Signalling scaffolds and local organization of cellular behaviour

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Abstract | Cellular responses to environmental cues involve the mobilization of GTPases, protein kinases and phosphoprotein phosphatases. The spatial organization of these signalling enzymes by scaffold proteins helps to guide the flow of molecular information. Allosteric modulation of scaffolded enzymes can alter their catalytic activity or sensitivity to second messengers in a manner that augments, insulates or terminates local cellular events. This Review examines the features of scaffold proteins and highlights examples of locally organized groups of signalling enzymes that drive essential physiological processes, including hormone action, heart rate, cell division, organelle movement and synaptic transmission.

This Review is dedicated to the memory of Tony Pawson, our friend, our colleague and a pioneer in this field.

A well-worn adage, rediscovered by successive generations of researchers, is that 'the longer a topic is investigated, the more complex the questions become'. This is certainly true for biomedical scientists, who grapple with the ever-accumulating molecular details of cell regulation. Within the perceived chaos of the cell, local organization of signalling enzymes guarantees the fidelity of information processing. Breakdown of this molecular order can result in disease. The concept of protein scaffolds as control centres for the integration and dissemination of subcellular information has evolved considerably during the past 25 years.

The protein scaffold model originates from three conceptual and technical breakthroughs in the late 1980s and early 1990s. These were: the realization that protein-interaction modules are the building blocks of macromolecular assemblies[1–6]; the use of yeast two-hybrid and proteomic analyses as a universal means to systematically identify protein–protein interactions[7,8]; and innovative genetic screens in lower organisms that uncovered functional relationships between signalling elements[9–11]. As investigators from different disciplines exploited these advances, it was soon recognized that a substantial proportion of human genes encode enzyme-binding proteins[12,13]. We now know that these ancillary signalling elements participate in the organization of MAPK cascades; the subcellular targeting of second-messenger-regulated protein kinases and phosphoprotein phosphatases; and the temporal control of rapid signalling events, such as muscle contraction, synaptic transmission and retrograde transport of organelles along microtubules.

At the dawn of the twenty-first century, many of these enzyme-binding proteins had been classified as adaptor, docking, anchoring or scaffold proteins[14]. Although it is cumbersome and confusing, this arbitrary terminology is now firmly entrenched in the signalling community. However, despite its utility, it is often difficult to assign an individual protein to a single class. Therefore, for the purposes of this Review, we propose that adaptor proteins are soluble proteins that contain several modular protein-interaction domains within their structure (FIG. 1a). Prototypical examples include growth factor receptor-bound protein 2 (GRB2) and SHC1, both of which are composed of SRC homology 2 (SH2) and SH3 domains and enable selective and simultaneous recruitment of several signalling elements[15,16] (FIG. 1a). Similarly, docking proteins are composed of modular protein-interaction domains, with a distinguishing feature being that they sequester signalling components at the cell membrane next to an activating receptor (FIG. 1b). Proteins emblematic of this class include insulin receptor substrate 1 (IRS1), IRS2, IRS3 and IRS4 (FIG. 1b), which help to transmit signals from insulin to various intracellular PI3K–AKT and MAPK cascades; and fibroblast growth factor receptor substrate 2α (FRS2α), which links its receptor to several downstream signalling pathways[17–19].

In contrast to the aforementioned examples of adaptor proteins and docking proteins, the delineation between anchoring and scaffold proteins is much less clear[12,20,21].

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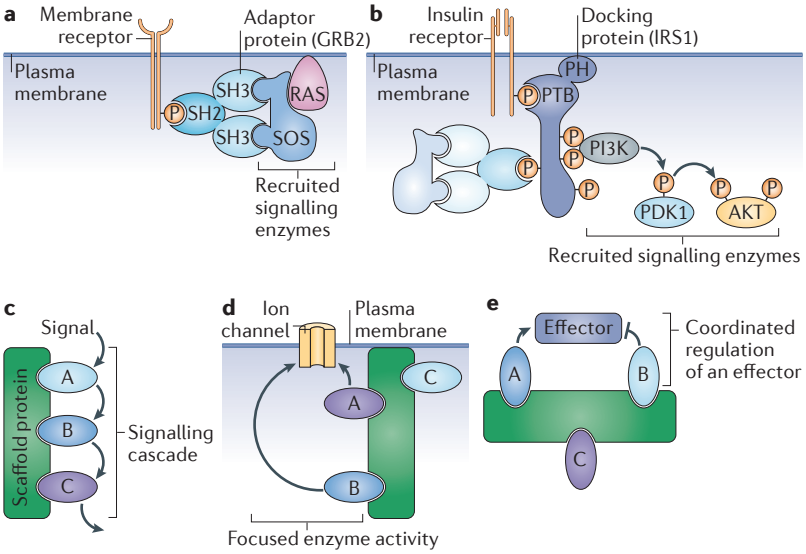


Figure 1 | **Properties of adaptor, docking and scaffold proteins.** The figure depicts the distinguishing features of signal-organizing proteins and of scaffold proteins. Adaptor proteins, such as growth factor receptor-bound protein 2 (GRB2) (part **a**), and membrane-associated docking proteins, such as insulin receptor substrate 1 (IRS1) (part **b**), are both composed of protein-interaction modules that recruit signalling enzymes near transmembrane receptors[21]. Essential features of scaffold proteins (shown in green) include the ability to hold in place successive members of a signalling cascade (part **c**), focus enzyme activity at a particular site of action (part **d**) and provide a molecular platform for the coordinated regulation of a particular effector protein by signal transduction and signal termination enzymes (part **e**). Phosphate groups are depicted as orange circles. PDK1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology domain; PTB, phosphotyrosine-binding domain; SH2, SRC homology 2; SOS, son of sevenless.

In fact, these terms have been used interchangeably to describe the same molecule; in this Review, we use the term scaffold protein. Here, we identify three features that are often attributable to this burgeoning class of signal-organizing proteins (FIG. 1c–e). First, they can be multivalent binding proteins that hold members of a transduction cascade in place to optimize signal relay (FIG. 1c). Second, they can be non-catalytic organizational elements that focus enzyme activity at a particular site of action (FIG. 1d). Third, they can be structural components that provide a molecular platform for the recruitment of signal transduction and signal termination enzymes to enhance the bidirectional control of cellular processes (FIG. 1e).

As these three features are generic characteristics of this expanding protein class, it is important to note that individual scaffold proteins may only fulfil one or two of these functional roles (FIG. 1c–e). Recent technical advances are leading to a rapid expansion of our understanding of the composition and structure of these signalling scaffolds. Data regarding the nature of macromolecular complexes are accumulating, and it is becoming clear that protein–protein interactions that induce conformational changes, confer catalytic activity or create an entirely new structural surface are as important for cell signalling as protein post-translational modifications. Specific examples of how scaffold proteins exhibit all of these characteristics are discussed in the following sections.

Allosteric modulators
Proteins that bind to a site that is distinct from the orthosteric agonist-binding site. They usually induce a conformational change within the protein structure.

