Review

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Allosterism in Targeting p53 Tumor Suppressor for Improved Cancer Therapy – Can AI Solve the Puzzle?

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Abstract

Intrinsically disordered p53 is a promising yet difficult target for precision oncology. p53 undergoes conformational changes in response to binding to the protein partners, amino acid substitution, or posttranslational modifications. These shifts often provoke the long-chain fluctuations which underlie allosteric transformations associated with the regulation of p53 function. Allosteric shifts regulate p53 translation, protein stability, and transcription activity. The 2024 Nobel Prize in Chemistry recognises advancements in computational protein design and structure prediction through AI innovations. Yet, current AI-based tools still fall short in unravelling the complexity of allosteric regulation in p53, a knowledge which is scarce yet essential for turning p53 into a feasible therapeutic target. Here, we highlight the challenges and the avenues to decipher the structure of locally misfolded p53, a multipotent transcription factor and the most abundantly inhibited tumour suppressor in human cancers.

Keywords: Intrinsically disordered protein; p53; allosteric modulation; molecular dynamic simulation; AlphaFold; mutant p53; SAXS

Rethinking Protein Structure: Challenges in Understanding Protein Order and Disorder

The thermodynamic hypothesis, proposed by the Nobel Laureate in Chemistry 1972, Dr. Christian B. Anfinsen, asserts that a native protein's three-dimensional structure in its normal physiological environment corresponds to the state where the Gibbs free energy of the entire system is minimised. This implies that the complete array of interatomic interactions governs the native conformation, which is dictated by the amino acid sequence in a specific environment. The Swedish Royal Academy of Sciences granted the Prize for his ground-breaking 'studies on ribonuclease, in particular, the relationship between the amino acid sequence and the biologically active conformation' [1]. However, the intrinsically disordered proteins (IDPs) challenge the universality of this theory, posing a significant limitation in applying this theory to all proteins, especially those with context-dependent or dynamic structures.

IDPs are abundant in the human proteome and are characterized by the presence of extended regions that lack stable 3D structures. They adopt multiple conformations, often in proximity to their binding protein partners, which enables them to function as key regulatory elements in complex cellular processes such as signal transduction, cell cycle modulation, chromatin remodelling or gene transcription [2]. While Anfinsen's principle underpins much of our understanding of protein folding, it must be reconsidered in the context of IDPs, whose structural plasticity plays a key role in the plethora of cellular processes.

Due to the high flexibility of the regulatory regions, the current protein structure prediction tools fall short of delivering high-resolution structures, posing a major challenge for structure-based drug development.

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Thus, the efforts to predict protein structures have recently culminated in major advances in *in silico* protein design and the development of transformative AI-based tools like AlphaFold (AF) (**Box 1**).

In 2024, the Nobel Prize in Chemistry recognised these breakthroughs, awarding David Baker for computational protein design, and jointly honouring Demis Hassabis and John M. Jumper for their contributions to AI-driven protein structure prediction.

These technologies have already played a significant role in drug discovery by enabling accurate target protein modelling, *de novo* design of therapeutic peptides and *in silico* screening of large compound libraries.

However, a key limitation of the AF algorithm arises from its reliance on deep learning models, trained on the available dataset of experimentally solved structures in the Worldwide Protein Data Bank (**Box 1**).

BOX 1. Publicly available depositories and databases supporting structural analysis of biomolecules.

To unlock the structure of IDPs, combining the publicly available data from DisProt, Alphafold and SASBDB databases provides a comprehensive approach. DisProt provides a curated repository for IDPs, focusing on their structural and functional aspects (https://disprot.org). Alphafold generates highly accurate protein 3D structures' predictions from amino acid sequences (https://alphafold.ebi.ac.uk). The dataset of solved protein structures can be extracted from the Worldwide Protein Data Bank (https://www.wwpdb.org) and supports molecular replacement analysis. SASBDB (https://www.sasbdb.org) reveals protein dynamics in solution, closely representing its native state in the cell. Together, these resources provide valuable insights into IDP behaviour, helping refine structural predictions and improve our understanding of protein folding in biologically relevant environments.

As a result, the flexibility and dynamic behaviour of unfolded or partially disordered proteins remain beyond the predictive capabilities of current AI models. Yet, such flexibility proved to be essential for protein function. The protein universe is replete with multi-domain proteins composed of structured units connected by flexible linkers of varying lengths, the features that challenge experimental techniques such as X-ray crystallography and cryoEM, and consequently, the current AI models.

