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Intrinsic disorder in scaffold proteins: Getting more from less

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Abstract

Regulation, recognition and cell signaling involve the coordinated actions of many players. Signaling scaffolds, with their ability to bring together proteins belonging to common and/or interlinked pathways, play crucial roles in orchestrating numerous events by coordinating specific interactions among signaling proteins. This review examines the roles of intrinsic disorder (ID) in signaling scaffold protein function. Several well-characterized scaffold proteins with structurally and functionally characterized ID regions are used here to illustrate the importance of ID for scaffolding function. These examples include scaffolds that are mostly disordered, only partially disordered or those in which the ID resides in a scaffold partner. Specific scaffolds discussed include RNase, voltage-activated potassium channels, axin, BRCA1, GSK-3β, p53, Ste5, titin, Fus3, BRCA1, Titin, MAP2, D-AKAP2 and AKAP250. Among the mechanisms discussed are: molecular recognition features, fly-casting, ease of encounter complex formation, structural isolation of partners, modulation of interactions between bound partners, masking of intramolecular interaction sites, maximized interaction surface per residue, toleration of high evolutionary rates, binding site overlap, allosteric modification, palindromic binding, reduced constraints for alternative splicing, efficient regulation via posttranslational modification, efficient regulation via rapid degradation, protection of normally solvent-exposed sites, enhancing the plasticity of interaction and molecular crowding. We conclude that ID can enhance scaffold function by a diverse array of mechanisms. In other words, scaffold proteins utilize several ID-facilitated mechanisms to enhance function, and by doing so, get *more* functionality from *less* structure.

Keywords

intrinsic disorder; scaffold; signaling

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Intrinsically disordered proteins

A simplified version of the dominant view in current biochemistry textbooks is that there are two broad types of non-membrane proteins. The first support the structure of the cell or organism and these are typically fibrous. The second carry out functions by means of folding into globular 3D structures required to form functional active sites. Most often the active sites carry out catalysis while less often they bind small molecules. The active-site concept can be summarized as the sequence → structure → function paradigm. This paradigm underlies attempts to understand enzymology and provides the basis for drug discovery efforts based on protein structure. Because this understandable bias towards structure has dominated for so long, intrinsically disordered proteins are scarcely mentioned in biochemistry textbooks.

Despite the limited attention given to intrinsic protein disorder in the past, it is now recognized that many functional proteins or protein regions lack rigid 3-D structure under physiological conditions *in vitro*, existing instead as dynamic ensembles of inter-converting structures (Daughdrill et al., 2005; Dunker et al., 2002a; Dunker et al., 2002b; Dunker et al., 2001; Iakoucheva et al., 2002; Radivojac et al., 2007; Romero et al., 1998; Tompa, 2002; Uversky, 2002a; Uversky, 2003b; Uversky et al., 2000; Uversky et al., 2005; Wright and Dyson, 1999). These proteins have been variously referred to as *rheomorphic* (Holt and Sawyer, 1993), *natively denatured* (Schweers et al., 1994), *natively unfolded* (Uversky et al., 2000; Weinreb et al., 1996), *intrinsically unstructured* (Tompa, 2002; Wright and Dyson, 1999), *intrinsically disordered* (Dunker et al., 2001), regions with *nonregular secondary structure* (NORS) (Liu et al., 2002; Schlessinger et al., 2007) and *natively disordered* (Daughdrill et al., 2005) among other names. We will use the terms “intrinsic disorder” (ID), “intrinsically disordered” (IDed), “intrinsically disordered proteins” (IDPs) and “intrinsically disordered regions” (IDRs) as this nomenclature seems to adequately describe the observed phenomenon (i.e., intrinsic lack of ordered structure). ID is manifested in a variety of contexts and affects various levels of protein structure. IDPs and IDRs have been grouped into at least two broad structural classes – compact (molten globule-like) and extended (random coil-like) (Daughdrill et al., 2005; Dunker et al., 2001; Dunker and Obradovic, 2001; Uversky, 2002a; Uversky, 2002b; Uversky, 2003b). A further complication is that some random coils can be collapsed rather than extended (Vitalis et al., 2007) in a structural form previously called the “pre-molten globule” (Uversky and Ptitsyn, 1994; Uversky and Ptitsyn, 1996).

The prevalence of ID in the literature is attested by the DisProt database which contains over 400 and 1000 experimentally characterized IDPs and IDRs, respectively (www.disprot.org) (Sickmeier et al., 2007; Vucetic et al., 2005).

Prediction of ID

The composition of amino acid sequences encoding ID is significantly different from those of ordered proteins on the basis of local amino acid composition, flexibility, hydropathy, charge, coordination number and several other factors (Dunker et al., 1998; Dunker et al., 2001; Li et al., 1999b; Radivojac et al., 2007; Romero et al., 1997a; Romero et al., 1997b; Romero et al., 2001; Uversky et al., 2000). For example, ID is significantly depleted in bulky hydrophobic (Ile, Leu, and Val) and aromatic (Trp, Tyr, and Phe) amino acid residues, which are highly represented in the hydrophobic core of globular proteins, and also possess fewer Cys and Asn residues. These residues were proposed to be called order-promoting amino acids (Campen et al., 2008; Williams et al., 2001). On the other hand, ID is substantially enriched in polar amino acids (Arg, Gln, Ser, Glu, and Lys), in the structure-breaking residues (Gly and Pro), and in Ala (Campen et al., 2008; Dunker et al., 2001; Radivojac et al., 2007; Romero et al., 2001; Vucetic et al., 2003; Williams et al., 2001). This collection of residues has been called disorder promoting (Campen et al., 2008; Williams et al., 2001). Many of the differences mentioned above were utilized to develop, in part, various ID predictors, including PONDR® (Predictor

of Naturally Disordered Regions) (Li et al., 1999b; Romero et al., 2001), charge-hydropathy plots (CH-plots) (Oldfield et al., 2005a; Uversky et al., 2000), the NORS Predictor (Liu and Rost, 2003; Liu et al., 2002; Schlessinger et al., 2007), GlobPlot (Linding et al., 2003a; Linding et al., 2003b), FoldIndex© (Prilusky et al., 2005), IUPred (Dosztanyi et al., 2005), DISOPRED (Jones and Ward, 2003; Ward et al., 2004a; Ward et al., 2004b) and NORSnet (Schlessinger et al., 2007).

The most accurate whole-protein predictor of ID is the PONDR® VLS1 family of predictors (www.pondr.com). The PONDR® VSL1 algorithm (Peng et al., 2006) (Obradovic et al., 2005) was judged to be among the most accurate predictor of ID evaluated in both CASP6 (Obradovic et al., 2005) and CASP7 (Bordoli et al., 2007). In this review, VLS1 PONDR® plots are used to illustrate whole-protein characteristics.

The other PONDR® predictor used in this review is VL-XT (Li et al., 1999b; Romero et al., 2001). Although VL-XT is a first generation ID predictor, it became evident early on that a certain type of characteristic prediction consisting of a short region of predicted order bounded by predictions of extended regions of ID corresponded to binding sites that involved disorder-to-order transitions upon complex formation (Garner et al., 1999). In direct comparisons with several commonly used predictors of disorder, the VL-XT still appears to be the best at indentifying binding regions within IDRs (Cheng et al., 2007). Thus the VL-XT predictor has proven useful for investigations of protein-protein interactions (see below).

Within this review, these PONDR® ID predictors are used to illustrate: 1) that the propensity for ID is predictable, 2) ID profiles that could be considered typical for the function being discussed and 3) that the accuracy of current predictors makes them useful tools for many types of research. PONDR® ID predictors take primary sequence as input and generate a PONDR® score for each residue. Typically, PONDR® scores are displayed on the y-axis an x–y plot with the residue number on the x-axis. PONDR® scores greater than 0.5 indicate regions that are predicted to be IDed and those with scores less than 0.5 are predicted to be ordered (Obradovic et al., 2005). PONDR® scores, although reported per-residue, are not based on single residues but rather comprised of the averaged attributes of a series of residues within a moving window that is centered on the scored residue (Li et al., 1999b; Peng et al., 2006; Romero et al., 2001) (Obradovic et al., 2005). In addition, two state prediction methods, CH-plot (Oldfield et al., 2005a) and cumulative distribution function (CDF) (Oldfield et al., 2005a) are used to illustrate the ID-promoting composition of mostly disordered proteins.

Functionality of ID

In recent years the dramatic increase in the interest in ID has been evidenced by the exploding literature base related to the subject (Sickmeier et al., 2007). This intensified interest is consistent with crucial findings related to the functionality of these proteins as well as with bioinformatics studies indicating that about 20 to 33% of eukaryotic proteins are mostly IDed (Liu et al., 2002; Oldfield et al., 2005a; Ward et al., 2004b), that more than half of eukaryotic proteins have long IDRs (Dunker et al., 2000; Oldfield et al., 2005a; Romero et al., 1998; Uversky et al., 2000), and that more than 70% of signaling proteins have long IDRs (Iakoucheva et al., 2002).

To increase the resolution of such studies and to link ID to specific functions and proteins, a comprehensive bioinformatics investigation into the functional roles of ID utilizing the highly annotated Swiss-Prot database was recently carried out (Xie et al., 2007b). By applying data-mining tools to over 200,000 proteins and associated functional keywords in the Swiss-Prot database, it was shown that, of the 710 functional keywords that were associated with more than 20 proteins, 238 were found to have a strong positive correlation with long IDRs, whereas 302 showed strong negative correlation with such regions (Vucetic et al., 2007; Xie et al.,

2007a; Xie et al., 2007b). These findings strengthen the supposition that structured proteins and IDPs participate, for a large part, in distinct functional roles.

At a fundamental conceptual level, it has been suggested that ID-related function may arise from the specific disorder type, from inter-conversion of IDed forms or from transitions between IDed and ordered forms (Dunker et al., 2002a; Dunker and Obradovic, 2001; Uversky, 2002a).

Although IDPs fail to form fixed 3D-structures under physiological conditions, they carry out numerous important biological functions (Daughdrill et al., 2005; Dunker et al., 2002a; Dunker et al., 2002b; Dunker et al., 2005; Dunker et al., 1998; Dunker et al., 2001; Dunker and Obradovic, 2001; Dyson and Wright, 2002; Dyson and Wright, 2005; Fink, 2005; Fuxreiter et al., 2004; Iakoucheva et al., 2002; Radivojac et al., 2007; Tompa, 2002; Tompa, 2005; Uversky, 2002a; Uversky, 2003b; Uversky et al., 2000; Uversky et al., 2005; Vucetic et al., 2007; Wright and Dyson, 1999; Xie et al., 2007a; Xie et al., 2007b). These IDP-associated functions can be grouped into four broad classes: (1) molecular recognition, (2) molecular assembly, (3) protein modification, and (4) entropic chain activities (Dunker et al., 2002a). For example, in living organisms, proteins are involved in complex interactions, which represent the mechanistic foundation for much of the organism's physiology and function. Regulation, recognition and cell signaling involve the coordinated actions of many players. To achieve this coordination, each participant must have unique identification features that can be recognized by the others. For signaling proteins, these features are often within IDRs (Uversky et al., 2005). Thus, IDRs are typically involved in regulation, signaling and control pathways in which interactions with multiple partners and high-specificity/low-affinity interactions are often requisite. Other schemes for classification of IDP-associated functions provide complementary insights into these proteins (Tompa, 2002; Tompa, 2005).

Molecular recognition features

Molecular recognition features (MoRFs) are short motifs within IDRs that impart specificity to protein-protein interactions. A prototypical MoRF could be characterized as a short region of order promoting residues flanked by IDRs. Important aspects of MoRFs are: they fold upon binding, they contain some degree of preexisting (preformed) structure and some examples can be predicted from sequence. These three aspects are discussed in the following sections.

Coupled folding and binding—A feature of many IDRs is their ability to fold under certain conditions. Although ID often exists as dynamic structural ensembles without fixed tertiary structures, there is evidence that many IDRs undergo coupled folding and binding (Daughdrill et al., 2005; Dunker et al., 2002a; Dunker et al., 2002b; Dunker et al., 2005; Dunker et al., 2001; Dyson and Wright, 2002; Dyson and Wright, 2005; Fink, 2005; Fuxreiter et al., 2004; Iakoucheva et al., 2002; Mohan et al., 2006; Oldfield et al., 2005b; Tompa, 2002; Tompa, 2005; Uversky, 2002a; Uversky, 2003b; Uversky et al., 2000; Uversky et al., 2005; Vacic et al., 2007; Wright and Dyson, 1999). These conformational changes in IDRs may be initiated by alterations in environmental or cellular conditions (e.g. disorder-to-order transition upon signal transduction)(Dunker and Obradovic, 2001; Romero et al., 2004; Uversky, 2003b).

A large decrease in conformation entropy that accompanies such disorder-to-order transitions leads to a combination of high specificity and weak affinity (Schulz, 1979). This phenomenon has the effect of making highly specific interactions that are easily reversible, which are traits highly suitable for interactions involved in signaling and regulation. It has been emphasized that a helical IDR containing a compact hydrophobic cluster (Callebaut et al., 1997) on one face (i.e., a MoRF) is a more suitable candidate for specific interaction with a binding partner than, a relatively featureless, extended IDed region (Cheng et al., 2006b). This is because the hydrophobic cluster would convert one face of the helix into a highly convex surface while its

structured binding partner would necessarily elaborate a complementary concave binding surface such as a hydrophobic pit (Cheng et al., 2006a).

Concomitant binding and folding—Binding by a disordered protein can involve the interaction of a localized region, followed by concomitant binding and folding (Pontius, 1993), which was also called zippering (Dunker et al., 1998). The idea of zippering was proposed because some 3D structures simply appeared not to be able to form all at once, but seemed required some type of step-wise association. Indeed, interactions such as those involving $p27^{kip1}$ (Russo et al., 1996) and the protein phosphatase inhibitor 2 (Hurley et al., 2007) must involve this type of mechanism because the disordered protein wraps around its partner, and so the many sites of interaction simply cannot happen without concomitant binding and folding.

Shoemaker et al. (Shoemaker et al., 2000b) studied concomitant binding and folding interactions from a theoretical point of view and suggested the name “fly-casting effect.” Kinetic studies on the associations of p27kip1 (Lacy et al., 2004) and CREB (Sugase et al., 2007) with their binding partners both provide strong support for such mechanisms.

Preformed structure—An alternative to concomitant binding and folding is a conformational selection mechanism in which the disordered protein samples a number of conformations, and the binding partner selects the member of the ensemble that has the best fit to its own structure (Karush, 1950; Pauling, 1940). If the structure preferred for binding is substantially over-represented in the structural ensemble, then this mechanism is said to involve preformed structure.

Recent studies of ID-mediated binding, both experimental and computational, have shown that the IDed partner often exhibits such a conformational preference for the structure it will adopt upon binding and that most of these so-called “preformed elements” studied to date have been helices (Bourhis et al., 2004; Cheng et al., 2007; Fuxreiter et al., 2004; Garner et al., 1999; Kriwacki et al., 1996; Lacy et al., 2004; Mohan et al., 2006; Oldfield et al., 2005b; Sivakolundu et al., 2005; Vacic et al., 2007).

Based on these observations it was concluded that, although folding of IDRs could be template-driven (i.e., folding upon a structured partner), the IDRs often possess inherent conformational preferences that could determine the binding affinity. This means that many IDPs do not necessary undergo extensive and energetically demanding structural rearrangements prior to binding to their partner, as they possess specific residual structure pertinent to their final conformations in the bound state (Fuxreiter et al., 2004). Interestingly, it has been recently shown that two mechanisms for the induced folding, folding before binding and binding before folding, can realized for a single protein, depending on the conditions (Onitsuka et al., 2008). Because preformed structure decreases the conformational entropy that subtracts from the binding energy (i.e., decreased disorder-to-order transition), the binding affinity of such interactions would vary depending on the amount of preformed structure present (Schulz, 1979). Thus it is likely that binding affinities of MoRFs extend along a continuum of values in a direct relationship with the amount of preformed structure present.

