

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33

# Reconciling *in vitro* and *in vivo* activities of engineered, LacI-based repressor proteins: Contributions of DNA looping and operator sequence variation

Sudheer Tungtur<sup>#a</sup>, Kristen M. Schwingen, Joshua J. Riepe<sup>#b</sup>, Chamitha J. Weeramange, and Liskin Swint-Kruse\*

Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, KS, 66160

<sup>#a</sup>Current address: School of Medicine, Cardiovascular Division, Lillehei Heart Institute, University of Minnesota, 2231 6th Street SE, Minneapolis, Minnesota 55455

<sup>#b</sup>Current address: Center for Neural Science, New York University, 4 Washington Place, Room 809, New York, NY 10003

\*To whom correspondence should be addressed:

Email: [lswint-kruse@kumc.edu](mailto:lswint-kruse@kumc.edu)

Phone: 913-588-0399

Fax: 913-588-9896

## 34 **Abstract**

35           One way to create new components for synthetic transcription circuits is to re-purpose  
36 naturally occurring transcription factor proteins and their cognate DNA operators. For the  
37 proteins, re-engineering can be accomplished via domain recombination (to create chimeric  
38 regulators) and/or amino acid substitutions. The resulting activities of new protein regulators  
39 are often assessed *in vitro* using a representative operator. However, when functioning *in vivo*,  
40 transcription factors can interact with multiple operators. We compared *in vivo* and *in vitro*  
41 results for two LacI-based transcription repressor proteins, their mutational variants, and four  
42 operator sequences. The two sets of repressor variants differed in their overall *in vivo*  
43 repression, even though their *in vitro* binding affinities for the primary operator spanned the  
44 same range. Here, we show that the offset can be explained by different abilities to  
45 simultaneously bind and “loop” two DNA operators. Further *in vitro* studies of the looping-  
46 competent repressors were carried out to measure binding to a secondary operator sequence.  
47 Surprisingly, binding to this operator was largely insensitive to amino acid changes in the  
48 repressor protein. *In vitro* experiments with additional operators and analyses of published data  
49 indicates that amino acid changes in these repressor proteins leads to complicated changes in  
50 ligand specificity. These results raise new considerations for engineering components of  
51 synthetic transcription circuits and – more broadly – illustrate difficulties encountered when  
52 trying to extrapolate information about specificity determinant positions among protein  
53 homologs.

54

## 55 **Keywords**

56 Lactose repressor protein, purine repressor protein, galactose repressor protein, operator,  
57 specificity determinant, looping

58

## 59 Introduction

60 As proteins are designed for biotechnological applications, one challenge can occur  
61 when *in vivo* outcomes do not match those of *in vitro* characterizations. We have encountered  
62 such an apparent discrepancy when making chimeras from the LacI/GalR transcription factors  
63 to be used in “logic gates” for bacterial computing [1-4].

64 Our design goal was to create repressor proteins that bound the same *lacO*<sup>1</sup> operator  
65 DNA sequence but responded to different small-molecule, allosteric ligands. To that end,  
66 chimeric repressors were created by joining the DNA binding domain of the *Escherichia coli* (*E.*  
67 *coli*) lactose repressor protein (“LacI”, UnitProtKP P03023) to the regulatory domains of  
68 paralogs, such as the *E. coli* purine repressor and galactose repressor proteins (respectively  
69 “PurR”, UnitProtKP P0ACP7; and “GalR, UnitProtKP P03024; Fig 1) [2, 5, 6]. The paralogous  
70 regulatory domains also mediated the dimerization needed to create a high affinity binding site  
71 for one DNA operator [7-9]. *In vivo* assays with these chimeras showed the desired outcomes:  
72 All chimeras repressed the natural *lac* operon and responded to the small molecule recognized  
73 by the paralogous regulatory domain (see Fig 2 in Meinhardt *et al.* [2]).

74  
75 **Figure 1. Ribbon and cartoon structures of LacI/GalR homologs. (A)** The homodimer of the lactose repressor  
76 protein (LacI) (PDB ID 1EFA [7]) is shown with one subunit as a gray ribbon and the other in green. On the “green”  
77 monomer, the linker region is shown in magenta. The protein dimer is bound to DNA, which is depicted as a blue  
78 ladder. Allosteric effector is bound in the regulatory domain and represented as black spheres. The figure was  
79 rendered using UCSF Chimera [10]. **(B)** The LacI protein structure has been rotated and zoomed to show positions  
80 48, 52, 55, 58 and 61 in the linker region. Amino acids nearest the plane of the viewer are shown in magenta ball-  
81 and-stick; those facing towards the rear of the structure (on the partner linker region) are in green wireframe. **(C)** The  
82 domain structure of the wild-type LacI homodimer is represented as a green cartoon; the PurR homodimer is  
83 represented in purple; and the GalR homodimer is represented in teal. These color schemes are used to indicate the  
84 source of the DNA binding domains (small ovals; LacI positions 1-44), linkers (bars; LacI positions 45-61), and  
85 regulatory domains (large ovals; PurR positions 60-340 or GalR positions 60-343) in the chimeric repressors “LLhP”  
86 and “LLhG”. All variants of LLhG in this manuscript contain the E62K mutation (“+K”), indicated by the yellow  
87 asterisk, as well as the “E230K” mutation (described in Materials and Methods).

88  
89 Next, in exploring the outcomes that arose from amino acid changes in the interface  
90 between the DNA-binding and regulatory domains [5, 6, 11, 12], we purified sets of variants for  
91 the LacI:PurR chimera (“LLhP” [6]) and the LacI:GalR chimera (“LLhG+K” [5]) for biophysical  
92 studies [3, 4]. This allowed us to compare *in vivo* repression with *in vitro* DNA binding affinities

93 for the primary operator of the *lac* operon (*lacO*<sup>1</sup>). Reassuringly, both sets of variants showed  
 94 the expected relationship, which for the high protein concentrations in the *in vivo* assays should  
 95 be linear [3, 13]. However, when the two studies were compared to each other, LLhP variants  
 96 had weaker repression than LLhG+K variants, even though their *K*<sub>d</sub> values for binding to the  
 97 natural *lacO*<sup>1</sup> operator spanned the same range (Fig 2). This was unexpected: To our  
 98 knowledge, the published literature about the *lac* operon, PurR, and GalR does *not* indicate that  
 99 direct interactions with heteroproteins are expected to affect LLhG or LLhP repression in this  
 100 setting. Thus, we explored the contributions that might arise from interactions of engineered  
 101 repressors with alternative operators *in vivo* (Table 1).

102  
 103 **Figure 2 Comparison of LLhP and LLhG+K variants binding operator *lacO*<sup>1</sup>.** *In vitro* binding to *lacO*<sup>1</sup> versus  
 104 values from *in vivo* repression assays for variants of LLhP (magenta squares) and LLhG+K (green circles). Lines  
 105 represent the best fit to the data and correlation coefficients are consistent with the linear relationship expected for  
 106 these *in vivo* concentrations [3, 13]. Both X and Y error bars represent the standard deviations of averages  
 107 determined from at least three separate experiments. Repression data were taken from [11]; for comparison among  
 108 multiple chimeras, these published values were reported with a different normalization scale than the separate  
 109 normalizations previously used for LLhP and LLhG+K in [5, 6]; error propagation was also revised. The arrows  
 110 outside the axes indicate that repression was enhanced as DNA binding affinity became tighter. In addition to altered  
 111 affinity from amino acid changes, LLhP had enhanced binding in the presence of 0.4 mM co-repressor hypoxanthine  
 112 [4, 6]; values were determined +/- this effector; “plus” data are indicated with black-outlined squares.

113  
 114  
 115

**Table 1.** Relevant *lac* operator sequences<sup>a</sup>

Name	Sequence
<i>lacO</i> <sup>sym</sup>	t g t t g t g t g g <b>A A T T G T G A G C</b>   G <b>C T C A C A A T T</b> t c a c a c a g g
<i>lacO</i> <sup>1</sup>	t g t t g t g t g g <b>A A T T G T G A G C</b>   G <b>G A T A A C A A T T</b> t c a c a c a g g
<i>lacO</i> <sup>2</sup>	t g t t g t g t g g <b>A A A T G T G A G C</b>   G <b>A G T A A C A A C C</b> t c a c a c a g g
<i>lacO</i> <sup>disC</sup>	t g t t g t g t g g <b>A A T T G T T A T C</b>   C G <b>G A T A A C A A T T</b> t c a c a c g g
<i>lacO</i> <sup>3</sup>	t g t t g t g t g g <b>A A C A G T G A G C</b>   G <b>C A A C G C A A T T</b> t c a c a c a g g

116  
 117 <sup>a</sup>The *lacO*<sup>sym</sup> operator is an engineered, symmetric DNA binding site constructed from the *lacO*<sup>1</sup> proximal half site  
 118 [14]; *lacO*<sup>1</sup>, *lacO*<sup>2</sup> and *lacO*<sup>3</sup> are naturally occurring operators in the *lac* operon [15-18]; *lacO*<sup>disC</sup> is also an engineered  
 119 DNA binding site constructed from the *lacO*<sup>1</sup> distal half site with additional central base pairs [19]. Base pairs shown  
 120 in bold are protected from DNase footprinting by LacI binding [20, 21]; sequences shown in lower case comprise the  
 121 flanking sequences of the 40-mer oligos used in binding assays. The base pairs shown in red differ from the  
 122 analogous positions in *lacO*<sup>1</sup>. The black vertical lines separate the point of symmetry between the two DNA half-sites.