The Prize Laurate and colleagues tried to address that issue by establishing a computational design pipeline to develop specific and high-affinity binders for a wide range of disordered peptides or proteins [3].

Deep-learning-based methods typically predict a single, most stable protein conformation, but they often struggle with fold-switching proteins or homologs that adopt distinct folds.

The major drawbacks of predicting alternative structures include a strong dependence on available training datasets, potential misalignment of coevolutionary signals, and errors in aligning structures of training proteins with those of distinctly folded homologs. Also, recent studies report significant discrepancies in residue positioning when comparing AI-predictions to experimental structures for multi-domain proteins [4].

Thus, there is a need to validate and complement the AF predictions with experimental techniques.

Addressing the knowledge gaps in characterising the conformational ensembles of IDPs is crucial for advancing our understanding of protein folding and function to develop new therapeutic strategies for precision medicine.

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Intrinsically Disordered p53 and the Yet Undiscovered Phenomenon of Its Allosteric Modulation

In IDPs, conformational transitions to folded states often occur either through posttranslational modifications (PTMs) or upon binding to a target ligand or binding protein partner, thereby enhancing target specificity [5].

The most extensively studied IDP in the human proteome is the tumor suppressor p53, which contains approximately 40% of disordered regions (126 076 PubMed entries by July 27th 2025) (**Fig 1**).

p53 is a vital tumor suppressor frequently mutated or inactivated in many human cancers. It plays a key role in controlling cell fate under cellular stress like DNA damage, hypoxia or oncogene activation. It also has non-canonical roles in antioxidant response, invasion, and immune regulation. As a pleiotropic transcription factor, p53 regulates the expression of over 100 target genes [6]. It functions as a homotetramer featuring essential domains: an intrinsically disordered N-terminal transactivation domain (NTD), a proline-rich domain (PRD), a folded DNA-binding domain (DBD), a nuclear localisation signal (NLS), a tetramerisation domain (TET), and an unfolded C-terminal domain (CTD). Each domain is integral to the protein's functionality (**Fig. 1a**).

