

Proteins without 3D structure: definition, detection and beyond

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ABSTRACT

Motivation: Predictions, and experiments to a lesser extent, following the decoding of the human genome showed that a significant fraction of gene products do not have well-defined 3D structures. While the presence of structured domains traditionally suggested function, it was not clear what the absence of structure implied. These and many other findings initiated the extensive theoretical and experimental research into these types of proteins, commonly known as intrinsically disordered proteins (IDPs). Crucial to understanding IDPs is the evaluation of structural predictors based on different principles and trained on various datasets, which is currently the subject of active research. The view is emerging that structural disorder can be considered as a separate structural category and not simply as absence of secondary and/or tertiary structure. IDPs perform essential functions and their improper functioning is responsible for human diseases such as neurodegenerative disorders.

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1 DEFINITION AND CLASSIFICATION

There is currently a lack of consensus regarding the definition, terminology or nomenclature of proteins without well-defined 3D structure in their native (functional) state. These proteins lack a stable equilibrium conformation but exist as dynamic ensembles within which atom positions exhibit extreme temporal fluctuations without specific equilibrium values (Uversky and Dunker, 2010). The intrinsically disordered proteins (IDPs), the most frequently used term, just as others do not always differentiate whether the whole or (a) significant segment(s) of the sequences of these proteins are without defined structures. The Database of Protein Disorder (*DisProt*) defines IDP as ‘a protein that contains at least one experimentally determined disordered region’ (Sickmeier *et al.*, 2007). There are proteins disordered in full length while others contain both ordered and disordered parts termed as intrinsically disordered regions (IDRs; Obradovic *et al.*, 2005). These proteins are often named also as IDPs; however, the name ‘proteins with IDR(s)’ appears to be more correct.

A number of terms have been used to indicate the disordered characteristics of these proteins which are as follows: natively

denatured (Schweers *et al.*, 1994), natively unfolded (Weinreb *et al.*, 1996), intrinsically unfolded (Baskakov *et al.*, 1999), intrinsically unstructured (Wright and Dyson, 1999), intrinsically disordered (Dunker *et al.*, 2000), exceptionally flexible (Ahmed *et al.*, 2007), natively unstructured (Schlessinger *et al.*, 2007a), naturally flexible (Uversky *et al.*, 2009), etc. The most often used synonyms are intrinsically unstructured/disordered proteins (IUPs/IDPs), although the pioneers of this field (Dunker *et al.* 2008, Uversky *et al.*, 2009) consider IUPs as a subset of IDPs without hydrophobic core and significant amounts of stable secondary structure.

The central dogma of the new protein structure–function paradigm, the *protein trinity/quartet*, consisting of the folded (ordered) state, the molten globule and the random coil (Dunker *et al.*, 2001), plus the pre-molten globule as the fourth unique thermodynamic state (Uversky, 2002), is that *any of these states may be the native state*, which is relevant to the biological function of a protein. Accordingly, the three types of intrinsic disorder can be classified as the native coil, native pre-molten globule (both considered as intrinsically unstructured) and native molten globule (Uversky *et al.*, 2009). Native coils, characterized by *extended disorder*, arise from chains having repulsion arising from a net charge, and these proteins and regions resemble the more classical idealized random coil. Pre-molten globules have no well-defined tertiary structure, may contain regions with transient and small amount of secondary structure and 3-fold larger hydrodynamic volume as expected for the folded state. Molten globules (*collapsed disorder*) possess secondary structure and folding pattern similar to the folded state (Uversky, 2002), with loosened, i.e. molten, tertiary interactions and exhibit an increase in hydrodynamic volume of no more than 50% (Uversky, 2002; Wright and Dyson, 1999). This classification, which is slightly artificial, is useful from conceptual and practical point of view, e.g. for the development of specific disorder predictors. However, we emphasize that according to our concept, in agreement with recently published ones (Rauscher and Pomés, 2010; Xue *et al.*, 2009), there is a *continuum of these states* with various degrees of compactness due to the different amounts and distribution of secondary and tertiary structure. Nevertheless, it is important to remember that this view does not influence the fact that each of these states, not only the folded one, can occur as the native state.

