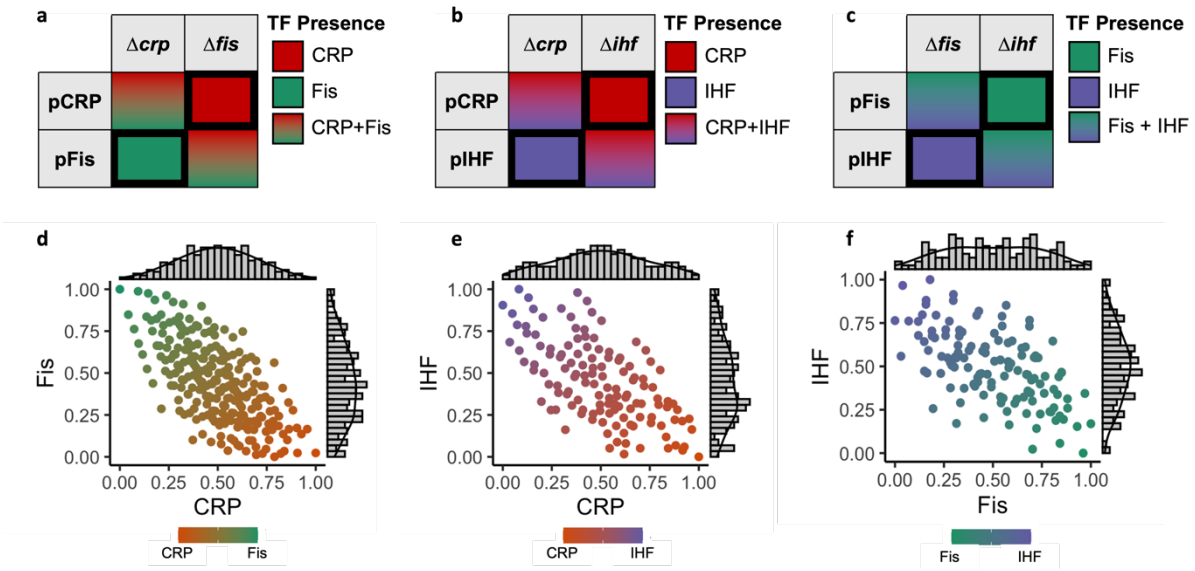

Figure 2. Characterization of regulation strengths for individual TFs. a-c. Specific plasmid-strain combinations ensure the presence of only one of two TFs during our measurements. Each table schematically represents the intracellular presence of TFs for combinations of a reporter plasmid encoding one of the TFs (rows) transformed into a mutant *E. coli* strain lacking the gene encoding one of the TFs (columns). Pure colors represent the presence of a single TF in the cell (CRP: red; Fis: green and IHF: purple), and color gradients represent the presence of both TFs. We avoided plasmid-strain combinations in which both TFs were present in the cell, because such combinations did not allow us to unambiguously measure the regulation conveyed by one TF's binding to each of the TFBSs in an exaptation landscape. The plasmid-strain combinations we used are highlighted with thick black borders. **d-f. Distribution of regulation strengths for three TFBS libraries.** Each panel shows data from a different TF1-TF2 library (d. CRP-Fis; e. CRP-IHF; f. Fis-IHF). Each circle represents a single TFBS variant in a landscape. The horizontal and vertical axes represent the regulation strengths of each variant in the respective landscape to the TF labelled on the axes. Pure colors represent the regulation strengths of each TF (CRP: Red; Fis: Green: IHF, blue), and intermediate colors represent binding by two TFs. The histograms on the top and right of each plot show the distribution of regulation strengths for each TF.

Exaptation landscapes are smooth and reveal abundant crosstalk

Next, we used our sort-seq-based data to map all three exaptation landscapes. We study each such landscape as a network of genotypes (TFBSs) where two genotypes are connected by an edge (straight line) if they differ by a single nucleotide (**Figure 3**). This data already contains important clues to the question of whether exaptation could occur by natural selection alone. The first clue is that the distribution of regulatory strengths appears to change gradually and smoothly with increasing distance from each wild-type TFBS (**Figures 3a-c**).

We quantified crosstalk for each TFBS as a the geometric mean of regulatory strengths by TF1 and TF2 (**Figures 3d-f**, see **Methods**). While the wild-type TFBS is regulated predominantly by the cognate TF in any pair of TFs (**Figure 3d-f, Supplementary Figure S5**), all intermediate TFBSs show at least some ability to be regulated by the other TF in the pair (**Figures 3d-f**). This means that even single mutations away from the wild type can be favored by natural selection. A dimensionality reduction analysis (**Supplementary Figure S14, Supplementary Methods 7.6**), as well as an analysis of nucleotide frequency changes during TFBS evolution (**Supplementary Figure S15**), shows that some nucleotide changes are more important than others in driving changes of regulation strength during exaptation.

A second clue comes from an analysis of the landscapes' peaks – TFBSs for which all neighbouring genotypes regulate transcription less strongly than the peak itself. Any one such peak (different from the wild-type) may hinder exaptation, because an evolving population could become trapped on local suboptimal peaks with low fitness[17,37]. However, for each TF1-TF2 landscape, there are only two peaks, one for each TF, corresponding to the two wild-type sequences (**Figures 3a-c**).

Third, we also studied the fraction of evolutionary paths to a wild-type sequence that would be accessible to selection, i.e., a series of mutational steps in which each step increases regulation strength. In all three exaptation landscapes, most paths starting from the wild-type TFBS of TF1 and leading to the wild-type sequence of the TF2 are accessible (ranging from 65% to 100%, **Supplementary Figure S16)**. The reason why some paths to the peak are not accessible in two landscapes is that these landscapes show (reciprocal) sign epistasis (**Supplementary Methods 7.5**, **Supplementary Tables S6-S8** and **Supplementary Figure S16**). These are

forms of non-additive interactions among sequence variants, in which one or two individual mutations reduce regulation strength but both mutations together increase it. Specifically, in the CRP-Fis landscape, 4% of double mutants show (reciprocal) sign epistasis. In the CRP-IHF landscape, 11% of double mutants show (reciprocal) sign epistasis.

