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Intrinsically disordered proteins: Ensembles at the limits of Anfinsen's dogma

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ABSTRACT

Intrinsically disordered proteins (IDPs) are proteins that lack rigid 3D structure. Hence, they are often misconceived to present a challenge to Anfinsen's dogma. However, IDPs exist as ensembles that sample a quasi-continuum of rapidly interconverting conformations and, as such, may represent proteins at the extreme limit of the Anfinsen postulate. IDPs play important biological roles and are key components of the cellular protein interaction network (PIN). Many IDPs can interconvert between disordered and ordered states as they bind to appropriate partners. Conformational dynamics of IDPs contribute to conformational noise in the cell. Thus, the dysregulation of IDPs contributes to increased noise and "promiscuous" interactions. This leads to PIN rewiring to output an appropriate response underscoring the critical role of IDPs in cellular decision making. Nonetheless, IDPs are not easily tractable experimentally. Furthermore, in the absence of a reference conformation, discerning the energy landscape representation of the weakly funneled IDPs in terms of reaction coordinates is challenging. To understand conformational dynamics in real time and decipher how IDPs recognize multiple binding partners with high specificity, several sophisticated knowledge-based and physics-based *in silico* sampling techniques have been developed. Here, using specific examples, we highlight recent advances in energy landscape visualization and molecular dynamics simulations to discern conformational dynamics and discuss how the conformational preferences of IDPs modulate their function, especially in phenotypic switching. Finally, we discuss recent progress in identifying small molecules targeting IDPs underscoring the potential therapeutic value of IDPs. Understanding structure and

function of IDPs can not only provide new insight on cellular decision making but may also help to refine and extend Anfinsen's structure/function paradigm.

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I. INTRODUCTION

For well over half a century, Anfinsen's dogma enunciated that a protein sequence/function paradigm constituted the foundation of our understanding of the protein universe. Anfinsen postulated that under favorable conditions, a protein will fold consistently into a native state structure, that is, effectively encoded in its amino acid sequence. The contextual nature (*under favorable conditions*) was implicit in the proclamation, "*the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment*" (Anfinsen *et al.*, 1961 and Anfinsen, 1973). Per this view, proteins have unique tertiary structures characterized by the fixed positions of their atoms and backbone dihedral angles that vary slightly around their equilibrium positions because of the low-amplitude thermal fluctuations.

However, the discovery that a significant portion of the proteome in all domains of life and all viral proteomes examined comprise intrinsically disordered proteins (IDPs) (or regions within ordered proteins, referred to as intrinsically disordered regions or IDRs) that lack rigid structure in the native state has drastically changed our perception of proteins (Ward *et al.*, 2004; Uversky, 2010; Schad *et al.*, 2011; Dyson, 2011; Xue *et al.*, 2012; Pancsa and Tompa, 2012; Midic and Obradovic, 2012; Korneta and Bujnicki, 2012; Hegyi and Tompa, 2012; Di Domenico *et al.*, 2013; van der Lee *et al.*, 2014; and Peng *et al.*, 2015). Furthermore, numerous computational studies have also revealed that the proportion of disorder increases with organism complexity (Dunker *et al.*, 2001; Ward *et al.*, 2004; Uversky, 2010; and Xue *et al.*, 2012). Thus, while the fraction of sequences predicted to have long IDPRs ($\geq$30 residues) is approximately equal in bacteria and archaea, it is significantly higher in eukaryotes (Dunker *et al.*, 2000; Ward *et al.*, 2004; Xue *et al.*, 2010; Xue *et al.*, 2012; Na *et al.*, 2013; and Peng *et al.*, 2015).

Despite the ubiquitous presence and evolutionary conservation, the IDP field was met with skepticism in the early years. In fact, the then unique, perhaps, tantalizing, observation that some proteins lacked ordered structures in isolation was considered as a mere artifact. On the contrary, it was tacitly assumed that, in the crowded cellular environment, such proteins would assume the native state. Thus, disorder was hardly considered as being important in orchestrating several of the molecular events in cell and developmental biology (Uversky and Dunker, 2010; Dyson and Wright, 2019; Fusco and Gianni, 2021; and for an interesting historical perspective, see Uversky and Kulkarni, 2021). Furthermore, because of the "apparent" lack of structure, IDPs are often misinterpreted by some as posing a challenge to Anfinsen's dogma (Potenza *et al.*, 2015; Das *et al.*, 2018; and Baul *et al.*, 2019). However, contrary to this view, IDPs abide by the Anfinsen's postulate albeit at its extreme limits (Vila, 2020 and Kulkarni, 2021). Therefore, despite lacking a single, well-defined equilibrium structure, IDPs exist as heterogeneous ensembles whose conformational properties do not abide by a single set of coordinates or backbone Ramachandran angles (Dunker *et al.*, 2013).

A. Order and disorder represent a structural and dynamic continuum rather than binary states

The description of proteins as ordered (folded) or disordered (unfolded) is predicated on their conformational ensembles. Ordered proteins tend to have thermally accessible states that resemble the ensemble average; however, disordered proteins sample an ensemble of dissimilar conformations during their biological lifetime (Wright and Dyson, 1999). Thus, while the native state of an ordered protein corresponds to a global energy minimum, that is, distinct from a large number of high energy states, disordered proteins display energy surfaces that contain multiple local energy minima that are separated by

low energy barriers. This helps ensure the rapid exchange between dissimilar states during the lifetime of the protein (Csermely *et al.*, 2010; Jensen *et al.*, 2014; Burger *et al.*, 2016; Schneider *et al.*, 2019; and Adamski *et al.*, 2019). Furthermore, the disorder spectrum covers a range of different entities from almost completely disordered and molten globules, to folded domains connected by disordered linkers and folded proteins flanked by disordered tails (Dyson and Wright, 2005; Uversky, 2013a; and van der Lee *et al.*, 2014). Therefore, although order and disorder are typically thought of as binary states, they form a continuum (DeForte and Uversky, 2016). Thus, in contrast to the lock-and-key analogy that represents a protein as highly ordered molecule, in reality, a protein molecule represents a complex system with remarkable spatiotemporal heterogeneity. Thus, a protein is likely to embody fragments with different structural complexities and folding complicities, such as foldons, inducible foldons, morphing inducible foldons, semi-foldons, and non-foldons (Uversky, 2013b; Uversky, 2016a; Uversky, 2016b; and Uversky, 2019b). This spatiotemporal heterogeneity of IDPs/IDRs is manifested in their multifunctionality with different (dis)ordered regions engaging in different functions (Uversky, 2015 and Uversky, 2016a). It also defines a structure-function continuum concept (Uversky, 2016b; Uversky, 2016c; Uversky, 2019a; and Uversky, 2019b); instead of the "one gene–one protein–one structure–one function" model, a protein molecule is a highly dynamic conformational ensemble with remarkable multifunctionality and binding promiscuity (Kulkarni *et al.*, 2018; Fonin *et al.*, 2019; Uversky, 2016b; and Uversky, 2019b).

The continuum also includes the specificity of interaction. This may explain the larger interactomes of the IDPs that are enabled by their flexibility and propensity to engage in promiscuous interactions. These properties allow IDPs to explore novel mechanisms such as facilitated exchange through trimer formation and ultra-sensitivity via threshold effects, and ensemble redistribution (Teilum *et al.*, 2021). Indeed, emerging evidence indicates that, like their ordered counterparts that exhibit allostery wherein the binding of the ligand stabilizes specific states and shifts the conformational ensemble (Kern and Zuiderweg, 2003; Gunasekaran *et al.*, 2004; and Tsai and Nussinov, 2014), IDPs can also exhibit allosteric effects (Garcia-Pino *et al.*, 2010; Motlagh *et al.*, 2012; Tompa, 2012; Ferreon *et al.*, 2013; Motlagh *et al.*, 2014; Krishnan *et al.*, 2014; Choi *et al.*, 2015; Garcia-Pino *et al.*, 2016; Zhang *et al.*, 2018; Li *et al.*, 2018; Berlow *et al.*, 2018; and Rehman *et al.*, 2019). The glucocorticoid receptor that contains a large IDR characteristic of the hormone receptor family of proteins further illuminates the degree of sophistication adopted by IDPs. In this case, genetically tunable "energetic frustration" controls allostery. Here, energetic frustration is defined the activity of the protein results from the combination of the opposing interactions, while the term genetically tunable refers to the splice variants with varying degrees of intrinsic disorder. Furthermore, the disordered regions can have opposing effects on other regions of the protein. Thus, energetic frustration can be envisaged as a "tug-of-war" whereby protein activity is predicated on a combination of the opposing interactions (Li *et al.*, 2017).

B. Structural plasticity enhances functionality

Because of the enormous conformational plasticity, IDPs occupy key nodal (hub) positions in cellular protein interaction networks (PINs) (Dunker *et al.*, 2005; Haynes *et al.*, 2006; Gsponer and Babu, 2009; Patil *et al.*, 2010; and Hu *et al.*, 2017). PINs that adopt a scale-free architecture serve as a conduit for channeling information flow within the cell (Barabasi and Albert, 1999; Barabási and Oltvai, 2004; and Barabási, 2009). However, because IDPs engage in promiscuous interactions when overexpressed (Vavouri *et al.*, 2009), they can also rewire PINs affording the system a robust degree of plasticity (Buljan *et al.*, 2013). It is, therefore, not surprising that the organization and properties of the PINs appear to be evolutionarily conserved (Rangarajan *et al.*, 2015).

In addition to channeling information, IDPs play important roles in numerous biological processes, including transcriptional regulation, splicing, signaling and development, and differentiation (Uversky *et al.*, 2008; Wright and Dyson, 1999; Dunker *et al.*, 2002; Uversky and Dunker, 2010; Xue *et al.*, 2012; Oldfield and Dunker, 2014; Uversky, 2015; Wright and Dyson, 2015; Berlow *et al.*, 2015; Fung *et al.*, 2018; Deiana *et al.*, 2019; and Csermely *et al.*, 2020). The physical characteristics of IDPs such as interactions characterized by high specificity but low affinity, and kinetic advantages in signaling, allow for an exquisite level of control of cellular signaling processes (Pontius, 1993; Wright and Dyson, 1999; Dyson and Wright, 2005; Oldfield *et al.*, 2005; and Wright and Dyson, 2015). Post-translational modifications (PTMs), for example, phosphorylation, further fine-tune the functions of IDPs to behave as sophisticated and sensitive switches and rheostats in the regulatory circuits they modulate (Dyson and Wright, 2005; Gsponer and Babu, 2009; Lee *et al.*, 2010; Van Roey *et al.*, 2012; and Van Roey *et al.*, 2013).

In addition, IDPs play important roles in many cellular processes such as regulation of the cell division cycle (Galea *et al.*, 2008; Yoon *et al.*, 2012; Mitrea *et al.*, 2012; Buske *et al.*, 2015; and Tsytlonok *et al.*, 2019), circadian rhythmicity (Hurley *et al.*, 2013; Dong *et al.*, 2016; Pelham *et al.*, 2018; Pelham *et al.*, 2020; and Diernfellner and Brunner, 2020), stress response (Boothby *et al.*, 2017), and phenotypic plasticity (Mooney *et al.*, 2016; Jia *et al.*, 2017; and Kulkarni *et al.*, 2020). Furthermore, several IDPs are reported to prion-like functions to actuate protein-based molecular memories underlying the emergence and inheritance of biological traits (Chakrabortee *et al.*, 2016), underscoring their importance in phenotype switching. Indeed, based on these observations, IDPs have also been implicated as playing a role in multicellularity, a cornerstone in major evolutionary transitions (Kulkarni and Uversky, 2018a and Kulkarni, 2021). Moreover, when dysregulated, IDPs can also cause pathological states (Iakoucheva *et al.*, 2002; Uversky *et al.*, 2008; Uversky, 2014; and Uversky *et al.*, 2014) (Vavouri *et al.*, 2009 and Marcotte and Tsechansky, 2009). Consistent with these observations, IDPs are dysregulated in several chronic human diseases, including cancer, diabetes, neurodegenerative diseases, and several genetic diseases (Uversky *et al.*, 2008; Babu *et al.*, 2011; Uversky, 2014; Kulkarni and Uversky, 2019; Santofimia-Castaño *et al.*, 2020; Brocca *et al.*, 2020; Midic *et al.*, 2009; and Uversky *et al.*, 2009). It is, therefore, not surprising that cellular IDP levels are tightly regulated from synthesis to degradation (Gsponer *et al.*, 2008 and Edwards *et al.*, 2009).

