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From Fuzzy to Function: The New Frontier of Protein–Protein Interactions

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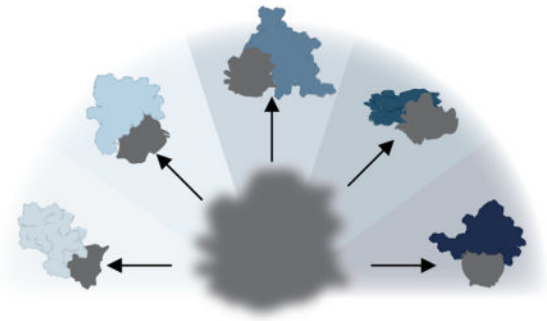
Abstract

Conformationally heterogenous or “fuzzy” proteins have often been described as lacking specificity in binding and in function. The activation domains, for example, of transcriptional activators were labeled as negative noodles, with little structure or specificity. However, emerging data illustrates that the opposite is true: conformational heterogeneity enables context-specific function to emerge in response to changing cellular conditions and, furthermore, allows a single structural motif to be used in multiple settings. A further benefit is that conformational heterogeneity can be harnessed for the discovery of allosteric drug-like modulators, targeting critical pathways in protein homeostasis and transcription.

Graphical Abstract



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The notion that many proteins have a high degree of disorder has been in the literature for decades.[1] Such sequences were commonly regarded as a nuisance: they caused aggregation and precipitation and often eluded structural and mechanistic studies. These motifs were also regarded as largely useless from a functional standpoint, perhaps because few believed that floppy motifs could encode information that facilitated specific function.

This viewpoint was particularly strong in the field of transcription. Transcription factors minimally consist of two domains: a DNA binding domain that localizes the protein to DNA and a transcriptional activation domain that assembles the transcriptional machine at the relevant gene location within the DNA.[2] Transcriptional activation domains rarely fold independently; because of their propensity for floppiness and sequences that often contain a surplus of negatively charged side chains, they were somewhat disparagingly labeled as "negative noodles" (Figure 1).[3] The prevailing view was that these motifs functioned simply as molecular Velcro, sticky patches that used the hydrophobic regions within the sequences to interact with various coactivator proteins in the transcriptional machinery with modest affinity. In this model, the functional specificity of transcriptional activators arose purely from the DNA binding motifs that targeted particular sequences within the DNA. An additional contributor to specificity came from interactions between transcription factors through the formation of hetero- and homomultimeric complexes, increasing the size and complexity of the protein–DNA interfaces.

At the turn of the 20th century, floppy motifs such as transcriptional activation domains received a new label of intrinsically disordered. The identification of amino acid sequences correlated with disorder and their prevalence in the genome lead to the growing recognition that such motifs underpin key cellular functions.[1,4] In transcription, for example, complexes formed between transcriptional activators and similarly disordered coactivator proteins in the transcriptional machinery underpin precisely timed gene-specific up-regulation. [5] Furthermore, dysregulation of these motifs is emerging as a critical element for many diseases, including cancer, metabolic disorders, and diseases of protein homeostasis. For example, genetic disruption of the ternary complex formed between transcriptional activators Myb and MLL and the KIX motif within the coactivator p300 leads to leukemogenesis.[6]

At first glance, intrinsically disordered motifs would seem to be puzzling choices to encode specificity, but this is often exactly the case.[7] Taking the KIX motif of p300 as an example, it forms ternary complexes not just with MLL and Myb as described above, but also with more than 10 other transcriptional activators using just two binding sites.[8] These sites are connected through an allosteric network of amino acids, and each ternary complex has a distinct cooperativity constant. This means that when MLL binds, it limits the range of conformations sampled by KIX and reinforces preferential interactions with CREB or cMyb over other activators that utilize that same binding surface (Figure 2A).[9,10] Thus, the intrinsic disorder of the system provides specificity through allosteric coupling. A second important advantage of conformationally heterogeneous structures is that a single motif is adaptable to many different contexts and thus there is not a need for a unique transcriptional activator–coactivator pairing for each gene or gene cluster.

