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Docking interactions in protein kinase and phosphatase networks

Attila Reményi, Matthew C Good and Wendell A Lim

To achieve high biological specificity, protein kinases and phosphatases often recognize their targets through interactions that occur outside of the active site. Although the role of modular protein–protein interaction domains in kinase and phosphatase signaling has been well characterized, it is becoming clear that many kinases and phosphatases utilize docking interactions — recognition of a short peptide motif in target partners by a groove on the catalytic domain that is separate from the active site. Docking is particularly prevalent in serine/threonine kinases and phosphatases, and is a versatile organizational tool for building complex signaling networks; it confers a high degree of specificity and, in some cases, allosteric regulation.

Addresses
Department of Cellular and Molecular Pharmacology, Program in Biological Sciences, University of California San Francisco, 600 16th Street, San Francisco, CA 94143-2240, USA

Corresponding author: Lim, Wendell A (lim@cmp.ucsf.edu)

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Introduction

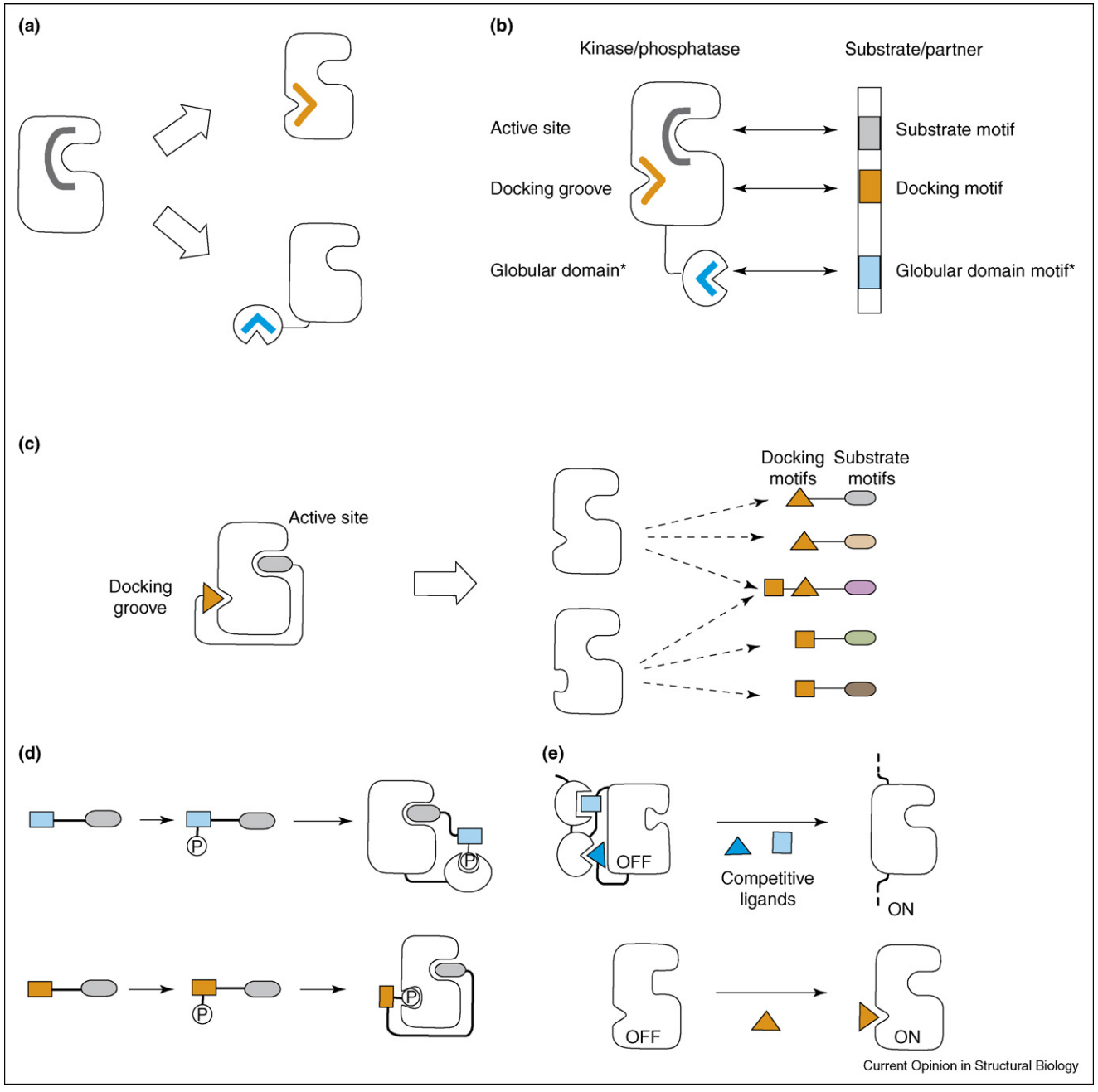
Biochemists dating back to Emil Fischer have traditionally assumed that the substrate specificity of an enzyme was determined primarily by stereochemical complementarity with its active site. Although protein kinase and phosphatase active sites do possess preferred target phosphorylation or dephosphorylation sequences, these preferences are not stringent enough; often they alone cannot explain *in vivo* specificity. In addition to substrate target site preferences, many protein kinases and phosphatases use dedicated protein–protein interaction surfaces (Figure 1a). One common strategy is to use modular protein–protein recognition domains, such as SH2 and SH3 domains — numerous kinases contain such domains fused to their core catalytic domain. However, a second strategy is to utilize docking interactions — interactions involving binding surfaces of the catalytic domain but distinct from the catalytic active site. These docking grooves bind to short peptide docking motifs that are separate from the substrate motif that is chemically modified by the enzyme. These two supplemental recognition strategies are not mutually exclusive (Figure 1b). The development of these alternative modes of recognition provides a simple way to meet the ever-increasing evolutionary requirement for specificity in protein–protein recognition within complex networks. Related kinases and phosphatases, for example, can develop slightly different docking grooves without compromising the strict stereochemical requirements for efficient catalysis performed at the active site (Figure 1c) [1].

