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Review Article

# Eukaryotic transcription factors: paradigms of protein intrinsic disorder

**Lasse Staby, Charlotte O'Shea, Martin Willemoës, Frederik Theisen, Birthe B. Kragelund and Karen Skriver**

The Linderstrøm-Lang Centre for Protein Science, Department of Biology, University of Copenhagen, 5 Ole Maaloes Vej, Copenhagen DK-2200, Denmark

Correspondence: Karen Skriver (kskriver@bio.ku.dk)

Gene-specific transcription factors (TFs) are key regulatory components of signaling pathways, controlling, for example, cell growth, development, and stress responses. Their biological functions are determined by their molecular structures, as exemplified by their structured DNA-binding domains targeting specific *cis*-acting elements in genes, and by the significant lack of fixed tertiary structure in their extensive intrinsically disordered regions. Recent research in protein intrinsic disorder (ID) has changed our understanding of transcriptional activation domains from 'negative noodles' to ID regions with function-related, short sequence motifs and molecular recognition features with structural propensities. This review focuses on molecular aspects of TFs, which represent paradigms of ID-related features. Through specific examples, we review how the ID-associated flexibility of TFs enables them to participate in large interactomes, how they use only a few hydrophobic residues, short sequence motifs, prestructured motifs, and coupled folding and binding for their interactions with co-activators, and how their accessibility to post-translational modification affects their interactions. It is furthermore emphasized how classic biochemical concepts like allostery, conformational selection, induced fit, and feedback regulation are undergoing a revival with the appreciation of ID. The review also describes the most recent advances based on computational simulations of ID-based interaction mechanisms and structural analysis of ID in the context of full-length TFs and suggests future directions for research in TF ID.

## Introduction

Composite molecular networks regulate gene transcription, and gene-specific transcription factors (TFs) are key components of these networks. TFs regulate the basal transcriptional apparatus by binding to specific target gene promoters as terminal points of signaling pathways controlling, for example, cell growth, development, and stress responses. Eukaryotic TFs are proteins composed of at least a family-designating DNA-binding domain (DBD) and a transcription regulatory domain [1] that can mediate induction or repression of gene transcription. The transcription regulatory domains bind co-activators of the transcriptional machinery or participate in remodeling or modification of chromatin [2]. Because of their lack of sequence similarity and their high degree of low-complexity sequences, these domains have been classified according to their amino acid profile as acidic, or glutamine-, proline-, or serine/threonine-rich [1]. Intrinsically disordered proteins/regions (IDPs/IDRs) are enriched in these profile residues, which promote structural disorder [3]. Accordingly, transcriptional activation domains (ADs) were early on known as acid blobs, negative noodles [4], and polypeptide lassos [5]. IDRs do not by themselves adopt well-defined structures but form dynamic conformational ensembles [6], yet they possess biological activity [7–9]. This review focuses on TFs as paradigms of the complex mechanisms associated with protein intrinsic disorder (ID) that is poised to mediate the dynamic events of transcriptional regulation.

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## Large-scale analysis of TF ID

About a decade ago, several genome-scale analyses indicated that the degree of ID is extremely high in proteins involved in signal transduction and transcription [10–12]. Later, large-scale analyses suggested that extended IDRs are enriched in DNA-binding proteins [13] in eukaryotes [14]. Using proteomic approaches, it was shown that IDRs are overrepresented in the nucleus compared with the whole cell, and that nuclear TFs are significantly enriched in IDRs [15]. Most eukaryotic TFs contain extended (>30 residues) IDRs, and the degree of ID in the ADs, annotated by Swiss-Pro and exhibiting varying lengths and composition characteristics, is at least 73% [11]. Thus, it is likely that eukaryotes with well-developed gene transcription processes depend on the properties of ID.

## TFs as hubs and hub partners

Many TFs are themselves so-called hub proteins or interact with hub proteins. Hub proteins integrate cellular responses from several cues and thus have many interaction partners. They form centers of protein–protein interaction networks (interactomes), where they connect network modules and can be essential to organisms [16,17]. In a recent analysis of network controllability, TFs were shown to be overrepresented among proteins located in strategically important positions where they efficiently regulate interacting network proteins [18]. Furthermore, proteins localized in more than one subcellular compartment, especially nuclear/cytoplasmic proteins, tend to be hubs, and most of these are TFs or regulators of signaling pathways [19], emphasizing the importance of TFs to interactomes. p53 is a prominent hub [20], and more than 500 direct interaction partners were curated from the STRING database (Figure 1A) [21], whereas the NAC TF, ANAC019, has seven experimentally verified interaction partners (Figure 1B). These TF hubs use IDRs for their many interactions. However, hubs can also use structured domains for binding to IDRs in partner proteins. This is the case for CREB (cAMP response element-binding protein)-binding protein (CBP) and for the stress-associated hub radical-induced cell death1 (RCD1), which both are large proteins with structured domains interrupted by linker regions which may be IDRs [22,23]. CBP and RCD1 have 103 and 29 direct interaction partners, respectively (Figure 1C,D), many of which are TFs (Figure 1D). In both hub types, ID flexibility probably underlies the numerous interactions.

## ID in prototypical TF structures

Three prototypical TF families with well-characterized IDRs form the core of the review. The first family is the no apical meristem, *Arabidopsis* transcription activation factor, cup-shaped cotyledon (NAC) family. The plant-specific NAC TFs consist of an N-terminal NAC DBD [24] and mostly disordered ADs which vary in size (Figure 2A) [25–28]. They play important roles in development and stress responses [29,30] and are of great applied interest [31]. The second family is the nuclear receptor (NR) family with specific focus on typical members of the steroidal-type subfamily. They consist of three major domains, a large and variable N-terminal domain (NTD), a zinc finger DBD, and a ligand-binding domain (Figure 2B). Whereas the last two domains are folded, the NTD is mostly disordered [32]. They have two ADs: AF1 in the NTD and AF2 on the surface of the ligand-binding domain [32,33]. The NR family includes important TFs such as androgen receptor (AR) [34], glucocorticoid receptor (GR) [35], and progesterone receptor (PR) [36]. The last family given special attention in this review is the p53 family. The tumor suppressor p53 acts as a homotetramer, with each monomer consisting of a mostly disordered AD, divided into AD1 and AD2, a proline-rich domain, a DBD, a tetramerization domain, and a basic C-terminal regulatory domain (Figure 2C) [37,38]. p53 is regulated by both the human/mouse double minute protein 2 (H/MDM2) E3 ubiquitin ligase and the transcriptional co-activator CREB-binding protein (CBP)/p300 [22,39–41]. In unstressed cells, p53 levels are kept low by MDM2-mediated proteasomal degradation, but genotoxic stress results in accumulation of p53, arrest of cell growth, and apoptosis [42,43]. Members of the NAC, the steroid sub-type NR, and the p53 families together illustrate many of the significant features associated with protein ID.

## Experimental demonstration of ID in TFs

A growing number of methods, including different types of spectroscopy [32,44–47], are used for structural investigation of IDRs. Nuclear magnetic resonance (NMR) has become a key technique that provides atomic resolution information on residual structure, dynamics, and interactions of IDPs [48]. More recently, hydrogen–deuterium exchange coupled with mass spectrometry has been used for the mapping of IDRs [49], just as

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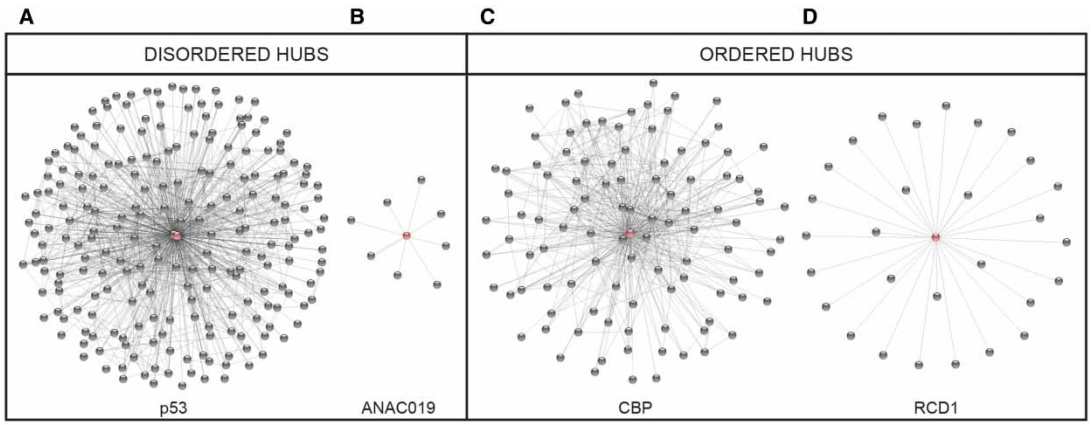


**Figure 1. TF-containing interactomes.**

ID enables numerous interactions within interactomes. Disordered hubs refer to hub proteins for which IDRs are mainly responsible for the many interactions of the hub as in the case of the TFs p53 (**A**) and ANAC019 (**B**). Ordered hubs refer to hub proteins for which ordered (structured) domains are mainly responsible for the many interactions of the hub as in the case of CBP (**C**) and RCD1 (**D**). The networks were derived from the STRING database [21]. Only direct hub interaction partners experimentally verified with a confidence score of >70 are shown. Red nodes represent query proteins, while gray nodes represent interaction partners.