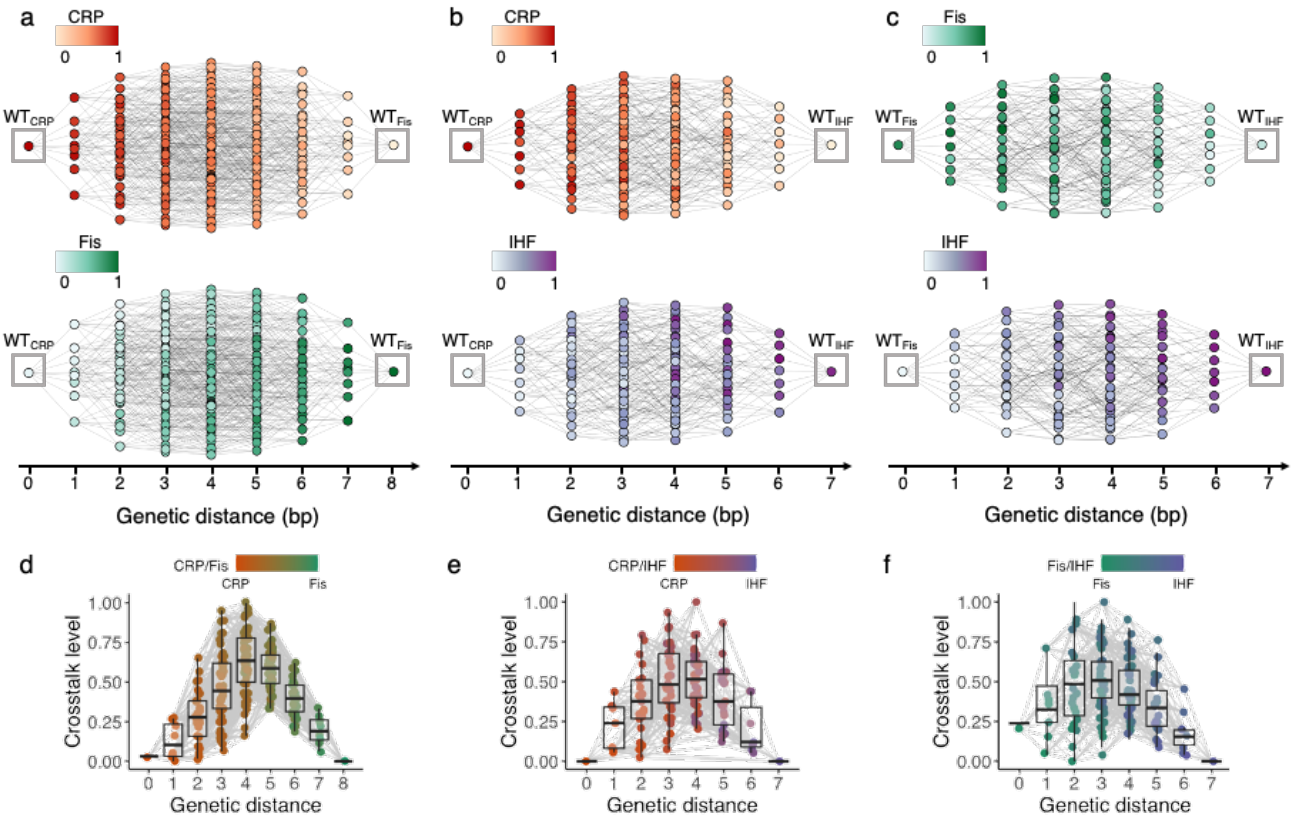

Figure 3. Exaptation landscapes are smooth with many intermediates subject to transcriptional crosstalk. Panels a-c depict exaptation landscapes as networks whose nodes (circles) represent binding site variants connected through single nucleotide changing mutations (straight lines). The x-axis of each panel shows the genetic distance (in number of nucleotide differences) from each TFBS variant to one of the wild-type sequences (at x = 0). Grey squares indicate WT sequences for each TF, which are peaks in their respective landscapes. **a. The CRP-Fis exaptation landscape.** Both panels represent the CRP-Fis exaptation landscape (N = 256) with nodes ordered by their distance to the CRP wild-type sequence. Colors represent regulation strengths of each TFBS variant to CRP (top panel, red heatmap) and to Fis (bottom panel, green heatmap). **b. The CRP-IHF exaptation landscape.** Both panels represent the CRP-IHF exaptation landscape (N = 128) with nodes ordered by their distance to the CRP wild-type sequence. Colors represent regulation strengths of each TFBS variant to CRP (top panel, red heatmap) and to IHF (bottom panel, purple heatmap). **c. The Fis-IHF exaptation landscape.** Both panels represent the Fis-IHF exaptation landscape (N = 128) with nodes ordered by their distance to the Fis wild-type sequence. Colors represent Fis (top panel, green heatmap) and IHF (bottom panel, purple heatmap) regulation strengths for each sequence. Light and dark colors in panels a-c represent

weak and strong binding, respectively. Note that all regulation strengths in panels a-c are normalized by the regulation strength of the wild-type sequence. **d-f. Crosstalk levels for binding sites in each exaptation landscape.** Each panel shows, for all TFBSs in an exaptation landscape, the ratio of binding to TF1 and TF2 (y-axis) as a function of the TFBSs genetic distance from the wild-type sequence (x-axis), which is a strong TFBS. Each circle symbolizes one genotype. Grey circles connect genotypes that differ in a single nucleotide. Boxplots indicate the distribution of crosstalk levels (see **Methods**) for each mutational distance. Each box covers the range between the first and third quartiles (interquartile range, IQR). The horizontal line within the box represents the median value, and whiskers span 1.5 times the IQR. Heatmap colors reflect the regulation strength ratio for each TFBS. That is, sequences binding predominantly to CRP, Fis or IHF are displayed in red, green, and purple, respectively. Intermediate colors indicate binding at intermediate strength to two TFs. Note that crosstalk levels are normalized by the maximum crosstalk value measured, resulting in a scale that ranges from 0 (no crosstalk) to 1 (maximum crosstalk). We generated networks with the *Gephi*[38] software using a combination of both the *3DForceAtlas* and *Network3DSplitter* algorithms.

In silico analysis suggests crosstalk between two closely related bacterial species

To study the exaptive evolution of TFBSs, one would ideally like to infer their evolutionary history with phylogenetic methods, an approach that has proven successful for eukaryotic TFBSs[39–42]. In order to explore if we could find genomic signatures of TFBS exaptation in bacteria, we analyzed the genomes of two closely related species, *Escherichia coli* K12 / MG1655 and *Salmonella typhimurium* LT2, which diverged approximately 100 million years ago[43] (see **Supplementary Methods 7.7**). By comparing noncoding DNA sequences upstream of orthologous genes and operons between the two species, we found evidence of both *de novo* evolution and exaptive evolution in TFBSs for eight *E. coli* global regulators (CRP, Fis, IHF, ArcA, FNR, Cra, Lrp, Fur)[23]. Any instance where the location of a TFBS for one TF within an intergenic region in one species overlapped with the location a TFBS for a different TF in the other species, we considered a potential candidate for exaptive evolution. Specifically, we observed an average gain of 3.2±0.3 (mean±s.d.) TFBSs per intergenic region through either *de novo* or exaptive evolution, with 34% of these gains potentially driven by exaptation. (**Supplementary Figure S17, Supplementary Methods 7.7**).

Rapid exaptation when Darwinian evolution favors strong binding by one TF

Motivated by the potential for naturally occurring TFBS exaptations in bacteria, we examined how populations would evolve on our experimentally mapped landscapes. We did so by simulating population evolution in the strong selection weak mutation (SSWM) regime (**Supplementary Methods 8**) [44–47]. We focused on this regime due to its relevance for bacteria with large populations, such as *E. coli* [48] (>10^8 individuals), where genetic drift is minimal, and even small fitness differences are visible to natural selection [44–47]. Given the low probability of mutations at individual TFBS nucleotide positions (2.2×10^{-10} per generation)[48], one beneficial mutation in this regime typically reaches fixation before the next mutation arises. The result is a population that is monomorphic most of the time – concentrated at a singular genotype on the landscape – and that adapts through stepwise fixations, i.e., through an adaptive walk on the landscape [44–47 47,49.]

We simulated 10^4 adaptive walks per TF1-TF2 landscape, starting from each of the 10 strongest TFBSs for TF1 for a total of 10^5 walks. We defined the fitness of each genotype (TFBS variant) as the regulation strength mediated by TF2. Fixation probabilities depend on population size, as prescribed by population genetic theory [50,51] (**Supplementary Methods 8**). Additionally, we simulated adaptive walks in the opposite direction, starting from the highest-regulation genotypes for TF2. All adaptive walks incorporated known *E. coli* mutation biases [52,53] (**Supplementary Methods 8**).

In large populations (10^8), where selection is strong and drift is weak [54,55], all adaptive walks reached the wild-type TFBS for TF2 or TF1 (CRP-Fis: **Figures 4a-b**; CRP-IHF: **Supplementary Figures S18a-b**; Fis-IHF: **Supplementary Figures S19a-b**). The length of these walks depended on the direction of the walk (e.g., CRP to Fis: 5.3±0.9 mutational steps

vs. Fis to CRP: 5±0.9 steps, mean±s.d., $p<0.001$, Welch Two Sample t-test). This slight asymmetry is likely a result of sign epistasis [17,56,57] (**Supplementary Tables S9-S11**). It also exists in the CRP-IHF landscape (**Supplementary Figures S18 c-f**). Overall, however, adaptive walks were nearly as short as the minimal genetic distance, reflecting landscape smoothness and navigability (CRP-Fis: **Figures 4e-f**; CRP-IHF: **Supplementary Figures S18e-f**; Fis-IHF: **Supplementary Figures S19e-f**). Contingency, defined as the dependence of a historical process on chance events[58–60], was also evident, as the same peak could be reached by multiple alternative paths (**Supplementary Figures S20-S21**). In smaller populations ($N=10^2$), where selection is weaker and drift is stronger [54,55], fitness at the end of a walk was only modestly reduced (by ~1%, $p<2.2\times10^{-16}$, One-Sample t-test, $n=10^5$, **Supplementary Figures S23a-f**), and walks were only slightly longer (by 10%, $p<2.2\times10^{-16}$, Welch Two Sample t-test, $n=2\times10^5$, **Supplementary Figures S23g-l**).