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This is true not only in transcription but in other multicomponent machines in the cell. The protein folding machinery is an excellent example. Hsp70 is a molecular chaperone that recognizes misfolded regions in proteins. Based on peptide array experiments and NMR studies, Hsp70 binds to a consensus peptide motif composed of any 7–8 nonpolar amino acid residues, typically with a cationic residue nearby.[11] This remarkably degenerate binding motif is found in virtually every protein in the proteome,[12] and indeed, it is thought that Hsp70 helps fold most polypeptides independent of their ultimate fold or function. However, in order for Hsp70 to bind, this motif must be displayed in a disordered context, and like in transcription, the affinity values are typically weak (often 1–10 μM). How is selectivity achieved against this backdrop of weak, staggeringly promiscuous interactions? One major clue is found in the close partners of Hsp70, the J proteins (also called Hsp40s). The human genome includes ~50 Hsp40 genes that all contain a highly conserved J domain.[13] The J domain binds directly to Hsp70, allowing allosteric communication and cooperation between the two chaperones (Figure 2B). Outside the J domain, some of the Hsp40s bind directly to hydrophobic regions of misfolded polypeptides. Thus, Hsp70 and Hsp40 often bind similar types of motifs in disordered regions at the same time they bind to each other, driving formation of a ternary complex with the misfolded “client” protein. This ternary complex is critical to function; for example, removing the peptide-binding region of Hsp40 or Hsp70 blocks the ability of the complex to refold denatured proteins *in vitro*.[14] Most importantly for this discussion, it is in the ternary complex that selectivity begins to emerge. The folding of specific clients is known to be highly dependent on Hsp70 and specific Hsp40s.[13] Thus, ternary complex selectivity is thought to arise from the composite of multiple, individual, weak binding events. As with the transcription machinery described above, the intrinsic disorder of the system expands its function and specificity, especially when combined with multivalency.

A characteristic of PPIs mediated by conformationally heterogeneous motifs is they are often short-lived and relatively weak in terms of affinity. As with structural dynamics, these characteristics are related to function. Transcription factors need to assemble the transcriptional machinery by the staged recruitment of smaller complexes and individual proteins and the assembled machine then must progress along the DNA. High affinity, long-lived PPIs would stall this process. Similarly, Hsp70 needs to guide polypeptides down folding trajectories, in which they are sampling multiple conformers to arrive at the stable, native fold. Tight interactions would defeat the purpose of chaperones and block, rather than facilitate, folding. Nonetheless, the transient nature of the complexes combined with the conformational gymnastics of the binding partners raises a significant challenge for the discovery and characterization of these functionally critical complexes, especially in the cellular environment.[15] Without detailed knowledge regarding the structure(s) of the individual complexes and the PPI networks they engage in, it becomes more difficult to design small-molecule modulators.

One way to elucidate PPIs, even within the cell, is with covalent chemistries that capture snapshots of these ensembles. Pioneering nonsense suppression work by Schultz and coworkers[16] opened the door to placing a photoactivatable, unnatural amino acid (UAA) site-specifically into a protein of interest. Activation of the UAA with light leads to a reactive species (such as the diradical from benzophenone) that can form covalent linkages

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with interacting protein partners, thus covalently capturing them. Importantly, this allows interactions to be interrogated in their native environment, the cell, which is especially important since many conformationally dynamic proteins or motifs function as part of larger complexes (Figure 3A).

