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RPPR Final Report
as of 06-Jun-2022

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Proposal Number: 70370LS **Agreement Number: W911NF-17-1-0043**

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Final Report for Period Beginning 09-Dec-2016 and Ending 08-Apr-2020
Title: Radical redesign of an allosteric biosensor to respond to new ligands
Begin Performance Period: 09-Dec-2016 **End Performance Period:** 08-Apr-2020
Report Term: 0-Other

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Major Goals: Biosensors to detect small molecule ligands (metabolites) is a highly valuable tool with applications in synthetic biology, medical diagnosis, environmental monitoring, bioremediation, and bioenergy. Nature has evolved proteins as small molecule biosensors with exquisite specificity and sensitivity. Our inability to design a biosensor for a desired molecule is a major hurdle to further expanding the use of biosensors. We currently rely almost exclusively on natural biosensors. Although thousands of sequences are marked as putative biosensors, the ligand partner for most is unknown. We are limited to 10-15 well-characterized natural biosensors; this leaves large chemical classes with no biosensors. For instance, anthropogenic chemicals (synthetic, human-made chemicals) such as explosives and pesticides are not detectable by natural biosensors, but will require new biosensors specifically designed for these chemicals. Biosensors also do not exist for microbial metabolites that are biomarkers of pathogenicity, environmental pollutants, human wellness indicators like hormones, and complex natural products of medicinal and industrial value.

Our overall goal is to redesign allosteric transcription factors (aTF) to sense new ligands. The specific aims of the proposal are:
Aim 1: Building mutation-tolerant scaffolds by stabilizing the wild type aTF
Aim 2: Mapping allosteric hotspots around the ligand-binding pocket by deep mutational scanning
Aim 3: Designing aTF to bind to new ligands radically different from the native ligand.

Accomplishments: We chose two aTFs (LacI and TtgR) as candidates for the redesign. Both aTFs belonged to large families of bacterial transcription factors.

We have three major accomplishments during the grant period

1. Design of a highly specific TtgR biosensor
2. Understanding the role of epistasis in the design of allosteric proteins
3. Redesigning LacI to make novel sugar biosensors

Design of a highly specific TtgR biosensor

TtgR is a TetR-family allosteric transcription regulator activated by two inducers: naringenin and resveratrol. We used computational design (Rosetta software suite) to engineer TtgR specificity by generating function-switching mutations that directly interact with the ligand. To increase resveratrol specificity, we redesigned the ligand-

contacting residues for greater affinity for resveratrol, assuming greater affinity may result in greater specificity. Since Rosetta is a structure-based design tool, the absence of a resveratrol-bound TtgR crystal structure made the design task challenging because the correct position of the ligand in the binding pocket was not known a priori. We computationally generated approximately 19,000 unique TtgR design variants (Fig.1). After design, each output variant comes with a set of Rosetta-calculated scores that reflect physical properties such as stability, repulsion, hydrogen bonds, and protein-ligand affinity. The best variants for library construction can be selected from the distribution of all scores of output designs based on user-defined preferences. The variants were curated Rosetta scoring metrics to yield a final list of approximately 3,500 unique sequences with an average of 5 mutations per variant for experimental testing (Supplementary Figs. 1, 2). A few positions such as 96, 137, 168, and 175 have mutations that are more abundant than the wildtype amino acid. We synthesized oligonucleotides encoding approximately 3,500 designed variants as a pool of exact chip-DNA sequences (Twist Bioscience Inc). To determine the activity of TtgR variants, we designed a pooled screen by sorting E. coli cells containing a GFP reporter system regulated by a TtgR operator adapted for E. coli. We isolated a resveratrol-specific TtgR variant with four mutations: C137I, I141W, M167L, and F168Y. All four mutations were in close proximity to the ligand and no mutations were found elsewhere on TtgR. The quadruple mutant gave 92- and 6.5-fold induction with 100 µM resveratrol and 1mM naringenin, respectively, compared to 55- and 55-fold of wildtype TtgR. This result showed that computation-guided could be used to enhance specificity of allosteric biosensors.

Fig 1: Design of resveratrol-specific TtgR variant.