In this review, we will focus on outlining our current understanding of docking interactions in both protein kinases and phosphatases. Although docking was originally discovered as a mechanism to increase enzyme–substrate specificity, docking interactions can also govern the binding of kinases and phosphatases to each other and other effectors. There are now numerous well-characterized examples of both kinases and phosphatases that utilize docking interactions. The majority are serine/threonine phosphatases, such as PP1 and calcineurin, and serine/threonine protein kinases, including CDK–cyclins, MAP kinases, PDK1 and GSK3. However, at least one protein tyrosine kinase (Csk) has recently been shown to also utilize docking.

Two solutions for increased network specificity: modular recognition domains and docking

The separation of partner recognition and partner modification in signaling circuits based on reversible phosphorylation is an evolutionarily advantageous process. It enables a high degree of transferability of protein recognition independent of catalytic function [1]. This separation appears to have taken two independent routes during the evolution of complex signaling pathways. On one hand, dedicated interaction domains separate from the catalytic domain are often used to establish links with other signaling protein partners. Examples of this include the non-receptor protein tyrosine kinases and phosphatases that contain, for example, SH3 and SH2 domains, such as the Src, Abl, Hck and Csk protein kinases and the SHP protein phosphatases [2]. On the other hand, kinase and phosphatase domains can also acquire surface grooves — referred to as docking sites — that are capable of establishing specific connections via small peptide motifs residing in interaction partners. Although domain-mediated and docking interactions are distinct, they serve a similar functional purpose in targeting catalytic domains to particular substrates, partners and cellular locations.

Figure 1



Comparison of interactions mediated by modular domains and docking grooves for protein kinases and phosphatases. **(a)** Separation of catalytic function from protein–protein recognition. A kinase or phosphatase catalytic domain, in addition to the substrate preferences of the active site, can acquire additional protein–protein recognition modes through a dedicated surface called the docking groove (orange) or through a dedicated modular protein–protein interaction domain (blue). **(b)** Substrates or other protein partners in turn can acquire simple peptide motifs that will interact with the docking groove or the globular protein recognition domain. (*It is also possible that substrates or kinase/phosphatase partners acquire the globular domain and the kinase/phosphatase contains the globular domain motif.) This panel demonstrates three protein recognition interactions that have been identified for protein kinases and phosphatases. **(c)** Docking motifs can direct the specificity of enzymes. Protein kinase and phosphatase active sites generally have limited substrate recognition capabilities. Although a relatively large array of substrates may fit the stereochemical requirements for catalysis, those with appropriate docking motifs will be selected through interactions mediated by the docking groove. **(d)** Like certain modular-domain-mediated interactions, certain docking-motif-mediated interactions can be phosphorylation dependent. **(e)** Docking interactions can also participate in allosteric regulation of kinase or phosphatase domain catalytic activity by directly modulating the catalytic domain structure. Modular domains often allosterically regulate their catalytic domains via autoinhibitory interactions.

They can govern protein association in a phosphorylation-dependent manner, and can influence kinase and phosphatase activity through allosteric mechanisms as well (Figure 1d,e).

Interestingly, enzymes that regulate serine/threonine phosphorylation and those that regulate tyrosine phosphorylation appear to have chosen different strategies for building supplemental specificity interactions (Figure 2). Analysis of the human genome reveals that the majority of serine/threonine kinases and phosphatases do not contain any recognizable modular recognition or targeting domains (including protein–protein, protein–lipid and transmembrane motifs). Conversely, most examples of docking interactions have been identified in serine/threonine kinases and phosphatases [3]. In contrast, the majority of tyrosine kinases and phosphatases contain one or more recognizable modular targeting elements outside of the catalytic domain. Overall, these observations are consistent with a model in which serine/threonine phosphorylation, which is thought to have evolved earlier as a signaling system, has primarily utilized a docking interaction strategy for achieving higher levels of partner discrimination, whereas the later-evolving tyrosine phosphorylation systems began to utilize the strategy of recombination with multiple alternative modular interaction domains. One possible exception to this rule is the tyrosine kinase Csk. New work suggests that the interaction of Csk with its substrates Src or Yes is mediated through a docking groove, indicating that modular domain and docking peptide interactions might co-function in some tyrosine kinases [4,5].

Identifying linear recognition elements

Most domain-mediated and docking interactions recognize short linear recognition elements rather than folded globular partners. These linear motifs are short, between three and ten amino acids, of which usually just a few residues are important for function. Linear-motif-mediated protein association tends to be more transient in nature and therefore is ideal for signaling networks. Whereas globular domains mainly arise by gene duplication, linear motifs, because of their short length, can arise convergently [6].

