Mechanisms of specificity in protein phosphorylation

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Abstract | A typical protein kinase must recognize between one and a few hundred bona fide phosphorylation sites in a background of ~700,000 potentially phosphorylatable residues. Multiple mechanisms have evolved that contribute to this exquisite specificity, including the structure of the catalytic site, local and distal interactions between the kinase and substrate, the formation of complexes with scaffolding and adaptor proteins that spatially regulate the kinase, systems-level competition between substrates, and error-correction mechanisms. The responsibility for the recognition of substrates by protein kinases appears to be distributed among a large number of independent, imperfect specificity mechanisms.

Phosphorylation site
(P-site). By convention, residues that are situated N-terminally of the P-site residue are numbered P–1, P–2, P–3 and so on, whereas residues that are situated C-terminally of the P-site are numbered P + 1, P + 2, P + 3 and so on.

Mitogen-activated protein kinase
(MAPK). A member of a family of protein kinases that are activated in response to diverse mitogens, stresses and developmental signals. MAPKs are the terminal components of three-kinase cascades.

Cyclin-dependent kinase
(CDK). A Ser/Thr-specific kinase that depends on the binding of a cyclin for full activity. CDKs are essential for cell-cycle progression.

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Protein phosphorylation is the most widespread type of post-translational modification used in signal transduction. It affects every basic cellular process, including metabolism, growth, division, differentiation, motility, organelle trafficking, membrane transport, muscle contraction, immunity, learning and memory[1,2]. Protein kinases catalyse the transfer of the γ-phosphate from ATP to specific amino acids in proteins (FIG. 1a); in eukaryotes, these are usually Ser, Thr and Tyr residues.

Considering their broad importance in signal transduction, it is not surprising that protein kinases are one of the largest families of genes in eukaryotes — making up ~2% of the genome — and have been extensively studied[1,3,4]. Furthermore, it has been estimated that 30% of all cellular proteins are phosphorylated on at least one residue[5,6]. If it is assumed that there are ~10,000 different proteins in a typical eukaryotic cell, with an average length of ~400 amino acids (~17% of which are Ser (8.5%), Thr (5.7%) or Tyr (3.0%) residues[7]), then there are ~700,000 potential phosphorylation sites (P-sites) for any given kinase.

Non-systematic studies and biochemical lore suggest that kinases vary greatly in the number of these sites that they phosphorylate. For example, the mammalian protein kinase MEK1 (mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) kinase-1) probably phosphorylates only four sites, two in ERK1 and two in ERK2, whereas calcium–calmodulin (CaM) kinases and cyclin-dependent kinases (CDKs) probably phosphorylate hundreds of sites. Systematic *in vitro* proteomic studies in *Saccharomyces cerevisiae* support this proposal: in one study, kinases phosphorylated between 1 and 256 substrates, and in a second study, cyclin-dependent kinase-1 (Cdk1) from yeast phosphorylated hundreds of substrates[8,9]. Even the most promiscuous kinases can select their many targets from among the 700,000 potential phosphorylation sites that they might encounter. Admittedly, some of the potential phosphorylation sites might be buried and, therefore, would be inaccessible to a kinase. In addition, biological systems might tolerate some off-target phosphorylation, particularly in view of the fact that phosphorylation is a reversible modification. Nevertheless, the challenge of discriminating among potential substrates seems a daunting task, especially as many of the substrates can be low-abundance proteins.

A further challenge is the fact that most eukaryotic protein kinases are structurally similar[10,11]. Classical protein kinases have a canonical catalytic domain of ~250 amino acids in length, which consists of a small N-terminal lobe of β-sheets and a larger C-terminal lobe of α-helices[12–19]. ATP binds in a cleft between the two lobes so that the adenosine moiety is buried in a hydrophobic pocket with the phosphate backbone orientated outwards towards the solution. The protein substrate binds along the cleft and a set of conserved residues within the kinase catalytic domain catalyse the transfer of the terminal γ-phosphate of ATP to the hydroxyl oxygen of the Ser, Thr or Tyr residue of the substrate (FIG. 1a,b). Although all classical protein kinases share a common fold, they differ in terms of the charge and hydrophobicity of surface residues (FIG. 1c). As discussed below, these differences are important for kinase specificity.

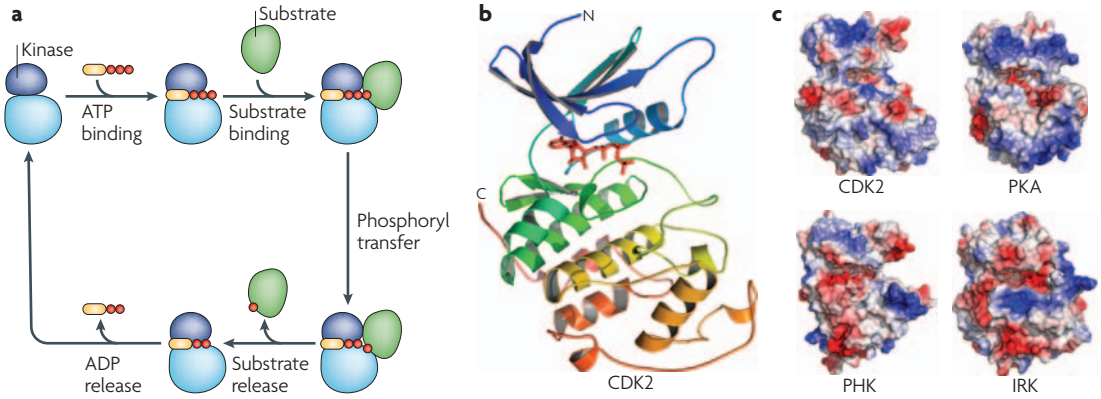

Figure 1 | **Protein kinases share a common mechanism and fold. a** | The basic catalytic cycle for substrate phosphorylation by a kinase. Starting top left, ATP binds to the active site of the kinase. This is followed by binding of the substrate to the active site. Once bound, the γ-phosphate of ATP (red) is transferred to a Ser, Thr or Tyr residue of the substrate. After phosphorylation, the substrate is released from the kinase. The last step shown is the release of ADP from the active site. The order of the steps differs for different kinases. For example, some kinases bind to their protein substrates before binding ATP and others release ADP before releasing the protein substrate. The rate-limiting step can also vary between different kinases. **b** | All protein kinases have a similar protein fold that comprises two lobes: one lobe consists of mainly β-sheet structure (blue) and the other lobe consists of α-helices (green, orange and yellow). This lobe structure forms an ATP-binding cleft that constitutes the active site. The crystal structure of cyclin-dependent kinase-2 (CDK2) (Protein Data Bank (PDB) ID: 1QMZ) shows this representative fold. ATP is modelled bound in the cleft (red ball and stick model). **c** | Despite sharing a common fold, kinases bind to and phosphorylate different protein substrates, partly due to differences in the charge and hydrophobicity of surface residues. Electrostatic surface representations of four different kinases are shown; three Ser/Thr kinases: CDK2, PKA (cAMP-dependent protein kinase, PDB ID: 1ATP) and PHK (phosphorylase kinase, PDB ID: 2PHK), and the Tyr kinase domain of the insulin receptor (IRK, PDB ID: 1IRK). Positively charged surfaces, blue; negatively charged surfaces, red. Three-dimensional structures were created using PyMol[134].

Previous excellent reviews have discussed the mechanisms that kinases use to phosphorylate their proper substrates selectively in order to maintain the specificity that is observed in signalling pathways[5,20–27]. Here, we discuss the local determinants around the active site of the kinase that can direct protein kinases to particular consensus phosphorylation sequences. We then consider how the distal parts of the kinase provide further interactions that contribute to specificity through binding, allostery or restricted localization. Finally, we discuss how systems-level effects, such as competition between substrates, multisite phosphorylation and kinetic proofreading, can contribute to specificity in the complex protein mixtures that are found in cells.

The active site of the kinase

The first level of substrate specificity arises from the structural characteristics of the kinase active site. Specific features of the active site, including its depth and perhaps charge or hydrophobicity, often greatly increase its binding preference for substrates with complementary characteristics.

Structure of the catalytic cleft. Protein kinases can be separated into two main groups: the Ser/Thr-specific kinases, which constitute about 80% of the protein kinases (BOX 1), and the Tyr-specific kinases[1]. Although these kinase groups phosphorylate different residues, they have similar catalytic domain structures (FIG. 1b). However, protein Tyr kinases have a deeper catalytic cleft than Ser/Thr kinases: a Tyr residue can span the distance between the peptide backbone and γ-phosphate of ATP, but the smaller side groups of Ser and Thr residues cannot[28,29] (FIG. 2). Interestingly, this same feature contributes to the specificity of Tyr phosphatases and Ser/Thr phosphatases[30–32] (BOX 2). The specificity imparted by the cleft depth is not absolute — there are several examples of Ser/Thr kinases that can phosphorylate Tyr residues to some extent[4]. The converse — a Tyr kinase that can phosphorylate Ser or Thr residues — appears to be rarer.

The interaction between the P-site of the substrate and the kinase can make a significant contribution to the overall binding energy. For example, the seven-amino-acid pseudosubstrate peptide LRRAALG has a K_i value of 490 μM for cyclic AMP (cAMP)-dependent protein kinase (also known as protein kinase A (PKA)), whereas the substrate peptide LRRASLG has a K_m value of 16 μM (and, presumably, an even smaller K_d)[12,33], which demonstrates that the presence of a phosphorylatable residue in the phosphorylation site adds a substantial amount of binding energy to the interaction.

Consensus sequences. The amino acids that are situated immediately N-terminal and C-terminal to the P-site often contribute substantially to kinase–substrate recognition. Free amino acids are poor substrates for protein kinases[34,35], and the minimal substrate for most kinases is a short peptide. In most cases, the active site of the kinase interacts with four amino acids on either side of the P-site. Sequences that are further away from the P-site can interact with portions of the protein kinase that are just outside the active site[12].

Insulin receptor
The heteromeric tyrosine kinase receptor for the anabolic hormone insulin.

Allostery
The regulation of protein activity through phosphorylation, or through the binding of a small molecule or protein, at a site distinct from the active site. Communication between the allosteric site and the active site usually occurs through a conformational change.

cAMP-dependent protein kinase
A Ser/Thr-specific protein kinase that is activated by the cAMP-induced dissociation of a regulatory subunit.

Box 1 | Serine versus Threonine — are they the same?