Pseudokinases and pseudophosphatases

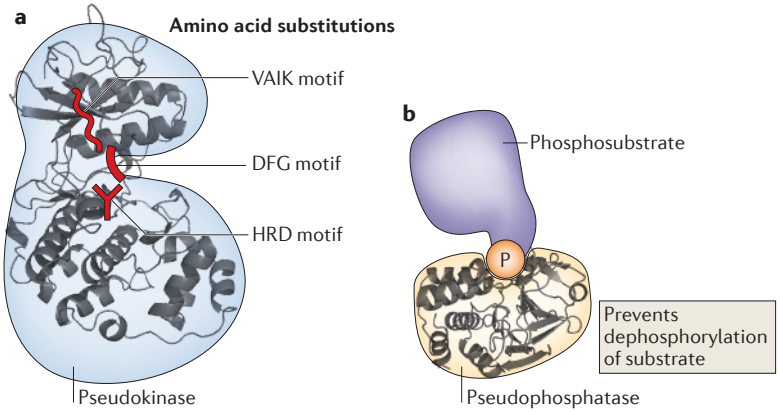
Of the 544 protein kinases (G. Manning, personal communication) that constitute the human kinome, 55 are currently known to be pseudokinases — kinase-like proteins that lack the key residues essential for catalytic activity[22–24] (BOX 1). Rather than functioning enzymatically, pseudokinases and their counterparts, pseudophosphatases, regulate phosphorylation events by functioning as allosteric modulators of other signalling enzymes[25,26]. Another important function of pseudokinases and pseudophosphatases is in kinase scaffolding, either directly by acting as kinase scaffolds or indirectly by functioning together with other scaffold proteins. As a result, these seemingly inert signalling elements are increasingly viewed as integral components of signalling pathways. Although there is considerable interest in the propagation of oncogenic signals by receptor tyrosine kinase-like pseudokinases — such as the epidermal growth factor (EGF) receptor family member HER3 (also known as ERBB3)[27,28] — we limit our discussion here to serine/threonine kinase-like pseudokinases.

Pseudokinases as scaffold proteins. One exciting new concept is that the activation of certain serine/threonine kinases requires the formation of a larger complex in which a pseudokinase functions cooperatively as a scaffold protein (FIG. 2a,b). Liver kinase B1 (LKB1; also known as STK11) is a tumour suppressor protein kinase that regulates cellular energy status, cell proliferation and cell polarity[29–31]. A defining feature of LKB1 is that it is not activated by the phosphorylation of its A loop, but instead it is induced into the active state through interaction with the pseudokinase STRAD[32] (FIG. 2a). Structural studies have shown that STRAD adopts an active kinase-like conformation through its interactions with nucleotides and with the horseshoe-shaped calcium-binding protein MO25 (also known as CAB39)[33,34]. The closed conformation of the ATP-bound STRAD engages LKB1, and the concomitant binding of MO25 to LKB1 stabilizes its active conformation[35] (FIG. 2a). These studies have elucidated distinct regulatory roles for each component of the STRAD–LKB1–MO25 ternary complex.

Pseudokinases with multiple functions. Although many pseudokinases have been identified as allosteric modulators or scaffold proteins, both properties often reside within the same protein. For example, kinase suppressor of RAS (KSR) is a multifunctional binding protein that brings together and modulates members of the conventional ERK cascade: rapidly accelerated fibrosarcoma (RAF) kinases, MEK and ERK[36,37]. This RAF–MEK–ERK triad relays signals that emanate from cell surface receptors and that are transmitted through the small guanosine triphosphatase RAS to the nucleus (FIG. 2b). Phosphorylation-dependent activation of ERK stimulates the expression of genes that drive proliferation, differentiation and other cellular processes.

Although early biochemical studies suggested that the pseudokinase scaffold protein KSR lacks the ability to catalyse phosphorylation[36], recent *in vitro* work indicates

Box 1 | **Pseudokinases and pseudophosphatases**



Pseudokinases

Pseudokinases contain amino acid substitutions within the conserved kinase catalytic core that impair phosphoryl transfer[23]. As indicated in part **a** of the figure (structural data from the RCSB Protein Data Bank (PDB) code: 3C9W), many of these substitutions reside within one of three invariant motifs: the VAIK motif, which engages ATP and positions it for phosphoryl transfer; the DFG motif, which binds to Mg^{2+} to coordinate ATP binding; and the HRD catalytic motif, which participates in proton transfer[22]. Recent structural studies indicate that some pseudokinases retain the ability to tightly bind nucleotides for delivery to a binding partner with kinase activity[30]. Thus, nucleotide release from these pseudokinases has a direct effect on the catalytic efficiency of the active kinase binding partners. These findings have contributed to the recent notion that serine/threonine-like pseudokinases can allosterically regulate their associated active kinases. Consequently, some pseudokinases are now considered to be viable drug targets.

Interest in the signalling pathway that is scaffolded by kinase suppressor of RAS (KSR), a pseudokinase, has been stimulated by clinical evidence that activating mutations in *RAS* are found in ~30% of all human tumours[131]. Understandably, the RAS–RAF–MEK–ERK signalling cascade is a prime target for therapeutic intervention. However, the outcome of several drug-discovery ventures has been quite surprising. Although certain ATP analogue drugs that inhibit BRAF — such as sorafenib (Nexavar; Bayer and Onyx Pharmceuticals) and dabrafenib (Tafinlar; GlaxoSmithKline) — effectively combat renal and hepatic carcinomas and melanomas, other BRAF inhibitors, such as vemurafenib (Zelboraf; Plexxikon and Genetech), paradoxically stimulate tumour growth in cells with normal (non-mutant) BRAF. Accordingly, vemurafenib can only be prescribed to patients with melanoma who have BRAF-V600E or BRAF-V600K substitutions, which affect the active site of this kinase[132–134]. Some of these anomalies may be explained by the complex interplay between RAFs, other members of the ERK cascade and its activator, KSR.

Pseudophosphatases

The concept of pseudophosphatases was first proposed to account for the unexpected functional roles of additional phosphotyrosine phosphatase homology domains that were identified in CD45, RPTPγ and RPTPζ[44]. These structured regions seemed to have little or no phosphatase activity against *in vitro* substrates[43,44]. A combination of biochemistry and bioinformatics has suggested that ~8% of the human phosphatome (17 out of 215 members) encodes pseudophosphatases[135]. A putative role for this burgeoning family of pseudoenzymes is to regulate signalling by functioning as conformational clamps that bind to phosphosubstrates and prevent their dephosphorylation (see the figure, part **b**; structural data from PDB code: 3N5U), thus indirectly augmenting kinase activity in various cellular contexts. The discovery of small molecules that can either function as inhibitors of activation or directly target these protein–protein interfaces is taking hold as a future health objective.

that, in certain contexts, KSR can phosphorylate MEK[38]. This provides an interesting new twist to the function of KSR, which was previously considered simply as an inert signalling component. Although this classic pseudokinase is now thought by some to have intrinsic phosphotransferase activity, it should be noted that the function of these KSR-mediated phosphorylation sites on MEK is unclear[38]. A combination of X-ray crystallography and chemical genetic approaches has determined that formation of a KSR–RAF–MEK ternary complex displaces a key helix within KSR, which derepresses its kinase activity[38]. Support for this allosteric activation mechanism was provided by evidence that the recruitment of the serine/threonine protein kinase BRAF substantially increases the kinase activity of KSR towards MEK. Experiments using a nucleotide-binding mutant of KSR that is specific for a particular synthetic ATP analogue confirmed that, in this case, MEK1 phosphorylation could only have been catalysed by KSR. Thus, small molecules that target the active conformation of KSR could have therapeutic benefit as potential modulators of the ERK cascade. Although more attention has focused on the complexity of RAF–KSR interfaces in the modulation of ERK signalling, other kinases are also indirectly recruited to this signalling unit. For example, the A-kinase anchor protein, AKAP-Lbc (also known as AKAP13), functions as an enhancer of ERK signalling by securing RAF in the vicinity of MEK1 and synchronizing the cAMP-dependent protein kinase A (PKA)-mediated phosphorylation of Ser838 on KSR[39]. This discovery not only offers mechanistic insight into the cAMP-responsive control of ERK signalling events but also shows how the coincident processing of discrete chemical signals can be integrated within this macromolecular complex.