Examples of preformed structure have been observed by many groups using a variety of methods. For example, using solution NMR spectroscopy it has been shown that the IDed $p27^{Kip1}$ kinase inhibitory domain (p27-KID) (Bienkiewicz et al., 2002; Lacy et al., 2004; Russo et al., 1996) possesses several segments of preformed structure (Sivakolundu et al., 2005). This example seems to involve a mixture of conformational selection, concomitant binding and folding, and preformed structural elements. Residual secondary structure has been demonstrated for a number of IDPs, including α-synuclein (Bussell and Eliezer, 2001; Uversky,

2003a; Uversky et al., 2001), β- and γ-synucleins (Uversky, 2002b), FlgM (Daughdrill et al., 1997; Daughdrill et al., 1998), CREB (Hua et al., 1998), p53 (Lee et al., 2000), GCN4 (Hollenbeck et al., 2002; Zitzewitz et al., 2000) and calpastatin (Mucsi et al., 2003).

The well-studied p53-Mdm2 interaction (Wasylyk et al., 1999) nicely illustrates the concept of preformed structure. Mdm2 binds to a short stretch of the p53 transactivation domain. Although X-ray crystallographic studies of the p53-Mdm2 complex reveal that the region of p53 that binds Mdm2 forms a helical structure that is situated in a deep groove on the surface of Mdm2 (Kussie et al., 1996), NMR studies show that the unbound N-terminal region lacked fixed structure, although a region within it does form of an amphipathic helix some fraction of the time (Lee et al., 2000). The structure observed in solution was highly similar that of the bound state (Lee et al., 2000).

Computational MoRFs and experimentally determined preformed structure are related features but the degree of overlap between the two sets has not yet been determined. On one hand, there are examples of predicted MoRFs that also have preformed structure (i.e., p53, above). But on the other hand, not enough preformed structures have been elucidated to date to allow a conclusive study. It is likely that the performed structure content of MoRFs exists along a continuum from little or none to that almost completely matching the bound state.

Prediction of molecular recognition features—Extension of the observation mentioned above that short regions of PONDR® VL-XT predicted order bounded by predictions of regions of ID (i.e., MoRFs) often corresponded to protein-protein interaction sites (Garner et al., 1999) led to the development of a predictor of α- helical molecular recognition elements (α-MoREs, a subset of MoRFs) (Oldfield et al., 2005b). α-MoREs form α-helical structure in the bound state. Application of this algorithm to proteomes and protein databases revealed that α-MoREs likely play important roles in protein-protein interactions involved in signaling (Oldfield et al., 2005b). Furthermore, eukaryotic proteins were found to contain ~7- and 10-fold more predicted α-MoRFs than bacteria and archaea, respectively, consistent with the increased complexity of signaling networks in eukaryotes. Indeed, 27% of the human proteome and > 50% of signaling proteins were predicted to contain α-MoREs (Oldfield et al., 2005b). Recently, by enlarging the training set through the identification of increased numbers of structured α-MoRF examples in PDB and by enlarging the training set still further by addition of homologous sequences, a second-generation α-MoRF predictor was constructed. This newer predictor achieved a sensitivity of 0.87 ± 0.10, a specificity of 0.87 ± 0.11, and an overall accuracy of 0.87 ± 0.08 as estimated ten-cross-validation (Cheng et al., 2007). Further studies have revealed the existence of structural subtypes in addition to α-MoRFs. A survey of RCSB Protein Data Bank yielded abundant examples of β- MoRFs and ι-MoRFs that form β-strands and irregular structures upon binding, respectively (Mohan et al., 2006; Vacic et al., 2007).

An example of how the α-MoRE predictor has been applied to help elucidate sites of protein-protein interaction is discussed in relation to RNase E (below).

Summary of ID

Taken together, the studies cited above suggest that, in addition to fibrous and globular water-soluble proteins, there is another functional group, namely proteins or regions of proteins that are IDed and that are more appropriate and more frequently used for signaling, regulation, and control. Thus, a second paradigm was proposed to augment the one that opened this section: sequence → IDed-ensemble → function for proteins and protein regions involved in signaling, regulation and control (Dunker and Obradovic, 2001; Uversky et al., 2005). (Note that, as discussed above, folding upon binding and the existence of preformed structure modify the transition from IDed ensembles to function.) Taken together, these two paradigms illustrate

the complementarily of the functional diversity provided by IDRs with the structural diversity provided by ordered protein regions.

Scaffold proteins

Scaffold proteins selectively bring together specific proteins within signaling pathways to facilitate and promote interactions between them. More specifically, 'bringing together' includes temporal, spatial, orientational and contextual aspects such that interactions between the bound proteins reflect the specific needs of the cell at a particular point in time. Scaffolding can influence the specificity and kinetics of signaling interactions. While the term scaffold conjures up a rigid structure, and in many cases this is true, this review focuses on the IDed aspects of scaffold proteins. To this end, several examples will be presented that illustrate the many ways that ID contributes to scaffold function.

Scaffold proteins are a hub subclass that have a modest number of interacting partners but additionally provide selective spatial orientation and temporal coordination to facilitate interactions among bound partners. Scaffold proteins can play a role in modulating the activation of alternative pathways by promoting interactions between various signaling proteins as observed for Rho GTPases (Jaffe et al., 2004; Marinissen and Gutkind, 2005) and p53 (Hohenstein and Giles, 2003).

Scaffold proteins typically contain several domains for protein-protein interaction and control the flux through signal transduction pathways and interactions between pathways by modifying the activity of individual partners, the relative position between bound partners, the composition of bound proteins and the localization of the holoscaffold. When involved in multiple pathways, scaffolds often provide coordination of alternate signal paths. Scaffolds can bind simultaneously to multiple participants in a particular pathway and facilitate and/or modify the specificity of pathway interactions (Liu et al., 2006). Scaffolds may act on individual proteins by changing their conformation and thus their activity and on interaction partners by providing proximity and spatial orientation (Liu et al., 2006). Some scaffolds create focal points for spatial and temporal coordination of enzymatic activity of kinases and phosphatases (Dhanasekaran et al., 2007). Modulation of the phosphorylation state of downstream members of signal transduction pathways is a primary mode of their action. Compartmentalization is provided by the fact that the activity of bound members is directed towards neighboring substrates that may or may not be bound to the scaffold. Enzymes may be activated or inhibited upon association with the scaffold. Associations are dynamic and may serve to coordinate the responses among pathways.

Hub proteins and ID

Metabolic and protein signaling networks within living cells, the so called 'interactome', share a commonality with airline routes and the World Wide Web: they are all examples of so-called scale-free or small-world networks [reviewed in (Goh et al., 2002)]. The basic feature that separates these networks from completely regular (resembling lattices with invariant nodes and links) or completely random networks (which are characterized by the stochastic variations in the number of links at the nodes and in the distances from one node to the next) is the presence of hubs; i.e., highly connected nodes that have hundreds or thousands of links and "ends" that have only a single connection (Goh et al., 2002; Watts and Strogatz, 1998). This high connectivity allows signals to move from any point of origin to any other node in the network with minimal internode traversals. However, although the error tolerance of such networks is high, the failure of hubs quickly leads to the breakdown of connectivity, suggesting that the scale-free networks are particularly resistant to random node removal but are extremely sensitive to the targeted removal of hubs (Albert et al., 2000; Jeong et al., 2001).

It was established that ID was a key feature of some hub proteins and the partners of some structured hub proteins (Dunker et al., 2005). The rationale that drove this hypothesis was that the plasticity of IDPs enables a single protein to bind to a large number of partners (Dunker et al., 1998; Dunker and Obradovic, 2001; Dyson and Wright, 2002; Dyson and Wright, 2005; Romero et al., 1998; Wright and Dyson, 1999) or facilitate the binding of multiple proteins to a common partner (Dunker et al., 2005; Dyson and Wright, 2005; Uversky et al., 2005). The general importance of ID in hubs or hub partners has since been further supported by additional bioinformatic studies (Bustos and Iglesias, 2006; Dosztanyi et al., 2006; Ekman et al., 2006; Haynes et al., 2006; Higurashi et al., 2008; Kim et al., 2008; Oldfield et al., 2008; Patil and Nakamura, 2006; Radivojac et al., 2006; Singh et al., 2007).

Using a bioinformatic approach, it was recently shown that there was a positive correlation between the number of partners and the presence of predicted IDRs in a complete genome (Schlessinger et al., 2007). In this study, IDRs were predicted using the established DISOPRED2 (Jones and Ward, 2003; Ward et al., 2004a; Ward et al., 2004b), IUPred (Dosztanyi et al., 2005) and a neural net-based predictor, NORSnet, that was trained to recognize the differences between loops with and without structure (Schlessinger et al., 2007). A set of 2,622 *Caenorhabditis elegans* proteins that had been characterized as interacting with at least one partner (Kerrien et al., 2007; Li et al., 2004) was analyzed using the three predictors. The results indicated that the presence of IDRs and the number of interacting partners was positively correlated in the dataset. It was estimated that 50 – 70% of proteins with seven or more interaction partners contained IDRs. The researchers concluded that the attribute most relevant to hub function was the presence of unstructured loops (Schlessinger et al., 2007). This finding of linkage of ID and interactivity likely applies to scaffold proteins as well as hubs in general.

Structure/ID interplay in scaffolds

ID and structure are often complementary partners in protein-protein interactions. Examples of this interplay are frequently found among scaffold proteins and their partners.

Modular domains that bind short linear motifs—Interactions between scaffold and individual partners are often mediated by domains that bind to small linear motifs such as Src-homology-2 (SH2), Src-homology-3 (SH3) and PDZ domains. These domain are encoded by 50 – 100 amino acids, recognize sequences ~5–10 residues in length and fold independently such that they retain full and specific binding activity when expressed by themselves (Hung and Sheng, 2002; Pawson, 1995).

Sh2 domains recognize and bind selectively to phosphotyrosine-containing sequences. They have high affinity to peptides in which the residues C-terminal to the phosphorylated tyrosine match the target specificity of the particular SH2 domain, much less affinity to phosphorylated peptides that do not match the target sequence and essentially no affinity to non-phosphorylated peptides (Pawson, 2004). SH2 domains are explicitly involved in pathways that utilize tyrosine kinase signaling (Pawson, 2004).

Target sequences of SH3 domains do not need to be phosphorylated but instead must contain one or two prolines (Pawson, 2004). The SH3 binding interaction is pseudo-symmetrical in that it has one binding mode in which the interacting chains are parallel and another in which the chains are antiparallel (Pawson, 2004). This pseudo-symmetry is enabled by the requirement that the target sequence adopt a polyproline II helical conformation (Pawson, 2004). Each SH3 domain/peptide conformation has its own preferred binding mode but some combinations will bind in either mode under physiological conditions (Ma et al., 2006). Individual SH2 and SH3 domains may have multiple physiological partners and a single protein

may contain both types of domains, thus the simple binding modes of these domains can be multiplexed to meet the requirements of complex signaling pathways (Pawson, 2004).

The PDZ domain binds in a sequence specific manner to the four C-terminal residues of a protein chain although, in some instances it can bind internal recognition sequences (Brenman et al., 1996a; Brenman et al., 1996b). One feature of PDZ domains is that several of them may be present in a single protein, often arranged in tandem arrays. It is thought that multiple PDZ domains can cooperate to enhance binding of two partners (Hung and Sheng, 2002). PDZ domains are among the commonest of known domains with the human proteome containing at least 440 PDZ domains in 259 different proteins (Hung and Sheng, 2002).

All three of these linear sequence-binding domains are obviously very important and useful for intracellular signaling and, not surprisingly, examples of their application are found amongst the IDed scaffold examples herein. An example of a PZD-mediated interaction is included in the section describing voltage-activated potassium channels and examples of SH3-mediated binding are included in the section describing the MAP2 and titin scaffolds.

Helical repeats and IDed scaffolds—Helical repeats are a class of protein structures especially suited for binding linear motifs and thus can contribute to scaffold functionality when used in conjunction with IDRs.

Helical repeat types include the armadillo (Peifer et al., 1994), the HEAT (Andrade and Bork, 1995), the tetratricopeptide (Zhang et al., 1991). As the name implies, the structural unit of helical repeats consists of an assembly of tandem repeats of alpha-helical structure that form super helical tertiary structures about a common axis (Andrade et al., 2001; Groves and Barford, 1999). While details of these super helical structure differ among the different types, all contain an extended and conformationally rigid core which elaborates extensive internal and external surfaces that can mediate protein-protein interactions (Andrade et al., 2001; Groves and Barford, 1999). While each type exhibits conserved motif packing, the residues not involved in structure formation can vary independently such that multiple specific binding sites can be encoded by a single helical repeat structure. In this way, helical repeats lend themselves to scaffold function (albeit of the structured variety)(Main et al., 2003). Implications for signaling function is evident in the higher incidence of helical repeat structures in eukaryotes compared to prokaryotes (Marcotte et al., 1999).

Examples of the use of helical repeats in scaffold interactions described in this review are the interactions between ATM and ATR (both HEAT repeat proteins) with the internal IDR of BRCA1 and PP2A (HEAT) and β-catenin (armadillo repeat) with the internal IDR of the axin scaffold. In all these interactions, the structured partner interacts with an IDR of the scaffold. Helical repeat domains are generally quite large. Members of the phosphatidylinositol-3 -OH kinase-like kinase (PIKK) family, of which ATM and ATR are members, are typically 270 to 450 kDa but the kinase domain accounts for only 5 to 10% of the total sequence with the remainder forming the tandem HEAT repeats (Perry and Kleckner, 2003). This arrangement serves to reduce the size and complexity required of the scaffold, which only needs to provide an IDed fragment to the interaction, thereby allowing increased functionality for a given-sized protein.

Thus helical repeats and modular interaction domains coupled with short IDRs represent a natural pairing of structure and ID, which together facilitate scaffold functionality encompassing at least one IDed partner.

ID-facilitated scaffold function

The following examples examine the different roles that ID plays in the function of scaffold proteins. Examples of ID-based scaffold function range from simple linkers to major conformation changes in large IDed scaffolds.

Molecular recognition features: RNase E

High-specificity low-affinity interactions are enabled by IDRs that contain short stretches of order-promoting residues. RNase E provides a well-documented and clear example of a scaffold with multiple binding regions within a predominantly IDR that contains multiple short stretches of residues with a propensity for structure formation. As mentioned above, these regions are known as MoRFs.

RNase E is a prokaryotic endonuclease that functions as the scaffold for the RNA degradosome and is involved in RNA processing and degradation. While the N-terminal half of Rnase E encodes the ribonucleolytic activity, the C-terminal region (CTR) serves as a scaffold for organizing the other main degradosome components (Carpousis, 2002). The CTR has been shown by far-UV circular dichroism and small-angle x-ray scattering to be IDed (Callaghan et al., 2004) while the catalytic N-terminal domain is likely structured (Callaghan et al., 2003). This is structural profile is apparent in the PONDR® VL-XT prediction of RNase E (Fig. 1). Despite being mostly unstructured, the CTR contains four regions that are predicted by PONDR® VL-XT to have increased propensity for structure formation (Fig. 1)(Callaghan et al., 2004). Characteristic downward pointing spikes within predicted IDRs of PONDR® VL-XT plots indicate stretches of order propensity (i.e., MoREs) and have long been recognized to represent regions likely to be involved in protein-protein interactions (Garner et al., 1999). These four regions hold the key to the scaffolding function of the CTR. While the intervening IDed residues provide flexibility, the regions with structural propensity serve as binding sites for other members of the degradosome. Indeed, an enolase, a polynucleotide phosphorylase, RhlB and homodimerization have been associated with the four structured segments (Fig. 1)(Callaghan et al., 2004). The presence of these small structured binding regions within a larger segment of ID makes RNase E an especially well-documented example of ID-facilitated molecular recognition (Uversky et al., 2005). In RNase E, the flexibility imparted by the interspersed IDRs is thought to persist in the assembled complex (Callaghan et al., 2004). This attribute may be required for the degradosome to function across a diverse range of RNA structural forms.