Additionally, we investigated the impact of deviations from the SSWM regime on our observations. When mutation rates are high or populations are even larger than we consider, populations are polymorphic most of the time [49,61,62]. In consequence, they experience clonal interference where the fittest among several simultaneously segregating mutations is likely to fixate [49,61,62]. High mutation rates and clonal interference, simulated via "greedy" adaptive walks [37,45,63,64] (**Supplementary Methods 8**), revealed no significant exaptation rate differences compared to the SSWM regime (**Supplementary Figures S24-S26**). In sum, the smooth, single-peaked landscapes we mapped enable rapid exaptation via selection, regardless of genetic drift or clonal interference.

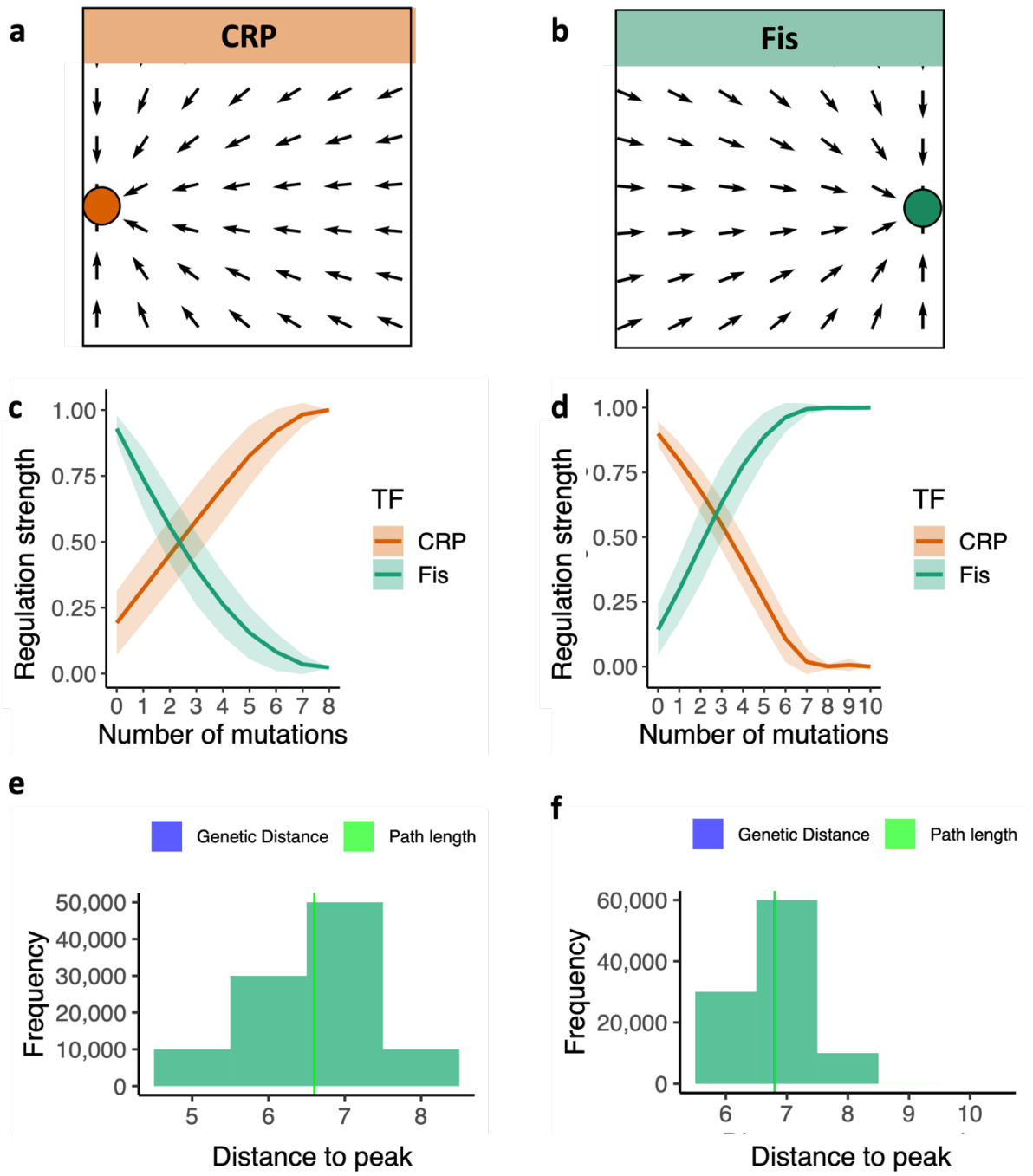

Figure 4. Rapid exaptation on the CRP-Fis landscape. All data presented is based on a population of 10^8 individuals. Data for each plot is based on 10^5 simulated adaptive walks, i.e., 10^4 adaptive walks for each of the 10 starting genotypes with the highest regulation strength for the opposite TF. **a-b. Vector field illustrations of adaptive walks in genotype space.** The illustrations show the direction of adaptive walks in genotype space when strong binding to a specific TF (CRP or Fis) is favored. Each panel represents a single TF landscape, with the x and y axes representing coordinates in genotype space. Each vector indicates the direction of an adaptive walk starting from a distinct genotype and ending at the colored circle peak ("attractor"), which signifies strong regulation by the TF. **a. Scenario 1: Adaptive trajectories favoring strong binding to CRP.** In the CRP landscape, adaptive walks favor strong binding to CRP, indicated by the orange circle (CRP peak). **b. Scenario 2: Adaptive trajectories favoring strong binding to Fis.** In the Fis landscape, adaptive walks favor strong binding to

Fis, indicated by the green circle (Fis Peak). **c-d. Adaptive walks readily attain peaks.** Each plot represents how regulation strengths (vertical axis) for both TFs (CRP in orange and Fis in green) change along the transversed path length (horizontal axis) from the initial sequence to the attained peak during an adaptive walk. Curves represent averages over 10^5 adaptive walks, and shading indicates one standard deviation of regulation strength. **c. Adaptive walks attain the CRP peak.** The data is based on 10^4 walks starting from each of the 10 strongest binding sites for Fis, favoring increasing CRP binding. **d. Adaptive walks attain the Fis peak.** Like panel (c), but for adaptive walks starting from the 10 strongest binding sites for CRP, favoring increasing Fis binding. **e-f. Evolutionary paths to a peak are not much longer than the minimal genetic distances.** Each composite histogram shows the distribution of path lengths (green) for all pairs of starting (non-peak) and ending (peak) genotypes during 10^5 adaptive walks. Blue histograms represent the distribution of genetic distances for each pair of non-peak and peak genotypes. The green and blue horizontal lines represent average path lengths and mutational distance, which are indistinguishable. We used a t-test of the null hypothesis that the means of these distance distributions are statistically indistinguishable. **e. Distribution of accessible path lengths and genetic distances to the CRP peak.** We observed no significant difference between mean genetic distance and path length (mean ± s.d.: 6.6 ± 0.7 for both genetic distance and path length; Welch Two Sample t-test: t = 31.973, df = 196,700, p-value = 0.7, $N_1 = 10^5$ genetic distances, $N_2 = 10^5$ path lengths). **f. Distribution of accessible path lengths and genetic distances to the Fis peak.** We observed no significant difference between mean genetic distance and path length (mean ± s.d.: 6.9 ± 0.8; Welch Two Sample t-test: t = 35.113, df = 196,700, p-value = 0.82, $N_1 = 10^5$ genetic distances, $N_2 = 10^5$ path lengths).

In silico analysis suggests extensive crosstalk between TFBSs in the *E. coli* genome

We next focused on crosstalk between our three TFs, and first asked whether crosstalk might exist between them in the *E. coli* genome. To this end, we used Position-Weight Matrices (PWMs). Each such matrix encodes information on experimentally verified TFBSs for any one TF and represents the probability that any one of the four DNA nucleotides occurs at each specific position of these TFBSs[65,66]. PWMs are well-established computational tools to score how strongly an unknown DNA sequence may be bound by a given TF [65,66]. For our three TFs and the TFBSs in our libraries, these computational PWM scores are correlated with our experimentally measured regulation strengths (mean Pearson R = 0.56±0.1, mean±s.d., N= 3, see **Supplementary Methods 7.8 and Supplementary Figure S27**). We thus used PWMs together with 756 experimentally validated *E. coli* TFBSs from the RegulonDB database[28] for our three TFs, to predict the potential for crosstalk between these TFs in the *E. coli* genome. This potential is indeed very high (**Figure 5a, Supplementary Methods 7.8**).

