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Emerging maps of allosteric regulation in cellular networks

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Abstract

Allosteric regulation is classically defined as action at a distance, where a perturbation outside of a protein active site affects function. While this definition has motivated many studies of allosteric mechanisms at the level of protein structure, translating these insights to the allosteric regulation of entire cellular processes - and their crosstalk - has received less attention, despite the broad importance of allostery for cellular regulation foreseen by Jacob and Monod. Here, we revisit an evolutionary model for the widespread emergence of allosteric regulation in colocalized proteins, describe supporting evidence, and discuss emerging advances in mapping allostery in cellular networks that link precise and often allosteric perturbations at the molecular level to functional changes at the pathway- and systems-level.

Introduction

Cellular networks are highly interconnected: in a single cell, thousands of different proteins and their ligands interact to perform diverse biological functions, including metabolism, biogenesis of cellular structures, differentiation, motility, and self-replication. Seemingly distinct processes must be coordinated in space and time, and in response to intra- and extracellular signals. This interconnected regulation requires mechanisms to integrate multiple signals by dynamically tuning one pathway's activity in response to changes in other processes. Six decades ago, Jacob and Monod famously put a name to one such means of integration: allosteric control [1].

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Christopher Mathy: Conceptualization, Writing – original draft, Writing – review & editing, Visualization.

Tanja Kortemme: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest statement

Nothing declared.

The concept of allosteric control - first described within a pathway - arose from observations of endpoint inhibition by Novick, Szilard [2] and Umbarger [3], in which the final product of a metabolic pathway inhibited the activity of the pathway's first enzyme but not downstream enzymes. The chemical dissimilarity between the final product and the first enzyme's substrate suggested the inhibitor did not act as a steric analogue of the substrate but rather bound at an alternative site [4]. The two-site argument was supported by results from Monod's student, Changeux, who found that certain regions of l-threonine-deaminase could be modified to selectively block inhibitor activity without full loss of substrate catalysis [5]. Allosteric control was immediately recognized to align with other emerging structural mechanisms in cellular regulation such as Pauling's model of cooperativity in oxygen binding by hemoglobin [6,7] and Koshland's theory of induced fit [1,8]. These powerful models provided starting points for a wealth of methodological and conceptual advances on structural mechanisms of allostery; we refer the reader to several excellent recent reviews on the more general ensemble-based descriptions of allostery [9] and methods for predicting [10,11] and engineering [12] allosteric coupling in protein structures.

Here, we focus on the broad potential for allostery to drive regulation of any cellular pathway (Figure 1), as theorized by Jacob and Monod in their original concept: they posited that since "there is no obligatory correlation between specific substrates and inhibitors of allosteric enzymes, the [allosteric] effect need not be restricted to 'endproduct' inhibition", and instead "*any* physiologically useful regulatory connection, between any two or more pathways, might become established by adequate selective construction of the interacting sites on an allosteric enzyme" (emphasis in original) [1]. This proposed universality is tantalizing, to the point that Monod famously told a colleague, "I think I have discovered the second secret of life" [13]. Yet such widespread occurrence of allosteric mechanisms in cellular regulation remains largely unexplored, despite its potential prevalence and importance. Allosteric regulation in a pathway enables conditional responses, feedback, and crosstalk with other pathways. The presence or absence of allosteric regulation thus influences our selection of appropriate quantitative models for predicting pathway activity in differing conditions, or in response to perturbations such as mutations, posttranslational modifications (PTMs), or therapeutic interventions.

In this review, we first examine theoretical arguments and recent experimental results in support of the likelihood of allosteric regulation emerging in virtually any cellular pathway; next we outline a general approach uniting molecular perturbations and phenotyping at the cellular level to identify allostery; finally, we highlight the role of systematic mutagenesis and technological advancements in high-throughput quantitative protein biochemistry in supporting unbiased mapping of allosteric regulation in cellular networks.

The evolutionary likelihood of allostery: models and evidence

In principle, allosteric regulation might not only be likely but expected in any pathway if (i) there is a general process by which allosteric coupling can emerge between distal and functional sites of a protein in the pathway; and (ii) there exists a molecular mechanism linking the regulation of that protein function to evolutionary selection.

