

Review

The 14-3-3 Protein Family, Beyond the Kinases and Phosphatases

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Abstract

Eukaryotic phosphorylation of serine and threonine residues is a central regulatory mechanism in cell signalling, carried out by more than 500 kinases and a diverse array of phosphatases. Traditionally understood as a two-component system driven by writers (kinases) and erasers (phosphatases), this regulatory network is now appreciated to involve additional proteins that modulate or interpret phosphorylation-dependent changes. Among them, the 14-3-3 protein family has emerged as a prominent example due to its ability to bind phosphorylated serine/threonine motifs—typically located within intrinsically disordered regions—and influence the activity, stability, or localization of its partners. In this review, we discuss the importance, evolution, structure, and dynamics of 14-3-3 proteins, as well as their interactions with small molecules—both natural and designed—that bind to them. We highlight several underexplored aspects of their molecular behaviour, integrate recent discoveries, and emphasize how these insights contribute to a broader understanding of phosphorylation-dependent regulation across eukaryotes.

Keywords: 14-3-3 proteins family; phosphorylation reader; protein structure; allosteric regulation



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1. Introduction

The 14-3-3 protein family comprises highly conserved regulatory polypeptides that influence diverse cellular processes. They function as protein kinase-dependent activators of tyrosine and tryptophan hydroxylases while simultaneously acting as endogenous inhibitors of protein kinase C. Several isoforms have been identified in all eukaryotes, including β (YWHAB), γ (YWHAG), ϵ (YWHAE), ζ (YWHAZ), θ (YWHAQ), σ (SFN), and η (YWHAH). This protein family has emerged as a key mediator of signalling pathways that govern mitosis, cellular proliferation, cortical development, and neuronal migration [1]. Molecularly, the 14-3-3 proteins bind phosphorylated serine/threonine residues within defined motifs, Mode I (RSXpSXP), Mode II (RX Φ XpSXP), and the C-terminal Mode III (pS/pT-X₁₋₂-COOH), where X is any amino acid and Φ is a hydrophobic amino acid [2]. Binding is primarily phosphorylation-dependent, although a few examples of unphosphorylated targets exist, as the phosphate group is stabilized within the amphipathic groove of 14-3-3 through the formation of three hydrogen bonds. Another key structural feature is the presence of approximately 30 consecutive amino acids in an intrinsically disordered region that contains the phosphorylated serine/threonine [3]. Notably, these motifs frequently overlap with kinase consensus sequences, particularly, but not exclusively, those

of AGC kinases (see Table 1). AGC kinases are a conserved family of serine/threonine protein kinases, including PKA (Protein Kinase A), PKG (Protein Kinase G), PKC (Protein Kinase C), and Akt/PKB (Protein Kinase B), that regulate diverse cellular processes such as metabolism, growth, proliferation, and survival.

Table 1. Representative kinase families, their consensus phosphorylation motifs, and recognition by 14-3-3 proteins. Information compiled in Table 1 was retrieved from the Scansite 4.0 database. Scansite “<https://scansite4.mit.edu/#motifs> (accessed on 22 July 2025)” integrates experimentally validated phosphosites with computational motif analysis to generate high-confidence consensus sequences. These patterns were extracted directly from the database and used to summarize the sequence preferences characteristic of each kinase family.

Often Recognized by 14-3-3. Y/N	Consensus Phosphorylation Motif	Notes/Function	Representative Kinases	Kinase Family
Y	RXXS/T or RXRXXS/T	Ser/Thr phosphorylation	PKA, PKG, PKC, Akt/PKB	AGC
Y	S/TP	Proline-directed Ser/Thr kinases; regulate cell cycle and signalling	CDKs, MAPKs, GSK3	CMGC
Decrease affinity of binding—N	D/E XXS/T(p)	Often requires pre-phosphorylated substrate (p)	CK1α, CK1δ	CK1
N	S/TD/E X D/E	Acidophilic; targets negative residues near Ser/Thr	CK2α	CK2
Y (Inhibition of binding)	S/T XXS/T	Often upstream in MAPK cascades	MEKK1, ASK1	STE (MAPKKK family)
Y	RXXS/T	Activated by Ca ²⁺ /calmodulin; Ser/Thr phosphorylation	CaMKII, AMPK	CAMK
N	S/TQ	DNA damage or nutrient response signalling	mTOR, ATM, ATR	Atypical
variable	S/Ta	Diverse motifs; context-dependent	Various	Other Ser/Thr

