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Unstructural biology coming of age

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It is now generally accepted that many proteins or protein domains (intrinsically disordered proteins, IDPs) lack a well-defined tertiary structure under functional conditions. Due to recent concerted activity, a critical transition in this field is gaining momentum, in which qualitative observations are turned into quantitative structural models of IDPs. Here, it is suggested that the transition is set up by the synergy of: (i) more advanced bioinformatic tools for the prediction of disorder and function of IDPs, (ii) ensemble description of their structure and dynamics in both free and bound states, down to the single molecule level, (iii) advent of in-cell approaches for characterizing their structure and function *in vivo*, and (iv) generation of small-molecule inhibitors both against their binding partners and IDPs themselves. In all, we suggest that due to steady advance in these areas, the field of 'unstructural' biology is rapidly maturing to a state where it can provide quantitative models of proteins functioning without well-defined three-dimensional structures.

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Introduction

The second half of the last century witnessed the continuous advance of structural biology, glorifying the notion that structure is the prerequisite of function. Arguably, the recent most exciting development in structural biology, however, is not the solution of yet another giant protein or complex, but the recognition that many proteins or regions of proteins exist and function without a well-defined structure. These IDPs demand a radical change in concept for describing biological events at the molecular level. Whereas this field has raised eyebrows for a decade, it is now becoming evident that a critical transition is taking place by 'unstructural' biology getting into the mainstream of molecular biology. Rapid growth of the field has been marked by several excellent recent reviews [1–5], including a textbook of comprehensive coverage [6]. Here, the most important recent developments of the field are surveyed.

Computational studies: prediction of disorder and functional sites

Bioinformatic predictions still play a decisive role in studies of structural disorder. Whereas the latest release (2010 November) of the DisProt database [7] contains 1342 disordered regions in 627 IDPs (www.disprot.org), there is still a very wide gap between experimentally demonstrated and expected structural disorder, which leaves much room for bioinformatics in large-scale functional association studies. Based on the compositional bias of IDPs, several dozen predictors of different principles have been developed [8]. To handle limitations inherent in prediction accuracy due to distinct flavors of disorder, however, different predictors are recently combined into metapredictors, such as metaPrDOS [9] or PONDR-FIT [10]. These combined predictors do show improved performance over their composite ones.

Predicting function and/or functional sites of IDPs is a task even more difficult. Recently, significant advance has been made in this direction, based on the observation that interactions of IDPs are often mediated by short linear motifs [11]. Because linear motifs are 3–15 residues in length, they contain very little sequence information and their prediction from sequence alone is fraught with very high false positive rates. A critical advance in this direction has been made by applying context-filters, which significantly increase prediction accuracy by taking into consideration motif enrichment in proteins that share the same binding partner or evolutionary history (e.g. SLiMFinder [12]). Completely different logic forms the basis of ANCHOR, which predicts disordered binding sites by estimating their interaction energy with a general partner [13], and of the molecular recognition feature predictor (α-MoRF-PredII), which uses patterns of order/disorder prediction [14].

Toward describing the structural ensemble of IDPs

It is becoming evident that IDPs are not fully disordered, but they have all sort of function-related transient short- and long-range structural organization. The major techniques toward describing the ensuing structural ensemble apply structural calculations restrained by NMR and small-angle X-ray scattering (SAXS) data (Table 1). Residue-level parameters carrying information mostly on the local structure of the IDP, such as chemical shifts,

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Table 1

Methods developed to describe the structural ensemble of IDPs. The methods usually rely on a set of structural constraints determined by NMR residual dipolar coupling (RDC), various chemical shift values, amide proton relaxation rate ($^{15}N\ R_2$), distance restraints from paramagnetic relaxation enhancement (PRE), small-angle X-ray scattering (SAXS, usually Kratky plot), dynamic light scattering (DLS) or analytical ultracentrifugation (AUC). In each method an ensemble of conformations is generated and iterated to match the experimental restraints as closely as possible