The small size of linear motifs makes bioinformatic identification and analysis challenging. The identification of globular domains (SH3, SH2, PTB, WW, PDZ, etc.) in signaling proteins is now straightforward using resources such as SMART and Pfam [7,8]. Efforts are also underway to catalogue linear motifs. The short length of these motifs makes them difficult to detect through sequence comparison; however, a set of proteins that all interact with a certain protein will normally share common features (Figure 3). The DILIMOT and SLiMDisc programs, for example, can find statistically over-represented motifs in non-homologous sequences that bind to a certain kinase or phosphatase [9,10]. This approach successfully rediscovered known motifs and predicted several others using genome-scale interaction data sets [11••].

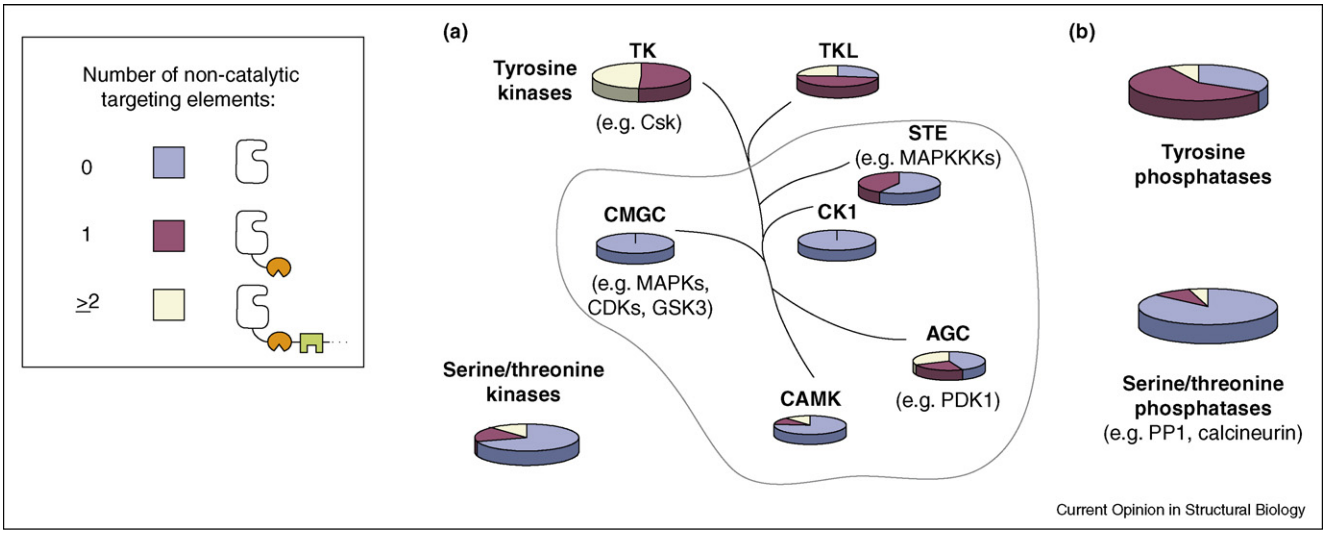
The small size of docking motifs potentially enables experimental screening of focused or randomized peptide libraries to discover novel docking peptide sequences. Docking-peptide-mediated interactions, however, are often weak and might function synergistically with interactions mediated by the active site. To overcome low-affinity binding, synthesis of multiple peptides on membranes was used to map synergistic components of weakly interacting protein–protein motifs for MAP kinases and tyrosine phosphatases [12,13].

Docking interactions in protein phosphatases

The serine/threonine phosphatase protein phosphatase 1 (PP1) has evolved effective catalytic machinery, but lacks strong substrate specificity in its active site. PP1 finds its targets via a large number of regulatory subunits, which influence the activity and cellular localization of the phosphatase. To date, more than 50 PP1–protein interactions have been identified [14]. Many of the PP1-interaction partners possess one or two of the following sequence motifs: FxxRxR or RVxF [15]. Co-crystallization of PP1 with a synthetic peptide encompassing the RVSF sequence established the RVxF motif as a conserved binding sequence that associates with a hydrophobic pocket on the surface of the PP1 catalytic subunit [16,17•]. Recently, a systematic analysis of structural elements that mediate the binding specificity of PP1-interacting proteins proposed a refined consensus for high-affinity PP1 ligands [18••]. Application of the results of this study to protein sequence database searches enabled the authors to predict PP1-interaction partners with high accuracy. Furthermore, binding studies with several PP1 partners and mutational analysis have demonstrated that differences in peptide–protein interactions dictate the affinity of PP1 for cellular regulators and control the dynamic physiological regulation of PP1 functions in the cell [19].

