**Integrate omics data and molecular dynamics simulations toward better understanding of
human 14-3-3 interactomes and better drugs for cancer therapy**

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Abstract

The 14-3-3 protein family is among the most extensively studied, yet still largely mysterious protein families in mammals to date. As they are well recognized for roles in apoptosis, cell cycle regulation, and proliferation in healthy cells, the aberrant 14-3-3 expression has unsurprisingly emerged as instrumental in the development of many cancers and in prognosis. Interestingly, while the 7 known 14-3-3 isoforms in humans have many similar functions across cell types, evidence of isoform-specific functions and localization has been observed in both healthy and diseased cells. The strikingly high similarity amongst 14-3-3 isoforms has made it difficult to delineate isoform-specific functions and for isoform-specific targeting. Here, we review our knowledge of 14-3-3 interactome(s) generated by high-throughput techniques, bioinformatics, structural genomics and chemical genomics and point out how integrating the information with molecular dynamics (MD) simulations may bring us new opportunity to the design of isoform-specific inhibitors that can not only be used as powerful research tools for delineating distinct interactomes of individual 14-3-3 isoforms, but also can serve as potential new anti-cancer drugs that selectively target the aberrant 14-3-3 isoform.

1. Introduction

First discovered by nervous system protein analysis through ion-exchange chromatography and gel electrophoresis, the 14-3-3 proteins were so named as a description of the enriched fraction number (Moore and Perez, 1968). Later 14-3-3 protein family was observed in almost all eukaryotic organisms from yeast to plants to humans, although the number of isoforms varies greatly between species. In humans, there are seven known 14-3-3 proteins: β (identical to α when phosphorylated), γ , ϵ , η , ζ (identical to δ when phosphorylated), θ , and σ . Although they are not the results of alternative splicing, the seven human isoforms have highly

similar primary sequences (Fig. 1). Not only is each isoform coded by a different gene, but also the gene for each isoform is located on a different chromosome. Due to their widespread presence in multiple subcellular locations and involvement in various signaling pathways, misregulated 14-3-3 expression can contribute to, or be the cause of, many disease phenotypes such as neurological diseases and cancers (Fu et al., 2000a; Zhao et al., 2011b). Thus, work to elucidate the involvement of 14-3-3 proteins in both diseases has been pursued for decades (Berg et al., 2003; Mhawech, 2005). In this review, we aim to summarize the currently known functions of the human 14-3-3 isoforms and their contribution to cancers. We touch on their unique properties and omics studies using high-throughput genomics, as well as structural and chemical genomics. Here, we borrow the term “interactome” and refer to “14-3-3 interactome(s)” as the whole set of proteins that directly interact with 14-3-3 proteins or certain 14-3-3 isoform. We suggest the integration of these data through the study of the energetics and dynamics of 14-3-3 proteins could be an approach to generate isoform-specific 14-3-3 inhibitors, which not only have potential for drug development with regards to cancer treatment, but also as a powerful research tool to study different 14-3-3 isoforms.

2. General Cellular Function of 14-3-3 Proteins and Their Clinical Implications in Cancer

The 14-3-3 protein family has a vast range of activity in eukaryotic cells, including functions in signaling pathways for cell cycle control, proliferation, and apoptosis (Messaritou et al., 2010). To overcome functional redundancy and compensate from different family members by classical genetics study for overall 14-3-3 protein function, Nguyen et al. (2004) performed functional knock-out (FKO) studies using a chemical genetics approach. Activity of all isoforms of 14-3-3 was antagonized simultaneously upon activation of the caged phosphopeptides through exposure to UV-A. Not only was cell death significantly increased in U2OS cells in response to

phosphopeptide activation, but also the rate at which cells undergoing division reach mitosis was significantly faster. These observations, coupled with the noted decrease in the fraction of non-dividing cells in stable G1 phase, imply a modulatory role of 14-3-3s in the cell's entrance to the mitotic stage of cell division (Clapp et al., 2012). In 2001, Masters and Fu observed that elimination of 14-3-3/Raf-1 interaction through use of the pan isoform 14-3-3 antagonist, difopein, resulted in death of HEK293 cells (Masters and Fu, 2001). This apoptotic response appears to be the consequence of lost signal transduction between pro-survival kinases, such as protein kinase C, phosphatidylinositol 3-kinase, and MAPKKK1, and apoptotic proteins, such as Bad, in the COS-7 cell line (Masters and Fu, 2001). Use of difopein on glioma cell line U251 also generated an apoptotic response as suggested by DNA ladder marker experiments. Decreased levels of pro-survival factor *Bcl-2* mRNA and increased levels of pro-apoptotic factor *Bax* mRNA upon cell transfection with difopein-containing plasmid further indicate the importance of 14-3-3 in pro-survival signal transduction process (Yang et al., 2009). These observations paint 14-3-3s as signal integrators: the connection between phosphorylation by kinases such as PKC and decreased apoptotic protein activity.

To trace their individual functions, knockdown/knockout or overexpression of individual 14-3-3 isoforms, especially in the context of cancer, has been extensively investigated. For example, the σ isoform appears to provoke cellular migration through forming a complex with cytoskeletal components and allowing them to be readily available during migration (Boudreau et al., 2013). In the case of 14-3-3 ζ , activity from both the c-Jun N-terminal kinase (JNK) and p38/MAPK pathways appear to be involved in the negative regulation of hepatoma cell proliferation in the absence of 14-3-3 ζ expression (Choi et al., 2011). When HepG2 liver hepatocellular carcinoma cells were transfected with siRNA against 14-3-3 ζ , the amount of cell

growth, as well as the expression levels of proliferating cell nuclear antigen(PCNA), a proliferation marker, exhibited a dose-dependent reduction. The authors also generated a cell line with stably removed 14-3-3 ζ , Huh7, to examine the effects on apoptotic and cell cycle phase progression(Choi et al., 2011). Apoptotic rate for Huh7 cells that were further infected with sh14-3-3 ζ by lenti-viral technique was unaltered when compared to control Huh7 cells. However, upon analysis of cell cycle phases, the number of lenti-viral infected Huh7 cells in the G1 phase was significantly higher than the number of control Huh7 in G1. The accumulation of cells lacking 14-3-3 ζ in the G1 phase speaks to the importance of 14-3-3 ζ in the advancement of the cell cycle. Additionally, a marked reduction in soft agar colony formation assay for Huh7 cells transfected with the si14-3-3 ζ supports the potential role of 14-3-3 ζ in tumor formation. Perhaps most therapeutically relevant, 14-3-3 ζ -transfected HepG2 cells treated with chemotherapeutic agent *cis*-diamminedichloridoplatinum (CDDP) showed a marked decrease in PCNA proliferation marker and an increase in cell death dose-dependently. Interestingly, phosphorylation of JNK and p38/MAPK, two downstream kinases activated by MAPK, was increased upon silencing of 14-3-3 ζ . The authors postulated that the increased sensitivity to chemotherapy to be related to increased activation of the JNK and p38/MAPK pathways upon reduction of 14-3-3 ζ levels(Choi et al., 2011).