This example of the utility of applying ID prediction to the discovery of binding regions within RNase E illustrates the possibility using bioinformatic methods to drive the elucidation and characterization of protein-protein interaction sites.

Fly-casting: Voltage-activated potassium channels

The fly-casting effect, first hypothesized as a mechanism for folding of IDRs upon binding, promotes interactions between the IDR and its partner by first binding weakly and non-specifically to its partner then folding as it approaches its cognate binding conformation (Shoemaker et al., 2000a). The mechanism has recently been explored experimentally in detail using the phosphorylated kinase inducible activation domain of CREB as a model (Sugase et al., 2007).

The example of ID-related scaffold function presented here does not pertain to the scaffold itself but rather to one of the proteins organized by the scaffold. Post-synaptic density protein 95 (PSD-95) organizes receptors and other signaling proteins in the postsynaptic density of neuronal excitatory synapses and ultimately determines the size, structure and plasticity of the

synapse by modulating the organization of the postsynaptic signaling complex (Kim and Sheng, 2004). Multimerization of PSD-95 provides for the clustering of large assemblies of proteins involved in synaptic function (Kim and Sheng, 2004). Of particular interest here is the well-documented association of voltage-activated potassium channels (Kv) channels with the PSD-95 scaffold.

A fly-casting effect is elaborated when a binding site is located at one end of an IDed chain that extends from the main body of the protein. The functional consequence of this combination has been likened to fishing rod that consists of an extended string (the IDed chain) with a hook (the binding site) at its tip (Magidovich et al., 2006; Magidovich et al., 2007). Well-documented examples of this structural motif can be found among voltage-activated potassium (Kv) channels. A high proportion of the Kv channels in families 1, 3 and 4 contain a C-terminal PDZ binding motif separated from the pore-forming domain by an IDR (Magidovich et al., 2006).

Kv channels are crucial for the formation of action potential in neurons. These gated channels are abundant in axons, pre-synaptic terminals, nodes of Ranvier and dendrites (Kim et al., 1995). Kv channels are modular proteins composed of several domains including a ball-and-chain inactivation domain, a tetramerization domain, pore-forming domains and a predominantly IDed intracellular C-terminal region. The pore is composed of six transmembrane segments, one of which serves as a voltage sensor that triggers the conformational change that opens the pore in response to a change in membrane potential. The C-terminal region of the Shaker Kv channel monomer is predicted to be IDed (Fig. 2 B). This prediction agrees with the lack of observed electron density in this region of the crystal structures of the prototypical shaker Kv channel (Long et al., 2005; Sokolova et al., 2003) and with hydrodynamic and spectroscopic findings (Magidovich et al., 2007).

The terminal C-terminal PDZ motif was shown to be responsible for the interaction of the channel with the PSD-95 scaffold, an association that promotes the channel clustering that is crucial for the proper synapse function (Kim et al., 1995; Tejedor et al., 1997; Zito et al., 1997). The result of this interaction is that the Kv channel is anchored at a specific location in the membrane via an IDed linker attached to the internal scaffold (Fig. 2 A) (Magidovich et al., 2006; Magidovich et al., 2007). This model was recently supported by the structural and functional analysis of the C-terminal segment (residues 513–655) of the archetypical *Shaker* Kv channel (ShB) (Magidovich et al., 2007). This analysis revealed that, although the isolated C-terminal region of ShB is IDed protein and behaves as a random coil, it was able to bind its protein scaffold partner and support protein clustering *in vivo*, indicating that the observed ID is compatible with PDZ-mediated binding (Magidovich et al., 2007).

Although the fly-casting mechanism is primarily thought of as a means of increasing the speed of association (Shoemaker et al., 2000a), ID chains have a larger hydrodynamic radius than ordered chains and therefore manifest increased capture radii. Here, the increased capture radius may be relevant for forming Kv channel/PSD-95 contacts. If the distance between the membrane-bound Kv-channel and the internal scaffolded complex containing PSD-95 may be such that the two proteins would not achieve the proximity necessary for contact without the increased capture radii provided for my the fly-casting mechanism Fig. 2 A).

It is interesting to note that ID also plays a crucial role in Kv channel inactivation. This inactivation relies on an intra-molecular ball-and-chain timing mechanism (Hoshi et al., 1990) in which an N-terminal inactivation ball, tethered by a long IDed chain, randomly searches for the open activation gate and subsequently binds in the hydrophobic binding pocket of the pore's central cavity, thus blocking potassium current from moving through the channel (Fig. 2 A) (Zhou et al., 2001). Therefore, the above described N- and C-terminal IDed segments

of the Kv channel (Fig. 2 B) exemplify two distinct roles of ID in the function of this protein: whereas the N-terminal IDed inactivation segment serves as an entropic clock that modulates the kinetics of inactivation, an IDR at the C-terminus provides orientational freedom for searching and connecting to scaffold protein partners (Magidovich et al., 2006).

Ease of encounter complex formation: Voltage-activated potassium channels

The ID in the C-terminal region of Kv channels makes an additional contribution to binding dynamics. The C-terminal IDR of a Kv channel was shown to modulate the dynamics of PSD-95 binding (Magidovich et al., 2007). The fact that ID in the C-terminal segment of ShB modulates its interaction with the PSD-95 protein was further supported by pull-down experiments involving C-terminal chains that differed in flexibility or length.

How can ID proximal to binding motifs serve to modulate protein-protein interactions? IDRs can serve to increase the association rate of the partners by providing weak non-specific interactions that serve to keep the two partners in an ‘encounter complex’ until subsequent spatial rearrangements result in the proper orientation for specific binding (Pontius, 1993; Shoemaker et al., 2000a). In addition, unstructured polymeric domains are more likely to provide the weak attractive forces necessary to maintain an encounter complex regardless of the orientation in which the molecules initially collide (Pontius, 1993). Obviously, this trait would play less of a role in the binding interactions between two rigid partners.

Structural isolation of partners: Voltage-activated potassium channels

In addition to contributing to binding dynamics, the IDed linker/PDZ domain functional motif is hypothesized to serve as a means to tether Kv channels together in membrane rafts (Kim et al., 1995). This clustering may be essential for synapse function. PSD-95 scaffold is associated with a large number of proteins of the scaffolded postsynaptic signaling complex (Kim and Sheng, 2004). The relatively rigid nature of this complex may not be compatible with synapse function. Since Kv channels function in the fluid membrane but are organized by the more rigid PSD-95 and associated intracellular scaffold components, the IDed linker between the scaffold-ound PDZ domain and the pore body may provide the structural isolation necessary for the membrane and associated proteins to form and maintain a functional synaptic cleft (Fig. 2 A). Testing this hypothesis could be accomplished by applying a similar approach used to ascertain the timing function of the IDed N-terminal linker of Kv channels (Hoshi et al., 1990) (i.e., by changing the length of the linker and assaying for altered function).

Modulation of interactions between bound partners: Axin

The flexibility of IDRs between sites where partners are bound can allow scaffolds to undergo conformational changes that result in promotion of interactions between bound partners. Two of the pathways that axis inhibition protein 1 (axin) functions as a scaffold likely make use of this ID-mediated functionality.

Axin is a multi-domain tumor suppressor that functions as a scaffold for four different pathways: the Wnt (Hart et al., 1998), the transforming growth factor β (TGF-β) (Furuhashi et al., 2001), the c-Jun NH2-terminal/stress-activated protein kinase (JNK) (Zhang et al., 1999) and the p53 signaling pathways (Luo and Lin, 2004; Rui et al., 2004; Salahshor and Woodgett, 2005; Tolwinski and Wieschaus, 2004). Here we review the roles that ID plays in support of the scaffolding functions of axin.

There are several lines of evidence that axin contains a substantial amount of ID. The IDed nature of the N-terminal half of axin was exploited to isolate the crystallizable fragment that binds adenomatous polyposis coli protein (APC). An axin fragment spanning residues 9 through 316 was produced by thrombin digestion and was further digested with elastase, a

frequent cutter, to arrive at a 147-residue fragment that crystallized alone and with the corresponding binding region of APC (Spink et al., 2000). This region of structure evidently resides in an otherwise IDed region that is sensitive to proteolytic cleavage (Fig. 3). Additionally, the region spanning amino acids 500 to 800 was found to be protease sensitive and to run at a larger molecular weight than expected size on gel filtration columns (Bill Weis, personal communication). Proteins and fragments with substantial amounts of extended ID tend to migrate slower than globular proteins when subjected to gel filtration (Iakoucheva et al., 2001). The regions of PONDR® VSL1 predicted ID in axin are in good agreement with the regions of experimentally characterized order and ID described above (Fig. 3).

Although not used here to illustrate contributions of ID to scaffold function, for completeness we mention that axin serves as a scaffold is for TGF-β signaling. TGF-β signaling is a key pathway involved in the regulation of developmental processes such as cell proliferation, differentiation, apoptosis, and determination of cell fate (Fig. 3)(Furuhashi et al., 2001). The three other pathways that axin is known to function a scaffold are described below along with a discussion of the functionality provided by ID.

Axin plays a critical role as a scaffold for the canonical Wnt growth factor signaling pathway. The Wnt signaling pathway is important for regulation of developmental processes such as polarity, cell proliferation, differentiation, motility and morphogenesis in vertebrates and invertebrates (Wodarz and Nusse, 1998). It follows that dysregulation of the Wnt signaling pathway has been implicated in a large variety of cancers (Polakis, 2000; Salahshor and Woodgett, 2005). Axin acts a scaffold to coordinate interactions among several proteins involved in the canonical Wnt signaling pathway, including β-catenin, APC, casein kinase I (CKI), protein phosphatase 2A (PP2A), and glycogen synthase kinase 3 β (GSK-3 β) in order to regulate the stability of β-catenin.

The potential role of ID in the Wnt signaling pathway becomes apparent when the location of the majority of binding sites is taken into consideration along with observations of ID within axin. APC binds to an ordered region near the N-terminus, GSK-3β and β-catenin at two sites in the central region and CKI and PP2A interact with regions in the C-terminal third of axin (Fig. 3). The substantial amount of ID in axin may contribute to scaffolding functionality by allowing conformational changes that serve to bring together the members of the Wnt signaling pathway in the proper orientations required to facilitate efficient interactions.

ID likely plays a role the scaffolding function of axin in the p53 signaling pathway as well. Like the Wnt and TGF-β signaling pathways, p53 signaling is known for its roles in cell growth, development, apoptosis and tumor suppression (Rui et al., 2004). DNA damage induces the formation of a ternary complex of axin, homeodomain-interacting protein kinase-2 (HIPK2) and p53. Subsequent phosphorylation of p53 by HIPK2 stimulates its activity (D'Orazi et al., 2002; Hofmann et al., 2002). Scaffolding by axin has been shown to greatly increase the p53-dependent transcription and apoptosis in response to DNA damage (Rui et al., 2004). In the scaffolded complex, axin interacts with p53 from two distinct pools: the first being the p53 bound independently by HIPK2 that, in turn, binds to the N-terminal half of axin and the second being the p53 that binds directly to a binding region located in the C-terminal half of axin (Fig. 3 and Fig. 4) (Rui et al., 2004). Given the distance between these two regions, it was proposed that a conformational change in axin occurs upon binding one or both pathway members that brings the two into the proximity and spatial orientation necessary to promote their interaction (Fig. 4) (Rui et al., 2004). Thus the putative IDed and therefore flexible nature of the central region of axin likely facilitates the conformational changes that contribute to this scaffolding function.

Masking of intramolecular interaction sites: Axin

In addition to enabling conformational changes that promote interactions between bound partners, ID likely allows axin to mask binding sites depending on what other proteins are already bound to the scaffold.

The JNK pathway is the fourth pathway in which axin participates as a scaffold (Luo et al., 2005; Zhang et al., 2000). Axin-mediated JNK activation requires mitogen-activated protein kinase kinase kinase (MAP3K) activity, which can be provided by either MEKK1 or MEKK4. These two kinases compete for binding to axin even though their respective binding sites, which are separate and distinct (Fig. 3)(Luo et al., 2003). Additionally, a region between the two MAP3K binding sites was shown to be essential for activity (Luo et al., 2003). The redundancy of the MAP3K requirement for JNK signaling likely reflects a subtle variation in signaling that has yet to be elucidated (Luo et al., 2003). This observation, along with the competitive nature of MAP3K binding and the distance between the two binding sites, again suggests the possibility that significant conformational changes in axin can be induced by binding events, with the flexibility of the IDRs playing a key role in scaffold function.

Binding of MEKK1 to axin can also be inhibited by GSK-3 β and CK1 (Zhang et al., 2001). In the case of GSK-3 β, the mechanism is evidently not binding site competition because the binding sites are distinct (Luo et al., 2005). A conformational change in axin upon GSK-3 β binding, possibly mediated by ID, may inhibit MEKK1 binding by modifying the accessibility of recognition features of the binding site or by physically blocking access to the binding site (Zhang et al., 2001). These inhibitory activities may function to coordinate the separate pathways that axin scaffolds (Zhang et al., 2001).

Speculations by researchers that axin undergoes conformational changes in order to mediate interaction or exclusion of proteins bound at different regions of the protein (Luo et al., 2005; Rui et al., 2004; Wong et al., 2004) lead us to suggest that this largely IDed and therefore highly flexible molecule functions at a higher level than would be expected from an assemblage of binding sites connected by linkers or so-called ‘beads on a string’ (Evans and Owen, 2002). Association of the flexible central region of axin with various protein partners likely imparts a certain amount of defined, but dynamic, structure to the complex. Axin probably adopts different structural conformations depending on which partners are bound. These conformations may serve to exclude or promote binding of additional partners within the same pathway (i.e., MEKK1 and MEKK4 in the JNK pathway) (Luo et al., 2003) or from other pathways (i.e., GSK-3 β and MEKK1 in the Wnt and JNK pathways) (Luo et al., 2005).

The concept of a scaffold undergoing significant functional conformational changes can be thought of as extending the scope of the phenomenon of polymorphisms in the bound state in bi-molecular interactions as described in the section 3.9. These axin examples document conformational changes in a scaffold protein that serve to modulate interactions between scaffold partners that take place at distinctly different sites.

Maximized interaction surface per residue: Axin and BRCA1

Another large IDed scaffold merits discussion along with axin in the context ID-mediated maximization of surface area per residue. Axin was described in the previous section and BRCA1, introduced below, shares many similarities. A discussion of the implications of the large IDRs within these proteins on their scaffolding functionality follows the description of BRCA1.

The differential response of breast cancer associated gene 1 product (BRCA1) to different types of DNA damage is an excellent example of how signaling scaffolds function to modify signal flow in pathways. BRCA1 activates one of two kinases as appropriate depending on the type

of damage detected. BRCA1 is involved in many diverse biological signaling processes such as DNA damage response (DDR) (Wang et al., 2000), transcription (TRANS), cell-cycle checkpoint control (CCCC), tumor suppression (TS), oncogenesis (ONCO), stress response and apoptosis (Fig. 5)(Deng, 2006; Venkitaraman, 2002). It is important to realize that efficient DDR necessitates that every instance of damaged DNA must trigger cell cycle arrest (Harrison and Haber, 2006) and thus tight association of upstream sensors and sensitive initial response are required. Both these requirements can be achieved by scaffolding.

BRCA1, like axin, consists of structured domains at the N- and C-termini that are separated by a large internal ID region that comprises 80% of the residues (Mark et al., 2005) (Fig. 5). This 1480 amino acid region has been structurally characterized as being IDed using NMR and CD spectroscopy (Mark et al., 2005). The PONDR® VLS1 prediction of ID in BRCA1 is in good agreement with these experimental data (Fig. 5). The IDed central region contains binding sites for DNA as well as several protein partners such as p53, retinoblastoma protein, BRCA2; the oncogenes c-Myc and JunB; DNA damage repair proteins such as Rad50 and Rad51; and the Fanconi anemia group A protein (Mark et al., 2005) (Fig. 5).