Calcineurin (known as protein phosphatase 2B, PP2B) is a serine/threonine phosphatase whose substrate selectivity is also determined in part by docking interactions. Calcineurin plays an important role in T-cell activation by directly regulating the activity of NFAT (nuclear factor of activated T cells) transcription factors via dephosphorylation. Calcineurin–NFAT signaling depends on the transient and reversible recognition of the N-terminal regulatory domain of NFAT by calcineurin. This interaction is mediated by a conserved PxIxIT motif present in NFAT proteins and a docking groove on the surface of calcineurin [20]. Peptide cross-linking, *in silico* docking and experimental analysis later revealed the structural

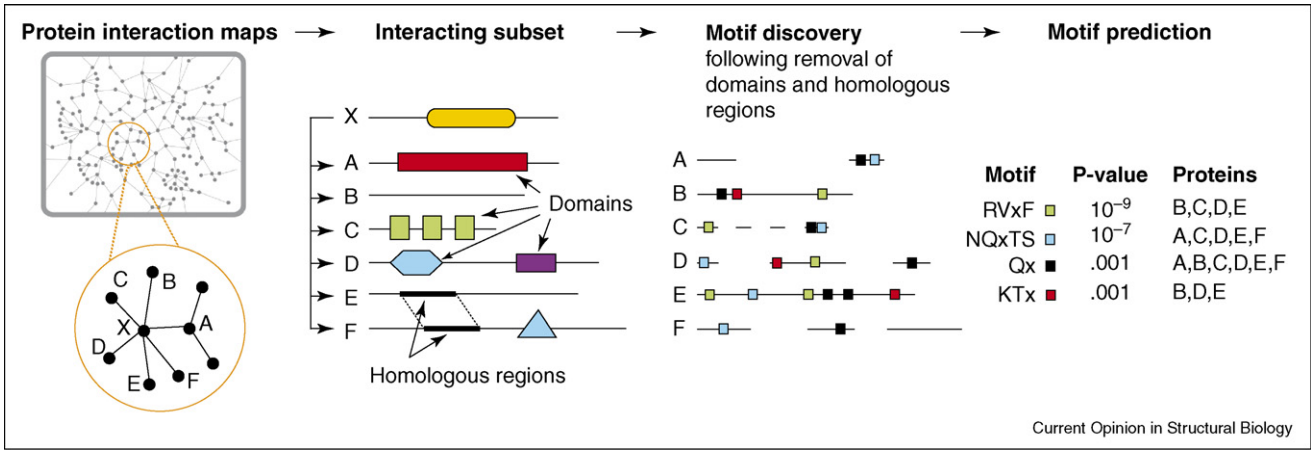
Figure 2



Distribution of non-catalytic targeting domains in human kinases and phosphatases. Human **(a)** kinases and **(b)** phosphatases containing 0, 1 or ≥2 types of recognizable targeting domains (in addition to the core catalytic domains) are shown. This analysis demonstrates that tyrosine kinases and phosphatases tend to use more targeting elements compared to serine/threonine kinases and phosphatases. Furthermore, members of certain serine/threonine kinase groups (e.g. the CMGC group) appear to entirely rely on the specificity of the kinase catalytic domain (they lack any additional, identifiable domains). Interestingly, this group includes kinase families such as MAPKs, CDK–cyclins and GSK3, for which many docking-groove- mediated interactions have been described. TKL (tyrosine kinase like) is a diverse group of families that resemble both tyrosine and serine/threonine kinases. Kinase sequences were obtained from the human kinome [50] and analyzed using the SMART database [7]. For phosphatases, only proteins fully annotated in SMART were utilized. The analysis involved a representative sample of about 270 known kinases and 65 phosphatases, corresponding to approximately half of the human kinome or phosphatome. Only intracellular targeting domains were counted in this analysis. Non-catalytic domains found include: protein–protein interaction (SH3, SH2, PDZ, SAM, ANK, FHA, Ig like, IGc2, FN3); membrane targeting (transmembrane regions, PH, PX, C2, B41); and miscellaneous (PBD, RBD, RGS, CNH, HR1, RhoGEF, CaM, IQ, C1, PB1, IG, DCX, TPR, LRR, UBA, RING, FCH, DEATH, RWD, CARD, GS, LIM).

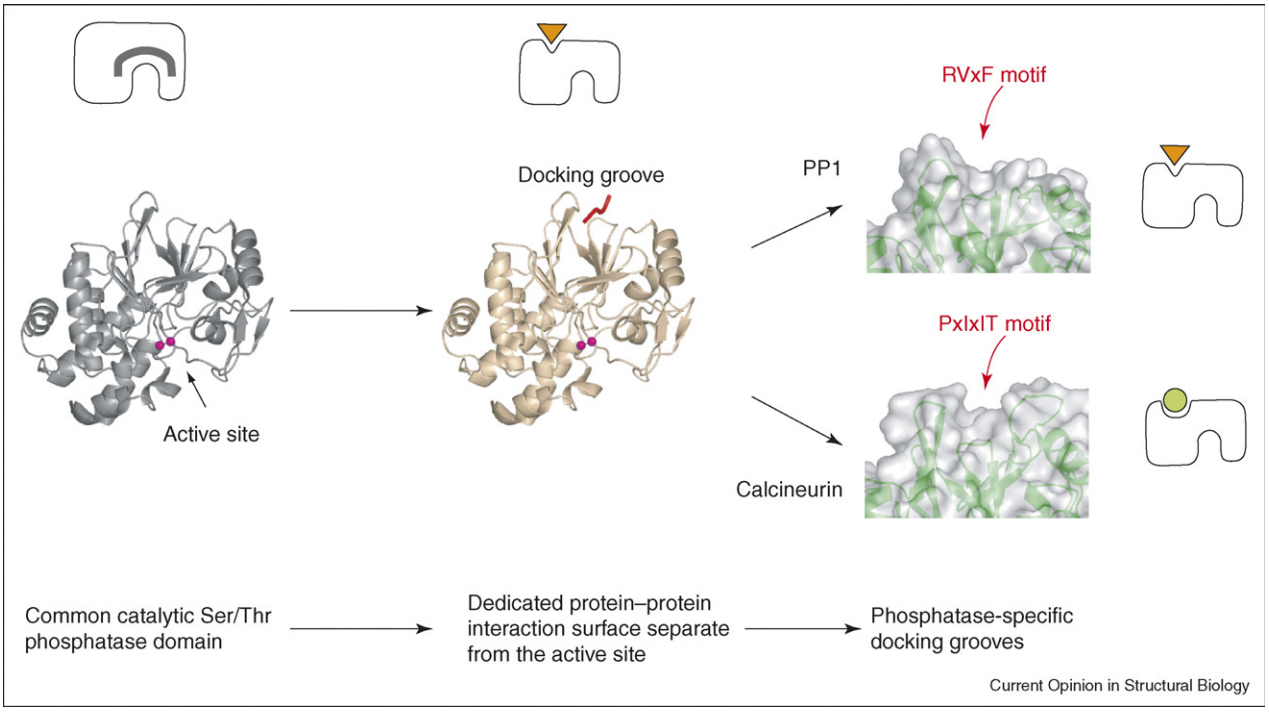
basis of calcineurin–PxIxIT docking motif interaction, and suggested an unexpected evolutionary parallel with the PP1–RVxF motif interaction [21•] (Figure 4). Finally, the importance of calcineurin–PxIxIT motif docking has also been established in other targets; the phosphatase binds to a K^+ channel through a consensus PxIxIT motif, which is indispensable for the regulation of channel activity [22].