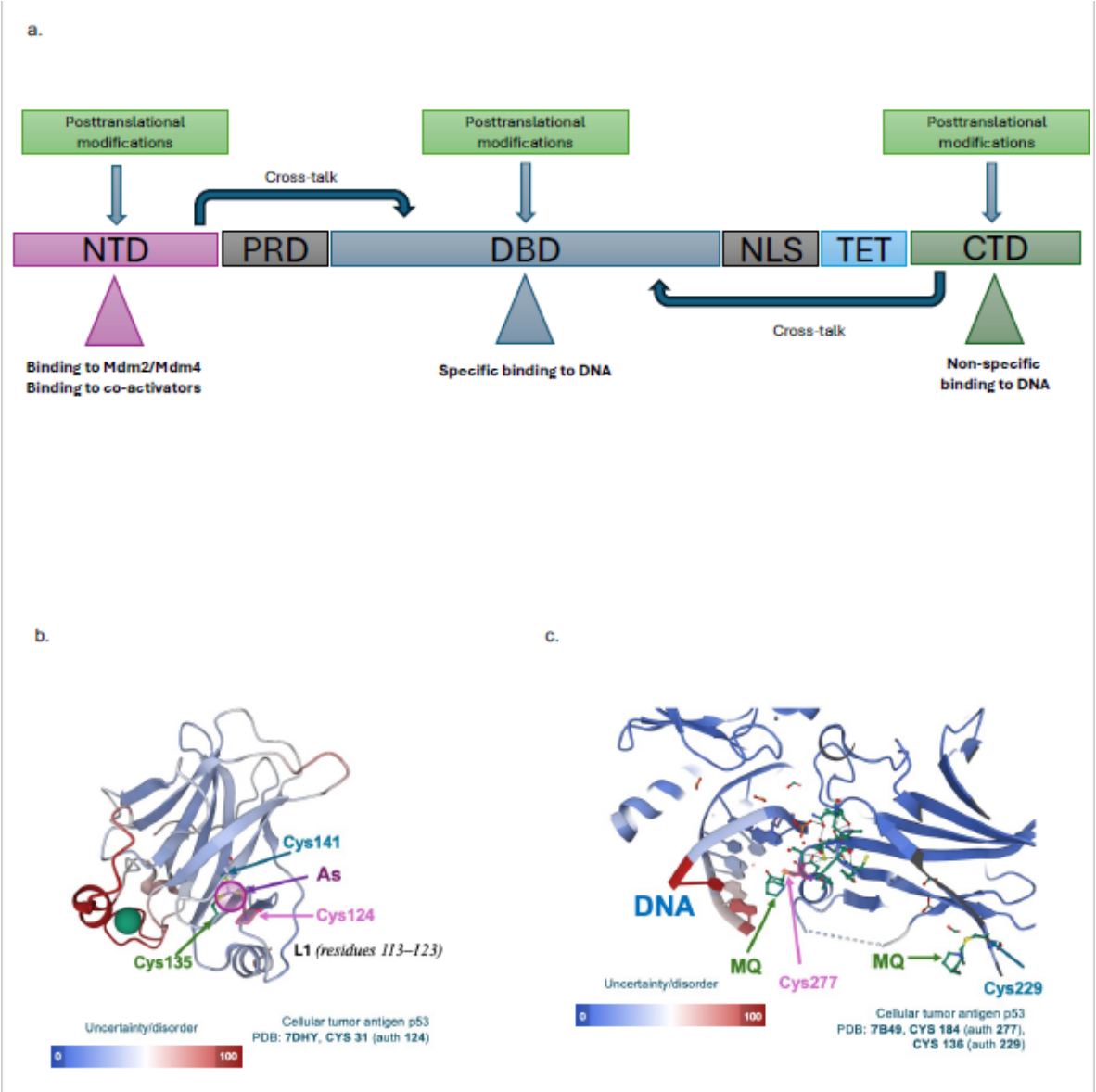

Figure 1. p53 tumor suppressor has a domain structure linked by inter-domain allosteric shifts, which can be induced by post-translational modifications or small molecules to convey the DNA binding activity of mutant

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p53. a. A simplified model of the primary domain structure of p53 and the allosteric crosstalk between domains induced by posttranslational modifications b. structure of the DNA binding domain of G245S mutant p53 core domain in complex with arsenic ion (PDB ID: 7DHY, https://doi.org/10.2210/pdb7DHY/pdb) c. structure of the DNA binding domain of R273H mutant p53 core domain in complex with MQ and the DNA (PDB ID: 7B49, https://doi.org/10.2210/pdb7B49/pdb). Color representation indicates uncertainty and disorder of an element's position, such as B-factor or RMSF, based on available data and experimental technique.

Depending on the type and severity of the stress, p53 undergoes multiple PTMs that allosterically modulate its structure. These modifications induce conformational changes in p53's functional domains. For example, the phosphorylation at Thr^{18} or Ser^{15} alters the structure of the MDM2 binding site in p53 (Phe^{19}, Trp^{23}, and Leu^{26}), disrupting p53/MDM2 interactions. This inhibition prevents p53 ubiquitination and subsequent proteasomal degradation (in the cytoplasm) or potentiates p53 transcription activity (in the nucleus). PTMs also facilitate p53's interactions with transcription co-factors or co-activators (**Fig. 1a**). Additionally, PTMs-induced long-chain and/or direct interactions between NTD and CTD of p53 and the DBD have been reported (described in more detail below). These allosteric modulations enhance p53 stability, promote binding to the canonical DNA sequence and drive the activation of target gene expression.

Since its discovery in 1979, the structure of p53 has been extensively studied. However, the NTD and CTD are intrinsically disordered, and the full-length p53 exists in multiple, unstable conformations, which makes crystallization of the entire protein impossible. For instance, a 13-residue segment within the NTD (residues 17–29), adopts a helical structure upon binding to the hydrophobic pocket of MDM2 [7,8]. These studies showed that the amino acid triad pocket (F19, W23, and L26) is responsible for the interactions with the MDM2 hydrophobic pocket [9].

Other interacting proteins were shown to influence the allosteric shifts in the TAD domain of p53, forming complexes stable enough to be crystallized. These include: MDM4, p300, high mobility group B1, CREB-binding protein, and components of RNA polymerase II assembly (reviewed in [9]).