a corresponds to a basic or acidic residue and X corresponds to any amino acid. AGC kinases include PKA, PKG, and PKC families; CMGC kinases encompass CDKs, MAPKs, GSKs, and CLKs; CK1 and CK2 refer to Casein Kinase 1 and 2 families; STE kinases (named *Sterile* in yeast) correspond to the MAPKKK family; MAPK: Mitogen-activated protein kinase; CaMKII: Calcium/Calmodulin-dependent protein kinase II; AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; mTOR: mechanistic target of rapamycin; ATM: Ataxia-telangiectasia mutated; ATR: Ataxia Telangiectasia and Rad3-related; Atypical kinases fall outside canonical ePK families and include PI3K-related, alpha-, and RIO kinases; and Other Ser/Thr kinases are additional serine/threonine kinases that do not neatly fit into the established groups.

This table summarizes major kinase families, their typical substrate motifs, and how these phosphorylated motifs serve as docking sites for 14-3-3 proteins, highlighting the integration of kinase signalling with 14-3-3-mediated regulation of protein localization, stability, and complex assembly.

These kinases phosphorylate substrates at specific consensus sequences, and these phosphorylation events generate docking sites for 14-3-3 proteins, linking kinase signalling to 14-3-3-mediated regulation of protein localization, stability, and complex assembly. This mechanism ensures that phosphorylation both modulates substrate activity and creates docking sites for 14-3-3. Examples include Akt-mediated phosphorylation of Bad, which promotes 14-3-3 binding and cytosolic sequestration, and Chk1/Chk2-dependent phosphorylation of Cdc25, which drives 14-3-3-mediated cytoplasmic retention and cell cycle arrest [4]. This superposition of kinase and 14-3-3 motifs establishes a direct mechanistic link between kinase signalling and 14-3-3-dependent regulation of protein localization, stability, and complex assembly. When 14-3-3 is viewed as a reader of a specific phos-

phoserine/phosphothreonine motif, it transforms the signalling pathway's step into a regulatory checkpoint.

2. Functional Divergence and Evolutionary Conservation of 14-3-3 Protein Paralogs in Mammals

Although 14-3-3 proteins were originally discovered and characterized at the protein level [5], and many researchers still refer to them as isoforms, advances in genomics have clarified their genetic basis. It is now well established that each 14-3-3 variant is encoded by a distinct gene, and should therefore be described as paralogs rather than isoforms. This distinction is important, as it reflects their evolutionary divergence and helps avoid confusion when comparing their structural, regulatory, and functional specificities across different biological systems.

The 14-3-3 protein family plays a central role in eukaryotic signal transduction by binding to hundreds of phosphorylated client proteins, modulating their localization, stability, activity, and interaction networks [6]. These proteins are essential regulators of diverse biological processes including cell cycle progression, differentiation, apoptosis, stress responses, metabolism, DNA damage repair, and cytoskeletal dynamics [7]. In mammals, the family was originated from ancient gene duplication events early in vertebrate evolution, likely during two rounds of whole-genome duplication (1R/2R) at the base of the vertebrate lineage. Indeed, the 14-3-3s protein targets are also 2R-ohnologues, which suggest that 14-3-3s coevolved with their targets [8].

Although these paralogs share a conserved tertiary and quaternary structure—characterized by a nine-helix bundle forming a dimeric, cup-like phosphopeptide-binding groove—their primary sequences, interaction networks, post-translational modification (PTM) landscapes, and biological roles have diverged significantly among the seven mammalian paralogs [9,10]. This divergence is particularly notable in six surface-exposed variable regions (V1–V6), located in flexible loops and terminal α -helices, which modulate client binding selectivity. Despite these important differences between 14-3-3 paralogs, with significant specificity and functional consequences, 14-3-3 orthologs of each paralog are highly conserved across mammals, with >99% sequence identity, for example, between human and murine 14-3-3 γ [10]. This extremely high conservation across mammals indicates that the structure of the protein and its function are under strong evolutionary constraint. Such strong purifying selection highlights the essential, non-redundant roles that each paralog fulfils, preserving specific functional repertoires across species. The strong purifying selection is negative selection pressure that acts to eliminate deleterious mutations and preserve essential biological functions. In other words, mutations that alter key residues involved in dimerization, phosphopeptide binding, or allosteric regulation are selected against—they reduce fitness—and are therefore removed from the population over time. This ensures that orthologous genes maintain their role in critical signalling pathways, such as insulin signalling pathways, cell cycle checkpoints, cell proliferation and differentiation (and many others), in which 14-3-3 proteins participate.