In an early example, the Mapp lab demonstrated that with an optimized tRNA/synthetase pairing, the UAA *p*-benzoylphenylalanine (pBpa) could be incorporated in a range of positions in the canonical eukaryotic transcription factor, Gal4, in a eukaryote (Baker's yeast or *Saccharomyces cerevisiae*), enabling the *in vivo* interrogation of a binding interface between this protein and its natural inhibitor, Gal80.[17] Nonetheless, this interaction is at least 2 orders of magnitude stronger than those Gal4 makes with the transcriptional machinery components, and there was some doubt that weaker, more transient interactions could be captured. Fortuitously, the benzophenone diradical proved capable of capturing those weaker interactions, and when coupled with shotgun proteomics, the identification of entirely new dynamic PPI networks was possible. In one study, direct PPIs between the transcriptional activator Gal4 and the Snf1/AMPK complex were pinpointed at a specific stress-response gene promoter in living cells.[18] This revealed that Gal4 makes two contacts within the Snf1/AMPK complex. One is with the enzymatic core unit, Snf1/AMPK, a kinase that phosphorylates both DNA-bound repressor proteins to facilitate their removal and the tail of RNA polymerase II to stimulate its activity. The second target of the activator is an exchangeable subunit, Gal83, that is only present when the complex is in the nucleus. Thus, the activator Gal83 PPI is present only in particular functional contexts, and modulating this PPI would provide an additional level of specificity relative to targeting only the kinase.

Small-molecule modulators of dynamic PPIs are also powerful chemical genetic tools for dissecting PPI networks in living cells and serve as the starting points for the development of chemotherapeutic agents. They are also among the most challenging to discover because traditional screening and structure-based design techniques are largely ineffective for targeting conformationally dynamic proteins. Indeed the number of validated small-molecule modulators of such PPIs lags behind those of well-defined PPI targets by more than an order of magnitude.[19]

Nonetheless, a recognition that conformationally heterogeneous PPIs offer some significant advantages as small molecule targets is emerging. Perhaps most significant is that small molecules can interact with distinct conformations of the protein. The binding free energy (ΔG_{bind}) of this interaction then favors a portion of the structural ensemble, which has downstream effects on all of the other PPIs that converge on that protein. For example, some partners might be directly (i.e., orthosterically) inhibited from interacting with the protein because the small molecule is bound at the normal interaction motif. Alternatively, the new structural ensemble might allosterically disfavor binding to other partners because distal sites are no longer available. On top of these possibilities, the impact of the new, chemically induced ensemble could either inhibit or favor the broader PPI network of the target protein. It is clear that conformationally dynamic regions provide many prospects for small-molecule discovery, often going beyond what is thought possible for more static targets. The challenge is to build discovery tools that allow us to survey the landscape of potential small-molecule PPI modulators.

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One strategy for such exploration is to reconstitute *in vitro* the cellular components that are involved in competitive and cooperative PPIs. In the Hsp70 system, the Gestwicki lab found it useful to recombine purified Hsp70, Hsp40, and peptide "client" at the half-maximal concentration of their normal PPI strengths,[20,21] effectively forming a heterogeneous series of complexes that might be expected to exist in the cell. Hsp70 is an ATPase and its enzymatic activity can be measured using malachite green assays. Although Hsp40 and the client do not have enzyme activity, they both allosterically activate Hsp70, increasing turnover by many fold. Accordingly, we found that chemical screens against the recombined Hsp70/Hsp40/client machinery revealed compounds that either activate or inhibit each of the PPIs (e.g., Hsp70 binding to the J protein) (Figure 3B). Some of these molecules bound orthosteric sites, while others were allosteric.[22] This approach has proven to be one way to find inhibitors within conformationally dynamic complexes, especially when they are linked to an enzymatic activity that is readily monitored. When there is not an enzymatic activity explicitly linked to the protein complex, such as for many transcriptional activator–coactivator complexes, the Mapp group has found that iterative screens against related but conformationally distinct complexes produces modulators with high specificity for the intended target and a mixed orthosteric/allosteric mechanism.[23]

