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Selective modulation of dynamic protein complexes

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Abstract

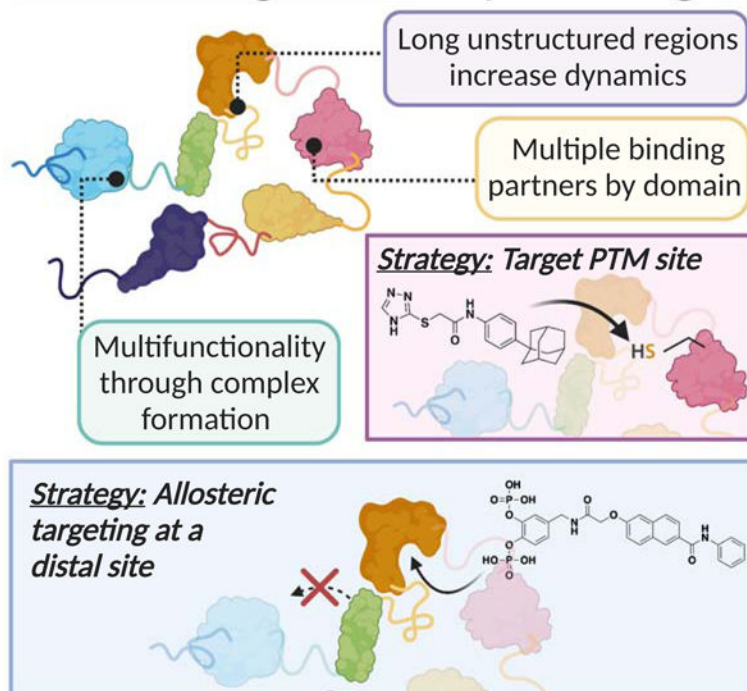
Dynamic proteins perform critical roles in cellular machines, including those that control proteostasis, transcription, translation, and signaling. Thus, dynamic proteins are prime candidates for chemical probe and drug discovery but difficult targets because they do not conform to classical rules of design and screening. Selectivity is pivotal for candidate probe molecules due to the extensive interaction network of these dynamic hubs. Recognition that the traditional rules of probe discovery are not necessarily applicable to dynamic proteins and their complexes, as well as technological advances in screening, have produced remarkable results in the last 2–4 years. Particularly notable are the improvements in target selectivity for small molecule modulators of dynamic proteins, especially with techniques that increase the discovery likelihood of allosteric regulatory mechanisms. We focus on approaches to small molecule screening that appear to be more suitable for highly dynamic targets and have the potential to streamline identification of selective modulators.

Graphical Abstract

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Overcoming selectivity challenges



Introduction

Conformationally dynamic proteins underpin all biological processes, from signaling to gene expression (Barrios-Rodiles et al., 2005; Kholodenko, 2006; Yeger-Lotem et al., 2004). These proteins often form short-lived protein-protein interactions (PPIs) that allow cellular machines to assemble and disassemble as needed to regulate key pathways and events (Stein et al., 2009). Efforts to decipher the principles that govern PPI formation have been extensive (Heo et al., 2011; Jones and Thornton, 1996; Khan et al., 2011). However, most work has been focused on PPIs between stable, well-defined partners and the emergent rules governing assembly, molecular recognition, and modulation of PPIs are not generally applicable to dynamic proteins. Members of this protein class tend to have high flexibility and structural plasticity (Mittag et al., 2010). Malleable structures allow them to perform context-dependent regulatory functions and interact with a multitude of binding partners (Figure 1A). For example, the master coactivator CBP/p300 (Figure 1B) contains six conformationally dynamic domains that interact with hundreds of transcriptional activators, which are linked by intrinsically disordered regions, thus increasing the overall flexibility of the protein (Dyson and Wright, 2016).

Dynamic proteins are often observed in higher order cellular machines with a functional need to rapidly form and exist only transiently, such as the multiprotein complexes involved in chromatin remodeling (Euskirchen et al., 2011) or protein folding (Walter and Buchner, 2002). Thus, dynamic proteins are often hubs, interacting with subunits within a complex as well as other proteins and ligands (Batada et al., 2006). The composition of a dynamic protein complex can dictate enzymatic subunit activity or even change the function of the

complex. For example, combinatorial assembly of Hsp70 (Figure 1C) allows the complex to switch between a folding system and a degradation complex (Muller et al., 2013). Transcription is another process that relies on dynamic proteins. It is important that individual components of the machinery, such as activators, coactivators, general transcription factors, and RNA polymerase, are able to assemble efficiently at one promotor and then move on to mediate expression of another gene (Fuxreiter et al., 2008). There are extensive reviews regarding the function and composition of these dynamic machines (Gavin and Superti-Furga, 2003; Kasahara et al., 2019; Marsh and Teichmann, 2015; Thompson et al., 2012).

The qualities that make dynamic proteins and their complexes effective cellular machines also make them challenging to target. Due to conformational flexibility, it is often difficult to obtain the structural and biophysical data useful for the rational design and/or optimization of chemical modulators. The PPIs of dynamic proteins are typically lower affinity, utilize a relatively large surface area, and have minimal topology, thus representing a significant challenge for orthosteric inhibition (Cossins and Lawson, 2015; Smith and Gestwicki, 2012). High throughput screening (HTS) with dynamic protein targets can also be challenging due to the difficult expression and isolation of the full-length proteins. Thus, in many cases, it is necessary to utilize simplified systems such as isolated domains that may not recapitulate the structure, dynamics, and/or activity of the full-length complexes. Finally, selective targeting of a particular PPI or specific binding interface is difficult because dynamic proteins typically interact with a multitude of binding partners, which is a challenge we focus upon in this review (Arkin et al., 2014; Cesa et al., 2015; Ran and Gestwicki, 2018).