Evolution of Human 14-3-3 Protein Family

Although the 14-3-3 proteins coexist in an organism, phylogenetic trees show that each paralog is more closely related to its orthologs in other species than to its sister paralogs [11]. For example, 14-3-3 γ from humans is genetically and structurally more like mouse or zebrafish 14-3-3 γ than to human 14-3-3 β . This pattern reflects ancient gene duplication events followed by paralog-specific divergence—a process that has allowed each paralog to evolve unique structural or regulatory features while maintaining a conserved core that anchors 14-3-3 to its ancestral protein interactions. Thus, functional specializations in the family,

such as phospho-target preferences, stability of dimers, or regulatory post-translational modifications are more paralog specific than by co-expression within a single organism.

The signalling pathways in which 14-3-3 proteins participate are deeply conserved in vertebrates and depend on the precise molecular recognition provided by the different 14-3-3 paralogs. Disruption in 14-3-3-mediated regulation can lead to cell death, severe developmental defects, neurological disorders, or cancer, which explains the strong evolutionary pressure to conserve their sequence and structure [12,13]. The high conservation of 14-3-3 orthologs in mammals reflects intense negative selection pressure, which maintains their essential role in cell signalling and protein regulation. This is a classic example of how selection pressure preserves protein function across species, particularly when that function is embedded in critical and ancient molecular networks. The functional importance of these 14-3-3 paralogs is evidenced by the fact that even their protein dosage is critical. For example, a mutation in only one allele of the 14-3-3 γ gene (YWHAG), that results in a mis-sense variant, leads to 14-3-3 γ haploinsufficiency in humans and mice [14,15]. This genetic disorder is phenotypically expressed as a syndrome named YWHAG, as the 14-3-3 γ gene, and affects a growing number of children and adults worldwide. Patients suffer many neurological issues, such as intellectual disability with ataxia, epilepsy, autism, hypotonia, and variable behavioural phenotypes like ADHD. Indeed, there is a YWHAG foundation to support families, and research focused on this syndrome “<https://www.ywhagfoundation.org/> (accessed on 22 July 2025)”. Table 2 resume 14-3-3 paralogs and its links to human diseases.

Table 2. Overview of human 14-3-3 Family. Members Including Chromosomal Location, Loss-of-Function Phenotypes, and Disease Links.

Key References	Human Disease Associations	Knockout/Loss-of-Function Phenotype	Chromosome (Human)	Paralog (Human)
[16,17]	Linked to neuropsychiatric disorders; expression altered in cancers	Full KO is embryonic lethal; partial reduction produces context-dependent phenotypes	7q11.23	YWHAB/(β)
[17,18]	De novo LoF variants cause neurodevelopmental syndromes	KO or conditional KO causes severe neurodevelopmental abnormalities; essential for neuronal migration; male infertility in mice	17p13.3	YWHAE/(ϵ)
[17,19]	Associated with schizophrenia and autism	Haploinsufficiency causes hyperactivity, anxiety-related behaviour, and neuronal signalling alterations	7q11.23	YWHAG/(γ)
[16,18]	Associated with Parkinson’s disease and schizophrenia	Single KO usually mild due to redundancy; combined loss with other paralogs causes synaptic and behavioural defects	22q13.2	YWHAH/(η)
[20]	Colorectal cancer and association with metastasis and poor survival of patients	KO increases susceptibility to colorectal cancer	1p36.11	SFN/(σ)
[16]	Expression altered in Alzheimer’s disease and multiple cancers	KO produces mild neurological traits; strong functional redundancy with $\beta/\epsilon/\zeta$	22q13.1	YWHAQ/(θ)
[21,22]	Linked to schizophrenia, CJD, Alzheimer’s, cancers	KO or KD causes neuronal migration defects, cognitive impairment, altered spine density, hyperactivity	8q22.3	YWHAZ/(ζ)

KO describes the complete inactivation or deletion of a gene, resulting in the absence of the corresponding protein. KD indicates a partial reduction in gene expression. LoF broadly encompasses any mutation or perturbation that diminishes or abolishes normal gene function, including both complete gene disruption (as in KO) and partial impairment (as in KD or hypomorphic variants).