Structural disorder can be considered as a separate structural category, and not merely as a lack of secondary and/or tertiary structure (Tompá and Kalmar, 2010). The length distribution of disorder in the human proteome is scale free (follows a power law), with many short regions and also a significant incidence of very long disordered regions. This is in sharp contrast with the length distribution of conventional secondary structural elements, which

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shows a length limit near 50 residues, however, is highly reminiscent of the distribution of tertiary structural units (domains) in proteins. This behavior was correlated with the direct functional involvement of disorder (Section 5), which place structural disorder as a unique 'structural' level between secondary and tertiary structures (Tomba and Kalmar, 2010). In line with this, the sequences of intrinsically disordered domains differ significantly from random sequences; the deviation is comparable with that found between ordered and disordered domains (Teraguchi *et al.*, 2010) suggesting a 'structure–function' relationship for disordered regions.

2 OCCURRENCE

Bioinformatics predictions (Section 4) suggest that intrinsic structural disorder is a widespread phenomenon, especially in eukaryotes, where conservative estimations suggest that 5–15% of proteins are disordered in full sequence (IDPs), and about 35–50% of proteins have at least one IDR (more than 30 residues) (Ward *et al.*, 2004). It was interpreted that more disorder is needed for signaling and coordination among the various organelles of eukaryotes being more complex than prokaryotes (Dunker and Obradovic, 2001). Indeed, in mammals, 75% of signaling proteins are predicted to contain long disordered regions (Dunker *et al.*, 2008). In general, the more complex the organism is the more frequent occurrence of disorder can be found. [An interesting exception is that some protist parasites have the highest prevalence of disorder (Feng *et al.*, 2006)]. The various genome wide *in silico* studies based on Gene Ontology annotations and Swiss-Prot functional keywords suggest that the biological processes involving IDPs are as follows (Tomba, 2009; Tomba *et al.*, 2006; Ward *et al.*, 2004; Xie *et al.*, 2007): (i) transcription and its regulation; (ii) signal transduction and cell-cycle regulation; (iii) functioning of nucleic acid containing organelle; (iv) mRNA processing and splicing; and (v) cytoskeleton organization. These results from genome-wide predictions of intrinsic disorder and the results from other bioinformatics studies drew attention to these proteins. However, prediction of disorder foreruns its experimental identification and only relatively few experimentally characterized examples are known. There is experimental evidence for the structural disorder of about 1100 regions within 500 proteins, which is collected in the DisProt database (Sickmeier *et al.*, 2007).

3 EXPERIMENTAL IDENTIFICATION

Disordered proteins can be identified and characterized by using wide arsenal of the experimental methodologies (Daughdrill *et al.*, 2005; Eliezer, 2009; Mittag and Forman-Kay, 2007; Receveur-Bréchet *et al.*, 2006; Uversky and Longhi, 2010). Nevertheless, the absence of a well-defined structure in disordered proteins complicates their investigation, since the determination of unique high-resolution structure of IDPs is frequently not attainable. Instead, the goal usually is to obtain experimental constraints on the ensemble of states, including the detection of residual secondary structure, transient long-range contacts and regions of restricted or enhanced mobility (Eliezer, 2007). There is neither time nor space to cover these important experimental results. Here, we give only a brief description of the techniques which are most successfully used for identification of IDPs and/or IDRs. Most of them provide information for the global structures and do not identify the specific

disordered region(s) within the molecule. For a more detailed description of specific methods, please see Supplementary Material File 1.

4 PREDICTION

Since the first predictors of protein disorder were published (Li *et al.*, 1999; Romero *et al.*, 1997), almost 60 predictors have been developed so far. The properties and the advantages/disadvantages of these predictors are summarized in several papers (Dosztányi and Tomba, 2008; Dosztányi *et al.*, 2010; Feng *et al.*, 2006, Ferron *et al.*, 2006; He *et al.*, 2009; Tomba, 2009; Uversky and Dunker, 2010). A very comprehensive recent review has been published by He *et al.* (2009), which contains detailed descriptions of the most often used predictors and references to the publicly available ones. The majority of the programs are accessible via public servers; links to many of them can be found in the Disordered Protein Database (<http://www.DisProt.org>) (Sickmeier *et al.*, 2007). Here, we shortly introduce merely some representative and frequently used disorder predictors (cf. also Supplementary Material File 2).