Notably, dynamic proteins are often dysregulated in disease and dynamic multiprotein complexes often contain specific subunits that are attractive therapeutic targets. For example, the tumor suppressor BRCA1, an important target for breast cancer prevention and therapy, associates with the Swi/Snf chromatin remodeling complex (Semmler et al., 2019; Takaoka and Miki, 2018). CBP/p300, p53, and other transcription factors are all also dynamic proteins that are potential targets for a variety of diseases (Lee and Young, 2013; Wang et al., 2013). While dynamic PPIs do not conform to typical “rules” used for drug discovery, in recent years significant achievements have been made when it comes to modulating some of these challenging targets. Here we review key principles that govern selectivity in binding and function for dynamic proteins and their complexes. Additionally, we present notable recent success stories in small-molecule modulation of these proteins, examples that either intentionally or serendipitously mimic natural regulatory mechanisms of dynamic proteins. Finally, we highlight emerging strategies that facilitate the discovery of selective chemical modulators of conformationally dynamic proteins.

Controlling the interactions and function of dynamic proteins: Lessons from Nature

As hubs associated with many complex cellular machines, dynamic proteins are precisely regulated. These regulatory processes often control the access of binding surface(s) within

the dynamic protein either directly or indirectly. This can occur through masking of binding surfaces via intra- or intermolecular interactions, through induced conformational changes of the protein, and, most commonly, combinations of both mechanisms.

Masking of binding surfaces within dynamic proteins occurs through intra- and intermolecular complexes that have a variety of functional outcomes. Masking interactions are common in transcriptional activators, where the highly disordered transcriptional activation domain is sequestered by a high affinity interaction until needed. Classic and well-studied examples include the Gal4•Gal80 (Gill and Ptashne, 1988; Wightman et al., 2008) and p53•Mdm2 (Zaika et al., 1999) complexes that provide temporal control of the transcription factors. Masking of dynamic proteins and/or protein domains is similarly important in many other cellular contexts. The scaffolding 14-3-3 protein, for example, regulates the subcellular localization and function of Caspase-2 through stabilizing two dynamic regions in individual subunits. 14-3-3 masks both the nuclear localization sequence of pro-Caspase2 and the dimerization interface, inhibiting pro-Caspase2 activation (Kalabova et al., 2020) (Figure 2A). 14-3-3 also regulates FOXO transcription factors by masking their nuclear localization sequences (Silhan et al., 2009). In all of these examples, the masking interaction provides fine temporal control of the dynamic protein. Obstruction of key binding surfaces in a dynamic protein is also associated with spatial control. For example, until the unfolded protein response is activated in a cell, the transcription factor ATF6 is sequestered at the ER membrane. Once the pathway is stimulated, ATF6 is cleaved from the ER membrane, enabling its translocation to the nucleus, where its potential binding partners reside (Wang et al., 2000).

Emerging data regarding the composition and function of biomolecular condensates indicates that masking of dynamic proteins likely plays a crucial role in the compartmentalization that such condensates afford. More recent examples of compartmentalization focus on phase separation, which increases the strength and specificity of interactions that otherwise appear promiscuous, while still allowing the proteins involved to retain their dynamic nature (Hahn, 2018). The transcription factors TAZ, OCT4, GCN4, and the estrogen receptor (ER), have been shown to form phase-separated condensates with Mediator via its coactivator subunits (Boija et al., 2018). TAZ has also been observed to form nuclear condensates with its DNA binding cofactor TEAD4 as well as the coactivator BRD4 (Lu et al., 2020). In all of these cases, unmasked transcriptional activation domains appear necessary for condensate formation.

Posttranslational modifications (PTMs) and distal binding events that influence structure are also mechanisms of binding surface control. PTMs occur at many PPI interfaces to promote or inhibit binding. For example, phosphorylation of the coactivator p300 at S89 inhibits its interaction with peroxisome proliferator-activated receptor γ (PPAR- γ) and retinoic acid receptor (RAR) (Yang et al., 2001). PTMs can also alter stability, folding, or conformation as a mechanism to regulate dynamic protein interactions (Duan and Walther, 2015). PTMs, such as ubiquitination, acetylation, phosphorylation, and glycosylation, have been observed to be allosteric regulators (Nussinov et al., 2012). For example, N-linked glycosylation of interleukin-7 receptor α allosterically enhances binding to human IL-7 nearly 300-fold (Walsh, 2010). In another example, a conserved cysteine in the palmitate binding pocket of

TEAD proteins undergoes palmitoylation that allosterically stabilizes the TEAD-YAP interaction (Chan et al., 2016) (Figure 2B). Nature often utilizes a combination of PTMs to fine-tune protein function, allowing a single protein to perform diverse cellular roles. A recent study of Hsp90, for example, identified a group of conserved PTMs that globally mediates dynamics and allosteric communication in the Hsp90 structures (Stetz et al., 2018).

Allosteric crosstalk via inter and intra-molecular interactions can lead to broader conformational changes, redistribution of conformers, and alterations in kinetic/thermodynamic barriers that also regulate protein interfaces (Swain and Gierasch, 2006; Tsai et al., 2009). For example, the binding of a cognate ligand or cofactor can lead to structural changes that influence protein function (Abrusán and Marsh, 2019). A classic example of this is G-protein coupled receptors (GPCRs). Ligand binding results in a conformational change in the GPCR, leading to activation of its associated G protein (Thal et al., 2018). PPIs can play a similar role. For example, the ubiquitinase BAP must interact with another protein, ASXL2, via its nonenzymatic ULD domain to allow ubiquitin to bind and be cleaved via its hydrolase (UCH) domain. Thus, allosteric changes induced in BAP1 via ASXL2 interaction with the ULD domain are critical for its enzymatic function. Biophysical studies suggest that a loop within the UCH domain of BAP1 is stabilized by ASXL2 interaction and this allows for ubiquitin to bind (Peng et al., 2018) (Figure 2C).

