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REVIEW

The role of protein disorder in the 14-3-3 interaction network†

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Disordered regions are segments of a protein that do not fold completely and thus remain flexible. These regions have key physiological roles, particularly in phospho-proteins, which are enriched in disorder-promoting residues surrounding their phosphorylation sites. 14-3-3 proteins are ordered hubs that interact with multiple and diverse intrinsically disordered phosphorylated targets. This provides 14-3-3 with the ability to participate in and to regulate multiple signalling networks. Here, I review the effect of structural disorder on the mechanism involved in 14-3-3 protein–protein interactions and how 14-3-3 impacts cell biology through disordered ligands. How 14-3-3 proteins constitute an advantageous system to identify novel classes of biological tools is discussed with a special emphasis on a particular—and innovative—use of small molecules to stabilize 14-3-3 protein complexes, useful to study gene expression, cancer signalling and neurodegenerative diseases.

Introduction

The first protein kinase activity was observed in 1954.[1] Thirteen years later, Moore and Perez named 14-3-3 an abundant mammalian brain protein family due to its particular elution and migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis.[2] It was not until three decades later, in 1996, that it was discovered that interactions of this family with their partners were mediated by phosphoserine/threonine interaction motifs, such as RXSPXP.[3]

The 14-3-3 protein family is constituted by 28–33 kDa acidic proteins found in all eukaryotic organisms in which they have been searched for.[4] They are functionally different from phospho-binding domains such as WD40, PDZ or WW. Two highly conserved family members are present in yeast, seven in mammals, and up to 15 isoforms in plants. These isoforms self-assemble into homo- or hetero-dimers that interact with a diverse array of cellular proteins; hundreds of 14-3-3 ligands have been reported in the literature and in theory they could represent 0.6% of the human proteome.[5] This ability to interact with many different proteins is in part the result of their specific phospho-serine/phospho-threonine binding activity.

Three high-affinity 14-3-3 binding motifs have been initially described in 14-3-3 target proteins: RSXpS/TXP (mode 1), RXXXpS/TXP (mode 2) and pS/T-X(1–2)-COOH (mode 3), where pS/T represents phospho-serine/threonine and X is any amino acid.[4] Structural analyses of 14-3-3 dimers have revealed that each monomer contains an independent ligand-binding channel and, as a result, the dimer can interact with two motifs simultaneously, found either on a single target or on separate binding partners.[6] 14-3-3 dimers are highly rigid structures and binding can induce conformational changes in their protein ligands. This might alter the stability and/or catalytic activity of the ligand.[7] In addition, 14-3-3 binding can hide intrinsic localization motifs, prevent molecular interactions and/or modulate the accessibility of a target protein to modifying enzymes such as kinases, phosphatases or

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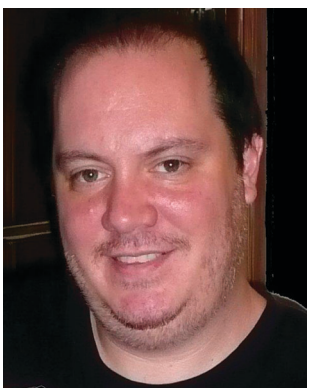

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I obtained my MSc Thesis in 1998 at the Universidad Nacional de Rosario, Argentina. Then I moved to the Instituto Tecnologico de Chascomus and obtained my PhD degree in 2002 at the Universidad de Buenos Aires, Argentina, as a Doctoral fellow from the Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET). I spent two years (2002–2004) in France at the Université Victor Segalen Bordeaux 2 as a Postdoctoral fellow from the Centre National de la Recherche Scientifique (CNRS) and the Foundation pour la Recherche Medicale. I returned to Argentina as a scientist from CONICET and started my research on structural disorder in 14-3-3 partners. Recently I did a second postdoctoral stage at the National Institutes of Health, National Institute of Child and Human Development, USA (2007–2009). At my return I established the Laboratorio de Biologia Estructural y Celular de 14-3-3 at the Instituto Tecnologico de Chascomus.

proteases.[4,7] Like domains in signalling networks, 14-3-3 generally interact with proteins that involve in one of three major functions: regulation, localization or catalysis.