In all, more than 50 proteins interact with BRCA1, including a variety of DNA damage sensors, DNA repair proteins and signal transducers (Deng, 2006; Venkitaraman, 2002; Wang et al., 2000). Two important proteins in the DNA damage checkpoint pathways are ATM and ATR. Both are members of the phosphatidylinositol 3’-kinase-related kinase (PIKK) family and are responsible for initial phosphorylation events in response to various DNA lesions. BRCA1 plays a crucial role in the p53 response with the decision between apoptosis or cell-cycle arrest being mediated by the presence or absence of additional interacting proteins on the BRCA1 scaffold (Hohenstein and Giles, 2003). Double-stranded breaks activate ATM while lesions producing single-stranded breaks activate ATR. Both these PIKKs associate with the BRCA1 scaffold and interact with a subset of their non-DNA-associated downstream phosphorylation targets. The increase in signaling efficiency provided by scaffolding is illustrated by the finding that the phosphorylated forms of several of BRCA1 partners were either undetectable or substantially delayed in BRCA1 deficient cells subjected to damaging levels of either UV or IR radiation (Foray et al., 2003). Additionally, efficient phosphorylation of essentially the same set of substrates by two different PIKKs, ATM and ATR, through association of the upstream and downstream pathway components, suggests that conformational changes such as occur in axin may play a role in BRCA1 function.

Both axin and BRCA1 are large mostly IDed scaffolds that accommodate a substantial number of partners. (Fig. 3 and Fig. 5). Only a few of the interaction sites of axin and BRCA1 have been highly resolved by structural studies. Nevertheless, it is obvious that the sites provided for the diverse partners that assemble on these scaffolds must, in general, be specific and distinct. It seems counterintuitive that a highly structured protein could accommodate the requirements for the large numbers of distinct binding sites that have been identified for these two scaffolds, however, such an expectation is consistant with the capabilities of IDPs.

What structural feature endows axin and BRCA1 with the ability to bind and coordinate a large number of partners and consequently participate in diverse multiple pathways? The IDR of these proteins demonstrate how ID can economically, in both mass and structure, endow proteins with a high density of sites for protein-protein interactions (Gunasekaran et al., 2003). Many of the known binding sites of axin and BRCA1 are arrayed throughout their large central IDRs (Fig. 3 and Fig. 4). This high density of binding sites is possible because IDed protein-protein interaction sites can be comprised of a few residues within a short linear stretch of sequence. These residues can be either adjacent or separated by a few intervening non-participating residues with additional economies being achieved when adjacent sites overlap [see (Bax et al., 2001; Dajani et al., 2003; Dunker et al., 2005]. Because of their small size,

many such sites can be arranged serially in regions of intrinsic disorder {Dunker, 2002 #70), just as observed in the central region of axin and BRCA1.

A fundamental physical basis for the ability of IDRs of a given length to participate in more interactions than an ordered region of the same length is that, by being extended, unstructured regions expose more surface area per residue (Gunasekaran et al., 2003; Gunasekaran et al., 2004). Calculations reveal that the size of protein required to present a given sized interface area would need to be 2 – 3 times larger for ordered than for IDed proteins (Gunasekaran et al., 2003). This means that IDRs have more surface area available for recognition and binding interactions. Although information content based on spatial position and orientation cannot, in large part, be encoded in an IDR because of constant resampling of different backbone angles and sidechain orientations, increased contribution of atomic recognition features per residue can be realized because of the extended structure. Backbone and sidechain atoms in globular proteins must necessarily contribute to structure and thus are partially buried and therefore unavailable for intermolecular binding interactions. Recognition and binding sites within ID can be composed of sequential residues or adjacent residues upon formation of secondary structure (α- helix, β- sheet, polyproline II helix). Binding sites of globular proteins are most often composed of residues that are adjacent in space but distant in sequence thereby limiting the number of sites that can be formed by a given number of residues (Gunasekaran et al., 2004).

Both axin and BRCA1 may have similar overall structures with the internal regions of each protein, being IDed (Fig. 3 and Fig. 5). Both these scaffolds have a large number of documented interacting partners and it is the authors' belief that this capability is facilitated by ID. Future study of these two scaffolds will yield additional information on ID-mediated scaffold function. So far, different aspects of each scaffold have been studied in detail, i.e., the structural aspects for BRCA1 (Mark et al., 2005) and the interactions between multiple pathways and partners for axin (Luo and Lin, 2004;Luo et al., 2005;Rui et al., 2004).

Toleration of high evolutionary rates: BRCA1

That IDed regions could evolve more rapidly than ordered regions has been demonstrated previously (see below). Relevant here is that BRCA1 is an example that this relationship also plays a role in scaffold evolution.

A bioinformatic study conducted by Brown et al. first revealed that ID may be more permissive to amino acid substitution and thereby be more tolerant to higher evolutionary rates than order (Brown et al., 2002). The study included 464 proteins in 26 families in which at least one member had an experimentally characterized region of ID 30 residues or longer. By aligning the proteins within a family and segregating the IDed regions from the ordered (or uncharacterized) regions and calculating evolution rates for the two region types, it was shown that the IDed regions of 19 of the 26 families evolved more rapidly than the ordered regions (Brown et al., 2002). A thorough study of a single protein family, FG nucleoporins, showed that ID and amino acid composition was conserved but that the sequences of the IDRs diverged at twice the rate of yeast proteins in general (Denning and Rexach, 2007). The FG regions of yeast nucleoporins were previously shown to be IDed by biophysical methods and sensitivity to proteolysis (Denning et al., 2003). In the case of FG nucleoporins, influence of expression level, interactivity and essentiality, additional factors that are known to influence the evolutionary rate of proteins, was ruled out as causes for their rapid evolution (Denning and Rexach, 2007).

As part of a study of the complete BRCA1 loci of human and several non-human primates, it was revealed that the central IDed region evolved more rapidly than the structured RING and BRCT domains (Pavletich, 1999). The overall structure of BRCA1 loci of rhesus macaque,

orangutan, gorilla, chimpanzee and human is conserved in terms of number of exons, exon-intron boundaries and length of longest encoded product (1863 residues). While DNA conservation of the coding region of BRCA1 of five primate species remains above 93% identity over the entire open reading frame, the percentage of amino acid substitutions in the IDed region range from ~95% at the ends to ~85% in the middle of the region. This reduced level of conservation contrasts to the > 95% identity for the structured RING and BRCT regions of BRCA1. Moreover, the ratio of non-synonymous/synonymous substitutions was higher in the human and chimpanzee lineages indication a positive (diversifying) selection. The influence of codon adaptation was ruled out as a driver for this selection. This finding extends a more limited study that found that a large portion of the internal IDed region of BRCA1 encoded by exon 11 had undergone positive selection (Huttley et al., 2000).

The diversifying selection uncovered in these studies indicates that the IDed region of BRCA1 has experienced a higher level of adaptive evolution than the structured regions. One factor that contributes to the higher evolutionary rates that are observed in IDed regions is the fact that the IDed nature (i.e., its flexibility and dynamics) of these regions can be conserved in spite of high amino acid sequence divergence (Daughdrill et al., 2007). This allows tolerance and potential accumulation of mutations that could lead to added functionality. For scaffolds in particular, this could be a major contributor to the complexity and interactivity that are among their most important traits.

Binding site overlap

GSK-3β—The GSK-3 β region that binds to the axin scaffold (discussed above) illustrates how ID regions can adopt specific bound conformations according to templates provided by structured partners. In the case of GSK-3β, this is embodied as an IDed loop with the ability to bind to either of two different structurally diverse partners: FRAT or axin (see below).

The Wnt signaling pathway discussed previously includes another example of ID-based scaffold function but in the context of one of the proteins that binds to the scaffold. GSK-3 β is one of several proteins that assemble on the axin scaffold to form the β-catenin destruction complex (Luo and Lin, 2004; Xing et al., 2003). In this role, axin facilitates the phosphorylation of β-catenin by GSK-3β in the Wnt signaling pathway. Phosphorylation leads to ubiquitination and subsequent degradation of β-catenin thereby keeping its concentration low in non-proliferating differentiated cells (Hart et al., 1998). However, GSK-3β also binds to a second protein, frequently rearranged in activated T-cells (FRAT), with the same 15-residue IDed loop that it uses to bind axin (Bax et al., 2001; Dajani et al., 2003; Dunker et al., 2005). In the context of scaffold function, FRAT competes with axin for binding this loop when stimulated by Wnt signaling. This binding reduces the availability of GSK-3β to the destruction complex and, since GSK3-β is a necessary component of the β-catenin destruction complex, β-catenin will become unregulated.

Several instances of preformed structure in IDRs have been observed (Dyson and Wright, 2002; Kriwacki et al., 1996; Lacy et al., 2004; Sivakolundu et al., 2005; Tsai et al., 2001). Given that IDRs exist as structural ensembles and that the bound states of these regions may often be the most prevalent of the conformer population, the question arises about the conformer populations of IDRs that bind to more than one partner. It would be interesting to determine if the population of conformers that make up the structural ensemble of this IDed loop of GSK-3β contains predominant conformers that are representative of the bound states of both FRAT and axin.

While the versatility of binding displayed by the IDed loop of GSK-3β illustrates that the same IDR can accommodate different structurally diverse partners, the mutual exclusivity of this

binding mode (Dajani et al., 2003) illustrates an elegant way that ID-mediated protein-protein interactions can modulate multiple signaling pathways while avoiding crosstalk between them.

p53—p53 was discussed above as a binding partner of both axin and BRCA1. Here, further examples of the promiscuous binding of this protein are discussed. Binding of the IDed C-terminal regulatory domain of p53 to four different partners serves as an illustrative example of one-to-many signaling interactions. Our recent computational analysis of structures currently in the RCSB Protein Data Bank shows that the same IDR of p53 (residues 374 to 388) forms three major secondary structure types in the bound state depending on which partner it binds: an α-helix when associating with S100ββ (Rustandi et al., 2000), a β-sheet with sirtuin (Avalos et al., 2002), an irregular structure with CBP (Mujtaba et al., 2004), and a different irregular structure with cyclin A2 (Lowe et al., 2002; Oldfield et al., 2007). The set of residues involved in these interactions exhibit a high degree of overlap along the linear sequence. However, because the secondary structures are distinct, it is likely that p53 utilizes different residues for the four different interactions. To examine this hypothesis, the buried surface area for each residue in each interaction was quantified by calculating the change in solvent accessible surface area (ΔASA) upon binding on a per-residue basis (Oldfield et al., 2007). ASA was calculated using the method of Connoly (Connolly, 1983) and the buried ASA upon binding was estimated by the method of Jones (Jones and Thornton, 1997). The contribution of each residue to the four interactions was compared by plotting the ΔASA for each residue. This analysis indicated that the same residues participate to different extents in the four interfaces, suggesting that the same sequence is “read” by the different partners in different ways and ultimately represents binding interactions with distinctly different characteristics (Oldfield et al., 2007).

Allosteric modification: Ste5

Alternate binding modes among the members of the yeast mating pathway illustrate how a flexible IDed linker connecting two halves of a bipartite binding sites can serve to allosterically modify the bound partner and thereby alter its activity.

Mitogen activated protein kinase (MAPK) cascades are central cores of complex signal transduction pathways used by all eukaryotic cells to respond to a wide variety of external stimuli and regulate numerous cellular responses (Cobb, 1999; Dhanasekaran et al., 2007; Gustin et al., 1998; Schaeffer and Weber, 1999; Widmann et al., 1999). A typical MAPK cascade is a module composed of three protein kinases arranged as a phosphorylation cascade. The same MAPK cascade can perform different functions in response to different stimuli or different levels of the same stimulus (Dhanasekaran et al., 2007; Elion, 1998) and different MAPK components can also participate in other MAPK cascades (Caffrey et al., 1999; Dhanasekaran et al., 2007). In this complex picture, signaling specificity is achieved via many levels of control, including preferred kinase-kinase interactions within the MAPK cascade, preferred MAPK-substrate interactions, the use of pathway-specific MAPKs, cross-regulation between MAPK modules, controlled localization of the MAPKs, and control of activation strength (Dhanasekaran et al., 2007; Elion, 2001). The formation of a given MAPK cascade on its associated scaffold allows the embodiment of many of these control mechanisms. These scaffold proteins not only facilitate interactions between the associated kinases, but additionally link them to receptors, G proteins and cytoplasmic components. They also provide means for kinase regulation through localization of upstream signaling components, allosteric control of the associated kinases, control of MAPK substrate availability and feedback attenuation mechanisms (Burack and Shaw, 2000; Elion, 2001; Elion et al., 2005; Whitmarsh and Davis, 1998).

Ste5 is a MAPK scaffold that interacts with G proteins and several specific kinases of the mating MAPK pathway of *Saccharomyces cerevisiae* (Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994). In its scaffold role, Ste5 contains separate binding sites for MAPKKK Ste11, MAPKK Ste7, and MAPK Fus3, three kinases involved in the mating pathway. However, in addition to assembling the pathway members, Ste5 also serves to regulate pathway activity through a mechanism employing an IDed linker {Bhattacharyya, 2006 #777}.

The most downstream member of the MAPK cascade, Fus3, is recruited primarily by the upstream member, MAPKK Ste7 and secondarily by the Ste5 scaffold in a competitive manner. Interaction of Fus3 with Ste7 results in efficient signal propagation but interaction with the Ste5 scaffold serves to down-regulate overall signal throughput. The interaction between Fus3 and Ste7 occurs via a canonical MAPK docking interaction between either of two redundant sites on Ste7 and the C-terminal lobe of Fus3 (site B) (Fig. 6 B). In contrast, Ste5 binding differs from canonical MAPK binding by being bipartite and contacting both lobes of Fus3 (Fig. 6 A)(Bhattacharyya et al., 2006). The N-terminal portion of the Ste5 fragment contacts the N-terminal lobe (site A) while the C-terminal portion contacts the C-terminal lobe of the Fus3 at site B. Neither half of the bipartite Ste5 interacting fragment alone is sufficient to mediate the interaction (Bhattacharyya et al., 2006). The eight-residue linker connecting the two Ste5 binding sites were deemed to be IDed as they were not visible in the crystal structure of the Ste5/Fus3 complex.

The competitive nature of Fus3 binding to Ste5 and Ste7 is explained by the fact that site B of Fus3 participates in both the Ste5 bipartate interaction and the canonical interaction with Ste7 (Bhattacharyya et al., 2006). Regulation of pathway throughput is affected by the outcome of this competitive binding. Binding of Fus3 to Ste5 enhances autophosphorylation of Fus3, which in turn, promotes the phosphorylation of Ste5 by Fus3. The net effect of these phosphorylation events serves to decrease the output of the pathway (Bhattacharyya et al., 2006). On the other hand, binding of Fus3 to Ste7 does not promote phosphorylation of Ste5 or Fus3 nor does it result in decreased pathway throughput (Bhattacharyya et al., 2006).

A model for how Ste5 mediates the autophosphorylation activity of Fus3 was proposed in which the IDed linker between the bipartite sites of Ste5 involved in binding Fus3 is critical for the modification of Fus3 activity. The separate binding of Ste5 to both the N- and C-terminal lobes of Fus3 in conjunction with the constraint imposed by the IDed linker between the two sites results in a perturbation of kinase activity by altering the relationship between the two lobes (Fig. 6 A & B). Indeed, lengthening or shortening the linker serves to reduce the level of auto-activation of Fus3 (Bhattacharyya et al., 2006). Changes in the linker length may also affect the flexibility of the activation loop of Fus3 and the ability of the phosphorylation substrate to enter the active site (Bhattacharyya et al., 2006).

Thus, the combination of a IDed linker between two halves of a bipartite binding site and the competition for binding between partners that require either one or both binding sites determines whether or not the activity of the MAPK component of the pathway and indeed the throughput of the module will be modified. Throughput of the pathway is thereby dependent on how the scaffold module is assembled and likely reflects the state of the cell prior to pathway assembly.