Several stapled peptides were designed and evaluated for anti-cancer efficacy based on the p53 NTD helix formed in the proximity of the MDM2 and MDM4 binding pocket. The most advanced development in this field is a cell-permeable stapled peptide, ATSP-7041, developed by Aileron Therapeutics. Its clinically tested analog, ALRN-6924 (sulanemadlin), binds to MDM2 and MDM4 with nanomolar affinities and demonstrated tumor growth inhibition *in vivo* [10,11]. The latest report indicates that optimization of stapled peptide targeting MDM2 can be further enhanced by combining the foldamer approach with side-chain cross-linking. This strategy produces more conformationally constrained, cell-permeable inhibitors that can effectively interact with the intracellular targets [12]. Thus, using the optimized structure derived from the flexible region of the p53 NTD led to the development of a potent candidate therapeutic. It remains to be seen if the *in vitro* efficacy will be reflected in the ongoing clinical studies.

Likewise, the CTD transitions to the helical structure when subject to PTMs like acetylation or when interacting with the DNA, promoting the allosteric shifts and playing a regulatory role. Such structural adaptation allows p53 to bind non-specifically to DNA and slide along it in search of target genes [13–17].

The structural flexibility of the DBD and its crosstalk with the CTD were demonstrated in the studies investigating the intrinsic regulatory mechanism of mutant p53 DBD. Mutant p53 can be reactivated by C-terminus-derived peptides [13,14] or by specific antibodies. The binding of these molecules likely triggers long-range conformational fluctuations that refold the DBD into a wild-type-like conformation. Thus, interaction between DBD and C-terminus regulates DNA binding and p53 latency [18].

This inherent flexibility of the p53 functional domains provides opportunities for the development of novel therapeutic approaches that reactivate p53 in cancers either by: (1) preventing its interactions with negative regulators to stabilise the wild-type protein; and (2) correcting the

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folding of mutant or unfolded DBD to enhance specific DNA binding. These approaches are described in more detail below.

Beyond X-ray Crystallography: Uncovering Hidden Structural Elements of p53 for Targeted Cancer Therapy

Despite significant progress in AI-driven protein structure prediction, resolving the structures of IDPs remains a critical challenge and hampers the connection of structures to biological functions. Traditional X-ray crystallography is inadequate for IDPs, as they rarely crystallize, and their inherent flexibility demands solution-based techniques for structural analysis.

In p53, NTD is unstructured in solution but plays a critical function in protein stability and transcriptional activation. It adopts a partial helical structure upon interacting with molecular partners as described in detail above**.**

Recent Monte Carlo simulations based on cryogenic electron microscopy models of full-length p53 tetramer showed that targeting residues 33-37 in the NTD with a small molecule or repurposed drug can induce an allosteric shift, leading to the shielding of the MDM2 binding residues from the solution.

Complementary studies using ion-mobility mass spectrometry (IM-MS) combined with circular dichroism demonstrated that the drug binding promotes structure compaction of the NTD and formation of a 3_{10} helix-like structure. These structural effects were supported by functional studies, which confirmed drug-induced inhibition of the p53/MDM2/MDM4 interactions in both yeast-based reporter assays and in cancer cell models [19].

The binding of a small molecule of RITA to the region spanning residues 33 – 37 was found to be reversible and likely driven by hydrophobic interactions between the furan rings present in the RITA structure and prolines within the SPLPS amino acid motif. The hydroxyl groups of RITA were essential for the association to occur under non-denaturing conditions.

Interestingly, fusing the NTD of p53 with the N-terminus of the spider silk protein, spidroin, also resulted in a more compact NTD conformation, improved p53 translation and increased protein stability in cells. Molecular dynamics (MD) simulations, nuclear magnetic resonance (NMR), and IM-MS analyses indicated that the fused NTD maintains a folded conformation likely no longer available for binding to MDM2 or MDM4. The structural stability leads to a compact arrangement of the chimeric construct [20]. These novel observations further support our observations that compaction of the NTD into helix structure can prevent p53/MDM2 interactions in cancer cells.

Thus, based on the examples described above, methods that allow for the analysis of intact protein structures in solution complemented by *in silico* approaches, such as MD simulation, might deepen our knowledge of structural conformers in p53 to design a novel approach to inhibit p53/MDM2 interactions.

In cancers, p53 is present in a plethora of oncogenic variants enriched in mutations within the DNA-binding domain. The selection of specific *TP53* mutations shows a high incidence of p53 variants predominantly harbouring missense aberrations, with a potential gain of novel antimorphic or neomorphic functions. According to Joerger et al., the mutational landscape is primarily influenced by the extreme structural fragility of the p53 protein [21].

