

REVIEW

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Phosphotyrosine recognition domains: the typical, the atypical and the versatile

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Abstract

SH2 domains are long known prominent players in the field of phosphotyrosine recognition within signaling protein networks. However, over the years they have been joined by an increasing number of other protein domain families that can, at least with some of their members, also recognise pTyr residues in a sequence-specific context. This superfamily of pTyr recognition modules, which includes substantial fractions of the PTB domains, as well as much smaller, or even single member fractions like the HYB domain, the PKC δ and PKC θ C2 domains and RKIP, represents a fascinating, medically relevant and hence intensely studied part of the cellular signaling architecture of metazoans. Protein tyrosine phosphorylation clearly serves a plethora of functions and pTyr recognition domains are used in a similarly wide range of interaction modes, which encompass, for example, partner protein switching, tandem recognition functionalities and the interaction with catalytically active protein domains. If looked upon closely enough, virtually no pTyr recognition and regulation event is an exact mirror image of another one in the same cell. Thus, the more we learn about the biology and ultrastructural details of pTyr recognition domains, the more does it become apparent that nature cleverly combines and varies a few basic principles to generate a sheer endless number of sophisticated and highly effective recognition/regulation events that are, under normal conditions, elegantly orchestrated in time and space. This knowledge is also valuable when exploring pTyr reader domains as diagnostic tools, drug targets or therapeutic reagents to combat human diseases.

Keywords: Posttranslational modification, Phosphotyrosine signaling, Ligand recognition specificity, Cancer therapeutics, Signaling circuit

Background

Phosphotyrosine signaling

Intracellular communication is transmitted via networks of molecules that execute information transfer using protein-mediated interactions. Post-translational modifications (PTMs) such as protein phosphorylation, acetylation, methylation and ubiquitination confer spatiotemporal dynamics to cell signaling [1]. Among these PTMs, the tyrosine phosphorylation signaling system in eukaryotes, especially in mammalian species, has been extensively studied owing to its importance in numerous cellular functions including differentiation, proliferation, motility and apoptosis as well as its therapeutic potential. In

particular, mutations and aberrant expression of kinases are frequently associated with tumourigenesis [2,3].

Signaling proteins often possess a cassette-like architecture made up of catalytic domains and/or protein interaction modules [4]. One important group of protein-protein interaction modules are the autonomous domains that recognize phosphorylated tyrosine (pTyr) residues at specific sites on their target molecules [5]. These pTyr-binding protein modules and their targets are a part of an elaborate pTyr signaling system that consists of three major components that help relay molecular messages [6]. The pTyr signaling system is activated when a stimulus reaches catalytic proteins that act as “writers” of phosphorylation, the protein tyrosine kinases (PTKs). Most PTKs are phosphorylated on themselves to attain an active state, and subsequently phosphorylate other substrate proteins. A second group of proteins that contain modular domains are capable

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of recognizing or “reading” this phospho-modification information and thereby linking the kinase signal to downstream molecules. The phosphorylation can be subsequently “erased” by a third group of proteins, the protein tyrosine phosphatases (PTPs), therefore terminating the signal [7,8].

The human genome harbours 90 PTKs [9], hundreds of pTyr-recognition domains that include 121 members of the Src homology 2 (SH2) domain family [10-12], and more than 10,000 tyrosine phosphorylation sites [12,13]. These signaling components form an enormous network of pTyr signaling that is both robust and dynamic. Nature is equipped with multiple strategies to reduce possible misfiring of pTyr signals due to the complexity of pTyr signaling network. First, PTKs can remain inactive until they are stimulated by a proper cue, most typically by association of a specific ligand molecule to the PTK. For example, receptor tyrosine kinases (RTKs), transmembrane proteins consisting of an extracellular ligand binding site and an intracellular tyrosine kinase domain, are designed inactive until a ligand binds to the extracellular site of the RTK, which often induces RTK oligomerization [14]. Structural studies have also revealed the presence of both active and inactive conformations for many cytoplasmic and receptor kinases [15,16]. Moreover, the level of PTP activity is very high in cells, thereby ensuring that pTyr sites can be rapidly dephosphorylated [17].

Second, each component of the pTyr signaling circuit, i.e., a PTK, PTP or pTyr-binding module, possesses substrate or ligand recognition specificity to narrow down potential interaction partners [18-23]. Since all three components are the modules that bind to linear motif peptides, the specificity at the molecular or atomic level is defined by the ligand peptide sequence and can also be contributed by conformation of the peptide. The interaction specificity is further enhanced by spatiotemporal regulation of the network components, including tissue-specific or cell cycle-dependent protein expression, protein localization to subcellular compartments or a scaffold protein, and protein inactivation involving receptor internalization (endocytosis) and/or protein degradation. These multiple layers of regulatory mechanisms are essential for coordinating such a complex functional network [1,15,24,25]. However, an unintended activation of pTyr signaling, or misfiring, may occur when a circuit component malfunctions, most commonly due to mutations, overexpression or loss of a component or an element, as we will discuss some cases below.

This review will focus on the pTyr “reader” proteins contributing to this complex system. In addition to the SH2 and phosphotyrosine-binding (PTB) domains, the two archetype domain families known for pTyr

binding, recent studies have shown that at least a handful of additional protein modules are capable of reading out the tyrosine phosphorylation. Here we review and explore the structure, function, specificity and therapeutic potentials of a number of typical and atypical members of the superfamily of pTyr-binding protein modules.

The SH2 domain

The Src homology 2 (SH2) domains, a non-catalytic module containing ~100 amino acids, was first discovered by insertion-mutation analysis of the *v-fps/fes* oncogene from the Fujinami sarcoma virus [26] (for historical perspectives of tyrosine phosphorylation studies, refer to reviews by Pawson [27] and Hunter [3]). Soon thereafter, the SH2 domain was identified in oncogenes such as *v-crk* and in the endogenous cytoplasmic proteins phospholipase C γ 1 (PLC γ 1) and the Ras GTPase activating protein (RasGAP) [28,29]. SH2 domains have since been identified in a wide range of eukaryotic species, including yeast, but primarily in metazoans [7,11]. A recent tally finds 121 SH2 in 111 proteins in the human genome [11]. Proteins containing SH2 domains include those that function as kinases, adaptors, phosphatases, ubiquitin ligases, transcription factors, guanine nucleotide exchange factors and phospholipid-based secondary signaling molecules [5,12,30]. Studies in almost two decades have demonstrated the tyrosine phosphorylation-dependent nature of typical SH2 domain-ligand interactions [31-34], the central role played by SH2 domains in connecting activated receptor tyrosine kinases, such as the epithelial growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR), to cytoplasmic signaling molecules [29,35]. In addition, kinase SH2 domains are essential in regulating the catalytic activity of cytoplasmic kinases as exemplified for the Src family as well as the Fes and Abl kinases [15]. A growing picture illustrates that kinase SH2 domains may regulate catalytic activity utilizing diverse mechanisms [16,36]. These and other lines of work establish the SH2 domain as a key player in the cellular signaling system in a pTyr-dependent manner [37].

Architecture of the SH2 domain

As represented by the *v-Src* SH2 domain (Figure 1A), the structure of an SH2 domain features two α -helices (α A and α B) sandwiching a β -sheet consisting of seven anti-parallel strands (β A- β G) [38,39]. Based on the experimentally determined structures of ~70 unique SH2 domains in the Protein Data Bank (PDB), the N-terminal region of the SH2 domain that provides a pTyr-binding pocket is more conserved than the C-terminal half of the SH2 domain that exhibits greater structural variability (Figure 1B). For instance, sequence

