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Why Does Binding of Proteins to DNA or Proteins to Proteins Not Necessarily Spell Function?

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ABSTRACT Studies of binding are often question: first, is the observed binding functional, and second, if it is, which function? Is it activation or repression? The first question relates to binding at *different* sites; the second relates to binding at *similar* sites. These questions apply to transcription factors binding to genomic DNA and to protein interaction domains binding to their partners. Here, we explain that both can be understood in terms of allostery and the cellular (or *in vitro*) environment. The idea is simple yet powerful; it emphasizes the role of allostery in defining whether binding between transcription factors and (cognate or noncognate) DNA sequences will lead to function and to the type of function. Allosteric effects are the outcome of dynamically shifting populations; thus binding to even slightly different DNA sequences will lead to different transcription factor conformations that can be reflected in the binding sites to their co-regulators. Currently, allostery is not considered when trying to understand how binding phenomena determine the functional outcome. Allosteric effects can enhance the binding specificity in a function-oriented manner. Here we provide a biological rationale that considers cellular crowding effects.

To be functional, proteins need to bind their partners; expressing function in the cell entails a network of binding events. Yet, *in vivo* and *in vitro*, binding of transcriptional control (TC) proteins to their cognate DNA response elements (REs) or of protein molecules to their protein partners does not imply function. Function is based on events taking place following binding, that is, whether the binding leads to specific subsequent binding events as specified by the cellular program. Consequently, identification of the location of the REs in the genome by chromatin immunoprecipitation (ChIP) that obtains protein−DNA interactions is often coupled with microarray readout of these experiments (ChIP-chip) (*1*). Such a combination assists in defining the *in vivo* utilization of genomic sequences by the TC proteins and the functional consequences. Toward this aim, the higher resolution and greater coverage ChiP-seq technique for genome-wide profiling of DNA-binding proteins is also gaining momentum (*2*). In genomic DNA, not all REs are chromatin-available (*3*). However, even for chromatin-available REs, binding of a TC protein does not necessarily indicate function. Similarly, detection of protein−protein interactions by co-immunoprecipitation *via* endogenous (not overexpressed and not tagged) proteins with subsequent Western blotting implies direct or indirect (*via* a bridging protein) binding; however, again, the binding may or may not specify function. Moreover, REs that are very similar, with only a single base pair (bp) change, can lead to vastly different functional consequences (*4–8*). Yet while it is broadly accepted that binding, whether of a TC protein to its RE or between proteins, does not indicate function, the reasons are not entirely understood.

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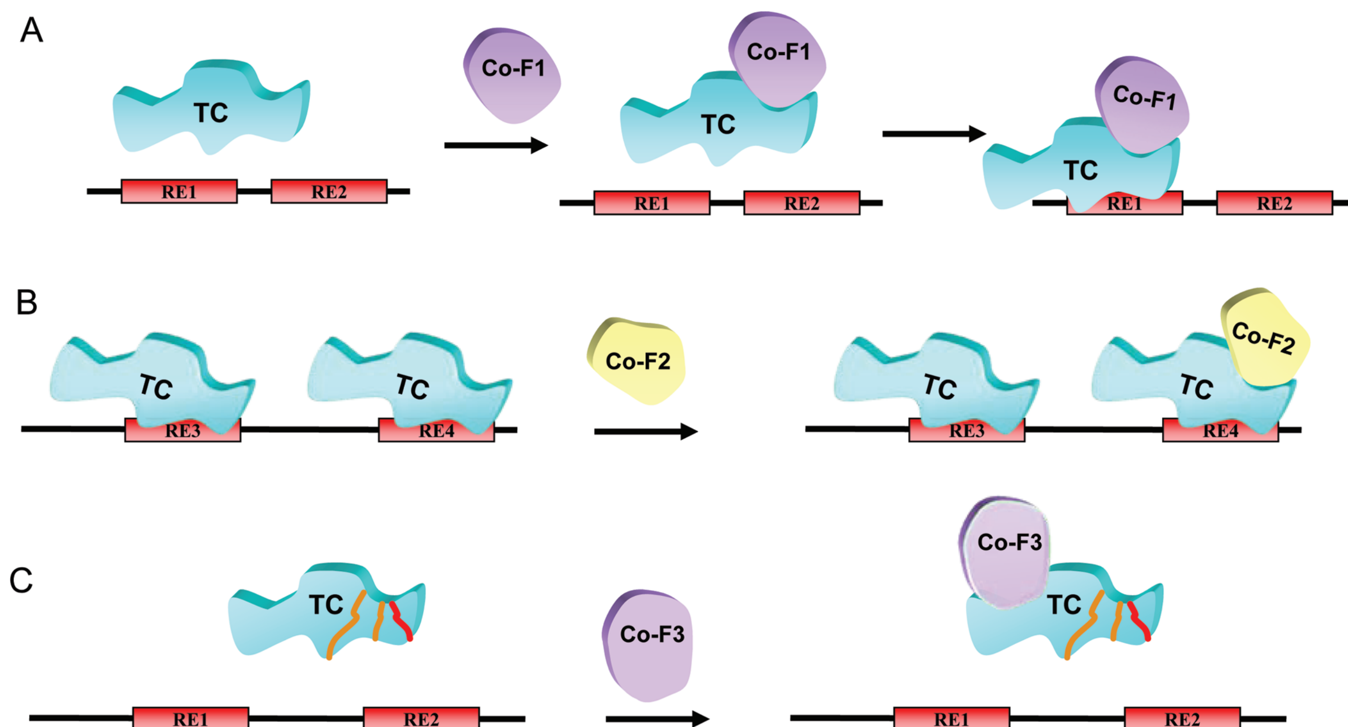