Impact of disordered interaction in the 14-3-3 biology

14-3-3 binding partners have neither structural nor functional relatedness

Low- and high-throughput studies identified many 14-3-3 interacting proteins in human and rodent cells and tissues, hydra, yeast and plants. Some of these studies were focused on specific biological process selecting one or a few proteins that were analysed further in detail.[8] In general, these studies could identify a subset of proteins involved in some specific processes like regulation of the cytoskeleton, GTPase function, membrane signalling, cell fate determination; response to insulin and TNF-alpha; phases of the cell cycle; apoptosis (for details see references in ref. 8).

However, analysis and clustering techniques of the full list of 14-3-3 partners failed to establish a rational classification (structural or functional) of these proteins.[9] A domain-based classification of all freely available 14-3-3 partners makes it difficult to understand how 14-3-3 can accommodate so many different structural domains. It was proposed that the different partners' side chains follow the 14-3-3 binding groove structure by an induced-fit mechanism.[10] Also, interactions of 14-3-3 with their partners show characteristics, such as hydrogen bonds between side chains of 14-3-3 and the backbone of the partners and hydrogen bonds between the backbone of the partners and water molecules, indicating that partners are disordered in solution just prior to association with 14-3-3.

14-3-3 proteins bind disordered partners

Intrinsic disorder, *via* diversity arising from structural plasticity or flexibility, enables proteins to interact with numerous partners.[11–17] According with the current estimations, more than 90% of the 14-3-3 protein partners do not adopt a defined three-dimensional structure totally or in part. Moreover, almost all the high-affinity 14-3-3 binding motifs are contained in these intrinsic disorder regions showing the importance of disorder in 14-3-3 binding.[18] The number of proteins with structural disorder in the 14-3-3 binding partners is significantly high compared with other studies, for example, on cell signalling and cancer-related proteins or RNA chaperons.[18] Similar conclusions relating to 14-3-3 binding sites and disorder were achieved later by Collins and co-workers.[19]

According to the classical theory, 14-3-3 proteins discriminate between the phosphorylated *vs.* the non-phosphorylated states, based on the conformational changes induced by the presence of a negatively-charged phosphate group in the basal state of the binding motif.[20] NMR studies for the KH—domain 1 of the RNA binding K—homology splicing regulator protein and its interaction to 14-3-3 proteins showed that adding a phosphate group leads to partial unfolding of previously structured domains, making them more accessible to the recognition domains.[21] Jacobson and co-workers provided insight into localized conformational changes driven by phosphorylation at near-atomic accuracy, which can be used to create hypotheses about mechanisms of regulation by phosphorylation.[22]

Disordered regions can bind their targets with high specificity and low affinity. Phosphorylation-dependent transitions among a native, disordered state and a globular structure may also provide thermodynamic regulation of binding.[16] Indeed, we demonstrated that a disorder-to-order transition occurs after the binding to 14-3-3 proteins.[18] Although this kind of transition is disfavoured in terms of entropy,[23] the formation of the complex is driven by a large enthalpy change associated with the favourable hydrogen bonding interactions involving the phosphoryl group.[18] Analysis of the crystallographic structures of 14-3-3 in complex with a variety of peptides also supports this hypothesis.[10] Because of the backbone of the peptides is highly hydrated in the bound state, this indicates that the binding peptide is likely to be unstructured prior to the binding. This uncouples the binding strength from specificity and renders highly specific reversible interactions, which are fundamental in cell signalling and regulation.[10,23]

Intrinsic disorder change among 14-3-3 isoforms' specific partners

Studies of 14-3-3 isoforms are technologically difficult, most studies reported on 14-3-3 proteins do not distinguish between isoforms and, in many cases, isoforms are assumed to be functionally equivalent while they are not.[24]