Paradoxically, however, some IDPs are important in protein folding (Lermyte, 2020). For example, several stress-response proteins and chaperone proteins are IDPs (Tompa and Kovacs, 2010; Uversky, 2011; and Webster *et al.*, 2019). Consistent with a chaperone function, deleting a disordered 23-residue C-terminal portion of GroEL that faces the central cavity of the bacterial GroEL-GroES complex in which folding occurs compromises chaperone function (Machida *et al.*, 2008). In tardigrades that can endure extreme conditions, IDPs

are found to respond to these challenges (Janis *et al.*, 2018 and Hesgrove and Boothby, 2020). Similarly, several stress response proteins in plants (Covarrubias *et al.*, 2017; Balcerowicz, 2020; and Rae *et al.*, 2014) and proteins that mediate plant immune responses to pathogens are IDPs (Sun *et al.*, 2014). Furthermore, IDPs play a role in regulation of plant growth (Sun *et al.*, 2010), development, and signaling, often by integrating signals from multiple plant growth regulatory inputs (Sun *et al.*, 2011 and Sun *et al.*, 2013). Similarly, in the human, IDRs are found in the small heat shock proteins Hsp22 and αB-crystallin (Kazakov *et al.*, 2009 and Sudnitsyna *et al.*, 2012), highlighting the link between stress and IDPs both in plants and animals.

C. Mechanisms underlying IDP interactions

In light of the incredible functional repertoire of the IDPs, understanding how they interact with partners in spite of (the perceived) lack of structure is of significant interest. It is now evident that some IDPs can undergo transitions from disorder to order upon binding to their cognate targets, a phenomenon referred to as "coupled folding and binding" (Dyson and Wright, 2002; Oldfield *et al.*, 2005; Mohan *et al.*, 2006; Cheng *et al.*, 2007; Vacic *et al.*, 2007; and Oldfield *et al.*, 2008). Two models have been advanced supporting this concept. While the "induced fit" mechanism postulates that folding occurs after association of the IDP with the target, the "conformational selection" mechanism suggests that all potential conformations of the ensemble exist *a priori* and the ligand then "selects" the most favored prefolded state from this preexisting pool (Boehr *et al.*, 2009). However, in studies on the interaction between pKID/KIX and KIX/Myb (Arai *et al.*, 2015) and the C-terminal domain (CTD) of the measles virus nucleoprotein (Wang *et al.*, 2013), some combination of both these mechanisms may also be applicable, suggesting that the exact binding mechanism is determined by the intrinsic secondary structure propensities of the IDPs (Wright and Dyson, 2015).

Coupled folding and binding is a complex process involving at least two steps—binding to the partner and folding of the IDP (Wright and Dyson, 1999; Uversky, 2002; Wright and Dyson, 2005; Tompa and Fuxreiter, 2008; Tompa, 2011; Kiefhaber *et al.*, 2012; Habchi *et al.*, 2014; and Gianni *et al.*, 2016). However, it is important to recognize that mechanistically, there are distinct differences between the classical spontaneous folding of globular proteins and binding-induced folding of IDPs. More specifically, while globular proteins fold via a robust mechanism consolidated by the presence of a loosely formed yet specific nucleus (Fersht, 1995 and Itzhaki *et al.*, 1995), IDPs appear to fold by heterogeneous nucleation via an overall mechanism, that is, induced by interaction with the partner (Rogers *et al.*, 2014a; Rogers *et al.*, 2014b; Toto and Gianni, 2016; Toto *et al.*, 2016; and Bonetti *et al.*, 2018). A recent study on protein folding employing molecular dynamics simulations with all-atom force fields, with folding pathways interpreted in terms of soliton structures, examined the presence of systematic dynamical patterns of self-organization that may govern protein folding. Simulations were performed on the conformational transformations of three different proteins, namely, the ordered region of the oncoprotein MYC, amylin, and indolicidin (IDPs with different length and binding dynamics). Interestingly, the authors observed the emergence of soliton-mediated secondary motifs only in the case of IDPs suggesting that, indeed, the folding mechanisms in IDP folds are different, and that soliton-like quasi-ordered conformations may serve as an important intermediate stage in this process (Ilieva *et al.*, 2016). Consistent with this observation, a previous study (Austin *et al.*, 2009) on the protein myoglobin showed that there is no long-lived Davydov soliton, at least in this highly ordered protein. Similarly, a theoretical study on intrinsic localized modes (ILMs), which are members of the large soliton family (Nicolaï *et al.*, 2015), found that the probability of ILMs playing a significant functional role in the flexible regions of the proteins and in proteins in a non-native state is significantly higher than in folded proteins/regions lending further credence to the idea that soliton-mediated structural events may be prevalent in IDPs. Furthermore, in model systems, it was demonstrated that structural disorder facilitates transmission of solitons (Kartashov *et al.*, 2011).

Thus, it follows that the mechanisms of disorder-to-order induced folding in IDPs could be intrinsically different from the mechanisms seen in globular proteins. However, there are important similarities. For example, the cooperative nature of the reaction underlying disorder-to-order transitions in IDPs is comparable to that of ordered proteins. However, their folding pathways are strikingly more malleable because of the heterogeneous nature inherent in their folding nuclei (Toto *et al.*, 2020). Furthermore, the timescale that governs the conformational dynamics is an important factor in the binding mode for IDPs (Choi *et al.*, 2019). In the induced fit model, rapid conformational dynamics play an important role. In many cases, the energy from order transitions is coupled to the recognition event. On the other hand, the disorder persists even in the bound state in some IDPs (Borgia *et al.*, 2018 and Tsytlonok *et al.*, 2019). If the conformational dynamics are slow, then the binding mode is limited to conformational selection. In this case, interactions can only occur in the presence of the binding-competent configuration.

Another model is the "extended conformational selection," which is a repertoire of selection and adjustment processes (Csermely *et al.*, 2010). Here, the contribution of induced fit that constitutes a subset of this repertoire is affected by the bonds, which stabilize the interaction and the differences between the partners. Per this model, segments, or regions of the proteins with dynamics distinct from the rest of the molecule referred to as "discrete breathers," can impact conformational transitions and the propagation of allosteric signals that occur along with the binding processes.

Aside from the scenarios described above, some IDPs do not appear to assume any discernable structure even when bound to a cognate ligand. For example, regions of caldesmon (Permyakov *et al.*, 2003 and Permyakov *et al.*, 2015), anhydrin (Chakrabortee *et al.*, 2010), c-Myc (Andresen *et al.*, 2012), prostate-associated Gene 4 (PAGE4) (He *et al.*, 2015), and the transcription factors (TFs) Sp1 and TAF4 (Hibino and Hoshino, 2020) remain largely disordered even while interacting with their cognate partners. Such interactions are described as "fuzzy complexes" (Tompa and Fuxreiter, 2008) suggesting yet another molecular mechanism underlying IDP interactions (Choi *et al.*, 2011 and Latysheva *et al.*, 2015). Therefore, IDPs can form tight complexes in the absence of any ordered structure (Borgia *et al.*, 2018) while retaining long-range flexibility and highly dynamic character (Mittag *et al.*, 2010 and Borgia *et al.*, 2018). Thus, the conformational equilibria present in even the bound states facilitate pleiotropic functions of IDPs (Tompa *et al.*, 2005) underscoring their importance in regulatory processes (Berlow *et al.*, 2015).

D. IDP interactions involve specific motifs

Interaction of IDPs with binding partners involves short sequence motifs referred to as short linear motifs (SLiMs) and molecular recognition features (MoRFs) as well as low-complexity sequences (Mohan *et al.*, 2006; Davey *et al.*, 2012; and van der Lee *et al.*, 2014). Frequently, two or more of such motifs are found in the same IDP underscoring multivalent interactions (Davey *et al.*, 2012; van der Lee *et al.*, 2014; Van Roey *et al.*, 2014; Krystkowiak and Davey, 2017; and Bhowmick *et al.*, 2015) and increasing the overall avidity of the interaction by exploring conformational ensembles that are recognized by distinct binding partners (Fung *et al.*, 2018 and Uversky *et al.*, 2008). Thus, the same binding region can bind to several different partners with very similar affinities (Oldfield *et al.*, 2008).

Interactions of IDPs that involve SLiMs often involve contributions from the flanking regions and/or such interactions are contextual. These contributions are typically electrostatic acting through either highly negatively charged proteins, for example Rb binding proteins (Palopoli *et al.*, 2018), or positively charged proteins such as the PCNA binding PIP-box (Prestel *et al.*, 2019). However, some flanking regions have also been observed to be hydrophobic (Alanen *et al.*, 2011). Furthermore, the structure and dynamics of the flanking regions can contribute to competition, cooperativity, and allosteric regulation (Berlow *et al.*, 2017) in addition to ensuring proper orientation and the velocity with which interactions occur (Fuxreiter *et al.*, 2007). Nonetheless, the underlying thermodynamics and the exact structural requirements of such interactions are not fully understood (Bugge *et al.*, 2020).

E. Conformational dynamics and conformational noise

In addition to the energetics of binding reactions, conformational dynamics also enables IDPs to control and regulate their hydrodynamic volume and spacing. For example, conformational exchange allows IDPs to explore a large volume while seeking binding partners appropriately dubbed as the "fly-casting" model (Shoemaker *et al.*, 2000; Hoffman *et al.*, 2006; and Metskas and Rhoades, 2015). Similarly, the entropic clock model demonstrates how the degree of extension of an IDP linker region between a pore and its blocking domain modulates timing of an ion channel (Podlaha and Zhang, 2003). Finally, the entropic bristle model revealed how IDPs regulate protein interactions by exploring large search space before populating an appropriate conformation (Hoh, 1998). Together, these unique aspects of IDPs underscore how the timescale and the range of conformational sampling within the ensemble modulates their structural properties.

In addition to affecting structural properties of the IDPs, conformational dynamics also results in noise referred to as "conformational noise" (Mahmoudabadi *et al.*, 2013; Kulkarni and Kulkarni, 2019; and Kulkarni, 2020) that it is distinct from transcriptional noise (Eldar and Elowitz, 2010 and Hansen *et al.*, 2018). In biology, noise is defined as the random variability in biomolecular quantities. Such variation arises even in the absence of any genetic contribution, and as a result, even cells that are isogenic can exhibit significant stochastic fluctuations in protein levels that are leveraged to facilitate probabilistic bet-hedging decisions (Jolly *et al.*, 2018 and Hansen and Weinberger, 2019). Indeed, transcriptional noise that arises due to stochasticity in gene expression is well documented (Raj and van Oudenaarden, 2009 and Hansen *et al.*, 2018), and isogenic cells in a population are observed to switch states (phenotypes) and behave differently in response to the same stimulus (Brock *et al.*, 2009 and Huang, 2009). Indeed, noise-driven phenotypic switching is now acknowledged to play an important role in development, stress response, disease pathological, and evolution (Mahmoudabadi *et al.*, 2013). Furthermore, stochasticity in phenotypic switching has been reported to modulate differentiation (Eldar and Elowitz, 2010 and Simon *et al.*, 2018), stem cell reprogramming (MacArthur *et al.*, 2008; Yamanaka, 2009; Wakao *et al.*, 2013; Chung *et al.*, 2014; Lin *et al.*, 2018a; Lin *et al.*, 2018b; and Raina *et al.*, 2021), and the conversion of cancer cells to cancer stem-like cells (Gupta *et al.*, 2011 and Sehl *et al.*, 2015).