Parameters	Ensemble method	Protein	Reference
RDC, SAXS	Flexible-Meccano, Accelerated Molecular Dynamics (AMD)	p53	[58]
RDC	Flexible-Meccano	Sendai virus PX	[59]
Chemical shifts, R_h (DLS), $^{15}N\ R_2$, SAXS	ENSEMBLE	Inhibitor-2, spinophilin, DARPP-32	[34]
PRE, RDC	ASTEROIDS	α-Synuclein	[60]
Chemical shifts, $^{15}N\ R_2$, RDC, PRE, SAXS	ENSEMBLE	Sic1	[16•]
SAXS, $^{15}N\ R_2$,	Ensemble optimization method	Ribosomal L12	[61]
Chemical shifts, R_h (AUC) SAXS	Molecular dynamics	$p27^{Kip1}$	[62]
PRE	Molecular dynamics	α-Synuclein	[15••]

hetNOE values, relaxation parameters and dipolar couplings, are determined in NMR. To obtain long-range structural constraints, spin probes are applied in paramagnetic relaxation enhancement (PRE) measurements. SAXS, on the other hand, mostly contributes information on the hydrodynamic behavior and topology of the polypeptide chain. To interpret the values by either technique, a large number of random conformers are generated, the parameters for each conformer are calculated and an optimization procedure is carried out to select a limited number (around 50) of conformers which together satisfy the constraints (for details and references, see Table 1).

If such an ensemble description of structure can be related to the function of IDP, we may state that 'unstructural' biology approaches the descriptive power we ascribe to traditional structural biology at present. For example, in a recent study on α-synuclein PRE distances were incorporated into molecular dynamics (MD) simulations to map the free energy landscape of the structural ensemble of the protein [15••]. In another key study, it was shown for pSic1 binding to Cdc4 [16•] that the ensemble can even be solved for the bound state (Figure 1), which provides insight into intricate details of binding and ubiquitination of this protein. These types of studies may represent the first steps toward quantitative structure–function models of IDPs.

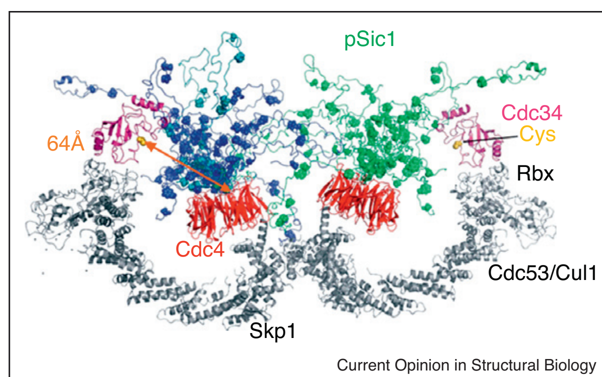
Single-molecule studies

Single-molecule studies of IDP structure, such as atomic-force microscopy (AFM) pulling studies of unfolding transitions [17], fast tapping AFM visualization of structural changes [18•,19], and single-molecule fluorescence resonance energy transfer (smFRET) measurements [20,21,22••] of the range and dynamics of global conformational changes may even surpass the descriptive power of ensemble methods. These approaches allow the observation of transient intermediates and both static and dynamic heterogeneity of structure.

Single-molecule mechanical unfolding by AFM was used to study the conformational heterogeneity of wild-type and mutant α-synuclein [17]. The molecule was found to have three main conformations, disordered, some soluble oligomeric state and 'β-like', which might be important in the transition of the protein toward its pathological amyloid state. AFM can also be used in an ultrafast scanning mode, when it can provide 'movies' of the topological details and conformational transitions of an IDP, such as that of myosin V motor molecules moving along actin tracks enabled by disordered linker regions [18•].

SmFRET is also unparalleled in its spatial and dynamic resolution, as demonstrated by studying of α-synuclein membrane association, structural distributions and dynamics [20]. In a similar study of p53 [21], it was found that its N-terminal domain has multiple preferred

Figure 1



Structural model of pSic1 bound to the SCF^{Cdc4} dimer/Cdc34 complex. The dynamic ensemble of pSic1–Cdc4 complex is calculated by ENSEMBLE using structure constraints obtained by NMR and SAXS. The ensemble is superimposed on a structural model of the SCF^{Cdc4} ubiquitin ligase dimer (the E3) bound to Cdc34 (the E2). Cdc4 is shown in red, Cdc34 in magenta, and the other subunits Skp1, Cdc53/Cul1 and Rbx1 in grey. One pSic1 ensemble is in blue, with the pSic1 ensemble binding to the other Cdc4 subunit shown in green.Adapted from [16•].

