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Review

# Modularity and functional plasticity of scaffold proteins as p(*l*)acemakers in cell signaling

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ABSTRACT

Cells coordinate and integrate various functional modules that control their dynamics, intracellular trafficking, metabolism and gene expression. Such capacity is mediated by specific scaffold proteins that tether multiple components of signaling pathways at plasma membrane, Golgi apparatus, mitochondria, endoplasmic reticulum, nucleus and in more specialized subcellular structures such as focal adhesions, cell–cell junctions, endosomes, vesicles and synapses. Scaffold proteins act as "pacemakers" as well as "placemakers" that regulate the temporal, spatial and kinetic aspects of protein complex assembly by modulating the local concentrations, proximity, subcellular dispositions and biochemical properties of the target proteins through the intricate use of their modular protein domains. These regulatory mechanisms allow them to gate the specificity, integration and crosstalk of different signaling modules. In addition to acting as physical platforms for protein assembly, many *professional* scaffold proteins can also directly modify the properties of their targets while they themselves can be regulated by post-translational modifications and/or mechanical forces. Furthermore, multiple scaffold proteins can form alliances of higher-order regulatory networks. Here, we highlight the emerging themes of scaffold proteins by analyzing their common and distinctive mechanisms of action and regulation, which underlie their functional plasticity in cell signaling. Understanding these mechanisms in the context of space, time and force should have ramifications for human physiology and for developing new therapeutic approaches to control pathological states and diseases.



**Contents**





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## 1. Introduction

### 1.1. Scaffold proteins define a unique class of signaling organizers

Cell is an autonomous machine that can coordinate and integrate various functional modules that control processes such as cytoskeleton rearrangement, intracellular trafficking, organelle and membrane dynamics, cell metabolism and immune response, gene expression and protein synthesis and stability, leading ultimately to cell motility, cell growth, cell death and cell differentiation (Fig. 1). Fundamental to all these distinct functional modules are extensive and iterative networks of protein–protein interactions that are executed and regulated with precise locality and timing [1,2]. These signaling modules, rely not only on the reactivity of the interacting partners but also depend on where, when and how efficiently these interacting proteins can be recruited and organized into higher order functional units in response to chemical and force perturbations in the environments. To build multi-component signaling networks efficiently, cells have evolved a unique class of *organizer* proteins, generally termed as scaffold proteins that: (a) recruit several proteins to a specific locality, (b) organize them into a higher order macromolecular complex, (c) facilitate the interaction and to fine-tune the activity and crosstalk among the proteins within the entire assembly and (d) coordinate functions of different molecular assemblies in different parts or "microdomains" of the cell (Figs. 1 and 2). The recruitment action can also be achieved by much simpler modular devices such as the adaptor or docker proteins. The adaptor protein simply bridges two proteins together, and in some instances dockers can undergo certain modifications such as phosphorylation for the purpose of localization or recruitment of target proteins. In general, adaptors and dockers do not directly affect the property of their target proteins. However, there is an emergent class of *professional* scaffold proteins which use their modular domains to assemble more than two other proteins. The scaffold proteins use multiplicity of their domain architecture, specific compartmentalization and intrinsic protein dynamics, not only to facilitate the interaction amongst multiple incoming partners, but in some instances also to modify the properties of the assembled complexes. Such an activity of scaffold proteins results in a plethora of emergent signaling properties including changes in dose–response of the ligand-activated pathway, threshold sensitivity, insulation against inactivating signals, crosstalk, integration and feedback regulation among different signaling nodes. Furthermore, scaffold proteins can also be modified by their interacting partners, thus providing important feedback circuits that in turn affect their spatial localization, their ability to recruit and retain substrates or the protein turnover/degradation rates. Such a complicated modular design and dynamic function of scaffold proteins in specific space and time help ensure that desired biochemical signals and mechanical forces are effectively sensed and transduced to their downstream molecules in a well-controlled manner. These intricate physical assemblies allow for signaling specificity and result in stimulatory or inhibitory signals transmitted by major biological pathways and networks.

Many scaffold proteins have been identified and functionally characterized. While majority of them are involved in controlling functions of core pathways, several also function at the nodes of signaling traffic or are present in different cellular locales, providing further versatility for signaling crosstalk. Examples of scaffold proteins include regulators of: (i) the Ras/mitogen-activated protein kinases (e.g. KSR, MP1, CNK1, JIP, IQGAP1, paxillin, SEF, β-arrestins, Axin, CARMA, RGS), (ii) G-protein coupled receptors (e.g. RGS, β-arrestins), (iii) small GTPases and their regulators (e.g. GIMAPs, MEKK1, MEK2, BNIP-2), (iv) cyclic AMP and protein kinase A (e.g. AKAPs, β-arrestins, RACK1, DISC1), (v) calcium signaling (e.g. IQGAP, PSD-95), (vi) Hippo tumor suppressor pathway (e.g. Sav, Mats) and (vii) cytoskeleton network, mechanosensing and mechanotransduction (e.g. FAK, RACK1, ILK, paxillin, p130Cas, IQGAP) (see Table 1). As such, deregulation of scaffold proteins have

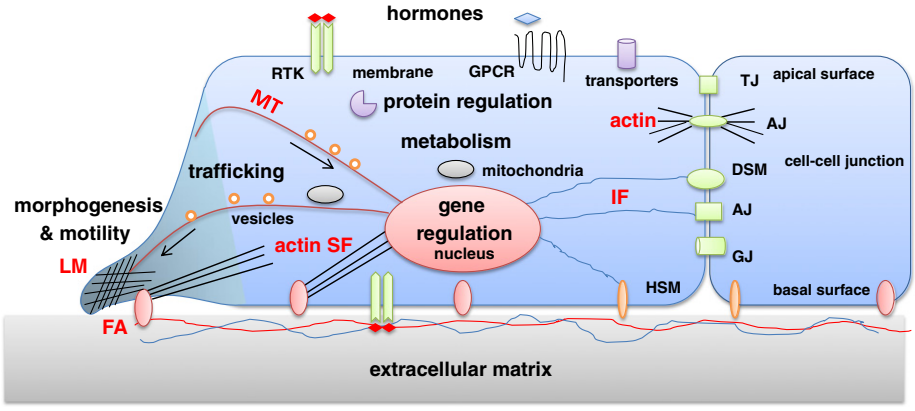


**Fig. 1.** The perfect links — coordination and integration of signaling networks define cell fates. The cell, as a machine, can execute distinct biological functions through an intricate network of protein–protein interactions that are assembled at specific place and time through their unique scaffold proteins. These events are tightly controlled at the cell membrane, cell–cell junction, cell-matrix, cytoplasm, nucleus, vesicles, organelles and cytoskeleton, as exemplified by this epithelial cell model. Signaling outputs from one of more of these signaling hubs can lead to cell morphogenesis, intracellular trafficking, metabolism control, regulation of gene and protein expression, protein stability and their post-translational modifications. These distinct functional modules are coordinated further to achieve the desired physiological outcomes under the influences of biochemical (hormones/chemicals) and physical (surface rigidity/force/geometry) perturbations in systemic and micro-environments. This intricate coordination, integration and feedback help determine their dynamics, growth, death and differentiation. *Abbreviations*: LM, lamellipodia; FA, focal adhesion; SF, stress fibers; MT, microtubules; IF, intermediate filaments; TJ, tight junction; AJ, adherens junctions; DSM, desmosome; GJ, Gap junction; HSM, hemidesmosome; RTK, receptor tyrosine kinase; and GPCR, G-protein coupled receptor. Names of specific scaffold proteins and other organelle structures are omitted for clarity.

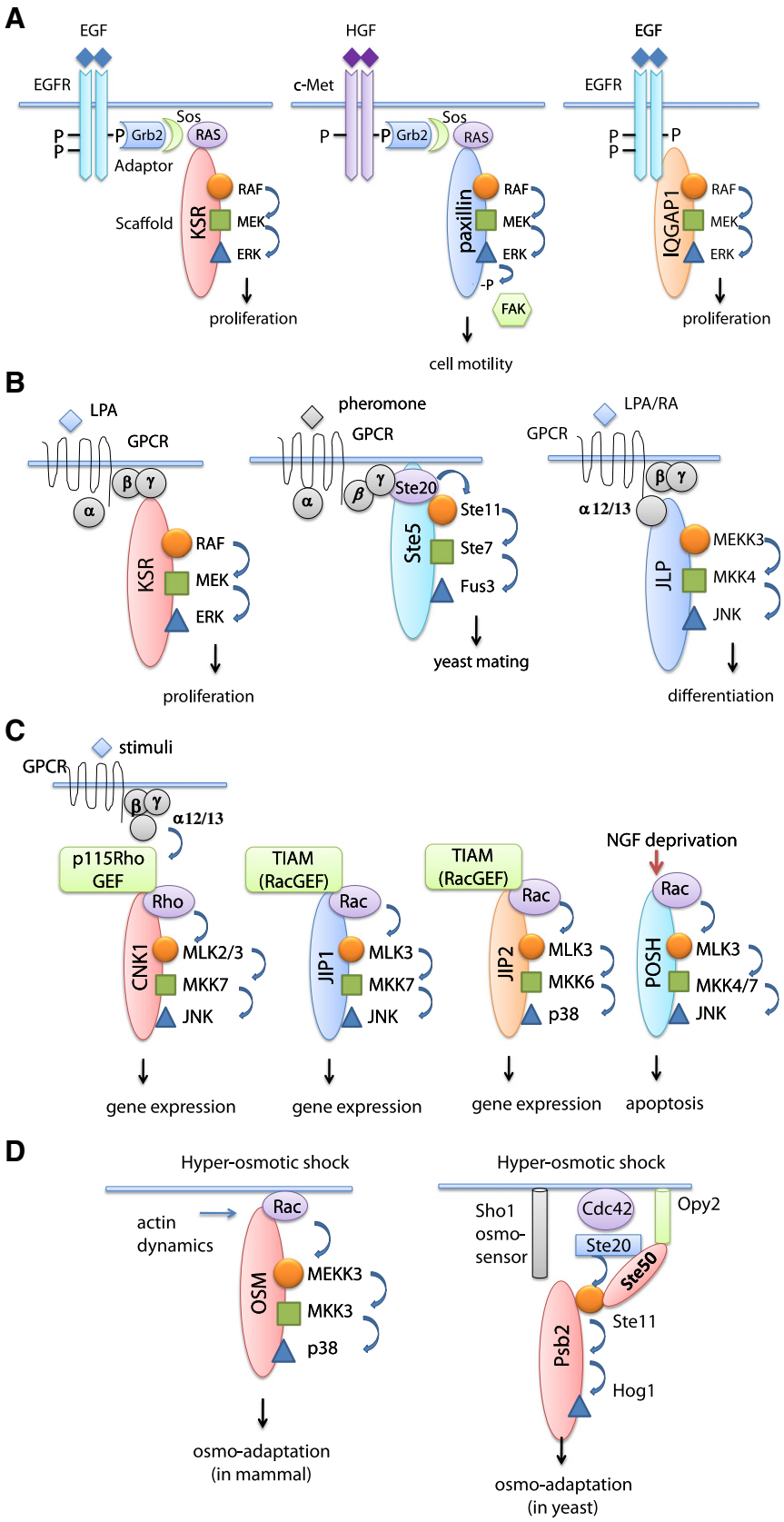


**Fig. 2.** Variations of a common theme — multiple scaffolds, common targets. The "3-tier kinase" scaffold proteins adopt a common platform design to control different signaling output downstream of receptor tyrosine kinases (A), G-protein coupled receptors (B), and those linking Rho small GTPases (C) and osmolarity sensors (D). Please refer to text for details.

**Table 1**
Scaffold proteins are the "pacemakers" as well as "placemakers" that regulate the localization, assembly, integration, activities and crosstalk of signaling proteins at precise locations and timing by utilizing their unique protein domains and undergoing multiple modes of regulation. These mechanisms help maintain the dynamics of key signaling pathways leading to the activation of various MAPKs (ERK, JNK, p38), G-proteins (GPCR, small GTPases), cAMP and calcium-dependent signaling networks, HIPPO pathway and mechanosensing and mechanotransduction pathways, among many others. Please refer to the text for more detailed discussion on selected scaffold proteins.

*The abbreviations used for the selected scaffold proteins indicated in the Table and in the text are*: Ste5, Sterile5; KSR, kinase suppressor of Ras; CNK, connector enhancer of KSR; MEK2, mitogen-activated protein kinase/ERK kinase 2; IQGAP1, IQ-motif-containing GTPase-activating protein 1; Sef, similar expression to FGF; MP1, MEK binding partner 1; JIP1–4, JNK-interacting partner 1–4; JLP, JNK-associated leucine zipper protein; MEKK1, MEK kinase 1; POSH, plenty of SH3 domains; CARMA1, CARD-MAGUK1 (also known as CARD11 or Bimp3); Pbs2p, polymyxin B sensitivity 2p; OSM, osmosensing scaffold for MEKK3; RGS, regulators of G-protein signaling; Axin, axis inhibition protein 1; BNIP-2, BCL2/adenovirus E1B 19 kDa protein-interacting protein 2; GIMAP, GTPase of the immunity-associated protein; mAKAP, muscle A-kinase anchoring protein; AKAP-LBC, A-kinase anchoring protein-lymphoid blast crisis; InaD, inactivation no after potential D; PSD-95, postsynaptic density-95; MTG, myeloid translocation gene; Mats, Mob-as-tumor suppressor; MOBKL1B, Mps one binder kinase activator-like 1B; FAK, focal adhesion kinase; RACK1, receptor for Activated C-Kinase1; p130Cas, Crk-associated substrate; ILK, integrin-linked kinase; Hpo, HIPPO (human orthologue MST1/2); Wts, Warts/LATS kinase (human orthologue LATS1/2), p75 NTR, p75 neurotrophin receptor; and DISC1, disrupted in schizophrenia 1.

*The abbreviations for the protein domains or motifs indicated in the table and used in the text are*: PH, pleckstrin homology; CA1–5, conserved areas 1–5; SAM, sterile alpha motif; CRIC, conserved region in CNK; PDZ, post synaptic density protein (PSD95), *Drosophila* disk large tumor suppressor (Dlg1), zonula occludens-1 (ZO-1); CHD, Calponin-homology domain; GRD, RasGAP-related domain; NLS, nucleus localizing signal; NES, nucleus exporting signal; JBD, JNK binding domain; PTB, Phosphotyrosine-binding; SH3, Src-homology domain 3; CARD, caspase Recruitment Domain; PID, phosphotyrosine interaction domain; BCH, BNIP-2 and Cdc42GAP Homology; DH-PH, Db1 homology–pleckstrin homology; DAG, diacylglycerol; GUK, guanylate kinase; FBM, FERM binding motif; SARAH, Salvador/RASSF1A/Hippo; FERM, F for 4.1 protein, E for ezrin, R for radixin, M for moesin; and LIM, Lin11, Isl-1, Mec-3.