Aside from transcription, noise also significantly affects information transduced in cellular PINs (Ladbury and Arold, 2012), especially noise contributed by random protein interactions (Kuwahara and Gao, 2013) due to promiscuous interactions (Kontogeorgaki *et al.*, 2017 and Azpeitia *et al.*, 2020). Consistent with this argument, most transcription factors (Liu *et al.*, 2006; Niklas *et al.*, 2015; Strzyz, 2018; and Brodsky *et al.*, 2020) and hub proteins in cellular PINs are IDPs (Haynes *et al.*, 2006; Dosztányi *et al.*, 2006; Gsponer and Babu, 2009; and Patil *et al.*, 2010). Therefore, in response to extrinsic or intrinsic perturbations, IDPs can unmask latent interactions to cause phenotypic switching (Mahmoudabadi *et al.*, 2013 and Kulkarni and Kulkarni, 2019). IDP conformational noise is implied as noise due to the random variability in sampling ensemble. Further, although interconversions of IDP conformations are in fast exchange, they are typically modified by post-translational modifications such as phosphorylation, which can result in variant conformational ensembles to have significantly longer half-lives (Kulkarni and Kulkarni, 2019).

Though conformational switching and even fold switching are well documented in folded/metamorphic proteins (Kulkarni *et al.*, 2018), some IDPs can switch between discrete conformational ensembles even while remaining disordered in both states (Choi *et al.*, 2011 and Choi *et al.*, 2019). While such transitions possess many common features, several IDPs stochastically switch between distinct states within the entire conformational space or display dynamics on slow timescales. Therefore, an in-depth understanding of the conformational dynamics beyond just the minimum energy states that characterize the ensemble both in terms of the landscape, that is, accessible, and the timescales is necessary to gain more insight into IDP structure and function.

F. Characterizing IDPs

Experimental characterization of IDPs, especially large proteins or regions within proteins, remains a challenge. X-ray crystallography and cryo-EM, which recover high-resolution images of proteins in their crystalline and frozen states, respectively, and provide a static view, are not well suited (Kaptein and Wagner, 2019). However, techniques, such as nuclear magnetic resonance (NMR), small-angle x-ray scattering (SAXS), single-molecule Förster resonance energy transfer (FRET), dynamic light scattering (DLS), and two-focus fluorescence correlation spectroscopy (2f-FCS), atomic force microscopy (AFM), circular dichroism (CD), fluorescence, Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy, and mass spectrometry (MS), are adapt at identifying the conformational transitions sampled by IDPs (Kjaergaard *et al.*, 2010a; Kjaergaard *et al.*, 2010b; Jurneczko

et al., 2012; Camilloni et al., 2012; Bernadó and Svergun, 2012; Jensen et al., 2013; Sterckx et al., 2014; Borgia et al., 2016; Khan et al., 2017; Cordeiro et al., 2017; Gomes and Gradinaru, 2017; LeBlanc et al., 2018; Dyson and Wright, 2019; Chan-Yao-Chong et al., 2019; Bax and Clore, 2019; Metskas and Rhoades, 2020; Casuso et al., 2020; and Dyson and Wright, 2021) since they perform measurements of protein molecules as they fluctuate in their "natural" environment. On the other hand, the measurements provided by the above techniques are of limited resolution. Therefore, they are inadequate to discern the distribution of the multiple distinct IDP conformational ensembles. Molecular simulations that can complement and even validate the experimental observations, have emerged as increasingly important tools to elucidate IDP conformational ensembles (Bhattacharya and Lin, 2019; Hsu et al., 2020; Dokholyan, 2020; Zhao et al., 2020; and Wang, 2021). Nonetheless, visualizing their energy landscape presents a formidable challenge.

In this review, using specific examples of IDPs, we highlight recent advances in molecular dynamics simulations and in energy landscape visualization techniques that have shed new light on their conformational dynamics and its functional implications at a systems level. In addition, we also discuss the emerging role of IDPs as therapeutic targets that, until recently, were regarded as "undruggable." Thus, a deeper understanding of the IDPs can not only provide new insight on cellular decision making with wider implications in biology and medicine but may also help to refine and extend the structure/function paradigm beyond Anfinsen's postulate.

II. ENERGY LANDSCAPE

A. Molecular dynamics simulations (MD)

The energy landscape theory has provided a general conceptual framework to understand the folding and functional properties of proteins (Frauenfelder et al., 1991; Onuchic and Wolynes, 2004; and Thirumalai et al., 2010). Based on the principle of minimal frustration (Bryngelson and Wolynes, 1987 and Ferreiro et al., 2018) and grounded on statistical mechanics principles, this approach led to the description of the protein folding funnel (Leopold et al., 1992 and Bryngelson et al., 1995), which has brought a comprehensive understanding of biomolecular processes, bridging theory and experiments (Hills and Brooks, 2009; Onuchic et al., 1997; and Chung et al., 2009). Indeed, these techniques not only aid the study of protein folding but also help elucidate the functional dynamics, which can involve large-scale conformational changes (Takada et al., 2015), motor-like energy transfer, and assembly (Hirokawa et al., 2009).

The general goal of this approach has been to describe the dynamics and thermodynamics of biological molecules in the context of funnel-like landscapes, which takes into account the interplay of topological, energetic, and entropic aspects (Koga and Takada, 2001). Despite the fact that protein folding and functional dynamics are intrinsically multidimensional, the energy landscape approach accurately describes the kinetic and thermodynamic properties in terms of a few key quantities that are used as reaction coordinates. The computational description is highly simplified when there are reference conformations, such as native or functional state, which are used as reaction coordinates and correlated with measurable experimental variables.

The analysis of MD trajectories seeks to capture properties of a system, such as the dominant kinetics and structural features of the transition state ensembles, as a function of few-dimensional reaction coordinates. Beyond the straightforward structure-based coordinates, such as the fraction of native contacts and the root mean square distance (RMSD) from reference structures, there are alternative strategies for inferring suitable reaction coordinates to describe the energy landscape. For instance, transition-path analysis can be used to find the coordinates that best portray the underlying free-energy barrier (Best and Hummer, 2016). On the other hand, time-correlation analysis (Noé and Clementi, 2017) allows defining classes of collective variables (CVs) associated with the slowest motions. A common limitation of these techniques is that they require, in general, *a priori* definition of coordinates, which can be time-consuming and computationally expensive. Moreover, applying putative coordinates may hide the richness of the dynamics.

Other approaches for representing the landscape involve determining a connectivity map between long-lived states, which can be inferred by Markov state models (MSMs) (Chodera and Noé, 2014; Zimmerman et al., 2017; and Jacobs and Shakhnovich, 2018). Local minima can be individually addressed and go beyond one-dimensional representation (Wales, 2010), and the visualization of distances between local minima in a hierarchical representation is also an appealing way to probe the energy landscape (Wales, 2018). The above methods suit well to investigate funnel-like landscapes with well-defined energy basins. However, IDPs are far more challenging systems due to the high disorder, shallow energy minima, and lack of reference structures.

B. Energy landscape visualization method (ELViM)

A novel approach called the energy landscape visualization method (ELViM) (Oliveira et al., 2014 and Oliveira et al., 2019) that relies upon a multidimensional scaling (MDS) method to examine IDP structure appears quite promising. This method is a reaction coordinate-free method, and it is based on pairwise distances between all structures of the ensemble (Ragonnet-Cronin et al., 2013). Using a local structural similarity metric (Hardin et al., 2000), one can survey and triangulate a high-dimensional conformational phase space and project the ensembles to two optimal dimensions but, at the same, preserve the local proximities. Thus, ELViM permits an intuitive visual analysis of the energy landscape. Moreover, different ensembles can be mapped into a single phase-space, which allows comparisons of ensembles investigated under different physical and chemical conditions.

Using the ELViM method, the authors focused on prostate-associated Gene 4 (PAGE4) in which three different phosphorylated versions of the PAGE4 protein were analyzed [wild type (WT)-PAGE4, HIPK1-PAGE4, and CLK2-PAGE4)] (Oliveira et al., 2021). In the ELViM 2D projection, each conformation is represented by a point, which can be fully examined, calculating any desirable variable, such as radius of gyration or specific distances between residues [Fig. 1(a)]. Moreover, ensembles can be analyzed separately, from which the density of states and free energies can be estimated [Fig. 1(b)]. In another study, the same group leveraged ELViM to amyloid-β (Aβ) monomer variants, all IPDs, to discern their propensities for fiber formation (Sanches et al., 2022). Figure 2 shows the ELViM projection of different ensembles of amyloid-β monomers, all projected in the same 2D effective phase space. Each conformation of the dataset can be examined individually, and any chosen variable can

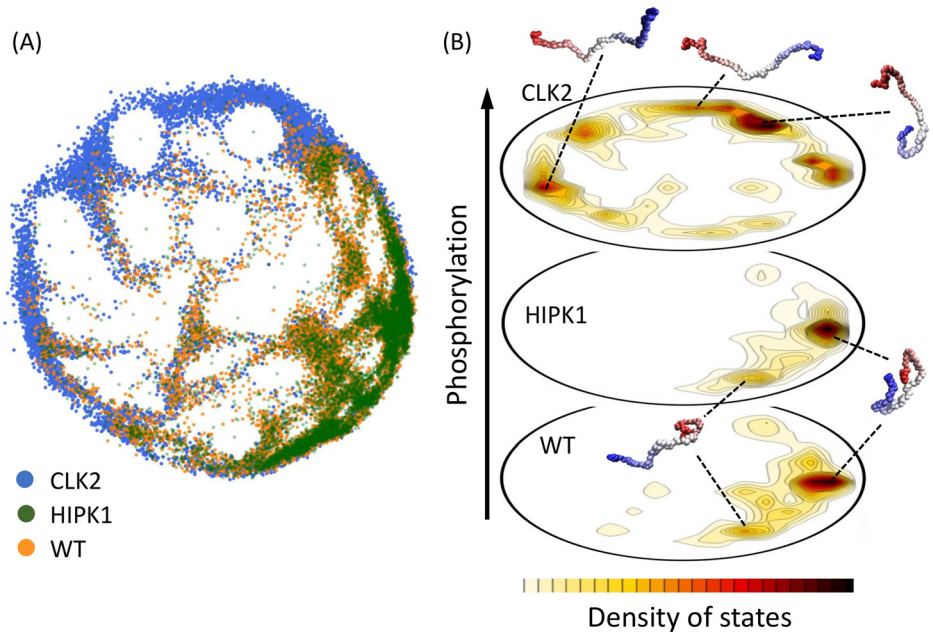

FIG. 1. The different PAGE4 ensembles represented in a single conformational phase space using the energy landscape visualization method. (a) Each conformation displayed by a point in the effective phase space. (b) Contour plots showing the density of states of the wild type (WT) and phosphorylated PAGE4. Each free energy valley is characterized by specific conformations that entail particular binding affinities. For WT-PAGE4, through a fly-casting mechanism, the C-terminal region is extended, facilitating its interaction with partner protiens. In the case of HIPK1-PAGE4, the lower free energy of the compact state decreases the affinity for c-Jun, while the extended conformations of CLK2-PAGE4 due to hyperphosporylation inhibit this interaction.

be tracked and colored accordingly. As an example, Fig. 2(b) shows all conformations of the amyloid-β monomers colored according to their radius of gyration (R_g), and examples of the conformations from different regions of the phase space are also shown. Therefore, one would expect that if such projection makes sense, meaningful coordinates would be "well behaved" and vary continuously throughout the 2D representation, which is shown by R_g in Fig. 2(b). Considered together, this MDS strategy appears to provide an insightful representation of IDP energy landscape.