The predictors are based on different principles and can be classified into three main categories (Csizsók and Tomba, 2009; Tomba, 2009): (i) propensity-based predictors; (ii) machine learning algorithms; and (iii) algorithms based on interresidue contacts. These categories are not absolute since some of the methods use more than one of these features. Moreover, combined meta-servers also exist.

4.1 Propensity-based predictors

IDPs are significantly depleted in so-called order-promoting residues, including bulky hydrophobic (Ile, Leu and Val) and aromatic amino acid residues (Trp, Tyr and Phe), which would normally form the hydrophobic core of a globular protein, as well as Cys and Asn. On the other hand, there are so-called disorder-promoting amino acids, namely, Ala, Arg, Gly, Gln, Ser, Pro, Glu and Lys, which are substantially overrepresented in IDPs (Dunker *et al.*, 2001; Romero *et al.*, 2001). This specific amino acid composition is usually indicative for disorder. This propensity of IDPs was used to develop sophisticated prediction methods as well (Section 4.2).

Several methods are based on simple amino acid propensity scales. Their advantage is that they are easy to calculate and to interpret; however, they are limited to a single property. For example, due to their amino acid composition, low overall mean hydropathy and high mean net charge represent a unique structural feature of IDPs/IDRs (Uversky *et al.*, 2000). The mean hydropathy is defined as the sum of the hydropathies of all residues divided by the number of residues in the polypeptide. The mean net charge is the net charge, at pH 7.0, divided by the total number of residues. A plot of mean net charge versus mean hydropathy (the CH plot or Uversky plot) separates ordered and disordered proteins into distinct regions (Uversky, 2002). By calculating the distribution of these features for a pre-defined sequence window, Prilusky *et al.* (2005) used this idea to design a per-residue disorder predictor, *FoldIndex*. Another predictor, the *GlobPlot* algorithm (Linding *et al.*, 2003a), uses the relative propensity of amino acid residues to be in an ordered or disordered state applying an amino acid scale based on the difference in the probability for a given amino acid to be in regular secondary

structure or to be in random coil. The basic algorithm behind GlobPlot is simple and very fast, representing a sum function.

4.2 Machine learning algorithms

The prediction of protein disorder can be viewed as a classic binary classification problem and can be addressed by standard machine learning techniques as *artificial neural networks* (NNs) and *support vector machines* (SVMs). The majority of the methods developed belong to these categories. They are trained on datasets of disorder and order and evaluate intrinsic disorder on a per-residue basis. Their underlying assumption is that sequence features calculated from a local sequence window can be directly mapped into the property of order or disorder.

The Dunker's lab was the pioneer of predictors (Romero *et al.*, 1997); then their *PONDR*® family of algorithms has been continuously developed and improved (Li *et al.*, 1999; Obradovic *et al.*, 2005; Peng *et al.*, 2005, 2006; Romero *et al.*, 1997, 2001). They typically use NNs, although in a few cases SVMs are also included. *PONDR*® algorithms based on the fact that the amino acid composition in a window of N amino acids for ordered proteins are distinguishable from the composition for disordered proteins. Beside merely amino acid composition itself, they use as inputs some attributes derived from composition as well. These various types of attributes are weighted and combined in a non-linear manner. The training datasets are different in the various family members (missing residues of X-ray structures; variously characterized long disordered regions; DisProt). Accordingly, there are predictors for short and long (>30 amino acid) regions, and for N- and C-terminal and internal ones. For their detailed descriptions, see the authors' recent review (He *et al.*, 2009) and the group's home page (<http://www.pondr.com>).

DisEMBL designed by Linding *et al.* (2003b) consists of three separate NN predictors, to predict three kinds of disordered structures in proteins, which represent residues within 'loops/coils', 'hot loops (loops with high B-factors, i.e. with high degree of mobility)' or those that are missing from the PDB X-ray structures. Thus, it performs better on short disordered regions.

Another NN algorithm, *RONN*, developed by Yang *et al.* (2005) is based on 'functional alignments'. The main idea is that if two proteins have similar biological functions, in this case the similar tendencies to be ordered/disordered, then their sequences are also similar. In the training process, the similarity of sequences is evaluated by sequence alignment techniques using a mutation matrix to score the similarity. These scores of sequence alignments are then used for training.