| Scaffold proteins | Target proteins | Domains and motifs | Regulation |
|---|---|---|---|
| **ERK signaling** | | | |
| Ste5 | Ste11, Ste7 and Fus3 in yeast mating | PH, RING-H2 | Localization (membrane and nuclear-cytoplasmic shuttling), phosphorylation, homo- and heterodimerization, oligomerization, degradation |
| KSR | RAF, MEK and ERK for cell proliferation and transformation. Also catalyzes MEK1 activation and binds to iNOS and Hsp90 to increase iNOS activity. Also interacts with AKAP-Lbc, another scaffold protein responsible for cAMP/PKA signaling | CA1–5, kinase domain | Localization (cytosol, membrane, and nuclear-cytoplasmic shuttling); phosphorylation, homo- and heterodimerization |
| CNK | RAF, MEK and ERK for cell proliferation, growth and differentiation; also for JNK activation by p115RhoGEF and crosstalk with Rho signaling | SAM, CRIC, PDZ, PH | Phosphorylation, ubiquitination |
| MEK2 | Promotes binding of peptidyl-prolyl isomerase PIN1 with the BCH domain-containing RhoGAP, the BPGAP1 | Proline-rich sequence, kinase domain | Phosphorylation |
| IQGAP1 | Binds actin, $Ca^{2+}$/calmodulin, E-cadherin, EGF receptor, B-Raf, MEK1/2, ERK2 and Cdc42; may regulate signaling from the two main glutamate receptors in the central nervous system, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, and N-methyl-D-aspartate (NMDA) receptor. | CHD, WW, IQ, GRD | Phosphorylation, $Ca^{2+}$/calmodulin |
| SEF | MEK and ERK in Golgi | Membrane-spanning domain, extracellular and intracellular domain (ICD) | Localization (Golgi, endocytic vesicles), phosphorylation, homophilic interaction via ICD |
| MP1 | Binds MEK1 and ERK1 in late endosomes and lead to sustained ERK activation; also regulates PAK1-dependent ERK activation and inhibit Rho/ROCK during adhesion and cell spreading | No distinctive domains identified | Localization to late endosome is mediated by adaptor protein p14 |
| β-Arrestin | Linked to many signaling receptors and it stabilizes JNK and ERK in early endosome; also as a scaffold for AKT1 and regulator of cAMP signaling pathways | NLS, clathrin binding motif (amino acid residues LIEF) and RxR-binding motifs. β-arrestin2 contains NES | Localization (cell membrane, endosome, nucleus, nucleocytoplasmic shuttling), phosphorylation, ubiquitination, monomeric, homo- and hetero-oligomerization, forming an alliance with CARMA |
| **JNK signaling** | | | |
| JIP1–4 | JNK, p38 (via JIP2/4) and RacGEF (via JIP1) | JBD, JIP1/2-(SH3, PTB) JIP3/4-(Coiled-coil domain, leucine zipper) | Phosphorylation, ubiquitination, homodimerization, degradation, forming alliances with other scaffold proteins such as POSH and FAK |
| JLP (variant of JIP4) | MEKK3, MKK4 and JNK for retinoic-acid induced differentiation; also links Gα12 and Gα13 to the JNK signaling; for p38α/β activation in myogenic differentiation by coupling to Cdo and BNIP-2 | JBD2, leucine zipper, coiled coil domain | Crosstalk with scaffold protein BNIP-2 |

**Table 1** (*continued*)

| Scaffold proteins | Target proteins | Domains and motifs | Regulation |
|---|---|---|---|
| MEKK1 | RAF-1, MEK1 and ERK2; JNK/SAPK; crosstalk with Rho | Kinase domain, N-terminal regulatory domain | Phosphorylation, alliance with another scaffold, Han11 to regulate HIPK2 and MEKK1 |
| POSH | Rac1 and JNK to promote apoptosis in neurons. Shroom3 downstream of Nogo66 to suppress axon outgrowth | RING finger, SH3 | Phosphorylation, degradation |
| CARMA1 | Induces oligomerization (via CARD domain) with JIP-like scaffold, Bcl10, for the assembly of JNK2, MKK7 and TAK1 | CARD domain, Guanylate kinase-like domain, PDZ (DHR) domain | Localization (cytosol, membrane raft), phosphorylation; alliance with Bcl10 |
| **p38 signaling** | | | |
| Pbs2p | Ssk2p, Ssk22p and Hog1p in osmo-sensing | Proline-rich motif | Phosphorylation |
| OSM | Rac, MEKK3, MKK3 and p38 in osmosensing; but involves Rac1-PLC-γ1 instead of MKK3-p38 pathway in activating osmoprotective transcription factor NFAT5.<br>Other scaffold proteins mediating p38 activation include JIP2/4, JLP (see above under JNK signaling) | PID | Localization to membrane ruffles |
| **GTPases-related signaling** | | | |
| RGS | Negative regulators of G protein signaling. RGS12 also assembles TrkA, activated H-Ras, B-Raf and MEK2 in endosome while RGS14 assembles H-Ras and Raf kinases to inhibit PDGF-induced ERK activation. | RGS domain RGS12: PDZ, PID, Ras-binding domain RGS14: GPR/GpLoco, Ras-binding domain | Localization (cytosol, plasma membrane, endosome, nucleus), phosphorylation, glycosylation, sumoylation, palmitoylation, cross-talk with $IP_3$-mediated $Ca^{2+}$ signaling (via RGS14) |
| Axin | For activation of MKK4/7 and JNK. It also targets Tip60, HIPK2 and p53 to induce p53 activation and apoptosis and acts as a co-activator for the nuclear-matrix associated scaffold, the pro-myelocytic leukemia protein, in p53 activation; causing β-catenin degradation and negatively regulates Wnt for survival. | RGS homologous domain, binding sites for MEKK1, GSK3β, β-catenin and PP2A/MEKK4; DIX domain; also contains intrinsic disordered regions. | Localization (cytosol, nucleus), phosphorylation, ADP-ribosylation, ubiquitination, sumoylation, homodimerization |
| BNIP-2 | Cdc42, Rho, p50RhoGAP/Cdc42GAP, BPGAP1 and other BCH domain-containing proteins. Also binds RhoGEF, FGF receptor, Cdo receptor, Bcl-2 and E1B 19 kDa viral proteins. Its brain-specific homolog, BNIP-H (Caytaxin) transports glutaminase KGA on kinesin-1 towards neurite termini | BCH domain at its C-terminus | BCH domain mediates homophilic or heterophilic interactions with identical or similar BCH domains and it also binds to Cdc42, Rho, RhoGEF and RhoGAP; Fragments containing BCH domain can be released from the full-length proteins upon cleavage by caspases and granzyme |
| GIMAP | GTP-dependent protein scaffold at the surface of lipid droplets to regulate apoptosis | Guanine nucleotide binding domain | Oligomerization might assemble pro- and anti-apoptotic Bcl2 family proteins |
| AKAP-LBC/AKAP13/Proto-Lbc | Links Gα13 to Rho signaling via its RhoGEF activity; scaffold for cyclic AMP/PKA and calcium signaling (see below) | DH–PH domain, phorbol-ester/DAG-type zinc finger | Phosphorylation by anchored PKA regulates activation of PKD via PKC but inhibits its RhoGEF activity upon its sequestration by 14-3-3 |
| **Cyclic AMP, PKA and calcium signaling** | | | |
| AKAP12/Gravin | Targets PKC, PKA, cyclins, calmodulin, Src, β-adrenergic receptor and cAMP-degrading phosphodiesterase-4 (PDE4) to modulate cyclic AMP signaling. It also associates with actin or tubulin cytoskeleton by binding directly or indirectly to profilin, dynein and cytokinesis components; as a tumor suppressor and anti-metastatic agent. | Nuclear localization signals, AKAP domain, nuclear exclusion domain. | Localization (membrane, perinuclear), lipid modification by myristoylation, phosphorylation |
| mAKAP | Muscle-specific AKAP sequesters PKA and EPAC (an exchange protein that activates small GTPases Rap1 and Rap2), ERK5, PDE4D3, and PP2A to modulate the cAMP and calcium ion-sensitive signaling. Also controls calcium release from the sarcoplasmic reticulum via ryanodine receptors | Spectrin-like repeats, RIIα binding site | Localization (membrane, sarcoplasmic reticulum, nucleus membrane), lipid modification |
| AKAP-LBC/AKAP13/Proto-Lbc | Links $G_{\alpha 13}$ to Rho signaling via its RhoGEF activity. It binds PKA, PKC and PKD. It also associates with KSR-1 and upon activation by cAMP, the bound PKA phosphorylates Ser838 on KSR-1, leading to sustained ERK activation. | DH–PH domain, phorbol-ester/DAG-type zinc finger | Phosphorylation by anchored PKA regulates activation of PKD via PKC but inhibits its RhoGEF activity upon its sequestration by 14-3-3 |

(*continued on next page*)

**Table 1** (*continued*)

| Scaffold proteins | Target proteins | Domains and motifs | Regulation |
|---|---|---|---|
| AKAP79/150 | PKA, PKC and PP2B-calcineurin proteins at post-synaptic density. Also binds to mGluR1/5. Crosstalk with scaffold proteins β-arrestin2 for MAPK signaling and PSD-95 for endocytosis of synaptic AMPA receptors. Also binds IQGAP1. | Polybasic domain interaction with PtdIns(4,5)$P_2$ | Phosphorylation; calmodulin binding; requiring actin remodeling for its anchorage on membrane ruffle and post-synapse |
| AKAP18/AKAP15 (α, β, γ, δ) | Localizes PKA to plasma membrane and coupled to L-type calcium channel. Modulates $Ca^{2+}$ re-uptake into the sarcoplasmic reticulum via SERCA in cardiomyocytes. Also modulates $ca^{2+}$ uptake via DHPR. | Membrane localization motif (α, β, myristoylation, dual palmitoylation); cytosolic and nuclear (γ, δ NLS) | Distinct localization for different isoforms by lipid modification and targeting motifs |
| AHNAK | Calcium channels, PLCγ and PKC; interacts with actin and annexin-2/S100A10 complex to regulate cortical actin cytoskeleton and cell membrane structure | PDZ | Localization (membrane, nucleus, nucleocytoplasmic shuttling); translocated from the nucleus to plasma membrane upon increase in calcium levels or phosphorylation by PKB/Akt |
| InaD | TRP, TRPL, NORPA, INAC, Calmodulin in *Drosophila* visual signal transduction to trigger opening of TRP channels for membrane depolarization and calcium entry into the rhodopsin. | PDZ domains | Phosphorylation, oligomerization with PDZ |
| PSD-95 | Glutamatergic receptors (NMDA and AMPA), potassium channels and other signaling proteins in synapses; also tethers glycine transporter-1 and NMDA receptors; links nNOS to NMDA receptor. It binds to two other scaffold proteins, i.e. IQGAP1 to regulate spine density and cognitive processes and to AKAP150 for regulating endocytosis of synaptic AMPA receptors | PDZ, SH3, GUK, disordered linker regions | Lipid modification, interacts with endosomal ATPase, Hrs (for synaptic clustering of PSD-95β), multiple scaffold assembly with PSD-93 |
| β-Arrestin | Inhibits β2-adrenoreceptor coupling to Gs, hence desensitizing activation by adenylyl cyclases; it also sequesters PDE4D5 to degrade cAMP for cells survival and also engaging CD28 and recruited to T-cell lipid rafts.<br>It is also a scaffold for RAF-MEK–ERK module (see above) | NLS, clathrin binding motif (amino acid residues LIEF) and RxR-binding motifs. β-arrestin2 contains NES | Localization (cell membrane, endosome, nucleus, nucleocytoplasmic shuttling), phosphorylation, ubiquitination, monomeric, homo- and hetero-oligomerization |
| RACK1 | PDE4D5, also as a scaffold for focal adhesion complex (see below) | WD repeats | Localization (cytosol, cell membrane/periphery, perinuclear region, focal adhesion-via integrin), phosphorylation, monomer and oligomerization |
| DISC1 | Regulates cAMP (via PDE4) and Wnt (via inhibiting the negative regulator, GSK3β). Anchors several targets such as NDEL1, LIS1, PCM1 and BBS in centrosome and microtubules networks. At synapse, DISC1 augments the binding of PSD-95 and KAL-7 while DISC1 interacts with TNIK to regulate turnover of synapse structure. DISC1 also regulates RacGEF, TRIO for axon guidance. | Putative coiled-coil regions, dimerization and oligomerization domain at the C terminus | Possibly via phosphorylation (at S710 switches from progenitor proliferation to migration in neural development), sumoylation, oligomerization and degradation (proteolysis), differential splicing isoforms. |
| **HIPPO pathway** | | | |
| Salvador (WW45) | Forming the core Hpo Kinase Cassette with kinases Hpo (MST1/2) and Wts (LATS1/2) and another scaffold protein, Mats (MOBKL1B); facilitates Wts phosphorylation by Hpo to inactivate transcriptional co-activator, Yki (YAP1). | FBM motif, SARAH, WW domain | Phosphorylation, homophilic, heterophilic interaction (with MST), competing with RASSF for HPO |
| Mats (MOBKL-1B) | Stimulates catalytic activity of Wts (LATS1/2) kinase. Hpo-phosphorylated Mats promote Mats–Wts complex formation. | No obvious domains identified | Localization, phosphorylation |
| **Cytoskeleton and mechanobiology** | | | |
| FAK | Links integrins to F-actin by assembling paxillin, ILK, p130Cas, talin, vinculin, RhoGAP, RhoGEFs, isomerase, kinases and phosphatases at focal adhesions. Links to growth factor receptors c-MET, IGF-1R and phospholipids, PtdIns(4,5)$P_2$; promotes assembly of p53-MDM2 in the nucleus, leading to p53 ubiquitination and degradation | FERM domain, proline-rich regions, SH3, catalytic domain, focal adhesion targeting | Localization (cytosol, focal adhesion-via FERM domain, nucleus), phosphorylation, sumoylation; mechanical forces; intramolecular interaction; interacts with ezrin and β-integrins clustering and interaction with PtdIns$(4,5)_2$ for activation. |

**Table 1** (*continued*)

| Scaffold proteins | Target proteins | Domains and motifs | Regulation |
|---|---|---|---|
| RACK1 | FAK, PKC, Src, integrin, NMDA receptor and PDE4D5 at nascent adhesions. Also recruits PKC to ribosome for local protein translation | WD repeats | Localization (cytosol, cell membrane/periphery, perinuclear region, focal adhesion-via integrin), phosphorylation, monomer and oligomerization |
| Paxillin | Binds RAF–MEK–ERK upon hepatocyte growth factor stimulation; can also shuttle to nucleus to act as trans-activator | LIM domains | Localization (cytosol, cytoskeleton, focal adhesion, nucleus), phosphorylation |
| p130Cas | Recruits Crk and GEFs-NSP and DOCK180, zyxin and Ajuba to the focal adhesion | SH3, proline-rich region, serine-rich region, intrinsic disordered regions | Phosphorylation, mechanical forces (stretched-induced phosphorylation), caspase cleavage |
| ILK | Assembles β-integrin subunits, paxillin, actopaxin family and the adaptor protein PINCH at focal adhesion | NLS, NES, ankyrin repeats, PH-like domain, catalytic domain | Localization (cytosol, focal adhesion, nucleus, centrosome), phosphorylation, forming alliance with FAK, PINCH |

been implicated in cancers (e.g. KSR, IQGAP, AKAP12/SSeCKS/Garvin, Axin, SAV1, MOB1, FAK, p130Cas), diabetes and obesity (e.g. JIP, AKAP), cardiac diseases (e.g. AKAP13, ILK, FAK, IQGAP1), kidney, muscular, inflammatory/immunological disorders (e.g. KSR, SLP-76, AKAP12/SSeCKS/Garvin) and neuronal disorders (e.g. KSR, JIP, PSD-95, DISC1). For detailed descriptions of these scaffold proteins and their roles in specific signaling pathways, we refer readers to recent reviews [2–26].

Despite of rather good understanding of the general functions of scaffold proteins, many questions are still outstanding, such as: (a) How do scaffolds use their diverse modular protein domains to recruit, organize and regulate their binding partners in concert? (b) How scaffolds influence the sensitivity threshold, the strength, the amplitude, and the duration of signals they mediate? Do they do it passively as physical platforms only, actively as catalysts or via combination of both kinds of interactions? (c) Which of the multicomponent complexes assembled on scaffolds convey discrete signals and which are redundant? (d) How could functions of scaffold proteins be regulated by biochemical and mechanical cues? (e) How, despite their intrinsic complexity, the different scaffolds crosstalk with each other, leading to higher order of circuitry control? Do they propagate the signals by directly forming a new complex by shuttling between different localities or more intermediates are required to relay signals between different scaffolds? (f) Last but not least, how can one particular type of scaffold protein act on multiple sets of substrates whereas one particular type of substrates can become targets of different families of scaffold proteins?

Here, we elected to highlight some of the emerging and unifying themes of signaling by scaffold proteins at the molecular and cellular levels. We will first examine their properties that provide the basis for their functional plasticity in signaling and facilitate cross-talk among signaling pathways. We will also discuss the dynamic localizations of scaffolds, their conformational changes, their functional specificity, integration and crosstalk, their assembly, including their self-oligomerization and their interaction with other scaffold proteins that generate higher-order regulatory networks. We will emphasize that scaffold proteins do not merely act as passive molecular platforms but can also actively recruit modifiers to the complex or possess unique catalytic functions that directly act on their targets, leading to either activation or suppression of the signals. We will stress how functions of scaffold proteins can be regulated by various post-translational modifications such as phosphorylation, ubiquitination, sumoylation, lipid modification, as well as mechanical perturbation. Finally, we will propose approaches which can be employed to better understand the molecular function of scaffold proteins in the context of space, time and force in order to reveal an integrated picture, if not partial *blue-print*, of cell function. This review aims to identify new signaling principles that would illuminate actual cellular design and would allow re-wiring of signaling networks toward new cellular functions for therapeutic interventions.