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conformations, some but not all interacting with the DNA-binding domain. The technique even enabled structural studies in live cells, as recently demonstrated for individual SNARE proteins [22••], which became incorporated into folded complexes at the cell membrane.

Structure of IDPs in the bound state

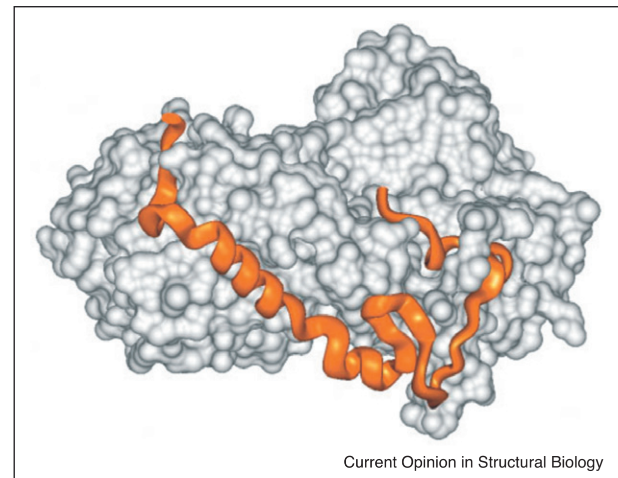
Binding of IDPs to their partners via linear motifs is often weak and is of limited specificity [11]. When stronger, more specific binding is required, IDPs use two distinct strategies. For one, they may use disordered domains for recognition [23], which are longer than 20 residues and conform to all three domain definitions, that is they are autonomous structural, functional, and evolutionary units. These long disordered regions (Figure 2) should be recognized as novel structural–functional elements of IDPs [23]. The other strategy is the combined action of several motifs, as observed in the case of the tripartite binding of calpastatin to calpain [24] or inhibitor 2 to protein phosphatase 1 [25].

This mode of binding (Figure 3a) may add binding strength and specificity, but may also mediate remote initial interaction, in accord with the 'fly casting' model [26]. Such an interaction has been observed in nonsense-mediated decay (NMD) that degrades mRNAs carrying a premature stop codon. NMD is triggered by the assembly of a multiprotein complex that includes three up-frameshift factor (UPF) proteins. Assembly of the NMD complex is initiated by the long disordered C-terminal domain of UPF2 initially binding UPF1 by separate α-helical and β-hairpin elements (Figure 3b) from a distance, thereby 'catching' UPF1 in an encounter complex and bringing various parts of the complex in proximity via fly casting [27].

Interestingly, the mechanism of fly-casting is somewhat confused with 'induced folding' in the literature. For example, when KID domain of transcription factor CREB binds to the KIX domain of CBP [28] or the regulatory domain of p53 binds to S100B(ββ) [29], folding does occur after binding, but an initial weak and long-range interaction may not occur. In fact, kinetic data on the interactions of ordered and disordered proteins [30] show very little difference, probably because their slower diffusion compensates for the larger capture radius of IDPs.

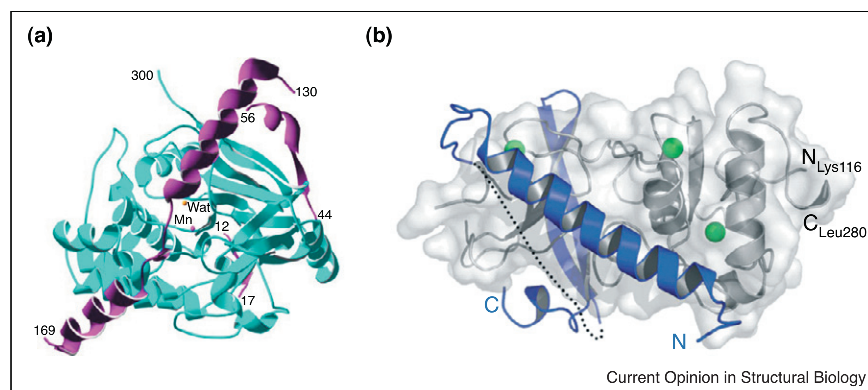
Structural, functional and kinetic dissection of the assembly of multicomponent complexes provides important functional insight into complex regulatory phenomena

Figure 2



Structure of an intrinsically disordered domain bound to its partner. Longer binding regions of IDPs conform to the structural, functional and evolutionary definition of domains. Here the kinase-inhibitory domain (KID) of Cdk inhibitor p27^{Kip1} (orange) bound to the CycA/Cdk2 complex (grey) is shown.
Adapted from [23].