Knockdown of gamma isoform of 14-3-3 by siRNA treatment led to significantly diminished metastasis rates in non-small cell lung cancer cells (Raungrut et al., 2014). Overexpression of 14-3-3 ϵ led to decreased E-cadherin and increased N-cadherin and vimentin protein levels as seen by Western blot. These cadherin expression changes are indicative of epithelial to mesenchymal transition, and ultimately the initiation of metastasis. Decreased E-cadherin levels were also detected at cell-cell junctions under conditions with increased 14-3-3 ϵ

in hepatocellular carcinoma cells(Liu et al., 2012).Further, metastatic progression is not loyal to the 14-3-3 ϵ isoform. Overexpression of the 14-3-3 γ isoform also displayed significant correlation with metastasis in non-small cell lung cancer as reported by Raungrut et al.(2014). Complimentary experiments using siRNA treatment against 14-3-3 γ in non-small lung cancer cells led to significantly diminished metastasis rates. While these results indicate the necessity of 14-3-3 γ for metastasis within the system, overexpression of 14-3-3 β , σ , and τ are also observed in lung cancers, raising the question of overlapped and distinct function of 14-3-3 isoforms within given forms of cancer(Qi et al., 2005; Raungrut et al., 2014).

Increases in 14-3-3 η and 14-3-3 γ transcription were seen in response to heart tissue damage, and were tentatively attributed to a 14-3-3 modulated protection mechanism (He et al., 2006). This protective mechanism, activated in response to stress or injury, is stipulated to prevent cell death. Following heart tissue injury, ischemia or hypoxia may occur, potentially leading to the development of infection from circulating intestinal bacteria. Infection may then trigger the increase of pro-inflammatory factors, which can lead to the initiation of cell death in infected heart tissue. The induction of some 14-3-3 isoforms in response to heart tissue injury suggests the protein's involvement in the body's response to increased levels of pro-inflammatory signals, such as cytokines, which are also linked to an increased risk for cancer development and tumor microenvironment establishment (Colotta et al., 2009).

Due to the robust documentation of aberrant 14-3-3 expression levels in a multitude of cancer types, some isoforms have been proven themselves to be potential valuable diagnostic and prognostic biomarkers(Zhao et al., 2011b).The sigma form has been claimed as a biomarker for basal breast cancer (Hermeking, 2003; Dim et al., 2014). Not only does 14-3-3 σ contribute to cancer development and metastasis, but also it plays a role in drug resistance in cancer cells,

providing potential prognosis prediction. In a multidrug resistant cancer cell line MCF7/AdVp3000, 14-3-3 σ overexpression was observed and its knockdown using siRNA resulted in reduction of drug resistance (Liu et al., 2006). 14-3-3 σ over-expression was also observed in a gemcitabine-resistant pancreatic cancer cell line, G3K, derived from MiaPaCa-2 cells and contributes to gemcitabine resistance (Qin et al., 2014a), further indicating the potential prognosis value of 14-3-3 σ . Indeed, 14-3-3 σ expression has been shown to associate with poor prognosis of pancreatic cancer patients (Li et al., 2010). 14-3-3 ζ has also generated much interest as a potential biomarker, as well as small molecule target in cancer. The 14-3-3 ζ isoform showed overexpression in 42% of breast cancer tissues tested by Neal et al. (2009) and its experimental overexpression led to increased cell growth and decrease in apoptosis resulting from stress stimulus. Similarly, not only is it involved in cancer progression and development, but also it has been linked to chemotherapy resistance, presenting a substantial hurdle to successful cancer treatment (Matta et al., 2012). Thus, 14-3-3 ζ may also associate with poor prognosis.

3. Uniqueness of Individual 14-3-3 Isoforms

While 14-3-3 proteins as a family have well-characterized roles in cell apoptosis and proliferation as presented above, detailed information on each isoform's exact functions remains elusive. The fact that the human genome contains seven isoforms of 14-3-3 makes it extremely difficult to trace their shared and individual functions and their contribution to cancer subtypes. Even in the simpler case of the *Saccharomyces cerevisiae* genome, which encodes only two highly homologous 14-3-3 proteins, bmh1 and bmh2, genomic studies suggest that they have secretive common and individual functions in yeast. Double deletion is usually inviable unless in yeast strains with a distinct genetic background such as Σ 1278b (Roberts et al., 1997), while single deletion is viable, demonstrating that bmh1 and bmh2 have common functions, which is

consistent with their largely overlapping interactome determined by global genome analysis(Panni et al., 2011).It was observed that *Bmh1/2* double mutant leads to global changes in gene expression in yeast when comparing the transcriptional profiles of the wild type using microarrays and proteomics studies. In the absence of *bmh1/2* proteins, the expression of 220 genes, including eight categories of functions, are significantly affected(Ichimura et al., 2004). Meanwhile, distinct phenotypes have been described in single deletion of *Bmh1* or *Bmh2* gene in yeast. For example, *Bmh1* and *Bmh2* play different roles in endoplasmic reticulum localization signal process (Michelsen et al., 2006) and differentially regulate rapamycin-mediated transcription in yeast (Trembley et al., 2014).To better understand the contribution of the seven human isoforms to cancer, it is imperative to dissect their individual functions and individualinteractomes in both normal and diseased states. Here we summarize our limited understanding of the uniqueness of each of the seven isoforms in terms of their localization, expression levels, and specialized functions.

3.1 14-3-3 β

The 14-3-3 β isoform has many characterized functions, including roles in stress response, proliferation, and anti-apoptotic signaling. The β/α isoform has been linked to increased levels of Bcl-2, a known anti-apoptotic protein (Jasinski-Bergner et al., 2014). 14-3-3 β has previously been observed in high levels in human astrocytomas, indicating its potential role in neurological disease phenotypes. Overexpression of β isoform increased proliferation rates in SVGp12 normal human astrocyte cell line, while removal of 14-3-3 β from U87 glioma cell line resulted in the prevention of proliferation (Gong et al., 2013).

3.2 14-3-3 γ

The γ isoform exhibits a striking affinity for membranes upon ligand stimulation, a phenomenon made evident through the favorable electrostatic potential of the amphipathic helices at the dimerization domain of γ , when compared to the ζ isoform, in the phosphopeptide-bound state (Bustad et al., 2012). The observed association of 14-3-3 γ with membranes upon binding to phosphopeptide ligand indicates the possible role of the γ isoform in the substrate localization to the membrane for signaling. The loss of 14-3-3 γ also correlated with a loss of cell-cell adhesion and desmosome assembly in both the HCT116 human colon cancer cell line and *in vivo* in a mouse model (Sehgal et al., 2014). *In vivo*, the loss of 14-3-3 γ also led to defective sperm generation and testicular development in the mouse model (Sehgal et al., 2014). Ajjappala and colleagues performed a study on the 14-3-3 γ isoform using IL-3 as a stimulant for proliferation in Ba/F3 hematopoietic progenitor cell line. Upon stimulation with IL-3, 14-3-3 γ mRNA and protein levels were upregulated in Ba/F3 cells. Conversely, cells treated with siRNA against endogenous 14-3-3 γ showed absence of proliferation, even after IL-3 application, implying the requirement of 14-3-3 γ to initiate IL-3 mediated proliferation (Ajjappala et al., 2009). Recently, a study on Cdt2, an E3 ubiquitin ligase complex substrate recognition adaptor, also noted a decrease in cell proliferation after depletion of 14-3-3 γ , which may be partially caused by the increases in E3 ligase's substrate within the cells. From this observation, Dar and colleagues concluded that 14-3-3 γ may have a role not only in advancement of cell division, as previously reported by other groups, but also in the stabilization of Cdt2 (Dar et al., 2014).