123 Here, we report that the tighter LLhG+K repression is consistent with this repressor  
124 protein looping two DNA operator sites, most likely *lacO*<sup>1</sup> and *lacO*<sup>2</sup>, that were present in the *in*  
125 *vivo* assays. *In vitro* experiments were also carried out to determine whether amino acid  
126 changes in the LLhG+K variants altered  $K_d$  for *lacO*<sup>2</sup> in addition to the previously-measured  
127 changes in *lacO*<sup>1</sup>  $K_d$  values [3]. Surprisingly, binding to *lacO*<sup>2</sup> showed very little sensitivity to any  
128 of the amino acid variants tested in LLhG+K. To further assess the ligand specificity of these  
129 variants, additional experiments showed that binding to the tight-binding *lacO*<sup>sym</sup> operator was  
130 sensitive to the LLhG+K amino acid changes, whereas binding to the *lacO*<sup>disC</sup> operator was  
131 weaker than the limit of the assay. These unexpected changes in specificity raise new  
132 considerations for engineering components of synthetic transcription circuits and – more broadly  
133 – for extrapolating information about specificity determinant positions among protein homologs.

134

## 135 **Materials and Methods**

### 136 **Ruling out “trivial” sources of repression differences**

137 The discrepancy between LLhP and LLhG+K repression shown in Fig 2 could arise if  
138 LLhP variants were expressed at lower levels than the LLhG+K variants. However, *in vivo*  
139 protein concentrations were previously estimated to be >2500 copies per *E. coli* cell for all  
140 LLhP and LLhG+K variants [2, 11]. This is in vast excess over the single *lac* operon per  
141 genome, which makes it unlikely that differences in LLhP and LLhG+K repression are due to  
142 altered protein expression.

143 Another possible source of the discrepancy could be the *in vivo* presence of endogenous  
144 allosteric effectors. However, PurR is only known to have natural co-repressors – hypoxanthine  
145 and guanine – which enhance DNA binding/repression [22, 23]. Likewise, when surveyed with  
146 a variety of small molecules, LLhP repression only responded to the known PurR co-repressors  
147 and no gratuitous inducers have been identified to date [2, 4, 6]. In contrast, wild-type GalR  
148 responds to the natural inducer galactose and the gratuitous inducer fucose, which weaken  
149 DNA binding and repression [24]. Again, LLhG+K showed a similar response profile [2, 3, 5],  
150 and no gratuitous co-repressors have been identified to date. Thus, even if allosteric effectors  
151 were endogenous in the *in vivo* repression assays, their known influences are opposite to the  
152 discrepancy illustrated in Fig 2.

153 We also considered differences in the *in vitro* binding conditions of LLhG+K and LLhP.  
154 Binding affinities for LacI and LLhP variants were assayed in “FBB” buffer (10 mM Tris-HCl, pH  
155 7.4, 150 mM KCl, 5% DMSO, 0.1 mM EDTA, and 0.3 mM DTT), but LLhG+K variants appeared  
156 to aggregate in this buffer over the course of the assay [3]. Relative to FBB, the successful  
157 LLhG+K binding buffer had a slightly lower pH, more reducing equivalents, and lacked DMSO  
158 (see below). However, LLhP DNA binding in the LLhG+K binding buffer produced essentially  
159 identical values to those previously reported [4]. Thus, the *in vitro* buffer differences were  
160 unlikely to be the source of the discrepancy illustrated in Fig 2.

## 161 **Proteins and purification**

162 Plasmids expressing the coding regions of full-length LacI (plasmid numbers #31490  
163 and #90058), LLhP (#90038), and LLhG (#90051) are available from addgene  
164 (<https://www.addgene.org/>). Variants of the LLhG/E62K protein (“LLhG+K”) were purified and  
165 DNA binding was carried out as described in Tungtur *et al.* [3]. This variant was previously  
166 chosen for mutagenesis because it repressed transcription more tightly than the parent “LLhG”  
167 chimera [5]. As before, all LLhG+K variants also carried the “E230K” mutation, which was  
168 required to alleviate bacterial toxicity [5]. Notably, DNA looping occurred in the parent LLhG+K  
169 chimera, despite the presence of the E230K substitution [2], which diminished looping in wild-  
170 type GalR [25]. Additional amino acid changes assessed in this study were located in the linker  
171 region of LLhG+K, as indicated in the figures and tables.

172 A brief description of LLhG+K purification is as follows: Variants were constitutively  
173 expressed from the plasmid pHG165a [5] and grown overnight in BLIM cells [26] in 2xYT media.  
174 Cell pellets were resuspended in cold breaking buffer (12mM HEPES, 200mM KCl, 1mM EDTA,  
175 5% glycerol, 0.3 mM DTT, pH to 8.0) with 1 protease inhibitor tablet (ROCHE Diagnostics,  
176 Indianapolis, IN, USA) and frozen at -20°C. Following (i) cell lysis *via* freeze/thaw with lysozyme  
177 (Fisher Scientific) and DNA degradation *via* DNase (Sigma-Aldrich Chemical Company), (ii)  
178 centrifugation, (iii) 37% ammonium sulfate precipitation and (iv) dialysis, the final purification  
179 step comprised a phosphocellulose (Whatman P-11) ion exchange column. LLhG+K proteins  
180 were eluted from the column using a linear gradient of Buffer A (12mM HEPES, 50mM KCl, 1mM  
181 EDTA, 5% glycerol, 0.3 mM DTT, pH to 8.0) and Buffer B (12mM HEPES, 500mM KCl, 1mM

182 EDTA, 5% glycerol, 0.3 mM DTT, pH to 8.0). Protein elution occurred near conditions of 50%  
183 buffer A/50% buffer B. Aliquots of purified protein were stored at -80°C.

184

## 185 **DNA binding assays**

186 Prior to DNA binding assays, purified LLhG+K variants required exchange into reducing  
187 conditions [3]. Protein variants were dialyzed against in HEPES/DTT buffer (12 mM Hepes, pH  
188 7.53, 150 mM KCl, 0.1 mM EDTA buffer and 3 mM DTT) for 30 minutes in each of two buffer  
189 volumes; a third buffer exchange was into Tris/DTT buffer (10 mM Tris, pH 7.13, 150 mM KCl,  
190 0.1 mM EDTA, and 3 mM DTT). The high concentrations of DTT precluded using  $A_{280}$  to  
191 determine concentrations of the LLhG+K variants. Therefore, protein concentration was  
192 estimated using the Bradford assay (BioRad, Inc., Hercules, CA), with bovine serum albumin  
193 (Fisher Biotech, Fair lawn, NJ, 07410) as a standard. In order to more precisely determine the  
194 concentration of protein competent for binding DNA, the activity of each protein preparation was  
195 determined by stoichiometric assays [27] to be between 70 and 99%. Activities were used to  
196 correct  $K_d$  values determined from binding titrations.

197 DNA binding affinities for LLhG+K and variants were measured by binding protein to  $^{32}\text{P}$ -  
198 labelled *lacO*<sup>2</sup>, *lacO*<sup>sym</sup>, and *lacO*<sup>disC</sup>. For most variants,  $K_d$  values for *lacO*<sup>1</sup> were reported in  
199 [3]; binding data for a variant new to this work is shown in S5 Fig. All operator sequences  
200 (Table 1) comprised the central region of a 40 basepair, double-stranded DNA oligomer [28] and  
201 were synthesized by Integrated DNA Technology (Coralville, IA) and radiolabeled as in Zhan *et*  
202 *al.* [28]. After mixing protein and DNA, a 30-minute equilibration was allowed prior to filtration  
203 through nitrocellulose filter paper using a 96 well dot blot apparatus. Pseudo-equilibrium  
204 measurements were made by quickly separating the free and protein-bound DNA through  
205 nitrocellulose filter paper, which has been well-established for wild type *Lacl* (e.g. [28]) and  
206 LLhP [4]. For affinity assays, the DNA concentration was fixed at least 10-fold below the value  
207 of  $K_d$  [27].

208 DNA binding affinities were determined in both the absence and presence of 10 mM  
209 inducer sugar fucose. Results were analyzed with nonlinear regression using the program  
210 GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) to determine values of  $K_d$ , using:

211

212



213 
$$Y_{obs} = \left( Y_{max} * \frac{[Prot]}{K_d + [Prot]} \right) + c$$

214

215

216 where “ $Y_{obs}$ ” is the observed signal from  $^{32}\text{P}$ -DNA, “ $Y_{max}$ ” is the signal observed at saturation,  
217 “[Prot]” is the concentration of the LLhG+K variants, “ $c$ ” is baseline value of the  $^{32}\text{P}$ -DNA signal,  
218 and “ $K_d$ ” is the equilibrium dissociation constant.

219 Reported values in Table 2 are the average and standard deviation for at least three  
220 separate determinations, using at least two different protein preparations. Note that the values  
221 of standard deviations were larger than the errors of the fit.

222

223 **Table 2.** Operator binding by LLhG+K variants<sup>a</sup>

	<i>lacO</i> <sup>2</sup> $K_d$ (x 10 <sup>-9</sup> M)	<i>lacO</i> <sup>sym</sup> $K_d$ (x 10 <sup>-11</sup> M)
LLhG + K	3.3 ± 1.8	3.7 ± 1.1
I48S	3.8 ± 1.8	6.7 ± 1.0
V52P	7.1 ± 1.1	24 ± 11
Q55V	9.9 ± 5.9	4.6 ± 2.8
Q55I	9.2 ± 1.1	n.d.
G58K	4.1 ± 0.3	n.d.
G58L	3.2 ± 0.6	79 ± 34
S61N	3.0 ± 1.1	3.3 ± 0.7
S61A	5.9 ± 2.0	11 ± 3

224

225 <sup>a</sup> $K_d$  values for LLhG+K and variants binding to *lacO*<sup>2</sup> and *lacO*<sup>sym</sup> operators in the absence of inducer fucose.

226 Reported error values represent one standard deviation of the mean. Binding experiments were completed in

227 Tris/DTT buffer (10 mM Tris pH 7.13, 150 mM KCl, 0.1 mM EDTA and 3 mM DTT). Values were determined using at

228 least three independent determinations comprising at least two independent protein purifications.