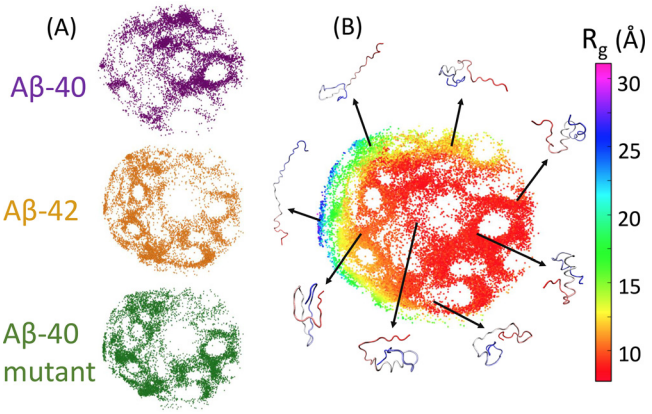

FIG. 2. Conformational phase space of the simulated amyloid-β (Ab) structures. (a) Distinct Ab ensembles present in the projection with the Ab-40 (purple), Ab-42 (beige), and the Ab-40 mutants (green). (b) Each point represents a conformation, which is colored as function of radius of gyration. Typical conformation examples of each region are displayed around the projection.

C. Parallel tempering (PT)

IDPs exist in shallow rugged free energy landscapes with multiple conformational populations that are in dynamic equilibrium with each other. As such, it is difficult to structurally resolve them at high-resolution with experimental techniques. Of late, in order to elucidate the structural and dynamical features of IDPs at higher resolution, molecular simulation is being routinely used in conjunction with low-resolution ensemble-averaged data (Lindorff-Larsen *et al.*, 2012; Bonomi *et al.*, 2017; Best, 2017; Peterson *et al.*, 2017; Bottaro and Lindorff-Larsen, 2018; Kasahara *et al.*, 2019; Gomes *et al.*, 2020; and Kassem *et al.*, 2021) from experiments such as SAXS (Henriques *et al.*, 2015; Hub, 2018; Hermann and Hub, 2019; Chan-Yao-Chong *et al.*, 2019; Ahmed *et al.*, 2021; and Kassem *et al.*, 2021), NMR (Fawzi *et al.*, 2008; Robustelli *et al.*, 2010; Fisette *et al.*, 2012; Fu and Vendruscolo, 2015; Salvi *et al.*, 2016; Papaleo *et al.*, 2018; Chan-Yao-Chong *et al.*, 2019; Heller *et al.*, 2020; and Kassem *et al.*, 2021), FRET (LeBlanc *et al.*, 2018 and Lerner *et al.*, 2021), and cryo-electron microscopy (Bonomi and Vendruscolo, 2019 and Nierzwicki and Palermo, 2021). Despite the many advances, extracting experimentally consistent ensemble for an IDP remains highly challenging. This, in large part, is due to the presence of diverse conformational states in an ensemble that render the experimental data noisy, sparse, and/or ambiguous. On the other hand, the molecular simulations typically sample only a tiny phase space of an IDP ensemble despite the underlying free energy landscape being shallow in nature. The presence of significant entropic barriers between different population clusters is an often-overlooked aspect of IDPs sampling and the primary reason for samples not reproducing the ensemble and thermodynamic averages of experiments. Also, modeling extremely fuzzy IDPs with very low hydrophobicity and high net charge is yet another example of entropically stabilized systems that are not sampled properly and need to be addressed post haste due to emerging roles of such IDPs in

interactions, both at single molecule recognition level (Jephthah *et al.*, 2019; Schuler *et al.*, 2020; and Sottini *et al.*, 2020) and in assemblies (Li *et al.*, 2001 and Rauscher and Pomès, 2017).

The determination of experimentally consistent ensemble data from simulation mandates adequate sampling, which is generally achieved in advanced sampling approaches by either applying structural restraints using collective variables or by reweighting the obtained conformations to arrive at the Boltzmann weighted populations (Cavalli *et al.*, 2013; Rangan *et al.*, 2018; and Köfinger *et al.*, 2019). Parallel tempering (PT) sampling is an attractive alternative since it can be used effectively without any reweighing and restraining and without the need to have a low-dimensional collective variable (CV) to define the ensemble states. Moreover, instances when sampling outcomes are not commensurate with experimental data, PT can be coupled seamlessly with other CV-based restraining methods or reweighted appropriately to solve the problems of interest (Do *et al.*, 2014; Zerze *et al.*, 2015; Awasthi and Nair, 2017; and Liu *et al.*, 2020). In the classical version of this method (Sugita and Okamoto, 1999) called temperature replica exchange MD (TREMD), multiple replicas are simulated simultaneously at a series of low and high temperatures and neighboring replicas are stochastically swapped at regular intervals based on criteria that honor detailed balance. These random walk swaps allow the broader phase space explored at the high temperature replicas to be accessed by the low temperature replicas, thereby generating an unbiased Boltzmann-weighted ensemble of conformations at a given temperature. The acceptance probability of swapping depends on the extent of potential energy overlap between the adjacent replicas and as the system size increases, more and more numbers of replicas are required for effective sampling (Baumketner and Shea, 2007; Wang *et al.*, 2013; Zerze *et al.*, 2015; and Jain *et al.*, 2021). This problem is particularly exacerbated when simulating large proteins in explicit solvent where the bulk of solvent molecules contributes majorly toward the poor overlap. For instance, about 96 replicas were needed to sample a 20-residues long disordered N-tail of measles envelop virus protein in solvent, and 180 replicas were needed for effectively sampling a 63-residues long alpha-synuclein (Wang *et al.*, 2013 and Baumketner and Shea, 2007).

Several variants of PT have evolved in recent years to alleviate the huge computational expenses of classical TREMD. One of the popular approaches is the replica exchange with solute tempering/scaling (REST/REST2 and gREST), where the Hamiltonian is designed in such a way that it effectively heats up the solute while keeping the solvent at room temperature (Liu *et al.*, 2005; Wang *et al.*, 2011; and Kamiya and Sugita, 2018). This transformation drastically reduces the required number of replicas as the exchange probability now depends only on the solute degrees of freedom and forgoes the calculations from the expensive solvent self-interactions. This method has found tremendous applications in sampling IDPs (Musiani *et al.*, 2013; Brown *et al.*, 2014; Smith *et al.*, 2016; Peng *et al.*, 2017; Shrestha *et al.*, 2019; Liu and Chen, 2019; and Shrestha *et al.*, 2021) and also on studies related to IDPs binding to their cognate partners (Miller *et al.*, 2014; Smith *et al.*, 2019; Khayat *et al.*, 2020; Noda *et al.*, 2020; Zhao *et al.*, 2021; and Gopal *et al.*, 2021).

The success of REST2, the evolved version of REST, is highly dependent on the choice of forcefield. In most of the cases, the combined application of REST2 with a99SB-disp forcefield (Robustelli *et al.*, 2018) recapitulated almost all the experimental measurements (Liu and Chen, 2019 and Shrestha *et al.*, 2021). For instance, in p53-TAD, the REST2-a99SB-disp duo generated ensemble captures multiple local and long-range structural properties, including chain dimension, residual secondary structures, and transient long-range contacts in consistent with measurements from NMR, smFRET, and TR-FRET experiments. Whereas with other state-of-the-art forcefields including Charmm36m (Huang *et al.*, 2017) and Amberff99SB-ILDN/TIP4PD (Lindorff-Larsen *et al.*, 2010 and Piana *et al.*, 2015), the REST2 either suffered with inadequate convergence or over-compaction issue (Liu and Chen, 2019). REST2 was also used in studying the coupled folding induced binding in c-Myb/KIX (Gopal *et al.*, 2021) and Bcl-XL/PUMA (Liu *et al.*, 2017) complexes. In these simulations, the REST2-a99SB-disp combination provided high-precision accurate structural properties when compared to circular dichroism and secondary chemical shifts. However, the predicted nuclear Overhauser effect (NOE)-like distances show significant violations from the NMR derived values particularly at the interface residues. This indicates the formidable challenge of sampling tertiary packing of IDP segments likely originated from inadequate sampling and convergence in these simulations (Smith *et al.*, 2016 and Liu *et al.*, 2017).

In addition to poor convergence and inadequate sampling in some cases, the REST2 also suffers with poor mixing of replicas between the high and low temperature regimes in complex proteins (Huang *et al.*, 2007 and Smith *et al.*, 2016), where friction between the protein and solvent determines the rate of conformational transition, and most existing methods suffer in overcoming the solvent-imposed (entropy driven) free-energy bottlenecks. This aspect is amply highlighted in a recent study where REST2-derived conformations at high and low temperatures are shown trapped in local temperature basins with nominal exchange taking place between them (Appadurai *et al.*, 2021). In this work, the entropic lock problem is solved by enabling differential tempering of both the solute and solvent in the Hamiltonian (Fig. 3). This scheme, called as replica exchange with hybrid tempering (REHT), specifically allows faster decay of water reorientation dynamics at non-base replica that, in turn, facilitates the faster and converged conformational sampling at the base replica (Appadurai *et al.*, 2021). REHT is able to reproduce SAXS and Chemical Shift data for a range of proteins with a variety of free-energy landscape (folded, metamorphic as well as IDPs) without any need to restrain or reweight the ensemble. The details of REHT simulation setup and scripts for generating the input files are available at the github repository, https://doi.org/10.5281/zenodo.4361714.

Across these different sampling methods and forcefields, it is clear that the correct modeling of protein–solvent interactions is critical for generating ensembles to a level of precision sufficient to draw physical conclusions. At this point, it is important to point out again how sampling and forcefield are very interconnected and need to be addressed simultaneously. Here, we would like to mention how the popular IDP forcefield Charmm36m, which does not work well with REST2, seems to generate excellent results with REHT. REHT has its origin from REST2, and it solves an old problem arising out of "cold solvent" effects in REST2 while keeping the computational requirements tractable. There is a substantially shortened round trip of replicas in REHT with the same forcefield, and this is due to the way it treats the water self-interaction (in non-base replicas). This is, in some sense, equivalent to the ideas in work with optimized forcefields of water interactions for better sampling (Best *et al.*, 2014; Piana *et al.*,

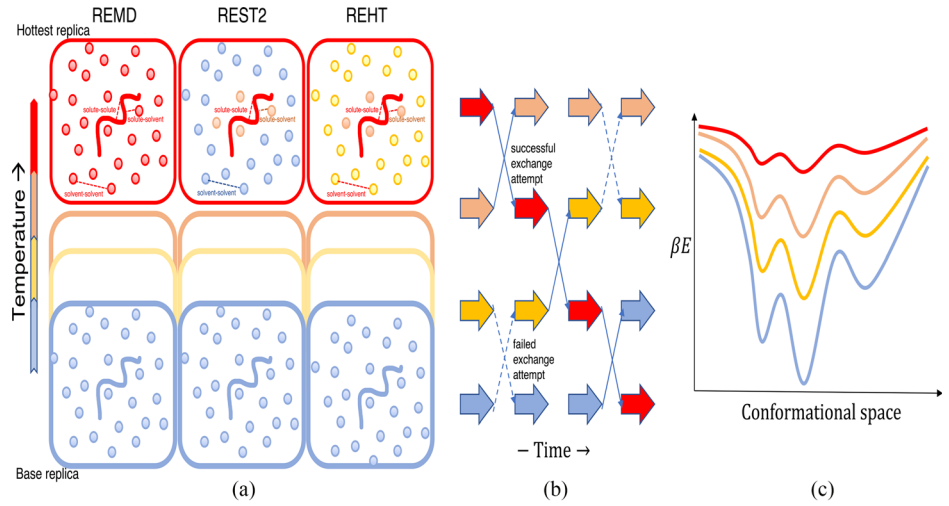

FIG. 3. Schematic diagram of different parallel tempering simulations. (a) In parallel tempering simulations, a series of low and high temperature replicas are simulated. The replicas in the conventional REMD differ by increasing bath temperatures across the ladder. Thus, the probability of accepting the exchange between adjacent replicas depends on the difference in the complete Hamiltonian of the system, including solute–solute, solute–solvent, and solvent–solvent contributions, which results in poor scaling in large systems. REST2 scales the energy function in a particle-wise manner, such that the solute is effectively heated up while keeping the solvent cold. Thus, the exchange acceptance probability depends on the energy difference in solute–solute interactions mainly and solute–solvent interactions subtly. The imbalance between hot solute and cold solvent causes entropic trap. REHT optimally heats the solute as well as the surrounding solvent by associating the replicas to different bath temperatures in addition to scaling down the potential function. Note that the base replica in all the cases is unbiased and has the same forcefield parameters and temperature conditions. (b) Stochastic swapping of replicas at regular intervals facilitates the equilibrium sampling at the base replica. (c) Schematic of energy landscape illustrating accessibility of broader conformational space facilitated by the high temperature replica.