Understanding the role of epistasis in the design of allosteric proteins

Allostery is a fundamental mechanism by which proteins recognize environmental cues (such as binding of an inducer or effector) within a localized region resulting in modulation of function at a distal site. Mutations in the binding pocket that trigger the allosteric network have the potential to create new nonspecific epistatic interactions at the level of protein function beyond the physical interactions commonly seen in specific epistasis and can create complex nonspecific interactions. For an allosteric transcription factor (aTF), function is the outcome of three parameters: affinity for the inducer ligand, affinity for DNA, and allosteric changes that accompany binding to the ligand. Each of these parameters will have its own fitness function mapped over the same sequence space, creating unique fitness landscapes. An aTF simultaneously traverses these multiple fitness landscapes, which collectively govern the evolutionary trajectory of the aTF under selective pressure.

To investigate how the same set of binding pocket mutations might uniquely affect each parameter, we constructed the fitness landscape of each parameter individually. We quantified the number of viable pathways in the resveratrol landscape by requiring that each additional mutation must increase parameter fitness if the quadruple mutant performs better than wildtype or decrease parameter fitness if the quadruple mutant performs worse than wildtype. There are 24 possible pathways from wildtype to quadruple mutant (Fig. 2). Each functional parameter shows distinctive patterns of epistasis, although some are closely related.

Fig. 2: Fitness landscapes for multiple functional parameters in response to induction with resveratrol. Fitness landscapes of (a) fold induction, (b) basal gene expression, (c) maximum gene expression, and (d) EC50 parameters for all 16 TtgR variants in response to resveratrol with each variant shown as a node in the graph.

Redesigning LacI to make novel sugar biosensors

LacI is a transcription regulator activated by disaccharide allolactose. We wanted to expand the specificity of LacI to recognize new sugars. A goal of this grant proposal was to improve the stability of scaffolds for design. We combined evolutionary sequence selection and Rosetta-based energy filtering to choose stable scaffolds and found that is dramatically-reduced inactive LacI variants. Over 80% of LacI variants mutated without evolutionary selection could not bind to DNA either due to allosteric dysfunction or loss of stability. We find that only 5% of LacI variants based on evolutionary sequence selection are not able to bind to DNA (Fig. 3). This demonstrates that evolution-guided Rosetta design can select functional scaffolds for biosensor design. We tested this new design protocol that combined evolutionary information with Rosetta design to avoid mutating hotspot residues that may affect allostery or thermodynamic stability. We successfully redesigned LacI to bind to four sugars – melibiose, raffinose, mannobiose and trehalose (Fig 3a). We solved the crystal structure of redesigned LacI bound to melibiose (Fig. 3b).

Fig. 3: Expanding specificity of LacI. (a) Redesigned LacI responding to four new sugars – melibiose, raffinose, mannobiose and trehalose. (b) crystal structure of redesigned LacI bound to melibiose

Training Opportunities: This grant supported the training of two graduate students – Kyle Nishikawa and Xiangyang Liu and one undergraduate Nicholas Hoppe.

Results Dissemination: Presentation – Vatsan Raman

Oct 2018 Istanbul Technical University, Turkey
"Designing highly specific protein-based small molecule biosensors"
Jun 2018 Protein Engineering Canada, Vancouver, BC
"Designing highly specific protein-based small molecule biosensors"

Jun 2018 University of Oregon, Eugene, OR
"Designing highly specific protein-based small molecule biosensors"

May 2018 University of Minnesota, Minneapolis, MN
"Designing highly specific protein-based small molecule biosensors"

Jan 2018 US Army Research Office, Cocoa Beach, FL
"Designing highly specific protein-based small molecule biosensors"

2017

Dec 2017 PLATO Frontiers in Life Sciences, Madison, WI
"Synthetic biology for biomanufacturing, environmental sustainability and health"

Aug 2017 American Chemical Society, Washington DC
"Designing highly specific protein-based small molecule biosensors"

Apr 2017 University of Notre Dame, South Bend, IN
"Designing highly specific protein-based small molecule biosensors"