Allosteric sites often reside within the most flexible regions of any protein. Dynamic proteins and the complexes they form rely heavily on unstructured regions such as loops and linkers to influence interactions. Loops are often directing components of selectivity and linkers play an important role in crosstalk between multidomain proteins. Linkers also can play a key role in multiprotein complex assembly and intradomain interactions (Papaleo et al., 2016). A flexible glycine rich linker region in NFkB allows for the formation of an interaction between the p50 and SWI6/ANK domains to regulate intracellular transport (Henkel et al., 1992). Allosteric sites tend to be less conserved between closely related proteins, subfamily members, or homologues (Furnham et al., 2012; Lu et al., 2014; Nussinov et al., 2011). Thus, allosteric inhibitors are a potential route to selective targeting of challenging PPIs as well as control over multiprotein, dynamic complexes. Perhaps more than any other class of proteins, nature suggests that dynamic proteins are prime targets for allosteric regulation.

Achieving selectivity with chemical probes

Assessing the native mechanisms that dictate interaction between dynamic proteins and their binding partners can illuminate possible routes or methods to target such challenging interactions. Natural regulation of dynamic proteins suggests that a possible route to successful modulators of their interactions is via allosteric sites. Allosteric modulators have the ability to shift the conformational ensemble of proteins to favor the formation of specific complexes or toggle active/inactive conformers without the need to directly target the PPI interface. This strategy is particularly attractive when it comes to targeting large surface area interactions because orthosteric modulation, particularly with small molecules, is challenging due to the lack of topological characteristics that allow for high affinity ligand binding. Allosteric modulation can be achieved -even for these challenging targets- with

small molecules that have more drug-like properties, such as lower MW (<600), greater structural rigidity, and hydrophobicity (Cossins and Lawson, 2015). Another advantage that comes with targeting allosteric regions of dynamic proteins is that they tend to be less conserved between the same protein family and homologous proteins, therefore, suggesting an avenue for selective modulation (Huang et al., 2013; Nussinov et al., 2011; Panjkovich and Daura, 2010). Figure 3 summarizes the allosteric compounds, all published since 2018, that will be discussed in this section and spotlights their selectivity profiles.

Allosteric regulators have demonstrated high selectivity for protein targets of many other classes, suggesting that similar results are possible with dynamic proteins. For example, the ability to selectively target kinases, GPCRS, and bromodomains (BRDs) has improved with our understanding of allostery in these systems (Fang et al., 2013; Filippakopoulos and Knapp, 2014; Wootten et al., 2013). Extensive research on selectivity within kinase families has shown that molecules that bind outside the catalytic domain display high selectivity on both a family and subtype level while still functioning as potent inhibitors of enzymatic activity (Fang et al., 2013). Allosteric modulation of GPCRs and the impact on selectivity is also well documented (Gao and Jacobson, 2013; Leach et al., 2007) For example, a group from Merck recently published work on MK-7622 a novel positive allosteric modulator of the muscarinic acetylcholine receptor M1 for the treatment of Alzheimer's disease. This compound was highly selective for the M1 receptor, with no potentiation or agonism of M2–M4 subtypes in an overexpression model conducted in CHO cells (Beshore et al., 2018). Furthermore, compounds that are able to distinguish between the most homologous BRDs, BRD4/7/9, take advantage of subtle conformational differences in non-conserved regions distal to the acetyl lysine binding site (Karim et al., 2020; Olp et al., 2020).

Covalent small molecule modulators have been successful at allosterically modifying dynamic proteins in a manner that mimics covalent PTMs found in nature. In a study informed by a naturally occurring PTM, Bum-Erdene et al. targeted a conserved cysteine residue in TEAD family proteins known to undergo palmitoylation in order to stabilize interaction with YAP. Starting with the FDA approved compound flufenamic acid, shown to weakly bind TEAD2 non-covalently near the thiol of the conserved cysteine, a chloromethylketone moiety was incorporated to make it a covalent inhibitor. This compound, TED-347, allosterically inhibited YAP-TEAD PPIs both in vitro and in mammalian cells ($K_i = 10 \mu\text{M}$). TED-347 also demonstrated selectivity for the TEAD family over other related proteins (Bum-Erdene et al., 2019). The recently published compound MGH-CP1 covalently modifies and inhibits TEAD family proteins similarly to TED-347, but with increased potency. MGH-CP1 inhibits autopalmitylation of TEAD2/4 but does not affect autopalmitylation of ZDHCC family palmityl acetyltransferases, suggesting that the compound is selective for TEAD proteins (Li et al., 2020). The authors also find that TEAD2 adopts similar conformations when bound to palmitate or MGH-CP1, further suggesting that mimicking regulation by naturally occurring PTMs can be a successful strategy for selectively targeting dynamic proteins. In another example, covalent allosteric inhibitors were designed to selectively target isoforms of protein kinase Akt. There are three different isoforms of Akt, each with a unique intracellular location and function; thus, highly selective probe compounds are needed to study these proteins. The covalent compound borussertib alkylates a cysteine residue located within an interdomain pocket between the

PH domain and kinase domain of Akt proteins, irreversibly stabilizing the inactive conformation. Quambusch et al. observed slight differences around this allosteric pocket between isoforms and designed a library of compounds based on the pyrazinone scaffold of borussertib that could engage these subtle sequence changes. With this approach, they were able to identify compounds with high selectivity for Akt1 and Akt2 (Quambusch et al., 2019). This study highlights paths to attain high selectivity and potency with allosteric modulators.