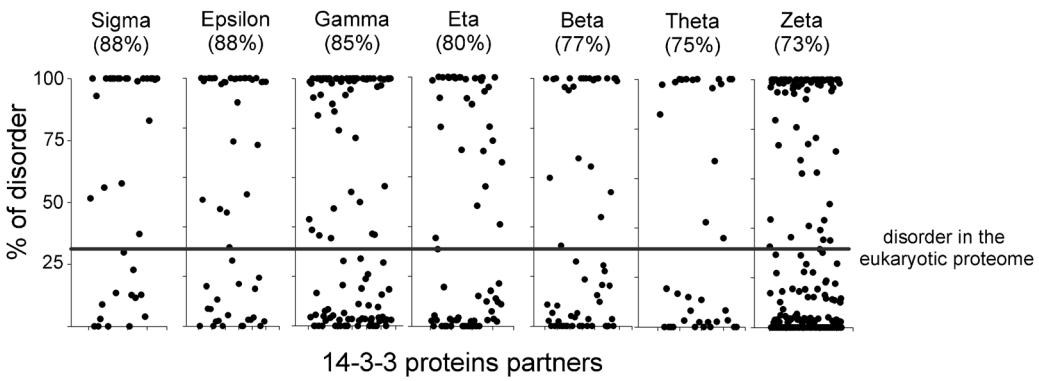

Fig. 1 Percentage of disorder in the binding partners of each 14-3-3 isoforms. Each black dot represents a 14-3-3 binding partner. The percentage number below each isoform name is the percentage of disorder partners for each of them.

Comparison of 14-3-3 PPIN with the Disprot (www.disprot.org)[25] and prediction of intrinsic disorder show that the 14-3-3 isoform specific partners have different levels of disorder (Fig. 1). 14-3-3ζ is the most interacting among the isoforms and also is the one with less content of disorder within its partners. 14-3-3σ reaches 88% of disordered partners, it is probably the highest percentage of disorder when compared with other studies.[18] Although initially the different mammalian 14-3-3 isoforms were thought to be functionally redundant, there are a growing number of examples that evidence specific roles for them.[24] Structural data show little divergence in the phosphopeptide-binding pockets of different 14-3-3s,[6] and because most 14-3-3 binding motifs conform to a few consensus sequences, it seems that isoform specificity does not reside in the binding site sequence of the binding partner. Indeed, it most likely depends on additional contacts with the partner proteins probably involving residues, such as anchors,[26] outside the 14-3-3 binding motifs[27] (Fig. 2).

The binding mechanism of 14-3-3 proteins and their disordered partners require anchor residues outside the 14-3-3 binding motif

Disordered proteins can have the advantage of a greater capture radius for a specific binding site than the folded protein state. The fly-casting mechanism hypothesizes that unfolded proteins bind weakly at a large distance followed by folding as the proteins approach to the binding sites.[28] However, the conformational search during the binding appears to be too long and some other mechanisms to reduce it would be necessary.[26] Rajamani and co-workers showed that for globular proteins, molecular recognition requires one of the interacting proteins to anchor a specific side chain in a structurally constrained binding groove of the other protein, providing a steric constraint that helps to stabilize a native-like bound intermediate.[26] They identified the anchor residues in 39 protein–protein complexes and verified that, even in the absence of their interacting partners, the anchor side chains are found in conformations similar to those observed in the bound complex. These ready-made recognition motifs include surface side chains that bury the largest solvent-accessible surface area after forming the complex (>100 Å^2). The existence of such anchors implies that binding mechanisms can avoid kinetically costly structural rearrangements at the core of the binding interface, allowing for a relatively smooth recognition process.[26] Once anchors are docked, an induced fit process further contributes to form the final high-affinity complex. This later stage involves flexible (solvent-exposed) side chains that latch to the encounter complex in the periphery of the binding pocket.[26] The mechanism emerging from the dynamics of solvated proteins indicates that anchor residues provide most of the specificity necessary for protein–protein recognition, whereas latch residues regulate the stability for protein function.[18,26,29]

Recently, we showed for the first time the existence of remote (to the 14-3-3 binding motif) residues in a 14-3-3 phosphorylated partner essential for the binding to 14-3-3.[29] Results support that phosphorylation, although necessary, is not sufficient for 14-3-3's complex formation, as structurally constrained anchor residues play a critical function in stabilizing the protein–protein interaction[29] (Fig. 2). These residues are