229

## 230 Results

231 The stronger repression for LLhG+K variants, as compared to LLhP variants (Fig 2),  
232 could be explained by several phenomena. Thus, we first ruled out the “trivial” explanations of  
233 different protein expression levels and *in vitro* buffer conditions (see Materials and Methods).  
234 Next, we considered two other possible differences: Either the LLhP variants were competed  
235 away from the *lac* operator, or the local concentration of LLhG+K was enhanced by some



236 mechanism. The first possibility could arise if LLhP variants showed tighter non-specific DNA  
237 binding than did LLhG+K variants. Although this remains a formal possibility, it would be very  
238 difficult to test since every base pair in a DNA sequence is the start of a distinct binding site, and  
239 different nonspecific DNA binding sequences can have different binding affinities [29-32].

240 In addition, we had prior experimental evidence [2] for the second option (enhanced  
241 LLhG+K local concentration). Local concentration can be increased when one protein (or  
242 protein complex) simultaneously binds two distal binding sites on DNA, “looping out” the  
243 intervening DNA sequence (Fig 3 A-C) [33]. Looping by LacI/GalR repressors requires  
244 tetramerization (since a homodimer is the unit for binding one DNA operator), and several  
245 homologs exhibit various tetramerization mechanisms.

246  
247 **Figure 3. Looping in the *lac operon*.** (A) When dimeric repressor is bound to the *lacO*<sup>1</sup> DNA operator, transcription  
248 of the downstream *lacZYA* genes are repressed. (B) Dimeric repressor protein is capable of binding other sites in the  
249 *E. coli* genome such as *lacO*<sup>2</sup>, *lacO*<sup>3</sup> and non-specific genomic DNA. (C) Tetrameric LacI can simultaneously bind two  
250 operator sites, leading to DNA looping. The regulatory domains of two wild-type GalR dimers also have the capability  
251 to form protein-protein interactions *via* its regulatory domains, which provides another means to facilitate  
252 tetramerization and DNA looping. (D) Prior experiments indicated that LLhG+K has looping capabilities, similar to its  
253 parent protein GalR [2]. Since DNA looping depends highly on inter-operator spacing (*x*-axis), *in vivo* repression can  
254 be altered by changing this distance. In the experiments shown, repression of the reporter gene was assessed using  
255 four strains of *E. coli*, containing a *lacZ* gene under control of the *lacO*<sup>sym</sup> and *lacO*<sup>2</sup> operators. Values were  
256 normalized to a “no repressor” control, and higher values represent increased repression. Note that LLhG+K  
257 repression was sensitive to operator spacing, whereas LLhP was not.

258  
259 For example, LacI has an additional C-terminal tetramerization domain that mediates  
260 formation of a dimer-of-dimers [34-37] and can simultaneously bind two operators [33]. For  
261 wild-type LacI, looping enhanced *in vivo* repression ~50-fold [20, 33, 38-44]. In *in vitro* studies,  
262 LacI binding to DNA containing two operators had tighter affinity than expected from the sum of  
263 binding two, single operators [15]. In another example, full length GalR exhibited  
264 tetramerization when it participated in a “repressosome” complex with the hetero-protein “HU”.  
265 HU facilitated repressosome formation and looping *via* DNA bending; the repressosome  
266 complex facilitated and was stabilized *via* homomeric contacts between the regulatory domains  
267 of two GalR dimers [25, 45-50]. (Although GalR may directly interact with HU under some  
268 conditions [51], the heteroprotein interaction did not appear to occur in the repressosome and

269 individual GalR dimers can repress transcription [52].) Notably, PurR and LLhP lack  
270 tetramerization domains; furthermore, no tetramerization has been observed to occur among  
271 the PurR or LLhP regulatory domains, even at high concentrations used in small angle X-ray  
272 scattering experiments [4].

273 *In vivo*, repressor-mediated looping can be detected by monitoring transcription from a  
274 promoter that is controlled by two operators. Changing the spacing between the two DNA  
275 binding sites rotates the binding sites around the DNA helix relative to each other. Thus, some  
276 spacings are better for tetramer binding – and have better repression – than others [38, 53-56].  
277 Using a second *in vivo* assay (comprising different cells strains and operators), we previously  
278 tested looping for the parent LLhP and LLhG+K chimeras [2]. Assays were carried out in *E. coli*  
279 strains that contained modified *lac* operons under control of the engineered *lacO<sup>sym</sup>* [14] and  
280 natural *lacO<sup>2</sup>* operators [2, 55]. Consistent with the known tetramerization propensities of GalR  
281 and PurR, LLhG+K exhibited changes consistent with looping whereas LLhP did not (Fig 3D)  
282 [2].

283 Thus, we considered whether both the offset and the slope differences between LLhP  
284 and LLhG+K variants (Fig 2) could be explained by LLhG+K looping in the original *in vivo*  
285 repression assay. These assays were carried out in an *E. coli* strain that contained a nearly  
286 wild-type *lac* operon (only *lacI* was interrupted). This operon comprises multiple DNA operators  
287 [57] – *lacO<sup>1</sup>*, *lacO<sup>2</sup>*, and *lacO<sup>3</sup>* (Fig 3; Table 1). Of these three natural operators, *lacO<sup>1</sup>* showed  
288 the highest affinity for wild-type LacI, *lacO<sup>2</sup>* exhibited 30-100 fold weaker binding (S1 Fig [19, 28,  
289 58]), and *lacO<sup>3</sup>* binding was weaker still [15-18].

290 Following the example of wild-type LacI [20, 33, 38-41, 43, 44], LLhG+K looping two *lac*  
291 operators should lead to enhanced repression relative to non-looping LLhP, and thus the overall  
292 offset seen in Fig 2. The difference in LLhP and LLhG+K slopes (Fig 2) could be explained by  
293 changes in the local concentration of repressor that would coincide with altered  $K_d$  for *lacO<sup>1</sup>* [59]:  
294 When tetramer stochastically dissociates from one of the two operator sites, binding to the other  
295 site would keep the repressor in the local vicinity, impeding competition by nonspecific genomic  
296 DNA. Thus, increasing affinity for *lacO<sup>1</sup>* would increase both the residence time at *lacO<sup>1</sup>* and  
297 the local concentration of repressor at auxiliary operators. This in turn would lead to the  
298 increased slope for LLhG+K relative to non-looping LLhP.

299 Next, we considered which of the two auxiliary operators ( $lacO^2$  or  $lacO^3$ ) was most likely  
300 to contribute to *in vivo* repression. Based on the very weak binding of wild-type LacI to  $lacO^3$   
301 (S1 Fig, [16, 17, 44, 59]), we reasoned that  $lacO^2$  was most likely to be involved in LLhG+K  
302 looping. Thus, equilibrium dissociation constants for this operator were determined using  
303 purified proteins and operator. The nanomolar binding affinities observed (Table 2; S2 Fig) are  
304 sufficiently strong to contribute to repression. However, among the nine LLhG+K variants  
305 assessed,  $lacO^2$  binding showed at most ~4-fold change (Fig 4), which was a much narrower  
306 range than expected from  $lacO^1$  measurements. Indeed, when correlated to binding affinities for  
307  $lacO^1$ , binding affinities for  $lacO^2$  showed a slope that approached zero (Fig 4). Finally, for  
308 several variants,  $lacO^2$  binding showed little effect from the addition of 10 mM fucose inducer  
309 (S2 Fig, closed squares).

310

311 **Figure 4. Comparison of *in vivo* repression and *in vitro* binding for LLhG+K variants binding to various**  
312 **operators.** For LLhG+K variant proteins, the  $K_d$  values for binding to operators  $lacO^2$  (magenta circles),  $lacO^{sym}$   
313 (black squares), and  $lacO^{disC}$  (green triangles) are plotted against  $K_d$  values for binding to operator  $lacO^1$ . The large  
314 green triangle highlights the V52P variant that had tighter  $lacO^{disC}$  binding than the other variants.  $K_d$  values for  
315 LLhG+K binding operator  $lacO^1$  are from [3].  $K_d$  values for  $lacO^{sym}$  and  $lacO^2$  are summarized in Table 2. For  
316  $lacO^{disC}$ , most  $K_d$  values were out of range for the binding assay and a lower limit is shown. The lines are to aid visual  
317 inspection of the data. Error bars on both the X and Y parameters represent one standard deviation of the average  
318 values.

319

320 These findings were unexpected and led us to wonder how amino acid changes among  
321 the LLhG+K variants altered binding to other operators, such as the engineered operators  
322  $lacO^{sym}$  and  $lacO^{disC}$  (Table 1; S3 Fig and S4 Fig). Binding to these operators was previously  
323 characterized for both LacI and LLhP variants (Fig 5). Most proteins bound  $lacO^{sym}$  more tightly:  
324 Variants of LacI bound  $lacO^{sym}$  up to 10-fold more tightly than  $lacO^1$  [28, 58], as did five LLhP  
325 variants [4]; however, two LLhP variants exhibited very poor binding ( $K_d > 10^{-7}$  M) [4]. For  
326  $lacO^{disC}$ , most LacI variants bound ~100-fold more weakly than  $lacO^1$  [19, 58], whereas LLhP  
327 variants bound  $lacO^{disC}$  5-10-fold more weakly than  $lacO^1$  [4].