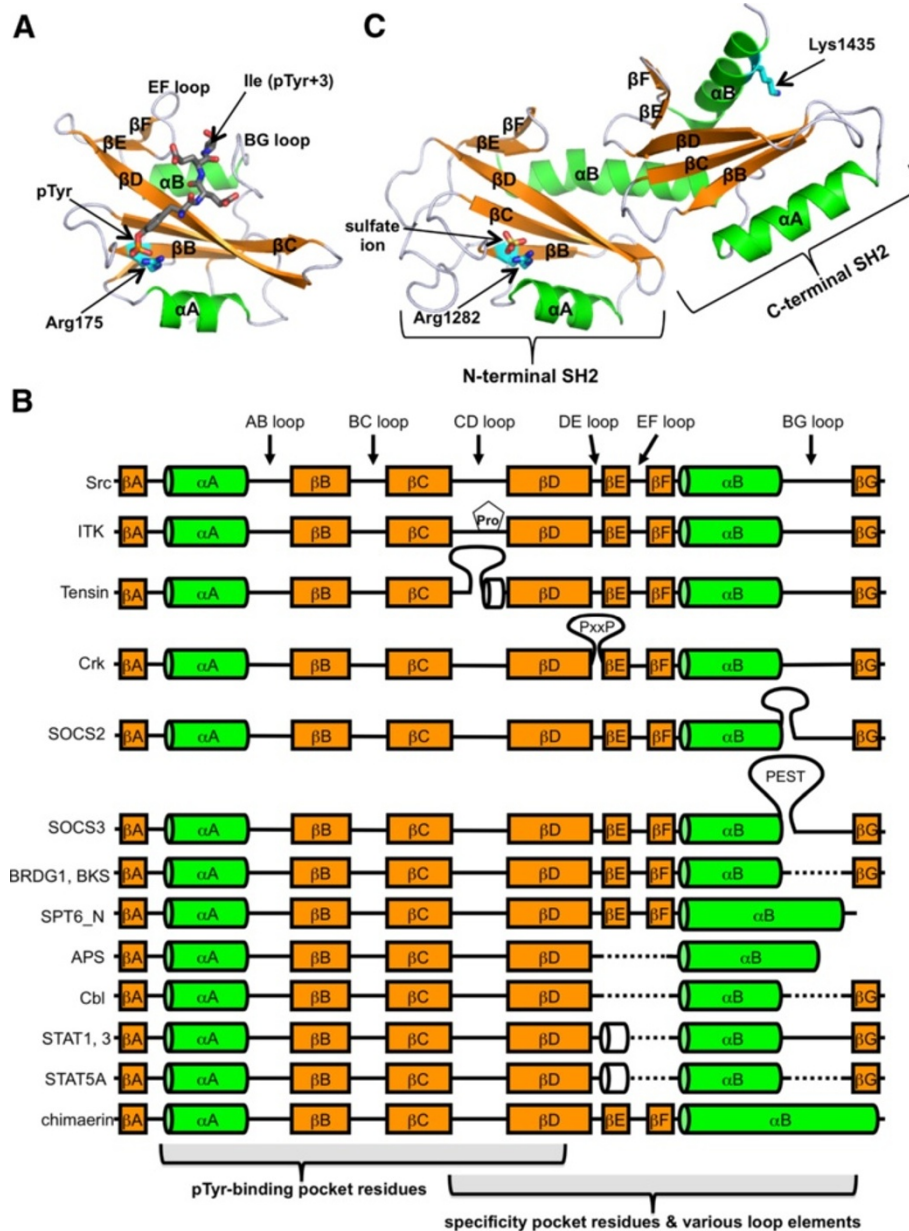


Figure 1 Structure and sequence patterns of the SH2 domain. (A) Structure of the v-Src SH2 domain in complex with the pYEEI peptide (PDB ID: 1SPS). The two conserved α -helices are coloured green, and the seven β -strands are coloured orange. The peptide is shown as grey sticks. The phosphate group of pTyr binds to Arg175 located on the β B strand of the SH2 domain. The pTyr+3 Ile side chain is captured by a hydrophobic pocket provided between the EF and BG loops. (B) Conservation and variation in the secondary structural elements of SH2 domains based on experimentally determined structures. Refer to [40] for a list of SH2 domain structures. The N-terminal half of an SH2 domain is dedicated to pTyr recognition and is much less variable than the C-terminal half where the specificity pocket is located. A dashed line indicates that the element does not exist in an SH2 domain. Structural variations are observed more often in the C-terminal half. For example, the BG loop of the STAP family (BRDG1 & BKS) and the Cbl family SH2 domains are much shorter than in other SH2 domains, which results in an open pocket capable of binding a hydrophobic pTyr +4 residue [40,43]. Pro287 of the ITK SH2 domain is susceptible to *cis-trans* isomerization via its CD loop, which leads to a switch of binding partners [44-46]. The long, proline-rich DE loop insertion in the Crk SH2 domain is the binding site for the Abl SH3 domain [47]. (C) The tandem SH2 domains of the transcription factor Spt6. Four research groups have reported crystal and solution structures, which are essentially identical to each other [48-51]. Shown here is the crystal structure of the *Saccharomyces cerevisiae* Spt6 with a sulfate ion located in the "canonical" phospho-residue binding pocket of the N-terminal SH2 domain (PDB ID: 3PSK) [48]. Mutagenesis studies and NMR titration analysis suggested that this pocket, involving Arg1282, as well as a positively charged patch, including Lys1435 of the C-terminal SH2 domain (residues shown as cyan sticks), are the binding sites of the phosphorylated CTD peptides [49,50,52].

deletion or insertion is found primarily in the β E- β F and BG loop regions (Figure 1B). In addition, structure-based sequence alignment revealed that the most conserved residues are clustered on the β B strand [40]. For the majority of experimentally solved SH2-ligand complex structures, the bound pTyr-peptide forms an extended conformation and binds perpendicular to the central β -strands of the SH2 domain (Figure 1A). Specific residues in the N-terminal region (α A to β D) form a positively charged pocket for binding of the pTyr residue [38,39,41]. A conserved arginine residue on the strand β B (Arg175 in the v-Src SH2 domain) (Figure 1A) plays the central role in forming bi-dentate hydrogen bonds with the phosphate moiety of pTyr. In contrast, a hydrophobic pocket provided by the second half of the domain (i.e. β D to β G) engages residues C-terminal to the pTyr of a ligand peptide to confer specificity [12,30,42].

Specificity of the SH2 domain

SH2 domains are equipped for the specific recognition of a subset of pTyr-containing ligands [20,22,53-55]. A number of studies have established that binding affinity of an SH2 domain to a pTyr-containing ligand is moderate, with the typical affinity range between 0.1 μ M and 10 μ M for equilibrium dissociation constant values (K_D) [56-60]. This moderate affinity is considered to be crucial for allowing transient association and dissociation events in cell signaling. Indeed, artificially increased affinity using an engineered SH2 domain (called the pTyr superbinder) has been shown to cause detrimental consequences to cells [61]. While the pTyr-binding pocket, which is present in the N-terminal half and highly conserved in the SH2 domain, provides the basal affinity for ligand binding with approximately a half of the total binding free energy [62], the hydrophobic pocket present in the C-terminal half of an SH2 domain provides specificity towards a hydrophobic residue in a peptide ligand. Recent studies suggest that the major positional specificity of an SH2 domain is conferred by the EF and BG loops which regulate ligand access to specificity pockets in an SH2 domain. Thus, distinct loop composition and configuration determines whether an SH2 domain has specificity for a residue at the second, third or fourth position C-terminal to the pTyr residue [10,11,20,40,63,64]. The wealth of experimentally solved SH2 domain-ligand complex structures allows the systematic ultrastructural investigation into how variations in the specificity pocket leads to distinct specificities with bioinformatics tools. Meanwhile, additional subtlety and sophistication in pTyr-peptide discrimination have been demonstrated by a recent study highlighting the importance of permissive and non-permissive residues proximal to pTyr in the ligand sequence [65]. The study provided evidence that local

sequence context provides an additional layer of specificity enhancement beyond the general sequence motifs uncovered by regular degenerate peptide library screens [20,66].

The Spt6 SH2 domain: a common ancestor of pTyr recognition?

The yeast genome encodes only one SH2 domain-containing protein, the transcription factor Spt6 [7]. The C-terminal region of the protein, initially predicted to contain a single SH2 domain, binds to the C-terminal domain (CTD) of the RNA polymerase II [52]. The CTD consists of an abundance of repeats (52 in human, 26 in yeast) of the heptad sequence YSPTSPS, in which each tyrosine, serine, and threonine residues can be phosphorylated. Furthermore, prolines are subjected to *cis-trans* isomerization, adding another layer of complexity and dynamics to the CTD [67,68]. The SH2 domain of Spt6 has been considered a prototypical SH2 domain for several reasons. (I) Spt6 is present in yeast that does not contain a PTK, (II) Yoh et al. demonstrated that the Spt6 SH2 domain region binds to Ser-phosphorylated CTD, and (III) Spt6 is conserved in eukaryotes including slime moulds and plants [7,11,52,69]. Indeed, the recently solved structures of the C-terminal region of Spt6 revealed that the region actually contains two SH2 domains in tandem that are intimately associated with each other (Figure 1C) [48-51]. The phospho-binding pocket of the N-terminal SH2 domain, which contributes to CTD phosphopeptide binding, contains an arginine that is invariant among eukaryotic SH2 domains [49,50,52]. In contrast, the corresponding pocket in the C-terminal SH2 domain lacks an arginine, and NMR titration studies suggest that this pocket is not used for peptide binding. Instead, a positively charged patch on the surface of the C-terminal SH2 domain participates in CTD binding (Figure 1C) [49,50]. Interestingly, the tandem SH2 domains have shown low affinities (with dissociation constants in the millimolar range) for both pTyr- and pSer-containing peptides derived from the CTD [50]. It is proposed that the binding of the Spt6 tandem SH2 domains to the polymerase may be significantly enhanced *in vivo* as the CTD contains numerous repeats of the phosphorylated heptad sequence that can increase the effective local concentration of the binding target for the tandem SH2 domains [50]. From an evolutionary standpoint, it is likely that the Spt6 SH2 domains provided the prototype for a family of modular domain for the phospho-specific interaction that have later evolved to be specific for phosphotyrosine. Notwithstanding this viewpoint, SH2 domains have been identified in abundance in protozoans such as choanoflagellates [7,8,70].

Atypical ligand recognition modes

Although SH2 domains are the largest group of pTyr-binding modules [5,10], it has been shown that certain SH2 domains have the ability to bind ligands in a tyrosine phosphorylation-independent manner [10,34,71-78]. For example, the SH2 domains of tensins and SAP bind to both phosphorylated and non-phosphorylated forms of ligand peptides [73-75,78,79]. Some SH2 domains feature a secondary site located outside of the primary ligand-binding site to engage a pTyr-ligand protein using two sites [76,80]. In this regard, Anderson and colleagues identified a second binding site on the C-terminal SH2 domain of the phosphoinositide 3-kinase p85 α subunit that binds the Raf family kinase member A-Raf in a phosphorylation-independent manner [80]. The authors posit that the second binding site increases the target selectivity of the SH2 domain. Biochemical and structural analysis illustrated pTyr-independent interaction between the N-terminal SH2 domain of PLC γ 1 and the tyrosine kinase domain of the fibroblast growth factor receptor (FGFR) via a secondary binding site, in addition to the canonical pTyr-binding primary site [76]. In another scheme, an SH2 domain may contain sequence motifs that are recognized by distinct types of modular domains. A long, proline-rich insertion into the DE loop of the Crk SH2 domain (Figure 1B) is recognized by the Src homology 3 (SH3) domain of the Abl kinase [47]. Sequence analysis of Crk orthologs suggests that this insertion is of recent evolutionary origin as it is identified only in mammalian species [11]. This provides an example of loop evolution that enriches the pTyr signaling network by introducing a novel interaction.

