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# Engineering allosteric communication

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Protein allostery is a vitally important protein function that has proven to be a vexing problem to understand at the molecular level. Allosteric communication is a hallmark of many protein functions. However, despite more than four decades of study the details regarding allosteric communication in protein systems are still being developed. Engineering of LacI and related homologues to confer alternate allosteric communication has shed light on the pre-requisites for the *de novo* design of allosteric communication. While the *de novo* design of an allosteric pathway and complementary functional surfaces has not been realized, this review highlights recent advances that set the stage for true predictive design for a given protein topology.

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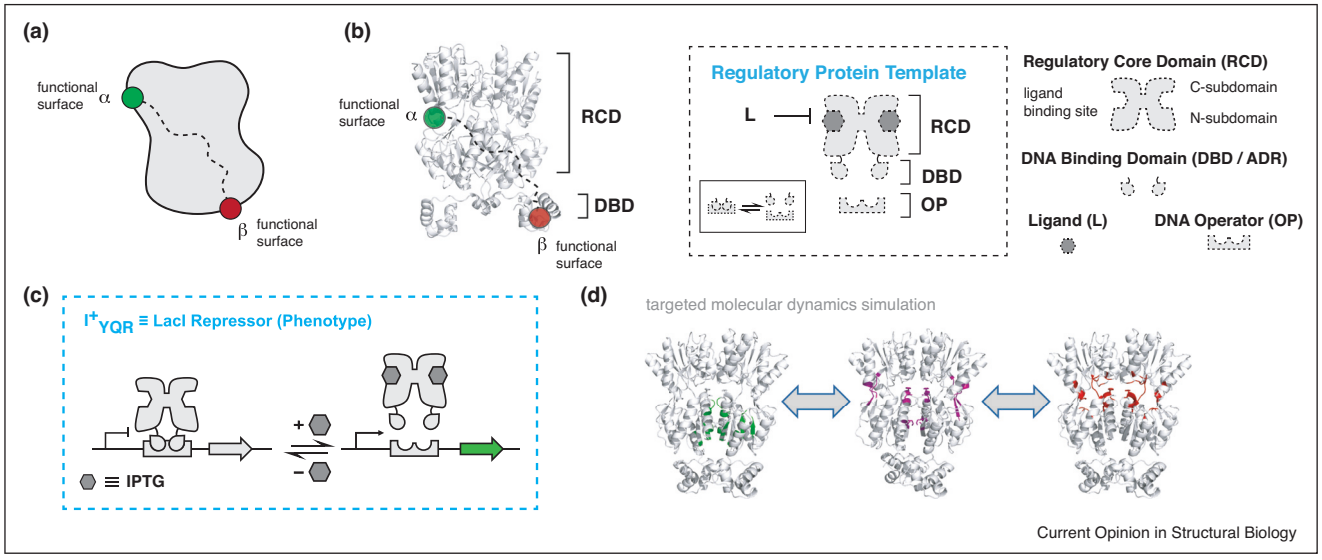
## Introduction

Allostery is broadly defined as the signal propagation between at least two functional surfaces upon external stimulus (Figure 1a) [1,2]. Purportedly, the signal propagation mechanism that influences a given allosteric conformational state follows a defined path along a unique network of residues [3]. Mechanistically, for a two-surface system: (i) an effector interacts with one functional surface (α) causing a disturbance to the surface residues, (ii) this disruption is propagated through a residue network, and (iii) this interaction typically results in a conformational change in the protein, and subsequent activation of the second functional surface (β). Allosteric communication enables a variety of important biological functions such as cell signaling, cooperativity, enzyme catalysis, and gene regulation [4•]. The pioneering work of Monod *et al.* revealed how small molecules can serve as effectors; however, allosteric communication can be initiated by a broad range of input signals — for example, light [5], post-translational modification [6], metals [7], in addition to interaction(s) with other proteins or peptides [8]. Accordingly, the *de novo* design of allosteric communication will have a broad and significant impact on many technological fields. Here we develop a brief roadmap: (i) illustrating an example of allosteric communication via the LacI system, (ii) demonstrating how allosteric communication can be blocked and subsequently conferred in the same protein topology, (iii) exemplifying how a modular engineering strategy can be used to pair alternate β functional surfaces to alternate allosteric routes that share a common α functional surface, and (iv) illustrating how modular engineering can be extrapolated to protein homologues leading to a broad range of allosteric outcomes. Finally, we leverage these studies to shed light on the requirements for the *de novo* design of functional systems that employ allosteric communication.