Several recent reports show that MEK activation can occur through two separate mechanisms: either phosphorylation by BRAF in a complex with another RAF orthologue or phosphorylation by KSR (as described above)[37,38,40]. *In vitro* structure–function analyses and inhibitor studies have addressed these provocative findings. The introduction of a bulky phenylalanine residue within the ATP-binding pocket of KSR abolished nucleotide binding by KSR and the kinase activity of MEK[40]. Nevertheless, this KSR mutant retained its scaffold function, as it was able to bind to BRAF. Using an *in vitro* assay, it was shown that the catalytic activity of KSR persisted even with the addition of the RAF inhibitor sorafenib or the MEK inhibitor PD0325901 (REF. 38). However, other investigators have used these and other related compounds to determine an alternative mechanism to explain the effect of KSR on the ERK cascade[37,41]. More recently, this same group has shown that RAF dimers function in tandem, whereby one partner functions as an activator kinase that transmits activation signals to its binding partner, the receiver kinase[42]. These findings provide a potential explanation as to why changes in KSR expression alter the effects of some of the aforementioned RAF inhibitors on oncogenic RAS–ERK signalling. In the presence of activated RAS at the cell membrane, RAF inhibitors stabilize the side-by-side dimerization of BRAF and CRAF and thus ERK activation (FIG. 2b). However, independently of RAS or BRAF activation, RAF inhibitors promote the heterodimerization of BRAF with KSR, which positions the associated MEK for phosphorylation[37]. In the absence of RAF inhibitors and RAS activation, the formation of KSR–BRAF dimers may function as a buffer to BRAF activation, as it competes with the CRAF–BRAF dimerization.

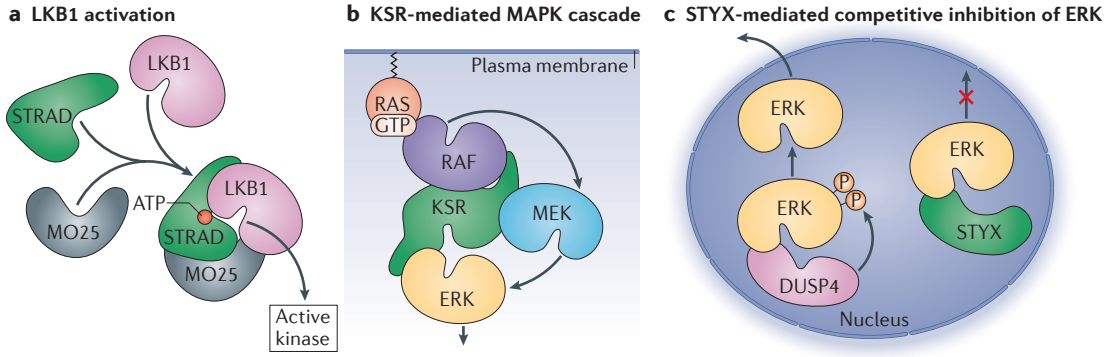


Figure 2 | **Scaffold functions of pseudokinases and pseudophosphatases.** Three prototypical examples of pseudokinase and pseudophosphatase function are shown. These non-catalytic proteins (shown in green) function as enzyme scaffolds and also allosterically modulate the activity of signalling enzymes. **a** | The tumour suppressor protein liver kinase B1 (LKB1) and the pseudokinase scaffold protein STRAD form a complex with MO25 (REF. 22). This heterotrimeric complex induces the ATP-bound STRAD to adopt a closed conformation that is reminiscent of an active protein kinase and that is able to activate LKB1. Interaction with MO25 stabilizes the active conformation of LKB1 (REF. 33). **b** | Kinase suppressor of RAS (KSR) is a multifunctional binding protein that brings together the three members of the conventional RAF–MEK–ERK–MAPK cascade[36,136]. **c** | The pseudophosphatase serine/threonine/tyrosine-interacting protein (STYX) competes with the active dual-specificity phosphatase 4 (DUSP4) for binding to ERK1 and ERK2 in the nucleus[135]. Through this mechanism, STYX traps active elements of the ERK cascade in the nucleus[47]. Phosphate groups are depicted as orange circles.

Clearly, the RAF–MEK–ERK cascade can be activated through multiple pathways, and KSR has been functionally implicated as a participant with multiple roles — as a scaffold protein, a non-catalytic allosteric modulator, an active kinase and a facilitator of hetero-oligomerization. Irrespective of which of these functions of KSR predominates in an individual cellular context, it is clear that the scaffold and pseudokinase protein KSR functions at pivotal points in the multiple activation routes of this MAPK pathway.

Pseudophosphatases as scaffold proteins. The concept of pseudophosphatases originated from the realization that the second phosphotyrosine phosphatase domain of proteins such as CD45, RPTPγ and RPTPζ has little or no phosphatase activity against various *in vitro* substrates[43,44] (BOX 1). However, it was subsequently realized that pseudophosphatases also participate in substrate trapping[45,46]. Perhaps the best example of this is the serine/threonine/tyrosine-interacting protein (STYX), a catalytically inactive member of the dual-specificity phosphatase (DUSP) family[47] (FIG. 2c). One role for this bona fide pseudophosphatase is to compete with the active enzyme DUSP4 for binding to ERK1 and ERK2 in the nucleus (FIG. 2c). STYX also functions as a nuclear scaffold that traps elements of the ERK1 and ERK2 signalling cascade. Cellular and molecular modelling approaches have led to the proposal that depletion of STYX redirects ERK activity to the cytosol, where it can augment ERK-dependent fragmentation of the Golgi apparatus[47]. Thus, one way in which STYX and other pseudophosphatases seem to regulate signalling is by functioning as substrate traps that sequester phosphosubstrates.

As highlighted by numerous examples, the interaction of key signalling enzymes with pseudokinases and pseudophosphatases provides a mechanism for the fine-tuning of several kinase-signalling pathways. Similarly, other types of scaffold proteins that occupy pivotal positions within signalling networks are being investigated. The non-catalytic AKAPs are a classic example of this type of scaffold protein.

AKAPs

Over the past 20 years, our understanding of AKAPs has grown from the discovery of a few simple binding proteins that were purported to direct PKA to specific subcellular locations, to a large family of multivalent enzyme scaffold proteins that organize complex signalling events[48–53]. Constraining broad-specificity enzymes, such as PKA, in customized macromolecular units enables cells to respond with efficiency and accuracy to the ebb and flow of diffusible second-messenger signals[54]. Most recently, various approaches have shown that AKAPs are dynamic participants in local signalling as a result of their flexibility in structure, transient interactions with other proteins and combinatorial assembly of binding partners[55] (FIG. 3).

AKAPs tether PKA holoenzymes. A defining feature of AKAPs is their ability to associate with PKA holoenzymes, which are composed of two regulatory subunits and two catalytic subunits. Nuclear magnetic resonance spectroscopy and X-ray crystallography studies have shown that the regulatory type I (RI) or type II (RII) subunits of PKA homodimerize to create a four-helix bundle that functions as a binding groove for AKAPs[56–59]. An amphipathic helix within the AKAP forms the reciprocal binding surface that binds to PKA with subnanomolar affinity[60,61]. Despite high-resolution structural information about this protein–protein interface, attempts by structural biologists to crystallize entire AKAP–PKA holoenzyme complexes have been unsuccessful. One explanation could be that both proteins have recognized regions of structural disorder