A key mechanism underlying the functional diversification of 14-3-3 paralogs is their specialization in distinct signalling pathways and cellular processes. For example, 14-3-3 σ is known for its role in epithelial cell cycle arrest and tumour suppression, acting as a downstream effector of p53 and participating in G2/M checkpoint regulation. Its expression is often lost in epithelial tumours due to promoter methylation, distinguishing it functionally from the other paralogs [4]. In contrast, 14-3-3 ϵ is heavily involved in neuronal development, interacting with proteins that regulate neurogenesis, axon guidance, and microtubule stability [22]. Meanwhile, 14-3-3 ζ has been implicated in metabolic homeostasis and insulin signalling, interacting with components of the MAPK/ERK and PI3K/Akt pathways [23]. Interesting is the 14-3-3 γ (YWHAG) paralog, which exhibits a strong enrichment in protein–protein interaction (PPI) networks associated with mRNA processing, neuronal signalling, GTPase-mediated signal transduction, and actin cytoskeleton organization, as revealed in comprehensive network analysis by Uhart [9,24].

The evolutionary mechanisms behind the retention and divergence of these paralogs can be explained through models of sub-functionalization (partitioning of ancestral functions), or a combination with neofunctionalization (acquisition of novel functions) called sub-neo-functionalization [9]. Following gene duplication, selective pressure tends to favour the retention of duplicates when each acquires a unique regulatory or structural feature not fully redundant with the others. The 14-3-3 paralogs exemplify this principle: despite functional overlap in binding motifs (e.g., RSXpSXP), their specificity arises through differential protein–protein interaction (PPI) networks connectivity, tissue-specific expression, and posttranslational modifications, all hallmarks of divergent retained paralogs under purifying selection [9]. Differences in these attributes reflect that each paralog has tailored regulatory or structural roles. Evolutionary analyses suggest that these genes have been retained under strong purifying selection, with divergence focused on regions that alter binding specificity, protein dynamics, and specific allosteric regulation [24].

We recently explored the molecular dynamics of the ligand-binding grooves of three 14-3-3 paralogs: γ , ϵ , and ζ [10]. Despite sharing nearly identical static structures, the three paralogs exhibited distinct groove-opening kinetics, affecting accessibility to client peptides. These dynamic differences were driven by paralog-specific aliphatic interactions between internal α -helices, revealing a layer of conformational regulation that may underlie differences in partner affinity and responsiveness to upstream signalling events. This research highlights the importance of protein dynamics, beyond primary sequence, in shaping paralog functionality.

Importantly, the functional divergence of 14-3-3 paralogs extend to post-translational modifications (PTM). In example, we found that the levels of 14-3-3 β (but not 14-3-3 γ) acetylation in a specific lysine residue (Lys 49/51) increase during osteogenic differentiation of mesenchymal stem cells [25]. 14-3-3 β is suspected to inhibit this process, and this specific acetylation inactivates the protein. Also, some other paralogs, such as 14-3-3 ζ and ϵ , are heavily phosphorylated and acetylated at regulatory sites that alter their dimerization or client affinity [26]. Moreover, these paralogs exhibit isoform-specific PTM crosstalk, as exemplified by their well-documented interaction with phospho-acetylated histone H3 and their involvement in transcriptional regulation [27].

The coexistence and evolutionary conservation of all seven 14-3-3 paralogs in mammals are probably the result of a neo-functionalization that allows each paralog to occupy “a specialized niche” within complex regulatory networks. Our work, based on state-of-the-art network analysis, has been critical in demonstrating that despite high sequence and structural conservation, 14-3-3 paralogs differ significantly in interaction specificity, biophysical behaviour, and signalling roles, offering a compelling example of how regulatory protein families evolve to support the complexity of eukaryotic life [9].

3. A Structural and Functional Journey from the N-Terminal to the C-Terminal Region of 14-3-3 Proteins

The 14-3-3 family shares architectural principles with the tetratricopeptide repeat superfamily of α -helical proteins, both functioning as multiprotein complex mediators [28]. The first two high-resolution structures of 14-3-3 date back to 1995, when Xiao [29] and Liu [30] published the crystal structures of θ and ζ paralogs, respectively. Both determinations describe dimers formed by two monomers, each having an arrangement of nine antiparallel α -helices connected by turns. Dimers present an omega-like shape, with two phosphopeptide binding regions on their concave face, that are highly conserved between paralogs and species [31]. Structural reviews on 14-3-3 have drawn major attention to this binding site, also known as the amphipathic groove (AG). This focus selection is justified by its major functional implications on PPIs, which make 14-3-3 a hub protein in cellular biology. Here, we will address less-visited aspects of the AG, and shift our attention towards other regions, such as the dimer interphase and other secondary binding sites of the protein.