the use of small-angle X-ray scattering (SAXS), which provides low-resolution information about the shape and oligomeric state of a protein, is emerging [50,51]. These methods are all time-averaged, meaning that they are limited to describing overall properties of entire populations [52,53]. In contrast, single-molecule techniques,

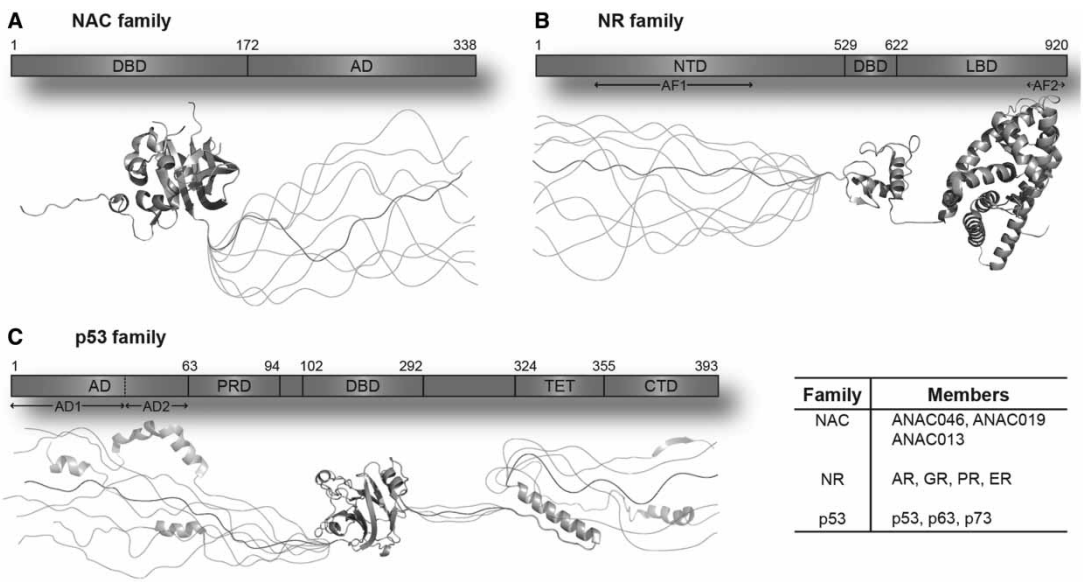


**Figure 2. Prototypical TF structures.**

(Top) Schematic domain structure (not drawn to scale) and (bottom) tertiary structures (ribbon representations) and IDRs (lines) for: (**A**) ANAC046 consisting of the NAC DBD (PDB code 1UT7) and the intrinsically disordered AD [25]. (**B**) AR consisting of the NTD with AF1, the DBD (PDB code 1R4I), and the AF2-containing ligand-binding domain (LBD; PDB code 2AM9). (**C**) p53 consisting of the AD, divided into AD1 and AD2, the proline-rich region (PRD), the DBD (PDB code 1TSR), the tetramerization domain (TET; PDB code 1C26), and the C-terminal regulatory domain (CTD) [37,38]. Regions which fold upon binding are shown for p53 only (PDB codes 1YCR; 2B3G; 2B3G; 1DT7; 1MA3; 2GS0) (shadowed ribbons). The TFs are shown as extended structures only to match the schematic domain structures. Inset: relevant members of the NAC, NR, and p53 TF families.

Biochemical Journal (2017) 474 2509–2532
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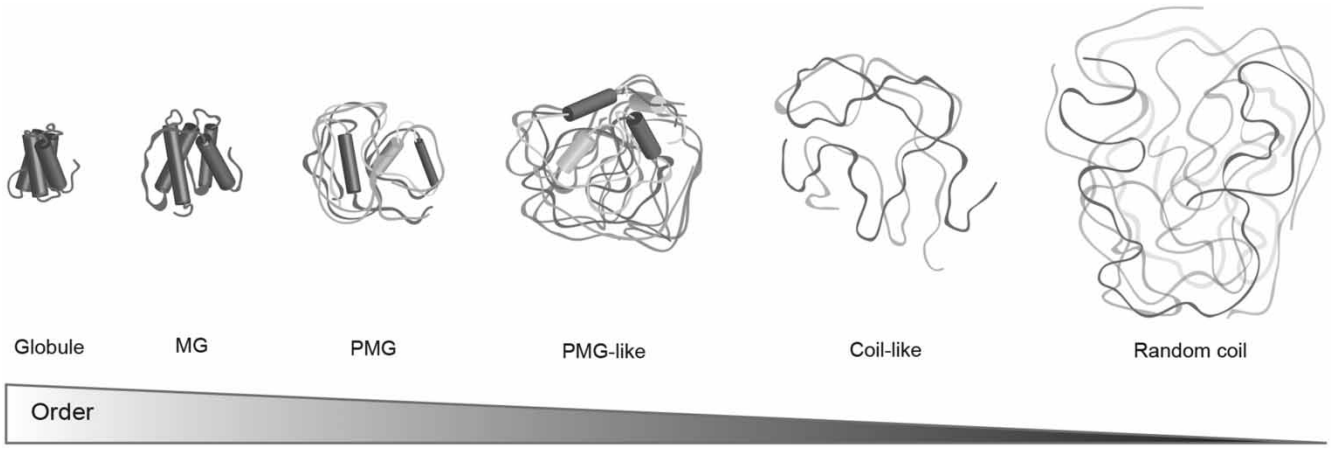


**Figure 3. Conformational states of proteins.**

Different conformational states for proteins: globule, molten globule (MG), pre-molten globule (PMG), and random coil for folded proteins and PMG-like and coil-like only defined for IDRs, which can also be in a more extended state than random coil. The different sizes of the states, not drawn to scale, illustrate that they may have different characteristic hydrodynamic dimensions [70]. However, the shapes of all states vary and large IDRs may contain regions present in different states.

such as single-molecule Förster resonance energy transfer and atomic force microscopy, enable detailed population-weighted studies and together with NMR can provide detailed information about mechanisms of IDRs [54,55]. This is also addressed in kinetic studies, which monitor binding and conformational changes occurring along the reaction path, typically performed using stopped-flow and temperature jump in combination with fluorescence spectroscopy [56–59]. When the reaction timescales are appropriate, kinetics can also be obtained from NMR experiments [60,61]. As a promising complementation of experimental studies, molecular dynamics (MD) simulations provide atomic scale resolution of mechanisms, and new force fields developed for IDRs are emerging [57,62]. Furthermore, integrative approaches that combine multiple structural biology methods are increasingly used to investigate IDRs [63].

Already before the first bioinformatic investigations of TF-ID, NMR, circular dichroism (CD), and Fourier-transform infrared spectroscopy and limited proteolysis were used to demonstrate that regions in the NTD of GR [44,45], estrogen receptor (ER) [46], and AR (Figure 2B) [32,47] are mostly disordered when unbound. Early experimental evidence for ID also exists for the kinase-inducible AD (KID) of CREB [64], which interacts with CBP to mediate cAMP-inducible gene expression [65], and for the AD of p53 (Figure 2C) [38,66]. Recently, ID in full-length TF contexts has also been addressed. Thus, studies by hydrogen exchange mass spectrometry confirmed ID properties of the PR-NTD within the full-length PR [49], and the NTD of full-length retinoid receptor α was shown by NMR and SAXS to remain highly flexible when bound to DNA [67]. For p53, single-molecule and ensemble fluorescence resonance energy transfer studies indicated that multiple conformations exist in the full-length TF [68]. By now, numerous studies have addressed TF ID. The NAC-ADs (Figure 2A) were shown by NMR and CD to be disordered [26–28], and the acidic AD subregion, TA2, of the NF-κB TF RelA/p65, which interacts with CBP, was unstructured when unbound as observed from limited dispersion of peaks in NMR spectra [69].

Beyond simply appreciating ID, it is important to describe function-related transient structure. Different protein conformational classes—native (globule), molten globule, pre-molten globule, and denaturant-unfolded (random coil) for globular proteins, and pre-molten globule-like and coil-like for IDRs—have been suggested to have characteristic hydrodynamic dimensions (Figure 3) [70]. However, the shapes of all states vary and large IDRs may have regions present in different states. Thus, computational and experimental studies indicate that the amino acid composition of IDRs affects their conformational state. IDRs with high net charge and low hydrophobicity tend to adopt an extended coil, and the ratio between hydrophobic residues and net charge can modulate IDR compaction [71,72]. Furthermore, the charge pattern may also determine if IDRs adopt extended coils or collapsed globular conformations [73]. Analysis of the structural state of five NAC-ADs by size-exclusion chromatography revealed hydrodynamic dimensions most similar to those of pre-molten

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globules (Figure 3), and they underwent structuring in the presence of osmolytes [26,27]. However, it should be kept in mind that this does not reveal aspects of the overall shapes or the secondary structure contents of the large NAC-ADs. The NR-NTDs also have significant levels of secondary structure [74,75], and native AR-AF1 exists in a collapsed disordered conformation, distinct from both a random coil state and a globular fold [76]. The dimensions of the NTD of the *Drosophila* ecdysteroid receptor isoform EcRB1 are smaller than those of the EcRA isoform, suggestive of structural diversity of the isoforms [77]. The native state of the p53-AD has dimensions similar to a random coil, but the chemically unfolded state is more extended, meaning that the native state of p53-AD has a degree of compactness that is disrupted upon denaturation [78]. In accordance with this, NMR analysis showed that the native state contains several regions of transient secondary structure [79]. Thus, the TF-ADs span widely with respect to dimensions and levels of function-related transient structure.