Figure 3



Schematic of a linear motif discovery strategy. Large-scale interaction maps are probed for interacting subsets of interest. Partners of proteins with multiple interactions are grouped. Domains and homologous regions are identified and subsequently removed before running extensive pattern-discovery algorithms. Finally, a list of motifs is ranked by their probabilities. Predicted motifs are depicted as colored boxes. Adapted from [11••].

Figure 4



Divergent evolution of a docking groove. PP1 and calcineurin are two serine/threonine phosphatases that have a homologous catalytic domain, but interact with different sets of proteins through a related docking groove. The similar location but clearly different topology of the RVxF and PxIxIT motif docking grooves on these two phosphatases suggests divergent evolution. The positions of Mn ions near the active site are shown as spheres in magenta and the middle panel shows an RVxF motif peptide from the PP1–MYPT1 complex crystal structure in red [17•]. The calcineurin PxIxIT motif docking groove has been mapped in [21•].

Docking interactions in protein kinases

In mitogen-activated protein kinase (MAPK) networks, extracellular input leads to the phosphorylation and activation of a three-tiered kinase cascade (MAPKKK-MAPKK-MAPK), which in turn stimulates a specific transcriptional response. Within the signaling cascade, the MAPKKK must first recognize the correct MAPKK and phosphorylate it, and the activated MAPKK in turn must locate and activate its cognate MAPK. Structural, biochemical and genetic data have all shown that docking motifs from interacting proteins are necessary for MAPK to bind to and phosphorylate its targets. MAPK docking is ubiquitous, having been demonstrated in yeast and mammals [23]. The best-characterized MAPK-docking motifs are the so-called D-motifs. The consensus D-motif [$(R/K)_{1–2}$-$(X)_{2–6}$-Φ-x-Φ; where Φ denotes a hydrophobic residue] is found in activators (MAPKKs), negative regulators (phosphatases) and various substrates. MAPK docking occurs in all mammalian MAPK families (ERK, p38 and JNK) and crystal structures now exist for most of these MAPK docking complexes [24–27]. Comparison of the MAPK docking interactions from yeast to humans reveals a conserved mechanism of interaction, whereby basic residues of the D-motif bind to a negatively charged surface area (CD-site) and the hydrophobic residues bind to a hydrophobic groove on the MAPK (Φ-x-Φ groove). However, there is also a second class of MAPK docking (found only in ERK signaling), whereby a so-called DEF motif with an FxFP consensus sequence binds to a separate MAPK surface [28•,29•,30]. Hydrogen-exchange mass spectrometry (HXMS) data have located the DEF-docking groove near the kinase active site. FxFP motif binding to ERK2 is coupled to the positioning of its activation loop; it has been demonstrated that the phosphorylated MAPK binds this docking motif better than the inactive kinase [29•]. Kinase docking also occurs at other levels in the MAPK cascade. The C-terminal region of mammalian MAPKKs (which contains a so-called DVD motif) is necessary for interacting with and discriminating between various MAPKKKs [31]. There is also evidence that MAPKKK–MAPKK docking occurs in yeast [32].

Similar to the domain-mediated recognition of phosphorylated peptides (e.g. SH2, PTB, 14-3-3 and FHA domains), docking motifs can also act as regulatory elements when the docking interactions are themselves phosphorylation dependent. 3-phosphoinositide-dependent kinase-1 (PDK1) interacts with several downstream AGC kinases that contain a conserved docking motif known as the PDK1-interaction fragment (PIF) [33,34].