414

415 For example, among 756 TFBSs with regulation strengths for both CRP and Fis predicted by
416 PWMs, 94% (706/756) exhibited modest crosstalk, i.e., regulation strengths above 0.25 for
417 each transcription factor (**Figure 5b)**. In the same dataset, 78% (591/756) and 82% (619/756)
418 of the TFBSs surpassed this threshold for the CRP-IHF (**Figure 5c**) and Fis-IHF (**Figure 5d**)
419 pairwise comparisons, respectively. Furthermore, 44% (332/756) of the sequences
420 demonstrated intermediate to high cross-talk – predicted regulation strengths above 0.5 for
421 CRP and Fis. Additionally, within the same dataset, 8% and 9% of the TFBSs exceeded the 0.5
422 threshold for CRP-IHF and Fis-IHF pairwise comparisons, respectively.

423

424 **Rapid exaptation also occurs when selection favors crosstalk between two TFs**

425 Motivated by our observations that crosstalk may be rampant, we speculated that it may be
426 adaptive for at least some regulated genes. We thus asked how readily crosstalk could evolve
427 adaptively from crosstalk-free TFBSs in our exaptation landscapes.

428

429 Under the SSWM regime, we performed 10^4 random walks per genotype across each
430 landscape, totaling N = 256 x 10^4, N = 128 x 10^4, and N = 128 x 10^4 for the CRP-Fis, CRP-IHF,
431 and Fis-IHF landscapes, respectively. Fitness was defined as the sum of regulation strength for
432 both TFs (CRP-Fis: **Figures 5e-g**; CRP-IHF: **Supplementary Figures S30 a,c,e**; Fis-IHF:
433 **Supplementary Figures S30 b,d,f**; **Supplementary Methods 8**). Both CRP-Fis and Fis-IHF
434 landscapes remained single-peaked, with intermediate regulatory strengths for each TF (CRP-
435 Fis: 0.63 for CRP, 0.75 for Fis; Fis-IHF: 0.68 for Fis, 0.76 for IHF). However, CRP-IHF had
436 two peaks: 87% of walks reached the first peak (CRP: 0.68, IHF: 0.68), and 13% reached the
437 second (CRP: 0.87, IHF: 0.32).

438

Consistent with our previous simulations without crosstalk, paths to each peak were as short as the minimal genetic distance, highlighting each landscape's smoothness and navigability. For instance, in CRP-Fis, evolutionary paths and mutational distances were not statistically different (Welch Two Sample t-test, p-value = 0.85, **Figures 5 f-g**). Similar observations hold for CRP-IHF (**Supplementary Figures S30 a,c,e**) and Fis-IHF (**Supplementary Figures S30 b,d,f**).

In small populations ($N=10^2$), genetic drift had variable effects across landscapes. In CRP-Fis, drift increased CRP regulation by 10% and decreased Fis by 14% (**Supplementary Figure S31a**). For CRP-IHF, the most accessed peak reduced CRP by 13% and IHF by 8%. In Fis-IHF, drift increased Fis by 8% and reduced IHF by 13% (**Supplementary Figure S31 d**). Drift did not impact peak attainment time, except for the most accessed peak in the CRP-IHF landscape, where the average number of mutations increased to reach such peak increased from 3.4 to 5 (45% increase, $p<2.2 \times 10^{-16}$, Welch Two Sample t-test, $n = 256 \times 10^4$, **Supplementary Figures S31 e-h**). We also simulated deviations from the SSWM regime using adaptive "greedy" walks, confirming that clonal interference did not alter exaptation rates, as high-fitness peaks were reached with similar steps as in the SSWM regime (**Supplementary Figure S32**). In sum, selection-driven evolution for crosstalk progresses rapidly on these single-peaked landscapes, with minimal interference from genetic drift or clonal interference.

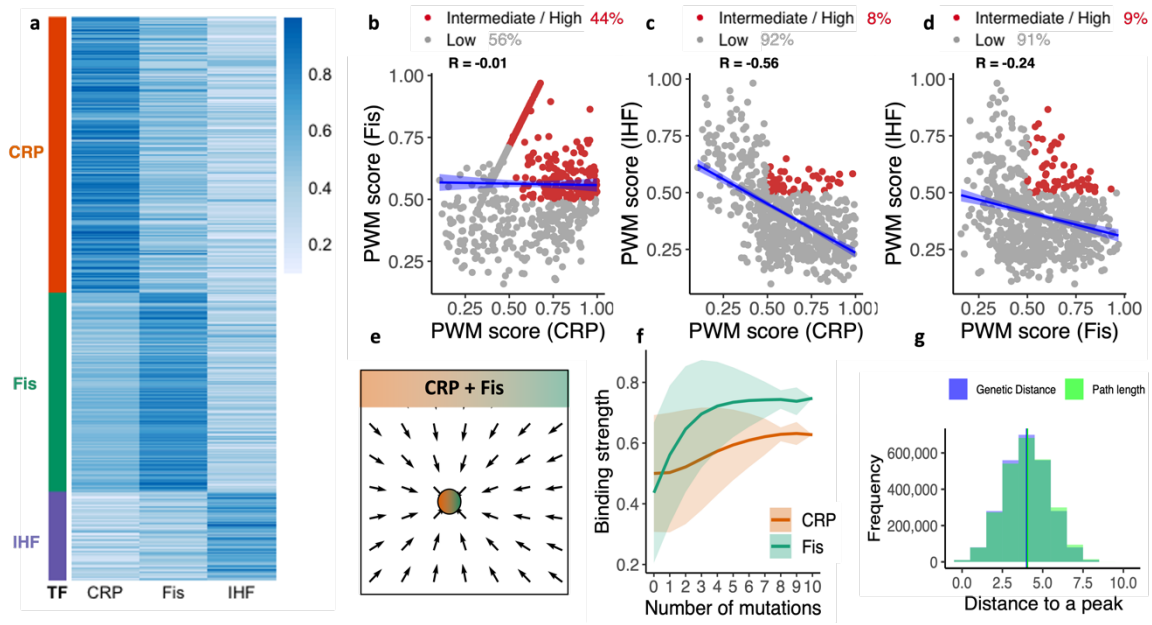

Figure 5. Transcriptional crosstalk and its consequences for evolutionary dynamics. a. PWM scores for experimentally characterized TFBSs from the *E. coli K-12* genome suggest ample crosstalk between TFs CRP, Fis, and IHF. The heatmap is based on 756 TFBS sequences in the *E. coli K-12* genome, as curated in the RegulonDB[67] database. Each row corresponds to one TFBS. The dataset includes 370 TFBSs for CRP (orange in left-most column), 267 for Fis (green), and 119 for IHF (purple). Each of the remaining columns quantifies the predicted binding strength (PWM score) for each of the 756 TFBSs with respect to the transcription factors CRP, Fis, and IHF, as indicated below each column. We normalized predicted binding strength by the maximum possible score for each TF, resulting in a scale from 0 to 1, as indicated by the color gradient in the heatmap color legend. We clustered the rows of the heatmap using a combination of Euclidean distance and hierarchical clustering with complete linkage to reveal patterns of similarity in predicted binding strengths among the sequences. **b-d. Association between predicted binding strengths for CRP, Fis and IHF and their genomic *E. coli* TFBS.** We computed for each pair of TFs the statistical association between predicted binding strength (CRP-Fis, CRP-IHF, Fis-IHF). In the scatter plots, red circles represent TFBSs whose average predicted binding strength for both TFs is higher than 0.5, indicating intermediate to high crosstalk. Grey circles represent TFBSs with an average predicted binding strength below this threshold, indicating low crosstalk. The percentage of sequences with intermediate to high crosstalk (red circles) and low crosstalk (grey circles) is shown at the top of the plots in red and gray. The blue line is a linear regression line (blue shading: 95% confidence interval; R: Pearson correlation coefficient.). **b. Association between CRP and IHF.** No significant association between CRP and Fis predicted binding strengths ($R = -0.01$ and t-statistic $p = 0.71$). The straight gray and red line results from some sequences with very similar predicted binding strength values, and does not materially affect this association. **c. Association between CRP and IHF.** $R = -0.56$ and t-statistic p-value $< 2 \times 10^{-6}$. **d. Association between Fis and IHF.** $R = -0.24$ and t-statistic p-value $< 2 \times 10^{-6}$. **e-g. Rapid evolution of crosstalk in the CRP-Fis landscape. e. Schematic of evolution towards TFBSs with high crosstalk.** The vector field illustrates the direction of adaptive walks in genotype space when strong binding towards a CRP and Fis is favored. Each vector indicates the direction of an adaptive walk starting from a distinct genotype and ending at a peak ("attractor", colored circle) strongly binding both TFs. **f. Adaptive walks readily attain TFBSs binding to both CRP and Fis with intermediate strengths.** We performed 10^4 adaptive walks starting