When considering the specificity of Ser/Thr kinases and phosphatases, it is often assumed that there is little preference for one residue over the other. However, this assumption is incorrect[5,127]. In proteins from human, fly, worm and yeast, ~8.5% of residues are Ser, 5.7% are Thr and 3.0% are Tyr[7]. If Ser/Thr kinases and phosphatases were unbiased towards potential Ser and Thr residues, one would expect a ~1.5:1 ratio of phosphorylated (p)Ser:pThr sites. Partial acid hydrolysis and phosphoamino-acid analysis of ^{32}P-labelled cells typically yields 90% pSer, 10% pThr and 0.05% pTyr (REF. 128), which gives a ratio of 9:1 pSer:pThr. Recent global mass-spectrometry studies of human protein phosphorylation have found the distribution of pSer, pThr and pTyr sites to be around 79.3%, 16.9% and 3.8%, respectively, or a 5:1 ratio of pSer:pThr (REFS 41,128). Interestingly, most Ser/Thr kinases appear to have a preference for phosphorylating Ser residues and most Ser/Thr phosphatases show a striking bias towards dephosphorylating pThr residues[5,127]. These biases may account for the over-representation of Ser residue phosphorylation in cells, and the existence of such biases raises interesting questions — for example, are off-target phosphorylation sites generally dephosphorylated more quickly than on-target sites?

Edman degradation
A method of sequencing proteins in which the N-terminal residue is chemically labelled, cleaved from the peptide and then identified chromatographically. The process can be repeated to obtain the sequence of the first ~10–50 amino acids in the protein or peptide.

The idea that protein kinases recognise a primary-structure sequence consensus came from mutational analysis of known substrates[36–38]. An important step forward was the introduction of orientated peptide library screens[39], in which a kinase of interest is added with ATP to a soluble mixture of 2.5 billion distinct peptides, each peptide being of identical length with only a single phosphorylatable residue. The kinase reaction is allowed to occur for a short period of time before the phosphorylated peptides are separated from non-phosphorylated peptides; the mixture is then sequenced by Edman degradation. Information about the preferred amino acid at each position can be obtained by comparing the abundance of amino acids at each position in the phosphorylated fraction with the starting mixture.

More sophisticated methods have been developed using biotinylated dual-orientated peptide libraries that contain a Ser/Thr residue in a fixed position and a second fixed amino acid[40]. Peptide mixtures are incubated with the kinase in 96-well plates and then transferred to an avidin-coated membrane that enables phosphorylation to be analysed by autoradiography. This method eliminates many of the artefacts that are seen with immobilized substrates and does not require Edman degradation. Using this and other methods, consensus phosphorylation sites have been determined for several protein kinases[5,38,41] (TABLE 1). Genome mining for proteins that contain these consensus phosphorylation sites is useful to predict whether a substrate is phosphorylated by a kinase of interest[42]. However, the presence of a consensus phosphorylation site in a protein does not guarantee that the protein is a substrate *in vivo*, and authentic phosphorylation sites do not always conform to the consensus[9].

Kinetic and structural studies have revealed the mechanistic basis for the local substrate specificity of several kinases. Frequently, the kinase and substrate have complementary sequences that interact on the basis of charge, hydrogen bonding or hydrophobic interactions. For example, in CDK2 (in which the consensus phosphorylation site is S/T-P-X-K/R, where X is any amino acid), the binding of any substrate that does not have a Pro residue at the P+1 position is unfavourable because of an unsatisfied hydrogen bond from the nitrogen atom in the main chain of the substrate[29]. This nitrogen atom in Pro is unique among the 20 amino acids because it is unable to form a hydrogen bond owing to the cyclic binding of the three-carbon side chain back to the nitrogen atom of the backbone (BOX 3). CDK2 has specificity for a basic residue in the P+3 position that stems from the interaction between this basic residue and a phospho-Thr in the kinase (Thr 160)[29,43] (FIG. 3a).

The crystal structure of PKA reveals structural elements that restrict the characteristics of potential substrates by charge and hydrophobicity. Two Glu residues in PKA create anionic binding sites for the P–3 and P–2 position of the substrate and there is a hydrophobic pocket in the kinase that favours a hydrophobic residue in the P+1 position[44,45] (FIG. 3b). The consensus sequence for PKA (R-R-X-S/T-Φ, where Φ represents a hydrophobic residue[39]) fits perfectly into the local environment of the active site and provides the first level of substrate specificity to the kinase.

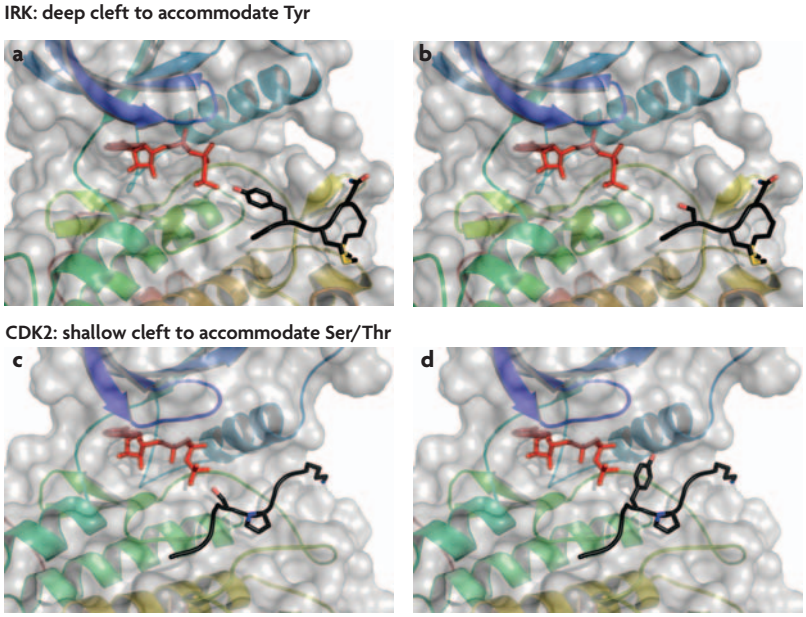

Figure 2 | **The depth of the catalytic cleft determines phosphorylation site (P-site) amino-acid specificity.** The catalytic clefts of Tyr kinases are deeper than those of Ser/Thr kinases and this determines their specificities for Tyr or Ser/Thr. **a** | The structure of the Tyr kinase domain of the insulin receptor (IRK) bound to a Tyr substrate peptide (Protein Data Bank (PDB) ID: 1IR3) and **b** | a modelled Ser substrate peptide. Unlike Ser, Tyr extends far enough into the catalytic cleft to be efficiently phosphorylated. **c** | The structure of the Ser/Thr kinase cyclin-dependent kinase-2 (CDK2) bound to a Ser substrate peptide (PDB ID: 1QMZ) and **d** | a modelled Tyr substrate peptide. Tyr is too large to fit into the catalytic cleft. Structures and modelled substrates were created using PyMol[134]. ATP is shown in red. Most of the peptide substrate is black, with hydroxyl side-chain oxygens shown in red.

Distal docking sites

The next level of substrate specificity often involves interactions between docking motifs on the substrate with interaction domains on the kinase[24,46]. These additional binding motifs increase the affinity of kinases for specific substrates by many times, and are often spatially separate from the kinase active site and the substrate P-site.

Box 2 | **Phosphatase structure and specificity**

As with protein kinases, protein phosphatases can be subdivided into three main families, depending on the residues that they dephosphorylate: Ser/Thr phosphatases, Tyr phosphatases and dual-specificity phosphatases. The human genome encodes ~500 protein kinases (~400 Ser/Thr, ~90 Tyr, ~40 dual-specificity) and ~200 protein phosphatases (~40 Ser/Thr, ~100 Tyr, ~50 dual-specificity)[129–131].

The discordance between the number of Ser/Thr phosphatases (~40) and Ser/Thr kinases (~400) raises interesting questions about specificity and regulation. Specificity appears to arise mainly through the association of phosphatase catalytic domains with particular regulatory subunits. For example, phosphatase-1C (PP1C) can form complexes with >50 regulatory subunits that target the catalytic core to different cellular locations and target substrates[132]. The regulatory subunits provide myriad opportunities for regulation, although our understanding of when and how dephosphorylation reactions are regulated generally lags far behind our understanding of the regulation of kinases.

Tyr kinases and phosphatases are similar in number: the phosphatases do not need to handle more substrates than the kinases do. Similar to the Tyr kinases, Tyr phosphatases tend to be modular proteins with separate catalytic and targeting domains[129]. Some classes of protein-interaction domain (for example, Src-homology-2 (SH2) domains) are found in both kinases and phosphatases, whereas others are found only in one (SH3 domains in kinases) or the other (FYVE domains in phosphatases)[129]. Catalytically inactive phosphatase domains (STYX domains) are a distinctive means of targeting Tyr phosphatases to particular substrates, and perhaps function in a similar way to SH2 domains (which are common in Tyr kinases and less common in Tyr phosphatases).

These docking interactions have been identified in various kinases, including c-Jun N-terminal kinases (JNKs)[47], phosphorylase kinase (PHK)[19], ERK[22], MEK[48,49], glycogen synthase kinase-3 (GSK3)[50], phosphoinositide-dependent kinase-1 (PDK1)[51], CDK2 (REFS 52,53), and transforming growth factor-β receptor (a Tyr kinase)[54,55], indicating that this is a general mechanism for enhancing substrate specificity.

Structure of docking motifs. The docking sites on MAPKs are particularly well studied and demonstrate the role that these distal sites have in enhancing substrate specificity. One of the first docking motifs to be discovered was the D domain (also known as the D box or DEJL domain) on MAPK substrates[47]. These domains are typically 50–100 residues away from the P-site and increase the affinity of the kinase for the substrate, thereby increasing the efficiency of phosphorylation. These docking domains are ubiquitous in MAPK substrates[56]: activating transcription factor-2 (ATF2) contains a docking site for JNK[57,58], the transcription factor ELK1 contains an ERK docking site[59] and myocyte enhancer factor-2A and -2C (MEF2A and MEF2C) contain docking sites for p38 MAPK[60].

The alignment of MAPK docking sites on substrates shows that they conform to an $(R/K)_{1-2}$-$(X)_{2-6}$-Φ-X-Φ pattern. The basic residues of the D domains bind to a negatively charged area (CD-site) that is located just C-terminally of the kinase domain, and the hydrophobic residues bind to a hydrophobic Φ-X-Φ groove that is located in the β-sheets β7–β8 and helices αD–αE of the MAPK[56,61] (FIG. 4a). Differences in the composition and spacing of residues in the distal docking site and the local preferences of the catalytic core for different amino acids around the P-site work together to increase the overall selectivity of kinase–substrate interactions[62].

Studies of the ETS-domain transcription factor LIN-1 (abnormal cell lineage-1) from *Caenorhabditis elegans* identified a second distinct ERK-docking motif, called the docking site for ERK, F/Y-X-F/Y-P motif (DEF) domain (also known as the FXF motif)[63]. DEF domains have been identified in other MAPK substrates, including many other transcription factors, MAPK phosphatases and other MAPK regulators, and are almost always located ten amino acids downstream of the phosphorylation site[25,63]. The two Phe residues in the DEF domain have crucial roles in kinase recognition but other residues that immediately surround the motif are also important. In LIN-1, the DEF domain is FQFP and the Pro residue is also important in substrate binding[64]. However, in isoforms of the cAMP-specific phosphodiesterase-4D (PDE4D), this Pro is replaced by a Glu residue[65]. In all cases, however, the DEF domain binds a hydrophobic pocket near the active site between helix αG and the activation lip of the kinase[61,66] (FIG. 4a). The existence of two distinct docking domains in MAPK substrates — D domains and DEF domains — underscores the importance of this type of interaction.