328

329 **Figure 5. Altered fold-change in operator binding indicates altered DNA specificity.** Fold-change for binding to  
330 the indicated operators was calculated relative to the  $K_d$  for binding  $lacO^1$ . To aid recognition of specificity changes,  
331 the ranges of the left and right y axes were chosen so that fold-change for  $lacO^{sym}$  (left y axis) and  $lacO^2$  (LLhG+K,  
332 left y axis) and  $lacO^{disC}$  (LLhP and LacI, right y axis) were visually similar for the parent proteins. For LLhP variants at

333 position 61, *lacO<sup>sym</sup>* binding was also diminished and is also plotted on the right y axis. Error bars were propagated  
334 from the standard deviations of average  $K_d$  values reported in this manuscript and in previous publications [3, 4, 28,  
335 58]. The dotted line is to aid visual comparison of the parent proteins with their amino acid variants. For each  
336 variant, (i) if fold-change for one operator deviates from the dotted line, or (ii) if fold-change of the two bars deviate  
337 from each other, then the DNA specificity of the variant has changed relative to the parent repressor protein.  
338

339 Analogous to results for LacI and LLhP, LLhG+K variants binding to *lacO<sup>sym</sup>* was  
340 enhanced and responded to inducer (Fig 4 black squares; S3 Fig; Table 2). The increase in  
341 *lacO<sup>sym</sup>* binding over *lacO<sup>1</sup>* binding was not perfectly uniform (*i.e.* scatter observed for Fig 4  
342 black squares). Nevertheless, mutational outcomes for *lacO<sup>sym</sup>* and *lacO<sup>1</sup>* were much better  
343 correlated (slope approaching 1; Fig 4, black dashed line) than those for *lacO<sup>2</sup>* and *lacO<sup>1</sup>* (slope  
344 near zero; Fig 4, magenta dashed line). For *lacO<sup>discC</sup>*, binding was above the limit of the filter  
345 binding assay for most LLhG+K variants (Fig 4; S4 Fig), which was a much larger fold-change  
346 than previously observed for LLhP variants. Nevertheless, LLhG+K V52P had measurable  
347 binding to *lacO<sup>discC</sup>* (Fig 4; S4 Fig). Although it seems surprising that a proline in the middle of  
348 the helix (Fig 1B) allowed DNA binding, a similar outcome was observed for V52P in wild-type  
349 LacI [28].  
350

## 351 Discussion

352 *In vivo* activity is usually the sum of many protein activities. In our attempts to dissect  
353 the parameters relevant to *in vivo* repression of the Lac-based transcription repressors, we  
354 unexpectedly discovered that – while amino acid changes in LLhG+K did alter *lacO<sup>1</sup>* and *lacO<sup>sym</sup>*  
355 binding – they had very little impact on *lacO<sup>2</sup>* binding (Fig 4). This phenomenon was not simply  
356 a property of weaker binding for LLhG+K and *lacO<sup>2</sup>*: LLhP variant binding to *lacO<sup>1</sup>* and *lacO<sup>discC</sup>*  
357 spanned a similar magnitude yet showed the expected sensitivity to amino acid variation [4].

358 These results raise the question as to how these outcome is expected to generalize to  
359 other LLhG+K variants or to other LacI/GalR homologs. The amino acid changes in the current  
360 study were located throughout the LLhG+K linker structure (Fig 1B); thus, we expect that *lacO<sup>2</sup>*  
361 binding may generally lack sensitivity to changes in this region of this protein. However,  
362 whether *lacO<sup>2</sup>* mutational insensitivity is unique to LLhG+K or a general property of any LacI-  
363 based repressor remains to be seen. Such studies have not been carried out even for variants  
364 of full-length LacI, and the three homologs and their variants studied to date have enough  
365 differences (Fig 5 and discussed further below) to preclude extrapolating binding behaviors from

366 one protein.

367 Another consideration raised by the current results is the comparison of *lacO*<sup>2</sup> binding to  
368 nonspecific binding. LLhG+K binding to *lacO*<sup>2</sup>, with its similar binding affinities of variants/lack  
369 of induction, is reminiscent of LacI binding to non-specific (genomic) DNA [30]. However,  
370 LLhG+K binding affinities were up to five orders of magnitude tighter than expected for non-  
371 specific binding, which is estimated to be  $3 \times 10^{-4}$  M for wild-type LacI [29]. Furthermore, non-  
372 induction is not a general property of *lacO*<sup>2</sup>, since wild-type LacI binding to *lacO*<sup>2</sup> was  
373 diminished in the presence of IPTG (S1 Fig). More experiments would be required to assess  
374 non-specific binding by LLhG+K. Likewise, although LacI binding to *lacO*<sup>3</sup> was much weaker  
375 than the detection limits of the assay used in the current study (S1 Fig) and thus not pursued,  
376 some LLhG+K variants might have unexpected interactions with *lacO*<sup>3</sup>.

377 These results raise several points that should be kept in mind when constructing  
378 synthetic transcription circuits. First, one should be aware whether or not alternate operators  
379 are present. If the LacI/LacZ combination is used as the reporter protein for circuit  
380 development, *lacO*<sup>2</sup> will naturally present at the start of the *lacZ* gene [16]. (Since remnants of  
381 the *lacZ* gene might also contain the *lacO*<sup>2</sup> operator sequence, discrepancies could arise even if  
382 another reporter gene is used.) Second, in fine-tuning circuits for desired output, one could  
383 mutate the operator sequence to alter baseline or induced expression levels. If, for example, a  
384 multi-input circuit was built using LacI-based chimeras (e.g. [1]) and the operator sequence was  
385 changed to reduce baseline expression, one should not assume that the repressor-operator  
386 interaction will be equally altered for all chimeras. Third, we expect this phenomenon could be  
387 observed for broad range of transcription factors that bind to alternative engineered or natural  
388 operator sequences.

389 More broadly, these results lead us to look at the criteria for quantitatively assessing  
390 ligand specificity changes. We previously used the rank order of ligand affinities to assess  
391 whether changes in the region altered ligand specificity [4, 60]. The current work shows that  
392 this definition was too narrow. In his seminal textbook, Creighton stated “Specific binding by a  
393 protein of one ligand, and not another, depends on their relative affinities, their concentrations,  
394 and whether they bind at the same site” [61]. By this definition, a specificity change would also  
395 be indicated by differences in the fold-change among ligands, even if the rank order stayed the  
396 same. Interestingly, fold-change among variant operators was similar for most LLhP variants

397 studied, in contrast to the fold-changes differences observed among the LLhG+K variants and  
398 variants at Lacl position 52 (Fig 5) [3, 4, 28]. Thus, this comparison provides another example  
399 for which the functional attributes of one protein cannot be extrapolated to other family  
400 members.

401 This extrapolation limitation is especially relevant when considering algorithms that  
402 predict ligand specificity from sequence alignments. Indeed, the linker positions mutated in this  
403 study were predicted to be specificity determinants (that is, locations that can be substituted to  
404 alter specificity) for the naturally occurring Lacl/GalR homologs (discussed in [62]). We  
405 previously concluded from the LLhP studies that changes at these linker positions affected  
406 overall binding affinity more often than specificity. However, in LLhG+K, variants at linker  
407 positions show fold-change differences indicative of specificity changes (Fig 5). Perhaps our  
408 LLhP studies were too limited in scope to detect specificity changes. Alternatively, one unified  
409 set of “specificity determinants” may not be appropriate for defining ligand specificity across the  
410 whole family. This conclusion is consistent with previous analyses of individual Lacl/GalR  
411 subfamilies, which predicted that the locations of positions important to each subfamily fall in  
412 different places on the common Lacl/GalR structure [63].

413 The complexity of the observed specificity changes may be analogous to the non-  
414 additive outcomes that often arise when multiple amino acids are substituted in one protein  
415 (epistasis). In the Lacl-based repressors, we noted considerable epistasis arose from  
416 combinatorial changes in the linker region [11, 12]. Ligand variation could be thought of as one  
417 more mechanism for changing the chemical environment that, in turn, alters the outcome of  
418 chemical changes that accompany amino acid substitution.

419

## 420 **Acknowledgements**

421 We thank Ms. Edina Kosa for assistance with DNA binding assays and Dr. Sarah Bondos  
422 (Texas A&M Health Science Center) for discussions about DNA binding and comments on the  
423 manuscript. We thank Drs. Ernesto Fuentes (University of Iowa), Brian Baker (Notre Dame  
424 University), and Marina Ramirez-Alvaredo (Mayo College of Medicine) for helpful discussions  
425 about the definition of “ligand specificity”.  
426

427



## 428 **References**

- 429 1. Shis DL, Hussain F, Meinhardt S, Swint-Kruse L, Bennett MR. Modular, Multi-Input  
430 Transcriptional Logic Gating with Orthogonal LacI/GalR Family Chimeras. *ACS Synthetic*  
431 *Biology*. 2014;3(9):645–51.
- 432 2. Meinhardt S, Manley MW, Becker NA, Hessman JA, Maher LJ, Swint-Kruse L. Novel  
433 insights from hybrid LacI/GalR proteins: family-wide functional attributes and biologically  
434 significant variation in transcription repression. *Nucleic Acids Research*. 2012;40(21):11139-54.
- 435 3. Tungtur S, Skinner H, Zhan H, Swint-Kruse L, Beckett D. In vivo tests of thermodynamic  
436 models of transcription repressor function. *Biophysical Chemistry*. 2011;159:142-51.
- 437 4. Zhan H, Taraban M, Trewhella J, Swint-Kruse L. Subdividing repressor function: DNA  
438 binding affinity, selectivity, and allostery can be altered by amino acid substitution of  
439 nonconserved residues in a LacI/GalR homologue. *Biochemistry*. 2008;47(31):8058-69.
- 440 5. Meinhardt S, Swint-Kruse L. Experimental identification of specificity determinants in the  
441 domain linker of a LacI/GalR protein: bioinformatics-based predictions generate true positives  
442 and false negatives. *Proteins*. 2008;73(4):941-57.
- 443 6. Tungtur S, Egan SM, Swint-Kruse L. Functional consequences of exchanging domains  
444 between LacI and PurR are mediated by the intervening linker sequence. *Proteins*.  
445 2007;68(1):375-88.
- 446 7. Bell CE, Lewis M. A closer view of the conformation of the Lac repressor bound to  
447 operator. *Nat Struct Biol*. 2000;7(3):209-14.
- 448 8. Chen J, Matthews KS. Subunit dissociation affects DNA binding in a dimeric lac  
449 repressor produced by C-terminal deletion. *Biochemistry*. 1994;33(29):8728-35.
- 450 9. Chakerian AE, Matthews KS. Characterization of mutations in oligomerization domain of  
451 Lac repressor protein. *J Biol Chem*. 1991;266(33):22206-14.
- 452 10. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al.  
453 UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of*  
454 *computational chemistry*. 2004;25(13):1605-12.
- 455 11. Meinhardt S, Manley MW, Jr., Parente DJ, Swint-Kruse L. Rheostats and toggle  
456 switches for modulating protein function. *PloS one*. 2013;8(12):e83502.