## Allosteric communication in a canonical transcription factor LacI

Allosteric communication is a hallmark of many transcription factors used to control gene expression and have enabled synthetic biologist to reprogram cells. The lactose repressor (LacI) transcription factor has been a workhorse involved in the development of many of these synthetic systems [9••,10••,11–13]. LacI is a canonical allosteric system in which a signal is propagated between two functional surfaces [14••] (Figure 1b). As illustrated, in the absence of the signal isopropyl-β-D-thiogalactoside (IPTG), LacI binds to operator-DNA. Conversely, upon binding of IPTG (functional surface α) LacI undergoes a conformational shift that reduces the repressor's affinity for operator-DNA (functional surface β) [14••,15•] (Figure 1c). In a computational study conducted by Flynn *et al.* putative allosteric routes were determined in detail for LacI via targeted molecular dynamics simulation (TMD) [15•,16]. The simulated trajectories revealed that the allosteric signal originates asymmetrically in the inducer-binding site of one monomer and propagates to the other monomer through various non-covalent interactions of three interconnected pathways (Figure 1d). Overall, the results from the simulated trajectories are in agreement with a wide range of experimental biochemical and genetic data. However, in order to effectively simulate the putative allosteric routes *via* TMD, high-resolution structural inputs for both the repressed and induced states must be available. Moreover, the β functional surface is unresolved in the induced form.