In the previous example, the structural differences exploited by selective small molecules at the allosteric site of Akt isoforms mainly occurred within a loop formed by residues 259–273. Often the most dynamic components of a protein structure, such as loops, linkers, and flexible helices, are effective sites for allosteric modulation and improved selectivity. Recent work targeting the dynamic coactivator Mediator subunit Med25 also highlights this point. Henderson et al. show that a small molecule, compound 22, can allosterically regulate binding of transcriptional activators to Med25 via covalent modification of a distal cysteine residue. This cysteine residue is located adjacent to regions predicted to be highly flexible via structural modelling. Additionally, compound 22 influences the flexible substructures within Med25, also known to be perturbed by interactions with transcriptional activation domains (Henderson et al., 2018). Thus, by targeting dynamic regions of a protein, even large PPI interfaces can be inhibited.

Another example illustrating that targeting loop modules can improve inhibitor selectivity is the discovery of LY3154207, a potent inhibitor of the Human Dopamine receptor, D1, presented by Lilly. Classical approaches to targeting D1 receptors have mainly been orthosteric, and candidate modulators bind similarly to the natural substrate dopamine (Zhang et al., 2009). However, as D1 and D5 receptors have high structural similarity, this approach leads to selectivity issues. The authors found that LY3154207 and its analogs bound to a novel binding site in the intracellular loop 2 (ICL2), allosterically inhibiting binding at the orthosteric D1 site. Excitingly, this inhibitor was highly selective for the D1 receptor over the closely related D5 receptor (>2700-fold). LY3154207 demonstrated over 1000-fold selectivity for the D1 receptor when tested against a panel of 40 additional targets (Hao et al., 2019). In another recent example targeting a transcription factor, fosfosal, a well-documented clinical prodrug, was found to inhibit the SH2 domain of STAT5b over its homologue STAT5a. Mutational analysis showed this selectivity was dictated by residues within an adjacent linker domain rather than within the SH2 domain itself (Gräb and Berg, 2020). This work illustrates the role that the STAT linker region in SH2 domain function, and targeting the linker with small molecules is thus an avenue for selective inhibition of STAT PPIs. This same group has identified additional molecules selective for both STAT5a and STAT5b utilizing the same concept (Elumalai et al., 2015; Gräb et al., 2019).

Emerging techniques for identification of selective dynamic protein complex modulators

The examples discussed above demonstrate the power and promise of allosteric regulators for modulating dynamic proteins, particularly in terms of selectivity. However, in the

majority of these examples, the mechanism of action was not the result of the discovery method but rather serendipitous. Here we outline emerging techniques and strategies that facilitate screens more likely to produce allosteric and ultimately selective inhibitors.

Inhibitor identification from well-structured proteins allows for easier structural characterization and lead compound optimization. Dynamic proteins, by definition alone, provide a steep challenge, with many of the common experimental techniques requiring improvements before being applied to proteins that are highly mobile and lack definitive structure. Thus, advancements in structural biology and biophysics are key when it comes to understanding selectivity in dynamic protein complexes. Recent improvements in techniques such as mass spectrometry (Ishii et al., 2018), NMR (Huang and Kalodimos, 2017), and cryogenic electron microscopy (cryo-EM) (Merk et al., 2016; Schmidt and Urlaub, 2017) have allowed a high-resolution view of multiprotein complexes and aided in our understanding of composition as well as identification of key PPIs. For example, Khattabi et al. recently reported a 5.9 Å cryo-EM structure mapping the entire mammalian Mediator complex (~4 mDa). This included, for the first time, mapping of the exchangeable and conformationally dynamic proteins within the tail region of the complex (El Khattabi et al., 2019). Many of the tail proteins are the primary targets of transcription factors and these results provide key insights into how to target this class of proteins successfully. In another exciting example, time resolved cryoEM was used to obtain a near-atomic-resolution view of the conformational changes that drive and regulate subunit assembly, initiation factor dissociation, and fMet-tRNA positioning during the formation of the 70S elongation-competent complex in bacteria (Kaledhonkar et al., 2019). This type of approach allows dissection of the specific timing and order of conformational changes contributing to the mechanism and regulation of large and dynamic multiprotein systems. These examples highlight how improved techniques for understanding dynamic protein complexes will allow researchers to identify more promising regions to target.

Increased complexity of an assay system is characteristic of strategies that allow for enrichment of selective hits from screening. Grey box screening is a prominent example. In this method, multiprotein complexes are reconstituted in vitro and subjected to HTS with the goal of identifying compounds that effect biochemical properties, such as enzymatic activity, of the complex. This approach has identified specific chemical modulators of dynamic proteins within complexes such as Hsp70 (Cesa et al., 2013; Taylor et al., 2018), Hsp90 (Patwardhan et al., 2014), and regulators of G-protein signaling (Monroy et al., 2013). This method has been thoroughly reviewed in other sources (Cesa et al., 2015; Thompson et al., 2012). Mass spectrometry approaches can also allow for multiplexing of protein assays. Collision-induced unfolding (CIU) can distinguish individual protein and protein complexes through their distinct unfolding pathways in the gas phase, allowing for analysis of multiprotein and protein-ligand complexes (Niu and Ruotolo, 2015). Combining CIU with collision induced dissociation (CID) mass spec analysis distinguished ATP competitive from allosteric kinase inhibitors of the tyrosine kinase Abl (Rabuck-Gibbons et al., 2018). There are also many other examples utilizing mass spectrometry to analyze intact multiprotein complexes (Ishii et al., 2018). Application of these techniques to dynamic targets in high throughput screens can streamline identification of selective modulators. Molecules displaying selectivity, as seen with the examples in the previous section, often act

allosterically, and thus characterization of their effects on target proteins will provide insights into allosteric regions to focus on in future studies.