3.3 14-3-3 ϵ

14-3-3 ϵ is known to be expressed in neuronal outgrowths and cardiomyocytes. Superbly diverse, this isoform functions as a chaperone protein in mitochondrial substrate localization (Liu et al.,

2012), a responder to RNA virus infection(Marzinke et al., 2013), and a cell cycle mediator (Kosaka et al., 2012). Initiation of immune defense response against RNA virus infection is incurred through the stabilization of RIG-I and TRIM25 by 14-3-3 ϵ (Liu et al., 2012). As a mediator of the cardiomyocyte cell cycle, 14-3-3 ϵ sequesters the cyclin-dependent kinase inhibitor p27^{Kip1} in the cytoplasm, leading to its eventual demise *via* ubiquitin-mediated mechanisms, and ultimately allowing the cyclin E-Cdk2 complex to facilitate the G1/S phase transition (Kosaka et al., 2012).

3.4 14-3-3 η

Pro-mitotic signaling(Wanzel et al., 2005), chaperoning in neurons(Sato et al., 2006), and prevention of DNA damage-induced cell cycle arrest (Lee et al., 2013)are a few processes involving the 14-3-3 η isoform. The η isoform has been shown to negatively regulate both Miz1, a protein involved in DNA repair, and parkin, an E3 ubiquitin ligase which is commonly mutated in the autosomal recessive juvenile Parkinson's disease(Wanzel et al., 2005; Sato et al., 2006).

3.5 14-3-3 θ

Like most other 14-3-3s, 14-3-3 θ is expressed abundantly in the brain, and its misregulation is often involved in the development and progression of neurological disorders such as Parkinson'sdisease(Hsu et al., 2012; Ding et al., 2013). This isoform may also play a role in other diseases, such as leukemia, by increasing cell survival and inhibiting the activity of pro-apoptotic proteins (Scheibner et al., 2012). 14-3-3 θ is also a direct binding partner of the Eag potassium channel in mammals, and significantly inhibited K⁺ current in rats upon its overexpression (Hsu et al., 2012).

3.6 14-3-3 σ

The σ isoform is the only isoform that strictly forms homodimers while other six homodimers can form either homodimer or heterodimer within the family (Fu et al., 2000b; Aitken et al., 2002; Benzinger et al., 2005a; Wilker et al., 2005; Zoete et al., 2010). Studies on 14-3-3 σ revealed a vital hydrophobic phenylalanine (Phe²⁵) promotes dimerization between two 14-3-3 σ monomers. This phenylalanine assists with the correct placement of hydrophobic and hydrophilic elements within 14-3-3 σ to support the formation of the metabolically active homodimer (Liu et al., 2011). The σ isoform localizes to myoepithelial cells in healthy breast tissue and has attracted much interest in the fields of breast cancer and drug resistance. 14-3-3 σ is down-regulated in mammary carcinoma cells and serves as a human mammary epithelial-specific marker (Prasad et al., 1992). In addition, 14-3-3 σ is distinct from other 14-3-3 isoforms in that it can be induced by tumor suppressor protein p53, which is expressed commonly upon exposure to γ irradiation and other DNA-damaging agents (Hermeking et al., 1997).

3.7 14-3-3 ζ .

Arguably one of the most well-studied isoforms, 14-3-3 ζ plays a role in a vast array of cellular processes. Somewhat uniquely, the 14-3-3 ζ isoform displays response to stimulation by androgen (Murata et al., 2012). An androgen receptor binding site in the 14-3-3 ζ gene was identified, leading to the use of androgen as a cellular stimulant in LNCaP prostate cancer cells. Upon exposure to androgen, LNCaP cells displayed an increase in proliferation rate along with increased 14-3-3 ζ expression. Follow-up experiments with siRNA treatment against 14-3-3 ζ resulted in significant decrease in cell growth after 2 and 4 days, providing supporting evidence for earlier observations regarding proliferation (Murata et al., 2012). Upon phosphorylation on Ser²⁴⁷, HNF1 α , a liver-specific transcription factor, directly interacts with 14-3-3 ζ , indicating this isoform's cell type-specific activity (Yu et al., 2013). Interestingly, while the 14-3-3 dimers have

highly characterized activity, it has been revealed recently that de-dimerization of 14-3-3 ζ can be achieved through the phosphorylation of Ser⁵⁸ within the dimer interface and the monomer has partially retained activity (Sluchanko et al., 2012). Since this Ser is not ubiquitous in all 14-3-3 isoforms, raising the possibility of different regulatory mechanisms for different isoforms (Fig. 1).

The seven functionally overlap but different 14-3-3 isoforms in humans have made it exceptionally difficult to study their functional divergence and contributions to different types of cancers, especially when considering not only genetic, but also epigenetic and environmental factors. A better understanding of the role of each individual 14-3-3 protein and the cooperativity between them is imperative to understanding the etiology of cancer and designing more successful drugs in targeted cancer therapy. Achieving this goal relies on building global models of overlapped and distinct isoform interactomes both in normal and diseased states. Evidently this is a very challenging task.

4. Contribution of Interactome(s) of 14-3-3 Proteins by High-throughput Techniques and Bioinformatics

Globally, it is well-known that the 14-3-3 protein network shows interaction with motifs containing phosphorylation marks on serine and threonine residues, and as a family, certain members have shared protein partners as well as functions. High-throughput screening of synthetic phosphopeptides coupled with subsequent peptide sequencing determined two types of binding motifs of 14-3-3 protein mode 1: RSXpSXP and mode 2: RXF/YXpSXP (Yaffe et al., 1997). Later, motif 3, X-pS/T-X-COOH, was identified, with which 14-3-3 binds the penultimate phosphorylated threonine or serine (Ganguly et al., 2005). In 2008, a peptide fragment library containing various phosphorylated serine and threonine combinations was utilized to test

potential isoform-distinctive binding amongst 14-3-3 proteins by Lu et al. (2008). The resultant peptide microarray generated binding profiles for all 7 isoforms of 14-3-3. While the binding profiles exhibited high semblance, the use of dual-color ratiometric microarray delivered 14-3-3 isoform-selective peptide sequences. Two peptides, B1: LFGpSLLR and B2: LFGpSLVR, bind with at least two-fold higher preference for the 14-3-3 σ isoform, with B1 having a K_d of 0.54 $\mu\text{mol/L}$ for 14-3-3 σ when compared to the other 6 isoforms with a K_d of greater than 10 $\mu\text{mol/L}$ (Lu et al., 2008). This approach presents a method for delineating minute distinctions among isoform binding sequences, which is especially relevant in the case of the 14-3-3 proteins, where each isoform presents a unique but overlapping roles and functions within signaling networks while maintaining similar physical characteristics to each other. However, it is worth to note that the binding is determined not only by primary sequence around the phosphorylated binding sites, but also by overall steric fit of the protein partner, which is another factor that bestows specificity of 14-3-3 isoforms and is under investigated.