- 457 12. Tungtur S, Meinhardt S, Swint-Kruse L. Comparing the functional roles of nonconserved  
458 sequence positions in homologous transcription repressors: Implications for sequence/function  
459 analyses. *Journal of Molecular Biology*. 2010;395(4):785-802.
- 460 13. Bain DL, Connaghan KD, Maluf NK, Yang Q, Miura MT, De Angelis RW, et al. Steroid  
461 receptor–DNA interactions: toward a quantitative connection between energetics and  
462 transcriptional regulation. *Nucleic Acids Research*. 2014;42(2):691-700.
- 463 14. Sadler JR, Sasmor H, Betz JL. A perfectly symmetric lac operator binds the lac  
464 repressor very tightly. *Proc Natl Acad Sci U S A*. 1983;80(22):6785-9.
- 465 15. Hsieh WT, Whitson PA, Matthews KS, Wells RD. Influence of sequence and distance  
466 between two operators on interaction with the lac repressor. *J Biol Chem*. 1987;262(30):14583-  
467 91.
- 468 16. Pfahl M, Gulde V, Bourgeois S. "Second" and "third operator" of the lac operon: an  
469 investigation of their role in the regulatory mechanism. *J Mol Biol*. 1979;127(3):339-44.
- 470 17. Winter RB, Von Hippel PH. Diffusion-driven mechanisms of protein translocation on  
471 nucleic acids. 2. The *Escherichia coli* lac repressor-operator interaction: equilibrium  
472 measurements. *Biochemistry*. 1981;20(24):6948-60.
- 473 18. Reznikoff WS, Winter RB, Hurley CK. The location of the repressor binding sites in the  
474 lac operon. *Proc Natl Acad Sci U S A*. 1974;71(6):2314-8.
- 475 19. Falcon CM, Matthews KS. Engineered disulfide linking the hinge regions within lactose  
476 repressor dimer increases operator affinity, decreases sequence selectivity, and alters allostery.  
477 *Biochemistry*. 2001;40(51):15650-9.
- 478 20. Brenowitz M, Mandal N, Pickar A, Jamison E, Adhya S. DNA-binding properties of a lac  
479 repressor mutant incapable of forming tetramers. *J Biol Chem*. 1991;266(2):1281-8.
- 480 21. Pfahl M, Hendricks M. Interaction of tight binding repressors with lac operators. An  
481 analysis by DNA-footprinting. *J Mol Biol*. 1984;172(4):405-16.
- 482 22. Choi KY, Zalkin H. Structural characterization and corepressor binding of the  
483 *Escherichia coli* purine repressor. *J Bacteriol*. 1992;174(19):6207-14.
- 484 23. Meng LM, Nygaard P. Identification of hypoxanthine and guanine as the co-repressors  
485 for the purine regulon genes of *Escherichia coli*. *Mol Microbiol*. 1990;4(12):2187-92.
- 486 24. Majumdar A, Rudikoff S, Adhya S. Purification and properties of Gal repressor:pL-galR  
487 fusion in pKC31 plasmid vector. *J Biol Chem*. 1987;262(5):2326-31.

- 488 25. Geanacopoulos M, Adhya S. Genetic analysis of GalR tetramerization in DNA looping  
489 during repressosome assembly. *J Biol Chem.* 2002;277(36):33148-52.
- 490 26. Wycuff DR, Matthews KS. Generation of an Ara-C-*araBAD* promoter-regulated T7  
491 expression system. *Analytical Biochemistry.* 2000;277:67-73.
- 492 27. Swint-Kruse L, Matthews KS. Thermodynamics, protein modification, and molecular  
493 dynamics in characterizing lactose repressor protein: strategies for complex analyses of protein  
494 structure-function. *Methods Enzymol.* 2004;379:188-209.
- 495 28. Zhan H, Swint-Kruse L, Matthews KS. Extrinsic interactions dominate helical propensity  
496 in coupled binding and folding of the lactose repressor protein hinge helix. *Biochemistry.*  
497 2006;45(18):5896-906.
- 498 29. Lin S, Riggs AD. The general affinity of lac repressor for *E. coli* DNA: implications for  
499 gene regulation in prokaryotes and eukaryotes. *Cell.* 1975;4(2):107-11.
- 500 30. Lin SY, Riggs AD. Lac repressor binding to non-operator DNA: detailed studies and a  
501 comparison of equilibrium and rate competition methods. *J Mol Biol.* 1972;72(3):671-90.
- 502 31. Kao-Huang Y, Revzin A, Butler AP, O'Conner P, Noble DW, von Hippel PH. Nonspecific  
503 DNA binding of genome-regulating proteins as a biological control mechanism: measurement of  
504 DNA-bound *Escherichia coli* lac repressor in vivo. *Proc Natl Acad Sci U S A.* 1977;74(10):4228-  
505 32.
- 506 32. von Hippel PH, Revzin A, Gross CA, Wang AC. Non-specific DNA Binding of Genome  
507 Regulating Proteins as a Biological Control Mechanism: 1. The lac Operon: Equilibrium Aspects.  
508 *Proceedings of the National Academy of Sciences.* 1974;71, No. 12:4808-12.
- 509 33. Matthews KS. DNA Looping. *Microbiological Reviews.* 1992;56(1):123-36.
- 510 34. Alberti S, Oehler S, von Wilcken-Bergmann B, Müller-Hill B. Genetic analysis of the  
511 leucine heptad repeats of Lac repressor: evidence for a 4-helical bundle. *The EMBO journal.*  
512 1993;12(8):3227-36.
- 513 35. Chen J, Matthews KS. Deletion of lactose repressor carboxyl-terminal domain affects  
514 tetramer formation. *J Biol Chem.* 1992;267(20):13843-50.
- 515 36. Chakerian AE, Tesmer VM, Manly SP, Brackett JK, Lynch MJ, Hoh JT, et al. Evidence  
516 for leucine zipper motif in lactose repressor protein. *J Biol Chem.* 1991;266(3):1371-4.

- 517 37. Lewis M, Chang G, Horton NC, Kercher MA, Pace HC, Schumacher MA, et al. Crystal  
518 structure of the lactose operon repressor and its complexes with DNA and inducer. *Science*.  
519 1996;271(5253):1247-54.
- 520 38. Krämer H, Niemöller M, Amouyal M, Revet B, von Wilcken-Bergmann B, Müller-Hill B.  
521 lac repressor forms loops with linear DNA carrying two suitably spaced lac operators. *The*  
522 *EMBO journal*. 1987;6(5):1481-91.
- 523 39. Eismann E, von Wilcken-Bergmann B, Müller-Hill B. Specific destruction of the second  
524 lac operator decreases repression of the lac operon in *Escherichia coli* fivefold. *J Mol Biol*.  
525 1987;195(4):949-52.
- 526 40. Eismann ER, Müller-Hill B. lac repressor forms stable loops in vitro with supercoiled wild-  
527 type lac DNA containing all three natural lac operators. *J Mol Biol*. 1990;213(4):763-75.
- 528 41. Müller J, Oehler S, Müller-Hill B. Repression of lac promoter as a Function of Distance,  
529 Phase, and Quality of an Auxiliary lac Operator. *J Mol Biol*. 1996;257:21-9.
- 530 42. Amouyal M, von Wilcken-Bergmann B. Repression of the *E. coli* lactose operon by  
531 cooperation between two individually unproductive "half-operator" sites. *C R Acad Sci III*.  
532 1992;315(11):403-7.
- 533 43. Oehler S, Eismann ER, Kramer H, Müller-Hill B. The three operators of the lac operon  
534 cooperate in repression. *The EMBO journal*. 1990;9(4):973-9.
- 535 44. Mossing MC, Record MT, Jr. Upstream operators enhance repression of the lac  
536 promoter. *Science*. 1986;233(4766):889-92.
- 537 45. Geanakopoulos M, Vasmatzis G, Zhurkin VB, Adhya S. Gal repressosome contains an  
538 antiparallel DNA loop. *Nat Struct Biol*. 2001;8(5):432-6.
- 539 46. Geanakopoulos M, Vasmatzis G, Lewis DE, Roy S, Lee B, Adhya S. GalR mutants  
540 defective in repressosome formation. *Genes Dev*. 1999;13(10):1251-62.
- 541 47. Lyubchenko YL, Shlyakhtenko LS, Aki T, Adhya S. Atomic force microscopic  
542 demonstration of DNA looping by GalR and HU. *Nucleic Acids Res*. 1997;25(4):873-6.
- 543 48. Aki T, Adhya S. Repressor induced site-specific binding of HU for transcriptional  
544 regulation. *The EMBO journal*. 1997;16(12):3666-74.
- 545 49. Choy HE, Adhya S. Control of gal transcription through DNA looping: inhibition of the  
546 initial transcribing complex. *Proc Natl Acad Sci U S A*. 1992;89(23):11264-8.