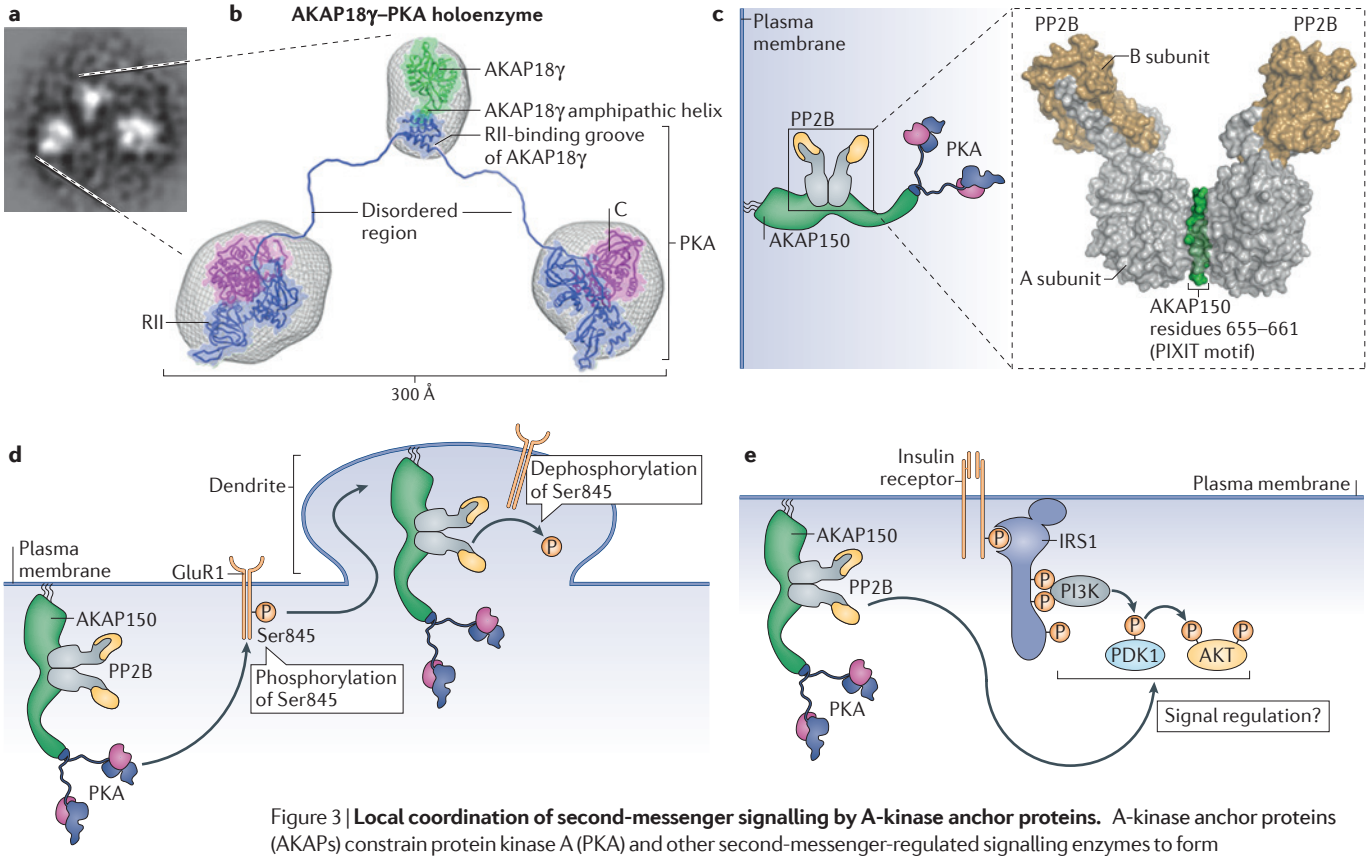


Figure 3 | **Local coordination of second-messenger signalling by A-kinase anchor proteins.** A-kinase anchor proteins (AKAPs) constrain protein kinase A (PKA) and other second-messenger-regulated signalling enzymes to form macromolecular units[137]. **a** | A composite negative-stain electron microscopy image (class average) of the intact type II PKA–AKAP18γ complex is shown. **b** | Three-dimensional reconstructions reveal that the complex has a heteropentameric protein assembly (containing one AKAP18 subunit, two regulatory type II (RII) subunits of PKA and two catalytic (C) subunits of PKA[55]). The flexible tripartite configuration enables the associated catalytic subunits to have a radius of motion of up to 300 Å. **c** | Mouse AKAP150 is a multifunctional anchor protein that coordinates different combinations of second-messenger-regulated enzymes. The figure shows an assembly of PKA and serine/threonine protein phosphatase 2B (PP2B) maintained by AKAP150. A structural model of the interface between AKAP150 and a pair of PP2B holoenzymes shows that protein–protein interactions occur through a modified PIXIT phosphatase-interaction motif[123,138]. **d** | An established role for AKAP150 is the modulation of the phosphorylation events that control glutamate receptor ion channels. AKAP150-associated PKA and PP2B provide bidirectional regulation of glutamate receptor 1 (GluR1) phosphorylation at Ser845. Anchored PKA-mediated phosphorylation of GluR1 at Ser845 augments the membrane insertion of GluR1 at dendrites[70]. PP2B-mediated dephosphorylation of GluR1 at this site reverses this process[70]. **e** | In other cellular contexts, AKAP150 coordinates metabolic signalling events. AKAP150-associated PP2B activity modulates aspects of the insulin-responsive PI3K–phosphoinositide-dependent kinase 1 (PDK1)–AKT signalling cascade in skeletal muscle to control insulin sensitivity[79]. Scaffold proteins are depicted in green; phosphate groups are depicted as orange circles. IRS1, insulin receptor substrate 1. Parts **a** and **b** are adapted from REF. 55, eLife Sciences Publications. Part **c** is adapted with permission from REF. 79, John Wiley & Sons.

that are refractory to conventional structural biology approaches[62,63]. Thus, in conjunction with X-ray crystallography, alternative approaches — including protein mass spectrometry and single-particle fluorescence imaging — have been used to establish the composition, stoichiometry and architectural arrangement of selected AKAP complexes[55] (FIG. 3a–c). Recently, electron microscopy and 3D reconstructions of intact type II PKA–AKAP18γ complexes showed that they have a heteropentameric structure (consisting of one AKAP18γ, two PKA RII and two PKA catalytic subunits) and can adopt a range of flexible tripartite configurations[55]. Intrinsically disordered regions within each PKA regulatory subunit impart the molecular plasticity that enables the associated catalytic subunits to have a radius of motion of ~16 nm. One mechanistic implication of these structural analyses is that flexibility within the PKA complex could enable precise orientation of the anchored catalytic subunit towards substrates. Thus, AKAP can be thought of as a scaffolding catalyst that physically brings the reactants together, and the flexibility within the anchored PKA holoenzyme enables the precise orientation of the enzyme and substrate that is required for optimal reactivity.

AKAP scaffolds in neuronal and endocrine pathways. Many biological insights into anchored signalling mechanisms have come from the investigation of human AKAP79 and its murine orthologue AKAP150, both of which are encoded by *AKAP5* (REF. 64). The members

of this AKAP family (AKAP79/150) scaffold different combinations of protein kinases and phosphatases at the inner face of the plasma membrane, where they are positioned to respond to intracellular changes in the levels of second messengers, such as cAMP, Ca^{2+} and phospholipids[65–67] (FIG. 3). An amphipathic helix on AKAP79/150 provides a binding site for PKA, a modified PIXIT motif on AKAP79/150 tethers the protein phosphatase 2B (PP2B; also known as calcineurin) heterodimer, and PKC interacts with AKAP79/150 in a phosphatidylserine-dependent manner through an amino-terminal basic and hydrophobic sequence (FIG. 3b,c). Much work has focused on how different combinations of these AKAP79/150-anchored enzymes modulate transmembrane receptors and ion channels in neuronal, endocrine and muscle tissues.

Long-term potentiation (LTP) and long-term depression (LTD) are electrophysiological indices of synaptic transmission that are related to hippocampal learning and memory[68]. These phenomena are regulated in part through the phosphorylation of glutamate receptor 1 (GluR1; also known as GluA1), a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR) for glutamate, which primes extrasynaptic receptors for synaptic insertion (FIG. 3d). Depletion of AKAP150 through siRNA in murine hippocampal neurons not only promotes mislocalization of PKA but also leads to reduced phosphorylation of GluR1 on Ser845 and the concomitant impairment of synaptic transmission and memory[69,70]. This model was further substantiated by analyses of AKAP150-knockout mice, in which displacement of both PKA and PP2B from postsynaptic sites altered hippocampal synaptic transmission. These studies were extended in PP2B-anchoring-deficient AKAP150ΔPP2B knock-in mice, in which the LTD-induced dephosphorylation and displacement of AMPAR and the AKAP150 signalling complex from the synapse were impaired[70]. Thus, it is clear that in these cells the AKAP150-anchored PP2B counterbalances the phosphorylation of GluR1 by the anchored PKA to control synaptic plasticity.