Figure 3



Multipartite binding of IDPs. IDPs often bind their partners via short motifs used in combination to enhance specificity and/or binding strength. **(a)** Inhibitor 2 (purple) wraps around protein phosphatase 1 (green) and binds via three discontinuous binding motifs by nanomolar affinity. Adapted from [25]. **(b)** In assembling the nonsense-mediated decay (NMD) complex, UPF2 (blue ribbon) binds on two opposite surfaces of the CH domain of UPF1 (grey). Adapted from [27]. Please note that parts of IDPs remain disordered in the bound state, which represent a case of fuzziness [33••].

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[31,32•]. In the case of bacterial toxin/antitoxin (T/A) pairs, the binding of the disordered antitoxin (CcdA or Phd) to the toxin (CcdB or Doc) results in inhibition of the toxin, allosteric release (rejuvenation) of inhibition, and the transition of the T/A operon from a repressed to derepressed state depending on T:A stoichiometry (conditional cooperativity). The subtle interplay between these events demonstrate the inherent complexity of regulation by IDPs [31,32•].

Fuzziness: structural ambiguity in the bound state

Many observations suggest that the dominant mode of IDP function is binding to a partner and concomitant folding [4]. This notion, however, contains a significant element of simplification, because IDPs hardly ever become fully ordered in the bound state [33••], and often their region(s) that remain disordered are important for function. This phenomenon termed 'fuzziness' represents the extension of structural disorder to the bound state. Fuzziness may turn out to be a general structural–functional phenomenon, as suggested by many important cases, such as Sic1 binding to Cdc4 (Figure 1 [16•]), inhibitor 2 binding to PP1c (Figure 3a [34]), and UPF2 binding to UPF1 (Figure 3b [16•]).

What about *in vivo*?

Understanding how IDPs exist and function in cells is complicated by crowding elicited by extreme macromolecular concentrations [35] and binding partners [4], both of which may strongly favor folded states. Several recent studies addressed this question. By applying extremely high concentrations of macromolecular crowding agents, disordered dehydrins of *Arabidopsis thaliana* were found to maintain their disordered character *in vitro* [36]. In addition, functional studies have corroborated that their chaperone function associated with structural disorder *in vitro* is also witnessed *in vivo*, which underlines their structural disorder in a living cell [37•]. In-cell NMR studies addressed the structural state of tau protein in Xenopus oocytes [38], where its microtubule-binding region became ordered, whereas its long projection domain remained largely disordered. On the contrary, α-synuclein, which becomes compacted by crowding conditions *in vitro* [39], remains largely disordered when overexpressed in *Escherichia coli* [40].

Indirect approaches also provide important information on how IDPs behave in a living cell. Recently, it was shown that a simple 'operational' definition of structural disorder can be provided by ubiquitin-independent degradation of IDPs by the 20S proteasome [41]. This relation enables the identification of IDPs *in vivo*, as shown through the regulation of p53 degradation by NAP(P)H quinine oxidoreductase 1 (NQO1) [42]. Similar studies confirm that other IDPs are also disordered and susceptible to 20S-proteasomal degradation *in vivo*, probably regulated by specialized accessory proteins termed 'nannies' [42]. The regulation of IDPs *in vivo* has also been addressed in a bioinformatic study of high-throughput datasets of transcripts and proteins [43•]. It was found that proteins of a high level of disorder are more tightly regulated than proteins of a low level of disorder at all levels of transcription, mRNA clearance, protein synthesis and degradation.