**Figure 1**



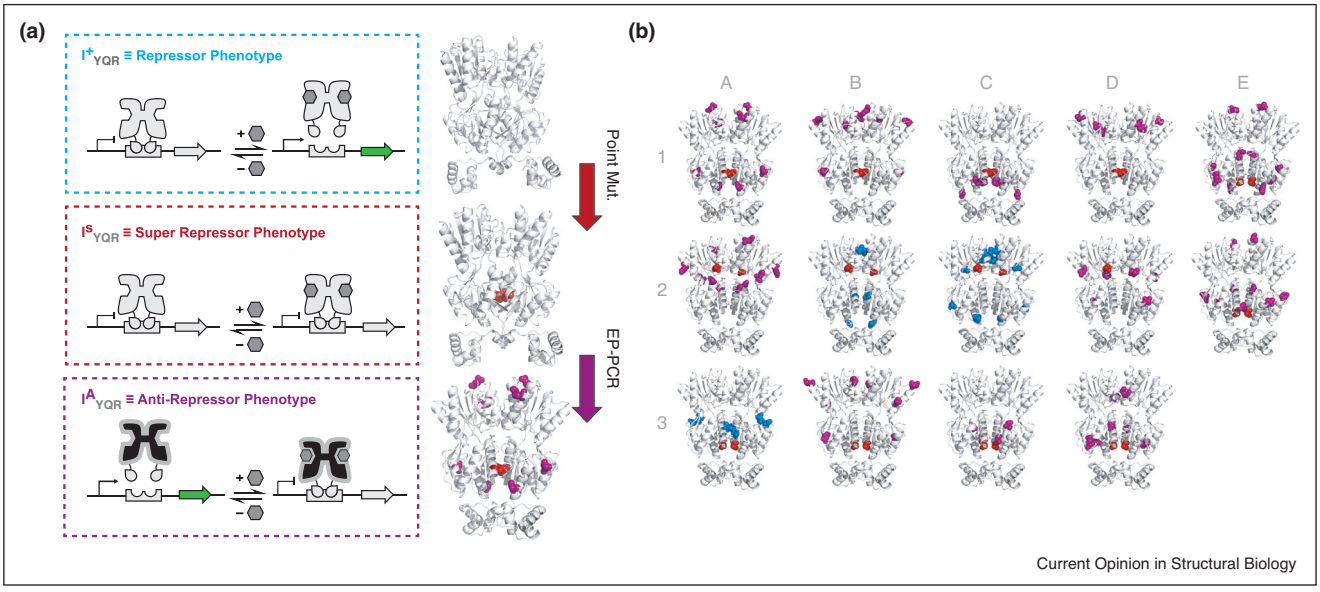

**(a)** Allostery as communication between two functional surfaces. Energy changes on the α surface (usually due to ligand-binding) propagate along a residue network to change properties of the β surface (altering its binding affinity to a substrate). **(b)** Functional surface overlay onto a lactose repressor dimer where the α surface is the ligand-binding domain and the β surface is the DNA-binding domain. Also shown are the modular template of a regulatory protein and its DNA operator. **(c)** Description of the wild-type ($I^+$) lactose repressor phenotype on its cognate DNA operator ($O^1$) and natural YQR amino acids at positions 17, 18, and 22 on the DNA-binding domain. In the absence of ligand, the protein represses. With ligand (IPTG), the gene is expressed due to a conformational shift induced by the ligand. **(d)** Allosteric pathway in LacI proposed using TMD simulations. Pathway 1 (green) starts at the ligand-binding pocket and causes shifts in β sheet motifs to alter the monomer-monomer interface. The signal then follows Pathway 2 (in purple) and disrupts paired residue interactions along the monomer-monomer interface (residues 74-74′, 77-77′, 78-78′, 84-84′) forcing residues connecting the N-subdomains and C-subdomains to pivot, interrupting DNA-binding. Pathway 3, in red, finishes the process by propagating the signal to the inducer binding pocket on the opposing monomer, increasing affinity for a second IPTG molecule.

Accordingly, TMD (as is) cannot be used to design or predict new allosteric functions. TMD is only one of many *in silico* strategies used to explore allosteric communication. A recent CECAM (Center Européen de Calcul Atomique et Moléculaire) workshop composed of computational biophysicists, protein modelers, and bioinformaticians sought to discuss and display the most recent advances in *in silico* approaches used to reconcile allosteric communication — a summary of the notable topics from this workshop are given in a recent review [4•].

## Conferring alternate allosteric communication in LacI

Allosteric communication in LacI can be blocked via the introduction of a single point mutation between the two functional surfaces (Figure 2a) — conferring super repression ($I^S_{YQR}$). This is proven by way of biophysical and biochemical studies that show both functional surfaces are unaffected by the point-mutation [17,18••]; however, the communication between the two surfaces is disrupted. Richards *et al.* demonstrated that one or more rounds of laboratory evolution (i.e., error-prone PCR (EP-PCR)) can be used to introduce additional compensatory mutations (complementary to the two functional surfaces) resulting in either: (i) a rescued repressive phenotype ($I^+_{YQR}$), or (ii) conferred anti-repressor phenotype ($I^A_{YQR}$) (Figure 2) [18••]. In this study, 14 functional variants were observed *via* three distinct allosteric blocks ($I^s$). The engineered systems were composed of three alternate repressors and 11 anti-repressors. None of the mutations introduced via EP-PCR changed the super repressor mutation (i.e., the initial allosteric block). Mutations were observed in both the N-subdomain and C-subdomain of the regulatory core domain (RCD) (Figures 1b and 2b), and in many cases these mutations overlap with residues identified in the allosteric trajectories observed via TMD (Figure 1d). However, there are at least two examples of engineered systems that have no overlap with the putative TMD allosteric routes. This demonstrates that there are multiple solutions to confer allosteric communication in the LacI scaffold. Likewise, the current supposition is that the compensatory mutation (s) are responsible for conferring alternate allosteric communication in the LacI scaffold. This observation suggests that allosteric communication in a given scaffold can be regarded as plastic, rather than as a fixed hard-wired path. Independently, Poelwijk *et al.* also illustrated that alternate LacI allosteric networks could be conferred *via* an $I^S$ intermediate in the LacI scaffold by way of laboratory evolution using a selection, rather than a screen [19].

**Figure 2**




**(a)** Lactose repressor phenotypes. Starting with the wild-type repressor ($I^{+}_{YQR}$), point mutations via site-saturation mutagenesis led to the discovery of the super repressor phenotype ($I^{S}_{YQR}$) which represses regardless of ligand-binding. Shown here is a K84A mutation in red. $I^{S}$ mutants were then used as templates for error-prone PCR, evolving the anti-repressor phenotype ($I^{A}_{YQR}$) which acts in an anti-fashion to the wild-type repressor phenotype. Additional mutations are shown in green. This resulted in the working hypothesis that a block in allosteric communication ($I^{S}$) was required before rerouting to an $I^{A}$ phenotype. **(b)** Fourteen LacI variants evolved using error-prone PCR with $I^{S}$ mutants as templates. Point mutations resulting in $I^{S}$ phenotype are shown in red, additional mutations from error-prone PCR are shown in blue or purple if the mutations resulted in a $I^{+}$ or $I^{A}$ phenotype respectively. For example, A1 started with a K84A point mutation that resulted in an $I^{S}$ phenotype. Additional mutations shown by the purple spheres at sites 113, 132, 230, and 267 resulted in an $I^{A}$ phenotype.