## Sequence motifs as functional elements in TF-ADs

What features determine the function of IDRs? As described below, functionally important features are prevalent at both the primary and the secondary structure levels. Several of these features are strictly associated with ID and an ID-specific nomenclature. IDRs play a significant role in protein–protein interactions, which are often mediated by short linear motifs (SLiMs), also known as eukaryotic linear motifs or linear motifs (Figure 4A) [80,81]. SLiMs are 3–11 residues long [82,83], and their small binding surface may result in transient interactions with low affinity [82,84,85], well suited for dynamic transcriptional networks. SLiMs are difficult to identify due to the challenge of obtaining robust statistical assessment [86], probably rooted in sequential and structural context variations, which hampers TF-AD definition.

ADs from, e.g., NAC TFs, p53, and NRs need one to a few hydrophobic residues for activation activity, and the bulky hydrophobic properties of these are more important than residue identity [26,87–90]. The hydrophobic residues serve as contact sites, as in the case of phosphorylated KID (pKID) binding to CBP [60], and increasing the hydrophobic content of the GR-AD enhanced activity [91]. For pKID, recent simulation studies indicated equal contributions of several hydrophobic residues and the phosphorylated Ser133 to the free energy of binding [92]. For Erwing sarcoma fusion proteins (EFPs), which are potent cancer-causing transcriptional activators [93], tyrosine residues present in the repetitive SLiM SYGQQS of the AD (Figure 4A) were shown by substitution studies to be essential for activity [90], possibly through binding to the co-activator complex TFIID [94]. In contrast, the effect of the disordered background sequence is limited [90]. Likewise, individual acidic residues within acidic ADs are generally not critical for function as long as a sufficient number is retained [95,96]. Determinants of AD function were addressed in structure and design studies of the acidic yeast TF Gcn4 [96,97]. Hydrophobic and aromatic residues within the Gcn4-AD interact with residues in a hydrophobic groove of the Mediator co-activator subunit Gal11/Med15. Strikingly, an α-helix induced in Gcn4 upon complex formation bound Gal11/Med15 in multiple orientations supported by a purely hydrophobic protein–protein interface [96]. Furthermore, rather than using a pattern of generic hydrophobic residues, in transactivation assays the activation activity of Gcn4 was dependent on the SLiM ΦΦWΦΦLF (Figure 4A), where Φ is any hydrophobic residue [97].

ΦXXΦΦ, where X is any residue, is an important generic AD-SLiM (Figure 4A) [22,98]. Its presence has been biochemically and structurally demonstrated for AD1 and AD2 of p53 [87,99,100], where it overlaps with the nine amino acid transactivation domain (Figure 4B) [101]. However, AD1 and AD2 have different binding specificities [99,102,103], supporting the notion that IDR contexts are more than passive scaffolds and possess SLiM-regulating features [104]. Both AD1 and AD2 can bind several co-activators [100,105], including the transcriptional adapter zinc finger (TAZ)2 domain of CBP (Figure 4C), although AD2 has higher affinity than AD1 [105–107] due to more hydrophobic contacts [108]. Furthermore, AD1 and AD2 bind TAZ2 synergistically, suggesting that studies performed with isolated AD motifs may be ambiguous [40,108]. Interestingly, AD1 binding to the secondary site on TAZ2, promoted by AD2 binding to the primary site, may protect p53 from interactions with MDM2, thereby hindering its degradation [107].

The two TF-binding sites of CBP-KIX—the pKID/c-Myb and the mixed-lineage leukemia (MLL) binding sites [109]—also interact via ΦXXΦΦ, which is often LXXLL preceded by an acidic residue, ζ [98,100,109–111]. In the E-protein TF, E2A, ζLXXLL is fused to the LDFS motif, and all residues throughout the extended motif (Figure 4A) are important for both KIX-binding, as demonstrated by both biochemical and structural studies, and for bone marrow immortalization, linking SLiMs and biological function [110,111]. ADs with two SLiMs, such as FOXO3a-AD and p53-AD (Figure 4B), form heterogeneous complexes with KIX [100,112]. For

Biochemical Journal (2017) **474** 2509–2532
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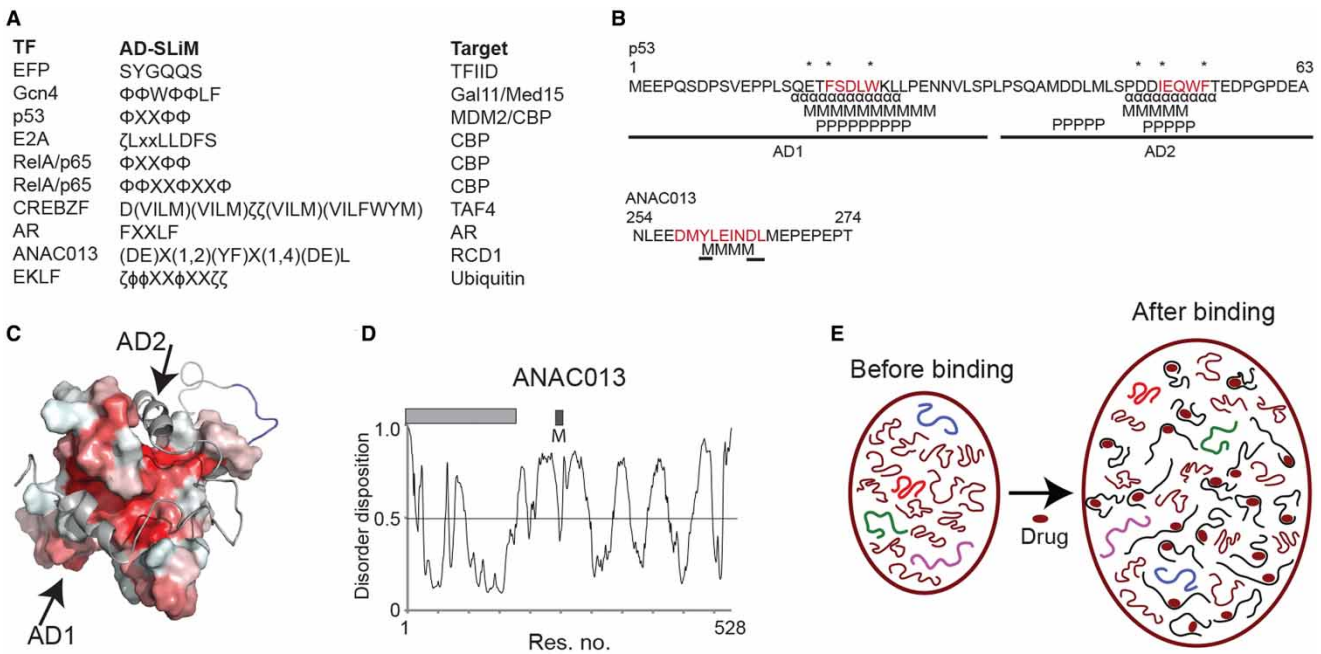


**Figure 4. Sequence motifs and TF binding.**

(**A**) Experimentally identified SLiMs in TF-ADs. From left to right: Representative TF-AD, SLiM, and representative interaction partner. All targets are experimentally identified except TFIID, which is a proposed target. (**B**) (Top) Sequence of the p53-AD with the AD1 and AD2 SLiMs shown in red. Residues of the nine amino acid autonomous transactivation domain are shown by * at the top of the sequence. α, M, and P indicate α-helices formed in complex with CBP-TAZ2, predicted MoRFs [28] and PreSMos determined by NMR [79]. (Bottom) ANAC013 peptide responsible for RCD1 binding. Residues of the bipartite SLiM, which are essential for binding, are shown in green. M as above. (**C**) Structure of the CBP-TAZ2 domain (PDB code 5HPD) in complex with p53-AD (gray cartoon). The artificial linker of the engineered CBP–p53 hybrid protein is shown in deep blue [108]. The surface of CBP-TAZ2 is colored according to hydrophobicity from white (polar) to red (nonpolar). (**D**) The RCD1-interacting SLiM is present in IDRs as shown for ANAC013. The positions of the DBD and the RCD1-binding SLiM are shown as a light and a dark gray bar, respectively. Disorder was predicted using PONDR-FIT and assigned to values ≥0.5 (black bar). M indicates a predicted MoRF [28]. (**E**) Expansion of the conformational space of a TF-IDR such as that of c-Myc upon binding of a small-molecule drug. (Left) Conformational space of free TF-IDR and (right) remodeling of the conformational space by drug (red) binding. Conformations induced by drug binding are shown in black, whereas the original conformations are shown in other colors (redrawn from [122]).

FOXO3a, one sub-site of its AD binds to the pKID/c-Myb site and the other to the MLL site in one conformer, and *vice versa* in the other [112]. Experimental data also showed that RelA/p65 uses ΦXXΦΦ and ΦΦXXΦXXΦ (Figure 4A) for binding of CBP-TAZ1, and this interaction affects gene expression *in vivo* [69]. The SLiM D(VILM)(VILM)ζζ(VILM)(VILFWYM), where ζ is any hydrophilic residue, is responsible for TF binding to TBP-associated factor 4 of TFIID as revealed using selection [113], demonstrating variance of the TF-SLiMs (Figure 4A).