A second approach that shows significant promise is to use a reversible covalent ligand discovery method such as Tethering, originally reported by Wells and co-workers at Sunesis.[24] In this screening approach, the binding surface of interest has an existing cysteine (or one is installed) nearby, and the protein target is screened against a library of small-molecule fragments, each of which bears a reactive substituent capable of forming a disulfide bond with the protein (Figure 3C). This is done under reversible conditions such that after each small molecule–protein combination has reached equilibrium, only ligands that have sufficient affinity for the protein of interest and a properly positioned disulfide will form a covalent bond that can be detected by mass spectrometry or other methods. As the Mapp group demonstrated with the KIX motif, the resulting fragments serve as chemical cochaperones that stabilize a variety of protein conformations, enabling their characterization and, depending on the conformation, selectively stabilize or destabilize the formation of KIX ternary complexes.[25] The value of these molecules as mechanistic probes is high; again focusing on KIX, we have used various chemical cochaperones to define the mechanism of ternary complex formation for KIX, producing a mechanistic model that connects the conformational changes of the protein with function inside the cell.[26] Further, replacement of the redox-sensitive disulfide moiety with a less-reversible electrophile produces structures that can be used in the cellular environment.[27]

Although fuzziness and function are firmly linked for many cellular machines, the PPI field is wide open for chemical innovations to create both detailed maps of dynamic PPI networks in healthy or diseased tissues and to intervene therapeutically.[15,19] There is a great need for high resolution and high throughput methods that allow imaging of the components of conformational ensembles. Ion-mobility mass spectrometry has emerged as a technology that allows detailed analysis of complex conformational ensembles but the approach is currently challenging to implement in a higher throughput manner.[28] As computational methods have advanced, approaches such as single-molecule spectroscopy, small-angle Xray scattering, and cryo-electron microscopy have provided increasing information about

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structural dynamics.[31–33] In the small-molecule realm, the incorporation of moieties that interact with a broader range of reactive side chains is an exciting frontier. Additionally, the combination of UAA technology with amino acids possessing novel reactivity will increase the resolution and dynamic range of covalent capture technologies.[29] Finally, recent advances in molecular dynamics simulations indicate that theory is taking a leading role in dissecting allosteric networks and predicting conformational changes to better understand mechanisms and to discover small molecules.[30] We anticipate that the next decade will bring a significantly enhanced understanding of how to modulate these functionally critical PPIs.