Interplay between SH2 and kinase domains

Among the 90 PTKs in the human genome, 32 are cytoplasmic tyrosine kinases [81]. Notably, 28 of them also contain an SH2 domain in tandem with the kinase domain (with the exceptions of TNK1, ACK, FAK and Pyk2). This suggests a strong physical-functional relationship between the kinase and SH2 domains. Mayer et al. demonstrated that a kinase-associated SH2 domain promotes phosphorylation of substrates, which they termed processive phosphorylation [82]. In the course of processive phosphorylation, the kinase domain of a cytoplasmic tyrosine kinase phosphorylates a substrate on the tyrosine site, that is then tightly bound by the SH2 domain, allowing the associated kinase domain to carry out further phosphorylation of the substrate (or a second substrate molecule associated with the first substrate) [19,83]. Thus the assembly of SH2-kinase domain cassettes allows the physical association required for function of the two domains [4,5,15]. Moreover, recent bioinformatics analysis of protein sequences for 330 *bona fide* SH2-binding motifs revealed that tyrosine

phosphorylation sites in human proteome are significantly enriched in the vicinity of the SH2 domain-binding sites [84], which may indicate that processive phosphorylation is a rather common phenomenon. Processive phosphorylation is perhaps unique to the SH2-kinase domain combination, because the PTB domain (see below) does not coexist with a tyrosine kinase domain in any human protein [85]. As we discuss later, direct intramolecular interaction between the kinase and SH2 domains is essential for activity in some PTKs, which represents another physical and functional interaction between the two domains and a potential target for treatment in cancer cells.

Multifunctional loops for the SHP SH2 domains

Interplay between an SH2 domain and a catalytic domain has also been observed for phosphatases. Pei et al. reported that the SH2 domains of tyrosine phosphatase SHP-1 regulates its catalytic activity via an auto-inhibition mechanism [86]. Since then, structural studies of SHP-1, and its paralog phosphatase SHP-2, have revealed multiple conformations for the SHP phosphatases. The first crystal structure of SHP-2 demonstrated its inactive conformation, in which Asp61 of the DE loop from the N-terminal SH2 domain mimics pTyr moiety and directly blocks the catalytic pocket of the phosphatase domain (Figure 2A) [87]. Interestingly, in this inactive conformation, the cleft between the EF and BG loops of the N-SH2 domain is closed and ligand binding is disabled. A similar inhibitory conformation was also observed for SHP-1 [88]. A recent model proposed that activation of the SHP-1 phosphatase requires binding of a pTyr ligand to the SH2 domains and a subsequent large structural rearrangement of the C-terminal SH2 domain to allow dissociation of the N-terminal SH2 domain from the catalytic pocket [89]. A further structural study illustrated that the N-terminal SH2 domain of SHP-2 possesses the ability to act either in the single-peptide or in the double-peptide binding mode, depending on the peptide sequence [90]. The single-peptide binding mode follows a canonical ligand binding mechanism, i.e., binding to an open cleft between the EF and BG loops (Figure 2B). In the double-peptide binding mode, one of the peptides binds the canonical pocket in a pTyr-dependent manner whereas the other pairs up with the first peptide to form a short antiparallel β -sheet (Figure 2C). The authors propose that such a property of the SH2-dual peptide interaction suggests the SH2 domain may serve as a scaffold for two ligand molecules.

Phosphorylation-dependent binding partner switching

Post-translational modification such as tyrosine phosphorylation may act as a switch for some proteins. For instance, the presence or absence of phosphorylation

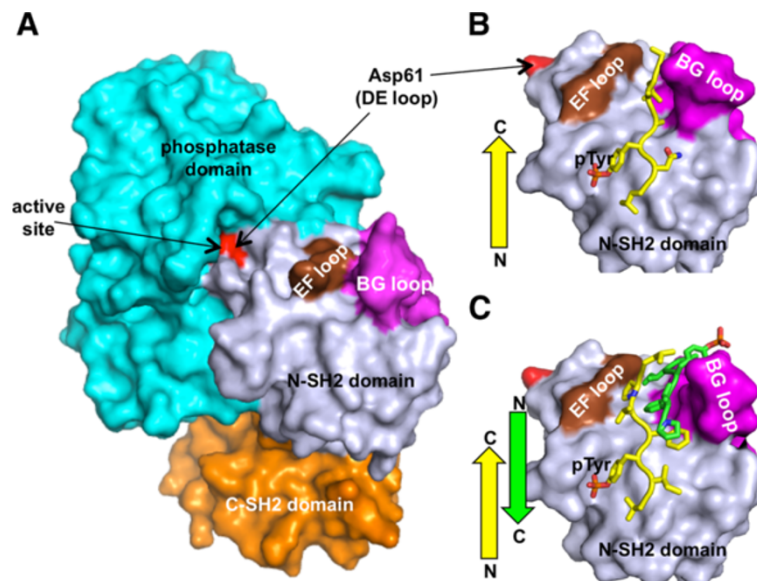


Figure 2 Surface loops in the SH2 domain confer multiple binding modes to the tyrosine phosphatase SHP2. The N-terminal SH2 (N-SH2), C-terminal SH2 (C-SH2) and the phosphatase domains are coloured in light blue, orange, and cyan, respectively. The EF and BG loops of the N-SH2 domain are coloured brown and magenta, respectively. Molecular orientation is aligned for the N-SH2 domain, and drawn to scale. **(A)** The inhibitory state of SHP2 (PDB ID: 2SHP) [87]. The DE loop region of the N-SH2 domain, including the side chain of Asp61 (coloured red), mimics a pTyr substrate and blocks the active site of the phosphatase domain. In this conformation, the BG loop contacts the EF loop and inhibits ligand binding. **(B)** The 1:1 binding mode (PDB ID: 3TL0) [90]. The bound LNpYAQLW peptide is coloured yellow. The C-terminal region of the single peptide binds to a cleft between the EF and BG loops. **(C)** The 1:2 binding mode, in which the two identical peptides, with a sequence VlpYFVPL, form a short, antiparallel β -sheet and bind to a single SH2 domain (PDB ID: 3TKZ) [90]. The BG loop is positioned to accommodate the two peptides.

may provide a mechanism for alternative binding to distinct protein partners [84]. This type of regulation is underscored in the multifaceted interaction between the T-cell receptor subunit CD3 ϵ and either the ZAP-70 tandem SH2 domains or the Eps8L1 and the N-terminal NCK SH3 domains (Figure 3) [91-93]. CD3 ϵ harbours both an immunoreceptor tyrosine-based activation motif (ITAM) and a PxxDY motif. These two motifs overlap at Tyr166, which may be phosphorylated (Figure 3). While phosphorylation of Tyr166, along with Tyr177 in the

ITAM motif, promotes the binding of the tandem ZAP-70 SH2 domains, the phosphorylation of Tyr166 also abrogates SH3 domain binding via the PNPDY motif (Figure 3) [91-93]. Bioinformatics analysis suggests that there exist a plethora of such phosphorylation sites on signaling proteins which could act as regulatory switches for selective protein-protein interactions [84]. Moreover, phosphorylation-dependent partner switching has been documented to also occur on other modular domain-mediated interactions, as elucidated below for the

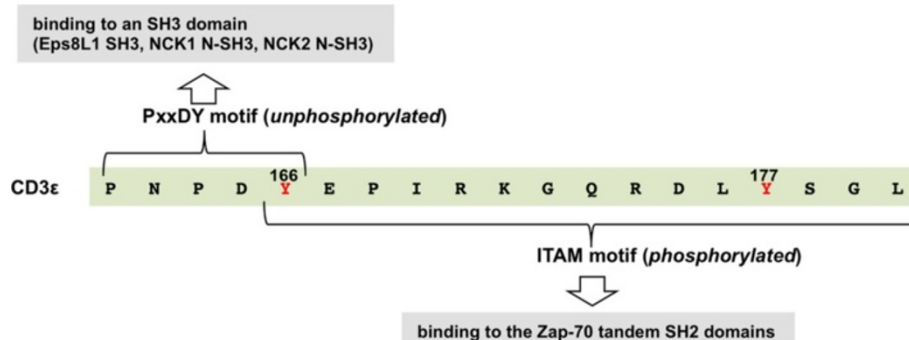


Figure 3 Binding partner switch induced by tyrosine phosphorylation. The sequence of the T-cell receptor subunit, CD3 ϵ , contains two interaction motifs overlapping at a tyrosine phosphorylation site. The two phosphorylation sites Tyr166 and 177 are coloured red. The binding partners of the motifs, depending on the phosphorylation state, is schematically depicted as highlighted boxes.

interaction between E-cadherin and the PTB and HYB domains [94-96].