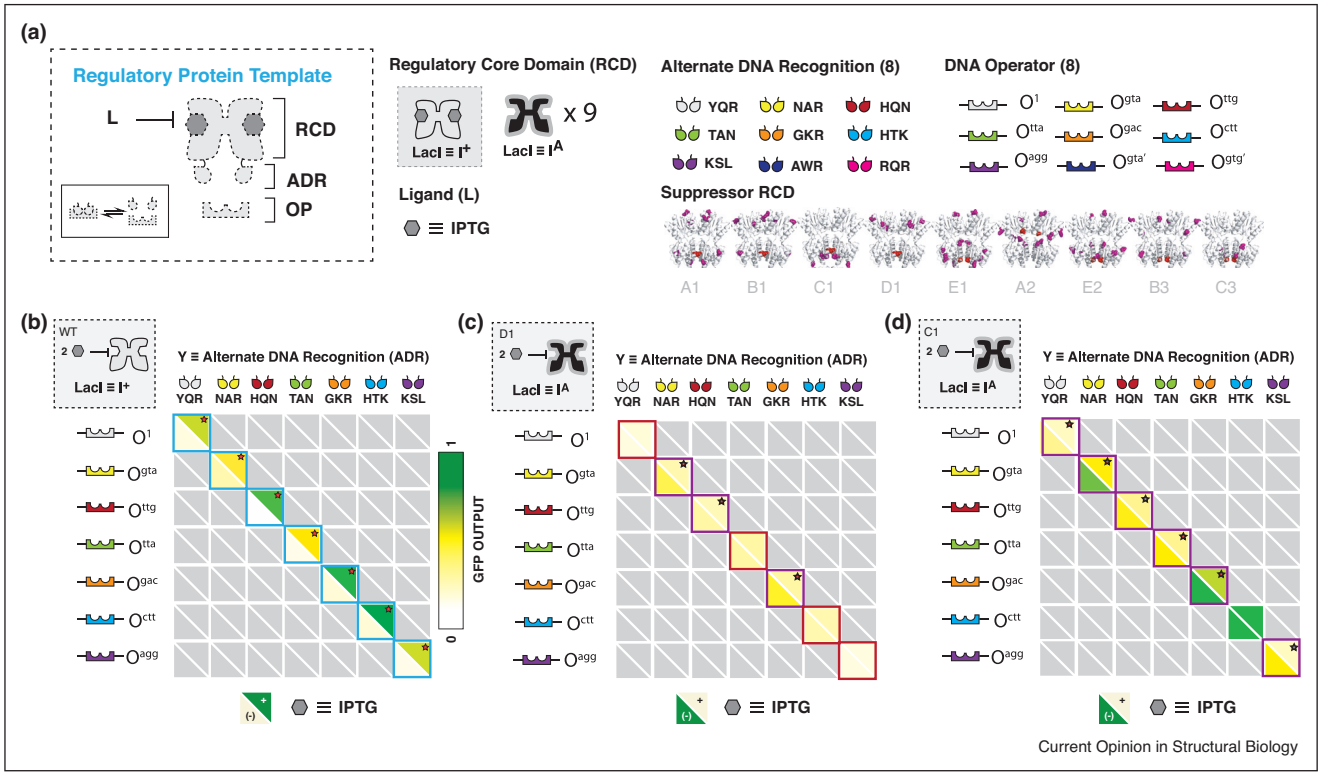
## Variable β functional surfaces paired with alternate allosteric routes with fixed α surface