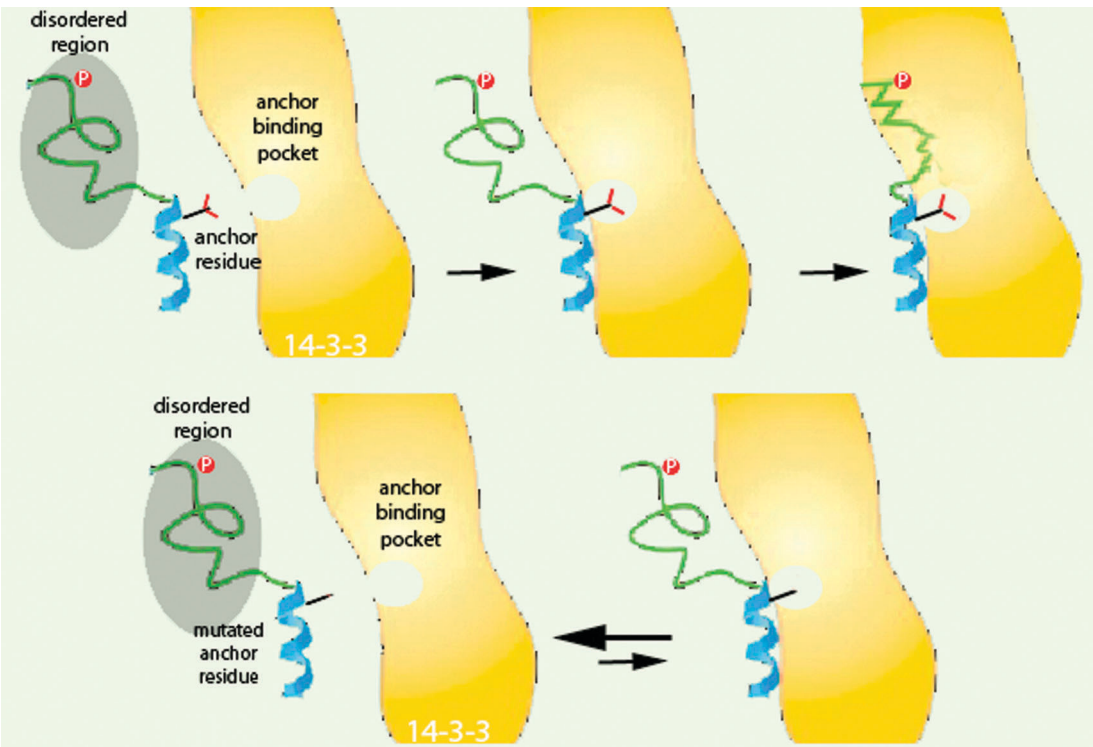

Fig. 2 A schematic representation of the mechanism of the binding to 14-3-3. Anchor residue is an initial contact that reduces the conformational time search, and the disordered region suffers a transition from disorder to order after the binding to 14-3-3 proteins. Adapted from Fuxreiter *et al.*[80]

within a globular region of the partner, whereas the 14-3-3 binding motif is in a disordered region. Besides their stabilizing role, these residues may be related to 14-3-3 binding specificity, which remains to be established.

Disordered ligands and 14-3-3 impact on cell biology

Many-to-one signalling in the 14-3-3 network

The clustering analysis of *in vivo* 14-3-3 binding proteins suggests that 14-3-3 proteins can impinge simultaneously on multiple facets of cellular behaviour, consistent with the protein–protein interaction network (PPIN) available through the following link: http://csbi.ltdk.helsinki.fi/pina/interactome.listUserNetwork.do. At the moment, the 14-3-3 PPIN contains 874 proteins interconnected by 1535 interactions (Table 1 resumes some 14-3-3 PPIN's characteristics). Each 14-3-3 isoform interacts with a different number of partners with a moderate degree of superposition (Table 1B and C). The 14-3-3ζ is the most interacting isoform with 432 partners, with 225 (52%) of each are specific for this isoform, whereas only 102 partners (20 specific) have been found at the moment for 14-3-3ε (Table 1).