These mutations induce significant variability in the functional integrity of the DBD**,** impairing p53's ability to bind its target promoters and, in consequence, to maintain tumor suppressor function.

Targeting structural p53 mutants is a daunting task due to their inherent instability, frequent unfolding, and resulting technical difficulties of *in vitro* studies caused by DBD protein precipitation at higher concentrations. However, a new frontier for therapeutic intervention seems to lie in exploiting allosteric modulation within the p53 DBD. This might help in designing staple peptides or small molecules rescuing mutant p53 through targeting a unique binding site or pocket, as exemplified by the recent discovery of Y220C targeting compound, rezatapopt [22].

For example, a study by Chen et al., identifies arsenic trioxide (ATO) as a potential rescue compound for structural mutant p53 proteins by using *in silico* analysis of the available sensitivity

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screens in NCI-60 cell lines [23]. The data from the screens was complemented with the adapted conformation-specific immunoprecipitation in cell lysates and differential scanning fluorimetry to assess the shift to the wild-type-like conformation and thermostabilisation of the mutant p53 variants – hallmarks of mutant p53 rescue. Of note, the major focus of the screen was on the most unfolded missense R175H mutant p53, which requires a high precision and bespoke approach to evaluate the effects on the change of the conformational and thermostablization. Despite rather implausible buffer conditions and insufficient controls, the authors claimed to identify ATO as a rescue agent of several missense mutant p53. The X-ray crystallography analysis of the G245S mutant p53 DBD was performed, which identified a novel allosteric site, called an arsenic-binding pocket, where the arsenic ion (ATO) interacts covalently with cysteine triad (C^{124}, C^{135} and C^{141}) and with M^{133} through the Van der Waals interactions, stabilizing the critical DNA-binding loop-sheet-helix motif (**Fig. 1 b**) [23]. The obtained structures were derived from crystals grown without the DNA target sequence; therefore, it remains unclear whether or not ATO rescues the binding capacity of mutant p53 DBDs *in vitro*.

Stabilization by ATO seems to restore the transcriptional activity across a broad spectrum of structural p53 mutants and prevents aberrant protein-DNA aggregation associated with certain mutations, such as Y220C. However, given ATO's known interactions with multiple cellular targets, further studies are essential to determine whether its effects are specific to a distinct subset of p53 mutants. Nonetheless, targeting this novel allosteric site may offer a comprehensive strategy for restoring p53 activity in cancers by enhancing mutant p53 protein refolding and promoting p53 transcription activity.

Another approach to reactivate mutant p53 is to modify the cysteine residues within the mutant p53 DBD. Eprenetapopt/APR-246 is a first-in-class mutant p53 reactivating compound, tested in several precision oncology clinical trials in hematological and solid tumors. The active form of the drug, methylene quinuclidinone (MQ), binds to cysteine residues and thermostabilizes DBD *in vitro* and rescues the tumor suppressor activity in $mutp53^{R175H}$ [24]. Recent long-term follow-up Phase 2 results conveyed that eprenetapopt is well-tolerated in combination with azacytidine, yielding the synergistic response rates in mut*TP53* MDS/AML [25]. The investigators reported that overall survival (OS) was significantly better in allo-HCT patients who achieved complete remission/*TP53* next-generation sequencing (NGS) negativity ($P = 0.00085$; 2-year OS of 54%).

Our biochemical and cell-based assays, which were conducted under stringent conditions securing protein stability at the concentrations tested, revealed thermal stability of wild-type p53, mutant R273H and R175H DBD proteins after treatment with MQ [24].

Site-directed mutagenesis replacing cysteines at positions 124, 277, 182, 229 and the double mutant 124/277 with alanines revealed that substitution of C^{277} led to the largest reduction in MQ-mediated thermal stabilization.

It was concluded that MQ binding to C^{277} contributes to thermo-stabilization of the mutant p53 core domain, while in cancer cells, both C^{124} and C^{277} were required for MQ-mediated reactivation of $mutp53^{R175H}$.

The crystallography studies with MQ-modified p53-DNA complexes showed that three MQ-modified residues, C^{124}, C^{229} and C^{277}, contribute to mutant p53 reactivation [26].