Figure 1. An illustration to explain why binding does not necessarily spell function. The response elements (REs, red boxes) of a given transcriptional control protein (TC) have very similar DNA sequences, with small base pair (bp) changes, yet they regulate genes whose functions can be vastly different. A key question is how the TC selectively binds a given RE among all the similar ones in the genome. A) Affinity of the TC to its REs is low. Binding of a cofactor (Co-F1) to the TC *allosterically* alters slightly the binding site of the TC to the DNA, leading to higher affinity binding to a specific RE. Which cofactor is available and binds depends on the cellular network. In this case, binding already implies specific function. B) The TC has high affinity to its REs; thus it binds to all chromatin-available ones. However, binding is insufficient. Since REs with even slightly different DNA sequences have slightly different conformations, binding to a specific RE *allosterically* leads to changes in the cofactor binding site of the TC, which now selectively binds a cofactor (Co-F2). Which cofactor binds again depends on the cellular network. C) Binding of a protein cofactor (Co-F3) has no functional consequences, since this binding event does not affect the allosteric communication pathways between the functional RE and transcriptionally relevant binding sites.

At one end of the spectrum, some types of specific binding are linked to specific functions (Figure 1, panel A); at the other, specific binding is insufficient. Function is determined by subsequent events (Figure 1, panel B). There are examples for both. For the first (*4*), p53 has a very large number of similar REs in the genome. Binding to certain REs activates DNA repair, cell-cycle arrest, senescence, or apoptosis; binding to others represses or inactivates pathways *via* either involvement of the histone deacetylase, inactivation of other DNA-bound and DNA-unbound activators, or other routes (*5*). The transcriptional repressor REST (NRSF), which encodes DNA binding affinity hierarchies contributing to regulation during lineage-specific and developmental programs, provides another example (*9*). Canonical REST REs bind strongly and control REST targets common to all cell types, whereas atypical motifs involve weak interactions in cell- or tissue-specific targets. Hence, selective binding of a certain RE already determines the functional outcome. The nuclear receptor (NR) provides an example for the other end of the spectrum; NR binding to its REs is insufficient. The functional outcome is determined by subsequent co-regulator, co-activator, or co-repressor, binding events at different sites (*6, 7*). The Apak (ATM and p53-associated KZNF protein), a Krüppel-associated box (KRAB)-type protein (*10*) that regulates p53-dependent apoptosis, provides an example for a yet different mechanism: binding to both p53 TC and to DNA *via* its zinc-finger motif. At the same time, not all binding events away from the co-activator