Apr 2017 American Society of Biochemistry and Molecular Biology, Chicago, IL
"Designing highly specific protein-based small molecule biosensors"

Jan 2017 International Conference on Biomolecular Engineering, San Diego, CA
"Designing highly specific protein-based small molecule biosensors"
2016

Nov 2016 DuPont Inc, Wilmington, DE
"Accelerating design-build-test cycles with small molecule biosensors"

Aug 2016 RosettaCon, Leavenworth, WA
"Accelerating design-build-test cycles with small molecule biosensors"

Jul 2016 Society for Industrial Microbiology, New Orleans, LA
"Designer biosensors that respond to new small molecules"

Jul 2016 Protein Society (Young Investigator Talk), Baltimore, MD
"Designing highly specific protein-based small molecule biosensors"

May 2016 Great Lakes Bioenergy Research Center Annual Symposium, Lake Geneva, WI
"Accelerating design-build-test cycles with small molecule biosensors"

Honors and Awards: Vatsan Raman – NIH New Innovator Award and Shaw Scientist Award
Kyle Nishikawa – Biochemistry Department Fellowship

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Technology Transfer: These results will contribute to a pending invention disclosure.

PARTICIPANTS:

Participant Type: PD/PI
Participant: Srivatsan Raman
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Kyle Nishikawa
Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Xiangyang Liu
Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Undergraduate Student
Participant: Nicholas Hoppe
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Authors: Srivatsan Raman
Keywords: allostery, synthetic biology, deep mutational scanning
Abstract: The study of allostery has a central place in biology because of the myriad roles of allosteric proteins in cellular function. As technologies for probing the spatiotemporal resolution of biomolecules have become increasingly sophisticated, so has our understanding of the diverse structural and molecular mechanisms of allosteric proteins. Studies have shown that the allosteric signal is transmitted a through a network of residue-residue interactions connecting distal sites on a protein. Linking structural and dynamical changes to the functional role of individual residues will give a more complete molecular view of allostery. In this work, we highlight new mutational technologies that enable a systems-level, quantitative description of allostery that dissect the role of individual residues through large-scale functional screens. A molecular model for predicting allosteric hot spots can be developed by applying statistical tools on the resulting large sequence-structure-function data se
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Article Title: Design of a Transcriptional Biosensor for the Portable, On-Demand Detection of Cyanuric Acid
Authors: Xiangyang Liu, Adam D. Silverman, Khalid K. Alam, Erik Iverson, Julius B. Lucks, Michael C. Jewett, Sri
Keywords: biosensors, cell-free systems, transcription regulators
Abstract: Rapid molecular biosensing is an emerging application area for synthetic biology. Here, we engineer a portable biosensor for cyanuric acid (CYA), an analyte of interest for human and environmental health, using a LysR-type transcription regulator (LTTR) from Pseudomonas within the context of Escherichia coli gene expression machinery. To overcome cross-host portability challenges of LTTRs, we rationally engineered hybrid Pseudomonas-E. coli promoters by integrating DNA elements required for transcriptional activity and ligand-dependent regulation from both hosts, which enabled E. coli to function as a whole-cell biosensor for CYA. To alleviate challenges of whole-cell biosensing, we adapted these promoter designs to function within a freeze-dried E. coli cell-free system to sense CYA. This portable, on-demand system robustly detects CYA within an hour from laboratory and real-world samples and works with both fluorescent and colorimetric reporters.
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Publication Location:
Article Title: Epistasis shapes the fitness landscape of an allosteric specificity switch
Authors: Kyle K. Nishikawa, Nicholas Hoppe, Robert Smith, Craig Bingman, Srivatsan Raman
Keywords: biosensor, allosteric transcription factor, ligand specificity, epistasis
Abstract: Epistasis is a major determinant in the emergence of novel protein function. In allosteric proteins, direct interactions between inducer-binding mutations propagate through the allosteric network, manifesting as epistasis at the level of biological function. Elucidating this relationship between local interactions and their global effects is essential to understanding evolution of allosteric proteins. We integrate computational design, structural and biophysical analysis to characterize the emergence of novel inducer specificity in an allosteric transcription factor. Adaptive landscapes of different inducers of the designed mutant show that a few strong epistatic interactions constrain the number of viable sequence pathways, revealing ridges in the fitness landscape leading to new specificity. The structure of the designed mutant shows that a striking change in inducer orientation still retains allosteric function.
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Authors: Xiangyang Liu
Acknowledged Federal Support: **N**