Each β functional surface in the LacI system is defined by two parts: (i) a DNA-binding domain, and (ii) a complementary DNA operator (Figure 3a — inset). In a recent study Rondon *et al.* engineered systems of LacI repressors and anti-repressors with alternate DNA-binding functions — that is, alternate β functional surfaces [20••] (Figure 3a). Initially, the wild-type regulatory core (encompassing the first functional surface, and the allosteric domain) was paired with eight alternate β functional surfaces that is, eight alternate DNA recognition (ADR) domains (Figure 3a and b). When paired with the wildtype RCD, three β functional surfaces resulted in non-cognate interactions, and one second surface failed to interact with any operator DNA. However, six alternate β functional surfaces only interacted with cognate DNA, when paired with the naturally occurring α functional surfaces, and in all cases the repressor phenotype was observed (Figure 3b). In turn, Rondon and Wilson selected 9 out of 14 of the engineered LacI anti-repressors from a previous study [18••,20••], and used the same modular design strategy to introduce alternate DNA functions (i.e., alternate β functional surfaces) (Figure 3a). However, only the six cognate alternate β functional surfaces were used, resulting in 54 putative anti-repressors. Out of the 54 putative anti-repressors, 46 functioned as cognate anti-repressors. Overall for the non-functional systems, four systems resulted in the super repressor phenotype, and three systems were unresponsive. Two example matrixes illustrate how alternate allosteric networks can influence functional outcomes (Figure 3c and d). These data illustrate that: (i) alternate allosteric communication in a given topology (with a fixed α functional surface) can accommodate a variety of β functional surfaces; (ii) all alternate allosteric networks are not necessarily compatible with a given β functional surface, even if that functional surface has allosteric communication variants that are proximal in sequence space; (iii) variation in allosteric topology alone can confer different degrees of dynamic (functional) range. Chen *et al.* have also demonstrated that tuning the properties of the DNA element that the β functional surfaces interacts with can result in fine control over the dynamic range — thus should be considered as an additional criteria for allosteric design with regard to transcription factors [9••].

## Engineering allosteric communication with variation in functional surfaces and topology

Our understanding of the LacI structure–function relationship has been expanded to the study and identification of more than 1000 homologous proteins, commonly