As mentioned above, the Ste5 and Ste7 peptides that interact with site B of Fus3 compete for the same site thus are an example of binding site overlap as discussed in the preceding section. This sharing of binding sites is one way to ensure that activities are clearly separated. In the case of Fus3, exclusion mediated by binding site overlap likely ensures that kinase activity is altered in when partnering with Ste5 but not with Ste7.

Another substantial distinction of peptides that bind to Fus3 site B is that individual interactions may occur in either orientation. This aspect is discussed in the following section.

Palindromic binding

Titin—Titin provides and excellent example of how ID can facilitate palindrome binding, in which a binding site can recognize interacting peptides in either orientation.

Titin is an extremely large (3–4 mDa) elastic protein found in the sarcomeres of vertebrate striated muscle. Titin associates with over 20 sarcomere and non-sarcomere proteins including nebulin, ankyrin, alpha crystallin, calpain, obscurin, calmodulin and actin (http://www.hprd.org) (Mishra et al., 2006). Besides providing structural framework and maintaining the structural integrity of the sarcomeres of myofibrils, titin acts as a hub for stress-related signaling (Hoshijima, 2006; Ma et al., 2006; Ma and Wang, 2002). Another important function of titin is to provide passive elastic force to restore myofibril length during the resting state (Erickson, 1997). This elasticity function is imparted, along with the immunoglobulin-like and N2B segments, by PEVK regions (Watanabe et al., 2002). As the designation implies, PEVK regions are rich in proline, glutamate, valine, and lysine. Enrichment in these residues endows PEVK elements with three important functional traits: flexibility (Romero et al., 2001), elasticity and an abundance of Src homology 3 (SH3) binding elements (Ma et al., 2006) that facilitate one component of the scaffolding capability of titin.

The IDed nature of the titin PEVK region was demonstrated by biophysical analyses of several signature peptides derived from the PEVK region. All possessed random coil-like far-UV CD spectra and had 2–4 times larger Stokes radii than expected for their molecular mass, thus were deemed to be IDed (Duan et al., 2006). This finding is supported by the VLS1 PONDR® prediction that indicates an amino acid composition favoring ID exists almost the entire length of titin (Fig. 7 C). Furthermore, it was shown that the PEVK region acts as an entropic spring that supplies some of the passive force necessary to restore muscle cells to their resting state after stretching (Trombitas et al., 1998). This extension and contraction of the PEVK region likely plays a role in myocyte signaling by providing inputs of muscle usage and extent of stretching. These inputs can be transmitted by the modulation of bound signaling proteins such as nebulin and myopalladin that bind to the abundant SH3 elements found in the PEVK region of titin (Ma and Wang, 2002).

SH3 domains are modular adaptors composed of ~60 residues that bind to SH3 elements using two orthogonally oriented ®-sheets that form a hydrophobic cleft (Musacchio et al., 1994; Rath et al., 2005). SH3 domains bind to SH3 elements that are composed of a core recognition motif of 7 – 10 residues with polyproline II (PPII) secondary structure. These peptide ligands have a variety of motifs that often include conserved prolines, lysines, tyrosines, and arginines (Ma et al., 2006; Ma and Wang, 2002). SH3 domains can bind in two different binding modes, class I and II, that represent opposite orientations (polarity) of the linear ligand sequence with respect to the SH3 domain (Feng et al., 1994). SH3 binding sites are ideally suited for presentation by IDed proteins like titin because the PPII conformation is often sampled by IDed segments (Rath et al., 2005). Two properties of the PPII helical structure make it highly suitable for presentation and orientation of recognition residues (Rath et al., 2005). First, the side chains and backbone amide and carbonyl groups are solvent exposed in the PPII conformation and therefore available for participation in protein-protein interactions. The lack of intra-chain hydrogen backbone atom bonding and the wide spacing between side chains that precludes intra-chain interactions boosts the per-residue reactive surface area of short linear peptides – a property that can be exploited for inter-protein recognition and interaction. Additional functionality is provided by the second property: Because PPII helices are twofold rotationally pseudosymmetric, any protein designed to bind this structure will have a possibility to recognize peptides in a reverse orientation (Zarrinpar et al., 2003).

The functionality of titin as a scaffold for SH3 domain-containing proteins was investigated in the context of the SH3-containing protein nebulin (Ma et al., 2006). The scaffolding function of titin arises from the dual binding mode of SH3 binding proteins and the proximity, overlap and dual-polarity of the SH3 binding sites (Fig. 7 A & B). The fact that SH3 domain-containing proteins (i.e., nebulin) can bind in either orientation increases the modes of interaction between adjacent bound partners and contributes to the scaffolding functionality of titin (Fig. 7 A & B) (Ma et al., 2006).

The titin example of a scaffold based on the dual binding mode of the SH3 domain leads one to speculate on the existence of others. A recent study of RCSB Protein Data Bank structures found 53 instances of PPII stretches of 4 – 12 residues in length in a dataset of 372 non-redundant short chains (< 70 residues) that were bound to larger proteins (Mohan et al., 2006). This set of sequences could provide a starting point for the search for additional PPII-mediated scaffold interactions.

Fus3—Fus3, discussed above in the context of its role as the MAPK in the yeast mating pathway, also exhibits palindromic binding. Site B of Fus3, is able to bind peptides oriented in either direction (Fig. 6 A & B). Structural analysis of Fus3 complexed with the canonical MAPK docking peptides of Ste5 and Far1 and the interacting peptide of Ste7 revealed that site B can accommodate peptides that are parallel or anti-parallel to the Fus3 chain (Fig. 6 C) (Bhattacharyya et al., 2006; Remenyi et al., 2005). This is a further example that ID facilitates the utilization of binding palindromes. The central regions of both Far1 and Ste5 peptides bound to Fus3 adopt a PPII helical conformation and present conserved (albeit in opposite polarity) arginine, proline and leucine to the same regions of site B (Fig. 6 D)(Bhattacharyya et al., 2006; Remenyi et al., 2005). The ability of the Site B binding groove to recognize peptides in either orientation is typical of SH3 domains and other recognition domains that interact with proline-rich peptides, although inclusion of proline is not a requirement for the PPII conformation (Rath et al., 2005).

Reduced constraints for alternative splicing

BRCA1—Another feature of BRCA1 (discussed above) is that the majority of alternative splicing (AS) occurs within the region coding for the central IDed region of the gene products (Fig. 5). This is consistent with the hypothesis that AS of regions coding for ID incurs less structural penalties than AS in regions coding for order (Romero et al., 2006).

AS of pre-mRNA generates two or more protein isoforms from a single gene, thereby increasing protein diversity. This, coupled with the fact that ID is highly represented in proteins involved in signaling and regulation (Iakoucheva et al., 2002; Uversky et al., 2005), makes AS an effective and efficient means of modifying the signaling specificity of scaffolds. By mapping AS to regions of ID, changes enabling diversity can be made while avoiding structural constraints, which would arise if ordered parts of a protein were removed or added by AS (Romero et al., 2006). This hypothesis was tested on a set of AS genes encoding structurally characterized human proteins containing similar amounts of structure and ID (Romero et al., 2006). In the majority of examples, AS was associated with fully and partially IDed regions and concomitantly led to modulation of the functional profile of the various isoforms.

BRCA1 is an illustrative and well-characterized example of functional modulation by removal of functional domains and binding sites via AS (Romero et al., 2006). In particular, the IDed central region of BRCA1 contains binding sites for both DNA and protein partners (Fig. 5) (Mark et al., 2005). BRCA1 has at least 24 alternatively spliced variants (Orban and Olah, 2003), with many of them maping to the mRNA fragment encoding the central IDR. Different AS isoforms lack single or multiple functional domains, thereby creating diverse functional profiles (Romero et al., 2006). Thus, the combination of AS and ID discussed above can enable

the AS-mediated time-, tissue-, and developmental-specific modulation of scaffold function required for cell differentiation and perhaps for the evolution of multicellular organisms.

Titin—Titin is also an excellent example of how reduced constraints for AS of ID can contribute to scaffolding function. Alternative splicing of exons can place different SH3 binding motifs, whether uni- or bi-directional, in proximal or distal relationships and thus tailor titin signaling to the specific needs of the cell. Being predominantly IDed, there are few structural ramifications for rearrangements within the PEVK region. Therefore AS can be applied to produce many alternate forms. Alternative splicing of IDRs is less complex compared to ordered regions because IDRs offer little or no folding constraints over the multiplicity of alternative isoforms (Romero et al., 2006). Indeed, upwards of 116 of titin exons code for different PEVK elements (Bang et al., 2001; Ma et al., 2006). In regards to the signaling functionality of the titin PEVK region, the combination of ID-facilitated AS and bi-directional binding modes combine to enhance functional diversity of the titin scaffold.

Efficient regulation via posttranslational modification: MAP2

Three A-kinase anchoring proteins (AKAPs) are discussed in the following sections. A general background that applies to all three AKAP scaffolds discussed is presented here.

The AKAP scaffold family of proteins contains more than 70 members (Malbon et al., 2004b; Michel and Scott, 2002). Although AKAPs are structurally diverse, each AKAP nevertheless performs similar functions. Several in-depth reviews provide a wealth of details about AKAP proteins and their functional mechanisms (Burns-Hamuro et al., 2005; Gold et al., 2006; McConnachie et al., 2006; Michel and Scott, 2002; Smith et al., 2006; Wong and Scott, 2004).

AKAPs are prototypical scaffold proteins and, as is true for most scaffolds, AKAPs provide for the physical association for combinations of proteins that, depending on the cellular environment and exogenous signals, can undergo dynamic rearrangements in order to modulate signaling in associated downstream pathways (Wong and Scott, 2004). The key enzyme associated with AKAP proteins is cyclic-AMP-dependent protein kinase A (PKA). PKA is tetramer composed of two catalytic subunits that are held in an inactive conformation when bound to a regulatory dimer (Taylor et al., 2005). When cAMP binds to the regulatory subunits, the catalytic subunits are released. PKAs are broad-spectrum serine-threonine kinases and are responsible for a multitude of diverse downstream responses. PKA is the primary target for cAMP generated by G-protein-coupled receptors (GPCRs). GPCRs are a family of receptors located in specific areas of the membrane that respond to a wide repertoire external stimuli including light and a wide repertoire of intercellular signaling molecules such as neurotransmitters, odorants, biogenic amines, lipids, proteins, amino acids, hormones, nucleotides, chemokines (Bar and Hechter, 1969; Kroeze et al., 2003; Marshall, 2001). Upon binding the appropriate ligand, GPCRs activate associated heteromeric G proteins that, in turn, activate adenylate cyclase that then converts ATP to cAMP. This locally generated cAMP pool targets AKAP-bound PKAs.

Beyond binding PKA, the multivalency of AKAP scaffolds facilitates the integration of signaling pathways by integrating additional enzymes. These companion enzymes can include Ras GTPase-activating-like protein (Nauert et al., 2003), Ca^{2+}/calmodulin-dependent phosphatase PP2B (Coghlan et al., 1995), protein kinase C (Klauck et al., 1996), Rho-activated protein kinase N (Takahashi et al., 1999), casein kinase-1 (Sillibourne et al., 2002), protein phosphatase PP2A (Takahashi et al., 1999), glycogen synthase kinase-3β (Tanji et al., 2002) among many others (Wong and Scott, 2004). This diverse assortment of enzymes reflects the importance of AKAP scaffolds as a point of convergence for signaling pathways. Not only do AKAP scaffolds complex with many partners simultaneously, the complement of bound

proteins can be varied thereby modifying associated signaling pathways in a context-specific manner (Wong and Scott, 2004). As the abundance of many signaling molecules (e.g. protein kinase A, protein kinase C and Src) is relative low in the cell, the major role of AKAPs is the creation of the locally increased concentrations of these signaling elements, which has great impact on the dynamics and temporal character of signaling (Malbon et al., 2004b).

Microtubule-associated protein 2 (MAP2) illustrates how the increased accessibility of IDRs provides for efficient regulation via posttranslational modification. In the case of MAP2, the rigidity of the molecule is altered by the phosporylation state thereby modulates the cytoskeletal and scaffolding roles of MAP2.

MAP2 was shown to contain a predominantly IDed region using circular dichroism, hydrodynamic methods and Far-UV CD spectroscopy (Hernandez et al., 1986). These findings supported the hypothesis that MAP2 is flexible, non-globular and non-compact in solution (Hernandez et al., 1986). These findings agree with ID predictions from sequence (Fig. 8 A).

MAP2, one of the most abundant MAPs in the brain, was the first AKAP to be identified (Vallee et al., 1981) and belongs to a family of cytoskeletal proteins and is predominantly expressed in dendrites of neurons (Matus, 1994). Early studies distinguished two functional domains in MAP2: the microtubule-binding domain (MTBD) that promotes tubulin polymerization and a long projection arm that harbors cAMP-dependent protein kinase (PKA) via binding the regulatory (RII) subunit (Theurkauf and Vallee, 1982). The MTBD of MAP2 consists of ~100–130 residues near the carboxyl terminus (Lewis et al., 1988). The projection arm was shown to mediate interactions with other cytoskeletal elements such as neurofilaments (Leterrier et al., 1982) and actin (Selden and Pollard, 1983).

For a long time it was generally accepted that MAP2 acted a cross-linker to stabilize the dendritic architecture (Matus, 1988; Wiche et al., 1991). Models of neuronal plasticity necessarily invoked abolishment the cross-linking function of MAP2 during cytoskeletal rearrangements (Aoki and Siekevitz, 1985; Friedrich, 1990). It was later discovered, however, that the function of MAP2 as a cross-linker and a simple stabilizer of the dendritic cytoskeleton is not as trivial as first thought (i.e., cross-linking by disulfide bonds or isopeptide bonds formed by transglutaminase) (Friedrich and Aszodi, 1991). It was later proposed that similar to tau protein, MAP2 could be heavily phosphorylated and that, upon phosphorylation, MAP2 lengthens and becomes more rigid (Friedrich and Aszodi, 1991). Therefore, MAP2 could be considered as a spacer whose length and rigidity can be altered in a graded manner by the orchestration of second messenger-regulated kinases and phosphatases (Friedrich and Aszodi, 1991; Halpain and Greengard, 1990). This regulated adjustability in length and stiffness might contribute to neuronal plasticity and morphology. In agreement with this model, the phosphorylation state of MAP2 is known to be modulated by neural activity (Aoki and Siekevitz, 1985; Halpain and Greengard, 1990; Montoro et al., 1993; Philpot et al., 1997; Quinlan and Halpain, 1996a; Quinlan and Halpain, 1996b).

In its scaffold role, MAP2 interacts with the non-receptor protein tyrosine kinase c-Src and the adaptor protein Grb2 (Lim and Halpain, 2000). These interactions are mediated through the SH3 domains of Src and Grb2 and the MTBD of MAP2 and regulated by phosphorylation by the mitogen-activated protein kinase extracellular signal-regulated kinase 2 (Lim and Halpain, 2000). Therefore, with MAP2 acting as a scaffold protein for Src, other cellular proteins can interact with P*XX*P motifs on MAP2, and become potential substrates for the MAP2-bound Src tyrosine kinase activity. Furthermore, since microtubule binding and SH3 domain binding are mutually exclusive, only the non-microtubule-bound pool of MAP2 plays a role as a scaffold for Src and Grb2. As both microtubule and SH3 domain binding are regulated by phosphorylation, differential phosphorylation could presumably regulate the scaffold *versus*

cytoskeletal roles of MAP2 *in vivo* (Lim and Halpain, 2000). The phosphorylation-dependence of MAP2 function provides a potential mechanism for modulation of the neuronal cytoskeleton by extracellular signals and the local organization of signaling complexes in the neuron (Lim and Halpain, 2000).