3.1. Alpha-Helices 1 to 4, the Dimeric Interphase

Dimers are stabilized by intermolecular contact between the first 4 alpha-helices of each monomer in an antiparallel fashion. These interactions can be grouped into two fundamental regions: one formed by polar/charged residues and the other by hydrophobic residues. The first one, found near the geometric centre of the dimer, contains two highly conserved salt bridges across the seven paralogs (Asp21-Lys85 and Arg18-Glu89, human ζ numbering). The implication of this polar region on 14-3-3's dimeric stability has been proven with point mutations on the above-mentioned residues [32], but more pronounced effects were seen with site-specific phosphorylation of Ser58 [33], which decreases 14-3-3 ζ dissociation constant (K_d) by six orders of magnitude [34]. The hydrophobic interphase is located at the external faces of the dimer, where Leu12 and Met78 participate in many contacts (Figure 1A). The double point mutant (Leu12Glu, Met78Lys), generated by Jandova et al., also disrupted the dimer, modifying 14-3-3 ζ K_d value in the same order as in the phosphoserine variant [35]. Phosphorylation of Ser58 has been observed in vivo by different kinases, like PKA [36], the Ste20/oxidant stress response kinase 1 (SOK-1) [37], or PKB/Akt [38], remarking the biological functions of the monomeric species. On this subject, Zhu and collaborators generated a non-hydrolysable phosphoresidue at Ser58 in 14-3-3 ζ , isolated its interactome, and differentiated client proteins that are specific for monomers or dimers [39]. Other less conserved residues located at the dimer interphase define their capacity to form homo- or heterodimers. Such is the case of 14-3-3 σ that exclusively forms homodimers, because of the presence of Phe25, which forms π -stacking interactions with Tyr84, and an additional salt-bridge between Lys9 and Glu83 [40]. On the other hand, 14-3-3 ϵ homodimers are less stable due to their unique Met in helix 4, and homodimers end up lacking one of the above-mentioned conserved salt bridges (Asp21-Lys85, ζ numbering). The rest of the paralogs have a conserved Lys residue in this position, resulting in higher contents of ϵ -heterodimers [41].

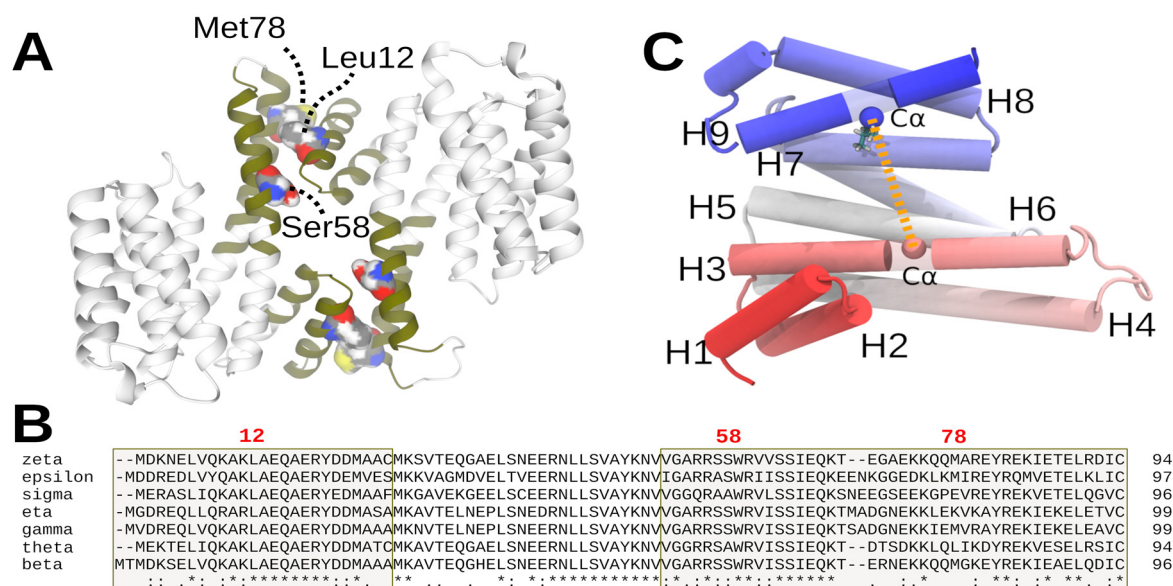


Figure 1. (A) Cartoon representation of 14-3-3 ζ highlighting its dimerization interface. Residues which mutations lead to monomerization are represented with surfaces and labelled [35,42]. (B) Multiple sequence alignment fragment of the seven 14-3-3 human paralogs. A green transparent square highlights their dimerization interphase residues. Adapted from [11]. (C) Simplified cartoon representation of one 14-3-3 monomer. On spheres, Gly52 and Leu220 C α s, showing the interhelix distance that defines the AG opening. Helices are labelled by their number and colored by index number [10]. (A) was made using VMD v1.9.4.