An elegant evolutionary model of the emergence of allostery following protein-protein interaction formation was introduced by Kuriyan and Eisenberg (Figure 2A) [14]. They argued that colocalization - and the resulting high local concentration - enables weak, promiscuous interactions between two proteins to nonetheless result in complex formation at sufficient frequency for subsequent mutations to exert a fitness advantage, enabling evolutionary selection. Importantly, these new interactions can be local or distal to existing functional sites. Additional mutations that could establish new or modulate existing allosteric coupling between an active and a distal interface site are then in principle also accessible to functional selection.

Recent experimental studies using ancestral sequence reconstruction support this model. In ancestral sequence reconstruction, homologous sequences of a protein of interest are identified from diverse species and aligned. Phylogenetic algorithms then estimate likely ancestral sequences at various evolutionary timepoints, which can then be recombinantly expressed and functionally characterized. Hadzipasic and colleagues used ancestral sequence reconstruction to investigate the emergence of allosteric activation of human Aurora A kinase (AurA) by the microtubule-associated spindle assembly factor TPX2 [15]. The results suggest that AurA autophosphorylation preceded the appearance of TPX2, that a high-affinity interaction between AurA and TPX2 evolved soon after the appearance of TPX2, and finally that additional mutations in AurA established allosteric activation of AurA by TPX2 binding (Figure 2B). The authors suggest that colocalization drove this evolutionary trajectory, with the AurA-TPX2 interaction providing an initial fitness advantage through delivery of AurA to spindle microtubules, and additional allosteric mutations accumulating thereafter.

A related study tracked the acquisition of different oligomerization states and cooperativity in ancestors of the model allosteric protein, hemoglobin (Hb) [16]. Pillai and colleagues suggest that the last common ancestor of both tetrameric Hb and monomeric myoglobin (Mb) was a monomer, and evolution proceeded first through variants forming non-cooperative heterodimers of the alpha and beta subunits (paralogs resulting from a gene duplication event), only then followed by tetrameric variants showing allostery-mediated cooperativity (Figure 2C). Unlike the AurA example above where allosteric coupling evolved after complex formation, here the authors found that the residues mediating coupling between the heme-oxygen binding site and the oligomeric interface were already present in the reconstructed monomeric ancestor. Taken together, the AurA and Hb cases illustrate both possible orders of events for the acquisition of allostery: In the globin fold the structural mechanism of allostery was present before functional selection for physiological hemoglobin activity, whereas allostery evolved in AurA through functional selection after complex formation.

In a third study of note, Fauré and colleagues developed a high-throughput approach using experimental deep mutagenesis combined with machine learning to distinguish the effects of mutations on binding affinity and stability in two small protein interaction domains, a PDZ domain and an SH3 domain [17]. Their finding of broad allosteric coupling between distal and peptide binding sites in these common domains lends further support to the model for widespread emergence of allostery, and provides a generalizable platform for identifying

allosteric sites coupled to a specific interaction interface. Furthermore, such a platform enables large-scale predictions of allostery prevalence drawn from computational [18] and structural techniques [19] to be tested in systems closely matching the native biological networks.

While the Kuriyan-Eisenberg model establishes how allosteric mechanisms could readily build on newly formed interactions driven by colocalization, there also must be a sufficiently strong selective pressure for allosteric regulation. That is, although a new interface may be allosterically coupled to a distal functional site it does not necessarily follow that a perturbation (such as binding) at that new interface will provide a fitness advantage. The hemoglobin study is an example of this principle: allosteric regulation is readily accessible in the globin fold, but the functional requirements of the organism determined whether evolution drove a protein towards allosteric regulation, as seen in hemoglobin, or not, as in myoglobin. Additional examples of how allosteric regulation can provide such a fitness advantage for newly evolved protein interactions have been reported for the evolution of oligomeric state [20,21] and the activation of MAP kinases by scaffolding proteins [22].