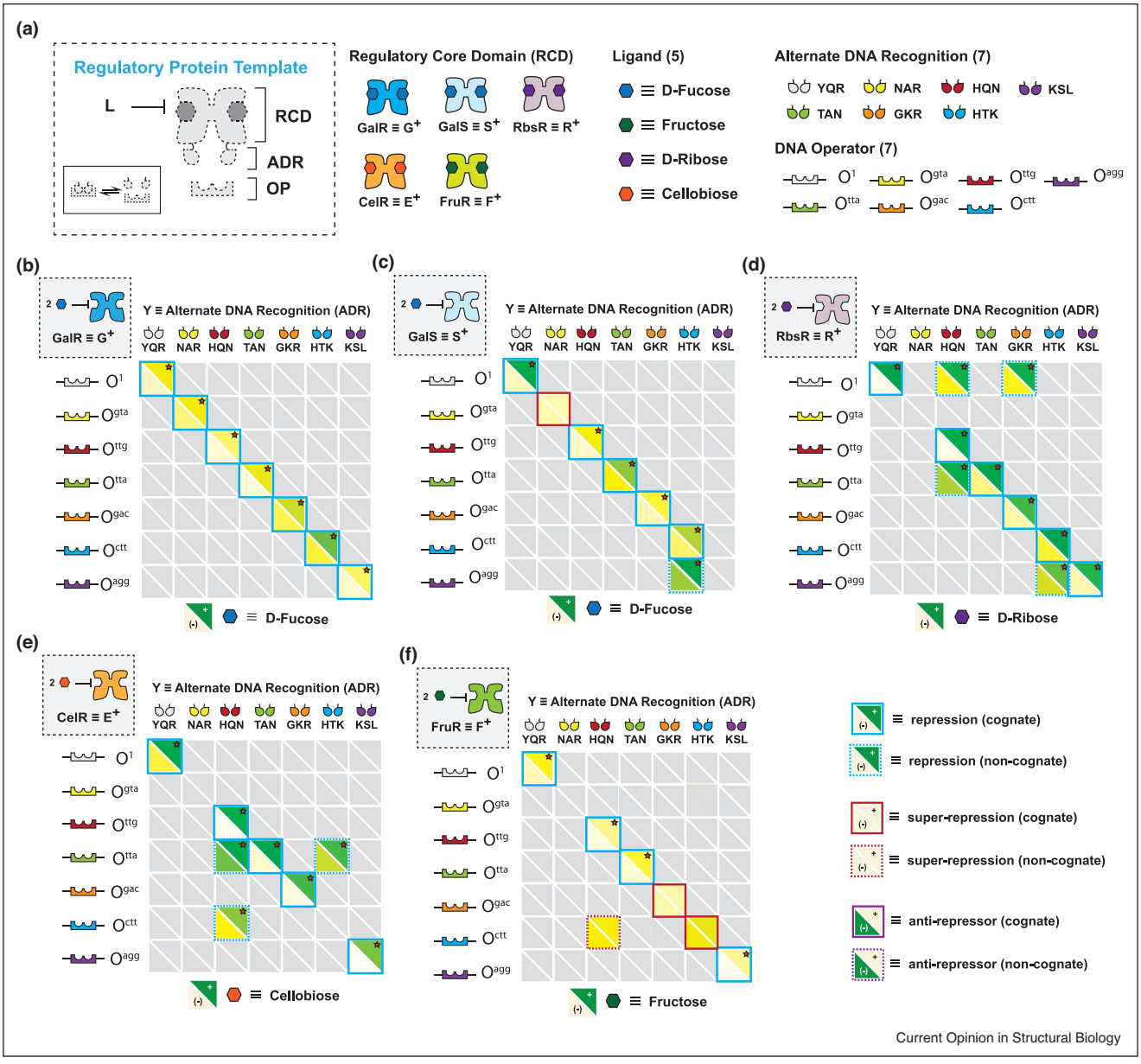
**Figure 3**




Engineering allostery in transcription factors using alternate DNA recognition. **(a)** Nine RCD topologies (structures labeled per Figure 2) with an $I^A$ phenotype were combined with eight alternate DNA recognition (ADR) modules. These ADR modules involved point mutations at sites 17, 18, and 22, replacing the native Y, Q, and R residues. Generated TFs were tested on eight different DNA operators. Cognate ADR modules and DNA operators share the same color, ex. TAN (green) domain's cognate DNA operator is $O^{tta}$. **(b)** Functional map of the wild-type lactose repressor with ADR. **(c)** Functional map using D1 RCD ($I^A$ function) with ADR. **(d)** Functional map using C1 RCD ($I^A$ function) with ADR. Functional maps contain bisected squares that show gene expression levels with and without IPTG. Green indicates maximum reporter expression, white represents minimal reporter expression. Stars indicate statistically significant differences between the two induction states. Grey boxes represent no interaction with the operator. $I^+$ phenotypes are boxed in blue, $I^S$ phenotypes are boxed in red, $I^A$ phenotypes are boxed in purple.

referred to as the LacI/GalR family [21–23]. The communal function of this protein family features allosteric regulation of DNA-binding to modulate transcription, similar to LacI. Each LacI homologue has evolved a unique variation in ligand specificity (α functional surface) and affinity for specific DNA targets (β functional surface), and a unique solution to allosteric communication *via* variations in topology of the RCD. The previous section illustrates how moderate changes in the allosteric route can result in unique functional solutions that are distinctive to a set of functional surfaces. Accordingly, a reasonable supposition is that the design of an allosterically regulated transcription factor requires simultaneous and reciprocal consideration of all three modules that is, α functional surface(s), β functional surface(s), and allosteric topology/medium. To demonstrate the proof of concept of the generalizability of the modular design (engineering) strategy, Rondon *et al.* paired disparate α functional surfaces and allosteric topologies (i.e., ligand-binding sites and complementary RCDs), with a set of alternate β functional surfaces (ADR) to create a collection of non-natural transcription factors [10••] (Figure 4a). In this study, five regulatory cores were selected, representing five allosteric topologies — with four α functional surfaces, and seven alternate DNA recognition units (β functional surfaces) (Figure 4a). Collectively, this represents a design space of 35 putative non-natural transcription factors. 27 out of 35 of the putative transcription factors were functional as non-natural repressors (Figure 4b–f). Six ADR (β functional surfaces) interacted with non-cognate DNA operators, post α functional surface interaction. In general, no two repression matrixes resulted in the same set of outcomes (Figure 4b–f). Two of the RCDs (GalR and GalS — 54.23% identical) utilize the same α functional surfaces, thus interact with the same signal (i.e., D-fucose) (Figure 3b and c); however, though the primary topologies (amino-acid compositions) are different. These differences in primary topology between GalS and GalR present an opportunity to evaluate how changes in the allosteric medium can influence

**Figure 4**




Engineering allostery in transcription factors using alternate regulatory core domains. **(a)** Five alternate regulatory core domains (RCD) were combined with seven alternate DNA recognition domains (ADR) and their cognate operators. **(b)** Functional map of the galactose repressor (GalR) RCD with ADR. **(c)** Functional map of the GalS repressor RCD with ADR. **(d)** Functional map of the ribose repressor (RbsR) RCD with ADR. **(e)** Functional map of the cellobiose repressor (CelR) RCD with ADR. **(f)** Functional map of the fructose repressor (FruR) RCD with ADR. Bisected squares show reporter gene expression levels with and without ligand, which varies with RCD. Green indicates maximum reporter expression, white represents minimal reporter expression. Stars indicate statistically significant differences between the two induction states. Grey boxes represent no interaction with the operator. $X^+$ phenotypes are boxed in blue, $X^S$ phenotypes are boxed in red, $X^A$ phenotypes are boxed in purple. Dashed boxes represent non-cognate ADR/operator interactions.