Structural disorder in disease-associated and 'less-evolved' proteins

Structural disorder is enriched in proteins involved in diseases, such as cancer, diabetes, cardiovascular disease and neurodegenerative diseases [44,45]. Disease state caused by IDPs may result not only from protein misfolding [46], but also misidentification, missignaling, and unnatural or nonnative folding, as summarized in the novel D2 (disorder in disorders) concept [45]. Several recent studies provided further details of this correlation.

One observation pertains to chromosomal translocations, which fuse segments of distinct genes and generate oncogenic protein chimeras in cancer. In a comprehensive bioinformatic analysis of 406 translocation-related human proteins, such as BCR-ABL and CBP-MLL [47], these proteins, and their translocation breakpoints in particular, were shown to be significantly enriched in disorder. Apparently, structural disorder enables these chimeras to evade cellular surveillance mechanisms and exert their deleterious functions. Another recent paper addressed the related phenomenon of dosage sensitivity [48•], that is asked what renders gene products harmful when they are overexpressed. It was found that predicted intrinsic protein disorder is the strongest determinant of this effect [48•], suggesting that the likely cause of dosage sensitivity is binding promiscuity of IDPs. Dosage-sensitive genes were also found to be tightly regulated at the transcriptional, RNA and protein levels, as reported for IDPs in general [43•]. Structural disorder is also apparent in viruses. Every step of the viral cell cycle is orchestrated through interactions with cellular proteins for the epigenetic reprogramming of the cell. In most cases viruses use motif-mimicry for this purpose, that is short motifs in disordered regions that compete off similar interactions of the host [49••]. Due to the pressure on the viral genome for compaction, such motifs also represent a very economic solution for high functional density, as demonstrated in the case of the adenoviral E1A oncoprotein [50].

A corollary of the foregoing observations is that structural disorder seems to enable the rapid appearance of novel, 'less-evolved' proteins that have not undergone a long evolutionary selection. For example, alternative splicing (AS) may shift translation reading frame resulting in dual coding, which is only conceivable if the protein product is

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disordered in at least one of the frames. Comparison of genomic sequences and transcripts has led to the identification of 67 human genes with dual-coding regions at least 75 nucleotides in length [51•]. Predictions did show either a high disorder in both frames, or a significant tendency to become more disordered upon shifting the frame.

Drug development: the new frontier

As seen, IDPs are often involved in disease [44–46], and it is of no doubt that proteins such as p53, BRCA1, CFTR or α-synuclein are preferred targets in drug development. Because the binding pockets of IDPs resemble the actives sites of enzymes, the binding partners of IDPs have been suggested as targetable proteins [44]. The potency of this approach has been demonstrated by nutlins, which can inhibit p53-MDM2 interaction and reactivate p53 pathway in cancer cells [52].

The recent buzz, however, is aroused by the observation that IDPs themselves can be targeted by small molecules [53••,54], as demonstrated in the case of the oncoprotein c-Myc, which can form a heterodimeric complex with Max. In a systematic search several small-molecule inhibitors were found that bind to distinct disordered regions of c-Myc, promote its disordered state and prevent its interaction with Max [54]. This concept probably can be generalized, because small molecules have also been found against other important IDP targets, such as Aβ, EWS-Fli1 and various peptides [53••]. Given the frequent involvement of IDPs in disease [45], the ability to interfere with their action represents tremendous potential in drug discovery, as also suggested in a recent excellent review [55]. An independent corroboration of this generalization has come from analyzing small-molecule γ-secretase modulators (GSM) aimed at selectively lowering Aβ42 levels in Alzheimer's disease [56]. GSMs were found to cross-link to the substrates amyloid precursor protein (APP) and Aβ, rather than γ-secretase, which suggests 'substrate targeting' by small-molecule effectors. Because substrate sites of enzymatic modifications correlate strongly with local disorder [57], this observation is highly relevant to the IDP field.

Conclusions

Although only about a decade old, the field of IDPs has already brought many surprises. The very idea of structural disorder rocked the building of structural biology, and the prevalence of IDPs in normal cell function and importance in pathology has brought the field into the limelight. With a steady advance in our ability to describe their structure and function in detail, now the next transition in the field is gaining momentum in which the generation of quantitative structural models of IDP function becomes possible. We reckon this transition will bring 'unstructural' biology the full recognition and appreciation it deserves, to be surely witnessed in the coming years.