A key constraint in this model of allostery evolution is that small energetic changes induced by allosteric mutations must be amplified to cause large enough cellular effects to increase fitness. While the thermodynamic effect of a single mutation is boosted for symmetrical oligomers by the simple fact of each mutation occurring multiple times, more nuanced mechanisms of signal amplification have been described for monomeric allosteric proteins. For example, ultrasensitive protein switches amplify small changes in activity due to covalent modifications [23], often further magnified by linking several switches serially in a “cascade” [24]. Several cyclin-dependent kinase (Cdk) switches undergo allosteric regulation, such as the cooperative increase in Cdk1 activity after priming by an initial phosphorylation event [25] or the allosteric coupling between cyclin binding, phosphorylation, and inhibitor binding that determine cyclin and inhibitor selectivity in Cdk2 [26].

Linking allostery from the molecular to the cellular scale

While the above models and experimental results support an expectation of widespread allostery, the true incidence of allosteric regulation in any cellular network is unknown. Since allosteric control of cellular processes by definition spans the molecular and cellular scales, we advocate for an approach of constructing unbiased allosteric regulatory maps by uniting molecular perturbations with comprehensive systems-level profiling (Figure 3A). Such an approach builds on long-standing efforts to map gene regulatory [27], metabolic [28], genetic [29], and protein-protein [30,31] networks using gene perturbations, but with one key extension: by employing targeted structural perturbations such as point mutations, the cellular effects of perturbations at potential allosteric sites can be directly compared to perturbations at active sites or other known functional sites.

Such an approach was recently described by Perica, Mathy, and colleagues to probe the cellular effects of targeted perturbations to the molecular switch Ran/Gsp1 [32], a small GTPase that simultaneously controls distinct cellular pathways via a large network of

partner proteins, dependent on GTPase cycling between active and inactive conformations. Amino acid point mutations targeting binding interfaces with all structurally characterized Gsp1 partners were introduced into the *S. cerevisiae* GSP1 gene, and the resulting strains were assayed by genetic and physical interaction profiling to measure the systems-level cellular effects of each perturbation. Unexpectedly, the functional effects of the mutations could not be inferred in a simple manner from their position on the GTPase structure: mutations in distinct Gsp1 interaction interfaces with different partners could show more similar systems-level functional outcomes than those targeting the same interface, and, strikingly, mutations in the same interface or even at the same sequence position could affect different subsets of cellular processes. Instead, biophysical experiments (nuclear magnetic resonance and enzyme kinetics) revealed that the perturbations in partner interfaces distal from the GTPase active site allosterically affected the kinetics of switching of the GTPase between its on- and off-states, and that these kinetic changes determined the functional cellular outcomes.

Each scale of measurements in this study provided complementary insight (Figure 3B–D). Structural and biochemical characterization were essential for distinguishing allosteric from local perturbations, for quantifying the relative thermodynamic effects of different perturbations, and for discovering that sites in more than one interface allosterically regulated the GTPase switch. Nuclear magnetic resonance experiments confirmed that distal point mutations - more than 18 Å away from the active site - shifted the relative populations of two conformations in the active site, and that this population shift explained allosteric effects on GTP hydrolysis. Concurrently, the data on genetic and physical interactions revealed distinct pathway changes that resulted from different allosteric perturbations to the kinetics of switching, thereby linking the molecular perturbations to several distinct regulatory functions of Gsp1. Integrating molecular perturbations, biochemical characterization, and cellular systems-level profiling was thus essential for defining the mechanism of how Gsp1 achieves coordinated regulation of downstream pathways via allosteric control, and provides a proof-of-concept for this multi-scale approach that could be used to uncover similar principles in other cellular pathways.

Moreover, once such a regulatory map is established for a set of key sites in a protein of interest, the map can be used to interpret the functional effects of additional perturbations. For instance, high-throughput mutational scanning assays could provide the scalability to build comprehensive maps of allosteric sites important for cellular function across an entire protein structure. In the case of Gsp1, fitness measurements of all possible single point mutations revealed over twenty previously unknown sites in the GTPase fold that are allosterically coupled to cellular switch function [33].