Efficient regulation via rapid degradation: MAP2

The increased accessibility of ID regions to modifying enzymes can be clearly illustrated by the example of ID-facilitated regulation of MAP2 via rapid degradation. One aspect of the phosphorylation state of MAP2 is that it can be rapidly degraded when not highly phosphorylated (Sanchez et al., 2000). MAP2 is rich in PEST sequences that mark it for rapid degradation. The rate of proteolysis, primarily mediated by calpain (Fischer et al., 1991), is controlled by the level of phosphorylation. Higher phosphorylation states protect MAP2 from degradation while less phosphorylated forms, which are bound to microtubules, are more susceptible to proteolysis (Sanchez et al., 2000). Thus the level of phosphorylation of MAP2 allows the fine control of turnover and steady state levels necessary during development and cytoskeleton remodeling (Sanchez et al., 2000).

Protection of normally solvent-exposed sites: D-AKAP2

Dual-specific A-kinase anchoring protein 2 (D-AKAP2) is expressed in nearly all tissues and functions as a coordinator of cAMP-mediated signaling. It is so named because it can bind the two subtypes of regulatory PKA subunits, RI and RII. Unlike MAP2 and AKAP250, the two mostly IDed AKAP scaffolds discussed elsewhere in this review, D-AKAP2 is predicted by PONDR® VLS1 to contain a substantial amount of order (Fig. 8 C). However, one IDed region of D-AKAP2 illustrates that the flexibility provided by ID allows it to protect an adjacent site. Like other AKAPs, D-AKAP2 serves as a regulator of PKA specificity, which in turn serves to phosphorylate a diverse set of proteins involved in numerous signaling pathways. AKAPs tether PKAs via A-kinase binding (AKB) domains (Burns-Hamuro et al., 2005). Binding of specific PKAs to specific AKAPs indirectly regulates the catalytic activity of the kinase by restricting subcellular distribution (Colledge and Scott, 1999;Edwards and Scott, 2000). Because D-52 AKAP2 can bind to both subtypes of regulatory PKA subunits, it represents a subfamily of AKAPs that can recruit PKAs of both regulatory subtypes. In addition to binding PKA regulatory subunits (Huang et al., 1997) it also binds PDZK1, an adapter protein via a PDZ binding domain (Gisler et al., 2003b;Gisler et al., 2001).

The AKB and PDZ binding motifs of D-AKAP2 are located in the C-terminal 40 amino acids of the protein (Burns-Hamuro et al., 2005; Gisler et al., 2003a; Huang et al., 1997). The C-terminal 152 residues of D-AKAP2 containing the AKB and PDZ domains was identified as being largely IDed by enhanced deuterium exchange-mass spectrometry (Hamuro et al., 2002). The AKB domain was unexpectedly found to be highly protected from deuterium exchange. In contrast to the high level of protection of the AKB domain, the adjacent C-terminal PDZ-binding motif was very solvent-accessible. Additional exchange experiments conducted on the isolated AKB domain indicated that the protection observed was the result of tertiary interactions between this domain and the rest of the protein, not secondary structure formation or intramolecular interactions within the AKB domain (Hamuro et al., 2002). Based on these observations it was hypothesized that the part of the C-terminal IDed region in D-AKAP2 functions as a flexible linker that allows association of the AKB binding surface with the rest of the protein for the purpose of stabilization. This association may also provide a mechanism for modulation of intermolecular associations. In support of this hypothesis, it has been shown that PKA can phosphorylate D-AKAP2 at a serine residue located N-terminal to the AKB domain. The effect of this posttranslational modification on the accessibility of the PKA binding site is currently under investigation but may represent a mechanism for modulating the accessibility of the AKB binding surface to PKA (Hamuro et al., 2002).

Enhancing the plasticity of interaction: D-AKAP2

D-AKAP2, being dual-specific, binds the dimerization/docking domains (DD) of either RI or RII regulatory units of PKA. The PKA holoenzyme is composed of two catalytic domains and two regulatory domains. Within the holoenzyme, the two regulatory DD domains combine to form a stable a hydrophobic groove that interacts with the hydrophobic ridge provided by the AKAP (Kinderman et al., 2006). The interacting surface of both RI and RII consists primarily of an anti-parallel four-helix bundle with two helices contributed by each DD of the two regulatory subunits. Although the DD domain is conserved in all PKA regulatory subunits, differences exist that that allow AKAP proteins to discriminate between RI and RII regulatory subunits (Kinderman et al., 2006). Additionally, analysis of structural data is only just beginning to allow the prediction of specificity of PKAs for AKAPs from sequence (Kinderman et al., 2006).

The crystal structure of a 22-residue D-AKAP2 peptide in complex PKA RIIα revealed the formation of a tight solvent-excluded interface upon binding. In addition, the first five residues of the IDed N-terminus (see above) of one of the RII subunits was observed to be ordered and in contact with D-AKAP2. Indeed, two residues in this region, Ile3 and Ile5, make multiple contacts with D-AKAP2 and are essential for binding. The contribution of these two residues is such that mutation of either prevents in vitro pull-downs of AKAP partners (Hausken et al., 1994; Li and Rubin, 1995). The N-terminus of the other RII subunit remains IDed and does not participate in the interaction. In contrast to RII, the N-terminus of RI subunits is ordered. This difference likely influences the binding specificity of different AKAP sequences for RI versus RII subunits. Additional support for this determinant of specificity is provided by the fact that mutations in the region of the AKAP binding surface that interacts with the IDed N-terminus of PKA regulatory subunits are more disruptive to RI binding than to RII binding (Kinderman et al., 2006). The IDed N-terminal tail of the RII PKA regulatory subunit is thought to increase the flexibility of the interaction and thus broaden the range of AKAP sequences that can be bound (Kinderman et al., 2006). Thus the flexibility of the IDed N-terminus of the RII subunit of PKA is likely crucial for the ability of RII to bind multiple AKAPs and also contributes to the dual specificity of D-AKAP2.

Other considerations

Molecular crowding I: An evolutionary imperative?: AKAP250 and AP2

A background description of AKAP scaffolds was presented previously. Relevant to this section, at least two AKAP proteins, AKAP250 and MAP2, have been characterized experimentally as predominantly IDed. The mostly IDed nature of these AKAP scaffold proteins is reflected in their VLS1 PONDR® prediction scores (Fig. 8 A & B). In addition, both are predicted to be mostly IDed by two binary ID prediction methods, CH-plot analysis (Oldfield et al., 2005a; Uversky et al., 2000) and cumulative distribution function (CDF) analysis (Oldfield et al., 2005a). MAP2 was discussed previously and AKAP250 is described below.

Analysis of AKAP250 in solution revealed that it was largely IDed and lacked secondary structure except, perhaps, for the interaction sites for other proteins (Malbon et al., 2004a). Based on these observations, was been proposed that the structure of AKAP250 was similar to a “necklace”, where small ordered domains appear as “beads” on the IDed “string”. This necklace-like organization facilitates dynamic membrane association and targeting of signaling assemblies to various regions of the cell (Malbon et al., 2004a).

Aside from allowing scaffolds to be more easily targeted to different cellular locations, the almost total IDed nature of several scaffolds in this review (AKAP250, MAP2, axin, BRCA1

and titin) lends itself to another discussion. That is, what is the fundamental basis for the existence of ID?

As mentioned in the introduction, a significant portion of all proteomes are thought to be IDed with the percentage increasing with complexity, i.e., prokaryotes < archaea < eukaryotes (Dunker et al., 2000; Liu et al., 2002; Ward et al., 2004b). Given that some functional requirements can be better satisfied by ID and thus can provide some specific positive evolutionary selection, is there a yet more fundamental benefit of that drives selection towards ID in general?

While no research to date links molecular crowding to scaffold function, it could be argued that the higher density of interaction sites that can be accommodated by a protein of a given size, the higher level of total functionality that can be provided by a given proteome. Thus, a fundamental benefit that could drive evolution towards the use of ID is minimization of molecular crowding. Proteins are a main contributor to this crowding as they compose about 40% of cell volume (Fulton, 1982; Luby-Phelps, 2000; Zimmerman and Minton, 1993). Evolutionary pressure towards the fulfillment of as many functions as possible with IDPs and IDRs instead of structured proteins could be provided by molecular crowding. As organism and cell complexities increase, new functions are necessarily added. Added functions require additional proteins and this increases molecular crowding. As there are limits and controls on cell size (Cossins, 1991; Jakab et al., 2002), spatial conservation is likely a component of evolutionary pressure that directs existing and as well as new functions toward efficient use of space (Berg, 1990).

Molecular crowding II: Structure induction of IDRs and IDPs?

Bringing the discussion of efficiency of employing ID for protein-protein interactions full circle, here we return to one of the points discussed early in this review: the presence of residual or pre-formed binding structures in IDRs. It is believed that induced structure is more likely to occur in the crowded conditions of the cellular environment (upwards of 500 g/l) (Fulton, 1982; Zimmerman and Minton, 1993) than in the relatively dilute conditions under which most in vitro structural studies are performed (Luby-Phelps, 2000). This prediction is based on the theoretical standpoint that molecular crowding will favor folded structure in proteins as a result of the preferential stabilization of compact states (Minton, 1981; Minton, 2000a; Minton, 2000b). In agreement with this prediction, the ability of crowded environment to fold globular proteins unfolded by chemical means (Davis-Searles et al., 1998; Ping et al., 2004; Tokuriki et al., 2004; van den Berg et al., 2000; Zhou, 2004) and to promote intermolecular association (Tellam et al., 1983; van den Berg et al., 1999) was demonstrated. A potential expected outcome of crowding effects for IDPs and IDRs is that, being IDed in ideal solutions, they may adopt compact or globular conformations in a crowded environment. This hypothesis that IDPs and IDRs may adopt stable structure under crowded conditions in which excluded volume was predicted to stabilize compact, ordered conformations was tested for such IDed proteins as C-terminal activation domain of c-Fos (Flaugh and Lumb, 2001), the kinase-inhibition domain of $p27^{Kip1}$ (Flaugh and Lumb, 2001), α-synuclein (McNulty et al., 2006; Shtilerman et al., 2002; Uversky, 2002a) and FlgM (Dedmon et al., 2002). Surprisingly, all these proteins (except for the C-terminal half of FlgM) were shown to devoid any significant conformational change in the model crowded environment in vitro. Furthermore, using in-cell NMR, α-synuclein (Shtilerman et al., 2002) and N-terminal half of FlgM (Dedmon et al., 2002) were shown to remain substantially IDed even inside the living cell. However, the ability of α-synuclein to aggregate and to form amyloid fibrils was dramatically accelerated in crowded environment (Mohan et al., 2006; Shtilerman et al., 2002; Uversky, 2002a). Therefore, molecular crowding effects are not necessarily sufficient to induce ordered structure in IDed proteins. However, they might promote the effective formation of signaling complexes.

Concluding remarks

Several reviews have enumerated various means by which ID contributes to interactions between proteins (Dunker et al., 2002a; Dunker et al., 2001; Dyson and Wright, 2005; Fink, 2005; Tompa and Csermely, 2004; Uversky, 2002a). In general, the ID-associated functional benefits presented here are not found in highly structured interaction sites. Here, we have reviewed the experimental findings that illustrate a variety of ID-mediated functionalities observed in scaffold proteins. In fact, the functionalities included here provide a nearly comprehensive list of the functional roles of ID in regards to protein-protein interactions. It follows that the efficiency (on a per residue basis) is addressed by the application of ID to these functions and that this efficiency can serve to enable reasonably-sized signaling scaffolds that nevertheless have the ability to interact with a multitude of different partners.

IDPs are highly abundant in nature, are involved in various cellular functions and possess unique biophysical characteristics that are well suited for those roles. The most intriguing is the overwhelming involvement of such proteins in recognition, regulation and cell signaling functions. Signaling scaffold proteins are uniquely positioned within complex signaling networks and pathways and serve to integrate signaling pathways. Therefore, they play crucial roles in regulation of signaling events by providing a means for the coordinated and specific signaling interaction. One of the common structural peculiarities unifying many scaffolds is the presence of ID that is exploited in various ways to enhance the multivalent interactivity of scaffold proteins.

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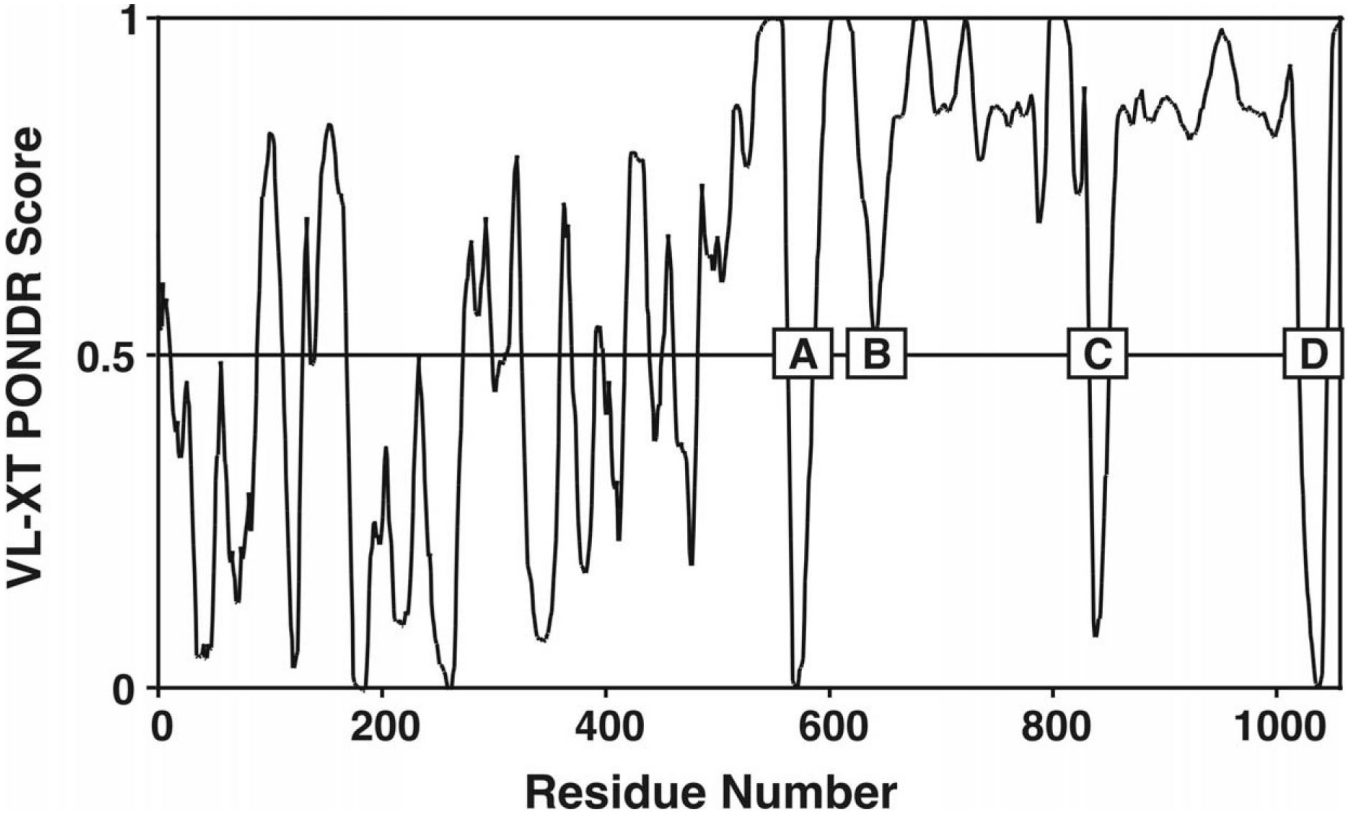

Fig. 1.

PONDR® VL-XT prediction of RNase E showing that the C-terminal half of the protein (residues 483–1061) is predicted to largely IDed (PONDR® scores > 0.5) except for four short regions (A–D) that contain order-promoting residues. These regions correlated to experimentally determined minimal motifs required for self-association (region A) and for binding structured RNAs (region B), enolase (region C) and PNPase (region D).