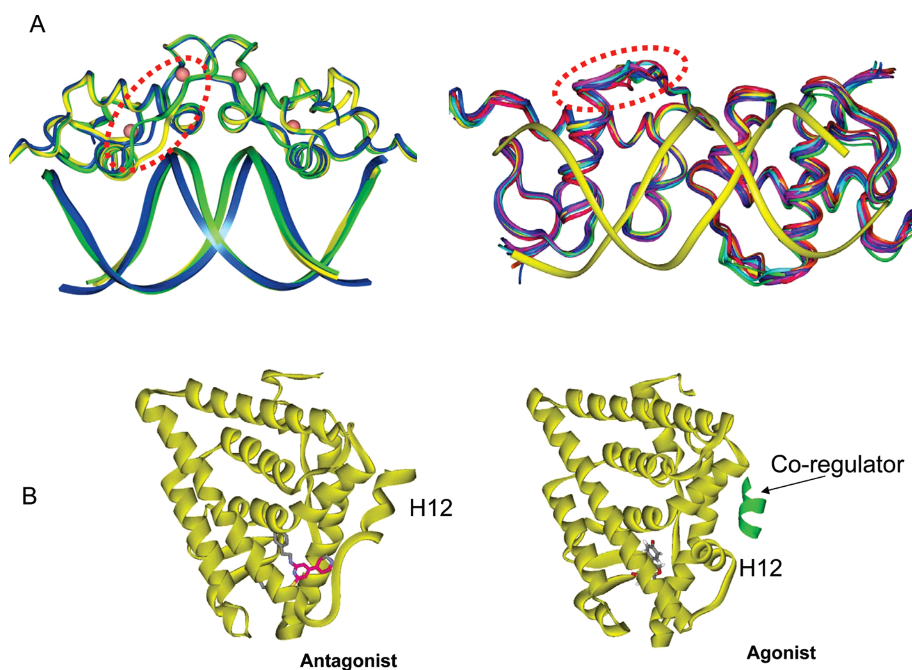
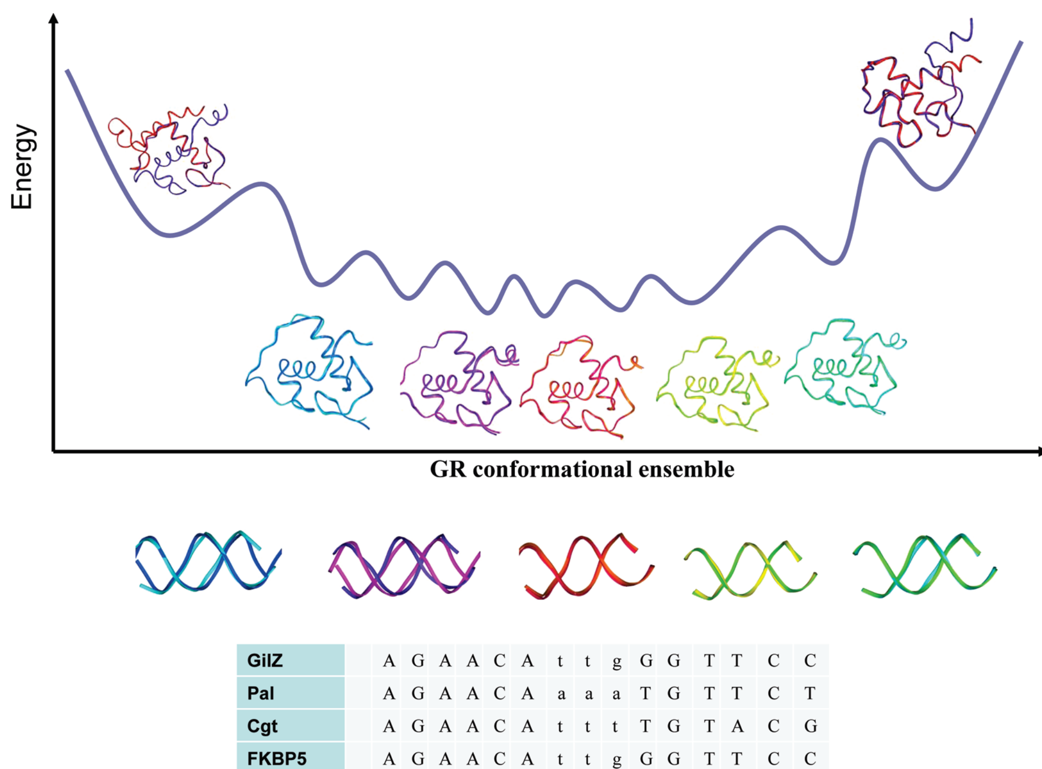