Classical approaches, such as Forster resonance energy transfer (FRET) and fluorescence polarization (FP), can be utilized for HTS of dynamic protein targets where allosteric sites are unknown. To assess the selectivity of lead compounds, secondary screening against a selected panel of alternate targets is necessary (Lea and Simeonov, 2011). This iterative screening process is highlighted in work from Majmudar et al. Hits from a FP-based HTS for inhibitors of CBP KIX were subjected to multiple rounds of secondary screening against increasingly relevant PPIs, from non-transcriptional PPIs to DNA-binding domain•DNA interactions to related coactivator•activator interactions. This led to the identification of the CBP KIX selective molecule lobaric acid (Majmudar et al., 2012). Library complexity is also key for screening dynamic proteins. Diversity oriented synthesis and DNA Encoded Libraries (DEL) have allowed for the generation of huge billion-trillion compound libraries that can be screened in a one pot format (Chan et al., 2015; Jr et al., 2016; Salamon et al., 2016). Other notable library developments with the ability to impact dynamic protein probe discovery include diverse natural product focused libraries, protein-protein interaction focused libraries, and cheminformatic based design and optimization (Gong et al., 2017; Jin et al., 2018; Moret et al., 2019)

Targeted fragment approaches have been successful in discovering allosteric sites and modulators of diverse proteins. Fragment based screening does not rely on identification of a single high affinity hit, rather it provides the opportunity to diversify lead scaffolds into directed libraries or link together lower affinity binders, acting at unique sites on the target protein, to construct a more potent compound (Doak et al., 2016; Kirsch et al., 2019). The Cravatt lab has pioneered cell-based approaches to fragment screening, providing the advantage of being able to study a protein of interest in its native environment (Backus et al., 2016; Parker et al., 2017). This would be particularly useful for dynamic proteins that are challenging to work with in vitro. Tethering is a site-directed fragment technique that utilizes a disulfide moiety within each fragment to facilitate localization to protein binding sites via covalent bond formation with adjacent cysteine residues. Tethering has enabled the identification of allosteric modulators for many dynamic protein targets, from enzymes such as PDK1 and Ras to coactivators such as CBP and Med25 (Henderson et al., 2018; Ostrem et al., 2013; Sadowsky et al., 2011; Wang et al., 2013) In a recent example targeting the scaffolding protein 14-3-3 PPI, tethering was used to identify orthosteric stabilizers of the 14-3-3•ER α complex. These stabilizers were able to enhance the interaction by up to 40-fold while demonstrating selectivity for ER α over other 14-3-3 interacting proteins (Sijbesma et al., 2019).

Screening methods that provide mechanistic insight into small molecule binding have proven highly useful for conformationally dynamic proteins, providing the ability to select for molecules that influence desired regions of the target. NMR-based methods, including Protein Observed Fluorine (PrOF) and $^1\text{H}^{15}\text{N}$ HSQC can provide information on both binding site location and impact on protein dynamics and structure (Arntson and Pomerantz, 2016; Dalvit and Vulpetti, 2019; Furukawa et al., 2016). Thus, one can select for compounds during screening that show specific alterations in structure and/or dynamics. Because

throughput in NMR based screening methods can be a limitation, this approach is extremely powerful when coupled with computational screening methods. Recent work by Gupta et al. combined computational and NMR screening approaches with the goal of targeting known allosteric sites on mutant and wildtype KRAS. Gupta et al. started from a virtual library of 76 million compounds and identified nine compounds that lead to $^1\text{H}^{15}\text{N}$ HSQC chemical shift perturbations of residues near the functionally responsive switch loop. Although the authors do not analyze the functional effect of their lead compound, E22, they find that it binds nearly 100-fold tighter to GNP-bound Ras compared to GDP bound Ras and hypothesize that the molecule may affect GEF-mediated GDP/GTP exchange, similar to other indole compounds that target KRAS (Gupta et al., 2019). Combinations of computational and experimental approaches, as this example illustrates, have and will continue to be important for dynamic protein probe discovery. Notably, computational studies of allostery, with a focus on identification of allosteric sites and discovery of potent allosteric inhibitors, have been extensively performed in recent years with many successes (De Vivo et al., 2016; Greener and Sternberg, 2018; Ma et al., 2016)

Thermal stability assays, such as differential scanning fluorimetry (DSF), are powerful screening options for dynamic protein targets (A. Senisterra and Patrick J. Finerty, 2009; Gao et al., 2020; Niesen et al., 2007). While DSF is a technique dating back to 1991, it recently has been used to screen some challenging targets such as STAT proteins (STAT1, STAT3, and STAT5) (Attarha et al., 2020; Desroses et al., 2018), nuclear receptors (DeSantis et al., 2012), and chaperone proteins (Mac Sweeney et al., 2018; Shao et al., 2020). Not only is this method simple, inexpensive, and amenable to HTS, it provides insights into the effect of a compound on the thermal stability of the target protein, making it possible to select for compounds with the desired stabilizing or destabilizing effect. Thermal stability assays have the added benefit of not requiring knowledge of binding partners. Recently, groups have been working to improve understanding of the theoretical underpinnings of DSF and further interrogate the potential information that can be obtained from DSF melting curves (Sun et al., 2020; Wu et al., 2020). This work has made it clear that more than just a melting temperature (T_m) emerges from these experiments. For example, the shape of the melt curve can provide some insight into ligand binding even if T_m shifts are not observed. Additionally, innovations to the technique suggest the ability to multiplex. DSF-GFP, a technique pioneered in 2012, involves labelling the target protein of interest with GFP and using the change in GFP fluorescence over a temperature gradient as a readout rather than solvatochromic dye (Sorenson and Schaeffer, 2020). This approach allows for the selective measurement of target protein T_m in the presence of other proteins.