PIF motifs, however, must be phosphorylated before they bind effectively to the PIF pocket located on PDK1 (PIF motif: FxxFS/TF/Y, where S/T has to be phosphorylated) [35]. Therefore, downstream AGC kinase substrates must be primed by phosphorylation before interaction with and phosphorylation by PDK1. A similar priming event is required for phosphorylation of some substrates by glycogen synthase kinase-3 (GSK3), which is part of the insulin signaling pathway. GSK3 substrates must be phosphorylated on a residue that is C terminal to the serine/threonine site to be modified by GSK3. This priming phosphorylation motif binds to a phospho-recognition docking groove adjacent to the active site of GSK3 [36]. The priming phosphorylation scheme observed in the GSK3 and PDK1 pathways provides a mechanism for making signal processing dependent on catalytically distinct phosphorylation events, thereby increasing the specificity and complexity of control.

The activities of cyclin-dependent kinases (CDKs) are governed by the cyclins and linked to phases of the cell cycle. However, cyclins not only serve as activatory subunits for the kinase but also may function in substrate recognition. Many CDK substrates contain an RxL or KxL docking motif that is remote from the site of phosphorylation. Interestingly, the RxL-docking groove is located not on the kinase but on the interacting cyclin, providing a slightly different mechanism of substrate recognition, whereby the cyclin functions as an adaptor [37,38•].

Allostery versus tethering

A fundamental question is how docking motif binding influences kinase and phosphatase activity: is it simply tethering or is allostery also important? Overall, the primary role of docking interactions appears to be tethering. However, there are a few cases in which docking interactions play an important role in altering protein conformation and allosterically regulating activity. The binding of PIF motifs to PDK1 increases kinase activity four- to six-fold [33,34]. Studies of MAP kinases, however, suggest that the main function of D-motif docking interactions is tethering the kinase to partners; the Fus3 MAPK structure, for example, changes very little upon binding to D-motif peptides from various interaction partners [39••]. In one example, however, the activity of the same MAPK is allosterically activated by a peptide whose binding site includes the docking groove for D-motifs [40•]. A recent structure of ERK2 in complex with a D-motif also demonstrates that peptide binding to the docking groove can have long-range effects, including reordering of the kinase activation loop [27] (Figure 5). Hydrogen-deuterium exchange data have also indicated that some flexibility is induced in the activation loop of p38 upon D-motif binding [29•].

In the case of phosphatases, binding of an RVxF motif to PP1 does not change the structure of the phosphatase domain. Similarly, allosteric effects of docking peptide binding on the catalytic activity of calcineurin have not been found. The catalytic domains of these two phosphatases may be too rigid and docking interactions apparently cannot augment structural changes at the active site. Therefore, it is likely that, in these examples, the localization or tethering of the effector phosphatase domain to the protein partner is sufficient to explain the physiological roles of docking. However, in the case of calcineurin–NFAT binding, it is possible that the association of the phosphatase with its PxIxIT docking peptide itself is controlled by an allosteric site. This site, bound by some small-molecule inhibitors of calcineurin–NFAT association, is separate from both the docking groove and the active site [41].

Specificity of MAP kinase docking interactions

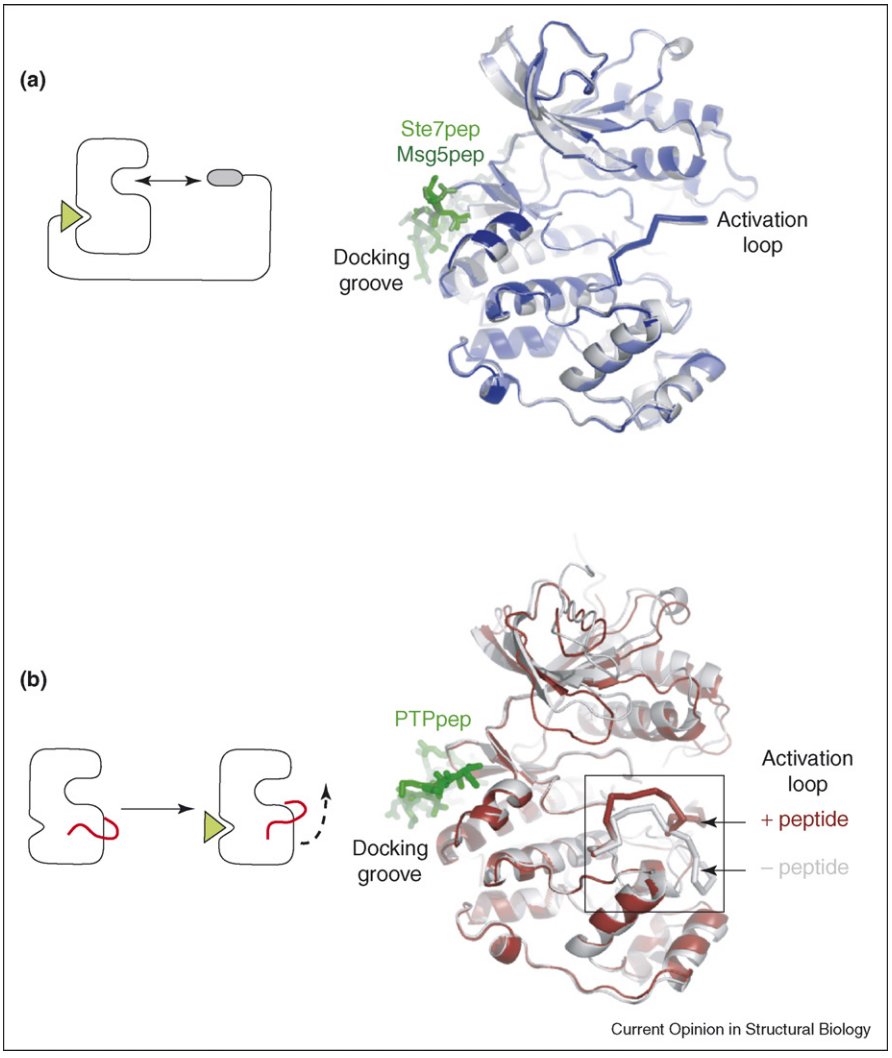
Although docking provides a simple way to generate new connectivity in circuits relying on kinase or phosphatase activity, the recurrent use of the same surface grooves presents a new problem: how can docking surfaces encode specific information about kinase or phosphatase interactions in the context of many related peptide motifs (Figure 6)? Signaling events regulated by MAP kinase docking interactions display a remarkable degree of fine-tuned specificity: simple sequence variation in yeast D-motifs can influence signal flow between distinct but closely related MAPKs and their interaction partners, and it appears that induced-fit recognition allows docking peptides to achieve discrimination by exploiting subtle differences in kinase flexibility [39••].