Docking motifs are found in Ser/Thr kinases and Tyr kinases, but they usually occur in different parts of the kinase molecules. In Tyr kinases, docking motifs tend to be found in modular domains that are separate from the kinase domains. Non-receptor Tyr kinases, for example, almost always have well-defined interaction domains, such as Src-homology-2 (SH2), SH3, integrin binding, focal adhesion binding, DNA binding, F-actin binding, pleckstrin homology, or Janus tyrosine kinase (JAK) homology domains[67] (FIG. 4c). In Ser/Thr kinases, the docking domains are often part of the kinase domain and additional modular protein–protein interaction domains seem to be less common. From a genetics perspective, the modular structure of the docking domain and the catalytic domain of Tyr kinases offer more flexibility than the specialized domains of Ser/Thr kinases. Conversely, the non-modular structure of Ser/Thr kinases might be more economical because more functions are built into a smaller protein.

Function of docking motifs. Docking motifs could function simply to increase the local concentration of the substrate around the kinase. If it is considered that the presence of a docking domain positions a substrate within a 10 nm sphere of the kinase active site, then the effective concentration of the substrate would be 3 mM[68]; this alone could be sufficient to greatly increase the rate of phosphorylation of a scarce substrate. This hypothesis assumes that there is a flexible region between the catalytic site of the kinase and its docking domain, between the phosphorylation site of the substrate and its docking domain, or both. If these regions are inflexible, then the interaction of the docking sites might serve to align the kinase precisely with the phosphorylation site, providing an additional enhancement in the rate of phosphorylation beyond what would be predicted from the local concentration effect.

Glycogen synthase kinase-3
A Ser/Thr kinase that is important for insulin and Wnt signalling. It was initially identified in studies of metabolic regulation and also has roles in development.

D domain
A distal docking site that is ubiquitous in mitogen-activated protein kinase substrates. The sequence of the D domain usually conforms to an $(R/K)_{1-2}$-$(X)_{2-6}$-Φ-X-Φ pattern, where Φ is a hydrophobic residue.

DEF domain
A distal docking site that is located ten amino acids downstream of the phosphorylation site, and is found in many, but not all, mitogen-activated protein kinase substrates.

Src
A non-receptor Tyr kinase proto-oncogene product. Src is normally kept inactive by intramolecular interactions between its kinase domain and its SH2 and SH3 domains, and can be activated by interaction with other SH2- and SH3-domain-binding proteins.

Table 1 | **Consensus phosphorylation sites of some protein kinases**

Kinase	Full name	Consensus phosphorylation site	Refs
PKA	Protein kinase A or cAMP-dependent protein kinase	R-R-X-S/T-Φ	5,39
CDK	Cyclin-dependent kinase	S/T-P-X-K/R	5,39
ERK2	Extracellular-regulated kinase-2	P-X-S/T-P	5,136
CK1*	Casein kinase-1	pS-X-X-S/T	5,137
CK2‡	Casein kinase-2	S/T-D/E-X-E/D	5,138
GSK3	Glycogen synthase kinase-3	S-X-X-X-pS	5,139
CaMK2	Calmodulin-dependent protein kinase-2	R-X-X-S/T	5,136
ABL	Abelson murine leukaemia virus tyrosine kinase	I/V/L-Y-X-X-P/F	5,140
EGFR	Epidermal growth factor receptor	E-E-E-Y-F	5,141
Src	Rous sarcoma virus tyrosine kinase	E-E-I-Y-E/G-X-F	5,141
IRK	Insulin receptor tyrosine kinase	Y-M-M-M	5,141
PKB/AKT	Protein kinase B	R-X-R-X-X-S/T	142
PKD	Protein kinase D	L/I-X-R-X-X-S/T	40
PIM1–3	Proviral integration site kinases 1–3	R-X-R-X-X-S/T	40,143

*CK1 is a well-conserved Ser/Thr-specific protein kinase, the regulation and function of which are incompletely understood. ‡CK2 is also a well-conserved Ser/Thr kinase that is unrelated to CK1 and is implicated in the regulation of diverse biological phenomena. pS, phosphorylated Ser; X, any residue; Φ, hydrophobic residue.

Several docking interactions have allosteric effects that either positively or negatively regulate kinase activity. For example, certain D-domain-containing peptides can stimulate MAPK activity or autophosphorylation[69], and the binding of FXF motifs to ERK appears to be coupled to full activation of the kinase[61]. Interestingly, other docking-site interactions appear to negatively regulate the docked kinase[70].This combinatorial control, in which a docking site allosterically regulates a kinase and directs it to specific substrates, greatly enhances specificity more than either mechanism could do alone.

Targeting subunits

Some Ser/Thr kinases use modular binding partners that contain docking domains that help to target the kinase to specific substrates. Moving the targeting domain from the kinase to a binding partner might allow a single kinase to interact with different substrates in different contexts.

For example, CDKs form stable, slowly exchanging complexes with several different cyclin proteins. The cyclins allosterically activate the CDK but, in addition, cyclins have a docking domain that helps to recruit the CDKs to their correct substrates. This docking domain is a small hydrophobic patch that is located on the cyclin partner about 40 Å away from the active site of the CDK[17,53,71,72] and that interacts with an RXL motif on specific substrates (FIG. 4b). The hydrophobic patches of different cyclins have minor sequence differences that might impart specificity for slightly different RXL motifs[53,73]. Importantly, the hydrophobic patch appears to be required *in vivo* for proper substrate recognition. For example, the hydrophobic patch of the yeast S-phase cyclin Clb5 has an important role in regulating DNA replication[74,75], presumably by directing Cdk1 to specific S-phase substrates.

Similar to the D domain of MAPKs, the hydrophobic patch appears to enhance substrate specificity by increasing the affinity of the kinase for the substrate[53]. This suggests that the hydrophobic patch either increases the local concentration of the substrate around the kinase[53] or that it correctly orientates the substrate in the kinase active site[76]. Consistent with the first hypothesis, it is often the case that substrates have a single RXL motif and multiple phosphorylation sites. It is unclear if the binding of the RXL motif occurs synchronously with the binding of the phosphorylation site. The exact mechanism by which the hydrophobic patch–RXL interaction enhances specificity is still uncertain, although it clearly targets specific cyclins to specific substrates and is important *in vivo*.

In addition to the activating cyclin, the specificity of CDKs is further influenced by the cyclin-dependent protein kinase regulatory subunit-1 (CKS1; also known as Suc1). CKS1 proteins are small (9–18 kDa) CDK-binding proteins that can also bind phosphorylated amino acids in CDK substrates[77,78]. The function of CKS1 in enhancing the substrate specificity of CDKs is not entirely clear, but it does play an important part in the phosphorylation of at least some essential substrates[79] and might have a role in enhancing the affinity of CDKs to substrates that have been previously phosphorylated by CDK1 or other kinases[80].

Conditional docking sites

The recruitment of kinases to substrates that have been previously phosphorylated (or primed) is a recurring theme. This priming can occur by phosphorylation of a residue that is close to or distant from the second phosphorylation site[5]. For example, the polo-like kinase PLK1 contains a polo-box domain (PBD)[81] that binds to phosphorylated substrates that have the consensus sequence S-pS/pT-P/X (REFS 81,82), where pS and pT stand for phospho-Ser and phospho-Thr, respectively. Because this is similar to the consensus sequence for CDK1 phosphorylation, an intriguing hypothesis is that active CDK1 primes a substrate for further phosphorylation and regulation by PLK1. Furthermore, in the absence of any substrate, the PBD binds to the kinase domain of PLK1 and inhibits it[82]. It is not until the PBD binds to a phosphorylated substrate that it moves out of the active site of PLK1, which allows PLK1 to phosphorylate the substrate at a second site. This mechanism ensures that the free kinase is held in an inactive state until it binds the correct substrates.

A similar priming event is required for some substrates of GSK3. GSK3 requires previous phosphorylation of a Ser residue in the P+4 site of the substrate for efficient phosphorylation[83]. The primed phosphorylation motif binds to a docking groove on GSK3 that is adjacent to the catalytic cleft of GSK3 (REFS 84–86). Interestingly, GSK3 is

RXL motif
A distal docking site that is found in substrates of cyclin-dependent kinases (CDKs). The RXL motif interacts with the hydrophobic patch that is found on the cyclin partner of the CDK.

Polo-like kinase
A conserved Ser/Thr kinase that is involved in mitotic progression. Polo-like kinases are activated by binding to peptide epitopes (often phosphoepitopes).

Box 3 | Proline-directed kinases and phosphatases

The vast majority of Ser/Thr kinases and phosphatases disfavour substrates that contain a Pro in the P+1 position. However, more than a quarter of the sites identified in global phosphorylation studies are phosphorylated (p)Ser-Pro or pThr-Pro sites (pSer-Pro:pThr-Pro = 5:1). This suggests that Pro-directed kinases are more promiscuous or regulate a larger number of proteins than non-Pro directed kinases. Pro-directed kinases such as cyclin-dependent kinase-2 (CDK2) and Pro-directed phosphatases such as CDC14 both use a similar strategy for accommodating the Pro ring by creating an apolar pocket that cannot satisfy the hydrogen-bonding requirements of the amide nitrogen of other residues[133]. Pro is disfavoured in the P+1 position by most kinases and phosphatases because the kinase or phosphatase residues that interact with the P+1 position expect to encounter a hydrogen-bonding partner for the backbone of the substrate.

itself phosphorylated and inactivated by phosphorylation on an N-terminal Ser residue by protein kinase B (PKB or AKT). This phosphorylated Ser residue loops back and binds the docking groove of GSK3, which inhibits the kinase from binding other primed substrates. A further example is PDK1, which interacts with several downstream AGC kinases (PKA, protein kinase G (PKG) and protein kinase C (PKC)) that contain a conserved PDK1-interaction fragment motif (PIF motif)[51,87]. PIF motifs require phosphorylation before they can bind to the PIF pocket that is located on PDK1 (REF. 88).

Priming phosphorylation adds a level of control beyond what is possible with non-conditional binding sites. Priming can function as an AND gate, making phosphorylation dependent on the activity of both the priming kinase and the ultimate kinase, and can help to establish the proper timing of substrate phosphorylation.

Localization of the kinase

A further layer of substrate specificity is achieved through the localization of protein kinases to distinct subcellular compartments or structures. Localization can increase the rate of substrate phosphorylation by increasing the concentrations of the reactants and can promote specificity by limiting the number of substrates to which a kinase has access.