Systematic mutagenesis as a key tool for investigating allostery

The success of employing mutations to identify allosteric regulation lies in their precision: unless they notably destabilize the targeted protein, their effects can distinguish between different functions of pleiotropic proteins, unlike gene knockouts which affect all functions of a given gene. Because amino acid mutations are introduced into a specific gene, the perturbation is also linked to a single protein of interest, unlike promiscuous drugs

or environmental perturbations which can act system-wide. And the ability to track the differential effects of 20 different amino acid chemical groups at a given structural position provides further functional variety with which to probe mechanistic questions.

A necessary qualifier for the use of mutations to study allostery is their physiological appropriateness, as cells do not regulate protein activity using mutations, but rather through processes like binding, localization, and PTMs. Yet, mutations can be functionally analogous to physiological perturbations, when the effects induced by a mutation are similar to the effects induced by binding or chemical modification (for example, aspartate is often used as an, albeit imperfect, mimic of phospho-serine). More formally, double mutant cycles can be used to determine whether residues at any two sites in a protein are thermodynamically coupled, meaning that the change in stability or function upon a mutation at one site changes in the background of a mutation at the other site [34]. It is this coupling which can be exploited by allosteric binding interactions or PTMs. In this way, mutations can not only probe for sites of allosteric regulation, but also provide mechanistic insight.

Given the usefulness of mutational analysis, technologies that enable precise mutagenesis and quantitative and functional variant profiling in high-throughput are essential tools to investigate allosteric regulation. The rapidly growing field of deep mutational scanning has developed from early studies of short regions of proteins in prokaryotes and fungi [35,36] to now enable systematic full-length scanning of proteins in diverse cellular contexts [33,37,38], including in mammalian cells [39] for interrogation of disease-associated proteins [40], and challenging-to-study membrane proteins [41,42]. Recent breakthroughs in genome editing for targeted mutation of endogenous genomic loci [43,44] allow for stable mutant cell lines to be assayed for regulatory differences, laying the groundwork for information-rich phenotypes [45] to be collected in deep mutational scanning analyses.

At the same time, more complex and scalable *in vitro* systems hold great potential for measuring the quantitative functional effects of allosteric mutations in carefully controlled biochemical contexts. For example, a new platform developed by Markin, Mokhtari, and colleagues named HT-MEK (High-Throughput Microfluidic Enzyme Kinetics) uses *in vitro* transcription-translation in microfluidic chips and fluorescently-labeled reactants to measure the catalytic activities of over a thousand enzyme variants in a single experiment [46]. By assaying variants of bacterial alkaline phosphatase PafA harboring mutations at sites throughout its structure, the authors identified several surface sites that are allosterically coupled to catalytic activity. The platform is amenable to repeated measurements of multiple substrates at different concentrations, allowing for rate constants to be measured in the context of candidate allosteric protein or small molecule binders. Such biochemical precision can enable improved modeling of metabolic networks in response to allosteric perturbations, and also comparison of the magnitude of effects of different classes of structural perturbations such as ligand binding or PTMs.

Integrative approaches to uncover allostery

Jacob and Monod foresaw that allosteric regulation, being by nature less constrained by structural requirements than orthosteric regulation, would need to be studied by systematic,

unbiased methods. They wrote that “it is to be hoped that, in future years, systematic attempts will be made to verify whether or not certain hormones may not actually act as allosteric inhibitors, inducers, or repressors of certain enzyme systems. The main difficulty of this research will be that no guiding chemical principle (based on steric analogy, reactivity, etc.) will help the investigator in the selection of which enzyme systems to test, since again the specificity of induction-repression and of allosteric inhibition is apparently completely independent of the structure and specificity of the controlled enzyme itself” [1].

This challenge of selecting which enzymes to test rings true to this day, but fortunately interdisciplinary approaches can help leapfrog the problem entirely: we could perhaps test them all. In a recent study of note, Cappelletti and colleagues used limited proteolysis followed by mass spectrometry (LiP-MS) of whole cell lysates to identify regions of proteins undergoing conformational change (inferred by differential proteolysis) in response to environmental stress in *S. cerevisiae* and nutrient adaptation in *E. coli* [47,48]. By mapping peptides with altered proteolysis patterns onto protein structures, the authors recapitulated known allosteric sites of metabolic enzymes as well as active sites undergoing structural changes in other proteins. LiP-MS provides an unbiased approach for globally discovering several types of functional sites, such as ligand-binding, protein-binding, and PTM sites, and can directly distinguish between orthosteric and allosteric regulation when coupled with structural information. Structural information for entire proteomes, with increasingly useful accuracy, has been generated using deep-learning based structure prediction methods [49,50].