2015; and Robustelli *et al.*, 2018). Entropic barrier is more acute in larger IDPs (>100 residues long), and REHT has opened the door to extract the experimentally commensurate conformational ensemble at atomic resolutions for very long IDPs.

III. CONFORMATIONAL DYNAMICS AND PHENOTYPIC SWITCHING

A. IDP dynamics and stochasticity

Conformational noise can have important implications in cellular behavior. Our current understanding of noise in biological systems is largely focused on stochasticity arising due to the low copy number of biomolecules (Balázsi *et al.*, 2011). Stochasticity can be seen at various regulatory levels: signaling cascades, transcription (DNA binding/unbinding), translation (microRNA-mRNA binding/unbinding), chromatin organization (Guillemin and Stumpf, 2021), eventually impacting cellular decision-making, and enabling phenotypic heterogeneity. Similar implications of stochasticity can also arise due to conformational noise. With IDPs acting as hubs in PINs, the interaction strength between members of the PIN and even the connections between them becomes a dynamical variable instead of fixed parameters in the "static" regulatory networks we often imagine. Thus, conformational noise can drive time-varying PIN(s) and/or regulatory network(s) where the interactions among the nodes change dynamically over time (Fig. 4). Such traits can amplify any preexisting cell-to-cell variability, despite identical genotype, facilitating non-genetic heterogeneity. Thus, it becomes important to delineate the impact of conformational noise and IDPs on phenotypic heterogeneity and plasticity (ability of cells to reversibly switch to a different cell-state, often as an emergent property of underlying network dynamics) (Hari *et al.*, 2020).

Various molecules involved in cell-state transitions and cellular transformation are known to be IDPs. For instance, various oncogenes and tumor-suppressor genes have IDRs. Similarly, transcription factors (TFs) such as ZEB1, SNAII, and OVOL1 and OVOL2 that are involved in phenotypic plasticity during cancer metastasis and therapy resistance have been shown to be IDPs (Mooney *et al.*, 2016). These

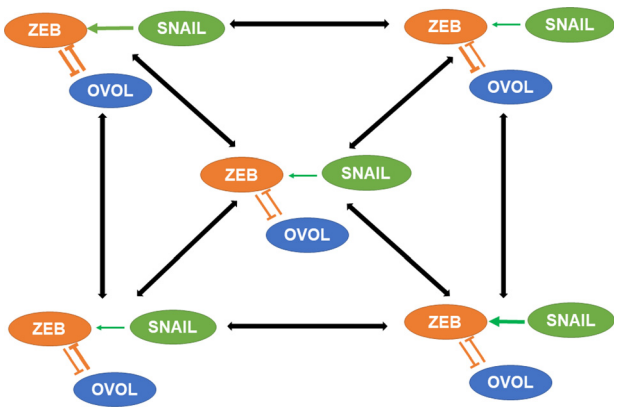

FIG. 4. Dynamic protein interaction networks. A "static" network consisting of IDPs (center) can be altered in terms of relative strengths of interactions across the nodes, in the presence of conformational noise. Orange hammer shows inhibition; green arrows show activation. Thicker lines show stronger activation or inhibition than weaker ones. These "dynamic" networks can interchange among themselves as well due to changes in conformational structure of IDPs/IDPRs involved.

TFs are the master regulators of epithelial-mesenchymal transition (EMT) and its reverse mesenchymal-epithelial transition (MET)—cellular processes, which enable cancer cells to alter their adhesion, migration, and invasion traits dynamically during different steps of the metastatic cascade. Even, the crucial drug targets for prostate and breast cancer, respectively—androgen receptor (AR) and estrogen receptor (ER)—contain IDRs (Myung *et al.*, 2013 and Peng *et al.*, 2019). EMT/MET can also impact drug resistance in cells by influencing the levels and/or activity of ER and AR (Graham *et al.*, 2010; Anose and Sanders, 2011; and Sahoo *et al.*, 2021) and *vice versa*. This crosstalk can lead to a dynamic PIN between these key nodes and can impact cancer cell fitness dynamics.

PAGE4, yet another IDP implicated in PCa, can show various conformations (He *et al.*, 2015). It can be phosphorylated at two residues (S9 and T51) by the kinase HIPK1; phosphorylation of PAGE4 enables its interactions with AP-1 transcription factor complex (Mooney *et al.*, 2014). PAGE4 can also be phosphorylated by another kinase CLK2, and these two phosphorylated versions of PAGE4 (HIPK1-PAGE4 and CLK2-PAGE4) have opposing functions due to their different conformational dynamics. HIPK1-PAGE4 has a compact conformational ensemble that can bind AP-1 and potentiate c-Jun, but CLK2-PAGE4 has a reduced affinity for AP-1 due to its random coil-like structure (Kulkarni *et al.*, 2017 and Lin *et al.*, 2018a). Because c-Jun potentiation can indirectly enhance CLK2 levels through AR, a negative feedback loop is formed, which can lead to oscillations [Fig. 5(a)] in the levels of AR and those of differently phosphorylated versions of PAGE4 (Kulkarni *et al.*, 2017). Such oscillations can generate non-genetic heterogeneity in a clonal prostate cancer cell population and also manifest in dynamic levels of AR in individual cells, impacting their therapeutic sensitivity.

B. Non-genetic heterogeneity due to conformational noise

Upon investigating the coupled dynamics of this negative feedback loop with that of EMT, a wider repertoire of cellular behavior can be realized. A core EMT circuit comprised of a mutually inhibitory loop between ZEB1 and microRNA family miR-200, driven by SNAI1, can lead to three distinct interconverting cell-states: epithelial (E; high miR-200 and low ZEB1), mesenchymal (M; low miR-200 and high ZEB1), and hybrid E/M (medium miR-200 and medium ZEB1) (Jolly *et al.*, 2017). Also, ZEB1 and AR can inhibit each other (Singh *et al.*, 2021). On coupling EMT and PAGE4/AR circuits, we see that these oscillations of PAGE4 circuit can convert to bistable behavior. Depending on the interaction strength between AR and ZEB, this coupled circuit can show both oscillations (sustained or damped) and

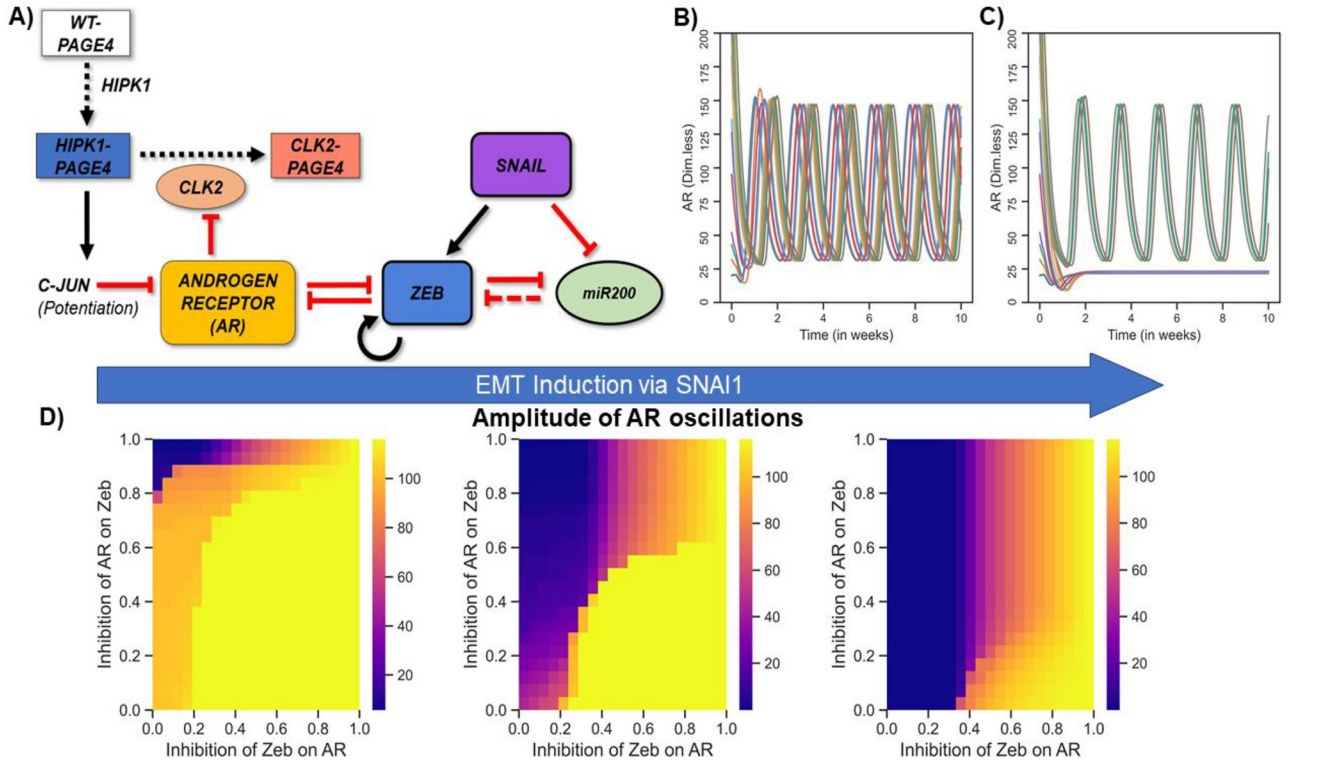

FIG. 5. Coupled dynamics of EMT and PAGE4 circuits containing IDPs. (a) Coupled network of EMT and PAGE4 circuit. Solid red hammer heads correspond to transcriptional inhibition, and dotted red hammer heads correspond to post transcriptional inhibition due to micro-RNA interaction. Solid black arrows correspond to transcriptional activation, and dotted arrows stand for phosphorylation. (b) and (c) Dynamics of AR: AR levels over time for two different values of interaction strength with ZEB. (d) Induction of EMT via SNAI1 leads to oscillations converting to bistability. Phase plot between interaction strengths of AR and Zeb, as EMT is induced via SNAI1, Zeb inhibits AR more strongly and leads to oscillations converting to bistability. [Adapted from Singh *et al.*, Entropy (Basel) **23**(3), 288 (2021). Copyright 2021 MDPI].

multistability—both of which are different examples of non-genetic heterogeneity in cancer cell populations [Fig. 5(b)].