Localization of an active kinase. Cyclins not only activate CDKs but often localize them to different subcellular compartments — this is thought to enhance their specificity greatly. For example, in vertebrate cells there are two mitotic cyclin B–CDK complexes: cyclin B1–CDK1 and cyclin B2–CDK1. Cyclin B2-containing complexes are localized primarily to the Golgi apparatus[89]. Cyclin B1 is localized in the cytoplasm during interphase and moves to the nucleus just before nuclear-envelope breakdown, where it accumulates on

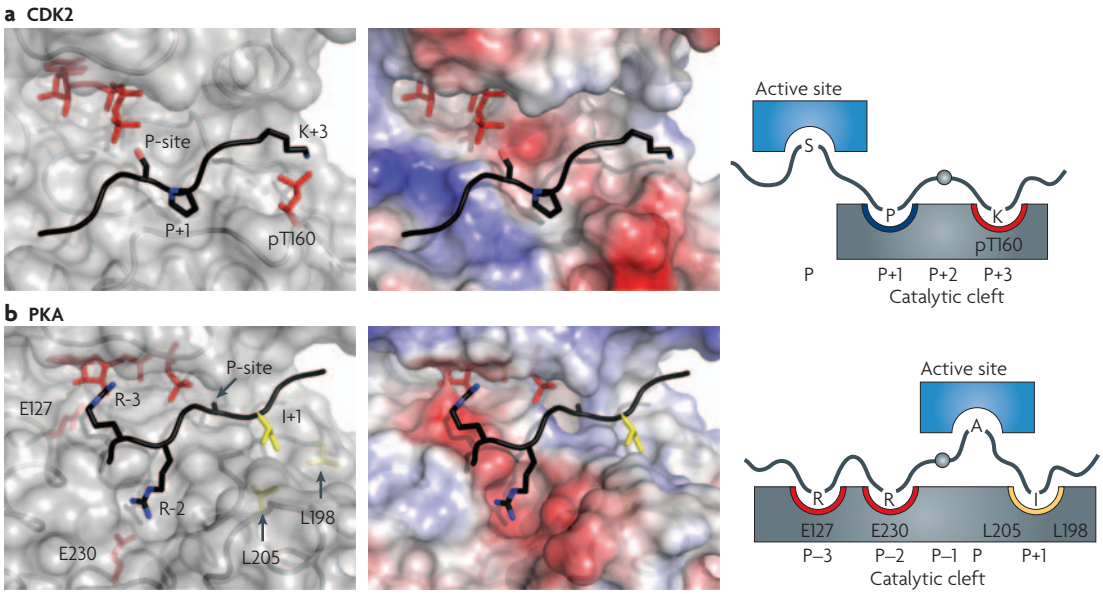

Figure 3 | **Local interactions are important in establishing specificity.** Ribbon diagrams, electrostatic surface representations and schematic diagrams of the substrate-binding site of cyclin-dependent kinase-2 (CDK2) bound to a substrate peptide (Protein Data Bank (PDB) ID: 1QMZ) (**a**); or the cyclic AMP-dependent protein kinase PKA bound to the non-phosphorylatable substrate mimetic PKI (PDB ID: 1ATP) (**b**). For CDK2, a Pro in the P+1 position of the substrate is greatly favoured over any other amino acid because there is no hydrogen-bonding partner available between the kinase and the P+1 position. The nitrogen atom in Pro is unique among the 20 amino acids because it is unable to form a hydrogen bond due to the cyclic binding of the three-carbon side chain back to the nitrogen atom of the backbone. The positively charged Lys in the P+3 position interacts strongly with the negatively charged phosphate on phospho-Thr160 of CDK2. If CDK2 is not phosphorylated at Thr160, it has a much lower binding affinity for peptide substrates and shows no preference for a positively charged amino acid in the P+3 position[33]. For PKA, the positively charged Arg residues at P–3 and P–2 of PKI interact with the negatively charged Glu127 and Glu230 of PKA. The hydrophobic Ile at P+1 fits into a hydrophobic pocket formed by Leu198 and Leu205 of PKA. These interactions contribute to the binding energy of PKI for PKA. Additional residues in PKI also contribute to high-affinity binding by interacting with residues further from the active site[45]. In the electrostatic surface representations, positively charged surfaces are in blue and negatively charged surfaces are in red.

Protein kinase C
(PKC). Classical PKC isoforms are activated by the presence of two second messengers: membrane-associated diacylglycerol and cytosolic calcium.

AND gate
A basic logic circuit in which two inputs together yield a high output, but either input alone yields no output.

the mitotic spindle[89–91]. Overexpression of either cyclin together with a form of CDK1 that is active in G0–G1 (CDK1AF) gives remarkable phenotypes. Cyclin B2 overexpression causes the Golgi to disassemble as it normally would during mitosis, but causes little else in the way of phenotypes[92]. Overexpressing cyclin B1 causes chromatin condensation, nuclear-envelope breakdown and microtubule reorganization, as well as Golgi disassembly. However, localizing cyclin B1 to the Golgi and cyclin B2 to the cytoplasm reverses their overexpression phenotypes. This suggests that the location of the two cyclin B–CDK1 complexes, rather than their intrinsic substrate specificity, dictates which mitotic events they regulate[92]. Similarly, cyclin B1 cannot normally support DNA replication in *Xenopus laevis* egg extracts that are depleted of cyclin E, but it does support replication if it is artificially targeted to the nucleus, where cyclin E normally functions[93]. Finally, in *S. cerevisiae*, reversing the localizations of cyclin-2 (Cln2) and Cln3 swaps their functions in promoting G1 progression[71,94].

Using localization to enhance specificity is not limited to CDKs. The localization of ERK2 has been shown to have a key role in the differentiation of PC12 cells[95]. Treatment of PC12 cells with nerve growth factor (NGF) drives neurite outgrowth that is dependent on ERK2 activation and translocation from the cytoplasm to the nucleus. An active form of ERK2 in the nucleus is enough to drive neurite outgrowth in the absence of NGF[96]. The treatment of PC12 cells with epidermal growth factor (EGF) promotes mitogenesis through ERK2 but does not appear to drive the nuclear localization of ERK2. These data indicate that ERK2 has two separate sets of substrates: cytoplasmic ones that bring about mitogenesis and nuclear ones for driving differentiation.

As another example, the peptide-substrate specificity of the many isoforms of PKC are similar but the isoforms are distributed to different subcellular compartments, which indicates that localization might be important in generating specificity[21,97]. Many PKC-binding proteins have been discovered and are termed receptors for activated C-kinase (RACKs)[98]. RACKs bind and localize active PKC to specific cellular locations, thereby limiting the number of substrates that PKCs are able to encounter and phosphorylate. Some RACKs also appear to be allosteric activators of PKC activity: binding of a RACK to some PKC isoforms enhances their activity by several fold[98].

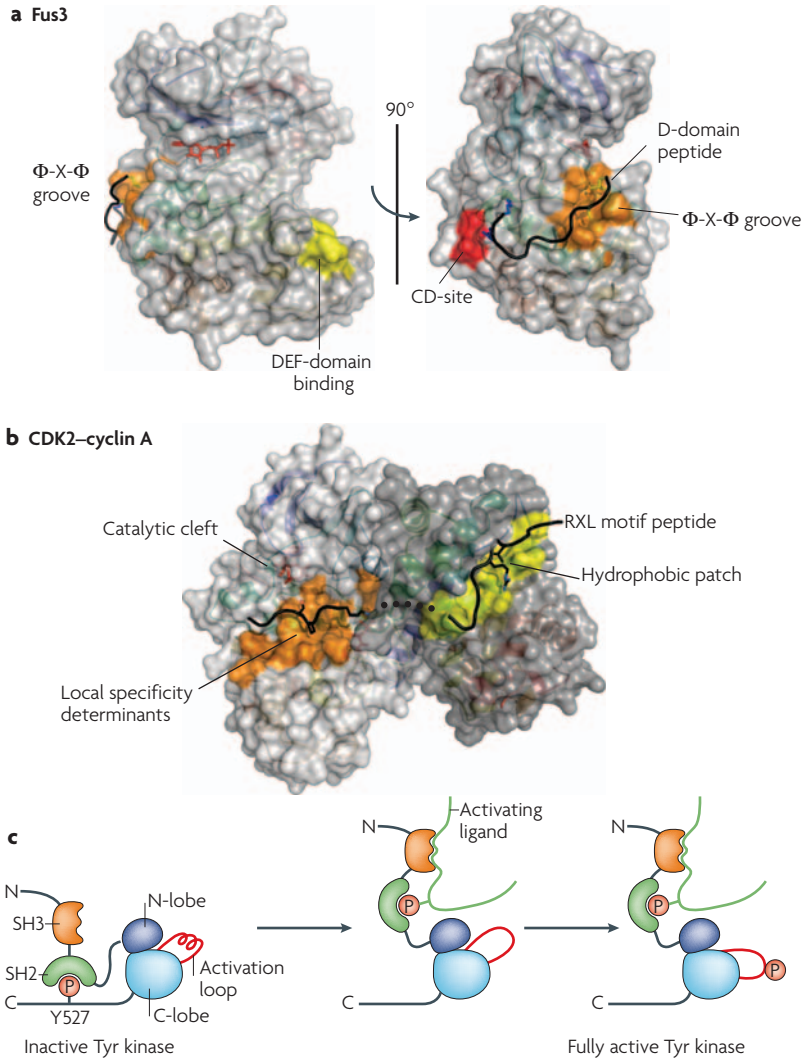

Figure 4 | **Distal docking sites have an important role in substrate recognition.** **a** | Surface representation of the yeast mitogen-activated protein kinase (MAPK) cell fusion-3 (Fus3) in complex with a D-domain-containing peptide substrate from the cyclin-dependent kinase inhibitor Far1 (black strand) (Protein Data Bank (PDB) ID: 2B9J). The negatively charged site (CD-site) in Fus3 (red area) binds the basic residues on the Far1 peptide. The Φ-X-Φ groove (where Φ is a hydrophobic residue and X is any residue) is shown in orange and binds the LXL residues. The region of the kinase that binds the FXF sequence of DEF-domain-containing substrates is shown in yellow. ATP is shown as a red ball and stick model. **b** | Surface representation of cyclin A (dark grey, right) bound to cyclin-dependent kinase-2 (CDK2; light grey, left) in complex with a substrate with an RXL docking motif (PDB ID: 2CCI). Local specificity determinants near the catalytic cleft of CDK2 are shown in orange. The hydrophobic patch on cyclin A is shown in yellow. The substrate peptide is shown in black. The middle of the substrate is disordered and is shown as a dotted line. **c** | Src is inactive because of an activation loop that blocks the catalytic site of the kinase between the N-terminal (dark blue) and C-terminal (light blue) lobes. Activation of Src requires dephosphorylation of a C-terminal Tyr residue (Y527), which allows the Src homology-2 (SH2) domain and/or SH3 domains to dissociate from the kinase and associate with a substrate[135]. The activation loop of Src is then phosphorylated (P) (at Y416) and the kinase becomes fully active. Part **c** is modified with permission from REF. 135 © (1999) Elsevier.