## 2. General principles of scaffold designs, functions and coordination

### *2.1. Modularity in cell signaling circuitry and protein domains*

#### *2.1.1. Signaling specificity*

Scaffold proteins carry discrete modular protein domains and regions that are intrinsically disordered. These features help them recruit, organize, and regulate the activity of target proteins at strategic locations. By doing so, scaffold proteins convert an otherwise distributive signaling process (where many independent interactions are needed to generate multiple modifications on these target proteins) to a well-coordinated "one-stop" processive center that is still capable of modulating the function of multiple targets [27]. Consequently, scaffold proteins ensure specificity of signaling if these target proteins belong to the intermediates from one particular pathway. For example, in the classic Ras/mitogen-activated protein kinase (MAPK) cascade, activated Ras triggers a sequential "3-tier kinases" signaling module upon activation of the growth factor receptors; firstly by activating the RAF (a MAPK kinase kinase; MAPKKK) which then activates the MEK (a MAPK kinase, MAPKK) and finally, the ERK (a MAPK), all on a single scaffold, the Kinase Suppressor of Ras (KSR). The signals are eventually transduced to the nucleus to regulate cell proliferation. Specifically, the adaptor protein Grb2 directly links to the active and phosphorylated epidermal growth factor (EGF) receptor tyrosine kinase, and it positions the SOS exchange factor to catalyze the exchange of Ras from its inactive GDP-form to active GTP-bound form, and the KSR scaffold complex translocates to the membrane. There, they collectively elicit the phosphorylation relay from RAF to MEK and then to ERK, leading to ERK activation (Fig. 2A). A similar mechanism is also employed downstream of the heterotrimeric G-protein/G-protein coupled receptor (GPCR). Here, KSR1 interacts with $G_{\beta\gamma}$ via the γ subunit instead and is recruited to the plasma membrane upon LPA stimulation [28] (Fig. 2B).

Indeed, the "3-tier kinase" modular design of the KSR scaffold appears to have undergone convergent evolution whereby components which carry no obvious protein sequence similarity could also adopt very similar modularity but for specific signaling functions (Fig. 2A–D). In an analogous system in the yeast, Ste5 provides signaling specificity for the yeast mating by anchoring the relay for the MAPKKK Ste11 and MAPKK Ste7 towards MAPK Fus3. This also helps prevent the crosstalk

between the mating process and the filamentation process that also shares the same Ste11 and Ste7. The signal is therefore transduced to Kss1 without the need for any scaffold proteins during filamentation [29].

In other distinct MAPKKK/MAPKK/MAPK modules, activation of the Jun-N-terminal Kinase (JNK) and p38 (both of which are different MAPKs from ERK and are involved mainly in response to environmental stress, inflammatory cytokines, differentiation, neuronal apoptosis, vesicular transport and metabolism) are mediated by some distinct and some common scaffold proteins. For examples, JLP (a variant of the JIP4 scaffold protein) assembles the MAPKKK MEKK3 and the MAPKK MKK4 for the activation of JNK, leading to retinoic-acid induced differentiation (Fig. 2B). Other subtypes of JIP are also involved for the different combinations of mixed lineage kinase (MLK) and MKK proteins. For example, JIP3 recruits multiple MAPKKKK (MLK, MEKK1) and MAPKK (MKK4 and MKK7) to activate JNK under growth factor withdrawal or endotoxin insults whereas JIP1 recruits MLK–MKK7–JNK upon excitotoxic stress and obesity [19] or upon activation of Rac by its exchange factor, Tiam [30,31]. In addition, JNK is activated by two other scaffold proteins, firstly, the Plenty of SH3 (POSH) upon apoptosis stimuli or growth factor withdrawal, and mediated via Rac1–MLKs–MKK4/7–JNK relay module [32]. And secondly, by the Connector Enhancer of KSR-1 (CNK1) (Fig. 2C) that links active p115RhoGEF and Rho, downstream of Gα12/13, to the trio of MLK3, MKK7 and JNK for gene regulation [8,33]. Indeed, unique scaffold proteins that facilitate complex formation of Rho with RhoGEF or/and RhoGAP could help dictate the choice of downstream effectors, thus ensuring signaling specificity [8].

The p38 MAPK, on the other hand, can be activated by JIP2, JIP4 and JLP. While JIP4 provides an important platform for activating p38 that requires MKK3 and MKK6 [34], JLP links the myogenic receptor Cdo to activate p38 [35] in a process that also requires signaling input from the novel scaffold BNIP-2/Cdc42 pathway [36]. Furthermore, p38 can be activated upon Rac stimulation by Tiam and the recruitment of MLK3, MKK6 and p38 to JIP2 [30]. Alternatively, it can be activated after hyper-osmotic shock which activates Rac, followed by the recruitment and translocation of MEKK3, MKK3 and p38 by the scaffold protein, Osmosensing Scaffold for MEKK3 (OSM), to the Rac-enriched membrane ruffles (Fig. 2D) [37]. Interestingly, this osmo-regulatory mechanism is analogous to that of the *Saccharomyces cerevisiae* where the CDC42–STE50–STE11–Pbs2 module of the Hog1 MAPK pathway is activated in response to hyperosmolarity [37]. In *S. cerevisiae*, active CDC42 binds to STE20 where it also leads to STE50 forming a stable complex with STE11 in order to translocate the complex to the plasma membrane. STE20 then phosphorylates STE11 which would in turn act on the scaffold protein Psb2 that is already linked to the osmosensor Sho1. Hog1 is then activated to elicit osmo-adaptation.

Since the "3-tier kinase" MAPK pathways are essential in the yeast's response to pheromone, filamentous growth and osmo-stress and that all these responses use the same Ste20 and Ste11 MAPK kinase kinase (MAPKKK), an efficient "insulation" for these distinct pathways would become highly desirable. This can be achieved in part by unique interactions amongst the components of the signaling module (e.g. Ste11–Ste7–Fus3 in mating versus Ste11–Psb2–Hog1 in osmoregulation) and with their specific scaffold proteins as described above. Furthermore, the insulations can be regulated by the ability of one pathway to cross-inhibit the other, and also by the kinetic insulation [38]. On the other hand, the JNK stress-activated protein kinase-associated protein 1 (JSAP1), positively regulates the JNK while suppressing the ERK signaling pathway by directly preventing MEK1 phosphorylation and activation by RAF1. These results indicate that JSAP1 serves as a scaffolding factor for JNK, and also as a suppressor for ERK pathway [39]. Apart from acting as an insulator against undesirable inactivation, some scaffold proteins can also discriminate between isoforms to selectively assemble specific signaling components. The MAPK scaffold protein, MEK Partner 1 (MP1), for example, interacts with MEK1 and ERK1 but not with MEK2 and ERK2 [40]. In comparison, ERK2 enhances interaction of MEK2 with IQGAP1 without altering binding of MEK1, whereas the EGF stimulation enhances MEK1 interaction while reducing MEK2 binding to

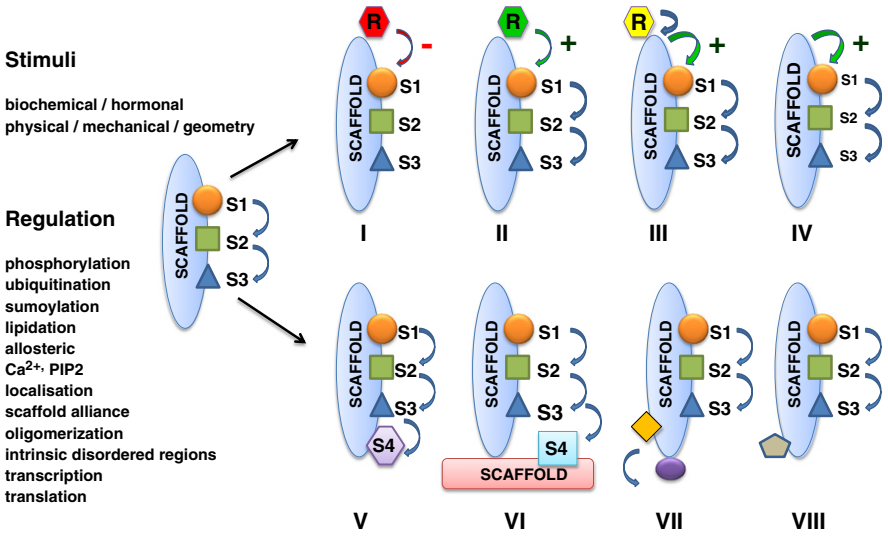


**Fig. 3.** Plasticity of scaffold proteins as the pacemakers and "placemakers" for the assembly, integration, crosstalk and regulation of signaling. Under basal conditions, scaffold proteins assemble their substrates or target proteins (S1 to S3) and allow their activation to proceed (shown by arrows). Upon biochemical and hormonal stimulation or physical and mechanical stress, the extent of the relay and signal output can be modified by the recruitment of a regulator (R) that can either suppress the initiation of the reaction at S1 or at any of the substrates (*Scenario I*) or directly promotes this reaction by acting on the substrates (*Scenario II*), or indirectly changing the conformation of the scaffold protein so the latter can catalyze the reaction further (*Scenario III*). In certain cases, a scaffold protein might regulate the reaction without the need of a regulator (*Scenario IV*). When protein(s) from other signaling node (e.g. S4) binds to the scaffold protein directly (*Scenario V*) or indirectly via the alliance of its own scaffold protein (*Scenario VI*), they can become modified by one of the substrates S1 to S4, leading to a novel integration or crosstalk of signaling. In other cases, one scaffold protein could serve as a platform for other reaction(s) to occur (*Scenario VII*) or it simply possesses other function unrelated to its scaffolding activity (*Scenario VIII*). Adding to such dynamic roles of scaffold proteins are various mechanisms by which these scaffold proteins can be modified and their functions regulated through multiple means. These mechanisms include but not limited to their protein phosphorylation, ubiquitination, sumoylation, allosteric regulation, binding by ions and phosphoinositides, specific localization, formation of scaffold alliance or its oligomerization, presence of intrinsic disordered regions, and changes in their gene expression and protein synthesis levels.

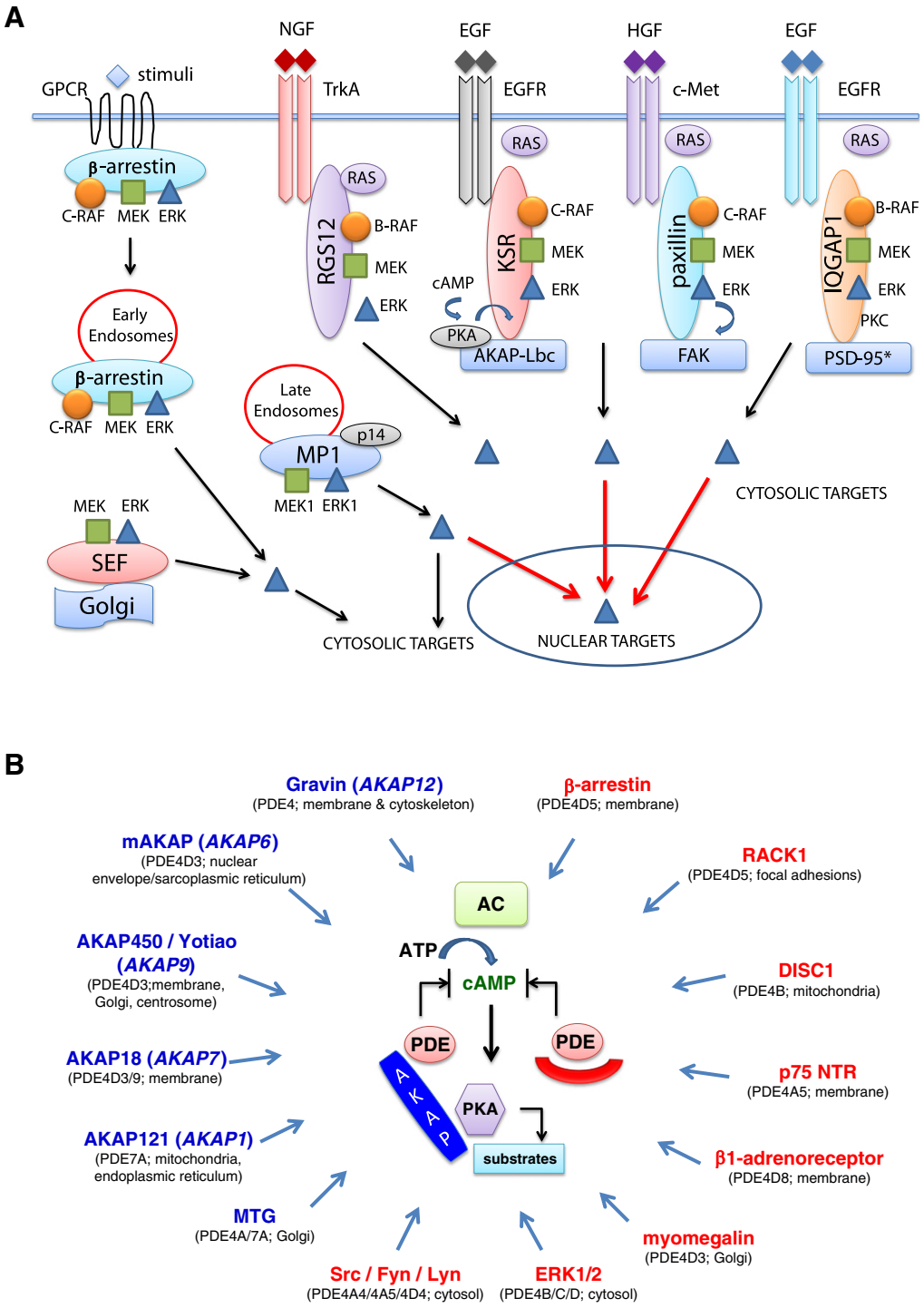


**Fig. 4.** Multiplicity and versatility of scaffold proteins. (A) Functions of the "3-tier" kinases, RAF–MEK–ERK and the "2-tier" kinases, MEK–ERK, are determined by their specific compartmentalization mediated by distinct scaffold proteins. This mechanism ensures that active ERK acts on different cytosolic or nuclear pools of substrates, leading to distinct biological functions. Some of the scaffold proteins (e.g. KSR, paxillin, IQGAP1) could also interact with other scaffold proteins (e.g. with AKAP-Lbc, focal adhesion kinase and PSD-95, respectively), thereby increasing the dynamics of signalling networks. For example, when the second messenger cAMP is generated in response to agonists, the PKA anchored on AKAP-Lbc will be activated and phosphorylates KSR-1, leading to sustained ERK activation. In comparison, activated ERK on the paxillin will phosphorylate FAK and together they regulate focal adhesion dynamics in cell motility. PSD-95, on the other hand, can couple IQGAP1 in regulating spine density and cognitive process in neurons, albeit in a process not necessarily linked to EGF receptor signaling (marked by *). β-Arrestin is another multifunctional scaffold protein that regulates the strength and duration of diverse signaling downstream of various receptors such as GPCR, Wnt, Notch, Hedgehog and TGFβ, and also downstream kinases such as the RAF–MEK–ERK (shown here) and Akt/PI3K cascades. It also plays an important role in regulating cAMP levels by recruitment of phosphodiesterase PDE4D5 (see next section, B). For clarity, Grb2 and SOS have been omitted from the network downstream of various receptors activation. (B) Sculpting local cAMP gradients by compartment-specific targeting of phosphodiesterases (PDEs) through diverse scaffold proteins and cellular partners. Signaling is propagated through the second messenger cAMP that is generated by activation of adenylyl cyclase (AC), acting downstream of stimulated membrane receptors. This second messenger will activate the effector protein, the protein kinase A (PKA) which subsequently phosphorylate its various substrates. This process is tightly regulated by precise spatiotemporal regulation on the gradients of cAMP at specific microdomains such as near the plasma membrane (usually associated with the membrane receptors), cytosol, mitochondria, endoplasmic reticulum, sarcoplasmic reticulum, Golgi, cytoskeleton, centrosome and nuclear envelope. Central to this regulation is the presence of the cAMP-degrading phosphodiesterases (PDEs) that are recruited to various compartments by specific AKAP scaffold proteins (indicated in *blue letters and blue scaffold cartoon*) or by other non-PKA binding scaffold proteins or other cellular proteins, as indicated in *red letters and red scaffold cartoon*. The plasticity of this versatile cAMP-PKA-PDE signaling node is further enhanced by the involvement of selective PDE isoforms, some of which are more commonly used than the others and they are differentially located (indicated in *brackets*). For the AKAPs, the more commonly used names are indicated followed by the recommended gene name listed as *italics* in the *brackets*. Please see text for more details and the references therein.