Recently, global and network studies shed new lights on disparities among human isoform interactomes (Uhart and Bustos, 2013). A network of 14-3-3 binding partners, including information on posttranslational modifications such as phosphorylation and acetylation, was generated. Distinctions were discovered about the substrates in the networks of the 14-3-3 ϵ and 14-3-3 ζ isoforms. When compared to other isoforms, 14-3-3 ζ binds to substrates of significantly lower disorder, and also higher acetylation levels. The 14-3-3 ϵ isoform tends to associate more often with phosphorylated tyrosine-containing substrates when compared to other isoforms. These results reveal potential mechanisms for distinction when targeting specific 14-3-3 isoforms with small molecules (Uhart and Bustos, 2013). Of equal interest, Tinti et al. (2012) show the human 14-3-3-binding phosphoproteome to contain a large amount of 2R-ohnologues,

defined as protein families with between two and four members resultant of two cycles of whole genome duplication dating back to the origin of the vertebrates. Moreover, they identified 2R-ohnologue families with members sharing a “lynchpin”, or 14-3-3-binding phosphosites that are conserved throughout a given 2R-ohnologue protein family (Tinti et al., 2012). Integrating their findings in an algorithm, a web resource named ANIA (ANnotation and Integrated Analysis of the 14-3-3 interactome), which integrates data concerning 14-3-3-binding phosphoproteins from various information sets, was developed to globally analyze interactions involving 14-3-3 binding (Tinti et al., 2014). In addition, ANIA could perform analysis on somatic mutations in cancer databases to predict possible cancer phenotypes that map to 14-3-3-binding phosphosites. These are good attempts to predict the individual interactome of the isoforms more precisely by addressing the important question of how interactions with 14-3-3 proteins has diverged structurally and functionally between different members of the family (Tinti et al., 2012, 2014).

5. Contribution of Interactome(s) of 14-3-3 Proteins by Structural Genomics

Proper function of 14-3-3 proteins is closely tied to their physical structures. The prosperous development of structural and chemical genomics brings us 76 structures of human 14-3-3 proteins to date in the Protein Databank (PDB), with all seven isoforms having at least one crystal structure available (Table 1). These structures include apo-14-3-3 proteins, peptide- and inhibitor-bound 14-3-3 complexes. The phosphopeptide-bound structures draw much attention and comparison of the structures of all 14-3-3 isoforms shows multiple striking similarities. In the following, although only the structures of two isoforms are chosen to compare for the sake of clarity, these similarities do exist in phosphopeptide-bound complexes of all 14-3-3 human isoforms. Firstly, all phosphopeptide-bound isoforms have very similar overall structure. All isoforms exist as a dimer with nine α -helices in each monomer, namely H1-H9, arranged in

antiparallel fashion (Fig. 2A). Fig.2A shows an overlaid view of the σ and ζ isoforms complexed with their phosphopeptide (PDBcode: 1YWT and 1QJB, respectively). The two chains of the σ isoform are depicted by blue and dark blue, respectively, and the ζ isoform is shown in pink. The rmsd is 0.85 Å when 308 atom pairs from these two structures are aligned using the Needleman-Wunsch algorithm. At the dimerization domain, the amino-terminal of helix H1 and H2 in one monomer associates with the H3 and H4 helices of the opposing monomer. Secondly, all isoforms have a similar Ser/Thrphosphopeptide-binding groove (Fig. 2B). Within each monomer of 14-3-3 resides an amphipathic groove, which serves to bind Serine/Threonine phosphopeptides. As shown in Fig.2B, not only the types, but also the conformations of the interacting residues are nearly identical. In both structures, the phosphate group is stabilized by the positively charged groups from Lys⁴⁹, Arg⁵⁶, Arg¹²⁹, and Tyr¹³⁰ with residue numbers seen in the σ isoform.

On one hand, the crystal structure of phosphopeptide-bound 14-3-3 complexes provide us the structural insight of the association between 14-3-3 proteins and their partners and lay a good foundation for structure-based drug design targeting 14-3-3 proteins which will be discussed in the next section; on the other hand, the comparison does not seem to help to understand the distinct interactome of 14-3-3 isoforms. However, we need to be aware the limitation of three aspects with the crystal structures. First, in all of these structures, information about the most variable region, the C-terminus (Fig. 1), is not available (Table 1). The C-terminus is believed to be responsible for different properties in different isoforms (Sluchanko and Uversky, 2015). Second, as pointed out earlier, the specificity and affinity between two proteins are determined not only by the consensus sequence, but also by the overall structural fit of the two interacting proteins. Therefore, the phosphopeptide-bound 14-3-3 complexes contain only partial

information of the association. Lastly, X-Ray crystallography measures protein structures averaged in time and space in solid state, and therefore very little information is available on protein dynamics. Recently, using molecular dynamics simulation approach, Hu et al. (2015) recorded the apo-14-3-3 σ in an open state, where helices H5 to H9 are much more flexible in comparison to the phosphopeptide bound 14-3-3 σ complex observed in their 80 ns molecular dynamics (MD) simulations. The hydrophobic contacts and H-bond network between helices H5 and H7 in apo-14-3-3 σ differ from those in the peptide bound state, indicating a possible conformational change that apo-14-3-3 σ has to undergo to bind with its phosphopeptide partner. This raises the question that, although the phosphopeptide bound complex structures of 14-3-3 isoforms are highly similar, their apo structures and dynamic properties could be divergent. These distinctions may determine their different binding preferences to select protein partners. This point of view receives support from the structural alignment of the apo 14-3-3 isoforms. Fig.2C shows the alignment between the apo-structure of the σ and ζ isoforms (PDB code: 1YZ5 and 1A40, respectively). Compared with 0.85 Å of the two complexed structural alignment, the rmsd is 1.54 Å when 308 atom pairs from the two apo-structures are aligned, indicating these two structures have greater disparity. When examining the structural alignment, it is clear H1 to H4 of the two structures are aligned very well, while H5 to H9 are not, with H7 to H9 having the highest dissimilarity as emphasized in the black frames.

6. Contribution of Interactome(s) of 14-3-3 Proteins by Chemical Genomics

Aided by crystal structures, the development of inhibitors targeting 14-3-3 proteins has been pursued due to their high clinical relevance. Currently, the major drawback to 14-3-3 inhibitors is the lack of isoform specificity. Overwhelming evidence supports the specificity of given 14-3-3 isoform misregulation with certain cancer types or chemotherapeutic resistance,

suggesting that broad 14-3-3 inhibition will most likely produce undesired effects. In addition, isoform specific inhibitors are valuable research tools to meet the high demand for research dissecting 14-3-3 isoform specific functions and interactomes.

The first generation inhibitors are peptide inhibitors targeting the 14-3-3 conserved active site, such as R18 and difopein(Bartel et al., 2014). Both R18 and difopein are used to understand the potential interactome members and the involved signaling pathways. As expected, R18 resulted in apoptosis in Ba/F3 and K562 human leukemia cells expressing BCR-ABL fusion protein (Dong et al., 2008) while transfection with myc-tagged difopein in cos-7 cells generated a reduction in survival as indicated by both a morphological assay and a lacZ expression β -galactosidase activity assay (Masters and Fu, 2001). Difopein also caused similar decreases in survival in A549 lung cancer cells, DU145 prostate cancer cells, and HeLa cervical carcinoma cells (Dong et al., 2008). In addition, difopein was successfully utilized to sensitize COS-7 cells to cisplatin treatment and initiate apoptosis (Dong et al., 2008). Thesedata confirm 14-3-3 proteins are ideal targets for cancer therapy. However, it is not surprising that peptides, including peptide derivatives as developed later by Lu et al. (2008), are pan isoform inhibitors since these peptides bind and replace 14-3-3 phosphopeptide ligand at their strictly conserved binding groove. This can be easily confirmed by comparing the R18-complexed 14-3-3 ζ (PDBcode: 1A38) with the phosphopeptide complexed one (PDBcode: 1QJB). Out of a total 209 α -carbon pairs, the alignment of 207 α -carbon atom pairs between these two structures has an RMSD value of 0.73, indicating 14-3-3 ζ adopts a nearly identical overall conformation between these two structures. Fig.2D shows the peptide R18 occupies the phosphopeptide binding groove with its Glu residue interacting with the surrounding residues in the same mode as phosphoserine.