functional outcomes, in a second scaffold, other than LacI. As with the LacI suppressor with ADR, the Gal RCDs (with fixed α functional surfaces and variable β functional surfaces) have different functional outcomes. As before, this implies that this set of β functional surfaces are not uniformly compatible with a given allosteric medium and corresponding α functional surface. Moreover, when the α and β functional surfaces are the same, but the topology and composition of the allosteric media vary, as well as the overall functional outcome can vary significantly — that is, in terms of dynamic range and β functional surface specificity (Figure 3d–f).

Shis *et al.* [24] and Chan *et al.* [25] have also used similar modular design strategies to engineer non-natural transcription factors from the same family of protein homologs. Moreover, Meyer *et al.* have demonstrated that a two-part laboratory evolution strategy can be used to tune the allosteric properties of LacI (and 11 other gene regulators), simultaneously selecting for lower background, high dynamic range, increased sensitivity, and low cross-talk [26••]. We posit that this strategy also represents engineering of allosteric communication, given that the perturbations do not involve functional surfaces. Accordingly, a similar two-part strategy could be used to confer similar outcomes in non-natural systems. Collectively, these studies outline yet another set of design criteria.

## Moving from engineered allosteric communication to predictive design

A prerequisite to the *de novo* design of an allosteric route is first to hone our ability to identify allosteric positions a priori in the given protein topology. The challenge in the prediction of allosteric routes in a given protein is that allosteric communication typically occurs between sites that are not in direct contact, which limits canonical pairwise molecular mechanics interpretations frequently used in computer-aided protein design [4•]. In addition to the engineering strategies outlined above, it may be possible to use bio-informatics strategies on large families of proteins to develop non-pairwise scoring functions that can be used to design allosteric communication between two functional surfaces. Toward this end, Suel *et al.* developed a sequence-based statistical mapping (a non-pairwise approach) to potentially identify networks of residues that mediate allosteric communication in proteins [27–30]. These statistical coupling analysis (SCA) studies revealed that non-allosteric residues (most sites in a given protein) act in an evolutionarily independent manner and are uninfluenced by perturbations (mutation). However, allosteric residues (a small number of positions in a given protein) form co-evolving linked networks throughout the structure — that is, producing architectures for mediating long-range communication in proteins. A hallmark of these allosteric positions is an extreme sensitivity to perturbation. This is proven (in part) via the LacI systems using deep-mutational scanning to test conferred allosteric positions for mutational tolerance [17], though assessment of co-evolution of these residues has not been evaluated.

The importance of SCA with regard to allosteric communication is best illustrated by its application in network discovery, design, and use in network elucidation. Recently, elastic network models have been developed that give rise to the identification of the underlying origins of putative allosteric sectors using amino acid sequence and mutational effect parameters [31]. However, this model has only been used to identify previously observed correlations between sectors and contacts, and in general lacks sufficient granularity to be used as a design tool. In another example, allosteric control was engineered using surface sites identified through SCA. Briefly, Lee *et al.* used SCA to reveal networks of co-evolving amino acids that functionally link two discrete functional surfaces. In turn, the allosteric networks of the two proteins were joined across their surface sites such that the activity of one protein controlled the activity of the other. This resulted in a light-sensing signaling domain from the Per/Arnt/Sim family of proteins and a dihydrofolate reductase that were combined at their functional surface sites, forming a chimera with light-dependent catalytic activity [30]. Another study used SCA to identify allosteric hotspots before testing them for functionality and regulatory potential. Termed Rational Engineering of Allostery at Conserved Hotspots (REACH), this process promises the ability to design proteins to respond to novel inputs [32]. In yet another example, sequence-based statistical coupling analysis was used to identify conserved residues that confer allosteric functional properties (functional sectors) in pancreatic-type ribonucleases (ptRNase) [33]. Likewise, functional sectors were identified *via* SCA and were used to guide the development of a mutant luciferase family, helping reveal synergistic residues within functional networks [34]. SCA has also provided important insights with respect to the putative process of proton transfer in a microbial laccase [35]. While this list of accomplishments for SCA are impressive, none of these examples represent the *de novo* design of an allosteric route.