The PTB domain

The phosphotyrosine-binding (PTB) or phosphotyrosine-interacting domain, first identified in the adaptor protein Shc [97-99], is the second largest family of pTyr-binding modules. Approximately 60 human proteins contain a PTB domain [85]. Although none have been identified from yeast or plants so far, two PTB domains have been found in the slime mould *Dictyostelium discoideum* [85], and 31 in the choanoflagellate *Monosiga brevicollis* [100]. The proteins harbouring a PTB domain strictly act as adaptors or molecular scaffolds [101], with possible exceptions at least in *M. brevicollis*, where multiple tyrosine kinases that contain a PTB domain have been identified [6]. Indeed, biochemical studies indicate that the PTB domain of the *M. brevicollis* tyrosine kinase HMTK1 assists in targeting of a pTyr-containing substrate peptide [102]. While a third of all PTB-containing proteins identified contain a single copy of the domain, the remaining two-thirds feature a PTB domain occurring in combination with other modular domains such as SH2, SH3, PDZ or SAM [85]. PTB domain-containing proteins are involved in a host of signaling processes, including those involving receptor tyrosine kinases, cytokines and lipoprotein receptors, and cellular functions such as cell division and cell-cell adhesion [101].

Architecture of the PTB domain

Johnson and colleagues divided the PTB domain family into three classes, namely Shc-like, IRS-like and Dab-like, based on the domain structure [85]. Although, ligand recognition by the Shc-like and IRS-like PTB domains is considered to be tyrosine phosphorylation-dependent, the majority of the remaining PTB domains, classified as the Dab-like domains, are phosphorylation-independent in ligand binding [85]. Structural analysis has revealed that the PTB domains are characterized with the pleckstrin homology (PH) domain "superfold", although they share little sequence identity with PH domains [103]. All PTB domains encompass a minimal fold containing two orthogonally arranged β -sheets composed of seven anti-parallel β -strands (Figure 4A). The β -sheets pack against two α -helices, α 2 and α 3 (nomenclature of the secondary structures follows that defined in [104]). The Shc-like and Dab-1-like PTB domains have an additional N-terminal helix (α 1 helix), whereas the IRS-1-like domains have an extremely truncated α 2 helix, which represents the minimal domain fold (Figure 4A) [105-109]. The PTB domains bind peptide ligands in an L-shaped hydrophobic groove contoured by the β 5 strand and the α 3 helix [110]. The peptide usually docks with its N-terminal residues forming an

anti-parallel β -strand and the C-terminal of the peptide arranged as a type I β -turn.

Tyrosine phosphorylation-dependent and independent ligand binding

Similar to the SH2 domain, PTB domains may bind tyrosine phosphorylation sites in cellular proteins. Proteomic studies have revealed that the two domain families may in fact target overlapping pTyr sites with micromolar affinities [57-59,113]. However, unlike SH2 domains, the specificity of a PTB domain is primarily focused towards amino acids N-terminal to the pTyr residue in a peptide, most commonly in an NPXpY or NPXY sequence motif [114]. Moreover, the majority of PTB domains prefer a non-phosphorylated tyrosine residue. In fact, phosphorylation of a peptide is inhibitory to binding to some members of the Dab-1-like PTB domain group [85]. Interestingly, a number of PTB domains can bind the head groups of inositol phosphates with varying affinities, a function observed also in some of the structurally similar PH domains [85,110]. PTB domains appear to commonly bind to phospholipids through a patch of basic residues on the surface of the domain, although the actual residues that bind to the phospholipid are variable or not resolved for most PTB domains [85,103]. Interestingly, a phosphopeptide and a phospholipid can compete against each other in binding to a PTB domain, as has been shown for the Shc PTB domain [115].

The versatile Numb PTB domain

Numb is an adaptor and endocytic protein that plays an important role in asymmetric cell division and embryogenesis [116]. It contains a Dab-1-like PTB domain indispensable for its function. Biochemical and structural studies have suggested that the Numb PTB domain is capable of binding to either non-phosphorylated sequences that contain an NXX[Y/F] motif or pTyr-containing sequences [104,111,116,117]. Structural analysis unraveled the molecular basis of promiscuous binding by the Numb PTB domain to peptides that possess distinct primary and secondary structures [104,111]. Figure 4B shows the complex structure of the Numb PTB domain in complex with a non-phosphorylated peptide that contains the sequence NMSF derived from the Numb-associated kinase (NAK) [111]. Interestingly, mutation of the sequence from NMSF to NAAF resulted in 15-fold increase in binding affinity, implying that the physiological association between the Numb PTB domain and NAK may not be optimized for high affinity [111]. Moreover, the Numb PTB domain provides a binding site for another domain in an isoform-specific manner. The p72 and p66 isoforms of Numb contain an 11-residue insert within the PTB domain after the

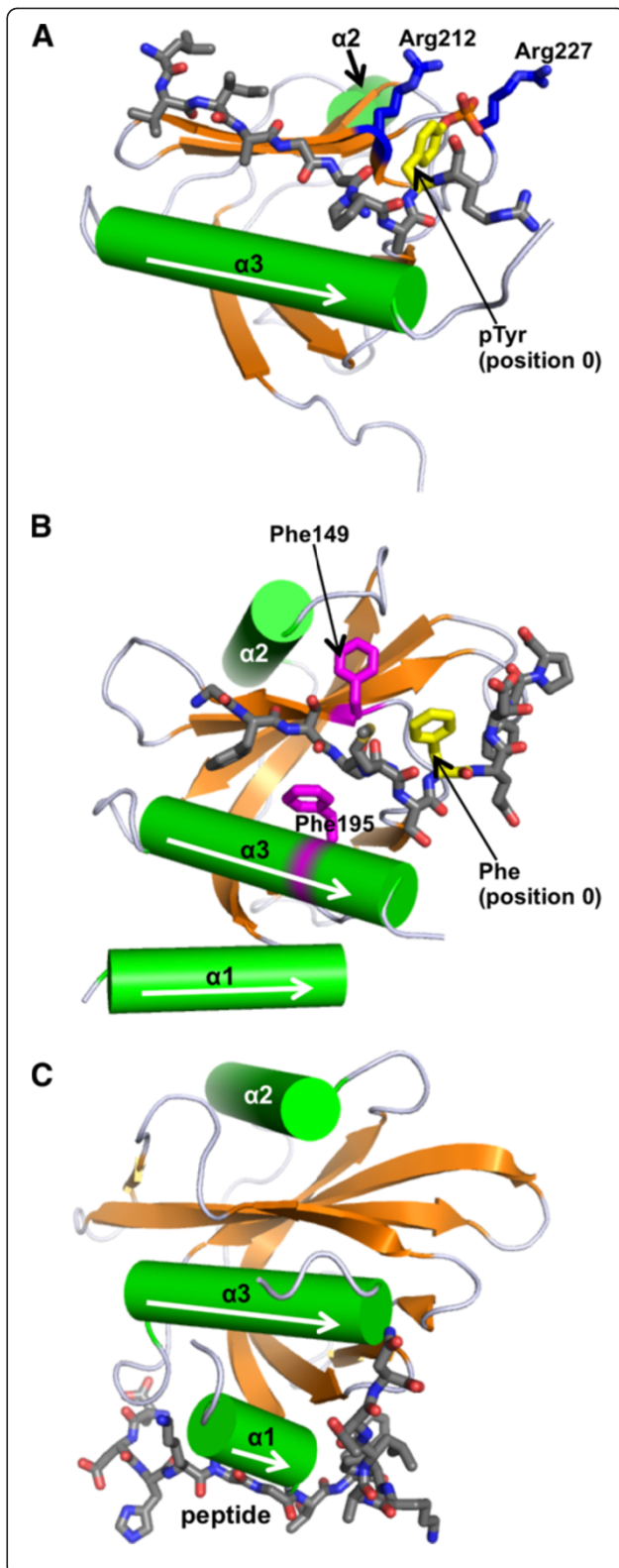


Figure 4 Diversity in ligand recognition in the PTB domain family.

PTB domains are shown in ribbon representations, with α -helices in green, and β -strands in orange. Bound peptides are drawn as gray sticks. **(A)** The PTB domain of IRS-1 bound to a pTyr-containing peptide derived from interleukin 4, containing an NPpY sequence (PDB ID: 1IRS) [105]. The two arginine residues, Arg212 and Arg227 (coloured blue), provide electrostatic contacts with pTyr at position 0 (coloured yellow). **(B)** The Numb PTB domain bound to an NAK-derived peptide (PDB ID: 1DDM) [111]. The peptide contains an NMSF sequence, but not a tyrosine. Phe149 and Phe195 (coloured magenta) of the PTB domain are essential for peptide binding. **(C)** The tensin2 PTB domain bound to a peptide derived from DLC-1 (PDB ID: 2LOZ) [112]. The peptide, which does not contain an NXX [Y/F] motif, binds to a novel site on the PTB domain that involves the $\alpha 1$ helix.

$\alpha 2$ helix, which serves as a binding site for the PDZ domain of LNX (Ligand-of-Numb), an E3 ubiquitin ligase required for isoform-specific Numb degradation [118]. Recently, we found that Numb serves as a new player in epithelial to mesenchymal transition, a critical step in cancer progression and metastasis [94,119]. The Numb PTB domain binds to the N⁷⁵¹VYYY motif located in the cytoplasmic region of E-cadherin, but phosphorylation of the motif by Src results in dissociation of the PTB domain, suggesting that Src activation negatively regulates the interaction between Numb and E-cadherin [94].

