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Review

AI-driven prediction of 14-3-3–intrinsically disordered protein interactions: From SLiM discovery to structural modeling of dynamic binding interfaces

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Abstract: Artificial intelligence (AI) has revolutionized structural biology by enabling the prediction of protein structures, biomolecular complexes, and interaction interfaces with unprecedented accuracy. The field has evolved from sequence motif searches and conventional machine learning algorithms to deep learning architectures and protein models capable of learning structural and functional representations directly from protein sequences. Recent advances, including protein language models (PLMs), AlphaFold2/3, ESMFold, and Boltz-2, have transformed the prediction of protein–protein interactions by integrating sequence, evolutionary, and structural information while reducing reliance on experimental structural data. These methods have expanded the ability to identify binding interfaces, characterize biomolecular complexes, and investigate interactions involving intrinsically disordered proteins and short linear motifs (SLiMs), although accurately modeling dynamic and context-dependent interactions remains a significant challenge. In this review, we summarize the evolution of AI-driven approaches for predicting protein–protein interactions, from classical machine learning to modern foundation models, highlighting their strengths, limitations, and emerging applications. The 14-3-3 protein family serves as a representative example to illustrate how AI has progressively improved the identification of phosphorylation-dependent binding motifs and interaction partners. Finally, we discuss future perspectives for integrating protein language models, structural prediction, and experimental data to achieve a more comprehensive understanding of protein interaction networks and to accelerate the discovery of regulatory mechanisms and therapeutic targets.

Keywords: artificial intelligence; protein binding prediction; 14-3-3 proteins; intrinsically disordered proteins

1. Introduction

Intrinsically disordered protein or regions (IDP/IDR) make up approximately 40% of the eukaryotic proteome [1], playing major roles in processes such as cell signaling and metabolic control. Unlike globular proteins, IDP/IDRs lack a stable tertiary structure in time, which gives them a unique interaction versatility [2]. These proteins or protein regions engage with partners through an ensemble of conformations, which usually contain short linear motifs (SLiMs) and molecular recognition features (MoRFs), which often undergo disorder-to-order transitions upon binding [3]. Furthermore, some IDRs form "fuzzy" complexes, where the region maintains high structural dynamism and heterogeneity even in the bound state.

The flexibility of IDP/IDRs has historically made their experimental structural characterization a big challenge [4]. However, the rapid progress of artificial intelligence, particularly AlphaFold3 [5], ESMFold [6] and Boltz-2 [7] (see Table 1 for details), has revolutionized structural biology by predicting protein complexes with accuracy previously reserved for experimental methods. While these models were primarily trained on independently folded domains, recent research has explored whether they can capture the complexity of IDR-mediated interactions.

Recent articles have benchmarked AlphaFold-Multimer predictions from many bound IDR structures with a success rate higher than 76% across different datasets [8]. Beyond just generating a structure, the model's intrinsic confidence scores, such as the combined score, predicted aligned error (PAE), and residue-ipTM (ripTM), have proven highly effective at distinguishing true biological interactions from decoys (candidate model that approximates the native structure but is not necessarily correct) and identifying regions of high structural heterogeneity. Despite these successes, the quality of AlphaFold-Multimer's prediction declines for interfaces with low hydrophobicity and high coil content, which are hallmark features of transient and multimodal interactions where one of the interactors is IDP/IDR.

As the field moves toward proteome-scale interaction discovery, identifying functional IDP/IDRs within full-length proteins presents a new challenge. To address this, the introduction of the "minD" metric, derived from AlphaFold-Multimer's distogram, offers a powerful tool to address potential interaction sites within large sequences, reducing the computational costs and false-positive discovery rates associated with traditional fragment-based scanning [8].

Beltrao et al. demonstrated that AlphaFold2 (AF2) extends beyond monomeric protein structure prediction by accurately modeling protein complexes and identifying biologically relevant protein–protein interfaces [9]. Unlike conventional docking approaches, AF2 simultaneously predicts protein folding and intermolecular interactions directly from sequence information, performing better than classical docking methods in the prediction of heterodimers. The study also showed that AF2 predicts correctly oligomeric complexes and can model many interactions involving IDRs, particularly those mediated by short linear motifs (SLiMs). However, performance decreases for highly flexible or multivalent IDRs and symmetric complexes. Importantly, the authors emphasized that confidence metrics such as predicted aligned error (PAE) provide valuable information for assessing interface reliability and distinguishing well-defined interactions from flexible or disordered regions. Overall,

AF2 represents a major advance for studying protein–protein interactions by enabling accurate prediction of binding interfaces directly from sequence, thereby expanding opportunities for structural interactomics and the characterization of dynamic protein complexes.

Table 1. Comparison of AlphaFold2, ESMFold, and Boltz-2, illustrating their capabilities of for modeling biomolecular complexes.