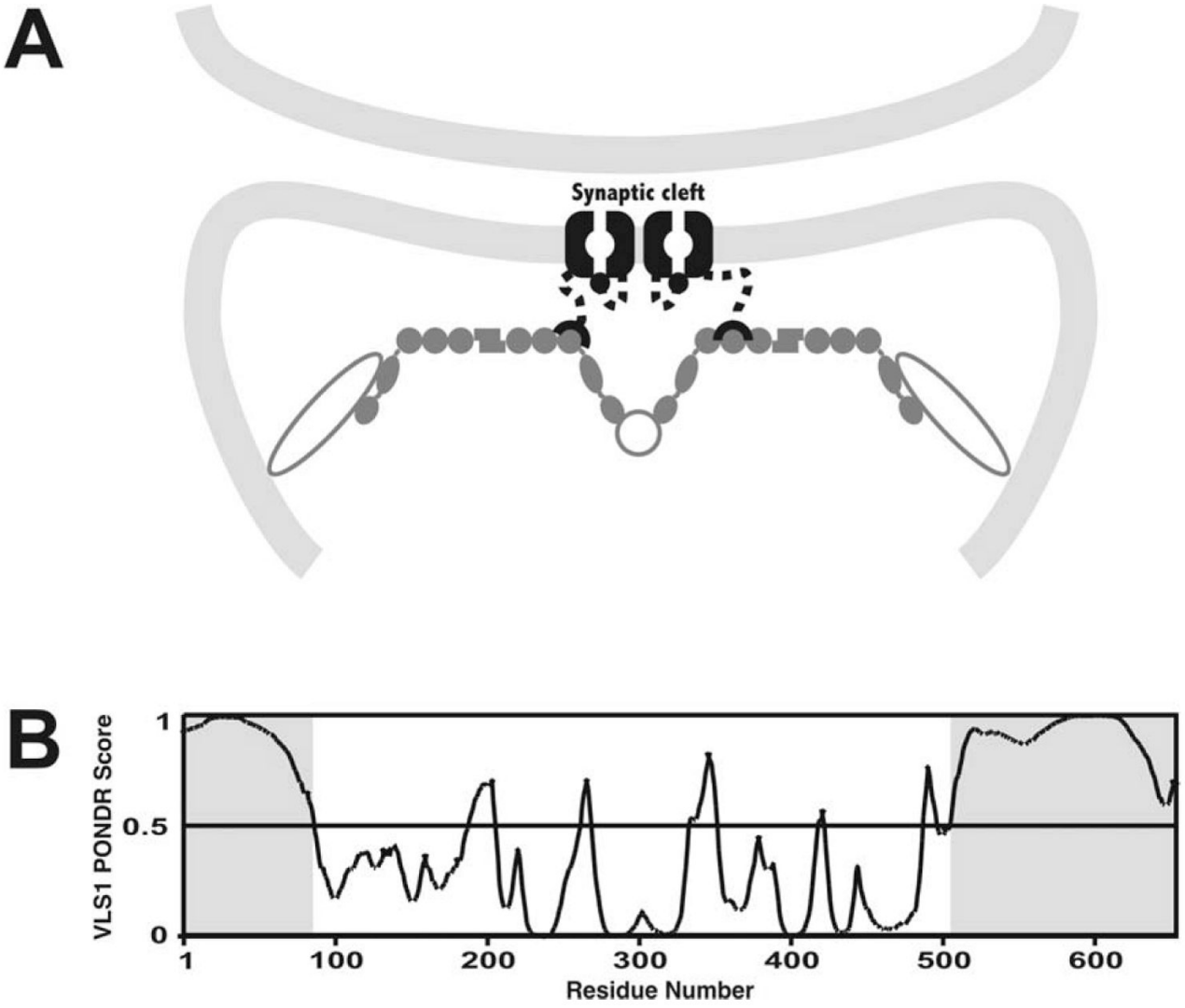

Fig. 2. ID-mediated association of PSD-95 and Kv channels

A. The Kv channels (black) are embedded in the postsynaptic membrane (light grey) and attached to the PSD-95 scaffold\(dark grey) by C-terminal PDZ domains (half circles) that are attached to the main body of the channel by IDed linkers (dotted lines). Open grey circles represent a much reduced subset of additional PSD-95-associtated proteins. Portions of the figure were based on Kim et al. (Kim and Sheng, 2004) and Magidovich et al. (Magidovich et al., 2006). The N-terminal ball and chain domains are depicted in the channel blocking position with the IDed linker also depicted as dotted lines. **B**. VLS1 PONDR® prediction of Shaker potassium voltage-gated channel protein (SwissProt accession number P08510) illustrating the substantial length of predicted ID (PONDR® scores > 0.5) located at both termini. The IDed regions depicted as dotted lines in panel A are highlighted with grey shading.

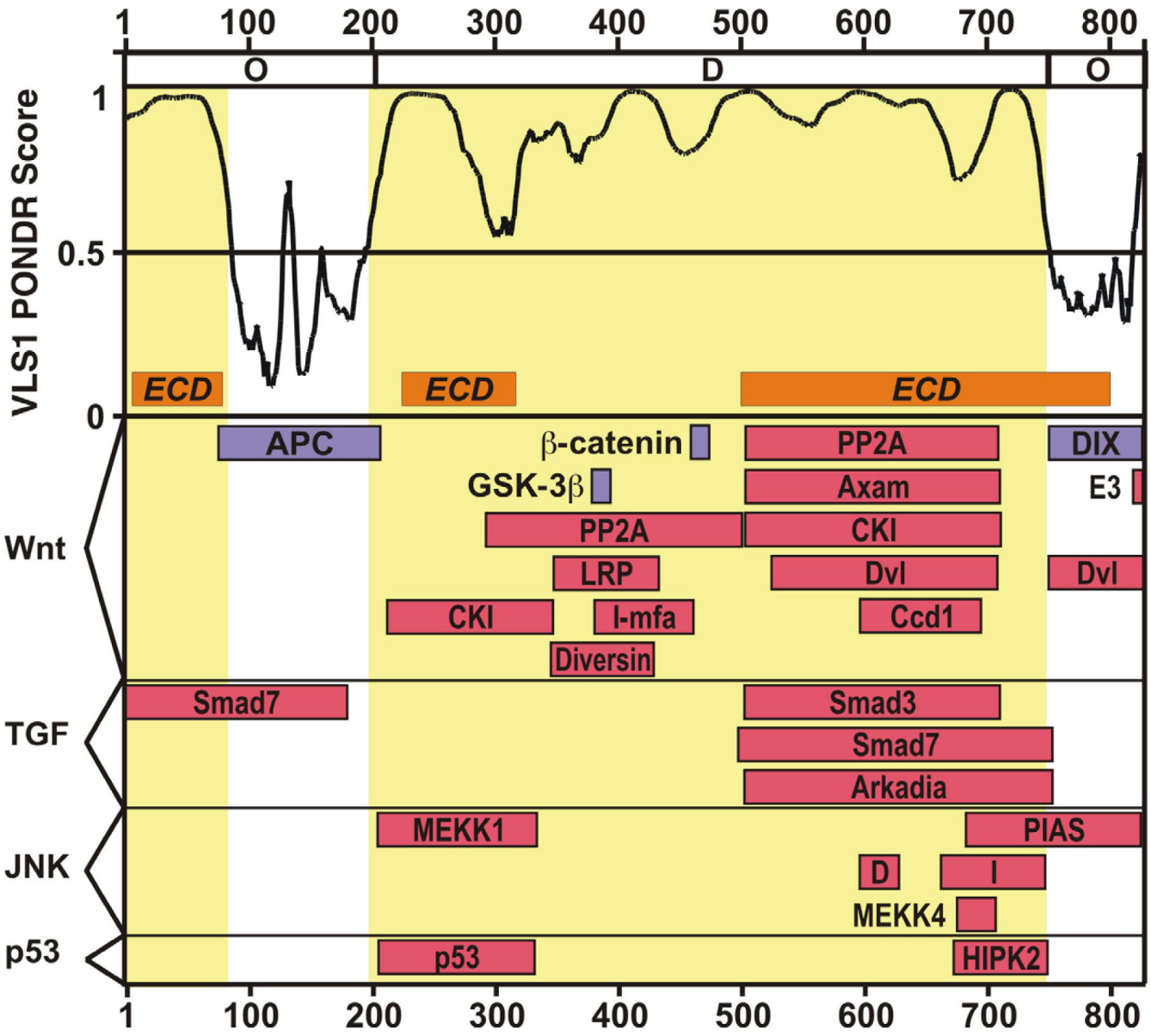

Fig. 3. ID and interaction regions for axin

All data are plotted to scale by residue number (x-axis). Predictions of ID are shown in the upper half of each panel. Combined output from charge/hydropathy and CDF two-state prediction analysis (see text) is depicted in the topmost bar with predicted order and predicted ID labeled O and D, respectively. Regions that have been *E*xperimentally *C*haracterized as *D*isordered (see text) are indicated with orange bars labeled *ECD*. The large central IDR predicted by VLS1 is highlighted in yellow. Experimentally determined binding regions derived from structures (blue) and molecular biology methods (red) are shown in the bottom half of each panel. Binding regions are grouped by associated pathway as labeled on the y-axis. Pathway abbreviations are explained in the text. Note that regions are shown only once but may participate in more that one pathway. The following regions are from experiments using from mouse isoform 2 (the shorter, more prevalent form, Swiss-Prot accession number O35625-2) or mapped onto that sequence using bl2seq (human and mouse axin share 86% identity and 90% similarity)(Tatusova and Madden, 1999): β-catenin (Xing et al., 2003);

GSK-3 β (Dajani et al., 2003); APC (Spink et al., 2000); Dix (homodimerization)(RCSB Protein Data Bank ID 1WSP, Shibata et al. 2006); Smad7 (Liu et al., 2006); PP2A, CKI, Axam, Smad3, dishevelled (Dvl), protein inhibitor of activated STAT (PIAS), Diversin, I-mfa, low density lipoprotein receptor-related protein 5 (LRP5) and LRP 6 from the excellent review by Luo (Luo and Lin, 2004); Arakadia and Smad7 (Liu et al., 2006); homeodomain-interacting protein kinase-2 (HIPK2) and p53 (Rui et al., 2004); Ccd1 (Wong et al., 2004); D and I (homodimerization) (Luo et al., 2005); mitogen-activated protein kinase kinase kinase 1 (MEKK1) and MEKK4 (Luo et al., 2003); small ubiquitin-related modifier conjugating enzymes (E6) (Rui et al., 2002).

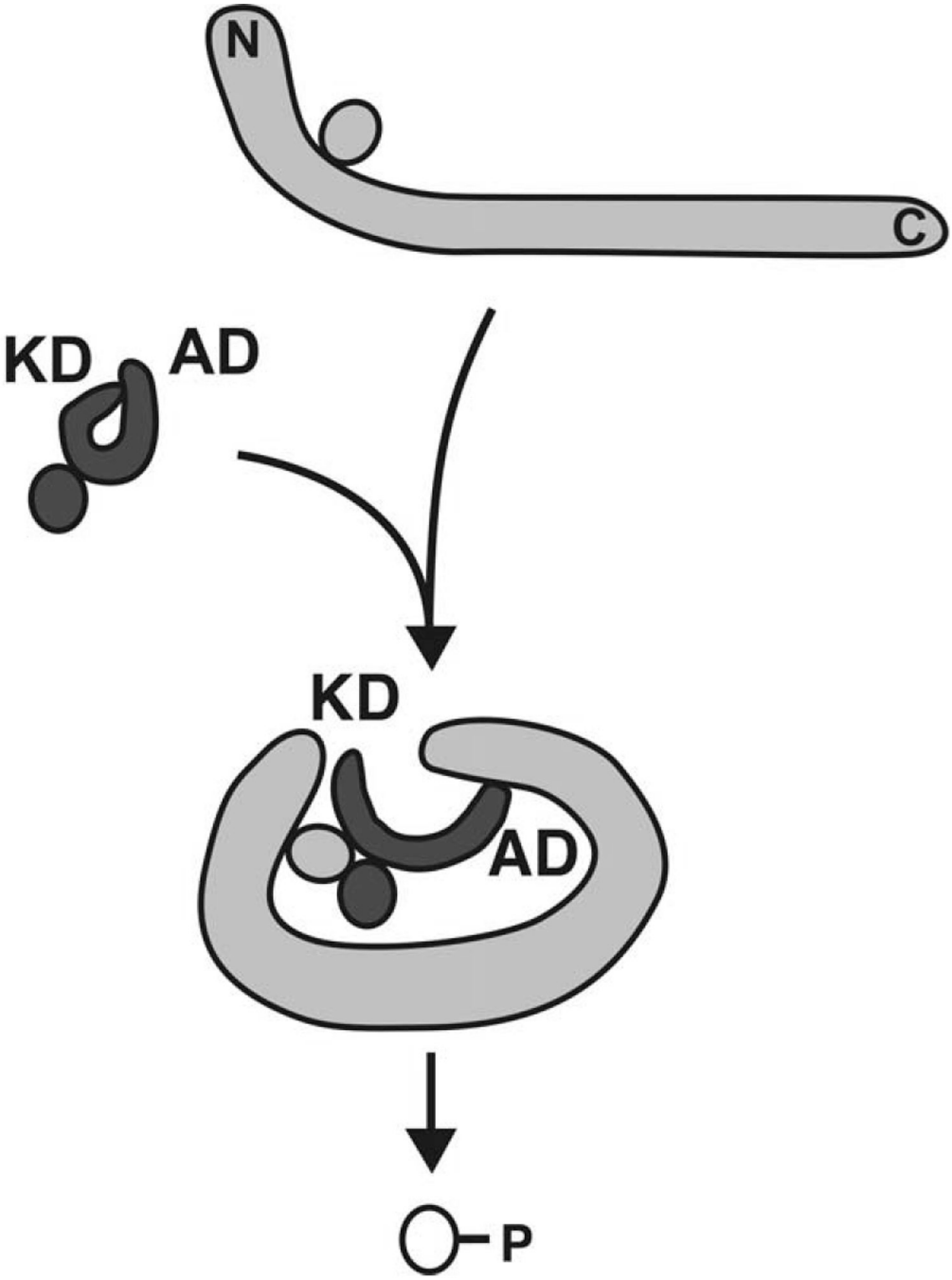

Fig. 4. Mechanism of axin-dependent activation of HIPK2 kinase activity

HIPK2 is held in an inactive state by interactions between its kinase and axin-binding domains (KD and AD, respectively). Upon binding axin, inhibition is relieved and the KD is able to phosphorylate both the HIPK2-bound p53 molecule and the one bound to axin. Binding sites of HIPK2 and p53 on axin are depicted approximately to scale (see Fig. 3). Based on Fig. 8 of Rui et al. (Rui et al., 2004).