Acknowledgements

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- •• of outstanding interest

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The complete structural description of the ensemble of IDP structures is an important development of the field. This paper is a nice illustration of even one step further, because here a combination of PRE NMR distance restraints are used in molecular dynamics simulations to obtain a mapping of the relative weight of conformations in the ensemble providing the

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A key recent transition in the description of IDP structures is the application of single-molecule techniques, which may allow the observation of both static and dynamic heterogeneity in IDP structure without ensemble averaging. This paper describes the invention of one such technique, high-speed atomic force microscopy, which allows direct visualization of conformational transitions in structural disorder. The dynamic behavior of myosin V molecules translocating along actin filaments enabled by disordered linker regions has been directly visualized. The 'movie' provides direct evidence of dynamic molecular behavior, leading to a comprehensive understanding of the motor mechanism. This technique may become one of the most powerful approaches to studying the structure and dynamics of IDPs in action.

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It is generally thought that IDPs undergo induced folding to their final, function-related structured state when they function by molecular recognition. In such a case, their function can be interpreted in terms of (bound) structure, which is very similar to the classical structure–function paradigm. On the contrary, it is demonstrated in this review that IDPs are never fully ordered even in the bound state, but show a significant amount of structural disorder or polymorphism in protein complexes. This function-related phenomenon termed 'fuzziness' represents the extension of the paradigm of structural disorder to the functional-bound-state. Due to the central role of disorder in protein–protein interactions and in regulatory processes, fuzziness may turn out to be a mechanism of fundamental importance in the interactome.

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A critical issue of the IDP field is how much *in vitro* structural and functional observations can be extrapolated to live cells. Here in-cell functional characterization of an anhydrin thought to provide desiccation tolerance in an anhydrobiotic nematode, *Aphelenchus avenae*, is carried out. When the protein is expressed in cells, it can reduce protein aggregation, in which a loose association with its client protein could be shown. This function is consistent with a physiological role of this protein as a molecular shield.

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An ambitious computational study of multiple high-throughput datasets aimed at elucidating the regulation of the level and availability of IDPs in cells. It was found that regulation of transcript clearance, proteolytic degradation and translational rate contribute to controlling the abundance of IDPs, some of which are only present in low amounts and for short periods of time. Fidelity in signaling of IDPs indicated by low stochasticity in transcription and translation rates indicate fine-tuning of the availability of IDPs, which may ensure that most IDPs are only available in appropriate amounts and only for the time they are needed.

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Structural disorder is known to correlate with disease, where mutations are thought to impair function or expression of the IDP, and disease results from loss of function. In this paper it is asked why certain genes are harmful when they are overexpressed. By analyzing overexpression phenotypes in yeast, intrinsic protein disorder is identified as an important determinant of dosage sensitivity. This inference is also validated in other species, fruit fly, worm, mouse and humans. It is suggested that disordered regions are prone to make promiscuous molecular interactions at elevated concentrations, which is the likely cause of pathology when genes are overexpressed.

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A central theme of the IDP field is that disordered proteins/regions function by molecular recognition, in which their short binding elements (linear motifs) bind their partner and undergo induced folding. In this important paper it is reviewed that viruses also use this mechanism very frequently for interactions with host proteins and epigenetic reprogramming of the host cell for their own needs. As shown by adequate examples, viruses use motif mimicking for this purpose, which has many advantages for them, such as rapid evolution, stronger binding their host competitor and high functional density.

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It is shown in this paper that there are many human genes in which alternative splicing shifts the reading frame, that is generates mature transcripts in which a segment is translated in two distinct frames. It is shown that the protein folding problem caused by dual coding is resolved by an elevated level of structural disorder in at least one of the frames. It is also shown that dual coding is under adaptive evolution and it may be an effective mechanism for the evolutionary appearance of novel functions.

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Despite increasing investments, the pace of discovery of new drugs is leveling off, which demands novel approaches of drug development. Binding partners of IDPs have recently been suggested as attractive drug targets. In this review it is shown through the example of c-Myc and other IDPs that this idea can be taken one step further because IDPs themselves can be targeted by small-molecule interactors. Because structural disorder is very frequent in disease-associated proteins, this approach may be one of the fastest-developing areas in the IDP field.

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