Since this mode of interaction is strictly conserved in all 14-3-3 isoforms, there is no surprise at R18's lack of specificity among 14-3-3 isoforms.

As a result of thriving chemical biology and chemical genomics, peptide inhibitors have given way to small molecule compound inhibitors in recent years. The small molecule inhibitors B2 (Lu et al., 2008) and Fobisin101 (Zhao et al., 2011a) contain phosphate groups (Fig. 3A) which mimic the phosphate group of the phosphoserine. As aforementioned, the binding mode of the phosphoserine is strictly conserved across all seven human 14-3-3 isoforms, therefore it is not surprising these inhibitors are pan isoform inhibitors as well. Recently, a supramolecular ligand of 14-3-3 that contains two phosphate groups was reported (Fig. 3A) (Bartel et al., 2014). Unlike other phosphate-containing ligands of 14-3-3 that occupy the peptide binding groove, the supramolecular ligand wraps itself around Lys²¹⁴ of 14-3-3 σ , located on the groove's edge as demonstrated by X-Ray crystallography (PDBcode: 4HQW). Although not located in the active peptide binding groove, it successfully inhibits the interaction of 14-3-3 with FAM-labeled peptides which present c-Raf and ExoS binding motifs. In addition, the combination of binding assays, X-ray crystallography and molecular simulations revealed rules depicting the lysine binding sites on protein surfaces to be highly favored. This novel mode of inhibitory action and the generated rules are encouraging. It increases the likelihood of designing isoform specific binders because these inhibitors do not have to work at the conserved active site. Unfortunately, since Lys²¹⁴ is strictly conserved in all seven human 14-3-3 isoforms (Fig. 1), the current supramolecular ligand is unlikely to be isoform specific.

The rich structural information of the 14-3-3 proteins and their corresponding partners makes *in silico* screening a reasonable and effective approach for inhibitor searching. Corradi et al. (2010) employed pharmacophore-based virtual screening using the 14-3-3 σ structure

complexed with mode I phosphopeptide (PDBcode: 1YWT) and identified a small molecule compound BV02 that can inhibit 14-3-3 with anti-proliferative activity. Although BV02, and subsequently BV01 and BV101, do not contain phosphate groups, the compounds may still bind the conserved phosphopeptide binding groove. This is supported by the superposition of BV02 with a phosphopeptide showing good agreement (Corradi et al., 2010; Mancini et al., 2011), which points to the possible pan isoform properties of these compounds.

As discussed, the search for chemical compounds that can specifically target one isoform, especially in the company of conserved active sites across family members, has proven to be a most arduous task. Recently, it was found that 14-3-3 proteins negatively regulate the dimerization of estrogen receptor α (ER α) *via* binding with the phosphorylated ER α penultimate Threonine (T⁵⁹⁴), namely mode III motif (De Vries-van Leeuwen et al., 2013). Moreover, the small-molecule fusicoccin (FC) can stabilize the interaction between ER α and 14-3-3 σ and reduce the estradiol-stimulated ER α dimerization and activation, resulting in decreased proliferation of the MCF-7 breast cancer cell line. Co-crystallization of the trimeric ER α -derived-peptide/14-3-3 σ /FC complex (PDBcode: 4JDD) revealed that the ER peptide binds to 14-3-3 σ at the conserved peptide binding groove while FC selectively binds at the ER α -derived-peptide/14-3-3 σ interface that is away from the active binding groove of 14-3-3 σ (Fig. 4). FC works as a stabilizer to increase the affinity between ER α and 14-3-3 σ . The discovery is exciting for three reasons. First, it shows that besides the conserved phosphopeptide binding groove, there are other sites that can be utilized to modulate protein-protein interactions involving 14-3-3 proteins. These off-sites may not be as highly conserved as the phosphopeptide binding groove across all isoforms, and therefore could potentially be targeted to generate 14-3-3 modulators unique to certain isoform. Second, it suggests different groups of 14-3-3 protein partners,

e.g., protein partners with mode III motif, can be possibly separately targeted and therefore can be individually dissected. Thirdly, it provides the rationale to design not only inhibitors but also stabilizers targeting 14-3-3 in cancer drug discovery and development.

Beside 14-3-3 protein inhibitors, another approach is to inhibit 14-3-3 proteins at the mRNA level by using siRNAs. Compared with protein inhibitors, this approach is easier to achieve targeting specificity because mRNA sequences are more different than protein sequences. Indeed, siRNAs specifically targeting certain 14-3-3 isoform have been successfully developed and have already served as a powerful tool (Liu et al., 2006; Ajjappala et al., 2009; Choi et al., 2011; Murata et al., 2012; Raungrut et al., 2014). However, siRNA also has some limitations both as research tools and new therapeutics. As research tool, because siRNA knocks down the total expression level of the protein, this approach does not provide the flexibility of inhibiting certain post-translational modified form such as phosphorylated form and therefore is not a good approach to dissect regulation and function at post-translational level. Another limitation is siRNA lacks the potential to silent only one type of unwanted interactions while keeping the wanted interactions active. This may not only limit siRNA as a research tool, but also limit its use as therapeutics. In addition, siRNA as new therapeutics still faces the challenges of drug delivery, stability and immune response *in vivo*, which is not as much an issue with chemical inhibitors (Gavrilov and Saltzman, 2012).

As reviewed, while there are many promising *in vitro* 14-3-3 inhibitors, currently there has no compound that is documented to specifically inhibit one type or one group of 14-3-3 isoform(s). In addition, the current absence of complimentary *in vivo* support makes it hard to evaluate whether these chemicals offer any hints for new drug development in cancer therapy, which is troubling. However, our increasing knowledge of isoform-specific 14-3-3 roles within

the cell does suggest these compounds may produce undesired side effects in *in vivo* or clinical applications due to the pan-inhibitory nature of these compounds (Dong et al., 2008). First, unselective inhibition of all 14-3-3 isoforms may create neurological disorder. FKO of all 14-3-3 proteins using a chemical genetics approach in the forebrain in difopein-YFP transgenic mice created difficulties in learning processes in mice (Nguyen et al., 2004). In humans, extensive deletions were observed in the chromosomal segment where 14-3-3 resides in a population of patients with severe lissencephaly (Kato and Dobyns, 2003). It has also been shown in human decreased level of 14-3-3 γ in the cortex of embryos is related to Down's syndrome (Peyrl et al., 2002). Second, unselective inhibition of all 14-3-3 isoforms may result in side effect corresponding to the specific function of each individual isoform. For example, the inhibition of the γ isoform has been shown to alter sperm generation in male mice (Sehgal et al., 2014), presenting the possibility of impotence or infertility as a side effect to pan-14-3-3 inhibition. The ϵ isoform is partially responsible for immune defense responses, especially to RNA virus infections, as well as mitochondrial substrate localization, indicating potential loss of energy and weakened immune system response as possible side effects upon this isoform's inhibition (Liu et al., 2012; Marzinke et al., 2013). Therefore, we believe continued work on the development of isoform-specific inhibitors will not only prove highly beneficial in the fields of cancer treatment and drug resistance, but also provide important tools that can shed light on the individual functions and interactomes of different 14-3-3 isoforms, and consequently a better understanding of the etiology of different cancer types that may or may not directly involve 14-3-3s.