Partners
Crystallography Facility, UW-Madison
Madison, WI USA
4
Dr. Craig Bingman helped with solving the structure of resveratrol-specific TtgR design

I certify that the information in the report is complete and accurate:
Signature: Srivatsan Raman
Signature Date: 5/25/22 11:41AM

Major Goals

Biosensors to detect small molecule ligands (metabolites) is a highly valuable tool with applications in synthetic biology, medical diagnosis, environmental monitoring, bioremediation, and bioenergy. Nature has evolved proteins as small molecule biosensors with exquisite specificity and sensitivity. Our inability to design a biosensor for a desired molecule is a major hurdle to further expanding the use of biosensors. We currently rely almost exclusively on natural biosensors. Although thousands of sequences are marked as putative biosensors, the ligand partner for most is unknown. We are limited to 10-15 well-characterized natural biosensors; this leaves large chemical classes with no biosensors. For instance, anthropogenic chemicals (synthetic, human-made chemicals) such as explosives and pesticides are not detectable by natural biosensors, but will require new biosensors specifically designed for these chemicals. Biosensors also do not exist for microbial metabolites that are biomarkers of pathogenicity, environmental pollutants, human wellness indicators like hormones, and complex natural products of medicinal and industrial value.

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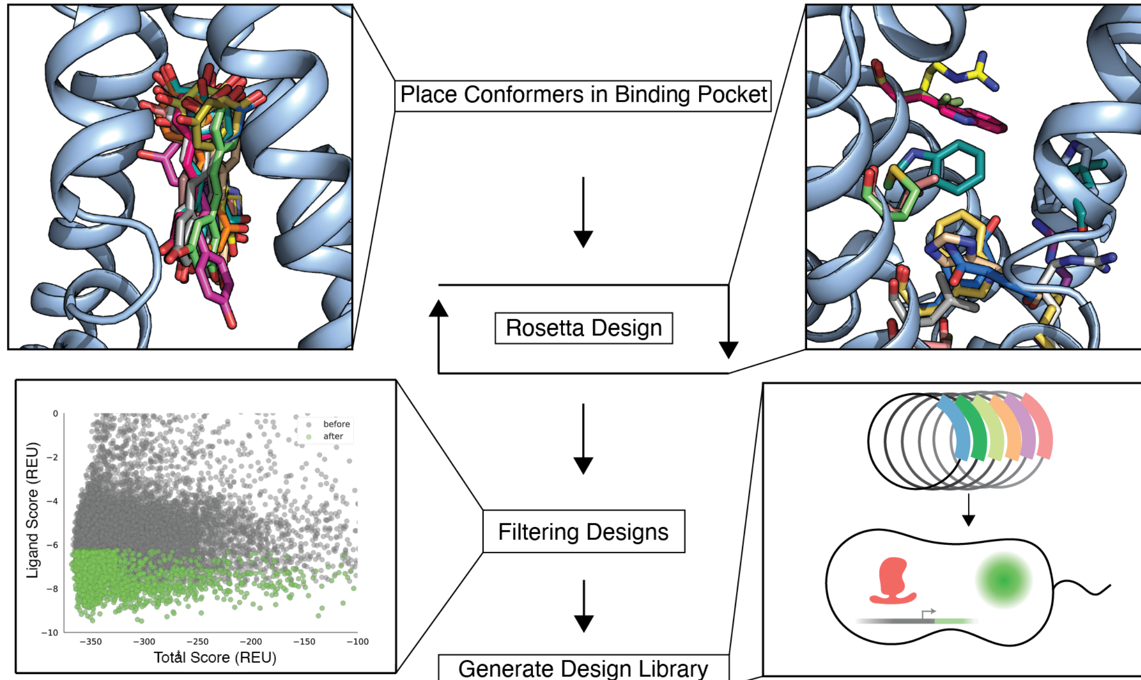