Feature	AlphaFold2	ESMFold	Boltz-2
Year	2021	2023	2025
Developer	DeepMind	Meta AI	MIT/Recursion
Input	Sequence + MSA + templates (optional)	Sequence only	Sequence ± ligands ± DNA/RNA ± post-translational modifications
MSA required	Yes	No	No
Architecture	Evoformer + Structure Module	Protein Language Model (ESM-2) + Folding Head	Multimodal transformer for biomolecules
Speed	Moderate	Very high	High
Accuracy	Very high for globular proteins	Good, lower than AF2	State-of-the-art for biomolecular complexes
Complex prediction	AlphaFold2-Multimer	Limited	Yes
Small-molecule ligands	No (requires external tools)	No	Yes
DNA/RNA	Limited	No	Yes
Post-translational	Limited	No	Yes
Main applications	Individual proteins and protein complexes	Rapid prediction of large proteomes	Protein–ligand complexes and AI-assisted molecular design

More recently, the emergence of next-generation AI-based structure prediction methods has further expanded the capabilities to obtain protein–protein structures. Unlike AF2, which relies heavily on multiple sequence alignments (MSAs), ESMFold employs a large protein language model to predict structures directly from amino acid sequences, enabling rapid proteome-scale analyses while maintaining competitive accuracy [6]. In 2025, Boltz-2 extended AI-driven modeling beyond protein structure prediction to include protein–ligand, protein–nucleic acid, and other biomolecular complexes, providing a unified framework for modeling diverse biological complexes [7]. All together, these advances broaden the applicability of AI to the study of IDP/IDRs, offering new opportunities to investigate their interactions, functional mechanisms, and potential pharmacological targeting at unprecedented scale. Using Boltz-2, researchers generated a structural atlas of the human interactome by modeling thousands of experimentally supported binary protein–protein interactions from the IntAct database (https://www.ebi.ac.uk/intact/) [10]. The predicted complexes were evaluated using Boltz-2 confidence metrics to identify reliable binding interfaces and integrated with protein domain

annotations to infer interaction mechanisms. This approach enabled the reconstruction of functional interaction networks, including cancer-associated pathways centered on proteins such as p53, which is ~ 45% disordered, and its cognate partner MDM2, demonstrating that Boltz-2 can provide structural insights into protein–protein interfaces at proteome scale. These findings highlight the potential of Boltz-2 as a powerful tool for structural interactomics, enabling the characterization of binding interfaces and facilitating the interpretation of biological and disease-related protein interaction networks. Table 1 summarizes a comparison between AI tools for modeling biomolecular complexes.

2. Biological and structural foundations of the 14-3-3 family

The 14-3-3 proteins constitute a family of highly conserved and ubiquitously expressed cellular regulators in eukaryotes [11]. The name 14-3-3 has a historical origin and derives from the procedure used for their initial isolation from bovine brain homogenates [12]. Although, in the early literature, members of the 14-3-3 family were frequently referred to as isoforms [13], they are currently regarded as paralogs, since they represent products of distinct genes arising from gene duplication events rather than protein variants derived from a single gene [14]. The monomeric unit of 14-3-3 proteins consists of nine antiparallel α-helices (H1–H9) [15]. From a functional perspective, these proteins form stable homo- or heterodimeric complexes in vitro [16]. The dimeric structure of 14-3-3 proteins is characterized by the presence of a wide and deep central channel, approximately 40 Å in width, flanked by two amphipathic grooves [17]. Binding within these amphipathic grooves occurs, in most cases, with high affinity toward client proteins containing phosphorylated serine or threonine motifs [18]. However, it has also been demonstrated that 14-3-3 proteins can recognize and interact with a few non-phosphorylated target proteins, thereby expanding the repertoire of their functional interactions [19].

Collectively, these mechanisms enable 14-3-3 paralogs to regulate multiple functional aspects of thousands of target proteins, contributing decisively to the organization and dynamics of the eukaryotic interactome [20,21]. The recognition of 14-3-3 proteins as potential therapeutic targets largely depends on a comprehensive characterization of their interactome, as the specificity and functional consequences of their interactions determine both their involvement in physiological processes and in a wide range of pathological conditions. This has driven growing interest in systematically identifying their client proteins and the motifs responsible for these interactions [22].

3. Intrinsically disordered proteins (IDPs) and short linear motifs (SLiMs)

IDPs and IDRs exist as dynamic ensembles of conformations, a property that confers high functional plasticity and enables them to participate in multiple cellular signaling and regulatory processes [2,23]. Within intrinsically disordered regions, the most frequent functional modules correspond to short linear motifs (SLiMs), short amino acid sequences that act as recognition sites for numerous regulatory proteins [24,25]. The 14-3-3 proteins represent a paradigmatic example of this recognition mechanism, binding phosphorylated SLiMs present in their client proteins and regulating multiple cellular processes [26]. Most of these recognition motifs are located within intrinsically disordered regions, where the absence of a stable three-dimensional structure promotes dynamic interactions regulated by post-translational modifications [25]. The high conformational plasticity of IDP/IDRs enables them to establish broader and more dynamic interaction networks than globular

proteins, thereby favoring more complex molecular regulatory mechanisms [27]. Taken together, the dynamic nature of intrinsically disordered regions and the transient character of SLiM-mediated interactions make their identification very challenging using only conventional experimental approaches [28]. In this context, the systematic identification of SLiMs is essential for understanding regulatory networks mediated by protein–protein interactions [25]; in particular, the characterization of 14-3-3 ligands is fundamental for reconstructing their interactome and facilitating the development of therapeutic strategies targeting these interactions [29]. However, the transient, context-dependent, and often low-affinity nature of these interactions complicates their experimental characterization, driving the development of computational strategies for their prediction and prioritization [28].