The tensin family SH2 and PTB domains

Unlike SH2 domains, which often coexist with a kinase or phosphatase domain in a protein, the human PTB domain-containing proteins invariably lack a catalytic domain, with the single exception of the presence of a PTP-like domain in the tensin family (see a later section for detail) [85,120,121]. Tensins 1–4 are focal adhesion proteins that contain an SH2 domain and a PTB domain in tandem [122]. Similar to the tensin-like lipid and tyrosine phosphatase PTEN [123], tensins 1–3 have been identified as tumour suppressors. Tensins interact with another tumour suppressor, deleted in liver cancer 1 (DLC1), and suppress focal adhesions and cell migration in various cancers [122,124]. The SH2 domain of tensin3 is itself phosphorylated by the Src kinase [125]. Lowy and colleagues suggest that tyrosine phosphorylation on the tensin3 SH2 domain provides a mechanism for controlling ligand binding and that phosphorylation of the SH2 domain by the Src kinase endows tensin3 with proto-oncogenic properties [125]. The study also indicated that binding to DLC1 was not dependent on tyrosine phosphorylation of the SH2 domain of tensin3, whereas binding to some other ligands such as the focal adhesion kinase was enhanced by the SH2 phosphorylation. Another study showed that the SH2 of tensin2 has the ability to bind non-phosphorylated DLC1 [78]. These two observations indicate that the phosphorylation state

of the ligand proteins as well as that of the SH2 domain can regulate SH2-ligand interactions in tensins [78,125]. Moreover, the PTB domain of tensin2 displays a novel peptide binding mode. Although it was observed that the tensin1 PTB domain can bind an NPXY peptide in a canonical manner [126], as well as a DLC1 peptide via a yet uncharacterized mode [75], the tensin2 PTB domain, which can also bind the NPXY motif [127], has been determined to utilize its N-terminal helix (α 1) to engage a non-NPXY site in DLC1 [112]. The binding surface in the latter PTB domain is opposite to the canonical NPXY peptide binding site and the peptide ligand adopts an elongated conformation rather than the conventional β -turn structure (Figure 4C) [112]. These observations indicate the presence of peptide binding surface specializations among the tensin family PTB domains.

Atypical pTyr recognition domains and proteins

The HYB domain

Hakai, a protein that binds to E-cadherin in a tyrosine phosphorylation-dependent manner, serves as an E3 ubiquitin ligase for the latter and induces endocytosis and degradation of E-cadherin [95]. Hakai is also known to interact with an RNA-binding protein, the PTB-associated splicing factor, that targets mRNAs encoding cancer-related proteins [128]. Hakai resembles the E3 ligase c-Cbl in that they both harbour a pTyr binding domain (an SH2 domain in the case of c-Cbl), a region homologous to the RING finger motif and a proline-rich region [95]. The pTyr binding region in Hakai was initially thought to be an SH2 domain, but this assumption was proven to be incorrect by recent studies showing that a unique, zinc co-ordinated domain formed by the dimerization of two Hakai monomers is responsible for pTyr recognition [95,96]. This novel pTyr recognition domain, now named the HYB domain, is formed via the dimerization of a 100-residue stretch, including the RING finger motif, in an anti-parallel orientation (Figure 5A). The dimer coordinates six zinc ions with a total of 24 Cys or His residues (four residues per zinc ion; Figure 5A). Crystallographic and NMR titration experiments revealed that the positively charged pTyr-binding pocket, which is responsible for binding phosphorylated E-cadherin, is situated in the interface of the dimer. In particular, phosphorylation of the N⁷⁵¹VYYY motif on the Tyr residues and acidic residues flanking the motif are necessary for binding to the HYB domain, with a K_D value of 7.2 μ M for the N⁷⁵¹VYPYY containing E-cadherin peptide [96]. Because this motif also mediates the binding to the Numb PTB domain when it is not phosphorylated [94], it provides yet another example of the tyrosine phosphorylation-dependent binding partner switch. Interestingly, the HYB domain has also been identified in the testis-specific ubiquitin ligase

ZNF645, but its target specificity is apparently distinct from that of the Hakai HYB domain [96]. Moreover, sequence inspection suggests that the Numb-associated E3 ligase LNX may contain an HYB domain [96]. Thus, the HYB domain appears to be a recurring feature in certain E3 ligases and its presence in cell adhesion regulatory proteins such as Hakai suggests a role for this domain in cell-cell adhesion and cancer metastasis [129]. The HYB domain is likely conserved through evolution as a sequence search identifies HYB-like domain sequences in different animals and plants with complete conservation of the zinc-coordinating residues (Figure 5B).

The GEP100 PH domain

The PH domain was first described in pleckstrin, a substrate of protein kinase C (PKC) [131,132]. It has since been identified in a large number of signaling and cytoskeleton-associated proteins. By virtue of its ability to bind phospholipids, in particular inositol phosphates, the PH domain plays an important role in targeting the corresponding protein to the plasma membrane [103,133]. Interestingly, some PH domains are found to bind to proteins in a phosphorylation-dependent manner [110,133-137]. The PH domain of GEP100 (also known as BRAG2 or IQSEC1), a guanine nucleotide exchange factor for the small GTP-binding protein Arf6, binds to the EGF receptor residues pTyr1068 and pTyr1086, both of which are part of a YXNQ motif [138]. These two pTyr sites are also known to recruit the adaptor proteins Grb2 and Shc as well as the transcription factor STAT3 via their SH2 and/or PTB domains [139,140]. However, it has been shown that the GEP100 pathway may not necessarily interfere with the Grb2 pathway in breast cancer [141]. Co-expression of GEP100 and Arf6 turned non-invasive MCF7 cells to become invasive upon EGF stimulation [138,142]. Therefore, this pathway is a potential new target for breast cancer therapeutics. The crystal structure of the GEP100 PH domain alone has been solved (PDB database entry 3QWM), but a PH domain – pTyr-peptide complex structure is still missing, since synthetic pTyr1068 and 1086 peptides immobilised on a carrier membrane can directly bind to the GEP100 PH domain, but have a surprisingly low binding affinity to it in solution (S.F. et al., unpublished data). The reason for this currently remains unclear. By contrast, the native EGFR receptor is effectively precipitated by the GEP100 PH domain upon activation, i.e., when phosphorylated on pTyr1068 and/or pTyr1086.

The PKC δ and PKC θ C2 domains

The C2 domain belongs to one of the largest domain families with over 200 members in human [103,143]. This ~130 residue module primarily binds to phosphatidylserine in the cell membrane in a calcium-dependent