from each of the 256 TFBS variants in the CRP-Fis landscape, with selection favoring strong binding for both CRP and IHF. The panels show the regulation strength (vertical axis) for CRP (orange) and Fis (green) plotted against the transversed path length of an evolving sequence to the attained peak (horizontal axis). Curves represent averages over all 256 x 10^4 adaptive walks and shading indicates one standard deviation of regulation strengths. **g. The distribution of accessible path lengths and genetic distances to peaks.** The green and blue horizontal lines show the average length of traversed paths during 256 x 10^4 walks between pairs of non-peaks and peaks, and the respective mutational distances between such pairs, respectively. The means of the distributions are statistically indistinguishable (3.31±1, mean ± s.d., for both distance metrics, Welch Two Sample t-test t = -5.5782, df = 2029535, p-value = 0.7, N_1= 256 x 10^4, N_2= 256 x 10^4).

506 **Discussion:**

507 We experimentally demonstrated that exaptation landscapes for three pairs of *E. coli* TFs (CRP-
508 Fis, CRP-IHF, Fis-IHF) are smooth, with only two peaks, one for each regulator. Despite this
509 smoothness, sign epistasis exists in some of the landscapes and restricts evolutionary paths.
510 Evolutionary dynamics simulations show that strong TFBSs for TF1 can readily evolve into
511 strong TFBSs for TF2 through few and individually adaptive mutational steps. Neither strong
512 genetic drift nor clonal interference would alter this conclusion.

513

514 Each of the global regulators we study controls hundreds of *E. coli* genes and co-regulates
515 complex *E. coli* promoters[68–71]. In specific environments, it may be advantageous for some of
516 these genes to become rewired and regulated by a different global regulator. While gene
517 regulatory rewiring is common across bacteria [72–76], documented cases of TFBS exaptation are
518 rare, likely due to challenges in observing them across species amid frequent horizontal gene
519 transfers and recombination [72,77–79]. Our results suggest that regulatory exaptation could be a
520 frequent mechanism in bacterial gene regulation diversification.

521

522 Although most studies of TFBS exaptation are theoretical [17,80–82], recent experimental work has
523 shed light on this process [17,80–82]. For example, a synthetic biology study engineered
524 overlapping TFBSs to accommodate up to three TFs [83,84]. Another study investigated the
525 potential of two distinct TFs (Lambda CI and P22 C2) to bind a small library of TFBS
526 mutants[85]. The study found that Lambda CI could recognize new TFBSs more easily when their
527 evolution started from P22 C2's TFBS than from CI TFBSs [85]. A possible reason for this
528 asymmetry may be a greater incidence of epistasis in the landscape of one TF, as we observed
529 in our landscapes (**Figures 4e-f**, **Supplementary Figures S18-S19**).

530

In contrast to smooth adaptive landscapes, rugged (multi-peaked) landscapes can hinder Darwinian evolution by entrapping populations on local suboptimal peaks, and thus prevent a population's ascendance of the highest fitness peak[17]. With some exceptions[31,86,87], empirical landscapes of gene regulation are smooth[17]. The smoothness observed likely results from low epistasis among mutated nucleotide positions.

A growing body of theoretical studies predicts that transcriptional crosstalk is a global phenomenon in nature[19–22,88], and our empirical and in silico analysis further support this hypothesis. We observed extensive crosstalk potential for intermediate genotypes across our landscapes. However, despite this potential, the influence of crosstalk on exaptation is still poorly understood. On the one hand, Darwinian evolution requires that all intermediates of an evolutionary process are functional, and crosstalk can facilitate the evolution of intermediate genotypes if regulation by both TFs is beneficial. Crosstalk can be adaptive when a gene needs to be expressed in multiple environments and by different TFs [19,22,88,89]. Such adaptive crosstalk can simplify gene regulation and coordinate cellular responses across diverse environments. Furthermore, in the compact genomes of bacteria, where space is limited, transcriptional crosstalk can help reduce the size of regulatory regions [13,71,83,84,90,91], as evidenced by the overlap of many TFBSs in bacterial promoters [71,89]. On the other hand, crosstalk may cause conflict between different regulators and impair adaptive evolution when precise gene regulation is paramount [19–21]. For example, genes whose protein products engage in many interactions inside the cell are less likely to undergo horizontal gene transfer, because the fortuitous regulation of many genes by an exogenous TF can reduce organismal fitness by globally rewiring the host's regulatory network[92–95].

These considerations motivated us to ask whether adaptive evolution on our landscapes could easily create crosstalk when such crosstalk is adaptive. The answer is yes, because our landscapes remained smooth under this selection regime, such that evolution rapidly finds TFBSs with intermediate strengths for two TFs and does so through short evolutionary paths of merely three mutations, on average. Because most *E. coli* genomic TFBSs for CRP, Fis, and IHF exhibit the potential for low to intermediate crosstalk levels (**Figure 5a**), crosstalk can also be quickly tuned up or down in evolution, depending on its adaptive value.

Our study has some limitations. First, we quantified gene regulation strength and crosstalk, but did not measure fitness directly. However, other studies suggest that strong gene regulation is associated with high fitness for at least some genes [81,96–99]. Whenever weak TFBSs are beneficial for regulation [100,101], the smoothness of the landscapes we mapped suggests that weak regulation can also readily evolve by individually adaptive steps. Second, we used a simple regulatory architecture, while native promoter architectures can involve multiple TFs and combinatorial regulation [70,71]. Such complex promoter architectures could potentially modulate crosstalk levels and should be further investigated. Lastly, we examined only a small subset of the TFBS sequence space. A broader sequence space may present more rugged, challenging landscapes for Darwinian evolution.

Within these limitations, we have shown that exaptive evolution in gene regulation can be easily accomplished, because the three exaptation landscapes we study are smooth. Examples of regulatory exaptation[6,15,102–104] and the existence of crosstalk in the wild[88,105] suggest that this may be a general principle of exaptation in gene regulation. Further corroboration will require studying more transcription factors, measuring fitness and crosstalk directly, exploring a larger sequence space, and doing so in the chromosomal context in which TFs operate. Lastly,

our study suggests that regulatory evolution does not need to be confined to an isolated landscape but has the potential to unfold in an interconnected space of adaptive landscapes, where each mutational step can simultaneously modulate the regulatory potential of multiple TFs.

584 **Methods**

585

586 The Supplementary methods contain extended details of experimental procedures and data
587 analysis.

588

589 **Media and reagents.** To prepare SOB medium, we mixed 25.5g from the solid medium stock
590 (VWR J906) with 960 ml of purified water and subjected the resulting suspension to
591 autoclaving. To prepare SOC medium, we dissolved 20 ml of 1 M D-glucose (Sigma G8270)
592 and 20 ml of 1 M magnesium sulfate (Sigma 230391) in 960 ml of the pre-prepared SOB
593 solution. For LB medium, we combined 25g of solid medium stock (Sigma-Aldrich L3522)
594 with 1 liter of purified water and then autoclaved it. To prepare M9 minimal medium, we
595 diluted M9 minimal salt sourced from Sigma (M6030) in distilled water as per the
596 manufacturer's guidelines, autoclaved the solution, and added 0.4% glucose (Sigma G8270),
597 0.2% casamino acid (Merk Millipore, 2240), 2 mM magnesium sulfate (Sigma 230391), and
598 0.1 mM calcium chloride (Sigma C7902). Where required, we supplemented growth media
599 with chloramphenicol (50 μg/mL), anhydrotetracycline (100 ng/mL, Cayman-chemicals
600 #10009542), and/or glucose (0.4% w/v final concentration). We prepared anhydrotetracycline
601 by diluting the dried chemical in absolute ethanol, from a stock concentration (1000X) of 100
602 μg/mL to a working concentration of 100 ng/mL.