Short linear motifs (SLiMs) are typically 3–10 amino acids in length. Their biological role is frequently regulated by post-translational modifications (PTMs), particularly phosphorylation on serine or threonine residues, which can create, disrupt, or modify binding motifs [27]. Correa Marrero et al. performed a large-scale structural comparison of phosphorylated and non-phosphorylated proteins using experimentally determined structures from the Protein Data Bank. Their analysis of 225 proteins and 347 phosphosites showed that phosphorylation generally induces modest conformational changes while predominantly stabilizing preexisting structural states. However, the low numbers of IDP/IDRs in the PDB limits such analyses and further highlights the need for next-generation AI methods capable of modeling post-translational modifications and dynamic protein conformations [30].

4. Evolution of computational tools and deep learning

AI-based approaches for the prediction of 14-3-3/IDP/IDR interaction can be broadly organized into two complementary steps: interaction-site identification and structural resolution of the resulting complexes. These steps address different questions and should not be regarded as competing solutions to the same prediction problem. Sequence-based methods, including SLiM-oriented predictors, are primarily designed to identify candidate short linear motifs and potential interaction regions from sequence information. Their principal value is therefore in the discovery and prioritization of candidate interactions. In contrast, structure-based approaches aim to determine the three-dimensional organization of a protein–protein complex, including the relative orientation of the interacting partners and the molecular contacts that may stabilize the interface.

This distinction is particularly relevant for 14-3-3/IDP/IDR interactions, in which motif recognition and structural organization represent interconnected but experimentally and computationally distinct problems. A practical AI-assisted workflow can therefore combine these approaches sequentially: candidate motifs or interaction regions can first be identified from sequence-level information and subsequently subjected to structure-based modeling to generate and evaluate possible complex architectures. Structural predictions can then be assessed against biochemical, biophysical, or mutational data, providing an iterative framework in which sequence-based discovery and structural modeling inform one another. Thus, rather than selecting a single universally superior predictor, the appropriate method depends on the stage of the interaction-discovery pipeline and the biological question being addressed.

Conventional protein–protein docking methods show limited performance in the study of IDP/IDRs, as they rely on well-defined initial structures and have difficulties in modeling the conformational heterogeneity, transient interactions, and dynamic binding interfaces that characterize these proteins [31]. Because many IDPs/IDRs undergo disorder-to-order transitions upon binding,

traditional docking approaches often fail to accurately capture the conformational ensembles and alternative conformations involved in molecular recognition. To overcome these limitations, early computational approaches focused on identifying short linear motifs (SLiMs) and phosphorylation-dependent interaction sites directly from protein sequences. These sequence-based methods provided a valuable framework for identifying potential interaction regions in disordered proteins, where conventional structure-based approaches are often limited by the absence of a well-defined three-dimensional structure. However, their predictive power was constrained by the short, degenerate, and context-dependent nature of SLiMs, as well as by the dynamic conformational ensembles of IDPs/IDRs.

Initial bioinformatic approaches developed to identify 14-3-3 binding sites lay primarily on position-specific scoring matrices (PSSMs) and support vector machines (SVMs), exploiting predefined sequence features such as amino acid composition, positional residue frequencies, motif conservation, and evolutionary information surrounding phosphorylated serine and threonine residues [32]. These methods represented an important improvement over consensus motif searches by incorporating statistical learning algorithms capable of identifying non-obvious sequence patterns associated with 14-3-3 recognition.

Subsequent generations of machine learning algorithms ameliorate predictive performance by integrating multiple sources of information, including evolutionary conservation, amino acid physicochemical properties, structural propensity, disorder predictions, and functional annotations [33]. Before the emergence of deep learning, random forests, gradient boosting, and other ensemble classifiers became widely used for predicting post-translational modification sites and protein–protein interactions. By combining multiple decision trees or machine learning models, these approaches achieved higher sensitivity and specificity than earlier motif-based methods, demonstrating the value of integrating heterogeneous biological features. However, their reliance on manually engineered descriptors limited their ability to capture complex structural and contextual relationships and to generalize to novel interaction patterns, motivating the development of deep learning–based approaches.

The recent emergence of deep learning has fundamentally transformed computational structural biology. Instead of relying on handcrafted features, deep neural networks learn hierarchical representations directly from protein sequences, capturing what in our opinion is the most fundamental feature in AI methods for protein modeling: understanding the importance of the long-range dependencies and complex contextual relationships between amino acids. Convolutional neural networks, recurrent neural networks, attention mechanisms, and, more recently, Transformer [34] architectures have dramatically improved the prediction of SLiM-mediated protein–protein interactions. Deep learning frameworks based on ensemble models can accurately identify phosphorylated 14-3-3 binding sites from short peptide sequences by automatically extracting informative sequence features without requiring explicit structural information.