7. Concluding Remarks

Considering the important roles of 14-3-3 proteins in apoptosis, cell cycle, and proliferation and its clinical relevance in cancers, it is not surprising that great interest has been

aroused identifying molecules targeting 14-3-3 proteins. However, so far there has no documented 14-3-3 inhibitors that can specifically target one isoform with no or little effect on the others. Broad-targeting of the 14-3-3 family may prove more problematic than beneficial because different isoforms may be misregulated in different cancers. Since many isoforms can be expressed simultaneously in human tissues, the reduction of the activity of an aberrant isoform in tandem with other normally-expressed isoforms may generate new issues due to altered activity of the normally expressed isoform. Additionally, increased drug-target specificity allows for higher effectiveness at lower doses, leading to a subsequent decrease in toxic off-target effects. For these reasons, the development of isoform-specific 14-3-3 inhibitors would greatly benefit patients by curbing potential negative side effects while decreasing dosage amount. Furthermore, the development of isoform-specific inhibitors would provide a valuable research tool to increase understanding of the specific roles of each 14-3-3 isoform. The ability to specifically inhibit one isoform while sparing the others would provide an unparalleled tool for the documentation of isoform-specific activity.

Here we propose structure and molecular dynamics (MD) simulation based approach may integrate low- and high-throughput data and provide insight on their differences in protein dynamics which could be used to enhance the possibility for identifying isoform-specific 14-3-3 inhibitors. The complex structure of 14-3-3 isoforms and protein partners of interest, which is determined by combination of low- and high-throughput data, can be established by molecular modeling and docking utilizing information from structural genomics. Epigenetics variations such as phosphorylation on 14-3-3 isoforms or their protein partners can be modeled in at this time. More importantly, the missing C-terminus can also be built in the model. MD simulations can be performed to these protein complexes to gain their different dynamic properties and the

refined differences in their binding mode. One can lay especial emphasis on the C-terminus and interrogate how the differences in structure and dynamics may link to the functions and specificity of 14-3-3 isoforms. Energy calculations can be performed and be decomposed to residues of interest, which can help to identify critical residues to target. The structural frames of the MD simulations can be collected and clustered. The clustered structures that are more diverged should be examined and be selected as the initial structural model to guide the design of new inhibitors. We believe inhibitors acquired by this approach will likely lock 14-3-3 isoforms in their distinct conformation and therefore can be more specific.

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Table 1.PDB entries for human 14-3-3 proteins.

Isoform	PDBcode	Ligand	Peptide sequence	Mutant/WT	Resolution (Å)	14-3-3 length	Reference
Alpha/beta	4DNK	No	n/a	WT	2.20	1-233	To be published
	2C23	Yes – exoenzyme S peptide	GLLDALD LASK	WT	2.65	3-232	Yang et al., 2006
	2BQ0	No	n/a	WT	2.50		Yang et al., 2006
Gamma	4O46	Yes – influenza NS1 C-terminal tail phosphorylated at S228	PKQKRK MARTAR pSKV	WT	2.90	2-236	Qin et al., 2014b
	4J6S	Yes – tyrosine hydroxylase with pSer19	MPTPDAT TPQAKGF RRAVpSE LDAKQA EAIMSPR FIGRRQS LIE	WT	3.08	1-236	Skjevik et al., 2014
	3UZD	Yes – histone deacetylase 4 with pSer350	LPLYTSPp SLPNITLG LP	WT	1.86	2-234	Xu et al., 2012
	4E2E	No	n/a	WT	2.25	2-234	To be published
	2B05	Yes – pSerine peptide	RAIpSLP	WT	2.55	2-234	Yang et al., 2006
Theta/Tau	2BTP	Yes – 14-3-3 protein consensus peptide	RQRpSAP	WT	2.80	2-230 (- 8-230 w His tag)	Yang et al., 2006
Sigma	4FL5	Yes – microtubule-associated protein tau peptide surrounding pS214	SRTpPSLP TPP	WT	1.90	1-231	To be published
	4IEA	Yes – phosphorylated c-Raf peptide	RSAPSEPS L	WT	1.70	1-231	Molzan et al., 2013
	4DHM	Yes – [2-(2-oxo-2-{[3-(trifluoromethyl)phenyl]amino}ethoxy)phenyl]phosphonic acid	n/a	WT	1.70	1-231	Thiel et al., 2013
	4DHN	Yes - {2-[2-(cyclopentylamino)-2-oxoethoxy]phenyl}phosphonic acid	n/a	WT	1.80	1-231	Thiel et al., 2013
	4DHO	Yes - (2-{2-[(3-methoxyphenyl)amino]-2-oxoethoxy}phenyl)phosphonic acid	n/a	WT	1.70	1-231	Thiel et al., 2013
	4DHP	Yes - [2-(2-{[2-chloro-5-(trifluoromethyl)phenyl]amino}-2-	n/a	WT	1.75	1-231	Thiel et al., 2013

		oxoethoxy}phenyl]phosphonic acid					
4DHQ	Yes - (2-{2-[(3-chlorophenyl)amino]-2-oxoethoxy}phenyl)phosphonic acid	n/a	WT	1.75	1-231	Thiel et al., 2013	
4DHR	Yes - (2-{2-[(2-chlorophenyl)amino]-2-oxoethoxy}phenyl)phosphonic acid	n/a	WT	1.40	1-231	Thiel et al., 2013	
4DHS	Yes - (2-{2-[(3,5-dichlorophenyl)amino]-2-oxoethoxy}phenyl)phosphonic acid	n/a	WT	1.74	1-231	Thiel et al., 2013	
4DHT	Yes - {2-[2-(cyclohexylamino)-2-oxoethoxy]phenyl}phosphonic acid	n/a	WT	1.80	1-231	Thiel et al., 2013	
4DHU	Yes - (2-{2-[(2,3-dichlorophenyl)amino]-2-oxoethoxy}phenyl)phosphonic acid	n/a	WT	1.67	1-231	Thiel et al., 2013	
4JC3	Yes - estrogen receptor peptide	TGEAEGF PApTV	WT	2.05	1-231	De Vries-van Leeuwen et al., 2013	
4JDD	Yes – estrogen receptor peptide and Fusicoccin	TGEAEGF PApTV	WT	2.10	1-231	De Vries-van Leeuwen et al., 2013	
4FR3	Yes – Fusicoccin and TASK-3 peptide	KRRKpSV	WT	1.90	1-231	Anders et al., 2013	
4HQW	Yes – lysine-specific molecular tweezers	n/a	WT	2.35	1-231	Bier et al., 2013	
4HRU	Yes – lysine-specific molecular tweezers	n/a	WT	3.15	1-231	Bier et al., 2013	
3UX0	Yes – Fusicoccin H and TASK-3 peptide	KRRKpSV	WT	1.75	1-231	Anders et al., 2013	
3T0L	Yes - [2-(2-oxo-2-{[2-(trifluoromethyl)phenyl]amino}ethoxy)phenyl]phosphonic acid	n/a	WT	1.60	1-231	Thiel et al., 2013	
3T0M	Yes - (2-{2-[(2,5-dimethoxyphenyl)amino]-2-oxoethoxy}phenyl)phosphonic acid	n/a	WT	1.62	1-231	Thiel et al., 2013	
3SMK	Yes – cotylenin A and TASK-3 peptide	KRRKpSV	Mutant C38V/N166H	2.10	1-231	To be published	
3SML	Yes –Fusicoccin A Aglyoneand TASK-3	KRRKpSV	Mutant C38N/N166H	1.90	1-231	To be published	