603

604 **Strains and plasmids**

605 Bacterial strains and plasmds used in this work are listed in **Supplementary Tables S1- S2**.
606 We obtained electrocompetent *E. coli* cells (strain SIG10-MAX®) from Sigma Aldrich
607 (CMC0004). The genotype of this strain (**Supplementary Table S4**) is similar to DH5α
608 (Sigma Aldrich commercial information, see **Supplementary Table S4**). The strain is resistant

609 to the antibiotic streptomycin. Due to its high transformation efficiency, we used strain SIG10-
610 MAX® for molecular cloning and library generation.
611
612 We amplified plasmid libraries in DH5α, extracted their DNA, and transformed them into the
613 *E. coli* K-12 BW25113 wild-type strain and its derivative mutants to study gene regulation. We
614 obtained the *E. coli* wild type (BW25113) strain and its mutants, which harbor deletions of
615 CRP, IHF, or Fis global regulators from the KEIO collection [106], and used them for sort-seq
616 experiments. The design, genetic parts, and assembly of the plasmid vectors we used in this
617 study are available in the Supplementary material. All primers, TFBS sequences/libraries,
618 strains and plasmids are listed in **Supplementary Tables S1-S5**.
619
620 **Sort-seq procedure**.
621 We performed our sort-seq experiments as previously described[31]. We designed TF binding site
622 libraries in the Snapgene® software (snapgene.com). The libraries were synthesized by IDT
623 (Coralville, USA) as a single-stranded DNA Ultramer® of 140bp (4nmol). We resuspended
624 each library in nuclease-free distilled water and serially diluted it to a concentration of 50ng/uL.
625 We PCR-amplified library oligonucleotides, ligated them into the PCR-amplified pCAW-Sort-
626 Seq-V2 plasmid backbone (a derivative of pCAW-Sort-Seq [31], a low-copy number, multi-host
627 plasmid with the pBBR1 replication origin) by Gibson assembly, and transformed cells with
628 the resulting library after column-based purification with a commercial electroporation kit
629 (NEB #T1020L). We grew populations of *E. coli* cells hosting each library in LB medium
630 supplemented with 50 µg/mL chloramphenicol to saturation (overnight growth, 16 hours, 200
631 rpm, 37°C). After overnight growth, we diluted the overnight culture in LB medium
632 supplemented with 50 µg/mL chloramphenicol and anhydrotetracycline (100 ng/mL) in a 1:100
633 ratio (v/v) and grew the culture for 8 h until it had reached stationary phase. Before sorting, we

634 diluted 50μL of the culture in 1mL of cold Dulbecco's PBS (Sigma-Aldrich #D8537) in a 15
635 mL FACS tube. We performed FACS-sorting on a FACS Aria III flow cytometer (BD
636 Biosciences, San Jose, CA). We sorted and binned cells according to their GFP-mediated
637 fluorescence (FITC-H channel, 488nm laser, emission filter 502LP, 530/30). To this end, we
638 recorded the fluorescence of 10^6 cells and created 4 evenly spaced gates ("bins") on a binary
639 logarithmic ($\log_2$) scale spanning the observed range of fluorescence values (**see Methods,**
640 **Supplementary Figures S6-S8**). The number of cells sorted into each bin corresponded to the
641 fraction of the 10^6 cells recorded for that bin. Thus, the total number of sorted cells was 10^6.
642 We performed each sorting in triplicate from the same overnight culture. We isolated plasmids
643 from cells sorted into each bin (Qiagen, Germany) after overnight growth, and used a
644 polymerase chain reaction (PCR) to amplify the TFBS region from each plasmid for Illumina
645 sequencing. The distribution of library variants among the different bins allowed us to map
646 each individual genotype to a measure of how strongly it regulates gene expression.
647
648 The Supplementary material has additional details on library construction and sort-seq
649 procedures.
650
651 **Sort-Seq procedure for dissecting the effects of individual TFs**
652 To reduce the effects of extrinsic noise (cell-specific variation)[107] we followed common
653 practice[108–110] and normalized our *gfp* fluorescence values by *mscarlet* fluorescence values after
654 flow-cytometry assays (**see Supplementary Methods 5**). To dissect the distinct effects of
655 individual transcription factors within a shared pool of sequences (libraries), we employed a
656 sort-seq procedure that involved a combination of plasmids expressing specific TFs in specific
657 mutant bacterial strains. Below, we outline the key steps in this procedure for each TF pair.
658

659 **Sorting the CRP-Fis library**

660

661 **Characterizing the effect of Fis binding on gene regulation.** To study the influence of Fis
662 binding on our CRP-Fis library sequences independent from that of CRP binding, we proceeded
663 as follows: We cloned the CRP-Fis library onto the plasmid pCAW-Sort-Seq-V2-Fis, which
664 expresses the Fis transcription factor upon Atc induction. Next, we introduced this plasmid into
665 the *E.coli* Δ*crp* strain, in which the *crp* gene has been deleted (**Supplementary Table S4**,
666 **Figure 2a**). We then cultured the transformed bacterial cells overnight in LB medium
667 supplemented with Atc, which induces the expression of plasmid-encoded Fis within the
668 bacterial population. By doing so, we guaranteed complete absence of CRP and that Fis was
669 expressed. We then subjected the CRP-Fis library to the sort-seq procedure under these
670 conditions, allowing us to specifically examine the effects of Fis binding on our library
671 sequences.

672

673 **Characterizing the Effect of CRP Binding.** To study the impact of CRP binding on gene
674 regulation in isolation from that of Fis binding, we cloned the same CRP-Fis library on plasmid
675 pCAW-Sort-Seq-V2-CRP, expressing CRP in the Δ*fis* strain. We induced Fis expression with
676 Atc, ensuring that of the two transcription factors, only CRP is present. After overnight growth,
677 we sorted cells to measure the strength of CRP binding on the CRP-Fis library sequences.

678

679 **Sorting the CRP-IHF Library**. We adopted the same procedure we just described for the
680 CRP-Fis library, except that we cloned the CRP-IHF library into the plasmid pCAW-Sort-Seq-
681 V2-IHF, which expresses the IHF transcription factor upon Atc induction, and transformed this
682 library into a Δ*crp* strain. Briefly, to study the effect of IHF binding independent from that of
683 CRP binding, we performed sort-seq after library growth in LB supplemented with Atc. To

684 study the effect of CRP binding independent from that of IHF binding, we cloned the CRP-IHF
685 library into the plasmid pCAW-Sort-Seq-V2-CRP, which expresses the CRP transcription
686 factor upon Atc induction, and transformed this library into a Δ*fis* strain. We performed the
687 sort-seq procedure after library growth in LB supplemented only with Atc.
688
689 **Sorting the Fis-IHF Library**. To study the effect of Fis binding in isolation from that of IHF,
690 we cloned the Fis-IHF library into the plasmid pCAW-Sort-Seq-V2-Fis, and transformed the
691 resulting construct into the Δ*ihf* strain. Inducing the expression of Fis with Atc in this strain
692 ensured the expression of Fis in the absence of IHF. To study the effect of IHF binding in
693 isolation from that of Fis-binding, we cloned the same (Fis-IHF) library into the plasmid
694 pCAW-Sort-Seq-V2-IHF, and transformed the resulting plasmid library into the Δ*fis* strain. By
695 inducing the expression of IHF with Atc in this strain, we ensured the expression of IHF in the
696 absence of Fis.
697
698 **Replication of experiments**
699 Data from high-throughput methods to measure gene expression are intrinsically noisy. The
700 reasons include biological cell-to-cell expression variation [107], as well as technical [111–114]
701 variation (e.g. pipetting errors, equipment biases, limit of detection thresholds etc.). To account
702 for such variability, we performed sort-seq in three replicates, i.e., we performed three
703 independent sorting procedures for the same overnight culture.
704
705 **Regulation strengths**
706 Due to gene expression and measurement noise, individual TFBS variants in a sort-seq
707 experiment usually appear in more than a single bin, and their read count (frequency) varies
708 among bins[115–118]. Following established practice[31,117,119], we used a weighted average of these