These developments have culminated in the appearance of protein foundation models trained on hundreds of millions of protein sequences. Protein language models (PLMs), such as ESM, and similar others like ProtT5, and ProGen, learn evolutionary and structural representations from sequence alone, enabling accurate predictions of protein properties without the need for multiple sequence alignments. In parallel, structure prediction systems such as AlphaFold3 [5], RoseTTAFold [35], ESMFold [6], and, more recently, Boltz-2 [7] have revolutionized structural biology by providing highly accurate models of proteins and biomolecular complexes. Together, these advances have shifted the field from

manually engineered, feature-based prediction methods toward end-to-end deep learning approaches capable of integrating sequence, structure, and interaction information, opening new opportunities for investigating the molecular mechanisms governing IDP/IDR function and interactions. An important advance in the evaluation of AlphaFold-Multimer for IDR-mediated protein interactions was reported by Omidi et al. (2024) [8], who investigated whether the model could capture the structural heterogeneity characteristic of fuzzy complexes. Although AlphaFold-Multimer accurately predicts the overall architecture of many IDR-containing complexes, its performance decreases for highly heterogeneous interfaces. More importantly, the authors demonstrated that the model's intrinsic confidence metrics, including PAE, pLDDT and ripTM, correlate with experimentally observed structural heterogeneity. These findings indicate that AlphaFold-Multimer confidence scores provide information beyond prediction accuracy, enabling the identification of IDR segments that participate in structurally heterogeneous (*fuzzy*) interactions (Figure 1).

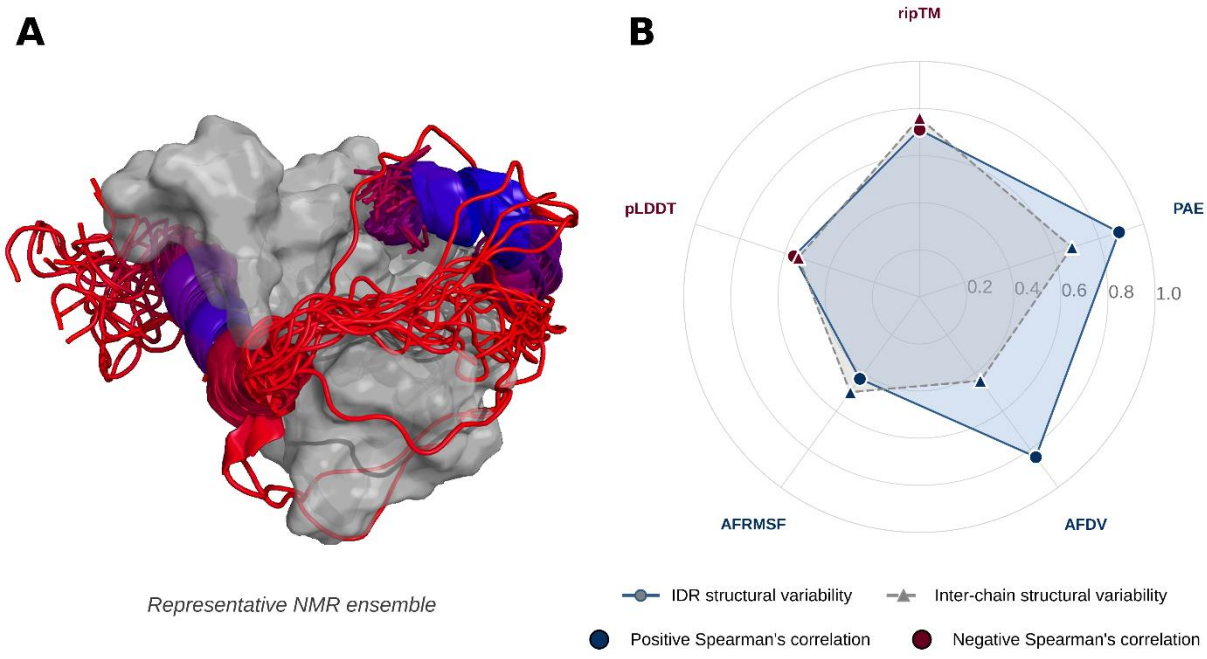

Figure 1. AlphaFold-Multimer confidence metrics reflect structural heterogeneity in IDR-mediated protein interactions. (A) Schematic representation of a *fuzzy complex* based on an experimental NMR conformational ensemble, showing the multiple conformations adopted by an intrinsically disordered region (red) while interacting with a folded receptor (gray). (B) Radar plot summarizing the Spearman correlation coefficients reported by Omidi et al. for AlphaFold-Multimer confidence metrics (PAE, pLDDT, and ripTM) and AlphaFold-derived descriptors of structural variability (AFDV and AFRMSF), evaluated in two structural contexts. Together, these results demonstrate that AlphaFold-Multimer confidence scores capture experimentally observed conformational heterogeneity, enabling the identification of flexible regions within IDR-mediated interfaces. Adapted and simplified from [8].