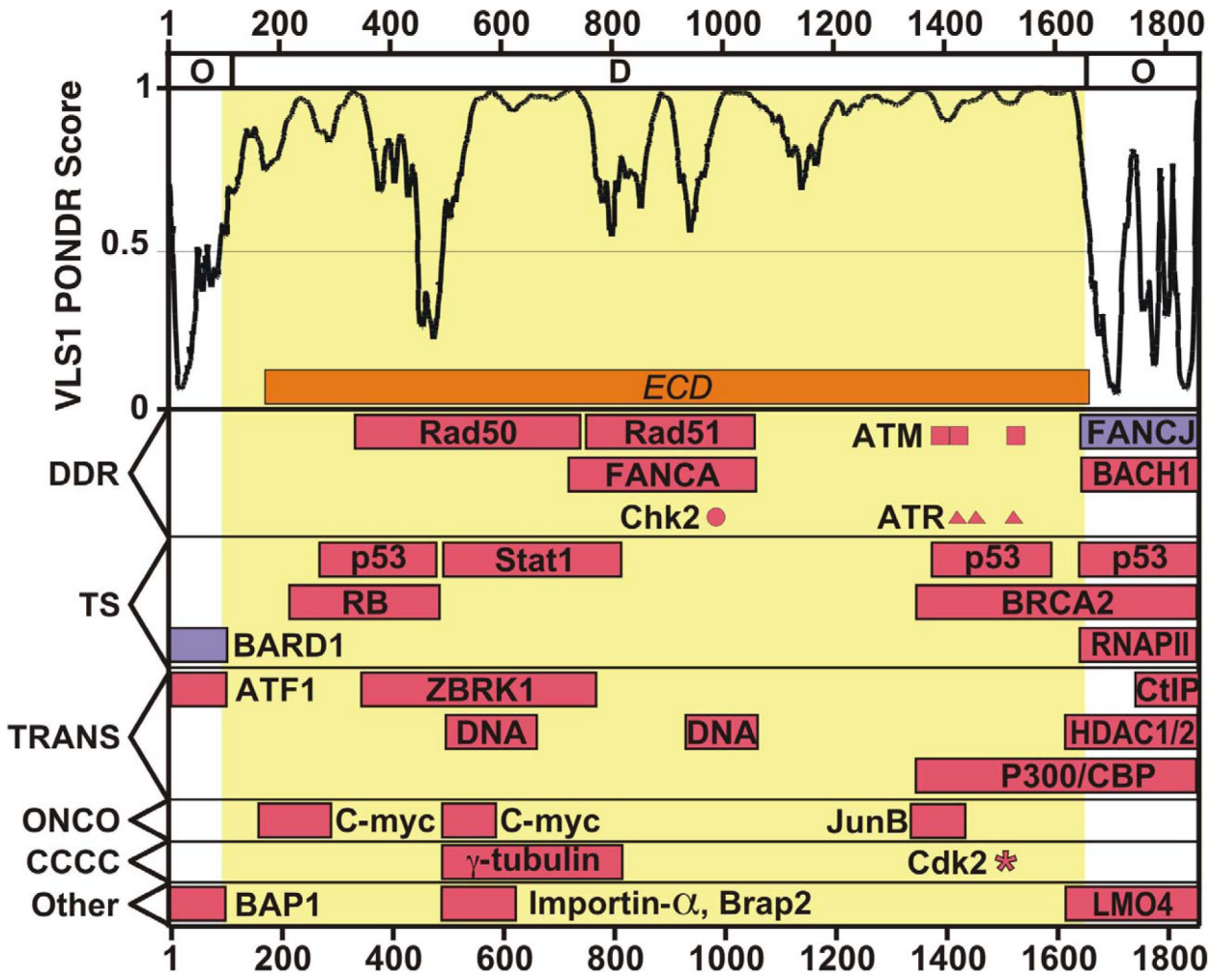

Fig. 5. ID and interaction regions for BCRA1

Layout and annotations are the same as for Fig. 3. Two domains of BRCA1, BRCT (which binds FNACJ) (Clapperton et al., 2004;Gaiser et al., 2004;Litman et al., 2005) and RING (which bind BARD1) (Brzovic et al., 2003), have known 3-D structures. The remaining evidence for protein-protein interactions is based on molecular biology evidence. These regions are: BRCA2 (Chen et al., 1998); HDAC1 & 2 (Yarden and Brody, 1999); BAP1 (Jensen et al., 1998); CtIP (Li et al., 1999a); p53 (Chen et al., 2006); ZBRK1 (Zheng et al., 2000); c-Myc, retinoblastoma protein (RB), DNA, Rad 50 & 51, FANCA, and JunB (Mark et al., 2005); Importin-α, BRAP2, STAT1, BACH1, ATF1, LMO4, p300/CBP RNA polemerase II (RNAPII) and γ-tubulin (Thompson and Schild, 2002). Sites phosphorylated by Chk2, ATM, ATR and CDK2 are shown as is indicated by a circle, boxes triangles, and an asterisk, respectively (Mark et al., 2005;Ruffner et al., 1999).

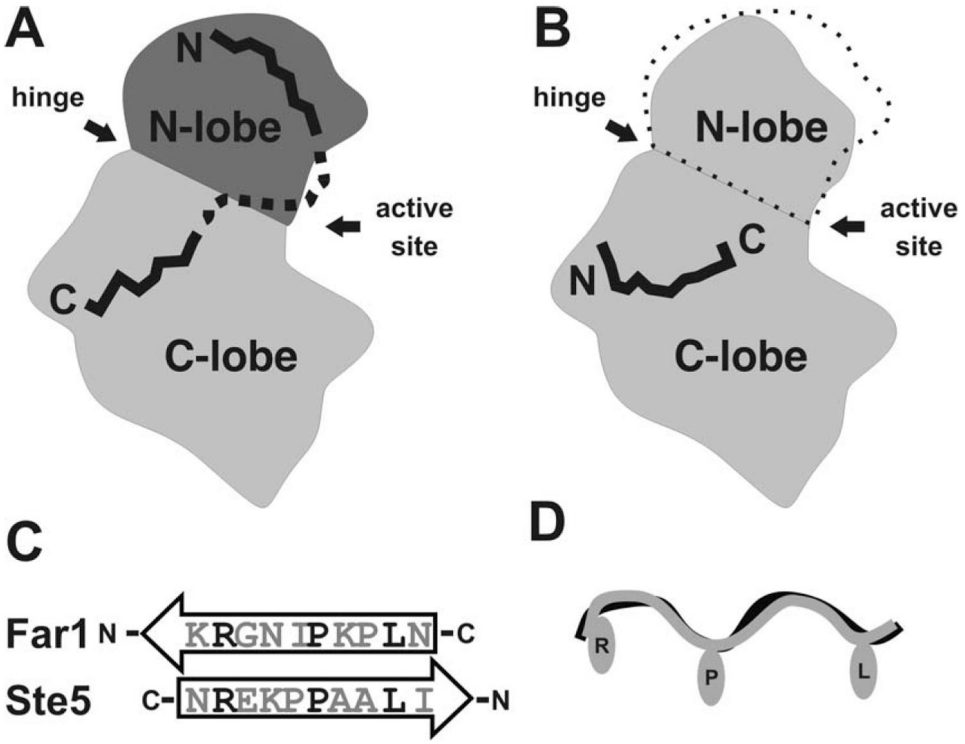

Fig. 6. Differential and competitive binding modes of MAPKK Ste7 and the Ste5 scaffold for sites on Fus3

A. Ste5 binds Fus3 in a bipartite manner. The two Ste5 interacting regions are separated by a IDed linker. By binding to both the N- and C-lobes of Fus3 and being restricted by the length of the linker, Ste5 alters the positioning of the lobes and consequently the active site configuration which results in altered Fus3 activity. The IDed linker connecting the bipartite binding peptides is depicted as a dotted line. **B**. Canonical binding of Ste7 to the C-lobe of Fus3 does not alter the relationship between the lobes or alter the conformation of the active site and thus does not alter Fus3 activity. Dotted line represents the position of the upper (N-lobe) of Fus3 as depicted in panel A. **C**. Sequence comparison of the structurally characterized binding modes of two peptides that bind the C-lobe of Fus3. Far1 binds in a canonical orientation as does the Ste7 peptide. However, the Ste5 peptide that binds to the C-lobe does so in an opposite orientation. **D**. Both peptides display arginine, proline and leucine side chains as recognition features on one face of the polyproline II helix. This figure is based on Figure 2 of Bhattacharyya et al. (Bhattacharyya et al., 2006).

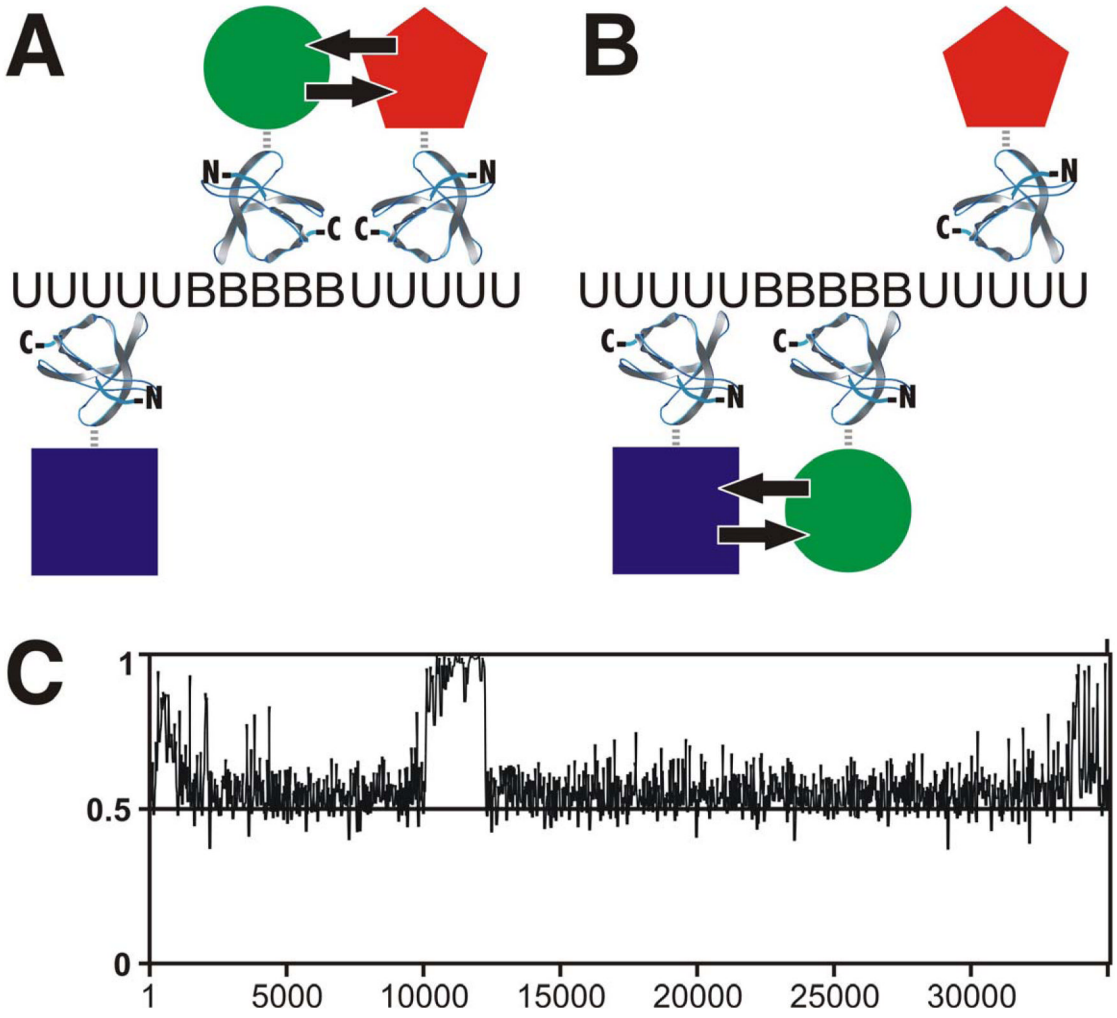

Fig. 7.

The SH3 domains (ribbon structures) of three different hypothetical interacting proteins (green circle, blue square and red pentagon) binding in either class I or class II modes to SH3 elements in the PVEK region of a titin molecule. The PVEK regions of titin isoforms can include both bipolar SH3 elements, which encode overlapping antiparallel ligand sequences (BBBBBBBBB), or unipolar binding sites (UUUUUUUU). Nebulin has a demonstrated ability to bind bipolar ligand sequences in either orientation and is represented here by the green circle. In translation from the class I binding mode (**A**) to the class II binding mode (**B**), the SH3 domain flips and rotates. Note that, due to the PPII conformation of the SH3 element, the per-residue offset between the antiparallel class I and class II domains would entail a 60° rotation about the centerline of the ligand sequence. In this simple cartoon, inter-protein interactions (black arrows) can take place between adjacent SH3-containing proteins (green and red in A and blue and green in B). Note that, in living cells, it is likely that such relationships would be dynamic with fractional stoichiometric distributions among alternate binding sites (Ma et al., 2006). **C**. PONDR® VLS1 prediction for titin (SwissProt accession number Q8WZ42) reflecting homogeneity of residue composition favoring ID (PONDR® scores > 0.5) along most of its length.

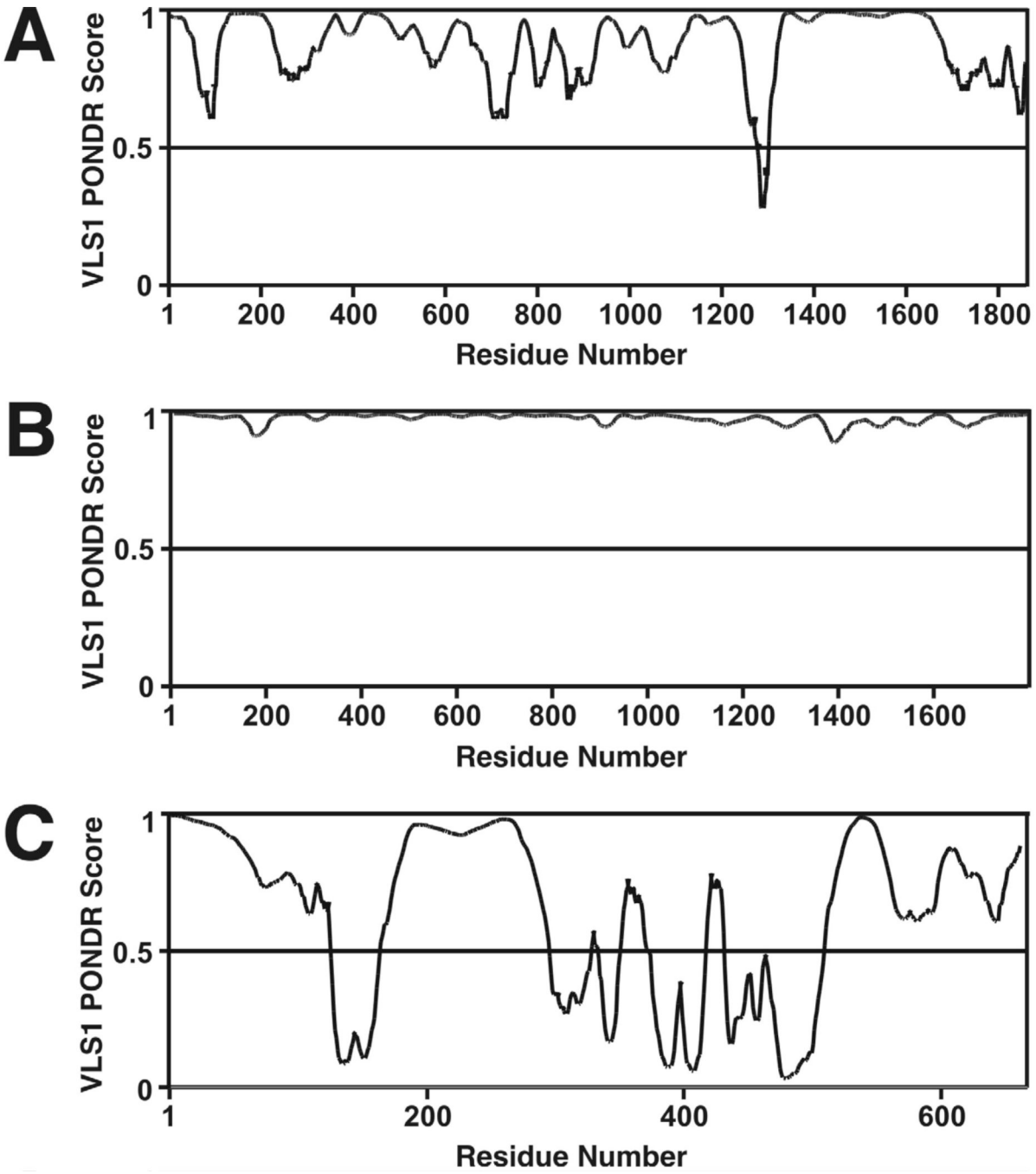

Fig. 8. PONDR® VLS1 predictions for three AKAP scaffolds

A. AKAP250, **B**. MAP2 and **C**. D-AKAP2 (Q02952, P15146 and O43572 SwissProt accession numbers respectively).