3.2. Alpha-Helices 3, 5, 7, and 9, the Amphipathic Groove

The characteristic binding site of the 14-3-3 family presents two well defined surfaces formed by hydrophobic and basic residues from α -helices 3, 5, 7, and 9. The protein partners have been thoroughly characterized, with phosphorylated Ser or Thr residues, flanked by different consensus sequences (Motif I, II and III). Nevertheless, the AG is not absolutely constrained to these motifs, and other sequences are also capable of binding, such as R18, a peptide identified through a phage display campaign [43] that contains the Trp-Leu-Asp-Leu-Glu motif, with two phosphomimic residues and also increased hydrophobic interactions. Another example of non-phosphopartners is the exoenzyme S-derived peptide [44]. Many phospho-targets have been described in detail in previous reviews from Somsen [45] and Obsilova [46]. Another distinctive feature of 14-3-3's AG is its preference for proteins containing intrinsically disordered regions (IDRs) [2], not only because phosphorylated residues are often found in IDRs [3], but also due to the steric restriction of the narrow AG, accessible to random coil elements but not to voluminous globular domains. This dual structural conception of the protein complexes, where the protein clients are characterized by being flexible and 14-3-3, rigid, has been contrasted by a handful of studies describing alternative conformations of 14-3-3, showing the opening of the AG. Yang and collaborators obtained a crystal structure of the β -paralog (PDB:2BQ0) [47], presenting one monomer with a shallow and exposed AG, with distances between its flanking alpha-helices (Figure 1B) of ~ 25 Å, compared to the ~ 18 Å of its closed counterpart. This plastic behaviour has also been identified with molecular dynamics simulations of paralogs σ [48], ζ [49], and in a recent publication of our group that describes the flexibility of ζ , γ , ϵ , and different 14-3-3 single point mutants [10]. Additional open structures have been solved for β -(PDBids: 8EQ8 and 4DNK) and σ -paralogs (PDBids: 5OMA and 6Y7T), all of them in apo states. The PDB entry 8EQ8 [39] corresponds to a genetically encoded 3-nitrosilation of Tyr130 that, together with Lys47, Arg58, and Arg129 (human β -paralog numbering), form a residue tetrad that recognizes the phosphate groups of the different protein clients. This

PTM ablated the capacity to bind client proteins but did not irreversibly open the AG since the other monomer was found in a closed conformation. Regarding the opened σ -paralogs, PDB:5OMA contains the crystal structure of a 14-3-3 chimera with its physiological partner StARD1, occupying the AG of a neighbouring subunit. The remaining monomer lacks a protein partner and presents interhelix distances of ~ 24 Å [42].

3.3. Alpha-Helices 4 to 9, Secondary Binding Sites

The challenges in obtaining high-resolution structures of multidomain complexes, in the case of 14-3-3, have led to the use of synthetic phosphopeptides, supplying a cropped view of their PPIs. By virtue of the Cryo-EM revolution, several full-length structures have been solved, indicating how secondary interactions stabilize the globular domains of different partners, explaining the adaptor, scaffolding, and sequestering functions of the protein family [45]. One example is the complex between the PEAK3 pseudokinase homodimer and the ϵ/β 14-3-3 heterodimer (PDB:8DP5). This structure is one of its kind, featuring interactions with α -helices 4, 6, and 8 at the convex side of the ϵ -protomer. This paralog binding preference was explained by the insertion of residues Met160 and Thr161 located at helix 6 inside a basic cavity from PEAK3. On the remaining paralogs, these positions present two conserved lysines, destabilizing a putative complex formation by electrostatic repulsion. The interaction with 14-3-3 did not alter the dimerization capacity of PEAK3; instead exerted its function, retaining the pseudokinase at the cytosol [50]. Secondary interaction sites at α -helices 8 and 9 are more commonly found in the PDB, as in the case of the BRAF-MEK1-14-3-3 complexes (PDBids: 6NYB and 6Q0J). Here, 14-3-3 regulates the switch between the autoinhibited form of the kinase and its active dimeric form. In the autoinhibited form, the BRAF kinase domain interacts with α -helices 8 and 9, and cysteine-rich domains at the centre of the concave face, all having highly conserved residues across all 14-3-3 paralogs. Instead, the active form interacted only with helix 9, allowing the BRAF dimerization [51]. In a similar way the Leucine-rich repeat kinase 2 (LRRK2), associated with Parkinson's, interacts with α -helices 8 and 9, this time inhibiting its function by occluding its dimerization interphase, also in a paralog nonspecific manner [52]. Other full-length 14-3-3 complexes interacting with α -helices 8 and 9 are deposited under PDB ids: 1IB1, 2O98, 3AXY, 5LTW, 6GN0.