For mammalian MAPK systems, substrates are phosphorylated by specific subsets of MAPKs depending on the number and sequence of the docking motifs. DEF and D-motifs form a modular system in which a different arrangement of these two docking motifs on c-Jun and JunD transcription factors governs the differential response of these two ERK2 targets upon EGF stimulation [30]. In another study that highlighted the restrictive and selective nature of docking interactions, discrete ERK2 docking groove mutations differentially affected the binding and inactivation of two different tyrosine phosphatases. Importantly, some of these mutations still allowed efficient phosphorylation of ERK2 by MEK1/2 (a MAPKK) [42].

Docking and drug design

The prevalence of protein kinases and phosphatases that are involved in disease has led to intensive efforts to develop specific inhibitors for use as therapeutics. Most kinase and phosphatase inhibitors currently target active sites. However, protein interactions, as outlined earlier, also play an essential role in linking kinases and phosphatases with their signaling partners. Thus, blocking docking interactions holds promise as an alternative

Figure 5

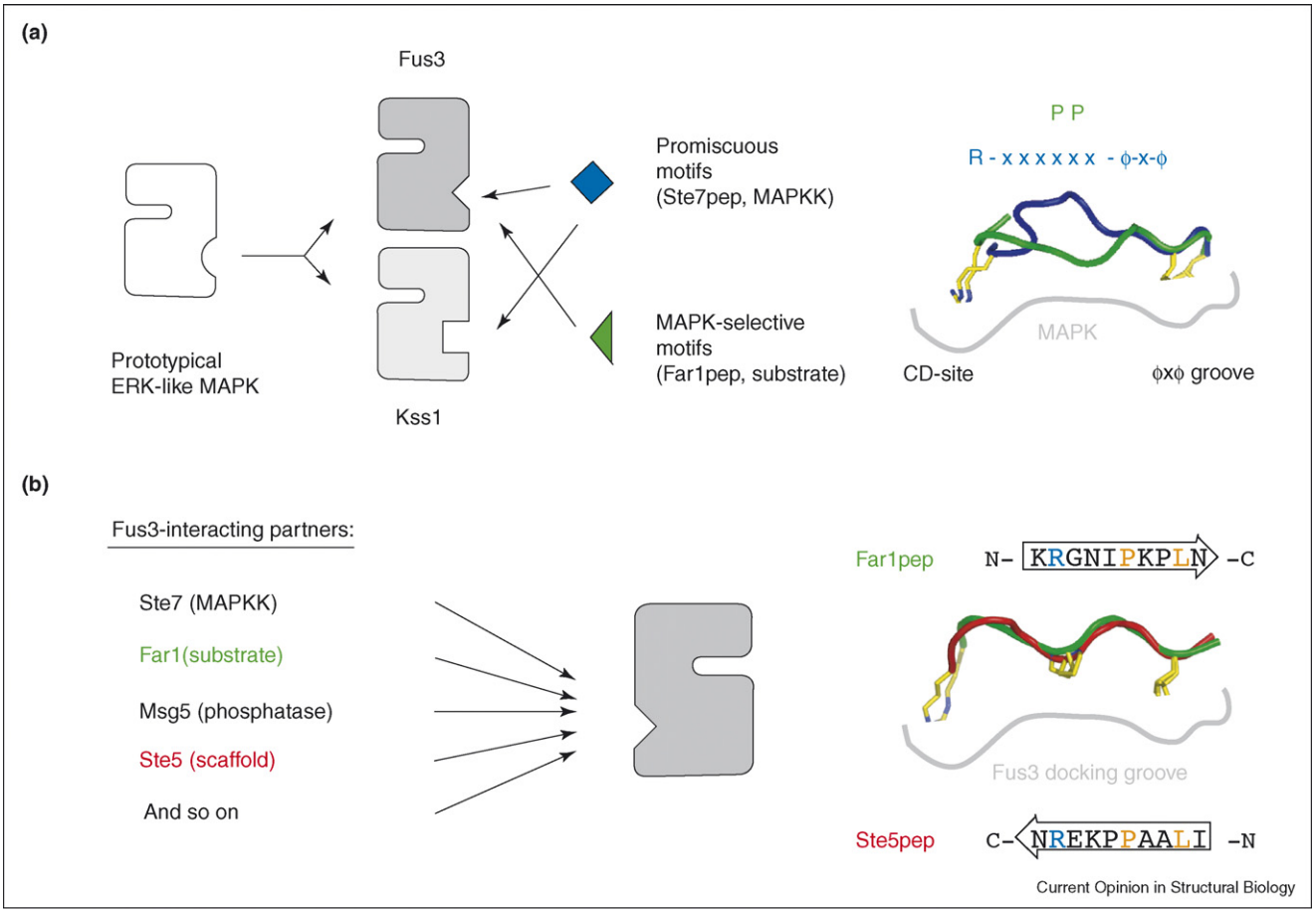


Mechanisms by which docking motifs can influence MAPK signaling. **(a)** The 'tethering' model: in the case of the yeast MAPK Fus3 and, in some cases, the mammalian MAPK ERK, docking peptide binding creates no obvious changes in structure or kinase activity. Structural depiction of the MAPK Fus3 in the apo state (gray) and bound to the D-motifs of its activator, Ste7 (dark blue), and its negative effector, the phosphatase Msg5 (light blue) [39••]. Docking peptides are shown in green. **(b)** Long-range conformational changes induced by D-motif docking: the 'allosteric' model. Peptide binding to the MAPK docking groove induces an allosteric effect, including reordering of the activation loop. These conformational changes are postulated to affect MAPK activity (the positioning of the activation loop is also controlled by phosphorylation and thus relates strongly to enzymatic function). Structural alignment of the mammalian MAPK ERK2 with (red) and without (gray) a D-motif peptide from the PTP phosphatase [27]. The activation loop in the ERK2–PTP D-motif complex moves into a new position that more closely matches the conformation in phosphorylated (and active) ERK2.

strategy for selectively inhibiting kinase and/or phosphatase signaling. For example, cell-permeable peptides containing docking motifs were shown to selectively modulate MAPK and PP1 activity *in vivo* [43–45].

Another approach to docking-based drug design is using high-throughput experimental or computational structure-based screens to identify small chemical compounds. The existence of a specialized substrate-targeting mechanism in calcineurin–NFAT signaling enabled the identification of small-molecule inhibitors that act by interfering with phosphatase–substrate docking rather than with the calcineurin catalytic site [46••,47]. As three-dimensional structures of docking groove surfaces on kinases and phosphatases become available, structure-based screens will be increasingly practical [48]. Taking advantage of the recently identified ERK MAPK docking surface, a computer-aided drug design study identified novel small-molecule ERK inhibitors that showed a dose-dependent reduction in the proliferation of several cancer cell lines [49]. Moreover, downstream branches of ERK signaling that are based on DEF- or D-motif-mediated