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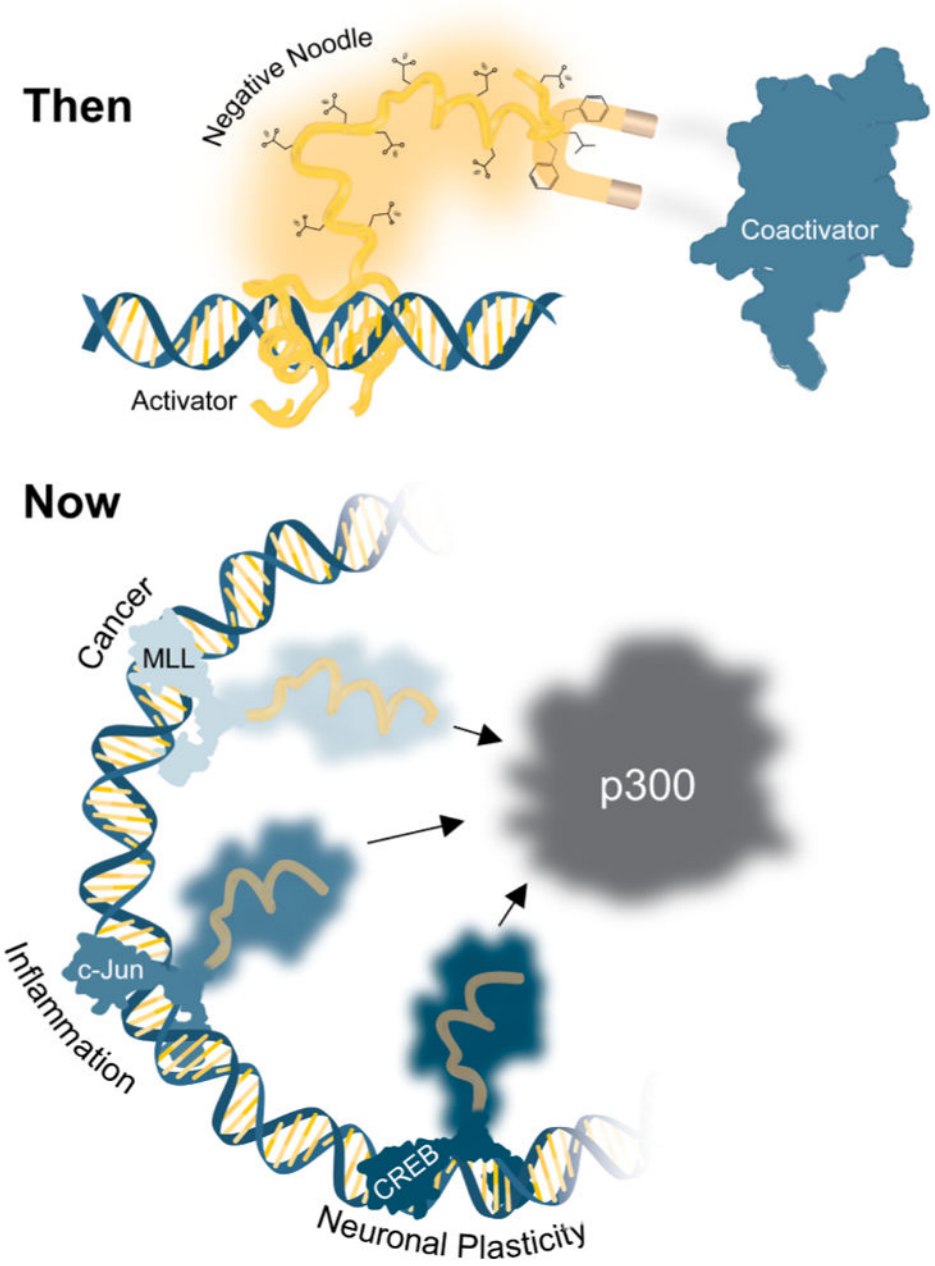

Figure 1.

Structure–function view of transcriptional activators in the 1970s and 1980s (“Then”) regarded the protein–protein interactions between activators and coactivators in the transcriptional machinery as largely nonspecific. The current model (“Now”) reflects the reality that the fuzzy domains of transcriptional activators and coactivators are integral to regulated, gene-specific transcription. Key reviews include refs 4, 5, and 7.

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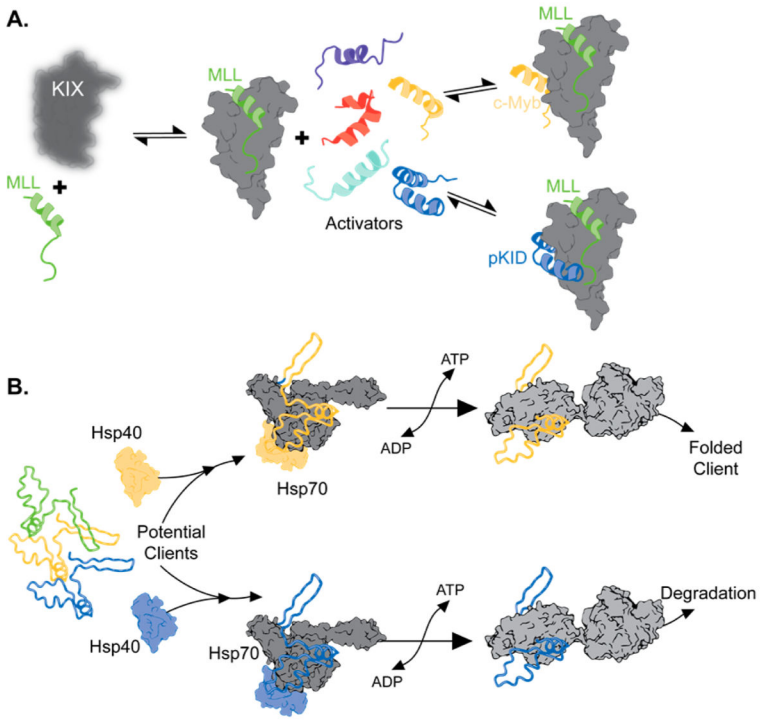

Figure 2.