The most often used SVM method is *DISOPRED2* (Ward *et al.*, 2004) where the input data are generated by sequence alignment using PSI-BLAST, and which is trained on a database of amino acids missing from PDB structures. Thus, the prediction is better on short disordered regions in the context of globally ordered proteins. The fact that the database contain much more ordered than disordered residues (176 550 versus 4590) is balanced by formulating the SVM to place greater cost of misclassification for points from the minority (disordered) class than from the majority (ordered) class. This is the reason for the low false positivity of *DISOPRED2*. Compared with other disorder predictors, the main difference is that *DISOPRED2* is directly trained on the whole sequence rather than measures of amino acid composition, sequence complexity or biophysical properties.

Prediction accuracy of this method depends on the number of homologs used for sequence alignment.

Additional methods which apply a second level of prediction using the output of the first level prediction as an input are the *POODLE* algorithms. They employ SVMs with radial basis kernels for training; the input is constructed from physico-chemical properties using PSI-BLAST profiles. *POODLE-S* (Shimizu *et al.*, 2007) and *POODLE-L* (Hirose *et al.*, 2007), which aim to predict disordered segments shorter and longer than 40 residues, respectively, calculate the input vector by using physico-chemical features and a reduced amino acid set of position-specific scoring matrices or from hydrophathy, average contact density propensity, mean net charge, sequence complexity, amino acid compositions relative to the composition of disordered and ordered training sets and secondary structure preferences.

4.3 Prediction methods based on interresidue contacts

Limitations due to the biased and insufficient databases can be overcome by the methods based on structural and energetic considerations, which do not rely on experimental data on protein disorder. The prominent representatives of these methods are *FoldUnfold* (Galzitskaya *et al.*, 2006; Garbuzynskiy *et al.*, 2004), *IUPred* (Dosztányi *et al.*, 2005a, b), and *Ucon* (Schlessinger *et al.*, 2007a). The main idea of these methods is that the disorder of proteins is originated from the lack or low level of the *interresidue contacts* which cannot compensate the large decrease in conformational entropy during folding (Tompa, 2009). The importance of interresidue contacts, especially that of the heavily interacting residue clusters (stabilization centers) is essential in the maintenance of the folded protein structure (Dosztányi *et al.*, 1997). Intuitively, it can be thought that the lack of them favors protein disorder, as it was found indeed in several cases (Orosz *et al.*, 2004).

FoldUnfold based on the statistical analysis of residue contact numbers. The summation of the interresidue contact numbers of the amino acids of a protein is indicative for its folded/unfolded character. Two residues are considered in contact if any pair of their heavy atoms is within 8.0 Å to each other. To express the average contact number of residues within a given distance in a protein structure, the mean packing density of residues is calculated. It was demonstrated that regions with low-expected packing density correspond to the disordered segments.

Ucon combines a former predictor, *PROFcon*, for long-range protein-specific internal contacts (Punta and Rost, 2005) with a generic pairwise potential to predict unstructured regions longer than 30 amino acids. It combines information from alignments, from predictions of secondary structure and solvent accessibility, from the region between two residues and from the average properties of the entire protein.

The core of *IUPred* is a method that renders the direct estimation of the interaction energies using exclusively the protein sequence possible. In this approach, the estimated energy for each residue depends on the amino acid type and on the amino acid composition of the sequential neighborhood. Generally, residues with less favorable predicted energies are more likely to be disordered. The parameters of this method are derived exclusively from a globular protein dataset without the use of specific datasets of disordered proteins. As globular protein datasets are considerably larger than that of disordered proteins, this stabilizes the method substantially if

compared with methods where a large number of parameters are trained on a limited and sometimes biased disordered protein dataset. IUPred performs comparatively well for predicting long disordered segments, and has a good sensitivity, i.e. does not miss a significant number of disordered residues.

However, a problem arises by the presence of conserved cysteines and/or of metal-binding motifs which can cause uncertain local predictions of disorder within these regions using the methods based on interresidue contacts. These predictors may display features typifying disorder while the protein region gains structure upon disulfide formation or binding to metal ions (Ferron *et al.*, 2006). This problem can in part be handled using methods predicting metal-binding sites and disulfide bridges of proteins from their sequence (Lippi *et al.*, 2008).