In sympathetic cervical ganglion neurons, loss of AKAP150 reduces muscarinic acetylcholine receptor-mediated suppression of inhibitory M currents through M channels[71]. These ion channels generate an outward potassium current that is required for the firing of an action potential. This process is thought to occur through the AKAP-mediated targeting of PKC to the KCNQ2 subunit of the M channel, the phosphorylation of which inhibits potassium permeability to enhance neuronal excitability[67,72]. Paradoxically, loss of this vital sympathetic neuronal response affords AKAP150-knockout mice some protection from seizures induced by the muscarinic agonist pilocarpine[73]. A secondary discovery that may be related to this phenomenon is that binding to AKAP150 rendered PKC insensitive to the ATP-competitive inhibitor bisindolylmaleimide I[74]. This unforeseen observation indicated that AKAP79 and AKAP150 not only control the access of binding partners to their substrates but also influence how binding partners such as PKC respond to certain pharmacological inhibitors. A broader interpretation of this result is that scaffold proteins such as AKAPs also function as allosteric modifiers that shape the activity of the kinase or phosphatase under their control. This may have important implications for drug discovery and research projects predicated on the selectivity of pharmacological protein kinase inhibitors.

In pancreatic β-islets, AKAP150 directs cAMP-dependent PKA and Ca^{2+}/calmodulin-dependent PP2B towards elements of the insulin release machinery that are controlled by changes in cAMP and Ca^{2+} levels. The scaffold protein AKAP150 directly binds to the cytoplasmic tail of L-type Ca^{2+} channels, where the associated enzymes (PKA and PP2B) control the phosphorylation-dependent modulation of channel activity. Loss of AKAP150 suppresses the activity of the Ca^{2+} channels, and the disrupted Ca^{2+} influx in these cells is accompanied by perturbation of the submembrane oscillations of cAMP levels that are crucial for insulin release[75,76]. This result may also be indicative of the compromised modulation of AKAP150-associated Ca^{2+}-sensitive adenylyl cyclases that generate cAMP and that are under the control of other enzymes within the AKAP150 complex[77,78]. An important new concept that has arisen from these studies is that decreased insulin release from AKAP150-deficient islets is not a consequence of interrupted signalling at a single intracellular locus but rather the result of a multifaceted failure of discrete molecular events at vulnerable points in the secretory cascade that are normally coordinated by the AKAP scaffold[79].

A logical consequence of reduced insulin release from β-islets is impaired glucose tolerance but, unexpectedly, AKAP150-knockout mice have improved glucose handling[79]. This confounding but metabolically favourable phenotype could be explained by one of two phenomena: a compensatory reduction in the level of the counter-regulatory hormone glucagon (which controls the release of stored glycogen), or enhanced insulin sensitivity. Metabolic profiling of AKAP150-deficient mice showed that compensatory mechanisms, such as reduced glycogen mobilization, do not contribute to their improved glucose tolerance. However, metabolic profiling of PP2B-anchoring-deficient AKAP150ΔPP2B knock-in mice provided an intriguing observation: deletion of seven amino acids (PIAIIIT) from AKAP150 not only disrupted PP2B targeting *in vivo* but also enhanced insulin sensitivity in skeletal muscle and the liver[79] (FIG. 3e). Therefore, small molecules that perturb the tethering of the phosphatase PP2B to AKAP150 might boost insulin sensitivity. Such a therapeutic approach could be of particular value to recipients of organ transplants who require immunosuppressive phosphatase-inhibitor drugs, such as cyclosporin and FK506. One drawback is that these drugs have considerable adverse effects, including increased blood glucose levels and hypertension, which are hallmarks of a newly defined clinical syndrome known as new-onset diabetes after transplant. Hence, compounds that target anchored PP2B to boost insulin action in peripheral tissues could be used in combination with immunosuppressive drugs to restore glucose homeostasis in these patients.

Long-term potentiation
(LTP). A process whereby brief periods of synaptic activity can produce a long-lasting increase in the strength of a neuronal synapse. LTP has an important role in learning and memory.

Long-term depression
(LTD). A long-lasting decrease in the synaptic response of neurons to stimulation of their afferents following a long patterned stimulus.

M channels
Muscarinic receptor-sensitive potassium channels that coordinate the depolarization of neurons and prepare these cells for the next action potential.

L-type Ca^{2+} channels
Ion channels that control Ca^{2+} influx into a large number of cell types. They are one of the most studied types of ion channels.