IQGAP1 [41]. In TCR costimulation, MEKK1 predominantly induces JNK1 activation while the related kinase MEKK2 regulates ERK5 activation. Furthermore, only the activated MEKK1, but not MEKK2, can recruit E3 ligase Itch for its function [42].

### 2.1.2. Signaling integration, crosstalk and feedback

While it is important to maintain the specificity of a signaling pathway by confining their hierarchical signaling intermediates within a closed circuit or microdomains, scaffold proteins can also promote crosstalk among multiple signaling networks. The crosstalk is facilitated by the multi-domain architecture of scaffolds, which allow them to interact with multiple components from diverse signaling pathways. This leads to an integration of multiple responses which can either be positively or negatively regulated (Figs. 3 and 4). For example, the MAPK scaffold protein, β-arrestin-2 can interact with several signaling components, resulting in the activation of ERK1/2, JNK3, p38, NF-κB, AKT, and many of their downstream responses [3,43,44]. It can stabilize phopshorylated JNK and ERK in early endosome, creating the second wave of sustained ERK activation outside of nucleus, thereby reducing gene expression. The β-arrestin also regulates the clathrin-dependent internalization, desensitization and resensitization of the GPCR, brings E3 ligases to the GPCR [6,7,45] and regulates cAMP signaling by recruiting the cAMP-degrading phosphodiesterase [23]. Interestingly, β-arrestin-2 but not β-arrestin-1 is required for LPA-induced NF-κB activation and interlukin-6 expression possibly via the recruitment of another scaffold CARMA3 to the LPA receptor during the GPCR-induced NFκB activation [46].

The regulators of G-protein signaling (RGS) comprise a large multifunctional protein family initially known to bind and deactivate $G_\alpha$ subunit upon stimulation of G-protein coupled receptors (GPCRs). However, several RGS proteins possess a multidomain architecture that adds complexity to their roles in addition to their GTPase-activating activity. Indeed, several RGS have been shown to act as scaffolds that link G-protein signaling to other pathways [9]. For examples, RGS14 integrates G-protein and Ras/RAF signaling pathways by modulating ERK activation [47]. RGS14 selectively inhibits PDGF but not EGF-stimulated ERK activation. It binds H-Ras and RAF on the membrane and this inhibition is reversed by co-expressing $G_{i\alpha1}$ that competes with RAF binding. In contrast, RGS4 regulates $G_{q/11}$ and $Ca^{2+}$ signaling [9] whereas RGS12 is an endosomal MAPK scaffold that assembles TrkA, activated H-Ras, B-RAF and MEK2 for prolonged ERK activation in NGF-mediated differentiation [48]. Interestingly, some RGS protein can act as a signaling switch. For example, upon sumoylation, the neuronal RGS-Rz protein switches from its GAP activity to scaffolding function for the $G_\alpha$ subunits for Mu-opioid receptors desensitization [49]. On the other hand, IQGAP1 also contains different signaling domains to regulate and provide crosstalk for $Ca^{2+}$/calmodulin, microtubule (via CLIP170), actin cytoskeleton (via Rac1, Cdc42 and APC), cell–cell adhesion (E-cadherin and β-catenin) and RAF–MEK–ERK signaling pathway, including its close proximity with the EGF receptor [12,41,50,51].

Axin is another master regulatory scaffold that interacts with many signaling components downstream of the Wnt pathway including the transcription regulator β-catenin, serine/threonine kinase GSK3, phosphatase PP2A, casein kinase CK and tumor suppressor APC, the TGF-β pathway (e.g. transcription regulators SMAD3 and SMAD7) and MEKK1/4, leading to activation of MKK4/7 and JNK [14,52]. It also forms a distinct complex with Tip60, HIPK2 and p53 to induce p53 activation and apoptosis [53] and acts as a co-activator for the nuclear-matrix associated scaffold, the promyelocytic leukemia protein, in p53 activation [54]. Like other RGS proteins, the RGS domain of Axin is able to directly interact with the α subunit of heterotrimeric G protein $G_{12}$ and it preferentially binds the activated form of $G\alpha_{12}$. However, unlike other RGS proteins, the RGS domain of Axin does not affect intrinsic GTP hydrolysis by $G\alpha_{12}$. Instead, it can compete for and inhibits $G\alpha_{12}$ binding with the RGS domain of p115RhoGEF that links $G_{12}$ signaling to activation of the small G protein Rho [55]. Such variation in the theme could provide additional level of signaling diversity.

In comparison, CNK1, which usually interacts with Ras/RAF and Ras/JNK signaling components, also plays a significant role in bridging Rho GTPases to its two RhoGEFs, Net1 and p115RhoGEF by binding to their Dbl-homology–pleckstrin-homology (DH–PH) domains. Consequently, CNK1 bridges p115RhoGEF-Rho to the JNK signaling [33]. Although *Drosophila* CNK was first described as a scaffold for the Ras/ERK pathway where it interacts with *Drosophila* RAF, only one of its human homologs, hCNK2, can target the mammalian RAF. Instead, both hCNK1 and hCNK2 interact with the GEFs of Ral, RalGDS and Rlf, respectively [8]. Furthermore, CNK1 can also cooperate with Rac to activate JNK, suggesting that CNK1 might interact with yet an unidentified RacGEF. Such versatility of CNK1 should therefore help integrate the signaling from different Rho GTPases to the JNK pathway [8]. Interestingly, functional crosstalk between ERK and Rho has also been report for the MP1 scaffold whereby ERK activation by PAK1 is linked to inhibition of Rho/Rho kinase, leading to turnover of focal adhesion for cell spreading [56].

The A Kinase Anchor Proteins (AKAPs) comprise a large family of structurally unrelated yet important class of scaffold proteins that tether protein kinase A (PKA) in different locales. Some of them also carry the phosphodiesterases (PDEs) that degrade the levels of second messenger, cAMP [22,24], the significance of which will be discussed in more details next. Indeed, as multivalent proteins, many AKAPs serve to engage more than just their common target, the PKA. For example, AKAP-Lbc is also associated with KSR-1, such that the flux of cAMP could activate the PKA pre-bound on AKAP-Lbc and that in turn phosphorylates Ser838 of KSR1 and helps sustain the ERK activation [57,58] (Fig. 4A). More recently, it has been shown that integrin $\alpha_6\beta_4$ cooperates with LPA signaling to stimulate Rac through AKAP-Lbc-mediated RhoA activation. This process is under distinct regulation by PKA, PDE and PI3-Kinase [59]. AKAP11 (or AKAP220), on the other hand, recruits active Rac and another scaffold protein IQGAP2 to regulate membrane ruffling [60] whereas AKAP12/Gravin is associated with actin or tubulin cytoskeleton by binding directly or indirectly to profilin, dynein, or even with cytokinesis components and PKC, and rendering it a tumor suppressor and anti-metastatic agent [20]. In addition to their individual localization signal [11,20], many of the AKAP genes undergo alternative RNA splicings which result in multiple variants that can be targeted to different parts of cells [61]. This mechanism restricts its broad-spectrum substrate PKA to distinct sets of substrates that help establish highly localized signaling specificity. However, AKAPs are not confined to specific cellular compartments since there are other mechanisms to regulate AKAP spatially. For example, modification of the lipid moiety of AKAPs via depalmitoylation, could dissociate AKAP18α and AKAP18β from the plasma membrane, resulting in translocation and inactivation of PKA [62,63] whereas AKAP12/SSeCKS/Gravin which also targets PKC, PKA and other cytoskeletal proteins, contain 3 polybasic domains that are likely to bind phosphoinositides and other phospholipids, or using its N-terminal myristoylation to associate with plasma and vesicle membranes [20]. In contrast to the existence of multiple scaffold proteins that regulate single signaling nodes, such as those described for the RAF–MEK–ERK module, or the use of unique members to target PKA to different localities as exemplified by AKAPs, many other scaffold proteins could exhibit their versatility by engaging multiple signaling nodes. Under such "single scaffold, multiple node" regime, these scaffold proteins serve as hubs for signals integration or crosstalk as already indicated above for β-arrestins, IQGAPs, Axin and RACK1 and others.

Increasing evidence suggest that some signaling intermediates such as MEKK1 and MEK2 could also act as scaffold proteins to promote signaling crosstalks. While predominantly activated to induce JNK1 activation, MEKK1 is also phosphorylated on multiple sites and polyubiquitinated following TCR co-stimulation where it recruits E3 ligase Itch. This interaction is specific to MEKK1 and not MEKK2, and is dependent on MEKK1 phosphorylation within the kinase domain and an intact MEKK1 PHD/RING finger motif [42]. MEKK1 also

interacts with active Rho via this PHD domain, potentially providing a bridge for the crosstalk between Rho-dependent cytoskeletal organization and MEKK signaling [64]. However, it remains to be examined whether Rho-binding and Itch recruitment would affect each other or work in parallel by different pools of MEKK1.

### 2.1.3. Multiplicity and versatility of scaffold proteins — gating compartmentalized activation of RAF–MEK–ERK and inactivation of cAMP signaling networks

*2.1.3.1. The RAF–MEK–ERK module.* To ensure signaling specificity, multiple scaffold proteins can be employed to restrict their signaling intermediates at specific subcellular structures or compartments. For examples, activation of ERK can lead to diverse outcomes such as cell motility, gene expression, cell proliferation, metabolism and differentiation, depending on where and how ERK itself is activated and regulated and how it interacts with other signaling pathways. Therefore, ERK can adopt different "states" of activity both spatially and temporally when it is co-regulated by different scaffold proteins and their partners. Indeed, the 3-tier kinase modules of "RAF–MEK–ERK" or 2-tier kinase of "MEK–ERK" are often localized by different scaffolds and regulated differentially, including positioning themselves for potential crosstalk by interacting with other signaling partners or other signaling scaffolds (Fig. 4A). For examples, upon growth factor receptor activation, KSR is translocated from the cytosol to the cell membrane to facilitate rapid and transient ERK activation whereas MP1 recruits MEK1–ERK1 to the late endosomes with the help of the adaptor protein p14, leading to sustained ERK1 activation [65,66]. The different thresholds and durations of ERK activation could lead to different cell fates such as cell proliferation, apoptosis and differentiation in different cell types [67]. Interestingly, KSR could also form a complex with another scaffold protein, AKAP-Lbc which is a RhoGEF that binds PKA. Upon its activation by cAMP that is generated by activated adenylyl cyclase, this bound PKA can phosphorylate Ser838 on KSR-1, leading to enhanced ERK activation [57].

The RAF–MEK–ERK module can also be recruited by paxillin, one of the several integral scaffold proteins at the focal adhesion, to the adhesion complexes in response to hepatocyte growth factor (HGF) stimulation [68] whereas RGS12 recruits RAF–MEK–ERK to the nerve growth factor receptor tyrosine kinase TrkA [48]. RGS12 binds Ras, RAF and MEK but not ERK directly. Binding of ERK is enhanced only in the presence of RAF and MEK. Interestingly, RGS12 translocates from the endosomes to the active Trk receptor, tethering the 3-tier kinase there to induce neurites outgrowth. On the other hand, RAF–MEK–ERK module can be recruited by IQGAP1 to the EGF receptor and IQGAP1 becomes phosphorylated at Ser1443 by PKC. Interestingly, EGFR autophosphorylation upon EGF stimulation is enhanced by S1443D mimicry, implying that PKC could modify function of IQGAP1 in providing a feedback activation for EGFR [51]. However, it is unclear how this feedback mechanism is linked to the activation of RAF–MEK–ERK module by IQGAP1.

The β-arrestins, which are first described for their ability to bind and desensitize agonist-stimulated GPCRs, could turn on the second wave of ERK activation by recruiting ERK modules to the activated receptors. This module is subsequently desensitized and internalized via the clathrin-coated pits. This occurs after the release of free Gβγ subunits that recruits GRK2 to phosphorylate the activated receptor which in turn become the target of β-arrestins. These β-arrestins recruit and sequester the RAF–MEK–ERK module in early endosome, preventing them from going into the nucleus to phosphorylate nuclear substrates, thereby reducing gene expression [3,69]. In contrast, Sef recruits active MEK/ERK to the Golgi complexes, preventing active ERK from interacting with nuclear substrates such as Elk-1 but not cytosolic substrate, RSK2 (p90 ribosomal S6 protein kinase 2) [70]. CNK, on the other hand, facilitates the assembly and membrane localization of Ras–MEK–ERK for cell proliferation, growth and cell differentiation [71]. It can also interact with the effector of Rho GTPases but does not impact on Rho-induced stress fiber formation, suggesting that it could act as a specific effector for transcriptional control. CNK also associates with Rho and Ras effectors (Rhophilin and Ral GDS, respectively), possibly mediating cross-talk between Rho and Ras signaling [72,73]. CNK which acts downstream of Gα12/13 also provides crosstalk between the Rho and JNK signaling by linking active p115RhoGEF and Rho complex to MLK3, MKK7 and JNK for gene regulation (Fig. 2C) [33,72].

In other related MAP kinase pathways, MEKK1 interacts with both the JNK/SAPK and the ERK1/2 modules whereby it binds ERK at the cytoskeleton [74,75]. This suggests that MEKK1 could function as a dual scaffold for two separate MAP kinases. Similarly, different scaffold proteins can also be mobilized to support both JNK and p38 MAPKs in immune cells. For example, the scaffold proteins B-cell lymphoma 10 (BCL-10) and MEKK1 and disks large homologue 1 (DLG1) are important regulators of the JNK and p38 pathways in immune cells [76]. In comparison, the focal adhesion scaffold protein, paxillin contains the LIM2/3 domain which helps its targeting to focal adhesions [77–79]. As indicated earlier, a targeting moiety can also be contributed by an adaptor protein that is closely associated with the scaffold protein. For example, the adaptor protein, p14 contains the endosomal targeting domain that facilitates the localization of scaffold protein MP1 to endosomes in order to provide sustained ERK activation [65,66].

POSH is another 3-tier kinase that binds to active Rac1 and assembles JNK modules (which includes MAP3K10,11, MAP2K4,7 and MAPK8,9) in order to promote apoptosis in neurons [32]. Furthermore, POSH forms a complex with ALG-2 (Apoptosis-linked gene-2) and ALIX/AIP1 (ALG-2-interacting protein) in a calcium-dependent manner [80]. As an E3 ubiquitin-protein ligase, POSH ubiquitinates few substrates which include Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) on early endosomes [81], Herp (that regulates calcium homeostasis) [82] and the potassium channel, ROMK1 [83]. However, it remains unclear whether this function is linked to its scaffold function. Furthermore, the axon outgrowth inhibitor Nogo66 transduces signals to POSH which then acts as a molecular scaffold to recruit leucine zipper kinase (LZK) and Shroom3 (which localizes F-actin and myosin II to regulate apical constriction) to suppress axon outgrowth [84]. As indicated earlier, an analogous system to that of yeast Hog1 signaling was identified in mammalian cells in response to hyperosmotic shock. This scaffold protein, OSM, assembles small GTPase Rac with MEKK3 and MKK3 to the Rac-enriched membrane ruffle in response to sorbitol, thereby activating the p38 MAPK in certain mammalian cells [37,85]. However, involvement of OSM in osmosensing appears to be versatile and is context-dependent as knockdown of Rac1 or OSM could lead to an increase instead of a reduction in the p38 MAPK activity in HEK293 cells. Furthermore, when challenged with high (hypertonic) levels of NaCl, Rac1/OSM activates the transcriptional activity of the osmoprotective NFAT5 (also known as TonEBP/OREBP) via the activation of phospholipase C-γ1 instead of p38 [86].

Taken together, it is clear that all these "3-tier kinase" scaffold proteins (KSR, CNK, OSM, JIP/JLP, paxillin, IQGAP1, Ste5, RGS12, POSH) or the "2-tier kinase" scaffolds (SEF and MP1) have each evolved to retain an optimal principle of design in signaling circuitry. This ensures that specificity of MAPKKK–MAPKK–MAPK signaling module is protected while allowing different forms of external stimuli to be detected and their functions regulated.

*2.1.3.2. The cAMP/phosphodiesterase (PDE)-linked scaffold proteins.* The original concept of controlling intracellular signaling by specific compartmentalization of the reactants and products came from the realization that the local concentrations and gradients of the second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) are tightly controlled by the interplay of the signal-generator adenylyl cyclase and its signal terminator, the cAMP-degrading phosphodiesterases

(PDEs). These processes as well as the ensuing effectors function mediated by cAMP-dependent Protein Kinase A (PKA) and Exchange Protein Activated by Cyclic AMP (EPAC, an exchange protein that activates small GTPases Rap1 and Rap2), are both subjected to tight control mediated by discrete scaffold proteins. Consequently, this interplay leads to precise spatial and temporal regulation of PKA and EPAC activities and activation of their downstream target proteins.