Finally, the usefulness of allosteric regulatory maps rely strongly on our ability to comprehensively interrogate altered cellular functions, increasingly using high-throughput imaging approaches [51–54], and on the use of new informative models of the effects of allostery to extract mechanistic knowledge from large-scale data. These efforts could be aided by integrative structural biology [55–58], mechanistic kinetic models of signaling which explicitly represent allosteric regulation [59], and formalized representations of protein structural information in cellular interaction network models [60,61].

Conclusion

While the allosteric regulatory networks of cellular systems may very well be as vast and intricate as Jacob and Monod imagined, we believe the concepts outlined here and increasing technological advances provide a promising outlook for systematic, unbiased interrogation of allosteric regulation. The resulting allosteric maps will drive better understanding of how our cellular networks respond to environmental stimuli and therapeutic interventions in both healthy and diseased states, and provide new ways to predictively control cellular behaviors.

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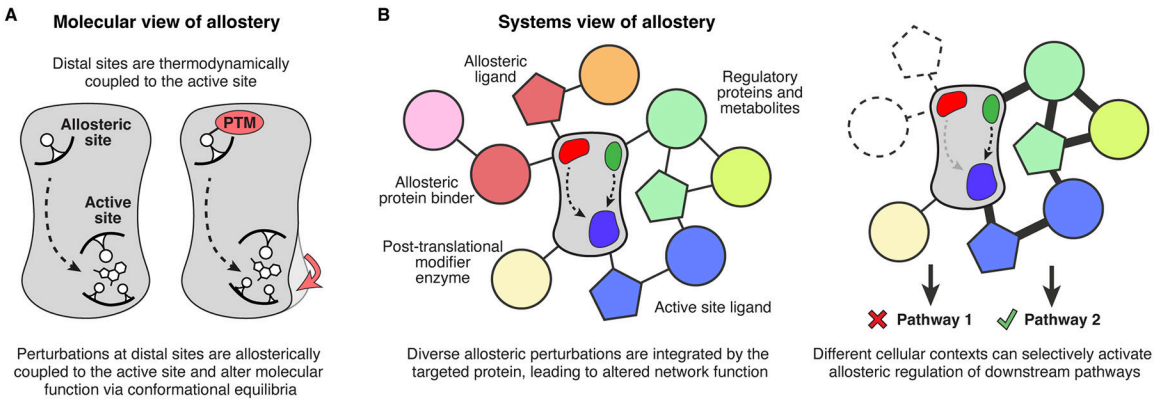

Figure 1. An integrative view of allostery.

(a) At the molecular level, allostery requires thermodynamic coupling between two distinct sites of a protein (left), for example an active site for binding of a small molecule ligand and a distal residue targeted by a post-translational modification (PTM, right). Perturbations at the regulatory site alter biochemical function at the active site. **(b)** In cellular networks, allostery enables independent regulatory mechanisms to control a central switch protein (left). Allosteric proteins can thus serve as network hubs, integrating signals from regulatory proteins (colored circles) and ligands (colored pentagons), and transmitting the signals to one or more downstream pathways. A key feature of network hubs is the selective activation of downstream pathways via independent regulation events (right) [32,62].