4.4 Metapredictors

Generally, it is a good idea not to rely on one single algorithm when predicting disorder. Instead, as these algorithms all capture different aspects of the structural properties of proteins, they can complement each other to give a more complete picture. Recently, a new direction in the development of disorder predictors based on the creation of metapredictors has attracted attention. These metapredictors (*metaPrDOS*, *MeDor*, *MD* and *PONDR-FIT*) combine the outputs of several individual predictors (Ishida and Kinoshita, 2008; Lieutaud *et al.*, 2008; Schlessinger *et al.*, 2009; Xue *et al.*, 2010a). They can be applied either at the residue level or at the whole sequence level. The individual predictors constituting metapredictors are based on different philosophies, the strength and weakness of which can be balanced by their combination. *MetaPrDOS* (Ishida and Kinoshita, 2008) uses SVM to integrate residue-level predictions from several algorithms and was trained on a group of PDB-extracted proteins that all have regions of missing electron density in their crystal structures, and the sequence identities among these proteins are <20%. *Meta-Disorder predictor (MD)* (Schlessinger *et al.*, 2009) uses NN and the training datasets were proteins from PDB and DisProt. The metapredictors improved the prediction accuracies which were several percentage points higher (max. 10 %) on various datasets in comparison with the values estimated for the individual predictors.

4.5 Future directions

The performance of disorder predictors has been compared in the CASP (Critical Assessment of Structure Prediction) experiments (Bordoli *et al.*, 2007; Jin and Dunbrack, 2005; Melamud and Moulton, 2003; Noivirt-Brik *et al.*, 2009). However, these comparisons are considered to be rather biased since ‘the performance of the methods depends on both the type of disorder and evaluation criteria’ (Tompa, 2009) as discussed in details in several other papers as well (Dosztányi *et al.*, 2010; Schlessinger *et al.*, 2007b). Moreover, the predictors focus on different type (‘flavors’) of disorder, thus predictors trained on disorder of one type of protein often achieve poor accuracy on disorder of proteins of a different type, as recognized already at the advent of IDP research (Vucetic *et al.*, 2003).

Currently, the per-residue prediction accuracies of these methods have risen to about 80% (Dunker *et al.*, 2008). The limitation to further improvement comes from inaccuracy in the ordered and disordered protein data (Dosztányi *et al.*, 2010; He *et al.*, 2009). The

performance of disorder prediction methods critically depends on the dataset used for testing and the type of disorder (e.g. extended or collapsed) studied (Dosztányi *et al.*, 2010; Schlessinger *et al.*, 2007b; Vucetic *et al.*, 2003). Datasets of experimentally verified ordered and disordered regions contain many mis-classified segments; moreover, the latter ones are not sufficiently large for prediction of very high level of accuracy. Various datasets of disordered protein sequences exhibit variations in their sequential bias. Differences can be observed depending also on the experimental method used for identification of the disordered regions (Dosztányi *et al.*, 2010), on their length, and on the location in the sequence (N- and C-terminal, middle regions) (Li *et al.*, 1999). Although these differences are smaller compared with the differences observed between ordered and disordered proteins, they should be taken into account during the development of prediction methods. Thus, it was suggested that predictors that go beyond the binary classification of proteins as ordered or disordered are necessary (Dosztányi *et al.*, 2010; He *et al.*, 2009).

5 FUNCTION

Disordered proteins can be separated into two main functional classes, based on their *in vivo* activities: *entropic chains* and IDPs involved in *molecular recognition* (Tompa 2002, 2005). There are IDPs which do not have a folded or ordered state under any known conditions, while others are capable of folding under certain circumstances, i.e. upon binding to a partner, termed as ‘*non-folders*’ and ‘*folders*’, respectively (Rauscher and Pomés, 2010). Entropic chains are necessarily ‘*non-folders*’, since their functions rely on their high-conformational entropy: their functions are derived by populating many accessible conformations without well-defined folded structure (Tompa, 2009). On the contrary, IDPs involved in molecular recognition due to their interacting potencies are generally ‘*folders*’ that become (partly) ordered upon binding to their targets. The binding can be permanent (*scavengers*, *effectors* and *assemblers*) or transient (*display sites* and *chaperones*) (Tompa and Kovacs, 2010). Scavengers and assemblers usually bind to multiple partners. Scavengers store and neutralize small molecules, while assemblers support the assembly of multi-protein complexes. Effectors regulate the activity of partner proteins. Display sites expose sites for post-translational modifications such as phosphorylation or limited proteolysis, whereas chaperones bind to the partner molecule to facilitate its correct folding preventing its aggregation or proteolysis.