Quantitative benchmarks illustrate important differences among current AI-based structure prediction frameworks. AlphaFold-Multimer achieved medium or higher interface accuracy (DockQ

≥ 0.49, a composite metric for assessing the accuracy of predicted protein–protein complex structures) for 13 of 17 heterodimeric targets and high accuracy (DockQ ≥ 0.80) for 7 of 17 targets in an early template-free benchmark. In a larger set of 4446 recent protein complexes, approximately 70% of heteromeric interfaces reached DockQ ≥ 0.23, whereas only 26% reached the high-quality threshold of DockQ ≥ 0.80. Importantly, subsequent evaluation on homology-reduced datasets reported overall success rates of approximately 40%–60%, depending on oligomeric state, illustrating the sensitivity of apparent performance to benchmark composition and sequence redundancy [36,37]. These results emphasize why leakage-controlled evaluation is particularly important when assessing complex prediction.

ESMFold provides a useful quantitative contrast because it predicts structures from a single sequence without explicitly generating a multiple-sequence alignment. In a recent leakage-controlled benchmark of 1337 proteins deposited after the relevant training period, ESMFold achieved a median TM score of 0.95, compared with 0.96 for AlphaFold2, while median RMSD values were 1.74 and 1.30 Å, respectively [8]. In CASP14, ESMFold-like single-sequence prediction achieved a mean LDDT of approximately 0.68 compared with approximately 0.85 for AlphaFold2, while CAMEO benchmarks yielded mean TM scores of approximately 0.90, 0.79, and 0.45 for easy, medium, and hard targets, respectively. These results indicate that removal of explicit MSA generation does not preclude accurate prediction of many folded proteins, although performance remains dependent on protein class and structural complexity [38].

For intrinsically disordered regions, however, global complex metrics must be interpreted cautiously. A dedicated evaluation of AlphaFold-Multimer on complexes containing intrinsically disordered regions reported correct complex predictions for more than 76% of the tested samples, but with substantial variation in structural quality. Thus, a model may correctly identify the overall association between a folded receptor and an IDR while still failing to reproduce the conformational heterogeneity of the disordered region or the precise ensemble of binding configurations. This distinction is particularly relevant to 14-3-3/IDP interactions, where recognition can be dominated by a short phosphorylated motif, while the remainder of the ligand remains conformationally heterogeneous [39].

Boltz-2 should be interpreted somewhat differently. Its diffusion-based generative framework allows multiple structural hypotheses to be sampled, but the number of generated structures should not be equated with the number of experimentally populated conformational states. Current structural benchmarks demonstrate that Boltz-2 can produce medium-quality protein–protein interfaces, but direct experimentally resolved post-training comparisons remain limited. For example, one recent interactome-scale evaluation identified only three suitable post-cutoff binary complexes, for which Boltz-2 achieved DockQ values of 0.755, 0.348, and 0.210. These results illustrate both its potential and the current limitations of quantitative generalization claims. Consequently, for 14-3-3/IDP systems, AF2-Multimer, ESMFold, and Boltz-2 should be regarded primarily as complementary generators of structural hypotheses rather than as methods that directly resolve the equilibrium conformational ensemble of an intrinsically disordered ligand [40].

Table 2. Benchmark performance of AI-based methods for protein structure and protein–protein interaction prediction.

Model	Task	Benchmark	Metrics	Results
AF2-Multimer	Complex structure	17 heterodimers	DockQ ≥ 0.49	13/17 ≥ medium
AF2-Multimer	Complex structure	17 heterodimers	DockQ ≥ 0.80	7/17 high-quality
AF2-Multimer	Complex structure	4446 complexes	DockQ ≥ 0.23	70% of interfaces
AF2-Multimer	Complex structure	4446 complexes	DockQ ≥ 0.80	26% of interfaces
AF2-Multimer	Complex structure	Homology-reduced benchmark, 2–6 chains	DockQ > 0.23	~ 40–60%
ESMFold	Monomer structure	CASP14	Mean LDDT	~ 0.68
AF2	Monomer structure	CASP14	Mean LDDT	~ 0.85
ESMFold	Monomer structure	CAMEO, easy	TM score	~ 0.90
ESMFold	Monomer structure	CAMEO, medium	TM score	~ 0.79
ESMFold	Monomer structure	CAMEO, hard	TM score	~ 0.45
Boltz-2	Complex structure	2315 recent protein complexes	Structural accuracy/DockQ	Competitive with AF3
Boltz-2	Binding affinity	CASP16 affinity challenge	Pearson's *r*	Best-performing method
Boltz-2	Binding affinity	OpenFE / FEP benchmark	Pearson's *r*	~ 0.66
Boltz-2	Virtual screening	MF-PCBA	AUPRC/enrichment	Competitive with specialized methods

4.1. Sequence-based and short linear motif prediction methods

Sequence-based approaches provide a first layer for identifying candidate 14-3-3-binding sites before structural modeling or experimental validation. Classical motif-based methods such as Scansite represent experimentally determined peptide-binding preferences as position-specific scoring matrices (PSSMs), derived from oriented peptide-library and phage-display experiments. The input is therefore primarily a local amino-acid sequence window, and candidate sites are ranked according to the residue preference at each position relative to the central phosphosite. Scansite 2.0 incorporated PSSMs for

multiple modular signaling domains, including 14-3-3, and was designed for proteome-wide scanning of protein sequences. The Eukaryotic Linear Motif (ELM) resource follows a related motif-centric strategy but uses manually curated, experimentally validated SLiM instances rather than a single universal classifier. Its prediction pipeline combines regular expression–like motif definitions with contextual filters, including evolutionary conservation, cellular localization, and structural/disorder information, to distinguish potentially functional motifs from random sequence matches. The ELM database is therefore both a training/knowledge resource and a motif annotation framework rather than a conventional machine-learning predictor. These approaches are computationally inexpensive and interpretable, but their dependence on short consensus patterns can result in substantial false-positive rates because SLiMs are short, degenerate, and frequently occur by chance in proteomes [41−43].