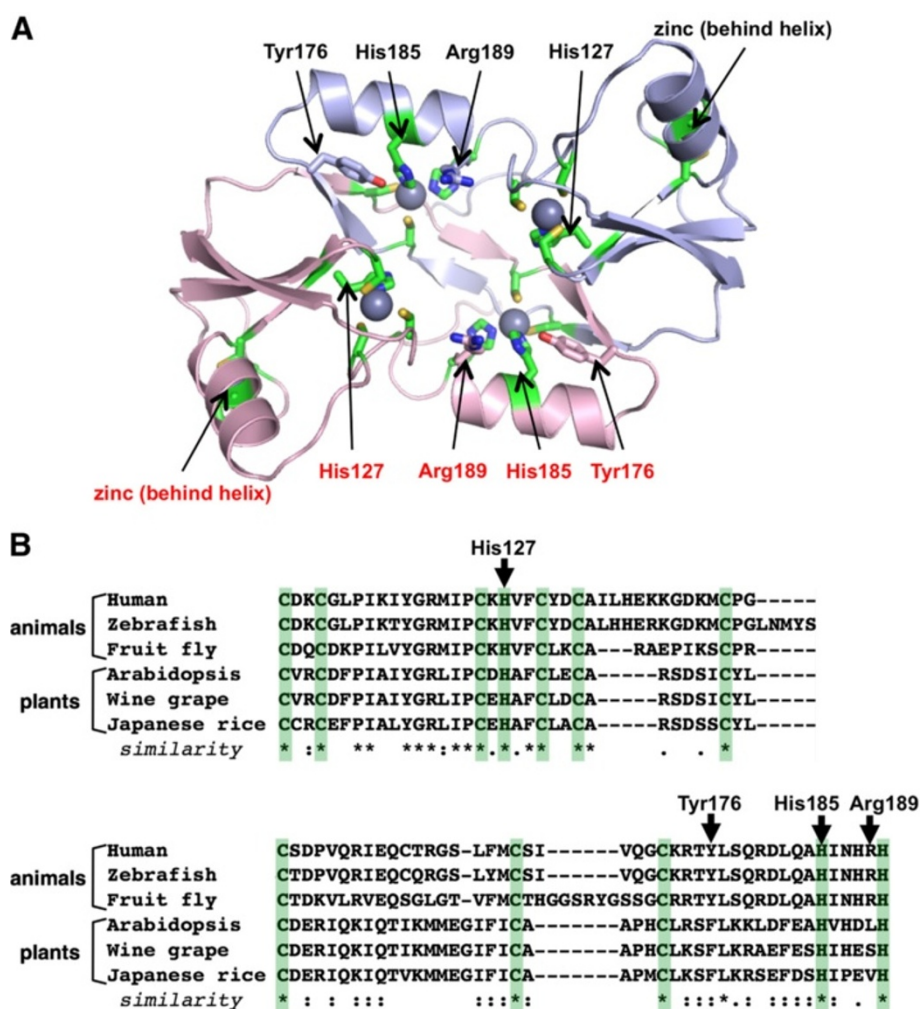
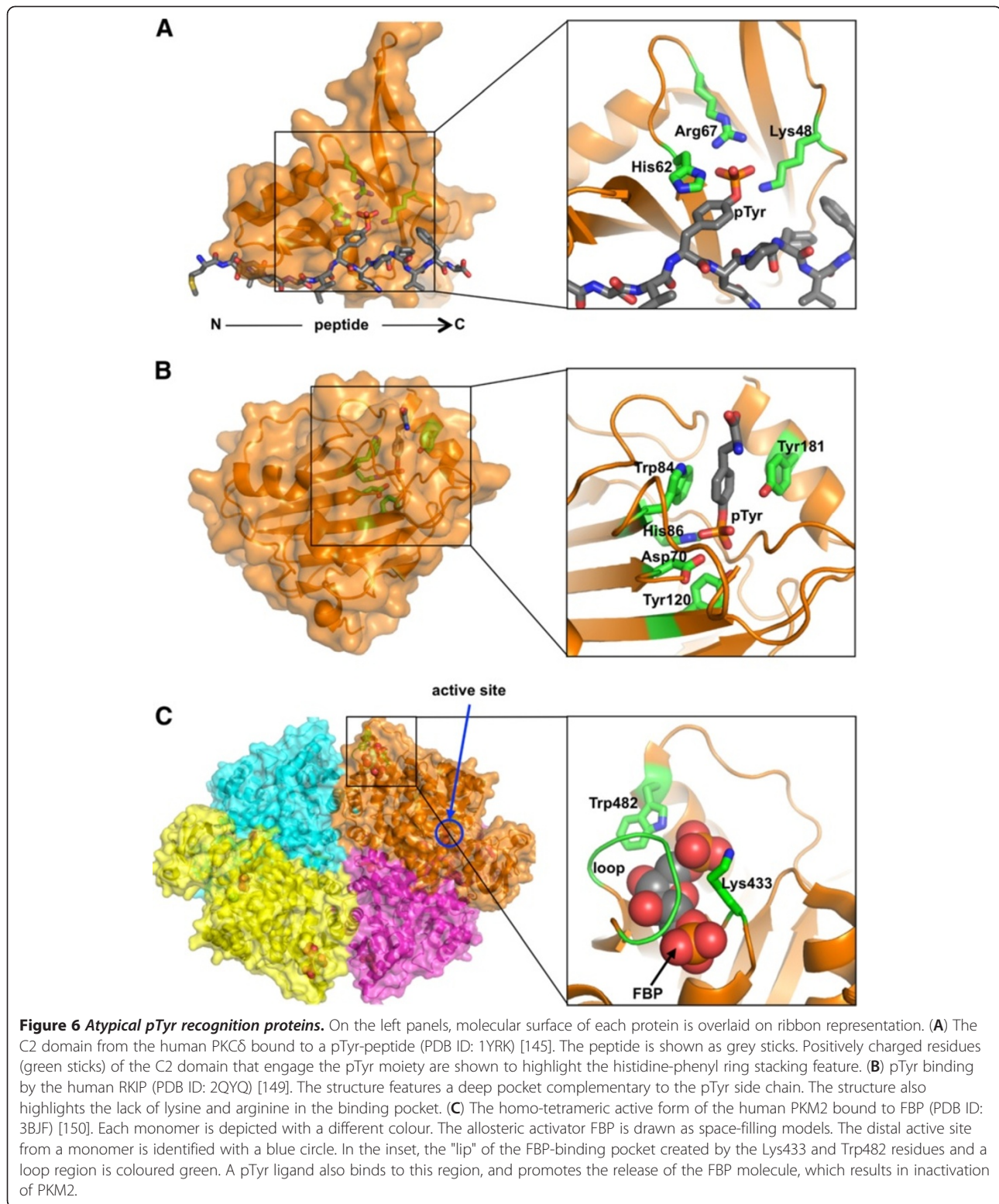


Figure 5 The structure and sequence conservation of the HYB domain. (A) The HYB domain is coordinated with six zinc ions. Shown is the homo-dimeric structure of the HAKAI HYB domain (showing the two chains with different colours) (PDB ID: 3VK6) [96]. Zinc ions are depicted as spheres. The 24 residues (12 per monomer) coordinating the zinc ions are shown as green sticks. The four residues, His127, Tyr176, His185, and Arg189 from each monomer, identified as sticks, mainly contribute to pTyr binding by providing a positively charged pocket. (B) HYB domain-like sequences identified in animals and plants. The alignment was generated by the program MAFFT [130]. The 12 zinc-binding residues are shaded green, showing that all of them are strictly conserved within these species. The human HAKAI HYB domain was aligned with following sequences with UniProt IDs: zebrafish (Q5RGV5), fruit fly (Q9VIT1), *Arabidopsis thaliana* (Q9LFC0), wine grape (F6HX7), and Japanese rice (Q0IWO6).

manner. The C2 domain has a core structure of a β -sandwich formed by eight antiparallel strands (Figure 6A). The calcium binding sites are located in the inter-strand loops [103]. It has been shown that C2 domains have disparate calcium dependency for activity and a weak affinity for most phospholipids, suggesting that they may have other binding functions [103,144]. Benes et al. demonstrated that the C2 domain of the Ser/Thr kinase PKC δ can recognize a pTyr peptide derived from CDCP1 (CUB domain-containing protein 1) in a sequence-specific manner, with a K_D of 240 nM [145,146]. CDCP1, a transmembrane protein overexpressed in a number of cancers, is a substrate of the Src family kinases [147]. The C2 domain-containing PKC δ is

the first example of a Ser/Thr kinase displaying a pTyr binding capability. Subsequently, the C2 domain of PKC θ , which shares 70% sequence identity with that of PKC δ [145], was also found to bind to the tyrosine-phosphorylated CDCP1 peptide with a similar affinity [148]. Importantly, this binding is the key for activation of PKC θ from an autoinhibitory state, which is mediated by an intramolecular interaction involving the C2 domain. The crystal structure of the PKC δ C2 domain in complex with an optimal pTyr peptide demonstrates that the pTyr-peptide binds in an elongated conformation across two β -sheets (Figure 6A). The C2 domain coordinates the phosphate group of pTyr in a deep pocket using positively charged lysine and arginine



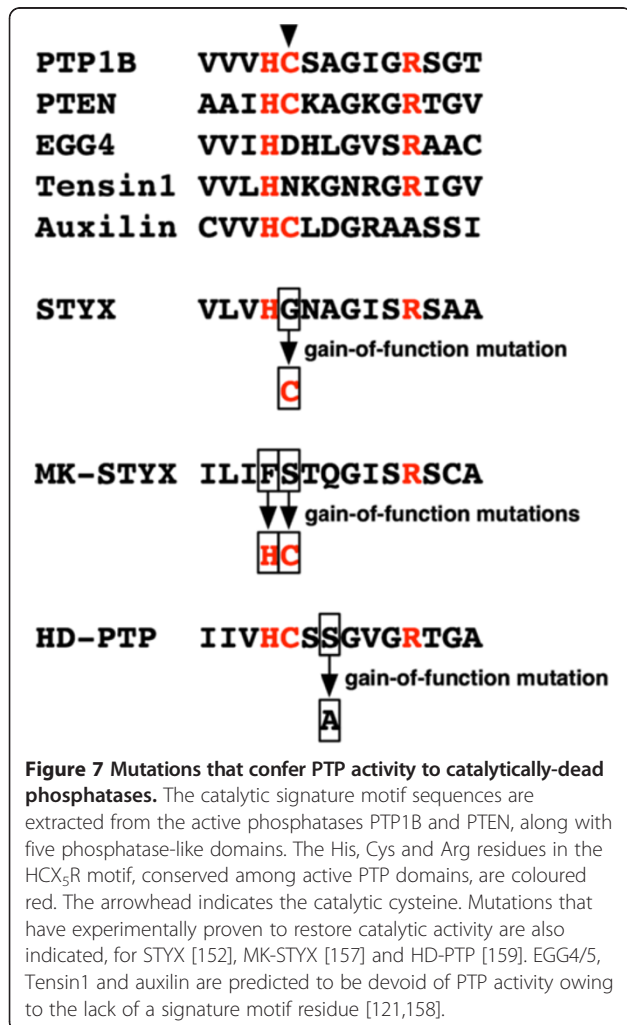
residues (Figure 6A). Moreover, the phenyl ring of pTyr is stabilized by a unique ring-stacking interaction with a histidine residue that is proximal to the phosphate-binding arginine residue (Figure 6A) [145].