The cellular thermal shift assay (CETSA) is another technique that interrogates changes in protein stability and can also be extremely useful for screening dynamic proteins (Molina et al., 2013). A benefit of this approach is the ability to screen the protein in a more native environment, such as in cell lysates. Recent advances to CETSA techniques have allowed for use in HTS. To date, there have been multiple HT-CETSA approaches published, all focusing on altering protein detection methods to allow for the classic CETSA western blot readout to be adapted to 384 or 1536 well format (Henderson et al., 2020). For example, Shaw et al. applied HT-CETSA via AlphaScreen technology to conduct a screen for modulators of BRAF and PARP1 through screening 896 and 6288 compounds, respectively

(Shaw et al., 2019). In a recent success story with a challenging target, a small molecule, NPD10084, was found to thermally destabilize PKM2, which can function as both a kinase and a transcriptional coactivator. This interaction with PKM2 leads to inhibition of PPIs with STAT3 and beta-catenin as well as altered expression of PKM2 regulated genes. Notably, the authors outlined a novel assay, which they termed 2D gel electrophoresis-based proteome-wide CETSA, or 2DE-CETSA, that allowed for proteome wide screening for small molecule target identification (Nagasawa et al., 2020)

The Outlook

As can be seen from the outlined examples, it is a particularly exciting time for those who study and/or target dynamic proteins. Discovery methods that are tailored for the distinct biophysical characteristics of dynamic proteins, as well as those that more closely recapitulate the native context, have been highly successful. In addition to these screening techniques, thoroughly interrogating the selectivity of lead compounds on a global scale will be integral in deriving effective biological probes of dynamic protein targets. Techniques such as thermal proteome profiling, activity based protein profiling and Drug Affinity Responsive Target Stability (DARTS) allow determination of potential off target effects for a chemical probe in a cellular context (Jessani and Cravatt, 2004; Pai et al., 2015; Savitski et al., 2014). The resulting generation of more selective and potent small molecule modulators now enable the chemical genetic dissection of critical cellular machines. For example, studies with full length p300 and other various multidomain constructs show BRD inhibitors can affect HAT activity, suggesting interdomain crosstalk does occur (Shrimp et al., 2018; Zucconi et al., 2016). Interdomain crosstalk has been observed in other dynamic systems, particularly in nuclear receptors such as the thyroid hormone receptor and ER α , where the DNA binding via the DNA binding domain (DBD) can be enhanced by changes in the ligand binding domain (LBD) (Huang et al., 2018). Further exciting studies focusing on the global effects of selective dynamic protein modulators on full length proteins or intact protein complexes are sure to come in the future and improve our understanding of interdomain communication in these systems.

Another interesting avenue for dynamic proteins that has yet to be fully explored is proteolysis targeting chimeras (PROTACs). PROTACs could be particularly advantageous in the field of dynamic protein complexes because the starting scaffold does not need to functionally affect any interactions, as it can be challenging to find modulators with low IC₅₀s or EC₅₀s for certain targets. Additionally, even non-specific inhibitors can be starting points for PROTACs. Recent work has shown specificity can be engineered into a PROTAC starting from a multi-target warhead by exploiting differences in the interface between the protein of interest and the E3 ligase (Bondeson et al., 2018; Gadd et al., 2017). It is possible that through PROTACs, some of the challenges that come with identifying selective modulators can be averted. With regards to dynamic protein complexes, one could imagine using a PROTAC to completely alter complex composition or skew complexes to favor one exchangeable subunit over others. These and other novel selective mechanisms to chemically target dynamic proteins are sure to be on the horizon and we look forward to the exciting discoveries to come.

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Garlick et al. summarizes the opportunities and difficulties of selectively targeting dynamic proteins. Reviewing natural mechanisms of regulation and highlighting recent examples of selective inhibitors from literature, the authors suggest avenues to overcome the challenge. Discussion of approaches with potential to streamline discovery of selective chemical modulators is also provided.

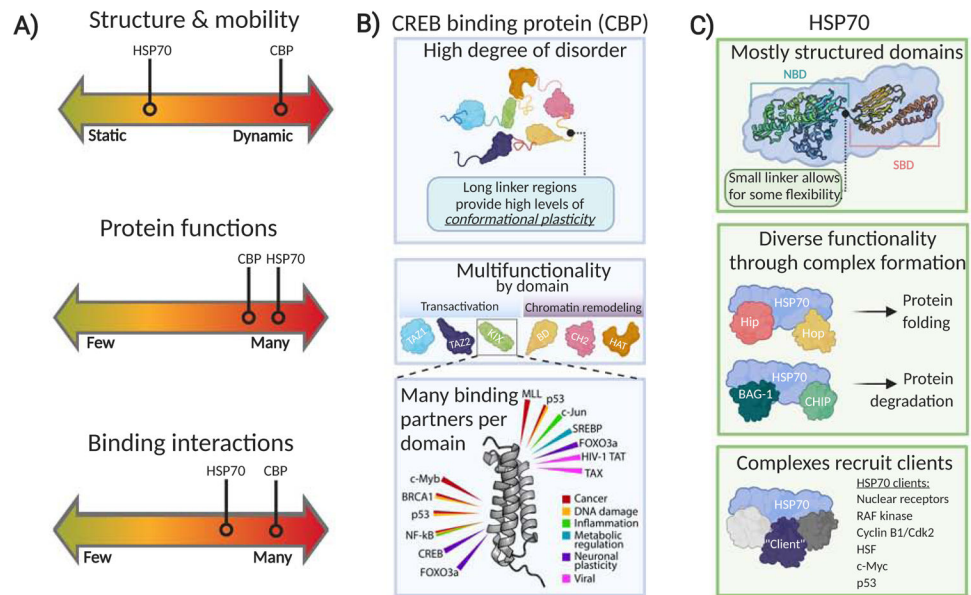


Figure 1. Key attributes of dynamic proteins with representative examples.