In this context, there are generally two broad categories of PDE-carrying scaffold proteins that allow sculpting the cAMP gradients at different locales by specific isoforms of PDE (Fig. 4B). They include various members of the A Kinase Anchoring Proteins (AKAP) family proteins that bind to both PKA and PDE and localized to certain receptors or complex on the membrane. For examples, AKAP12/Gravin, AKAP18, Yotiao, AKAP450, and mAKAP each links to β2-adrenoreceptor, aquaporin-2, NMDA receptor/KNCQ1 subunit of the potassium channel, centrosome and sarcoplasmic reticulum/nuclear envelope, respectively. PDEs can also be targeted by a diverse class of non-PKA binding scaffold proteins or other proteins to the plasma membrane (β-arrestins, β1-adrenoreceptors, p75NTR), in the cytosol (Src/Fyn/Lyn family non-receptor tyrosine kinases, ERK1/2), at nascent focal adhesion (RACK1), sarcoplasmic reticulum (ryonodine receptor), Golgi (myomegalin) and mitochondria (DISC1). The presence of such a huge repertoire of scaffold proteins in regulating cAMP–PKA–PDE network at various microdomains and the selectivity through the specific isoforms of PDEs, all highlight their importance as the "pacemakers" as well as the "placemakers" in gating the thresholds, amplitude, duration, localities and crosstalk of cAMP signaling — all in a context-dependent manner to govern our body physiology (Fig. 4B) [22–24,61,87,88]. Therefore it is not too surprising that deregulation of DISC1, mAKAP and p75NTR have been linked to schizophrenia, heart failure and fibrosis, respectively. In this regard, there are promising prospects of developing therapeutics against these scaffold-based PDEs (e.g. PDE4) to tackle inflammatory diseases such as asthma, chronic obstructive pulmonary disease, psoriasis, depression or even as enhancers for cognitive behavior [22,89].

Interestingly, some of these PDE-targeting AKAPs and also the non-PKA binding scaffold proteins can also interact with other signaling intermediates or regulators, such as other protein kinases, protein phosphatases, GTPases and adaptor proteins, and also with other scaffold proteins, thus providing further means of crosstalk and regulation [11,20,22–24,90]. For examples, mAKAP interacts with ERK5, PDK1 and PDE4D to modulate the cAMP signaling and it also interacts with the ryanodine receptors (RyRs) which control the flux and release of intracellular $Ca^{2+}$ that mediates excitation–contraction coupling in the muscles. The β-arrestin, which also serves as scaffold for the RAF–MEK–ERK module, regulates β2-adrenoreceptor signaling by inhibiting the coupling of adenylyl cyclase as well as targeting the PDE4D5 to degrade the local cAMP downstream of the receptor activation. β-Arrestin also engages CD28 and is recruited to T-cell lipid drafts upon T-cell receptor activation [23]. In line with their high level of specificity, β1-adrenoreceptor directly engages another isoform of PDE, PDE4D8 leading to regulation of contraction. RACK1, on the other hand, is a scaffold protein for the adhesion complex (see later). The recent findings on its association with PDE4D5 offer a novel insight into the functional coupling between focal adhesion dynamics and cAMP signaling.

In comparison, DISC1 regulates cAMP (via PDE4B) and Wnt (via inhibiting the negative regulator, GSK3β) where it anchors NDEL1 (nuclear distribution protein nudE-like 1), LIS1 (lissencephaly protein 1), PCM1 (pericentriolar material 1) and BBS (Bardet–Biedl Syndrome) in the centrosome and microtubules networks [91,92]. At synapses, DISC1 augments the binding of another neuronal scaffold, PSD-95 and KAL-7 and it also interacts with TNIK to regulate the turnover of synapse structure. Furthermore, DISC1 regulates RacGEF, TRIO for axon guidance [87,89]. Taken together, regulation of cAMP–PKA–PDE signaling node involves extensive network of scaffolding proteins that not only target specific forms of PDEs to sculpt the local cAMP gradients, but they also engage other signaling nodes to enrich for their functional plasticity.

### 2.1.4. Coordination and alliance of different scaffold modules

Multiple signal integration and crosstalk can be elicited by scaffolds that cooperatively assemble multiple signaling components into an even larger functional complex, or indirectly linked via other intermediates that relay signals from one scaffold protein to the others. This facilitates better spatial and temporal integration and control of the magnitude and duration of the signaling output. Recent advances using biosensors of Rho, Cdc42 and Rac elegantly showed that these small GTPases undergo cyclical loop of activation and inactivation in a mutually antagonistic manner both temporally and spatially [93]. In advancing fibroblasts, Rho is activated at the edge and necessary for initiating cell protrusions whereas Cdc42 and Rac1 are activated 2 μm behind the edge with a delay of 40 s in order to reinforce and stabilize the newly expanded protrusions. While it remains unclear how these GTPases are located and activated (or inactivated) in such a precise spatiotemporal manner, it is tempting to speculate that single or multiple scaffold proteins associated with either the activating guanine nucleotide exchange factor (e.g. GEF) or inactivating GTPase-activating protein (GAP) are likely to be key determinants. Here we highlight several examples where several scaffold proteins form functional alliances that help determine cell dynamics, cell fates and tissue/organ development. Increasing evidences suggest that this emergent property of scaffold function is likely to be more common than previously thought.

*2.1.4.1. The Hippo tumor suppressor pathway.* In the highly conserved Hippo signaling pathway, Salvador (Sav; human orthologue SAV1 or WW45) and its co-activator, Mats (human orthologue MOBKL1B) are two central scaffold proteins that help assemble two core serine–threonine kinases, the Hpo (human orthologue MST1/2) and Wts (human orthologue LATS1/2) into a ternary Hpo kinase cassette, leading to restriction in cell growth and proliferation and enhanced apoptosis [13,94–96] (Fig. 5A). Similar to other kinase signal transduction pathways, both Hpo and Wts are activated by phosphorylation. Upon activation of Hpo, presumably downstream of the engagement between two atypical cadherins Ft and Ds and via the Kibra/Expanded/Merlin scaffold complex at the apical junction, Hpo phosphorylates Wts, Sav and Mats. However, Wts activation depends on the concerted actions of both scaffolds Sav and Mats, such that Sav facilitates Wts phosphorylation by Hpo whereas Hpo-phospshorylated Mats promotes Mats–Wts complex formation. Activated Mts will in turn phosphorylate the transcriptional co-activators Yki (or Yes kinases-associated protein, YAP), leading to their binding with 14-3-3 and cytoplasmic retention, thus inhibiting them from activating transcriptional factors and growth target genes such as cyclinE and diap1. Interestingly, one unique feature of the Hippo pathway is that many of the components, ranging from the core kinase cassette and upstream regulators to its downstream nuclear effectors, possess the well conserved arrays of proline-directed WW domains and the corresponding proline-rich target motifs [13]. Since they serve as major devices for protein–protein interactions, including their ability to form WW domain homodimers or heterodimers, the domains raise an interesting prospect that this pathway could be subjected to complex crosstalk with and regulation by other signaling networks that harbor the WW domains or/and proline-rich motifs [13,21,97]. In this regard, other scaffold proteins have also been identified as regulators of the Hippo pathway. For examples, Kibra scaffold (similar to SAV1 in harboring 2 WW domains) activates Hippo by interacting with Expanded and Merlin [13,95,98–100] whereby Kibra, Ex and Merlin can form a complex with SAV1 [98]. Kibra interacts and activates Wts to inhibit Wts ubiquitination thereby stabilizing it [101]. In mammalian and *Drosophila* cells, Ajuba LIM protein/dJub, a LIM

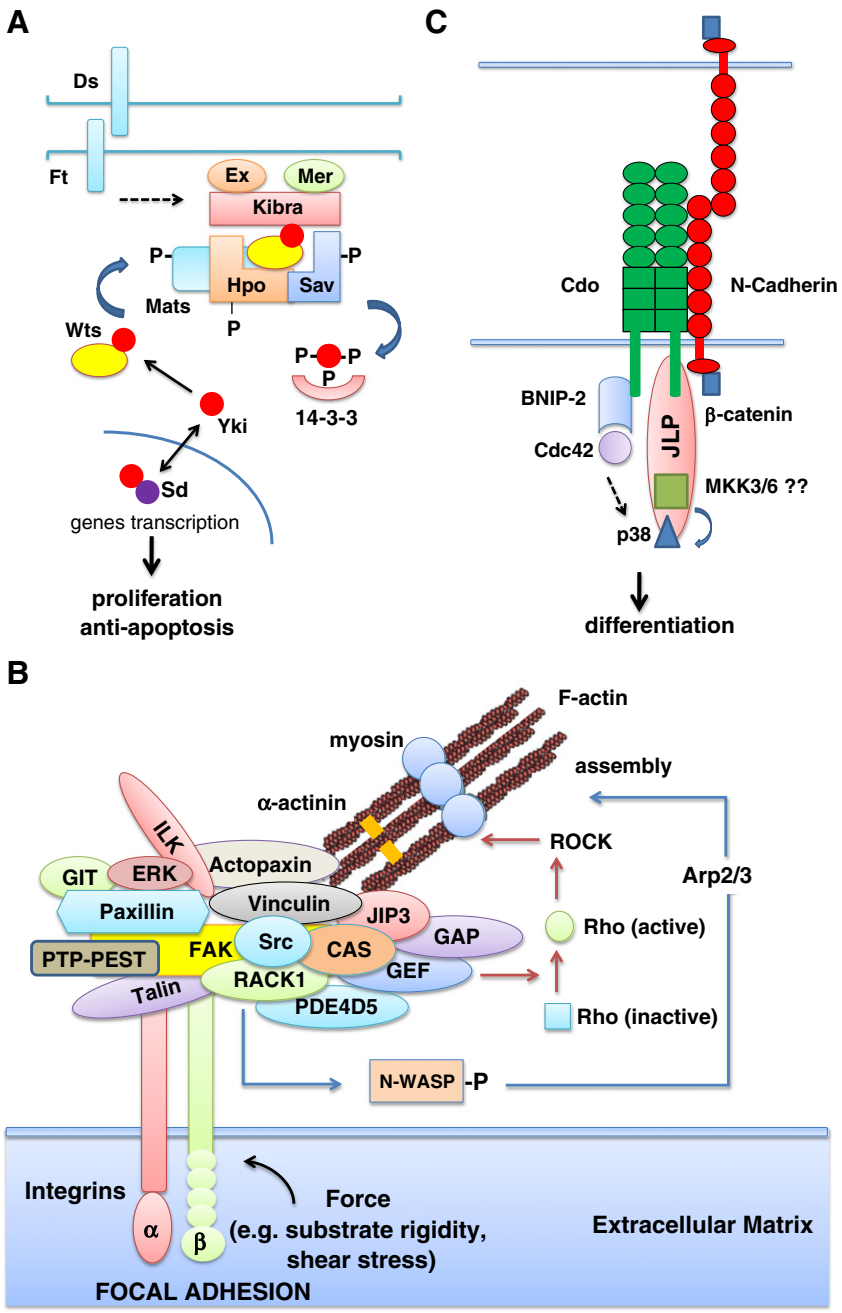


**Fig. 5.** Forming strategic scaffold alliances. Increasing evidence suggest that some scaffold proteins do not exist as distinct "insulators" that only allow the assembly of specific signaling intermediates. Instead, these scaffold proteins can interact with one another directly or bridged by other proteins to form a higher-order macromolecular complex. This organization would facilitate better integration, regulation, crosstalk and feedback of the target proteins, as shown schematically in the following examples here. (A) In the *Drosophila* Hippo pathway, upon engagement of atypical cadherins Ft and Ds at the apical junction, the scaffold protein Kibra recruits the Hippo Kinase Casette (HKC) comprising two other scaffold proteins, Sav and Mats and two central serine/threonine-specific kinases, Hpo and Wts. HKC is formed upon autophosphorylation and activation of Hpo, which in turn phosphorylates and activates Sav, Mats and Wts. However, Wts activation depends on the concerted actions of Sav and Mats, such that Sav facilitates Wts phosphorylation by Hpo whereas Hpo-phospshorylated Mats promotes Mats–Wts complex formation. Activated Mats then recruits Wts (prebound with Yki) to HKC where it now gets phopshorylated by Wts and become sequestered in the cytoplasm by 14-3-3 protein. This mechanism ensures that Yki does not shuttle to the nucleus that would otherwise act as a transcriptional co-activator for Sd to elicit growth and anti-apoptosis. (B) Focal adhesions comprise a dynamic macromolecular assembly of several key scaffold proteins including the FAK, Paxillin, ILK, p130CAS, RACK1, JIP3 and PTP-PEST, together with other adaptor proteins such as talin, vinculin, actopaxin and tensin that bridge the integrins with F-actin network. Present within this complex and contributing to the fine-tuning of focal adhesion signaling and impacts are various regulators of small GTPases (e.g. p190RhoGEFs, p190RhoGAPs), kinases (e.g. Src, MEK, ERK), phosphatases (e.g. PTP-PEST), protein isomerase (e.g. Pin1), cAMP-degrading phosphodiesterase PDE4D5 and N-WASP. The intricate alliance of these scaffold proteins with diverse families of regulators help sense and transduce the mechanical signals arising from the impacts of force (e.g. substrate rigidity and shear stress) that exert on the integrins, which act as the frontline mechanosensor. Activation of certain growth factor receptors can also lead to integrin activation (omitted for clarity here). As an example, FAK at the focal contacts can activate RhoGEF and Rho, leading to enhanced actomyosin stress fibers formation while activating N-WASP for the F-actin assembly. For clarity, this simplified schematic diagram highlights only part of the complex interactome for the focal contacts and matured focal adhesions not drawn according to the sequential steps of mechanism. In the "environment-probing" nascent or sometimes called "early-spreading" adhesions, another scaffold protein RACK1 is also present in this supra-complex [139] whereas FAK can be activated by integrins independently of talin [125]. Please see text for more details on the functions of other scaffold proteins at focal adhesions. (C) The pro-myogenic cell surface receptor, Cdo binds to both MAPK-p38α/β pathway scaffold protein, JLP and a novel regulatory scaffold protein for Rho small GTPase, BNIP-2, that tethers and activates Cdc42 via its conserved BCH domain. Although any direct interaction between BNIP-2 and JLP remains unclear, both scaffolds are functionally linked by Cdo upon N-cadherin ligation, leading to BNIP-2/Cdc42-dependent p38α/β activation and stimulation of myogenic and neuronal differentiation.

domain-containing adaptor/scaffold protein has been shown to interact with SAV1/Sav, LATS/Wts and possibly YAP/Yki, leading to inhibition of YAP/Yki phosphorylation [102]. In addition, the *Drosophila* ortholog of RASSF, another tumor suppressor protein, can act as a negative regulatory scaffold by directly competing with Sav for Hpo [103], by forming heteromeric interactions via their coiled-coil SARAH domains [104].

In addition to playing a key role in the Hippo core kinase cassette, Mst2 and Sav1 also directly interact with Nek2A and regulate its ability to localize to centrosomes and to phosphorylate C-Nap1 and rootletin, establishing a novel role of Sav1–Mst2–Nek2A pathway in controlling centrosome disjunction and bipolar spindle formation [105]. The importance of Hippo core in providing multiple crosstalk to other signaling nodes is a further demonstration of the unexpected role of Mst2 in supporting RAF/ERK activation by maintaining high levels of catalytic phosphatase 2A that suppresses the inhibitory RAF-1 phosphorylation [106]. How Hippo scaffolds provide the links to the Nek2A and Ras/MAPK network remains to be determined but these observations clearly exemplify its versatility in regulating multiple signaling modules.