3.4. The Intrinsic Disordered C-Terminal Region

A general limitation of structure-based analyses of 14-3-3 proteins is the dependence on PDB entries, which typically lack the highly flexible C-terminal tails. These regions are intrinsically disordered, vary among paralogs, and often play regulatory roles that cannot be captured in crystallographic structures. As a result, current structural models provide only a partial view of the full conformational landscape of 14-3-3 proteins. Additionally, because this review does not introduce new experimental validation, the discussion relies entirely on previously published data.

The 14-3-3 proteins do not end at helix 9, continuing with a disordered region that contains two conserved phosphorylation sites (Ser229 and Thr230; human ζ -numbering), except for the σ -paralog with only one phosphosite at the conserved Ser residue [26]. This fragment has been associated with autoinhibitory mechanisms, presenting multiple anionic amino acids that confer the capacity to occupy the active site, as proposed by spectroscopic experiments and molecular dynamics simulations [53,54]. The length of this region also varies between paralogs, being ϵ and η the ones with the longest (27 residues) and shortest (16 residues) tails, respectively; presenting only the first 4 residues highly conserved across the seven human paralogs [46]. The scarce structural information of the C-terminal is directly related to its intrinsic disordered characteristic, as well as the use of C-terminal

truncated forms due to their easier crystallizable nature [55]. The functional effect of such deletion was studied using in vitro binding assays, showing increased affinities for R18, a phospho-Ser Raf peptide, and a hypophosphorylated mutant of the proapoptotic protein Bad [56]. The only structure deposited in the PDB Bank (PDB id: 3EFZ) with electron density for its C-terminal corresponds to a *Cryptosporidium parvum* 14-3-3 protein. Inspired by its sequence, Sluchanko found optimal linker lengths to design chimeric versions that fuse 14-3-3 with different peptide partners [42] with potential application on biosensor design.

3.5. 14-3-3's Small Molecule Modulators

The regions described above can also be identified by their ligandability, with small compounds targeting the dimerization interphase, the AG, and secondary binding sites (Figure 2). Ehlers et al. performed an in silico design of derivatized peptides and obtained X-ray diffraction structures of a ligand targeting the 14-3-3's central pore, with binding constants at the low micromolar range [57]. This region could also be targeted after a fragment screening made by Brink et al., who found a small compound stabilizing 14-3-3 dimers [58].

Once again, the AG stands out for its notoriety, with several ligands that can be divided into stabilizers or inhibitors of PPIs. The latter includes small molecules containing a phosphate group that binds into the conserved basic tetrad of the AG, competing with the protein clients [59–61]; and modified peptides designed to include the classical binding motifs but also generating additional stabilizing interactions [62–65]. As regards PPI stabilizers, also known as molecular glues, several efforts have been made to design ligands that interact with the interphase generated between the AG and different phosphopeptides, in an analogous form of the diterpenic toxin fusicoccin A [66]. For a detailed description of this compound family, we refer the readers to the review made by Somsen [45].