## Conclusions

The benefit of using SCA techniques when engineering allostery lies in its minimal resource requirements. Structural data are not needed for analysis, though comparison to functionally important protein domains can help with analysis. However, statistical mapping and related strategies are 'thermodynamic' in nature, and therefore provide no intrinsic information regarding the underlying mechanism of the interactions between residues. Moreover, sequence-based statistical mapping (and similar approaches) lacks the necessary granularity to identify dormant positions that have been important in alternate allosteric communication when such positions become activated without change in identity, thus limiting the algorithms use as a design tool. In addition, SCA studies suggest that allosteric residues may also overlap with residues that are important for protein stability — convoluting the problem of defining and quantifying a given allosteric network. What is clear from the case studies presented in this review is that the full a priori design of a functional allosteric protein will require the simultaneous design of both functional surfaces along with the corresponding allosteric topology. Using systematic workflows could potentially simplify the allosteric design problem – that is, hierarchical design of functional surfaces, followed

by the design of the corresponding allosteric topology. These workflows will likely benefit from SCA parametrization of multi-dimensional energy functions that can be used to accurately predict high-resolution tertiary allosteric structures.

## Conflict of interest statement

Nothing declared.

## Acknowledgements

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- •• of outstanding interest

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This review is a compilation of ideas presented at the interdisciplinary CECAM workshop, bringing together computational biophysicists, protein modelers, and bioinformaticians. The purpose is to inform on the current state of allostery, allosteric modeling and design, and the limitations of these models. Summaries of simulation algorithms attempting to account for thermodynamic parameters are discussed and the effect of enhanced sampling on these models. Additionally, purely physical interactions were modeled to provide mechanical insight into allosteric communication. These led to experimental techniques for engineering specific protein function by leveraging sensitive sites discovered by the models.

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In this article, the authors sought to tune the dynamic range of promoters (i.e., ligands that complement the β functional of a transcription factor) surface controlled by AraC and LacR. By engineering them with −10 and −35 sites from synthetic and *E. coli* promoters, the authors created transcriptional logic gates with four sequence combinations of promoters. These logic gates were multi-input in nature and allow for designed control of the regulatory elements.

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The authors describe a biological programming structure that leverages engineered transcription factors (with variation in α and β functional surfaces and allosteric topology and complementary genetic architectures). Rondon *et al.* demonstrate that variation in one or more modules can significantly influence the overall functional outcome (dynamic range). The logic functions AND, OR, NOT, NOR, and half-AND were produced. The parallel and series architectures made with these functions are the foundation for future use in transcriptional programs with both digital and analog performance.

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This review explores the structure-function relationship of LacI in response to induction ligands. Additionally, it investigates how the structure of LacI can be used to understand other regulatory proteins, the tunability of the expression of these proteins, and leveraging the modularity of LacI for the engineering of other regulatory elements.

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This review examines different modes of biomolecular assemblies and how engineering them could lead to major advances in the biotechnology space. Protein assemblies are the focus with chapters discussing mechanisms of nucleation, designer peptide assemblies, functional proteins, transcription factor assembly, self-assembled protein scaffolds, and amyloids and prions. Many of these systems involve allosteric communication. The review provides the basis for the relationship between allostery and stability. Fundamental aspects of these topics are discussed along with their potential uses in engineered biosystems.

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In this paper, the author use an error-prone PCR on previously produced LacI super repressors to create 14 new regulatory elements. This followed the hypothesis that an $I^S$ block was required before allostery could be rerouted to the anti-lac phenotype. This study shows that allosteric communication in a given scaffold can be regarded as plastic, rather than as a fixed hard-wired path. Variants with alternate allostery produced both wild-type (repressive) and anti-lac (suppressive) functions with differing dynamic ranges of gene expression.

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In this work, the authors used previously produced anti-lac (suppressive) scaffolds to produce new regulatory elements by mutating the DNA-binding domain and matching this alternate DNA domain to cognate DNA operators (i.e., variation in the β functional surface). 46 anti-lac variants were produced that are orthogonal to the natural $O^1$ operator. These variants were then used to create genetic toggle switches with two distinct fluorescent outputs.

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This research presents a directed laboratory evolution strategy for cellular sensors that attempts to remove background noise while simultaneously selecting for high dynamic range, an increase in sensitivity, and minimal cross-talk. Both random and selected bases were mutated on a double selection plasmid. With each round of selection, interventions were performed to bias toward desired functions. A total of 12 high-performance sensors were created that can be controlled by 12 small-molecule inducers. This study marks important guidelines for the *de novo* design of allosteric communication.

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