*2.1.4.2. Scaffold proteins as mechanosensors and mechanotransducers.* Scaffold proteins play a critical role in sensing external environment and stimuli such as rigidity of extracellular matrix and forces exerted on the cells and tissues. They transduce the mechanical signals to regulate intracellular states and nuclear responses. The processes of mechanosensing and mechanotransduction are mediated by modular associations of multiple scaffold proteins at specialized structures such as the focal adhesions, podosomes, invadopodia, adheren junctions and synapses. Central to the regulation of cell adhesion dynamics is the extensive network of scaffold proteins comprising at least the focal adhesion kinase, FAK [107,108], RACK1 [15], integrin-linked kinase [16], paxillin [109,110] and p130Cas [17,110]. They provide the link between trans-membrane integrins and actin that act as mechanosensors through various adaptors such as talin, vinculin and tensin, eventually to the actin cytoskeleton. Through their rich and dynamic repertoire of protein domains, they recruit many effectors and regulators to the complex. These include protein and lipid kinases (e.g. SRC, PKC, PI3K, MEK, ERK, PI(4)P5K1), phosphatases (PEST, PP2A), isomerase (e.g. PIN1), phosphodiesterase (e.g. PDE4D5), small GTPases (e.g. Rho, Cdc42, Rac, Rap), guanine nucleotide exchange factors (e.g. C3G, DOCK180, p190RhoGEF), and GTPase-activating proteins (e.g. DLC1, p190RhoGAP) [18,111,112] (Fig. 5B). Their actions lead to changes in cell morphogenesis, motility, polarity, cell growth, cell death or differentiation. In many of these cases, it is the generation of force and the dynamics of actin filament assembly that are converted to biochemical signals.

The FAK scaffold protein is a tyrosine kinase autoinhibited by the intramolecular interaction between the kinase domain and the highly conserved, multifunctional four-point-one, ezrin, radizin, moesin (FERM) domain. This FERM domain protects the active and autophosphorylation sites and hinders recruitment of Src. Upon integrin clustering or growth factor stimulation, this autoinhibition can be released by binding of the FERM domain to phospholipids PtdIns(4,5)$P_2$ enriched at the cell membrane. This displacement triggers the autophosphorylation at Y397 and recruitment of Src tyrosine kinase which in turn phosphorylates Y576 and Y577 at the linker region between the two domains, thus ensuring full activation [107,113]. This auto-inhibition can also be disrupted upon tension-induced conformation changes in FAK, similar to other focal adhesion resident proteins such as p130Cas, talin and integrins [114]. Through this FERM domain, FAK assembles diverse protein partners near the cell membrane and cell cortex, including the β-integrins, growth factor receptors c-MET, EGFR, actin nucleation complex subunit Arp3, N-WASP, and other scaffold proteins JIP3 and RACK1. Interestingly, phosphorylation at Y397 destabilizes FAK–Arp2/3 complex, resulting in the release and relocalization of Arp2/3 from the focal adhesions to regions of extending lamellipodia and protrusions [115]. FAK also causes assembly of the p53–MDM2 complex in the nucleus, leading to p53 ubiquitination and degradation [116]. Adding to the nuclear connection, FAK regulates heterochromatin remodeling at the promoter of Myogenin via its interaction with MBD2 [117].

Outside the FERM domain, FAK binds to another scaffold protein paxillin and several RhoGEFs (PDZ-RhoGEF, p190RhoGEF) and RhoGAPs (PSGAP, GRAF, and indirectly p190RhoGAP via p120RasGAP) [114,118]. By activating p190RhoGAP initially (indirectly via p120RasGAP) [114] followed by its association with p190RhoGEF [119], FAK is thought to control the switch for Rho inactivation and Rho activation that drives cell spreading on fibronectin and subsequently cell polarization [114,120]. At the later stages of cell spreading, FAK/p190RhoGEF forms a complex with paxillin that is localized to the focal adhesions. As indicated above, paxillin is another "3-tier kinase" scaffold that assembles RAF–MEK–ERK at the focal adhesion, mediated by the binding of its LIM domain to the cytoplasmic tail of β-integrins. In addition, paxillin also plays a central role in coordinating the spatial and temporal actions of Rho/Cdc42/Rac GTPases by recruiting a large number of GTPase activators, suppressors and effectors to cell adhesions [121]. Upon HGF stimulation and phosphorylation by active ERK, paxillin recruits FAK and activates PI3K and Rac at the cell leading edge, leading to enhanced cell spreading and adhesion [4,68,122,123]. The LD4 motif of paxillin is particularly important because it helps recruit a large molecular complex, GIT–PIX–PAK–NCK, comprising the G-protein coupled receptor kinase-interacting proteins (GIT1 or GIT2), PAK-interacting exchange factor (PIX), p21-activated serine/threonine kinase (PAK) and Nck adaptor, to the adhesion sites and activating Rac1 in migrating cells. Since phosphorylation of LD4 motif at S273 by PAK promotes GIT1 binding but also reduces the affinity of FAK for the motif [124], paxillin can regulate the local activity of FAK and Rac1 through the phosphorylation of its LD4 motif [121]. Therefore, by coupling to paxillin, talin, N-WASP, RhoGEFs and RhoGAPs, FAK can regulate the levels of Rho and Cdc42/Rac activities, leading to F-actin assembly, contractility and cell polarity [118] (Fig. 5B). However, such a complex assembly can undergo dynamic rearrangements. For example, in the "environment-probing" nascent or sometimes called "early-spreading" adhesions, another scaffold protein Receptor for Activated C-Kinase (RACK1) is present in this supra-complex (see next) but it is absent from the matured adhesions. During the early stage of adhesions, FAK can be activated by β1-integrins independently of talin [125]. This is in contrast to the general view that FAK would require talin for its recruitment to integrins during the matured adhesions [126–129].

On the other hand, acting downstream of the Ras and Fgd1–Cdc42–PAK1–MEK–ERK signaling cascade, ERK phopshorylates FAK at S910 [130] and yet another scaffold protein phosphatase, PTP-PEST at S571 [131], leading to recruitment of the peptidyl-prolyl isomerase PIN1 and possibly the prolyl isomerization of both proteins. This dual effect results in their enhanced interaction and dephosphorylation of FAK Y397 which results in high turn-over of focal adhesions that promotes Ras-induced cell migration, invasion, and metastasis. Therefore, the orchestrated crosstalk between the integrin, FAK, Src, Rho/Rac and Ras–MAPK signaling nodes is needed to ensure effective remodeling of focal adhesion complexes [132]. Furthermore, shear stress is known to activate FAK which in turn activates ERK2 and JNK1 [133,134] whereas increasing matrix stiffness increases both the FAK-Rho signaling for invasion and activation of Ras–MAPK signaling for enhanced proliferation [133,135]. Interestingly, activated FAK forms a complex with IQGAP1 and melusin, a heart-specific chaperone protein, to enhance the recruitment of MEK1/2 and activation of ERK1/2 during mechanical stress in hearts [136]. All these examples highlight the potency and multifunctional roles of FAK in regulating cell dynamics and nuclear events.

In addition to binding to FAK, RACK1 also interacts with talin and vinculin depending on the adherence status of the cell. RACK1 interacts with vinculin in attached cells but it switches to interacting with talin in suspended cells [137,138]. During early cell spreading, RACK1 co-localizes with vinculin in areas of early spreading but not in the mature

focal adhesion. By binding to the FERM domain of FAK, RACK1 bridges FAK and recruits the cAMP-degrading phosphodiesterase PDE4D5 to nascent adhesions and regulates initiation of cell spreading and polarity of migrating cells [139]. Interestingly, the spreading initiation center is also the site for assembly of ribosomes and mRNA attachment [140] and RACK1 has previously been shown to be the ribosomal scaffold protein that supports local translation at the spreading initiation center [137] where it recruits PKC to the 40S subunit of ribosome to stimulate translation via phosphorylation of initiation factor 6 eIF6 [137]. It remains to be seen how the local production of specific proteins would impact on focal adhesion dynamics.

ILK assembles β-integrin subunits, paxillin, actopaxin family protein and the adaptor protein PINCH at focal adhesion and it regulates adhesion-mediated cell survival (anoikis), apoptosis, proliferation and mitosis, cell migration and invasion as well as cardiac and smooth-muscle contractility [16]. ILK also undergoes auto-phosphorylation [141] and can be phosphorylated by PAK1 in a process that correlates with increased ILK signaling [142]. While it remains a controversy whether ILK is indeed a functional serine/threonine kinase inside the cells, ILK has been shown to phosphorylate β-integrin subunits (β1 and β3), Akt, GSK-3β, myosin-targeting MYPT1 and MLC-20 in vitro. In addition, ILK also phosphorylates and activates the CPI-17 family of protein phosphatase inhibitors to act on Merlin, PP1 and other phosphatases, thus increasing the overall kinase activity in the cells [16,143,144]. On the other hand, studies have also shown that JSAP1 (JIP3) serves as a cooperative scaffold for the activation of JNK at the leading edge of migrating cells in response to fibronectin stimulation. It promotes complex formation of JNK with FAK, resulting in augmentation of JNK and FAK activity and the phosphorylation of both JSAP1 and another scaffold protein, p130 Crk-associated substrate (p130Cas) [145]. Unlike other scaffold proteins that are enzymatic in nature, p130Cas undergoes changes in conformation owing to mechanical forces and stretching, revealing effector binding sites and motifs for kinases [146]. Indeed, p130Cas provides a unique platform for the integrins and receptor tyrosine kinases to recruit other kinases, phosphatases, Crk and also the GEFs such as NSP and DOCK180 for the activation of Rap (that leads to ERK activation) and Rac1, leading to actin polymerization and assembly of high affinity integrin receptors for lamellipodia extension [17,147–149]. Therefore, FAK, RACK1, paxillin, JIP3 and p130Cas all constitute a supra-scaffold alliance that governs the dynamics of focal adhesions. Unraveling molecular mechanisms underlying this aspect of mechanobiology in the context of space, time and force, could help to understand the regimes involved in stem cell differentiation and controlling cancer metastasis [18].

*2.1.4.3. BCH domain as a regulatory scaffold for GTPases and MAPKs.* The ability of multiple scaffold proteins to form a functional alliance among themselves is further exemplified by the ability of pro-myogenic cell surface receptor, Cdo in binding to both MAPK-p38α/β pathway scaffold protein, JLP and a novel regulatory scaffold protein for GTPase, BNIP-2 [36]. BNIP-2 tethers and activates Cdc42 via its conserved BNIP-2 and Cdc42GAP Homology (BCH) domain [150]. Although any direct interaction between BNIP-2 and JLP remains unclear, both scaffolds are functionally linked by Cdo receptor upon N-cadherin ligation, leading to BNIP-2/Cdc42-dependent p38α/β activation and stimulation of myogenic and neuronal differentiation [36,151,152] (Fig. 5C). Indeed, BCH domains are highly versatile. Not only do they target different small GTPases such as Cdc42, Rho and Ras, they also interact with their immediate regulators such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and form homophilic interactions with themselves or interact with other homologous BCH domains [150,153–158]. Further, BCH domain-containing proteins also interact with diverse cellular proteins such as fibroblast growth factor receptors [158], peptidyl-prolyl isomerase [159], kinesin-1 motors [160] (also our unpublished data), caspases [161,162] and also metabolic enzymes such as the glutaminase [163] — all via their comprehensive composite of binding motifs leading to their functional plasticity. For more details, please see review in [164].

We recently showed that active MEK2 could act as a regulatory scaffold that greatly stimulates the interaction of BPGAP1 (a BCH-containing homolog of p50RhoGAP) with PIN1 peptidyl-prolyl isomerase. Binding of active MEK2 to BPGAP1 helps release an autoinhibition on BPGAP1 and exposes the proline-rich region for the binding by the WW domain of PIN1, leading to suppression of BPGAP1-induced acute ERK activation and cell migration [165]. Interestingly, through the BCH-mediated heterophilic alliance between BNIP-2 and BPGAP1, BNIP-2 could facilitate BPGAP1-induced Rho inactivation. As we had recently shown that the BCH domain of p50RhoGAP can sequester Rho from its inactivation by the adjacent RhoGAP domain [156], it is possible that BCH-mediated alliance between BNIP-2 and BPGAP1 could compete for Rho-binding on the BCH domain of BPGAP1. This could in turn prevent the sequestration of Rho and making Rho more accessible to inactivation by BPGAP1 (unpublished data). Interestingly, our further studies suggest that the BCH domain of BPGAP1 can also target Ras and one of its immediate regulators, leading to enhanced ERK/MAPK activation (unpublished data). Therefore, it is possible that the BCH domain of BPGAP1 regulates signaling crosstalk between Ras and Rho within a single complex. Since BPGAP1 also harbors a proline-rich region that targets cortactin and endophilin for morphogenesis, motility and receptor endocytosis [166,167], it is likely that the interplay of BPGAP1–MEK2–PIN1 signalome could impact on various functions of BPGAP1 and BNIP-2.

Indeed, increasing numbers of scaffold proteins have been found to form their strategic alliances. These include, as discussed earlier, the 3-tier kinase scaffold proteins, IQGAP1, KSR and paxillin which engage PSD-95, AKAP-Lbc and FAK, respectively for executing their unique functions at different locales (Fig. 4A). PSD-95 can also interact with AKAP150 to regulate endocytosis of synaptic AMPA receptors [168] whereas AKAP220 partners with IQGAP1 to integrate calcium and cAMP signaling [25].

### *2.2. Scaffold proteins are not mere physical platforms — they activate or suppress signaling by recruiting modifiers or acting as catalysts*

Scaffold proteins are usually considered as passive physical platforms that assemble specific groups of signaling intermediates into various functional assemblies. However, increasing evidence indicates that some scaffold proteins can regulate the binding and activity of their cognate substrates in the complex. These can be achieved either by (i) indirectly recruiting specific regulators to the complex, or (ii) themselves directly acting as catalysts that modify properties and activity of their cognate substrates (Fig. 3). For example, the prototypical MAPK scaffold protein in *S. cerevisiae*, Ste5 plays a co-catalytic-active role to unlock Fus3 (MAPK) for phosphorylation by Ste7 (MAPKK) [169,170]. Ste5 also recruits phosphatase Msg5 to specifically inactivate Fus3 but not the other MAPK, Kss1p [171]. KSR contains a pseudokinase domain that lacks the catalytic lysine residue usually conserved among the kinases. However, this domain still retains structural features crucial for its heterodimerization with the RAF, leading to an allosteric activation of RAF isoforms in a manner similar to the stimulatory effect induced by B-RAF/C-RAF heterodimers [172]. Interestingly, structural studies revealed that binding of B-RAF to KSR allosterically activate KSR's kinase activity towards MEK in vitro, by inducing a shift of the crucial αC helix of KSR into an active configuration [173]. Mutational studies further show that retaining the scaffold function without the kinase activity in KSR failed to augment ERK signaling [174]. Taken together, these results confirm the importance of KSR's kinase activity in transducing ERK signaling although its exact physiological substrate(s) remain unknown.

The scaffold protein tyrosine phosphatase, PTP-PEST negatively regulates lymphocyte activation through the dephosphorylation of

Shc, Pyk2, FAK and Cas and inactivation of the Ras pathway [175] whereas some scaffold proteins could also regulate either the same or different signaling cascade(s) in both positive and negative manners. For example, the JNK-interacting proteins, JIP1, recruits and coordinates specific MAPK kinases to positively regulate the JNK and p38 MAPK pathways. However, JIP1 also interacts with the MAPK phosphatase 7, suggesting that JIP1 could promote or limit the JNK pathway by targeting both MAPK kinases and phosphatases [176]. As discussed earlier, one of the key functions of AKAPs is to determine where, when and how much cAMP is to be maintained for any one particular cellular process. This is achieved via the recruitment of phosphodiesterases that breaks down cyclic AMP, thereby locally inactivating the PKA [11,22,23,61,177,178]. Some AKAPs also recruit the cAMP-generating adenylyl cyclase while others, such as AKAP450, recruit all the 3 kinases, PKA, PKC and PKN and several other phosphatases to dephosphorylate the signaling components efficiently [179,180]. As a further example, AHNAK1, which links the calcium channels with phosphoslipase Cγ (PLC-γ) and PKC [76], potentiates PKC activation to turn on MAPK/ERK pathway by disrupting the binding between the phosphatase PP2A and PKC [181].