5.1 Binding to partners

The folding of IDPs during molecular recognition is analogous to protein folding of globular proteins, since both processes involve a thermodynamically stable folded state and an unfolded state of higher conformational entropy (Verkhivker *et al.*, 2003, 2005). Since many IDPs and IDRs fold upon binding to their targets (Wright and Dyson, 2009), a challenging question is whether folding occurs before binding or binding occurs before folding? The two extremes are induced folding and conformational selection (Wright and Dyson, 2009). In the case of the first mechanism, the protein associates with its binding partner in a disordered state and subsequently folds in association with the target protein. In the conformational selection mechanism, the target protein ‘selects’ a

conformation closely approximating that of the bound form from the ensemble of conformations populated by the IDP when free in solution. This question is analogous to and generalization of the induced fit—fluctuation fit (conformational selection) duality of molecular recognition (Vértessy and Orosz, 2011). In real systems, one or another or both mechanism(s) can be favored (Wright and Dyson, 2009).

In general, the binding of IDPs differs from that of ordered proteins since they often bind their partner via short recognition elements [*MoRFs* (*molecular recognition features*)] (Fuxreiter *et al.*, 2007; Mohan *et al.*, 2006; Oldfield *et al.*, 2005) in a structurally adaptive process termed *disorder-to-order transition* (Verkhivker *et al.*, 2003). Structural disorder may confer significant functional advantages for IDPs, such as rapid binding to the partner molecule, the combination of high specificity with weak and reversible interaction and the ability to carry out more than one function either via multiple interaction sites or through regions specific to distinct partners (Tompa, 2002, 2005; Tompa *et al.*, 2005). They can fold into different structures on binding to different target proteins (*‘one-to-many binding mode’*), with different functional outcomes (functional promiscuity or moonlighting) (Tompa *et al.*, 2005). In contrast to this, the *‘many-to-one binding mode’* was also suggested, when many different IDPs may bind alternatively to one site on a single ordered partner, by which different IDPs fold into similar conformations (Uversky *et al.*, 2009). It was suggested that these binding features of IDPs may explain their *‘organizing role’* in protein–protein interaction networks as so-called *‘hub’* proteins (Dunker *et al.*, 2008).

Some binding sites can be found as linear motifs (LMs; Puntervoll *et al.*, 2003), short segments involved in the molecular recognition of proteins. It was shown that there is a connection between LMs and molecular recognition elements of IDPs. LMs are embedded in locally unstructured/highly flexible regions, while their amino acid composition exhibits a mixture characteristic of folded and disordered proteins (Fuxreiter *et al.*, 2007).

As we discussed above, there are many algorithms for predicting IDPs; however, the methods for predicting regions undergoing disorder-to-order transition upon protein binding is rather limited. An algorithm proposed by the Dunker’s and Uversky’s labs, *α-MoRF-PredII*, combines two bioinformatic tools, sequence alignment and disorder prediction, to find possible binding partners in protein databases and identify the interaction sites. The method is based on the identification of the above-mentioned MoRFs, which are short segments expected to have a high propensity for folding upon binding and that are located within regions of disorder (Cheng *et al.*, 2007; Mohan *et al.*, 2006; Oldfield *et al.*, 2005). Very recently, the authors hypothesized that not only MoRFs with similar sequences can be aligned but also those of with reversed sequential order (*‘retro-MoRFs’*). Applying this theory, they developed a software package named *PONDR-RIBS*, which aligns protein segments, predicts disorder and interaction regions (Xue *et al.*, 2010b). However, experimental verification of this new method is needed.

A recent method, *ANCHOR*, based on the principles behind the IUPred algorithm (Section 4.3), has been developed for this aim (Mészáros *et al.*, 2009). The essential feature of these binding segments is that they exist in a disordered state in isolation, but they can favorably interact with a globular protein and adopt a rigid conformation upon binding. Based on this model, the combination

of the high disordered tendency of the sequential environment, the unfavorable intrachain interaction energies and high energetic gain by interacting with a globular protein partner indicates the presence of a disordered binding region (Dosztányi *et al.*, 2010).

For a discussion of the connection of intrinsic disorder and alternative splicing and diseases, respectively, please see Supplementary Material File 1.

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