Figure 2. Two examples illustrating how binding at other sites—by an RE (A) or agonist/antagonist ligand (B)—can *allosterically* alter the respective co-regulator binding site conformation leading to activation or inhibition. In the left panel of A, three crystal structures (*6*) of the glucocorticoid receptor (GR) bound to three REs whose sequences are very similar to each other are superimposed. Binding *allosterically* leads to a conformational change at the co-regulator binding site (PDB ids: yellow, 3G99; blue, 3G6P; green, 3FYL). In the right panel, all 15 crystallized GR structures (3FYL, 3G6P, 3G6Q, 3G6R, 3G6T, 3G6U, 3G8U, 3G97, 3G8X, 3G99, 3G9I, 3G9J, 3G9M, 3G9O, and 3G9P) are superimposed and viewed from a different angle showing that the conformational change is far away from the DNA. Here only one RE (CGT in 3FYL) is shown, for clarity. Panel B illustrates the effects of the binding of an antagonist (left panel, PDB 1nde) and an agonist (right panel, PDB 1nde) on coactivator binding to the estrogen receptor (ER) ligand binding domain (LBD) (*14*). The binding of the agonist and antagonist are at the same ER site; however, the antagonist leads to an allosteric displacement of H12 to occupy roughly the same position as the co-activator. Hence, co-activator binding to the LBD is blocked. Binding of the agonist exposes the co-regulator binding site.

binding site are functional (Figure 1, panel C). Why then does binding not necessarily imply function? We propose that to be functional, binding either should be a consequence of allosteric amplification of a minor conformational change, as in p53 and REST, or should lead to it, as in NR, or both, as in Apak. A similar situation can be seen in protein interaction domains (PID) such as PDZ, 14-3-3, Bromo, SH2, SH3, and LRR (*11*). Ligand binding to PID, mutations, or post-translational modifications away from the binding site allosterically alter the PID binding site conformation (*12, 13*). Figure 2 provides two examples illustrating how binding at other sites by an RE (*6*) or agonist/antagonist ligand (*14*) can alter the respective co-regulator binding site conformations leading to activation or inhibition. Cellular fluctuations play key roles in all, either in the initial allosteric event or in subsequent binding events. Similar conformational changes can be elicited by perturbation (binding, post-translational modification) events on major allosteric pathways elsewhere in the structure (*12*). On the other hand, in nonfunctional binding the perturbation site is not on a major pathway between the RE's (or agonist's) and the co-regulator's (Figure 1, panel C). Thus chromatin immunoprecipitation obtains binding; however, microarray readout may not present functional change. This emphasizes the shortcomings in cellular network diagrams: pathways are neither simply sequential nor "yes/no" contingent events. Mechanistically,



GilZ	A	G	A	A	C	A	t	t	g	G	G	T	T	C	C
Pal	A	G	A	A	C	A	a	a	a	T	G	T	T	C	T
Cgt	A	G	A	A	C	A	t	t	t	T	G	T	A	C	G
FKBP5	A	G	A	A	C	A	t	t	g	G	G	T	T	C	C

Figure 3. Simple illustration of conformational selection in terms of the free energy landscape. Several crystal structures of the glucocorticoid receptors and their corresponding REs are used here as examples. GR samples the conformational space around the native state. Conformers lie in distinct local minima separated by low barriers, and their energies differ slightly. All conformers *pre-exist* prior to binding, and each *selectively* binds an RE (*15–19*). NMR has recently validated this theoretical proposition, illustrating that the bound conformers *pre-exist* in the unbound state (*19, 35–37*). Here five superimposed pairs of crystal conformers are shown presenting minor conformational changes. The conformers at the left and right have been manually slightly unfolded to depict higher energy states. At the bottom are the corresponding five superimposed GR REs with very similar DNA sequences. The corresponding PDB structures and REs (from left to right) are 3FYL-3G6P (DNA, CGT-FKBP5), 3G6Q-3G6R (DNA, FKBP5-FKBP5), 3G6T-3G6U (DNA, FKBP5-FKBP5), 3G8U-3G8X (DNA, GILZ-GILZ), and 3G97-3G99 (DNA, GILZ-PAL). The aligned RE sequences (*6*) are also presented, illustrating the minor base pair changes.

pathway steps are the outcome of allosteric response reflected in conformational selection (*15–20*). This mechanistic picture rests on a dynamic view of molecules as ensembles of conformations.