generated approximately 19,000 unique TtgR design variants (Fig.1). After design, each output variant comes with a set of Rosetta-calculated scores that reflect physical properties such as stability, repulsion, hydrogen bonds, and protein-ligand affinity. The best variants for library construction can be selected from the distribution of all scores of output designs based on user-defined preferences. The variants were curated Rosetta scoring metrics to yield a final list of approximately 3,500 unique sequences with an average of 5 mutations per variant for experimental testing. A few positions such as 96, 137, 168, and 175 have mutations that are more abundant than the wildtype amino acid. We synthesized oligonucleotides encoding approximately 3,500 designed variants as a pool of exact chip-DNA sequences (Twist Bioscience Inc). To determine the activity of TtgR variants, we designed a pooled screen by sorting E. coli cells containing a GFP reporter system regulated by a TtgR operator adapted for E. coli. We isolated a resveratrol-specific TtgR variant with four mutations: C137I, I141W, M167L, and F168Y. All four mutations were in close proximity to the ligand and no mutations were found elsewhere on TtgR. The quadruple mutant gave 92- and 6.5-fold induction with 100μM resveratrol and 1mM naringenin, respectively, compared to 55- and 55-fold of wildtype TtgR. This result showed that computation-guided could be used to enhance specificity of allosteric biosensors.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Rosetta Design
Filtering Designs
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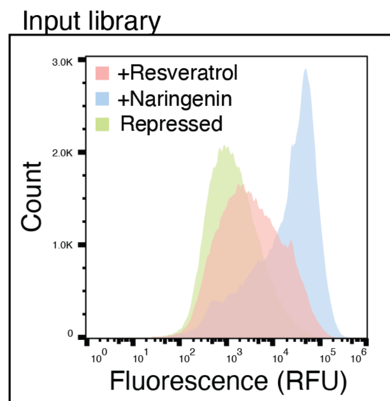
Generate Design Library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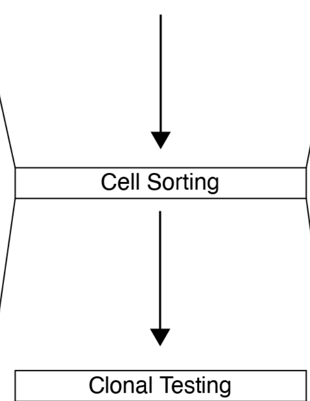
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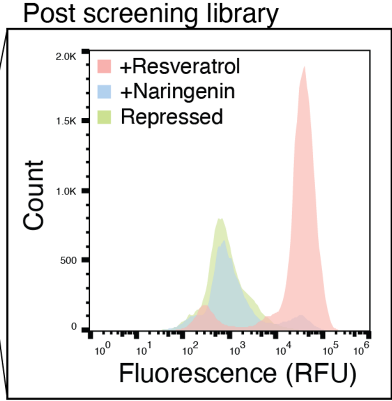
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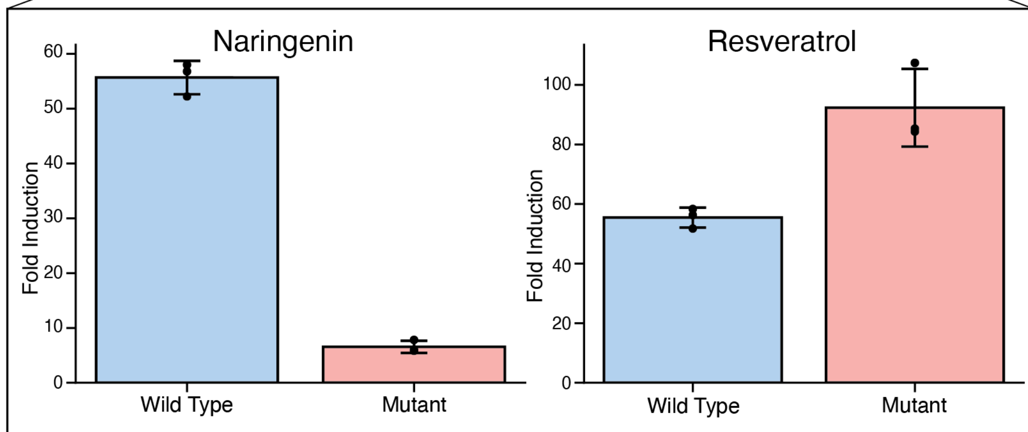
Naringenin
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Resveratrol
Fold Induction
Wild Type
Mutant