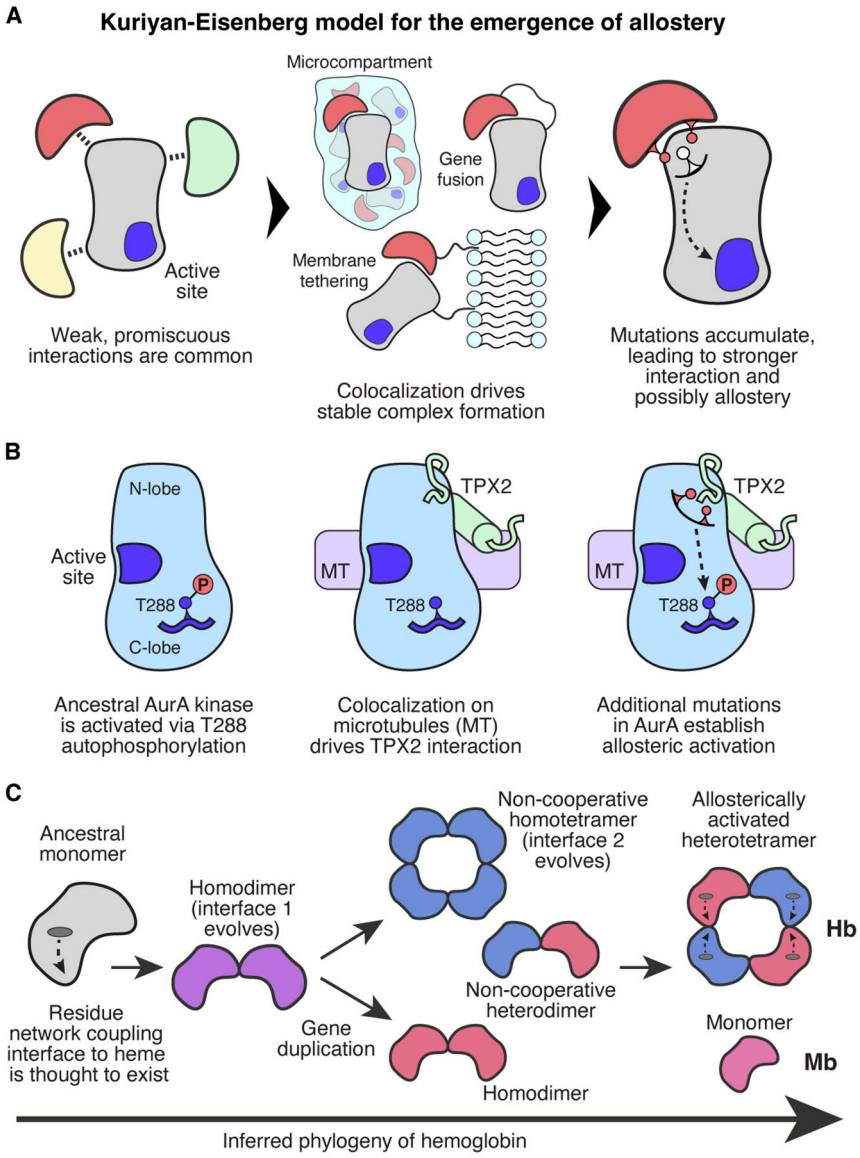

Figure 2. A model for the widespread emergence of allostery in cellular networks.

(a) A general process by which proteins readily evolve new interactions and allosteric regulation was introduced by Kuriyan and Eisenberg [14]. Proteins that interact only weakly in solution can form complexes when colocalized due to an increase in effective concentration. Additional mutations that strengthen the interaction or establish allosteric regulation can evolve. **(b)** Inferred emergence of allosteric regulation of AurA kinase by TPX2, as described by [15]. **(c)** Inferred emergence of heterotetramerization and allosteric regulation in hemoglobin, as described by [16].