The catalytically inactive PTP domain

The protein tyrosine phosphatase (PTP) family proteins dephosphorylate a pTyr residue of substrate proteins ("erasers" in the toolkit). There are 107 PTPs in human,

of which 61 phosphatases are classified as the dual-specificity phosphatase subfamily, which also possess Ser/Thr phosphatase activity in addition to pTyr dephosphorylation [151]. The PTP family phosphatases share a conserved domain fold and possess a catalytic cysteine in the HCX₅R motif located on the active site loop [152-154]. However, sequence analysis led to the identification of PTP domain homologs that lack the catalytic cysteine or another essential residue in the motif (Figure 7) [153,154]. Such findings were first described for the protein named STYX (phospho-serine or threonine or tyrosine interaction protein), which is similar in sequence to PTPs, except for a Cys to Gly substitution in the signature motif (Figure 7) [152,155]. This renders the STYX protein unable to catalyze dephosphorylation. However, the STYX Gly-to-Cys mutant conferred catalytic activity to the protein and the mutant demonstrated phosphatase activity for both the pTyr and pThr residues [152]. Since then, many more PTP-like domains have been identified [153,154,156-159]. For example, EGG4

and EGG5, two almost identical proteins in *Caenorhabditis elegans*, contain a PTP-like domain without a catalytic Cys residue (Figure 7). Cheng, et al. reported that the domain binds to tyrosine residues in the activation loop of a kinase that regulates the oocyte-to-embryo transition [158]. This interaction is enhanced, although not absolutely necessary, by phosphorylation of the tyrosines. Haynie and Ponting have proposed that the N-terminal regions of two proteins, tensin1 and auxilin, are PTP-like domains, in which the former lacks the catalytic cysteine, whereas the latter lacks the arginine of the HCX₅R motif (Figure 7) [121]. Interestingly, the PTP-like domain is immediately followed by a C2 domain in both proteins, and the PTP-C2 unit is homologous to the tumour suppressor PTEN [160,161]. The PTP domain of PTEN possesses an intact signature motif, and it displays phosphatase activity for both phospholipids and phosphotyrosine [162]. It is therefore possible that the PTP-like domain of tensin1 and auxilin may be a phosphotyrosine or phospholipid binding domain. Many PTP-like domains are awaiting functional characterization.



Raf-1 kinase inhibitory protein

Raf-1 kinase inhibitory protein (RKIP), also known as phosphatidylethanolamine binding protein 1, suppresses Raf-1 kinase activity in the MAP kinase pathway [163]. RKIP binds to a 24 amino acid stretch in the N-terminal of the Raf-1 kinase which has a central element (S³³⁸SY³⁴¹) regulated by phosphorylation [164]. Substitutions in the ligand binding site of RKIP compromise the stability of the phosphorylated Raf-RKIP complex. Additionally, mutation of the Ser residues or Tyr341 on the Raf-1 kinase motif disrupts its interaction with RKIP. This suggests that RKIP contains a novel phospho-amino acid binding domain [165]. The structure of RKIP bound to pTyr shows that RKIP indeed contains a deep pocket molded complementary to the shape of the pTyr side chain (Figure 6B) [149]. NMR titration studies further confirmed that the pocket region is the binding site for the tri-phosphorylated Raf-1 peptide, pS³³⁸pSYpY, with a K_D value of 45 μM [166]. However, direct, physiological or structural, evidence for complex formation is required before RKIP can be classified as a *bona fide* pTyr binder.

Searching for novel pTyr recognition proteins

In the above sections, we have explored that, in addition to the SH2 and PTB domains, there are more than a handful of examples of atypical domains that can recognize and bind pTyr-containing target proteins. So does nature have more domain members capable of pTyr-epitope binding that remain to be identified? Recent advances in proteomic technologies are starting to shed light on this question. Christofk et al. employed the

SILAC (stable isotope labeling of amino acids in cell culture) technique in combination with a pTyr-peptide library to identify novel pTyr-binding proteins from mammalian cell lysates [150]. Whereas the majority of proteins identified were SH2 or PTB domain-containing proteins, as expected, the screening also identified pyruvate kinase, a protein that does not have any known pTyr-recognition domain. Pyruvate kinase (PK) regulates the final rate-limiting step in glycolysis that converts cellular metabolism from an anaerobic to an aerobic process. The M2 isoform of the PK (PKM2) is the only form of PK used for glycolysis in cancer cells, and it is also the only one of the four isoforms that has the ability to bind to pTyr targets [167,168]. The structure of hPKM2 illustrates a homo-tetramer with each monomer binding a fructose-1,6-bisphosphate (FBP) molecule in a site distal to the active site (Figure 6C) [150,169]. Phosphotyrosine ligand binding may be a key event in modulating PK activity regulated by the allosteric activator FBP [150]. The study demonstrated that PKM2 binds pTyr peptides on the lip of the FBP binding pocket and acts as a negative regulator of PK activity (Figure 6C) [150]. Binding of the phosphopeptide releases FBP from the active tetrameric form of PKM2 and dissociates the enzyme into inactive dimers [168].

More recently, Christofk et al. identified a handful of potential pTyr-binding proteins from SILAC experiments. These include the serine/threonine-protein kinase WNK1, 5-formyltetrahydrofolate cyclo-ligase, glycerol-3-phosphate dehydrogenase, vimentin, 2,4-dienoyl-CoA reductase, and the T-complex protein 1 subunit η [170]. Although biochemical characterization is required to ascertain if these candidates are true pTyr-binders, results from this study suggest that there may be more pTyr recognition domains or proteins in nature that await to be discovered.

Phosphotyrosine recognition domains and therapeutic applications

New insights into the molecular basis of cancer were generated already decades ago by the analysis of SH2-domain containing oncoproteins such as v-Src [27,171]. SH2-domain containing proteins have been implicated in many diseases, including immune-related disorders, metabolic syndromes, osteopathologic conditions and different cancers [10,172]. Mutations in the SH2 domains that cause malignancies fall usually into one of three categories: missense mutations of amino acids involved in target binding, mutations in residues that regulate catalytic activity or mutations on the SH2 domain, distal to the module core, that affect the architectural integrity of the SH2 domain [16]. A number of disease-causing mutations on SH2 domains have been reported as compiled by Liu et al. [10] and Lappalainen,

et al. [173], including mutations related to the Noonan syndrome, juvenile myelomonocytic leukemia and the X-linked lymphoproliferative syndrome. X-chromosome-linked agammaglobulinemia (XLA) is an example of a disease where mutations are present in the SH2 domain of the Bruton's tyrosine kinase (BTK), and many of these mutations are located on the pTyr-ligand binding site, including the indispensable arginine of the pTyr-binding pocket as well as BG loop residues [16,173]. Moreover, Hong et al. demonstrated that the BTK SH2 domain binds to phospholipids and the XLA-causing mutations alter lipid binding selectivity [174]. In another example, multiple point mutations within the transcription factor STAT3 SH2 domain have been identified and linked to large granular lymphocytic leukemia and the hyper-IgE syndrome [175,176]. In conjunction with the identification and study of SH2 domain-related diseases, small molecule inhibitors of SH2 domains, SH2 domain-containing proteins or SH2 binding partners, are being developed with some success as therapeutic reagents, although the development of phosphomimetics faces hurdles due to the strong charge of the phosphate group [177,178]. Besides SH2 domains, mutations in the PTB domain have also been linked to diseases such as coronary heart disease and type II diabetes [85].

Biochemical and structural studies have demonstrated that direct intramolecular interactions between the SH2 and kinase domains are required for kinase activation in some PTKs [15,16,36]. In the Fes kinase, electrostatic interactions and shape complementarity between residues from the SH2 domain and the α C helix of the kinase domain stabilize the active state of the kinase. Similarly, in the Abl kinase, interaction between Ile164 of the SH2 domain and Thr291/Tyr331 in the kinase domain N lobe is essential for activity [179]. Subsequently, this interface has been investigated intensively as a target for cancer intervention. A successful approach was the creation of a protein-based agent called the monobody, derived from the fibronectin type III (FN3) domain with engineered loops designed for high affinity binding to a specific target molecule [180]. Koide and colleagues created a monobody engineered to selectively bind the SH2 domain of the Abl kinase [181]. They further generated another monobody that targets the SH2-kinase interface that involves Ile164 and then connected the two monobodies with a linker. The resulting tandem monobody disrupted the interface between the SH2 and kinase domains to inhibit the catalytic activity of the deregulated fusion kinase Bcr-Abl, both *in vitro* and *in vivo* [179].

Due to its independently folding nature, a modular domain can often be successfully used as a tool in proteomic research. Jadwin et al. termed this "domainomics" [182]. In particular, applications of phosphotyrosine-

recognition domains have been reported for therapeutic and diagnostic purposes. SH2 and PTB domain-based probes have been developed and used to profile global pTyr landscapes using innovative assays such as the peptide dot blotting, Far Western blotting and oligonucleotide tagged multiplex (OTM) assays [183-186]. The OTM assay is designed for quantitative multiplexed profiling of tyrosine-phosphorylated proteins from cell extracts using DNA-tagged SH2 domains, and has been successfully harnessed to discriminate tyrosine phosphorylation states in tumour cell lines and leukemia samples from patients [184]. Similarly, Machida et al. reported application of domain-based probe technology to profile lung cancer cell lines, and demonstrated significant correlation between EGFR mutations and the Grb2 SH2 and ShcA PTB domain probe signals, suggesting a diagnostic value [187]. A potential for application of SH2 domains as non-invasive intracellular imaging or as inhibitor reagents has also been demonstrated. An SH2 domain from Grb2 or PLC γ 1 fused with a protein transduction tag (the TAT tag) [188] was used for SH2 domain delivery into cells. These TAT-tagged SH2 domains showed anti-tumour effects [189,190]. In addition, the TAT-tagged Grb2 SH2 domain can be used as a molecular probe for monitoring EGFR localization in cells [191].