The MAPK scaffold protein MEKK1 contains the kinase activity to phosphorylate both MEK1 and MEK2 while tethering RAF-1, MEK1 and ERK2 into a three-tier complex [74]. But, it also provides a dynamic negative feedback loop by acting as an E3 ubiquitin ligase that mediates the ubiquitination and degradation of ERK1/2 via its PHD domain (RING finger-like structure) [182]. Interestingly, the same PHD region is also thought to provide a crosstalk to the Rho signaling pathway [64]; however, the significance of this overlapping target binding remains unclear. In comparison, the JNK signaling scaffold protein POSH also confers an E3 ubiquitin ligase activity to the Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) at the early endosome [81]. It is also unclear whether this ubiquitination function is directly linked to its scaffold function. In addition to MEKK1, other components of kinase cascade can also act as scaffold proteins. As described earlier, an active MEK2 (a MAPKK) acts as a dynamic scaffold to release the autoinhibition of the Rho GTPase-activating protein, BPGAP1 and exposes its proline-rich region to bind the WW domain of PIN1, leading to suppression of ERK1/2 activation and cell motility [165]. Interestingly, the osmoregulatory scaffold protein in *S. cerevisiae*, Pbs2p (also another MAPKK) provides a platform to tether Sho1p, Ste11p (MAPKKK), and the Hog1p (MAPK) [183] under high osmotic stress conditions. In other scenarios, the MAPK phosphatase SAPK acts as a scaffold to enhance the interaction between MKK7 (MAPKK) and ASK1 (MAPKKK) in the JNK pathway [184] whereas another RAF–MEK–ERK 3-tier kinase scaffold PAK1 facilitates both the Akt stimulation by PDK1 and also the recruitment of Akt to the membrane [185]. Adding to the versatility of scaffold proteins, KSR1 has recently been shown to recruit inducible nitric oxide synthase (iNOS) and heat shock protein-90 to enhance iNOS activity and to release NO upon infection [186]. On the other hand, in the phototransduction of *Drosophila* photoreceptor cells, INAD contains multiple PDZ domains thereby enhancing the specificity and efficiency to generate pulses of ion flux via PLCβ (phospholipase Cβ)–DAG (diacylglycerol)–PKC (protein kinase C) pathway on the time-scale of milliseconds, one of the fastest known heterotrimeric G-protein-based signaling cascades [187,188]. Taken together, many scaffold proteins are no longer viewed as passive binding platforms. Indeed, they control the duration and magnitude of signaling output by recruiting other regulators to their close proximity or possessing unique enzymatic or regulatory motifs that directly influence the properties of their cognate substrates.

### 2.3. Dynamic signaling output: from graded, digital to oscillating responses

Scaffold proteins facilitate the assembly of multiple signaling components and they can directly or indirectly regulate the activity of assembled targets. However, the precise dynamics of assembly and disassembly of these complexes in the signaling processes is not fully understood. Mathematical modeling suggests that coordination of feedback loops could allow signaling pathways to display an oscillatory signaling behavior only by tethering the signaling components on the scaffold proteins [76]. Indeed, instead of acting as passive binding platforms, some scaffold proteins can convert a graded stimulus (i.e. signal output is proportional to the input) into a digital/switch-like response (i.e. signaling occurs after a certain threshold is reached and the output is maximal) simply by undergoing modifications such as phosphorylation or changes in their subcellular localization. For example, in yeast, the dynamic role of Ste5 is determined by the competition between the MAPK Fus3 and phosphatases Ptc1 [189]. Such graded to switch-like conversion can be further regulated by the localization of Ste5. When present in the cytosol Ste5 results in ultrasensitive digital response. However, the response would remain graded when Ste5 is localized to the plasma membrane [190]. In addition, compartmentalization of signaling components by scaffold proteins could result in distinct output signals. For example, KSR tethers the three-tier RAF–MEK–ERK kinase module at the membrane, resulting in acute transient activation of ERK [191]. In contrast, MP1 which is localized at late endosomes and constitutively binds to MEK with its adaptor protein p14, will lead to sustained ERK activation instead [65,66].

Adding to their functional complexity, some scaffold proteins do not necessarily lead to signal amplification. Instead, depending on certain cellular context, they coordinate signaling components positively and negatively (i.e. contextual signaling) and that could reshape the signaling output in an oscillating manner over time, as supported by mathematical modeling and studies using engineered scaffolds [192,193]. For example, the Ste5 scaffold protein that is engineered to incorporate feedback loops reshapes the MAPK signaling output via the recruitment of positive or negative regulators [194]. Similarly, the MAPK scaffold proteins, JIP1/2 can interact with MKP7 and M3/6 phosphatases to dampen MLK3-dependent activation of JNK [5,176]. In addition, scaffold proteins could insulate/protect the signaling complex from premature inactivation. Mathematical modeling predicts that in the absence of scaffold proteins, the activated signaling proteins (such as the kinases) are likely to be deactivated by the abundant levels of phosphatase, thereby dampening the signaling output [192]. Any duality and contextual signaling output can be regulated by the concentration of the scaffold proteins. At low concentration of the scaffold protein, functional complexes cannot be formed while excess scaffold proteins sequester the individual signaling components and therefore disrupting the formation of functional signaling complex. Optimal scaffold concentration is therefore important to establish stable and functional signaling complexes. In this regard, simulation of the EGFR–ERK signaling with changes in the levels of KSR and MP1 show that they could affect the ligand-sensitivity of the signaling when co-regulated by two endosomal regulators, Cbl-CIN85 and endophilin [195]. Taken together, scaffold proteins can dynamically regulate the localization, amplitude and duration of signaling output either by acting alone or in alliance with other scaffold proteins and modifiers.

## 3. Regulation of scaffold functions

As the unique domain architectures of scaffold proteins facilitate their interaction with diverse cellular partners, the versatility of scaffold proteins is further augmented by their ability to undergo various forms of modifications. These include their phosphorylation, ubiquitination, sumoylation or lipidation. These modifications can alter the biochemical, physical and mechanical properties of scaffold proteins and regulate their interactions with other protein partners (including substrates or inhibitors) or metabolites. They also regulate the ability of scaffolds to undergo mechanical sensing, protein

degradation and changes in localization and also their ability to act as co-catalysts.

### 3.1. Phosphorylation

Phosphorylation is one of the most common forms of post-translational modifications that generate uniquely modified motif sequence that mediates or inhibits specific protein–protein interaction or lead to changes in protein conformations. It provides an important feedback or crosstalk mechanisms for several scaffold proteins. For example, β-arrestin binds to clathrin-coated pits and recruits c-Src for the activation of ERK signaling [196], which in turn phosphorylates β-arrestin to down-regulate the pathway. Similarly, upon promoting RAF/MEK/ERK signaling, KSR1 is subjected to feedback regulation by active ERK, leading to dissociation of signaling components from KSR1 and the release of KSR1 from the plasma membrane [197]. KSR1 is also subjected to phosphorylation at S838 by the PKA bound to another scaffold protein AKAP-Lbc that forms a complex with KSR1. This PKA is activated by the flux of cAMP upon stimulation of GPCR and adenylyl cyclase, thus promoting an important crosstalk to sustain the ERK activation [57].

Similarly, the yeast MAPK mating scaffold protein, Ste5 is also phosphorylated by its MAPK substrates, Fus3p and Kss1p to down-regulate the signaling output [169,170,198–200]. Phosphorylation also modifies the ability of scaffold proteins to interact with their partners, inhibitors or metabolites. For example, phosphorylation of the JNK/MAPK scaffold protein, JSAP1 (or JIP3) by ASK1 (Apoptosis signal-regulating kinase 1) promotes the interaction of JSAP1 with its MAPK signaling components, SEK1/MKK4, MKK7 and JNK3 [201]. In comparison, the phosphorylation of AKAP-Lbc by one of its tethered signaling components, PKA, induce its interaction with 14-3-3, leading to the suppression of the RhoGEF activity of AKAP-Lbc [202,203]. Such phosphorylation also releases the activated PKD from AKAP-Lbc for signal propagation [61,204]. In contrast, phosphorylation of AKAP79/150 by PKC abolishes its interaction with phospholipids, particularly PtdIns(4,5)P2, thus preventing the recruitment of AKAP79/150 to the plasma membrane [205]. AKAP12, on the other hand, undergoes FAK-dependent tyrosine phosphorylation upon EGF or PDGF stimulation that leads to impaired binding to F-actin. While its phosphorylation by mitogen-induced kinases inhibits its scaffolding function, phosphorylation of AKAP12 by kinases associated with differentiation, such as PKA, enhances its scaffolding activity [20]. Similarly, in the presence of mating stimulus, Cln/CDK phosphorylates Ste5 at regions proximal to the membrane binding motif, disrupting Ste5 localization to the plasma membrane and that lead to the inactivation of Ste5 signaling [206]. Recently, it has been shown that IQGAP binding to the kinase domain of EGFR leads to the phosphorylation of IQGAP1 at Ser1443 by PKCα. Interestingly, IQGAP1-null cells exhibit reduced EGFR autophosphorylation upon EGF stimulation but this level is enhanced by S1443D mimicry. This result implies that PKC could modify function of IQGAP1 in providing a feedback activation for EGFR [51]. However, it is unclear how this feedback mechanism is linked to the activation of RAF–MEK–ERK module by IQGAP1.

Phosphorylation can also affect the localization of scaffold proteins via protein sequestration. The constitutive phosphorylation of KSR1 at S297 (by unknown kinases) and S392 [by c-TAK1 (Cdc25C-associated kinase 1) and Nm23 (nucleoside diphosphate kinase, mitochondrial-23)] [207], create the docking sites for 14-3-3 and sequester the inactive KSR1 in the cytosol during the quiescent state. KSR1 can also be inhibited by IMP (impedes mitogenic signal propagation), a Ras-responsive E3 ubiquitin ligase that undergoes auto-polyubiquitination and degradation upon Ras activation. This allows KSR to assemble Raf–MEK–ERK modules. [208]. Both 14-3-3 and IMP mask the C1 domain of KSR1 and prevent KSR localization to the plasma membrane [208–210]. Upon growth factor stimulation, the phosphatase PP2A dephosphorylates KSR and that leads to the release of KSR from 14-3-3 and also its degradation by IMP. This favors KSR recruitment to the plasma membrane again [211].

During vegetative growth of the yeast, the nuclear-cytoplasmic shuttling of Ste5 leads to its accumulation and degradation in the nucleus [212]. This mechanism may help suppress non-specific activation of the mating MAPK, Fus3, and allow the specific activation of another MAPK, Kss1 to induce filamentation under starvation conditions [3]. This is further supported by the finding that active Fus3 induces the degradation of filamentation-specific transcription factor Tec1p for MAPK signaling specificity during mating [213–215]. Upon activation by pheromone, the inactive Ste5 is phosphoylated, dimerized and shuttled from the nucleus via the nuclear exportin, Ste21/Msn5 and recruited to the plasma membrane. This recruitment is facilitated by interacting with activated Gβγ (i.e. localized on the membrane) and with the plasma membrane via the amphipathic α-helix domain of Ste5 [216,217]. Once Ste5 is present in the cytosol or at the plasma membrane, its half-life is markedly prolonged, allowing its bound MAPK cascade components to sustain the signaling. Adding to the complexity of Ste5 regulation, Ste5 and Pbs2 (also a scaffold protein) interact with the kinase Ste11 under different conditions [183,218]. Under pheromone activation, Ste11 will phosphorylate Ste7 leading to the mating response. However, when Ste11 is recruited to Pbs2, it will activate Hog1 to elicit the osmotic-response.

Phosphorylation can also regulate the turnover of scaffold proteins. For example, in cell cycle arrest, phosphorylation of Ste5 by G1-Cdk1 (G1 cyclin-dependent protein kinase), initiates $SCF^{Cdc4}$ (E3 ubiquitin ligase)-dependent and proteasome-mediated destruction of Ste5 in the nucleus. Intriguingly, such phosphorylation by G1-Cdk1 could negatively regulate Ste5 in different cellular compartments. In the cytosol, G1-Cdk1 phosphorylation of Ste5 blocks the membrane binding motif, thus preventing Ste5 from localizing to the plasma membrane. This control keeps the level of Ste5 below the threshold to ensure that yeast cells that have not exited G1 phase cannot be stimulated by pheromone during this cell cycle dependent process. This mechanism also ensures that activation only occurs in the presence of appropriate stimuli since uncontrolled hyperactivation of Ste11 is lethal to the cell [212,219].

Intriguingly, phosphorylation can be an important developmental switch. For example, phosphorylation of the neuronal scaffold for cAMP, DISC1 (disrupted in schizophrenia 1; Fig. 4B) at Ser710, switches its role from maintaining proliferation of mitotic progenitor cells to migration of neurons in mice [88]. Therefore, DISC1 is a versatile scaffold that can act as a molecular switch to regulate different cellular dynamics via its post-translational modification. Besides exerting an immediate response to regulate the activity and spatial distribution of scaffold proteins, phosphorylation also provides a gradual but cumulative means to allow cellular adaptation to external stimuli. For example, in the *Drosophila* photoreceptor cells, the scaffold INAD acts as a memory device that quantitatively regulates the high-light input threshold through cycles of conformational change. Under low-light conditions, the fifth PDZ domain of INAD adopts an open conformation. However, under high-light conditions, the cumulative activation of protein kinase C leads to phosphorylation of INAD which allosterically switches INAD to the closed conformation and also forms disulphide bonds. These modifications lead to reduced binding of PLC-β to INAD that is necessary for the activation of TRP cation channel. Such adaptive feedback regulation via the conformation change of INAD can effectively reduce the signal strength under high-light conditions and resulting in long-term adaptation [187,220,221].

Last but not least, phosphorylation plays important roles in mechanosensing and mechanotransduction, mediated by the close alliance of scaffold proteins such as the FAK, ILK, paxillin and p130Cas that occurs at the cell adhesion complex or mediated by other protein machineries in podosomes, invadopodia, adherens junctions and

synapses. By physically coupling to the integrins that attach to external cellular matrices and to the internal F-actins that are linked to focal adhesions, these proteins can experience mechanical stretching under different substrate rigidities, geometrical constraints and/or mechanical forces. Such mechanical perturbations could lead to conformational changes of the scaffold proteins that further results in their phosphorylation as a reinforced biochemical signal to propagate downstream signaling via protein–protein interactions, leading to immediate changes in cell morphogenesis and motility or for the long-term gene regulation and cell fate control [18]. As described in the earlier section, the auto-inhibition of FAK can be released upon tension [114] and through its FERM domain, FAK assembles diverse protein partners including paxillin, which can also be recruited to FAK upon phosphorylation by ERK. Acting downstream of Ras and Cdc42-PAK1, ERK can phosphorylate FAK [130] and another scaffold protein, PTP-PEST [131], leading to the recruitment of prolyl–peptidyl isomerase PIN1 and possibly prolyl isomerization of both proteins. This dual effect leads to high turn-over of focal adhesions that promotes Ras-induced cell migration, invasion, and metastasis. Possibly related to this, p130Cas also undergoes changes in conformation induced by mechanical forces and stretching that exposes multiple phosphorylation sites and motifs for kinases, adaptors and effectors [18,146].

### 3.2. Ubiquitination and sumoylation

Upon phosphorylation by G1-Cdk1 during the exit of G1 phase, Ste5 is "marked" and destined for the ubiquitin ligase, $SCF^{Cdc4}$ where it is subsequently ubiquitinylated for proteasome-mediated degradation in the nucleus. However, upon stimulation by pheromone, exportin Msn5 mediates the exit of Ste5 from the nucleus and protects Ste5 against degradation [212]. Besides proteasome-mediated degradation, ubiquitination of scaffold proteins can also influence its ability to interact with other proteins or protein trafficking. For example, ubiquitination of β-arrestin at its cytoplasmic tail enables its interaction with the G-protein coupled receptor (GPCR) at the membrane, leading to enhanced receptor internalization and an increase in the level of stable endosome-localized AT1aR (GPCR)–β-arrestin complex. This mechanism leads to sustained ERK activation in the endosomes [222]. In contrast, de-ubiquitination of β-arrestin leads to its dissociation from the GPCR, allowing GPCR to be redirected to clathrin-coated pits for rapid recycling [223,224]. Besides ubiquitin, scaffold proteins can also be covalently attached with SUMO (Small Ubiquitin-like Modifiers) that lead to changes in the protein stability, protein–protein interaction, proteins targeting and genes transcription. The scaffold RGS contains the GTPase-activating protein (GAP) activity to accelerate the hydrolysis of GTP to GDP in the activated GαGTPs subunits [225]. However, sumoylation of RGS-Rz proteins leads to a reduction in the GAP activity and it switches to become a scaffolding protein that sequesters the activated Gα subunits and G-protein coupled receptors (Mu-opioid receptors) in synaptosomes [49]. Axin, the scaffold protein for WNT and JNK signaling, also contains two sumoylation sites at the C terminus such that its sumoylation-defective mutants failed to activate JNK but not the WNT signaling [226]. In contrast, FAK could be sumoylated at the FERM domain, leading to enhanced FAK activation [227]. Interestingly, some fractions of sumoylated FAK can also be translocated to the nucleus [227], and this can possibly explain its roles in enhancing gene transcription of Prx1 [228] and cell-cycle progression [229].