Figure 6



Specificity and evolution of MAPK docking networks. **(a)** Divergent evolution of MAPK–D-motif interactions. The D-motif–MAPK interactions of two yeast MAPKs demonstrate how a common and highly conserved docking groove can dictate interaction specificity. Ste7, a MAPKK, activates two related yeast MAPKs: Fus3 and Kss1. This interaction is mediated by a D-motif that can bind to Fus3 as well as to Kss1 (promiscuous). A separate D-motif of the substrate Far1 is specific for Fus3 (selective). Promiscuous and selective peptides bind the docking groove in conformationally distinct modes and, interestingly, it is the sequence of the intervening region between the basic (R) and the Φ-x-Φ motif that appears to influence the binding mode. Insertion of two proline residues into this region renders a promiscuous D-motif MAPK selective [39••]. **(b)** Convergent evolution of MAPK–D-motif interactions. Many Fus3-interaction partners bind to the Fus3 docking groove. Two proteins, the Far1 substrate and the Ste5 scaffold, contain Fus3-selective docking motifs that bind the docking groove in what appears to be a similar conformation: they both interact with the negatively charged CD-site using an arginine, use a leucine to bind the Φ-x-Φ groove and have a proline in a pivotal position that favors a Fus3-specific peptide conformation [39••,40•]. However, these motifs are clearly unrelated: the orientations of the peptide chains are opposite (note that the N-C orientation is different). It appears that both proteins evolved a similar but unrelated solution to interact with Fus3 in a specific manner, suggesting an example of convergent evolution for docking motif design.

docking can be selectively inhibited without blocking total pathway activity [28•]. These studies demonstrate that interference with docking interactions is a viable alternative to the direct catalytic inhibition of kinase or phosphatase activity.

Conclusions

A growing number of substrates as well as many regulator proteins have been shown to bind kinase or phosphatase catalytic domains through docking groove interactions. Docking-mediated interactions are particularly prevalent in serine/threonine kinases. The search for new docking peptide sequences and their corresponding protein–protein interaction surfaces on kinase/phosphatase domains is an active area of research. Computational approaches, in combination with systematic experimental analysis, will aid the identification of new examples of protein–protein interactions mediated by docking. The next challenge is to discover the molecular principles behind the specificity mediated by individual docking grooves and their corresponding peptide motifs. This new knowledge will be helpful in the design of biological tools to modulate protein–protein interactions *in vivo* and in elucidating the specific roles of this ubiquitous class of enzymes in diverse cellular processes. Such studies will set the stage for the selective regulation of kinase/phos-

phatase network activity by small-molecule inhibitors, whereby the focus would be on targeting connections rather than catalytic activity.

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- •• of outstanding interest

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