(A) While no single definition can be used to identify dynamic proteins, useful metrics for successful classification include: 1) the structure and mobility of the protein and/or the individual domains; 2) the number of functional roles the protein can fulfill; and 3) the number binding interactions the protein can participate in. Dynamic proteins tend to be multifunctional, participating in many pathways and performing multiple cellular roles, either through the presence of multiple domains or by the formation of dynamic transient complexes that vary in function. The functional multiplicity can in part be attributed to the number of binding partners the protein engages with, which oftentimes leads to conformational changes either within a single domain or domain rearrangement as an entire protein to elicit variable functional effects. (B) The master coactivator CREB binding protein (CBP) analyzed as a representative dynamic protein. CBP is highly disordered, with more unstructured and dynamic regions than structured domains (Dyson and Wright, 2016). The protein has high conformational plasticity, as its domains are connected by long unstructured linkers. The individual domains within CBP allow it to perform multiple functions. For example, CBP has four activator binding domains, KIX, TAZ1, TAZ2, and IBiD, that interact with transcriptional activators to regulate gene expression. It also contains a histone acetyltransferase domain (HAT) and bromodomain (BD) that it utilizes for perform chromatin remodeling functions (Breen and Mapp, 2018). Each of these individual domains has its own network of interactions, summing up to 100s of potential PPIs made by CBP. The KIX domain is shown here as an example – it interacts with a suite of 15+ activator proteins, all which can be implicated in a variety of diseases (Mapp et al., 2015). (C) The chaperone HSP70 analyzed as a representative dynamic protein. HSP70 contains much more secondary structure than CBP. It is comprised of two main domains: the nucleotide binding domain (NBD) and substrate binding domain (SBD). These two domains are connected by a small dynamic linker (Fernández-Fernández and Valpuesta, 2018). Substrate binding can induce domain rearrangement to elicit different functional effects. HSP70 itself functions as an ATPase, however it is able to perform many diverse cellular functions by forming

different multiprotein complexes. For example, Hsp70 in complex with cochaperones Hip and Hop acts as a protein folding complex, while Hsp70 in complex with BAG-1 and CHIP functions as a degradation complex (Muller et al., 2013). Finally, Hsp70 complexes also interact with many other proteins. Many regulatory proteins are known to be controlled via transient association with Hsp70. Thus, Hsp70 is a potential point of therapeutic intervention for conditions such as cancer, autoimmune and neurodegenerative diseases (A. Assimon et al., 2013).

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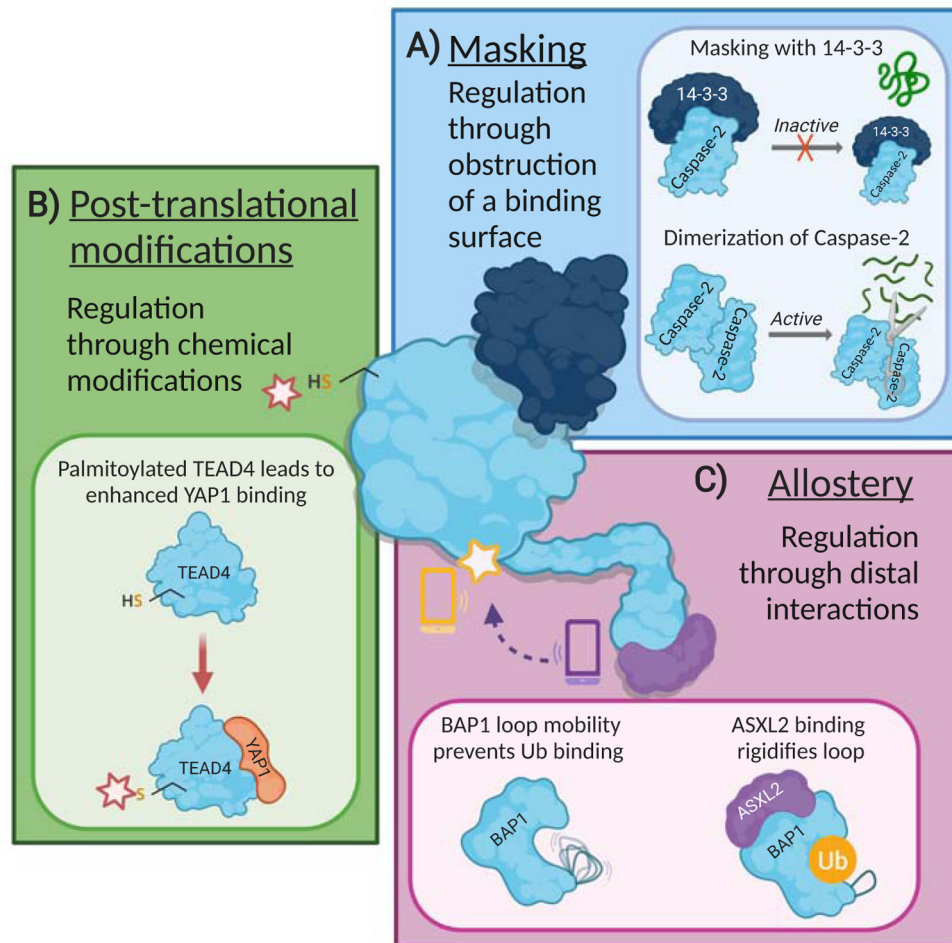


Figure 2. Nature uses multiple mechanisms to dictate interaction and function of dynamic proteins.