- 547 50. Haber R, Adhya S. Interaction of spatially separated protein-DNA complexes for control  
548 of gene expression: operator conversions. *Proc Natl Acad Sci U S A*. 1988;85(24):9683-7.
- 549 51. Kar S, Adhya S. Recruitment of HU by piggyback: a special role of GalR in  
550 repressosome assembly. *Genes Dev*. 2001;15(17):2273-81.
- 551 52. Semsey S, Virnik K, Adhya S. Three-stage regulation of the amphibolic gal operon: from  
552 repressosome to GalR-free DNA. *J Mol Biol*. 2006;358(2):355-63.
- 553 53. Becker NA, Peters JP, Lionberger TA, Maher LJ. Mechanism of promoter repression by  
554 Lac repressor–DNA loops. *Nucleic Acids Research*. 2013;41(1):156-66.
- 555 54. Peters JP, Becker NA, Rueter EM, Bajzer Z, Kahn JD, Maher LJ, 3rd. Quantitative  
556 methods for measuring DNA flexibility in vitro and in vivo. *Methods Enzymol*. 2011;488:287-335.
- 557 55. Becker NA, Kahn JD, Maher LJ, 3rd. Bacterial repression loops require enhanced DNA  
558 flexibility. *J Mol Biol*. 2005;349(4):716-30.
- 559 56. Krämer H, Amouyal M, Nordheim A, Müller-Hill B. DNA supercoiling changes the  
560 spacing requirement of two lac operators for DNA loop formation with lac repressor. *The EMBO*  
561 *journal*. 1988;7(2):547-56.
- 562 57. Bachmann BJ. Pedigrees of some mutant strains of *Escherichia coli* K-12.  
563 *Bacteriological Reviews*. 1972;36(4):525-57.
- 564 58. Falcon CM, Matthews KS. Operator DNA sequence variation enhances high affinity  
565 binding by hinge helix mutants of lactose repressor protein. *Biochemistry*. 2000;39(36):11074-  
566 83.
- 567 59. Müller-Hill B. The function of auxiliary operators. *Mol Microbiol*. 1998;29(1):13-8.
- 568 60. Swint-Kruse L. Using Evolution to Guide Protein Engineering: The Devil IS in the Details.  
569 *Biophysical journal*. 2016;111(1):10-8.
- 570 61. Creighton T. *Proteins: Structures and Molecular Properties*. 2nd edition ed. New York:  
571 W. H. Freeman and Company; 1993.
- 572 62. Tungtur S, Parente DJ, Swint-Kruse L. Functionally important positions can comprise the  
573 majority of a protein's architecture. *Proteins: Structure, Function, and Bioinformatics*.  
574 2011;79(5):1589-608.
- 575 63. Parente DJ, Swint-Kruse L. Multiple Co-Evolutionary Networks Are Supported by the  
576 Common Tertiary Scaffold of the LacI/GalR Proteins. *PloS one*. 2013;8(12):e84398.

577

## 578 **Supporting Information**

579 **S1 Fig.** Representative curves for LacI binding to *lacO*<sup>2</sup> and *lacO*<sup>3</sup> operators.

580 **S2 Fig.** Representative curves for LLhG+K variants binding to operator *lacO*<sup>2</sup>.

581 **S3 Fig.** Representative curves for LLhG+K variants binding to operator *lacO*<sup>sym</sup>.

582 **S4 Fig.** Representative curves for LLhG+K variants binding to operator *lacO*<sup>disC</sup>.

583 **S5 Fig.** Representative curve for LLhG+K S61A binding to operator *lacO*<sup>1</sup>.



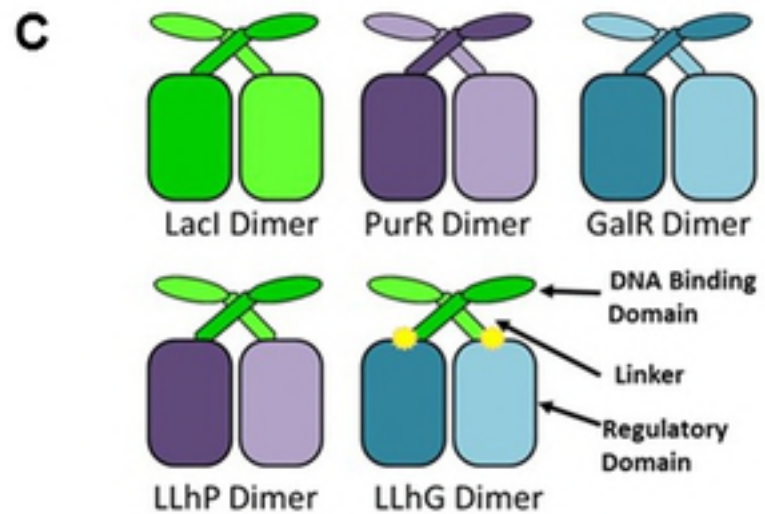
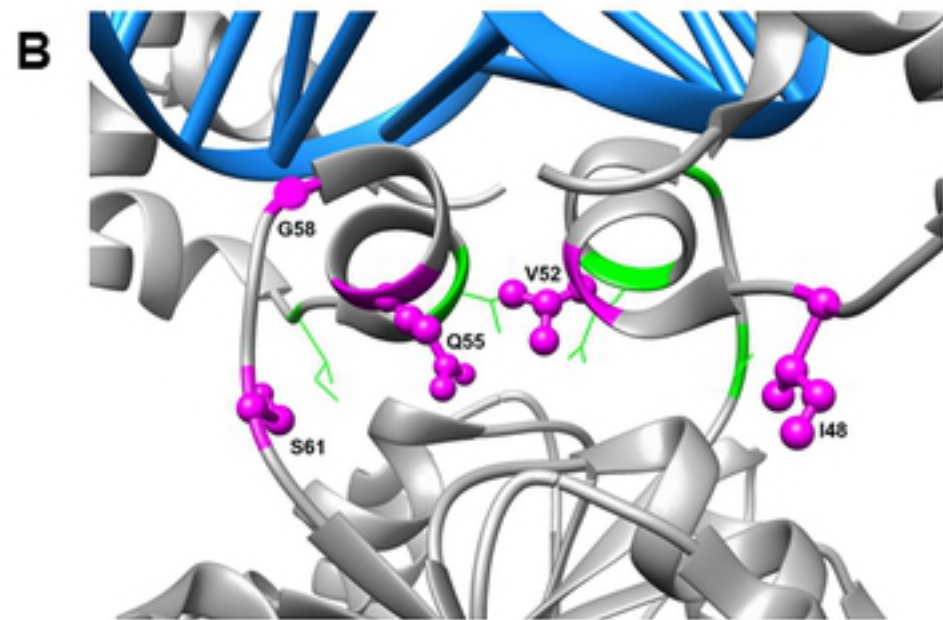
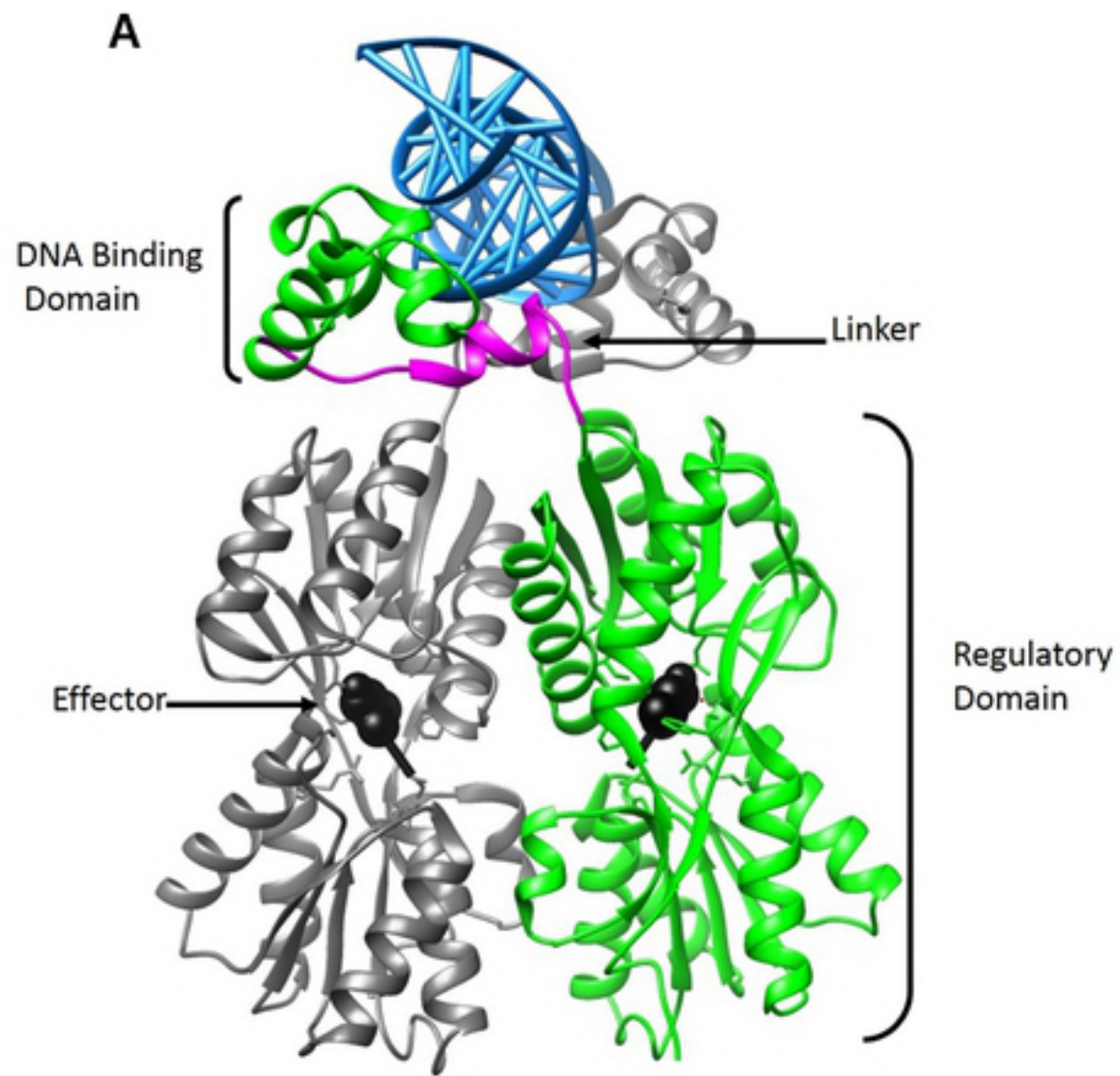