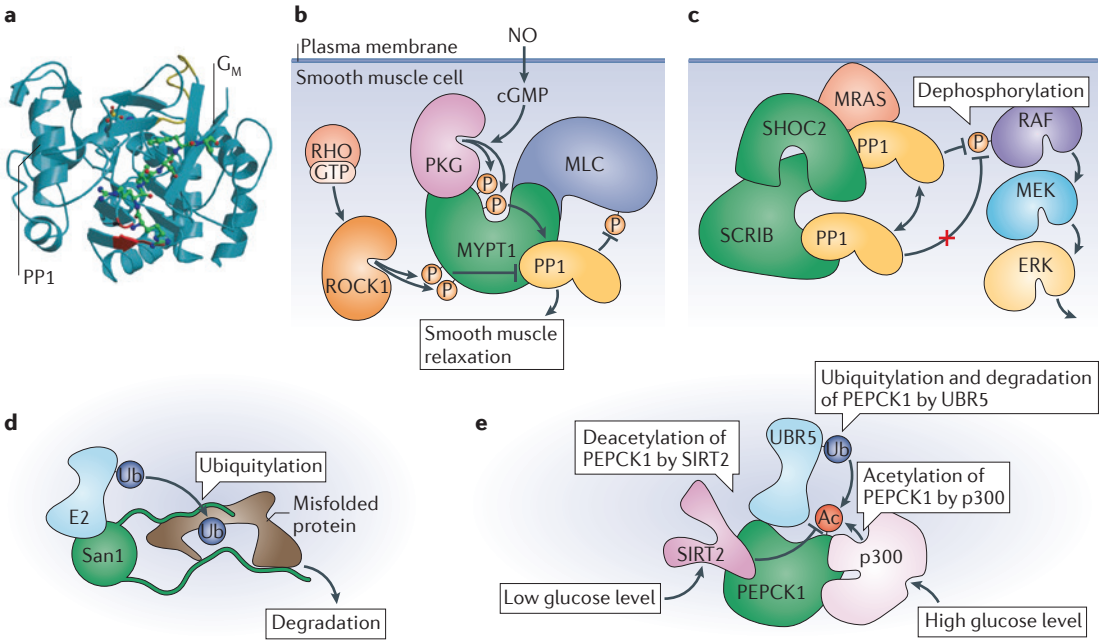


Figure 4 | **Signal termination scaffolds.** Scaffold proteins that target phosphatases and the enzymes that control protein ubiquitylation, acetylation and deacetylation are shown. **a** | A ribbon diagram of serine/threonine protein phosphatase 1 (PP1) catalytic subunit (turquoise) in a complex with a KVXF-motif peptide derived from the glycogen-targeting subunit G_M (green)[25] is shown. Important regions for peptide binding are shown in red. The peptide-binding channel of PP1 lies at the interface of the two β-sheets of a β-sandwich. **b** | An example of a higher-order PP1 scaffold is depicted. A phosphatase–kinase scaffold tightly modulates smooth muscle contraction[87]. The proximity of the activator protein kinase G (PKG) and the inhibitory RHO-associated protein kinase 1 (ROCK1) provides bidirectional control of PP1 when tethered to its scaffold protein, the myosin-targeting PP1 subunit MYPT1, and the myosin light chains (MLCs). **c** | A second example of a higher-order PP1 scaffold is shown. When in a complex with MRAS, the phosphatase-targeting subunit SHOC2 competes with another scaffold protein, SCRIB, for interaction with PP1. Thus, ERK signalling is antagonized by the SHOC2-mediated dephosphorylation of RAF and is facilitated when the phosphatase is sequestered by SCRIB[95]. Ubiquitylation and acetylation are important molecular switches controlled by the protein–protein interactions within scaffolds. **d** | By recognizing the exposed hydrophobicity of misfolded proteins, the yeast E3 ligase San1 functions as a scaffold and is able to target misfolded proteins for ubiquitylation and proteasomal degradation[101]. **e** | The scaffold formed by the phosphoenolpyruvate carboxykinase 1 (PEPCK1) efficiently responds to changes in glucose levels through its associated metabolic enzymes[109]. In the presence of high levels of glucose, the associated acetyltransferase p300 acetylates PEPCK1, targeting it for ubiquitylation and subsequent degradation by the E3 ubiquitin protein ligase UBR5 that is recruited to the complex. When the glucose level is low, the deacetylase sirtuin 2 (SIRT2) blocks this process. Acetyl groups are depicted as red circles, phosphate groups are depicted as orange circles, and ubiquitin groups are depicted as blue circles. NO, nitric oxide. Part **a** is adapted with permission from REF. 25, John Wiley & Sons.

Although AKAPs have no intrinsic enzymatic activity themselves, it is their complement of associated enzymes that determines the function of the scaffold. Moreover, as mentioned above, several recent studies suggest that AKAPs allosterically modulate the enzymatic activity of their binding partners. Consequently, within AKAP–enzyme scaffolds there are multiple protein interfaces that could be examined as potential therapeutic targets for the disruption of pathological signalling.

Signal termination scaffolds

Enzymes that inactivate substrates or promote the degradation of key elements in signalling pathways often function as 'off' switches. Similarly to signal activation mechanisms, these homeostatic mechanisms are also more effective when organized into protein scaffolds. Three examples of this are the scaffolds that target phosphatases, those that control protein ubiquitylation and those that control acetylation and deacetylation (FIG. 4).

Phosphatase scaffolds. Generally, the greater catalytic efficiency of phosphoprotein phosphatases affords them a competitive advantage over their kinase counterparts. Consequently, the dephosphorylation of substrates often predominates when phosphatases are recruited to kinase-containing scaffolds. The targeting of some phosphatases may be quite simple, whereas others are constrained within larger signalling scaffolds. The regulatory phosphatase subunits PP2A, PP4 and PP6 have been shown to restrict their respective catalytic subunits to defined subcellular locations, where the catalytic subunits dephosphorylate a subset of nearby substrates[80–82]. However, our understanding of how PP1 is directed towards its substrates is much more complete. More than 200 PP1-interacting proteins have been identified that scaffold specific protein complexes with the phosphatase catalytic subunit (PP1c) to modulate all aspects of PP1 action[83]. Subcellular targeting is mediated by a short conserved binding motif (RVxF or variants) in the scaffold

protein, often preceded by further basic residues, which docks on a small hydrophobic groove surface on PP1 (REFS 25,84) (FIG. 4a). Another key facet of PP1 scaffolding is that the association of PP1 with targeting subunits allosterically modulates enzymatic activity and determines substrate specificity, as first demonstrated for the glycogen-targeting subunit G_M and glycogen-associated phosphosubstrates[85]. Accordingly, these PP1 interactions determine the regulation of diverse cellular events, such as the onset of hypertension and tumour growth.

The myosin light-chain phosphatase complex controls the dephosphorylation of proteins to promote the relaxation of smooth muscle in arterial walls[86,87]. Myosin light-chain phosphatase is a desirable therapeutic target for the management of hypertension, as drugs that can reduce vascular tone will ultimately normalize blood pressure. Current interest in myosin light-chain phosphatase centres on the modulation of its PP1-targeting subunit, MYPT1 (also known as PPP1R12A)[88,89]. Mobilization of the nitric oxide (NO) pathway — either naturally or pharmacologically with nitrovasodilators — produces the second messenger cGMP (FIG. 4b). This leads to the activation of PKG, another component of the myosin light-chain phosphatase complex[88]. PKG-mediated phosphorylation of MYPT1 activates PP1 to promote rapid smooth muscle relaxation through myosin light chain dephosphorylation. By contrast, phosphorylation of the MYPT1 scaffold protein by the RHO-associated protein kinase 1 (ROCK1) inhibits myosin light-chain dephosphorylation[90,91]. Thus, combining nitrovasodilators with ROCK1 inhibitors to manage smooth muscle contraction is being considered for the treatment of hypertension.

MAPK cascades comprising RAF–MEK–ERK transmit signals from growth factors to cell proliferation through mobilization of the GTPase RAS[92]. Interest in this signalling pathway is motivated by clinical evidence that activating mutations in *RAS* are found in ~30% of human tumours[93]. In addition, several 'RASopathies' have been identified. One of these disorders, a Noonan-like syndrome with loose anagen hair, is caused by a mutation in the gene encoding the phosphatase-targeting protein SHOC2 that results in aberrant targeting of SHOC2 (REF. 94). Recent evidence indicates that SHOC2 requires interaction with the RAS-related GTP-binding protein MRAS for high-affinity binding to PP1c and the subsequent dephosphorylation of an inhibitory site on RAF[95,96]. One unusual feature of the SHOC2–PP1 scaffold is the recruitment of an additional PP1-interacting protein, SCRIB (FIG. 4c). Formation of this larger complex, which contains multiple phosphatase-targeting components, promotes internal competition between SHOC2 and SCRIB for PP1c binding, thus controlling the phosphorylation and activation of RAF[95]. This complicated PP1-dependent mechanism has been postulated to fine-tune the frequency and amplitude of ERK activity pulses during the establishment of cell polarity[95].

Ubiquitylation scaffolds. It is well known that protein ubiquitylation regulates protein degradation and enables the control of protein quality — a cellular phenomenon in which damaged or inappropriately folded proteins are eliminated[97]. However, ubiquitylation is also an effective means of modifying protein–protein interactions[98]. Accordingly, ubiquitylation is increasingly being recognized as a dynamic modulator of molecular scaffolds[99]. Nowhere are these roles more relevant than in the nucleus, where the integrity of nuclear proteins is essential for transcriptional fidelity and cell division[97]. In yeast, the nuclear E3 ligase San1 is a highly selective sensor that can discriminate between folded and misfolded forms of the same protein[100]. Although San1 is a bona fide RING-finger E3 ligase, it also functions as a scaffold protein (FIG. 4d). Flexible and intrinsically disordered regions of San1 confer a plasticity that enables this enzyme to recognize, bind to, ubiquitylate and thereby signal the removal of a diverse collection of misfolded substrates[101].

During the mammalian cell cycle, cullin–RING ligase (CRL) functions as a molecular scaffold that brings together one of several RING-finger proteins (RBX1, RBX2 or RO52 (also known as TRIM21)) plus its active component, a specific ubiquitin-conjugating enzyme (UBC), and the adaptor S phase kinase-associated protein 1 (SKP1), which binds to an F-box protein (SKP2) and its associated substrate[102]. These scaffolds direct the UBC towards an appropriate cyclin-dependent kinase (CDK) inhibitor to target it for ubiquitylation and degradation. Thus, by using interchangeable F-box, RING-finger and UBC proteins, an individual SKP1–cullin 1 (CUL1)–F-box protein complex can be tailored for cell cycle progression.