Disordered proteins are subject of tight regulation and targeted protein degradation,[30] and they are substrates of twice as many kinases as are ordered proteins.[31] On average, 51% of protein substrates of kinases are highly unstructured, whereas only 19% are highly structured. This is a significant bias as compared with the expected genome wide distribution of ~30% of highly disordered proteins.[32–35] Overall, disordered regions have a much higher frequency of phosphorylation sites than ordered regions, either known or predicted. Comparing the B-factors of phosphorylated residues in crystal structures, Dunker and co-workers concluded that protein phosphorylation occurs predominantly within disordered regions and not merely on surface residues.[32,36]

Recently we linked phosphorylation, protein–protein interaction and protein disorder in a biologically relevant context.[29] 14-3-3 proteins bind multiple disordered and phosphorylated partners, being a typical example of the many-to-one signalling network, where disordered regions with different sequences use their flexibility to bind to a common binding site.[13] The role of disorder on protein–protein interactions networks was not examined on the initial studies.[37] However, it is widely accepted today that intrinsic disorder is an important feature in protein networks, and contributes to build highly connected networks.[37] An interesting classification was postulated by Gerstein and co-workers whom distinguished between singlish-interface and multi-interface hubs in the context of structural interaction networks.[37] They found that singlish-interface hubs have a much higher fraction of disordered residues than multi-interface hubs. However, if the interface of the singlish-interface hub itself is structured, as in 14-3-3 proteins, the promiscuous binding is led by promoting disorder residues on the binding partners. In other words, binding partners of structured singlish-interface hubs are significantly more disordered than the binding partners of multi-interface hubs.

Table 1 Characteristics of 14-3-3 protein–protein interaction network. (A) Number of partners and number of interactions for each isoform. (B) Number of partners in common between the different isoforms (intersection). (C) Number of specific partners for each isoform

A

Isoform	Gene name	Protein number	Interaction number
14-3-3 eta	*ywhaq*	151	150
14-3-3 zeta	*ywhaz*	432	431
14-3-3 gamma	*ywhag*	351	350
14-3-3 epsilon	*ywhae*	102	101
14-3-3 beta	*ywhab*	201	200
14-3-3 theta	*ywhah*	154	153
14-3-3 sigma	*Stratifin*	164	163
All	Full network	874	1535

B

Isoform Name	Beta	Epsilon	Gamma	Eta	Sigma	Theta	Zeta
Beta		47	96	42	50	68	87
Epsilon	47		40	41	26	27	58
Gamma	96	40		80	59	57	123
Eta	42	41	80		32	38	80
Sigma	50	26	59	32		34	50
Theta	68	27	57	38	34		56
Zeta	87	58	123	80	50	56	

C

Isoform name	Number of specific partners
Beta	32
Epsilon	20
Gamma	130
Eta	25
Sigma	72
Theta	30
Zeta	225

Hence, promiscuous binding is partly mediated by disorder, but not on the interface in the singlish-interface hub itself, rather on the interacting partners.[37,38] 14-3-3 proteins are ordered hubs and interact with intrinsically disordered binding partners, such interactions play crucial roles in the regulation and coordination of 14-3-3 hub activities.[10] Intrinsically disordered regions provide hubs with the ability to bind multiple and diverse targets, thereby enabling them to participate in and regulate multiple networks.[38]