In solution proteins exist as conformational ensembles, which can be described by statistical mechanical laws, and their populations follow statistical distributions (*21–23*). The number of states is vast, the conformational differences are generally small, and the barriers are low (Figure 3). The more flexible the proteins are (as in the case of transcription factors that are often disordered), the larger the number of states. Ample data from single molecule, NMR, and other techniques (*24–34*) validate this description (*19, 35–37*). During binding, higher energy lower population conformers that are most complementary to the ligand are selected and the equilibrium shifts toward these conformers (*15–18*). This validated (*19*) "conformational selection and population shift" model (*15–18*) for molecular recognition provides an alternative to the 50-year old "induced fit" hypothesis (*38*). Relating this description to binding and function, in our first case type (Figure 1, panel A), binding implies function; hence a key question is how the TC protein selects a particular RE among all similar and available REs in the genome (*4*) and similarly, how the PID (*11*), which can have hun-

dreds of partners binding at the same site (*39*), selects a specific one. Since here the affinities are generally low, selection is dictated by prior binding (or post-translational modification) events. These allosterically shift the ensemble toward specific conformations. At the other end of the spectrum (Figure 1, panel B), the TC already binds with high affinity many REs. One or two bps changes are amplified *via* population shifts in the TC (Figure 2, panel A). Similar shifts are reflected in the PID (*11*) following phosphorylation/acetylation or ligand (*e.g.*, agonist/antagonist (*14, 40, 41*), Figure 2, panel B) binding. The outcome is surfaces complementary to a specific co-repressor or co-activator (Figure 1, panel B) (*14*). This explains the fundamental question of how the minor differences elicited by substitutions of single bps among REs or mutational, post-translational modification or ligand-binding events can lead to vastly different functional effects. This question is particularly crucial since protein factors and DNA generally present only small conformational changes. We note that here we assume that one RE is recognized by a specific TC. Although to date no cases of one RE recognized by multiple TCs has been observed, in principle this can happen; under such circumstances the RE can block the TC binding site, mimicking an antagonist.

Gene expression is controlled by cellular networks, which consist of linked processes. Text books, such as *Cell Biology* (*42*) depict processes as diagrams of series of binding events, where one follows the other or is contingent on the other. However, events like those of the nuclear receptor (*5, 6*) question such simple descriptions (Figure 4). Conformational changes elicited by one RE differ from those of another. Eventually, which co-regulator, *e.g.*, acetylase or deacetylase, is selectively recruited depends on co-regulator concentration, post-translational modification states, *etc*., that is, on the network. In turn, the network reflects the cellular environment. Similarly, a PID can bind similar ligands but elicit different conformational changes in the partner-binding site (Figure 1, panel B). Hence, here DNA (or, ligand) binding observed by experiment does not indicate whether it activates or inhibits expression; function depends on sufficiently high concentration of protein factors that recognize a specific binding site conformation amplified by a certain RE (or ligand). On the other hand, in the first case (*e.g.*, of p53 (*4*) or REST (*9*), Figure 1, panel A), binding implicates function. Such a description further sheds light on the often observed low affinity binding: affinity measurements may not reflect *in vivo* scenarios.

From the mechanistic standpoint, we face two problems: first, if binding is at different sites, is it functional or nonfunctional, and second, if at similar sites, what is the outcome, activation or inhibition? Experiments reflect steady-state concentrations; they do not follow the time course of the immense fluctuations in the cellular environment and the consequent allosteric effects. This problem transcends into cellular network binding diagrams that do not reflect this changing selectivity. Yet affinity is a function of allosteric effects, and "yes/no" contingencies cannot mirror such changes. Binding can be a function of concentration or selectivity; in turn, selectivity is the outcome of shifts of the ensemble of conformational states following perturbation events, that is, the outcome of allostery. To increase binding selectivity is then the key role of allosteric events, and allosteric effects are not accounted for in cellular network diagrams.