Fig 1: Design of resveratrol-specific TtgR variant. Resveratrol conformers are docked into TtgR followed by Rosetta-based computational design of the binding pocket. Candidates with favorable Rosetta score metrics (green points) are synthesized and cloned into an expression vector. Distribution of fluorescence in cells containing uninduced TtgR variant library (light green), induced with naringenin (light blue) and resveratrol (red) before sorting (Pre-Sort) and after three rounds of sorting (Post-Sort) are shown. Colony screening identified a quadruple mutant showing resveratrol specificity: C137I/I141W/M167L/F168Y. The quadruple mutant phenotype was compared to wildtype in biological triplicate (n=3) by inducing each with either 1000μM naringenin or 100μM resveratrol. The error bars denote the standard deviation of the fold induction for the triplicate measurements.

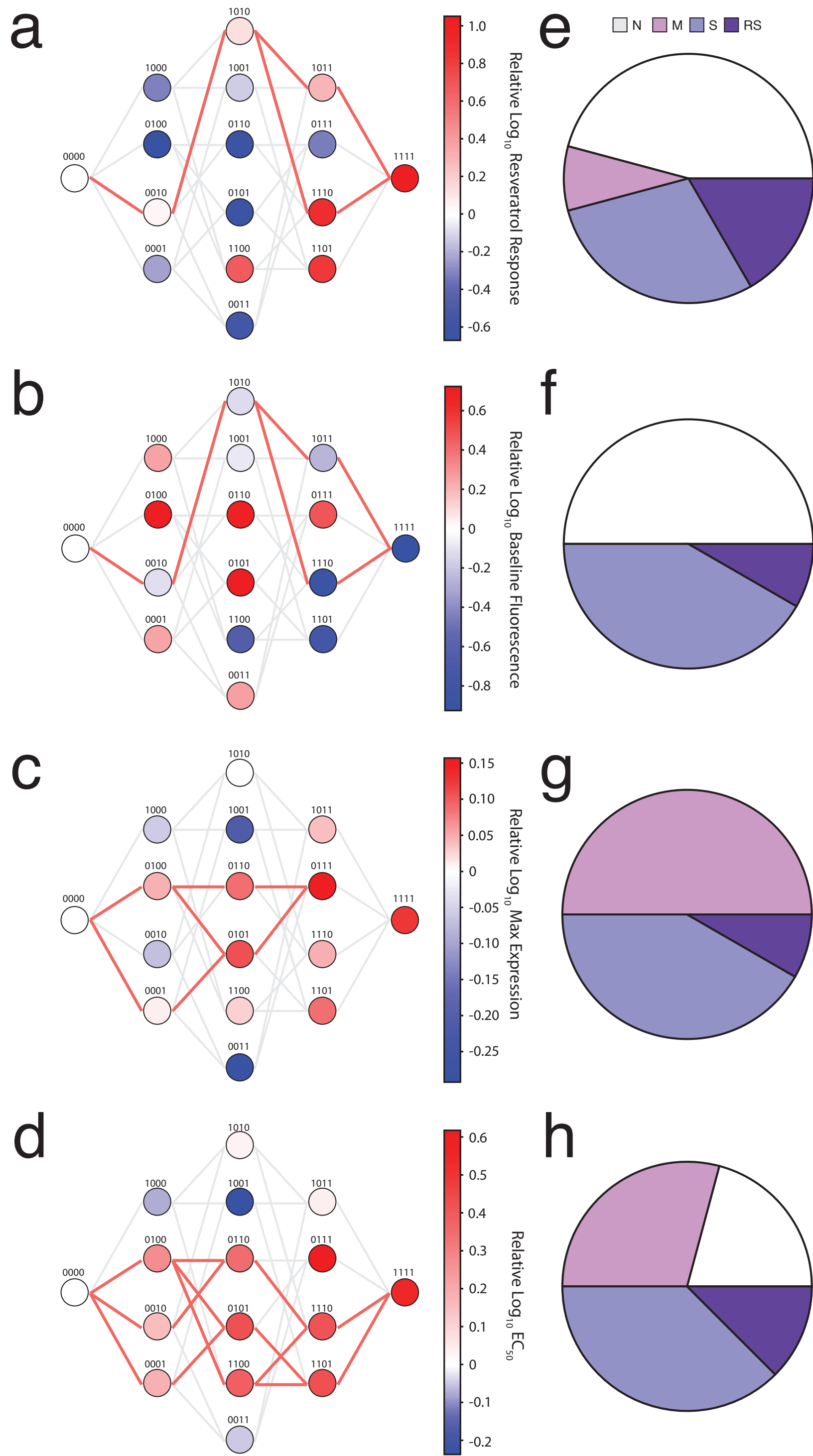
Understanding the role of epistasis in the design of allosteric proteins