14-3-3 bind and regulate disordered transcription factors

Based on a comprehensive analysis of the preference of disorder, Camacho and co-workers classified the genomes in different types. Higher eukaryotic genomes show no strong preference for ordered structures in binding proteins but preference for disorder in transcription factor proteins.[39] Also, the role of the disorder in transcription factors' binding mechanisms was analytically resolved by molecular simulations.[40] The 14-3-3 interactome has provided a list of transcription factors (see http://csbi.ltdk.helsinki.fi/pina/interactome.listUser Network.do). The length of this list reveals that the regulation of transcription factors by 14-3-3 proteins is unexpectedly complex, and that many transcription factors probably act in concert with 14-3-3 proteins and kinases to control the cell functions. Some of the transcription factors are p53,[41] TAZ,[42] YAP,[43] FOXO1, 3a and 4,[44] MIZ1,[45] and others. Among these, the interaction of p53 and 14-3-3 was exhaustively studied. Intrinsic disorder is important in the mechanism of binding, in the regulation and to facilitate multiple recognitions.[10,41,46–48] The p53 protein has three different domains, the N-terminal domain (the trans-activation domain), the C-terminal domain (the regulatory domain, from residue 374 to 388), and the DNA binding domain. The terminal domains have been characterized as intrinsically disordered and the DNA binding domain as structured. 14-3-3 (isoforms ε and γ) interact with p53 phosphorylated in the C-terminal at S366, S378 and T387 *in vitro* and *in vivo*. This increases the transcriptional activity of p53 mediated by stabilizing p53 levels in cells.[41,46,47] The most significant and general feature that correlates with intracellular degradation is protein disorder.[30,49] The presence of disorder renders proteins sensitive to degradation irrespective of the actual length of the chain. It is possible that the presence of protein disorder is mandatory for the recognition of classical short degradation signals, such as the destruction-box or the KEN-box, but it is also possible that a disordered segment serves as a kind of conformational recognition element, which is recognized due to its lack of a stable fold, irrespective of its actual sequential content.[30,49] One of the recognized functions of 14-3-3 is the protection against proteasomal proteolysis,[50] which explains the effect of 14-3-3 stabilizing p53 protein levels in the cell. The intrinsically disordered C-terminus of p53 binds to 14-3-3 and also to other proteins such as cyclin A, sirtuin, CBP and S100ββ,[10] highlighting the importance of intrinsic disorder to generate highly connected protein–protein interaction networks.

14-3-3 bind and regulate enzymes

Since the catalytic activity of enzymes imposes highly structured domains, from their discovery, 14-3-3 proteins have been characterized as regulators of the activities of a number of signalling and metabolic enzymes, including Raf-1,[51] protein kinase C,[52] tyrosine and tryptophan hydroxylases[53] and nitrate reductase.[54] Probably, the most studied interaction of 14-3-3 with an enzyme is its interaction with the serotonin *N*-acetyl transferase (AANAT).[50,55–57] The resolution of the crystal structure of the near-full-length complex arranged between ovine AANAT and the human 14-3-3ζ contributed to understand the binding process.[56] oAANAT is a 14-3-3 binding partner with two canonical motifs located in disordered regions and a globular domain. It is one of the 50 so far reported 14-3-3 binding proteins with two binding sites. Before the binding, the N- and C-terminal regions containing the 14-3-3 binding motifs are fully disordered and hydrated. After the binding to 14-3-3, the N terminal 14-3-3 binding motif follows the binding groove in 14-3-3. Unfortunately, oAANAT must be truncated in its C-terminal region in order to obtain the crystals and thus similar information to the N terminal was impossible to observe. Besides the induced-fit on the disordered region, other residues within the globular region of oAANAT contribute to the binding to 14-3-3 by stabilizing a native-like intermediate. These amino acids, not surrounding the pS/pT in the phosphorylated partner's 14-3-3 binding motif, are essential in the interaction between these proteins (Fig. 2). This means that a phosphorylated 14-3-3 motif is necessary but not enough as a target for the binding. If these residues are mutated the binding is impaired, although the 14-3-3 binding motif is phosphorylated.[29]

Specific 14-3-3 isoforms impact in cell cycle/cell death control

A significant number of genetic and biochemical studies (in several organisms) that isolated a set of genes associated with cell cycle regulation and cell death showed that 14-3-3 proteins play a key role in cell cycle control.[41,58] The most studied relationship between a member of 14-3-3 family and proteins linked to these cellular functions involve the σ isoform.[58] However, it is important to note that genes associated also with these two biological functions have been found in databases as partners of other 14-3-3 isoforms (η and ε). These may reflect the specific biology of these isoforms. Studying the hyper-connected loops of the 14-3-3's protein network we discover that certain cell functions are specifically linked to one 14-3-3 isoform and not to the others. The 14-3-3ε isoform has a centrality in cell death; the eta isoform in the cell cycle with a particular motif loop with Mdm4, Chk2 and Cdc25c that wasn't previously fully described. Similar analysis shows that γ's partners are enriched in RNA splicing.