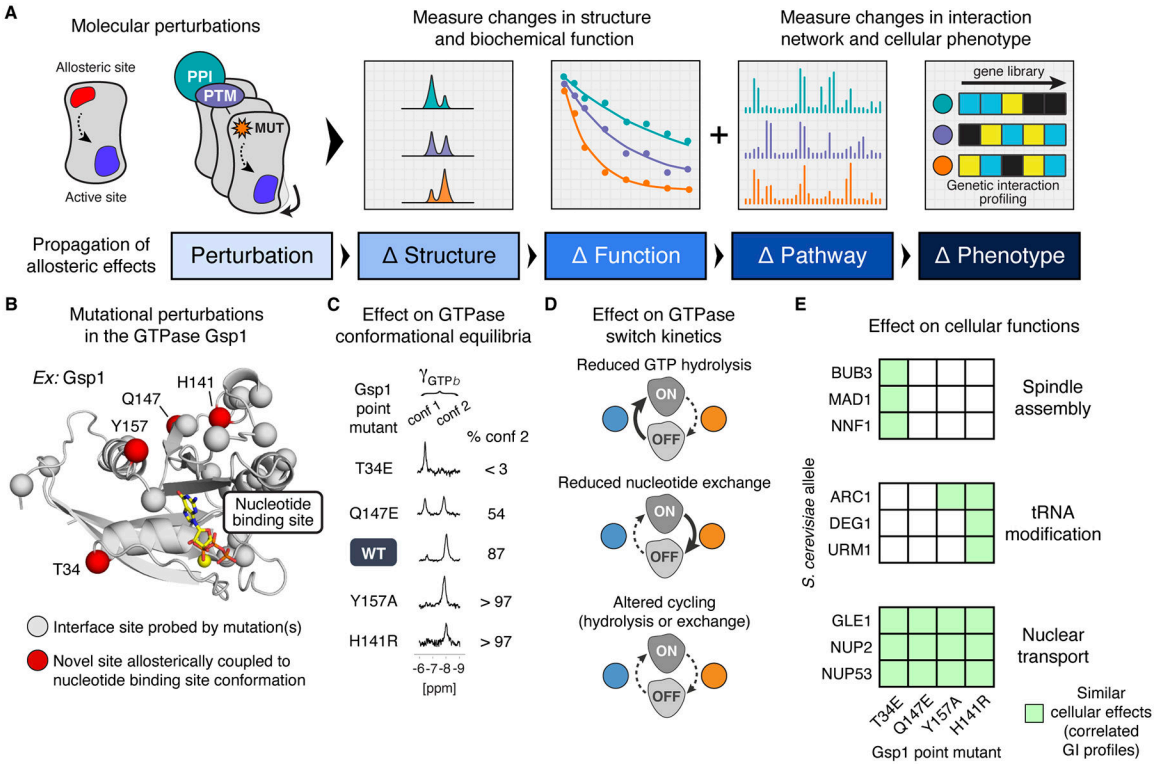

Figure 3. A general approach for unbiased mapping of allosteric regulation by uniting molecular perturbations with systems-level profiling.

(a) Interrogation of allosteric regulation in cellular networks is achieved by systematically introducing molecular perturbations and measuring changes at each of the following scales: protein structure and dynamics (e.g. population of distinct conformations), biochemical activity (e.g. enzymatic rates or binding affinities), protein interaction networks (e.g. protein complex abundance and localization), and cellular phenotype (e.g. genetic interactions, cell morphology, and fitness). **(b-d)** Implementation of the approach for the eukaryotic small GTPase Ran/Gsp1, an allosterically regulated and multi-specific model protein switch, as described by [32]. **(b)** A series of 55 point mutations at 24 sites were introduced individually to the endogenous *GSP1* gene in *S. cerevisiae*, targeting structurally characterized protein interaction interfaces outside of the nucleotide binding site. The unbiased approach identified four previously uncharacterized sites (shown in red) to be allosterically coupled to the nucleotide binding site conformation. Mutation of these sites changed conformational equilibria at the active site (**c**), thereby altering the kinetics of the GTPase cycle (**d**) and selectively perturbing pathways regulated by Gsp1 (**e**). **(c)** 1D ^{31}P NMR spectra of Gsp1 variants bound to GTP showing that mutations at novel allosteric sites perturbed the relative populations of two conformations differing in the chemical shift of the terminal γ-phosphate of the bound GTP ligand (γ_{GTPb}). (**d**) Gsp1 mutations have differential effects on the GTPase switch kinetics. (**e**) The effects on kinetics selectively determined the effect on systems-level pathways as measured by genetic interaction (GI) profile correlations, which annotate the cellular effects of Gsp1 mutants by comparing them to the effects of knockdown of genes in various pathways: mutants with primarily reduced GTP hydrolysis (dotted arrow next to the orange GAP regulator in (**d**)) affected spindle assembly, mutants

with primarily reduced nucleotide exchange rates (dotted arrow next to the blue GEF regulator in (**d)**) affected RNA modification, and all mutants altering cycling (hydrolysis and/or exchange) affected nuclear transport.