Figure 1

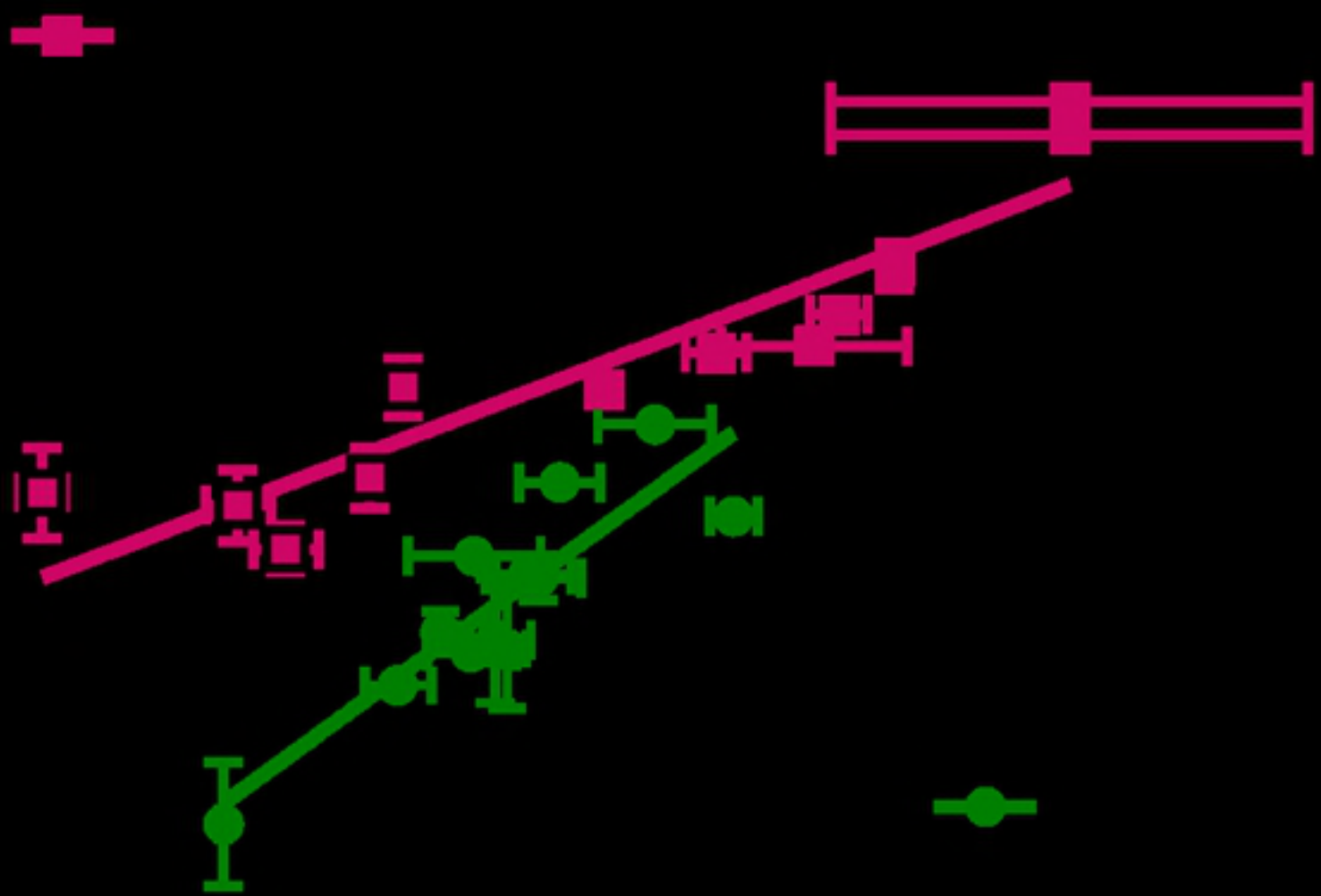


Figure 2



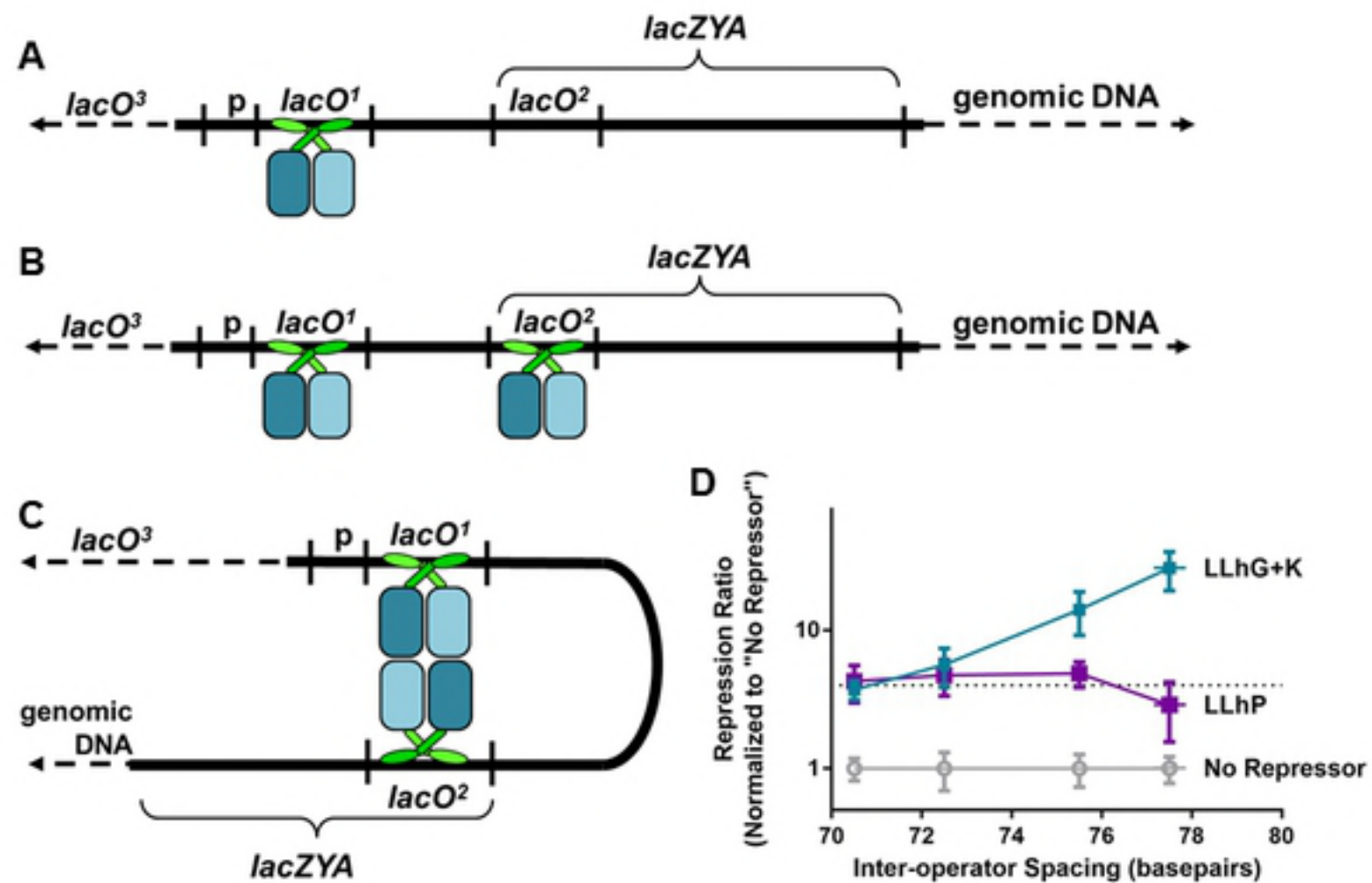


Figure 3

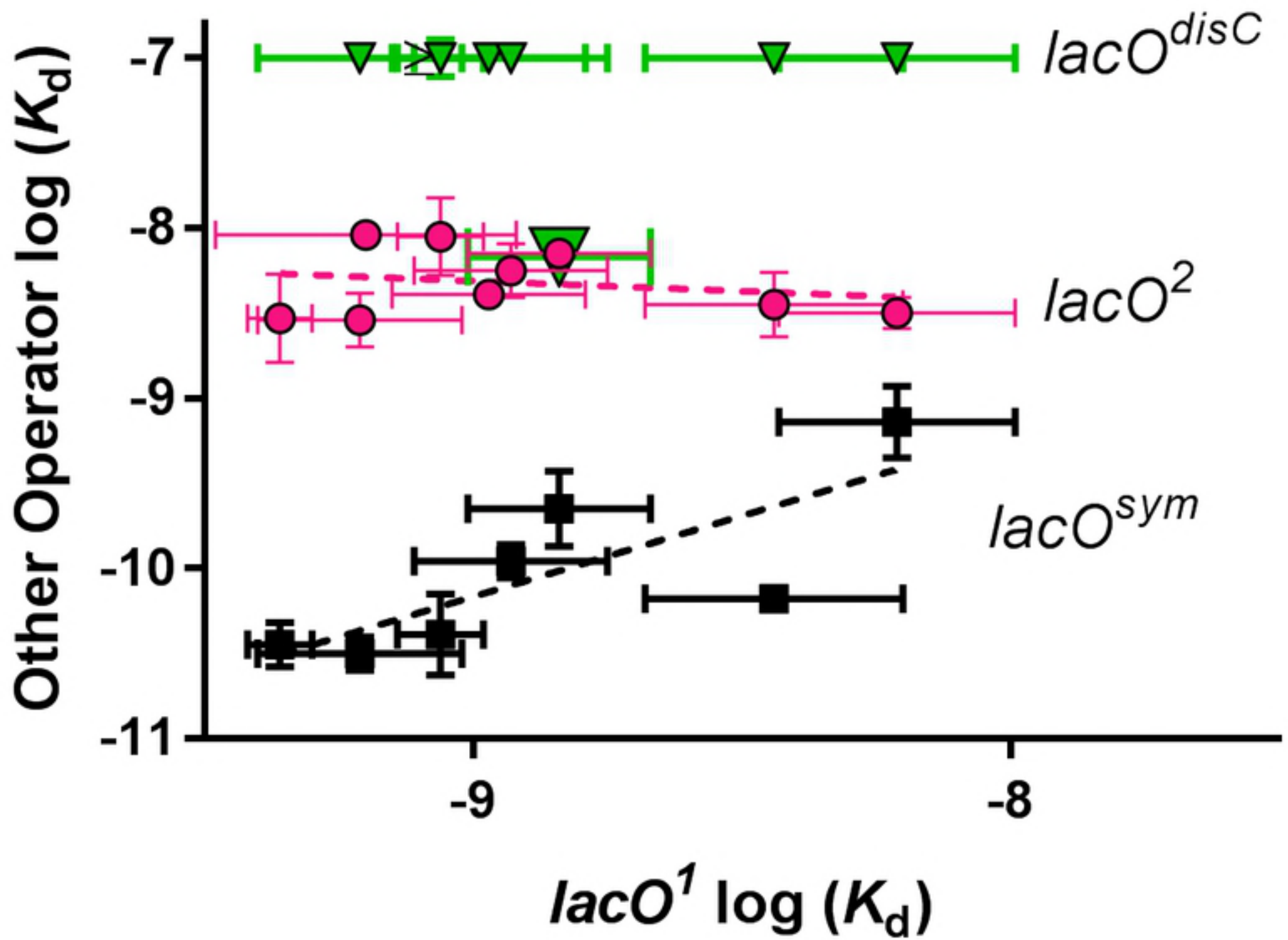