709 frequencies for each variant to represent the mean expression level driven by the variant. To
710 facilitate interpretation, we converted this expression level into a regulation strength relative to
711 the wild type binding site (regulation strength of one) for that specific TF. Values below one
712 indicate weaker TF binding (higher GFP expression), whereas values above one indicate
713 stronger regulation strength (lower GFP expression).
714
715 **Calculating crosstalk levels:**
716 Crosstalk levels were calculated as the geometric mean of the regulatory strengths of both
717 transcription factors for each sequence. Each TF's regulatory strength was normalized to a
718 maximum value of 1, corresponding to its highest observed regulation. Consequently, the
719 crosstalk levels were bounded between 0 and 1.
720
721 **Validating regulation strengths with plate reader measurements**
722 To further validate our regulation strengths thus inferred, we chose 10 DNA binding sites that
723 cover a wide range of measured regulation strengths. We cloned these sequences into the
724 appropriate vector pCAW-Sort-Seq-V2-TF (TF: Fis or IHF) and transformed them into the
725 appropriate mutant strain to ensure that only Fis or IHF are expressed. We picked individual
726 colonies and grew them overnight (16 hours, 37°C, 220 rpm) in liquid LB supplemented with
727 50 µg/mL of chloramphenicol and anhydrotetracycline. We diluted the cultures to 1:10 (v/v) in
728 cold Dulbecco's PBS (Sigma-Aldrich #D8537) to a final volume of 1 mL. We transferred 200
729 µl of the diluted cultures into individual wells in 96-well plates and measured GFP fluorescence
730 (emission: 485nm/excitation: 510nm, bandpass: 20nm, gain: 50) as well as the optical density
731 at 600nm (OD_{600}) as an indicator of cell density. We then normalized fluorescence by the
732 measured OD_{600} value to account for differences in cell density among cultures and compared
733 the obtained ratios to the previously inferred regulation strengths for the 10 selected variants.

We performed all such measurements in biological and technical triplicates (three colonies per sample and three wells per colony, respectively).

Data availability: The DNA sequencing data generated in this study have been deposited in the NCBI database (BioProject) under the accession code: PRJNA1162486. The flow cytometry and plate reader data generated in this study have been deposited in the Zenodo public repository and are accessible publicly via the following DOI: 10.5281/zenodo.14008581.

Code availability: The computer code generated in this study has been deposited in the Zenodo public repository and is publicly accessible via the following DOI: 10.5281/zenodo.14008581

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Figure legends:

Figure 1. Experimental workflow. a. The plasmid system. We redesigned a previously developed plasmid[31] (**Supplementary Methods 2-3**) that encodes a chloramphenicol resistance gene as a selective marker (CmR, grey, right-oriented arrow) and features a broad-host low-copy replication origin (pBBR1, grey, left-oriented arrow). **b. The regulatory modules.** The plasmid harbors three modules interconnected through regulatory interactions. The first module comprises the constitutively expressed *tetR* gene (purple). The TetR regulator controls the second regulatory module (red box) through the *plTetO1* promoter[32]. This second module encompasses a bicistronic operon, comprising both the TF of interest (orange) and a reporter *mScarlet-I* gene[33] (red). In the third module (green box in b), the TF interacts with its cognate TFBS or a variant from the appropriate library. This interaction results in the transcriptional repression of the fluorescent reporter gene *sfgfp*[34] through steric hindrance, blocking RNA polymerase activity. The addition of anhydrotetracycline (Atc) alleviates repression by TetR, prompting the expression of the TF of interest, which leads to repression of *sfgfp*. To safeguard against the unintended effects of 5'- untranslated regions (UTRs) created by some TFBS library variants on the expression of the *sfgfp* mRNA, we used the RiboJ[35,36] transcriptional insulator (hairpin structure, in grey). Ribosome binding sites (RBSs) are highlighted as grey semi-circles. **c. Basic reporter dynamics.** As the TetR inducer (Atc) levels increase, more TetR regulator is sequestered from its binding site, allowing the expression of the TF and the mScarlet-I reporter (red). Increasing TF concentration causes a decrease in green fluorescence, because the TF causes increasing repression of the sfGFP reporter. **d. Library generation.** Alignment of two strong-affinity ("wild-type") TFBSs for individual TFs, indicating positions (dots) at which the two aligned sites differ from each other. We created a binding site library by varying the nucleotides at all nucleotide positions that differ between the two sites. The library comprises 2^n sequences, where n is the number of positions at which the sites differ (See **Supplementary Methods 4, Supplementary Table S3** and **Supplementary Figure S3** for library details). **e. Transformation of the reporter system harboring the library into two distinct bacterial strains.** One strain expresses TF1 (+TF1) but not TF2 (ΔTF2), and the other TF2 (+TF2) but not TF1(ΔTF1). We then performed fluorescence-activated cell sorting (FACS) independently for each strain, separating cells (green rectangles) into specific bins according to their fluorescence. High-throughput sequencing of the library variants from each bin reveals the mutational differences in each

variant relative to the reference TFBS, indicated as red and blue bars inside grey cylinders (TFBS), corresponding to data from the two TF-producing strains. The strength of regulation (binding) conveyed by each binding site is measured from the site's read count across bins (**Supplementary Methods 7.2**). **f. Mapping an Exaptation landscape.** Each TFBS is characterized by two regulation strengths, one for each TF. The regulation strength of each genotype defines an adaptive landscape for each TF (shown as the central-colored network, with the color gradient representing the regulation strengths for TF1, red, and TF2, blue). These can be interpreted as two entangled adaptive landscapes (red and blue above and below the network), where a single sequence is mapped to two phenotypes.

Figure 2. Characterization of regulation strengths for individual TFs. a-c. Specific plasmid-strain combinations ensure the presence of only one of two TFs during our measurements. Each table schematically represents the intracellular presence of TFs for combinations of a reporter plasmid encoding one of the TFs (rows) transformed into a mutant *E. coli* strain lacking the gene encoding one of the TFs (columns). Pure colors represent the presence of a single TF in the cell (CRP: red; Fis: green and IHF: purple), and color gradients represent the presence of both TFs. We avoided plasmid-strain combinations in which both TFs were present in the cell, because such combinations did not allow us to unambiguously measure the regulation conveyed by one TF's binding to each of the TFBSs in an exaptation landscape. The plasmid-strain combinations we used are highlighted with thick black borders. **d-f. Distribution of regulation strengths for three TFBS libraries.** Each panel shows data from a different TF1-TF2 library (d. CRP-Fis; e. CRP-IHF; f. Fis-IHF). Each circle represents a single TFBS variant in a landscape. The horizontal and vertical axes represent the regulation strengths of each variant in the respective landscape to the TF labelled on the axes. Pure colors represent the regulation strengths of each TF (CRP: Red; Fis: Green: IHF, blue), and intermediate colors represent binding by two TFs. The histograms on the top and right of each plot show the distribution of regulation strengths for each TF.