Binding is not necessarily highly selective, as in the case of the transcriptional repressor CoR or NotchIC binding to CSL (*43*), where binding reflects cellular concentrations; at the same time, cellular events reflect allosteric effects that amplify minor conformational changes, thus spelling higher selectivity. Cellular network descriptions should mirror both. Network modules have been featured from the topological standpoint (*44, 45*). Recent work highlighted the relationship between protein conformational fluctuations and their "promiscuous functions" and how they can greatly facilitate the evolution of new functions. Such mechanisms have been delineated both experimentally (*46*) and theoretically (*47*). Phenomena described here further apply to the biological functions of protein disordered states where similar conformational principles apply (*48, 49*). Here we suggest a mechanistic conformational level description where cellular processes consist of independent components governed by dynamically shifting populations. Such a description accounts for molecular level binding selectivity and incorporates the immense fluctuations in cellular conditions. It is based on a picture of molecules as dynamic conformational ensembles and at the same time explains how evolution minimizes errors in molecular recognition. Development increases functional complexity. We speculate that evolution faced the question of how to exert efficient response to the environment: engineer new control proteins or make use of the conformational space of

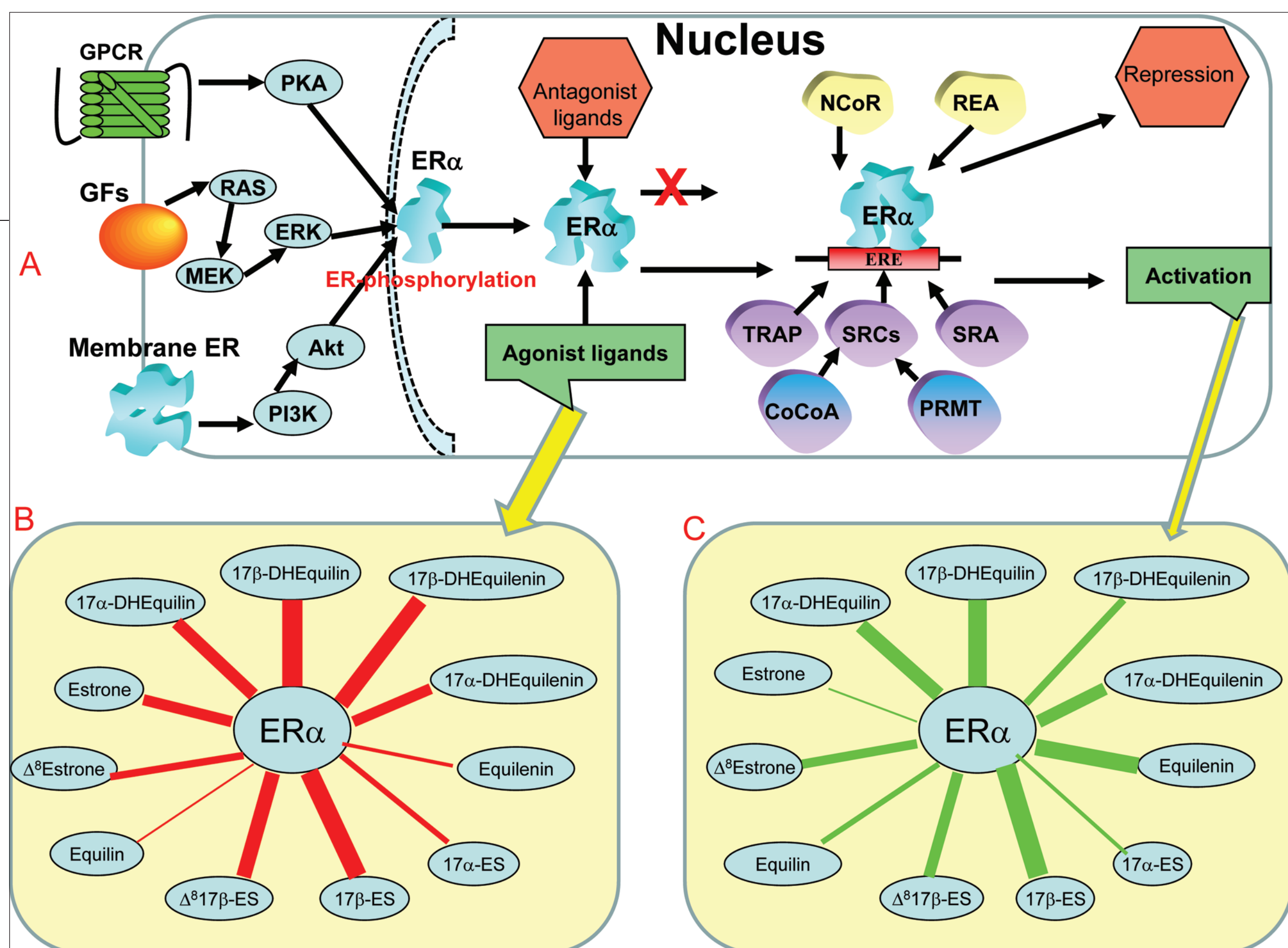


Figure 4. Allosteric regulation underlies the complex binding–function relationship in cellular networks. The figure highlights the inadequacy of current cellular diagrams that depict series of binding events. As an example. we depict the estrogen receptor, for which there are experimental data. Estrogen receptors (Era and ERb) can be selectively activated by ligand binding, with allosteric control of ligand selectivity and function (*55*). A) Schematic "textbook" network diagram of the estrogen receptor (ER) signaling pathways. ER activation is controlled by extracellular signals, hormone and cofactor binding events (*56*). Extracellular signals lead to phosphorylation of the ER monomer. Examples of the extracellular signals are (i) dopamine and cAMP binding to GPCR can activate PKA; (ii) growth factors (GFs) activate their receptors with subsequent activation of the RAS-RAF-ERK pathway; and (iii) nongenomic action of ER in the membrane activates the PI3K-Akt pathway. Both antagonist and agonist ligands can prompt ER dimerization with different allosteric consequences for the helix H12 position (see Figure 2, panel B). The shift in the H12 position triggered by antagonist ligands blocks subsequent cofactor binding, while agonist ligands allostericaly change the ER conformations to allow cofactor recruitment. Cofactors (*57*) like the nuclear receptor co-repressor (NCoR) and the repressor of the estrogen receptor activity (REA) lead to repression of ER response elements (ERE). Examples of direct activators are the thyroid hormone receptor (TRAP), steroid receptor activator (SRA), and steroid