Localization of the inactive kinase. Interestingly, it is not always the active form of the kinase that is localized to specific sites. A-kinase anchoring proteins (AKAPs) are a family of >30 proteins with little sequence homology, but which all bind inactive PKA through the regulatory subunits (reviewed in REF. 99). Individual AKAPs have distinct subcellular targeting sequences or mechanisms that localize PKA to different locations in the cell (FIG. 5a). AKAPs could allow the generation of cAMP only in particular parts of the cell — those parts where the AKAPs concentrate — to result in PKA activation. AKAPs could also ensure that the activated PKA acts only on substrates that reside close to the AKAP. Either of these localization effects would depend on the rates of diffusion and destruction of the species involved. For example, the production of a substantial gradient of active PKA requires that the inactivation of the released PKA must occur quickly (relative to the

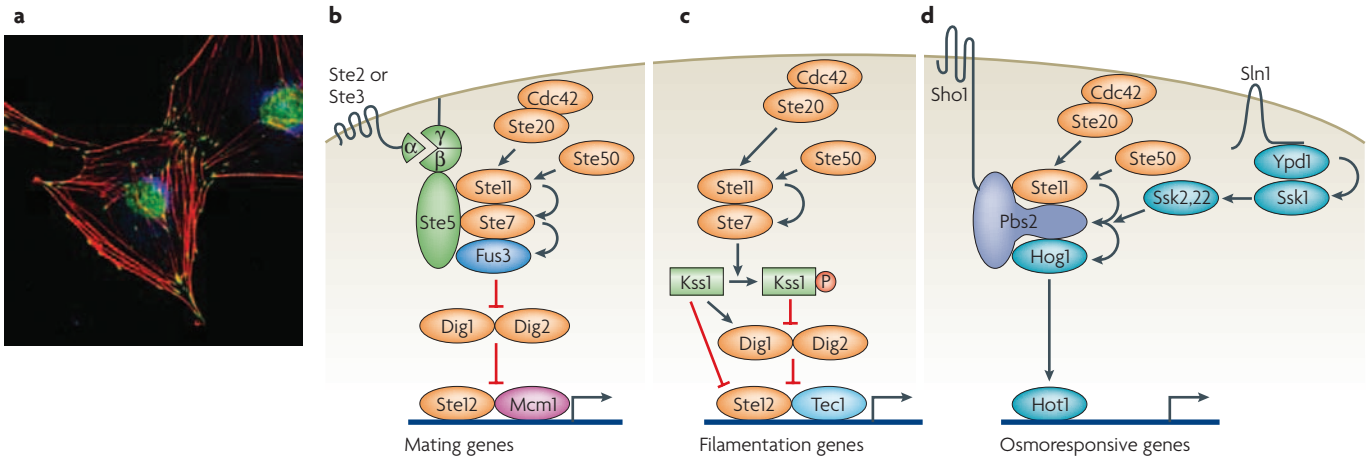

Figure 5 | **Scaffolds help to provide specificity. a** | The A-kinase anchoring protein WAVE1 assembles signalling complexes in specific cellular domains. Immunostaining of a Swiss 3T3 fibroblast shows WAVE1 (green) and the regulatory subunit of the cyclic AMP-dependent protein kinase PKA (blue). The actin cytoskeleton is stained red. **b** | In *Saccharomyces cerevisiae*, the mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) Ste11 (sterile-11) is involved in the mating, filamentation and osmolarity pathways, and the scaffolds it binds determine specificity. For the mating pathway, mating pheromone binds and activates a G protein-coupled receptor (Ste2 or Ste3), which releases a Gβγ complex (Ste4 and Ste18) from the inhibitory Gα subunit (Gpa1). Gβγ then recruits the Ste5-scaffolded complex (which comprises Ste11, the MAP kinase kinase (MAPKK) Ste7 and the MAP kinase (MAPK) cell fusion-3 (Fus3)) to the plasma membrane. Here, the signal-transducing kinase Ste20 (which is activated by Cdc42) activates Ste11 with the help of the adaptor protein Ste50. Ste11 activates Ste7, which indirectly activates mating-gene transcription and promotes cell-cycle arrest through the transcription factor Mcm1. Dig1 and Dig2 are constitutively expressed and pheromone-inducible regulatory proteins, respectively, that inhibit pheromone-responsive transcription. **c** | The filamentation pathway shares many of the same components as the mating pathway. Ste20 activates the MAPKKK Ste11, which activates the MAPKK Ste7. Ste7 phosphorylates (P) the filamentation MAPK Kss1 (kinase suppressor of Sst2 mutations-1), which indirectly activates transcription factors for filamentation genes (Tec1). **d** | The high osmolarity pathway has two redundant input branches (the Sho1 and Sln1 receptors) that, together with the MAPKKK Ste11, activate the MAPKK Pbs2 (polymyxin B sensitivity-2). Pbs2 also serves as a scaffold for the cascade. Active Pbs2 activates the MAPK Hog1 (high osmolarity glycerol response-1), which then activates transcription factors for osmoresponsive genes (such as high-osmolarity-induced transcription-1 (Hot1)). Ssk1, suppressor of sensor kinase-1; Ssk2, suppressor of sensor kinase-2; Ssk22, suppressor of sensor kinase-22; Ste12, transcription factor that activates genes involved in mating; Ypd1, Tyr (Y)-phosphatase-dependent-1. Part **a** is reproduced with permission from REF. 99 © (2004) Macmillan Publishers Ltd. Parts **b–d** are modified with permission from REF. 103 © (2004) Annual Reviews.

rate at which PKA diffuses away from the AKAP)[100]. Assuming a diffusion coefficient of ~10 $\mu m^2 sec^{-1}$ (a typical value for a cytoplasmic protein), then PKA must be inactivated within a second or so of its release from the AKAP to produce a gradient of active PKA molecules. It would be of interest to measure the diffusion rate and inactivation rate of PKA in a living cell.

Scaffolds

In addition to the direct interactions between protein kinases and their substrates, sometimes the two proteins interact through the intermediacy of adaptors or scaffolds, which act as organizing platforms that recruit both the kinase and the substrate to the same complex[25,101]. In some respects, scaffolds are similar to targeting subunits and there might be some overlap between the two categories. However, whereas targeting subunits tend to associate stably with the kinase, the interaction between a kinase and a scaffold might be more dynamic[102]. Also, whereas targeting subunits often mediate the interaction of kinases with terminal effector proteins, scaffolds are best known for coordinating the interactions of kinases with other kinases and phosphatases.

Many MAPK cascades and PKA pathways are dependent on scaffolding proteins to maintain kinase specificity. Scaffolds allow protein kinases to achieve different substrate specificity depending on the composition of the scaffolding complex. For example, the yeast MAPK kinase kinase (MAPKKK) Ste11 (sterile-11) is a component of three distinct MAPK cascades that are involved in three different biological processes: the mating, invasive growth and high-osmolarity responses[103] (FIG. 5b–d). The choice of scaffold determines, at least in part, the process that Ste11 regulates and ensures that active Ste11 activates only one of the three pathways to prevent cross-talk with other MAPK cascades[103].

Several studies have shown that artificial scaffolds can be engineered to generate novel kinase–substrate interactions. For example, chimeric scaffolds made from components of the yeast mating and high osmolarity MAPK pathways can convert a mating signal into an osmolarity response[104]. In mammalian cells, chimeric adaptor proteins that link growth-factor receptors to apoptotic signalling proteins can be used to convert proliferative signals into pro-apoptotic responses[105]. Differential localization or expression of scaffolding

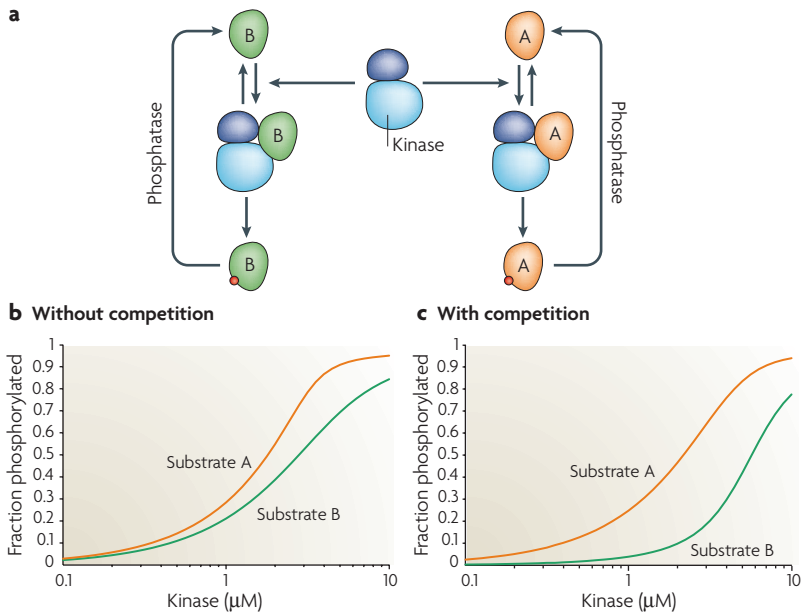

Figure 6 | **Substrate competition for phosphorylation. a** | Reaction diagram that shows a protein kinase that can phosphorylate two substrates, good substrate A (orange) and bad substrate B (green), in the face of a constitutive phosphatase. The small red circle represents phosphorylation. **b** | Steady-state stimulus–response curves for A and B are similar if only one of the two substrates is present. **c** | The phosphorylation of the poorer substrate (substrate B) is dramatically reduced in the presence of A. The steady-state levels of phosphorylation of A and B were modelled using a set of ordinary differential equations with the following rate constants and initial conditions: [Kinase] = 0.1–10 μM, [A] = 100 μM or 0 μM, [B] = 100 μM or 0 μM, k_{on}(A) = 1.0×10^6 (s^{-1} M^{-1}), k_{off}(A) = 1.0×10^6 (s^{-1}), k_{cat}(A) = 3 (s^{-1}), k_{on}(B) = 1.0×10^6 (s^{-1} M^{-1}), k_{off}(B) = 30.0×10^6 (s^{-1}), k_{cat}(B) = 3 (s^{-1}), $k_{phosphatase}$ = 0.1 (s^{-1}). The salient properties of this system are that the kinetic constants are realistic and that the k_{off} for substrate B is 30 times greater than the k_{off} for substrate A.

proteins in specific cell types can also help to enhance substrate specificity. Splice variants of scaffolds that lack specific interactions or localization motifs have also been identified[106,107], which allow different tissue types to use the same basic cascade with minor changes in complex composition[108].