Acetylation scaffolds. Reversible protein acetylation has emerged as a versatile form of post-translational modification, the importance of which rivals phosphorylation and ubiquitylation. This process is controlled by the opposing actions of acetyltransferases and deacetylases. In the nucleus, histone acetyltransferases (HATs) are frequently associated with transcriptional activation, whereas histone deacetylases (HDACs) are often linked to the inactivation of genes. The formation of a larger scaffold that can simultaneously target HATs and HDACs within the same complex has been shown to sustain transcriptional competence during myogenesis and transcriptional events that proceed through the tumour suppressor p53 (REF. 103). Higher-order HAT complexes can also control nucleosome remodelling[104]. Of particular interest are larger macromolecular scaffolds that contain protein kinases as well as acetyltransferases[105]. For example, the ADA2-containing (ATAC) HAT complex constrains a three-tier JUN kinase cascade in proximity to target genes. In this context, the ATAC complex functions as both a transcriptional co-activator of and a scaffold for JUN N-terminal kinase (JNK) signalling[105]. The integration of these signalling enzymes within the ATAC scaffold facilitates a timely response to osmotic stress.

Recent evidence also suggests that, in the cytoplasm, phosphoenolpyruvate carboxykinase 1 (PEPCK1) functions as a scaffold for the acetylation and deacetylation enzymes that regulate PEPCK1 activity in response to blood glucose levels[106]. Acetylation of PEPCK1 by the acetyltransferase p300 in the complex promotes recruitment of the E3 ubiquitin ligase UBR5 and proteasomal

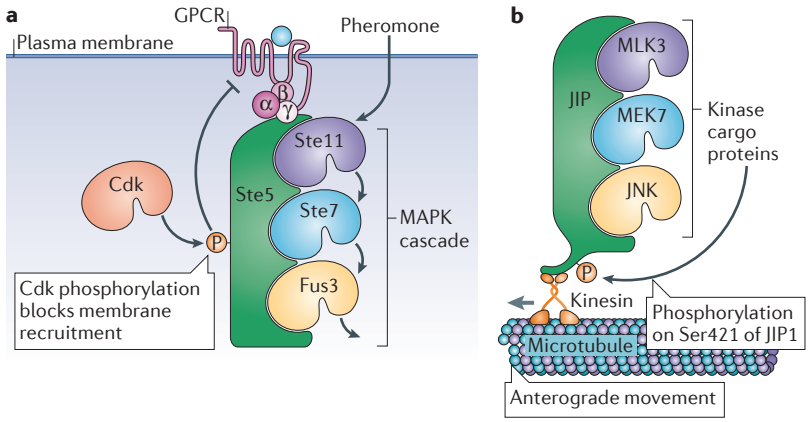


Figure 5 | **Scaffold proteins that function as molecular switches.** MAPK scaffold proteins in yeast and mammals are depicted. **a** | The yeast scaffold protein Ste5 assembles a MAPK cascade that includes Ste11, Ste7 and Fus3 at the cell membrane. However, phosphorylation of Ste5 by cyclin-dependent kinase (Cdk) inhibits this process[112,115]. **b** | The JUN amino-terminal kinase (JNK) and p38 MAPK scaffold proteins of the JNK-interacting protein (JIP) family associate with motor proteins to transport various cargo proteins along microtubules[139]. Phosphorylation of Ser421 on JIP1 links the mixed lineage kinase 3 (MLK3)–MEK7–JNK cascade to the motor protein kinesin for forward motion along the microtubule, whereas dephosphorylation of this residue functions as a switch for retrograde movement facilitated by interaction with dynactin (not shown)[118]. Phosphate groups are depicted as orange circles. GPCR, G protein-coupled receptor.

degradation of PEPCK1 (FIG. 4e). The deacetylase sirtuin 2 (SIRT2) that is contained within the complex counteracts this process to prolong the half-life of PEPCK1. Interestingly, this homeostatic process is regulated by the metabolic state of the cell. High glucose levels induce the expression of p300 and suppress SIRT2 activity, which favours the acetylation and degradation of PEPCK1. However, under low-glucose conditions, SIRT2-mediated deacetylation stabilizes PEPCK1 to drive gluconeogenesis (FIG. 4e). Thus, it has been postulated that controlling the acetylation status and half-life of PEPCK1 has the potential to manage aspects of diabetes and metabolic syndrome[106]. In a broader context, proteomic studies have shown that the acetylation profile and stability of proteins can be quite different in many cell types, including liver and leukaemia cells[107–109]. Thus, scaffolded acetyltransferase- and deacetylase-mediated regulatory circuits that control the half-life of the transcriptional machinery or of metabolic enzymes may prove to be therapeutic targets for the management of metabolic disorders and certain cancers.

The above examples are just a few of the protein scaffolds that incorporate signal termination enzymes within their repertoire of binding proteins. In the following section, we discuss recent studies that have identified other scaffold proteins that are able to rapidly toggle between on and off signals in response to the rapidly changing intracellular environment.

The switch function of scaffold proteins

By constraining successive enzymes in a signalling cascade, scaffold proteins simultaneously facilitate the efficient relay of chemical messages and segregate individual signalling units for the accurate processing of molecular information. These macromolecular assemblies have also been shown to enhance enzymatic activity and to identify and mark proteins for degradation. However, an emerging role for some of these scaffold proteins is as ultra-sensitive switches that determine alternative cell fates. New discoveries regarding two prototypical MAPK scaffolds underlie this new perspective.

In yeast, pheromone-induced recruitment of the sterile 5 gene product (Ste5) to the plasma membrane promotes its active conformation[110,111]. This induces binding of all three members of a MAPK cascade (the MEKK Ste11, the MEK Ste7 and the MAPK Fus3) to propagate the mating response[112] (FIG. 5a). However, at certain stages of the cell cycle, this constellation of enzymes can have other biological effects. Phosphorylation of the scaffold protein Ste5 by the cell cycle regulator kinase Cdk blocks accumulation of Ste5 at the cell membrane[113]. Recent advances in synthetic biology, combined with structural studies, indicate that conformational changes in Ste5 enable the constrained MAPK complex to switch between alternative fates[114,115] (FIG. 5a). For example, recruitment of Fus3 to another binding site on Ste5 mediates feedback phosphorylation events that initiate a yeast morphological response known as 'shmooing', in which opposite mating types of haploid yeast cells migrate towards each other in order to fuse and form a diploid cell[113].

In mammals, the JNK-interacting protein (JIP) family coordinates three-tier JNK and p38 MAPK modules[116]. JIPs were initially identified as elements that contribute to glutamate transporter type 2 gene expression and β-cell function, but members of this scaffold protein family can also oligomerize to create pockets of concentrated kinase activity[117]. In highly polarized cells such as neurons, JIP1 has a unique role in the directional movement of its cargo of signalling enzymes along microtubules (FIG. 5b). This is accomplished through association with kinesin motor proteins that move towards the plus end of growing microtubules or with dynactins that track towards the minus end of microtubules[118]. Moreover, JIP1 can also interact with the dual-specificity phosphatases MKP7 (also known as DUSP16) and M3/6 to switch off the JNK activation module[119]. More recent evidence suggests that JIP1 coordinates the retrograde axonal transport of autophagosomes. During neuronal development, these organelles deliver cytoplasmic materials from the growth cone to the cell body, where they are degraded by lysosomes[120]. Phosphorylation of JIP1 on Ser421 increases binding to kinesin, whereas dephosphorylation favours binding to dynactin[120]. Thus, covalent modification of this scaffold protein not only determines which binding partners preferentially associate with JIP1 but also switches the directional movement of this organellar signalling scaffold (FIG. 5b).