More recent methods have incorporated supervised machine learning and deep learning to capture sequence features that are not adequately represented by simple consensus motifs. SLiMPred, for example, uses machine-learning models trained on experimentally annotated SLiM instances from the ELM database and integrates sequence-derived structural, biophysical, and biochemical features to identify potential motifs directly from primary protein sequences, including motifs located within intrinsically disordered regions. For 14-3-3-specific prediction, 14-3-3-Pred compared position-specific scoring matrices, support-vector machines, and artificial neural networks trained using experimentally characterized 14-3-3-binding phosphopeptides and non-binding phosphopeptides. The predictors used peptide sequence windows centered on the phosphosite, with the optimal window extending from −6 to +4, and achieved Matthews correlation coefficients of up to 0.60. More recently, the 14-3-3 site-finder incorporated machine-learning features beyond the local consensus sequence, including intrinsic disorder and phosphosite detection frequency from proteomic datasets, thereby improving recognition of non-canonical 14-3-3 docking sites. A further deep-learning framework, trained specifically for 14-3-3 binding-site prediction, reported 75% balanced accuracy on external sequences and experimentally confirmed five of eight top-ranked peptide predictions, with measured binding affinities ranging from approximately 1.6 to 70 μM. Together, these developments illustrate a transition from fixed motif representations toward models that integrate sequence context, disorder, post-translational modification information, and learned nonlinear sequence features [32,44−46].

5. Specific AI tools for 14-3-3/IDP/IDR predictions

Recent advances in deep learning have significantly improved the prediction of 14-3-3 protein-mediated interactions by moving beyond conventional motif-based approaches and manually engineered features. A recent study developed an ensemble deep learning framework trained on experimentally validated phosphopeptides to predict novel 14-3-3 binding sites directly from sequence information. By combining different encoding strategies and neural network architectures, including convolutional and recurrent models, this approach achieved improved predictive performance over previous state-of-the-art tools, demonstrating that sequence-derived representations are enough to capture critical determinants of phospho-dependent 14-3-3 recognition. Particularly, the study by Madeira, F. et al. (2015) highlighted the importance of residues flanking the phosphorylated serine or threonine, especially at positions −3 and +2, in agreement with the canonical consensus motifs previously described for 14-3-3 binding [32].

Importantly, this computational framework was validated experimentally through biophysical and structural analyses. Several high-confidence candidate phosphopeptides identified by the model were

shown to bind 14-3-3 proteins with micromolar affinities, and crystallographic studies confirmed binding conformations consistent with previously characterized complexes. These findings demonstrate the capacity of deep learning models not only to improve ligand discovery but also to expand the known 14-3-3 interactome. More broadly, this work illustrates how integrating sequence-based artificial intelligence approaches with structural and biochemical validation can be a powerful strategy for uncovering novel regulatory interactions, particularly in intrinsically disordered regions where conventional structural approaches remain very limited.

A recent study by van Weesep et al. (2025) developed a deep learning–based framework for the prediction of phosphopeptide-mediated interactions with 14-3-3 proteins, addressing the persistent challenge of identifying novel client binding sites at proteome scale [46]. The authors trained and benchmarked multiple neural network architectures, including convolutional neural networks (CNNs), multilayer perceptrons (MLPs), and gated recurrent units (GRUs), using curated datasets of experimentally validated phosphopeptides. Their optimized ensemble model achieved high predictive performance and enabled large-scale prospective screening of candidate 14-3-3 binding motifs across disease-relevant proteins. This study combined computational prediction with experimental validation through fluorescence anisotropy assays, confirming several previously uncharacterized phospho-dependent 14-3-3 interactions with important proteins in relevant pathologies such as FOXO3, Tau, c-Myc, and BAD. Structural validation by X-ray crystallography further revealed canonical binding conformations within the amphipathic binding groove of 14-3-3, while molecular dynamics simulations provided additional insights into the contribution of flanking residues to binding stability. This integrative strategy significantly expands the known 14-3-3 interactome and provides a powerful framework for systematic discovery of phospho-regulated protein–protein interactions.