Furthermore, some scaffolds appear to have more active roles beyond the passive recruitment of a kinase and substrate. The yeast Ste5 scaffold allosterically activates the autophosphorylation of a Tyr in the activation loop of the MAPK Fus3 (cell fusion-3)[109]. The active form of Fus3 then phosphorylates Ste5 and appears to be part of a negative feedback loop that downregulates signalling output from the mating pathway. Therefore, scaffolds not only bring kinases and substrates together in space, but they also precisely tune the quantitative characteristics of the pathway.

In several cases, scaffolds have been found to recruit other regulators of kinases. For example, many AKAPs not only control the localization of inactive PKA (discussed above), but they also serve as scaffolds[99] and recruit other important signalling proteins, including phosphatases such as PP2B[110], kinases such as PKC[111] and PDEs such as PDE4 that breaks down local concentrations of cAMP to inactivate PKA[112]. PDE4 recruitment potentially helps to maintain a gradient of PKA activation. Similarly, the scaffold protein JIP1 (JNK interacting protein-1) recruits the MAPK JNK and its upstream MAPK kinase and MAPKKK[113,114]. It also recruits the JNK phosphatase MKP7, which targets it to dephosphorylate JNK[115]. Another JNK scaffold, β-arrestin-2, dynamically binds MKP7. MKP7 dissociates from the scaffold on pathway stimulation and then rebinds after removal of or adaptation to stimulation[116]. Scaffolds might, therefore, modulate positive and negative feedback loops between the kinases that are bound to them. By recruiting phosphatases or enforcing positive or negative feedback, scaffolds could modulate the output of signalling pathways into sudden pulses of activity, ultrasensitive responses and hysteretic responses.

Systems-level effects

Kinase–substrate interactions are often studied *in vitro* using a purified kinase and a purified substrate mixed in a buffer of the researcher's choice. However, in a cell the substrates of kinases are present in a mix of thousands of non-target proteins. Often, a kinase will have more than one substrate *in vivo*, and each substrate acts as a competitive inhibitor for other substrates. Furthermore, there are phosphatases that oppose the phosphorylation of substrates. Taking all of these other components into consideration can lead to complex phosphorylation behaviours that are referred to as systems-level effects.

Competition. A theoretical situation can be considered whereby a protein kinase phosphorylates two substrates, A and B, in the face of a constitutive phosphatase (FIG. 6). Rate constants can be chosen such that the steady-state stimulus–response curves for A and B are similar if only one of the two substrates is present, but the phosphorylation of the poorer substrate (substrate B) is dramatically reduced if A is present as well. In this situation, the presence of A suppresses the phosphorylation of B through competition for access to the kinase. The suppression is particularly dramatic at low kinase concentrations: the presence of A builds a kinase-level threshold and a temporal lag into the phosphorylation of B.

This type of systems-level improvement in specificity arises when a kinase is saturated by its preferred substrates, which keeps the levels of free kinase available to other 'second-tier' substrates low. In principle, competition for a saturated kinase could produce switch-like, ultrasensitive steady-state responses in second-tier substrates, and could generate temporal ordering in substrate phosphorylation, where the kinase shifts to second-tier substrates once the phosphorylation of the best substrates is near completion. Such mechanisms have been invoked to explain how specificity arises in the interaction of yeast CDKs with their substrates[117], and how thresholds arise in the phosphorylation of vertebrate WEE1 by CDKs[118].

It will be important to establish how often protein kinases run close to the substrate saturation conditions in which these competition effects can be significant. In one particular case, Loog and Morgan estimated that the total concentration of Cdk1 consensus sites in *S. cerevisiae* is approximately 1 mM, whereas typical K_m values are

Ultrasensitive response
A response to an increasing stimulus that is described by a sigmoidal dose-response curve. Low levels of stimulus produce a poor response but, as the stimulus level increases, there is an abrupt increase in the response to near-maximal levels.

Steady state
A condition that is reached when the concentrations of reactants and products in a complex system do not change with time.

1–100 μM[117], which suggests that Cdk1 is probably saturated by substrates. This indicates that competition among substrates could be an important contributor to the specificity of CDKs.

Error correction. Many substrates need to be phosphorylated at multiple sites before their function is altered. This requirement can allow phosphatases to correct an errant phosphorylation before the occurrence of the crucial second (or *n*th) phosphorylation that alters the function of a substrate. This idea of iterative discrimination lies at the heart of kinetic proofreading, a concept that was proposed by Hopfield to explain the high specificity of biosynthetic reactions[119] and which is also applicable to protein phosphorylation reactions[120,121]. Therefore, if off-target phosphorylation occurs — despite the many mechanisms described above for ensuring correct kinase–substrate pairing — the combination of active phosphatases and a requirement for multisite phosphorylation can minimize the consequences of off-target phosphorylation.

Other error-correction mechanisms have evolved to ensure that signalling specificity is preserved in kinase signalling pathways. The kinase cascades shown in FIG. 5b are not completely insulated and signals from one pathway can leak into other pathways that share components. For example, pheromone addition to yeast leads to Ste5-scaffold-mediated activation of the Ste11–Ste7–Fus3 MAPK cascade as well as significant activation of the filamentation-specific MAPK Kss1 (kinase suppressor of Sst2 mutations-1)[122,123]. The system seems to have evolved a cross-pathway inhibition mechanism to ensure that this leak does not lead to the transcription of genes that are involved in the activation of filamentation. This is achieved by Fus3 phosphorylating the filamentation-specific transcription factor Tec1, which targets Tec1 for degradation and prevents the transcription of filamentation-specific genes[124,125]. Expression of a non-phosphorylatable Tec1 results in a loss of signalling specificity such that the pheromone signal now activates filamentation gene expression[124,125]. There is also cross-pathway inhibition between the pheromone pathway and the high-osmolarity pathway, although the molecular mechanism is unclear[126]. It is likely that kinase signalling *in vivo* is not 100% specific at the level of kinase–substrate interactions, but organisms have probably evolved mechanisms to suppress the activation of pathways in which cross-talk would be detrimental.

Conclusions and future outlook

In summary, kinases use many different mechanisms to phosphorylate their proper substrates selectively to maintain the specificity that is observed in signalling pathways. The consensus phosphorylation site and Ser/Thr or Tyr specificity is determined by the structure of the catalytic cleft of the kinase and local interactions between the kinase cleft and the substrate phosphorylation site. Distal binding interactions between the kinase and the substrate often provide additional binding interactions, and sometimes provide allosteric regulation and localization to specific cellular compartments or structures. Systems-level effects such as competition, multisite phosphorylation and kinetic proofreading help to regulate specificity in complex mixtures of proteins. Although not all kinases make use of all of these mechanisms, we propose that many protein kinases use at least some of them to achieve the specificity required for robust signal transduction. Important open questions remain as to exactly which mechanisms are important for which kinases, and what degree of specificity is required and achieved for protein phosphorylation.

1. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298**, 1912–1934 (2002).
This study catalogues and classifies the complete complement of protein kinases in the human genome.
2. Manning, G., Plowman, G. D., Hunter, T. & Sudarsanam, S. Evolution of protein kinase signaling from yeast to man. *Trends Biochem. Sci.* **27**, 514–520 (2002).
3. Caenepeel, S., Charydczak, G., Sudarsanam, S., Hunter, T. & Manning, G. The mouse kinome: discovery and comparative genomics of all mouse protein kinases. *Proc. Natl Acad. Sci. USA* **101**, 11707–11712 (2004).
4. Zhu, H. *et al.* Analysis of yeast protein kinases using protein chips. *Nature Genet.* **26**, 283–289 (2000).
5. Pinna, L. A. & Ruzzene, M. How do protein kinases recognize their substrates? *Biochim. Biophys. Acta* **1314**, 191–225 (1996).
6. Cohen, P. The regulation of protein function by multisite phosphorylation — a 25 year update. *Trends Biochem. Sci.* **25**, 596–601 (2000).
7. Echols, N. *et al.* Comprehensive analysis of amino acid and nucleotide composition in eukaryotic genomes, comparing genes and pseudogenes. *Nucleic Acids Res.* **30**, 2515–2523 (2002).
8. Ptacek, J. *et al.* Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679–684 (2005).
The authors test the ability of 75% of all yeast kinases to phosphorylate 4,400 yeast proteins that were spotted on high-density protein arrays.
9. Ubersax, J. A. *et al.* Targets of the cyclin-dependent kinase Cdk1. *Nature* **425**, 859–864 (2003).
This study screens a yeast proteomic library for proteins that are directly phosphorylated by Cdk1 in whole-cell extracts and identifies ~200 substrates.
10. Hanks, S. K., Quinn, A. M. & Hunter, T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42–52 (1988).
11. Hanks, S. K. & Hunter, T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9**, 576–596 (1995).
12. Knighton, D. R. *et al.* Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 407–414 (1991).
This first crystal structure of the cAMP-dependent protein kinase PKA is the touchstone for all subsequent structural studies of kinases.
13. Knighton, D. R. *et al.* 2.0 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with a peptide inhibitor and detergent. *Acta Crystallogr. D* **49**, 357–361 (1993).
14. Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H. & Huber, R. Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 Å structure of the complex with Mn^{2+} adenylyl imidodiphosphate and inhibitor peptide PKI(5–24). *EMBO J.* **12**, 849–859 (1993).
15. De Bondt, H. L. *et al.* Crystal structure of cyclin-dependent kinase 2. *Nature* **363**, 595–602 (1993).
16. Zhang, F., Strand, A., Robbins, D., Cobb, M. H. & Goldsmith, E. J. Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. *Nature* **367**, 704–711 (1994).
17. Jeffrey, P. D. *et al.* Mechanism of CDK activation revealed by the structure of a cyclin A–CDK2 complex. *Nature* **376**, 313–320 (1995).
18. Xu, W., Harrison, S. C. & Eck, M. J. Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**, 595–602 (1997).
19. Lowe, E. D. *et al.* The crystal structure of a phosphorylase kinase peptide substrate complex: kinase substrate recognition. *EMBO J.* **16**, 6646–6658 (1997).
20. Faux, M. C. & Scott, J. D. More on target with protein phosphorylation: conferring specificity by location. *Trends Biochem. Sci.* **21**, 312–315 (1996).
21. Sim, A. T. & Scott, J. D. Targeting of PKA, PKC and protein phosphatases to cellular microdomains. *Cell Calcium* **26**, 209–217 (1999).
22. Sharrocks, A. D., Yang, S. H. & Galanis, A. Docking domains and substrate-specificity determination for MAP kinases. *Trends Biochem. Sci.* **25**, 448–453 (2000).
23. Adams, J. A. Kinetic and catalytic mechanisms of protein kinases. *Chem. Rev.* **101**, 2271–2290 (2001).
A terrific, chemically-orientated review that includes a detailed discussion of the kinetic and catalytic mechanisms of protein kinases.
24. Biondi, R. M. & Nebreda, A. R. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem. J.* **372**, 1–13 (2003).