Figure 4

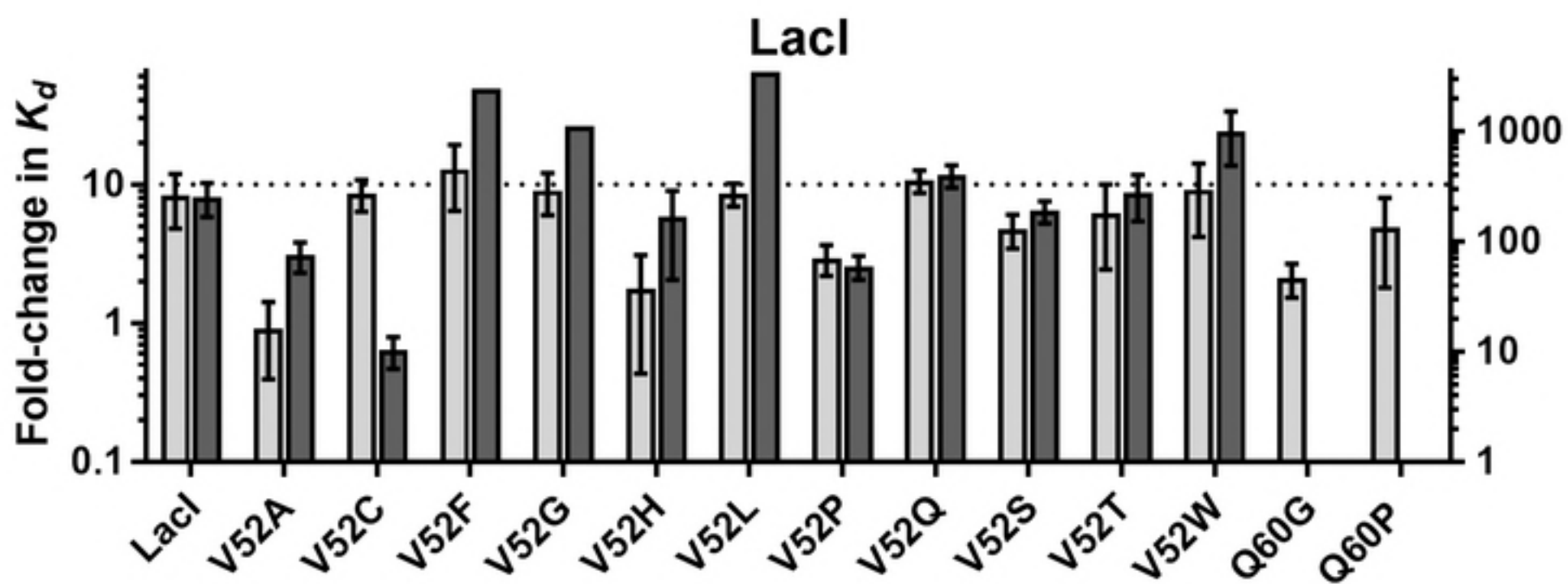
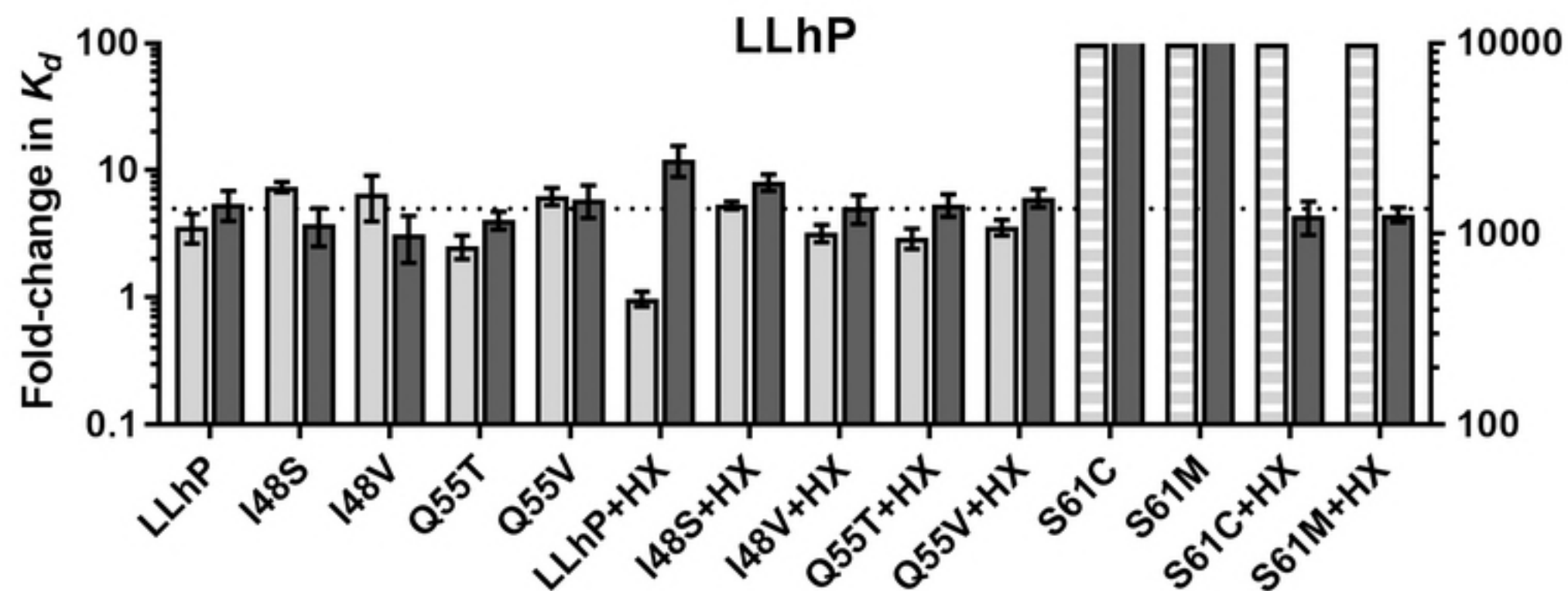
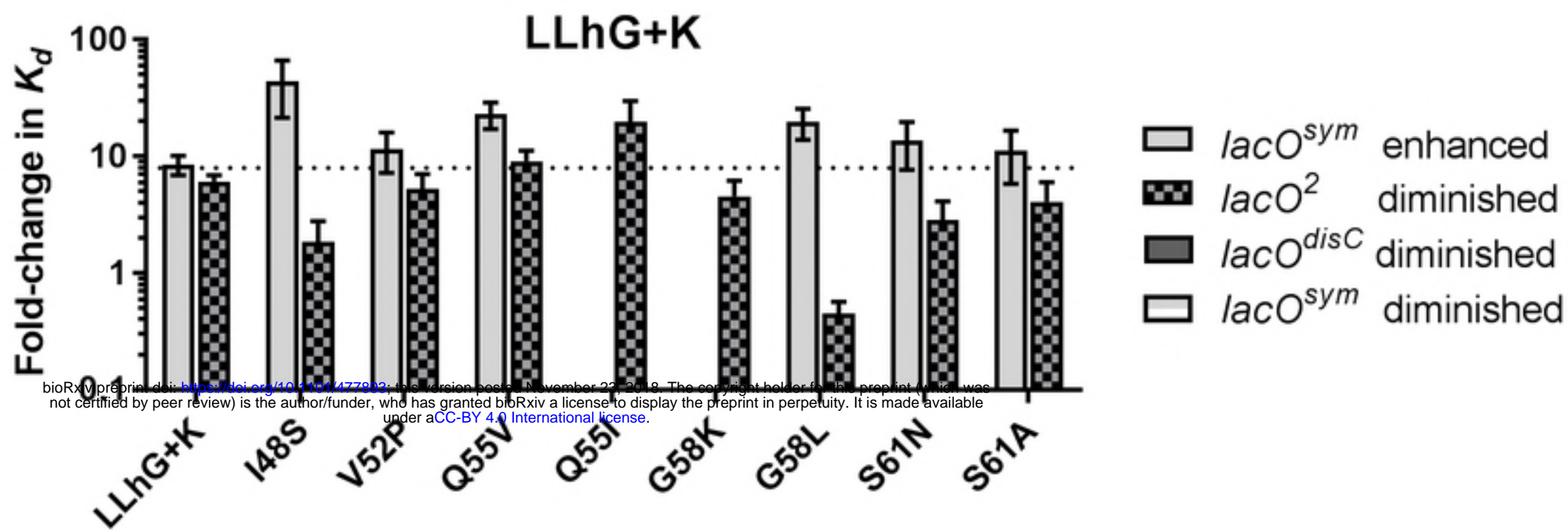


Figure 5