Conclusions and future directions

As discussed above, scaffold proteins regulate a multitude of cellular responses — at the plasma membrane, on organelles and in the nucleus. They coordinate enzyme location and activation, and substrate

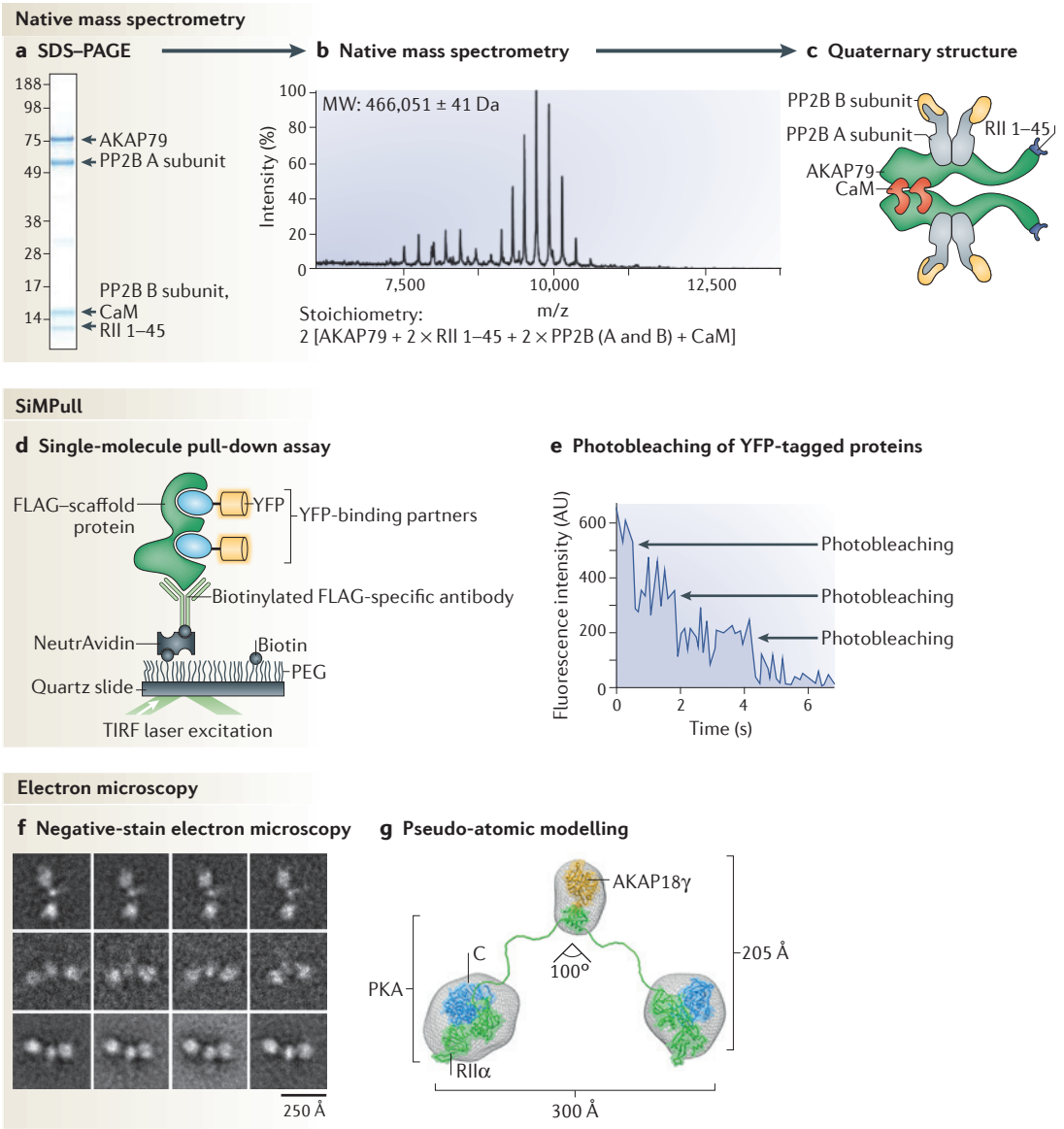


Figure 6 | **Emergent technologies for the analysis of signalling scaffolds.** Three emergent biophysical approaches have the potential to enhance the molecular dissection of macromolecular signalling scaffolds. Native mass spectrometry enables the investigation of intact protein complexes[121,140]. This top-down mass spectrometry approach probes the quaternary structure of protein complexes suspended in volatile buffers, thus enabling accurate calculation of their mass[123]. An A-kinase anchoring protein 79 (AKAP79)–kinase–phosphatase sub-complex was first assessed by SDS–PAGE (part **a**). Next, the molecular mass of the sub-complex was derived by native mass spectrometry (part **b**). The quantitative information that was obtained was used to determine a more refined model of the quaternary structure of this macromolecular complex (part **c**). Single-molecule pull-down photobleaching (SiMPull) is a sensitive new assay that combines a modified pull-down assay (part **d**) with single-molecule photobleaching of fluorescently tagged proteins (part **e**) to enable direct analysis of individual protein complexes[124]. SiMPull can be used to derive the ratio of distinct protein complexes assembled on a specific population of scaffold protein. Electron microscopes have sufficient resolving power for structural studies of macromolecules. Recent technical innovations, including a new generation of direct-detection camera and the development of more sophisticated data-processing packages, have markedly increased the resolution of cryo-electron microscopy for higher-order macromolecular complexes[127,128]. Smaller complexes (molecular weight (MW) <300,000 kDa) can be resolved by negative-stain electron microscopy (part **f**). Class averages of negative-stain electron microscopy particles show three perspectives of the AKAP18γ–protein kinase A (PKA) complex[55]. The density map of the AKAP18γ–PKA holoenzyme complex can be overlaid with structural models from the RCSB Protein Data Bank (PDB) coordinates for AKAP18γ (PDB code: 3J4Q), and the type IIα regulatory (RIIα) and catalytic (C) subunits of PKA to enable pseudo-atomic modelling (part **g**). CaM, calmodulin; FLAG, DYKDDDDK polypeptide tag; m/z, mass-to-charge ratio; PEG, polyethylene glycol; PP2B, protein phosphatase 2B; TIRF, total internal reflection fluorescence; YFP, yellow fluorescent protein. Parts **a** and **b** are adapted from REF. 123, US National Academy of Sciences; part **e** is adapted from REF. 125, US National Academy of Sciences; and part **g** is adapted from REF. 55, eLife Sciences Publications.

selectivity. They also function as allosteric modulators of the enzymes under their influence, facilitate the transport of cargo and segregate alternative cellular fates. Consequently, they are prime research targets for therapeutic objectives.

Emergent technologies often open the door to new biological insights. In this final section, we highlight three biophysical applications that provide the means to establish the stoichiometry of enzyme scaffolds, the range of enzyme combinations assembled on an individual scaffold protein and the near-atomic structural details of intact macromolecular complexes (FIG. 6).

First, native mass spectrometry is an exciting new technique that probes the quaternary structure of intact protein complexes that are suspended in volatile buffers[121,122]. This powerful quantitative approach can accurately establish the composition and stoichiometry of macromolecular complexes[123]. Native mass spectrometry can even be used to monitor the binding of a drug to different configurations of a signalling scaffold and to establish whether the complex under investigation exists in multimeric forms (FIG. 6a–c).

Second, single-molecule pull-down photobleaching (SiMPull) is an exquisitely sensitive pull-down assay that monitors the number of sequential photobleaching steps to accurately calculate the stoichiometry of the individual fluorescent enzyme–scaffold complexes that are captured from cell lysates[124]. This approach has been successfully used to calculate the stoichiometry of several PKA–AKAP complexes *in situ*[125]. Another innovative modification of the SiMPull technique monitors two proteins labelled with different fluorophores. This has been used to calculate the occupancy of the enzyme-binding site on a scaffold protein (FIG. 6d,e).

Third, the complexity, size and intrinsic disorder of many macromolecular complexes preclude analysis by X-ray crystallography[62]. The cutting-edge approaches of negative-stain electron microscopy and cryo-electron microscopy can be applied to resolve near-atomic structures of enzyme scaffolds (FIG. 6f,g). Moreover, recent innovations in detection sensors and processing algorithms have enabled the collection and analysis of cryo-electron microscopy data sets that extend the effective resolution of reconstructed macromolecular assemblies to a resolution of <4 Å[126–130]. This level of resolution produces density maps that will enable investigators to pinpoint the conformational elements of these large and flexible signalling scaffolds that are crucial for their function. Undoubtedly, the increasing use of these three technical breakthroughs will yield new insights into the inner workings of enzyme scaffolds as local mediators of cellular control.

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Competing interests statement
The authors declare no competing interests.

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