Conformational heterogeneity enforces specificity and function. (A) The KIX motif of p300 interacts with more than 10 distinct transcriptional activators to form numerous conformationally unique ternary complexes. The structure and function of the KIX motif was recently reviewed in ref 8. (B) The combination of Hsp70 with distinct Hsp40 partners allows differential folding or degradation of clients in the cell.

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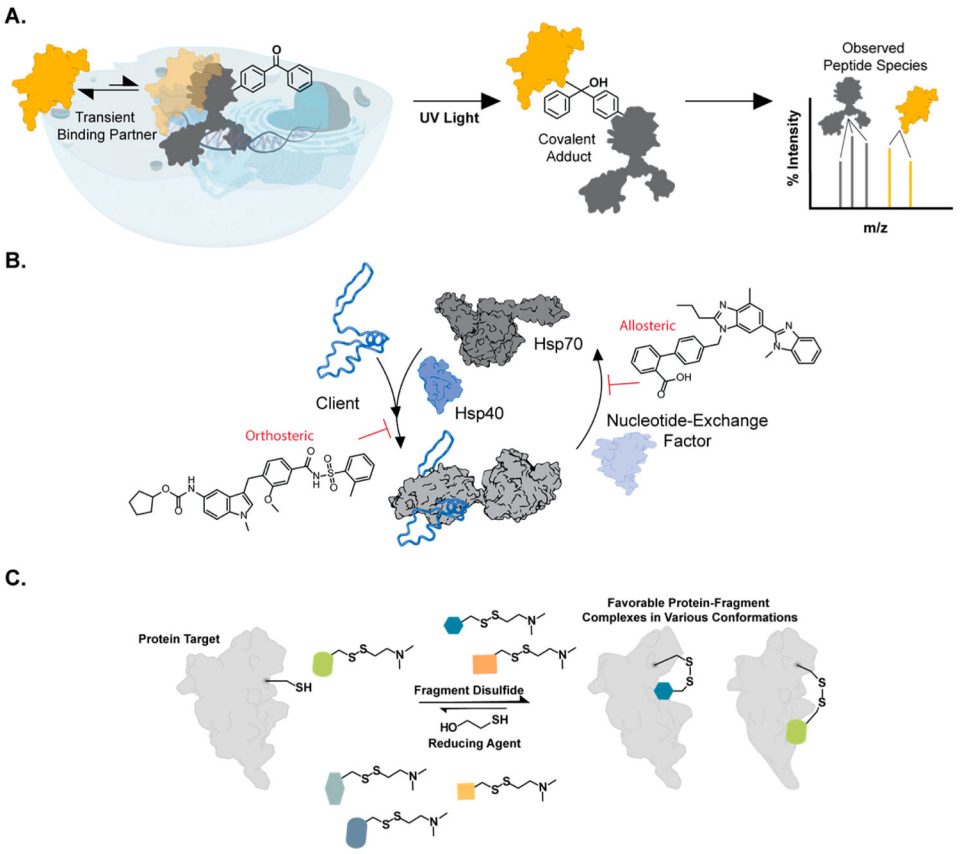

Figure 3.

Methods for "capturing" conformationally heterogeneous proteins and PPIs via genetic and chemical genetic means. (A) Site-specific incorporation of unnatural amino acids enables covalent chemical capture of even weak PPIs in the context of the cell.[17,18] (B) Screening against different Hsp70 complexes can provide orthosteric and allosteric modulators of Hsp70 function.[22] (C) Site-directed fragment screening can produce small-molecule ligands called chemical cochaperones that stabilize particular conformations of the protein target.[24–26]