		peptide					
3SMM	Yes – Fusicoccin J Aglycone and TASK-3 peptide	KRRKpSV	Mutant C38N/N166H	2.00	1-231	To be published	
3SMO	Yes – Fusicoccin J Aglycone and TASK-3 peptide	KRRKpSV	Mutant C38V/N166H	1.80	1-231	To be published	
3SPR	Yes – Fusicoccin and TASK-3 peptide	KRRKpSV	Mutant C38V/N166H	1.90	1-231	To be published	
4DAT	Yes – PADI6 binding motif II	SSFYPpSA EG	WT	1.40	1-231	Rose et al., 2012	
4DAU	Yes – PADI6 binding motif I	MVSVEG RAMpSFQ S	WT	2.00	1-231	Rose et al., 2012	
3U9X	No	n/a	WT	1.80	1-231	Roglin et al., 2012	
3P1N	Yes – 6mer peptide from potassium channel subfamily K member 9	KRRKpSV	WT	1.40	1-231	To be published	
3P1O	Yes – 6mer peptide from potassium channel subfamily K member 9 and Fusicoccin	KRRKpSV	WT	1.90	1-231	To be published	
3P1P	Yes – 6mer peptide from potassium channel subfamily K member 9	KRRKpSV	Mutant C38N/N166H	1.95	1-231	To be published	
3P1Q	Yes – 6mer peptide from potassium channel subfamily K member 9 and Fusicoccin A	KRRKpSV	Mutant C38N/N166H	1.70	1-231	To be published	
3P1R	Yes – 6mer peptide from potassium channel subfamily K member 9	KRRKpSV	Mutant C38V/N166H	1.70	1-231	To be published	
3P1S	Yes – 6mer peptide from potassium channel subfamily K member 9 and Fusicoccin A	KRRKpSV	Mutant C38N/N166H	1.65	1-231	To be published	
3O8I	Yes – c-Raf peptide and 6,6-dihydroxy-1-methoxyhexan-2-one	QRSTpST PNVH	WT	2.00	1-231	Molzan et al., 2010	
3MHR	Yes – YAP pS127 peptide	RAHpSSP ASLQ	WT	1.15	1-231	Schumacher et al., 2010b	
3IQJ	Yes – Raf1 10mer peptide	QRSTpST PNVH	WT	1.15	1-231	Molzan et al., 2010	
3IQU	Yes – Raf1 6mer peptide	QRSTpST	WT	1.05	1-231	Molzan et al., 2010	
3IQV	Yes – Raf1 6mer and Fusicoccin	QRSTpST	WT	1.20	1-231	Molzan et al., 2010	
3LW1	Yes – p53 pT387	FKpTEGP	WT	1.28	1-231	Schumacher	

		peptide	DSD				et al., 2010a
	1YZ5	No	n/a	WT	2.80	1-233	Benzinger et al., 2005b
	1YWT	Yes – pSerine mode-1 peptide	MARSHpSYPAGKK	WT	2.40	1-231	Wilker et al., 2005
Zeta/delta	1A38	Yes – R18	WLDLE	WT	3.35	1-228	Petosa et al., 1998
	4N7G	Yes – ExoS derived peptide	QGLLDALDLAS	WT	2.25	1-230	Glas et al., 2014
	4WRQ	Yes – Chibby peptide	KTPPRKS ApSLSNL HSLDR	WT	2.41	1-230	Killoran et al., 2015
	4HKC	Yes – Integrin α -4 peptide phosphorylated at S1011	GFFKRQY KpSILQEE NRRDSW SYINSKS NDD	WT	2.20	2-230	Bonet et al., 2013
	4N7Y	Yes – ExoS derived 8-carbon linker cyclic peptide	QGXLDDL DLAS	WT	2.16	1-230	Glas et al., 2014
	4N84	Yes – ExoS derived 12-carbon linker peptide	GXLDXL DLAS	WT	2.50	1-230	Glas et al., 2014
	4IHL	Yes – diphosphorylated c-Raf peptide and Cotylenin A	QHRYpST PHAFTFN TSSPSSEG SLSQRQR STpSTPN VH	WT	2.20	1-230	Molzan et al., 2013
	4BG6	Yes – farnesylated Rnd3 peptide	DLRKDK AKpSC	WT	2.30	1-231	Riou et al., 2013
	4FJ3	Yes – diphosphorylated c-Raf peptide	QHRYpST PHAFTFN TSSPSSEG SLSQRQR STpSTPN VH	WT	1.95	2-230	Molzan and Ottmann, 2012
	3RDH	Yes – FOBISIN101	n/a	WT	2.39	1-230	Zhao et al., 2011a)
	3NKX	Yes – RAF proto-oncogene serine/threonine protein kinase peptide fragment	QRSTpST PNVH	WT	2.40	1-230	Molzan et al., 2010
	2WH0	Yes – synthetic diphosphorylatedPKC ε V3 region	DRSKpSA PTSPCDQ EIKELEN NIRKALp SFDNR	WT	2.25	2-229	Kosteletzky et al., 2009
	3CU8	Yes – RAF proto-oncogene serine/threonine protein kinase fragment	RSTpSTP NVH	WT	2.50	1-230	To be published
	2V7D	Yes – Integrin β 2	KSAPTTT	WT	2.50	1-229	Takala et al.,

		pThr758 peptide	VMNP				2008
	2O02	Yes – ExoS peptide	GHGQGL LDALDLA S	WT	1.50	1-230	Ottmann et al., 2007
	2C1J	Yes- phosphorylated and phosphoacetylated Histone H3	ARKpSTG GK	WT	2.60	1-230	Macdonald et al., 2005
	2C1N (mono mer)	Yes-phosphorylated and phosphoacetylated Histone H3	ARKpSTG GK	WT	2.00	1-230	Macdonald et al., 2005
	1IB1	Yes – Serotonin N-acetyltransferase	*STPSVH CLKPSPL HLPSGIP GSPGRQR RHpTLPA NEFRCLT PEDAAG	WT	2.70	2-228	Obsil et al., 2001
	1A4O	No	n/a	WT	2.80	1-228	Liu et al., 1995
	1QJA	Yes – pSerine mode-2 peptide	RLYHpSL PA	WT	2.00	1-230	Rittinger et al., 1999)
	1QJB	Yes – pSerine mode-1 peptide	ARSHpSY PA	WT	2.00	1-232	Rittinger et al., 1999
Epsilon	3UAL	Yes – Myeloid leukemia factor 1 fragment	MIRSFpSE PFGRDL	WT	1.80	3-232	Molzan et al., 2012
	3UBW	Yes – Myeloid leukemia factor 1 fragment and 3-pyrrolidinol	MIRSFpSE PFGRDL	WT	1.90	3-232	Molzan et al., 2012
	2BR9	Yes – 14-3-3 protein consensus peptide I	RRQRpSA P	WT	1.75	3-232	Yang et al., 2006
Eta	2C63	Yes – 14-3-3 protein consensus peptide II	RSIpSLP	WT	2.15	3-235	Yang et al., 2006
	2C74	Yes – 14-3-3 protein consensus peptide I	RRQRpSA P	WT	2.70	2-236	Yang et al., 2006

n/a refers to not applicable; WT refers to wild type; *partial sequence of serotonin N-acetyltransferase is shown.