Allostery is a fundamental mechanism by which proteins recognize environmental cues (such as binding of an inducer or effector) within a localized region resulting in modulation of function at a distal site. Mutations in the binding pocket that trigger the allosteric network have the potential to create new nonspecific epistatic interactions at the level of protein function beyond the physical interactions commonly seen in specific epistasis and can create complex nonspecific interactions. For an allosteric transcription factor (aTF), function is the outcome of three parameters: affinity for the inducer ligand, affinity for DNA, and allosteric changes that accompany binding to the ligand. Each of these parameters will have its own fitness function mapped over the same sequence space, creating unique fitness landscapes. An aTF simultaneously traverses these multiple fitness landscapes, which collectively govern the evolutionary trajectory of the aTF under selective pressure.

To investigate how the same set of binding pocket mutations might uniquely affect each parameter, we constructed the fitness landscape of each parameter individually. We quantified the number of viable pathways in the resveratrol landscape by requiring that each additional mutation must increase parameter fitness if the quadruple mutant performs better than wildtype or decrease parameter fitness if the quadruple mutant performs worse than wildtype. There are 24 possible pathways from wildtype to quadruple mutant (Fig. 2). Each functional parameter shows distinctive patterns of epistasis, although some are closely related.

Our results highlight the dependence of epistasis on protein function and the prevalence of distinctive adaptive landscapes for multiple functions within the same set of mutations. This process highlights the functional tradeoffs that occur during an evolutionary process and raises the implication that proteins with multiple functions may readily traverse nonoptimal sequence space through varying selective pressures. These landscapes can thus become interconnected by changing selection pressures between different protein functions. On an evolutionary scale, simultaneously changing protein sequence and selection pressure may enable improbable trajectories by bypassing epistatic barriers to

reach previously inaccessible mutational states. In our case, higher order epistasis which prevents access to the quadruple mutant in the naringenin fold induction landscape, could be bypassed by toggling between naringenin and resveratrol selection pressures. The evolution of allosteric proteins is inherently dependent on epistasis and the interactions arising between mutations in these proteins uniquely affects multiple adaptive landscapes.



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Relative Log10 Max Expression
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-0.15
-0.20
-0.25
0.5
0.3
0.1
-0.1

Figure 2: Fitness landscapes for multiple functional parameters in response to induction with resveratrol. Fitness landscapes of **(a)** fold induction, **(b)** basal gene expression, **(c)** maximum gene expression, and **(d)** EC_{50} parameters for all 16 TtgR variants in response to resveratrol with each variant shown as a node in the graph. Each variant is labeled with a binary string corresponding to the presence (1) or absence (0) of a mutation at position 137, 141, 167, or 168 in order. Nodes separated by a single mutation are connected by edges showing viable (bold red) and unviable paths (light gray) through sequence space. Nodes are shaded by $\log_{10}$ of the fitness parameter at 250μM resveratrol normalized to the fitness of wildtype TtgR. Number of epistatic subnetworks in the resveratrol **(e)** fold induction, **(f)** basal gene expression, **(g)** maximum gene expression, and **(h)** EC_{50} landscape determined by Bahadur expansion. Non-epistatic subnetworks (N) are shown in white, magnitude epistasis (M) in pink, sign epistasis (S) in light purple, and reciprocal sign epistasis (RS) in dark purple.