(A) Masking particular sequences or binding surfaces on proteins provides control over function, localization, and ligand binding. 14-3-3 regulates activity of Caspase-2 by masking its dimerization interface. Without 14-3-3 bound, Caspase-2 dimerizes and proteolytically cleave other proteins (Kalabova et al., 2020). (B) Post-translational modifications can both orthosterically and allosterically regulate protein by covalent modification of amino acid residues. Palmitoylation of transcription factor TEAD4 at C367 acts as an allosteric switch to enhance interaction with the coactivator YAP1 (Chan et al., 2016). (C) Allostery, via intra or intermolecular interactions, regulates structure and function of proteins. The Polycomb group protein ASXL2 interacts with the deubiquitinase BAP1, stabilizing a distal dynamic loop and allowing ubiquitin to bind and be hydrolyzed. Without ASXL2 interaction, BAP1 is unable to perform its enzymatic function (Peng et al., 2018).

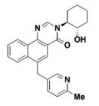
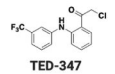
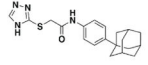
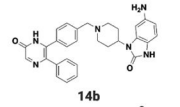
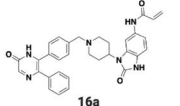
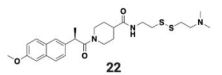
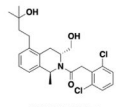
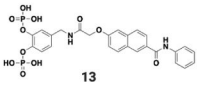
Compound	Target	Affinity / Activity	Selectivity
 MK-7622	Muscarinic acetylcholine receptor M1 (GPCR)	IP = 19 nM	No potentiation or agonism of M2-M4 muscarinic subtypes in CHO cell overexpression model (Beshore et al., 2018)
 TED-347	TEAD family proteins	EC ₅₀ = 3.5 ± 0.3 μM (TEAD4) EC ₅₀ = 4.3 ± 0.3 μM (TEAD2)	Does not inhibit other PPIs via FP assay (urokinase receptor and voltage-gated calcium channel Cav2.2) (Bum-Erdene et al., 2019)
 MGH-CP1	TEAD family proteins	IC ₅₀ = 710 nM (TEAD2); IC ₅₀ = 672 nM (TEAD4)	Did not affect autopalmitylation of several ZDHHC family palmitoyl acetyltransferases (Li et al., 2020)
 14b	Akt2	IC ₅₀ = 1237 ± 142 nM	10 fold selective over Akt1 >20 fold selective over Akt3 (Quambusch et al., 2019)
 16a	Akt1	IC ₅₀ = 39 ± 5 nM	334 fold selective over Akt2 >500 fold selective over Akt3 (Quambusch et al., 2019)
 22	Med25	Reduces <i>k_{off}</i> of activators by 25%	No selectivity data (Henderson et al., 2018)
 LY3154207	Dopamine receptor D1	EC ₅₀ = 3.0 ± 0.3 nM	>2700 fold selective over D5 receptor; >1000 fold selective over 40 other targets from the standardized Lilly selectivity panel (Hao et al., 2019)
 13	STAT5b	<i>K_i</i> = 44 ± 1 nM	55 fold selectivity over STAT5a (Gräb et al., 2019)

Figure 3. Recent allosteric modulators.

Quantitative affinity data for each target as well as any selectivity data from the literature is reported. Abbreviations: IP, inflection point of potentiation curve; EC₅₀, half maximal effective concentration; IC₅₀, half maximal inhibitory concentration; *k_{off}*, off-rate constant; *K_i*, inhibitory constant – the concentration required to produce half maximum inhibition.

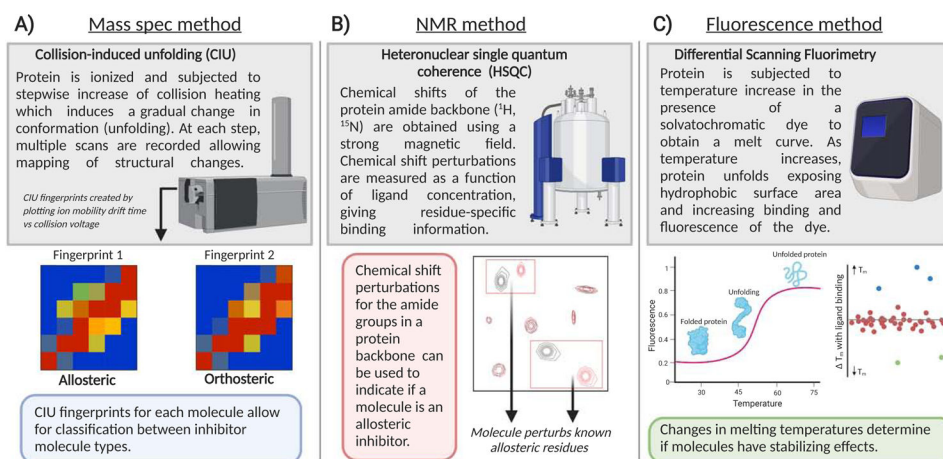


Figure 4. Examples of techniques for identification of dynamic protein modulators.

(A) Collision induced unfolding (CIU) can be utilized as a medium-high throughput approach to determine binding profiles protein•ligand complexes. If the CIU profile is known for a certain type of protein•ligand complexes, for example an allosteric signature or orthosteric signature, a screen can be conducted and molecules can be sorted by distinct fingerprints (Dixit et al., 2018). (B) $^1\text{H}^{15}\text{N}$ HSQC NMR can be utilized as a low-medium throughput approach to determine structural effects of small molecule binding to proteins. If a particular amino acid can be associated with allostery, a library of molecules can be screened looking for perturbation of chemical shifts associated with that residue. This method is particularly powerful when used in combination with computation data (as seen in Gupta et al.). (C) Differential scanning fluorimetry (DSF) is a high throughput method for screening proteins. This method monitors protein unfolding as a function of temperature. As the protein unfolds, a solvatochromatic dye binds and fluorescence increases. The melting temperature (T_m) of the protein is interpreted as the inflection point of the resulting melt curve (Niesen et al., 2007). While allosteric effects of small molecules cannot be determined using this method, their effect on protein stability can be determined. In a HTS, molecules can be sorted by their ability to increase or decrease protein T_m .