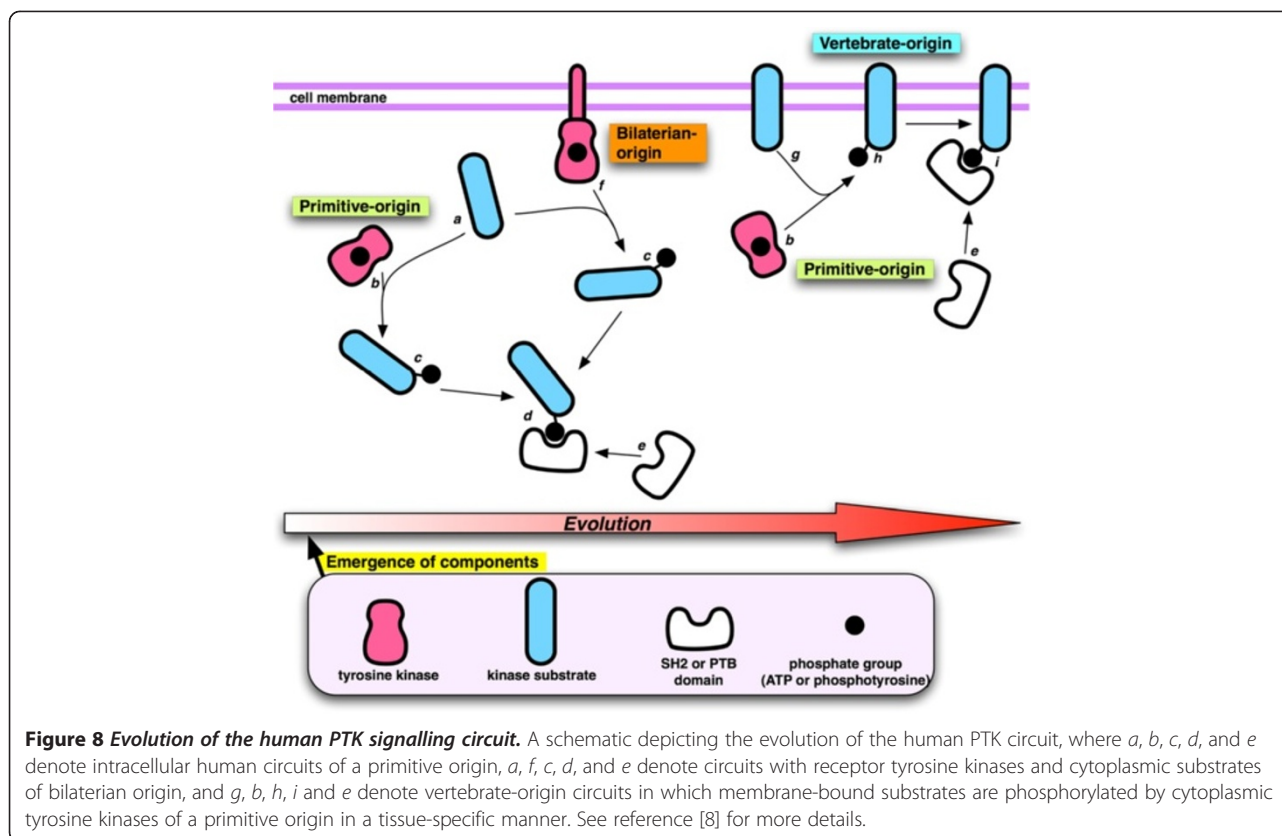
SH2 domains have also been incorporated as a biosensor molecule that functions in live cells. Wang et al. created a bipartite Src reporter biosensor protein that contains an SH2 domain and a Src kinase substrate fused, respectively, with the cyan and green fluorescent proteins [192]. Upon phosphorylation of the substrate by endogenous Src kinase, the SH2 domain of the biosensor binds to the substrate in *cis*, and produces a change in the emission spectrum. Using the Src reporter, the authors successfully visualized kinase activity in cells. In another example, an engineered adenocarcinoma-derived cell line that expresses an EGFR biosensor has been developed [193]. This cell line expresses the Grb2 SH2 domain fused to the green fluorescent protein, and can be used to monitor EGFR internalization upon EGF stimulation. The cell line may provide a useful tool for high-throughput drug screening since effects of drug candidates on modulating EGFR activity can be monitored in live cells.

Lastly, understanding the evolution of phosphotyrosine recognition domains may inform cancer research and treatment. In general, the degree of network complexity in the phosphotyrosine signaling system has increased during the course of evolution, and genetic events, such as gene duplications to create paralog proteins and gene fusions to create multi-domain proteins, played a major role in expanding the network scale [4,11]. Robustness of the signaling network is partly conferred

by network redundancy and a collection of feedback loops or cross-talks [194]. The network evolution creates highly connected conserved nodes, called network hubs, and cancer-causing mutations tend to be observed for the network hub proteins [194]. Besides, Kitano argues that cancer cells hijack the cellular signaling mechanisms for their network robustness [195]. We have recently analyzed the evolutionary origins of human pTyr signaling circuits in 19 eukaryotic species by identifying ortholog proteins of the human circuit components in each species (Figure 8) [8]. The 19 species were classified into three groups (primitive organisms, bilaterians, and vertebrates) based on their evolutionary distances from humans. In this study, a pTyr signalling circuit is defined to comprise a tyrosine kinase, a substrate of the tyrosine kinase, and an SH2 or PTB domain that binds to the tyrosine-phosphorylated site. Statistical analysis showed that the circuits for intracellular (cytoplasmic) signalling (Figure 8: *a*, *b*, *c*, *d*, and *e*) largely originate from primitive species. Circuits that involve receptor tyrosine kinases which phosphorylate cytoplasmic substrates (Figure 8: *a*, *f*, *c*, *d*, and *e*) mainly originate from bilaterians. Conversely, vertebrate-origin circuits are enriched with membrane protein substrates that are phosphorylated by primitive-origin cytoplasmic kinases (Figure 8; *g*, *b*, *h*, *i* and *e*) in a tissue-specific manner. This study underlined the importance of network hubs as hotspots for tumorigenesis, as high frequency cancer pTyr sites are involved in more circuits than low frequency sites, and kinase substrate proteins that contain a PTK, SH2 or PTB domain are more frequently recruited for cancer signalling [8].

Future perspectives

The genome sequence analysis of *M. brevicollis*, a unicellular choanoflagellate that possesses a PTK signalling system [70], brought a big surprise since the numbers of PTKs, PTPs and SH2 domains are comparable to or greater than those in mammals [6,100]. Numerous PTK, PTP, SH2 and PTB domain-containing proteins have been also identified in other pre-metazoan species [196,197], challenging the simple notion of "expansion from yeast to man" in terms of the evolution of phosphotyrosine signalling. Since *M. brevicollis* contains a plethora of putative PTK toolkit proteins, many of which have no orthologs in humans [6,100,196], it suggests that multiple evolutionary roads, which remain to be elucidated, may have been used to mix and match different pTyr signalling components in order to build the PTK signalling pathways and networks unique to a particular species. For example, bacteria may possess their own phosphotyrosine signalling systems as bacterial tyrosine kinases are found to be unrelated to eukaryotic PTKs [198,199]. Nevertheless, bacteria do not only rely on



their own repertoire of pTyr signalling components. Some pathogenic bacteria, for example the enteropathogenic *Escherichia coli* (EPEC) and *Helicobacter pylori*, are known to hijack pTyr signalling system components of their host by injecting a virulence factor into the host cell during infection [200,201]. The injected bacterial protein is then phosphorylated by a host PTK, and subsequently recruits SH2 domains of host proteins to rewire the host signalling network for the bacteria's benefit. The injected bacterial effectors have evolved to be readily phosphorylated by host PTKs, and to recruit a number of host SH2 domains after being phosphorylated [201,202]. For instance, EPEC injects a transmembrane effector protein Tir (translocated intimin receptor) to host cells upon infection. The cytoplasmic YDXV motif of Tir is then phosphorylated and recruits the SH2 domain of the adaptor protein NCK, which results in pathological actin reorganization [200,203]. It is noteworthy that a similar scenario is seen in the vaccinia virus. A tyrosine residue in the viral membrane protein named A36R is phosphorylated by host PTKs, and the viral protein subsequently recruits the NCK and Grb2 SH2 domains to manipulate downstream actin regulation of the host cell [204,205]. Intervention of pTyr-mediated host-pathogen interactions could be a novel therapeutic strategy, if sufficiently selective agents can be developed.

Conclusions

Much of what has been learnt in past decades about pTyr reader domains, their interactions and the networks that they serve can now be utilised and also warrants further exploration into several directions. Firstly, we need to make an effort to put more 'puzzle pieces' together to start seeing a more complete picture. This includes analysing in much more detail how the compartmentalisation of pTyr writers and readers, i.e. the architecture of the pTyr signalling machinery, generates highly specific signals *in vivo*. Protein modification reader domains should also be useful in a range of clinical settings, for example for molecular diagnoses and monitoring, but this potential remains untapped in clinical routine settings. As demonstrated for the SH2 domains, e.g., specificity-switching mutants or affinity-enhanced "pTyr superbinders" [40,61,63], engineering of pTyr-binding domains may yield protein-based reagents with desired specificities and affinities towards tyrosine phosphorylated targets, which may provide an array of novel agents for research and clinical purposes. Furthermore, we are just beginning to realise that at least some protein modification reader domains have the capacity to decipher combinations of modifications rather than recognising just single site changes, and further research in this area promises to provide additional clues

to understanding the elegant ways in which cells manage to read and process a huge number of incoming signals and to translate them into a wide spectrum of biochemical and biological responses. Hence, typical and atypical pTyr reader domains should be considered to be 'old dogs up to new tricks' which we are just starting to grasp.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RJ and TK drafted the first version of the manuscript. All authors edited and approved the final version.

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