Functional synergy in AR is mediated by the association of the FXXLF-SLiM of AF1 and AF2 of the ligand-binding domain (Figure 2B) [33,75]. Prevention of this N:C interaction by substitution of a conserved phenyl-alanine in the FXXL-SLiM resulted in a delay of disease onset in an animal model [114], demonstrating the functional importance of the SLiM. For the RCD1 interactome, SLiM identification has proved challenging [27,28,115]. Therefore, combinations of bioinformatic and biochemical approaches were used together with considerations of parameters such as disorder, context, charges, and pI to identify the loose consensus sequence (DE)X(1,2)(YF)X(1,4)(DE)L as the RCD1-interacting SLiM. The SLiM is bipartite with essential contributions to binding from aromatic, acidic, and leucine residues from its two sub-sites [27,28] (Figure 4B). The SLiM is also present in the RCD1-interacting TF dehydration-responsive element-binding protein 2A (DREB2A), and a DREB2A splice variant, which lacks the SLiM, cannot bind RCD1 [115]. It was proposed that removal of RCD1 or the loss of the SLiM in DREB2A is required for proper DREB2A function under stress conditions. Alternative splicing is frequent in IDRs [116], and its functional importance is demonstrated from the

Biochemical Journal (2017) **474** 2509–2532
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DREB2A:RCD1 system. As typical for TF SLiMs [97], the RCD1-binding SLiM must be present in an IDR to be functional (Figure 4D) [28].

AD-SLiMs can have several functions. For example, SLiMs of acidic ADs contribute to both activation activity and ubiquitin-mediated regulation [117]. In the case of erythroid-Krüppel-like factor, its AD α-helix also forms the recognition interface for ubiquitin using the SLiM ζΦΦXXΦXXζζ as shown by structure determination (Figure 4A) [118]. Such combinatory SLiMs may further increase complexity in identification.

Thus, although difficult to identify, SLiMs are emerging as important for the function of TF-ADs. The generic SLiM ΦXXΦΦ is prominent among AD-SLiMs and, furthermore, forms the core of expanded SLiMs (Figure 4A). The SLiMs reflect the importance of hydrophobic and acidic residues for TF–co-activator interactions (Figure 4A). ADs with two SLiMs (Figure 4B) may use both for binding to the same target domain (Figure 4C) and for bridging two different proteins in ternary regulatory complexes [40].

## Thermodynamics of TF–SLiM interactions and effect of contexts

The affinities of TF–SLiM interactions vary greatly [28,59,105]. For example, the affinities between RCD1 and different NAC peptides vary from high with a $K_d$ of 9 nM to medium with a $K_d$ of 609 nM [27,28]. Although these differences may have *in vivo* consequences, detailed thermodynamic analysis demonstrated a significant effect of peptide length and context on affinity. The SLiM core residues have been suggested to contribute ~80% of the binding energy [119]. However, a more complex pattern is emerging. Whereas truncation of the large ANAC013-AD to the minimal SLiM resulted in a 60-fold affinity increase, suggesting a negative allosteric effect of the context, an opposite positive effect was observed for the same SLiM present in DREB2A. For ANAC013, truncation reversed the entropic contribution to binding from negative to positive, whereas DREB2A truncation had the opposite effect [28], supportive of regulatory functions of SLiM ID contexts [104]. It is the general observation that IDRs pay an entropic cost upon binding because of restriction of the conformational space [120,121]. However, as demonstrated for the NAC TFs, IDRs can also use entropy for binding, which could be explained by solvent-mediated entropic effects or by relief of charge–charge interactions [27,28]. Binding by SLiMs may also result in less compaction of the surrounding IDR, or, as suggested for TF-AD binding of small-molecule drugs, in increased conformational flexibility of the IDR (Figure 4E) [122], thereby inducing favorable entropic changes. To conclude, the affinities of SLiM-mediated protein interactions vary and may be context-dependent with complex and unpredictable thermodynamics.

## Secondary structural elements as features of TF IDRs

Whereas SLiMs are found not just in IDRs, molecular recognition features (MoRFs) are found exclusively within IDRs and are functional IDR features apparent at the secondary structure level. They are identified by computational analysis and are relatively short, interaction-prone disordered segments that form secondary structures upon binding (Figure 5A) [123,124]. The level of α-helix-forming MoRFs (α-MoRFs) [123] is much higher for TFs than for other proteins [11], and several MoRFs were predicted for p53 [125], with both ΦXXΦΦ SLiMs of its AD coinciding with a MoRF (Figure 4B). Interestingly, divergence in the p53 family, which also includes p63 and p73, primarily evolved by insertions and deletions within IDRs connecting structured or structure-prone (MoRF) functional regions [125].

Several MoRFs were also predicted for the NAC-ADs, and they often coincide with SLiMs and dips in disorder profiles (Figure 4D) [25,27]. Although the NAC-AD sequences are diverse, reflecting the generally fast evolution of IDRs [126], a correspondence between MoRF, ID, and SLiM patterns was identified for several NAC subgroups associated with specific functions suggestive of conserved disorder-related functions [25,127]. This, together with evidence obtained from studies of the Myc-TFs [128], suggests that ID predictions may eventually complement multiple sequence alignments for construction of dendrograms and thus for dissection of evolutionary relationships. Whereas most NAC-ADs have complex MoRF patterns, the ANAC046-AD has only a single MoRF at the C-terminal end of the long ANAC046-AD that may function as an entropic chain suitable for catching interaction partners [27]. Multiple MoRFs were also predicted for the AD of the plant TF TCP8, suggesting that TCP8 interacts with many partners in the fine-tuning of transcription in response to various stimuli [129], possibly as a scaffold. For the EFP-AD, its array of tyrosine residues is also associated with MoRFs conferring effective transcriptional function [90]. The above examples illustrate how MoRF predictions can pinpoint functional hotspots.

Biochemical Journal (2017) **474** 2509–2532
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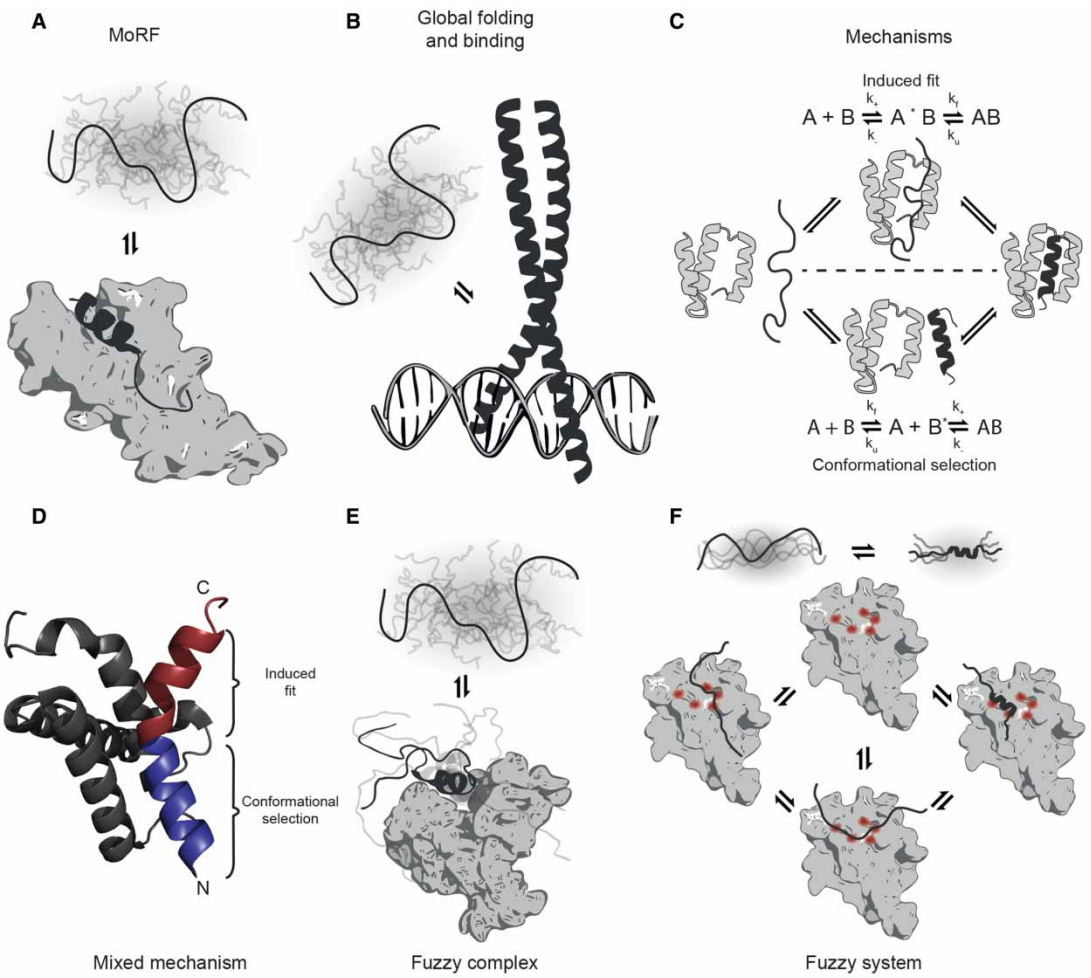