5.1. Structural and solution-based validation of AI-predicted complexes

High-confidence AI-predicted protein complexes should ideally be subjected to orthogonal experimental validation, particularly when the predicted interaction involves intrinsically disordered regions or flexible binding interfaces. Cryo-electron microscopy (cryo-EM), including time-resolved cryo-EM (TR-cryo-EM), can provide structural information for sufficiently stable and well-populated complexes and, in favorable cases, can resolve alternative conformational states. Time-resolved approaches further allow transient structural states to be captured following a defined perturbation, extending cryo-EM toward the characterization of molecular dynamics on millisecond-to-second timescales [47,48]. NMR spectroscopy provides a complementary approach that is particularly valuable for protein–IDP interactions because it interrogates proteins in solution and can characterize conformational ensembles and exchange processes at residue-level resolution. Chemical-shift perturbations, relaxation measurements, residual dipolar couplings, paramagnetic relaxation enhancement, and relaxation-dispersion experiments can identify interaction interfaces while simultaneously reporting on local and global conformational dynamics [49,50]. Importantly, NMR can also resolve coexisting cis and trans populations associated with proline peptide-bond isomerization and characterize their relative populations, including minor conformers that may be invisible to conventional static structural methods. For example, residue-specific NMR approaches have detected minor proline isomers representing approximately 4%–15% of the population in intrinsically disordered p53 transactivation-domain constructs and demonstrated changes in the cis/trans equilibrium following phosphorylation [51,52]. Such states cannot be represented adequately by a

single static structure generated by an AI prediction algorithm. This distinction is particularly relevant to 14-3-3/IDP complexes, where phosphorylation-dependent recognition, local disorder, and proline isomerization may influence binding kinetics and the distribution of bound conformations.

Single-molecule fluorescence and solution-phase biophysical approaches can provide a complementary bridge between static structural predictions and the dynamic behavior of the biological complex. Single-molecule Förster resonance energy transfer (smFRET) directly reports distance distributions and their temporal fluctuations between selected labeling sites, allowing predicted conformational states to be tested at the level of individual molecules and enabling transitions between conformational states to be characterized [50,53]. Broad or multimodal FRET distributions can therefore reveal structural heterogeneity that may be obscured by ensemble-averaged measurements or inadequately represented by a single AI-generated model. Hydrogen–deuterium exchange mass spectrometry (HDX-MS) provides a complementary ensemble-sensitive measurement of solvent accessibility and backbone protection, allowing regions predicted to form an interface or undergo conformational stabilization upon complex formation to be experimentally tested. HDX-MS can monitor structural fluctuations and conformational changes under solution conditions and has been applied to both folded protein complexes and intrinsically disordered proteins [54,55]. Other solution-based methods, including small-angle X-ray scattering (SAXS), fluorescence anisotropy, native mass spectrometry, and analytical ultracentrifugation, can provide complementary information on global dimensions, oligomeric state, binding stoichiometry, and conformational changes. Together, these approaches establish a validation continuum in which AI models provide structural hypotheses, cryo-EM and NMR test molecular architecture, and smFRET, HDX-MS, SAXS, and related solution methods determine whether the predicted structures are consistent with the dynamic ensemble and molecular behavior of the experimentally observed complex.

6. Challenge in the 14-3-3/IDP/IDR interface recognition; proline isomerization: an underexplored determinant

Among the conformational processes that remain difficult to capture with current AI-based approaches, peptidyl-prolyl cis/trans isomerization is particularly relevant to 14-3-3/SLiM recognition. L-Proline is structurally unique among the 20 canonical amino acids because its side chain forms a covalent bond with the backbone nitrogen, generating a five-membered pyrrolidine ring that restricts backbone conformational freedom [56]. Unlike most peptide bonds, which strongly favor the trans conformation, Xaa–Pro bonds have a relatively small free-energy difference between cis and trans states [57–59]. In intrinsically disordered proteins, both conformations can coexist and expand the conformational landscape available for molecular recognition [60]. However, the high activation barrier between them makes spontaneous cis/trans interconversion kinetically slow, allowing prolyl isomerization to act as a rate-limiting step in protein folding and as a regulator of protein–protein interactions [58,60,61].

Within the 14-3-3 family, a conserved proline at the +2 position relative to the phosphorylated serine or threonine is a characteristic feature of canonical binding motifs [18,58]. Structural studies indicate that this proline introduces a sharp bend in the peptide backbone, facilitating ligand positioning and exit from the amphipathic binding groove [59]. Importantly, recent work demonstrated that cis/trans isomerization of this conserved proline can substantially alter the binding affinity of

phosphorylated SLiMs for 14-3-3, identifying proline conformation as an underappreciated determinant of molecular recognition [56].

The cis/trans state of proline residues can be characterized by NMR through chemical-shift analysis of the pyrrolidine ring [47,53]. The physiological relevance of prolyl isomerization is further illustrated by peptidyl-prolyl cis/trans isomerases such as Pin1, which catalyze conformational switching in phosphorylated Ser/Thr–Pro motifs and regulate protein signaling [61]. Although Pin1 and 14-3-3 recognize distinct sequence motifs, this mechanism provides a paradigm for how proline isomerization can regulate phosphorylation-dependent molecular recognition. Whether the conserved +2 proline in canonical 14-3-3 motifs is subject to enzymatic catalysis or predominantly undergoes spontaneous thermal isomerization remains unknown.

This mechanism also highlights an important limitation of current protein foundation models. Although these models can predict protein–protein interfaces involving intrinsically disordered regions, they generally represent discrete structural states rather than dynamic conformational processes. Proline cis/trans isomerization is a clear example in which a single peptide-bond conformation can substantially alter binding affinity without changing the underlying amino acid sequence [56]. Incorporating explicit proline conformational states into future protein language models and structural prediction frameworks could therefore improve the prediction of SLiM-mediated interactions and contribute to more realistic representations of dynamic 14-3-3 interaction networks.