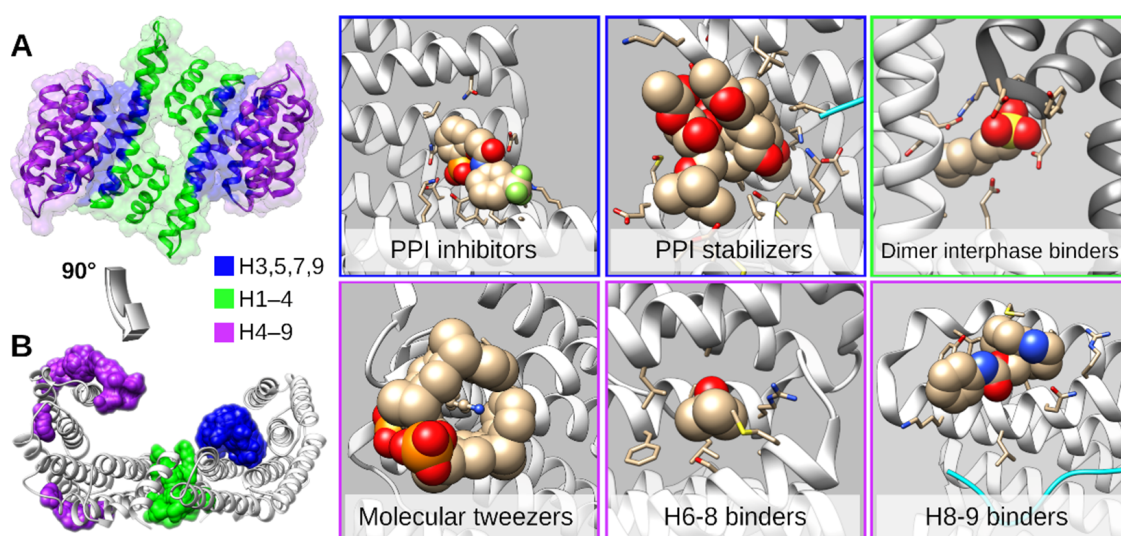


Figure 2. (A) “Top” view of a 14-3-3 dimer, represented in cartoons and semi-transparent surfaces, colours are chosen according to the regions corresponding to the AG (blue), dimer interphase (green), and secondary binding sites (magenta). (B) “Side” view of the 14-3-3 dimer with superimposed molecular surfaces of interacting ligands. Inset: examples of PPI inhibitors [60], PPI stabilizers [67], dimer interphase binders [68], molecular tweezers [69], H6–H8 binders [70], and H8–H9 binders [71]. Ligands are shown as spheres, and residues within 5 Å as sticks. The colour code for each region is maintained across the figure. (A,B) were made using UCSF ChimeraX v1.10.1.

We conclude this quest, revisiting the convex face of 14-3-3. Secondary interactions have also been targeted by small molecules. This is the case of lysine-specific molecular tweezers, compounds that contain fused norbornadiene and benzene rings with two

phosphate groups at their centre. This chemical moiety was found to wrap a conserved Lys at the beginning of helix 9, inhibiting its interaction with C-Raf, and the exoenzyme-S [69]. On top of α -helices 8 and 9, two additional binding sites have been found, one located near the C-terminal and the other close to the loop connecting both α -helices. These binding sites are part of the interacting residues that stabilize protein partners like the serotonin N-acetyltransferase (AANAT), the Flowering Lotus T protein from rice (Hd3a), and the plant H⁺-ATPase PMA [72]. Andlovic and collaborators found a PPI stabilizer for the 14-3-3/TAZpS89 complex, combining X-ray crystallography and differential scanning fluorimetry. This small molecule binds on the surface of α -helices 8 and 9, increasing the binding affinity towards the phosphopeptide in an allosteric fashion. This mechanism of action was proposed considering that no secondary interactions outside the AG were observed due to the use of the TAZ derived peptide instead of its full-length structure [71]. Over the convex face, in a small cavity formed by α -helices 6 to 8, recently identified in an *in silico* approach by our group, different organic solvent molecules were found (PDBids: 3UAL, 3UBW, 7V9B, 2WH0, 8P1H). Unlike the surfaces formed by α -helices 8 and 9, this small cavity is less conserved, making it a promising candidate for paralog-specific modulators.

4. Conclusions and Perspectives

Given their gene duplication origin, the 14-3-3 protein variants are now referred to as paralogs. A structural and functional exploration of this family, from the N-terminal dimerization interface to the C-terminal secondary binding sites, highlights their remarkable versatility as central signalling integrators. While the amphipathic groove remains the primary hub for phospho-dependent PPIs, other regions—including the dimer interface and convex secondary sites—contribute to the adaptor, scaffolding, and sequestering functions of the family. The 14-3-3 proteins themselves are subject to differential regulation via phosphorylation, post-translational modifications, and small molecule interactions, demonstrating that their activity and client specificity are dynamically modulated. This multi-level regulation underscores their pivotal role in coordinating complex cellular signalling networks, making them attractive therapeutic targets with high translational value. Dysregulation of 14-3-3 binding is implicated across numerous disease indications, including neurodegenerative disorders (like Alzheimer's, Parkinson's and infant epilepsy), cardiovascular diseases, and cancers (where overexpression and client sequestration often promote oncogenic survival and drug resistance). This recognition has driven the urgent need for paralog-selective chemical probes, small molecules capable of differentiating between the seven highly homologous paralogs (e.g., targeting unique surface loops or quaternary structures) to precisely dissect their individual functions and liabilities in specific disease contexts. However, the physiological consequences of inhibiting or activating a single paralog, and whether such interventions will demonstrate acceptable therapeutic windows, remain open questions.

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