It was proposed that the DNA binding of both DNA contact and structural mutants is supported by a general mechanism mediated by MQ bound to those three cysteines. In addition, R273H bound to both DNA and MQ (**Fig. 1c**), and it is speculated that the DNA-binding activity of other DNA-contact mutants, like R248Q, or R273C, might be restored by the same mechanism.

The authors put forward the concept that the loss of hydrogen bonds with DNA is compensated for by new MQ-mediated p53-DNA and protein-protein interactions. In structural mutants, the binding of MQ stabilizes the structure of DBD, likely through an allosteric shift, which enables the binding to DNA [26].

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However, further studies are needed to elucidate the precise mechanisms underlying the cysteine-targeted allosteric regulation by MQ, especially in structural mutant p53, which may require the solving of the full tetramer crystal structure for the accuracy of the findings.

p53 functions as a tetramer; and its intrinsically disordered regions pose challenges for structural analysis by X-ray crystallography. Nonetheless, the quaternary structure of the modified human p53 has been successfully studied in solution using small-angle X-ray scattering (SAXS) and NMR.

Fragments or full-length p53 proteins, harbouring stabilizing mutations in the core domain (M133L/V203/N239Y/N268D), with or without DNA, have been studied. Structural models generated using SAXS, supported by NMR, showed transient interactions between core domains. These analyses reveal that p53 exists as an open tetramer in solution that transitions into a fully folded conformation upon binding to DNA.

Erroneously, electron microscopy of immobilised, super stable pseudo-wild-type p53 showed a predominant closed conformation that must open to allow DNA binding. These findings suggest that structural insights obtained from SAXS and NMR take precedence over those from electron microscopy and might provide critical mechanistic understanding for reconstituting the transcriptional activity of mutant p53 [27].

Complementing AF Structure Prediction with Biophysical Approaches

Growing evidence supports the value of SAXS in providing a unique perspective into the structure and dynamics of macromolecular complexes, in solution, revealing their size, shape, and conformational flexibility. Notably, SAXS can detect subtle structural transitions in IDPs as they adopt more ordered conformations when interacting with their molecular counterparts. These capabilities demonstrate the advantage of SAXS over traditional X-crystallography, particularly in capturing the dynamic nature and functional versatility of flexible proteins.

AF allows for exceptional accuracy in predicting the structure of well-folded proteins; however, it falls short in modelling intrinsically disordered regions and flexible linkers.

A recent analysis of selected entries from the Small-Angle Scattering Biological Data Bank (SASBDB) (**Box 1**) and their corresponding AF-predicted structures revealed that the AF-predictions often failed to reproduce the experimental SAXS profiles. In contrast, strong agreement was found with ensemble models incorporating flexible linkers between structured domains. The authors used the Monte Carlo method to generate a pool of structural models by adjusting backbone dihedral angles. Through rapid ensemble modelling, they fitted pair distance distribution functions [P(r) versus r] and intensity profiles [I(q) versus q] to experimental SAXS data. Their findings underscored the complementarity value of combining AF predictions with solution SAXS and MD simulation for modelling proteins composed of both structured and flexible regions [28].

This integrated approach enables the structural characterisation of proteins with mixed content, specifically, well-folded domains connected by flexible linkers, using AF predictions alongside SAXS constraints.

In classical crystallography, the molecular replacement (MR) approach uses known crystal structures from the repositories to solve given biomolecular crystal structures. When no similarity is found, experimental phase evaluation is needed. A recent study developed an automated MR workflow that leverages AF's high prediction accuracy, integrating Phaser, Rosetta and AutoBuild to correct AF-generated conformations. Such an approach allowed for an MR solution even from the incomplete data [29].

Concluding Remarks

With the development of the combined AI-aided approaches, it is becoming increasingly feasible to capture the dynamic nature of IDPs. Deconvoluting the allosteric shifts within the p53 functional domains might help to develop a structure-function-guided drug discovery approach, where a highly precise, desirable effect can be imposed through targeting a specific allosteric site in p53.

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Summary Points

1. An allosteric shift in p53 functional domains is an established yet overlooked paradigm in p53 protein regulation.
2. Current AI models do not go beyond predicting the 3D protein structures based on the available data, failing to capture IDPs's dynamics.
3. A combined approach, using an AI algorithm and a bespoke experimental, solution-based approach, is needed to address p53 structure flexibility to accelerate the drug discovery process.

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