Such non-genetic heterogeneity can often subvert the efficacy of therapeutic treatments. Because the interaction strength between members of these dynamic PINs can change, some cells may exhibit oscillatory dynamics for AR, while others may enable bistability (enabling cells to spontaneously switch from AR-high to AR-low state and *vice versa*). This diverse arsenal of cellular dynamics makes it difficult to design targeted therapies aimed to kill these cancer cells, thus aggravating disease progression in many patients.

IV. INTRINSICALLY DISORDERED REGIONS IN CELLULAR FUNCTIONING AND MALFUNCTIONING: CLASSIC CASE OF KIRSTEN RAT SARCOMA VIRUS (KRAS)

In addition to IDPs, proteins containing IDRs are abundantly found in modulating cellular functioning (Romero *et al.*, 2006 and Oldfield and Dunker, 2014). Most IDR regions are involved in membrane-associated activities and cell signaling (Buljan *et al.*, 2013; Wright and Dyson, 2015; Nussinov *et al.*, 2018; and Cornish *et al.*, 2020). The Ras superfamily of small GTPases represents a classic example where such signaling proteins act like binary molecular switches that regulate cell growth, proliferation, and differentiation (Colicelli, 2004 and Cox and Der, 2010). The switching function of Ras regulates an inactive GDP-bound off-state and active guanosine-5′-triphosphate (GTP)-bound on-state. Hyperactivation of RAS signaling is often triggered by direct mutations leading to Ras-induced cancer development (Biankin *et al.*, 2012 and Wood *et al.*, 2016).

A. Dynamics of disordered regions influence catalytic activity of KRAS: Insights from experimental data

Amongst RAS isoforms, KRAS is the most frequently mutated oncogene found in human cancers (Pleasance *et al.*, 2010 and Prior *et al.*, 2012). Only in the activated GTP-bound state, KRAS can associate with its effector protein like RAF-kinases, PI3K, and RalGDS to activate them (Pantsar, 2019 and Nussinov *et al.*, 2019). The activation of KRAS, however, depends on the guanosine exchange factor (GEF) that helps to replace GDP with GTP when the cellular concentration of GTP is higher [Fig. 6(a)]. On the other hand, despite having low intrinsic GTPase activity, the inactivation of KRAS is often induced by GTPase activating proteins (GAP) that catalyze GTP hydrolysis to GDP (Milburn *et al.*, 1990 and Bos *et al.*, 2007). For their catalytic function, all RAS isoforms have a very similar catalytic domain (residue: 1–166), including the N-terminal residues. This catalytic domain contains highly disordered functionally critical switch regions (switch 1: residues 25–40 and switch 2: residues 57–75). In particular, the positively charged hypervariable region (HVR), (residues 167–179) in the C terminus, and the flexible switch regions of small GTPases have drawn much attention of the recent investigations on the effects of such IDRs on the regulation and modulation of signaling output compared to its wild type (WT) and oncogenic variants (Gorfe, 2010; Abraham *et al.*, 2010; and Hunter *et al.*, 2014). The HVR mainly mediates membrane association [Fig. 6(b)]. A recent paramagnetic relaxation enhancement (PRE) NMR study has provided mechanistic insight into membrane-dependent RAS dimerization and the implications of the HVR region with membrane association (Lee *et al.*, 2020). In both the monomeric and the dimeric states of KRAS4B, the basic poly-lysine stretch in the C-terminal HVR through electrostatic interactions helps to anchor the anionic lipid head groups of the membrane [Fig. 6(b)]. Although RAS dimerization has been proposed as an essential step in the cascade of RAS signaling, the oligomerization state of KRAS remains elusive. It has been proposed that its membrane association occurs only in the monomeric state (Chung *et al.*, 2018); however, other suggestions include dimers, trimers, and even oligomers (Muratcioglu *et al.*, 2015; Sarkar-Banerjee *et al.*, 2017; and Barklis *et al.*, 2019).

Dynamical behavior of the flexible switch regions was first obtained via NMR spectroscopy where switch-I is found in two different conformations: open and closed states (Spoerner *et al.*, 2001). The closed state is essentially found when it is bound to other effector proteins. Specific mutations in the switch regions, such as D33E in the switch-I and A59G in the switch-II regions, have the potential to lock the conformation in its open form when one considers only the catalytic G-domain (Lu *et al.*, 2018). However, for the full-length RAS the equilibrium shifts toward the closed state. While such mutated open conformations are stated as an inactive GTP-bound state, these mutants show similar affinity to the RAS binding effector protein, RAF, when compared to the WT KRAS. This possibly occurs as the overall structure eventually moves toward the closed conformation leaving the open state presumably as an intermediate functional conformation where the disordered dynamics of switch regions still helps to maintain the association and affinity toward other effector proteins. NMR data also showed that the equilibrium-shift toward the open state is attainable if one perturbs Y32 position by replacing it with other amino acids in the dynamic switch-I region (Spoerner *et al.*, 2010). As dynamic switch-I is in the immediate close region of the catalytic cavity, this region is identical in all RAS isomers.

The allosteric behavior of RAS has been well-studied previously (Buhrman *et al.*, 2010 and Buhrman *et al.*, 2011) to elucidate its allosteric function. In WT RAS-GTP, an allosteric switch is found to promote disorder to order transition of switch-II through a network of H-bonding interactions connecting the allosteric site to switch-II involving key residues crucial for catalysis. These studies revealed that an "on" state of the allosteric switch may enhance the hydrolysis rate in a GAP-independent pathway with the signal being turned off. Again, when the allosteric switch is in the "off" state, GTP-hydrolysis is deprived and signaling remains on. While RAS and its effector RAF are crucial driver proteins to control the RAS/RAF/MEK/ERK (extracellular signal-regulated kinase 1) signaling pathway, several oncogenic mutations including Gly12 and Gln61 are found to impair the GTPase activity of RAS and are abundantly found in human cancer (Prior *et al.*, 2012). Thus, the allosteric mechanism helped to explain how such oncogenic mutations could affect the catalytic process.

B. Advancement of drug development to target oncogenic mutations of KRAS and drug resistance

Until recently, KRAS was considered undruggable. AMG510 was one of the first KRAS (G12C) inhibitors that was efficacious against KRAS G12C tumors (AMG510, 2019 and Canon *et al.*, 2019). Soon after, MRTX849 was found to be a potent mutant selective covalent inhibitor of KRAS G12C. MRTX849 is highly efficacious in tumor regression in KRAS G12C mutant cell lines, patient-derived xenograft models from multiple tumor types, and in lung and colon cancer patients.

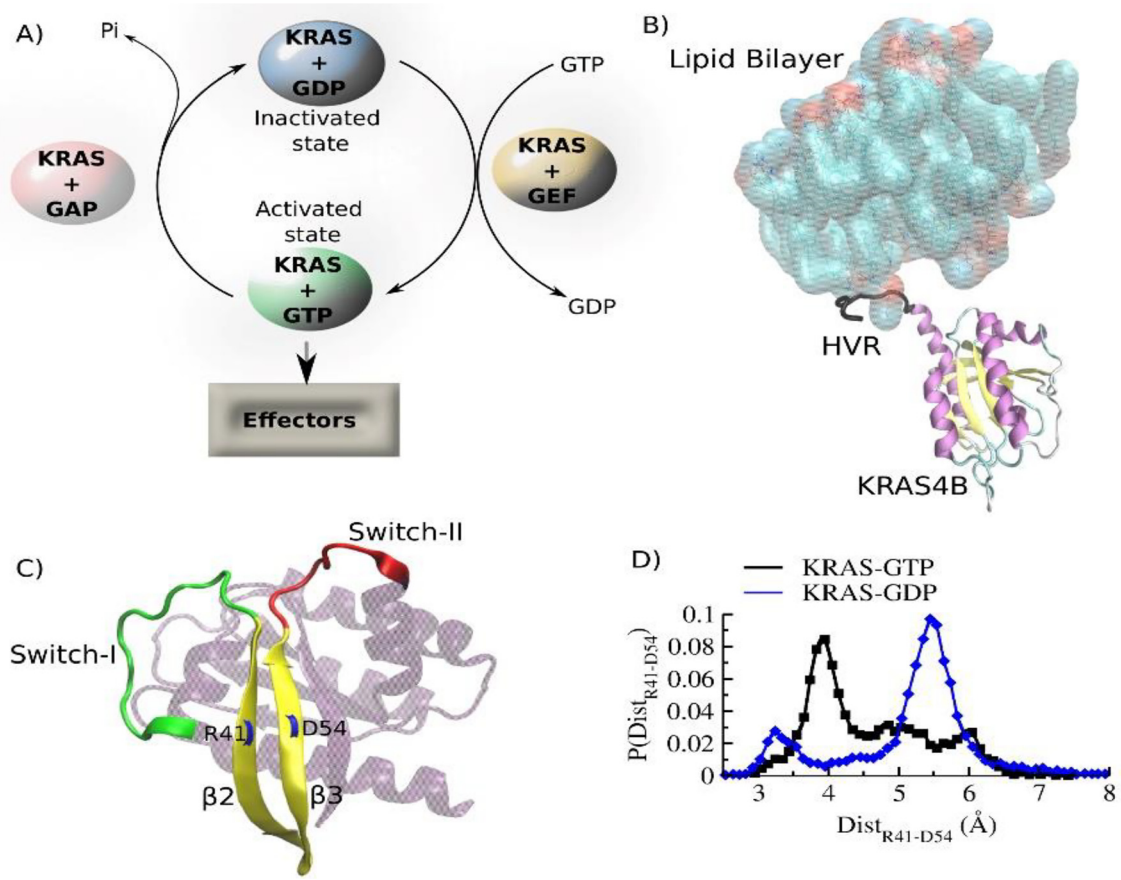

FIG. 6. The structure/function cycle of KRAS. (a) Activation/deactivation cycle of KRAS GTPase. GDP/GTP exchange in this cycle is mediated by two other proteins: guanine nucleotide-exchange factors (GEFs) and GTPase activating proteins (GAPs). While GEFs catalyze the exchange from GDP to GTP, GAPs enhance the rate of exchange from GTP to GDP. (b) NMR-driven structure of KRAS4B-GTP on a lipid bilayer (pdb id:6W4E) (Lee *et al.*, 2020). The positively charged intrinsically disordered hypervariable region (HVR) is shown in gray to highlight its mode of association with the lipid membrane. (c) Three-dimensional structure of inactive GDP-bound human KRAS highlighting the dynamic switch regions: Switch I (green) and Switch II (red) (pdb id: 4OBE) (Hunter *et al.*, 2014). These two switch regions are connected via two parallel β strands: β2 and β3. (d) The switch dynamics and their correlation with β2-β3 fluctuation are compared in GDP and GTP-bound states. The dynamics are assessed by quantifying the distance between two residues R41 (located in β2) and D54 (located in β3). The distance distribution indicates enhanced conformation fluctuation of the switches in the GDP-bound state. [Adapted from Vatansever *et al.*, Sci. Rep. **6**, 37012 (2016). Copyright 2016 Author(s), licensed under a Creative Commons Attribution 4.0 License.]