receptor co-activators (SRCs). The secondary co-activators (like CoCoA and PRMT) also bind ERS indirectly through association with SRCs. Thus, the network diagram provides simple binding events but misses the allosteric regulation of binding and function (*57*), which are highlighted in panels B and C. B) Affinities between the central node (here the human ERa) and its binding partners (agonists, connected *via* the edges), where line thickness indicates the binding strength (*58*) thus specifying its rank. For example, the 17b-dihydroequilenin (17b-DHEquilenin) ranks third, and Equilenin ranks tenth in binding strength. C) The rank of human ERa functional activity (*58*), where line thickness indicates the strength of the functional activity. Unlike the binding strength, 17b-DHEquilenin only ranks eighth and Equilenin ranks third in binding strength for ERa. For ERb, the Equilenin ranks last in binding strength but is the most active for ERb (*58*). Comparison of the widths of corresponding edges between panels B and C illustrates that the extent of the affinity does not necessarily correspond to the degree of function. Thus the allosteric control of estrogen receptors strongly supports the notion that allostery should be considered when trying to understand how binding phenomena determine the functional outcome.

existing ones? Developing new molecules is risky and slower, yet expanding existing ones is wasteful, requiring high concentrations with only some of the binding events being productive. To minimize the chance of errors, evolution nonetheless chose this route. The number of similar REs and similar PID partners increased dramatically (*3, 39, 50, 51*), leading to numerous nonproductive binding events as in the case of the NR. However, at the same time, evolution embraced allostery: through environment-triggered conformational changes that lead to enhanced specificity, allosteric effects enforce an ordered sequence of events in multimolecular associations, leading to complex yet less error-prone structures as in the case of viral capsids. Allostery limits the conformational space of the association, reducing the chances of nonproductive associations inherent to diffusion-collision-type processes, thus offering advantageous solutions. We further note that our

definition of function as binding that leads to specific subsequent events explains why interface design is so difficult: the binding should be such that it would elicit an allosteric transition culminating with (far away) binding sites that have "correct" conformations. To conclude, here we present a perspective of protein−DNA binding that suggests possible criteria that can be used to discriminate between functional and nonfunctional binding events. A key ingredient of the discriminant criteria is the presence of allosteric effects that are capable of enhancing the binding specificity in a function-oriented manner. Allostery plays a key role in determining whether a binding event is functional and the type of function (*4, 8, 13, 15–20, 52, 53*). This provides a new definition of function and as such of biophysical events that qualify as "functional".

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