**Figure 5. ID-associated structure features of TFs.**

(**A**) MoRFs are short regions identified by computational analysis as interaction-prone disordered segments that form secondary structures upon binding. Shadows represent conformational ensembles of TF-IDRs. (**B**) Larger IDRs may also undergo large-scale coupled folding and binding. (**C**) Two models describe the mechanisms of coupled folding and binding *Conformational selection* and *Induced fit*. In conformational selection, a conformation from the ensemble is drawn by binding to the target partner. In induced fit, the TF recognizes its partner in a disordered state that then folds over the surface of the partner (**D**). Structure of the c-Myb-AD:CBP-KIX complex (PDB code ISB0). The N-terminal half (blue) of the c-Myb α-helix binds KIX by conformational selection, whereas binding of the C-terminal half (red) occurs by induced fit [61]. (**E**) Some IDRs form fuzzy complexes retaining conformational heterogeneity in the complexes. The model is inspired by the Gcn4:Gal11/Med15 complex (PDB code 2LPB). (**F**) Cartoon model of RCD1 interactions with different TFs (redrawn from ref. [28]). The gray surface domain depicts the unknown structure of the RST domain of RCD1. Five hydrophobic binding anchor points, shown in red on the RCD1 surface, are assumed. RCD1 can bind both unstructured and α-helical peptides. Since it remains unknown whether the interactions take place via conformer selection or induced fit, both possibilities are suggested by the equilibrium between conformations of the free ligand.

Transient secondary structural elements, referred to as, e.g., pre-structured motifs (PreSMos) [130] or pre-formed structural elements [131], may be present in IDRs prior to target binding as revealed by NMR analysis. Heteronuclear multidimensional NMR was used to analyze for PreSMos in the p53-AD [79], which resulted in the identification of three structured motifs, an amphipathic helix, which was populated in ∼25% of unbound p53-AD, and two turns (Figure 4B). Using p53 as a well-studied case, the different approaches for predicting binding sites are in accordance and supplement each other.

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## Kinetics of binding

Kinetic analysis represents a key tool for unraveling the mechanisms of ID-based interactions. Association rate constants ($k_{on}$) of IDR interactions vary greatly [132], but it is not yet clear if they differ from those of structured proteins. IDRs can have larger capture radii than folded proteins, and the fly-casting mechanism states that the flexibility and extensibility of IDRs allow them to catch distant target proteins [133]. However, the large radius of gyration of IDRs may diminish the diffusion coefficient and thereby cancel out the advantage [134]. High $k_{on}$ values approaching the diffusion limit of $10^9$–$10^{10}$ $M^{-1}$ $s^{-1}$ for folded proteins have been determined for ADs, indicating that ID in association with favorable electrostatics is efficient in accelerating protein–protein interactions [105,135–137]. Even when corrected for the effects of electrostatic attraction, $k_{on}$ values were still in the diffusion-limited range [135,136,138]. Both $k_{on}$ and dissociation rate constants ($k_{off}$) play roles in enabling the short-lived protein–protein complexes needed for rapid switching in gene regulatory networks [84,139]. However, with similar affinities in complexes involving an IDP compared with complexes with two globular proteins [121], a high $k_{off}$ was hypothesized to be most important for IDRs considering the relationship between the thermodynamics and kinetics ($K_a = k_{on}/k_{off}$) and the diffusion-determined upper limit of $k_{on}$ [84]. In support of this, experimental data showed that $k_{off}$ values are indeed generally larger for IDRs than for structured proteins [138], which for the pKID:KIX system was suggested to be due to a reduction in the activation free energy of dissociation [140].

## Mechanism of binding

Disorder to order transition (Figure 5B) is a major functional paradigm for ID, and TF-Ads, in particular, contribute to the adaptability of IDRs. For PR, the same subregions of its NTD are required for folding and activation [49], linking induced folding to activity. Two three-state mechanisms of coupled folding and binding are hotly debated (Figure 5C) [22,56,59]. In *conformational selection*, a conformation from the ID ensemble, which is structurally similar to the bound form—implicating a structured transition state—is drawn from the ensemble, thus permitting binding to the target partner, and is followed by a population shift. In *induced fit*, the transition state is unstructured and the protein recognizes its partner in a disordered state, which then folds over the surface of the partner. Kinetic tests are critical for distinguishing between the two mechanisms, although still challenging [58,59,141]. It is now becoming evident that the processes rarely occur purely by one mechanism but involve combinations [58,84], as described below.

The complicated nature of the mechanisms is demonstrated from studies of p53. NMR and MD simulation studies indicated that the MDM2-bound conformation of an AD1 peptide was also significantly populated in the unbound state, suggestive of conformational selection (Figure 5C) [79,142]. In support of this, substitution of Pro27 in p53 with serine significantly increased the affinity for MDM2 as well as the α-helix content of unbound p53 peptide [143], pre-organizing it for binding [79,142]. This substitution also had an *in vivo* effect by de-balancing interactions involving p53 [144]. In contrast, MD analysis indicated global conformational selection and local induced folding for interactions between p53-AD and the NR co-activator-binding domain (NCBD) of CBP [145].

Studies of TF binding to CBP-KIX have been crucial for improving the understanding of the binding mechanisms. Already in 2007, NMR titrations and relaxation dispersion experiments indicated that KIX-pKID association takes place by induced fit (Figure 5C) [60]. This mechanism was supported by MD simulations [146] and kinetic studies [138]. However, recent simulation studies suggested that the pKID:KIX association takes place by a mixture of the two mechanisms [92]. c-Myb-AD forms a long α-helix with a kink upon binding to KIX (Figure 5D) [109,147], and kinetic measurements suggested that the c-Myb-AD binds KIX in a fast two-state process with no accumulation of intermediates [136,148]. In contrast, Φ value analysis, which provides information about structure formation in the binding transition state [59], indicates that this is only slightly more disordered than the bound state, suggestive of conformational selection [149]. Contributing to the confusion, kinetic studies, not only of c-Myb-AD binding to KIX, but also of several additional TF-ADs, suggested poor correlation between helicity and $k_{on}$ values [138,148]. Recently, NMR analysis provided evidence that the N-terminal half of the c-Myb α-helix binds KIX by conformational selection, whereas the C-terminal half was suggested to bind by induced fit (Figure 5D) [61], and MD simulations suggested c-Myb:CBP association to involve both structured and unstructured transition states, explaining previous disagreeing results [150]. Interestingly, the c-Myb-AD was recently shown to fold in a template-dependent manner when binding KIX that dictated the folding transition state of c-Myb, meaning that substitutions in KIX affected c-Myb

Biochemical Journal (2017) **474** 2509–2532
DOI: 10.1042/BCJ20160631

folding [151]. Thus, although pKID and c-Myb bind to the same site on KIX, their binding mechanisms appear different, which was suggested to reflect their different roles in transcription. Whereas KID depends on phosphorylation for high-affinity interaction with KIX, c-Myb is a constitutive activator [61]. Taken together, the studies described above reveal that the mechanisms of AD binding are complex and difficult to address experimentally.

## Fuzzy complexes involving TF-ADs

Adding to the structural complexity, some IDRs retain some of their conformational heterogeneity after binding [152,153]. In such cases, the complexes have been termed *fuzzy complexes* [154], which refer to complexes where ensembles of short-lived bound conformations are needed to describe the bound state, as discussed recently (Figure 5E) [155,156]. Despite fast interconversions, high local concentrations may still allow a productive outcome *in vivo* [156]. Furthermore, the flexibility of the binding interface decreases the entropic penalty of binding [120], also promoting complex formation.

Bioinformatic and biophysical analyses indicated that fuzziness is significant in the diverse RCD1:TF complexes. DREB2A forms an α-helix with flanking disorder upon complex formation, whereas ANAC046 and ANAC013 either form an extended structure or a fuzzy complex (Figure 5F) [26–28]. Fuzziness has also been suggested for p53 complexes, since several residues of the p53-AD remain disordered in complex with CBP-NCBD [157]. For c-Myc, AD binding to the SH3 domain of the tumor suppressor Bin1 [158] is mediated by multivalent interactions, resulting in a highly dynamic complex. Bin1 sterically hinders interactions present in unbound c-Myc, and the flexibility and dynamics of the complex enables phosphorylation of c-Myc, required for proper function [159]. Molecular recognition by the EFP-ADs involves multiple aromatic contacts facilitated by a flexible peptide backbone and weak contributions from amenable side chains, also representing a fuzzy complex [160]. As a last example, the Hox TFs may form fuzzy complexes with both DNA and other proteins [161]. Different Ultrabithorax isoforms, resulting from alternative splicing of an IDR, potentially contribute to fuzziness and increase the functional spectrum in gene regulation [161].

Gcn4 binds Gal11/Med15 via multiple SLiM-mediated low-affinity interactions (Figure 4A), which additively contribute to activation activity [96,97]. Interestingly, insertion of additional hydrophobic residues in positions surrounding the SLiM resulted in potent ADs that bound Gal11/Med15 with high affinity. In contrast with Gcn4 itself, the activity of the synthetic ADs did not strongly depend on specific residues. The additional hydrophobic residues may enable interactions between multiple faces of the Gcn4-AD and Gal11/Med15, optimizing fuzziness in the interface [97].

Thus, flexibility and dynamics of TFs are important also in the bound state, and fuzzy complexes of TF ADs add to the functional repertoire by allowing for regulation even in bound-state ensembles.