7. Limitations, data leakage, and training bias in protein AI models

An important limitation in evaluating AI-based protein prediction models is data leakage between training and test sets. In structure-prediction and protein–protein interaction benchmarks derived from the PDB, sequence or structural homology between training and evaluation complexes can lead to substantial overestimation of predictive performance. This is particularly problematic when highly similar proteins, homologous interaction partners, or related complexes are present in both sets, as models may exploit evolutionary or structural information that is effectively shared between training and test examples rather than demonstrating genuine generalization. Consequently, benchmark accuracy should be interpreted with caution when redundancy is not explicitly controlled at the sequence, protein-family, structural-fold, or interaction-complex level. This issue is especially relevant for 14-3-3/IDP prediction, where many experimentally characterized ligands and binding motifs are evolutionarily or structurally related, making conventional random train–test splits potentially optimistic.

A related concern is training bias, arising from the uneven representation of protein families and interaction types in available training corpora. Well-studied proteins and extensively characterized interaction motifs are more likely to be represented in structural databases and experimentally curated datasets than novel or weakly characterized systems. The overrepresentation of 14-3-3 proteins and their known ligands may therefore favor recognition of already-characterized binding patterns while limiting generalization to previously unseen SLiMs or atypical interaction modes. These limitations highlight the importance of leakage-resistant evaluation strategies, including homology-reduced or clustering-based cross-validation, protein family–level data splitting, and, where appropriate, temporal or time-split benchmarks in which models are evaluated on structures or interactions deposited after the training dataset was established. Such approaches provide a more stringent assessment of whether

AI models can identify genuinely novel 14-3-3/IDP interactions rather than reproducing patterns already represented in their training data.

8. Conclusions and future perspectives

AI-predicted protein interaction structures are increasingly valuable for generating mechanistic insights into protein function, complex organization, and disease mechanisms. By integrating structural predictions with functional annotation and disease-associated mutations, computational approaches can guide experimental studies and contribute to a broader understanding of the human interactome.

The computational study of 14-3-3-mediated interactions has evolved from consensus motifs and manually engineered features toward deep learning approaches capable of extracting complex sequence representations. These advances have improved the identification of canonical binding sites and expanded the discovery of potential ligands. Because 14-3-3 recognition depends strongly on phosphorylated serine or threonine residues, protein language models (PLMs) may further contribute to identifying functional motifs and prioritizing candidate ligands within the 14-3-3 interactome.

A major challenge for next-generation AI approaches is to integrate sequence, structural, and experimental information while explicitly accounting for conformational heterogeneity. PLMs capture sequence-level relationships but do not explicitly describe the conformational ensembles of intrinsically disordered proteins. Conversely, structure-prediction methods such as AlphaFold-Multimer and diffusion-based models can generate plausible three-dimensional complexes but provide limited descriptions of conformational heterogeneity and experimentally observable kinetic populations. Experimental approaches including NMR, HDX-MS, SAXS, and smFRET provide complementary information, but their observations differ substantially in dimensionality and physical meaning. Future frameworks should therefore combine such heterogeneous data rather than treating them as equivalent structural constraints.

One possible strategy is a hierarchical workflow in which PLMs identify candidate SLiMs and sequence determinants, structure-prediction models generate alternative complex architectures, and experimental data are incorporated during ensemble refinement. Rather than selecting a single predicted structure, such approaches could maintain multiple structural hypotheses and assign probabilities according to their agreement with independent experimental observations.

An additional challenge is the representation of conformational dynamics and uncertainty. In 14-3-3/IDP interactions, the biologically relevant state may comprise an ensemble of interconverting bound and partially bound conformations rather than a unique structure. Integrating molecular-dynamics simulations and Markov state models (MSMs) with AI-generated structures could provide a route toward identifying kinetically distinct states and connecting them with experimentally measurable observables such as NMR populations, smFRET distance distributions, HDX protection patterns, and SAXS profiles. Bayesian and maximum-entropy approaches could further enable ensemble reweighting while limiting overfitting.

Ultimately, future AI systems for 14-3-3/IDP interactions may evolve from static structure predictors into iterative hypothesis-generation and experimental-updating frameworks. Sequence models could identify interaction determinants, structural models could generate candidate states, MD/MSMs could characterize their dynamics, and experiments could test and refine these predictions. Such closed-loop approaches would be particularly valuable for 14-3-3 systems, where

phosphorylation, motif degeneracy, intrinsic disorder, and proline isomerization generate conformational landscapes that cannot be adequately represented by a single static structure.

Use of generative-AI tools declaration

The authors declare they have used Artificial Intelligence (AI) tools in the creation of this article.
AI tools used: ChatGPT (OpenAI).

How were the AI tools used? AI tools were used to assist with language editing, grammar correction. All scientific content was reviewed and validated by the authors, who take full responsibility for the final article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SM and DMB conceived and designed the article, prepared the first draft. IMG conceived and design the Figure 1. SM, IMG and DMB contributed to the revision and final approval of the manuscript.

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