Figure 3. Exaptation landscapes are smooth with many intermediates subject to transcriptional crosstalk. Panels a-c depict exaptation landscapes as networks whose nodes (circles) represent binding site variants connected through single nucleotide changing mutations (straight lines). The x-axis of each panel shows the genetic distance (in number of nucleotide differences) from each TFBS variant to one of the wild-type sequences (at x = 0). Grey squares indicate WT sequences for each TF, which are peaks in their respective landscapes. **a. The CRP-Fis exaptation landscape.** Both panels represent the CRP-Fis exaptation landscape (N = 256) with nodes ordered by their distance to the CRP wild-type sequence. Colors represent regulation strengths of each TFBS variant to CRP (top panel, red heatmap) and to Fis (bottom panel, green heatmap). **b. The CRP-IHF exaptation landscape.** Both panels represent the CRP-IHF exaptation landscape (N = 128) with nodes ordered by their distance to the CRP wild-type sequence. Colors represent regulation strengths of each TFBS variant to CRP (top panel, red heatmap) and to IHF (bottom panel, purple heatmap). **c. The Fis-IHF exaptation landscape.** Both panels represent the Fis-IHF exaptation landscape (N = 128) with nodes ordered by their distance to the Fis wild-type sequence. Colors represent Fis (top panel, green heatmap) and IHF (bottom panel, purple heatmap) regulation strengths for each sequence. Light and dark colors in panels a-c represent weak and strong binding, respectively. Note that all regulation strengths in panels a-c are normalized by the regulation strength of the wild-type sequence. **d-f. Crosstalk levels for binding sites in each exaptation landscape.** Each panel shows, for all TFBSs in an exaptation landscape, the ratio of binding to TF1 and TF2 (y-axis) as a function of the TFBSs genetic distance from the wild-type sequence (x-axis), which is a strong TFBS. Each circle symbolizes

one genotype. Grey circles connect genotypes that differ in a single nucleotide. Boxplots indicate the distribution of crosstalk levels (see **Methods**) for each mutational distance. Each box covers the range between the first and third quartiles (interquartile range, IQR). The horizontal line within the box represents the median value, and whiskers span 1.5 times the IQR. Heatmap colors reflect the regulation strength ratio for each TFBS. That is, sequences binding predominantly to CRP, Fis or IHF are displayed in red, green, and purple, respectively. Intermediate colors indicate binding at intermediate strength to two TFs. Note that crosstalk levels are normalized by the maximum crosstalk value measured, resulting in a scale that ranges from 0 (no crosstalk) to 1 (maximum crosstalk). We generated networks with the *Gephi*[38] software using a combination of both the *3DForceAtlas* and *Network3DSplitter* algorithms.

Figure 4. Rapid exaptation on the CRP-Fis landscape. All data presented is based on a population of 10^8 individuals. Data for each plot is based on 10^5 simulated adaptive walks, i.e., 10^4 adaptive walks for each of the 10 starting genotypes with the highest regulation strength for the opposite TF. **a-b. Vector field illustrations of adaptive walks in genotype space.** The illustrations show the direction of adaptive walks in genotype space when strong binding to a specific TF (CRP or Fis) is favored. Each panel represents a single TF landscape, with the x and y axes representing coordinates in genotype space. Each vector indicates the direction of an adaptive walk starting from a distinct genotype and ending at the colored circle peak ("attractor"), which signifies strong regulation by the TF. **a. Scenario 1: Adaptive trajectories favoring strong binding to CRP.** In the CRP landscape, adaptive walks favor strong binding to CRP, indicated by the orange circle (CRP peak). **b. Scenario 2: Adaptive trajectories favoring strong binding to Fis.** In the Fis landscape, adaptive walks favor strong binding to Fis, indicated by the green circle (Fis Peak). **c-d. Adaptive walks readily attain peaks.** Each plot represents how regulation strengths (vertical axis) for both TFs (CRP in orange and Fis in green) change along the transversed path length (horizontal axis) from the initial sequence to the attained peak during an adaptive walk. Curves represent averages over 10^5 adaptive walks, and shading indicates one standard deviation of regulation strength. **c. Adaptive walks attain the CRP peak.** The data is based on 10^4 walks starting from each of the 10 strongest binding sites for Fis, favoring increasing CRP binding. **d. Adaptive walks attain the Fis peak.** Like panel (c), but for adaptive walks starting from the 10 strongest binding sites for CRP, favoring increasing Fis binding. **e-f. Evolutionary paths to a peak are not much longer than the minimal genetic distances.** Each composite histogram shows the distribution of path lengths (green) for all pairs of starting (non-peak) and ending (peak) genotypes during 10^5 adaptive walks. Blue histograms represent the distribution of genetic distances for each pair of non-peak and peak genotypes. The green and blue horizontal lines represent average path lengths and mutational distance, which are indistinguishable. We used a t-test of the null hypothesis that the means of these distance distributions are statistically indistinguishable. **e. Distribution of accessible path lengths and genetic distances to the CRP peak.** We observed no significant difference between mean genetic distance and path length (mean ± s.d.: 6.6 ± 0.7 for both genetic distance and path length; Welch Two Sample t-test: t = 31.973, df = 196,700, p-value = 0.7, $N_1 = 10^5$ genetic distances, $N_2 = 10^5$ path lengths). **f. Distribution of accessible path lengths and genetic distances to the Fis peak.** We observed no significant difference between mean genetic distance and path length (mean ± s.d.: 6.9 ± 0.8; Welch Two Sample t-test: t = 35.113, df = 196,700, p-value = 0.82, $N_1 = 10^5$ genetic distances, $N_2 = 10^5$ path lengths).

Figure 5. Transcriptional crosstalk and its consequences for evolutionary dynamics. a. PWM scores for experimentally characterized TFBSs from the *E. coli K-12* genome suggest ample crosstalk between TFs CRP, Fis, and IHF. The heatmap is based on 756 TFBS sequences in the *E. coli K-12* genome, as curated in the RegulonDB[67] database. Each row

corresponds to one TFBS. The dataset includes 370 TFBSs for CRP (orange in left-most column), 267 for Fis (green), and 119 for IHF (purple). Each of the remaining columns quantifies the predicted binding strength (PWM score) for each of the 756 TFBSs with respect to the transcription factors CRP, Fis, and IHF, as indicated below each column. We normalized predicted binding strength by the maximum possible score for each TF, resulting in a scale from 0 to 1, as indicated by the color gradient in the heatmap color legend. We clustered the rows of the heatmap using a combination of Euclidean distance and hierarchical clustering with complete linkage to reveal patterns of similarity in predicted binding strengths among the sequences. **b-d. Association between predicted binding strengths for CRP, Fis and IHF and their genomic *E. coli* TFBS.** We computed for each pair of TFs the statistical association between predicted binding strength (CRP-Fis, CRP-IHF, Fis-IHF). In the scatter plots, red circles represent TFBSs whose average predicted binding strength for both TFs is higher than 0.5, indicating intermediate to high crosstalk. Grey circles represent TFBSs with an average predicted binding strength below this threshold, indicating low crosstalk. The percentage of sequences with intermediate to high crosstalk (red circles) and low crosstalk (grey circles) is shown at the top of the plots in red and gray. The blue line is a linear regression line (blue shading: 95% confidence interval; R: Pearson correlation coefficient.). **b. Association between CRP and IHF.** No significant association between CRP and Fis predicted binding strengths ($R = -0.01$ and t-statistic $p = 0.71$). The straight gray and red line results from some sequences with very similar predicted binding strength values, and does not materially affect this association. **c. Association between CRP and IHF.** $R = -0.56$ and t-statistic p-value $< 2 \times 10^{-6}$. **d. Association between Fis and IHF.** $R = -0.24$ and t-statistic p-value $< 2 \times 10^{-6}$. **e-g. Rapid evolution of crosstalk in the CRP-Fis landscape. e. Schematic of evolution towards TFBSs with high crosstalk.** The vector field illustrates the direction of adaptive walks in genotype space when strong binding towards a CRP and Fis is favored. Each vector indicates the direction of an adaptive walk starting from a distinct genotype and ending at a peak ("attractor", colored circle) strongly binding both TFs. **f. Adaptive walks readily attain TFBSs binding to both CRP and Fis with intermediate strengths.** We performed 10^4 adaptive walks starting from each of the 256 TFBS variants in the CRP-Fis landscape, with selection favoring strong binding for both CRP and IHF. The panels show the regulation strength (vertical axis) for CRP (orange) and Fis (green) plotted against the transversed path length of an evolving sequence to the attained peak (horizontal axis). Curves represent averages over all 256×10^4 adaptive walks and shading indicates one standard deviation of regulation strengths. **g. The distribution of accessible path lengths and genetic distances to peaks.** The green and blue horizontal lines show the average length of traversed paths during 256×10^4 walks between pairs of non-peaks and peaks, and the respective mutational distances between such pairs, respectively. The means of the distributions are statistically indistinguishable (3.31 ± 1, mean ± s.d., for both distance metrics, Welch Two Sample t-test $t = -5.5782$, $df = 2029535$, p-value $= 0.7$, $N_1 = 256 \times 10^4$, $N_2 = 256 \times 10^4$).