## ID in DNA binding

Although TF-ID is mostly associated with ADs, DBDs can themselves have high contents of ID [11,12,162], and a recent analysis of 76 classes of DBDs indicated that DBD-flanking regions also exhibit significant ID [162]. IDRs can directly interact with DNA [163,164], making the development of high-throughput methods for prediction of DNA binding by IDRs timely [165]. Already in 1990, the basic regions of several bZip-DBDs were shown to be disordered when unbound and to form α-helices when interacting with DNA (Figure 5B) [166–168]. Folding upon DNA binding was also suggested from thermodynamic studies of the GR-DBD [169]. NMR spin relaxation analysis of the Gcn4-DBD suggested a two-step mechanism in which partially structured Gcn4-DBD conformations form encounter complexes with DNA followed by rearrangement to a high-affinity folded state [170].

The effects of IDRs on DNA binding have been studied in detail for the Hox TFs. The N-terminal arm directly flanking the homeodomain DBD significantly affects both the affinity and the specificity of DNA binding [171–173], and full-length Ubx binds alternate DNA sequences with a much wider range of affinities than the DBD alone [173]. Since remote IDRs may fine-tune DNA binding [163], both the NAC DBD and full-length ANAC092 were used in a large-scale analysis of the DNA-binding-site landscape and regulatory network of NAC TFs [174]. Full-length ANAC092 bound with higher affinity to an expanded range of k-mers compared with the isolated NAC DBD, but the DNA-binding specificities were not significantly different (Figure 6A). The large NAC-IDRs (Figure 2A) may assist DNA binding through modulation of conformation, flexibility, or spacing within the NAC:DNA complex or via additional fuzzy interactions [163,174]. The p53-AD (Figure 2C) plays an essential role in inhibiting p53 target gene activation through interactions with the oncoprotein

Biochemical Journal (2017) **474** 2509–2532
DOI: 10.1042/BCJ20160631

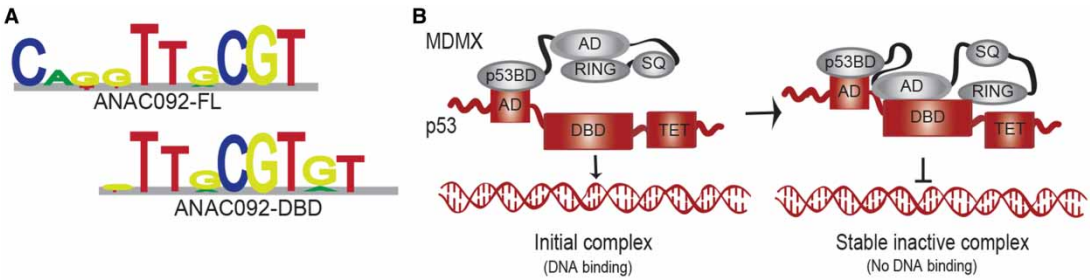


**Figure 6. ID in DNA binding.**

(**A**) Logo of ANAC092-DBD and full-length (FL) ANAC092 target DNA sequences (inspired by [174]). (**B**) A model of p53 inhibition by MDMX. Following formation of the initial p53-AD:MDMX complex, the MDMX AD and RING domains establish interactions with the p53-DBD blocking p53-DNA binding (redrawn from ref. [177]).

MDMX, which is related to MDM2, but does not mediate p53 degradation [175,176] (Figure 6B). Initial interactions between the p53-AD and MDMX are followed by strong interactions between p53-DBD and the AD and RING domains of MDMX which thereby block p53-DNA binding [177], providing a delicate mechanism of ID-mediated regulation of DNA binding.

Rapid binding of TFs in response to stimuli is required, and TFs use a search model in which weak and non-specific binding is followed by migration by one-dimensional sliding along non-specific DNA, three-dimensional jumping and intersegment transfer before high-affinity binding occurs (reviewed in ref. [178]). Disordered ID tails play significant roles here [164,179]. For p53, single-molecule experiments [180] and simulation studies [181] indicated that its positively charged C-tail increases the sliding speed in a manner dependent on its positive charge [164] and affects the DBD:DNA interactions by influencing the orientation of the DBD relative to DNA [181]. The positively charged tails of the homeodomains from HoxD9, *Antennapedia*, and NK-2 vary in size and boost sliding by increasing the affinity for non-specific DNA, but also slow diffusion along DNA. The tails furthermore facilitate intersegment transfer by bridging two different DNA segments in a *monkey-bar* mechanism [179]. Studies of designed NK-2 and Antp variants demonstrated that the number of intersegmental transfers depends on both charge composition and distribution of the tails. This is reflected in wild-type tails, suggestive of evolutionary selection for optimized function [182]. Thus, not only affinity and specificity, but also kinetics are important and strongly influenced by disordered TF tails. For the TF Egr-1/Zif268, the balance between affinity and speed in target DNA search may be optimized by modulation of the TF ensembles [183], suggesting an applicative potential of TF ensembles.

## Regulatory functions of TF-ID

With the appreciation of protein ID, the classic biochemical concepts of allostery and co-operativity have been revived to generate models that account for ID in a regulatory context. Originally, the definition of co-operativity according to the MWC model [184], and later extended [185], refers to sigmoid binding curves. In most cases, it is derived from binding of a ligand to two or more identical sites in a concerted equilibrium that exists between low- and high-affinity forms. The binding of a second ligand, an allosteric effector, which binds to a site distinct from that of the first ligand [186], may also control the equilibrium between low- and high-affinity forms. For IDRs and their interaction with two or more ligands, the literature often uses the terms *interaction* or *coupling* [187,188] in the description of allosteric effects that preserves the original meaning of co-operativity. Allostery plays a central role in the regulation of cellular signaling networks [189], and several proteins with functionally distinct but coupled IDRs were recently described [190–192]. Theoretical considerations suggest that allosteric modulation is enhanced via many conformers when one or both of the coupled binding sites are intrinsically disordered [187,193]. Thereby, the flexibility of IDRs allows complex allosteric behavior of IDRs in fine-tuning regulatory interactions [188].

Allosteric mechanisms involving ID have evolved in many TFs such as the NRs [194]. Upon DNA binding, structural changes of NRs are not limited to local effects in the DBDs, but additional structure may be formed in the NTD and the overall NR structure may also be influenced [195–197]. In such cases, DNA itself can be said to be an allosteric effector promoting the conformer of the NR, where its ID mediates the allosteric

Biochemical Journal (2017) **474** 2509–2532
DOI: 10.1042/BCJ20160631

responses. Allosteric regulation has also been inferred from studies, showing that overexpression of TATA-binding protein in cells enhances transcriptional activation by the PR-NTD by increasing the interactions between steroid receptor co-activator 1 and the PR-NTD. Thus, TATA-binding protein may mediate allosteric structural reorganization of the NTD to facilitate the binding of co-activators required for maximal transcriptional activity [49].

A model was proposed according to which the GR-NTD can be divided into a regulatory and a functional region that are thermodynamically coupled (Figure 7A, I). Removal of the regulatory region, as in a GR splice variant, resulted in stabilization of the functional region, and suggested that the two regions are negatively coupled (Figure 7A, II). Thus, IDRs may have ID subregions that are coupled, enabling allosteric modulation of the stability of the functional site [187,198]. Expanding on this idea, a regulatory subregion could also bind an effector ligand, which in turn could affect the stability of the functional region by redistribution of the conformational ensemble (Figure 7A, I) [199]. The advantage of IDRs is that the heterogeneity of their ensembles makes them functionally pluripotent, meaning that they can fine-tune and reverse the amplitude of a signal caused by binding of effectors [191,199].

TF binding to the KIX domain of CBP also illustrates TF-ID involvement in allosteric regulation, and several studies have reported coupled binding of TFs to KIX [200]. MLL and c-Myb or MLL and pKID can bind simultaneously to interconnected KIX-binding sites to form ternary complexes with allosteric binding affinity enhancement (Figure 7B) [109,201,202]. Binding of MLL stabilizes KIX and decreases dynamics in local regions [109,138,203,204] through repacking of the hydrophobic KIX core connecting the two binding sites [201]. Although both $k_{on}$ and $k_{off}$ are reduced by TF pre-binding, stabilization of the ternary complex is due to a larger reduction in $k_{off}$ than of $k_{on}$ [138,203,205]. It was suggested that binding of one TF results in a reduced entropic cost of binding the second TF [138,203], again demonstrating the importance of entropy, now also in allosteric regulation involving TF-ID.