Redesigning LacI to make novel sugar biosensors

LacI is a transcription regulator activated by disaccharide allolactose. We wanted to expand the specificity of LacI to recognize new sugars. A goal of this grant proposal was to improve the stability of scaffolds for design. We combined evolutionary sequence selection and Rosetta-based energy filtering to choose stable scaffolds and found that is dramatically-reduced inactive LacI variants. Over 80% of LacI variants mutated without evolutionary selection could not bind to DNA either due to allosteric dysfunction or loss of stability. We find that only 5% of LacI variants based on evolutionary sequence selection are not able to bind to DNA (Fig. 3). This demonstrates that evolution-guided Rosetta design can select functional scaffolds for biosensor design. We tested this new design protocol that combined evolutionary information with Rosetta design to avoid mutating hotspot residues that may affect allostery or thermodynamic stability. We successfully redesigned LacI to bind to four sugars – melibiose, raffinose, mannobiose and trehalose (Fig 3a). We solved the crystal structure of redesigned LacI bound to melibiose (Fig. 3b).

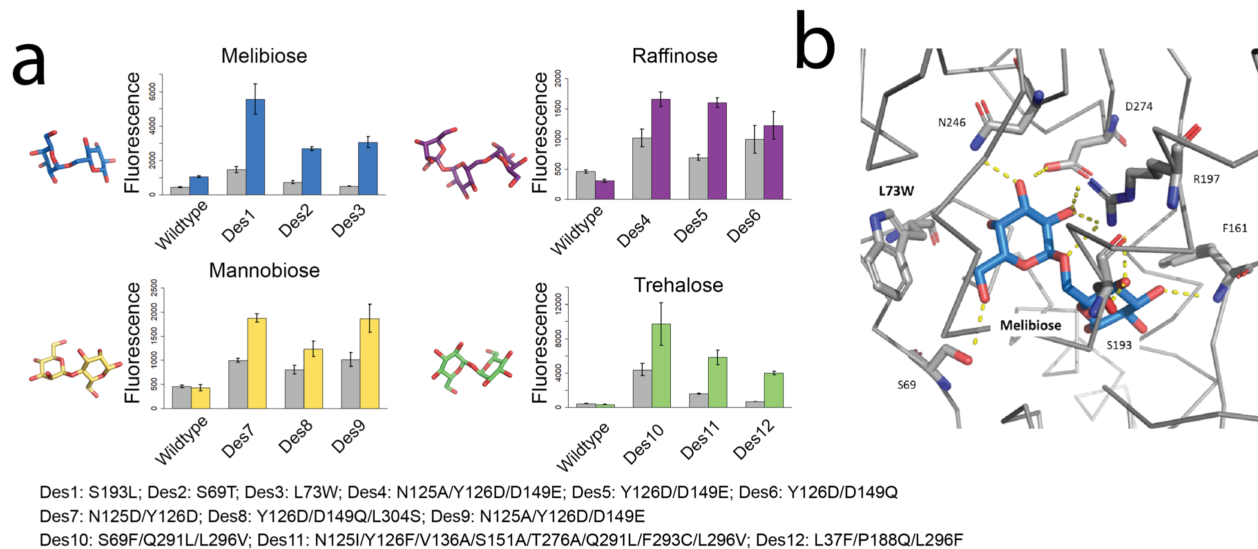

Fig. 3: Expanding specificity of LacI. (a) Redesigned LacI responding to four new sugars – melibiose, raffinose, mannobiose and trehalose. Barplots show fluorescence reporter levels without (gray) and with ligand (colored). The different design sequences are shown below the figure. (b) crystal structure of redesigned LacI bound to melibiose