Mutations in KRAS are frequently observed in lung, pancreatic, and colorectal cancers. Lung adenocarcinoma has the highest percentage of KRAS mutations, and the most frequent mutations include substitution of glycine 12 with either cysteine, valine, aspartic acid, alanine, serine, or asparagine. Each of these substitutions leads to conformational changes in the KRAS molecule, which impinge on its biophysical property. For example, Moghadamchargari *et al.* (2019) reported that KRAS has intrinsic GTPase activity, that is, also involved in the conversion of the KRAS-GTP active form to KRAS-GDP inactive form, and this activity is higher in the native (WT), G12C, or G12D KRAS mutants, but lower in the G12A, G12V, G12S, and G12R mutants. Similarly, based on the affinity for Ras binding domain (RBD), KRAS can be grouped into a high affinity group (WT, G12A, and G12C) and a low affinity group (G12V, G12R, and G12D) (Hunter *et al.*, 2015). Since KRAS lacks a groove or pocket except the GTP binding domain for the small molecules to bind, GTP analogs were used to compete against the cellular GTP for the GTP binding pockets of KRAS but that approach did not work (Noonan *et al.*, 1991). The other option to target KRAS signaling was by targeting the upstream and downstream signaling pathways. Thus, various inhibitors of the RAF-MEK-ERK and AKT serine/threonine kinase 1-mTOR pathways were developed that were able to suppress the growth of KRAS driven tumor. Unfortunately, the activation of overly complex network of positive and negative feedback loops associated with KRAS signaling reduced the efficacy of these drugs and, eventually, caused tumor relapse. Therefore, a more directed approach was tried to target the KRAS molecules and block its activation.

The proposal was to block the KRAS function by developing the covalent inhibitors, which could covalently interact with KRAS and block its conversion from KRAS-GDP (inactive sate) to KRAS-GTP (active state). In the initial study, the small molecules were designed to covalently interact with the thiol group of cysteine (G12C) residue and lock KRAS in GDP-bound state. The inhibitors like SML-10–70-1 appeared selective for the KRAS G12C compared to the WT, inhibited

activation of AKT and ERK, and increased accumulation of KRAS-GDP (Lim *et al.*, 2014 and Hunter *et al.*, 2014). However, its ability to inhibit tumor expressing KRAS G12S mutants raised questions about its specificity. Other inhibitors such as vinyl sulfonamide and acrylamide analogs were developed by either changing the positions or altering the electrophilic group for efficient interaction with KRAS G12C. Compound 12 was developed, which could interact with the new allosteric pocket and change the preference of KRAS G12C for GDP compared to GTP (Ostrem *et al.*, 2013). The compound was selective for KRAS G12C but had poor pharmacological properties. Compound 12 was further modified and developed to ARS853, which had 600-fold more affinity for KRAS G12C and locked it in the inactive GDP bound state. However, the compound had lower metabolic stability in the plasma and poor oral bioavailability in mice, which restricted its use for *in vivo* studies.

Janes *et al.* (2018) reported a new covalent inhibitor ARS1620, which was based on the structure of ARS-853 by scaffold optimization. X-ray crystallography studies showed the binding on the ARS1620 to the allosteric pocket region located beneath the switch II loop of KRAS-GDP. It is a biochemically stable and orally bioavailable compound shown to inhibit KRAS G12C activity *in vitro* and *in vivo*, but it has suboptimal potency owing to small volume of pocket being occupied (Canon *et al.*, 2019). The crystallographic structure of ARS1620-KRAS G12C revealed a hydrogen bonding between the ARS1620 and histidine 95 residue. Canon *et al.* (2019) reported that this histidine residue could flip up and reveal a hidden groove, which could be targeted by covalent inhibitors leading to the discovery of the KRAS inhibitor AMG510 (Canon *et al.*, 2019). ARS1620 and AMG510 have structural similarity but enhanced the interaction of AMG510 with the H95 groove, which increases its potency by ten-fold compared to ARS1620.

MRTX849 also binds to cysteine 12 residue irreversibly and locks it in an inactive GDP-bound state inhibiting the KRAS driven downstream signaling pathways. It is highly selective against the KRAS G12C, and *in vivo* data demonstrate that it is effective against several solid tumors, including lung, pancreas, and colon. In *in vitro* studies, the drug was also shown to be effective against cell lines that have co-mutations in genes like P53, STK11, KEAP1, HER, or CDKN2A (Hallin *et al.*, 2020).

C. Investigations of conformational ensembles and disordered dynamics of KRAS by computer simulation methods

Several microsecond simulations have been performed for different RAS isoforms (HRAS, NRAS, and KRAS) to sample their conformational ensembles and understand the conformational dynamics in their GDP- and GTP-bound states (Kapoor and Travesset, 2015 and Prakash and Gorfe, 2013). These simulations capture the high flexibility of the switch regions, and the range of flexibility differs in different RAS isoforms. In wild-type KRAS, dynamics of switch regions are observed to influence the closure of two immediate parallel β-strands located between switch-I and switch-II regions [Fig. 6(c)]. The differential switch dynamics in GDP- and GTP-bound states are reflected when the distance closure between these two parallel β-strands (β2 and β3) is measured [Fig. 6(d)]. The distance distribution between β2 and β3 indicates that GTP-binding increases KRAS stiffness by restraining the switch dynamics, which possibly helps enable its GTPase activity (Vatansever *et al.*, 2016). However, recent NMR analysis of GDP-bound G12V-HRAS and G12V-HRAS (GMPPNP, a stable GTP analog) obtained a different result, which shows that the latter is more flexible (Chen *et al.*, 2021). Investigations on WT-HRAS, WT-KRAS, and other RAS mutants are consistent with that recent NMR analysis (Kraulis *et al.*, 1994; Araki *et al.*, 2011; O'Connor and Kovrigin, 2008; Vo *et al.*, 2013; Fetics *et al.*, 2015; Matsumoto *et al.*, 2016; and Yin *et al.*, 2017). Moreover, HRAS (GMPPNP) is more susceptible to proteolytic cleavage by an engineered subtilisin protease than the GDP form. Protease recognition occurs specifically at the Switch II YSAM site, with cleavage right after the methionine. This region is in the alpha2 helix in the GDP form but is more disordered in the GTP form, thus making it more accessible to proteolysis (Chen *et al.*, 2021). Early MD simulations showed that these intrinsically flexible switch regions belong to an evolutionarily conserved nucleotide-binding lobe-1 (residue 1–86), which has an isoform-specific communication pathway with C-terminal lobe-2 (residue 87–171) (Gorfe *et al.*, 2008).

Apart from classical MD, accelerated MD and targeted MD approaches have also been employed to probe the large timescale and extensive length-scale conformational dynamics that are associated with GDP and GTP binding processes (Milburn *et al.*, 1990; Diaz *et al.*, 1997; and Grant *et al.*, 2009). It was proposed that the conformation selection and the population shift mechanisms might play an important role where allosteric interference is also associated in such ligand-binding phenomena. Allosteric regulations have an immense role in post-translation modifications (PTMs) of such signaling proteins (Nussinov *et al.*, 2012 and Ahearn *et al.*, 2018). PTMs that occur away from the functional site yet propagating through conformational and dynamical changes are called allosteric PTMs, while PTM events taking place at the functional site via direct recognition are often known as an orthosteric PTMs (Nussinov *et al.*, 2012 and Clausen *et al.*, 2015). However, a theoretical dynamic energy landscape combining equilibrium fluctuation concepts has been proposed to explain such dynamic conformational changes of the substrate regulated by allosteric event (Kar *et al.*, 2010). Such a concept also shows promises with the allosteric drugs that allow modulation of signal and responses in comparison to targeted drug binding at active sites.

The full-length KRAS including the IDR dynamics of HVR regions in solution along with their oncogenic mutations was also investigated using large microsecond simulation data (Chavan *et al.*, 2015; Jang *et al.*, 2016; Sayyed-Ahmad *et al.*, 2017; and Pantsar *et al.*, 2018). The orientational dynamics of KRAS has also been studied including the membrane, and the results correlated well with the experimental findings (Li and Buck, 2017). Apart from early membrane-associated simulation with KRAS displaying distinct rotational conformations, a recent microsecond long membrane-associated simulation of G12V KRAS shows three unique conformations (Prakash *et al.*, 2019). These conformations are also found in the case of G12D and Q61H mutants but in a different population (Prakash and Gorfe, 2019). Coarse-grained simulation approaches have also been adopted to model clusters of inactive or lipid anchored RAS embedded in a phase-separating lipid mixture of DPPC, cholesterol (CHOL), and DLiPC (Janosi *et al.*, 2012). The lipid mixture was found to segregate between CHOL/DPPC containing the ordered domain and DLiPC containing disordered domain to form a raft and

non-raft like domains, respectively, indicating how asymmetric RAS-binding induces bilayer deformation.

Among different computational and theoretical studies, most investigations have focused on the G-domain. Conformational dynamics comparing WT KRAS and its different oncogenic mutants, namely, G12C, G12D, G12V, G13D, and Q61H, have been studied using microsecond long simulations, and differences were assessed using a residual contact probability network (Lu *et al.*, 2016 and Vatansever *et al.*, 2020). Simulation studies have been performed on all KRAS G12 missense mutants, and analyses were made using Markov state models (MSMs). MSMs highlight seven metastable conformational ensembles indicating different dynamic states and conformational plasticity of the flexible switch regions. MSMs also help evaluate the transition probabilities of those conformational ensembles (Husic and Pande, 2018). Comparing different oncogenic mutated KRAS variants, it appears that the dynamical shift in KRAS results in an allosteric manner, and that a mutation can rewire the crosstalk between the switch regions maneuvering the switch flexibility. However, current understanding is still not adequate to discern the driving force behind such allosteric communication and mutation-induced re-wiring mechanism, which is required for targeted inhibition of mutated KRAS.

MD simulations have also rationalized our understanding of how KRAS interacts with its effector proteins to instigate their activation process. To study the KRAS induced P13Kα activation mechanism, KRas4B and its interaction with the Ras binding domain (RBD) of PI3Kα in solution were investigated through extensive atomistic simulation of 10 μs. This study suggests that Ras recruitment shifts conformational ensemble of PI3Kα in such a way that it is likely to determine the recruitment and restriction of the PI3Kα population at the membrane (Zhang *et al.*, 2019). Recent computational modeling also provided mechanistic insight into how farnesylated/methylated KRAS4B interacts with calmodulin (CaM). Due to multiple interaction modes, various conformational ensembles of the KRas4B-CaM complex have been distinguished, effectively helping to activate PI3Kα/AKT signaling by recruiting PI3Kα to the plasma membrane (Jang *et al.*, 2019).

V. RATIONAL DRUG DISCOVERY TARGETING IDPs

As discussed above, IDPs constitute a significant portion of the human proteome, and their involvement in multiple diseases has been well documented (Uversky *et al.*, 2008 and Babu *et al.*, 2011). The pathological role of IDPs is related to their altered PTMs and their expression and lifetime in the cell since they can rewire PINs, leading to the activation of latent pathways (Babu, 2016 and Salgia and Kulkarni, 2018). Aggregation of IDPs such as the tau-protein is associated with neurodegenerative diseases. Moreover, dysregulated splicing in certain cancers such as chronic lymphocytic leukemia and colorectal carcinoma can produce novel spliced proteins that behave as IDPs (Sciarrillo *et al.*, 2020 and Romero *et al.*, 2006). These proteins are, therefore, considered promising yet challenging drug targets. Currently, some of the major obstacles involved in rational drug discovery targeting IDPs are (i) the identification of structurally stable druggable pockets (Ruan *et al.*, 2019; Joshi and Vendruscolo, 2015; and Cheng *et al.*, 2006), (ii) weak affinity of binders (Metallo, 2010), and (iii) lack of selectivity to the target (Metallo, 2010).