Fig. 1. Multiple sequence alignment of all seven human 14-3-3 isoforms by Clustal 2.1.

Asterisks indicate identical residues across all seven isoforms. Positions of helices 1-9 are indicated by solid bars on the top.

Fig. 2. Structural alignment of 14-3-3 proteins.

A. Representative structural alignment of the σ and ζ isoforms complexed with phosphopeptides (PDB code: 1YWT and 1QJB, respectively). The two chains of the σ form are depicted by blue and dark blue, respectively, and the ζ isoform is shown in pink. The nine α -helices in each monomer, H1 to H9, were labeled. These two structures can be aligned very well with an rmsd 0.85 Å when 308 atom pairs from these two structures are aligned. **B:** Zoom-in view of the phosphopeptide-binding groove of the same structural alignment as in **A**. The phosphopeptide is

shown in stick representation and the interacting residues are shown in ball-and-stick. Not only residue types, but also the conformations of the interacting residues are nearly identical. In both structures, the phosphate group is stabilized by the positively charged groups from K49, R56, R129, and Y130 with residue numbers labeled as in σ form. **C:** Structural alignment between the apo-structures of the σ and ζ isoforms (PDB code: 1YZ5 and 1A40, respectively). Compared with the phosphopeptide bound structural alignment, the rmsd is 1.54 Å when 308 atom pairs from the two apo-structures are aligned. Examination of the structural alignment shows H1 to H4 of the two structures are aligned very well, while H5 to H9 are not, with H7 to H9 having the highest dissimilarity as emphasized in the black frames. **D:** The binding groove of the 14-3-3 ζ structures complexed with R18 (PDB code: 1A38) and complexed with phosphopeptide (PDB code: 1QJB). For clarity, only the main chain atoms of R18 and phosphopeptide are shown with exception of E5 residue on R18 and phosphoserine residue on the phosphopeptide. The Glu residue mimics the phosphoserine and interacts with the same group of residues, R56, R60, R127 and Y128, which all adopt almost identical conformations.

Fig. 3. Chemical structures of 14-3-3 inhibitor or modulator.

Fig. 4. The binding of the FC and ER α -derived-peptide to 14-3-3 σ (PDB code: 4JDD).

FC binds to 14-3-3 σ in a different site from the phosphopeptide and increases the affinity between the peptide and 14-3-3 σ . 14-3-3 σ monomer is shown by ribbons. ER α -derived-phosphopeptide (carbon atoms shown in cyan) and FC (carbon atoms shown in gold) are depicted by ball-and-stick representation. Oxygen atoms are shown in red and phosphate atom is shown in orange.

Figure 1

1

	H1	H2	H3	H4
14-3-3β/α	-MVDREQLVQKARLAEQAERYDDMAAAMKNVTELNEPLSNEERNLLSVAYKNVVGARRSSWRVISSIEQKTSADGNEKKIEMVRAYREKIEKELEAVCQD			99
14-3-3γ	-MGDREQLLQRRARLAEQAERYDDMASAMKAVTELNEPLSNEDRNLLSVAYKNVVGARRSSWRVISSIEQKTMADGNEKKLEKVKAYREKIEKELETVEND			99
14-3-3ε	MTMDKSELVQKAKLAEQAERYDDMAAAMKAVTEQGHLSNEERNLLSVAYKNVVGARRSSWRVISSIEQKT--ERNEKKQMGKEYREKIEAELQDICND			98
14-3-3η	--MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNERNLLSVAYKNVVGARRSSWRVSSIEQKT--EGAEEKQGMAREYREKIEIETELRDICND			96
14-3-3θ	--MEKTELIQKAKLAEQAERYDDMATCMKAVTEQGAELSNERNLLSVAYKNVVGARRSSWRVSSIEQKT--DTSDKKLQLIKDYREKVESELRSICTT			96
14-3-3σ	--MERASLIQKAKLAEQAERYEDMAAFMKGAVEKGEELSCEERNLLSVAYKNVVGQRAAWRVLSIEQKSNEEGSEEKGPEVREYREKIVETELQGVCDT			98
14-3-3ζ/δ	-MDDREDLVYQAKLAEQAERYDEMVESMKKVAGMDVELTVEERNLLSVAYKNVIGARRASWRISSIEQKEENKGGEDKLKMIREYRQMVETELKLICCD			99
	* * * * *	* * * * *	* * * * *	* * * * *
	H5	H6	H7	H8
14-3-3β/α	VLSLLDNYLIKNCSETQYESKVFYLMKMGDYRYLAEVATGEKRAVTVESSEKAYSEAHEISKEHMQPHTPIRLGLALNYSVFYYEIQNAPEQACHLAKT			199
14-3-3γ	VLSLLDKFLIKNCNDFQYESKVFYLMKMGDYRYLAEVASGEKKNSVVEA SEAAYKEAFEISKEHMQPHTPIRLGLALNFSVFYYEIQNAPEQACLLAKQ			199
14-3-3ε	VLELLDKYLIPNATQP--ESKVFYLMKMGDYFRYLSEVASGDNKQTTVSN SQQAYQEAFEISKKEMQPHTPIRLGLALNFSVFYYEILNSPEKACSLAKT			196
14-3-3η	VLSLLEKFLIPNASQA--ESKVFYLMKMGDYRYLAEVAAGDDKKGIVDQ SQQAYQEAFEISKKEMQPHTPIRLGLALNFSVFYYEILNSPEKACSLAKT			194
14-3-3θ	VLELLDKYLIANATNP--ESKVFYLMKMGDYFRYLAEVACGDDRKTIDN SQGAYQEAFDISKKEMQPHTPIRLGLALNFSVFYYEILNNPELACTLAKT			194
14-3-3σ	VLGLLDShlikeAGDA--ESRVFYLMKMGDYRYLAEVATGDDKKRIIDS ARSAYQEAMDISKKEMPPTNPPIRLGLALNFSVFHYEIANSPPEAISLAKT			196
14-3-3ζ/δ	ILDVLDKHLIPAANTG--ESKVFYLMKMGDYHRYLAEFATGNDRKEAAEN SLVAYKAASDIAMTELPHTPIRLGLALNFSVFYYEILNSPDRACRLAKA			197
	* * * * *	* * * * *	* * * * *	* * * * *
	H9			
14-3-3β/α	AFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDDDG---GEGNN----- 247			
14-3-3γ	AFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDEEA---GEGN----- 246			
14-3-3ε	AFDEAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSENQGDG-DAGEG-EN----- 246			
14-3-3η	AFDEAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDTQGDEA-EAGEGGEN----- 245			
14-3-3θ	AFDEAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDSAGEEC-DAAEGAEN----- 245			
14-3-3σ	TFDEAMADLHTLSEDSYKDSTLIMQLLRDNLTLWTADNAGEEGGEAPQEPQS----- 248			
14-3-3ζ/δ	AFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDMQGDGEEQNKEALQDVEDENQ 255			
	* * * * *			

A

B

C

D

Figure 3

Fobisin101

Supramolecular Tweezer

B2

BV02

FC

Figure4