25. Bhattacharyya, R. P., Remenyi, A., Yeh, B. J. & Lim, W. A. Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. *Annu. Rev. Biochem.* **75**, 655–680 (2006).
26. Remenyi, A., Good, M. C. & Lim, W. A. Docking interactions in protein kinase and phosphatase networks. *Curr. Opin. Struct. Biol.* **16**, 676–685 (2006).
27. Shi, Z., Resing, K. A. & Ahn, N. G. Networks for the allosteric control of protein kinases. *Curr. Opin. Struct. Biol.* **16**, 686–692 (2006).
28. Hubbard, S. R. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J.* **16**, 5572–5581 (1997).
29. Brown, N. R., Noble, M. E., Endicott, J. A. & Johnson, L. N. The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. *Nature Cell Biol.* **1**, 438–443 (1999).
30. Jia, Z., Barford, D., Flint, A. J. & Tonks, N. K. Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science* **268**, 1754–1758 (1995).
31. Yang, J. *et al.* Structural basis for substrate specificity of protein-tyrosine phosphatase SHP-1. *J. Biol. Chem.* **275**, 4066–4071 (2000).
32. Andersen, J. N. *et al.* Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol. Cell. Biol* **21**, 7117–7136 (2001).
33. Kemp, B. E., Graves, D. J., Benjamini, E. & Krebs, E. G. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **252**, 4888–4894 (1977).
34. Hunter, T. Synthetic peptide substrates for a tyrosine protein kinase. *J. Biol. Chem.* **257**, 4843–4848 (1982).
35. Foulkes, J. G., Chow, M., Gorka, C., Frackelton, A. R. Jr & Baltimore, D. Purification and characterization of a protein-tyrosine kinase encoded by the Abelson murine leukemia virus. *J. Biol. Chem.* **260**, 8070–8077 (1985).
36. Kemp, B. E., Bylund, D. B., Huang, T. S. & Krebs, E. G. Substrate specificity of the cyclic AMP-dependent protein kinase. *Proc. Natl Acad. Sci. USA* **72**, 3448–3452 (1975).
37. Daile, P., Carnegie, P. R. & Young, J. D. Synthetic substrate for cyclic AMP-dependent protein kinase. *Nature* **257**, 416–418 (1975).
38. Pearson, R. B. & Kemp, B. E. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.* **200**, 62–81 (1991).
39. Songyang, Z. *et al.* Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr. Biol.* **4**, 973–982 (1994).
The authors developed a technique for determining the substrate specificity and consensus phosphorylation sites of protein kinases using an orientated library of >2.5 billion peptide substrates.
40. Hutti, J. E. *et al.* A rapid method for determining protein kinase phosphorylation specificity. *Nature Methods* **1**, 27–29 (2004).
41. Olsen, J. V. *et al.* Global, *in vivo*, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**, 635–648 (2006).
42. Yaffe, M. B. *et al.* A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nature Biotech.* **19**, 348–353 (2001).
43. Holmes, J. K. & Solomon, M. J. The role of Thr160 phosphorylation of Cdk2 in substrate recognition. *Eur. J. Biochem.* **268**, 4647–4652 (2001).
44. Zheng, J. *et al.* 2.2 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MnATP and a peptide inhibitor. *Acta Crystallogr. D* **49**, 362–365 (1993).
45. Taylor, S. S. *et al.* Dynamics of signaling by PKA. *Biochim. Biophys. Acta* **1754**, 25–37 (2005).
46. Holland, P. M. & Cooper, J. A. Protein modification: docking sites for kinases. *Curr. Biol.* **9**, R329–R331 (1999).
47. Kallunki, T. *et al.* JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.* **8**, 2996–3007 (1994).
48. Bardwell, L., Cook, J. G., Chang, E. C., Cairns, B. R. & Thorner, J. Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. *Mol. Cell. Biol.* **16**, 3637–3650 (1996).
49. Bardwell, L. & Thorner, J. A conserved motif at the amino termini of MEKs might mediate high-affinity interaction with the cognate MAPKs. *Trends Biochem. Sci.* **21**, 373–374 (1996).
50. Williams, D. D., Marin, O., Pinna, L. A. & Proud, C. G. Phosphorylated seryl and threonyl, but not tyrosyl, residues are efficient specificity determinants for GSK-3β and Shaggy. *FEBS Lett.* **448**, 86–90 (1999).
51. Biondi, R. M. *et al.* Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *EMBO J.* **19**, 979–988 (2000).
52. Adams, P. D. *et al.* Identification of a cyclin–CDK2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Mol. Cell. Biol.* **16**, 6623–6633 (1996).
53. Schulman, B. A., Lindstrom, D. L. & Harlow, E. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc. Natl Acad. Sci. USA* **96**, 10453–10458 (1998).
54. Chen, Y. G. *et al.* Determinants of specificity in TGF-β signal transduction. *Genes Dev.* **12**, 2144–2152 (1998).
55. Lo, R. S., Chen, Y. G., Shi, Y., Pavletich, N. P. & Massague, J. The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-β receptors. *EMBO J.* **17**, 996–1005 (1998).
56. Tanoue, T., Adachi, M., Moriguchi, T. & Nishida, E. A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nature Cell Biol.* **2**, 110–116 (2000).
57. Gupta, S., Campbell, D., Derijard, B. & Davis, R. J. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**, 389–393 (1995).
58. Livingstone, C., Patel, G. & Jones, N. ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.* **14**, 1785–1797 (1995).
59. Yang, S. H., Yates, P. R., Whitmarsh, A. J., Davis, R. J. & Sharrocks, A. D. The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. *Mol. Cell. Biol.* **18**, 710–720 (1998).
60. Yang, S. H., Galanis, A. & Sharrocks, A. D. Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol. Cell. Biol.* **19**, 4028–4038 (1999).
61. Lee, T. *et al.* Docking motif interactions in MAP kinases revealed by hydrogen exchange mass spectrometry. *Mol. Cell* **14**, 43–55 (2004).
This study used hydrogen-exchange mass spectroscopy to identify conformational changes in MAPK on binding of DEF-domain and D-domain peptides.
62. Remenyi, A., Good, M. C., Bhattacharyya, R. P. & Lim, W. A. The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. *Mol. Cell* **20**, 951–962 (2005).
Structural analysis of MAPK bound to three different binding partners, which demonstrates how different D-domain peptides can selectively bind different MAPKs through a common docking groove and have different effects on kinase activity.
63. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J. & Kornfeld, K. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev.* **13**, 163–175 (1999).
This paper identified two important ERK docking sites through the analysis of *lin-1* mutations in *Caenorhabditis elegans*.
64. Jacobs, D., Beitel, G. J., Clark, S. G., Horvitz, H. R. & Kornfeld, K. Gain-of-function mutations in the *Caenorhabditis elegans lin-1* ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. *Genetics* **149**, 1809–1822 (1998).
65. MacKenzie, S. J., Baillie, G. S., McPhee, I., Bolger, G. B. & Houslay, M. D. ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases. The involvement of COOH-terminal docking sites and NH_2-terminal UCR regions. *J. Biol. Chem.* **275**, 16609–16617 (2000).
66. Dimitri, C. A., Dowdle, W., MacKeigan, J. P., Blenis, J. & Murphy, L. O. Spatially separate docking sites on ERK2 regulate distinct signaling events *in vivo*. *Curr. Biol.* **15**, 1319–1324 (2005).
67. Hubbard, S. R. & Till, J. H. Protein tyrosine kinase structure and function. *Annu. Rev. Biochem.* **69**, 373–398 (2000).
68. Deshaies, R. J. & Ferrell, J. E. Jr. Multisite phosphorylation and the countdown to S phase. *Cell* **107**, 819–822 (2001).
69. Chang, C. I., Xu, B. E., Akella, R., Cobb, M. H. & Goldsmith, E. J. Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. *Mol. Cell* **9**, 1241–1249 (2002).
70. Heo, Y. S. *et al.* Structural basis for the selective inhibition of JNK1 by the scaffolding protein JIP1 and SP600125. *EMBO J.* **23**, 2185–2195 (2004).
71. Miller, M. E. & Cross, F. R. Cyclin specificity: how many wheels do you need on a unicycle? *J. Cell Sci.* **114**, 1811–1820 (2001).
72. Cheng, K. Y. *et al.* The role of the phospho-CDK2/cyclin A recruitment site in substrate recognition. *J. Biol. Chem.* **281**, 23167–23179 (2006).
73. Archambault, V., Buchler, N. E., Wilmes, G. M., Jacobson, M. D. & Cross, F. R. Two-faced cyclins with eyes on the targets. *Cell Cycle* **4**, 125–130 (2005).
74. Cross, F. R., Yuste-Rojas, M., Gray, S. & Jacobson, M. D. Specialization and targeting of B-type cyclins. *Mol. Cell* **4**, 11–19 (1999).
75. Wilmes, G. M. *et al.* Interaction of the S-phase cyclin Clb5 with an 'RXL' docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. *Genes Dev.* **18**, 981–991 (2004).
76. Takeda, D. Y., Wohlschlegel, J. A. & Dutta, A. A bipartite substrate recognition motif for cyclin-dependent kinases. *J. Biol. Chem.* **276**, 1993–1997 (2001).
77. Arvai, A. S., Bourne, Y., Hickey, M. J. & Tainer, J. A. Crystal structure of the human cell cycle protein CksHs1: single domain fold with similarity to kinase N-lobe domain. *J. Mol. Biol.* **249**, 835–842 (1995).
78. Bourne, Y. *et al.* Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. *Cell* **84**, 863–874 (1996).
79. Patra, D. & Dunphy, W. G. Xe-p9, a *Xenopus* Suc1/Cks protein, is essential for the Cdc2-dependent phosphorylation of the anaphase-promoting complex at mitosis. *Genes Dev.* **12**, 2549–2559 (1998).
80. Tang, Y. & Reed, S. I. The Cdk-associated protein Cks1 functions both in G1 and G2 in *Saccharomyces cerevisiae*. *Genes Dev.* **7**, 822–832 (1993).
81. Elia, A. E., Cantley, L. C. & Yaffe, M. B. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science* **299**, 1228–1231 (2003).
82. Elia, A. E. *et al.* The molecular basis for phosphodependent substrate targeting and regulation of Plks by the polo-box domain. *Cell* **115**, 83–95 (2003).
References 81 and 82 discover and explain the binding of polo-box domains to phosphorylated peptides.
83. Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W. & Roach, P. J. Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *J. Biol. Chem.* **262**, 14042–14048 (1987).
84. Dajani, R. *et al.* Crystal structure of glycogen synthase kinase 3β: structural basis for phosphate-primed substrate specificity and autoinhibition. *Cell* **105**, 721–732 (2001).
85. Frame, S., Cohen, P. & Biondi, R. M. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Mol. Cell* **7**, 1321–1327 (2001).
86. ter Haar, E. *et al.* Structure of GSK3β reveals a primed phosphorylation mechanism. *Nature Struct. Biol.* **8**, 593–596 (2001).
87. Frodin, M., Jensen, C. J., Merienne, K. & Gammeltoft, S. A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. *EMBO J.* **19**, 2924–2934 (2000).
88. Biondi, R. M. *et al.* High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site. *EMBO J.* **21**, 4219–4228 (2002).
89. Jackman, M., Firth, M. & Pines, J. Human cyclins B1 and B2 are localized to strikingly different structures: B1 to microtubules, B2 primarily to the Golgi apparatus. *EMBO J.* **14**, 1646–1654 (1995).