A. Computer aided drug discovery

Computer aided drug discovery relies on well-defined protein structures with druggable pockets that are deep, with a fair number of hydrophobic patches to facilitate partitioning of drug molecules from solvent (Volkamer *et al.*, 2012), although exceptions to these rules exist (Nisius *et al.*, 2012; and Zheng *et al.*, 2013). It is conceivable that IDPs, due to their inherent flexibility, may not fit into the above paradigm. Many IDPs show significantly higher fraction of hydrophilic residues in the sequence compared to folded proteins, implying that hydrophobic pockets that can bind drugs may be rare among IDPs. Yet, the possibility of stable hydrophobic pockets has been shown in certain IDPs such as the nuclear protein 1 (NUPR1) (Neira *et al.*, 2017). In other cases, such as the oncogenic transcription factor c-Myc, small molecules have been shown to bind to the disordered regions of the protein (Follis *et al.*, 2008 and Hammoudeh *et al.*, 2009). In the case of NUPR1, the binding compounds targeted the part of the protein sequence with lower flexibility than the rest of the protein. In a more recent study, a compound (epigallocatechin gallate or EGCG) was reported to bind to the disordered N terminal domain (NTD) of P53, which disrupted its interaction with the ligase MDM2 and stabilized P53 for enhanced antitumor activities (Zhao *et al.*, 2021). SAXS and NMR experiments showed that EGCG introduced subtle conformational changes to the P53 NTD, leading to a more compact conformational ensemble. The NMR and enhanced replica exchange MD simulations further revealed that EGCG interacts with the NTD through many dynamic contacts, as opposed to a few stable ones. Such reports regarding the interaction of small molecules with IDPs or IDRs are becoming increasingly frequent (Santofimia-Castaño *et al.*, 2020), while only a few years back, disordered proteins such as transcription factors were considered undruggable (Henley and Koehler, 2021).

In addition to small molecules, several alternative strategies have been promising in targeting IDPs. One such approach involves the use of peptide aptamers, which are short peptide sequences as part of a loop within a protein scaffold. Aptamers can be designed through a directed evolutionary process in live cells, where the aptamer sequences that result in the best desired phenotypes can be selected over several rounds of optimization. Since the aptamer sequences are constrained within a protein loop, they suffer less entropic loss upon binding compared to free peptides and, therefore, can achieve high binding affinities. Using yeast-based screening assays and in-cell NMR, the peptide aptamers were designed to bind to the disordered region of the ubiquitin-like protein Pup with nanomolar affinity (Cobbert *et al.*, 2015). These three aptamers were shown to interact with a disordered segment in Pup that folds into an alpha-helix upon binding to the partner Mpa. Despite targeting the same protein and roughly similar regions, the three aptamers led to different functional effects, underscoring the complexity in targeting IDPs for functional inhibition.

In computational drug discovery, IDPs as drug targets offer unprecedented opportunities, but with significant challenges. Some likely improvizations necessary for developing *in silico* pipelines for designing IDP binders are (i) methods to account for entropy loss upon binding of drugs, (ii) machine learning approaches, (iii) consideration of folding upon binding of IDPs to partner proteins, and (iv) application of MD, enhanced MD and NMR generated protein ensembles in the binder screening, among others. Recently, a virtual

screening method was reported that utilized the mechanism of disorder-to-order transition in IDPs to screen for inhibitors (Na *et al.*, 2020). The method involves generating short (20 AA) peptide conformations from the IDP region that undergoes order-to-disorder transition and docking small molecule libraries to these peptide conformations. Using a discriminatory score that combines peptide-compound interactions with peptide structural stability, the authors successfully identified the known inhibitor for the proto-oncogene Myc from among thousands of negatives.

B. *In silico* strategy to design peptide sequences

In contrast to the disorder-to-order mechanism, where a specific segment of the IDP interacts with the partner protein, many IDPs remain disordered upon binding to the partner (Freiberger *et al.*, 2021). In such cases, multiple residues in the disordered segment typically form transient contacts with a focused region in the partner protein, which is normally folded. This mechanism is referred to as the many-to-one mode of interaction. Such interactions can be targeted for inhibitor design, if the interaction hotspots in the partner protein cavity are known and the IDP conformations that interact with the partner protein can be resolved using NMR, MD, or other approaches. Recently, utilizing the above-described principle, Bhattacharya and coworkers designed an inhibitory peptide for the carbohydrate binding protein galectin-3 (Bhattacharya *et al.*, 2021). Galectin-3 consists of a disordered N terminal domain (NTD) that interacts with a folded C terminal domain (CTD). By combining accelerated MD simulations of full-length galectin-3 with available chemical shift perturbations, the ensemble of NTD that interacts with the CTD was determined. This ensemble was used to derive peptide scaffolds, and a hierarchical *in silico* strategy was used to design peptide sequences that were predicted to disrupt the NTD–CTD interaction. The sensitivity of this approach was demonstrated where one out of only three tested peptides was found to be a positive hit. Such approaches can be easily applied to other IDPs, which bind to folded partners, for which experimental structural data in the form of NMR or SAXS are available. Moreover, the inhibitory peptide sequences and their bound structures can be used to construct pharmacophores for searching large libraries of small molecules for potential lead compounds.

The purpose of a therapeutic agent is to modulate the biological function of its target. Since IDPs are multi-functional proteins, targeting IDPs requires the consideration not only of the thermodynamic and structural aspects of the drug binding, but also of the specific function, that is, being targeted. Examples include targeting a transcription factor for disrupting DNA binding vs preventing phosphorylation via interaction with kinases or inhibiting the formation of liquid–liquid separated granules. A single IDP can carry out each of these functions, through different structural regions. Targeting such specific functions not only improves the selectivity of the therapeutic agent but also requires deeper structural understanding of the mechanisms governing IDPs. Rational designing of agents targeting an IDP and its specific function should, therefore, begin with the accurate determination of the segment of the protein sequence, that is, responsible for the functional effect. Next, a variety of methods can be applied to search for binders, which can include both *in silico* methods and directed evolution to find aptamers. Another promising avenue is to search for small molecule fragments (molecular weight < 200 Da) that bind to the region of interest using high throughput screening methods such as differential scanning fluorimetry, solution small angle x ray scattering, and isothermal titration calorimetry (Murray and Rees, 2009). The most promising fragments can then be linked together through appropriate linkers to develop drug molecules with high affinity. Such approaches have been proposed as viable avenues for targeting IDP related pathologies, such as aggregation (Joshi *et al.*, 2016).

VI. CONCLUSIONS AND FUTURE DIRECTIONS

Since their discovery >20 years ago (see Dyson and Wright, 2019 and Uversky and Kulkarni, 2021, for historical accounts), there has been an explosion in the IDP field. Almost 10 000 papers dedicated to IDPs were published by the end of 2021 with more than 350 000 citations (Fig. 7). These statistics confirm the increased attention that the IDPs have attracted and will undoubtedly continue to do so in the years to come. The challenges they pose have led to new thinking such as IDPs and dynamical systems theory (Uversky, 2014 and Kulkarni, 2020) and new technical advances such as mass spectrometry technologies for protein structure analysis, "footprinting" studies, and cryo-electron microscopy (Nwanochie and Uversky, 2019 and Chance *et al.*, 2020). Concomitant with these developments, we have also seen significant advances in computational methods such as new developments in force field strategies (Huang and MacKerell, 2018; Masetti *et al.*, 2020; Hsu *et al.*, 2020; Ahmed *et al.*, 2020; Mu *et al.*, 2021;

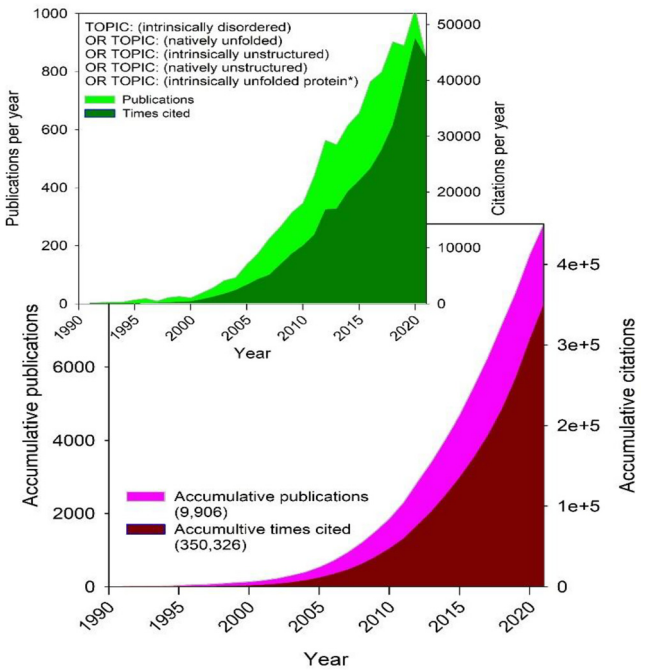

FIG. 7. An explosion in the protein intrinsic disorder literature. The plots represent the time-courses of the increase in the number of publications dealing with the intrinsic disorder and the number of papers citing those publications (inset). Plot shows total publications per year and accumulative number of publications. Inset shows the data for the sum of times cited per year and accumulative sum of times cited. Data for these plots were retrieved from Web of Science on November 17, 2021 using the following search criteria: TOPIC: (intrinsically disordered) OR TOPIC: (natively unfolded) OR TOPIC: (intrinsically unstructured) OR TOPIC: (natively unstructured) OR TOPIC: (intrinsically unfolded protein).

Wang, 2021; and Gopal *et al.*, 2021) and physics-based computational and theoretical approaches (Shea *et al.*, 2021 and Sieradzan *et al.*, 2021).

Aside from the biological functions discussed here, IDPs are important constituents of proteinaceous membrane-less organelles (PMLOs). PMLOs are formed by liquid–liquid phase separation when a polypeptide coalesces into a dense phase in an aqueous solution (Uversky, 2021). PMLOs play important roles in myriad cellular processes from responding to stress to transcriptional regulation of gene expression. Furthermore, it is also postulated that PMLOs very likely played a critical role in prebiotic evolution of the predecessor of the first universal common ancestor (Kulkarni and Uversky, 2018b). We suspect that these aspects of the IDPs would be intensely investigated going forward. Finally, since several proteins associated with drug-resistance in cancer and prion proteins associated with neurodegenerative disease are IDPs (Kulkarni and Uversky, 2019 and Salahuddin *et al.*, 2021), a deeper understanding of IDPs can help better understand their role in phenotypic switching and adaptive evolution via non-genetic, protein-based mechanisms (Kulkarni, 2020).

To further inspire work on IDPs, we put forth the Janus challenge (Kulkarni and Uversky, 2018b). We believe that meeting this challenge is likely to lead to technological advances with important biomedical applications. Finally, IDPs, for example, c-Myc and KRAS, that were once considered as "undruggable" are emerging as favorite therapeutic targets. Thus, it is very likely that IDPs including many transcription factors (Tsafou *et al.*, 2018), will be targeted for therapeutic development. The availability of several dedicated databases to the community that house a wealth of information related to IDPs (Hatos *et al.*, 2020; Lazar *et al.*, 2021; Piovesan *et al.*, 2021; and Quaglia *et al.*, 2021) as well as powerful tools to analyze big data that are designed using machine learning and artificial intelligence (Katuwawala *et al.*, 2019; Ramanathan *et al.*, 2021; Lindorff-Larsen and Kragelund, 2021; and Strodel, 2021) should help realize the full potential of IDPs.

Although IDPs are incorrectly perceived to lack structure and, hence, presumed to defy Anfinsen's dogma, IDPs are not random coils but exist as conformational ensembles. However, IDP ensembles have conformational preferences. Therefore, IDPs do have "structure" (or a set of interconverting structures), albeit subtle perhaps, at the limit of Anfinsen's dogma. In fact, being dynamical multifunctional systems, IDPs represent a logical extension to the Anfinsen's dogma, since different members of their conformational ensembles might have different functions.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors declare no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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