Autoinhibition is another classic biochemical concept that is reinterpreted with the appreciation of ID. For regulation of the TF Ets-1, autoinhibition of DNA binding is essential. It involves the marginally stable helical

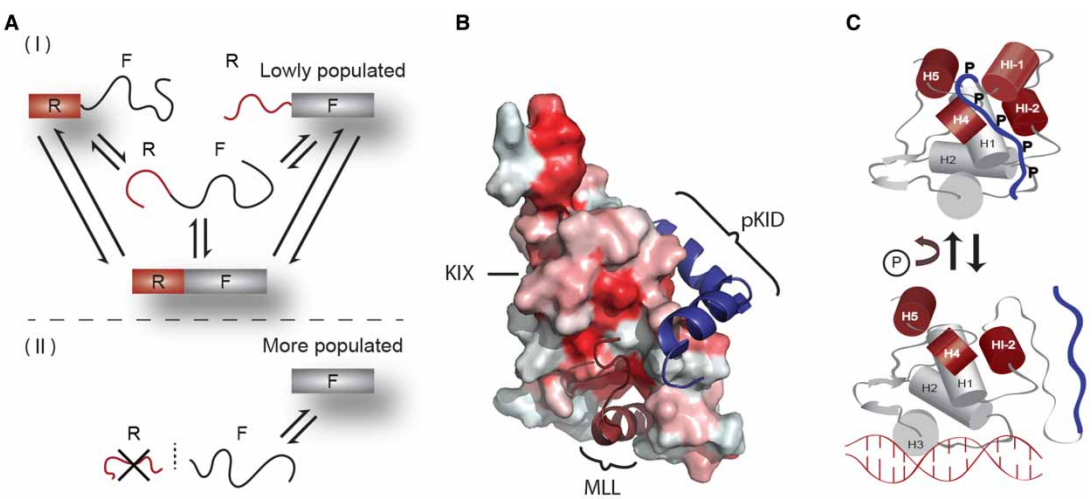


**Figure 7. Regulatory functions of TF IDRs.**

(**A**) (I) Division of a TF-IDR into a regulatory (R) and functional (F) subregion, which can be intrinsically disordered or folded (boxes). Stabilization of the folded R subregion by, e.g., an allosteric ligand may stabilize the F subregion by redistribution of the conformational ensemble (redrawn from ref. [199]). The arrows show the equilibriums between the different forms. (II) For GR, the lack of the R region as in GR splice variants results in stabilization of the folded F subregion due to negative coupling between the subregions [198]. (**B**) The allosteric network of hydrophobic residues in the KIX domain (PBB ID 2LXT) connecting the two remote TF-binding sites. The surface of KIX is colored according to hydrophobicity from white (polar) to red (nonpolar). (**C**) Phosphorylation-dependent inhibition of DNA binding by Ets-1. Phosphorylation of the disordered serine-rich region (blue string) results in electrostatic interactions which stabilize the inhibitory conformation of Ets-1. The ETS-DBD and inhibitory module are shown in gray and red. The model is based on structure determinations ([265]; PDB code 1R36), although the position of the serine-rich region is not known.

Biochemical Journal (2017) **474** 2509–2532
DOI: 10.1042/BCJ20160631

inhibitory module that packs against the DBD (Figure 7C) and is allosterically disrupted upon DNA binding [206]. The disordered serine-rich region is required for full inhibition by the inhibitory module, which also depends on phosphorylation, as described below [207–209]. Through transient interactions, this region mediates the inhibition of DNA binding [207,210]. For C/EBPβ, DNA binding is autoinhibited by multiple ϕXXϕϕ SLiMs that also mediate association with CBP (Figure 4A). It was proposed that intramolecular interactions between the SLiMs and an autoinhibitory domain of C/EBPβ generate a hydrophobic core that reciprocally inhibits DNA binding and transactivation [211]. The MDMX–p53 interaction is also regulated by autoinhibition involving intramolecular interactions in MDMX mediated by a region with compositional similarities to the p53-AD1, thus representing p53 mimicry (Figures 4A,B and 6B) [212]. The above examples based on TF-ID illustrate how biochemical concepts accepted many years ago for structured proteins are undergoing a revival with the appreciation of ID.

## Post-translational modifications—phosphorylations

The flexibility and conformational plasticity of IDRs make them readily accessible for post-translational modifications such as phosphorylation, acetylation, and ubiquitination [213–215]. Modifications of IDRs may result in changed signaling outputs, thereby increasing the complexity of regulatory networks. Phosphorylation is one of the most studied post-translational modifications and takes place predominantly in IDRs [213], and is therefore the focus of this section describing the effect of post-translational modifications of TF-IDRs. Essentially, a phosphorylation changes the energy landscape of the IDR, as described below, which has a bearing on the interactome.

Upon cellular stress, p53 is modified by numerous post-translational modifications [214], and the p53-AD1 has by itself nine phosphorylation sites [216,217]. Phosphorylation of Thr18 and Ser20 mediates the rapid uncoupling of p53 from MDM2 (Figure 8A), thereby stabilizing p53 against degradation [40,218,219]. The effect has been suggested to be caused by increased electrostatic repulsion between the negatively charged phosphorylated Thr18 of p53 and anionic regions on the MDM2 surface [220,221], by the long-range dynamical properties of the p53-AD and thereby its interactions, or by changes in the α-helix content of the unbound p53-AD increasing its CBP affinity [222,223]. According to MD simulations, p53 phosphorylation results in changes in the topology of the interaction landscape of a diffusively bound encounter complex which prevents the p53 peptide from binding to the MDM2 surface and in a reduction in residence time, hindering final complex formation [224]. These different explanations reflect the challenges of understanding the malleability of IDRs.

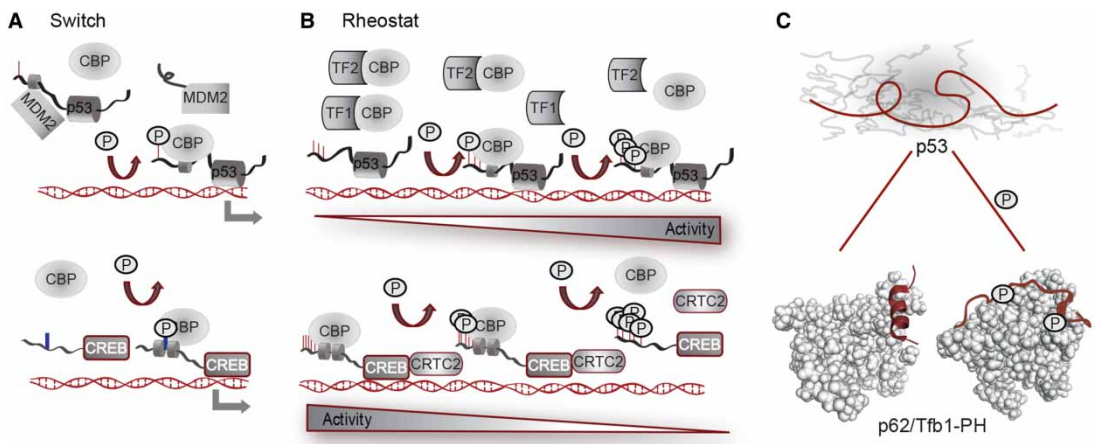


**Figure 8. Structural and functional effects of TF AD phosphorylation.**

(**A**) Single-site phosphorylation switch exemplified by the p53-AD:MDM2 and the pKID:KIX complexes. (**B**) Phosphorylation as a rheostat response with each successive phosphorylation event increasing or decreasing interaction affinity as in the case of the p53:CBP and CREB:KID:CRTC2 complexes, respectively. (**C**) Phosphorylated p53-AD2 binds to the PH domain of the human TFIIH subunit p62/Tfb1 as an extended string (PDB code 2RUK) allowing extensive electrostatic interactions with p62-PH. In contrast, p53-AD2 binds to yeast p62/Tfb1-PH in an α-helical conformation (PDB code 2GS0).

Biochemical Journal (2017) **474** 2509–2532
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In contrast with the single-site phosphorylation switch (Figure 8A), p53:CBP association is modulated by a phosphorylation rheostat where each phosphorylation of the p53-AD increases the binding affinity to CBP due to a change in bulk electrostatics (Figure 8B) [41,157]. p53 thereby outcompetes other TFs from CBP binding [157]. Phosphorylation of Ser15, Thr18, and Ser20 of p53 specifically enhances its affinity for CBP-TAZ2 [225], which may be explained by changes in the bound structure compared with the unbound ensemble, allowing new and specific contacts [226]. Interestingly, the p53-AD2 is extended in complex with the pleckstrin homology (PH) domain from the human TFIIH subunit p62/Tfb1. This string-like binding mode is independent of—but enhanced by—phosphorylation. It allows extensive electrostatic interactions with lysine residues of the p62/Tfb1-PH domain [227] and is different from the helical-binding mode of the unphosphorylated p53-AD2 to yeast p62/Tfb1-PH (Figure 8C) [228]. As an important IDR characteristic, this demonstrates conformational malleability that is controllable by chemical modifications [227].

Activity-dependent phosphorylation of the CREB-KID-AD represents a much-cited example of TF-AD regulation by a simple phosphorylation switch. Formation of the pKID:KIX complex involves folding of pKID, and phosphorylation of CREB-KID-Ser133 is critical for stabilization of the complex (Figure 8A) [64,65]. Maximal target gene induction by CREB depends on recruitment of both CBP and another co-activator, CREB-regulated transcription co-activator (CRTC) [229]. A cluster of five phosphorylation sites, termed the ataxia-telangiectasia-mutated (ATM)/casein kinase (CK) cluster within the N-terminus of KID, are co-ordinately phosphorylated by different kinases, resulting in weaker CBP binding. Proportionally to the number of phosphorylated residues, phosphorylation of the ATM/CK cluster in response to genotoxic stress results in inhibition of DNA binding by CREB and CRTC2 association [230,231], highlighting another example of rheostat regulation (Figure 8B). The attenuation may involve allosteric communication between KID and the DBD of CREB, here initiated by phosphorylation.