14-3-3 interact with structural cell proteins

Cytoskeleton and structural proteins represent the group with less intrinsic disorder and it might be reasonably to suspect that binding to 14-3-3 proteins is limited in this class of proteins. However, Pawson and collaborators found that numerous proteins involved in regulation of the actin cytoskeleton, polarity, focal adhesions, and endocytosis bind to 14-3-3.[5] The significance of these proteomic data was supported by the effects of inhibiting 14-3-3 phosphopeptide binding in living cells, which markedly affects actin polymerization, perturbs cell morphology,

membrane dynamics, cell protrusions, and ability to establish tight junctions. The molecular mechanism by which 14-3-3 regulates all these processes has not been determined.

14-3-3 impact on chromatin biology *via* interaction with histones disordered N-tails

Histones comprise the major protein component of chromatin, and are subject to phosphorylation, acetylation, methylation and other types of post-translational modifications on their disordered N-tail.[59] These modifications constitute a 'histone code' and could be used to manage epigenetic information extending the genetic message beyond DNA. The first report of 14-3-3 binding a phosphorylated histone was in 1994.[60] The 14-3-3 proteins bind histone tails in a strictly phosphorylation-dependent manner[61] and acetylation of lys9 and 14 does not impede the binding.[62]

It is known that histone tri-methylation on Lys9 is recognized as a signal for repression and binding of the transcriptional repressor HP1. Histone phosphorylation on Ser10 (and 28) promotes the recruitment of 14-3-3 and leads to the dissociation of HP1, allowing the transcription of specific genes. The 14-3-3 proteins are able to recognize this mark (phosphorylation on Ser 10 and/or 28) to bring histone acetyl transfereases to the promoters for gene activation *via* acetylation on Lys9. A second model was also suggested, 14-3-3 proteins mediate cross-talk between histone phosphorylation and acetylation during transcriptional elongation by creating a bridge between the Ser10 kinase and the Lys9 acetylase.[63]

However, a complete mechanism from the removal of the methylation mark to the 14-3-3 recruitment remains elusive.

Future directions

The extraordinarily high sequence conservation between 14-3-3 protein isoforms, characterized by the presence of highly conserved sequence blocks of invariant amino acids, poses a significant technological challenge to researchers working with 14-3-3 proteins. A systems-level approach is ultimately necessary to map 14-3-3 network's additional components and to understand their functions. Comprehension of phosphorylation-dependent signalling networks and integration of that information with information about kinases-substrates,[33,35,64,65] acetylation[66] and ubiquitination could provide insights on the cellular behaviour and what could occur when those regulatory circuits become dysfunctional or are modified in response to changing environmental conditions.

It is clear that 14-3-3-partners interactions are embedded in a much rich network of interactions with a wide variety of other components, showing extensive cross-talk with other signalling activators and inhibitors of signalling, through its regulatory or scaffold mechanisms.[67] Due to its size, a complete network map, although informative, offers little insight into its large-scale characteristics. It is necessary to identify the architecture of the network, determining whether it is best described by an inherently uniform exponential topology, with proteins on average possessing the same number of links, or by a highly heterogeneous scale-free topology, in which proteins have a widely different number of partners.[68] Small molecules libraries represent a powerful and promising approach to decipher intricate phosphorylation-based cellular signalling networks[69,70] and to analyse the role of every new highly connected motif that could be identified in the 14-3-3 network. Unlike in the case of classical domain–domain interactions,[71,72] the binding mode of 14-3-3 proteins with their partners enables small molecules to compete in the binding,[73] which raises the opportunity to develop potential drug molecules that can specifically interfere with key signal transduction pathways involved in disease states.[74–76]

A very interesting idea is to use small molecule stabilizers[75] of protein–protein interactions, which, targeting the interaction surface of two proteins, are able to modulate a precise target-protein function. The addressed binding site (pocket) will be constituted by two protein partners simultaneously (protein–protein interface). This allows a higher specificity, because the actual binding pocket exists not *per se* as part of a single polypeptide but it is formed by the specific interaction of the two protein partners. Additionally these combined sites are likely to be distinct from each other, since protein surfaces in general are much more variable than enzyme active sites. The 14-3-3-proteins provide an ideal and advantageous system to identify novel classes of biological tools which can be used in new ways to the study of gene expression, and cancer signalling pathways, as well as in the understanding of neurodegenerative diseases.[74,75,77–79]

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