### 3.3. Lipidation and calcium signals

Scaffold proteins can be covalently attached with different lipid moieties to target to specific membrane microdomains. Such lipid modifications include prenylation and myristoylation which usually irreversibly target proteins to the membrane or palmitoylation. This could reversibly regulate the protein's subcellular localization and protein–protein interaction [230]. For examples, the scaffold protein in cAMP signaling, AKAP18/AKAP15 (α- and β-isoforms) can be myristoylated and/or palmitoylated for membrane targeting. However, mutations in these palmitoylation or myristroylation sites fail to localize them to the plasma membrane [63]. Similarly, PSD-95, the neuronal scaffold protein that tether signaling enzymes at post-synapse, requires palmitoylation to induce clustering of the AMPA receptors at the excitatory synapse and for the steady synaptic transmission via the AMPA receptor [231,232]. Intriguingly, such palmitate cycling on PSD-95 itself is further regulated by the dynamic synaptic activity [231]. In addition to modification by lipids, calcium levels can also regulate the spatial dynamics of scaffold protein such as AHNAK that tethers the calcium channels and phospholipase Cγ (PLCγ). At low calcium concentration, AHNAK is primarily located in the nucleus via the nuclear localization signal at its C-terminus. With increasing intracellular calcium concentrations, AHNAK is translocated to the plasma membrane [76,233–235]. Furthermore, AHNAK can also be translocated from the nucleus to the plasma membrane via its phosphorylation by PKB/Akt, during the formation of $Ca^{2+}$-dependent cell–cell adhesion and development of polarity in epithelial cells. Upon translocation, AHNAK interacts with actin and annexin-2/S100A10 complex to regulate cortical actin cytoskeleton and cell membrane structure [236]. Calcium levels also regulate ubiquitination-mediated proteasome degradation of another MAPK scaffold protein, IB1/JIP1 (islet-brain 1/JNK interacting protein 1) [237].

### 3.4. Compartmentalization and trafficking

As the primary function of scaffold proteins is to anchor specific biochemical reactions at distinct locales, it is expected that they possess unique localization signal peptides or targeting domains. Indeed, different alternate splicing of AKAP genes result in multiple variants that can be targeted to different parts of the cells. This mechanism not only restrict its broad-spectrum substrate, PKA, to distinct sets of substrates, hence specificity [61] but it also helps increase the repertoire of its effects in response to cAMP signaling. Similarly, the cAMP-degrading phosphodiesterases are brought to sculpt the gradients of cAMP by multiple scaffolds [22] some of which also bind PKA (Fig. 4B). Together, they provide crucial roles in controlling the threshold, amplitude and duration of this signaling. Similarly, β-arrestin 2 but not β-arrestin 1, contains a classical leucine rich nuclear export signal can undergo nucleocytoplasmic shuttling to restrict the subcellular distribution of JNK3 to cytoplasm and not to nucleus [224,238,239]. Interestingly, two key scaffold components in cell adhesion complex, the FAK and paxillin, have been shown to function in the nucleus. For example, FAK promotes the assembly of the p53-MDM2 complex in the nucleus, leading to p53 ubiquitination and degradation [116] and it also regulates heterochromatin remodeling at the promoter of Myogenin by interacting with MBD2 [117]. In comparison, Paxillin interacts with Hic-5 to function as transactivators for androgen and glucocorticoid receptors and it also binds poly(A) binding protein-1, perhaps for the movement of specific mRNAs to the leading edge to facilitate their translation [109].

On the other hand, instead of being targeted to specific compartments to carry out the scaffold functions there, the dynamic disposition of scaffold proteins may act to carry their signaling partners. For example, studies have shown that the MAPK scaffold, JIP1/2 and JSAP1 binds to conventional kinesin [240]. Furthermore, JIP1 has been shown to be a cargo of the motor protein, kinesin-1 [241,242] whereas the *Drosophila* homolog of JIP-1, APLIP1 can regulate the two opposing motors (i.e. kinesin-1 and dynein) [243]. Therefore, JIP1 plays an important role in axonal transport. In addition, the functional relationship between JSAP1 and convention kinesin is confirmed in *C. elegans* and *Drosophila* [244,245]. Intriguingly, JSAP1 can determine the traffic direction of kinesin in neuronal axon [246], although such transportation to growth cones of neurites is independent of JNK signaling pathways [247]. Such interactions therefore suggest that kinesin-1 might help localize

activated JNK in growth cones involved in neurite extensions and neuron migration.

Although the BCH domains of BNIP-2 family proteins function to regulate GTPase signalome, we and others have shown that the brain-specific homolog, BNIP-H (Caytaxin) can traffic glutaminase and mitochondria towards neurite termini [163]. Since the transport of mitochondria by BNIP-H involves kinesin-1 [160], we have further shown that BNIP-H could indeed act as a scaffold to bridge glutaminase to kinesin-1 in a process regulated by the interplay of a specific kinase and an isomerase (our unpublished data). Glutaminase is a metabolic enzyme necessary for the production of neurotransmitter glutamate. We therefore speculate that loss of BNIP-H function, hence the production of localized neurotransmitters such as glutamate, could in part contribute to the etiology of neurological disorders seen in human Cayman ataxia [248] and also in the mice and rat models of dystonia and ataxia [249–251]. Analogous to this mechanism, BNIP-2 is also found to undergo dynamic disposition along microtubules and it also associates with certain species of phosphoinositides in vitro (our unpublished data). And, similar to BNIP-H, BNIP-2 and the extended form of another homolog, BNIP-XL (BMCC1/PRUNE2) harbor putative kinesin-binding motifs [164]. These observations raise the possibilities that some of the BNIP-2 family proteins could act as mobile signaling scaffolds that not only target their substrates to specific compartments but they could also regulate their activities in situ there.

In comparison, the MAPK scaffold, MP1 and its adaptor, p14 are structurally similar to the membrane trafficking SNARE-like proteins. This result suggests that the scaffold protein that is enriched in lipid raft [252] might be involved in trafficking MEK1 between membrane structure [253]. Interestingly, the adaptor p14 displays sequence homology to the Roadblock/LC7 family of dynein-binding proteins, thereby suggesting that the localization of the MP1/p14 complex may be regulated by dynein motor activity [254,255]. Taken together, scaffold proteins are dynamic rather than a static binding platform.

### 3.5. Modular interaction, allosteric regulation, oligomerization and intrinsic disordered regions

Scaffold proteins must adopt an optimal conformation to effectively support and regulate the activity of various signaling components. Therefore, any changes in their conformation will have significant impact on their binding and activity towards their substrates or regulators. These can be mediated via multiple mechanisms such as the modular domain interaction, allosteric binding that includes their intrinsically disordered regions, their ability to undergo auto-inhibition and/or to form a larger functional complex through oligomerization. These can be further regulated by some of the post-translational modifications as discussed above. For example, in the yeast mating pathway, interaction between the scaffold protein, Ste5 and the Gβγ subunit of heterotrimeric G-protein, Gpa, may induce conformation changes in Ste5. This helps bring the N and C terminal of Ste5 closer to facilitate recruitment of Ste7 to the C-terminal of Ste5 and Fus3 to the N-terminal of Ste5 in right orientation for the activation of Fus3 by Ste7 [256]. Furthermore, conformation changes in scaffold proteins also regulate their activity. As discussed above, the scaffold protein for visual signaling in photoreceptors of *Drosophila*, INAD, is regulated by conformation switch via formation/disruption of disulphide bond at one of the PDZ domain [220]. Similarly, MEK2, which also acts as a catalytic scaffold protein, is also regulated via the switch between active and inactive conformation. MEK2 must be in its active conformation to promote the interaction of peptidyl-prolyl cis/trans isomerase PIN1 with the RhoGAP protein, BPGAP1, to suppress BPGAP1-induced acute ERK1/2 activation and cell migration [165].

In facilitating proteins assembly, a scaffold protein would interact with its target proteins on an equimolar basis. However, such equilibrium can be affected by the level of that particular scaffold protein relative to its targets. It also depends on how fast the scaffold protein catalyzes the assembly and its disassociation from the complex in order to re-initiate the next round of reaction. In the presence of excessive scaffolds, formation of separate scaffold-target entities is preferred over the multimeric functional complex. This is due to the greater tendency of the overcrowding scaffold proteins in preventing the different target proteins from coming to close proximity and interacting with one another. To help minimize such undesirable mutual sequestration and to ensure that the local concentrations of the target proteins remain favorably high for their subsequent interaction, scaffold proteins can form multiple protein complex via their oligomerization domains/motifs, resulting in a more efficient signaling output. For example, KSR1 contains RING-H2 domain which is required for homo- or heterophilic oligomerization (e.g. with the inhibitor, IMP). It also contains the pseudokinase domain that binds to RAF, forming a functional KSR–RAF dimer that leads to their mutual activation via allosteric control [173,174]. Most significantly, this leads to the activation of the KSR kinase activity necessary for the subsequent ERK activation. Similarly, the dimerization of Ste5 requires both its RING-H2 domain and the distal region that overlaps with the leucine zipper domain and Ste11p binding site [219,257].

Besides, another MAPK scaffold protein, CNK contains the SAM (Sterile alpha motif) domain that is crucial for the homophilic interaction; while the MAPK–JNK scaffold protein, JNK-interacting protein (JIP) can form homo-oligomers or hetero-oligomers with other JNK scaffold proteins such as POSH via its SH3/SH3 domains dimerization [258]. Interestingly, Ste5 dimerization in the presence of pheromone could facilitate trans-phosphorylation between Ste11, Ste7 and Fus3 [219,257]. Intriguingly, conformational changes in the scaffold proteins could also result in scaffold dimerization and/or oligomerization, thereby enriching the architecture for signaling output. For example, upon stimulation in the yeast mating pathway, the monomer inactive Ste5 that predominantly resides in the nucleus, interacts with nuclear exportins or the heterotrimeric G-protein, Gpa, particularly the Gβγ subunit (Ste4/Ste18) which induce conformation changes of Ste5. This increases the accessibility of RING-H2 domain of Ste5 for dimerization and/or oligomerization in the cytoplasm [257,259]. On the other hand, the homodimerization of the JNK/MAPK scaffold protein, Islet-brain 1 (IB1 or JIP1) via its SH3 domain could be important for the scaffold function as impaired IB1 homodimerization affects the insulin secretion process in pancreatic β-cells [260]. Such scaffold dimerization and/or oligomerization may cooperatively orientate their MAPK signaling components for more efficient phosphorylation relay of Ste11, Ste7 and Fus3 during yeast mating [3,219].

Similarly, the MAPK scaffold protein, β-arrestins can form both homo- and hetero-oligomers. β-arrestin 1 oligomers are primarily localized in cytoplasmic while β-arrestin 1 monomers are localized in the nucleus. Intriguingly, co-expression of β-arrestin 2 and β-arrestin 1, prevents the accumulation of β-arrestin 1 in the nucleus. Therefore, the oligomerization of β-arrestins regulates the cellular localization of β-arrestin1 and β-arrestin2. Interestingly, scaffold proteins can also induce the oligomerization of other scaffold proteins. For example, the scaffold protein of the TCR (T-cell receptor) signaling, CARMA1, once activated, it can induce the oligomerization of the JIP-like scaffold, B-cell lymphoma 10 (Bcl10), in order to recruit and assemble the three-tier kinases, JNK2, MKK7 and TAK1 for signal transduction [261]. Intriguingly, such scaffold oligomerization can be induced upon interaction with other proteins. For example, the MAPK scaffold protein in yeast, the RING-H2 domain of Ste5 interacts with Ste4 which is a prerequisite for Ste5–Ste5 self-association and transduction/activation of the MAPK signaling [262].

Although many functions and regulations of scaffold proteins are centered on the dynamic interplay of their protein modules, increasing evidences point to the emergent roles of the intrinsically disordered regions (IDR) present in many of them. Unlike protein modules that

adopts well-defined, three-dimensional structures to accommodate specific protein partners for a particular function, IDR do not conform to any organized structures or folds when they are present alone. Yet, they can offer greater interaction surface area and conformational flexibility to allow interactions with multiple proteins and hence increasing the repertoire of re-using the same protein in multiple pathways [263]. IDR can also undergo post-translational modifications such as phosphorylation and when IDR do fold upon binding to their targets, such interactions are usually associated with high specificity for the initiation of a signaling process but with low affinity to allow rapid dissociation when the process is completed [264]. It has been proposed that IDR could play an important role in force-sensing and stretching of proteins during mechanosensing and mechanotransduction, for example, via the substrate regions on the scaffold protein p130Cas [18,146,265]. Indeed, the master scaffold protein Axin, has been identified to carry an important IDR within its critical scaffolding region [266]. Similarly, several other scaffold proteins such as Ste5, BRCA1, AKAP250 and others all contain their IDR that further provide the plasticity of scaffold proteins [267].

## 4. Conclusion and future perspectives

Scaffold proteins represent a specialized group of signaling organizers that mediate specific signaling events in a spatial and temporal manner through bringing components together. As active platforms of protein assembly, scaffold proteins also facilitate crosstalk among various signaling pathways and integrate various biochemical and physical stimuli. With their signature architecture of juxtaposed modular protein domains, their precise subcellular localization and their ability to undergo protein modifications, scaffold proteins are no longer perceived as mere passive physical platforms but active signaling centers. Such versatile properties not only underlie their central roles in controlling normal physiology but they also offer us exciting prospects of exploiting them as potential targets of therapeutic intervention. It is not too farfetched to think about creating novel cellular processes and functions by re-wiring specific networks or modifying their responses using synthetic scaffold proteins. Several recent studies have shown the feasibility of re-wiring signaling output. For example, Ste5 is involved in both yeast mating and osmoresponse via the assembly of different components of the three-tier MAPK kinases. By assembling a hybrid MAPK kinase it was shown that upon treatment with pheromone, instead of the mating response, the signaling could be re-directed to activation of the osmo-stress [268]. On the other hand, re-engineering Ste5 scaffold with synthetic positive- and negative-feedback loops could re-wire the sensitivity, response time and tunable adaptation of Ste5 [194]. Knowing the detailed mechanism(s) underlying the functions and regulation by scaffold proteins can therefore offer new alternatives to modify signaling networks by mimicry agents or inhibitors, or by re-engineering new regulatory loops.

To achieve this goal, the intricate network designs for their protein domain architecture and circuitry as well as the function and regulation of scaffold proteins by their immediate substrates, regulators and their interconnectivity must all be fully understood. This should be interrogated in the context of space, time and the physical forces that the cells experience and various types of modifications that scaffold proteins would undergo. These should also be examined with more quantitative and integrative approaches across all levels: tissue, cellular, biochemical, biophysical, molecular, or even at the atomic levels. And it is now possible to engineer new versions of scaffold proteins using the modern synthetic biology approaches to obtain newly improved or completely novel functionality [269,270]. All these studies should also incorporate super-resolution bioimaging tools to track precise localization of scaffold proteins with their target proteins and their dynamic behavior there, and by monitoring their dynamic movement within and between different cellular compartments. In this regard, it is now possible, albeit not quite routine yet, to directly manipulate and monitor the local activity of proteins (in this case, the scaffold proteins) or/and their interacting partners through light and chemical means [271] such that their local concentrations and activation (or inactivation), their specific localization and their proximity to some or all interacting partners, could all be more "subtly" perturbed instead of resorting to gene knockout or knockdown that would have destroyed a whole complex because it is so central and essential. Hopefully, such behaviors could then be traced to immediate downstream functional readouts such as the local activity of Rho, the cAMP gradients (all of which can be monitored by local biosensors) and the more distal gene expression profiling. In particular, by imaging scaffold proteins and their molecular assembly in cells expressing efficient biosensors as their functional read-out, and by culturing cells on functionalized micropillars, one can collectively measure force-dependent activities of scaffold proteins and establish their precise force, spatial and temporal relationship. And by mapping their conformational shifts using NMR, by scrutinizing the effect of protein modifications on the assembly function, as well as by analyzing their gene expression and activities, one could learn a lot more about the integrative nature of signaling systems present in both cells and tissues. At the higher organism level, key signaling events mediated by wild type and mutant scaffold proteins could be visualized and analyzed in appropriate animal models using knock-in with appropriate mutant proteins and inducible systems. It is anticipated that with such detailed understanding of modular design of scaffold proteins and how their protein–protein interaction networks work and regulated, we will have much better control in rewiring signaling pathways using the modern synthetic biology approach for better therapeutic purposes.

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