NR function is also regulated by phosphorylation, and the effect of GR phosphorylation has been demonstrated both *in vitro* and *in vivo*. Several years ago, the degree of phosphorylation of Ser203 and Ser211 of GR-AF1 was shown to increase upon treatment with a GR agonist, and transcriptional activity of GR correlated with the degree of Ser211 phosphorylation [232]. Furthermore, substitution of Ser211 with alanine had a diminishing effect on apoptosis [233]. Phosphorylation of Ser211 and Ser226 of GR-AF1 regulates GR: co-activator interactions, which according to molecular modeling may result in a conformational change facilitating cofactor interaction with Ser211 [234]. Later, phosphorylation of Ser211 was shown by CD analysis to induce secondary structure in GR-AF1, indicating that this post-translational modification is crucial for adoption of functionally active conformations of the disordered GR-AF1. The structural changes facilitated the interaction of the GR-AF1 with several co-regulators including CBP and TBP [235]. Recently, the TAZ2 domain of CBP was shown to be the target of phosphorylated GR-AF1, and phosphorylation of Ser211 and Ser226 enhanced CBP-TAZ2 binding synergistically, suggesting that phosphorylation promotes CBP recruitment and thereby enhances GR activity *in vivo* [236].

The serine-rich region of Ets-1 contains five phosphoserine residues (Figure 7C), which incrementally control DNA binding through decreased conformational flexibility of the inhibitory module and the DBD [207–209]. The interactions of the serine-rich region are fuzzy (Figure 5E), with this region rapidly interconverting within an ensemble of conformations that are restrained by transient interactions within the region itself and with the DBD [210]. These interactions were suggested to involve the simultaneous association of adjacent phosphoserines and aromatic residues with basic side chains in the DNA-recognition interface of Ets-1 via ion-pair and π-cation interactions, respectively [210], thus resulting in co-operative, multivalent binding of the serine-rich region to the DBD. Alternatively, the negatively charged phosphates could interact with the solvent and thereby promote hydrophobic clustering of the neighboring aromatic residues [210]. A similar role was suggested for the multiple tyrosine residues of the EFP-ADs, which are essential for phosphorylation-dependent binding of RNA polymerase II [237]. The tyrosine residues were hypothesized to form fuzzy polycation–π interactions with positively charged partner proteins [238]. Ets-1 is also phosphorylated in its N-terminal IDR, which increases its affinity for CBP [239]. The modifications may cause a shift in the conformational landscape towards substrates with high CBP affinities, because the phosphate charges disrupt salt bridges, resulting in exposure of hydrophobic residues of Ets-1 [240].

The above examples illustrate how phosphorylation can influence TF-IDRs and thereby whole organisms. Most studied examples are from human systems. However, TF-IDRs may also affect plants and food quality. Thus, changes in the predicted phosphorylation sites of the IDR of *Arabidopsis* WRINKLED1 affect its stability and enhances oil accumulation, suggesting that design of this IDR has an applicative potential [241].

Biochemical Journal (2017) **474** 2509–2532
DOI: 10.1042/BCJ20160631

## Competition for binding?

For several of the described systems, a hub protein interacts with numerous TFs (Figure 2C,D), making *in vivo* competition scenarios modulated by overlapping spatiotemporal expression likely. Microarray analyses and focused TF studies revealed similar expression changes in several *NAC* genes and *DREB2A* from the RCD1 interactome (Figure 2D) in response to different stress exposures [27,242–244]. Thus, important TFs of the RCD1 interactome are involved in stress-related signaling and senescence similarly to RCD1 [23], suggestive of regulatory interactions between these proteins. Interestingly, although these TFs use the same SLiM for binding (Figure 4A), their complexes with RCD1 differ (Figure 5F) [28]. Future studies will show whether these TFs compete for or allosterically affect binding of each other to RCD1, as in the case of the CBP-interacting TFs [109,201,202].

As a central node in eukaryotic transcriptional regulatory networks, CBP interacts with more than 103 proteins (Figure 2C), which probably compete for binding to CBP in the cell (Figure 8B). For example, the ADs of STAT1, C/EBP and p53 bind the same surface of TAZ2 [108,245,246]. The high-affinity and phosphorylation-dependent interaction of full-length p53-AD with the two sub-sites on TAZ2 (Figure 4C) may help p53 compete for binding [107]. Also, of relevance to *in vivo* competition, the viral oncoproteins E1A and E7 also bind to the same surface on TAZ2, but with higher affinity than the TF-ADs [22,40,247,248], thus outcompeting their binding to CBP and thereby inhibiting their activity. How *in vivo* concentrations and localization by, e.g., scaffolding are modulators of interactome selection is currently not known.

## Drugs and interference with binding

IDRs are commonly involved in disease [7,249–251]. However, due to their broad ensemble distributions and dynamics, they are highly underrepresented as drug targets [122]. Thus, the development of therapeutics that target or affect IDRs is still in its infancy despite successful proof-of-concept studies [250,252], making this a promising field.

MDM2 and MDMX inhibit the tumor suppressor function in many human tumors expressing a wild-type p53 gene. Reactivation of p53 by inhibition of its interaction with MDM2 and MDMX is therefore an important therapeutic strategy in oncology [253,254]. Nutlin [255], a small-molecule inhibitor of the MDM2–p53 interaction, is a promising anticancer lead candidate [256]. MD simulations suggested that the initial interaction of p53 with MDM2 is long-lived and takes place at the N- and C-termini of MDM2, away from the binding site, suggestive of an allosteric mechanism. In contrast, nutlin interacts with MDM2 directly at the binding site [257]. Other drugs have been proposed to function by expanding the conformational space of the target IDR, resulting in a corresponding increase in entropy (Figure 4E) [122]. This proposal was based on simulation studies, suggesting that the interaction between the c-Myc-IDR and the small-molecule inhibitor 10058-F4 results in an increased number of lowly populated states of the c-Myc peptide [258].

Current modulators of NRs, which mainly target the ligand-binding domain, suffer from lack of selectivity and the development of resistance [259], making the development of modulators targeting the NR-NTDs an obvious goal [260]. Indeed, the NR-NTDs are emerging as suitable direct or allosterically modulated drug targets for certain cancers [260–262]. The small-molecule inhibitor EPI-001 alters the conformational ensemble of the AR-AF1, thereby inhibiting both its interactions with specific co-regulators and the allosteric N:C interaction of AR [114,263]. EPI-001 targets the AR-AF1 subregion Tau5 that is important for prostate cells to proliferate in the absence of androgens—a distinctive feature of castration-resistant prostate cancer [264]. Since Tau5 is partially folded, conformational selection (Figure 5C) in EPI-001-binding is possible, although the mechanism is not yet known. Thus, the possibility of therapeutically targeting the intrinsically disordered NR-NTD surfaces and TFs in general leaves a huge unexplored area.

## Conclusion and future work

We have here reviewed how ID contributes to the molecular function of eukaryotic TFs. SLiMs are emerging as important to the function of intrinsically disordered TF-ADs, and they have hydrophobic and acidic residues which are essential for TF:co-activator interactions. TFs must compete for co-activator binding in their large regulatory interactomes *in vivo*, and such scenarios are regulated by post-translational modifications, such as phosphorylation, and by alternative splicing. Both events are frequent in IDRs due to their flexibility, conformational plasticity, and low degree of sequence conservation. Despite low sequence similarities between TF-IDRs, their disorder–order pattern may be conserved, making analysis of evolutionary relationships based on

Biochemical Journal (2017) **474** 2509–2532
DOI: 10.1042/BCJ20160631

disorder–order patterns a future goal. Classic biochemical concepts are revised with the appreciation of ID, and studies of TF-ADs have contributed significantly to the understanding of the mechanisms of coupled folding and binding, which can involve a mixture of conformational selection and induced fit. For TF-IDRs, redistribution of conformational ensembles and energetic coupling is important for allosteric regulation, and a promising future goal may be the development of drugs directed toward TF-IDRs to control TF function by regulating their degree of disorder. The properties of TF-IDRs are probably modulated by their intracellular environment, and quantitative data of in-cell coupled binding and folding are the next experimental challenge. Thus, experiments in living cells, involving entire biological systems, represent an important road ahead.

### Abbreviations

AD, activation domain; AR, androgen receptor; ATM, ataxia-telangiectasia-mutated; CBP, CREB-binding protein; CD, circular dichroism; CK, casein kinase; CREB, cAMP response element-binding protein; CRTC, CREB-regulated transcription co-activator; DBD, DNA-binding domain; DREB2A, dehydration-responsive element-binding protein; EFP, Erwing sarcoma fusion protein; ER, estrogen receptor; GR, glucocorticoid receptor; H/MDM2, human/mouse double minute protein 2; ID, intrinsic disorder; IDP/R, intrinsically disordered protein/region; KID, kinase-inducible activation domain; MD, molecular dynamics; MLL, mixed-lineage leukemia; MoRF, molecular recognition feature; NAC, no apical meristem, *Arabidopsis* transcription activation factor, cup-shaped cotyledon; NCBD, nuclear receptor co-activator-binding domain; NMR, nuclear magnetic resonance; NR, nuclear receptor; NTD, N-terminal domain; PH, pleckstrin homology; pKID, phosphorylated KID; PR, progesterone receptor; PreSMos, pre-structured motif; RCD1, radical-induced cell death1; SAXS, small-angle X-ray scattering; SLiM, short linear motif; TAZ, transcriptional adapter zinc finger; TF, transcription factor.

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### Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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