90. Hagting, A., Jackman, M., Simpson, K. & Pines, J. Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. *Curr. Biol.* **9**, 680–689 (1999).
91. Jackman, M., Lindon, C., Nigg, E. A. & Pines, J. Active cyclin B1–Cdk1 first appears on centrosomes in prophase. *Nature Cell Biol.* **5**, 143–148 (2003).
92. Draviam, V. M., Orrechia, S., Lowe, M., Pardi, R. & Pines, J. The localization of human cyclins B1 and B2 determines CDK1 substrate specificity and neither enzyme requires MEK to disassemble the Golgi apparatus. *J. Cell Biol.* **152**, 945–958 (2001).
References 89–92 provide compelling evidence for the importance of localization in CDK function.
93. Moore, J. D., Kirk, J. A. & Hunt, T. Unmasking the S-phase-promoting potential of cyclin B1. *Science* **300**, 987–990 (2003).
94. Miller, M. E. & Cross, F. R. Distinct subcellular localization patterns contribute to functional specificity of the Cln2 and Cln3 cyclins of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **20**, 542–555 (2000).
95. Vaudry, D., Stork, P. J., Lazarovici, P. & Eiden, L. E. Signaling pathways for PC12 cell differentiation: making the right connections. *Science* **296**, 1648–1649 (2002).
96. Robinson, M. J., Stippec, S. A., Goldsmith, E., White, M. A. & Cobb, M. H. A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. *Curr. Biol.* **8**, 1141–1150 (1998).
97. Disatnik, M. H., Buraggi, G. & Mochly-Rosen, D. Localization of protein kinase C isozymes in cardiac myocytes. *Exp. Cell Res.* **210**, 287–297 (1994).
98. Mochly-Rosen, D., Khaner, H. & Lopez, J. Identification of intracellular receptor proteins for activated protein kinase C. *Proc. Natl Acad. Sci. USA* **88**, 3997–4000 (1991).
99. Wong, W. & Scott, J. D. AKAP signalling complexes: focal points in space and time. *Nature Rev. Mol. Cell Biol.* **5**, 959–970 (2004).
100. Brown, G. C. & Kholodenko, B. N. Spatial gradients of cellular phospho-proteins. *FEBS Lett.* **457**, 452–454 (1999).
101. Pawson, T. & Scott, J. D. Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075–2080 (1997).
102. van Drogen, F., Stucke, V. M., Jorritsma, G. & Peter, M. MAP kinase dynamics in response to pheromones in budding yeast. *Nature Cell Biol.* **3**, 1051–1059 (2001).
103. Schwartz, M. A. & Madhani, H. D. Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **38**, 725–748 (2004).
104. Park, S. H., Zarrinpar, A. & Lim, W. A. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. *Science* **299**, 1061–1064 (2003).
This study demonstrates that the scaffold Ste5 tethers kinase–substrate pairs in space, and that changing the proteins to which Ste5 binds can produce non-natural input–output properties.
105. Howard, P. L., Chia, M. C., Del Rizzo, S., Liu, F. F. & Pawson, T. Redirecting tyrosine kinase signaling to an apoptotic caspase pathway through chimeric adaptor proteins. *Proc. Natl Acad. Sci. USA* **100**, 11267–11272 (2003).
106. Feliciello, A., Gottesman, M. E. & Avvedimento, E. V. The biological functions of A-kinase anchor proteins. *J. Mol. Biol.* **308**, 99–114 (2001).
107. Sierralta, J. & Mendoza, C. PDZ-containing proteins: alternative splicing as a source of functional diversity. *Brain Res. Brain Res. Rev.* **47**, 105–115 (2004).
108. Muller, J., Cacace, A. M., Lyons, W. E., McGill, C. B. & Morrison, D. K. Identification of B-KSR1, a novel brain-specific isoform of KSR1 that functions in neuronal signaling. *Mol. Cell. Biol.* **20**, 5529–5539 (2000).
109. Bhattacharyya, R. P. *et al.* The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. *Science* **311**, 822–826 (2006).
This study demonstrates that scaffolds are not passive participants in signalling cascades but can allosterically activate binding partners.
110. Coghlan, V. M. *et al.* Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. *Science* **267**, 108–111 (1995).
111. Klauck, T. M. *et al.* Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. *Science* **271**, 1589–1592 (1996).
112. Dodge, K. L. *et al.* mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. *EMBO J.* **20**, 1921–1930 (2001).
113. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J. & Davis, R. J. A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science* **281**, 1671–1674 (1998).
114. Yasuda, J., Whitmarsh, A. J., Cavanagh, J., Sharma, M. & Davis, R. J. The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol. Cell. Biol.* **19**, 7245–7254 (1999).
115. Willoughby, E. A., Perkins, G. R., Collins, M. K. & Whitmarsh, A. J. The JNK-interacting protein-1 scaffold protein targets MAPK phosphatase-7 to dephosphorylate JNK. *J. Biol. Chem.* **278**, 10731–10736 (2003).
116. Willoughby, E. A. & Collins, M. K. Dynamic interaction between the dual specificity phosphatase MKP7 and the JNK3 scaffold protein β-arrestin 2. *J. Biol. Chem.* **280**, 25651–25658 (2005).
117. Loog, M. & Morgan, D. O. Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature* **434**, 104–108 (2005).
This study shows how the intrinsic biochemical properties of different cyclins help to promote the correct timing of CDK substrate phosphorylation during the cell cycle.
118. Kim, S. Y. & Ferrell, J. E. Jr. Substrate competition as a source of ultrasensitivity in the inactivation of WEE1. *Cell* **128**, 1133–1145 (2007).
119. Hopfield, J. J. Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity. *Proc. Natl Acad. Sci. USA* **71**, 4135–4139 (1974).
This important study describes how kinetic proofreading, driven by phosphate hydrolysis, can increase the specificity of biological processes beyond the level available from free-energy differences in intermediates or kinetic barriers.
120. Swain, P. S. & Siggia, E. D. The role of proofreading in signal transduction specificity. *Biophys. J.* **82**, 2928–2933 (2002).
121. Ferrell, J. E. Jr. & Bhatt, R. R. Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. *J. Biol. Chem.* **272**, 19008–19016 (1997).
122. Breitkreutz, A., Boucher, L. & Tyers, M. MAPK specificity in the yeast pheromone response independent of transcriptional activation. *Curr. Biol.* **11**, 1266–1271 (2001).
123. Sabbagh, W. Jr, Flatauer, L. J., Bardwell, A. J. & Bardwell, L. Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation. *Mol. Cell* **8**, 683–691 (2001).
124. Bao, M. Z., Schwartz, M. A., Cantin, G. T., Yates, J. R. & Madhani, H. D. Pheromone-dependent destruction of the Tec1 transcription factor is required for MAP kinase signaling specificity in yeast. *Cell* **119**, 991–1000 (2004).
125. Chou, S., Huang, L. & Liu, H. Fus3-regulated Tec1 degradation through SCFCdc4 determines MAPK signaling specificity during mating in yeast. *Cell* **119**, 981–990 (2004).
126. McClean, M. N., Mody, A., Broach, J. R. & Ramanathan, S. Cross-talk and decision making in MAP kinase pathways. *Nature Genet.* **39**, 409–414 (2007).
References 124–126 demonstrate that input signals will sometimes leak along pathways with shared signalling components; the studies also identify additional mechanisms that ensure that these leaks do not lead to aberrant outputs.
127. Pinna, L. A. & Donella-Deana, A. Phosphorylated synthetic peptides as tools for studying protein phosphatases. *Biochim. Biophys. Acta* **1222**, 415–431 (1994).
128. Hunter, T. & Sefton, B. M. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl Acad. Sci. USA* **77**, 1311–1315 (1980).
129. Alonso, A. *et al.* Protein tyrosine phosphatases in the human genome. *Cell* **117**, 699–711 (2004).
130. Arena, S., Benvenuti, S. & Bardelli, A. Genetic analysis of the kinome and phosphatome in cancer. *Cell Mol. Life Sci.* **62**, 2092–2099 (2005).
131. Moorhead, G. B., Trinkle-Mulcahy, L. & Ulke-Lemee, A. Emerging roles of nuclear protein phosphatases. *Nature Rev. Mol. Cell Biol.* **8**, 234–244 (2007).
132. Cohen, P. T. Protein phosphatase 1 — targeted in many directions. *J. Cell Sci.* **115**, 241–256 (2002).
133. Gray, C. H., Good, V. M., Tonks, N. K. & Barford, D. The structure of the cell cycle protein Cdc14 reveals a proline-directed protein phosphatase. *EMBO J.* **22**, 3524–3535 (2003).
134. DeLano, W. L. *The PyMOL molecular graphics system* [online] < http://www.pymol.org > (2002).
135. Xu, W., Doshi, A., Lei, M., Eck, M. J. & Harrison, S. C. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol. Cell* **3**, 629–638 (1999).
136. Songyang, Z. *et al.* A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol. Cell. Biol.* **16**, 6486–6493 (1996).
137. Flotow, H. *et al.* Phosphate groups as substrate determinants for casein kinase I action. *J. Biol. Chem.* **265**, 14264–14269 (1990).
138. Meggio, F. & Pinna, L. A. One-thousand-and-one substrates of protein kinase CK2? *FASEB J.* **17**, 349–368 (2003).
139. Fiol, C. J., Wang, A., Roeske, R. W. & Roach, P. J. Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates. *J. Biol. Chem.* **265**, 6061–6065 (1990).
140. Till, J. H., Chan, P. M. & Miller, W. T. Engineering the substrate specificity of the Abl tyrosine kinase. *J. Biol. Chem.* **274**, 4995–5003 (1999).
141. Songyang, Z. *et al.* Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature* **373**, 536–539 (1995).
142. Obata, T. *et al.* Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J. Biol. Chem.* **275**, 36108–36115 (2000).
143. Friedmann, M., Nissen, M. S., Hoover, D. S., Reeves, R. & Magnuson, N. S. Characterization of the proto-oncogene *pim-1*: kinase activity and substrate recognition sequence. *Arch. Biochem. Biophys.* **298**, 594–601 (1992).

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Competing interests statement
The authors declare no competing financial interests.

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ERRATUM

Mechanisms of specificity in protein phosphorylation

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On page 533 of the article, left column, it was incorrectly stated that transforming growth factor-β receptor is a Tyr kinase. Transforming growth factor-β receptor is a Ser/Thr kinase.